Prog. Biophys. molec. Biol., Vol. 35, pp. 103–134.

0079–6107/79/0301–0103$05.00/0

THE NMR STUDIES OF WATER IN BIOLOGICAL SYSTEMS

R. MATHUR-DE VRÉ

Université Libre de Bruxelles, Laboratoire de Radiobiologie Moléculaire, rue des Chevaux 67, 1640 *Rhode St. Genèse, Belgium and*
Institut d'Hygiène et d'Epidémiologie, Département de l'Environnement, rue Juliette Wytsman 14, 1050 *Bruxelles, Belgium*

CONTENTS

I. INTRODUCTION

The state of water in the body constituents of living organisms and in the vicinity of biological macromolecules differs significantly from the state of water in solutions of simple molecules and in pure water. Nuclear magnetic resonance (NMR) spectroscopy is a powerful technique for studying in detail the structure, mobility, and extent of ordering of water molecules in various biological systems. The NMR spectra of water present in a sample can be obtained either from the total water content, or specifically from the hydration water or intracellular water depending on the nature of the sample. In order to fully appreciate the unique contribution of NMR spectroscopy in the domain of hydration studies, it is important to note that the behaviour of a water molecule  can be investigated by looking at the nuclear magnetic resonance of four different nuclei; these are the three isotopes of hydrogen: proton (^{1}H), deuterium (^{2}H), tritium (^{3}H), and oxygen ^{17}O. Of these, the most widely and extensively studied nucleus is ^{1}H; the applications of ^{2}H and ^{17}O NMR of water have been limited to the study of relatively few systems. The NMR studies of tritiated water in biological systems apparently have been neglected so far, but in the course of time they are likely to become a centre of intensive and growing research as a result of the recent progress in the development of tritium NMR spectroscopy. The importance of the NMR studies of tritiated water lies in the fact that such investigations are expected to offer a new and promising method for exploring the biological hazards of tritium (the radioactive isotope of hydrogen) present in the environment. The widespread risks

arise because tritium released from nuclear reactors inevitably pervades the environment as tritiated water: a form in which tritium can be readily absorbed by plants and animals.

The NMR parameters most useful in the study of water are the relaxation times, T_1, T_2 and $T_{1\rho}$. The diffusion constant and the distribution of correlation times for water can generally be evaluated from relaxation times measured under specific conditions. It is well known that the relaxation processes for protons, deuterons, and ^{17}O, are influenced by different factors. Therefore, for a comprehensive study of the state of water in any system, using NMR, it is important to compare the relaxation time data for more than one nucleus.

The present article comprises: a brief discussion of the characteristics of hydration water, an outline of the theory of NMR relaxation times with emphasis on the factors relevant to the study of water, and finally an extensive review covering the NMR studies of the different water nuclei in various biological systems and in the damage induced by γ-irradiation in the aqueous medium. Different theories related to either the structure of water in general, or to cell water in particular, have not been described at any length in this review. Several monographs have covered these subjects in considerable detail (Ling, 1962, 1969; Cope, 1970; Kavanau, 1964; Eisenberg and Kauzmann, 1969; Franks, 1972–1975). A variety of physical techniques other than NMR spectroscopy are also highly suitable for the study of the hydration of biological macromolecules. A number of methods which have been applied to nucleic acids are discussed explicitly in a recent article by Texter (1978).

II. HYDRATION WATER

Biological macromolecules induce a characteristic water structure in their close vicinity due to weak macromolecular–water interactions. The solvent water molecules interact with the solute species by electrostatic forces (dispersion and induction forces) because of the high dipole moment of water, as well as through extensive hydrogen bonding by virtue of the potentially available proton donor and proton accepting sites. Consequently, macromolecules form a well-defined hydration layer in solution, in hydrated fibres (DNA, collagen) and in all biological samples such as intracellular water, tissues, muscle, and membrane. The water molecules contributing to the hydration layer are dynamically oriented, and exhibit restricted motion due to a significant decrease in the translational and rotational modes of motion caused by macromolecular–water interactions. As a result, the mobility and the extent of ordering of hydration water molecules are distinctly different from those characterizing the fast and random motion of the bulk water. Furthermore, the overall behaviour of water molecules in the hydration layer is influenced by factors other than simple molecular interactions. Berendsen (1975) has distinguished three aspects of hydration water: thermodynamic aspects, dynamic aspects, and structural aspects. A complete description of hydration should necessarily comprise all three aspects.

The NMR studies of water illustrate that certain coherent and cumulative processes come into effect when biological macromolecules interact with water, either in solution or in biological systems. These processes (outlined below) are favoured by the extended, ordered, and complex structure of macromolecules (such as proteins and nucleic acids), and they govern different dynamic states of the hydration water molecules. (1) The restricted motion of water in different fractions of the hydration layer is not uniform, and all the dynamic modes of these water molecules cannot be described completely by a single correlation time but require a distribution of correlation times. (2) The diffusion of water molecules in the hydration layer occurs in an anisotropic manner. (3) The hydrogen bonded interactions between water molecules and several proton donor and proton accepting groups of macromolecules give rise to a continuous hydrogen bonded path in the hydration layer. This process promotes enhanced proton transfer along the

macromolecular chain in a continuous and an extensive path having a well-defined structure.

From the temperature-dependent changes in the relaxation times of water protons, the activation energy values of the order of 10.0 and 5.0 kcal/mole were calculated for the processes of proton transfer and diffusion, respectively (Berendsen and Migchelsen, 1966; Migchelsen and Berendsen, 1973; Mathur-De Vré *et al.*, 1976). These processes involve the breaking of two hydrogen bonds for the proton transfer and one hydrogen bond for the diffusion process. In general, the hydrogen bonds are very sensitive to the nature and orientation of groups interacting with water, and to small changes in the temperature. Therefore, the relaxation processes that are influenced predominantly by proton transfer in the hydrogen bonded path are also expected to exhibit a characteristic dependence on the above mentioned factors. When macromolecules are present in a medium containing ^2H_2O and ^3H_2O, the exchangeable or labile protons undergo isotopic substitution by exchange. This leads to incorporation of ^2H and ^3H throughout the system in preferential sites on the macromolecular chain. Furthermore, there is evidence suggesting that the isotopic distribution in the hydration layer is not random.

At temperatures well below the freezing point of solvent, the hydration water molecules remain unfrozen or mobile on the NMR time scale. This phenomenon is distinctly different from the physical processes such as freezing point depression and formation of eutectic mixtures. For the macromolecular solutions and all biological samples, the NMR spectra of water observed between -5 and $\sim -60°C$ arise from only a fraction of the total water content, i.e. the hydration water. From the temperature-dependent changes in these spectra, activation energy values for the relaxation processes have been calculated. In frozen samples, the extra-hydration layer water freezes to form a rigid ice-like structure (as in free water) giving rise to a very broad signal. The area under the water proton signals obtained from frozen solutions was shown to vary linearly with the concentration of macromolecules (Kuntz *et al.*, 1969; Mathur-De Vré *et al.*, 1976). This indicates that the water NMR signals observed in frozen samples arise from water associated with macromolecules. Kuntz and Kauzmann (1974) defined hydration water as the unfrozen water. The potential of this phenomenon for detailed studies of the properties of water in biological systems was soon recognized. This is evident from the numerous examples cited in Section IV.

Franks (1977) has described water–protein interactions in solutions as illustrated diagrammatically in Fig. 1. The A shell includes solvent molecules which are in the neighbourhood of the protein side chains or the backbone. The motion of water molecules in the primary hydration sphere (A shell) is determined by the motion of protein molecules or their localized groups. The region C represents the water molecules that are unperturbed by macromolecules. Finally, "incompatibility of the hydrogen

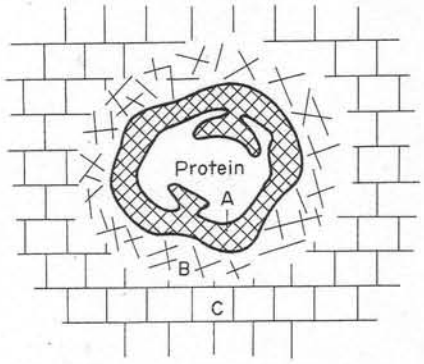

FIG. 1. (Franks, 1977) Diagrammatic representation of the protein environment in solution. The protein can be regarded as a hydrodynamic sphere with a primary hydration shell A in which the molecular motions are largely determined by those of the polar protein sites. C is the unperturbed water "structure" and region B arises from the spatial and orientational mismatch between regions A and C.

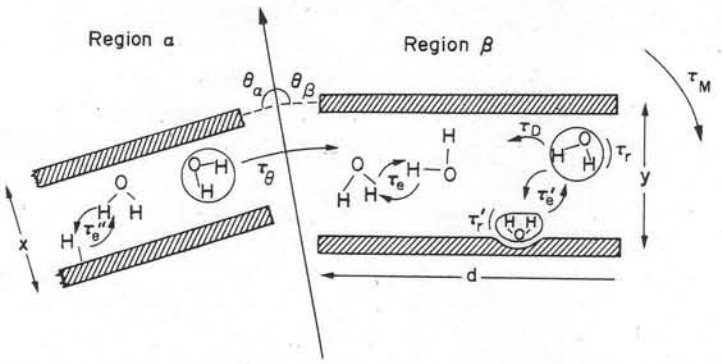

FIG. 2. (Packer, 1977) A schematic illustration of small-scale heterogeneity and various dynamic processes which may be experienced by water molecules in such a system. The shaded regions represent macromolecular structures characterized by dimensions d, x, y, etc., orientations θ_α, θ_β etc. with respect 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''.

bonding in regions A and C" gives rise to the region B. Franks indicated that water in regions A and B markedly influences the NMR spectra and contributes to the unfrozen fraction. In other words, regions A and B represent the hydration layer, and region C represents the extra-hydration water or the frozen fraction.

Berendsen (1975) defined the hydration of macromolecules in terms of specific and nonspecific hydration. The interactions between water and specific binding sites on the macromolecular chains result in specific hydration. Nonspecific hydration is the amount of water affected by the macromolecules in such a manner that it exhibits slightly lower rate of rotation than molecules in the bulk liquid state, and it also contributes to the unfrozen fraction of water. The regions of specific and nonspecific hydration may be compared with regions A and B in Fig. 1.

Packer (1977) has represented diagrammatically (see Fig. 2) different modes of motion of dynamically oriented water near a macromolecular surface. Distribution of correlation times for water protons arise because water molecules are subjected to largely diverse dynamic processes as a result of their interactions with a variety of sites and groups constrained in different environments on the macromolecular chain. In Fig. 2, α and β represent two components of a heterogeneous macromolecular surface, oriented by θ_α and θ_β with respect to an external reference axis. Packer proposed that water molecules near the macromolecular surface move in an anisotropic potential whose spatial properties remain unchanged during the reorientation of a water molecule; as a result the anisotropic potential experienced by each water molecule is not averaged out during its reorientation time. On the other hand, in liquid and bulk water the potential experienced by a molecule at any instant is also anisotropic but its axis undergoes a rapid change during the reorientation of water molecules, consequently the effects due to anisotropic potential are averaged out

III. NMR AND ITS APPLICATION TO WATER STUDIES

Several authors have discussed in detail the theory and techniques of NMR spectroscopy (Emsley *et al.*, 1965; Carrington and McLachlan, 1969; Ferrar and Becker, 1971; Shaw, 1976). Therefore, in this section only the basic principles of the NMR phenomenon will be described briefly and concisely, with special emphasis on the relaxation processes.

1. *Basic Concepts*

A nucleus with spin I is characterized by a spin angular momentum and possesses a magnetic moment (μ). In addition, a nucleus also has an electric quadrupole moment Q

if $I > 1/2$. For the NMR studies of water, the nuclei of interest are: proton ($I = 1/2$), deuteron ($I = 1$), tritium ($I = 1/2$), and oxygen ^{17}O ($I = 5/2$). In the presence of a large magnetic field H_0, the nuclear energy levels split into $2I + 1$ states. The distribution of spins between these energy levels attains thermal equilibrium with the lattice in such a manner that the lower energy level has a slight excess of spins. At equilibrium there is no net transfer of spins. However, in the presence of a second oscillating radiofrequency field $H_1(W)$ (RF field) whose magnetic component is perpendicular to H, the absorption of energy occurs and induces spin transfer from the lower to the upper energy level, provided the frequency (ν) of oscillating field satisfies the resonance condition: $h\nu = g_N\beta_N H_0$; where g_N is the nuclear g factor and β_N is nuclear magneton. This phenomenon gives rise to the nuclear resonance absorption signals. It is clear that the process of spin transfer would eventually lead to a state of saturation if there were no relaxation processes to re-establish the excess spin population in the lower level. However, spins in the higher energy level can relax to the lower level by means of relaxation processes that are induced by: (i) coupling interactions between the nuclear spin and the local fluctuating fields arising from thermal motion of the lattice, (ii) molecular motion, (iii) proton exchange processes and (iv) paramagnetic centres. The ultimate effects of the interactions (i)–(iii) on different nuclear magnetic relaxation processes are a function of the time scale and the nature of fluctuations in motion. Herein lies the basis of the application of the NMR relaxation times as a sensitive tool to monitor dynamic processes.

The process of energy exchange between the magnetic nuclei and the lattice is known as spin–lattice relaxation, designated by the time constant T_1. In addition, the magnetic nuclei also interact with each other, therefore each nuclear magnet experiences an additional small local field H_{loc} produced by the neighbouring nuclear magnets. Only the nearest neighbours exert an important influence because H_{loc} falls off rapidly with increasing distance. The spread of the steady magnetic field experienced by each nucleus results in dipolar broadening. The spin–spin interaction time constant T_2 represents the lifetime (or phase memory time) of a nuclear spin state. T_1 and T_2 are also defined as longitudinal and transverse relaxation times, respectively. This follows from the fact that when the RF field applied initially to a system of nuclear spins is removed, the magnetization aligned along H_0 (M_z component) returns exponentially to the equilibrium value with a time constant T_1, whereas the M_{xy} component decays with the time constant T_2. A third relaxation time constant $T_{1\rho}$ known as spin–lattice relaxation in rotating frame corresponds to the decay of magnetization aligned along $H_{(1\rho)}$ at right angles to H_0, rather than along H_0. Due to fast rotational and translational motions persisting in the liquid state, the local fluctuating fields of the surrounding lattice molecules are averaged thereby reducing the T_1 relaxation rates, while the averaging of the dipolar coupling interactions between nuclei decreases the T_2 relaxation rates.

In liquid samples very sharp water NMR signals are observed because magnetic interactions are averaged to zero. However, due to restricted motion in more rigid states (e.g. frozen samples, macromolecular–water interface) the fluctuating fields and dipolar interactions are not totally averaged, their residual effects on the NMR spectra constitute a valuable source of information concerning the mechanisms of relaxation rates and molecular interactions. At low temperatures, the averaging of dipolar and quadrupolar interactions is less effective because of reduced thermal motion, orientation, and ordering of water molecules. Ferrar and Becker (1971), and Frey *et al.* (1972) showed that the relaxation time T_1, T_2 and $T_{1\rho}$ are sensitive to dynamic processes occurring at different frequencies. For instance, T_1 is most sensitive to motions corresponding to the Larmor frequency $\omega_0 = \gamma H_0$, $T_{1\rho}$ detects motions corresponding to RF frequency $\omega_1 = \gamma H_{(1\rho)}$, and T_2 exhibits sensitivity to motions characterized by frequency $\omega_2 = \gamma H_{loc}$. For protons, $T_1, T_{1\rho}$, and T_2 were shown to be sensitive to motions occurring with frequencies of the order of 30 MHz, 50 kHz and 10 Hz, respectively (Frey *et al.*, 1972). Generally, proton exchange processes contribute to T_2 and $T_{1\rho}$. High

frequency processes affect both T_1 and T_2, but the low frequency processes (such as chemical exchange and slow diffusion) influence mainly the T_2 relaxation, causing T_2 to be much shorter than T_1 provided $\omega_0\tau_c \gg 1$.

The line broadening is an approximate measure of spin–spin relaxation time; T_2 may be obtained from the half-linewidth ($\Delta 1/2$) of the signals observed in the steady state, $\Delta 1/2 = 1/\pi T_2$. The relaxation times are measured accurately by the pulse techniques; for a detailed discussion of the theory and experimental pulse techniques one may refer to Ferrar and Becker (1971) and Shaw (1976). In this method a strong RF field is applied for short durations, i.e. in pulses. The effects of pulses depend on their magnitude and duration. The principle of the pulse method is that by applying either an initial 90° RF pulse M_z is reduced to zero, or it is reversed by a 180° pulse. A second pulse is used to tip M_z into the xy plane where the exponential regrowth of magnetization can be detected, and the relaxation time constants T_1 and T_2 measured. T_2 is generally obtained by the "spin echo" experiment (Carr–Purcell method), i.e. by applying a 90° pulse followed by a succession of 180° pulses. T_1 can be measured by applying a series of pulse sequences of the type 180°–τ–90° or by monitoring the amplitude of the induction decay signal M_z following a 90° pulse. The advent of the pulsed Fourier transform technique has greatly facilitated precise measurements of the relaxation times, particularly of nuclei present in low quantities.

2. *The Relaxation Rates*

The relaxation rates of the water molecules are governed by two important factors: (i) the strength of local magnetic interactions between water nuclei; (ii) the molecular motion and proton exchange rates.

(i) The important interactions between water nuclei are: nuclear magnetic dipole–dipole coupling (inter- and intramolecular), and nuclear quadrupole coupling for deuterium and ^{17}O. Quadrupole interactions result because the electric moment Q interacts with the neighbouring electric field gradient. Within the hydration layer, the magnetic interactions are partially averaged by specific processes depending on the interactions of water with macromolecules such as proton transfer, dynamic orientation and diffusion of water molecules through regions of different orientations. Whereas, in free or bulk water, motional averaging of magnetic interactions dominate the relaxation behaviour.

(ii) The effects of molecular motion are generally incorporated into the theory of nuclear magnetic relaxation in terms of the correlation time, (τ_c). τ_c is considered as the time taken by a molecule to translate through a molecular distance, or the average time between molecular collisions for a molecule in its actual state of motion. For fast motion, i.e. when $1/\tau_c \gg \omega_0$, T_1 and T_2 are equal. For slow and restricted motion, both T_1 and T_2 decrease and may not necessarily be identical as already mentioned in the previous section. The nuclear spin relaxation times are sensitive to molecular motions of 10^{-8}–10^{-12} sec. Water molecules tumble in liquid solutions at a rate of about 10^{-12} sec; this motion is considerably slowed down when water molecules interact with biological macromolecules in solution, in muscle, or in cells, but still falls within the limits of NMR sensitivity. For example, the rotational motion of water molecules associated by hydrogen bonds with polar groups on the macromolecular chains are reduced so that their correlation time is of the order of 10^{-6} sec instead of $\sim 10^{-12}$ sec for the remaining water (Fung, 1977a). Under the conditions of rapid exchange between the hydration and bulk water in liquid solutions of macromolecules, the observed relaxation rate $(1/T_1)_{obs}$ is given by:

$$(1/T_1)_{obs} = X_f(1/T_1)_f + X_h(1/T_1)_h. \tag{1}$$

Even though the fraction of free water X_f is much greater than the fraction of hydration water X_h, the term $X_h(1/T_1)_h$ can still make an important contribution to $(1/T_1)_{obs}$, since $(1/T_1)_h > (1/T_1)_f$ because of the restricted motion of water molecules in the hydration layer. If the rates of exchange of water between different regions in a

sample is fast compared with the regional relaxation rates, then an average water resonance signal is observed (fast-exchange condition); on the other hand, if the exchange rate is slow compared with relaxation rates in each site, then a resonance signal characteristic of water in each site may be observed (slow-exchange condition).

Assuming the motions involved in intramolecular relaxation, the following expressions were reported for $1/T_1$, $1/T_2$ and $1/T_{1\rho}$ in terms of the correlation time τ (Finch and Homer, 1974; Knispel *et al.*, 1974; Belton *et al.*, 1973; Fung and McGaughy, 1974).

$$1/T_1 = C\left(\frac{2\tau}{1+\omega_0^2\tau^2} + \frac{8\tau}{1+4\omega_0^2\tau^2}\right) \tag{2}$$

$$1/T_2 = C\left(3\tau + \frac{5\tau}{1+\omega_0^2\tau^2} + \frac{2\tau}{1+4\omega_0^2\tau^2}\right) \tag{3}$$

$$1/T_{1\rho} = C\left(\frac{3\tau}{1+4\omega_1^2\tau^2} + \frac{5\tau}{1+\omega_0^2\tau^2} + \frac{2\tau}{1+4\omega_0^2\tau^2}\right), \tag{4}$$

where ω_0 is the Larmor angular frequency in the constant magnetic field H_0 and is related to the resonance frequency ν_0 by the relation $\omega_0 = 2\pi\nu_0$, ω_1 is the Larmor frequency in the rotating frame in the presence of a RF field ($H_{1\rho}$), and C is a constant having the form $k'\gamma^4\hbar I(I+1)r_{jk}^{-6}$ for protons. A similar expression for deuteron $1/T_1$ was given where the constant C is equal to $k''\pi^2(1+\eta^2/3)(e^2qQ/h)^2$ (Fung *et al.*, 1975a); γ is the gyromagnetic ratio, $\hbar$ = Planck constant, $h/2\pi$, r_{jk} is the internuclear distance, e^2qQ/h is the nuclear quadrupolar coupling constant, η is the asymmetry parameter, and finally k' and k'' are the numerical values. Both ω_0 nd ω_1 are variable parameters. It can be seen from eqns (2)–(4) that when $\omega_0\tau > 1$, information about τ (and motion) can be obtained by studying the ω_0 dependence of relaxation times. In pure water, τ is very small ($<10^{-11}$ sec) and $\omega_0\tau \ll 1$ even at very high ω_0 values, therefore T_1 and T_2 values are independent of the ω_0 values.

Berendsen has shown that in the hydration layer of biopolymers, T_2 can be related to the diffusion of water molecules and the exchange lifetime of protons in the following manner (Berendsen, 1975; Mathur-De Vré *et al.*, 1976):

$$1/T_2 = (\gamma^2/20)(\Delta H)^2\tau_c, \tag{5}$$

where γ is the proton gyromagnetic ratio; ΔH is the maximum splitting of the proton resonance in the event that the biopolymer is completely aligned with the magnetic field, and $\tau_c = (6D/a^2) + \tau_{exch}^{-1}$; D is the diffusion constant; a is the measure for the length over which the macromolecules are oriented; and τ_{exch} is the exchange lifetime of a proton on the water molecule. It was shown that in frozen DNA solutions the diffusion process determines the behaviour of T_2 or $(\Delta 1/2)$ at low temperatures, while at higher temperatures the exchange rate dominates the relaxation process: where $(\Delta 1/2) = 1/\pi T_2$.

In free or bulk water, the rotational and translational motions of water molecules are strongly coupled, as a result the motion of water molecules can be defined by a single correlation time (Eisenberg and Kauzmann, 1969). Whereas, in the hydration layer the translational and rotational motions are no longer appreciably coupled due to molecular interactions, restricted motion and ordering of water molecules. Under the influence of the decoupling effect, the motion of the entire mass of water cannot be expressed by a single correlation time but requires a distribution function. At temperatures below the freezing point of the solvent, the molecules of unfrozen water in biological samples have been treated as spherical molecules undergoing translational and rotational motion, governed by a distribution of correlation times (Fung and McGaughy, 1974). The normalized log-Gaussian distribution function is given by the relation:

$$g(\tau) = \frac{\alpha}{\sqrt{\pi\tau}} \times \exp\left[-(\alpha \ln \tau/\tau_0)^2\right]. \tag{6}$$

The parameters τ_0 and α are temperature-dependent, τ_0 is the median of distribution and α is a parameter that determines the width of the distribution. The distribution of correlation times leads to different frequency dependence of T_1, $T_{1\rho}$ and T_2 from that given by eqns (2)–(4).

At temperatures above the freezing point of water, the following distribution function was suggested:

$$g'(\tau) = X \times g(\tau_1) + (1 - X)\delta(\tau_2), \tag{7}$$

where X = fraction of molecules at any instant having a log-Gaussian distribution of the correlation times. Equation (7) signifies that above the freezing temperatures, each water molecule spends part of its time behaving as isotropic water with a single correlation time.

The correlation between the motion of protons from the water molecules and from macromolecules gives rise to the process of cross-relaxation or spin diffusion. The observed relaxation rates of water protons can be influenced significantly by the cross-relaxation rates. Spin diffusion occurs by way of mutual exchange of spin magnetization between water protons of the hydration layer and protons on the macromolecular chain. The contribution of cross-relaxation becomes very important under the conditions of slow molecular diffusion and when $T_2 \ll T_1$ (such as in many biological systems). Under these conditions, the energy exchanges much more rapidly within the system of spins than between the surrounding lattice and the spin system (Berendsen, 1975).

3. *Diffusion Constant*

The self-diffusion coefficient is a measure of the interchange of identical molecules by way of thermal movements. The diffusion constant (D) of water can be evaluated from the spin-echo decay of the NMR signals by applying a known magnetic field gradient. Several authors have discussed in detail the NMR methods of calculating the self-diffusion constant of water in biological systems (Andrasko, 1976; Packer, 1973; Chang *et al.*, 1972; Hazlewood *et al.*, 1974b; Cleveland *et al.*, 1976). Theoretically, the contribution of diffusion to the spin-echo decay is given by the expression:

$$A(\tau, G) = \exp\left[-2/3\gamma^2 G^2 D_s \tau^3\right], \tag{9}$$

where $A(\tau, G)$ is the echo amplitude for a 90°–180° pulse separation τ in the presence of an applied field gradient G, γ is the gyromagnetic ratio (Cleveland *et al.*, 1976).

The interest in the theory and measurements of the diffusion constant of water in muscle and cell water has centred mainly on the following two objectives: (i) to study the extent of ordering of water molecules by comparing the self-diffusion constant of water (H_2O, ^2H_2O) in various biological samples and in free water; (ii) to investigate any possible effects of diffusion of water molecules through the internal field gradients (generated by the heterogeneity of the magnetic susceptibility in biological samples) on the relaxation processes of water nuclei (Packer, 1973; Hazlewood *et al.*, 1971; Chang *et al.*, 1972). Diffusion of water in the hydration layer is an activation process in which a molecule must attain sufficient energy to cross over a potential barrier; the values of (D) depend on molecular interactions in the system under consideration.

4. *A Comparison of Different Water Nuclei*

Certain characteristic nuclear properties of proton, deuteron, tritium and ^{17}O are given in Table 1 (Emsley *et al.*, 1965, p. 589). The relaxation of protons is influenced by inter- and intramolecular dipolar interactions; whereas, the relaxation behaviour of deuterium and ^{17}O is dominated by the quadrupolar interactions. Glasel (1967) has reported the following equations for relaxation rates of ^1H, ^2H and ^{17}O.

TABLE 1. NUCLEAR PROPERTIES OF ^1H, ^2H, ^3H, ^{17}O (EMSLEY *et al.*, 1965)

Isotope	NMR frequency in Mc/sec for a 10 kG field	Natural abundance (%)	Relative sensitivity for equal number of nuclei: At constant field	At constant frequency	Magnetic moment μ, in multiples of the nuclear magneton ($eh/4\pi$ Mc)	Spin I, in multiples of $h/2\pi$	Electric quadrupole moment Q, in multiples of $e \times 10^{-24}$ cm^2
^1H	42.577	99.9844	1.000	1.000	2.7927	1/2	—
^2H	6.536	1.56×10^{-2}	9.64×10^{-3}	0.409	0.85738	1	2.77×10^{-3}
^3H	45.414	—	1.21	1.07	2.9788	1/2	—
^{17}O	5.772	3.7×10^{-2}	2.91×10^{-2}	1.58	−1.8930	5/2	-4×10^{-3}

Protons:

$$(1/T_1)_{D,\text{intra}} = \frac{3\gamma^4\hbar^2}{2r^6}\tau_c$$

$$(1/T_1)_{D,\text{inter}} = \frac{\pi N\gamma^4\hbar^2}{5aD} \tag{10}$$

Deuterons:

$$(1/T_1)_Q = \frac{3}{8}\left(\frac{e^2qQ}{\hbar}\right)^2_{^2H}\tau_c \tag{11}$$

^{17}O:

$$(1/T_1)_Q = \frac{3}{125}\left(\frac{e^2qQ}{\hbar}\right)^2_{^{17}O}\tau_c, \tag{12}$$

where τ_c is correlation time; a = radius of a molecule; D = microscope diffusion coefficient; and N = number density of molecules.

In water, deuterons relax about 10-times faster than protons, and ^{17}O nuclei relax about 100-times faster than deuterons. Such fast relaxation rates of ^{17}O satisfy the slow-exchange condition in the studies of cell water. Whereas, in general the relaxation behaviour of protons and deuterons is governed by the fast-exchange condition. The spin-lattice relaxation time T_1 for deuterons in ^2H_2O is governed completely by rotational time of the individual ^2H_2O molecules; as a result the ^2H NMR easily detects the anisotropic motion of water. The dipolar interactions of water protons are sensitive to rotational and translational motion, proton exchange, and the presence of paramagnetic centres in a given sample. The quadrupolar interactions are higher in magnitude than the magnetic dipolar interactions; therefore, for small changes in the nuclear environment the deuteron relaxation times exhibit much greater sensitivity than the proton relaxation times. However, the "NMR" sensitivity for the detection of deuteron resonance signal is much lower than for the proton signal (see Table 1). The NMR studies of tritiated water in biological systems should be favoured by high sensitivity to detect tritium resonance (Table 1); eventually such measurements face a great drawback because low quantities of ^3H are required to be present in samples due to its radioactivity. The NMR spectroscopy of tritium has been developed and applied successfully during the past few years mainly by the research group of Professor Elvidge (Bloxsidge *et al.*, 1971; Al-Rawi *et al.*, 1974; Al-Rawi *et al.*, 1975).

IV. THE NMR STUDIES OF WATER IN DIFFERENT BIOLOGICAL SYSTEMS

A large variety of biological systems whose hydration properties have been investigated by NMR spectroscopy may be classified broadly into two groups on the basis of the NMR spectra of water.

1. Randomly oriented systems such as macromolecular solutions, cell suspensions, muscle, tissues and membranes give rise to water proton and deuteron resonance signals consisting of a single peak even at low temperatures in frozen samples.
2. Oriented samples: the water spectra from oriented fibres of DNA and collagen appear either as a single broad resonance line or a signal split into two lines. The characteristic feature of these spectra, which distinguishes them from the group (1) spectra, is that the linewidth or the splitting of proton and deuteron resonance signals are a function of the orientation of samples with respect to the magnetic field.

1. *Randomly Oriented Systems*

(a) *Biopolymer solutions and hydrated proteins*

The initial efforts to study water by NMR had shown that the water signal from DNA solutions was much broader than the signal from pure water. In order to explain these results, Jacobsen *et al.* (1954) proposed that line-broadening was due to increased ordering in the water structure and the formation of hydration shells near DNA. This early concept of hydration water in terms of a static model describing shells of water molecules near macromolecules proved inadequate. The current theories and the proposed models for water in macromolecular solutions or in different biological systems consider hydration water in terms of dynamic processes. Several examples of dynamic models of water in hydrated biological systems are discussed in different sections of this article. Lubas and Wilczok (1966, 1967, 1971) studied the hydration of DNA in solutions by measuring the relaxation times, using the spin-echo technique. They also interpreted the results in terms of firmly bound hydration shells (non-rotational binding), and rotationally bound water (freedom of movement as in free water). Lower ionic strength was found to favour an increase in the hydration of DNA (Lubas and Wilczok, 1970).

Further studies revealed that the relaxation behaviour of water was characterized by the conformation and structure of different biopolymers in solution. Glasel (1970) investigated the role of water in conformational changes of several biological macromolecules by studying the deuteron magnetic relaxation of water at 31°C. The following polymers were studied: poly(L-glutamic acid), poly(L-lysine), poly(adenylic acid), poly(uridylic acid), poly(methacrylic acid), poly(vinyloxazolidinone methyl), and poly(vinylpyrrolidone). He gave the following equation for the observed relaxation rate under the fast-exchange condition:

$$(1/T_1)_{\text{obs}} = (1/T_1)_{\text{free}} + C\omega K\tau_a, \tag{13}$$

where: $(1/T_1)_{\text{obs}}$ is the measured relaxation rate for solutions; $(1/T_1)_{\text{free}}$ is the relaxation rate for pure solvent; C is the concentration of the polymer; ω is the time-independent weight of water associated per gram of polymer; K is the quadrupole coupling term; and τ_a is the rotational reorientation time of water molecules associated with the polymer. Straight line plots of $(1/T_1)_{\text{obs}} - (1/T_1)_{\text{free}}$ vs C were observed.

In this work, the importance of polymer–water interactions by hydrogen bonding was pointed out; for example, poly(U) did not show any interaction, whereas strong interaction was recorded for poly(A) and poly(L-glutamic acid). It was observed that strong water interactions were favoured by the stable topology of polymers.

The magnetic field dependence of the relaxation rates of protons, deuterons, and ^{17}O nuclei of water (termed as relaxation dispersion) was investigated in detail for a variety of proteins in liquid solutions (Hallenga and Koenig, 1976; Koenig and Schillinger, 1969; Koenig *et al.*, 1975: Koenig *et al.*, 1978; Grösch and Noack, 1976). Koenig *et al.* (1975) showed that for lysozyme and haemocyanin solutions, the relaxation dispersion for ^1H, ^2H and ^{17}O nuclei of water were essentially the same. In general, these authors observed that the plots of $1/T_1$ vs H_0 for ^1H, ^2H and ^{17}O show an inflexion at a value of H_0 that corresponds to the Larmor frequency ν_c given by the relation $\nu_c = \sqrt{3}/(2\pi\tau_R)$; where τ_R is the rotational relaxation time of the protein molecule. It was proposed that the correlation time for orientation of water molecules in the neighbourhood of

protein macromolecules was determined by the Brownian motion (tumbling rate) of macromolecules. A hydrodynamic mechanism describing a small transfer of the protein τ_c to the solvent molecules was considered to be the common cause for the observed dispersion in T_1 for all three nuclei. By studying the dispersion behaviour of protein solutions in partially deuterated water, Koenig *et al.* (1978) were led to conclude that cross-relaxation between solvent and solute protons makes an important contribution to the proton relaxation rates, but not to the deuteron relaxation rates of water. The rate of cross-relaxation between protein and solvent protons shows a similar dispersion behaviour as the T_1 relaxation rates. Proton transfer between water protons and the titrable groups on the protein surface was suggested as a possible mechanism for the cross-relaxation, and the cross-relaxation term was shown to be proportional to the protein–solvent interface. A detailed description of the cross-relaxation effects within proteins is given by Kalk and Berendsen (1976), Sykes *et al.* (1978). The exchange of spin-magnetization was considered to occur at a rate faster than the rate of spin-lattice relaxation of protons.

Finally, Grösch and Noack (1976) interpreted the frequency-dependent changes of the proton relaxation rates (T_1, T_2) of BSA solutions in terms of a three-state model for water in protein solutions.

The original work of Kuntz *et al.* (1969) showed that when solutions of proteins or nucleic acids were frozen, a relatively sharp and distinct signal was observed from hydration water at temperatures as low as -35°C. The area under this signal gave a measure of the unfrozen water. The activation energy values of the processes influencing the relaxation rates were calculated from the temperature-dependent changes in the linewidth of water signal. These interesting observations reported by Kuntz *et al.* were immediately elaborated and applied by several groups of workers for studying extensively the hydration water characteristics in different biological systems. Using the same procedure, Kuntz (1971a,b) investigated the hydration of several polypeptides. He demonstrated that the linewidth of water signals observed in frozen solutions was very sensitive to the polypeptide conformation. All those systems known to be in random coils at room temperature exhibited sharper lines than the corresponding systems in the helical conformation. Kuntz indicated that the hydration of globular proteins could be estimated from the hydration of appropriate polypeptides, but the linewidth of water signal could not be calculated from the amino acid composition. The results of Mathur-De Vré *et al.* (1975) have shown clearly that the nature and structure of polynucleotides: poly(A), poly(U), poly(C) and their complexes: poly(A + U), poly(A + 2U) and poly(AH^+) exert a marked influence on the linewidth of water proton signals in frozen solutions $(\Delta 1/2)_{-5°}$. The formation of double-stranded helical complexes from single-stranded polynucleotides is accompanied by a large increase in the $(\Delta 1/2)_{-5°}$ values. This observation was explained by considering that proton transfer in the hydration layer of polynucleotides decreases due to the formation of inter-chain links in the complexes. The increase in the rigidity of the macromolecular structure accompanying the formation of complexes is expected to influence the water spectra when measurements are performed in the liquid state. The NMR studies of frozen macromolecular solutions show clearly that the diffusion motion, proton transfer, and macromolecular–water interactions are important factors influencing the relaxation behaviour of hydration water protons.

The wide-line proton magnetic resonance spectra of ribonuclease and BSA were studied in the hydrated and vacuum dried states over the temperature range of -140 to -180°C (Blears and Danyluk, 1968). Considerable translational and rotational motion of water was shown to persist at such low temperatures by comparing the second moment of ice with that of water in proteins. The hydration of ribonuclease and total ribosomal RNA, as studied from the water spectra at -35°C, was found to increase steadily as a 70S particle was successively broken into smaller and more expanded fragments (White *et al.*, 1972). Fuller and Brey (1968) reported the NMR spectra of water sorbed on serum albumin as a function of temperature and water content. They explained that sorbed water could exist in different states depending on the water

content: water up to 75 mg/g protein represented water strongly bonded to polar groups of the protein, the "primary water". The NMR signal from the primary layer was strongly influenced by the protein–water interactions, further addition resulted in strongly hydrogen bonded water to the primary layer which is much less influenced by the protein; these two states may be compared with regions A and B in Fig. 1. The amount of water strongly bound to a solid protein is less than that for protein in solution. The water deuteron relaxation studies of the protein elastin (ligamentum nuchae of mature beef) soaked in excess ^2H_2O showed that the deuteron relaxation exhibited a multi-component behaviour, thereby suggesting the existence of distinct regions of water in the sample. These are: water contained within the bulk elastin and water surrounding the bulk elastin. The hydration of elastin is particularly important in rendering it rubber-like properties (Ellis and Packer, 1976). Hilton and Bryant (1977) studied the relaxation times of hydrated lysozyme powder as a function of temperature and water content. A model based on cross-relaxation was found to account satisfactorily the behaviour of proton relaxation rates.

(b) *Effects of γ-irradiation on hydration water*

When biological systems are subjected to γ-irradiation, the induced radiation damage is known to localize on the DNA molecule (Blok and Loman, 1973; Latarjet, 1972). For a full understanding of the radiation effects on DNA in an aqueous medium and the role of water at molecular level in mediating the overall damage, it is essential to study the effects of irradiation on the characteristics of hydration water, e.g. on macromolecular–water interactions, on water mobility and proton transfer along the hydration layer. A detailed investigation of the effects of γ-irradiation on the hydration water of DNA and polynucleotides in H_2O, ^2H_2O, $X\%H_2O/Y\%^2H_2O$ solvents was undertaken by Mathur-De Vré *et al.* (1976), Mathur-De Vré and Bertinchamps (1977a,b). The solutions were irradiated at 0, −80, and −196°C, and the linewidth of the water proton NMR signals was measured from −5 to −45°C. It is known that when aqueous solutions are subjected to γ-irradiation, free radicals are generated that are stable at low temperatures but decay rapidly at about 0°C. The solutions irradiated at −80 and −196°C were thawed at 0 to +5°C and refrozen before the NMR measurements. This process curtailed the possible line-broadening effects of paramagnetic free radicals trapped in the frozen irradiated solutions, on the linewidth of water signals observed from the irradiated solutions below −5°C.

It was shown by comparing linewidths at −5°C, that the irradiation of DNA solutions at 0 and −80°C resulted in a decrease in linewidth of water proton signal as compared with that of the corresponding non-irradiated solution; furthermore, the observed decrease was greater when irradiation was performed at −80°C. No significant change in linewidth was recorded after irradiating the solutions at −196°C, by irradiating the dry solid (at −196°C) before dissolution, or by sonication of the DNA solutions. An interesting observation was that irradiation at 0°C of poly(A + U) and poly(A + 2U) complexes resulted in a large broadening, whereas much sharper signals were observed by irradiating the same solution at −80°C. Above 0°C, the bulk water is liquid and highly mobile, and the segments of macromolecular chains possess considerable flexibility. In frozen samples at −80°C, the hydration water remains unfrozen and mobile but the bulk water is frozen and the segmental mobility of the macromolecular chains is restricted. Consequently, when the solutions of macromolecules are irradiated at different temperatures, the process of either radiation-induced cross-linking or separation of the chains (causing the linewidth of water proton signals to increase or decrease) predominates depending on the dynamic states of the hydration and bulk water molecules, and on the flexibility of the segments of the macromolecular chains, favoured under the conditions of irradiation. It was suggested that the striking differences observed in the hydration water proton spectra after γ-irradiation were largely due to important changes in the proton transfer along the hydration layer resulting from the modifications induced in the structure of macromolecules.

TABLE 2. RELAXATION TIMES FOR A FEW SELECTED SYSTEMS

Sample	Temp (°C)	Frequency (MHz)	Nucleus	Relaxation times (msec) T_1	$T_{1\rho}$	T_2	Reference
Pure water	25	4	^1H	2300		2300	Cope (1969)
				2830		2830	James and Gillen (1972)
			^2H	470		450	Cope (1969)
			^{17}O		3.7	6.5	Swift and Barr (1973)
Ice			^1H			6 (μsec)	Foster *et al.* (1976)
Muscle	25	4	^2H	92		9	Cope (1969)
Brain				131		22	
Frog skeletal muscle	30		^{17}O			1.22	Swift and Barr (1973)
	10					1.18	
Frog gastrocnemius muscle	22.7		^1H	700	180	44	Finch and Homer (1974)
Plasma	25	25	^{17}O	3.9			Fabry and Eisenstadt (1975)
Red cell inertia				1.7			
Membrane bound water phase	30	23.3	^1H	91.9	22.2		Finch and Schneider (1975)
Random population of HeLa cells	25		^1H	667			Beall *et al.* (1976)
Cell pellets				1.0–1.10			Shporer and Civan (1975)
Cell suspension in the presence of Mn^{2+}	22–24	8.13	^{17}O	1.0–1.05			
Partially hydrated muscle fibres 1 g H_2O/g protein	25	51.6	^1H	200		42	Cooke and Wien (1971)
Normal cells Oxy	22	44.4	^1H	560		88	Zipp *et al.* (1976)
Deoxy				550		84	
Sickle cells Oxy				530		56	
Deoxy				550		26	
Liver Normal	26	100	^1H	570			Damadian *et al.* (1974)
Tumorous				832			
Lung Normal				788			
Tumorous				1110			
Breast Normal				367			
Tumorous				1080			
Healthy C3H mice Liver		30	^1H	386	47	25	Frey *et al.* (1972)
Lung				641	117	47	
Mice with large MC-1 Tumour ($8–37\ cm^2$) Liver				461	56	33	
Lung				665	124	49	
Tumour				853	208	47	

The NMR studies of water in frozen samples proved to be a very informative method in revealing that hydration water molecules make a distinct contribution in mediating the overall radiation-induced damage to the structure of DNA and polynucleotides in solution.

(c) *Muscles and tissues*

Considerable evidence has accumulated leading to the general conclusion that water in muscles and tissues exists in more than one fraction, and that fast exchange of water molecules occurs between different regions. One of the fractions has been assigned to a well-defined and ordered water phase. In addition, it was affirmed that the motion of all the water molecules cannot be described satisfactorily by a single correlation time.

In general, the relaxation times and the diffusion constant for muscle water were found to be reduced with respect to the values for pure water. The values for a few selected systems are included in Table 2.

Bratton *et al.* (1965) postulated a two-state model for water in muscle. Hazlewood *et al.* (1969) concluded that at least two ordered phases of water (major and minor) exist in muscle; water molecules in the major phase were considered to exhibit greater motional freedom than in the minor phase and exchange rapidly with free water. Cooke and Wien (1971) fitted their data of T_1 and T_2 measurements (33°C) on live muscle fibres with a model in which 4–5% of the total water was associated with proteins and represented the fraction with fast relaxation rate, while the bulk of the water inside a muscle was considered to be free. It was suggested that fast exchange of water protons could occur between these two phases. Three-state models for water in muscle were proposed by Hazlewood *et al.* (1974b) and by Belton *et al.* (1972).

Deuteron magnetic resonance studies of Cope (1969) also indicated the existence of two distinct fractions of tissue water in muscle and brain of adult rats. He suggested that each fraction may be composed of multiple subfractions. The ^{17}O spectra of $H_2{}^{17}O$ in frog skeletal muscle has further provided evidence in favour of the restricted motion in muscle water as compared with pure water (Swift and Barr, 1973). In both these studies, the reported T_2 and T_1 values were found to be much smaller than the values for pure water ($T_1 = T_2$), see Table 2.

In an attempt to describe the relaxation behaviour of water in muscles, tissues and cells, most authors explained the observed shortening of relaxation times by considering the existence of an ordered phase of water. In this phase, the motional freedom of individual water molecules is restricted by their interactions with cellular macromolecules reducing the relaxation time (Hazlewood *et al.*, 1969; Hazlewood *et al.*, 1971; Hazlewood *et al.*, 1974b). Hansen and Lawson (1970) and Hansen (1971) pointed out that the line-broadening was induced, at least partially, by diffusion of water molecules through microscopic magnetic field inhomogeneities present in the heterogeneous samples. Cooke and Wien (1971) measured T_1 and T_2 for solutions of F-actin and G-actin. These authors reported a decrease in the T_2 values when actin solutions were polymerized, and they emphasized that diffusion through increased magnetic field heterogeneity contributes significantly to the relaxation behaviour of water. Chang *et al.* (1972) have contested the discussion of Hansen and Lawson; Chang *et al.* (1972) argued that unusually large values of the local field inhomogeneity must be assumed in order that the proposed mechanism be effective. On the basis of the detailed calculations, Packer (1973) concluded that the effects of diffusion of water through inhomogeneous internal field gradients in striated muscle were negligible. He pointed out analogies between the effects of restricted diffusion, the motional narrowing of resonance lines, and diffusion through periodically heterogeneous and structured systems. Yet another mechanism describing the water relaxation rates was proposed based on the effects of cross-relaxation between the water protons and macromolecular protons of muscle (Edzes and Samulski, 1978).

Ratković and Sinadinović (1977) investigated the relaxation times for water protons in tissues of the thyroid glands of rats. They obtained evidence indicating that the relaxation times decrease (as compared with free water) by long-range interactions of water with macromolecules and the effects of compartmentalization, rather than due to diffusion of water through the microscopic magnetic field inhomogeneity inside the sample. It may be important to differentiate between the physical compartmentalization of water in macroscopic regions of complex biological samples and the distinguishable fractions of water structured at the molecular level. Both these phenomena may produce similar effects on the relaxation rates of water nuclei.

Hazlewood *et al.* (1974b) demonstrated, by decomposing the spin-echo decay curves at 24°C, that at least three distinguishable fractions of water protons were required to fit the data for skeletal muscle: water associated with macromolecules represents approximately 8% of the total tissue water and it does not exchange rapidly with

the remaining intracellular water (T_2 less than 5 msec), myoplasm fraction 82% ($T_2 = 45$ msec), extracellular space ~10% ($T_2 = 196$ msec), T_2 of pure water or Ringer's solution = 1.6 sec. These authors showed that water in different fractions did not exchange rapidly and considered the possibility that each fraction may be composed of fast-exchanging sub-fractions. Hazlewood *et al.* (1974b), and Belton *et al.* (1972) attributed the fastest relaxing fraction to the closely bound water: three fractions detected by Belton *et al.* correspond to T_2 values of 250, 40 and 9 msec, respectively. This interpretation has since been contested by Foster *et al.* (1976) and Fung (1977b); both these groups of workers have postulated that the fastest relaxing fraction (4–9 msec) of protons arises from the nonrigid protons of macromolecules rather than from "bound" water.

The relaxation studies performed at a single frequency furnished ample evidence to show that water in muscles and tissues is not homogeneous but exists in more than one fraction. Further new and revealing details about the dynamic states of water, e.g. distribution of correlation times and dispersion of proton relaxation rates, were brought into evidence by careful measurements of the relaxation times over a wide range of frequency and at varied temperatures. Outhred and George (1973a,b) described a method for analysing the frequency-dependent behaviour of relaxation rates. They analysed the distribution of correlation times for toad muscle water from measurements at three frequencies: 2.3, 8.9 and 30 MHz (1973b). A very clear and detailed treatment of the dispersion of water proton spin–lattice relaxation times T_1 and $T_{1\rho}$ at 25°C for selected mouse tissues was given by Knispel *et al.* (1974). The dispersion (frequency dependence of relaxation times) of $T_{1\rho}$ was attributed to proton exchange between water molecules, whereas the major contribution to T_1 came from processes such as molecular rotational and translational diffusion. The correlation times for exchange, molecular rotation and fast diffusion processes were given as 7×10^{-6}, 2×10^{-8} and $\sim 10^{-10}$ sec. Figure 3 shows the dispersion of the total relaxation for muscle water. In a subsequent paper, Diegel and Pintar (1975b) recognized the exchange process as arising from the slow-exchange diffusion of water molecules between the hydration layer and free water and not from the exchange of protons as discussed earlier (Knispel *et al.*, 1974; Thompson *et al.*, 1973). Figure 4 illustrates different relaxation processes discussed by Diegel and Pintar (1975b). One may compare the dynamic states of H_2O molecules participating in the slow-exchange diffusion and undergoing slow reorientation as defined by Diegel and Pintar, with the water molecules designated by the lifetime τ'_e and τ'_r, respectively, in Packer's diagram (see Fig. 2).

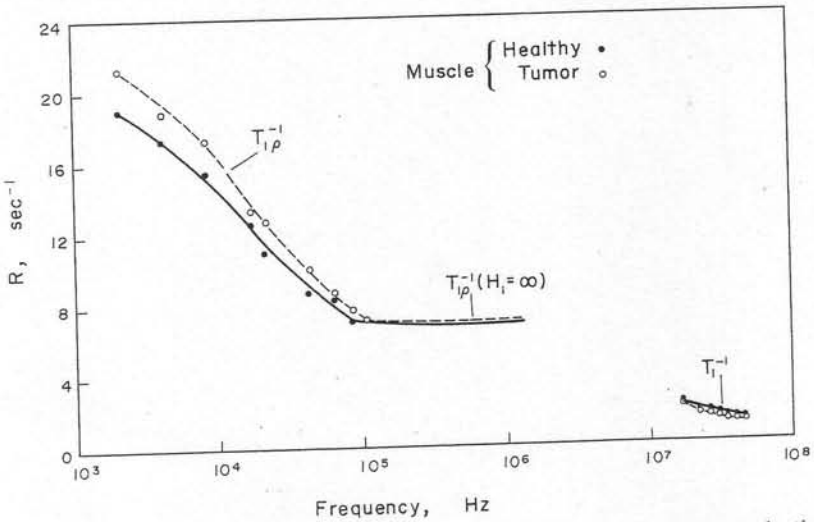

FIG. 3. (Knispel *et al.*, 1974) Dispersion of T_1 and $T_{1\rho}$ in samples of mouse muscle tissue. R = relaxation rate.

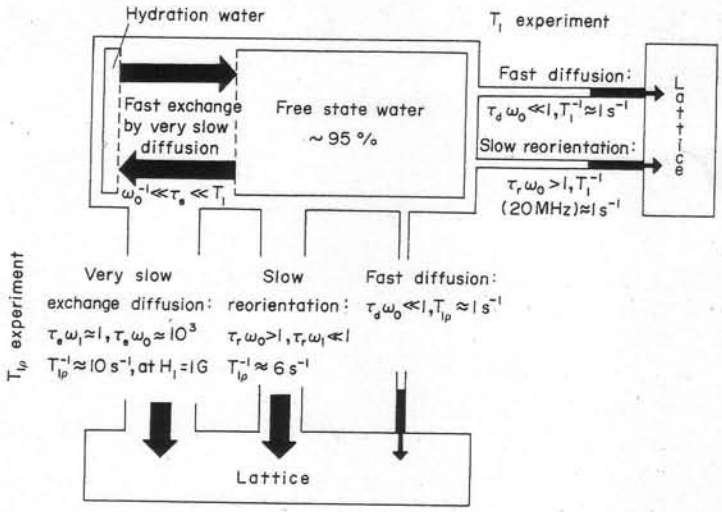

FIG. 4. (Diegel and Pintar, 1975b) The three relaxation processes and their contribution to the high field and the rotating field Zeeman relaxation. The numerical values shown for the relaxation rates are approximative.

Finch and Homer (1974) reported the values of T_1, T_2 and $T_{1\rho}$ for frog muscle water protons at different temperatures above 0°C and over a wide range of frequency. They obtained a distribution of correlation times for muscle water, ranging from $\sim 10^{-5}$ to $\sim 10^{-11}$ sec. The results were interpreted in terms of exchange of water molecules between two fractions: one with a distribution of different degrees of restricted motion, and the other with unrestricted motion like ordinary water (97%). Fung (1977a) measured T_1 of water protons in mouse muscle in the frequency range from 10^4 to 10^8 Hz, and the deuteron T_1 from 2.0×10^3 to 1.54×10^7 Hz. He proposed relaxation mechanisms for hydration water protons and deuterons based on the observed frequency and temperature-dependent behaviour of relaxation times above 0°C, and the isotope substitution effects (discussed in a subsequent section).

The NMR studies of muscle water performed on frozen samples have revealed that the unfrozen fraction of water is characterized by the frequency-dependent variations of relaxation times. From the pulsed NMR measurements of the transverse relaxation times of water protons in striated frog muscle, Belton *et al.* (1972) showed that the bound water did not freeze; as a result, below -7 to -10°C about 20% of the signal was observed. In another paper, Belton *et al.* (1973) investigated in detail the spin-lattice relaxation times and the dynamics of the unfrozen fraction of water in muscle at two frequencies (30 and 60 MHz) and over a wide range of temperature ($+10$ to -75°C). A distribution of correlation times was indicated for the unfrozen water. Fung and McGaughy (1974) measured the relaxation times of water in rat gastrocnemius muscle at frequencies ranging from 4.5 to 60 MHz at $+37$ to -70°C. The T_1 values of H_2O and ^2H_2O for muscle and liver were also reported at different frequencies and in the temperature range $+37$ to -70°C (Fung *et al.*, 1975a). In samples at sub-freezing temperatures, the unfrozen fraction of water was shown to exhibit a distribution of correlation times; while, above -8°C a single correlation time was observed which was short enough to render T_1 independent of frequency. Based on these results, Fung and McGaughy (1974) and Fung *et al.* (1975a) supported a two-phase model: one phase exhibiting a distribution of correlation times (the unfrozen fraction) and the other with a single correlation time (the frozen fraction). It may be noted that a similar model was also proposed by Finch and Homer (1974) from the results obtained above 0°C (discussed in the previous section). Of the various models suggested to describe the state of water in muscle (and other biological systems), this model accounts in the most satisfactory manner the behaviour of water. Contrary to Cope (1969), Fung *et al.* concluded that all the ^2H_2O was "NMR" visible. Duff and Derbyshire (1974) also reported a complex

behaviour of relaxation times (T_1, T_2, $T_{1\rho}$) of the bound or unfrozen fraction of water in frozen porcine muscle.

Several authors have demonstrated that the water content of muscles and tissues exerts a determining role on the relaxation behaviour of water nuclei observed in a variety of samples. Cooke and Wien (1971) measured T_1 and T_2 of partially hydrated various muscle proteins by the pulsed spin-echo technique. The relaxation times decreased as the ratio of water to protein decreased, and $1/T_1$ was found to be directly proportional to the protein concentration. Fung (1977c) measured T_1 of water protons for dehydrated mouse muscle at three frequencies (5, 30, 100 MHz) down to very low water contents. At all three frequencies, a decrease in T_1 values with decreasing water content (X) was observed, followed by an increase in T_1 at very low contents ($X \leqq 0.07$). This phenomenon may have arisen because of a change in the structure of hydration layer at low water contents.

Belton and Packer (1974) undertook a detailed study of the effects of water content on the water proton relaxation times T_1 and T_2. The stepwise dehydration of a muscle was found to correlate with changes in the transverse relaxation times in a manner shown in Fig. 5. Dehydration of the muscle followed by rehydration was also investigated. A very important contribution of this experiment was to show that the amount of "unfrozen" water for fresh and rehydrated muscles is the same, but the relaxation behaviour at low temperatures is quite different in these two cases. The fact that the amount of unfrozen water is similar in the two cases reflects that it depends on the concentration rather than on the state of macromolecules in muscle. However, the difference in the relaxation behaviour arises from marked changes in the distribution of water in different fractions.

An interesting study making use of the correlation between the water content and relaxation times was conducted to investigate the action of cholera toxin (Udall *et al.*, 1975). These authors measured the water content and T_1 and T_2 values from the control and cholera-infected small intestinal tissues of rats. It was found that the relaxation times of water in cholera-infected tissues were longer and the tissue hydration was greater than in control tissue samples:

	Control	Cholera
Percentage of tissue water	79.49%	84.52%
T_1 (msec)	521.22	667.96
	±69.5	±119.25
T_2 (msec)	62.34	80.35
	±9.59	±21.46

The abovementioned results suggest that cholera-treated tissues exhibit greater motional freedom of water than the control samples. This observation was considered to support the general view that cholera enterotoxin acts by influencing the intracellular protein–water interactions, giving rise to increased hydration. As a result, the permeability of cells to water is increased, leading to enhanced secretory activity of small intestines.

The importance of the relationship between water content of muscles and different tissues, and the relaxation times for explaining the increase in T_1 when tumours develop will be discussed in a subsequent section.

A very important contribution of the NMR studies of water in tissues and muscles has been to reveal that the relaxation rates of water nuclei can be correlated with the actual state of muscle caused by strain and death. Bratton *et al.* (1965) reported that T_2 of muscle water protons increased with contraction and exhaustion, whereas T_1 remained insensitive to changes in the state. They explained that T_2 increased because the change in tension released 20% of water; part of the bound water was released reversibly during isometric contraction and irreversibly in death. Chang *et al.* (1976) studied the relaxation time of water protons in skeletal muscle (gastrocnemius) at different time intervals after taking the sample from the animal. They obtained two

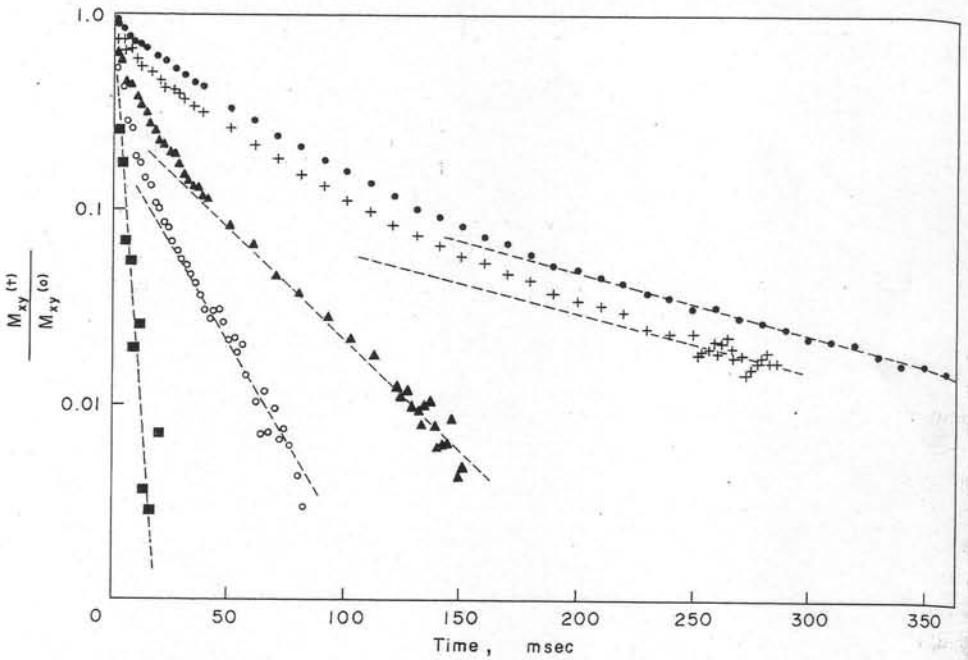

FIG. 5. (Belton and Packer, 1974) The variation of the transverse relaxation behaviour of the water protons in frog gastrocnemius muscle as a function of water content at ~300 K. The measurements were made using a Carr–Purcell/Gill–Meiboon pulse sequence and the symbols correspond to different water contents which, relative to that of fresh muscle taken as 100%, are: ● 100%, + 54%, △ 18%, ○ 10%, ■ 6%. Not all the measured points are shown for clarity.

relaxation times: T_{1B} and T_{1A}. T_{1B} (characterized by the slow relaxation rate) was influenced by the early post-mortem changes, and its value increased with time after the removal of tissues from the muscle. T_{1A} (representing the weighted average of all water protons) remained practically unchanged with the lapse of time. These authors stated that cellular water molecules "recognize" a change of environment as the physiological state of cells undergoes a change. Furthermore, post-mortem changes were observed to be relatively slow, taking about 4 hr for completion. Hazlewood *et al.* (1971) classified T_1 and T_2 of water (28°C) as a function of age. The muscles from animals less than 10 days old were defined as immature muscles, and those from animals greater than 40 days old were considered as mature muscles. The following relaxation times were reported:

	T_1 (sec)	T_2 (sec)
Immature muscle	1.206 ± 0.055	0.127 ± 0.009
Mature muscle	0.723 ± 0.049	0.047 ± 0.004

It was proposed that the fraction of ordered water increased in the post-natal development of muscle. They also suggested that the extent of ordering of muscle water tends to increase with maturation of muscles.

By plotting the amplitude of the spin-echo train of water protons of mouse muscle (at 37°C) as a function of time, Fung (1977b) observed that the decay curve was exponential soon after the dissection, but with time it changed into a non-exponential curve during the first 40 min as illustrated in Fig. 6. On the contrary, for brain tissues very little change in the spin-echo decay curves at 37°C was observed during the first hour after death. He concluded that changes in the relaxation times of hydration water protons observed after death were caused by changes in the conformation of muscle proteins, rather than by a rapid redistribution of water in different parts of the tissue. Shporer *et al.* (1976) showed that the relaxation of ^{17}O from $H_2{}^{17}O$ in rat lymphocytes was non-exponential in the fresh state but became exponential after cell death. Contrary to the opinion of Fung, Shporer *et al.* suggested that necrosis could

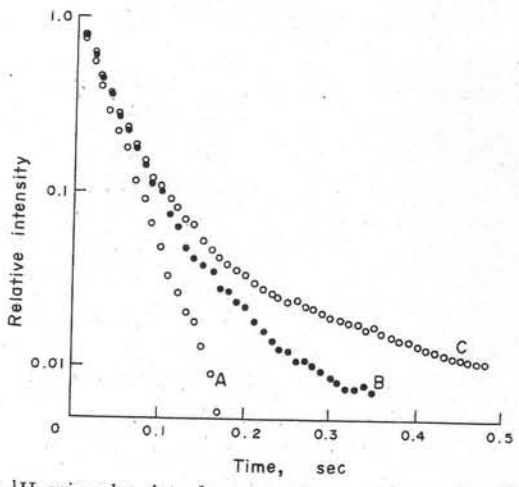

FIG. 6. (Fung, 1977b) ^1H spin-echo data for mouse water at 37°C. The initial intensities are not included because the contribution from organic protons is not negligible. (A) 5 min, (B) 10 min, (C) 30 min.

lead to mixing of water in different compartments of water in tissues, or between nuclear and cytoplasmic water.

The magnetic moment of deuteron is much smaller than the magnetic moment of protons (Table 1), this causes reduced dipolar coupling interactions between protons and deuterons as compared with proton and proton. Therefore, it is expected that partial substitution of H_2O by ^2H_2O in a system should result in sharper water proton signals. Such a behaviour is observed in free water. For water in striated muscle of frog; Civan and Shporer (1975) reported that T_1 of water protons was unaffected after partial substitution of H_2O by ^2H_2O. Resing *et al.* (1977) and Fung (1977a) also reported that deuterium substitution had very little effect on the relaxation times of muscle water. Fung explained this isotope substitution effect by considering that the major relaxation mechanism is the intermolecular dipole–dipole coupling interactions between water protons in the hydration layer and protons in the relatively immobile macromolecules, assisted by the slow water diffusion of the type defined by Knispel *et al.* (1974), and Diegel and Pintar (1975b). The importance of cross-relaxation in explaining the relaxation behaviour of water protons in protein solutions and in collagen has been discussed elsewhere (see Sections IV.1(a) and IV.2(a). In addition, it was observed that for DNA and polynucleotide solutions in $H_2O/^2H_2O$ solvents of various compositions, unlike at +5°C, in each case the linewidth at −5°C was independent of the $H_2O/^2H_2O$ composition but dependent on the nature of macromolecules. Furthermore, for DNA solutions between −5 and −35°C, the temperature-dependence of the linewidth of water proton signal decreased with increasing ^2H_2O content (Mathur-De Vré and Bertinchamps, 1977a,b). These results could be better explained by taking into account the influence of cross-relaxation and indicate that dipolar interactions between macromolecular protons and water protons dominate the T_2 process of unfrozen water. Furthermore, Civan and Shporer (1975), and Civan *et al.* (1978) have reported the following important results from a comparative study of the three nuclei (^1H, ^2H, ^{17}O) in muscle water. (i) The T_1 relaxation rates of ^{17}O, ^2H and ^1H nuclei of muscle water exhibit identical frequency dependence. (ii) The ratio $(T_1)_{^2H}/(T_1)_{^{17}O}$ of muscle water and pure water are closely similar while the ratio $(T_1)_{^1H}/(T_1)_{^{17}O}$ in pure water is 2.1-times greater than for muscle water. (iii) The ratio $(T_1)/(T_2)$ for ^2H and ^1H was found to be in the range of 9–11, whereas this ratio for ^{17}O was approximately 1.5–2.0. Rapid exchange motion between a small immobilized fraction and a large fraction of free water was proposed.

(d) *Membranes and cell water*

The erythrocyte membrane is highly permeable to water, this results in a fast exchange between water in cells and plasma (exchange time of the order of 10 msec).

The NMR technique is particularly fitting for studying such fast-exchange processes. Water molecules present in these two compartments are constrained in widely different environments. Consequently, each type of water exhibits a characteristic and distinct relaxation behaviour. The use of Mn^{2+} in these systems has proved to be of particular value for the following two reasons: (i) in the presence of Mn^{2+}, the relaxation rates of water nuclei can be enhanced by the paramagnetic contribution to such an extent that their NMR signals become unobservable; (ii) the cell membrane is known to be effectively impermeable to Mn^{2+}. Consequently, the addition of Mn^{2+} to a cell suspension results in an enhancement of the relaxation rates of water nuclei in the extracellular region. Therefore, it is possible to selectively resolve the contribution of the intracellular water to the observed spectra. A similar effect can equally well be obtained by freezing samples below 0°C; in this case, only the intracellular water remains unfrozen and therefore contributes to the resulting spectra. It is surprising to note that applications of the freezing technique in this domain is very limited. The only example is that of water in haemoglobin solutions reported by Zipp *et al.* (1976). Certain groups of workers have used the pellets of cells to eliminate the contribution of extracellular water.

Andrasko (1976) measured the water diffusion permeability of human erythrocytes by using pulsed magnetic field gradient techniques. The following values were reported for the diffusion constant (D_1) and the lifetime (τ_B) of water within red blood cells.

	D_1 (cm^2/sec)	τ_B (sec)
blood	1.16×10^{-5}	0.017
blood + *p-Cl. $HgBzO^-$	7.75×10^{-6}	0.048

*p-Chloromercuribenzoate: known to drastically reduce the osmotic water permeability of human red cells.

It was shown that deoxygenation of normal and sickle erythrocyte results in a considerable decrease in the T_2 values but causes no change in the T_1 values of water (Cottam *et al.*, 1974; Thompson *et al.*, 1975; Zipp *et al.*, 1976). A three-state model was proposed to explain the relaxation data, each state exhibiting a characteristic correlation time: bulk water $\cong 10^{-11}$ sec, water hydrated to macromolecule ($10^{-7} > \tau_c > 10^{-11}$ sec), finally the third region of water which is tightly bound exhibits a correlation time similar to that of protein ($\tau_c \geqq 10^{-7}$ sec) (Thompson *et al.*, 1975; Zipp *et al.*, 1976). Zipp *et al.* reported that upon deoxygenation of sickle cells and haemoglobin S solutions the T_2 values at room temperature decreased by a factor of 2; whereas after deoxygenation of normal cells and haemoglobin A solutions, no change in T_2 was observed. The low temperature studies of linewidth at (−15 to −36°C), and T_1 at −20 to −80°C, for oxy- and deoxy-haemoglobin A and haemoglobin S solutions suggested that the characteristics of bound water were similar for all four species. On the basis of the three-state model, Zipp *et al.* proposed that the sickling process altered the irrotationally bound water. Lindstrom and Koenig (1974) and Lindstrom *et al.* (1976) investigated the effects of oxygenation, and aggregation of haemoglobin (HbA) and sickle haemoglobin (HbS) solutions by studying the frequency dependence of water proton relaxation rate ($1/T_1$) (dispersion curves). They calculated τ_c, the correlation times for the rotational motion, of haemoglobin molecule from the inflexion frequency ν_c (see Section IV.1(a), p. 112). It was shown that under the conditions of complete oxygenation, HbS molecules interact with each other more strongly than do the HbA molecules. The orientation time of oxy-HbS molecules was shown to be larger than that of HbA molecules. The T_1 values of water obtained at low frequency gave much more information about the state of aggregation and rotational motion of the haemoglobin macromolecule as compared with the T_1 values obtained at a single high frequency.

A simple NMR technique for measuring the water exchange between erythrocytes

and plasma labelled with Mn^{2+} was described by Conlon and Outhred (1972). The use of paramagnetic ions as a tool for distinguishing the proton NMR signal of intracellular water from that of extracellular water was initially described by Fritz and Swift (1967). These authors successfully applied this method to investigate the state of water in polarized and depolarized frog nerves. The nerves were depolarized by the chemical and electrical stimulation. It was apparent from the results that depolarization of nerves is accompanied by marked changes in the state of intracellular water.

Shporer *et al.* (1976) reported the relaxation behaviour (at 26.5°C) of ^{17}O from $H_2{}^{17}O$ in rat lymphocytes using samples in the form of packed cells (pellets) and supernatant In the fresh state, the non-exponential behaviour of the relaxation data of pellets reflected the presence of two (or more) distinct types of water. The slowly relaxing fraction of water was ascribed to nucleus ($T_1 = 5.1$ msec), and the more rapidly relaxing population to cytoplasm ($T_1 = 3.1$ msec). The T_1 of ^{17}O in supernatant was appreciably longer ($T_1 = 7.5$ msec). The results of temperature effects on the relaxation times led these authors to conclude that the exchange rate of water between these two phases is slower than the relaxation rate of ^{17}O (slow-exchange condition). Two different methods were used to study the NMR of ^{17}O in $H_2{}^{17}O$ enriched human erythrocytes (Shporer and Civan, 1975): (1) direct comparison of relaxation rates of ^{17}O in isolated pellets and supernatant, (2) relaxation rates measured in the presence of Mn^{2+}. It was noted that T_1 for intracellular water ws 4–5-times shorter than for the supernatant. The values of rate constants (k_x) at 25 and 37°C were found to be 60 and 107 sec^{-1}, and the activation energy for k_x as equal to 8.7 ± 1.0 kcal/mole. The authors emphasized the importance of the interaction between water and membrane during the transport of water. Fabry and Eisenstadt (1975) investigated the exchange of water between human red blood cells and the plasma phase by studying water proton NMR in the presence of Mn^{2+}, and by measuring ^{17}O relaxation times of $H_2{}^{17}O$ in the absence of added Mn^{2+} The half-life for cell water at 25°C was found to be 15 msec ± 2 msec; and the exchange time equal to 0.046 msec. The relaxation time values are reported in Table 2. The results were analysed in terms of the classical two-compartment exchange model.

Finch and Schneider (1975) measured T_1 and $T_{1\rho}$ for water protons from 0 to 30°C, and the ω_1 dependence of $T_{1\rho}$ for aqueous dispersion of red cell membrane (see Table 2). From the available data these authors could not specify whether the water detected by NMR was associated with erythrocyte membrane, lipids or polysaccharides, or all three. Nevertheless, they pointed out the importance of the membrane-bound water in defining the structure and functions of membranes. Simple lecithin systems have been used as models for the biological membranes. Klose and Stelzner (1974) reported on the NMR study of specific amounts of water in lecithin–benzene systems. They postulated that water–membrane interactions are limited to three regions: (1) the interaction of water with the phosphate groups, (2) water interacting by additional weak interactions, and (3) water molecules beyond both these regions.

With a view to studying the effects of different cellular materials on the state of water, James and Gillen (1972) measured T_1, T_2 and self-diffusion constant (D) values of water from the unfertilized chicken egg. Only one resonance signal was observed from the mobile and immobile water. The values of relaxation times and diffusion constant are given below:

	Egg yolk	Egg white	Egg albumin (10% solution sealed in vacuum)	Distilled H_2O
Diffusion constant with respect to pure water	0.25	0.80	0.88	
T_1 (msec)	67.0	1180	1270	2830
T_2 (msec)	27.0	—	340	2830

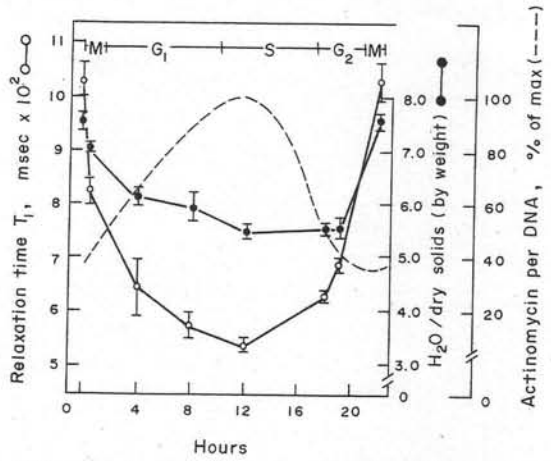

FIG. 7. (Beall *et al.*, 1976) T_1 and water content as a function of HeLa cell cycle. (○——○) T_1 during the cell cycle (mean of eight–ten experiments). Bars denote standard error of the mean. (●——●) Water content during the cell cycle. (----) Actinomycin-D binding ability of the chromatin. Data for the dashed line are from Pederson and Robbins.

Significant portions of cellular water are known to exhibit reduced mobility, which can be attributed to the obstruction or hydration effects. On the basis of T_1 measurements, these authors proposed that probably the hydration effects are more important. Fung *et al.* (1975a) also reported that the T_1 values for egg yolk were considerably shorter than for egg white.

Some authors have given evidence showing that the changes in T_1 of cell water are due to alterations in the configuration of macromolecules. T_1 in mammalian cells and tissues was measured as a function of the external ion concentration and total cell water content (Raaphorst *et al.*, 1975). The changes in the fraction of bound and unbound water were shown to be associated with changes induced in the macromolecular configuration by varying the salt concentration and the amount of water. Beall *et al.* (1976) investigated T_1 of water protons (at 25°C) and the total water content as a function of HeLa cell cycle (Fig. 7). They demonstrated the effects of biological and physiological alterations during the growth and division of cells on the T_1 of water. It was proposed that the relation between T_1 and the cyclic pattern of cell growth and division was influenced probably by the conformational changes of macromolecules (associated with the morphological changes during cell division), and by the water content.

(e) *Tumours and cancerous cells*

An important development in the NMR studies of water in biological systems has been to show that the measurements of T_1, T_2 and $T_{1\rho}$ of water in tissues and organs have a potential use in the cancer research. The relaxation times are closely related to the structure, mobility and the content of water in normal and cancerous tissues and cells. As a result, the effects of the presence and growth of tumours on the behaviour of water can be investigated by the NMR technique. Nevertheless, the eventual use of this method for the diagnostic purpose remains a highly controversial issue. The basic problem arises because, even though it is now fairly well established that there is a marked difference between the relaxation behaviour of water protons in the normal and cancerous states, there is no certitude that such a behaviour is strictly specific for the cancerous state.

In his pioneer paper, Damadian (1971) reported that the water proton relaxation times T_1 and T_2 for malignant and normal tissues were distinctly different. Water in malignant tissues exhibited longer relaxation times or a higher motional freedom. These observations were interpreted in terms of the postulate of Szent-Györgyi (1957): it states that the degree of organization of water in the cancerous tissues is much lower than in normal

tissues. The results reported by Damadian were soon confirmed by several groups of workers and initiated extensive research in this field.

Frey *et al.* (1972) showed that many nonmalignant tissues from spleen, kidney, liver, heart, muscle, intestines, stomach, skin and lung for mice with a tumour on hindleg had significantly longer relaxation times (T_1 and $T_{1\rho}$) as compared with the corresponding tissues from healthy mice (see Table 2). They did not observe any systematic variations in the T_2 values. It was proposed that water was more ordered in tissues with tumours. Inch *et al.* (1974) measured the water content and T_1 for neoplastic and non-neoplastic tissues from mice and humans. The T_1 values for tissues of liver, spleen and kidney from animals with large, rapidly growing tumours were longer than T_1 for similar tissues from healthy animals. Whereas, for slowly growing tumours this difference was negligible, i.e. T_1 was found to be related to the rate of growth rather than to malignancy. These authors emphasized that the T_1 values were related directly with the water content. Hollis *et al.* (1975) also observed that the slowly growing and well-differentiated tumours gave shorter T_1 values as compared with more rapidly growing tumours. Schara *et al.* (1974) concluded that proton spin-lattice relaxation (at room temperature) can be a valuable tool for the characterization of the pathological changes in the thyroid gland tissue in case of thyroid gland cancer.

Hazlewood *et al.* (1974a) studied in great detail the relationship between hydration and the relaxation times of water protons in tissues from mice with and without tumour. It was shown that T_1 and T_2 of water protons, and diffusion constant (D), increased monotonically and distinguishably from the normal, to nodule and finally to tumour cells in the development of mammary tumours in mice. These results indicated that macromolecular–water interactions were altered by the presence of a tumour or onum in the host. The changes in T_1 and T_2 were independent of changes in organ hydration. A comparison of the T_1 and T_2 of neoplasms from the breast of normal and diseased tissues showed that the values of relaxation times were correlated with, but not strictly dependent on the hydration of tissues (Medina *et al.*, 1975; Hazlewood *et al.*, 1974a). The tissues could be classified as fibrocystic or neoplastic depending on the values of the pair of T_1, T_2. If $T_1 \leqq 792$ and $T_2 \leqq 58.1$, then the tissue was classified as fibrocystic; if $T_1 > 792$, $T_2 > 58.1$, then the tissue was classified as neoplastic. The T_2 values were considered to be more discriminating than T_1 in certain diseased states. Hazlewood *et al.* (1974a) and Medina *et al.* (1975) pointed out that NMR spectroscopy could be employed as a useful tool for the detection of cancer, and in cancer research. Saryan *et al.* (1974), and Bovée *et al.* (1974) indicated that the increase in NMR relaxation time T_1 (at $\sim$25°C) is determined, in part, by the increased water content of cancerous tissues.

Block and Maxwell (1974) studied the behaviour of water proton T_1 for normal and tumorous tissues of rats. These authors considered a model in which tumorous tissues had lesser amount of water with restricted mobility than the normal tissues. Three mouse cell populations (EL 4 ascitis tumour cells, normal spleen leukocytes, and normal erythrocytes) were studied at 13.56 and 100 MHz by Block *et al.* (1977). At each frequency, the T_1 values for tumour cells were found to be greater than the values for either of the normal cell type (see Table 2). Qualitatively, they attributed this effect to the binding of a fraction of water (exhibiting restricted mobility) to slowly moving macromolecules. The $1/T_1$ values were found to vary approximately linearly with the total water content over the range investigated.

It has been demonstrated by several authors that T_1 of water protons in tumours is considerably longer than in healthy tissues. Furthermore, Damadian *et al.* (1973) have pointed out that the discrimination between the relaxation times of water in normal and cancerous tissues appears to improve at lower frequency, i.e. the overlapping is reduced at low frequencies. Diegel and Pintar (1975a) defined the resolution "r" as:

$$\text{"}r\text{"} = \frac{T_1\,(\text{tumorous}) - T_1\,(\text{healthy})}{T_1\,(\text{tumorous})} = \left(\frac{1/T_1 - R_f}{1/T_1}\right)\frac{\delta_b}{b}$$

3×10^{-4} sec with an activation energy of 4.8 kcal/mole, and the proton exchange time was calculated to be 1.3×10^{-4} sec with an activation energy of 10.0 kcal/mole. The addition of NH_4Cl enhanced the proton exchange rate. It may be remarked that the value of correlation time noted above for collagen is much longer than the value of 2×10^{-8} sec that was reported earlier for the correlation time of molecular rotation of water in muscles (Knispel *et al.*, 1974). The rate of proton exchange that greatly influences the water proton signal from collagen was found to depend on the temperature, pH, and buffer salts (Migchelsen and Berendsen, 1973; Bieńkiewicz *et al.*, 1977).

In order to explain the non-averaging of dipolar and quadrupolar interactions which are responsible for the splitting of ^1H and ^2H signals, respectively, Dehl and Hoeve (1969) assumed a model in which certain preferential hydrogen bonded structures of water were formed. The water molecules could diffuse rapidly between the highly oriented strands of collagen fibres, but their motion was anisotropic. Chapman and McLauchlan (1969) also proposed a continuous chain model for water in collagen. Fung and Trautmann (1971) proposed that the observed dipolar or quadrupolar splittings for water in collagen were the average of two types of water: (1) water molecules adsorbed or bound to the collagen triple helix (the oriented water), and (2) the remaining free water molecules, that undergo rapid reorientation. These authors reported the effects of ions on collagen hydration by studying the ion effects on the deuteron quadrupole splitting. Migchelsen and Berendsen (1973) were led to the conclusion that the chain model was not sufficient to account completely for the hydration of collagen; their results also favoured the specific binding model. Field-dependent splitting of the water signal was also reported for sciatic nerves of rabbits (Chapman and McLauchlan, 1967). The maximum splitting was observed when the nerve axis was parallel to the applied field.

Dehl (1970) described a method for estimating the amount of unfrozen water in frozen fibres of collagen. The method is based on the fact that line separation is given by $K\ (\cos^2\theta - 1)$, where the splitting constant K decreases with increasing ^2H_2O content, and θ is the angle between the fibre axis and the magnetic field axis. Fung and Wei (1973) applied this method to study in detail the effects of water content and salts on quadrupolar splitting of ^2H_2O in hydrated collagen for the maximum splitting ($\theta = 0°$). The amount of "unfrozen water" decreased in the presence of salts. They pointed out that hydrated ions block the binding sites for water in collagen. Only the water molecules bound directly to collagen were oriented and resulted in the dipolar or quadrupolar splitting for H_2O and ^2H_2O, respectively.

The NMR studies of collagen water discussed so far were performed by the continuous wave technique at a single frequency. Fung *et al.* (1974) applied the pulse technique to measure the T_1 values for water in hydrated collagen at different frequencies and over a wide temperature range (25 to $-80°C$). They observed that T_1 was strongly dependent on the temperature and frequency. The correlation times could be described by a distribution function in a manner similar to that for water in muscles, discussed earlier in Section IV.1(c). Edzes and Samulski (1977) used the FT pulse technique to study the proton spin-lattice relaxation decay of hydrated collagen under the conditions when the dipolar splitting is zero (fibre axis perpendicular to magnetic field). By studying the effects of partial substitution of H_2O by ^2H_2O, these authors proposed that dipolar coupling between water protons and collagen protons, i.e. cross-relaxation and spin diffusion make an important contribution to the water proton relaxation mechanism. Edzes and Samulski (1978) further confirmed, by using the method of selective inversion of water proton magnetization with longer 180° pulses, that cross-relaxation contributes to the relaxation of water protons in hydrated collagen.

(b) *DNA fibres*

A study of water from the hydrated DNA (salmon sperm) in the form of oriented fibres was initially reported by Berendsen and Migchelsen (1965). They observed that the second moment of the water proton signal varied qualitatively with the angle between the fibre direction and magnetic field. In the case of oriented DNA, the anisotropy of

water molecules in a direction perpendicular to the fibre axis was proposed in contrast to the model which was put forth for collagen water. Rupprecht (1966) prepared samples of calf thymus DNA (NaDNA) by the wet spinning method. He plotted peak-to-peak amplitude of the derivative signal recorded as a function of the angle between the direction of molecular orientation and the magnetic field. From these results, Rupprecht was led to conclude that the hydration structure in DNA is similar to the structure present in hydrated collagen.

Finally, Migchelsen *et al.* (1968) investigated the proton NMR spectra of water in oriented NaDNA in the A form, and LiDNA in B and C forms. Similar to the results reported by Rupprecht (1966), Migchelsen *et al.* (1968) also observed a single proton signal at room temperature for NaDNA whose linewidth depended on the angle between the fibre direction and the field. However, for LiDNA in the B and C forms, angular dependent splitting was recorded at room temperatures. The proton exchange process was considered to be an important factor influencing the water spectra of NaDNA.

V. SUMMARY AND CONCLUSIONS

This article presents a general perspective of the multifold NMR studies of water performed in various biological systems. A considerable effort has been devoted to investigate the relaxation times of water nuclei (^1H, ^2H, ^{17}O) for a variety of biological samples as a function of temperature and frequency. One common and striking feature observed in nearly all cases studied is that the relaxation times of water nuclei and the diffusion constants of water molecules are much lower than the values observed for free water. Generally, these results can be interpreted in terms of: (1) the restricted and anisotropic motion of water molecules and enhanced proton transfer in the hydration layer; (2) the preferential and dynamic orientations of water molecules in the vicinity of biological macromolecules. The characteristics (1) and (2) arise because a fraction of the total water content (in solution or in biological samples) is associated with proteins and nucleic acids, forming a hydration layer in their close vicinity. In other words, water molecules in the hydration layer exhibit distinctly different properties from those observed for free or extra-hydration layer water. Furthermore, the behaviour of the hydration water molecules was found to depend strongly on the nature of the hydrated species. Two-state and three-state models were proposed to account for the relaxation behaviour of water nuclei.

An important characteristic of hydration water is that it remains unfrozen or mobile (on the NMR time scale) at temperatures much lower than the freezing point of free solvent. This phenomenon proved to be very valuable for investigating the state of water in systems such as muscle, collagen, tissues, membranes; as well as for studying the changes in macromolecular–water interactions induced by external factors (γ-irradiation), and the changes in water structure which result during natural, biological and physical processes such as growth and division of cells, muscle strain, cancerous growth. The proton NMR spectra obtained from hydration water in frozen samples furnish unprecedented information concerning the macromolecular–water interactions and the state of water in biological systems.

There is a vast scope for the application of the NMR studies of hydration water to explore and study in detail the effects produced by certain toxins, drugs, carcinogens and radiations: to investigate the sensitivity and specificity of different organs to these and other related perturbing factors.

There is increasing evidence showing that cross-relaxation between the protons of water molecules and of the macromolecular chain contributes to the relaxation rates of water protons. Eventually, it may become necessary to revise and reconsider the interpretation of certain previously published results in the light of cross-relaxation.

Water constitutes the major component of all living systems, for example it represents about 70–80% of the total cell constituent. There is conclusive evidence showing that water does not simply serve as an inert medium, but it participates at the molecular

level in basic biological interactions and in fundamental biological processes. In fact, the hydration water molecules constitute an integral part of any macromolecular or cellular system under consideration. The importance of water in maintaining the structural integrity of proteins is well-established. Nevertheless, investigators in different domains have not fully recognized the important and crucial role that hydration water molecules may play in various biophysical and radiobiological processes. While postulating ingenious theories and mechanisms to explain such processes, many authors have either totally neglected the participation of water or considered it simply in terms of the overall medium effects. Hopefully, the NMR studies of water carried out very extensively in different laboratories would largely contribute to unravel the vital functional and structural roles played by water at the molecular level in many biological interactions and biophysical processes.

ACKNOWLEDGEMENTS

The author expresses her gratitude to Dr A. J. Bertinchamps (Euratom-ULB) for help and encouragement that made it possible to accomplish this article. Thanks are acknowledged to Dr P. Lejeune (Institut d'Hygiène et d'Epidémiologie) for many useful discussions and his keen interest in this work. Finally, grateful thanks are offered to Dr W. Stone (Université de Louvain) and Dr K. Hallenga (Vrije Universiteit Brussel) for reading the manuscript with interest, and giving several invaluable suggestions.

REFERENCES

AL-RAWI, J. M. A., BLOXSIDGE, J. P., O'BRIEN, C., CADDY, D. E., ELVIDGE, J. A., JONES, J. R. and EVANS, E. A. (1974) Tritium nuclear magnetic resonance spectroscopy. II. Chemical shifts, referencing, and an application. *J. Chem. Soc. Perkin Trans* **2,** 1635–1638.

AL-RAWI, J. M. A., ELVIDGE, J. A., JONES, J. R. and EVANS, E. A. (1975) Tritium nuclear magnetic resonance spectroscopy. III. Coupling constants and isotope effects. *J. Chem. Soc. Perkin Trans* **2,** 449–452.

ANDRASKO, J. (1976) Water diffusion permeability of human erythrocytes studied by a pulsed gradient NMR technique. *Biochim. biophys. Acta* **428,** 304–311.

BEALL, P. T., HAZLEWOOD, C. F. and RAO, P. N. (1976) NMR patterns of intracellular water as a function of HeLa cell cycle. *Science, N.Y.* **192,** 904–907.

BELTON, P. S., JACKSON, R. R. and PACKER, K. J. (1972) Pulsed NMR studies of water in striated muscle. I. Transverse nuclear spin relaxation times and freezing effects. *Biochim. biophys. Acta* **286,** 16–25.

BELTON, P. S., PACKER, K. J. and SELLWOOD, T. C. (1973) Pulsed NMR studies of water in striated muscle. II. Spin-lattice relaxation times and the dynamics of the non-freezing fraction of water. *Biochim. biophys. Acta* **304,** 56–64

BELTON, P. S. and PACKER, K. J. (1974) Pulsed NMR studies of water in striated muscle. III. The effects of water content. *Biochim. biophys. Acta* **354,** 305–314.

BERENDSEN, H. J. C. (1962) Nuclear magnetic resonance study of collagen hydration. *J. chem. Phys.* **36,** 3297–3305.

BERENDSEN, H. J. C. and MIGCHELSEN, C. (1965) Hydration structure of fibrous macromolecules. *Ann. N. Y. Acad. Sci.* **125,** 365–379.

BERENDSEN, H. J. C. and MIGCHELSEN, C. (1966) Hydration structure of collagen and influence of salts. *Fedn Proc.* **25,** 998–1002.

BERENDSEN, H. J. C. (1975) Specific interactions of water with biopolymers. In *Water: A Comprehensive Treatise.* (ed. F. FRANKS), Vol. 5, pp. 293–349. Plenum Press, New York.

BIEŃKIEWICZ, K. J., BERENDSEN, H. J. C. and ANDREE, P. J. (1977) Properties of water in native and modified collagen. *Ann. Soc. Chim. Polonorum.* **51,** 149–158.

BLEARS, D. J. and DANYLUK, S. S. (1968) Proton wide-line nuclear magnetic resonance spectra of hydrated proteins. *Biochim. biophys. Acta* **154,** 17–27.

BLOCK, R. E. and MAXWELL, G. P. (1974) Proton magnetic resonance studies of water in normal and tumor rat tissues. *J. magn. Reson.* **14,** 329–334.

BLOCK, R. E., MAXWELL, G. P., PRUDHOMME, D. L. and HUDSON, J. L. (1977) High resolution proton magnetic resonance spectral characteristic of water, lipid and protein signals from three mouse cell populations. *J. natn. Cancer Inst.* **58,** 151–156.

BLOK, J. and LOMAN, H. (1973) The effects of γ-irradiation in DNA. *Curr. Top. Radiat. Res. Q.* **9,** 165–245.

BOVÉE, W., HUISMAN, P. and SMIDT, J. (1974) Tumor detection and nuclear magnetic resonance. *J. natn. Cancer. Inst.* **52,** 595–597.

BLOXSIDGE, J., ELVIDGE, J. A., JONES, J. R. and EVANS, E. A. (1971) Tritium NMR spectroscopy. I. Technique, internal referencing and some preliminary results. *Org. magn. Reson.* **3,** 127–138.

BRATTON, C. B., HOPKINS, A. L. and WEINBERG, J. W. (1965) Nuclear magnetic resonance studies of living muscle. *Science, N. Y.* **147,** 738–739.

CARRINGTON, A. and MCLACHLAN, A. D. (1967) *Introduction to Magnetic Resonance.* Harper and Row, New York, and John Weatherhill, Tokyo.

CHANG, D. C., HAZLEWOOD, C. F., NICHOLS, B. L. and RORSCHACH, H. E. (1972) Spin echo studies on cellular water. *Nature, Lond.* **235,** 170–171.

CHANG, D. C., RORSCHACH, H. E., NICHOLS, B. L. and HAZLEWOOD, C. F. (1973) Implications of diffusion coefficient measurements for the structure of cellular water. *Ann. N. Y. Acad. Sci.* **204,** 434–443.

CHANG, D. C., HAZLEWOOD, C. F. and WOESSNER, D. E. (1976) The spin-lattice relaxation times of water associated with early post mortem changes in skeletal muscle. *Biochim. biophys. Acta* **437,** 253–258.

CHAPMAN, G. and MCLAUCHLAN, K. A. (1967) Oriented water in the sciatic nerve of rabbit. *Nature, Lond.* **215,** 391–392.

CHAPMAN, G. and MCLAUCHLAN, K. A. (1969) The hydration structure of collagen. *Proc. R. Soc.* B. **173,** 223–234.

CIVAN, M. M. and SHPORER, M. (1975) Pulsed nuclear magnetic resonance study of ^{17}O, ^{2}D and ^{1}H of water in frog striated muscle. *Biophys. J.* **15,** 299–306.

CIVAN, M. M., ACHLAMA, A. M. and SHPORER, M. (1978) The relationship between the transverse and longitudinal nuclear magnetic resonance relaxation rates of muscle water. *Biophys. J.* **21,** 127–136.

CLEVELAND, G. G., CHANG, D. C., HAZLEWOOD, C. F. and RORSCHACH, H. E. (1976) Nuclear magnetic resonance measurement of skeletal muscle. *Biophys. J.* **16,** 1043–1053.

CONLON, T. and OUTHRED, R. (1972) Water diffusion permeability of erythrocytes using an NMR technique. *Biochim. biophys. Acta* **288,** 354–361.

COOKE, R. and WIEN, R. (1971) The state of water in muscle tissue as determined by proton nuclear magnetic resonance. *Biophys. J.* **11,** 1002–1017.

COPE, F. W. (1969) Nuclear magnetic resonance evidence using D_2O for structured water in muscle and brain. *Biophys. J.* **9,** 303–319.

COPE, F. W. (1970) The solid state physics of electron and ion transport in biology. *Adv. biol. med. Phys.* **13,** 1–35.

COTTAM, G. L., VALENTINE, K. M., YAMAOKA, K. and WATERMAN, M. R. (1974) The gelation of deoxyhemoglobin S in erythrocytes as detected by transverse water proton relaxation measurements. *Archs Biochem. Biophys.* **162,** 487–492.

DAMADIAN, R. (1971) Tumor detection by nuclear magnetic resonance. *Science, N. Y.* **171,** 1151–1153.

DAMADIAN, R., ZANER, K., HOR, D., DIMAIO, T., MINKOFF, L. and GOLDSMITH, M. (1973) Nuclear magnetic resonance as a new tool in cancer research: human tumors by NMR. *Ann. N. Y. Acad. Sci.* **222,** 1048–1076.

DAMADIAN, R., ZANER, K., HOR, D. and DIMAID, T. (1974) Human tumors detected by nuclear magnetic resonance. *Proc. natn. Acad. Sci. U.S.A.* **71,** 1471–1473.

DAMADIAN, R., MINKOFF, L., GOLDSMITH, M., STANFORD, M. and KOUTCHER, J. (1976) Field focussing nuclear magnetic resonance (FONAR): visualization of a tumor in a live animal. *Science, N. Y.* **194,** 1430–1432.

DAMADIAN, R., GOLDSMITH, M. and MINKOFF, L. (1977) NMR in cancer: XVI FONAR image of the live human body. *Physiol. Chem. Phys.* **9,** 97–100.

DEHL, R. E. and HOEVE, C. A. J. (1969) Broad-line NMR study of H_2O and D_2O in collagen fibers. *J. chem. Phys.* **50,** 3245–3251.

DEHL, R. E. (1970) Collagen: mobile water content of frozen fibers. *Science, N. Y.* **170,** 738–739.

DIEGEL, J. G. and PINTAR, M. M. (1975a) A possible improvement in the resolution of proton spin relaxation for the study of cancer at low frequency. *J. natn. Cancer Inst.* **55,** 725–726.

DIEGEL, J. G. and PINTAR, M. M. (1975b) Origin of the non exponentiality of the water proton spin relaxation in tissues. *Biophys. J.* **15,** 855–860.

DUFF, I. D. and DERBYSHIRE, W. (1974) NMR investigations of frozen porcine muscle. *J. magn. Reson.* **15,** 310–316.

EDZES, H. T. and SAMULSKI, E. T. (1977) Cross relaxation and spin diffusion in the proton NMR of hydrated collagen. *Nature, Lond.* **265,** 521–523.

EDZES, H. T. and SAMULSKI, E. T. (1978) The measurement of cross-relaxation effects in the proton NMR spin-lattice relaxation of water in biological systems: hydrated collagen and muscle. *J. magn. Reson.* **31,** 207–229.

EGGLESTON, J. C., SARYAN, L. A. and HOLLIS, D. P. (1975) Nuclear magnetic resonance investigations of human neoplastic and abnormal non neoplastic tissues. *Cancer Res.* **35,** 1326–1332.

EISENBERG, D. and KAUZMANN, W. (1969) *The Structure and Properties of Water.* Clarendon Press, Oxford.

ELLIS, G. E. and PACKER, K. J. (1976) Nuclear spin-relaxation studies of hydrated elastin. *Biopolymers* **15,** 813–832.

EMSLEY, J. W., FEENEY, J. and SUTCLIFFE, L. (1965) *High Resolution Nuclear Magnetic Resonance Spectroscopy.* Vols I and II. Pergamon Press, London.

FABRY, M. E. and EISENSTADT, M. (1975) Water exchange between red cells and plasma. Measurements by nuclear magnetic relaxation. *Biophys. J.* **15,** 1101–1110.

FERRAR, T. C. and BECKER, E. D. (1971) *Pulse and Fourier Transform NMR.* Academic Press, New York.

FINCH, E. D. and HOMER, L. D. (1974) Proton nuclear magnetic resonance relaxation measurements in frog muscle. *Biophys. J.* **14,** 907–921.

FINCH, E. D. and SCHNEIDER, A. S. (1975) Mobility of water bound to biological membranes. A proton NMR relaxation study. *Biochim. biophys. Acta* **406,** 146–154.

FOSTER, K. F., RESING, H. A. and GARROWAY, A. N. (1976) Bounds on "bound water": transverse nuclear magnetic resonance relaxation in barnacle muscle. *Science, N. Y.* **194,** 324–326.

FRANKS, F. (1972–1975) *Water: A Comprehensive Treatise,* Vols 1–5. Plenum Press, New York.

FRANKS, F. (1977) Solvation interactions of proteins in solution. *Phil. Trans. R. Soc.* B. **278,** 89–96.

FREY, H. E., KNISPEL, R. R., KRUUV, J., SHARP, A. R., THOMPSON, R. T. and PINTAR, M. M. (1972) Proton spin-lattice relaxation studies of non malignant tissues of tumorous mice. *J. natn. Cancer Inst.* **49,** 903–906.

FRITZ, O. G. JR and SWIFT, T. J. (1967) The state of water in polarized and depolarized frog nerves. A proton magnetic resonance study. *Biophys. J.* **7,** 675–686.

FULLER, M. E. and BREY, W. S. JR (1968) Nuclear magnetic resonance study of water sorbed on serum albumin. *J. biol. Chem.* **243,** 274–280.

FUNG, B. M. and TRAUTMANN, P. (1971) Deuterium NMR and EPR of hydrated collagen fibers in the presence of salts. *Biopolymers* **10,** 391–397.
FUNG, B. M. and WEI, S. C. (1973) The effects of alkali and alkaline earth salts on the structure of hydrated collagen fibers as studied by deuterium NMR. *Biopolymers* **12,** 1053–1062.
FUNG, B. M. (1974) Non-freezable water and spin-lattice relaxation time in muscle containing a growing tumor. *Biochim. biophys. Acta* **362,** 209–214.
FUNG, B. M., WITSCHEL, J. JR and MCAMIS, L. L. (1974) The state of water on hydrated collagen as studied by pulsed NMR. *Biopolymers.* **13,** 1767–1776.
FUNG, B. M. and MCGAUGHY, T. W. (1974) The state of water in muscle as studied by pulsed NMR. *Biochim. biophys. Acta* **343,** 663–673.
FUNG, B. M., DURHAM, D. L. and WASSIL, D. A. (1975a) The state of water in biological systems as studied by proton and deuterium relaxation. *Biochim. biophys. Acta* **399,** 191–202.
FUNG, B. M., WASSIL, D. A., DURHAM, D. L., CHESNUT, R. W., DURHAM, N. N. and BERLIN, K. D. (1975b) Water in normal muscle and muscle with tumor. *Biochim. biophys. Acta* **385,** 180–187.
FUNG, B. M. (1977a) Proton and deuteron relaxation of muscle water over wide ranges of resonance frequencies. *Biophys. J.* **18,** 235–239.
FUNG, B. M. (1977b) Carbon-13 and proton magnetic resonance of mouse muscle. *Biophys. J.* **19,** 315–319.
FUNG, B. M. (1977c) Correlation of relaxation time with water content in muscle and brain tissues. *Biochim. biophys. Acta* **497,** 317–322.
GLASEL, J. A. (1967) A study of water in biological systems by ^{17}O magnetic resonance spectroscopy. II. Relaxation phenomena in pure water. *Proc. natn. Acad. Sci., U.S.A.* **58,** 27–33.
GLASEL, J. A. (1970) Participation of water in conformational changes of biopolymers as studied by deuteron magnetic resonance. *J. Am. Chem. Soc.* **92,** 375–381.
GOLDSMITH, M., KOUTCHER, J. and DAMADIAN, R. (1978) NMR in cancer XI. Application of the NMR malignancy index to human gastro-intestinal tumors. *Cancer* **41,** 183–191.
GRÖSCH, L. and NOACK, F. (1976) NMR relaxation investigation of water mobility in aqueous bovine serum albumin solutions. *Biochim. biophys. Acta* **453,** 218–232.
HALLENGA, K. and KOENIG, S. H. (1976) Protein rotational relaxation as studied by solvent ^{1}H and ^{2}H magnetic relaxation. *Biochemistry* **15,** 4255–4263.
HANSEN, J. R. and LAWSON, K. D. (1970) Magnetic relaxation in ordered systems. *Nature, Lond.* **225,** 542–543.
HANSEN, J. R. (1971) Pulsed NMR study of water mobility in muscle and brain tissue. *Nature, Lond.* **230,** 482–486.
HAZLEWOOD, C. F., NICHOLS, B. L. and CHAMBERLAIN, N. F. (1969) Evidence for the existence of a minimum of two phases of ordered water in skeletal muscle. *Nature, Lond.* **222,** 747–750.
HAZLEWOOD, C. F., NICHOLS, B. L., CHANG, D. C. and BROWN, B. (1971) On the state of water in developing muscle: a study of the major phase of ordered water in skeletal muscle and its relationship to sodium concentration. *Johns Hopkins Med. J.* **128,** 117–131.
HAZLEWOOD, C. F., CLEVELAND, G. and MEDINA, D. (1974a) Relationship between hydration and proton nuclear magnetic resonance relaxation times in tissues of tumor-bearing and non tumor-bearing mice: implications for cancer detection. *J. natn. Cancer Inst.* **52,** 1849–1853.
HAZLEWOOD, C. F., CHANG, D. C., NICHOLS, B. L. and WOESSNER, D. E. (1974b) Nuclear magnetic resonance transverse relaxation times of water protons in skeletal muscle. *Biophys. J.* **14,** 583–606.
HILTON, B. D., HSI, E. and BRYANT, R. G. (1977) ^{1}H nuclear magnetic resonance relaxation of water on lysozyme powders. *J. Am. Chem. Soc.* **99,** 8483–8490.
HOLLIS, D. P., SARYAN, L. A., EGGLESTON, D. and MORRIS, H. P. (1975) Nuclear magnetic resonance studies of cancer. VI. Relationship among spin-lattice relaxation times, growth rate and water content of Morris hepatomas. *J. natn. Cancer Inst.* **54,** 1469–1472.
HOULT, D. I. (1977) Zeugmatography: a criticism of the concepts of a selective pulse in the presence of a field gradient. *J. magn. Reson.* **26,** 165–167.
HUTCHINSON, J. M. S., SUTHERLAND, R., MALLARD, J. R. and FOSTER, M. A. (1977) In *NMR in Biology* (eds R. A. DWEK, I. D. CAMPBELL, R. E. RICHARDS and R. J. P. WILLIAMS), pp. 368–369. Academic Press, London.
INCH, W. R., MCCREDIE, J. A., KNISPEL, R. R., THOMPSON, R. T. and PINTAR, M. M. (1974) Water content and proton spin relaxation time for neoplastic and non-neoplastic tissues from mice and humans. *J. natn. Cancer Inst.* **52,** 353–356.
JACOBSEN, B., ANDERSON, W. A. and ARNOLD, T. (1954) A proton magnetic resonance study of the hydration of deoxyribonucleic acid. *Nature, Lond.* **173,** 772–773.
JAMES, T. L. and GILLEN, K. T. (1972) NMR relaxation time and self-diffusion constant of water in hen egg white and yolk. *Biochim. biophys. Acta* **286,** 10–15.
KALK, A. and BERENDSEN, H. J. C. (1976) Proton magnetic relaxation and spin diffusion in proteins. *J. magn. Reson.* **24,** 343–366.
KAVANAU, J. L. (1964) *Water and Solute–Water Interactions.* Holden-Day, London.
KLOSE, G. and STELZNER, F. (1974) NMR investigations of the interaction of water with lecithin in benzene solutions. *Biochim. biophys. Acta* **363,** 1–8.
KNISPEL, R. R., THOMPSON, R. T. and PINTAR, M. M. (1974) Dispersion of proton spin-lattice relaxation in tissues. *J. magn. Reson.* **14,** 44–51.
KOENIG, S. H. and SCHILLINGER, W. E. (1969) Nuclear magnetic relaxation dispersion in protein solutions. *J. biol. Chem.* **244,** 3283–3289.
KOENIG, S. H., HALLENGA, K. and SHPORER, M. (1975) Protein–water interaction studied by solvent ^{1}H, ^{2}H and ^{17}O magnetic relaxation. *Proc. natn. Acad. Sci. U.S.A.* **72,** 2667–2671.
KOENIG, S. H., BRYANT, R. G., HALLENGA, K. and JACOB, G. S. (1978) Magnetic cross-relaxation among protons in protein solutions. *Biochemistry* **17,** 4348–4358.

KOUTCHER, J. A., GOLDSMITH, M. and DAMADIAN, R. (1978) NMR in cancer X. A malignancy index to discriminate normal and cancerous tissue. *Cancer* **41,** 174–182.

KUMAR, A., WELTI, D. and ERNST, R. R. (1975) NMR Fourier zeugmatography. *J. magn. Reson.* **18,** 69–83.

KUNTZ, I. D., BRASSFIELD, T. S., LAW, G. D. and PURCELL, G. V. (1969) Hydration of macromolecules. *Science, N. Y.* **163,** 1329–1331.

KUNTZ, I. D. (1971a) Hydration of macromolecules. III. Hydration of polypeptides. *J. Am. Chem. Soc.* **93,** 514–516.

KUNTZ, I. D. (1971b) Hydration of macromolecules. IV. Polypeptide conformation in frozen solution. *J. Am. Chem. Soc.* **93,** 516–518.

KUNTZ, I. D. and KAUZMANN, W. (1974) Hydration of proteins and polypeptides. *Adv. Protein Chem.* **28,** 239–345.

LATARJET, R. (1972) Interaction of radiation energy with nucleic acids. *Curr. Top. Radiat. Res. Q.* **8,** 1–38.

LAUTERBUR, P. C. (1973) Image formation by induced local interactions: examples employing NMR. *Nature, Lond.* **242,** 190–191.

LAUTERBUR, P. C. (1974) Magnetic resonance zeugmatography. *Pure Appl. Chem.* **40,** 149–157.

LAUTERBUR, P. C. (1977) Spatially-resolved studies of whole tissue, organs and organisms by NMR zeugmatography. In *NMR in Biology* (eds. R. A. DWEK, I. D. CAMPBELL, R. E. RICHARDS and R. J. P. WILLIAMS), pp. 323–335. Academic Press, London.

LINDSTROM, T. R. and KOENIG, S. H. (1974) Magnetic-field dependent water proton spin-lattice relaxation rates of hemoglobin solutions and whole blood. *J. magn. Reson.* **15,** 344–353.

LINDSTROM, T. R., KOENIG, S. H., BOUSSIOS, T. and BERTLES, J. F. (1976) Intermolecular interactions of oxygenated sickle hemoglobin molecules in cells and cell-free solutions. *Biophys. J.* **16,** 679–689.

LING, G. N. (1962) In *A Physical Theory of the Living State: The Association-Induction Hypothesis.* Blaisdell, New York.

LING, G. N. (1969) A new model for the living cell: a summary of the theory and recent experimental evidence in its support. *Int. Rev. Cytol.* **26,** 1–61.

LUBAS, B. and WILCZOK, T. (1966) Spin-echo technique study of the non-rotational hydration of deoxyribonucleic acid. *Biochim. biophys. Acta* **120,** 427–433.

LUBAS, B. and WILCZOK, T. (1967) Thermal transition of DNA measured by NMR spin-echo technique. *Biopolymers* **5,** 967–974.

LUBAS, B. and WILCZOK, T. (1971) NMR study on molecular mobility of DNA molecules in solution. *Biopolymers* **10,** 1267–1276.

MANSFIELD, P., MAUDSLEY, A. A. and BAINES, T. (1976) Fast scan proton density imaging by NMR. *J. Phys. E.* **9,** 271–278.

MATHUR-DE VRÉ, R., BERTINCHAMPS, A. J. and BERENDSEN, H. J. C. (1976) The effect of γ-irradiation on the hydration characteristics of DNA and polynucleotides. I. An NMR study of frozen H_2O and D_2O solutions. *Radiat. Res.* **68,** 197–214.

MATHUR-DE VRÉ, R. and BERTINCHAMPS, A. J. (1977a) The effects of γ-irradiation on the hydration characteristics of DNA and polynucleotides. II. An NMR study of mixed H_2O/D_2O frozen solutions. *Radiat. Res.* **72,** 181–189.

MATHUR-DE VRÉ, R. and BERTINCHAMPS, A. J. (1977b) The effects of γ-irradiation on the hydration characteristics of DNA and polynucleotides. III. A comparative NMR study of frozen and liquid solutions. *Radiat. Environ. Biophys.* **14,** 311–315.

MEDINA, D., HAZLEWOOD, C. F., CLEVELAND, G. G., CHANG, D. C., SPJUT, H. J. and MOYERS, R. (1975) Nuclear magnetic resonance studies on human breast dysplasias and neoplasms. *J. natn. Cancer Inst.* **54,** 813–818.

MIGCHELSEN, C. and BERENDSEN, H. J. C. (1967) Deuteron magnetic resonance on hydrated collagen. In *Magnetic Resonance and Relaxation* (ed. R. BLINC), pp. 761–766. North-Holland, Amsterdam.

MIGCHELSEN, C., BERENDSEN, H. J. C. and RUPPRECHT, A. (1968) Hydration of DNA. Comparison of nuclear magnetic resonance results for oriented DNA in the A, B and C form. *J. molec. Biol.* **37,** 235–237.

MIGCHELSEN, C. and BERENDSEN, H. J. C. (1973) Proton exchange and molecular orientation of water in hydrated collagen fibers. An NMR study of H_2O and D_2O. *J. chem. Phys.* **59,** 296–305.

OUTHRED, R. K. and GEORGE, E. P. (1973a) A nuclear magnetic resonance study of hydrated systems using the frequency dependence of the relaxation processes. *Biophys. J.* **13,** 83–96.

OUTHRED, R. K. and GEORGE, E. P. (1973b) Water and ions in muscles and model systems. *Biophys. J.* **13,** 97–103.

PACKER, K. J. (1973) The effects of diffusion through locally inhomogeneous magnetic fields on transverse nuclear spin relaxation in heterogeneous systems. Proton transverse relaxation in striated muscle tissue. *J. magn. Reson.* **9,** 438–443.

PACKER, K. J. (1977) The dynamics of water in heterogeneous systems. *Phil. Trans. R. Soc.* B. **278,** 59–87.

RAAPHORST, G. P., KRUUV, J. and PINTAR, M. M. (1975) Nuclear magnetic resonance study of mammalian cell water. *Biophys. J.* **15,** 391–402.

RANADE, S. S., CHAUGHULE, S. R., KASTURI, S. R., NADKARNI, J. S., TALWALKAR, G. V., WAGH, U. V., KORGAONKAR, K. S. and VIJAYARAGHAVAN, R. (1975) Pulsed nuclear magnetic resonance studies on human malignant tissues and cells *in vitro*. *Indian J. Biochem. Biophys.* **12,** 229–232.

RATKOVIĆ, S. and SINADINOVIĆ, J. (1977) Nuclear magnetic resonance studies on the thyroid gland. II. On the state of tissue water in normal thyroid gland. *Studia Biophys.* **63,** 25–39.

RESING, H. A., FOSTER, K. R. and GARROWAY, A. N. (1977) "Bound water" in barnacle muscle as indicated in nuclear magnetic resonance studies. *Science, N. Y.* **198,** 1180–1182.

RUPPRECHT, A. (1966) Hydration of DNA, a wide line NMR study of oriented DNA. *Acta Chem. scand.* **20,** 582–585.

SARYAN, L. A., HOLLIS, D. P., ECONOMOU, J. S. and EGGLESTON, J. C. (1974) Nuclear magnetic resonance studies of cancer. IV. Correlation of water content with tissue relaxation times. *J. natn. Cancer Inst.* **52,** 599–602.

SCHARA, M., SENTJURC, M., AUERSPERG, M. and GOLOUH, R. (1974) Characterization of malignant thyroid gland tissue by magnetic resonance methods. *Br. J. Cancer* **29,** 483–486.

SHAW, D. (1976) *Fourier Transform NMR Spectroscopy*. Elsevier, Amsterdam.

SHPORER, M. and CIVAN, M. M. (1975) NMR study of ^{17}O form $H_2{}^{17}O$ in human erythrocytes. *Biochim. biophys. Acta* **385,** 81–87.

SHPORER, M., HAAS, M. and CIVAN, M. M. (1976) Pulsed nuclear magnetic resonance study of ^{17}O from $H_2{}^{17}O$ in rat lymphocytes. *Biophys. J.* **16,** 601–610.

SINADINOVIĆ, J., RATKOVIĆ, S. KRAINČANIC. M. and JOVANIĆ, M. (1977) Relationship of biochemical and morphological changes in rat-thyroid and proton spin-relaxation of the tissue water. *Endokrinologie* **69,** 55–66.

SWIFT, T. J. and BARR, E. M. (1973) An oxygen magnetic resonance study of water in frog skeletal muscle. *Ann. N. Y. Acad. Sci.* **204,** 191–196.

SYKES, B. D., HULL, W. E. and SNYDER, G. H. (1978) Experimental evidence for the role of cross-relaxation in proton nuclear magnetic resonance spin lattice relaxation time measurements in proteins. *Biophys. J.* **21,** 137–146.

SZENT-GYÖRGYI, A. (1957) *Bioenergetics*. Academic Press, New York.

TEXTER, J. (1978) Nucleic acid–water interactions. *Prog. Biophys. molec. Biol.* **33,** 83–97.

THOMPSON, R. T., KNISPEL, R. R. and PINTAR, M. M. (1973) A study of the proton exchange in tissue water by spin relaxation in the rotating frame. *Chem. Phys. Lett.* **22,** 335–337.

THOMPSON, B. C., WATERMAN, M. R. and COTTAM, G. L. (1975) Evaluation of the water environments in deoxygenated sickle cells by longitudinal and transverse water proton relaxation rates. *Archs Biochem. biophys.* **166,** 193–200.

UDALL, J. N., ALVAREZ, L. A., NICHOLS, B. L. and HAZLEWOOD, C. F. (1975) The effects of cholera enterotoxin on intestinal tissue water as measured by nuclear magnetic resonance spectroscopy. *Physiol. Chem. Phys.* **7,** 533–539.

WALMSLEY, R. H. and SHPORER, M. (1978) Surface induced NMR line splittings and augmented relaxation rates in water. *J. chem. Phys.* **68,** 2584–2590.

WHITE, J. P., KUNTZ, I. D. and CANTOR, C. R. (1972) Studies on the hydration of *Escherichia coli* ribosomes by nuclear magnetic resonance. *J. molec. Biol.* **64,** 511–514.

ZIPP, A., JAMES, T. L., KUNTZ, I. D. and SHOHET, S. B. (1976) Water proton magnetic resonance studies of normal and sickle erythrocytes. Temperature and volume dependence. *Biochim. biophys. Acta* **428,** 291–303.