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A New Interpretation of Proton NMR Relaxation Time Measurements of Water in Food

B. P. Hills, S. F. Takacs* & P. S. Belton

AFRC Institute of Food Research, Norwich Laboratory,
Colney Lane, Norwich NR4 7UA, UK

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ABSTRACT

The transverse water proton relaxation in three widely differing types of sample, native lysozyme solutions, skimmed milk and apple, has been analysed. The relaxation times show characteristic variations with CPMG pulse spacing and morphology which can be interpreted in terms of chemical exchange and molecular diffusion without recourse to popular concepts such as various amounts and types of 'bound' water. Our results suggest that transverse water proton relaxation might be used as a sensitive probe of changes in water distribution during cellular growth and differentiation, freeze–thawing and dehydration–rehydration in food systems.

INTRODUCTION

The role and state of water in food is generally agreed to be of importance in a range of properties such as texture, microbial growth rates, storage deterioration, etc. (Duckworth, 1975). Proton NMR relaxation times have been much used to investigate the state of water under a wide variety of conditions and materials (Belton, 1984). The interpretations given by various authors have been based on a range of models. However, the common features of such interpretations are that water exists in a number of 'states' or 'sites' and that these 'sites' correspond to water that is 'bound' in various ways. These conclusions are based on a number of observations

* On secondment from: Center of Advanced Food Technology, Rutgers University, Cook College, PO Box 231, New Brunswick, NJ 08903, USA

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(Belton, 1984). Two important ones are that transverse relaxation is often multiexponential and that it is always faster than the corresponding relaxation rate in bulk water. The chain of logic is essentially that each component of the multiexponential relaxation must represent water experiencing some different correlation time for motion, which hence must be in a different state or site. Since each site has a faster relaxation rate than in bulk water the correlation times must be slower than in bulk water; hence, the water is 'bound' in some way, or in exchange with bound water.

The interpretation of multiple exponential relaxation has been challenged on the basis of the spatial heterogeneity of the sample (Browstein & Tarr, 1979; Lillford *et al.*, 1980; Belton & Ratcliffe, 1985). Lillford and co-workers proposed that, in a heterogeneous system, a molecule far from a surface at which fast relaxation was taking place would have to diffuse there in order to relax, if the diffusion time was longer than the intrinsic relaxation time of the molecule then no fast relaxation would be observed. At the other extreme, molecules very close to the surface would have very short diffusion times and hence fast relaxation. Since there would be a continuum of distances there would be a continuum of relaxation times. Independently Brownstein and Tarr (1979) undertook a mathematical analysis of the situation where there was surface relaxation and concluded that a sum of exponentials would be expected. A more general treatment for a variety of situations has recently been developed (Belton & Hills, 1987) and applied to gels (Belton *et al.*, 1988*b*) and Sephadex bead systems (Hills *et al.*, 1989*c*). An important result of this work has been to predict and observe the onset of multiple exponential relaxation as the scale of the heterogeneity changes.

Even though diffusion in a heterogeneous medium can explain multiple exponential relaxation it does not, of itself, explain why transverse relaxation is faster than in bulk water. Analysis of ^{17}O relaxation data in sucrose solutions (Belton & Wright, 1986) and protein solutions (Halle *et al.*, 1981) suggests that the increase in the rotational correlation time of the water hydrating the solute is relatively small: about three times in the case of sucrose and up to ten times in the case of proteins. This would not account for the dramatic increases in proton relaxation rates observed when protein is dissolved in water (Oakes, 1976). An explanation might therefore lie in exchange between water protons and exchangeable protons on the solute since, in general, the solute will have a much longer correlation time, hence faster relaxation rate, than the water. There is independent evidence from high resolution spectral lineshape studies for exchange of water protons with NH groups in polypeptides (Woodhouse *et al.*, 1975). More recently this has been used to explain connectivities between resonances of exchangeable and non-exchangeable protein protons and water observed in two-dimensional NOESY measurements (Van de Ven, 1988).

If these explanations of the origins of multiple exponential, enhanced relaxation processes are correct then they imply that proton relaxation measurements can give limited information on bound water and the number of sites or states but may serve as a useful indicator of morphology and the state of the biopolymers or other species having exchangeable protons. In the remainder of the paper we offer evidence to suggest that this is the case and suggest that much of the published data in this area may be in need of reinterpretation. To illustrate the effects of exchange on proton relaxation, a pulse spacing dependence of the transverse relaxation rate of water protons in a lysozyme solution is reported which may be interpreted purely in terms of chemical exchange. The effects of morphology on multiexponential relaxation processes are illustrated by examination of the effects of particle size on water proton relaxation in dried skimmed milk suspensions and apple.

MATERIALS AND METHODS

NMR measurements were carried out on an MSL100 spectrometer operating at 100 MHz for protons. Transverse relaxation times were measured using a Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence with a 90°–180° pulse spacing which was varied between 25 μs amd 10 ms. Ninety degree pulse widths were typically between 1 and 2 μs. Data were accumulated with eight scans and long recycle delays of 15–20 s were used to avoid saturation. Sufficient echoes were acquired to give a zero baseline. Experiments were carried out at a thermostated probe temperature of 23 ± 1°C.

Lysozyme was purchased from the Sigma Chemical Company (Lot No. L6876; Poole, UK) and was used without further purification. Solutions were made in distilled, deionized water and adjusted to pH 8 with NaOH. The concentration (108 mg/ml) was determined spectrophotometrically using an absorbance coefficient at 280 nm of 2·58 ml/mg cm (Halford, 1975).

Low-fat spray-dried skimmed milk was purchased from a retail source. A suspension was obtained by adding known amounts to distilled, deionized water. Both non-compacted fresh and compacted year-old samples were used. Casein micelles were isolated by centrifugation at 18 000 g for 30 min followed by resuspension in a solution 20 mM in $CaCl_2$ and 80 mM in KCl.

RESULTS AND DISCUSSION

The dependence of the transverse relaxation rate of a lysozyme solution on the pulse spacing of the CPMG sequence is shown in Fig. 1. At very short

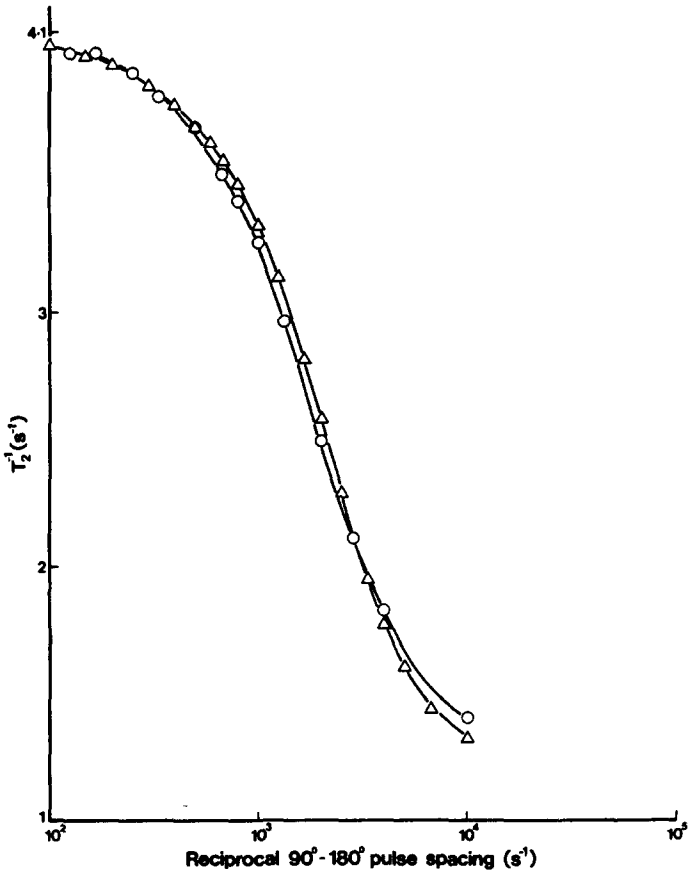

Fig. 1. Transverse water proton relaxation rates at 100 MHz for a 108 mg/ml native lysozyme solution of pH8 and 296°K plotted against reciprocal pulse spacing. ○, Experiment. △, Theoretical fit with the parameter values; $P_b = 6{\cdot}297 \times 10^{-3}$, $k_b = 3{\cdot}0 \times 10^3\,s^{-1}$, $T_{2a} = 2s$, $T_{2b} = 8{\cdot}4\,ms$, $\delta\omega = 2{\cdot}171$ ppm, $\omega_0 = 100$ MHz. See text for parameter definitions.

pulse spacings double exponential relaxation was observed. This arises from the contribution of the protein protons which have short relaxation times and consequently are only observable at short pulse spacings. The origin of a CPMG dispersion is well understood and documented in the literature (Carver & Richards, 1972). It arises from the exchange of the nuclei between chemically shifted sites. When the pulse spacing is long compared to the exchange rate the exchanges between the two chemically shifted sites cause dephasing of the spins, hence enhanced relaxation. When the pulse spacing is short compared to the exchange rate there is no time for exchange between pulses hence there is no exchange contribution to dephasing and relaxation times are long. A general expression in terms of the relaxation rates in the

two sites, the exchange rate and the chemical shift difference, has been obtained by Carver and Richards (1972). After some slight algebraic modification this expression can adequately describe such behaviour (Hills *et al.*, 1989*a*). Unfortunately, analysis of T_2 dispersion curves using the modified Carver–Richards equations requires numerical methods and the importance of the various terms is not very clear. The expression derived by Swift and Connick (1962) shows the interplay of the various terms but is only applicable when the fraction of protons in one site is much less than that in the other site. That is for two sites, *a* and *b*:

$$P_b \ll P_a \qquad \text{and} \qquad P_a + P_b = 1$$

P_j is the population in site *j*. If k_b^{-1} is the mean lifetime in the *b* site and the relaxation times in the two sites are T_{2a} and T_{2b}, then provided $t_{cp} \gg \frac{1}{k_b}$ (t_{cp} is the 180°–180° pulse spacing in the CPMG sequence):

$$T_2^{-1} = T_{2a}^{-1} + P_b k_b \left\{ \frac{T_{2b}^{-2} + T_{2b}^{-1} k_b + (\delta\omega)^2}{(T_{2b}^{-1} + k_b)^2 + (\delta\omega)^2} \right\} \tag{1}$$

Here $\delta\omega$ is the chemical shift difference in frequency units between sites *a* and *b*. T_2^{-1} is the observed relaxation rate.

Equation (1) clearly demonstrates the interplay between the relaxation rates, exchange rate and chemical shift differences. Some useful limits can be derived which show the effects of the various parameters.

If $k_b \gg \delta\omega$, T_{2b}^{-1} then:

$$T_2^{-1} = T_{2a}^{-1} + P_b T_{2b}^{-1} + \frac{P_b(\delta\omega)^2}{k_b} \tag{2}$$

In practice since $k_b \gg \delta\omega$ the last term in eqn (2) will be small. The expression then can be approximated:

$$T_2^{-1} = T_{2a}^{-1} + P_b T_{2b}^{-1} \tag{3}$$

This is the same expression as to be expected in the short pulse spacing limit. Hence, under the conditions $k_b \gg \delta\omega$, T_{2b}^{-1}, a small or zero dispersion will be seen. Similarly, if $T_{2b}^{-1} \gg k_b, \delta\omega$ expression (1) in the long pulse spacing limit becomes:

$$T_2^{-1} = T_{2a}^{-1} + P_b k_b \tag{4}$$

In this case the relaxation is dominated by the exchange term since it is the rate at which the nuclei experience the very fast relaxing site that will determine the relaxation rate. Again under these circumstances no dispersion would be observed. It is important to note therefore that the absence of a dispersion does not imply the absence of exchange processes, merely that certain limits are met.

Whilst it is possible to explain the general shape of the dispersion it is important to see if the detailed fit gives physically reasonable values. In order to do this it is useful to fix one or more of the parameters first. We have chosen to fix the population of the exchangeable protein protons. These will not be backbone protons but side chain protons from NH_2, H_3N^+ and OH. The exchangeable proton population may therefore be calculated from the amino acid composition. This gives 82 protons per protein molecule which, in our experiment, gives $P_b = 6{\cdot}297 \times 10^{-3}$. The values of the parameters in the fit are given in Fig. 1. The rate constant and chemical shift are close to the values obtained in the literature. The chemical shift (Wutrich, 1986) of NH protons on side chains is from 6·6 to 7·6 ppm compared to water at about 4·8 ppm. A reasonable value of chemical shift difference would therefore be between 2 and 3 ppm. Since some of the exchangeable protons are from OH groups, which are less shifted, a value on the low side may be expected. The value for k_b similarly falls comfortably within the reported range (Wutrich, 1986) of 3×10^3–$2 \times 10^4\ s^{-1}$. T_{2b} is similar to the value obtained from fitting the double exponential curve observed at very short pulse spacing; this gave a value of 9·6 ms. The calculated value is a little shorter than this but, given that it reflects a slightly different set of protons and the problems of exponential curve fitting, agreement is good.

It is important to note that, in the analysis, no consideration has been given to a contribution from 'bound' water, T_{2a} at 2 s is the relaxation time for bulk water. The implication of this is that if bound water exists relaxation times are not far from that of bulk water.

Oxygen-17 relaxation time measurements are not complicated by exchange processes (Halle *et al.*, 1981) and can be used to give an indication of rotational correlation times of water near to the protein surface. They are therefore one way of finding out about bound water. Halle *et al.* (1981) have analysed ^{17}O relaxation times in lysozyme solutions and conclude the correlation times for rotational motion are about ten times as long as for bulk water. Proton relaxation depends both on rotational and translation motions; it would be expected that the translational correlation times scale as the translational diffusion coefficient. Polnaszek and Bryant (1984) have measured translational diffusion coefficients close to the surface (within 10 Å) of bovine serum albumin in aqueous solution; they conclude that there is a decrease of between five and ten times in the diffusion coefficient. Both results suggest therefore that a tenfold increase in relaxation rate would be expected in water close to the surface. Assuming such water was present in the order of 0·5 g per g of protein (a fairly generous allowance) then the effective relaxation time, T_{2a}, would drop from 2 s to 1·3 s, an insignificant change.

We conclude therefore that the presence of water where motion is affected

by the protein does not materially affect the observed proton relaxation rate and that it is dominated by exchange. The values of diffusion coefficient close to the protein surface (Polnaszek & Bryant, 1984) also preclude any possibility that diffusive exchange between 'bound' and 'free' water contributes to the relaxation process. Both forms are exchanging in the fast exchange limit.

A fairly simple way of quantifying the importance of the protein water exchange process is to compare the excess relaxation rates of $^{17}0$ and protons.

The excess relaxation rate is given by:

$$R_{ex} = T_{2ex}^{-1} = T_{2obs}^{-1} - T_{2w}^{-1} \tag{5}$$

T_{2obs} is observed transverse relaxation time; T_{2w} is the transverse relaxation time of pure water; R_{ex} is the excess transverse relaxation rate. In order to compare $^{17}0$ and ^{1}H we define a relative excess relaxation rate as:

$$R_r = \frac{R_{ex}}{R_w}, \; R_w = \frac{1}{T_{2w}} \tag{6}$$

For $^{17}0$ in 100 mg/ml lysozyme solution R_r is about 0·8 (Halle *et al.*, 1981). From the proton data reported here at the shortest pulse spacing, $R_r \simeq 1{\cdot}4$ and, at the longest, $R_r \simeq 7$. Thus the enhancement in proton relaxation times due to exchange is of the order of two to ten times. Estimates of correlation times based on proton relaxation rates that do not take account of exchange could therefore be up to an order of magnitude in error.

Whilst lysozyme solutions are good models for demonstrating the importance of exchange in proton transverse relaxation rates they are homogeneous solutions and thus do not represent the important feature of heterogeneity that is present in many food stuffs. Skimmed, fat-free milk powder dispersed in water represents the next level of complexity. Fresh uncompacted powder produces a dispersion of casein micelles having a diameter of about 0·1 μm. This mean diameter is close to literature values for casein micelles (Glaser *et al.*, 1980) and was obtained using both scanning and transmission electron micrographs of the skimmed milk suspended in an agarose gel (Fig. 2). Besides the casein micelles there are substantial quantities of carbohydrates and proteins such as lactose, β-lactoglubulin and α-lactalbumin in solution. The transverse water proton relaxation is single exponential and shows a dispersion similar to lysozyme indicating exchange between two sites differing in resonance frequency. We first consider the contribution of the casein micelles to the relaxation. The

Fig. 2. Transmission electron micrograph of a skimmed milk suspension in an agarose gel matrix. The sample was fixed in gluteraldehyde, stained with osmium and mounted in Spurr resin.

criterion for the observation of multiexponential relaxation in heterogeneous systems is dependent on the detailed geometry; however, the limit:

$$\frac{a^2}{D}|\Delta\gamma| \geq 1 \tag{7}$$

is correct to an order of magnitude approximation (Belton *et al.*, 1988*b*). a is the characteristic dimension of the inhomogeneity; in this case the micelle radius $\simeq 0{\cdot}05\,\mu$m, D is the diffusion coefficient and $\Delta\gamma$ is the difference between the relaxation rates in the two sites. Typically casein micelles contain about 3 g water/g casein (Dalgleisch, 1982) and it would be expected, under such circumstances, that the diffusion coefficient would be of the order of that for bulk water i.e. $\simeq 2 \times 10^{-5}\,\text{cm}^2\,\text{s}^{-1}$. This means that the casein micelles could only give multiexponential relaxation if $|\Delta\gamma| \geq 10^5\,s^{-1}$. This implies a relaxation time of water in the micelle less than 10 μs. Such a value is unreasonably short and would not be typical of protein solutions. The Free Induction Decay of the milk suspension shows an initial fast decaying gaussian component superimposed on the more slowly decaying exponential. This gaussian component is expected for micelle proteins protons which are immobilized (Belton *et al.*, 1988*a*), and has a relaxation time of roughly 50 μs. This means that the condition $|\Delta\gamma| \geq 10^5\,\text{s}^{-1}$ cannot be met and single exponential relaxation is predicted for the skimmed milk suspension. The shortness of the micelle protein relaxation time does suggest that exchange between the water and micelle protein protons has no significant chemical shift contribution and that, in this limit, eqn (4) applies. The observed dispersion in T_2 arises therefore, from the soluble components such as lactose, β-lactoglobulin and α-lactalbumin and not from the casein micelles. We confirmed this by observing a relaxation dispersion from the whey obtained by filtering off the coagulated casein micelles after lowering the pH to 4·8 (Fig. 3). In another experiment the casein micelles were separated by centrifugation at 18 000*g* and resuspended in a solution containing 20 mM $CaCl_2$ and 80 mM KCl. The resuspended micelles showed only a very weak pulse spacing dependence which probably arose from residual amounts of sugars and soluble proteins as well as from the polydispersity of the suspension which contained clumps of aggregated micelles up to 1 μm in diameter. In all these cases the relaxation remained single exponential.

A different situation arose when a wet paste of compacted skimmed milk powder was examined. This paste contained particles of aggregated casein visible to the naked eye having radii of at least 400–500 μm. Figure 4 shows that the transverse relaxation was multiexponential with three relaxation components. This result is consistent with the slow diffusive exchange of water between the inside of the compacted protein particles and interstitial solution. Indeed, taking a particle radius of 400 μm, the ratio $(a^2/D)|\Delta\gamma|$ is

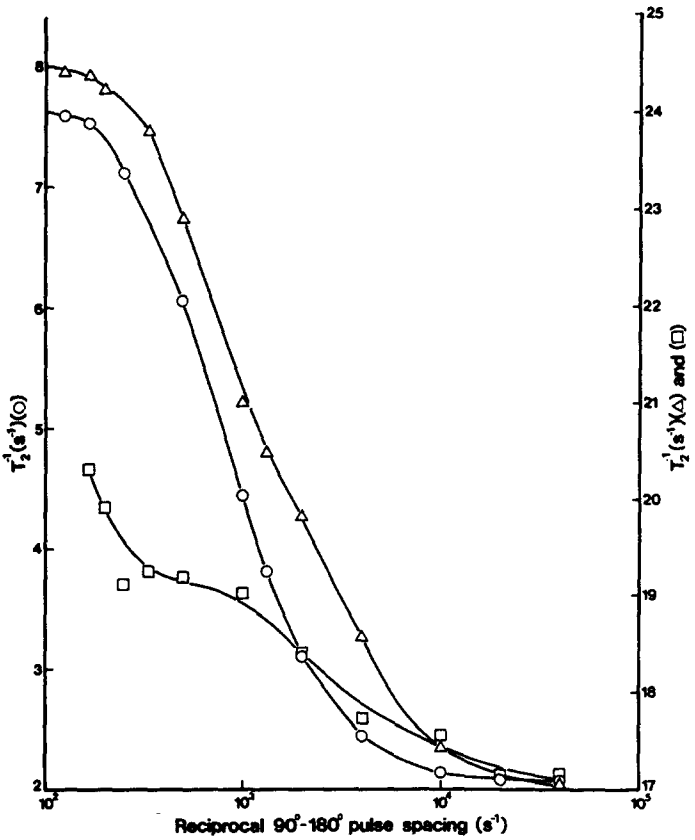

Fig. 3. Transverse water proton relaxation rates at 100 MHz plotted against reciprocal pulse spacing at 298 K. △, Aqueous skimmed milk suspension (~42% w/w). ○, Whey (pH 6·5) from skimmed milk ~15% w/w. □, Suspension of casein micelles (~80% w/w) containing 20 mM $CaCl_2$ and 80 mM KCl.

$\gg 1$ for all reasonable values of the relaxation times for water protons inside the compacted protein particles.

By grinding up the dry compacted skimmed milk powder before suspending it in water the particle size could be reduced to less than 400 μm. This finer suspension gave a biexponential decay whose component relaxation times decrease monotonically with decreasing pulse spacing (Fig. 5) and whose populations also vary with pulse spacing. The observation of a decrease in the number of multiexponential components as the particle size falls is consistent with observations and calculations in Sephadex bead systems (Hills *et al.*, 1989*c*). The biexponential decay undoubtedly arises from slow diffusive exchange between the particulate and solution phases while the pulse spacing dependence could originate from the dispersion in the solution phase analogous to that of whey. Diffusion through magnetic

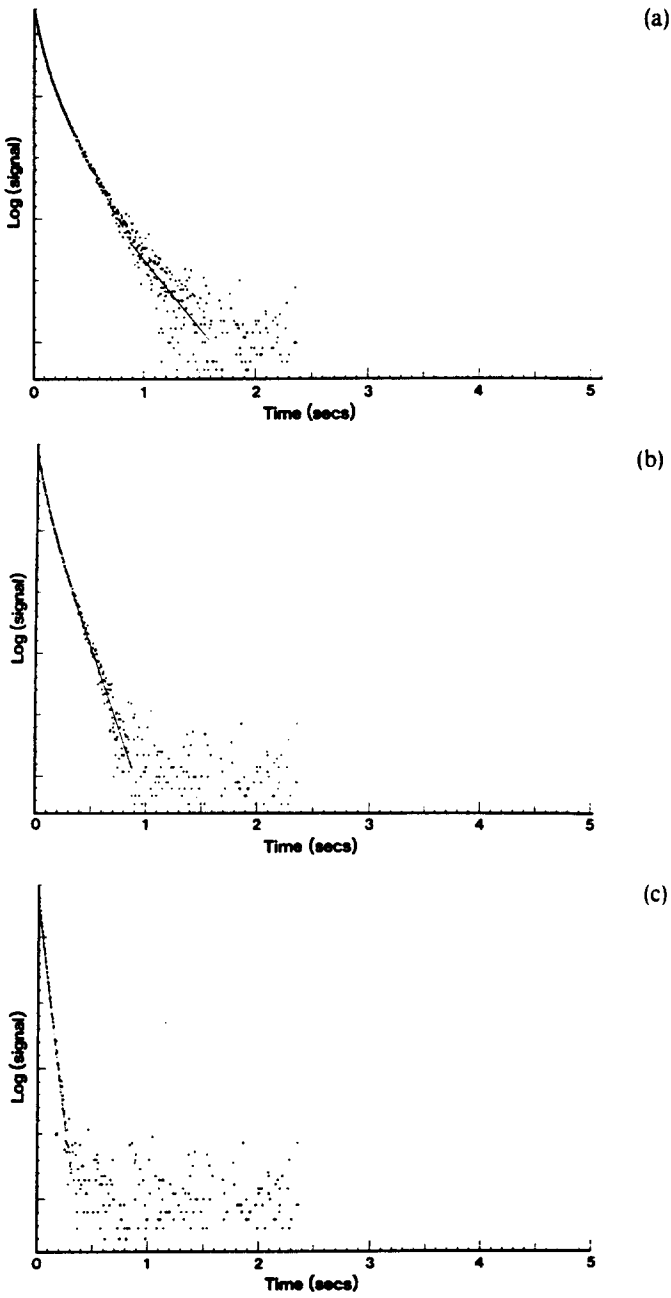

Fig. 4. CPMG echo decay envelope of water proton relaxation in an aqueous suspension of compacted spray-dried skimmed-milk powder. A pulse spacing of 2 ms was used at a spectrometer frequency of 100 MHz. (a) Total decay envelope showing best fit for the first decay component. $T_2 = 0{\cdot}392$ s, population = 12·7%. (b) Best fit for the second component. $T_2 = 0{\cdot}167$ s, population = 44·9%. (c) Best fit for the third component, $T_2 = 62{\cdot}19$ ms, population = 42·4%.

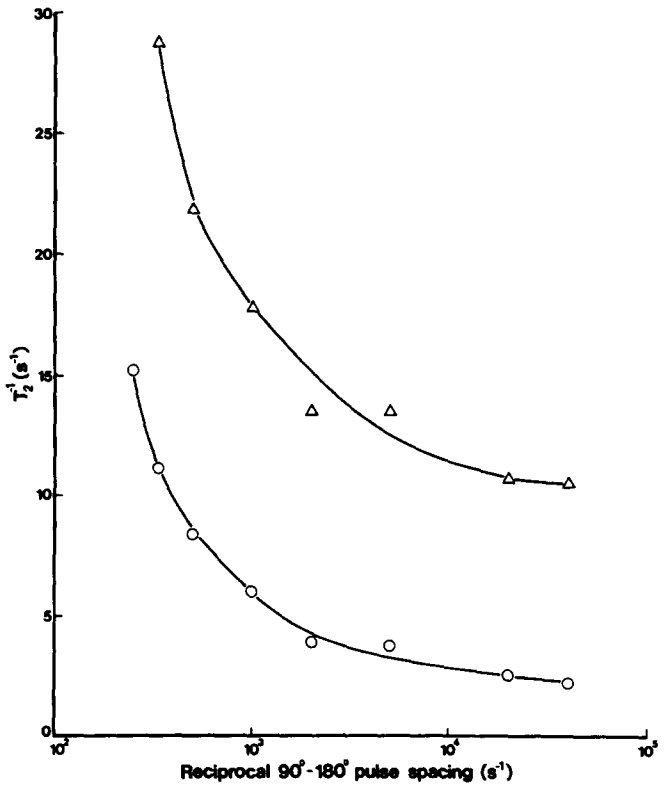

Fig. 5. The variation of the fast and slow water proton relaxation components with reciprocal pulse spacing for an aqueous suspension of finely ground, compacted spray-dried skimmed-milk. Fast (△) and slow (○) decay components of the biexponential CPMG echo envelope.

field gradients, generated at the water–particle interfaces, could also give a monotonic increase in relaxation rate as the pulse spacing is increased (Abragam, 1961; Glasel & Lee, 1974).

The variations observed in suspensions of dried milk powder can therefore be explained in terms of two exchange mechanisms: chemical exchange and diffusive exchange. Chemical exchange gives rise to a dispersion of T_2 and diffusive exchange gives rise to multiexponential behaviour. Diffusion through internally generated field gradients can also contribute to a pulse spacing dependence of T_2. An important feature to note is that the number and relative populations of the multiple exponential components are not necessarily linked with different sites but are reflections of the interactions of diffusion coefficient, morphology and relaxation rate.

Apple fruit obtained after removal of the skin and core is a system in which the typical distance scale arising from the cells is of the order of a hundred microns. The intracellular water is found mainly in large vacuoles

surrounded by a thin layer of cytoplasm pressed against the cell wall. This intracellular water is in diffusive exchange with extracellular water associated with the cell wall. A crude cell wall extract rehydrated to 85% by weight of water gave single exponential relaxation with a relaxation time of 30 ms at all pulse spacings. This short relaxation time is to be expected if there is fast chemical exchange between water and hydroxyl protons on the rigid cell wall polysaccharides such as cellulose and hemicellulose. The hydroxyl protons of these biopolymers have very short relaxation times (T_{2b}) of the order of tens of microseconds so that the condition $T_{2b}^{-1} \gg k_b$ or $\delta\omega$ is valid and eqn (4) is expected to account for the short relaxation time of the extracellular water. The relaxation time of the intracellular water is expected to be much longer, of the order of 0·5–1 s, which was measured for the juice extracted by crushing the apple and filtering it through a 0·22 μm millipore. The diffusion coefficient would be close to that for water; hence the condition $(a^2/D)|\Delta\gamma| \gg 1$ is met and slow diffusive exchange and multiple exponential relaxation is predicted if the cellular dimensions typify the distance scale for diffusive exchange. In slow diffusive exchange, mixing of nuclei from the two sites is slow on the relaxation time scale and hence each site should show a characteristic relaxation curve.

The data for apple fruit show a double exponential relaxation; however, the time constants and relative populations of these components vary with pulse spacings (Figs 6(a) and 6(b)). This variation in population implies that the slow limit has not been completely reached and this may arise because the characteristic dimensions of the cytoplasm and extracellular spaces are significantly smaller than those of the vacuole and thus diffusive mixing may be underestimated by condition 7. It should also be noted that, as the pulse spacing is decreased, the relaxation rates of both components converge. Under these circumstances it may be that the changes in population represent more the difficulties of fitting multiple exponentials than true trends. Even given this difficulty, it does remain true that relaxation rates and populations converge at short pulse spacings, indicating a trend away from strongly double exponential behaviour.

One possibility is that the effect arises due to exchange with solutes, as in the milk samples. However, the filtered juice shows only a very weak dispersion, consistent with a dilute solution (Fig. 6(a)). When the fibre from the crushed apple is repeatedly washed to remove the cytoplasm and the wet cell wall extract is examined, double exponential relaxation is recovered. However, no dispersion is observed and the relaxation consists of a slower decaying component with a rate similar to that of the extracted juice and a faster relaxing component of the order of $\frac{1}{3}$ of the rate of fast relaxation at long pulse spacing (Fig. 6(b)). The observation of a relaxation dispersion in the whole fruit but not in the juice or the wet cell wall extract could be

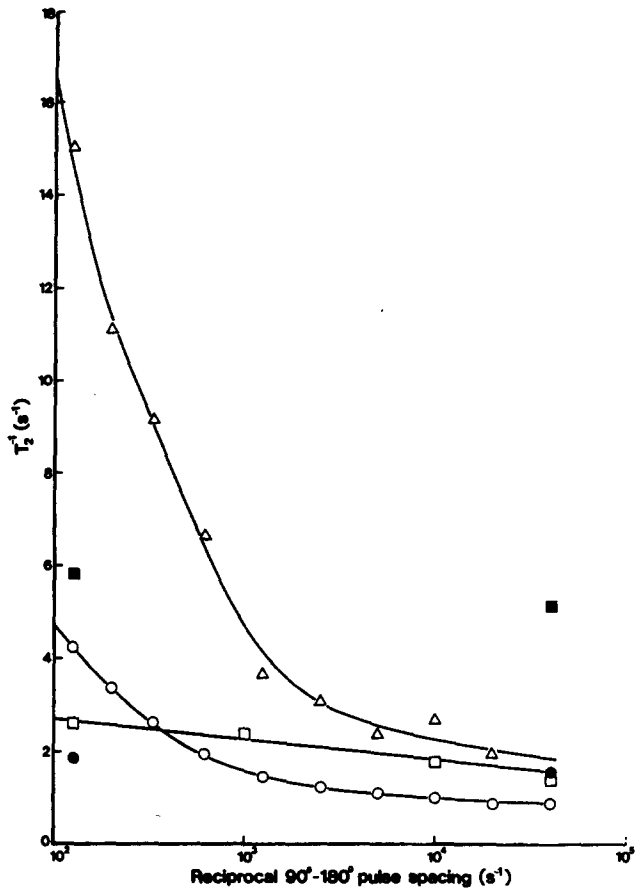

Fig. 6. (a) Transverse water proton relaxation rates at 100 MHz and 298°K for various fractions of apple plotted against reciprocal pulse spacing. Fast (△) and slow (○) components of biexponential decay from apple fruit. Fast (■) and slow (●) components of biexponential decay from washed, crushed apple fibre. (□) Single exponential relaxation rate of the juice from crushed apple.

explained if water protons exchange with some cell wall component such as pectin or protein that is removed by the cell wall extraction procedure. However, a more probable explanation is diffusion of water molecules through internally generated magnetic field gradients. It is known that up to 25% by volume of ripe apple tissue consists of intercellular air gaps (Bollard, 1970). The change in bulk magnetic susceptibility at these curved air–gap water interfaces would be expected to generate quite intense local field gradients. These would be absent in both the juice and cell wall extracts. The dephasing effect of diffusion through these field gradients would be removed at the shortest pulse spacing but would increase monotonically with increasing pulse spacing. There is clearly a need for more detailed

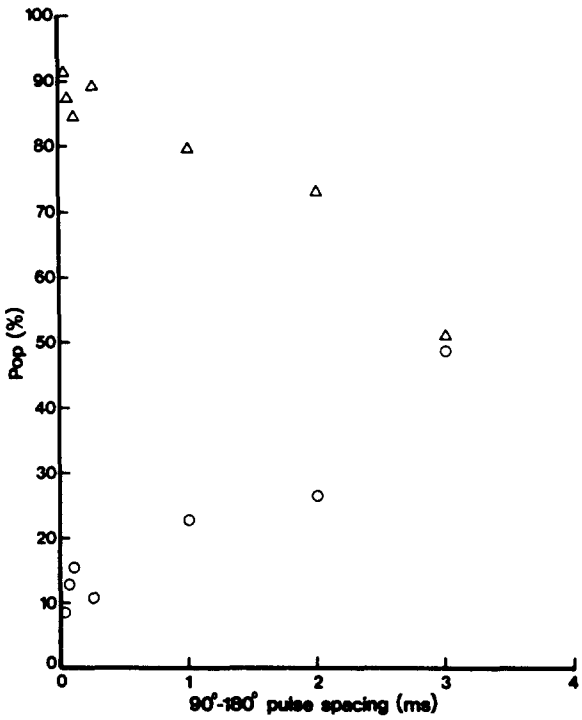

Fig. 6—*contd.* (b) Populations (%) of the biexponential decay components corresponding to Fig. 6(a). Fast (○) and slow (△) components.

experimentation; however, the phenomena observed in the apple system can comfortably be explained with the diffusion-chemical exchange model proposed without recourse to consideration of the state or nature of the water in the system.

CONCLUSIONS

A systematic approach to proton NMR relaxation in aqueous systems requires an understanding of the effects of chemical and diffusive exchange. A quantitative theory of these phenomena is now available (Belton & Hills, 1987; Belton *et al.*, 1988*b*; Hills *et al.*, 1989*b*,*c*) which permits a self consistent explanation of many of the phenomena which are observed. Significantly, the explanation does not involve hypothetical bound water, whose properties are hard to define, but variables available from other measurements such as exchange rates and diffusion coefficients.

Whilst it is true that proton relaxation measurements in aqueous systems may not give useful information about the state of water, they do give very

valuable information about the state of the biopolymers in the system (Hills *et al.*, 1989*b*) and some information about morphology. If this is the case, then the abundant water protons may be considered as an amplifier of the relatively few biopolymer protons and thus, considerably ease the process of measurement.

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