State of water in gelatin solutions and gels: An ^1H n.m.r. investigation

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According to a now well established interpretation, the network growth in gelatin gels results from a conformational coil–helix transition leading to partial renaturing of native collagen. Proton magnetic resonance has been used in order to elucidate the role of water in this process. Proton spin-lattice T_1 and spin-spin T_2 relaxation times, have been measured at various concentrations and quenching temperatures. The results have been interpreted within the framework of a multiphase model involving three populations of water protons in rapid exchange which are affected differently by the macromolecular network growth in the course of gelation. In particular, the model is adequate to explain the time dependence of T_2 after quenching. Our results concerning the spin-lattice relaxation of the bound water protons are in good agreement with those measured in hydrated native collagen or in agarose gels.

(Keywords: gelatin; nuclear magnetic resonance; water; exchange model; network growth)

INTRODUCTION

The gelatin–water system forms elastic gels at room temperature at relatively low concentrations (a few per cent gelatin in water). As in most of the physical gels (agarose, poly(vinyl alcohol) gels, etc.), a three-dimensional network of polymer chains grows during the gelation process. In the case of gelatin (denatured collagen) gels the junction zones of the network are the result of partial renaturing of native collagen as the temperature of the system is lowered below 35°C[1]. Experimental methods such as polarimetry[2], rheology and thermal analysis[1,9] have been used to investigate the properties of these polymer networks.

As this paper is concerned with the ^1H n.m.r. relaxation of water in gelatin gels we shall first briefly describe the collagen structure in order to show how this experimental method could be relevant in the study of gelation of aqueous gelatin solutions.

The collagen unit is a rod of approximately 280 nm length made of three strands, each one being twisted into a left-handed helix of about 0.9 nm pitch and all three being wrapped into a super-right-handed helix with a pitch of 8.6 nm. It has been established that the collagen structure is stabilized by interchain hydrogen bonds, which are of two types: either between CO and NH groups of two polypeptide backbones, or via a water molecule between two CO groups or between CO and NH groups[3]. This led us to search for the existence of a solvent fraction involved in the formation of the network, which is due to helix renaturing.

The proton nuclear spin relaxation times T_1 and T_2 provide information about the dynamics of water molecules and their local environments. We have undertaken in this work ^1H n.m.r. studies to investigate the role of water in the gelation process.

The effects of concentration, temperature and ageing are considered and our results are examined in terms of the Zimmermann–Brittin exchange theory.

In the first section, we describe the preparation of our samples and the n.m.r. measurements. In the second section we report the results obtained from pulsed and wide-line n.m.r. measurements. Finally we propose, in the last section, an interpretation of the results according to a multiphase model.

EXPERIMENTAL

Preparation of the samples

Our gelatin samples come from lime-processed, demineralized ossein kindly provided by Société Rousselot (Isle-sur-Sorgue). Sample characterization has been described elsewhere[2].

For the preparation of the gels, the samples were firstly swollen for about 24 h at 4°C in a solution of water and 0.1 M NaCl (to fix the ionic strength) and a small amount of sodium azide, NaN_3, was added to prevent bacterial contamination. The samples were then dissolved at 45°C (or 60°C for concentrations $C \geqslant 15\%$ g/g) for ~ 30 min, and the pH adjusted to 7 by adding a solution of water and NaOH. The sols were then transferred to the sample tubes (9 mm o.d.), using a dropping pipette. After preparation all the sample tubes were sealed.

N.m.r. measurements

Pulsed n.m.r. experiments were carried out using a Bruker SXP 4–60 MHz spectrometer and the signals were observed on a Tektronix type 468 storage oscilloscope. The n.m.r. absorption signal was obtained from a Varian wide-line spectrometer operating at 7.5 MHz. Spin-lattice

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relaxation times were measured by using the progressive saturation and the inversion–recovery methods.

Spin-spin relaxation times were measured using the Gill–Meiboom sequence because of the large influence of diffusion. With pulse spacings $\tau \simeq 0.4$ ms, the effect of diffusion was then negligible.

A gas-flow cryostat was used to control temperature to within ± 0.1 K.

RESULTS

Spin-lattice relaxation

The gelatin–water system, at all the concentrations studied, is characterized in the sol, as in the gel state, by a single proton spin-lattice relaxation time, with a value very similar to that for pure water. When quenching and annealing the system at temperatures for which it is expected to gel, we found no change in T_1 with time, between the first ten minutes and several months, whatever the concentration of the gel.

Figure 1 shows the temperature dependence of T_1 for $10°C \leqslant T \leqslant 50°C$ at three concentrations: 5%, 11% and 21% g/g. The T_1 values for pure water are also plotted on the same graph. One can see that the temperature dependence is qualitatively the same as in pure water. This feature has already been reported for agarose gels[4,5]. The difference between T_1 for pure water and the gelatin solutions increases with increasing concentration of gelatin. We did not observe any discontinuity in T_1 when lowering the temperature from sol to gel. It should also be noticed that the temperature dependence is exactly the same for both decreasing and increasing the temperature. This is quite different from what is observed for agarose gels[5,6]. Therefore, we can conclude that for gelatin gels, T_1 is dependent only on temperature and concentration and is independent of the thermal treatment or ageing.

Figure 2 reports T_1 values in gels at room temperature (22°C) for twelve concentrations ranging from 1.5% g/g to 21% g/g. The T_1 values decrease rapidly as the concentration of gelatin increases.

In order fully to characterize the system, we measured T_1 as a function of the temperature below 0°C. Below −20°C, the free induction decay signal actually splits into

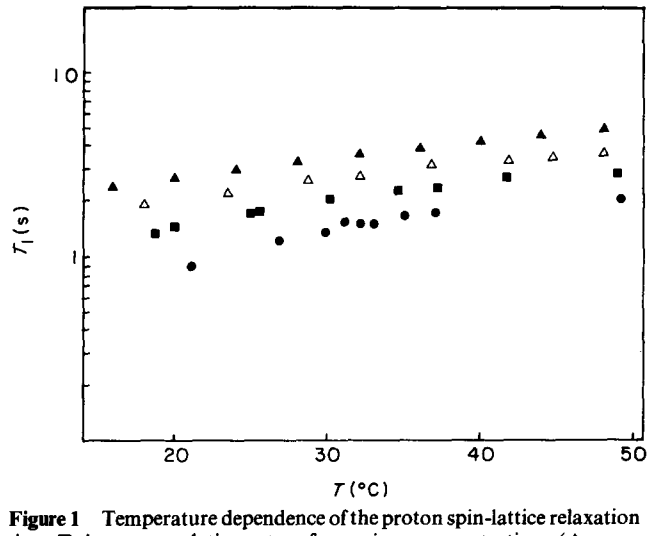

Figure 1 Temperature dependence of the proton spin-lattice relaxation time T_1 in water–gelatin system for various concentrations (▲, pure water; △, $C = 5\%$ g/g; ■, $C = 11\%$ g/g; ●, $C = 21\%$ g/g). Resonance frequency = 40 MHz

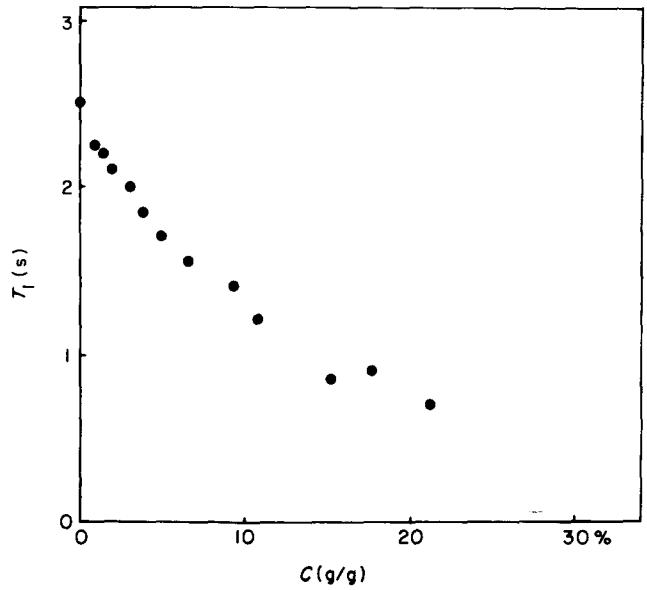

Figure 2 Concentration dependence of the proton spin-lattice relaxation time T_1 in gelatin gels at room temperature (22°C). Resonance frequency = 15 MHz

two components: a fast decay (corresponding to $T_2 \leqslant 20$ μs) and a slow decay ($T_2 \simeq 50$ ms). This sudden change may be attributed to the freezing of a part of the solvent, as in the case of agarose gels[7]. The fast decay represents mostly the ice protons and the slow decay the unfrozen water (see later). Because of the value of the dead time of the spectrometer (20 μs), the fast component could not be investigated. Therefore, below −20°C only the temperature dependence of the spin-lattice relaxation time of the slow component has been measured. We have plotted the results as a function of the reciprocal temperature in *Figure 3* for the whole temperature range and different concentrations and one can observe a minimum for T_1 around $T \simeq -37°C$.

Wide-line n.m.r. results

In agarose gels[4,5], hydrated native collagen[8,9] or gelatin[10,11], the presence of a fraction of water bound to the macromolecules has been proved and its physical properties, compared with those of pure water, are modified. In particular, the bound water does not freeze at the freezing point of bulk water. We have shown that our results, obtained using pulsed n.m.r. techniques, agree with the existence of such a water fraction. The wide-line measurements of the proton absorption spectrum in gels also corroborate this statement. Below −8°C, we can detect on the c.w. spectra a narrow line superimposed on a broad line. In *Figure 4*, we report the spectrum at −34°C. The temperature dependence of the linewidth of the broader line in the range −110 to −8°C, is very similar to that of ice. Therefore, we have assigned the broad line to the frozen water and we shall call it: free water. The width of the narrow line is 65 mG, corresponding approximately to the magnet inhomogeneity. Such mobile protons may be those of the bound water and of the gelatin macromolecules. Indeed it has been proved that not only bound water but also part of the protons of the chemical structure of gelatin are still characterized in the gel state by a mobility much greater than that of ice protons[12,13]. Woessner *et al.*[6] obtained similar results on the agarose gels by pulsed n.m.r.

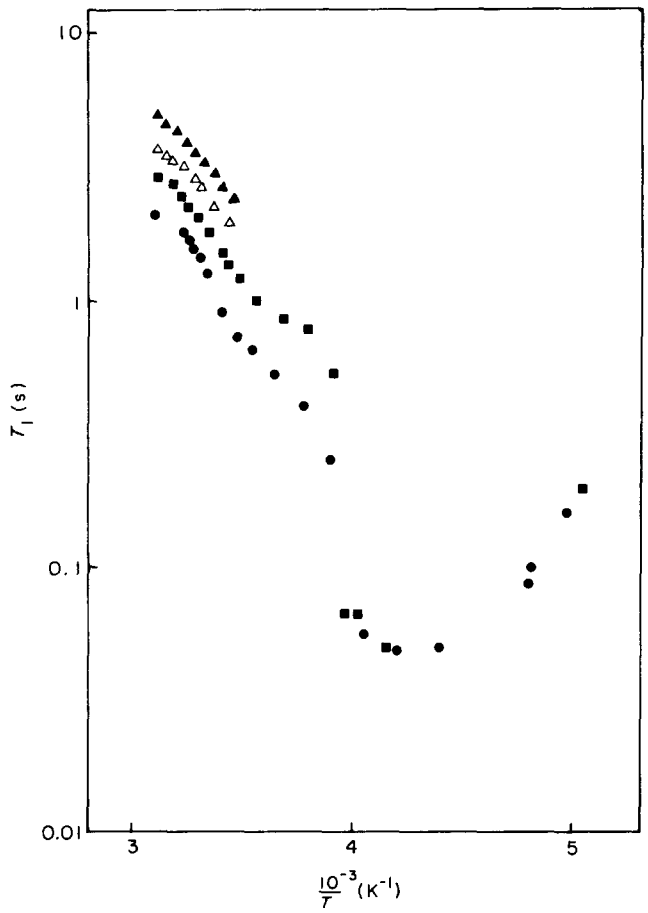

Figure 3 Dependence of the proton spin-lattice relaxation time T_1 on the reciprocal temperature for the same concentrations as in *Figure 1*. Resonance frequency = 40 MHz

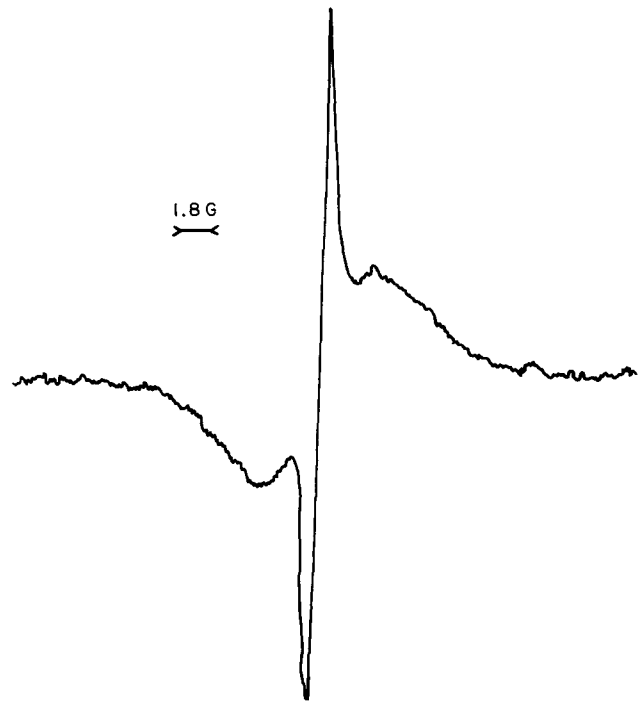

Figure 4 Wide-line n.m.r. spectrum of the protons in a gel ($C = 11\%$ g/g) at low temperatures (-34°C). Resonance frequency = 7.5 MHz

We performed a study of the proton absorption spectrum at 7.5 MHz for six concentrations ranging from 1.5% g/g to 21% g/g at $T = -34$°C. For each concentration we estimated the ratio r of the mobile protons fraction to the ice protons fraction by measuring the area under each line. We have a linear plot $r = f(c)$ with a slope of 0.63 (*Figure 5*). The linearity of the plot shows that the amount of bound water is proportional to the gelatin concentration. However we are not able to deduce from the slope of the plot the value of the bound water population because some gelatin protons contribute to the narrow line, as seen before.

Spin-spin relaxation

The gelatin water system is characterized by a single spin-spin relaxation time in the gel as in the sol state ($10°C \leqslant T \leqslant 50°C$). When quenching the system at temperatures below $+35$°C, we found that the proton spin-spin relaxation time decreased progressively during the course of gelation. We report this behaviour in *Figure 6* for a solution with $C = 21\%$ g/g quenched from 45°C to 24°C. The decrease of T_2 is extremely rapid during the first few minutes, and starts while thermal equilibrium is not completely acheived, T_2 going down from 660 to 200 ms within 15 min. Later, the evolution is much slower, and even after one week we could not find any equilibrium value.

There is a striking analogy between the kinetics of T_2 and χ, the fraction of renatured helices measured by polarimetry[2]. The optical rotation measurement detects the growth of the left-handed helices and gives the amount of helices χ (or the fraction of chains in the helical conformation).

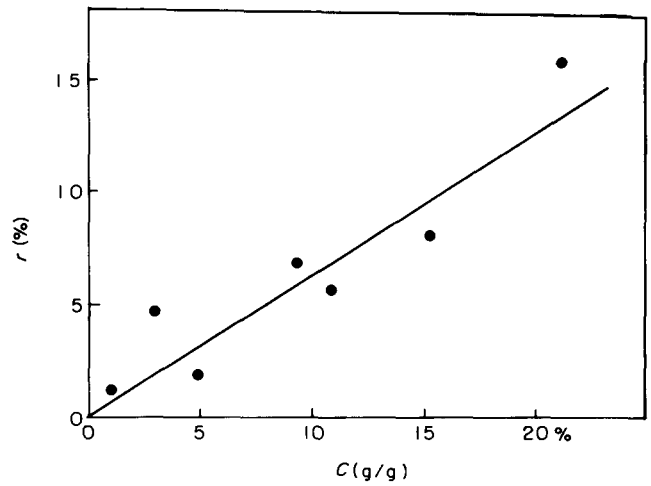

Figure 5 Ratio r of the mobile to the ice protons *versus* concentration as derived from the absorption spectra

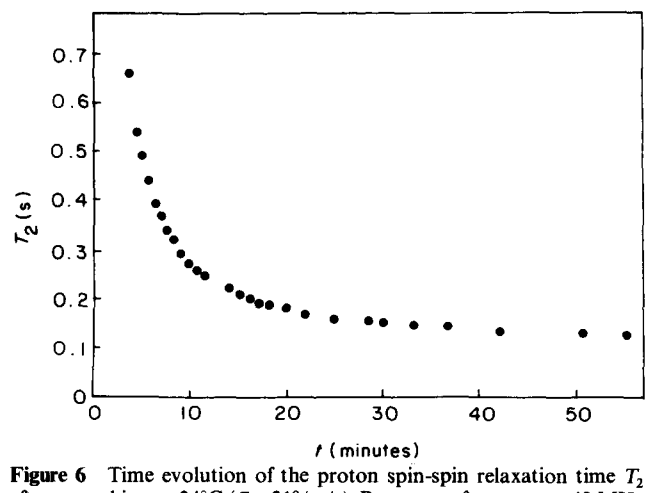

Figure 6 Time evolution of the proton spin-spin relaxation time T_2 after quenching at 24°C ($C = 21\%$ g/g). Resonance frequency = 40 MHz

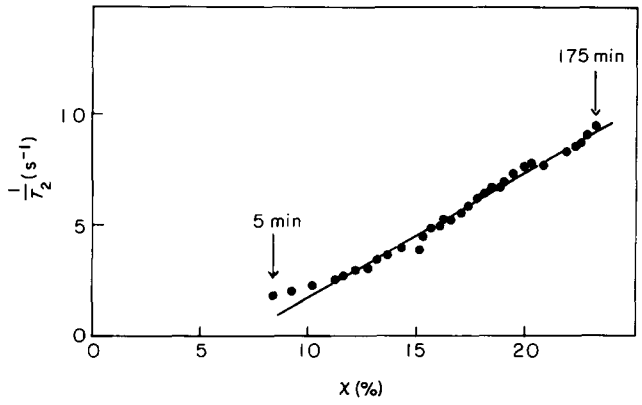

Figure 7 Proton spin-spin relaxation rate T_2^{-1} *versus* the helix amount χ, after quenching at 24°C ($C = 21\%$ g/g)

In *Figure 7*, we have plotted the T_2^{-1} values *versus* χ. It can be seen that between the 7th and the 175th minutes after the beginning of the quenching, the graph is linear to a good approximation. The χ dependence of T_2^{-1} does not seem to be the same before and after the 7th minute after quenching. This difference might be attributed to the uncertainty of the time scale origin in the two types of measurements: the thermal equilibrium conditions are not the same in the polarimetry and n.m.r. experiments. For concentrations $C < 15\%$ g/g, T_2 is a decreasing function of the concentration and it extrapolates to the spin-spin relaxation time of pure water for $C \rightarrow 0$. No kinetic effect could be detected at these low concentrations, although we know, from polarimetry measurements, that helices are renatured.

INTERPRETATION

Exchange model

Free water and bound water. Our study of the gelatin–water system at low temperatures reveals the existence of a fraction of water which does not freeze. As for many water–protein systems[5,10,14,15] this is due to the fact that some water molecules are bound to certain groups, such as hydrophilic groups of the macromolecules. In the range of temperature $T > 10$°C, bound water protons are expected to relax faster than the free water: as their motions are restricted they are less effective in averaging the dipolar interactions.

In spite of the existence of two fractions of water with different relaxation times, we observed in the high temperature range only one spin-lattice and one spin-spin relaxation time. This led us to the assumption of a rapid exchange between free water and bound water protons. For a system consisting of several spin populations with different relaxation times, Zimmermann and Brittin[16] have shown that if a rapid exchange occurs between the nuclei of the different populations, then, only one relaxation time is observed. Rapid exchange is defined as occurring in a time scale much shorter than the characteristic relaxation times of each population. The observed relaxation rate—reciprocal of the relaxation time—is a population-weighted average of the relaxation rates.

Such a model allows us to reconcile the existence of bound water and the uniqueness of the observed relaxation times.

To explain the time dependence of the spin-spin relaxation during the sol–gel transition, we must postulate that a third population arises, characterized by a T_1 much larger than T_2. Such a situation is realized in two principal cases and has been described by Woessner[17,18].

In the first case, a large T_1/T_2 ratio appears which is due to the slowing down of the motion of some bound water molecules adsorbed on strongly adsorbing sites such as hydroxyl groups. Their motion is characterized by a correlation time τ_c sufficiently long, so that it obeys the condition $\omega_0 \tau_c \gg 1$, where ω_0 is the resonance frequency. In the case of gelatin, the strongly adsorbing sites may be the bridging sites of the helices. We shall call the water molecules, bound in these sites, the structural water molecules.

In the second case, the large T_1/T_2 ratio is caused by the presence of an anisotropy in the motion even if the allowed molecular reorientations are rapid compared with ω_0. Therefore, we can assume that the growth of the gel network may cause a diffusion of preferentially oriented water molecules among the domains of partially ordered macromolecules.

Analysis of relaxation during gelation in terms of an exchange model between three water populations (third population is structural water). **'Structural water'**: We recalled in the Introduction section the different collagen structure models proposed up to now. They suppose that the formation of collagen triple helices from α-chains of gelatin is the result of hydrogen bonds between either –NH or –CO groups of the polypeptidic backbone to two different chains or via water molecules[3].

In the gelatin–water system the renaturing of the helices below 30°C leads to the aggregation of the chains and to network growth[19,20]. The network junctions have been described in the literature[21] as rodlike fibrils which are comparable with the native collagen fibrils.

Thus, when quenching a sol below 30°C, part of the water molecules are incorporated into the triple helices as the network is growing. We shall call this fraction 'structural water' and suppose rapid exchange between free, bound and structural water protons. Such a hypothesis has already been proposed[22] in a study of collagen fibril denaturation in solutions of collagen in water (protein concentrations ranging from 0.1% to 1.5%) using ^1H n.m.r. and microcalorimetry. Similar descriptions of proteins solutions, cellular suspensions and tissues have been also proposed[23].

Exchange model: In the sol-state the water molecules of the gelatin–water system can be divided in two fractions, with different relaxation times:

(i) the free water,

(ii) the water bound to the gelatin macromolecules in the coil conformation.

As a gel is forming we shall distinguish between: (a) the free water; (b) the water bound to the coils or to the aggregates of triple helices exposed to the free solvent; (c) the structural water made up of molecules which are bound to the chains in order to stabilize the triple helices, or the aggregates of triple helices.

Let us now apply the Zimmermann–Brittin theory to the gelatin–water system as described in the preceding sections. Let P_f, P_b and P_{st}, T_{if}, T_{ib} and T_{ist} be the populations and the respective relaxation times of free water (f), bound water (b) and structural water (st). Zimmermann and Brittin[16] have shown that, provided

the mean life-time of a proton in regions f, b or st is much shorter than its relaxation time in these regions (rapid exchange hypothesis), the following formula gives the observed relaxation times:

$$\left(\frac{1}{T_i}\right)_{obs} = \frac{P_f}{T_{if}} + \frac{P_b}{T_{ib}} + \frac{P_{st}}{T_{ist}}; \qquad i=1,2 \qquad (1)$$

Our n.m.r. study of gelatin gels at low temperature does not allow us, as stated before, to estimate P_b. So, we have estimated it from the results obtained by different groups in their ^1H n.m.r. studies of bound water in hydrated native or denatured collagen (gelatin). We have reported in *Table 1* the estimated amounts of bound water defined as that part of the water content which does not freeze when the temperature is lowered to a value at which the remainder of the water freezes. A fairly good agreement among the different results can be observed. We chose the value 0.45 g of water per g of gelatin, such a choice being confirmed in the following section. Let us call h this fraction: $h=0.45$ g of H_2O/g gelatin.

The bonding of part of the solvent to the gelatin chains in solution is due mainly to the presence of many polar groups such as OH, NH_2, CO, CONH or ionic groups as COO^-, NH_3^+.

In the course of gelation, a fraction of the bound water molecules is involved in the stabilization of the helices and becomes the structural water population. Thus we can predict that the fraction of bound water molecules decreases while the aggregates grow. We may also take into account a contribution of free water to the structural water population, but we shall show that it would have a second order effect on the relaxation time values.

To conclude we shall suppose the constancy of $P_b + P_{st}$ in the course of gelation.

Time dependence of the spin-lattice and spin-spin relaxation: Let us suppose that the gelatin–water system is in the sol state at any time $t<t_0$. At time $t=t_0$, we quench the sample at a given temperature. At time $t>t_0$, gelation and so formation of macromolecular lattice starts. Let $x(t)$ be the ratio of the fraction of structural water protons to the fraction of bound water protons. We can write equation (1) in the following form:

$$\frac{1}{T_i} = \frac{P_f}{T_{if}} + \frac{P_b[1-x(t)]}{T_{ib}} + \frac{P_b x(t)}{T_{ist}} \qquad i=1,2 \qquad (2)$$

where $x(t_0)=0$ (sol state).

We can see that taking into account the contribution of free water to the structural water population, would consist in multiplying P_f by a term $[1-y(t)]$. The relative variation of P_f as the gel is forming would be nevertheless negligible.

Table 1 Hydration amounts, h, for native collagen and gelatin from literature

	Ref. 21	Ref. 8	Ref. 22	Ref. 9
h (g/g)	0.50	0.54	0.45	0.50

For $t>t_0$ the relaxation times may be written:

$$\frac{1}{T_i} = \frac{1-hc}{T_{if}} + \frac{hc}{T_{ib}} + hc\,x(t)\left[\frac{1}{T_{ist}} - \frac{1}{T_{ib}}\right] \qquad (3)$$

where $P_b = hc$ and $P_f = 1-hc$, h being the amount of bound water per unit mass of the macromolecule.

From the plot of T_2^{-1} *versus* χ in *Figure 7*, we shall assume that $x(t)$ is proportional to $\chi(t)$. So:

$$x(t) = m\chi(t) \qquad (4)$$

An estimate of m can be obtained in the following way:

$$m = \frac{x}{\chi} = \frac{\text{No. of structural } H_2O \text{ protons}}{\text{No. of helical residues}} \times \frac{\text{total number of residues}}{\text{No. of bound } H_2O \text{ protons}}$$

The first factor of equation (3) can be evaluated from ref. 3, equal to 2/3; the second factor, called f, can be calculated with $h=0.45$ g of water/g of gelatin and taking the molar weight of a residue equal to 100 g.

So we have:

$$f^{-1} = \frac{0.45 \times 100 \times 2}{18} = 5$$

So the order of m is $\frac{2}{15}$.

In the temperature range $20°C < T < 25°C$, it is reasonable to assume that $T_{1b} \sim T_{2b}$. For pure water, $T_{1f} = T_{2f}$, thus equation (3) can be rewritten in the form:

$$\frac{1}{T_i} = \frac{1}{T_0} + hcm\chi(t)\left[\frac{1}{T_{ist}} - \frac{1}{T_b}\right], \qquad i=1,2 \qquad (5)$$

Let us make some further assumptions concerning T_1: $T_{1st} \geqslant T_{1b}$; taking $T_{1b} \simeq 300$ ms and $C=20\%$ g/g, $m=0.13$ and $\chi=25\%$ the second term in equation (5) is, at maximum, equal to $10^{-2}\,s^{-1}$ which is negligible compared with T_0^{-1}, equal to $0.6\,s^{-1}$. So we cannot expect to see any kinetic effect on T_1.

On the other hand, however, assuming that the spin-spin relaxation time of the structural water is much smaller than that of the bound water, $T_{2st} \ll T_{2b}$, we can predict that the second term of equation (5) will vary considerably with time.

From the slope of T_2^{-1} *versus* χ (*Figure 7*), we can deduce an estimation of $T_{2st} \simeq 0.2$ ms. During the first 175 min, T_2 varies between

$$70\text{ ms} < T_2 < 1.66\text{ s}$$

which corresponds to the values measured experimentally.

Finally we can conclude that the expressions of T_1^{-1} and T_2^{-1} in the framework of our model are the following:

$$\frac{1}{T_1} \simeq \frac{1-hc}{T_{1f}} + \frac{hc}{T_{1b}} \qquad (6)$$

$$\frac{1}{T_2} \simeq \frac{1-hc}{T_{2f}} + \frac{hc}{T_{2b}} + hcm\chi(t)\left[\frac{1}{T_{2st}} - \frac{1}{T_{2b}}\right] \qquad (7)$$

Analysis of relaxation during gelation in terms of an exchange model between three water populations (third population is a fraction of bound water submitted to an anisotropic motion). We may assume that the bound water molecules which diffuse along the macromolecules (in the helix conformation) have a preferential orientation. So, the diffusion motion of the bound water in the helices is strongly anisotropic, indicating that the interproton vector moves rapidly along a preferential axis, this axis itself undergoing a much slower reorientation, or wobbling with a fairly low frequency. Woessner[18] has shown that the contributions of these two types of motions to the relaxation phenomena are not independent. However, under certain conditions, the spin lattice relaxation time, T_{1bh}, of the bound water in the helix conformation is mainly dominated by the fast reorientation, while the spin-spin relaxation of these molecules, T_{2bh}, is determined by the slower motion. In this case:

$$T_{1bh} \gg T_{2bh}$$

In the gelatin solution, this situation can be understood in the following way: in the sol-state the macromolecules are in the coil conformation. The anisotropic character of the interproton vector motion of the bound water is cancelled either by the fast change of orientation of the residues, or by the translational diffusion along the random oriented residues of a protein chain. One can fairly assume that:

$$T_{1bc} = T_{2bc}$$

and the relaxation times are equal in the coil conformation.

During gelation, the anisotropic character of the bound water motion appears in the region of triple helix aggregates. The population of water molecules undergoing an anisotropic motion is given by the fraction of water bound to the residues in the helical conformation, i.e. $hc\chi$. One may write:

$$\left(\frac{1}{T_i}\right)_{obs} = \frac{1-hc}{T_{if}} + hc(1-\chi)T_{ibc} + \frac{hc\chi}{T_{ibh}} \quad , \qquad i=1,2 \quad (8)$$

In the temperature range $20°C < T < 25°C$, we can assume that:

$$T_{1bh} \sim T_{1bc} \sim T_{2bc} = 300 \text{ ms}$$

while

$$T_{1bh} \gg T_{2bh}$$

Finally, we can conclude that

$$\frac{1}{T_{1,obs}} \simeq \frac{1-hc}{T_{1f}} + \frac{hc}{T_{1bc}} \qquad (9)$$

$T_{1,obs}$ is independent of the helix amount χ, and

$$\frac{1}{T_{2,obs}} \simeq \frac{1-hc}{T_{2f}} + \frac{hc}{T_{2bc}} + \frac{hc\chi}{T_{2bh}} \qquad (10)$$

From the slope of T_2^{-1} *versus* χ (*Figure 7*) we can deduce an estimation for $T_{2bh} \simeq 1.5$ ms.

Concentration dependence of the spin-lattice relaxation. In *Figure 8* we have plotted the spin-lattice relaxation rates *versus* concentration derived from *Figure 2*. The graph is linear in all the concentration ranges and the T_1^{-1} value for $C=0$ is that of pure water. In *Figure 9*, we plot T_1^{-1} *versus* concentration at various temperatures. In the sol (48°C, 36°C) as well in the gel state (24°C, 30°C) the graph is linear and for $C=0$ we obtain the pure water relaxation rate at the considered temperature.

These results confirm our analysis of the observed spin-lattice relaxation time in terms of exchange between free and bound water.

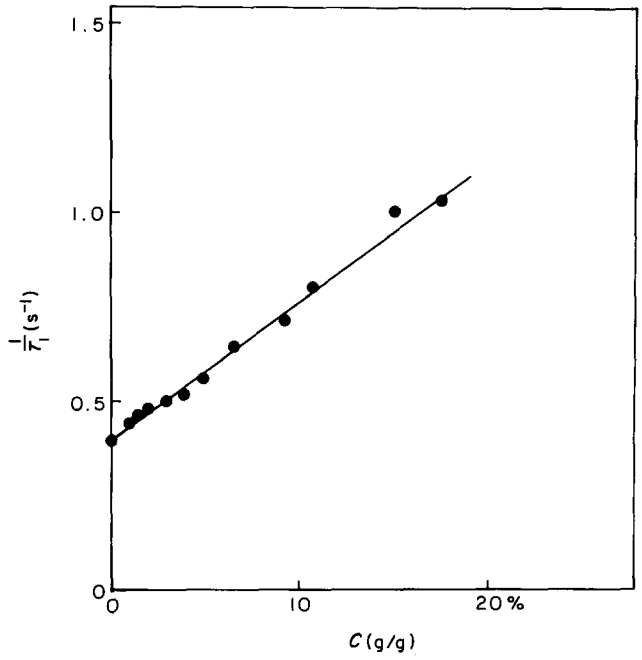

Figure 8 Concentration dependence of the proton spin-lattice relaxation rate T_1^{-1} at 22°C. Resonance frequency = 15 MHz

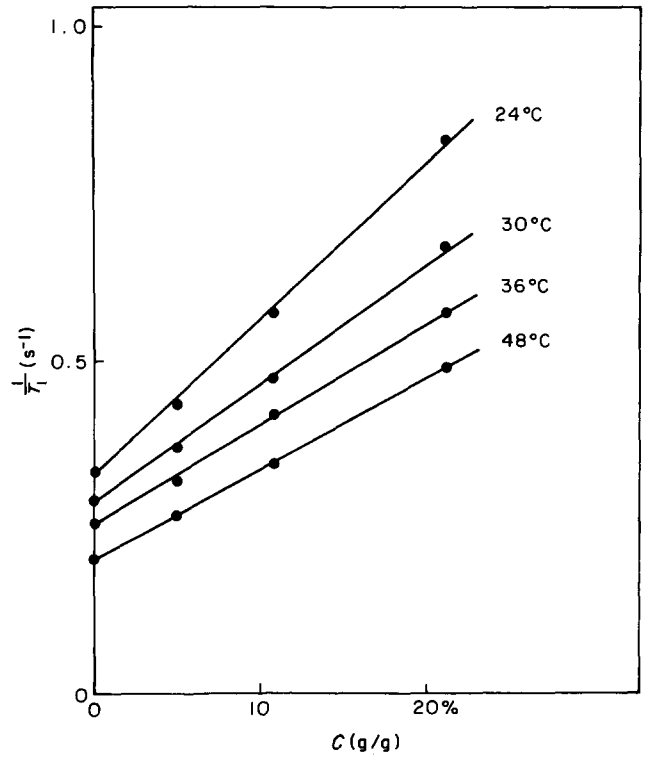

Figure 9 Concentration dependence of the proton spin-lattice relaxation rate T_1^{-1} for various temperatures corresponding to the sol and gel states. Resonance frequency = 40 MHz

Making the assumption that the model still applies at temperatures as low as $-20°C$, we deduced T_{1b} from the measured T_1 in the temperature range $-20°C \leqslant T \leqslant +50°C$, for three concentrations: 5%, 11%, 21%. We report in a semilog plot in *Figure 10*, the calculated values of T_{1b} using equation (6) in the range $-20°C \leqslant T \leqslant +50°C$ and those measured directly for $-80°C \leqslant T \leqslant -20°C$, *versus* the reciprocal of the temperature.

We can see that T_{1b} values obtained for different concentrations coincide with one another. The order of magnitude of T_{1b} is characteristic of water adsorbed on proteins[24].

The graph of T_{1b} *versus* T^{-1} in the range $-80°C$ to $+50°C$ (*Figure 10*), forms a continuous curve from high to low temperatures. Turning back to *Figure 3*, we can see that the discontinuity observed in the plot $\ln T_1(T^{-1})$ is due to the fact that, below $-20°C$, the exchange between bound water and free water protons suddenly slows down, so that the free water does not contribute to the T_1 value any more. Such a phenomenon is known as an 'apparent phase transition' and is often observed in the n.m.r. studies of systems in which exchange between several populations of nuclei occurs[25].

The combining in *Figure 10* of the plots obtained by different methods (bound water at high temperature and water that does not freeze at low temperature) agrees with the value of the hydration coefficient $h = 0.45$ g/g, which was measured by other workers.

Figure 10, the semilog plot of T_{1b} *versus* T^{-1} presents a minimum (48 ms) at $T = -37°C$ for $\omega = 40$ MHz. This minimum is much shallower than would be expected from the BPP theory[26] where it is assumed that relaxation is due to one type of molecular motion characterized by one correlation time τ_c. It is well known in the n.m.r. study of adsorption[27] that such a fact can be explained within the framework of the BPP theory by the existence of a distribution of correlation times. In such a case the shallowness of the T_{1b} minimum depends upon the spreading of τ_c values around their mean value $\langle \tau_c \rangle$. In the case of the bonding of water molecules to the α-chains of gelatin or the collagen fibrils, it would be undoubtedly an oversimplification to consider the molecular motions as being characterized by one correlation time. On the contrary, we expect the numerous macromolecular binding sites to be characterized by different activation energies and correlation times spread around a mean value.

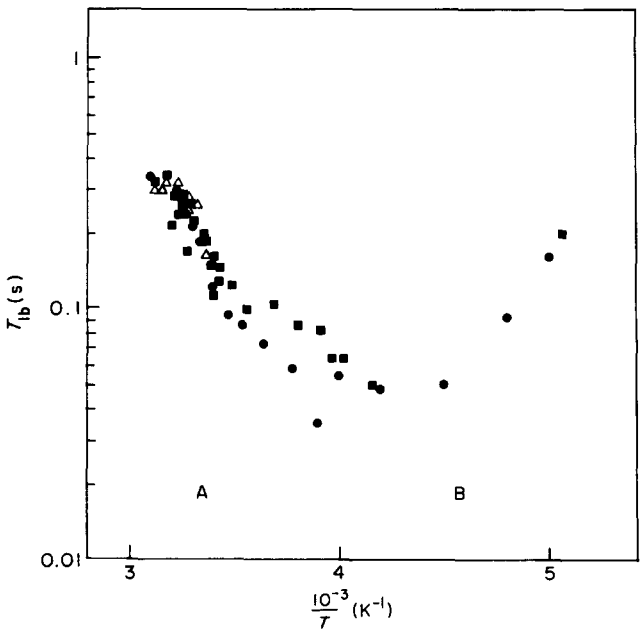

Figure 10 Temperature dependence of the proton spin-lattice relaxation time T_{1b} of the bound water for three concentrations. In the temperature range (A) T_{1b} is calculated using equation (6); in the temperature range (B) T_{1b} is directly measured (see *Figure 3*)

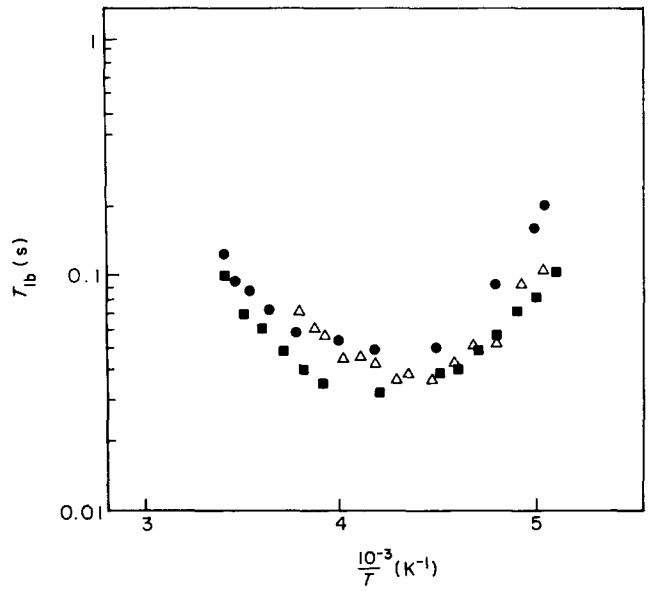

Figure 11 Comparison of the temperature dependences of the proton spin-lattice relaxation time T_{1b}: ■, in hydrated native collagen (ref. 8); △, in agarose gels (ref. 6); ●, in our gelatin samples

In *Figure 11* the results concerning the spin-lattice relaxation of the bound water on hydrated native collagen (from ref. 8) and in agarose gels (from ref. 6) are presented for comparison. The definition of the bound water is the same as ours and very good agreement can be seen concerning the value of the T_{1b} minimum, as well as its temperature position and the shallowness of the curve near it.

CONCLUSION

The results presented in this study reveal the interest of an ^1H n.m.r. study of gelation in elucidating the role of a solvent. Application of a simple exchange model allows us to explain our results as a whole. Moreover the similarity of the relaxation times proves the importance of the exchange phenomenon in gelatin gels, contrary to what is observed in some studies of agarose gels[4].

The most important result that we have obtained was to prove the correlation between polarimetry and ^1H n.m.r. data. We have proposed an interpretation of the n.m.r. data in terms of a third water population growth when the gelation starts. We have made two alternative hypotheses for the origin of this third population, which is always a fraction of the bound water. In the first case, the third population is the bridging water of the gel structure, while in the second case, it is constituted by the bound water molecules subjected to anisotropic motions inside the gel network. It is difficult to make a choice between these two descriptions, both of them being in agreement with the n.m.r. data. Nevertheless, the first model (the structural water) has the advantage of being in accordance with the well-known structure of collagen, which implies the existence of bridging water in the triple helices.

Moreover, it has also been proved that in the presence of heavy water, the helix renaturing of denatured collagen in solution is accelerated[28]

Further investigation of the gelation of aqueous gelatin solutions is under way, particularly using high resolution ^1H n.m.r. in order to observe the evolution of the spectrum with time and to determine which residues contribute to the gel growth.

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