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^{1}H NMR studies: dynamics of water in gelatin

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Abstract Proton magnetic resonance was used to characterize the dynamics of water in gelatin. Both sol and gel states were investigated. Transverse relaxation rates (R_2) were dependent on the proton frequency measurement. (R_2) measured with the Carr-Purcell-Meiboom-Gill pulse sequence was dependent on pulse spacing. These observations were interpreted in terms of chemical exchanges between water protons and those of the macromolecules in the sol state, whereas in the gel state the contribution of diffusion through microheterogeneities in the sample seems to provide an additional transverse relaxation mechanism.

Key words Gelatin · Sol · Gel · Relaxation rates · Exchange rate

Introduction

Gelatins are water soluble products of thermal or chemical degradation of collegenous tissues. Their amino acid sequences are almost identical to their parent collagens. A reversible sol-gel phase transition is one of the main characteristics of water-gelatin systems. The gelling of gelatin results in the formation of a three-dimensional network of cross-links and is considered as a partial reformation of the collagen structure (Harrington and Von Hippel 1961). The specific interactions between water and gelatin chains make an important contribution to the stabilization of gelatin gel. NMR provides information on water dynamics from longitudinal ($R_1 = 1/T_1$) and transverse ($R_2 = 1/T_2$) relaxation rates, and diffusion coefficients (D) of water protons. The studies performed on the relaxation rates of water protons in protein solutions have been interpreted in terms of exchange between water belonging to the hydration layer and free water (Blinc et al. 1995; Edzes and Samulski 1977, 1978; Maquet et al. 1986). The dispersion observed when the transverse relaxation rate R_2 is plotted versus the Carr-Purcell-Meiboom-Gill (CPMG) pulse repetition rate was explained by chemical exchange between water protons and those exchangeable on the macromolecules (Hills 1992a, b). Santyr et al. (1988) explained this dependency of R_2 in gelatin in terms of the "spin-locking" effect. Spin-locking using a very short echo time measures the rotating-frame spin-relaxation rate (Farrar and Becker 1971). In this experimental condition, the measured parameter did not correspond to R_2. Brown and Fantazzini (1993) assumed that the only source of this dependence was diffusion in the presence of field gradients due to susceptibility differences. Our aim was to determine the relaxation mechanism responsible for the R_2 behavior. The effects of various experimental variables such as gelatin concentration, gel strengths expressed in Bloom, pH, temperature, and measurement frequency were taken into account to characterize the water-protein interactions.

Materials and methods

Gelatin powders (Sigma) of two gel strengths (60 and 300 Bloom) from porcine skin were dissolved in deionized distilled water at 60 °C. Sodium azide (400 ppm) was added to prevent microbiological growth. Four pH values were used: 4.85, 6, 7.15, and 8. The final concentrations were in the range 5–20% (weight of dry gelatin per total weight).

^{1}H NMR measurements were carried out at 400 and 20 MHz on Bruker AMX400 Bruker PC20 spectrometers respectively. Field frequency lock was not required.

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The temperature ranged from −20 to 60 °C. The temperature was controlled to ±0.1 °C. The CPMG pulse sequence was used to determine the transverse relaxation rates. At time τ after the 90° excitation pulse, a train of 180° refocusing pulses at a frequency $1/2\tau$ was used to generate echoes for the transverse relaxation measurements. The 90–180° pulse spacing (τ) was varied between 50 µs and 2 ms. At 20 MHz the acquisition time was maintained constant (800 ms) irrespective of the interpulse delay by varying the echo number between 40 and 160. At 400 MHz the echo number ranged between 2048 and 16 382 for a 1682 ms constant acquisition time.

Results

R_2 values were measured for different echo times. Figure 1 illustrates the variation in R_2 (400 MHz) for 300 Bloom gelatin as a function of $\Gamma = 1/\tau$ for different concentrations (5, 10, 15, and 20%) at 40 °C (sol state). The experimental values decreased markedly with increasing Γ. For the highest concentration, the R_2 value measured for 500Γ was six times that measured for 20 000Γ. It is noteworthy that the dispersion increased with the concentration. Figure 2 shows R_2 as a function of Γ for different pH values (6, 7.15, and 8). The curve shapes remained similar to those obtained previously. The R_2 values increased with increasing pH. However, the R_2 dispersions at acid pH were less than those at pH values above 7.

The state of the gelatin sol or gel was investigated for two gelatins (15%; pH 6) at two strengths (60 and 300 Bloom). Figure 3 shows their R_2 values as a function of Γ for the gel and sol state (10 and 40 °C). The shapes of the experimental curves were very similar. However, for all the gel strengths the dispersion was greater at 40 °C than at 10 °C. At a given temperature, R_2 values for 60 Bloom gelatin were higher than for 300 Bloom gelatin. At high Γ the R_2 values were lower at 40 °C than at 10 °C, whereas the opposite was observed at low Γ.

At 400 MHz, for 1000 $s^{-1}\Gamma$, R_2 decreased from 40 °C to 10 °C whereas for 20 000 $s^{-1}\Gamma$, R_2 increased (Table 1). Likewise, for 1000 $s^{-1}\Gamma$ the R_2 values were always lower at 20 MHz than at 400 MHz at any state of the gelatin. The sol-gel transition in gelatin solutions induced an increase in R_2 at 20 MHz. The values measured at 20 MHz for 1000 $s^{-1}\Gamma$ were similar to that observed at 400 MHz for 20 000 $s^{-1}\Gamma$.

Discussion

With the CPMG sequence, the echo amplitude detected at time t is given by:

$$M(t) = M_0 \times \exp\left(-t \times \left[R_2 + \frac{D\gamma^2 G^2}{3\Gamma^2}\right]\right) \quad (1)$$

where γ is the gyromagnetic ratio and D the molecular self diffusion constant, G is the magnetic field gradient, which can result either from the static magnetic field B_0 inhomogeneity or from the susceptibility difference across the sample. In a highly homogeneous field and in a homogeneous sample, the behavior of R_2 as a function of Γ may be explained by the exchange of protons between chemically shifted sites (Carver and Richards 1972; Luz and Meiboom 1963; Swift and Cornick 1962). The decay rate of the echoes for Γ longer than 200 s^{-1} can be described by the Luz-Meiboom relation (Luz and Meiboom 1963) as:

$$R_2 = (R_2)_0 + k_e^{-1} \times \left[1 - \Gamma k_e^{-1} \times \tanh\left(k_e \Gamma^{-1}\right)\right] \times p_b \delta_b^2 \omega^2 \quad (2)$$

$$(R_2)_0 = R_w + p_b \times (R_{2b} - R_w) \quad (3)$$

where k_e is the rate of proton exchange, p_b the exchangeable proton population, δ_b the chemical shift difference in ppm, between water and exchangeable protons, and ω the measurement frequency; $(1 - p_b)$ is the fraction of bulk water protons with the relaxation rate R_w. R_{2b} is the weighted sum of spin-spin relaxation rates of water molecules at the protein interface that have lifetimes dependent on the number of binding hydrogens (Koenig et al. 1993).

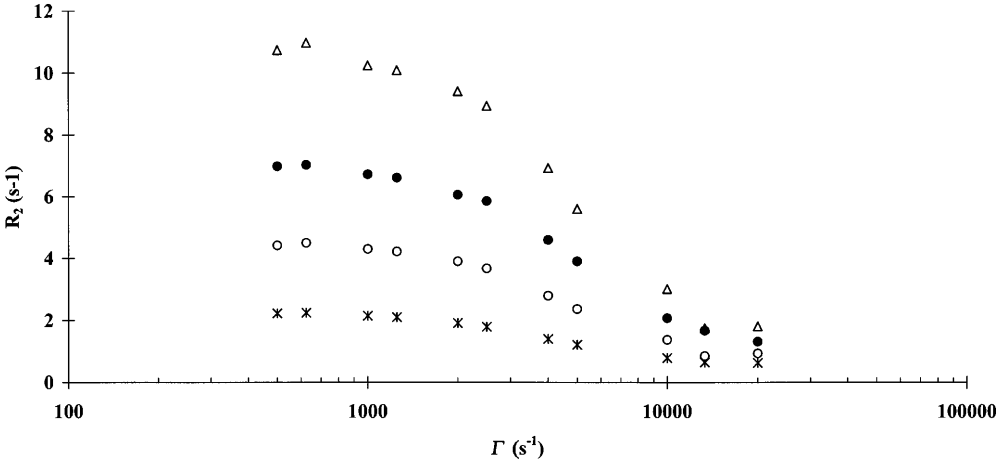

Fig. 1 Variation of the relaxation parameter R_2 as an inverse function of interpulse delay Γ for gelatin (300 Bloom; pH 4.85) at 40 °C for four concentrations: (⁎) 5%; (○) 10%; (●) 15%; (△) 20%

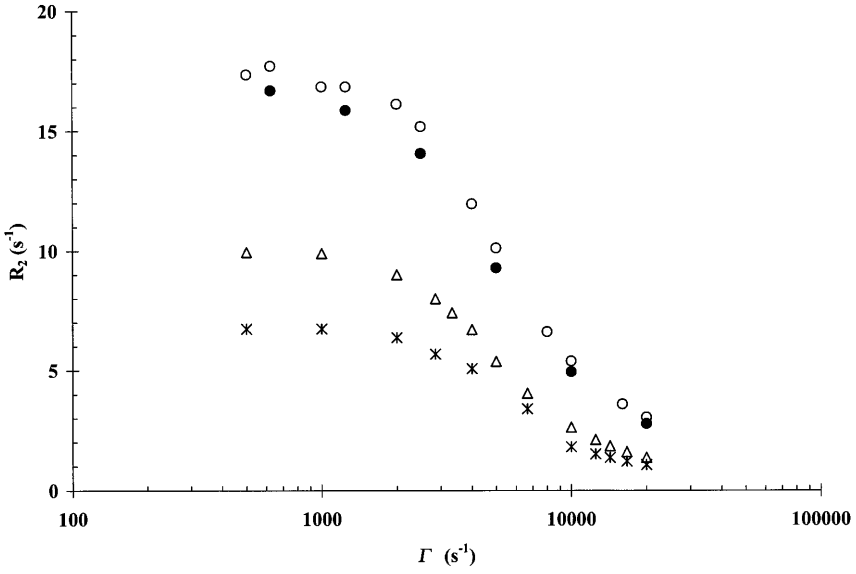

Fig. 2 Variation of the relaxation parameter R_2 as an inverse function of interpulse delay Γ for gelatin (300 Bloom; concentration 15%) at 40 °C for four pH values: (⁎) 4.85; (△) 6; (●) 7.15; (○) 8

In the limit of the fast echo rate (Γ) with $k_e \gg R_{2b}, R_2$ is not dependent on the exchange process and is expressed as R_{2F}:

$$R_2 = R_{2F} = (R_2)_0 \tag{4}$$

and in the limit of the slow echo rate with $k_e \gg \Gamma$, R_2 is expressed as R_{2S}:

$$R_2 = R_{2S} = (R_2)_0 + \frac{p_b \delta_b^2 \omega^2}{k_e} \tag{5}$$

These equations agree with those obtained by Hills et al. (1990), using the Carver and Richards equation (Carver and Richards 1972). The amplitude of the dispersion ΔR_2, which is the difference between R_{2S} and R_{2F}, is equal to $p_b \delta_b^2 \omega^2 / k_e$.

From our experimental results, the different parameters k_e, $(R_2)_0$, and $p_b \delta_b^2 \omega^2$ were determined and are given in Tables 2–4. The rate of exchange k_e did not vary significantly with different gelatins except for the 5% concentration. In the sol and gel states of gelatin the k_e values were very similar. Hills (1992b) has already reported this observation. However, our k_e values were twice those of Hills: $\approx 5 \times 10^3$ s^{-1}. This difference may be due to the difference in Bloom number. Moreover, Hills' gelatin came from human skin collagen.

The R_2 values depended on concentration and pH. Assuming a number of exchangeable gelatin protons equal to 0.33 per 100 g of gelatin at 4.85 pH (Veis 1964), the p_b was calculated for each concentration. From this assumption, a linear relationship was found between $(R_2)_0$ and p_b ($R^2 = 0.993$; $F = 297$). The intercept of 0.39 s^{-1} corresponds to R_w and is in agreement with the relaxation rate of free water. The slope of 64 s^{-1} corresponds to R_{2b}, which is the weighted sum of spin-spin relaxation rates of water molecules at the protein inter-

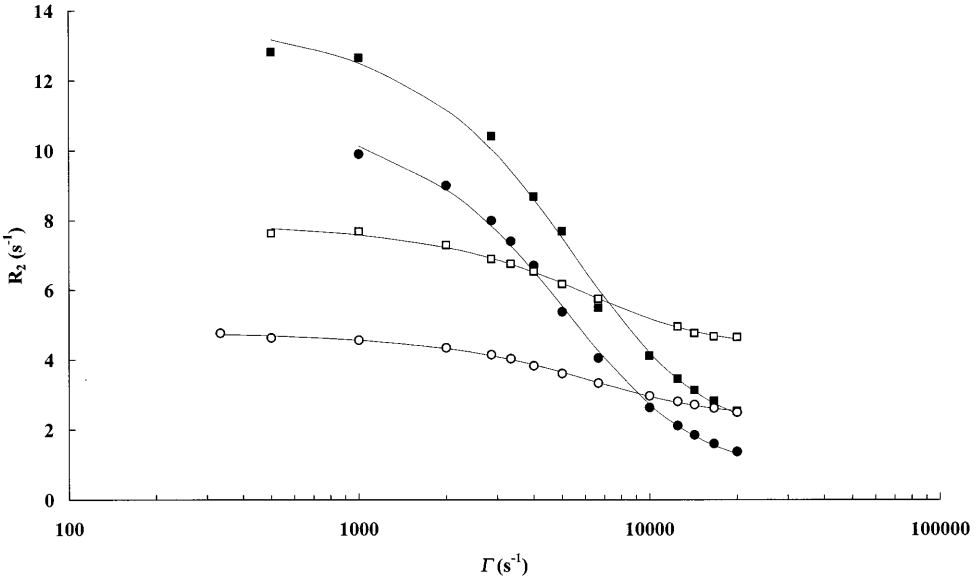

Fig. 3 Variation of the relaxation parameter R_2 as an inverse function of interpulse delay Γ at two temperatures for two strengths of gelatin: (■) 60 Bloom, 40 °C; (●) 300 Bloom, 40 °C; (□) 60 Bloom, 10 °C; (○) 300 Bloom, 10 °C at the same concentration (15%)

Table 1 R_2 as a function of measurement frequency at two temperatures for two Γ for gelatin; 300 Bloom, 15% concentration, pH 6

Γ (s^{-1})	20 MHz	400 MHz	
	1000	1000	20 000
10 °C	2.65	4.57	2.54
40 °C	0.82	9.9	1.31

Table 2 Parameters determined from Eq. (2) for water protons of gelatin at different concentrations; 300 Bloom gel strength, 40 °C, pH 4.85

Concentration (%)	k_e ($\times 10^3$ s^{-1})	$(R_2)_0$ (s^{-1})	$p_b\delta_b^2\omega^2$ ($\times 10^3$ s^{-2})
5	7.31 ± 0.29	0.49 ± 0.03	13.9 ± 0.6
10	8.31 ± 0.61	0.56 ± 0.11	35.4 ± 3.0
15	8.86 ± 0.40	0.81 ± 0.10	59.5 ± 3.3
20	8.84 ± 0.63	0.82 ± 0.30	95.7 ± 8.1

Table 3 Parameters determined from Eq. (2) for water protons of gelatin at different pH values, at two temperatures; 15% concentration, 300 Bloom gel strength

Temperature (°C)	pH	k_e ($\times 10^3$ s^{-1})	$(R_2)_0$ (s^{-1})	$p_b\delta_b^2\omega^2$ ($\times 10^3$ s^{-2})
10	4.85	7.4 ± 0.6	3.80 ± 0.3	14.2 ± 1.1
	6	10.3 ± 0.9	2.41 ± 0.2	25.2 ± 2.4
	7.15	8.9 ± 0.7	9.34 ± 0.7	68.7 ± 3.4
	8	12.7 ± 1.1	9.43 ± 0.7	121.3 ± 10.8
40	4.85	8.9 ± 0.7	0.81 ± 0.07	59.5 ± 3.3
	6	8.6 ± 0.6	0.71 ± 0.06	91.7 ± 7.5
	7.15	10.4 ± 0.9	0.97 ± 0.07	228.8 ± 18.4
	8	9.5 ± 0.8	1.47 ± 0.12	163.3 ± 12.2

face. Koenig et al. (1993) proposed that water molecules held by four hydrogen bonds and those held by two bonds have lifetimes τ_{hyL} and τ_{hyM}:

$$R_{2b} = p_{hyL} \times R_{2hyL} \times p_{hyM} \times R_{2hyM} \quad (6)$$

R_{2hyL} and R_{2hyM} are defined as the relaxation rates associated with τ_{hyL} and τ_{hyM}, respectively. p_{hyL} and p_{hyM} are the corresponding populations. From τ_{hyL} and τ_{hyM} values of 10^{-6} and 2×10^{-10} s (Koenig et al. 1993) and from the 64 $s^{-1}R_{2b}$, the population associated with τ_{hyL} was less than 1% of the hydration layer, consistent with previous results obtained on bovine serum albumin (Koenig et al. 1993). This determination assumes the general theory of protein hydration (Liepinsh and Otting 1996; Otting and Liepinsh 1995).

The $p_b\delta_b^2\omega^2$ values correlated closely with $p_b(R^2 = 0.991; F = 228)$. Hence δ_b did not vary within the gelatin concentration range and from the slope δ_b was about 1.5 ppm. This value agrees with previous results of Hills (1992b). This low δ_b value forecasts a small contribution to the exchange process at 20 MHz. According to the Eq. (2) the exchange process is expected to vary with the square of the frequency (ω). In this condition, $\delta_b^2\omega^2$ becomes 400 (20^2) times lower when ω changes from 400 to 20 MHz. The R_2 values at 20 MHz (Table 1) were very close to $(R_2)_0$ determined from measurements at 400 MHz (Table 4). The difference in R_2 values at two frequencies correspond to the term $p_b\delta_b^2\omega^2/k_e$. R_2 at 20 MHz was also very similar to that measured at 400 MHz for 20 000 $s^{-1}\Gamma$. At this Γ value for this frequency, R_2 was expected not to be dependent on the exchange process. Santyr et al. (1988) assumed that the CPMG sequence could be equivalent to a spin-locking sequence for the short delays between the 180° refocusing pulses. They argued from an unreasonably large value for the chemical shift of about 1000 ppm required to explain the dispersion curve obtained at 20 MHz by Gruker et al. (1986). Our δ value is far from this one. Moreover, R_2 was measured using the Carr-Purcell-Freeman-Hill (CPFH) sequence (Freeman and Hill 1971) to avoid possible spin-locking; the results were identical to those measured with the CPMG sequence (Fig. 4). This linearity ($R^2 = 0.998$; $F = 17975$) and the slope value (1.01 ± 0.01) allowed us to reject the spin-locking effect as the source of the observed dispersion in the R_2 versus Γ plot.

By increasing the pH from 4.85 to 8, the amplitude of variation R_2 showed a dispersion (Fig. 2 and Table 3). Both $(R_2)_0$ and $p_b\delta_b^2\omega^2$ increased with a pH value higher than 6. If the values of R_w and R_{2b} are assumed to be constant within the pH range, the R_2 behavior is related to p_b since δ is expected to increase with pH (Wüthrich 1986). Hence p_b went through a minimum at the pH which corresponds to gelatin pI. The k_e constant value was not expected according to the observation of Liepinsh and Otting (1996). These authors observed a minimum for the exchange rate for hydroxyl and amino protons of amino acid side chains in solution (Liepinsh and Otting 1996). Our result can be explained by the presence of hydrogen bonds between amino acids, which stabilize the macromolecules structure, unlike amino acids in solution. These hydrogen bonds also explains the decrease in $(R_2)_0$ and $p_b\delta_b^2\omega^2$ with the increase in gel

Table 4 Parameters determined from Eq. (2) for water protons of gelatin at 10 and 40 °C and for 60 and 300 Bloom gel strength; 15% concentration, pH 6

Temperature (°C)	Gel strength (Bloom)	k_e ($\times 10^3$ s^{-1})	$(R_2)_0$ (s^{-1})	$p_b\delta_b^2\omega^2$ ($\times 10^3$ s^{-2})
10	300	10.3 ± 0.8	2.41 ± 0.20	25.2 ± 0.18
10	60	10 ± 0.7	4.32 ± 0.36	36.5 ± 0.29
40	300	8.6 ± 0.7	0.71 ± 0.05	91.7 ± 7.8
40	60	9.1 ± 0.8	1.68 ± 0.13	110.9 ± 9.3

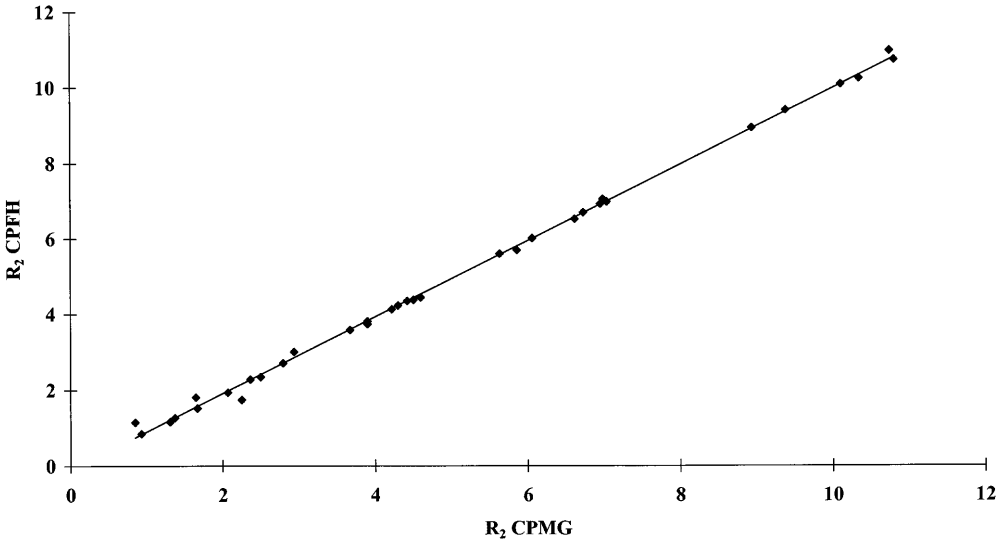

Fig. 4 R_2 measured from CPFH as a function of R_2 measured from CPMG for gelatin (pH 4.85) at 40°C

strength. The increase in Bloom number is derived from the formation of covalent intermolecular cross-links. These protein-protein interactions increased at the expense of the water-protein interactions and induced the decrease in p_b. At low temperature, the interchain hydrogen bonds stabilize the gelatin gel and many hydroxyl or amino groups become inaccessible for water interactions. The $p_b\delta_b^2\omega^2$ values decreased threefold from 40 °C to 10 °C (Tables 3 and 4). The contribution of exchange to the transverse relaxation rate should depend on p_b, which represents the availability of exchangeable sites on the macromolecule. When the temperature decreases from 40 °C to 10 °C, no significant variation of k_e was observed, in agreement with previous results (Hills 1992b), while $(R_2)_0$ increased as expected by the Arrhenius equation.

Diffusion through internal field gradients (G) can also contribute to a pulse spacing dependence of R_2 (Brown and Fantazzini 1993; Carr and Purcell 1954; Hills et al. 1990). When the field gradient is not constant (non-linear field distribution) throughout a sample, Eq. (1) is modified. For a bounded or restricted diffusion case, the spin-echo amplitude at time t is given by (Yu 1993):

$$M(t) = M_0 \times \exp\left[-\frac{a^4\gamma^2G^2}{120D}\left(1 - \frac{17a^2}{112D\tau}\right) \times t\right] \qquad (7)$$

when the time 2τ of echo formation satisfies the condition $\tau \gg a^2/\pi^2D$, where a is the apparent size of restriction and D the diffusion constant of nuclear spin magnetization. Then the decay rate of the CPMG measurement becomes (Yu 1993):

$$R_2(\tau) = R_2(\tau_\infty) - \frac{17}{13440}\frac{a^6\gamma^2G^2}{D^2\tau} \qquad (8)$$

$$R_2(\tau_\infty) = \frac{a^4\gamma^2G^2}{120D} \qquad (9)$$

Fitting the $R_2(\tau)$ data for long τ using Eq. (8), and knowing D, one obtains a and G. Assuming a water D value of 2×10^{-5} cm^2 s^{-1} and 10^{-5} cm^2 s^{-1} in gelatin sol and gel states, respectively (Blinc et al. 1995), the average values of a thus obtained are 1.2 and 0.6 μm for the sol and gel, respectively. The 35–70 μs values of the ratio a^2/π^2D are consistent with the required condition of τ for the analysis. The field gradient G ranged between 200 and 2000 G cm^{-1} for the different gelatins studied and is about two times greater for the gel state than for the sol state. Although the restriction distances obtained are greater than the 0.02–0.1 μm of the nucleated junction zones in gelatin gel reported by Djabourov (1986) from an electronic microscopy study, the small size of the restrictions and the intense local field gradients are an indicative signature of the microscopic origin of the inhomogeneous fields. Figure 5 displays the variations of G and a as a function of the gelatin concentration (C). In the gelatin concentration range (5–20%) used, linear relationship are found. The decrease of a with the increase of C is consistent with an increase of the density of the three-dimensional network of the cross-links. The concomitant increase of the internal field gradient strength may be the result of an increase of the d-dimensionality of the gelatin percolating network, gelatin being regarded as a fractal random medium (Derrida et al. 1984; Kveder et al. 1988).

Conclusion

The analysis of R_2 measured at 400 MHz versus Γ showed, for the gelatin sol state, the dominant role of chemical exchange between water protons and the exchangeable macromolecule protons in the water transverse relaxation mechanism. The exchange rate of 10 000 s^{-1} was determined from the Luz-Meiboom relation. The variation of the physico chemical conditions (pH, gelatin strength, concentration, and temperature) confirmed the exchange process. The shape of the gel-

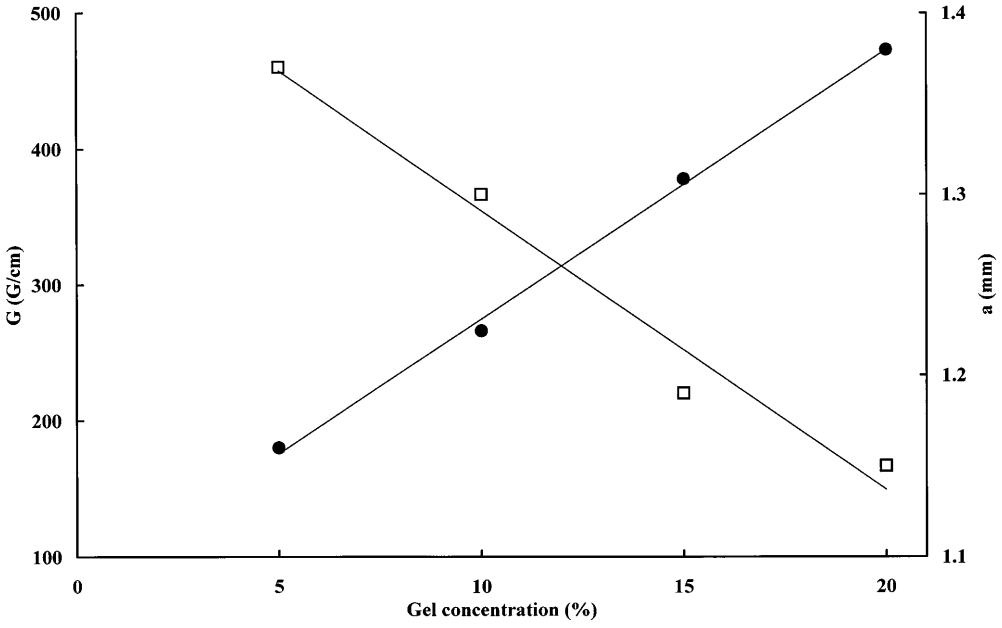

Fig. 5 The internal field gradient G (●) and the apparent size of restriction a (□) as a function of concentration in gelatin of 300 Bloom at pH 6

form dispersion curves was less well described by the exchange model and a contribution of diffusion through an inner magnetic field gradient was assumed. These results suggest that gelatin gels, although macroscopically homogeneous, display heterogeneity at the microscopic level.

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