Effect of Water Mobility on Drug Hydrolysis Rates in Gelatin Gels

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The stability of drugs incorporated in gelatin gels was studied, with a focus on the water mobility in the gels. Trichlormethiazide hydrolysis and kanamycin-catalyzed flomoxef hydrolysis in gelatin gels were chosen as models for apparent first-order and second-order hydrolysis, respectively. The mobility of water in gelatin gels was determined by NMR, ESR, and dielectric relaxation spectroscopies. The amount of bound water in the gels was determined from dielectric relaxation spectra. Spin-lattice relaxation time of water determined by ^{17}O NMR and rotational correlation time of an ESR probe determined by an ESR probing method were useful in determining the microviscosity of the gels. The hydrolysis rate of trichlormethiazide in the gels was found to depend on the amount of free water available for the reaction, while that of flomoxef depended on the microviscosity of the gels, which reflected the mobility of water molecules. Thus the dependence of hydrolysis rates on the water mobility was influenced by the hydrolysis mechanism.

KEY WORDS: gelatin; gel; hydrolysis; mobility; nuclear magnetic resonance (NMR); electron spin resonance (ESR); dielectric relaxation spectroscopy; trichlormethiazide; flomoxef.

INTRODUCTION

Polymeric gels consisting of hydrophilic polymers are useful vehicles for drug delivery systems. While the release kinetics of pharmaceuticals from gel matrices have been thoroughly studied (1–6), the drug stability in gels has been neglected. Polymeric gels can be glassy solids in the dehydrated state, while they absorb a significant amount of water to form elastic gels in the presence of water. Water in gels is supposed to be present as different types of water possessing different physical properties, which are described as free water (normal bulk water) and bound water (perturbed water) (7). Since drug stability in gels may be affected by mobility of water molecules as well as amount of water present in the system, as reported for drug stability in the solid state, evaluation of the dynamic properties of water in gels is required to predict the stability of drugs incorporated in gels.

The present study was undertaken to examine the effect of the dynamic properties of water molecules present in gelatin gels on the stability of drugs incorporated in gelatin gels. These gels have promise as vehicles for controlled delivery systems. The mobility of water in gelatin gels was studied by nuclear magnetic resonance (NMR), electron spin resonance (ESR), and dielectric relaxation spectroscopies. Trichlormethiazide (TCM) and flomoxef (FMX) were chosen as model drugs. TCM is known to be hydrolyzed in aqueous solution by apparent first-order kinetics (8), while FMX undergoes general base-catalyzed hydrolysis in aqueous solution (9). The hydrolysis rate of FMX in the presence of a general base depends on the diffusion rate of the drug and the base as well as their concentrations. Kinetics of TCM hydrolysis and kanamycin-catalyzed FMX hydrolysis in gelatin gels were studied as models for apparent first-order and second-order hydrolysis, respectively.

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EXPERIMENTAL

Materials

Gelatin powder (G-0252P) was obtained from Nitta Gelatin Co. (Osaka). The water content of the gelatin was determined to be 14.3%. Pulverized gelatin was suspended in water-free methanol and kept at room temperature. The water dissolved in the methanol was measured by the Karl Fisher method (684 KF Coulometer, Switzerland). Measurement was repeated until a constant value was obtained. Dissolution of the gelatin powder in distilled water produced a pH of 5.6.

FMX was kindly donated by Shionogi Seiyaku Co. (Osaka). TCM and kanamycin were purchased from Sigma (St. Louis, MO) and Wako Chemical Industry Co. (Osaka), respectively.

Preparation of Gelatin Gels Containing TCM

Amounts of 5, 10 and 15 g of gelatin were dissolved in 30, 25, and 20 g of pH 5.4 phosphate buffer (10 m*M*), respectively, with vigorous stirring at 60°C. Fifteen milliliters of TCM solution (33 μg/ml of pH 5.4 phosphate buffer), which contained 30 mg of methyl *p*-hydroxybenzoate, was added with stirring to produce 50 g of a viscous gelatin solution containing 10 μg/g of TCM. The pH of this solution was 5.4. The solution was transferred into a petri dish (10-cm diameter) and kept at 4°C until a gel structure was formed (about 1 hr). The gel was then cut into pieces (10 × 10 × 5 mm). The water content of the gel was calculated to be 0.91, 0.83, and 0.74 g/g, respectively, taking the water content of the original gelatin powder into consideration.

Gels of lower water content were prepared by drying the gels with 0.74 g/g of water at a reduced pressure for various periods. The water content was calculated from the decrease in weight during the drying. Although condensation of buffer components accompanied by water elimination could affect the hydrolysis rate, this effect and the effect of a possible change in local pH are considered negligible, since the rate of hydrolysis is small in highly condensed gels.

Preparation of Gelatin Gels Containing FMX

Kanamycin solution was prepared by dissolving 780 mg of kanamycin and 30 mg of methyl *p*-hydroxybenzoate in 20 ml of disodium phosphate solution (100 m*M*) and adding an appropriate aliquot of 1 *N* sodium hydroxide to adjust the pH to 7.4. Various amounts (5, 10, 15, 20, and 25 g) of gelatin were dissolved in 20 ml of the kanamycin solution with vigorous stirring at 60°C. Five milliliters of FMX solution (10 mg/ml of pH 7.4 phosphate buffer; 100 m*M*) and an appropriate aliquot of pH 7.4 phosphate buffer were added with

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stirring to make 50 g of viscous gelatin solution containing 1 mg/g of FMX and 15.6 mg/g of kanamycin. The solution was kept at 4°C and pieces of gel were obtained in the same way as for the TCM gels. The water content of the gel was calculated to be 0.89, 0.81, 0.72, 0.64, and 0.56 g/g, respectively.

For the gels used to study the effect of temperature on hydrolysis rate, gelation of the gelatin solution was carried out at the same temperature (10, 15, 20, 27, and 30°C) as the kinetic studies. The sol obtained before gelation was used in kinetic studies at 40, 50, and 60°C.

Kinetic Studies

Two pieces of the TCM or FMX gel of various water contents were put in a sample tube with a tight stopper and kept at 27°C in a thermostatic chamber. The sample gels were taken at appropriate intervals for the assay of remaining TCM or FMX. Hydrolysis of TCM and FMX was also determined in pH 5.4 and pH 7.4 phosphate buffer solutions, respectively, without gelatin at 27°C.

In the experiment to study the effect of temperature on kanamycin-catalyzed hydrolysis, FMX gels containing 0.81 g/g of water were kept at 10, 15, 20, 27, and 30°C. Hydrolysis of FMX in sols of the same water content was also followed at 40, 50, and 60°C.

The hydrolysis rate of FMX in gels without kanamycin was determined in gels containing 0.82 and 0.57 g/g of water as well as in buffer solution (0.99 water content) at 27°C.

Assay of TCM and FMX in Gels

TCM was assayed by high-performance liquid chromatography (HPLC). Two pieces of gel (about 1 g) were dissolved in 3 ml of pH 5.4 phosphate buffer (10 m*M*) at 60°C. Three milliliters of ethyl acetate solution of ethiazide (internal standard) (2.5 μg/ml) was added. After vigorous stirring and centrifugation at 3000 rpm, 2 ml of the ethyl acetate phase was dried under nitrogen gas, and the residual was dissolved in 1 ml of acetonitrile. After 3 ml of acetic acid solution (10 m*M*) was added, the solution was injected through a 20-μl loop to a column (ODS-80TM, 15 cm × 4.6 mm, TOSOH, Tokyo) maintained at 35°C. The mobile phase was a mixture of acetonitrile and 10 m*M* acetic acid solution (1:3), delivered at a rate of 1 ml/min. The column eluate was monitored at 280 nm. The HPLC equipment consisted of a Hitachi Model 655 system (Tokyo), a Tosoh Model AS-8000 autosampler (Tokyo), and a Shimadzu Model C-R3A computing integrator (Kyoto).

FMX was also assayed by HPLC. Two pieces of gel were dissolved in 2 ml of pH 7.4 phosphate buffer (100 m*M*). Eight milliliters of acetonitrile solution of *p*-chlorobenzoic acid (internal standard; 50 μg/ml) was added with shaking. After centrifuging at 3000 rpm, 1 ml of the solution was added to 4 ml of pH 4.5 acetate buffer (10 m*M*) containing 20 m*M* tetrabutylammonium chloride. The solution was analyzed by HPLC in a similar way as TCM. The mobile phase was a mixture of methanol and pH 4.5 tetrabutylammonium acetate buffer (3:4).

ESR Measurement

The gels used for ESR measurement were prepared using pH 5.4 and pH 7.4 phosphate buffers in a similar way as those for kinetic studies. 4-Hydroxy-2,2,6,6-tetramethylpiperidinyloxy (TEMPOL) was dissolved in gelatin solution instead of TCM or FMX. The final concentration of TEMPOL was 1 mmol/g. The sample gel was put in a capillary tube (1-mm diameter). ESR spectra were recorded with a JEOL spectrometer (JES-FE2XG, Tokyo) at ambient temperature, with a power level of 1 mW and a modulation amplitude of 0.5 gauss.

^{17}O NMR Measurement

The gels used for ^{17}O NMR measurement were prepared using pH 5.4 and pH 7.4 phosphate buffer without TCM or FMX. A Varian NMR spectrometer (VXR-400S) was operated at 54.2 MHz for the ^{17}O NMR measurements. A spin-lattice relaxation time of ^{17}O was obtained by using the inversion recovery method. A 90° ^{17}O pulse width of 50 μsec and a recycling time of 250 msec were used.

Dielectric Relaxation Measurement

The gels used for dielectric measurements were prepared using pH 5.4 phosphate buffer in a similar way as those for kinetic studies of TCM hydrolysis, except for the addition of TCM. Dielectric measurements were performed using the time domain reflectometry method developed by Mashimo *et al.* (10–12). The spectrometer setup used was the same as reported by Mashimo *et al.*

RESULTS

Figures 1 and 2 show the time courses of TCM and kanamycin-catalyzed FMX hydrolysis, respectively, in gelatin gels. The water content of 1.0 g/g for TCM and that of 0.97 g/g for FMX represent buffer solutions of TCM and FMX without gelatin. Hydrolysis appeared to follow first-order kinetics for both drugs. The solid lines in the figures were calculated from the parameters obtained by nonlinear regression analysis according to a first-order kinetic expression. The hydrolysis rate decreased with decreasing water content in the gels. The hydrolysis rate of FMX in gels with-

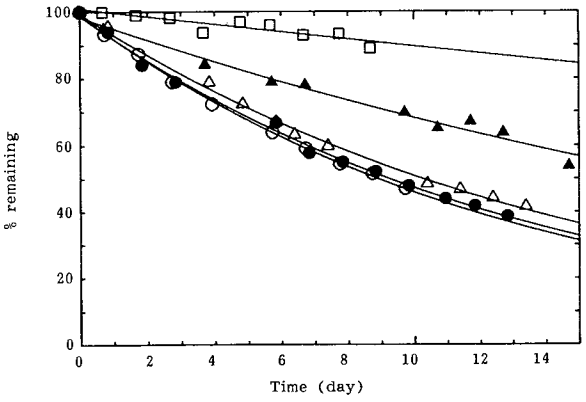

Fig. 1. Hydrolysis of trichlormethiazide in gelatin gel at 27°C, as a function of water content: (○) 1.0, (●) 0.83, (△) 0.74, (▲) 0.49, and (□) 0.29 g/g. A pH 5.4 phosphate buffer was used for gel preparation.

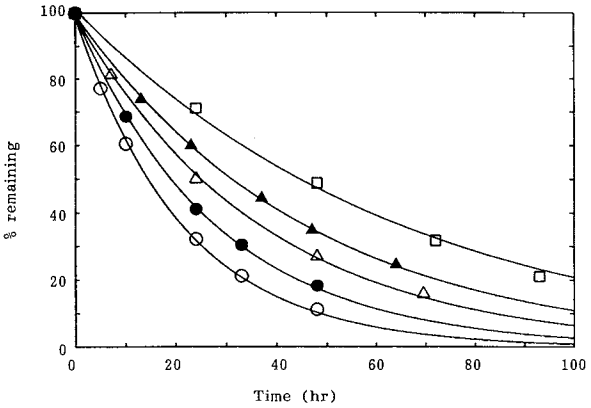

Fig. 2. Kanamycin-catalyzed hydrolysis of flomoxef in gelatin gel at 27°C, as a function of water content: (○) 0.97, (●) 0.89, (△) 0.81, (▲) 0.72, and (□) 0.56 g/g. A pH 7.4 phosphate buffer was used for gel preparation.

out kanamycin was much lower than that of kanamycin-catalyzed hydrolysis and showed no significant dependence on water content (data not shown).

Figure 3 shows typical ESR spectra of gelatin gel containing TEMPOL, the spin probe. The signal for the gelatin-free solution of TEMPOL consisted of three narrow symmetrically spaced peaks of equal height, indicating that the spin probe molecule was moving rapidly and isotropically. A differential line broadening was observed in the spectra for the gels of lower water content. This indicates that the spin probe was not moving freely in the gels. The rotational correlation time of the probe, τ_c, was calculated for the TCM gel and the FMX gel according to Eq. (3) described under Discussion and is plotted against water content in Figs. 4 and 5, respectively. The τ_c increased with decreasing water content of the gels.

The spin-lattice relaxation times, T_1 of $H_2{}^{17}O$ were determined for both TCM and FMX gels and are plotted against water content in Figs. 4 and 5, respectively. The T_1 decreased with water content for both gels. The reciprocal of T_1 was related linearly to τ_c as shown in Fig. 6.

Two dielectric relaxation peaks were found for the gels with a water content of more than 0.5 g/g. The lower frequency peak around 100 MHz was due to water molecules bound to gelatin, and the peak at a 100-fold higher frequency was due to bulk water in the gels. The amount of bound water was determined according to Eq. (2) described under Discussion, and the amount of free water was calculated by subtracting the amount of bound water from the theoretical total water present in the gel. The amount of free water calculated for the gel is plotted against water content in Fig. 4. The free water decreased with the total water content and approached zero at a water content of 0.14 g/g as shown by the extrapolated line.

The apparent first-order rate constant calculated for TCM degradation in the gels is plotted against water content in Fig. 4, as well as τ_c, T_1, and amount of free water. At water contents greater than 0.8 g/g, the apparent rate constant appeared to be constant, as usually observed for apparent first-order degradation in solution. At water contents lower than 0.8, the rate constant decreased with water content and approached zero at a water content of about 0.14 g/g. The rate constant seemed to be correlated to the amount of free water determined by the dielectric relaxation method.

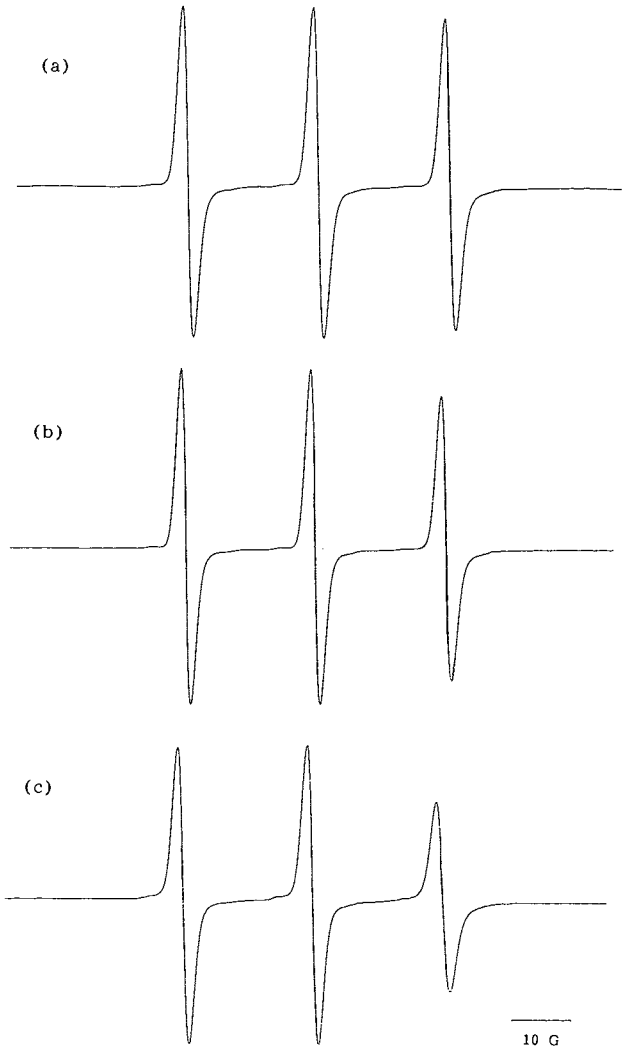

Fig. 3. ESR spectra of gelatin gel containing TEMPOL, as a function of water content: (a) 0.99, (b) 0.74, and (c) 0.57 g/g. A pH 7.4 phosphate buffer was used for gel preparation.

The apparent second-order rate constant of FMX hydrolysis is plotted against total water content in Fig. 5. The constant was calculated by dividing the apparent first-order rate constant by the concentration of kanamycin, which was regarded as constant throughout the observation period. The rate constant decreased with water content even at water contents greater than 0.8 g/g and appeared to be correlated with the decrease in T_1.

The FMX gel with 0.81 g/g of water revealed sol-gel transition around 30°C. Figures 7 and 8 show the effect of temperature on the hydrolysis rate of FMX in the gel and sol states, respectively. The apparent first-order rate constant was determined by curve-fitting and the Arrhenius plots are shown in Fig. 9, which shows the T_1 determined at corresponding temperatures as well. Neither the rate constant nor the T_1 showed any marked discrepancy in the temperature dependence between the gel and the sol states.

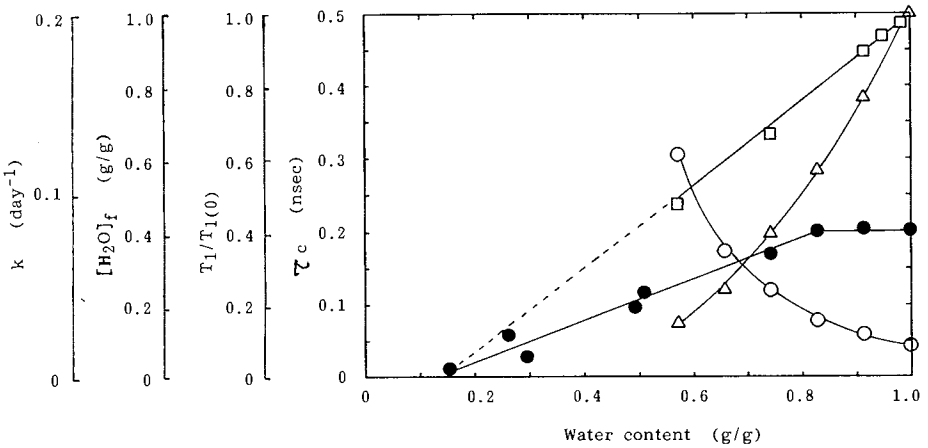

Fig. 4. The water content dependence of (●) the apparent first-order rate constant of trichlormethiazide hydrolysis at 27°C, (□) the amount of free water, (△) the spin-lattice relaxation time of $H_2{}^{17}O$, and (○) the rotational correlation time of TEMPOL.

DISCUSSION

NMR spectroscopy has been used to study the mobility of water in a variety of systems (7,13–16). Spin-lattice relaxation time of water, T_{obs}, observed by ^{17}O NMR can be related to the relaxation time of bound water, T_b, and that of free water, T_f, according to Eq. (1):

$$1/T_{obs} = P_b/T_b + P_f/T_f \tag{1}$$

where P_b and P_f are the fractions of bound and free water in the system, respectively (14). The T_{obs} depends on the ratio of bound water as well as the strength of bonding, which is inversely proportional to the mobility of bound water.

Dielectric relaxation spectroscopy can also provide information on the amount of bound water and the strength of bonding (10–12). The strength of bonding is indicated by the frequency at which the dielectric constant changes, and the amounts of bound and free water can be determined from the changes in the dielectric constant observed at low and high frequencies, respectively. Mashimo *et al.* (12) have reported the number of water molecules bound to collagen, n, which was calculated according to Eq. (2):

$$n = \frac{9kT}{4\pi\langle\mu^2\rangle} \frac{3\Delta\epsilon_l}{(\epsilon_\infty + \Delta\epsilon_h + 2)^2} \tag{2}$$

where ϵ_∞ is the high-frequency limiting permittivity; $\Delta\epsilon_l$ and $\Delta\epsilon_h$ are the relaxation strengths for low- and high-frequency processes, respectively; $\langle\mu^2\rangle$ is the square of the effective dipole moment of the water molecules; and T and k are the absolute temperature and Boltzmann constant, respectively.

On the other hand, ESR spectroscopy has been used to measure microscopic viscosities of various polymer systems. Broadening of the signal of a spin probe can be related to reduced mobility of the probe, which indicates increased microviscosity of the system. Microviscosity can be represented by the rotational correlation time of the probe, τ_c. Armstrong *et al.* have calculated τ_c for glycerogelatin gels according to Eq. (3):

$$\tau_c = 6.5 \times 10^{-10} H_o (\sqrt{h_o/h} - 1) \tag{3}$$

where h_o and h are the amplitudes of the central and high-field lines, respectively; and H_o is the line width of the central line in gauss (17,18).

In the present study, the amount of bound water in the

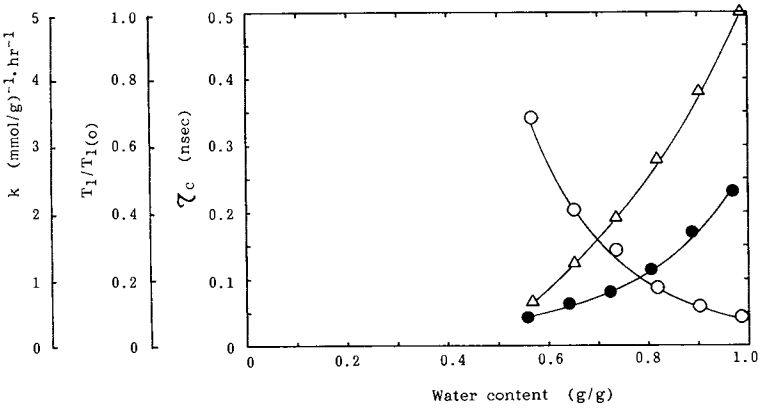

Fig. 5. The water content dependence of (●) the apparent second-order rate constant of kanamycin-catalyzed flomoxef hydrolysis in gelatin gel at 27°C, (△) the spin-lattice relaxation time of $H_2{}^{17}O$, and (○) the rotational correlation time of TEMPOL.

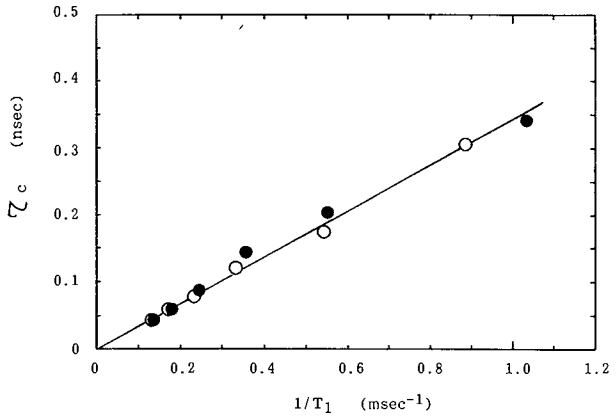

Fig. 6. Relationship between reciprocal of spin-lattice relaxation time of $H_2{}^{17}O$ and rotational correlation time of TEMPOL. The pH of the phosphate buffer used for gel preparation was (○) 5.4 and (●) 7.4.

TCM and FMX gels was successfully determined from the dielectric relaxation spectra. The amount of free water could be calculated from the amount of bound water and the theoretical total water content. The free water decreased with increasing gelatin content (Fig. 4). The gels were found not to contain free water at water contents lower than 0.14 g/g. This coincides with the water content of the original gelatin powder.

The spin-lattice relaxation time of water, T_1, in the TCM and FMX gels was determined by ^{17}O NMR. A decrease in T_1 was observed with increasing gelatin content (Figs. 4 and 5). This result can be ascribed to the increase in the amount of less mobile water molecules which were bound by gelatin molecules. Thus T_1 can be used as a measure of water mobility. T_1 was related to temperature in a manner analogous to that expressed by the Arrhenius equation. No marked discrepancy was observed between the gel and the sol states (Fig. 9). This suggests that gelatin molecules attract water molecules and decrease the mobility of water molecules regardless of the rigidity of the fiber structure.

The reciprocal of T_1, that is, the relaxation rate, can be a measure of the decrease in the mobility of water molecules. The reciprocal of T_1 correlated well with the microviscosity

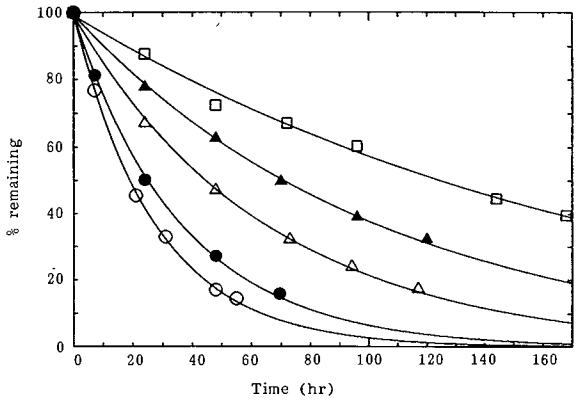

Fig. 7. Kanamycin-catalyzed hydrolysis of flomoxef in gelatin gel as a function of temperature: (□) 10, (▲) 15, (△) 20, (●) 27, and (○) 30°C.

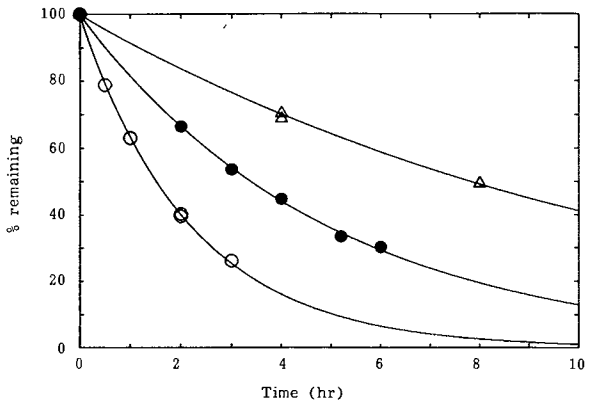

Fig. 8. Kanamycin-catalyzed hydrolysis of flomoxef in gelatin sol as a function of temperature: (△) 40, (●) 50, and (○) 60°C.

determined from the rotational correlation time of the ESR probe molecules (Fig. 6). The mobility of water molecules in the gels can be reflected in the mobility of the ESR probe.

TCM in the gels was hydrolyzed in the range of water content greater than 0.14 and the hydrolysis rate increased with the amount of free water (Fig. 4). This indicates that bound water cannot participate in hydrolysis, and the hydrolysis rate depends on the amount of free water present as described by Eq. (4). The hydrolysis rate did not vary when the water content exceeded 0.8 g/g. This can be explained by assuming that at a water content above 0.8 g/g, there are sufficient water molecules to surround each drug molecule, so the hydrolysis rate becomes independent of the amount of water. The kinetics can then be described as first-order hydrolysis in aqueous solutions, i.e.,

$$k_{obs} = k[H_2O]_f \tag{4}$$

The apparent second-order rate constant for kanamycin-catalyzed hydrolysis of FMX in the gels, which is the

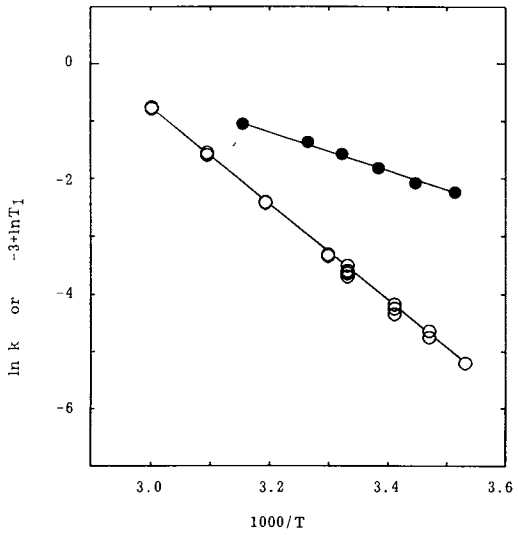

Fig. 9. The Arrhenius plots of (○) the apparent first-order rate constant of kanamycin-catalyzed flomoxef hydrolysis and (●) the spin-lattice relaxation time of $H_2{}^{17}O$ in gelatin gel. The gels with 0.81 and 0.82 g/g water were used for rate constant and relaxation time, respectively.

term $k[H_2O]_f$ in Eq. (5), decreased with decreasing T_1 and increasing τ_c (Fig. 5). This suggests that the apparent second-order rate constant depends on the microviscosity, which determines the diffusion rates of both FMX and kanamycin. No large discrepancy was observed in the temperature dependence of the apparent first-order rate constant between the sol and the gel states. This suggests that interstices are too large to act as physical barriers to diffusing molecules, and the hydrolysis rate depends on the microviscosity in a similar way in both sol and gel states.

$$k_{obs} = k[H_2O]_f\,[\text{kanamycin}] \quad (5)$$

In conclusion, the hydrolysis rates of drugs in gelatin gels are determined by the amount of free water available for the reaction and/or the microviscosity related to the mobility of water molecules, depending on the hydrolysis mechanism. When hydrolysis is a reaction between a drug molecule and a water molecule, as for the TCM hydrolysis in gelatin gels, the rate is determined by the amount of free water present in the gel. If diffusion of molecules is rate-determining as the kanamycin-catalyzed hydrolysis of FMX in gelatin gels, the rate depends on the microviscosity, which is determined by the mobility of water molecules in the gel, as well.

NMR relaxation spectroscopy, dielectric relaxation spectroscopy, and ESR spectroscopy were found to be useful to study the mobility of water in gelatin gels, which affects the hydrolysis rates of drugs loaded in gelatin gels.

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