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Nuclear Magnetic Resonance Study of the Binding of Tolbutamide and Chlorpropamide to Bovine Serum Albumin[1,2]

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The bindings of tolbutamide and chlorpropamide to bovine serum albumin and to acetylated bovine serum albumin were studied by nuclear magnetic relaxation techniques in order to determine which protons of the drug molecules are involved in the interaction. The effects of the bovine serum albumin concentration and pH on the relaxation rate of each proton were examined. A marked increase in the relaxation rate of each proton was observed with the addition of bovine serum albumin or acetylated bovine serum albumin. Relatively large effects on the relaxation rates of the α-methylene group of tolbutamide and that of chlorpropamide were observed upon addition of bovine serum albumin and change of pH. It is suggested that the sulfonylurea moiety (–SO_2NHCONH–) of both drugs is the major binding site, so that the relaxation rates of the nearby α-methylene protons are most affected. The next largest increase was in the relaxation rates of phenyl protons of both drugs, suggesting that the phenyl moiety might be another site of drug-protein interaction.

Keywords——protein binding; nuclear magnetic relaxation technique; tolbutamide; chlorpropamide; bovine serum albumin; acetylated bovine serum albumin; relaxation rate

The binding of sulfonylureas to protein, and especially the competitive binding of the drugs to available protein sites, has been investigated by many workers.[4–8] In addition, this phenomenon has been related to clinical use of the drugs, namely, the problem of drug interaction.[9–11]

Various methods have been used for such binding studies. However, it is difficult to decide which method is most reliable, and the mechanisms of binding of sulfonylureas to bovine serum albumin are not fully understood.[5,6]

Recent studies have established the usefulness of nuclear magnetic resonance (NMR) spectroscopy as a tool for conformational determination of pharmacologically active molecules in solution[12–14] and for assessing the extents to which various functional groups on a small molecule participate in drug-protein interactions.[15–17] A detailed theoretical basis for this

1) This paper forms part XXXII of "Physico-chemical Approach to Biopharmaceutical Phenomena." The preceding paper, Part XXXI: H. Ueda and T. Nagai, *Chem. Pharm. Bull.*, **26**, 1353 (1978).
2) A part of this work was presented at the 98th Annual Meeting of the Pharmaceutical Society of Japan, Okayama, April 1978.
3) Location: *Ebara 2-4-41, Shinagawa-ku, Tokyo, 142, Japan.*
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NMR method has been given by Fischer and Jardetzky.[18,19] Numerous investigators have utilized the extreme sensitivity of the spin-spin relaxation rate ($1/T_2$) to analyze small variations in the molecular environment.

In this study, determination of the spin-lattice relaxation rate in the rotating frame ($1/T_{1\rho}$)[20] by Fourier transform NMR (FT-NMR), was used to investigate the binding of sulfonylureas to BSA and to acetylated BSA. It was assumed that $T_{1\rho}=T_2$ in solution; this is reasonable on theoretical grounds.[21,22] $T_{1\rho}$ could be obtained more easily by the spin-locking method than could T_2 by the spin-echo FT method.

Experimental

Materials——BSA powder was obtained from Armour Pharmaceutical Company; 99.8% deuterium oxide and the deuterated compounds Na_2DPO_4 and KD_2PO_4 were from Merck. Highly purified sulfonylureas, which conformed to the officially registered descriptions, were obtained from the following sources: chlorpropamide from Taito Pfizer Co., Ltd., mp 127—129°; tolbutamide from Hoechst Japan Co., Ltd., mp 128.5—129.5°. All chemicals were used without further purification.

Acetylated BSA was prepared according to the procedure described by Frankel-Conrat *et al.*[23] The reaction product was dialyzed against 18 l of deionized distilled water to remove ions and excess reagents at 4°, changing the deionized water six times.

Methods——The drugs (M) and the protein (%(w/v)) in D_2O were subjected to NMR spectroscopy. The pH of the solution was adjusted with KD_2PO_4 and Na_2DPO_4, using a Hitachi-Horiba F-7 pH meter with microelectrodes calibrated with standard buffer solution. All pH values given are actual meter readings and are uncorrected for deuterium isotope effect.

Spectra——All spectra were obtained on a JEOL FX-100 spectrometer with spin-locking units. Temperatures were maintained at $24.5\pm0.5°$ during all experiments. Tetramethylsilane (TMS) was used as an external reference. The values of $1/T_{1\rho}$ for the various protons were obtained from the spin-locking sequence according to Eq. 1[20,21]:

$$M_\rho(t) = M_0\exp(-t/T_{1\rho}) \qquad \text{(Eq. 1)}$$

where $M_\rho(t)$ is the macroscopic magnetization at t, M_0 is the equilibrium magnetization at $t=0$, t is the spin-locking time, and $T_{1\rho}$ is the spin-lattice relaxation time in the rotating frame. $T_{1\rho}$ was determined under the following conditions: $\pi/2$ pulse, 6 sec; locking pulse ($H_{1\rho}$), 2.5 gauss; spin-locking times changing several times in 0—5 sec.

Results and Discussion

Effects of Various Concentrations of BSA on the Spin-Lattice Relaxation Rates in the Rotating Frame($1/T_{1\rho}$) of 0.01 M Tolbutamide and 0.01 M Chlorpropamide

The proton magnetic resonance spectrum of 0.01 M tolbutamide in D_2O at pH 7.0 is shown in Fig. 1. The aromatic protons were easily identified from their characteristic low-field position (AA'BB'). The α-methylene protons were identified as a triplet (299.8 Hz), p-methyl protons as a sharp single peak (239.7 Hz), β,γ-methylene protons as a multiplet (132.5 Hz), and δ-methyl protons as a triplet (83.9 Hz).

In the case of chlorpropamide, each proton was easily identified in similar positions. The aromatic protons were identified from their low-field position (AA'BB'). The α-methylene protons were identified as a triplet (296.1 Hz), β-methylene protons as a multiplet (135.5 Hz), and γ-methyl protons as a triplet (81.3 Hz).

The chemical shifts of 0.01 M tolbutamide and 0.01 M chlorpropamide at several concentrations of BSA are shown in Table I. Small changes (0.04—0.05 ppm) in chemical shifts of all

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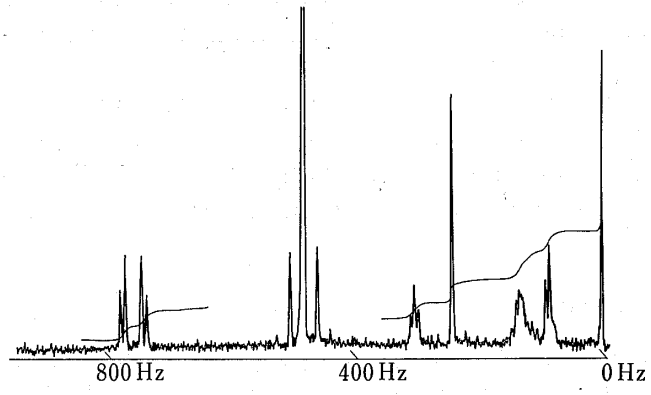

Fig. 1. Proton Magnetic Resonance Spectrum of 0.01 M Tolbutamide at pH 7.0

the spectra were observed in the present concentration range. However, these may result from changes in magnetic susceptibility due to changes in the viscosity of the solution with addition of various amounts of BSA. Consequently, a detailed study of the drug-protein interactions cannot be carried out on the basis of the changes in chemical shifts.

As an example of a spin-locking sequence, Fig. 2 shows the spin-lattice relaxation traces obtained by Fourier transformation of a spin-locking sequence on the protons of 0.01 M chlorpropamide. The relaxation rates of 0.01 M tolbutamide in D_2O at pH 7.0 were as follows: phenyl protons, 0.41(sec^{-1}); α-methylene protons, 0.64(sec^{-1}); p-methyl protons, 0.78(sec^{-1}); β,γ-methylene protons, 0.62(sec^{-1}); δ-methyl protons, 0.58(sec^{-1}). The relaxation rates of 0.01 M chlorpropamide in D_2O at pH 7.0 were: phenyl protons, 0.35(sec^{-1}); α-methylene protons, 0.64(sec^{-1}); β-methylene protons, 0.63(sec^{-1}); γ-methyl protons, 0.57(sec^{-1}).

Table I. Effect of BSA on the Chemical Shifts of 0.01 M Tolbutamide and 0.01 M Chlorpropamide

a: Tolbutamide

Concentration of BSA (w/v%)	Chemical Shifts from TMS (ppm)				
	P-CH_3	Phenyl protons	α-CH_2▲	β,γ-$(CH_2)_2$▲	δ-CH_3▲
0	2.406	7.539	3.009	1.329	0.843
0.25	2.384	7.519	2.989	1.296	0.823
0.5	2.374	7.513	2.978	1.283	0.813
1.0	2.364	7.511	2.972	1.279	0.804
2.0	2.352	7.506	2.964	1.276	0.801

b: Chlorpropamide

Concentration of BSA (w/v%)	Chemical Shifts from TMS (ppm)			
	Phenyl protons	α-CH_2▲	β-CH_2▲	γ-CH_3▲
0	7.666	2.972	1.359	0.816
0.25	7.646	2.947	1.340	0.791
0.5	7.641	2.939	1.336	0.786
1.0	7.636	2.934	1.335	0.782
2.0	7.630	2.928	1.334	0.774

Phenyl protons: the average of the four peaks.
▲: the central peak of the triplet or multiplet.

The effects of BSA concentration on the relaxation rates of 0.01 M tolbutamide and 0.01 M chlorpropamide at pH 7.0 are summarized in Figs. 3 and 4 as plots of $1/T_{1\rho}$ for each proton *versus* the concentration of BSA. Figures 3 and 4 show that the relaxation rates of all peaks for tolbutamide and chlorpropamide increased with the addition of BSA. The effect was particularly marked in the case of the α-methylene protons of both molecules. In connection with the penicillin/BSA system, Fisher and Jardetzky postulated that a marked effect on the

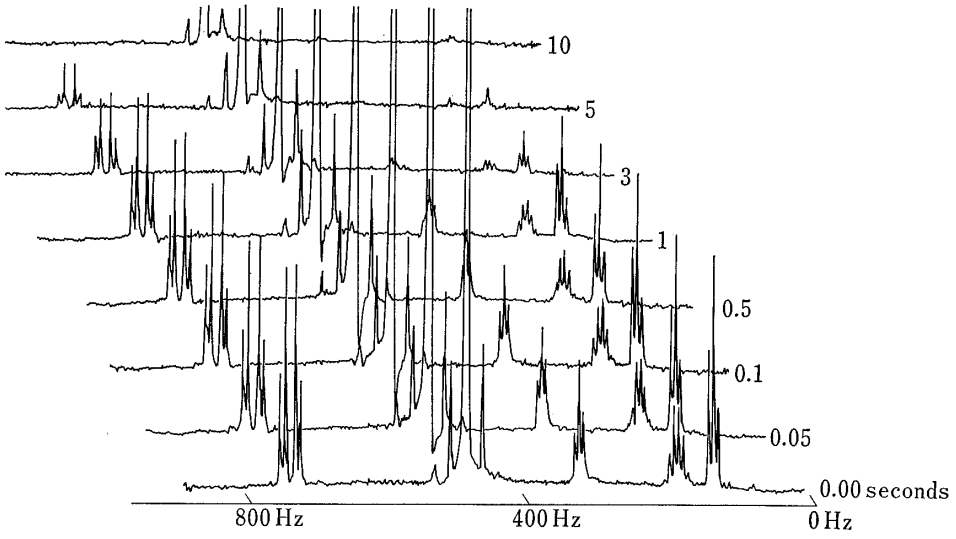

Fig. 2. Spin-Lattice Relaxation Traces in the Rotating Frame, obtained by Fourier Transformation of the Spin-Locking Sequence for Protons of Chlorpropamide

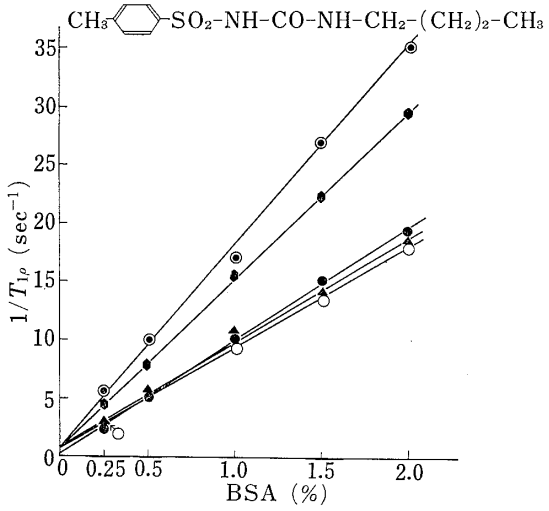

Fig. 3. Effect of BSA on the Proton Relaxation Rates of 0.01 M Tolbutamide at pH 7.0

Key: ⊙=α-CH_2, ●=p-CH_3, ○=δ-CH_3, ▲=β-γ-$(CH_2)_2$, and ●=phenyl protons.

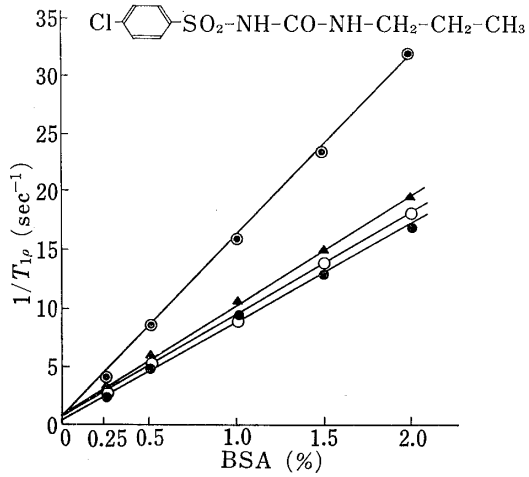

Fig. 4. Effect of BSA on the Proton Relaxation Rates of 0.01 M Chlorpropamide at pH 7.0

Key: ⊙=α-CH_2, ▲=β-CH_2, ○=γ-CH_3, and ●=phenyl protons.

relaxation rate of a particular portion of molecule might imply the existence of a specific type of intermolecular interaction differing from such nonspecific effects as viscosity and intermolecular interaction of the penicillins themselves. In other words, nonspecific mechanisms should make the $1/T_{1\rho}$ of all protons in a given molecule increase to the same extent. If the increase in relaxation rates is caused by binding, the bound part of the molecule may exhibit the greatest change. However, the absolute values of $1/T_{1\rho}$ for the bound molecules reflect to a large extent the intrinsic differences in the relaxation of the different groups, methylene, phenyl, *etc.* Thus, in order to compare the changes in correlation time that accompany binding, it is necessary to compare the ratio of the relaxation rate for the molecule bound with BSA to that for the free molecule for each peak.

The results reported in this study indicate that specific tolbutamide-BSA and chlorpropamide-BSA interactions take place and also that α-methylene protons are involved in the binding processes.

Furthermore, as shown in Fig. 3 and Fig. 4, the next largest increase in the relaxation rate of both molecules was that of the phenyl protons. This results suggests that these positions are another site of drug-protein interaction.

Effect of pH on the Relaxation Rates of Tolbutamide-BSA Solution and Chlorpropamide-BSA Solution

Solutions of 0.01 M tolbutamide–1% BSA and 0.01 M chlorpropamide–1% BSA were examined to study the effect of changes in pH on the relaxation rates in both systems. The study could be carried out only above pH 6.8 because of the low solubility of both drugs below that pH.

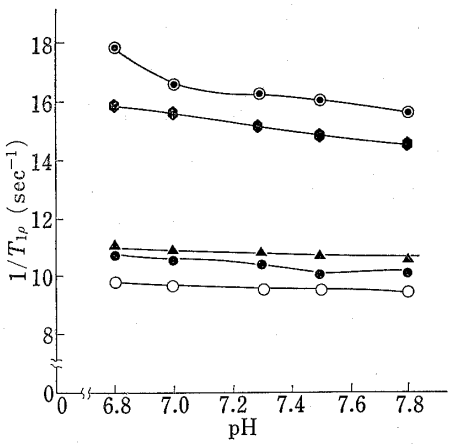

Fig. 5. Effect of pH on the Proton Relaxation Rates in 0.01 M Tolbutamide–1% BSA Solution

Key: ⊙=α-CH_2, ●=p-CH_3, ▲=β-γ-$(CH_2)_2$, ◉=phenyl protons, and ○=δ-CH_3.

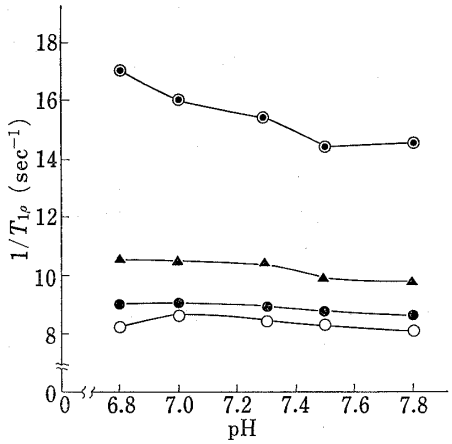

Fig. 6. Effect of pH on the Proton Relaxation Rates in 0.01 M Chlorpropamide–1%BSA Solution

Key: ⊙=α-CH_2, ▲=β-CH_2, ○=γ-CH_3, and ◉=phenyl protons.

The plots of pH *versus* $1/T_{1\rho}$ for the protons of tolbutamide and chlorpropamide are shown in Fig. 5 and Fig. 6. The relaxation rate of each proton generally decreased with increase of pH. However, the effect was greatest in the case of the α-methylene protons of both molecules. The present data are insufficient to elucidate the exact mechanism of the decrease in relaxation rates. However, it seems likely that an increase in pH affects the conformational characteristics of the protein and thus decreases the cationic charge of the amino groups of BSA. This reasoning is based on the supposition that, in the present concentration range, the determining factor of binding is the number of protein binding sites, not the quantity of ionized drug. In the range of pH used, sufficient ionized molecules should exist for both drugs in view of the reported values of pK_a.[6]

Thus, it seems possible that the sulfonylurea moiety (–SO_2NHCONH–) of both drugs is the binding site, so that the relaxation rates of the nearby α-methylene protons would be strongly affected.

Effect of Concentration of Acetylated BSA on the Spin-Lattice Relaxation Rates in the Rotating Frame ($1/T_{1\rho}$) of 0.01 M Tolbutamide and 0.01 M Chlorpropamide

In order to elucidate further the mechanism of the tolbutamide-BSA and chlorpropamide-BSA interactions, BSA was acetylated in order to block partially the ε-ammonium side chains in it and thus to increase the net negative charge on the protein. The solubility of acetylated BSA at the present pH was too low to carry out experiments at concentrations over 1%.

Fig. 7 and Fig. 8 show that the relaxation rates of the tolbutamide-acetylated BSA and chlorpropamide-acetylated BSA systems were low compared with those of the tolbutamide-BSA and chlorpropamide-BSA systems (Figs. 3 and 4), respectively.

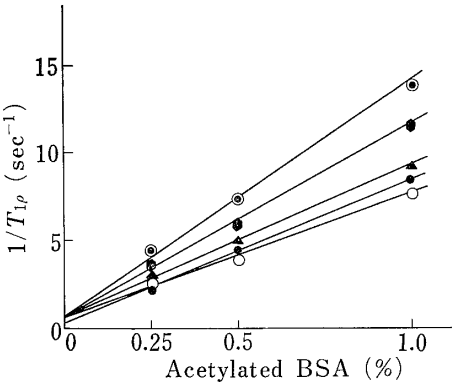

Fig. 7. Effect of Acetylated BSA on the Proton Relaxation Rates of 0.01 M Tolbutamide at pH 7.0

Key: ⊙=α-CH_2, ●=p-CH_3, ○=δ-CH_3, ▲=β-γ-$(CH_2)_2$, and ●=phenyl protons.

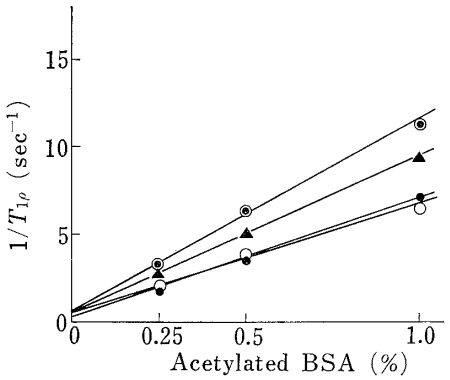

Fig. 8. Effect of Acetylated BSA on the Proton Relaxation Rates of 0.01 M Chlorpropamide at pH 7.0

Key: ⊙=α-CH_2, ▲=β-CH_2, ○=γ-CH_3, and ●=phenyl protons.

This decrease of relaxation rates was considered to be due to a decrease in the affinity between BSA and the anion forms of both drugs which might result from the decrease in the positively charged sites in BSA.

As shown in Figs. 7 and 8, the effect was large in the case of α-methylene and phenyl protons of both molecules. The magnitude of the effect for α-methylene protons was the same as, or a little less than, that for phenyl protons. This result supports the view that a specific interaction between the sulfonylurea moiety of both molecules and BSA is predominant in the binding. Futhermore, this is consistent with the proposal[5] that tolbutamide and chlorpropamide may bind to BSA electrostatically.

The exact mechanism of sulfonylurea-BSA interaction will shortly be investigated in more detail by studying the effects of temperature and ionic strength on the relaxation rates.