Binding Position of Tolbutamide to Human Serum Albumin[1)]

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The interaction between drugs (tolbutamide (1), 1-butyl-3-(methylsulfonyl)urea (2)) and human serum albumin (3) was investigated by equilibrium dialysis and NMR spectroscopy. The binding of 1 and 2 to 3 was concluded to be hydrophobic and hydrophilic, respectively, on the basis of the dependence of the binding constants on temperature, ionic strength, and chain length of fatty acid added. In ^{1}H-NMR spectra of 1, there were no significant shifts with change in concentration or addition of 3. The spin–lattice relaxation time (T_1) and spin–spin relaxation rate ($1/T_2$) of the respective protons of 1 were independent of concentration, but depended on the concentration of 3 added. The binding position was determined from the ratio of $1/T_2$ of 1 bound to 3 and free 1. 1 and 2 were found to bind to 3 through the tolyl group and sulfonylurea group, respectively. The binding property of 1 was considered to be governed by the competition between the hydrophobic effect of the tolyl group and the hydrophilic effect of the sulfonylurea group in the molecule.

Key words tolbutamide; human serum albumin; hydrophobic interaction; spin–spin relaxation rate; binding position

In a series of studies concerning the interaction between drugs and water-soluble polymers,[2–9)] NMR, especially the ratio of the spin–spin relaxation rate ($1/T_2$) of the free drug to that of the bound drug, was the most useful parameter to determine the binding position of the drug to protein. The Carr–Purcell–Meiboom–Gill (CPMG) method[10)] was the most useful one to determine the T_2 value, though the peak width at half height[11)] and the spin–lattice relaxation time in the rotating frame ($T_{1\rho}$)[12)] were used. There were two controversial points in the CPMG method, however. One was the deformation of phase by J-modulation, and the other was the modulation induced by repeated application of the π pulse. In our experiments, the π pulse was applied at time intervals short enough to prevent the modulation. In the series of studies, the following findings were reported: binding position of phenylbutazone[3)] and ibuprofen[4)] to bovine serum albumin (BSA) (**4**) were mainly at the phenyl group; that of azathioprine[5)] was at the methylimidazole group; that of indomethacin[6)] and naproxen[9)] were at the aromatic part; and 5-methyl-2-methyl-indolacetic acid[6)] was at the carboxyl and aromatic group.

In the present article, the binding position of tolbutamide (**1**) to human serum albumin (HSA) (**3**) was investigated by NMR and equilibrium dialysis. Crooks and Brown[13)] reported that the two kinds of binding sites on **3** for **1** were ionic and non-ionic. Ueda *et al.*[12)] suggested based on $T_{1\rho}$ measurement that the sulfonylurea moiety of **1** to **4** was the major binding site, and that the phenyl group of **1** was another site. On the other hand, Goto *et al.*[14)] reported that the binding mechanism of sulfonylurea drugs to **4** was hydrophobic from the results of thermodynamic data. It was found in the present article that the phenyl group of **1** was the principal binding position to **3**. In addition, the interaction between 1-butyl-3-(methylsulfonyl)urea (**2**) and HSA (**3**) was studied by the same methods to compare with **1** and suggested to be weak hydrophilic and non-specific interaction. The competition between the hydrophobic effect of the phenyl group and the hydrophilic effect of the sulfonylurea moiety was concluded to be the main factor governing the binding property of **1**.

Experimental

Materials **1** was of special reagent grade from Sigma and used without further purification. **3** with molecular weight of 6.9×10^4 was from Sigma (product number: A1887, essentially fatty acid free). Other reagents were from commercial sources and were used without further purification. **2** was prepared according to the method reported by Cassady *et al.*[15)]

Equilibrium Dialysis The equilibrium dialysis method was described previously.[2)] Temperatures were regulated within 0.2 °C during all experiments. The drug concentration was determined by absorbance on a Shimadzu UV-190 spectrometer connected to an Iwatsu VOAC-7513 digital multimeter and an NEC PC-9801RX microcomputer. **1**, UV λ_{max} (pH 7) nm (ε): 225.5 (11400). **2**, UV λ_{max} (pH 7) nm (ε): 205.5 (4660).

NMR Measurement The NMR spectra were measured in deuterium oxide (D_2O, phosphate buffer, 0.1 M, pH 7) on a JEOL GX-400 spectrometer (radio frequency, 400 MHz; $\pi/2$ pulse, 11.1 μs) at 40.0 ± 0.5 °C. The spin–lattice relaxation time (T_1) was obtained by the inversion recovery method[16)] (Eq. 1):

$$\ln(M_0 - M_t) = -t/T_1 + \ln(2M_0) \quad (1)$$

where t is the interval between π and $\pi/2$ pulse, and M_0 and M_t represent equilibrium magnetization at $t=0$ and macroscopic magnetization at t, respectively. The spin–spin relaxation time (T_2) was determined according to the CPMG method[10)] (Eq. 2):

$$\ln(M_t) = \ln(M_0) - t/T_2 \quad (2)$$

where t is the time when a free induction decay (FID) is observed after irradiation of $\pi/2$ pulse, and M_t is the intensity of a spin echo at t. The pulse delay time (20 s), when the next pulse was applied after observation of FID, had to be longer than the relaxation time T_1 by a factor of 5 or above. Homo-gated irradiation technique was used to depress the HDO peak in D_2O.

Results and Discussion

Binding of Tolbutamide (1) to HSA (3) The binding of **1** to **3** was examined by equilibrium dialysis at 20—40 °C. It is safely assumed that **3** is stable enough at these temperatures since the homologous protein, BSA (**4**) is not denaturated up to 42 °C.[17)] The free drug concentration (Df) was determined from the residual drug concentration, and the number of mol of the drug binding to one mol of **3** (r) was estimated from the decrease in drug

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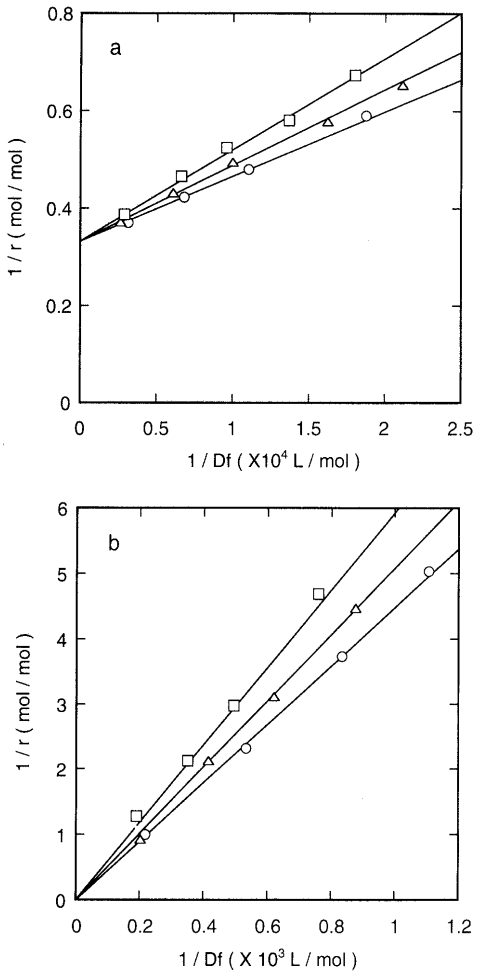


Fig. 1. Klotz Plots for the Binding of Drugs (a, **1**; b, **2**) and **3** (a, 7.25×10^{-5} M; b, 7.25×10^{-4} M) in 0.1 M Phosphate Buffer (pH 7) at 20 °C (○), 30 °C (△) and 40 °C (□)

concentration. The plot of $1/Df$ *vs.* $1/r$ produced a linear relationship, as shown in Fig. 1, and satisfied Eq. 3 proposed by Klotz[18]:

$$\frac{1}{r} = \frac{1}{nKDf} + \frac{1}{n} \tag{3}$$

where n is the number of binding sites per mol of **3** and K is the binding constant between **1** and **3**. The values of n and K can be calculated from the intercept and slope of the straight line (Table 1). Fig. 1a revealed that the binding site was of one class and the number of sites was $n=3$. Crooks and Brown[13] reported that the interaction between **1** and **3** involved two classes of binding sites ($n_1 = 2.27$, $K_1 = 2.186 \times 10^5\ \mathrm{M}^{-1}$, $n_2 = 8.21$, $K_2 = 1.71 \times 10^2\ \mathrm{M}^{-1}$) in the wide range of concentration ($Df = ca.\ 4 \times 10^{-5}$—$6 \times 10^{-3}$ M). Muller *et al.*[19] reported that **1** bound with **3** on the hydrophobic binding site I. However, in view of the small range of high concentration used in this work (0.05—0.5 mM), it would be better to take into account the specific and non-specific binding site.

The number of binding sites ($n=3$) was independent of temperature (20—40 °C). The thermodynamic parameters were calculated from the linear relationship between $\ln K$ and the reciprocal absolute temperature (Table 1, Fig. 2). For the **1**–**3** system, the free energy changes ($\Delta G°$) and the standard increase of enthalpy ($\Delta H°$) were large and negative, and the standard entropy changes ($\Delta S°$) were positive. These results indicated that the decrease of energy was due to the hydrophobic interaction between **1** and **3**, and that the increase of entropy was based on the destruction of the iceberg structure induced by the hydrophobic interaction. As shown in Fig. 3a, the binding constant was independent of ionic strength in **1**–**3** system, and decreased with the addition of short-chain fatty acid (Table 2). The binding constant markedly decreased with the increase of the alkyl chain length of fatty acid, though the dissociation constants of fatty acid used were similar.[20] These results suggested that the fatty acids competed with **1** for the protein binding, which might be hydrophobic rather than hydrophilic.

Table 1. Thermodynamic Data for the Binding of Drugs with **3**[a]

Drug	Temp. (°C)	K ($\times 10^4\ \mathrm{M}^{-1}$)	nK ($\times 10^3\ \mathrm{M}^{-1}$)	$\Delta G°$ (kJ/mol)	$\Delta H°$ (kJ/mol)	$\Delta S°$ (J mol^{-1} K^{-1})
1[b]	20	2.38		−24.55	−11.63	44.08
	30	2.07		−25.04		44.25
	40	1.76		−25.44		44.11
						(Av.) 44.11
2[c]	20		2.22		−10.39	
	30		1.97			
	40		1.69			

a) [Phos. buf.] = 0.1 M, pH = 7. *b*) [**3**] = 7.25×10^{-5} M. *c*) [**3**] = 7.25×10^{-4} M.

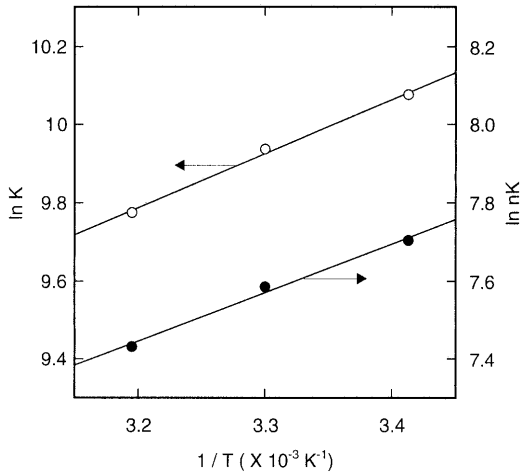


Fig. 2. Relationship between $\ln K$, $\ln nK$ and Reciprocal Absolute Temperature (○, **1**/**3**; ●, **2**/**3**)

Chemical Shift of 1 As shown in Table 3, both 10 fold dilution and addition of **3** resulted in slight differences in the chemical shift of **1**. Therefore, it was difficult to determine the binding position from the change in this shift; even the largest shift of 3-CH_2 was only 0.016 ppm.

Spin–Lattice Relaxation Time of 1 Spin–lattice relaxation time (T_1) of **1** was independent of the concentrations (Table 4). The addition of **3** (7.25×10^{-5} M) to **1** (10 mM) caused a significant decrease in the relaxation time. It was predicted that the phenyl group of **1** mainly interacted with **3**, since the T_1 values of aromatic protons decreased more than those of aliphatic protons.

Spin–Spin Relaxation Rate Spin–spin relaxation time (T_2) of **1** was measured by the CPMG method (Fig.4). Spin–spin relaxation rates ($1/T_2$) of **1** were almost independent of the concentrations (1—10 mM) (Table 5).

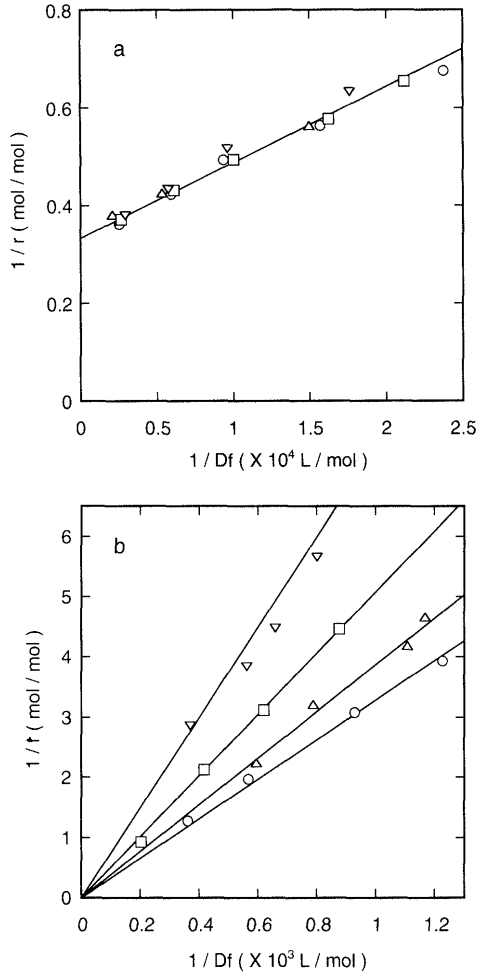


Fig. 3. Klotz Plots for the Binding of Drugs (a, **1**; b, **2**) and **3** (a, 7.25×10^{-5} M; b, 7.25×10^{-4} M) in Phosphate Buffer (○, 0.2 M; △, 0.1 M; □, 0.05 M; ▽, 0.025 M, pH 7) at 30 °C

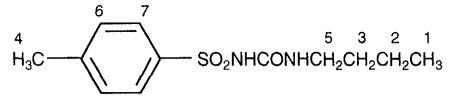
Table 2. Effect of Fatty Acid for the Binding of **1** to **3**[a]

Fatty acid	C_n	K_a[b] ($\times 10^{-5}$)	K ($\times 10^4$ M^{-1})
None			2.07
Butyric acid	C_4	1.50	1.35
Valeric acid	C_5	1.56	1.00
Caproic acid	C_6	1.46	0.71
Enanthic acid	C_7	1.46	0.37
Caprylic acid	C_8	1.44	0.30

a) [Fatty acid] = 1 mM, [**3**] = 7.25×10^{-5} M, [Phos. buf.] = 0.1 M, pH = 7, 30 °C. *b*) Acid dissosiation constant.[20]

The addition of **3** (7.25×10^{-5} M) to **1** (10 mM) caused a significant increase in the relaxation rate. Owing to the lack of effect of **3** on chemical shift, slow exchange between free and bound states was reported to cause the superposed narrow and broad peaks in the NMR spectrum.[11] In this work, however, rapid exchange between both states was understood, since one peak was observed as the weighted average of the two states. In a slow exchange system, T_2 measurement by the CPMG method was found to be difficult because of modulation action by repeated irradiation of the π pulse.[21] In this work, a series of echoes measured by the CPMG method decayed according to Eq. 2 without modulation. Therefore, the exchange between

Table 3. Chemical Shifts (δ) of **1**[a]



	1-CH_3 (t)	2-CH_2 (m)	3-CH_2 (m)	4-CH_3 (s)	5-CH_2 (t)	6-CH (d)	7-CH (d)
1 mM **1**	0.580	0.971	1.110	2.144	2.747	7.112	7.455
10 mM **1**	0.578	0.968	1.109	2.142	2.746	7.111	7.456
10 mM **1**/7.25×10^{-5} M **3**	0.571	0.960	1.093	2.134	2.743	7.102	7.454

a) External reference, tetramethylsilane; pH = 7; 40 °C; (s), singlet; (d), doublet; (t), triplet; (m), multiplet.

Table 4. Spin–Lattice Relaxation Time (T_1, s) of **1**[a]

		1-CH_3	2-CH_2	3-CH_2	4-CH_3	5-CH_2	6-CH	7-CH
1 mM **1**		2.532	1.988	1.491	1.505	1.397	3.249	4.034
10 mM **1**	(A)	2.709	2.076	1.609	1.548	1.388	3.442	4.373
10 mM **1**/7.25×10^{-5} M **3**	(B)	1.309	1.124	1.032	1.107	0.973	1.534	1.719
B/A (%)		48	54	64	72	70	45	39

a) pH = 7; 40 °C.

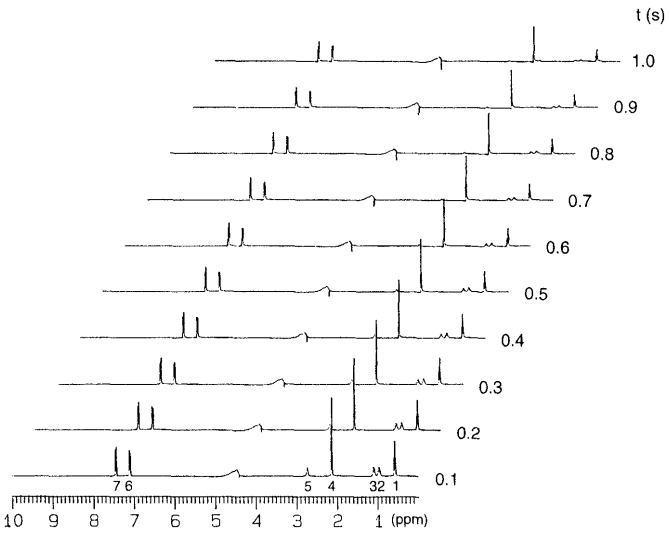


Fig. 4. Spin–Spin Relaxation Traces Obtained by the Carr–Purcell–Meiboom–Gill Method for the Protons of **1** at 40 °C

free and bound states in our system was suggested to be rapid, similar to the case reported by Jardetzky.[22] The spin–spin relaxation rate of drug bound to albumin, $(1/T_2)_b$, was calculated according to the equation proposed by Jardetzky[22]:

$$1/T_2 = (1-B)(1/T_2)_f + B(1/T_2)_b \quad (4)$$

where $(1/T_2)_f$ is the spin–spin relaxation rate of the free drug, and B is the proportion of the drug bound to albumin. The B value can be calculated from the binding constant (K) and the number of binding sites (n) obtained by equilibrium dialysis. Fehske *et al.*[23] reported that the binding sites of two hydrophobic drugs, warfarin and diazepam, were tryptophan and tyrosine residues on **3**, respectively, and that a maximum 10.7 units of 18 units of tyrosine in **3** were modified. Therefore, the largest number (n) of hydrophobic binding sites on **3** was assumed to be

Table 5. Spin–Spin Relaxation Rates ($1/T_2$, s^{-1}) of **1**[a]

Peak	$(1/T_2)_{f1}$ (s^{-1})	$(1/T_2)_{f2}$ (s^{-1})	$1/T_2$ (s^{-1})	$n=3$		$n=12$	
				$(1/T_2)_b$ ($\times 10^2\,s^{-1}$)	$(1/T_2)_b/(1/T_2)_{f2}$ ($\times 10^2$)	$(1/T_2)_b$ ($\times 10^2\,s^{-1}$)	$(1/T_2)_b/(1/T_2)_{f2}$ ($\times 10^2$)
1-CH_3	1.125	1.541	6.71	2.41	1.56	0.62	0.41
2-CH_2	1.672	3.413	7.87	2.10	0.61	0.56	0.16
3-CH_2	2.119	4.425	9.62	2.45	0.55	0.66	0.15
4-CH_3	0.796	0.733	9.62	4.11	5.61	1.05	1.44
5-CH_2	2.506	1.887	10.53	4.01	2.13	1.04	0.55
6-CH	0.431	0.409	7.58	3.32	8.11	0.85	2.07
7-CH	0.356	0.329	3.98	1.69	5.17	0.43	1.33

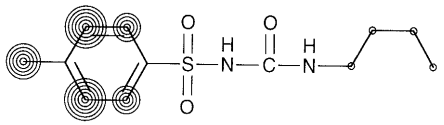
a) $(1/T_2)_{f1}$, free **1** (1 mM) observed; $(1/T_2)_{f2}$, free **1** (10 mM) observed; $1/T_2$, overall observed (10 mM **1**/7.25×10^{-5} M **3**); $(1/T_2)_b$, **1** bound to **3** calculated; n, number of binding sites on **3**.

Table 6. Spin–Spin Relaxation Rates ($1/T_2$, s^{-1}) of **2**[a]

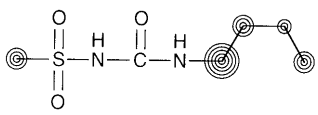
4 5 3 2 1
$CH_3SO_2NHCONHCH_2CH_2CH_2CH_3$

Peak	$(1/T_2)_{f1}$ (s^{-1})	$(1/T_2)_{f2}$ (s^{-1})	$1/T_2$ (s^{-1})	$(1/T_2)_b$ ($\times 10\,s^{-1}$)	$(1/T_2)_b/(1/T_2)_{f2}$ ($\times 10$)
1-CH_3	0.749	0.835	4.73	4.92	5.89
2-CH_2	0.855	0.845	3.31	3.44	4.08
3-CH_2	0.903	0.986	4.82	5.01	5.09
4-CH_3	0.489	0.678	3.57	3.71	5.48
5-CH_2	0.617	0.691	4.91	5.11	7.40

a) $(1/T_2)_{f1}$, free **2** (1 mM) observed; $(1/T_2)_{f2}$, free **2** (10 mM) observed; $1/T_2$, overall observed (10 mM **2**/7.25×10^{-5} M **3**); $(1/T_2)_b$, **2** bound to **3** calculated.



tolbutamide (**1**)



1-butyl-3-(methylsulfonyl)urea (**2**)

Fig. 5. Contour Plots of the Ratio of the Spin–Spin Relaxation Rate of Bound Drug to Free Drug

12, which consisted of 1 unit of tryptophan and 11 units of tyrosine. The values of $(1/T_2)_b$ were calculated according to Eq. 4. However, the discussion of the binding position on the basis of $(1/T_2)_b$ values was not reasonable, since the relaxation rates of the respective protons of **1**, $(1/T_2)_{f2}$, were different from each other. The ratio $(1/T_2)_b/(1/T_2)_{f2}$ has a significant meaning for determination of the binding position, since the sequence of the ratio was independent of the arbitrary n-values (3 or 12), although the absolute values of the ratio depended on these n-values. As can be seen from Table 5, the 6-CH proton had the largest value of $(1/T_2)_b/(1/T_2)_{f2}$, followed by the 4-CH_3 proton. It was therefore concluded that the binding position of **1** to **3** was at the tolyl group.

Binding of 1-Butyl-3-(methylsulfonyl)urea (2) to HSA (3) The binding of **2** to **3** was examined by the same methods as used for **1** to **3**. Linear Klotz's plots were obtained (Fig. 1b). However, the n-value was numerically large and could not be estimated accurately because the intercept was nearly zero; this system was therefore believed to have nonspecific binding. Takagishi and Kuroki[24] evaluated the magnitude of binding in terms of the nK-value (binding capacity) rather than the K-value estimated from the uncertain n-value. The nK-values are estimated in Table 1. The standard increase of enthalpy (ΔH°) was calculated from the linear relationship between $\ln nK$ and the reciprocal absolute temperatures, assuming independence of the n-value on temperature (Table 1, Fig. 2). The binding capacity was reduced by increase of the ionic strength (Fig. 3b). These results suggested that the binding of **2** to **3** was hydrophilic.

The binding sites on **3** for **2** were thought to be the basic moieties such as lysine, arginine and histidine since **2** was an acidic drug. Therefore, the largest number (n) of binding sites was assumed to be 99 which consisted of 59 units of lysine, 24 units of arginine and 16 units of histidine. The values of $(1/T_2)_b$ were then calculated (Table 6), and the 5-CH_2 proton had the largest value of $(1/T_2)_b/(1/T_2)_{f2}$. This observation suggested that **2** bound to **3** through the sulfonylurea moiety of **2**, which was hydrophilic.

Conclusion

The contour plots of $(1/T_2)_b/(1/T_2)_{f2}$ values are illustrated in Fig.5. These illustrations are a useful method to visibly represent the binding position. As described above, the bindings of **1** and **2** to **3** were due to hydrophobic and hydrophilic interactions, respectively. **1** and **2** exhibited different binding properties, though both drugs have a common structure, sulfonylurea. The competition between the hydrophilic effect of the sulfonylurea moiety and the hydrophobic effect of the tolyl group was concluded to be the main factor governing the binding property of **1**.

References and Notes

1) This report constitutes Part IX of the series entitled "Interaction between Drugs and Water-Soluble Polymers." This work was presented at the 116th Annual Meeting of the Pharmaceutical Society of Japan, Kanazawa, 1996.
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