Binding Position of Phenylbutazone with Bovine Serum Albumin Determined by Measuring Nuclear Magnetic Resonance Relaxation Time[1)]

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The binding of phenylbutazone (PB) to bovine serum albumin (BSA) was considered to be predominantly due to hydrophobic interaction based on the thermodynamic parameters obtained by an equilibrium dialysis method. Little variation of proton nuclear magnetic resonance (^{1}H-NMR) chemical shift of PB was found with change in the concentration of PB (0.5—5 mM) or upon the addition of BSA (7.25×10^{-5} M). The NMR spectrum of PB in 0.1 M phosphate buffer solution at pH 7 showed that PB existed as a mesomeric anion. The spin–lattice relaxation time (T_1) of PB was almost concentration-independent, but decreased in the presence of BSA to 36—38% for the phenyl group and 48—100% for the butyl group. The spin–spin relaxation time (T_2) of PB was also almost independent of concentration, but was remarkably decreased in the presence of BSA to *ca.* 2.5% for the phenyl group and *ca.* 6—9% for the butyl group. The ratios of the spin–spin relaxation rate ($1/T_2$) of the free PB to that of the bound PB were *ca.* 5000—11000 for the butyl group and *ca.* 23000 for the phenyl group. The binding of PB to BSA was considered to involve primarily the phenyl group.

Keywords equilibrium dialysis; hydrophobic interaction; nuclear magnetic resonance; spin–lattice relaxation time; spin–spin relaxation time; spin–spin relaxation rate; phenylbutazone; bovine serum albumin; binding position

In the previous paper,[2)] the interaction between warfarin (WF) and water-soluble polymers such as bovine serum albumin (BSA), polyvinylpyrrolidone (PVP), *etc.* was studied by equilibrium dialysis, and it was concluded that hydrophobic binding played an important role. Our next aim was to determine the binding position of the drug molecule to the polymer. However, it seemed impossible to solve the problem by means of general methods such as dialysis, gel filtration, or electronic, fluorescence or circular dichroism spectroscopy, *etc.*[3)] Nuclear magnetic resonance (NMR) spectroscopy, which yields information on each proton of a molecule, seemed to be the most appropriate method for this study. Previously, Jardetzky[4)] pointed out that NMR spin–lattice relaxation time (T_1) and spin–spin relaxation time (T_2) were available for the study of drug–protein interaction. However, few applications of T_1 and T_2 have been attempted[5)] because the measurement was troublesome. In the early studies,[5)] the T_2 value for a singlet peak was roughly estimated from the half-width. Recently, Ueda *et al.*[6)] examined the binding position of sulfonylurea drugs to albumin by using the spin–lattice relaxation time in the rotating frame ($T_{1\rho}$) instead of T_2. Though we tried to apply this method to the study of the interaction between WF and BSA, the measurement was difficult because the NMR spectrum of WF gave many overlapping peaks.

In the present paper, the T_2 values of phenylbutazone (PB), which competed with WF for the binding to BSA,[2)] were determined directly by means of the Carr-Purcell-Meiboom-Gill (CPMG) method,[7)] and it was elucidated that the binding of PB to BSA involves mainly the phenyl group of PB.

Experimental

Materials PB was of special reagent grade from Sigma, and was used without further purification. BSA was from Wako and its average molecular weight was 6.9×10^4. Other reagents were from commercial sources and were used without further purification.

Equilibrium Dialysis The equilibrium dialysis method was the same as described previously.[2)]

NMR Spectroscopy The NMR spectra were measured in deuterium oxide-d_2 (D_2O, phosphate buffer, 0.1 M, pH = 7) on a JEOL GX-400 spectrometer at 40 °C. Tetramethylsilane (TMS) was used as an external reference. The ^{1}H-NMR spectrum in chloroform-d_1 ($CDCl_3$) was measured at room temperature by using TMS as an internal reference. The spin–lattice relaxation time (T_1) was obtained by the inversion recovery method[8)] according to Eq. 1:

$$\ln(M_0 - M_t) = -\frac{t}{T_1} + \ln(2M_0) \quad (1)$$

where t is the interval between the π pulse and the $\pi/2$ pulse, M_0 is the equilibrium magnetization at $t=0$, and M_t is the macroscopic magnetization at t. The spin–spin relaxation time (T_2) was obtained by the CPMG method[7)] according to Eq. 2:

$$\ln(M_{t_2}) = \ln(M_{t_1}) - \frac{\tau}{T_2} \quad (2)$$

where t_1 and t_2 are the times when a free induction decay (FID) is observed after the $\pi/2$ pulse, M_{t1} and M_{t_2} are the macroscopic magnetization at t_1 and t_2, respectively, and τ is the interval between t_1 and t_2. The pulse delay time (20 s) when the next pulse was applied after the observation of FID was selected to be more than five times T_1. Homogated irradiation was carried out to depress the HDO peak in D_2O.

Results and Discussion

The Binding Constant of PB to BSA Determined by Equilibrium Dialysis The binding of drug (PB) to polymer (BSA) was examined by equilibrium dialysis at 20—40 °C. The free drug concentration (Df) was determined from the residual drug concentration and the number of mol of drug binding to one mol of the polymer (r) was estimated from the decrease of drug concentration. The plot of r *vs.* r/Df gave a linear relationship, as shown in Fig. 1, and satisfied Eq. 3 proposed by Scatchard[9)]:

$$\frac{r}{Df} = Kn - Kr \quad (3)$$

where n is the number of binding sites per mol of the polymer and K is the binding constant between the drug and the polymer. The n- and K-values were calculated from the intercept on the abscissa and the slope of the line. Brown and Crooks[10)] reported that the interaction between

PB and BSA involved two classes of binding sites ($n_1=3.18$, $K_1=2.78\times10^5\ M^{-1}$, $n_2=4.21$, $K_2=2.29\times10^3\ M^{-1}$) by using a dynamic dialysis method. However, in view of the small range of concentration used in this work (0.05—0.5mM), it seemed sufficient to consider only one class of binding site, as Fig. 1 showed a linear relationship. Furthermore, since the binding constant of this work was smaller than that of Brown and Crooks,[10] it is considered that only the secondary site was observed in the range of drug concentration used in this work. The number of binding sites ($n=3$) was independent of the temperature (20—40 °C). The thermodynamic parameters were calculated from the linear relationship between ln K and the reciprocal absolute temperature ($1/T$) (Table I). The values of the standard increases of enthalpy ($\Delta H°$) and free energy ($\Delta G°$) were negative and large, and the value of the standard increase of entropy ($\Delta S°$) was positive and small. These results can be explained on the basis of the decrease of energy owing to the hydrophobic interaction between PB and BSA, and the increase of entropy by the destruction of the iceberg structure.

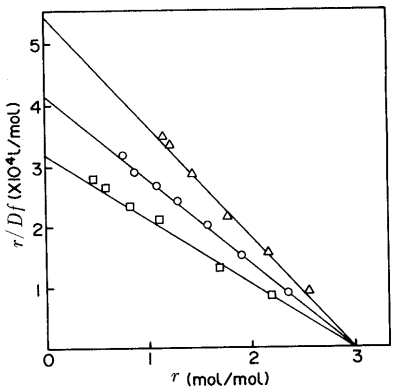

Fig. 1. Scatchard Plots for the Binding of Phenylbutazone (PB) to BSA (7.25×10^{-5} M) in 0.1 M Phosphate Buffer (pH 7) at 20 °C (△), 30 °C (○) and 40 °C (□)

TABLE I. Thermodynamic Parameters for the Binding of PB to BSA

Temp. (°C)	K ($\times10^4\ M^{-1}$)	$\Delta G°$ (kJ/mol)	$\Delta H°$ (kJ/mol)	$\Delta S°$ (J/mol/K)
20	1.82	−23.89	−20.80	10.59
30	1.38	−24.02		10.63
40	1.06	−24.10		10.54
				(Av.) 10.59

[BSA]=7.25×10^{-5} M, pH=7, [Phos. buf.]=0.1 M.

The Chemical Shifts of PB The spectrum of PB in $CDCl_3$ gave peaks corresponding to all the protons. However, in 0.1 M phosphate buffer solution (both in H_2O and D_2O) at pH 7, the peak of the methine proton (5-CH) in the pyrazolidine ring disappeared and the peak of the adjacent mehylene (4-CH_2) in the butyl group was transformed from a quartet to a triplet (Table II). PB (I) exists as a mesomeric anion (II) at pH 7 since PB is a weak acid ($pK_a=4.2$) and the methine signal on the NMR spectrum disappears in the aqueous solution at pH 7. Therefore it is predicted that the hydrophobic part in II is the diphenyl groups rather than the butyl group.

When the solution of PB was diluted from 5 to 0.5 mM, the variation of chemical shift was less than 0.003 ppm. Furthermore, the variation of chemical shift was less than 0.039 ppm in the presence of BSA (7.25×10^{-5} M). Therefore, it is difficult to determine the binding position from the change of chemical shift of PB.

Spin–Lattice Relaxation Time The spin–lattice relaxation time (T_1) of PB under typical conditions was measured by the inversion recovery method (Table III). When the solution of PB was diluted from 5 to 0.5 mM, little variation of T_1 was observed. In the presence of BSA (7.25×10^{-5} M), the T_1-values of the phenyl group decreased to 36% (*p*-H) and 38% (*o*,*m*-H), and those of the butyl group decreased to 49% (1-CH_3), 63% (2-CH_2) and 81% (3-CH_2), respectively, whereas little change was observed in that of 4-CH_2. From these results, it is con-

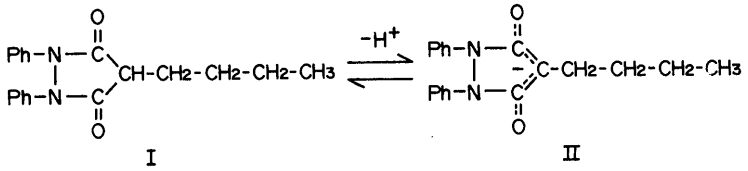

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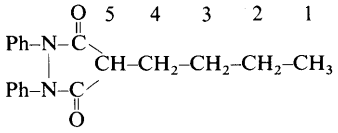
Chart 1. Dissociation of Phenylbutazone

TABLE II. Chemical Shifts (δ) of PB



Concentration	Solv.	1-CH_3	2-CH_2	3-CH_2	4-CH_2	5-CH	*o*,*m*-H	*p*-H
		t	m	m	t		m	m
5 mM PB	D_2O[a]	0.834	1.263	1.382	2.118		7.276	7.146
0.5 mM PB		0.835	1.262	1.383	2.117		7.279	7.149
5 mM PB/7.25×10^{-5} M BSA		0.822	1.265	1.384	2.111		7.262	7.107
		t	m	m	q	t	m	m
5 mM PB	$CDCl_3$[b]	0.900	1.356	1.479	2.084	3.384	7.314	7.170

a) From TMS (external reference), pH=7, [Phos. buf.]=0.1 M, 40 °C. *b*) From TMS (internal reference), room temperature.

TABLE III. Spin–Lattice Relaxation Time (T_1, s) of PB

Concentration	1-CH_3	2-CH_2	3-CH_2	4-CH_2	*o*,*m*-H	*p*-H
5 mM PB	2.06	1.39	0.96	0.70	2.74	2.92
0.5 mM PB	1.92	1.29	0.93	0.64	2.67	2.94
5 mM PB/7.25×10^{-5} M BSA	1.00	0.87	0.78	0.71	1.05	1.04

pH = 7, [Phos. buf.] = 0.1 M, 40 °C

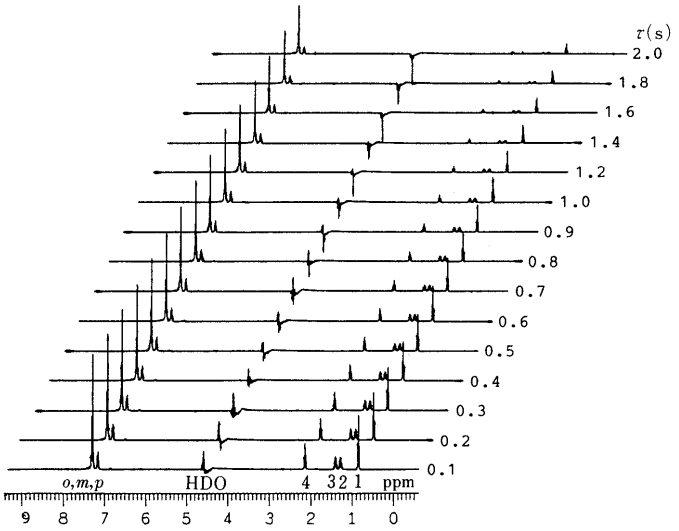


Fig. 2. Spin–Spin Relaxation Traces Obtained by the Carr-Purcell-Meiboom-Gill Method for Protons of PB

sidered that the phenyl group mainly interacts with BSA.

Spin–Spin Relaxation Time The spin–spin relaxation time (T_2) of PB was measured by the CPMG method and a series of spectra were shown in Fig. 2. Furthermore, the T_2-values under typical conditions were listed in Table IV. When the solution of PB was diluted from 5 to 0.5 mM, little variation of T_2 was observed. In the presence of BSA (7.25×10^{-5} M), the T_2-values of the phenyl group and butyl group decreased to 2.5% and 6—9%, respectively. This also supports the binding at the phenyl group. The decreases of T_2 values were far larger than those of T_1 values. Thus, spin–spin relaxation time (T_2) is an useful parameter to detect a small change of the molecular environment.

Effect of Concentration of BSA on the Spin–Spin Relaxation Rate Linear relationships between the spin–spin relaxation rates ($1/T_2$) for all the protons and the concentration of BSA were obtained in the range of 0 to 7.25×10^{-5} M BSA (Fig. 3). The slopes decreased in the order of 4-CH_2, *p*-CH, *o*,*m*-CH, 3-CH_2, 2-CH_2 and 1-CH_3. The increase of the overall rate ($1/T_2$) of the 4-CH_2 group by BSA was larger than those of the phenyl group and the other part of the butyl group. This result is in conflict with the above conclusion that the phenyl group is the binding position. The reason for this discrepancy may be a difference of the rate for the free drug $(1/T_2)_f$.

Spin–Spin Relaxation Rate of the Bound Drug The spin–spin relaxation rate of the bound drug ($(1/T_2)_b$) was measured by a modification of the method proposed by Jardetzky,[4] based on the following equation:

$$\frac{1}{T_2}=\left(\frac{1}{T_2}\right)_f+B\left[\left(\frac{1}{T_2}\right)_b-\left(\frac{1}{T_2}\right)_f\right] \tag{4}$$

TABLE IV. Spin–Spin Relaxation Time (T_2, s) of PB

Concentration	1-CH_3	2-CH_2	3-CH_2	4-CH_2	*o*,*m*-H	*p*-H
5 mM	1.22	1.04	1.01	0.51	2.12	2.09
0.5 mM PB	1.12	1.23	1.00	0.62	2.04	2.20
5 mM PB/7.25×10^{-5} M BSA	0.078	0.081	0.077	0.045	0.052	0.051

pH = 7, [Phos. Buf.] = 0.1 M, 40 °C.

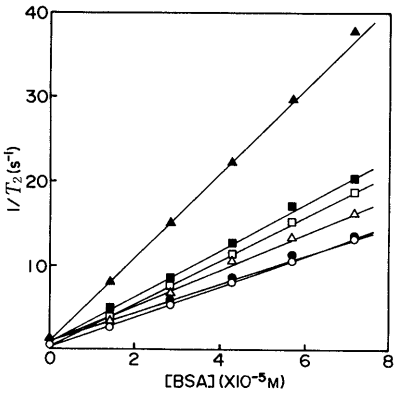


Fig. 3. Effect of BSA on the Proton Relaxation Rate ($1/T_2$) of PB

[PB] = 5 mM, pH = 7, at 40 °C. ○, 1-CH_3; ●, 2-CH_2; △, 3-CH_2; ▲, 4-CH_2; □, *o*, *m*-H; ■, *p*-H.

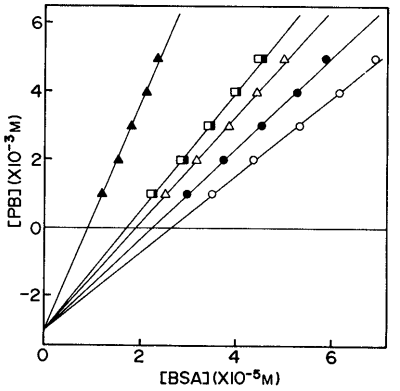


Fig. 4. Linear Relationships (Eq. 7) between Concentrations of Free PB and BSA Estimated from the Proton Relaxation Rate ($1/T_2$) of PB

○, 1-CH_3; ●, 2-CH_2; △, 3-CH_2; ▲, 4-CH_2; □, *o*, *m*-H; ■, *p*-H.

where $(1/T_2)_f$ is the spin–spin relaxation rate of the free drug and B is the proportion of the drug (PB) bound to protein (BSA). The binding constant (K) is calculated by means of Eq. 5:

$$K=\frac{B[\mathrm{PB}]}{[\mathrm{PB}](1-B)(n[\mathrm{BSA}]-B[\mathrm{PB}])} \tag{5}$$

where [PB] and [BSA] are the concentrations of free PB and BSA, respectively and n is the number of binding sites on BSA. The modification of Eq. 5 gives Eq. 6.

$$[\mathrm{PB}]=\frac{1}{K(B-1)}+\frac{n}{B}[\mathrm{BSA}] \tag{6}$$

As the proportion of the drug bound to BSA is small ($B\ll1$), Eq. 7 is valid.

$$[\mathrm{PB}]=-\frac{1}{K}+\frac{n}{B}[\mathrm{BSA}] \tag{7}$$

TABLE V. Spin–Spin Relaxation Rates ($1/T_2$) of PB

Peak	$1/T_2$ (s^{-1})	$(1/T_2)_f$ (s^{-1})	n/B ($\times 10^3$)	$n=3$ (Dialysis) $(1/T_2)_b$ ($\times 10^3 s^{-1}$)	$n=3$ (Dialysis) $(1/T_2)_b/(1/T_2)_f$ ($\times 10^3$)	$n=100$ (Assumed) $(1/T_2)_b$ ($\times 10^2 s^{-1}$)	$n=100$ (Assumed) $(1/T_2)_b/(1/T_2)_f$ ($\times 10^2$)
1-CH_3	12.8	0.823	1.15	4.58	5.57	1.39	1.69
2-CH_2	12.4	0.958	1.35	5.13	5.35	1.55	1.62
3-CH_2	13.0	0.988	1.59	6.38	6.46	1.92	1.94
4-CH_2	22.2	1.974	3.35 (1.52)[a]	22.6 (10.3)[a]	11.4 (5.22)[a]	6.80 (3.09)[a]	3.44 (1.57)[a]
o,m-H	19.2	0.473	1.77	11.0	23.3	3.32	7.08
p-H	19.6	0.478	1.76	11.2	23.4	3.37	7.05

$1/T_2$, overall observed; $(1/T_2)_f$, free PB observed; $(1/T_2)_b$, PB bound to BSA calculated; n, number of binding sites on BSA; B, ratio of PB bound to BSA. *a*) Assumed $n/B=1.52\times10^3$.

Therefore, in the linear plot of [PB] *vs.* [BSA], the slope and the intercept on the ordinate correspond to n/B and $-1/K$, respectively. As the n-value is obtainable from an equilibrium dialysis experiment, the B-value can be calculated. When the calculated value of B and the found values of $1/T_2$ and $(1/T_2)_f$ are substituted into Eq. 4, $(1/T_2)_b$ can be evaluated.

We measured $1/T_2$ for all the protons of PB under the conditions ([PB]=5 mM, and [BSA]=0—7.25×10^{-5} M) and obtained a linear relationship between $1/T_2$ and [BSA] (Fig. 3). Similar experiments were carried out at the various concentrations of PB (1—4 mM). Based on a series of experiments, an arbitrary value ($12\,s^{-1}$) near the minimum of $1/T_2$ was chosen. From the linear relationship of $1/T_2$ and [BSA], pairs of [PB] and [BSA] providing a constant value of $1/T_2$ ($12\,s^{-1}$) were obtained. Since a linear relationship between [PB] and [BSA] was observed (Fig. 4), Eq. 7 was confirmed to be valid. The values of $(1/T_2)_b$ were calculated by means of Eqs. 7 and 4 (Table V).

The n/B value of 4-CH_2 was about twice as larger as the others ($1.52\pm0.27\times10^3$). This discrepancy is presumably caused by the effect of the ionization (I⇌II) on the relaxation time (T_2) of the neighboring 4-CH_2. Since all n/B values should be the same (1.52×10^3), the $(1/T_2)_b$ value of 4-CH_2 was estimated to be $10.3\times10^3\,s^{-1}$. Furthermore the $(1/T_2)_b/(1/T_2)_f$ value of 4-CH_2 was calculated to be 5.22×10^3, which was close to those of the other butyl protons. Namely the ratio for the phenyl group was 3—4 times that for the butyl group. These results again confirmed that the binding position of PB to BSA is mainly the phenyl group. The binding constant determined from the NMR relaxation rate was *ca.* $3.33\times10^2\,M^{-1}$, which was somewhat smaller than that obtained by the equilibrium dialysis method. Since the drug (PB) concentration for the NMR measurement (1—5 mM) was higher than that in the equilibrium dialysis method (0.05—0.5 mM) and the drug/protein ratio was of the order of one hundred, it is considered that the binding site examined by NMR was secondary (in other words, nonspecific) as compared with that by the equilibrium dialysis method. If the number of the nonspecific sites (n) was assumed to be 100, the relative values of $(1/T_2)_b/(1/T_2)_f$ did not vary, although the absolute values decreased as shown in Table V. Therefore, even if the binding was nonspecific, the conclusion mentioned above stands.

Otagiri and Uekama[11] reported that the binding site of human serum albumin to PB was a wide region in the vicinity of the tryptophan residue. A similar binding site was also predicted for the PB–BSA system. However the binding of PB to nonspecific sites on BSA should be considered because the drug/protein ratio was very large in this work.

As mentioned above, the NMR relaxation rate generally gives useful information, which cannot be obtained by other methods, with respect to the binding position of a drug molecule to a polymer such as a protein. Even in the case of a high drug concentration or the large drug/protein ratio, the binding position of the drug on the secondary (that is nonspecific) sites of the protein is the same as that on the primary site.

References and Notes

1) This reports constitutes Part II of the series entitled "Interaction between Drugs and Water-Soluble Polymers". This work was presented at the 107th Annual Meeting of the Pharmaceutical Society of Japan, Kyoto, 1987.
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