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Characteristics of Protein Binding of Thiobarbiturates and 6-*n*-Propyl-2-thiouracil[1,2]

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To investigate the protein binding characteristics of thiamylal (TA), thiopental (TP) and 6-*n*-propyl-2-thiouracil (PTU), the effects of pH, ionic strength and temperature on the binding of these three drugs were compared by equilibrium dialysis.

The bindings between each of the three drugs and bovine serum albumin (BSA) were similarly dependent on pH. However, the binding constants of TA and TP at pH 7.4 were scarcely influenced by changes of the ionic strength made by the addition of KCl or K_2SO_4, while that of PTU decreased with increase of the ionic strength. On the basis of these results and the considerable changes in the pK_a of bound PTU (previous paper), it is possible that the binding of PTU involves a larger contribution from the interaction with the charged region on BSA than those of TA and TP.

A thermodynamic study showed major contributions of $\Delta S°$ in the cases of TA and TP and of $\Delta H°$ in the case of PTU to $\Delta G°$, suggesting that the binding sites of TA and TP might be different from those of PTU.

The pH dependency of binding was also studied by fluorometric titration employing 8-anilinonaphthalene-1-sulfonate (ANS) as an indicator. The displacement ratios of TA and TP increased with increase of pH, in accord with the results of equilibrium dialysis, but that of PTU was only slightly dependent on pH.

Keywords——protein binding; bovine serum albumin; thiopental; thiamylal; 6-*n*-propyl-2-thiouracil; 8-anilinonaphthalene-1-sulfonate; pH dependency; equilibrium dialysis; fluorometric titration

Introduction

The drug–albumin interaction has been extensively studied in pharmacy and pharmacology because of its possible influence on the biological activity or pharmacokinetic behavior of drugs. The study of binding forces in the interaction may be useful for the consideration of displacement phenomena between drugs. Recently, the molecular nature of the interaction has been considered in terms of the hydrophobicity and pK_a of the drugs.[4] The electrostatic forces and hydrophobic forces in the interaction are generally considered to be important, though Klotz[5] emphasized the contribution of the Van der Waals forces to the binding affinity. For example, with benzoates[4a] and sulfonamides[4b] electrostatic interaction contributes to the binding forces, but with phenothiazines,[4c] sulfonylureas and warfarin,[4d] hydrophobic bonding is a major contributor.

The binding of thiamylal (TA), thiopental (TP) and 6-*n*-propyl-2-thiouracil (PTU) to bovine serum albumin (BSA) is accompanied by a pK_a shift to the acidic side[6] and an

1) This forms part II of "Protein Bindings of Thiobarbiturates" by T. Sakurai.
2) Presented at the 97th Annual Meeting of the Pharmaceutical Society of Japan, Tokyo, Japan, April 1977.
3) Location: *1432-1 Horinouchi, Hachioji, Tokyo.*
4) *a*) I. Moriguchi, *Chem. Pharm. Bull.*, **16**, 597 (1968); *b*) I. Moriguchi, S. Wada, and T. Nishizawa, *ibid.*, **16**, 601 (1968); *c*) H. Zia and J.C. Price, *J. Pharm. Sci.*, **64**, 1177 (1975); *d*) S. Goto, H. Yoshitomi, and M. Nakase, *Chem. Pharm. Bull.*, **26**, 472 (1978).
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6) T. Sakurai, H. Matsumaru, and S. Tsuchiya, *Yakuzaigaku*, **37**, 169 (1977).

electrostatic interaction[7] was suggested. In this study, in order to investigate the nature of the binding of these three drugs, the effects of pH, ionic strength and temperature on the binding of the drugs were studied. 8-Anilinonaphthalene-1-sulfonate (ANS) was used as an indicator of binding at various pH values, and the results were compared with those of equilibrium dialysis.

Experimental

Materials——TA and TP were extracted from a commercially available injective preparation,[6] and PTU was of guaranteed reagent grade, from Kanto Kagaku Co. ANS was obtained from Tokyo Kasei Kogyo Co., Ltd. Sörensen phosphate buffer (1/15 M, pH 6.7—8.1) and Kolthoff borate-sodium carbonate buffer (pH 9.2—10) were employed. BSA, fraction V, was purchased from Armour Pharmaceutical Co., and its molecular weight was assumed to be 69000.

Equilibrium Dialysis——Drug solution (2 ml) was dialyzed against 2 ml of BSA solution for 20 hr in polymethyl methacrylate cells. The binding studies on pH dependency were carried out using 1% BSA solution and a fixed amount of the drugs (1×10^{-4} M) at 30°. To calculate the thermodynamic parameters for the binding process, dialysis was conducted at 10°, 15° and 30° over the concentration range from 1×10^{-4} M to 5×10^{-4} M of the drug against 1% BSA solution. To obtain the binding constant, Langmuir plots were prepared. To vary the ionic strength of the buffer solution, KCl and K_2SO_4 were dissolved in pH 7.4 phosphate buffer. After equilibration, the concentration of the free drug in the drug compartment was analyzed with a Hitachi 124 spectrophotometer at 275 nm for PTU, and at 305 nm in the cases of TA and TP, after adding 0.1 N NaOH to 2 ml of the sample.

Fluorometric Titration——The fluorescence measurements were made on a Hitachi 204 fluorescence spectrophotometer at 25±1°. The excitation and emission wavelengths were 365 nm and 465 nm, respectively. The fluorometric titration[8] was carried out as follows. BSA solution (0.01%, 3 ml) was titrated by successive additions of 2 μl of 1×10^{-3} M ANS dissolved in the buffer solution. A separate titration was performed for BSA at the same concentration but containing the drug (2.5×10^{-4} M). BSA solution (0.1%) in which all the ANS was bound was also titrated. Blank titration was performed in the absence of BSA. The fraction of bound ANS (X) was calculated from the equation $X=(I_0-I_f)/(I_b-I_f)$ where I_0 and I_f refer to the fluorescence intensity of a given concentration of ANS in the 0.01% BSA solution and in the solution without BSA, respectively, and I_b is the fluorescence intensity of the same concentration of ANS in the 0.1% BSA solution. Using the value X, the free ANS concentration (D_f) and the number of ANS molecules bound per BSA molecule (r) can be calculated for each point along the titration curve.

Results and Discussion

Effects of pH and Ionic Strength

The amount of bound drug increased with increase of pH (Fig. 1) for all three drugs. To investigate the participation of the dissociated forms of the drugs in binding, the amounts of bound drug at two pH values, pK_1+1 and pK_1-1, were calculated (Table I). The ionic forms of TA and TP were bound preferentially, though in the case of PTU both the ionic and nonionic forms were bound similarly. However, as the ionic form of each drug participates in binding, the effect of ionic strength was investigated at pH 7.4 using KCl and K_2SO_4 which have different hydration numbers. Langmuir plots in the absence and presence of the salts for each drug had the same intercept on the ordinate. The numbers of binding sites (n) for TA, TP and PTU were 10, 10 and 1.8, respectively. Fig. 2 shows the relationships between the binding constants and ionic strength. The binding constant of PTU decreased more sharply with increase of ionic strength than those of TA and TP; no differences between KCl and K_2SO_4 were noted. This suggests that the binding sites for PTU are more located in a more ionic region than those for TA and TP.

The degree of participation of the ionic form of PTU was estimated by a consideration of the changes of pK_a. ΔpK_a, which is the apparent decrease in the pK_a of the bound drug, decreased in the order PTU, TA and TP; the values of ΔpK_a were 0.76, 0.17 and 0.15, respec-

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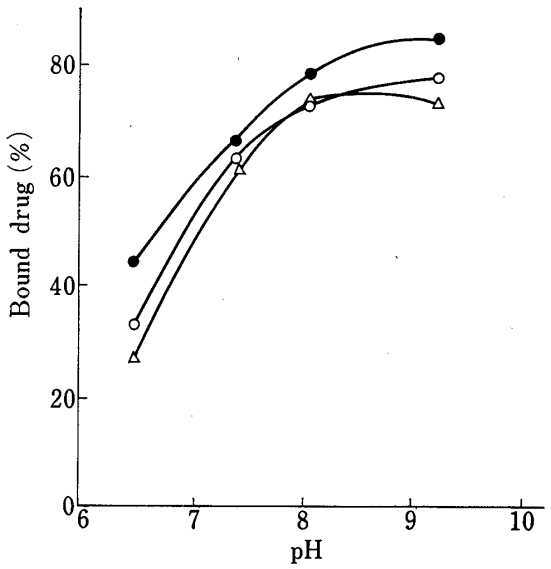

Fig. 1. Effect of pH on the Bound Drug (%)[a] at 30° as Determined by Equilibrium Dialysis

—●—, thiamylal; —○—, thiopental;
—△—, 6-*n*-propyl-2-thiouracil. 1×10^{-4} M drug solution was used at each pH.
a) bound drug (%)=[amount of the bound drug/amount of the total drug (in BSA compartment)] ×100.

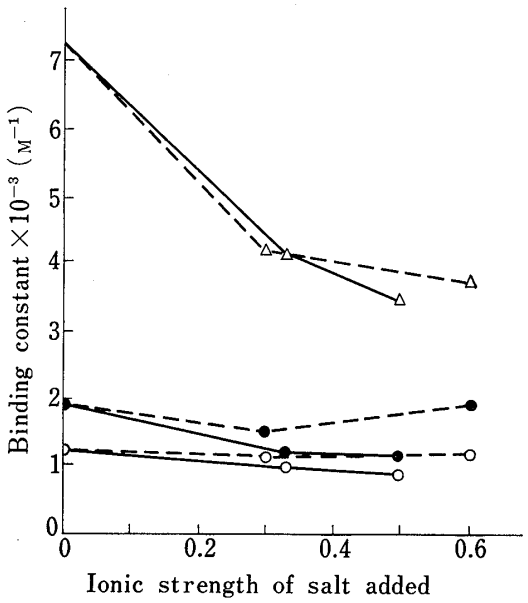

Fig. 2. Effect of Ionic Strength on the Binding Constants of Thiamylal, Thiopental and 6-*n*-Propyl-2-thiouracil at pH 7.4

—●—, thiamylal; —○—, thiopental;
—△—, 6-*n*-propyl-2-thiouracil.
Solid lines and dashed lines indicate the presence of KCl and K_2SO_4, respectively.

TABLE I. Estimated pK_1 Value and Bound Drug (%) for Thiamylal, Thiopental and 6-*n*-Propyl-2-thiouracil at [$pH=pK_1\pm1$]

Drug	pK_1[a]	Bound drug (%) at	
		$pH=pK_1+1$	$pH=pK_1-1$
Thiamylal	7.58	81	45
Thiopental	7.66	75	35
6-*n*-Propyl-2-thiouracil	8.34	69	60

a) T. Sakurai, H. Matsumaru, and S. Tsuchiya, *Yakuzaigaku*, **37**, 169 (1977).

tively.[6] This pK_a shift was well simulated in the presence of quaternary ammonium ion micelles. Montal[9] *et al.* showed that the pK_a of bromothymol blue and methyl red adsorbed onto micelles shifted towards the acidic side with cetyltrimethylammonium bromide micelles, and towards the alkaline side with sodium laurylsulfate micelles, indicating that the changes of pK_a of the dyes are influenced by the surface charges on the micelles, and that the value of ΔpK_a reflects the degree of penetration into the charged region. Thus, it may be considered that PTU is located closer to the charged regions of BSA than TA and TP. The pK_a of PTU at the surface of BSA, which differs from that in bulk solution, may be 7.58 (8.34—0.76). On the other hand, it seems plausible that the decrease of bound PTU at pH 9.2 (Fig. 1) indicates that PTU binds in a region that readily undergoes structural changes at alkaline pH.[10]

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Table II. Thermodynamic Parameters for Thiamylal, Thiopental and 6-*n*-Propyl-2-thiouracil bound to BSA at pH 7.4

Drug	Temp. (°C)	log $K^{a)}$	$\Delta G°$ (kcal·M^{-1})	$\Delta H°$ (kcal·M^{-1})	$\Delta S°$ (kcal·M^{-1}°K^{-1})
Thiamylal	10.0	3.40	−4.40	0.00	15.60
	14.6	3.41	−4.49	0.00	15.60
	30.0	3.40	−4.71	0.00	15.60
Thiopental	10.0	3.18	−4.11	−0.64	12.30
	14.6	3.17	−4.18	−0.64	12.30
	30.0	3.20	−4.43	−0.64	12.30
6-*n*-Propyl-2-thiouracil	10.0	4.44	−5.75	−5.87	0.00
	15.6	4.32	−5.70	−5.87	0.00
	30.0	4.22	−5.85	−5.87	0.00

a) Logarithm of the binding constant.

Effect of Temperature

The thermodynamic parameters are presented in Table II. Those of TA and TP were scarcely influenced by temperature, and the main source of free energy change ($\Delta G°$) was the contribution of the entropy change ($\Delta S°$). However, PTU showed a large negative enthalpy change ($\Delta H°$) which contributed to $\Delta G°$. If $\Delta S°$ derives from changes of the water structure, the binding of TA and TP will increase "free" water in comparison with that before binding, and consequently a decrease in free energy may occur. This is not incompatible with strong hydrophobicity of TA and TP.[6] Klotz[3] indicated that the molecular mechanism must be consistent with the thermodynamic features, but the thermodynamic parameters in themselves do not provide a diagnostic criterion for distinguishing the types of forces involved in ligand binding by protein. However, taking the pH dependency, the effect of ionic strength and the pK_a shift into consideration, it seems reasonable to say that both hydrophobic bonding and some electrostatic forces are involved in the binding of TA and TP.

As it is well known that hydrophobic bonding is an entropy-driven interaction, the binding of PTU can not be a result of hydrophobic interaction, since $\Delta S°$ was nearly O (Table II). According to Oster *et al.*,[11] a marked difference between the binding forces for two drugs raises some doubt as to the coincidence of their binding sites on the albumin molecule, so the binding sites for TA and TP may be different from those for PTU.

pH Dependency Employing ANS as an Indicator

ANS is a well-known fluorescence probe, showing strong fluorescence emission at 465 nm when bound to protein. In the study of ANS binding by BSA, Kolb *et al.*[12] observed that two types of binding sites exist, so-called "inner" sites, which are sufficiently inaccessible to water to prevent its interaction with ANS during the fluorescence lifetime, and the other, "outer" sites, at which water is able to quench the fluorescence.

Initially, we investigated changes of the emission spectrum of ANS bound to BSA in the presence of the drug in the pH range from neutral to alkaline. The results shown in Fig. 3 are the data obtained at pH 6.7 and pH 8.1. At each pH, the fluorescence intensities decreased, but the peak position did not change. This indicates that ANS is displaced by coexisting drug,[13] so TA, TP and PTU may bind to the inner ANS binding sites competitively with ANS.

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12) D.A. Kolb and G. Weber, *Biochem.*, **14**, 4476 (1975).
13) S. Ozeki and K. Tejima, *Yakugaku Zasshi*, **98**, 320 (1978).

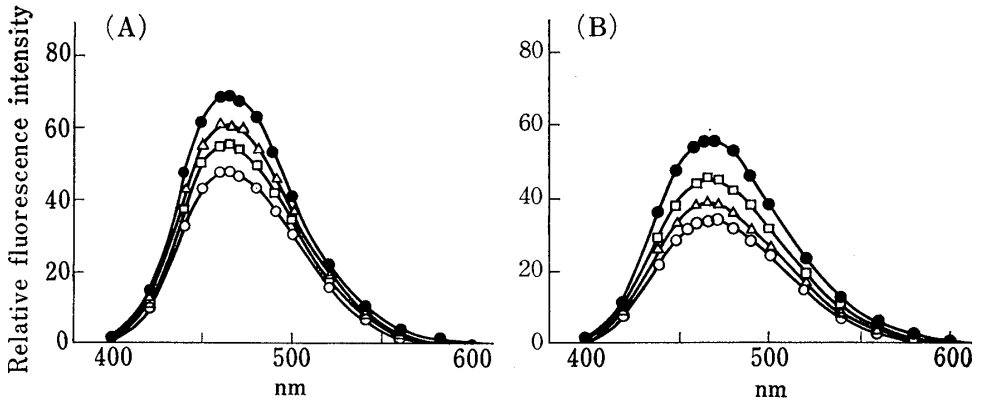

Fig. 3. Fluorescence Spectra of ANS-BSA in the Absence and Presence of Each Drug at pH 6.7 and pH 8.1

—●—, ANS-BSA; —○—, ANS-BSA+thiamylal; —△—, ANS-BSA+thiopental; —□—, ANS-BSA+6-*n*-propyl-2-thiouracil.
excitation, 365 nm; drug concentration, 2.5×10^{-4} M The concentrations of ANS and BSA were 7.9 μmol/3 ml and 0.01%, respectively, in (A) and 6.3 μmol/3 ml and 0.012%, respectively, in (B).

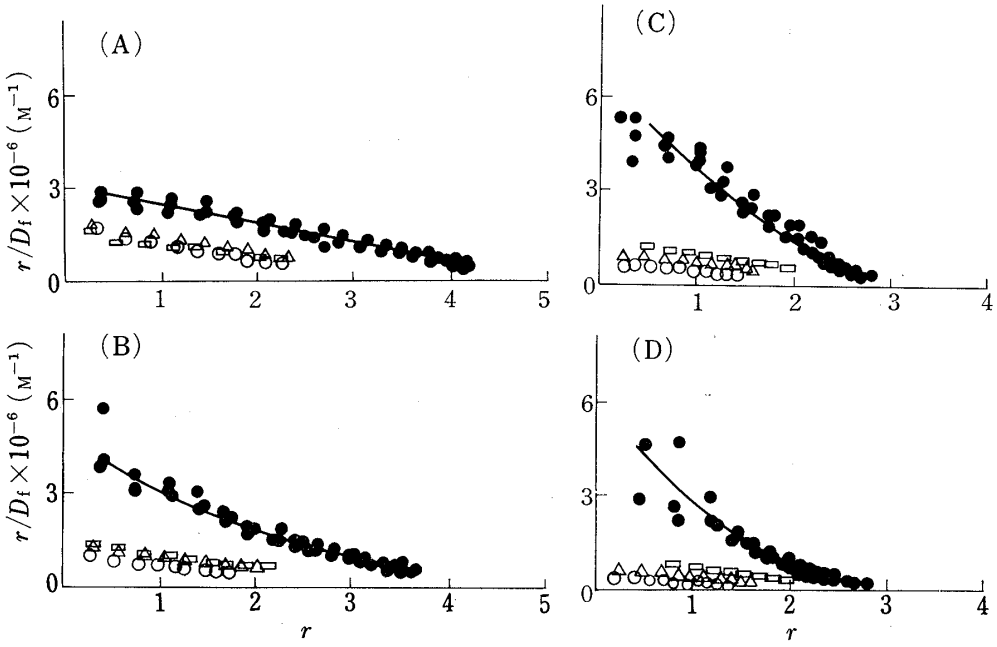

Fig. 4. Scatchard Plots for ANS-BSA in the Absence and Presence of Drug at pH 6.7 (A), 7.4 (B), 8.1 (C) and 9.3 (D) as Determined by Fluorometric Titration

—●—, ANS-BSA; ○, ANS-BSA+thiamylal; △, ANS-BSA+thiopental; □, ANS-BSA+6-*n*-propyl-2-thiouracil.

Next, the amounts of ANS bound to BSA in the absence and presence of a constant amount (2.5×10^{-4} M) of each drug as a competitor were calculated by fluorometric titration at each pH. The results are illustrated in Fig. 4 as Scatchard plots. With increase in pH, the Scatchard plots of ANS in the absence of the drug showed an increase in the value of the extrapolated intercept on the ordinate, though the corresponding value on the abscissa decreased. If the Scatchard plot for ANS is linear, the binding constant of each coexisting drug can be calculated using the Klotz equation. However, as nonlinear plots for ANS were observed above pH 7.4 in the absence of the drug, it could not be applied in this case.

It is interesting to calculated the displacement ratio, which is defined as the ratio of the difference in the relative fluorescence intensity of ANS–BSA in the absence and presence

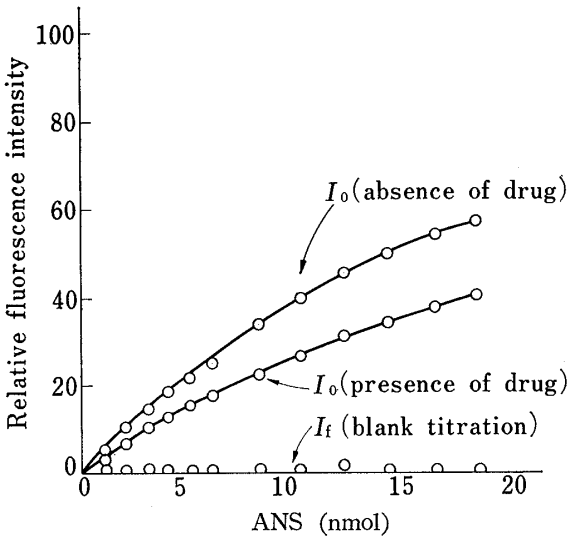

Fig. 5. Fluorometric Titration Curves employed for Calculating the Displacement Ratio

Taking (I_0-I_f) in the absence and presence of the drug as F_0 and F_d, respectively, the displacement ratio is $(F_0-F_d)/F_0\times100$.

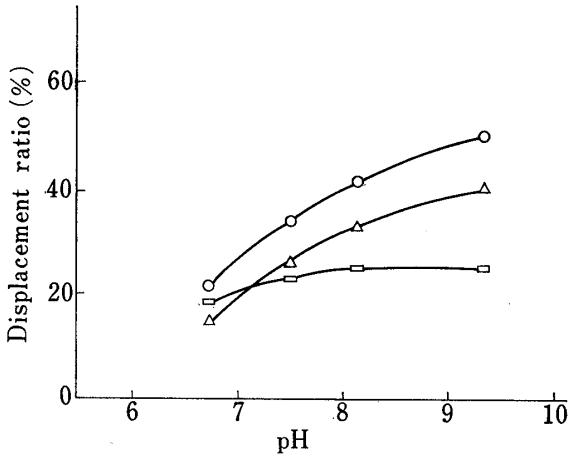

Fig. 6. pH Profile of the Displacement Ratios of Thiamylal, Thiopental and 6-*n*-Propyl-2-thiouracil

—○—, thiamylal; —△—, thiopental; —□—, 6-*n*-propyl-2-thiouracil. The displacement ratio is the mean value at each titration point.

of the drug to the relative fluorescence intensity in the absence of the drug at a given point on the fluorometric titration curve (Fig. 5). Fig. 6 shows plots of the mean values of the displacement ratio at every titration point against pH. The displacement ratios of TA and TP rose with increasing pH. This pH dependency is similar to the results of equilibrium dialysis. This suggests that most TA and TP molecules share the inner sites with ANS. On the other hand the displacement ratio of PTU at pH 6.7 was similar to those of TA and TP. However, no increase in the displacement ratio with increase of pH could be seen, in contrast to the results of equilibrium dialysis. This suggests that most PTU molecules may bind to sites other than the inner sites, at which electrostatic forces may govern the interaction. It has been shown that the fluorescence of ANS bound to BSA is quenched by many drugs,[14] but it seems clear from the binding studies of PTU that it may be misleading to discuss the nature of the binding simply on the basis of the displacement of ANS. Luzzi *et al.*[14b–e] concluded that the compounds which displace ANS bind to the hydrophobic sites on BSA. However, ANS is an amphipathic compound[15] and may bind to regions in the neighborhood of the surface on the protein where positively charged amino acid residues are backed by residues with a nonpolar side chain.[16] It is not incompatible with the results obtained in this study to consider that TA and TP bind to these regions of BSA, as the interaction of these two drugs results from hydrophobic bonding with some electrostatic contribution.

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