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Protein Binding of Sulfonylureas. II. Interaction of Some *p*-Toluenesulfonyl-*n*-alkylureas with Bovine Serum Albumin

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To evaluate the substituent effect, the binding of *p*-toluenesulfonyl-*n*-alkylureas to bovine serum albumin was studied by equilibrium dialysis and fluorescence titration. The binding constants obtained by fluorescence titration were nearly equal to the geometric means of the primary and secondary binding constants obtained by equilibrium dialysis. The binding constants of *p*-toluenesulfonyl-*n*-butylurea were about ten times greater than those of the other compounds tested.

Keywords——protein binding; *p*-toluenesulfonyl-methylurea; *p*-touenesulfonyl-ethylurea; *p*-toluenesulfonyl-*n*-propylurea; *p*-toluenesulfonyl-*n*-butylurea; equilibrium dialysis; fluorescence titration; bovine serum albumin; dansylglycine;

Derivatives of benzenesulfonylurea are some of the most widely used oral antidiabetic agents. Protein binding is considered to be a major factor affecting the bioavailability of these drugs. Many binding studies of the antidiabetic agents to bovine serum albumin (BSA) have been reviewed.[2] However, the protein binding of these drugs were studied by various methods, and the binding parameters are generally not comparable. In the previous paper,[3] we reported the protein binding of some 4-substituted benzenesulfonyl-*n*-propylureas. The binding affinities were affected by the substituent at the 4-position of benzene. It has been found that 4-chloro- and 4-iodobenzenesulfonyl-*n*-propylurea were bound to BSA more strongly than benzenesulfonyl-, *p*-toluene-sulfonyl- and 4-aminobenzenesulfonyl-*n*-propylureas at the primary binding sites on BSA.

In the present work, the interactions of *p*-toluenesulfonyl-methylurea (TMU), -ethylurea (TEU), -*n*-propylurea (TPU) and -*n*-butylurea (TBU) were studied by equilibrium dialysis and fluorescence titration with dansylglycine as a fluorescent probe. On fluorescence titration, the fluorescent probe competes with the drug for the binding sites on BSA. For the competitive binding of two ligands to protein, Klotz *et al.*[4] derived equation (2), which corresponds to equation (1) for single species binding.

$$r = \frac{nk_a[A]}{1+k_a[A]} \tag{1}$$

$$r = \frac{nk_a[A]}{1+k_a[A]+k_b[B]} \tag{2}$$

Here, r is the number of moles of bound ligand A, n is the number of binding sites on the protein, and k_a, k_b and [A], [B] represent the binding constants and the concentrations of the indicated species. The binding constant of the competitive ligand, k_b, was obtained as the mean of the values calculated from each probe concentration according to Jun *et al.*[5]

The binding parameters of *p*-toluenesulfonyl-*n*-alkylureas are listed in Table I and II. Although the binding parameters depend somewhat on the experimental range selected for

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calculation, these values are of same order as the reported values.[3,6-8] The binding constants of TBU are about ten times greater than those of other *p*-toluenesulfonyl-*n*-alkylureas. Since the fluorescence titration calculation is based on a single class of binding sites on BSA, the binding constants of *p*-toluenesulfonyl-*n*-alkylureas obtained by fluorescence titration are nearly equal to the geometric means of the primary and secondary binding constants obtained by the equilibrium dialysis method assuming two classes of binding sites. This suggests that the binding to primary and secondary classes of binding sites on BSA may be competitive with respect to dansylglycine.

Table I. Binding Parameters of *p*-Toluenesulfonyl-*n*-alkylureas with Two Classes of Binding Sites on BSA

Urea	n_1	$k_1 \times 10^{-4}(\mathrm{M}^{-1})$	n_2	$k_2 \times 10^{-2}(\mathrm{M}^{-1})$	Method	Reference
TMU	1.14	1.4	3.52	9.7	E	6
	0.623	7.30	6.18	8.31	E	This study
TEU	0.84	1.7	4.83	13.6	E	6
	1.31	1.18	8.54	4.46	E	This study
TPU	1.40	5.01	5.53	7.72	E	3
TBU	2.98	24.82	8.12	3.39	D	7
	0.75	24.9	5.10	56.3	E	6
	0.50	48.2	6.22	43.6	E	This study

D: Dynamic dialysis, E: Equilibrium dialysis.

Table II. Binding Parameters of *p*-Toluenesulfonyl-*n*-alkylureas with a Single Class of Binding Sites on BSA

Urea	$k_b \times 10^{-4}(\mathrm{M}^{-1})$	Method	Reference
TMU	0.799	F	This study
TEU	0.771	F	This study
TPU	0.697	F	This study
TBU	6.23	F	This study
	9.04	F	8
	16.5	S	9

F: Fluorescence titration, S: Difference spectrophotometry.

Experimental

Materials——TMU and TEU were synthesized in this laboratory according to the reported method.[10] TMU was synthesized from *p*-toluenesulfonamide and methyl isocyanate, mp 172° (lit. 172—174°,[11] 171—172°,[12] 174—175.5°[13]). *Anal.* Calcd for $C_9H_{12}N_2O_3S$: C, 47.36; H, 5.30; N, 12.27. Found: C, 47.23; H, 5.36; N, 12.55. TEU was synthesized from *p*-toluenesulfonamide and ethyl isocyanate, mp 136° (lit. 138—140°,[11] 140—141°,[12] 139—142°[13]). *Anal.* Calcd for $C_{10}H_{14}N_2O_3S$: C, 49.57; H, 5.82; N, 11.56. Found: C, 49.07; H, 5.82; N, 11.27. TPU was prepared as reported previosly.[3] TBU was purchased from Toyama Chem. Ind., Tokyo. Dansylglycine and BSA (fraction V) were purchased from Sigma Chem. Co., St. Louis, U.S.A. Methanol was of spectroscopic grade. All other chemicals were of reagent grade.

Measurements——Equilibrium dialysis was carried out at 10° for three days using Visking cellulose tubing. Dialysis bags which contained five ml of BSA solution (1.43×10^{-5} M) dissolved in 1/15 M phosphate

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buffer (pH 7.4) were immersed in five ml of various concentration of the drug solution (1×10^{-5}—1×10^{-3} M) dissolved in the same buffer solution. The concentration of free TMU and TEU were determined spectrophotometrically with a Shimadzu UV-200 spectrophotometer at 228 and 226 nm, respectively. That of TBU was determined with a Hitachi double-wavelength spectrophotometer, model 356, using a wavelength pair of 226—280 nm. The data in the form of moles of bound drug per mole of BSA, r, and equilibrium concentration of free drug, c, were analyzed by nonlinear least-squares fitting as described previously.[3]

Fluorescence titration was carried out at room temperature. Two ml of BSA (9.85×10^{-6} M) or BSA (9.60×10^{-6} M) and *p*-toluenesulfonyl-*n*-alkylurea (9.90×10^{-6} M) solution in 1/15 M phosphate buffer (pH 7.4) was titrated with dansylglycine dissolved in methanol. The final concentration of dansylglycine was varied from 5×10^{-7} to 2×10^{-5} M. The fluorescence intensity was measured a 480 nm with excitation at 352 nm, using a Hitachi fluorescence spectrometer, model MPF-3. The binding paramters, n and k_a, of dansylglycine for BSA were 1.027 and 5.316×10^{-5} M^{-1}, respectively.

The pH measurements were carried out with a Hitachi-Horiba pH meter, model M-7, calibrated with standard buffer solutions.

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Microcrystallization Method for Amobarbital and the Physicochemical Properties of the Products[1]

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A method for the microcrystallization of amobarbital was developed. This method consists of the following procedures: (1) dissolution of amobarbital (J.P. IX) in a hot solvent such as a glycol or glycol derivative, (2) pouring this solution into cold water (3°) while stirring to precipitate microcrystals, and (3) collection and drying of the precipitated microcrystals. The crystalline properties of the pulverized crystals of amobarbital were also studied. The degree of pulverization of amobarbital by recrystallization from glycols and their derivatives decreased with increase in the number of carbon atoms in the hydrophobic chain of these solvents.

Examination of pulverized amobarbital was carried out by IR, X-ray diffraction, and DSC–TG, and two kinds of polymorphs were detected.

Keywords——amobarbital; microcrystallization; glycols; glycol derivatives; mean particle diameter; polymorphism

It is generally known that the absorption of drugs is often influenced by their physicochemical properties and dosage form, and it has been reported in many papers that practically insoluble drugs sometimes show high serum concentrations only when administered in a pulverized form.[3–6]

One of the authors (Y. Kato) previously reported higher serum concentration and increased urinary excretion as a result of pulverization of aspirin,[7,8] and the pulverization of aspirin,[9]

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