

Protein Binding of 2-Benzenesulfonamide-5-(β -methoxyethoxy)pyrimidine

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Binding affinity of 2-benzenesulfonamide-5-(β -methoxyethoxy)-pyrimidine (GL) to bovine serum albumin (BSA) was investigated by equilibrium dialysis and fluorescence titration. Binding parameters of GL to BSA was calculated from the results of equilibrium dialysis assuming two classes of binding sites on BSA. The values for n_1 , n_2 and k_1 , k_2 were 0.109, 2.47 and $3.71 \times 10^5 \text{ M}^{-1}$, $4.78 \times 10^3 \text{ M}^{-1}$, respectively. The association constant of GL which was obtained by fluorescence titration would be the secondary association constant calculated from the results of equilibrium dialysis.

Keywords—protein binding; 2-benzenesulfonamide-5-(β -methoxyethoxy)-pyrimidine; equilibrium dialysis; fluorescence titration; Scatchard plot

The protein binding of drug is considered as an important factor of drug availability, transport and distribution. Many drugs exhibit their biological activity through the interactions of drugs with proteins in cell membrane. A number of investigations concerning with protein binding of oral antidiabetic agents have been reviewed.²⁾ While 2-benzenesulfonamide-5-(β -methoxyethoxy)-pyrimidine (GL), one of the oral antidiabetic agents, differs from benzenesulfonyl ureas structurally, eighty percents of GL bind to human plasma albumin (HSA)³⁾ as well as tolbutamide (97%⁴⁾) and chlorpropamide (84%,⁴⁾ 83—86%⁵⁾). However, these percentage of bound drugs would change with protein and drug concentrations. To calculate the binding affinity of drugs to protein, many techniques are adopted. The equilibrium dialysis method is very simple and convenient technique for this purpose. The fluorescence spectroscopic method would also appear to be one of the most useful techniques due to their high sensitivity. Competitive binding of drugs with a fluorescent probe dye to protein is quite convenient to study the binding of drugs to protein.

To evaluate further accurate binding parameters, the binding of GL to bovine serum albumin (BSA) was investigated by the equilibrium dialysis method and the fluorescence spectroscopic method using dansylglycine as a fluorescent probe.

Experimental

Materials—2-Benzenesulfonamide-5-(β -methoxyethoxy)pyrimidine (sodium salt)⁶⁾ was given from Bayer Yakuhin Ltd., Osaka, Japan. Dansylglycine⁷⁾ and bovine serum albumin (fraction V)⁷⁾ were obtained from commercial sources. Methanol was of spectroscopic grade. All other chemicals were of reagent grade.

Spectral Experiments—Concentration of free GL was determined spectrophotometrically at 242 nm with a Shimadzu-Baush and Lomb spectrophotometer, model Spectronic 700. Fluorescence intensity was measured at 480 nm excited at 352 nm with a Hitachi fluorescence spectrophotometer, model MPF-3.

Methods—a) Equilibrium Dialysis Method: Equilibrium dialysis experiments were carried out at 10° for four days using Visking cellulose tubing. Dialysis bags which contained five ml of BSA solution

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2) J.J. Vallner, *J. Pharm. Sci.*, **66**, 447 (1977).

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6) Common name: Sodium Glymidine.

7) Sigma Chem. Co., St. Louis, Mo., U.S.A.

(1.52×10^{-4} M) dissolved in 1/15 M phosphate buffer at pH 7.4 were immersed in a five ml of various concentrations of GL (2×10^{-5} — 2×10^{-3} M) dissolved in the same buffer solution.

b) Fluorescence Titration: A two ml of BSA (1.05×10^{-5} M), or BSA (1.07×10^{-5} M) and GL (5×10^{-5} M) solution in 1/15 M phosphate buffer was titrated with successive addition of dansylglycine dissolved in methanol according to the work of Jun *et al.*⁸⁾ Final concentration of dansylglycine was varied from 5×10^{-7} to 8×10^{-5} M.

Treatment of Data—The fraction of bound fluorescent probe (X) was calculated from the following equation⁹⁾

$$X = \frac{(I_o/I_f) - 1}{(I_b/I_f) - 1} \quad (1)$$

Where I_o and I_f refer to the fluorescence intensities of a given concentration of probe in solutions of low protein concentration, and in solutions without protein, respectively. I_b refers to the fluorescence intensity of the same concentration of probe in solutions of high protein concentration. To determine the values of I_b for the given concentration of the probe, fluorescence titration was carried out for several protein concentrations. The value of I_b was determined as the value extrapolated to the intercept of plot of $1/I$ versus $1/[P_t]$, where I refers to the fluorescence intensity of a given concentration of BSA, and $[P_t]$ represents the total concentration of BSA.⁹⁾

The data in the form of moles of bound drug per mole of BSA (r), and equilibrium concentration of free drug (c) were subjected to nonlinear least squares fit described previously.¹⁰⁾

Results and Discussion

For a simple reversible binding of a ligand to protein, equation (2) is derived on the assumption that there is a single class of independent binding sites on protein, and all sites are equivalent in binding affinity.

$$r = \frac{nk_c}{1 + k_c} \quad (2)$$

Where, n is the number of binding sites on protein and k is the association constant of ligand-protein complex. The equation (2) is rearranged to the Scatchard equation (3) which is the most used manner of plotting binding data and calculating the values of n and k .

$$r/c = nk - rk \quad (3)$$

The Scatchard plots of GL to BSA is non-linear as shown in Fig. 1 over the initial concentration range of GL from 2×10^{-5} to 2×10^{-3} M. The curved line of this plot is generally considered to indicate that there are more than one class of binding sites on BSA. A good fit of calculated values to experimental ones was obtained assuming that there are two classes of binding sites on BSA as shown in Fig. 1. For this type of binding, the equation (2) is rewritten as follows

$$r = \frac{n_1 k_1 c}{1 + k_1 c} + \frac{n_2 k_2 c}{1 + k_2 c} \quad (4)$$

Where, n_1 , n_2 and k_1 , k_2 represent the number of binding sites and the association constants of each class, respectively. The binding parameters of GL to BSA (1.52×10^{-4} M) in 1/15 M phosphate buffer at pH 7.4 obtained by the equation (4) are listed in Table I. The primary association constant of GL was the same magnitude as that determined for the binding of tolbutamide to HSA ($k_1 = 8.504 \times 10^5 \text{ M}^{-1}$ ¹¹⁾) and chlorpropamide to HSA ($k_1 = 1.849 \times 10^5 \text{ M}^{-1}$ ¹¹⁾), to BSA ($k_1 = 1.23 \times 10^5 \text{ M}^{-1}$ ¹⁰⁾). However, the secondary association constant of GL was greater than those for tolbutamide ($k_2 = 6.65 \times 10^2 \text{ M}^{-1}$ ⁹⁾) and chlorpropamide ($k_2 = 6.52 \times 10^2 \text{ M}^{-1}$ ¹¹⁾, $8.96 \times 10^2 \text{ M}^{-1}$ ¹⁰⁾). Since the number of primary binding sites on BSA is too small ($n_1 = 0.109$), secondary binding sites would contribute mainly to the protein binding of GL.

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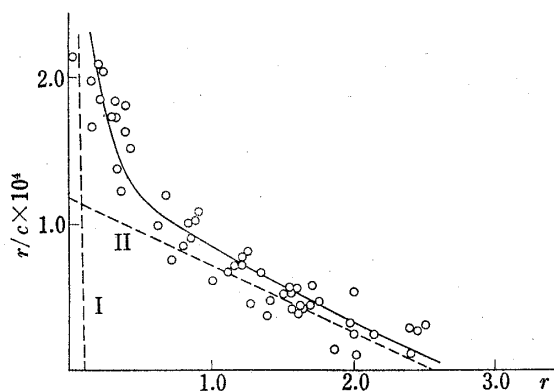
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TABLE I. Binding Parameters of 2-Benzenesulfonamide-5-(β -methoxyethoxy)-pyrimidine to BSA at pH 7.4

n_1	$k_1 \times 10^{-5} \text{ (M}^{-1}\text{)}$	n_2	$k_2 \times 10^{-3} \text{ (M}^{-1}\text{)}$
0.109	3.71	2.47	4.78


 Fig. 1. Scatchard Plot for Binding of 2-Benzenesulfonamide-5-(β -methoxyethoxy) pyrimidine to BSA

Broken line I; plot for primary class, $n_1=0.109$, $k_1=3.7 \times 10^5 \text{ M}^{-1}$. Broken line II; plot for secondary class, $n_2=2.47$, $k_2=4.78 \times 10^3 \text{ M}^{-1}$. Solid line; sum of above lines.

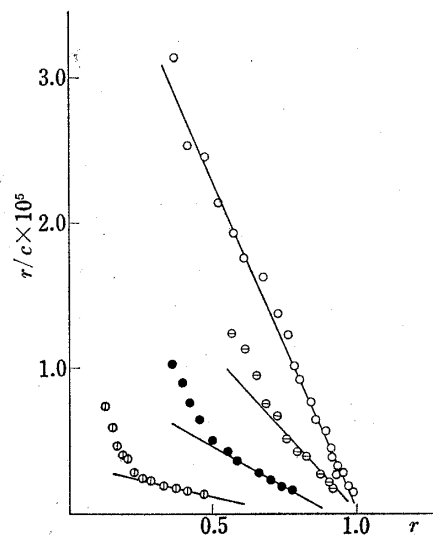


Fig. 2. Scatchard Plot of Binding of Dansylglycine to Various Concentration of BSA

(\circ), $1.05 \times 10^{-5} \text{ M}$; (\odot), $1.97 \times 10^{-5} \text{ M}$; (\bullet), $3.94 \times 10^{-5} \text{ M}$; (\oplus), $9.85 \times 10^{-5} \text{ M}$

On the fluorescence spectroscopic method, the fluorescent probe competes with a drug in the binding on protein. For these competitive binding of two ligands to protein, Klotz *et al.*¹²⁾ derived equation (5) corresponding to the equation (2).

$$r = \frac{nk_a[A]}{1+k_a[A]+k_b[B]} \quad (5)$$

Where, k_a , k_b and $[A]$, $[B]$ represent the association constant and the concentration of respective species. Jun *et al.*⁸⁾ calculated the association constant of competitive drug, k_b , from following equation which is based on the equation (5).

$$k_b = \frac{n[P_t]k_a[A]-k_a[A][PA]-[PA]}{[B_t]k_a[A]-[P_t]k_a[A]+k_a[A][PA]+[PA]} \times \frac{k_a[A]}{[PA]} \quad (6)$$

Where, k_a is the association constant of probe and n is the number of binding sites. $[P_t]$, $[B_t]$, $[A]$ and $[PA]$ represent the concentrations of total protein, total drug, free and bound probe, respectively. The value of k_b was obtained as a mean value of ones which were calculated from each probe concentration.

For another method to evaluate k_b , the equation (5) is rewritten as follows

$$r = \frac{n[A]}{1/k_a+[A]+k_b[B]/k_a} \quad (7)$$

If the total concentration of drug, $[B_t]$, is higher than that of protein, $[P_t]$, the concentration of free drug, $[B]$, is assumed to be constant because of few moles of drug which binds to the lower concentration of protein. Then the equation (7) can be simplified as follows

12) I.M. Klotz, H. Triwush, and F.M. Walker, *J. Am. Chem. Soc.*, **70**, 2935 (1948).

$$r = \frac{n[A]}{K+[A]} \quad (8)$$

Where

$$K = (1+k_b[B])/k_a \quad (9)$$

The equation (8) is rearranged as follows

$$r/[A] = n/K - r/K \quad (10)$$

The equation (10) is a Scatchard type equation. K represents the reciprocal of slope of the Scatchard plot in the presence of competitive drug. Thus the association constant of drug, k_b , is calculated by the equation (9) using K and k_a values which are obtained statistically from the Scatchard plots.

To evaluate the accurate association constant of probe, k_a , fluorescence titration was carried out at various concentrations of BSA. The Scatchard plots were not linear in all range of concentration as shown in Fig. 2. The curvature of these lines increased as the concentration of BSA increased. For these curved lines, the low values of r have been discounted to obtain a straight line.¹³⁾ The same treatment can be applied to the data of Fig. 2 to find the values of k and n for the range of concentration within the linear portion of the plot. Table II shows the values of k and n which are obtained from the linear portion of the plot in Fig. 2. The number of binding sites equals to unity within the concentration range of BSA studied. However, the association constant varied with the concentration of BSA. At higher concentration of BSA, it was difficult to obtain the accurate values of k and n due to insufficient number of data along the linear portion of the plot.

TABLE II. Binding Parameters of Dansylglycine to BSA at pH 7.4

Conc. of BSA $\times 10^5$ (M)	n	$k_a \times 10^{-5}$ (M^{-1})
1.05	1.02	4.46
1.97	1.01	2.19
3.94	0.916	1.10
9.85	0.731	0.466

To compare the results determined by the fluorescence spectroscopic method with those by the equilibrium dialysis method, fluorescence titration was carried out for the solution of BSA (1.07×10^{-5} M) and GL (5×10^{-5} M), since the Scatchard plot would be expected to be linear over a wider range of r . Using the association constant of probe in Table II ($k_a = 4.46 \times 10^5 M^{-1}$), the association constant of GL, k_b , was calculated as $7.36 \times 10^3 M^{-1}$ by the equation (6). This value is smaller than the value, $8.30 \times 10^3 M^{-1}$, calculated by the equation (9).

However, it has been shown that the Scatchard plot for lower molar ratio of probe to protein indicates the lower number of binding sites and higher binding affinity for a single class of binding site.¹⁴⁾ Therefore, the binding parameters would depend on a range of the molar ratio studied. The Scatchard plot in Fig. 3 showed apparently curved line. It is more reasonable to consider that there are two classes of binding sites with no interaction between sites and between free and bound drugs. In the competitive binding on two classes of binding sites, the equation (5) is written as¹⁵⁾

$$r = \frac{n_1 k_{a1}[A]}{1 + k_{a1}[A] + k_{b1}[B]} + \frac{n_2 k_{a2}[A]}{1 + k_{a2}[A] + k_{b2}[B]} \quad (11)$$

13) J.K.H. Ma, H.W. Jun, and L.A. Luzzi, *J. Pharm. Sci.*, **62**, 2038 (1973).

14) H.W. Jun, L.A. Luzzi, and J.K.H. Ma, *J. Pharm. Sci.*, **64**, 493 (1975).

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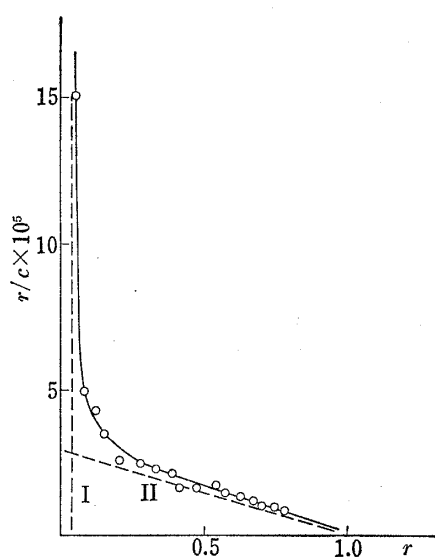


Fig. 3. Scatchard Plot of Binding of Dansylglycine to BSA ($1.07 \times 10^{-5} \text{ M}$) in the Presence of 2-Benzenesulfonamide-5-(β -methoxyethoxy)-pyrimidine ($5 \times 10^{-5} \text{ M}$)

Broken lines I and II are plots for primary and secondary classes, respectively. Solid line is sum of above lines.

Using the equation (9), the equation (11) can be simplified as follows

$$r = \frac{n_1[A]}{K_1 + [A]} + \frac{n_2[A]}{K_2 + [A]} \quad (12)$$

The binding parameters of probe are listed in Table III in the absence and presence of GL. Using the values in Table III ($k_{a1} = 1.74 \times 10^8$, $k_{a2} = 4.01 \times 10^5 \text{ M}^{-1}$), the association constants of GL, k_{b1} and k_{b2} , were also calculated by the equations (9) and (12). Since the product of $K_1 \times k_{a1}$ is smaller than unity, k_{b1} takes negative value. The value of n_1 in the Table II decreases as the ratio of probe to BSA increased. This may be supported by the reports that the molecular environment of the binding sites is not identical,¹⁴⁾ and that the capacity of binding sites increased at higher drug concentration.¹⁶⁾ Therefore, negative k_{b1} value would suggest that the protein binding of GL in these range of probe concentration may not be simple competitive one.

By the equation (9), the secondary association constant, k_{b2} , was obtained as $7.82 \times 10^3 \text{ M}^{-1}$ using $k_a = 4.46 \times 10^5 \text{ M}^{-1}$ (Table II), and as $8.45 \times 10^3 \text{ M}^{-1}$ using $k_{a2} = 4.01 \times 10^5 \text{ M}^{-1}$ (Table III). When a range

of concentration is selected to obtain a straight line of the Scatchard plot, the slope of the line, k_a , was greater than the secondary association constant, k_{a2} . Therefore, the association

TABLE III. Binding Parameters of Dansylglycine to BSA at pH 7.4 in the Absence (A) and Presence (B) of 2-Benzenesulfonamide-5-(β -methoxyethoxy)-pyrimidine

n_1	$k_1 (\text{M}^{-1})$	n_2	$k_2 (\text{M}^{-1})$
A	6.87×10^{-2}	1.74×10^8	4.01×10^5
B	3.33×10^{-2}	4.40×10^{11}	2.88×10^5

constant of GL obtained by fitting a single straight line method, would be smaller than the value which was calculated with whole range of concentration. Though the value by fluorescence spectroscopic method was one-half greater than that by equilibrium dialysis method, the association constant of GL which was obtained with the linear portion of the Scatchard plot would be that of the secondary binding sites on BSA which should mainly contribute to protein binding of GL.

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