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Interaction of Theophylline with Bovine Serum Albumin and Competitive Displacement by Benzoic Acid

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The interaction of theophylline with bovine serum albumin (BSA) was studied at pH 6.85, using the equilibrium dialysis method. The investigation was carried out at three different temperatures, 15, 25, and 37°C. The data were analyzed by assuming two types of independent binding sites, and the binding parameters and thermodynamic parameters were determined to be as follows; $\bar{n}_1 = 0.61$, $\Delta H_1 = -9.72$ kcal/mol, $\Delta S_1 = -19.0$ (e.u.) ($\Delta G_1 = -3.93$ kcal/mol at 25°C), $\bar{n}_2 = 1.19$, $\Delta H_2 = -2.86$ kcal/mol, $\Delta S_2 = 1.6$ (e.u.) ($\Delta G_2 = -3.34$ kcal/mol at 25°C). It was also found that benzoic acid binds to BSA in competition with theophylline. The interaction of benzoic acid with BSA was studied in the same way as that of theophylline and the binding parameters and thermodynamic parameters were determined to be as follows; $\bar{n}_1 = 0.91$, $\Delta H_1 = -8.81$ kcal/mol, $\Delta S_1 = -7.5$ (e.u.) ($\Delta G_1 = -6.59$ kcal/mol at 25°C), $\bar{n}_2 = 2.94$, $\Delta H_2 = -3.32$ kcal/mol, $\Delta S_2 = 4.7$ (e.u.) ($\Delta G_2 = -4.71$ kcal/mol at 25°C). The general features of the thermodynamic parameters for both theophylline and benzoic acid were similar to those of salicylic acid. Quenching of the fluorescence of tryptophan residues on BSA in the presence of theophylline or benzoic acid was observed. On the basis of all the experimental results, it is considered that theophylline and benzoic acid bind primarily to tryptophan residue on BSA by stacking due to van der Waals forces and secondarily bind to other amino acid residues on BSA through ionic and hydrophobic interactions.

Keywords—theophylline; bovine serum albumin; Scatchard plot; binding parameter; thermodynamic parameter; equilibrium dialysis; benzoic acid; competitive replacement

We have already studied the interaction of theophylline with bovine serum albumin (BSA) at 5°C in pH 6.85 phosphate buffer, utilizing the equilibrium dialysis method,¹⁾ and we suggested that the binding of theophylline to BSA is exothermic. In the present paper, we deal with the thermodynamic properties of the binding of theophylline to BSA, the replacement reaction with benzoic acid, and the binding sites of theophylline. The binding mechanism of theophylline to BSA is discussed.

Experimental

Materials—Bovine serum albumin, fraction V (BSA), (purchased from Armour Pharmaceutical Co., U.S.A.), was used without further purification and its molecular weight was assumed to be 66000. KH_2PO_4 and Na_2PO_4 were used to prepare pH 6.85 buffer solution (1/15 M). Theophylline and benzoic acid were used after purification by recrystallization from water. [Carboxyl-¹⁴C]benzoic acid was purchased from New England Nuclear Co. The radiochemical purity of the labeled benzoic acid was greater than 99% and its specific activity was 29.4 mCi/mmol. Water that had been deionized and doubly distilled in all-glass apparatus was used. Other chemicals were of reagent grade. All drug and BSA solutions were prepared immediately before use for equilibrium dialysis, in the pH 6.85 buffer solution mentioned above.

Apparatus—Absorbance was measured with a Shimadzu spectrophotometer, type 200. Fluorescence spectra were measured on a Shimadzu RF-510 spectrophotometer.

Equilibrium Dialysis—i) Theophylline-BSA System: The general approach and technique were the same as described previously.¹⁾ After equilibrium had been reached, the drug concentration in the cell in the absence of BSA

was determined by measuring the absorbance at 272 nm against 1/15 M phosphate buffer as a blank.

ii) Benzoic Acid-BSA System: Experimental conditions were the same as those used in the theophylline-BSA system, except that determination of benzoic acid was done by radioisotopic analysis. [Carboxyl- ^{14}C]benzoic acid (0.1 μCi) was added to the cell which received the drug. The amount of [carboxyl- ^{14}C]benzoic acid used was 3.4×10^{-9} mol, which was *ca.* 1/1000 of the amount of cold benzoic acid, and therefore was negligible quantitatively. After equilibrium dialysis had been completed, one ml of solution from each side of the dialysis membrane was taken into vials, 10 ml of Aquasol-2 (New England Nuclear Co.) was added, and mixing was carried out with a Vortex-genie. The radioactivity was measured to determine the content of benzoic acid.

Results

Figure 1 shows a Scatchard plot of the results obtained by the equilibrium dialysis of theophylline at 15 °C, where r is the molar ratio of protein-bound drug, and C_f is the free drug concentration. On the assumption that each BSA molecule has two types of independent binding sites, the data were analyzed on the basis of a Langmuir-type adsorption isotherm (1);

$$r = \frac{n_1 K_1 C_f}{1 + K_1 C_f} + \frac{n_2 K_2 C_f}{1 + K_2 C_f} \quad (1)$$

where K_1 and K_2 are the association constants corresponding to n_1 and n_2 , the numbers of primary and secondary sites. The most probable values for n_1 , n_2 , K_1 , and K_2 were calculated by the same procedure as described previously¹⁾ and the values obtained are given in Table I. The solid line in Fig. 1 is the theoretical line obtained by using the parameters in Table I. Then, the interaction of theophylline with BSA was studied at different temperatures, 25 and 37 °C. Figure 2 shows Scatchard plots at these temperatures. The binding parameters obtained from Fig. 2 are also included in Table I. The binding constants decreased with increase in temperature. The binding constant for the primary site (K_1) showed a greater decrease than that for the secondary site (K_2). The thermodynamic parameters were evaluated from these results. Enthalpy changes ΔH for the primary and the secondary sites were

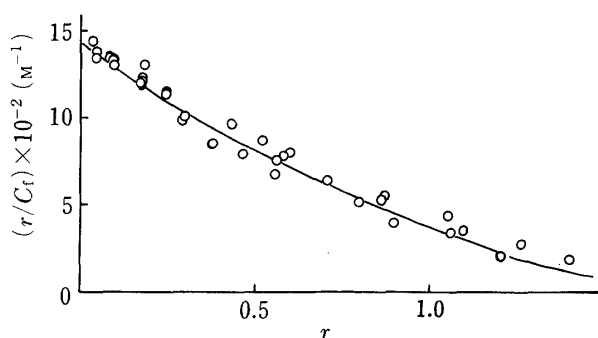


Fig. 1. Scatchard Plot for the Binding of Theophylline to BSA at 15 °C

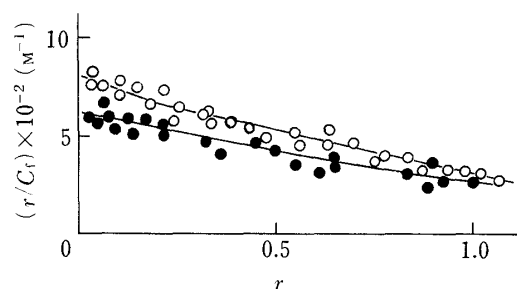


Fig. 2. Scatchard Plots for the Binding of Theophylline to BSA at 25 °C (○) and 37 °C (●)

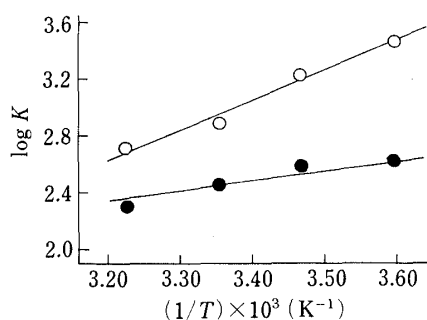


Fig. 3. Van't Hoff Plots of the Data for the Primary (○) and Secondary (●) Sites of Theophylline

TABLE I. Binding and Thermodynamic Parameters for the Interaction of Theophylline with BSA

Temp. (°C)	\bar{n}_1^a	\bar{n}_2^a	$K_1 \times 10^{-3}$ (M ⁻¹)	$K_2 \times 10^{-2}$ (M ⁻¹)	ΔG_1 (kcal/mol)	ΔH_1 (kcal/mol)	ΔS_1 (e.u.)	ΔG_2 (kcal/mol)	ΔH_2 (kcal/mol)	ΔS_2 (e.u.)
5 ^{b)}	0.48	1.34	2.89	4.03	-4.41			-3.32		
15	0.63	1.01	1.66	3.91	-4.25	-9.72	-19.0	-3.42	-2.86	1.6
25	0.60	1.21	0.76	2.79	-3.93			-3.34		
37	0.72	1.19	0.52	2.00	-3.85			-3.27		

a) $\bar{n}_1 = 0.61$, $\bar{n}_2 = 1.19$. b) Ref. 1.

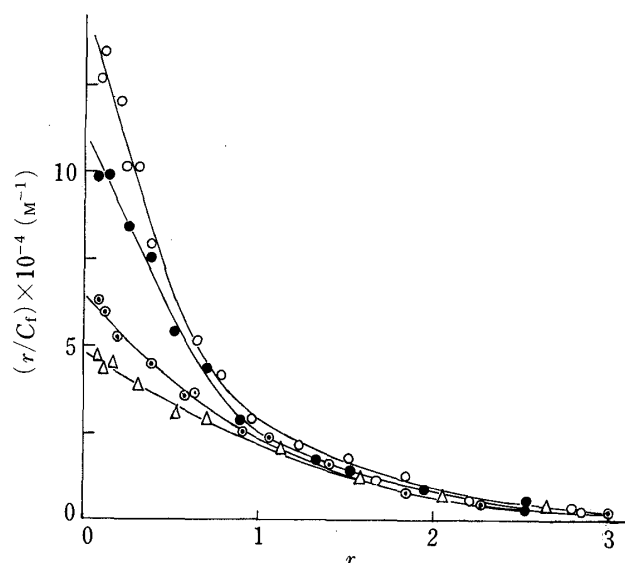


Fig. 4. Scatchard Plots for the Binding of Benzoic Acid to BSA

○, 5°C; ●, 15°C; ⊙, 25°C; △, 37°C.

calculated from Eq. 2

$$\log K = -\frac{\Delta H}{2.303RT} + \text{constant} \quad (2)$$

by plotting K_1 and K_2 against $1/T$ and obtaining the slope of each line (Fig. 3). Free energy changes ΔG and entropy changes ΔS for both sites were calculated by using Eqs. 3 and 4;

$$\Delta G = -2.303RT \log K \quad (3)$$

$$\Delta G = \Delta H - T\Delta S \quad (4)$$

where R is the gas constant, T is absolute temperature, and K is the binding constant. The obtained values are shown in Table I. The above study clarified that binding of theophylline to BSA at the primary site is accompanied by large negative changes both in enthalpy and entropy, while the binding at the secondary site is accompanied by a small negative change in enthalpy and a small positive change in entropy.

Next, the effect of benzoic acid on the binding of theophylline and BSA was studied. Benzoic acid is known to form a soluble complex with theophylline²⁾ and to interact with BSA.³⁾ Before performing the experiment, the interaction of benzoic acid with BSA was studied by equilibrium dialysis. The results obtained at 5, 15, 25, and 37°C are shown as Scatchard plots in Fig. 4, from which the parameters were calculated (Table II). The increase in temperature results in a decrease in the association constants. Of the two association constants, that at the primary site showed a greater decrease. The thermodynamic parameters, obtained by the same method as used for theophylline (Fig. 5), are included in Table II.

TABLE II. Binding and Thermodynamic Parameters for the Interaction of Benzoic Acid with BSA

Temp. (°C)	\bar{n}_1^a	\bar{n}_2^a	$K_1 \times 10^{-5}{}^b$ (M ⁻¹)	$K_2 \times 10^{-3}$ (M ⁻¹)	ΔG_1 (kcal/mol)	ΔH_1 (kcal/mol)	ΔS_1 (e.u.)	ΔG_2 (kcal/mol)	ΔH_2 (kcal/mol)	ΔS_2 (e.u.)
5	0.70	2.71	1.84	4.39	-6.70			-4.64		
15	0.84	2.80	1.23	3.35	-6.71	-8.81	-7.5	-4.64	-3.32	-4.7
25	0.97	2.96	0.68	2.82	-6.59			-4.71		
37	1.12	3.29	0.37	2.40	-6.49			-4.78		

a) $\bar{n}_1 = 0.91$, $\bar{n}_2 = 2.94$. b) The literature value³⁾ is 5×10^4 M⁻¹ at room temperature and pH 7.4.

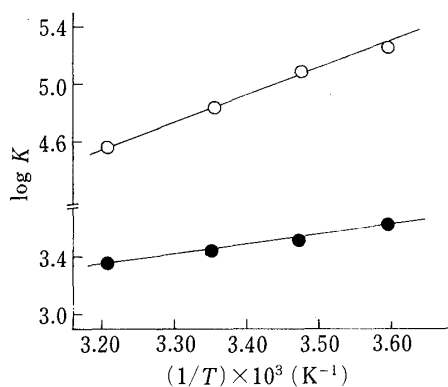


Fig. 5. Van't Hoff Plots of the Data for the Primary (○) and Secondary (●) Sites of Benzoic Acid

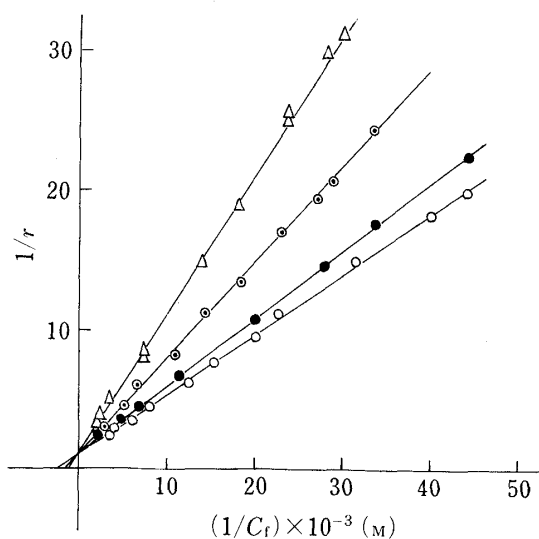


Fig. 6. Klotz Plots for the Binding of Theophylline to BSA in the Presence of Benzoic Acid at 5°C

○, in the absence of benzoic acid. ●, in the presence of 3.8×10^{-4} M benzoic acid. ○, in the presence of 1.5×10^{-3} M benzoic acid. △, in the presence of 3.2×10^{-3} M benzoic acid.

Binding of benzoic acid to the primary site caused large negative changes in both enthalpy and entropy, whereas binding to the secondary site was accompanied by a small negative change in enthalpy and a positive change in entropy; the alterations of the thermodynamic parameters were similar to those observed in the case of theophylline.

The effect of benzoic acid on the binding of theophylline to the protein at 5°C was studied by using Klotz's Eq. 5, which is useful for the investigation of inhibition types in protein binding.⁴⁾ Figure 6 shows a Klotz plot of the binding of theophylline to BSA at low theophylline concentrations, assuming one type of binding site experimental point, ○.

$$\frac{1}{r} = \frac{1}{nKC_f} + \frac{1}{n} \quad (5)$$

The experimental points ●, ○, and △ in Fig. 6 are the results obtained in the presence of 3.8×10^{-4} , 1.5×10^{-3} , and 3.2×10^{-3} M benzoic acid, respectively. The straight line obtained with theophylline only and the lines obtained in the presence of benzoic acid cross at the same point on the ordinate. Therefore, benzoic acid binds to the same primary site as theophylline in a competitive manner, thereby replacing theophylline. These results are consistent with the facts that the thermodynamic parameters for the binding of theophylline and benzoic acid

with BSA are similar.

Discussion

The thermodynamic parameters seem to be divisible into two major categories as regards the primary binding site:^{5,6)} one with relatively large negative enthalpy and negative entropy changes, and the second with small negative enthalpy and positive entropy changes. The former class is represented by the bindings of tryptophan and its derivatives, salicylate, and ethacrynic acid. The second group is represented by long-chain fatty acid, amino azo dyes, and sulfonylureas. In comparing the thermodynamic parameters of theophylline binding to the primary and secondary binding sites with the two major categories mentioned above, it appears that the primary binding resembles the former class and the secondary binding, the latter class. That is to say, the general pattern of the thermodynamic parameters for theophylline binding is similar to that of salicylate⁵⁾ ($\Delta G_1 = -6.45$ kcal/mol at 25 °C, $\Delta H_1 = -9.81$ kcal/mol, $\Delta S_1 = -11.2$ e.u., $\Delta G_2 = -4.72$ kcal/mol at 25 °C, $\Delta H_2 = -4.69$ kcal/mol, $\Delta S_2 = 0.1$ e.u.). It has been postulated that the binding of salicylate involves both hydrophobic and ionic contributions. However, from the thermodynamic parameters for the primary binding site of salicylate, it is considered that van der Waals forces are most important, and both hydrophobic and ionic forces contribute to the binding secondarily. As judged from the thermodynamic parameters, the forces contributing to the binding of benzoic acid may be the same as those for the binding of theophylline.

It is also possible to consider the mechanism of theophylline binding to BSA from a different point of view. At pH around 6.8, under the conditions used in the present study, theophylline is considered to be largely unionized in view of its pK_a , 8.64⁷⁾ at 25 °C. Therefore, ionic forces cannot play a major role in the binding. This view is also supported by the rather large negative value of ΔH_1 for the primary binding site. If the nature of the interaction is largely electrostatic, the main source of ΔG_1 should be a large contribution of the ΔS_1 term with little contribution from ΔH_1 .⁶⁾ Instead, the binding characteristics suggest an alternative mechanism. It was considered that theophylline and benzoic acid bind to the same primary site on BSA in view of the thermodynamic parameters and the competition between theophylline and benzoic acid. It is known that theophylline forms a soluble complex with benzoate by stacking in aqueous solution. Thus, the possibility arises that theophylline binds to phenylalanine, tyrosine, and/or tryptophan residues of BSA by stacking. Tryptophan residue is the most probable candidate for the theophylline binding site judging from the facts that n_1 is smaller than 2 and that tryptophan residue has the largest overlapping area⁸⁾ with

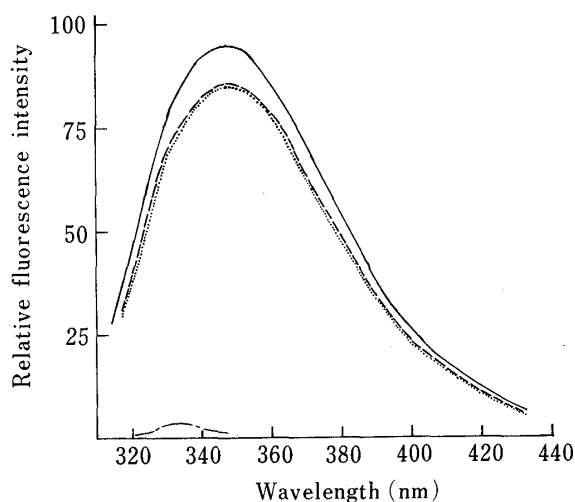


Fig. 7. Emission Spectra (Excited at 300 nm)
 —, 1.40×10^{-6} M BSA. ---, 1.40×10^{-6} M BSA
 in the presence of 1×10^{-4} M theophylline. ----, 1.40×10^{-6} M BSA
 in the presence of 1×10^{-4} M benzoic acid. - · - ·, represent both 1×10^{-4} M theo-
 phylline and 1×10^{-4} M benzoic acid.

theophylline of the three residues. In order to examine whether or not there is an interaction between theophylline and tryptophan residue, the fluorescence spectra of tryptophan residue were measured under the conditions shown in Fig. 7. As shown in Fig. 7, quenching was observed in the presence of theophylline. This observation supports the view that theophylline binds to tryptophan residues in the partial sequences $-\text{Lys}-\text{Phe}-\text{Trp}-\text{Gly}-\text{Lys}-\text{Tyr}-\text{Leu}-$ and $-\text{Lys}-\text{Ala}-\text{Trp}-\text{Ser}-\text{Val}-\text{Arg}-$.⁹⁾ The tryptophan residue in the former sequence is adjacent to a phenylalanine residue and these residues are likely to stack with each other, so theophylline probably binds to the tryptophan residue in the latter sequence. The latter sequence is very similar to the partial sequence around the tryptophan residue of human serum albumin, $-\text{Lys}-\text{Ala}-\text{Trp}-\text{Val}-\text{Ala}-\text{Arg}-$.

Benzoic acid is also considered to bind to a tryptophan residue judging from the experimental results mentioned above. To confirm this, fluorescence spectra were measured in the same way as in the case of theophylline. The results are also shown in Fig. 7. Quenching was observed in the presence of benzoic acid, and therefore it is clear that benzoic acid interacts with tryptophan residue. In this connection, aspirin has been reported to bind to a tryptophan residue of BSA based on measurement of the fluorescence spectrum.¹⁰⁾ It can thus be presumed that theophylline and benzoic acid bind to the primary site on BSA by stacking interactions between tryptophan residue and these drugs. The smaller binding constant K_1 of theophylline than of benzoic acid is presumably due to the difference in the degree of ionization. Thus, theophylline is mostly in unionized form under the experimental conditions and it has practically no negative charge. Therefore, theophylline is less attracted by positively charged lysine and arginine residues around the tryptophan residue than benzoic acid, which in contrast is more than 90% ionized at around pH 6.8.¹¹⁾ It is presumed that this difference in the electrostatic effect contributes greatly to the difference in the binding constant K_1 between two compounds.

The foregoing considerations lead us to consider that theophylline (also benzoic acid) binds to the tryptophan residue on BSA by van der Waals forces due to stacking (in this case, stacking is considered to occur more strongly through van der Waals forces than by hydrophobic bonding) and the electrostatic interaction between the positive charges of lysine and arginine residues on BSA and the negative charge of theophylline takes part in the binding. Accordingly the thermodynamic parameters for theophylline may be interpreted as follows; large negative changes in enthalpy and entropy for the primary binding site can be attributed to the binding due to van der Waals forces and a negative change in enthalpy and a positive change in entropy, to the effects of ionic and hydrophobic bonding.

References and Notes

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