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Effect of Salicylate on the Binding of Sulfonamides to Bovine Serum Albumin

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Displacement of eight sulfonamides by sodium salicylate from the binding sites of bovine serum albumin (BSA) was studied by the equilibrium dialysis method at pH 7.4 and 37°.

Amount of sulfonamides displaced by the addition of salicylate (1 or 3 mM) were linearly correlated with their amount bound to the binding sites of BSA in the absence of salicylate.

Double-reciprocal plots of the binding of sulfamethoxazole to BSA with and without salicylate, following Langmuir's isotherms, resulted in linear curves with a common ordinate intercept. This result suggests that the inhibitory effects of salicylate on the binding of sulfamethoxazole to albumin were competitively performed at the same binding site on the protein.

Keywords——sulfonamides; salicylate; protein-binding; displacement; drug interaction; equilibrium dialysis method

It has been well established that the binding of drugs to circulating proteins in the living body can influence on their pharmacological activities[2] as well as their pharmacokinetic parameters.[3] Many studies[4] have demonstrated that the extent of protein binding of drugs is influenced by the presence of other drugs which also bind to proteins. The increased pharmacologic effect of a drug observed *in vivo* when it is concurrently given with non-steroidal anti-inflammatory drugs is frequently subjected to the increase in free concentration of the drug in blood.[5,6] Derivatives of salicylic acid are clinically used as non-steroidal anti-inflammatory drug. Salicylic acid binds to circulating proteins,[7,8] in particular to the serum albumin, in many species including man, and displaces many drugs from the binding site on serum proteins.

Presently, the effect of salicylate on the binding of eight sulfonamides to bovine serum albumin was studied *in vitro*. And it was shown that salicylate can displace sulfonamides from the binding site of albumin and the nature of displacement was also discussed on the basis of the competitive binding to the protein employing sulfamethoxazole and salicylate.

Experimental

Materials——Commercially available eight sulfonamides, sulfanilamide (S), sulfamethoxazole (SMX), sulfathiazole (ST), sulfadimethoxine (SDM), sulfisoxazole (SIX), sulfisomidine (SIM), sulfadiazine (SD),

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2) P.M. Aggeler, R.A. O'Reilly, L. Leong, and P.E. Kowitz, *N. Engl. J. Med.*, **276**, 496 (1967).
3) A.H. Anton, *J. Pharmacol. Exp. Ther.*, **134**, 291 (1961); K. Kakemi, H. Sezaki, T. Komuro, and K. Ikeda, *Chem. Pharm. Bull.* (Tokyo), **18**, 2386 (1970).
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7) C.J. Moran and W.H.C. Walker, *Biochem. Pharmacol.*, **17**, 153 (1968).
8) C.A. Cruze and M.C. Meyer, *J. Pharm. Sci.*, **65**, 33 (1976).

sulfapyridine (SP), and sodium salicylate (SA) were used without further purification. Bovine serum albumin (Fraction V) (BSA), was obtained from the Armour Pharmaceutical Company and cellophane tubing (Visking Company, 27/32 inch inflated diameter) was used as a dialyzing membrane. All other chemicals were analytical grade and distilled water was used throughout.

Binding to Albumin——BSA was made up as 1% (w/v) solution in a phosphate buffer, pH 7.4, and ionic strength 0.1. Sulfonamides and sodium salicylate were dissolved in the phosphate buffer. The binding of drugs to BSA was determined with an equilibrium dialysis method employing the cellophane bag. Ten ml of the BSA solution was taken into the dialysis bag which was placed in a glass tube containing 10 ml of the drug solution. After continuous shaking of 90 cycle/min for 8 hr at 37° employing a water-bath shaker, the concentration of drug outside the bag was measured. It took 6 and 4 hr for sulfonamides and salicylate to establish a dialysis equilibrium, respectively. In the preliminary experiments, it was determined that neither sulfonamides nor salicylate was bound to the cellophane tubing over the range of concentration studied. The value of 69000 was used for the molecular weight of BSA.[9]

Analytical Methods——Sulfonamides were colorimetrically assayed employing a modified Bratton-Marshall method with Tsuda's reagent.[10]

For the assay of salicylate, 5 ml of a sample solution were acidified with 1 ml of 1 N HCl and shaken with 4 ml of $CHCl_3$. After complete separation of the mixture with centrifugation, 3 ml of the $CHCl_3$ layer was taken and extracted with 7 ml of a glycine-HCl buffer, pH 2.5, which contained 0.025% $Fe(NO_3)_3$. The aqueous layer was spectrophotometrically measured at 530 nm.

Results and Discussion

Effect of Salicylate on the Sulfonamide-Albumin Interaction

Ten ml of the buffer solution of 0.2 mM sulfonamide without or with 1 or 3 mM salicylate was placed outside the dialysis bag which contained 10 ml of 1% BSA buffer solution. The amount of sulfonamide bound to BSA at 37° was obtained in terms of the bound amount to 0.1 g BSA. The amount of sulfonamide displaced by the addition of salicylate was obtained from the differences of the bound amounts of sulfonamide between with and without salicylate. In every experiment, the presence of salicylate increased the unbound concentration of each sulfonamide.

It was noted that the displaced amounts of sulfonamides by the addition of salicylate were linearly correlated with the intrinsic affinity of the respective sulfonamide for BSA (Fig. 1). It was interesting to note that, when the sulfonamide solution with 1 mM salicylate was equilibrated with the 1% BSA solution, 50% of the bound sulfonamide was forced to released from the binding site of BSA by the addition of salicylate. Similarly 70% was released in the presence of 3 mM of salicylate.

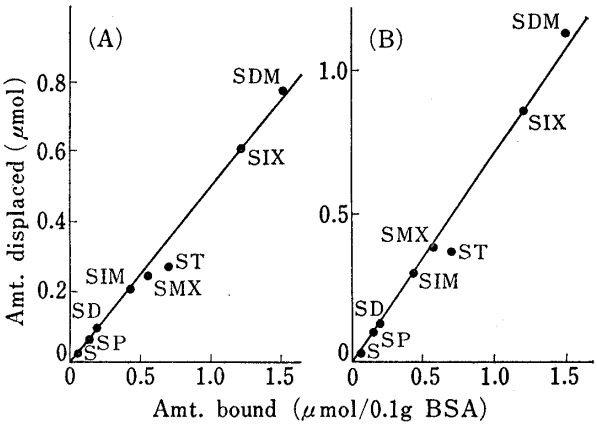

Fig. 1. Relations between the Amounts of Sulfonamides Bound to BSA in the Absence of Salicylate and the Displaced Amounts of Sulfonamides in the Presence of Salicylate

(A) in the presence of 1 mM salicylate,
(B) in the presence of 3 mM salicylate.

Logarithms of the association constants of sulfonamides to BSA are reported to be linearly correlated with their molecular weights with a correlation coefficient of 0.93.[11] Thus, the

9) G. Scatchard, A.C. Batchelder, and A. Brown, *J. Am. Chem. Soc.*, **68**, 2320 (1946).
10) M. Yamazaki, M. Aoki, A. Kamada, and N. Yata, *Yakuzaigaku*, **27**, 37 (1967).
11) M. Yamazaki, N. Kakeya, T. Morishita, A. Kamada, and M. Aoki, *Chem. Pharm. Bull.* (Tokyo), **18**, 708 (1970).

displaced amount of sulfonamide by 1 and 3 mM salicylate is considered to be estimated by equations (1) and (2).

$$\text{Displaced Amt.} = -0.984\,\text{M.W.} + 0.005 \quad (1\ \text{mM}) \tag{1}$$
$$n = 8,\ r = 0.728$$
$$\text{Displaced Amt.} = -1.415\,\text{M.W.} + 0.007 \quad (3\ \text{mM}) \tag{2}$$
$$n = 8,\ r = 0.734$$

where, M.W. is the molecular weight of sulfonamides. But poor correlations were obtained. Further study is considered to be required for the present analysis by increasing the number of sulfonamides.

Nature of the Mutual Displacement of Sulfamethoxazole and Salicylate

In the presence of salicylate, less amount of sulfonamide was bound to BSA. To clarify the inhibitory effect of salicylate on the protein binding of sulfonamides, the mutual displacement for the BSA binding was studied employing sulfamethoxazole and salicylate.

Binding parameters of sulfamethoxazole and salicylate to BSA were measured at pH 7.4 and 37° employing 1% BSA solution (Table I). It was reported that the interaction of sulfonamides with albumin took place at one class of binding site with about 2 sites per albumin molecule.[12] The present results of salicylate-BSA binding were similar to those reported by Moran *et al.*[7] employing human serum albumin. They resolved binding sites into two components, one corresponding to 1.5 primary binding sites per albumin molecule, n_1, with the association constant, K_1, of 14300 and the other corresponding to 4.5 binding sites, n_2, with the association constant, K_2, of 670.

Table I. Binding Parameters of Sulfamethoxazole and Salicylate

Compounds	n_1	$K_1(M^{-1})$	n_2	$K_2(M^{-1})$
Sulfamethoxazole	2.1	3000	—	—
Salicylate	2.0	20000	5.1	520

Binding of 0.2 mM sulfamethoxazole to BSA was markedly reduced by the addition of salycylate (Fig. 2). But the binding of 0.5 mM salicylate to BSA was slightly influenced by

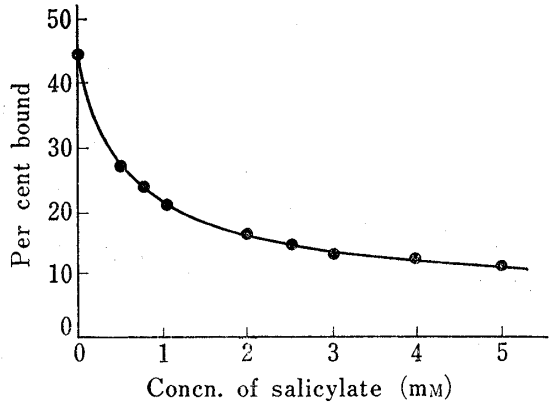

Fig. 2. Effect of Salicylate on the Binding of Sulfamethoxazole to BSA

Initial concentration of sulfamethoxazole: 0.2 mM.

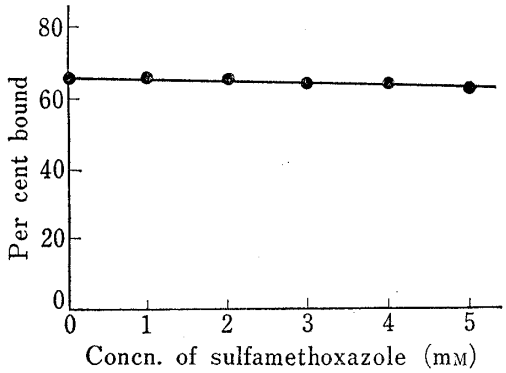

Fig. 3. Effect of Sulfamethoxazole on the Binding of Salicylate to BSA

Initial concentration of salicylate: 0.5 mM.

12) M. Yamazaki, M. Aoki, A. Kamada, and N. Yata, *Yakuzaigaku*, **27**, 40 (1967); I. Moriguchi, S. Wada, and J. Nishizawa, *Chem. Pharm. Bull.* (Tokyo), **16**, 601 (1968); S. Goto, T. Ohki, S. Kiryu, and S. Iguchi, *Yakuzaigaku*, **31**, 247 (1971).

the addition of sulfamethoxazole (Fig. 3). To explain the present results, a possible existence of interaction between sulfamethoxazole and salicylate was studied employing solubility and spectrophotometric methods. No direct interaction between two drugs was observed within the concentration range studied. Thus, it was neglected for the following consideration.

The molar ratio, r, of bound sulfamethoxazole to albumin and the unbound sulfamethoxazole concentration (C_f) were obtained employing 1% BSA and without or with 1 mM salicylate. A plot of $1/r$ against $1/C_f$ enables a graphic estimation of the maximum association constant and the number of binding sites. As shown in Fig. 4, two curves were linear with a common ordinate intercept, indicating that sulfamethoxazole and salicylate compete at the same binding site of BSA and the binding parameters, n and K_{app}, are maintained constant in the presence of 1 mM salicylate. Thus, it may be considered that salicylate competitively inhibits the binding of sulfamethoxazole at the same binding sites of BSA which are the primary binding site for salicylate.

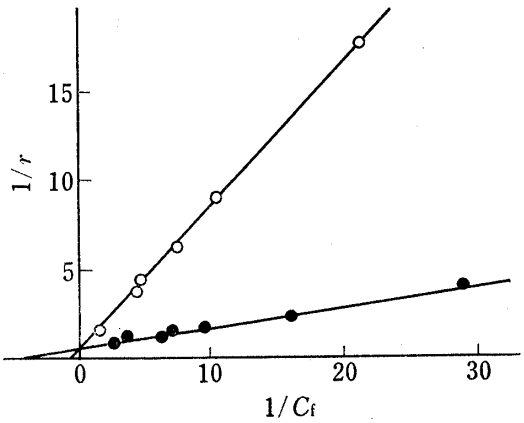

Fig. 4. Double-reciprocal Plots of Sulfamethoxazole-BSA Interactions with and without Salicylate

—●—: without salicylate,
—○—: with 1 mM salicylate.

The competitive inhibition of drug-protein interaction by two drugs, A and B, is expressed by equation (3).

$$\frac{1}{r} = \frac{1}{nK_A} \cdot \frac{1}{[A]} \cdot \{1 + K_B \cdot [B]\} + \frac{1}{n} \quad (3)$$

Where, [A] and [B] are the free concentrations of two drugs A and B, respectively. K_A and K_B are the binding constants of A and B, to BSA, respectively.

If plots of $1/r$ *versus* $1/[A]$ yield a linear line for the competitive interaction of A and B, an apparent association constant between A and BSA in the presence of B (K_{app}) is expressed by equation (4).

$$K_{app} = K_A/\{1 + K_B \cdot [B]\} \quad (4)$$

When the values of K_A and K_B are preliminarily determined and [B] is measured for the experiments of competitive interaction of two drugs, the association constant can be calculated employing equation (4) (K_{cal}).

In the present study, it was found that the amount of salicylate bound to BSA was little influenced by the addition of sulfamethoxazole being subjected to the strong protein affinity of salicylate. Thus, the concentration of salicylate unbound to BSA, [B], is considered to be constant over the range of sulfamethoxazole concentration studied. In the presence of 1 mM salicylate, the values of K_{app} and n graphically obtained from Fig. 4 were 520 and 2.3, respectively. A similar value of 420 was obtained for K_{cal} employing 0.29 mM of [B], supporting that the inhibitory effects of salicylate on the binding of sulfamethoxazole to albumin were competitively performed at the same binding site of the protein.

Goto *et al.*[13] studied on the binding of sulfisoxazole to BSA employing a dynamic dialysis method and found an uncompetitive inhibition of salicylate for the binding of sulfisoxazole. The opposite results are considered to be partially subjected to the difference in methods and the kind of sulfonamides employed. But further study is required to clarify the mechanisms of the protein binding of drugs on the basis of their competitive interactions.

13) S. Goto, T. Hara, and H. Yoshitomi, *Yakuzaigaku*, **35**, 51 (1975).

The binding sites of sulfonamides and salicylate to albumin were reported to be ε-amino moiety of the protein with an electrostatic interaction.[14] Thus, it may be considered that sulfamethoxazole and salicylate competitively bind to the same cationic binding sites on BSA.

Displacement of a drug from the binding sites on protein may change pharmacokinetic parameters of the drug and produce an increased pharmacologic effect as well as a toxic effect.

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Studies on Furan Derivatives. III.[1] Synthesis of 4-Substituted 5-(5-Nitro-2-furyl)thiazol-2-ones and Some of Their Derivatives

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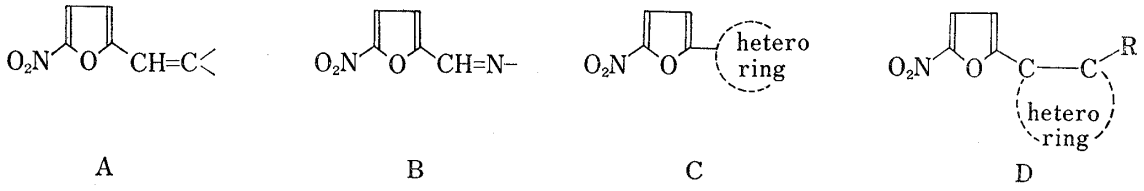
Seven kinds of 4-substituted 5-(5-nitro-2-furyl)thiazol-2-ones were obtained by the reaction of α-substituted β-(5-nitro-2-furyl)vinylamines (Ia—g) with chlorocarbonylsulfenyl chloride. Reaction of Ia and c with trichloromethylsulfenyl chloride gave 3-substituted 4-(5-nitro-2-furyl)-5-chloroisothiazoles.

Keywords——nitrofuran derivatives; 5-(5-nitro-2-furyl)thiazol-2-ones; 2-chloro-5-(5-nitro-2-furyl)thiazoles; 4-(5-nitro-2-furyl)-5-chloroisothiazoles; cyclization of β-(5-nitro-2-furyl)vinylamines; antibacterial activity

A number of nitrofuran derivatives have hitherto been prepared with a view to obtaining antibacterial compounds. They may be grouped into three types with respect to their structure; (5-nitro-2-furyl)vinyls[3] (A), (5-nitro-2-furyl)azomethines[4] (B), and (5-nitro-2-furyl)heterocycles[5] (C).

Many compounds of type A exhibit higher antibacterial activity *in vitro* but the activity usually tends to decrease *in vivo*. Such a behavior is believed to be due to the instability



A B C D

Chart 1

1) Part II of this series, A. Tanaka, T. Usui, and S. Yoshina, *J. Heterocyclic Chem.*, **15**, 555 (1978).
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5) W.R. Sherman, *J. Med. Chem.*, **8**, 25 (1965); S. Yoshina, I. Maeba, and K. Asai, *Yakugaku Zasshi*, **88**, 984 (1968); P.J. Islip and M.R. Johnson, *J. Med. Chem.*, **16**, 1308 (1973); H.A. Burch and L.E. Benjamin, *ibid.*, **17**, 451 (1974).