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# Displacement of Sulfonamides from Bovine Serum Albumin by Nonsteroidal Anti-inflammatory Drugs[1,2]

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The effects of six nonsteroidal anti-inflammatory drugs, phenylbutazone, flufenamic acid, salicylic acid, indomethacin, flurbiprofen, and naproxen, on the binding of sulfonamides to bovine serum albumin were examined using the equilibrium dialysis method at pH 7.4 and 37°. Lineweaver–Burk plots suggested that these anti-inflammatory drugs inhibit the binding of sulfonamides competitively. Anti-inflammatory drugs could be ranked in the following order of diminishing ability to inhibit the binding of sulfonamides to bovine serum albumin: phenylbutazone, flufenamic acid, salicylic acid, indomethacin, flurbiprofen, and naproxen; the inhibitory activities of the first three drugs were especially high. Furthermore, the competitive binding of sulfisoxazole and salicylic acid was studied in detail on the basis of a theoretical equation. The data for the inhibition of the binding of sulfisoxazole by salicylic acid satisfied the equation well, confirming that this theoretical equation is suitable to describe such competitive binding phenomena.

**Keywords**——nonsteroidal anti-inflammatory drugs; sulfonamides; protein binding; displacement; competitive binding; equilibrium dialysis method

In recent drug-interaction studies, a number of investigations[4] have demonstrated that the pharmacological response to a drug can be enhanced as a result of its displacement from binding sites on protein molecules by another coadministered drug or an endogenous substance. Furthermore, in many combinations of drugs the possibility of drug displacement *in vitro* or *in vivo* has been reported.[5] Nevertheless, there are few detailed quantitative studies of such drug displacement.

We reported previously[1,6] that acidic nonsteroidal anti-inflammatory drugs have a high binding affinity to bovine serum albumin and displace salicylate to a considerable extent from its binding sites on the albumin molecules. On the other hand,

1) This paper forms Part III of "Protein Binding of Nonsteroidal Anti-inflammatory Drugs." Preceding paper (Part II): Y. Kaneo, A. Nishikawa, Y. Kato, and S. Kiryu, *Yakugaku Zasshi*, **98**, 1452 (1978).

2) A part of this work was presented at the 98th Annual Meeting of the Pharmaceutical Society of Japan, Okayama, April, 1978.

3) Location: *a*) *Ichigaya Funagawara-machi, Shinjuku-ku, Tokyo*; *b*) *Maidashi 3-chome, Higashi-ku, Fukuoka.*

4) L.K. Christensen, J.M. Hansen, and M. Kristensen, *Lancet*, **2**, 1298 (1963); P.M. Aggeler, R.A. O'Reilly, L. Leong, and P.E. Kowitz, *N. Engl. J. Med.*, **276**, 496 (1967); A.H. Anton, *Cin. Pharmacol. Ther.*, **9**, 561 (1968); A.H. Anton and W.T. Corey, *Acta Pharmacologica*, **29**, suppl. 3, 134 (1971); I. Sjöhlm, A. Kober, I. Oder-cederlof, and O. Borga, *Biochem. Pharmacol.*, **25**, 1205 (1976).

5) C.M. Kunin, *Clin. Pharmacol. Ther.*, **7**, 166 (1966); P.M. Keen, *Brit. J. Pharmacol.*, **26**, 704 (1966); H.M. Solomon and J.J. Schrogie, *Biochem. Pharmacol.*, **16**, 1219 (1967); S. Goto, T. Hara, and H. Yoshitomi, *Yakugaku Zasshi*, **96**, 565 (1976); *a*) H.M. Solomon, J.J. Schrogie, and D. Williams, *Biochem. Pharmacol.*, **17**, 143 (1968); S. Goto, T. Hara, and H. Yoshitomi, *Yakuzaigaku*, **35**, 51 (1975).

6) Y. Kaneo, A. Kai, S. Kiryu, and S. Iguchi, *Yakugaku Zasshi*, **96**, 1412 (1976).

protein binding of sulfonamides has been intensely investigated by Klotz,[7] Scholtan,[8] Nakagaki,[9] Elofsson,[10] and many other workers.

Thus, in order to examine drug displacement more quantitatively, the effects of nonsteroidal anti-inflammatory drugs on the protein binding of sulfonamides were investigated in this study. Binding data were plotted according to Klotz's equation, and the types of displacement were identified by means of Lineweaver–Burk plots. Furthermore, the competitive binding of sulfisoxazole and salicylic acid was analyzed in detail on a theoretical basis.

## Experimental

**Materials**——Bovine serum albumin (BSA, fraction V, Armour Co.) was assumed to have a molecular weight of 69000. Phenylbutazone, flufenamic acid, indomethacin, flurbiprofen, and naproxen were provided by the companies described in the previous paper.[6] Salicylic acid of the purest reagent grade was obtained commercially. Sulfadimethoxine, sulfisoxazole, sulfamethomidine, sulfaphenazole, sulfamethoxazole, and sulfisomidine were obtained from commercial sources, and were recrystallized before use. [Carboxyl-$^{14}$C] salicylic acid was purchased from The Radiochemical Centre Ltd., Amersham, and had a specific activity of 59 mCi/mmol.

**Equilibrium Dialysis Method**——The equilibrium dialysis procedure was carried out as described previously;[1,6] Four ml of $1.95 \times 10^{-4}$ M BSA solution was placed in the dialysis bag, then it was immersed in 10 ml of buffer solution containing both a drug and a displacing drug (inhibitor). The binding of sulfonamides at various concentrations was measured in the presence of a fixed amount of each anti-inflammatory drug. In the experiments on the binding of salicylic acid, a trace amount of [carboxyl-$^{14}$C] salicylic acid (about 0.2 $\mu$Ci) was added to the external buffer solution. In all experiments 1/15 M phosphate buffer (pH 7.4, isotonic, ionic strength=0.24) was used. All dialysis experiments were carried out at 37°.

**Analytical Methods**——The concentration of sulfonamides in the equilibrated external solution was determined by diazotization and coupling with Tsuda's reagent according to the Bratton–Marshall method.[11] The concentrations of salicylic acid in the internal and external solutions were determined as follows. One ml of a sample solution was added to 10 ml of Aquazol II (New England Nuclear Co.), and [carboxyl-$^{14}$C] salicylic acid in the medium was determined with a liquid scintillation counter (Aloka, LSC-651).

## Results and Discussion

### Types of Drug Displacement

Three types of displacement have been presented, as shown in Fig. 1.[5a] These models are derived from enzyme kinetics, and are defined by reference to Lineweaver–Burk plots as described below. (a) Competitive inhibition: an inhibitor competes with a drug for the same binding sites on a protein molecule, so that the same intercept on the ordinate ($1/r$) is obtained graphically. (b) Noncompetitive inhibition: an inhibitor binds to different sites, inhibiting the binding of a drug noncompetitively. In this case the same intercept on the abscissa ($1/C$) is obtained graphically. (c) Uncompetitive inhibition: an inhibitor binds reversibly to the drug–protein complex.

The effects of 400 $\mu$g and/or 800 $\mu$g of anti-inflammatory drugs on the binding of sulfonamides to BSA were examined. Binding data for displaced sulfonamides were plotted according to Klotz's equation (double-reciprocal plot), expressed as

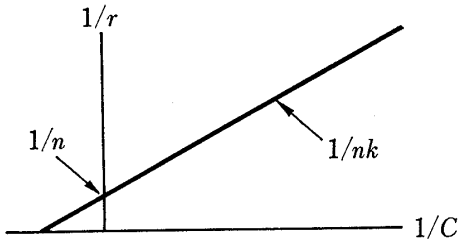
$$1/r = 1/nk \cdot 1/C + 1/n \quad (1)$$

where $r$ is the number of bound drug molecules per protein molecule, $C$ is the concentration of unbound drug, and $n$ and $k$ are the maximum number of binding sites and the binding

7) I.M. Klotz, F.M. Walker, and R.B. Pivan, *J. Am. Chem. Soc.*, **68**, 1486 (1946); I.M. Klotz and F.M. Walker, *ibid.*, **70**, 943 (1948); I.M. Klotz and J.M. Urguhart, *ibid.*, **71**, 847 (1949).
8) W. Scholtan, *Arzneimittel-Forsch.*, **14**, 348 (1964); W. Scholtan, *ibid.*, **14**, 469 (1964); W. Scholtan, *ibid.*, **18**, 505 (1968).
9) M. Nakagaki, N. Koga, and H. Terada, *Yakugaku Zasshi*, **83**, 586 (1963); M. Nakagaki, N. Koga, and H. Terada, *ibid.*, **84**, 516 (1964).
10) R. Elofsson, S.O. Nilsson, and A. Ågren, *Acta Pharm. Suecica*, **7**, 473 (1970); R. Elofsson, S.O. Nilsson, and B. Kluczykowska, *ibid.*, **8**, 465 (1971); A. Ågren, R. Elofsson, and S.O. Nilsson, *ibid.*, **8**, 475 (1971).
11) A.C. Bratton and E.K. Marshall, *J. Biol. Chem.*, **128**, 537 (1939).

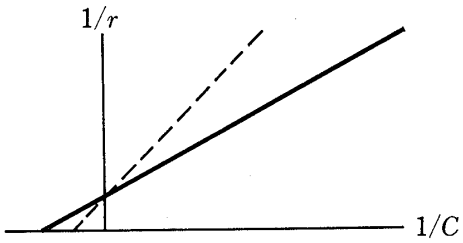
Klotz's equation

$$1/r = 1/nk \cdot 1/C + 1/n$$



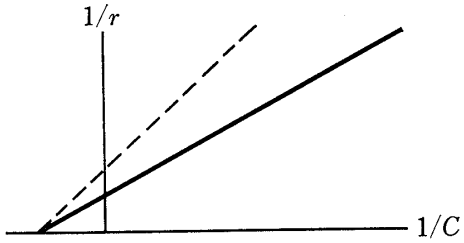

(a) competitive inhibition

$$1/r = 1/nk \cdot 1/C \cdot (1+k'C') + 1/n$$




(b) noncompetitive inhibition

$$1/r = 1/nk \cdot 1/C \cdot (1+k'C') + 1/n \cdot (1+k'C')$$




(c) uncompetitive inhibition

$$1/r = 1/nk \cdot 1/C + 1/n \cdot (1+k'C')$$

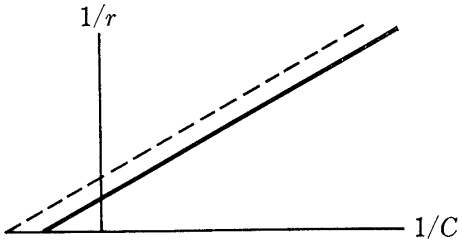



Fig. 1. Types of Inhibition in Protein Binding

where
$r$: number of bound drug molecules/protein molecule,
$C$: conc. of unbound drug,
$C'$: conc. of unbound inhibitor,
$n$: maximum number of binding sites,
$k$: binding const. of drug,
$k'$: inhibitory const. of inhibitor.

constant, respectively. Typical inhibitions of the binding of sulfadimethoxine by flufenamic acid and of the binding of sulfisoxazole by phenylbutazone and salicylic acid are shown in Figs. 2—4, respectively. The binding regression lines clearly intercept the ordinate ($1/r$) at the same point in each case, so it appears from these findings that the types of inhibition are all competitive. Similar results were obtained with the other sulfonamides.

Since it appeared that anti-inflammatory drugs inhibit the binding of sulfonamides to BSA competitively, the inhibitions were next examined more quantitatively. Rippie discussed the displacement of warfarin and phenylbutazone from human albumin by free fatty acids, calculating the value of $\Delta\Delta G^{\circ}_{app}$.[12] Instead of this value, we calculated $-\log(k_{app}/k)$, where

12) E.G. Rippie, *Biochem. Pharmacol.*, **25**, 1215 (1976); The apparent inhibitor induced free energy change in drug binding, $\Delta\Delta G^{\circ}_{app}$, can be determined from the relationship:

$$\Delta\Delta G^{\circ}_{app} = -RT \ln (k_{app}/k)$$

where $R$ is the gas constant, $T$ is the absolute temperature, $k$ is the binding constant, and $k_{app}$ is that measured in the presence of inhibitor. It is apparent from the equation that the positive value of $\Delta\Delta G^{\circ}_{app}$ increases in proportion to the extent of inhibition.

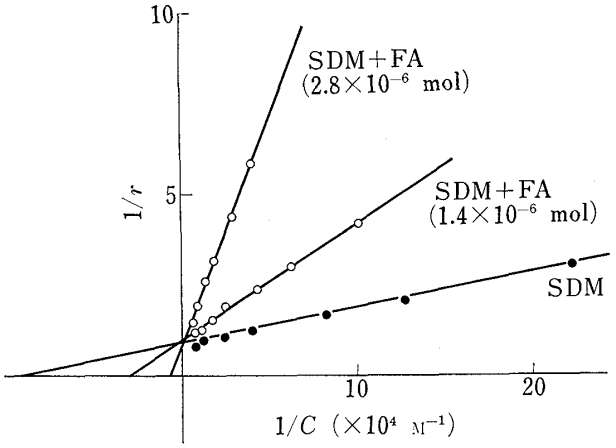



Fig. 2. The Effect of Flufenamic Acid (FA) on the Binding of Sulfadimethoxine (SDM) to BSA

The number in parentheses is the amount of flufenamic acid added to each sample in that series of experiments.

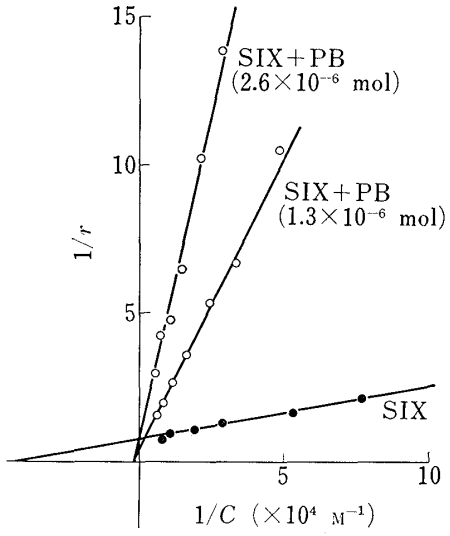



Fig. 3. The Effect of Phenylbutazone (PB) on the Binding of Sulfisoxazole (SIX) to BSA

The number in parentheses is the amount of phenylbutazone added to each sample in that series of experiments.

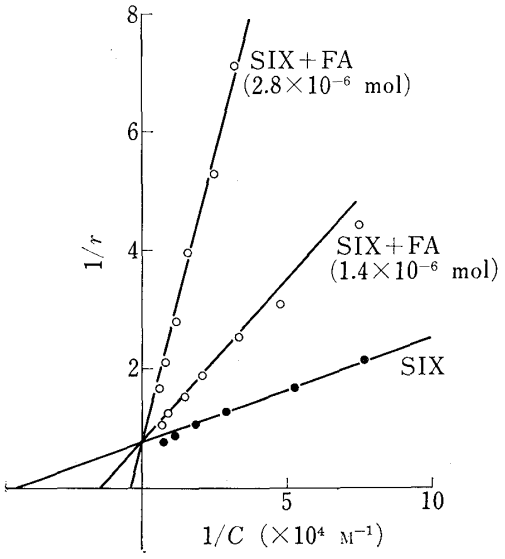



Fig. 4. The Effect of Flufenamic Acid (FA) on the Binding of Sulfisoxazole (SIX) to BSA

The number in parentheses is the amount of flufenamic acid added to each sample in that series of experiments.

Table I. Binding Parameters[a] of Sulfonamides

| Sulfonamides | $n$ | $k$ ($\times 10^4$ M$^{-1}$) | $nk$ ($\times 10^4$ M$^{-1}$) |
|---|---|---|---|
| Sulfadimethoxine | 1.2 | 8.36 | 10.0 |
| Sulfisoxazole | 1.3 | 4.39 | 5.75 |
| Sulfamethomidine | 1.5 | 2.39 | 3.58 |
| Sulfaphenazole | 1.9 | 0.702 | 1.32 |
| Sulfamethoxazole | 3.0 | 0.159 | 0.475 |
| Sulfisomidine | 3.4 | 0.117 | 0.394 |

a) Binding parameters were determined by the equilibrium dialysis method at 37° with $1.95 \times 10^{-4}$ M BSA in 1/15 M phosphate buffer (pH 7.4).

$k_{app}$ is the binding constant measured in the presence of inhibitor, in order to estimate the extent of inhibition of the binding of sulfonamides to BSA by anti-inflammatory drugs (Fig. 5). The binding parameters of sulfonamides used in this study are shown in Table I. The anti-inflammatory drugs could be ranked in the following order of diminishing ability to inhibit the binding of sulfonamides: phenylbutazone, flufenamic acid, salicylic acid, indomethacin, flurbiprofen, and naproxen. The first three drugs showed especially high inhibitory activities (Fig. 5).

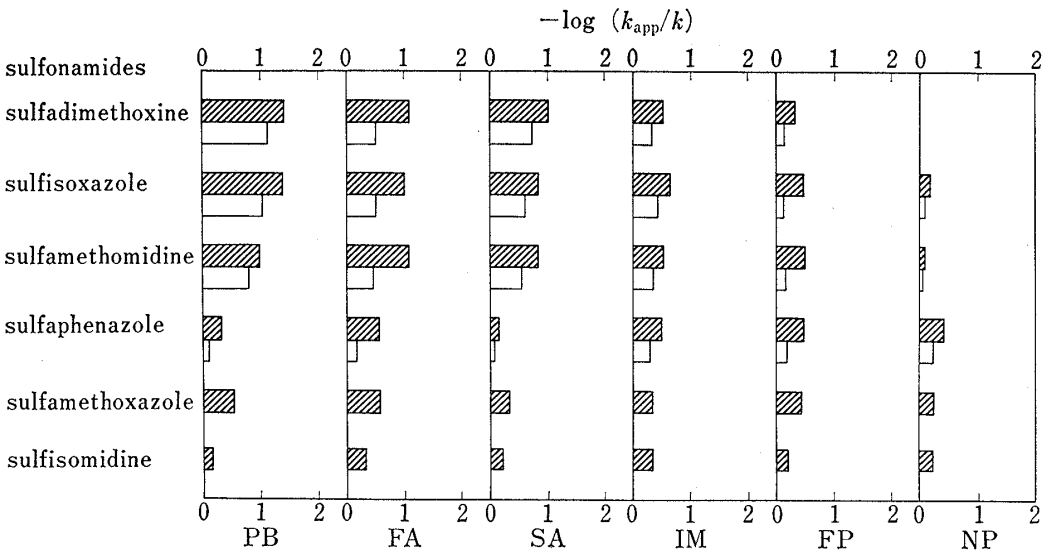



Fig. 5. The Extent of Inhibition of the Binding of Sulfonamides to Bovine Serum Albumin by Anti-inflammatory Drugs (AID)

▨: 800 μg of AID added, □: 400 μg of AID added.
PB: phenylbutazone, FA: flufenamic acid,
SA: salicylic acid, IM: indomethacin,
FP: flurbiprofen, NP: naproxen.

The inhibitions of the binding of sulfadimethoxine, sulfisoxazole, and sulfamethomidine to BSA by these anti-inflammatory drugs in terms of $-\log(k_{app}/k)$ were approximately equivalent. However, the values of $-\log(k_{app}/k)$ for the other three sulfonamides, which have relatively low binding affinities to BSA, were low, especially in the presence of phenylbutazone, flufenamic acid, or salicylic acid (Table I and Fig. 5). Nakagaki[9] and Elofsson[10] reported that the binding of sulfonamides to albumin is due to electrostatic interaction. Scholtan[8] demonstrated that there is also a hydrophobic interaction. Thus, it may be assumed that both interactions contribute to the protein binding of sulfonamides, and if the proportions of the two interactions vary according to the binding affinity of the sulfonamides, different values of $-\log(k_{app}/k)$ may be obtained as shown in Fig. 5.

**Analysis of Competitive Binding of Sulfisoxazole (SIX) and Salicylic Acid (SA) to BSA**

Competitive binding of the two drugs can be described theoretically by the following equation.[13]

$$r = nkC/(1+kC+k'C') \tag{2}$$

Equation (2) can be rearranged to yield:

$$1/r = 1/nk \cdot 1/C \cdot (1+k'C') + 1/n \tag{3}$$

where $k'$ is the binding constant of inhibitor (inhibitory constant), $C'$ the concentration of unbound inhibitor, and $n$, $k$, $r$ and $C$ have the same meaning as in equation (1). When $1/r$ was plotted as a function of $1/C$ on the basis of equation (1), straight lines were obtained as shown in Figs. 2—4. This indicates that $C'$ is almost constant when a fixed amount of inhibitor is added to a series of drug binding systems. The apparent binding constant of drug, $k_{app}$, determined in the presence of an inhibitor can thus be represented by the following equation:

$$k_{app} = k/(1+k'C') \tag{4}$$

This equation can be rearranged to give:

$$k_{app} = k - k_{app} \cdot C' \cdot k' \tag{5}$$

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

then,

$$nk_{app} = nk - nk_{app} \cdot C' \cdot k' \quad (6)$$

When $nk_{app}$ is plotted against $nk_{app} \cdot C'$, a straight line is obtained if the binding is competitively inhibited. The ordinate intercept corresponds to $nk$ for that particular drug–protein binding. The slope of the line, $-k'$, which should theoretically be equal to the binding constant of inhibitor, is characteristic for a particular competitive inhibition system.

In the previous study,[6] we reported that a Scatchard plot for the binding of SA to BSA gave a curve which corresponded to the summation of two straight lines representing $n_1=1.81$, $k_1=1.47\times10^4\,\mathrm{M}^{-1}$; $n_2=5.85$, $k_2=3.61\times10^2\,\mathrm{M}^{-1}$, where $k_1$ and $k_2$ are the binding contants corresponding to $n_1$ and $n_2$, the numbers of primary and secondary binding sites, respectively. When the binding of SIX as well as SA was examined over a wide range of concentration, a curved Scatchard plot was also obtained as shown in Fig. 6, and its binding parameters were: $n_1=1.07$, $k_1=6.11\times10^4\,\mathrm{M}^{-1}$; $n_2=2.69$, $k_2=7.45\times10^2\,\mathrm{M}^{-1}$. However, in the low plasma concentration range of the drugs, such as would be obtained in clinical use, the plot obtained could be regarded as a straight line, and subsequent data analysis was done as described previously.

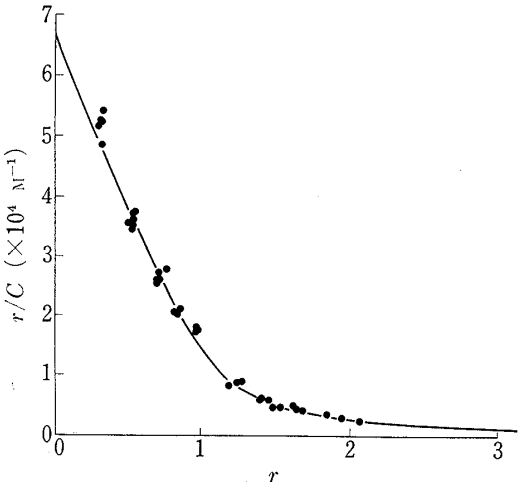



Fig. 6. Scatchard Plot for the Binding of Sulfisoxazole to $1.95\times10^{-4}$ M BSA in 1/15 M Phosphate Buffer (pH 7.4) at 37°

All points are experimental data while the solid line shows the values computed from the binding parameters.

Table II. Typical Binding Data for Sulfisoxazole (SIX) in the Presence of $1\times10^{-6}$ mol of Salicylic Acid (SA)

| $r$ | Free SIX $C$ ($\times10^{-5}$ M) | Free SA $C'$ ($\times10^{-5}$ M) |
|---|---|---|
| 0.212 | 0.815 | 3.41 |
| 0.385 | 1.76 | 3.75 |
| 0.516 | 3.03 | 3.91 |
| 0.621 | 4.30 | 4.16 |
| 0.758 | 5.72 | 4.27 |
| 1.05 | 14.2 | 4.64 |

Table III. $nk_{app}$ and $nk_{app}C'$ for the Binding of Sulfisoxazole in the Presence of Various Amounts of Salicylic Acid (SA)

| SA added ($\times10^{-6}$ mol) | $nk_{app}$ ($\times10^4\,\mathrm{M}^{-1}$) | $C'$[a] ($\times10^{-5}$ M) | $nk_{app}C'$ |
|---|---|---|---|
| 0 | 5.99 | 0 | 0 |
| 0.5 | 4.58 | 1.82 | 0.834 |
| 1.0 | 3.52 | 3.98 | 1.40 |
| 2.0 | 2.23 | 8.77 | 1.96 |
| 3.0 | 1.71 | 14.4 | 2.46 |
| 4.0 | 1.21 | 20.0 | 2.42 |
| 5.0 | 1.15 | 25.9 | 2.98 |
| 6.0 | 0.981 | 32.0 | 3.14 |

a) Means of experimental values.

Typical binding data for SIX in the presence of $1\times10^{-6}$ mol of SA are shown in Table II. The value of $C'$ varied a little with the concentration of SIX. This indicates that the action of the inhibitor, SA is also inhibited reversibly by SIX. However, the variation of $C'$ was negligibly small compared with the range of the unbound drug concentration, $C$, so that we regarded $C'$ as essentially constant, and used the mean value. Double-reciprocal plots for the binding of SIX in the presence of $0.5\times10^{-6}$—$6\times10^{-6}$ mol of SA are shown in Fig. 7. The values of $nk_{app}$ and $nk_{app}\cdot C'$ derived from these treatments are listed in Table III. On

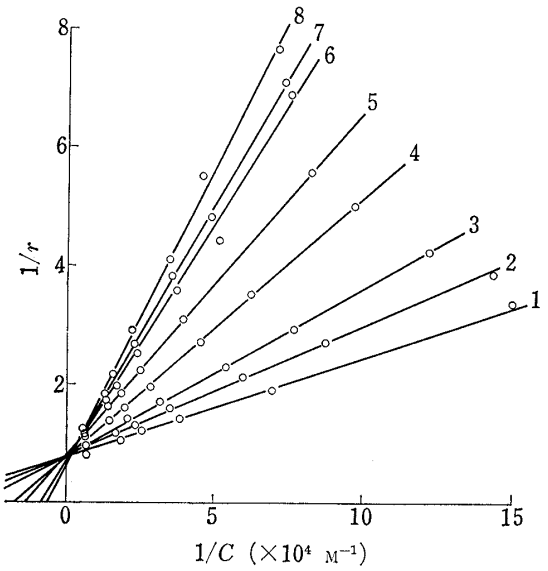



Fig. 7. The Effect of Salicylic Acid (SA) on the Binding of Sulfisoxazole (SIX) to BSA

1: SIX alone, 2: $0.5\times10^{-6}$ mol SA added,
3: $1.0\times10^{-6}$ mol SA added, 4: $2.0\times10^{-6}$ mol SA added,
5: $3.0\times10^{-6}$ mol SA added, 6: $4.0\times10^{-6}$ mol SA added,
7: $5.0\times10^{-6}$ mol SA added, 8: $6.0\times10^{-6}$ mol SA added.

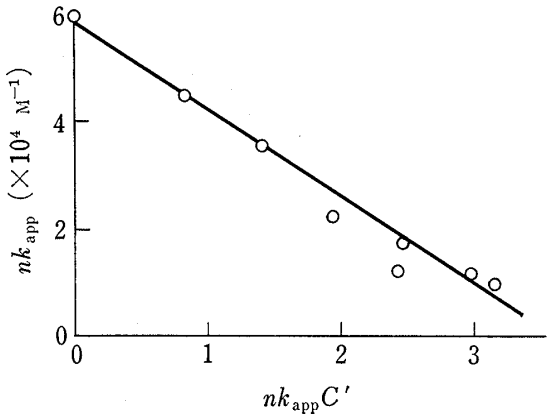



Fig. 8. Plot of $nk_{app}$ *versus* $nk_{app}C'$ for the Binding of Sulfisoxazole in the Presence of Various Amounts of Salicylic Acid

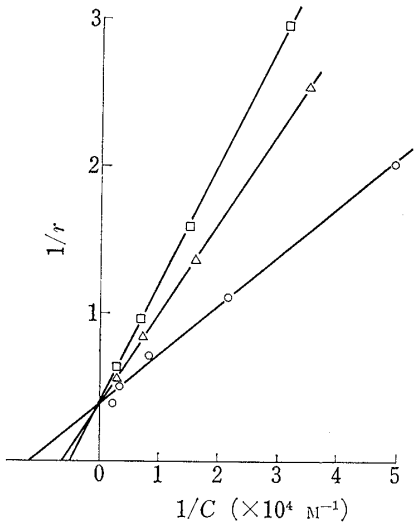



Fig. 9. The Effect of Sulfisoxazole (SIX) on the Binding of Salicylic Acid (SA) to BSA

○——○: SA alone,
△——△: $1.0\times10^{-6}$ mol SIX added,
□——□: $2.0\times10^{-6}$ mol SIX added.

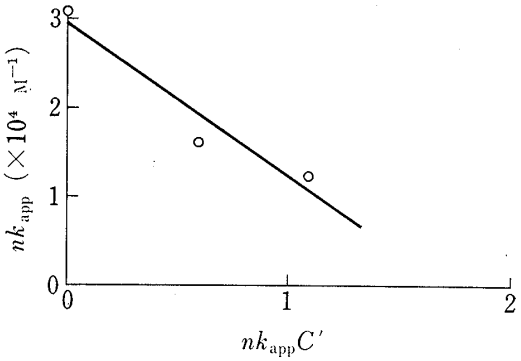



Fig. 10. Plot of $nk_{app}$ *versus* $nk_{app}C'$ for the Binding of Salicylic Acid in the Presence of Various Amounts of Sulfisoxazole

plotting these values a linear relationship between $nk_{app}$ and $nk_{app} \cdot C'$ was obtained, as shown in Fig. 8. It was thus confirmed that the binding of SIX, at least in the presence of $0.5 \times 10^{-6}$—$6 \times 10^{-6}$ mol of SA, obeys the theoretical relationship (3). On the other hand, to investigate the inhibition of SA by SIX, the binding of SA in the presence of $1 \times 10^{-6}$ mol of SIX and in the presence of $2 \times 10^{-6}$ mol of SIX was measured. Figure 9, Table IV and Fig. 10 show the data obtained, treated as described above.

Table IV. $nk_{app}$ and $nk_{app}C'$ for the Binding of Salicylic Acid (SA) in the Presence of Various Amounts of Sulfisoxazole (SIX)

| SIX added ($\times 10^{-6}$ mol) | $nk_{app}$ ($\times 10^{4}$ M$^{-1}$) | $C'$[a] ($\times 10^{-5}$ M) | $nk_{app}C'$ |
|---|---|---|---|
| 0 | 3.07 | 0 | 0 |
| 1.0 | 1.63 | 3.64 | 0.593 |
| 2.0 | 1.24 | 8.81 | 1.09 |

a) Means of experimental values.

The binding parameters for the competitive binding of SIX and SA obtained in this study are summarized in Table V. Comparison of the inhibitory constant, $k'$, with the corresponding binding constant of the inhibitor can characterize the competitive binding more clearly. As shown in Table V, the inhibitory constant of SA against the binding of SIX was $1.47 \times 10^{4}$ M$^{-1}$, and was nearly equal to its binding constant, $1.23 \times 10^{4}$ M$^{-1}$. In contrast, the value of $k'$ against the binding of SA, which was $1.70 \times 10^{4}$ M$^{-1}$, was lower than the binding constant of SIX, $4.39 \times 10^{4}$ M$^{-1}$.

Table V. Binding Parameters for the Competitive Binding of Sulfisoxazole (SIX) and Salicylic Acid (SA)

| Drug | $n$ | $k$ ($\times 10^{4}$ M$^{-1}$) | $k'$ ($\times 10^{4}$ M$^{-1}$) |
|---|---|---|---|
| SIX | 1.3 | 4.39 | 1.47 |
| SA | 2.5 | 1.23 | 1.70 |

However, in this study, the data for the inhibition of the binding of SIX by SA satisfied equation (6) well. Therefore, it was confirmed that the theoretical equation (3) is suitable to describe these competitive binding phenomena.