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Effect of Simultaneous Administration of Drugs on Absorption and Excretion. VI.[1] Effect of Protein Binding on Membrane Permeability of Sulfonamides

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Effect of protein binding on permeation of sulfonamides through a cellophane membrane was investigated by use of a dynamic dialysis technique.

The membrane permeation of sulfadimethoxine and sulfamethoxypyridazine that showed high binding to bovine serum albumin (BSA) was significantly increased by the presence of BSA in the receptor compartment, while the membrane permeation of sulfanilamide that showed little binding to BSA was not changed at all by the presence of BSA. Further the increase in the membrane permeation of sulfadimethoxine and sulfamethoxypyridazine in the presence of BSA was significantly depressed by the addition of salicylic acid. Also, salicylic acid strongly displaced sulfadimethoxine or sulfamethoxypyridazine from its protein binding sites.

These results suggest that the depressive effect of salicylic acid on the membrane permeation of sulfadimethoxine and sulfamethoxypyridazine in the presence of BSA is due to its displacing activity.

Alteration of the kinetics of penetration through membrane may result from the binding of a drug with plasma proteins, since the bound drug cannot penetrate through the membrane. Displacement of one drug from its protein binding sites by another causes the significant increase in unbound fraction of the drug. Therefore the kinetics of drug transfer across the membrane may be further affected by displacement. The purpose of the present study is to elucidate the role of drug-protein interaction with relation to gastrointestinal absorption.

Nakagaki and Terada[3] showed that permeability constant of methyl orange through a cellophane membrane greatly changed in the presence of bovine serum albumin (BSA), while permeability constant corrected for the binding of methyl orange with BSA remained constant in the presence of BSA and agreed with that of methyl orange alone. Recently, Kamada *et al.*[4] have reported that adsorption to the rat blood is one of the important physicochemical factors in the absorption of sulfonamides from the rat small intestine. Previous reports in this series[5,6] demonstrated that the acidic drugs such as salicylic acid and phenylbutazone could displace highly bound sulfonamides from the binding sites, resulting in a marked increase in the antibacterial activities of sulfonamides *in vitro* and *in vivo*.

In the present report, we studied the effect of protein binding on the diffusion of sulfonamides through a cellophane membrane, using a dynamic dialysis technique. The experimental variables which affected the kinetics of permeation of sulfonamides were also described.

Experimental

Materials——Sulfadimethoxine (Daiichi Seiyaku Co., Ltd.), sulfamethoxypyridazine (Takeda Pharmaceutical Industry Co., Ltd.), sulfisomidine (Daiichi Seiyaku Co., Ltd.), sulfanilamide (Katayama Chemical Co.,

1) Part V: Y. Imamura, K. Shigemori, and H. Ichibagase, *Chem. Pharm. Bull.* (Tokyo), **23**, 1377 (1975).
2) Location: *5–1 Oe-honmachi, Kumamoto, 862, Japan.*
3) M. Nakagaki and H. Terada, *Yakugaku Zasshi*, **88**, 1596 (1968).
4) T. Morishita, M. Yamazaki, N. Yata, and A. Kamada, *Chem. Pharm. Bull.* (Tokyo), **21**, 2309 (1973).
5) Y. Imamura and H. Ichibagase, *Yakugaku Zasshi*, **93**, 1206 (1973).
6) Y. Imamura, K. Shigemori, and H. Ichibagase, *Chem. Pharm. Bull.* (Tokyo), **22**, 2324 (1974).

Ltd.), sulfapyridine (Tokyo Kasei Kogyo Co., Ltd.), salicylic acid (J. P. VIII grade), phenylbutazone (Fujisawa Pharmaceutical Industry Co., Ltd.), aminopyrine (J. P. VIII grade), bovine serum albumin, Fraction V (Armour Pharmaceutical Co., Ltd.), seamless cellulose tubing (Visking Co., Ltd., size 27/32).

Equipments[7,8]——Figure 1 shows a schematic diagram of the system. The system is consisted of the following parts: 1) Two glass cells with the same size (5.0 cm in diameter, 12.0 cm in height). 2) The rubber stopper with the glass tubing. 3) A constant-temperature water bath. 4) Two magnetic stirrers. 5) A variable speed stirring motor. 6) A perfusion pump.

Procedures——A dialysis tubing was knotted at one end with cotton thread to form a bag and attached with a rubber band to the glass tubing. It was dried at room temperature for 90 minutes. Two hundred ml of 1/15 M phosphate buffer, pH 7.4 was placed in the two cells, which were connected to the perfusion pump with rubber tube, and immersed in a water bath ($30 \pm 0.1°$). Fifteen ml of drug solution prepared with the same buffer was placed in the bag and the rubber stopper with the bag attached was fitted on a cell. The internal and external liquid levels were adjusted to the same. The total volume of the external solution was 205 ml, since the syringe of the perfusion pump contained 5 ml of solution. The drug solution in the bag was stirred with a glass rod which was connected to a stirring motor. The external solution was stirred with a magnetic stirring bar and circulated with a perfusion pump. At suitable time intervals, 0.1 ml samples were removed from the bag for subsequent assay. The volume of the drug solution in the bag was maintained constant by adding buffer immediately after sample removal. Such a sampling method causes the problems of the mass balance and the lag time, that is, the period during which the motor and pump had to be stopped for sampling. However, preliminary experiments showed that this method had good reproducibility as compared with the one that the samples were removed from the external solution. This sampling method also has an advantage when the external solution contained BSA. The bag was changed every each experiment, since the dialysis rate was found to decrease with the repeated use of it.

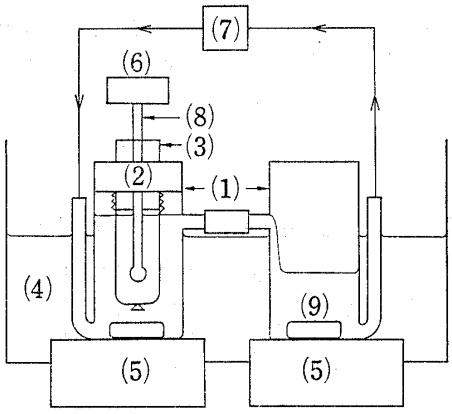

Fig. 1. Schematic Diagram of System Used for Dynamic Dialysis

(1) glass cell, (2) rubber stopper, (3) glass tubing, (4) water bath with thermostated heater, (5) magnetic stirrer, (6) stirring motor, (7) perfusion pump, (8) glass rod for stirring, (9) magnetic stirring bar

Equilibrium Dialysis Experiment——Dialysis experiment was carried out as described previously.[9] The binding constant and the number of binding sites for sulfonamides were calculated according to the method of Klotz.[10]

Analytical Methods——Sulfonamides were analyzed by the method of Bratton and Marshall.[11] Salicylic acid, phenylbutazone, and aminopyrine were assayed spectrophotometrically at 303, 265, and 265 mμ, respectively.

Theoretical

If there is a linear fall of concentration within the membrane, flux of solute across unit area, J is given by[12]

$$J = D\frac{C_1 - C_2}{h} \tag{1}$$

where D is the diffusion constant, C_1 and C_2 the concentrations of solute in the donor and receptor compartments, respectively and h the effective thickness of membrane. Eq. (1) may be rewritten as

$$-\frac{dC_1}{dt} = \frac{A \cdot D}{V_1 \cdot h}(C_1 - C_2) = K(C_1 - C_2) \tag{2}$$

where V_1 is the volume of donor compartment, A the effective area of membrane, and K the dialysis rate constant. Integration of Eq. (2) leads to

7) M.C. Meyer and D.E. Guttman, *J. Pharm. Sci.*, **57**, 1627 (1968).
8) S. Goto, T. Ohki, S. Kiryu, and S. Iguchi, *Yakuzaigaku*, **31**, 247 (1971).
9) H. Ichibagase, Y. Imamura, A. Kinoshita, and S. Kojima, *Chem. Pharm. Bull.* (Tokyo), **20**, 947 (1972).
10) I.M. Klotz, "The Protein," Vol. 1, part B, ed. by H. Neurath, K. Bailey, Academic Press, New York, 1953, p. 727.
11) A.C. Bratton and E.K. Marshall, *J. Biol. Chem.*, **128**, 539 (1939).
12) G.L. Flynn, S.H. Yalkowsky, and T.J. Roseman, *J. Pharm. Sci.*, **63**, 479 (1974).

$$\log \frac{C_1-C_2}{C_1^0-C_2^0} = -\frac{V_1+V_2}{2.303V_2}K\cdot t \tag{3}$$

where C_1^0 and C_2^0 are the respective concentrations of the two compartments at $t=0$, and V_2 is the volume of receptor compartment.

Eq. (2) may be rewritten as

$$-\frac{dC_1}{dt} = K\cdot\left(1-\frac{C_2}{C_1}\right)\cdot C_1 = K\cdot F(t)\cdot C_1 \qquad (0<F(t)<1) \tag{4}$$

where $F(t)$ is the dialysis efficiency coefficient. If $F(\phi)$ is relatively invariant with ϕ, the slope of ln C_1 *versus* t is given by[13]

$$K_{app} = K\cdot F(\phi) \qquad (0<\phi<t) \tag{5}$$

where K_{app} is an apparent first order rate constant.

In this report, we analyzed experimental data using Eq. (3) and Eq. (5).

Results and Discussion

Effect of Experimental Conditions on the Dialysis Rate Constant, K

1. Stirring Rate of Internal Solution——The dialysis of sulfisomidine was carried out at 0, 120, 222, and 366 rpm. The results are illustrated in Fig. 2. The rate constant increased with the rate of stirring and approached a constant value over about 300 rpm. A similar observation was reported by Ginzburg and Katchalsky,[14] and Goto, *et al.*[8] The observed results may be explained on the basis of the existence of the unstirred layer adjacent to the membrane.[15] It is assumed that the thickness of the unstirred layer decreases with an increase of the rate of stirring and approaches a limiting value over about 300 rpm.

2. Temperature——The rate constants for sulfisomidine and salicylic acid at 10, 20, 30, and 40° were found to be as follows. Sulfisomidine; 0.724, 0.988, 1.27, and 1.54×10^{-2} min^{-1}: Salicylic acid; 1.14, 1.76, 2.36, and 3.00×10^{-2} min^{-1}, respectively. The results indicate that the temperature control is an important factor in determining the membrane permeability of drugs as pointed out by Meyer and Guttman.[16] Plots according to the Arrhenius equation are shown in Fig. 3. The activation energies calculated from the slope determined by least squares were 4.42 kcal/mole for sulfisomidine, and 5.63 kcal/mole for salicylic acid.

3. Initial Concentration——The dialysis rate constant of sulfisomidine or salicylic acid was essentially invariant with changes in initial concentration. The results are follows. Initial concentration of drug designated in parentheses. Sulfisomidine: 0.0127 min^{-1} (200 μg/ml), 0.0124 min^{-1} (1000 μg/ml), 0.0129 min^{-1} (2000 μg/ml); Salicylic acid: 0.0235 min^{-1} (200 μg/ml), 0.0236 min^{-1} (2000 μg/ml).

4. Flowing Rate of External Solution——The flow of the external solution was adjusted to deliver 11.5 ml/min or 58.0 ml/min. The rate constants for sulfisomidine were 0.0122 min^{-1} and 0.0127 min^{-1}, respectively. The difference of the two rate constants was not significant, but the flowing rate of 58.0 ml/min had good reproducibility.

5. pH of Solution——It was pointed out by Yamazaki, *et al.*[17] that the permeability for unionized sulfonamides was about 10% greater than that for ionized one, but no data was presented. To clarify the effect of pH on the dialysis rate constant of sulfisomidine, the dialysis was conducted at pH 6.2, 7.4, and 8.4. When both the unionized and ionized form are assumed to penetrate the membrane, dialysis rate constant may be given by

13) A. Suzuki, W.I. Higuchi, and N.F.H. Ho, *J. Pharm. Sci.*, **59**, 645 (1970).
14) B.Z. Ginzburg and A. Katchalsky, *J. Gen. Physiol.*, **47**, 403 (1963).
15) G.L. Flynn, O.S. Carpenter, and S.H. Yalkowsky, *J. Pharm. Sci.*, **61**, 312 (1972).
16) M.C. Meyer and D.E. Guttman, *J. Pharm. Sci.*, **59**, 33 (1970).
17) M. Yamazaki, M. Aoki, A. Kamada, and N. Yata, *Yakuzaigaku*, **27**, 37 (1967).

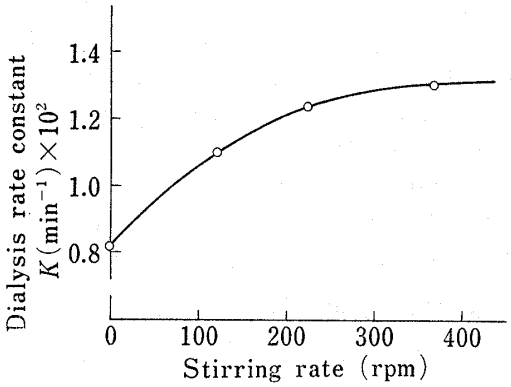

Fig. 2. Dialysis Rate Constant, K, of Sulfisomidine as a Function of Stirring Rate

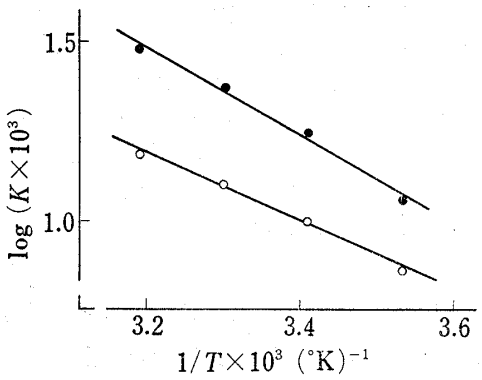

Fig. 3. Arrhenius Plots for Permeation of Sulfisomidine and Salicylic Acid through a Cellophane Membrane

—○—: sulfisomidine
—●—: salicylic acid

$$K = K_u f_u + K_i f_i \tag{6}$$

where K_u and K_i are the dialysis rate constant of unionized and ionized form, respectively, and f_u and f_i are the fraction of unionized and ionized form, respectively. Eq. (6) can be rewritten

$$K = K_i + f_u(K_u - K_i) \tag{7}$$

in which f_u has been substituted for f_i, since $f_i = 1 - f_u$. Therefore a plot of K *versus* f_u should yield a straight line with a slope of $K_u - K_i$ and intercept of K_i. The ratio of K_u/K_i can be calculated from slope and intercept. Plots according to Eq. (7) for permeation of sulfisomidine are shown in Fig. 4. The value of K_u and K_i were 1.44×10^{-2} and 1.16×10^{-2} min^{-1}, respectively, and the ratio of K_u/K_i was 1.24. The lower rate constant for ionized sulfisomidine may be due to the greater degree of interaction with the solvent as discussed by Withington and Collet.[18] They observed the similar effect of pH on the dialysis rate constant of salicylic acid through a cellophane membrane. Meyer and Guttman[16] also found that the dialysis rate of phenol red through a cellophane membrane was affected by the pH of solution.

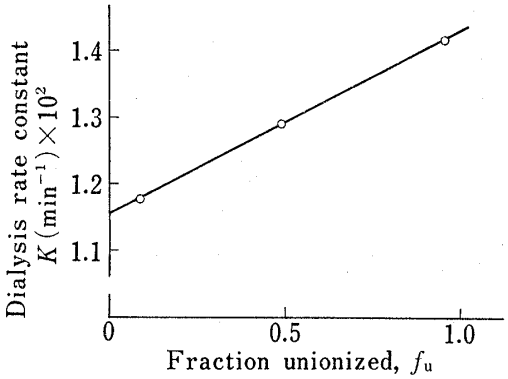

Fig. 4. Plots of the Dialysis Rate Constant, K, of Sulfisomidine *versus* the Fraction, f_u, of Drug Unionized

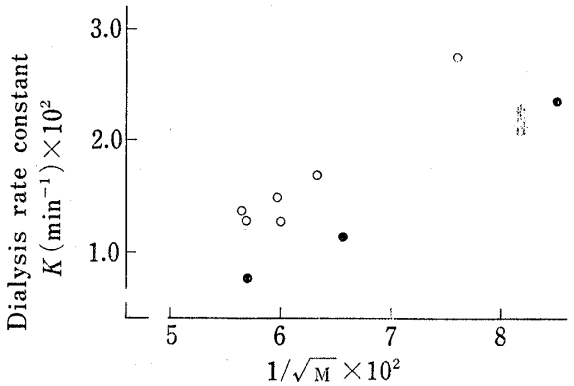

Fig. 5. Plots of Dialysis Rate Constant, K, *versus* the Reciprocal Square Root of Molecular Weight, $1/\sqrt{M}$, of Drug

○: sulfonamides
●: salicylic acid, aminopyrine and phenylbutazone

18) R. Withington and J.H. Collet, *J. Pharm. Pharmacol.*, **25**, 273, (1973).

6. Molecular Weight——Plots of the dialysis rate constant, K *versus* the reciprocal of square root of molecular weight are given in Fig. 5. Obviously high molecular weight drugs penetrated through the membrane more slowly than those of lower molecular weight. Some compounds, however, penetrated through membrane more slowly than expected. It may be due to the difference in diffusional size as discussed by Craig.[19]

In the subsequent experiments, the internal solution was stirred at 366 rpm and the external solution was stirred at a constant rate and circulated at 58.0 ml/min. Initial concentration of drug was 200 μg/ml and pH of solution was 7.4 and temperature was 30°.

Effect of the Protein Binding on an Apparent First Oder Rate Constant, K_{app}

The representative data for sulfadimethoxine is shown in Fig. 6. In the presence of BSA, Eq. (3) was inapplicable to our study, since the slope increased with time as shown in the figure. Such a deviation from linearity was also observed for sulfamethoxypyridazine, sulfisomidine, salicylic acid, and phenylbutazone, but not for sulfanilamide and aminopyrine. On the other hand, the plots according to Eq. (5) were found to be linear both in the absence and in the presence of BSA, when the dialysis was conducted up to 50 to 150 minutes for each drug. Accordingly, we used the Eq. (5) instead of Eq. (3) and compared the apparent first order rate constant (K_{app}) of drug in the presence of BSA with that in the absence of BSA.

When BSA was contained in the external solution, K_{app} of sulfadimethoxide, sulfamethoxypyridazine, and sulfisomidine were significantly increased as compared with their control values. However, the rate constant of sulfanilamide was not affected by the presence of BSA. In addition, the membrane permeation of salicylic acid, phenylbutazone, and aminopyrine was studied both in the absence and in the presence of BSA. While K_{app} of salicylic acid and phenylbutazone that showed high binding to BSA was significantly increased as compared with their control values, the rate constant of aminopyrine that showed little binding to BSA was not changed at all in the presence of BSA. These results are summarized in Table I.

In order to elucidate the mechanism of BSA effect on K_{app} of sulfonamides, the percentage of bound sulfonamide in the presence of BSA was calculated with the following equation.

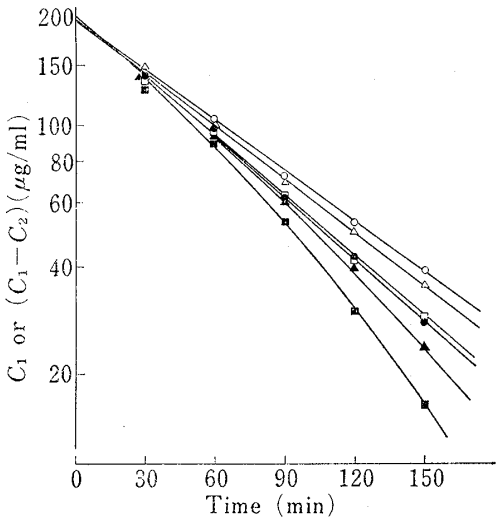

Fig. 6. Plots of Logarithms of C_1 or (C_1-C_2) *versus* Time of Dialysis for Sulfadimethoxine

(○,●) sulfadimethoxine alone, (□,■) sulfadimethoxine in the presence of BSA, (△,▲) sulfadimethoxine with salicylic acid in the presence of BSA,; (○,□,△) according to Eq. (5), (●,■,▲) according to Eq. (3).

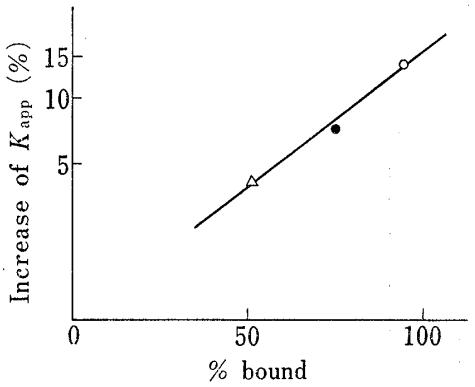

Fig. 7. Effect of Binding of Sulfonamides with BSA on the Apparent First Order Rate Constant, K_{app}

○: sulfadimethoxine
●: sulfamethoxypyridazine
△: sulfisomidine

19) L.C. Craig, *Science*, 144, 1093 (1964).

Table I. Effect of BSA[a] on Drug Permeation through a Cellophane Membrane

Drugs	K_{app} (min^{-1}) $\times 10^2$	P values
Sulfanilamide	2.42 ±0.074	
Sulfanilamide+BSA	2.40 ±0.083	N.S.[b]
Sulfisomidine	1.17 ±0.025	
Sulfisomidine+BSA	1.22 ±0.041	$P<0.05$
Sulfamethoxypyridazine	1.36 ±0.015	
Sulfamethoxypyridazine+BSA	1.46 ±0.023	$P<0.001$
Sulfadimethoxine	1.12 ±0.024	
Sulfadimethoxine+BSA	1.28 ±0.036	$P<0.001$
Aminopyrine	1.07 ±0.013	
Aminopyrine+BSA	1.06 ±0.001	N.S.[b]
Phenylbutazone	0.720±0.001	
Phenylbutazone+BSA	0.769±0.016	$P<0.02$
Salicylic acid	2.13 ±0.039	
Salicylic acid+BSA	2.38 ±0.017	$P<0.01$

a) concentration of BSA: 1.0% (w/v)
b) not significant

$$\beta = \frac{P_t+1/nK+D_t/n-\sqrt{(P_t+1/nK+D_t/n)^2-4(D_t/n)P_t}}{2D_t/n} \quad (8)$$

where β is per cent bound, P_t the total molar concentration of BSA, D_t the total molar concentration of sulfonamides, n the number of binding sites, and K the binding constant. Meyer and Guttman[20] pointed out that β is a useful characterization of intrinsic binding strength only when it approaches a maximum value as follows.

$$\beta = \frac{1}{1+1/nKP_t} \quad (9)$$

There was a good agreement between values obtained from calculation by Eq. (8) and Eq. (9). The calculated values are summarized in Table II. A plot of mean values of per cent bound sulfonamides *versus* logarithms of per cent increase of K_{app} was found to give a linear relationship as shown in Fig. 7. These results indicate that the increase of K_{app} in the presence of BSA is due to the binding of a drug with BSA. If the effect of BSA on K_{app} of drug was due to either change of the viscosity of the solution or the osmotic pressure of the solution, K_{app} of all the drugs will be affected more or less by the presence of BSA. However, K_{app} of sulfanilamide and aminopyrine that showed little binding to BSA were not affected by the presence of BSA.

Table II. Characteristics of Binding of Sulfonamides to BSA

Sulfonamides	n[a]	K (M^{-1})[b]	β[c] $\times 100$ (%)	
			Calculated with Eq. (8)	Calculated with Eq. (9)
Sulfanilamide	2.0	1.97×10^2	5.4— 6.5	5.3
Sulfisomidine	1.9	4.09×10^3	50.5—51.8	52.4
Sulfamethoxypyridazine	1.7	1.38×10^4	74.4—76.1	76.8
Sulfadimethoxine	1.7	8.14×10^4	94.3—94.9	95.1

a) number of binding sites
b) binding constant
a,b) The values of n and K were determined by the equilibrium dialysis method. The dialysis experiment was carried out in 1/15M phosphate buffer, pH 7.4, ionic strength 0.267, temperature 5—7°.
c) fraction bound

20) M.C. Meyer and D.E. Guttman, *J. Pharm. Sci.*, **57**, 895 (1968).

Dialysis efficiency coefficient, $F(t)$, is always changing with time as shown in Fig. 8. Rate constant, K_{app}, estimated graphically was, however, apparently constant with time in early period, since it was less sensitive to time than calculated value as pointed out by Suzuki, *et al.*[13] The value of $F(t)$ in the presence of BSA was much closer to unity than control value for sulfadimethoxine or sulfamethoxypyridazine, but it was almost the same as control one for sulfanilamide. This may be reflected in determination of K_{app}.

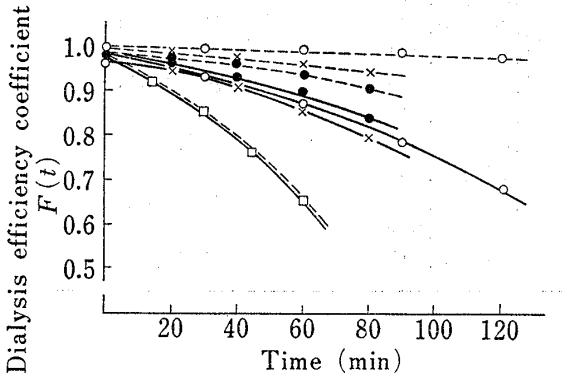

Fig. 8. Change in the Dialysis Efficiency Coefficient, $F(t)$, with Time

○: sulfadimethoxine
×: sulfamethoxypyridazine
●: sulfisomidine
□: sulfanilamide
——: $F(t)$ in the absence of BSA
------: $F(t)$ in the presence of BSA, 1.0% (w/v)

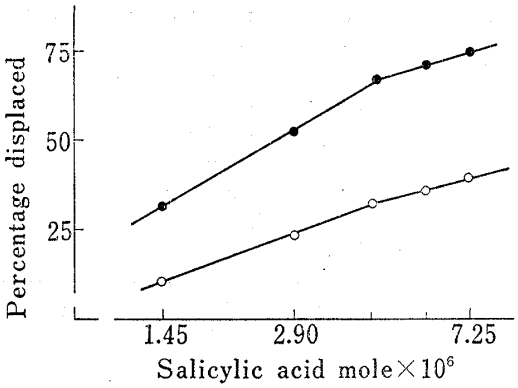

Fig. 9. Effect of Salicylic Acid on Binding of Sulfadimethoxine and Sulfamethoxypyridazine with BSA

initial concentration of sulfonamides: 2×10^{-6} mole
concentration of BSA: 1% (w/v)
○: sulfadimethoxine
●: sulfamethoxypyridazine

In the absence of BSA, control experiments were carried out to confirm the effect of salicylic acid on K_{app} of sulfonamides. As shown in Table III, K_{app} of sulfadimethoxine, sulfamethoxypyridazine, and sulfanilamide was not affected by salicylic acid.

In the presence of BSA, the increase in K_{app} of sulfadimethoxine and sulfamethoxypyridazine described above was significantly depressed by salicylic acid, while K_{app} of sulfanilamide was not influenced (see Table III). Fig. 9 shows that the binding of sulfadimethoxine or sulfamethoxypyridazine with BSA is strongly inhibited by salicylic acid. Further, in the dynamic dialysis experiment, concentration of total salicylic acid in the receptor compartment was found to be more than ten times that of total sulfadimethoxine or sulfamethoxypyridazine. These results suggest that the depressive effect of salicylic acid on K_{app} in the presence of BSA is due to its displacing activity.

Table III. Effect of Salicylic Acid on Permeation of Sulfonamides through a Cellophane Membrane both in the Absence and in the Presence of BSA

Sulfonamides	K_{app} (min^{-1}) $\times 10^2$		P values[c]
	Control[a]	Salicylic acid[b]	
Sulfanilamide	2.41±0.074	2.41±0.093	N.S.[e]
BSA[d]	2.40±0.083	2.39±0.089	N.S.
Sulfamethoxypyridazine	1.36±0.015	1.37±0.020	N.S.
BSA	1.46±0.023	1.40±0.021	$P<0.02$
Sulfadimethoxine	1.12±0.024	1.10±0.033	N.S.
BSA	1.28±0.036	1.19±0.013	$P<0.02$

a) sulfonamides alone
b) with salicylic acid, initial concentration: 2000 μg/ml
c) compared to control
d) in the presence of BSA, 1.0% (w/v)
e) not significant

Studies are in progress to evaluate the possibility of correlation between the *in vitro* finding and *in vivo* situation.