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Protein Bindings. III.[1] Binding of Sulfonamides to Bovine Serum Albumin

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Binding constant, K, for 19 sulfonamides with bovine serum albumin was evaluated spectrophotometrically utilizing the competition between 2-(4′-hydroxyphenylazo)-benzoic acid and sulfonamides. The values of log K well correlated with degree of the decrease in *in vitro* bacteriostatic activities by the addition of bovine serum. A linear relationship was also observed between log K and $p\mathrm{K_a}$ for the sulfonamides. The relationships between log K and *in vitro* bacteriostatic activities against *Escherichia coli* 0-55 and *Staphylococcus aureus* 209P were expressed with parabolic curves, which indicated the optimum values of log K being 3.5—4.5 for the potent sulfonamides.

Binding of drugs to serum albumin produces a number of divergent biological effects.[3] Especially since several long-acting sulfonamides have recently appeared, the meaning and importance of blood concentration of the drugs have been discussed and the binding of sulfonamides to serum albumin has become a subject of considerable interest.

Numerous studies have been made on the binding by using equilibrium dialysis[4,5] or ultrafiltration.[6,7] But compared with these methods, a new spectrophotometric method using 2-(4′-hydroxyphenylazo)benzoic acid (HABA) reported in the previous paper[1] is simple in the procedure, rapid in the measurement, free from the trouble caused by the adsoprtion of components in the test solution on the semipermeable membrane, and exceeds in reproducibility. In this work, the new method was utilized for estimating the binding constant for 19 sulfonamides, and correlations of the constant with physico-chemical and biological properties of the sulfonamides were investigated.

Assuming that the binding data of sulfonamides to bovine serum albumin are represented by a modified Langmuir-type equation[8] with the same values of m, a parameter dependent on the experimental conditions, and n, the number of binding sites on a single molecule of albumin, as those in HABA-albumin binding, and that sulfonamides and HABA compete for the same binding sites on albumin molecules, logarithm of the binding constant for sulfonamides may be expressed as[1]

$$\log K=\log K_A+\log\{(a-x)/(b-y)\}+(1/m)\log(y/x) \qquad (1)$$

where K and K_A represent the intrinsic binding constant for sulfonamide and HABA to each site on albumin molecules, and a and b the initial concentrations of HABA and sulfonamide, respectively, and x, the concentration of bound HABA, and y, the concentration of bound sulfonamide, may be given by Eqs. (2)[8] and (3),[1] respectively.

1) Part II: I. Moriguchi, *Chem. Pharm. Bull.* (Tokyo), **16**, 597 (1968).
2) Location: *Takadaminami-cho, Toshima-ku, Tokyo*; *a*) Present address: *School of Pharmaceutical Sciences, Showa University, Hatanodai, Shinagawa-ku, Tokyo.*
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$$x=\Delta E/\Delta\varepsilon d \qquad (2)$$

$$y=np-(\Delta E/\Delta\varepsilon d)\{1+1/K_A{}^m(a-\Delta E/\Delta\varepsilon d)^m\} \qquad (3)$$

In these equations, ΔE is the difference between the absorbances at 482 mμ of HABA in the presence and the absence of albumin, $\Delta\varepsilon$ the difference between the molar extinction coefficients at 482 mμ of bound and unbound HABA, and p the total concentration of albumin. Hence the values of the binding constant for sulfonamides with serum albumin can be obtained by measurement of the values of ΔE.

Fig. 1 shows ΔE values against the concentrations of 19 sulfonamides added to a solution containing bovine serum albumin and HABA. The extents of the reversion of the albumin-induced spectral change of HABA increase with the increasing concentrations of the sulfonamides added. This may suggest the occurence of competition between HABA and sulfonamides for binding sites on albumin molecules.

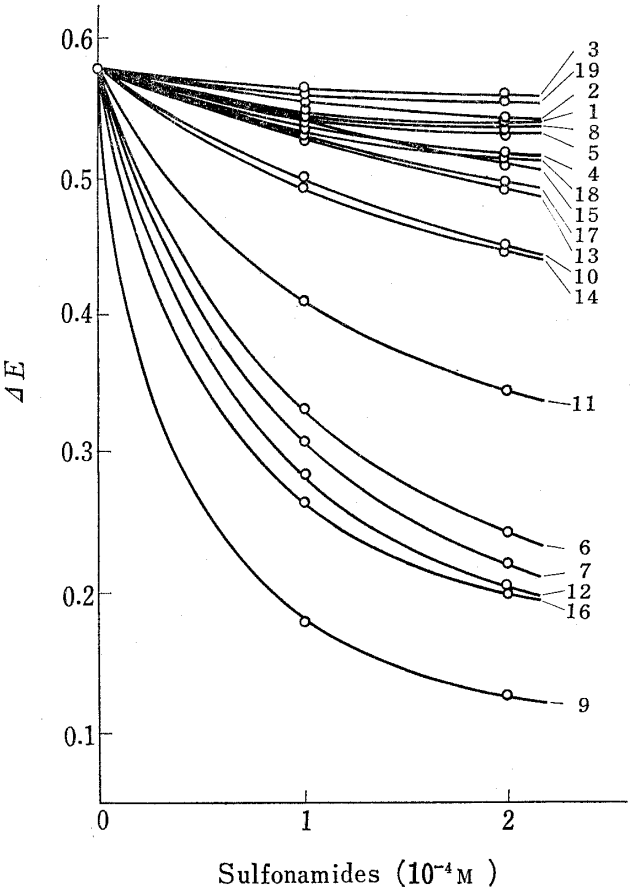

Fig. 1. ΔE of HABA–Albumin Solutions with Varying Concentration of Sulfonamides

1×10^{-4}M HABA and 5×10^{-5}M bovine serum albumin with 0—2×10^{-4}M sulfonamides in 0.15 M tris. buffer solution at pH 7.4 and 37°.
For numbering, see Table 1.

The values of log K for the sulfonamides were obtained from the values of ΔE by Eqs. (1), (2), and (3) utilizing the calculation curves in Fig. 1 of the previous report.[1] They are listed in Table I, together with other data estimated by using equilibrium dialysis or ultrafiltration for comparison. Table I indicates that the values of log K are nearly constant irrespective of the initial concentration of the sulfonamides and are similar to the other data listed together. This may justify the procedure and the assumptions involved in the spectrophotometric method.

The relations of the log K–value so estimated to pK_a, half-life for elimination from blood plasma, and *in vitro* bacteriostatic activities and their decrease by addition of serum were investigated with the sulfonamides.

Correlation between log K and pK_a values for sulfonamides is shown in Fig. 2. Coefficient of the correlation is −0.707 (18 samples); the correlation is highly significant at the 1-percent level. This may support the general view[3] that electrostatic force is dominant in the binding of acidic compounds to serum albumin.

Fig. 3 shows the correlation of log K with half-life for elimination from human blood plasma of sulfonamides. Coefficient of the correlation was 0.412 (11 samples); the correlation was not significant.

Relations between log K and bacteriostatic activities of sulfonamides were investigated. The antibacterial activities were tested *in vitro* against gramm-negative *Escherichia coli 0-55* and gramm-positive *Staphylococcus aureus 209P*. The relationships were generally

TABLE I. log K–Values[a] for Sulfonamides with Bovine Serum Albumin

No.	Sulfonamides	1×10^{-4} M[b]	2×10^{-4} M[b]	Average	Other Data[c]
1	N^1–Phenyl–sulfanilamide	2.78	2.91	2.8	
2	N^1–*p*–Methoxyphenyl–sulfanilamide	2.72	2.87	2.8	
3	N^1–*o*–Tolyl–sulfanilamide	2.72	2.46	2.6	
4	N^1–*m*–Tolyl–sulfanilamide	3.17	3.15	3.2	
5	N^1–*p*–Tolyl–sulfanilamide	3.19	2.96	3.1	
6	Xyloyl–sulfanilamide	4.73	4.61	4.7	
7	Sulfamethoxypyridazine	4.86	4.76	4.8	4.0—5.3
8	Sulfadiazine	2.87	2.91	2.9	2.9—3.9
9	Sulfadimethoxine	5.64	5.24	5.4	4.5—5.4
10	Sulfamerazine	3.66	3.67	3.7	3.3—4.5
11	Sulfamethizole	4.29	4.23	4.3	
12	Sulfamethomidine	4.99	4.83	4.9	
13	Sulfaphenazole	3.26	3.37	3.3	4.1—5.0
14	Sulfisomezole	3.72	3.69	3.7	3.2—3.5
15	Sulfisomidine	3.05	3.21	3.1	3.9—4.2
16	Sulfisoxazole	5.08	4.86	5.0	3.7—4.8
17	Sulfamonomethoxine	3.34	3.32	3.3	3.4—4.2
18	Sulfathiazole	3.17	3.12	3.1	3.1—3.8
19	Sulfanilamide	2.83	2.61	2.7	2.6—3.2

a) In 0.15 M tris. buffer solution at pH 7.4 and 37°. Unit of K is liter/Avogadro number of binding sites on albumin.
b) Initial concentration of sulfonamides.
c) Calculated from the data of the following literatures. They were estimated by using equilibrium dialysis or ultrafiltration, and including the binding with human serum albumin.
B.D. Davis, *Science*, **95**, 78 (1942); *idem*, *J. Clin. Invest.*, **22**, 753 (1943); P. Bünger, W. Diller, J. Führ, and E. Krüger-Thiemer, *Arzneimittel-Forsch.*, **11**, 247 (1961); J. Rideder, *ibid.*, **13**, 81 (1963); W. Scholtan, *ibid.*, **14**, 348 (1964); M. Nakagaki, N. Koga, and H. Terada, *Yakugaku Zasshi*, **83**, 586 (1963); and *idem*, *ibid.*, **84**, 516 (1964).

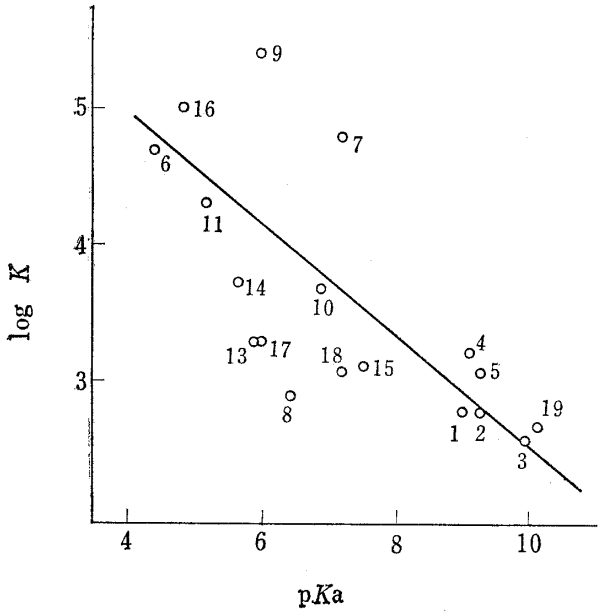

Fig. 2. Correlation between log K and pK_a[a] for Sulfonamides

For numbering, see Table I.
a) P.H. Bell and R.O. Roblin, Jr., *J. Am. Chem. Soc.*, **64** 2905 (1942); M, Yoshioka, K. Hamamoto, and T. Kubota, *Bull. Chem. Soc. Japan*, **35**, 1723 (1962); *idem Yakugaku Zasshi*, **84**, 90 (1964); J. Rieder, *Arzneimittel Forsch.*, **13**, 81 (1963).

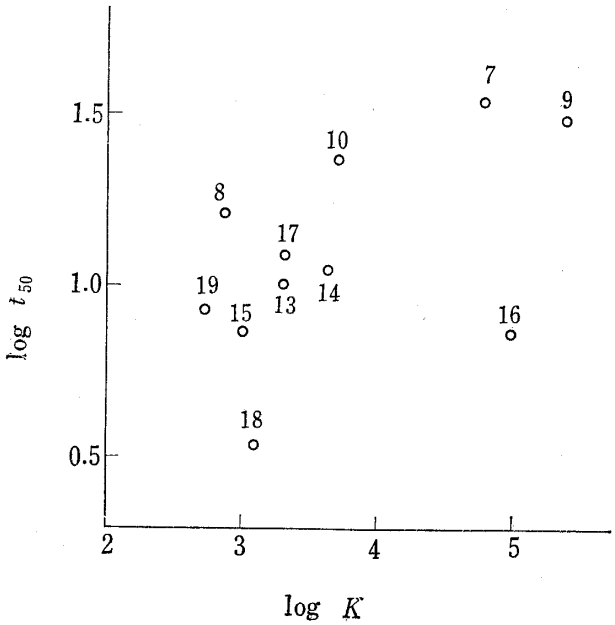

Fig. 3. Correlation of log K with Half-Life[a] (t_{50}, in hr) for Elimination from Human Blood Plasma of Sulfonamides

For numbering, see Table I.
a) J. Rieder, *Arzneimittel-Forsch.*, **13**, 81 (1963).

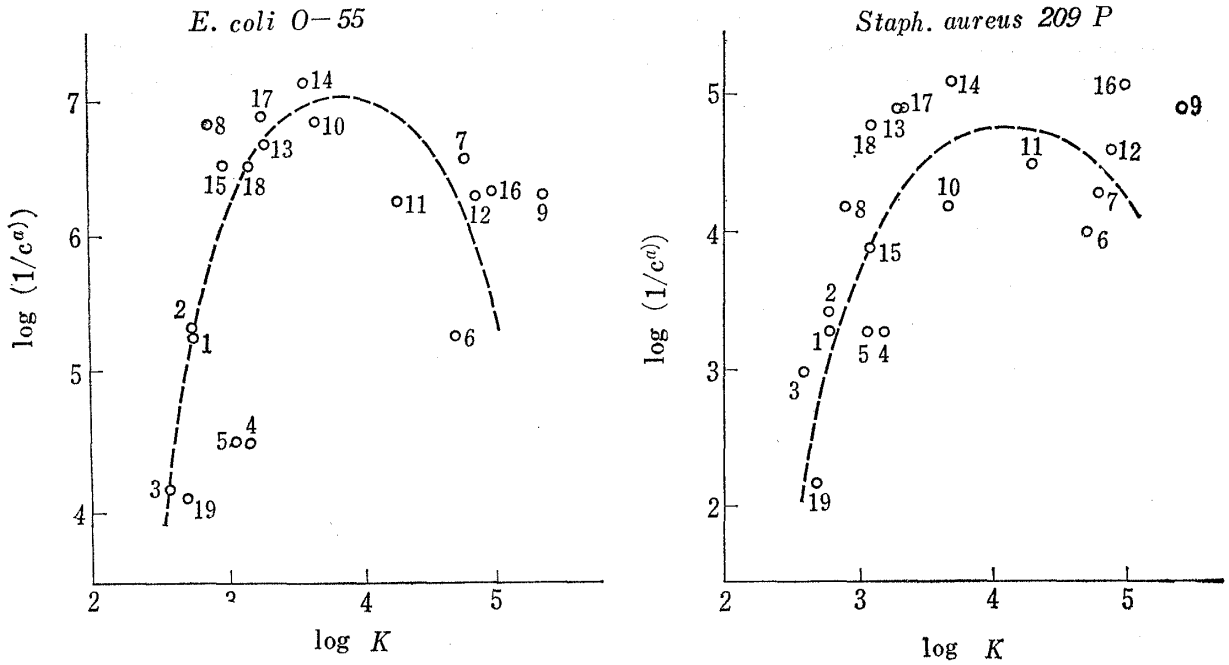

Fig. 4. Relationships between log K and *in vitro* Bacteriostatic Activities against *Escherichia Coli* 0–55 and *Staphylococcus Aureus* 209 P

For numbering, see Table I.
a) c is the minimum bacteriostatic concentration in M.

expressed with parabolic curves as shown in Fig. 4. From this it may be supposed that too high affinities between sulfonamides and proteins prevent the drugs from reaching their target sites of action in bacteria, and that too low affinities does not enable the drugs to bind so intimately with the sites of action on enzyme proteins or the like to reveal their bacteriostatic action, assuming that affinity of sulfonamides to serum albumin is in parallel with affinities to proteins in bacteria.

Fig. 5 shows correlations between log K and decrease in *in vitro* bacteriostatic activities of sulfonamides against *Escherichia coli* 0–55 and *Staphylococcus aureus* 209 P by the addition of bovine serum. Coefficients of the correlations were 0.761 and 0.805 (19 samples) in case

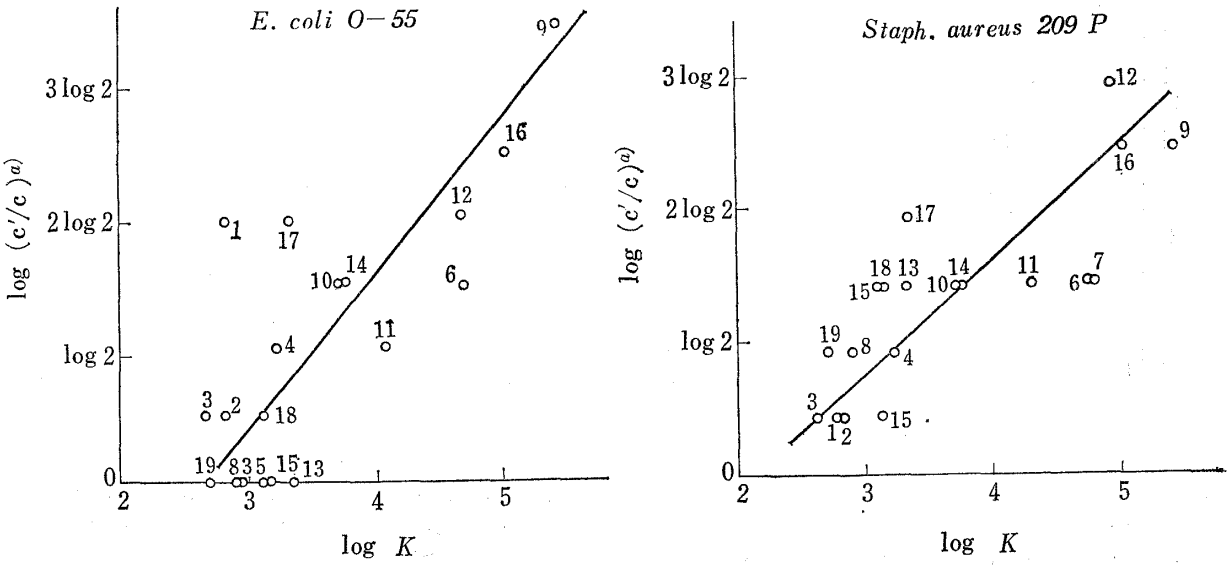

Fig. 5. Correlations between log K and Degree of Decrease in *in vitro* Bacteriostatic Activities of Sulfonamides against *Escherichia Coli* 0–55 and *Staphylococcus Aureus* 209 P by Addition of Bovine Serum

For numbering, see Table I.
a) c and c' are the minimum bacteriostatic concentrations in M in the media without and with bovine serum, respectively.

of the *E. coli* and the *Staph. aureus*, respectively; the correlations were highly significant at the 1-percent level. This may support the general view[3] that drugs bound to plasma proteins are pharmacologically inactive.

Experimental

Materials——Commercial products for medical use were used after recrystallization for the following 12 sulfonamides: xyloylsulfanilamide (Geigy–Hujisawa Yakuhin Kogyo Co.), sulfamethoxypyridazine (Japan Lederle Co.), sulfadiazine and sulfadimethoxine (Chugai Seiyaku Co.), sulfamerazine and sulfamethomidine (Tanabe Seiyaku Co.), sulfamethizole (Eizai Co.), sulfaphenazole and sulfisomidine (Dainihon Seiyaku Co.), sulfisomezole (Shionogi Seiyaku Co.), sulfisoxazole (Yamanouchi Seiyaku Co.), and sulfamonomethoxine (Daiichi Seiyaku Co.). Sulfathiazole and sulfanilamide were of Guaranteed Reagent Grade, Tokyo Kasei Kogyo Co. Five N^1-arylsulfanilamides were synthesized by the usual method.[9] All other materials were used as previously described.[1] All the test solutions for photometry were prepared with 0.15 M tris. buffer solution of pH 7.4.

Measurement of ΔE——Optical absorption (E') of the solutions containing 1×10^{-4} M HABA and 5×10^{-5}M bovine serum albumin with 0, 1×10^{-4} M, and 2×10^{-4} M sulfonamides, absorption (E) of the solution of 1×10^{-4} M HABA, and absorption (E'') of 5×10^{-5} M bovine serum albumin solution were measured at 482 mμ as previously described.[1] The values of ΔE were calculated as

$$\Delta E=E'-(E+E'')$$

Test of *in vitro* Bacteriostatic Activities——Bacteriostatic activities of sulfonamides were tested against *Escherichia coli* 0-55 in a modified Koser's medium[10] without[11] L-tryptophan and against *Staphylococcus aureus* 209 P in a modified Kuwabara's medium[12] without[11] choline, base components of nucleic acids, folic acid, biotin, riboflavin, calcium pantothenate, pyridoxine, Mg^{2+}, and Ca^{2+}. For the test in the presence of serum, calf serum prepared by Chiba Kessei Kenkyujo was used 5% and 7% to the media against the *E. coli* and the *Staph. aureus*, respectively. In all cases the minimum bacteriostatic concentrations of the sulfonamides were estimated from the turbidity of the test solutions incubated for 24 hours at 37°.

Acknowledgement The authors are very grateful to Professors Z. Tamura and M. Tsuboi of the University of Tokyo for valued criticisms and advices, and to Dr. Akiba, Director, and Dr. H. Sano, Chief Member of these Laboratories, for continuing encouragement.

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10) S.A. Koser and F. Saunders, *J. Infectious Disease*, **56**, 305 (1935).
11) These omissions of some components from the media were recommended by Dr. Oya of these Laboratories.
12) S. Kuwabara, *Shindan to Chiryo*, **48**, 114 (1964).