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Protein Bindings. VI.[1] Binding of Phenols to Bovine Serum Albumin

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Binding constant, K, for 23 phenols with bovine serum albumin was evaluated spectrophotometrically utilizing the albumin-induced metachromasia of 2-(4′-hydroxyphenylazo)-benzoic acid. In the binding, electrostatic force seems dominant but hydrophobic binding may not be negligible with 2,4-dichlorophenol and 2,4,5-trichlorophenol. The values of log K generally correlated with pK_a, *in vitro* bacteriostatic activity against *Staphylococcus aureus* 209 P, action of uncoupling oxidative phosphorylation at mitochondria, and π-electron-density for the oxygen-atom of phenolic hydroxy group of phenols,

The binding of drugs to proteins is one of the most important factors related to biological activities of the drugs directly or indirectly. For estimation of binding constant for drugs to serum albumin, the authors[3] established a spectrophotometric method by utilizing the albumin-induced metachromasia of 2-(4′-hydroxyphenylazo)benzoic acid (HABA), and bindings of aromatic carboxylic acids and sulfonamides to bovine serum albumin were investigated by using the new method. The present work was intended to obtain binding constant for phenols with bovine serum albumin, and to investigate the relations of the constant to pK_a, the bacteriostatic activity, action in uncoupling phosphorylation, and π-electron-density for the oxygen-atom of phenolic hydroxy group of the phenols.

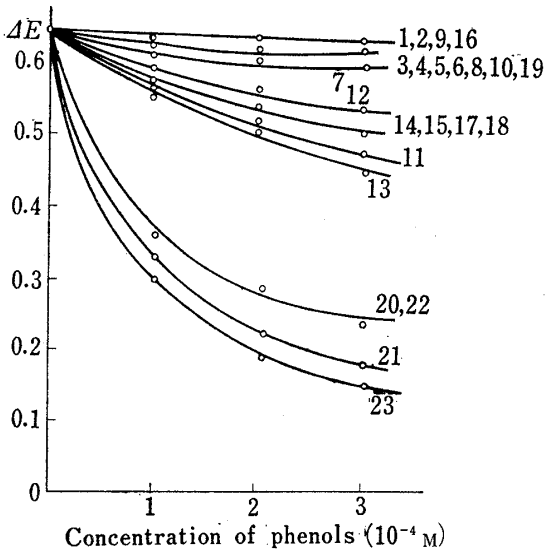

Fig. 1. ΔE of HABA-Albumin Solution with Varying Concentrations of Phenols

1×10^{-4}M HABA and 5×10^{-5}M bovine serum albumin with 0—3×10^{-4}M phenols in 0.15M Tris buffer solution at pH 7.4 and 37°. For numbering, see Table I.

Assuming that HABA and phenols compete for the same binding sites on serum albumin molecules, intrinsic binding constant for phenols with bovine serum albumin, K, may be calculated from Eq. (1)—(3) as described in the previous paper,[3b]

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

$$x=\Delta E/\Delta\varepsilon d \quad (2)$$

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

where x is the concentration of bound HABA, ΔE the difference between the absorbances at 482 mμ of HABA in the presence and in the absence of albumin, $\Delta\varepsilon$ the difference between the molar extinction coefficients at 482 mμ of bound and of unbound HABA, d the depth of the optical path, y the

1) Part V: S. Wada and I. Moriguchi, *Chem. Pharm. Bull.* (Tokyo), **16**, 1440 (1968).
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3) *a*) I. Moriguchi and H. Sano, *Chem. Pharm. Bull.* (Tokyo), **16**, 592 (1968); *b*) I. Moriguchi, *ibid.*, **16** 597 (1968); *c*) I. Mori- guchi, S. Wada, and T. Nishizawa, *ibid.*, **16**, 601 (1968)

concentration of bound phenol, n the number of binding sites on a single molecule of albumin, p the total concentration of albumin, K_A the intrinsic binding, and m a parameter dependent on the experimental conditions, and a and b represent the initial concentrations of HABA and phenols, respectively.

Fig. 1 shows that values of ΔE decrease with increasing concentrations of the phenols added, and the degree of the decrease depends on the kind of phenols.

The values of log K for phenols with bovine serum albumin were calculated from Eq. (1), (2), and (3), the values of ΔE in Fig. 1, and the values of $\Delta\varepsilon$, K_A and m previously determined,[3a] and were listed in Table I. Table I indicates that the values of log K are nearly

Table I. log K–Values for Phenols with Bovine Serum Albumin

No.	Phenols	log K		
		1×10^{-4}M	2×10^{-4}M	Average
1	phenol	2.98	2.92	3.0
2	*o*-aminophenol	3.15	3.21	3.2
3	*m*-aminophenol	3.39	3.31	3.3
4	*p*-aminophenol	3.22	3.33	3.3
5	*o*-methylphenol	3.29	3.25	3.3
6	*m*-methylphenol	3.35	3.30	3.3
7	*p*-methylphenol	3.34	3.39	3.4
8	*o*-methoxyphenol	3.22	3.34	3.3
9	*m*-methoxyphenol	3.16	3.19	3.2
10	*p*-methoxyphenol	3.35	3.24	3.3
11	*o*-nitrophenol	3.85	3.80	3.8
12	*m*-nitrophenol	3.50	3.44	3.5
13	*p*-nitrophenol	3.76	3.61	3.7
14	*o*-chlorophenol	3.50	3.57	3.5
15	*m*-chlorophenol	3.44	3.49	3.5
16	*p*-chlorophenol	3.20	3.19	3.2
17	*o*-bromophenol	3.55	3.41	3.5
18	*m*-bromophenol	3.50	3.43	3.5
19	*p*-bromophenol	3.29	3.25	3.3
20	2,4-dichlorophenol	4.48	4.46	4.5
21	2,4,5-trichlorophenol	5.09	4.96	5.0
22	2,4-dinitrophenol	4.55	4.42	4.5
23	2,4,6-trinitrophenol	5.28	5.20	5.2

a) in 0.15M Tris buffer solution at pH 7.4, 37°
Unit of K is liter/Avogadro number of binding sites on albumin.
b) initial concentration of phenols

constant irrespective of the initial concentration of the phenols. Correlation between log K and pK_a values for phenols is shown in Fig. 2. Coefficient of the correlation is −0.794 (23 samples); the correlation is highly significant at the 1 percent level. It may be said from this that the larger amount of the phenols may bind to serum albumin as the acidity of the phenols is stronger, and that electrostatic force is dominant in the binding. But 2,4-dichlorophenol and 2,4,5-trichlorophenol show higher ability to bind to serum albumin than that predicted from their pK_a-values. This may suggest that hydrophobic binding reported by Hansch, *et al.*[4] is not negligible. The *ortho* effect observed in the case of benzoic acids[3b] is not recognized with phenols. This may be because the binding of phenols to serum albumin is loose owing to the weak acidity of phenols.

Relation between log K and bacteriostatic activity of the phenols was investigated. The bacteriostatic activity was tested *in vitro* against *Staphylococcus aureus* 209 P. Fig. 3

4) C. Hansch, K. Kiehs, and G.L. Lawrence, *J. Am. Chem. Soc.*, **240**, 1811 (1965).

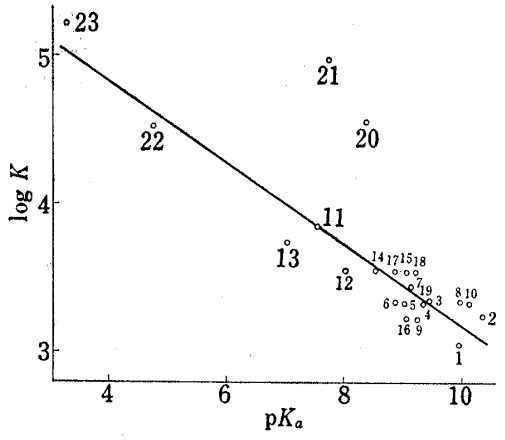

Fig. 2. Correlation between log K and pK_a for Phenols
For numbering, see Table I.

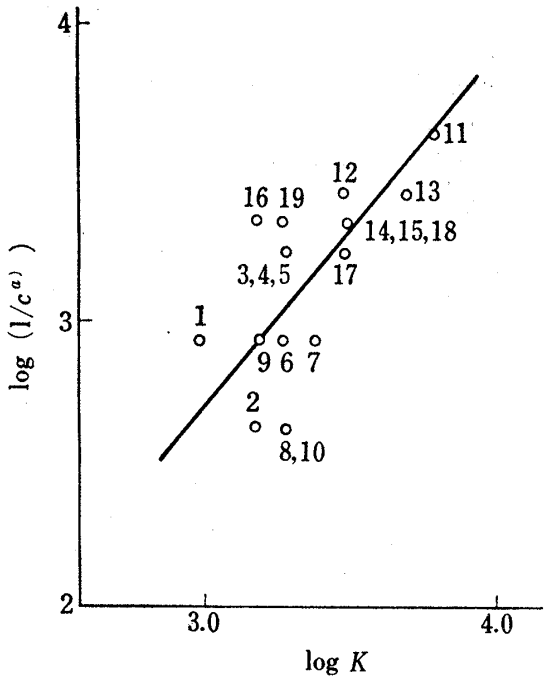

Fig. 3. Correlation between log K and *in Vitro* Bacteriostatic Activity against *Staphylococcus aureus* 209 P for Phenols

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

shows the relationship. c in this Figure is the minimum bacteriostatic concentration of the phenols in M. The bacteriostatic activity becomes stronger with the larger values of log K of phenols. The correlation of log K with log $(1/c)$ is highly significant at the 1 percent level with the coefficient of 0.643 (19 samples).

Weinbach and Garbus[5] reported that various phenols which uncouple oxidative phosphorylation in isolated mitochondria produce the uncoupling effect by the interaction with mitochondrial protein. Therefore, relation between log K and uncoupling activity was investigated. Fig. 4 shows this relation. Coefficient of the correlation was 0.920 (6 samples); the correlation was highly significant at the 1-percent level.

In the previous paper[3c] relations between log K and bacteriostatic activities of sulfonamides were expressed with a parabolic curve, but relations of log K to bacteriostatic activity and to action of uncoupling phosphorylation of phenols were generally simple and linear as shown in Fig. 3 and 4. It may seem from this that non-specific protein bindings participate in these actions of phenols.

These physicochemical and biological properties of phenols are thought to concern with the electronic structure of the molecules. As the parameter for the electronic structure, π-electron-density, q, for oxygen-atom of phenolic hydroxy group was calculated by using the Hückel molecular orbital method.[6] Fig. 5 shows relationship between calculated q-value and log K of phenols. Coefficient of the correlation was -0.673 (23 samples); the correlation was highly significant at the 1 percent level. But 2,4-dichlorophenol and 2,4,5-trichlorophenol show larger value of log K than expected from their q-values, as in the case of relation of log K to pK_a previously described. q-value may be intimately related to the electrostatic properties, but scarcely related to the hydrophobic character of molecules. It seems necessary to consider the distributions of not only π-electron but σ-electron in order to elucidate and furthermore, to predict physicochemical and biological properties of drugs.

5) E.C. Weinbach and J. Garbus, *J. Biol. Chem.*, **240**, 1811 (1965).
6) E. Hückel, *Z. Physik*, **70**, 204 (1931); *ibid.*, **76**, 628 (1932).

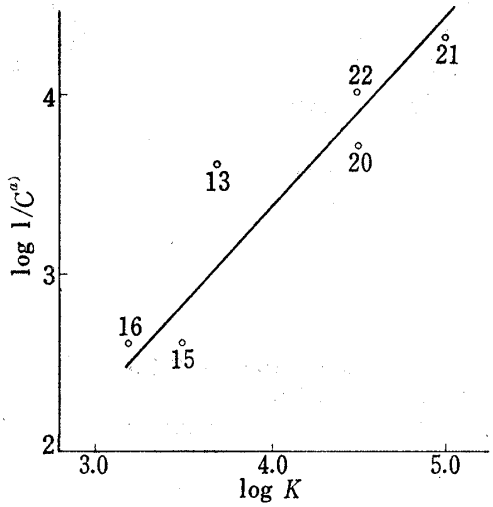

Fig. 4. Correlation between $\log K$ and Uncoupling Phosphorylation for Phenols

For numbering, see Table I.
a) *C* is M in medium required for complete uncoupling.
See, ref. 5).

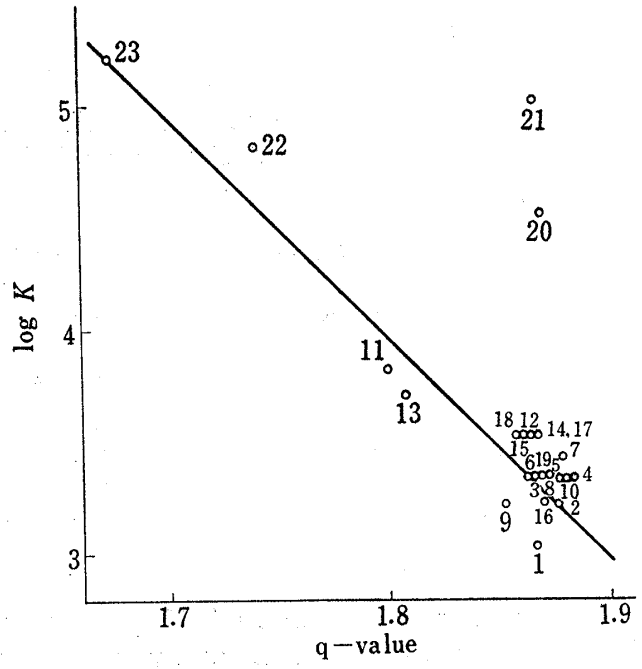

Fig. 5. Correlation between π-Electron-Density of Oxygen-Atom of Phenolic Hydroxy Group and $\log K$ for Phenols

For numbering, see Table I.

Experimental and Calculation Method

Materials——HABA was of reagent grade for clinical analysis, Daiichi Kagaku Yakuhin Co. All phenols used were of Guaranteed Reagent Grade, Tokyo Kasei Kogyo Co. Bovine serum albumin was Armour Laboratories Co. "Fraction V," and corrections for water content and molecular weight were as described previously.[3a)]

Measurements of ΔE——Optical absorption (E') of the solutions containing 1×10^{-4}M HABA and 5×10^{-5}M bovine serum albumin together with 0, 1×10^{-4}M, 2×10^{-4}M, and 3×10^{-4}M phenols, absorption (E) of the solution of 1×10^{-4}M HABA, and absorption (E'') of 5×10^{-5}M bovine serum albumin were measured at 482 mμ at 37° as previously described.[3b)] The values of ΔE were calculated as $\Delta E=E'-(E+E'')$. All the test solutions for photometry were prepared with 0.15M Tris buffer solution of pH 7.4.

Test of *in Vitro* Bacteriostatic Activity——Bacteriostatic activity of phenols was tested against *Staphylococcus aureus* 209 P in a modified Kuwabara's medium as previously described.[3c)] In all cases the minimum bacteriostatic concentration of the phenols was estimated from the turbidity of the test solutions incubated for 24 hours at 37°.

Measurement of Apparent Dissociation Constant——10^{-4}M of phenols was dissolved in 95 ml of a mixture solvent of water–methanol (1:1) and titrated by 0.02N NaOH at 25° with recording of pH by model HM-5A pH Meter of Tōa Denpa Kōgyō Co. Apparent dissociation constant was obtained from the pH at half-neutralization.

Calculation of π-Electron-Density by the Hückel Approximation Method——π-electron-density of oxygen-atom of phenolic hydroxy group calculated by using the Hückel LCAO-MO method[6)] and the parameters recommended by Yonezawa, *et al.*[7a)] The variation or the perturbation method[7b)] were employed.

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7) *a*) T. Yonezawa, T. Nagata, H. Katō, A. Imamura, and K. Morokuma, "Ryoshi-Kagaku Nyūmon," Vol. 1, Kagaku-Dōjin, Kyōto, 1963, p. 55; *b*) *Ibid.*, p. 15.