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# Protein Bindings. II[1]. Binding of Aromatic Carboxylic Acids to Bovine Serum Albumin

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Binding constants for benzoic acid, its monosubstituted derivatives, and pyridinecarboxylic acids with bovine serum albumin were evaluated spectrophotometrically by utilizing the competition with 2-(4′-hydroxyphenylazo)benzoic acid (HABA). A modified Langmuir-type equation which quantitatively represented the binding of HABA to albumin was expanded to express the competitive system in which HABA and a noncolored compound competed for the same binding sites on albumin molecules in the same manner. The values of the binding constant obtained by the use of the expanded equation were irrespective of the three levels of initial concentration of the acids. A linear relation was observed between logarithm of the binding constant and p$K_a$ for the acids except *ortho*-substituted benzoic acids and pyridine-2-carboxylic acid.

Competition between a few anionic dyes such as methyl orange and colorless compounds for binding sites on protein molecules has been demonstrated by spectral methods.[3,4] Unfortunately the spectral changes of these dyes induced by serum albumin are not so remarkable as that of 2-(4′-hydroxyphenylazo)benzoic acid (HABA) investigated in the previous work.[1] The author recently observed that addition of any one of noncolored carboxylic acids, phenols, and sulfonamides to a solution containing serum albumin and HABA reversed the effect of albumin on the spectrum of HABA. If such a reversion is also caused by the competition, it may be possible to evaluate the amount of noncolored compounds bound to serum albumin from the extent of the spectral reversal of HABA with rapidity and considerable accuracy.

The present work was intended to establish the spectrophotometric method for evaluating the binding constant for noncolored compounds by utilizing HABA as a reference dye, and to investigate the relation between affinity to serum albumin and structural features of aromatic carboxylic acids.

In the binding of HABA, represented by $A$, to bovine serum albumin, represented by $P$, concentration of the complex $PA_i$ is expressed as[1]

$$(PA_i)={}_nC_iK_A^{im}(A)^{im}(P) \qquad (1)$$

where $K_A$ represents the association constant of HABA with each site on albumin, $m$ a parameter dependent on the experimental conditions, and $i$ may be any integer from 1 to $n$, the number of binding sites on a single molecule of $P$.

Assuming that binding of noncolored compound, represented by $B$, to serum albumin are expressed by Eq. (3) of the previous paper[1] with the same values of $m$ and $n$ as those for HABA-albumin binding, concentration of the complex $PB_j$ may be given similarly by

$$(PB_j)={}_nC_jK_B^{jm}(B)^{jm}(P) \qquad (2)$$

1) Part I: I. Moriguchi, S. Wada, and H. Sano, *Chem. Pharm. Bull.* (Tokyo), **16**, 592 (1968).
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3) I.M. Klotz, *J. Am. Chem. Soc.*, **68**, 2299 (1946).
4) I.M. Klotz, H. Triwush, and F.M. Walker, *J. Am. Chem. Soc.*, **70**, 2935 (1948).

where $K_B$ represents the association constant of B with each site on albumin, and $j$ may be any integer from 1 to $n$.

When HABA and noncolored compound B compete for the same binding sites on albumin molecules, it follows from Eqs. (1) and (2) that

$$(PA_iB_j)=\{n!/i!j!(n-i-j)!\}K_A{}^{im}K_B{}^{jm}(A)^{im}(B)^{jm}(P) \qquad (3)$$

In the competitive binding, $r_A$, the moles of bound HABA per mole of total albumin may be expressed as

$$r_A=\sum_{i=1}^{n}\sum_{j=0}^{n-i} i(PA_iB_j)/\sum_{i=0}^{n}\sum_{j=0}^{n-i}(PA_iB_j) \qquad (4)$$

Thus we may substitute Eq. (3) into Eq. (4) and obtain.

$$r_A=nK_A{}^m(A)^m/\{1+K_A{}^m(A)^m+K_B{}^m(B)^m\} \qquad (5)$$

Similarly, $r_B$, the moles of bound $B$ per mole of total albumin may be written as

$$r_B=nK_B{}^m(B)^m/\{1+K_A{}^m(A)^m+K_B{}^m(B)^m\} \qquad (6)$$

From Eqs. (5) and (6), logarithm of $K_B$ is given by

$$\log K_B=\log K_A+\log\{(a-x)/(b-y)\}+(1/m)\log(y/x) \qquad (7)$$

where $a$ and $b$ represent the initital concentrations of $A$ and $B$, and $x$ and $y$ the concentrations of bound $A$ and $B$, respectively. The value of $x$ can be obtained by[1]

$$x=\Delta E/\Delta\varepsilon d \qquad (8)$$

where $\Delta E$ is the difference between the absorbances at 482 mμ of HABA solution in the presence and the absence of albumin, $\Delta\varepsilon$ the difference between the molar extinction coefficients at 482 mμ

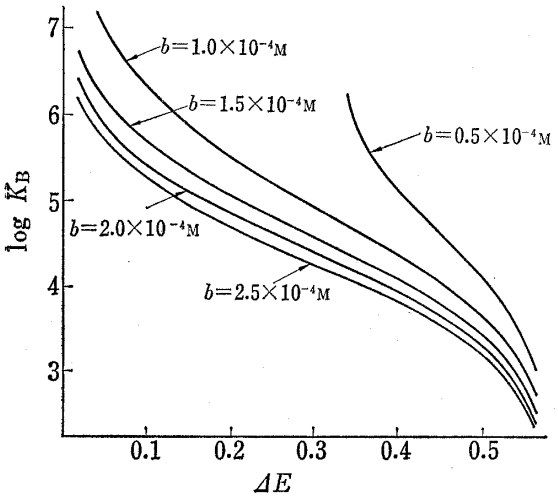


Fig. 1. Theoretical Curves Plotted log $K_B$ *versus* $\Delta E$

The following values were employed for the calculation by using Eqs. (7), (8), and (9); $p=5\times10^{-5}$M, $a=1\times10^{-4}$M, $b=5\times10^{-5}$—$2.5\times10^{-4}$M, log $K_A=4.27$, $m=0.81$, and $n=2$.

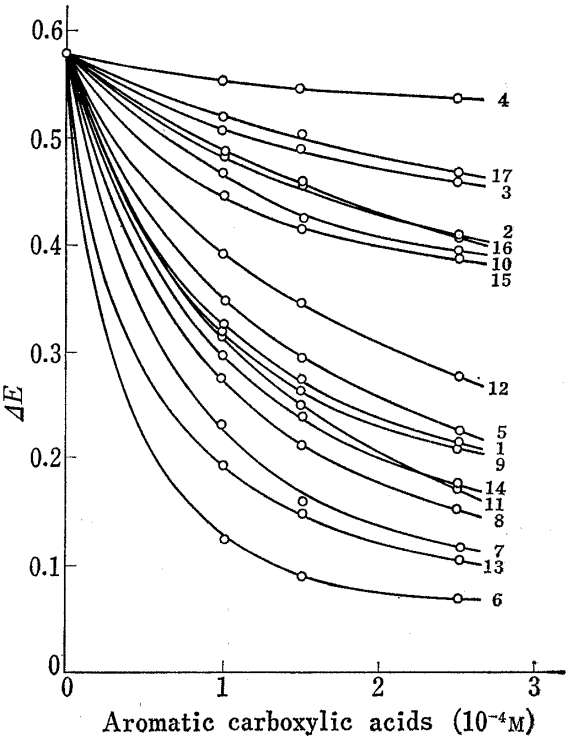


Fig. 2. $\Delta E$ of HABA–Albumin Solutions with Varying Concentrations of Aromatic Carboxylic Acids

$1\times10^{-4}$M HABA and $5\times10^{-5}$M bovine serum albumin with 0—$2.5\times10^{-4}$M aromatic carboxylic acids in 0.15M tris. buffer solution at pH 7.4 and 37°.
For numbering, see Table I.

of bound and unbound HABA, and $d$ the depth of the optical path. From Eqs. (5), (6), and (8), $y$ may be given by

$$y=np-(\Delta E/\Delta\varepsilon d)\{1+1/K_A{}^m(a-\Delta E/\Delta\varepsilon d)^m\} \quad (9)$$

where $p$ represents the total concentration of serum albumin. Since values of $a$, $b$, $d$, and $p$ are given, and the values of $K_A$, $m$, $n$, and $\Delta\varepsilon$ can be estimated by the method previously described,[1] the value of log $K_B$ can be obtained by measurement of the value of $\Delta E$.

Examples of theoretical curves in the form of log $K_B$ *versus* $\Delta E$ are shown in Fig. 1. The curves of this kind are convenient for selection of experimental conditions and for evaluation of the value of log $K_B$ from $\Delta E$.

Table I. log $K$-values[a] for Aromatic Carboxylic Acids with Bovine Serum Albumin

| No. | Aromatic carboxylic acids | $1.0\times10^{-4}$M[b] | $1.5\times10^{-4}$M[b] | $2.5\times10^{-4}$M[b] | Average |
|---|---|---|---|---|---|
| 1 | Benzoic acid | 4.77 | 4.70 | 4.66 | 4.7 |
| 2 | *o*-Aminobenzoic acid | 3.85 | 3.80 | 3.78 | 3.8 |
| 3 | *m*-Aminobenzoic acid | 3.61 | 3.51 | 3.50 | 3.5 |
| 4 | *p*-Aminobenzoic acid | 2.92 | 2.86 | 2.80 | 2.9 |
| 5 | *o*-Bromobenzoic acid | 4.64 | 4.62 | 4.60 | 4.6 |
| 6 | *m*-Bromobenzoic acid | 6.06 | 5.73 | 5.54 | 5.8 |
| 7 | *p*-Bromobenzoic acid | 5.30 | 5.26 | 5.20 | 5.3 |
| 8 | *o*-Hydroxybenzoic acid | 5.05 | 4.99 | 4.93 | 5.0 |
| 9 | *m*-Hydroxybenzoic acid | 4.81 | 4.74 | 4.65 | 4.7 |
| 10 | *p*-Hydroxybenzoic acid | 3.91 | 3.91 | 3.84 | 3.9 |
| 11 | *p*-Methoxybenzoic acid | 4.79 | 4.77 | 4.77 | 4.8 |
| 12 | *o*-Methylbenzoic acid | 4.42 | 4.36 | 4.37 | 4.4 |
| 13 | *m*-Methylbenzoic acid | 5.52 | 5.33 | 5.21 | 5.4 |
| 14 | *p*-Methylbenzoic acid | 4.92 | 4.88 | 4.83 | 4.9 |
| 15 | Pyridine-2-carboxylic acid | 4.09 | 4.01 | 4.09 | 4.1 |
| 16 | Pyridine-3-carboxylic acid | 3.81 | 3.76 | 3.79 | 3.8 |
| 17 | Pyridine-4-carboxylic acid | 3.52 | 3.46 | 3.48 | 3.5 |

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 aromatic carboxylic acids.

Fig. 2 shows the influence of unsubstituted and monosubstituted benzoic acids and isomeric pyridinecarboxylic acids on $\Delta E$.

The values of $\Delta E$ decrease with increasing concentrations of the acids added, and the degree of the decrease depends on the kind of the acids. The curves may suggest the occurrence of competition between HABA and the carboxylic acids for binding sites on serum albumin.

The values of log $K$ for the carboxylic acids with bovine serum albumin were obtained from the values of $\Delta E$ in Fig. 2 by utilizing the curves in Fig. 1. They are listed in Table I.

Table I indicates that the values of log $K$ for the carboxylic acids are nearly constant irrespective of their initial concen-

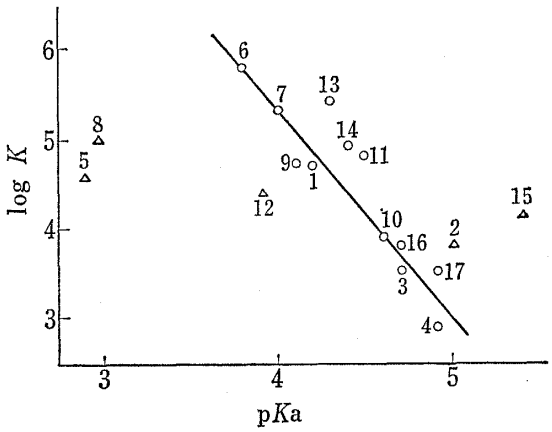


Fig. 3. Correlation between log $K$ and p$K_a$[a] for Aromatic Carboxylic Acids

△ *ortho*- and 2-substituted acids.
For numbering, see Table I.
a) A. Albert and E.P. Serjeant "Ionization Constants of Acids and Bases," Methuen & Co., London, 1962, 127, 134, 148.

trations, and this may justify the procedure and the assumptions involved in the spectrophotometric method.

A linear relationship between log $K$ and $pK_a$ values for the aromatic carboxylic acids is observed in Fig. 3 except for *ortho*–substituted benzoic acids and pyridine–2–carboxylic acid, which suggest the presence of *ortho* effect. Hence the value of log $K$ can be predicted from the value of Hammett constant for *meta*– and *para*–substituted benzoic acids, generally.

The increase in the log $K$ in proportion to decrease in the $pK_a$ may support the general view[5] that in the binding of acidic compounds to serum albumin electrostatic interactions between acidic groups of the compounds and cationic groups of albumin are dominant among several possible sources for the bonding force. But *p*–methoxybenzoic acid and *m*– and *p*–methylbenzoic acid show somewhat higher affinities to serum albumin than those predicted from their $pK_a$–values or Hammett constant. This may suggest that other kinds of forces such as hydrophobic bonding[6] are not negligible.

## Experimental

**Materials**——All benzoic acids and pyridinecarboxylic acids were of Guaranteed Reagent Grade, Tokyo Kasei Kogyo Co. Bovine serum albumin was Armour Laboratories Co. "Fraction V," and the correction for water content and the molecular weight were as described previously.[1] HABA was of reagent grade for clinical analysis, Daiichi Kagaku Yakuhin Co. All the test solutions were prepared with 0.15 M tris. buffer solution of pH 7.4.

**Measurement of $\Delta E$**——Optical absorbance ($E'$) of the solutions containing $1\times10^{-4}$ M HABA and $5\times10^{-5}$ M bovine serum albumin with 0, $1\times10^{-4}$, $1.5\times10^{-4}$, and $2.5\times10^{-4}$ M aromatic carboxylic acids, absorbance ($E$) of the solution of $1\times10^{-4}$ M HABA, and absorbance ($E''$) of the solution of $5\times10^{-5}$ M bovine serum albumin were measured in 1 cm cells at 482 m$\mu$ and 37° with a model EPU–2 Hitachi spectrophotometer one hour after preparing the solutions in duplicate. The values of $\Delta E$ were calculated as,

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

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5) I.M. Klotz, "The Proteins," Vol. I, Academic Press, New York, 1953, p. 727.
6) C. Hansch, K. Kiehs, and G. L. Lawrence, *J. Am. Chem. Soc.*, **87**, 5770 (1965).