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Protein Bindings.[1] I. Binding of 2-(4'-Hydroxyphenylazo)benzoic Acid to Bovine Serum Albumin

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Binding of anionic dye 2-(4'-hydroxyphenylazo)benzoic acid (HABA) to bovine serum albumin at pH 7.4 was studied spectrophotometrically. The relationship between albumin-bound ratio of HABA and concentration of the unbound dye was expressed by a modified Langmuir-type equation involving a repulsing interaction. The binding becomes more difficult with the increasing bonds. The binding constant and the number of binding sites on albumin were evaluated in 0.05 M phosphate buffer solution and in 0.15 M tris. buffer solution at 25° and 37°.

The albumin-induced changes in spectral absorption of dyes are well known.[3] Especially the spectral change of anionic dye 2-(4'-hydroxyphenylazo)benzoic acid (HABA) is remarkable and specific to serum albumin[4,5] and hence HABA has been utilized for the quantification of serum albumin.[6,7] However the quantitative relation among the concentrations of bound and unbound species in HABA-albumin solution has not been cleared yet. The present work was undertaken to determine the equation which expressed the law governing HABA-albumin interaction and to evaluate the binding constant for the dye to serum albumin for the succeeding investigations on albumin-drug bindings by utilizing the spectral change of HABA.

In the binding of small molecule A to serum albumin the dependence of albumin-bound ratio of A on the concentration of unbound A has been reported to follow Freundlich-type equation[8] (1) or Langumuir-type equation[9] (2),

$$r=K(A)^m \qquad (1)$$

$$r=nK(A)/\{1+K(A)\} \qquad (2)$$

where r represents the ratio of moles of bound A per mole of total albumin, (A) the concentration of unbound A, K the binding constant which indicates the strength of the bond, m an empirical parameter, and n the number of binding sites on a single molecule of albumin.

In addition to the two equations, Eq. (3) is presented here for comparative investigation.

$$r=nK^m(A)^m/\{1+K^m(A)^m\} \qquad (3)$$

The physico-chemical meaning of this equation will be discussed later.

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To compare these equations, the amount of albumin-bound HABA must be measured. Binding of HABA to bovine serum albumin causes a remarkable change in optical absorption of the dye—the appearance of a new band at 482 mμ at the normal blood pH of 7.4 (Fig. 1). This spectral change occurs rapidly and reversibly.

The development of the light absorption induced by albumin may be expressed by

$$\Delta E=\Delta\varepsilon xd \qquad (4)$$

where ΔE is the difference between the absorbance 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 of bound and unbound HABA, x the concentration of bound HABA, and d the depth of the optical path. Hence, the concentration of bound HABA may be calculated by

$$x=\Delta E/\Delta\varepsilon d \qquad (5)$$

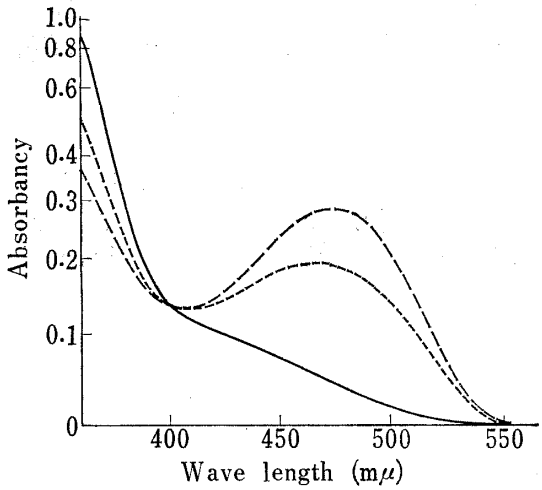

Fig. 1. Spectral Change of HABA Bound to Bovine Serum Albumin

——— 5×10^{-5}M HABA,
- - - - - - 5×10^{-5}M HABA and 1.8 g/dl bovine serum albumin,
..... 5×10^{-5}M HABA, 1.8 g/dl albumin, and 3×10^{-5}M sulfadimethoxine,

in 0.05M phosphate buffer solution at pH 7.4 and 25°. The reversal effect of sulfadimethoxine will be discussed on in part III of this series.

In practice, it is necessary to estimate $\Delta\varepsilon$ for the calculation of x. Taking cognizance of Eq. (5) leads Eqs. (1), (2), and (3) to Eqs. (6), (7), and (8), respectively.

$$\Delta E/p=\Delta\varepsilon^{1-m}a^mK(\Delta\varepsilon-\Delta E/a)^m \qquad (6)$$

$$\Delta E/p=naK(\Delta\varepsilon-\Delta E/a)/\{1+\Delta\varepsilon^{-1}aK(\Delta\varepsilon-\Delta E/a)\} \qquad (7)$$

$$\Delta E/p=\Delta\varepsilon^{1-m}na^mK^m(\Delta\varepsilon-\Delta E/a)^m/\{1+\Delta\varepsilon^{-m}a^mK^m(\Delta\varepsilon-\Delta E/a)^m\} \qquad (8)$$

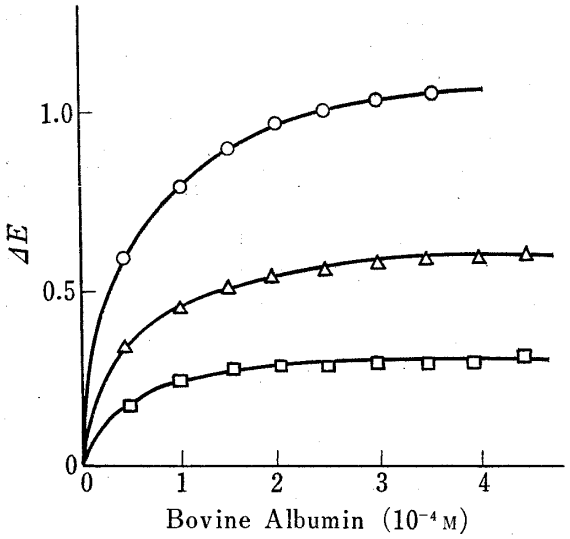

Fig. 2a. Spectral Change of HABA Solutions with Varying Concentrations of Bovine Serum Albumin

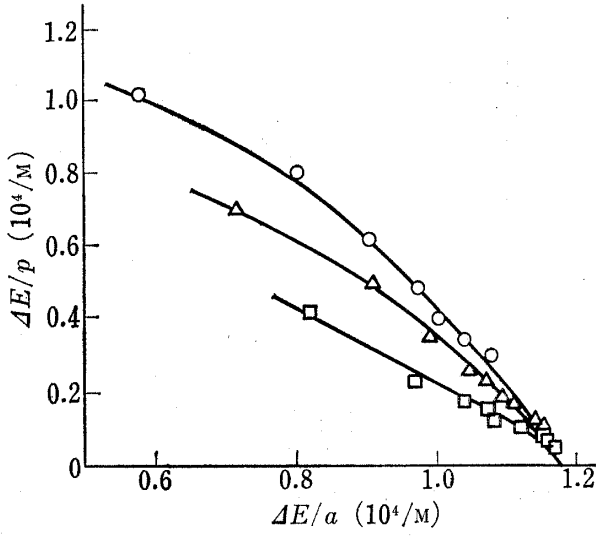

Fig. 2b. Curves Plotted *ΔE/p versus ΔE/a* for the Data in Fig. 2a.

—○— 1.0×10^{-4}, —△— 0.5×10^{-4}, and —□— 0.25×10^{-4}M HABA with 0—4.5×10^{-4}M of bovine serum albumin in 0.05M phosphate buffer solution at pH 7.4 and 25°.

In these equations, p represents the total concentration of albumin and a the total concentration of HABA. From any of Eqs. (6), (7), and (8) $\Delta\varepsilon$ is given by

$$\Delta\varepsilon = \lim_{\Delta E/p \to 0} \Delta E/a \qquad (9)$$

Figure 2a shows ΔE of three levels of HABA solutions with varying concentrations of bovine serum albumin. When the same data are plotted in the form of $\Delta E/p$ *versus* $\Delta E/a$, all curves converge on the abscissa to a point which may indicate the value of $\Delta\varepsilon$ as shown in Fig. 2b. Thus we could estimate $\Delta\varepsilon$, and then calculate the amount of HABA bound to albumin using Eq. (5).

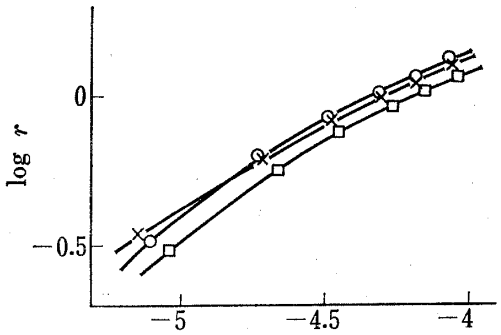

Fig. 3a

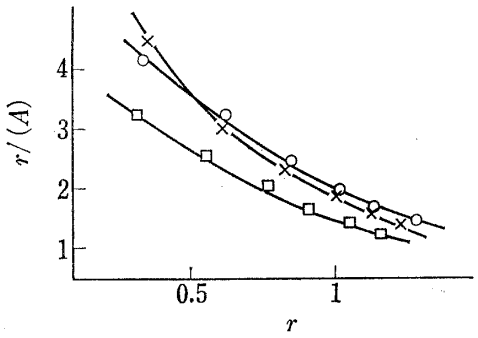

Fig. 3b

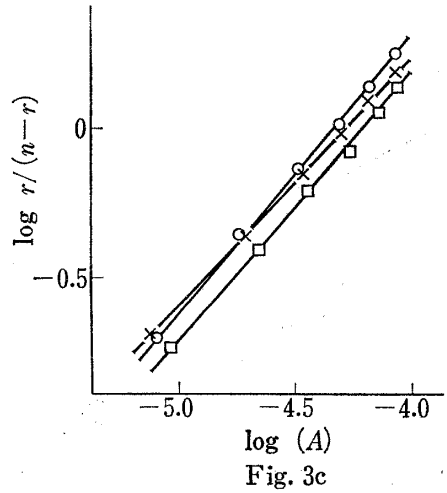

Fig. 3c

Fig. 3a—3c. Three Kinds of Plotting for the Data on Binding of HABA to Bovine Serum Albumin

0.25×10^{-4}—1.5×10^{-4}M HABA and 5×10^{-5}M bovine serum albumin in —⊙— 0.05M phosphate buffer solution at 25°, —□— at 37°, and —×— 0.15M tris. buffer solution at 37°, and at pH 7.4.

For the sake of convenience to graphical investigation, Eqs. (1), (2), and (3) are rewritten as Eqs. (10), (11), and (12), respectively.

$$\log r = m \log (A) + \log K \qquad (10)$$

$$r/(A) = nK - rK \qquad (11)$$

$$\log\{r/(n-r)\} = m \log (A) + m \log K \qquad (12)$$

Figure 3a shows some examples of graphs of $\log r$ *versus* $\log (A)$, Fig. 3b $r/(A)$ *versus* r, and Fig. 3c $\log\{r/(n-r)\}$ *versus* $\log (A)$ in HABA–albumin binding.

Equations (10), (11), and (12) demand straight lines on the graphs in Fig. 3a, 3b, and 3c, respectively, but this is fulfilled only in Fig. 3c. It follows from this that Eq. (12), or (3), is the best of the three equations to represent the binding data.

Constants for Eq. (3) in HABA–bovine albumin binding under various conditions are shown in Table I. By trial and error n was estimated so that the graph of $\log\{r/(n-r)\}$ *versus* $\log (A)$ might be linear, and m and $\log K$ were evaluated from the slope and the intercept on the ordinate of the graph. The binding constant and the number of binding sites on albumin were almost constant over the range of the experimental conditions; the parameter m varied according to the concentration of albumin, the kind of buffering agent, and temperature.

Equation (3) was originally empirical but let us give some consideration on its physicochemical meaning. The multiple equilibria in which a single molecule of the protein, P, may combine with many molecules of A are expressed as the following general form

$$PA_{i-1} + A \rightleftarrows PA_i \qquad (13)$$
$$i = 1, 2, \cdots\cdots, n.$$

Table I. Constants for the Modified Langmuir-type Equation on Binding of HABA[a] to bovine Serum albumin

Buffer	Conc. of Albumin (10^{-4} M)	Temp. (°C)	n	m	log K[b]
0.05 M Phosphate	0.25	25	2.0	0.96	4.4
	0.50	25	2.0	0.91	4.3
	0.75	25	2.0	0.88	4.3
	1.00	25	2.0	0.86	4.2
	0.50	37	2.0	0.89	4.2
0.15 M Tris.	0.50	25	2.0	0.80	4.3
	0.50	37	2.0	0.81	4.3

a) The concentrations of HABA were 0.25×10^{-4} to 1.5×10^{-4} M in each run of the experiments.
b) Unit of K is liter/Avogadro number of binding sites on albumin.

The equilibrium constant, K_i, was shown by Klotz[9] for the ideal situation of no other interaction, as

$$K_i=(PA_i)/(PA_{i-1})(A)=\{(n-i+1)/i\}K \tag{14}$$

where K is the intrinsic binding constant, or the association constant for A with each site on P. If some other interactions influencing the reaction (13) are not negligible, Eq. (14) may be modified as

$$K_i=\{(n-i+1)/i\}Kf \tag{15}$$

where f is a coefficient modifying the binding constant K for the interactions. From Eqs. (14) and (15), (PA_i) is given by

$$(PA_i)={}_nC_iK^i(A)^i(P)f^i \tag{16}$$

The ratio, r, of the moles of bound A per mole of total protein is expressed as

$$r=\sum_{i=1}^{m}i(PA_i)/\sum_{i=0}^{m}(PA_i) \tag{17}$$

In view of Eqs. (3) and (17), (PA_i) may be shown as

$$(PA_i)={}_nC_iK^{im}(A)^{im}(P) \tag{18}$$

From Eqs. (3), (16), and (18), f is given by

$$f=\{r/(n-r)\}^{(m-1)/m} \tag{19}$$

In Eq. (19), $r/(n-r)$ is the ratio of bound fraction per unbound fraction of the sites on albumin. Accordingly, the coefficient f depends on degree of the binding. If $m=1$, f equals unity, and Eq. (3) reduces to Eq. (2) involving no other interaction. But in the HABA–albumin binding, the experimental values of m are less than 1 as shown in Table I. Hence it follows that the binding may become more difficult with increase of the bond. The causes of such a kind of repulsing effect are not cleared, but electrostatic interactions between successively bound dye anions, and occurrence of competition between the dye and buffer anions may be accounted.

Experimental

Materials——For all experiments involving albumin was utilized Serum Albumin Fraction V, Armour Laboratories Co. Correction for the water content was made by drying a small sample at 60° and 14 mmHg

until constant weight was attained. For the molecular weight, 69000[10] was used. HABA was of reagent grade for clinical analysis, Daiichi Kagaku Yakuhin Co. All the test solutions were prepared by using 0.05 M phosphate or 0.15 M tris. buffer solution of pH 7.4.

Measurement——Optical absorption of the dye–albumin solutions prepared in duplicate was measured in 1 cm cells at 25° and 37° with a model EPU–2 Hitachi spectrophotometer one hour after preparing the solutions.

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