A New Equation to Express Drug–Protein Interaction and Its Application to Spectrophotometric Titration

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We introduced a new binding equation to express drug–protein interaction and applied it to a spectrophotometric titration method. We also discussed its relation to other equations for spectrophotometric titration.

Keywords spectrophotometric titration; protein binding; Langmuir equation

The binding of drugs with serum albumin has long been studied in detail, because the binding is directly associated with drug action including side effects. There are therefore a variety of methods[1] in common use to determine the binding constants between a drug (ligand) and macromolecules, such as equilibrium dialysis, ultrafiltration, and gel-filtration. Each of these methods, however, has serious drawbacks.[1,2] For example, equilibrium dialysis is tedious and considerable time is required before results can be obtained. The spectrophotometric method[1] is more convenient than others, because it allows rapid analysis of the binding. The absorption spectrum of a certain drug changes on addition of proteins or other large molecules, because of metachromasy. The apparent molar extinction coefficient, ε, at a certain wavelength then decreases, as shown in Fig. 1, or increases. In a solution containing a total drug concentration $[L]_T$, let the molar extinction coefficients for a non-bound (free) and a bound drug be expressed as ε_f and ε_b, respectively, the bound concentration $[L]_b$ and the free concentration $[L]$ can then be expressed by Eqs. 1 and 2.[1-5]

$$[L]_b=\frac{\varepsilon_f-\varepsilon}{\varepsilon_f-\varepsilon_b}[L]_T \quad (1)$$

$$[L]=[L]_T-[L]_b \quad (2)$$

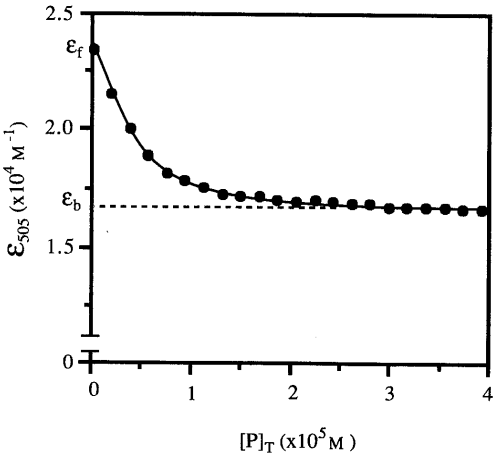

Fig. 1. Effect of BSA on the Absorbance of Ponceau 3R at 505 nm (pH 6.0)

Absorption spectra of 4.10×10^{-5} M ponceau 3R at pH 6.0 were measured in the presence of various concentrations of BSA.

In Eq. 1, one can determine ε_f from the absorbance of the drug solution without protein, but it is difficult to determine the exact value of ε_b. Peacocke and Skerrett[3] determined ε_b from ε at higher concentrations of macromolecules, where the absorbance took a constant value (Fig. 1). However, this method cannot always be used under the conditions where the constant optical absorbance is not attained such as due to limited solubility of the macromolecules.[1,2] The value of ε_b is also determined by the methods of Westphal *et al.*,[4] Terada *et al.*[2] and Moriguchi *et al.*[5] by extrapolation of the absorbances to the value at an infinite concentration of the macromolecules before the absorbance attains the constant value. These methods are practically useful for determination of ε_b, but their accuracy is dependent on the macromolecular concentrations used. As a small difference in the value of ε_b dramatically affects the value of binding constant, determination of the exact ε_b value is very important for analysis of the binding data. In this study, we introduce a basic equation useful for producing these graphical methods and develop a new analytical method for determining the exact ε_b value.

Theory

Binding Theory Interaction of protein P with drug L can be described by multiple stepwise reactions[1] as follows; $P+L\rightleftarrows PL$, $PL+L\rightleftarrows PL_2,\ldots,PL_{i-1}+L\rightleftarrows PL_i,\ldots,PL_{n-1}+L\rightleftarrows PL_n$. Then, equilibrium constants are given by Eq. 3.

$$K_i=\frac{[PL_i]}{[PL_{i-1}][L]} \quad (i=1,\ldots,n) \quad (3)$$

Here the relation $[PL_0]=[P]$ was used. The bound drug concentration $[L]_b$ and the total protein concentration $[P]_T$ are then expressed by Eqs. 4 and 5, respectively.

$$[L]_b=[P](K_1[L]+2K_1K_2[L]^2+\cdots+nK_1K_2\cdots K_n[L]^n) \quad (4)$$

$$[P]_T=[P](1+K_1[L]+K_1K_2[L]^2+\cdots+K_1K_2\cdots K_n[L]^n) \quad (5)$$

Consequently, the mean number of drug molecules bound with one molecule of the protein, *i.e.*, the binding number $r(=[L]_b/[P]_T)$, is expressed by Eq. 6.

$$r=\frac{K_1[L]+2K_1K_2[L]^2+\cdots+nK_1K_2\cdots K_n[L]^n}{1+K_1[L]+K_1K_2[L]^2+\cdots+K_1K_2\cdots K_n[L]^n} \quad (6)$$

This is the most general binding equation,[1,2] including

1) cooperativity in the binding, and 2) the creation of new sites in the process of the binding. Fletcher *et al.*[6] have proven that Eq. 6 can be expressed in the form of the Langmuir equation 7 for the binding of drugs to a protein with a single class of independent binding sites of which the number is N, and that Eq. 6 further becomes Eq. 8, if the protein has two classes of binding sites, N_1 and N_2.

$$r=\frac{NK[\mathrm{L}]}{1+K[\mathrm{L}]} \tag{7}$$

$$r=\frac{N_1K_1[\mathrm{L}]}{1+K_1[\mathrm{L}]}+\frac{N_2K_2[\mathrm{L}]}{1+K_2[\mathrm{L}]} \tag{8}$$

Equation 7 gives the linear relationship in the Scatchard plot, but Eq. 8 gives the inward concave curve.

On the other hand, the binding ratio of the drug, *i.e.*, the molar ratio of the bound one to the total drug, s, $(=[\mathrm{L}]_b/[\mathrm{L}]_T)$ in the stepwise binding can be expressed by Eq. 9.

$$s=\frac{[\mathrm{P}](K_1[\mathrm{L}]+2K_1K_2[\mathrm{L}]^2+\cdots+nK_1K_2\cdots K_n[\mathrm{L}]^n)}{[\mathrm{L}]+[\mathrm{P}](K_1[\mathrm{L}]+2K_1K_2[\mathrm{L}]^2+\cdots+nK_1K_2\cdots K_n[\mathrm{L}]^n)} \tag{9}$$

From Eqs. 5, 6 and 9, Eq. 10 is obtained.

$$s=\frac{[P]_Tr}{[\mathrm{L}]+[\mathrm{P}]_Tr} \tag{10}$$

Equations 9 and 10 are the most basic equations for expressing the binding ratio of the drug, s. In these cases, s is dependent on the total protein concentration $[\mathrm{P}]_T$.

When the binding obeys the Langmuir-type equations 7 and 8, Eq. 10 becomes Eq. 11 and 12, respectively.

$$s=\frac{NK[\mathrm{P}]_T}{1+K[\mathrm{L}]+NK[\mathrm{P}]_T} \tag{11}$$

$$s=\frac{[\mathrm{P}]_T(N_1K_1+N_2K_2+N_1K_1K_2[\mathrm{L}]+N_2K_1K_2[\mathrm{L}])}{(1+K_1[\mathrm{L}])(1+K_2[\mathrm{L}])+[\mathrm{P}]_T(N_1K_1+N_2K_2+N_1K_1K_2[\mathrm{L}]+N_2K_1K_2[\mathrm{L}])} \tag{12}$$

Spectrophotometric Titration Curve Let Eq. 10 be substituted into Eq. 1, then the apparent molar extinction coefficient, ε, is expressed by Eq. 13.

$$\varepsilon=\varepsilon_f-(\varepsilon_f-\varepsilon_b)\frac{[\mathrm{P}]_Tr}{[\mathrm{L}]+[\mathrm{P}]_Tr} \tag{13}$$

Equation 13 is equivalent to Eq. 14 when one class of binding sites is present.

$$\varepsilon=\varepsilon_f-(\varepsilon_f-\varepsilon_b)\frac{NK[\mathrm{P}]_T}{1+K[\mathrm{L}]+NK[\mathrm{P}]_T} \tag{14}$$

Equation 14 is readily applicable to obtain the parameters ε_f, ε_b, N and K using non-linear least-squares calculation, in which the free drug concentration [L] is successively approximated by Eqs. 1 and 2. Furthermore, Eq. 14 can be transformed to Eq. 15.

$$\frac{[\mathrm{P}]_T}{\varepsilon_f-\varepsilon}=\frac{(1+K[\mathrm{L}])/NK}{\varepsilon_f-\varepsilon_b}+\frac{[P]_T}{\varepsilon_f-\varepsilon_b} \tag{15}$$

Equation 15 gives a graphical method to provide ε_b: *i.e.*, when $[\mathrm{P}]_T/(\varepsilon_f-\varepsilon)$ is plotted *versus* $[\mathrm{P}]_T$, the slope of the linear line at higher $[\mathrm{P}]_T$ gives ε_b.

Experimental

Materials Ponceau 3R and bovine serum albumin (BSA, Fraction V) were purchased from Wako Pure Chemical Industries, Ltd. The molecular mass of BSA was assumed to be 69 kDa.

Binding Experimental Ponceau 3R was dissolved in 1/15 M phosphate buffer (pH 6.0) to give 4.1×10^{-5} M. The absorbances of the Ponceau 3R solution in the presence of various concentrations of BSA (0—1.45×10^{-4} M) were measured on a Shimadzu spectrophotometer, model 300, at 505 nm.

Results and Discussion

We obtained the new basic Eqs. 10 and 13 for drug-protein binding based on multiple stepwise reactions and applied them to spectrophotometric titration. Figure 1 shows the changes in the absorbance of ponceau 3R with various concentrations of BSA at its λ_{max} (505 nm). The value of ε_f was determined to be $2.339\times10^4\,\mathrm{M}^{-1}$ in the ponceau 3R solution without BSA. The value of ε_b was determined by graphical methods, as shown in Figs. 1 and 2, and also by the new method for simulating the data in Fig. 1 using Eq. 14. The binding constants were

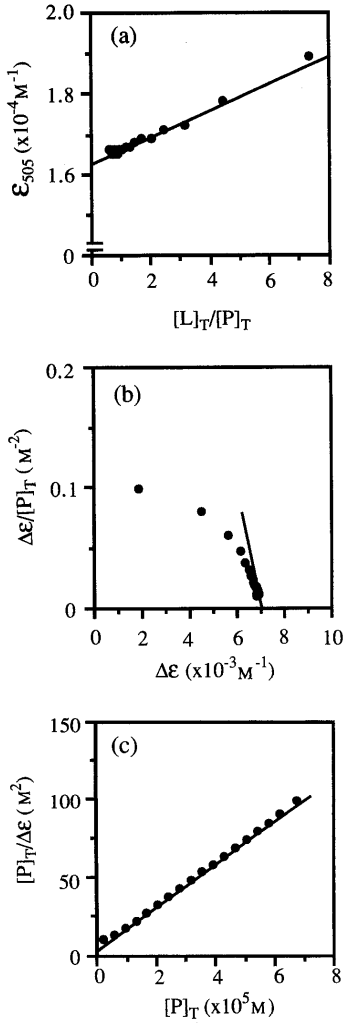

Fig. 2. Determination of Molar Extinction Coefficient ε_b of Bound Drug

(a) plot of ε *versus* $[\mathrm{L}]_T/[\mathrm{P}]_T$; (b) plot of $\Delta\varepsilon/[\mathrm{P}]_T$ *versus* $\Delta\varepsilon$; (c) plot of $[\mathrm{P}]_T/(\varepsilon_f-\varepsilon)$ *versus* $[\mathrm{P}]_T$.

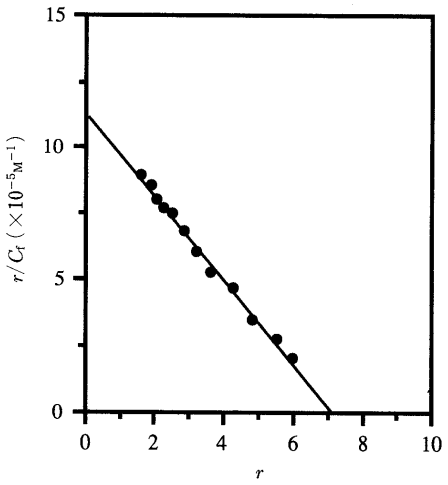

Fig. 3. Scatchard Plots for the Binding between Ponceau 3R and BSA at pH 6.0

The solid line was drawn using the binding constants; $n=7.18$, $K=1.51\times10^5\,M^{-1}$.

determined by the Scatchard plots. Figure 3 shows the Scatchard plot produced by an ε_b from Eq. 14. These results are summarized in Table I and showed that it is very important to obtain the value of ε_b as exactly as possible, because the shifts of ε_b reflect on the binding constants, as mentioned above.

Next, Eq. 16 is obtained from Eq. 11 at the limit of $[P]_T\rightarrow\infty$, *i.e.* $[L]\rightarrow0$.

$$s=\frac{NK[P]_T}{1+NK[P]_T} \tag{16}$$

Dimicoli and Helene[7] studied drug–DNA interaction and applied Eq. 16 to the chemical shifts in NMR spectra. Applying Eq. 16 to Eq. 14, one can obtain Eq. 17.

$$\varepsilon=\varepsilon_b+(\varepsilon_f-\varepsilon_b)\frac{NK[P]_T}{1+NK[P]_T} \tag{17}$$

Because it can also be assumed that $[L]_T/[P]_T\ll NK[L]_T$, Eq. 17 can be reformulated to Eq. 18.

$$\varepsilon=\varepsilon_b+\frac{(\varepsilon_f-\varepsilon_b)}{NK[L]_T}[L]_T/[P]_T \tag{18}$$

Equation 18 is the equation introduced by Terada *et al.*[2] Therefore, a plot of the apparent molar extinction coefficient ε *versus* $[L]_T/[P]_T$ provides ε_b at the intercept of the vertical axis, as shown in Fig. 2(a). Equation 18 can readily be reformulated to Eq. 19.

$$\frac{1}{\varepsilon-\varepsilon_b}=\frac{1/NK}{\varepsilon_f-\varepsilon_b}\cdot\frac{1}{[P]_T}+\frac{1}{\varepsilon_f-\varepsilon_b} \tag{19}$$

Equation 19 corresponds to the Ketelaar modification[8b] of the Benesi-Hildebrand equation,[8a] which is appropriate to graphically determine the equilibrium constants for associations or complex formations between small molecules. However, Eq. 19 should be extended to Eq. 15 for multiple stepwise reactions. Denoting that

TABLE I. The Values of ε_b Determined by Various Methods and the Binding Constants between Ponceau 3R and BSA

Method	$\varepsilon_b\times10^{-4}\,M^{-1}$	N	$K\times10^{-5}\,M^{-1}$	r
Terada *et al.*[2]	1.658	7.06	1.62	0.988
Moriguchi *et al.*[5]	1.658	7.06	1.62	0.988
Peacocke and Skerrett.[3]	1.659	7.18	1.51	0.989
Eq. 15[a]	1.660	6.94	1.74	0.972
Eq. 14[b]	1.656	7.18	1.51	0.990

a) Graphical method. *b*) Least-squares method ($\varepsilon_b\pm0.093\times10^4\,M^{-1}$).

$\Delta\varepsilon_b=\varepsilon_f-\varepsilon_b$, $\Delta\varepsilon=\varepsilon_f-\varepsilon$ and $[L]=[L]_T(\Delta\varepsilon_b-\Delta\varepsilon/\Delta\varepsilon_b)$, rearrangement of Eq. 14 further leads to Eq. 20.

$$\frac{\Delta\varepsilon}{[P]_T}=\frac{NK(\Delta\varepsilon_b-\Delta\varepsilon)}{1+K[L]_T(\Delta\varepsilon_b-\Delta\varepsilon)/\Delta\varepsilon_b} \tag{20}$$

Equation 20 then provides $\Delta\varepsilon=\Delta\varepsilon_b$ at the limit of $\Delta\varepsilon/[P]_T\rightarrow0$, and thus, in the plot of $\Delta\varepsilon/[P]_T$ *versus* $\Delta\varepsilon$, the intercept on the horizontal axis is $\Delta\varepsilon_b$, as shown in Fig. 2b. Equation 20 corresponds to the equation of Moriguchi *et al.*[5] Equations 13 and 14 gave very successful results for the spectrophotometric titration, including Eqs. 15, 18, 19 and 20. In these graphical methods, the binding data at a limited range of $[P]_T$ is taken into account for determination of ε_b, but in non-linear least-squares calculation ε_b is determined from all the binding data. Therefore, as shown in Table I, Eq. 14 is useful to analyze the binding between drug and protein, which has a single class of independent binding sites.

When the protein has two classes of binding sites, Eq. 21 should be available from Eqs. 12 and 13.

$$\varepsilon=\varepsilon_f-(\varepsilon_f-\varepsilon_b)\cdot\frac{[P]_T(N_1K_1+N_2K_2+N_1K_1K_2[L]+N_2K_1K_2[L])}{(1+K_1[L])(1+K_2[L])+[P]_T(N_1K_1+N_2K_2+N_1K_1K_2[L]+N_2K_1K_2(L)} \tag{21}$$

This equation was found to be useful for the analysis of binding data, and will be detailed in the subsequent paper.

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