Determination of Equilibrium Constants and Binding Capacities Using a Modified Scatchard Method in Drug–Protein Binding Studies

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Abstract ☐ The equilibrium constants and the number of binding sites for the binding of 1-anilinonaphthalene-8-sulfonate to human and bovine serum albumin, studied by a fluorescent method, were determined by an equation derived from the Scatchard multiple-equilibrium treatment. The results and the equation are discussed and compared with other equations used in drug–protein binding studies. Treatment of data by the modified method allows a clear determination of the number of binding sites.

Keyphrases ☐ Drug–protein binding—equilibrium constants and binding capacities using modified Scatchard method, equations ☐ Equilibrium constants—determined using a modified Scatchard method, drug–protein binding studies, equations ☐ Binding capacities—determined using a modified Scatchard method, drug–protein binding studies, equations ☐ Scatchard multiple-equilibrium treatment, modified—equation derived to determine equilibrium constants and binding capacities, drug–protein binding studies

In drug–protein binding studies, several equations have been used for binding constant calculation (1–3). One frequently used equation is the Langmuir-type equation:

$$\bar{V}/(D) = nK - \bar{V}K \qquad \text{(Eq. 1)}$$

where $\bar{V}$ represents the ratio of moles of bound drug per mole of total protein, (D) is the concentration of unbound drug, K is the binding constant, and n is the number of binding sites on a single protein molecule, assuming that all sites are of equal binding strength. However, the plot of $\bar{V}/(D)$ *versus* $\bar{V}$ frequently gives a downward curve rather than a convincing straight line. Such curvature may be indicative of binding to more than one class of sites (4). Mathematical treatments for the determination of binding parameters along this line were also reported (5, 6).

Recently, Eq. 1 was modified (7, 8) by the introduction of an empirical parameter:

$$\log [\bar{V}/(n - \bar{V})] = m \log (D) + m \log K \qquad \text{(Eq. 2)}$$

By this method, the value of n is estimated by trial and error so that the plot of $\log [\bar{V}/(n - \bar{V})]$ *versus* $\log (D)$ might be linear, and m and $\log K$ are evaluated from the slope and the intercept of the graph. When $m = 1$, Eq. 2 may be reduced to Eq. 1. For the binding of 2-(4′-hydroxyphenylazo)benzoic acid to bovine serum albumin, it was shown (7, 8) that m is not equal to 1 and that it varies with factors such as the concentrations of albumin and buffering agent and temperature.

In the present study, the binding of 1-anilinonaphthalene-8-sulfonate to human and bovine serum albumins was studied by a fluorescent method (9). The data were treated using a modified Scatchard equation for multiple equilibria.

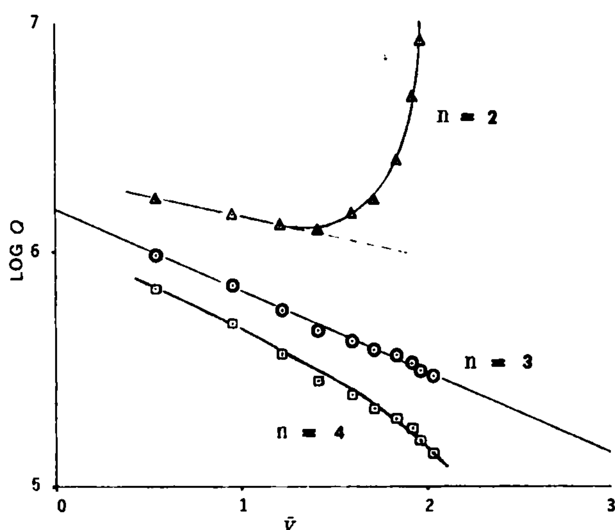

Figure 1—*Plots of log* Q versus $\bar{V}$ *for the binding of 1-anilinonaphthalene-8-sulfonate to human serum albumin.*

EXPERIMENTAL

Materials—1-Anilinonaphthalene-8-sulfonate[1], crystalline human serum albumin[2], bovine serum albumin[3], and spectroscopic grade methanol (as a solvent) were used. All other chemicals were of reagent grade.

Instruments and Methods—All fluorescence measurements were made on a spectrophotofluorometer[4] equipped with a 150-w. xenon lamp and 1P21 photomultiplier tube. Fluorescence titrations over a range of protein concentrations were carried out at the emission maxima of 474 and 467 nm. for human and bovine serum albumins, respectively, with the excitation for both systems at 375 nm. Two milliliters of each protein solution in pH 7.4 phosphate buffer was titrated with successive additions of 2 μl. of 1×10^{-3} *M* 1-anilinonaphthalene-8-sulfonate. The probe was dissolved in methanol and delivered with microsyringes[5]. The temperature of all samples was 25 ± 0.5°.

RESULTS AND DISCUSSION

The relative fluorescence intensities of solutions containing various amounts of 1-anilinonaphthalene-8-sulfonate in the absence and presence of human and bovine serum albumins are shown in Table I. During the titration, above the critical protein concentrations of 29.10×10^{-6} *M* for human serum albumin and 61.41×10^{-6} *M* for bovine serum albumin, there was no further enhancement of the fluorescence upon increases in protein concentration. It was then assumed that all of the drug was bound to protein. The fractions of bound and unbound drug and the molar ratio of the drug bound to protein were calculated by a method previously used in these laboratories (10). Determinations of binding constants were made for solutions containing 1.45×10^{-6} *M* human serum

[1] Aldrich Chemical Co.
[2] Control No. 4619, Nutritional Biochemicals Corp.
[3] Control No. 1693, Nutritional Biochemicals Corp.
[4] Aminco-Bowman, American Instrument Co., Silver Spring, Md.
[5] Hamilton.

Table I—Relative Fluorescence Intensity of Solutions Containing Varying Amounts of 1-Anilinonaphthalene-8-sulfonate

$(D_t) \times 10^6$, M[a]	No Protein	Human Serum Albumin, 1.45×10^{-6} M	Human Serum Albumin, 29.10×10^{-6} M	Bovine Serum Albumin, 1.23×10^{-6} M	Bovine Serum Albumin, 61.41×10^{-6} M
1	1.9	6.5	7.8	8.5	11.1
2	3.5	11.6	15.4	15.6	22.2
3	5.2	15.7	23.0	21.2	32.8
4	6.8	18.8	30.3	25.7	43.4
5	7.0	21.4	38.2	28.9	53.7
6	7.3	23.3	46.0	31.0	63.8
7	7.5	24.8	43.2	33.0	74.7
8	7.7	26.0	60.3	34.8	86.0
9	7.9	26.6	67.0	35.6	95.9
10	8.0	27.3	73.5	36.3	105.7

[a] The total 1-anilinonaphthalene-8-sulfonate concentration.

albumin and 1.23×10^{-6} M bovine serum albumin with varying concentrations of 1-anilinonaphthalene-8-sulfonate.

To calculate the equilibrium constants, a function, Q, is defined by the relation:

$$Q = \bar{\nu}/(n - \bar{\nu})(D) \qquad \text{(Eq. 3)}$$

or:

$$\log Q = \log [\bar{\nu}/(n - \bar{\nu})] + pD \qquad \text{(Eq. 4)}$$

Equation 3 was developed by Scatchard and first employed by Edsall *et al.* (11). The method of Edsall *et al.* was used for the present study to determine the binding parameters. The value of n was not obtained experimentally, however, since it was previously reported (12) that each mole of bovine serum albumin was bound by 3 moles of 1-anilinonaphthalene-8-sulfonate; it was decided to introduce this number first as the probable value for n. The following equilibrium processes ($n = 3$) were chosen for the determination of the equilibrium constants:

$$P + D = PD \qquad k_1 = (PD)/(P)(D) \qquad \text{(Eq. 5)}$$

$$PD + D = PD_2 \qquad k_2 = (PD_2)/(PD)(D) \qquad \text{(Eq. 6)}$$

$$PD_2 + D = PD_3 \qquad k_3 = (PD_3)/(PD_2)(D) \qquad \text{(Eq. 7)}$$

where P represents protein and k_1, k_2, and k_3 are the successive equilibrium constants.

A plot of calculated values for log Q *versus* $\bar{\nu}$ will give a straight line (13, 14), although the points as $\bar{\nu}$ approaches zero or the maximum number of binding sites may show some curvature due to experimental error. For $n = 3$, the extrapolated log Q values at $\bar{\nu} = 0$ and $\bar{\nu} = 3$ are essentially equal to log K_1 and log K_3, respectively. K_1 and K_3 are the intrinsic association constants, which are related to the equilibrium constants by the following equations:

$$K_1 = k_1/3 \qquad \text{(Eq. 8)}$$

$$K_2 = 2k_2/2 \qquad \text{(Eq. 9)}$$

$$K_3 = 3k_3 \qquad \text{(Eq. 10)}$$

To obtain k_2, the following procedure (13) can be used. From the plot of log Q *versus* $\bar{\nu}$, one can determine log Q at $\bar{\nu} = 1.5$ as:

$$\log Q = \log [1.5/(3 - 1.5)] + pD \qquad \text{(Eq. 11)}$$

The value of pD can be easily calculated and is taken to be the value of log k_2.

In the present study, three sets of log Q values were calculated using Eq. 4 by letting $n = 2$, 3, and 4, respectively. These values for log Q were then plotted as a function of $\bar{\nu}$. The results seen in Fig. 1 represent the plots for $n = 2$, 3, and 4 when human serum albumin is the protein. Figure 2 is a similar representation of data when bovine serum albumin is the protein. The plots are linear for both systems at $n = 3$. For $n = 2$ and 4, however, two opposing curves, above and below the straight line, were obtained. These results indicate that both human and serum albumins probably have three binding sites for 1-anilinonaphthalene-8-sulfonate. This is reinforced when one considers previously reported values for n in similar systems.

The successive equilibrium constants determined from Figs. 1 and 2 are listed in Table II. Human serum albumin has a larger log k_1 value but smaller values of log k_2 and log k_3 than those of bovine

Table II—Successive Logarithmic Equilibrium Constants of the Binding of 1-Anilinonaphthalene-8-sulfonate to Human and Bovine Serum Albumins

Protein	Concentration	n	log k_1	log k_2	log k_3
Human serum albumin	1.45×10^{-6}	3	6.66	5.66	4.66
Bovine serum albumin	1.23×10^{-6}	3	6.48	5.84	5.20

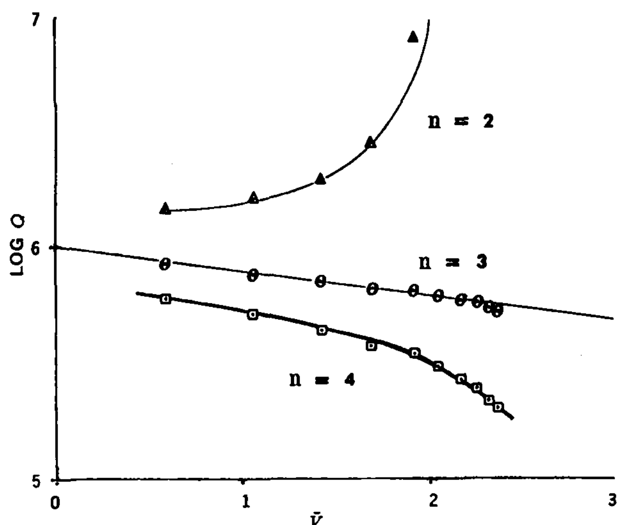

Figure 2—*Plots of log* Q versus $\bar{\nu}$ *for the binding of 1-anilinonaphthalene-8-sulfonate to bovine serum albumin.*

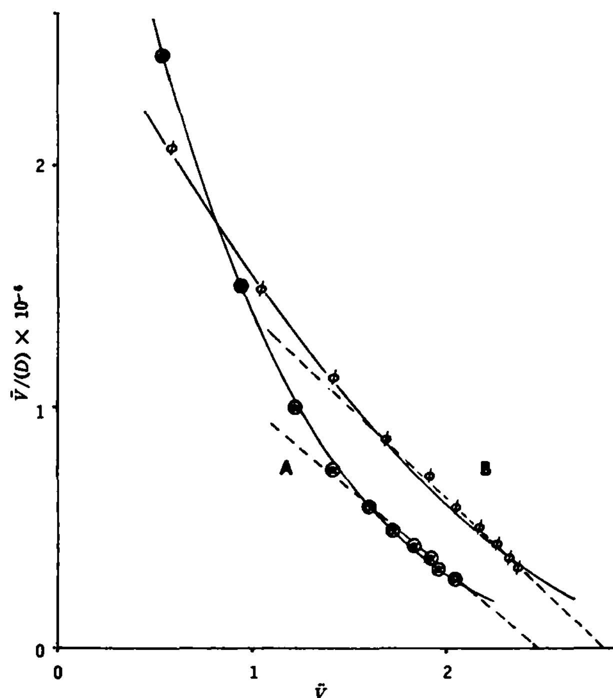

Figure 3—*Plots of* $\bar{\nu}$/(D) versus $\bar{\nu}$ *for the binding of 1-anilinonaphthalene-8-sulfonate to (A) human serum albumin and (B) bovine serum albumin.*

serum albumin. This suggests that the binding of one 1-anilinonaphthalene-8-sulfonate molecule to human serum albumin discourages other 1-anilinonaphthalene-8-sulfonate molecules from combining in a greater extent than the binding of 1-anilinonaphthalene-8-sulfonate to bovine serum albumin.

Equation 1 was suggested by Scatchard (3) for the special case when the intrinsic association constants are all equal. He also pointed out that, if the plot of $\bar{\nu}/(D)$ *versus* $\bar{\nu}$ does not give a straight line, it may be inferred that the intrinsic constants are not equal and/or there is interaction among the bound ions. The former effect is well demonstrated by the results of this study. For comparison, the plots of $\bar{\nu}/(D)$ *versus* $\bar{\nu}$ for both protein systems are shown in Fig. 3; these plots are not quite linear. If one were to discount the low values of $\bar{\nu}$, one could plot a straight line with a high significance factor. The n values for the human and bovine serum albumin systems obtained from the straight-line portion of the Scatchard plots are 2.5 and 2.8, respectively. When using Eq. 3, the results seem to be much more satisfactory. It is possible to detect the number of sites with greatest affinity as well as to detect dramatically the total number of binding sites over a range of small molecule concentrations.

Equation 2 is an empirical equation in which a parameter, m, is introduced into Eq. 1 to obtain a straight-line plot. The fact that m is varied by the protein concentration, buffer agent, and temperature (7) may indicate the effect of these factors on the binding. However, the physicochemical meaning of m is still not clear.

The determination of the binding parameters may also depend on the experimental methods used. The results obtained from the fluorescence technique are different from those of other methods, probably in the manner that only the primary (strong) binding sites are detected, whereas the results obtained from other methods such as dialysis (15, 16) often show larger numbers of binding sites. The curvature of the Scatchard plot has been interpreted (4) as the binding of the small molecule to more than one class of sites on the protein. However, it is possible that the intrinsic association constants of the binding of these sites are not equal even in the same class.

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Low Temperature Maintenance of Test Organism Suspensions for Antibiotic Assays

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Abstract ☐ The survival rates of 14 test organisms that had been suspended in saline, distilled water, peptone water, or nutrient broth were compared after storage at −70°. Viable cell counts and dose–responses to various antibiotics were determined prior to freezing and at 3-month intervals for 2 years after freezing. Most test organisms were successfully maintained in this manner using water, peptone water, or broth with little or insignificant losses in viable cells or change in dose–response in antibiotic assay systems.

Keyphrases ☐ Microorganisms—low temperature maintenance for use in antibiotic assays ☐ Antibiotic assays—low temperature maintenance of test organism suspensions ☐ Test organisms—low temperature maintenance for use in antibiotic assays

This laboratory maintains a number of test organism suspensions for daily use in turbidimetric and agar diffusion assays of antibiotics (1). Detailed procedures for the preparation of these suspensions are described in the *Code of Federal Regulations* (2). Because of the variety of antibiotics tested and the volume of samples to be assayed, preparing fresh suspensions on a weekly or biweekly basis is a time-consuming task attended by a number of laboratory problems. Ideally, the growth characteristics and specific antibiotic dose–response of any given suspension are reproducible if the suspension has been prepared according to the official method and stored under refrigeration at temperatures just above freezing. In actual practice, even the most exacting