Method for Measuring the Binding of Small Molecules to Proteins from Binding-Induced Alterations of Physical–Chemical Properties†

Clarke J. Halfman‡ and Toshiro Nishida*

ABSTRACT: A method is presented for obtaining binding data in any ligand–protein system for which binding induces a measurable change in a physical property of the system. The relationship between the equivalents of ligand bound and the physical property change need not be a known theoretical function, nor need it be empirically determined at an impractically high protein concentration. Rather, the physical property change is measured at a number of total ligand–protein mole ratios at two or more protein concentrations. The number of equivalents of bound ligand and the concentration of free ligand is computed from data obtained at a minimum of two protein concentrations. As an example of the utility of the method, the binding of dodecyl sulfate to the 12 primary sites of bovine serum albumin was measured from a binding-induced fluorescence change. Results were obtained at two values of pH and ionic strength and were analyzed in terms of a two set binding model.

Binding of small molecules to proteins is described by the same mass action law that applies to the association of simpler monovalent substances (Von Muralt, 1930), *i.e.*

$$\bar{\nu} = \frac{nKc}{1 + Kc} \tag{1}$$

where $\bar{\nu}$ = average mole ratio of bound ligand to protein, c = equilibrium free-ligand concentration, n = number of binding sites on a protein molecule, and K = intrinsic association constant of the binding sites.

This relationship describes the binding when the intrinsic association constants of all n sites are equal and when binding to any one site does not influence binding to any other site. When more than one equivalent set, m, of sites exists, the appropriate relationship is

$$\bar{\nu} = \sum_{i=1}^{m} \frac{n_i K_i c}{1 + K_i c} \tag{2}$$

An ideal analysis of data from binding studies would allow m and each n_i and K_i and be determined unambiguously. When all sites are equivalent, $m = 1$ and n and K may be determined by plotting $1/\bar{\nu}$ *vs.* $1/c$ as suggested by Klotz (1946) or by plotting $\bar{\nu}/c$ *vs.* $\bar{\nu}$ as suggested by Scatchard *et al.* (1950). The plots are not linear when more than one set of sites is present. Curvature, indicative of heterogeneous sites, is more obvious in the Scatchard plot which therefore provides a more sensitive analysis of the data. A nonlinear plot is also obtained by the presence of cooperative or destructive interactions between sites.

Nonchromophoric ligands do not possess the advantage of exhibiting a measurable spectral change upon binding. However, a change in a physical property of the protein may be induced by the binding. This communication presents a general method which allows binding to be quantitated from any measurable physical property that is significantly altered by the binding.

Experimental Section

Solvents. All aqueous solutions were prepared in glass distilled water. Reagent grade organic solvents and inorganic salts were used throughout, without further purification. The buffers employed were 0.01 M K_2HPO_4 containing 0.03 or 0.15 M KCl with the pH of the mixture adjusted to 7.5 with HCl and 0.01 M dipotassium succinate containing the same amounts of KCl with the pH similarly adjusted to 6.0.

Bovine Serum Albumin. Sigma crystallized, lyophilized bovine serum albumin (lot 79B-8080) was used in these studies. Both the defatting procedure for removing bound impurities and method of albumin stock solution preparation are described elsewhere (Halfman and Nishida, 1971a). The concentration of the albumin solutions was determined by recording the absorption peak at 278 nm with a Cary 14 spectrophotometer. Concentration in units of molarity was calculated using a molecular weight of 66,700 (Squire *et al.*, 1968) and $E_{1\,cm}^{1\%} = 6.67$ (Sterman, 1955).

Sodium Dodecyl Sulfate. The sodium dodecyl sulfate used in these studies was prepared in this laboratory (Wong, 1968) after the procedure of Dreger *et al.* (1944). Sulfur assay showed the material to be at least 98% pure. From a stock solution in absolute ethanol, aqueous dodecyl sulfate solutions were prepared immediately before use by evaporating the appropriate volume of stock solution in a volumetric flask with a Rinco rotary evaporator and redissolving the dodecyl sulfate in water.

Fluorescence Measurements. Fluorescence measurements were performed with an Aminco Bowman spectrophotofluorometer and recorded with an X–Y recorder (Model

† From the Burnsides Research Laboratory, Department of Food Science, University of Illinois, Urbana, Illinois 61801. *Received February 4, 1972.* Supported by funds from the National Institutes of Health, U. S. Public Health Service (HL 03063), the Illinois and Chicago Heart Associations, and the Illinois Agricultural Experiment Station. The material represents a part of the dissertation submitted to the University of Illinois in partial fulfillment of the requirement for the Ph.D. degree of C. J. H., whose fellowship was provided in part by National Institutes of Health Training Grant GM 0653.

‡ Present address: Clinical Chemistry Laboratory, Georgetown University, Washington D. C. 20007.

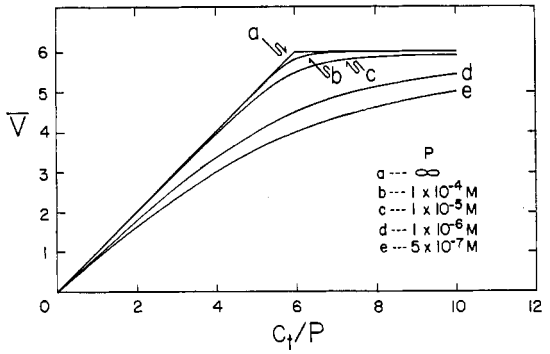

FIGURE 1: Computed plots of $\bar{V}$ *vs.* C_t/P for $n = 6$, $K = 10^6$ as a function of protein concentration.

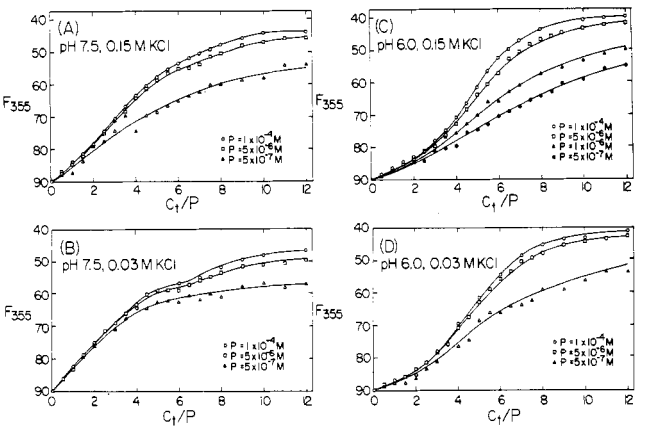

FIGURE 2: Measurements of the fluorescence intensity of bovine serum albumin (λ_F = 355 nm, λ_{ex} = 275 nm) as influenced by the presence of dodecyl sulfate. Measurements are with respect to a solution not containing dodecyl sulfate whose fluorescence intensity was arbitrarily set to 90 for each protein concentration. Lines through data points are a best visual fit.

300, Electro Instruments, Inc.). The method used to obtain 300- to 400-nm full scale across the wavelength axis on the recorder, calibration of the spectrophotofluorometer and associated techniques were previously described (Halfman and Nishida, 1971a,b). All measurements were performed in a square, 10-mm cell (Pyro cell) at room temperature, with water at approximately 25° circulating through the sample holder. No sample or reference solution was placed in the light beam more than once in order to minimize photodecomposition by the high-intensity light of the xenon lamp.

Calculations. Complex calculations were performed with a Hewlett-Packard programable calculator. The drawing of many theoretical curves was also done with this calculator and its associated plotter.

Results

When binding data are plotted as $\bar{V}$ *vs.* the mole ratio of total ligand to protein, C_t/P, at sufficiently high protein concentration, the number of binding sites, n, can be obtained (Figure 1, curve a). Equilibrium free-ligand concentration, c, corresponding to any value of $\bar{V}$ may be obtained from plots at sufficiently low protein concentrations (Figure 1, curve d or e). At a sufficiently low protein concentration, the fraction of bound to total ligand is significantly less than 1 and c may be determined from the difference between the mole ratio of total ligand to protein and $\bar{V}$ multiplied by the protein concentration, *i.e.*,

$$c = P\left(\frac{C_t}{P} - \bar{V}\right) \tag{3}$$

A more representative quantity for the ordinate would be values of the binding-induced physical property change, Δ, since $\bar{V}$ is not measured directly but is determined from measurements of Δ. The relationship between $\bar{V}$ and Δ is generally obtained at a protein concentration as high as is practical so that a plot of Δ *vs.* C_t/P is essentially a plot of Δ *vs.* $\bar{V}$. From a plot obtained at a sufficiently low protein concentration, c is computed from eq 3 for corresponding values of $\bar{V}$. To obtain reliable results from this treatment of the data, it is necessary that the binding does not involve protein concentration effects and/or that the binding-induced physical property change is not dependent upon protein concentration.

It should be noted that the relationship between Δ and $\bar{V}$ need not be linear when data are plotted in the above manner. However, when the relationship between Δ and $\bar{V}$ is not linear, it is difficult to be assured that data obtained at the highest protein concentration provides a stoichiometric relationship between Δ and $\bar{V}$, *i.e.*, C_t/P may not be equivalent to $\bar{V}$ for all values of C_t/P. Higher protein concentrations may, of course, be employed until a limit of concentration is attained where an apparent shift in the plot of Δ *vs.* C_t/P no longer occurs. However, when the association constant is not sufficiently high, this protein concentration limit may be unattainable before solubility limits are approached. The expense and availability of a protein would also preclude measurements at too high a concentration. Although not immediately obvious, it is unnecessary to use an extremely high protein concentration to empirically determine the stoichiometric relationship between Δ and $\bar{V}$ when data are collected at several protein concentrations and plotted as Δ *vs.* C_t/P. Since eq 3 is valid for any protein concentration, we may equate two expressions at protein concentrations, P_a and P_b, for c and solve for $\bar{V}$. It follows that

$$\bar{V} = \frac{(C_t/P)_a - \frac{P_b}{P_a}(C_t/P)_b}{1 - \frac{P_b}{P_a}} \tag{4}$$

$$c = \frac{P_a P_b}{P_a - P_b}[(C_t/P)_b - (C_t/P)_a] \tag{5}$$

where $(C_t/P)_a$ and $(C_t/P)_b$ are the total equivalents of ligand added at protein concentrations P_a and P_b, respectively, which gives the same value of Δ corresponding to a particular value of $\bar{V}$. Equation 4 shows that a stoichiometric relationship between $\bar{V}$ and Δ may be computed from data acquired at protein concentrations where a significant fraction of the total ligand added is not bound. Results may be computed from eq 4 and 5 without the determination of a stoichiometric curve. This method has been used successfully for measuring the binding of ionic ligand to proteins where the measured response is based on the influence of charge on the hydrogen ion equilibria of protein (Halfman and Steinhardt, 1971). In this communication, the concepts for the derivation of eq 4 are fully described and the relationship is generalized to include any binding-induced change.

The binding of long-chain ligands to bovine serum albumin alters the fluorescence of the protein (Spector and John, 1968). The nature of the binding-induced fluorescence alter-

TABLE I: Calculation of Binding Data from Figure 2A.[a]

	C_t/P Protein Concentration				
ΔF_{355}	10^{-4} M	5×10^{-6} M	5×10^{-7} M	$\bar{\nu}$	c ($\times 10^6$/M)
8.5	1.50	1.5	1.85	1.50	0.175
11.8	2.00		2.55	2.00	0.275
15.7	2.50		3.35	2.50	0.425
19.5	3.00		4.25	3.00	0.625
23.2	3.50		5.20	3.49	0.85
26.7	4.00		6.40	3.99	1.20
29.5	4.50		7.70	4.49	1.60
32.1	5.00		9.30	4.98	2.15
34.6	5.50		10.95	5.48	2.72
36.6	6.00	6.68		5.97	3.55
38.0	6.50	7.33		6.55	4.40
39.6	7.00	8.03		6.95	5.40
40.7	7.50	8.83		7.44	6.75
42.2	8.00	9.70		7.92	8.95
43.3	8.50	10.85		8.39	12.30
44.5	9.00	12.50		8.82	18.40

[a] Values of $\bar{\nu}$ and c were computed from eq 4 and 3 or 5 using values of C_t/P at two protein concentrations corresponding to the same value of ΔF_{355}.

ation has been studied previously (Halfman and Nishida, 1971b) and is characterized by a decrease in intensity at approximately 355 nm. The decrease in the fluorescence intensity of bovine serum albumin at pH 7.5 in 0.15 M KCl effected by the addition of dodecyl sulfate was measured at various protein concentrations (Figure 2A). From the solid line curves drawn through these data, values of $\bar{\nu}$ and c were computed by eq 4 and 5. Table I shows the method of calculation by which the results presented as an isotherm (Figure 3A) were obtained. The decrease in fluorescence intensity produced by the addition of dodecyl sulfate to bovine serum albumin was also measured at three other conditions of pH and KCl concentrations (Figure 2B, C, and D). Results were computed in a like manner and isotherms were plotted (Figure 3B, C, and D). The results obtained at pH 6 in 0.03 M KCl may be compared to equilibrium dialysis results obtained at pH 5.6 in phosphate buffer (μ = 0.033) (Reynolds *et al.*, 1967; Cassel and Steinhardt, 1969). Differences which exist (Figure 3D) were most likely caused by differences in the commercial preparations employed in each study.

A total of 12 sites are indicated for each pH and KCl concentration from Scatchard plots of the results (Figure 4). Curvature in the plots shows that the binding is heterogeneous, as does the nonlinear decrease of the fluorescence intensity (Figure 2). The binding isotherms (Figure 3) did not exhibit any clear inflections from which the number of sets of sites, or the number of sites in each set, could have been estimated. When heterogeneous binding is encountered, one generally assumes a certain number of sets and the number of sites in each set and their corresponding association constants are varied in a trial and error manner to obtain a best theoretical fit to the data. Without auxiliary information, the minimum number of parameters which provides a resonable fit to the data can be considered to adequately characterize the binding.

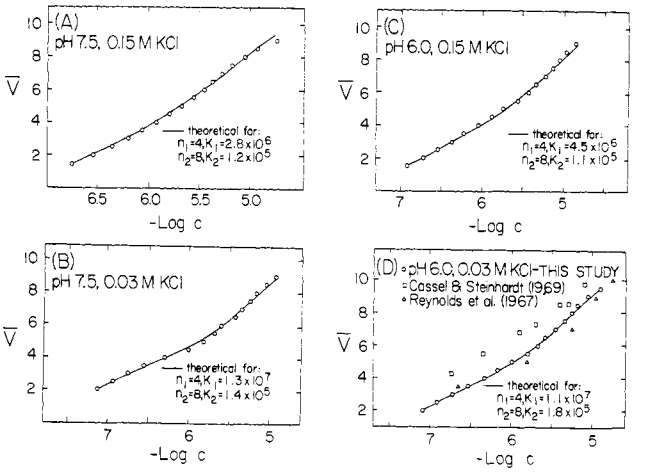

FIGURE 3: Binding data computed from Figure 2 by eq 4 and 3 or 5 as illustrated in Table I. Lines drawn through data points are curves computed from values of the parameters given in Table III.

If two sets of sites would prove to fit the data satisfactorily, trial and error, with its inherent subjectivity, may be eliminated from the analysis by using the intercepts from the Scatchard plot and any two pairs of data points. The intercepts are: $N_T(=n_1 + n_2)$ and $A(=n_1K_1 + n_2K_2)$. Since each set of sites binds independently and a particular value of $\bar{\nu}$ exists for each set corresponding to a particular value of c, *i.e.*, $\bar{\nu}_1 = n_1K_1c/(1 + K_1c)$ and $\bar{\nu}_2 = n_2K_2c/(1 + K_2c)$ it follows that $c = \bar{\nu}_1/[K_1(n_1 - \bar{\nu}_1)] = \bar{\nu}_2/[K_2(n_2 - \bar{\nu}_2)]$. From the above, eq 6 may be derived

$$n_1 = \frac{c(A - K_2N_T)\left[N_TK_2 - \frac{\bar{\nu}}{c}(1 + K_2c)\right]}{\frac{\bar{\nu}}{c}(1 + K_2c)^2 - (cN_TK_2^2 + A)} \qquad (6)$$

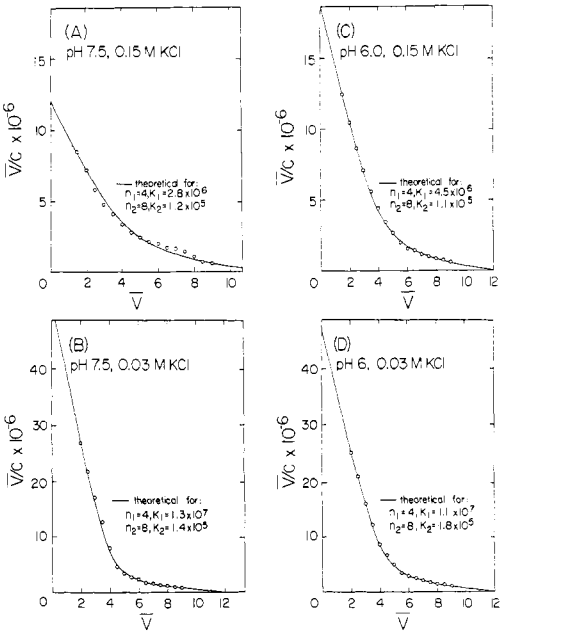

FIGURE 4: Scatchard plots of binding data. Estimated intercepts of these plots were used to determine parameters as in Table II Solid line curves are computed from values of the parameters given in Table III.

TABLE II: Determination of Binding Parameters for Two-Set Binding Model.[a]

$\bar{\nu}$ / c	3 6.25×10^{-7}	4 1.2×10^{-6}	5 2.16×10^{-6}	6 3.55×10^{-6}	7 5.40×10^{-6}	8 9.00×10^{-6}	9 2.80×10^{-5}
2	4.0	4.5	4.3	4.1	4.0	4.1	4.3
2.75×10^{-7}	2.3×10^{6}	2.6×10^{6}	2.6×10^{6}	2.7×10^{6}	2.7×10^{6}	2.7×10^{6}	2.6×10^{6}
	6.7	7.5	7.8	7.9	8.0	7.9	7.7
	1.0×10^{5}	7.5×10^{4}	1.0×10^{5}	1.2×10^{5}	1.3×10^{5}	1.3×10^{5}	9.4×10^{4}
3		3.5	3.6	3.5	3.4	3.5	4.0
6.25×10^{-7}		3.1×10^{6}	3.0×10^{6}	3.1×10^{6}	3.2×10^{6}	3.1×10^{6}	2.8×10^{6}
		8.5	8.4	8.5	8.6	8.5	8.1
		1.4×10^{5}	1.4×10^{5}	1.5×10^{5}	1.6×10^{5}	1.4×10^{5}	1.0×10^{5}
4			3.7	3.4	3.3	3.6	4.2
1.2×10^{-6}			3.0×10^{6}	3.1×10^{6}	3.2×10^{6}	3.0×10^{6}	2.7×10^{6}
			8.3	8.6	8.7	8.4	7.8
			1.3×10^{5}	1.5×10^{5}	1.6×10^{5}	1.4×10^{5}	9.6×10^{4}
5			n_1	3.0	2.8	3.4	4.4
2.16×10^{-6}			K_1	3.5×10^{6}	3.7×10^{6}	3.1×10^{6}	2.5×10^{6}
			n_2	9.0	9.2	8.6	7.6
			K_2	1.7×10^{5}	1.7×10^{5}	1.4×10^{5}	9.2×10^{4}
6					2.6	3.8	5.1
3.55×10^{-6}					3.9×10^{6}	2.9×10^{6}	2.2×10^{6}
					9.4	8.2	6.9
					1.8×10^{5}	1.3×10^{5}	7.9×10^{4}
7						5.0	5.8
5.40×10^{-6}						2.3×10^{6}	1.9×10^{6}
						7.0	6.2
						1.0×10^{5}	6.0×10^{4}
8							4.7
9.00×10^{-6}							1.6×10^{6}
							7.3
							4.1×10^{4}

[a] Binding parameters for the interaction of dodecyl sulfate with bovine serum albumin at pH 7.5 in the presence of 0.15 M KCl were computed from eq 6 and 7 from paired values of $\bar{\nu}$ and c from Scatchard plot intercepts in Figure 4A.

Using two corresponding values of $\bar{\nu}$ and c gives

$$n_1 = \frac{c_i(A - K_2N_T)\left[N_TK_2 - \frac{\bar{\nu}_i}{c_i}(1 + K_2c_i)\right]}{\frac{\bar{\nu}_i}{c_i}(1 + K_2c_i)^2 - (c_iN_TK_2^2 + A)}$$

$$= \frac{c_j(A - K_2N_T)\left[N_TK_2 - \frac{\bar{\nu}_j}{c_j}(1 + K_2c_j)\right]}{\frac{\bar{\nu}_j}{c_j}(1 + K_2c_j)^2 - (c_jN_TK_2^2 + A)}$$

The only unknown in the last two equalities is K_2. Performing the proper algebraic operations gives

$$aK^3 + bK^2 + cK + d = 0 \qquad (7)$$

where

$$a = -N_T[c_i\bar{\nu}_j(N_T - \bar{\nu}_i) - c_j\bar{\nu}_i(N_t - \bar{\nu}_j)]$$

$$b = c_iN_T(N_T - \bar{\nu}_i)\left(A - \frac{\bar{\nu}_j}{c_j}\right) + A\bar{\nu}_j(\bar{\nu}_ic_j + c_iN_T) - c_jN_T(N_T - \bar{\nu}_j)\left(A - \frac{\bar{\nu}_i}{c_i}\right) - A\bar{\nu}_i(\bar{\nu}_jc_i + c_jN_T)$$

$$c = N_T\left(\frac{\bar{\nu}_j}{c_j} - A\right)(c_iA + \bar{\nu}_i) + Ac_i\bar{\nu}_i\left(A - \frac{\bar{\nu}_j}{c_j}\right) - N_T\left(\frac{\bar{\nu}_i}{c_i} - A\right)(c_jA + \bar{\nu}_j) - Ac_j\bar{\nu}_j\left(A - \frac{\bar{\nu}_i}{c_i}\right)$$

$$d = A\left[\bar{\nu}_i\left(A - \frac{\bar{\nu}_j}{c_j}\right) - \bar{\nu}_j\left(A - \frac{\bar{\nu}_i}{c_i}\right)\right]$$

By using pairs of corresponding values of $\bar{\nu}$ and c, the coefficients are calculated and K is determined by solving the cubic equation. Three roots are obtained; two of which are K_1 and K_2; the third is of no significance. Upon establishing suitable values for K_1 (or K_2), n_2 (or n_1) may be determined from eq 6. All combinations of pairs of the data points will give values of n_1, K_1, n_2, and K_2 from which average values are computed. If the theoretical curves determined by these average parameters fit the data, two sets of sites adequately describe the binding; if not, a greater degree of heterogeneity is indicated.

An example of results analyzed by this procedure is presented in Table II. From combinations of pairs of $\bar{\nu}$ and c, values of n_1, K_1, n_2, and K_2 were obtained. Average values appear in Table III together with average values of the parameters determined in the same manner for the three other conditions of pH and KCl concentration. The solid line curves

TABLE III: Binding Parameters for the Interaction of Dodecyl Sulfate with Bovine Serum Albumin Determined from a Two-Set Binding Model.[a]

pH	KCl Concn (M)	n_1	K_1	n_2	K_2
7.5	0.03	3.8 ± 0.3	$1.3 \times 10^7 \pm 0.1 \times 10^7$	8.2 ± 0.3	$1.4 \times 10^5 \pm 0.3 \times 10^5$
7.5	0.15	3.9 ± 0.7	$2.8 \times 10^6 \pm 0.5 \times 10^6$	8.0 ± 0.8	$1.2 \times 10^5 \pm 0.3 \times 10^5$
6.0	0.03	3.9 ± 0.2	$1.1 \times 10^7 \pm 0.1 \times 10^7$	8.1 ± 0.2	$1.8 \times 10^5 \pm 0.2 \times 10^5$
6.0	0.15	4.0 ± 0.4	$4.5 \times 10^6 \pm 0.4 \times 10^6$	8.0 ± 0.4	$1.1 \times 10^5 \pm 0.2 \times 10^5$

[a] Parameters are an average ± sd computed from 24 paired values of $\bar{\nu}$ and c, except for the condition, pH 7.5, 0.15 M KCl, for which 28 paired values were used.

in Figures 3 and 4 are plots calculated with these parameters. Agreement with the data is quite good for all four cases. Two sets of sites appear to adequately describe the binding process. The number of sites in each set, four and eight, respectively, was not influenced by pH or electrolyte concentration. A change in these conditions influenced the value of K_2 only slightly. The value of K_1 was influenced strongly by electrolyte concentration and only slightly by pH.

Discussion

The binding of dodecyl sulfate to bovine serum albumin was quantitated from measurements of the reduction in fluorescence intensity of the protein (ΔF_{355}) induced by the binding of the ligand. Fluorescence techniques, previously used to study binding, have primarily relied on increased fluorescence intensities of bound ligands (Laurence, 1952; Anderson and Weber, 1965; Daniel and Weber, 1966). Only a few investigators have taken advantage of binding-induced alterations in the fluorescence of the protein to obtain binding data (Brewer and Weber, 1966; Lehrer and Fasman, 1966). In these studies, however, data were obtained from a linear stoichiometric relationship between an altered fluorescence intensity and the degree of binding, based on initial limiting slopes which were acquired at as high a protein concentration as was practicable. If the protein concentration is not high enough, the limiting slope and the extrapolated stoichiometric relationship will depend upon protein concentration. Furthermore, when more than one site is involved the entire stoichiometric relationship may not be linear. In the present study, eq 4 enabled binding data to be obtained without determining a stoichiometric relationship at high protein concentrations. When data are acquired at two or more protein concentrations and results are computed from eq 4, no presumptions are made regarding the shape of the stoichiometric relationship and the results will not be influenced by protein concentration.

Quantitation of the binding of small molecules to proteins by eq 4 may be used with any binding-induced physical property change of sufficient magnitude. Of course, the measured response to binding and/or the binding process itself must not depend on protein concentration for the equation to be valid. One response to ionic ligand binding, ΔpH, was shown to depend on protein concentration and would yield valid results only with measurements above a certain minimum protein concentration (Halfman and Steinhardt, 1971).

The binding of long-chain ionic compounds to albumins has been shown to occur in three stages (Decker and Foster, 1966; Pallansch and Briggs, 1954). The first stage involves simple statistical binding to approximately 12 sites followed by two stages of cooperative binding; approximately 40 and 80 sites are involved in the second and third stages, respectively. Fluorescence intensity measurements are apparently limited to quantitation of the first 12 primary binding sites which, by Scatchard plot analysis of the results, yielded two sets of four and eight sites for all four conditions studied (Table III).

Characterization of the binding of dodecyl sulfate to the 12 primary sites in terms of two sets of sites is consistent with the observation that the alteration of the fluorescence of bovine serum albumin produced by dodecyl sulfate binding occurs in two stages, *e.g.*, quenching, followed by a blue shift (Halfman and Nishida, 1971b). Quenching would be produced by binding to the first set of four stronger sites and the blue shift would result from binding to the eight weaker sites. Even though the second stage involves a blue shift, the difference spectra are still characterized by a maximum difference in the neighborhood of 355 nm. Although our binding results were successfully analyzed by the Scatchard procedure in terms of two sets with four and eight sites, a greater degree of heterogeneity is indicated from a quantitative examination of the shape of the stoichiometric curves. Such resolution of the binding is not possible from a mathematical analysis of the binding data alone. A quantitative examination of the stoichiometric plots and the heterogeneity which is indicated will be presented in a succeeding communication.

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