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Theory of Weak Molecular Complexes. III. Observation Equations for Multiple Equilibria and an Application to Protein Charge-Transfer Titrations[1]

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Abstract: The classical treatment of multi-site titration analysis is extended to include the specific experimental method used in the investigation of a binding system by means of a set of general *observation equations*. The observation equations are formulated for both statistical and intrinsic binding parameters and reduce to the classical equations in the special case of *direct* experimental methods, where the number of free and/or bound adsorbate molecules can be uniquely counted. For *indirect* experimental methods such as light absorption, an intensive parameter (*e.g.*, extinction coefficient) appears in the equations and cannot be factored from the sums unless independent information concerning the relationship of this parameter to the number of bound adsorbate molecules is available. Thus, in the general case of an indirect method, it is not possible to deduce the order of the reaction from titration experiments utilizing the intensive parameter alone. Multiple-site protein charge-transfer complexes are used as examples of an indirect titration method, and approximate treatments of the binding data are considered for these systems. Association constants and extinction coefficients obtained from 1:1 homomorphic model complexes of the protein donor amino acids with a suitable acceptor can be used to interpret the protein data and to extract information concerning both the number and kind of sites available provided that the protein data are analyzed in terms of intrinsic association constants and extinction coefficients. The present communication gives the mathematical basis for such an interpretation, as well as for less specific interpretations based on the classical straight-line technique for charge-transfer systems.

The analysis of data from studies of weak molecular complexes between donors and acceptors has been considered extensively by several authors,[2] most notably by Person.[3] A few years ago the present author attempted to give a theoretical explanation of Person's limit criteria for complexes in which complete saturation could not be reached and, following an earlier treatment by Weber,[4] extended the limit criteria on the basis of the amount of information obtained in a binding experiment.[5] In addition, the effects of second-order interactions on the calculated values of the extinction coefficients and association constants of charge-transfer complexes were examined.[6] More recently, we have been studying protein surface topography by probing for exposed aromatic amino acid donor residues with a suitable charge-transfer acceptor (1-methyl-3-carbamidopyridinium chloride).[7-9] The simple one- and two-site binding models previously considered do not provide an adequate description of these systems; each protein examined so far appears to have more than a single binding site for the probe, and, furthermore, some proteins have more than one type of site available for complexation (*e.g.*, both tryptophan and tyrosine side chains). In such cases the observed charge-transfer spectrum is composed of several nonidentical overlapping bands, each of which depends on the type of residue forming the complex and on the degree of saturation of the individual donor residues. The overlapping spectral distributions can be used to advantage in identifying the specific types of donors involved in the complex, but because of the very weak binding of the probe, it is not possible to obtain saturation of any of the donor sites. The inability to attain saturation requires the use of extrapolation techniques and other approximations to arrive at quantitative estimates of the extent of interaction and the number of residues participating in the binding.

Since the two-site model previously considered for charge-transfer complexes is inadequate for the description of multiple charge-transfer complexes on proteins, it was necessary to consider generalized equations for multiple equilibria. Such generalized equations are well known for methods in which the number of free and/or bound adsorbate molecules can be counted directly (*e.g.*, equilibrium dialysis), but general equations have apparently not been considered where an indirect method such as light absorption is used to estimate the extent of interaction. The key to a general analysis for any given experimental technique lies in the formulation of the relevant *observation equation*—the equation relating the desired molecular parameters to the experimental variable used to study the interaction.

This communication presents a simple derivation of a general observation equation for multi-site titration analysis and considers in some detail various forms of the equation, the slopes and intercepts of relevant graphs, and approximate methods of solution for weak complexes. Where appropriate, protein charge-transfer complexes are used as specific examples to clarify the discussion. The general equations reduce to classical equations involving only the number of sites and their respective association constants when certain assumptions concerning the relationship between the number of sites and the experimental variable are satisfied. In many instances these assumptions may change the character of the analysis and give rise to misleading or false interpretations of the data, as well as to erroneous values of the various molecular parameters deduced from otherwise appropriate graphs. In addition to the specific inclusion of the experimental variable used to study the interaction, the general equations quantitatively account for the nature of the assumptions involved in applying the classical equations for multiple equilibria.

Observation Equation for Multi-Site Titration Analysis. In each case of an equilibrium reaction, say $P + nX \rightleftharpoons PX_n$, we can define an intensive quantity $\bar{\Phi}$ which is the statistical average of the individual quantities Φ_j associated with the molecular species PX_j as follows

$$\bar{\Phi} \equiv \sum_{j=0}^{n} \Phi_j w_j [PX_j] / \sum_{j=0}^{n} w_j [PX_j] \tag{1}$$

Here the brackets indicate molar concentration, and the w_j are weight factors (*e.g.*, activity coefficients or intensity factors or both) appropriate to the particular experimental method being used. Examples of Φ_j include the number of binding sites, the molar extinction coefficient, the nmr chemical shift, molar ellipticity in circular dichroism, the rate constant for a chemical reaction, or any other intensive property of the complex. Introducing the macroscopic (statistical) association constants K_j defined by

$$[PX_j] \equiv K_j [PX_{j-1}][X] = \mathbf{K}_j [P][X]^j \tag{2}$$

where the $\mathbf{K}_j = K_0 K_1 K_2 \ldots K_j$ are the so-called *titration constants* and $\mathbf{K}_0 = K_0 = 1$, eq 1 becomes, with [P] not dependent on j

$$\bar{\Phi} = \sum_{j=0}^{n} \Phi_j w_j \mathbf{K}_j [X]^j / \sum_{j=0}^{n} w_j \mathbf{K}_j [X]^j = \frac{\Phi_0 w_0 + \Phi_1 w_1 K_1 [X] + \Phi_2 w_2 K_1 K_2 [X]^2 + \ldots}{w_0 + w_1 K_1 [X] + w_2 K_1 K_2 [X]^2 + \ldots} \tag{3}$$

Equation 3 is the *general observation equation* connecting the statistical average value of the intensive parameter Φ with its macroscopic values for the individual chemical species and the statistical equilibrium constants of the system. When $\Phi = \bar{\nu}$, the average number of X molecules bound per molecule of P, $\Phi_j = \nu_j = j$ and with unit weight factors eq 3 becomes identical with the classical equation first proposed by Adair in 1925 to explain the binding of oxygen to hemoglobin[10]

$$\bar{\nu} = \sum_{j=0}^{n} j \mathbf{K}_j [X]^j / \sum_{j=0}^{n} \mathbf{K}_j [X]^j \tag{4}$$

In contrast to Adair's eq 4, the general observation equation specifically includes the intensive parameter used to study the properties of an equilibrium distribution, and we turn next to an examination of the possible correspondence between eq 3 and 4.

Assignment of the Φ_j. In a direct method of binding analysis, where the number of bound (or unbound) adsorbate molecules can be counted independently, it is always possible to make the assignment $\bar{\Phi} = \bar{\nu}$ (and thus $\Phi_j = j$). Examples of such direct methods include equilibrium dialysis or the use of specific ion electrodes, in which the equilibrium distribution between two or more compartments separated by a membrane freely permeable to one of the components can be measured separately and unequivocally. In the majority of binding studies—largely spectroscopic in nature—it is not possible to count the number of free or bound molecules independently unless the Φ_j are all known to be uniquely related to one another.[11] In these cases recourse is had to some measurable quantity *other than* $\bar{\nu}$ which is presumed to be proportional to the amount of complex formed (absorbance, chemical shift, quenching of fluorescence, etc.). Furthermore, it is very often assumed that "proportional to" is to be interpreted as meaning the Φ_j are given by the simple arithmetic progression $\Phi_j = j\Phi$. This assumption is certainly not justified in many instances; yet, the desire to express the binding equations in terms of the number of sites occupied is compelling. Without loss of generality, we can introduce the definition $\Phi_j = j\Phi - \delta_j$ in eq 3, obtaining

$$\bar{\Phi} = \Phi \frac{\sum_{j=0}^{n} j w_j \mathbf{K}_j [X]^j}{\sum_{j=0}^{n} w_j \mathbf{K}_j [X]^j} - \frac{\sum_{j=0}^{n} \delta_j w_j \mathbf{K}_j [X]^j}{\sum_{j=0}^{n} w_j \mathbf{K}_j [X]^j} \tag{5}$$

The δ_j are the differences between the actual value of Φ_j and the value expected on the basis of additivity, and when the differences are all zero, setting all $w_j = 1$ and dividing (5) through by Φ gives Adair's equation in terms of the *experimentally measured ratio*

$$\bar{\Phi}/\Phi = \sum_{j=0}^{n} j \mathbf{K}_j [X]^j / \sum_{j=0}^{n} \mathbf{K}_j [X]^j \equiv \bar{\nu} \tag{6}$$

Here $0 \leq (\bar{\Phi}/\Phi) \leq n$, and it is this equation which has most often been used in the literature to evaluate allosteric and other types of multiple equilibria.[12]

Clearly this procedure is of limited applicability, requiring prior knowledge of the additivity relationships *and* the value of Φ. For protein charge-transfer systems, where the Φ_j are the extinction coefficients of the complexes PX_j, such an assignment $\epsilon_j = j\epsilon$ would be wholly inappropriate because at least two different kinds of donor–acceptor complexes with markedly different spectral properties can contribute to the extinction of a given complex. Large site-specific changes in Φ_j are not uncommon in other methods as well (*e.g.*, fluorescence), and they are not necessarily accompanied by large concomitant changes in the respective association constants. Figure 1 shows the effect of nonzero δ_2 values on the graph of $\bar{\Phi}/[X]$ *vs.* $\bar{\Phi}$ (double intercept or Scatchard plot) for a hypothetical two-site system with identical *microscopic* association constants.[6] Both positive and negative changes arising from the nonzero δ_2 result in curved lines on the plot and could be interpreted[6] as being due to positive cooperative effects which are in fact absent. Furthermore, the association constants and the number of sites deduced (respectively) from the slope and intercepts of the plot on the basis of eq 6 will not reflect the actual molecular situation save when the δ_j are all very small. When independent information concerning the magnitude of the δ_j is not available, analysis of the experimental data is best accomplished *via* the actual observation eq 3, which is free of potentially restrictive assumptions.

To show the use of the Φ_j for a specific example of an indirect method, consider the formation of multiple charge-transfer complexes between a suitable acceptor X and various donors on the surface of a protein P. From eq 1 the ob-

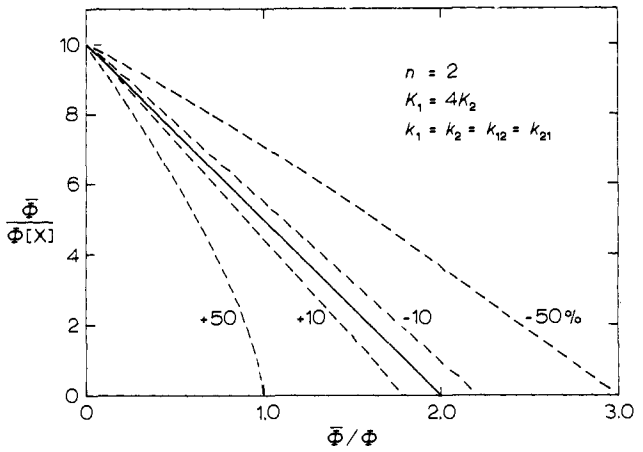

Figure 1. Double intercept plots of a hypothetical titration of two sites with identical microscopic association constants, showing the effects of changes in δ_2 on the shape of the curves. The number next to each curve represents the per cent change in the expected value of Φ_2 ($100\delta_2/2\Phi$). Constants used correspond to $\Phi = 1$, $n = 2$, $\delta_1 = 0$, $w_1 = w_2 = 1$, $K_1 = 4K_2 = 10$. See text for discussion.

served average extinction coefficient is given directly as

$$\bar{\epsilon} = \sum_{j=0}^{n} \epsilon_j[PX_j]/\sum_{j=0}^{n}[PX_j] \quad (7)$$

since the weighting factors in this case are all unity. The numerator of this expression is the definition of the absorbance $A = \Sigma_j A_j$ for unit path length (Beer's law), and the denominator is the conservation of mass equation for the total protein concentration [P]. Thus by definition $\bar{\epsilon} = A/[P]$, and using this identity in eq 3 in a spectral region where $\epsilon_0 = 0$ yields the observation equation for the formation of multiple charge-transfer complexes on a protein[13]

$$A/[P] = \sum_{j=1}^{n} \epsilon_j \mathbf{K}_j[X]^j/\sum_{j=0}^{n} \mathbf{K}_j[X]^j \quad (8)$$

The last equation is the generalization, to any number of sites, of the macroscopic two-site model previously discussed in detail.[6]

Use of the Weighting Factors w_j. In spectroscopic studies involving the return of excited molecules to their ground states, the intensive parameter of interest may depend not only on the concentration of the species PX_j but also on the amount of light absorbed by the complex. As an illustration, we consider the fluorescence intensity F of a given complex in very dilute solution, which is proportional to the product of the quantum efficiency η and the extinction coefficient and concentration of the complex. The fluorescence per mole of total P is given by

$$\sum_{j=0}^{n} \eta_j\epsilon_j[PX_j]/\sum_{j=0}^{n}[PX_j] = \overline{\eta\epsilon} = F/[P] \quad (9)$$

and does not involve weighting coefficients in the sense of eq 1, whereas the average quantum yield is weighted according to the extinction coefficients of the various species

$$\sum_{j=0}^{n} \eta_j\epsilon_j[PX_j]/\sum_{j=0}^{n} \epsilon_j[PX_j] = \bar{\eta} = F/A \quad (10)$$

These and other examples of weighted and unweighted observation equations for indirect methods are listed in Table I. The list is not intended to be exhaustive.

In all real systems, the association constants given in eq 2 may be concentration dependent by reason of changes in activity, and it may be preferable to use the concentration-independent constants defined by

$$\gamma_j[PX_j] = K_j^0\gamma_{j-1}\gamma_X[PX_{j-1}][X] = \mathbf{K}_j^0\gamma_0\gamma_X{}^j[P][X]^j \quad (11)$$

where γ_j is the activity coefficient of the species PX_j and $\mathbf{K}_j^0 = K_0^0K_1^0K_2^0 \ldots K_j^0$, $\mathbf{K}_0^0 = K_0^0 = 1$. The relation between the concentration-dependent and concentration-independent titration constants is $\mathbf{K}_j = \mathbf{K}_j^0\gamma_0\gamma_X{}^j/\gamma_j$ from eq 2 and 11, and the appropriate activity coefficients may be introduced as weighting coefficients in eq 1 and 3.[14]

Limiting Values and the Slope and Intercepts of Log and Double Intercept Plots. Graphical methods are commonly used to present and interpret data obtained in titration studies, and the advantages and disadvantages of several methods have been discussed previously.[5,6] Here we consider only the plots of $\bar{\Phi}$ *vs.* log [X] (formation function plot or titration curve) and $\bar{\Phi}/[X]$ *vs.* $\bar{\Phi}$ (double intercept or Scatchard plot). Furthermore, we assume for ease of discussion that the weighting coefficients can be included in the association constants in a manner similar to that described for activity coefficients above and that conditions can usually be found where $\Phi_0 = 0$. In this case eq 3 can be written in the simple form

$$\bar{\Phi} = \sum_{j=0}^{n} \Phi_j\mathbf{K}_j[X]^j/\sum_{j=0}^{n} \mathbf{K}_j[X]^j \quad (12)$$

Equation 12 will be referred to as the *reduced*, or corrected, observation equation and has the advantage that $\bar{\Phi}$ is zero when no complex is present. Note, however, that the $\mathbf{K}_j$ may be composite constants depending on the nature of the weighting coefficients.

The limiting values of the log plot of the reduced observation equation at low and high saturation are respectively zero and

$$\lim_{[X]\to\infty} \bar{\Phi} = \bar{\Phi}_\infty = \Phi_n \text{ (or } n\Phi - \delta_n) \quad (13)$$

On the double intercept plot, the high saturation limit is the intercept on the $\bar{\Phi}$ axis and is likewise given by eq 13. The low saturation limit is the intercept on the $\bar{\Phi}/[X]$ axis

$$\lim_{[X]\to 0} \bar{\Phi}/[X] = \Phi_1K_1 \text{ [or } (\Phi - \delta_1)K_1] \quad (14)$$

Table I. Representative Experimental Methods for Multi-Site Titration Analysis and Their Corresponding Observation Equations[a]

Measured quantity, definition	Observation eq	Wt factor
Absorbance (unit path length), $A_j = \epsilon_jc_j$	$\bar{\epsilon} = \Sigma_j\epsilon_jc_j/\Sigma_jc_j = A/[P]$	1
Circular dichroism (unit path length), $\Delta A_j = (\epsilon_{Lj} - \epsilon_{Rj})c_j$	$\bar{\Delta\epsilon} = \Sigma_j(\epsilon_{Lj} - \epsilon_{Rj})c_j/\Sigma_jc_j = \Delta A/[P]$	1
Fluorescence intensity (dilute solutions, $\Sigma_jA_j < 0.01$), $F_j = \eta_j\epsilon_jc_j$	$\bar{\eta} = \Sigma_j\eta_j\epsilon_jc_j/\Sigma_j\epsilon_jc_j = F/A$	ϵ_j
Anisotropy of fluorescence, $\mathfrak{A}_j = \dfrac{I_{\|i} - I_{\perp i}}{I_{\|i} + 2I_{\perp i}}$	$\bar{\mathfrak{A}} = \dfrac{\Sigma_j\mathfrak{A}_j\eta_j\epsilon_jc_j}{\Sigma_j\eta_j\epsilon_jc_j} = \dfrac{\Sigma_j\mathfrak{A}_jF_j}{F}$	$\eta_j\epsilon_j$
Chemical shift difference, $\bar{\delta}_j = \delta_j[PX_j]/[P]$	$\bar{\delta} = \Sigma_j\delta_jc_j/\Sigma_jc_j = \Sigma_j\delta_jc_j/[P]$	1
Velocity of a reaction catalyzed by a multi-site enzyme, $v_j = k_j^{(R)}c_j$	$\bar{k}^{(R)} = \Sigma_jk_j^{(R)}c_j/\Sigma_jc_j = v/[P]$	1

[a] Notation: c_j, molar concentration (or activity) of the species PX_j; ϵ, molar (decadic) extinction coefficient; η, quantum efficiency; $I_{\|}$, $I_{\perp}$, intensity components parallel and perpendicular to the electric field vector of the exciting radiation; $\Delta A = (360/8\pi \ln 10)\theta°$, where $\theta°$ is the observed ellipticity in degrees; $k^{(R)}$, rate constant.

It should be obvious from the last two equations that data evaluated at the intercepts alone cannot be used to determine the order of the reaction, even if the δ_j are all zero, since the product $n\Phi$ obtained at the high saturation limit of either plot cannot be separated. This is the basis for the earlier statement that Φ (or n) must be known in advance in order to perform an analysis in terms of Adair's equation. On the other hand, if the δ_j are all zero, dividing eq 14 by eq 13 gives K_1/n and n could be found from the ratio of the intercepts if K_1 were known. We now show that this procedure also fails to resolve the problem of determining n.

The log plot is generally an S-shaped curve, and the double intercept plot can take the form of a straight line or a curve concave up or down.[6] The slopes of these plots are obtained by differentiating the relevant form of eq 12. Performing this differentiation and evaluating the first few terms for $n \leq 2$ gives some idea of the complexity of the slopes and of the explicit dependence of each on both the concentration and the Φ_j

$$\text{log plot slope} = d\bar{\Phi}/d \log [\mathrm{X}] = \frac{\Phi_1 K_1[\mathrm{X}] + 2\Phi_2 K_1 K_2[\mathrm{X}]^2 + (K_1^2 K_2(\Phi_2 - \Phi_1) + \ldots)[\mathrm{X}]^3 + \ldots}{(1 + K_1[\mathrm{X}] + K_1 K_2[\mathrm{X}]^2 + \ldots)^2} \quad (15)$$

$$\text{double intercept plot slope} = d(\bar{\Phi}/[\mathrm{X}])/d\bar{\Phi} = \frac{\Phi_1 K_1 - \Phi_2 K_2 + 2(\Phi_1 K_1 K_2 - \ldots)[\mathrm{X}] + (\Phi_2 K_1 K_2^2 - \ldots)[\mathrm{X}]^2 + \ldots}{\Phi_1 + 2\Phi_2 K_2[\mathrm{X}] + (K_1 K_2(\Phi_2 - \Phi_1) + \ldots)[\mathrm{X}]^2 + \ldots} \quad (16)$$

Clearly it is not possible to obtain K_1 from these slopes by any simple procedure. At extremely low or extremely high saturation values, the concentration dependence of the slopes falls out, resulting in limiting slopes of zero at either end of the log plot, and limiting slopes of

$$-K_1 + K_2\Phi_2/\Phi_1 \qquad [\mathrm{X}] \longrightarrow 0 \quad (17)$$

$$-\Phi_n K_n/(\Phi_n - \Phi_{n-1}) \qquad [\mathrm{X}] \longrightarrow \infty \quad (18)$$

at the low and high saturation ends of the double intercept plot, respectively. In the simplest case, eq 13 and 14, together with eq 17 and 18, form a set of four equations in at least five unknowns, and no single constant save Φ_n can be uniquely determined from data obtained at the intercepts. In the general case, therefore, the only satisfactory procedure for analyzing the data from multi-site titrations using an indirect method is by curve fitting. However, the intercept data—when available—is useful in putting constraints on certain of the constants appearing in the reduced observation equation used to fit the data. The situation for weak complexes will be more difficult, for in these cases the high saturation limits may not be available, or worse, not enough of the curve can be obtained to ensure a representative fit. Setting $\Phi_j = \epsilon_j$ in eq 13–18 gives the general intercept and slope equations for charge-transfer systems. Notice that in no way can the observed slopes be considered as functions of the association constants alone, as is the case for the Adair equation.

Formulation in Terms of Intrinsic Association Constants. The Equivalent and Independent Site Approximation. In a variety of applications the statistical (macroscopic) association constants are not of primary interest in explaining the complexities of a binding system. Particularly with protein–ligand equilibria and with enzyme subunit interactions, the microscopic or site-specific association constants are the desired parameters, since they contain information about the individual (distinguishable) sites which may be of key importance for the explanation of modes of substrate binding or of allosteric interconversions. The actual microscopic constants are not generally available experimentally, for although they are defined in terms of the macroscopic constants, there are usually more microscopic constants than there are defining equations.[15]

A method for estimating the microscopic association constants which has proven to be extremely useful in protein studies, but which has never been rigorously applied in the general case to indirect titration methods, makes use of the concept of equivalent and independent sites. Here it is assumed that the individual sites of a given *class* (*e.g.*, tryptophanyl residues) are not only intrinsically identical but do not interact with each other or with sites of other classes. Following earlier treatments,[16] it can be shown that the macroscopic or statistical association constants for a set of *identical* sites are related to the *intrinsic* or site-specific association constants $k^{(j)}$ for the $\binom{n}{j}$ different microscopic species of the set having j bound X molecules by the relations

$$K_j = (n - j + 1)k^{(j)}/j$$

$$\mathbf{K}_j = K_1 K_2 \ldots K_j = \binom{n}{j} k^{(1)} k^{(2)} \ldots k^{(j)} \quad (19)$$

$$\binom{n}{j} = n(n-1)\ldots(n-j+1)/j = n!/j!(n-j)!$$

If the sites are *independent* as well as identical, the constants for PX_j must be the same as those for PX_{j-1}, etc.; thus $\Phi_j = j\phi$, $k^{(1)}k^{(2)} \ldots k^{(j)} = k^j$ and using these with eq 19 in eq 12 gives

$$\bar{\phi} = \phi[\mathrm{X}] \sum_{j=1}^{n} j\binom{n}{j} k^j [\mathrm{X}]^{j-1} \Big/ \sum_{j=0}^{n} \binom{n}{j} k^j [\mathrm{X}]^j = \phi[\mathrm{X}] d \ln \sum_{j=0}^{n} \binom{n}{j} k^j [\mathrm{X}]^j / d \ln [\mathrm{X}] = n\phi k[\mathrm{X}]/(1 + k[\mathrm{X}]) \quad (20)$$

for a single class of sites. The replacement $\bar{\phi} \equiv \bar{\Phi}$ is intended to emphasize that the resulting equations are based on the assumption of equivalent sites. Except for the appearance of $\bar{\phi}$ and ϕ in the equation, (20) has the same form as the classical equation for equivalent and independent sites. As before, when the δ_j are all zero, $\bar{\phi}/\phi \equiv \bar{\nu}$. If there are m classes total, $\bar{\phi}$ is given by the sum (average) over all classes i by

$$\bar{\phi} = \sum_{i=1}^{m} \bar{\phi}_i = \sum_{i=1}^{m} \frac{n_i \phi_i k_i [\mathrm{X}]}{1 + k_i[\mathrm{X}]} = \sum_{i=1}^{m} n_i \phi_i s_i \quad (21)$$

where

$$s_i = k_i[\mathrm{X}]/(1 + k_i[\mathrm{X}]), \quad 0 \leq s_i \leq 1 \quad (22)$$

is the saturation fraction of the ith class.

In principle each separate site can be taken as a unique class in itself ($n_i = 1$ for all "classes"), and the summation represented by eq 21 is thus equivalent to the description of the binding given by eq 12, provided only that the sites are *independent*. Expanding eq 21 in terms of a common denominator and collecting terms shows that, for example

$$\Phi_1 K_1 = \sum_{r=1}^{N} n_r \phi_r k_r$$

$$\Phi_2 K_1 K_2 = \sum_{s>r}^{N} \sum_{r=1}^{N-1} (n_r \phi_r + n_s \phi_s) k_r k_s$$

$$\Phi_j K_1 \ldots K_j = \sum_{j>u}^{N} \ldots \sum_{r=1}^{N-j+1} (n_r \phi_r + \ldots + n_j \phi_j) k_r \ldots k_j$$

$$K_1 = \sum_{r=1}^{N} k_r$$

$$K_1 K_2 = \sum_{s>r}^{N} \sum_{r=1}^{N-1} k_r k_s$$

$$K_1 \ldots K_j = \sum_{j>u}^{N} \ldots \sum_{r=1}^{N-j+1} k_r \ldots k_j \qquad (23)$$

where the subscripts $r, s, t, \ldots$ refer to the individual distinguishable sites. Equation 23 also shows the average or statistical character of the macroscopic quantities Φ_j in all the equations formulated in previous sections of this communication; since

$$\Phi_1 = \sum_{r=1}^{N} n_r \phi_r k_r / \sum_{r=1}^{N} k_r$$

$$\Phi_2 = \sum_{s>r}^{N} \sum_{r=1}^{N-1} (n_r \phi_r + n_s \phi_s) k_r k_s / \sum_{s>r}^{N} \sum_{r=1}^{N-1} k_r k_s \qquad (24)$$

$$\Phi_j = \sum_{j>u}^{N} \ldots \sum_{r=1}^{N-j+1} (n_r \phi_r + \ldots + n_j \phi_j) k_r \ldots k_j / \sum_{j>u}^{N} \ldots \sum_{r=1}^{N-j+1} k_r \ldots k_j$$

the Φ_j are weighted according to the intrinsic association constants. By analogy with the preceding discussion, the ϕ_i can be referred to as the intrinsic or site-specific values of the intensive parameter for sets of independent but not necessarily equivalent sites.

A plot of $\bar{\phi}$ *vs.* log [X] according to eq 21 has a lower saturation limit of zero and an upper saturation limit

$$\lim_{[X]\to\infty} \bar{\phi} = \bar{\phi}_\infty = \sum_i n_i \phi_i \qquad (25)$$

Equation 25 also gives the high saturation limit of the double intercept plot ($\bar{\phi}/[X]$ *vs.* $\bar{\phi}$), and the low saturation intercept is given by

$$\lim_{[X]\to 0} \bar{\phi}/[X] = \sum_i n_i \phi_i k_i \qquad (26)$$

Comparing eq 25 and 26 with eq 13 and 14 of the previous section, we find that

$$\Phi_n = \sum_i n_i \phi_i, \quad \Phi_1 K_1 = \sum_i n_i \phi_i k_i \qquad (27)$$

which leads to the definition

$$\Phi_1 K_1 / \Phi_n = \sum_i n_i \phi_i k_i / \sum_i n_i \phi_i \equiv \bar{k} \qquad (28)$$

The average value of the intrinsic association constant for the entire system, weighted according to the number of sites and their intrinsic ϕ_i, is thus uniquely determined by the high and low saturation intercepts of the double intercept plot. We note that while $\bar{k}$ is the "slope" of a straight line passing through both intercepts, the actual slope of the plot is a function of concentration

$$\frac{d(\bar{\phi}/[X])}{d\bar{\phi}} = \frac{\Sigma_i n_i \phi_i k_i^2 (1 + k_i[X])^{-2}}{\Sigma_i n_i \phi_i k_i (1 + k_i[X])^{-2}} = \bar{k}_X \qquad (29)$$

At the low and high saturation limits, the concentration dependence disappears and the limiting slopes are given by

$$\lim_{[X]\to 0} (\text{slope}) = \sum_i n_i \phi_i k_i^2 / \sum_i n_i \phi_i k_i = \bar{k}_0 \qquad (30)$$

$$\lim_{[X]\to\infty} (\text{slope}) = \sum_i n_i \phi_i / \sum_i n_i \phi_i k_i^{-1} = \bar{k}_\infty \qquad (31)$$

The observed slope of the plot (average tangent to the curve) thus changes from a second moment average (eq 30) to a reciprocal average (eq 31) during the course of a titration. The first moment average is defined by the intercepts alone (eq 28). The last three equations have been given previously, in slightly different form, for the specific case of protein charge-transfer systems.[17] For the sake of completeness, we should give the slope of the log plot ($\bar{\phi}$ *vs.* log [X])

$$d\bar{\phi}/d \ln [X] = \sum_i n_i \phi_i k_i [X](1 + k_i[X])^{-2} \qquad (32)$$

and mention it has upper and lower limits of zero.

Application to Weak Complexes. Protein Charge-Transfer Titrations. Two distinct problems arise in the analysis of data obtained with very weak multiple complexes, which are typical of protein charge-transfer systems.

(i) Because of very small association constants it is usually impossible to obtain a complete saturation curve. In particular, it will generally not be feasible to obtain experimental points at or near the high saturation intercept, where potentially useful information concerning the maximum value is available.

(ii) Curvature in the double intercept plot due to concentration or frequency effects may not be apparent, even if present, because of experimental scatter in the data. When curvature is readily apparent, curve fitting utilizing eq 12 can give satisfactory estimates of the statistical association constants and Φ_j for up to two or possibly three sites.[18] Beyond that, the fitting procedure becomes unwieldy, since the number of constants which must be determined is $2n$ ($\Phi_j \mathbf{K}_j$ and $\mathbf{K}_j$ for each j). The same reasoning applies to fitting data to eq 21, where $2m$ constants must be determined ($n_i \phi_i k_i$ and k_i for each i) but here it may be possible to group the sites according to class and thus effect an overall reduction in the number of fitted parameters. *When using homomorphic model systems as a basis for interpreting protein data, there is an added advantage to using intrinsic constants in fitting the data, since these will be directly comparable with the association constants determined for 1:1 model complexes.* This follows directly from inspection of eq 21, which reduces to eq 20 for any particular 1:1 model under discussion.

In the event that curvature is not apparent in the double intercept plot for weak complexes, or when it is so small as to be practically indistinguishable from experimental scatter, an approximate method of some usefulness for multisite titrations is the fit to a straight line. This is equivalent to the assumption that all sites are identical as well as independent, and the fitted line is described by a set of apparent constants through rearrangement of eq 20

$$\bar{\phi}/[X] = k_{app}(n\phi_{app} - \bar{\phi}) \qquad (33)$$

The slope k_{app} of such a line is the average tangent to the curve over the range of the experimental data, and *if this range is sufficiently large,*[5] the slope determined in this manner will be very roughly comparable with the slope $\bar{k}$ defined by the real intercepts (eq 28) and with the slope determined by averaging the actual slope $\bar{k}_X$ (eq 29) over the range of the available data. The high saturation intercept $n\phi_{app}$ of such a line will always satisfy the condition

$$n\phi_{app} \leq \bar{\phi}_\infty = \sum_i n_i \phi_i \qquad (34)$$

if there are no positive cooperative effects, since curvature in this case is always concave up.[6] The equality on the left-

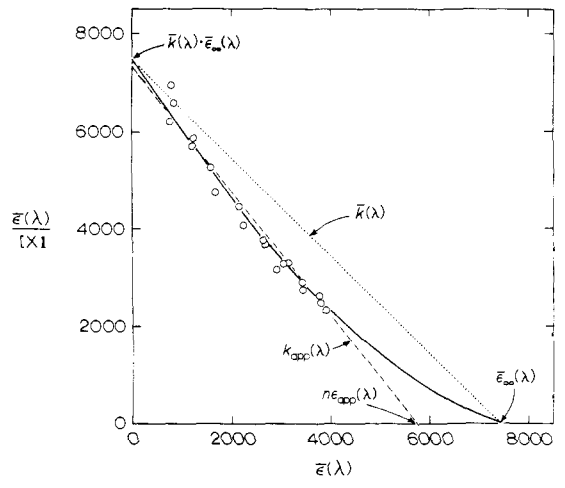

Figure 2. Double intercept plot of the charge-transfer titration of reduced and alkylated trypsin with 1-methyl-3-carbamidopyridinium chloride in 6 *M* guanidine hydrochloride at 350 nm. See text for details.

hand side of eq 34 applies when all ϕ_i save one are zero (1:1 complex or a set of completely identical sites).

These points are graphically illustrated in Figure 2 for the charge-transfer titration of exposed tryptophan and tyrosine donors in denatured trypsin (P) using 1-methyl-3-carbamidopyridinium chloride as an acceptor (X).[9] The theoretical curve was generated by means of eq 21 for two classes of sites, using $n_{Trp} = 4$, $k_{Trp} = 1.54$; $n_{Tyr} = 10$, $k_{Tyr} = 0.24$. The values for ϕ_i, in this case the extinction coefficients ϵ_{Trp} and ϵ_{Tyr}, were obtained from studies of 1:1 complexes of various tryptophanyl and tyrosinyl derivatives with the acceptor. The plot shows clearly that the actual value of $\bar{\epsilon}_\infty = \Sigma_i n_i \epsilon_i$ is underestimated by the straight line method, due to the small but significant curvature arising from nonidentical chromophores with differing association constants and extinction coefficients. The numerical values of interest, for comparison to the fitted intrinsic constants, are $k_{app} = 1.3$, $\bar{k} = 1.0$, $\bar{k}_0 = 1.4$, $\bar{k}_\infty = 0.5$; $n\epsilon_{app} = 5700$, $\bar{\epsilon}_\infty = 7430$. The intrinsic association constants used to fit the curve (1.54 and 0.24 for tryptophan and tyrosine, respectively) are both slightly smaller than the association constants of the 1:1 model complexes (1.7 and 0.26, respectively), undoubtedly reflecting the expectation that access to the sites in the denatured protein is randomly hindered by adjacent sections of the polypeptide chain. A point of more than passing interest is that in spite of the unfavorable tryptophan to tyrosine ratio, the characteristics of the measured part of the curve are practically all due to the tryptophan contribution in the example given.

It is appropriate to emphasize here that, depending on the method used, Φ_j and ϕ_i may be frequency-dependent quantities. Specific reference to this fact is made for charge-transfer complexes in Figure 2, where the wavelength dependence of the average extinction coefficient has been symbolically included in the form $\bar{\epsilon}(\lambda)$. As a consequence of the frequency dependence of Φ_j and ϕ_i, it is important to recognize that the observed slope, or average tangent to the curve, will be a function of frequency (wavelength) as well as of concentration. Thus, in eq 28–33, for example, we should denote the slopes by $\bar{k}(\lambda)$, $\bar{k}_X(\lambda)$, $\bar{k}_0(\lambda)$, etc. The frequency effect can be removed by integrating over all frequencies, using oscillator strengths in place of extinction coefficients for example. However, this does not remove the weighting due to the Φ_j or ϕ_i in the various definitions of the average slopes, and these quantities will vary from system to system.

Before leaving the discussion of various averages and approximations, we should mention one other average quantity of interest. When the high saturation limit $\bar{\phi}_\infty$ can be obtained experimentally or by curve-fitting to an appropriate model, the overall average saturation fraction can be calculated from a combination of eq 21 and 25, since

$$\bar{\phi}/\bar{\phi}_\infty = \sum_i n_i \phi_i s_i / \sum_i n_i \phi_i = \bar{s} \qquad (35)$$

Comparing the last with a similar relationship obtained from eq 12 and 13 shows that

$$\bar{\Phi}/\bar{\Phi}_\infty = \sum_{j=1}^{n} \Phi_j \mathbf{K}_j [X]^j / \Phi_n \sum_{j=0}^{n} \mathbf{K}_j [X]^j = \bar{s} \qquad (36)$$

because the quantities on the extreme left of both equations are identities ($\bar{\Phi} \equiv \bar{\phi}$, $\bar{\Phi}_\infty \equiv \bar{\phi}_\infty$). Under the assumption of linearly related Φ_j (all δ_j = zero), eq 36 becomes

$$\bar{\Phi}/\bar{\Phi}_\infty = \sum_{j=1}^{n} j\mathbf{K}_j [X]^j / n \sum_{j=0}^{n} \mathbf{K}_j [X]^j \equiv \bar{\nu}/n = \bar{s} \qquad (37)$$

It is therefore always possible to calculate $\bar{s}$ from an indirect method (if $\bar{\Phi}_\infty \equiv \bar{\phi}_\infty$ is known), but $\bar{\nu}$ cannot be obtained unless separate information concerning n is available (this is another way of stating that it is not generally possible to deduce the order of a reaction from an indirect method alone; see discussion following eq 14).

Different Protein Species in Equilibrium. The General Model. An equation of the form of eq 12 is necessary and sufficient to describe all of the species resulting from the equilibrium $P + nX \rightleftharpoons PX_n$, since each of the species PX_j is unique in the thermodynamic sense (average over a great many, possibly *allosteric* complexes of P with j molecules of bound X). In certain cases it can be shown that P itself exists as two or more discrete species in equilibrium with each other and with X, and the entire array of possible macroscopically distinct forms can be written in terms of the *species matrix*

$$\begin{vmatrix} P_1 & P_1X & P_1X_2 & \dots & P_1X_n \\ P_2 & P_2X & P_2X_2 & \dots & P_2X_n \\ & & & & \\ P_m & P_mX & P_mX_2 & \dots & P_mX_n \end{vmatrix} = C_{ij}$$

$$i = 1, 2, \dots m; \; j = 0, 1, 2, \dots n \qquad (38)$$

The reduced observation equation for C_{ij} is obtained from eq 12 by summing over all the i species

$$\bar{\Phi} = \sum_{i=1}^{m} \sum_{j=1}^{n} \Phi_{ij} [P_iX_j] / \sum_{i=1}^{m} \sum_{j=0}^{n} [P_iX_j] =$$

$$\sum_{i=1}^{m} \sum_{j=1}^{n} \Phi_{ij} \mathbf{K}_{ij} [P_i][X]^j / \sum_{i=1}^{m} \sum_{j=0}^{n} \mathbf{K}_{ij} [P_i][X]^j \qquad (39)$$

where we have used

$$[P_iX_j] = K_{ij}[P_iX_{j-1}][X] = \mathbf{K}_{ij}[P_i][X]^j \qquad (40)$$

with $\mathbf{K}_{i0} = K_{i0} = 1$ in place of eq 2 to define the set of association constants for the binding of X. The $[P_i]$ in eq 39 and 40 can be written in terms of the equilibria $P_1 \rightleftharpoons P_2 \rightleftharpoons \dots$ by making use of the definitions

$$[P_i] = K_i[P_{i-1}] = K_1K_2 \dots K_i[P_1] = \mathbf{K}_i[P_1] \qquad (41)$$

where $\mathbf{K}_1 = K_1 = 1$, so that eq 39 becomes

$$\bar{\Phi} = \sum_{i=1}^{m} \sum_{j=1}^{n} \Phi_{ij} \mathbf{K}_{ij} \mathbf{K}_i [X]^j / \sum_{i=1}^{m} \sum_{j=1}^{n} \mathbf{K}_{ij} \mathbf{K}_i [X]^j \qquad (42)$$

The last equation contains a complete set of independent constants describing the set of equilibria involved, indexed

according to the species matrix C_{ij} to avoid confusion. The set of association constants K'_{ij} for the reactions $P_{i-1}X_j \rightleftharpoons P_iX_j$ is not independent and can be found from the relations

$$K'_{ij} \equiv [P_iX_j]/[P_{i-1}X_j] = K_i\mathbf{K}_{ij}/\mathbf{K}_{i-1,j} \quad j > 1 \quad (43)$$

so that eq 42 and 43 together contain all of the possible macroscopic constants of the system. Comparing eq 42 with eq 12, we find that both have the same form provided that the constant terms in eq 12 are interpreted as

$$\mathbf{K}_j = \sum_{i=1}^{m}\mathbf{K}_{ij}\mathbf{K}_i, \quad \Phi_j\mathbf{K}_j = \sum_{i=1}^{m}\Phi_{ij}\mathbf{K}_{ij}\mathbf{K}_i \quad (44)$$

This is the observation equation equivalent of the statement that all possible forms of the indirect binding equations are special cases of Adair's equation.

The reduced observation eq 42 can also be written in terms of the intrinsic association constants for identical sites

$$K_{i1}\ldots K_{ij} = \binom{n_i}{j} k_i^{(1)}\ldots k_i^{(j)} \quad (45)$$

If the sites are equivalent and independent for a particular i, then $k_i^{(1)}\ldots k_i^{(j)} = k_i^j$ and $\Phi_{ij} = j\phi_i$ such that

$$\bar{\phi} = \sum_{i=1}^{m}\sum_{j=1}^{n} j\phi_i\binom{n_i}{j}k_i^j\mathbf{K}_i[X]^j \Big/ \sum_{i=1}^{m}\sum_{j=0}^{n_i}\binom{n_i}{j}k_i^j\mathbf{K}_i[X]^j \quad (46)$$

The last equation can be simplified if n_i and k_i are the same for all i (binding sites are not excluded by changing the form of P, and all forms of P have intrinsically identical sites); then

$$\bar{\phi} = \sum_{i=1}^{m}\phi_i\mathbf{K}_i[X]\Big(\sum_{j=1}^{n} j\binom{n}{j}k^j[X]^{j-1}\Big) \Big/ \sum_{i=1}^{m}\mathbf{K}_i\Big(\sum_{j=0}^{n}\binom{n}{j}k^j[X]^j\Big) = n\bar{\phi}_i k[X]/(1 + k[X]) \quad (47)$$

where $\bar{\phi}_i = \Sigma_i\phi_i\mathbf{K}_i/\Sigma_i\mathbf{K}_i$ is weighted according to the protein–protein association constants. With the exception of the appearance of the average quantity $\bar{\phi}_i$, eq 47 is the same as eq 20. Evidently in a system of completely equivalent and independent sites, the form of the protein is immaterial as long as binding sites are not created or excluded among the different protein species, and the correct value for the association constant will be obtained from a plot of $\bar{\phi}/[X]$ *vs.* $\bar{\phi}$. However, the intercepts may be a function of $\mathbf{K}_i$, depending on the particular intrinsic parameter used in the investigation. A specific example has been given for the case in which P_1 is a monomer, P_2 a dimer, etc.[19]

The assumption of completely equivalent and independent sites involved in deriving eq 47 does not necessarily represent a physically interesting case for proteins, but as before we can consider the equation to hold for an individual class of sites and sum over all classes p to obtain

$$\bar{\phi} = \sum_{p=1}^{M} n_p\bar{\phi}_{ip}k_p[X]/(1 + k_p[X]) = \sum_{p=1}^{M} n_p\bar{\phi}_{ip}s_p \quad (48)$$

As with the all identical and equivalent site model, eq 48 has an analogous form—eq 21—but the k_p are averages over all the i protein species with sites of class p, and the $\bar{\phi}_{ip}$ are similarly defined.

When the protein species are independent of one another (mixture) or are very slowly interconverting with respect to the time of measurement, the species matrix C_{ij} can be summed over the individual rows to give

$$\bar{\Phi} = \sum_{i=1}^{m}\Big(\sum_{j=1}^{n}\Phi_{ij}K_{ij}[X]^j \Big/ \sum_{j=0}^{n}K_{ij}[X]^j\Big) \quad (49)$$

If the equivalent and independent site approximation is applied to eq 49, it reduces identically to eq 21. Equation 21 is therefore applicable to mixtures of different protein species, each with a single set of equivalent and independent sites, as well as to several sets of equivalent and independent sites on a single protein species.

References and Notes

(1) Supported by NSF Grant GB 18016. For papers I and II of the series, see ref 5 and 6.

(2) For a review, see R. S. Mulliken and W. B. Person, "Molecular Complexes," Wiley-Interscience, New York, N.Y., 1969, and R. Foster, "Organic Charge Transfer Complexes," Academic Press, New York, N.Y., 1969.

(3) W. B. Person, *J. Amer. Chem. Soc.*, **87**, 167 (1965).

(4) G. Weber in "Molecular Biophysics," B. Pullman and M. Weissbluth, Ed., Academic Press, New York, N.Y., 1965.

(5) D. A. Deranleau, *J. Amer. Chem. Soc.*, **91**, 4044 (1969).

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(7) D. A. Deranleau, R. A. Bradshaw, and R. Schwyzer, *Proc. Nat. Acad. Sci. U.S.*, **63**, 885 (1969); R. A. Bradshaw and D. A. Deranleau, *Biochemistry*, **9**, 3310 (1970). (At the time of this work, it was believed that a single tryptophanyl residue was available for complexation on the protein studied [lysozyme], but subsequent studies indicate that both a tryptophan and a tyrosine are available; see ref 8.)

(8) L. M. Hinman, C. R. Coan, and D. A. Deranleau, *J. Amer. Chem. Soc.*, **96**, 7067 (1974).

(9) C. R. Coan, L. M. Hinman, and D. A. Deranleau, manuscript in preparation.

(10) G. S. Adair, *J. Biol. Chem.*, **63**, 529 (1925).

(11) In a molecular weight analysis of an aggregating protein system, for example, the Φ_i must all be integral numbers. The significance of a derivation in terms of the average number of bound molecules lies in the demonstration that most if not all other binding equations involving a direct determination of $\bar{\nu}$—the Scatchard–Klotz model for equivalent and independent sites, the "allosteric" models of Koshland, *et al.*, and Monod, *et al.*, and so forth—are all special mathematical cases of Adair's equation [M. E. Magar and R. F. Steiner, *J. Theor. Biol.*, **32**, 495 (1971)].

(12) An equivalent formulation can be given in terms of the average saturation fraction $\bar{s} \equiv \bar{\nu}/n$ by dividing eq 6 by the total number of sites. In either formulation the equation presupposes that Φ or $n\Phi$ are known in advance or can be determined independently. Note that information obtained at the high saturation limits of the titration curve is insufficient to distinguish between n and Φ, even if all δ are zero. Magar's rather complete discussion of the Adair equation (M. E. Magar, "Data Analysis in Biochemistry and Biophysics," Academic Press, New York, N.Y., 1972, Chapter 13) evidently assumes that the method used to investigate the binding is a direct one.

(13) Equation 8 can be written in the form of eq 5 by using $\epsilon_j = j\epsilon - \delta_j$ and dividing both sides of the result by ϵ gives

$$A/\epsilon[\mathbf{P}] = \bar{\nu} - \sum_{j=1}^{n}\delta_j\mathbf{K}_j[X]^j/\epsilon\sum_{j=0}^{n}\mathbf{K}_j[X]^j$$

which shows the nature of the correction term to be applied when it is desired to express the results in terms of the number of bound X molecules. As discussed in the text, the correction term will be significant, and in addition "ϵ" is a relatively meaningless quantity. Similar conclusions might well apply in a variety of methods where Adair's equation rather than the appropriate observation equation has been used to evaluate the experimental data.

(14) E. H. Lane, S. D. Christian, and J. D. Childs, *J. Amer. Chem. Soc.*, **96**, 38 (1974), give a discussion of the use of activity coefficients in charge-transfer systems.

(15) The relationships between the macroscopic and microscopic constants are discussed extensively by J. T. Edsall and J. Wyman, "Biophysical Chemistry," Vol. I, Academic Press, New York, N.Y., 1958, Chapter 11.

(16) I. M. Klotz in "The Proteins," Vol. 1B, H. Neurath and K. Bailey, Ed., Academic Press, New York, N.Y., 1953, p 727 ff.

(17) Reference 7, Appendix of second paper listed.

(18) A discussion numerical fitting procedures applicable to the Adair equation is given by M. E. Magar ("Data Analysis in Biochemistry and Biophysics," Academic Press, New York, N.Y., 1972), and G. Weber and S. R. Anderson [*Biochemistry*, **4**, 1942 (1965)] have devised a test as to whether a fit to Adair's equation is possible within experimental error.

(19) D. A. Deranleau, *J. Chem. Phys.*, **40**, 2134 (1964).