Multiplicity of Binding. Range of Validity and Practical Test of Adair's Equation*

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ABSTRACT: In this paper we discuss the theoretical aspects of binding necessary for the interpretation of the experimental results to be described in the second paper. Frequent reference will also be made to a recent publication by one of us (Weber, G., 1965, *in* Molecular Biophysics, Pullman, B., and Weissbluth, M., eds., New York, Academic) where the general problem of binding of small molecules to proteins is discussed in detail. To begin with we consider the information content of binding experiments and the form of presentation of the experimental data. Next we examine the problems of cooperative binding: in attempting a description of cooperative binding a distinction must be made between those cases in which the dissociation constants of the protein–ligand complexes are independent of the degree of saturation of the system and those in which they are functions of it. We shall demonstrate that binding of an arbitrary number of moles of ligand by a protein existing in an indefinite number of tautomeric forms in equilibrium belongs to the first type, and can always be described by an equation proposed by Adair (Adair, G. S., 1925, *J. Biol. Chem. 63*, 529).

We shall establish a new criterion to determine whether the experimental data conform to this equation. We will finally discuss the properties of the binding system that are implied by its failure to conform to Adair's equation.

A reversible equilibrium in which two components participate (protein and ligand in our case) is completely defined when the concentration of the three partners: free protein [P], free ligand [X], and protein–ligand complex [PX], are known, under fixed conditions of solvent composition and temperature.[1] If the total concentration of protein $[P_0]$ and the total concentration of ligand $[X_0]$ are given, the simple specification of the degree of saturation $\varphi = [PX]/[P_0]$ appears sufficient to define the system, since $[X] = [X_0] - \varphi[P_0]$ and $[P] = (1 - \varphi)[P_0]$. However, if [X] is smaller than $\delta\varphi[P_0]$ where $\delta\varphi$ is the standard error in the determination of φ, [X] is *experimentally* zero over the range of values of φ to which the uncertainty applies. The foreknowledge that there is always some free ligand in solution has often prevented the realization that for all practical purposes what is detected under these circumstances is not an equilibrium but the stoichiometric addition of the ligand to the protein. In a plot of φ against $[X_0]$, stoichiometric conditions are indicated by the existence of a straight line segment (Weber, 1965). If a dissociation constant K is determined from the data, it is only valid for the range of values outside the stoichiometric range. In cases in which more than 1 mole of ligand is bound/mole of protein, this implies that the value of K so computed is an average in which the weight of the moles bound at undetectable free ligand concentration is negligible in comparison with the weight of those bound at measurable free ligand concentration. When 1 single mole of ligand is bound/mole of protein the dissociation constant obtained is assumed to be valid for all φ values. While this is probably correct in equilibria between relatively simple molecules it can never be safely assumed in protein solutions where the homogeneity in the properties of the whole molecular population may be questionable.

Information Content of Binding Experiments. A probability of binding, p, may be defined (Weber, 1965) as p = (actual concentration of ligand–protein complexes)/(maximum possible concentration of ligand–protein complexes). Thus

$$p = [PX]/[P_0] \quad \text{if } P_0 \leqslant X_0$$
$$p = [PX]/[X_0] \quad \text{if } P_0 \geqslant X_0 \qquad (2)$$

If we prescribe that under our experimental conditions

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[1] We assume in this case the existence of a simple equilibrium $P + K \rightleftharpoons PX$. In the general case $P + NX \rightleftharpoons PX_N$, knowledge of the individual concentrations $[PX_1], \ldots, [PX_N]$ is required for the complete definition of the system. Exact determination of the distribution of the forms PX_i is not feasible; the most that can be expected in the general case is the knowledge of the average number $\bar{n}$ of moles of ligand bound/mole of protein. The degree of saturation $\varphi = \bar{n}/N$ equals in general,

$$\varphi = \frac{[PX_1] + 2[PX_2] + \ldots\ldots + N[PX_N]}{N\{[P] + [PX_1] + [PX_2] + \ldots + [PX_N]\}} = \frac{\sum_{i=1}^{i=N} i[PX_i]}{N \sum_{i=0}^{i=N} [PX_i]} \qquad (1)$$

P_0 should never exceed $[X_0]$, the degree of saturation $\varphi = p$, and therefore represents a true probability of binding. Apart from the practical value in avoiding altogether the ambiguous situation described in the last paragraph, our identification of φ and p enables (Weber, 1965): (1) the quantitation of the information obtained from a binding experiment; (2) the evaluation of the possibility of resolution of the over-all binding process into unitary components.

For these purposes we only require the introduction of the well-known relation between probability and information (*e.g.*, Brillouin, 1962; Yaglom and Yaglom, 1959):

$$I(p) = -p \log_2 p - (1 - p) \log_2 (1 - p) \qquad (3)$$

Since logarithms to the base 2 are used $I(p)$ is given in binary units or "bits." An immediate consequence of the above equation is that $I(p) = 0$ when $p = 1$ or $p = 0$. As may be recognized intuitively we do not possess any information about binding as a reversible process unless free and bound protein and free ligand are detectable. The total information in binary units obtained in an experiment in binding in which L different measurements of p are taken is

$$I(L) = \sum_{i=1}^{i=L} I(p_i) \qquad (4)$$

The value of $I(L)$ cannot be increased arbitrarily by simply increasing the number of measurements. Two values of p differing by less than $2\delta p$, where δp is the standard deviation of an independent measurement of p, convey essentially the same information. Therefore the maximum information obtainable in a study of binding, in which the standard deviation in the measurements of p is $\pm\delta p$, is given by

$$I_{max} = \sum_{i=1}^{i=1/2\delta p} I(p_i) \qquad (5)$$

and corresponds to $L = 1/2\delta p$ measurements of p separated by equal intervals $2\delta p$. Figure 1 gives the information in binary units as a function of L when the measurements are evenly spaced along the p coordinate.

When the binding experiments in the literature are reviewed from the point of view developed above, the incomplete character of most of them becomes apparent. In many cases a majority of the values of φ reported fall in the stoichiometric range, and therefore have no information content. In *most* experiments reported on the binding of NADH by dehydrogenases the information content ranges from 1.5 to 3 bits.

In our own experiments we have attempted to cover as wide a range of p as feasible, approaching $p = 1$ and $p = 0$ at the extremes and trying to reach the ideal $1/2\delta p$ evenly spaced measurements of p. Since in our case $\delta p = 0.02$–0.03 in the different experiments, this has necessitated the measurement of 10–20 significantly different values of p/experiment (8–15 bits).

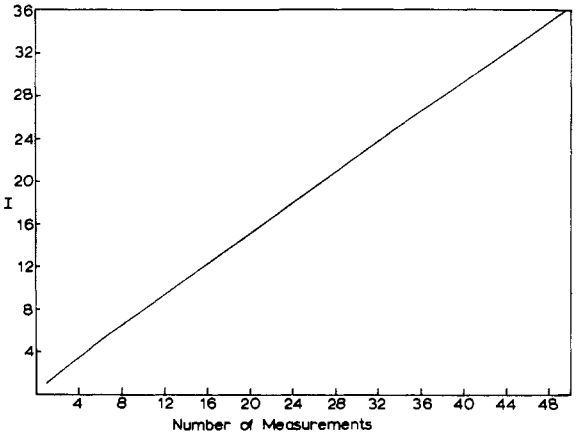

FIGURE 1: Information in binary units, I as a function of the number of measurements of the probability of binding. Equation (4) has been employed in the calculation. L values of I (p_i) have been added, spacing the L values of p_i evenly from 0 to 1.

In the presentation of the data we have plotted the concentration of the free ligand (log [X]) against $Np = \bar{n}$. In our opinion this plot should be used to the exclusion of other forms of presentation of the experimental data. The ideal form of presentation should be one that excludes values at which no free ligand is detectable, that is, those at which stoichiometric binding takes place. The use of log [X] for one of the coordinates fits this purpose since the point [X] = 0 is excluded from the graph. Two or more orders of magnitude in [X] have to be covered so that the use of functions like [X] or 1/[X] is impractical. For the other coordinate $\bar{n}$ or $p = \varphi$ is the natural choice.[2]

Plots in which the data are reduced to a linear form, with the purpose of evaluating a dissociation constant, ought to be particularly avoided. In the experimental studies that we shall describe here and in others in progress in our laboratory we would have been unable to detect many of the interesting regularities present had we resorted to reduction of the data to a linear form. This mode of operation followed in many binding studies results often in throwing away much of the information in the quest for a golden number, "the dissociation constant" of the system.

Determination of Stoichiometry. For the determinations of stoichiometry one requires measurements of the degree of saturation at undetectable free ligand concentrations, that is, under conditions in which p approaches unity. It appears convenient to use a separate treatment of the experimental data relating to stoichiometry and to the reversible binding process,

[2] Although a plot of this type has been repeatedly recommended on several grounds (*e.g.*, Scatchard *et al.*, 1957; Tanford, 1961) and is of common use in reporting results on O_2 binding by hemoglobin (*e.g.*, Rossi-Fanelli *et al.*, 1964), its use is far from general.

since the data appropriate for one of these purposes are unsuitable for the other (Weber, 1965). Dissociation constant and stoichiometry are sometimes obtained from one single graphical plot by the use of some extrapolation procedure; this is only possible if the binding process is assumed to follow some definite rule like the uniqueness or independence of the binding constants. If values of degree of saturation at which p approaches unity are alone used in the determination of stoichiometry, no hypothesis of any kind as to the dissociation constants of the system need be made.

The Problem of Cooperative Binding. The classical example, and until recently the only well-documented case, of cooperative effects is found in the binding of O_2 by hemoglobins [*e.g.*, Rossi-Fanelli *et al.* (1964); Wyman (1964)]. The characteristic feature of cooperative binding is that simple superposition of elementary independent binding processes is insufficient to account for it. Binding at independent sites is described by

$$n = \sum_{i=1}^{i=N} \frac{[X]}{[X] + K_i} \qquad 1 \leqslant i \leqslant N \tag{6}$$

where $\bar{n}$ is the average number of moles of ligand bound/mole of protein at free ligand concentration [X], and the K values are the dissociation constants characteristic of the N binding sites. If a single mole is bound or if all the K values are equal, in a plot of log $R = \log \bar{n}/(N - n)$ against log [X], a span of two logarithmic units in [X] is covered between $R = {}^1/_{10}$ and $R = 10$. Equation (6) predicts that this span of two units is lengthened, *never shortened*, if two or more independent dissociation constants are operative (*e.g.*, Weber, 1965). Cooperative binding on the other hand is characterized by a shortened logarithmic span. As shown by Adair in his classical studies (1925) these cases can be handled by an equation of the type

$$\bar{n} = \frac{\sum_{i=1}^{i=N} i \frac{[X]^i}{K_i}}{1 + \sum_{i=1}^{i=N} \frac{[X]^i}{K_i}} \tag{7}$$

now known as Adair's equation.

The belief that proteins can exist in more than one conformation has promoted as an explanation of cooperative binding the displacement in equilibria among the conformations due to ligand binding (Changeux 1965). We shall show first that an equation of the form of (7) is obtained for a system of an indefinite number m of protein tautomers in equilibrium, binding an arbitrary number N of moles of ligand.

Binding by Protein Tautomeric Forms. In the scheme (8) P_jX_i ($1 \leqslant j \leqslant m$; $0 \leqslant i \leqslant N$) designates the complex of the jth protein conformation with i moles of ligand. The concentrations of the conformations next to each other in any one row are related by the first-order constants

$$k_{j,i} = \frac{[P_{j+1}X_i]}{[P_jX_i]} \tag{9}$$

In any one column addition of 1 mole of ligand to the ith conformation is described by the second-order constants

$$K_{j,i+1} = \frac{[P_jX_i][X]}{[P_jX_{i+1}]} \tag{10}$$

From the above equations it appears that the concentration $[PX_i]$ ($= \sum^{m} [P_jX_i]$) of the complexes of protein with i moles of ligand, irrespective of the conformation involved, may be written as a function of the concentration of any arbitrary conformation $[P_rX_i]$ as follows:

$$[PX_i] = \sum_{j=1}^{j=m} [P_jX_i] = [P_rX_i] \left[\frac{1}{k_{1,i}\ k_{2,i} \ldots k_{r-1,i}} + \ldots + \frac{1}{k_{r-1,i}} + 1 + k_{r,i} + k_{r,i}\, k_{r+1,i} + \ldots + k_{r,i} \ldots k_{m,i} \right] = [P_rX_i]C_{r,i} \tag{11}$$

where $C_{r,i}$ depends upon the first-order constants alone. Similarly

$$[P] = \sum_{j=1}^{j=m} [P_j] = [P_r]C_{r,0} \tag{12}$$

We now introduce *apparent* dissociation constants $K_I, K_{II}, \ldots K_N$. These are the dissociation constants that would be observed for the binding of the first, second, ... nth mole of ligand respectively, regardless of the protein conformations involved in the binding. They are given by the equations:

$$K_I = \frac{\sum_{j=1}^{j=m} [P_j][X]}{\sum_{j=i}^{j=m} [P_jX]}$$

$$\begin{array}{ccccccccc}
P_1 & \overset{k_{1,0}}{\rightleftharpoons} & P_2 & \ldots\ldots & P_{r-1} & \overset{k_{r-1,0}}{\rightleftharpoons} & P_r & \overset{k_{r,0}}{\rightleftharpoons} & P_{r+1} \ldots P_m \\
K_{1,1}\downarrow\uparrow & & \downarrow\uparrow K_{2,1} & & & & & & \\
P_1X & \overset{k_{1,1}}{\rightleftharpoons} & P_2X & \ldots\ldots & P_{r-1}X & \overset{k_{r-1,1}}{\rightleftharpoons} & P_rX & \overset{k_{r,1}}{\rightleftharpoons} & P_{r+1}X \ldots P_mX \\
K_{1,2}\downarrow\uparrow & & \downarrow\uparrow K_{2,2} & & & & & & \\
\vdots & & \vdots & & & & & & \\
K_{1,N}\downarrow\uparrow & & \downarrow\uparrow K_{2,N} & & & & & & \\
P_1X_N & \overset{k_{1,N}}{\rightleftharpoons} & P_2X_N & \ldots\ldots & P_{r-1}X_N & \overset{k_{r-1,N}}{\rightleftharpoons} & P_rX_N & \overset{k_{r,N}}{\rightleftharpoons} & P_{r+1}X_N \ldots P_mX_N
\end{array} \tag{8}$$

$$K_{II} = \frac{\sum_{j=1}^{j=m}[P_jX][X]}{\sum_{j=1}^{j=m}[P_jX_2]} = \frac{\sum_{j=1}^{j=m}[P_j]}{\sum_{j=1}^{j=m}[P_jX_2]} \times \frac{[X]^2}{K_I}$$

$$K_N = \frac{\sum_{j=1}^{j=m}[P_jX_{N-1}][X]}{\sum_{j=1}^{j=m}[P_jX_N]} = \frac{\sum_{j=1}^{j=m}[P_j]}{\sum_{j=1}^{j=m}[P_jX_N]} \times \frac{[X]^N}{K_IK_{II}\ldots K_{N-1}} \quad (13)$$

By introduction of equations (11) and (12) into equation (13) the apparent dissociation constants may be written as

$$K_I = \frac{C_{r,0}}{C_{r,1}}K_{r,1};\ K_{II} = \frac{C_{r,1}}{C_{r,2}}K_{r,2};\ K_N = \frac{C_{r,N-1}}{C_{r,N}}K_{r,N} \quad (14)$$

Therefore $K_I \ldots K_N$ are true constants, independent of the degree of saturation of the system if the $C_{i,j}$ quantities are themselves constants. The average number n of moles of ligand bound/mole of protein is

$$\bar{n} = \frac{\sum_{i=1}^{i=N}\sum_{j=1}^{j=m} i\,[P_jX_i]}{\sum_{j=1}^{j=m}[P_j] + \sum_{i=1}^{i=N}\sum_{j=1}^{j=m}[P_jX_i]} \quad (15)$$

or

$$\bar{n} = \frac{\frac{[X]}{K_I} + 2\frac{[X]^2}{K_IK_{II}} + \ldots + N\frac{[X]^N}{K_IK_{II}\ldots K_N}}{1 + \frac{[X]}{K_I} + \frac{[X]^2}{K_IK_{II}} + \ldots + \frac{[X]^N}{K_IK_{II}\ldots K_N}} \quad (16)$$

The last is Adair's equation (7) in which $K_1 = K_I$; $K_2 = K_IK_{II}$; $K_N = K_IK_{II}\ldots K_N$. We can now assert that the binding of several moles of ligand by a system of protein conformations must always be described by an equation of the Adair type if the relative proportions of the conformations that make up each given molecular species (P, PX, PX_2, etc.) remain constant, independently of the average number of moles of ligand bound. The introduction of an arbitrary number of protein forms ensures the independence of the dissociation constants $K_{i,j}$ from $\bar{n}$. Therefore it is also true that if a system cannot be described by Adair's equation the proportions of the protein forms that make up each of the species P, PX, ... PX_n cannot remain constant for all n. If the quantities $k_{i,j}$ determining the proportions of the tautomers are truly first-order constants, the above statement implies that detailed balance is not operative in the system.

Practical Tests of Adair's Equation. From equation (7)

$$R = \frac{\bar{n}}{N - \bar{n}} = \frac{\sum_{i=1}^{i=N} i\frac{[X]^i}{Ki}}{N + \sum_{i=1}^{i=N}(N-i)\frac{[X]^i}{K_i}} \quad (17)$$

and consequently,

$$\frac{dR}{d\log[X]} = \frac{\left(\sum_{i=1}^{i=N} i^2\frac{[X]^i}{K_i}\right)\left(N + \sum_{i=1}^{i=N}(N-i)\frac{[X]^i}{K_i}\right) - \left(\sum_{i=1}^{i=N} i\,(N-i)\frac{[X]^i}{K_i}\right)\left(\sum_{i=1}^{i=N} i\frac{[X]^i}{K_i}\right)}{\left(N + \sum_{i=1}^{i=N}(N-i)\frac{[X]^i}{K_i}\right)^2} \quad (18)$$

$$\frac{1}{R} \times \frac{dR}{d\log[X]} = \frac{d\log R}{d\log[X]} = (R+1)\frac{\sum_{i=1}^{i=N} i^2\frac{[X]^i}{K_i}}{\sum_{i=1}^{i=N} i\frac{[X]^i}{K_i}} - NR \quad (19)$$

Replacing R by $\bar{n}/(N - \bar{n})$ in equation (19), and recalling that

$$\sum_{i=1}^{i=N} i^2\frac{[X]^i}{K_i} \leqslant N\sum_{i=1}^{i=N} i\frac{[X]^i}{K_i}$$

$$S = \frac{d\log R}{d\log[X]} = \frac{N}{N-\bar{n}}\left(\frac{\sum_{i=1}^{i=N} i^2\frac{[X]^i}{K_i}}{\sum_{i=1}^{i=N} i\frac{[X]^i}{K_i}} - \bar{n}\right) \leqslant N \quad (20)$$

Thus we have the well-known result that if the slope in the plot of log R against log [X] exceeds N the system cannot be described by an equation of Adair's type. In cases of multiple binding sites N is often 4 so that only the most extreme type of cooperative binding could be expected to approach the limiting conditions imposed. To obtain a further criterion of the applicability of Adair's equation we look at the behavior of S as a function of $\bar{n}$. From equation (20)

$$\frac{dS}{d\bar{n}} = \frac{S}{N-\bar{n}} - \frac{N}{N-\bar{n}} + \frac{N}{N-\bar{n}}\frac{df([x])}{d\bar{n}} \quad (21)$$

with

$$f([X]) = \left(\sum_{i=1}^{i=N} i^2 \frac{[X]^i}{K_i}\right) \Big/ \left(\sum_{i=1}^{i=N} i \frac{[X]^i}{K_i}\right)$$

Moreover,

$$\frac{df}{d[X]} = \frac{\left(\sum_{i=1}^{i=N} i^3 \frac{[X]^i}{K_i}\right)\left(\sum_{i=1}^{i=N} i \frac{[X]^i}{K_i}\right) - \left(\sum_{i=1}^{i=N} i^2 \frac{[X]^i}{K_i}\right)^2}{[X]\left(\sum_{i=1}^{i=N} i \frac{[X]^i}{K_i}\right)^2}$$

$$= \frac{\sum_{i \neq j}^{i=N} ij(i-j)^2 \frac{[X]^i}{K_i}\frac{[X]^j}{K_j}}{[X]\left(\sum_{i=1}^{i=N} i \frac{[X]^i}{K_i}\right)^2} \qquad (22)$$

In the extreme right-hand side of equation (22) all the terms are obviously positive so that $df([x])/d[X] > 0$. Moreover, $\bar{n}$ either increases or remains stationary with increase in [X], so that $d\bar{n}/d[X]$ is also always positive or zero. In conclusion

$$\frac{df([X])}{d\bar{n}} = \frac{df([X])}{d[X]} \times \frac{d[X]}{d\bar{n}} \geqslant 0$$

and

$$\frac{dS}{d\bar{n}} \geqslant \frac{S-N}{N-\bar{n}} \qquad (23)$$

An upper limit for $df/d\bar{n}$ is also easily derived: from equation (7) we find

$$\frac{d[X]}{d\bar{n}} = \frac{[X]\left(1 + \sum_{i=1}^{i=N} \frac{[X]^i}{K_i}\right)^2}{\left(\sum_{i=1}^{i=N} i^2 \frac{[X]^i}{K_i}\right)\left(1 + \sum_{i=1}^{i=N} \frac{[X]^i}{K_i}\right) - \left(\sum_{i=1}^{i=N} i \frac{[X]^i}{K_i}\right)^2} \qquad (24)$$

Multiplying this by the value of $df/d[X]$ in equation (22) and remembering that

$$\sum i^3 \frac{[X]^i}{K_i} \leqslant N \sum i^2 \frac{[X]^i}{K_i}$$

we have

$$\frac{df([X])}{d\bar{n}} < \frac{\left(1 + \sum_{i=1}^{i=N} \frac{[X]^i}{K_i}\right)}{\left(\sum_{i=1}^{i=N} i \frac{[X]^i}{K_i}\right)^2}$$

$$\times \frac{\left(N \frac{\sum_{i=1}^{i=N} i \frac{[X]^i}{K_i}}{\sum_{i=1}^{i=N} i^2 \frac{[X]^i}{K_i}} - 1\right)}{\left(1 + \sum_{i=1}^{i=N} \frac{[X]^i}{K_i}\right)\frac{\sum_{i=1}^{i=N} i \frac{[X]^i}{K_i}}{\sum_{i=1}^{i=N} i^2 \frac{[X]^i}{K_i}} - \left(\frac{\sum_{i=1}^{i=N} i \frac{[X]^i}{K_i}}{\sum_{i=1}^{i=N} i^2 \frac{[X]^i}{K_i}}\right)^2} \qquad (25)$$

The ratios

$$\frac{1 + \sum_{i=1}^{i=N} \frac{[X]^i}{K_i}}{\sum_{i=1}^{i=N} i \frac{[X]^i}{K_i}} = \bar{n}^{-1}; \quad \frac{\sum_{i=1}^{i=N} i \frac{[X]^i}{K_i}}{\sum_{i=1}^{i=N} i^2 \frac{[X]^i}{K_i}} = \rho^{-1}$$

are experimental quantities since according to equation (20)

$$\rho = \bar{n} + S\left(1 - \frac{\bar{n}}{N}\right) \qquad (26)$$

From equations (21), (23), (25), and (26), the limits of $dS/d\bar{n}$, or in practice the limits of the ratio of finite differences $\Delta S/\Delta\bar{n}$, are given by:

$$-\frac{N-S}{N-\bar{n}} < \frac{\Delta S}{\Delta\bar{n}} < \frac{N-S}{N-\bar{n}}\left(\frac{N}{S} + \frac{N}{\bar{n}} - 2\right) \qquad (27)$$

Equation (27) provides a considerably more refined criterion of the applicability of Adair's equation than that furnished by equation (20). As might be expected the amount of information on binding required to test the validity of equation (27) is greater than the amount required to test the validity of equation (20).

Implications of Departures from Adair's Equation. In cases in which, by the application of the criteria previously discussed, it becomes certain that Adair's equation cannot represent the experimental data, the quantities $K_{i,j}$ or $C_{i,j}$ must be themselves functions of the degree of saturation of the system. The dependence of the activity coefficient of the protein upon its concentration may be discarded if it can be shown that the degree of saturation of the system depends solely upon the concentration of free ligand. This may be safely concluded if the degree of saturation remains constant upon dilution with a solution of free ligand of the equilibrium concentration. If protein concentration effects are ruled out by this test, the quantities $C_{i,j}$, which depend upon first-order constants alone, must vary with the degree of saturation of the system. It seems possible to account for this behavior by assuming that following combination with the ligand the protein

molecules assume a new "conformation"[3] characterized by a decreased dissociation constant. The acquisition of this new conformation upon binding and its loss following dissociation of the ligand are supposed to take place over finite relaxation times. If the time for the assumption of a new protein conformation following combination (τ_+) and the time of relaxation of this conformation following dissociation (τ_-) are short compared to the average lifetime of the protein–ligand complex (ξ) and the average life of a free protein molecule (σ), respectively, the ratios of all protein conformations in either free or complexed state, and therefore the $C_{i,j}$ values, will be virtually constant and independent of $\bar{n}$. On the other hand if $\tau_+ > \xi$, or $\tau_- > \sigma$, or both, the distribution of the conformations in both free and bound protein will vary with $\bar{n}$, and so will the $C_{i,j}$ quantities: detailed balance (among the protein conformations) will not obtain in practice. Cooperative character could be observed in these cases in the binding of a single mole of ligand. At low free ligand concentration, when $\sigma \gg \xi$, the distribution of conformations in the free protein molecules would be that characterized by the larger dissociation constant K_1, which if valid for all φ should give rise to the titration curve 1 in Figure 2. At high free ligand concentration when the time spent by the protein molecules in the complexed condition is much larger than the time spent as free molecules, the prevalent conformation of the protein will be that characterized by the dissociation constant $K_2 < K_1$. If K_2 were operative at all φ values, the curve (2) in Figure 2 should result. The actual experimental curve, giving φ as function of log [X], will have some intermediate shape as shown in (3), since it has to approach curve 1 at low values of log [X] and curve 2 at high values of it. It is a relatively simple matter to derive an approximate descriptive theory of binding of a single mole of ligand by a protein that undergoes relaxation effects of the type described (*e.g.*, Weber, 1965). However, the simplest known cases of cooperative binding involve 4 moles of ligand bound/mole of protein. These would demand a much more complex theory, including not only the said relaxation of effects but also specific assumptions as to the influence of the binding of successive moles of ligand upon the binding properties of the remaining monomer units. Because of our lack of relevant experimental information in regard to the latter aspect, the development of a descriptive theory of relaxation effects applicable to polymer molecules does not appear at present profitable.

[3] The word "conformation" is used here in its widest sense. It may even be that the structural changes in the protein upon binding the ligand are very minor ones and that the main difference is in the functional properties of the molecule reflected by the change in dissociation constant.

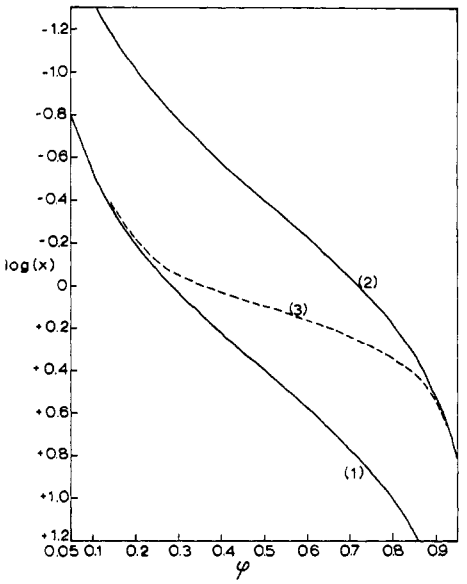

FIGURE 2: Cooperative character to be expected in the binding of a single mole of ligand by a protein that undergoes relaxation effects. (3) Explanation in text.

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