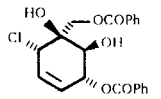
ceae),[52] neither this compound nor any of its relatives (bearing the cis diepoxide structure) has been found in the *Uvaria* plant. This has raised the suspicion that despite their rich pool of benzyl benzoates (e.g., **36–43**) and dienes (e.g. **18**), the *Uvaria* plants probably lack the biological means to synthesize the cis diepoxides. The possibility that these mono- and diepoxides should arise from different biogenetic pathways is considered unlikely since it has been established that crotepoxide (**1**), α-senepoxide (**2**), pipoxide **16**, zeylenol (**28**), and ferrudiol (**30**) all have identical 2*S*,3*R* absolute configurations, a fact highly suggestive of a unified biosynthesis via a common key intermediate, the diene **18**.[53] Results from the study of *Piper hookeri*, from which crotepoxide (**1**), pipoxide **16**, and pipoxide chlorohydrin **85** have been isolated,[26] lend further support to these deductions.

Deserving special mention among the *Uvaria* species is *Uvaria ferruginea* which was collected from northeastern Thailand. With the exception of the arene oxide **6b**, all of the compounds postulated in Scheme IV[27] have been isolated from this plant. These are the benzyl benzoates (**19** and **37**),[38] the *o*-(hydroxybenzyl)flavanones (**14** and **46**),[38] the missing link dienes (**22** and **23**),[31] and the cyclohexene oxides (**2**, **24**, and **25**)[31] as well as their various metabolites (**30–33**).[35,36] These findings have put the Cole and Bates biogenetic pathway (Scheme IV) on firm ground.

Last of all it might be mentioned that the latest addition (unpublished) to the family of naturally occurring cyclohexene oxides is a new cyclohexene diepoxide, boesenoxide **86**, from *Boesenbergia sp.* (Zingibereceae) from Thailand.[54]



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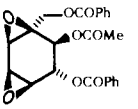

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(52) O. Pancharoen, V. A. Patrik, V. Reutrakul, P. Tuntiwachwuttikul, A. H. White, *Aust. J. Chem.* **1984**, *37*, 221.
(53) G. R. Shulte, B. Ganem, *Tetrahedron Lett.* **1982**, *23*, 4299.
(54) Personal communication with Dr. P. Tuntiwachwuttikul of the Department of Medical Science, Ministry of Public Health, Bangkok, Thailand.

Cooperative Binding to Macromolecules. A Formal Approach

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Many macromolecules are able to bind a variety of ligand molecules to one or more specific sites. The importance of this phenomenon lies in the fact that the binding of one ligand often influences the binding strength of the macromolecule toward a subsequent ligand (or ligands). When this happens, one speaks of *cooperative binding*. This effect is the basis of enzyme control and many other vital biological processes. If we were to elaborate on this theme, we could only repeat what has been ably presented elsewhere.[1-5]

In view of the importance of cooperative binding, it is not surprising that much effort should have been devoted not only to the elucidation of the mechanism by which the phenomenon might arise in a specific case[1] but also to the development of general methods by which cooperative binding can be recognized and subjected to mathematical or graphical representation. What *is* surprising is the seeming lack of coordination between papers dealing with different aspects of the topic, so that there is even some confusion about the very definition of the term "cooperativity". Wrong, or, at least, misleading statements seem never to have been challenged, and the interesting results of some theoretical treatments[6-8] have not yet found their way into the literature which deals with more practical aspects.

In this Account, we present a short review of the *formal* aspects of cooperative binding as we see them, confining ourselves to homotropic binding, i.e., the multiple binding of like molecules. We shall employ a consistent nomenclature and point out some of the various pitfalls presented by this subject. We also believe to have some original contributions to make to various aspects of the topic.

Stoichiometric Binding Constants and Binding Equations

We shall use the following definitions for the stoichiometric binding constants[3,5,9]

$$K_1 = \frac{[PX]}{[P][X]}, K_2 = \frac{[PX_2]}{[PX][X]}, \ldots, K_n = \frac{[PX_n]}{[PX_{n-1}][X]}, \ldots, K_t = \frac{[PX_t]}{[PX_{t-1}][X]} \quad (1)$$

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(1) D. E. Koshland, Jr., and K. Neet, *Annu. Rev. Biochem.*, **37**, 359 (1968).
(2) N. Citri, *Adv. Enzymol.*, **37**, 397 (1973). J. A. M. Karplus and J. A. McCammon, *CRC Crit. Rev. Biochem.*, **9**, 293 (1981).
(3) G. G. Hammes and C.-W. Wu, *Annu. Rev. Biophys. Bioeng.*, **3**, 1 (1974).
(4) G. Weber, *Adv. Protein Chem.*, **29**, 1 (1975).
(5) I. M. Klotz and D. L. Hunston, *Arch. Biochem. Biophys.*, **193**, 314 (1979).
(6) P. A. Baghurst, L. W. Nichol, and D. J. Winzor, *J. Theor. Biol.*, **74**, 523 (1978).
(7) W. G. Bardsley, *J. Theor. Biol.*, **67**, 407 (1977).
(8) W. G. Bardsley and R. D. Waight, *J. Theor. Biol.*, **72**, 321 (1978).

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where P and X designate the macromolecule and the ligand, respectively, and t is the total number of sites. (In a nonideal solution these constants are not thermodynamic constants but concentration quotients, a fact which is often glossed over. We shall assume that measurements are always carried out under conditions of constant activity coefficients.[3]) When the binding sites are identical and independent, then all the K_n's can be expressed in terms of a single intrinsic or site binding constant Q, namely[3,9]

$$K_n = Q(t - n + 1)/n \quad (2)$$

so that

$$K_{n+1}/K_n = n(t - n)/(n + 1)(t - n + 1) \quad (3)$$

Multiple binding of this kind is called *statistical.* When the sites are initially equivalent but the binding of the nth ligand causes a conformational change in the macromolecule such that its binding strength toward the $(n + 1)$th ligand is enhanced, then we speak of positive linkage[10] or, more commonly, *positive cooperativity.* Equation 3 then becomes an inequality, and K_{n+1}/K_n is higher than it would be for statistical binding. All the criteria given in the literature for the recognition of positive cooperativity are essentially based on this inequality; *we shall therefore adopt a positive deviation from the equality (3) as our criterium for positive cooperativity.* The condition for *initial* positive cooperativity is then $K_2 > K_1(t - 1)/2t$. Similarly, when the binding of a ligand impedes the binding of a subsequent ligand, then K_{n+1}/K_n is lower than it would be for statistical binding, *negative cooperativity* having taken place. Mixed cooperativity can of course also occur. However, we shall not deal with this phenomenon in our short Account.

In order to get some insight into the nature of the binding, the experimenter will in general interpret his experimental results in terms of the binding constants. The most straightforward presentation of the data is to calculate the occupancy r, that is to say, the average number of ligands bound per macromolecule, and to write the result in terms of the stepwise equilibrium model which is always applicable, whatever the nature of the binding[5,9]

$$r = \frac{[PX] + 2[PX_2] + ... + n[PX_n] + ... + t[PX_t]}{[P] + [PX] + [PX_2] + ... + [PX_n] + ... + [PX_t]} \quad (4)$$

or, introducing the stoichiometric binding constants (eq 1)

$$r = \{K_1x + 2K_1K_2x^2 + ... + nK_1K_2...K_nx^n + ... + tK_1K_2...K_tx^t\}/\{1 + K_1x + K_1K_2x^2 + ... + K_1K_2...K_nx^n + ... + K_1K_2...K_tx^t\} \quad (5)$$

where x is the concentration of free ligand. The denominator of this expression is the *binding polynomial* defined by Wyman.[10] The numerator of r is the derivative of the binding polynomial with respect to $\ln x$.[9]

For statistical binding the binding polynomial is easily seen to equal $(1 + Qx)^t$, so that the numerator of r becomes $tQx(1 + Qx)^{t-1}$; eq 5 reduces therefore to the Scatchard equation[11]

$$r = tQx/(1 + Qx) \quad (6)$$

When r and x have been determined, then Q and t can easily be obtained from reciprocal plots[9] or from a plot of r/x as a function of r, known as the Scatchard plot.[11]

On the basis of eq 5, the experimentally accessible parameters are the Adair[12] constants K_1, K_1K_2, $K_1K_2K_3$, and $K_1...K_t$, and not the binding constants defined in eq 1. With the exception of K_1, these latter constants are thus the result of dividing two experimental quantities by each other. If, for example, K_2/K_1 then turns out to fulfill the conditions for positive cooperativity, then it would be misleading to say[13] that the first ligand is bound less strongly than subsequent ligands. Rather, positive cooperativity means that in the *bicomplex both* ligands are bound more strongly than the first ligand is in the *monocomplex.* This is because, whatever the mechanism of the cooperativity, the conformation of the whole system has changed as a consequence of the binding of the first ligand. (It is only for heterotropic binding that the binding constants of one ligand in the presence or absence of another ligand can be compared and the interaction energy evaluated.[4])

Binding Curve and Scatchard Plot

A plot of r as a function of x is termed the binding curve. Equation 6 shows that, for statistical binding, this plot is "hyperbolic"; i.e., it is the positive section of a rectangular hyperbola with a horizontal asymptote at $r = t$, reached at high values of r, and a vertical asymptote at $x = -1/Q$. The tangent at $x = 0$ is Qt. This curve is useful for a qualitatively interpretation of experimental results, though, as we shall see, it is not a good diagnostic for cooperativity.

The plot of r/x as a function of r is a much better diagnostic. When the binding is statistical, then, again as shown by eq 6, this plot is a straight line. The intercept on the r/x axis is tQ, and the slope is $-Q$. Furthermore, r tends to t at saturation (when $r/x \rightarrow 0$). This enables the number of sites to be estimated by extrapolation in cases where measurements near saturation may not be feasible.

When there is cooperativity, the plot is no longer a straight line, but *the above features are preserved.* The intercept on the r/x axis is K_1, and the slope at high values of r approaches $-tK_t$ (which, for statistical binding, would equal tQ and $-Q$, respectively). The *initial* slope equals $2K_2$-K_1, *independent of* t, contrary to a recent statement by Dahlquist.[13] Therefore, provided experiments can be carried out in a sufficiently wide range of concentration, the plot yields quantitative information about four of the parameters of eq 5, namely,[14] K_1, K_2, K_t, and t. In the absence of initial cooperativity $2K_2 - K_1$ is again just equal to $-Q$ for any value of t (see eq 2). But when there is negative initial cooperativity, then $|2K_2 - K_1| > |Q|$ and the initial slope is more negative than the final slope—the plot is convex. By the same reasoning, initial positive cooperativity leads to a concave plot. Concavity is thus a sufficient criterium for positive cooperativity,[3,7,8] although the experimental data may have to be extremely

(9) I. M. Klotz, *Arch. Biochem.*, **9**, 109 (1946).
(10) J. Wyman, *Q. Rev. Biophys.*, **1**, 35 (1968).
(11) G. Scatchard, *Ann. N.Y. Acad. Sci.*, **51**, 660 (1949).
(12) G. S. Adair, *J. Biol. Chem.*, **63**, 529 (1925).
(13) F. W. Dahlquist, *Methods Enzymol.*, **48**, 270 (1978). The expression given by this author for the initial slope of the Scatchard plot with $t = 2$ is identical with that given here, his binding constants being divided by the statistical factor, so that his K_1 is $K_1/2$ and his K_2 is $2K_2$ of our eq 1.
(14) D. L. Hunston, *Anal. Biochem.*, **63**, 99 (1975).

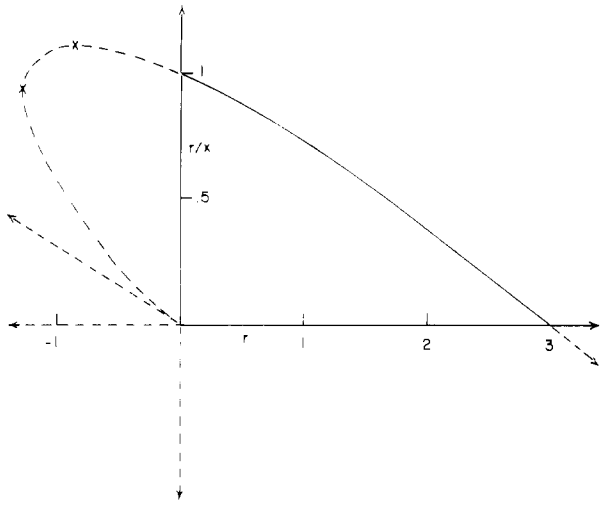

Figure 1. Scatchard plot (full curve) for $K_1 = 1$, $K_2 = 0.39$, and $K_3 = 0.13$. The plot is continued to negative values of r and r/x (broken curve) to show the mathematical behavior of the function r/x. The crosses indicate the maximum and the point of infinite slope, respectively.

good for this property to be recognized. This is illustrated in Figure 1 for $t = 3$, $K_1 = 1$, $K_2 = 0.39$, and $K_3 = 0.13$ (full curve). Cooperativity is of course much more apparent when $(2K_2 - K_1) > 0$ so that the plot has an actual maximum, but this is *not* the condition for initial positive cooperativity as is sometimes[15] stated.

Another criterium for positive cooperativity is often stated to consist in a point of inflection in the binding curve. It can be shown, however,[7] that the condition for this to occur is more stringent, namely, $(2K_2 - K_1) > 0$. But this is just the condition for a maximum in Scatchard plot! From the condition $d^2r/dx^2 = 0$, on the one hand, and from the initial slope of the Scatchard plot on the other, we found that only for $(2K_2 - K_1) = 0$ do the point of inflection of one curve and the maximum of the other occur at the same value of x, namely, $x = 0$ (independent of t). For other values of $(2K_2 - K_1)$ we solved $d^2r/dx^2 = 0$ only for $t = 2$ and found that for $(2K_2 - K_1) > 0$ the point of inflection and the maximum, respectively, occur at different values of x. When $(2K_2 - K_1) < 0$ while the condition for positive cooperativity continues to be fulfilled, then the binding curve still has a point of inflexion, and the Scatchard plot still has a maximum, but at negative (and, again, not coinciding) values of x. Clearly, these points cannot be realized experimentally (see Figure 1, broken line, for an example with $t = 3$). From the evidence presented by Baghurst et al.[6] we conclude that our result can be generalized, an exact correspondence existing between the critical points in the Scatchard plot and the points of inflection of the binding curve.

The Hill Plot

A plot of $\ln [r/(t - r)]$ as a function of $\ln x$ is called a Hill plot. The slope of the line obtained,

$$n_H = \frac{d \ln [r/(t - r)]}{d \ln x} = \frac{d \ln (r/t)}{d \ln x}\left(\frac{t}{t - r}\right) \quad (7)$$

is called the Hill coefficient; $[r/(t - r)]$ is the number of occupied sites divided by the number of free sites. If n_H were constant, we could integrate this equation to get $r/(t - r) = Kx^{n_H}$ (where K is an integration constant) or, solving for r/t

$$r/t = Kx^{n_H}/(1 + Kx^{n_H}) \quad (8)$$

which is the Hill equation[16] in our terminology. Experimentally, n_H is indeed often found to be constant over a wide range of x. Early workers seem to have been concerned when this was not the case, whereas it is now realized that eq 8 has no theoretical basis, and n_H can be constant only in two limiting cases. One is $n_H = 1$, when eq 8 reverts to eq 6 (no cooperativity, $K = Q$ of eq 6). In the second limit, $n_H = t$ (infinite cooperativity, $K = K_1K_2...K_t$). In this limit, all binding constants must be infinitely small in comparison with K_t. This is possible only when the corresponding site interaction becomes infinite.[10] Obviously, any *real* system can only approach this state of affairs; i.e., n_H can only *approach* t, the more closely, the higher the cooperativity.

In order to make use of n_H as a diagnostic for positive cooperativity we should like to know $(n_H)_{max}$, the highest value that n_H can take for a given system. A good estimate can be obtained from the value of r/t at the maximum of the Scatchard plot[13,17] where—from $d(r/x)/dr = (1/x)(1 - d \ln x/d \ln r) = 0$—we have $d \ln r/d \ln x = 1$. Introducing this condition into the second half of eq 7 we get

$$n_H = \left(\frac{r}{t - r}\right) \text{ (at max of Scatchard plot)} \quad (9)$$

Our calculations for $t = 2$ and $t = 3$ (again with initial cooperativity only) showed that n_H at the maximum of the Scatchard plot does not coincide mathematically with $(n_H)_{max}$. Nor does n_H always have its maximum at the midpoint $(r/t = 0.5)$ as is often stated. This is correct only for $t = 2$, and for higher values of t when the ratios between the binding constants fulfill certain conditions of symmetry.[18] It is only because the Hill plot is very nearly straight over a wide range of r that the various estimates for $(n_H)_{max}$ coincide for all practical purposes. The approximation expressed by eq 9 breaks down only when the cooperativity is so weak that the Scatchard plot has no maximum, and n_H differs only little from unity.

Not only can eq 9 thus serve to estimate $(n_H)_{max}$, but also it can be applied in reverse.[17] When n_H has been determined experimentally and found constant over a sufficiently wide range, then it can be used to estimate r at the maximum of the Scatchard plot. This is claimed[17] to give a better estimate for the maximum than that derived from an inspection of the experimental Scatchard plot itself.

In addition to the conclusions that can be drawn from n_H, the Hill plot has been shown by Wyman[10,19] to be an important tool for the estimation of the interaction energy. In order to understand this, we have to consider

(15) J. E. Fletcher, *J. Phys. Chem.*, **81**, 2374 (1977); A. A. Spector, J. E. Fletcher, and J. D. Ashbrook, *Biochemistry* **9**, 4580 (1970).

(16) A. V. Hill, *J. Physiol. (London)* **40**, IV (1910).

(17) F. W. Dahlquist, *FEBS Lett.*, **49**, 267 (1974).

(18) A. Cornish-Bowden and D. E. Koshland, Jr., *J. Mol. Biol.*, **95**, 201 (1975).

(19) J. Wyman, *Adv. Protein Chem.*, **4**, 407 (1948); **19**, 223 (1964); *J. Am. Chem. Soc.*, **89**, 2202 (1967). The simple result that the interaction energy equals RT times the vertical distance between the asymptotes is the limiting case of a more general treatment which considers the interaction energy at any intermediate stage of binding. For this purpose, a new coordinate system, at 45° to the original one, has to be introduced. The total interaction energy is therefore often given as $RT(2)^{1/2}$ times the length of a line connecting the two asymptotes and at a right angle to them.

the asymptotes of the plot at the limits of very high and very low values of x. We shall illustrate this again for $t = 3$, when

$$\frac{r}{t-r} = \frac{K_1x + 2K_1K_2x^2 + 3K_1K_2K_3x^3}{3 + 2K_1x + K_1K_2x^2} \quad (10)$$

As $x \rightarrow \infty$, $\ln [r/(t-r)] = \ln 3K_3 + \ln x$ and $n_H = 1$. As $x \rightarrow 0$, $\ln [r/(t-r)] = \ln K_1/3 + \ln x$ and $n_H = 1$. This bears out our statement that the Hill plot cannot be a straight line over the whole concentration range. Two lines of unit slope—corresponding to binding to only the first or only the last site, respectively[18]—are connected by an S-shaped curve.

Furthermore, we see that at any given value of $\ln x$ the vertical distance between the two asymptotes is $\ln K_1/3$ minus $\ln 3K_3$. This can be rewritten as $\ln K_3 - \ln K_1 + \ln 9$, or $(\Delta G_1^\circ - \Delta G_3^\circ)/RT + \ln 9$, where ΔG_1° and ΔG_3° are the standard free energies of formation of the first complex from its constituents, and of the last complex from its precursor, respectively. In the absence of site interaction, this distance is clearly equal to zero. However, when there is positive cooperativity, then ΔG_3° is more negative than it would be for independent binding, more pronouncedly so the higher the cooperativity. Therefore, the vertical distance times RT can be seen as the total interaction energy[10,19] between initially equal sites, and, as we shall see, the minimum total interaction energy between initially unequal sites. The general expression for this interaction energy is $RT \ln (K_1/t^2K_t)$.

Nonequivalent Sites and Site Binding Constants

In order to understand systems in which the sites are initially of different binding strength, we use the concept of *site binding constants*.[5] Let there be a number of sites a, b, c, ...; then the first ligand can bind to any of these sites, the second ligand can bind to any of the remaining free sites, and so on. Therefore, in the absence of site interaction

$$\begin{aligned} K_1 &= Q_a + Q_b + Q_c + \dots \\ K_1K_2 &= Q_aQ_b + Q_aQ_b + Q_aQ_c + Q_bQ_c + \dots \quad (11) \\ K_1K_2K_3 &= Q_aQ_bQ_c + \dots \\ &\dots \end{aligned}$$

The stoichiometric binding constants resulting from eq 11 can easily be calculated to exhibit the characteristics of negative cooperativity. This is intuitively obvious, because the stronger binding sites will tend to be occupied first, leaving the weaker sites to be occupied by the ligands which enter at a later stage. It must be emphasized that, although the formal condition for negative cooperativity is thus met, "negative cooperativity" is really a misnomer in this case, because no negative site interaction has taken place.

When, in addition to being initially non-equivalent, the occupied sites exhibit a negative influence on further binding, then the "negative cooperativity" will merely be enhanced.

When the site interaction is positive, this still need not give rise to positive cooperativity in the sense of our definition. This is because the two properties, inequality and positive interaction, have opposite effects on the stoichiometric binding constants.[10] A clear distinction should be made between the two concepts—positive *site* interactions on the one hand and positive *cooperativity* as expressed in our *formal* definition, on the other. *Failure to make this distinction*[15] *is apt to lead to confusion.*

In order to show the condition under which positive site interaction leads to a positive deviation from the equality (3), we shall confine ourselves to the simple case of $t = 2$. If the presence of one ligand on site a influences the binding strength of site b (and, necessarily, vice versa[3]), then K_1K_2 no longer equals Q_aQ_b as in eq 11. Following Fletcher et al.[15] we now define an interaction coefficient I_{int} by the expression $K_1K_2 = Q_aQ_bI_{int}$. Clearly, when the site interaction is positive, then $I_{int} > 1$ and vice versa. From the definition of I_{int} we calculate that $K_2/K_1 = Q_aQ_bI_{int}/(Q_a + Q_b)$,[2] whereas in the absence of cooperativity $K_2/K_1 = 1/4$ (eq 3). Therefore, for the formal condition for positive cooperativity to be fulfilled, the positive interaction must be strong enough so that $I_{int} > (Q_a + Q_b)^2/4Q_aQ_b$. Obviously, the greater the initial difference between sites a and b, the higher does I_{int} have to be for positive cooperativity to become manifest. It is important to note that the experimental measurement of K_1K_2 does not enable one to make any pronouncement about Q_a, Q_b, and I_{int}. Different combinations of these parameters can lead to the same value of K_2K_1.[5,15]

The Scatchard Equation and the Factorability of the Binding Polynomial

For the special case of independent, initially unequal sites, eq 6 can be extended to[11]

$$r = \frac{Q_ax}{1 + Q_ax} + \frac{Q_bx}{1 + Q_bx} + \frac{Q_cx}{1 + Q_cx} + \dots \quad (12)$$

When this equation is multiplied out, we obtain an expression of the type of eq 4, the constants appearing in this equation being given by eq 11. Conversely, when the experimental data resulting from the binding to independent sites of different strength are interpreted in terms of eq 4, then the binding polynomial can be factorized, the negative reciprocals of the roots[15] equalling Q_a, Q_b, Q_c, ... It turns out that it may be possible to factorize the binding polynomial even when the binding sites are *not* independent. In such cases, however, it is not justified to represent the experimental data in terms of eq 12 (although the resulting equation is claimed[15] to be more covenient to fit to experimental results than eq 4). The reciprocal roots have no physical meaning[5,15] because obviously one cannot assign a value to the binding constant of a site whose binding strength changes during the binding process. Therefore, the resulting values have been very aptly termed[20] the binding constants of "independent ghost sites". It is important to realize that *no* conclusion about the *origin* of negative deviations from statistical binding can be drawn from the fact that the binding polynomial has been successfully factorized.

However, when the sites are not independent, then it is sometimes impossible to factorize the binding polynomial in terms of real numbers. The conditions under which this occurs will be seen to be of special interest.

For $t = 2$ the roots of the binding polynomial turn out to equal $-(1/2K_2)[1 \pm (1 - 4K_2/K_1)^{1/2}]$.[5] We see

(20) I. M. Klotz, *Trends. Pharmacol. Sci.*, 4, 253 (1983).

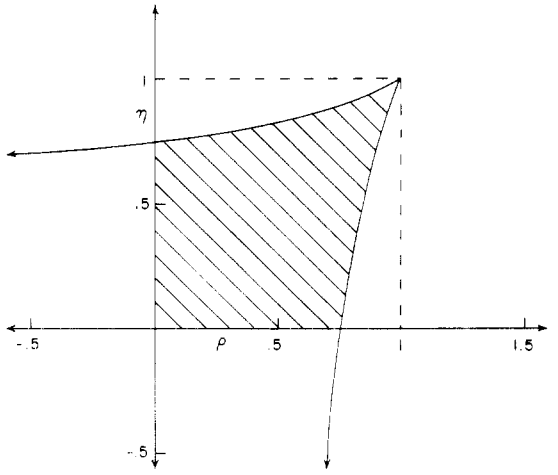

Figure 2. Curves representing the loci for three real roots of the binding polynomial (two of which are identical) in the coordinate system $\eta = 3K_2/K_1$ and $\rho = 3K_3/K_2$. Only positive values of η and ρ are physically meaningful. The polynomial is factorable for values of η and ρ within the hatched surface.

immediately that in the absence of cooperativity, when $4K_2/K_1$ equals unity, the polynomial has two identical roots, when there is negative cooperativity it has two real, different roots, whereas positive cooperativity leads to the appearance of a complex conjugate pair. For $t = 2$ the lack of factorability can thus serve as an unequivocal criterium for positive cooperativity. This criterium is often held to be generally valid.

It has recently been shown,[7] however, that for $t \geq 3$, positive cooperativity is a *sufficient*, but not a *necessary* condition for complex roots to appear. For $t = 3$, the problem is amenable to graphical representation in two dimensions. This is shown in Figure 2 where $\eta \equiv 3K_2/K_1$ and $\rho \equiv 3K_3/K_2$. For statistical binding, $\rho = \eta = 1$. The two curves in this figure represent the loci of those combinations of η and ρ values which correspond to three real roots, two of which are identical. The region for one real and two complex roots of the polynomial lies to one side of the loci, whereas the region to the other side—shown hatched in the graph—corresponds to factorability into three unequal roots. The graph is seen to be symmetrical with respect to η and ρ, the loci being determined by $\eta = (1/\rho^2)(3\rho - 2 \pm 2(1-\rho)^{3/2})$ and an identical equation in which η and ρ are interchanged.[21] We have continued the curves to negative values of η and ρ although these values have no physical meaning, in order to show that the function is continuous, the locus reaching the asymptotes of $\rho = 0$ and $\eta = 0$ for $\eta = -\infty$ and $\rho = -\infty$, respectively.

Figure 2 illustrates several interesting features. One of them is the well-known fact that positive cooperativity (η or ρ or both above unity) always leads to complex conjugate roots of the binding polynomial. On the other hand, when both η and ρ are smaller than 0.75, then the polynomial can always be factorized. When either η or ρ, or both, are above these values, but still *smaller than unity*, there is an infinite number of combinations of these parameters for which the polynomial is factorable and of others in which it is not. In particular, we note with some surprise that for a system which can be characterized by a point in the upper right-hand corner of the hatched area (η or ρ or both >0.75) not only *positive* site interaction (movement of the point upwards and/or to the right) but also *negative* site interaction (movement of the point downwards and/or to the left) can cause the polynomial to become nonfactorable. We have not been able to discern any obvious or intuitive difference between pairs of η and ρ which lead to factorability and those which do not.

Furthermore, it has been shown[8] that for $t = 4$ the mathematical conditions for two real and two complex, and even all-complex, roots are consistent with negative deviations of any kind from statistical behavior. The authors of ref 8 conclude this situation to hold also for still higher values of t. We may thus state that whereas factorability of the binding polynomial cannot be taken as a sign for the *absence* of site interaction, *lack* of factorability, in the presence of negative deviations from statistical binding, can be taken as an *unequivocal* indication for site interaction—negative when the sites are initially equivalent, negative *or* positive when they are unequal. This conclusion does not seem to have been drawn before.

Prior Isomerization as a Mechanism for Cooperative Binding

Just as the criteria for negative cooperativity may be met even in the total absence of negative site interaction, positive cooperativity may become manifest through a mechanism which has nothing to do with positive site interaction, namely, prior isomerization.[1] With $t = 4$, this is the mechanism proposed in the classical paper by Monod, Wyman, and Changeux for binding to hemoglobin.[22] However, all the relevant features can be illustrated with the aid of the simpler case of $t = 2$.

Consider a macromolecule which, in the absence of ligand, exists in two conformations, R and T, having different binding strengths. This can be written schematically in Scheme I, where we have assumed the binding of each of the two conformers R and T to be statistical. It is now intuitively obvious that when L is large, (i.e., when T is the dominant form) and, at the same time, the R form has the higher binding strength, then the stoichiometric binding constant for the first ligand may be small in comparison with that for the second ligand which already finds a conformation favorable to binding.[23] This kind of positive deviation from statistical binding is thus based on a shift of the equilibrium T → R. The stabilization of the more strongly binding form has early been recognized[24] and has been treated quantitatively for more general cases.[25]

Scheme I

$$\mathrm{R} \rightleftharpoons \mathrm{T} \quad [\mathrm{T}]/[\mathrm{R}] = L$$

$$\mathrm{R} + \mathrm{X} \rightleftharpoons \mathrm{RX} \quad K_{1,\mathrm{R}} = 2Q_{\mathrm{R}}$$

$$\mathrm{T} + \mathrm{X} \rightleftharpoons \mathrm{TX} \quad K_{1,\mathrm{T}} = 2Q_{\mathrm{T}}$$

$$\mathrm{RX} + \mathrm{X} \rightleftharpoons \mathrm{RX_2} \quad K_{2,\mathrm{R}} = Q_{\mathrm{R}}/2$$

$$\mathrm{TX} + \mathrm{X} \rightleftharpoons \mathrm{TX_2} \quad K_{2,\mathrm{T}} = Q_{\mathrm{T}}/2$$

(21) Bardsley[7] presents a graph similar to our Figure 2, but with axes K_2/K_1 and K_3/K_1 instead of our $2K_2/K_1$ and $3K_3/K_2$. The symmetry of the interaction between the first and second, and second and third sites is thus lost. Furthermore, the fact that positive interaction between the second and third sites *must* lead to lack of factorability becomes less immediately apparent from the graph.

(22) J. Monod, J. Wyman, and J.-P. Changeux, *J. Mol. Biol.*, **12**, 88 (1965).

(23) E. Nissani and B. Perlmutter-Hayman, *Int. J. Chem. Kinet.*, **17**, 591 (1985).

(24) J. F. Foster and K. Aoki, *J. Am. Chem. Soc.*, **80**, 5215 (1958).

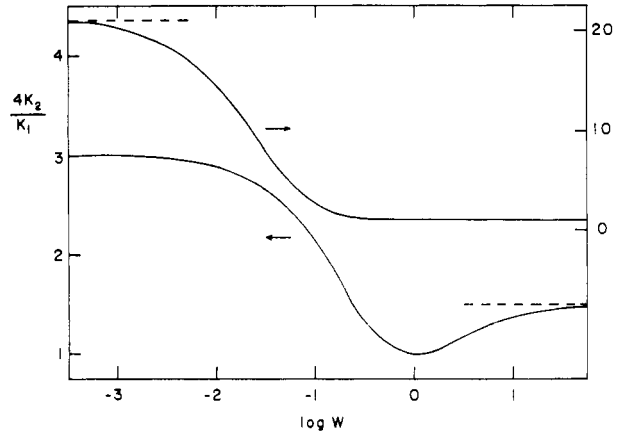

Figure 3. The dependence of $4K_2/K_1$ on log W (where W is the ratio of the intrinsic binding constants) at two fixed values of L (the ratio of the concentration of the two conformers): lower curve, $L = 2$; upper curve, $L = 20$. Where the limiting values are not reached, they are indicated by dashed lines. (At the higher value of L, the increase of $4K_2/K$ above unity as log W increases above zero is too small to show up in the graph.) Note the difference in scale for the two curves.

From Scheme I, the observed binding constants are easily derived to be

$$K_1 = 2(Q_T L + Q_R)/(1 + L)$$
$$K_1K_2 = [Q_T^2 L + Q_R^2]/(1 + L) \quad (13)$$

so that

$$4K_2/K_1 = 1 + L(W - 1)^2/(LW + 1)^2 \quad (14)$$

where we define $Q_T/Q_R \equiv W$. The second term on the right hand side of eq 14 cannot be negative. Therefore, *in a system which can be described by Scheme I, a positive deviation from statistical binding must always occur* (except in the trivial case when $L = 0$ or $L = \infty$, i.e., when there is only one conformer present). Furthermore, we see that, for a given L, the value of $4K_2/K_1$ starts at $1 + L$ for $W = 0$. With increasing W, the function decreases, reaching unity (the condition for statistical binding) in the trivial case when $W = 1$. As W continues to increase, our function increases, reaching a limiting value of $1 + 1/L$. Clearly, when $L > 1$, the limiting value at $W = 0$ will be high, whereas at high W it will be only slightly above its statistical value (and vice versa for $L < 1$). This is illustrated in Figure 3 where we show $4K_2/K_1$ as a function of log W for two fixed values of L, higher than unity.

Although our intuitive guess is thus confirmed, we must not simply conclude that high values of L paired with low values of W always entail a strong positive deviation. This is because eq 14 is not symmetrical with respect to W and L; whereas the cooperative effect reaches the highest value compatible with a given L (>1) as W tends to zero, differentiation of eq 14 with respect to L at constant W shows that (for $W < 1$) the effect does *not* increase monotonically with increasing L to some limiting value. Rather, $4K_2/K_1$ as a function of L passes through a maximum when

$$WL = 1 \quad (15)$$

Equation 14 shows that the highest value of $4K_2/K_1$ compatible with a given value of W is thus $1 + (W - 2 + 1/W)/4$, reached only at one specific value of L.

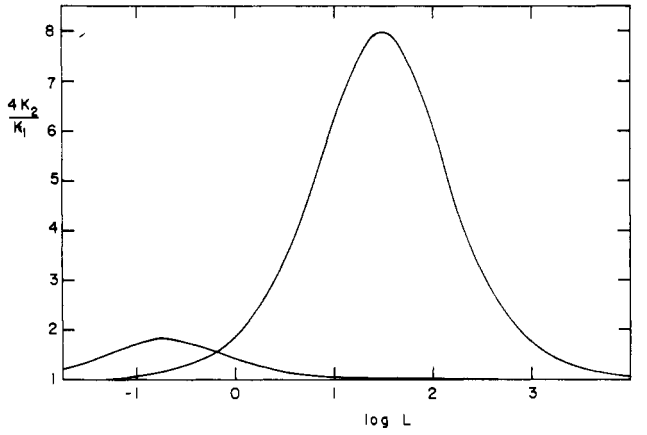

Figure 4. The dependence of $4K_2/K_1$ on log L, for two fixed values of W: left curve, $W = 5$; right curve, $W = 1/30$.

The behavior of $4K_2/K_1$ as a function of log L is shown in Figure 4 for two fixed values of W, one somewhat above unity and one considerably below. The curves are symmetric around log L = -log W, in accordance with eq 15. The peak is seen to be higher—in fact, it increases indefinitely—the more L and W differ from unity while obeying eq 15. On the other hand, the effect is seen to fall off rapidly as L deviates more and more from this equation. A *dramatic* positive effect thus occurs only when both *W and L* differ very much from unity while at the same time fulfilling, or nearly fulfilling, the stringent condition imposed by eq 15.

From the general formula give by Monod, Wyman, and Changeux[22] it can easily be derived that for $t = 4$ the condition for initial cooperativity is of exactly the same mathematical form as the condition for cooperativity in the simpler system treated above, and the same analysis would apply. The general statement that the cooperativity is more marked when L is large and W is small[3,23] is therefore misleading. It is true, of course, that the effect is small when L and W are both high or both low[3] but an *indefinite* increase of the effect with increasing L occurs *only* when W actually equals zero.

Concluding Remarks

In conclusion we should like to stress that the various methods of representing experimental results—binding equation, binding curve, Scatchard or reciprocal plots, and Hill plot—are all equivalent. For a given case, one may be more appropriate than others, but information that, *in principle*, cannot be garnered from one cannot be obtained in any other way. In particular, as we have seen, positive site interaction may pass undetected when the sites are initially different, whereas negative interaction between identical sites is usually indistinguishable from the presence of different independent sites. These conclusions hold however painstakingly the experiments may have been carried out, whatever formula is chosen for a representation of the data, and however sophisticated the method used to evaluate the parameters appearing in this formula.

In practice, however, the prospect may not be quite so bleak, because the experienced worker in the field is after all not confined to the measurement of homotropic binding! He may use his knowledge of the structure of the macromolecule to decide about the

(25) M. M. Rubin and J.-P. Changeux, *J. Mol. Biol.*, 21, 265 (1966); J. A. Reynolds, S. Herbert, H. Polet, and J. Steinhardt, *Biochemistry*, 6, 937 (1967).

likelihood of initially equivalent sites being present, or he may follow the change in some physical property of the macromolecule as a function of r in order to determine whether a conformational change has accompanied the binding. In simple cases, kinetic measurements may also be of help. Furthermore, the specific properties of the ligand can provide a clue; if the ligand is charged or polar, negative interactions are almost a certainty. Furthermore, the experimenter may be able to draw conclusions from measurements of heterotropic binding. For example, it has been shown[26] that a comparison between n_H in the presence of a competing ligand and that in its absence enables a decision between the two mechanisms of a negative deviation from eq 3 to be made.

It is a pleasure to acknowledge helpful suggestions by Prof. H. J. G. Hayman and Dr. T. Feldmann.

(26) Y. I. Henis and A. Levitsky, *Eur. J. Biochem.*, **102**, 449 (1979); *Ann. N.Y. Acad. Sci.*, **366**, 217 (1981).