On the Nature of Allosteric Transitions: Implications of Non-exclusive Ligand Binding

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Further predictions are derived from the model for allosteric transitions of Monod, Wyman & Changeux (1965) for the general case in which both the postulated conformational states of an allosteric protein bind a specified ligand with significant but unequal affinity (non-exclusive binding). In particular, the non-exclusive binding of one or more of the ligands, such as the substrate, inhibitor or activator of a regulatory enzyme, is expected to introduce limits on: (1) the extent to which the equilibrium between the conformational states of the protein may be shifted in their presence; (2) the degree of co-operativity in the saturation by each ligand (as measured by the Hill coefficient), and (3) the extent of co-operative or antagonistic interactions among the various ligands (partial and multivalent effects).

1. Introduction

Monod, Wyman & Changeux (1965) have proposed a model for allosteric interactions which postulates the existence of an equilibrium between two conformational states of the protein that can be shifted by the binding of different allosteric effectors. The general formulation of this model, in which the ligands may exhibit significant but differential affinity for both states of the protein (non-exclusive binding) was found by the authors to be suitable for the quantitative description of the homotropic§ interactions found for several well-defined regulatory proteins. To simplify their discussions of heterotropic effects, the authors considered only the case of exclusive binding of each type of ligand. However, recent observations on several allosteric proteins (Atkinson, Hathaway & Smith, 1965a,b; Atkinson, 1966) appear to be inconsistent with simple exclusive binding and thus have led us to re-examine the more general statement of the model. In particular, this paper focuses on the consequences of non-exclusive ligand binding for homotropic and heterotropic interactions. The experimental manifestations of these effects are discussed in terms of two parameters of the system: the ligand concentration required for half-saturation, and the maximum slope of the Hill plot (Brown & Hill, 1922-23) found by saturation experiments.

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§ The nomenclature used here is that of Monod *et al.* (1965): *homotropic* interactions are those between identical ligand molecules; *heterotropic* interactions are those between different ligands.

2. Graphical Analysis of the General Equations

(a) *Derivation and definition of parameters*

As proposed by Monod *et al.* (1965), homotropic interactions—for example, those of the substrate of an enzyme (S)—may be described mathematically by two functions: $\bar{R}$, the fraction of protein molecules in the conformational state (R) for which the specified ligand has higher affinity, and $\bar{Y}_s$, the fractional saturation of the sites for this ligand in the total protein population (both R and T states).

$$\bar{R} = 1 - \bar{T} = \frac{(1 + \alpha)^n}{(1 + \alpha)^n + L(1 + \alpha c)^n},\tag{1}$$

$$\bar{Y}_s = \frac{\alpha(1 + \alpha)^{n-1} + L\,\alpha c(1 + \alpha c)^{n-1}}{(1 + \alpha)^n + L(1 + \alpha c)^n}\tag{2}$$

where L is the intrinsic allosteric constant, i.e., the equilibrium constant for the transition $R \rightleftharpoons T$ in the absence of ligands; k_R and k_T are the microscopic dissociation constants of a ligand bound to a stereospecific site on the protein in the R and T states, respectively; c is $k_R/k_T < 1$; a is the substrate concentration relative to k_R (S/k_R); and n is the number of identical binding sites per protein molecule for each type of ligand, i.e., the number of protomers. A fundamental property of this model, which is apparent from the comparison of these two functions in Fig. 1, is
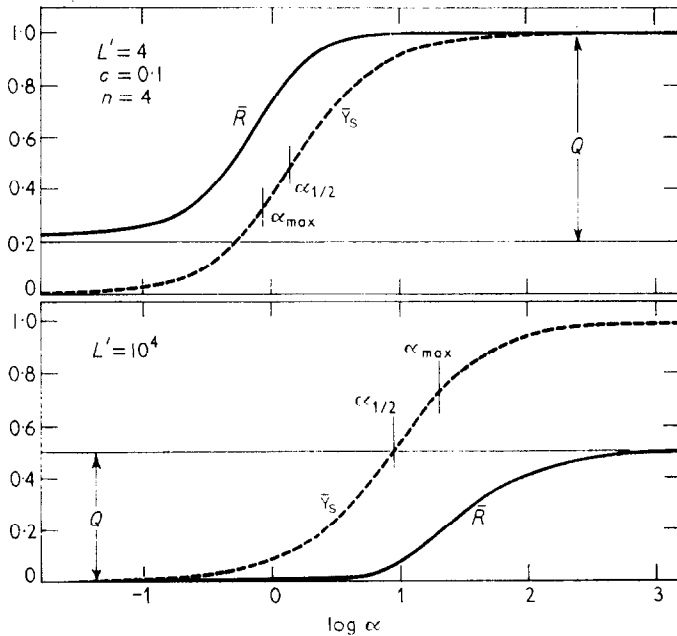

FIG. 1. The saturation function, $\bar{Y}_s$, and state function, $\bar{R}$, for two values of the apparent allosteric constant, L', equations (1), (2) and (3). Also indicated are the allosteric range, Q, equation (7), and the ligand concentrations corresponding to half-saturation, $\alpha_{1/2}$, and to the maximum slope of the Hill plot, α_{max}, equations (5) and (6).

that the extent of the conformational transition ($\bar{R}$) as a function of free ligand concentration (α) is not necessarily proportional to the amount of ligand bound ($\bar{Y}_s$)†.

Inherent in the model is the assumption that heterotropic ligands influence the homotropic interactions described above exclusively by shifting the conformational equilibrium. Mathematically, all heterotropic interactions may therefore be encompassed in a single parameter, L', the ratio of T to R states of the protein in the presence of all effectors except the ligand whose binding is being studied. This apparent allosteric constant is related to the intrinsic allosteric constant, L, by

$$L' = L \left(\frac{1 + \beta d}{1 + \beta} \cdot \frac{1 + \gamma e}{1 + \gamma} \right)^n \tag{3}$$

where the heterotropic effectors are classified as either inhibitors ($k_R/k_T = d > 1$) or activators ($k_R/k_T = e < 1$), and are present at concentrations β and γ, respectively, relative to the corresponding k_R‡. Substitution of the parameters L' in place of L in equations (1) and (2) produces general equations for the saturation and conformational state functions of allosteric proteins. Since either the addition of inhibitor or the depletion of activator concentration will tend to increase L', according to equation (3), the use of this parameter permits a systematic analysis of the heterotropic effects of any combination of ligands having either non-exclusive or exclusive affinity for one protein state.

While the apparent allosteric constant L' may be determined by numerical fitting of the saturation data or by physical chemical techniques, a more easily measured parameter of the effects of heterotropic ligands is the substrate concentration required for half-saturation ($\alpha_{1/2}$). In terms of the allosteric model, the following relationship between $\alpha_{1/2}$ and L' may be derived by replacing L by L' and $\bar{Y}_s$ by $1/2$ in equation (2):

$$L' = \frac{\alpha_{1/2} - 1}{1 - \alpha_{1/2}c} \left(\frac{1 + \alpha_{1/2}}{1 + \alpha_{1/2}c} \right)^{n-1}. \tag{4}$$

Accordingly, $\log \alpha_{1/2}$ is a sigmoidal function of $\log L'$, when the value of c is greater than zero.

A standard measure of homotropic interactions in the saturation of multivalent proteins has been the slope of the Hill plot, n_H, (Brown & Hill, 1922-23). The physical significance of this parameter has been discussed by Wyman (1963). When the saturation function is defined by equation (2), the function

$$n_H = \frac{d \log [\bar{Y}_s / (1 - \bar{Y}_s)]}{d \log \alpha} \tag{5}$$

is found to reach a maximum, $n_{H \, max}$, at some substrate concentration, α_{max}, which does not necessarily coincide with $\alpha_{1/2}$, as indicated on Fig. 1. The direction and magnitude of the departure of α_{max} from $\alpha_{1/2}$, depend on the relative values of L' and $c^{-n/2}$. In the following analysis of the effects of heterotropic ligands, $n_{H \, max}$

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† This feature of the model of Monod et al. (1965) should be contrasted with the predictions of the "induced fit" theory (Koshland, 1963), in which ligand binding is requisite for a conformational transition.

‡ We exclude from this discussion direct interactions between distinct ligands such as substrates and substrate analogues. The extension to systems with more than one modifier of each class involves a simple product of expressions of the form $\left(\dfrac{1 + \beta d}{1 + \beta} \right)^n$.

rather than the slope of the Hill plot at half-saturation will be used as the parameter of homotropic interactions, since the former is a measure of the maximum degree of co-operativity under the specified conditions.
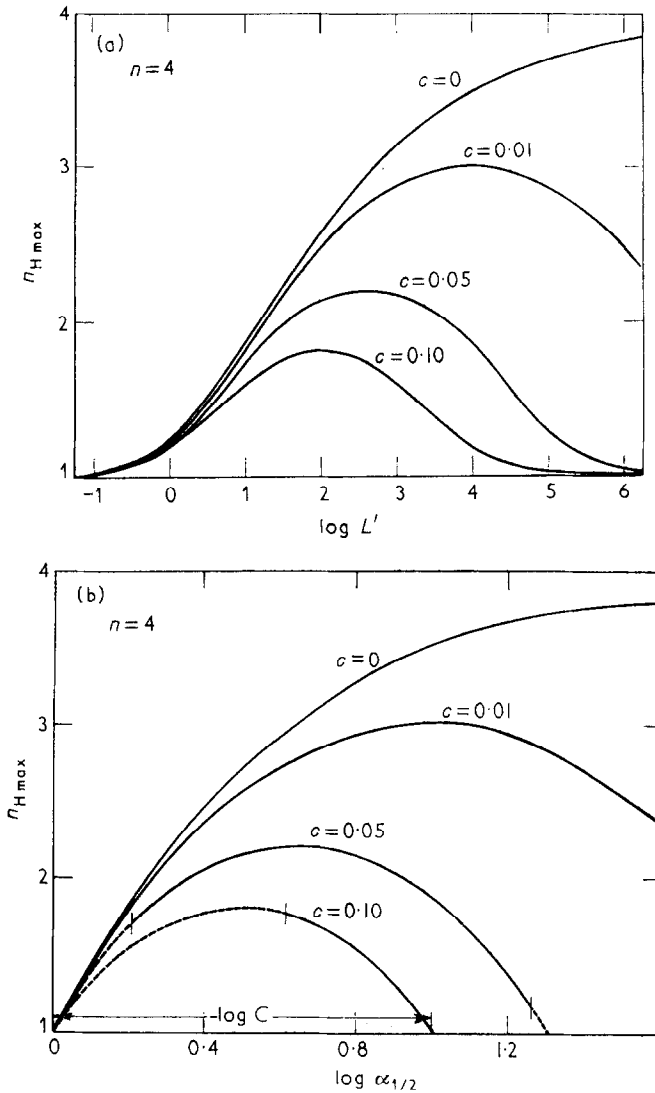

FIG. 2. Dependence of the maximum slope of the Hill plot, $n_{H\ max}$, on the ratio of affinities of the ligand for the R and T states, c.

(a) Variation of $n_{H\ max}$ with the apparent allosteric constant, L', equations (2), (3) and (5). For $c = 0$, $n_{H\ max}$ approaches n, the number of sites, as L' tends to infinity. For $c > 0$, the maximum value attainable by $n_{H\ max}$, $n_{H\ max\ max}$, is less than n and occurs at $L' = c^{-n/2}$.

(b) Variation of $n_{H\ max}$ with $\alpha_{1/2}$, the substrate concentration required for half saturation. Corresponding values of $n_{H\ max}$ and log $\alpha_{1/2}$ were obtained from Hill plots computed according to equations (2) and (5). For $L' = c^{-n/2}$, $n_{H\ max} = n_{H\ max\ max}$ and $\alpha_{1/2} = \alpha_{\ max} = c^{-1/2}$. In curves for $c = 0.05$ and $c = 0.1$, values of log $\alpha_{1/2}$ and $n_{H\ max}$ which are inaccessible as a consequence of limits on L' are indicated by *dashed lines*. Limits were calculated according to equation (9) for an inhibitor with $d = 5$ and an activator with $e = 0.4$ and values of the intrinsic allosteric constant $L = 400$ for the curve with $c = 0.05$ and $L = 10^4$ for the curve with $c = 0.1$.

(b) *Dependence of* $n_{H\ max}$ *and* $\alpha_{1/2}$ *on* L'

Heterotropic ligands influence both the shape and the position of the substrate-saturation function. In terms of the parameters introduced above, heterotropic effects on homotropic co-operativity in binding may be expressed as the variation of $n_{H\ max}$ with L', which is illustrated in Fig. 2(a). As seen in the curve for $c = 0$, the assumption of exclusive binding, discussed by Monod et al. (1965), implies that by decreasing L', the successive addition of activator reduces homotropic interactions until $n_{H\ max} = 1$, and, conversely, that the increase in L' concomitant with addition of inhibitor enhances homotropic interactions until $n_{H\ max} = n$, the total molar binding capacity. When c is not identically zero (non-exclusive binding), the results are qualitatively different: the computed curves of $n_{H\ max}$ as a function of log L' are bell-shaped, pass through a maximum, $n_{H\ max\ max}$, and tend at *both* extremes to 1. Although the value of the maximum depends on both n and c, the computed values of the "reduced Hill maximum" $[(n_{H\ max\ max} - 1)/(n - 1)]$ depend only on the value of c.

The bell-shaped dependence of $n_{H\ max}$ on log L' is correlated with the sigmoidal dependence of log $\alpha_{1/2}$ on log L' (when c is non-zero) in Fig. 2(b). The effects of a given heterotropic ligand on the shape of the substrate saturation curve ($n_{H\ max}$) as compared with its effects on the position ($\alpha_{1/2}$) are thus seen to depend *in both magnitude and direction* on the value of L'. In the region surrounding $n_{H\ max\ max}$, the value of $n_{H\ max}$ is least sensitive, while log $\alpha_{1/2}$ is most sensitive to changes in L'. At the extremes of L', on the other hand, all binding is to the predominant state of the protein. Consequently, the homotropic interactions which are mediated by transitions between states are not apparent and the corresponding saturation curves are hyperbolas characteristic of the intrinsic affinity of the ligand for either the R or T state, with $\alpha_{1/2} = 1$ or $1/c$.

3. Consequences of Non-exclusive Binding for the Range of $\bar{R}$, $n_{H\ max}$ and $\alpha_{1/2}$

The mass action law predicts that saturating concentrations of a ligand which is bound exclusively by one state of an allosteric protein will pull the R $\rightleftharpoons$ T equilibrium essentially to completion in the direction of that state. Conversely, the finite affinity of an effector for *both* states of the protein imposes limits on the extent to which the equilibrium may be shifted. In qualitative terms, the limits on the conformational ratio must depend on both the apparent allosteric constant and the ratio of affinities of the ligand for the R and T states.

To quantitate the limitations on the conformational equilibrium, we again use L' as the parameter of heterotropic interactions and represent homotropic effects by the state function, $\bar{R}$. Considering first the consequences of non-exclusive substrate binding, it is apparent from equation (1) that for a given value of L', the range of values of $\bar{R}$ between zero and infinite substrate concentrations,

$$[1/(L' + 1)] \leq \bar{R} \leq [1/(L'c^n + 1)] \tag{6}$$

may be considerably less than 1. As illustrated in Fig. 1, for low values of L', a large fraction of the protein molecules are in the R state in the absence of substrate, and conversely, for very high values of L', saturating amounts of substrate will not pull the conformational equilibrium completely in favor of the state for which the substrate

has preferential (but non-exclusive) affinity. Furthermore, the difference between the limits on $\bar{R}$ for this ligand, which has been designated the allosteric range, Q (Crick & Wyman, manuscript in preparation), given by:

$$Q = \bar{R}_{\alpha \to \infty} - \bar{R}_{\alpha = 0} = \frac{L'\,(1 - c^n)}{(L' + 1)\,(L'c^n + 1)} \tag{7}$$

is a convenient parameter for investigating the consequences of non-exclusive binding. The correlation of Q and $n_{H\ max}$ as functions of L', for example, provides insight into the occurrence of $n_{H\ max\ max}$ when c is non-zero. As observed in the computed curves (Fig. 3), it is precisely at the value of L' at which $n_{H\ max\ max}$ occurs ($L' = c^{-n/2}$), that the allosteric range itself reaches the maximum

$$Q_{max} = (c^{-n/2} - 1)/(c^{-n/2} + 1). \tag{8}$$

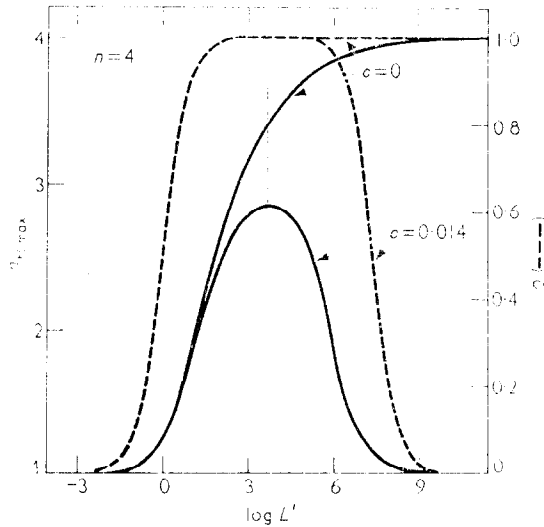In other words, *the maximum allosteric range allows the maximum homotropic co-operativity in binding.*

FIG. 3. Correlation of the maximum slope of the Hill plot, $n_{H\ max}$ (———) and the allosteric range, Q (– – –), as functions of the apparent allosteric constant, L', for exclusive ($c = 0$) and non-exclusive binding. The maxima of both functions, Q_{max} and $n_{H\ max\ max}$, occur at $L' = c^{-n/2}$ when c is non-zero, equations (7) and (8). The values $c = 0.014$ and $\log L' = 3.96$ are the parameters derived by Monod *et al.* (1965) for the saturation of human hemoglobin by oxygen at pH 7.0.

Extending the consideration of non-exclusive binding to both classes of heterotropic ligands, we determine the limiting ratios of T to R states in the absence of substrate, in other words, the range of L'. For a system containing a single effector of each type, it is apparent from equation (3) that the maximum value of L' occurs in the absence of activator and the presence of infinite inhibitor concentration ($\gamma = 0$, $\beta = \infty$), while the minimum corresponds, by symmetry, to $\beta = 0$, $\gamma = \infty$. For the more general case in which there may be several inhibitors and activators having relative affinities d_i and e_i, and concentrations β_i and γ_i, respectively, the range of L' is defined by

$$L\,e_1^n e_2^n \ldots e_i^n \ldots \leq L' \leq L\,d_1^n d_2^n \ldots d_i^n \ldots \tag{9}$$

These limitations on L', which results from non-exclusive binding of the heterotropic effectors, in turn place additional restrictions on the ranges of both $\alpha_{1/2}$ and $n_{H\ max}$ as indicated on Fig. 2(b).

To summarize the various restrictions on allosteric transitions discussed in this section, we have devised the grid, shown in Fig. 4, of the accessible distributions of the conformational states of a given allosteric protein (L, n) in the presence of a given complement of ligands (c, d_1, e_1). The ordinate of this grid corresponds to homotropic interactions, the height of the accessible region being the allosteric range of the substrate (equation (7)). The abscissa of the grid corresponds to heterotropic interactions, the horizontal extremes of the accessible region reflecting the relative affinities of the activators and inhibitors, according to equation (9).
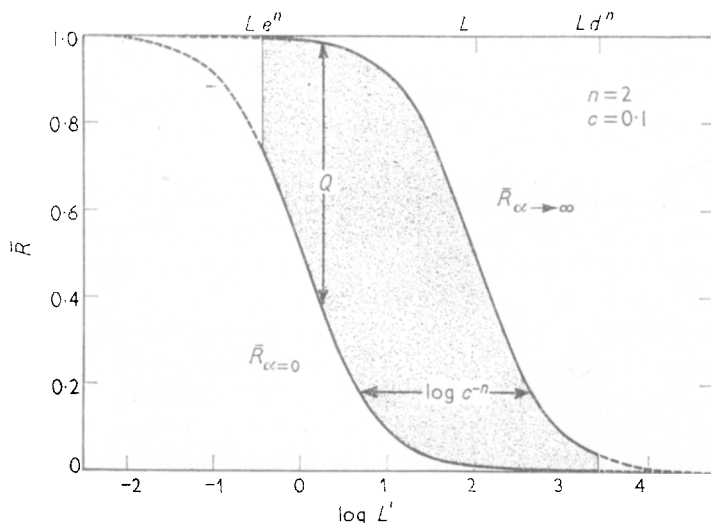

FIG. 4. Restrictions on allosteric transitions in the presence of non-exclusively bound ligands. The accessible region (shaded) is delimited by the state functions, $\bar{R}$, at infinite and zero substrate concentrations and the range of the apparent allosteric constant, L', equations (6) and (9). The grid illustrated corresponds to an intrinsic allosteric constant, $L = 100$, and relative affinities of the substrate, inhibitor and activator of $c = 0.1$, $d = 5$ and $e = 0.4$, respectively. The maximum allosteric range, Q_{max}, corresponding to these parameters is 0.82, equation (8).

4. Experimental Consequences

The application of the preceding theoretical developments to *binding* data obtained with real systems involves the assumption that the observed allosteric interactions are manifestations of a single conformational equilibrium between two states of the protein and that the concentrations of hybrid states (in which some protomers correspond to the R conformation and some to the T form) are negligible. The interpretation of *kinetic* data in these terms involves the additional assumption that both states of the protein have the same maximum reaction velocity, so that the reported initial velocities (v) are proportional to the fractional saturation of the enzyme by the substrate $(\bar{Y}_s)$.

Granting these assumptions, we consider first the observations that certain heterotropic ligands cause marked shifts in the v *versus* S curves but have negligible effects

on the slope of the corresponding Hill plots (Atkinson *et al.*, 1965a; Preiss, Shen, Greenberg & Gentner, manuscript in preparation). One explanation for such effects is that the values of c and L for these systems is such that the co-operativity of substrate-binding is maximal. Under these conditions, $n_{H\ max}$ would be close to $n_{H\ max\ max}$ and, as seen in Fig. 2(b), would vary only slightly with $\alpha_{1/2}$.

Certain aspects of the alkaline Bohr effect in hemoglobin may be similarly interpreted. In the case of human hemoglobin, for example, the reported slopes of Hill plots of oxygen saturation in buffers of pH 6·5 to 8·0 (without added salts) vary only slightly from 2·9, while the oxygen pressure for half-saturation $p_{1/2}$ varies by a factor of 5 (Antonini, Wyman, Rossi-Fanelli & Caputo, 1962). Surprisingly, for $n = 4$ and $c = 0·014$ (calculated by Monod *et al.*, 1965, from the data of Lyster), the computed curve of $n_{H\ max}$ as a function of L' (Fig. 3) shows that $n_{H\ max\ max}$ is 2·9 and occurs at a value of L' close to that actually found for this system (Monod *et al.*, 1965). As a consequence, the change of $p_{1/2}$ produced experimentally by a pH shift from 6·5 to 8·0 would be expected to be accompanied by a variation of $n_{H\ max}$ of only 6·5%. Accordingly, the Bohr protons may conceivably act as heterotropic ligands with preferential affinity for the state of hemoglobin to which oxygen is weakly bound.

A further prediction of the model of Monod *et al.* (1965) is that for certain combinations of the parameters ($L' > c^{-n/2}$) an increase of inhibitor or a depletion of activator concentration would decrease $n_{H\ max}$, although such seemingly paradoxical effects have not yet been found experimentally.

Non-exclusive binding of at least one of the allosteric ligands may also be indicated by the observation of various partial and multivalent effects (ref. in Stadtman, 1966). Partial antagonism of the binding of limited amounts of a given ligand by concentrations of an effector with *opposite* preferential affinity, for example, may result from non-exclusive binding of either or both of the ligands, as shown in Fig. 5.
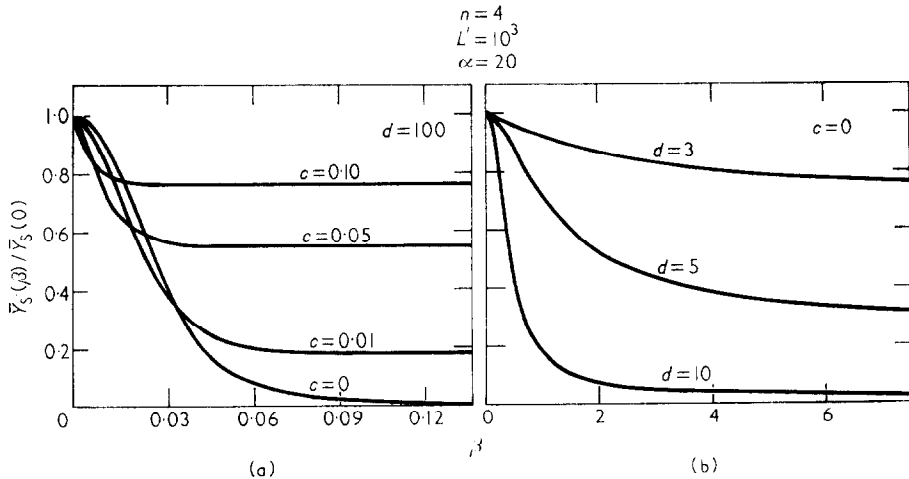

FIG. 5. Partial inhibition resulting from non-exclusive binding of the substrate or inhibitor. Substrate saturation in the presence of inhibitor concentration β relative to saturation in the absence of inhibitor, $\bar{Y}_s(\beta)/\bar{Y}_s(0)$, as a function of β for the given apparent allosteric constant, L', and substrate concentration, α.

(a) Preferential affinity of inhibitor, d, is fixed; that of substrate, c, is varied. (b) Exclusive substrate binding, $c = 0$; variable preferential affinity of inhibitor, d.

Such effects, computed on the basis of the model of Monod *et al.* (1965), have numerous parallels in experimental observations of partially competitive inhibition kinetics, which can be attributed neither to effects on the maximum reaction velocity nor to heterogeneity in the enzyme population (Segal, Kachmar & Boyer, 1952; Sanchez & Changeux, 1966; Cohen, Patte & Truffa-Bachi, 1965). In particular, Gerhart's (1964) interpretation of his results concerning the partial inhibition of aspartate transcarbamylase from *Escherichia coli* by several nucleoside triphosphates in terms of non-exclusive binding of the inhibitors is supported by the striking resemblance of his data to our computations in Fig. 5(b). The incomplete antagonism between an allosteric activator and an allosteric inhibitor is fundamentally analogous to the substrate-inhibitor interactions described above. For example, the inability of excesses of the activators L-norleucine or L-valine to overcome inhibition of L-threonine deaminase by L-iso-leucine has been well documented for the enzyme isolated from both *E. coli* (Changeux, 1962) and *Salmonella typhimurium* (Maeba & Sanwal, 1966).

The combined effects of several ligands with *similar* preferential affinity may also be analyzed in terms of the model of Monod *et al.* (1965). Thus, the observed inability of the activator fructose-1, 6-diphosphate to eliminate co-operativity in the kinetics of the phosphoenolpyruvate carboxylase reaction (Sanwal & Maeba, 1966) may be analogous to our predictions for $n_{H\ max}$ in Fig. 2(b). Similarly, the cumulative feedback inhibition of glutamine synthetase by saturating concentrations of eight specific metabolites (Woolfolk & Stadtman, 1964) may be interpreted by assuming that each type of inhibitor is non-exclusively bound at distinct sites on the enzyme molecule (equation (9)).

The preceding interpretations of some experimental data in terms of our extension of the model of Monod *et al.* (1965) illustrate the variety and complexity of effects which can derive from the simple physical hypotheses on which it is based. These results emphasize the difficulty of establishing or rejecting the applicability of the model to a given physical situation without a detailed evaluation of the parameters involved.

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