A Theoretical Study of the Binding of Small Molecules to a Polymerizing Protein System. A Model for Allosteric Effects*

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ABSTRACT: Previously established concepts of multiple equilibria have been applied to a system in which a protein monomer coexists with a single higher polymer and both bind a low molecular weight solute (S). The treatment permits the number of binding sites per mole of monomer and polymer to assume any integral values (including zero), but assumes that sites within a molecular entity are equivalent with regard to binding, except for a statistical effect. An equation is derived which relates the free equilibrium concentration of S in any equilibrium mixture to a binding function, r, defined on a weight-concentration scale as the concentration of S bound divided by the initial protein concentration. It is shown that equations of the same form describe binding in other types of systems, including those involving isomerization of the protein and a system in which a series of polymers coexist in equilibrium. In the special case where no binding sites are lost in successive polymerization reactions and the intrinsic binding constants are identical for all molecular entities it is shown that the plot of r *vs.* [S] is a rectangular hyperbola describing the binding to any polymer alone. Additional simultaneous equations (polynomials) are presented which permit numerical solution of cases where the phenomena of binding and polymerization are either competitive or noncompetitive. Binding curves have been calculated with specified sets of parameters, and methods are discussed for the distinction between competitive and noncompetitive situations. Consideration of a system in which monomer, but not higher polymer, binds S shows that sigmoidal binding curves result in the competitive case, this allosteric effect being favored by an initially large proportion of nonbinding polymer. It is also concluded that a polymerization (especially one involving a large polymer) is more effective than an isomerization in producing sigmoidality of the binding curve. Possible significance of these results in relation to several allosteric systems is discussed.

The biological implications of allosteric phenomena in relation to metabolic control have been considered extensively (Stadtman, 1966), but the basis of such effects has received far less attention. Originally it was suggested that sigmoidal binding curves obtained with the oxygen–hemoglobin system satisfied the postulate that oxygen bound to a dissociated form of hemoglobin, which existed in equilibrium with a polymeric, nonbinding form (Douglas *et al.*, 1912; Briehl, 1963; Schejter *et al.*, 1963). However, this idea has been replaced by the hypothesis proposed by Monod *et al.* (1965) that a model involving an equilibrium mixture of two conformational states of macromolecule with different affinities for small molecule, S, affords a plausible explanation of allosteric effects. Koshland *et al.* (1966) have subsequently presented several models which adequately describe the oxygen–hemoglobin data, but these were also based on isomerization. This recent emphasis on conformational changes has led to the situation where virtually all allosteric phenomena are attributed to the presence of isomeric forms of the macromolecule. This situation was not basically changed by the formulation of a detailed mechanism for the interconversion of isomeric forms of hemoglobin which includes a dissociation step (Benesch *et al.*, 1965,1966).

The present theoretical study of the binding of a small molecule to different polymeric species coexisting in equilibrium reemphasizes that this model also provides a plausible basis for allosteric effects. Furthermore, distinction between the isomer and polymer models should be possible from experimental data. Although this study is concerned primarily with the case of polymerization, and its comparison with the isomer model, it is also evident that systems of the types $A + B \rightleftarrows C$ and $A + B \rightleftarrows C + D$, where one or all of A, B, C, and D bind S, may also be invoked to account for allosteric phenomena. The present treatment may be extended readily to include these reactions.

The usual practice of attributing allosteric effects to the presence of isomeric forms of the macromolecule is obviously inadvisable without supporting experimental evidence for the validity of the postulate. With this viewpoint in mind the theoretical expressions describing binding curves are derived in a form suitable for experimental application. Numerical examples are presented to illustrate the effects of varying the various parameters for the polymer and isomer cases. In addition, brief mention is made of a few systems for which published experimental data suggest the possibility of mechanisms other than isomerization as the basis for the allosteric phenomena.

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Theory

General Formulation of the Binding Function (r). Consider first a system in which monomer (A) coexists in equilibrium with a single higher polymer (C), *i.e.*

$$nA \rightleftarrows C \tag{1}$$

The addition of solute (S) results in binding to A and C according to eq 2, where each equilibrium reaction is governed by the defined equilibrium constant.

$$\begin{aligned}
&A + S \rightleftarrows AS;\ L_1 = \frac{[AS]}{[A][S]} \\
&C + S \rightleftarrows CS;\ M_1 = \frac{[CS]}{[C][S]} \\
&\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots \\
&AS_{i-1} + S \rightleftarrows AS_i;\ L_i = \frac{[AS_i]}{[AS_{i-1}][S]} \\
&CS_{j-1} + S \rightleftarrows CS_j;\ M_j = \frac{[CS_j]}{[CS_{j-1}][S]} \\
&\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots \\
&AS_{p-1} + S \rightleftarrows AS_p;\ L_p = \frac{[AS_p]}{[AS_{p-1}][S]} \\
&CS_{i-1} + S \rightleftarrows CS_q;\ M_q = \frac{[CS_q]}{[CS_{q-1}][S]}
\end{aligned} \tag{2}$$

Concentrations are on a molar scale and there are p binding sites per mole of A ($i = 1, 2 \ldots p$) and q binding sites per mole of C ($j = 1, 2 \ldots q$). Since equilibrium concentrations of free A, C, and S, and all AS_i and CS_j are independent of the pathway of formation, the above equilibrium constants (all L_i and M_j) together with a constant relating to the equilibrium between A and C are sufficient to describe any equilibrium mixture.

An experimentally determinable value of the binding function (r) defined on a weight-concentration scale as the concentration of S bound divided by the initial protein concentration, is given by eq 3 for any equilibrium mixture. M_S is the molecular weight of S, and M_A that of monomer.

$$r = \frac{M_S\{[AS] + 2[AS_2] + \ldots + i[AS_i] + \ldots + p[AS_p] + [CS] + 2[CS_2] + \ldots + j[CS_j] + \ldots + q[CS_q]\}}{M_A\{[A] + [AS] + \ldots + [AS_i] + \ldots + [AS_p]\} + nM_A\{[C] + [CS] + \ldots + [CS_j] + \ldots + [CS_q]\}} \tag{3}$$

Substitution of expressions for all L_i and M_j from eq 2 into eq 3 gives

$$r = \frac{M_S[A][S]f_1' + M_S[C][S]f_2'}{M_A[A]f_1 + nM_A[C]f_2} \tag{4a}$$

where

$$f_1 = 1 + L_1[S] + \ldots + L_1L_2 \ldots L_i[S]^i + \ldots + L_1L_2 \ldots L_p[S]^p \tag{4b}$$

$$f_2 = 1 + M_1[S] + \ldots + M_1M_2 \ldots M_j[S]^j + \ldots + M_1M_2 \ldots M_q[S]^q \tag{4c}$$

$$f_1' = df_1/d[S] \tag{4d}$$

$$f_2' = df_2/d[S] \tag{4e}$$

By making the assumption that all binding sites within a molecular entity are equivalent except for a statistical effect in binding it is possible to define intrinsic binding constants, K_A and K_C, as follows (Klotz, 1946)

$$L_i = \frac{\{p - (i - 1)\}K_A}{i} \tag{5a}$$

$$M_j = \frac{\{q - (j - 1)\}K_C}{j} \tag{5b}$$

Without this assumption of equivalence of sites all L_i and M_j must be treated independently. Substituting eq 5a into eq 4b, and eq 5b into eq 4c gives on application of the binomial theorem

$$f_1 = (1 + K_A[S])^p \tag{6a}$$

$$f_2 = (1 + K_C[S])^q \tag{6b}$$

Thus eq 4a becomes

$$r = \frac{\{M_S[A][S]pK_A(1 + K_A[S])^{p-1} + M_S[C][S]qK_C(1 + K_C[S])^{q-1}\}}{M_A[A](1 + K_A[S])^p + nM_A[C](1 + K_C[S])^q} \tag{7}$$

In a more general case, where a series of polymers coexisting in equilibrium bind S, it may be shown by a similar treatment that

$$r = \frac{M_S \sum_{m=1}^{m=n} [m][S]\tau_m K_m(1 + K_m[S])^{\tau_m - 1}}{M_1 \sum_{m=1}^{m=n} m[m](1 + K_m[S])^{\tau_m}} \tag{8}$$

where m refers to the polymeric species ($m = 1$, monomer; $m = 2$, dimer, etc.), $[m]$ represents the free molar concentration of that polymer, K_m the intrinsic binding constant for that polymer, and τ_m the number of binding sites per mole of the respective polymer. It is also clear that an analogous approach provides expressions relating r to [S] when ligand binds to one or more macromoleuclar species undergoing reactions of the type $A + B \rightleftarrows C$ or $A + B \rightleftarrows C + D$.

Special Cases. We now wish to consider eq 7 and 8 as applied to specific systems.

A. A SINGLE MACROMOLECULE. By placing [C] (or [A]) equal to zero in eq 7 the physical situation described is the binding of S to equivalent sites on a single macromolecule. The plot of r *vs.* [S] is a rectangular hyperbola (Klotz, 1946) described by the equation (when [C] is zero)

$$r = \frac{M_S p K_A[S]}{M_A(1 + K_A[S])} \tag{9}$$

A double-reciprocal plot of the binding curve ($1/r$ *vs.* $1/[S]$) gives a straight line, the slope and intercept of which enable p and K_A to be estimated provided M_A is known.

B. AN ISOMERIZING SYSTEM. By substituting $n = 1$ into eq 1 the model simplifies to an isomerizing system governed by a dimensionless equilibrium constant. Monod *et al.* (1965) have already treated this case, but with the additional restriction that the number of binding sites per mole was the same for each isomer. Their eq 2 may be obtained from eq 7 above by substituting $n = 1$ and $p = q$, and noting the different symbolism adopted for the defined parameters; in particular it should be noted that $M_A r/M_S = p\bar{Y}_S$. This relation arises because Monod *et al.* (1965) have defined the binding function, Y_S, in terms of the number of moles of bound S per total number of potential binding sites. The denominator of r used in the present study is simply the weight concentration of protein employed.

C. A POLYMERIZING SYSTEM WITH IDENTICAL INTRINSIC BINDING CONSTANT FOR EACH POLYMER. For simplicity consider a monomer with p binding sites in equilibrium with a single higher polymer possessing q binding sites. Let $q = xp$, where x may assume any value including 0 and n. Substituting $K_A = K_C$ into eq 7, and factorizing, yields

$$r = \frac{[M_S p K_A[S]\{[A](1 + K_A[S])^{p-1} + x[C](1 + K_A[S])^{xp-1}\}]}{[M_A(1 + K_A[S])\{[A](1 + K_A[S])^{p-1} + n[C](1 + K_A[S])^{xp-1}\}]} \tag{10}$$

It is clear that eq 10 reverts to eq 9 if $x = n$. In other words, provided no sites are lost on polymerization ($q = np$) and the intrinsic binding constants are the same for all macromolecular entities the rectangular hyperbola obtained by plotting r *vs.* [S] is identical with that obtained if either monomer or polymer could be studied alone. On the other hand, if $x \neq n$ eq 10 shows that deviation from this ideal behavior may be expected.

When $x = n$ it follows that a double-reciprocal plot permits the evaluation of p and K_A even in environments where appreciable amounts of monomer and polymer coexist. The result may be generalized to a situation where a series of polymers coexist, provided no binding sites are lost in the successive polymerizations and all K_m in eq 8 are identical. Sarfare *et al.* (1966) have demonstrated that the binding of the inhibitor β-phenylpropionic acid to α-chymotrypsin in phosphate buffer (pH 6.1), ionic strength 0.2, is an example of this case, since weight-average molecular weights at several enzyme concentrations were unchanged by the presence of S for this monomer–dimer–trimer system.

D. A MONOMER–SINGLE HIGHER POLYMER SYSTEM WITH DIFFERENT INTRINSIC BINDING CONSTANTS. The initial concentration of protein on a weight scale is given by

$$\bar{c}_P = M_A[A](1 + K_A[S])^p + nM_A[C](1 + K_C[S])^q \tag{11}$$

For numerical calculations it is convenient to introduce the transforms $\alpha = K_A[S]$ and $\beta = K_C/K_A$. We note that selection of α as the abscissa for a binding curve permits the representation of several cases with different K_A by a single curve. In these terms eq 7 becomes

$$r = \frac{M_S[A]p\alpha(1 + \alpha)^{p-1} + M_S[C]q\alpha\beta(1 + \alpha\beta)^{q-1}}{\bar{c}_P} \tag{12}$$

For a fixed set of parameters M_S, p, q, β, and $\bar{c}_P$, r may be evaluated as a function of selected α values provided the free equilibrium concentrations [A] and [C] corresponding to the particular α value may be determined. This requires an additional relationship which expresses the law of mass action governing the polymerization equilibrium. Two cases seem pertinent.

In the first, a binding site is identical or closely associated with a polymerization site, so that the phenomena of binding and polymerization become competitive. The *constituent* concentration of monomer (Tiselius, 1930; Nichol and Ogston, 1965) varies with the total protein concentration and the concentration of S. For this competitive case the equilibrium constant, X, is given by

$$X = [C]/[A]^n \tag{13}$$

where X has the dimensions 1^{1-n} mole^{n-1}. Substitution of eq 13 into eq 11 leads to a polynomial in [A].

$$nXM_A(1 + \alpha\beta)^q[A]^n + M_A(1 + \alpha)^p[A] - \bar{c}_P = 0 \tag{14}$$

When $n = 2$ (monomer–dimer) eq 14 is a quadratic in [A] and only the positive root for each selected α is physically acceptable. Similarly, when $n = 3$ (monomer–trimer) there is only one real root for each α, since X and α are necessarily positive. The two conjugate, imaginary roots are rejected.

The second type of situation which may be visualized is one in which binding sites are distinct from polymerizing sites, and the two phenomena are noncompetitive. Thus, while the constituent concentrations of [A] and [C] depend on the value of $\bar{c}_P$ selected, they are not affected by the concentration of S added. In this case eq 11 and 12 again apply, together with eq 15

$$X = [\bar{C}]/[\bar{A}]^n \tag{15}$$

where

$$[\bar{A}] = [A](1 + \alpha)^p \tag{16a}$$

$$[\bar{C}] = [C](1 + \alpha\beta)^q \quad (16b)$$

Combination of eq 11, 15, 16a, and 16b gives

$$nXM_A(1 + \alpha)^{np}[A]^n + M_A(1 + \alpha)^p[A] - \bar{c}_P = 0 \quad (17)$$

For a selected value of α solution of the polynomial gives [A], from which the corresponding values of [C] and r (eq 7) may be calculated for the noncompetitive case. It is noted that the solution for this noncompetitive case when $q = np$ and $\beta = 1$ is identical with that obtained in C.

Another special example of a noncompetitive case involves the situation where two noninteracting solutes both bind S. Equation 7 applies noting that nM_A must be replaced by M_C, and that the law of mass action no longer applies. Equations 16a and b may still be applied, however, to give a complete solution, provided [$\bar{A}$] and [$\bar{C}$] may be estimated.

E. A SERIES OF POLYMERS COEXISTING IN EQUILIBRIUM. Equation 8 may be rewritten in expanded form for any sets of polymers, but contains the parameter [m], the equilibrium concentration of unbound polymer, in each term of the numerator and denominator. Three possibilities arise: (i) all successive polymerizations are competitive with binding of S, whereupon a polynomial in [m] may be written in a form similar to eq 14; (ii) all polymerizations are noncompetitive with binding reactions, in which case eq 17 is the relevant form of the polynomial; and (iii) competitive and noncompetitive phenomena coexist, whereupon polynomials of both types must be employed. In this complicated case there are sufficient simultaneous equations to solve for r as a function of α in numerical examples, but analysis of experimental results may be prohibitively difficult.

Application of Theory

Competitive Interactions. Initially we shall consider a situation where monomer coexists in equilibrium with a single higher polymer, which does not bind S. Throughout these examples fixed values have been assigned to the following parameters; $M_S = 200$, $M_A = 20{,}000$, $q = 0$, and $\beta = 0$. Figure 1 illustrates computed binding curves showing the effect of varying the number of binding sites per mole of monomer (p) in a monomer–dimer system with $X = 10^7$ l./mole at an initial protein concentration ($\bar{c}_P$) of 10 g/l. These binding curves are obviously sigmoidal, the values of r approaching the maximum limiting values, pM_S/M_A, at much lower values of α for higher values of p. The molar ratio of dimer to monomer defined by the parameters relevant to Figure 1 was 50:1 initially (in the absence of S). The same molar ratio of nonbinding form to binding form, and a value of 4 for p was selected to compare different polymeric systems. Values of X for isomer, monomer–trimer, and monomer–hexamer systems were computed to achieve this ratio at a fixed protein concentration of 10 g/l., all other relevant parameters being the same for each case. The solid curves of Figure 2 clearly show that the binding data assume a more sigmoidal character as the value of n increases progressively. For example, at low values of α, $dr/d\alpha$ is less for the monomer–trimer than the isomer model, but the curves intersect at higher α values so that r approaches the maximum limiting value at lower α for $n = 3$. On a molar basis it would therefore appear that polymerization is more effective than isomerization in leading to sigmoidal binding curves, this effect being more pronounced the higher the value of n. The broken line in Figure 2 refers to the isomer model with $X = 300$ for comparison with a monomer–hexamer system having the same initial weight ratio of inactive to active form. On this weight basis it would also appear that polymerization leads to binding curves with more pronounced sigmoidal character, since the two curves are essentially identical at low values of α, but r for the polymeric system then increases more rapidly with increasing α.

The effect of changing the polymerization association constant (X) in a monomer–dimer system with $p = 4$ and all other parameters the same as in Figure 1 is shown in Figure 3. Pronounced sigmoidality is favored by an initial excess of nonbinding dimer, and thus the allosteric effect becomes less noticeable as X is decreased. A similar decrease in the extent of sigmoidality would result in practice (where X is fixed) when a series of binding curves were determined at progressively lower total protein concentrations.

Thus far illustrations have been plotted in the form r *vs.* α, but for cases in which $p = 1$ a double-reciprocal plot ($1/r$ *vs.* $1/\alpha$) is required to demonstrate that the binding curves are not rectangular hyperbolas. Again the same parameters (with $p = 1$) were selected to illustrate the effect of changing $\bar{c}_P$ in a monomer–dimer system. The results are shown in Figure 4, from which it is evident that deviation of the curves from the straight line describing binding to monomer alone (solid line) increases with increase of $\bar{c}_P$, and is more noticeable at low α values. All curves intersect on the $1/r$ axis at $M_A/M_S p$.

Noncompetitive Interactions. The behavior of this type of system will first be illustrated by considering a monomer–dimer system. Equation 17 reverts to a quadratic, which may be solved for [A] by selecting the positive root, *viz.*

$$[A] = y/4X(1 + \alpha)^p \quad (18a)$$

where

$$y = -1 + (1 + 8\bar{c}_P X/M_A)^{1/2} \quad (18b)$$

Combining eq 16a, 16b, 18a, and 18b gives

$$[C] = y^2/16X(1 + \alpha\beta)^q \quad (19)$$

Substituting these expressions for [A] and [C] into eq 12 leads to the following expression for r

$$r = \frac{M_S\alpha}{4X\bar{c}_P}\left\{\frac{py}{1 + \alpha} + \frac{q\beta y^2}{4(1 + \alpha\beta)}\right\} \quad (20)$$

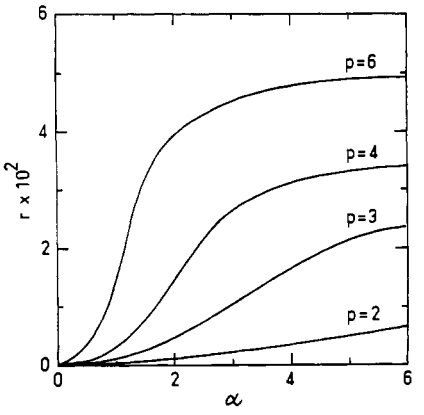

FIGURE 1: Binding curves for a competitive case illustrating the effect of varying the number of binding sites per mole of monomer (p) for a monomer–dimer system. Selected values of other parameters are M_S = 200, M_A = 20,000, q = 0, β = 0, X = 10^7 l./mole, and c_P = 10 g/l.

That eq 20 is consistent with the earlier treatment may be seen by placing $q = 2p$ and $\beta = 1$, whereupon eq 20 simplifies to eq 9, previously obtained under similar circumstances from eq 10. The solid line in Figure 5 is a plot of $1/r$ *vs.* $1/\alpha$ for this situation, with $p = 1$, $q = 2$, $n = 2$, $M_S = 200$, $M_A = 20{,}000$, and $\beta = 1$. The intercept on the $1/r$ axis is given by $M_A/M_S p$, and the line is

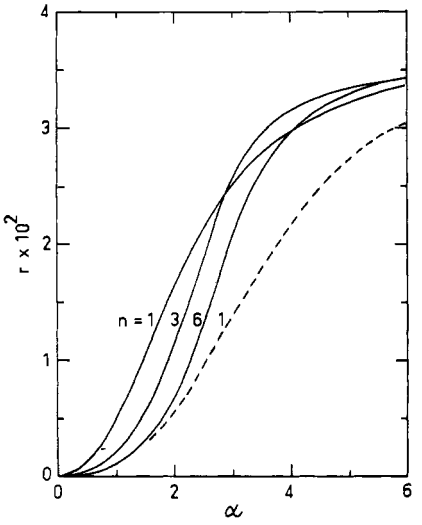

FIGURE 2: Plots of r *vs.* α for a competitive case illustrating the effect of changing n. In each case M_S = 200, M_A = 20,000, p = 4, q = 0, β = 0, and $\bar{c}_P$ = 10 g/l. The solid lines are obtained using a value of X such that the molar ratio of nonbinding to binding form of protein is 50:1, the number referring to the value of n. The dashed line refers to the isomer model with an initial weight ratio of 300:1, the situation existing in the case where n = 6.

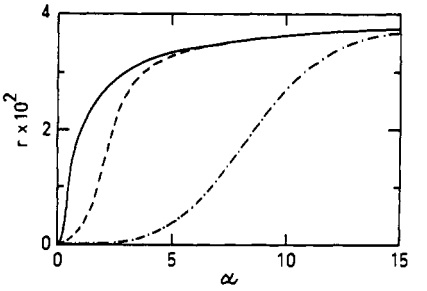

FIGURE 3: The effect of changing the polymerization association constant (X) upon binding curves for a competitive case. Parameters are assigned the following values: M_S = 200, M_A = 20,000, n = 2, p = 4, q = 0, β = 0, and $\bar{c}_P$ = 10 g/l. (———) X = 10^3 l./mole, (– – –) X = 10^7 l./mole, and (–·–·–) X = 10^{11} l./mole.

independent of $\bar{c}_P$ and X. Instances may arise, however, when the double-reciprocal plot for noncompetitive interactions is not linear.

Consider a monomer–dimer system in which binding sites are preserved on polymerization ($q = 2p$) and $\beta \neq 1$. Typical double-reciprocal plots are illustrated by the broken lines in Figure 5, computed using eq 20 and the same parameters selected for the computation of the solid line, except that β = 0.1 and the product $\bar{c}_P X$ was assigned different numerical values. The situation with the latter quantity taken as 6×10^4 could correspond, *e.g.*, to an initial protein concentration of 10 g/l. and a 1:1 molar ratio of monomer:dimer. The curvature of the relevant plot, evident from Figure 5, is in the opposite direction to that obtained in the competitive case (*cf.* Figure 4). Calculated examples (not shown) indicate that the curvature increases as β is decreased, provided $\beta \neq 0$. This behavior could also reflect binding to two different sites on the same macromolecule. However, binding curves at a series of protein concentrations suf-

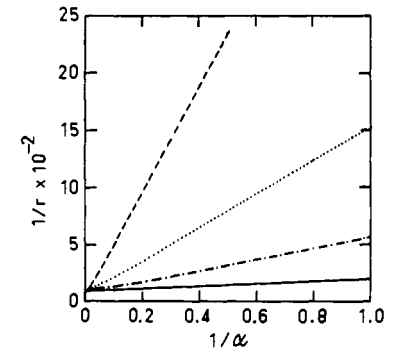

FIGURE 4: Double-reciprocal plots of binding curves for a competitive case where M_S = 200, M_A = 20,000, n = 2, p = 1, q = 0 β = 0, and X = 10^7 l./mole. (– – –) c_P = 2 g/l., (. . . . .) $\bar{c}_P$ = 0.2 g/l., and (–·–·–) $\bar{c}_P$ = 0.02 g/l. The solid line represents the ideal behavior of monomer alone.

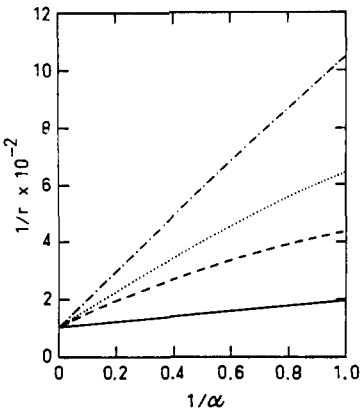

FIGURE 5: Double-reciprocal plots of binding curves for a noncompetitive case where $M_S = 200$, $M_A = 20{,}000$, $n = 2$, $p = 1$, and $q = 2$. The solid line refers to cases where $\beta = 1$, the broken lines to $\beta = 0.1$. (– – –) $X\bar{c}_P = 6 \times 10^4$, (. . . . .) $X\bar{c}_P = 6 \times 10^5$, and (–·–·–) $X\bar{c}_P = 10^6$.

fice theoretically to distinguish between the two possibilities, provided $n \neq 1$. The other curves in Figure 5, calculated with different values of the product $\bar{c}_P X$, demonstrate this point. As with the competitive case the double-reciprocal plot is shifted as $\bar{c}_P X$ increases, in contrast with the behavior exhibited when $q = np$ and $\beta = 1$. Figure 5 also indicates a further similarity between this noncompetitive and the competitive cases in that the intercepts on the $1/r$ axis are identical and given by $M_A/M_S p$. The latter point may be shown theoretically, for at large α, when $(1 + \alpha) \simeq \alpha$ and $(1 + \alpha\beta) \simeq \alpha\beta$, eq 20 for this case reduces to

$$r \sim \frac{M_S p}{8 X \bar{c}_P}(2y + y^2) = \frac{M_S p}{M_A} \qquad (21)$$

Different intercepts on the $1/r$ axis are obtained from a series of double-reciprocal plots at different values of $\bar{c}_P X$ in a noncompetitive situation ($q = np$) with K_C (or β) equal to zero. Combination of eq 7, 15, 16a, and 16b yields the following expression for r.

$$r = \frac{\alpha}{1 + \alpha} \frac{M_S p}{(M_A + n M_A X[\bar{A}]^{n-1})} \qquad (22)$$

Since $[\bar{A}]$ is not a function of [S] or α for the noncompetitive case eq 22 describes a rectangular hyperbola, and a plot of $1/r$ *vs.* 1/[S] is thus a straight line with intercepts $-K_A$ (when $1/r = 0$) and $(M_A + nM_A X[A]^{n-1})/M_S p$ (when 1/[S] = 0). The product $X[\bar{A}]^{n-1}$ equals $[\bar{C}]/[\bar{A}]$, which changes with $\bar{c}_P$ for a polymerizing system. Thus a series of experiments at different initial protein concentrations produces a family of straight lines with different points of intersection of the $1/r$ axis and a common intercept ($-K_A$) on the 1/[S] axis. For a noncompetitive isomerization with $\beta = 0$, however, $[\bar{C}]/[\bar{A}]$ does not vary with $\bar{c}_P$, and thus the slope and intercept of the linear double-reciprocal plot would be independent of $\bar{c}_P$.

Discussion

Experimental evaluation of r requires the determination of the concentration of ligand (S) bound to protein, this quantity being the difference between the initial and equilibrium concentrations of small solute. In the event that the free concentration of ligand may not be estimated in the presence of protein and complex, equilibrium dialysis offers a general, though time-consuming method of estimating binding curves for systems in which both S and protein are in solution form. A suitable liquid–liquid partition system for S would have the advantage of reducing the time factor, but precautions are necessary to ensure that the second phase causes no protein denaturation. Cross-linked dextran, polyacrylamide, or agarose gels afford suitable partition systems for this purpose since both phases have the same solvent composition. These experiments are conveniently performed chromatographically employing frontal analysis under conditions where the complex and protein migrate together ahead of S; the concentration of pure S which separates on the trailing side is a direct measure of its equilibrium concentration (Nichol and Winzor, 1964). This situation is realized fairly readily by choosing a gel such that the protein and complex are both excluded from the gel phase, most of which is accessible to the small solute (S). It is frequently reasonable to assume that binding of several molecules of ligand will not materially affect the sedimentation coefficient of the protein, in which case the slower boundary separating in sedimentation velocity of an equilibrium mixture also yields the free concentration of S directly (Gerhart and Schachman, 1965; Steinberg and Schachman, 1966). A large number of allosteric phenomena detected have involved enzymes, the data being presented in the form velocity *vs.* substrate concentration. In many cases allosteric kinetics may reflect binding phenomena, an analogy made by Monod *et al.* (1965), but the sigmoidality could also result from differences between the rates of product formation from the monomer and polymer forms of the enzyme–substrate complex. Equilibrium studies with a competitive inhibitor as S would be required to establish unequivocally that substrate binding is the source of the allosteric effect.

From this study of ligand binding to polymers coexisting in equilibrium it is clear that double-reciprocal plots of binding curves ($1/r$ *vs.* 1/[S]) at several concentrations of macromolecule suffice theoretically to distinguish between the various combinations of polymerization and ligand binding. Six possible forms that these plots may assume, and the type(s) of system yielding such data may be summarized as follows.

(*1*) *A straight line with slope and intercept independent of protein concentration:* a single protein or series of polymers with polymerization and binding occurring independently, all sites being equivalent, *i.e.*, $\beta = 1$ and $q = np$; or a series of noninteracting species with iden-

tical binding affinities; or noncompetitive isomerization with one form inactive, *i.e.*, $n = 1$ and $\beta = 0$.

(2) *A family of straight lines with different intercepts on the 1/r axis but common intercept on the 1/[S] axis:* noncompetitive polymerization and ligand binding, with one form of polymer binding no ligand ($\beta = 0$).

(3) *A curve convex to the 1/[S] axis, independent of protein concentration:* noncompetitive isomerization and binding, with one form having some, but less affinity for ligand than the other, *i.e.*, $n = 1, q = p$, and $0 < \beta < 1$; or a single protein with two sets of sites having different binding affinities; or two noninteracting proteins with different binding affinities.

(4) *A series of curves convex to the 1/[S] axis with common 1/r intercept:* noncompetitive polymerization and binding, with the polymeric species having some, but less affinity for ligand than monomer, *i.e.*, $n > 1$, $q = np$, and $0 < \beta < 1$.

(5) *A curve initially concave to the 1/[S] axis, independent of protein concentration:* competitive isomerization and ligand binding.

(6) *A series of curves initially concave to the 1/[S] axis with common 1/r intercept:* competitive polymerization and ligand binding. In categories 5 and 6 the curves may subsequently become convex to the 1/[S] axis (at low concentrations of ligand), reflecting pronounced sigmoidality of the binding curves.

Three further points should be noted. First, in the numerical examples chosen to illustrate competitive behavior (Figures 1–4) it was assumed for convenience that monomer was the form which bound S, *i.e.*, β was set equal to zero. The alternative situation involving an active polymer and inactive monomer may be readily examined by introducing into eq 7 the transforms $\alpha' = K_C[S]$ and $\beta' = K_A/K_C$ and setting $\beta' = 0$. Categories pertaining to the nature of double-reciprocal plots for these systems are similar to those outlined above. Secondly, in competitive cases sigmoidal effects may also be observed when both forms bind ligand to some extent, but become less pronounced as β approaches unity (Monod *et al.*, 1965). Finally, classification of a particular system may be difficult in practice due to limitations in the accuracy of experimental binding data. The possibility also exists that polymer and isomer forms of the protein may coexist.

Of the various features of ligand binding to polymers coexisting in equilibrium that have been illustrated, the most striking is the demonstration that a model based on competitive polymerization and ligand binding provides a plausible alternative to the simple isomer postulate of Monod *et al.* (1965) as a basis of some allosteric phenomena. This possible role of polymerization, as we have noted, has been recognized previously, but lacked extensive theoretical support. It is of interest that the same thermodynamic treatment may be used to predict binding curves for the polymerization and isomerization models, even though in their simplest forms they may be distinguished conceptually. In the model of Monod *et al.* (1965) the isomers are visualized as arising from stoichiometric polymerization of monomers (subunits) into two conformational states which coexist in equilibrium, whereas the present model is concerned primarily with situations in which the polymerization equilibrium is not displaced completely in favor of monomer or polymer. The existence of subunit structure in allosteric proteins, discussed by Monod *et al.* (1965) is compatible with both models. Furthermore, the theoretical demonstration (Figure 2) that competitive polymerization-binding systems are more effective than the isomer model in producing sigmoidal binding curves certainly cannot be taken as evidence that the former model pertains exclusively to all *in vivo* systems. Thus the basis of any particular allosteric effect may be attributed to the coexistence of polymers or isomers, or both: possible contributions from reactions involving dissimilar molecules, *e.g.*, the hybridization $A + B \rightleftarrows C + D$, have already been noted. The flexibility of the present treatment to include terms in the binding equation applying to any species which may bind S may prove of value in interpreting complicated cases.

In addition, the present theoretical treatment does suggest experimental approaches which would implicate as a basis of allosteric phenomena reactions characterized by equilibrium constants which are not dimensionless. For example, the existence of a polymerization competitive with ligand binding would be reflected by concentration dependence of the sigmoidal binding curve. If the latter effect were observed, a simple competitive isomerization characterized by a single, dimensionless equilibrium constant could not be the sole basis of the allosteric phenomenon. Since optimal conditions for allosteric binding require relatively small amounts of active polymer (Figure 3) failure to detect an equilibrium between polymeric forms in the absence of ligand does not necessarily preclude the polymerization model. On the other hand, when polymerization and binding are competitive the addition of a ligand (even of small molecular weight) may be expected to effect a change in the weight-average molecular weight of the system. Such changes would not be induced to any marked degree in noncompetitive polymerizing systems or in isomerizing systems of the noncompetitive or competitive type. In this connection the weight-average sedimentation coefficient of the system could be used as an alternative parameter to molecular weight on the assumption that possible isomeric forms are characterized by nearly identical sedimentation coefficients.

A system for which experimental evidence of the above type implicates polymerization equilibria as the basis of allosteric effects rather than isomerization alone is the H_4 isoenzyme of lactic dehydrogenase (Hathaway and Criddle, 1966). At low enzymic concentrations the sedimentation coefficient is that expected of the inactive dimer form, but successive increases in substrate (pyruvate) concentration from 0 to 1 mM result in corresponding increases in the sedimentation coefficient (s). At a substrate concentration of 1 mM, which is required for maximal activity, s corresponds to that of pure tetramer. As noted by Hathaway and Criddle (1966) the kinetics are non-Michaelian, a double-reciprocal replot of their data exhibiting the upward curvature predicted for competitive polymerization and substrate binding re-

actions. Rapidly attained equilibrium between polymeric species with different affinities for S also appears to be responsible for sigmoidal effects observed in the binding of oxygen to lamprey eel hemoglobin, monomer being the active form in this case (Briehl, 1963). The oxygen equilibrium curves are dependent on hemoglobin concentrations under the conditions where allosteric binding is observed, and independent of protein concentration under conditions where binding curves are hyperbolic. In addition, the sedimentation coefficient of the deoxygenated hemoglobin increases with increasing protein concentrations under conditions of allosteric behavior, no such effect being observed with s of the oxygenated form. Examples such as these indicate clearly the applicability of the present generalized theory to the interpretation of experimental systems.

Finally we note two examples where interpretation seems to require systems differing from the simple isomer or polymer models. The regulatory enzyme aspartyl transcarbamylase is a system in which a dissociated form appears to be the active species (Gerhart and Pardee, 1962), but in this case more recent evidence (Gerhart and Schachman, 1965) indicates that the reaction competitive with substrate binding is of the type $A + B \rightleftarrows C$, where A represents the catalytic subunit and B the regulating subunit. An example which seems to require an even more complex model is the binding of oxygen to hemoglobin. The original postulate that polymerization equilibria are involved (Douglas *et al.*, 1912; Briehl, 1963; Schejter *et al.*, 1963) was bypassed in favor of the isomer model (Monod *et al.*, 1965; Koshland *et al.*, 1966) despite experimental evidence that oxygen equilibrium curves depend on hemoglobin concentration (Barcroft, 1914). It is therefore interesting to note that Benesch *et al.* (1965, 1966) have reinstated a dissociation reaction, as well as postulating additional isomerization and hybridization ($A + B \rightleftarrows C + D$) reactions to account for the allosteric nature of the oxygen–hemoglobin system.

References

Barcroft, J. (1914), The Respiratory Function of the Blood, England, Cambridge University.

Benesch, R. E., Benesch, R., and MacDuff, G. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 535.

Benesch, R. E., Benesch, R., and Tyuma, I. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 1268.

Briehl, R. W. (1963), *J. Biol. Chem. 238*, 2361.

Douglas, C. G., Haldane, J. S., and Haldane, J. B. S. (1912), *J. Physiol. 44*, 275.

Gerhart, J. C., and Pardee, A. B. (1962), *J. Biol. Chem. 237*, 891.

Gerhart, J. C., and Schachman, H. K. (1965), *Biochemistry 4*, 1054.

Hathaway, G., and Criddle, R. S. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 680.

Klotz, I. M. (1946), *Arch. Biochem. 9*, 109.

Koshland, Jr., D. E., Némethy, G., and Filmer, D. (1966), *Biochemistry 5*, 365.

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

Nichol, L. W., and Ogston, A. G. (1965), *Proc. Roy. Soc. (London) B163*, 343.

Nichol, L. W., and Winzor, D. J. (1964), *J. Phys. Chem. 68*, 2455.

Sarfare, P. S., Kegeles, G., and Kwon-Rhee, S. J. (1966), *Biochemistry 5*, 1389.

Schejter, A., Adler, A. D., and Glauser, S. C. (1963), *Science 141*, 784.

Stadtman, E. R. (1966), *Advan. Enzymol. 28*, 41.

Steinberg, I. Z., and Schachman, H. K. (1966), *Biochemistry 5*, 3728.

Tiselius, A. (1930), *Nova Acta Regiae Soc. Sci. Upsaliensis [4] 7*, 1.