# LIGAND-INDUCED OLIGOMERIZATION AND REGULATORY MECHANISM

Author: **Allen T. Phillips**
Department of Biochemistry
Pennsylvania State University
University Park, Pennsylvania

Referee: D. Eugene Wampler
Department of Biochemistry
University of Connecticut
Farmington, Connecticut



## I. INTRODUCTION

There is a firm understanding at the present time that the oligomeric nature of proteins is a central and basic feature of allosteric mechanisms of regulation. The examples of enzymes subject to control by effector molecules are quite numerous, and in most, if not all, instances, such enzymes have been shown to possess a quaternary structure which is somehow altered by the allosteric control agents to produce a modulation of catalytic activity. It is the purpose of this review, however, to focus attention on only one aspect of this control process, namely ligand-induced oligomerization, and to attempt to place in proper perspective the importance which this phenomenon may have in the overall question of metabolic regulation of enzymatic action.

Even a casual examination of the protein chemistry and enzymology literature will reveal that a thorough discussion of oligomerizing systems is unfeasible because the number of individual examples is easily in excess of two score. Unfortunately, the more well-behaved systems from a physicochemical viewpoint, to cite the self-association of lysozyme, α-chymotrypsin, and hemocyanin as examples, are not of general interest from the standpoint of biological regulation by ligand control. On the other hand, although hemoglobin has served as the principal model for explaining cooperativity in ligand binding in terms of quaternary structure interactions, there is no sound basis for its inclusion in a discussion of oligomerizing systems. As the choice of topics, therefore, I have preferred to select from a dozen or so logical possibilities some enzymes which are far from being well understood, but which clearly exhibit ligand-induced oligomerization of possible regulatory significance. A partial listing of other enzymes which display more or less well-documented tendencies toward oligomerization under ligand control is provided in Appendix A.

In an enlightening review of the molecular basis for allosteric phenomena, Koshland[1] has argued that association-dissociation reactions are not themselves regulatory functions but, rather, are consequences of the conformational changes generated by ligand binding. Thus, he stated, "The phenomena of association-dissociation can enhance or dampen ligand-induced changes and play an important role in the allosteric effect." If one adopts this interpretation of the interplay between oligomerization and ligand-promoted alterations in

kinetic behavior, then the perennial question of whether or not association-dissociation reactions are biologically significant simplifies to the question of whether there is a need for amplification or moderation of ligand-induced conformational changes in order to effectively regulate the action of a given enzyme. For the examples I have selected to discuss, this question can be answered affirmatively. Whether this reply will be similar for other enzymes possessing oligomerizing tendencies remains to be examined.

## II. MOLECULAR PROPOSALS FOR ALLOSTERIC REGULATION

Before turning to the main theme of this paper, I should like to briefly review some pertinent features of those models for allosteric proteins which have formed the basis for so much of our present understanding in this area, with the primary aim being that of interlacing these ideas with the concept of oligomerization as an asset to regulatory function.

### A. The Concerted Model of Monod, Wyman, and Changeux

Although a great number of mechanisms have been proposed to explain the phenomena of cooperativity in ligand binding, with varying degrees of complexity, the most familiar of these is the Monod, Wyman, and Changeux model. This model is comparatively restrictive, having its foundations in three assumptions: (1) the existence of two (at the least) states which are reversibly accessible in an oligomeric multisite protein, the so-called R and T states, and which generally differ in their affinities for a ligand; (2) an equivalency of all binding sites for a given ligand in each conformation; and (3) an "all or none" change in the conformation of all subunits due to the constraint imposed on each protomer by its association with other protomers. I should now like to examine some details of this proposal as they have been described.[2]

For this model, it is postulated that the two states, $R_0$ and $T_0$, are in equilibrium through the expression $T_0 = LR_0$, where L is the "allosteric constant" relating the two states of the oligomeric protein. The binding of a ligand, F, to a stereospecific site on each protomer can then be written as:

$$\begin{array}{ll} R_0 + F \rightarrow R_1 & T_0 + F \rightarrow T_1 \\ R_1 + F \rightarrow R_2 \text{ and} & T_1 + F \rightarrow T_2 \\ R_{n-1} + F \rightarrow R_n & T_{n-1} + F \rightarrow T_n \end{array}$$

and dissociation constants $K_R$ and $K_T$ may be expressed for each of these binding equations. $K_R/K_T$ is, then, the ratio of the "affinities" of F for each of the two states, and is designated as c. For the ratio $F/K_R$, the designation $\alpha$ is used.

The fraction of protein in the R state can be expressed as

$$\bar{R} = \frac{(1+\alpha)^n}{L(1+c\alpha)^n + (1+\alpha)^n} \quad (1)$$

and the "saturation function," $\bar{Y}_F$, indicating the fraction of sites in R to which the ligand is bound becomes

$$\bar{Y}_F = \frac{Lc\alpha(1+c\alpha)^{n-1} + \alpha(1+\alpha)^{n-1}}{L(1+c\alpha)^n + (1+\alpha)^n} \quad (2)$$

This equation can, perhaps, be considered the fundamental relationship of the Monod, Wyman, and Changeux model, for it describes a variety of binding relationships ranging from hyperbolic to decidedly sigmoidal, depending on the magnitude of the constants, L, $\alpha$, and c, for an oligomer composed of n protomers. Thus, an apparent cooperativity in the binding of the ligand F can be made very obvious when L is large and c is small. Representative cases are shown in Figure 1 for n = 4. It is also important to recognize that in the limit case where L approaches 0 (no interconvertibility between states) and c approaches 1 (equal affinity of the states for ligand), hyperbolic binding curves result.

The situation just presented is that of a ligand acting as a homotropic effector. To extend the model to cover interactions between dissimilar ligands (i.e., heterotropic effects), it is only necessary to redefine L, the allosteric constant, in order to take into consideration a further displacement of the equilibrium between the R and T states. If an activator, A, and an inhibitor, I, are affecting the binding of the principal ligand, F (usually equated with substrate in the case of an enzyme), then their action can be viewed as a shift in the $R_0 \leftrightarrow T_0$ equilibrium through a preferential attachment of I (inhibitor) to the T state and A (activator) to the R state, assuming that F also favors R as in the above discussion (i.e., $c < 1$).

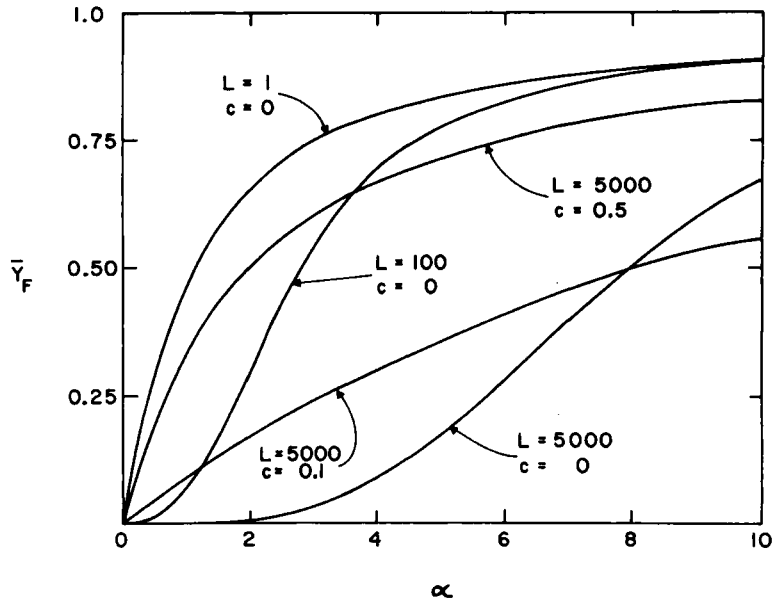



FIGURE 1. Calculated curves illustrating the extent of cooperative effects in the binding of a homotropic ligand at different values of the allosteric constant, L, and affinity ratio, c, for an allosteric tetramer protein.

It can be stated that

$$L' = L\frac{(1+\beta)^n}{(1+\gamma)^n} \qquad (3)$$

where $\beta = I/K_I$ and $\gamma = A/K_A$, with $K_I$ and $K_A$ defined as the microscopic dissociation constants of the inhibitor and activator with the T and R states, respectively.

Then, if only small (essentially zero) values of c are assumed,

$$\bar{Y}_F = \frac{\alpha(1+\alpha)^{n-1}}{L\frac{(1+\beta)^n}{(1+\gamma)^n} + (1+\alpha)^n} \qquad (4)$$

Because, as we have seen, L values determine the extent to which cooperativity in F binding is observed, values of $\beta$ and $\gamma$ will also influence the degree of cooperativity. High concentrations of an activator (i.e., large values of $\gamma$) will tend to abolish the sigmoidal binding response for ligand F, whereas large $\beta$ values will exaggerate any cooperativity in F binding. This is illustrated in Figure 2.

I have not introduced several complications which are very likely to be observed in dealing with real situations, as, for example, where a given ligand binds to more than one conformational state. These, of course, can be handled by appropriate mathematical adjustments of Equations 2 and 3, but all lead to much the same conclusion. Nor have I justified one of the more rigorous assumptions of the MWC model: namely, that symmetry in the bonding of the oligomer be conserved during the conformation transitions. This point, a fundamental one in the model, will be touched on later. It is important to reemphasize the ease with which this model explains cooperativity in binding as simply a consequence of preferential binding to one of two or more conformational states, and to point out that when any ligand, whether it be substrate, activator, or inhibitor, alters the equilibrium between the R and T states, cooperativity in its binding (homotropic effect) is to be expected, as well as heterotropic effects between different ligands.

In many instances, the MWC model has been directly translated into applications in enzyme kinetics, with the intent being to explain the quantitative action of various ligands on reaction velocities. The following generalizations may be offered at this point, with additional comments found in Section D:

1. Enzymes in which the substrate and a modulator ligand (activator or inhibitor) have different affinities towards the R and T states

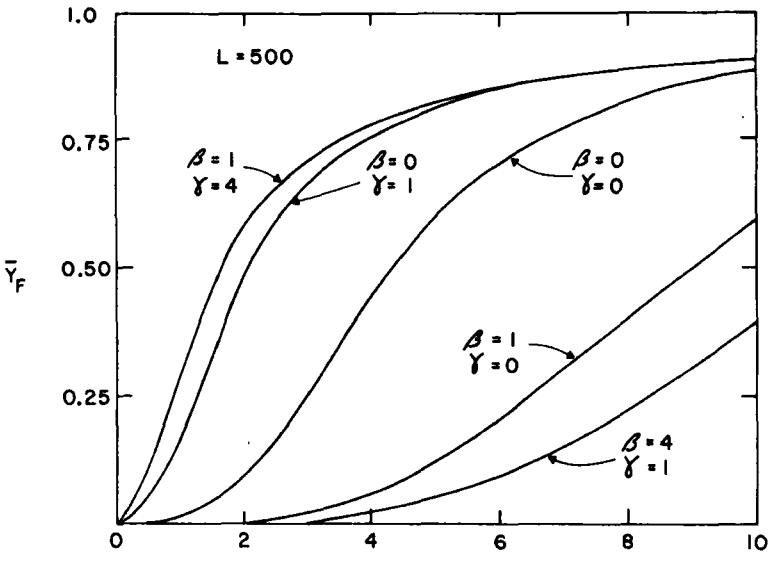



FIGURE 2. Calculated curves from Equation 4 describing the binding of a homotropic ligand, F, in the presence of an activator or an inhibitor with different affinities for the two states. The examples are shown for a tetramer (n = 4) in which c for ligand F is taken to be zero.

exhibit reciprocal effects in binding. That is, the modulator ligand, M, will alter the apparent affinity of the enzyme for substrate, S, and vice versa. These are termed "K systems," and may be positive (activator) or negative (inhibitor) types.

2. Enzymes in which only the substrate has a differential affinity for the R or T state, and with the modulator ligand being bound equally to both states, will not exhibit the reciprocal binding relationships mentioned above, but will display an altered maximum velocity if the two states of the protein differ in their catalytic activity. This will then be a "positive V system" if the modulator is an activator, or a "negative V system" if an inhibitor.

There have been observations of both K and V systems, including some which are apparently of a mixed type, but K systems appear to predominate. Either can demonstrate cooperativity in ligand binding and a resulting sigmoidal response, this feature being of greatest interest in a functional analysis of the consequences of ligand binding.

It seems clear that the Monod, Wyman, and Changeux model was, in part, an outgrowth of the failure of the original Adair concept[3] of direct heme-heme interaction in hemoglobin to be confirmed by X-ray analysis. Although the four equilibrium constants for the binding of oxygen to hemoglobin were shown by Roughton and coworkers[4] to be different for each of the successive oxygenation steps, as predicted by Adair, the work of Perutz conclusively established that the four heme groups were physically too distant from one another to permit any direct interaction.[5] The resulting impasse was avoided by the assumption that a reversible conformational alteration in the hemoglobin substructure was influenced by the binding of oxygen. From this point, and with an ever-increasing number of enzymes exhibiting cooperativity in ligand binding qualitatively analogous to oxygen binding in hemoglobin, the MWC model became the first formulated to account for the allosteric properties of proteins.

### B. The Sequential Models of Koshland, Nemethy, and Filmer

Probably the major criticism leveled at the MWC postulate concerns the conservation of symmetry – the so-called "all or none" change in conformation induced in all protomers by ligand binding to one state preferentially. The more general proposal of Koshland, Nemethy, and Filmer,[6] herein referred to as the KNF model, in its simplest form abandoned this assumption in favor of the idea that each individual protomer can exist in two conformations, and that a ligand binds principally to only one conformation. The contrasting pictorialization of these two models is shown in Figure 3 for a tetrameric protein. A basic

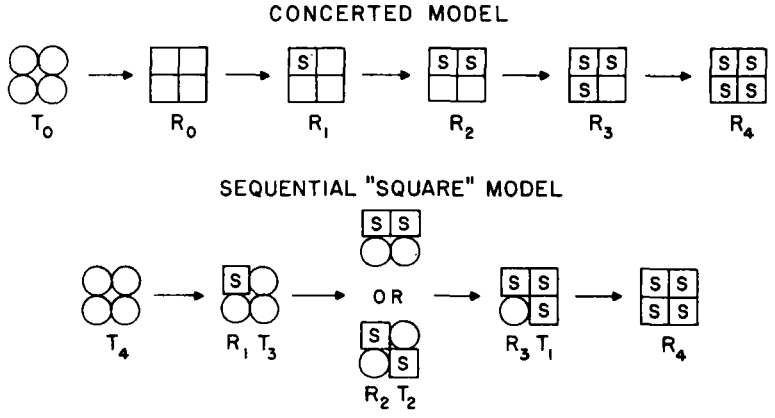



FIGURE 3. Schematic representation of the two principal binding mechanisms for a tetrameric protein. All subunits are represented as being identical, with ligand denoted as S. Although the "square" model is illustrated, in which each subunit is in contact with only two others, similar schemes can be written for a "tetrahedral" case, where each subunit contacts three others, and a "linear" case, where each subunit interacts with either one or two others depending on its position in a linear arrangement.

feature of this sequential model is the correlation of fractional saturation with a ligand to the extent of conformational change. For mathematical analysis, the equilibrium constant representing each step in the association of the ligand is composed of four terms: (1) the ligand binding constant, $K_S$, representing the intrinsic affinity of the ligand for an individual subunit; (2) a transformation constant, $K_T$, for the conformational change from the subunit in conformation T to conformation R; and (3) two interaction constants, one referring to oppositely conformed subunits, $K_{AB}$, and one for interaction between ligand-binding subunits, $K_{BB}$. The interaction constant, $K_{AA}$, between nonbinding conformations was assigned a unit value to represent a reference state.

The exact expression for the number of binding sites occupied, $N_S$ $(= n\bar{Y})$, is rather complex for any of the sequential forms because the number of ways of distributing ligand S and the interactions between AB and BB adjacent subunits must be considered for each form written. Interested readers will find the equation representing the situation of a "square" tetramer binding a ligand to individual subunits in the induced conformation B in Equation 18 of Reference 6.

Aside from the apparent greater generality of the KNF model, several important features should be mentioned. First, this model allows for the sequential binding of the ligand with a concomitant change in the conformation of only one subunit. Secondly, the intrinsic binding constants for a ligand to a four-site enzyme can be more independently related in various formulations of the KNF model than in the concerted MWC model. For example, in the concerted model, $K_1' < K_2' = K_3' = K_4'$, whereas in the simple sequential square model, $K_1'K_4' = K_2'K_3'$. Moreover, both positive cooperativity, $K_1' < K_2' < K_3' < K_4'$, and negative cooperativity, $K_1' > K_2' > K_3' > K_4'$, in addition to mixed situations can be encompassed in the KNF scheme. This ability of the sequential or induced-fit model to account for negative cooperativity has been one of its most useful aspects.

Numerous attempts have been made to distinguish which model is most appropriate for a given enzyme. Frequently, these attempts take the form of obtaining data on the binding of a ligand as a function of ligand concentration. When such data are plotted as $\bar{Y}$ vs [S], a sigmoidal curve results if positive cooperativity is present, but a hyperbolic curve will be obtained if either noninteracting sites (Michaelis-Menten behavior) or negative cooperativity is present. The Hill plot, however, has provided a sensitive method for distinguishing these latter situations, in that a plot of log $\bar{Y}$ vs log [S] gives a region of linearity whose slope is 1.0 for noninteraction, $> 1.0$ for positive cooperativity, and $< 1.0$ for negative cooperativity. Even more elaborate curve fitting procedures have been

described by Cornish-Bowden and Koshland,[7] and these permit saturation data to be somewhat diagnostic for a particular model.

Situations in which intermediary plateau regions are noted for plots of $\bar{Y}$ vs [S] or velocity vs [S] have been explained by Teipel and Koshland[8] as being due, in many instances, to mixed positive and negative cooperativity. When such an explanation is appropriate, this can be taken as strong presumptive backing for a ligand-induced conformational change model, as can any instance where negative cooperativity alone is observed.

### C. Cooperativity Inherent in Oligomerizing Systems

The MWC and KNF models are formulated around the assumption that conformational changes can occur within an oligomeric protein and that the equilibrium between states is influenced by preferential ligand attachment, leading to a cooperative binding nature in some instances. In proteins which exhibit association-dissociation phenomena, however, cooperativity can be explained without invoking conformational changes as an integral component.[5,8] A simple example of this would be a monomer-dimer equilibrium in which one of the forms is either inactive or less active towards ligand binding. One such situation is pictured in Figure 4, where each binding site is composed of elements of each monomer such that efficient binding is observed only when the protomers are in the proper juxtaposition. Dissociation results in a loss of binding to either site.

Mathematically, the system shown is quite analogous to the two-state MWC model, with the exceptions that L, the allosteric constant, would have to be redefined to include the monomer concentration as well as the equilibrium constant for association; and that c, the ratio of affinities of the ligand for monomer and dimer, would necessarily be zero in the example shown. Clearly, this situation will account for cooperative binding as well as almost normal hyperbolic binding, the determining influence being the magnitudes of the equilibrium constant and enzyme concentration. Conditions which favor dimer formation, e.g., large $K_{eq}$ or high enzyme concentration, will promote the appearance of hyperbolic binding curves, since in the limiting case only one enzyme form would exist. Many other variations on this general idea have also been considered by Nichol et al.[5,8]

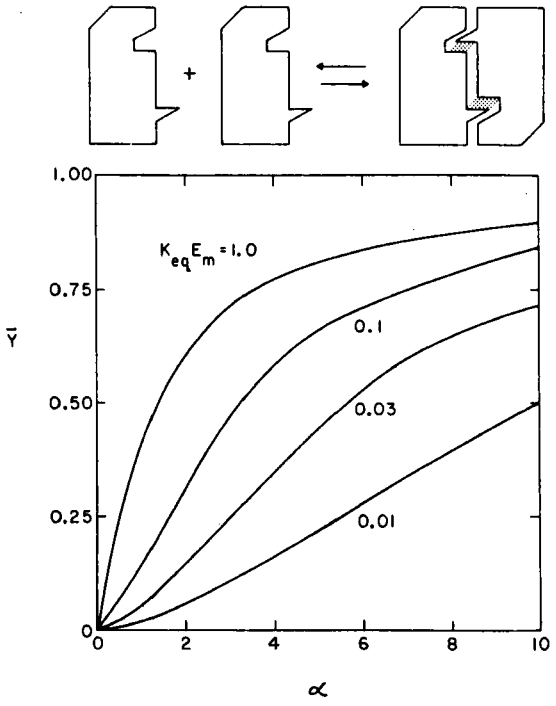



FIGURE 4. Binding curves for a simple monomer-dimer equilibrium in which ligand attachment is preferential for the dimer. In the top portion, a schematic illustration is given of the formation of binding sites (shaded region) upon contact of the monomers. The bottom portion depicts the theoretical relationship between fractional saturation ($\bar{Y}$) and $\alpha$, the reduced ligand concentration, for different values of equilibrium constant for association times monomer concentration. For this example, n = 2.

While there appear to be no situations known in which cooperative binding can be attributed solely to an equilibrium between oligomeric states, it should be obvious that either conformational changes within protomers or association-dissociation of an oligomeric protein can be invoked as a basis for cooperativity. In those instances where enzyme concentration influences binding behavior, however, the importance of association-dissociation phenomena cannot be discounted.

### D. Application of Enzyme Kinetic Data to the Analysis of Allosteric Models

Both of the aforementioned treatments deal with *binding* of ligands, and do not specifically concern reaction kinetics. All the same, there has been considerable success in the application of the ideas of these models to explain cooperativity

noted with effectors, whether they be of the homotropic or of the heterotropic type. Each model predicts that complex (non-Michaelis) kinetics are simple displacements of the $R \leftrightarrow T$ equilibrium where the substrate binds to one state in preference to the other. In a similar fashion, an allosteric inhibitor would exhibit preferential binding for the other conformational state. Depending, then, on the magnitude to the equilibrium constant for the T to R transition, cooperativity with respect to substrate binding will be quite marked if the substrate binds to the R state when the equilibrium constant for the unliganded forms favors the T conformation.

If $v/V_{max}$ is proportional to $\bar{Y}$, then the considerations presented earlier are interpretable in terms of kinetic data. However, it is clear that such a proportionality will not be observed in instances where conformational changes are not rapid or reversible relative to the rate-determining step in catalysis, or where the rate-determining step is not the same for all ES species. Fortunately, there are examples where velocity data do reflect binding quite well, and such situations can be analyzed in terms of the binding models.

### *1. Kinetic Treatments for Changes in Conformational States*

Frieden[9] has provided a detailed explanation of the additional considerations required when the MWC model for ligand binding is directly applied to data on initial reaction velocity. He has transformed Equation 2 into a kinetic one by replacing $\bar{Y}_F$ with the term $v_0/nV_{max}$, where $v_0$ is the initial velocity, n is the number of ligand (substrate) sites per mole of protein, and $V_{max}$ is the maximum initial velocity, giving the equation

$$\frac{v}{nV_{max}} = \frac{Lc\alpha(1+c\alpha)^{n-1} + \alpha(1+\alpha)^{n-1}}{L(1+c\alpha)^n + (1+\alpha)^n} \qquad (5)$$

In order for the equation to be valid, it must be assumed that there is a rapid equilibrium between all enzyme forms and enzyme containing species; that the rate-determining step lies in a late reaction step, such as breakdown of enzyme product complex; that $\alpha$ refers to the binding of the substrate rather than a modifier ligand; and that any modifier present affects only the ratio of the two conformational forms and not the specific binding of the substrate or the maximum catalytic activity observable at infinite substrate concentration.

It is obvious that simple data on the relation of velocity to substrate concentration alone will be insufficient to permit evaluation of all terms in the above equation. Generally, the procedure recommended by Frieden[9] is within the scope of most investigators who wish to assume the applicability of Equation 5 to their data. Velocity data should be carefully obtained over the region from 10% $V_{max}$ to 90% $V_{max}$, and even beyond these limits where possible. Plots of v/S vs are best used to illustrate the desired values. A linear v/S vs v plot will only be obtained when $c = 1$, or $L = 0$, or when L is very large and $c \neq 0$. Most often for a homotropic situation, parabolic curves result. Data displayed as v/S vs v can be used to approximate n by examination of the extent of the limiting slope at high values of v. The larger the region covered by this limiting slope, the larger is the value of n. Alternatively, n may be approximated from Hill plots or from knowledge of subunit composition. Estimates of c and L are then obtained from comparison of the experimental data with calculated plots made by assuming the n value determined earlier. The position of the maximum value of v/S along the abscissa is a function of L. Increasing values of c above zero diminish the parabolic steepness and result in relatively flat curves.

### *2. Additional Considerations for Reversible Oligomerization*

For illustrative purposes, we can examine how the concept of reversible conversion between monomer and dimer states can be treated as an extension of the MWC model. Frieden[9] has offered the following equation for this particular case.

$$\frac{v}{nV_{max}} = \frac{k_1\alpha(1+\alpha)^{n-1} + 2k_2K_{eq}Ed\alpha(1+d\alpha)^{2n-1}}{(1+\alpha)^n + K_{eq}E(1+d\alpha)^{2n}} \qquad (6)$$

In this equation, the allosteric constant, L, is replaced by the free enzyme concentration, E, and the equilibrium constant for polymerization, $K_{eq}$, defined as $[E_2]/[E]^2$. The additional terms $k_1$ and $k_2$ are rate constants for the breakdown of the enzyme-substrate complex of monomer and dimer, respectively, and d is the ratio of the dissociation constants of substrate combined with monomer and dimer, by analogy to the term c in the MWC approach.

Attempts to utilize this type of equation for higher oligomeric states are not especially fruitful,

since the concentration of free enzyme is not readily estimated. For the monomer-dimer case, however, E can be related to the total enzyme concentration, $E_0$, by the relationship

$$E = \frac{-(1+\alpha)^n + (1+\alpha)^{2n} + 4E_0 K_{eq}(1+d\alpha)^{2n-1}}{2K_{eq}(1+d\alpha)^{2n}} \quad (7)$$

Although some use has been made of this approach in treating kinetic data for glutamate dehydrogenase, it has not been widely exploited because of its obvious limitations. In the present context, however, the value of these equations lies in their demonstration that kinetic behavior of a rapidly oligomerizing enzyme will be a function of the total enzyme concentration. As we shall see later, this feature is a major property of systems in which oligomerization is an essential component.

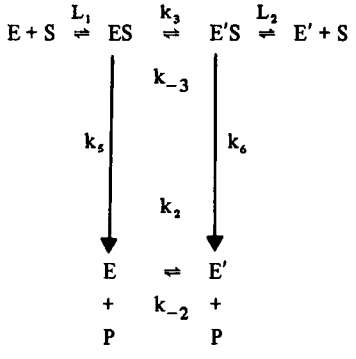
### E. Hysteresis in Enzymatic Reactions

Although the kinetic behavior with respect to the effects of ligand concentration on catalytic activity (reflected as a change in either maximum velocity or Michaelis constant) is adequately explained by one of the aforementioned models when applied to many allosteric enzymes, there are other instances where additional considerations must be introduced. The most common complexity encountered is a slow conformational response to a rapid change in the substrate or other ligand concentration, leading to an observed lag in the overall velocity of an enzymatic reaction. Frieden has termed such enzymes "hysteretic enzymes" and has offered a fundamental analysis of these systems.[10,11]

Assuming a single substrate reaction of the type,

$$E + S \rightleftharpoons ES \rightarrow E + P$$

the introduction of a two-state conformation leads to the formulation




in which $L_1$ and $L_2$ are equilibrium constants and E′ is the alternate conformation of E. If only ligand binding were of interest, so that $k_5$ and $k_6$ could be assumed to be zero, then this scheme would give the same expression for fractional saturation with substrate, $\bar{Y}$, as would be found in the MWC treatment for a single binding site (see Equation 2). However, when catalysis is occurring, $k_5$ and $k_6$ are not zero. If they are assumed to be the rate-determining steps, then the reaction velocity can be expressed as

$$\frac{v}{E_0} = \frac{k_5(1 + K_2 k_6 L_1/k_5 L_2)/(1 + K_2 L_1/L_2)}{1 + \frac{L_1}{S}\left(\frac{1 + K_2}{1 + K_2 L_1/L_2}\right)} \quad (8)$$

where $K_2 = k_2/k_{-2}$.

The velocity of such a reaction will be a hyperbolic function of substrate concentration when dealing with a single site enzyme, although sigmoidal relationships will be obtained when multisite enzymes are considered. The complexity of these multisite expressions deters me from presenting them here, however. Several representative cases are worked out in Reference 11. For our purposes, it is sufficient to consider how the velocity of a single site hysteretic enzyme changes as a function of the reaction time course.

Assuming that the isomerization steps $E \rightleftarrows E'$ and $ES \rightleftarrows E'S$ are slow relative to the binding of ligand, and further assuming that substrate (ligand) concentration does not change, the time dependence of the reaction velocity is given by the expression

$$v_t = v_f + (v_0 - v_f)e^{-k't} \quad (9)$$

where $v_t$ is the velocity at some time, t, between the initial and final states; $v_f$ is the velocity at infinite time relative to the initiation of the conformational change (but where substrate concentration is still unchanged); and $v_0$ is the velocity at zero time. The term k′ is a combination of terms defined for the case shown as

$$k' = \frac{k_3 S + k_2 L_1}{L_1 + S} + \frac{k_{-3} S + k_{-2} L_2}{L_2 + S} \quad (10)$$

Even for the simple case described here, it is clear that there will be time dependent velocity changes whose extent will depend on the concen-

tration of substrate, the relative values of $L_1$, $L_2$, and all rate constants defined in the original scheme. If, for example, $v_0$ is taken to be zero (i.e., $k_5 = 0$) and if E but not E′ is present at zero time, then there will be an initial lag in the attainment of a constant velocity, $v_f$, and the lag will increase with decreasing values of k′. If both E and E′ are present initially but differ in their kinetic properties, then immediately upon substrate addition the activity will be a time dependent weighted average of the two forms until an equilibrium of all enzyme species has been established.

Other situations, such as an activating or inhibitory modifier, displacement of one ligand by another, or even some monomer-oligomer changes, can be described by Equation 9 with appropriate modifications in the k′ term, provided that in each case there is a slow conversion of one enzyme form to another.

I hope to illustrate later that hysteretic behavior is not merely of theoretical interest, but is actually observed in kinetic studies of some regulatory enzymes. In addition, it may be important to point out that this concept has two features which make it of special significance for the present discussion.

First, hysteresis concerns only transient phenomena; that is, it describes the kinetic consequences which may result when a conformational change is brought on by the introduction of an appropriate regulatory ligand. It is principally of value when the conformational change is slow relative to other steps in catalysis, because were the change exceedingly rapid, the behavior upon ligand addition would be adequately explained by one or more of the allosteric models described earlier, which generally assume instantaneous equilibrium among enzyme species during catalysis. In short, then, hysteresis becomes a special consideration to be incorporated into the explanation of the changes in kinetic properties of an enzyme promoted by ligand attachment.

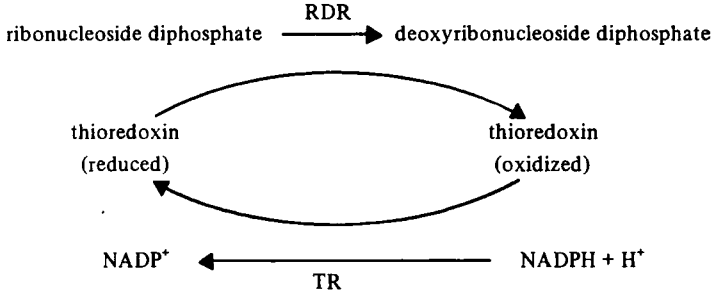
Secondly, hysteresis is especially likely to be encountered in association-dissociation systems because the bimolecular nature of such processes suggests that they may be slower than simple conformational changes of an isomerization type, particularly at low enzyme concentrations. The increased probability that oligomerizing enzymes will exhibit hysteresis would seem to be a uniquely important feature of such enzymes.

## III. RIBONUCLEOSIDE DIPHOSPHATE REDUCTASE AND CYTIDINE TRIPHOSPHATE SYNTHETASE

In the various pathways utilized for nucleotide biosynthesis, many examples of enzymes subject to allosteric regulation are recognized. This would hardly be unexpected, in view of the importance of these sequences for cellular growth and multiplication, as well as for economic considerations upon attainment of cell maturity. From the wealth of information available on the control of the biosynthesis of both pyrimidine and purine nucleotides, several clear examples of ligand-induced oligomerization have unfolded. I have selected two enzymes from this group to discuss: one, the *Escherichia coli* ribonucleoside diphosphate reductase, because of its complexity as a two-component enzyme; and the second, cytidine triphosphate synthetase, chosen for the completeness of information pertaining to our central theme.

### A. Ribonucleoside Diphosphate Reductase

The enzymatic reduction of ribonucleotides to deoxyribonucleotides in *E. coli* occurs through the coupled reaction sequence:



where RDR is ribonucleoside diphosphate reductase and TR is thioredoxin reductase. In contrast to the system from *Lactobacillus leichmannii*, which uses ribonucleoside triphosphates and contains a deoxyadenosyl cobalamin co-factor, the RDR of *E. coli* exhibits no requirement for a prosthetic group, although nonheme iron has been found. The enzyme acts on all four common ribonucleoside-5'-diphosphates, but its specificity for a particular substrate is closely linked to the presence of particular ribo- or deoxyribonucleoside triphosphates. Purified RDP can be separated into two nonidentical proteins, B1 and B2, each alone inactive in catalyzing reduction. In the active form, B1 and B2 combine in a 1:1 ratio when $Mg^{++}$ ions are present. Protein B1 appears to be a dimer of molecular weight 200,000, composed of similar if not identical polypeptide chains; protein B2 is smaller, having a molecular weight of approximately 80,000.

An exhaustive analysis of the binding of nucleoside triphosphate effectors to protein B1 and to the active B1-B2 complex has been conducted by Reichard's group.[12,13] Only protein B1 or the complex binds these allosteric effectors to an appreciable extent. A dual effect of dATP on the activity of RDR (measured as CDP reduction) has been observed; at low dATP concentration ($10^{-6}$ *M*) the effect was stimulatory, but higher concentrations are very inhibitory (Table 1). Scatchard plots of binding data for dATP with protein B1 obtained either by Sephadex G-50 gel filtration or by dialysis were biphasic, suggesting that 2 types of binding sites were present: a pair of high affinity sites (h-sites) with an average $K_d$ of $3 \times 10^{-8}$ *M* at 2° C, and a set of 2 low affinity sites (l-sites) of average $K_d = 5 \times 10^{-7}$ *M*. The presence of protein B2 (i.e., formation of the B1-B2 complex) enhanced dATP binding, particularly at higher temperatures, but otherwise did not alter these observations. A total of 4 mol of ligand per mol B1 was bound at saturating levels of dATP. The binding of other effectors revealed somewhat different results. Binding of ATP to protein B1 was of a complex, cooperative nature, but the limiting extent of binding was again 4 mol/mol B1. dTTP and dGTP were bound only to the extent of 2 mol/mol B1, but the magnitude of the average $K_d$ for each ($3 \times 10^{-7}$ *M* for dTTP, $8 \times 10^{-8}$ *M* for dGTP) and the competition binding described below between dTTP or dGTP and dATP suggested that these nucleotides bound mainly to h-sites.

A study of the sedimentation properties of a mixture of B1 and B2 revealed that a complex of $s_{20,w}$ 9.5 S was formed from the combination of B1 (7.8 S) and B2 (5.5 S) at pH 7.6 with 0.01 *M* $MgCl_2$ present.[12] Dissociation of the complex could be prevented by adding an excess of either B1 or B2. In an important observation, nucleotides such as ATP or dTTP, which were stimulatory for the reduction of CDP, were seen to produce no alteration in the sedimentation coefficient of the complex, whereas inhibitory concentrations of

TABLE 1

**Influence of Different Effectors on the Activity and Specificity of Ribonucleoside Diphosphate Reductase**

| Nucleotide (*M*) | Catalytic activity | Specificity for base |
|---|---|---|
| ATP ($2 \times 10^{-3}$) | Stimulation | Pyrimidines |
| dATP ($10^{-6}$) | Stimulation | Pyrimidines |
| dATP ($10^{-4}$) | Inhibition | Purines + pyrimidines |
| dGTP ($10^{-5}$) | Stimulation | Purines |
| dTTP ($10^{-5}$) | Stimulation | Purines + pyrimidines |
| dATP ($10^{-4}$) + ATP ($2 \times 10^{-3}$) | Stimulation | Pyrimidines |
| dATP ($10^{-6}$) + dTTP ($10^{-4}$) | Inhibition | Purines + pyrimidines |
| dATP ($10^{-6}$) + dGTP ($10^{-4}$) | Inhibition | Purines + pyrimidines |

The concentrations given are those for which the effect is fully developed. All catalytic activity measurements are relative to the activity seen in the absence of effectors.

(From Brown, N. C. and Reichard, P., *J. Mol. Biol.*, 46, 52, 1969. With permission of Academic Press, London.)

dATP produced an aggregation of the complex to a new species of 14.7 S, as shown in Table 2. These data were interpreted as indicating that the active form of RDR is a B1-B2 complex of 9 to 9.5 S size, and that inhibition of activity by high concentrations of dATP is associated with the formation of a higher molecular weight species, possibly a (B1-B2) dimer, of nearly 15 S.

To extend this conclusion required study of the effect of combination of nucleotides. For example, it was noted that dATP was no longer inhibitory when ATP was present in sufficient quantity to displace dATP from l-sites. Sedimentation analysis revealed that under these conditions, the complex migrated as the 9.5 S species. Competitive binding experiments involving dTTP and dATP indicated that dTTP was displaced from h-sites by dATP, and vice versa, but that dTTP was ineffective in competing with dATP for l-sites. These data were therefore interpreted as suggesting that the *level* of activity is regulated by compounds (dATP, ATP) which can bind to l-sites; dATP binding to these sites produces inhibition and leads to a corresponding oligomerization of the B1-B2 complex, whereas ATP binding to l-sites blocks dATP binding but is unable to bring about oligomerization. The h-sites were postulated to control the *specificity* of the complex for the type of nucleoside diphosphate reduced.

It should be mentioned that substrate saturation curves, either in the presence or absence of nucleotide effectors, show no evidence for homotropic effects,[14] and with one major exception, the binding of nucleoside triphosphates was also hyperbolic. The only exception to this was the cooperative binding of ATP to B1.

An analysis of these data leads to the following conclusions:

1. All binding sites, except for the l-sites of ATP, are of a noninteracting nature; that is, a conformational change upon binding is not transmitted to any other sites on the same or a different protomer. This would appear to eliminate the strict application of the MWC model to RDR.

2. Although no evidence for negative cooperativity exists, the fact that various nucleotide effectors cause changes in $V_{max}$, Km, and specificity for nucleoside diphosphate substrates suggests that definite conformational changes must occur upon binding of effectors. This, plus the above argument, lends support for the KNF model as being appropriate insofar as binding is concerned.

3. The competition between dATP and ATP for binding to l-sites appears to correlate with data which indicate that dATP binding at these sites is inhibitory, whereas ATP binding reverses the inhibition. High levels of dATP which produce inhibition also promote oligomerization to a 15 S species, and ATP can effectively block this association. Therefore, it is likely that a consequence of dATP binding to l-sites is the formation of an inactive (or less active) oligomer (15 S) of the 9S R1-R2 complex.

From a regulatory standpoint, the production

TABLE 2

**Sedimentation Coefficients of B1-B2 Complexes in the Presence of Effectors**

| Effectors used (*M*) | Sedimentation coefficient (S) |
|---|---|
| ATP ($2 \times 10^{-3}$) | 9.1 |
| dTTP ($5 \times 10^{-4}$) | 9.1 |
| dATP ($3 \times 10^{-6}$) | 11.8 |
| dATP ($1 \times 10^{-4}$) | 14.7 |
| ATP ($2 \times 10^{-3}$) + dATP ($1 \times 10^{-4}$) | 9.5 |
| dATP ($1 \times 10^{-4}$) + dTTP ($1 \times 10^{-3}$) | 14.8 |

Sedimentation coefficients were obtained by sucrose density gradient centrifugation with catalase as a marker enzyme. The migration of the complex was followed through the use of $^{59}$Fe-labeled B2 in the presence of excess B1 protein.

(From Brown, N. C. and Reichard, P., *J. Mol. Biol.*, 46, 33, 1969. With permission of Academic Press, London.)

of an inactive oligomeric complex by high levels of dATP is a logical control phenomenon, since dATP can be considered as an excellent indicator of the intracellular deoxynucleotide pool size. There is, however, no definite link at this time between the inactivation and oligomerization other than the data cited herein. It remains to be definitely established whether oligomerization is required in order for the inhibition of activity to be achieved. All the same, one can easily picture how substrate binding to catalytic sites could be altered when association to a dimeric form of the R1-R2 complex occurs.

### B. Cytidine Triphosphate Synthetase

Homogeneous preparations of *Escherichia coli* CTP synthetase consist of a 108,000 mol wt protein which is a dimer of apparently identical subunits.[15] The enzyme is capable of catalyzing the amination of UTP to CTP in the presence of ATP, $Mg^{++}$, and either glutamine or ammonia as a nitrogen donor. When glutamine is the nitrogen source, GTP is a potent allosteric activator (Table 3), but this nucleotide produces no effect on catalysis mediated directly by ammonia. The independence of the binding sites for glutamine and ammonia was established by Levitzki et al.[16] when they found that an enzyme treated with 6-diazo-5-oxo-L-norleucine had lost the ability to catalyze nitrogen transfer from glutamine but not from ammonia. Levitzki and Koshland[17] later demonstrated that GTP was not an activator of the entire reaction, but rather stimulated only the formation of a covalent glutamyl-enzyme complex which was produced during the transfer of amide nitrogen to an ammonia binding site of enzyme. Consistent with this interpretation was the fact that CTP synthetase served as a glutaminase in the absence of UTP and ATP, liberating glutamic acid and ammonia. GTP accelerated this activity approximately tenfold.

Despite the fact that direct measurements of molecular weight by several independent techniques all indicated a dimeric structure of 105 to 110,000 mol wt, certain observations were difficult to reconcile with this fact. An unusually high degree of cooperativity was obtained from kinetic analyses aimed at determining the extent of interaction between sites for either ATP or UTP; Hill coefficients were 3.8 and 3.4, respectively, somewhat higher than might be anticipated for a simple dimeric protein with only 2 sites for each of these nucleotides. Moreover, negative cooperativity was demonstrated in the cases of glutamine and GTP binding.[18] A mathematical treatment of kinetic data in which an intermediary plateau region exists in substrate saturation curves has led to the conclusion that such data can only be accounted for by a model in which more than two substrate binding sites are present, and where catalytic or binding constants first decrease, then increase as enzyme becomes saturated.[8]

Resolution of these data became possible upon finding that the enzyme aggregated to an active tetrameric form in the presence of ATP and UTP (Table 4). As seen in these results, only the simultaneous addition of ATP and UTP, each at 1 m*M*, produced the 210,000 mol wt species; glutamine was not essential. Data to be presented

TABLE 3

**Effects of FTP on CTP Synthetase Kinetics**

| | $S_{0.5}$ (*M*) | | $k_{cat}$ | |
|---|---|---|---|---|
| Substrate | –GTP | +GTP | –GTP | +GTP |
| Glutamine | $1 \times 10^{-3}$ | $1.6 \times 10^{-4}$ | 42 | 300 |
| $NH_3$ | $5.3 \times 10^{-3}$ | $5.3 \times 10^{-3}$ | 270 | 270 |

GTP concentration was $5 \times 10^{-4}$ *M*; ammonia was present at 0.025 *M*. $k_{cat}$ is expressed as moles of CTP formed per mole of enzyme active sites/min.

(Reprinted with permission from Levitzki, A. and Koshland, D. E., Jr., *Biochemistry*, 11, 242, 1972. Copyright by the American Chemical Society.)

TABLE 4

**Aggregation of CTP Synthetase**

| Substrates present | Molecular weight |
|---|---|
| Glutamine, $MgCl_2$ | 102,000 |
| Glutamine, $MgCl_2$, ATP | 110,000 |
| Glutamine, $MgCl_2$, UTP | 105,000 |
| Glutamine, $MgCl_2$, ATP, UMP | 110,000 |
| ATP, UTP, $MgCl_2$ | 210,000 |
| Glutamine, $MgCl_2$, ATP, UTP | 210,000 |

Molecular weights were estimated by Sephadex G-200 gel filtration, with appropriate standards. Substrates were used at the following concentrations: L-glutamine, 2 m*M*; $MgCl_2$, 10 m*M*; ATP, UTP, or UMP, 1 m*M* each.

(From Long, C. W., Levitzki, A., and Koshland, D. E., Jr., *J. Biol. Chem.*, 245, 80, 1970. With permission from the American Society of Biological Chemists.)

later, however, indicate that higher concentrations of a single nucleotide can promote some degree of tetramer formation. For the experiments reported in Table 4, a constant level of enzyme, approximately 0.8 mg, was applied to each column. Variations in enzyme concentration and in the state of purity, however, revealed no evidence of aggregation of the 105,000 mol wt native species unless ATP and UTP were present. This situation is, in itself, relatively unique, since some tendency towards aggregation even in the absence of effectors is usually observed at sufficiently high concentrations of unliganded enzyme.

A more detailed study of the dimer-to-tetramer transformation was reported recently by Levitzki and Koshland,[19] with the aim being to provide data which would permit a distinction among a concerted MWC model, or various versions of the sequential induced-fit models (KNF proposals). The depth of their probe was such that it provides an excellent illustration of what experimental data can shed light on this question.

Binding data for ATP and UTP are illustrated in Figures 5 and 6. When the binding of ATP was examined in the absence of UTP, a cooperative binding behavior was seen, with a Hill coefficient of 1.6 and a $S_{0.5}$ of 0.7 m*M*. Addition of UTP resulted in hyperbolic binding (Hill coefficient of 1.0) and a $S_{0.5}$ of 0.3 m*M*. The reciprocal experiment gave similar but not identical findings. The Hill coefficient for UTP was 2.0 and $S_{0.5}$ was 0.7 m*M*; upon ATP addition, $S_{0.5}$ changed to 0.06 m*M* and binding was again hyperbolic. These observations permitted the conclusion that the conformational changes induced by each ligand are not equal and that the second ligand is not merely attaching to a single protein form which is identical no matter whether UTP or ATP binds first. Furthermore, because hyperbolic binding is observed for the second ligand, this implies that if a conformational change occurs upon its attachment, this change is not transmitted to neighboring subunits.

The influence of ATP or UTP concentration on the association of CTP synthetase dimers was then examined. Measurements of peak position of the enzyme from Sephadex G-200 columns and the assumption that only dimer and tetramer forms were present allowed the calculation of the

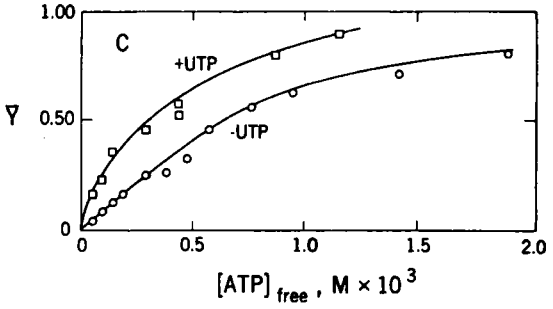



FIGURE 5. Binding of [$^{14}$C] ATP to CTP synthetase in the presence and absence of UTP. The ultrafiltration method was employed for binding measurements. Incubation mixtures contained enzyme at 0.4 $\mu M$ concentration (based on 210,000 mol wt). When added, UTP was 2 m*M*. (Reprinted with permission from Levitzki, A. and Koshland, D. E., Jr., *Biochemistry*, 11, 249, 1972. Copyright by the American Chemical Society.)

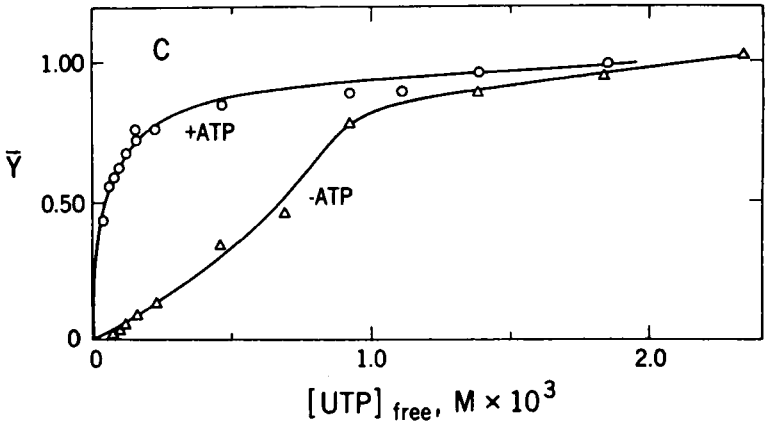



FIGURE 6. Binding of [$^{14}$C] UTP in the presence and absence of ATP. Enzyme concentration was 2.4 $\mu M$ and ATP was 2 m*M*. (Reprinted with permission from Levitzki, A. and Koshland, D. E., Jr., *Biochemistry*, 11, 250, 1972. Copyright by the American Chemical Society.)

percentage of tetramer contributing to the apparent weight-average molecular weight estimate. These data indicated that a ligand concentration of 1.3 m*M* for ATP and 1.2 m*M* for UTP was necessary to obtain a 50% conversion to tetramer in the absence of the other nucleotide. When both nucleotides were present, the midpoint in tetramer formation occurred at a total ligand concentration of approximately 0.4 m*M*. Thus, the combination resulted in a greatly enhanced degree of association compared to each ligand alone.

Conformational changes upon binding of ligands was studied by fluorescence quenching techniques. These data are illustrated in Figures 7 and 8. For quenching by ATP, a $S_{0.5}$ value of 0.5 m*M* and a Hill coefficient of 1.6 were found. On the other hand, UTP binding resulted in $S_{0.5}$ of 0.4 m*M* and a Hill coefficient of 1.0. Because these experiments were conducted with the companion nucleotide absent, but with glutamine present, tetramer formation should not occur to a large extent below nucleotide concentrations of 1 m*M*, and therefore, quenching is mainly due to conformational changes induced in the native dimeric enzyme molecule. In both cases it should be noted that the midpoint of the fluorescence change occurs at a lower concentration of ligand than was noted for the midpoint of binding, although the differences are not striking.

Levitzki and Koshland[19] interpreted these data collectively as follows. Because the midpoint of the binding curve is seen at a higher ligand

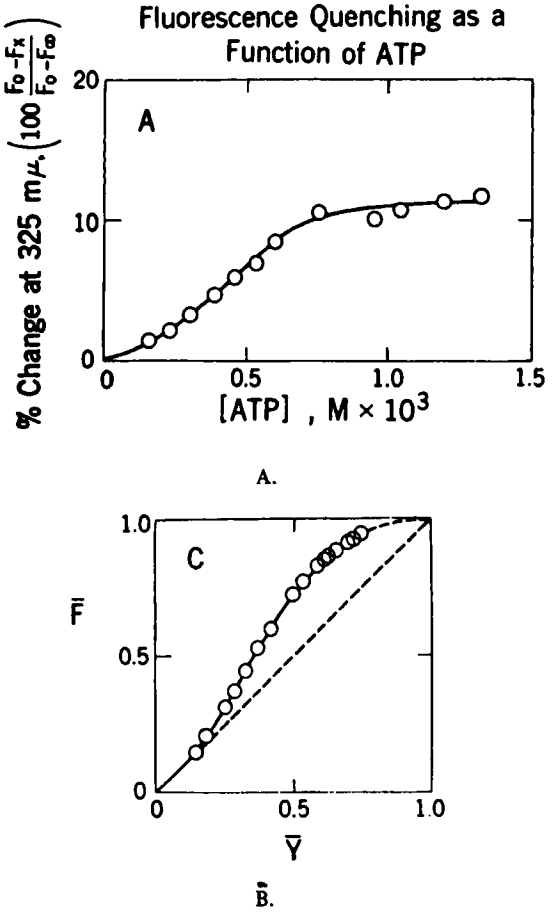



FIGURE 7. A. Fluorescence change as a function of ATP binding to CTP synthetase. Excitation was at 295 nm. Fluorescence decreases due to absorption by ATP have been corrected for. $F_0$ is the fluorescence value of the enzyme, $F_x$ is the corrected value at a given ligand concentration, and $F_\infty$ is the corrected fluorescence for the fully liganded protein. B. Data of Figure 7A expressed as fraction of fluorescence change (F) vs the fractional saturation ($\bar{Y}$) (Reprinted with permission from Levitzki, A. and Koshland, D. E., Jr., *Biochemistry*, 11, 250, 1972. Copyright by the American Chemical Society.)

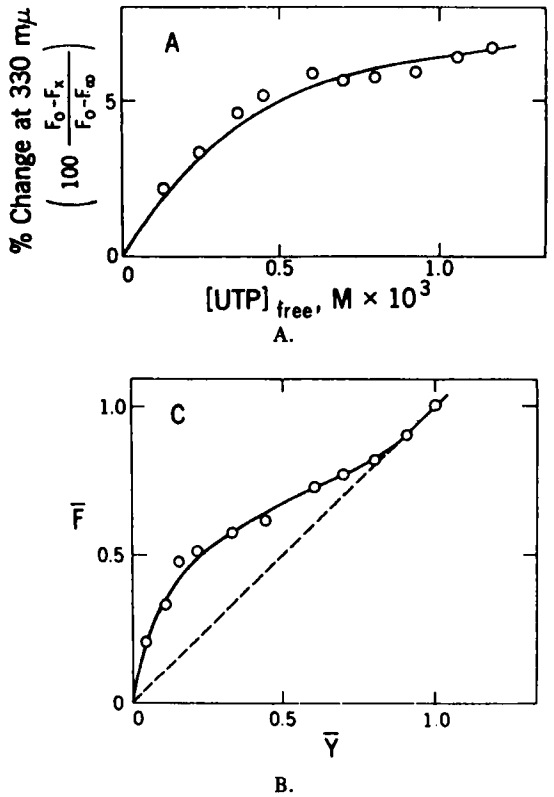



FIGURE 8. A. Fluorescence change of CTP synthetase as a function of UTP binding. Enzyme concentration was 0.15 μ*M*. Excitation was at 300 nm. Correction for UTP absorption has been applied. Other conditions were as employed in Figure 7. B. Data of Figure 8A expressed as fraction of fluorescence change ($\bar{F}$) vs fractional saturation ($\bar{Y}$). (Reprinted with permission from Levitzki, A. and Koshland, D. E., Jr., *Biochemistry*, 11, 250, 1972. Copyright by the American Chemical Society.)

concentration than the midpoint for the conformational change indicated by fluorescence quenching studies, binding of the first ligand would appear to induce a conformationally visible change in the neighboring, unliganded subunit of each dimer. This further substantiates the conclusion that a simple sequential model is inadequate for this enzyme. In addition, however, the conformational change midpoint is much earlier in terms of ligand concentration than is the midpoint for tetramer formation. This observation imposes the conclusion that there must exist an intermediate species in which the ligand-induced conformational change has not been rendered complete for all subunits. The concerted model is therefore inapplicable in this particular case, leaving only a more complex sequential model as the logical choice for CTP synthetase.

The foregoing data provide firm support for the concept of ligand-induced oligomerization in CTP synthetase. What is still not completely clear, however, is the significance of oligomerization insofar as catalytic activity is concerned. Undoubtedly, the conversion of dimer enzyme to tetramer is associated with the binding of ligands, preferably both UTP and ATP; but as was shown, some tetramer can exist with sufficiently high concentrations of either ligand. On the other hand, catalytic activity is observed only when both UTP and ATP are present, i.e., under conditions most favorable for oligomerization. Thus, it is apparent that tetramer formation is closely associated with maximal catalytic activity, although it cannot be ascertained directly whether tetramer formation is essential for the highest degree of activity.

One can make something of a case for this belief by considering the effect of ligands on the glutaminase activity of CTP synthetase under conditions where either dimer or tetramer species would predominate. The CTP synthetase-catalyzed hydrolysis of glutamine in the absence of ligands proceeds at a rate of 5 mol of substrate turned over per minute per mole of enzyme sites; enzyme is dimeric for this level of activity. If UTP and an ATP analog with a nitrogen atom in the $\beta$, $\gamma$ position (ADPNP) are added at 1 m*M* levels, tetramer is produced and the rate changes to 28 mol/min/mole of active sites. Addition of GTP to either reaction produces a ten fold increase in the rate over that seen without GTP.[17] These rates for tetrameric enzyme are the same as are found for CTP formation from UTP in the presence of ATP, in accordance with the interpretation that the formation of the enzyme-glutamyl complex is the rate-limiting step in catalysis. This may well mean then that maximal catalytic activity is only observed for CTP synthesis when tetrameric enzyme is present, but it is clear that the conformational changes induced upon the binding of all the various nucleotide ligands favor both full activity and tetramer formation. Oligomerization alone can occur without the full conformational alterations required for maximum catalytic activity.

## IV. ASPARTOKINASE AND HOMOSERINE DEHYDROGENASE

### A. Aspartokinase-Homoserine Dehydrogenase of *Escherichia coli*

In *Escherichia coli*, strain K-12, there exist three distinguishable aspartokinase activities. A threonine-inhibited enzyme (aspartokinase I) and a methionine-repressed enzyme (aspartokinase II) exhibit homoserine dehydrogenase activity as an integral part of their molecular structure.[20,21] A third, aspartokinase III, is repressed by lysine but lacks the associated dehydrogenase activity. Of this trio of aspartokinases, the most extensively examined from the point of view of molecular interactions with ligands is aspartokinase I – homoserine dehydrogenase I (AK-HSD-I). Several laboratories have presented rather complete evidence to suggest that the 2 activities exist on a single polypeptide chain of molecular weight 86,000, with the native enzyme being a tetramer composed of 4 identical subunits (360,000 mol wt).[22,69] Mutant analysis coupled with polypeptide fragmentation studies support the contention that gene fusion has resulted in the two activities being localized within the same polypeptide.[22,70] The $NH_2$-terminal portion carries the aspartokinase activity, while homoserine dehydrogenase activity presumably resides in the COOH-terminal part.

One of the earlier indications that a ligand-induced oligomerization occurred in AK-HSD-I came from studies on a mutant which lacked one activity, the HSD-I. The remaining aspartokinase possessed a molecular weight of only 180,000 instead of the usual 360,000, and although it showed normal inhibition by threonine, the enzyme was unstable in the absence of the ligand and dissociated into a threonine-insensitive but

active aspartokinase of roughly $4 \times 10^4$ mol wt.[23] Shortly thereafter, somewhat analogous findings were made in another strain (*E. coli* 9723) which, however, maintained both AK-I and HSD-I as the complex.[24] Elution of this AK-HSD-I from Sephadex G-100 columns in the presence and absence of 20 $\mu M$ L-threonine revealed that both activities were contained in a single species of 90,000 mol wt in the absence of threonine, but were unretarded on the same columns if threonine was present. Sucrose density gradient experiments confirmed these findings by showing that without threonine, the enzyme had an $s_{20,w}$ of 6.3 S, whereas the $s_{20,w}$ was 9.7 S in the presence of 20 $\mu M$ threonine. Intermediate threonine concentrations (e.g., 2 $\mu M$) gave intermediate elution patterns from Sephadex G-100 columns, and reversibility of association was demonstrated by removing threonine from the higher molecular weight form, whereupon the dissociated species was produced.

Although ATP and $Mg^{++}$ together reversed threonine inhibition of aspartokinase competitively, at respective concentrations of 10 m$M$ and 3.3 m$M$, they did not cause or prevent formation of the higher molecular weight complex seen in the presence of 20 $\mu M$ threonine. Threonine inhibition of HSD activity was unaffected by either aspartate or $Mg^{++}$. Probably the single most interesting feature of the analysis was the observation that threonine inhibited AK-I activity in a sigmoidal fashion, with no appreciable inhibition observed at 20 $\mu M$ threonine, and a 50% inhibition found at a threonine concentration of 700 $\mu M$. HSD-I activity was inhibited by threonine in a hyperbolic fashion, but again a relatively high level (500 $\mu M$) of threonine was required for 50% inhibition. (The hyperbolic inhibition by threonine differs from that observed in *E. coli* K-12, where a clearly sigmoidal inhibition curve has been reported.)[71] From these observations, it was concluded that the action of threonine on the AK-HSD-I complex was a 2-fold process: a reversible association of the complex into a high molecular weight form, followed by an inhibition of each activity when threonine concentrations are 50 to 100 times greater than are necessary for the association process. Clearly, in this case the dissociated form retains both catalytic activities but is insensitive to inhibition by threonine.

In more recent years, a number of investigators have devoted intensive efforts on the question of oligomerization in the *E. coli* K-12 AK-HSD-I. Wampler, Takahashi, and Westhead[25] reported that the tetrameric enzyme dissociated into smaller but catalytically active dimers in the absence of any of several ligands – threonine, aspartate, or $K^+$ ions – at pH 8. A more detailed analysis of this phenomenon was subsequently described by Wampler.[26] He reinvestigated the degree of dissociation as a function of pH, ionic strength, and buffer type, finding a rather wide range of sedimentation coefficients. Although these data are relatively difficult to generalize from, there seemed a marked tendency for dissociation as pH increased, with values ranging from 10 S in Pipes buffer at pH 6.2, to 5.2 S at pH 11 in lysine buffer. Sedimentation equilibrium experiments under the latter condition confirmed that dissociation was indeed occurring, and a monomer molecular weight of 90,000 was estimated. Moreover, Wampler employed the graphical method of Roark and Yphantis[27] in the analysis of these results to provide further evidence that the system was an equilibrium association-dissociation. Unfortunately, under the conditions used (pH 11), the two-species plot procedure did not coincide satisfactorily with that predicted for a simple monomer-tetramer equilibrium (Figure 9). In addition to the possible existence of a stable dimer intermediate, there was also the suggestion that very high molecular weight aggregates (30 to 120 S) can exist, all serving to detract from the application of the two-species plot to the data obtained.

Wampler also attempted to correlate the size of the catalytic species with the effects of ligand (threonine) binding. Two methods were employed for this purpose – gel filtration on Sephadex G-200 under assay conditions and the technique of active enzyme centrifugation. Because of the similarity of results obtained, only the latter will be described here. As seen in Table 5, the sedimentation coefficient of the catalytically active species in the forward homoserine dehydrogenase reaction appears to correspond to that of tetramer, although the values are slightly below the $s^\circ_{20,w}$ seen in boundary sedimentation under noncatalytic conditions (11.0 S,[26]; 11.4 S[28]). In addition, there was a positive concentration dependence, but the significance of this must be questioned in view of the fact that excessive enzyme in this technique leads to artificially high sedimentation coefficients.[29] The addition of

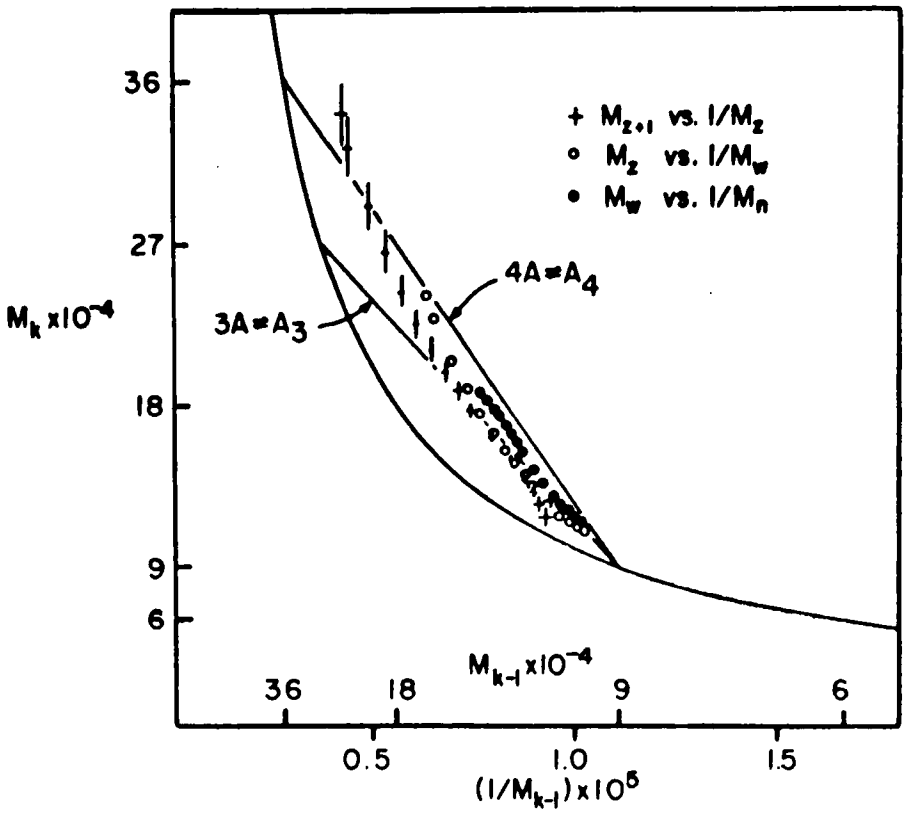



FIGURE 9. Two-species plot of data from equilibrium sedimentation at 18°C and 16,000 rpm. Buffer was 0.02 *M* lysine, 0.1 *M* NaCl, pH 11. The curved line indicates the locus of points which would be given by samples of homogeneous molecular weights ($M_k = M_{k-1}$). Data extrapolated to this line give estimates of the monomer and oligomer molecular weights. The two straight lines in the figure are calculated lines for a 90,000 subunit involved in monomer-trimer and monomer-tetramer associations. The $M_{z+1}$ vs $1/M_z$ symbols are drawn to indicate the size of the error estimate. (Reprinted with permission from Wampler, D. E., *Biochemistry*, 11, 4430, 1972. Copyright by the American Chemical Society.)

excess threonine did not alter these results. Surprisingly, however, the reverse homoserine dehydrogenase reaction, performed at pH 9.0 in contrast to pH 7.5 for the forward assay, revealed a much lower sedimentation coefficient. Threonine increased this value significantly, approaching the $s_{20,w}$ of the tetrameric species.

The latter observation did not go completely unchallenged. Mackall and Neet[30] reexamined this point in detailed fashion, laying particular emphasis on the role that pH could play in the association-dissociation phenomenon. While confirming the observations of Wampler,[26] they indicated that the apparent dissociation observed in the reverse homoserine dehydrogenase assay was essentially a pH effect, most noticeable in $H_2O$ but also found in 40% $D_2O$. As shown in Figure 10, the small change in pH from 7.6 to 8.0 produces a drastic decrease in $s_{20,w}$ when monitoring either the forward or reverse reaction. From this, they concluded that the size of the reacting species is totally independent of the direction of catalysis when the forward and reverse HSD reactions are conducted under the same conditions.

But these two studies did reveal several important aspects of oligomerization in AK-HSD-I. First, as seen from Table 6, careful analysis of sedimentation coefficients clearly indicates that dissociation to a form probably corresponding to an active dimer does occur at pH 8 or pD values above 8.5. This is irrespective of the direction of assay. Secondly, L-threonine can promote oligomerization to the tetramer (10.2 to 10.7 S species), even at the very low concentrations of enzyme employed, throughout the pH (or pD)

TABLE 5

**Sedimentation Coefficients of AK-HSD-I under Homoserine Dehydrogenase Reaction Conditions**

| Forward reaction | | | | Reverse reaction | | | |
|---|---|---|---|---|---|---|---|
| No Thr | | 5.6 m*M* Thr | | No Thr | | 5.6 m*M* Thr | |
| Conc. | $s_{20,w}$ (S) | Conc. | $s_{20,w}$ (S) | Conc. | $s_{20,w}$ (S) | Conc. | $s_{20,w}$ (S) |
| 3 | 9.2 | | | | | | |
| 6 | 10.1 | 6 | 9.9 | 6 | 7.2 | | |
| 15 | 10.7 | 15 | 10.4 | 15 | 7.0 | 15 | 9.2 |
| 150 | 10.3 | | | 60 | 7.3 | 30 | 9.2 |
| | | | | 150 | 7.6 | 150 | 10.4 |

*Concentrations are given as μg of enzyme/ml introduced into the assay.* Average concentration of enzyme in the sedimenting band is approximately 1/10 of the layered enzyme. Forward reaction = aspartic semialdehyde → homoserine; reverse reaction = homoserine → aspartic semialdehyde. Sedimentation coefficients were determined from the movement of the midpoint of the reaction boundary, as visualized at 340 nm.

(Reprinted with permission from Wampler, D. E., *Biochemistry*, 11, 4430, 1972. Copyright by the American Chemical Society.)

range studied. Thirdly, aspartate (plus $K^+$ ions present in the assay mixture) can also stabilize tetramer, at least over a limited range of pH. These observations are in keeping with the findings of Wampler et al.[25] that aspartate plus $K^+$ ions or threonine were necessary to protect the enzyme from loss of sensitivity to inhibition by threonine. It therefore can be concluded that the tetramer is the only species capable of retaining HSD sensitivity towards threonine. Probably a similar type of conclusion would also apply to inhibition of AK activity by threonine.

Both Wampler and Mackall-Neet were conservative in their positions concerning a biological function for the dimer-tetramer interconversion. Considering, however, the fact that dissociation can readily occur in the pH range of 7 to 8 (in $H_2O$), and recognizing that a reversible, concentration-dependent association is an inherent property of the system, it does not seem unreasonable to expect some threonine-insensitive dimeric form to exist in the cell, particularly under conditions of low intracellular threonine concentrations and intermediate states of derepression. But this is not to be construed as suggesting that any of the dissociated forms are of *major* importance in threonine biosynthesis. Clearly, the tetrameric species is the only one endowed with full regulatory control so essential to cellular homeostasis.

### B. Separate Aspartokinase and Homoserine Dehydrogenase Activities from Other Species

In concluding my discussion of aspartokinase and homoserine dehydrogenase, I should like to draw attention to the properties of these enzymes in two other organisms. The homoserine dehydrogenase of *Rhodospirillum rubrum* is distinct from that organism's aspartokinase. Early investigations of HSD by Datta and his co-workers[31] revealed that the enzyme could exist in forms having different sedimentation properties depending on the presence of modulators of activity. Full recognition of this molecular interconversion was provided by the studies of Mankovitz and Segal.[32,33] Dilution of the enzyme in 0.1 *M* KCl, pH 7.2, led to a loss of sensitivity toward threonine. Resensitization could be readily achieved by incubation with threonine. While the rate of desensitization was independent of enzyme concentration, resensitization was biphasic, with the initial rate being proportional to both enzyme and threonine concentration. The degree of resensitization was a sigmoidal function of threonine level, yielding a Hill coefficient of 2.2 for the

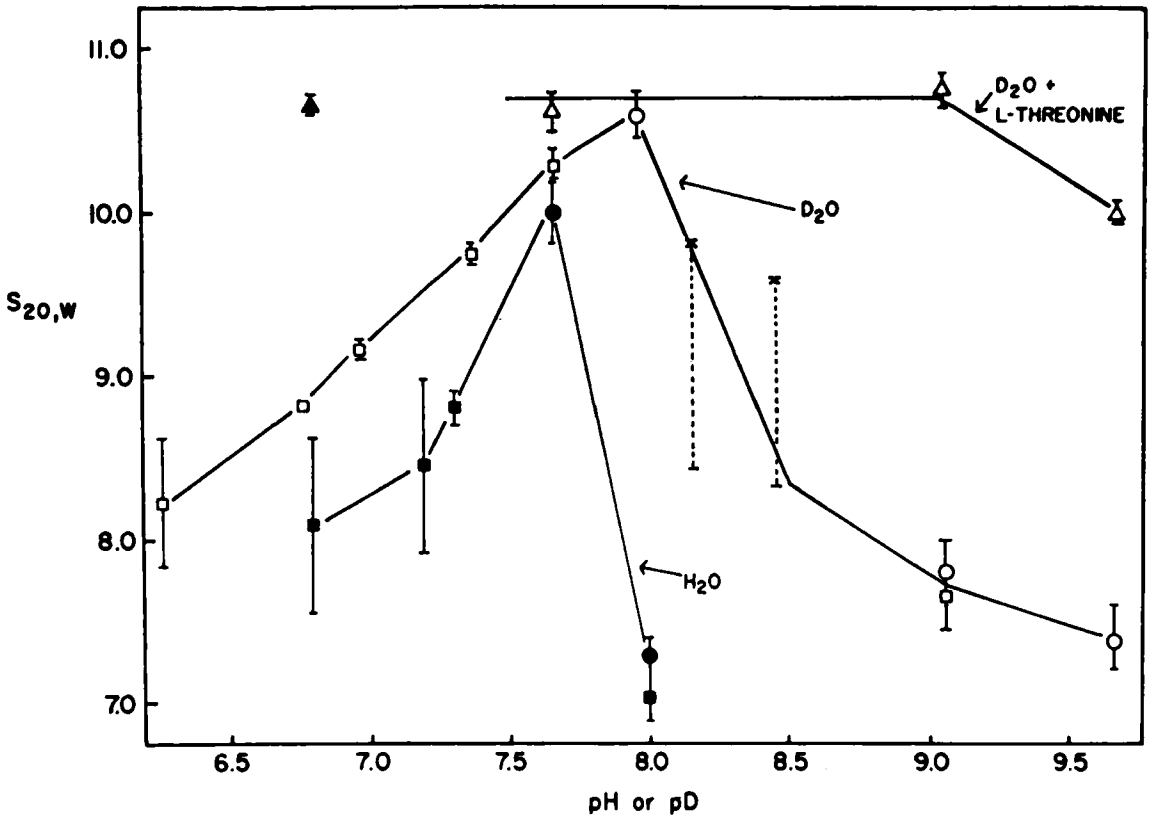



FIGURE 10. Sedimentation of AK-HSD–I as a function of pH or pD. Experiments in $D_2O$ with the HSD assay in the forward reaction (□); in $d_2O$ with assay in the reverse reaction (○); in $H_2O$ with assay in the forward reaction (■); with $H_2O$ with assay in the reverse reaction (●); in $D_2O$ with 20 m*M* threonine (△); and in $H_2O$ with 20 m*M* threonine (▲). The dotted arrows at pD 8.16 and 8.46 indicate the dependence of the sedimentation coefficient on concentration and represent the range of $s_{20,w}$ values obtained from 38 to 57 μg/ml (pD 8.16) and from 68 to 98 μg/ml (pH 8.46). (Reprinted with permission from Mackall, J. C. and Neet, K. E., *Biochemistry*, 12, 3487, 1972. Copyright by the American Chemical Society.)

process. Other ligands which were activators of the enzyme (methionine and isoleucine) could substitute for threonine in producing the resensitization toward threonine. The dissociated form was almost fully active, but was only weakly activated by isoleucine. $K_m$ for aspartic semialdehyde or NADPH was unchanged by dissociation. Data on the enzyme concentration dependence for the regaining of threonine sensitivity fit rather well to a simple model:

$$2E \overset{k_1}{\rightleftharpoons} E_2 \overset{k_2}{\underset{2\theta}{\rightleftharpoons}} E_2\theta_2$$

where $\theta$ represents threonine, and $k_1$ and $k_2$ are equilibrium constants for the respective reactions. Based on a molecular weight of 100,000, $k_1$ was calculated to be $10^{-8}$ *M*. While the number of active sites appeared to be independent of the aggregation state, binding of regulators was considered to occur only on associated subunits.

This conclusion must, however, be viewed as tentative, for there are alternative mechanisms which can adequately encompass the data presented. For example, threonine could be bound to either monomer or dimer, but with a greatly enhanced affinity for dimer. If threonine stabilizes dimer formation (as it appears to do), then with increasing threonine or enzyme levels, dimer concentration increases. The additional necessary consideration would be to explain why sensitivity to threonine is noted only for the dimer species. For this purpose one would only need to specify that the dimer is of the isologous type having a closed structure (Figure 11). In this instance, close interaction between threonine ($\theta$) sites and active sites ($\alpha$) would only be possible in the dimer form. A more complex postulate could also satisfy the

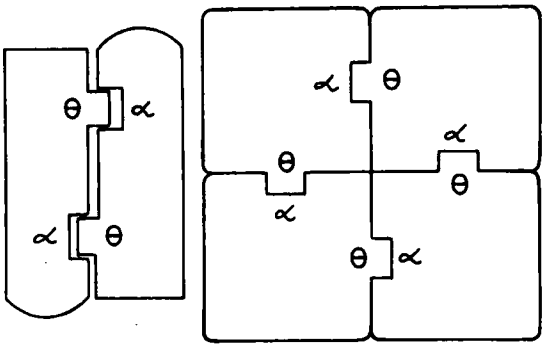
TABLE 6

**Sedimentation of Homoserine Dehydrogenase Activity in the Presence of Effector Molecules**

| pH (D) | Reaction[a] | Effector | $s_{20,w}$[b] (S) |
|---|---|---|---|
| 6.8 | F[c] | 20 m*M* Thr | 10.7 ± 0.07 (2) |
| 7.66 | F | – | 10.3 ± 0.1 (4) |
| 7.96 | R | – | 10.6 ± 0.14 (4) |
| 8.00 | F[c] | – | 7.03 ± 0.16 (2) |
| 9.06 | F | – | 7.63 ± 0.24 (4) |
| 9.06 | R | – | 7.78 ± 0.21 (8) |
| 9.06 | F | 1 m*M* Thr | 10.24 (1) |
| 9.06 | R | 10 m*M* Thr | 10.75 ± 0.11 (3) |
| 9.66 | R | 20 m*M* Thr | 10.1 ± 0.09 (2) |
| 7.96 | R | 20 m*M* Thr | 11.0 ± 0.2 (3) |
| 9.06 | R | 10 m*M* Asp | 10.47 ± 0.22 (4) |
| 9.66 | R | 20 m*M* Asp | 7.71 ± 0.25 (2) |

[a] F = assay in the forward reaction, R = assay in the reverse reaction.
[b] Average ± standard deviation (number of experiments).
[c] No $D_2O$; all others contained $D_2O$, 30 or 40%.

(Reprinted with permission from Mackall, J. C. and Neet, K. E., *Biochemistry*, 12, 3487, 1973. Copyright by the American Chemical Society.)




ISOLOGOUS HETEROLOGOUS

DIMER TETRAMER

FIGURE 11. Possible planar arrangements of two and four subunits applicable to homoserine dehydrogenase of *R. rubrum*.

limited data available. One could propose that the dimer so formed is of the open, heterologous type. This would then proceed to higher order oligomers, but could also stabilize into a heterologous tetramer of the type shown in Figure 11. In this instance, somewhat similar properties would be expected for dimer and tetrameric species, at least insofar as threonine sensitivity is concerned. A threonine bound dimer-tetramer equilibrium was also considered possible by Mankovitz and Segal[32] on the basis of their data and that presented earlier by Datta et al.[31] Until threonine binding data for monomeric and polymeric species become available, however, these points must be regarded as speculation.

Recently, Dungan and Datta have examined the oligomeric properties of the aspartokinase of *Pseudomonas fluorescens*.[34] This enzyme exhibits concerted feedback inhibition by threonine and lysine or threonine and methionine. Threonine alone is a weak inhibitor. The oligomeric state was profoundly influenced by the combination of threonine plus lysine, as shown in Table 7. Sedimentation velocity experiments confirmed these findings. Furthermore, they were able to provide rather convincing evidence that the larger molecular weight species (268,000) was catalytically inactive. Stopped-flow analysis provided an indication that inhibition by threonine plus lysine occurred over a wide range of protein concentrations up to 400 μg/ml. At this high value, similar to that used in gel filtration experiments, inhibition was proportional to the concentration of threonine plus lysine, up to 5 m*M* each. Figure 12 illustrates the correlation observed between oligomeric size and inhibition.

One rather puzzling aspect should be mentioned, however. The onset of inhibition seen in stopped-flow experiments was within 200 msec after initiation of the reaction. Although this point was not discussed, it might be anticipated that the feedback inhibitor effectiveness would be more time dependent, since oligomerization must occur (based on the results of Figure 12) *before* inhibition is seen. That such a delay in the inhibition process is not observed within 200 msec argues for an interpretation that the rate of oligomerization is extremely fast at high protein concentrations relative to catalytic rate, or that a conformational change occurs upon binding of ligands to the dimeric species to produce inhibition. This conformational change can then be followed by a slow oligomerization which results in no further effects on the catalytic process. To distinguish between these possibilities, additional experiments relating molecular size to the degree of inhibition must be performed at lower enzyme concentrations, as would be used in active enzyme centrifugation, to slow down the oligomerization process.

TABLE 7

**Stokes Radii of Aspartokinase as Influenced by Various Ligands**

| Additions | Stokes radius (A) | Approximate mol wt |
|---|---|---|
| None | 41 | $137 \times 10^3$ |
| + Lysine, 5 m*M* | 42 | $146 \times 10^3$ |
| + Methionine, 5 m*M* | 44 | |
| + Threonine, 5 m*M* | 47 | |
| + Threonine, Lysine, 0.1 m*M* each | 43 | $225 \times 10^3$ |
| + Threonine, Lysine, 0.5 m*M* each | 55 | |
| + Threonine, Lysine, 5 m*M* each | 57 | $268 \times 10^3$ |
| + Threonine, Methionine, 5 m*M* each | 54 | |

Aspartokinase (420 μg, >90% pure) was eluted through Sephadex G-200 columns (85 × 1.4 cm) containing 20 m*M* histidine – KOH buffer, pH 8.0, 10 m*M* $MgCl_2$, 200 m*M* KCl, and 5 m*M* aspartate.

(From Dungan, S. M. and Datta, P., *J. Biol. Chem.*, 248, 8544, 1973. With permission of the American Society of Biological Chemists.)

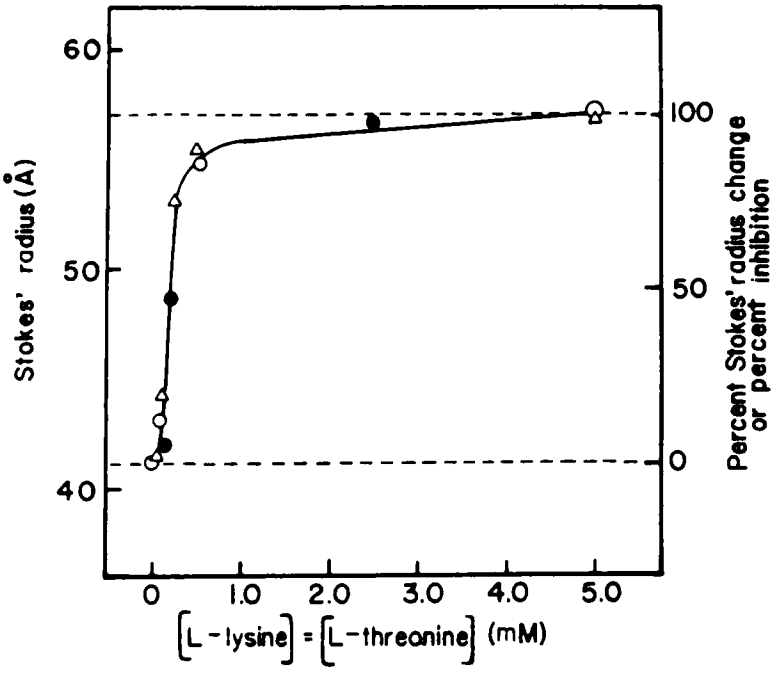



FIGURE 12. Relationship between the changes in the Stokes radius and percent inhibition of enzyme activity as a function of equimolar concentrations of threonine plus lysine. ○, Stokes radius values; △, percent inhibition of activity at 2.1 μg/ml enzyme; ●, percent inhibition of activity at 432 μg/ml enzyme. (Reprinted with permission from Dungan, S. M. and Datta, P., *J. Biol. Chem.*, 248, 8545, 1973. Copyright by the American Society of Biological Chemists.)

## V. GLUTAMATE DEHYDROGENASE

Over the past ten years or so, it has become apparent that bovine liver glutamate dehydrogenase activity is intimately associated with the enzymic state of aggregation. Many of the early observations concerning the relative activity of the enzyme towards glutamate or alanine were expressed in terms of altered states of aggregation, with dissociation leading to an inhibition of

glutamate dehydrogenase activity and an activation of alanine dehydrogenase activity.[35] Although these ideas were attractive, subsequent investigations revealed that this simplistic interpretation was incorrect and, in fact, both activities can occur on the enzyme in its "monomeric" state.[36]

I believe it is still instructive to consider this enzyme in a discussion of ligand-induced oligomerization for two reasons: (1) glutamate dehydrogenase activity, as influenced by purine nucleotide ligands (ADP, GTP), is strongly related to oligomeric state, and (2) more extensive data exist on the protein concentration-dependence of ligand-induced changes than are available for most other enzymes.

Glutamate dehydrogenase is a complicated enzyme.[37] Its normal "monomeric" form consists of 6 subunits of molecular weight 56,000. At protein concentrations in excess of 0.2 mg/ml, polymerization occurs to yield oligomers of molecular weights approaching $2 \times 10^6$. Its catalytic properties are equally complex. In general, it can be stated that the enzyme carries out the reaction

$$\text{L-glutamate} + \text{NADP}^+ + \text{H}_2\text{O} \rightleftharpoons \alpha\text{-ketoglutarate} + \text{NADPH} + \text{NH}_4^+ + \text{H}^+$$

but a wide variety of amino acids can be acted on, and NAD(H) can also be utilized very effectively. With NADP(H) as a coenzyme, the enzyme is activated towards dicarboxylic acid substrates by 5′-ADP and inhibited towards monocarboxylic substrates. An almost opposite catalytic effect is observed upon the replacement of ADP with GTP; reactions with glutamate are inhibited in either direction by GTP, whereas monocarboxylic acid substrate reactions are activated. For a complete picture of kinetic properties, the recent review by Fisher[37] should be consulted. It is sufficient at this time to point out that Frieden[38] and Fisher et al.[39] have presented evidence that ADP and GTP bind to different but mutually exclusive sites, although the most effective binding of GTP also requires the presence of NADP(H). $K_i$ for GTP was 0.4 $\mu M$ at an NADPH concentration of 0.1 m$M$, whereas it was 10 $\mu M$ in the absence of the coenzyme. $K_a$ for ADP was roughly 10 $\mu M$ in the presence 0.1 m$M$ NADPH, and was not greatly changed in the absence of coenzyme.

By way of contrast to the other enzymes described here, it has often been stated that the degree of association of glutamate dehydrogenase has no effect on its activity. This initially surprising fact was illustrated in the results of Fisher et al.[40] and Frieden and Colman.[41] In the latter study, use was made of an acetylated, nonassociating, but fully active enzyme plus stopped-flow assay techniques for following reaction velocities at high enzyme concentrations. Table 8 describes some of these results. Other data to be described shortly will provide molecular weight estimates corresponding to the native enzyme concentrations employed; but certainly, at the concentrations less than 0.1 mg/ml, the predominant form is the 340,000 mol wt monomer, whereas appreciable amounts of higher oligomeric forms exist above this concentration.

In order to assess the effectiveness of GTP binding to the various forms of glutamate dehydrogenase, direct binding measurements were conducted as a function of enzyme concentration. As shown in Figure 13a, the Scatchard plot of binding data indicates marked cooperativity between GTP sites with particularly evident deviations from the expected linearity at high levels of enzyme. These data can be readily understood in terms of a two-state model composed of monomer and oligomer in equilibrium, and in which GTP binds preferentially to the monomer state. As enzyme concentration changes, the ratio of the

TABLE 8

**Specific Activity as a Function of Enzyme Concentration**

| Native enzyme | | Acetylated nonassociating fully active enzyme | |
|---|---|---|---|
| Concentration, mg/ml | Specific activity | Concentration, mg/ml | Specific activity |
| <0.001 | 2.6 | <0.001 | 2.2 |
| 0.01 | 2.0 | 0.11 | 2.4 |
| 0.10 | 3.0 | 0.44 | 2.6 |
| 0.52 | 3.6 | 1.3 | 2.9 |
| 1.75 | 3.4 | | |

Assays were conducted by stopped-flow spectrophotometry at 25°C. Each assay contained 100 $\mu M$ NADPH, 5 m$M$ $\alpha$-ketoglutarate, and 50 m$M$ $NH_4Cl$. Specific activity is given in terms of optical density change/sec/mg of enzyme.

(From Frieden, C. and Colman, R. F., *J. Biol. Chem.*, 242, 1713, 1967. With permission of the American Society of Biological Chemists.)

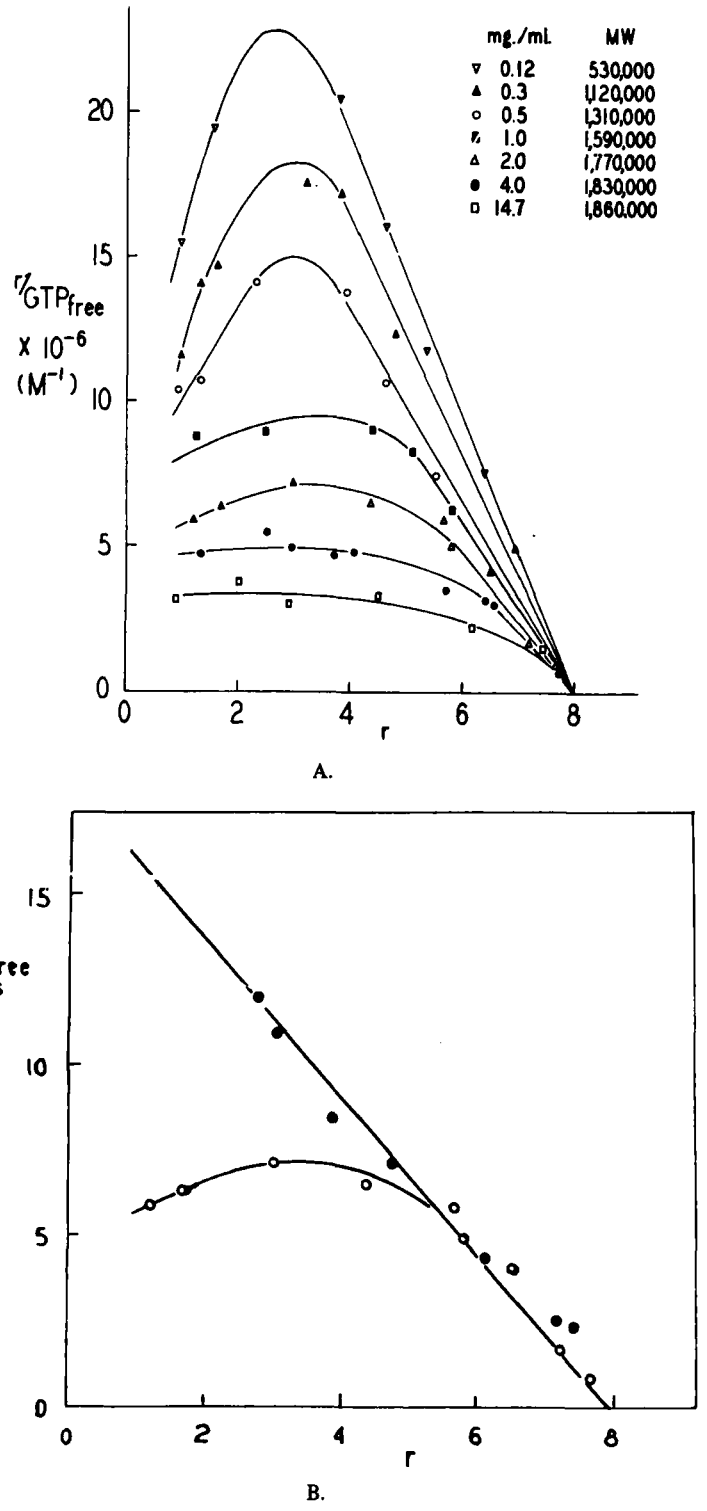



FIGURE 13. A. Scatchard plot of moles of ligand bound, r, vs r/$GTP_{free}$ at various concentrations of enzyme. Binding measurements were made by an ultracentrifugal technique separating free and bound labeled nucleotide. All experiments were performed at pH 7.1 in the presence of 400 $\mu M$ NADH. The limiting value of r (8) was based on a monomer molecular weight of 400,000 rather than the more recent value of 340,000. B. Scatchard plot for GTP binding to native (○) and acetylated nonassociating fully active enzyme (●), each at a concentration of 2 mg/ml. Other experimental conditions are as in 13a. (Reprinted with permission from Frieden, C. and Colman, R. F., *J. Biol. Chem.*, 242, 1709, 1967. Copyright by the American Society of Biological Chemists.)

two forms changes until essentially complete formation of polymer is achieved, whereupon increasing the enzyme level has no further effect. The observation that increases in the dissociation constant for GTP (as indicated by the negative reciprocal of the limiting slope at high $r$ values) occur as the fraction of polymer increases supports the interpretation that GTP is poorly bound to the polymer form compared to the monomer.

It is interesting to note that the cooperative nature of GTP binding is not reflected by kinetic inhibition analysis performed at extremely low enzyme levels ($< 0.001$ mg/ml). Thus, one must assume that the functional basis for the cooperativity resides in the association of monomer units to form oligomers, a situation not encountered at the levels of enzyme normally used for assays. Support for this conclusion is seen in Figure 13b, where GTP binding to native enzyme at a concentration of 2 mg/ml is contrasted to that seen with the acetylated enzyme preparation which cannot undergo polymerization. The binding to the nonassociating form is completely normal, with no evidence of cooperativity.

Comparative data for the binding of ADP to acetylated nonassociating enzyme and native enzyme are shown in Figure 14. Again, for the nonassociating enzyme, no cooperativity is seen, but likewise, normal binding is noted for ADP to native enzyme. The dissociation constant for ADP from acetylated enzyme is 19 $\mu M$, compared to 8 $\mu M$ for native enzyme. Kinetically determined dissociation constants at very low enzyme levels are the same for acetylated and native enzyme. Thus, it appears that ADP may bind more favorably to the polymeric form, but the data are somewhat less convincing than were those for GTP binding.

If we consider that ADP and GTP binding are mutually exclusive by steric hindrance on the enzyme, and accept the conclusions that GTP inhibits by preferentially binding to the monomeric forms while ADP activates by attaching principally to polymeric forms, then it can be predicted that increasing levels of ADP would displace GTP and promote association. Furthermore, at high enzyme levels, where association is naturally favored, the competitive influence of ADP in reversing GTP inhibition would be reduced by virtue of the fact that GTP binding is normally less under associative conditions. The results of such a competition experiment are shown in Figure 15, with GDP replacing GTP. The diphosphate derivative is less tightly bound than is the triphosphate compound, thereby permitting a more effective competition by lower levels of ADP. These data indicate that at a fixed concentration of GDP, the extent of activation by ADP is less as the enzyme concentration increases and approaches the fourfold activation observed when no GDP is present.

Throughout the preceding discussion, no attention has been paid to the role of NAD(H) or NADP(H) in the structural changes induced by GTP or ADP. Frieden,[38] however, has shown that depolymerization promoted by GTP requires the presence of coenzyme. This point has been emphasized more recently in a study of the rates of depolymerization induced by GTP and NADH or NADPH.[42] Depolymerization was measured by absorbance changes at 300 nm in a stopped-flow apparatus. It was noted that the rate of absorbance decrease was considerably greater upon addition of GTP to enzyme-NADPH compared to NADPH addition to enzyme-GTP. But a strictly ordered sequence of binding is inconsistent with another observation that GTP can bind to the enzyme in the absence of NADPH. Instead, it was concluded that binding is random, but that an ordered sequence of conformational changes occurs. These were visualized as first a conformational change upon the binding of NADPH (but no GTP), followed by a depolymerization of the enzyme-NADPH-GTP complex. The situation encountered when NADH replaces NADPH is more complex, owing to the fact that two binding sites exist for NADH on each subunit, but only one for NADPH.

As just presented, the extent of depolymerization can be monitored by following absorbance changes at 300 nm or by other direct methods such as light scattering.[43] Depolymerization can be effected by either GTP or, less rapidly, by GDP on the enzyme-NAD(P)H complex. The influence of enzyme concentration on GDP-mediated depolymerization of glutamate dehydrogenase was also examined by Huang and Frieden.[42] GDP concentrations of over 500 $\mu M$ can produce almost complete depolymerization, but as subsaturating levels the GDP-induced depolymerization is a function of enzyme concentration. For example, at enzyme levels of 0.5 mg/ml or less, GDP at 500 $\mu M$ can promote a complete depolymerization, whereas at 1.5 mg/ml, this same concentration of GDP accounts for only a 70% depolymerization,

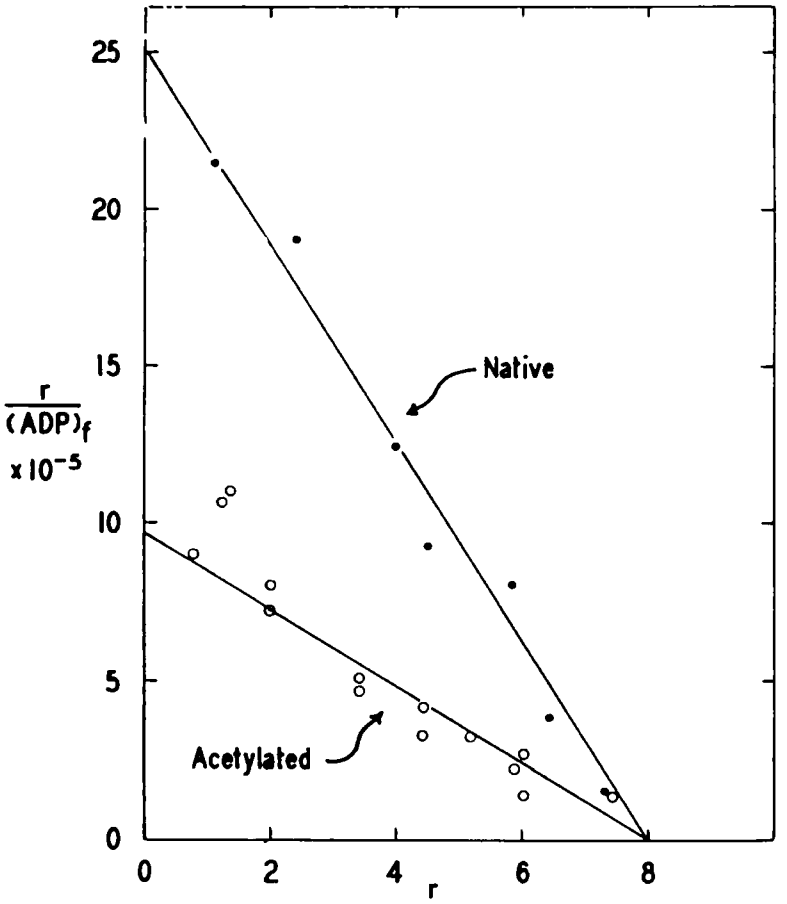



FIGURE 14. Scatchard plot of $r/ADP_{free}$ vs r for native and acetylated nonassociating fully active enzyme. Experiments were performed at an enzyme concentration of 2 mg/ml in the presence of 100 $\mu M$ NADH. Other details are as in Figure 13. (Reprinted with permission from Frieden, C. and Colman, R. F., *J. Biol. Chem.*, 242, 1710, 1967. Copyright by the American Society of Biological Chemists.)

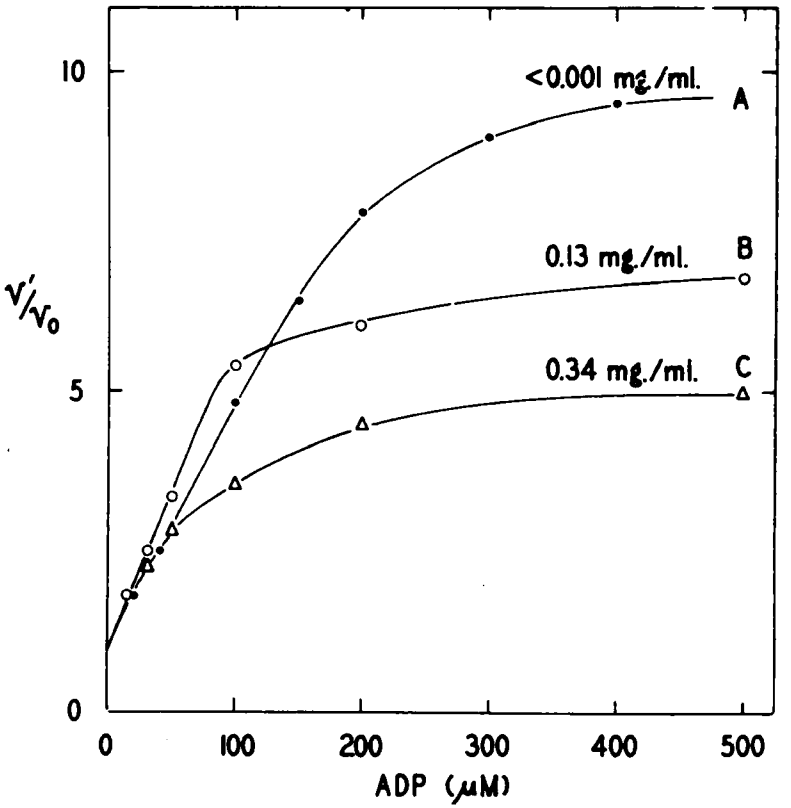



FIGURE 15. Activation as a function of ADP concentration in the presence of 50 $\mu M$ GDP at 3 levels of enzyme. Activity was measured at pH 8.1 in the presence of 200 $\mu M$ NADPH, $\alpha$-ketoglutarate, and $NH_4Cl$. In the absence of ADP, GDP inhibits 75%, 58%, and 25% at <0.001, 0.13, and 0.34 mg/ml, respectively. All rates in the absence of ADP have been normalized to the same value. (Reprinted with permission from Frieden, C. and Colman, R. F., *J. Biol. Chem.*, 242, 1712, 1967. Copyright by the American Society of Biological Chemists.)

and a 58% depolymerization is seen at an enzyme level of 3 mg/ml. These data, measured on the enzyme-NADH complex at a constant level of GDP, suggest that the ability of GDP (and probably GTP as well) to promote dissociation is dependent upon the extent of monomer present in the association-dissociation system.

## VI. BIODEGRADATIVE THREONINE DEHYDRATASE

For my last example, I would like to describe some recent investigations conducted on L-threonine dehydratase, also often referred to as threonine dehydrase or threonine deaminase. In *E. coli* and in certain plants,[44,45] two forms of this enzyme are known. One, a constitutive biosynthetic enzyme concerned with isoleucine formation, has long been the subject of studies pertaining to the mechanism of allosteric regulation by isoleucine and valine, and there appears to be some preliminary evidence to suggest that association-dissociation phenomena are important in this process.[46] On the other hand, the inducible biodegradative enzyme from *E. coli* is activated by 5′-AMP and undergoes a facile monomer-tetramer interconversion which has been well documented.[47] An analogous enzyme from *Clostridium tetanormorphum* is activated by 5′-ADP rather than AMP, but otherwise closely resembles its *E. coli* counterpart. Let us first consider the properties of the AMP-activated dehydratase from *E. coli.*

### A. Biodegradative Threonine Dehydratase from *Escherichia coli*

In 1964, Phillips and Wood[48] first established that this enzyme exhibited the properties of a monomer-oligomer system, with AMP promoting oligomer formation. That study, and a later, more complete report by Whanger et al.,[49] revealed that the principal kinetic effect of AMP was exerted on the $K_m$ for threonine, such that a $K_m$ of between 30 to 100 m*M* in the absence of AMP was decreased to 4 m*M* when AMP was present in saturating amounts. Although both of these investigations, plus studies conducted by Hirata et al.,[50] indicated that $V_{max}$ was also increased by AMP, a recent reexamination of this point by Dunne et al.[51] has convincingly established that $V_{max}$ for pure enzyme is 480 μmol/min/mg protein, irrespective of AMP presence. Measurements of activity conducted in the absence of AMP require significant corrections for inactivation of the enzyme upon dilution, without which $V_{max}$ appears to be altered from that seen with AMP present.

Rather extensive analysis of the specificity for the nucleotide activator revealed that 5′-AMP could be replaced by 5′-CMP but not by 3′-AMP, dAMP, ADP, or ATP.[52,53] From these findings, it must be concluded that, despite some degree of nonspecificity, 5′-AMP exhibits the lowest $K_a$ for activation and is probably the physiological regulator for this enzyme.

Crystalline preparations of threonine dehydratase contain 4 mol of pyridoxal phosphate per 147,000 g of protein and can bind AMP to the extent of 4 mol/mol of protein.[54] Sedimentation velocity ultracentrifugation and diffusion measurements gave a molecular weight value of 147,000 and an $s^0_{20,w}$ of 8.2 S for the enzyme from *E. coli* strain W, closely agreeing with values estimated for the enzyme from Crookes strain by sucrose density gradient centrifugation and gel filtration in the presence of AMP.[49] Precise molecular weight data over extremes of protein and AMP concentration are still incomplete, but general agreement has been reached on the conclusion that a species of approximately 8 S, corresponding to a globular molecular weight of roughly 150,000, is present at enzyme concentrations greater than 1 mg/ml with 5 m*M* AMP; and a monomeric species of 3.2 S (approximately 40,000 mol wt) exists at low dehydratase concentrations with AMP absent. The sedimentation rate in the absence of AMP is strongly dependent upon dehydratase concentration, and oligomerization can occur at extremely high enzyme levels even without AMP. In all instances, however, a single sedimenting peak has been observed, leading to the conclusion that the monomer-oligomer interconversion is quite rapid.[49,55]

As in the case of CTP synthetase, it has been possible to pinpoint the role of the nucleotide effector in the overall catalytic process. Rabinowitz et al.[56] found that AMP exerts its effect primarily on the earliest steps in threonine dehydration, probably on the binding of threonine to the enzyme. This conclusion was supported by observations that AMP could evoke changes in the affinity of enzyme for L-α-aminobutyrate, L-alanine, DL-β-hydroxy butyrate, or a variety of other compounds which could all bind competi-

tively with L-threonine, but which could not undergo dehydration. Furthermore, measurements of circular dichroism showed that the enzyme exhibits an absorption maximum at 415 nm, attributed to the phosphopyridoxal-lysyl aldimine, and that this absorption is gradually lost upon addition of increasing amounts of any of the α-amino-containing competitive inhibitors. If the assignment of the 415 nm signal is correct, then its loss corresponds to transaldimination between the ε-amino group of the lysyl residue and the α-amino group of threonine or an inhibitor. Since AMP decreased the intensity of the 415 nm signal over that drop which was observed when only L-threonine or L-α-aminobutyrate was added at subsaturating amounts, it was concluded that AMP affected the efficiency of binding *prior to* transaldimination.

Although it appears certain that AMP addition results in a 23-fold decrease in $K_m$ for threonine and is also capable of producing oligomerization from a monomer of 3.2 S to higher forms such as an 8.2 S tetramer, the question of whether oligomerization is itself responsible for the kinetic changes has long been raised. Gerlt et al.[55] sought to answer this for the AMP-activated dehydratase by examining the effect of changes in enzyme concentration on kinetic properties. Figure 16 illustrates the point mentioned earlier that even without AMP present, a significant amount of oligomer was formed at enzyme levels above 100 μg on the sucrose gradient, corresponding to a concentration of 0.2 to 0.5 mg/ml. Extrapolation of these data indicates that oligomerization should be essentially complete at concentrations exceeding 2 to 5 mg/ml. Recognizing that oligomerization could occur either with or without AMP, assays were conducted over a wide range of enzyme – 0.02 to 1,000 μg/ml. For the highest enzyme levels, stopped-flow measurements of initial reaction velocity replaced the usual spectrophotometric assay. Despite the presumed oligomerization at the highest enzyme concentrations, $K_m$ did not decrease, and instead increased significantly from 70 m*M* at 0.02 μg/ml to 225 m*M* at 1 mg/ml. Thus, it appears that oligomerization per se is inadequate to promote the formation of an activated species having a $K_m$ of 3 m*M*, and may

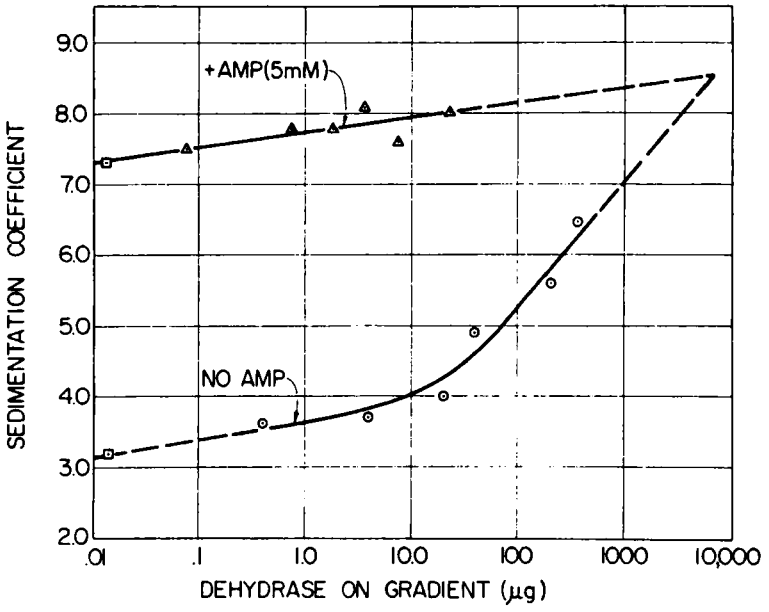



FIGURE 16. Change in sedimentation coefficient as a function of threonine dehydratase concentration. Sedimentation was performed on sucrose gradients (5 to 20%) buffered at pH 8. Calculations of $s_{20}$ were made from measurements of migration of internal marker proteins. The points denoted by □ correspond to the concentration of dehydratase normally employed in a coupled spectrophotometric assay. (Reprinted with permission from Gerlt, J. A., Rabinowitz, K. W., Dunne, C. P., and Wood, W. A., *J. Biol. Chem.*, 248, 8204, 1973. Copyright by the American Society of Biological Chemists.)

even interfere with substrate binding in the absence of AMP.

Unfortunately, it was not possible to evaluate any effect that L-threonine itself might exert on the oligomerization, either of a negative or positive nature; but it seems apparent that if threonine influences the system, it must be either to restrain oligomer formation or to interfere with the stability of the activated conformation which is responsible for the low $K_m$ value.

An important consideration in evaluating these data is whether AMP can bind directly to a monomeric species or if it promotes oligomer formation by virtue of selective binding to oligomeric states. That the former is more likely correct can be inferred from other data offered by these same investigators. Figure 17 shows that the addition of 20 m*M* threonine to a preparation of AMP-free enzyme at 0.1 μg/ml yielded a rate characteristic of that condition. Then, upon the addition of AMP to 5 m*M*, there occurred an instantaneous decrease in reaction velocity to near zero, followed by a slow return of velocity to a value finally exceeding the rate observed without AMP. Because the final concentration of AMP was tenfold over the value for the $K_a$ for AMP, it must be assumed that nearly complete binding of AMP should have occurred at all available regulatory sites on the monomer population. The observable result of this binding is a transient drop in velocity, which likely represents the formation of an AMP-monomer which is either inactive or has an exceedingly poor affinity for substrate. Following this state, the oligomerization of AMP-containing monomers takes place, with the oligomer-AMP form exhibiting a much decreased $K_m$ for threonine.

Further evidence to indicate that an oligomerization had occurred during the period of activation after AMP addition was obtained by taking the instantaneous slope (area B of Figure 17) and calculating the second derivative of replots of rates vs time at different enzyme concentrations. This method permitted the determination of reaction order for the process

$$nB \xrightarrow{k_a} A$$

where B is the inactive form (containing AMP), A is the low $K_m$ dehydratase, $k_a$ is a rate constant for activation, and n is a coefficient of stoichiometry. A value of n equal to 1.9 was obtained, eliminating the possibility that an isomerization process was occurring, and suggesting that a reaction second-order with respect to enzyme was taking place.

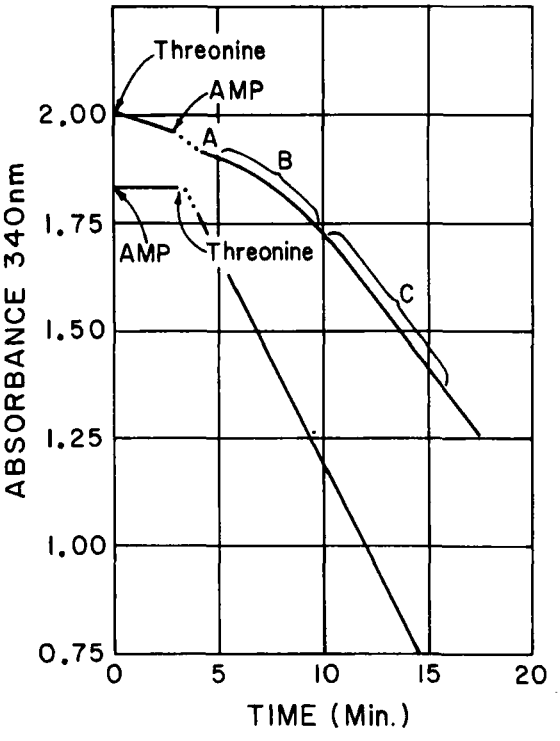



FIGURE 17. Effect of order of addition to the assay for threonine dehydratase. The lower curve represents a normal assay with 0.10 μg dehydratase/ml, with 5 m*M* AMP and L-threonine at 20 m*M*. The top curve illustrates a similar assay with the addition order reversed. Parts A, B, and C of the top curve represent different rate periods, as discussed in the text. (Redrawn and reprinted with permission from Gerlt, J. A., Rabinowitz, K. W., Dunne, C. P., and Wood, W. A., *J. Biol. Chem.*, 248, 8202, 1973. Copyright by the American Society of Biological Chemists.)

Data on the role of L-threonine in activation or oligomerization are scant and the question of possible homotropic effects has been sharply debated.[44,51] Although reconciliation of this matter is not possible, largely because the experimental data collected are not directly comparable, there is some reason to believe that L-threonine binding may not be strictly analyzable in terms of attachment to a single enzyme form.[57] This would appear all the more likely in view of the demonstrated equilibrium existing between monomer and oligomer forms.

### B. ADP-Activated Threonine Dehydratase from *Clostridium tetanomorphum*

Threonine dehydratase from *Clostridium tetanomorphum* shares many of the molecular properties exhibited by the *E. coli* degradative dehydratase. It is also a tetramer, having a molecular weight of 184,000[59,60] which dissociates upon dilution in the absence of ADP to a dimeric or possibly a monomeric form. Dissociation is considerably promoted by pH values above 9, whereas maximal catalytic activity is seen at pH 8.[61] Simon et al.[62] have recently described the subunit nature of the enzyme. Each subunit of molecular weight 45,000 is composed of two, nonidentical polypeptide chains (26,000 and 19,000) which are joined by disulfide linkages.

Of principal interest here are data collected which illustrate the role of ligands in the oligomerization process. The enzyme clearly exhibits reversible association and dissociation in the absence of any ligands.[63] As shown in Figure 18, the weight average molecular weight is strongly protein concentration-dependent, with an equilibrium constant of $3 \times 10^5\ M^{-1}$ estimated at 16°C. A shift in this equilibrium favoring tetramer formation is seen upon the addition of 0.1 m*M* ADP or by increasing the temperature for equilibrium ultracentrifugation. Vanquickenborne and Phillips[61] also observed that association was promoted by L-threonine, although less effectively than with ADP.

The reversibility of the association process has possibly been the source of much controversy concerning the kinetic properties of this enzyme.

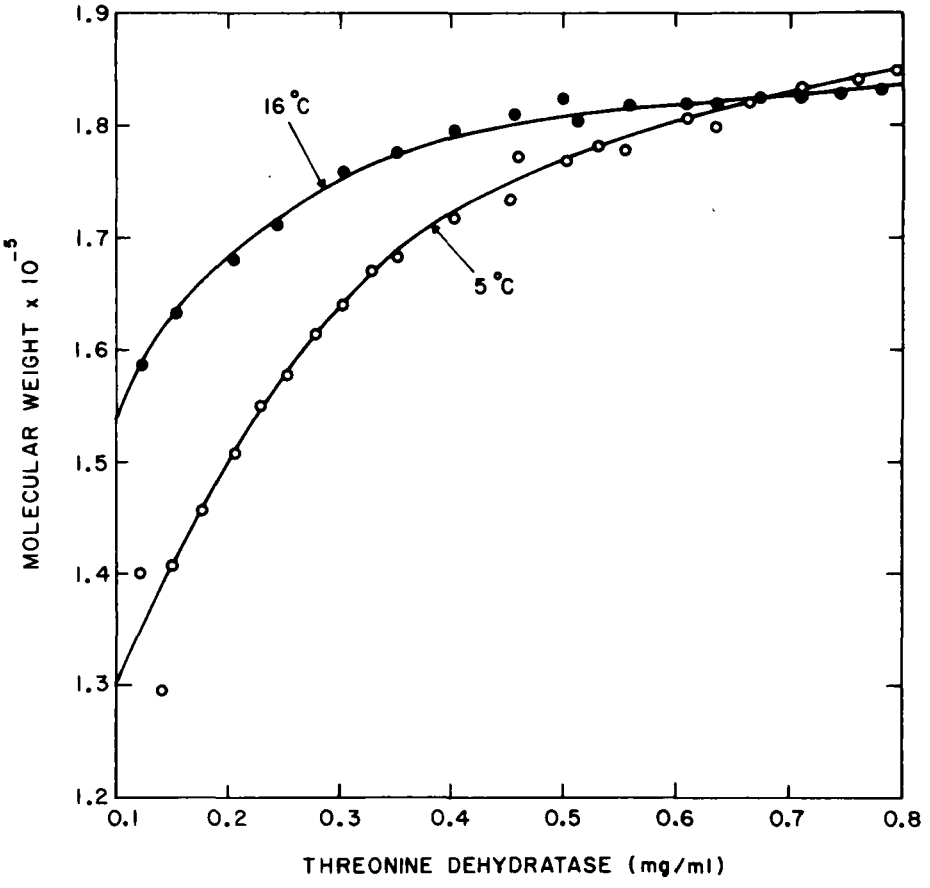



FIGURE 18. Concentration-dependent changes in molecular weight for clostridial threonine dehydratase. Each set of data at a given temperature was obtained from the analysis of equilibrium concentration vs distance profiles obtained with a single cell at 12,000 rpm in a specially modified analytical ultracentrifuge equipped with an ultraviolet light source (280 nm) and a digitized stepwise photoelectric scanner. Each concentration was calculated from the optical density value at a particular radial position. Point-to-point molecular weights were determined with a computer program kindly provided by Dr. Dennis Roark of Hershey Medical School.

For example, Whiteley and Tahara[64] reported a sigmoidal response of velocity to substrate concentration when assayed at pH 9.5 in the absence of ADP. This sigmoidicity was eliminated if ADP was present. Similar results were obtained by Nakazawa and Hayaishi[65] at pH 8.4. Both reports were based on data obtained in fixed-time spectrophotometric assays at very low enzyme concentrations. On the other hand, Vanquickenborne and Phillips[61] observed hyperbolic kinetics at pH 7.8, either with or without ADP, provided reaction velocities were monitored continuously and stable rates were used in the kinetic analysis. As illustrated in Figure 19 for velocities measured at pH 8.6, the reaction rates without ADP are not constant until some 30 min after reaction initiation. Use of "early" rates gave decidedly sigmoidal substrate-velocity plots, whereas "final" rates resulted in hyperbolic relationships. Of course, this behavior is only seen when ADP is absent. Proof that this hysteretic change was due to aggregation rather than a conformational isomerization could

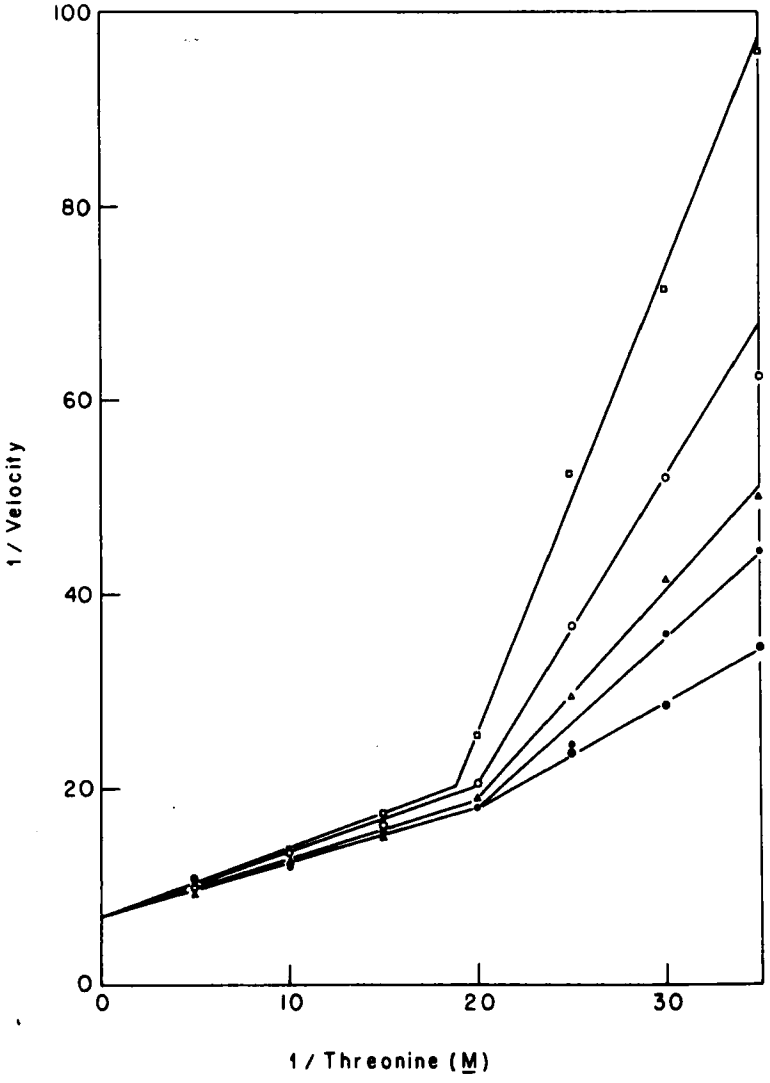



FIGURE 19. Double reciprocal plot of threonine concentration and velocity measured at different time intervals. Reactions were conducted at pH 8.6 without ADP. □, 5 to 10 min rate interval; ○, 10 to 15 min rate interval; △, 15 to 20 min rate interval; ●, 20 to 25 min rate interval; ⊙, 25 to 30 min rate interval. Enzyme concentration was approximately 0.05 μg/ml. (Reprinted with permission from Vanquickenborne, A. and Phillips, A. T., *J. Biol. Chem.*, 243, 1317, 1968. Copyright by the American Society of Biological Chemists.)

not be obtained. Nevertheless, it seems quite reasonable to infer this conclusion based on the known tendency to dissociate at low enzyme levels and to reassociate at high concentrations of threonine. Additional support is provided by the observation that the length of the lag period in such assays conducted without ADP is decreased appreciably at higher enzyme concentrations (J. P. Simon, unpublished observation).

As in the *E. coli* dehydratase, the kinetic consequences of ADP activation are a pronounced decrease in the $K_m$ for threonine, with no change in $V_{max}$.[61] This conclusion was not difficult to reach, for in contrast to the *E. coli* enzyme, the dissociated form(s) appear to be fairly stable, and consequently retain maximal catalytic activity for short periods in the absence of associative ligands. In keeping with the general properties of a K-type system in which ADP alters $K_m$ for threonine, it is found that threonine increases the affinity of the enzyme for ADP. $K_a$ for ADP is decreased from 30 $\mu M$ at a threonine concentration of 5 m$M$ to 3 $\mu M$ at a threonine concentration of 100 m$M$.[61] The midpoint dissociation constant ($K_{0.5}$) for ADP, estimated by direct binding studies without threonine present, was 70 $\mu M$, and the binding curve is decidedly sigmoidal with a Hill coefficient of 1.7.[66]

The rate of oligomerization for the clostridial enzyme appears to be considerably slower than was noted for the *E. coli* enzyme. This fact is reflected in the time-dependent velocity characteristics, where oligomerization is promoted by substrate, and in the observation that both tetrameric and dimeric forms were present in sucrose density gradients containing threonine at pH 9.4.[61] Confirmation of the oligomerizing effect of threonine at high pH under assay conditions was recently accomplished by Schorr.[66] He performed active enzyme ultracentrifugation experiments with enzyme concentrations of 1 $\mu$g/ml or less and observed that the sedimentation characteristics of the enzyme were markedly influenced by threonine concentration. Representative data are shown in Table 9. Particularly interesting are the results seen during centrifugation of dimeric (5.2S) enzyme in the presence of 150 m$M$ threonine. Although the enzyme migrated with a constant velocity corresponding to 5S for the first 6 min of observation, there then occurred an oligomerization to yield the 8S tetramer. The extent of oligomerization could not be readily established, since the technique measures only the sedimentation rate of the fastest migrating species, but presumably after the transition point the tetrameric species became the predominant form.

One additional aspect of threonine-induced oligomerization was established by Schorr.[66]

TABLE 9

**Structure – Kinetic Relationships for Threonine Dehydratase as Measured by Reactive Enzyme Centrifugation**

| Threonine m$M$ | ADP m$M$ | pH | $s_{20,w}$ Initial | $s_{20,w}$ Final | Kinetic behavior[a] Initial | Kinetic behavior[a] Final |
|---|---|---|---|---|---|---|
| 15 | 0 | 7.2 | 8.0 | 8.0 | Michaelis | Michaelis |
| 15 | 0 | 9.0 | 5.2 | 5.4 | Non-Michaelis | Non-Michaelis |
| 150 | 0 | 7.2 | 8.0 | 8.0 | Michaelis | Michaelis |
| 150 | 0 | 9.0 | 5.2 | 8.3 | Non-Michaelis | Michaelis |
| 15 | 1 | 7.2 | 8.1 | 8.1 | Michaelis | Michaelis |
| 15 | 1[b] | 9.0 | 5.1 | 8.0 | Non-Michaelis | Michaelis |
| 15 | 1[c] | 9.0 | 8.0 | 8.0 | Michaelis | Michaelis |

[a]Kinetic behavior was assessed separately under nearly identical conditions in a spectrophotometric assay, except that enzyme concentration was 0.05 $\mu$g/ml rather than 0.1 to 1.0 $\mu$g/ml used in the centrifugation experiments. Michaelis behavior refers to a linear double reciprocal plot of initial velocity data or of reaction velocity after 20 min. Non-Michaelis refers to a nonlinear plot (see Figure 19 for an example).

[b]ADP present during centrifugation only.

[c]ADP present during preincubation and centrifugation.

Enzyme treated with glyoxal became completely insensitive towards ADP. The $K_m$ for threonine was 80 m*M* even in the presence of 10 m*M* ADP; this contrasted sharply with the usual observation for native enzyme, where $K_m$ changes from 80 m*M* without ADP to 5 m*M* with ADP. Glyoxal treatment did not alter maximum velocity, however. When glyoxal-treated enzyme was subjected to active enzyme centrifugation at a threonine concentration of 125 m*M*, it underwent the identical transition from 5 to 8S after 6 min exposure to the assay mixture. These results were interpreted as further evidence that aggregation can occur without a concomitant change in $K_m$ towards threonine, and that aggregation can be influenced by threonine as well as by ADP or high enzyme levels. Furthermore, it was clear that threonine promoted aggregation, by binding to sites other than those normally occupied by ADP.

## VII. CONCLUSIONS

From the examples discussed here, there is sufficient evidence to permit establishing an argument that oligomerization is not merely a biological curiosity, but rather is an effective mechanism of regulation in itself. In the case of ribonucleoside diphosphate reductase, oligomerization of the R1-R2 complex in the presence of high concentrations of dATP appears to be correlated with the inhibition of reductase activity. Similarly, glutamate dehydrogenase undergoes oligomerization at high enzyme and ADP levels, with the result being an insensitivity to inhibition by GTP. This particular situation seems to support a physiological role for oligomerization by virtue of the fact that mitochondrial glutamate dehydrogenase concentration has been estimated to be 1 mg/ml,[41] an amount which is clearly sufficient for the existence of appreciable amounts of oligomer. Furthermore, the findings that GTP can bind only to the monomeric form in the presence of NAD(P)H, while ADP more often binds to polymeric forms, implies that a reversible association-dissociation process is required in order for effective control of glutamate dehydrogenase by purine nucleotides.

The aspartokinases, homoserine dehydrogenases, and CTP synthetase also appear to exhibit clear-cut differences in kinetic properties and sensitivity to ligands, depending on the state of aggregation of the enzymes. Although CTP synthetase could be shown to undergo conformational changes upon ligand binding which preceded oligomerization, thereby raising the question of the significance of oligomerization, it can be argued that oligomerization is a mechanism for stabilizing activated conformations, thereby rendering the changes more permanent and providing a buffer effect against rapid changes in ligand concentration.

Data presented on threonine dehydratase also point up the potential importance of oligomerization, for in this case, enzyme concentration has been shown to play a role in regulating the extent of oligomerization, especially in conjunction with adenine nucleotide effectors. Because this particular enzyme is inducible, its cellular concentration is highly variable, depending on the state and degree of inducibility. This would suggest that oligomerization can be a means of utilizing enzyme concentration as a regulatory function beyond the simple linear dependence of reaction velocity to enzyme level.

Arguments for a contrary point of view are best sustained by the persistent lack of evidence that association-dissociation phenomena are operative under physiological conditions, or even under conditions (themselves possibly nonphysiological) where regulatory kinetic or binding data are obtained. In some situations, polymerization or depolymerization can be demonstrated only upon pH change or by addition of ligands which have no regulatory significance. This fosters the conclusion that oligomerization is a nonspecific physical response. All such points have merit, but clearly cannot directly refute a possible regulatory significance for ligand-induced oligomerization.

One can then ask, "What types of information will be most useful in establishing a role for oligomerization in enzyme regulation?" The answer to this appears to lie at hand, through a combination of the approaches already discussed, but with added efforts made toward correlation of kinetic and binding data with the oligomeric state and with greater attention given to obtaining all data as a function of enzyme concentration.

Several extremely powerful techniques have recently been developed and exploited towards this end. I refer to such procedures as molecular sieve chromatography, reactive enzyme centrifugation, and the use of chemically altered species which exhibit an inability to associate or dissociate. Ackers[67] has refined molecular sieve chromatography to such a degree that complex mixtures

of interacting macromolecules can be analyzed for the number of components, their molecular weights, and association constants, either alone or in the presence of ligands. The reactive enzyme centrifugation techniques of Cohen and Mire [29], likewise, is useful for relating physical size to catalytic activity, although its application to mixtures of active components is limited, as is its use at higher enzyme levels. Godschalk[68] has also described a method for the enumeration of interacting components based on diffusion measurements in the ultracentrifuge. Light scattering measurements of molecular weight as influenced by ligands have also proven of value, particularly in those instances where relatively high enzyme concentrations are to be studied.

Unfortunately, there still exists no single method capable of relating ligand influence on reaction kinetics to enzymic quaternary structure in a direct fashion. Thus, one must presently rely on precise and complete descriptions of the oligomeric state as affected by ligands under a variety of conditions, and independent evaluation of kinetic parameters under comparable situations, in order to draw firm conclusions regarding the role of oligomerization.

At the outset of this article, the point was made that oligomerization can be viewed as a mechanism for enhancing the action of an allosteric effector, as well as for providing a buffer, in the form of a slow response, against large, rapid changes in metabolite levels. These two processes are not incompatible, since one is quantitative while the other is temporal, but both mechanisms may not be of significance for all oligomerizing enzymes. Clearly, the enhancement of ligand binding upon either association or dissociation implies a preferential binding by one form. When a conformational change preceding an association-dissociation process is slow, or when the rate constant for polymerization or depolymerization itself is small, then a hysteretic effect will also be noted, provided that the kinetic or binding properties of the various forms differ. One fact emerges, however, from consideration of the regulatory function of oligomerization. That fact is the potential for modulating the response of an enzyme to allosteric effectors as a function of enzyme concentration. This point takes on increasing importance in view of the large number of enzymes which are either inducible in nature or which exist in active and inactive forms interconvertible by covalent modification.

## APPENDIX A

**Enzymes Possessing Oligomerization Tendencies Under Ligand Control**

| Enzyme | Source | Ref. |
|---|---|---|
| Acetyl-CoA carboxylase | Chicken liver | 73 |
| Phosphoenol pyruvate carboxytransphosphorylase | *Propionibacterium shermanii* | 74 |
| Anthranilate synthetase | *Clostridium butyricum* | 75 |
| Isopropylmalate synthase | *Salmonella typhimurium* | 72 |
| Glycogen phosphorylase b | Rabbit muscle | 81, 82 |
| Glycogen phosphorylase a | Rabbit muscle | 80, 81 |
| Pyridine nucleotide transhydrogenase | *Pseudomonas aeruginosa* | 76 |
| Deoxythymidine kinase | *Escherichia coli* | 79 |
| Malate dehydrogenase (cytosol form) | Bovine heart | 78 |
| Glyceraldehyde-3-phosphate dehydrogenase | Rabbit muscle | 77 |
| Isocitrate dehydrogenase | Bovine heart | 84 |
| Glucose-6-phosphate dehydrogenase | Human erythrocyte | 83 |
| Phosphofructokinase | Rabbit muscle | 85 |
| Deoxycytidylate deaminase | Chicken embryo | 86 |

## REFERENCES


1. **Koshland, D. E., Jr.,** The molecular basis for enzyme regulation, in *The Enzymes,* Vol. 1, Boyer, P. D., Ed., Academic Press, New York, 1970, 341.
2. **Monod, J., Wyman, J., and Changeux, J. P.,** On the nature of allosteric transitions: a plausible model, *J. Mol. Biol.,* 12, 88, 1965.
3. **Adair, G. S.,** The hemoglobin system. VI. The oxygen dissociation curve for hemoglobin, *J. Biol. Chem.,* 63, 529, 1925.
4. **Roughton, F. J. W., Otis, A. B., and Lyster, R. L. J.,** The determination of the individual equilibrium constants of the four intermediate reactions between oxygen and sheep haemoglobin, *Proc. R. Soc. Lond.,* B144, 25, 1955.
5. **Perutz, M. F., Rossman, M. G., Cullis, A. F., Muirhead, H., Will, G., and North, A. C. T.,** Structure of haemoglobin. A three-dimensional Fourier synthesis at 5.5 A resolution, obtained by X-ray analysis, *Nature,* 185, 416, 1960.
6. **Koshland, D. E., Jr., Nemethy, G., and Filmer, D.,** Comparison of experimental binding data and theoretical models in proteins containing subunits, *Biochemistry,* 5, 365, 1966.
7. **Cornish-Bowden, A. and Koshland, D. E., Jr.,** A general method for the quantitative determination of saturation curves for multisubunit proteins, *Biochemistry,* 9, 3325, 1970.
8. **Teipel, J. and Koshland, D. E., Jr.,** The significance of intermediary plateau regions in enzyme saturation curves, *Biochemistry,* 8, 4656, 1969.
9. **Frieden, C.,** Treatment of enzyme kinetic data. II. The multisite case: comparison of allosteric models and a possible new mechanism, *J. Biol. Chem.,* 242, 4045, 1967.
10. **Frieden, C.,** Kinetic aspects of regulation of metabolic processes. The hysteretic enzyme concept, *J. Biol. Chem.,* 245, 5788, 1970.
11. **Frieden, C.,** Metabolic interconversions of enzymes: relation to the hysteretic response, in *Metabolic Interconversion of Enzymes,* Wieland, O., Helmreich, E., and Holzer, H., Eds., Springer-Verlag, Berlin, 1972, 391.
12. **Brown, N. C. and Reichard, P.,** Ribonucleoside diphosphate reductase. Formation of active and inactive complexes of proteins B1 and B2, *J. Mol. Biol.,* 46, 25, 1969.
13. **Brown, N. C. and Reichard, P.,** Role of effector binding in allosteric control of ribonucleoside diphosphate reductase, *J. Mol. Biol.,* 46, 39, 1969.
14. **Larsson, A. and Reichard, P.,** Enzymatic synthesis of deoxyribonucleotides. IX. Allosteric effects in the reduction of pyrimidine ribonucleotides by the ribonucleoside diphosphate system of *Escherichia coli, J. Biol. Chem.,* 241, 2533, 1966.
15. **Long, C. W., Levitzki, A., and Koshland, D. E., Jr.,** The subunit structure and subunit interactions of cytidine triphosphate synthetase, *J. Biol. Chem.,* 245, 80, 1970.
16. **Levitzki, A., Stallcup, W. B., and Koshland, D. E., Jr.,** Half-of-the-sites reactivity and the conformational states of cytidine triphosphate synthetase, *Biochemistry,* 10, 3371, 1971.
17. **Levitzki, A. and Koshland, D. E., Jr.,** Role of an allosteric effector. Guanosine triphosphate activation in cytosine triphosphate synthetase, *Biochemistry,* 11, 241, 1972.
18. **Levitzki, A. and Koshland, D. E., Jr.,** Negative cooperativity in regulatory enzymes, *Proc. Natl. Acad. Sci. U.S.A.,* 62, 1121, 1969.
19. **Levitzki, A. and Koshland, D. E., Jr.,** Ligand-induced dimer-to-tetramer transformation in cytosine triphosphate synthetase, *Biochemistry,* 11, 247, 1972.
20. **Umbarger, H. E.,** Regulation of amino acid metabolism, *Annu. Rev. Biochem.,* 38, 323, 1969.
21. **Truffa-Bachi, P. and Cohen, G. N.,** Some aspects of amino acid biosynthesis in microorganisms, *Annu. Rev. Biochem.,* 37, 79, 1968.
22. **Truffa-Bachi, P. and Cohen, G. N.,** Amino acid metabolism, *Annu. Rev. Biochem.,* 42, 113, 1973.
23. **Janin, J., Truffa-Bachi, P., and Cohen, G. N.,** Subunits of the complex protein carrying the threonine-sensitive aspartokinase activity in a mutant of *Escherichia coli* K-12, *Biochem. Biophys. Res. Commun.,* 26, 429, 1967.
24. **Cunningham, G. N., Maul, S. B., and Shive, W.,** Multiple interactions of threonine with an aspartokinase-homoserine dehydrogenase complex, *Biochem. Biophys. Res. Commun.,* 30, 159, 1968.
25. **Wampler, D. E., Takahaski, M., and Westhead, E. W.,** Active subunits of the aspartokinase-homoserine dehydrogenase I complex from *Escherichia coli, Biochemistry,* 9, 4210, 1970.
26. **Wampler, D. E.,** Threonine-sensitive aspartokinase-homoserine dehydrogenase complex from *Escherichia coli.* Subunit stoichiometry and size of the catalytic unit, *Biochemistry,* 11, 4428, 1972.
27. **Roark, D. and Yphantis, D.,** Studies on self-associating systems by equilibrium ultracentrifugation, *Ann. N.Y. Acad. Sci.,* 164, 245, 1969.
28. **Starnes, W. L., Munk, P., Maul, S. B., Cunningham, G. N., Cox, D. L., and Shive, W.,** Threonine-sensitive aspartokinase-homoserine dehydrogenase complex, amino acid composition, molecular weight and subunit composition of the complex, *Biochemistry,* 11, 677, 1972.
29. **Cohen, R. and Mire, M.,** Analytical-band centrifugation of an active enzyme-substrate complex. 1. Principal and practice of the centrifugation, *Eur. J. Biochem.,* 23, 267, 1971.
30. **Mackall, J. C. and Neet, K. E.,** Variation of the size of the reacting form of *Escherichia coli* K-12 threonine-sensitive aspartokinase-homoserine dehydrogenase with pH and effectors, *Biochemistry,* 12, 3483, 1973.

31. **Datta, P., Gest, H., and Segal, H. L.,** Effects of feedback modifiers on the state of aggregation of homoserine dehydrogenase of *Rhodospirillum rubrum, Proc. Natl. Acad. Sci. U.S.A.,* 51, 125, 1964.
32. **Mankovitz, R. and Segal, H. L.,** Dissociation-produced loss of regulatory control of homoserine dehydrogenase of *Rhodospirillum rubrum.* I. Factors which affect the interconversion of the regulatable and nonregulatable states, *Biochemistry,* 8, 3757, 1969.
33. **Mankovitz, R. and Segal, H. L.,** Dissociation-produced loss of regulatory control of homoserine dehydrogenase of *Rhodospirillum rubrum.* II. Some properties of the regulatable and nonregulatable forms, *Biochemistry,* 8, 3765, 1969.
34. **Dungan, S. M. and Datta, P.,** Concerted feedback inhibition. Modifier induced oligomerization of the *Pseudomonas fluorescens* aspartokinase, *J. Biol. Chem.,* 248, 8541, 1973.
35. **Tomkins, G. M., Yielding, K. L., and Curran, J.,** Steroid hormone activation of L-alanine oxidation catalyzed by a subunit of crystalline glutamic dehydrogenase, *Proc. Natl. Acad. Sci. U.S.A.,* 47, 270, 1961.
36. **Fisher, H. F., Cross, D. G., and McGregor, L. L.,** The independence of the substrate specificity of glutamate dehydrogenase on its state of aggregation, *Biochim. Biophys. Acta,* 99, 165, 1965.
37. **Fisher, H. F.,** Glutamate dehydrogenase-ligand complexes and their relationship to the mechanism of the reaction, in *Advances in Enzymology,* Vol. 39, Meister, A., Ed., John Wiley & Sons, New York, 1973, 369.
38. **Frieden, C.,** Glutamate dehydrogenase. V. The relation of enzyme structure to the catalytic function, *J. Biol. Chem.,* 238, 3286, 1963.
39. **Fisher, H. F., Gates, R. E., and Cross, D. G.,** A ligand exclusion theory of allosteric effects, *Nature,* 228, 247, 1970.
40. **Fisher, H. F., Cross, D. G., and McGregor, L. L.,** Catalytic activity of subunits of glutamic dehydrogenase, *Nature,* 196, 895, 1962.
41. **Frieden, C. and Colman, R. F.,** Glutamate dehydrogenase concentration as a determinant in the effect of purine nucleotides on enzymatic activity, *J. Biol. Chem.,* 242, 1705, 1967.
42. **Huang, C. Y. and Frieden, C.,** The mechanism of ligand-induced structural changes in glutamate dehydrogenase, *J. Biol. Chem.,* 247, 3638, 1972.
43. **Huang, C. Y. and Frieden, C.,** Rates of GDP-induced and GTP-induced depolymerization of glutamate dehydrogenase: a possible factor in metabolic regulation, *Proc. Natl. Acad. Sci. U.S.A.,* 64, 338, 1969.
44. **Umbarger, H. E.,** Threonine deaminases, in *Advances in Enzymology,* Vol. 37, Meister, A., Ed., John Wiley & Sons, New York, 1973, 349.
45. **Kagan, Z. S., Sinelnikova, E. M., and Kretovich, W. L.,** L-threonine dehydratases of flowering parasitic and saprophytic plants, *Enzymologia,* 36, 335, 1969.
46. **Calhoun, D. H., Rimerman, R. A., and Hatfield, G. W.,** Threonine deaminase from *Escherichia coli.* I. Purification and properties, *J. Biol. Chem.,* 248, 3511, 1973.
47. **Wood, W. A.,** Allosteric L-threonine dehydrases of microorganisms, in *Current Topics in Cellular Regulation,* Vol. 1, Horecker, B. L. and Stadtman, E. R., Eds., Academic Press, New York, 1969, 161.
48. **Phillips, A. T. and Wood, W. A.,** Basis for AMP activation of biodegradative threonine dehydrase from *Escherichia coli, Biochem. Biophys. Res. Commun.,* 15, 530, 1964.
49. **Whanger, P. D., Phillips, A. T., Rabinowitz, K. W., Piperno, J. R., Shada, J. D., and Wood, W. A.,** The mechanism of action of 5′-adenylic acid-activated threonine dehydrase. II. Protomer-oligomer interconversions and related properties, *J. Biol. Chem.,* 243, 167, 1968.
50. **Hirata, M., Tokushige, M., Inagaki, A., and Hayaishi, O.,** Nucleotide activation of threonine deaminase from *Escherichia coli, J. Biol. Chem.,* 240, 1711, 1965.
51. **Dunne, C. P., Gerlt, J. A., Rabinowitz, K. W., and Wood, W. A.,** The mechanism of action of 5′-adenylic acid-activation of threonine dehydrase. IV. Characterization of kinetic effect of adenosine monophosphate, *J. Biol. Chem.,* 248, 8189, 1973.
52. **Nakazawa, A., Tokushige, M., Hayaishi, O., Ikehara, M., and Mizuno, Y.,** Studies on the interaction between regulatory enzymes and effectors. I. Effect of adenosine-5′-monophosphate analogues on threonine deaminase, *J. Biol. Chem.,* 242, 3868, 1967.
53. **Rabinowitz, K. W., Shada, J. D., and Wood, W. A.,** The mechanism of action of 5′-adenylic acid-activated threonine dehydrase. III. Structural requirements for nucleotide allosteric activation, *J. Biol. Chem.,* 243, 3214, 1968.
54. **Shizuta, Y., Nakazawa, A., Tokushige, M., and Hayaishi, O.,** Studies on the interaction between regulatory enzymes and effectors. III. Crystallization and characterization of adenosine-5′-monophosphate-dependent threonine deaminase from *Escherichia coli, J. Biol. Chem.,* 244, 1883, 1969.
55. **Gerlt, J. A., Rabinowitz, K. W., Dunne, C. P., and Wood, W. A.,** The mechanism of action of 5′-adenylic acid-activated threonine dehydrase. V. Relation between ligand-induced allosteric activation and the protomer-oligomer interconversion, *J. Biol. Chem.,* 248, 8200, 1973.
56. **Rabinowitz, K. W., Niederman, R. A., and Wood, W. A.,** The mechanism of action of 5′-adenylic acid-activated threonine dehydrase. VI. Identification of the partial reaction activated by adenosine monophosphate, *J. Biol. Chem.,* 248, 8207, 1973.
57. **Shizuta, Y., Kurosawa, A., Tanabe, T., Inoue, K., and Hayaishi, O.,** Regulation of biodegradative threonine deaminase. II. Reaction mechanism as analyzed by spectrophotometric properties, *J. Biol. Chem.,* 248, 2413, 1973.

58. Nichol, L. W., Jackson, W. J. H., and Winzor, D. J., A theoretical study of the binding of small molecules to a polymerizing protein system. A model for allosteric effects, *Biochemistry*, 6, 2449, 1967.
59. Whiteley, H. R., Threonine deaminase of *Clostridium tetanomorphum*. II. Dissociation to subunits, *J. Biol. Chem.*, 241, 4890, 1966.
60. Simon, J. P., Schorr, J. M., and Phillips, A. T., Consideration of the quaternary structure of the ADP-activated threonine dehydratase, *Fed. Proc.*, 30, 1057, 1971.
61. Vanquickenborne, A. and Phillips, A. T., Purification and regulatory properties of the adenosine diphosphate-activated threonine dehydratase, *J. Biol. Chem.*, 243, 1312, 1968.
62. Simon, J. P., Schorr, J. M., and Phillips, A. T., Interactions of 5'-adenosine diphosphate and L-threonine with the quaternary structure of L-threonine dehydratase. I. Nature of the enzymic substructure, *J. Biol. Chem.*, 249, 1993, 1974.
63. Simon, J. P., Ph.D. dissertation, Pennsylvania State University, 1974.
64. Whiteley, H. R. and Tahara, M., Threonine deaminase of *Clostridium tetanomorphum*. I. Purification and properties, *J. Biol. Chem.*, 241, 4881, 1966.
65. Nakazawa, A. and Hayaishi, O., On the mechanism of activation of L-threonine deaminase from *Clostridium tetanomorphum* by adenosine diphosphate, *J. Biol. Chem.*, 242, 1146, 1967.
66. Schorr, J. M., Ph.D. dissertation, Pennsylvania State University, 1973.
67. Ackers, G. K., Molecular sieve studies of interacting protein systems. II. Enumeration of components and determination of molecular size distributions, *J. Biol. Chem.*, 243, 2056, 1968.
68. Godschalk, W., Enumeration of species in a self-associating protein system by diffusion measurements in the ultracentrifuge, *Biochemistry*, 10, 3284, 1971.
69. Falcoz-Kelly, F., Janin, J., Saari, J. C., Véron, M., Truffa-Bachi, P., and Cohen, G. N., Revised structure of aspartokinase I-homoserine dehydrogenase I of *Escherichia coli* K-12. Evidence for four identical subunits, *Eur. J. Biochem.*, 28, 507, 1972.
70. Véron, M., Falcoz-Kelly, F., and Cohen, G. N., The threonine-sensitive homoserine dehydrogenase and aspartokinase activities of *Escherichia coli* K-12. The two catalytic activities are carried by two independent regions on the polypeptide chain, *Eur. J. Biochem.*, 28, 520, 1972.
71. Cohen, G. N., Patte, J.-C., and Truffa-Bachi, P., Parallel modifications caused by mutations in two enzymes concerned with the biosynthesis of threonine in *Escherichia coli*, *Biochem. Biophys. Res. Commun.*, 19, 546, 1965.
72. Leary, T. R. and Kohlhaw, G. B., α-Isopropylmalate synthase from *Salmonella typhimurium*. Analysis of the quaternary structure and its relation to function, *J. Biol. Chem.*, 247, 1089, 1972.
73. Moss, J. and Lane, M. D., Acetyl coenzyme A carboxylase. III. Further studies on the relation of catalytic activity to polymeric state, *J. Biol. Chem.*, 247, 4944, 1972.
74. Haberland, M. E., Willard, J. M., and Wood, H. G., Phosphoenolpyruvate carboxytransphosphorylase. Study of the catalytic and physical structures, *Biochemistry*, 11, 712, 1972.
75. Baskerville, E. and Twarog, R., Regulation of a ligand-mediated association-dissociation system of anthranilate synthesis in *Clostridium butyricium*, *J. Bacteriol.*, 117, 1184, 1974.
76. Louie, D. D., Kaplan, N. O., and McLean, J. D., Allosteric effect of 2'-adenylic acid on the *Pseudomonas* pyridine nucleotide transhydrogenase, *J. Mol. Biol.*, 70, 651, 1972.
77. Hoagland, V. D., Jr. and Teller, D. C., Influence of substrates on the dissociation of rabbit muscle D-glyceraldehyde 3-phosphate dehydrogenase, *Biochemistry*, 8, 594, 1969.
78. Cassman, M. and King, R. C., Subunit interactions and ligand binding in supernatant malic dehydrogenase. Cooperative binding of reduced nicotinamide adenine dinucleotide associated with a monomer-dimer equilibrium of the protein, *Biochemistry*, 11, 4937, 1972.
79. Iwatsuki, N. and Okazaki, R., Mechanism of regulation of deoxythymidine kinase of *Escherichia coli*. I. Effect of regulatory deoxynucleotides on the state of aggregation of the enzyme, *J. Mol. Biol.*, 29, 139, 1967.
80. Huang, C. Y. and Graves, D. J., Correlation between subunit interactions and enzymatic activity of phosphorylase a. Method for determining equilibrium constants from initial rate measurements, *Biochemistry*, 9, 660, 1970.
81. DeVincenzi, D. L. and Hedrick, J. L., Gel filtration, aggregation and the enzymatic activity of glycogen phosphorylase, *Biochemistry*, 9, 2048, 1970.
82. Tu, J.-I. and Graves, D. J., Association-dissociation properties of sodium borohydride-reduced phosphorylase b, *J. Biol. Chem.*, 248, 4617, 1973.
83. Bonsignore, A., Cancedda, R., Lorenzoni, I., Cosulich, M. E., and DeFlora, A., Human erythrocyte glucose-6-phosphate dehydrogenase. Physical properties, *Biochem. Biophys. Res. Commun.*, 43, 94, 1971.
84. Plaut, G. W. E., DPN-linked isocitrate dehydrogenase of animal tissues, in *Current Topics in Cellular Regulation*, Vol. 2, Horecker, B. L. and Stadtman, E. R., Eds., Academic Press, New York, 1970, 1.
85. Paetkau, V. and Lardy, H. A., Phosphofructokinase correlation of physical and enzymatic properties, *J. Biol. Chem.*, 242, 2035, 1967.
86. Maley, F. and Maley, G. F., The regulatory influence of allosteric effectors on deoxycytidylate deaminases, in *Current Topics in Cellular Regulation*, Vol. 5, Horecker, B. L. and Stadtman, E. R., Eds., Academic Press, New York, 1972, 177.