METABOLIC INHIBITORS

A COMPREHENSIVE TREATISE

edited by

R. M. Hochster
Morris Kates
J. H. Quastel

volume IV

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Metabolic Inhibitors

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Contents

CHAPTER 3

Inhibitors of Amino Acid Activation

ROBERT B. LOFTFIELD

CHAPTER 4

Inhibitors of Protein Synthesis

C. T. CASKEY

CHAPTER 5

Inhibition of Photosynthesis

N. E. GOOD and S. IZAWA

CHAPTER 6

Inhibitors of Blood-Clotting Mechanisms

PETER G. BARTON and E. THYE YIN

CHAPTER 7

Inhibitors of Fatty Acid Oxidation and the Pathway of Fatty Acid Biosynthesis from Glucose

I. B. FRITZ and M. HALPERIN

CHAPTER 8

Inhibitors of Enzymes of Phospholipid and Sphingolipid Metabolism

SHIMON GATT

CHAPTER 9

Inhibitors and Activators of Enzymes Regulating the Cellular Concentration of Cyclic AMP

SHAIL K. SHARMA

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Preface

Volume IV of this treatise continues the coverage begun in Volume III of recent developments in studies of inhibition of metabolic and enzymic processes. Articles on inhibition of photosynthesis, blood clotting, protein synthesis, fatty acid metabolism, and phospholipid metabolism are included as well as on inhibition of specific enzyme reactions such as amino acid activation, amino acid hydroxylation, and cyclic AMP formation. This volume also contains a contribution dealing specifically with allosteric inhibition and allosteric inhibitors.

It is inevitable that some overlapping of subject matter will be found in Volume IV, redundancy is difficult to avoid or rectify in a treatise such as this. However, the reader will probably benefit from any inadvertent repetition since coverage of similar topics from different points of view is bound to make for a deeper understanding of the subject.

Much of the burden of preliminary organization of the articles was carried by our coeditor Rolf Hochster; we dedicate this volume to his memory.

Our sincere thanks are due the contributors for their efforts in preparing their articles and for their patience in the face of the unavoidable delays encountered. We are grateful to the many authors and publishers who gave permission to use previously published material and to the staff of Academic Press for their fine spirit of cooperation during production of this volume. Mr. Paul Deroo again prepared the Subject Index for which we express our sincere appreciation.

Morris Kates
J. H. Quastel

Contents of Other Volumes

Volume I

Volume II

CHAPTER 1

Allosteric Inhibitors

R. W. Miller

I. INTRODUCTION[1]

Feedback inhibition has become a well-established concept in the search for metabolic control mechanisms that regulate the rate of metabolite synthesis. The discovery that end products of biosynthetic pathways may inhibit directly the initial reactions of the pathway provides an attractive explanation for the precise metabolic homeostasis that is essential in all organisms. However, direct *in vivo* observations of enzymic feedback phenomena generally are not possible due to problems of transport of metabolites across membranes and the presence of other regulatory processes such as enzyme repression. Therefore, the study of the effects of inhibitors on the quaternary structure, tertiary structure, catalytic activity, and affinity for substrates of purified enzymes has produced a great deal of information as to how inhibitors may modulate the rates of catalyzed processes.

A particularly significant type of enzyme inhibition has been examined intensively in recent years and has emerged as a plausible mechanism for the general hypothesis of feedback regulation of metabolic transformations. Enzymes that display anomalous order (higher than one) with respect to a subtrate or coenzyme produce sigmoidal plots of reaction velocity versus ligand concentrations. Inhibitors are often observed to increase the order of the reaction with respect to substrate or to give inhibition patterns that lead to a change in the V_{max}, K_m (substrate concentration which gives half-maximal reaction velocity, referred to as $S_{0.5}$), or other parameters that describe binding and rates of catalysis by enzymes. Inhibitors that bind at a site remote from the catalytic site and produce effects of this type are referred to as allosteric inhibitors or negative effectors as opposed to compounds that modify, sterically block, bind at, or otherwise interfere directly with the catalytic site. Depending on the type of chemical transformation inhibited (i.e., single- or multiple-substrate reactions), characteristic and diagnostic kinetic data are obtained which may lead to detailed physical and chemical studies. These combined methods have greatly increased knowledge of regulatory phenomena. In this area it is somewhat artificial to distinguish between allosteric inhibitors and allosteric activators since similar mechanisms have been proposed (*1*) for both. However, since

[1] Standard abbreviations are used for purine and pyrimidine nucleotides. NAD and NADP are used for nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate, respectively.

allosteric inhibitors account for many cases of feedback regulation this chapter will emphasize the former. Table I lists allosteric inhibitors and the enzymes they may control. It is apparent that by far the largest number of compounds represented are purine and pyrimidine nucleotides. Indeed, Atkinson (*2, 3*) has proposed that many enzymes respond to the relative concentrations of adenine nucleotides (AMP, ADP, ATP, cyclic 3′,5′-AMP) rather than to the concentration of a single inhibitor or activator.

Many enzymes have more than one negative effector. While there are many well-known examples of feedback inhibition listed in Table I, there is no apparent limitation of allosteric inhibition to end products of metabolic pathways. Inorganic anions and cations, antibiotics, complex carbohydrates, and other seemingly unrelated compounds can bind at remote receptor sites and cause allosteric effects which, while they may represent control mechanisms in the general sense, are not associated with feedback control of metabolism. However, metabolic regulation is clearly an extremely complex and subtle process, and relationships between biosynthetic reactions and their allosteric inhibitors are not necessarily always as obvious as end-product inhibition.

II. LIMITING MODELS FOR ALLOSTERIC INHIBITION

Several limiting mechanistic models have been proposed to explain the behavior of allosteric enzymes. The models attempt to account for the sigmoidal shape of plots of reaction velocity (or fractional binding-site saturation) against substrate or effector concentration. Data of this type have generally suggested cooperative interactions between subunits of an oligomeric protein where the binding of effector molecules at a regulatory site may change the binding constant for substrate (K-type system) or may influence the activity of the catalytic site (V-type system) or both. Curve 2 of Fig. 1 (*4*) shows cooperative behavior for a well-documented allosteric enzyme, aspartate transcarbamylase. This enzyme catalyzes the first step in pyrimidine biosynthesis and is inhibited by the end product of that pathway, cytidine triphosphate. Cytidine triphosphate is a true allosteric inhibitor; that is, it decreases the reaction rate at low substrate concentrations by increasing the dependence of the rate on the concentration of substrate molecules.

Control curve 2 of Fig. 1 shows a dependence on substrate concentration that is anomalous since ordinary substrate dependence would

give a typical hyperbolic curve. An effector molecule could modify this cooperative effect so as to produce more or less cooperativity. The negative effector CTP increases the order of the dependence of activity on substrate concentration in the case of aspartate transcarbamylase (curve 3). The cooperative interactions associated with molecules of the same ligand are designated homotropic, while interactions involving the inhibitor (CTP) or the activator (AMP) and the substrate are heterotropic. The allosteric activator AMP changes the curve to a rectangular hyperbola which is the response expected in the absence of substrate-induced cooperative interactions. Heating the enzyme also eliminates cooperativity (Curve 1, Fig. 1). With the native enzyme the rate, in the absence of ligands other than substrate, is slower than expected from a normal hyperbolic response. This effect suggests that the substrate must

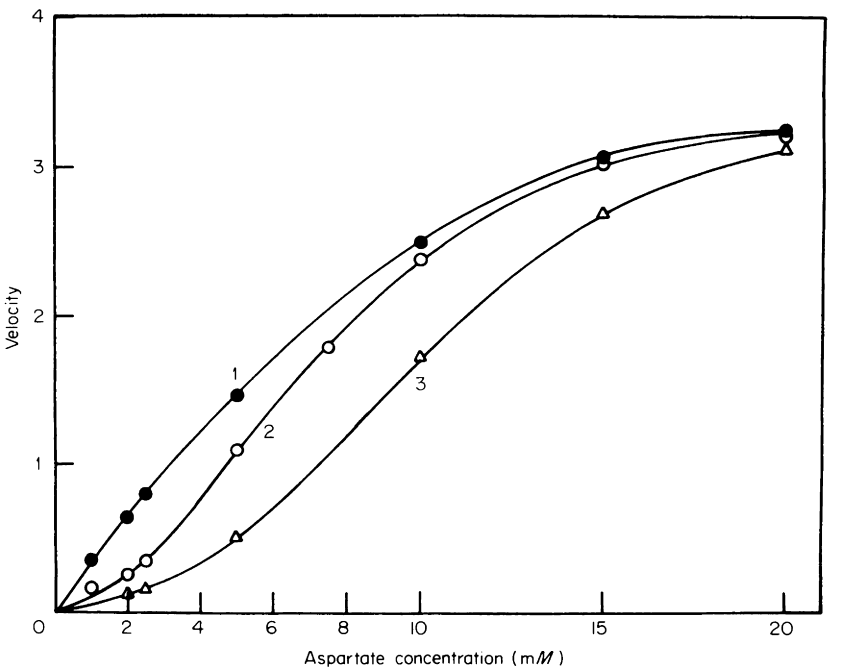

FIG. 1. Dependence of reaction rate on aspartate concentration at pH 7.0. Velocity expressed as units of activity per milligram of protein $\times 10^{-3}$. Legend: ○, native enzyme; ●, heated enzyme; and △, native enzyme in the presence of 0.1 m*M* CTP. Assay mixtures contained 0.05 *M* tris at pH 7.0, 3.3 m*M* carbamyl phosphate, asparatate as indicated, and 0.75 μg enzyme protein/ml. [Reprinted from Weitzman and Wilson (*4*) by permission of the copyright owner. Copyright (1966) by the American Society of Biological Chemists.]

convert the enzyme to a more active form for maximum reaction velocity to occur and is designated positive homotropic cooperativity. If in the presence of an activator the rate of reaction at low substrate concentration is greater than that expected from a hyperbolic relationship, the implication is that increased substrate concentrations convert the enzyme to a less active form. This type of interaction is labeled negative homotropism.

Since both types of behavior are reported for some enzymes it is important to diagnose the type of cooperativity present in a given system. Also, some models allow only positive homotropic cooperativity and kinetic analysis enables the investigator to distinguish between models. Allosteric inhibitors lead to more positive homotropic cooperativity. In this chapter inhibitors falling in the category of effectors that change cooperativity between substrate molecules by binding at a site remote from the catalytic center will be considered mainly. Inhibitors such as reaction products that bind or interfere directly at the catalytic site, some of which also may be described as feedback inhibitors, will not be considered. Also, noncompetitive inhibition (i.e., change of V_{max}), which is often observed with multisubstrate enzymes, will not be discussed unless evidence for interaction of the inhibitor at a separate allosteric site or for an effect on the cooperative behavior of one of the substrates is available. Noncompetitive inhibition can result from direct interaction with the active site in multisubstrate cases. Just as some allosteric activators may produce negative homotropic interactions for substrate molecules, allosteric inhibitor molecules may exhibit negative or positive homotropic interactions depending on whether increasing the concentration of the inhibitor produces more or less drop in activity than expected from a normal hyperbolic inhibitor response. Again, diagnosis of the type of cooperativity for the inhibitor being observed is important in ascribing kinetic phenomena to an appropriate mechanistic model.

Allosteric inhibition may be competitive, in the sense that it is completely overcome at high substrate concentrations (pure K-type system), or partially noncompetitive. Lineweaver–Burk plots obtained with allosteric enzymes may be straight lines but are generally curved [see Fig. 2 (*5*)]. Thus, in the presence or absence of heterotropic ligands a hyperbolic response of reaction velocity to substrate concentration yields a straight Lineweaver–Burk plot, while positive homotropic interactions give an upward curvature and negative homotropism causes a downward curvature. Mahler and Cordes (*5a*) and Farago and Denes (*5b*) give further graphical information that is useful in identifying the different types of cooperative interactions.

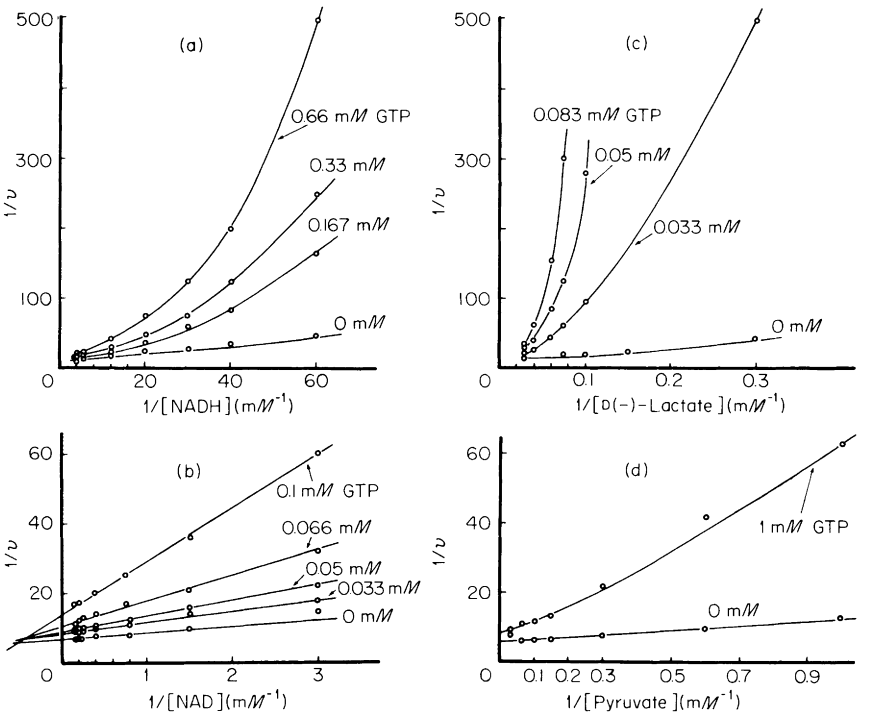

FIG. 2. Inhibition of *Pythiam* D(—)-lactate dehydrogenase by GTP. (a) Cooperative binding of NADH in the presence of GTP; (b) noncompetitive inhibition by GTP with respect to NAD as varied ligand; (c) cooperative binding of lactate in the presence of GTP; (d) noncompetitive inhibition by GTP with respect to pyruvate as varied ligand. The concentration of enzyme used in experiments (a) and (d) was 3 μg and in experiments (b) and (c), 12 μg. Concentration of GTP as specified in the figures. [Reprinted from LéJohn (*5*) by permission of the copyright owner. Copyright (1971) by the American Society of Biological Chemists.]

The cooperative binding of ligands to a protein such as hemoglobin is described by the empirical Hill equation, which relates the fractional saturation of binding sites to the ligand concentration and the Hill coefficient n. A commonly used method for assessing cooperativity from enzyme kinetic data employs a modified form of the Hill equation where the reaction velocity v is given by

$$v = V_{max}S^n/K + S^n \quad (1)$$

$$\log (v/V_{max} - v) = n \log (S) - \log (K) \quad (2a)$$

$$\log (v_i/V_{max} - v_i) = \log (K') - n' \log (I) \quad (2b)$$

Here, V_{max} is the maximum reaction velocity at infinite substrate concentration in the absence of inhibitors, and S is the substrate concentration. Reaction order is designated by the Hill coefficient n while K is a constant. Many sigmoidal curves yield straight lines when replotted by this method [Eq. (2a)]. The coefficient n is the slope of the line obtained when the log of the kinetic parameter is plotted as shown in Fig. 3 (*5c, 6*). Under certain conditions n could be interpreted as being equal to the number of cooperative binding sites. However, this assumption is theoretically sound only when the total number of effector sites on an enzyme molecule is large relative to the number that actually bind effectors under a given set of conditions (*7*). Since this prerequisite is seldom fulfilled, n is often not as large as the number of binding sites. In the case of aspartate transcarbamylase the number of substrate binding subunits was estimated to be four while later evidence obtained by complete dissociation (*8, 9*) showed that there were six.

The Hill equation may also be used to plot data obtained with negative effectors when substrate concentration is held constant at a saturating level and inhibitor concentration (I) is varied. In this case negative slopes are obtained [Eq. (2b)], but the magnitude of n remains greater than one for systems in which there is positive cooperativity between

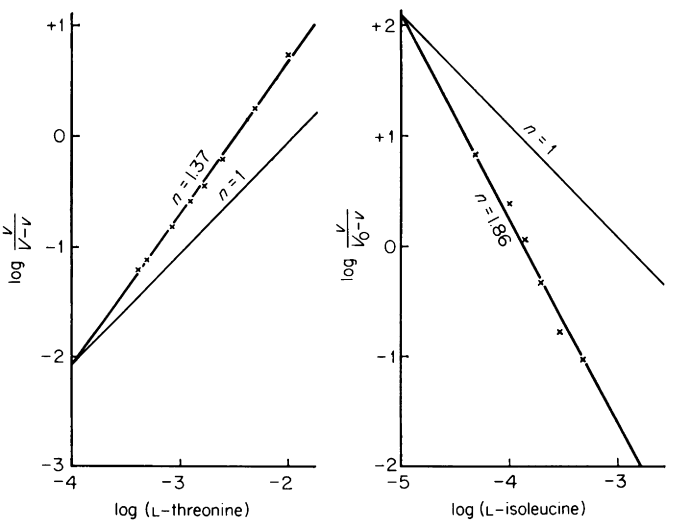

FIG. 3. L-Threonine deaminase activity as a function of the concentration of substrate (left) and allosteric inhibitor (right). [Reprinted from Monod *et al.* (*5c*) by permission of the copyright owner. Copyright 1963 by Academic Press.]

inhibitor molecules. Values of n less than one again would indicate negative homotropic cooperativity for the inhibitor.

Ligand binding functions may also be described by equations of the form

$$\frac{1}{y} = \frac{c + dS + eS^2}{aS + bS^2} \tag{3}$$

where y is the fractional saturation of binding sites and S is the free-ligand concentration (*10*). Reciprocal plots of $1/y$ against $1/S$ are curved upward in cases where positive homotropic cooperativity is present. The shape of the reciprocal plot is given by the sign of the second derivative (*10*),

$$\frac{d^2(1/y)}{d(1/S)^2} = 2FS^3/a + bS^3 \tag{4}$$

where $$F = a^2e + b^2c - abd$$

which is determined by the sign of the factor F, a combination of constants. If F is negative, downward curvature of plots is present and negative homotropic effects are indicated. Some site interaction hypotheses are symmetrical and allow negative values of the second derivative while others do not. An analysis of this type was applied to glutamate dehydrogenase where the coenzyme NAD gives downward-curved reciprocal saturation plots, indicating that the degree of saturation at low ligand concentration is higher than would be expected from a normal hyperbolic saturation phenomenon (*10*). Another interpretation of this negative homotropic process would be an apparent activation of the enzyme at high cofactor concentration. However, the rate of change of saturation or reaction velocity may be higher than expected throughout the entire concentration range.

In one type of model system the binding of effector molecules at specific regulatory sites is postulated to alter the observed rate of the catalyzed reaction through concerted or stepwise changes in three-dimensional structure and transitions between conformational states. Such changes may or may not be reflected in the state of aggregation of the subunits. The models generally are applied to enzymes that exhibit cooperative interactions between the subunit protomers. Ligands thus alter the interactions between the subunit peptide chains of an oligomeric enzyme. A second type of mechanism accounts for apparent cooperative effects through combinations of different reaction pathways (*11*) or as time dependence of enzyme activity (*12*). In theory these effects may be observed even with single-site enzymes.

A. Models Requiring Changes in Conformational State

Monod *et al.* (*1*) have proposed that data indicating cooperative effects fit a model that requires an existing equilibrium between two conformational states of an enzyme. This equilibrium may be shifted by binding of allosteric ligands to either form. The R state may represent the reactive form of the enzyme. A ligand that binds preferentially to the T state thus inhibits the catalyzed reaction by shifting this equilibrium to the right. A positive effector (substrate or activator) shifts the equilibrium to the active form by preferentially binding to the R state,

$$R \rightleftharpoons T$$

The protomers of the oligomeric enzyme may be identical and bind only substrates, or they may be nonidentical and have specific binding sites for substrate and negative or positive effectors. When a bound ligand molecule enhances the affinity of binding of further molecules of the same ligand, cooperativity is said to be positive homotropic, as mentioned previously. When a nonidentical ligand (effector) changes the affinity of binding of another ligand, the interaction is said to be heterotropic and may be positive or negative. Heterotropic interactions (synergistic effects) may be observed between two different ligands which are both inhibitors. Molecules of a single allosteric inhibitor would be expected to show homotropic interactions in bringing about a physical change in the quaternary structure of the enzyme. Some instances of product inhibition appear to be due to an allosteric effect. In this case the phenomenon still would be termed a heterotropic negative interaction, since the inhibitor is nonidentical with the starting substrates.

In the model of Monod *et al.* the transitions between the R and T forms are required to be concerted; that is, for a given enzyme molecule, the binding of an effector molecule encourages the transition in all of the protomers to take place without the formation of stable hybrid states containing mixtures of protomers in the R and T states. The concerted model does not allow negative homotropic interactions. The effectors may exert their influence through weak or noncovalent forces such as electronic forces or conceivably could even bind covalently where the binding reaction is enzyme catalyzed and equilibration is therefore rapid. Binding may cause small physical displacements in the positions of key amino acid residues which shift the equilibrium between the conformational states. Actual details of structural transitions are known in only a very few cases, although allosteric interactions often correlate with

alterations in bonding between the subunit protomers. In the original theory, the authors of this model considered only exclusive binding of effectors (*1*) in discussing heterotropic effects including negative interactions. That is, the effector ligand would bind to only one of the states. General nonexclusive binding (binding of heterotropic effectors to both states with unequal affinities) was later considered (*13*). This modification limits the extent to which the equilibrium may be shifted by effectors. The equilibrium would be pulled to completion in the case of exclusive binding in the presence of excess effector. Nonexclusive binding of effectors considerably extends the range of kinetic data that may be accommodated by this hypothesis. The postulation of a single conformational equilibrium is, of course, a limiting case. The presence of several concerted transitional equilibria in an enzyme system would be more complex but probably would be expected in most allosteric enzymes.

Koshland *et al.* (*14*) have proposed a different binding model to account for allosteric effects. According to the induced-fit hypothesis, effectors (negative or positive) may induce stepwise conformational changes in the enzyme protein which can in turn cause higher- or lower-affinity binding for further molecules of substrate. This model allows nonexclusive binding as well as hybrid, i.e. partially changed, states and also allows negative homotropic cooperativity (*14a*). The essential differences between these related models are illustrated in Fig. 4 (*15*).

B. Models Postulating Multiple Reaction Pathways or Time-Dependent Enzyme Activity (Transient Forms)

It has been shown that model mechanisms requiring a single, independent active site can account for nonhyperbolic kinetic data if there are multiple pathways leading to the binding of substrate molecules to the single active site (*11*), as shown in Fig. 5A. Therefore, multiple binding sites or subunit interactions are not required in some cases in order to explain anomalous kinetic order.

A general model for a two-substrate (G, P), two-product (G′, P′) enzyme-catalyzed reaction with an effector A allows multiple pathways to several enzyme–substrate complexes if association of the enzyme with substrates and products is random (not ordered) and multiple pathways are operating as shown in Fig. 5B (*16*). This scheme was applied to phosphorylase a. Rapid equilibration of substrates in the bimolecular associations with the enzyme was established by isotope exchange experi-

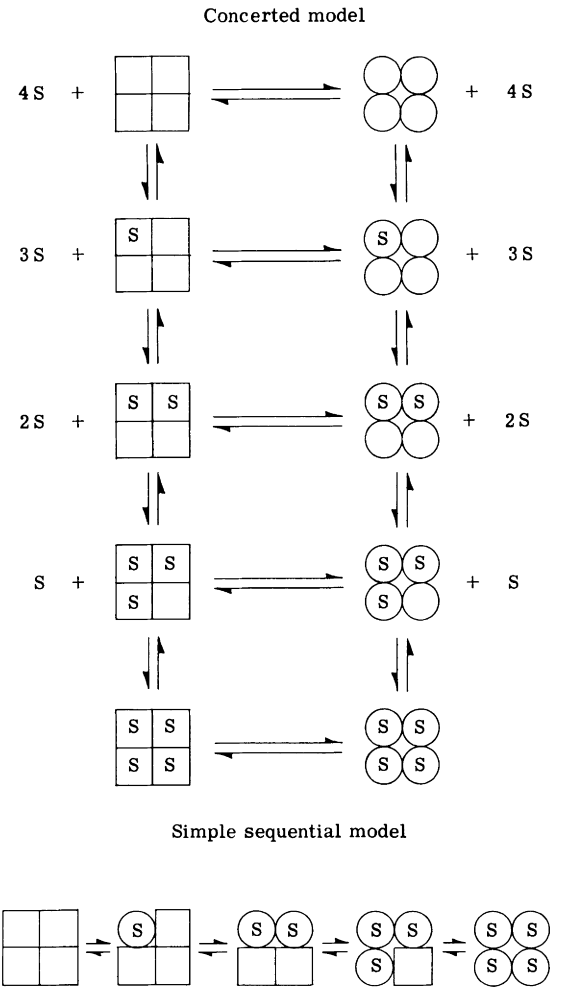

FIG. 4. Two models for allosteric inhibition that require changes in conformational states (represented by circles and boxes). [Reprinted from Hammes and Wu (*15*) by permission of the copyright owner. Copyright June, 1971, by the American Association for the Advancement of Science.]

ments. An agent that perturbed the rapid equilibration by favoring one set of complexes could produce curved Lineweaver–Burk plots even in the absence of cooperative interactions. However, in the case of phosphorylase, this does not occur and therefore cooperativity must be invoked. In the

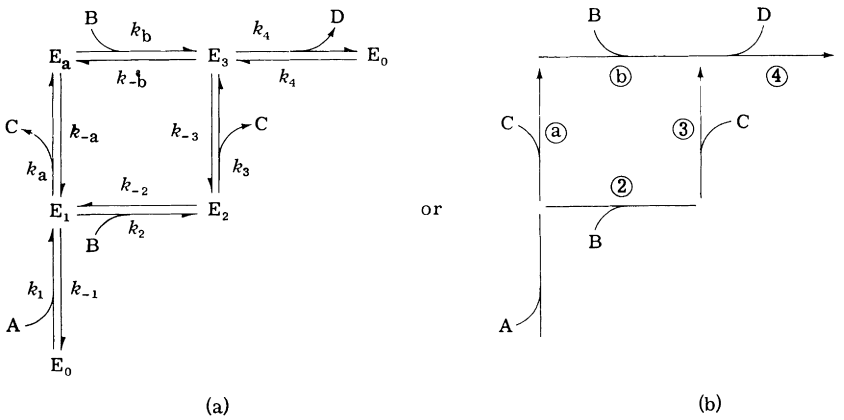

FIG. 5A. Multiple reaction pathways for an enzyme-catalyzed reaction of the type $A + B \rightleftharpoons C + D$. (a) Written in a completely reversible form, where E_0 is the free form of enzyme and E_1, E_2, E_3, and E_a are enzyme-substrate complexes or other forms of the enzymes. (b) A less complex representation with steps a, 3, and 4 considered to be irreversible because products (C and D) are omitted and initial velocities are measured. [Reprinted from Sweeney and Fisher (*11*) by permission of the copyright owner. Copyright 1968 by the American Chemical Society.]

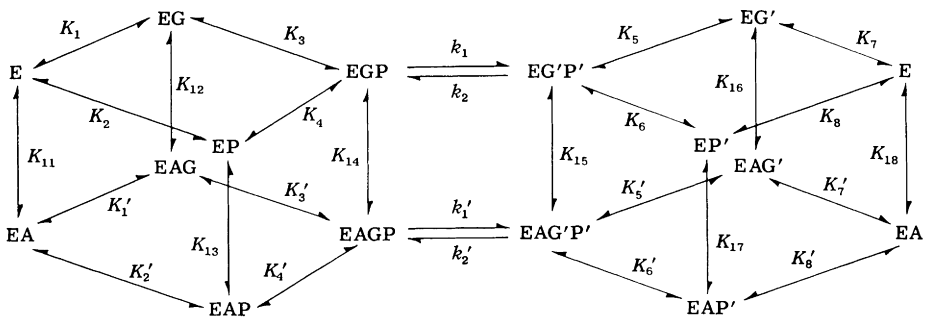

FIG. 5B. Proposed rapid-equilibrium, random kinetic mechanism for rabbit muscle phosphorylase a: A, E, G, and P are, respectively, 5-AMP, enzyme, glycogen, and P_i; G′ is glycogen with one less terminal glucose while P′ is glucose 1-phosphate. The 24 dissociation constants and four rate constants for enzyme in the presence or absence of AMP are indicated. [Reproduced from Engers *et al.* (*16*) by permission of the National Research Council of Canada from the *Canadian Journal of Biochemistry*.]

presence of the multiple equilibria which exist in multisubstrate systems, the relative magnitude of the rate constants must be determined in order to assess the importance of cooperativity in accounting for kinetic results.