Kinetic Aspects of Regulation of Metabolic Processes

THE HYSTERETIC ENZYME CONCEPT*

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SUMMARY

Hysteretic enzymes are defined as those enzymes which respond slowly (in terms of some kinetic characteristic) to a rapid change in ligand, either substrate or modifier, concentration. Such slow changes, defined in terms of their rate relative to the over-all catalytic reaction, result in a lag in the response of the enzyme to changes in the ligand level. Several mechanisms, including ligand-induced isomerization of the enzyme, displacement of tightly bound ligands by other ligands, or polymerization and depolymerization, are discussed and it can be shown that the description of the time-dependent change in enzyme activity is similar for many different cases. Examination of the literature reveals that a large number of enzymes fall into the category termed hysteretic, that such enzymes are frequently those which are important in metabolic regulation, that the time of conversion from one kinetic form to another may vary between seconds and minutes, and that there are experimental examples of all the mechanisms which are discussed theoretically. The possible relation between those enzymes which are hysteretic and regulation of complex metabolic processes is discussed in terms of the fact that the slow response of the hysteretic enzyme to changes in ligand level will lead to a time-dependent buffering of some metabolites and that this may be important with respect to pathways which utilize common intermediates or in which there are multiple branch points. It is suggested that the question of hysteresis in enzyme systems as defined here be systematically investigated in regulatory enzymes and that this concept may be of value in discussing the regulation of complex processes in vivo.

Current attempts to relate the regulation of metabolic processes to the known properties of enzyme systems are primarily centered around those characteristics attributed to allosteric or "regulatory" enzymes (1-5). Thus, schemes which propose preferential use of particular metabolic pathways under certain conditions are correlated with the unique characteristics of the enzymes which are concerned with those pathways. Those characteristics of particular importance in this type of correlation are of two types (a) the ability of specific metabolites to influence enzymatic activity by binding to specific sites distinct from the catalytically active site, and (b) the unusual (i.e. nonhyperbolic) dependence of the initial velocity on substrate of ligand concentration.

The original and elegantly simple idea of feedback inhibition, utilizing as it does both of these characteristics, demonstrates the ability to describe and understand concepts of metabolic regulation in terms of enzymatic properties and lends considerable support to the idea that much of metabolic regulation, even in complex cases, may be discussed in these relatively simple terms.

There has been, over the few years since this simple regulatory concept was presented, considerable elaboration of the basic and underlying theme of feedback inhibition. Enzymes, much more complicated than those originally pictured, have been characterized and terms such as concerted, cooperative, or cumulative inhibition, activation, multivalent inhibition, sequential (or induced fit) mechanisms have been coined to describe some of the unique kinetic properties of such proteins. Furthermore, the ideas discussed in the relationship of structure to function and which involved only conformation changes have been expanded to include systems in which different conformational forms of an enzyme do not have the same intrinsic specific activity or enzymes which undergo reversible association-dissociation reactions in which different molecular weight forms of the enzyme have different characteristics with respect to ligand binding or activity (6,7).

If there are any issues to be faulted with current approaches to the question of metabolic regulation in vivo, they are in the application of presently available equations (8-10), developed to describe ligand binding, to initial velocity kinetic data and the presumption that such equations reflect the instantaneous rate of substrate disappearance in a sequence of enzymatic steps. This presumption would be valid if all of the conformational forms of an enzyme had the same intrinsic activity and if rates involving all of the different enzyme complexes were rapid prior to that in which the product is released from the enzyme. Such an assumption may not be true and the rate of certain steps in the interconversion between enzyme-containing species may be rate determining, thus making the kinetic properties of the enzyme different from those predicted by the binding equations. So far, the attempts to include this kinetic argument have centered around questions relating to the derivation of the equation to represent a particular mechanism. Thus, the use of the "steady

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The changes discussed with respect to the enzyme systems are, of course, considered to be reversible.

Greek word appears to simply mean "to be behind, come late."

Magnetic induction of an iron bar on first increasing then decreasing the magnetic field strength, frequently represent irreversible energy losses. On the other hand, the definition of the hysteresis in physics, as exemplified by change in kinetic characteristics of the two (or more) conformational forms. Some of the concepts concerning slow and rapid conformational (or molecular weight) changes and their consequences were briefly considered previously (13). In this paper, the concept of the slow response to a rapid change in ligand concentration and the consequence of such slow changes with respect to enzymatic activity and metabolic regulation will be discussed in detail. The term "hysteretic enzyme" is introduced to refer to enzymes which respond (in terms of configurational or other changes) slowly to rapid changes in ligand concentration.

Development of Hysterisis Concept

Hysteretic Enzymes—In physics, hysteresis is defined as the time lag exhibited by a body in reacting to outside forces. By analogy, hysteresis in regulatory enzymes could be defined as the time lag exhibited in some kinetic or physical property of an enzyme in response to a rapid change in concentration of substances which influence those properties. Hysteretic enzymes are described here as those enzymes which respond slowly to rapid changes in ligand concentration. Slow and rapid, although they are relative terms, may be related for convenience to the time required to measure enzyme activity. This point will be discussed later.

That some enzymes may show this type of behavior, that is, that some enzymes undergo rather slow conformational changes, has been recognized previously. As shown later, many regulatory enzymes may exhibit such behavior, but the fact that this may have important consequences in terms of metabolic regulation has not been extensively discussed.

In general, there appear to be two mechanisms which may be primarily responsible for slow responses (a) isomerization processes and (b) displacement (under certain conditions) of a tightly bound ligand by another with a different effect on activity. A more complex process which may include either or both of these mechanisms is the polymerization or depolymerization of an enzyme.

Isomerization

In order to present the concepts relevant to the point of this paper, we shall start with simple enzyme models and develop the kinetic equations, listing along with this development the assumptions made and the reasoning for such assumptions.

**Single Substrate Case**—The simplest single substrate enzyme system is written as

\[ E + S \rightarrow ES \rightarrow E + P \]

**Mechanism I**

One can introduce more intermediates of the enzyme-substrate or enzyme-product type and make the reaction reversible. In all cases, and independent of the absolute value of any of the rate constants, the initial velocity shows a normal hyperbolic dependence on the substrate concentration. If the substrate level does not change as a function of time and the product concentration is low enough so that it does not inhibit the reaction (for example, if it is continuously removed, as may occur in a metabolic sequence involving a series of enzymatic steps), the velocity at any time is the initial velocity. This velocity \( v_i \) will also show the same substrate concentration dependence as the initial velocity (i.e. hyperbolic).

One property not exhibited by this system which appears to be inherent in regulatory enzymes is that the enzyme may exist in two or more conformational states which differ in some catalytic or binding sense. Thus, the simplest extension of Mechanism I to be considered is

\[
\begin{align*}
E + S &\rightarrow ES \\
ES &\rightarrow E + S
\end{align*}
\]

or, written in the form in which a solid line represents a reversible reaction

\[
E + S \xrightleftharpoons[k_{-2}]{k_2} E'S + S
\]

where \( L_1 \) and \( L_2 \) are equilibrium constants.

This mechanism assumes two conformational states of the enzyme, both of which can bind substrate (with different affinities). The rate constants \( k_2 \) and \( k_4 \) represent breakdown of the ES and E'S complex to give product.

**Derivation of the equation or equations which represent kinetic or binding data for this mechanism may be approached in several ways.**

If, for example, one assumes that \( k_3 = k_4 = 0 \) (i.e., if we are concerned with ligand binding only), and if the time of measurement is long relative to adjustment of equilibrium, \( \bar{P} \), the fraction of saturation, is

\[
\bar{P} = \frac{S}{L_1 + \frac{S}{L_2} \left( 1 + \frac{S}{L_3} \right)}
\]
where \( K_2 = k_s/k_{-s} \). This equation is identical to that derived by Monod, Wyman, and Changeux (8), for the case where there is only one binding site (\( n = 1 \)).

The kinetic equation which corresponds to (has the identical form as) this binding equation may be derived by assuming that \( k_1 \) and \( k_s \) are identical and that these steps are the rate-determining steps in the over-all reaction, i.e., that all steps prior to the breakdown to product are in rapid equilibrium. In this case (after rearranging)

\[
\frac{v}{E_0} = \frac{k_s}{1 + \frac{k_s}{S} \left( \frac{1 + K_1}{1 + K_1} \right)}
\]

(2)

where again \( K_1 = k_s/k_{-s} \).

If \( k_s \) and \( k_{-s} \) are not identical

\[
\frac{v}{E_0} = \frac{k_s \left( \frac{1 + k_s}{1 + k_s} \right) + \frac{L_2}{S} \left( \frac{1 + K_1}{1 + K_1} \right)}{1 + \frac{k_s}{S} \left( \frac{1 + K_1}{1 + K_1} \right)}
\]

(3)

In either case, the velocity shows a hyperbolic dependence on substrate concentration and there is no kinetically convenient way to distinguish between the two cases. The complete steady state derivation \([d(EX)/dt = 0]\) of Mechanism II gives a more complex rate expression including \( S^2 \) terms. As a consequence, it has been suggested several times that allosteric behavior may, in some cases, simply be a kinetic, rather than binding, argument (11, 12). Although this kinetic situation may arise whenever an alternate pathway for the reaction exists, there is good reason to believe that the complete steady state derivation is not the primary explanation for the kinetic abnormalities of such enzyme systems. Thus, there are a large number of conditions, including equilibration of only some of the steps, under which the more complex rate reduces to the same form of the equation derived by rapid equilibrium methods (14, 15). For example, the assumption of equilibration between substrate and enzyme leads to such a simplification. In view of the known rate constants for the step \( E + S \rightarrow ES (\sim 10^7 \text{ sec}^{-1}) \), and given reasonable binding constants, such steps may indeed equilibrate very rapidly. Simple calculations show, for example, that equilibration of binding steps may occur in less than \( 10^{-3} \text{ sec} \). But such an equilibration is not the only mechanism for obtaining the simple form of the kinetic equation. Identity of certain rate constants or ratios of rate constants will also serve this purpose (15). Although it is reasonable to expect that equilibration in binding steps may be very rapid, the conversion of one conformational form to another may be very slow.

If one assumes (a) the steps \( E + S \rightarrow ES \) and \( E' + S \rightarrow E'S \) are in rapid equilibrium, (b) that the over-all reaction is irreversible, (c) that the substrate concentration is maintained at a constant level, and (d) that there is no product inhibition, the velocity obtained will be a measurable function of time provided that the isomerization rate constants \( (k_2, k_{-2}, k_s, \text{and } k_{-s}) \) are small enough.

Under these conditions, the time dependence of the velocity for Mechanism II may be shown to be

\[
v(t) = v_f + (v_i - v_f)e^{-k't}
\]

where \( v_f \) is the velocity at time \( t = 0 \) relative to the change in conditions (at \( t = 0 \)) which initiates the conformational change (as for example addition of substrate), \( v_i \) is the velocity at \( t = 0 \) (remembering the assumption that the substrate level remains constant), \( v_0 \) is the velocity at \( t = 0 \) and \( k' \) is a complex rate constant which depends on the substrate concentration. For Mechanism II and, without making the assumption of microscopic reversibility (discussed below)

\[
k' = \frac{k_s \left( \frac{1 + k_s}{1 + k_s} \right) + \frac{L_2}{S} \left( \frac{1 + K_1}{1 + K_1} \right)}{1 + \frac{k_s}{S} \left( \frac{1 + K_1}{1 + K_1} \right)}
\]

(4)

Assuming, from the principle of microscopic reversibility, that \( k_{-s}k_sL_2 = k_{-s}k_sL_4 \), one of the rate constants can be eliminated. Thus

\[
k' = k_2 \left( \frac{1 + k_s}{k_2L_1} \right) \left( \frac{1 + \frac{S}{L_1}}{1 + \frac{S}{L_1}} \right) + \frac{L_2}{L_2 + \frac{L_2}{L_1}}
\]

(5a)

As may be seen from Equation 4, \( k' \) is easily evaluated from a plot of \( \log (v_f - v_i)/(v_i - v_f) \) versus \( t \) and is clearly dependent on \( S, L_1, L_2, \) and the other rate constants.

An example of this type of enzyme system may be taken from data involving phosphorylase \( a \). Fig. 1 shows a plot of data for the frog phosphorylase \( a \) measuring the increase in enzymatic activity during arsenolysis after adding glycerogen at zero time. The data, obtained at both 10 and 15°C, are plotted as \( \log (v_i - v_f)/(v_i - v_f) \) versus \( t \) and show reasonably good first order plots. The values of \( k' \) at 10 and 15°C are 2.3 and \( 4.8 \times 10^{-4} \text{ sec}^{-1} \), respectively, and correspond to half-times of 5.05 and 2.4 min at the two temperatures.

Equation 4 holds whether substrate is added to the enzyme alone or whether a new level of substrate is introduced into a system which already contains substrate, provided that the new substrate level remains unchanged during the time of the conversion from one form of the enzyme to another. Equation 4

\footnote{B. E. Metzger, L. Glaser, and E. Helmreich, unpublished data.}
is also independent of the relative values of \( k_3 \) and \( k_6 \) (including 0) since the total concentration of \( E + ES \) or \( E' + E'S \) is unchanged by \( ES \rightarrow E \) or \( E'S \rightarrow E \) because there is (by the previous assumptions) a rapid equilibration of these species. The rate constants \( k_1 \) and \( k_2 \) do appear, of course, in the velocity terms, \( v_O \) and \( v_F \), and it is instructive at this point to consider the substrate dependence of these velocities.

If the enzyme exists in the \( E \) form only before substrate addition, \( v_o \) is defined by

\[
\frac{v_o}{E_0} = \frac{k_2}{1 + L_2/S} \tag{6}
\]

Assuming microscopic reversibility, the final velocity, \( v_F \), is

\[
\frac{v_F}{v_o} = \frac{K_3(l + K_2 L_1 / k_2 L_2)/(l + K_2 L_1 / L_2)}{1 + L_1/S} \tag{7}
\]

as previously derived in Equation 3 for the velocity assuming rapid equilibration of all enzyme-containing forms.

The ratio \( v_F/v_o \) is then

\[
\frac{v_F}{v_o} = \frac{(S + L_1)(1 + L_2 / k_2)}{S(l + K_2) + L_1(1 + K_2)} \tag{8}
\]

where \( K_3 = k_3 / k_5 \) and \( K_5 = k_5 / k_2 \).

Clearly the velocity will change as a function of time but the extent of change will be dependent on the substrate level and the relative values of \( L_1, K_2 \) and \( K_5 \). The rate of change is still defined by Equation 4. If both \( E \) and \( E' \) exist at time zero or if the substrate concentration is rapidly changed from one level to another, the ratio \( v_F/v_o \) is

\[
\frac{v_F}{v_o} = \frac{(S + L_1)(1 + K_2)}{S(l + K_2) + L_1(1 + K_2)} \tag{9}
\]

where \( S_2 \) is the new level of substrate. The rate of change is again defined by Equations 4 and 5 with \( S = S_2 \).

As pointed out by Rabin (17), the progress curve (the product concentration as a function of time) for such a system may show a lag, but will then reach a constant value. It should be noted that the same is true of the case where \( k_s = 0 \) provided that \( v_F > v_o \). If one assumes substrate depletion, the curve will level off and the over-all progress curve would be sigmoidal. If, as is the assumption made previously in the derivation of these equations, the substrate level remains constant throughout the time involved, it becomes a relatively simple matter to express the change in product concentration as a function of time. Thus, according to Equation 4

\[
\frac{dP}{dt} = v_r - \left( v_o - v_F \right) e^{-k't} = \frac{1}{v_F} \left[ (v_F - v_o) e^{-k't} \right] \tag{10}
\]

where \( v_F \) is a substrate concentration, \( v_o, v_F, \) and \( k' \) are constants. Integration yields

\[
P_e = v_F - \frac{(v_F - v_o)e^{-k't}}{k'} \tag{11}
\]

The extent of the lag before reaching a constant rate of product formation clearly depends on \( k' \), larger values of \( k' \) yielding shorter lag times. Product formation becomes a linear function of time when \( e^{-k't} \approx 1 \). Under these conditions

\[
P_e = v_F \left( t - \frac{1}{k'} \right) \tag{12}
\]

Extrapolation of this linear portion of a \( P_e \) versus \( t \) curve yields

\[
P_e = -\left( v_F - v_o \right)/k' \tag{13}
\]

An equation similar to Equation 10 has recently been derived by Ray and Hatfield (19). The latter authors, however, used somewhat different assumptions for the mechanism as well as conditions under which the substrate level is assumed to saturate. Equation 10 as derived in this paper assumes only that the substrate level does not change during the time of the experiment. If the substrate level is saturating

\[
k' = k_3 + k_4 = k_{2a}(1 + K_2) \tag{14}
\]

while at low substrate levels

\[
k' = k_3 + k_4 = k_{2a}(1 + K_2) \tag{15}
\]

**Single Substrate-Single Modifier Case**—The single substrate Mechanism II can be expanded to include the modifier. The mechanism can then be represented as

\[
\text{MECHANISM III}
\]
The rate constants which define steps in the presence of modifiers are primed (i.e. $k_3$ versus $k'_3$, and so on). The dissociation constants for modifier binding $J_1$ to $J_4$ are of the type $J_1 = [(E)(M)]/EM$, etc.

The derivation of the equation for this system can be made relatively easily using the same assumptions as in Mechanism II: (a) all steps defined by dissociation constants equilibrate rapidly, (b) the reaction to give product is irreversible and product does not inhibit by competing with the substrate, and (c) the concentration of substrate is maintained at a constant level throughout the time course of measurement.

Using these assumptions, one can obtain
\[ v_t = v_f + (v_o - v_f)e^{-k't} \]
that is, an equation identical to Equation 4. In this case, however, $k'$ is a much more complex function. Invoking the principle of microscopic reversibility yields Equation 12 of Scheme 1. As before, the amount of product formed as a function of time is defined by Equation 10 which rests in turn on $v_f - v_o$ and on the value of $k'$. If $M$ binds preferentially to $E'$ or $E'S'$, the lag in achieving a constant rate of product formation will be longer than if $M$ bound preferentially to $E$ and $ES'$, since this, in essence, decreases the concentration of $ES$ and $ES'M$.

The values of $v_o$ and $v_f$ will, of course, depend upon the conditions chosen. For example, $v_o$ might be the velocity of the running system in the absence of modifier and might have the form of Equation 3. The final velocity, assuming microscopic reversibility, is shown in Equation 13 of Scheme 1. If it is assumed that modifier does not affect substrate binding ($J_1 = J_2 = J_3 = J_4$), then Equation 14 of Scheme 1 results. If it is further assumed that $k_6 = k'_6 = k_8 = k'_8 = 1$, then Equation 14a of Scheme 1, which is identical to the Monod Equation (8) for $n = 1$ in the presence of a modifier, results.

In any case, $v_o$ and $v_f$ could differ markedly so that the addition of modifier to an enzyme system which is already producing product may yield a hysteretic response with the apparent rate constant, $k'$, being defined by Equation 12 of Scheme 1.

**Multisite Systems**—Most regulatory enzymes, of course, exist as multiple polypeptide chains, each chain usually containing an active and perhaps a modifier site. Mechanism III would therefore be greatly expanded and becomes too complex to indicate pictorially. The expansion to multiple substrate or modifier sites is not too difficult to visualize conceptually, however. Assuming as before, that all ligand binding steps are essentially equilibrium situations, that the substrate concentration is maintained constant throughout the experiment, that the reaction is irreversible, and that the product, if it accumulates, does not inhibit, the system yields the same Equation 4, $v_t = v_f + (v_o - v_f)e^{-k't}$.
whether the principle of microscopic reversibility is valid or not does not arise.

The presence of modifier introduces terms in $M$ raised to the power $n$, the exact relation being determined by the assumptions made. If assumptions similar to those above are made, the time dependence of the velocity may still obey that described by Equation 4. At infinite substrate concentration,

$$k' = k_1 + k_2 + \frac{k_3 + k_4 M}{1 + M/M_1 + 1/M_2}$$

(16)

so that at infinite substrate and modifier levels $k' = k_1 + k_4$. For all of the mechanisms described so far, it should be emphasized that the substrate dependence of the initial and final velocities is that which would be calculated by assuming rapid equilibration of all the enzyme species involved. Thus the hysteretic response appears as a lag in the rate of product formation after substrate addition, the final velocity of the enzymatic reaction is that value obtained when the product formation becomes a linear function of time. This final velocity may show typical allosteric behavior as a function of substrate concentration. Furthermore, such behavior can be discussed in terms of the models which have been proposed by Koshland et al. (9) or by Monod et al. (8), and such a treatment of kinetic data would be perfectly valid. The hysteretic response observed, therefore, is not inconsistent with these models; it only indicates that a measurable amount of time is required to convert one conformation form of the enzyme to another.

Although it may be possible to fit either the initial or final velocity with equations derived for the Koshland et al. or Monod et al. models, kinetic data obtained at intermediate times may show an abnormal substrate dependence which is not easily described. Such data might be obtained, for example, if the time of the hysteretic response is strongly dependent on substrate concentration. In cases in which readily explicable kinetic data are not obtained, one should certainly investigate this type of possibility.

**Displacement Reactions**

If a ligand is bound to an enzyme surface quite tightly, albeit reversibly, its rate of dissociation from the enzyme could be relatively slow. Under these conditions, the step $E + M \rightleftharpoons EM$ cannot be assumed to be rapidly equilibrating and the previous assumptions concerning the equilibration of this process are not valid. Thus if a tightly bound inhibitor is displaced by an activator, the rate of increase in enzymatic activity could be a measurable function of time depending on how rapidly the inhibitor is released from the enzyme surface. This mechanism, in its simplest form, would be represented by

$$EM \xrightarrow{k_{EM1}} E \xrightarrow{k_{EM2}} EM_1$$

**Mechanism IV**

Under these conditions and applying the steady state assumption that $d(E)/dt = 0$, one can derive an expression for the concentration of $EM_1$ or $EM_2$ as a function of time.

$$EM_1 = EM_2 (k_{EM2} + k_{EM3} + k_{EM4})$$

(17)

and

$$EM_2 = EM_3 (k_{EM3} + k_{EM4})$$

(18)

where

$$k' = k_{EM1} + k_{EM2} + k_{EM3}$$

(19)

and

$$k'' = k_{EM3} + k_{EM4} + k_{EM5}$$

(20)

If $k_1$ and $k_4 < k_{EM1} + k_{EM3}$, which is likely, then

$$k' = k''$$

(21)

Since the steady state assumption is used, the concentration of $E$ is independent of time. If it is assumed that activity at saturating substrate levels measures relative concentration of $EM_1$ and $EM_2$, which is equivalent to assuming that $k_1$ and $k_4$ are << $k_{EM1} + k_{EM2}$ and that the concentration of free $E$ is small, then it may be shown that $v_E = v_F + (v_o - v_F) e^{-kt}$. Under these conditions, it will be noted that the velocity as a function of time is identical to the expression derived previously for the isomerization case. This case may therefore be treated in an identical way to the previous case where $k'$ may be obtained from a first order plot and where the product $P$ may be shown to be $P = v_P - (1/k') (v_F - v_o)$ (1 - $e^{-kt}$). That is, there will be a lag in the concentration of $P$ and the extent of the lag will depend upon $k'$ and $v_F - v_o$.

Fig. 2 shows an example of such a displacement reaction using glutamate dehydrogenase. The enzymatic activity is measured while the tightly bound inhibitor GTP is displaced by the activator ADP. Since the dissociation constant for GTP is about $4 \times 10^{-11}$ M under these conditions (20), its rate of dissociation is slow and the result is that the change in rate of product formation increases rapidly after an initial lag. Extrapolation of the linear portion of the curve to $P = 0$ yields, as indicated before, $v_o = (v_F - v_o)/k'v_F$. Assuming $v_o = 0$, $k'$ is calculated to be 0.3 sec$^{-1}$ and the apparent half-time is 2.3 sec for the displacement reaction under these conditions. The (—) in Fig. 2 is drawn according to Equation 11 using this value of $k'$.

The mechanism which should be used is, however, somewhat more complex than shown by Mechanism IV. Thus a more accurate mechanism would be
Some enzymes, especially those which are important in regulation of metabolic pathways, undergo reversible self-association reactions to higher molecular weight forms. Frequently, the equilibrium constant for the polymerization can be affected by ligands which are either substrates or modifiers of enzymatic activity. Previous papers have discussed the fact that anomalous (i.e. non-hyperbolic) binding of ligands may occur when the different molecular weight forms differ in affinity for the ligand or in the number of available binding sites (6, 7). The equation for the displacing type case quickly becomes more complex and takes a form different from Equation 4 when the enzyme involved is a multisite enzyme and the sites are not independent of each other. The treatment of such cases will not be pursued further in this paper.

**An equation identical to Equation 1 may be derived if it is assumed that substrate binding steps are equilibrated and that the conversion of ESM1 and ESM2 to ES is slow relative to their breakdown to give product, or if the substrate concentration is saturating so that all enzyme forms are forced into ESM1, ES, and ESM2.**

The equation for the displacement type case quickly becomes more complex and takes a form different from Equation 4 when the enzyme involved is a multisite enzyme and the sites are not independent of each other. The treatment of such cases will not be pursued further in this paper.

**Polymerizing and Depolymerizing Systems**

A number of enzymes, especially those which are important in regulation of metabolic pathways, undergo reversible self-association reactions to higher molecular weight forms. Frequently, the equilibrium constant for the polymerization can be affected by ligands which are either substrates or modifiers of enzymatic activity. Previous papers have discussed the fact that anomalous (i.e. non-hyperbolic) binding of ligands may occur when the different molecular weight forms differ in affinity for the ligand or in the number of available binding sites (6, 7). In particular, the former case holds for the bovine liver glutamate dehydrogenase with respect to GTP binding in the presence of reduced coenzyme (20). If the different molecular weight forms have different kinetic characteristics, and if conversion between them is slow, then there should be a measurable time-dependent change in the enzymatic activity or response to modifier. The situation with respect to the description for such systems could be considerably more complicated than the isomerization or displacement mechanisms considered earlier, although under some conditions, the equations could be analogous to those already developed.

Very few studies of the rate of polymerization or depolymerization in such enzyme systems as these have been carried out, but it is clear that, at least in some cases, the rate-controlling step is quite slow and could represent a conformational change within the protein with a molecular weight change resulting as the consequence. Certainly, for studies of the rate of depolymerization, one would expect that a conformational change would precede a dissociation forced by the addition of a ligand. In such cases, the system could be treated in a manner analogous to the development already presented for the isomerization case and it might be expected that the time dependence of the velocity would follow that described by Equation 4. The situation for a polymerizing case, however, may be somewhat more complex, depending upon whether the polymerization itself is rate determining or whether it is rapid relative to conformational change which allows the enzyme to polymerize. Which process is rate limiting may depend upon the concentration of the protein. For example, at an enzyme level of 10^{-5} M or higher, the rate of polymerization per se may be too rapid to conveniently measure, whereas, if the enzyme concentration was as low as 10^{-7} M, polymerization of two or more monomeric forms of the enzyme could be a rather slow process. In the former case, the time dependence of the change in velocity might be described by an equation similar to Equation 4, but in the latter case, the expression would be more complex. Experimentally the two cases could be distinguished by using a range of enzyme concentrations.

If polymerization per se is not a rate-limiting step in the conversion between different molecular weight forms, then the system might be treated as an isomerization case and the equations developed earlier would be applicable here.

Development of the equations for the more complex system in which polymerization is rate limiting is beyond the scope of this paper, since we are primarily concerned with the hysteresis concept as such and the various mechanisms which may be involved rather than the complete kinetic description.

**DISCUSSION**

The rapid response of an enzyme to a change in ligand concentration is a condition which is inherent in the models currently proposed to relate the kinetic characteristics of regulatory enzyme to their observed molecular thermodynamic or initial velocity properties. The possible physiological consequences of the current mechanisms have been discussed extensively in the literature and excellent correlation may be made between the kinetic characteristics of an enzyme and its role in metabolic regulation. However, complex regulatory processes, which include systems in which different pathways utilize common intermediates or in which there are many branch points within a given pathway, may require more complex kinetic regulation of the key enzymatic steps. For such cases, the hysteretic enzyme concept may be useful. It will be apparent from the discussion below that the physiological consequences of a slow response can be quite distinct from those of a rapid response and that
such responses may be important in the over-all regulation of complex metabolic processes.

Under the assumptions made in the body of this paper, and which presumably apply to an enzyme located within a sequence of events, there are several aspects of hysteretic enzyme systems which are relevant to metabolic regulation. One of these is the activity of the enzyme as a function of time at a given substrate or modifier level. If a modifier induces slow conformational change, for example, the kinetic characteristics of two extreme cases (at \( t = 0 \) and \( t = \infty \)) may be defined by velocity studies before and well after addition of the modifier. Clearly, if the kinetic characteristics of the different forms of the enzyme differ, the activity immediately after modifier addition will be time dependent. The activity at any given time will be some weighted average of the activity of the two different forms.

Thus, under conditions in which the addition of a modifier results in the slow conversion of one form of the enzyme to another, but the activity responds immediately to changes in substrate levels, the type of substrate dependence curve obtained will be a function of time. Further, if the conversion from one form to another is slow enough, then, at any given time, the substrate dependence curve could be described by the allosteric models which assume rapid responses (8, 9).

Since a hysteretic enzyme will retain, for some period of time, some of the characteristics of the enzyme before a change in ligand concentration, it acts as a time-dependent buffer, while at the same time slowly responding to changes which will eventually alter the kinetic characteristics of the enzyme to correspond to the altered level of metabolites in the cell. The buffering capacity of such an enzyme system, which depends upon the rate of conversion of one form of the enzyme to another, serves as a mechanism to prevent immediate changes in the concentrations of other metabolites in the particular pathway, perhaps allowing the rate of other interacting pathways which use a common starting metabolite to be maintained. One would expect that such buffering systems might be common in complex metabolic regulatory systems and perhaps less frequent in pathways which could be characterized as simple biosynthetic pathways controlled by feedback inhibitors.

In order of increasing complexity, we can consider the following possible processes

$$A \rightarrow B \rightarrow C \rightarrow D \rightarrow F$$

\( (P1) \)

$$A \rightarrow B \rightarrow C \rightarrow D \rightarrow F$$

\( (P2) \)

$$A \rightarrow B \rightarrow C \rightarrow D \rightarrow F$$

\( (P3) \)

$$A \rightarrow B \rightarrow C \rightarrow D \rightarrow F$$

\( (P4) \)

where, when indicated by an arrow, \( F' \) and \( F'' \) are feedback inhibitors. For purposes of discussion it is assumed (a) that the rate of formation of the substrate, \( A \), is constant, (b) that the hysteretic response is only to the feedback inhibitor and not to the substrate, and (c) that the utilization of the end products, \( F' \) and \( F'' \), is constant. In the cases to be discussed we will assume that the level of \( F \) rises rapidly either because of an increase in the concentration of \( F \) itself from some other pathway (or an intermediate metabolite preceding \( F \)) or because of a decreased rate of \( F \) utilization.

Pathway \( P1 \), the simplest feedback system, would normally be expected to show the behavior, that, as \( F \) rises, the rate of \( F \) formation decreases and more \( A \) is available for \( F' \) production. The predicted sequence of events as a consequence of an increase in the level of \( F \) is shown in Table I. The symbols in the table are defined in the legend. If the step \( A \rightarrow B \) does not show a hysteretic response to the feedback inhibitor, the sequence of events (shown by section labeled “None”) can be read as follows: as the rate of production of \( B \) falls rapidly, \( B \), the level of \( A \) rises rapidly, \( A \). As a consequence, the rate of formation of \( F \) decreases, the rate of formation of \( F' \) and \( F'' \) rise, and the level of \( F' \) rises. All changes occur rapidly. If, on the other hand, \( A \rightarrow B \) shows a hysteretic response to the inhibitor, \( F \), then, after a rapid rise in \( F \) level the decrease in the rate of \( F \) formation will lag behind the rise in the \( F \) concentration. As shown in Table I, the rate of \( B \) formation decreases slowly, \( (B \downarrow) \), and as a consequence the rate of \( F \) formation decreases slowly (slow

### Table I

**Sequence of events for pathway P1 after rapid rise in F level**

This table indicates the predicted sequence of events which may occur after a rapid rise in the level of end product \( F \). The time scale is read from left to right. Letters placed above each other indicate that the changes occur approximately simultaneously. The conventions for rapid or slow changes in the level or rate of production of a metabolite are indicated in the footnote to this table.

| Enzyme step with hysteretic response to modifier | Sequence of events after \( F \uparrow \) |
|-----------------------------------------------|---------------------------------------|
| None                                          | Rate of formation \( B \uparrow \) \( \{F', F'\uparrow\} \) \( \{E', E'\uparrow\} \) |
| Level                                         | \( A \uparrow \) \( F' \uparrow \) |
| \( A \rightarrow B \)                        | Rate of formation \( B \uparrow \) \( \{F', F'\uparrow\} \) \( \{E', E'\uparrow\} \) |
| Level                                         | \( A \uparrow \) \( F' \uparrow \) |

\(^a\) Italic letter followed by \( \uparrow \) or \( \downarrow \) = a rapid increase or decrease in level; italic letter enclosed in parentheses followed by \( \uparrow \) or \( \downarrow \) = a slow increase or decrease in level; italic letter with underbar followed by \( \uparrow \) or \( \downarrow \) = a rapid increase or decrease in formation; italic letter with underbar enclosed in parentheses followed by \( \uparrow \) or \( \downarrow \) = a slow increase or decrease in formation.
changes indicated by the parentheses). Concurrently, the level of \( A \) rises slowly and, as a consequence, the rate of \( F' \) formation and the \( F'' \) level rise slowly. It should be noted that the extent of the rise in \( F'' \) level in this case may depend on the rate of utilization of \( F' \), but we are assuming that the utilization rate is constant and independent of end product concentration.

The importance of a hysteretic enzyme in an isolated system like \( P_1 \) is questionable and the other systems, \( P_2 \) through \( P_4 \), are of more interest. Table II shows the predicted sequence of events for \( P_2 \) when either \( A \to B \) or \( A \to B' \) shows a hysteretic response to the inhibitor. Note that when \( A \to B' \) is hysteretic the level of \( F'' \) could rise rapidly followed by a slow decrease in the rate of \( F'' \) formation. On the other hand, the rate of \( F'' \) formation falls rapidly in this system. If the \( A \to B \) step is hysteretic, the rate of formation of both \( F' \) and \( F'' \) will change slowly.

Table III shows the sequence of events for pathway \( P_3 \) after a rapid rise in the level of \( F \). Note that if the hysteretic response is at the \( A \to B \) step, an increase in \( F \) will cause a rapid rise in the concentration of \( C \), but the rate of \( C \) formation will then decrease slowly. Thus a high level of \( C \) will be conserved. If the \( C \to D \) step is hysteretic, \( C \) is also conserved but at a lower level.

Pathway \( P_4 \) represents a more likely combination of metabolic steps than do the preceding three systems. In this case, there are multiple branch points and more than one feedback inhibitor. Table IV shows some possible predictions of the sequence of events after a rapid rise in \( F \). Examination of Table IV shows that it is possible to buffer out rapid changes in the formation of either \( F' \) and \( F'' \) or \( F'' \), or all of these, depending upon which enzymatic step responds slowly to its inhibitor.

The predictions listed in the tables must be viewed with a great deal of care. A number of assumptions have been made to help define the systems and different assumptions could give rise to different predictions. Thus, differences could arise if one of the inhibitor levels was initially high enough to block that pathway, if an increased \( A \) level could overcome the \( F \) inhibitor after the rise in \( F \) concentration, if the pool sizes of intermediates were different, if the hysteretic response were either to the inhibitor or to the substrate, or both, if the utilization of the end product or production of the initial substrate was not constant, or if an end product, or some other intermediate, could activate an enzymatic step or displace an inhibitor, and so on. The essential point, however, is that it is possible to buffer out rapid changes in end product concentrations and the exact prediction would depend upon the characteristics of the system under discussion. It is also to be noted in reference to these tables that the concepts of a slow increase or decrease is relative only to the rate of formation of the enzyme induced by the inhibitor and defined by Equation 4 and relative to the rate of enzymatic reaction. The tables indicate that qualitatively the same final results would be achieved regardless of whether the pathways contain a hysteretic enzyme step or not. In actuality, this may not be the case at all because of changing rates of utilization of the end products.

The diagram \( P_4 \) can be considered to be similar to the pathway involved in utilization of aspartate in *Escherichia coli* if \( A = \) aspartic semialdehyde, \( F'' = \) lysine, \( F'' = \) methionine, and \( F \) = threonine. (For the sake of accuracy, \( F \) should go on to another metabolite, isoleucine, which is a feedback inhibitor of the threonine deaminase, and the step \( A \to C' \) is controlled by \( F'' \).) Stadtman (2) has pointed out that feedback control of
TABLE IV

Sequence of events for pathway $P_4$ after rapid rise in $F$ level

Conventions used are defined in legend and footnote to Table I.

| Enzyme step with hysteretic response to modifier | Sequence of events after $P^+$
|-----------------------------------------------|--------------------------------|
|                                              | Rate of formation | Time |
| None                                         | $D_1 \frac{[F]}{[B]}$ | $C^+$ |
|                                              | $A\uparrow \frac{[A]}{[B]}$ | $F^\uparrow \frac{[F^\uparrow]}{[F^\downarrow]}$ |
| $A \rightarrow B$                            | $D_1 \frac{[F]}{[B]}$ | $C^+$ |
|                                              | $(A^\dagger)$ | $(F^\uparrow)$ |
|                                              | $(B^\dagger)$ | $(F^\downarrow)$ |
| $C \rightarrow D$                            | $(D_1) \frac{[F]}{[B]}$ | $(C^\dagger)$ |
|                                              | $(A^\dagger)$ | $(F^\uparrow)$ |
|                                              | $(B^\dagger)$ | $(F^\downarrow)$ |
| $B \rightarrow C^*$                          | $D_1 \frac{[F]}{[B]}$ | $C^+$ |
|                                              | $A\uparrow \frac{[A]}{[B]}$ | $F^\uparrow \frac{[F^\uparrow]}{[F^\downarrow]}$ |

* Unless piling up $C$ causes $B^+$, in which case the sequence would be $B^+, F^\uparrow, E^\downarrow, (B^1), (F^\uparrow)$.

$A \rightarrow B$ (homoserine dehydrogenase) by threonine is disadvantageous because it also blocks the production of methionine ($F^\uparrow$) and, in fact, methionine is required for normal growth rates when the organism is grown on excess threonine (21). If, on the other hand, the step $A \rightarrow B$ were controlled by a hysteretic enzyme, the methionine level, over a short period of time at least, would be conserved. Under such conditions normal protein biosynthesis with excess threonine might occur and the system might be able to catch up with itself without significantly decreasing the level of methionine. In other words, if homoserine dehydrogenase were a hysteretic enzyme, the change in the rate of formation of methionine would be buffered against changes in threonine levels. It is of interest that the threonine inhibition of the E. coli homoserine dehydrogenase is in fact time dependent (22) (see Table V). On the other hand, if the threonine deaminase is hysteretic, the concentrations of all the products, $F^\uparrow$, $F^\downarrow$, and $F^\uparrow$ (lysine, methionine, and threonine), are buffered against changes in isoleucine levels. In Bacillus subtilis, the threonine deaminase is a hysteretic enzyme, responding slowly to a rapid increase in the concentration of the inhibitor isoleucine (24).

These examples are presented to show the value of the hysteretic enzyme concept in control of metabolic regulation. There are undoubtedly many more possibilities and the pathways chosen above are certainly not the most complex ones. Furthermore, the tables give only what might be expected if only one enzyme of those involved shows hysteretic behavior. Better control, however, would be obtained if more than one enzyme was hysteretic.

Two examples of hysteretic enzymes are given above, but examination of the literature reveals a number of enzymes which might be classed as hysteretic enzymes. A partial list of such enzymes, and some of their characteristics, is shown in Table V. It is of interest that this table includes all of the mechanisms which have been proposed in this paper for hysteretic enzymes. For example, the increase in activity of the yeast glyceraldehyde 3-phosphate dehydrogenase after DPN addition is an example of isomerization, but a similar observation for phosphorylase $a$ on glycogen addition appears to result from depolymerization of the enzyme. The slow rise in activity of phosphofructokinase on raising the pH from 6 to 7.5 or above appears to be a result of polymerization of less active molecular weight forms; on the other hand the slow increase in activity (also shown in Fig. 2) on addition of ADP to GTP-inhibited glutamate dehydrogenase appears to be due to the slow release of GTP from the enzyme surface. Several other points can be made about the enzymes listed in the table. First, most, if not all, of the enzymes involved are regulatory enzymes in the sense that they occur at a sensitive point in a metabolic pathway and frequently show allosteric (i.e. sigmoidal) kinetic behavior. Second, the approximate times involved for the interconversion of different forms may vary from seconds to minutes (perhaps hours) and there appears to be no way to predict $a$ priori what these times will be. Third, the table does not include other possible slow interactions like those for hemocyanin (from changes in $P_{Ol}$) (33); for hemoglobin (after ATP addition to deoxy Hb) (34); or for the myosin-actin interactions (35). Nor does it include a rather large class of cold-labile enzymes which can be reactivated by warming (see Reference 36, for example) and for which the reactivation may be a relatively slow process. Furthermore, Table V does not include slow refolding processes which occur on renaturing enzymes from their denatured forms. These latter cases, of which there are many examples, probably fall into a very different class with respect to their importance.
undertaken in regard to the possibility of a hysteretic response in regulatory processes. Fourth, the large number of such enzymes is a surprise since investigators have not systematically examined regulatory enzymes for this type of effect.

It would be hoped that systematic investigation might be undertaken in regard to the possibility of a hysteretic response with respect to regulatory enzyme systems. Such investigation could take the form of examination for time-dependent changes in activity, either as a lag in the product formation after substrate or effector addition or after dilution of the enzyme, or a lag in the change in activity when displacing one allosteric modifier by another. Of particular interest is the question of whether preliminary incubation of the enzyme with a substrate or effector will alter any observed lag. However, it is possible for a hysteretic enzyme that even preliminary incubation would not decrease the lag time if both the substrate and modifier are required to induce the interconversion. Such an effect could be termed concerted hysteresis. While this paper discusses the cases where the substrate concentration is not maximal, experimentally, velocity measurements at very high substrate levels may be required if one is to watch the reaction over a long period of time. If it is known that different molecular weight species have significantly different kinetic or ligand-binding characteristics, an examination of the rate of molecular weight change may be in order. One can also examine the enzyme system for time-dependent conformational changes (as indicated by spectral shifts or change in reactivity of amino acid residues) but in such cases, a clear correlation between these changes and a change in the kinetic or ligand-binding characteristics should be established.

There are, of course, pitfalls in such experiments as these, since it is always possible that the changes one observes are not metabolically significant. For activity measurements one must avoid conditions in which product accumulation or substrate depletion would alter the rate of the reaction, but attempts to do so by using coupled assay systems may present difficulties inherent in the coupling system.

It may also be helpful at this point to discuss briefly the concept of slow and rapid changes as they have been used in this paper. Arbitrarily, it is most convenient to relate such changes to the time required to measure the actual rate of enzymatic

### Table V

Partial list of enzymes which appear to show a hysteretic response

The following list of enzymes are those which appear to show a hysteretic response as discussed in the text. The half-times are given only in terms of seconds or minutes since they depend upon a number of factors including temperature, buffer, pH, and so on. Not all of the hysteretic responses for a given enzyme are indicated in the table nor are all of the enzymes which show such a response listed here.

| Enzymes                                | Time dependent change                                                                 | Approximate half-times | Type                      | Reference |
|----------------------------------------|---------------------------------------------------------------------------------------|------------------------|---------------------------|-----------|
| Glyceraldehyde 3-phosphate dehydrogenase (yeast) | Addition of DPN Increase in velocity                                                  | Seconds                | Isomerization             | (23)      |
| Homoserine dehydrogenase E. coli        | Addition of threonine Enzyme dilution Decrease in velocity                             | Seconds                | Isomerization             | (22)      |
| Rhodospirillum rubrum                    |                                                                                       | Minutes                | Molecular weight change   | (25)      |
| Threonine deaminase E. coli             | Addition of AMP Increase in velocity                                                   | Minutes                | Molecular weight change   | (26)      |
|                                          | Addition of threonine to enzyme isoleucine complex or addition of isoleucine to running reaction |                        | Isomerization             | (24)      |
| B. subtilis                             |                                                                                       |                        | Isomerization             |           |
| Glutamate dehydrogenase (bovine liver)  | Addition of ADP to GTP-inhibited enzyme Increase in velocity                          | Seconds                | Displacement              | Fig. 2    |
| Phosphorylase a (rabbit muscle)         | Addition of glycogen Increase in velocity                                              | Minutes                | Depolymerization          | (27)      |
| Phosphofructokinase (rabbit muscle)     | Raising pH from 6 → 7.5 Increase in velocity                                           | Minutes                | Polymerization            | (13)      |
| Acetyl-CoA carboxylase (rat adipose tissue) | Addition of citrate Increase in velocity                                              | Minutes                | Polymerization            | (28)      |
| DPNH oxidase (Mycobacterium tuberculosis) | Dissociation of AMP Change in extent of AMP activation Increase in velocity           | Minutes                | Isomerization             | (29)      |
| UDPG Hydrolase (E. coli)                 | Addition of protein fraction Inhibition                                                | Minutes                | Isomerization of added protein? Isomerization | (30)      |
| Glutamine-PP-ribose-P amido transferase  | Addition of substrate Increase in velocity                                              | Minutes                | Isomerization             | (31)      |
| Lactic dehydrogenase                    | Addition of pyruvate as product Inhibition                                             | Seconds                | Aborting complex formation | (32)      |

in regulatory processes. Fourth, the large number of such enzymes is a surprise since investigators have not systematically examined regulatory enzymes for this type of effect.
reaction as measured by product formation or substrate disappearance under the particular conditions. It will be noted that the enzymes of Table V undergo changes which in general take at least seconds if not minutes or hours. At saturating levels of substrate and modifier, the terms fast and slow could, of course, be related to the turnover number of the enzyme under those conditions. Since turnover numbers vary greatly and since it is not a requirement, under the assumptions used, to saturate the system, relating rates of conversion of one form to another to the time required to make an initial velocity measurement is a convenient definition. It should also be pointed out that equilibration of certain steps in the mechanism, i.e., binding steps, is indeed probably quite rapid relative to the time chosen to define slow conversion of one form to another. Thus the kinetic treatment, as described in this paper, is probably valid and avoids some of the more complex equations which arise when no assumptions are made as to what might be a rate-limiting step in an enzymatic reaction.

The examples of the importance of hysteretic enzymes to metabolic pathways given above are discussed in terms of the assumption that the level of one of the end products or intermediates in a pathway rises rapidly. The term rapid as used here may be misleading in the sense that changes in each concentration need only be faster than the rate of the hysteretic response. Thus, the level of an intermediate which is common to two pathways may change as a consequence of metabolic process in one of the pathways and the change could be a relatively slow one. However, if the change is faster than the hysteretic response in the other pathway, it may be considered as a rapid change relative to that pathway. Table V shows that some of the responses of hysteretic enzymes may be quite slow, on the order of minutes or longer, and large changes in the flow of metabolites through a given pathway may occur in such times. In this regard, the question of fast or slow changes may only be relative to the rate of substrate flow through a given pathway under a given set of conditions. It is perhaps this latter information which will be of importance in correlating hysteretic response to complex metabolic processes.

The introduction of the concept of hysteretic enzymes clearly adds another dimension to the question of metabolic regulation in vivo. Although it is difficult at this time to establish any relation between the degree of hysteresis an enzyme may exhibit and the complexity of the regulatory process, it would appear that the concept is most important in relation to complex metabolic pathways. It is hoped that this concept, rather than complicating the issue of regulation in the cell, will eventually lead to some simplification of these complex systems.

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