The Kinetics of Enzyme Degradation of Mixed Polymer Systems. Endwise Cleavage*

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ABSTRACT: A steady-state analysis of endwise enzyme-catalyzed degradation of mixed polymers is presented. It is demonstrated that monomer heterogeneity in the polymer, conformation changes, competitive self-inhibition, repetitive attack, the type of polymer distribution, and other effects influence the observed Michaelis–Menten constants. In certain limiting cases it is possible to dissect the measured Michaelis parameters into sets of constants characteristic of certain processes; *e.g.*, competitive self-inhibition or repetitive attack. It is also demonstrated that polymers of average chain length x from either a most probable or a Poisson distribution will behave as pure polymers of chain length x as the number average degree of polymerization increases.

Polymers heterogeneous in both monomer composition and chain length have frequently been employed as substrates for the characterization of depolymerizing enzymes. A rigorous steady-state analysis of enzyme–mixed polymer systems does not appear to have been undertaken although Hanson (1962) has thoughtfully developed the theory for the enzyme kinetics of short chain polymer cleavage. In order to extend this treatment to mixed polymers, we have developed a general representational model based upon experimental observations and current notions about the pathway of endwise cleavage of biopolymers (Hanson, 1962; Bailey and French, 1957; Thoma and Koshland, 1960). These developments will aid in the interpretation of data and with experimental design.

It has been clearly established that degradation of biopolymers with enzymes can yield valuable information about the structure of these polymers. On the other hand, the fact that important information about enzyme action can be derived by studying the action of enzymes upon polymers of defined structure has not been as well recognized although a few studies of this type have been undertaken (for some pertinent references, see Hanson, 1962; Thoma and Koshland, 1960; Bailey and French, 1957). Recently it has been pointed out that measurement of the variations of the Michaelis–Menten parameters with chain length can aid in elucidating the mechanism of enzyme action as well as enzyme specificity patterns (Thoma and Koshland, 1960). Preferably experiments of this nature should be conducted with pure polymers of precisely defined composition, sequence, and structure. Unfortunately, because of technical difficulties, even homopolymers whose degree of polymerization (DP)[1] exceeds 10 cannot normally be fractionated (for pertinent references, see Hanson, 1962). A direct approach, therefore, is normally impossible. An alternate, but less desirable, solution to this problem is to investigate the enzyme kinetics using mixed polymer populations. In order to interpret the results of these investigations, the relationships between the empirical constants measured in the kinetic studies and the individual rate constants characteristic of the molecular processes should be established. This is particularly important for mixed polymers because the population distribution will depend upon the method of preparation (Tanford, 1961; Flory, 1953).

Since it is frequently observed that the enzyme kinetics of homopolymer populations or polymer mixtures heterogeneous with respect to chain length and monomer composition give linear double reciprocal plots (1/velocity *vs.* 1/substrate), it can be assumed that the simple Michaelis–Menten equation holds to a good approximation. Whether or not it is possible for the kinetics of these systems to conform to the Michaelis–Menten equation or whether at best the kinetics can give only an approximation of the Michaelis–Menten equation can be established only by a steady-state analysis of an appropriate representational model.

Additionally, it should be noted that there are two different types of assays for following the progress of enzyme degradation: chemical methods (such as end group analysis) and physical methods (viscosity change, weight loss, etc.). These methods give varying weights to the loss of polymers of different chain lengths (*e.g.*, viscosity is more sensitive to the loss of large molecules than to the loss of small ones). One must, therefore, inquire if and how the assay procedure might influence the empirical constants for enzyme action. The theoretical treatment of these situations is considered below.

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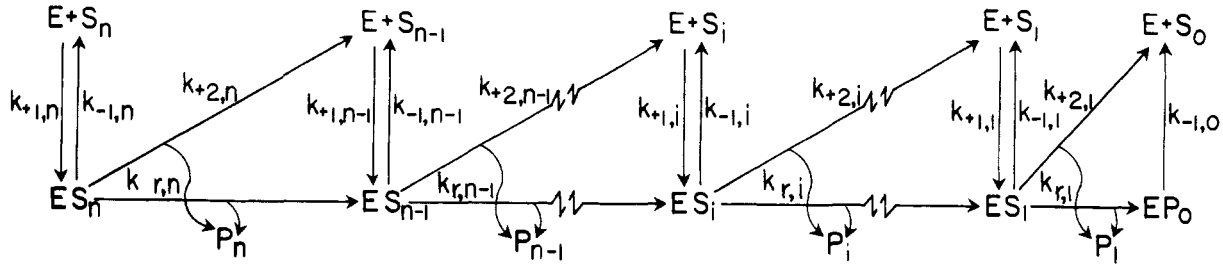
[1] Abbreviations used: DP, degree of polymerization.



SCHEME I: Endwise cleavage of a polymer mixture (S_n, S_{n-1}, . . . S_1, . . . S_0) composed of polymers of fixed sequence defined from the end not attached to the enzyme.

It is also pertinent to inquire into the effects that changes in the conformation of both substrate and enzyme may have on the Michaelis–Menten parameters. This is particularly true since large biopolymers may frequently assume shapes not thermodynamically stable for the smaller substrates.

In this paper the endwise cleavage of polymers of variable monomer composition and chain length is treated. Two limiting cases, cleavage of a single polymer of defined sequence but of mixed monomer composition, and cleavage of homopolymers of the most probable and the Poisson distributions, are examined. In addition the influence of substrate and of enzyme conformation changes and competitive self-inhibition on observed Michaelis–Menten parameters is discussed. The utility of the developments presented here will be exemplified in a forthcoming paper which describes an experimental study of the β-amylase–amylodextrin system.

Theoretical Section

Most of the following derivations are based upon Scheme I which depicts the manner in which an enzyme might induce endwise cleavage of a homopolymer mixture heterogeneous with respect to chain length or of a polymer mixture produced by endwise degradation of a given polymer of a specified sequence but heterogeneous with respect to monomer composition. Degradation of a homopolymer mixture will be treated first and a more general treatment will follow. A k_r process involves reaction followed by release of product and formation of a new reactive enzyme–substrate complex. A k_{+2} process involves reaction and subsequent dissociation of enzyme, substrate, and product. The largest cleavable polymer has a chain length of $n + 1$ and hence n positions capable of rupture. The index, i, corresponds to a particular bond in the polymer and the superscript, ~, indicates an experimentally measurable constant which may be composed of a combination of rate constants, equilibrium constants, mole fractions, and inhibitor concentrations. Definitions of unexplained symbols can be found in the text by Dixon and Webb (1958). Other symbols are explained when used. In the derivations it is assumed that steady-state condition (*i.e.*, $d[P_i]/dt \gg d[ES_i]/dt$) holds and that end product, P_i, inhibition (see Scheme I) is insignificant. Product inhibition can normally be circumvented experimentally by limiting the extent of degradation or by extrapolation techniques. However if products are introduced as part of the substrate mixture, their influence on the measured parameters can no longer be ignored. This special situation is considered below.

From Scheme I the concentrations of ES_n, ES_{n-1} and ES_i are found to be

$$[ES_n] = \frac{[E][S_n]k_{+1,n}}{k_{-1,n} + k_{+2,n} + k_{r,n}} = [E][S_n]\kappa_n \quad (1)$$

$$[ES_{n-1}] = \frac{[E][S_{n-1}]k_{+1,n-1} + [ES_n]k_{r,n}}{k_{-1,n-1} + k_{+2,n-1} + k_{r,n-1}} =$$

$$[E]([S_{n-1}]\kappa_{n-1} + [S_n]\kappa_n\phi_n) \quad (2)$$

where

$$\phi_n = \frac{k_{r,n}}{k_{-1,n-1} + k_{+2,n-1} + k_{r,n-1}}$$

and by analogy

$$[ES_i] = [E]([S_i]\kappa_i + [S_{i+1}]\kappa_{i+1}\phi_{i+1} + \cdots + [S_n]\kappa_n\phi_{i+1}\cdots\phi_n) =$$

$$[E]\sum_{p=i}^{n}[S_p]\kappa_p\theta_p \quad (3)$$

where θ_p is the appropriate product of ϕ_i's.

The conservation equation is given as

$$[E_0] = [E] + \sum_{i=1}^{n}[ES_i] \quad (4)$$

If the assay registers the cleavage of all bonds, then the observed initial velocity, $\tilde{v}$, is

$$\tilde{v} = \sum_{i=1}^{n}\frac{\partial[P_i]}{\partial t} = \sum_{i=1}^{n}(k_{+2,i} + k_{r,i})[ES_i] \quad (5)$$

The conservation equation, 4, can be combined with eq 3 and 5 and the free enzyme concentration, [E], eliminated to produce an equation for the normalized velocity $\tilde{v}/[E_0]$

$$\frac{\tilde{v}}{[E_0]} = \frac{\sum_{i=1}^{n}\left\{(k_{+2,i} + k_{r,i})\sum_{p=i}^{n}[S_p]\kappa_p\theta_p\right\}}{1 + \sum_{i=1}^{n}\sum_{p=i}^{n}[S_p]\kappa_p\theta_p} \quad (6)$$

The concentration of the individual polymer, $[S_i]$, is not normally measurable but the number average concentration, $[S_a]$, of all the polymers can readily be measured by standard techniques. To express eq 6 in terms of experimental quantities it is necessary to establish the relationship of $[S_i]$ to $[S_a]$ given by

$$\frac{[S_i]}{[S_a]} = f_i \quad (7)$$

where f_i is the functional relationship. This relationship can be readily evaluated

$$f_i = \left(\frac{\text{g of polymer}}{\text{l.}}(W_i)\right)\Big/\left(\frac{\text{g of polymer}}{\text{l.}}\right) = \frac{(W_i)(\overline{DP}_n)}{MW_i} \quad (8)$$

Canceling terms and substituting for W_i, the weight fraction of i-mer, and $\overline{DP}_n$, the number average degree of polymerization, gives

$$f_i = \left[\frac{\text{g of } i\text{-mer}}{\sum_{i=1}^{\infty}\text{g of } i\text{-mer}}\right]\left[\frac{\sum_{i=1}^{\infty}\text{g of } i\text{-mer}}{\text{total moles of polymer}}\right] \quad (9)$$

which on simplification gives

$$f_i = \frac{\text{g of } i\text{-mer}}{(MW_i)(\text{total moles of polymer})} \quad (10)$$

which will be recognized as the expression for the mole fraction of the i-mer so that for any polymer mixture where the total concentration is expressed as a number average concentration

$$f_i = N_i \quad (11)$$

The dependence of the normalized velocity on $[S_a]$ is then given by substituting for $[S_i]$ in (6) employing the relationships of eq 7 and 11 and leads to

$$\tilde{v} = \frac{[E_0][S_a]\tilde{k}}{1 + [S_a]/\tilde{K}_m} \quad (12)$$

which is homeomorphic with the Michaelis–Menten equation. In eq 12, $[E_0]\tilde{k}$ is a pseudo-first-order rate constant with respect to $[S_a]$ when the latter quantity is much smaller than $\tilde{K}_m$. If a physical system corresponds to Scheme I then the apparent Michaelis constants, $\tilde{K}_m$ and $\tilde{V}_{max}$, for that system are given by

$$\frac{1}{\tilde{K}_m} = \sum_{i=1}^{n}\sum_{p=i}^{n}N_p\kappa_p\theta_p \quad (13)$$

$$\tilde{V}_{max} = [E_0]\tilde{k}\tilde{K}_m = \frac{[E_0]\sum_{i=1}^{n}(k_{+2,i} + k_{r,i})\sum_{p=i}^{n}N_p\kappa_p\theta_p}{\sum_{i=1}^{n}\sum_{p=i}^{n}N_p\kappa_p\theta_p} \quad (14)$$

and the pseudo-first-order rate constant is

$$[E_0]\tilde{k} = [E_0]\sum_{i=1}^{n}\left\{(k_{+2,i} + k_{r,i})\sum_{p=i}^{n}N_p\kappa_p\theta_p\right\} \quad (15)$$

The results of a more general treatment will now be given. A polymer mixture isolated from a natural source, *e.g.*, a polypeptide or polynucleotide mixture, will normally be heterogeneous with respect to chain length and monomer composition. Each set of related polymers (identical monomer composition except for deletions or additions at one terminus of the chain) will be degraded according to Scheme I. If there are h sets of these polymers, q will designate the particular set to which a polymer belongs and n_q will define the number of cleavage points of the largest polymer in the qth set.

An analysis of this system following the line of reasoning presented in eq 1–11 again leads to the functional relationship, eq 12. The observable quantities for the mixed polymer systems are ascertained to be

$$\frac{1}{\tilde{K}_{m(\text{mixed polymer})}} = \sum_{q=1}^{h}\sum_{i=1}^{n_q}\sum_{p=i}^{n_q}N_{p_q}\kappa_{p_q}\theta_p \quad (16)$$

$$\tilde{V}_{max\ (\text{mixed polymer})} = [E_0] \times \frac{\left(\sum_{q=1}^{h}\left\{\sum_{i=1}^{n_q}(k_{+2,i,q} + k_{r,i,q})\sum_{p=i}^{n_q}N_{p_q}\kappa_{p_q}\theta_{p_q}\right\}\right)}{\sum_{q=1}^{h}\sum_{i=1}^{n_q}\sum_{p=i}^{n_q}N_{p_q}\kappa_{p_q}\theta_{p_q}} \quad (17)$$

$$[E_0]\tilde{k}_{(\text{mixed polymer})} = [E_0] \times \left(\sum_{q=1}^{h}\left\{\sum_{i=1}^{n_q}(k_{+2,i,q} + k_{r,i,q})\sum_{p=i}^{n_q}N_{p_q}\kappa_{p_q}\theta_{p_q}\right\}\right) \quad (18)$$

Equations 16–18 may be compared to eq 13–15. It may be noted that the rate constant $k_{+2,i,q}$, does not occur independently in the steady-state analysis but in combination with either $k_{r,i,q}$ or $k_{-1,i,q}$. Thus steady-state kinetics will not yield information about the relative values of $k_{+2,i,q}$ and $k_{r,i,q}$. It also follows that a k_{+2} process cannot be distinguished from a combination of a k_r and a k_{-1} process. Since the relationship between $\tilde{v}$ and $[S_a]$ takes on the form of the Michaelis–Menten equation, Lineweaver–Burk plots for these systems will always be linear and $\tilde{K}_{m(\text{mixed polymer})}$ and $\tilde{V}_{max(\text{mixed polymer})}$ will have the usual significance, the substrate concentration required to achieve half-

maximum velocity, and the observed velocity at enzyme saturation, respectively. The expressions for both $\tilde{V}_{max(mixed\ polymer)}$ and $\tilde{K}_{m(mixed\ polymer)}$ are very complex. In general $\tilde{V}_{max(mixed\ polymer)}$ will bear no simple relationship to the cleavage rate of a particular bond which may be saturating the enzyme if a pure polymer of specified structure and sequence was used as substrate. Similarly $\tilde{K}_{m(mixed\ polymer)}$ will normally not be directly related to the substrate concentration of a pure polymer required to achieve half-maximum velocity. These constants are more appropriately considered as weighted Michaelis constants. The weighted factors will be sensitive to such parameters of the system as the extent of repetitive attack, the type of polymer population, and the monomer distribution within a given polymer. In order to establish the weighting factors it would be necessary to use very well defined substrates under conditions which allow considerable simplification of the $\tilde{V}_{max(mixed\ polymer)}$ and $\tilde{K}_{m(mixed\ polymer)}$ expressions since these factors cannot be extracted from a steady-state kinetic analysis on mixed polymers (see below).

Hanson's (1962) propositions, developed for another system and relating various functions of the system, also apply to the present situation. They are: (a) that $\tilde{V}_{max(mixed\ polymer)}$ is less than or equal to the maximum velocity that would be observed if the bond most readily cleaved at high substrate concentration could be studied in the absence of all competitive effects (in this case cleavage of more slowly reacting bonds); and (b) that the observed rate of cleavage at high substrate concentration of a particular bond is smaller than would be observed if all competitive effects were excluded. In addition, proposition (c), that $\tilde{K}_{m(mixed\ polymer)}$ is equal to or greater than the substrate concentration of the monomer grouping with the lowest $\tilde{K}_m$ in the absence of other competitive effects, can easily be demonstrated.

Influence of Assay on Michaelis Constants. If a chemical assay registers cleavage only at certain bonds, say j, which may or may not be a terminal bond in any of the original polymers, then it is apparent that $\tilde{V}_{max(mixed\ polymer)}$ will be a function of the assay procedure and from proposition b it follows that

$$\tilde{V}_{max,j} \leqq \tilde{V}_{max(mixed\ polymer)} \tag{19}$$

where $\tilde{V}_{max,j}$ is the apparent maximum velocity of cleavage of the j type bond. The phenomenon will be reflected as a decrease in $\tilde{k}_{(mixed\ polymer)}$ but $\tilde{K}_{m(mixed\ polymer)}$ remains unaltered, and it follows that $\tilde{K}_{m(mixed\ polymer)}$ is independent of the assay procedure. If, on the other hand, physical rather than chemical methods are employed to measure the cleavage rate, $\tilde{V}_{max(mixed\ polymer)}$ will be expressed in arbitrary physical units but the value of $\tilde{K}_{m(mixed\ polymer)}$ still remains unaltered. Mathematically, this situation is somewhat analogous to competitive self-inhibition (see below), where the bonds that are not hydrolyzed act as competitive inhibitors of the reaction. This conclusion holds whether repetitive attack is operative or not.

Central Complexes. To this point it has been assumed that during reaction the enzyme–substrate complex, ES_i, does not pass through a series of central complexes (reaction intermediates) to give ES_{i-1}. It has been established for simpler schemes that isomerization of central complexes does not change the functional relationship of $\tilde{v}$ to [S] given by eq 12 (Alberty and Bloomfield, 1963; Cleland, 1963; Wong and Hanes, 1962). This conclusion is also correct for the more complicated system presented here and is demonstrated inductively in the Appendix. Introduction of isomerization of central complexes into Scheme I only adds to the complexity of $\tilde{k}_{(mixed\ polymer)}$, $\tilde{V}_{max(mixed\ polymer)}$ and $\tilde{K}_{m(mixed\ polymer)}$. Because of the great degree of kinetic complexities which may be hidden in apparent Michaelis constants for mixed polymer systems, it seems that their primary function may be limited to a simple characterization of the enzyme. It is doubtful that steady-state kinetics will be of much value in aiding the interpretation of, or adding significantly to, our understanding of enzyme kinetic data. Additional useful information can be derived from a time course study of the products of the reaction but such considerations remain beyond the scope of this paper.

Limiting Cases. In certain limiting situations, eq 12 can be extensively simplified and has proved very useful in our laboratory for the interpretation of steady-state enzyme kinetic data. Some of the interesting situations are examined below.

CASE 1. If only a single polymer with a specified monomer sequence of DP_n is present, the velocity expression simplifies to

$$\tilde{v} = \frac{[E_0][S] \sum_{i=1}^{n} (k_{+2,i} + k_{r,i})\theta_i}{1 + [S] \sum_{i=1}^{n} \theta_i} \tag{20}$$

which is identical with eq 34 specifically derived for this simple situation in Hanson's (1962) paper. The kinetics of pure polymer cleavage will not be further elaborated here since this scheme has been thoroughly treated elsewhere (Hanson, 1962). It should be noted, though, that θ_i and hence $\tilde{K}_m$ and $\tilde{V}_{max}$ will be functions of the monomer arrangement in the polymer. It is reasonable, then, that enzyme studies lead to useful information if the monomer composition and sequence are judiciously chosen.

CASE 2. The general expression (eq 12) for enzyme-catalyzed degradation yields useful results when applied to cleavage of mixtures of homopolymers. Ideally one should work with polymers of known DP, but unfortunately such polymers are only obtained with difficulty and their chain length generally is limited to a DP of 10 or less. Since a great deal can be learned about enzyme catalysis of homopolymers from a study of the variation of $\tilde{K}_m$, $\tilde{V}_{max}$, and derived functions with chain length, it is of great importance to relate the Michaelis parameters for a mixed population of homopolymers of average DP, x, to that of a pure homopolymer of DP, x.

In the simplest situation of endwise degradation of homopolymers, it may be envisioned that rate constants for all comparable processes are the same. For this situation it is readily shown that

$$[ES_i] = [S_a][E](\kappa)\{N_i + N_{i+1}\phi + N_{i+2}\phi^2 + \ldots + N_n\phi^{n-1}\} \quad (21)$$

and the total concentration of all enzyme substrate complexes is then found to be

$$\sum_{i=1}^{n}[ES_i] = [S_a][E](\kappa)\left\{\sum_{i=1}^{n}N_i + \phi\sum_{i=2}^{n}N_i + \phi^2\sum_{i=3}^{n}N_i + \ldots + N_n\phi^{n-1}\right\} \quad (22)$$

N_i will then be a function of polymer distribution and the situation for a most probable distribution and a Poisson distribution are examined below.

CASE 2A. MOST PROBABLE DISTRIBUTION. If a variable, g, is defined as the number of monomer units in a given polymer, then it follows that i ranges from 1 to $g - 1$. For a most probable distribution, $n = \infty$ and N_g is related to the number average DP, $\overline{DP}_n$, by the following relationship (Tanford, 1961; Flory, 1953)

$$N_g = \frac{(\overline{DP}_n - 1)^{g-1}}{\overline{DP}_n{}^g} \quad (23)$$

Substituting in eq 22 for N_i in terms of DP yields a rapidly converging infinite geometric series

$$\sum_{i=1}^{\infty}[ES_i] = [S_a][E](\kappa)\left\{\frac{\overline{DP}_n - 1}{\overline{DP}_n} + \phi\left(\frac{\overline{DP}_n - 1}{\overline{DP}_n}\right)^2 + \phi^2\left(\frac{\overline{DP}_n - 1}{\overline{DP}_n}\right)^3 + \ldots\right\} \quad (24)$$

which sums to

$$\sum_{i=1}^{\infty}[ES\,] = [S_a][E](\kappa)\left(\frac{\overline{DP}_n - 1}{\overline{DP}_n}\right)\times\left(\frac{1}{1 - \phi\left(\frac{\overline{DP}_n - 1}{\overline{DP}_n}\right)}\right) = [S_a][E](c)(\kappa) \quad (25)$$

If K_0 is the inhibition constant of the monomer and $1/\overline{DP}_n$ is the mole fraction of the monomer which will undoubtedly act as a competitive inhibitor for the reaction, then

$$\tilde{v} = \frac{[E_0][S_a](k_{+2} + k_i)(c)(\kappa)}{1 + [S_a]\left\{(c)(\kappa)\frac{1}{DP_nK_0}\right\}} \quad (26)$$

The relationship of $\tilde{V}_{max(MPD)}$ and $\tilde{K}_{m(MPD)}$ (MPD signifies a property of a most probable distribution) to $\tilde{V}_{max(x)}$ and $\tilde{K}_{m(x)}$ for a pure polymer of DP, x, are expressed by the following equations when $\overline{DP}_{n(MPD)} = x$

$$\frac{\tilde{V}_{max(x)}}{\tilde{V}_{max(MPD)}} = 1 + \frac{1}{(K_0)(\kappa)}\left(\frac{1}{x - 1} - \frac{\phi}{x}\right) \quad (27)$$

$$\frac{\tilde{K}_{m(x)}}{\tilde{K}_{m(MPD)}} = \frac{1 - \phi}{1 - \phi^{(x+1)}}\left\{\frac{1}{(K_0)(\kappa)(x)} + \frac{x - 1}{x - \phi(x - 1)}\right\} \quad (28)$$

$\tilde{V}_{max(x)}$ and $\tilde{K}_{m(x)}$ come directly from eq 20 which simplifies when the assumption is made that rate constants for comparable reactions are identical. It is apparent from inspection of eq 27 and 28 that the $\lim_{x\to\infty}(\tilde{V}_{max(x)}/\tilde{V}_{max(MPD)}) = 1$ and that the $\lim_{x\to\infty}(\tilde{K}_{m(x)}/\tilde{K}_{m(MPD)}) = 1$. For small values of x the ratios defined by eq 27 and 28 increase as $\phi \to 1$ and $1/[(K_0)(\kappa)]$ becomes large. In qualitative terms, as the $\overline{DP}_n$ decreases end effects play a more important role in determining the values of the various parameters. The discrepancies between the values of $\tilde{V}_{max}$ and $\tilde{K}_m$ determined for the homopolymer mixtures and pure homopolymers are graphically displayed in Figures 1 and 3.

CASE 2B. POISSON DISTRIBUTION. Another distribution commonly encountered in polymer populations is the Poisson distribution. If, for this distribution, we define a function, v, such that $v = \overline{DP}_n - 1$, then the mole fraction of a g-mer is given as (Tanford, 1961)

$$N_g = \frac{e^{-v}v^{g-1}}{(g - 1)!} \quad (29)$$

Remembering that i ranges from 1 to $g - 1$, we have, after substituting for N_i in eq 22

$$\sum_{i=1}^{\infty}[ES_i] = [S_a][E](\kappa)\{1 - e^{-v} + \phi[1 - e^{-v}(1 + v)] + \phi^2[1 - e^{-v}(1 + v + v^2/2!)] + \ldots\} \quad (30)$$

which after summing similar terms gives on rearrangement

$$\sum_{i=1}^{\infty}[ES_i] = [S_a][E](\kappa)\left\{\frac{1 - e^{v}}{1 - \phi} - \frac{e^{-v}}{1 - \phi}\times\left(v\phi + \frac{(v\phi)^2}{2!} + \frac{(v\phi)^3}{3!} + \ldots\right)\right\} \quad (31)$$

and sums to

$$\sum_{i=1}^{\infty}[ES_i] = [S_a][E](\kappa)\left\{\frac{1}{1 - \phi}\left(1 - e^{-v(1-\theta)}\right)\right\} \quad (32)$$

When $\overline{DP}_{n(PD)} = x$, it can be shown that

$$\frac{\tilde{V}_{\max(x)}}{\tilde{V}_{\max(\mathrm{PD})}} = 1 + \frac{1}{(K)(\kappa)}\left(\frac{(1-\phi)e^{-(x-1)}}{1-e^{-(x-1)(1-\phi)}}\right) \quad (33)$$

and

$$\frac{\tilde{K}_{m(x)}}{\tilde{K}_{m(\mathrm{PD})}} = \frac{1-\phi}{1-\phi^{(x+1)}}\left\{\frac{e^{-(x-1)}}{(K_0)(\kappa)} + \frac{1-e^{-(x-1)(1-\phi)}}{1-\phi}\right\} \quad (34)$$

where PD indicates a function characteristic of a population of molecules belonging to a Poisson distribution. Equations 33 and 34 for a Poisson distribution may be compared to eq 27 and 28 for the most probable distributions. Again the

$$\lim_{x\to\infty}\frac{\tilde{V}_{\max(x)}}{\tilde{V}_{\max(\mathrm{PD})}} = 1 \text{ and the } \lim_{x\to\infty}\frac{\tilde{K}_{m(x)}}{\tilde{K}_{m(\mathrm{PD})}} = 1$$

Substrate Conformation Effects. It is well established that many biopolymers are average, or flexible structures and assume time average or statistical conformations which may depend upon chain length. Thus at any particular instant only a fraction of the polymers will possess the conformation necessary for binding to the active center of an enzyme. The effective substrate concentration will then be less than the measured concentration. The influence of these effects on steady-state enzyme kinetics is quantitatively considered below. Because of the extreme complexity of handling the full steady-state analysis only two limiting cases are presented. In the first situation it is assumed that all the various conformers are in a state of equilibrium and in the second situation the rate of formation of active substrate is allowed to be rate controlling.

CASE 1. If $[S_i]$ and $[\sigma_i]$ are respectively the active and inactive forms of the substrate, governed by the following equilibrium

$$S_i \rightleftharpoons \sigma_i\,;\ K_i = \frac{[\sigma_i]}{[S_i]} \quad (35)$$

the conservation equation is

$$[S_{i,\mathrm{total}}] = [S_i] + [\sigma_i] \quad (36)$$

There will probably be a large number of active and inactive molecules of different conformation, but as long as they remain in equilibrium with each other eq 35 and 36 obtain and the effective concentration of S_i is

$$[S_i] = \frac{[S_{i,\mathrm{total}}]}{1+K_i} \quad (37)$$

For a single pure polymer, substitution for $[S_i]$ into eq 6 leads to an equation where $[S_i](\kappa_i)$ is replaced by $[S_i](\kappa_i)/(1+K_i)$. Although the dependency of $\bar{v}$ on substrate concentration is of increased complexity, the form of the equation is not altered, Michaelis–Menten kinetics are still obeyed, and it can be shown that $\tilde{V}_{\max}$ is unaltered but $\tilde{K}_m$ increases by the factor, $(1+K_i)$. This case is readily extended to the situation where substrates in different conformations are bound with varying degrees of avidity. For a polymer mixture the situation will be considerably more complicated when K_i is a function of chain length.

CASE 2. If conversion of inactive substrate to active substrate is very slow relative to the catalytic process, then the velocity (at sufficiently high enzyme concentration) will be governed by the first-order rate of formation of active substrate. As the enzyme concentration is lowered, however, the rate of reaction will ultimately be governed by the second-order rate of formation of enzyme–substrate complex. In the former case (high $[E_0]$) both a plot of $\bar{v}$ *vs.* [S] and a Lineweaver–Burk plot will be linear but the latter plot will have an intercept, $1/\tilde{V}_{\max}$, of 0. When the rate is governed by the bimolecular association of the enzyme and substrate, both plots will again be linear but will have slopes dependent upon the enzyme concentration and intercepts of 0.

Enzyme Effects. A number of years ago Koshland (1958) proposed the induced fit hypothesis as a modification of the classical lock and key theory of Fisher to more extensively account for the mechanism of enzyme binding of substrate. In this theory it was envisaged that the substrate induced the enzyme to assume its catalytic geometry. Thermodynamically these postulates can be expressed by Scheme II for the simplest system. In the scheme, ϵ and E represent enzyme in the inactive and active conformations, respectively. It is reasonable to assume that ϵ is in equilibrium

$$\epsilon \overset{K}{\rightleftharpoons} \mathrm{E} \underset{k_{-1}}{\overset{k_1 S}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k_{+2}} \mathrm{E} + \mathrm{P}$$

SCHEME II

with E since it has been demonstrated that protein conformation changes can occur at a rate faster than enzyme-catalyzed reactions (Eigen and Hammes, 1963). Granting this assumption, a steady-state analysis reveals that this situation cannot be distinguished from the situation where substrate undergoes a conformation change. $\tilde{V}_{\max}$ will not be affected but $\tilde{K}_m$ will be enhanced by the factor, $(1+K)$. This analysis can obviously be extended to more complicated cases.

Competitive Self-Inhibition. It now seems reasonably well established that substrates can act as their own competitive inhibitors by forming unproductive enzyme–substrate complexes (Thoma and Koshland, 1960; Hein and Neiman, 1961; Wolf and Neiman, 1963). Since the inhibitor concentration (in this case substrate) obviously cannot be varied independently of substrate concentration, the effect will normally go unnoticed and appear in disguise in an apparent Michaelis constant. In view of accumulating experimental evidence, it can be anticipated that this phenomenon will be encountered frequently. Fortunately in the case of polymers another

variable (chain length) can be used to dissect the inhibition constant from the observed Michaelis constant. This situation is formulated below. If it is assumed that the inhibited enzyme–substrate complexes are in equilibrium with free enzyme and substrate and that a substrate of i bonds can combine in j discrete ways to form inhibited complexes, the generalized reaction can be represented as

$$E_{I_j}S_i \underset{k_{+4,i,j}}{\overset{k_{-4,i,j}}{\rightleftharpoons}} E + S_i \tag{38}$$

where the subscript I represents an inhibited complex. The apparent self-inhibition constant, $\tilde{K}_{si}$, can then be defined as

$$\tilde{K}_{si} = \frac{[E]\sum_{i=1}^{n}\sum_{j}[S_i]}{\sum_{i=j}^{n}\sum_{j}[E_{I_j}S_i]} = \frac{[E][S_a]\sum_{i=1}^{n}\sum_{j}N_i}{\sum_{i=1}^{n}\sum_{j}[E_{I_j}S_i]} \tag{39}$$

Then to derive the dependence of $\tilde{v}$ on $[S_a]$ for this situation it is only necessary to add the term

$$\frac{[S_a]\sum_{i=j}^{n}\sum_{j}N_i}{\tilde{K}_{si}}$$

to the denominator of eq 12. When this substitution is made we find that

$$\frac{1}{\tilde{K}_{m,si}} = \frac{1}{\tilde{K}_m} + \frac{\sum_{i=1}^{n}\sum_{j}N_i}{\tilde{K}_{si}} \tag{40}$$

and that

$$\tilde{V}_{max,si} = \frac{\sum_{i=1}^{n}(k_{+2,i} + k_{r,i})\sum_{p=i}^{n}N_p\kappa_p\theta_p}{\sum_{i=1}^{n}\sum_{p=i}^{n}N_p\kappa_p\theta_p\left(\sum_{i=1}^{n}\sum_{j}N_i\right)\Big/\tilde{K}_{si}} \tag{41}$$

But $[E_0]\,\tilde{k}$ remains unaltered so that the inhibition effects are unobserved at very low substrate concentrations.

From eq 40 and 41 it is apparent that both $\tilde{K}_m$ and $\tilde{V}_{max}$ can be profoundly influenced by competitive self-inhibition. If other competitive inhibitors are added to the system, then a term of the form $[I]/K_I$ [where I is inhibitor concentration and K_I is the dissociation constant of the enzyme–(Linderstrøm-Lang, 1953)[2] inhibitor complex] will appear as a denominator term of eq 12 for each inhibitor and correspondingly influence $\tilde{V}_{max}$, $\tilde{K}_m$, and $\tilde{k}$. This inhibition constant, however, can be sorted out of the apparent Michaelis constant by conventional techniques if inhibitor concentration is varied independently of substrate concentration.

Again it is of interest to consider the situation for cleavage of a homopolymer population where all bonds and binding constants for internal inhibition are held to be constant. If the enzyme site spans m monomer units, then a polymer of DP, x, can form $x - m$ self-inhibited complexes but only one active complex. For this situation it has been demonstrated that for any polymer population (S. Dygert and J. A. Thoma, unpublished work)

$$\frac{\sum_{i=1}^{n}\sum_{j}N_i}{\tilde{K}_{si}} = \frac{x - m}{\tilde{K}_{si}} \tag{42}$$

so that

$$\frac{1}{\tilde{K}_{m,si}} = \frac{1}{\tilde{K}_m} + \frac{x - m}{\tilde{K}_{si}} \tag{43}$$

and

$$\tilde{V}_{max,si} = \frac{[E_0](k_{+2} + k_r)}{1 + \dfrac{\tilde{K}_m(x - m)}{\tilde{K}_{si}}} \tag{44}$$

In the absence of multiple attack, $\tilde{K}_m$ is a constant and independent of chain length so that plots of $1/\tilde{K}_{m,si}$ and $1/\tilde{V}_{max,si}$ *vs.* x will be straight lines with slopes of $1/\tilde{K}_{si}$ and $\tilde{K}_m/\tilde{K}_{si}$ and intercepts of $1/\tilde{K}_m$ and $1/E_0(k_{+2})$, respectively.

On the other hand, if a significant degree of multiple attack occurs (*i.e.*, as the ratio of $k_r/(k_r + k_{+2} + k_{-1}) \rightarrow 1$) then from eq 14 it is seen that $\tilde{K}_m$ is a function of chain length and plots of $1/\tilde{K}_{m,si}$ and $1/\tilde{V}_{max,si}$ *vs.* x are no longer linear. However the limiting slopes as $x - m \rightarrow \infty$ are $1/\tilde{K}_{si}$ and $\tilde{K}_{m(limit)}/\tilde{K}_{si}[E_0](k_{+2} + k_r)$, respectively, where $\tilde{K}_{m(limit)}$ is the limiting value of $\tilde{K}_m$ (*i.e.*, for the active complex) for a long chain. Since these reciprocal plots of the Michaelis functions *vs.* $x - m$ will be considered in greater detail in a forthcoming paper, additional comments about them will not be made here.

It should be pointed out, though, that both $\tilde{K}_m$ and $\tilde{V}_{max}$ for a pure homopolymer and for a homopolymer mixture will be weighted by the function $(x - m)/\tilde{K}_{si}$. Hence it is obvious that in the presence of competitive self-inhibition the ratios, $\tilde{V}_{max}/\tilde{V}_{max(polymer\ mixture)}$ and $\tilde{K}_m/\tilde{K}_{m(polymer\ mixture)}$, will approach unity more rapidly as x becomes large than in the absence of competitive self-inhibition.

Discussion

While the analysis presented here has been limited to considerations of systems of constant pH and temperature, it is obvious that these restrictions could be

[2] This paper has also been reprinted in the "Selected Papers" of Linderstrøm-Lang published by Academic and the Danish Science Press in 1962.

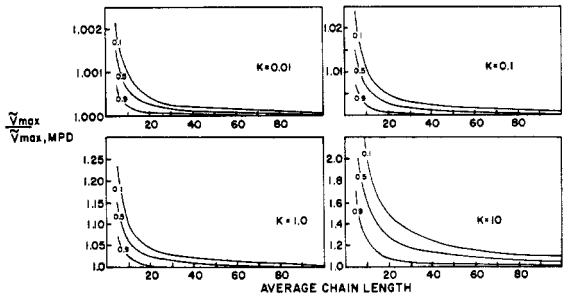


FIGURE 1: Dependence of the ratio of the apparent maximum velocity for a pure homopolymer and homopolymers of the most probable distribution upon chain length. The numbers in the figure correspond to the degree of repetitive attack, ϕ, as defined by (2). ϕ is assumed independent of chain length. $K = 1/K_0\kappa$ and is a measure of the effectiveness of monomer to act as a competitive inhibitor. Large values of K correspond to effective monomer inhibition; curves computed from eq 27.

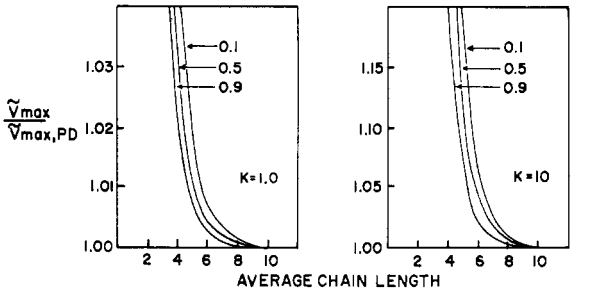


FIGURE 2: Dependence of the ratio of apparent maximum velocity for pure homopolymer and homopolymers of the Poisson distribution upon chain length; numbers and K as in Figure 1; computed according to eq. 33.

relaxed in a more complete treatment of this problem. Even within its limited framework, the developments presented here cannot be considered in any way complete but can only serve as an introduction to the steady-state analysis of enzyme–polymer systems. Because of the almost infinite variety of possible interactions between polymers and enzymes, additional ramifications are obviously too numerous to require further discussion here. Modifications and extensions of the schemes presented above will need to be implemented as experiments demand.

From these derivations, though, a number of general patterns have emerged indicating how $\tilde{K}_m$ and $\tilde{V}_{max}$ might vary in different systems. For example, it is apparent that an almost endless variety of phenomena occurring at the molecular level may be disguised in the simple Michaelis–Menten equation. More often than not these phenomena will affect $\tilde{V}_{max}$ and $\tilde{K}_m$ in independent ways. The various effects of changes in the system or molecules on the Michaelis–Menten equation or constants can be summarized as follows. Heterogeneity in monomer composition, variation in the type of polymer distribution, competitive self-inhibition, repetitive attack, conformation changes of both enzyme and substrate, and multiple intermediates will all affect the experimentally measured $\tilde{K}_m$. Competitive self-inhibition will always operate to produce a lower $\tilde{K}_m$ than would be observed in the absence of this effect. Contrariwise, enzyme conformation changes as well as an increase in the degree of repetitive attack will produce a larger $\tilde{K}_m$ than would be observed in the absence of these effects. Unfortunately the changes in $\tilde{K}_m$ which will occur as other factors are altered cannot necessarily be predicted *a priori*. All of the factors mentioned above except either conformation changes occurring at the terminal end of the substrate or conformation changes of the enzyme will influence $\tilde{V}_{max}$. That the conformation changes of the enzyme or conformation changes at the end of the polymers should not affect the maximum velocity (in the absence of competitive effects) is intuitively obvious, because this is the velocity measured at substrate saturation. At infinite substrate concentration all of the enzyme, because of appropriate shifts of equilibria, should be complexed with substrate. Conversely, shifts in equilibria will be reflected by changes in $\tilde{K}_m$.

From the above analysis one can anticipate that linear double reciprocal plots will be observed for mixed polymer systems regardless of the extent of heterogeneity within the polymers and regardless of the type of polymer distribution used as substrate. The only exception to this general rule could occur when the rate of a conformation change of a substrate is of the same order of magnitude as the enzyme-catalyzed degradation. To our knowledge this situation has never been experimentally encountered.

To disentangle experimentally the molecular events influencing $\tilde{K}_m$ and $\tilde{V}_{max}$, it will be necessary to vary the structure and the nature of the polymer. For a definitive interpretation of the data, molecules of precisely defined structures and composition should be employed. Such investigations are most conveniently performed with homopolymers. For this reason it is most encouraging that useful information about the degradation of pure homopolymers can be obtained from studies with mixed homopolymer populations. In the Theoretical Section it was established that for homopolymers (under reasonable limiting conditions) the Michaelis–Menten parameters for homopolymer mixtures of average DP, x, approached those of a homopolymer of DP, x, as the value of x increases. For practical purposes, however, especially for experimental design, it is important to ascertain how rapidly $\tilde{K}_{m(x)}/\tilde{K}_{m\text{(polymer mixture)}}$ and $\tilde{V}_{max(x)}/\tilde{V}_{max\text{(polymer mixture)}}$ ratios converge to unity. These ratios as a function of chain length are recorded in Figures 1–4 for different conditions. The computations were limited to polymers having an average chain length of four or more because homopolymers in the range of 1–10 can be readily ob-

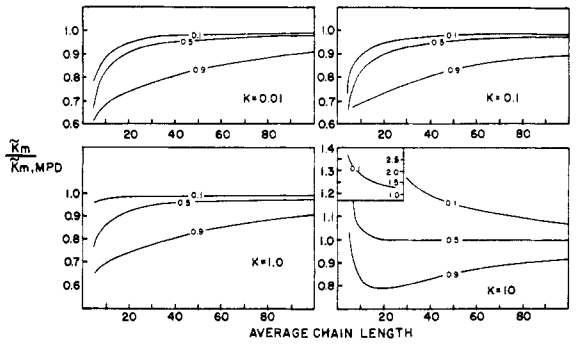


FIGURE 3: Dependence of apparent Michaelis constant for pure homopolymer and homopolymers of the most probable distribution upon chain length; numbers and K as in Figure 1; computed according to eq 28.

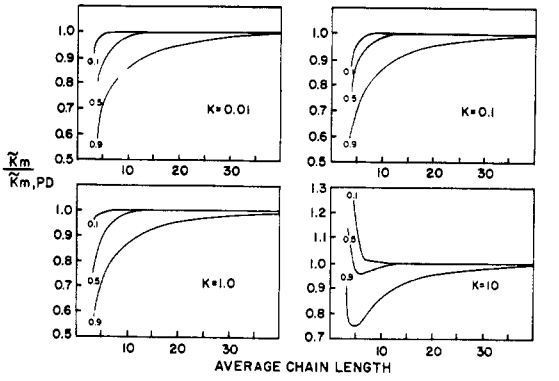


FIGURE 4: Dependence of ratio of apparent Michaelis constant for pure hompolymer and homopolymers of the Poisson distribution upon chain length; numbers and K as in Figure 1; computed according to eq 34.

tained by conventional laboratory techniques (for pertinent references, see Hanson, 1962). From these figures several important conclusions pertinent to experimental design emerge. Perhaps the most important generality is that the observed Michaelis–Menten constants for a polymer with a Poisson distribution of $\overline{DP}_n$, x, are always better estimates of the constants for a homopolymer of DP, x, than those obtained by a most probable distribution. This conclusion is intuitively satisfying because the polymers of a Poisson distribution cluster more closely about the average chain length than those of a most probable distribution. The latter distribution is heavily weighted by the smaller molecules of the series. The lower homologs, especially when they act as effective competitive inhibitors, will introduce large end effects and account for the slow convergence of the ratios plotted in Figures 1 and 4. It seems intuitively obvious that if the distribution were sharpened by removal of the small molecules, the ratios $\tilde{V}_{max(x)}/\tilde{V}_{max(polymer\ mixture)}$ and $\tilde{K}_{m(x)}/\tilde{K}_{m(polymer\ mixture)}$ would converge more rapidly as x is increased. It is also interesting to note (other things being equal) that the $\tilde{V}_{max(x)}/\tilde{V}_{max(polymer\ mixture)}$ ratio converges more rapidly to unity than the $\tilde{K}_{m(x)}/\tilde{K}_{m(polymer\ mixture)}$ ratio. As might be anticipated, both the ratio of the Michaelis constant to monomer inhibitor constant (K in the figures) and the degree of repetitive attack affect the rate at which $\tilde{V}_{max(x)}/\tilde{V}_{max(polymer\ mixture)}$ and $\tilde{K}_{m(x)}/\tilde{K}_{m(polymer\ mixture)}$ approach unity. These curves have been calculated assuming no competitive self-inhibition but the ratios will converge even more rapidly to unity as competitive self-inhibition becomes more important. Finally, it is interesting to note that the $\tilde{V}_{max(x)}/\tilde{V}_{max(polymer\ mixture)}$ ratio is always greater or equal to unity, whereas the ratio $\tilde{K}_{m(x)}/\tilde{K}_{m(polymer\ mixture)}$ may be equal to, less than, or greater than unity. $\tilde{K}_{m(polymer\ mixture)}$ may be smaller than $\tilde{K}_{m(x)}$ because the competitive inhibition constant for the monomer is disguised in the apparent Michaelis–Menten constant. When the monomer is bound more strongly than substrate, this will have the effect of decreasing the apparent Michaelis–Menten constant.

On the basis of these plots it would appear that polymers of the Poisson distribution or other narrow distributions may be substituted for pure polymers and give reliable estimates of $\tilde{K}_{m(x)}$ and $\tilde{V}_{max(x)}$ provided the average chain length is 20 or more and the degree of repetitive attack is not too large. The bias that would be introduced by assuming that the observed Michaelis–Menten parameters for the polymer mixture are the same as those of the pure polymer of comparable chain length would amount to only a few per cent in most instances. Since this bias is normally several times smaller than the standard error of experimentally measured Michaelis constants, the bias could easily be tolerated. When conducting kinetics studies on polymer mixtures, it should be remembered that the number average molecular weight rather than the weight average molecular weight or the z-average molecular weight should be used to compute $[S_a]$ to avoid distortion of $\tilde{K}_{m(polymer\ mixture)}$.

Appendix

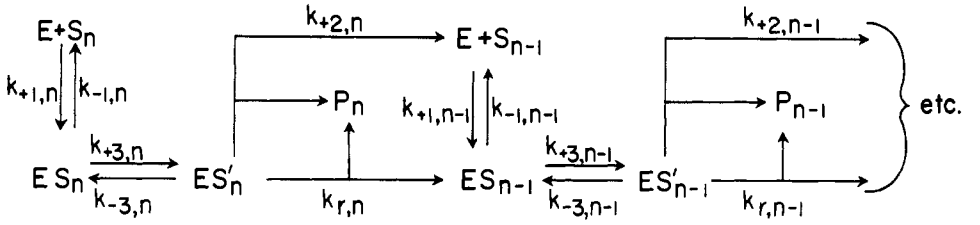
It will be demonstrated inductively here that isomerization of central complexes does not alter the functional relationship of $\tilde{v}$ to [S] given by eq 12. Straightforward analysis of Scheme III with the conventional steady-state approximation for homopolymers leads directly to expression for $[ES_n']$ and $[ES_{n-1}']$

$$[ES_n'] = [E][S_n]\kappa_n' \tag{45}$$

$$[ES_{n-1}'] = [E]\{[S_{n-1}](\kappa_{n-1}') + [S_n](\kappa_n')\phi_n'\} \tag{46}$$

where

$$\kappa_n' = \frac{k_{+3,n}k_{+1,n}}{k_{-1,n}(k_{-3,n} + k_{+2,n} + k_{r,n}) + k_{+3,n}(k_{+2,n} + k_{-,n})}$$



SCHEME III

and

$$\kappa_{n-1}' = \frac{k_{+1,n-1}k_{+3,n-1}}{k_{-1,n-1}(k_{-3,n-1} + k_{+2,n-1} + k_{r,n-1}) + k_{+3,n-1}(k_{+2,n-1} + k_{r,n-1})}$$

and

$$\phi_n' = \frac{k_{r,n-1}k_{+3,n-1}}{k_{-1,n-1}(k_{-3,n-1} + k_{+2,n-1} + k_{r,n-1}) + k_{+3,n-1}(k_{+2,n-1} + k_{r,n-1})}$$

The analysis is readily extended to more complicated situations. The expressions eq 45 and 46 are of the same form as those presented in eq 1 and 2. It is quite obvious that extension of the analysis (*i.e.*, 3–5) will lead to an expression of the form of eq 12 where the functions κ and θ_i are replaced by primed functions of greater complexity. This conclusion is unaltered by the inclusion of more central complexes and it follows that steady-state kinetics yields no information about the number or types of central complexes involved in enzyme cleavage.

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