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MODELS FOR DEPOLYMERIZING ENZYMES: CRITERIA FOR DISCRIMINATION OF MODELS

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ABSTRACT

Several models for the action of alpha amylase have been proposed to account for the nonrandom distribution of oligosaccharides in the amylase digests of polysaccharides. The preferred-attack model attempts to account for the nonrandom distribution by assuming that the probability for bond cleavage depends upon the position of the bond in the chain. The repetitive, or multiple-attack, model suggests that the nonrandom distribution of oligosaccharides arises because an amylase can form a cage-like complex with a substrate and attack it several times during a single encounter. The multiple-enzyme or dual-site model suggests that the nonrandom yield of oligosaccharides arises from the combined action of exo- and endo-enzymes. The effects of pH, inhibitors, and substrate chain-length on enzyme action have been studied in several laboratories to determine which of the three action-patterns best describes the action of alpha amylase. The influence of these variables on product distributions or enzyme action-patterns are mathematically modeled in the Appendix. The experimental data on porcine-pancreatic alpha amylase are discussed in the light of the derivations.

DISCUSSION

Despite voluminous evidence to the contrary, the notion that amylases operate exclusively by a random-attack mechanism persists in the literature and particularly in textbooks. Cleavage patterns of carbohydrases depend upon many factors, such as the structure and chain length of the substrate[1–4], pH[5,6], temperature[7], inhibitors[8], and the origin of the enzyme[4,6]. Consequently, the effects that these factors have on cleavage patterns of amylases have often been studied to gain insight into the molecular mechanisms of hydrolysis. (For recent reviews, see refs. 4, 6, 9). In spite of abundant experimental data, there are still conflicting interpretations of the molecular events underlying the behavior of amylases[4,5,8–11]. These alternative interpretations of data can in large measure, be traced to the incomplete theoretical analysis of the various models which purport to account for amylase behavior. The purpose of this paper is to place these conceptual models into a more-complete mathematical framework and to comment on published results for several amylases in the light of this

framework. The newly developed mathematical models for amylase action help to resolve the conflicting interpretation of experimental data by pinpointing the ambiguties inherent in certain experiments designed to discriminate between the various models for alpha amylolysis.

The models depicted in Fig. 1 that have been used to explain the action of alpha amylase are: (*a*) a random-attack model, (*b*) a preferred-attack model, (*c*) a multiple- or repetitive-attack model, and (*d*) a multiple-site model. In (*a*), the amylase binds to the substrate at random, hydrolyzes a single bond, and then dissociates from both substrate fragments. The enzyme then attacks a second substrate molecule. In (*b*), the enzyme shows a preference for rupturing certain bonds of the substrate (for instance, bonds near one terminus of the molecule). In (*c*), the enzyme cleaves a substrate at random but releases only one of the newly formed substrate fragments. The retained fragment suffers reattack one or more times near the newly formed terminus before the enzyme–substrate complex finally dissociates. In (*d*), an amylase possesses two active sites, one that operates by a random-hit mechanism, and another that attacks chain ends to produce oligosaccharides. However, the mechanisms are not mutually exclusive and an amylase may conceivably shift from one mechanism to another under different experimental conditions. For example, the multiple-site model predicts that, if the endo-amylase activity can be selectively inhibited, the amount of oligosaccharides in the amylase digest should be proportional to the concentration of inhibitor. In general, the yield of oligosaccharides generated by

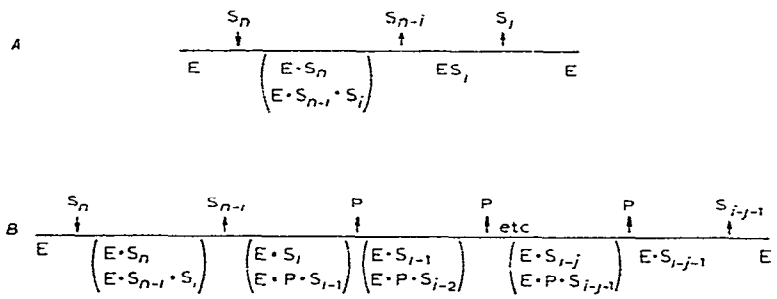

Fig. 1. Comparison of random-attack, preferred-attack, repetitive-attack, and multiple-site models for depolymerizing enzymes.

Random-attack model (Fig. 1*A*): An effective encounter (formation of enzyme–substrate complex that leads to products) is always followed by dissociation of both substrate fragments from the enzyme. E and S are, respectively, enzyme and substrate and n is the chain length, and i is a random number from 1 to $n-1$. Preferred attack, Fig. *A*, i is nonrandom and will depend upon the enzyme and the size of the original substrate n. Repetitive-attack model, (Fig. 1*B*): An effective encounter is followed by dissociation of only one substrate fragment, namely S_{n-i}. The other substrate fragment, S_i, remains associated with the enzyme. S_i is attacked at one terminus one or more times to yield D-glucose or small oligosaccharides (P) before it finally dissociates from the enzyme to give S_{i-j-1}. The value j is the number of repetitive attacks and i is essentially a random number from 1 to $n-1$.

In the multiple-site model, one active site cleaves substrate bonds at random. In *Fig. A*, i is random and the other active site cleaves oligosaccharides at the substrate terminus. In the latter case, i is normally restricted to an integer between 1 and 5 (Fig. *A*).

nonrandom hydrolysis under a variety of conditions was thought to give valuable clues about the molecular mechanisms of amylase action.

In the Appendix, equations are derived that show how the ratio of random to nonrandom hydrolysis depends on pH, chain length, substrate, and inhibitor concentrations for the different models. A ratio (R) is defined that is proportional to the degree of nonrandom hydrolysis; $R = [\dot{P}]/[\dot{S}]$, where $[\dot{P}]$ is the rate of production of D-glucose (or oligosaccharides) with time, and $[\dot{S}]$ is rate of change of the concentration of substrate fragments with time; $[\dot{S}]$ is proportional to random hydrolysis.

In the following sections, the effects of experimental conditions and reaction mechanisms on R are examined. The dependence of R on the degree of hydrolysis is a valuable guide for distinguishing between the mechanisms[9,11,12].

Effects of pH. — The effect of pH on the action pattern of amylases is normally monitored by (*a*) chromatographic examination of the reaction products [9] or (*b*) by examination of the concomitant change of the iodine-staining capacity (blue value) and the reducing value of the amylase digest[5,8]. A plot of blue value *vs.* reducing value gives slightly curved lines having a negative slope, whose magnitude is proportional to the extent of random degradation of the substrate[5,8]. Thus, a repetitive-attack enzyme that has a nonrandom component superimposed on a random component will give plots having smaller negative slopes than enzymes operating solely by a random-hit mechanism.

Blue value (BV) *vs.* reducing value (RV) plots for porcine-pancreatic, *Aspergillus oryzae*, and human-salivary alpha amylases acting near their pH optima all have slopes less negative than the plot for H^+ (random-catalyzed degradation[5]). However, when the pH is raised to 10.5, the slope for porcine-pancreatic alpha amylase becomes much more negative, and the enzyme appears to shift toward random hydrolysis[5]. Robyt and French interpreted these results to mean that these three enzymes operate by a repetitive-attack mechanism under optimum conditions. At highly alkaline pH values (near 10.5), the BV *vs.* RV curves for the amylases are quite similar to the curve produced by H^+. Robyt and French suggested that, under the adverse conditions of high pH, the nonrandom component disappears and the enzymes operate exclusively by random attack. This is equivalent to saying that, under optimum conditions, an enzyme–substrate complex has a finite probability for reacting more than once before it dissociates but, under adverse conditions, it almost always dissociates after hydrolysis.

However, Banks and coworkers offered an alternative interpretation of the shift in BV *vs.* RV plots at high pH[11]. They noted than an amylase may be a mixture of isoenzymes or have two active sites, one to attack a chain terminus and another to attack interior segments randomly. They reasoned that selective repression of the exo type of activity at extreme pH values would shift the overall action pattern toward random cleavage. In a more-recent paper, Banks and coworkers have agreed that porcine-pancreatic alpha amylase does operate by repetitive attack under optimum conditions and is not composed of a mixture of isoenzymes[13].

It is instructive to examine these two interpretations in the light of the

mathematical models presented in the Appendix. In the first place, it should be obvious that a random-attack model cannot account for any change in action pattern and is therefore inconsistent with the experimental data. The pH dependence of R, for the repetitive-attack model, was found to be (see Appendix)

$$R = \frac{\text{const } k_r'}{\frac{k_{\eta+1}[H^+]}{K_{es1}} + k_\eta + \frac{k_{\eta-1}K_{es2}}{[H^+]}}, \qquad (1)$$

where k_r' is the pH-independent hydrolysis coefficient, K_{es1} and K_{es2} are the first and second proton dissociation-coefficients of the enzyme–substrate (es) complex, η is the charge on the enzyme, and the k_η values are the rates of dissociation of the ES complexes having $\eta+1$, η, and $\eta-1$ charges.

At alkaline pH values, the term $k_{\eta-1}K_{es2}/[H^+]$ may dominate the denominator of Eq. *1* and force R to fall toward zero as the pH is raised. Normally, as $[H^+]$ increases, the term, $k_{\eta+1}[H^+]/K_{es1}$, should become dominant and force R toward zero. Experimentally, it is found that R decreased at high pH values but is independent of pH on the acid side of the pH optimum[5], down to pH 3.5 for porcine-pancreatic alpha amylase. In the light of Eq. *1*, these results must be interpreted to mean that the term $k_{\eta+1}[H^+]/K_{es1}$ makes inconsequential contributions to the denominator at all pH values tested. In other words, dissociation of the ES complex through a $k_{\eta+1}$ process (Fig. 2) rarely happens. Near the pH optimum, hydrolysis via a k_r' process completes very effectively with dissociation via any k_η pathway (Fig. 2). At high pH values, however, the enzyme–substrate complex tends to decay almost exclusively along the $k_{\eta-1}$ pathway. The lifetime of the complex is too short for a k_r' or hydrolytic

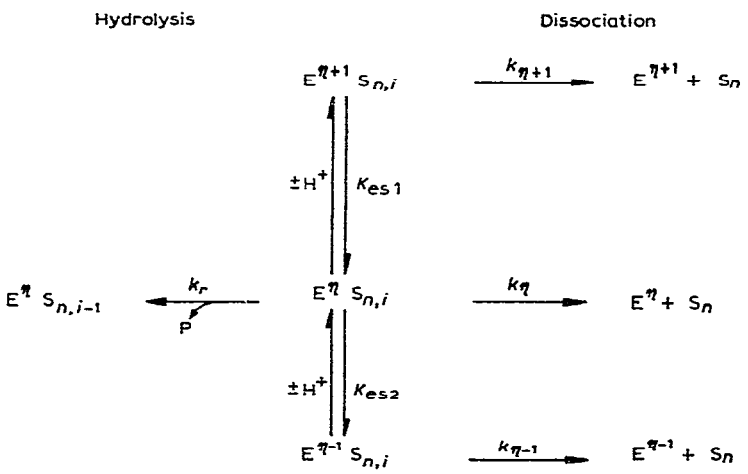

Fig. 2. Fate of an enzyme–substrate complex as a function of pH. The term η is the net charge on the protein. Proton binding occurs at the active site and it is assumed that only those enzymes mono-protonated at the active site catalyze bond rupture. K_{es1} and K_{es2} are respectively the first and second proton-dissociation constants for the enzyme–substrate complex. Modified after Dixon and Webb[14].

event to occur. Thus the repetitive-attack model can readily explain the pH-dependent action pattern of porcine-pancreatic alpha amylase.

For the multiple-site model, the following relationship holds at enzyme saturation. The *f*-functions are the Michaelis pH functions (see Appendix):

$$R = \frac{V_1' f_{\eta,2}}{V_2' f_{\eta,1}}, \tag{2}$$

defining that fraction of the enzyme properly protonated to perform hydrolysis. The prime refers to a pH-independent function, the subscripts 1 and 2 refer to exo- and endo-activity of the two active sites, and η is the charge on the enzyme. As the ratio $f_{\eta,2}/f_{\eta,1}$ may achieve a maximum or minimum at extreme pH values, the multiple-site model can also accommodate the observed shift in action pattern. At subsaturating substrate-concentrations, the pH dependence on both K_m and V come into play and R is a more-complex function than that given in Eq. *2*. Again maxima or minima at pH extremes are possible. However, in the case that the multiple activities were due to isoenzymes, one would expect compensation in the f_η values, and R would be essentially independent of pH.

For the preferred-attack model and a linear substrate of n glucose units, at enzyme saturation, R has the form:

$$R = \frac{\text{const } f_{\eta,i}}{n f_{\eta,1}}, \tag{3}$$

where the subscripts 1 and i represent the first and the ith bonds (Fig. 2A in Appendix) and η is the charge on the enzyme.

For a single-site amylase, $f_{\eta,1}$ would normally parallel $f_{\eta,i}$ as the pH is changed, and R would not be expected to be strongly dependent on pH. Although it is not impossible, it is highly unlikely that the pH shift in action pattern reported for porcine-pancreatic alpha amylase can be attributed to the preferred-attack mechanism. We conclude, then, that the shifts is the action pattern of porcine-pancreatic alpha amylase with pH are most satisfactoriiy, but not conclusively, interpreted in terms of the repetitive-attack model.

Effects of inhibitors. — To test the possibility that the shifts in action pattern of porcine-pancreatic alpha amylase could be explained by the multiple-site model, Banks and coworkers[8] examined the effect of methyl α-D-glucoside and erythritol on the behavior of the enzyme. Both inhibitors shifted the action pattern toward random attack. To interpret these experimental results, they assumed that the compounds acted as selective competitive-inhibitors of exo-amylase activity[8]. It is known though that simple sugars may act either as competitive or non-competitive inhibitors of endo-amylases[15]. Hence, either endo-type or exo-type of activity may have been selectively inhibited by these compounds, making it impossible to predict whether the action pattern would shift towards or away from random attack. In effect, then, any observed effect of these inhibitors on porcine-pancreatic alpha amylase would be consistent with the multiple-site model.

A strictly competitive inhibitor cannot influence the R values of any single-site enzyme. This type of inhibitor simply depresses the activity of the enzyme. In contrast, non-competitive inhibitors may shift the action pattern of any alpha amylase. For the repetitive-attack model, R becomes a function of [N], the concentration of the non-competitive inhibitor, which may or may not act as an alternative acceptor (see Fig. 3). For a repetitive-attack enzyme ,R was found to be

$$R = \frac{\text{const } k'_r K_{N,es}}{k'_{-1} K_{N,es} + (k'_t + k'_{-1,N})[N]}, \tag{4}$$

where $K_{N,es}$ is the dissociation constant for release of N from the ESN complex, k'_{-1}, k'_r, k'_t, and $k'_{-1,N}$ are, respectively, the rate coefficients for dissociation of ES, for hydrolysis of ES, for transfer of substrate to acceptor N and, for dissociation of S from ESN. The prime indicates a rate coefficient independent of [N]. The important point to note here is that R can only decrease as [N] increases, so that, in constrast to the multiple-site enzyme, a repetitive-attack enzyme must always shift to random attack at high [N]. This shift is observed with porcine-pancreatic alpha amylase[5] and is another piece of data consistent with its currently accepted mode of repetitive attack[12].

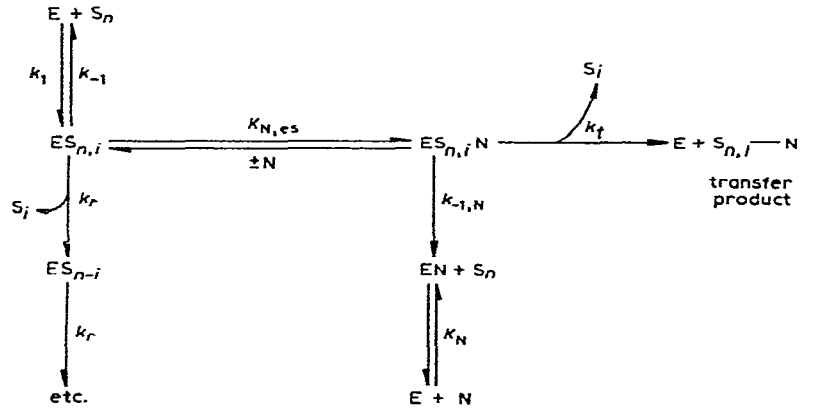

Fig. 3. Alternative pathways for decomposition of an enzyme–substrate complex in the presence of N, a noncompetitive inhibitor or an alternative acceptor if $k_t \neq 0$. N is assumed to bind at the alternative product-binding site and its dissociation constant K_N is the dissociation constant of the EN complex. The dissociation constant of the ternary complex to give the second fragment is $k_{-1,N}$, and the reaction coefficient for transfer to form the alternative product $S_{\overline{n-1}}N$ is k_t. The term i may assume any value less than $n-1$.

The preferred-attack model, like the multiple-site, predicts that the action pattern may shift either toward or away from random hits, depending on whether random or non-random cleavage is selectively inhibited. The extent of the shift will depend on the [N]/[S] ratio. Because of the flexibility of these two models, it will probally be impossible to distinguish between them by inhibition studies in which N

alone is varied. Thus, the observation that inhibitors depress the non-random component of hydrolysis of porcine-pancreatic alpha amylase at constant [S] is also consistent with these two models.

It is important to note, here, that theory predicts that the action pattern will be a function of not only inhibitor but also of substrate concentration. This concentration dependence of the action pattern on both S and N could help to distinguish between the various models.

Effects of chain length. — Because the mathematics of the various models has not been fully developed earlier, the fact that the dependence of an action pattern on chain length is a promising guide for classifying enzyme mechanism has not been fully realized. Let us consider the effect of n upon the behavior of the various models. Intuitively, it may be seen that the extent of repetitive attack should achieve a maximum when n is large. Contrariwise, both the multiple-site model and the preferred-attack model predict that the frequency of endwise bond-cleavage to random bond-cleavage will decrease as n increases.

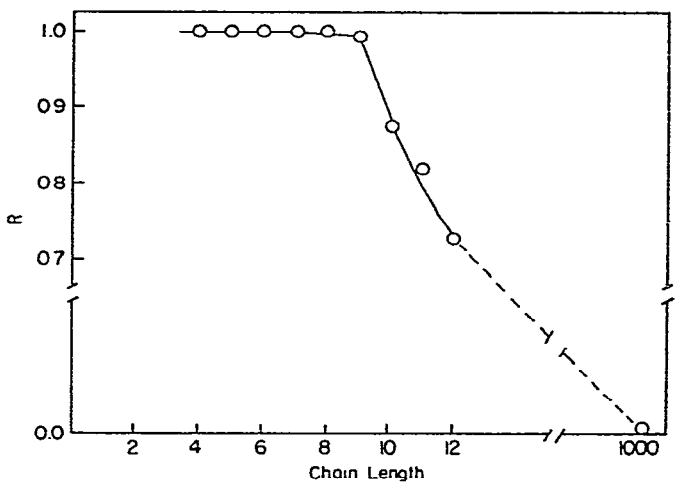

Fig. 4. Ratio of nonrandom/random attack for *Bacillus amyloliquefaciens* amylase as a function of chain length.

O, experimental data; ——, theoretical data. Experimental data from ref. 19 and Li and Thoma (unpublished). *B. amyloliquefaciens* operates exclusively by the preferred-attack mechanism and it tends to cleave the three bonds nearest the reducing end of an oligosaccharide substrate[2]. However, at longer substrate chain-lengths, random cleavage in the interior of the molecule tends to become dominant. Hence for this enzyme, R is defined as the probability of cleaving one of the three bonds at the reducing terminus. The theoretical line was computed with the aid of Eq. *8* in ref. 2. For porcine pancreatic alpha amylase, a repetitive-attack enzyme[12], 4 or 5 moles of oligosaccharides are released for each mole of bonds hydrolyzed at random for a substrate of chain-length 1000.

Mathematical modeling of the repetitive model show that the degree of repetitive attack (θ) does in fact approach a constant for long chains, and R takes the following

form (see Appendix):

$$R \cong \frac{\text{const}\ \theta}{(1-\theta)}. \tag{5}$$

For both the multiple-site model and the preferred-attack model, R simplifies to the following form:

$$R \cong \frac{\text{const}}{n}. \tag{6}$$

Eq. 6 has been verified by the observation that R is inversely proportional to chain length n, when *Bacillus amyloliquefaciens* hydrolyzes the malto-oligosaccharides (Fig. 4). *Bacillus amyloliquefaciens* amylase operates exclusively by preferred attack[2].

CONCLUSIONS

A summary of the theoretical conclusions reached in the Appendix, and the possible significance of the experiments that have been used in an attempt to elucidate the mechanism of alpha amylase actions, are shown in Table I. Some experiments designed to distinguish between the various mechanisms gave ambiguous results. When interpreting the results of such experiments, it is important to remember that, depending upon experimental conditions, a single enzyme may operate by a combination of mechanisms. The relative contribution of any mechanism to the overall action-pattern will depend on the substrate chain-length. For example, porcine-pancreatic alpha amylase acting on very long synthetic substrate will have a very low probability of finding chain ends. Under these conditions, the alpha amylase will be confined to ether a random-attack or repetitive-attack mechanism. As amylolysis

TABLE I

THEORETICAL INFLUENCE OF VARYING CHAIN LENGTH, pH, AND COMPETITIVE AND NONCOMPETITIVE INHIBITORS, ON R, THE YIELD OF OLIGOSACCHARIDES ÷ EXTENT OF RANDOM CLEAVAGE

Model	*Variable that increases*[a]			
	Chain length	*Distance from pH optimum*	*Competitive inhibitor*	*Noncompetitive inhibitor*
Repetitive-attack	↑ to constant value at large n	→ or ↓	→	↓
Preferred-attack	↓	↑, ↓, or →[b]	→	↑, ↓, or →[c]
Multiple-site				
S saturating	→	↑, ↓, or →	→	↑, ↓, or →
$S \ll K_1$ and K_2	↓	↑, ↓, or →	↑, ↓, or →	↑, ↓, or →
Random attack	→	→	→	→

[a] ↑, ↓, and → mean R increases, decreases, or remains constant, respectively, as the variable increases. [b] When proton dissociation is independent of positional isomerism. [c] When N combines with equal affinity to all positional isomers. K_1 and K_2 are respectively, Michaelis constants for E_1 and E_2.

proceeds and significant concentrations of chain ends build up in the digest, preferred attack will become increasingly important. In the terminal stages of the digestion, when oligosaccharides predominate, hydrolysis will occur almost exclusively by a preferred-attack route. Thus, any experiments designed to distinguish among mechanisms must take the effect of substrate size into consideration.

Table II summarizes the experiments designed to elucidate the action pattern of porcine-pancreatic alpha amylase. The results from experiments 4–6 definitely classify this amylase as a repetitive-attack enzyme. It is noteworthy that the results of these three experiments depend in some way on the chain length of the substrate, whereas the results of the experiments 1–3 that gave ambiguous results were not dependent on chain length.

TABLE II

SUMMARY OF EXPERIMENTAL DATA FOR PORCINE-PANCREATIC ALPHA AMYLASE

Experiment	*Result*	*Observation consistent with*			
		Repetitive attack	*Preferred attack*	*Multiple-site model*	*Random attack model*
1. Increase pH from 6.9 to 10.5 (refs. 5 and 8)	*R* decreases	Yes	Yes	Yes	No
2. Inhibitors added[11]	*R* decreases	Yes	Yes	Yes	No
3. Yield of oligosaccharides detected by paper chromatography at pH 6.9 and pH 10.5 (ref. 17)	marked decrease in oligosaccharides at pH 10.5	Yes	Yes	Yes	No
4. *R* plotted *vs.* degree of scission[12]	non zero intercept	Yes	No	No	No
5. Ratio, $\overline{DP}_n/\overline{DP}_w$[a] measured as a function of degree of scission[13]	ratio increases with degree of scission	Yes	No	No	No
6. Digestion of end labeled oligosaccharides[18]	Maltooctaose undergoes repetitive attack	Yes	No[b]	No	No

[a] $\overline{DP}_n$ and $\overline{DP}_w$ are respectively the number- and weight-average degree of polymerization. [b] Preferred attack must be followed by repetitive attack.

APPENDIX

The kinetic equations describing the enzymic breakdown of long-chain polymers are often cumbersome mathematical expressions[19,20]. In order to keep the expressions

manageable in this paper, only the hydrolysis of a single polymer, n units in length, is considered. Earlier it was shown that polymer mixtures having an average chain-length of n tend to behave as a pure n-mer when subjected to enzymic depolymerization[20]. However, if n is less than 20, end-group effects may become important. With substrates greater than 20 monomer units, it was found that the equations describing degradation of some polymer distributions of average chain-length n will be homeomorphic with those describing degradation of a homogeneous n-mer.

The effects of modifying chain-length, pH, and inhibitor concentrations on repetitive-attack, preferred-attack and multiple-site models are established in the following sections. The effects of these three factors on amylase action are discussed for each model. The representional schemata for these three models are embodied in Figs. 1, 1A, 2, and 3.

To simply the following analysis, P is allowed to represent a monomer released by repetitive attack, although P could equally well represent an oligomer or mixture of oligomers. Nonproductive enzyme–substrate complexes are ignored in the present derivations. Their presence simply changes the magnitude of apparent Michaelis constants. The magnitude of Michealis constants has no bearing on the conclusions reached. However, alternative product- and saccharide-acceptors that compete with water for transfer of the donor portion of the substrate may act as noncompetitive inhibitors of hydrolysis. The effects of these substances on the action pattern of amylases is considered.

Effect of chain length. — Repetitive-attack model. Repetitive attack occurs when an enzyme cleaves more than one bond, on the average, during an effective encounter with the substrate. An effective encounter is defined as the formation of an enzyme–substrate complex that results in bond cleavage. Following the development of Hanson[19] and Thoma[20], it can be shown that, for initial steady-state conditions (see Fig. 1A and 2A for nomenclature),

$$[ES_{n-i-j,i+j}] = [ES_{n,i}]\,\theta^j, \tag{1A}$$

where

$$\theta = \frac{k_r}{k_{-1}+k_r}, \tag{2A}$$

and θ^j represents that fraction of $ES_{n,1}$ leading to products (P and $ES_{n-i-j,i+j}$), and j is the number of moles of P released. Mathematically, the condition for repetitive attack is that (yield of monomers)÷(yield of substrate fragments) exceeds zero or

$$0 < \frac{[\dot{P}]}{[\dot{S}]} = \frac{k_r \sum_{i=1}^{n-1} \sum_{j=1}^{n-i-1} [ES_{n-i-j,i+j}]\,\theta^j}{k_{r,n} \sum_{i=1}^{n-1} [ES_{n,i}]}, \tag{3A}$$

where $[S]$ is $\sum_{s=1}^{s=\infty} [S]$ and the dot signifies a time derivative. The other symbols are

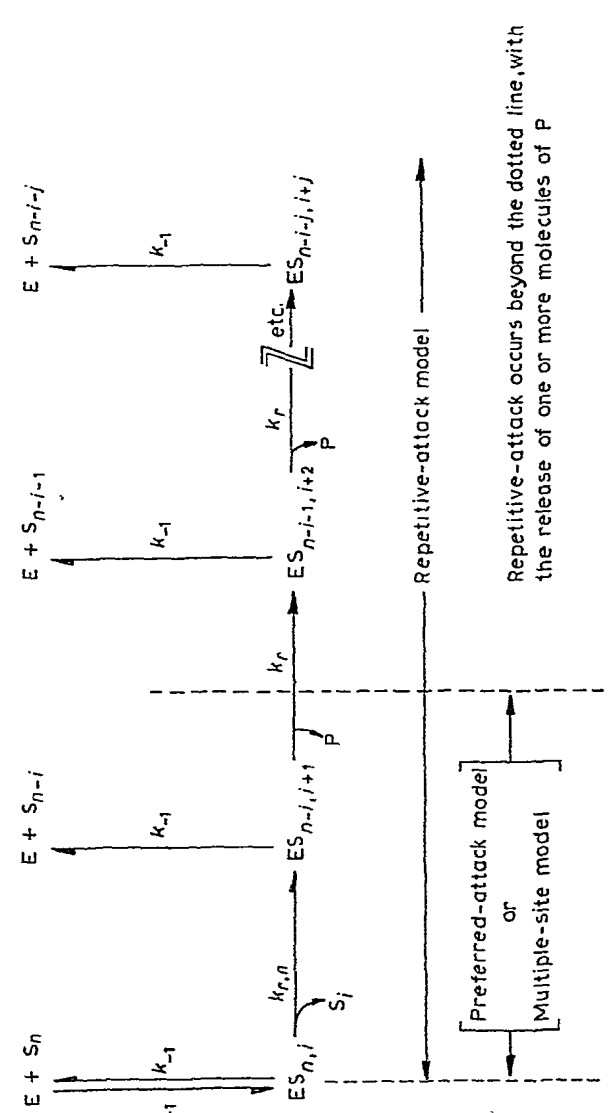

Fig. 1A. Representational model of depolymerizing enzymes acting on a homopolymer of chain length n. The enzyme associates at random with the polymer (k_{+1}), cleaves a bond, and may repetitively attack near the end of one of the fragments. E, S, and P represent free enzyme, free substrate, and product respectively. i is an index or substrate bond-number which specifies the position of the catalytic site of the enzyme on the substrate (see Fig. 2A) and n is the chain length. P is a monomer unit released from the chain terminus. The extent of reaction is limited so that $[S_{n-i-j}]$ is low enough to prevent its association with the enzyme. Relaxation of these restrictions complicates, but does not change, the form of the steady-state equations. A k_r process is initiated by the hydrolytic rupture of the substrate followed by diffusion of one of the fragments from the enzyme. The surviving non-productive complex $ES_{n,0}$ (Fig. 2A) then isomerizes to form a productive complex $ES_{n,2}$ or $ES_{n,3}$ (Fig. 2A) and thus a new bond in the second fragment is subject to hydrolysis. To a first approximation, k_{-1} is considered to be independent of positional isomerization.

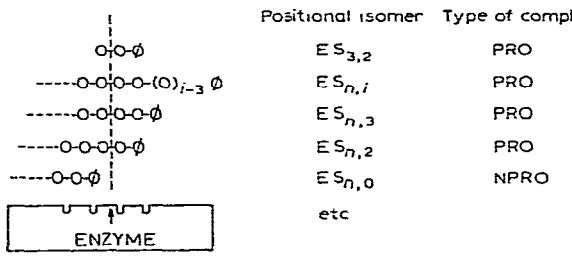

Fig. 2A. Illustration of positional isomerization of enzyme–polymer complexes. Monomer units are numbered from right to left for a chain of n monomer units.

U, ↑, O, ——, ∅ represent a monomer subsite, catalytic site, monomer, connecting bond, and reducing terminus, respectively. The dotted lines show the position of bond rupture. Substrates smaller than the number of subsites occupy only a portion of the active site. Complexes of the type such as $ES_{n,0}$ are ignored in the present analysis because their effect are hidden in apparent Michaelis constants. PRO and NPRO represent productive and nonproductive encounters, respectively.

defined in Fig. 1A. The polarity of repetitive attack in Fig. 1A is toward the nonreducing end of the substrate, but the actual direction is inconsequential to the theory. $[\dot{P}]/[\dot{S}]$ may be thought of as the ratio of nonrandom to random attack, or the ratio of exoactivity/endoactivity, and is replaced by the symbol R. Substituting for $[ES_{n-i-j,i+j}]$ in Eq. *3A* and cancelling common terms yields:

$$0 < R = \frac{k_r \sum_{i=1}^{n-1} [ES_{n,i}] \sum_{j=1}^{n-i-1} \theta^j}{k_{r,n} \sum_{i=1}^{n-1} [ES_{n,i}]} . \tag{4A}$$

For long substrates, $[ES_{n,j}]$ will be essentially independent of i so that Eq. *4A* reduces to

$$0 < R \cong \frac{k_r \sum_{i=1}^{n-1} \sum_{j=1}^{n-i-1} \theta^j}{k_{r,n}(n-1)} = \frac{k_r \theta}{k_{r,n}(1-\theta)} . \tag{5A}$$

Repetitive attack occurs when the numerator of Eq. *4A*, or $\sum_{j=1}^{n-i-1} \theta_j$, is measurably greater than zero. In terms of the molecular events depicted in Fig. 1A, this requirement specifies that there is a singificant probability that a substrate will be hydrolyzed more than once before it dissociates from the enzyme. Since $\theta < 1$, $\sum_{i=1}^{n-1} \sum_{j=1}^{n-i-1} \theta_j/(n-1)$ converges to $\theta/1-\theta$ for large values of n. In other words, for long substrates, the value of (moles of monosaccharide produced)/(moles of bonds cleaved) is independent of chain length.

Preferred-attack model. Greenwood and Milne[21] proposed that bonds near the polymer terminus are often of unusually high or of unusually low reactivity. If the bond at position 1 is sensitive to hydrolysis, then the $ES_{n,1}$ complex is hydrolyzed

more rapidly than the $ES_{n,i}$ complexes ($i \geq 2$), which are cleaved to yield larger products.

The rate of exo-cleavage (to give P) to endo-cleavage (to give S_i, $i \geq 2$) becomes

$$R = \frac{k_{r,1}[ES_{n,1}]}{k_{r,n}\sum_{i=2}^{n-1}[ES_{n,i}]}. \tag{6A}$$

For long chains, there are approximately n ways that the enzyme can bind internal segments. Substitution for $[ES_{n,i}]$ in terms of free [enzyme] and [substrate] and simplification gives:

$$R = \frac{k_{r,1}/K_1}{k_{r,n}/\sum_{i=2}^{n-1}K_i}, \tag{7A}$$

where K_1 and K_i are the microscopic Michaelis constants for formation of terminal and internal complexes, respectively; for long chains, K_i will be essentially independent of n so that Eq. *7A* takes the form

$$R \approx \frac{\text{const}}{n}. \tag{8A}$$

As the denominator in Eq. *8A* is proportional to the chain length of the substrate, the probability of finding P in the digest and, thus the degree of preferred attack, decreases to zero as n goes to infinity. This means that R (moles P or monosaccharide)/(moles of bonds cleaved) should decrease with increasing polymer size. This conclusion is intuitively obvious, because the single terminus of a chain cannot effectively compete for the specificity site with the very large number of internal segments of a long substrate.

Multiple-site model. In order to account for the appearance of small oligomers during the early stages of polymer hydrolysis, Banks and coworkers[13] have hypothesized the simultaneous action of isoenzymes on a single substrate. One isoenzyme is hypothesized to behave as an endo-enzyme and the other as an exo-enzyme. In another paper[8], they suggested that porcine-pancreatic alpha amylase may have two different sites; one that attacks substrates at chain ends and one that attacks randomly. Kinetically, it is impossible to distinguish two noninteracting sites on a single enzyme from two equivalent sites on separate enzymes. Therefore, we classify both possibilities as the multiple-site model. Dixon and Webb[14] have treated this situation in some detail, and this development is an extension of their theory. The total velocity, v_t, is the sum of the velocities of the two enzymes, where the subscripts refer to enzymes 1 and 2, respectively.

$$v_t = \frac{V_1[S]}{K_{m1}+[S]} + \frac{V_2[S]}{K_{m2}+[S]} \tag{9A}$$

When $K_{m1} \neq K_{m2}$, Eq. *9A* is second order in [S], and double-reciprocal plots for

multiple-site systems are curved although the curvature may be difficult to detect. Both the repetitive and preferred-attack models generate linear double-reciprocal plots. If E_1 is the exo-enzyme, then:

$$\frac{\text{exo-activity}}{\text{endo-activity}} = R = \frac{V_1(K_{m2}+[S])}{V_2(K_{m1}+[S])}. \tag{10A}$$

When $[S] \gg K_{m1}$ and K_{m2}, Eq. *10A* shows that R is independent of n. But when $[S] < K_{m1}$ and K_{m2}, then R becomes a function of chain length. As E_2 forms $n-1$ productive complexes with a substrate of chain length n, the measured Michaelis constant K_{m2} may be expressed as a function of chain length. Thus K_{m2} may be approximated by K'_{m2}/n for an $ES_{n,i}$ complex, where K'_{m2} is the microscopic Michaelis constant for a single positional-isomer. Substituting for K_{m2} in *10A* yields

$$R \approx \frac{V_1 K'_{m2}}{n V_2 K_{m1}} = \frac{\text{const}}{n}. \tag{11A}$$

Here R, or the relative amount of P in the digest, decreases as n increases. Thus when both enzyme sites are saturated with substrate, the multiple-site model like the repetitive-attack model shows no chain-length dependence at large n. But at very low substrate-concentration, the multiple-site model behaves like the preferred-attack model.

Effect of pH. — Bell-shaped pH–activity curves are almost universally interpreted in terms of the two-proton model[14]. The proton concentration controls the degree of repetitive attack by altering k_r and by partitioning the $ES_{n,i}$ complex between three states of ionization. The fate of $ES_{n,i}$ is shown in Fig. 2. Both the apparent hydrolysis coefficient, k_r, and the apparent dissociation coefficient, k_{-1}, are functions of the proton concentration. The discussion of the effect of pH on these apparent rate-coefficients can be facilitated by introducing the Michaelis pH functions. These functions describe how a dibasic acid, AH_2, will be distributed among the various states of ionization as a function of pH. The pertinent equilibria[14] are

$$AH_2 \underset{\pm H}{\overset{K_1}{\rightleftharpoons}} AH^- \underset{\pm H}{\overset{K_2}{\rightleftharpoons}} A^{2-}, \tag{12A}$$

and the pH functions are defined as:

$$f \quad = f_{\eta+1} = \frac{[A_t]}{[AH_2]} = 1 + \frac{K_1}{[H^+]} + \frac{K_1 K_2}{[H^+]^2}, \tag{13A}$$

$$f^- = f_\eta \quad = \frac{[A_t]}{[AH^-]} = 1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]}, \tag{14A}$$

$$\text{and} \quad f^{2-} = f_{\eta-1} = \frac{[A_t]}{[A^{2-}]} = 1 + \frac{[H^+]}{K_2} + \frac{[H^+]^2}{K_1 K_2}. \tag{15A}$$

When A represents an enzyme–substrate complex, then K_1 and K_2 in Eqs. *13A–15A* should be replaced by K_{es1} and K_{es2}, respectively, and η is the charge on the enzyme (see Fig. 2). These latter two constants are the first and second proton-dissociation constants for the enzyme–substrate complex. Following the analysis of Dixon and Webb[14], we find that k_r' (see Fig. 2), the pH-dependent coefficient for hydrolysis, is related to k_r', the pH independent coefficient, by:

$$k_r = k_r'/f_\eta \tag{16A}$$

The pH-dependent dissociation coefficient (k_{-1}) for the complexes in Fig. 2 is related to three pH-independent coefficients, k_η, $k_{\eta+1}$, and $k_{\eta-1}$, by the following formula:

$$k_{-1} = \frac{k_{\eta+1}}{f_{\eta+1}} + \frac{k_\eta}{f_n} + \frac{k_{\eta-1}}{f_{n-1}}. \tag{17A}$$

Repetitive-attack model. Substituting *13A–15A* into *2A*, we find the probability that hydrolysis occurs before ES dissociates, becomes

$$\theta = \frac{\dfrac{k_r'}{f_\eta}}{\dfrac{k_{\eta+1}}{f_{\eta+1}} + \dfrac{k_\eta}{f_\eta} + \dfrac{k_{\eta-1}}{f_{\eta-1}} + \dfrac{k_r'}{f_\eta}}. \tag{18A}$$

Multiplying numerator and denominator of Eq. *18A* by f_η, substituting for the Michaelis pH functions (Eqs. *13A–15A*), and simplifying, leads to

$$\theta = \frac{k_r'}{\dfrac{k_{\eta+1}[H^+]}{K_{es1}} + k_\eta + \dfrac{k_{\eta-1}K_{es2}}{[H^+]} + k_r'}. \tag{19A}$$

Substitution of *19A* into *5A* at substrate saturation gives the dependence of R on pH. Making the assumption that the pH dependence of k_r' and $k_{r,n}'$ are similar, we find that *5A* takes the form:

$$R = \frac{\text{const } k_r'}{\dfrac{k_{\eta+1}[H^+]}{K_{es1}} + k_\eta + \dfrac{k_{\eta-1}K_{es2}}{[H^+]}}. \tag{20A}$$

This is a normal, bell-shaped pH function.

Preferred-attack model. The influence of $[H^+]$ ion on the preferred-attack model is ascertained by substituting, for the pH-dependent rate constants in Eq. *6A*, the pH-independent coefficients, making use of Eq. *14A*. At substrate saturation, Eq. *6A* becomes:

$$R = \frac{k_{r,1}'[ES_{n,1}]/f_{\eta,1}}{k_{r,n}'\sum_{i=2}^{n-1}[ES_{n,i}]/f_{\eta,n}}. \tag{21A}$$

As there are approximately n equivalent complexes when n is large, Eq. *21A* takes the form:

$$R = \frac{\text{const } f_{\eta,n}}{n f_{\eta,1}}. \qquad (22A)$$

When proton equilibria regulating hydrolysis are the same for both enzymes, namely, when $f_{\eta,1} = f_{\eta,n}$, R is not influenced by $[H^+]$. When the proton dissociation-constants of the enzyme complexes with an internal segment and a terminal segment are different, $f_{\eta,1}/f_{\eta,n}$ will achieve either minimum or maximum values at extremes of pH, and the relative amount of P may rise or fall as pH is changed. If substrate binding is also affected by pH, more-complicated pH behavior of R may result.

Multiple-site model. If hydrolysis is conducted under saturating substrate-concentrations and V_1 and V_2 are replaced by their pH-independent analogs, Eq. *10A* becomes:

$$R = \frac{V_1' f_{\eta,2}}{V_2' f_{\eta,1}}. \qquad (23A)$$

If the pK_{es} values of the catalytic amino acids of the two enzymes are identical, the pH functions cancel from Eq. *23A*, and R does not depend on pH. When they are unequal, compensating but noncancelling effects with pH functions may be observed. As already noted, the ratio, $f_{\eta,2}/f_{\eta,1}$ may achieve either a maximum or a minimum at extreme pH values. Thus, the relative amount of P released may rise or fall as the pH is raised or lowered.

Effect of inhibitors. — Non-competitive inhibitors of hydrolytic enzymes often act as alternative acceptors and stimulate transfer-reactions and repress hydrolytic reactions[16]. They may also bind to the active site of the enzyme and prevent rearrangement of non-productive complexes to productive complexes. After a hydrolytic event, one of the substrate fragments may dissociate from the enzyme, leaving behind a non-productive enzyme–substrate complex. A non-competitive inhibitor may then occupy the site vacated by the departing fragment to form a ternary enzyme–substrate–inhibitor complex. The substrate fragment remaining bound to the enzyme cannot form a productive complex, and the only reaction route open to the substrate is dissociation from the enzyme.

Repetitive-attack model. In the presence of an alternative acceptor, θ (Eq. *2A*) becomes

$$\theta = \frac{k_r}{k_{-1} + k_r + k_t + k_{-1,N}}, \qquad (24A)$$

where k_t (Fig. 3) is the rate coefficient for transfer, and the k values are the apparent coefficients dependent of [N]. The term θ can be written as a function of [N] and k values that are independent of [N].

When N, a non-competitive inhibitor, binds to any enzyme–substrate complex to form the ternary complex (see Fig. 3), the concentration of ES is depressed by the

factor, $K_{n,es}/[N]+K_{n,es}$, and the apparent hydrolytic coefficient will be depressed the same amount. On the other hand, the concentration of ESN is proportional to the factor $[N]/[N]+K_{n,es}$, so that the apparent Michaelis constants will change by this same factor.

Thus substituting the N-independent functions into *23A* gives:

$$\theta = \frac{k'_r \dfrac{K_{N,es}}{[N]+K_{N,es}}}{k'_{-1}\left(\dfrac{k_{N,es}}{[N]+K_{N,es}}\right)+(k'+k'_{-1,N})\left(\dfrac{[N]}{[N]+K_{N,es}}\right)+k'_r\left(\dfrac{K_{N,es}}{[N]+K_{N,es}}\right)}, \qquad (25A)$$

which simplifies to

$$\theta = \frac{k'_r(K_{N,es})}{(k'_{-1}+k'_r)(K_{N,es})+(k'_t+k'_{-1,N})[N]}. \qquad (26A)$$

If the ratio $k_r/k_{r,n}$ (Eq. *5A*) is not dependent on [N], substitution of *25A* into *5A* gives:

$$R = \frac{k'_r K_{N,es}}{k'_1(K_{N,es})+(k'_t+k'_{-1,N})[N]}. \qquad (27A)$$

According to Eq. *24A* and *26A*, at a sufficiently high [N], θ, R, P and repetitive attack all vanish.

Competitive inhibitors only combine with free enzyme and do not influence the fate of either an enzyme–substrate complex or the degree of repetitive attack.

Preferred-attack model. A slope-linear competitive inhibitor (see Cleland for nomenclature[16]) will depress the concentration of free enzyme and all enzyme substrate complexes by the same account. Competitive inhibitors will depress the overall hydrolysis rate but will not alter the partitioning of substrate along a reaction pathway and cannot change the action pattern of an enzyme.

When an intercept, linear, non-competitive inhibitor combines rapidly and reversibly with enzyme-substrate complexes, it diverts a fraction of the enzyme proportional to $1+[N]/K_{N,es}$ from the hydrolytic patway[16]. In the simplest case, when the dissociation constant $K_{N,es}$ is independent of positional isomerization of the substrate, the same fraction of $ES_{n,1}$ and $ES_{n,i}$ is diverted from the hydrolytic pathway. The rate of overall reaction slows but the ratio R (Eq. *6A*) is unaffected. If the binding affinity of N to $ES_{n,1}$ differs from the binding affinity to $ES_{n,i}$ Eq. *6A* becomes

$$R = \frac{k_{r,1}[ES_{n,1}]\left(1+\dfrac{[N]}{K_{N,es,1}}\right)}{k_{r,n}\displaystyle\sum_{i=2}^{n-1}[ES_{n,i}]\left(1+\dfrac{[N]}{K_{N,es,i}}\right)}. \qquad (28A)$$

As $[N]\to\infty$, the ratio of internal to terminal rupture achieves a limiting value of

$K_{N,es,i}/K_{N,es,1}$. Thus N may either increase or decrease the apparent degree of internal cleavage, depending on the relative magnitudes of the dissociation constants $K_{N,es,1}$ and $K_{N,es,i}$.

Multiple-site model. Competitive inhibition disappears at substrate saturation, and hence competitive-inhibition terms are associated exclusively with the apparent Michaelis constant[16]. Thus, the presence of C, a competitive inhibitor, for slope-linear competitive inhibition, K_m will change by the factor, $1+[C]/K_C$. Substituting for K_m in *6A* gives:

$$R = \frac{V_1\left[K_2\left(1+\frac{[C]}{K_{C,2}}\right)+[S]\right]}{V_2\left[K_1\left(1+\frac{[C]}{K_{C,1}}\right)+[S]\right]}. \qquad (29A)$$

For a multiple-site model, the relative amount ot exo-cleavage will be a function of the ratio, $K_{C,2}/K_{C,1}$. We see that R may increase, decrease, or remain constant as C is changed at subsaturating concentrations of S.

In the presence of intercept-linear, non-competitive inhibitors, V apparently will change by the factor $1+[N]/K_N$. Incorporating this factor into Eq. *10A* shows that:

$$R = \frac{V_1\left(1+\frac{[N]}{K_{N,1}}\right)(K_2+[S])}{V_2\left(1+\frac{[N]}{K_{N,2}}\right)(K_1+[S])}. \qquad (30A)$$

Non-competitive inhibitors may raise, lower, or leave R unchanged at either saturating or nonsaturating levels. For very closely related enzymes, compensating effects in the numerator and denominator of Eqs. *27A* and *28A* would tend to minimize effects on R.

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