Biochemistry VOLUME 1, NUMBER 5

October 8, 1962

Enzyme Kinetics of Short-Chain Polymer Cleavage

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Received December 18, 1961

The properties of polysaccharide, polynucleotide, and polypeptide hydrolyzing enzymes may be studied with purified oligopolymers as substrates. An equation is derived, applicable to enzymes of endo-action-pattern, which relates the initial velocity of cleavage to the rate constants for the cleavage process at the various positions along the substrate chain. This equation has the Michaelis-Menten form. The simple derivation is extended to include the effects of competitive and noncompetitive self-inhibition, of *p*H, and of temperature. The application of these equations to the study of the specificity region of endo-acting hydrolytic enzymes is discussed. A model for the action of exo-cleaving enzymes in which the enzyme may attack repeatedly the same polymer chain is also examined and an experimental test for such action proposed.

In order to interpret at the molecular level the action-pattern of polymer-cleaving enzymes, kinetic studies on chemically defined substrates are essential. It is the purpose of this paper to consider the type of information that may be obtained from studies of the initial velocity of polymer cleavage as a function of substrate concentration, temperature, *p*H, and degree of polymerization of the substrate.

The short-chain homopolymers required as substrates for the polysaccharide and polynucleotide hydrolyzing, or phosphorolyzing, enzymes may be obtained in certain cases by stepwise synthesis (Peat *et al.*, 1960; Khorana, 1960). They are more readily obtained by separating the mixtures of oligopolymers produced by either chemical (Khorana *et al.*, 1961; Khorana and Vizsolyi, 1961; Turner and Khorana, 1959) or enzymatic synthesis (Peat *et al.*, 1956), or by the partial degradation of natural (Miller *et al.*, 1960; Whelan *et al.*, 1953; Whitaker, 1954) or enzymatically (Staehelin *et al.*, 1959) synthesized polymers. Oligosaccharides may be separated by adsorbtion chromatography on charcoal-celite (Miller *et al.*, 1960; Peat *et al.*, 1956; Taylor and Whelan, 1962; Whelan *et al.*, 1953; Whitaker, 1954), by partition chromatography on cellulose powder (Thoma *et al.*, 1959), by ion-exchange chromatography (amino sugars) (Lenk *et al.*, 1961), by molecular-sieve chromatography on dextran gel (Flodin and Aspberg, 1961), or by column electrophoresis in borate buffer (Porath and Waligora, personal communication.); oligonucleotides by ion-exchange chromatography on DEAE-cellulose (Khorana *et al.*, 1961; Khorana and Vizsolyi, 1961; Staehelin *et al.*, 1959).

For enzymes which are able to cleave the substrate at more than one position, the initial velocity most readily determined is the sum of the initial velocities of cleavage at the various positions along the polymer chain. Thus the hydrolysis of oligosaccharides may be followed by measuring the increase in the number of terminal reducing groups, and the hydrolysis of oligonucleotides, when the products of cleavage are not cyclic phosphate esters, by measuring the uptake of alkali at constant *p*H (Razzell and Khorana, 1959). Relationships are therefore derived linking the apparent constants (Michaelis constant *etc.*) obtained from such measurements, and the constants that would be observed if the cleavage of each polymer unit-unit bond could be studied without regarding the breaking of other bonds.

The various models examined apply directly to hydrolytic enzymes of endo-type action-pattern (near-random attack), although complications arising from the effect of metal ion concentration on the reactivity of oligonucleotides are not considered. Equations appropriate to the hydrolytic

and phosphorolytic enzymes of simple exo-type action-pattern (end attack) may readily be derived by modifying these models. A possible variant of the simple exo-type model, in which the enzyme may repeatedly cleave monomer units from the same polymer chain without any intermediate dissociation of enzyme and substrate taking place, is also examined. Although peptidases and proteolytic enzymes are not discussed in this paper the models examined should be applicable to the study of such enzymes.

Theoretical

In the following derivations, the number of cleavable bonds in the linear substrate molecule is represented by n. The degree of polymerization (D.P.) of the substrate unless otherwise indicated is therefore n + 1. The cleavable bonds of the substrate are numbered 1 to n, and the prior-index g is used to indicate some particular bond in this series—*i.e.*, g may have any value 1 to n. The superscript ~ is used to designate the empirical constants obtained by substituting the observed initial velocities of polymer cleavage $\bar{v}$ at various substrate concentrations in an equation of the Michaelis-Menten form, and also the complex values for the activation energy obtained by plotting the natural logarithm of the above constants against the reciprocal of the absolute temperature. Further symbols are explained as required. Those not explained will be found in general textbooks on enzyme kinetics (Dixon and Webb, 1958; Laidler, 1958), or in reviews of this subject (Segal *et al.*, 1959). The assumption is made, unless otherwise indicated, that before the enzyme can act again after cleaving a polymer bond, the substrate fragments and enzyme must completely dissociate.

Simple Endo-Cleavage.—For each point of cleavage the chemical relationship of Figure 1 applies, there being *n* such equations.[1] For rela-

$$n\left[\mathrm{S} + \mathrm{E} \underset{{}^{g}k_{-1}}{\overset{{}^{g}k_{+1}}{\rightleftharpoons}} {}^{g}\mathrm{ES} \xrightarrow{{}^{g}k_{+2}} \mathrm{E} + \text{Products}\right]$$

Figure 1

tively low values of n, the situation in which two enyzme molecules attack the same substrate chain simultaneously may be neglected. Since only initial velocities are to be considered, the further cleavage of the products of the reaction may also be neglected. For simplicity, models in which the enzymatic process requires more than three rate constants for its full definition are not examined (see below). If $[E_0]$ is the total concentration of added enzyme, $\bar{v}$ is the initial velocity of cleavage, and the substrate concentration $[S] >> [E_0]$, then

$$[E_0] = [E] + \sum_{g=1}^{n} [{}^{g}ES] \quad (1)$$

$$\bar{v} = \sum_{g=1}^{n} {}^{g}k_{+2}[{}^{g}ES] \quad (2)$$

For each separate point of cleavage the steady state assumption may be made:

$$[{}^{g}ES] = [E][S]{}^{g}k_{+1}/({}^{g}k_{-1} + {}^{g}k_{+2}) = [E][S]/{}^{g}K_m \quad (3)$$

On eliminating the unknowns $[{}^{g}ES]$ and [E] from equations (1) to (3) the following expression is obtained:

$$\bar{v} = \frac{[E_0][S]\sum_{g=1}^{n}({}^{g}k_{+2}/{}^{g}K_m)}{1 + [S]\sum_{g=1}^{n}(1/{}^{g}K_m)} \quad (4)$$

Equation (4) is of the Michaelis-Menten form and in the special case that n = 1 becomes identical with the standard Briggs-Haldane, Michaelis-Menten equation:

$$\bar{v} = \frac{[E_0][S]k_{+2}/K_m}{1 + [S]/K_m}$$

Since only $\bar{v}$ and [S] are observable quantities it is convenient to define the empirical constants $\tilde{k}_f$ and $\tilde{K}_m$ as follows:

$$\bar{v} = \frac{[S]\tilde{k}_f}{1 + [S]/\tilde{K}_m} \quad (5)$$

where $\tilde{k}_f$ is the velocity constant, first order with respect to substrate, observed when $[S] << \tilde{K}_m$. If then, the model under consideration is held to describe some particular experimental situation, the empirical constants of equation (5) may be equated with the algebraically equivalent expressions in equation (4):

$$1/\tilde{K}_m = \sum_{g=1}^{n} 1/{}^{g}K_m \quad (6)$$

$$\tilde{k}_f = [E_0]\sum_{g=1}^{n}({}^{g}k_{+2}/{}^{g}K_m) = \sum_{g=1}^{n} {}^{g}k_f \quad (7)$$

$$\tilde{V}_{max} = \tilde{k}_f\tilde{K}_m = [E_0]\sum_{g=1}^{n}({}^{g}k_{+2}/{}^{g}K_m) \div \sum_{g=1}^{n} 1/{}^{g}K_m \quad (8)$$

Equation (6) is equivalent to the definition (*cf.* equation 3) $\tilde{K}_m$ = [E][S]/[total concentration of enzyme-substrate complex]. Equation (7) indicates that $\tilde{k}_f$ is equal to the sum of the first-order velocity constants for each individual bond cleaved. This result is readily explained when it is remembered that, for the first order equation $\bar{v} = \tilde{k}_f[S]$ to apply, only a minute fraction of the added enzyme can be in the form of enzyme-substrate complex. Under these circumstances competition by the various substrate cleavage

[1] The rate constants are differentiated by the post-subscripts +1, −1, +2 in accordance with the recommendations of the Commission on Enzymes of the International Union of Biochemistry.

points for the active site of the enzyme may be entirely neglected.

At substrate concentrations other than $[S] << \tilde{K}_m$, competition for the active site of the enzyme must be taken into account. The significance of this competition may be expressed in the following two propositions:

(*a*) $\tilde{V}_{max}$ is not greater than 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 competition effects.

Let the particular bond for which ${}^{g}k_{+2}$ is greatest be designated by a, then since possible values of g include a, ${}^{g}k_{+2} \leqslant {}^{a}k_{+2}$. For any value of g

$${}^{g}k_{+2}/{}^{g}K_m \leqslant {}^{a}k_{+2}/{}^{g}K_m$$

Summing all values of g and introducing $[E_0]$

$$[E_0] \sum_{g=1}^{n} k_{+2}/{}^{g}K_m \leqslant [E_0]{}^{a}k_{+2} \sum_{g=1}^{n} 1/{}^{g}K_m$$

hence proposition (*a*):

$$\tilde{V}_{max} \leqslant [E_0]{}^{a}k_{+2} = {}^{a}V_{max}$$

In the special case that all values of ${}^{g}k_{+2}$ are identical and equal to ${}^{a}k_{+2}$, then $\tilde{V}_{max} = [E_0]\,{}^{a}k_{+2} = {}^{a}V_{max}$.

(*b*) The observed rate of cleavage at high substrate concentration of a particular bond is less than would be observed if all competitive effects could be ignored.

Let it be assumed that methods are available to follow the cleavage of some particular bond b, then, if equation (2) is replaced by ${}^{b}\bar{v} = {}^{b}k_{+2}[{}^{b}ES]$,

$${}^{b}\tilde{V}_{max} = [E_0]({}^{b}k_{+2}/{}^{b}K_m) \div \sum_{g=1}^{n} 1/{}^{g}K_m$$

$$= {}^{b}V_{max}\tilde{K}_m/{}^{b}K_m$$

Since $1/\tilde{K}_m > 1/{}^{b}K_m$, then $\tilde{K}_m/{}^{b}K_m < 1$ hence proposition (b):

$${}^{b}\tilde{V}_{max} < {}^{b}V_{max}$$

The derivation of equations (4), (6), (7), and (8) has been based on the assumption that the simple relationship of Figure 1 holds. If the enzyme-substrate complex first formed passes through a series of distinct forms before dissociation to enzyme and substrate, *e.g.*

$$n\left[E + S \underset{{}^{g}k_{-1}}{\overset{{}^{g}k_{+1}}{\rightleftharpoons}} {}^{g}ES_1 \underset{{}^{g}k_{-2}}{\overset{{}^{g}k_{+2}}{\rightleftharpoons}} {}^{g}ES_2 \xrightarrow{{}^{g}k_{+3}} E + P\right]$$

then it can be shown that the final relationship $\tilde{v} = f[S]$ is of the Michaelis-Menten form. The empirical constants may be related to the constants for the individual bond cleavage process as above. Thus if a sequence of two enzyme-substrate complexes are held to take part in the cleavage process

$$1/{}^{g}K_m = \frac{{}^{g}k_{-1}{}^{g}k_{-2} + {}^{g}k_{-1}{}^{g}k_{+3} + {}^{g}k_{+2}{}^{g}k_{+3}}{{}^{g}k_{+1}({}^{g}k_{-2} + {}^{g}k_{+2} + {}^{g}k_{+3})}$$

$${}^{g}k_f = \frac{{}^{g}k_{+1}{}^{g}k_{+2}{}^{g}k_{+3}}{{}^{g}k_{-1}{}^{g}k_{-2} + {}^{g}k_{-1}{}^{g}k_{+3} + {}^{g}k_{+2}{}^{g}k_{+3}}$$

then $1/\tilde{K}_m = \sum_{g=1}^{n} 1/{}^{g}K_m$, $\tilde{k}_f = \sum_{g=1}^{n} {}^{g}k_f$,

and $\tilde{V}_{max} = \tilde{k}_f\tilde{K}_m$.

Propositions *a* and *b* above may be shown to be correct in the more general case that a sequence of enzyme-substrate complexes are involved in the cleavage process.

The above treatment may be extended, by standard methods, to cover the cases (1) in which substrate molecules with different chain lengths are present, (2) in which inert polymer molecules are present and act as competitive inhibitors, or (3) in which cleavable polymers are added to act as competitive inhibitors for a simple cleavage process being followed by an independent method (Razzell and Khorana, 1959).

Competitive Self-Inhibition.—In the preceding section the self-inhibition of the enzyme arising from the competition of the various cleavable bonds for the active site has been examined. The model discussed in that section may readily be extended to include the case in which additional enzyme-substrate complexes are reversibly formed, but no cleavage of the substrate takes place. This form of competitive self-inhibition is algebraically equivalent to supposing that additional bonds are present for which ${}^{g}k_{+2} = 0$. Thus, if there are j such inactive enzyme-substrate complexes which may be formed, a given complex being designated by prior-superscript h, if ${}^{h}K_i = {}^{h}k_{-1}/{}^{h}k_{+1}$, and if in a given experimental situation this model applies, then the empirical constants of equation (5) may be defined as follows:

$$1/\tilde{K}_m = \sum_{g=1}^{n} 1/{}^{g}K_m + \sum_{h=1}^{j} 1/{}^{h}K_i \qquad (9)$$

$$\tilde{k}_f = [E_0] \sum_{g=1}^{n} ({}^{g}k_{+2}/{}^{g}K_m) \qquad (10)$$

$$\tilde{V}_{max} = \frac{[E_0] \sum_{g=1}^{n} ({}^{g}k_{+2}/{}^{g}K_m)}{\sum_{g=1}^{n} 1/{}^{g}K_m + \sum_{h=1}^{j} 1/{}^{h}K_i} \qquad (11)$$

Equation (10) is identical with equation (7); *i.e.* competitive self-inhibition has no effect on the velocity at low substrate concentration.

The above model is closely related to that proposed by Thoma and Koshland (1960) for the action of β-amylase on α-1→4-oligoglucosides. These authors considered the situation in which only one enzyme-substrate complex is susceptible to cleavage (n = 1), all non-cleavable complexes are equally stable (identical ${}^{h}K_i$ values) and involve the same number of substrate monomer units, and the number of inactive complexes j is equal to the number of monomer units in the oligoglucoside less the number of units involved

in forming the enzyme-substrate inactive complex. If the above substitutions are made in equations (9) to (11), the expression derived by Thoma and Koshland is obtained. The β-amylase model, therefore, represents a specific application of one member of the class of models described by the general model for competitive self-inhibition here considered.

Non-competitive Self-Inhibition or Self-Stimulation.—In the following scheme, the enzyme is assumed to have one additional site capable of binding substrate molecules, but incapable of cleaving the substrate. Complex formation at this point does not prevent the binding of substrate at the active site; it may either inhibit or stimulate the rate of cleavage. A given substrate position of binding at the inactive site is designated by prior-superscript r, and the total number of possible enzyme-substrate combinations at this site by q. A steady-state treatment of this problem would be exceedingly complex: in the derivation in Figure 2 the equilibrium assumption is therefore made.

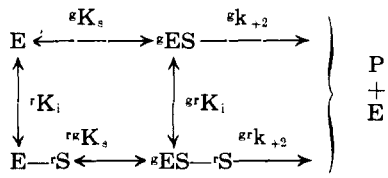

FIG. 2.—There are n possible complexes of the type ᵍES, q of the type E—ʳS, and nq of the type ᵍES—ʳS. There are n constants of the type ᵍKₛ and ᵍk₊₂, q of the type ʳKᵢ, and nq of the types ʳᵍKₛ, ᵍʳKᵢ and ᵍʳk₊₂.

The following relationships obtain:

$$[E_0] = [E] + \sum_{g=1}^{n} [{}^{g}ES] + \sum_{r=1}^{q} [E—{}^{r}S] + \sum_{g=1}^{n}\sum_{r=1}^{q} [{}^{g}ES—{}^{r}S] \quad (12)$$

$$\tilde{v} = \sum_{g=1}^{n} {}^{g}k_{+2}[{}^{g}ES] + \sum_{g=1}^{n}\sum_{r=1}^{q} {}^{gr}k_{+2}[{}^{g}ES—{}^{r}S] \quad (13)$$

$$[{}^{g}ES] = [E][S]/{}^{g}K_s \text{ and } [E—{}^{r}S] = [E][S]/{}^{r}K_i$$
$$[{}^{g}ES—{}^{r}S] = [E][S]^2/{}^{g}K_s{}^{gr}K_i \quad (14)$$

Since the species E, ᵍES, ᵍES—ʳS, and E—ʳS are in equilibrium it follows, from the definitions of the various equilibrium constants or from the first law of thermodynamics, that the concentrations of these species are defined by any three of the four constants, *i.e.* ʳKᵢ ʳᵍKₛ = ᵍKₛ ᵍʳKᵢ. In deriving equation (15) the class of constants ʳᵍKₛ is entirely omitted.

The form of equation (15) is unaffected by the number of cleavable bonds in the substrate (n), or the number of inactive enzyme-substrate complexes (q). Thus the complex situation is indistinguishable from the case when n = 1 and q = 1 by the type of experiment under consideration. In this respect equation (15) resembles equation (4).

At high substrate concentration when the terms containing $[S]^2$ are dominant, $\tilde{v}$ tends to a steady value, *viz.*

$$\tilde{v}_\infty = \lim_{[S]\to\infty} \tilde{v} = \frac{[E_0]\sum_{g=1}^{n}\sum_{r=1}^{q} ({}^{gr}k_{+2}/{}^{g}K_s{}^{gr}K_i)}{\sum_{g=1}^{n}\sum_{r=1}^{q} 1/{}^{g}K_s{}^{gr}K_i} \quad (16)$$

When the additional substrate molecule produces total inhibition of the enzyme, *i.e.* ᵍʳk₊₂ = 0, then $\tilde{v}_\infty = 0$. If the ᵍES—ʳS complexes precede group transfer only, and not cleavage, then the ᵍʳk₊₂ terms will effectively be zero since no net release of end groups takes place during transfer.

At intermediate substrate concentrations $\tilde{v}$ passes through a point of inflection (less than $\tilde{v}_\infty$), or a maximum (greater than $\tilde{v}_\infty$), which is determined by the parameters of the system. When ᵍʳk₊₂ = 0 the substrate concentration at which $\tilde{v}$ reaches a maximum is given by the relationship:

$$[S]_{max} = \left(\sum_{g=1}^{n}\sum_{r=1}^{q} 1/{}^{g}K_s{}^{gr}K_i\right)^{-1/2} \quad (17)$$

Provided the coefficients of $[S]^2$ are much smaller than those of [S], then when [S] is sufficiently low the terms containing $[S]^2$ may be neglected, and equation (15) reduces to the Michaelis-Menten form. This is equivalent to the condition that the amount of ᵍES—ʳS is zero. If Fig. 2 is modified to omit these species then the steady-state treatment may be applied.

$$\tilde{k}_f = [E_0]\sum_{g=1}^{n} ({}^{g}k_{+2}/{}^{g}K_m) \quad (18)$$

$$1/\tilde{K}_m = \sum_{g=1}^{n} 1/{}^{g}K_m + \sum_{h=1}^{j} 1/{}^{h}K_i + \sum_{r=1}^{q} 1/{}^{r}K_i \quad (19)$$

Thus where the h terms are added to include competitive self-inhibition effects. Equation (18) is identical with equations (7) and (10). The value of $\tilde{V}_{max}$ obtained by use of the Michaelis-Menten equation should not be confused with the observed maximum value of $\tilde{v}$ discussed in the preceding paragraph.

The above model, in which the enzyme posses-

$$\tilde{v} = \frac{[E_0]\left([S]\sum_{g=1}^{n} ({}^{g}k_{+2}/{}^{g}K_s) + [S]^2\sum_{g=1}^{n}\sum_{r=1}^{q} ({}^{gr}k_{+2}/{}^{g}K_s{}^{gr}K_i)\right)}{1 + [S]\left(\sum_{g=1}^{n} 1/{}^{g}K_s + \sum_{r=1}^{q} 1/{}^{r}K_i\right) + [S]^2\sum_{g=1}^{n}\sum_{r=1}^{q} 1/{}^{g}K_s{}^{gr}K_i} \quad (15)$$

ses only two substrate binding sites, one of which is the active site, is a member of the class of models in which there are x binding sites, only one of which is the active site. The derivation of this more general model follows closely that of the case in which x = 2. If x = 3, the inactive sites being designated by r and t, then equilibria between eight species of the type E, E—^{r}S, E—^{t}S, E—^{r}S^{t}S, ^{s}ES, ^{s}ES—^{r}S, ^{s}ES—^{t}S, ^{s}ES—^{r}S^{t}S, must be considered. In general the number of such species in equilibrium is 2^x, the minimum number of equilibrium constants of types $^{s}K_s$, $^{r}K_i$, $^{t}K_i$, etc. required to express the concentration of all enzyme-substrate complexes in terms of [E] and [S] is $2^x - 1$, and the number of types of $^{s}k_{+2}$, $^{sr}k_{+2}$, $^{st}k_{+2}$ etc. term is 2^{x-1}. Since for each species involving enzyme combined with u molecules of substrate $[ES_u] = C[E][S]^u$, where C is a constant composed of u equilibrium constants (see equation 14), the expression for $\bar{v} = f[S]$ must contain a series of $[S]^u$ terms. This expression has the following form:

$$\bar{v} = \frac{[E_0]([S]\sigma_1 + [S]^2\sigma_2 + [S]^3\sigma_3 + \ldots + [S]^x\sigma_x)}{1 + [S]\theta_1 + [S]^2\theta_2 + \ldots + [S]^x\theta_x} \quad (20)$$

where σ_1 ($= \bar{k}_f/[E_0]$) is identical with the corresponding term in equation (15) and

$$\theta_1 = \sum_{g=1}^{n} 1/{}^{g}K_s + \sum_{r=1}^{q} 1/{}^{r}K_i \text{ etc. to x summands.}$$

The number of summands present in the coefficients σ_1, σ_2, σ_3 etc. is given by the corresponding terms in the binomial expansion of $(1 + 1)^{x-1}$, and in the coefficients θ_1, θ_2, θ_3 etc., by the terms of the binomial expansion of $(1 + 1)^x$ beginning at the second term (=x). By analogy with equation (15) one may see that each coefficient σ_u and θ_u contains the reciprocal of the product of u equilibrium constants (see equation 15). If the additional assumption is introduced that the cleavage process involves a sequence of enzyme-substrate complexes in equilibrium, equations (15) and (20) may be derived provided that the various $^{g}k_f$, $^{g}K_s$, $^{gr}K_s$ etc. terms are understood as incorporating the several equilibrium constants of the cleavage process sequence (see discussion of the first model).

Since carbohydrates, polypeptides, and nucleic acids show a marked ability to complex with proteins it is not improbable that enzymes acting on these substrates will possess a number of regions capable of binding polymer. It will therefore be of interest to determine whether in a given experimental situation equation (20) x = 1 or x = 2 can be fitted to the experimental points, or whether additional terms corresponding to x = 3, x = 4, etc., must be employed. It should be emphasized that, as the equilibrium assumption was made in deriving equations (15) and (20), any interpretation of experimental results based on these equations also involves making the equilibrium assumption.

Effects of pH.—The study of the variation of $p\bar{K}_s$, $p\bar{V}_{max}$, and $p\bar{k}_f$ with pH provides valuable information about the ionizing groups present at the active site of the enzyme (Dixon and Webb, 1958; Laidler, 1958), or about groups which exercise a controlling influence on the conformation of the enzyme molecule. The standard treatment of this problem may be extended to polymer cleavage in the following manner: if the reaction of a non-ionizing polymer S with enzyme EH be considered, if only species of the type ^{g}EHS break down to give products and enzyme, and if the equilibria represented in Figure 3 apply, then the chemical relationships shown in that figure may be set down.

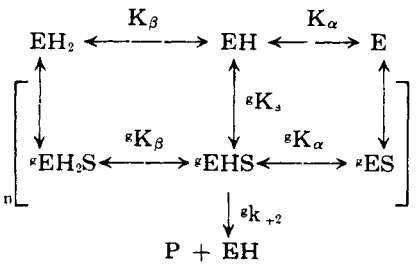

Figure 3

Accordingly,

$$[E_0] = [EH_2] + [EH] + [E] + \sum_{g=1}^{n} ([{}^{g}EH_2S] + [{}^{g}EHS] + [{}^{g}ES]) \quad (21)$$

$$\bar{v} = \sum_{g=1}^{n} {}^{g}k_{+2}[{}^{g}EHS] \quad (22)$$

$$[EH_2] = [EH][H^+]/K_\beta \text{ and } [E] = [EH]K_\alpha/[H^+]$$
$$[{}^{g}EH_2S] = [{}^{g}EHS][H^+]/{}^{g}K_\beta \text{ and}$$
$$[{}^{g}ES] = [{}^{g}EHS]{}^{g}K_\alpha/[H^+]$$
$$[{}^{g}EHS] = [E][S]/{}^{g}K_s \quad (23)$$

The equilibrium constants connecting the species EH_2 and $^{g}EH_2S$, E, and ^{g}ES are redundant and need not be introduced into the derivation (*cf.* Fig. 2).

For convenience the following acidity functions are introduced:

$$(A) = 1 + K_\alpha/[H^+] + [H^+]/K_\beta$$
$$({}^{g}B) = 1 + {}^{g}K_\alpha/[H^+] + [H^+]/{}^{g}K_\beta \quad (24)$$

The resulting expression for $\bar{v} = f[S]$ is of the Michaelis-Menten form. If, therefore, the present model is considered to apply in some experimental situation the empirical constants of equation (5) may be identified with the constants derived in terms of this model. The following three equations may be compared with equations (6), (7), and (8):

$$1/\bar{K}_m = \sum_{g=1}^{n} (({}^{g}B)/{}^{g}K_s) \div (A) \quad (25)$$

$$\tilde{k}_f = [E_0] \sum_{g=1}^{n} ({}^{g}k_{+2}/{}^{g}K_s) \div (A) \qquad (26)$$

$$\tilde{V}_{max} = [E_0] \sum_{g=1}^{n} ({}^{g}k_{+2}/{}^{g}K_s) \div \sum_{g=1}^{n} (({}^{g}B)/{}^{g}K_s) \qquad (27)$$

Inspection of these equations reveals that the acidity functions (^{g}B) are present in equations (25) and (27) but absent in (26). Information about the ionization of the free enzyme is, therefore, unambiguously obtainable (equation 26), but clear values for the ionization constants of the enzyme-substrate complex will only be obtained if the acidity function (^{g}B) does not vary with the site of association, *i.e.* with g. If, therefore, indeterminate plots of $p\tilde{V}_{max}$ against pH are obtained, the equilibrium conditions of Figure 3 may still prevail. The appropriate equations for the cases in which substrate ionization affects the binding of substrate to enzyme may readily be derived, and resemble the corresponding cases in which n = 1 provided that (^{g}B) is independent of g. The general steady-state treatment provided by Laidler (1958) does not appear to be directly applicable to the complex situation under discussion.

Apparent Activation Energies.—The apparent Arrhenius activation energy μ of a process characterized by the rate constant k is defined by equation (28), whatever the complexity of the rate constant k. If k is a known function of rate constants for simple molecular processes, then application of the theory of absolute reaction rates provides a precise thermodynamic interpretation of μ (Dixon and Webb, 1958; Laidler, 1958).

$$\frac{\partial \log_e k}{\partial 1/T} = \frac{-\mu}{R} \qquad (28)$$

For polymer-cleaving enzymes, the individual bond rate constants $^{g}k_f$ and $^{g}K_m$ correspond to the activation energy terms $^{g}\mu_f$ and $^{g}\mu_m$, while the observable constants $\tilde{k}_f$ and $\tilde{K}_m$ correspond to $\tilde{\mu}_f$ and $\tilde{\mu}_m$. In order to interpret $\tilde{\mu}_f$ and $\tilde{\mu}_m$, it is necessary to know the relationship of these terms to the corresponding terms for individual bond cleavage. For the three models discussed above, $\tilde{k}_f = \sum_{g=1}^{n} {}^{g}k_f$ (equations 7, 10, and 18); therefore:

$$\frac{-\mu_f}{R} = \frac{\partial \log_e \tilde{k}_f}{\partial 1/T} = \frac{1}{\tilde{k}_f} \frac{\partial \tilde{k}_f}{\partial 1/T} = \frac{1}{\tilde{k}_f} \sum_{g=1}^{n} \frac{\partial {}^{g}k_f}{\partial 1/T}$$

$$\partial {}^{g}k_f = {}^{g}k_f \partial \log_e {}^{g}k_f = -({}^{g}k_f {}^{g}\mu_f/R)\partial 1/T$$

hence

$$\tilde{\mu}_f = (1/\tilde{k}_f) \sum_{g=1}^{n} {}^{g}k_f {}^{g}\mu_f \qquad (29)$$

Similarly it may be shown for the first model

$$\mu_m = \tilde{K}_m \sum_{g=1}^{n} ({}^{g}\mu_m/{}^{g}K_m) \qquad (30)$$

and this result may be extended to the inhibition models by introducing terms containing $^{h}\mu_i/{}^{h}K_i$ and $^{r}\mu_i/{}^{r}K_i$ (equations 9, and 19).

These equations show that, provided the cleavage of each of the polymer bonds takes place in an identical manner, the observed $\tilde{\mu}_f$ will be equal to $^{g}\mu_f$. In all other cases $\tilde{\mu}_f$ will be closer to the value of $^{g}\mu_f$, for the more readily cleaved bonds. The same conclusion is reached with regard to $\tilde{\mu}_m$; however, if competitive or non-competitive self-inhibition takes place, the activation energies for these processes will be included in the $\tilde{\mu}_m$ term. It follows, even in the case when cleavage at all bonds is identical, that $\tilde{\mu}_m$ need not be characteristic of the individual bond cleavage process.

It was stated, in discussing the first model, that if a sequence of enzyme-substrate complexes is involved in the cleavage process, the additivity relationships for $\tilde{k}_f$ and $\tilde{K}_m$ (equations 7 and 6) are valid providing the terms $^{g}k_f$ and $^{g}K_m$ are understood to involve the appropriate set of rate constants. Since equations (29) and (30) are derived from these additivity relationships they must apply in the case of this more general model.

Exo-cleavage: Repeated Attack on Individual Molecules of Substrate.—In the above paragraphs it has been assumed that the enzyme must dissociate from the cleaved substrate before it can act again on the substrate. The possibility has been considered that some enzymes with exo-type action pattern do not behave in this manner. In particular, Bailey and French (1957) have discussed the action of sweet potato β-amylase on amylose in terms of a "multiple attack" model which they formulate as shown in Figure 4 (complexes and rate constants re-numbered; U = cleavage unit *i.e.* maltose). By repre-

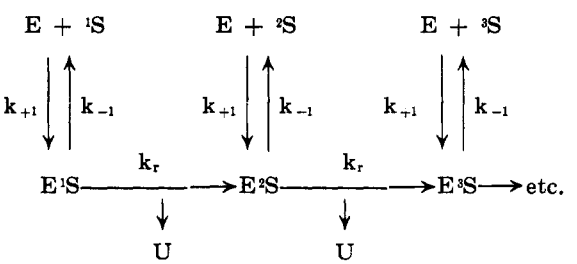

Figure 4

senting all corresponding rate constants as identical (all k_{+1} terms the same, etc.) the model in Figure 4 expresses the assumptions that all cleavable bonds are, from the point of view of the enzyme, indistinguishable. It also implies that cleavage is always accompanied by re-arrangement of the substrate on the enzyme surface (my interpretation). For short-chain polymers the former is unlikely to be correct while the latter confines all discussion to a special case of multiple attack. A more general treatment of this problem may be provided in the following manner.

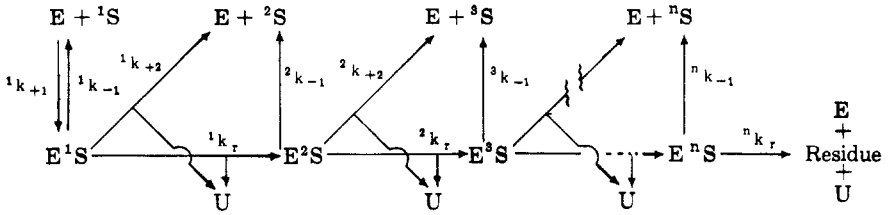

Figure 5

If the substrate is a polymer of n cleavable bonds, initial cleavage taking place at bond 1, and if each enzyme-substrate complex may (a) dissociate to give enzyme and substrate (k_{-1} process), (b) cleave the substrate to give the cleavage unit U plus substrate with one less cleavage unit (k_{+2} process), or (c) cleave and then rearrange to give cleavage unit plus enzyme-substrate complex with one less cleavage unit (k_r process), then the model shown in Figure 5 is obtained. Since the species ^2S, ^3S, to ^nS are not initially present in the reaction mixture, the recombination of E + ^2S etc. is neglected in the same way that the back reaction of the products with enzyme is neglected in deriving the Michaelis-Menten equation, and the recombination of the products of endo-cleavage with enzyme was neglected in deriving equation (4). The species E^1S, E^2S, E^3S, etc. are, therefore, assumed to reach steady state concentrations determined only by the constants in Figure 5. The following equations obtain:

$$[E_0] = [E] + [E^1S] + [E^2S] + \ldots + [E^nS] \quad (31)$$

$$\tilde{v} = ({}^1k_{+2} + {}^1k_r)[E^1S] + ({}^2k_{+2} + {}^2k_r)[E^2S] + \ldots + {}^nk_r[E^nS] \quad (32)$$

$$[E^1S] = [E][S]{}^1k_{+1}/({}^1k_{-1} + {}^1k_{+2} + {}^1k_r) = [E][S]{}^1\phi$$

$$[E^2S] = [E^1S]{}^1k_r/({}^2k_{-1} + {}^2k_{+2} + {}^2k_r) = [E^1S]{}^2\phi = [E][S]{}^1\phi{}^2\phi$$

$$[E^nS] = [E^{n-1}S]\,{}^{n-1}k_r/({}^nk_{-1} + {}^nk_r) = [E^{n-1}S]{}^n\phi = [E][S]{}^1\phi{}^2\phi \ldots {}^n\phi \quad (33)$$

hence

$$\tilde{v} = \frac{[E_0][S](({}^1k_{+2} + {}^1k_r){}^1\phi + ({}^2k_{+2} + {}^2k_r){}^1\phi{}^2\phi \text{ etc.})}{1 + [S]({}^1\phi + {}^1\phi{}^2\phi + {}^1\phi{}^2\phi{}^3\phi \text{ etc.})} \quad (34)$$

Equation (34) is of the Michaelis-Menten form and reduces to the standard expression for exo-cleavage without repeated attack provided that $^1k_r = 0$, *i.e.* $\tilde{k}_f = [E_0]{}^1k_{+2}$, and $\tilde{K}_m = ({}^1k_{-1} + {}^1k_{+2})/{}^1k_{+1}$. The present model is thus compatible with the model for sweet potato β-amylase action (no repeated attack) examined by Thoma and Koshland (1960) (see above under competitive self-inhibition). The equations extending this model to include competitive and non-competitive self-inhibition terms may be derived by making the necessary additions to equations (31) to (33).

If the substrate molecule is modified in such a way that the release of the terminal cleavage unit may be followed (*e.g.* by means of carbon-14 labeling), then the initial velocity of cleavage $\tilde{v}^*$ so determined may be compared to the total velocity of cleavage $\tilde{v}$. Since

$\tilde{v}^* = [E][S]\,({}^1k_{+2} + {}^1k_r){}^1\phi$ and

$\tilde{v} = [E][S](({}^1k_{+2} + {}^1k_r){}^1\phi + ({}^2k_{+2} + {}^2k_r){}^1\phi{}^2\phi + \ldots + {}^nk_r{}^1\phi{}^2\phi \ldots {}^n\phi)$

then for all values of [S]

$$\frac{\tilde{v}}{\tilde{v}^*} = 1 + \frac{({}^2k_{+2} + {}^2k_r)}{({}^1k_{+2} + {}^1k_r)}{}^2\phi + \frac{({}^3k_{+2} + {}^3k_r)}{({}^1k_{+2} + {}^1k_r)}{}^2\phi{}^3\phi \text{ etc.} \quad (35)$$

Since $^2\phi = {}^1k_r/({}^2k_{-1} + {}^2k_{+2} + {}^2k_r)$ then when $^1k_r = 0$, *i.e.* no repeated attack, $\tilde{v}/\tilde{v}^* = 1$. Any significant deviation of $\tilde{v}/\tilde{v}^*$ from unity may be interpreted as evidence for the repeated attack mechanism provided the present model is held to describe the experimental situation under investigation. This conclusion is independent of the presence or absence of self-inhibition terms in the expression for $\tilde{v}$.

If in some experimental situation 'single-chain digestion' is held to occur, then only E^1S may dissociate to give E + S (all k_{-1} terms = 0 except $^1k_{-1}$) and no cleavage to give E + S + U takes place (all k_{+2} terms = 0). These conditions lead to the relationships

$$\tilde{k}_f = [E_0]\,n{}^1k_r{}^1k_{+1}/({}^1k_r + {}^1k_{-1}) \quad (36)$$

$$1/\tilde{K}_m = \frac{{}^1k_r{}^1k_{+1}(1/{}^1k_r + 1/{}^2k_r + \ldots + 1/{}^nk_r)}{({}^1k_r + {}^1k_{-1})} \quad (37)$$

$$\tilde{v}/\tilde{v}^* = n \quad (38)$$

In this extreme situation, therefore, the total velocity of the reaction will be many times the velocity for removing the terminal unit of the chain.

If in some experimental situation all bonds are held to be equivalent (*e.g.* $k_r = {}^1k_r = {}^2k_r$ etc.), then

$$\tilde{k}_f = G[E_0](k_{+2} + k_r)k_{+1}/(k_{-1} + k_{+2} + k_r) \quad (39)$$

$$1/\tilde{K}_m = G\,k_{+1}/(k_{-1} + k_{+2} + k_r) \quad (40)$$

$$\tilde{v}/\tilde{v}^* = G = 1 + k_r/(k_{-1} + k_{+2} + k_r) + k_r{}^2/(k_{-1} + k_{+2} + k_r)^2 + \ldots + k_r{}^n/(k_{-1} + k_r)^n \quad (41)$$

Provided that k_{-1} and k_{+2} are not zero, then $k_r/(k_{-1} + k_{+2} + k_r) < 1$ and the above geometric series is convergent. The maximum value for

$\tilde{v}/\tilde{v}^* = G$ is thus determined by the situation in which $n = \infty$, viz.

$$\lim_{n\to\infty} \tilde{v}/\tilde{v}^* = \frac{1}{1 - k_r/(k_{-1} + k_{+2} + k_r)} = 1 + \frac{k_r}{k_{-1} + k_{+2}} \quad (42)$$

Under these circumstances

$$\lim_{n\to\infty} \tilde{k}_f = [E_0](k_{+2} + k_r)k_{+1}/(k_{-1} + k_{+2}) \quad (43)$$

$$\lim_{n\to\infty} 1/\tilde{K}_m = k_{+1}/(k_{-1} + k_{+2}) \quad (44)$$

Equation (44) will be valid only if self-inhibition effects may be neglected but equations (42) and (43) are independent of such effects. If, therefore one considers the digestion of a polymer which is long as compared with the specificity region of the enzyme, and if the rate constants are such that successive terms in equation (41) become negligible after only a few terms, then the above limits (equations 42 and 44) may be equated with the observed constants even though chains of infinite length are not present.

It is important to note that equations (43) and (44) can be derived by considering directly the situation in which a polymer chain that is long as compared with the specificity region of the enzyme is digested by repeated attack. Following Bailey and French (1957): (*a*) Let the polymeric products of a single cleavage, from the point of view of the law of mass action, be entirely equivalent to the starting material. (*b*) Let the rate constants be such that the frequency is vanishingly small with which a single chain is diminished, by repeated attack, to the point where the polymeric product of cleavage is not equivalent to the starting material. These conditions lead to the model represented in Figure 6.

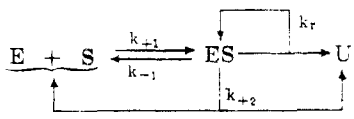

Figure 6

If, then, [ES] reaches a steady state, the following equations obtain:

$$[E][S]k_{+1} + [ES]k_r = [ES](k_{-1} + k_{+2} + k_r)$$

$$[ES] = [E][S]k_{+1}/(k_{-1} + k_{+2}) \quad (45)$$

$$[E_0] = [E] + [ES] \quad (46)$$

$$\tilde{v} = [ES](k_r + k_{+2}) \quad (47)$$

Equations (43) and (44) follow in the usual way. The same argument applies to a group of polymers of similar chain length. Since [S] and [ES] remain constant with time the model implies that the time course of monomer release is that of a zero order reaction.

The above alternative derivation of equations (43) and (44) serves as a check on the more elaborate model. It also allows the implications of omitting a k_{+2} term from the model for repeated attack to be separately examined. Thus the model (Fig. 4) discussed by Bailey and French (1957) may be re-written as Figure 6, $k_{+2} = 0$. Substituting $k_{+2} = 0$ in (44) $1/\tilde{K}_m = k_{+1}/k_{-1}$ as deduced by these authors. The more elaborate model possesses the advantage that an expression for $\tilde{v}/\tilde{v}^*$ may be derived (the simple model does not distinguish the first bond to be cleaved from any other). Also, the model is applicable to short-chain polymers, the primary concern of this paper.

The above treatment of exo-cleavage by repeated attack on single chain may be extended to cover the following more complex situations: in which (1) a group of polymers differing in length by one unit are present as substrate; (2) group transfer may take place (*e.g.* phosphorolysis) in place of, or in addition to, hydrolytic cleavage; (3) additional intermediate enzyme-substrate complexes are considered to take part in the cleavage and rearrangement processes; (4) competitive and non-competitive self-inhibition effects are taken into account.

Discussion

The models examined in the theoretical section of this paper employ certain familiar assumptions of enzyme kinetics, *viz.* the law of mass action, the steady state assumption, and the view that when initial velocities of reaction are studied the back reaction of the products to give the reactants may be neglected. Polymeric substrates, however, differ from the substrates normally considered in that the products of the reaction are themselves substrates. It is convenient therefore to distinguish four phases in the time course of enzyme action: (1) the transient phase, (2) a phase in which the steady state assumption applies and in which the reaction of the products of cleavage with the enzyme may be neglected, (3) a phase in which the reaction of the products with the enzyme must be considered, (4) a terminal phase in which the back reaction of products to give reactants is also of importance. In the present paper it is assumed that the transient phase is sufficiently brief and phase (2) of sufficient duration for the initial velocity of reaction $\tilde{v}$ to be defined in terms of phase (2). Although the kinetics of the transient phase and of phase (3) (see Whitaker, 1954) are of importance, their mathematical analysis is outside the scope of this study.

In applying the mathematical models to particular experimental situations, certain properties of polymeric substances must be borne in mind. Thus when long-chain polymers are attacked by enzymes, the rate of cleavage of the substrate may be limited by the rates of diffusion of the enzyme and substrate. Also, polymeric molecules may exist in solution not only as randomly oriented chains, but as a dynamic mixture of such extended molecules and molecules that have adopted

more stable conformations, either by association or by intra-molecular bond formation. Such ordered structures (*e.g.* the α-helical region in a polypeptide molecule) will be less readily cleaved than the randomly oriented polymer, which is free to adopt the configuration on the enzyme surface necessary for cleavage. The longer the polymer chain the more important will this effect become. It follows that the models developed in this paper may be applied to the cleavage of the first few members of a polymer series with reasonable confidence, but that care must be exercised in interpreting the cleavage of longer molecules. The degree of polymerization at which these effects become significant will vary according to the nature of the substrate.

The various models considered demonstrate, *inter alia*, that a multitude of rate constants may hide themselves behind the mask of the Michaelis-Menten equation. The simple models for endo-cleavage, and exo-cleavage by repeated attack, and the modifications of these models to include competitive self-inhibition give rise to equations of this form (4, 9 to 11, and 34). The models involving non-competitive self-inhibition also give rise to equations which may reduce to the Michaelis-Menten form as the substrate concentration is decreased (18 and 19). It follows that the empirical constants $\tilde{k}_f$ and $\tilde{K}_m$ [obtained by fitting the experimental data to an equation of this form (5)], and the variation of these constants with the degree of polymerization of the substrate, may be interpreted in terms of these theoretical models.

Endo-cleavage.—The various expressions for $\tilde{V}_{max}$ ($= \tilde{k}_f\tilde{K}_m$), $1/\tilde{K}_m$, and $\tilde{k}_f$ deduced from the models for endo-cleavage, show the following trend towards simplicity. The expressions for $\tilde{V}_{max}$ are complex, $\tilde{V}_{max}$ bearing no simple relationship to the rate constants for the cleavage processes at the individual bonds of the substrate (8, 11, 18, and 19, 27). The expressions for $\tilde{K}_m$ are much simpler (6, 9, 19, 25) and in the absence of self-inhibition effects $1/\tilde{K}_m = \sum_{g=1}^{n} 1/{}^gK_m$ (6). Unfortunately the extent to which self-inhibition terms contribute to $1/\tilde{K}_m$ is not readily determined. This uncertainty, however, does not arise in interpreting $\tilde{k}_f$. The relationship $\tilde{k}_f = \sum_{g=1}^{n} {}^gk_f$ (7) is obtained both for the simple model and for the models involving self-inhibition (10 and 18). The same equation is obtained whether one or several enzyme-substrate complexes are considered to take part in the cleavage process. Furthermore, the pH dependence of $\tilde{k}_f$ is a function of the ionization of the enzyme and does not depend on the ionization of the various enzyme-substrate complexes (26). The study of $\tilde{k}_f$ as a function of n is, therefore, of potential use in investigating the mode of action of endo-cleaving enzymes.

The above comments are of a mathematical nature. In the following paragraphs the mode of action of an enzyme of endo-action pattern is discussed in molecular terms. The properties of this representational model are related to the mathematical models already examined. Kinetic measurements may, therefore, be interpreted in terms of this representation and may be used to give information about the cleavage process so understood. The model may be usefully compared with that advanced by Thoma and Koshland (1960) to account for the action of the exo-acting enzyme β-amylase.

An enzyme exhibiting an endo action-pattern may be pictured as having on its surface, actually or potentially, a linear series of segments, each segment capable of binding a substrate monomer residue in some definite orientation. The enzyme surface is thus complementary to a fixed length of polymer chain. If a mixed population of enzyme molecules with specificity sequences of varying lengths are present in an apparently homogeneous preparation, the observed properties of the enzyme will be those of a hypothetical average enzyme molecule. The chemical groupings that constitute the active site of the enzyme, or that may give rise to the active site through substrate-induced folding of the enzyme (induced-fit hypothesis [Thoma and Koshland, 1960]), must be pictured as occupying some region along the line of the specificity sequence. Only substrate molecules that bind across the active site can be cleaved. Short polymers capable of complexing with the enzyme but incapable of inducing the formation of the active site will not be cleaved, but will act as competitive inhibitors towards longer polymers. Short polymers, or the ends of longer polymers, may bind to segments removed from the active site, and at high substrate concentration complexes may form in which two molecules of substrate are bound, neither group bridging the active site. In this circumstance, inhibition at high substrate concentration will be observed (equation 15). From equations (15) and (19) it follows that measurements of $\tilde{K}_m$ for a given substrate will record not only the true ${}^gK_m$ values, or ${}^gK_s$ values, for the cleavage process, but also the reciprocal of the affinity of the enzyme for the substrate at positions which do not promote cleavage. Measurements of $\tilde{k}_f$, on the other hand, give information about the properties of the enzyme-substrate complexes which form with the substrate bridging the active site, and about no other complexes.

The several affinities of the enzyme for the substrate $1/{}^gK_s$ ($= {}^gk_{+1}/{}^gk_{-1}$), $1/{}^hK_i$, and $1/{}^rK_i$, will be functions of both the length of the substrate molecule and the segments at which binding takes place. If the enzyme has to fold into some otherwise unfavorable conformation in order to bind substrate, the lowest members of the polymer series may be unable to complex with the enzyme and will therefore neither be cleaved nor act as

competitive inhibitors towards higher polymers. An increase in affinity with an increase in the degree of polymerization of the substrate should take place unless the bound substrates in the complexes considered lap over the end, or ends, of the specificity region. Provided that only the affinity constants are markedly affected by the position of complexing, and that the other rate constants for the cleavage process are relatively insensitive to the length of the substrate molecule, then the various ${}^{g}k_f$ and $1/{}^{g}K_m$ terms, as well as the $\tilde{k}_f$ and $1/\tilde{K}_m$ terms, should increase with the degree of polymerization for the first few members of the polymer series.

The results of studies of the mode of action of *Myrothecium verrucaria* cellulase by Whitaker and his associates (1959) are in keeping with the above picture. For the series of β-1→4-oligoglucosides, cellobiose to cello-pentaose, inhibition at high substrate concentration takes place, and both $\tilde{k}_f$ and $1/\tilde{K}_m$ increase with increase in the degree of polymerization of the substrate. The data indicate that the ${}^{g}k_f$ values for cellotriose are both greater than $\tilde{k}_f$ for cellobiose, but are less than ${}^{g}k_f$ for the center bond of cellotetraose (Whitaker, 1954, 1956; Whitaker and Merler, 1956). Salivary α-amylase acting on α-1→4-oligoglucosides (Walker and Whelan, 1960), the dextranase from *Lactobacillus bifidus* acting on α-1 → 6-oligoglucosides (Bailey and Clarke, 1959), and the nuclease from *Azotobacter agilis* acting on the oligoribonucleotides pApA to pApApApA (Stevens and Hilmoe, 1960), show action patterns which resemble that of cellulase.

In order to determine the length of the specificity region of a given enzyme and the position in the specificity sequence of the active site, two possible procedures present themselves: (1) If ${}^{g}k_f$ values for the terminal bonds, (${}^{1}k_f$ or ${}^{n}k_f$) of a series of polymers were determined by using substrates labeled in one or the other terminal residue and by following the release of radioactive monomer, then the ${}^{1}k_f$ (or ${}^{n}k_f$) values should increase with the length of the substrate until the substrate reaches to the end of the specificity region. For polymers of greater length, ${}^{1}k_f$ (or ${}^{n}k_f$) should be independent of the degree of polymerization. In this way both the length of the specificity region and the position of the active site could be measured. (2) If measurements of $\tilde{k}_f$ were made for a series of polymers, and if a length were established beyond which a constant increase in $\tilde{k}_f$ was observed for each unit increase in the polymer, then a direct measurement of the length of the specificity region would result. The latter argument when applied to the data on cellulase shows that the specificity region of this enzyme is at least 5 units in length. This lower limit may be compared with the 4 to 5 unit region postulated for sweet potato β-amylase (Thoma and Koshland, 1960), the 6 to 7 unit region present in antibodies to dextran and levan (Kabat, 1957), and the primer binding region of potato phosphorylase which is at least 4 units and may be 6 units in length (Whelan and Bailey, 1954).

The several enzymes cited above have been shown to cleave bonds near the end of short-chain polymer substrates less readily than those at the center. For the lowest members of the polymer series this effect is unlikely to be a consequence of the substrate extending beyond the specificity region of the enzyme. The induced-fit hypothesis, among other explanations, is capable of accounting for the variation.

In the preceding paragraphs the nature of substrate-enzyme binding has not been considered. For carbohydrates the difference between hydrogen bonding to water and hydrogen bonding to enzyme must be small. The major contribution to the free energy of binding may therefore be the increase in rotational freedom, *i.e.* entropy, of the water molecules displaced from the enzyme and substrate surfaces when the substrate is bound (Singer, 1957). The loss in rotational freedom of the polymer chain on binding should be small when compared with this increase. It follows that, in the special case in which the simple model represented in Figure 1 is applicable and the equilibrium assumption holds ($K_m = K_s$), $\tilde{\mu}_m$ will be very small and almost independent of the degree of polymerization of the substrate, and ${}^{g}\mu_f$ will be almost equal to ${}^{g}\mu_{+2}$. If, in addition, the ${}^{g}\mu_{+2}$ terms vary little with g and polymer size, then $\tilde{\mu}_f$ will be independent of the degree of polymerization of the substrate. It is of interest that the $\tilde{\mu}_f$ values for cellulase are constant, within the limits of experimental error, for the series cellobiose to -tetraose although the individual ${}^{g}k_f$ values in this series differ at least 100-fold (Whitaker, 1956).

Exo-cleavage.—Thoma and Koshland (1960) have ingeniously demonstrated that the non-terminal portion of the amylose chain may act as a competitive inhibitor in the cleavage, by sweet-potato β-amylase, of maltose units from the non-reducing end of the polymer. By determining $\tilde{K}_m$ for substrates of various chain lengths and by making a reasonable assumption about the specificity region of the enzyme (4 glucose units in length) they were able to estimate the value of $\tilde{K}_m$ that would have been observed in the absence of competitive self-inhibition effects. The finding that a plot of $1/\tilde{K}_m$ against the degree of polymerization of the substrate was linear within the limits of experimental error supported the view that K_m is independent of the length of the polymer chain, and agreed with the postulated representational model of the inhibition process. If non-competitive self-inhibition can arise for this enzyme by the binding of the ends of two substrate molecules on the specificity region, then the inhibition constants for this effect will make a small contribution to the estimated value of K_m. It would seem reasonable that competitive self-inhibition is general for enzymes of exo-action pattern (equations (9) to (11), n = 1).

In examining the hypothesis that a particular enzyme degrades by repeated attack upon single molecules of substrate it is essential to take into account competitive inhibition. In the mathematical model for repeated attack developed in the theoretical section only the functions for k_f (34, 36, 39, and 43) and $\bar{v}/\bar{v}^*$ (35, 38, 41, and 42) are independent of such effects. In the special case of "single-chain" digestion (36), $\tilde{k}_f$ increases linearly with the number of cleavable bonds in the substrate (n), but in all other cases $\tilde{k}_f$ will tend to some steady value as n increases (*e.g.* 43). A more general test for the repeated attack process is given by the expression $\bar{v}/\bar{v}^*$, *i.e.* the ratio of the initial velocity of polymer cleavage to the initial velocity for removing the terminal unit of the polymer. If $\bar{v}/\bar{v}^*$ is unity, then no repeated attack occurs, whereas if $\bar{v}/\bar{v}^* = n$, "single chain" degradation takes place (38). Values intermediate between 1 and n indicate an intermediate type of process. The above examination of repeated attack in terms of $\bar{v}/\bar{v}^*$ may be compared with the alternative statistical treatment developed by Bailey and French (1957). They conclude that the average number of maltose units removed from amylose by sweet potato β-amylase, during the period of association of enzyme and substrate, is 3·3 (see also French, 1960). A detailed comparison of the two approaches is outside the scope of the present paper.

An enzyme cleaving a polymer by means of a repeated attack process may be considered to be more efficient, as an agent of degradation, than an equivalent enzyme that operates by cleaving the terminal unit only. At low substrate concentration (first-order conditions) a measure of this greater efficiency is provided by the function $\bar{v}/\bar{v}^*$. Unfortunately the attractiveness of the hypothesis that degradation takes place in the above manner is nicely matched by the difficulty in advancing a convincing representational model for such a process. This difficulty may be illustrated by considering the following two models for repeated attack by a β-amylase. In the first model, after the terminal maltose unit has been removed from the end of the chain, the polymer remains bound to the specificity region of the enzyme by weak forces until either dissociation or rearrangement takes place. The polymer moves up to the position for cleavage by passing first to some secondary specificity region and then back to the primary region (a lifting process). In the second model, based on the mechanism for repeated attack proposed by Wurtz *et al.* (1962), the specificity region of the enzyme consists of two sets of two segments, each segment equivalent to a glucose unit; also two potentially formable active sites are present. After the terminal maltose unit has been removed the polymer may either dissociate from the enzyme or glucose units 4 and 5 may "roll-on" to occupy the vacated segments. The second active site now forms to cleave a second maltose unit from the polymer, and the situation before the first cleavage is reconstituted. The first model involves a "lifting" process and a lateral motion of the two sections of the enzyme of at least 10 Angstrom units. The second involves either an improbable folding of the amylose chain or a migration of vacated sites before the next stage in the helical "roll-on" process can take place. Both of these models may be interpreted in terms of the rate constants of the mathematical model. The question, therefore, of whether or not repeated attack takes place can be dissociated from the question of how it takes place.

Such detailed representational models would be required to account for a cleavage process that approached single-chain digestion. Some degree of repeated attack, however, would be observed if the enzyme were able to bind both the cleavable end of the polymer chain and some other part of the chain distant from the end. Dissociation of the cleaved end would not, in general, result in the complete separation of enzyme and substrate, and further cleavage from the same chain would therefore be likely. It is also possible that for large polymers repeated attack could arise as a result of slow diffusion processes: a molecule of bulky substrate once cleaved is for some finite time after cleavage more likely to be attacked by the same enzyme molecule than a molecule of substrate that is outside some particular diffusion radius. This last intuitive model has been invoked, in connection with studies on the endo-acting salivary α-amylase, by French *et al.* (1950) but is equally, if not more, applicable to enzymes of exo-action pattern.

In the theoretical section of this paper various suggestions have been made for the further development of the mathematical models discussed. Insofar as these developments are related to standard models for enzyme kinetics, reference may be made to the textbooks mentioned above (Dixon and Webb, 1958; Laidler, 1958). As experimental evidence is gathered it may be anticipated that more elaborate mathematical models will have to be considered and that the representational model for the action of endo-cleaving enzymes will need to be modified. A complete theory will require that the various stages in the chemical cleavage process for exo- and endo-acting enzymes be represented by distinct rate constants, and that methods to determine these constants be evolved.

Acknowledgments

The author wishes to thank Dr. D. R. Whitaker of the National Research Council Laboratories, Ottawa, Canada, for demonstrating to the writer that a more detailed theoretical analysis of enzyme polymer cleavage was necessary; Dr. Anthony F. Bartholomay of the Biophysics Research Laboratory, Peter Bent Brigham Hospital, Boston, for his valuable comments and for his close reading of the manuscript; and also Dr. H. B. Vickery for his aid in the preparation of this paper.

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