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# MATHEMATICAL MODELS FOR THE ACTION OF ALPHA-AMYLASE ON AMYLOSE*

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ABSTRACT

Mathematical treatments have been developed to describe the action of alpha-amylases on amylose. The treatments are based on the unique properties of the exponential (or most-probable) distribution of molecular weights of the substrate, namely, that (*a*) the principal averages are invariant to chain-end attack if the product molecules are ignored, and (*b*) the ratio of the principal averages is invariant to random attack. The relations so developed allow published, qualitative data for the alpha-amylolysis of amylose to be interpreted in a quantitative manner. As a result, it appears that multiple attack is of little or no significance in the action patterns of alpha-amylases, with the exception of those derived from the pancreas.

## INTRODUCTION

The linear substrate amylose is degraded by alpha-amylases from various sources, not by the same action pattern, but rather by a series of mechanisms, each of which is characteristic of the specific enzyme. For example, when the course of alpha-amylolysis is followed by measuring reducing powder as a function of the decrease in iodine stain, a family of quite distinct curves is produced[1]. The differences in action pattern have been explained on the basis of two theories, namely, those of multiple attack[2] and preferred attack[3].

The theory of *multiple attack* envisages an initial random encounter between enzyme and substrate, with ali the bonds in the latter molecule being equally susceptible to hydrolysis. After the initial random scission, only one of the two product molecules diffuses away from the enzyme, and the other is retainecd, to undergo a second hydrolytic event at a bond close to the newly-exposed reducing group[4], producing a small molecule such as maltose or maltotriose. This repetitive attack can take place a number of times before the ultimate separation of enzyme and amylose molecules. For the same number of bonds broken, the molecular size of the amylose

*Dedicated to the memory of Sir Edmund Hirst, C.B.E., F.R.S.

(and thus its ability to bind iodine) is decreased more rapidly by random hydrolysis than by the repetitive action. Therefore, by postulating that alpha-amylases differ in their degree of multiple attack, the existence of a family of reducing-power/iodine-stain curves may be explained.

The theory of *preferred attack* postulates only a single hydrolytic event during each effective encounter between enzyme and substrate. Differences in action pattern are then explained by the assumption that the glycosidic bonds of amylose are not equally susceptible to hydrolysis, so that the various alpha-amylases fragment the polysaccharide in different ways. Specifically, bonds near chain ends may be more resistant to hydrolysis. With a starting material containing $\sim 1000$ D-glucosyl residues, the effect of chain ends is negligible, so that, in the initial stages, the hydrolysis is approximately random in nature, but as the reaction progresses, the influence of chain ends becomes more pronounced and the action pattern changes to preferred attack.

Two experimental methods have been proposed to differentiate between multiple and preferred attack. Banks *et al.*[5] measured the ratio $\bar{P}_w/\bar{P}_n$, where $\bar{P}_w$ and $\bar{P}_n$ are the weight- and number-average d.p., respectively, during the initial stages of the action of various alpha-amylases on amylose. Assuming the amylose originally possessed a most probable distribution of molecular weights, they argued that multiple attack would be shown by a rapid increase in the ratio, whereas in the initial stages of preferred attack, the ratio would be constant, with a value of $\sim 2$. Robyt and French[2], on the other hand, studied a somewhat later stage of the hydrolysis. These authors measured the reducing power of the system, and then used an arbitrary fractionation procedure to separate the components into small product molecules, arising from repetitive action, and polymer molecules, arising from random hydrolysis; the ratio of the reducing power of the system to that of the polymer fraction then provided a measure of repetitive attack.

We now describe mathematical models for evaluating these two experimental methods. Thoma[6] has recently considered this problem independently, using a rather different approach.

## THEORY

### (*a*) *The properties of the most probable or exponential distribution*

These properties have been elucidated in detail by Gordon and co-worker[7–9], and hence only a summary is provided here. For linear condensation polymers, the number-fraction of molecules containing exactly $x$ units is designated $P_x$, and

$$P_x = (1-p)p^{x-1}, \tag{1}$$

where $p$ is the probability that a functional group has reacted. For such a system,

$$\bar{P}_n = 1/(1-p) \tag{2}$$

$$= 1/q. \tag{3}$$

Substituting (*2*) and (*3*) into (*1*) gives

$$P_x = q(1-q)^{x-1}, \tag{4}$$

which for the limiting case of $x \gg 1$ yields

$$P_x = q \exp(-qx). \tag{5}$$

The corresponding weight fraction, $w_x$, is given by

$$w_x = xq^2 \exp(-qx). \tag{6}$$

The function expressed by (*1*) is the most probable distribution; in the alternative form of (*5*), it is referred to as the exponential distribution. For practical purposes, the two are identical.

The distribution function, $f(x)$, is given by

$$f(x) = q \exp(-qx). \tag{7}$$

Equations (*5*) and (*7*) are identical, because the latter is normalized.

If the chains in the distribution given by (*7*) are subjected to chain-end attack with the removal of $m$ units, and these product molecules are taken from the system, a new polymer distribution, $f'(x)$, remains where

$$f'(x) = q \exp[-q(x+m)], \tag{8}$$

and

$$P'_x = q \exp[-q(x+m)]/q \int_{x=m}^{\infty} \exp(-qx) \tag{9}$$

$$= q \exp(-qx) \tag{10}$$

$$= P_x. \tag{11}$$

For a substrate having the exponential distribution of molecular weights, there exists the unique solution that chain-end attack causes no change in the distribution function, and therefore the principal averages of the distribution are invariant, assuming the product molecules to be removed from the system.

The exponential distribution is the function to which polymers having other distribution functions revert on random degradation. For example, when a single polymer molecule of infinite length is subjected to random hydrolysis, the weight-fraction of $x$-mer is approximated by[10]

$$w_x = xs^2(1-s)^{x-1}, \tag{12}$$

where $s$ is the fraction of bonds hydrolysed. For this model,

$$s = q. \tag{13}$$

Substituting (*13*) into (*12*) gives

$$w_x = xq^2(1-q)^{x-1}. \tag{14}$$

In the limiting case of $x \gg 1$, (*14*) reduces to (*6*). The theoretical treatment of Thoma[6] is based on the use of equation (*12*).

The corollary to the statement that other distributions revert to the exponential form on random hydrolysis is that the form of the exponential distribution is invariant to such degradation, although the value of $q$ increases. As a result, the ratios of the principal averages of the distribution do not change during random hydrolysis, and in particular

$$\bar{P}_w/\bar{P}_n = 2. \tag{15}$$

As already indicated, the initial stages of preferred attack are indistinguishable from random hydrolysis, so that equation (*15*) is applicable in the latter case.

(*b*) *The mathematical model for multiple attack during the initial stages of hydrolysis*

The properties of the exponential distributions are such that the principal averages are invariant to chain-end attack, if the product of low molecular weight is removed from the system, and the ratio of the principal averages is invariant to random hydrolysis. The theory of multiple attack envisages a combination of random and chain-end hydrolysis. The distribution function for amylose leached from potato starch is exponential in form[11]; therefore, the properties of the distribution described above can be incorporated in a mathematical model applicable to the hydrolysis of the polysaccharide by an alpha-amylase exhibiting multiple attack.

It is convenient to consider multiple attack as giving rise to two distinct distributions, one arising from chain-end attack (product distribution) and the other from the random hydrolytic component (polymer distribution). By definition, the principal averages of the system are then given by

$$\bar{P}_{n(\text{system})} = \left[\frac{w_p}{\bar{P}_{\text{product}}} + \frac{1-w_p}{\bar{P}_{n(\text{polymer})}}\right]^{-1}, \tag{16}$$

and $$\bar{P}_{w(\text{system})} = w_p P_{\text{product}} + (1-w_p)\bar{P}_{w(\text{polymer})}, \tag{17}$$

where $w_p$ and $P_{\text{product}}$ are the weight fraction and d.p. of the product, respectively; it is assumed that the difference between the number- and weight-average d.p. of the product is negligible.

The weight fraction of product molecules is given by

$$w_p = S\theta P_{\text{product}}/\bar{P}_{n(o)}, \tag{18}$$

where $\theta$ is the number of repetitive attacks per random scission. The degree of scission, $S$, is defined by either

$$S = (\bar{P}_{n(o)}/\bar{P}_{n(t)}) - 1, \tag{19}$$

or

$$S = (\bar{P}_{w(o)}/\bar{P}_{w(t)}) - 1, \tag{20}$$

where the subscripts $o$ and $t$ refer to measurements made at the time zero and time $t$, respectively. Only in the case of random degradation of a polymer having an exponential distribution of molecular weights are equations (*19*) and (*20*) valid simultaneously.

Substituting (*18*) into (*16*) gives

$$\bar{P}_{n(\text{system})} = \left[\frac{S\theta}{\bar{P}_{n(o)}}\left(1 - \frac{P_{\text{product}}}{\bar{P}_{n(\text{polymer})}}\right) + \frac{1}{\bar{P}_{n(\text{polymer})}}\right]^{-1}. \tag{21}$$

Equation (*21*) shows that as the size of the product molecule (*i.e.*, the molecule produced by chain-end attack) increases, the action pattern asymptotically approaches that predicted for random hydrolysis, which is intuitively obvious. However, in the initial stages of the hydrolysis, the ratio $P_{\text{product}}/\bar{P}_{n(\text{polymer})}$ can be neglected, reducing (*21*) to

$$\bar{P}_{n(\text{system})} = \left[\frac{S\theta}{\bar{P}_{n(o)}} + \frac{1}{\bar{P}_{n(\text{polymer})}}\right]^{-1}. \tag{22}$$

From equation (*20*),

$$\bar{P}_{n(\text{polymer})} = \bar{P}_{n(o)}/(S+1). \tag{23}$$

Substituting (*23*) into (*22*) gives

$$\bar{P}_{n(\text{system})} = \bar{P}_{n(o)}/(S\theta+S+1). \tag{24}$$

In the initial stages of the hydrolysis, the parameter $w_p$ is sufficiently small to reduce (*17*) to

$$\bar{P}_{w(\text{system})} = \bar{P}_{w(\text{polymer})} \tag{25}$$

$$= 2\bar{P}_{n(\text{polymer})} \tag{26}$$

$$= 2\bar{P}_{n(o)}/(S+1). \tag{27}$$

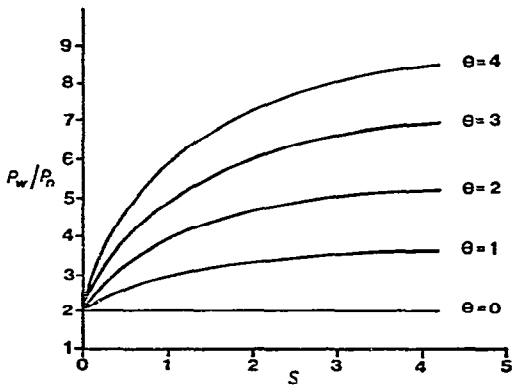


Fig. 1. The calculated variation in the ratio $\bar{P}_w/\bar{P}_n$ as a function of the degree of scission $S$ for various values of $\theta$, the degree of multiple attack. In each case, the product of low molecular weight resulting from non-random hydrolysis is assumed to be maltotriose.

Combining equations (*24*) and (*27*) gives

$$\bar{P}_{w(\text{system})}/\bar{P}_{n(\text{system})} = 2(S\theta+S+1)/(S+1). \tag{28}$$

Thoma[6], employing different assumptions and a rather more complex mathematical treatment, obtained a relation identical to equation (*28*).

Values of the ratio $\bar{P}_w/\bar{P}_n$, calculated from equation (*28*), are shown in Fig. 1 as a function of the degree of scission $S$ for various values of the parameter $\theta$, the number of repetitive attacks/random scission.

(*c*) *The mathematical model for multiple attack during the intermediate and later stages of hydrolysis*

Robyt and French[2] used fractionation with 67% alcohol to separate the components of the system into product (resulting from chain-end attack) and polymer (resulting from random hydrolysis). The parameter $r_1$ may be defined as

$r_1$ = (total increase in reducing end-groups of system)/(increase in reducing end-groups due to random hydrolysis) (*29*)

$$= \left[\frac{1}{\bar{P}_{n(\text{system})}} - \frac{1}{\bar{P}_{n(o)}}\right] \Big/ \left[\frac{1}{\bar{P}_{n(\text{polymer})}} - \frac{1}{\bar{P}_{n(o)}}\right]^{-1}. \tag{30}$$

Substituting (*23*) and (*24*) into (*30*) yields

$$r_1 - 1 = \theta. \tag{31}$$

In obtaining (*31*), the approximation that the term $\bar{P}_{\text{product}}/\bar{P}_{n(\text{polymer})}$ of equation (*21*) is negligible has been introduced. Whilst valid in the initial stages of the hydrolysis, calculation suggests that this approximation would lead to errors of up to $\sim 15\%$ in the values of $r_1$ in the range investigated by Robyt and French. The exact equation is obtained by substituting (*21*) and (*23*) into (*30*), to give

$$r_2 - 1 = \theta[1 - P_{\text{product}}(S+1)/\bar{P}_{n(o)}]. \tag{32}$$

Although analysing their results by means of equation (*31*), Robyt and French actually used a slightly different experimental parameter, $r_3$, where

$r_3$ = (increase in reducing power of system)/(increase in reducing power of polymer). (*33*)

The increase in reducing power of the system $\Delta R_s$ is given by

$$\Delta R_s = k\left[\frac{1-w_p}{\bar{P}_{n(\text{polymer})}} + \frac{w_p}{P_{\text{product}}} - \frac{1}{\bar{P}_{n(o)}}\right], \tag{34}$$

and that of the polymer, $\Delta R_p$, by

$$\Delta R_p = k\left[\frac{1-w_p}{P_{n(\text{polymer})}} - \frac{1}{\bar{P}_{n(o)}}\right], \tag{35}$$

where $k$ is a proportionality constant.

Substitution of (*18*), (*23*), (*34*), and (*35*) in (*33*) gives

$$r_3 - 1 = \theta \Big/ \left[ 1 - \frac{\theta P_{\text{product}}}{\bar{P}_{n(o)}} (S+1) \right]. \tag{36}$$

Effectively, Robyt and French used the approximation that the term $\theta P_{\text{product}}(S+1)/\bar{P}_{n(o)}$ could be discarded, reducing equation (*36*) to

$$r_3 - 1 = \theta. \tag{37}$$

The error introduced by this approximation obviously depends on the values ascribed to the parameters $\theta$, $P_{\text{product}}$, and $\bar{P}_{n(o)}$, and to the stage of the reaction. However, it is apparent that the use of equation (*37*) rather than (*36*) will lead to $r_3$ increasing as the hydrolysis proceeds, a phenomenon observed by Robyt and French[2]. In these circumstances, use of the average value of $r_3$, as did Robyt and French, must lead to an over-estimation of the degree of multiple attack.

Another source of error in the treatment of Robyt and French[2] concerns their assumption that the fractionation procedure separates the system into product and polymer molecules. They stated that the material not precipitated by ethanol has d.p. values in the range 1–20 D-glucosyl residues. This range, as they acknowledged, covers the low molecular weight end of the polymer distribution, and the assumption that it represents only the product becomes progressively less valid as the hydrolysis proceeds. For this reason, they restricted measurements to the region in which the intensity of the iodine stain had not fallen below 50% of its original value. Even in this range, however, the approximation cannot be justified. Banks and Greenwood[12] showed that, in the range of $S$ values used by Robyt and French[2], even a purely random hydrolytic process would generate an apparently finite degree of multiple attack. These calculations were based on the assumption that chains containing 20 or fewer D-glucosyl residues are not precipitated by 67% ethanol, and that the amylose initially has $\bar{P}_{n(o)} = 1000$ residues. Under these conditions, the weight fraction of polymer (*i.e.*, chains produced by random scission) not precipitated by 67% ethanol, $w'_{\text{LP}}$, will be given by

$$w'_{\text{LP}} = q_o^2(S+1)^2 \int_{x=0}^{1/50q_o} x \exp - q_o(S+1)x\,dx \tag{38}$$

$$= 1 - \left[ \frac{S+1}{50} + 1 \right] \exp - [(S+1)/50]. \tag{39}$$

Designating the number-average d.p. of the material precipitated by the alcohol as $\bar{P}_{n(\text{HP})}$ and that of the material not precipitated as $\bar{P}_{n(\text{LP})}$, there exists the relation

$$\bar{P}_{n(\text{polymer})} = \left[ \frac{w'_{\text{LP}}}{\bar{P}_{n(\text{LP})}} + \frac{1 - w'_{\text{LP}}}{\bar{P}_{n(\text{HP})}} \right]^{-1}. \tag{40}$$

For the example chosen above, $\bar{P}_{n(\text{LP})}$ can be ascribed an average value of 10 D-glucosyl residues over the entire hydrolysis without introducing a serious error.

By solving equation (*39*) for $w'_{LP}$ and using the derived value in (*40*), it is possible to relate $\bar{P}_{n(\text{polymer})}$ to $\bar{P}_{n(\text{HP})}$, the latter parameter being that quoted by Robyt and French[2]. Graphical solution of (*40*) shows that over the range of $S$ values employed by Robyt and French, there exists the approximate relation

$$\bar{P}_{n(\text{HP})} - \bar{P}_{n(\text{polymer})} \sim 16. \tag{41}$$

When the results of these workers are corrected according to equation (*41*), it becomes apparent that they extended their measurements to $\bar{P}_{n(\text{polymer})} = 21$ D-glucosyl residues when using the alpha-amylase from *Aspergillus oryzae*, although, even at that stage, the intensity of the iodine stain was still 51% of the original value.

In equations (*38*)–(*40*), the parameter $w'_{LP}$ refers to the weight fraction *of polymer* that is not precipitated by 67% ethanol. The weight fraction, $w_{LP}$, with respect to the system, that is not precipitated by ethanol is

$$w_{LP} = (1 - w_p) w'_{LP}. \tag{42}$$

The increase in reducing power, $\Delta R'_p$, measured by Robyt and French is thus given by

$$\Delta R'_p = k \left[ \frac{1 - (w_p + w_{LP})}{\bar{P}_{n(\text{HP})}} - \frac{1}{\bar{P}_{n(o)}} \right]. \tag{43}$$

Substituting (*42*) into (*40*) gives

$$\frac{1 - (w_p + w_{LP})}{\bar{P}_{n(\text{HP})}} = \frac{1 - w_p}{\bar{P}_{n(\text{polymer})}} - \frac{w_{LP}(1 - w_p)}{(1 - w_p)\bar{P}_{n(\text{LP})}}. \tag{44}$$

Substituting (*44*) into (*43*) yields

$$\Delta R'_p = k \left[ \frac{1 - w_p}{\bar{P}_{n(\text{polymer})}} - \frac{w_{LP}}{\bar{P}_{n(\text{LP})}} - \frac{1}{\bar{P}_{n(o)}} \right]. \tag{45}$$

Defining the ratio $r_4$ as

$$r_4 = \Delta R_s / \Delta R'_p, \tag{46}$$

and substituting (*34*) and (*45*) into (*46*) gives

$$r_4 = \left[ X + \frac{w_p}{\bar{P}_{\text{Product}}} \right] \Big/ \left[ X - \frac{w_{LP}}{\bar{P}_{n(\text{LP})}} \right], \tag{47}$$

where $X$ is defined by

$$X = \frac{1 - w_p}{\bar{P}_{n(\text{polymer})}} - \frac{1}{\bar{P}_{n(o)}}. \tag{48}$$

Further, from (*34*), (*35*), and (*36*), it follows that

$$\left[ X + \frac{w_p}{P_{\text{product}}} \right] \Big/ X = \theta \Big/ \left[ 1 - \frac{\theta P_{\text{product}}}{\bar{P}_{n(o)}} (S+1) \right] + 1. \tag{49}$$

One further refinement of (*47*) is required, due to the fact that D-glucose residues are transferred from the polymer distribution to the product distribution. Thus, when $\bar{P}_{n(\text{polymer})}$ reaches, for example, 40 D-glucose residues from an initial value of 1000, the degree of scission calculated from equation (*19*) is 24, *i.e.*, each polymer molecule that has survived has undergone an average of 24 random hydrolytic events. However, each random hydrolytic scission is followed by chain-end attack giving rise to product molecules, a process earlier shown to be equivalent to reducing the number of polymer chains. Thus, the proportion of D-glucose residues found in the polymer fraction decreases as the reaction proceeds, and the value of $S$ derived from equation (*19*) is no longer identical to that used in (*18*). It is therefore necessary to redefine the weight fraction of product, $w'_p$, as

$$w'_p = S'\theta P_{\text{product}}/\bar{P}_{n(o)}, \tag{50}$$

where $S'$ is the degree of scission corrected for the loss of polymer chains.

The parameter $S'$ can be obtained as follows. Consider a model in which polymer molecules are subjected to an average of one random hydrolytic scission per chain. As before, each random event is followed by $\theta$ non-random scissions to give a product of d.p., $P_{\text{product}}$, which is removed from the system, and the residual chains are then subjected repeatedly to the same process. The fraction of chains entering each cycle that survives as polymer, $F_i$, is given by

$$F_i = 1 - \frac{\theta P_{\text{product}}}{\bar{P}_{n(i)}}, \tag{51}$$

where $\bar{P}_{n(i)}$ is the number-average d.p. at the start of each cycle. In the above model, the molecular weight is halved in each cycle, so that $\bar{P}_{n(i)}$ takes successively the values $\bar{P}_{n(o)}$, $P_{n(o)}/2$, $P_{n(o)}/4$, etc. Therefore, the fraction of the original material exposed to random hydrolysis in each cycle is given by

$$\text{1st cycle} \quad S = 1 \quad F_1 = 1; \tag{52}$$

$$\text{2nd cycle} \quad S = 3 \quad F_2 = 1\left(1 - \frac{\theta P_{\text{product}}}{\bar{P}_{n(o)}}\right); \tag{53}$$

$$\text{3rd cycle} \quad S = 7 \quad F_3 = 1\left(1 - \frac{\theta P_{\text{product}}}{\bar{P}_{n(o)}}\right)\left(1 - \frac{2\theta P_{\text{product}}}{\bar{P}_{n(o)}}\right); \tag{54}$$

$$\text{4th cycle} \quad S = 15 \quad F_4 = 1\left(1 - \frac{\theta P_{\text{product}}}{\bar{P}_{n(o)}}\right)\left(1 - \frac{2\theta_{\text{product}}}{\bar{P}_{n(o)}}\right)\left(1 - \frac{4\theta P_{\text{product}}}{\bar{P}_{n(o)}}\right); \tag{55}$$

and so on. The value of $S'$ corresponding to $S = 15$, for example, is then obtained from

$$S' = 15F_4 + 7(F_3 - F_4) + 3(F_2 - F_1) + (F_1 - F_2). \tag{56}$$

The difference between $S$ and $S'$ is shown below for the case in which $\bar{P}_{n(o)} = 1000$, $\theta = 3$, and $P_{\text{product}} = 3$:

| | | | | | | |
|---|---|---|---|---|---|---|
| $S$ | 1.00 | 3.00 | 7.00 | 15.00 | 31.00 | 63.00 |
| $S'$ | 1.00 | 2.98 | 6.87 | 14.38 | 28.31 | 52.16. |

By plotting $S'$ as a function of $S$, it is possible to interpolate values of $S'$ in the range of results recorded by Robyt and French. Equation (*50*) then enables $w'_p$ to be calculated and used in preference to $w_p$ in (*47*).

## INTERPRETATION OF LITERATURE RESULTS

(*a*) *Results of Banks, Greenwood, and Khan*[5]. In this work, the ratio $\bar{P}_w/\bar{P}_{n(\text{system})}$ was measured in the presence of 40% aqueous glycerol, and also in its absence. Under the former condition, it was suggested that the four alpha-amylases examined (from *Bacillus subtilis*, human saliva, malted rye, and porcine pancreas) did not exhibit multiple attack. In the absence of glycerol, only the alpha-amylases from *Bacillus subtilis* and porcine pancreas were examined; in this case, the porcine enzyme exhibited a considerable degree of multiple attack, whereas the bacterial amylase again acted by a random, or multi-chain, mechanism. These conclusions were based on whether, as the hydrolysis proceeded, the measured ratio remained constant (indicating multi-chain attack), or increased (indicating multiple attack). Our previous data, analysed according to equations (*20*) and (*24*), are shown in Tables I and II for the hydrolysis of amylose carried out in the presence and absence, respectively, of glycerol.

Anomalous values of $\theta$ tend to occur at very low $S$. Re-writing equation (*28*), and substituting for $S$ from equation (*2*), gives

$$\theta + 1 = [(\bar{P}_{n(o)}/\bar{P}_{n(\text{system})}) - 1]/[(\bar{P}_{w(o)}/\bar{P}_{w(\text{system})} - 1] . \qquad (57)$$

When the ratios in both the numerator and denominator of equation (*57*) are close to unity, the potential error is high. The denominator is a measure of random scission,

TABLE I

VALUES FOR THE DEGREE OF MULTIPLE ATTACK ($\theta$) AS A FUNCTION OF THE DEGREE OF SCISSION ($S$) FOR VARIOUS ALPHA-AMYLASES ACTING ON AMYLOSE IN THE PRESENCE OF 40% OF GLYCEROL[a]

| B. subtilis | | *Human saliva* | | *Malted rye* | | *Porcine pancreas* | |
|---|---|---|---|---|---|---|---|
| S | $\theta$ | S | $\theta$ | S | $\theta$ | S | $\theta$ |
| 0.01 | 22 | 0.10 | 0.2 | 0.16 | 0.8 | 0.16 | 0.8 |
| 0.12 | 3.8 | 0.72 | −0.3 | 1.11 | 0.6 | 0.92 | 0.6 |
| 0.38 | 0.8 | 0.72 | 0 | 2.92 | 0.1 | 2.03 | 0.2 |
| 0.65 | 0.7 | 0.92 | 0.1 | 4.19 | 0 | 3.00 | 0.1 |
| 0.92 | 0.5 | 1.13 | 0 | 4.71 | 0.1 | 2.77 | 0.3 |

[a]Results of Banks, Greenwood, and Khan[5].

TABLE II

VALUES FOR THE DEGREE OF MULTIPLE ATTACK ($\theta$) AS A FUNCTION OF THE DEGREE OF SCISSION ($S$) FOR VARIOUS ALPHA-AMYLASES ACTING ON AMYLOSE[a]

| B. subtilis | | *Porcine pancreas* | |
|---|---|---|---|
| S | $\theta$ | S | $\theta$ |
| 0.10 | 2.3 | −0.06 | −3.9 |
| 0.30 | 0.5 | 0.14 | 4.5 |
| 0.56 | 0.2 | 0.30 | 2.1 |
| 0.80 | 0.2 | 0.40 | 2.6 |
| 1.13 | 0.1 | 0.63 | 2.7 |

[a]Results of Banks, Greenwood, and Khan[5].

whereas the numerator is compounded from both random and non-random activities. The overall error will therefore be greatest when $\theta$ is close to zero, and $S$ is small. Consequently, in both Tables I and II, the initial values of $\theta$ should be ignored.

In the presence of glycerol, therefore, the values for $\theta$ for the alpha-amylases from human saliva, malted rye, and porcine pancreas are sufficiently close to zero to conclude that multiple attack does not occur; only in the case of the enzyme from *B. subtilis* is $\theta$ finite. Unfortunately, the results were obtained for that alpha-amylase at rather lower $S$ values than for the other enzymes, which may affect the conclusion. Independent evidence[13] shows that the enzyme hydrolyses amylose by an essentially multi-chain action. Therefore, the value of $\theta \sim 0.5$ obtained from Table I must be regarded as an upper limit. Even accepting such a value, however, corresponds to a single non-random event being associated with two random attacks, which would generally be considered a fairly low level of multiple attack.

The results in Table II show that, in the absence of glycerol, the degree of multiple attack exhibited by *B. subtilis* alpha-amylase is small ($\theta \sim 0.3$), whereas that exhibited by the enzyme from porcine pancreas is large ($\theta \sim 3$).

Thus, the present quantitative analysis largely substantiates our previous qualitative conclusions, namely, that of the alpha-amylases examined, only the pancreatic enzyme is capable of a pronounced degree of multiple attack.

(*b*) *Results of Robyt and French*[2]. In this work, the parameter $r$, the ratio of the reducing power of the system to that of the polysaccharide precipitated by 67% alcohol, was measured. The values recorded by Robyt and French[5] are shown in Fig. 2 as a function of the degree of hydrolysis ($S$) of the polymer; the latter values were obtained from the tabulated data using equations (*41*) and (*19*). The solid lines are the expected theoretical relations, constructed using equation (*47*), modified by (*50*) in the case of the porcine pancreatic enzyme at pH 6.9. They attempt to fit both the experimental points and provide the theoretical change in $r$ with increasing $S$. In all cases, the agreement between experiment and theory is poor at low $S$, but, with the exception of the human salivary alpha-amylase, the values at high $S$ are compatible with the theories developed here.

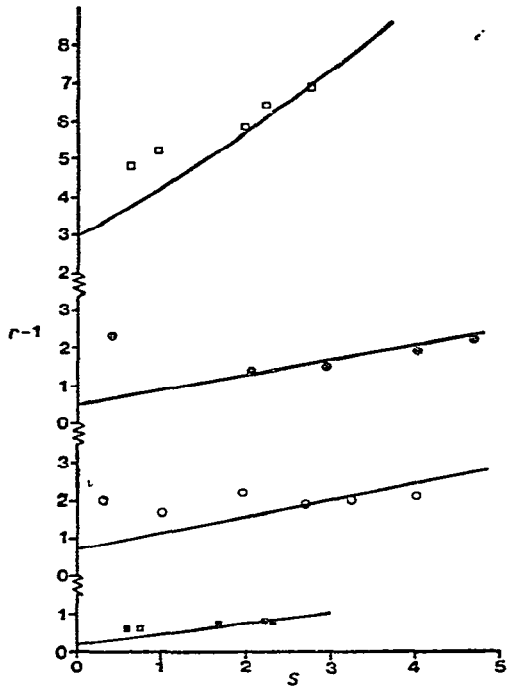


Fig. 2. The results of Robyt and French[2]; the parameter $r-1$, where $r$ is the ratio of the reducing power of the system to that of the polysaccharide precipitated by 67% alcohol, as a function of the degree of scission $S$ for various alpha-amylases: □, porcine pancreas at pH 6.9; ●, *Aspergillus oryzae* at pH 5.5; ○, human saliva; ■, porcine pancreas at pH 10.5. The solid lines are derived from equation (*47*), with $P_{product} = 3$, or equation (*47*) modified by (*50*) in the case of porcine pancreatic alpha-amylase at pH 6.9.

From the results shown in Fig. 2, porcine pancreatic alpha-amylase acting at pH 6.9 is capable of pronounced multiple attack ($\theta \sim 3$), whereas the facility for multiple attack is virtually absent at high pH values, and the action pattern reverts to a multi-chain action. The degree of multiple attack for the amylases of both human saliva and *A. oryzae* is not very prounounced, there being less than one non-random hydrolytic event/random scission in each case.

## DISCUSSION

The theories developed here allow values for the degree of multiple attack to be calculated from published data for the alpha-amylolysis of amylose. The results of Robyt and French[2] have been interpreted as showing that a number of alpha-amylases possess a significant multiple-attack component, whereas our own view has been that, with the exception of the porcine pancreatic amylase, multi-chain attack is the dominating factor, and the degree of multiple attack is very small. The mathematical equations developed in this work allow these opposing views to be rationalized.

By averaging their values of $\theta$ over the course of the reaction, Robyt and French[2] overestimated the degree of multiple attack. Thoma[6] realised that this was the case, and introduced an extrapolation procedure. The mathematical treatment for this procedure was effectively based on the combination of equations (*31*) and (*39*). Consequently, the resultant relation predicted the slope of the graph of $(r_3 - 1)$ as a function of $S$ to be independent of $\theta$. Because this conclusion could not be supported by the experimental results, Thoma[6] argued that a high error was associated with the derivation of the slope, and therefore the fact that it did not conform to its predicted value could be ignored. Hence, Thoma[6] merely used a least-squares treatment to obtain the extrapolated value of $\theta$. From the description of the experimental technique given by Robyt and French[2], we believe the choice of equation (*31*) as the basis of the mathematical treatment of Thoma to be in error, and we consider equation (*36*) to be the more appropriate starting-point. The combination of equations (*36*) and (*39*) predicts that the slope of the graph $(r - 1)$ as a function of $S$ should increase with increasing $\theta$, a conclusion compatible with the experimental results of Robyt and French at high $S$.

The extrapolated values of $\theta$ from Fig. 2 are much lower than the values given by either Robyt and French[2] or Thoma[6]. However, the extrapolations have been carried out by ignoring the value of $(r - 1)$ at low $S$, and might therefore be considered suspect; after all, the same results have been interpreted quite differently by Robyt and French[2], Thoma[6], and now ourselves. However, Robyt and French[2] provided sufficient details to enable an independent comparison to be made of the validities of the various $\theta$ values. Their tabulated values for the course of hydrolysis included the relative change in Blue Value (b.v), and the number-average d.p. of the polysaccharide fraction. The latter parameter corresponds to $\bar{P}_{n(\mathrm{HP})}$, from which $\bar{P}_{n(\mathrm{polymer})}$ can be calculated by using equation (*41*), and this value in turn enables $S$ to be derived from equation (*19*). Assuming $P_{\mathrm{product}} = 3$, the parameter $w_p$ (or $w_p'$) can be calculated

TABLE III

A COMPARISON OF EXPERIMENTAL AND CALCULATED VALUES OF REDUCING POWER (AS % APPARENT MALTOSE) FOR $\theta$-VALUES DERIVED BY VARIOUS WORKERS, USING THE RESULTS OF ROBYT AND FRENCH[2]

| *Enzyme* | *Experimental* | | *Calculated* | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *B.v.*[a] | *R.p.*[b] | *Robyt and French*[2] | | *Thoma*[6] | | *This work* | |
| | | | $\theta$[c] | *R.p.* | $\theta$ | *R.p.* | $\theta$ | *R.p.* |
| Porcine pancreas at pH 6.9 | 61 | 21.5 | 6.0 | 31.5 | 3.8 | 23.2 | 3.0 | 20.3 |
| Porcine pancreas at pH 10.5 | 64 | 5 | 0.7 | 7.7 | 0.5 | 7.2 | 0.2 | 5.6 |
| *Aspergillus oryzae* at pH 5.5 | 51 | 9 | 1.9 | 23.6 | 2.0 | 24.3 | 0.5 | 13.5 |
| Human salivary, pH 6.9 | 61 | 10 | 2.0 | 21.2 | 1.9 | 20.7 | 0.7 | 13.0 |

[a]B.v. = Percentage of initial absorbance of amylose–iodine complex [b]R.p. = Reducing power as % apparent maltose. [c]$\theta$ = Degree of multiple attack.

from the derived value of $S$ and the quoted value of $\theta$, and equation (*17*) solved for $\bar{P}_{n(\text{system})}$. When $\bar{P}_{n(\text{system})}$ is known, the reducing power (r.p.) can be calculated in terms of % apparent maltose, and compared with the experimental values obtained from interpolation of the various b.v./r.p. curves shown by Robyt and French[2]. The results of these calculations are shown in Table III for the $\theta$-values derived by the various workers, and compared with experimental values. For each enzyme, the lowest tabulated value of b.v. was used.

The $\theta$-values given by Robyt and French[2] lead to calculated values of r.p. which are quite inconsistent with the experimental b.v./r.p. relation in each case; the derivation of $\theta$ favoured by Thoma[6] leads to a marked improvement between theory and experiments only in the case of the porcine pancreas enzyme acting at pH 6.0. The mathematical treatment advanced in this work gives the best agreement between calculated and experimental values, but still tends to overestimate the production of reducing power by the enzymes from *A. oryzae* and human saliva. The error could be due to the assumed value of $P_{\text{product}}$ being low, or to $\theta$ being overestimated, or to a combination of both factors. Assuming $P_{\text{product}} = 5$ only reduces the calculated r.p. by 0.5 percentage unit. However, if one assumes a multi-chain action for both enzymes ($\theta = 0$), the calculated values of r.p. are 9.2% and 8.2% for the alpha-amylases from *A. oryzae* and human saliva, respectively, whereas the corresponding experimental values are 9% and 10% (see Table III). Thus the values of $\theta$ obtained in the present work for the *A. oryzae* and human salivary enzymes must be regarded as overestimates, so that in both cases $\theta < 0.5$.

Our interpretation of the work of Robyt and French is that it demonstrates multiple attack unambiguously only in the case of porcine pancreatic alpha-amylase acting at, or below, its pH of maximum activity. For this enzyme at pH 10.5, and for the alpha-amylases of *A. oryzae* and human saliva, the degree of multipe attack is so low ($\theta < 0.5$) that it is difficult to distinguish experimentally from a purely multi-chain action pattern. In the case of the results of Banks *et al.*[5], the conclusion is exactly the same—porcine pancreatic alpha-amylase can act by a mechanism involving multiple attack at its pH of maximum activity in the absence of glycerol, whereas under adverse conditions, *i.e.*, the presence of glycerol, it reverts to a multi-chain action. None of the other alpha-amylases (from *B. subtilis*, human saliva, and malted rye) used in that work showed any marked degree of multiple attack.

The present mathematical analysis shows that, for the experimental methods of both Banks *et al.*[5] and of Robyt and French[2], the derived values for the degree of multiple attack are the same ($\theta \sim 3$) for porcine pancreatic alpha-amylase. Given that the two methods cover quite different stages of the hydrolysis, such good agreement must be considered highly satisfactory.

We have consistently argued that, of those alpha-amylases subjected to detailed examination, only that from the porcine pancreas is capable of repetitive action. In this context, it is noteworthy that only the amylases derived from the pancreas (of pigs and cattle[14]) exhibit b.v./r.p. relations that are grossly dependent on the pH of digestion. We therefore suggest that those cases in which b.v./r.p. relations are

independent of digestion conditions signify that multiple attack is of little or no significance.

Other methods of investigating multiple attack have proved inconclusive. Thus, Abdullah, French, and Robyt[15], using a technique in which the alpha-amylase acted on cyclooctaamylose in the presence of an excess of beta-amylase, concluded that the porcine pancreatic enzyme showed pronounced multiple attack, whereas that from *A. oryzae* showed little or none. On the other hand, Suetsugu *et al.*[16], in a kinetic study of the alpha-amylolysis of cyclo-hexa-, -hepta-, and -octa-amyloses, concluded that the enzyme from *A. oryzae* exhibited multiple attack, with $\theta \sim 1.5$. This value is close to that derived by Robyt and French[2], but is quite incompatible with the b.v./r.p. relations discussed above.

## CONCLUSIONS

The mathematical analyses developed here have shown, when applied to literature results, that only pancreatic alpha-amylase is capable of pronounced multiple attack; the other alpha-amylases act essentially by a multi-chain mechanism.

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