524. *Studies on Carbohydrate-metabolising Enzymes. Part IV.* The Action of Z-Enzyme on Starch-type Polysaccharides.*

By W. L. CUNNINGHAM, D. J. MANNERS, A. WRIGHT, and (in part) I. D. FLEMING.

The Z-enzyme contaminant in a barley β-amylase preparation has no action on the anomalous linkages in amylose, but catalyses random hydrolysis of a small number of α-1,4-glucosidic linkages. Amylopectin and amylopectin β-dextrin are also slowly attacked, but under similar conditions, the rate of hydrolysis of glycogen and glycogen β-dextrin is not measurable. The activity, which is optimum at pH 5·6, is stabilised by calcium ions, and partly inhibited by EDTA and mercuric chloride, is attributed to a minute trace of α-amylase.

The Z-enzyme activity of soya-bean β-amylase preparations, and of almond emulsin, is also due to the presence of very small traces of α-amylase.

The methods available for the detection of α-amylase contaminants in carbohydrase preparations are discussed. The most sensitive assay is that using amylose β-limit dextrin as substrate and viscometry as the method of analysis.

THE action of purified β-amylase on most samples of amylose is incomplete, since only 65—80% conversion into maltose is observed.[1] This indicates the presence of a small number of enzymically resistant or anomalous structures in the substrate. Further, amylose is heterogeneous with respect to both degree of polymerisation ($\overline{DP}$) and behaviour on β-amylolysis;[2] with potato amylose of $\overline{DP}$ 3200, 40% (by weight) of $\overline{DP}$ *ca.* 2000 is completely hydrolysed by β-amylase, and the anomalous structures occur only in the remaining material of $\overline{DP}$ *ca.* 6000. For complete amylolysis a second enzyme, named Z-enzyme,[3] is required; this occurs together with β-amylase in soya-beans[3,4] and barley,[5] is inactivated at pH 3·6,[4] and has no action on α-1,3- or α-1,6-glucosidic linkages or on β-glucosidic linkages.[2] We now report evidence that the action of Z-enzyme involves

* Part III, Gunja, Manners, and Khin Maung, *Biochem. J.*, 1960, **75**, 441.

[1] For reviews see Manners, *Quart. Rev.*, 1955, **9**, 73; *R.I.C. Monographs and Lectures*, 1959, No. 2.
[2] Cowie, Fleming, Greenwood, and Manners, *J.*, 1957, 4430.
[3] Peat, Thomas, and Whelan, *J.*, 1952, 722.
[4] Peat, Pirt, and Whelan, *J.*, 1952, 705, 714.
[5] Manners, Ph.D. Thesis, Cambridge 1952; Bell and Manners, *J.*, 1952, 3641.

hydrolysis of a small number of non-terminal α-1,4-glucosidic linkages in amylose rather than selective hydrolysis of anomalous linkages and is due to the presence of a trace of an α-amylase in the β-amylase preparation. Z-Enzyme (α-amylase) also slowly degrades amylopectin and its β-dextrin.

In our earlier studies on α-1,4-glucosans,[2,6-8] a highly active preparation of barley β-amylase (Wallerstein Analytical reagent) was used. By conventional tests, α-amylase could not be detected: (*a*) the iodine-staining power of amylopectin β-dextrin measured at 680 mμ did not decrease within 24 hr.;[7] (*b*) the β-amylolysis limit of glycogen was independent of enzyme concentration;[7] (*c*) the molecular weight (13×10^6) of the β-limit dextrin of fœtal sheep liver glycogen was in good agreement with that calculated from the molecular weight (29×10^6) and β-amylolysis limit (49%) of the original glycogen;[9] (*d*) during enzyme action on amylose, the intermediate 50% conversion dextrin had the same molecular size as the original substrate.[10] The same enzyme preparation was considered to contain Z-enzyme since complete degradation of amylose at pH 4·6 but not at 3·6 was observed.[2]

The first indication that the apparent Z-enzyme activity might be due to traces of α-amylase was obtained during studies of the β-amylolysis of amylopectin, when an apparent relation between enzyme concentration and β-amylolysis limit was found.[11] With digests containing 33, 66, 99, and 128 units [12] of β-amylase per mg. of polysaccharide, the apparent β-amylolysis limits were 64, 65, 68, and 71 after 92 hours' incubation at pH 4·9 and 37°. Such a relation is characteristic of the α-amylases.[13] Since this finding was at variance with the previous results,[2,7,9,10] the enzymic homogeneity of the β-amylase preparation was investigated.

Degradation of Amylopectin and β-Dextrin by Barley Z-Enzyme.—The above possibility has been examined in detail with amylopectin β-dextrin as substrate, and (*a*) iodine staining, (*b*) viscosity, and (*c*) reducing-power measurements to follow enzyme action.

Peat, Pirt, and Whelan [4] carried out iodine-staining by measuring the decrease in absorption value (A.V.) of the polysaccharide–iodine complex at 680 mμ, the wavelength used for "blue-value" (B.V.) determinations. In our experiments, the wavelength of maximum absorption ($\lambda_{max.}$) has been used, namely, 530—540 mμ. Under these conditions, and with an increase in the relative enzyme concentration and time of incubation, a marked decrease in iodine-staining power was observed. For example, with 0·1% of substrate and 0·2% of barley preparation (equivalent to *ca.* 250 β-amylase units [12] per mg. of polysaccharide) at pH 4·6, an 87% decrease in A.V. occurred in 70 hr., and the residual polysaccharide–iodine complex then had $\lambda_{max.}$ at 420 mμ. This indicates random hydrolysis of non-terminal α-1,4-glucosidic linkages.[14] Under similar conditions the A.V. of glycogen β-dextrin at 470, 430, 420, and 410 mμ was unchanged.

The decrease in A.V. of amylopectin β-dextrin could be detected with only 0·03% barley preparations; at 480, 520, and 560 mμ, the A.V. fell by 17, 30, and 40% respectively after 70 hr. Further experiments (Table 1) showed that this activity was increased by pre-incubation of the enzyme with 5×10^{-3}M-calcium for 30 min., and was decreased by similar treatment with 5×10^{-2}M-ethylenediaminetetra-acetic acid (EDTA). The function of the calcium appears to be that of an enzyme-stabiliser rather than a specific activator. The presence of this ion decreased the rate of inactivation of the enzyme at pH 4·6 and 37° during incubation for 67 hr. in the absence of substrate, and did not restore the activity of a partly inactivated enzyme preparation. EDTA appears to lower the activity by

[6] Fleming, Hirst, and Manners, *J.*, 1956, 2831.
[7] Liddle, Ph.D. Thesis, Edinburgh 1956; Liddle and Manners, *J.*, 1957, 3432.
[8] Eddy, Fleming, and Manners, *J.*, 1958, 2827.
[9] Greenwood, Jones, and Manners, unpublished work.
[10] Cowie, Fleming, Greenwood, and Manners, *J.*, 1958, 697.
[11] King and Manners, unpublished work.
[12] For unit of β-amylase activity see Hobson, Whelan, and Peat, *J.*, 1950, 3566.
[13] Caldwell and Adams, *Adv. Carbohydrate Chem.*, 1950, **5**, 229.
[14] Manners, *Ann. Reports*, 1953, **50**, 288.

TABLE 1. *The effect of the barley β-amylase preparation on the A.V. of amylopectin β-dextrin.**

Barley prep. λ (mμ)	Fall in A.V. (%) Normal	Fall in A.V. (%) Pre-treated with Ca^{++}	Fall in A.V. (%) Pre-treated with EDTA
480	67	80	14
500	75	85	24
520	82	89	27
540	85	91	33
560	86	92	36
580	88	92	38

	Fall in A.V. (%) Normal	Fall in A.V. (%) Pre-treated with Ca^{++}	Fall in A.V. (%) Pre-treated with EDTA
Original $\lambda_{max.}$ (mμ)	530	530	530
Original A.V.	0·67	0·66	0·68
Final $\lambda_{max.}$ (mμ) ...	430	430	510
Final A.V.	0·22	0·23	0·49

* Digests contained 0·1% of substrate and 0·2% of enzyme preparation and were incubated at pH 4·6 and 35° for 72 hr.

partial removal of the calcium (cf. ref. 15). The protection from inactivation of other α-amylases, including malt α-amylase, by calcium ions has been noted by several workers.[16]

The activity towards β-dextrin was greater in sodium acetate buffer of pH 5·6 than at pH 4·8 or 6·5 (see Figs. 1 and 2); none was detected at pH 3·6. At pH 5·6, the activities in

FIG. 1.

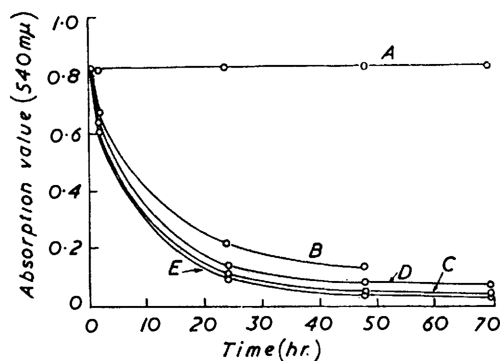


FIG. 2.

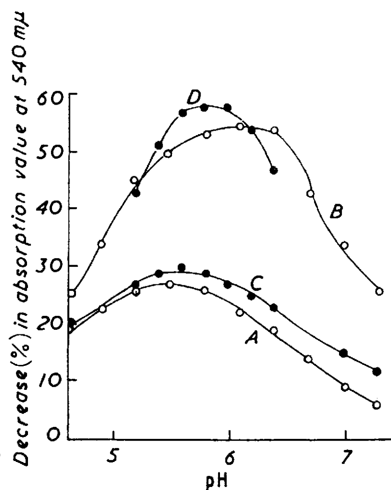


FIG. 1. *Effect of barley Z-enzyme on the iodine-staining power of amylopectin β-dextrin. Enzymic reactions carried out at* pH 3·6 (*A*), 4·6 (*B*), 5·6 (*C*), 6·5 (*D*), *and at* 5·6 *in presence of* 5 × 10^{-3}M-*borate* (*E*).

FIG. 2. *Effect of* pH *on barley Z-enzyme activity.*
Substrate: amylopectin β-dextrin; (*A*, *B*) *acetate buffer analysed after* 6 *and* 24 *hr.*; (*C*, *D*) *phosphate-citrate buffer analysed after* 8·75 *and* 27 *hr.*

acetate and B.D.H. Universal buffer were identical, in contrast to the behaviour of *Cladophora rupestris* amylase which is more active in the latter buffer.[17] Addition of 5 × 10^{-3}M-borate, which inhibits isoamylase [18] and activates *Cladophora* amylase,[17] to the acetate buffer had no effect; phenylacetate (7 × 10^{-3}M) and phosphate (7 × 10^{-3}M) likewise did not alter the rate of decrease of A.V. (at 540 mμ).

[15] Stein and Fischer, *J. Biol. Chem.*, 1958, **232**, 867.
[16] Kneen, Standstedt, and Hollenbeck, *Cereal Chem.*, 1943, **20**, 399; Caldwell and Kung, *J. Amer. Chem. Soc.*, 1953, **75**, 3132; Hanrahan and Caldwell, *ibid.*, p. 4030.
[17] Cunningham, Duncan, and Manners, unpublished work.
[18] Gunja, Manners, and Khin Maung, *Biochem. J.*, in the press.

In contrast to the above results (with *ca.* 250 units of β-amylase per mg. of substrate), the enzyme preparation caused only a slight decrease in the iodine-staining power of amylopectin when experimental conditions similar to those of Peat, Pirt, and Whelan [4] were used [*ca.* 40 units per mg. of substrate; A.V. (680 mμ)] as shown in Table 2. This result

TABLE 2. *Action of normal concentrations of β-amylase on amylopectin.**

	Time of incubation (hr.) 27	49		Time of incubation (hr.) 27	49
Barley β-amylase			Soya-bean β-amylase		
β-Amylolysis limit (%) ...	55	54	β-Amylolysis limit (%) ...	54	55
A.V. (680 mμ)	0·039	0·036	A.V. (680 mμ)	0·037	0·033
A.V. (540 mμ)	0·152	0·128	A.V. (540 mμ)	0·147	0·136

* Digests contained *ca.* 40 units of β-amylase per mg. of amylopectin.

illustrates the importance of varying the enzyme concentration when testing for contaminating enzymes.

A slow and limited degradation of 0·5% amylopectin β-dextrin solution by 0·5% barley preparation at pH 5·6 and 25° was also shown by viscosity measurements. After 5, 40, and 70 min., the specific viscosity (η_{sp}) values were 0·194, 0·186, and 0·179 respectively. At pH 3·6, the viscosity was unchanged.

Despite the limited degradation of β-dextrin in the above experiments, the effect of this initial α-amylolysis can be magnified by the presence of an excess of β-amylase. Hydrolysis of only a small number of interior α-1,4-linkages liberates non-reducing end-groups which are susceptible to this enzyme. The resultant increase in reducing power is then appreciable, whereas neither β-amylase nor low concentrations of α-amylase, acting separately on β-dextrin, yield measurable amounts of reducing sugars. The results summarised in Table 3 show the apparent percentage conversion into maltose (P_M) during degradation of β-dextrin by the barley preparation. In a further experiment, the relative P_M values at pH 3·6, 5·6, and 6·5 were 4, 45, and 33 respectively.

TABLE 3. *Action of barley preparation on amylopectin β-dextrin.*

	Apparent conversion (%) into maltose *		
Age of digest (hr.)	20	70	86
Digest conditions:			
(*a*) Acetate buffer pH 4·6	25	33	37
(*b*) ,, pH 5·6	35	54	55
(*c*) ,, pH 5·6 (with 5 × 10^{-3}M-borate)	35	54	56
(*d*) B.D.H. Universal buffer pH 5·6	31	49	52

* For composition of digests, see p. 2610.

Degradation of Amylose and β-Dextrin by Barley Z-Enzyme.—Although the highly branched amylopectin β-dextrin may be used to detect relatively high concentrations of Z-enzyme, the use of a linear substrate is preferable since the hydrolysis of only a small number of linkages will produce a more marked change in physical properties. The effect of certain inhibitors on barley Z-enzyme was therefore investigated by using amylose β-limit dextrin (prepared by the prolonged action of β-amylase at pH 3·6 on potato amylose): the results are summarised in Table 4.

TABLE 4. *Effect of inhibitors * on the action of barley Z-enzyme on amylose β-dextrin.*

Inhibitor	Inhibn. (%) †	Inhibitor	Inhibn. (%) †
10^{-2}M-Iodoacetate	100	10^{-5}M-*p*-Chloromercuribenzoate ...	7
1·5 × 10^{-5}M-Mercuric chloride	80	10^{-4}M-Silver nitrate	86
10^{-4}M-Phenylmercuric acetate	79		
10^{-5}M-Phenylmercuric acetate	73		

* All inhibitors caused 100% inhibition of β-amylase.
† Based on A.V. (600 mμ) measurements; see p. 2611.

Peat, Thomas, and Whelan[3] reported that Z-enzyme was not inhibited by *ca.* 1·5 × 10^{-6}M-mercuric chloride and concluded that this distinguished Z-enzyme from α- and β-amylase. Although the extreme sensitivity of β-amylase towards mercury compounds and related thiol-reactants is well known,[19] there is evidence that thiol groups are not essential for the activity of α-amylases.[20,21] We have examined the effect of various concentrations of mercuric chloride on the activity of a number of α-amylases, and the results (Table 5) show that only *partial* inactivation occurs with concentrations of

TABLE 5. *Effect of mercuric chloride on the activity of α- and β-amylases.*

	Inhibition (%)			
Concn. (M) of $HgCl_2$	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Barley β-amylase *	100	100	100	100
Salivary α-amylase †	84	66	44	22
Bacterial α-amylase †	87	67	35	26
Malt α-amylase ‡	—	96	50	19
Barley Z-enzyme ‡	—	86	70	6

* Assay under Hobson, Whelan, and Peat's conditions.[12]
† Assay under Bernfeld's conditions (*Methods in Enzymology*, 1955, **1**, 149).
‡ See p. 2611.

10^{-5} to 10^{-6}M. Further, the action of barley Z-enzyme is only partially inhibited by mercuric chloride (Table 4 and 5). Notwithstanding the qualitative nature of these studies (the concentration of α-amylase was not identical in the various assays), it is concluded that the use of mercuric chloride shows, in fact, a similarity between Z-enzyme and α-amylase.

p-Chloromercuribenzoate (1 × 10^{-5}M) completely inhibits β-amylase[19] and yet has less effect on barley Z-enzyme than have similar concentrations of mercuric chloride (Table 4). This reagent has therefore been used for the selective inactivation of β-amylase. On incubation of amylose (0·2%) with barley preparation (0·07%, corresponding to *ca.* 45 β-amylase units/mg.) and *p*-chloromercuribenzoate, a marked decrease in specific viscosity was observed, but without a concomitant decrease in iodine-staining power or increase in reducing power. This is attributed to the random hydrolysis of a small number of non-terminal linkages. Since the iodine-staining power of amylose as obtained by " blue-value " or $\lambda_{max.}$ measurements is approximately the same for samples of $\overline{DP}$ 500 or 2000,[22] this result emphasises the caution required in following *limited* α-amylolysis by iodine-staining. Measurements of the change in reducing power[13] or of the sedimentation constant[10] of the residual amylose are also unsatisfactory when the concentration of α-amylase is extremely low. It is clear that viscometry provides the only sensitive method when concentrations of β-amylase contaminated by Z-enzyme similar to those used in our previous studies[6-8] and by Peat and his co-workers[3,4] are employed.

The presence of traces of α-amylase in unpurified barley β-amylase preparations has been noted by earlier workers including Hopkins, Murray, and Lockwood.[23] The amount of α-amylase appears to depend upon the condition of the grain, and the variety of the barley.[24] Part of the α-amylase may represent precursors of the enzyme which develops in quantity during germination, and part may arise from contamination of the barley husk by amylase-secreting bacteria.[25] The α-amylase constituents of ungerminated and germinated barley are undoubtedly closely related, and we have found that the *initial*

[19] Weill and Caldwell, *J. Amer. Chem. Soc.*, 1945, **67**, 214; Englard, Sorof, and Singer, *J. Biol. Chem.*, 1951, **189**, 217.
[20] Caldwell, Weill, and Weill, *J. Amer. Chem. Soc.*, 1945, **67**, 1079.
[21] Fischer and Haselbach, *Helv. Chim. Acta*, 1951, **34**, 325.
[22] Kerr, Cleveland, and Katzbeck, *J. Amer. Chem. Soc.*, 1951, **73**, 3916; Whelan, personal communication.
[23] Hopkins, Murray, and Lockwood, *Biochem. J.*, 1946, **40**, 507; cf. Blom, Bak, and Braae, *Z. physiol. Chem.*, 1936, **241**, 273; Hanes, *New Phytol.*, 1937, **36**, 101, 189.
[24] Chrzaszcz and Janicki, *Biochem. J.*, 1936, **30**, 1298; Howard, personal communication.
[25] Hopkins and Cooper, *J. Inst. Brewing*, 1946, **52**, 188.

action of malt α-amylase on amylose β-dextrin is also not activated by calcium ions, and is partially inhibited by mercuric chloride ($1\cdot5 \times 10^{-5}$ and $1\cdot5 \times 10^{-6}$M). Further, the optimum pH of unpurified malt α-amylase is *ca.* 5·4,[21] a value similar to that shown in Fig. 2 and different from that of bacterial α-amylase [26] (*ca.* 6·5).

Z-Enzyme Activity of Soya-bean β-Amylase and Almond Emulsin.—Since Z-enzyme was originally detected in unpurified (" stock ") preparations of soya-bean β-amylase [3,4] and in almond emulsin [3,27] (a complex mixture of carbohydrases including β-glucosidases), samples of these have been examined for contamination with α-amylases.

Neither preparation had a significant effect on the production of maltose from amylopectin–β-amylase or Floridean starch–β-amylase systems (cf. Table 2 and ref. 6); by this criterion [13] gross contamination with α-amylase could be ruled out. However, when amylose, amylose β-dextrin, or amylopectin β-dextrin was used as substrate, and assay was by iodine-staining and reducing power or viscosity, the presence of a trace of α-amylase was established. Slight random degradation of the substrates occurred; for example, incubation of amylose (0·1%) with emulsin (0·5%) at pH 4·6 for 24 hr. reduced η_{sp} by 88% and increased the β-amylolysis limit from 75 to 95%. This α-amylolytic activity was increased by calcium ions (which again act as a stabiliser rather than activator), and partly inhibited by EDTA and mercuric chloride (10^{-4}—10^{-6}M), and was optimum in the region pH 5·8—6·1 (cf. Peat, Thomas, and Whelan [3] who reported the action of soya-bean Z-enzyme on amylose β-dextrin as maximum at pH 6). Typical results are shown in Table 6 and Figs. 3 and 4. In similar conditions, the extent of hydrolysis of glycogen

TABLE 6. *Effect of emulsin on the iodine-staining power of amylose β-dextrin.*

	Fall in A.V. (%)			
Wavelength (mμ)	580	600	640	680
Digest conditions : *				
(*a*) Control	56	60	65	69
(*b*) Mercuric chloride: $1\cdot5 \times 10^{-5}$M	33	35	38	41
$1\cdot5 \times 10^{-6}$M	44	47	55	51
(*c*) Pre-treated with $CaSO_4$ †	72	74	79	82
(*d*) Pre-treated with EDTA: (i) alone †	23	24	26	26
(ii) diluted with water	27	28	31	32
(iii) diluted with $CaSO_4$ †	47	50	51	55

* Incubated for 25 hr. at 35°. † Final concentration 2×10^{-4}M.

β-dextrin by emulsin was approximately one-half that of amylopectin β-dextrin (P_M 3·7 and 7·8 respectively). The properties of the α-amylase present in soya-beans and almond emulsin are therefore generally similar to, although not necessarily identical with, those of barley Z-enzyme.

Discussion and Conclusions.—The recognition of the presence of a trace of α-amylase in the barley and soya-bean β-amylase preparations, and in emulsin, provides an explanation for the observed increase in the β-amylolysis of amylose. The slight random hydrolysis will expose sufficient new non-reducing end-groups to enable further β-amylolysis to take place. If the presence of only one anomalous structure per amylose molecule is assumed, the random hydrolysis of only one α-1,4-glucosidic linkage will cause a 10—15% increase in β-amylolysis limit, *e.g.*, from *ca.* 75 to *ca.* 87%. The ability of Z-enzyme to increase the phosphorolysis limit of amylose, from 70 to 95% conversion into glucose 1-phosphate,[3] can now also be explained in terms of slight α-amylolytic activity.

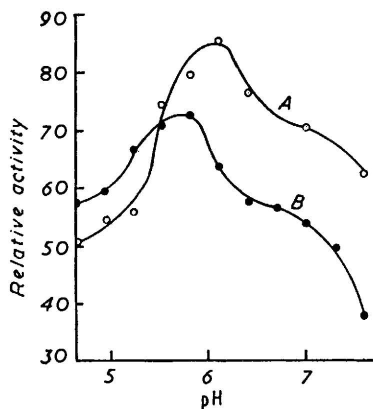
The nature of the structural anomalies in amylose is not yet known.* These may include one or more of the following possibilities: (*a*) an anomalous linkage (*i.e.*, a glucosidic

* Since the proportion of these is extremely low (probably less than 0·1%), their presence in enzymic hydrolysates of amylose cannot be revealed by available methods of analysis, and is inferred from the known specificity requirements of α- and β-amylase.

[26] Fischer and Stein, *Arch. Sci.*, 1954, **7**, 131.
[27] Neufeld and Hassid, *Arch. Biochem. Biophys.*, 1955, **59**, 405.

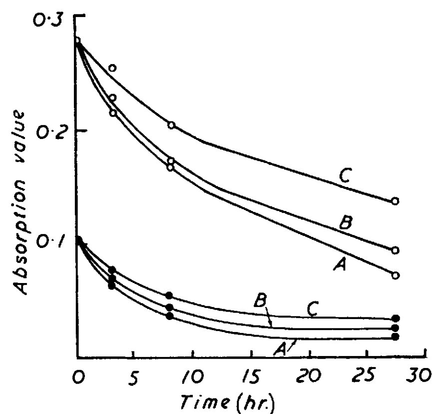
linkage other than the α-1,4-type) in the amylose chain or as a branch point; (*b*) an anomalous residue, *i.e.*, an α-1,4-linked hexose residue derived from D-glucopyranose by substitution with a phosphate group, probably at position 6, or by acylation or oxidation at position 2, 3, or 6; (*c*) both a residue and its linkage may be anomalous. Recent evidence [28] suggests that a small number of glucose residues in amylose may become modified by oxidation during isolation of the polysaccharide, becoming resistant to β-amylase and phosphorylase. Since Z-enzyme is an α-amylase, its action will involve the " by-passing " of such structural anomalies rather than their removal by selective hydrolysis. Similarly, any anomalous linkage which is present as a branch point will not be hydrolysed by Z-enzyme (or any other α-amylase), *i.e.*, Z-enzyme does not act as a " debranching " enzyme.

FIG. 3. *Effect of* pH *on the Z-enzyme activity of emulsin and " stock " soya-bean β-amylase preparation.*



Curve A represents action of soya-bean preparation on amylopectin β-dextrin (reducing-power measurements expressed as P_M *values); curve B shows the effect of emulsin on the* A.V. (640 mμ) *of amylose β-dextrin expressed as percentage decrease.*

FIG. 4. *Effect of soya-bean β-amylase on the* A.V. (540 mμ, ○; 680 mμ, ●) *of amylopectin β-dextrin.*



Concn. of mercuric chloride in the digests was 0 (*A*), 1·5 × 10^{-6}M (*B*), *and* 1·5 × 10^{-5}M (*C*).

Some anomalous structures are present in unfractionated starch since the β-amylolysis limits with purified and " stock " soya-bean β-amylase are 53 and 61% respectively.[4] This difference was attributed to the action of Z-enzyme on the amylose component. However, Hopkins and his co-workers [23] had previously shown that barley β-amylase prepared by a method involving pretreatment at pH 3·4 caused 56% conversion of soluble starch into maltose, and that if this treatment was omitted, or if a trace of bacterial α-amylase was added to the purified preparation, the β-amylolysis limit was 63%. These findings are in accord with our observations, and the view that " Z-enzyme " is a trace of α-amylase adequately explains the effect of pH on the β-amylolysis limit of unfractionated starch (cf. ref. 13).

The amount of α-amylase present in the barley preparation is too small to be assessed accurately but, in comparative experiments, a salivary α-amylase solution containing 34 units [26] was diluted 50,000 times and found to cause a decrease in the iodine-staining power of both amylose and amylopectin β-dextrin and to have only a limited action on glycogen β-dextrin. This suggests that the α-amylase activity of the barley preparation is of the order of 10^{-3} unit/mg. A further indication of the minute degree of contamination

[28] Baum, Gilbert, and Scott, *Nature*, 1956, **177**, 889; Gilbert, *Stärke*, 1958, **10**, 95; Banks, Greenwood, and Thomson, *Chem. and Ind.*, 1959, 928; Manners and Wright, unpublished work.

is shown by a comparison of the turn-over number [29] of the related malt α-amylase, equivalent to the hydrolysis of 19,000 bonds per min. per mole, with the limited degradation of amylose β-dextrin observed by viscometry in our experiments during incubation for some hours, and the previous failure to detect degradation of glycogen β-dextrin by light-scattering,[9] or of amylose by sedimentation measurements.[10] It is suggested that the inability of barley Z-enyme to cause appreciable degradation of glycogen is a consequence of (*a*) the low concentration of enzyme and (*b*) the lowered affinity of α-amylases in general for glycogen,[30] rather than to an absolute specificity requirement (cf. R-enzyme which hydrolyses 1,6-linkages in amylopectin but not in normal 12-unit glycogen [14]). The hydrolysis of glycogen or its β-dextrin with normal concentrations of other α-amylases, including preparations from ungerminated barley and soya-beans, can readily be detected.[31]

Our general conclusions are in accord with the findings of Hopkins and Bird,[32] who have emphasised the difficulty in detecting traces of α-amylase when using amylopectin rather than amylose as a substrate, and with the recent results of Baba and Kojima,[33] and of Banks, Greenwood, and Jones.[34] Baba and Kojima also showed the presence of α-amylase in emulsin, and Banks *et al.* independently proved random hydrolysis of starch components, using light-scattering and viscosity measurements, by the Z-enzyme contaminant of several unpurified β-amylase preparations.

It must be noted that the present results do not alter our earlier conclusions [2,6-8] on the molecular structure of starch and glycogen-type polysaccharides, or on the mechanism of β-amylase action,[10] which are derived, in part, from results obtained with the Wallerstein barley β-amylase preparation.

EXPERIMENTAL

Analytical Methods.—The general methods used were those described in earlier papers.[2,6-8] For viscometry, digests were prepared in modified Ubbelohde or Ostwald viscometers, and the viscosity at 25° was measured at intervals. Since the activity of an α-amylase is related to $d(1/\eta_{sp})/dt$,[35] graphs of $1/\eta_{sp}$ against t were prepared. With identical enzyme and substrate concentrations, the effect of added reagents could then be observed by a comparison of the slopes. The polysaccharide solutions were filtered through sintered glass (G4) before analysis.

In the iodine-staining experiments with amylopectin β-dextrin, measurements at 540 mμ increased the A.V. to *ca.* 0·5 in the most sensitive region of the spectrophotometer (Unicam S.P. 500) (cf. A.V. of *ca.* 0·1 at 680 mμ).

Enzyme Preparations.—The properties of the barley β-amylase are reported in ref. 7. " Stock " and purified soya-bean β-amylase were prepared by the methods of Bourne, Macey, and Peat [36] and Peat, Pirt, and Whelan.[4] Sweet-almond emulsin was isolated by Tauber's method; [37] a weighed amount was centrifuged in the stated volume of water, and insoluble material was discarded. Wallerstein malt diastase was used as a source of malt α-amylase, with short incubation periods to minimise the effect of β-amylase.

Substrates.—(a) *Amylose.* Various samples made by the fractionation of starch from potatoes (var. Kerr's pink) with thymol and butanol were used, together with amylose VI, VII, and VIII.[2] Amylose β-dextrin was prepared by incubating amylose VIII (500 mg.) with barley β-amylase (100 units/mg.) at pH 3·6 for 24 hr. The β-amylolysis limit was 72%. The digest was heated for 10 min., cooled, and filtered (G4 sinter), and the pH was adjusted to 5·6 with aqueous sodium hydroxide. The dextrin was stored under toluene at room temperature; the maltose present did not interfere with subsequent measurements.

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(b) *Amylopectin.* Fractionation of potato starch with thymol or pyridine gave samples I and II respectively. Waxy-maize and sorghum starch were commercial samples. Amylopectin β-dextrin was prepared from waxy-maize starch I (5 g.) treated with purified β-amylase (6000 units) in a total volume of 250 ml. at 35° for 48 hr. The β-amylolysis limit was 53%. After dialysis, the dextrin was isolated by freeze-drying. Samples of amylopectin β-dextrin were also prepared from waxy-sorghum starch and potato amylopectin by similar methods.

(c) *Glycogen β-dextrin.* This was isolated from a digest of *Ascaris lumbricoides* glycogen and β-amylase.

Action of Barley Z-Enzyme on Amylopectin β-dextrin.—(a) *Iodine-staining measurements.* Polysaccharide (25 mg.), barley preparation (52 mg.), 0·2M-sodium acetate buffer (pH 4·6; 3 ml.), and water to 20 ml. were incubated at 35° for 70 hr. Samples (2 ml.) were removed, heated to inactivate the enzyme, and stained with iodine solution (0·2% in 2% potassium iodide solution; 2·5 ml.) in a total volume of 25 ml. The A.V. (540 mμ) of amylopectin β-dextrin decreased from 0·740 to 0·097 and the product showed $\lambda_{max.}$ 420 mμ and A.V.$_{max.}$ 0·210. With glycogen β-dextrin, the initial and final A.V.'s were: at 470 mμ, 0·044 and 0·042; at 430 mμ, 0·073 and 0·074; at 420 mμ, 0·075 and 0·078; at 410 mμ, 0·067 and 0·069. When only 6·25 mg. of barley preparation were used, the following results were obtained:

λ (mμ)	480	500	520	540	560	580
Initial A.V.	0·545	0·622	0·674	0·680	0·630	0·552
Final A.V.	0·446	0·469	0·472	0·440	0·377	0·319

Barley preparation (*ca.* 50 mg.), pretreated with 5 × 10^{-3}M-calcium sulphate or 5 × 10^{-2}M-EDTA (pH 4·7) for 30 min. at 37°, was incorporated into similar digests. Samples (3 ml.) were removed after 72 hr.; the results are reported in Table 1. In a further experiment with 0·67 mg. of EDTA-treated enzyme, only a slight decrease in iodine-staining power was noted:

λ (mμ)	480	500	520	540	560	580
Initial A.V.	0·529	0·607	0·675	0·665	0·610	0·531
Final A.V.	0·529	0·573	0·600	0·575	0·517	0·436

The optimum pH for EDTA–calcium complex formation is 7·5; [38] hence, in the above experiments, the calcium ions may not have been completely removed.

(b) *Measurement of reducing power.* Digests (20 ml.) were prepared containing amylopectin β-dextrin (19·2 mg.), barley β-amylase (3800 units), buffer solution (3 ml.), and water. At intervals samples (5 ml.) were deproteinised, and the apparent maltose contents were determined. The results are shown in Table 3.

(c) *Viscometry.* Amylopectin β-dextrin (1% filtered solution; 10 ml.), 0·2M-acetate buffer (5 ml.) of pH 3·6 or 5·6, and barley β-amylase (100 mg. in 5 ml. of water) were mixed in a viscometer. η_{sp} was determined during 2 hr. At pH 5·6, $d(1/\eta_{sp})/dt$ indicated [35] a relative activity of 7·8 × 10^{-3} unit, and in presence of mercuric chloride (1·5 × 10^{-5}M), of 7·2 × 10^{-3} unit. At pH 3·6, there was no change in viscosity.

Effect of pH *and Various Ions on Activity.*—Amylopectin β-dextrin (*ca.* 30 mg.), barley preparation (6000 units), buffer (3 ml.), and water (to 25 ml.) were incubated at 35°. The buffers used were 0·2M-acetate of (*a*) pH 3·6, (*b*) pH 4·8, (*c*) pH 5·6, (*d*) pH 6·5, and (*e*) pH 5·6 containing borate to give a final concentration of 5 × 10^{-3}M. The A.V. of samples (2 ml.) was measured at intervals. The results obtained at 540 mμ are shown in Fig. 1. Similar results were obtained over the range 460—680 mμ.

For the pH–activity curves, β-dextrin (10 mg.) was incubated with β-amylase (1250 units) and 0·2M-acetate buffer (pH 4·6—7·6; 5 ml.) in a total volume of 15 ml. Samples (3 ml.) were removed after 6 and 24 hr. and the A.V.'s at both 540 and 680 mμ were determined. After 6 hr., at 540 mμ, the maximum decrease was at pH 5·5; after 24 hr., over the range pH 6·1—6·4 (see Fig. 2). The small change in pH is attributed to the decreased stability of the enzyme in acetate buffer at pH 4—6. The same results were obtained from A.V. determinations at 680 mμ. The experiment was repeated with phosphate–citrate buffer (pH 5·2—7·3; 0·1M-citric acid and 0·2M-disodium hydrogen phosphate; 3 ml.) in a 10 ml. digest. The maximum fall in A.V. (540 mμ) occurred at pH 5·6 after 8·75 hr. and at pH 5·8 after 27 hr.

Digests containing β-dextrin (10 mg.), barley preparation (2000 units), and buffer (5 ml.) in a total volume of 15 ml. were incubated at 35°. The following results were obtained.

[38] West and Sykes, "Analytical Applications of Diaminoethanetetra-acetic acid," B.D.H. Ltd. London, 1959, p. 11.

Buffer	Acetate (pH 5·6)	B.D.H. Universal (pH 5·6)	Phenyl-acetate *	Borate *	Phosphate *
Fall (%) in A.V. (540 mμ):					
after (*a*) 6 hr.	34	33	34	33	33
,, (*b*) 27 hr.	70	69	70	70	70
P_M, after 99 hr.	12·6	12·9	13·1	12·5	12·6

* These digests contained 5 ml. of sodium acetate buffer (pH 5·6) and 5 ml. of 2 × 10^{-2}M-anion.

The function of the calcium ion was examined by incubating enzyme solution (2 ml.) with β-dextrin (10 mg.) and acetate buffer (pH 5·8; 3 ml.) in a total volume of 10 ml. Digest no. 1 contained barley preparation pre-incubated at 37° and pH 5·8 for 67 hr.; digest no. 2 contained enzyme solution as above, but also 5 × 10^{-2}M-calcium acetate; digest no. 3 was as digest no. 1 except that the enzyme was added to a mixture of β-dextrin and calcium acetate.

Fall (%) *in* A.V. (540 mμ).

Incubation (hr.)	4·5	7	24
Digest no. 1	13	18	49
Digest no. 2	31	45	83
Digest no. 3	13	19	51

Action of Barley Z-Enzyme on Amylose β-Dextrin.—(a) *Iodine-staining measurements.* Digests containing amylose β-dextrin solution (0·46 mg./ml. by acid hydrolysis; 14 ml.) and β-amylase (14 mg. in 0·5 ml. of water; pre-incubated at 20° for 20 min. with 0·5 ml. of inhibitor solution) were incubated at 35°. Samples (4 ml.) were withdrawn after 2·5 and 4·5 hr., stained with iodine solution (1 ml.), and diluted with water to 25 ml. A.V.'s were measured at 560, 580, 600, and 640 mμ. The trend of results was the same at all wavelengths; the results at 600 mμ are given in Table 4.

The effect of mercuric chloride was examined in digests containing amylose β-dextrin (10 mg.), β-amylase (15 mg.), 0·2M-acetate buffer (pH 5·5, 3 ml.), mercuric chloride solution (1 ml.), and water (6 ml.). Samples (3 ml.), removed after 2·5 hr., gave the following results:

Concn. of $HgCl_2$ (M)	10^{-4}	10^{-5}	10^{-6}	Nil
Decrease (%) in A.V. at (600 mμ)	7	15	46	49

(b) *Viscometry.* A digest containing amylose solution (70 mg.; 20 ml.; with *p*-chloromercuribenzoate, 10^{-5}M) and β-amylase (25 mg. in 15 ml. of 0·2M-acetate buffer of pH 4·6; with *p*-chloromercuribenzoate, 10^{-5}M) was prepared in a viscometer. The following results were obtained:

Time (min.)	15	30	60	97	120	155	205	20 hr.	45 hr.
$1/\eta_{sp}$	3·13	3·32	3·73	3·94	4·20	4·48	4·65	6·25	6·76

Samples were also removed for the measurement of A.V. at both 540 and 680 mμ, and of the reducing power. No change was detected within 24 hr. A control experiment showed that 10^{-5}M-*p*-chloromercuribenzoate had no effect on the reaction of maltose with the Somogyi reagent; in the absence of this material the viscosity change is accompanied by a marked decrease in iodine-staining power and rapid production of reducing sugars.

Action of Normal Concentrations of β-Amylase on Amylopectin.—Digests were prepared containing waxy-maize starch I (30 mg.), 0·2M-acetate buffer (pH 4·6; 10 ml.), barley β-amylase or " stock " soya-bean β-amylase (1300 units), and water to a final volume of 50 ml. Samples (2 ml. for iodine-staining; 3 ml. for reducing-power measurements) were removed at intervals. The results are in Table 2.

Action of " Stock " Soya-bean β-Amylase on Amylopectin β-Dextrin.—Polysaccharide (23·9 mg.), 0·2M-acetate buffer (pH 4·6; 3 ml.), enzyme solution (3 ml.), and water (19 ml.) were incubated at 35°. [The enzyme solution was prepared by dissolving 50 mg. of powder (activity *ca.* 100 units/mg.) in 5 ml. of buffer and centrifuging the mixture.] Samples (2 ml.) were removed after 27 and 72 hr.: the results after 27 hr. were:

λ (mμ)	480	500	520	540	560	580	680
Initial A.V.	0·528	0·612	0·687	0·700	0·649	0·574	0·198
Final A.V.	0·034	0·039	0·039	0·038	0·038	0·038	0·020

Similar results were obtained after 72 hr.

The effect of pH was examined in digests containing β-dextrin (5 mg.), phosphate–citrate

buffer (pH 4·6—7·6; 5 ml.), 1% β-amylase solution (2 ml.) and water (3 ml.). The P_M values of 3 ml. portions were determined after 25 hr. at 35° (see Fig. 3).

Amylopectin β-dextrin (10 mg. in 5 ml. of water) was added to 0·3% β-amylase solution (4 ml.) pre-incubated as follows: (*a*) with 0·2M-acetate buffer (pH 6·1; 10 ml. containing 5×10^{-3}M-calcium acetate); (*b*) with buffer containing 5×10^{-3}M-EDTA; (*c*) with buffer alone. The total volume was 24 ml. The decreases (%) in A.V. (540 mμ) of samples (2 ml.) measured after 1, 13, and 42 hr. were: (*a*) 7, 66, and 92; (*b*) 0, 6, and 13; (*c*) 1, 24, and 50 respectively. The P_M values after 42 hr. were 19, 7, and 14 respectively.

Digests containing β-dextrin (10 mg.), 0·2M-acetate buffer (pH 6·0; 5 ml.), 0·25% β-amylase solution (2 ml.), water, and mercuric chloride (to give final concentrations of $1·5 \times 10^{-5}$ and $1·5 \times 10^{-6}$M severally) in a total volume of 15 ml. were prepared. The change in A.V. (540 and 680 mμ) is shown in Fig. 4. The P_M values determined after 8·5 and 27·5 hr. were: (*a*) without mercuric chloride, 15 and 18; (*b*) $1·5 \times 10^{-6}$M, 14 and 18; (*c*) $1·5 \times 10^{-5}$M, 5 and 9.

Action of " Stock " Soya-bean β-Amylase on Other Polysaccharides.—Potato amylopectin II (40 mg.), 0·2M-acetate buffer (pH 4·6; 9 ml.), 0·2% β-amylase solution (1 ml.), and water to 30 ml. were incubated at 35°. The β-amylolysis limit was 49 (0·5 hr.), 50 (1 hr.), 53 (4 hr.), and 53 (24 hr.). In similar conditions soluble starch had a β-amylolysis limit of 62% but with purified soya-bean β-amylase the value was 57%. When potato amylose VI was used, β-amylolysis limits of 77% were found after 4 and 22 hours' incubation with *ca.* 5 units per mg. of polysaccharide at pH 4·6; with higher enzyme concentrations, complete degradation occurred.

Action of Emulsin on Amylopectin.—Waxy-maize starch (20 mg.), barley β-amylase (50 units/mg.), 0·2M-acetate buffer (pH 5·0; 4 ml.), and water (to 25 ml.) were incubated at 35° for 48 hr. The β-amylolysis limit was 57%. Emulsin (20 mg.) was added; after a further 24 hr. the β-amylolysis limit was 58%. In a second digest in which β-amylase and emulsin acted together on waxy-maize starch, the β-amylolysis limit was 56 and 56% after 24 and 48 hr.

Action of Emulsin on Amylose.—Amylose VIII (30 mg.) was incubated at pH 3·6 with barley β-amylase (100 units/mg.) in a total volume of 50 ml. for 24 hr. The β-amylolysis limit was 75%. The enzyme concentration was then doubled, and after 24 hr. the β-amylolysis limit was 76%. The pH of the digest was then adjusted to 4·8, and to a 15 ml. portion 1% emulsin solution (5 ml.) was added. After 1 and 24 hr., the β-amylolysis limits were 88 and 95% and the A.V.'s (680 mμ) (measured on a 3 ml. sample stained with 1 ml. of iodine solution and diluted to 25 ml.) were 0·005 and 0·002 respectively compared with an original A.V. of 0·200.

A second 15 ml. portion of the digest was incubated with emulsin and 0·01M-mercuric chloride (0·5 ml.) in a total volume of 25 ml. (final concentration 2×10^{-4}M). The A.V.'s (680 mμ) were 0·202, 0·195, and 0·165 after 0, 1, and 24 hr. respectively.

For amylose VI, β-amylolysis limits of 73% before, and 101% after, addition of emulsin were obtained; the A.V. (680 mμ) of a sample fell from 0·21 to 0·08.

Amylose VII solution (2 mg./ml.; 25 ml.) was then incubated at pH 4·6 with 2% emulsin solution (12·5 ml.) in a total volume of 50 ml. After 24 hr. the digest was heated, then cooled, and denatured protein was removed at the centrifuge. The residual polysaccharide was precipitated with ethanol, washed, and dried. The specific viscosity at 25° of 25 mg. of polysaccharide dissolved in 20 ml. of 0·2N-potassium hydroxide was 0·025, and the β-amylolysis limit at pH 3·6 was 93%. Under similar conditions, amylose VII has a β-amylolysis limit of 75%.[2] In a control experiment with heat-denatured emulsin, the residual polysaccharide had a specific viscosity of 0·212.

Effect of pH *on Activity.*—Digests containing amylose β-dextrin (2·4 mg. in 2 ml. of water), phosphate–citrate buffer (pH 4·6—7·6; 2 ml.), and emulsin solution (15 mg. in 1 ml. of water) were incubated at 37°. Control digests (*a*) without enzyme and (*b*) without β-dextrin were also prepared. After 27 hr. samples (2·5 ml.) were withdrawn, heated, and centrifuged. Iodine solution (1 ml.) was added to 2 ml. of solution and the A.V.'s at 640 mμ were measured after dilution to 25 ml. The results are shown in Fig. 3. No correction was required for the enzyme control.

Effect of Calcium Ions and Inhibitors on Emulsin.—(a) *Iodine-staining measurements.* Digests were prepared containing 0·4% amylose solution (5 ml.), emulsin (0·5% in 0·2M-acetate buffer of pH 5·6; 5 ml.), and calcium acetate (5×10^{-3}M; 0·5 ml.) or water (0·5 ml.). Digest (*a*) contained newly prepared enzyme and substrate and water; digest (*b*) contained enzyme pre-incubated at 37° for 40 hr.; digest (*c*) contained enzyme pre-incubated with calcium, and digest (*d*) contained pre-incubated enzyme added to calcium. Samples (2 ml.) were withdrawn

Fall (%) *in* A.V.

Incubation (hr.)	(a)	(b)	(c)	(d)	Incubation (hr.)	(a)	(b)	(c)	(d)
5	11	—	—	—	21·5	—	9	29	12
6·5	—	2	4	2	40	52	—	—	—
16	35	—	—	—	47·3	—	12	47	15

at intervals, heated, and coagulated, protein was removed, and 1 ml. portions were used for A.V. (680 mμ) measurements. Results, tabulated, show that the calcium ions have a stabilising rather than an activating action.

(b) *Viscosity measurements.* Digests containing amylose β-dextrin (13·5 mg.), ~2% emulsin solution (4·5 ml.; pre-incubated for 20 min. with reagent), and water (total volume 15 ml.) were incubated in a viscometer. The relative activities [35] in two series of experiments were (*a*) 6·5 and 5·7 × 10^{-3} unit with water and mercuric chloride (1·5 × 10^{-6}M) respectively, (*b*) 9·3, 8·2, and 6·5 × 10^{-3} unit with water, calcium sulphate (2 × 10^{-4}M), and EDTA (2 × 10^{-4}M) respectively.

Action of Emulsin on Amylopectin and Glycogen β-Dextrin.—Digests were prepared containing either amylopectin β-dextrin (30 mg. in 15 ml. of 0·2M-acetate buffer of pH 5·8) or glycogen β-dextrin (20 mg. in 5 ml. of buffer) and 0·5% emulsin (5 ml.). The annexed results were obtained.

	Amylopectin β-dextrin		Glycogen β-dextrin
Time of incubation (hr.)	Decrease (%) in A.V. (540 mμ)	P_M	P_M
4	7	—	—
24	26	4·2	—
30	—	—	2·4
48	35	7·8	3·7

Action of Malt α-Amylase on Amylose β-Dextrin.—β-Dextrin (5 mg.), 0·2M-acetate buffer (pH 5·6; 9 ml.), 0·01% diastase solution (0·5 ml.), and water or reagent (0·5 ml.) were incubated at 37° for 30 min. The A.V. (640 mμ) of a sample (3 ml.) was then determined. The following results were obtained (expressed as % fall in A.V.); control, 68; calcium acetate (2·5 × 10^{-4}M), 67; EDTA (2·5 × 10^{-3}M), 66; mercuric chloride (1·5 × 10^{-4}M), 2; (1·5 × 10^{-5}M), 25; (1·5 × 10^{-6}M), 41; *p*-chloromercuribenzoate (10^{-5}M), 51. The last observation shows the effect caused solely by the α-amylase, and the results in Table 5 are calculated on this basis.

Action of Salivary α-Amylase on β-Dextrins.—Freeze-dried salivary α-amylase (34 units; [26] 1 mg. in 1 ml. of water) was diluted 50,000 times, and sodium chloride was added to a final concentration of 0·05M. Digests were prepared containing various β-dextrins (4·8—12·0 mg.) dissolved in 0·2M-acetate buffer of pH 5·8 (6 ml.) and diluted salivary amylase (1 ml.). Samples (1 or 3 ml.) were removed for analysis by iodine-staining or reducing-power measurements. After incubation for 23 and 42·5 hr., the following results were obtained: with amylose β-dextrin (4·8 mg.), the A.V. (640 mμ) fell by 25 and 53%; with amylopectin β-dextrin (12·0 mg.), the A.V. (540 mμ) fell by 24 and 39%; with glycogen β-dextrin (12·0 mg.), the P_M values were 0·9 and 1·8 respectively.

In additional digests containing amylopectin β-dextrin (17·0 mg.) and glycogen β-dextrin (17·8 mg.), and either (*a*) 6·8 × 10^{-4} unit of α-amylase or (*b*) 13·6 × 10^{-4} unit, in a total volume of 16—27 ml., the extents of degradation were:

	Incubation (hr.)	
Amylopectin β-dextrin	24·5	48·5
(*a*) Fall (%) in A.V. (540 mμ)	10	22
P_M	3·0	3·2
(*b*) Fall (%) in A.V. (540 mμ)	22	42
P_M	6·2	7·0

Glycogen β-dextrin:	Incubation (hr.)	
(*a*) P_M	0·5	1·4
(*b*) P_M	1·4	3·2

Under similar conditions, the diluted salivary α-amylase thus hydrolyses more than twice as many bonds in amylopectin β-dextrin as in glycogen β-dextrin.

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