135. *Physicochemical Studies on Starches. Part IX.* The Mechanism of the β-Amylolysis of Amylose and the Nature of the β-Limit Dextrin.*

By J. M. G. Cowie, I. D. Fleming, C. T. Greenwood, and D. J. Manners.

The mechanism of the hydrolysis of amylose by (*a*) pure β-amylase, and (*b*) β-amylase and Z-enzyme, has been studied by measuring the $\overline{D.P.}$ of the residual polymer at varying degrees of conversion into maltose. Amylose fractionated severally in presence and absence of air, and a sub-fraction obtained by aqueous leaching of the granule, have been used as substrates. The molecular properties of the various 50% conversion dextrins and the 77% β-limit dextrins were examined in detail. In all cases, hydrolysis proceeded by an essentially single-chain mechanism as there was no evidence of molecules other than amylose of $\overline{D.P.} \geqslant$ the original and maltose in the digest. The structure of the β-limit dextrin, which is thought to contain a randomly-situated barrier to β-amylolysis, is discussed.

The action of β-amylase on amylose, which commences at the non-reducing end of the molecule, involves hydrolysis of alternate α-1 : 4-glucosidic linkages with the production of maltose. In a recent paper,[1] we reported the β-amylolysis of an amylose of high molecular weight, prepared [2] by thymol fractionation of potato starch after complete disruption of the granules. Our results confirm that the *pure* enzyme degrades only *ca.* 75% of amylose, and that for complete conversion into maltose a second enzyme (Z-enzyme) is required. The specificity of the latter enzyme was examined, and evidence presented that the β-amylolysis limit for the pure amylose is not an artefact associated with the colloidal instability of the amylose substrate.[1] The nature of the barrier to β-amylolysis is not known.

The specific mode of action of the β-amylase has been in dispute.[3] Maltose may be produced by the enzyme either by (1) attachment to one amylose molecule and then, by stepwise removal of maltose units, complete degradation before attack on another amylose molecule (" single-chain " action), or (2) by removal of one maltose unit on each random collision with an amylose molecule, with the result that all chains in the system will be shortened simultaneously (" multi-chain " action). Reaction mechanisms between (1) and (2) are also possible. However, determination of the molecular weight of the residual polysaccharide at intermediate stages of β-amylolysis will indicate which mechanism is operative. Under normal experimental conditions, with a large substrate : enzyme ratio, the number-average degree of polymerisation ($\overline{D.P.}$) of the residual amylose at any time during a single-chain reaction will be the same as that for the original up to the stage when the number of substrate molecules is approximately equal to the number of enzyme molecules (at this point, a multi-chain mechanism is inevitable). For multi-chain action throughout, the $\overline{D.P.}$ of the amylose will decrease as the reaction proceeds, the reduction being proportional to the percentage conversion into maltose. Experiments of this type have been carried out by Kerr and Cleveland,[4] who found that the polymeric product isolated at about 50% conversion into maltose possessed virtually the same iodine affinity, limiting viscosity number, and $\overline{D.P.}$ as the original amylose. In our work (a preliminary account of which has appeared [5]), we have extended this type of experiment to include the measurement of the $\overline{D.P.}$ of the residual polymer at varying degrees of conversion into

* Part VIII, *J.*, 1957, 4640.

[1] Cowie, Fleming, Greenwood, and Manners, *J.*, 1957, 4430.
[2] Cowie and Greenwood, *J.*, 1957, 2862.
[3] See Greenwood, *Adv. Carbohydrate Chem.*, 1956, **11**, 335.
[4] Kerr and Cleveland, *J. Amer. Chem. Soc.*, 1951, **73**, 2421.
[5] Cowie, Fleming, Greenwood, and Manners, *Chem. and Ind.*, 1957, 634.

maltose resulting from the action of (*a*) pure β-amylase, and (*b*) β-amylase and Z-enzyme. Amylose fractionated severally in presence and absence of air, and a subfraction obtained by aqueous leaching of the granule, have been used as substrates. The molecular size of various 50% conversion dextrins and 77% β-limit dextrins has been examined, and the structure of the β-limit dextrin is discussed.

Experimental

Preparation of Amylose Samples and their Characterisation.—Potato starch (var. Arran Banner) was fractionated by (1) dispersion in the presence or absence of oxygen, and (2) aqueous leaching at 70°. These methods and those used to characterise the polymers have been described in detail previously in this Series.

Preparation of Enzymes.—Barley β-amylase and soya-bean β-amylase were used. Their preparation and properties have been described elsewhere.[1]

Digest Conditions.—At pH 4·6 and 35° soya-bean β-amylase showed no Z-enzyme activity and converted 77% of amylose samples of high $\overline{\text{D.P.}}$ into maltose, whilst under these conditions barley β-amylase hydrolysed all samples completely.[1] Amylose was dissolved directly in water from the well-centrifuged butan-1-ol complex and buffered with acetate to pH 4·6. Enzyme solution [1] was added and the reaction rate followed by withdrawal of aliquot parts at intervals and estimations of the liberated maltose. In all digests, the concentration of enzyme (100 units per mg. of amylose) was such that 50% conversion had occurred within 30 min., and hence retrogradation of amylose was unlikely.

Isolation of and Measurements on β-Amylolysis Products.—The $\overline{\text{D.P.}}$ of the polymeric product at different percentage conversions was obtained by withdrawing aliquot parts (2 ml.) of the digest and adding M-potassium hydroxide (0·5 ml.). The resultant 0·2M-potassium hydroxide solution (maximum concentration of amylose, 0·18 g./100 ml.) was examined directly in the Spinco ultracentrifuge. Each aliquot portion was studied at three dilutions; the limiting dilution was 0·02 g./100 ml. The 50% conversion and 77% β-limit dextrins were isolated by adding butan-1-ol, then heating the digest for 2 min. on a boiling-water bath (to complete deactivation of enzyme) and allowing the butan-1-ol complexes to be precipitated at room temperature during 24 hr. After removal by centrifugation, the complexes were thoroughly washed with butan-1-ol-saturated water to remove maltose.

Discussion

A study of the β-amylolysis of amylose is complicated by the fact that the β-limit depends on the method of preparation of the amylose. Our aqueous leaching experiments [1,2,6] have shown that potato starch granules contain an easily accessible amylose fraction of relatively low molecular weight, which is completely hydrolysed by pure β-amylase. In this work, the action pattern of β-amylase on the *whole* amylose has been studied in order to investigate the β-limit dextrin and also to use conditions equivalent to those of other workers. The action pattern under these conditions proved to be identical with that for the completely linear amylose prepared by aqueous leaching.

Action of Pure β-Amylase.—(a) *Whole amylose.* Preliminary trial digests showed that the sample of amylose used ($\overline{\text{D.P.}}$ 3200) gave 77% conversion into maltose. When aliquot parts were removed at intervals, and studied in the ultracentrifuge, the sedimentation constant (S_{20}) of the residual polymer as a function of the concentration (c) was as shown in Fig. 1*a*. (The sedimentation constant for amylose is concentration-dependent, as previously reported.[7]) All the points lie on the same curve, within experimental error, although there is a tendency for the values in the earlier stages of conversion to be slightly higher. Fig. 1*a* also shows the corresponding plot of S_{20} against $S_{20}.c$ as recommended by Gralén [8] to facilitate extrapolation to infinite dilution. The points again lie on the same curve. The limiting value of S_{20} for all the residual amyloses was therefore independent of the degree of conversion into maltose up to and including the 77% limit. This result would not be expected on the basis of multi-chain action.

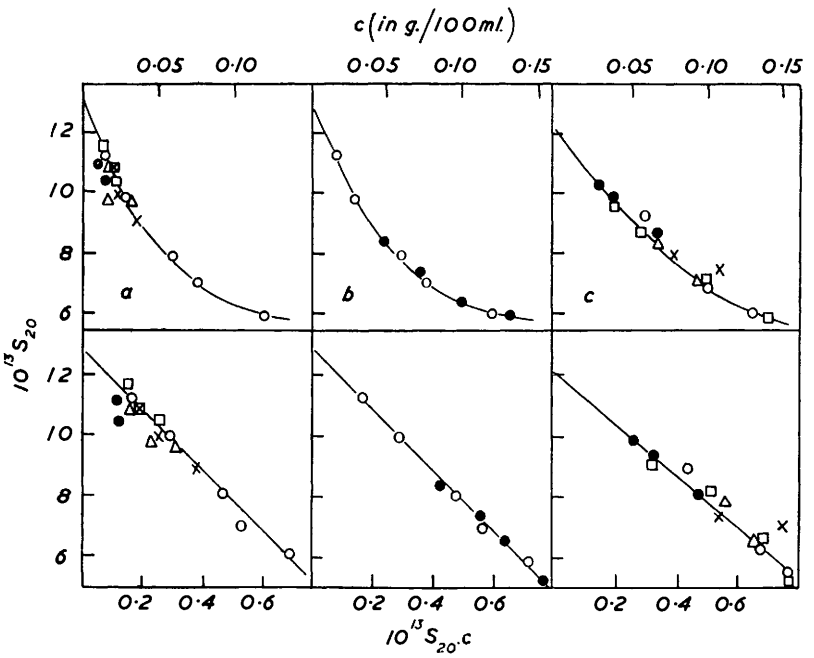
[6] Cowie and Greenwood, *J.*, 1957, 4640.
[7] Bryce, Cowie, and Greenwood, *J. Polymer. Sci.*, 1957, **25**, 251.
[8] Gralén, Inaugural Diss., Uppsala, 1944.

In order to confirm that the sedimentation constant of the β-limit dextrin was unchanged, it was isolated from a large-scale digest. A comparison of the properties with those for the original amylose were as shown:

	$[\eta]$	Iodine affinity	$10^{13}(S_{20})_0$
Original amylose	430	19·5	13·1
77% limit	415	19·2	13·1

The sedimentation results are shown in Fig. 1*b*. The agreement in sedimentation constants shows that the liberated maltose does not influence S_{20} when portions of the digest are studied directly. The properties of the two polymers are identical within experimental error.

FIG. 1. S_{20} versus *c and* S_{20} versus $S_{20}.c$ *for amyloses treated with pure β-amylase.*



(*a*) *Effect of enzymic hydrolysis*: ○ *original amylose*; × 35% *conversion*; △ 45% *conversion*; □ 55% *conversion*; ● 77% *conversion into maltose.*
(*b*) *Original amylose* ○ *and* 77% *limit dextrin* ●.
(*c*) *Effect of enzymic hydrolysis (oxygen-treated amylose)*: ○ *original amylose*; × 23% *conversion*; △ 34% *conversion*; □ 56% *conversion*; ● 75% *conversion into maltose.*

Further, paper chromatography of the digest showed that sugars other than maltose were not present. It was apparent that, in the hydrolysis of amylose by pure β-amylase at pH 4·6 and 35°, the hydrolysate contains only amylose with a $\overline{\text{D.P.}}$ greater than or equal to that of the original and maltose. Multi-chain action is therefore excluded.

If amylose is attacked only from the non-reducing end then, as has been stressed by Kerr and Gehman,[9] the rate of maltose production should be proportional to the molarity rather than the actual weight concentration of amylose. Accordingly solutions of amylose of varying $\overline{\text{D.P.}}$ but equal molarities should show the same rate of production of maltose. Results of experiments for two 1·4 × 10^{-6}M-solutions were as shown:

Conversion (%) *into maltose at a given time* (*min.*).

$\overline{\text{D.P.}}$ of amylose	5	10	15	30	60
3200	34·9	44·9	55·6	71·0	77·8 (const.)
2000	32·5	44·7	53·0	71·1	75·2 (const.)

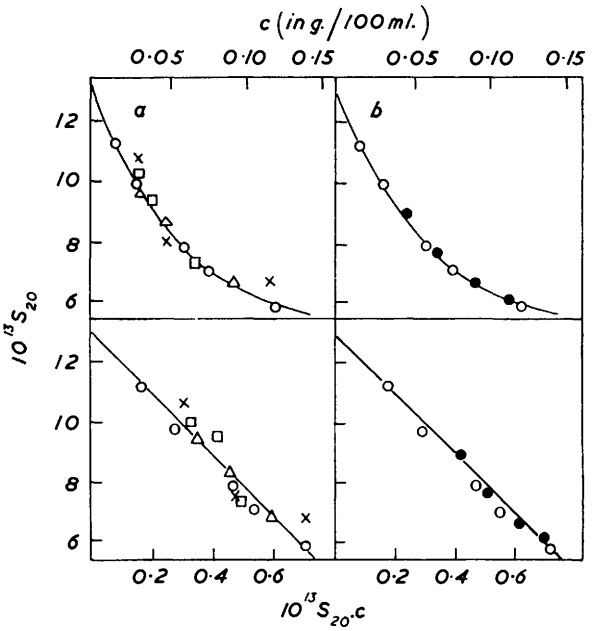
[9] Kerr and Gehman, *Stärke*, 1951, **3**, 271.

These results again substantiate an essentially single-chain action. Similar results have been reported by Kerr and Gehman.[9]

Although it has been reported [10] that oxygen-treatment can introduce barriers to the phosphorolysis of amylose, it was shown elsewhere [1] that amylose prepared in the presence of oxygen was hydrolysed as far as 75% conversion into maltose. The enzymic degradation of this amylose sample was also studied in detail. Fig. 1*c* shows the plots of S_{20} against c and against $S_{20}.c$ for the residual amylose at varying degrees of conversion into maltose. Again, within experimental error, the limiting value of S_{20} for the residual amylose is independent of the conversion up to, and including, the limit. Typical sedimentation diagrams are shown in Fig. 3. Essentially single-chain attack is therefore again established.

(b) *Aqueous-leached amylose.* To confirm the above action pattern, a 50% conversion product was prepared from a sample of amylose leached [2,6] at 70°. The limiting viscosity number of this product was the same as that for the original amylose, within experimental error (for the original amylose, $[\eta] = 270$; for the 50% conversion dextrin, $[\eta] = 265$).

FIG. 2. S_{20} versus c *and* S_{20} versus $S_{20}.c$ *for amyloses treated with β-amylase and Z-enzyme.*



(*a*) *Effect of enzymic hydrolysis*: ○ *original amylose*; × 35% *conversion*; △ 53% *conversion*; □ 64% *conversion into maltose.*
(*b*) *Original amylose* ○ *and* 50% *conversion dextrin* ●.

Action of β-*Amylase and Z-enzyme on Whole Amylose.*—A similar series of investigations was carried out under conditions involving the concurrent action of β-amylase and Z-enzyme and the complete hydrolysis of whole amylose. Fig. 2*a* shows the sedimentation results for the residual amylose at varying degrees of conversion, whilst the corresponding results for a 50% conversion dextrin (isolated from a large-scale experiment) are shown in Fig. 2*b*. Typical sedimentation diagrams are shown in Fig. 3. The properties of the 50% limit and the original amylose were identical, in agreement with Kerr and Cleveland's results,[4] as shown:

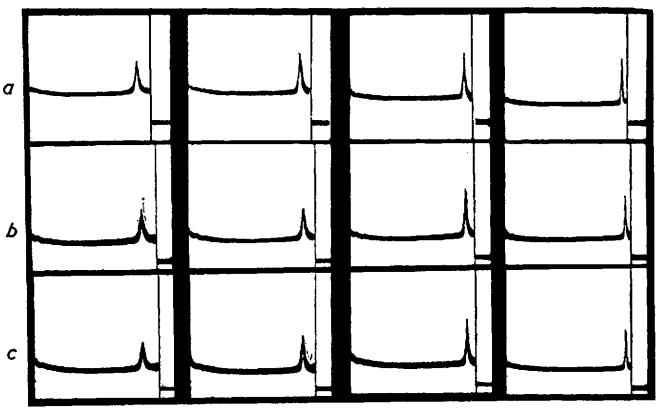
	$[\eta]$	Iodine affinity	$10^{13}(S_{20})_0$
Original amylose	430	19·5	13·1
50% limit dextrin	425	19·6	13·1

This again substantiates single-chain action.

[10] Baum, Gilbert, and Scott, *Nature*, 1956, **177**, 889.

Effect of β-Amylase Action on the Absorption Spectra of the Amylose–Iodine Complex.—Swanson [11] observed that the wavelength of maximum absorption ($\lambda_{max.}$) of the amylose–iodine complex was unaltered during β-amylolysis, thus indicating single-chain action. However, Bourne and Whelan [12] have criticised Swanson's iodine-staining conditions, and found a movement of $\lambda_{max.}$ from 660 to 580 mμ when equal weights of polysaccharide were stained during the formation of 86% of maltose. We have therefore incubated amylose of $\overline{D.P.}$ 3200 with β-amylase, and stained equal weights of residual amylose at intervals up to the production of 84% of maltose. In all samples, the $\lambda_{max.}$ remained unchanged at *ca.* 660 mμ. It now seems probable that Bourne and Whelan's results are due, in part, to contamination of amylose with amylopectin (the β-dextrin of amylopectin [13] has $\lambda_{max.}$ *ca.* 540 mμ), and are not the direct result of multi-chain action. (Amylose prepared recently in this Laboratory by the aluminium hydroxide method [14] contained only 81—87% of amylose.) It should also be noted that iodine-staining measurements on amylose of high $\overline{D.P.}$ (*ca.* 3000) do not enable the reaction mechanism to be determined. (With

FIG. 3. *Tracings of typical sedimentation diagrams. Schlieren wire assembly. In all cases, speed=* 60,000 *r.p.m.; movement is from right to left; times given are those after reaching full speed; the figures in parentheses after the times indicate the angle of the Schlieren wire.*



(*a*) *Original amylose*: *c* = 0·14 g./100 *ml. at* 5 (70°), 9 (65°), 13 (60°), *and* 17 (60°) *min.*
(*b*) 77% *limit dextrin*: *c* = 0·13 *g.*/100 *ml. at* 5 (70°), 9 (60°), 13 (60°), *and* 19 (60°) *min.*
(*c*) 50% *conversion dextrin*: *c* = 0·11 *g.*/100 *ml. at* 5 (70°), 9 (60°), 14 (60°), *and* 18 (60°) *min.*

single-chain action, no movement of $\lambda_{max.}$ is to be expected, whilst, with multi-chain action, the residual amylose at 84% β-amylolysis would have a $\overline{D.P.}$ of *ca.* 500; $\lambda_{max.}$ for this would be little altered.[3])

The Action Pattern of β-Amylase.—All the above results are inconsistent with the concept of multi-chain action. Rather, it appears that, under the conditions of our experiments, the amylose after making contact with a substrate molecule hydrolyses it completely before attacking another molecule, in agreement with Kerr and Cleveland's results.[4] This action is consistent with the remarkably high "turn-over number" (250,000) reported [15] for the enzyme.

Nevertheless, the action pattern appears to differ for short-chain amyloses. Recent studies by Bird and Hopkins [16] have shown that amylose-dextrins ($\overline{D.P.}$ 16—30) were degraded by multi-chain action, whilst Bailey and French [17] found that short-chain

[11] Swanson, *J. Biol. Chem.*, 1948, **172**, 825.
[12] Bourne and Whelan, *Nature*, 1950, **166**, 258.
[13] Fleming and Manners, unpublished results.
[14] Bourne, Donnison, Peat, and Whelan, *J.*, 1949, 1; Hobson, Pirt, Whelan, and Peat, *J.*, 1951, 801.
[15] Englard and Singer, *J. Biol. Chem.*, 1950, **187**, 213.
[16] Bird and Hopkins, *Biochem. J.*, 1954, **56**, 140.
[17] Bailey and French, *J. Biol. Chem.*, 1957, **226**, 1.

synthetic amyloses were attacked by an intermediary mechanism, whereby several glucosidic linkages are hydrolysed during the enzyme–substrate reaction. It is probable that the relative rate of diffusion of the substrate is a controlling factor in the reaction, since at higher temperatures multi-chain action predominates.[18]

The present study therefore indicates that, at pH 4·6 and 35°, β-amylase degrades amyloses of high molecular weight ($\overline{D.P.} \approx 10^3$) by an essentially single-chain mechanism.

Order of Reaction.—Under our experimental conditions, the rate of reaction was so fast that a detailed analysis was not possible. However, the reaction in its initial stages was not of a definite zero or first order (cf. refs. 19 and 20), but the value of k altered. For the overall reaction, the plot [21] of $1/\overline{D.P.}$ against t was not linear. This reaction is being investigated further.

Nature of the β-Limit Dextrin and the Structure of Amylose.—As indicated above, amylose in potato starch is heterogeneous both in $\overline{D.P.}$ and in behaviour on β-amylolysis. Our previous results indicate that there is 30—40% of amylose of $\overline{D.P.}$ 1800, which is completely hydrolysed to maltose by *pure* β-amylase. The sample of whole amylose used in this work had a $\overline{D.P.}$ of 3200, and a β-limit of 77%. It therefore follows that the $\overline{D.P.}$ of the presumably incompletely hydrolysed amylose is of the order of 6000, and that, to the first approximation, it has a β-limit of 50%. (This accounts for a final $\overline{D.P.}$ of *ca.* 3000.) A 50% limit suggests that the barrier to β-amylolysis is randomly distributed throughout the high-molecular-weight amylose.

Although the nature of the barrier has not been established, several possibilities have been considered. The barrier may be situated in the main amylose chain itself, or in a side-chain joined through position 2, 3, or 6 of a constituent glucose residue in the main chain. The former possibility would imply that phosphorylase is not completely specific for α-1 : 4-linked glucopyranose residues.

A side-chain formed by an ester-phosphate group is unlikely, since bone phosphatase, which dephosphorylates starch, does not remove the anomalous linkage.[22] Further, the suggestion by Peat and his co-workers [22] that single glucose residues are attached to a main amylose chain could not be verified experimentally by Hopkins and Bird.[23] An alternative possibility is that the molecule is branched, each branch containing several hundred glucose residues. Kerr and Cleveland [24] have, in fact, suggested that potato and tapioca amylose are singly branched, and contain 1—3 branches per molecule. Our previous studies [1] suggest that, if branching occurs, the interchain linkage is not of the α-1 : 3- or α-1 : 6-type.

It must be noted that amyloses from a wide variety of plant starches contain anomalies which are resistant to β-amylase.[25] Individual amyloses appear to differ in both $\overline{D.P.}$ and β-amylolysis limit, indicating that variations exist in the relative proportion and distribution of the barriers to β-amylolysis.

The authors thank Professor E. L. Hirst, F.R.S., for his interest in this work and the Rockefeller Foundation for financial support. Thanks are also offered to the D.S.I.R. for a maintenance grant (to I. D. F.).

CHEMISTRY DEPARTMENT,
THE UNIVERSITY, EDINBURGH, 9. [*Received, July 18th*, 1957.]

[18] Whelan, *Biochem. Soc. Sympos.*, 1953, **11**, 17.
[19] Hopkins and Jelinek, *Biochem. J.*, 1948, **43**, 32.
[20] Kerr and Severson, *J. Amer. Chem. Soc.*, 1943, **65**, 193.
[21] Bryce and Greenwood, *J. Polymer. Sci.*, 1957, **25**, 480.
[22] Peat, Thomas, and Whelan, *J.*, 1952, 722.
[23] Hopkins and Bird, *Nature*, 1953, **172**, 492.
[24] Kerr and Cleveland, *J. Amer. Chem. Soc.*, 1952, **74**, 4036.
[25] Manners, *Quart. Rev.*, 1955, **9**, 82; Neufeld and Hassid, *Arch. Biochem. Biophys.*, 1955, **59**, 405; Arbuckle and Greenwood, unpublished experiments.