978. *Studies on* Aspergillus Niger. *Part IX.*[1] *The Mechanism of Glucamylase Action.*

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The action of glucamylase, prepared by the standard method.[1] on some thirty oligo- and poly-saccharides is reported. It is shown that the attack of glucamylase on various amylosaccharides is multi-chain and consists of the stepwise removal of single glucose units from the non-reducing ends of the chains.

After the isolation [1] of a purified glucamylase from the strain of *Aspergillus niger* responsible for the synthesis of nigeran [2] the specificity and mechanism of its action were investigated. As a preliminary step, amylosaccharides (maltotriose, maltotetraose, maltopentaose, and maltohexaose) were isolated in a chromatographically pure state by fractionation of the partial acid hydrolysis products of amylose on a charcoal column.[3,4] The purity of each (95—98%) was determined by estimation of the glucose produced by complete acidic hydrolysis.

Maltose, maltotriose, maltotetraose, and maltopentaose were each converted quantitatively into glucose by glucamylase (Table 3). Paper chromatography showed that the main attack of the glucamylase was by end-wise removal of single glucose units. Thus, during the initial attack of the glucamylase on maltotetraose and maltopentaose, only the next lower member of the homologous series and glucose could be detected. The relative concentrations of the various amylosaccharides produced during the reactions suggested that the mechanism was primarily a multi-chain and not a single-chain attack.

Evidence that the glucamylase attacked the non-reducing end of oligosaccharides was obtained by studying the action of the enzyme on the isomeric trisaccharides produced by partial acid hydrolysis of nigeran. While *O*-α-D-glucopyranosyl-(1 → 3)-*O*-α-D-glucopyranosyl-(1 → 4)-D-glucopyranose (T1) was immune to glucamylase, *O*-α-D-glucopyranosyl-(1 → 4)-*O*-α-D-glucopyranosyl-(1 → 3)-D-glucopyranose (T2) gave nigerose and glucose. Again, calcium maltobionate was rapidly attacked, to give calcium gluconate and glucose. Further evidence in favour of an endwise attack by glucamylase was its inability to hydrolyse Schardinger cyclic dextrins (Table 4).

Table 1. *The specificity of glucamylase.*

Substrates not attacked: Glucose, methyl α-D-glucopyranoside, methyl β-D-glucopyranoside, dipotassium α-D-glucose 1-phosphate, αα-trehalose, nigerose, trisaccharide T1, isomaltose, dextran, α- and β-Schardinger dextrins, laminaribiose, gentiobiose, cellobiose, turanose, melibiose, lactose, *O*-α-D-mannopyranosyl-(1 → 6)-D-mannose, melezitose, maltulose.

Substrates attacked only very slowly: Sucrose, raffinose, isomaltotetraose, panose.

Substrates rapidly and completely hydrolysed: Maltose, maltotriose, maltotetraose, maltopentaose amylose, calcium maltobionate, trisaccharide T2.

Table 1 shows that of 29 linear substrates examined, in general only those containing a terminal α-glucopyranosyl residue linked to position 4 of a second glucopyranose unit were attacked at an appreciable rate. Clearly the two glucose units are concerned jointly in providing suitable sites for the enzyme. It is of interest, for example, that maltulose is not a substrate, whereas calcium maltobionate (the only exception to the generalisation) is a good one, presumably because the latter can adopt a shape in which the acyclic moiety approximates sufficiently closely to a second glucose unit to form a template for the enzyme. Panose and isomaltotetraose were degraded very slowly by the enzyme; the former gave glucose and maltose as the initial products, probably as a result of a slight enzymic

[1] Part VIII, Barker and Fleetwood, preceding paper.
[2] Barker, Bourne, and Stacey, *J.*, 1953, 3084.
[3] Whistler and Durso, *J. Amer. Chem. Soc.*, 1950, **72**, 677.
[4] Alm, Williams, and Tiselius, *Acta Chem. Scand.*, 1952, **6**, 826.

impurity. Likewise the enzyme had a very slow action on sucrose; minute amounts of glucose and fructose could be detected in the digest after 48 hr. A similar rate of hydrolysis was observed with raffinose.

Although the glucamylase preparation resembles β-amylase in its attack on the non-reducing end of the chain, it differs in its action on sodium starch glycollate. β-Amylase cannot hydrolyse the glycollate beyond the first substituted glucose residue and with this substrate showed only a very slow action up to 8·8% hydrolysis. Glucamylase produced considerably more reducing sugar than this and achieved a 35% conversion of sodium starch glycollate in 24 hr. (Table 6). Since this apparent ability of the glucamylase to hydrolyse α-1 : 4-glucosidic linkages beyond the substituted residues could be explained by the presence of a very small amount of α-amylase, the action of glucamylase on amylose was examined more closely in order to determine the proportion of this random attack.

A glucamylase-type enzyme could hydrolyse amylose molecules by random attack or by end-wise action; in the latter case it could be either a single-chain action (degradation of one chain at a time) or a multi-chain action (degradation of all chains simultaneously). The relation between the degree of polymerisation (D.P.) of the residual amylose and the degree of hydrolysis (measured as glucose) would be different in the various cases. The intrinsic viscosity $[\eta]$ of solutions of many polymers has been shown to be related to the average molecular weight $\overline{M}$ by the expression $[\eta] = K\overline{M}^a$ which was derived empirically by Flory.[5] Amylose (intrinsic viscosity 1·7) of D.P. about 2000, prepared under anærobic conditions,[6] was incorporated into a digest containing acetate buffer and glucamylase and incubated. Portions of this digest were withdrawn at intervals for the determination of A.V., reducing power, and intrinsic viscosity, measured in 0·5N-sodium hydroxide. A control digest, containing amylose and acetate buffer, but not glucamylase, was incubated alongside this digest so that the amount of degradation due to the acidity of the digest could be ascertained.

Table 7 shows that after 28% of the total number of bonds had been broken the intrinsic viscosity of the residual amylose was reduced to 33% of its original value. If a is taken as unity (the experimentally derived values of a for amylose and for cellulose esters vary between 0·83 and 1·05), then at this stage the intrinsic viscosity should have been 0·18, 72, and 100% of the original value, respectively, for random, multi-chain end-wise, and single-chain end-wise attack. Clearly a completely random attack was excluded. The discrepancy between the other two theoretical figures and the figure actually obtained may be attributed to a very small proportion of random fission (*e.g.*, by a trace of α-amylase). It would only be necessary to break by random attack 1—2 bonds per molecule in order to account for the discrepancy; for an initial D.P. of 2000 the number of bonds broken per molecule by end-wise attack at 28% conversion would be 560, and therefore only one random fission in 280—560 would cause the observed discrepancy. These conclusions are in agreement with absorption value–reducing power curves (see Part VIII).

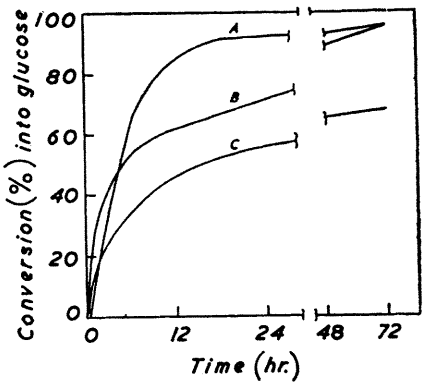
Unfortunately, even such a small amount of α-amylase activity is sufficient to make it impossible to distinguish between single-chain and multi-chain mechanisms on large molecules. The only direct evidence on this question, the chromatographic detection of a considerable amount of the next lower oligosaccharide when glucamylase is allowed to act on maltotetraose or maltopentaose, indicates a multi-chain mechanism. On the question of random or endwise attack, however, there seems little doubt that glucamylase acts by end-wise fission.

Amylopectin, limit β-dextrin (produced by the action of β-amylase on amylopectin), and amylose were compared with respect to both their rates of hydrolysis (see Figure) and their degree of susceptibility to glucamylase (Table 8). The Figure shows that, although the initial rate of hydrolysis of amylopectin was greater than that of amylose (in agreement with an end-wise attack), the rate of hydrolysis of amylopectin decreased sharply at about

[5] Flory, *J. Amer. Chem. Soc.*, 1943, **65**, 372.
[6] Baum and Gilbert, *Chem. and Ind.*, 1954, 490.

60% conversion, showing that the presence of 1 : 6-links hinders the normal action of glucamylase. The removal, by end-wise attack, of 60% of the total number of glucose residues would leave a molecule of the limit-dextrin type, and in agreement with this it is seen that the rate of hydrolysis of limit β-dextrin was similar to the rate of hydrolysis during the latter part of the hydrolysis of amylopectin. When the action of glucamylase on amylopectin, limit β-dextrin, or glycogen was followed on a paper chromatogram, isomaltose was detected in each case. Clearly these branched molecules were hydrolysed virtually completely after prolonged incubation, presumably because the inner chains were made accessible by the small amount of α-amylase impurity.

Additional evidence in favour of this conclusion was provided by the action on limit β-dextrin of glucamylase, which had been purified further on a chromatogram with an



The action of glucamylase on (A) amylose, (B) amylopectin, and (C) dextrin A.

aqueous-acetone solvent. The chromatogram was divided lengthwise into two parts; on one of these halves the position of the glucamylase was determined by its action on amylose; the other half of the chromatogram, when incubated with limit β-dextrin, caused no degradation of limit β-dextrin, either in the region which had been shown to contain glucamylase or in any other part of the chromatogram. It was shown that neither the paper, nor treatment with aqueous acetone, caused any loss in the activity of glucamylase on amylose or on limit β-dextrin. The failure of the chromatographed glucamylase to hydrolyse β-dextrin must therefore be due to the removal of an enzyme which either hydrolyses or by-passes the 1 : 6-links. Since it is known[1] that glucamylase and α-amylase are produced together by *A. niger* and that α-amylase has a greater mobility on paper in aqueous acetone, the most likely explanation is that the last trace of α-amylase in the glucamylase solution had been removed by chromatography.

Experimental

Preparation of Amylosaccharides.—Amylose (23 g.) was partially hydrolysed with 0·5N-sulphuric acid (2 l.) at 100° for 140 min. The reducing power of the hydrolysate then corresponded to a 34·2% conversion into glucose. After neutralisation with 5N-sodium hydroxide and removal of insoluble material (0·7 g.), the solution was concentrated *in vacuo* to 200 c.c., filtered, and dialysed against distilled water for 3 hr., the water being changed every hour. The saccharide mixture remaining in the dialysis tube was fractionated on a charcoal column (65 × 5·5 cm.), which was washed by gradient elution[4] with water containing increasing concentrations of ethanol. The eluates were filtered, concentrated, freeze-dried, and then analysed by paper chromatography.

The purity of each major fraction, measured by determination of the glucose formed after hydrolysis in 2N-sulphuric acid at 100° for 4 hr., was: maltotriose, 96%; maltotetraose, 95%; maltopentaose, 98%; maltohexaose, 95%.

Action of Glucamylase on Amylosaccharides.—A series of digests were prepared containing substrate (25 mg.), M-acetate buffer (pH 4·0; 2 c.c.), and glucamylase solution (5 c.c.) in a

total volume of 25 c.c. Each digest, in a glass-stoppered flask, was incubated at 50°, and aliquot parts (2 or 1 c.c.) were withdrawn at intervals for determination of reducing power.[7]

More concentrated digests were prepared containing substrate (25 mg.) dissolved in water (0·25 c.c.), M-acetate buffer (pH 4·0; 0·04 c.c.), and glucamylase solution (0·2 c.c.) and analysed by paper chromatography with the organic phase of a butan-1-ol–ethanol–water–ammonia mixture (40 : 10 : 49 : 1). The oligosaccharides were detected by spraying with aniline hydrogen phthalate.[8] Maltose gave progressively increasing amounts of glucose and was no longer detectable after 72 hr. In the initial stages (up to 8 hr.), maltotriose gave roughly equimolar quantities of maltose and glucose but thereafter the former rapidly disappeared. The maltotriose was completely converted into glucose after 72 hr. Maltotetraose gave maltotriose and

TABLE 2. *Isolation of amylosaccharides.*

Concn. (%) of EtOH in eluant	Rate of change of EtOH concn. (% per l.)	Sugar component	Yield (g.)	$[\alpha]_D^{17}$ in H_2O
10—15	2·5	Maltose	Trace	—
15—18	1·5	—	—	—
18—21·5	1	Maltotriose	1·865	+157°
21·5—23	1	Maltotriose + Maltotetraose	Trace	—
23—24·2	1	Maltotetraose	1·254	+169
24·2—25·2	1	Maltotetraose + Maltopentaose	1·192	—
25·2—26·2	0·5	Maltopentaose	0·350	+182
26·2—26·8	0·5	Maltopentaose + Maltohexaose	0·460	—
26·8—34	0·5	Maltohexaose	0·269	+177

glucose during the initial stages (up to 8 hr.) and no maltose was detectable. Thereafter the maltotriose was attacked to give maltose, and eventually all the amylosaccharides were hydrolysed enzymically to glucose. The initial products of the action of glucamylase on maltopentaose were maltotetraose and glucose. Thereafter the course of the reaction followed that just described with maltotetraose.

Action of Glucamylase on the Isomeric Trisaccharides from Nigeran.—A mixture containing

TABLE 3. *Action of glucamylase on amylosaccharides.*

Time (hr.)	Conversion (%) into glucose of: Maltose	Maltotriose	Maltotetraose	Maltopentaose
1	19·0	62·5	83·8	74·4
3	81·1	100·0	97·5	96·6
5	82·1	100·4	98·8	100·1
23	94·3	100·6	100·0	100·1
47	98·3	100·6	100·2	100·4

two trisaccharides (50 mg.), namely, *O*-α-D-glucopyranosyl-(1 ⟶ 4)-*O*-α-D-glucopyranosyl-(1 ⟶ 3)-D-glucopyranose (T2) and *O*-α-D-glucopyranosyl-(1 ⟶ 3)-*O*-α-D-glucopyranosyl-(1 ⟶ 4)-D-glucopyranose (T1), in water (0·6 c.c.) was incubated with M-acetate buffer (pH 4·0; 0·1 c.c.) and glucamylase (0·3 c.c.), and the progress of the reaction was followed by paper ionophoresis in borate buffer [9] (pH 10·0). Glucose and nigerose were progressively liberated from trisaccharide T2 which could not be detected after 17 hr. Trisaccharide T1 was not

TABLE 4. *Action of glucamylase on the Schardinger dextrins.*

Time (hr.)	0·067	1·5	4·0	22·0	48·0	72·0
Conversion (%) into glucose: α-dextrin	1·6	1·6	2·2	1·8	2·2	2·0
Conversion (%) into glucose: β-dextrin	3·1	2·5	3·1	2·5	2·7	3·1

attacked even after 92 hr. The M_G values of the possible components were T2, 0·62; T1, 0·32; maltose, 0·34; nigerose, 0·73.

Action of Glucamylase on Schardinger Dextrins.—Schardinger α(or β)-dextrin (25 mg.), after

[7] Shaffer and Hartmann, *J. Biol. Chem.*, 1921, **45**, 377.
[8] Partridge, *Nature*, 1949, **164**, 443.
[9] Foster, *J.*, 1953, 982.

being dissolved in 0·2N-sodium hydroxide (5 c.c.) and neutralised, was incorporated in a digest (total volume, 25 c.c.) which also contained M-acetate buffer (pH 4·0; 2 c.c.) and glucamylase (5 c.c.). The reducing power was measured at intervals (see Table 4). Chromatography of more concentrated digests failed to reveal any reducing sugar during an incubation period of 150 hr.

Action of Glucamylase on Various Substrates.—The action of glucamylase on a wide range of substrates (Table 1) was examined by paper chromatography. For this purpose digests, containing the substrate (50 mg.) in water (0·5 c.c.), M-acetate buffer (pH 4·0; 0·1 c.c.), and glucamylase solution (0·4 c.c.) were incubated at 50°, and aliquot portions (10 µl.) withdrawn at intervals for paper chromatography.

To see whether the glucamylase exhibited phosphatase activity, the enzyme (5 c.c.) was incorporated in a digest (total volume, 25 c.c.) containing dipotassium α-D-glucose 1-phosphate (50 mg.) and M-acetate buffer (pH 5·96; 2 c.c.). A similar digest, to which a 1% solution (0·1 c.c.) of soluble starch was added, was used for the detection of any phosphorylase activity. Both digests were incubated at 37° and aliquot portions (2 c.c.) withdrawn for the determination of inorganic phosphate [10] (see Table 5).

TABLE 5. *Action of glucamylase on glucose 1-phosphate.*

Time (hr.)		0·013	1·0	3·0	5·0	24	30
Hydrolysis (%) of G 1-P	no starch	2·4	2·7	2·7	3·0	—	3·9
	starch added	2·7	2·7	3·0	3·0	3·9	—

TABLE 6. *Action of amylases on sodium starch glycollate.*

	α-Amylase		β-Amylase		Glucamylase	
Time (hr.)	A.V. (680 mµ)	Apparent conversion (%) into glucose	A.V. (680 mµ)	Apparent conversion (%) into glucose	A.V. (680 mµ)	Apparent conversion (%) into glucose
0·033	0·074	6·9	0·285	4·1	0·279	4·1
2·0	0·011	33·8	0·254	7·4	0·195	14·5
24·0	0·010	40·2	0·168	8·8	0·032	35·6
48·0	0·010	43·0	0·132	9·0	0·014	44·8
72·0	0·012	44·9	0·125	8·8	0·011	48·1

Action of Glucamylase on Sodium Starch Glycollate.—Three digests (each 25 c.c.) were prepared containing sodium starch glycollate (95·5 mg.) and M-acetate buffer (2 c.c.) with glucamylase, salivary α-amylase, or soya-bean β-amylase. The digests containing α- and β-amylase were buffered at pH 5·0 and incubated at 28°, while that containing glucamylase was buffered at pH 4·0 and incubated at 50°. The reducing power and A.V. 680 mµ [11] were measured at intervals (see Table 6).

Relation Between the Intrinsic Viscosity and Degree of Hydrolysis of Amylose by Glucamylase.—Amylose (B.V. 1·12) was isolated under anærobic conditions by Baum and Gilbert's method.[6] The amylose (21·9 mg.) was dissolved in cold 0·5N-sodium hydroxide (12 c.c.) and filtered, through a grade-4 sintered-glass filter, into a carefully cleaned, and dried, dust- and fibre-free vessel. This solution (10 c.c.) was transferred to a modified Ubbelohde viscometer (flow time for 0·5N-sodium hydroxide at 20°, 179·2 sec.) and the viscosity measured; the utmost care was exercised during the measurements to prevent the entry of dust or fibres into the viscometer. The solution was diluted to $\frac{2}{3}$, $\frac{1}{2}$, and $\frac{1}{3}$ of the original concentration with 0·5N-sodium hydroxide, and the specific viscosity was determined at each concentration; the mean value of at least four flow-time measurements, which did not differ by more than 0·4 sec. from each other, was taken for the calculation. The intrinsic viscosity was obtained by extrapolating the value of η_{sp}/c to zero concentration (Table 7).

Amylose (120 mg.) was dissolved in cold 0·5N-sodium hydroxide (20 c.c.), and the solution neutralised with sulphuric acid; M-acetate buffer (pH 4·0; 2 c.c.) and glucamylase (2 c.c.) were added and the digest was diluted to 40 c.c. with water and incubated at 50°. Samples (10 c.c.) were withdrawn at intervals, inactivated with 6% trichloroacetic acid (1 c.c.), then centrifuged, and the supernatant solutions neutralised. 7·5N-Sodium hydroxide (2 c.c.) was added to the

[10] Allen, *Biochem. J.*, 1940, **34**, 858.
[11] Bourne, Haworth, Macey, and Peat, *J.*, 1948, 924.

supernatant solution which was then diluted to 15 c.c. with water. Part (10 c.c.) of this solution was used for the determination of the intrinsic viscosity of the residual amylose. Simultaneously with the withdrawal of the samples for the viscosity determinations, aliquot portions (0·33 and 1 c.c.) were withdrawn for the determination of A.V. and reducing power respectively.

TABLE 7. *Relation between the intrinsic viscosity and the degree of hydrolysis.*

Original amylose:				
Concn. (%)	0·182	0·122	0·091	0·061
η_{sp}/c	2·14	1·98	1·93	1·83
Amylose from control digest:				
Concn. (%)	0·200	0·133	0·100	0·067
η_{sp}/c	2·16	1·97	1·84	1·72
11·4% Hydrolysed amylose:				
Concn. (%):	0·177	0·118	0·089	0·059
η_{sp}/c	1·23	1·09	1·02	0·95
27·9% Hydrolysed amylose:				
Concn. (%)	0·144	0·096	0·072	0·048
η_{sp}/c	0·98	0·83	0·71	0·65

Time (hr.)	A.V. (680 mμ)	% of theor. reducing power	Intrinsic viscosity
0	0·97	—	1·70
0·017	0·94	0·6	—
0·167	0·82	11·4	0·80
0·67	0·40	27·9	0·52
1·33	0·03	78·0	—
			1·56 (control)

A control digest, similar to the above digest but without glucamylase, was prepared and incubated, inactivated, etc., as above, and the intrinsic viscosity determined.

Action of Glucamylase on Amylopectin and on Limit β-Dextrin.—The substrate (25 mg.) was dissolved in 0·2N-sodium hydroxide (5 c.c.) and neutralised with sulphuric acid. M-Acetate buffer (pH 4·0; 2 c.c.) and glucamylase (5 c.c.) were added and the digest was diluted, with water, to 25 c.c. The absorption value and reducing power were measured at intervals (see Table 8).

TABLE 8. *Action of glucamylase on amylopectin and limit β-dextrin.*

	Amylopectin		Limit β-dextrin	
Time (hr.)	A.V. (680 mμ)	Red_G	A.V. (680 mμ)	Red_G
0·033	0·183	0·6	0·142	0
1·0	0·120	28·7	0·114	16·0
4·0	0·054	44·6	0·051	28·1
24·0	0·013	71·2	0·017	55·8
48·0	0·011	89·2	0·013	65·7
72·0	0·012	95·7	0·011	68·4
192·0	0·010	96·7	0·011	74·7

Paper chromatography of more concentrated digests containing amylopectin and limit β-dextrin showed that the main reducing sugar produced was glucose. In addition, another reducing sugar was detected which had an R_F value identical with that of isomaltose in two different solvent systems. Its M_G value in borate buffer (pH 10·0) was identical.

Action of Chromatographically Purified Glucamylase on Limit β-Dextrin.—Glucamylase (0·2 c.c.) was applied to a strip of Whatman No. 1 filter paper, irrigated with 50% aqueous acetone at 0° for 7 hr., dried in cold air, and divided into 1 cm. strips. The position of the glucamylase was determined by estimating the reducing power produced after incubating half of each strip with a solution of amylose (1 mg.) for 24 hr. The other half of each strip was incubated with dextrin A (1 mg.) for the same time and the reducing power of each digest determined. Determinations were carried out in the presence of 0·12 mg. of glucose (see Table 9).

TABLE 9. *Action of chromatographically purified glucamylase on limit β-dextrin.*

Strip no:	1	2	3	4	5	6	7	8	9	10	11	12
	Reducing power as glucose (mg.)											
Amylose	0·15	0·12	0·25	0·38	0·35	0·25	0·12	0·12	0·10	0·08	0·10	0·10
Limit β-dextrin ...	0·14	0·14	0·12	0·15	0·12	0·12	0·10	0·10	0·12	0·08	0·12	0·10

Glucamylase solution (180 μl.) was applied to two strips of filter paper, which were put into two digests containing amylose (1 mg.) and limit β-dextrin (1 mg.) respectively. Two other similar digests were prepared containing glucamylase (180 μl.) but no filter paper. The four digests were incubated for 24 hr., after which the reducing power of each was determined:

Digest containing:	amylose + paper	amylose alone	limit β-dextrin + paper	limit β-dextrin alone
Reducing power as glucose (mg.) ...	0·63	0·65	0·26	0·25

A similar amount of glucamylase solution was applied to each of four strips of filter paper. Two of these strips were moistened with 50% aqueous acetone and kept in an atmosphere of the same solvent mixture for 4 hr. at 0°. These strips were allowed to dry in cold air and added to two digests containing amylose (1 mg.) and limit β-dextrin (1 mg.) respectively. The two other paper strips, not treated with acetone, were added to two similar digests, the four digests were incubated for 24 hr. and the reducing powers determined.

Digest containing:	acetone-treated enzyme + amylose	untreated enzyme + amylose	acetone-treated enzyme + β-dextrin	untreated enzyme + β-dextrin
Reducing power as glucose (mg.)	0·73	0·72	0·29	0·29

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