977. *Studies on* Aspergillus niger. *Part VIII.** *The Purification of Glucamylase.*

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The purification is described of an enzyme, glucamylase, which yields glucose as the sole product of its action on amylose and, when analysed on a paper chromatogram, shows only one component that exhibits amylolytic activity. Its optimum pH is 4·0 and optimum temperature 52°; the minimum concentration of acetate buffer necessary for full activity is 0·02M.

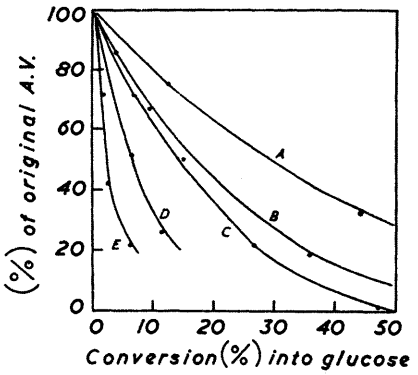
The existence of starch-degrading enzymes in *Aspergillus niger* has long been known, but considerable confusion has arisen in the nomenclature of the amylases from various *Aspergillus* species and other moulds. The principal amylase-type enzyme produced by moulds is an α-amylase, sometimes called a dextrinising enzyme. The deviations from the expected behaviour of an α-amylase by mould-enzyme preparations have usually been attributed to contamination with other enzymes such as β-amylase, maltase, and a saccharifying or glucogenic enzyme. In a study of the amylolytic enzymes of a number of moulds, especially of the *Aspergillus* and *Rhizopus* species, Corman and Langlykke [1] found that while in some cases there was a rapid liquefaction of starch solution with the production

* Part VII, *J.*, 1957, 3541.

[1] Corman and Langlykke, *J. Bact.*, 1947, **54**, 149.

of maltose and dextrins (α-amylase-type action), in others there was rapid production of glucose. In 1953, Barker and Carrington [2] reported that a cell-free extract of *A. niger* " 152," the strain responsible for the synthesis of nigeran, gave glucose as the major product of its action on starch. The purpose of the present communication is to describe the purification of this enzyme, termed glucamylase, which gives glucose as the sole product of its action on amylose. This enzyme appears to belong to a general group of enzymes, the glucamylases or amyloglucosidases, which probably include that isolated from *Clostridium acetobutylicum*,[3,4] and those reported in *Rhizopus delemar*,[5,6] *Aspergillus oryzae*,[7] *and A. niger* [8] (NRRL-330-1).

Purification of the glucamylase was followed by observing the changes in shape of the iodine-absorption value (A.V.)–reducing power curve obtained with each enzyme fraction after incorporation in a standard amylose digest devised so that neither the amylose nor the concentration of acetate buffer had any effect on the method of estimating the glucose produced. Such a curve is independent of the concentration of enzyme employed and characteristic of the type of enzyme(s) present.[9] With the crude cell-free extract of *A. niger*, the shape of the curve (see Figure) was reminiscent of the action of α-amylase



Enzyme fractions obtained during the purification of glucamylase.

A, Purified glucamylase; *B*, fraction 2 after treatment at pH 2·36; *C*, band A enzyme from crude extract; *D*, crude enzyme extract; *E*, enzyme in band B of crude extract.

on amylose, but was not reliable since the extract exhibited glucose oxidase and other activity towards glucose (Table 3). Glucose was, however, the only sugar detectable by paper chromatography throughout the incubation. As previously reported,[2] the crude extract also contains transglycosidases which act on sucrose and maltose. The crude enzyme extract could be stored for 1 month at 0° with only a small loss of amylolytic activity (Table 4) and was obtained with full activity in the freeze-dried form.

Addition of solid ammonium sulphate (20, 40, and 60 g.) to a solution of crude extract (100 c.c.) gave fractions 1, 2, and 3 respectively. Fraction 2 contained 85% of the activity recovered, and 40% of the original activity (Table 6). It was free from glucose oxidase (Table 6) and the transglucosidase which acts on maltose to give, *inter alia*, isomaltose and panose. The removal of glucose oxidase from the crude extract caused a shift in the absorption value–reducing power curve (Table 6), and again glucose was the only sugar detectable.

In order to determine whether this action was due to a single enzyme, fraction 2 and the crude enzyme extract were examined by paper chromatography with aqueous acetone as the irrigating solvent. To locate the amylolytic enzymes the chromatograms were

[2] Barker and Carrington, *J.*, 1953, 3588.
[3] Hockenhull and Herbert, *Biochem. J.*, 1945, **39**, 102.
[4] French and Knapp, *J. Biol. Chem.*, 1950, **187**, 463.
[5] Phillips and Caldwell, *J. Amer. Chem. Soc.*, 1951, **73**, 3559.
[6] Fukumoto, Sakazaka, and Minamii, *Symp. on Enz. Chem. (Japan)*, 1954, **9**, 94.
[7] Drews, Specht, and Olbrich, *Branntweinwirtschaft*, 1954, **76**, 21.
[8] Kerr, Cleveland, and Katzbeck, *J. Amer. Chem. Soc.*, 1951, **73**, 3916.
[9] Bourne, Macey, and Peat, *J.*, 1945, 882.

divided into narrow horizontal strips and, after incubation with a solution of amylose, the absorption value of each digest was determined. Amylolytic activity was located in two bands with both fraction 2 and the crude enzyme extract. The enzyme (R_F 0·036—0·143) of band A, eluted from a chromatogram of the crude extract, exhibited a marked change in the shape of the A.V.—reducing power curve (Figure). In addition, the wavelength of maximum absorption of iodine-stained aliquot portions of the digest did not shift to lower wavelengths throughout most of the time of incubation. This phenomenon was the opposite to that which had been observed with the crude extract, fraction 2, and α-amylase, and was typical of a β-amylase-type action except that, instead of maltose being progressively removed from the non-reducing ends of the amylose chains, the moiety removed was glucose. Attempts to elute the enzyme (R_F 0·475—0·571) from band B were unsuccessful and so the activity of this band was determined on paper. The results (Table 7) suggested that this band contained an α-amylase (random fission).

Purification of fraction 2 on a larger scale followed the example of other workers [5, 8] who freed glucamylase from α-amylase by treatment with acid. Treatment of fraction 2 at successively lower values of pH and determination of the nature of the residual activity showed a change, indicative of the removal of α-amylase, after treatment at pH 3·1 or below for 6 days (Table 8). The standard method adopted for the preparation of glucamylase was the treatment of fraction 2 at pH 2·9 for 12 days at 10°.

The shape of the A.V.–reducing power curve of this acid-treated enzyme (Figure) showed that it contained even less α-amylase than did the enzyme eluted from band A of the chromatogram of the crude extract. Moreover, chromatography of the acid-treated enzyme disclosed only one enzyme capable of hydrolysing amylose to products which were not stained by iodine; the R_F value of this enzyme was the same as that of band A.

The optimum pH and temperature of the acid-treated enzyme for its action on amylose were *ca.* pH 4·0 and 52° respectively. A minimum concentration (0·02M) of acetate buffer was necessary for the enzyme to exhibit its full activity. An attempt to find an alternative method of preparation by preferential inhibition of the α-amylase impurity in the crude extract was unsuccessful. Although mercuric chloride (50 parts per million) inhibited salivary α-amylase completely and had little effect on the acid-treated enzyme (Table 16), it did not inhibit appreciably the α-amylase in the crude extract (Table 5). The major portion of this mould α-amylase appears to be absorbed on the nigeran released during the disruption of the *A. niger* cells, since in the early stages of the action of a suspension of nigeran on amylose an appreciable concentration of maltose was formed, together with some maltotriose and higher oligosaccharides.

The action of glucamylase on amylose was not reversible (Table 14) and was not competitively inhibited by the product of its action, glucose (Table 15).

The glucamylase, purified by the procedures described, was thus seen to be substantially free from α-amylase, and also from other enzymes (including glucose oxidase and transglucosidase) capable of acting upon amylose or the possible products of the action of glucamylase on amylose.

Experimental

Standard Digest for the Measurement of Glucamylase Activity.—Amylose (80—100 mg.), moistened with ethanol, was dissolved in 0·2N-sodium hydroxide (10 c.c.) by warming at 60° for 2—3 min. After cooling, the solution was diluted to 25 c.c. and a portion containing 25 mg. of amylose (B.V. 1·24) was neutralised with 0·5N-sulphuric acid. This was incorporated in the standard digest (total volume, 25 c.c.) which also contained M-acetate buffer (2 c.c.) and glucamylase solution (5 c.c.). Before the isolation of the purified enzyme the digest was buffered at pH 6·0 and incubated at 37°; thereafter the buffer solution had pH 4·0, and the digest was incubated at 50° in a glass-stoppered flask. At intervals, aliquot portions (1 c.c.; equiv. to 1 mg. of amylose) were stained with iodine (2 mg.) and potassium iodide (20 mg.) in

a final volume of 100 c.c. and the A.V. 680 mμ measured.[10] Other portions (4 c.c.) were subjected to the Shaffer–Hartmann determination [11] of reducing sugar. Where the reducing power of the solution was suspected to be <0·5 mg., a solution (1 c.c.) containing a known amount of glucose was added before determination. Where reducing power (Red_G) is quoted it is expressed as a percentage of the theoretically possible amount of glucose liberated on complete hydrolysis. For the comparison of different enzyme preparations the activity is expressed as the Red_G after 4 hours' incubation under standard conditions.

(a) *Effect of amylose on the method of estimation.* The amounts of glucose (0·475 mg.; 0·950 mg.) estimated by the Shaffer–Hartmann method [11] in the absence of amylose were in good agreement with those (0·470 mg.; 0·945 mg.) determined in the presence of amylose of the concentration which would normally be present in a sample taken for the determination of glucamylase activity.

(b) *Effect of the concentration of acetate buffer on the method of estimation.* The reducing powers of glucose at two concentrations were determined in the presence of acetate buffer, pH 4·0, of the strengths given in Table 1.

TABLE 1. *Effect of varying concentrations of acetate buffer.*

Molarity of acetate buffer	0·0	0·002	0·02	0·2	0·5	0·0	0·002	0·02	0·2	0·5
Glucose determined (mg.)	0·58	0·59	0·60	0·59	0·45	1·21	1·20	1·19	1·20	0·54
Glucose taken			0·59 mg.					1·18 mg.		

Preparation of a Crude Aspergillus niger *Extract.*—Mycelia of *A. niger* (strain 152) were grown aerobically on a sterile solution containing sucrose (15%) and the mineral components of Currie's medium [12] and incubated at 30° for 6 days. The mycelia were removed from the growing cultures, any portions showing sporulation were discarded, and the remainder was washed in several changes of tap water, followed by three changes of distilled water. After shredding of the mycelium in an " Atomix " blender, with sufficient water to make a thick paste, the cells were disrupted with glass beads for 20 min. at 0° in a Mickle shaker or in a vibratory ball mill. The smashed cells were extracted twice with water, the combined extracts being centrifuged to remove cell debris. Microscopic examination of the extract, after staining with carbol-fuchsin, showed the absence of whole cells in each of twenty fields examined.

(a) *Action of the crude extract on amylose.* The crude extract (5 c.c.) was incorporated in a standard digest (25 c.c.) and aliquot portions were removed at intervals for determination of reducing power and A.V. The results are given in Table 2.

TABLE 2. *Action of the crude extract on amylose.*

Time (hr.)	0·013	0·13	1·33	5·25	10	22	46	60
A.V. (680 mμ)	1·120	1·114	1·042	0·798	0·552	0·215	0·033	0·014
Red_G	1·1	—	1·6	6·8	10·1	19·2	33·4	49·0

(b) *Action of the crude extract on glucose and maltose.* Portions (5 c.c.) of the crude extract were incorporated in standard digests in which the amylose was replaced by glucose (1·88 mg./c.c.) or maltose (1·60 mg./c.c.). Both digests contained the same amount of sodium sulphate as that present in the standard digest. The reducing power was determined at intervals (Table 3).

TABLE 3. *Action of the crude extract on glucose and maltose.*

(i) *Glucose*

Time (hr.)	0	0·013	0·13	1·0	2·0	18·0	36·0
Reducing power	1·88	1·83	1·64	1·475	1·39	1·30	1·60

(ii) *Maltose*

Time (hr.)	0·006	0·13	0·5	1·0	2·0	4·0	6·0	24·0
Reducing power as glucose (mg.) ...	0·69	0·72	0·87	1·26	1·29	1·07	1·10	0·99

(c) *Stability of the crude extract on storage.* The action of a fresh aqueous extract of *A. niger* cells on amylose was determined in a standard digest and then redetermined after being stored at 0° for 1 month (cf. Table 4).

[10] Bourne, Haworth, Macey, and Peat, *J.*, 1948, 924.
[11] Shaffer and Hartmann, *J. Biol. Chem.*, 1921, **45**, 377.
[12] Currie, *ibid.*, 1917, **31**, 15.

TABLE 4. *Stability of the crude extract.*

	Fresh extract		Extract stored for 1 month	
Time (hr.)	A.V. (680 mμ)	Red_G	A.V. (680 mμ)	Red_G
0·013	0·873	4·5	0·908	3·1
0·065	0·834	5·75	0·902	5·0
0·45	0·630	11·5	0·688	9·8
2·0	0·303	18·9	0·401	18·4

(d) *Effect of mercuric chloride on the crude extract.* Two standard amylose digests, into one of which mercuric chloride solution (2·5 c.c. containing 0·00125 mg. of this salt) was incorporated, were prepared and the activities of the crude extract determined (Table 5).

TABLE 5. *Effect of mercuric chloride on the crude extract.*

	Without $HgCl_2$		With $HgCl_2$	
Time (hr.)	A.V. (680 mμ)	% of theor. reducing power	A.V. (680 mμ)	% of theor. reducing power
0·05	1·04	5·0	1·02	5·2
0·5	0·58	9·9	0·57	10·1
1·25	0·13	16·2	0·25	17·1
4·0	0·03	29·2	0·06	32·4
22·0	0·02	51·3	0·02	52·2

(e) *Removal of glucose oxidase activity by ammonium sulphate fractionation.* The activity of a portion (5 c.c.) of a crude extract (50 c.c.) was determined and solid ammonium sulphate (9·5 g., equiv. to 20 g. per 100 c.c. of solution) added to the remainder. After being kept overnight at 0°, the precipitate (fraction 1), which formed a solid cake on the surface after centrifugation, was separated and redissolved in water (45 c.c.). The concentration of ammonium sulphate was increased to 40 g./100 c.c. and 60 g./100 c.c. of the original solution, giving precipitates (fractions 2 and 3 respectively) which were each redissolved in water (45 c.c.). The activities of each fraction were determined with respect to amylose and in the case of fraction 2 with respect to glucose also.

TABLE 6. *Fractionation of the crude extract with ammonium sulphate.*

	Crude extract		Fraction 1		Fraction 2		Fraction 3	
Time (hr.)	A.V. (680 mμ)	Red_G	A.V. (680 mμ)	Red_G	A.V. (680 mμ)	Red_G	A.V. (680 mμ)	Red_G
0·013	1·15	—	1·17	—	1·21	—	1·19	—
0·25	0·85	1·2	1·14	0·2	1·20	0·2	1·17	0·2
1·0	0·60	6·5	1·10	1·1	1·10	1·9	1·16	0·2
2·5	0·31	11·7	1·05	1·5	0·68	3·25	1·16	0·7
21·0	0·02	65·6	0·93	3·4	0·28	25·8	1·07	1·1

Action of fraction 2 on glucose.

Time (hr.)	0·013	0·5	1·0	2·0	18·0	114·0
Reducing power as glucose (mg.)	1·88	1·87	1·875	1·875	1·85	1·83
Glucose destroyed (% of theory)	0·0	0·53	0·26	0·26	1·60	2·66

Separation of Enzymic Components by Paper Chromatography.—(i) *Crude* A. niger *extract-activities of bands A and B.* Freeze-dried crude enzyme extract (100 mg.) was dissolved in water (0·4 c.c.) and placed, in two applications, along lines (20 cm. long) drawn on two strips of filter paper (23 cm. wide). After irrigation with 50% aqueous acetone for 7 hr. at 0°, the paper was dried in a current of cold air. Strips (3 cm. wide) were cut from each side of the chromatogram and the position of those enzymes exhibiting amylolytic activity determined by examination of portions (1 cm. long) of these strips. Each small strip was incubated with 0·05% amylose solution (1 c.c.), and 0·02M-phosphate (pH 6·18)–0·03M-sodium chloride buffer (5 c.c.) for 6 hr. at 37°. Aliquot portions (5 c.c.) of each digest were withdrawn, acidified with N-sulphuric acid (1 c.c.), and stained with 0·006% iodine solution (1 c.c.). The location of the enzyme was indicated by a decrease in the absorption value (680 mμ) measured in 1 cm. cells. Amylolytic activity was exhibited by two distinct bands on the chromatogram. Band A enzyme, which had R_F 0·036—0·143, could be completely eluted from the paper (4 cm. wide) with 6 c.c.

of 0·02M-phosphate buffer (pH 6·18). Band B enzyme, which had R_F 0·465—0·571, was not extracted from the paper under the same conditions and its activity was therefore determined with the enzyme still intact on the paper. The activities of both Band A and B enzymes, after incorporation in standard digests, are given in Table 7.

TABLE 7. *Activities of amylolytic enzymes in bands A and B.*

Enzyme in band A							
Time (hr.)	0·083	6·0	22·0	46·0	94·0		
A.V. (680 mμ)	1·13	0·82	0·58	0·31	0·03		
Red_G	0·7	6·7	15·0	26·8	47·3		
Enzyme in band B							
Time (hr.)	0·083	1·0	6·0	24·0	48·0	96·0	144·0
A.V. (680 mμ)	1·13	1·06	0·83	0·54	0·47	0·25	0·16
Red_G	0·45	0·9	1·6	2·7	3·8	6·3	13·3

(ii) *Ammonium sulphate-purified enzyme.* Fraction 2 was separated on filter paper as described above for the crude enzyme, and the amylolytic enzymes were located. The amylolytic activity was again located in two distinct bands, A (R_F 0·040—0·12) and B (R_F 0·40—0·52).

Purification of Fraction 2 by Acid-treatment.—(i) *Effect of storage at different* pH *values.* Portions (5 c.c.) of the enzyme preparation described as fraction 2 were adjusted to various pH values (Table 8) with N-sulphuric acid and stored at 0° for 6 days. Each solution was then adjusted to pH 6·0 with N-sodium hydroxide, and its activity determined by using standard amylose digests. A further portion (5 c.c.) of fraction 2 (pH 5·22), to which no acid was added, was similarly treated and served as a control.

TABLE 8. *Acid treatment of fraction 2.*

	pH 5·22		pH 3·98		pH 3·12		pH 2·36	
Time (hr.)	A.V. (680 mμ)	Red_G	A.V. (680 mμ)	Red_G	A.V. (680 mμ)	Red_G	A.V. (680 mμ)	Red_G
0·067	1·07	—	1·09	—	1·08	—	1·09	—
0·5	0·74	3·4	0·82	2·6	0·99	2·25	0·96	3·6
1·5	0·59	4·8	0·66	4·0	0·91	3·7	0·83	4·7
5·0	0·32	10·0	0·38	9·5	0·74	11·0	0·75	9·4
24·0	0·08	31·8	0·14	27·0	0·16	36·0	0·22	36·0
52·0	0·01	61·6	0·02	52·8	0·08	53·2	0·10	50·4

(ii) *Chromatography of acid-treated enzyme.*—A solution of glucamylase, obtained by treatment of fraction 2 at pH 2·0 and 10° for 12 days, was examined by paper chromatography as described for the crude extract. Only one band was now observed which had amylolytic activity and this had an R_F value (0·038—0·19) similar to that of band A. All glucamylase used below had been subjected to this acid-treatment.

Optimum pH *Value for Glucamylase Action.*—A number of standard amylose digests were prepared, incorporating M-acetate buffer (2 c.c.) of different pH values from 2·94 to 6·96. The activity of acid-treated glucamylase in each digest was determined in the usual way (see Table 9).

TABLE 9. *Activity of glucamylase at different* pH *values.*

	pH 2·94		pH 4·00		pH 5·00		pH 5·98		pH 6·96	
Time (hr.)	A.V. (680 mμ)	Red_G	A.V. (680 mμ)	Red_G	A.V. (680 mμ)	Red_G	A.V. (680 mμ)	Red_G	A.V. (680 mμ)	Red_G
0·026	1·09	0·7	1·08	0·4	1·09	0·9	1·10	0·9	1·09	0·7
1·0	0·95	8·3	0·86	10·8	0·85	11·0	0·95	5·6	1·06	3·8
4·0	0·44	21·6	0·28	32·2	0·29	34·2	0·65	17·1	0·99	7·0
24·0	0·02	63·0	0·02	70·1	0·02	54·9	0·03	47·7	0·64	20·2

Optimum Temperature of Glucamylase Action.—Glucamylase solution was incorporated into each of five standard amylose digests buffered at the optimum pH value (4·0). These digests were incubated at different temperatures between 37° and 70°. The results of activity determinations are given in Table 10.

TABLE 10. *Activity of glucamylase at different temperatures.*

	37°		44°		50°		60°		70°	
Time (hr.)	A.V. (680 mμ)	Red_G	A.V. (680 mμ)	Red_G	A.V. (680 mμ)	Red_G	A.V. (680 mμ)	Red_G	A.V. (680 mμ)	Red_G
0·026	1·13	2·2	1·11	2·5	1·11	2·5	1·12	2·5	1·14	2·5
1·0	0·71	14·6	0·67	18·9	0·47	24·5	0·65	19·1	0·89	5·9
4·0	0·34	32·8	0·29	31·6	0·03	43·8	0·18	37·4	0·86	6·3
24·0	0·01	62·6	0·01	67·5	0·01	83·6	0·01	59·0	0·86	6·1

Influence of Ionic Concentration on the Activity of Glucamylase.—A series of standard amylose digests was prepared incorporating acetate buffer (pH 4·0; 5 c.c.) of varying molarity so that the concentration of each digest with respect to acetate buffer was that given in Table 11. The effect on the activity of glucamylase was determined.

TABLE 11. *Influence of ionic concentration on activity.*

	0·2M		0·02M		0·002M		Nil	
Time (hr.)	A.V. (680 mμ)	Red_G	A.V. (680 mμ)	Red_G	A.V. (680 mμ)	Red_G	A.V. (680 mμ)	Red_G
0·067	1·14	0·9	1·14	0·4	1·15	0	1·15	0·9
1·0	0·89	9·2	0·80	13·5	1·05	5·4	1·10	4·0
4·0	0·38	28·8	0·32	32·9	0·80	11·7	0·90	7·4
22·0	0·01	67·6	0·01	71·1	0·01	50·0	0·09	44·1

Stability of Glucamylase to Acid.—Portions (5 c.c.) of glucamylase were acidified with sulphuric acid to pH 2·04, 1·50, 1·02, and 0·44 respectively. After 12 days at 10° they were neutralised to pH 6·0 ± 0·1 with sodium hydroxide, and the activity of each determined in a standard amylose digest (cf. Table 12).

TABLE 12. *Stability of glucamylase to acid.*

	pH 2·04		pH 1·50		pH 1·02		pH 0·44	
Time (hr.)	A.V. (680 mμ)	Red_G	A.V. (680 mμ)	Red_G	A.V. (680 mμ)	Red_G	A.V. (680 mμ)	Red_G
0·067	1·11	0·4	1·09	0·9	1·12	0·9	1·12	0·9
3·0	0·76	8·1	1·09	0·7	1·11	0·9	1·11	0·7
22·0	0·06	46·8	1·10	0·4	1·13	0·7	1·12	0·4
48·0	0·01	54·8	1·06	0·3	1·07	0·4	1·05	0·3

Action of Glucamylase on Amylose.—The amylose was dried over phosphoric oxide for 16 hr. at 60°/0·002 mm. A sample was hydrolysed in 2N-sulphuric acid for 5 hr. and the reducing power determined. After a correction for the loss of glucose during hydrolysis the purity of the amylose was calculated to be 96·5%. This amylose was incorporated into a standard digest and the activity of the glucamylase determined in the usual way (see Table 13).

TABLE 13. *Action of glucamylase on amylose.*

Time (hr.)	0·026	1·0	4·0	24·0	48·0	192·0
A.V. (680 mμ)	1·33	1·01	0·45	0·02	0·01	0·01
Red_G	0	12·5	44·2	91·8	93·2	98·0

Paper-chromatographic examination of a similar digest revealed only one component, with an R_F value equal to that of glucose. This was the only sugar which could be detected throughout the action of glucamylase on amylose.

Stability of Glucose to Glucamylase Action.—A digest was prepared containing glucose (17 mg.),

M-acetate buffer (pH 4·0; 2 c.c.), and glucamylase solution (5 c.c.) in a total volume of 25 c.c. The digest was incubated at 50° and aliquot portions (2 c.c.) were withdrawn at intervals for the determination of reducing power. When these were analysed paper-chromatographically, no sugar other than glucose was detected in the digest (Table 14).

TABLE 14. *Action of glucamylase on glucose.*

Time (hr.)	0·067	0·5	2·5	18·0	42·0	66·0	138·0	210·0
Reducing power as glucose (mg.)	1·35	1·35	1·36	1·35	1·36	1·36	1·35	1·34

Amylolysis in the Presence of Glucose.—Three standard digests were prepared, and glucose (10 mg.; 25 mg.) incorporated in two of them. The action of glucamylase was then determined under these conditions (see Table 15).

TABLE 15. *Amylolysis in the presence of glucose.*

	No added glucose		Glucose (10 mg.)		Glucose (25 mg.)	
Time (hr.)	A.V. (680 mμ)	Red_G	A.V. (680 mμ)	Red_G	A.V. (680 mμ)	Red_G
0·013	1·08	2·7	1·10	3·1	1·12	3·6
1·0	0·28	37·8	0·25	41·4	0·24	39·6
3·0	0·01	94·5	0·01	96·4	0·01	94·5
25·0	0·01	99·0	0·01	98·1	0·01	98·4

Action of Mercuric Chloride on Glucamylase and α-Amylase.—Saliva (4 c.c.) was diluted with water (6 c.c.) and the mucin removed by centrifuging the mixture. The supernatant liquid was diluted to 150 c.c. The activities of the salivary α-amylase and glucamylase were determined at pH 5·0 and 37° in standard digests in the presence and absence of mercuric chloride (0·00125 mg.). Results are in Table 16.

TABLE 16. *Effect of mercuric chloride on glucamylase and α-amylase.*

	Salivary α-amylase			Glucamylase	
Time (hr.)	Without $HgCl_2$ Red_G	With $HgCl_2$ Red_G	Time (hr.)	Without $HgCl_2$ Red_G	With $HgCl_2$ Red_G
0·067	4·2	0·2	0·067	1·6	1·6
1·0	5·9	0·0	1·0	17·5	17·5
3·5	7·2	0·0	4·0	57·8	54·4
21·0	10·8	0·2	20·0	62·9	64·8
45·0	19·8	0·2			

Amylase Adsorbed on the Intracellar Nigeran.—The rupture of *A. niger* cells, for the extraction of glucamylase, releases the nigeran which, on centrifugation, is obtained as a layer on top of the cell debris. This nigeran (600 mg.) was suspended in water (15 c.c.), and a portion (2 c.c.) incorporated in a digest containing amylose (50 mg.) [in 0·08N-sodium sulphate solution (2·5 c.c.)] and M-acetate buffer (pH 4·0; 0·5 c.c.) which was incubated at 37°. Aliquot portions (30 μl.) were withdrawn at intervals and examined by paper chromatography.

The same suspension (diluted 5 times; 5 c.c.) of nigeran was incorporated in a standard amylose digest and its activity determined (Table 17) at 37°.

TABLE 17. *Action on amylase.*

Time (hr.)	0·33	1·5	2·5	4·0	8·5	12·0	24·0
A.V. (680 mμ)	1·11	1·01	0·66	0·39	0·18	0·12	0·04
% of theor. reducing power as glucose	1·0	3·3	4·3	8·25	19·6	27·0	52·9

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