128. *Enzymic Synthesis and Degradation of Starch. Part XV.* β-Amylase and the Constitution of Amylose.*

By Stanley Peat, S. J. Pirt, and W. J. Whelan.

The action of Balls's crystalline β-amylase is shown to be different from that of amorphous preparations of β-amylase inasmuch as it converts potato amylose to the extent of only 70% into maltose. Evidence is presented that a standard preparation of β-amylase from soya beans contains, in addition to true β-amylase, an enzyme (Z-enzyme) which supplements the β-amylase in effecting the complete conversion of amylose into maltose. It appears that some degree of branching occurs in potato amylose and that this branching impedes the progress of β-amylolysis. The branch links of amylose are different from the α-1 : 6-links of amylopectin and are specifically attacked by Z-enzyme which is without action on the amylopectin branch links. At pH 3·6 the action of Z-enzyme is inhibited.

The literature relating to the β-amylolysis of whole starch reveals differences of opinion as to the true limit of conversion into maltose. Thus, Hopkins, Murray, and Lockwood (*Biochem. J.*, 1946, **40**, 507) state that the arrest point for starch is 56%, whereas Myrbäck (*Adv. Carbohydrate Chem.*, 1948, **3**, 265) gives the figure of 61%. We now report a study of the action of the crystalline β-amylase of sweet-potato and of soya-bean β-amylase on starch and its components, and offer a partial explanation of the previous inconsistencies. A preliminary account of this work has been published in *Nature*, 1949, **164**, 499.

Our specimen of the crystalline β-amylase of sweet potato was the generous gift of Dr. A. K. Balls and an account of its preparation and properties is given by Balls, Walden, and Thompson (*J. Biol. Chem.*, 1948, **173**, 9).

β-Amylase is generally supposed to convert amylose completely into maltose, although conversions which are quantitatively complete have seldom been reported. In the apparent completeness of this hydrolysis lies the strongest evidence for the view that amylose possesses a linear structure, free from branch linkages such as those which impede the hydrolysis of amylopectin by β-amylase. When, however, crystalline sweet potato β-amylase was allowed to act on amylose at pH 4·8 its rapid action ceased abruptly at 70% conversion into maltose (Fig. 1). Thereafter an exceedingly slow further action was observed, the rate of which was about 0·02% of the initial rate. Measurements at successive stages showed that the enzyme lost little of its activity during the incubation (Table 3, p. 710). There remained a polysaccharide which stained blue with iodine, exhibiting peak absorption at the same wave-length as the original amylose, *i.e.*, at 640—650 mμ. Possible causes of this incomplete conversion of amylose into maltose were investigated in turn. The possibilities that maltose, a product of the reaction, inhibited further enzyme action or that the enzyme established an equilibrium between amylose and maltose were discounted by methods described in the Experimental section (cf. Table 7, p. 711).

Another possibility was that part of the amylose was protected from enzyme action by the physical change which, following K. H. Meyer *et al.* (*J. Phys. Chem.*, 1949, **53**, 319), we will describe as " ageing." Ageing may well be the initial stage in a process of aggregation which ultimately results in the amylose becoming insoluble (retrogradation), but it is important to note that " ageing " and " precipitation " are not synonymous terms. Experiments were next designed to test the possibility that the ageing of amylose was responsible for the incompleteness of β-amylolysis with the crystalline enzyme.

(1) An amylose solution was stored for 15 days at pH 6·5 and 35·4° and portions were removed at intervals for the determination of the limit of hydrolysis by crystalline β-amylase at pH 4·8. The % conversion into maltose at the arrest point shifted upwards from 67·0 to 71·5 (Table 8, p. 712); ageing of the amylose solution would have been expected to increase the resistance to β-amylase not to decrease it.

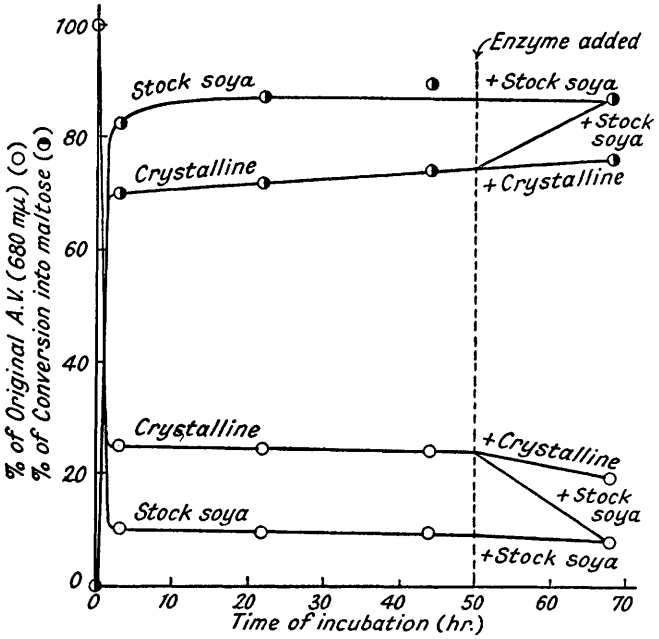
* Part XIV, *J.*, 1951, 1451.

(2) The ageing process can be reversed by treatment of an amylose solution with alkali. Amylose was therefore incubated with crystalline β-amylase at pH 4·8 until the arrest point was reached. A portion of the solution was then made alkaline (pH >12) and heated at 100° for one minute and, after adjustment of the pH to 4·8, fresh enzyme was added. The further slow action of the enzyme on the " rejuvenated " polysaccharide was indistinguishable from its action on the untreated residual polysaccharide (Table 9, p. 712).

Alternatively, Bernfeld and Gürtler (*Helv. Chim. Acta*, 1948, **31**, 106) eliminate the effects of ageing in digests by the device of a slow addition of the amylose in alkaline solution to the buffered enzyme. With a standard preparation of wheat β-amylase they observed a conversion of 100% under these conditions. When, however, we used this technique with crystalline β-amylase, the limit of conversion of the amylose remained at 72·3% (Table 10, p. 712).

(3) Meyer and Gonon (*ibid.*, 1951, **34**, 294) have shown that an increase in the amylase concentration minimises the effect of ageing. Nevertheless, we found that a nine-fold

FIG. 1. *Action of crystalline β-amylase and stock soya-bean β-amylase on amylose* (B.V. 1·24) *at* pH 4·8.



increase in the usual concentration of crystalline β-amylase did not materially influence the observed arrest point (Table 11, p. 712).

(4) The degree of ageing of an amylose sample is closely connected with the method of its preparation and storage, but it is found that the % conversion into maltose by the same sample of β-amylase does not vary from one amylose preparation to another (Part XIII, *J.*, 1951, 801) and, moreover, amyloses from different plant sources are converted to the same degree (*ca.* 70%) by a pure β-amylase (Part XVI, succeeding paper). Clearly the incompleteness of the hydrolysis of amylose by crystalline β-amylase cannot be associated with any of the recognised attributes of ageing.

(5) Finally, if the ageing of amylose were responsible for the resistance to β-amylolysis, then the addition of stock soya-bean β-amylase to an amylose already hydrolysed to the limit by crystalline β-amylase would not be expected to induce further hydrolysis. Such an addition did, however, cause the limiting conversion to increase rapidly from 70 to 86% (Fig. 1), the latter value being the normal limit of conversion of this amylose by the stock soya preparation.

We are left with the proposition that the incompleteness of the conversion by the

crystalline enzyme is due to the occurrence in amylose of structural anomalies which impede the progressive action of β-amylase along the amylose chains. In amylopectin, these " anomalies " are the branch linkages and it is reasonable to suppose that in amylose also they are glucosidic linkages different from the α-1 : 4-chain links. That these anomalous links have previously escaped detection would appear to be due to the presence in the usual β-amylase preparations of another enzyme which either hydrolyses the anomalous linkages or, like α-amylase, by-passes them and cleaves the " inner " chain-links, thereby exposing new non-reducing end groups to β-amylolytic action. A decision between these alternatives was made by a comparison of the actions of crystalline β-amylase and of a stock preparation from soya bean on amylopectin.

With potato amylopectin as substrate and at a pH of 4·8 the crystalline and stock soya-bean enzymes behaved identically; the same arrest point was observed, at 52% conversion into maltose (Table 6, p. 711). If the soya-bean preparation had contained an α-amylase capable at pH 4·8 of splitting the inner chains of amylopectin, the limiting amylolysis brought about by this preparation would have been greater than that achieved by crystalline β-amylase. The crystalline β-amylase shows the same well-defined arrest point also at pH 6·5 and 3·6 and moreover this limit of conversion is not further increased by the addition of fresh enzyme (Table 12, p. 713). On the other hand, the soya-bean preparation appears not to be quite free from α-amylase, the action of which on amylopectin becomes manifest at pH 6·5 (Table 6, p. 711), although as already stated, such action is entirely suppressed at pH 4·8.

These results with amylopectin having demonstrated that the action of the soya-bean preparation on amylose at pH 4·8 cannot be ascribed to the presence in it of α-amylase, the conclusions must be drawn (*a*) that amylose possesses a branched structure and that the branch links, although different from those in amylopectin, obstruct the action of β-amylase in the same manner and (*b*) that β-amylase as normally isolated from soya bean contains a factor capable of removing these anomalous links.

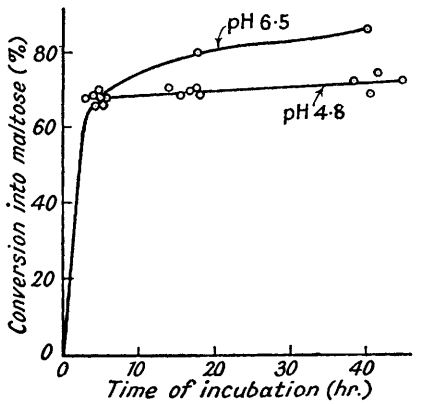
That this new factor is an enzyme was demonstrated in two ways First, its activity with respect to amylose varies with the hydrogen-ion concentration. At pH 3·6 the soya-bean preparation and the crystalline β-amylase behave identically inasmuch as hydrolysis of amylose ceases abruptly at 70% conversion in each case (Table 4, p. 710). These results suggest that the factor in the bean preparation which supplements β-amylolysis of amylose at pH 4·8 (see Fig. 1) is inactive at pH 3·6. Secondly, the factor is thermolabile. When an aqueous solution of the stock soya-bean preparation was heated at 63° for varying times and then allowed to hydrolyse amylose at pH 4·8 it was found that the degree of conversion varied inversely with the period of heating (Table 13, p. 713). Soya-bean β-amylase which had been held at 63° for 45 minutes converted only 72·5% of the amylose into maltose during 17 hours' incubation. The assumption that the factor is an enzyme is therefore justified and it will be referred to hereafter as Z-enzyme. Final proof of its enzymic nature has been obtained by its isolation from soya beans in a form which is free from α- or β-amylases and can function independently of β-amylase (Thomas, Whelan, and Peat, *Biochem. J.*, 1950, **47**, xl; Part XVII of this series).

When amylose was the substrate for crystalline β-amylase in solutions buffered to pH 3·6, 4·8, and 6·5, a clearly defined arrest point occurred in each case at a value between 67·5 and 70% conversion into maltose. Fig. 2 records the combined results of six experiments carried out at pH 4·8 and indicates the good reproducibility of the results; Table 4 (p. 710) shows that the same behaviour was apparent at pH 3·6. The rate of the end-point " drift " occurring at pH values of 3·6 and 4·8 is insignificant when compared with the rate of the initial hydrolysis. The magnitude of the " drift " at pH 6·5 is, however, more appreciable (Fig. 2) and it is suggested that this may be due to a trace of Z-enzyme in the crystalline preparation. Since the pH optimum for Z-enzyme action is 6·1 (See Part XVII) it might be expected to exert a greater effect at pH 6·5 than at pH 4·8. The results in Table 11 (p. 712) show that the renewal of enzyme in high concentration after the arrest point of β-amylase action on amylose at pH 4·8 had been attained caused some additional hydrolysis of the amylose limit β-dextrin. This did not happen at pH 3·6 (Table 4, p. 710) It appears therefore that the crystalline β-amylase, although free from α-amylase

(incidentally, we have shown it to be free also from maltase), was contaminated with a trace of Z-enzyme, the action of which became evident at pH 6·5 but not at pH 3·6 or 4·8. An attempt preferentially to destroy the impurity in the crystalline enzyme by the heat treatment described above was not successful (Table 14, p. 713), but it should be pointed out that whereas Z-enzyme could be heat-inactivated in the early stages of purification of β-amylase (Part XVI, succeeding paper), it could not be removed by this method when it was present in a more highly purified β-amylase preparation.

A cognate experiment was carried out by Potter, Hassid, and Joslyn (*J. Amer. Chem. Soc.*, 1949, **71**, 4075) who incubated amylose (from apple starch) with a high concentration of crystalline β-amylase at pH 4·7. The quantity of enzyme used is equal to 50 000 units on our scale and was, further, renewed after 24 hours. The reducing power was not measured until after further incubation for 24 hours and a conversion limit of 90%, as opposed to our 70%, was recorded. Since the amount of β-amylase added was sufficient to hydrolyse the amylose within a few minutes, it is not improbable that even a minute proportion of impurity (Z-enzyme) could considerably enhance the degree of hydrolysis at these concentrations. Experiments with unfractionated apple starch (G. J. Thomas, unpublished

FIG. 2. *Action of crystalline β-amylase on amylose* (B.V. 1·47) *at* pH 4·8 *and* pH 6·5.



results) indicate that Z-labile linkages are, in fact, present; the limiting conversions into maltose by β-amylase and β-amylase + Z-enzyme at pH 4·8 are, respectively, 54 and 60%, values which correspond closely to those obtained with whole potato starch (Table 1). Furthermore amylose preparations from three other plants all show the same definite arrest point as potato amylose when acted upon by pure β-amylase (see Part XVI).

TABLE 1. *Comparison of the actions of crystalline and stock soya-bean β-amylases on starch polysaccharides at different* pH *values.*

		Amylose (B.V. 1·47)		Potato starch (B.V. 0·470)		Amylopectin (B.V. 0·193)	
Enzyme	pH	% Convn.† at arrest point	A.V. (680 mμ) at arrest point	% Convn. at arrest point	A.V. (680 mμ) at arrest point	% Convn. at arrest point	A.V. (680 mμ) at arrest point
Crystalline	3·6	70·7	0·287 *	52·2	0·194	51·8	0·108
Stock soya	,,	69·7	0·293 *	52·3	0·205	51·8	0·103
Crystalline	4·8	67·5	0·411	53·2	0·208	52·1	0·110
Stock soya	,,	96·8	0·056	60·5	0·118	53·0	0·111
Crystalline	6·5	67·5	0·410	53·8	0·200	52·0	0·110
Stock soya	,,	93·0	0·057	—	—	>63·0 ‡	<0·09 ‡

* Amylose (B.V. 1·24). † % Convn. = % Conversion into maltose. ‡ See Table 6.

Table 1 summarises the results with respect to the action of β-amylase on potato starch and on its separated components. The values given for % conversion and A.V. (680 mμ) at the arrest points are averages of a number of experimental results.

Calculations made from the data in Table 1 indicate that whole starch may not be a

simple mixture of the amylose and the amylopectin obtained from it by the usual technique (Part XIII, *J.*, 1951, 801). In Table 2, the observed values for potato starch are contrasted with the values calculated for artificial mixtures of amylose and amylopectin in the stated proportions.

TABLE 2. *Action of β-amylase on whole potato starch.*

		Crystalline β-amylase		Stock soya-bean β-amylase	
	B.V.	% Convn.	A.V. (680 mμ)	% Convn.	A.V. (680 mμ)
Observed values † for whole starch	0·470	53·2	0·208	60·5	0·118
Calculated values for mixtures :					
Am/Ap * = 23/77	0·486	55·6	0·179	63·0	0·098
= 20/80	0·448	55·2	0·170	62·6	0·100
= 25/75	0·513	56·0	0·183	63·9	0·097

* Am/Ap = ratio of amylose to amylopectin by weight. † See Table 5.

It is clear from Table 2, that if the amylose and amylopectin used are mutually uncontaminated then the composition of starch cannot be accurately expressed in terms of two components alone (for evidence of the purity of these components see Part XIII, *J.*, 1951, 801). These results are explicable on the view that there is present in whole starch a third component which has a slightly higher blue value and a lower limit of β-amylolysis than has amylopectin. One is reminded here of the polysaccharides of structure intermediate between amylose and amylopectin, which are synthesised by mixtures of phosphorylase and Q-enzyme (Parts VIII and IX, *J.*, 1950, 3022, 3027). Lansky, Kooi, and Schoch (*J. Amer. Chem. Soc.*, 1949, **71**, 4066) have also advanced evidence for the existence in starch of from 5 to 7% of a polyglucose which is intermediate in structure between the linear and branched components.

In the succeeding communications reports are given of the properties of purified soya-bean β-amylase (the behaviour of which is identical with that of crystalline β-amylase) and also of the properties and function of Z-enzyme. Although we have provided evidence that ageing of amylose is not primarily responsible for the incompleteness (70%) of the conversion of amylose into maltose by pure β-amylase, we have been careful to employ conditions of β-amylase action under which ageing does not normally occur. We have found that amylose can be caused to age under appropriate conditions which will be defined in a later paper, and that when this happens the limit of conversion by pure β-amylase may be as low as 40%. This change is reversed by alkali treatment, the conversion then returning to the normal limit of not more than 70%. Two effects may therefore be responsible for the incompleteness of the hydrolysis of amylose by β-amylase, first, the existence in amylose of anomalous linkages, and secondly, the apparently physical change in the amylose described as ageing.

EXPERIMENTAL

Analytical Methods.—For the methods used in determination of iodine stains of polysaccharides see Bourne, Haworth, Macey, and Peat (*J.*, 1945, 924). The standard method of blue-value determination was occasionally modified by using an amylose solution containing acetate buffer (pH 4·8). Under these conditions the blue values of the purest amyloses (B.V. >1·40) were depressed by 0·05, those of less pure amyloses, *e.g.*, B.V. 1·24, were unaffected. Reducing sugar was determined by use of the Somogyi copper reagent (*J. Biol. Chem.*, 1945, **160**, 61) as in Pirt and Whelan's work (*J. Sci. Food Agric.*, 1951, **2**, 224, where the method of acid hydrolysis, which was also used in the analysis of starch polysaccharides, is described). α-Amylase activity was determined as in Part X (*J.*, 1950, 3566). In determinations of the activity of the enzyme at intervals during the β-amylolysis of a polysaccharide (Table 3) the scale of the experiment was reduced to one-tenth of that given in Part X (*J.*, 1950, 3566).

Preparation of Starch Fractions.—The amylose of B.V. 1·24 was prepared by the method of Bourne, Donnison, Peat, and Whelan (*J.*, 1949, 1). All other fractions were prepared as in Part XIII (*J.*, 1951, 801).

Preparation of Enzymes.—Stock soya-bean β-amylase, which contains Z-enzyme, was prepared as in Part II (*J.*, 1945, 882). Crystalline sweet potato β-amylase was prepared by Balls,

Walden, and Thompson (*loc. cit.*) and we express our thanks to Dr. A. K. Balls for the gift of the samples used in this work.

β-*Amylolysis of Starch Polysaccharides.*—(a) *Preparation of digests.* All digests contained the following constituents in the same relative amounts : polysaccharide (*ca.* 30 mg.), benzene (0·25 ml.), 0·25N-sodium hydroxide (10 ml.), phenolphthalein indicator (0·01%; 0·05 ml.), N-hydrochloric acid (to neutralise. the alkali), 0·2M-acetate buffer (6 ml.) or 0·053M-citrate buffer (12 ml.), β-amylase solution (0·5 ml. containing 1300 units of enzyme), and water to 50·5 ml. The digest was covered with a layer of toluene.

The dried polysaccharide was introduced into a dry 50-ml. flask and wetted with benzene, and sodium hydroxide solution was added. After shaking, dissolution was completed by heating the flask on a boiling water-bath for 3 minutes. The solution was cooled, indicator added, and the whole neutralised. Buffer solution and water were then added almost to the graduation mark. The flask was placed in the thermostat and allowed to attain the temperature of incubation (35·4°). After dilution of the solution to the mark the enzyme solution (see below) was added. Aliquot portions were removed at intervals for the determination of A.V. (680 mμ), maltose, and residual β-amylase activity. At the same time a control digest, used to determine the reducing power of the enzyme, was incubated. This digest contained all the above constituents except the polysaccharide. The control showed that only the stock soya-bean preparation possessed any reducing power. As a precaution against contamination by salivary amylase, the mouthpieces of all pipettes required for the digests were plugged with cotton wool.

(b) *Preparation of enzyme solutions.* When stock soya-bean β-amylase was used the enzyme powder was weighed into a centrifuge tube, and water (equal to half the required final volume) was added. The suspension was triturated with a glass rod and after 4—5 minutes an equal volume of the buffer solution used in the digest was added. The solutions were mixed and the sediment was removed on the centrifuge. This procedure eliminated the precipitate which formed when the enzyme preparation was buffered to an acid pH. The final enzyme concentrations of these stock solutions were 2600 units/ml. An unbuffered aqueous solution of the crystalline β-amylase was used, its concentration again being adjusted to 2600 units/ml.

(c) *Maltase activity of the enzymes.* Tests for maltase activity were made by incubating a digest as above but replacing the polysaccharide by maltose (25 mg.). Crystalline β-amylase had no action on maltose during 200 hours at pH 3·6, 4·8, 5·6, or 6·5. The specimen of stock soya-bean β-amylase used most frequently in this work did, however, cause an increase in reducing power of 2% in 87 hours at pH 4·8. Where necessary, the reducing power of a polysaccharide–β-amylase digest was corrected for this maltase activity. Tables 3—6 are self-explanatory.

Successive Actions of Crystalline and Stock Soya-bean β-Amylases on Amylose.—Two standard digests (*A* and *B*) of amylose (B.V. 1·24) were prepared and incubated respectively with crystalline and stock soya-bean β-amylases at pH 4·8. After the limiting conversion into maltose had

TABLE 3. *The stabilities of crystalline sweet-potato and stock soya-bean β-amylases at various* pH *values.*

	Crystalline β-amylase.		Stock soya-bean β-amylase.		
pH	4·8	6·5	3·6	4·8	6·5
Time (hr.)	Units of β-amylase per ml. of digest :				
0	26	26	26	26	26
5	—	—	20	—	—
24	20	16	—	18	24
48	—	9	—	13	—
72	16	—	—	10	—

TABLE 4. *Action of crystalline and stock soya-bean β-amylases on amylose* (B.V. 1·24) *at* pH 3·6.

	Crystalline β-amylase		Stock soya-bean β-amylase	
Time of incubation (hr.)	Conversion into maltose (%)	A.V. (680 mμ)	Conversion into maltose (%)	A.V. (680 mμ)
1	69·9	—	67·9	—
2	70·7	0·287	69·7	0·293
5	71·2	—	69·7	0·293
24	Enzyme added equivalent to 26 units/ml.		—	—
26	71·1	—	—	—
28	71·4	0·269	—	—

TABLE 5. *Action of crystalline and stock soya-bean β-amylases on potato starch* (B.V. 0·470) *at* pH 4·8.

Time of incubation (hr.)	Crystalline β-amylase		Stock soya-bean β-amylase	
	Conversion into maltose (%)	A.V. (680 mμ)	Conversion into maltose (%)	A.V. (680 mμ)
0·17	48·5	—	—	—
0·33	52·0	—	58·0	—
2·0	53·2	0·208	60·0	0·122
5·2	53·9	0·208	60·5	0·117
24·0	57·1	0·175	61·0	0·118
48·0	56·1	0·195	61·0	0·118

TABLE 6. *Action of crystalline and stock soya-bean β-amylases on amylopectin* (B.V. 0·193) *at* pH 3·6, 4·8, *and* 6·5.

	Time of incubation (hr.)	Crystalline β-amylase		Stock soya-bean β-amylase	
		Conversion into maltose (%)	A.V. (680 mμ)	Conversion into maltose (%)	A.V. (680 mμ)
At pH 3·6 :	1	—	—	51·5	—
	2	51·8	0·106	51·7	—
	3	—	—	51·8	0·103
	6	Enzyme added equivalent to 26 units/ml.		—	—
	6·4	51·8	0·108	—	—
At pH 4·8 :	0·17	48·0	—	51·5	—
	0·33	49·7	—	51·5	—
	2·0	52·1	0·110	52·4	0·114
	5·2	51·6	0·118	53·4	0·111
	24·0	54·0	0·104	54·8	0·112
	48·0	53·0	0·108	54·3	0·108
	72·0	54·0	0·109	54·5	0·111
At pH 6·5 :	0·5	—	—	51·2	—
	1·1	—	—	52·0	—
	2·2	—	—	54·5	0·110
	3	52·0	0·105	—	—
	24·7	—	—	62·7	0·089
	40	54·4	0·105	—	—
	48·8	—	—	69·6	0·059
	80	54·2	0·102	—	—

been attained in both digests, two portions (25 ml. each) were removed from digest *A* and were treated respectively with crystalline β-amylase (0·25 ml.; 650 units) and the equivalent amount of stock soya-bean β-amylase. To a portion (25 ml.) of digest *B* was added the same amount of the latter enzyme preparation. Further measurements of reducing power were then made (see Fig. 1).

Effect of Added Maltose or Amylose on the β-Amylolysis of Amylose.—Amylose (B.V. 1·24) was hydrolysed with crystalline β-amylase at pH 4·8 until the arrest point was attained. A portion of the digest (25 ml.) was then mixed with maltose solution (5 ml.; 51 mg.) and β-amylase solution (0·25 ml.; 990 units). The maltose concentration was thereby increased from 0·33 to 2·01 mg./ml. The lack of effect of this added maltose is shown in Table 7.

TABLE 7. *Effect of added maltose on the β-amylolysis of amylose.*

Time of incubation (hr.)	3·7	5·3	23·6	Maltose added here	27·8	29·2
Conversion into maltose (%)	66·9	67·5	67·6		68·0	68·0

In a similar manner it was shown that when amylose, instead of maltose, was added to a digest of β-amylase and amylose, in which limiting conversion had been attained, the limit of conversion of the added amylose was the same (69%).

β-Amylolysis Limits of an Amylose stored at pH 6·5.—A solution of amylose (B.V. 1·47; 0·598 mg./ml.) in citrate buffer (pH 6·5) was incubated at 35·4° under a layer of toluene. At intervals, portions of the solution were removed and incubated with crystalline β-amylase solution under standard conditions, the reducing power and A.V. (680 mμ) at the arrest point of action being determined (see Table 8).

TABLE 8. *β-Amylolysis of amylose kept at* pH 6·5 *and* 35·4°.

Age of solution (days)	At arrest point: Conversion into maltose (%)	At arrest point: A.V. (680 mμ)	Age of solution (days)	At arrest point: Conversion into maltose (%)	At arrest point: A.V. (680 mμ)
0	67·0	0·394	9	72·5	0·330
1	67·5	0·394	15	71·5	0·315
5	71·5	0·347			

Alkali-treatment of Amylose Limit β-Dextrin.—Amylose (B.V. 1·43) was hydrolysed under standard conditions by crystalline β-amylase at pH 4·8 and 35·4°, and after 2·75 hours a portion of the digest (25 ml.) was removed and N-sodium hydroxide (7·5 ml.) was added. The mixture was heated at 100° for 1 minute, then cooled, and 5N-sulphuric acid (1 ml.) added. β-Amylase solution (0·5 ml.; 650 units) was added at 3 hours from the commencement of the initial incubation and the digest was then incubated at 35·4° (see Table 9).

TABLE 9. *Alkali treatment of amylose limit β-dextrin.*

Initial β-amylolysis of amylose: Time of incubation (hr.)	Conversion into maltose (%)	A.V. (680 mμ)	β-Amylolysis of alkali-treated dextrin: Time of incubation (hr.)	Conversion into maltose (%)	A.V. (680 mμ)
0	0	1·43	0	—	0·360
0·5	58·1	—	0·25	70·1	0·355
2	69·2	0·381	1	71·0	0·348
5	69·4	0·379	21	71·7	0·341
24	70·3	0·361			

β-Amylolysis by Bernfeld and Gürtler's Method.—A solution of amylose (B.V. 1·46; 52·4 mg.) was prepared by dissolving it in 0·25N-sodium hydroxide (20 ml.) and dilution to 100 ml. A buffer solution of pH 4·52 was prepared by dissolving hydrated sodium acetate (18·5 g.) and acetic acid (10·2 ml.) in a total volume of 250 ml. The buffer solution (4 ml.), water (15·5 ml.), and crystalline β-amylase solution (0·5 ml.; 2500 units) were mixed, and alkaline amylose solution (28·08 ml.) was added at 15 drops/minute with shaking. The temperature was 23°. The addition occupied about 30 minutes and the final pH of the solution was 5·06. To complete the β-amylolysis the digest was incubated at 35·4° until the A.V. (680 mμ) attained a constant value. At this stage the conversion into maltose and residual β-amylase activity were determined (Table 10).

TABLE 10. *β-Amylolysis of amylose* (B.V. 1·46) *by Bernfeld and Gürtler's method.*

Time of incubation (hr.)	0·75	1·3	2·0
Conversion into maltose (%)	—	—	72·3
A.V. (680 mμ)	0·429	0·381	0·384
Units of β-amylase per ml. digest	—	—	33

Hydrolysis of Amylose (B.V. 1·47) *by Crystalline β-Amylase in High Concentration.*—A standard digest (50 ml.) containing amylose (B.V. 1·47) was prepared as above and to it was added 1 drop of the suspension of crystalline β-amylase (approx. 11 000 units). Two such additions were subsequently made as indicated in Table 11. Measurements of reducing power and A.V. (680 mμ) were made during the initial action, but the second and third additions of enzyme caused interference with the copper reagent and therefore only A.V. (680 mμ) was determined. The interference was not eliminated by deproteinisation (method of Somogyi, *J. Biol. Chem.*, 1945, **160**, 69).

TABLE 11. *Hydrolysis of amylose at* pH 4·8 *by crystalline β-amylase in high concentration.*

Time of incubation	Conversion into maltose (%)	A.V. (680 mμ)	Units of enzyme/ml. digest
0	0	1·47	220
2 mins.	53·6	—	—
5 ,,	61·4	—	—
11 ,,	63·0	0·420	—
1·0 hr.	66·1	0·381	—
2·7 hrs.	70·4	—	160
24·0 ,,	70·4	0·350	—
24·5 ,,		1 drop of enzyme suspension added	
25·8 ,,	—	0·327	500
26·0 ,,		1 drop of enzyme suspension added	
27·0 ,,	—	0·280	1000
50·0 ,,	—	0·217	—

A similar experiment was performed with potato amylopectin (B.V. 0·193). Fresh enzyme was added after 93 hours and occasioned no change in the degree of hydrolysis (Table 12). This quantity of enzyme was not sufficient to interfere with the measurement of reducing power.

TABLE 12. *Hydrolysis of amylopectin at* pH 4·8 *by crystalline* β*-amylase in high concentration.*

Age of digest (hr.)	13·5	18·5	35·5	84·5	93 *	110
Conversion into maltose (%)	52·0	54·2	53·4	53·4	—	53·9
A.V. (680 mμ)	—	—	0·110	0·112	—	0·110

* Enzyme added equivalent to 26 units/ml.

Amylolytic Action of Heat-treated Stock Soya-bean β*-Amylase.*—Stock soya-bean β-amylase (268 mg.) was dissolved in water (13·2 ml.), and the insoluble residue removed on the centrifuge; the enzyme concentration was approx. 1300 units/ml. Portions (3 ml.) of the solution were placed in 10-ml. centrifuge tubes and heated for varying times in a water-bath at 63° ± 1°. The tubes were cooled in cold water, toluene was added, and the solutions were stored for 4 days in the refrigerator to allow the *temporarily* inactivated β-amylase to recover its full activity (see succeeding paper, Part XVI). The β-amylase activities were measured and compared with that of an unheated specimen stored under the same conditions. Amylose (B.V. 1·24) was then digested at pH 4·8 with each of the solutions; standard digests were used and the enzyme activity in each was 28 units/ml. The small precipitates formed in the digests containing heated enzyme may have carried down some polysaccharide because the values of A.V. (680 mμ) at the arrest points (Table 13) were lower than was to be expected from the measured conversion into maltose (cf. Table 4).

TABLE 13. *Hydrolysis of amylose* (B.V. 1·24) *at* pH 4·8 *using heat-treated stock soya-bean* β*-amylase.*

Duration of heat treatment (min.)	β-Amylase activity remaining after heat treatment	Conversion of amylose after 17 hr.	A.V. (680 mμ) after 17 hr.
0	100	86·9	0·070
15	63	78·5	0·142
30	50	73·0	0·215
45	40	72·5	0·210

Heat-treatment of Crystalline β*-Amylase.*—Two portions (2·5 ml. each; 15 125 units) of crystalline β-amylase solution were heated at 59° in a water-bath for 10 and 30 minutes. They were cooled, then stored overnight in a refrigerator, and the enzyme activities remaining were found to be 66·5 and 43·9% of the original activity, respectively. The activity of a portion of the original solution stored for the same time was unchanged. This sample, and those which had been heated, were used to hydrolyse amylose (B.V. 1·47) at pH 4·8 and pH 6·5 under the standard conditions (see Table 14).

TABLE 14. *Action of heat-treated crystalline* β*-amylase on amylose* (B.V. 1·47).

	Time of incubation (hrs.)	Conversion into maltose (%)		
		Unheated enzyme	Enzyme at 59° for 10 min.	Enzyme at 59° for 30 min.
At pH 6·5	2·3	71·5	71·0	71·5
	14·0	73·3	74·3	77·5
	38·5	84·0	81·5	86·6
At pH 4·8	2·3	71·5	70·1	70·1
	14·0	71·1	67·6	70·8
	38·5	74·6	72·5	72·5

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