333. *The Enzymic Synthesis and Degradation of Starch. Part XIV. R-Enzyme.*

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An enzyme (R-enzyme), which catalyses the scission of branch links in amylopectin, has been isolated from the potato and the broad bean as a stable amorphous powder.

It is shown that R-enzyme operates by a purely hydrolytic process and that, unlike Q-enzyme, it has no link-synthesising function. It is a " debranching " enzyme with respect to the predominant type of branch linkage in amylopectin, the 1 : 6-glucosidic link. It neither makes nor breaks chain-forming 1 : 4-links and has no action on amylose (synthetic or natural) or on the linear dextrins related to amylose.

A convenient method of estimating the activity of R-enzyme is based on the fact that its action on amylopectin or limit β-dextrin is accompanied by a *rise* in the iodine-staining power of the substrate.

It has been shown in Part VII of this series (*J.*, 1950, 93) that, if the conversion of amylose into amylopectin by the agency of Q-enzyme is a reversible reaction, then at equilibrium the unbranched component must be present in a negligible proportion; in a practical sense, Q-enzyme action is irreversible. As reported in a preliminary notice (*Biochem. J.*, 1950, **47**, xxxix) we have been able to isolate from both the potato and the broad bean an enzyme (R-enzyme) which, in part, reverses the reactions catalysed by Q-enzyme. R-Enzyme is a " debranching " enzyme inasmuch as it effects the scission of the branch links in amylopectin. This appears to be a purely hydrolytic process and R-enzyme, unlike Q-enzyme, shows no synthetic activity.

R-Enzyme was first encountered during exploration of methods for the removal of the traces of α-amylolytic activity which persisted in the purest Q-enzyme preparations (see Part VII, *loc. cit.*). During this study an attempt was made, following Holmberg (*Biochem. Z.*, 1933, **258**, 134), preferentially to adsorb the α-amylase impurity on starch grains. The Q-enzyme of broad bean was isolated at the Q1-stage by the method described in Part X (*J.*, 1950, 3566) and a solution of this Q-preparation in 20% ethanol was stirred at 0° with potato starch. When treated in this way, the Q-preparation lost its α-amylolytic activity but, what was more

unexpected, its Q-enzyme activity also disappeared. Nevertheless the starch-treated solution (which was without action on amylose) contained an enzyme, which was characterised by the fact that it caused an *increase* in the blue values of limit β-dextrin (Table I) and amylopectin (Table III). This enzyme we describe, noncommittally, as R-enzyme. In our earlier work, a transitory rise in the blue value of a β-dextrin digest with Q-enzyme was occasionally observed and, after the discovery of R-enzyme, we were able to demonstrate that a Q3-preparation did contain the new enzyme. When β-dextrin was incubated with a bean Q-enzyme at an acid pH (5·2), the activity of any α-amylase was suppressed, and a definite increase in blue value was observed (Table XII).

R-Enzyme was isolated as a stable powder, the activity of which was quoted in terms of the time taken to increase the blue value of β-dextrin from 0·15 to 0·20, under standardised conditions. Its pH and temperature optima were found to be 6·5—7·0 and *ca.* 34° respectively. R-Enzyme is not a phosphorylase and its action on β-dextrin is not influenced by added phosphate (Table II) although most preparations contain traces of phosphorylase (*e.g.*, 1 unit in 430 mg.).

The data in Table III show that the increase in blue value of amylopectin (and of branched dextrins derived from it) effected by R-enzyme is coincident with an increase in the extent of conversion into maltose by β-amylase. For example, the action of R-enzyme on β-dextrin occasions a rise in the blue value from 0·145 to a constant value of 0·265 and at the same time the β-amylolysis limit increases from 2·8 to 61·6% conversion. Clearly the β-dextrin is much less highly branched (and therefore more susceptible to hydrolysis by β-amylase) after it has been acted upon by R-enzyme. This effect can only be due to the scission of the branch links in the β-dextrin and, since a large majority of these are hydrolysed by R-enzyme (Table III), it follows that they must be of the predominant 1 : 6-glucosidic type.

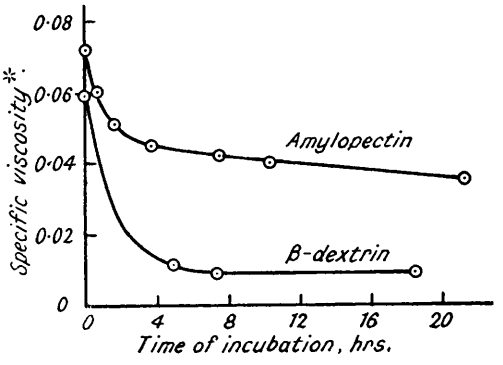
Only branched polysaccharides of the amylopectin type function as substrates for R-enzyme. Its action on an amylose of high blue value is negligible and, as can be calculated from the data in Table III, its activity with respect to whole starch or an imperfectly separated amylose is proportional to their amylopectin content. It is evident that R-enzyme, unlike Q-enzyme or the amylases, is incapable of hydrolysing 1 : 4-glucosidic chain links.

At first sight, the fact that R-enzyme effects an increase in the blue values of branched polysaccharides might be regarded as an indication of a synthetic function involving increases in chain length. If this were so, R-enzyme by cleaving 1 : 6-links and synthesising chain-forming 1 : 4-links would be exactly reversing Q-enzyme action, which is thermodynamically impossible unless a different mechanism is involved in the reversal. There is ample evidence, however, that R-enzyme does not catalyse the conversion of amylopectin into amylose, *i.e.*, reverse Q-enzyme action. (1) Although R-enzyme increases the blue value of amylopectin, the maximum blue value attained (0·25) never approaches that of amylose (1·4). (2) The conversion limit (with a stock β-amylase preparation) of the product (dextrin-R) of the action of R-enzyme on amylopectin is 66% (see Table III) whereas that of amylose is >90%. (3) Dextrin-R does not function as a substrate for the introduction of branches by Q-enzyme as is demonstrated by the negligible difference in the limits of β-amylolysis before and after Q-enzyme action (Table XI). It would seem that the linear chains (*ca.* 20 glucose units) derived from amylopectin by the debranching action of R-enzyme are too short to function as substrate for Q-enzyme (cf. Part XII, *J.*, 1951, 596). Analogous observations were made on a β-dextrin treated with R-enzyme (Table X). (4) A quantitative estimation showed that the phosphorylase-priming activity of amylopectin was essentially unchanged by treatment with R-enzyme (Table V). The priming activity of the amylopectin would have been lower if the proportion of *non-reducing* chain ends had been diminished by the synthesis of 1 : 4-links by R-enzyme. (5) A complete reversal of Q-enzyme action implies that R-enzyme effects the synthesis of a 1 : 4-link for every 1 : 6-link severed. If this were so, the proportion of *reducing* chain-ends in amylopectin or β-dextrin would remain unaltered. Actually the copper-reducing power of β-dextrin increased from 0 to 11·4% under the action of R-enzyme although the observed increase with amylopectin was much smaller (Table IV). (6) Similarly, if R-enzyme exchanges 1 : 4- for 1 : 6-linkages, then dextrin-R would have the same molecular weight as the amylopectin from which it was derived, but its structure would be more linear. It is to be expected therefore that, in solution, dextrin-R would show either the same, or a greater, viscosity, but Fig. 1 reveals that the relative viscosity of amylopectin is halved by R-enzyme action. The effect on the viscosity of β-dextrin is even more marked (85% fall). It is evident that R-enzyme does not catalyse the conversion of amylopectin into amylose and that it does not increase the length of the linear repeating chains of either amylopectin or β-dextrin. It is reasonable to conclude therefore that R-enzyme neither makes nor breaks 1 : 4-linkages.

It is important to note the bearing of this conclusion on the problem of the rise of blue value observed when R-enzyme acts on a branched amylaceous polysaccharide. Since R-enzyme does not synthesise 1 : 4-linkages, it follows that the rise in blue value is directly associated with the scission of branch linkages. In other words, the blue value of a polysaccharide composed of unbranched chains is higher than that of a structure in which chains of the same average length are mutually combined by branch links.

FIG. 1.

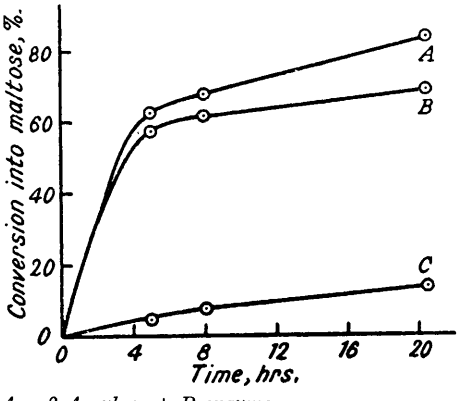
Action of R-enzyme on amylopectin and β-dextrin. Viscosity changes.



* *Relative to control digest without polysaccharide.*

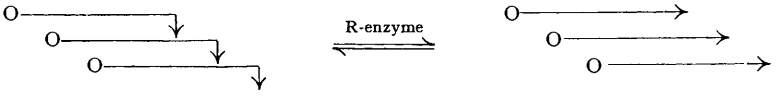
FIG. 2.

Simultaneous action of β-amylase and R-enzyme on β-dextrin.



A.—β-Amylase + R-enzyme.
B.—β-Amylase + R-enzyme, corrected for action of β-amylase alone.
C.—β-Amylase alone.

It remains to be decided whether R-enzyme catalyses a reversible reaction, *i.e.*, the synthesis as well as the scission of branch 1 : 6-linkages. It will be recalled (Table III) that only about two-thirds of the branch links in amylopectin (and slightly fewer in β-dextrin) are severed by R-enzyme. This could be caused by the establishment of an equilibrium between branched and unbranched structures of the same basal chain-length, thus



On such a view, the addition of β-amylase to the equilibrium mixture *after* the destruction of the R-enzyme by heat would result in the complete hydrolysis of the unbranched and the partial hydrolysis of the branched chains. If, however, the β-amylase were added while the R-enzyme were still present and active, the disturbance of the equilibrium, consequent upon the hydrolysis of the linear chains by the β-amylase, would result in the further debranching of the ramified structures by R-enzyme. Ultimately the whole of the β-dextrin would be converted into maltose. But this does not happen. The simultaneous action of R-enzyme and β-amylase on limit β-dextrin results in a conversion into maltose of about 60% only (Fig. 2), a limit which is essentially the same as that recorded for the successive actions of R-enzyme and β-amylase on the dextrin (Table III).

Another line of attack provided further evidence that R-enzyme is incapable of synthesising 1 : 6-linkages. A series of α-dextrins was prepared from amylose by the agency of α-amylase. These dextrins which consisted essentially of unbranched chains of widely varying lengths were submitted to the action of R-enzyme for 19 hours. Thereafter the enzyme was killed and the limit of hydrolysis of the product by β-amylase determined. Two α-dextrins, one of relatively high and one of low blue value, were used and in each case no significant difference in the degree of β-amylolysis was observed between the R-enzyme-treated dextrin and the original α-dextrin (Table VIII). In other words, R-enzyme had not effected the cross-linking of any of these linear α-dextrins. The confirmatory observation was also made that R-enzyme does not influence the shape of the curve, % fall of amylose value–% conversion into maltose, obtained

when amylose is submitted to α-amylolysis. The curves for α-amylase alone and for α-amylase and R-enzyme acting together were identical (Fig. 3).

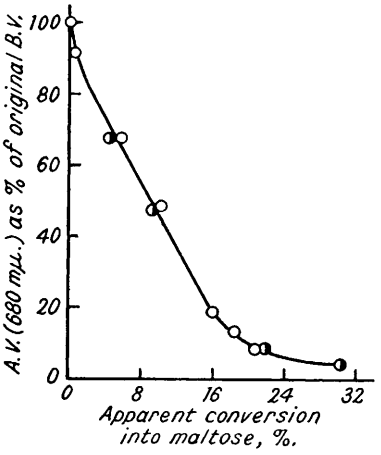
Further support came from a study of the effect of R-enzyme on the synthesis of polysaccharide from glucose-1 phosphate by the agency of phosphorylase. The development of iodine-staining power was followed quantitatively in two digests, one containing potato phosphorylase, glucose-1 phosphate, and an achroic primer, and the other containing R-enzyme in addition. The results given in Table IX show that in each case the rates of synthesis are the same and the products have practically the same blue values. Substrates with chain-lengths up to that of amylose were thus presented to R-enzyme and had it been capable of synthesising 1 : 6-linkages, a ramified structure of lower blue value would have been formed in the second digest (see above).

One question remains to be answered. Why does the debranching process initiated by R-enzyme cease before all the barriers to β-amylolysis (*i.e.*, the branch links) have been removed? This is not due to exhaustion of the R-enzyme as is shown by the fact that a digest of β-dextrin with R-enzyme which has attained maximum blue value is still active with respect to freshly added β-dextrin (Table I).

Experiments directed towards a solution of this problem are now in progress and will be

FIG. 3.

Action of R-enzyme and α-amylase on amylose.



○ *α-Amylase alone.* ◑ *R-enzyme + α-amylase.*

reported in a later communication together with an account of the isolation and the properties of the products of the action of R-enzyme on amylopectin and limit β-dextrin.

Hints have not been lacking in the recent literature of the existence of one or more polysaccharide-debranching enzymes in both plant and animal cells. K. H. Meyer and Bernfeld (*Helv. Chim. Acta*, 1942, **25**, 399) demonstrated the presence of an enzyme in yeast which degraded β-dextrin. This was regarded as a phosphorolysing enzyme. It was claimed by Petrova (*Biokhimiya*, 1948, **13**, 244; 1949, **14**, 155) that muscle contains an " amylose isomerase " which synthesises and hydrolyses polysaccharides with 1 : 6-linkages. This enzyme is probably akin to the " amylo-1 : 6-glucosidase " found in muscle by Cori and Larner (*Fed. Proc.*, 1950, **9**, No. 1). Knee and Spoerl (*Chem. Abs.*, 1949, **43**, 8407) make reference to a " limit dextrinase " which is associated with the amylases of malt in the conversion of starch into fermentable sugar. Tsuchiya, Montgomery, and Corman (*J. Amer. Chem. Soc.*, 1949, **71**, 3265) report the presence in the culture filtrate of *Aspergillus niger* of an enzyme capable of hydrolysing the α-1 : 6-glucosidic link in *iso*maltose. Maruo and Kobayashi (*J. Agr. Chem. Soc.*, 1949, **23**, 115) conclude that Nishimura's " amylosynthease " from autolysed yeast is a debranching enzyme in that it converts glutinous-rice starch into a product which is considered to have long glucose chains with fewer branching points. It is proposed by these authors (*Nature*, 1951, **167**, 606) to change the name " amylosynthease " to " iso-amylase." Finally should be mentioned the discovery of a mould dextranase which is capable of hydrolysing with equal ease almost all the 1 : 6-links in dextran (Hultin and Nortström, *Acta Chem. Scand.*, 1949, **3**, 1405) and the

" maltase " isolated from *Clostridium acetobutylicum* by French and Knapp (*J. Biol. Chem.*, 1950, **187**, 463). This maltase hydrolyses the branch links and the chain links of starch as well as the 1 : 4-linkage in maltose.

Experimental.

Analytical Methods.—For the methods used in measurement of the iodine-stains of polysaccharides, inorganic and ester phosphorus, and phosphorylase activity see Part IV of this series (*J.*, 1949, 1705). The methods of determining reducing power and β-amylase activity and of preparing starch fractions and limit β-dextrin (dextrin-A) are given in Part X (*J.*, 1950, 3566).

Preparation of R-Enzyme.—A Q1-precipitate was prepared from " Prolific Longpod " broad-bean flour (300 g.) as described in Part X. The precipitate was dissolved in ice-cold water (100 c.c.), then centrifuged to remove a little gummy residue, and to the supernatant liquid anhydrous sodium sulphate (1·0 g.), absolute ethanol (25 c.c.), and potato starch (56 g.) were added at 0° to 2°. The whole was slowly stirred at +2° for 24 hours, and the starch removed on the centrifuge in pots previously cooled to 0°. To the supernatant liquid (77 c.c.), cooled to 0° to −3°, cold acetone (154 c.c.) was added, with stirring, and the precipitate rapidly centrifuged off in pots previously cooled to <0°. The precipitate was dried by washing it in the centrifuge pots with two volumes (*ca.* 60 c.c. each) of cold acetone and finally in a vacuum-desiccator over phosphoric oxide. The dry powder (*ca.* 1·5 g.) was stored in the refrigerator. The phosphorylase activity of the preparation was equivalent to 1 unit in 430 mg. of the powder.

Determination of the Activity of R-Enzyme Preparations.—β-Dextrin (B.V. 0·145; dry wt. *ca.* 48 mg.) was moistened with ethanol, 0·012N-sodium hydroxide (15 c.c.) added, and dissolution completed by warming for 1 minute on a boiling water-bath. The solution was cooled, exactly neutralised to phenolphthalein with dilute sulphuric acid, and diluted to 25 c.c. (all subsequent polysaccharide solutions were prepared in the same manner). A portion, such that the remaining solution contained 46·7 mg. of β-dextrin, was removed, and the remainder diluted to 25 c.c.

A digest containing β-dextrin solution (3·75 c.c.), 0·2M-citrate buffer (pH 7·0; 1·5 c.c.), water (0·75 c.c.), and R-enzyme solution (0·028 g.; 1 c.c.) was incubated at 21°. At intervals portions (0·5 c.c. ≡ 0·5 mg. of β-dextrin) were removed and stained with iodine (1 mg.)–potassium iodide (10 mg.) in a final volume of 50 c.c. for determination of the blue value. The time taken for the blue value to increase from 0·150 to 0·200 was taken as a measure of the activity.

The first sample of R-enzyme retained 85% of its original activity after storage for 11 weeks.

Experiments to show that the R-Enzyme is still Active when the β-Dextrin has reached the Maximum Blue Value.—A β-dextrin digest, prepared as described above, was incubated at 21° and, at intervals, portions (0·5 c.c.) were removed for determination of the blue value, the results being shown in Table I.

Table I.

The Action of R-enzyme on β-dextrin.

Digest A. β-Dextrin + R-enzyme.
Digest B. β-Dextrin + a portion of digest A after incubation for 28 hours.

Digest A	Time of incubation (hrs.)	0	1	3	4·8	10·5	22·7	34
	Blue value	0·142	0·178	0·218	0·242	0·265	0·270	0·265
Digest B	Time of incubation (hrs.)	0	2	19·5	23·5	46·3	—	—
	A.V. (680 mμ.)	0·183	0·198	0·249	0·253	0·270	—	—

After 28·3 hours a portion (1 c.c.) of this digest was used as enzyme for a second similar digest. Portions (0·5 c.c.) of this were removed and the A.V. (680 mμ.) determined after staining with iodine (1 mg.)–potassium iodide (10 mg.) in a total volume of 50 c.c. The results are also shown in Table I.

Determination of the Optimum pH.—A freeze-dried Q3-preparation (1·11 g., isolated from 75 g. of beans) was dissolved in water (50 c.c.), and ammonium sulphate solution (50 g./100 c.c.; pH 7·0) added, at 0°, to a concentration of 19 g./100 c.c. After storage for 30 minutes at 0° the precipitate was removed in cold centrifuge pots, dissolved in water (25 c.c.) containing sodium sulphate (0·25 g.), and centrifuged. The solution was then cooled to +2°, cold absolute ethanol (6·25 c.c.) and potato starch (14 g.) were added, and the whole was slowly stirred at +2° for 20¼ hours. The starch was removed on the centrifuge, and portions (1 c.c.) of the supernatant liquid were added to digests containing β-dextrin (7 mg.; 3·75 c.c.), sodium acetate–sodium veronal buffer (1·5 c.c., the pH being adjusted to six values between 2 and 9 by the addition of 0·1N-sulphuric acid), and water (0·75 c.c.) to give a total volume of 7 c.c. The digests were incubated at 21°, and at intervals portions (1 c.c.) removed for determination of the blue values. It is thus shown that the optimum pH is 6·5—7·0.

Determination of the Optimum Temperature.—β-Dextrin digests, prepared as under " Determination of the Activity " were incubated at four temperatures between 10° and 45°. At intervals portions (1 c.c.) were removed for determination of blue values. The optimum temperature is *ca.* 34°.

Action of R-Enzyme on β-Dextrin in the Presence of Phosphate Ions.—An R-enzyme solution (140·1 mg.; 5 c.c.) was centrifuged to remove a little insoluble residue, and portions (2 c.c.) were added to two digests: (A) contained β-dextrin (14 mg.; 7·5 c.c.), 0·2M-citrate buffer (pH 7·0; 3 c.c.), and water (1·5 c.c.) to give a final volume of 14 c.c. In (B) the citrate buffer was replaced by 0·2M-phosphate buffer (pH 7·0; 3 c.c.). The digests were incubated at 21°, and at intervals portions (1 c.c.) were removed for determination of the blue values. No difference was observed (Table II).

TABLE II.

The action of R-enzyme on β-dextrin in the presence and absence of inorganic phosphate ions.

Time of incubation (hrs.).	Blue values. Phosphate buffer.	Citrate buffer.	Time of incubation (hrs.).	Blue values. Phosphate buffer.	Citrate buffer.
0	0·142	0·142	26	0·247	0·255
2	0·190	0·185	32½	0·259	0·260
6¾	0·226	0·226			

Successive Actions of R-Enzyme at pH 7·0, *and β-Amylase at* pH 4·8 *on Starch and Starch Fractions.*—β-Amylase, prepared from soya beans by the method given in Part II (*J.*, 1945, 882), was dissolved in 0·2M-acetate buffer, pH 4·8, to give an activity of 440 units/c.c., the insoluble residue being removed on a centrifuge. All solutions of β-amylase used in this work were prepared in the same manner.

A neutral solution of the polysaccharide under examination (1 mg./c.c.) was incorporated in digests as follows, all of which contained 0·2M-acetate buffer (pH 7·0; 3 c.c.):

Digest 1. Polysaccharide solution (10 c.c.); R-enzyme solution (2 c.c.; 56 mg.).

Digest 1*a*. The same.

Digest 2. Polysaccharide solution (10 c.c.); water (2 c.c.).

Digest 3. β-Dextrin (10 mg.; 10 c.c.); R-enzyme solution (2 c.c.).

The digests were incubated at 21°, and at intervals portions (0·75 c.c.) of (1*a*) and (3) were removed for determination of the blue values. At 21 hours the blue values of digests (1) and (2) were similarly determined to ensure that all the digests were proceeding at the same rate, and these digests were then heated on a boiling water-bath for 10 minutes to deactivate the enzyme. After cooling, the pH was adjusted to 4·8 by the addition of N-acetic acid (0·35 c.c.), and β-amylase solution (2 c.c.) and water to 25 c.c were then added. The digests were incubated at 35·5° and at given times portions (5 c.c.) were removed and the reducing power, as maltose, was determined after deproteinisation.

Blank digests, corresponding to (1) and (2), but containing no polysaccharide, were treated in the same way and used for the determination of the reducing power of the enzyme solutions. The results are recorded in Table III.

TABLE III.

The successive actions of R-enzyme and β-amylase on potato starch fractions.

Fraction.	Action of R-enzyme (21 hrs.) at pH 7·0. Initial B.V.	Final B.V.		Conversion into maltose by β-amylase (%) at pH 4·8. Time (hrs.). 7.	24.	33.	46.
β-Dextrin	0·145	0·265	(*a*)	2·8	5·4	6·0	7·0
			(*b*)	61·6	65·9	68·9	72·7
Amylopectin	0·160	0·246	(*a*)	48·3	51·1	52·3	53·5
			(*b*)	66·0	71·0	72·6	74·0
Starch	0·435	0·490	(*a*)	56·9	60·3	60·8	61·1
			(*b*)	71·0	77·7	78·4	79·2
Amylose I	0·910	0·860	(*a*)	66·5	70·3	71·3	71·3
			(*b*)	71·3	72·8	74·1	75·0
Amylose II	1·30	1·13	(*a*)	82·6	83·0	83·0	82·6
			(*b*)	78·4	81·0	82·7	85·3

Conversions: (*a*) Before the action of R-enzyme.
(*b*) After the action of R-enzyme.

Simultaneous Action of R-Enzyme and β-Amylase on β-Dextrin at pH 5·3.—Digests were prepared containing (1) β-dextrin (10 mg.; 10 c.c.), and β-amylase in aqueous solution (2 c.c.), (2) β-dextrin (10 c.c.), β-amylase (2 c.c.), and R-enzyme (2 c.c.; 56 mg.), and in addition both contained 0·2M-acetate buffer (pH 5·3; 3 c.c.) and water to 25 c.c. The digests were incubated at 35·5°, and at intervals the reducing power, as maltose, was determined on portions (5 c.c.) after neutralisation and deproteinisation. The results are recorded in Fig. 2.

Determination of the Reducing Power developed by R-Enzyme acting on β-Dextrin and Amylopectin.—Two digests were prepared containing (1) β-dextrin (28 mg.; 15 c.c.) and (2) amylopectin (B.V. 0·160; 28 mg.; 15 c.c.); in addition each contained R-enzyme (4 c.c.; 90·8 mg.), 0·2M-citrate buffer (pH 7·0; 6 c.c.), and water (3 c.c.).

The digests were incubated at 21°, and at intervals portions (0·5 c.c.) were removed for determination of the blue value, and further portions (4 c.c.) for determination of the reducing power as maltose after deproteinisation (Table IV). A control digest, as above, but containing water in place of the polysaccharide solution, was incubated in parallel for determination of the reducing power of the enzyme.

Change in Viscosity of β-Dextrin and Amylopectin Solutions during Incubation with R-Enzyme.—A solution of R-enzyme (27·6 mg./c.c.) was prepared, centrifuged, and placed in a thermostat at 21°. Two digests containing 0·2M-citrate buffer (pH 7·0; 1·5 c.c.), water (0·75 c.c.), and respectively amylopectin and β-dextrin (7 mg.; 3·75 c.c.) were also placed in the thermostat. When all had attained 21° the R-enzyme solution (1 c.c.) was added to the digests and, 1 minute after mixing, portions (2 c.c.) were transferred from each digest to Ostwald viscometers in the same thermostat. At intervals, portions (0·5 c.c.) were removed

from the main bulk of each digest for determination of blue value, and at the same time the time of flow of the liquid in the viscometer was observed. In addition to these digests, the times of flow of water and a control digest containing no polysaccharide were determined. See Fig. 1.

Amylopectin, before and after Incubation with R-Enzyme, as a Primer for Phosphorylase Synthesis.—Two digests were prepared both containing amylopectin (7 mg.; 3·75 c.c.), 0·2M-citrate buffer (pH 7·0; 1·5 c.c.), and water (0·75 c.c.). In addition (1) contained R-enzyme solution (29·4 mg.; 1 c.c.), and (2) water (1 c.c.). Both digests were incubated at 35·5° for 10½ hours, and a portion (0·5 c.c.) was removed from each for determination of blue value. A further portion (1 c.c.) of each was incorporated in a digest containing in addition 0·1M-glucose-1 phosphate (1 c.c.), 0·5M-citrate buffer (pH 6·0; 0·5 c.c.), and bean phosphorylase (4·4 units; 1 c.c.). The digests were incubated at 35·5° for 12 minutes, the enzyme was destroyed by the addition of 10% trichloroacetic acid (5 c.c.), the protein precipitate removed on the centrifuge, and the inorganic phosphate in a portion (1 c.c.) of the supernatant liquid determined. A control digest was used to determine the initial inorganic phosphate concentration. Table V shows the priming action to be unchanged.

TABLE IV.

The reducing power developed by R-enzyme alone acting on β-dextrin and amylopectin.

	β-Dextrin.		Amylopectin.	
Time of incubation (hrs.).	B.V.	Conversion into maltose (%).	B.V.	Conversion into maltose (%).
0	0·145	0·0	0·158	0·0
2	0·170	0·0	0·185	0·0
8	0·220	7·0	0·218	1·0
21½	0·266	11·2	0·245	1·3
25½	0·259	11·4	0·246	1·0

TABLE V.

The priming action of amylopectin in phosphorylase synthesis before and after treatment with R-enyzme.

Primer.	Inorganic phosphorus (mg.) liberated per c.c. of inactivated digest.
Amylopectin (B.V. 0·170)	0·036
Amylopectin treated with R-enzyme (B.V. 0·293)	0·030

Simultaneous Action of α-Amylase and R-Enzyme on Amylose. B.V.–% Conversion into Maltose.—A solution of amylose (B.V. 1·20) of concentration 14 mg./7·5 c.c. was incorporated in digests containing (1) amylose solution (15 c.c.) and salivary α-amylase (1 : 500 dilution; 4 c.c.), and (2) amylose solution (15 c.c.), a solution of R-enzyme (167·3 mg.), and α-amylase (4 c.c.). In addition each contained 0·2M-citrate buffer (pH 7·0; 6 c.c.) and water (3 c.c.). At intervals, portions (4 c.c.) were removed for determination of the reducing power as maltose after being deproteinised, and further portions (0·5 c.c.) for determination of blue values. Corrections were made for the intrinsic reducing powers of the enzymes. The results recorded in Fig. 3 show that R-enzyme does not influence the normal course of α-amylolysis.

Successive Actions of α-Amylase, R-Enzyme, and β-Amylase on Amylose.—Digest (1): Amylose (B.V. 1·20; 53·6 mg.) dissolved as usual; 0·2M-acetate buffer (pH 7·0; 5 c.c.); α-amylase (saliva diluted 1 : 100; 3 c.c.); and water to 50 c.c. The digest was incubated at 21° and at intervals portions (1 c.c.) were removed and stained with iodine (2 mg.)–potassium iodide (20 mg.) in a total volume of 100 c.c. for determination of the A.V. (680 mμ.). The results are given in Table VI.

TABLE VI.

Time (mins.)	0	5	17	55	75	195
A.V. (680 mμ.)	1·28 *	0·955	0·800	0·293	0·192	0·053

* Calculated value.

At the times shown in Table VII, portions (10 c.c.) were removed, placed in flasks (2), (3), (4), and (5) and heated in boiling water (10 minutes) and then cooled. To (2) and (4), 0·2M-acetate buffer (pH 7·0; 2 c.c.) and water (3 c.c.) were added, and to (3) and (5) 0·2M-acetate buffer (pH 7·0; 2 c.c.), water (1 c.c.), and R-enzyme (56 mg.; 2 c.c.). The digests were incubated at 21° for 19 hours.

TABLE VII.

Digests.	Time of removal of aliquot from digest (1) (mins.).	A.V. (680 mμ.) of digest (1).
(2) and (3)	14	0·835
(4) and (5)	65	0·237

At 19 hours, digests (2), (3), (4), and (5) were heated in boiling water (10 minutes), then cooled, and N-acetic acid (0·35 c.c. to adjust to pH to 4·8), β-amylase (2 c.c.), and water to 25 c.c. were added. The digests were incubated at 35·5°, and at intervals portions (5 c.c.) were removed for determination of the reducing power as maltose, after deproteinisation. Controls containing water in place of the polysaccharide

gave the reducing power of the enzymes alone. To ensure that the R-enzyme was fully active, it was incubated with β-dextrin. The results are recorded in Table VIII.

TABLE VIII.

The successive actions of α-amylase, R-enzyme, and β-amylase on amylose.

B.V. of dextrins produced by α-amylase at pH 7·0.		Conversion into maltose by β-amylase at pH 4·8, %. 5 hrs.	8 hrs.
0·827	*a*	78·7	81·2
	b	83·1	85·6
0·221	*a*	85·6	86·7
	b	81·8	83·1

Conversions: (*a*) Before the action of R-enzyme at pH 7·0.
(*b*) After the action of R-enzyme at pH 7·0.

Synthesis of a Polysaccharide from Glucose-1 Phosphate by Phosphorylase in the Presence of R-Enzyme.—A primer was prepared by hydrolysing amylose (B.V. 1·43; 20 mg.) with sulphuric acid to the achroic point. The solution was neutralised and diluted to 10 c.c. Two digests were prepared, both containing 0·5M-citrate buffer (pH 6·0; 2 c.c.), potato phosphorylase (P4, activity 3·3 units; 2 c.c.), primer solution (0·25 c.c.), 0·1M-glucose-1 phosphate (1 c.c.). In addition, digest (2) contained water (0·75 c.c.) and R-enzyme (28·0 mg.; 1 c.c.), and digest (1) water (1·75 c.c.). The digests were incubated at 21°, and at intervals portions (1 c.c.) stained with iodine (2 mg.)–potassium iodide (20 mg.) in a total volume of 100 c.c. for determination of A.V. The results are recorded in Table IX.

Successive Actions of R-Enzyme, Potato Q-Enzyme, and Soya β-Amylase on β-Dextrin.—A new β-dextrin preparation was used in this experiment. The results are therefore not directly comparable with those given in Table III.

TABLE IX.

The synthesis of a polysaccharide from glucose-1 phosphate by phosphorylase in the presence of R-enzyme.

Time of incubation (hr.).	A.V. (680 mμ.). Enzymes. P- + R-.	P- alone.	incubation (hr.).	A.V. (680 mμ.) Enzymes. P- + R-.	P- alone.
1	0·148	0·134	10·7	1·48	1·48
2·5	0·512	0·496	22·7	1·67 *	1·75
4·5	0·875	0·860	34·3	1·33 *	1·62 *

* Precipitate forming.

The R-enzyme solution contained 28 mg./c.c. and the Q3-solution 109·9 mg./4·5 c.c. Digests were prepared, all of which contained β-dextrin (18·7 mg.; 10 c.c.), 0·2M-acetate buffer (pH 7·0; 3 c.c.), and water (3·7 c.c.). In addition digest (1) contained water (2 c.c.), and digests (2) and (3) R-enzyme solution (2 c.c.). The digests were incubated at 21° for 21 hours, then portions (0·5 c.c.) were removed for determination of B.V. and the enzyme was destroyed by heat. After cooling, to each was added 0·2M-acetate buffer (pH 7·0; 1 c.c.); in addition, to digest (2) was added Q-enzyme solution (2 c.c.), and to each water to 25 c.c. These digests were incubated at 21° for 6 hours and after removal of portions (0·69 c.c.) for determination of B.V., the digests were heated in boiling water for 10 minutes. The β-amylolysis limits at pH 4·8 of portions (20 c.c.) of each were determined in the usual way. The results are recorded in Table X.

TABLE X.

The successive actions of R-enzyme, Q-enzyme, and β-amylase on β-dextrin.

	B.V.	β-Amylolysis (% conversion) after: 5 hrs.	7 hrs.	16 hrs.
(*a*) β-Dextrin	0·172	4·9	4·9	4·9
(*b*) R-Treated β-dextrin	0·308	68·7	71·4	71·4
(*c*) After Q-enzyme action on (*b*)	0·207	65·9	66·8	67·7

Successive Actions of R-Enzyme, Potato Q-Enzyme, and β-Amylase on Amylopectin.—The procedure given above was followed throughout, the β-dextrin being replaced by amylopectin. The results are given in Table XI.

TABLE XI.

	B.V.	β-Amylolysis (% conversion) after: 4 hrs.	18 hrs.	20 hrs.
(*a*) Amylopectin	0·148	50·5	51·1	—
(*b*) R-Treated amylopectin	0·255	67·3	65·8	—
(*c*) After Q-enzyme action on (*b*)	0·140	61·2	63·2	63·2

Action of Bean Q-Enzyme on β-Dextrin at pH 5·2.—A freeze-dried Q3-preparation (176·4 mg.) was dissolved in 0·2M-acetate buffer (pH 4·8; 3 c.c.), and after being centrifuged a portion (2 c.c.) was added to a β-dextrin digest of the same volumes as the standard digest but containing 0·2M-acetate buffer (pH 4·8) in place of the citrate buffer. The presence of citrate buffer in the freeze-dried Q3-preparation gave a final pH value of 5·2. The digest was incubated at 21° and portions (1 c.c.) were removed at intervals for the determination of A.V. (680 mμ.). The results in Table XII show the presence of R-enzyme in the bean preparation.

TABLE XII.

The action of bean Q-enzyme on β-*dextrin at* pH 5·2.

Time of incubation (mins.)	1	30	58	109	927
A.V. (680 mμ.)	0·326	0·406	0·448	0·460 *	0·248 *

* Precipitate forming in digest.

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