292. *The Enzymic Synthesis and Degradation of Starch. Part XVIII.* The Minimum Chain-length for Q-Enzyme Action.*

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The minimum length of an unbranched amylose-type chain which functions as substrate for Q-enzyme has been determined by two methods. It is shown that the minimum length for *rapid* transglucosidation by Q-enzyme is about 40 glucose units. A slow branching action is observed when Q-enzyme acts upon substrates of shorter chain-length. Thus, a synthetic, linear, 28-unit dextrin was branched at a much slower rate than was a 58-unit dextrin.

THE plant enzyme (Q-enzyme) responsible for the synthesis of the ramified polysaccharide amylopectin is a transglucosidase (see Parts VII and XII, *J.*, 1950, 93; 1951, 596). The initial substrate for this action consists of unbranched amylose-type chains, and a proportion of the α-1 : 4-links of these chains are converted into α-1 : 6-links by the transferring action. The length of the linear chains must exceed a certain determinable minimum if the rate of branching is to be comparable with that occurring with natural amylose, the chain-length of which is *ca.* 1000 units. It was shown in Part XII (*loc. cit.*) that this minimum length was greater than 25 units since α-dextrins (from amylose) having this average length were unattacked by Q-enzyme within the normal period of completed action on amylose. This is the same molecular size as was originally postulated for "pseudo-amylose" (Part I, *J.*, 1945, 877) which was considered to be an intermediate in amylopectin synthesis. The present work is concerned with a precise determination of the minimum length of linear chain which can serve as substrate for rapid Q-enzyme action. Since the publication of our preliminary account (*Biochem. J.*, 1952, **51**, xxxiv), Nussenbaum and Hassid have reported that potato Q-enzyme is without action on linear dextrins of average chain-lengths 23, 30, and 42 glucose units, but converts a 116-unit dextrin into amylopectin (*J. Biol. Chem.*, 1952, **196**, 785).

* Part XVII, *J.*, 1952, 722.

As a preliminary to this work an investigation was made to define the pattern of action of potato phosphorylase and it was proved that the synthesis of amylose induced by this enzyme proceeds by the apposition of glucose residues (from glucose-1 phosphate) simultaneously to all the primer molecules (Bailey and Whelan, *Biochem. J.*, 1952, **51**, xxxiii). The successful separation and purification of suitable primers, the maltodextrins (Bailey, Whelan, and Peat, *J.*, 1950, 3692), thus made possible the enzymic synthesis of linear molecules of accurately known chain-length which could serve as substrates for Q-enzyme action.

One method of approach was to prepare a series of linear amylose-type molecules of known chain-length and to test each individually for susceptibility to Q-enzyme action. Alternatively, by incorporating Q-enzyme in a digest in which synthesis by phosphorylase was proceeding and comparing the course of reaction with that effected by phosphorylase acting alone, the onset of Q-enzyme action could be readily determined; the average chain-length of the substrate molecule at that point is calculated from a knowledge of the initial molar concentration of primer and the amount of phosphate liberated from the glucose-1 phosphate. The detection of Q-enzyme activity makes use of the facts that the branched product of its action has a lower intensity of iodine stain and a lower degree of β-amylolysis than has the linear substrate.

The results of experiments of the second type are shown by Fig. 1 which correlates the intensity of iodine stain (at 680 mμ) and the degree of polymerisation (equivalent to chain

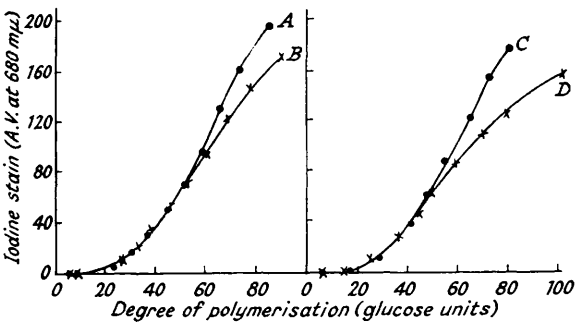


FIG. 1. *Synthesis of polysaccharides by* (1) *phosphorylase* (*curves A and C*) *and* (2) *phosphorylase and Q-enzyme* (*curves B and D*).

length in the case of unbranched polysaccharides) of polysaccharides synthesised (i) by phosphorylase and Q-enzyme acting together (curve *B*) and (ii) by phosphorylase alone (curve *A*). It will be seen that the two curves are coincident until the degree of polymerisation is greater then 49 glucose units, at which point Q-enzyme begins to synthesise branched products exhibiting a lower intensity of iodine stain than do the linear products of phosphorylase synthesis. Obviously the value, 49, represents the minimum chain-length of a linear substrate on which the branching action of Q-enzyme is detectable in this experiment. The light-absorption curves of the iodine-stained polysaccharides also confirmed that the onset of Q-enzyme action was marked by a lowering of $\lambda_{max.}$ relative to that observed in the absence of Q-enzyme. It should be reiterated that in the presence of Q-enzyme, measurements of " chain-length " beyond this point have no real significance since Q-enzyme operates against the chain-lengthening action of phosphorylase by its branching action. The numerical results of this experiment might well have depended on the relative amounts of the two enzymes present, since in the presence of an excess of phosphorylase, Q-enzyme action might not become detectable until the chain-length of the synthetic linear substrate had been appreciably increased beyond the minimum. In a second similar experiment, the ratio of Q-enzyme to phosphorylase was increased five-fold and the results confirmed the above conjecture since the apparent minimum length of chain susceptible to rapid attack by Q-enzyme was lower, namely 42 units (Fig. 1, curves *C* and *D*). Experimental difficulties prevented a higher enzyme ratio from being employed but the alternative means, described below, of following Q-enzyme action provided a more accurate estimate of this minimum chain-length for rapid branching activity. At intervals during the second experiment portions were removed from both digests, the enzymes were

inactivated by heat, and β-amylase was added. After 10 minutes' incubation the new digests were stained with iodine. The results (Fig. 2) indicated that at no stage during synthesis by phosphorylase acting alone did any iodine-staining β-dextrin remain after the subsequent action of β-amylase. When both phosphorylase and Q-enzyme were present, however, the β-amylolysis was incomplete (as indicated by the presence of iodine-staining β-dextrin) if the degree of polymerisation (calculated) of the initial linear product was greater than 40 units.

These results indicate that the minimum chain-length for *rapid* Q-enzyme action is about 40 glucose units. This does not preclude the possibility that the enzyme may exert

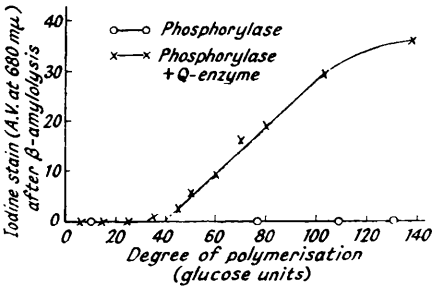
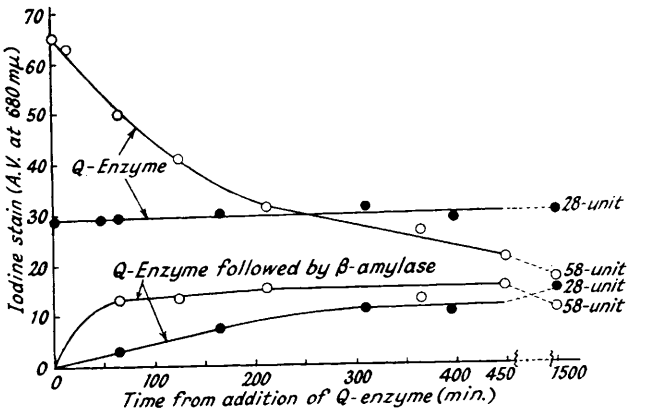


FIG. 2. *Branching by Q-enzyme.*

a slow action on shorter chains, and in fact the figure of 40 units must be an approximation since the factors governing synthesis by phosphorylase dictate that an appreciable distribution of chain-length around the average must always exist and become wider as synthesis proceeds even though the action of phosphorylase follows, as already stated, the " simultaneous " pattern. Thus with chains averaging 40 units in length the distribution,

FIG. 3. *Action of Q-enzyme on 28-unit and 58-unit synthetic maltodextrins.*



within practical limits, is of the order of ±10 units (Dr. A. W. Boyne, personal communication).

In a third approach two amylose-dextrins of average length 28 and 58 units respectively were synthesised by phosphorylase action and, after deactivation of the phosphorylase, Q-enzyme was allowed to act on the individual polysaccharides. Fig. 3 shows the progress of each reaction with respect to changes in (i) the intensity of iodine stain of the products of Q-action and (ii) the extent of production of β-limit dextrin by the successive actions of Q-enzyme and β-amylase, also measured by the iodine-staining method. With respect to the changes under (i), it will be seen that, although Q-enzyme effected a steady diminution in the intensity of stain of the 58-unit substrate, no change in that of the 28-unit dextrin took place. The latter observation would, in terms of all previous experience, have suggested that Q-enzyme had no action on the 28-unit dextrin. Nevertheless when portions of the digests were subjected to β-amylolysis it was obvious, from the persistence of β-limit dextrins, that Q-enzyme was, in fact, utilising the 28-unit as well as the 58-unit dextrin.

In a similar experiment, a comparison of the relative rates of action of Q-enzyme on the two linear polysaccharides, in terms of the initial rates of formation of β-limit dextrin (measured by iodine-staining) when Q-enzyme and β-amylase were allowed to act successively, showed that the 58-unit dextrin was branched by Q-enzyme 25 times as fast as the 28-unit dextrin. This is an indication that what appears to be the minimum chain-length value (40 units) is really the substrate length at which Q-enzyme action becomes sufficiently rapid to be perceptible under the given conditions.

A more precise formulation of the action-pattern of Q-enzyme may now be envisaged. Two possibilities have been considered. In type (i), two chain molecules take part, a " donor " chain, in which an α-1 : 4-linkage is severed, and an " acceptor " chain. A glucose member of the acceptor chain provides a primary hydroxyl group for the formation of an α-1 : 6-linkage with the reducing group liberated from the " donor " chain. Type (ii) requires only one chain and involves three stages : first, the scission of an α-1 : 4-link; secondly, the movement of the severed portion along the remainder of the original chain; and finally the establishment of an α-1 : 6-linkage between the two chain fragments. The following evidence may be cited in favour of the first mechanism. It has been shown that substances which are not, in the usual sense, substrates for Q-enzyme action may take part in the reaction. This was demonstrated for the Q-enzyme of *Polytomella coeca* by Barker, Bebbington, and Bourne (*Nature*, 1951, **168**, 834) who found that the addition of amylopectin, glycogen, dextrins, or even maltose to a digest of amylose and this Q-enzyme increased the rate of reaction. Rees and Hanes (unpublished experiments with the Q-enzyme of potato) have observed that maltodextrins appear to be incorporated in the synthetic polysaccharide if they are incubated with a mixture of the enzyme and amylose. These experiments make clear that a distinction must be drawn between the linear substrate (the " donor "), the length of which, as we have shown, must be at least 40 glucose units for rapid Q-enzyme action, and the " acceptor " molecule, the characteristics of which are differently defined, since maltose will fulfil this rôle. Obviously any molecule which can act as a donor in this sense will also function as an acceptor, but it does not follow that the reverse proposition is true.

Experimental

Analytical Methods.—(*a*) *Determination of inorganic phosphate.* Allen's method (*Biochem. J.*, 1940, **34**, 858) was used in a modified form because it was found that when inorganic phosphate was determined in presence of glucose-1 phosphate appreciable hydrolysis of the latter occurred within the period of development of the colour. This hydrolysis was almost completely eliminated by the use of 1 ml. instead of 2 ml. of " Amidol " reagent in a final volume of 25 ml., the other reagents, namely perchloric acid and ammonium molybdate, being used in the prescribed amounts. In consequence of this change the sensitivity of the method was lower by 8%. When a Spekker photometer, Ilford 608 filters, and 1-cm. cells were used, 0·1 mg. phosphorus ≡ 0·635 scale reading, the mean deviation being ±0·001. The possibility that the digest components used in polysaccharide synthesis might interfere with the determination of phosphate was tested as follows. The free phosphate in a solution of potassium phosphate (P, 0·1 mg./ml.) was estimated by the modified Allen procedure with and without the addition of a boiled digest containing potato phosphorylase, glucose-1 phosphate, maltohexaose (primer), ammonium molybdate, and mercuric chloride. The results of the experiment, given in the Table, show that the digest components did not interfere with the determination of phosphate.

	Phosphate solution (ml.) :	0	0·1	0·3	0·4	0·5
		Colour intensity (" Eel " colorimeter units)				
I.	Phosphate solution	0	9·9	29·7	39·7	49·5
II.	Phosphate solution +0·2 ml. of digest	14·0	23·4	44·0	53·7	63·8
III.	(II) less 14·0	0	9·4	30·0	39·7	49·8

(*b*) *Measurement of iodine stain.* The synthetic polysaccharides were stained with a 0·2% solution of iodine in 2% potassium iodide. The light absorption at 680 mμ [A.V. (680 mμ)] was measured with the " Eel " colorimeter, and light-absorption curves with the Unicam spectrophotometer.

Preparation of Enzymes.—(a) *Phosphorylase and Q-enzyme.* The use of iron-containing vessels and apparatus was avoided and the ammonium sulphate solution was freed from iron

by heating it to 70—80°, adjusting it to pH 10 with ammonia (*d* 0·880), stirring in 1 g. of silica/l., and filtering the mixture through a Seitz filter. The pH was readjusted to 7·0 with sulphuric acid, and the salt concentration to 50 g./100 ml. The juice of Kerr's Pink potatoes was used to prepare a lead complex eluate (Part IV, *J.*, 1949, 1705) which was fractionated as follows. For phosphorylase the protein precipitated at between 19 g. and 35 g. of ammonium sulphate per 100 ml. was collected, dissolved in 100 ml. of water, and reprecipitated twice, the 19—35 g. and 22—35 g. fractions being collected successively. The final precipitate was dissolved in 0·2M-citrate buffer (20 ml.; pH 7·0), then centrifuged, and the solution freeze-dried. The yield (from 500 ml. of juice) was 5 g. and the activity 28 units/g. (Green and Stumpf, *J. Biol. Chem.*, 1942, **142**, 355). Q-Enzyme was precipitated from the lead eluate at an ammonium sulphate concentration of 19 g./100 ml., redissolved in 100 ml. of water, centrifuged, and reprecipitated at the same salt concentration. After dissolution in citrate buffer (50 ml.) the solution was freeze-dried, yielding 4·8 g. of powder from 560 ml. of juice.

(b) β-Amylase was prepared from soya beans as in Part XV (*J.*, 1952, 705).

Polysaccharide Synthesis in Presence and Absence of Q-Enzyme.—(a) *Low Q/P ratio.* Two digests were prepared containing maltohexaose primer (0·575 ml.; 2·3 mg.; prepared as in *J.*, 1953, 1293, 0·1M-glucose-1 phosphate (4 ml.; pH 7·0), 8% ammonium molybdate (0·5 ml.; see Bailey *et al.*, *Biochem. J.*, 1951, **49**, lvi), 0·2M-citrate buffer (5 ml.; pH 7·0), and phosphorylase (1 ml.; 75 mg.). The second digest contained also Q-enzyme (1 ml.; 100 mg.), and the volume of each digest was adjusted to 25 ml. with water. The phosphorylase was added last, and immediately after dilution a portion of each digest (1 ml.) was used in determination of the initial inorganic phosphate content, the enzymes being inactivated by the perchloric acid used in this determination. Prior removal of the enzyme protein by trichloroacetic acid precipitation was found to be unnecessary; the coloured solutions were very slightly turbid but a precipitate was not formed. The digests were incubated at 35° and measurements of inorganic phosphate were made at intervals by using progressively smaller aliquots. The average chain-length (in glucose units) of the polysaccharide synthesized at each stage was calculated from (i) the inorganic phosphate liberated and hence the molar amount of glucose-1 phosphate decomposed and (ii) the molar concentration of the primer, maltohexaose. Portions (0·5 ml. each) of the digests were also removed and stained with iodine solution (0·5 ml.) in a total volume of 25 ml., containing 1 drop of 6N-sulphuric acid. The experimental results are plotted in Fig. 1 (curves *A* and *B*).

(b) *High Q/P ratio.* Two digests were prepared, identical in composition with those given above except in respect of enzyme concentration. The first contained phosphorylase (1 ml.; 40 mg.), and the second, phosphorylase (0·85 ml.; 34 mg.) and Q-enzyme (1 ml.; 250 mg.). The smaller amount of phosphorylase in the second digest was to compensate for the slight phosphorylase activity of the Q-enzyme preparation, the correction being necessitated by the high Q/P ratio. The course of polysaccharide synthesis was followed as in the first experiment and, in addition, portions (1 ml. each) were incorporated in digests containing 0·2M-acetate buffer (1 ml.; pH 3·6), purified soya-bean β-amylase (0·05 ml.; 200 units), and water (3 ml.). Under these conditions phosphorylase and Q-enzyme activities were entirely suppressed and a control experiment showed that β-amylolysis of the synthetic polysaccharide was complete within 6·5 min. The digests were incubated for 15 min. at room temperature; then iodine (0·5 ml.), 6N-sulphuric acid (1 drop), and water (to 10 ml.) were added. The A.V. (680 mμ) was then measured with the " Eel " colorimeter. The results of the above experiments are plotted in Figs. 1 and 2.

Synthesis of Amyloses of Known Chain-length.—A digest was prepared containing maltohexaose primer (6·9 ml.; 27·6 mg.), 0·1M-glucose-1 phosphate (30 ml.; pH 7·0), 0·2M-citrate buffer (5 ml.; pH 7·0), 8% ammonium molybdate (3 ml.), potato phosphorylase (110 mg.), and water (to 100 ml.). The digest was incubated at 35° and portions (0·3 ml.) were used in determination of inorganic phosphate. When the calculated chain-length of the synthetic polysaccharide was approx. 30 units a portion (50 ml.) of the digest was withdrawn and the enzyme inactivated by being heated at 100° for 5 min. Determination of the inorganic phosphate content of the cooled solution showed that the polysaccharide chain-length was 28 units. When, in the remainder of the digest, the chain-length had reached approx. 60 units, a second portion (40 ml.) was withdrawn and treated similarly The resulting polysaccharide had an average chain-length of 58 units. Coagulated protein was removed on a Seitz filter. Some retrogradation occurred when the solutions were kept (within 3 hr. for the 58-unit dextrin and overnight with the 28-unit dextrin). The 58-unit polysaccharide was redissolved by treating the solution (30 ml.) with 6N-sodium hydroxide (3 ml.) and heating. The cooled solution was

neutralised with 6N-hydrochloric acid and diluted to 50 ml. with water (solution *A*). The solution (40 ml.) of the 28-unit dextrin was treated similarly (solution *B*).

Action of Q-Enzyme on the Synthetic Amyloses.—Solutions *A* and *B* were incorporated in three digests of the following composition. Each contained Q-enzyme (250 mg.) and 0·2M-citrate buffer (2 ml.; pH 7·0); in addition, digest (1) contained solution *A* (20 ml.); digest (2), solution *A* (10 ml.) + solution *B* (10 ml.); and digest (3), solution *B* (20 ml.). Each digest was diluted to 25 ml. with water and incubated at 21°. Portions [0·5 ml. of digests (1) and (2) and 1 ml. of digest (3)] were withdrawn at intervals and stained with iodine (0·5 ml.) before dilution to 25 ml. with water. The A.V. (680 mμ) of each solution was measured by using the "Eel" colorimeter. At similar intervals, portions (2 ml.) from each digest were incubated for 10 min. at room temperature with purified soya-bean β-amylase (0·01 ml.; 50 units) and 0·2M-acetate buffer (1 ml.; pH 3·6). Portions (1 ml.) were stained with iodine (0·5 ml.) in a final volume of 10 ml., and the A.V. (680 mμ) of each was measured. The results with digests (1) and (3) are plotted in Fig. 3. The relative rates of action of Q-enzyme on the two polysaccharides were more accurately compared by a repetition of the above experiment with two digests, each containing Q-enzyme (50 mg.) and 0·2M-citrate buffer (2 ml.; pH 7·0). The first contained the 28-unit dextrin (9·1 mg.), and the second the same weight of the 58-unit dextrin. The rates of formation of β-dextrin were measured from the tabulated results.

Successive actions of Q-enzyme and β-amylase on synthetic amyloses.

Period of Q-enzyme action (min.)	A.V. (680 mμ) 58-Unit dextrin	28-Unit dextrin
12	3·4	—
28	4·8	0·2
66	7·0	0·7
243	9·9	2·9

The authors are grateful to the Department of Scientific and Industrial Research for a maintenance grant to one of them (J. M. B.) and to Imperial Chemical Industries Limited for financial assistance.

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