587. *The Enzymic Synthesis and Degradation of Starch. Part XXII.* Evidence of Multiple Branching in Waxy-maize Starch. A Correction.*

By STANLEY PEAT, W. J. WHELAN, and GWEN J. THOMAS.

The yield of maltose + maltotriose obtained when waxy-maize β-dextrin is treated with R-enzyme is 12·8%. In our previous Note,[1] the yield was erroneously given as 5·3%.

Additional evidence confirms that the molecule of the β-limit dextrin, and hence that of the parent amylopectin, is multiply branched on the random pattern postulated by K. H. Meyer.

The different modes of action of R-enzyme on waxy-maize amylopectin, potato amylopectin, and animal glycogen allow the α-1 : 6-branch linkages to be classified in three groups, according to their positions in the molecule.

An enzymic method of assay of basal-chain length is described.

IN 1952 we briefly reported [1] evidence for multiple branching in waxy-maize starch, which is an amylopectin virtually free from amylose. An error made in a calculation in this paper has been discovered by Miss E. F. Neufeld (Berkeley, California) and communicated to us by Professor E. L. Hirst. It is the purpose of this paper to give publicity to the correction of this error and to report further details of the relevant experiments.

The products of the action of R-enzyme [2] on waxy-maize β-limit dextrin (4·19 g.) were separated into diffusible (1·77 g.) and non-diffusible (2·40 g.) fractions. Sub-fractionation of the former on charcoal–Celite [3] yielded maltose (0·278 g.), maltotriose (0·282 g.), and higher maltodextrins. The percentage conversion of the β-dextrin into maltose + maltotriose was erroneously calculated to be 5·3. The correct figure is 12·8%. The miscalculation does not invalidate the conclusion we reached, namely, that amylopectin is a multiply branched structure. Indeed, the true yield of maltose + maltotriose in this experiment indicates an even greater degree of multiple branching than that inferred from the figure, 5·3%.

The structural formulæ which have been proposed for amylopectin are reproduced in the Figure. Three types, *A*, *B*, and *C*, of linear " basal " chain units are distinguishable. In a type *A* chain only the reducing-end glucose unit is involved in 1 : 6-linkage; a *B* chain is linked at its reducing end to another *B* or to a *C* chain while at the same time it carries one or more basal chains as branches; the *C* chain carries the only reducing group in the molecule.

If the amylopectin molecule contains n basal chains, then the singly branched Haworth molecule (Fig. *a*) is constituted of the unique *C* chain, one *A* chain, and $(n - 2)$ *B* chains. The Staudinger formula (Fig. *c*) has one *C* chain, $(n - 1)$ *A* chains, and no *B* chains. Between these two extremes stands the Meyer formula (Fig. *b*) which depicts a ramified structure in which the branching is multiple and completely random. Such a structure contains equal numbers of *A* and *B* chains, if the single *C* chain is neglected.[4]

The yield of maltose + maltotriose liberated, as described, by the debranching action of R-enzyme on the β-limit dextrin is a measure of the proportion of *A* chains in the original amylopectin. β-Amylase degrades the *A* chains of amylopectin to two-unit or three-unit " stubs " according as the original chains contain an even or odd number of glucose units respectively.[3,5] The " stubs " are then liberated by R-enzyme as maltose or maltotriose and it is to be noted that these sugars are derived from *A* chains only (see below). Statistically it is to be expected that the numbers of " odd " and " even " *A* chains will be equal and in consequence the average length of the stubs in the β-dextrin will be 2·5 glucose units.

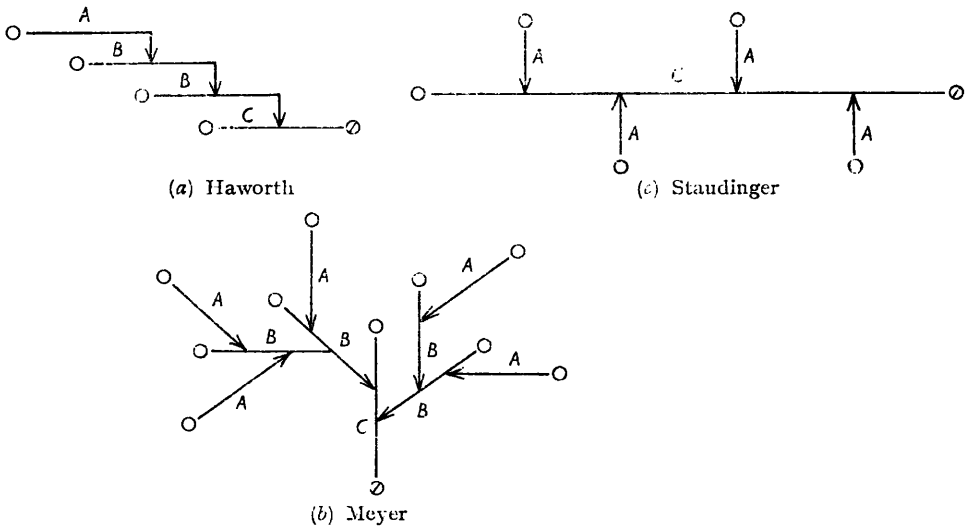
* Part XXI, *J.*, 1956, 53.

[1] Peat, Whelan, and Thomas, *J.*, 1952, 4546.
[2] Hobson, Whelan, and Peat, *J.*, 1951, 1451.
[3] Whelan, Bailey, and Roberts, *J.*, 1953, 1293.
[4] Hirst and Manners, *Chem. and Ind.*, 1954, 224.
[5] Whelan and Roberts, *Biochem. J.*, 1954, **58**, 569.

Since the average length (calculated from the proportion of non-reducing end groups) of the basal chains of the amylopectin is 24·4 and the degree of β-amylolysis is 52%, their average length in the β-dextrin is 12. In the Meyer formula of the β-dextrin the numbers of *A* and *B* chains are equal and since the average *A*-chain length is 2·5, the average *B*-chain length is 21·5 and, in terms of glucose residues, the proportion of *A* chains is (100 × 2·5/24)% = 10·4%. Similar calculation shows the Staudinger β-dextrin to contain 20·8% of *A* chains. In the Haworth molecule the proportion is vanishingly small.[1]

The actual yield of maltose + maltotriose (12·8%) eliminates the Haworth structure, leaving those of Meyer and Staudinger for consideration. The Staudinger formula cannot be rejected solely on the ground that the yield of the two sugars is less than 20·8%, since the data in the Table prove that the β-dextrin was not completely debranched. The maximum yield of maltose + maltotriose may not therefore have been obtained. Nevertheless the Staudinger formulation may be discarded since it requires that maltose and maltotriose be the only low-molecular products of R-enzyme action on the β-dextrin. It is shown below that this is not the case. The evidence therefore points to the probable correctness of the Meyer formula in the sense that approximately equal numbers of *A* and

Possible structural formulæ for amylopectin.



(*a*) Haworth (*c*) Staudinger

(*b*) Meyer

○ = Non-reducing chain end. ↓ = α-1 : 6-Link. ⊘ = Free reducing chain end.

B chains are present, as required by this structure in its completely random form.* It is to be noted, however, that there are three variables concerned in the " randomness " of the Meyer model, namely, (i) the ratio of *A* to *B* chains, (ii) the relative spacing of the branch linkages, and (iii) the relative length of the basal chains. The present proof of randomness with respect to one of these variables does not preclude a degree of orderliness in respect of the other two. Bearing on this question is the composition of the diffusible fraction of the R-treated β-dextrin. Sub-fractionation of the diffusible fraction on charcoal yielded maltose and maltotriose, as stated, but neither malto-tetraose nor -pentaose was isolated. The first sugar to be eluted after the maltotriose was maltohexaose, followed by a continuous series of higher maltodextrins, the degree of β-amylolysis of which indicated the absence of branch linkages (see Experimental section). This discontinuity in the series of diffusible products establishes the validity of the assumption that the maltose and maltotriose originated only from *A* chains. If these sugars had been formed from *B* chains it is difficult to imagine why four-unit and five-unit *B* chains should not also have

* Professor Hirst has asked us to point out that the ratio of *A* to *B* chains, calculated by the method of Hirst and Manners,[4] is approximately unity when the corrected analytical figures are used and that he and Dr. Manners accept the new figure for the degree of multiple branching in waxy-maize starch.

been liberated. We therefore conclude either that the β-dextrin contains no *B* chains shorter than six units or that such chains are present but are not liberated by R-enzyme. Taken in conjunction with the recent demonstration that the length of an outer *B* chain in a β-dextrin is either zero or one glucose unit,[6] the first alternative implies that five is the *minimum* number of glucose units interposed between pairs of C_6-substituted glucose members of different basal chains in waxy-maize amylopectin.

Steric Factors influencing R-Enzyme Action.—The degree of β-amylolysis of waxy-maize starch is 52% before, and 64% after treatment with R-enzyme, and the corresponding values for the β-dextrin are 0 and 73%. Clearly R-enzyme has a greater debranching action on the β-dextrin than on the parent amylopectin, an indication that some at least of the outer chains of the amylopectin (which are degraded by β-amylase) constitute a barrier to R-enzyme. When, however, R-enzyme and β-amylase act simultaneously on waxy-maize starch, conversion into maltose and maltotriose is complete. Incidentally, the relative yields of the two sugars enable the average basal-chain length to be calculated (see below). This contrast between the successive and the simultaneous action of R-enzyme and β-amylase is explicable if it is assumed (i) that R-enzyme, attacking the amylopectin molecule at its surface, penetrates inwards only as the surface is eroded by the removal of some of the outer branches and (ii) that a further number of branch linkages become accessible to R-enzyme when the outer chains are degraded by β-amylase. Complete debranching occurs when β-amylase and R-enzyme act simultaneously because R-enzyme exposes what were previously inner chains to the action of β-amylase which, in turn, degrades these newly exposed chains and thus allows R-enzyme to penetrate to the innermost branch linkages.

The properties of the non-diffusible fraction of R-treated β-dextrin support this view (see Table). Although the components of this fraction contain the R-resistant branch links, each was completely debranched by R-enzyme and β-amylase acting together. The less soluble part of the non-diffusible fraction (fraction 2 in the Table) was also completely debranched by the *successive* action of β-amylase and R-enzyme.

Action of R-enzyme and β-amylase on waxy-maize and potato amylopectin and their sub-fractions.

Substrate		Apparent conversion into maltose (%)		
			R-Enzyme and β-amylase	
		β-Amylase	Successive	Simultaneous
R-Treated waxy-maize β-dextrin	Diffusible fraction	87	—	—
	Non-diffusible fraction 1	61	—	93
	Non-diffusible fraction 2	65	98	97
Waxy-maize amylopectin		52	64	101
Waxy-maize β-dextrin		0	73	—
Potato amylopectin		51·5	78	86
Potato β-dextrin		0	55·5	—

The branch links in waxy-maize amylopectin therefore fall into two classes, according to their position in the molecule. The first type is directly accessible to R-enzyme; the second becomes accessible only after initial β-amylolysis. That 1 : 6-branch linkages are to be found in yet a third situation with respect to R-enzyme action is shown by the failure of this enzyme alone, or in combination with β-amylase, to attack the branch linkages of glycogen (rabbit-liver or oyster).[7] The glycogen molecule is much more compact than amylopectin, the basal chains being shorter and the branch linkages nearer together (the average basal-chain length is half that of amylopectin). Nevertheless the inaccessibility of the branch links in glycogen cannot be due entirely to the shorter distance between them, because when glycogen is fragmented by α-amylase, the fragments are readily debranched by R-enzyme, although the relative spacing is the same in the fragments as in the parent glycogen.[8]

The amylopectin of potato presents an interesting contrast to that of waxy maize.

[6] Summer and French, *Abstracts Amer. Chem. Soc. Meeting*, 1955, 36C.
[7] Peat, Whelan, Hobson, and Thomas, *J.*, 1954, 4440.
[8] Whelan and Roberts, *Nature*, 1952, **170**, 748.

Thus, the simultaneous action of R-enzyme and β-amylase completely degrades the latter polysaccharide to maltose and maltotriose whereas the conversion of the former ceases at 86% (see Table), from which it is inferred that there are resistant centres in the structure of potato amylopectin which are not present in the waxy maize. The conversion limits of the successive action of R-enzyme and β-amylase (Table) demonstrate that the same proportion of branch linkages are broken by R-enzyme in potato β-dextrin as in the amylopectin. It is evident that the outer chains of potato amylopectin do not obstruct R-enzyme action as does to some degree the superficial structure of waxy-maize amylopectin.

The resistance of the " core " of potato amylopectin to the combined action of R-enzyme and β-amylase suggests that some of the interior branch links are protected from R-enzyme action by a high degree of ramification, such as is associated with the whole molecule of glycogen. Another factor must, however, be considered. Potato amylopectin contains ester phosphate [9] and such groups are known to obstruct β-amylolysis. Nevertheless this is unlikely to be the main cause of the resistance since the phosphate content (0·1%) corresponds to only one phosphate group per 200 glucose units.

Calculation of the Basal-chain Length of Waxy-maize Starch.—By the simultaneous action of R-enzyme and β-amylase, waxy-maize starch (6·24 g.) was completely converted into maltose (5·97 g.) and maltotriose (0·358 g.), the percentage recovery of the two sugars being 96·2. Only the " odd " basal chains will yield maltotriose. If the amylopectin consists of equal numbers of chains of odd and even degree of polymerisation and if the average number of units in a basal chain is p, then 3 in every $2p$ glucose units appear as maltotriose. The ratio, yield of maltotriose : combined yield of maltose and maltotriose (in terms of glucose), is thus 3 : $2p$. The value of p so calculated, namely, 26·1, is in fair agreement with that determined by periodate oxidation (24·4).

Experimental

Analytical Methods.—These are described in Part XIX.[7] All buffers contained sodium acetate–acetic acid, and all incubations were carried out at 35°.

Unless otherwise stated, the results recorded in the Table were obtained by the following general procedures. In successive actions of R-enzyme and β-amylase, incubation with R-enzyme (2·5—5·5 mg. of enzyme per mg. of polysaccharide) was carried out at pH 7·0. The progress of reaction was followed by measurement of blue value,[11] this becoming constant in about 6—8 hr. and remaining constant overnight; the enzyme was then inactivated by heat. The pH was lowered to 6·0 and crystalline sweet-potato or purified soya-bean β-amylase (15—30 units per mg. of polysaccharide) added. The reducing powers became constant in about 6 hr. and did not change appreciably overnight. In simultaneous actions the two enzymes (concentrations as above) were incubated with the polysaccharide at pH 6·0, reducing powers becoming constant overnight. When the action of β-amylase alone was studied, the pH was 4·8. In one instance (non-diffusible fraction 2 of R-treated waxy-maize β-dextrin) the successive actions of β-amylase, R-enzyme, and β-amylase were examined. The first treatment (β-amylase) was at pH 4·8, the second and third treatments (R-enzyme, β-amylase) at pH 6·0.

Enzymes.—R-Enzymes was prepared as in Part XIX,[7] and purified soya-bean β-amylase as in Part XVI.[12]

Amylopectins.—Genetically pure waxy-maize starch was defatted with boiling 80% methanol.[13] Potato amylopectin was prepared as in Part XIII of this series.[14]

β-Dextrins.—Waxy-maize starch (11·70 g.) was treated with purified soya-bean β-amylase (10,300 units) in 0·02M-acetate buffer (pH 4·8; 1 l.) for 19 hr. The enzyme was heat-inactivated, the digest dialysed for 48 hr. against running distilled water, then concentrated, and the enzyme treatment was repeated (4120 units of enzyme; 800 ml. of digest; 0·012M-buffer); in 16 hr. the increase in reducing power corresponded to only 0·25% conversion. Dialysis of the heated digest for 144 hr. reduced the apparent maltose content to 4·8 mg./l. The dextrin was not isolated but was used as described below.

[9] Posternak, *J. Biol. Chem.*, 1951, **188**, 317.
[10] Peat, Whelan, and Bailey, *J.*, 1953, 1422.
[11] Bourne, Haworth, Macey, and Peat, *J.*, 1948, 924.
[12] Peat, Pirt, and Whelan, *J.*, 1952, 714.
[13] Schoch, *J. Amer. Chem. Soc.*, 1942, **64**, 2954.
[14] Hobson, Pirt, Whelan, and Peat, *J.*, 1951, 801.

Potato amylopectin β-dextrin was similarly prepared.

Action of R-Enzyme on Waxy-maize β-Dextrin.—A portion of the β-dextrin solution containing 4·30 g. was treated with R-enzyme 1 g.) in 1 l. of 0·05M-acetate buffer (pH 7·0). The reaction was followed by staining portions (0·5 ml.) of the digest with 0·2% iodine in 2% potassium iodide solution (2·5 ml.) in 100 ml. and measuring the A.V. (680 mμ) in 4 cm. cells. The initial value of 0·281 changed to 0·441 after 23·5 hr. when more enzyme (0·36 g.) was added, the A.V. at 27·8 hr. being 0·449. Addition of a further 0·5 g. of enzyme caused the A.V. to reach 0·475 at 35·8 hr.; then all but a small portion of the digest was heated to inactivate the enzyme. The unheated portion had A.V. (680 mμ) 0·473 at 48 hr. The heated portion was diluted to 1 l., the polysaccharide content (4·205 g.) determined by acid hydrolysis, and an aliquot removed for treatment with purified soya-bean β-amylase (60 units per mg. of polysaccharide); the apparent conversion into maltose was 73% after 8 hr. The remainder was concentrated in a vacuum to 400 ml. and dialysed in Cellophane bags against five changes of aqueous 0·08M-mercuric chloride (2 l. each) for successive 24-hr. periods. The combined diffusates were concentrated to 250 ml. of solution, estimated by acid hydrolysis to contain 1·77 g. of polyglucose.

The non-diffusible part was freed from protein by coagulation at 100° for 15 min. and concentrated to 50 ml. The carbohydrate precipitate was removed, suspended in water, and freeze-dried (fraction 2; 0·954 g.; blue value, 0·320). The supernatant solution was also freeze-dried, yielding fraction 1 (1·451 g.; blue value, 0·251).

Properties of the Fractions from R-Treated Waxy-maize β-Dextrins.—Action of β-amylase. The diffusible fraction was treated with purified soya-bean β-amylase (6 units per mg. of polysaccharide) at pH 4·8. The percentage conversions (as maltose) were 86·5 and 87 at 5 and 22·5 hr. respectively. An 8-fold increase in the enzyme concentration raised the % conversion to 108 (constant after 97 hr.). The conversion limit of the " linear " diffusible fraction (87%) with the dilute β-amylase corresponds to the production of maltose and maltotriose in the ratio 2·22 : 1. Addition of a large amount of β-amylase causes the hydrolysis of maltotriose into maltose and glucose [3] and would raise the apparent conversion to 112%, in reasonable agreement with the experimental value of 108%.

The non-diffusible fractions were treated with crystalline β-amylase (9 units per mg. of polysaccharide) at pH 4·8. The percentage conversions recorded in the Table were those obtaining at 6 hr. (constant).

Fractionation of the Diffusible Debranched Waxy-maize β-Dextrin.—A portion of the solution of the diffusible fraction equivalent to 1·70 g. was fractionated on a charcoal–Celite column (72 × 4 cm.) as already described.[3] Elution was with water (2·2 l.), and 100 ml. fractions were collected; measuring their optical rotation in a 4 dm. tube showed that no optically active material was removed. Further elution was continued with stepwise increasing concentrations of aqueous ethanol.[3] Maltose (0·267 g.) and maltotriose (0·271 g.) were thereby obtained, the weights being estimated by evaporation of the appropriate fractions to dryness and measurement of glucose liberated after acidic hydrolysis of the products. Identification was based on the following properties. The maltose had $[\alpha]_D$ +135° in H_2O, maltotriose +160° (cf. ref. 3), and the respective reducing powers were 100% and 104% of those of authentic specimens. The maltose was not attacked by crystalline β-amylase but the maltotriose underwent 96% conversion into maltose and glucose when treated with a large amount of enzyme. The R_F values of the two sugars in butan-1-ol–acetic acid–water (4 : 1 : 5, v/v) were the same as those of authentic specimens.

Further perfusion of the column with 19%, 21%, and 23% ethanol did not elute optically active material. 30% Ethanol (2 l.) eluted 95 mg. of carbohydrate having $[\alpha]_D$ +179° in H_2O, a degree of polymerisation of 6·3 (measured by copper reducing power [3]), and a percentage conversion by β-amylase of 92 (as maltose). Paper chromatographic fractionation showed it to consist of two sugars having the R_F values of malto-hexaose and -heptaose. 40% Ethanol and 50% ethanol eluted 263 and 79 mg. respectively of carbohydrate. The degrees of polymerisation were 9·0 and 12·0.

Simultaneous Treatment of Waxy-maize Starch with R-Enzyme and β-Amylase.—Waxy-maize starch (6·67 g.) was treated with R-enzyme (275 mg.) and crystalline β-amylase (5000 units) in 1 l. of solution containing 100 ml. of 0·2M-acetate buffer (pH 7·0). The percentage conversion into maltose was 100 after 33 hr. and 101 after 46 hr. After 48 hr. the enzymes were heat-inactivated, and the solution was concentrated to about 100 ml. and then dialysed successively for 24-hr. periods against 2, 1, and 1 l. of distilled water. The combined diffusates were evaporated to dryness and made up to 100 ml. with water, and a portion (95 ml., equiv. to 6·24 g. of original starch) was fractionated on a charcoal–Celite column (100 × 4 cm.) which

was irrigated successively with water and stepwise increasing concentrations of aqueous ethanol. No glucose was obtained, but maltose was eluted with 7·5% ethanol and maltotriose with 15% ethanol. The appropriate fractions were combined, evaporated to dryness, and dissolved in water for measurement of yield (by acid hydrolysis), $[\alpha]_D$, reducing power, and β-amylolysis limits. Portions of the solutions were evaporated to dryness and the solid residues acetylated with sodium acetate–acetic anhydride. The maltotriose acetate was obtained crystalline only after fractionation on Magnesol–Celite (cf. ref. 15). The reducing powers of the two sugars were identical with those of corresponding authentic specimens. Other properties of the isolated maltose and maltotriose were: $[\alpha]_D$ in H_2O, +141°, +158°; β-amylolysis limits, 0%, 98%; m. p. of acetate 160°, 136°; $[\alpha]_D$ of acetate in $CHCl_3$, +63°, +87°.

We thank Dr. T. J. Schoch for the waxy-maize starch, Dr. A. K. Balls for the crystalline β-amylase, and the Department of Scientific and Industrial Research for a maintenance grant (to G. J. T.).

UNIVERSITY COLLEGE OF NORTH WALES, BANGOR.
THE LISTER INSTITUTE, LONDON, S.W.1.

[*Received, January 6th,* 1956.]

[15] Whelan and Roberts, *J.*, 1953, 1298.