676. *The Structure of Floridean Starch. Part II.*[1] *Enzymic Hydrolysis and Other Studies.*

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The Floridean starch of *Dilsea edulis* has been hydrolysed by β-amylase and α-amylase, in the absence and in the presence of R-enzyme. The products contain nigerose, from which it is concluded that the Floridean starch molecule contains a small proportion of α-1 : 3-links as an integral part of its structure. Evidence shows that Floridean starch is more closely related in structure to the plant amylopectins than to the animal glycogens. In particular, R-enzyme, which is without significant action on glycogens, causes debranching of Floridean starch to about the same extent as it does of waxy maize starch.

The average basal chain length of Floridean starch is found by periodate oxidation to be 15 glucose residues; comment is made on the variability of results determined by periodate oxidation methods.

The view that Floridean starch is a glucan of the amylopectin (or glycogen) type is supported by a study [1] of the di- and tri-saccharides liberated when the polysaccharide is partially hydrolysed by acid. It possesses a ramified structure in which the main chain-forming links are α-1 : 4-glucosidic, the branch links being α-1 : 6. The isolation of nigerose from the partial acid hydrolysate is a strong indication of the presence also of a small proportion of α-1 : 3-links. Account has to be taken, however, of the possibility that the nigerose had been produced by action of acid on the other sugars present and, although the nigerose was obtained in greater quantity than might be expected from reversion synthesis, further evidence was sought by a study of the hydrolysis of the starch by appropriate enzymes. It was expected that this investigation would shed light also on the question of the distribution of branch points in the Floridean starch structure, *i.e.*, the question as to whether this starch more nearly resembled glycogen or amylopectin in regard to degree of branching.

In preliminary experiments, Floridean starch (the same specimen as was used for acid hydrolysis, when digested with purified soya-bean β-amylase was converted into maltose to a limit of 42—43%. The sugar formed was shown by paper-chromatographic and by electrophoretic analysis to be primarily maltose. Nevertheless it contained a trace of a second disaccharide which had the R_F and M_G values of nigerose.

The same digest after inactivation of the β-amylase was subjected to the debranching action of R-enzyme (from broad bean [2]). It has been shown [3] that R-enzyme hydrolyses the branch links of amylopectin but those of glycogen are largely resistant to the enzyme. It became evident that at least a proportion of the branch linkages of Floridean starch were hydrolysed by the debranching enzyme when the successive actions of R-enzyme and β-amylase increased the β-amylolysis limit from 42 to 52% (see Table).

In a larger-scale experiment the starch was incubated with β-amylase until maximum conversion was attained (43%). From the digest the β-limit dextrin was isolated in good yield by removing the maltose by dialysis and precipitating the dextrin with ethanol. The β-limit dextrin ($[\alpha]_D$ +169°; B.V. 0·046) was now subjected to the successive actions of R-enzyme and β-amylase, and analysis of the digest at this second β-amylolysis limit showed that the dialysable sugar products included nigerose, the components of the mixture being identified as maltose, glucose, maltotriose, and nigerose, in diminishing order of yield.

The observation that only a trace of nigerose is liberated in the β-amylolysis of Floridean

[1] Part I, preceding paper.
[2] Hobson, Whelan, and Peat, *J.*, 1951, 1451.
[3] Peat, Whelan, Hobson, and Thomas, *J.*, 1954, 4440.

starch, suggests either that α-1 : 3-linkages do not occur in significant amounts in the starch or that the bulk of these linkages are to be found in the interior chains of the molecule. (An " interior chain " is defined as a chain, or part of a chain, between two adjacent branch points [4].) This question could be resolved by taking advantage of the facts that α-amylase, unlike β-amylase, fragments amylaceous polysaccharides by the scission of " inner " as well as " outer " α-1 : 4-linkages, and that the α-1 : 6-links in the fragments are more susceptible to the debranching action of R-enzyme than they are in the intact molecule. Accordingly, Floridean starch was digested successively with salivary α-amylase, R-enzyme, and again α-amylase. Dialysis of the final digest yielded a mixture of sugars in which, by chromatographic comparison with authentic specimens, panose, maltotriose, maltose, isomaltose, and nigerose were shown to be present. Separation of this mixture on thick filter paper gave a zone containing maltose and nigerose only. The presence of nigerose in this zone was confirmed by a comparison of R_F and M_G values with those of the authentic disaccharide. The conclusion is unavoidable that the nigerose isolated is not an artifact but that the α-1 : 3-glucosidic link is an integral, though minor, part of the structure of Floridean starch.

Comparison of Floridean starch, amylopectin (waxy maize) and animal glycogen.

	Common animal glycogens	Floridean starch	Waxy maize starch
Blue value [a]	0·02	0·065	0·16
* $\lambda_{max.}$ (mμ)	420—490 [b]	530	520 [e]
β-Amylolysis limit (% maltose)	40—50 [c]	42	52 [f]
Increase in β-amylolysis limit by R-enzyme (%)	0	10	12 [f]
Basal chain length (glucose units)	10—14 [d]	15	20 [g]

* Wavelength of peak absorption of iodine complex.

[a] Bourne, Haworth, Macey, and Peat, *J.*, 1948, 924. [b] Manners, *Adv. Carbohydrate Chem.*, 1957, **12**, 278. [c] Liddle and Manners, *J.*, 1957, 3432. [d] Abdel-Ahker and Smith, *J. Amer. Chem. Soc.*, 1951, **78**, 994. [e] Barker, Bourne, and Peat, *J.*, 1949, 1712. [f] Peat, Whelan, and Thomas, *J.*, 1956, 3026. [g] Anderson, Greenwood, and Hirst, *J.*, 1955, 225.

In the Table, Floridean starch is compared with an average glycogen on the one hand and waxy maize starch (*i.e.*, amylopectin) on the other. Too much importance can be attached to comparisons of this kind but the evidence favours the view that Floridean starch is better described as an amylopectin than as a glycogen. In particular, we draw attention to the effect of R-enzyme and to the comparison of basal chain lengths.

If susceptibility to the debranching action of R-enzyme is accepted as a criterion,[3] then Floridean starch is an amylopectin and not a glycogen. Fleming and Manners however, describe [5] an 18-unit glycogen (from rabbit liver) which is attacked by R-enzyme (β-amylolysis limit raised from 51 to 58%) and these authors express the view that " the specificity of R-enzyme is controlled not by the degree of branching in the substrate, or the exterior chain length, but by the average length of the interior chains." This minimum interior chain length is calculated to be 5 glucose units. If this view is accepted then it would follow that the average number of glucose units between branch points in the Floridean starch molecule is greater than 5 (see below). It should be mentioned that the debranching enzyme of yeast, isoamylase,[6] also raises the β-amylolysis limit of Floridean starch but this enzyme is less specific than R-enzyme inasmuch as it also has a debranching action on normal animal glycogens.

The comparative values given in the Table for basal chain length need some comment. " Basal (or ' repeating ') chain length " is a convenient term for expressing the number of monosaccharide units per non-reducing end unit in a polysaccharide molecule. Since in a large branched molecule, the number of non-reducing ends is the same as the number

[4] Manners, *Ann. Reports*, 1953, **50**, 289.

[5] Fleming and Manners, *Chem. and Ind.*, 1958, 831.

[6] Maruo and Kobayashi, *Nature*, 1951, **167**, 606; Manners and Khin Maung, *Chem. and Ind.*, 1955, 950.

of branch points, it follows that the basal chain length is a measure of the degree of branching. The value of 15 was determined by periodate oxidation of the Floridean starch under the conditions described by Peat, Whelan, and Turvey [7] and estimation of the formic acid produced. The periodate method continues to present difficulties of interpretation because the course of oxidation is strongly influenced by the experimental conditions employed. For example, the basal chain length of Floridean starch was found by Barry, Halsall, Hirst, and Jones [8] to be 18 and by O'Colla [9] to be 12. More recently, Fleming, Hirst, and Manners,[10] who find a value of 9 for the basal chain length of Floridean starch, attributed the variability of earlier results to the varying degree of completeness of oxidation achieved by different experimental methods. The position is further complicated by the ease with which over-oxidation can occur (with the consequent liberation of additional formic acid) in certain circumstances.[11] It was thus clearly desirable that comparisons should be made, when possible, under identical experimental conditions. For this reason the glycogen of fœtal sheep liver, kindly supplied by Dr. D. J. Bell, was oxidised by sodium metaperiodate by exactly the same procedure as we used in the oxidation of the Floridean starch. The basal chain length was thus estimated as 12, in close agreement with the value (13) previously reported by Bell and Manners [12] for the same specimen of glycogen. The figures (10—14) given in the Table for the basal chain lengths of animal glycogens are those of Abdel-Ahker and Smith,[13] who examined, by periodate oxidation, 37 specimens of glycogen from different sources. The limits of deviation from the average value of 12 glucose units were 10 and 14.

The value we find by periodate oxidation for the average basal chain length of Floridean starch is supported by the argument that if for R-enzyme action the minimum number of glucose residues separating adjacent branch points averages 5 (Fleming and Manners [5]), and the observed β-amylolysis limit is 42—43% then it follows that the average basal chain length of our specimen of Floridean starch is at least 15.

It is concluded that if α-1 : 3-linkages are not an integral part of the structure of Floridean starch then the isolation of nigerose means that this disaccharide is synthesized from some other sugar, not only by preparations of β-amylase but also of α-amylase and R-enzyme, a highly unlikely proposition, and secondly, that in respect of degree of branching, Floridean starch is more closely related to plant amylopectins than to animal glycogens.

Experimental

General Methods.—The methods of paper chromatography and electrophoresis were as described.[1] The determination of reducing power and of blue value, and the oxidation of polysaccharides with periodate followed the methods described by Peat *et al.*[7] Purified soya-bean β-amylase was prepared by the method of Peat *et al.*[14] and contained no detectable Z-enzyme or α-amylase. R-Enzyme, prepared from broad beans by the method of Hobson *et al.*,[2] was free from detectable amylase impurity. During enzymic hydrolysis, all digests were covered with toluene to prevent contamination with micro-organisms. The concentration of polysaccharide in a solution was determined by Pirt and Whelan's method.[15] The Floridean starch sample was that described previously.[1]

Light-absorption Curves.—The starch (3 mg.) was dissolved in warm water and cooled, and 3N-hydrochloric acid (0·5 ml.) was added. Iodine solution (2 ml., containing 2 mg. of iodine and 20 mg. of potassium iodide per ml.) was added and the solution diluted to 100 ml. The light absorption was measured in a Unicam S.P. 500 spectrophotometer in 1 cm. cells at various

[7] Peat, Whelan, and Turvey, *J.*, 1956, 2317.
[8] Barry, Halsall, Hirst, and Jones, *J.*, 1949, 1468.
[9] O'Colla, *Proc. Roy. Irish Acad.*, 1953, **55**, *B*, 321.
[10] Fleming, Hirst, and Manners, *J.*, 1956, 2831.
[11] Whelan, " Encyclopedia of Plant Physiology," Springer-Verlag, Berlin, 1956, Vol. VI, p. 164.
[12] Bell and Manners, *J.*, 1952, 3641.
[13] Abdel-Ahker and Smith, *J. Amer. Chem. Soc.*, 1951, **78**, 994.
[14] Peat, Pirt, and Whelan, *J.*, 1952, 714.
[15] Pirt and Whelan, *J. Sci. Food Agric.*, 1951, **2**, 224.

wavelengths, and the wavelength of peak absorption found by interpolation (533 mμ). Increasing the amount of iodine solution from 2 to 8 ml. did not affect the position of the peak. Two other samples of Floridean starch were also examined, one isolated by Lawley [16] and the other a gift from Professor E. L. Hirst. Both samples showed $\lambda_{max.}$ 527 mμ.

The Action of β-Amylase and R-Enzyme.—The initial digest contained Floridean starch (82 mg.), 0·2M-acetate buffer (pH 4·8; 5 ml.), and β-amylase (800 units) in 50 ml. A digest containing the reagents but without starch was used throughout as a blank. The digests were incubated at 35°, and portions (2 ml. each) examined at intervals. Between 2 and 44 hr. the reducing power remained practically constant, corresponding to a conversion into maltose of 42·1%. After 44 hr. a portion (20 ml.) of each digest was heated at 100° for 3 min. to inactivate the β-amylase, and cooled; the pH was then adjusted to 7·0 with 1N-sodium hydroxide. R-Enzyme (50 mg.; 1 ml.) was added to each digest and the volumes were adjusted to 25 ml. After incubation at 35° for 6 hr., the digests were heated at 100° for 10 min. and cooled. A portion (4 ml.) was removed from each digest for estimation of reducing power (after deproteinisation) and these indicated a conversion (as maltose) of 43·1%. The pH of a second portion (20 ml.) was adjusted to 4·8 with 1N-acetic acid, β-amylase (800 units) was added, and the digests were diluted to 25 ml. Further incubation at 35° and estimation of reducing power indicated that the conversion (as maltose) remained constant after 21 hr. at 53·8%.

Large-scale β-Amylolysis.—Floridean starch (1·26 g., corr. as $C_6H_{10}O_5$) was incubated at 35° with β-amylase (8000 units) and 0·2M-acetate buffer (pH 4·8; 30 ml.) in a total volume of 500 ml. Portions (2 ml. each) were examined at intervals for reducing power. After 6 hr. the reducing power remained constant at a conversion into maltose of 43·3%. After 24 hr. the digest was heated at 100° for 5 min., cooled, and dialysed against water (4 × 400 ml.) during 36 hr. The dialysate was concentrated to dryness at pH 6—7, the sugars extracted with 80% methanol, the extracts evaporated to dryness, and the residues examined by paper chromatography and electrophoresis (see discussion). The impermeate was concentrated to 300 ml., the concentrate poured into ethanol (1 l.), and the precipitated β-limit dextrin collected (centrifuge) washed, and dried (yield 0·64 g.). The blue value and specific optical rotation (*c* 0·16 in water) were determined. The β-limit dextrin (*ca.* 40 mg.) was incubated at 35° with R-enzyme (100 mg.; 2 ml.) and 0·2M-acetate buffer (pH 7·0; 3 ml.) in a total volume of 50 ml. After 6 hr. the digest was heated at 100° for 5 min. and then cooled, and the pH adjusted (acetic acid) to 4·8. The digest was incubated at 35° for a further 40 hr. with β-amylase (800 units). The digest was dialysed against water (4 × 100 ml.) and the sugars were isolated from the dialysate and examined as before.

Action of α-Amylase and R-Enzyme.—The α-amylase was prepared by diluting saliva with an equal volume of water and centrifuging to remove precipitated mucins. Floridean starch (200 mg.) was incubated at 35° with α-amylase (2 ml.) and 0·2M-acetate buffer (pH 7·0; 6 ml.) in a total volume of 100 ml. After 30 min. the digest was heated at 100° for 5 min., cooled, and incubated at 35° with R-enzyme (300 mg.) for 6 hr. The digest was again heated at 100° for 5 min. and then cooled, and α-amylase (5 ml.) added. After 1 hr. at 35° the digest was dialysed against water (2 × 600 ml.), and the sugars were isolated from the dialysate as before. Paper chromatography indicated the presence of maltotriose, panose, isomaltose, maltose, nigerose, and glucose. The mixture was fractionated by thick-paper chromatography in the butan-1-ol–pyridine–water solvent system in which isomaltose can be separated from maltose and nigerose.[1] The zone containing maltose and nigerose was cut out and eluted with water, and the constituent sugars examined by paper chromatography in the butan-1-ol–acetic acid–water solvent system and by electrophoresis in borate buffer.[1] In both cases a zone migrating with the same mobility as nigerose was detected in addition to the maltose zone.

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[16] Lawley, Ph.D. Thesis, Wales, 1955.