653. *The Structure of Floridean Starch. Part I. Linkage Analysis by Partial Acid Hydrolysis.*

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We have isolated the Floridean starch of *Dilsea edulis* completely free from the accompanying galactan sulphate. The starch has been submitted to partial acid hydrolysis; isolation from the hydrolysate of isomaltose and trisaccharides containing the α-1 : 6-glucosidic link demonstrates that the main branch linkage in this polysaccharide is the same as it is in amylopectin and glycogen.

Conclusive evidence is afforded of the presence in the hydrolysate, in small quantity, of nigerose. Furthermore, nigerose was detected in the partial acid hydrolysate of the trisaccharide fragments. This is strong indication that the α-1 : 3-glucosidic link is an integral part of the structure of this starch, since the nigerose is produced in greater amounts than would be expected by reversion synthesis.

THE constitution of Floridean starch, the iodophilic polysaccharide present in red algæ (*Rhodophyceae*), has been much investigated. Early workers [1,2] showed that this glucan was present in granular form in the algæ, that it was stained by iodine (red to violet), and

[1] Kylin, *Z. physiol Chem.*, 1913, **83**, 171; 1915, **94**, 337.
[2] Colin and Augier, *Compt. rend.*, 1933, **197**, 423.

was hydrolysed by malt diastase to maltose. Although some evidence suggested that the starch might contain 1 : 3- in addition to 1 : 4-linkages,[3] it was later shown that few, if any, 1 : 3-linkages were present.[4,5] That the starch did not consist entirely of α-1 : 4-linkages was indicated by the incomplete (*ca.* 50%) conversion into maltose by β-amylase.[4,6] More recently, periodate oxidation of Floridean starch has suggested that the repeating chain unit consists of 9 glucose members joined by 1 : 4-linkages and that the interchain, *i.e.*, branch, linkages are α-1 : 6-glucosidic, since isoamylase (an α-1 : 6-glucosidase from yeast) increased the β-amylolysis limit.[7]

This communication describes a structural analysis of Floridean starch by partial acid hydrolysis and identification of the oligosaccharide fragments. A preliminary account has already been published.[8]

Properties of the products of partial hydrolysis of Floridean starch.

Sugar	Yield [a] (g.)	[α]$_D$ [b]	Acetyl derivative [b] [α]$_D$	Acetyl derivative [b] m. p.
Glucose	4·97	+52·4° (52·6°)	+101·7° (101·6°)	112—113° (114°)
Maltose	1·69	+134·5 (136)	+63·1 (62·7)	158—159 (159—160)
Isomaltose	0·289	+123·8 (120—122)	+96·9 (97·8)	142—143 (143) [c]
Nigerose	0·035	+136 (136—139)	+86·6 (80—85)	147—148 (149—153) [c]
Panose	0·087	+152·5 (154)	+118·5 (120) [d]	147—148 (148—150) [c,d]
6-*O*-α-Maltosylglucose	0·079	+148·4		

[a] From 12·5 g. of Floridean starch. [b] Other reported values are given in parentheses. [c] Undepressed on admixture with an authentic specimen. [d] Panitol dodeca-acetate.

The Floridean starch, which was isolated by Lawley [6] from *Dilsea edulis*, contained a galactan sulphate. Selective precipitation [9] of the starch with iodine gave a galactan-free product but there was some loss of starch, more of which was isolated by selective precipitation of the galactan sulphate with cetyltrimethylammonium bromide. This reagent forms insoluble complexes with many acidic polysaccharides but does not precipitate neutral polysaccharides such as starch from aqueous solutions.[10] This procedure also gave a galactan-free product and the two samples were combined for subsequent examination. The product contained 92·6% of polyglucose as determined by acid hydrolysis, and paper chromatography of the hydrolysate indicated the presence of glucose only. An aqueous solution had [α]$_D$ +173° (as $C_6H_{10}O_5$, +187°), a value similar to that found by Lawley [6] but higher than other reported values (+105° to +163°),[2,3,7] and akin to that of the glycogens (184—201°).[11]

The starch was hydrolysed in 0·33N-sulphuric acid at 100° to an apparent conversion (into glucose) of 53% (130 min.), and the products were fractionated by chromatography on charcoal–Celite. The only monosaccharide eluted was glucose. The disaccharides were eluted in two major fractions, the first consisting mainly of isomaltose, which was separated from minor contaminants and characterised (see Table). The second disaccharide fraction contained much maltose but a small amount of another disaccharide was also detected. The latter was separated from maltose by use of its higher electrophoretic mobility than that of maltose in borate buffer. Glucose oligosaccharides having the reducing-end glucose unit linked at $C_{(3)}$ or $C_{(6)}$ will have [12] a higher electrophoretic mobility (M_G value) in borate buffer than have those with the end unit linked at

[3] Barry, Halsall, Hirst, and Jones, *J.*, 1949, 1468.
[4] O'Colla, *Proc. Roy. Irish Acad.*, 1953, **55**, *B*, 321.
[5] Barry, McCormick, and Mitchell, *J.*, 1954, 3692.
[6] Lawley, Ph.D. Thesis, Wales, 1955.
[7] Fleming, Hirst, and Manners, *J.*, 1956, 2831.
[8] Peat, Turvey, and Evans, *Nature*, 1957, **179**, 261.
[9] Steiner and Guthrie, *Ind. Eng. Chem. Anal. Edn.*, 1944, **16**, 736.
[10] Scott, *Chem. and Ind.*, 1955, 168.
[11] Manners and Ryley, *Biochem. J.*, 1955, **59**, 369.
[12] Foster, *Adv. Carbohydrate Chem.*, 1957, **12**, 81.

$C_{(2)}$ or $C_{(4)}$. The minor disaccharide component with high M_G value would thus appear to be either a 1 : 3- or a 1 : 6-linked diglucose. Comparison with the R_F values of isomaltose, gentiobiose, and laminaribiose made it unlikely that the diglucose was any of these. This strong indication that the minor disaccharide is nigerose was confirmed by a comparison of the properties of the sugar and its acetyl derivative with those of authentic nigerose. The yields of nigerose (34·6 mg.) and isomaltose (289 mg.) from Floridean starch (12·5 g.) are many times larger than would be expected by acid reversion from an equivalent weight of glucose (2 mg. and 7 mg., respectively).[13] However, as pointed out previously,[13] the amounts of acid reversion products arising from glucose may not be the same as those arising from a similar weight of an oligosaccharide treated under the same conditions (see later).

The trisaccharide components of the partial hydrolysate were eluted from the column in two main portions, each of which was a mixture. The first mixture migrated as a single substance with the R_F value of 4-*O*-α-isomaltosylglucose (panose) on a paper chromatogram, but was resolved into two trisaccharides by preparative paper electrophoresis in borate buffer. The component with lower M_G value was identified as panose and that with higher M_G value as 6-*O*-α-maltosylglucose (Table), both of which have been obtained by partial acid hydrolysis of potato starch and of yeast glycogen.[14,15] Confirmation of these structures was furnished by reduction with sodium borohydride, partial acid hydrolysis of the trisaccharide alcohols so produced, and chromatographic identification of the derived disaccharides and alcohols. Thus panose gave a mixture of isomaltose and maltitol as the only " disaccharides " and 6-*O*-α-maltosylglucose gave maltose and isomaltitol. The second portion of the original eluate was collected in two parts, *A* and *B*, each of which contained maltotriose, but differed in their content of other trisaccharides. Mixture *A* was separated by paper electrophoresis into two fractions having high and low M_G values (A_H and A_L respectively). Fraction A_L (150 mg.) was shown by paper chromatography to consist of sugars with the R_F value of the main constituent, maltotriose, and it was slowly hydrolysed to a mixture of maltose and glucose by β-amylase, a characteristic of maltotriose. Partial acid hydrolysis of A_L showed however that the maltotriose was not pure, since isomaltose and nigerose, in addition to maltose, were detected in the hydrolysate. The presence of isomaltose in the hydrolysate of A_L, coupled with the low M_G value of this fraction, indicated that a trisaccharide containing an α-1 : 6-linkage with an α-1 : 4-linkage at the reducing end was present in the mixture. Two trisaccharides fulfil this requirement, namely 4-*O*-α-isomaltosylglucose and 4 : 6-di-*O*-glucosylglucose. The former, panose, can be eliminated as a possibility on the grounds that this sugar has a different R_F value from that of maltotriose and would therefore not be found in the mixture A_L. Consequently it is inferred that the " branched " trisaccharide 4 : 6-di-*O*-α-glucosylglucose is a constituent of mixture A_L. Similar evidence for the presence of this trisaccharide in acid hydrolysates of potato starch has previouslv been recorded.[14] Mixture *B* was also resolved by paper electrophoresis into two fractions with high and low M_G values (B_H and B_L respectively). Fraction B_L (254 mg.) was examined by the method outlined for fraction A_L, and gave similar results except that more nigerose and less isomaltose were detected in a partial acid hydrolysate of the fraction. The derivation of significant amounts of nigerose from A_L and B_L is an indication of the presence of the trisaccharide, 4-*O*-α-nigerosylglucose, in these fractions. It is improbable that nigerose was produced by reversion synthesis from the trisaccharides during acid hydrolysis, since it has been found that the partial hydrolysis of such trisaccharides as panose and 6-*O*-α-maltosylglucose does not give rise to nigerose.

Fractions A_H and B_H were combined and examined by the same procedure. The combined fraction had an R_F value similar to that of maltotriose but had a high M_G value

[13] Peat, Whelan, Edwards, and Owen, *J.*, 1958, 586.
[14] Edwards, Ph.D. Thesis, Wales, 1955.
[15] Peat, Whelan, and Edwards, *J.*, 1955, 355.

indicating the presence of 3- or 6-linked reducing end units. The disaccharide components of the partial acid hydrolysate were maltose, isomaltose, and nigerose, the last two being present in greater amounts than was the maltose, as judged by visual examination of paper chromatograms.

The conclusion is reached that Floridean starch is structurally related to glycogen or amylopectin inasmuch as the main polymeric linkage is α-1 : 4-glucosidic and that a small proportion of α-1 : 6-linkages also occurs. The isolation of panose and 6-*O*-α-maltosylglucose, and the inferred presence of 4 : 6-di-*O*-α-glucosylglucose in the hydrolysate, support the view that the 1 : 6-linkages constitute points of branching. Furthermore, the isolation of nigerose and of trisaccharides containing α-1 : 3-linkages indicates the presence of a small, but significant, proportion of these linkages in the structure. It is to be noted that small proportions of α-1 : 3-linkages have been reported in waxy-maize starch and calf-liver glycogen.[16] It is not yet known whether the nigerose and related trisaccharides isolated could be entirely the products of acid reversion but the balance of evidence is against this view. First, no trisaccharides have been detected as reversion products when glucose is treated with acid under similar conditions, and the disaccharides produced from glucose by reversion are mixtures of α- and β-isomers whereas no saccharides containing β-linkages have been detected in this study. Secondly, as reported in the following communication, nigerose has been found also in enzymic hydrolysates of Floridean starch.

Experimental

General Methods.—Paper chromatography was conducted in the solvent systems (*a*) butan-1-ol–acetic acid–water (4 : 1 : 5; by vol.) and (*b*) butan-1-ol–pyridine–water (6 : 4 : 3; by vol.) as described by Peat *et al.*[17] With solvent (*a*) nigerose can be detected in presence of maltose and isomaltose; with (*b*) isomaltose in the presence of maltose and nigerose, and panose in the presence of maltotriose. Paper electrophoresis, determination of specific optical rotations, and partial acid hydrolysis of oligosaccharides were carried out as described by Peat *et al.*[17] The determination of the polyglucose content of the starch was as described by Pirt and Whelan.[18] Acetyl derivatives were prepared by the sodium acetate–acetic anhydride method unless otherwise stated.

Purification of Floridean Starch.—The sample (79 g.) was isolated by Lawley [6] from *Dilsea edulis* using the method of Barry *et al.*[3] As it was contaminated with a galactan, it was treated in 1 g. portions by the iodine-precipitation method.[9] Complete acid hydrolysis of the product (22 g.) gave glucose alone. The polysaccharide not precipitated by iodine was recovered by addition of N-sodium thiosulphate to remove excess of iodine and precipitation in ethanol (3 vol.); it still contained some starch as indicated by the iodine stain. This starch was isolated by adding to an aqueous solution (2% w/v), a solution of cetyltrimethylammonium bromide (4% w/v) until the point of flocculation of the complex [10] was reached. The precipitate was removed by centrifugation and the supernatant solution was freed from excess of reagent by repeated extraction with chloroform. The aqueous layer was poured into ethanol (3 vol.), and the precipitated starch washed with ethanol and ether and dried over phosphoric oxide. Complete acid hydrolysis of a small portion confirmed that the product (5 g.) contained no galactan. The combined starch fractions contained 15% of ash. Accordingly, the starch (27 g.) was dissolved in hot water (1 l.), dialysed against running water for 72 hr., recovered by pouring into ethanol (3 vol.), and dried as before. The Floridean starch (20 g.) contained 0·25% of ash, 0·24% of nitrogen, and 92·6% of polyglucose, and had $[\alpha]_D^{18}$ +173° (*c* 0·1 in water).

Partial Hydrolysis of Floridean Starch and Fractionation of the Products.—A solution of the starch (12·5 g.) in 0·33N-sulphuric acid (1·25 l.) was heated on a boiling-water bath. After 130 min., the apparent conversion into glucose was 53%, as measured by the copper-reducing power of a sample. After neutralisation (sodium hydroxide) and concentration to 800 ml., the hydrolysate was adsorbed on charcoal–Celite (112 × 5 cm.), which was eluted with water (4·4 l.), 7·5% aqueous ethanol (8·2 l.), and 15% aqueous ethanol (8 l.), in that order. The

[16] Wolfrom and Thompson, *J. Amer. Chem. Soc.*, 1956, **78**, 4116; 1957, **79**, 4214.
[17] Peat, Whelan, and Roberts, *J.*, 1957, 3916.
[18] Pirt and Whelan, *J. Sci. Food Agric.*, 1951, **2**, 224.

course of the elution was followed by collecting fractions (*ca.* 200 ml. each) and measuring their optical rotations (4 dm. tube). Fractions were combined as experience suggested and evaporated to dryness at pH 6—6·5, and the residue dissolved in hot 80% methanol, and the solution filtered and re-evaporated to dryness.

Examination of Hydrolysis Products.—(a) *Monosaccharides.* The only sugar eluted by water (fractions 10—24) was glucose (4·97 g.), identified by R_F value and by preparation of the α-acetyl derivative (acetic anhydride–perchloric acid method).

(b) *Disaccharides.* Two main products were eluted by 7·5% ethanol. The first (fractions 40—48) consisted of isomaltose with traces of glucose and maltose. Fractionation by preparative paper electrophoresis furnished pure isomaltose, a portion of which was acetylated (see Table). The second (fractions 49—64) when fractionated by preparative paper chromatography and electrophoresis furnished two sugars with the R_F and M_G values of maltose and nigerose, the latter being present mainly in the later fractions (52—64) of this batch. Each sugar was acetylated and the properties are reported in the Table.

(c) *Trisaccharides.* Two products were eluted by 15% ethanol. The first consisted mainly of a sugar with the R_F value of panose. After removal of traces of maltotriose by paper chromatography, the panose zone was resolved by preparative paper electrophoresis into zones of high and low M_G values. Partial acid hydrolysis of a small portion of each indicated that each yielded maltose and isomaltose. Reduction of small portions (7—8 mg.) with sodium borohydride,[15] followed by partial acid hydrolysis and paper-chromatographic identification of the disaccharides liberated indicated that the trisaccharide with the low M_G value gave isomaltose and maltitol, while that with the high M_G value gave maltose and isomaltitol. These are the products expected from panose and 6-*O*-α-maltosylglucose, respectively. The panose was further characterised by reduction to panitol and preparation of the acetyl derivative [15] (Table).

The second trisaccharide band was further separated into *A* (fractions 87—91) and *B* (fractions 92—99), each of which contained sugars with the R_F value of maltotriose but which were separated by paper electrophoresis into minor components, A_H and B_H, with high M_G value, and major components A_L (520 mg.) and B_L (284 mg.) of low M_G value. Portions of A_L and B_L (5 mg. each) were incubated separately with purified soya bean β-amylase [19] (4000 units) in 0·02M-acetate buffer (pH 4·8; 5 ml.) for 3 days at 35° under toluene. Paper chromatography of the hydrolysates indicated glucose and maltose in both, in addition to unchanged trisaccharide. Examination of the partial acid hydrolysate of each by paper chromatography and electrophoresis showed that the disaccharides consisted of isomaltose and nigerose in addition to the main component, maltose, and that fraction A_L gave more isomaltose than nigerose, while the reverse was true for fraction B_L. Fractions A_H and B_H were combined (37 mg.) and examined by partial acid hydrolysis. The disaccharides produced by hydrolysis again consisted of a mixture of maltose, isomaltose, and nigerose, but visual examination of the paper chromatograms indicated that isomaltose and nigerose were present in greater amounts than was the maltose.

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[19] Peat, Pirt, and Whelan, *J.*, 1952, 714.