553. α-1 : 4-*Glucosans. Part IV.* A Re-examination of the Molecular Structure of Floridean Starch.*

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The structure of Floridean starch from *Dilsea edulis* has been re-investigated. The available evidence, from periodate oxidation and enzymic degradation studies, shows that it resembles the amylopectin–glycogen class of polysaccharides. It is hydrolysed by β-amylase and does not contain 1 : 3-glucosidic linkages (cf. Barry, Halsall, Hirst, and Jones, *J.*, 1949, 1468).

The molecular structure of Floridean starch, an iodophilic glucose polysaccharide present in various red algæ, has been the subject of several investigations. Early studies by Colin *et al.*[1] showed that the glucosan was stained violet with iodine, and was dextrorotatory, and Kylin [2] found that it was degraded by a dialysed malt extract to maltose. Barry, Halsall, Hirst, and Jones [3] examined a specimen of Floridean starch (referred to below as sample I) isolated from *Dilsea edulis*; it was a glucosan, $[\alpha]_D$ +156° in H_2O, which resisted attack by crystalline sweet-potato β-amylase. On oxidation with potassium metaperiodate, one mol. of formic acid was liberated per 18 glucose residues; the observed uptake was 0·6 mol. of periodate per glucose residue, suggesting that 40% of 1 : 3-glucosidic linkages were present. A second sample of Floridean starch, also from *Dilsea*, was investigated by O'Colla.[4] This material, which was contaminated with galactan (18%), had $[\alpha]_D$ +166° in H_2O, gave 50% of maltose on treatment with wheat β-amylase, and on periodate oxidation an uptake of 0·77 mol. per glucose residue was observed: one mol. of formic acid was released per 12 glucose residues. The material was partially methylated (OMe 28·2%) and, on hydrolysis, 2 : 3 : 4 : 6-tetra- (3·3%) and 2 : 3 : 6-tri-*O*-methylglucose

* Part III, *J.*, 1955, 867.

[1] Colin and Augier, *Compt. rend.*, 1933, **197**, 423; Colin, *ibid.*, 1934, **199**, 968.
[2] Kylin, *Z. physiol. Chem.*, 1913, **83**, 171.
[3] Barry, Halsall, Hirst, and Jones, *J.*, 1949, 1468.
[4] O'Colla, *Proc. Roy. Irish Acad.*, 1953, **55**, *B*, 321.

(42%) were obtained. A third sample of Floridean starch, free from galactan, was subjected to periodate oxidation by Barry, McCormick, and Mitchell;[5] the observed periodate uptake was 0·97 mol. per glucose residue, and this figure was confirmed by analysis of the thiosemicarbazide and isoniazid derivatives of the periodate-oxidised Floridean starch. Further, an acid hydrolysate of the latter did not contain glucose (cf. Hirst, Jones, and Roudier[6]). This sample could not, therefore, contain an appreciable proportion of 1 : 3-glucosidic linkages.

The present communication is concerned with a re-investigation of sample I, and the results of a preliminary examination of two further samples of Floridean starch, which has been carried out in an attempt to resolve the discrepancies in the above investigations.

Paper chromatography of an acid hydrolysate of sample I showed the presence of glucose and a barely detectable trace of galactose. The latter is not significant since the traces of contaminating galactan can be removed on further purification. The polysaccharide failed to react with the acid resorcinol (ketose) reagent, or with the naphtharesorcinol (uronic acid) reagent. By cuprimetric titration, the reducing sugar content was 93%; the remaining constituents are inorganic material and protein, which have no structural significance. In the following section, analytical figures have been corrected for the presence of 7% of non-carbohydrate material in the sample; the small destruction of glucose (*ca.* 1—2%) during acid hydrolysis has been neglected.

An aqueous solution of sample I had $[\alpha]_D$ +163° (corr. as pure glucosan, +176°), and was stained reddish-brown with iodine; the iodine complex gave an absorption spectrum with a maximum at 500 mμ. On incubation with salivary α-amylase, a rapid decrease in iodine-staining power was observed. The apparent percentage conversion into maltose (R_m) after enzyme action had ceased was 65, the main end-product being a reducing sugar with the same paper chromatographic mobility as maltose. Under identical conditions, the R_m values for waxy maize starch and rabbit-liver glycogen were 88 and 70 respectively. A solution of crystalline sweet-potato β-amylase rapidly degraded sample I, giving 46% conversion into maltose, whilst an amorphous preparation of β-amylase gave an R_m value of 44—45%. The latter β-amylase, from soya beans, contained the Z-factor[7] (a mixture of a group-specific β-glucosidase, an endo-β-1 : 4-glucosanase, and an endo-β-1 : 3-glucosanase[8]). The barriers to β-amylase in sample I are hydrolysed by yeast *iso*amylase[9] (which hydrolyses the α-1 : 6-glucosidic interchain linkages in glycogen and amylopectin), since after incubation with *iso*amylase, a 9% increase in β-amylolysis limit was observed. Sample I is also degraded by potato phosphorylase; in the presence of inorganic phosphate, 35% of the polysaccharide was degraded, yielding glucose 1-phosphate. Oxidation of sample I by sodium metaperiodate at 1° resulted in the consumption of 1·05 mol. of periodate per glucose residue; under similar conditions, waxy maize starch consumed the normal amount of periodate (1·04 mol. per glucose residue). Determination of the maximum amount of formic acid liberated by potassium metaperiodate indicated an average chain length of nine glucose residues.

The above properties suggest, in general, that sample I belongs to the amylopectin–glycogen class of polysaccharides (see Table). Thus, enzymic degradation shows that the main repeating linkage is of the α-1 : 4-glucosidic-type, and that the molecule is branched; further, the branch points are not adjacent to the non-reducing terminal groups. Since the iodine complex showed little absorption at 620 mμ, the presence of a linear amylose-type component in the sample is excluded. The extent of periodate oxidation indicates the absence of any appreciable proportion of 1 : 3-glucosidic linkages; it now seems probable that the earlier[3,4] periodate uptake values of 0·6 and 0·77 mol. per anhydroglucose unit resulted from incomplete oxidation of the Floridean starch. In the earlier experiments, an oxidation period (with potassium metaperiodate) of only 8 days was used, whereas it has been found that at least 12 days are required for the complete oxidation of normal glycogens

[5] Barry, McCormick, and Mitchell, *J.*, 1954, 3692.
[6] Hirst, Jones, and Roudier, *J.*, 1948, 1779.
[7] Peat, Thomas, and Whelan, *J.*, 1952, 722.
[8] Manners, *Biochem. J.*, 1955, **61**, xiii.
[9] Manners and Khin Maung, *Chem. and Ind.*, 1955, 950.

and starches.[10,11] Under such conditions, the production of formic acid follows the periodate uptake.[11] The increased yield of formic acid, and hence the decrease in average chain length reported in the present study, is in accord with this view.

The specific rotation (+176°) of sample I, which is slightly lower than that of glycogens [12] (184° to 201°), is an unusual property, for which no explanation is yet available. The presence of β-glucosidic linkages has been considered; however, since β-glucosidases have no effect on the β-amylolysis limit, the barriers to β-amylase action cannot be β-glucosidic linkages. The possibility that sample I is contaminated with a small proportion of a β-glucosan, or contains a small number of β-glucosidic linkages in the interior parts of the molecule, is being investigated.

A preliminary investigation of two additional samples of Floridean starch has been carried out. Sample II was kindly provided by Dr. V. C. Barry, and sample III by Dr. A. G. Ross. Both contained small amounts of a contaminating galactan, and the analytical data are therefore recorded only in terms of the weights of material analysed. The presence of this galactan does not affect the mode of action of the several enzymes used in this study and, hence, the general conclusions drawn from their use.

Floridean starches II and III, which did not react with the resorcinol reagent, formed iodine complexes with absorption spectra showing a single maximum at 525 mμ. The samples were rapidly attacked by the following enzymes: (*a*) salivary α-amylase (R_m *ca.* 57); (*b*) crystalline sweet-potato β-amylase, yielding *ca.* 33% of maltose; (*c*) amorphous soya-bean β-amylase, giving *ca.* 37% of maltose; and (*d*) potato phosphorylase, with a 27% conversion into glucose 1-phosphate. These observations, which represent mean values for samples II and III, show the presence of branched chains of α-1 : 4-linked glucose residues in the molecules. The barriers to β-amylase in sample II appear to be similar to those in glycogen; after incubation with yeast *iso*amylase, the β-amylolysis limit was increased to 57%. There is no evidence for the presence of β-glucosidic linkages in the outer chains * of the Floridean starches; emulsin has no action on these samples. After oxidation of the samples with sodium metaperiodate, the observed uptakes were 0·96 and 0·86 mol. of periodate per anhydrohexose unit, whilst the formic acid liberation during potassium metaperiodate oxidation corresponded to average chain lengths of 12 and 13 glucose residues respectively. If a correction is applied for the contaminating galactan, the chain length values would be even smaller.

The three samples of Floridean starch appear to have similar molecular structures; they are branched α-1 : 4-glucosans, are partially hydrolysed by β-amylase, and do not contain appreciable proportions of 1 : 3-glucosidic linkages. In these properties, they resemble the glucosans which have been isolated [13] from other algæ, *e.g.*, Odonthalia and *Ulva expansa*.

Comparison of the properties of glycogen, Floridean starch, and amylopectin.

Property	Glycogen [12]	Floridean starch	Amylopectin [12]
$[\alpha]_D$ in H_2O	+196°	+176°	+212°
Iodine coloration	Reddish-brown	Deep reddish-brown	Purple
$\lambda_{max.}$ (mμ) of absorption spectrum of iodine complex	460	500	540
β-Amylolysis limit [a]	45	46	54
Potato-phosphorolysis limit [b]	—	35	41
α-Amylolysis limit [a]	70	65	88
Periodate uptake (mols. per anhydroglucose unit)	1·08	1·05	1·04
Average chain length (glucose residues)	12	9	20

[a] Percentage conversion into maltose. [b] Percentage conversion into glucose 1-phosphate.

* *I.e.*, those parts of a chain between the branch point and the non-reducing terminal group.

[10] Bell and Manners, *J.*, 1952, 3641.
[11] Anderson, Greenwood, and Hirst, *J.*, 1955, 225.
[12] Manners and Ryley, *Biochem. J.*, 1955, **59**, 369; Manners, *Adv. Carbohydrate Chem.*, in preparation.
[13] Meeuse and Kreger, *Biochem. Biophys. Acta*, 1954, **13**, 593.

Experimental

Analytical Methods.—(a) *Reducing-sugar determination.* Somogyi's improved method [14] was used.

(b) *Nitrogen.* Total nitrogen was determined by Johnson's method,[15] the reagents being calibrated by use of a mixture of soluble starch and lysozyme.

(c) *Iodine absorption spectra.* Polysaccharide (2·5 mg.) and iodine solution (0·2% of iodine in 2% aqueous potassium iodide; 2·5 ml.) in a total volume of 25 ml. were mixed. The absorption spectrum in the range 400—700 mμ was then determined using a Unicam SP. 600 Spectrophotometer (1 cm. cells; iodine–water blank).

The other methods used were those described in Part III of this series.

Enzymic Reactions.—Except as stated, the enzyme preparations have been described in Part III and earlier papers.[10,12] Enzymic reactions were carried out at 35° (with the exception of the digests containing *iso*amylase), in the presence of toluene. Acetate buffer pH 4·6 and phosphate–citrate buffer pH 7·0 were used for β- and α-amylolysis, respectively.

Properties of the Floridean Starches.—(a) *Sample I.* Hydrolysis with 1·5N-sulphuric acid at 100° for 2 hr. gave glucose and a trace of galactose (paper chromatography). By quantitative acid hydrolysis, sample I had a reducing sugar content of 92·7%. No reaction was observed with the acid resorcinol or naphtharesorcinol reagent; ketoses and uronic acids were therefore absent. Sample I had $[\alpha]_D^{15}$ +163° (*c* 1·30 in H_2O) (Found: N, 0·92%, equiv. to 5·7% of protein). Corrected for the presence of this protein and 0·8% of ash, the glucosan has $[\alpha]_D$ +176°. Sample I had a very slight reducing power towards the Somogyi reagent, equivalent to D.P. 67; no evidence of molecular size can be deduced from this figure. An aqueous solution of sample I showed no appreciable opalescence; the molecular weight is therefore much lower than that of glycogen ($\sim 10^6$).

(b) *Samples II and III.* Qualitative acid hydrolysis showed the presence of glucose and a small proportion of galactose. The samples did not react with the acid resorcinol reagent, and the reducing-sugar contents by cuprimetric titration, as glucose, were 87·9 and 84·1% respectively. Aqueous solutions of the starches were unsuitable for polarimetric observation. Sample II had an apparent D.P. of 86 (Somogyi reagent) (Found: N, 0·34%). Sample III was also analysed (Found: N, 0·53%).

Degradation by Salivary α-Amylase.—Enzymic digests were set up containing Floridean starch (*ca.* 20 mg.), buffer (1 ml.), water (24 ml.), and maltase-free salivary α-amylase solution (5 ml.). Aliquot portions (3 ml.) were analysed, at intervals, for reducing sugar (as maltose). After 48 hours' incubation, the apparent percentage conversions into maltose (R_m) were: sample I, 65; II, 56; III, 58. In similar experiments with rabbit-liver glycogen (20·7 mg.) and waxy maize starch (25·6 mg.) the R_m values were 70 and 88 respectively.

Qualitatively, salivary α-amylase–Floridean starch digests become achroic within a few minutes. Paper chromatography of these digests showed the presence of maltose (intense spot) together with smaller amounts of glucose (R_G 1·0) and a series of higher saccharides of low chromatographic mobility ($R_G \leqslant 0{\cdot}17$).

Action of Crystalline Sweet-potato β-Amylase on Floridean Starches.—Sample I (15·5 mg., ≡14·4 mg. of glucosan) was incubated with buffer (4 ml.), water (20 ml.), and β-amylase solution (1 ml.). Samples were analysed, at intervals, for maltose. After 22 and 48 hr., 46% conversion into maltose was obtained.

Sample II (45·0 mg.) was added to buffer (10 ml.), water (38 ml.), and β-amylase solution (2 ml.). The percentage conversion into maltose was 38 and 37 respectively, after 24 and 48 hr. A portion (7 ml.) of the digest was added to a solution of sample II (16·7 mg.; 3 ml.) and incubated for a further 48 hr. At this time, 37% of the newly added sample II had been hydrolysed to maltose. A second portion (7 ml.) of the main digest was added to a solution of sample III (23·1 mg.; 3 ml.). After incubation for a further 48 hr., 28% conversion into maltose was observed.

It follows, therefore, that all three samples of Floridean starch are susceptible to hydrolysis by crystalline sweet-potato β-amylase.

Action of Amorphous Preparation of β-Amylase on Floridean Starches.—A solution of β-amylase was prepared by dissolving soya-bean β-amylase (50 mg.) in 0·2M-acetate buffer (pH 4·6; 20 ml.), and removing insoluble material by centrifugation; the supernatant liquid had an activity [16] of 196 units/ml. Enzyme digests containing Floridean starch (*ca.* 20 mg.),

[14] Somogyi, *J. Biol. Chem.*, 1952, **195**, 19.
[15] Johnson, *ibid.*, 1941, **137**, 575.
[16] Hobson, Whelan, and Peat, *J.*, 1950, 3566.

β-amylase (980 units; 5 ml.), and water (20 ml.) were set up. Samples (3 ml.) were analysed, at intervals, for maltose, with the following results :

	β-Amylolysis limit		
Time of incubation (hr.)	4	24	48
Sample I (*a*)	—	43	44
(*b*)	—	44	45
Sample II	36	37	—
Sample III	35	37	—

Paper chromatography of β-amylase–Floridean starch digests showed that maltose was the sole reducing sugar present. Control experiments showed that the β-amylase had no maltase activity, and slowly hydrolysed salicin and laminarin.

The Floridean starches were also hydrolysed by an amorphous preparation of β-amylase from barley, yielding 30—40% of maltose.

Action of iso*Amylase and* β*-Amylase on Floridean Starches*.—*iso*Amylase [9] was isolated from brewer's yeast by Manners and Khin Maung. Sample I (10·0 mg.), 0·2M-acetate buffer (pH 5·89; 1 ml.), water (4·5 ml.), and *iso*amylase (40 mg. per ml.; 0·5 ml.) were incubated at 18° for 24 hr. The enzyme was then inactivated by heating the digest to 100° for 2—3 min. Barley β-amylase (500 units; 1 ml.) was added. After 24 hours' incubation at 35°, a 3 ml. sample was withdrawn, and after deproteinisation (zinc sulphate–barium hydroxide) analysed for maltose. The β-amylolysis limit was 54%. In a similar experiment with sample II, the β-amylolysis limit of the *iso*amylase-treated starch was 54% after 24 hours' incubation with β-amylase, and 57% after 48 hours'.

Phosphorolysis of Floridean Starches [with A. Margaret Liddle].—Digests were prepared containing Floridean starch I, II, III (*ca.* 25 mg.), potato phosphorylase (100 mg., 6 Green and Stumpf [17] units), 0·5M-phosphate buffer (pH 6·8; 2 ml.), and water to 10 ml. Aliquot portions (2 ml.) were analysed at intervals for glucose 1-phosphate, as described previously :

Conversion (%) *into glucose* 1-*phosphate*.

Time of incubation (hr.)	10	24	40
Sample I	—	33	35
Sample II	23	24	—
Sample III	—	27	28

In a control experiment with waxy maize starch, a phosphorolysis limit of 41% was obtained. Similar limits have been obtained by other workers.[7]

Action of Emulsin on the Floridean Starches.—Digests were set up containing samples II or III (49·8 or 46·8 mg.), 0·2M-acetate buffer (pH 5·0; 3·5 ml.), 0·01M-mercuric chloride (0·5 ml.), distilled water (18·0 ml.), and emulsin solution (40 mg./ml.; 3·0 ml.). Aliquot portions (3 ml.) were analysed, after deproteinisation (zinc sulphate–barium hydroxide), for reducing sugar. No significant increase in reducing power occurred within 48 hr. In control experiments, the emulsin rapidly attacked laminarin and salicin, and had no action on soluble starch.

Sodium Metaperiodate Oxidation of Floridean Starches.—Sample I (96·8 mg., ≡90·0 mg. of glucosan) was oxidised with sodium metaperiodate (3·2% w/v; 10 ml.) at 1°. Samples (2 ml.) were removed at intervals, and the periodate uptake determined by Fleury and Lange's method.[18] After 72 and 168 hr., 0·93 and 1·05 mol. respectively of periodate were consumed per anhydroglucose unit.

Sample II [(*a*) 152·7 and (*b*) 152·0 mg.] were oxidised under similar conditions. The periodate consumption, after 96 and 192 hr., was (*a*) 0·90 and 0·98 and (*b*) 0·95 and 0·94 mol. per anhydrohexose unit.

Sample III [(*a*) 147·4 and (*b*) 151·0 mg.] were oxidised as above. After 90 and 216 hr., the periodate uptakes were (*a*) 0·82 and 0·87 and (*b*) 0·83 and 0·85 mol. per anhydrohexose unit.

If reducing-sugar contents of 88 and 84% are assumed for samples II and III, respectively, these figures are equivalent to periodate uptakes of 1·03 and 1·02 mol. per anhydrohexose unit.

In a control experiment waxy maize starch (211·5 mg.) was oxidised at 1° with sodium metaperiodate (4·0% w/v; 20 ml.). After 50 and 120 hr., 1·03 and 1·04 mol. of periodate were consumed per anhydroglucose unit.

Control titrations of arsenite in the presence of Floridean starches against iodine showed that

[17] Green and Stumpf, *J. Biol. Chem.*, 1942, **142**, 355.
[18] Fleury and Lange, *J. Pharm. Chim.*, 1933, **17**, 107.

the impurities present (galactan and protein) did not interfere with the titration, and hence with measurement of the periodate uptake.

Potassium Metaperiodate Oxidation of Floridean Starches.—Floridean starch (55—100 mg.) was dissolved in 3% potassium chloride solution (40 ml.) and the pH adjusted to 5·8 (glass electrode) by the addition of sodium hydroxide. 4% w/v Sodium metaperiodate solution (10 ml.) was added; 10 ml. portions were withdrawn at intervals for determination of formic acid.[10] A mixture of potassium chloride and sodium metaperiodate was analysed similarly. The following results were obtained:

Sample	I	II	III
Weight oxidised (mg.)	90·9	98·8	55·8
Total formic acid produced (mg.)	2·80	2·43	1·22
Apparent chain length (glucose residues)	9·2	11·6	13·0

The formic acid release was complete after 210 hours' oxidation at room temperature (15—17°); similar titres were obtained after 260 hr.

The oxidised sample I was isolated by freeze-drying (after decomposition of excess of periodate with ethylene glycol, and dialysis), and hydrolysed with acid. Paper chromatography of the neutralised and de-ionised hydrolysate showed the presence of galactose (moderate spot) and glucose (faint spot). The amount of glucose is less than that of the insignificant quantity of galactose present in the original acid hydrolysate (p. 2832) and represents less than 1% of the original molecule.

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