575. α-1 : 4-*Glucosans. Part IX.* The Molecular Structure of a Starch-type Polysaccharide from* Dunaliella bioculata.

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A glucose-containing polysaccharide, isolated from the unicellular salt-water alga *Dunaliella bioculata*, has been examined by chemical and enzymic methods.

It resembles plant starches in many respects, but contains only 13% of amylose, and the amylopectin component has an average chain length of 15—16 glucose residues. Both components are incompletely degraded by β-amylase, the barriers to this enzyme being hydrolysed by Z-enzyme and isoamylase, respectively.

Although the polysaccharides isolated from red and brown seaweeds (Rhodophyceae and Phaeophyceae) have been extensively studied,[1,2] present knowledge of similar materials from green and blue-green algae (Chlorophyceae and Myxophyceae) is limited. The last two are, on the whole, less readily available. It is generally accepted [3] that the reserve carbohydrate of *Chlorella* is a starch, but precise structural details have not been reported. The green alga *Ulva expansa* contains an iodophilic polysaccharide which, unlike normal starches, shows no appreciable birefringence and gives an abnormal *X*-ray powder diagram.[4] Polysaccharides from *Oscillatoria* have been characterised as amylopectin [5] or glycogen [6] in type, and *Nitella* [5] contains a cellulose-type material. In contrast, *Nostoc* [5] yields a complex mucilage composed of uronic acids, rhamnose, xylose, galactose, and glucose, whilst green algae of the *Chlamydomonas* species synthesise soluble extracellular polysaccharides with galactose and arabinose as the main constituents.[7] This paper describes an examination of the storage polysaccharide from the halophytic unicellular alga *Dunaliella bioculata* (class, Chlorophyceae; order, Volvocales; family, Polyblepharidaceae). The food value of this organism has been reported elsewhere.[8]

A preliminary study of the dried algal cells showed the presence of glucosan which was extracted with water at 70° or 100°, or with potassium hydroxide solution (5% or 24%) at 20°. Since this material was stained blue-black with iodine, and was degraded by α-amylase, the presence of a starch-type polysaccharide was indicated. A sample of polysaccharide material extracted with hot water contained glucose (69%) and protein (23%). Attempts to separate these by extraction with chloral hydrate [9] or trichloroacetic acid and toluene were not successful. Although the protein content decreased to 6% and 3% respectively, some degradation of the polysaccharide (content 78 and 82% respectively) occurred. As an alternative method of extraction, the procedure of MacWilliam and his co-workers [10] was examined.

The dried cells were extracted with 30% aqueous perchloric acid at room temperature, and the resulting polysaccharide was then purified *via* the iodine complex. The product was amorphous, contained 99% of glucose and *ca.* 0·1% of protein, and an aqueous solution, which had $[\alpha]_D$ +169°, was stained blue with iodine, exhibiting maximum absorption at 600 mμ. On incubation with salivary α-amylase, an apparent percentage conversion into maltose (P_M) of 85 was observed; under similar conditions, potato starch had a P_M value

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[9] Meyer and Bernfeld, *Helv. Chim. Acta*, 1940, **23**, 875.
[10] MacWilliam, Hall, and Harris, *J. Inst. Brewing*, 1956, **62**, 227.

of 88. The polysaccharide was rapidly degraded by soya-bean β-amylase, giving 62% conversion into maltose; this degradation was increased to 83% by prior incubation with yeast isoamylase. The latter enzyme hydrolyses α-1 : 6-glucosidic inter-chain linkages in amylopectin and glycogen.[11]

The polysaccharide was oxidised at room temperature with potassium metaperiodate;[12] the production of formic acid corresponded to an average chain length ($\overline{CL}$) of 18 glucose residues, and the absence of glucose in an acid hydrolysate of the periodate-oxidised polysaccharide indicated that 1 : 2- or 1 : 3-glucosidic linkages were absent.[13]

The presence of an amylose-type component was shown by potentiometric titration[14] of the iodine complex, and the iodine binding power, kindly determined by Mr. J. M. G. Cowie, indicated an amylose content of 12—14%. The $\overline{CL}$ value of the amylopectin component was therefore 15—16 glucose residues. The small amount of formic acid which is liberated from the end-groups of amylose can, to a first approximation, be neglected. The polysaccharide was fractionated by the thymol method,[15] in the absence of oxygen, and the resulting fractions treated with β-amylase. The amylose component, with pure β-amylase, had a β-amylolysis limit of 73%, and on addition of barley β-amylase (which contains Z-enzyme[16]) the limit increased to 93%. The amylopectin fraction had β-amylolysis limits of 60% before, and 76% after, pre-treatment with isoamylase. The polymeric chains are therefore mainly composed of α-1 : 4-glucosidic linkages, and the outermost inter-chain linkages in the amylopectin are of the α-1 : 6-type.

The above evidence shows that the polysaccharide isolated from *Dunaliella bioculata* resembles normal plant starches in many respects. The interaction with iodine, the degradation by α- and β-amylase, and the separation into two components by thymol are typical properties. It is of interest that the algal amylose, like that from the potato and the wrinkled pea,[16] contains a small number of anomalous linkages which are not attacked by β-amylase.

The amylose content of the algal starch is unusually low (plant starches normally contain 20—30% of amylose[17]) and is similar to that of the starch synthesised by the flagellated protozoan *Polytomella coeca*.[18] Further, the amylopectin component has a relatively high degree of branching ($\overline{CL}$ values of 20—25 are usual[19]), which is similar to that of the polysaccharide from *Oscillatoria princeps*.[6] Both these organisms show high Q-enzyme (*i.e.*, amylo-1 : 4 ⟶ 1 : 6-transglucosidase) activity, and the present study suggests that *D. bioculata*, which can be readily cultivated under laboratory conditions, may also be a convenient source of this enzyme.

Experimental

The analytical methods and enzyme preparations used have been described in Parts IV,[20] VI,[21] and VII [16] of this series.

Isolation of Polysaccharide.—The algal cells, grown in artificial double-strength sea-water, were harvested by centrifugation, dried, and stored *in vacuo* over P_2O_5. Before extraction, the dried cells were disintegrated in a ball-mill and sieved (200 mesh).

In a preliminary experiment, ethanol was added to a hot-water extract of the powdered cells, to give an impure polysaccharide material [Found: reducing sugar (as glucose), 69·4%; N, 3·7%, equiv. to 23% of protein]. A portion was extracted with aqueous 33% chloral hydrate to give sample I [Found: glucose (by paper chromatography and cuprimetric titration),

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[18] Bourne, Stacey, and Wilkinson, *J.*, 1950, 2694.
[19] Bell, *Ann. Reports*, 1947, **44**, 223.
[20] Fleming, Hirst, and Manners, *J.*, 1956, 2831.
[21] Liddle and Manners, *J.*, 1957, 3432.

77·7; N, 0·9%]. A second portion was largely freed from protein by shaking it with 10% aqueous trichloroacetic acid and toluene, to yield sample II (Found: glucose, 81·7; N, 0·46%). Analysis by periodate oxidation and potentiometric iodine titration indicated that both samples had been degraded during purification.

A pure sample of undegraded polysaccharide was finally prepared by purification of a perchloric acid extract of the dried cells.[10] The latter (5 g.) were extracted three times with water (80 ml.) and 72% perchloric acid (60 ml.) by shaking for 30 min. at room temperature. Cell debris was removed by centrifugation, and the crude polysaccharide was precipitated and washed with acetone. An aqueous solution was then dialysed for 48 hr. against running tap-water. The volume was adjusted to 100 ml. and, to this, 20% sodium chloride solution (25 ml.) and iodine solution (3% in 3% potassium iodide; 10 ml.) were added with stirring. After several hours, the polysaccharide–iodine complex was collected, washed with alcoholic sodium chloride (2% in 75% ethanol), and decomposed by treatment with alcoholic sodium hydroxide (25 ml. of 5N-aqueous solution in 125 ml. of ethanol). The polysaccharide was washed again with alcoholic sodium chloride, dissolved in water, dialysed for 48 hr., and precipitated with acetone. This gave sample III (1 g.) (Found: glucose, 99·5; N, 0·02%). Sample III was a white amorphous powder which dissolved in warm water. The aqueous solution had $[\alpha]_D$ +169° (*c* 0·54) and was stained blue with iodine, exhibiting maximum absorption at 600 mμ.

The iodine-binding power of sample III was determined by Mr. J. M. G. Cowie using the method of Anderson and Greenwood.[14] It gave a typical starch titration curve, and extrapolation indicated the presence of 12—14% of amylose.

Enzymic Degradation.—(a) *Salivary α-amylase.* Polysaccharide (27·8 mg.), 0·1M-citrate–phosphate buffer (pH 7·0; 5 ml.), sodium chloride (5 mg.), and freeze-dried salivary α-amylase [21] (15 mg.) in a total volume of 50 ml. were incubated at 35° for 48 hr. The P_M value was 85. In a control experiment, potato starch (31·4 mg.) gave a P_M value of 88.

(b) *Soya-bean β-amylase.* Sample III (15·1 mg.) was incubated with 0·2M-acetate buffer (pH 4·6; 3 ml.), containing soya-bean β-amylase solution (0·05 ml.; 1000 units), in a total volume of 25 ml. After 24 and 48 hr., the β-amylolysis limit was 62%.

(c) *Isoamylase and β-amylase.* Sample III (29·1 mg.) in acetate buffer (pH 5·9; 5 ml.) and water (8 ml.) was treated with isoamylase solution (80 mg. in 2 ml.) at room temperature for 24 hr. (The isoamylase was extracted from brewer's yeast by Miss Zeenat H. Gunja.) After inactivation of the isoamylase by heat, soya-bean β-amylase solution (0·05 ml.) and water (to 50 ml.) were added. The β-amylolysis limit, after 24 hours' incubation at 35°, was 83%.

Potassium Metaperiodate Oxidation.—Sample III (174·5 mg.), dissolved in 3% potassium chloride solution (50 ml.), was oxidised with 8% sodium metaperiodate solution (8 ml.) at room temperature. Portions (10 ml.) were analysed at intervals:

Time of oxidn. (hr.)	50	100	200	300
Total formic acid prodn. (mg.)	1·99	2·50	2·76	2·80
Apparent $\overline{CL}$ (glucose residues)	24·9	19·8	18·0	17·7

Since the starch contains 13% of amylose, the amylopectin component has a $\overline{CL}$ value of 15—16 glucose residues. The remaining solution of periodate-oxidised starch was treated with ethylene glycol (5 ml.), dialysed for 48 hr., and freeze-dried. After acid hydrolysis with 2N-sulphuric acid for 2 hr. the neutralised hydrolysate was examined by paper chromatography. Glucose could not be detected.

Fractionation of the Starch.—Polysaccharide (*ca.* 200 mg.) was dissolved in boiling water (150 ml.) in an atmosphere of nitrogen. The solution was allowed to cool to 70° and powdered thymol (100 mg.) added. The temperature was maintained at 70° for 30 min. with continuous stirring, then the solution was set aside at room temperature for 2 days. The thymol–amylose complex was collected and dissolved in water (30 ml.). The supernatant solution was treated with acetone, and the precipitated polysaccharide collected, washed with acetone and dried (yield, *ca.* 170 mg.). This material gave a solution which was stained with iodine, showing maximum absorption at 560 mμ.

Enzymic Degradation of Starch Components.—(a) *Amylose.* The above solution of amylose (0·62 mg./ml. determined by acid hydrolysis) was incubated with acetate buffer (pH 4·6; 5 ml.) and soya-bean β-amylase solution (0·1 ml.) in a 50 ml. digest. After 24 hr. the β-amylolysis limit was 73%. Barley β-amylase (which contains Z-enzyme; 1500 units; 15 mg.) was then added. After a further 24 hr. the β-amylolysis limit was 93%.

(b) *Amylopectin.* The above precipitate (48·1 mg.) was incubated at pH 4·6 with barley β-amylase (3000 units; 30 mg.) in a final volume of 50 ml. The β-amylolysis limit was 60%.

[With ZEENAT H. GUNJA] Amylopectin (30·1 mg.) was incubated with isoamylase (50 mg.) at pH 5·9 and 20° in a 15 ml. digest for 18 hr. The isoamylase was inactivated by heat, β-amylase solution (3 ml.) added, and the maltose content determined. After 24 hr., the β-amylolysis limit was 76%.

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