790. *Starch-type Polysaccharides Isolated from the Green Seaweeds,* Enteromorpha compressa, Ulva lactuca, Cladophora rupestris, Codium fragile, *and* Chaetomorpha capillaris.

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Starch-type polysaccharides have been isolated, and fractionated into an amylose and an amylopectin component, from four genera of green seaweed. The conditions of isolation from *Chaetomorpha* were such as to destroy any amylose, and only the amylopectin of this genus was separated and characterised. Although apparently considerably smaller molecules, the algal amyloses and amylopectins resembled potato amylose and amylopectin, respectively, in many of their properties.

The only reported isolations of characterised starch-type polysaccharides from green seaweeds are those of an amylopectin-type polymer from *Caulerpa filiformis* [1] and a starch-type polymer from *Codium fragile*.[2] The former of these was fractionated with cetyltrimethylammonium bromide from the water-soluble polysaccharides, and the latter was precipitated as the starch–iodine complex from the hot-water extract.[3] We have now succeeded in fractionating starch-type polymers as the starch–iodine complex from the water-soluble polysaccharides of *Enteromorpha compressa, Ulva lactuca,* and *Chaetomorpha capillaris.* Owing to gelling of *Cladophora rupestris* polysaccharide, it was advantageous to separate the starch from the aqueous extracts before isolation of the

[1] Mackie and Percival, *J.*, 1960, 2381.
[2] Love and Percival, *Biochem. J.*, 1962, **84**, 29P.
[3] McKinnell and Percival, *J.*, 1962, 3141.

sulphated polysaccharides and *ca.* 50-g. batches of dry weed gave the highest percentage yield of starch. The same procedure was also used for *Codium fragile*.[2] The yields of the respective starches based on the dry weight of weed are: *C. fragile*, *ca.* 1·0%; *E. compressa*, 0·6%; *U. lactuca*, 1·2%; *C. capillaris*, 0·3%; *C. rupestric*, 1·0%. An acid hydrolysate of each product contained glucose as the sole carbohydrate (paper chromatography and treatment with glucose oxidase). The glucose contents were determined by cuprimetric titration, and all the analytical figures of the different fractions are based on their glucose content. Most of the starches were contaminated with ash (0·9—2·0%) and protein (up to 5%).

Apart from the material from *C. capillaris*, which gave a purple colour, all the products gave a blue colour with iodine and were fractionated by the thymol method, and the resulting amylose complexes were purified by two precipitations with butanol (for percentage of amylose see Table 1). In each fractionation a proportion of material was lost, probably during dialysis of the solution containing the amylopectin.

The intrinsic viscosity, blue value (B.V.),[4] and $\lambda_{max.}$ (mμ) (wavelength of peak absorption of iodine complex) for each of the amyloses and for potato amylose measured under identical conditions are given in Table 1, and those of the respective amylopectins in Table 2. An estimate of the degree of polymerisation ($\overline{DP}$) of the amyloses was made from the approximate relation, $\overline{DP} = 7{\cdot}4[\eta]$ [5] (see Table 1).

The quantity of periodate reduced [6] corresponds to *cf.* 1 mole for every anhydroglucose unit, for each of the amyloses (see Table 1) and amylopectins (Table 2), and the oxopolysaccharides were devoid of unattacked glucose units. This provides qualitative evidence that 1,2- and 1,3-glucosidic linkages are absent and that the polysaccharides contain 1,4-linked glucose units with possible branch points at C-6. Confirmation of this structure was obtained for the amylopectins from *E. compressa*, *C. rupestris*, and *C. fragile*, from methylation studies.

TABLE 1.

Properties of amylose-type molecules.

	Cladophora rupestris	*Enteromorpha compressa*	*Ulva lactuca*	*Codium fragile*	Potato
% Amylose in starch	20	22	37	16	25
$[\eta]$	78	44	41	36	300
$\overline{DP}$	577	325	302	266	2220
Blue value	1·2	0·66	1·14	0·96	1·21
$\lambda_{max.}$	635	610	630	610	640
$[\alpha]_D$	+158° *	+177°	+161° *	+197°	+157° *
β-Amylolysis: pH 3·6, P_M	73	—	71	84	80
pH 4·6, P_M	88	76	90	96	100
Reduction IO_4^-/anhydro-unit	0·97	1·08	1·01	1·10	1·01

* Measured in M-KOH.

Parallel experiments with β- and α-amylase on each of the algal glucans and on potato amylose and amylopectin were carried out. The apparent percentage conversion into maltose (P_M) was measured and the results are given in Tables 1 and 2. The β-amylolysis limits with a Wallerstein β-amylase preparation, which was known to be contaminated with *Z*-enzyme, were measured at pH 3·6 and 4·6. At pH 3·6 the action of *Z*-enzyme is inhibited.[7] In the present experiments the action of the contaminating *Z*-enzyme is so slight that only a minute proportion of the α-1,4-glucosidic linkages in amylopectin are cleaved, so that the resultant increase in β-amylolysis limit is very small. On the other hand, amyloses are essentially linear and slight *Z*-enzyme activity in the case of potato

[4] Bourne, Haworth, Macey, and Peat, *J.*, 1948, 924.
[5] Cowie and Greenwood, *J.*, 1957, 2658, 2862.
[6] Aspinall and Ferrier, *Chem. and Ind.*, 1957, 2168.
[7] Cowie, Fleming, Greenwood, and Manners, *J.*, 1957, 4430.

amylose was sufficient to result in complete fission to maltose. Considerable difficulty was encountered in carrying out the amylolyses at pH 3·6 with the algal amyloses which readily retrograde from solution. Additional experiments with a purified β-amylase which was free from *Z*-enzyme and maltase activity were carried out on the amylopectins of *C. rupestris*, *U. lactuca*, *C. fragile*, and potato, glutathione being used as an activator. Since glutathione interferes with cuprimetric estimation, the maltose concentrations were measured by ferricyanide–ceric sulphate titration.[5] Addition of *Z*-enzyme to the amylopectin digests increased the β-amylolysis limits to a small extent (see Table 3).

TABLE 2.

Properties of amylopectin-type molecules.

	Cladophora rupestris	*Enteromorpha compressa*	*Ulva lactuca*	*Codium fragile*	*Chaetomorpha*	Potato	Glycogen
$[\eta]$	41	35	58	24	—	161	10
Blue value	0·196	0·140	0·220	0·220	0·108	0·176	—
$\lambda_{max.}$	565	550	560	560	540	560	460
$[\alpha]_D$	+197°	+190°	+205°	+192°	—	+197°	+196°
α-Amylolysis, P_M	92	90	91	84	81	92	70
β-Amylolysis: pH 3·6, P_M	55	—	55	—	—	53	
pH 4·6, P_M	57	58	56	—	62	56	45
Reduction IO_4^-/anhydro-unit	1·05	0·900	1·05	1·2	1·11	1·04	1·0

TABLE 3.

	β-Amylolysis			β-Amylolysis	
Amylopectin	β-Limit P_M	β + *Z*-Limit P_M	Amylopectin	β-Limit P_M	β + *Z*-Limit P_M
C. rupestris	50	55	*C. fragile*	51	53
U. lactuca	51	55	Potato	53	57

The present study has shown that four different genera of green seaweed synthesise a starch which can be fractionated into essentially linear (amylose) and branched (amylopectin) components. Apart from that in *U. lactuca* which is higher (37%), the proportion of amylose (16—22%) in the other genera is comparable with that of the majority of plant starches (20—30%), although certain varieties of pea [8] and maize [9] starches have a much higher amylose content. The algal amyloses have a low iodine-binding power and this may be correlated with their low $\overline{DP}$, since there is evidence that $\lambda_{max.}$ of amylose-type chains is directly related to the $\overline{DP}$ [10] if this is lower than 500. Amylose is readily degraded by oxygen and alkali, and for this reason the present starches were extracted and fractionated under the mildest possible anaerobic conditions. It seems very probable, therefore, that they are smaller molecules than plant amyloses. Microscopic examination certainly indicates a less organised granule. The difficulty of obtaining large weights of *C. capillaris* precluded separate extraction of the starch and the sulphated polysaccharide, and consequently the method used, which gave a relatively pure yield of the latter (boiling water in contact with air and then treatment overnight with 4% trichloroacetic acid) was sufficiently drastic to destroy any amylose present in the native starch and it was only possible to isolate and examine an amylopectin component. That the native *Chaetomorpha* contained an amylose component was deduced from the deep blue colour given with iodine by solutions obtained by extraction of the weed for 15—20 minutes with cold water; successive samples taken during prolonged extraction with boiling water showed a progressive change in the colour given with iodine to the typical purple colour produced with amylopectins. Similar hot-water extractions of *C. rupestris* in contact with air also

[8] Potter, Silveira, McCready, and Owens, *J. Amer. Chem. Soc.*, 1952, **75** 1335.
[9] Wolff, Hofreiter, Watson, Deatherage, and MacMasters, *J. Amer. Chem. Soc.*, 1955, **77**, 1654.
[10] Bailey and Whelan, *J. Biol. Chem.*, 1961, **236**, 969.

produced a polysaccharide mixture which gave a purple rather than the blue colour with iodine solution.

The high positive rotation of each of the amyloses, their ready retrogradation from aqueous solution, and the reduction by them of *ca.* 1 mole of periodate for every anhydroglucose unit all confirm their essentially linear α-1,4-linked structure. The β-amylolysis limits, apart from those of *E. compressa* amylose, are only slightly lower than that of potato amylose and are further evidence of their similarity with the latter.

The algal amylopectins apart from their low intrinsic viscosities show even more striking similarity with potato amylopectin. Their rotations, $\lambda_{max.}$, and α- and β-amylolysis limits are the same as those of potato amylopectin and, like the latter, they reduce *ca* 1 mole of periodate for every anhydroglucose unit. The hydrolysate from the methylated amylopectin from *E. compressa* contained tetra- to tri-*O*-methylglucose in the ratio of 1 : 26. This indicates an average chain-length for *E. compressa* amylopectin of *ca.* 27, a value slightly higher than that of the majority of plant amylopectins whose average chain-length is *ca.* 25. The tetra- and tri-*O*-methylglucoses from methylated *Cladophora* amylopectin were separated and characterised as crystalline sugars.

Experimental

The analytical methods used have been described by O'Donnell and Percival.[11] Blue values refer to 680 mμ throughout.

Isolation of the Starches.—Finely powdered *C. rupestris* weed (30—40 g. batches) was freed from colouring matter by immersion in butanol half-saturated with water overnight, followed by several extractions with cold acetone. The decolorised weed in water (500 ml.) was thoroughly saturated with nitrogen and the mixture heated to 90—95° with continuous stirring and passage of nitrogen. After 1 hr. the mixture was cooled and the residual weed removed by centrifugation. The residue, after being washed with warm water, was re-extracted as before. The extract and washings were concentrated to 200 ml. and the starch-type polysaccharide was separated from this solution as the starch–iodine complex.[3] The Celite was reduced to 1·5 g. for each 40 g. of weed extracted. Precipitation of the starch–iodine complex was repeated before removal of the Celite, and the combined precipitate and Celite were then suspended in 95% ethanol (200 ml.) before dropwise addition of thiosulphate solution. When the blue colour had disappeared, the ethanol concentration was reduced to 70% with water and the starch–Celite mixture filtered off and washed with 70% ethanol (200 ml.). The starch–Celite was made into a paste with water and plunged into water (100 ml.) at 90° in an atmosphere of nitrogen. After vigorous stirring for 15 min. the Celite was removed by high-speed centrifugation. The supernatant solution was dialysed before freeze-drying. The yield of a white amorphous powder was 0·4 g. from 40 g. of weed.

The starch-type polysaccharides from the other genera were separated, essentially as above,[3] from aqueous solutions of the isolated water-soluble polysaccharides. An acid hydrolysate of each of the products contained glucose only (paper chromatography and glucose oxidase treatment).

Separation of Amylose and Amylopectin from the Starch-type Polysaccharides.—The starch (800 mg.), made into a paste with water, was plunged with vigorous stirring into water (100 ml.) at 95° in an atmosphere of nitrogen. After 20 min. the solution was allowed to cool to 60° and then saturated with powdered thymol (0·13 g.). The mixture was stirred for 30 min. at 55—60° and finally allowed to cool to room temperature with constant stirring and in an atmosphere of nitrogen throughout. After 2 days a copious white thymol–polysaccharide precipitate had been deposited. This was removed on a high-speed centrifuge and dispersed with stirring under nitrogen in distilled water at 90°. The centrifugate was freed from thymol by extraction with ether, and amylopectin (400 mg. from *C. rupestris*) was isolated from the aqueous solution by freeze-drying after dialysis. It gave a purple colour with iodine and had $[\alpha]_D$ +197° (*c* 0·4) [Found: glucose by cuprimetric titration [12] of a hydrolysate (2N-sulphuric acid at 100° for 2·5 hr.), 81%; ash, 2·0%; protein-N, 0·8%]. Amylopectins from *E. compressa*

[11] O'Donnell and Percival, *J.*, 1959, 2168.
[12] Somogyi, *J. Biol. Chem.*, 1952, **195**, 19.

(Found: glucose, 91%; ash, 0·9%; protein-N, nil), *Ulva lactuca* (Found: glucose, 86%; ash, 1·0%; protein-N, 0·4%, and *C. fragile* (Found: glucose 81%; whole starch ash, 1·8%; protein-N, 0·8%), were similarly obtained. Compare amylopectin (Found: glucose 95%); potato (Found: glucose, 95%).

The dispersed amylose solution was saturated with butanol (10%) at 60°, the mixture allowed to cool to room temperature with stirring, then stoppered and set aside overnight. The deposited butanol–amylose complex was removed at the centrifuge and recrystallised again with butanol. The derived amylose (160 mg. from *C. rupestris*) gave a blue colour with iodine and had $[\alpha]_D$ +158° (*c* 0·7 in N-NaOH). The starch from *U. lactuca* (400 mg.), which was insoluble in water, was dissolved in 0·5N-sodium hydroxide with stirring under nitrogen. The alkaline solution was dialysed against running water until neutral (6 days) and then concentrated at 40° to 100 ml. before fractionation with thymol. By cuprometric titration as for the amylopectins the percentage glucose contents of the amyloses were found to be *C. rupestris* 83, *E. compressa* 80, *C. fragile* 95.

Viscosity Determination.[5]—The specific viscosity (η_{sp}) of N-potassium hydroxide solutions of the polysaccharides (0·5—1·5%) saturated with nitrogen were determined at several concentrations at 25° in a Ubbelohde viscometer and the limiting viscosity numbers determined (see Tables 1 and 2).

Enzymic Degradation.—(*a*) *Salivary α-amylase.* Algal amylopectin and potato amylopectin solutions (0·05—0·1%) in 0·04M-citrate–phosphate buffer (pH 7·0), sodium chloride (1 mg.), and purified salivary α-amylase [13] (0·1%) in a total volume of 20 ml. each were incubated separately at 37° for 48 hr. and analysed for their maltose content by cuprimetric titration.[12] The P_M values are given in Table 2.

(*b*) *β-Amylase.* (i) Digests of the respective algal amyloses, amylopectins, and potato amylose and amylopectin solutions (0·05—0·1%), were prepared in acetate buffer (pH 4·6; 0·04M), serum albumin (0·01—0·05%), and Wallerstein β-amylase preparation (0·05—0·1%; 110 units/mg. assayed by the method of Hobson, Peat, and Whelan [14]), in a total volume of 20—25 ml. and incubated at 37°. The P_M values are given in Tables 1 and 2.

(ii) Similar digests were prepared of *C. rupestris*, *U. lactuca*, *C. fragile*, and potato amylopectins, except that purified β-amylase (3000 units/mg.[14]) free from *Z*-enzyme and maltase activity in 0·5M-glutathione was used. The P_M was determined after a further 24 hr.[5] (see Tables 1 and 2).

Periodate Oxidation.—Polysaccharide samples (10 mg.) were oxidised with aqueous 0·015M-sodium metaperiodate (25 ml.) in the dark at 4°. Aliquot parts (0·1 ml.) were analysed at intervals for the amount of periodate reduced.[6] Chromatographic examination of a hydrolysate of the reduced ($NaBH_4$) oxo-polysaccharides after oxidation for 98 hr. showed the absence of glucose.

Periodate oxidation of the starch fractions. Moles IO_4^- reduced/anhydro-unit.

Time (hr.)	20	44	68	98
Potato amylose	0·684	0·891	0·991	1·01
amylopectin	0·844	0·964	1·03	1·04
C. rupestris amylose	0·488	0·699	0·90	0·97
amylopectin	0·840	0·890	1·02	1·05
E compressa amylose	0·800	0·890	0·95	1·08
amylopectin	0·70	0·79	0·81	0·90
U. lactuca amylose	0·424	0·748	0·94	1·01
amylopectin	0·700	0·996	1·03	1·05
C. fragile amylose	0·84	0·95	1·00	1·10
amylopectin	1·02	1·19	1·21	1·21

Methylation of the Amylopectin Fractions.—(a) *From* C. fragile. Amylopectin (120 mg.) in water (10 ml.) was treated overnight at room temperature with potassium borohydride (50 mg.). 60% Sodium hydroxide solution (10 ml.) and dimethyl sulphate (6 ml.) were then added dropwise during 6 hr. Throughout these additions the mixture, saturated with nitrogen, was stirred vigorously and kept below 2°. It was then stirred at room temperature overnight. The methylation was repeated twice under exactly the same conditions. The partially

[13] Fischer and Stein, *Arch. Sci.*, 1954, **7**, 131.
[14] Hobson, Peat, and Whelan, *J.*, 1950, 3566.

methylated product (100 mg.) was isolated by freeze-drying after dialysis. The above methylation procedure was repeated thrice more and the polysaccharide isolated after each methylation as before. The product was extracted overnight with chloroform under reflux and the chloroform-soluble material dried overnight at 60° *in vacuo*. It (*ca.* 30 mg.) was subjected to two Purdie methylations with methyl iodide (10 ml.) and dry silver oxide (2·0 g.) added during 5 hr.

(*b*) *From* E. compressa. The amylopectin (52 mg.) was mixed with Filter-Cel and methylated with potassium and methyl iodide in liquid ammonia.[15] After four complete methylation cycles the products were extracted with dry boiling chloroform (20 ml. × 5) for 2 hr.

(*c*) *From* C. rupestris. The amylopectin (100 mg.) was subjected to three complete methylations as under (*b*), and the product isolated from the chloroform solution as a pale yellow mobile syrup (105 mg.).

Hydrolyses of the Methylated Polysaccharides.—(i) The methylated amylopectins (*ca.* 15 mg.) were refluxed separately with 2% methanolic hydrogen chloride for 8 hr. After neutralisation with silver carbonate, filtration, and concentration, the methyl glucosides were examined by gas chromatography.[16] Methyl 2,3,4,6-tetra-*O*-methyl-α- and -β-glucoside, methyl 2,3,6-tri-*O*-methyl-α- and -β- glucoside, and two small peaks characteristic of methyl di-*O*-methylglucosides were detected.

(ii) The methylated glucosides from *E. compressa* were hydrolysed by N-sulphuric acid at 100° for 6 hr., and the derived syrups separated on Whatman No. 1 paper eluted with ethyl methyl ketone saturated with water containing 0·1% of ammonia. The paper was dried after elution for 3 hr. and then eluted with the same solvent for a further 6 hr. The portions of paper containing the tetra- and tri-*O*-methyl sugars, together with comparable areas of blank paper, were eluted with water and methanol. The eluates, after concentration to dryness, were made up to a standard volume with water, and the sugar contents of aliquot parts were determined [17] after the preparation of standard graphs. Values given by the blanks were subtracted from the respective sugars. The ratio of tetra- to tri-*O*-methylglucose was 1 : 26.

(iii) The methylated amylopectin from *C. rupestris* (72 mg.) was hydrolysed with 90% formic acid (4 ml.) in an atmosphere of carbon dioxide in a sealed tube at 100° for 6 hr. The solution was cooled and neutralised with Amberlite IR-4B(OH^-) resin, and a portion of the hydrolysate (60·0 mg.) was separated on Whatman 3 MM paper in solvent 6.[11]

Fraction I was crystalline 2,3,4,6-tetra-*O*-methylglucose (5·0 mg.), chromatographically and ionophoretically identical with an authentic specimen run as a control. It had m. p. and mixed m. p. 84°; the derived anilide had m. p. 137°, undepressed on admixture with authentic material.

Fraction II was crystalline 2,3,6-tri-*O*-methylglucose (50 mg.), m. p. and mixed m. p. 115° (from ether), $[\alpha]_D$ +68° (*c* 1·5). The derived diethyl mercaptal had m. p. 70°.[18]

Fraction III was syrupy di-*O*-methylglucose which was not characterised.

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[16] Bishop and Cooper, *Canad. J. Chem.*, 1960, **38**, 388; Beattie and Percival, *Proc. Roy. Soc. Edinburgh*, 1962, **68**, 177.
[17] Dubois, Gilles, Hamilton, Rebers, and Smith, *Analyt. Chem.*, 1956, **28**, 350.
[18] Wolfrom and Georges, *J. Amer. Chem. Soc.*, 1937, **59**, 602.