559. *Polysaccharides of the Characeae. Part I. Preliminary Examination of a Starch-type Polysaccharide from* Nitella translucens.

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The fresh-water green alga, *Nitella translucens*, gives an iodophilic glucan in 4% yield (dry-weight basis). This material, which is very difficult to extract and purify satisfactorily, has been examined by chemical, enzymic, and physical methods.

Extraction by chloral hydrate yields a severely degraded product which has an abnormal potentiometric iodine-titration curve. Extraction by perchloric acid gives a more satisfactory product having a limiting viscosity number of 40; the amylose content is 12%, and the amylopectin component, which has an average chain length of 19 glucose units, appears to be similar in structural properties to plant amylopectins.

The fresh-water green alga, *Nitella translucens* (class, Chlorophyceae; order, Charales; family, Characeae) is of current interest since its long, filamentous, internodal cells (up to 40 cm. long) facilitate certain biophysical and physiological experiments.[1,2] Little is known of the chemical constitution of the Characeae; at the request of the Biophysics Department of this University, the polysaccharide components of both *Nitella translucens* and *Chara australis* have been studied. This paper reports a preliminary study of the iodophilic glucan isolated from *Nitella translucens*.

Both salt-water and fresh-water green algae are included in the Chlorophyceae, and authorities[3] consider species of protozoa, such as *Polytoma*, *Polytomella*, and *Prototheca*, to be colourless members of the Chlorophyceae. Although much is known of the metabolism of algae,[3] few studies of the carbohydrate systems in such materials have been

[1] Mercer, Hodge, Hope, and Maclean, *Austral. J. Biol. Sci.*, 1955, **8**, 1.
[2] Walker, *Austral. J. Biol. Sci.*, 1955, **8**, 476.
[3] Fogg, "The Metabolism of Algae," Methuen, London, 1953.

reported.[4,5] Investigations of algal cell components[5,6] must be distinguished from studies of the soluble extracellular materials exuded by some algae.[7]

According to Fogg,[3] the Chlorophyceae tend to be ancestral to land flora, having starch and fat as reserve materials; the starch is considered to be essentially similar to that from land plants (Fogg, p. 93). Although it is clear that the " paramylon granules "[8] reported in early studies may be starch-like (*e.g.*, for *Polytoma uvella*[9] and *Polytoma obtusum*[10]), recent work has shown that the " paramylon " from *Ochromonas malhamensis*[11] and from *Euglena* species[12] (which do not belong to the Chlorophyceae) resemble laminarin. Recent chemical investigations on *Polytomella coeca*[13] and *Dunaliella bioculata*[5] however support Fogg's statements, but it must not be presumed that the polysaccharide systems of all members of the Chlorophyceae will be similar to those of land plants. Indeed, *Cladophora rupestris*[6,14] is reported to contain laminarin rather than starch. This is of interest since Cronshaw *et al.*[15] found, by *X*-ray and electron-microscopical studies, that *Cladophora rupestris* and *Chaetomorpha melagonium*, from four green marine algae examined, had cell-wall structures resembling those of land plants: it was also observed that chemically differing carbohydrates can give the same *X*-ray diffractogram.[15] Recent *X*-ray evidence[16,17] has suggested the presence of differing " degrees of order " in tuber and algal starches, including an unidentified species of *Nitella*.

An early study[18] of an unnamed species of *Nitella* showed a cellulose-type polyglucan to be present; tests for starch were negative. However, iodophilic starch-like granules, enclosed within a membrane, were later observed[1] in *Nitella* species.

Experimental and Results

Analytical Methods.—Conventional carbohydrate techniques were used throughout.

Collection of Material.—*Nitella translucens* (authenticated by Dr. A. J. Brook) was collected on May 19th, 1959, from Loch Cardney, Dunkeld, Perthshire. The filamentous algal cells were easily snapped, with resultant loss of the cell inclusions, and were therefore handled as carefully as possible during collection. The cells were individually freed by hand from traces of debris and other pond-weeds. The raw material contained: N 2·3, 2·1%; ash 14·1, 13·7%; uronic anhydride 24·1, 24·4%. A methoxyl content of $<0{\cdot}2\%$ may arise from the chlorophyll present.

Preparation of Crude Starch.—The cells were homogenised in water chilled to 0° by brief treatments in an " Atomix " homogeniser. The turbid fluid was poured through a double layer of muslin. The combined residues were re-homogenised with chilled water; this extraction process was carried out 4 times in all.

The dark-green sediment was exhaustively defatted (Soxhlet), giving material which was stained blue-black with iodine solution (yield, 21%, dry-weight basis) (Found: N, 4·6; uronic anhydride, 13·0; ash, 24·4; SO_4'', 0·8%). Paper chromatography of a hydrolysate showed that galactose, glucose, arabinose + mannose, and xylose were present in the ratio 1·5 : 92 : 5·5 : 1; cuprimetric determinations[19] of the total reducing power after hydrolysis indicated 21% of free sugar (as glucose). Calculation therefore shows that the total amount of starch present was $>4\%$ (dry weight basis).

[4] Hirst, *Proc. Chem. Soc.*, 1958, 177.
[5] Eddy, Fleming, and Manners, *J.*, 1958, 2827.
[6] Fisher and Percival, *J.*, 1957, 2666.
[7] Lewin, *Canad. J. Microbiol.*, 1956, **2**, 665.
[8] Gottlieb, *Ann. Chem. Pharm.*, 1850, **75**, 50.
[9] Pringsheim, *Naturwiss.*, 1935, **23**, 120.
[10] Brechot, *Compt. rend. Soc. Biol.*, 1937, **126**, 555.
[11] Archibald and Manners, *Chem. and Ind.*, 1958, 1516.
[12] Clark and Stone, *Biochim. Biophys. Acta*, 1960, **44**, 161.
[13] Bourne, Stacey, and Wilkinson, *J.*, 1950, 2694.
[14] Kylin, *Kgl. Fysiograf. Süllskap. Lund Forh.*, 1944, **14**, 221.
[15] Cronshaw, Myers, and Preston, *Biochim. Biophys. Acta*, 1958, **27**, 89.
[16] Meeuse and Kreger, *Biochim. Biophys. Acta*, 1954, **13**, 593.
[17] Meeuse and Kreger, *Biochim. Biophys. Acta*, 1959, **35**, 26.
[18] Hough, Jones, and Wadman, *J.*, 1952, 3393.
[19] Somogyi, *J. Biol. Chem.*, 1952, **195**, 19.

Attempted Deproteinisation.—The action of (*a*) proteolytic enzymes, (*b*) trichloroacetic acid, and (*c*) a modified Sevag denaturation process, previously found effective in freeing cereal starches from protein,[20] failed to reduce the nitrogen content significantly. Since other protein precipitants had been ineffective on material of somewhat similar origin,[6] methods of extracting the glucan preferentially from the inorganic and proteinaceous material were applied.

(1) *Alkali-swelling and leaching with hot water.* These gave a cream-coloured powder (Found: N, 1·8%). An aqueous solution was stained blue with iodine ($\lambda_{max.}$ 585 mμ). Hydrolysis gave glucose with very small amounts of xylose and arabinose; cuprimetric titration showed that the glucan was 76% pure. On α-amylolysis,[21] conversion into maltose (P_M) = 60% in 6 hr., 76% in 24 hr., and addition of iodine–potassium iodide solution then gave no reaction. On β-amylolysis, P_M = 54 (24 hr.), 59 (48 hr.). 1·15 mol. of periodate were reduced [22] per anhydro-glucose unit after 190 hr.

(2) *Extraction by chloral hydrate.* Exhaustive extraction [23] with 33% aqueous chloral hydrate at 70° under nitrogen gave a pale cream-coloured product (26%). A solution in hot water did not reduce Fehling's solution, had $[\alpha]_D^{16}$ +163° (*c* 1·0% in water), and gave a blue colour with iodine solution (Found: N, 0·8%). Hydrolysis and paper chromatography gave only glucose and xylose (98 : 2). Cuprimetric determinations,[19] with correction for the trace of xylose present, gave the purity of the glucan as 91, 90, 92, 92% (control determinations on purified oat starch [20] gave 96, 99, 97, 96%). On α-amylolysis, P_M = 70% (4 hr.), 82% (24 hr.), 83% (48 hr.). On β-amylolysis at pH 4·6, P_M = 56% (24 hr.), 62% (48 hr.). Viscosity measurements were made [24] in 0·1M-potassium chloride solution in a modified Ubbelohde viscometer. Extrapolation to zero concentration of the usual viscosity graph gives $[\eta]$ = 24·4.

The glucan (51·2 mg.) was oxidised at 0° with sodium metaperiodate [25] in a total volume of 50 ml. with the following results:

Time of oxidation (days)	1	3	6	9	11
Periodate (mol.) reduced per anhydroglucose unit (ref. 26)	0·91	0·95	0·99	1·01	1·03
0·00714N-Formic acid (ml.) released per 5 ml. (ref. 27)	0·25	0·29	0·30	0·30	0·31

Hence the average chain length = 15 anhydro-glucose units, if no amylose was present.

The " blue-value " [28] (B.V.) of the glucan and of ten starches (whose amylose content had earlier been found by the potentiometric method [29]) was determined. The values found are shown in Table 1.

TABLE 1.

Source of starch *	Amylose (%) *	Blue value	Source of starch *	Amylose (%) *	Blue value
Oat	26·0	0·365	Pearl manioc	15·7	0·241
Barley	22·0	0·370	Parsnip	11·1	0·159
Potato I	20·4	0·372	Oat amylopectin	3·2	0·071
Sweet potato	17·8	0·287	Waxy maize	1·4	0·044
Banana	16·8	0·294	*Nitella translucens*	?	0·161
Tapioca	16·7	0·270			

* Origin of samples and amylose contents as quoted in ref. 29.

The graph of B.V. against amylose content (see Fig. 1) indicated that the *Nitella* starch contained 10—11% of amylose. Although there is no simple general relation between blue value and amylose content, an approximately linear relation apparently holds for amylose contents of <18%.

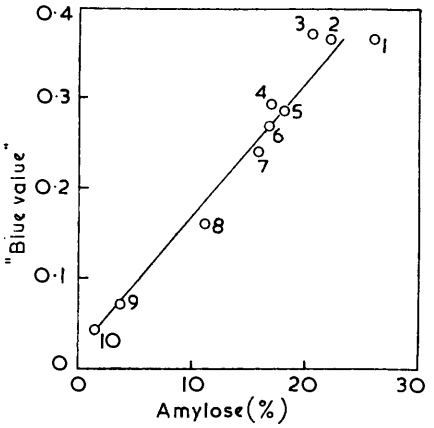
Potentiometric iodine titrations [29] of the glucan gave curve A in Fig. 2, from which it was not possible to obtain the amylose content by extrapolation. The glucan gave no reaction with " concanavalin A " (result by courtesy of Dr. D. J. Manners and Dr. A. Wright).

(3) *Exhaustive extraction with* 30% *aqueous perchloric acid.* Such extraction at room

[20] Anderson and Greenwood, *J. Sci. Food Agric.*, 1955, **6**, 587.
[21] Liddle and Manners, *J.*, 1957, 3432.
[22] Aspinall and Ferrier, *Chem. and Ind.*, 1957, 1216.
[23] Meyer, Brentano, and Bernfeld, *Helv. Chim. Acta*, 1940, **23**, 845.
[24] I.U.P.A.C., *J. Polymer Sci.*, 1952, **8**, 257.
[25] Potter and Hassid, *J. Amer. Chem. Soc.*, 1948, **70**, 3488.
[26] Halsall, Hirst, and Jones, *J.*, 1947, 1399.
[27] Anderson, Greenwood, and Hirst, *J.*, 1955, 225.
[28] Bourne, Haworth, Macey, and Peat, *J.*, 1948, 924.
[29] Anderson and Greenwood, *J.*, 1955, 3016.

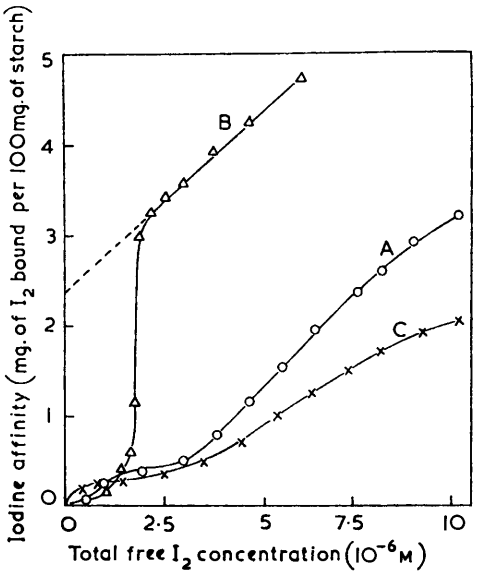
temperature [30] gave a product which was precipitated and purified as iodine complex. The final product was freeze-dried (yield, 5%); it had $\lambda_{max.}$ 585 mμ, $[\alpha]_D^{16}$ +170° (*c* 1% in water) (Found: N = 0·8%). After hydrolysis, glucose + xylose (98 : 2) were detected by paper chromatography; cuprimetric determination [19] showed that the glucan was 90% pure. On α-amylolysis, P_M = 53% (3 hr.), 77% (24 hr.); β-amylolysis at pH 4·6 gave P_M = 50% (24 hr.), 61% (48 hr.). From viscosity measurements, extrapolation of the graph gave $[\eta]$ = 40. Viscosity determinations on parsnip starch [31] and on a commercial sample of waxy maize starch gave $[\eta]$ = 44 and 40 respectively.

FIG. 1. *Plot of amylose content (potentiometric titration) against "blue value" for materials of low amylose content.*



1, oat starch; 2, barley starch; 3, potato starch; 4, banana starch; 5, sweet potato starch; 6, tapioca; 7, pearl manioc starch; 8, parsnip starch; 9, oat amylopectin; 10, waxy maize starch: origins as quoted in ref. 29.

FIG. 2. *Potentiometric iodine-titration curves (standard conditions as in ref. 29) for*: A, *N. translucens* glucan (chloral hydrate), B, *N. translucens* glucan (perchloric acid), C, Protozoal glucan (chloral hydrate) (see ref. 36).



The glucan (101·2 mg.) was oxidised [27] at room temperature with potassium metaperiodate (final vol. 50 ml.) with the following results:

Time of oxidation (days)	3	6	9	11
Periodate (mol.) reduced per anhydroglucose unit (ref. 26)	0·60	0·76	0·95	0·97
0·00714N-Formic acid (ml.) released per 5 ml. (ref. 27)	0·28	0·38	0·42	0·43

Hence the average chain length is 21 units, and that of the amylopectin component (if the glucan contains 12% of amylose) is 19 glucose units. The product resulting from periodate oxidation for 11 days was isolated, dialysed, and hydrolysed. Paper chromatography revealed a small amount of xylose but no glucose.

Potentiometric iodine titration [29] of the glucan (20·02 mg.) gave curve B in Fig. 2. This curve is of similar shape to that given by plant starches and indicates the presence of 12% of amylose.

DISCUSSION

The crude material extracted as a cold-water sediment was very difficult to purify. Initial attempts to deproteinise the starch consumed considerable amounts of material without success (cf. ref. 6). Extraction with cold alkali and hot water gave, in poor yield, a glucan of 76% purity, a result similar to that (69%) reported [5] for *D. bioculata*.

Chloral hydrate gave a relatively pure glucan (90%; cf. ref. 32) which was severely degraded (periodate oxidation and viscosity results) and gave an abnormal potentiometric iodine titration curve (see below).

[30] Pucher, Leavenworth, and Vickery, *Analyt. Chem.*, 1948, **20**, 850.
[31] Anderson and Greenwood, *J.*, 1956, 220.
[32] Archibald, Hirst, Manners, and Ryley, *J.*, 1960, 556.

Although the yield was poor, the glucan extracted by perchloric acid was, comparatively, much less degraded. Indeed, its limiting viscosity number and amylose content were similar to that of parsnip starch which is therefore included in Table 2, together with the present results and the values reported for commercial waxy maize starch, a salt-water alga,[5] and a green seaweed.[33] The properties of algal starches can now be compared [32,33,34] with those of glycogens and floridean, plant, and protozoal starches, though only a preliminary investigation of our glucan could be made with the material available.

TABLE 2.

Source of glucan	*N. translucens* * (fresh-water alga)		*D. bioculata* [5] (salt-water alga)	*C. filiformis* [33] (green seaweed)	Parsnip starch [31]	Waxy maize starch ¶
Method of extraction	perchloric acid	chloral hydrate	perchloric acid	see ref. 33	cold water	commercial sample
$[\alpha]_D$ (c 1% in H_2O)	+170°	+163°	+169°	+154°	+166°	+170°
$\lambda_{max.}$ of I_2 complex (mμ)	585	590	600	540	590	555
Blue value	0·16	0·16	—	—	0·16	0·04
Amylose (%) (I_2 titrn.)	12	?	12—14	0	11 [29]	1 [29]
β-Amylolysis limit	61	62	62	57	72 ‡	54 [34]
α-Amylolysis limit	77	82	85	90	85	88 †
IO_4^- reduction (mole per anhydroglucose unit)	0·97	1·03	—	0·95	1·03 ‖	1·05 [27]
Av. chain length ($\overline{C.L.}$)	21	15	18	21	23 [27]	20 [27]
Hence amylopectin $\overline{C.L.}$	19	13	15—16	21	20·4 [27]	20 [27]
Limiting viscosity no. [η]	40	24	—	15	44	40
Av. internal C.L.§	5—6	3—4	3—4	6—7	6—7	6—7 [34]
Av. external C.L.§	13—14	9—10	12	14—15	13—14	14—15 [34]

* Analytical values are based on the determined glucose content (cf. ref. 32). † Data from *J.*, 1956, 2831. ‡ Data from *Stärke*, 1960, **12**, 169. § As calculated in ref. 21. ‖ D. M. W. Anderson, Ph.D. Thesis, Edinburgh, 1956. ¶ Same sample as used in refs. 27 and 29. A different sample, having $[\alpha]_D$ 212°, [η] 150, is presumably referred to in refs. 33 and 34.

The differences between the products obtained by use of chloral hydrate and perchloric acid suggest that the amylose-type component in algal starches may be highly labile and very easily degraded. Although chloral hydrate was stated [13] to be the best extractant for similar materials, it was later found [5] to cause more extensive degradation (cf. ref. 32) than perchloric acid.

Abnormal iodine-titration curves, similar in shape to that given by the chloral hydrate product, have been noted previously [35] for proteinaceous, floridean, and protozoal starches, for degraded samples, and for certain amylopectins obtained by fractionation; degradation, contamination with protein or waxy materials (cf. ref. 29) and possibly also the presence of abnormal linkages can all distort the normal shape of the titration curve. A value for the amylose content, in good agreement with that found by iodine titration for the perchloric acid product, was, however, deduced from the "blue-value" of the chloral hydrate material. This suggests that the degradation, although severe, had not proceeded below the achroic limit. Since both glucans had very similar nitrogen contents, it is possible that in this instance the abnormal curve resulted primarily from the degradation caused during extraction. The iodine-titration curve (Fig. 2, curve C) given by a

[33] Mackie and Percival, *J.*, 1960, 2381.
[34] Manners and Ryley, *Biochem. J.*, 1955, **59**, 369.
[35] Anderson and Greenwood, unpublished results.

protozoal starch [36] is strikingly similar in shape; since the amylose content was stated—on the basis of a low B.V.—to be negligible,[36] re-examination of protozoal starches extracted [37] with chloral hydrate may now be desirable. It is unusual for no amylose to be detectable in amylopectin-type glucans, *e.g.*, in the " waxy " starches or in fractionation products.

On this basis, the results quoted for an amylopectin-type glucan [33] may also bear re-investigation since the mode of extraction involved addition of a quaternary ammonium salt. Amylose is precipitated by such reagents,[38] and some fractionation may inadvertently have been effected.

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[36] Forsyth and Hirst, *J.*, 1953, 2132.
[37] Forsyth, Hirst, and Oxford, *J.*, 1953, 2030.
[38] Fishman and Miller, *J. Colloid Sci.*, 1960, **15**, 232.