1298. *The Carbohydrates of* Phaeodactylum tricornutum. *Part I. Preliminary Examination of the Organism, and Characterisation of Low Molecular Weight Material and of a Glucan* *

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A water-soluble glucan and low-molecular carbohydrates were separated from an aqueous extract of *Phaeodactylum tricornutum*. After mild chlorite and cold alkali treatment of the residual material a glucuronomannan was extracted with hot dilute alkali.

The low molecular weight material was separated. Laminaribiose, glucose, and laminitol (*C*-methylinositol) were fully characterised, and laminaritriose, galactosyl(1 ⟶ 3)laminaribiose, myoinositol, and scyllitol were identified.

After purification, the glucan was subjected to partial hydrolysis, to methylation, to periodate oxidation, and to Smith degradation. The results showed this polysaccharide to be a typical chrysolaminarin and to comprise β-1,3-linked glucose units with branching at C-6 on some of the units.

P. tricornutum is a microscopic marine diatom belonging to the Bacillariophyceae class of algae and the Phylum Chrysophyta. It was earlier confused with *Nitzschia closterium* var *minutissima* but is now classed in the order Bacillariales in a new sub-order *Phaeodactylineae* of which it is the only member.[1] Since the isolation of the original culture [2] its nutritional and environmental requirements have been widely studied.[3] It is apparently a food of zooplankton and a major constituent in the diets of mussels and oysters.[4] It is pleomorphic, producing ovoid, fusiform, triradiate, and cruciform cells.[5]

The *P. tricornutum* used in this investigation was grown under bacteria-free conditions by Michael Droop at the Marine Biological Station, Millport, in culture medium S68 [6] except that potassium nitrate and not L-valine was the nitrogen source, and atmospheric carbon dioxide and not sodium acetate was the carbon source. The organisms were centrifuged from 630 litres of culture medium and freeze-dried to a dark green solid (85 g.; containing 10·6 g. of inorganic salts). Preliminary investigation revealed a carbohydrate content of *ca.* 20% tentatively identified as comprising glucose, mannose, and smaller amounts of acidic material, xylose, rhamnose, and ribose.

* A brief account of this work and that of the following Paper was given at the International Carbohydrate Symposium, Münster, July 1964.

[1] J. C. Lewin, *J. Gen. Microbiol.*, 1958, **18**, 427.
[2] E. J. Allen and E. W. Nelson, *J. Marine Biol. Assoc.*, 1910, **8**. 421.
[3] C. P. Spencer, *J. Marine Biol. Assoc.*, 1954, **33**, 265; J. E. G. Raymont and M. N. E. Adams, *Limnology and Oceanography*, 1958, **3**, 118.
[4] P. R. Walne, *J. Marine Biol. Assoc.*, 1963, **43**, 778.
[5] N. I. Hendey, *J. Marine Biol. Assoc.*, 1954, **33**, 335.
[6] M. Droop, *J. Marine Biol. Assoc.*, 1959, **38**, 615.

The best method for complete extraction of the carbohydrate was found to be initial rupturing of the cell walls by thorough grinding, after freezing the organism in liquid nitrogen. Decolourisation without removal of carbohydrate was achieved by extracting the powdered organism with butan-1-ol. Exhaustive cold-water treatment of the decolourised material extracted both polysaccharide and low molecular weight carbohydrate. The former was separated by alcohol precipitation. It consisted mainly of glucose units and is known hereinafter as the glucan. The residual aqueous ethanolic solution on concentration and freeze-drying gave a pale green solid (20% of the dry weight of the organism) comprising inorganic material, *ca.* 20% of reducing carbohydrate, colouring matter, and other impurities.

Further extraction of the residual organism with hot water under nitrogen gave additional quantities of the glucan and of the same low molecular weight material (6%). They were therefore combined with the respective cold-water extracts. Mild chlorite treatment of the residual *P. tricornutum* material removed a high proportion of protein and negligible carbohydrate. Subsequent treatment with cold alkali also removed little carbohydrate, and it was found that the residual solid still contained about 18% of carbohydrate. This residue was found to dissolve completely in hot 4% alkali. Addition of ethanol to the neutralised solution precipitated polysaccharide material which comprised mainly mannose with less glucuronic acid and is hereinafter known as the glucuronomannan (see Part II).

The pale green solid separated from the combined cold and hot water extracts was freed from colouring matter with activated charcoal, some of the inorganic salts were deposited on concentration, and the rest removed by treatment with resins. Chromatographic examination of the derived syrup revealed the presence of four reducing and two non-reducing compounds. After separation, these were identified as glucose, laminitol (*C*-methylinositol), laminaribiose, laminaritriose, galactosyl(1 ⟶ 3)laminaribiose, myoinositol, scyllitol, and a disaccharide containing glucose and xylose residues. The first three of these substances were fully characterised.

Owing to their microscopic size and hence the difficulty of collecting appreciable quantities of diatoms, very little previous chemical investigation of these organisms has been carried out. The identification of these substances in *P. tricornutum* is therefore of considerable biological importance. Two oligosaccharides comprising two and three glucose residues were previously reported [7] from a mixture of diatom species. But this is the first reported occurrence of laminari-biose and -triose in diatoms. The former has been found in the brown seaweed *Laminaria cloustoni* and is a possible intermediate in the biosynthesis of laminarin in the brown algae and of the β-1,3-linked glucan isolated from the present organism. Laminitol was previously isolated from a number of brown seaweeds and from the red seaweeds *Gelidium cartilagineum* and *Porphyra umbilicalis*.[8] Myoinositol occurs widely in algae and has been suggested [9] as the intermediate in the biosynthesis of uronic acid and pentose-containing polysaccharides, and is a possible precursor of the glucuronomannan which appears to constitute the cell-wall polysaccharide of *P. tricornutum* (see Part II).

The food reserve polysaccharide of the Chrysophyta to which *P. tricornutum* belongs has been given different names in the different classes, but it appears that leucosin, volutin, and chromatin are the same substance.[10] Leucosin has been regarded as a polysaccharide since 1893,[11] but the difficulty of obtaining sufficient quantity of a pure species of any of these organisms precluded structural investigations of this polysaccharide, and it has been variously described as a polyfructose, a polyglucose, etc. Quillet [12] found that leucosin

[7] G. K. Baraskov, *Doklady Akad. Nauk S.S.S.R.*, 1956, **111**, 148.
[8] B. Lindberg and J. McPherson, *Acta Chem. Scand.*, 1954, **8**, 1875; B. Lindberg, *ibid.*, 1955, **9**, 168, 1097, 1323.
[9] F. A. Loewus, *Nature*, 1964, **203**, 1175.
[10] G. M. Smith, "Fresh Water Algae of the U.S.A.," McGraw-Hill, London, 1933, p. 199; P. Gavaudin, *Compt. rend.*, 1932, **194**, 2075.
[11] G. Klebs, Flagellatenstudien II, *Z. wiss. Zool.*, 1893, **55**, 401.
[12] M. Quillet, *Compt. rend.*, 1955, **240**, 1001.

from *Hydrurus foetidus* had $[\alpha]_D$ −6°, gave no colour with iodine, and gave only glucose on hydrolysis. Paper-chromatographic analysis of a partial hydrolysate showed six evenly spaced spots from which this author deduced that the polysaccharide comprises at least eight anhydroglucose units. In view of the ambiguity of the term leucosin he suggested the name chrysolaminarin. A large scale extraction and crystallisation of this material from mixed species of Chrysophyceae by von Stosch,[13] and a comparative study of its structure with that of laminarin,[14] established that it was an essentially linear β-1,3-linked glucan with a small degree of branching at C-6, but that it differed from laminarin in the apparent absence of mannitol residues.

The crude glucan isolated from the aqueous extract of *P. tricornutum* constituted *ca.* 14% of the dry weight of the organism. It contained about 55% of carbohydrate, and purification by fractional precipitation with ethanol, by extraction with toluene, or with phenol proved unsatisfactory, low yields of only slightly purer material being obtained. Gradient elution on a DEAE-cellulose column gave 35% yield of a pure glucan, containing 90% of carbohydrate and about 10% of residual moisture. It gave only glucose on hydrolysis.

The essential similarity of this material to the β-1,3-linked glucan, laminarin, was established by parallel partial acidic and enzymic hydrolyses and by periodate oxidation studies; the major difference being the absence of mannitol in the present polysaccharide.

The greater oxidation of periodate by *P. tricornutum* glucan could be due to the fact that over-oxidation occurs in all the chains whereas the mannitol-ended chains in laminarin resist over-oxidation. The slower oxidation of the former in very dilute periodate, particularly at 2° (Table 2) could again be due to the mannitol end-groups in laminarin which are probably oxidised more readily than the glucopyranose units under these mild conditions.

Methylation studies on *P. tricornutum* glucan supported the above results. The presence of gentiobiose in both acid and enzymic hydrolysates, and of 2,4-di-*O*-methylglucose in the methylated sugars, indicates some branching at C-6.

The fact that glycerol was the sole cleaved product of Smith degradation (periodate oxidation, reduction, and mild hydrolysis) was further evidence that this polysaccharide is indeed essentially the same as the chrysolaminarin found in other diatoms, and comprises a β-1,3-linked glucan with some branching at C-6.

It should be pointed out that we examined a sample of *P. tricornutum* grown in seawater tanks in California (through the courtesy of J. C. Lewin and Jim Love) and found that it contained negligible amounts of chrysolaminarin. It appears that the artificial conditions used in the culture of the material in Scotland favoured the storage of this polysaccharide.

Experimental

Analytical Methods.—Paper chromatography was carried out on Whatman No. 1 paper with the following solvents (v/v): A, ethyl acetate–acetic acid–formic acid–water (18 : 3 : 1 : 4); B, ethyl acetate–pyridine–water (10 : 4 : 3); C, butan-1-ol–ethanol–water (40 : 11 : 19); D, ethyl methyl ketone–water (10 : 1 + 1% NH_4OH); E, butan-1-ol–acetic acid–water (2 : 1 : 1); F, butan-1-ol–acetone–water (5 : 3 : 2); G, butan-1-ol–pyridine–water (6 : 4 : 3). Papers were sprayed with (1) saturated aqueous aniline oxalate, (2) a solution of *p*-anisidine hydrochloride (5%) in butan-1-ol (for both of these sprays the paper was heated at 120° for 5 min.), (3) silver nitrate, (4) sodium metaperiodate followed by potassium permanganate,[15] (5) Bromocresol Green (0·1% solution in 95% ethanol made just alkaline with sodium hydroxide. R_g = chromatographic mobility relative to that of glucose. M_{GA} = distance on ionophoresis at pH 7 between the sugar and glucose relative to the distance between glucuronic acid and glucose. Ionophoresis was carried out in the following buffers: borate (pH 10),[16] phosphate (pH 7), and sodium tungstate (pH 5).[17]

[13] H. A. von Stosch, 3rd International Seaweed Symposium, Galway, 1958.
[14] A. Beattie, E. L. Hirst, and E. Percival, *Biochem. J.*, 1961, **79**, 531.
[15] R. U. Lemieux and H. F. Bauer, *Analyt. Chem.*, 1954, **26**, 920.
[16] A. B. Foster, *Chem. and Ind.*, 1952, 1050.
[17] H. J. F. Angus, E. J. Bourne, and H. Weigel, *J.*, 1965, 21.

Gas chromatography (g.l.c.) was carried out on a Pye Argon Chromatograph. The stationary liquid phase was supported on Celite and was either (1) butanediol succinate polyester or (2) polyphenyl [*m*-bis-(*m*-phenoxyphenoxy)benzene], at an operating temperature of 175°. Retention times are relative to that of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside.[18]

Unless otherwise stated acid hydrolysis was with N-sulphuric acid at 100° for 4 hr., and the hydrolysate was neutralised with barium carbonate, filtered, and concentrated. Reductions were with sodium borohydride buffered with 0·5M-boric acid. Methanolysis and glycosidation were carried out by refluxing the sugars with 3% dry methanolic hydrogen chloride for 6 hr. The solution was neutralised with silver carbonate, filtered, the silver salts were washed thoroughly with dry methanol, and the combined filtrate and washings concentrated to a syrup.

Preliminary Hydrolysis of the Whole Organism.—*P. tricornutum* organism (200 mg.) was hydrolysed, and, after neutralisation, the resulting solution was treated with Amberlite IR-120 (H^+) resin and then made up to 10 ml. with water. Quantitative analysis [19] of an aliquot gave a carbohydrate content of *ca.* 20% of the dry weight of the organism,. Chromatography (solvents A and B, spray 1) of the syrup derived from the rest of the solution gave spots with the mobilities and colours of xylose, mannose, glucose, ribose (trace), and rhamnose (trace), together with a number of slower acidic and neutral spots. The solution obtained on cold-water extraction of the organism (100 mg.) had a reducing power [19] equivalent to 2·3% of sugar, and this, after acid hydrolysis, rose to 9·5%.

Extraction of Carbohydrate.—The organisms (10 g.) were frozen in liquid nitrogen and then thoroughly ground to a fine powder. This was stirred with butan-1-ol (250 ml.). The liquid quickly became dark green and was replaced by fresh butan-1-ol after 1, 4, 12, and 24 hr. A further two 24-hr. extractions were necessary before the butan-1-ol remained colourless. The extracts contained negligible carbohydrate.

Extraction of the decolourised material with water (6 × 200 ml.) during 3 days with stirring at room temperature was followed by concentration of the combined extracts and addition of ethanol (10 vol.) to the solution. The derived precipitate was isolated by freeze-drying (1·43 g.) (Found: SO_4^{2-}, nil; uronic anhydride,[20] nil; N_2, 1·95; ash, 4·2%). Analysis gave a carbohydrate content of 55%,[21] and chromatography of a hydrolysate revealed glucose with trace quantities of xylose, mannose, and rhamnose.

The aqueous alcoholic supernatant was concentrated to dryness, the residue taken up in water and freeze-dried to a pale green solid (A) (2·01 g.). This contained 20% carbohydrate,[21] and chromatography revealed glucose and other slower reducing spots.

The residual material from the cold-water extraction was stirred with water (250 ml.) in an atmosphere of nitrogen on a boiling-water bath for 5 hr. The extracts treated in the same way as the cold-water extract gave alcohol-insoluble material (460 mg., containing 31% of carbohydrate), and from the supernatant a solid (620 mg., containing 13% of carbohydrate). Both products, the former after hydrolysis, gave chromatograms similar to the respective materials obtained from the cold-water extraction.

The solid remaining from the hot-water extraction was given a mild chlorite treatment [22] and the derived chlorite solution contained negligible carbohydrate and comprised a high proportion of protein as shown by a nitrogen content of 5% and the detection of amino-acids (paper chromatography, solvent C, spray ninhydrin) in a hydrolysate.

Treatment of the bleached residual solid with 4% sodium hydroxide solution (200 ml.) for 24 hr. with stirring at room temperature in an atmosphere of nitrogen left residual material (1·79 g., containing *ca.* 18% carbohydrate [19]) which was isolated by freeze-drying after washing free from alkali. Addition of acetic acid and of ethanol to the alkaline solution gave precipitates (0·75 and 0·79 g., respectively) which were devoid of carbohydrate. On treating the residual material with 4% sodium hydroxide under nitrogen on a boiling-water bath it almost completely dissolved (residue about 15 mg.). Neutralisation with glacial acetic acid failed to yield a precipitate. The solution was dialysed and concentrated. Addition of ethanol (10 vol.) to the concentrate gave a precipitate which was isolated, after dissolution in water, by

[18] G. O. Aspinall, *J.*, 1963, 1676.
[19] M. Somogyi, *J. Biol. Chem.*, 1952, **195**, 19.
[20] B. Tollens, *Ber.*, 1908, **41**, 1788.
[21] M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Analyt. Chem.*, 1956, **28**, 350.
[22] L. E. Wise, *Ind. Eng. Chem.* (*Analyt. Ed.*), 1946, 290.

freeze-drying (450 mg.). This had $[\alpha]_D$ +34° and gave, on hydrolysis, mannose and glucuronic acid (paper chromatography), and is hereinafter known as the glucuronomannan. Its structural investigation is described in Part II.

Low Molecular Weight Carbohydrates.—Solid (A) from a number of aqueous extracts of *P. tricornutum* (total 40 g.) was dissolved in aqueous ethanol (10% v/v ethanol) and treated with activated charcoal. The filtrate was concentrated (*ca.* 5 ml.) and set aside for some days. Inorganic material crystallised out and was removed by centrifugation. This procedure was repeated until no more crystals were deposited. The final solution was concentrated to a syrup which was dissolved in water and finally freed from inorganic ions by passage through columns of IR-120 (H^+) and IR-45 (OH^-) resins, and the eluate was concentrated to a colourless syrup (1·5 g.). Chromatography (solvent A, spray 1) revealed spots with R_g 1·15, 1·0, 0·51, 0·22, and 0·13, and in addition (spray 3) 0·67 and 0·45.

Separation (750 mg.) was attempted on a cellulose column with solvent G and on a second column with solvent A. Partial separation was achieved with the latter solvent, and mixed fractions were re-separated on 3MM paper with solvent A.

Fraction 1. A syrup (20 mg.), R_g 1·15 (solvents A, B, and F; brown spot, spray 1), 0·33 (tungstate-impregnated paper; pH 8 in solvent F [23]); M_G (pH 10) 0·82. Chromatography of a hydrolysate revealed spots with the mobilities of glucose and xylose (solvents A and B, sprays 1 and 3). Reduction of an aliquot was followed by hydrolysis, and the resulting syrup contained xylose and an unidentified non-reducing substance R_g 1·0 (solvent A), 0·81 (solvent B); M_G (pH 10) 0·86.

Fraction 2. A syrup (12 mg.), R_g 1·0 (solvents A, B, and F); M_G (pH 10) 1·0. After incubation of an aliquot (5 mg.) at 36° with the enzyme glucose oxidase the derived syrup was chromatographically (solvents A and B, spray 3) and ionophoretically (pH 7) identical with gluconic acid. Reduction of a second aliquot (6 mg.) and chromatography (tungstate-impregnated paper, solvent F, spray 3 [23]) and ionophoresis (sodium molybdate, pH 5) of the reduced material revealed a single spot $R_{sorbitol}$ 1·0, $M_{sorbitol}$ 1·0, respectively.

Fraction 3. Crystallised from 50% aqueous ethanol as colourless prisms (3·5 mg.), R_g 0·67 (solvent A), M_G (pH 10) 0·82, and m. p. and mixed m. p. with laminitol (*C*-methylinositol) 260—262° (kindly donated by Dr. B. Lindberg), and was chromatographically and ionophoretically identical with this authentic material.

Fraction 4. A chromatographically pure syrup (4 mg.) which had D.P. 2·1,[24] R_g 0·51 (solvent A), and M_G (pH 10) 0·57, both identical with laminaribiose, and gave only glucose on hydrolysis. Incubation of an aliquot (2 mg.) with β-1,3-glucanase at 36° for 24 hr. and chromatographic examination of the resulting solution revealed only glucose.

Fraction 5. A syrup (10 mg.), R_g 0·45 (solvent A, revealed only by spray 3), 0·35 and 0·30 (solvent F, the latter on tungstate-impregnated paper pH 8 [23]) (identical with myoinositol and/or scyllitol run as controls). Ionophoresis at pH 10 revealed two components with M_G 0·04 and 0·46 (slow development of spots, spray 3) identical with myoinositol and scyllitol, respectively. In 0·2M-arsenite buffer [25] at pH 9·6 a single red spot was detected with the same mobility as myoinositol and different from (+)-inositol. These were detected [26] by dipping the dried ionophoretogram in a mixture of concentrated nitric acid and ethanol (1 : 9) and heating at 95° for 10 min., and spraying with a 5% solution of calcium chloride in 50% aqueous ethanol and re-heating at 95° for 15 min. Scyllitol does not react [25] with nitric acid–calcium chloride.

Fraction 6. A chromatographically pure syrup (52 mg.), R_g 0·22 (solvent A), M_G (pH 10) 0·53, D.P.[24] 3·1, which gave on hydrolysis glucose and galactose (solvents A and F, sprays 1, 2, 3, and 4) in the ratio of *ca.* 3 : 1 (visual examination). Paper chromatograms impregnated with sodium tungstate buffer at pH 8 were used with solvent F to give an improved separation of glucose and galactose.[23] After reduction, hydrolysis gave glucose, galactose, and sorbitol. Incubation of an aliquot (5 mg.) with a specific β-1,3-glucanase at 36° gave glucose and a second spot, R_g 0·5 (paper chromatography). Prolonged incubation did not alter the chromatographic pattern. Reduction of the incubation solution with borohydride, and ionophoretic examination (pH 5) of the product gave spots with the mobilities $M_{sorbitol}$ 1·0 and 0·0 (spray 3) (cf. reduced 1,3-linked disaccharides which have $M_{sorbitol}$ 0·0 [23]). An aliquot (5 mg.) of the initial

[23] N. Sufi and H. Weigel (unpublished results).
[24] T. E. Timmell, *Svensk Papperstidn.*, 1960, **63**, 668.
[25] J. L. Frahn, *Aust. J. Chem.*, 1964, **17**, 274.
[26] See H. G. Fletcher, *Adv. Carbohydrate Chem.*, 1948, **3**, 45.

syrup was dissolved in dimethylformamide (1 ml.) and methylated in dimethylformamide with methyl iodide and silver oxide.[27] G.l.c. of the methanolysed product gave peaks with the retention times of methyl 2,3,4,6-tetra-*O*-methyl-glucosides, -galactosides, and methyl 2,4,6-tri-*O*-methylglucosides. Acid hydrolysis of the methanolysate and separation of the derived sugars on Whatman No. 1 paper with solvent C gave: *fraction* (i), a syrup, R_G 1·0, which on demethylation [28] gave only glucose; *fraction* (ii), R_G 0·92, which was chromatographically identical with 2,3,4,6-tetra-*O*-methylgalactose; *fraction* (iii), R_G 0·85 and M_G (pH 10) 0·0, chromatographically and ionophoretically identical with 2,4,6-tri-*O*-methylglucose (cf. 2,3,6-tri-*O*-methylglucose, R_G 0·88); *fraction* (iv), a trace quantity, R_G 0·77, identical in mobility and colour with 2,4,6-tri-*O*-methylgalactose.

These results indicate that fraction 6 is probably a mixture of *O*-D-glucosyl(1 ⟶ 3)-D-glucosyl(1 ⟶ 3)-D-glucose (laminaritriose) and *O*-D-galactosyl(1 ⟶ 3)-D-glucosyl(1 ⟶ 3)-D-glucose (galactosyl-laminaribiose).

Purification of the Glucan.—The crude glucan (950 mg.) isolated from the cold-water extract, dissolved in the minimum quantity of water, was applied to the top of a DEAE-cellulose column (55 g.) in the chloride form,[28] and eluted with increasing concentration of potassium chloride (0 ⟶ 0·5N). Eluate was collected in 25-ml. fractions and tested for the presence of carbohydrate.[21] The main fraction (250—425 ml.) was concentrated, dialysed, and the solution freeze-dried to a white solid (340 mg.). It had a carbohydrate content of 90%,[21] and gave only glucose on hydrolysis (Found: SO_4^{2-}, nil; N_2, nil). It had $[\alpha]_D$ +42° (*c* 0·58) and gave no colour with iodine. A minor fraction (450—650 ml.) (25 mg.) had a carbohydrate content of 65% [21] (based on glucose) and gave on hydrolysis glucose, xylose, and mannose (paper chromatography). This was not examined further. Subsequent gradient elution of the column with 0·5N-potassium chloride ⟶ 0·5N-potassium hydroxide failed to elute any more carbohydrate.

Examination of the Purified Glucan.—(a) *Characterisation of the glucose.* The hydrolysed polysaccharide (5 mg.) after neutralisation was incubated with the enzyme glucose oxidase at 35° for 24 hr. Chromatography (solvents A and B, sprays 3 and 5) and ionophoresis (pH 7, spray 3) of the resulting syrup revealed a single spot with the mobility of gluconic acid.

(b) *Partial acid hydrolysis.* The glucan (5 mg.) and laminarin (5 mg.) were hydrolysed with 0·1N-sulphuric acid at 100° for 1 hr. After cooling, neutralisation, and concentration to a syrup, chromatography (solvent A, spray 1) revealed spots from both polysaccharides with R_g 1·0, 0·53, 0·38, and 0·30, identical with those given by glucose, laminaribiose, gentiobiose, and laminaritriose, respectively. Material with smaller mobility was also visible.

(c) *Enzymic hydrolysis.* The glucan (2 mg.) and laminarin (2 mg.), each in citrate buffer (pH 5) (0·1 ml.), were separately treated with endo-β-1,3-glucanase, and the solutions kept at 38° for 12 hr. Chromatography revealed spots with the mobility of glucose, laminari-biose, -triose, and gentiobiose. Incubation for 72 hr. with the enzyme left only glucose, R_g 1·0, and gentiobiose, R_g 0·38.

(d) *Methylation.* The glucan (200 mg.), after being stirred at room temperature overnight with sodium borohydride (1%; 20 ml.), was treated dropwise at 2° in an atmosphere of nitrogen with sodium hydroxide (20 ml.; 30%) and dimethyl sulphate (8 ml.) during 6 hr. with vigorous stirring. Thereafter the stirring was continued at room temperature overnight. The procedure was repeated four times at room temperature. After dialysis and concentration, the partly methylated polysaccharide was isolated as a white solid (250 mg.) by freeze-drying. A second series of five similar methylations gave a freeze-dried solid (223 mg.) (Found: OMe, 31·5%) which on infrared analysis showed a small amount of free hydroxyl. It was therefore subjected to four Purdie methylations and the product shown by infrared spectroscopy to be devoid of free hydroxyl groups. Methanolysis and gas chromatography gave peaks with the retention times of methyl 2,3,4,6-tetra-*O*-methyl-, 2,4,6-tri-*O*-methyl- (major), and di-*O*-methyl-glucosides. The methanolysed material was hydrolysed with N-sulphuric acid at 100° for 6 hr. After neutralisation, the derived mixture of methylated sugars was separated on 3MM paper with solvent C. The tetra-*O*-methylglucose had the correct chromatographic mobility, but the quantity was too small for further characterisation. The 2,4,6-tri-*O*-methylglucose crystallised and had m. p. and mixed m. p. 122—123°, and was chromatographically identical in solvents C and D with

[27] O. Perila and C. T. Bishop, *Canad. J. Chem.*, 1961, **39**, 815.
[28] M. A. Jermyn, *Austral. J. Biol. Sci.*, 1962, **15**, 787.

authentic material. A trace quantity of material with the mobility of a dimethylglucose was separated. Demethylation [29] gave only glucose. Ionophoresis (pH 10) identified it as 2,4-di-*O*-methylglucose, authentic di-*O*-methylglucoses being run as controls.[30]

(e) *Periodate oxidation.* (i) *Phaeodactylum* glucan (18·2 mg.) and laminarin (22·7 mg.) were oxidised in the dark with 0·015M-sodium metaperiodate (20 ml.) at 2° for 2 days, and thereafter the oxidation was allowed to proceed at room temperature. Aliquots were removed from time to time and the quantity of periodate reduced measured.[31]

TABLE 1

Moles of periodate reduced per C_6 anhydro unit

Time (days)	2°	1	2	Room temp.	3	4	5	6	7
P.t. glucan ...		0·211	0·225		0·277	0·416	0·484	0·526	0·538
Laminarin ...		0·217	0·228		0·358	0·389	0·389	0·389	

(ii) Oxidation was repeated under the same conditions with 0·0015M-periodate and *Phaeodactylum* glucan (21·3 mg.) and laminarin (20·9 mg.).

TABLE 2

Moles of periodate reduced per C_6 anhydro unit

Time (days)	2°	1	2	Room temp.	3	4
P.t. glucan		0·068	0·094		0·147	0·172
Laminarin		0·154	0·171		0·206	0·213

(f) *Smith degradation.* *Phaeodactylum* glucan (57·5 mg.) was oxidised with sodium metaperiodate (168·5 mg.) in the dark in acetate buffer (pH 6; 5 ml.) for 110 hr. After destruction of excess periodate with sulphur dioxide, the oxopolysaccharide was precipitated with ethanol (10 vol.). It was dissolved in boric acid buffer [32] (0·05M; 3 ml.), and reduced with sodium borohydride (300 mg. in 2 ml.) during 12 hr. The recovered polyalcohol was treated with N-sulphuric acid (2 ml.) at room temperature for 48 hr. Addition of ethanol (10 vol.) led to the isolation, by freeze-drying, of polysaccharide (30 mg.). The supernatant, after neutralisation, was concentrated, and showed on a paper chromatogram only a trace of glycerol (solvent B, spray 3). Chromatography of the hydrolysate of the precipitated polysaccharide revealed a single spot with the mobility of glucose.

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[29] T. G. Bonner, E. J. Bourne, and S. McNally, *J.*, 1960, 2929.
[30] I. M. Mackie and E. Percival, *J.*, 1959, 1151.
[31] G. O. Aspinall and R. J. Ferrier, *Chem. and Ind.*, 1957, 1216.
[32] M. L. Wolfrom and B. O. Juliano, *J. Amer. Chem. Soc.*, 1960, **82**, 1675.