1052. *Polysaccharides of the Characeae. Part IV.*[1] *A Non-esterified Pectic Acid from* Nitella translucens.

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The pectic complex extractable from the fresh-water green alga *Nitella translucens* contains a non-esterified pectic acid. This is best purified by precipitation as the copper complex, followed by incubation with α-amylase to remove contaminating glucans. The pectic acid had $[\alpha]_D$ +245° and contained 74% of uronic anhydride; hydrolysis gave galacturonic acid, with galactose, arabinose, xylose, and rhamnose in the ratio 4 : 6 : 3 : 1. Partial hydrolysis indicated that a high proportion of the arabinose and xylose residues were labile, the galactose being resistant to hydrolysis. Passage through diethylaminoethylcellulose gave one main fraction only. Methylation studies established the presence of chains of 1,4′-linked-α-D-galacturonic acid. This pectic acid is of interest in studies of the ion-exchange capacity of the algal cell-wall.

Recent studies [1] revealed that the fresh-water green algæ *Nitella translucens* and *Chara australis* have clear similarities to higher terrestrial plants on the basis of their carbohydrate content. This is of interest since the botanical relationship of the *Charaphytae* to (*a*) *Chlorophytae* and (*b*) higher terrestrial plants is debated.[1]

The cell-walls of *Chara* and *Nitella* contain significant quantities of pectic materials;[1] these appear to perform ion-exchange functions similar to those of pectins in the roots of

[1] Parts II and III, Anderson and King, *Biochim. Biophys. Acta*, 1961, **52**, 441, 449,

higher terrestrial plants.[1] Pectin has so far not been isolated from marine sources.[2] The precise function of pectic materials in plant cells has long been in question.[2] When it was found that the pectic acid in these Characeae was non-esterified, it became important to establish the extent of its differences from the known plant pectic acids. Algal polysaccharides have previously provided many novel structural features:[3,4] indeed, *N. translucens* contains also an interesting labile starch-type glucan.[5]

The origin and collection of the alga, analyses of the dried cells (MeO $\gtrsim$ 0·1%), and the extraction procedures have been described.[1] Although the material extracted by water at 90° (fraction N5, ref. 1) contained 10·6% of galacturonic acid and had no methoxyl content, it was shown (Table 1, ref. 1) that this fraction accounted for only 1% of the total uronic anhydride content of the cells. Negligible amounts of pectic substances were therefore extracted in the hot-water and other preliminary treatments. In general, the lower the methoxyl content, the more difficult is the hot-water extraction of pectic substances from plant tissues.[6]

Extraction of the algal residues with hot aqueous ammonium oxalate–oxalic acid solution [7] gave ammonium pectate. Paper chromatography of a hydrolysate showed that galacturonic acid, galactose, glucose, arabinose, xylose, and rhamnose were present (Found: $[\alpha]_D$ +205°; uronic anhydride, 51%; Ac, SO_4, and OMe, 0). Despite attempted purification by precipitation with organic solvents, cetylpyridinium bromide, ammonium sulphate, as the calcium salt, and as the copper complex, no fractionation was achieved and none of the neutral sugars originally present was eliminated. Regeneration from the copper complex gave the best product, having $[\alpha]_D$ +240°, uronic anhydride 72% (both as free pectic acid). Subsequent incubation with salivary α-amylase removed the glucose-containing contaminant; the incomplete extraction of starch by cold and hot water [8] and the contamination of pectin by starch are well known.[9,10] The glucose-free pectic acid, which contained 74% of uronic anhydride, together with galactose, arabinose, xylose, and rhamnose in the ratio 4 : 6 : 3 : 1, was used in the experiments reported in this paper. Decarboxylation studies made later indicated [11] that this material had probably undergone about 8% of decarboxylation during its extraction (these studies assumed that all the carbon dioxide evolved came from 6-carboxyl groups).

Fractionation on a column of diethylaminoethylcellulose [12] gave one major component (see Table) which accounted for 74% of the material recovered from the column and for which the optical rotation, uronic anhydride, and proportions of neutral sugars were almost identical with those of the unfractionated material. Seventeen other fractions, none of which accounted for more than 2·5% of the material recovered, were also investigated. The significance of these must await further investigation with larger quantities of material; however, hydrolysis of some of the fractions eluted before the main peak gave two sugars of high chromatographic mobility (cf. refs. 13 and 14), and one of the fractions eluted after the main peak gave only galactose on hydrolysis, indicating that minor quantities of a galactan were present in the pectic complex (cf. ref. 2).

Hydrolysis at pH 5·0 with fungal pectinase gave the crystalline sodium–calcium double salt [15] of galacturonic acid in good yield. Partial hydrolysis (0·1N-hydrochloric

[2] Hirst and Jones, *Adv. Carbohydrate Chem.*, 1946, **2**, 235.
[3] Smith, *Ann. Reports*, 1956, **53**, 259.
[4] Hirst, *Proc. Chem. Soc.*, 1958, 177.
[5] Anderson and King, *J.*, 1961, 2914.
[6] Kertesz, "The Pectic Substances," Interscience Publ. Inc., New York, 1951.
[7] Williams and Bevenue, *J. Off. Agric. Chemists*, 1956, **39**, 901.
[8] Anderson and Greenwood, *J. Sci. Food Agric.*, 1955, **6**, 587.
[9] Taufel and Feldman, *Chem. Tech. (Berlin)*, 1954, **6**, 525.
[10] Bock, Baum, Döring, and Wardsack, *Ernährungsforsch.*, 1960, **5**, 539.
[11] Anderson, Bews, Garbutt, and King, *J.*, 1961, 5230.
[12] Neukom, Deuel, Heri, and Kundig, *Helv. Chim. Acta*, 1960, **43**, 64.
[13] Aspinall and Cañas-Rodriguez, *J.*, 1958, 4020.
[14] Aspinall and Fanshawe, 1961, 4215.
[15] Isbell and Frush, *J. Res. Nat. Bur. Stand.*, 1944, **32**, 77.

Fractionation of pectic acid on diethylaminoethylcellulose.

Fraction	Total vol. eluted (ml.)	Wt. of fraction (mg.)	Sugars identified					$R_{rhamnose}$ of unidentified sugars (solvent C)
			Gal. Acid	Gal	Ara	X	Rha	
1	40	2	++	++	++		+	
2	220	7	+	++	++		+	
3	420	7	+	++	++		+	
4	670	4	+	++	++		+	
5	690	3	+	++	++			1·21(+), 1·89(+)
6	830	5	+	++	++		+	1·24(+)
7	960	6	+	+	+			
8	1130	7	+	+	+		+	1·74(+)
9	1150	3	+	+++	+			
Elution by sodium hydroxide begun.								
10	1350	10	+	+++	+	+	+	1·22(+)
11	1410	11	+	++++	+	++		oligos. ++
12	1560	3	++	++	+	+		oligos. ++
13	1610	9	++	++	+			oligos. +
14	1840	295	71%	+	+			oligos. +++
15	1930	9	+	+++	+			oligos. +
16	2150	8	+	+++				
17	2250	9	—	+++				
18	2400	3	—	+++	+			
		401						

acid, 2 hr., 100°) gave an insoluble, degraded polysaccharide having 83% of uronic anhydride; the sugars in the hydrolysate were galactose, arabinose, xylose, and rhamnose (3 : 10 : 6 : 1), with galacturonic acid also present. The pectic acid therefore contained labile arabinose and xylose. Further hydrolysis (2N-sulphuric acid, 6 hr., 100°) of the degraded polysaccharide gave galacturonic acid, and galactose, arabinose, xylose, and rhamnose (3 : 1 : 1 : 1), but hydrolysis was incomplete. Hydrolysis with 90% formic acid was also incomplete.

Periodate oxidation of the ammonium pectate required 1·05 moles of periodate per anhydrogalacturonic acid unit. The oxidised polysaccharide was reduced with sodium borohydride; chromatography of the hydrolysed product gave arabinose, xylose, and small amounts of galacturonic acid in addition to the expected threonic acid. The presence of arabinose and absence of galactose is in agreement with the structures proposed [2] for the araban and galactan components of pectic complexes. The unoxidised galacturonic acid may equally have arisen from incomplete periodate oxidation or from some galacturonic acid residues linked other than in the α-1,4′-manner commonly found in pectic acids.

Methylation of the pectic acid was difficult and was accompanied by extensive degradation: similar difficulties have been encountered previously.[2,16,17] Reasonable yields and methoxyl contents greater than 37—39% appear to be difficult to achieve with pectic materials (cf. refs. 13 and 16). After successive repeated treatments with thallium hydroxide–methyl iodide, dimethyl sulphate–sodium hydroxide, silver oxide–methyl iodide, a product having 37·4% of methoxyl (88% of the theoretical) was eventually obtained. After methanolysis, the product was reduced with lithium aluminium hydride, and the reduction product was hydrolysed. Chromatographic separation of the resultant mixture of sugars gave 7 fractions; from the two major fractions, crystals of the aniline derivative of 2,3-di-*O*-methyl-D-galactose were prepared.

Supplies of the authenticated alga are difficult to procure; only a limited amount of information could be obtained with the amount of material available, and it is hoped to obtain a larger supply in due course. It is apparent, however, that the pectic complex in *Nitella translucens* is similar in many respects to that from land plants, containing a pectic acid which has chains of 1,4′-linked α-D-galacturonic acid residues; these are

[16] Bishop, *Canad. J. Chem.*, 1955, **33**, 1521.
[17] Neukom and Deuel, *Chem. and Ind.*, 1958, 683.

not, however, present as methyl esters. This is clearly not the result of inadvertent demethylation or de-esterification during extraction, since the methoxyl content of the dried alga was $>0{\cdot}1\%$. The significance of this interesting pectic acid has been discussed [1] in relation to the lack of auxin-type hormonal activity in the *Nitellae* and to the possible ion-exchange function of these materials in the algal cell. The material may be of interest in investigations of the validity of current theories which relate the stability of pectins to their ester content.[18,19]

Experimental

Paper partition chromatography was carried out on Whatman No. 1 paper with the following solvent systems (v/v): (A) butan-1-ol–benzene–pyridine–water (5 : 1 : 3 : 3, upper layer); (B) pyridine–ethyl acetate–acetic acid–water (5 : 3 : 1 : 3); (C) butan-1-ol–ethanol–water (4 : 1 : 5, upper layer); (D) butan-2-one, half saturated with water containing 1% of ammonia. Optical rotations were measured at 18° ± 2°. Uronic anhydride was found by Anderson's decarboxylation apparatus; [20] methoxyl by the vapour-phase infrared method; [21] nitrogen by Kjeldahl semimicro-determinations; ash by heating to constant weight at 550—600° (muffle).

Isolation and Examination of the Water-soluble Polysaccharides.—The origin and pre-treatment of the algal cells have been described, and the extraction scheme used has been detailed.[1] The yields and analytical results obtained for the cold- and hot-water extracts [1] show that insignificant amounts, if any, of the pectic content were extracted in these pre-treatments.

Isolation and Examination of the Pectic Substances.—The pectic substances were extracted from the cold- and hot-water residues by aqueous oxalate solution (0·25% with respect to both oxalic acid and ammonium oxalate [7]) at 80—90° for 1 hr. (three treatments) [yield, 31 g. of ammonium pectate from a batch of algal cells (dry wt. estimated at 320 g.)]. This crude product had $[\alpha]_D$ +205° (*c* 0·2 in H_2O), uronic anhydride 51%. After hydrolysis, chromatography showed that galacturonic acid was present, and also galactose, glucose, arabinose, xylose, and rhamnose (3 : 4·5 : 3 : 3 : 1).

Purification and Attempted Fractionation of Ammonium Pectate.—(1) The crude product (5 g.) was dissolved in water (400 ml.); ethanol, acidified with acetic acid (5 ml. per l.), was slowly added with stirring. Two fractions, which were not sharp, were collected: (A) at 40% ethanol, 3·7 g., $[\alpha]_D$ +220° (uronic anhydride, 60%); (B) at 60% ethanol, 0·8 g., $[\alpha]_D$ +220° (uronic anhydride, 65%). Reprecipitation failed to increase the uronic acid content significantly. Hydrolysis gave the same sugars as had been found in the crude product; the two fractions were therefore combined in view of their similarity.

(2) These combined fractions (3·8 g.) were dissolved in water, and 10% calcium chloride solution was added until precipitation of calcium pectate was complete. The precipitate was heated in water at 80° for 30 min. with a slight excess of ammonium oxalate; calcium oxalate was removed by filtration and the solution dialysed against distilled water. Pectic acid was precipitated by addition of acidified ethanol, isolated, then re-dissolved and re-precipitated a further three times. The product (3·4 g.; uronic anhydride, 64%) gave a cloudy solution, even after filtration through Celite on a No. 2 glass sinter. This was probably due to colloidal calcium oxalate; clarification was achieved by making the solution slightly alkaline, then adding sufficient ethylenediaminetetra-acetic acid to make the solution 0·05N in it. The ammonium pectate was precipitated after 30 min. by addition of ethanol, isolated, redissolved, and reprecipitated. This material had $[\alpha]_D$ +240° (*c* 0·3% in H_2O) (uronic anhydride, 69%); hydrolysis gave galacturonic acid, with galactose, glucose, arabinose, xylose, and rhamnose (4 : 1 : 5 : 4 : 1).

(3) Pectic acid (5 g.), a product of procedures 1 and 2 above, was dissolved in water and Fehling's solution added until precipitation of the copper complex was complete. Attempted decomposition of this complex, by adding it to 50% aqueous ethanol which was 4N with respect to acetic acid, removed only a little of the copper. The complex was therefore re-dispersed in water which was made just alkaline by sodium hydroxide; disodium ethylenediaminetetra-acetate was added and the mixture stirred at room temperature until the copper complex

[18] Whistler and BeMiller, *Adv. Carbohydrate Chem.*, 1958, **13**, 289.
[19] Albersheim, Neukom, and Deuel, *Arch. Biochem. Biophys.*, 1960, **90**, 46.
[20] Anderson, *Talanta*, 1959, **2**, 73.
[21] Anderson and Duncan, *Talanta*, 1961, **8**, 241.

dissolved. The sodium pectate was precipitated with ethanol, redissolved, and reprecipitated twice. The pectic acid (4·6 g.) finally isolated had $[\alpha]_D$ +240° (*c* 0·3% in H_2O) (uronic anhydride, 72; N, 0·16%).

(4) Ammonium sulphate (100 g.) was stirred into an aqueous solution (100 ml.) of a portion (2 g.) of the product from procedure (3) above. The solution was kept at 0° for 3 days. The precipitate was isolated, dissolved in water, and dialysed till free from salts. The pectic acid 1·6 g.), isolated in the usual way, had $[\alpha]_D$ +238° (*c* 0·2% in H_2O) (uronic anhydride, 68%).

(5) Cetylpyridinium bromide (1% aqueous solution) was added to a solution of ammonium pectate (a product of treatments 1 + 2) until precipitation was complete. This complex was very stable. Attempted regeneration of the pectic material by stirring it with 4N-acetic acid (400 ml.) for 1 hr. (three treatments) or 15% aqueous sodium chloride failed to dissolve the complex. Heating it at 60° with 0·5% aqueous ammonium oxalate was finally successful. The pectic acid finally obtained had $[\alpha]_D$ +205° (*c* 0·4% in H_2O) (uronic anhydride, 54%).

Removal of Glucose.—Each of the purified products described above gave a positive reaction with iodine solution and gave, on hydrolysis, galacturonic acid with galactose, glucose, arabinose, xylose, and rhamnose. Only the amounts of glucose present were variable. Digestion with salivary α-amylase, followed by dialysis, removed all the glucose-containing contaminant,[6,10] leaving the amounts of other sugars unchanged. The polysaccharide having the highest uronic anhydride content was obtained by treatment with α-amylase of the product from procedure (3) above. This pectic acid had $[\alpha]_D$ +245° (*c* 0·3% in H_2O) (uronic anhydride, 74%); hydrolysis and paper chromatography gave galactose, arabinose, xylose, and rhamnose (4 : 6 : 3 : 1), and galacturonic acid. The subsequent experiments were made on this material.

Chromatography on Diethylaminoethylcellulose.[12]—Pectic acid (420 mg.) was treated on a column (22 × 3 cm.) of diethylaminoethylcellulose, with gradient elution with phosphate buffer (pH 6, 0·5M, 750 ml. siphoning into 500 ml. of water, flow-rate 40 ml. per min.) followed by gradient elution with aqueous sodium hydroxide. The fractions (20 ml.) were screened for uronic anhydride content by the carbazole method.[22] The Table shows the sugars found in each fraction after dialysis, freeze-drying, hydrolysis, and chromatography: 401 mg. of material were recovered.

Enzymic Hydrolysis.—Crude fungal pectinase (50 mg.) was shaken with acetate buffer (0·1M, pH 5·0, 50 ml.); the filtrate was added to an aqueous solution of ammonium pectate (300 mg. in 100 ml.). After incubation at 37° for 2 days, the solution was concentrated to about 30 ml., then fractionated on a charcoal–Celite column [23] [2 × 30 cm.; Celite + ultrasorb ZF charcoal (1 : 1)]. Gradient elution was by aqueous ethanol (0·5—15%; total vol. 1·25 l. in 150 ml. fractions), then by aqueous pyridine (0·5—3%). The pyridine fractions were found to contain only galacturonic acid and were therefore combined and reduced in volume. After addition of sodium hydrogen carbonate (to pH 4·0) and calcium carbonate (1 g.), the mixture was heated at 80° for 10 min. Reduction in volume after filtration gave a thin syrup which crystallised (92 mg. of sodium calcium galacturonate hexahydrate [15]). From the free acid, the 2,5-dichlorophenylhydrazone, m. p. 179°, and mucic acid, m. p. 205°, were prepared; these derivatives had infrared spectra identical with those of authentic specimens.

Periodate Oxidation.—Ammonium pectate (190 mg.; $[\alpha]_D$ +240°, uronic anhydride, 69%) was oxidised at 2° in darkness with 0·025M-sodium metaperiodate (100 ml.). The reduction of periodate [24] (mol. per uronic anhydride unit) was: 0·32 (17 hr.); 0·47 (42 hr.); 0·77 (95 hr.); 0·94 (138 hr.); 1·05 (173 hr.); 1·06 (320 hr.). The periodate oxidation was repeated on a larger sample of pectate (490 mg.). Ethylene glycol (5 ml.) was added after 320 hr. and the oxidised polysaccharide was then treated with sodium borohydride (0·8 g.) overnight. The product was precipitated with ethanol, reprecipitated twice, then taken to dryness several times with small portions of methanol to remove borate {yield, 520 mg., $[\alpha]_D$ +36° (*c* 1·2% in H_2O)}. Dialysis against distilled water gave a dialysable fraction {160 mg., $[\alpha]_D$ +18° (*c* 1·6% in H_2O)} and a non-dialysable fraction {140 mg., $[\alpha]_D$ +10° (*c* 1·0% in H_2O)}. On hydrolysis by 0·5N-hydrochloric acid for 4 hr. at 100° each fraction gave the same products (paper chromatography; solvents A, B, and C)—galacturonic acid, arabinose, and xylose (galactose absent). With solvent C, the presence of threonic acid ($R_{rhamnose}$ 1·27) was indicated.

[22] Dische, "Methods of Biochemical Analysis," Interscience Publ. Inc., New York, 1951, Vol. II, p. 313.
[23] Whistler and Durso, *J. Amer. Chem. Soc.*, 1950, **72**, 677.
[24] Halsall, Hirst, and Jones, *J.*, 1947, 1399, 1427.

Methylation of Ammonium Pectate.—Complete methylation was difficult to achieve and several experiments were conducted with little success. Eight treatments with dimethyl sulphate and sodium hydroxide gave, in 26% yield, a product having 14·6% of methoxyl; three treatments with silver oxide and methyl iodide then gave, in 15% yield, a product having 32·3% of methoxyl. The best result (OMe, 37·4%; yield, 8%) was obtained by a procedure involving the use in turn of repeated treatments with thallium hydroxide–methyl iodide, dimethyl sulphate–sodium hydroxide, and silver oxide–methyl iodide.

Fractionation of Methylated Methyl Pectate.—Light petroleum (b. p. 40—60°) was added to a solution of the methylated polysaccharide (380 mg.) dissolved in chloroform (20 ml.), giving the following fractions:

Ratio of chloroform to light petroleum	Fraction no.	Wt. of fraction (mg.)	$[\alpha]_D$
2 : 1	I	33	+180°
1 : 1	II	57	—
1 : 2	III	154	+186
Unprecipitated	IV	110	+123

Methanolysis of Methylated Methyl Pectate; Reduction, Hydrolysis, and Separation of Methylated Sugars.—Fraction III (154 mg.) was heated in a sealed tube with methanolic 6% hydrogen chloride (3 ml.) at 100° for 12 hr. After neutralisation (silver carbonate) and removal of solvent, the residue was extracted several times with chloroform. The resulting syrup was dissolved in dry tetrahydrofuran (15 ml.), and lithium aluminium hydride in tetrahydrofuran (0·2 g. in 10 ml.) was added dropwise to the boiling solution. After refluxing for 30 min., the solution was cooled and the excess of hydride destroyed by addition of ethyl acetate and water. The mixture was taken to dryness under reduced pressure, and the residue extracted with acetone and ethanol. The extracts were diluted with water, de-ionised, and reduced in volume to a syrup (107 mg.). This was hydrolysed with N-hydrochloric acid (5 ml.) at 100° for 4 hr. Neutralisation by silver carbonate, filtration, and evaporation gave a syrupy mixture of sugars (89 mg.). This was chromatographed on thick paper (50 × 15 cm., Whatman seed-test grade), upward development with solvent B being used to give seven fractions. After elution with hot 7 : 3 aqueous methanol, these fractions were reduced in volume and examined by paper chromatography and solvent B: two of the fractions contained three components and the remainder contained two components (cf. ref. 13) as shown below:

Fraction	$R_{rhamnose}$, colour with aniline oxalate, and intensity			
a	0·62 R–B +++	0·80 Y–B +		
b	0·63 R–B +	0·81 R–B ++		
c	0·63 R–B +	0·80 R–B +++		
d		0·83 R–B +++	1·14 O–R +++	
e	0·60 R–B +	0·86 R–B +	1·17 O–R ++++	
f		0·86 R–B +	1·10 O–R +	1·45 O–R +++
g		0·82 R–B +		1·76 O–R +++

R–B, red-brown; Y–B, yellow-brown; O–R, orange-red.

The largest individual fractions were f and g. These were combined (total 35 mg.) and refluxed with aniline (0·5 ml.) in methanol (5 ml.) in darkness. From this, crystals, m. p. 151° (20 mg.), were obtained which were identical (infrared spectroscopy) with the aniline derivative of 2,3-di-*O*-methyl-D-galactose.

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