232. *The Constitution of Alginic Acid.**

By D. W. Drummond, E. L. Hirst, and Elizabeth Percival.

The isolation of L-threaric acid from the hydrolysate of alginic acid that had been oxidised first with periodate and then with bromine confirms the presence of 1,4′-linked L-guluronic acid units in the alginic acid molecule. Epimerisation experiments on mannuronolactone and glucuronolactone confirm the ready conversion of these two uronic acids into their $C_{(5)}$ epimers in alkaline solution, but evidence is presented that the L-guluronic acid residues in alginic acid do not arise during the alkaline extraction of this polyuronide.

Further periodate-oxidations under conditions which avoid over-oxidation have revealed that alginic acid reduces *ca.* 0·55 mole of periodate for every anhydrouronic acid unit, and it is shown that a high proportion of these units are not cleaved by the periodate. The possible reasons for this immunity are discussed.

Before 1955, the available evidence indicated a straight chain of 1,4′-linked β-D-mannuronic acid residues [1] as the structure of alginic acid. However, with improved chromatographic techniques Fischer and Dorfel [2] showed that two uronic acids, D-mannuronic and L-guluronic acid, were present in the hydrolysates of commercial alginic acid, and also of alginic acid from twenty-two species of European brown algæ. It is noteworthy that one of the fractions isolated from reduced methylated alginic acid by Hirst *et al.*[1] and tentatively identified as a di-*O*-methylglucose could have been a methylated L-gulose derived from L-guluronic acid units.

Crystalline D-mannuronolactone had previously been isolated from acid hydrolysates of alginic acid by a number of workers,[3] the highest recorded yield being 45% of the theoretical.[3e] Fischer and Dorfel [2b] were able to separate two crystalline lactones, L-guluronolactone and D-mannuronolactone, from their hydrolysates, and concluded from infrared measurements that both the manuronolactone and the guluronolactone were pyranose 3,6-lactones. The ratio of D-mannuronolactone to L-guluronolactone varied according to the starting material and the conditions of hydrolysis. Prolonged heating with 0·5N-sulphuric acid caused more decomposition of L-guluronolactone than of D-mannuronolactone. These authors dismissed the possibility that the former had arisen from the latter by epimerisation at $C_{(5)}$ on the grounds that the proportions of the two lactones resulting from one set of hydrolysis conditions were reproducible.

In the present investigations chromatographic analysis of formic acid hydrolysates of alginic acid freshly extracted from *Laminaria digitata* confirmed the presence of two lactones and separation on a cellulose column of a hydrolysate of a guluronolactone-rich fraction of alginic acid gave crystalline D-mannuronolactone with the high melting point and rotation recorded by Fischer and Dorfel. Syrupy L-guluronolactone, with the rotation and chromatographic mobility recorded by these workers, was also separated; all attempts to crystallise this lactone were, however, unsuccessful. Reduction of the ester glycoside of this fraction and hydrolysis gave a syrup which was chromatographically and ionophoretically identical with gulose. While this work was in progress Whistler and Kirby [4] confirmed the presence of L-guluronic acid in sulphuric acid hydrolysates of alginic acid isolated from *Macrocystis pyrifera*. In contrast with the present results these

* A brief account of part of this work has already been published in *Chem. and Ind.*, 1958, 1088.

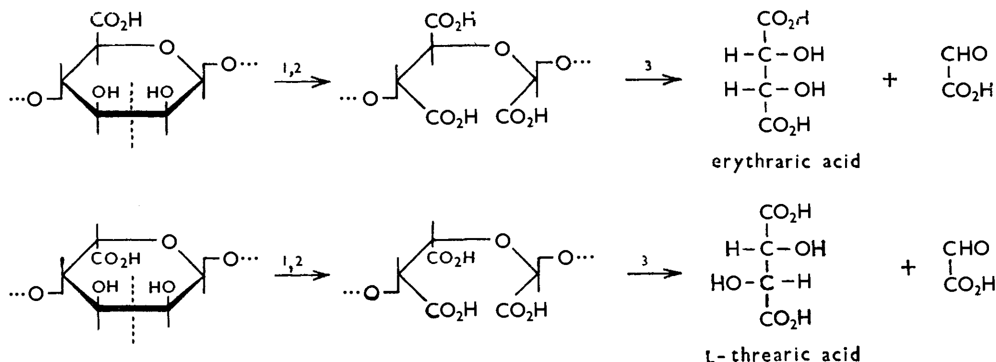
[1] Hirst, Jones, and Jones, *J.*, 1939, 1880; Chanda, Hirst, and Percival, *J.*, 1952, 1833.
[2] (*a*) Fischer and Dorfel, *Z. physiol. Chem.*, 1955, **301**, 224; (*b*) **302**, 186.
[3] (*a*) Nelson and Cretcher, *J. Amer. Chem. Soc.*, 1930, **52**, 2130; (*b*) Isbell and Frush, *J. Res. Nat. Bur. Stand.*, 1946, **37**, 321; (*c*) Bird and Haas, *Biochem. J.*, 1931, **25**, 403; (*d*) Schoeffel and Link, *J. Biol. Chem.*, 1932, **95**, 213; **100**, 397; (*e*) Spoehr, *Arch. Biochem.*, 1947, **14**, 153.
[4] Whistler and Kirby, *Z. physiol. Chem.*, 1959, **314**, 49.

workers were unable to detect L-guluronolactone after hydrolysis of alginic acid with hot formic acid.

Since the L-guluronic acid residues might have arisen by epimerisation at $C_{(5)}$ of some of the D-mannuronic acid units during the alkaline extraction of the alginic acid from *L. digitata*, D-mannuronolactone was subjected to these conditions. Chromatographic analysis of the resulting syrup revealed the presence of a considerable proportion of guluronic and glucuronic acid [5] and their lactones in addition to mannuronic acid and its lactone. However, proof that guluronic acid units are indeed present in the native alginic acid was obtained by acidic hydrolysis of the weed itself. The resulting syrup contained a considerable proportion of guluronic acid in addition to mannuronic acid, glucuronic acid, and fucose. At the same time it should be stressed that when mannuronolactone was subjected to the same hydrolytic conditions as the seaweed it was recovered unchanged in good yield, and chromatographic examination of the recovered crystalline mannuronolactone and of the mother liquors showed the absence of guluronic acid or its lactone. Recent hydrolysis experiments on alginic acid by Vincent [6] agree with the present results.

Further confirmation that the guluronic acid units in alginic acid had not arisen by epimerisation of the mannuronic acid units during the alkaline extraction of the polyuronide was obtained from the fact that subjection of methyl methyl mannosiduronate to the conditions of alkaline extraction caused no epimerisation. This derivative of mannuronic acid with its substituted reducing group more closely resembles the units in alginic acid than does unsubstituted mannuronolactone, since the reducing groups of the individual residues in the polyuronide are substituted by linkage with adjacent units. It seems unlikely from this evidence that epimerisation at $C_{(5)}$ takes place at the polysaccharide level and it is possible that the interchange of D-mannuronolactone to L-guluronolactone may resemble the transformation envisaged for D- into L-galactose,[7] that is the simultaneous enzymic reduction and oxidation at $C_{(6)}$ and $C_{(1)}$, respectively.

In order to provide additional evidence that the guluronic acid was not an artefact of acidic hydrolysis and also to obtain evidence of its mode of linkage in the macromolecule, periodate oxidation, followed by bromine oxidation of the derived aldehydic to carboxylic groups, and hydrolysis of the polytricarboxylic acid was carried out. Any 1,4'-linked



L-guluronic acid residue in the alginic acid chains should under this treatment be degraded to L-threaric acid. If these units are 1,3'-linked then they should be immune to periodate and bromine oxidation and appear in the final solution as guluronic acid, and finally 1,2'-linked residues should yield two three-carbon acids when subjected to this treatment.

It has been shown by Lucas and Stewart [8] that 25% of *meso*-tartaric acid (erythraric

[5] Fischer and Schmidt, *Ber.*, 1959, **92**, 2184.
[6] Vincent, *Chem. and Ind.*, 1960, **38**, 1109.
[7] Hirst, *Proc. Chem. Soc.*, 1958, 177.
[8] Lucas and Stewart, *J. Amer. Chem. Soc.*, 1940, **62**, 1792.

acid) from 1,4′-linked D-mannuronic acid residues could be isolated from hydrolysates of alginic acid which had been oxidised in this manner. In the absence of chromatographic techniques it is not surprising that these authors failed to detect L-threaric acid in the mother liquors. In the present investigations freshly prepared alginic acid was oxidised with periodate under the conditions recorded by Lucas and Stewart,[8] and this was followed by bromine oxidation in the presence of barium carbonate to prevent development of acidity. Care was taken to isolate a pure polymer after each oxidation, and the polytricarboxylic acid was allowed to hydrolyse until the solution had a positive rotation (36 hr.). In contrast the earlier workers stopped the hydrolysis when the solution became optically inactive (16 hr.). From this hydrolysate a small proportion of L-threaric and erythraric acids was separated, the former being characterised by rotation and *X*-ray powder photograph of the potassium hydrogen salt. Although the isolation of L-threaric acid confirmed the presence of L-guluronic acid residues in alginic acid and provided the first evidence that at least some of these units are 1,4′-linked the low yield of these acids necessitated careful examination of the action of periodate on this polyuronide.

The oxidation was repeated under the same conditions, and after the theoretical amount of periodate (one mole for every anhydro-uronic acid unit) had been reduced the reaction was stopped. The derived oxo-alginic acid was oxidised with bromine, and the polytricarboxylic acid hydrolysed. Chromatographic analysis of the derived syrup revealed the presence of L-threaric and erythraric acids, glyoxylic acid, guluronolactone, and mannuronolactone, together with a single spot with the speed of guluronic and mannuronic acids. After these experiments had been completed, Whistler and Schweiger[9] published an account of the oxidation of alginic acid from *M. pyrifera* with hypochlorite, and the isolation of low yields of oxalic, L-(+)-tartaric (L-threaric) and DL-tartaric acids from the oxidised and hydrolysed products. These authors also obtained evidence for the presence of a high proportion of unoxidised uronic acid residues.

Reduction of a portion of the above syrup from the hydrolysed tricarboxylic acid, after conversion into the ester glycosides, was followed by hydrolysis. The resulting syrup was shown by chromatography and ionophoresis to contain appreciable quantities of gulose and mannose. Although the theoretical quantity of periodate had been reduced the presence of glucuronic and mannuronic acids in the hydrolysate of the oxo-alginic acid is proof that a proportion of the residues in the alginic acid was unattacked by the oxidant. In view of the fact that the only other recognisable products in the hydrolysate were the two expected four-carbon acids and glyoxylic acid, it seems reasonable to assume that some of the periodate had been consumed in over-oxidation of the alginic acid chains at the reducing ends.

The work of Hough and his colleagues[10] shows that unless oxidation with periodate is carried out in dilute solution at controlled temperatures and pH, anomalous results can be expected, owing to hydrolysis of formyl esters and consequent over-oxidation of the polysaccharide from the reducing end. Formyl esters are most stable at pH 3·6[11] and 2°.

Sample (1 g.)	Temp.	pH	Buffer	Periodate reduced (moles/anhydro unit)	Oxo-polyuronide (g.)
A	2°	1·65	0·1N-H_2SO_4	0·40	0·41
B	2	3·72	Acetate	0·55	0·83
C	2	3·2[a]	Unbuffered	0·54	1·08
D	25	3·2[a]	,,	0·50	0·99

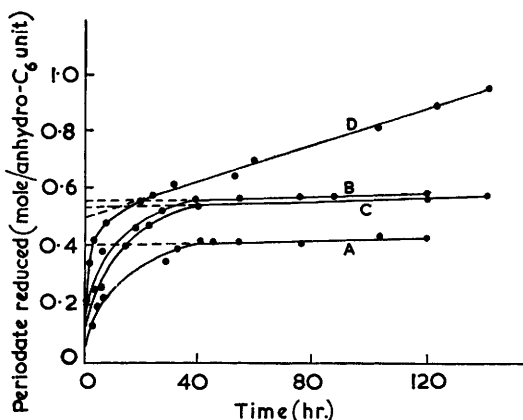
[a] After 48 hr.

Four samples of alginic acid were therefore oxidised with dilute periodate under different conditions of acidity and temperature (see Table and Fig.). At a pH of 1·65 and 2° the reaction reached completion after the reduction of 0·4 mole of periodate for each uronic acid residue, but in this experiment the yield of oxo-polysaccharide (*ca.* 40%) was

[9] Whistler and Schweiger, *J. Amer. Chem. Soc.*, 1958, **80**, 5701.
[10] Hough *et al.*, *Chem. and Ind.*, 1956, **34**, 768; 1959, **37**, 1126; *J.*, 1958, 1212.
[11] Schopf and Wild, *Ber.*, 1954, **87**, 1571; Hughes and Nevell, *Trans. Faraday Soc.*, 1948, **44**, 941.

low. It is very probable that under these acidic conditions some of the oxoalginic acid was hydrolysed and degraded. In each of the other experiments in buffered (pH 3·72) and unbuffered solution, at 2° and 25° the oxidation was complete after the reduction of about 0·55 mol. of periodate, and only in the reaction at 25° did slow over-oxidation occur beyond this value (see Fig.).

The free aldehydic groups in each of the oxo-alginic acids (isolated in not less than 83% yield) were reduced to primary alcoholic groupings,[11, 12] and the acidic polymers isolated as white powders. Esterification was achieved with diazomethane. Attempted esterification with anhydrous 4% methanolic hydrogen chloride at room temperature for 3 days or under reflux for 6 hr. led to extensive degradation. Reduction of the carboxyl



groups to primary alcoholic groups by sodium borohydride was followed by acidic hydrolysis of the derived polymers (I). Chromatographic analysis of each of the hydrolysates revealed their essential similarity and the presence of mannose, gulose, erythritol, threitol, and glycerol. The mannose and gulose could only have arisen from unoxidised mannuronic and guluronic acid units in the alginic acid. Erythritol is derived from D-mannuronic acid units cleaved between $C_{(2)}$ and $C_{(3)}$, and threitol from L-guluronic acid units similarly cleaved. Glycerol is produced from the non-reducing ends of the polymer chains. The absence of organic acids in these hydrolysates indicated that complete reduction had been achieved.

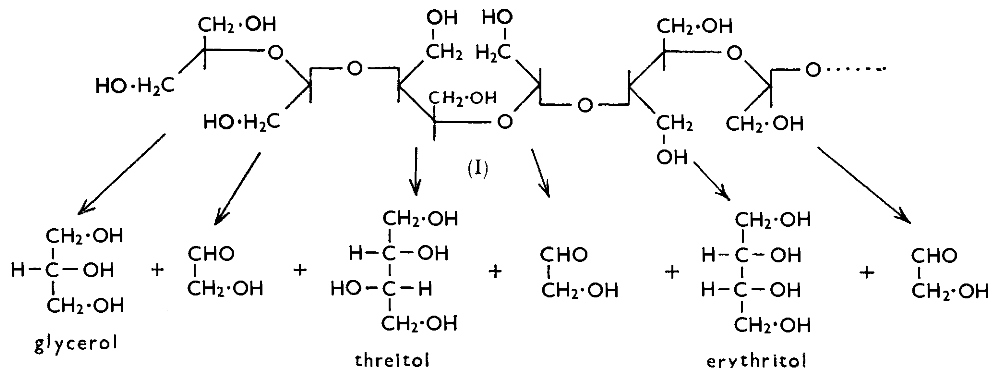
The hydrolysates from the different oxidations were combined, and a portion separated on thick paper, into three fractions: fraction (1) glycerol (1 part); fraction (2) mannose + gulose (8 parts); fraction (3) erythritol + threitol (11 parts). Crystalline mannose phenylhydrazone was separated from fraction (2). No glycollaldehyde separated, probably owing to the fact that this substance was lost during the removal of borate with methanol.

Alginic acid readily yields fibres, and this, together with the results of *X*-ray diffraction measurements,[13] provides evidence that this polymer has an essentially unbranched molecule. Molecular-weight determinations[14] gave a minimum value of 200 anhydrouronic acid units, and the evidence is in favour of a considerably larger molecule. Bearing these facts in mind there does not appear to be any explanation for the high proportion of glycerol separated from the reduced oxo-alginic acid hydrolysate. It should, however, be noted that Goldstein and his co-workers[15] were unable to account for glycerol in a hydrolysate derived from similarly treated cellulose.

The low reduction of periodate by alginic acid is surprising since all the evidence

[12] Smith *et al.*, *J. Amer. Chem. Soc.*, 1956, **78**, 5910; 1957, **79**, 6469.
[13] Astbury, *Nature*, 1945, **155**, 667; Palmer and Hertzog, *J. Amer. Chem. Soc.*, 1945, **67**, 1865.
[14] Donnan and Rose, *Canad. J. Res.*, 1950, *B*, **46**, 441; Cook and Smith, *Canad. J. Biochem. Physiol.*, 1954, **32**, 277.
[15] Goldstein, Hamilton, Montgomery, and Smith, *J. Amer. Chem. Soc.*, 1957, **79**, 6469.

indicates that the residues are 1,4'-linked and therefore vulnerable at $C_{(2)}$–$C_{(3)}$. It has been found,[16] however, that failure to undergo periodate oxidation does not necessarily imply the absence of adjacent hydroxyl groups. 1,6-Anhydro-β-D-glucofuranose, for example, is not oxidised because the adjacent hydroxyl groups are locked in a *trans*-position. Alginic acid, however, contains no free *trans*-hydroxyl groups if the residues are



1,4'-linked. Moreover, models of 1,4'-linked chains comprising both D-mannuronic and L-guluronic acid units show no evidence of steric hindrance, and both types of hexuronic acid residue should be equally vulnerable to periodate attack.

Kaye and Kent[17] estimated the proportion of lactones in dried samples of various polyuronides by formation of hydroxamic acids and concluded that dry alginic acid consisted of a proportion of 3,6-lactone residues. In order to form the 3,6-lactone, D-mannuronic acid units must assume the unstable 1C conformation (in which hydroxyl on $C_{(3)}$ and carboxyl on $C_{(6)}$ are both axial) which is then stabilised by the presence of the lactone ring. Cleavage of the lactone ring would probably result in a return to the stable C1 conformation. It has been pointed out to us by Dr. P. Schwarz that this is a possible explanation of the difference in the *X*-ray diffraction measurements for alginic acid and sodium alginate. In alginic acid the projection per pyranose unit along the fibre axis is 4·37 Å (corresponding to the 1C conformation) whereas in the sodium salt this is 5·0 Å units (C1 conformation).[13] It follows that a possible explanation of the immunity of a high proportion of the mannuronic acid residues in alginic acid to oxidation by periodate is that they are lactonised, the 3,6-lactone ring being stable to the oxidation conditions. Nevertheless, this does not explain the presence of unoxidised gulopyranuronic acid units since, in spite of the findings of Fischer and Dorfel[2b] the formation of gulopyranurono-3,6-lactones either in the C1 or the 1C conformation is, in our opinion, sterically impossible since the carboxyl and $C_{(3)}$ hydroxyl groups are *trans* to one another. On the other hand gulofuranurono-3,6-lactone scale models can readily be built and the possibility of such a structure cannot be ruled out, in which case it would be necessary to consider the possibility of the occurrence in alginic acid of furanose uronic acid residue linked through the 1,5'-positions.

Another possible explanation for the immunity of some of the uronic acid units to periodate oxidation could be the presence of ester linkings between adjacent chains, the carboxyl group of one chain being linked to the hydroxyl group of $C_{(2)}$ or $C_{(3)}$ of another chain. In fact the infrared spectrum of alginic acid in Nujol mull in the carbonyl region favours the presence of ester rather than lactone groups (1745—1750 cm.$^{-1}$). Although a small absorption at higher wave-numbers could be present indicating a 3,6-lactone, there is no visible indication of this in the spectrum.

Finally, the reason for the non-oxidation of some of the uronic acid units in the alginic

[16] See Bobbit, *Adv. Carbohydrate Chem.*, 1956, **11**, 11; Dimler, *ibid.*, 1952, **7**, 46.
[17] Kaye and Kent, *J.*, 1953, 79.

acid molecule might be that some of the residues are present in 1,3′-linkage. A mixture of 1,4′- and 1,3′-linked residues is not unique in polysaccharides. The xylan isolated from the red seaweed *Rhodymenia palmata* comprises 80% of 1,4′- and 20% of 1,3′-linked xylose units,[18] and lichenin and isolichenin isolated from *Cetraria islandica* contain both 1,4′- and 1,3′-linked glucose residues.[19] No evidence of 1,3′-linkage has been found in any of the earlier studies on alginic acid, but in view of the inability of any group of workers to obtain theoretical yields from this polysaccharide the possibility cannot be dismissed. A final decision regarding the presence of lactones, ester linkages, or 1,3′-linked units in the alginic acid molecule must await further evidence.

Experimental

The analytical methods used have been described by O'Donnell and Percival.[20] In addition the following chromatographic solvents were used: (9) ethyl methyl ketone–acetic acid–water (9 : 1 : 1) saturated with boric acid; (10) pentyl alcohol–acetic acid–water (4 : 1 : 5); (11) pyridine–ethyl acetate–acetic acid–water (5 : 5 : 1 : 3) as descending eluant equilibrated with pyridine–ethyl acetate–water (11 : 40 : 60) in the bottom of the chromatography tank.[2b] Uronic acids and lactones were detected with aniline oxalate, uronic and organic acid with Bromocresol Green,[21] and erythritol, threitol, and glycerol with silver nitrate [21] sprays. L-Threaric acid has R_{Gl} 2·5, 4·0, 10, and 0·63 in solvents (3), (4), (10), and (11), respectively, and erythraric acid R_{Gl} 2·1, 3·3, 7·1, and 0·72 in these solvents.

Extraction of Alginic Acid from Laminaria digitata.—The milled dried weed, collected at Thorntonloch in December, was extracted by dilute sodium carbonate and precipitated as the insoluble calcium salt by the method of Black, Cornhill, and Dewar.[22] After conversion into the free acid by stirring for 8 hr. with 2N-hydrochloric acid at room temperature, the product was washed free from calcium ions with ethanol containing 10% of N-hydrochloric acid and then free from chloride ion with ethanol, and isolated as a white fibrous solid (yield 26% of dry weight of frond), $[\alpha]_D$ −136° (*c* 1·0 in 0·05N-sodium hydroxide) [Found: Ash, 0·96%; Equiv., 170 (by titration). Calc. for a hexuronic acid polymer: Equiv., 176].

Fractionation of Alginic Acid.[23]—A 1% solution of sodium alginate was prepared by dissolving freshly extracted alginic acid (10 g.) and sodium hydrogen carbonate (5 g.) in water (1200 c.c.). An equal volume of saturated aqueous potassium chloride was added and the mixture set aside overnight. The precipitate (mannuronolactone-rich fraction) was removed and the residual alginic acid precipitated from the supernatant liquid by the addition of concentrated hydrochloric acid. This precipitate was freed from chloride ion with water and acetone. After being dried, it was refractionated twice, yielding from successive supernatant liquids a guluronolactone-rich fraction (5·5 g.).

Separation of D-*Mannuronolactone and* L-*Guluronolactone from a Hydrolysate of Alginic Acid.*—The guluronolactone-rich fraction (5 g.) was hydrolysed with 90% formic acid (300 c.c.) at 100° for 10 hr. The mixture was cooled, and undissolved alginic acid was removed, washed with water and ethanol, and dried (1·6 g.). Formic acid was removed from the solution by repeated distillation with water under reduced pressure at 50°. The residual syrup was hydrolysed with N-sulphuric acid at 100° for 3 hr. After neutralisation with barium carbonate and decolorisation with charcoal a pale yellow syrup (1·8 g.) was obtained. Separation on a cellulose column with solvent (4) yielded the following fractions [R_{Gl} are recorded for solvent (4)]:

Fraction (1). A non-reducing syrup (14 mg.) which was not examined further.

Fraction (2). A syrup (128 mg.) which had $[\alpha]_D$ +46·4° (*c* 0·54). Chromatography gave two spots identical with guluronolactone, R_{Gl} 4·8, and guluronic acid. Attempted crystallisation from ethanol was unsuccessful both before and after lactonisation at 100° for 4 hr. Separation of the lactone spot from a portion of the syrup on 3MM paper yielded another syrup (30 mg.), $[\alpha]_D$ +80° $\longrightarrow$ +56° (*c* 0·33, 2 hr.). A portion of the original syrup (20 mg.) after conversion into methyl methyl glycosiduronate (refluxing with 4% anhydrous methanolic hydrogen

[18] Chanda and Percival, *Nature*, 1950, **166**, 787.
[19] Meyer and Gürtler, *Helv. Chim. Acta*, 1947, **30**, 751; Chanda, Hirst, and Manners, *J.*, 1957, 1953; Peat, Whelan, and Roberts, *J.*, 1957, 3916.
[20] O'Donnell and Percival, *J.*, 1959, 2168.
[21] Beattie and Percival, *Biochem. J.*, 1961 **79**, 531.
[22] Black, Cornhill, and Dewar, *J. Sci. Food Agric.*, 1952, **3**, 542.
[23] Haug, *Acta Chem. Scand.*, 1959, **13**, 1250.

chloride for 7 hr.) was reduced by adding solid sodium borohydride (20 mg.) to the glycosiduronate in water (1 c.c.). After 16 hr. the solution was neutralised with acetic acid, treated with IR 120(H) and IR 4B(OH) resins, and concentrated to a syrup. The glycosidic group was removed by hydrolysis of the syrup with N-sulphuric acid (2 c.c.) for 4 hr. at 100°. Chromatographic analysis (solvent 9) of the neutralised product revealed the presence of a single spot, R_{Gl} 1·88 (cf. mannose R_{Gl} 1·36), and ionophoresis in borate buffer [20] gave a single spot, M_G 0·59 (cf. mannose M_G 0·46).

Fraction (3). A syrup (438 mg.) consisting of a mixture of guluronolactone, mannuronolactone, and their respective acids.

Fraction (4). Crystalline D-mannuronolactone (600 mg.), m. p. and mixed m. p. 188°, R_{Gl} 2·8, had $[\alpha]_D$ +92° (*c* 0·72).

Fraction (5) (water washings): a syrup (500 mg.) which had $[\alpha]_D$ +81° (*c* 1·0). Chromatographic examination showed an elongated spot corresponding to mannuronic and guluronic acids, and unhydrolysed material on the starting line of the chromatogram.

The Effect of Heat on Aqueous Solutions of Uronic Acids at Different pH (*with* MR. J. K. WOLD).—(a) *At* pH 4·5. 0·01M-Solutions of D-mannuronolactone and D-glucuronolactone (200 c.c. each) in distilled water were refluxed for 24 hr. The solutions slowly became brown, and dark-coloured precipitates were deposited. At intervals the pH of samples (2 c.c.) was determined.

Period of heating (hr.)	0	3	8	24
Mannuronolactone solution, pH	4·30	3·10	2·95	2·90
Glucuronolactone solution, pH	5·15	3·10	2·90	2·85

After 24 hr. a portion of each solution (50 c.c.) was withdrawn and filtered, and the filtrate was neutralised to pH 6 with sodium hydroxide solution and then freeze-dried to a cream-coloured solid (50 mg., *ca.* 50%).

(b) *At* pH 7·0. A further 50 c.c. of the above solutions were withdrawn and filtered, and the filtrate, after adjustment to pH 7 with sodium hydroxide solution, was reheated at 100° for 2 hr. The resulting solutions were decolourised with charcoal and concentrated to dryness.

The four solids were examined chromatographically in solvents (2), (3), and (11) and in each case the main spot was that of the original acid; a faint spot corresponding to the lactone could be observed in the chromatogram of the solid derived from mannuronolactone which had been heated for 24 hr. in distilled water, and in addition a spot R_{GA} 1·47 (solvent 3) could be detected from both mannuronolactone and glucuronolactone on paper sprayed with aniline oxalate or with Bromocresol Green when more than the usual quantity of substance was eluted on the chromatogram. No spots corresponding to guluronic acid or its lactone could be detected in the materials derived from mannuronolactone or to a second substance from glucuronolactone.

About half of each solid (dried overnight in a vacuum desiccator) was converted into the methyl methyl glycosiduronate, reduced with borohydride, and hydrolysed as for fraction (2) from the alginic acid hydrolysate. Each of the products was examined chromatographically (solvent 9) and ionophoretically [20] in borate buffer. The two products from mannuronolactone gave a single spot identical with that of mannose, run as a control, and those derived from glucuronolactone gave a single spot corresponding to glucose.

(c) *Hot* 98% *formic acid on mannuronolactone*. Crystalline mannuronolactone (64 mg.) was heated at 100° in 98% formic acid (5 c.c.) for 1 hr. The solution was diluted to 10 c.c. with distilled water and evaporated to dryness. More formic acid (5 c.c.) was added and the process repeated. After treatment with charcoal, filtration and evaporation gave a solid (50 mg.) which on recrystallisation from water had m. p., and mixed m. p. with mannuronolactone, 188°. Chromatographic examination in solvents (4) and (11) of the mother liquors from which the crystals had separated revealed a single spot with the mobility of mannuronolactone together with a trace of material on the starting line.

(d) *Conditions used in the extraction of alginic acid from* L. digitata. D-Mannuronolactone (0·25 g.), methyl methyl mannosiduronate (0·1 g.) and glucuronolactone (0·25 g.) in water (100 c.c.) were stirred with an equal weight of calcium hydroxide under nitrogen for 30 min. at 60°. Sulphuric acid was then added until the solution was 0·2N with respect to acid, and the mixture was shaken for 1 hr. Sodium carbonate was then added until the final solution contained 3% of sodium carbonate. The mixture was kept at 50—60° for 3 hr. under nitrogen and then brought to pH 6 with sulphuric acid and concentrated to small volume. After partial

neutralisation with barium carbonate and filtration, the filtrate was freed from sulphate ions by the addition of barium chloride. De-ionisation was then achieved with IR 120(H) resin, silver carbonate, hydrogen sulphide, and finally Ultrasorb S.C. charcoal.[24] The resulting syrups were analysed chromatographically; that derived from mannuronolactone showed two spots corresponding to mannuronic and guluronic and/or glucuronic acids, respectively (solvent 12, 60 hr.), and three additional spots corresponding to the respective three lactones (solvent 11, 6 hr.). The syrup from the ester glycoside on similar chromatographic analysis gave before hydrolysis a single faint spot for mannuronolactone and, after acid hydrolysis of the glycosidic methoxyl group, two strong spots corresponding to mannuronic acid and its lactone. From glucuronolactone one spot identical with glucuronic acid and a second spot, R_{GA} 1·26 (solvent 12, 60 hr.). (No authentic iduronic acid was available for comparison.)

Hydrolysis of Dried Laminaria digitata *Frond* (*with* MR. J. K. WOLD).—(*a*) The dried powdered frond (0·5 g.) was hydrolysed with sulphuric acid under the conditions described by Fischer and Dorfel.[2b] Chromatographic analysis of the derived syrup (solvent 11, 60 hr.) revealed the presence of mannuronic and guluronic and/or glucuronic acids. Three spots corresponding to the lactones of these three acids were detected on papers run for 6 hr. Glucose and fucose could also be detected.

(*b*) Powdered frond (1·0 g.) was set aside with 0·2N-sulphuric acid (20 c.c.) overnight. The residual weed after being washed with water was hydrolysed with 75% formic acid (30 c.c.) at 100° for 12 hr. After removal of the residue the solution was treated as described in the formic acid hydrolysis of alginic acid except that neutralisation with barium carbonate was omitted and the sulphuric acid was removed with *N*-methyldioctylamine,[25] and traces of amine removed with IR 120(H) resin. The resulting syrup gave strong spots for guluronic/glucuronic acid in addition to mannuronic acid on chromatographic analysis (solvent 11, 60 hr.). The three respective lactones were detected on papers eluted for 6 hr., the spot corresponding to gulurono-lactone being considerably stronger than the analogous spot on the chromatogram from the sulphuric acid hydrolysis of the weed.

Oxidation of Alginic Acid and Isolation of L-*Threaric Acid*.—Alginic acid (10 g.) was oxidised with a saturated solution of sodium metaperiodate under the conditions used by Lucas and Stewart.[8] After 26 hr. the oxo-alginic acid was precipitated with t-butyl alcohol, forming a fibrous white solid. After dissolution in water and dialysis to remove inorganic ions the oxo-polyuronide, isolated by freeze-drying, had $[\alpha]_D$ +108° (8·2 g.). It was dispersed in water (350 c.c.) and rapidly stirred with bromine (16 g.) and barium carbonate (35 g.) at room temperature for 24 hr. Excess of bromine was removed by aeration and barium was precipitated by addition of the calculated quantity of sulphuric acid. The filtrate was dialysed until free from ions (3 days) and the polytricarboxylic acid isolated by freeze-drying as a white solid (2·4 g.), $[\alpha]_D$ −32·5° (*c* 1·18). A portion (0·74 g.) was heated with 0·05N-sulphuric acid (100 c.c.) at 100° and the hydrolysis followed by measurement of the rotation (α_D −0·15° ⟶ +0·07°; 36 hr., const.). After the solution had been cooled the sulphuric acid was removed with *N*-methyl-dioctylamine.[25] (In a trial experiment, this reagent did not extract threaric or erythraric acid from aqueous solution.) The residual aqueous solution after being shaken with IR 120(H) resin was concentrated to a syrup (108 mg.). Chromatographic analysis revealed the presence of erythraric and threaric acids (R_{GI} 4·0 and 3·3, respectively, in solvent 4, and 10·0 and 7·1 in solvent 10). (To remove completely the solvent it was necessary to dry the papers at room temperature for at least 24 hr.) Separation on Whatman 3MM paper (solvent 4) gave syrupy erythraric acid (27 mg.), $[\alpha]_D$ ±0° (*c* 2·0), chromatographically identical with an authentic sample in solvents 3, 4, 10, and 11), and syrupy L-threaric acid (23 mg.) which, after purification, yielded a glass (14 mg.), $[\alpha]_D$ +10·8 (*c* 1·4) ⟶ +66° on addition of ammonium molybdate,[26] and chromatographically identical with authentic material in solvents 3, 4, 10, and 11. After being dried (P_2O_5) at 61° for 3 days a partly crystalline mass was obtained which gave an *X*-ray powder photograph essentially identical with one of authentic L-threaric acid, and very different from the *X*-ray powder photograph of erythraric acid and of D-mannuronolactone. Addition of the calculated quantity of potassium hydroxide to a solution of the syrup and evaporation to dryness yielded the crystalline potassium hydrogen salt. This gave an *X*-ray powder photograph identical with that of potassium hydrogen L-threarate.

[24] Hughes and Whelan, *Chem. and Ind.*, 1958, **36**, 884.
[25] Smith and Page, *J. Soc. Chem. Ind.*, 1948, **67**, 48T.
[26] Richardson and Gregory, *J. Soc. Chem. Ind.*, 1903, **22**, 405.

Oxidation with Periodate and Measurement of the Periodate Reduced.—Alginic acid (8·5 g.) was stirred with 0·43M-sodium metaperiodate solution (500 c.c.) at room temperature. At intervals portions (2 c.c.) were withdrawn and the extent of reduction measured: [27]

Time (hr.)	0·5	1·0	2·0	4·0	5·0	6·0	8·5	26·0
Periodate reduced (moles/anhydro-C_6 unit)	0·64	0·67	0·70	0·72	0·75	0·78	0·85	0·96

After 26 hr. the oxidation was stopped by blowing sulphur dioxide through the solution, and the excess of sulphur dioxide removed by aeration. The solution was then oxidised with bromine as before and the polytricarboxylic acid hydrolysed. Chromatographic analysis of the resulting syrup (4·1 g.) revealed the presence of L-threaric and L-erythraric acids, glyoxylic acid, mannuronolactone, guluronolactone, and a spot with the mobility of the two hexuronic acids. It was possible to separate glyoxylic acid from L-threaric acid only in solvent (3). The former acid could be distinguished by the bright yellow spot it gave with aniline oxalate spray.

A portion of the syrup was converted into the ester glycosides and then reduced with sodium borohydride as before. Chromatographic analysis (solvent 9) and ionophoresis of the hydrolysate of the product revealed mannose and gulose as predominant components.

Periodate Oxidation under Carefully Controlled Conditions.—Samples (1 g.) were oxidised by 0·015M-sodium metaperiodate (800 c.c.) under the conditions given in the table on p. 1210. Buffered solutions were made by dispersing the alginic acid in the buffer (400 c.c.) and adding 0·03M-periodate (400 c.c.). Each solution contained 2·11 moles of periodate for every anhydrohexuronic acid residue. At intervals, portions (1 c.c.) were withdrawn and the amount of periodate reduced measured.[28] The results are collected in the table and shown in the Figure.

After the oxidation had reached completion (50 hr. A, B, C; 20 hr. D) excess of periodate was destroyed with sulphur dioxide, the solutions were dialysed, and the oxo-alginic acids precipitated with t-butyl alcohol. The precipitates were washed with acetone and ether and dried *in vacuo* before being weighed.

Reduction of the Oxo-alginic Acids.—A portion (*ca.* 0·5 g.) of each of the above oxo-alginic acids was added to 0·4M-boric acid (50 c.c.), and the mixture cooled to 0° and treated dropwise with sodium borohydride (1 g.) in water (40 c.c.) with stirring at 0°. The solution was kept overnight at 2°. After acidification with acetic acid, dialysis, and treatment with IR 120(H) resin, the reduced oxo-alginic acid was isolated by freeze-drying as a fluffy white powder (yield from B, C, and D *ca.* 330 mg. each) which was acid in aqueous solution.

The reduced oxo-alginic acids (from B, C, and D), dissolved in methanol (50 c.c.), were titrated with an ethereal solution of diazomethane at room temperature, until a persistent yellow colour indicated an excess of diazomethane. Distillation at 60° removed that excess and ether. Addition of an equal volume of water to the residual methanolic solution gave a neutral liquid, which was reduced with excess of sodium borohydride overnight at 2°. Acidification with dilute sulphuric acid was followed by hydrolysis in N-sulphuric acid for 6 hr. at 100°. After neutralisation with barium carbonate and repeated evaporation with methanol the resulting products were freed from ions by treatment with IR 120(H) and IR 4B(OH) resins and concentrated. The derived syrups from each of the reduced oxo-alginic acids (B, C, and D) on chromatographic examination in solvents 2, 4, and 9 appeared to be identical and each gave spots corresponding to mannose, gulose, erythritol, threitol, and glycerol. After the repeated evaporation with methanol a portion of the hydrolysate was treated with IR 120(H) resin only and chromatographically examined for the presence of organic acids. No spots were detected. The syrups from the different hydrolysed reduced oxo-alginic acids B, C, and D were combined (780 mg.) and separated into 3 fractions on a number of sheets of 3MM paper and eluted with solvent (4) giving: fraction (1) glycerol (26 mg.), fraction (2) mannose + gulose (200 mg.), and fraction (3) erythritol + threitol (290 mg.). Mannose was isolated from fraction (2) as the crystalline phenylhydrazone, m. p. and mixed m. p. 188°.

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[27] Fleury and Lange, *J. Pharm. Chim.*, 1933, **17**, 107.
[28] Aspinall and Ferrier, *Chem. and Ind.*, 1957, **35**, 1216.