284. *The Structure of Alginic Acid. Part IV.*[1] *Partial Hydrolysis of the Reduced Polysaccharide.**

By E. L. Hirst, Elizabeth Percival, and J. K. Wold.

Alginic acid, extracted under mild conditions from freshly harvested *Laminaria digitata*, was converted into di-*O*-propionylalginic acid. Reduction and de-esterification were followed by partial acid hydrolysis. L-Gulose (characterised as crystalline 1,6-anhydrotri-*O*-benzoyl-L-gulose), 4-*O*-β-D-mannosylgulose, and mannobiose were isolated from this hydrolysate.

Alginic acid constitutes the principal carbohydrate of the brown seaweeds but the content varies within a range of *ca.* 10—30% in different species, and from season to season, especially in the fronds.[2] It is a polyuronide comprising D-mannuronic acid[3] and L-guluronic acid,[4] the relative proportions of which vary in different species. Fractionation into two homopolyuronides has not been achieved but partial fractionation into gulurone- and mannurone-rich materials has been brought about by precipitation with manganous

* A brief account of part of this work has already been published in *Chem. and Ind.*, 1963, 257.

[1] Part III (taken as), Drummond, Hirst, and Elizabeth Percival, *J.*, 1962, 1208.
[2] Black, *J. Soc. Chem. Ind.*, 1948, **67**, 165.
[3] Nelson and Cretcher, *J. Amer. Chem. Soc.*, 1932, **54**, 3409.
[4] Fischer and Dörfel, *Z. physiol. Chem.*, 1955, **302**, 186.

chloride followed by calcium chloride,[5] and by the addition of aqueous potassium chloride [6] to a sodium alginate solution. In the present studies fractionation of alginic acid was attempted on a column of diethylaminoethyl(D.E.A.E.)-cellulose [7] by elution with potassium chloride solutions of increasing concentration. Although the polyuronide was eluted in two distinct peaks, paper chromatography of hydrolysates of the material from the respective peaks indicated the same relative proportions of the two acids.

Evidence that a high proportion of the mannuronic acid units are 1,4-linked in the polymer has been derived from methylation studies.[8] Support for this was the isolation of erythraric acid [9] from the products of periodate oxidation of alginic acid followed by bromine oxidation and hydrolysis. Indirect evidence that at least some of the guluronic acid units are 1,4-linked was the isolation of L-(+)-threaric acid [1] from a similar hydrolysate obtained after periodate and bromine oxidation.

Vincent [10] subjected alginic acid to partial hydrolysis and separated from the hydrolysate syrupy oligouronic acids containing both guluronic and mannuronic acid residues, but he offered no evidence, apart from chromatography, that the separated fragments were single oligouronic acids and not mixtures of acids with the same chromatographic mobilities. In view of the extreme difficulty encountered in separating pure oligouronic acids, coupled with the high acid stability of alginic acid, these results do not provide unequivocal proof that the two uronic acids occur in a single molecule. The isolation, by continuous electrophoresis of a partial acid hydrolysate of alginic acid, of a crystalline biouronic acid (considered to be a di-mannuronic acid) has been reported,[11] but its detailed structure was not established.

Neutral polysaccharides are considerably more amenable to partial acid hydrolysis than are polyuronides, and furthermore the derived neutral oligosaccharides are more readily separated by standard chromatographic techniques than are the corresponding oligouronic acids. The present studies are concerned with the reduction of alginic acid to a neutral polysaccharide, and the separation and characterisation of some of the fragments of partial acid hydrolysis. The alginic acid was extracted from freshly harvested weed under the mildest possible conditions [12] and isolated as the free acid either by freeze-drying or by washing with ethanol–water then ethanol, and drying with ether. The isolated material was further dried for several weeks *in vacuo* over phosphoric oxide at 0°, since alginic acid stored at room temperature depolymerises.[13]

Alginic acid is rather inert chemically and appears to require " activation " before acylation can be achieved. Kristensen and Percival [14] found that by swelling alginic acid for 15 minutes in strong mineral acid (concentrated hydrochloric acid or 50% sulphuric acid), washing with water and then glacial acetic acid, and acetylating with acetic anhydride in the presence of sulphuric acid (0·3%) as a catalyst, a fully acetylated product was obtained. Schweiger [15] followed a similar procedure but used perchloric acid as the catalyst. In the present studies the alginic acid was activated by being swollen in formamide,[16] and then suspended in pyridine and acylated with propionic anhydride. The acylation was repeated until constant rotation was attained. The dipropionate was prepared, rather than the diacetate, as Smith and Stephen [17] reported that the latter is not

[5] McDowell, *Chem. and Ind.*, 1958, 1401.
[6] Haug, *Acta Chem. Scand.*, 1959, **13**, 601.
[7] Elizabeth Percival and Wold, *J.*, 1963, 5459.
[8] Hirst, Jones, and Jones, *J.*, 1939, 1880; Chanda, Hirst, E. G. V. Percival, and Ross, *J.*, 1952, 1833.
[9] Lucas and Stewart, *J. Amer. Chem. Soc.*, 1940, **62**, 1792.
[10] Vincent, *Chem. and Ind.*, 1960, 1109.
[11] Jayme and Kringstad, *Chem. Ber.*, 1960, **93**, 2263.
[12] Black, Cornhill, and Dewar, *J. Sci. Food Agric.*, 1952, **3**, 542.
[13] McDowell, " Properties of Alginates," Alginate Industries Ltd., London, 1961, p. 5.
[14] Kristensen and E. G. V. Percival, 1949, unpublished results.
[15] Schweiger, *J. Org. Chem.*, 1962, **27**, 1786.
[16] Carson and Maclay, *J. Amer. Chem. Soc.*, 1946, **68**, 1015.
[17] Smith and Stephen, *Tetrahedron Letters*, 1960, No. 7, 17.

sufficiently soluble in the ether-type solvents used in diborane reductions. By using a large excess (*ca.* × 10) of diborane and a long reaction time (2 days), 90% of the carboxyl groups in alginic acid dipropionate were reduced to primary alcohol groups.

A hydrolysate of this reduced material revealed, in addition to mannose and gulose, trace spots of xylose and of slow-moving oligouronic acids, and three faint fast spots; the fastest of these was not revealed by alkaline triphenyltetrazolium chloride spray, a 2-substituted derivative being therefore indicated. It had been reported [18] that acetylated hydroxyl groups in steroid derivatives are converted by diborane reduction into the corresponding ethoxy-groups ($R{\cdot}O{\cdot}CO{\cdot}CH_3 \longrightarrow R{\cdot}O{\cdot}CH_2{\cdot}CH_3$) and it is considered that this has occurred to a slight extent in the present experiments, with the formation of acid-stable mono- and di-propyl derivatives. Such compounds would be expected to have a high chromatographic mobility. Indeed, analysis indicated a propoxyl content of 5·2%, corresponding to one propyl group for every seventh sugar unit. Whilst this is not significant in the present structural studies, it may be of importance in other reductions of carbohydrates by this method.

The apparent presence of xylose in the hydrolysate of the reduced material, also reported by Smith and Stephen,[17] is difficult to explain, since the starting material gave no indication of any pentose sugar. Other workers [19] have reported the presence of xylose in hydrolysates of alginic acid but, in every instance, paper-chromatographic evidence only has been advanced for the identity of this sugar. Decarboxylation of either mannuronic or guluronic acid during the drastic conditions necessary for hydrolysis of alginic acid would yield lyxose, not xylose. The "apparent" xylose present in the above hydrolysate was quite distinct from lyxose run as a control. In the absence of more rigid proof, however, the identity of the present spot as xylose, rather than a degradation product, can only be tentatively assumed.

Unlike the water-insoluble, acid-resistant, β-1,4-linked mannans studied by other workers,[20] the reduced alginic acid was water-soluble and rather labile even to dilute acid. These properties may possibly be an indication that at least part of the alginic acid is not composed of separate chains of β-1,4-linked mannuronic acid and of guluronic acid, respectively, but that both acids are present in a single molecule.

As partial hydrolysis of the reduced alginic acid with commercial hemicellulase [21] gave only traces of oligosaccharides, the action of dilute sulphuric and oxalic acids for different periods was studied; the best yield was given by 0·1N-oxalic acid at 90° for 2 hour. Residual polymeric material (*ca.* 33%) was precipitated with ethanol, and this appeared to consist almost entirely of mannose residues. However, in view of the ready conversion of gulose into the 1,6-anhydride under acid conditions, the proportion of gulose in probably double that revealed on the paper chromatogram.

The material of low molecular weight, recovered from the alcoholic supernatant liquor as a syrup, was separated into its constituents on Whatman 3MM paper. Since mannose had previously been separated from reduced alginic acid and characterised,[1] the mannose-containing fraction was discarded. The gulose was isolated as a syrup and converted into the 1,6-anhydrogulose,[22] but this too failed to crystallise. The tri-*O*-benzoyl derivative was obtained crystalline and gave an *X*-ray powder photograph identical with that of 1,6-anhydrotri-*O*-benzoyl-D-gulose synthesised from D-gulose. This substance apparently crystallised with a molecule of acetone of crystallisation. This is the first authenticated crystalline derivative of L-guluronic acid derived from alginic acid. It is worthy of note that 1,6-anhydrotri-*O*-benzoyl-D-gulose which had been recrystallised from chloroform–pentane [22] had a higher melting point and gave a different *X*-ray powder photograph.

[18] Pettit and Kasturi, *J. Org. Chem.*, 1961, **26**, 4553.
[19] Massoni and Duprez, *Chimie et Industrie*, 1960, **83**, 79.
[20] Jones, *J.*, 1950, 3292; Aspinall, Hirst, E. G. V. Percival, and Williamson, *J.*, 1953, 3184.
[21] Perila and Bishop, *Canad. J. Chem.*, 1961, **39**, 815.
[22] Stewart and Richtmyer, *J. Amer. Chem. Soc.*, 1955, **77**, 1021.

The major oligosaccharide (S) was obtained crystalline and gave, on hydrolysis and paper chromatography, three spots corresponding to mannose (strongest spot), gulose, and 1,6-anhydrogulose. Pure D-gulose was partly converted into the 1,6-anhydride on being subjected to the same hydrolytic conditions,[22] and, since the degree of polymerisation (DP) of (S) was found [23] to be 2·1, it is concluded that a proportion of the gulose is converted into the 1,6-anhydride during the hydrolysis. Reduction of (S) with borohydride, followed by hydrolysis, gave mannose and gulitol (= sorbitol) indicating that gulose is the reducing moiety in (S). Paper chromatography of (S), and spraying with triphenyltetrazolium hydroxide,[24] indicated that the units were not 1,2-linked. A blue-grey spot, which developed at the same speed and had the same colour as that from mannobiose, was revealed on spraying a second paper containing (S) with periodate and *p*-rosaniline.[24] Paper chromatography of (S), after reduction with borohydride, gave a spot with the same colour and speed of development as cellobiitol.[25] All these results indicate a 1,4-linkage and suggest that (S) is 4-*O*-β-D-mannosyl-L-gulose, the β-linkage being inferred from the negative rotation. However, neither (S) nor reduced (S) moved on ionophoresis in molybdate buffer, although slight streaking occurred with (S). Whilst this is to be expected for the reduced material,[26] since 4-mannosylgulose becomes 3-mannosylsorbitol on reduction, in the non-reduced form, if the gulose is in the C1 conformation, it should form a complex with the molybdate and therefore move on ionophoresis. However, in the disaccharide it is possible that the gulose is held in the 1C or a boat conformation neither of which would complex readily with the molybdate, and some streaking might be expected. This is the first time that a crystalline oligosaccharide containing both mannuronic and guluronic acid has been obtained from alginic acid and therefore the first direct evidence that both acids are present in a single molecule.

Crystalline mannobiose (4-*O*-β-D-mannosyl-D-mannose) and a trace of a syrupy third disaccharide ($R_{\text{mannobiose}}$ 1·2) were also separated. The latter appeared to contain only mannose; after reduction it had $R_{\text{cellobiitol}}$ 1·4 and gave a greyer spot than cellobiitol but with the same speed of development (periodate–*p*-rosaniline spray).[25] The immobility on ionophoresis in molybdate, both before and after reduction, is indicative of 1,3-linkage.[26] The amount separated was too small to permit complete characterisation. Further work will be necessary to determine whether this latter material plays any significant part in the structure of alginic acid or whether it is an artefact. The isolation of mannobiose provides evidence that in some of the molecules adjacent mannuronic acid units occur.

Although alginic acid is thought to constitute the structural polysaccharide of the brown algæ, evidence has also been advanced that it is an active metabolite.[27] That it is not a homogeneous polymer has been clearly demonstrated by fractionation experiments, and it seems probable that there are several molecular species. Frei and Preston [28] suggest, from extraction experiments in which they isolated mannuronic and guluronic acid-rich fractions, that the latter occurs mainly in the cell wall while the former is located in the intercellular mucilage of the weed. On the basis of the available evidence, however, it seems probable that the material hitherto designated as alginic acid is to be regarded as a family of polymers containing varying proportions of the two acids.

Experimental

The analytical methods, including details of the solvents used for chromatography, have been described by Drummond, Hirst, and Percival.[1] Unless otherwise stated, paper chromatograms were sprayed with saturated aniline oxalate.

[23] Peat, Whelan, and Roberts, *J.*, 1956, 2258; Timell, *Svensk. Paperstidn.*, 1960, **63**, 668.
[24] Bell, "Modern Methods of Plant Analysis," ed. Peach and Tracey, Vol. II, Springer Verlag, Berlin-Göttingen, Heidelberg, 1955, p. 1.
[25] Baddiley and Wicken, *Biochem. J.*, 1963, **87**, 34.
[26] Bourne, Hutson, and Weigel, *Chem. and Ind.*, 1959, 1047.
[27] Bidwell, Craigie, and Krotkov, *Canad. J. Bot.*, 1958, **36**, 581.
[28] Frei and Preston, *Nature*, 1962, **196**, 130.

Laminaria digatata (2 kg. wet weight), collected in North Berwick in November 1961, was immediately cut into 1 in. pieces and immersed in water containing calcium hydroxide (20 g.), and the mixture was stirred for 30 min. at 60°. The greenish liquid was removed by decantation and the weed washed with water until neutral. Sulphuric acid (0·2N; .2·5 l.) was stirred with the weed for 10—15 min. and then discarded, and fresh acid (2·5 l.) was added and the mixture set aside overnight at 0°. The residual weed was then washed with water until neutral and digested with sodium carbonate (3%; 5 l.) at 40—60° with occasional stirring for 3 hr. The resulting jelly was diluted with water (20 l.) and stirred overnight at room temperature. Portions (2—4 l.), after dilution with an equal volume of water, were stirred until homogeneous solutions were obtained. Centrifugation and subsequent filtration under suction gave a clear, light yellow solution. Alginic acid was precipitated from this solution by pouring it into an excess of N-hydrochloric acid with stirring. The precipitate was washed exhaustively with water until free from chloride ions (2—3 days) and the alginic acid isolated either by freeze-drying an aqueous suspension or by sequential washing of the precipitate with aqueous ethanol of increasing alcohol content, then with alcohol–ether, and finally triturating with ether. The dried product (52·3 g.) was a cream-coloured powder, $[\alpha]_D$ −139° (*c* 1·16 in 0·1N-NaOH), Equiv., 199 (phenolphthalein).

Preparation of the Di-O-propionyl Ester of Alginic Acid.[17]—Alginic acid (20 g.; dried *in vacuo* for 2 weeks over phosphoric oxide) was added slowly to dimethylformamide (150 g.) and stirred for 1 hr. at 40—50°. To the resulting thick jelly, pyridine (200 g.) was added during 30 min. with stirring, and the mixture was allowed to cool to 30°. Propionic anhydride (200 g.) was added with continuous stirring during 4 hr. and the stirring continued for a further 4—5 hr. The viscous brown solution, after standing overnight at room temperature, was poured on to a mixture of ice (500 g.) and N-hydrochloric acid (5 l.). The precipitated cream-coloured flocculent ester, after filtration, was washed with 0·1N-hydrochloric acid and water, and dried *in vacuo*, to give a cream-coloured crumbly solid (26 g.). It was dispersed in pyridine (300 g.) and re-esterified with propionic anhydride (50 g.). The mixture was kept at room temperature for 1 week and poured into an excess of light petroleum (b. p. 60—80°) (1·5 l.). The resulting ester was an off-white powder (28 g.), $[\alpha]_D$ −166° (*c* 0·50 in pyridine). Re-esterification gave no increase in weight or change in rotation, and the product was soluble in dry diethyleneglycol dimethyl ether (diglyme).

Diborane Reduction of Alginic Acid Dipropionate.[17]—Di-*O*-propionylalginic acid (12 g.) in freshly distilled (twice over sodium wire and once over lithium aluminium hydride), peroxide-free, diglyme (200 ml.) was mixed with sodium borohydride (20 g.) in diglyme (100 ml.). Boron trifluoride etherate (100 g.) in diglyme (250 ml.) was added in nineteen portions during 2—2·5 hr. Between the additions the mixture was continuously shaken. It was then set aside at room temperature with occasional shaking for 2 days. Thereafter ice and water were added (400 ml.) and the mixture was neutralised with dilute sodium hydroxide solution. The solution was concentrated at 40°/1 mm. to *ca.* 150 ml. and mixed with N-sodium hydroxide (150 ml.); this gave a final pH of *ca.* 10. The mixture was kept at 60—70° for 2 hr., the small amount of precipitate was removed by filtration, and the filtrate was dialysed and concentrated. Methanol was added to the residual solution several times, with subsequent evaporation, to remove the last traces of borate. Finally the mixture was concentrated to *ca.* 100 ml., and the neutral polysaccharide precipitated by pouring this solution into a stirred 1 : 3 mixture of ether in methanol (900 ml.). After drying, the product (4·9 g.) had $[\alpha]_D$ −87·5° (*c* 0·40 in 0·1N-NaOH). Conversion of an aliquot part into the free acid by treatment with Amberlite IR-120 (H^+) resin, dissolution in carbon dioxide-free water, and titration with 0·05N-sodium hydroxide gave an equivalent weight of 2000. This corresponds to a uronic anhydride content of 8·8% [Found: uronic anhydride (decarboxylation) 9·6%]. Chromatography of a hydrolysate (N-sulphuric acid for 6 hr. at 100°) revealed two main spots with the chromatographic mobility of mannose and gulose (solvent 9), a few weak, slow-moving, acid spots (Bromocresol Green spray), a trace spot with the colour and mobility of xylose, and three faint spots with $R_{mannose}$ 1·65, 2·00, and 2·50, and R_F 0·56 (brown), 0·66 (brown), and 0·84 (pink), respectively, in solvent (2). The neutral polysaccharide had n-propoxyl = 5·2% (kindly determined by Mr. S. S. H. Zaidi by the specific method of infrared spectroscopy [29]). Chromatographic examination of hydrolysates (N-sulphuric acid for 8 hr. at 100°) of the alginic acid used as starting material and

[29] Anderson and Duncan, *Talanta*, 1961, **8**, 1.

of the seaweed in solvents (2) and (3) revealed a trace spot corresponding to xylose in the latter, but that from the alginic acid was devoid of any trace of this pentose.

Partial Acid Hydrolysis of the Reduced Polysaccharide.—(*a*) Portions of the reduced polysaccharide (10 mg.) were heated with sulphuric acid (1 ml.) of various strengths (0·5N, 0·25N, 0·1N, and 0·05N) at 100 or 80° for various intervals (1, 2, and 5 hr.). Paper chromatography of the products revealed the presence of mannose and gulose, the latter being present in proportionately larger amounts in those from the weaker acid treatments. In no case was the yield of oligosaccharides appreciable. Hydrolysis with oxalic acid appeared to give a slightly higher yield of oligosaccharides.

(*b*) Reduced alginic acid (3 g.) was hydrolysed with 0·1N-oxalic acid (300 ml.) at 90° for 2 hr. Some brown undissolved material was filtered off and dried [0·15 g. = (A)]. The filtrate, after concentration to *ca.* 75 ml., was treated with ethanol (455 ml.), and the mixture set aside overnight. The precipitated polysaccharide was filtered off and dried [0·98 g. = (B)]. After concentration of the supernatant liquor to 100 ml., neutralisation with calcium carbonate, and filtration, the filtrate was shaken with Amberlite IR-120 (H^+) resin and evaporated to a syrup (C) (1·80 g. after drying over phosphoric oxide). Paper chromatography of hydrolysates of (A) and (B) revealed that, apart from a trace of mannose, (A) was devoid of carbohydrate, whereas that from (B) gave a strong spot corresponding to mannose and a weak spot for gulose. Paper chromatography of (C) revealed approximately equal quantities of mannose and gulose, two distinct oligosaccharide spots, one with the mobility of mannobiose in solvents (2) and (3) and the other (S), the stronger spot having $R_{\text{mannobiose}}$ 0·79 and 0·82, respectively, in the two solvents. Some low-mobility higher oligosaccharides, and faint indication of the three fast spots detected in the original hydrolysate of the reduced material, were also revealed.

Separation and Characterisation of the Major Fragments from the Partial Hydrolysate.—The syrup (C) (1·8 g.) was separated on Whatman 3MM paper into gulose (4 sheets) and oligosaccharides (*ca.* 20 sheets) (23 × 45 cm.) in solvent (2) [2 days elution for gulose, and 3 days for mannobiose and oligosaccharide (S)].

Gulose. The syrupy gulose was refluxed for 13 hr. in 0·5N-sulphuric acid (10 ml.).[22] Unchanged gulose was then destroyed by heating with an excess of barium hydroxide in an open beaker on a steam-bath for several hours. The non-reducing solution was freed from Ba^{2+} by precipitation with sulphuric acid, and filtered. The filtrate was deionised with Amberlite IR-120 (H^+) and Duolite 45 (OH) resins. Concentration of the filtrate gave a chromatographically pure syrup of 1,6-anhydrogulose (silver nitrate spray [1]) which failed to crystallise. It was dispersed in dry pyridine and treated with 2 drops of benzoyl chloride, and the mixture set aside overnight. Two drops of water were then added and the mixture left for 15 min. It was extracted with chloroform, and the chloroform solution was washed with an ice-cold dilute sulphuric acid, and saturated sodium hydrogen carbonate, dried (Na_2SO_4), and concentrated. The derived syrup was taken up in alcohol and the solution treated with charcoal. Removal of the alcohol gave crystals, m. p. 122° (change of crystal form 100—105°) (from aqueous acetone). An *X*-ray powder photograph was identical with authentic material prepared as described below from D-gulose (mixed m. p. 110—115°).

Synthesis of 1,6-*Anhydrotri*-O-*benzoyl*-D-*gulose.* Authentic D-gulose (150 mg.) was partially converted into the 1,6-anhydro-derivative by refluxing with acid, as in the previous experiment. The mixture was then separated on Whatman 3MM paper and the chromatographically pure syrupy 1,6-anhydrogulose converted into the tri-*O*-benzoyl derivative, m. p. (from aqueous acetone), 122° (change of crystal form 100—105°), $[\alpha]_D$ +120° (*c* 0·2 in $CHCl_3$) (Found: C, 67·25; H, 4·96. Calc. for $C_{27}H_{22}O_8,C_3H_6O$: C, 67·64; H, 5·26%).

Oligosaccharides.—*Oligosaccharide* (*S*). After extraction from the appropriate section of the filter paper with water, oligosaccharide (S) was not completely pure and was rechromatographed in the same solvent. It was then obtained as a chromatographically and ionophoretically [1] (M_G 0·62) pure white solid (60 mg.) which could be detected as a pink spot on a paper chromatogram sprayed with triphenyltetrazolium hydroxide.[24] Paper chromatography and development with periodate–*p*-rosaniline hydrochloride sprays [25] slowly gave a greyish purple spot which developed at the same speed and had the same colour as that from mannobiose. After reduction, the derived alcohol had $R_{\text{cellobiitol}}$ 1·43, and gave a reddish purple spot similar in colour and time of development to that from cellobiitol. Ionophoresis in 1·5% sodium molybdate [26] (pH 5, 2000v) of (S) and reduced (S) (kindly carried out by Dr. D. H. Hutson) gave single spots which did not migrate from the starting line although slight streaking

occurred with (S). The material (S), as eluted, appeared to be contaminated with some water- and alcohol-soluble non-carbohydrate material from the filter paper. This was removed by refluxing for 15—20 min. with methanol–chloroform (1 : 1 v/v). The undissolved oligosaccharide (25 mg.), after recrystallisation from ethanol–ethyl acetate (1 : 1), had m. p. 201—203°, $[\alpha]_D$ −23·2° (*c* 0·83 in H_2O). A hydrolysate, on paper chromatography (solvent 9), revealed spots corresponding to mannose and gulose, the latter being fainter than the former. However on development with silver nitrate and ethanolic sodium hydroxide,[30] or with periodic acid–benzidine,[31] an additional faster spot, $R_{\text{mannose/gulose}}$ 1·80 (solvent 2), slowly appeared. Pure gulose, when heated with acid under the conditions used for the hydrolysis of (S) gave the same fast-moving spot with the mobility of 1,6-anhydrogulose,[22] in addition to the gulose spot, only the latter being revealed with aniline oxalate spray.

The DP of (S) was estimated according to Timell's modification of the method of Peat, Whelan, and Roberts.[23] Oligosaccharide (S) (3·4 mg.) was dissolved in water (25 ml.). To 1·00 ml. of this solution was added 1% aqueous sodium borohydride (1·00 ml.), and to a second portion (1·00 ml.) 1% sodium borohydride in N-sulphuric acid (1·00 ml.) " inactive borohydride " was added. The two solutions were set aside for 1 hr. at room temperature. Aqueous phenol (4%; 1 ml.), followed by concentrated sulphuric acid (5·00 ml.), was then added to each solution.[32] After 0·5 hr. at room temperature the optical densities were read at 490 mμ against a blank, prepared in the same way as the test solutions but with water replacing the oligosaccharide solution. A number of tests were carried out and the average optical densities of the solutions from the reduced and the non-reduced oilgosaccharide were 0·374 and 0·705, respectively. The DP is $Q/Q - 1$, where Q is the ratio of the optical densities of the two solutions. In the present instance $Q = 1{\cdot}89$ and the DP = 2·1.

To determine the sequence of the two sugars in (S) a sample (*ca.* 3 mg.) was dissolved in water (0·5 ml.) and left overnight at room temperature with sodium borohydride and boric acid (*ca.* 3 mg. of each). After neutralisation with Amberlite IR-120 (H^+) resin, removal of borate (methanol), and concentration of the solution, the residue was hydrolysed. Paper chromatography in solvent (9) revealed only mannose. Silver nitrate [30] spray on a second chromatogram revealed also L-gulitol (=D-sorbitol) clearly distinguished from mannitol, both alcohols being run as controls.

Mannobiose. The syrupy mannobiose, $R_{\text{cellobiose}}$ 0·92 (solvent 2), eluted from the 3MM paper was contaminated with a trace of another disaccharide, $R_{\text{mannobiose}}$ 1·19 (solvent 2). It was rechromatographed on Whatman 3MM paper and the mannobiose, after keeping for some weeks, crystallised. It had m. p. and mixed m. p. 190—193°.

Unknown disaccharide. The disaccharide, $R_{\text{mannobiose}}$ 1·19, after reduction, gave a single spot, $R_{\text{cellobiitol}}$ 1·4 (solvent 2) definitely more grey (periodate–*p*-rosaniline spray) than that given by cellobiitol, but with the same speed of development. Ionophoresis of the disaccharide and its reduction product in 1·5% sodium molybdate (pH 5; 2000v) [26] for 1 hr. (kindly carried out by Mr. Frances Searle) gave spots which did not move, although some streaking occurred with the reduced material. Hydrolysates of the disaccharide before and after reduction showed mannose, and possibly mannose and mannitol, respectively (paper chromatography).

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Department of Chemistry, University of Edinburgh.

[Present address (E. P.): Chemistry Department, Royal Holloway College, Englefield Green, Surrey.] [*Received, September 13th*, 1963.]

[30] Trevelyan, Proctor, and Harrison, *Nature*, 1950, **166**, 444.
[31] Gordon, Thornburg, and Werum, *Analyt. Chem.*, 1956, **28**, 849.
[32] Dubois, Gilles, Hamilton, Rebers, and Smith, *Analyt. Chem.*, 1956, **28**, 350.