

**394.** *The Structure of Brachychiton diversifolium Gum*  
(*Sterculia caudata*).

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The gum exudate from *Brachychiton diversifolium* is an acetylated acidic polysaccharide which contains rhamnose (1 part), galactose (1 part), and glucuronic acid (2 parts). Graded hydrolysis of the gum gives D-glucurone, an aldobiuonic acid, identified as 2-O- $\alpha$ -D-glucuronosyl-L-rhamnose, and unidentified oligosaccharides of high uronic acid content.

The methylated gum gives on hydrolysis 2 : 3 : 4 : 6-tetra-O-methyl- and 2 : 3 : 6-tri-O-methyl-D-galactose, 3 : 4-di-O-methyl- and 3-O-methyl-L-rhamnose, 2 : 3 : 4-tri-O-methyl-D-glucuronic acid, and a mixture of methylated uronic acids.

THROUGH the kindness of Mr. V. Grenning, Director of Forests in Queensland, Australia, a sample of gum from *Brachychiton diversifolium* collected in the Atherton district of Northern Queensland was made available for study. This tree was formerly known as *Sterculia caudata*. Gum exudates of the *Sterculia* trees previously examined<sup>1</sup> have been found to be polymers of rhamnose, galactose, and galacturonic acid residues, partly acetylated in two instances at least. The gum from *Sterculia setigera* also contains residues of tagatose. The three species so far investigated, *S. urens*, *S. tormentosa*, and *S. setigera*, are, however, all characterised by a high uronic acid content and are consequently very resistant to hydrolysis.

Investigation of three separate nodules of *Brachychiton* exudate indicated that the sample was essentially homogeneous, and contained residues of galactose, rhamnose, and glucuronic acid. Traces of xylose and arabinose were also present in the crude gum, and

<sup>1</sup> Beauquesne, *Compt. rend.*, 1946, **222**, 1056; Hirst, Hough, and Jones, *J.*, 1949, 3145; Hough and Jones, *J.*, 1950, 1199; Rao and Sharma, *Proc. Indian Acad. Sci.*, 1957, **45**, A, 24.

were detected in large-scale work on the pure polysaccharide. The proportions present, however, are so low they can have very little structural significance. It was noticeable that gum contaminated with bark had more pentoses than did the purer sample. The presence of ash (4.1%) was due at least in part to metal salts of the uronic acid; the aqueous mucilage was neutral to litmus and non-reducing to Fehling's solution. The small amount of methoxyl (1.4%) was present as methyl ester since the purified polysaccharide, isolated after alkaline treatment of the gum, was free from methoxyl groups. The acetyl content (19.3%) resembled that of *Sterculia setigera*<sup>1</sup> and *Cochlospermum gossypium*.<sup>2</sup>

The gum was slightly soluble in water from which it was best precipitated with acetone. Three extracts were obtained of identical composition. Further attempts at fractionation by dissolution of the gum in alkali and precipitation of the "free-acid" form by addition of ethanol were unsuccessful. These results are in harmony with the electrophoretic behaviour of the pure polysaccharide in a borate buffer at pH 10, where a single peak was obtained.

The purified gum,  $[\alpha]_D +69^\circ$ , had an equivalent weight of 342 and a uronic anhydride content of 50.1% (calculated for a substance of equivalent weight of 342, 51.5%). The proportion of uronic anhydride is unusually high for a plant gum, but finds parallels in *Sterculia setigera*<sup>1</sup> gum and *Khaya grandfolia*<sup>3</sup> gum. In keeping with this high uronic acid content, and the extreme stability of the glycosiduronic acid linkage, the material was very resistant to hydrolysis. Complete hydrolysis was accompanied by degradation: the solutions invariably darkened during hydrolysis and the yield of hydrolysate was never higher than 80%.

Although a complete quantitative hydrolysis was impossible, estimation<sup>4,5</sup> of the neutral sugars indicated the presence of approximately equal amounts of galactose and rhamnose. The quantity of rhamnose was substantiated by determination<sup>6</sup> of the proportion (24%) of this sugar in the unhydrolysed polysaccharide. A partial hydrolysate (N-sulphuric acid at 100° for 7 hr.), after neutralisation with barium hydroxide, was concentrated to an amorphous solid (A) consisting of neutral sugars and barium uronates (B). The neutral sugars were extracted with methanol and partitioned on a cellulose column. Crystalline L-rhamnose (3.2% of the original weight of gum) and D-galactose (10%) were separated, and further characterised as crystalline derivatives. On further hydrolysis, the acidic fraction (B) (72% of the original weight of gum) liberated more galactose and rhamnose.

Conversion of the hydrolysate (A), after de-ionisation, into the methyl ester methyl glycoside, reduction with potassium borohydride, and hydrolysis gave a syrup (C) containing galactose, rhamnose, and glucose (paper chromatography), the last being eliminated by treatment of the hydrolysate with D-glucose oxidase. Partition of syrup (C) on a paper column led to the isolation and characterisation of the following sugars in the approximate proportions given: rhamnose (27.5%), galactose (54%), and glucose (18.5%). An alternative method of estimation<sup>4,7</sup> of the proportions of the sugars present in syrup (C) gave rhamnose (24%), galactose (58%), and glucose (17.7%); these figures are in reasonable agreement with the amount of the sugars separated from this syrup. As glucose was not found in any of the gum hydrolysates, it was not present in the original gum, and the glucose isolated from syrup (C) was clearly produced by reduction of glucuronic acid residues by potassium borohydride. Support for this deduction was obtained by the separation and characterisation (as the crystalline *p*-nitroaniline derivative) of glucurone from the acid fraction of the gum hydrolysate.

<sup>2</sup> Hirst and Dunstan, *J.*, 1953, 2332.

<sup>3</sup> Aspinall, Hirst, and Matheson, *J.*, 1956, 989.

<sup>4</sup> Hirst and Jones, *J.*, 1949, 1659.

<sup>5</sup> Pridham, *Analyt. Chem.*, 1956, **28**, 1967.

<sup>6</sup> Dische and Shettles, *J. Biol. Chem.*, 1948, **175**, 595.

<sup>7</sup> Manners, unpublished work.

The presence of D-glucuronic acid in *Brachychiton diversifolium* exudate is in contrast to the positions with the other species of *Sterculia* gums examined, with *Cochlospermum gossypium*, and with *Khaya grandifolia*, all of which contain D-galacturonic acid, although the last of these contains in addition 4-O-methyl-D-glucuronic acid. The possible presence of galacturonic acid was considered and the question was raised whether the higher proportion of galactose found in the reduced syrup (C) compared with the amount estimated in the gum hydrolysate might have been produced by reduction of galacturonic acid residues. However, no evidence could be obtained for the presence of this acid in any of the gum hydrolysates by chromatography, by standard colour tests, or by the isolation of mucic acid from the oxidised acid hydrolysate. It might be argued that the inability to isolate free galacturonic acid was due to its degradation under the hydrolytic conditions used since Fischer and Dorfel<sup>8</sup> record 77% and 97% destruction of galacturonic acid by N-hydrochloric acid at 100° in 20 and 40 hours respectively. Against this argument, however, is the fact that the present authors failed to isolate any oligosaccharides or methylated oligosaccharides containing galacturonic acid residues from partial acid hydrolysates of the gum or methylated gum respectively. The present indications are that galacturonic acid residues, if present in the gum, are only there in very small proportion and have escaped detection.

The gum was converted into its fully methylated derivative (OMe, 42.3%) which, like the parent material, was extremely resistant to hydrolysis. Methanolysis with 8% methanolic hydrogen chloride and then with aqueous hydrochloric acid yielded 2 : 3 : 4 : 6-tetra-O-methyl-D-galactose (12 parts), 3 : 4-di-O-methyl-L-rhamnose (1 part), 2 : 3 : 6-tri-O-methyl-D-galactose (10 parts), 3-O-methyl-L-rhamnose (4 parts) (characterised as crystalline derivatives), and a mixture of methylated barium uronates (69% of the hydrolysate). The barium uronates were hydrolysed further with 2N-sulphuric acid, and the product was separated into three fractions. Each fraction was converted into the methyl ester methyl glycoside, reduced with lithium aluminium hydride, and hydrolysed. From fraction U1 2 : 3 : 4-tri-O-methyl-D-glucose and 3 : 4-di-O-methyl-L-rhamnose were separated. The former was characterised by its analytical constants and as the N-phenyl-D-glucosylamine, and the latter by its analytical constants, demethylation, electrophoretic mobility, and oxidation with potassium periodate.<sup>9</sup> As tri-O-methylglucose was not present among the neutral sugars isolated on direct hydrolysis of the methylated gum, it must have arisen by reduction of 2 : 3 : 4-tri-O-methyl-D-glucuronic acid. The hydrolysates from the second and third fractions both contained 2 : 3 : 4-tri-O-methyl-D-glucose, 3-O-methyl-L-rhamnose, rhamnose, and a mono-O-methylhexose. In addition, the third fraction contained 2 : 3 : 6-tri-O-methylgalactose and a di-O-methylhexose (paper chromatography).

The hydrolysate of the methylated, reduced, remethylated polysaccharide was found to contain 2 : 3 : 4 : 6-tetra-O-methylglucose in addition to all the methylated derivatives found above (paper chromatography).

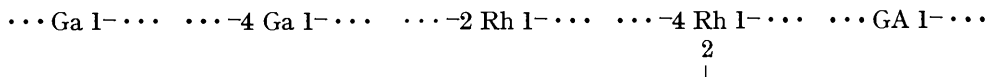
Attempted fractionation of the partly hydrolysed acidic material (B) on an ion-exchange resin column gave pure fractions corresponding to glucurone and an aldobiuronic acid. The aldobiuronic acid,  $[\alpha]_D +63^\circ$ , gave rhamnose and glucuronic acid (paper chromatography) on hydrolysis and had the correct equivalent weight (332) for an aldobiuronic acid containing these two sugar residues. Reduction with potassium borohydride eliminated rhamnose, indicating that the free reducing group was carried by this sugar. Conversion of the aldobiuronic acid into the methyl ester methyl glycoside, reduction, and remethylation gave a methylated disaccharide which on hydrolysis and separation of the hydrolysate on thick paper gave 2 : 3 : 4 : 6-tetra-O-methyl-D-glucose and 3 : 4-di-O-methyl-L-rhamnose, both sugars being identified by chromatography, ionophoresis, and demethylation. A trace of a third component corresponding chromatographically

<sup>8</sup> Fischer and Dorfel, *Z. physiol. Chem.*, 1955, **301**, 224; **302**, 186.

<sup>9</sup> Butler, Lloyd, and Stacey, *J.*, 1955, 1531.

to a tri-*O*-methylhexose was considered to have arisen through under-methylation. These results indicate that the aldobiuronic acid is 2-*O*- $\alpha$ -D-glucuronosyl-L-rhamnose. This is supported by the isolation of 3 : 4-di-*O*-methyl-L-rhamnose from the hydrolysate of the methylated polysaccharide, and of 2 : 3 : 4-tri-*O*-methyl-D-glucose from the reduced methylated uronic acids, and the chromatographic detection of 2 : 3 : 4 : 6-tetra-*O*-methylglucose in the hydrolysate of the methylated, reduced, and remethylated polysaccharide. Although 2-*O*-galacturonosyl-L-rhamnose has been isolated from other plant gums,<sup>2,3,10</sup> and from okra mucilage,<sup>11</sup> this is the first reported isolation of glucuronic acid glycosidically linked to C<sub>(2)</sub> of L-rhamnose.

It is not possible to formulate a unique molecular structure for the gum. The monomethylrhamnose isolated from the hydrolysate of the methylated polysaccharide and the free rhamnose, and di- and mono-*O*-methylhexoses in the methylated acidic hydrolysate, must have arisen from residues which were triply linked in the gum. It is clear, therefore, that the gum possesses a highly branched structure with galactose and glucuronic acid residues at the ends of the branches. Sugar residues, the presence of which has been definitely established, are:



(Ga = D-galactopyranose, Rh = L-rhamnopyranose, GA = D-glucopyruonic acid).

The isolation and characterisation of an aldobiuronic acid indicate that the residue GA I-2 Rh is also a structural feature of the gum.

#### EXPERIMENTAL

*Analytical Methods.*—All solutions were evaporated under reduced pressure below 60°. Unless otherwise stated, hydrolyses were done with 2*N*-sulphuric acid for 24 hr. at 100°. The cooled hydrolysates were neutralised with barium carbonate, and the filtrates concentrated to syrups. Paper-partition chromatography was carried out on Whatman No. 1 paper at a constant temperature of 20° with the upper layers of the following solvent systems (v/v): (1) butan-1-ol-benzene-pyridine-water (5 : 1 : 3 : 3); (2) ethyl acetate-pyridine-water (10 : 4 : 3); (3) butan-1-ol-ethanol-water (4 : 1 : 5); (4) ethyl acetate-acetic acid-formic acid-water (18 : 3 : 1 : 4); (5) butan-1-ol-acetic acid-water (4 : 1 : 5); (6) benzene-ethanol-water (169 : 47 : 15); (7) ethyl methyl ketone half saturated with water plus ammonia (99 : 1). Papers were sprayed with saturated aqueous solution of aniline oxalate, and 3% *p*-anisidine hydrochloride in butan-1-ol containing a little stannous chloride.  $R_G$ ,  $R_{Gal}$ , and  $R_F$  are the rates of travel relative to tetramethylglucose, galacturonic acid, and the solvent front respectively. Electroionophoresis<sup>12</sup> was carried out in borate buffer of pH 10. Whatman 3MM sheets after preliminary extraction with hot benzene-ethanol (1 : 1) were used for the chromatographic separation of small amounts of material. Specific rotations were measured in water at 18°.

*Preliminary Examination of the Gum.*—The gum, obtained as light-brown translucent nodules, was contaminated with bark and had a resinous odour. Three separate nodules were powdered, dried to constant weight over phosphoric oxide at 60° (loss 17%), and analysed. They were not significantly different in composition and had ash 4.1; N, 0; OMe, 1.4; AcO, 19.3%. Chromatography of acid hydrolysates (solvent 1) revealed spots corresponding in position and colour with glucurone, rhamnose, galactose, and barium uronates, the last remaining at the starting line.

*Purification and Attempted Fractionation of the Material.*—(1) Repeated extraction of the crude gum with water and precipitation of the polysaccharide from each extract by the addition of acetone gave identical fractions (paper chromatography of the hydrolysates).

(2) The crude material (4 g.) was dissolved in chilled *N*-sodium hydroxide (200 c.c.) in an atmosphere of nitrogen. After acidification (pH 2) of the solution with 50% hydrochloric

<sup>10</sup> Hirst, Hough, and Jones, *J.*, 1949, 3145.

<sup>11</sup> Whistler and Conrad, *J. Amer. Chem. Soc.*, 1954, **76**, 3544.

<sup>12</sup> Foster, *Chem. and Ind.*, 1952, 1050.

acid, and removal of fragments of bark at the centrifuge, the polysaccharide was precipitated in four fractions by the gradual addition of ethanol (1.3 vols. gave 1.81 g.; 2 vols., 0.30 g.; 5 vols., 0.06 g.; 7 vols., 0.03 g.). Each fraction, freed from chloride ion by trituration with ethanol, was washed with ether and dried over phosphoric oxide (60°/12 mm.) to constant weight. Chromatography of the hydrolysate of each fraction (solvent 1) showed spots identical with those given by the crude gum. Very faint spots corresponding to xylose and arabinose were also detected from the last two fractions.

The recombined fractions were dissolved in water, and the polysaccharide, precipitated with ethanol (3 volumes), was washed and dried as before. It was a white fibrous material (2.2 g.) which had  $[\alpha]_D + 69^\circ$  ( $c$  0.4 in *N*-NaOH) [Found: equiv., 342 (by titration): uronic anhydride, 50.1 (by decarboxylation); ash, 0.83%; AcO, 0; OMe, 0]. A portion (0.05 g.) in borate buffer of pH 10 (10 c.c.) gave a symmetrical peak when examined in the Antweiler micro-electrophoresis apparatus (1.5 mA, 35 v, 10 min.). Autohydrolysis of the polysaccharide for 24 hr. at 100° showed traces of galactose and rhamnose in the concentrated hydrolysate. After hydrolysis with *N*-sulphuric acid at 100° for 13 hr. ( $[\alpha]_D + 104^\circ$ , 2 hr.  $\rightarrow + 77^\circ$ , 13 hr.) the solution still contained unhydrolysed polysaccharide. Heating with 2*N*-sulphuric acid at 100° for 24 hr. was necessary to hydrolyse the polysaccharide completely. The solution then became dark brown and it was necessary to decolorise the neutralised filtrate with charcoal. Quantitative estimation, by the method of Hirst and Jones,<sup>4</sup> of the sugars produced on complete acid hydrolysis of the gum gave, after allowance for a uronic anhydride content of 50%, a 60% recovery of neutral sugars (calc. as percentage of anhydro-sugars in the original gum) comprising 23.5% of rhamnose and 26.5% of galactose. Pridham's colorimetric method<sup>5</sup> gave the proportion of galactose : rhamnose = 1 : 1.09. After allowance for the uronic acid present, this corresponds to 23.8% of anhydrogalactose and 26.0% of anhydrorhamnose in the gum. Colorimetric estimation of rhamnose by the method of Dische and Shettles<sup>6</sup> gave 23.9% of anhydrorhamnose in the purified gum.

*Partial Hydrolysis of the Gum and Characterisation of the Neutral Sugars.*—The purified gum (9.45 g.) was heated at 100° with *N*-sulphuric acid (500 c.c.) for 7 hr. The cooled solution was made slightly alkaline with barium hydroxide solution, the excess of alkali being removed immediately with carbon dioxide and the filtrate concentrated to a solid (A) consisting of neutral sugars and barium uronates. The cold aqueous extracts of this material were poured into well-stirred methanol (10 volumes). The precipitated barium salts were exhaustively extracted with hot methanol, and the total methanolic liquors evaporated to a syrup (3.11 g.). The barium uronates (B) (5.38 g.) were left as an amorphous solid. The syrup was fractionated on a cellulose column<sup>13</sup> (85 × 2.7 cm.) with butan-1-ol two-thirds saturated with water as the eluant:

Fraction 1 (1.125—2.175 l.) (0.021 g.),  $R_F$  0.78 (solvent 1), gave a positive Selivanoff reaction.

Fraction 2 (2.325—2.925 l.): L-rhamnose (0.26 g.),  $R_F$  variable but identical with that of authentic rhamnose,  $[\alpha]_D^{20} + 8.4^\circ$  ( $c$  1.0). Recrystallisation from butan-1-ol gave L-rhamnose hydrate, m. p. and mixed m. p. 88—89°. The benzoylhydrazone<sup>10</sup> had m. p. and mixed m. p. 186—189°.

Fraction 3 (2.930—6.225 l.) (0.046 g.),  $R_F$  0.34—0.39. Three components were observed in this fraction (paper chromatography) corresponding to rhamnose, xylose, and arabinose. Visual inspection suggested that rhamnose comprised the bulk of the fraction.

Fraction 4 (8.250—12.027 l.): D-galactose (0.951 g.), m. p. and mixed m. p. 163—165°,  $[\alpha]_D^{20} + 83^\circ$  ( $c$  3.5). The diethyl mercaptal<sup>14</sup> had m. p. and mixed m. p. 140—141°.

Fraction 5 (1.43 g.), eluted partly with butan-1-ol-acetic acid-water (4 : 1 : 5) and finally with water, was added to the amorphous barium uronates (B) (total yield 6.81 g.).

*Isolation and Characterisation of Glucose after Reduction of the Polysaccharide Hydrolysate.*—The mixture of neutral sugars and barium uronates (A) (8.2 g.), after de-ionisation with ion-exchange resin (Amberlite IR-120, H<sup>+</sup> form), was refluxed with 4% methanolic hydrogen chloride for 6 hr. The neutralised filtrate gave on evaporation a solid which was dissolved in water (50 c.c.) and added slowly with stirring to a solution of potassium borohydride (4 g.) in water (60 c.c.). After 2 hr. excess of acetic acid was added and ions were removed on a mixed-bed resin column (Amberlite IR-120 H<sup>+</sup> and IR-4B, OH<sup>-</sup>). The eluant was evaporated to

<sup>13</sup> Hough, Jones, and Wadman, *J.*, 1949, 2511.

<sup>14</sup> Wolfrom, *J. Amer. Chem. Soc.*, 1930, 52, 2466.

dryness, traces of borate being removed by repeated evaporation with methanol. The derived syrup (5.1 g.) was heated at 100° with *n*-sulphuric acid for 7 hr. Neutralisation of the cooled solution, with barium carbonate, filtration, and evaporation gave a syrup (C) (4.4 g.) containing glucose, galactose, and rhamnose (paper chromatography). Glucose was eliminated on treatment of the syrup (C) (10 mg.) with the specific enzyme *D*-glucose oxidase. Partition of the syrup (C) (2.19 g.) on a Grycksbo filter paper column (LKB 3391) eluted with butan-1-ol under-saturated with water (prepared by saturating butan-1-ol with water at 0° and allowing the separated organic phase to warm to room temperature) gave:

Fraction (a): *L*-Rhamnose (0.508 g.), characterised as the 2 : 5-dichlorophenylhydrazone, m. p. and mixed m. p. 171°.

Fraction (b): *D*-Glucose (0.150 g.), m. p. and mixed m. p. [after recrystallisation of the isolated material and of authentic *D*-glucose from ethanol and light petroleum (b. p. 100—120°)] 148—149°,  $[\alpha]_D + 53^\circ$  (*c* 0.70).

Fraction (c): *D*-Galactose (0.631 g.), characterised as the 2 : 5-dichlorophenylhydrazone, m. p. and mixed m. p. 196—197°. An overlap fraction (0.563 g.) containing galactose and glucose (2 : 1) (paper chromatography, visual estimation) was also separated.

The quantity of glucose in syrup (C) was estimated by determination of the reducing power<sup>15</sup> before and after treatment with *D*-glucose oxidase<sup>7</sup> (commercial "Dee-O" manufactured by the Takamine Corporation), an enzyme which catalyses the oxidation of glucose to the non-reducing gluconic acid.<sup>16</sup> Synthetic mixtures containing glucose (a) 0.25 mg., (b) 0.41 mg., galactose (a) 1.49 mg., (b) 1.30 mg., and rhamnose (a) 0.60 mg., (b) 0.55 mg. per 5.0 c.c. portion, and the syrup C (2.26 mg. in 5.0 c.c. of water) were each treated with *D*-glucose oxidase (7.5 mg. in water 1 c.c.) for 3 hr. at 36°. As it was necessary to deproteinise the mixture before measuring the reducing power the mixture was then treated with water (0.40 c.c.), zinc sulphate solution (0.30 c.c.), and barium hydroxide solution (0.30 c.c.) which had been prepared according to Nelson's directions.<sup>17</sup> The precipitate was removed on the centrifuge, a portion of the supernatant solution (5.00 c.c.) withdrawn, and its reducing power estimated. A "blank" determination was carried out simultaneously in the same manner, except that the enzyme was inactivated by heating it at 100° for 10 min. before use. Reducing powers are expressed as c.c. of 0.01*N*-sodium thiosulphate and in each instance have been calculated so as to refer to a 5 c.c. aliquot part of the original solution:

	(a)	(b)	C
Reducing power of "blank" (c.c.) .....	6.55	6.41	5.29
Reducing power of incubated solution (c.c.) .....	5.65	4.90	3.98
Decrease in reducing power after incubation (c.c.) .....	0.90	1.51	1.31
Glucose (mg.) per 5.0 c.c. ....	0.26	0.44	0.39

Hence the syrup (C) contained 17.7% (average of two determinations) of glucose.

Quantitative estimation<sup>4</sup> of the rhamnose and total hexose in syrup (C) gave 24.2 and 75.8% respectively.

Syrup (C), therefore, comprises *D*-glucose 17.7, *L*-rhamnose 24.2, and *D*-galactose 58.1%. The syrup had  $[\alpha]_D + 55^\circ$  (*c* 1.1). The above composition requires  $[\alpha]_D + 58^\circ$ .

*Examination and Fractionation of the Uronic Acids on Ion-exchange Resin.*—The free uronic acids (D) (4.66 g.) obtained by treatment of the barium salts (B) (6.81 g.) with ion-exchange resin (Amberlite IR-120 H<sup>+</sup>) had equivalent weight 338. Complete hydrolysis of the acids (D) (20 mg.) liberated more rhamnose and galactose (paper chromatography).

A column of anion-exchange resin (Amberlite IRA-400, acetate form) was prepared,<sup>18</sup> and the uronic acids (D) (3.39 g.) were absorbed on it. After removal of neutral sugars (0.074 g.) by elution with water, the acids were removed with increasing concentrations of aqueous acetic acid. Only two pure fractions were obtained; four other fractions were isolated but paper chromatography showed them to be mixtures of oligosaccharides which on hydrolysis gave galactose, rhamnose, and glucuronic acid:

Fraction E (0.008 g.), eluted with 2—3% acetic acid, was chromatographically identical with glucurone.

<sup>15</sup> Shaffer and Sömogyi, *J. Biol. Chem.*, 1933, **100**, 695; Hanes and Cattle, *Proc. Roy. Soc.*, 1938, **B**, 125, 387.

<sup>16</sup> Keilin and Hartree, *Biochem. J.*, 1948, **42**, 230.

<sup>17</sup> Nelson, *J. Biol. Chem.*, 1944, **153**, 375.

<sup>18</sup> Matheson, Ph.D. Thesis, Edinburgh, 1955.

Fraction F (0.355 g.), eluted with 3.5–5% acetic acid, had  $[\alpha]_D + 63^\circ$  ( $c$  1.5),  $R_{Gal}$  0.24,  $M_G$  0.72 [Found: equiv., 332 (by titration)]. Complete acid-hydrolysis gave rhamnose and glucuronic acid (paper chromatography). Partial hydrolysis, by *N*-sulphuric acid at  $100^\circ$  for 4 hr., gave rhamnose, glucuronic acid, and unhydrolysed material. Reduction by aqueous sodium borohydride followed by hydrolysis, neutralisation, and de-ionisation gave a product containing only glucuronic acid (paper chromatography). Methylation of Fraction E (0.320 g.) three times with 40% aqueous sodium hydroxide (7.5 c.c.) and dimethyl sulphate (3.0 c.c.) in an atmosphere of nitrogen at room temperature was followed by two further methylations, one at  $50^\circ$  and another at  $70^\circ$ . After acidification, the solution was extracted with cold chloroform. Removal of the chloroform gave a syrup (0.148 g.) which was methylated with methyl iodide (2.3 c.c.) and silver oxide (1.2 g.) under reflux. The product (0.140 g.), dissolved in dry methylal (2.0 c.c.), was reduced with lithium aluminium hydride (0.1 g. in 1 c.c. of methylal). Excess of reagent was destroyed by cautious addition of water and the product (0.098 g.) isolated by methylal-extraction. Two further methylations with Purdie's reagents gave a syrup (0.088 g.) which was hydrolysed by *N*-sulphuric acid at  $100^\circ$  for 6 hr., and the product (0.060 g.) was separated on thick paper with solvent (3). The first component was identical with 2 : 3 : 4 : 6-tetra-*O*-methylglucose chromatographically and ionophoretically. Demethylation gave glucose. The second component was identical with 3 : 4-di-*O*-methylrhamnose chromatographically and ionophoretically ( $M_G$  0.36). Demethylation gave rhamnose. A very small quantity of a third component ( $R_G$  0.76) gave glucose on demethylation.

*Isolation of Glucurone after Complete Hydrolysis of the Polysaccharide.*—Free uronic acids (0.20 g.) isolated as before from a completely hydrolysed sample of the gum were separated on sheets of thick paper (solvent 4) into two fractions:

Fraction 1 (0.039 g.) was chromatographically identical with glucurone. The derived yellow crystalline *p*-nitroaniline derivative<sup>19</sup> (0.020 g.) had m. p. and mixed m. p.  $128$ – $130^\circ$  (after recrystallisation from methanol).

Fraction 2 (0.116 g.) had  $[\alpha]_D + 33.6^\circ$  ( $c$  1.16) and gave a single spot on chromatography identical with glucuronic and/or galacturonic acid. Treatment with basic lead acetate<sup>20</sup> gave a yellow-brown precipitate similar to that given by authentic glucuronic acid and distinct from the brick-red precipitate given by a control sample of galacturonic acid. A portion (0.10 g.) in water was oxidised with bromine (1 c.c.) for 4 days at  $30$ – $35^\circ$  (nitric acid oxidation was avoided because it would convert any trace of galactose, which might have persisted in this fraction, into mucic acid). The residue obtained after removal of bromine by aeration and evaporation of the solution to dryness was dissolved in *N*-sodium hydroxide. Filtration followed by acidification of the filtrate by dropwise addition of hydrochloric acid failed to yield mucic acid even on prolonged storage.

*Methylation of the Polysaccharide.*—The pure polysaccharide (22 g.) was dissolved in 30% sodium hydroxide solution (600 c.c.) in an atmosphere of nitrogen which was maintained during all further methylations involving sodium hydroxide. The solution was cooled to  $10^\circ$  and dimethyl sulphate (270 c.c.) was added during 8 hr. with vigorous stirring, the temperature being kept below  $15^\circ$ . The mixture was set aside for 18 hr., after which the methylation was repeated twice. After cooling to  $5^\circ$  the solution was acidified by cautious addition of 50% sulphuric acid, and the mixture dialysed against running water for 24 hr. Concentration to 200 c.c. was followed by three methylations as before, acidification, and dialysis for 4 days. The pH was adjusted to 2 with sulphuric acid and a further equal volume of acid added. Dialysis (until the solution was free from sulphate ions) and evaporation gave an amorphous solid (14.0 g.) (Found: ash, 5.0; OMe, 28.4%). A portion (9.0 g.) of this material, dissolved in water, was de-ionised with Amberlite IR-120  $H^+$  resin and then stirred overnight with an 8–10-fold excess of silver carbonate. Filtration and freeze-drying gave an amorphous silver salt which was triturated with methyl iodide (50 ml.); the mixture was then refluxed with the addition of silver oxide (4 g.) in portions (0.5 g.). The product obtained on filtration and evaporation was methylated three times according to Purdie's directions. The fully methylated polysaccharide (6.2 g.) had  $[\alpha]_D^{18} + 68.4^\circ$  ( $c$  1.17 in  $CHCl_3$ ) (Found: OMe, 42.3%).

*Hydrolysis of the Methylated Polysaccharide.*—Methanolysis of the polysaccharide (6.0 g.) with 8% methanolic hydrogen chloride (150 c.c.) at  $100^\circ$  in a sealed tube for 18 hr. was followed by hydrolysis with 4% hydrochloric acid for 6 hr. at  $100^\circ$ . Neutralisation with silver carbonate

<sup>19</sup> Hamilton, Spriestersbach, and Smith, *J. Amer. Chem. Soc.*, 1957, **79**, 443.

<sup>20</sup> Ehrlich, *Ber.*, 1932, **65**, 352.

and removal of silver ions with hydrogen sulphide gave a solution which was made alkaline with barium hydroxide, excess of alkali being then removed by passage of carbon dioxide through the solution. A viscous syrup (5.73 g.) was isolated from which the neutral methylated sugars were extracted by refluxing dry diethyl ether ( $4 \times 100$  c.c.). Removal of the ether gave a syrup (1.54 g.). The residual barium salts (4.0 g.) were further hydrolysed with 2*N*-sulphuric acid at 100° for 21 hr. A brown amorphous solid was obtained which gave a further quantity of neutral sugars (0.20 g.) on extraction with dry ether. The residue (2.10 g.) consisted of methylated barium uronates.

*Fractionation of the Neutral Methylated Sugars.*—The methylated sugars (1.74 g.) were partitioned on a cellulose column (850  $\times$  30 cm.), eluted with butan-1-ol–light petroleum (b. p. 100–120°) (30 : 70; v/v) saturated with water. After the collection of 2.3 l. the proportions in the eluant were changed to (60 : 40; v/v) and after 12 l. had been collected the eluant was changed to water. Fractions were as follows,  $R_G$  values being for solvent (3):

Fraction G (1.768–1.92 l.): Syrupy 2 : 3 : 4 : 6-tetra-*O*-methyl-D-galactose (0.127 g.),  $R_G$  0.88,  $[\alpha]_D^{18} + 113^\circ$  (*c* 1.14) (Found: OMe, 51.9. Calc. for  $C_{10}H_{20}O_6$ : OMe, 52.5%). The derived anilide had m. p. 190–191°,  $[\alpha]_D^{18} - 69^\circ \longrightarrow + 35^\circ$  (*c* 0.75 in acetone).

Fraction H (1.928–2.230 l.): A syrup (0.307 g.),  $R_G$  0.86. Electroionophoresis showed two components of  $M_G$  0.36 and 0.0 respectively. Separation was achieved by partition on a column of "Celite 535" (50  $\times$  3.0 cm.) developed with solvent (6). The syrup (0.305 g.), dissolved in the organic phase of solvent (6) (3 ml.), was absorbed on dry Celite (3 g.), and the mixture was packed on top of the column, giving:

Fraction (i) (775 c.c.—1025 c.c.), syrupy 2 : 3 : 4 : 6-tetra-*O*-methylgalactose (0.214 g.),  $R_G$  0.88,  $[\alpha]_D^{18} + 112^\circ$  (*c* 2.1) (Found: OMe, 52.0%).

Fraction (ii) (1275–1300 c.c.), crystalline 3 : 4-di-*O*-methyl-L-rhamnose (0.036 g.),  $R_G$  0.85,  $M_G$  0.36, m. p. 93–94°  $[\alpha]_D^{18} + 23^\circ$  (*c* 2.3) (Found: OMe, 32.9. Calc. for  $C_8H_{16}O_5$ : OMe, 32.3%) [demethylation gave rhamnose (paper chromatography)].

Fraction J (4.96–6.84 l.): Syrupy 2 : 3 : 6-tri-*O*-methyl-D-galactose (0.436 g.),  $R_G$  0.71,  $[\alpha]_D^{20} + 96^\circ$  (*c* 1.8) (Found: OMe, 40.9. Calc. for  $C_9H_{18}O_6$ : OMe, 41.9%). The derived lactone had m. p. 97–99°,  $[\alpha]_D^{18} - 40^\circ \longrightarrow - 30^\circ$  (*c* 1.2) (constant), and gave one component on chromatography in solvents (4) or (5) (detected by spraying with alkaline hydroxylamine hydrochloride, then ferric chloride<sup>21</sup>).

Fraction K (10.8–11.84 l.): Crystalline 3-*O*-methyl-L-rhamnose (0.140 g.),  $R_G$  0.56, m. p. and mixed m. p. 114–115°,  $[\alpha]_D + 30^\circ$  (*c* 1.0) (Found: OMe, 17.0. Calc. for  $C_7H_{14}O_5$ : OMe, 17.4%). The X-ray powder photograph was identical with that of the authentic material and distinct from that of 4-*O*-methyl-L-rhamnose. The derived lactone had  $[\alpha]_D^{18} - 20^\circ$  (*c* 1.0).

Fraction L: Water-washings; methylated barium uronates (0.24 g.).

*Examination of the Methylated Barium Uronates.*—The combined methylated barium uronates (2.34 g.) were converted into the free uronic acids (1.70 g.) by treatment with Amberlite IR-120 H<sup>+</sup> resin. Partition on "Celite" as for fraction H with butan-1-ol–*n*-butyl acetate–acetic acid–water (50 : 6 : 4 : 40) as eluant gave three fractions:

(a) Fraction U 1 (0.35–0.65 l.): A syrup (1.0 g.),  $[\alpha]_D + 59^\circ$  (*c* 0.93). Paper chromatography showed a single spot (solvent 4) identical with that of 2 : 3 : 4-tri-*O*-methylglucuronic acid, but development of a paper chromatogram with solvent 7 revealed two spots. Conversion of a portion into the methyl ester glycoside by treatment with 3% methanolic hydrogen chloride was followed by dissolution in dry tetrahydrofuran (5 c.c.) and reduction by the dropwise addition of a saturated solution of lithium aluminium hydride in dry tetrahydrofuran. The solution was refluxed gently for 30 min., then cooled and the excess of hydride was destroyed by ethyl acetate. Addition of water, evaporation to dryness, extraction of the residues with boiling acetone, then with dry chloroform, and evaporation of the extracts gave a syrup. Hydrolysis with *N*-hydrochloric acid (5 c.c.) at 100° for 6 hr. gave a syrup (0.279 g.) which was partitioned on a cellulose column (25  $\times$  42 cm.) with solvent 7. Solvent was removed from the respective fractions at 30–40° in a stream of nitrogen.

Fraction M (0.054 g.) was purified by separation on Whatman 3MM paper with solvent 7. A syrup (0.028 g.) was isolated which had  $R_G$  0.88 (solvent 3),  $R_F$  0.83 (solvent 7),  $M_G$  0.36,  $[\alpha]_D + 17^\circ$  (*c* 0.36) (Found: OMe, 29.5. Calc. for  $C_8H_{16}O_5$ : OMe, 32.3%). Demethylation of a portion of the syrup gave rhamnose (paper chromatography). The syrup was oxidised in aqueous solution with potassium periodate.<sup>9</sup>

<sup>21</sup> Abdel-Akher and Smith, *J. Amer. Chem. Soc.*, 1951, **73**, 5859.



Fraction *N*: A syrupy mixture (0.137 g.) of fractions *M* and *P* (paper chromatography, solvent 7).

Fraction *P*: 2 : 3 : 4-Tri-*O*-methyl-*D* glucose (0.062 g.),  $[\alpha]_D + 65^\circ$  (*c* 0.18),<sup>22</sup>  $R_G$  0.85 (solvent 3),  $R_F$  0.72 (solvent 7),  $M_G$  0.0 (Found: OMe, 41.3. Calc. for  $C_9H_{18}O_6$ : OMe, 41.9%). The derived *N*-phenyl-*D*-glucosylamine had m. p. and mixed m. p. with 2 : 3 : 4-tri-*O*-methyl-*N*-phenyl-*D*-glucosylamine 145—146°.<sup>22</sup>

(b) Fraction *U* 2 (0.85—1.6 l.) (0.239 g.): This fraction was contaminated with neutral sugars from which it was separated by elution on Whatman 3MM paper with solvent 7. The uronic acid remained on the starting line and was eluted with water, de-ionised (Amberlite IR-120  $H^+$ ), and evaporated to a syrup (0.083 g.) which had  $[\alpha]_D + 80^\circ$  (*c* 0.33). A portion (0.060 g.) was converted into the ester glycoside, reduced, and hydrolysed as before. Chromatography (solvent 3) showed 2 : 3 : 4-tri-*O*-methyl-*D*-glucose  $R_G$  0.85, 3-*O*-methyl-*L*-rhamnose  $R_G$  0.56, rhamnose  $R_G$  0.28, and a mono-*O*-methylhexose  $R_G$  0.23. The material of  $R_G$  0.85 was separated on Whatman 3MM paper and examined electroionophoretically: a single spot of  $M_G$  0.0 was detected.

(c) Fraction *U* 3 (1.625—2 l.) (0.10 g.) had  $[\alpha]_D^{20} + 93^\circ$  (*c* 0.38). Treatment as for the previous fraction gave a syrup containing six components (paper chromatography, solvents 3 and 7). These included the four sugars present in the previous fraction, together with 2 : 3 : 6-tri-*O*-methyl-*D*-galactose  $R_G$  0.71 and a di-*O*-methylhexose  $R_G$  0.46.

*Reduction and Remethylation of the Methylated Polysaccharide.*—The methylated gum (OMe, 42.3%) (0.75 g.) in dry tetrahydrofuran (1.5 c.c.) was reduced with lithium aluminium hydride as for fraction *U* 1. The solid product (0.61 g.) had  $[\alpha]_D + 75^\circ$  (*c* 1.03 in  $CHCl_3$ ). This material (0.41 g.) after four methylations with Purdie reagents had  $[\alpha]_D + 45.8^\circ$  (*c* 2.6 in  $CHCl_3$ ) (Found: OMe, 41.8%). Methanolysis with 4% methanolic hydrogen chloride for 8 hr. under reflux, followed by hydrolysis with *N*-hydrochloric acid for 16 hr. at 100°, gave a syrup (0.238 g.). Chromatography (solvents 3 and 7) showed all the components of fraction *U* 2 except the 2 : 3 : 4-tri-*O*-methylglucose and in addition 2 : 3 : 4 : 6-tetra-*O*-methyl-*D*-glucose  $R_G$  1.0, 2 : 3 : 4 : 6-tetra-*O*-methyl-*D*-galactose and 3 : 4-di-*O*-methylrhamnose  $R_G$  0.85. Tetramethylgalactose has  $R_F$  0.92 in solvent 7.

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<sup>22</sup> Peat, Schlucterer, and Stacey, *J.*, 1939, 581.