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A POLYSACCHARIDE FROM *Aeodes orbitosa*

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INTRODUCTION

Red-seaweed polysaccharides have received attention[1] from many workers, but, because of variations in their structures, many more will have to be investigated before definitive generalisations can be made.

Aeodes orbitosa, a red seaweed belonging to the *Grateloupiaceae* and easily recognised by its broad, flat fronds, grows abundantly on the Atlantic coast of the Cape Peninsula.

RESULTS AND DISCUSSION

Hot-water extraction of the fresh weed yielded a highly sulphated polysaccharide, aeodan, which was purified by conventional methods. The pure polysaccharide (sodium salt) exhibited a single, sharp peak in an ultracentrifuge, indicating that it probably was homogeneous, and an extended type of molecule. Chromatography of a hydrolysate revealed the presence of D-galactose and 2-*O*-methyl-D-galactose as major components, together with small proportions of D-xylose and glycerol. All four components were isolated and characterised. In demonstrating the presence of glycerol as a polysaccharide component, special care (see Experimental) was taken to ensure that it had not arisen as a contaminant from an exterior source.

D-Galactose (58%, calc. as $C_6H_{10}O_5$) was determined by the Somogyi[2] micro-method after separation by chromatography. This method was unsatisfactory for the determination of 2-*O*-methyl-D-galactose (10%, calc. as $C_7H_{12}O_5$), the concentration of which was calculated from the methoxyl content (1.9%) of the purified polysaccharide. Attempts to determine the glycerol have so far been unsuccessful. However, on the amounts isolated from large-scale hydrolyses of the polysaccharide, it is estimated to be present to the extent of 1–2%. From the above values and the sulphate content (27.5%, calc. as $NaSO_3^-$), the approximate molecular ratio of D-galactose:2-*O*-methyl-D-galactose:sulphate ($NaSO_3^-$) is 12:2:9. Such a repeating unit has an equivalent weight of 366 (found 375) and a methoxyl content of 1.88%. No account has been taken of glycerol in these calculations.

Treatment of aeodan with alkali and sodium borohydride[3] resulted in the liberation of *ca.* 16% of the sulphate, and the formation of an equivalent amount of

3,6-anhydro-D-galactose, indicating that *ca.* 5.7% of the D-galactose was substituted in either the 2- or 4-positions, or partly in each. This substitution could be interpreted as (1→2)- or (1→4)-linked D-galactose 3(or 6)-sulphate.

The infrared spectrum showed peaks at 1240 (characteristic of carbohydrate sulphates) and 815 cm^{-1} (sulphated primary hydroxyl group[4]). The latter peak was very broad, the peak area spreading well past the 830 cm^{-1} wavelength, and possibly masking a second peak in this area, which would be indicative of equatorial secondary sulphate (possibly at position 2).

No conditions were found for the complete desulphation of the molecule. The lowest sulphate values were achieved by shaking with 0.15M methanolic hydrogen chloride[5] for 114 h at room temperature, which gave 36% of degraded polysaccharide having 3.45% of sulphate. Paper chromatography of an acid hydrolysate of the methanol-soluble material revealed that desulphation had removed some of the D-galactose and 2-*O*-methyl-D-galactose residues. The infrared spectrum of the desulphated polysaccharide displayed a peak at 1240 cm^{-1}, but no other well-defined peaks.

Oxidation of aeodan with periodate ceased after 48 h at room temperature (Table I), when 0.170 mole of periodate had been consumed per hexose residue. In

TABLE I

AMOUNT (MOLE) OF PERIODATE REDUCED PER HEXOSE RESIDUE

Time (h)	*3*	*24*	*48*	*72*	*96*	*120*
Aeodan	0.051	0.142	0.170	0.170	0.170	
Desulphated aeodan (SO_4^{2-}, 6.3%)	0.456	0.610	0.675	0.698	0.718	0.723
Desulphated aeodan (SO_4^{2-}, 3.45%)	0.444	0.545	0.607	0.634	0.641	0.659

the case of each specimen of desulphated aeodan, no definite end-point was reached even after 120 h. However, the rate of reduction was then extremely low. After this time, both specimens had consumed about four times as much periodate per hexose residue as aeodan. The low consumption of periodate by aeodan suggests the presence of (*a*) a large proportion of (1→3)-links; (*b*) (1→3)-links, together with units containing other glycosidic links, but carrying sulphate or methoxyl groups in such positions as to render these units immune to periodate; or (*c*) a large proportion of units containing other glycosidic links, and carrying sulphate or methoxyl groups in such positions as to render these units immune to periodate.

Aeodan and desulphated aeodan were oxidised with periodate, and the resulting oxopolysaccharides were reduced with borohydride[6]. Complete, acid hydrolysis of the polyalcohols, followed by paper chromatography, revealed the presence of threitol, glycolaldehyde, and a trace of glycerol as degradation products of aeodan, together with unchanged D-galactose and 2-*O*-methyl-D-galactose, whereas only

threitol and the above two sugars were detected in the case of the polyalcohol from desulphated aeodan. No xylose was detected on any of the chromatograms. Most of the threitol and glycolaldehyde are considered to have arisen from D-galactose residues substituted at position 4, probably by glycosidic links, and unsubstituted at positions 2, 3, and 6. Support for this inference is provided by the presence of 2,3,6-tri-*O*-methyl-D-galactose in the hydrolysis products of methylated aeodan and methylated, desulphated aeodan. In addition, alkali treatment of aeodan indicates that only a small portion of the D-galactose residues carry sulphate on position 6 (or 3). The presence of glycerol in the degradation products of aeodan could have arisen from glycerol glycosidically linked through position 2 as an end group. On the other hand, it could have arisen through oxidation of 6-substituted D-galactose residues having positions 2, 3, and 4 free, a view partly supported by the presence of a trace of 2,3,4-tri-*O*-methylgalactose in the hydrolysate of methylated aeodan. However, not only could the trace of tri-*O*-methylgalactose have arisen from undermethylation, but no glycerol was detected in the desulphated aeodan hydrolysate, results that tend to rule out the second explanation for the presence of glycerol in the hydrolysate. The only other possibility, *viz*, 2-substituted galactose having positions 3, 4, and 6 free, is ruled out by the absence of glyceraldehyde in the hydrolysate.

Methylation of desulphated aeodan was accomplished by a modification of Kuhn and Trischmann's method[7], followed by several treatments with Purdie's reagents[8]. Hydrolysis of this material, followed by separation of the products by paper and charcoal–Celite column chromatography, revealed the presence of 2,3,6- and 2,4,6-tri-*O*-methyl-D-galactoses as the main components with 2,6- and 4,6-di-*O*-methyl-D-galactoses and 2,3,4,6-tetra-*O*-methyl-D-galactose present in small proportions. The presence of 2,4,6-tri-*O*-methyl-D-galactose provides additional, strong evidence for the presence of (1→3)-linkages, whereas the presence of the 2,3,6-isomer confirms the presence of (1→4)-linkages in the molecule.

In common with other sulphated polysaccharides, aeodan was difficult to methylate, and a maximum methoxyl content of 19.8% only was achieved. The final product contained 16.5% of sulphate and gave a faint, positive reaction for a 3,6-anhydrogalactose. The methylated polysaccharide was hydrolysed with N sulphuric acid, and the components were separated by column chromatography on cellulose and charcoal–Celite. 2,3,4,6-Tetra-*O*-methyl-D-galactose, 2,3,6- and 2,4,6-tri-*O*-methyl-D-galactoses, 2,6- and 4,6-di-*O*-methyl-D-galactoses, and 4-*O*-methyl-D-galactose were isolated. In addition to the above sugars, a trace of a sugar chromatographically identical with 2,3,4-tri-*O*-methylgalactose was detected.

The results of the methylation of aeodan must be treated with some caution because of the uncertainty of the extent of methylation. The isolation of 4,6-di-*O*-methyl-D-galactose suggests the presence of (1→3)-linked units sulphated at position 2. The isolation of 2,4,6-tri-*O*-methyl-D-galactose would appear to indicate that not all of the (1→3)-linked D-galactose residues are sulphated. The 2,6-di-*O*-methyl-D-galactose could have arisen from (1→3)-linked units sulphated at position 4, although the spectroscopic evidence[4] (no band at 850–860 cm^{-1} for axial secondary sulphate)

is against this. On the other hand, it may represent a genuine (1→4)-linked unit or, possibly, undermethylation. 4-*O*-Methyl-D-galactose, isolated in good yield from the methylation of the polysaccharide but not detected in the methylation of the desulphated polysaccharide, is added evidence for a (1→3)-link, and possibly arises from (1→3)-linked units sulphated at positions 2 and 6.

At present, no unique structure can be proposed for aeodan. It is fairly clear, however, that the molecule is composed of D-galactose residues linked (1→3) and (1→4), with, apparently, the former in greater concentration. Aeodan resembles the λ-fraction of carrageenan[9] in several respects. It is not precipitated from solution in the presence of potassium chloride, it is highly sulphated, and it contains (1→3)-linked residues of D-galactose 4-sulphate. On the other hand, aeodan differs from λ-carrageenan in having most of the (1→4)-linked D-galactose units free from sulphate and therefore a lower proportion of alkali labile sulphate.

Evidence from a study of the partial hydrolysis products of aeodan will be presented in a further paper.

EXPERIMENTAL

Unless otherwise stated, concentration of solutions was carried out at 40°/20 mm, and specific rotations were measured in water. Paper chromatography was carried out with Whatman Nos. 1 and 20 filter papers. The following solvent systems were used: (1) ethyl acetate–acetic acid–formic acid–water (18:3:1:4), (2) butyl alcohol–pyridine–water (9:2:2) and (3) butyl alcohol–ethanol–water (40:11:19). *p*-Anisidine hydrochloride[10], periodate–benzidine[11], and aniline–diphenylamine–phosphoric acid[12] are sprays *a*, *b*, and *c*, respectively. Thin-layer chromatography (t.l.c.) was carried out on plates coated with Silica Gel G containing calcium sulphate as binder, employing methyl ethyl ketone–water (85:7) as solvent. Infrared spectra were recorded on a Beckman IR-8 instrument, using KBr discs.

Isolation and purification of aeodan. — Wet *Aeodes orbitosa* (6 kg) was mixed with hot water, and acetic acid was added to pH 2–3. The acid caused the rapid disintegration of the weed and did not appear to degrade the polysaccharide. The mixture was heated for 0.5 h with constant stirring after the disintegration of the weed had begun, during which time the pH rose to between 6 and 7. The extract was strained through muslin and centrifuged while still hot, yielding a murky, pale-brown liquid. Steam was passed into the mixture for 10 min., and the crude extract was centrifuged a second time. This afforded a clear, pale, yellow-brown liquid. A colloidal precipitate which appeared on cooling was removed by centrifugation. Precipitation into ethanol (5 vol.) and washing with ethanol and finally ether afforded an off-white product (570 g, 9.5% on a wet wt. basis) [Found (on material dried at 60°/0.5 mm): ash (sulphated), 16.8%]. Analysis of the ash indicated the presence of sodium as the main cation. Further purification of the polysaccharide for analysis was effected by repeated (5 times) dissolution in water, centrifugation of the solution, and precipitation in ethanol (5 vol.). Finally, a solution was passed through Amberlite IR-120 resin,

and the acid eluate was exactly neutralised with aqueous sodium hydroxide, after which the solution was concentrated, and precipitated in ethanol (10 vol.). The polysaccharide was collected in a centrifuge, washed with ethanol, and dried; $[\alpha]_D^{18}$ +79° (*c* 0.53) [Found (on material dried at 60°/0.5 mm): 3,6-anhydrogalactose[13], 0.1; ash (sulphated), 16.9; OMe, 1.9; N, 0.0; SO_4^{2-}, 25.59%; equiv. wt. (from SO_4^{2-} detn.), 375; ν_{max} 1240 and 815 cm^{-1} (KBr disc)]. The polysaccharide failed to precipitate from solution when mixed with potassium chloride solution.

Chromatographic examination (solvents 1, 2, and 3) of the hydrolysate (N sulphuric acid for 16 h at 100°) revealed spots corresponding to galactose (major), 2-*O*-methylgalactose (minor), and xylose (trace) with spray *a*. Spray *b* revealed, in addition to the above monosaccharides, the presence of a very faint spot having the mobility of glycerol.

Separation and characterisation of the components of aeodan. — The polysaccharide (70 g) was hydrolysed with N sulphuric acid (400 ml) for 16 h. After neutralisation with barium carbonate, the solution was deionised with Amberlite IR-120 (H^+) and Amberlite IR-4B (OH^-) resins. The neutral, aqueous effluent, after evaporation to a partially crystalline syrup (33.5 g), was applied to a cellulose column (61 × 5.4 cm), which was eluted with butyl alcohol half saturated with water, and fractions (*ca.* 50 ml) were collected. On the basis of paper chromatography, the fractions were combined into five fractions.

Fraction 1. The syrup (500 mg), chromatographically indistinguishable from glycerol, was decolourised with charcoal in water. After filtration and freeze drying, there was obtained a colourless syrup, which yielded glycerol tris(*p*-nitrobenzoate), m.p. and mixed m.p. 188–190° (Kofler hot-stage). Nunn and von Holdt[14] reported m.p. 191–193°.

Fraction 2. An aqueous solution of the syrup (212 mg) was decolourised with charcoal, filtered, and evaporated, and the residue was recrystallised from methanol–ethyl acetate, yielding colourless prisms of 2-*O*-methyl-D-galactose, m.p. 148–149°, $[\alpha]_D^{16}$ +84.9° (final) (*c* 0.53) (Found: C, 42.9; H, 7.1, $C_7H_{14}O_6$ calc.: C, 43.3; H, 7.2%). Oldham and Bell[15] reported m.p. 147–149°, $[\alpha]_D$ + 53→ +82.6°. This sugar had the mobility of authentic 2-*O*-methyl-D-galactose in solvent systems 1 and 2, and gave spots of the same colour as given by the authentic sugar with spray *a*. When a similar paper was sprayed with triphenyltetrazolium chloride, neither the sugar derived from the polysaccharide nor authentic 2-*O*-methyl-D-galactose were revealed, whereas authentic 3-*O*-methyl-D-galactose, eluted on the same paper, was readily revealed (pink spot).

Demethylation[10] of the sugar gave D-galactose and unchanged 2-*O*-methyl-D-galactose (paper chromatogram). The derived "anilide" had m.p. and mixed m.p. (with authentic 2-O-methyl-*N*-phenyl-D-galactosylamine[16]) 164–165°.

Fraction 3. The syrup (2.2 g) was shown to be a mixture of 2-*O*-methylgalactose (major component) and xylose. A portion (500 mg) of this fraction was separated on Whatman 3MM paper, using solvent 1. Extraction of the appropriate portion of the papers with methanol yielded a product, which, on recrystallisation (charcoal) from

ethanol, gave D-xylose (19 mg), m.p. and mixed m.p. 145–146°; $[\alpha]_D^{16}$ +18.1° (*c* 0.55). Percival and Wold[17] reported m.p. 144–145°; $[\alpha]_D$ +18.3°.

Fraction 4. The syrup (730 mg) was shown by chromatography to be a mixture of galactose (trace), xylose (trace), and 2-*O*-methylgalactose.

Fraction 5. A portion of this fraction (19.2 g) was recrystallised from methanol (charcoal), giving D-galactose, m.p. and mixed m.p. 166–167, $[\alpha]_D^{16}$ +90.1° (*c* 0.71). It gave mucic acid, m.p. and mixed m.p. 212–213°, on oxidation with nitric acid–water (1:1).

Ether extraction of a hydrolysate of aeodan. — In order to ensure that the glycerol was not a contaminant in the above large-scale hydrolysis, and that it represents a true component of the polysaccharide, the highly purified polysaccharide (0.967 g) was heated on a boiling-water bath with N sulphuric acid (5 ml) for 16 h, and the neutralised hydrolysate was diluted and then extracted with ether continuously for 24 h. Removal of the ether from the dried (Na_2SO_4) solution, followed by paper chromatography of the residue, showed spots having the mobility of glycerol (spray *b*).

Action of alkali on aeodan. — Polysaccharide (3 g) in water (200 ml) containing sodium borohydride[3] (0.4 g) was kept for 48 h at room temperature. Sodium hydroxide (20 g) and sodium borohydride (2 g) were then added, and the mixture was maintained at 80°. After 4 h, a further amount of sodium borohydride (2 g) was added, and, after 7 h, the solution was cooled and made slightly acid with hydrochloric acid. The mixture was dialysed against frequently changed distilled water, concentrated, and freeze-dried, yielding a white foam (2.1 g), $[\alpha]_D^{17}$ +76° (*c* 0.5) (Found: SO_4^{2-}, 21.57; 3,6-anhydrogalactose[13], 5.1%). Chromatography (solvent 1) of a hydrolysate showed spots corresponding to galactose, 2-*O*-methylgalactose, and xylose.

Desulphation of aeodan by treatment with methanolic hydrogen chloride[5]. — Polysaccharide (1.99 g) was shaken with 0.15M methanolic hydrogen chloride (100 ml) for 72 h at room temperature. Undissolved material was centrifuged off, and the supernatant liquid, after neutralisation with silver carbonate and concentration, gave a non-reducing syrup (582 mg). Paper chromatography of an acid hydrolysate showed the presence of galactose and 2-*O*-methylgalactose.

The insoluble material was washed with methanol, dissolved in water, and dialysed against distilled water. After concentration, the polysaccharide (1.14 g, 57%) was isolated by freeze drying (Found: SO_4^{2-}, 7.65%). The peak at 815 cm^{-1} recorded in the infrared spectrum of the parent polysaccharide was absent, and the peak at 1240 cm^{-1} was much smaller.

Decreasing the strength of the methanolic hydrogen chloride to 0.09M resulted, after a single treatment (48 h), in an 83% yield of polysaccharide, $[\alpha]_D^{18}$ +77° (*c* 0.64) (Found: SO_4^{2-}, 24.8%). Further shaking with 0.15M methanolic hydrogen chloride for 114 h gave a 36% yield of polysaccharide, $[\alpha]_D^{18}$ +94° (*c* 0.34) (Found: SO_4^{2-}, 3.45%). Paper chromatograms of both an acid hydrolysate of the polysaccharide and an acid hydrolysate of the non-reducing, methanol-soluble syrup revealed the presence of galactose and 2-*O*-methylgalactose.

In a third experiment, the polysaccharide was shaken with 0.15M methanolic hydrogen chloride for 48 h at room temperature and isolated (yield, 81%) as above.

(Found: SO_4^{2-}, 16.8%). After a further 48-h treatment, the yield was 37% (Found: SO_4^{2-}, 6.3%).

Periodate oxidation of polysaccharides. — Aeodan (29.5 mg) and desulphated polysaccharides (SO_4^{2-}, 6.3 and 3.45%; 24.7 and 24.2 mg, respectively) were dissolved separately in water (5 ml), and 0.288M sodium metaperiodate (5 ml) was added to each solution. The solutions were set aside at room temperature in the dark, and, at intervals, aliquots (0.10 ml) were removed, and the reduction of periodate was measured[18] (Table I).

Reduction of periodate-oxidised polysaccharide[6]. — (*a*) A solution of polysaccharide (3 g) and sodium metaperiodate (21.4 g) in water (500 ml) was kept in the dark at room temperature for 36 h, when reduction of periodate was complete. After removal of excess of periodate with ethylene glyclol, sodium borohydride (5 g) was added, and the mixture was set aside overnight. The solution was dialysed (4 days) against frequently changed, distilled water, and concentrated, and the polysaccharide alcohol (2.55 g) was isolated by freeze drying. The polysaccharide alcohol (500 mg) was heated with N sulphuric acid on a boiling-water bath for 12 h, and the neutralised ($BaCO_3$) hydrolysate was filtered, and concentrated by freeze drying to a syrup. Paper chromatography revealed the presence of galactose and 2-*O*-methylgalactose with spray *a*, whereas chromatograms treated with spray *b* revealed spots having the mobilities of glycolaldehyde and glycerol (trace), in addition to the above two sugars. The syrup was diluted with water (10 ml) and shaken with Amberlite IRA-400 (OH^-) until the solution gave a negative Molisch test (removal of reducing sugars). The solution was then filtered, and concentrated to a colourless syrup by freeze-drying. Paper chromatography of an aliquot revealed spots having the mobilities of glycerol (trace) and threitol (spray *b*); similar chromatograms failed to reveal any spots with spray *a*. T.l.c. of a second aliquot (ethyl acetate–propyl alcohol–water, 2:7:1) revealed spots having the mobilities of glycerol and threitol (alkaline permanganate spray).

(*b*) A solution of desulphated, degraded polysaccharide (SO_4^{2-}, 6.3%; 0.32 g) and sodium metaperiodate (2.14 g) in water (30 ml) was kept for 96 h and then treated as in (*a*). The oxidation in this case took much longer. Paper chromatograms of a neutralised hydrolysate of the oxopolysaccharide (recovered, 0.1 g) revealed the presence of galactose and 2-*O*-methylgalactose. The oxopolysaccharide, after reduction with borohydride and subsequent treatment as in (*a*), yielded a solution, which exhibited a single spot having the mobility of threitol on chromatography.

Methylation of aeodan. — Polysaccharide (24.7 g) in water (500 ml) was treated slowly and simultaneously with methyl sulphate (150 ml) and sodium hydroxide solution (30% w/v; 450 ml) with vigorous stirring during 7 h, after which the mixture was stirred for a further 17 h. This was repeated a further four times, when the final solution was dialysed against running water (2 weeks), and the polysaccharide was isolated by freeze-drying. Methylation was incomplete as shown by the presence of a large proportion of galactose on chromatography of a neutralised hydrolysate. After a further fourteen additions of the above reagents, a hydrolysate was found to contain only a minute trace of galactose. The product was isolated by concentration

of the dialysed solutions, followed by freeze-drying (yield, 11.2 g) (Found: OMe, 19.8; SO_4^{2-}, 16.5%).

Hydrolysis of methylated polysaccharide and separation of the products. — Methylated polysaccharide (11.0 g) was hydrolysed on a boiling-water bath with N sulphuric acid (100 ml) for 16 h. The hydrolysate was neutralised with barium carbonate, centrifuged, and concentrated to a brown syrup, which was applied to a cellulose column (60 × 5.4 cm), and eluted with butyl alcohol–water (95:5). On the basis of paper chromatography, appropriate fractions were recombined into the following seven fractions.

Fraction 1 contained traces of tetra-*O*-methylgalactose (paper chromatography), together with degradation products arising from the hydrolysis.

Fraction 2, a syrup, chromatographically identical with tetra-*O*-methylgalactose, gave a crystalline "anilide", m.p. and mixed m.p. (with 2,3,4,6-tetra-*O*-methyl-*N*-phenyl-D-galactosylamine) 188–189°. Clingman and Nunn[19] reported m.p. 189–190°.

Fraction 3, a syrup, shown by t.l.c. (spray *c*) to be a mixture of 2,4,6- and 2,3,6-tri-*O*-methylgalactose, together with a trace of the 2,3,4-isomer, was applied, in the minimum volume of water, to a charcoal–Celite column (60 × 5.4 cm). Linear-gradient elution was effected with aqueous methyl ethyl ketone, initially at 1% and finally at 5% of the latter component over 10 litres of eluant. On the basis of t.l.c., the fractions were recombined into three fractions.

Fraction 3a, a syrup, $[\alpha]_D^{18}$ +91.5° (*c* 0.5), chromatographically (t.l.c., spray *c*) identical with 2,4,6-tri-*O*-methylgalactose, yielded an "anilide" which, after recrystallisation from ethanol–ethyl acetate, had m.p. and mixed m.p. (with 2,4,6-tri-*O*-methyl-*N*-phenyl-D-galactosylamine) 171–172°. Clingman and Nunn[19] reported m.p. 170.5–171.5°. The phenylosazone[9], on recrystallisation from aqueous ethanol, had m.p. 154–155°.

Fraction 3b, a syrup, was shown by chromatography (t.l.c., spray *c*) to be a mixture of 2,3,6- and 2,4,6-tri-*O*-methylgalactose.

Fraction 3c, a syrup, $[\alpha]_D^{18}$ +90° (*c* 0.37), was chromatographically identical with 2,3,6-tri-*O*-methyl-D-galactose. The derived 2,3,6-tri-*O*-methyl-D-galactono-1,4-lactone[20] had m.p. 97–99°.

Fraction 4 contained a mixture of 2,6- and 4,6-di-*O*-methyl-D-galactose, which was fractionated on a cellulose column (60 × 5.4 cm) by elution with solvent 1. The 2,6-di-*O*-methyl-D-galactose, after crystallisation from ethyl acetate, had $[\alpha]_D^{18}$ +86° (*c* 0.5), m.p. and mixed m.p. (with an authentic sample[19]) 129–130°.

Fraction 5 contained 4,6-di-*O*-methyl-D-galactose which, after several recrystallisations from ethyl acetate, had m.p. 136–138°, $[\alpha]_D^{16}$ +117.6 (5 min) → +73.8° (*c* 0.87) (Found: C, 46.1; H, 7.9. $C_8H_{16}O_6$ calc.: C, 46.1; H, 7.8%). Dolan and Rees[9] reported m.p. 146–147°, $[\alpha]_D$ +120 (5 min) → +74°, whereas Bell[21] reported m.p. 131–133° for 4,6-di-*O*-methyl-D-galactose. Demethylation[10], followed by paper chromatography, showed the presence of galactose. It readily gave a phenylosazone (on treatment with redistilled phenylhydrazine in the presence of sodium metabisulphite), m.p. and mixed m.p. (with the phenylosazone derived from fraction *3a*) 154–155°;

Dolan and Rees[9] reported m.p. 155°. This sugar, when treated with aniline and a trace of glacial acetic acid, gave a crystalline "anilide" which, after recrystallisation from ethanol, had m.p. 149–150°. Hirst and Jones[22] reported m.p. 207° for 4,6-di-*O*-methyl-*N*-phenyl-D-galactosylamine.

Fraction 6 crystallised when triturated with methanol, and was a mixture of 4,6-di-*O*-methylgalactose and 4-*O*-methylgalactose (paper chromatography).

Fraction 7 crystallised from ethanol or ethanol–water, yielding prisms of 4-*O*-methyl-D-galactose, m.p. 202–209°, $[\alpha]_D^{20}$ +63.5 (5 min) → +82.5° (*c* 1.26). The "anilide" crystallised from ethanol as needles, m.p. 166–167°. Hirst and Jones[23] reported m.p. 207° for 4-*O*-methyl-D-galactose, and 168° for 4-*O*-methyl-*N*-phenyl-D-galactosylamine.

Methylation of desulphated aeodan. — Polysaccharide (4.8 g; SO_4^{2-}, 6.3%) was dissolved in methyl sulphoxide (60 ml) with gentle heating, and then *N,N*-dimethylformamide (60 ml) was added. After cooling in ice for 0.5h, barium hydroxide octahydrate (60 g) was added with stirring. This was followed, after a further 0.5 h, by methyl sulphate (10 ml). Further additions of methyl sulphate (10 ml) were made after 1, 1.5, 2, and 2.5 h. The mixture was stirred in a closed system for 72 h at room temperature, and conc. ammonia solution (25 ml) was then added, followed by vigorous shaking for 0.5 h to decompose the excess of methyl sulphate. After the addition of water (300 ml), the mixture was dialysed against running tap water for two weeks. The dialysate was centrifuged, and extracted with chloroform (6 × 400 ml). Evaporation of the combined chloroform solutions yielded a gum (2.4 g) that was incompletely methylated as revealed by the infrared spectrum. The aqueous solution containing the chloroform-insoluble material, on concentration and freeze-drying, yielded a partially methylated polysaccharide (2.15 g). This fraction was not further investigated.

The chloroform-soluble gum (2.4 g) was dissolved in *N,N*-dimethylformamide (15 ml), methyl iodide (20 ml) and silver oxide (20 g) were added, and the mixture was shaken for 48 h. After a further three treatments with methyl iodide (15 ml) and silver oxide (15 g), the methylated polysaccharide (1.25 g) was isolated (Found: SO_4^{2-}, 1.65; OMe, 35.0%). The infrared spectrum of the product showed a very small hydroxyl peak. Further treatment with Purdie's reagents failed to increase the methoxyl content.

Hydrolysis of desulphated, methylated polysaccharide, and separation of the products. — Hydrolysis of the methylated polysaccharide (OMe, 35%; 1.2 g) with N sulphuric acid for 16 h, followed by neutralisation with barium carbonate, filtration, and evaporation, afforded a syrup (0.7 g), chromatographic examination of which revealed the presence of tetra-*O*-methylgalactose, two tri-*O*-methylgalactoses, and two di-*O*-methylgalactoses. The hydrolysate appeared to be devoid of monomethylgalactoses and unmethylated sugars.

A portion of the hydrolysate (500 mg) was fractionated on Whatman No 20 paper by elution with solvent 2 for 36 h, and the fractions were examined as follows.

Tetra-O-methyl fraction. The syrup (25 mg) was chromatographically (t.l.c.)

identical with 2,3,4,6-tetra-*O*-methylgalactose. The derived aniline derivative[19], after recrystallisation from ethanol, had m.p. and mixed m.p. 188–189° with 2,3,4,6-tetra-*O*-methyl-*N*-phenyl-D-galactosylamine.

Tri-O-methyl fraction. The syrup (250 mg) was shown by chromatography (t.l.c., spray *c*) to be a mixture of 2,4,6- and 2,3,6-tri-*O*-methylgalactose. This syrup was separated into two fractions on a charcoal–Celite column (60 x 5.4 cm) by gradient elution with aqueous methyl ethyl ketone (1 to 5%) over ten litres of eluant. The major fraction, $[\alpha]_D^{18}$ +90° (*c* 0.47), on treatment with aniline yielded an "anilide" which, after recrystallisation from ethanol–ethyl acetate, had m.p. and mixed m.p. (with 2,4,6-tri-*O*-methyl-*N*-phenyl-D-galactosylamine[19]) 171–172°. The minor fraction gave a single spot on chromatography (t.l.c.), which was identical both in colour (grey) and mobility with that of authentic 2,3,6-tri-*O*-methylgalactose, but different from 2,4,6- and 2,3,4-tri-*O*-methylgalactoses eluted on the same plate.

Di-O-methyl fraction. The syrup (50 mg) was shown by chromatography to be a mixture of approximately equal parts of 2,6- and 4,6-di-*O*-methylgalactoses.

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The ultracentifruge examination of aeodan was done by Dr. F. J. Joubert of the National Chemical Research Laboratory, Pretoria. The microanalyses were performed at the same laboratory.

SUMMARY

A highly sulphated, methylated galactan, aeodan, isolated from *Aeodes orbitos a* was shown to contain D-galactose, 2-*O*-methyl-D-galactose, and glycerol. It was desulphated with methanolic hydrogen chloride. Both aeodan and desulphated aeodan were studied by periodate oxidation and methylation. In addition, aeodan was subjected to alkaline degradation. Evidence is presented for the presence of (1→3)- and (1→4)-glycosidic linkages with sulphate on positions 2 and 6.

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THE ACID HYDROLYSIS OF GLYCOSIDES

VI. EFFECT OF SUBSTITUTION AT C-3 AND C-5

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INTRODUCTION

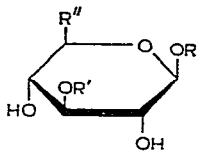
In a previous study[1], it was found that the 2-, 3-, and 6-methyl ethers of methyl β-D-glucopyranoside and gentiobiose are hydrolyzed in acid solution somewhat more slowly than the unsubstituted compounds. Höök and Lindberg[2] noted a stabilization towards acid when methyl α- and β-D-glucopyranosides are substituted with isopropyl at O-5, but a considerable destabilization when an isopropyl group is introduced at each of the three other hydroxyl groups. Theander[3] found that methyl β-D-*gluco*-hexodialdo-1,5-pyranoside is hydrolyzed 20 times, and its 4-methyl ether 70 times, as fast as methyl β-D-glucopyranoside. He attributed this behavior to the formation of a cyclic hemiacetal between O-5 and O-3, which caused the hexodialdose to assume the *1C* (D) conformation, in which all of the hydroxyl groups are axially attached.

A 5,3-lactone might be formed by D-glucopyranosiduronic acids; it would be expected to influence the acid-catalyzed hydrolysis of such compounds. It was therefore of interest to establish the rate of hydrolysis of D-glucopyranosiduronic acids methylated at O-3 and incapable of giving a 5,3-lactone. The aglycons chosen were isopropyl and 2-chloroethyl, which are the least and the most stable, respectively, of the D-glucopyranosiduronic acids previously investigated[4]. Comparison was made with the corresponding β-D-glucopyranosides. For the isopropyl and 2-chloroethyl β-D-glucopyranosides, the effect of a methyl group on O-6 was also studied.

RESULTS

Isopropyl (**1**) and 2-chloroethyl (**2**) 3-*O*-methyl-β-D-glucopyranosides were synthesized by conventional methods. The corresponding β-D-glucopyranosiduronic acids (**3** and **4**) were obtained by catalytic oxidation of the D-glucopyranosides. Isopropyl (**5**) and 2-chloroethyl (**6**) 6-*O*-methyl-β-D-glucopyranosides were prepared by standard methods. Hydrolyses were conducted as previously described[1,4–7]. Pseudo-first-order rate-coefficients, and energies and entropies of activation, are presented in Table I, which also contains data for the hydrolysis of methyl 6-deoxy-6-fluoro-β-D-glucopyranoside.

It was found that the 3-*O*-methyl-D-glucosides **1** and **2** are hydrolyzed at



	R	R'	R''
1	$CH(Me)_2$	Me	CH_2OH
2	CH_2CH_2Cl	Me	CH_2OH
3	$CH(Me)_2$	Me	CO_2H
4	CH_2CH_2Cl	Me	CO_2H
5	$CH(Me)_2$	H	CH_2OMe
6	CH_2CH_2Cl	H	CH_2OMe

slightly higher rates than the unsubstituted D-glucosides[4], whereas the acids **3** and **4**, are hydrolyzed twice as fast as their unsubstituted analogs, indicating that the rapid hydrolysis of these D-glucopyranosiduronic acids is not attributable to formation of 5,3-lactones. The energies and entropies of activation of acids **3** and **4** were lower than those of the D-glucosides **1** and **2**. The 6-methyl ethers **5** and **6** were hydrolyzed slightly more slowly than their unsubstituted analogs[4]. The methyl, isopropyl (**5**), and 2-chloroethyl (**6**) 6-*O*-methyl-β-D-glucopyranosides did not differ considerably in their rates of hydrolysis, in notable contrast to the corresponding D-glucopyranosiduronic acids[4,6]. Methyl 6-deoxy-6-fluoro-α-D-glucopyranoside was hydrolyzed at approximately the same rate as the 6-chloro and 6-iodo compounds studied previously[6].

DISCUSSION

It was noted previously[1] that methyl 3-*O*-methyl-β-D-glucopyranoside is hydrolyzed slightly more slowly than methyl β-D-glucopyranoside, as would be expected from the considerations of Edward[8]. Compounds **1** and **2**, on the other hand, are hydrolyzed slightly more rapidly than the corresponding, unsubstituted D-glucopyranosides. The differences in rates are, however, quite small, and do not warrant further speculation.

The fact that the 3-methyl ethers **3** and **4** are hydrolyzed twice as fast as the corresponding, unmethylated D-glucopyranosiduronic acids was unexpected. It is

TABLE I

RATE COEFFICIENTS AND KINETIC PARAMETERS FOR THE HYDROLYSIS OF MONO-*O*-METHYL-β-D-GLUCOPYRANOSIDES, MONO-*O*-METHYL-β-D-GLUCOPYRANOSIDURONIC ACIDS, AND METHYL 6-DEOXY-6-FLUORO-β-D-GLUCOPYRANOSIDE IN 0.5M SULFURIC ACID

Glycoside	$k \times 10^{-6}$, *sec*$^{-1}$			*E* (*kcal mole*$^{-1}$)	$\Delta S^{\ddagger}$ *at 60°* (*cal deg*$^{-1}$ *mole*$^{-1}$)
	60°	*70°*	*80°*		
3-*O*-Methyl-β-D-glucopyranoside					
isopropyl (**1**)	2.95	12.2	49.6	33.0	+13.7
2-chloroethyl (**2**)	2.13	9.39	37.1	33.4	+14.2
3-*O*-Methyl-β-D-glucopyranosiduronic acid					
isopropyl (**3**)	41.7	144.5	430.5	27.3	+ 1.7
2-chloroethyl (**4**)	0.365	1.32	4.75	30.0	+ 0.5
6-*O*-Methyl-β-D-glucopyranoside					
isopropyl (**5**)	2.10	9.17	36.3	33.3	+13.9
2-chloroethyl (**6**)	1.51	6.60	26.2	33.3	+13.2
6-Deoxy-6-fluoro-α-D-glucopyranoside					
methyl	—	0.55	2.56	37.1	+19.1

possible that introduction of a substituent at O-3 facilitates the catalytic action of the carboxyl group, as postulated previously[4]; if this is the case, the effect of the 3-*O*-methyl substituent must be conformational, and similar to the influence of the 4-*O*-methyl group in the compounds studied by Theander[3]. Interestingly, he found[3] that introduction of a methyl group at O-4 of methyl β-D-glucopyranosiduronic acid had no noticeable effect on the rate of hydrolysis.

The similar rates of hydrolysis of the methyl, isopropyl (5), and 2-chloroethyl (6) 6-*O*-methyl-β-D-glucopyranosides shows that, on substitution at C-5 with methoxymethyl, a group having the same size as the carboxyl group, the rate of hydrolysis remains independent of the polarity of the aglycon. It was shown previously[4] that D-glucopyranosiduronic acids are hydrolyzed at rates that are strongly affected by the electron affinity of the aglycon. Obviously, this effect is not associated with the size of the carboxyl group on C-5.

The influence, on the rate of hydrolysis, of different substituents at C-5 was studied previously[6], and it was concluded that inductive and conformational factors are both probably operative. In Fig. 1, the logarithm of the rate coefficient at 80° for various, 5-C-substituted methyl α-D-xylopyranosides has been plotted agains

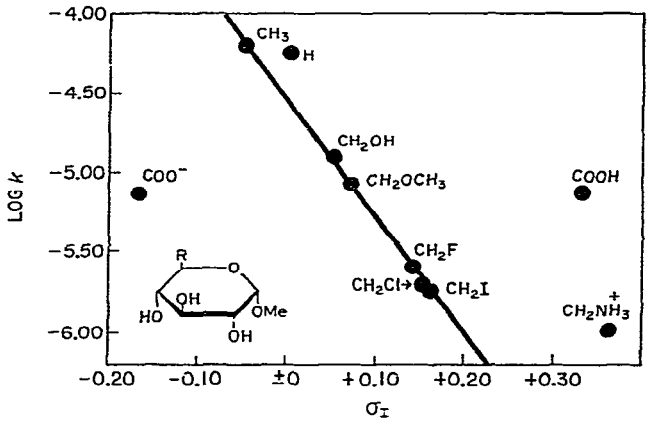


Fig. 1. Relation between the electron affinity of the substituent at C-5 and the rate of hydrolysis of 5-*C*-substituted methyl α-D-xylopyranosides.

Charton's[9] polar substituent constant (σ_I), using values reported here and in a previous study[6]. In most cases, there is an inverse relationship between the rate of hydrolysis and the electron affinity of the substituent at C-5. For hydrogen, the measured rate is somewhat higher than that expected, undoubtedly because of the lower conformational stability of this glycoside[8]. The size of the other substituents at C-5 has no influence on the rate of hydrolysis.

In Fig. 1, there are three notable exceptions to the linear relationship between log k and σ_I. Methyl 6-amino-6-deoxy-α-D-glucopyranoside is hydrolyzed 10 times, and methyl α-D-glucopyranosiduronic acid 44 times, more rapidly than calculated. The recent results of Capon and Ghosh[10] show the significant contribution made by

the carboxylate anion at higher pH values. In the past, it has been generally assumed that, in acid solutions of the concentration used here, protonation of the carboxylate anion is complete, but this is not correct. The data in Fig. 1 permit estimation of the relative rates of ionized D-glucopyranosiduronic acid, D-glucopyranoside, and un-ionized D-glucopyranosiduronic acid. The values obtained at 80°, namely, 2820:74:1, are of the same order of magnitude as those reported by Capon and Ghosh[10], namely, 1580:78:1. If pK_a for D-glucuronic acid is assumed[11] to be 3.2, and if the rate is assumed to be equal to k_1 [$RCOO^-$] + k_2 [RCOOH], where $k_1 = 480 \times 10^{-6}$ sec^{-1} and $k_2 = 0.17 \times 10^{-6}$ sec^{-1} (see Fig. 1), it is found that, in an 0.05M solution of sugar in 0.5M aqueous sulfuric acid, the rate will be $(15.8+8.5) \times 10^{-9}$ mole $liter^{-1}$ sec^{-1}. Clearly, in this case, the contribution of the carboxylate anion, despite its low concentration, cannot be ignored. The rate thus obtained, namely, 24.3×10^{-9} mole $liter^{-1}sec^{-1}$, is 15 times lower than that measured, namely 370×10^{-9} mole $liter^{-1}$ sec^{-1}. The possibility of an increase in rate through anchimeric assistance of the carboxyl group, as suggested previously[4], is thus indicated. Such intramolecular catalysis could involve the carboxyl group on C-5 in its ionized or un-ionized forms, or both[12]. Polar and conformational factors and intramolecular catalysis might thus influence the acid hydrolysis of D-glucopyranosiduronic acids.

EXPERIMENTAL

General experimental conditions, kinetic measurements, and calculations were the same as in a previous investigation[4]. Purity of noncrystalline compounds was checked by paper, thin-layer, and gas–liquid chromatography. Experimental and analytical data for all compounds synthesized are summarized in Tables II and III.

Isopropyl 3-O-methyl-β-D-glucopyranosiduronic acid (3). — 1,2:5,6-Di-*O*-isopropylidene-α-D-glucofuranose, m.p. 109–110°, $[\alpha]_D$ −14°, was methylated, and the product was hydrolyzed to give 3-*O*-methyl-D-glucose[13]; yield 85%, m.p. 161–163°, $[\alpha]_D$ +56°. This was converted into 2,4,6-tri-*O*-acetyl-3-*O*-methyl-α-D-glucosyl bromide[14,15] (42 g); a solution thereof in purified chloroform (100 g) was added to a mixture[16] of isopropyl alcohol (200 ml), chloroform (250 g), mercuric oxide (20 g), mercuric bromide (1.5 g), and Drierite (60 g). The mixture was stirred for 36 h at room temperature, the suspension was filtered through Celite, the filtrate was evaporated to dryness, the residue was mixed with chloroform, the suspension was filtered, and the filtrate was evaporated to dryness. The resulting syrup was dissolved in isopropyl alcohol (500 ml), and the glycoside was allowed to crystallize, giving 33.2 g. Deacetylation was conducted with sodium methoxide in methanol, affording crystalline isopropyl 3-*O*-methyl-β-D-glucopyranoside (**1**).

Compound **1** (9 g) was dissolved in water (200 ml) in a 3-necked flask, and 10% platinum-on-charcoal (2 g) was added. Oxygen was introduced with rapid stirring for 6 h at 55°, while M sodium hydrogen carbonate (52 ml) was added occasionally to maintain alkalinity. The suspension was filtered through Celite, the filtrate was treated with Amberlite IR-120 (H^+) ion-exchange resin, the suspension was filtered, and the filtrate was added to the top of a column of Dowex 1-X4 (OAc^-) ion-exchange

TABLE II

EXPERIMENTAL AND ANALYTICAL DATA FOR THE MONO-*O*-METHYL-β-D-GLUCOPYRANOSIDE AND MONO-*O*-METHYL-β-D-GLUCOPYRANOSIDURONIC ACID PERACETATES

Glycoside	*Yield, %*	*M.p., degrees*	$[\alpha]_D$, *degrees*	*Carbon, %*		*Hydrogen, %*	
				Calc.	*Found*	*Calc.*	*Found*
2,4,6-Tri-*O*-acetyl-3-*O*-methyl-β-D-glucopyranoside							
isopropyl	79	107–108.5	−32.5	53.03	53.22	7.23	7.30
2-chloroethyl[a]	62	61.5–62.5	−22.9	47.06	46.67	6.06	5.91
Methyl (2,4-di-*O*-acetyl-3-*O*-methyl-β-D-glucopyranosid)uronate							
isopropyl	60	103–104	−52.1				
2-chloroethyl	65	94–95	−38.4				
2,3,4-Tri-*O*-acetyl-6-*O*-methyl-β-D-glucopyranoside							
isopropyl	75	130–131	−23.6	53.03	52.21	7.23	7.10
2-chloroethyl[b]	37	102–103	−11.4	47.06	47.63	6.06	6.26

[a]Calc.: Cl, 9.26. Found: Cl, 9.16. [b]Calc.: Cl, 9.26. Found: Cl, 9.34.

TABLE III

EXPERIMENTAL AND ANALYTICAL DATA FOR THE MONO-*O*-METHYL-β-D-GLUCOPYRANOSIDES AND MONO-*O*-METHYL-β-D-GLUCOPYRANOSIDURONIC ACIDS

Glycoside	*M.p., degrees*	$[\alpha]_D$, *degrees*	*Carbon, %*		*Hydrogen, %*	
			Calc.	*Found*	*Calc.*	*Found*
3-*O*-Methyl-β-D-glucopyranoside						
isopropyl (1)	78–81	−37.7	50.83	50.81	8.53	8.68
2-chloroethyl[a] (2)	110–111	−28.1	42.11	41.80	6.68	6.91
3-*O*-Methyl-β-D-glucopyranosiduronic acid						
isopropyl (3)		−54.0	47.98	46.06	7.25	7.46
2-chloroethyl (4)		−42.2				
6-*O*-Methyl-β-D-glucopyranoside						
isopropyl (5)		−35.3				
2-chloroethyl (6)		−25.5				

[a]Calc.: Cl, 13.81. Found: Cl, 13.53.

resin. Elution with water (5 liters), followed by evaporation of the eluate, gave crystalline isopropyl 3-*O*-methyl-β-D-glucopyranoside (3.8 g). Further elution with 50% aqueous acetic acid (2 liters), followed by evaporation of the eluate, afforded a glass (6.9 g) which had an equivalent weight of 273 and was isopropyl 3-*O*-methyl-β-D-glucopyranosiduronic acid (3).

This compound (1 g) was converted into the methyl ester with diazomethane in ethanol–ethyl ether, and the ester was acetylated with acetic anhydride in pyridine. The acetate was recrystallized from ethanol.

Compounds **2** and **4** were synthesized in a similar manner.

Isopropyl (**5**) *and 2-chloroethyl* (**6**) *6-O-methyl-β-D-glucopyranosides.* — 6-*O*-Methyl-D-glucose[17,18], m.p. 139–142°, $[\alpha]_D$ +63°, was converted in the usual way into 2,3,4-tri-*O*-acetyl-6-*O*-methyl-α-D-glucosyl bromide[15]. Condensation as above, but in the presence of silver carbonate, with isopropyl alcohol and 2-chloroethanol, respectively, followed by deacetylation, gave the desired compounds, **5** and **6**.

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SUMMARY

Isopropyl and 2-chloroethyl 3-*O*-methyl-β-D-glucopyranosiduronic acids, but not the corresponding β-D-glucopyranosides, are hydrolyzed in acid solution at a rate higher than that for the unmethylated compounds. The hydrolysis of 6-*O*-methyl-β-D-glucopyranosides is independent of the polarity of the aglycon. Several 5-*C*-substituted methyl α-D-xylopyranosides are hydrolyzed at rates inversely proportional to the electron affinity of the substituent on C-5. It was shown that, in N sulfuric acid, the contribution of the carboxylate anion to the rate cannot be ignored. The rate of hydrolysis of methyl α-D-glucopyranosiduronic acid is 15 times that calculated on the basis of polar effects, suggesting anchimeric assistance of the carboxyl group.

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METHYL 4,6-*O*-BENZYLIDENE-*O*-ETHYL- AND -*O*-VINYL-α-D-GLUCOPYRANOSIDES

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ABSTRACT

Methyl 4,6-*O*-benzylidene-3-*O*-vinyl-α-D-glucopyranoside (**2**), methyl 4,6-*O*-benzylidene-2,3-di-*O*-vinyl-α-D-glucopyranoside (**6**), and methyl 4,6-*O*-benzylidene-2,3-di-*O*-ethyl-α-D-glucopyranoside (**7**) have been prepared and characterized. The 2-vinyl ether (**3**) rearranges on alumina chromatography to methyl 4,6-*O*-benzylidene-2,3-*O*-ethylidene-α-D-glucopyranoside (**4**).

INTRODUCTION

Studies of the synthesis, optimum conditions for preparation, and structure of vinyl ethers of carbohydrates have been the subject of previous papers[1–4]. It was found that these vinyl ethers are usually quite hydrophilic, and, when polymerized, do not yield products having the desired properties. The vinyl ethers of methyl 4,6-*O*-benzylidene-α-D-glucopyranoside appeared more suitable as monomers, and their synthesis by vinylation with acetylene was undertaken.

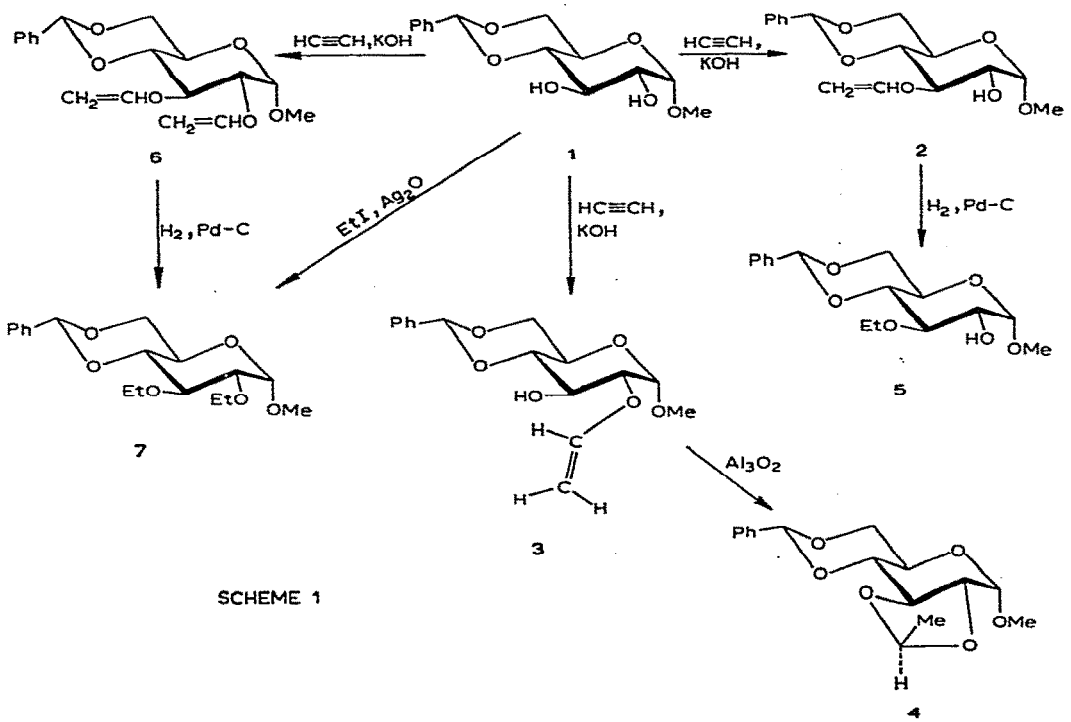
DISCUSSION

Mono-*O*-vinylation of methyl 4,6-*O*-benzylidene-α-D-glucopyranoside (**1**) was accomplished by using a *p*-dioxane–water solvent system and relatively mild reaction conditions[2,5], and a crude mixture (a) was obtained by recrystallization from ether. T.l.c. showed two widely separated spots, the faster-moving of which probably contained two compounds having R_F values almost identical with that of methyl 4,6-*O*-benzylidene-3-*O*-ethyl-α-D-glucopyranoside (**5**), and the slower-moving spot corresponded to the starting material (**1**).

When a portion of the original reaction mixture was recrystallized repeatedly from ether, a mixture (b), m.p. 107–109°, was obtained, which showed only the faster-moving spot on t.l.c. From the n.m.r. spectra of this mixture, obtained in methyl sulfoxide-d_6 and chloroform-*d*, it was possible to estimate the ratio of free 3-OH and 2-OH present. This estimate was based on the area under the 3-OH and 2-OH lines, and their field position and line-width, by analogy with the spectra of methyl 4,6-*O*-benzylidene-2- and -3-*O*-ethyl-α-D-glucopyranosides[5] (**3** and **2**). The chemical

shifts of the 2-OH and -3-OH in the starting material (**1**) appear as doublets at τ 5.35 (J 2.3 Hz) and τ 5.08 (J 6.7 Hz) respectively, whereas the 3-ethyl ether (**5**) exhibits a doublet at τ 5.35 (J 1.8 Hz) attributed to the 2-OH resonance. The total line-widths are 6 and 12 Hz., respectively, and this is assumed to be the case for compounds **2** and **3**.

Chromatography of mixture (a), and subsequent recrystallization of the eluted solid from ether, yielded pure methyl 4,6-*O*-benzylidene-3-*O*-vinyl-α-D-glucopyranoside (**2**), m.p. 134–134.5°, $[\alpha]_D^{22}$ +95.6° (*c* 1, chloroform). The n.m.r. spectra in chloroform-*d* and methyl sulfoxide-d_6 confirmed the position of the vinyl group. Satisfactory chemical evidence for the identity of this compound was obtained by hydrogenation of **2** to methyl 4,6-*O*-benzylidene-3-*O*-ethyl-α-D-glucopyranoside (**5**), which was identical in all respects with an authentic sample.

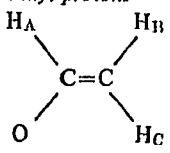


SCHEME 1

Concentration of the mother liquors from which mixture (a) had crystallized gave a crude solid (mixture c). The n.m.r. spectrum in methyl sulfoxide-d_6 indicated that c was a mixture of starting material with the 2- and 3-vinyl ethers (**3** and **2**). The last two were present in the ratio of 3 to 2. Chromatography of mixture (c) on Woelm alumina gave three fractions, A, B, and C. The most polar component (C) was the starting material **1**. Fraction B was a mixture of several compounds that was not examined further. Fraction A, the least polar, was a single compound, m.p. 139–140°. Its i.r. spectrum indicated that there was no hydroxyl group in the molecule.

TABLE I

N.M.R. SPECTRA OF METHYL 4,6-*O*-BENZYLIDENE-*O*-VINYL-α-D-GLUCOPYRANOSIDES[a]

Compound	*Ph*	*Low-field Vinyl protons* 	*PhCH*	*H-1*	*H-2, H-3, H-4, H-5, H-6, vinyl protons*	*OMe*	*2-OH or 3-OH*
1[b]	2.53 s		4.42 s	4.87 d, $J_{1,2}$ 4	5.8–6.6 e	6.70 s	5.35 d, $J_{2,OH}$ 2.3, 5.08 d, $J_{3,OH}$ 6.7
2[c]	2.48 e	3.47 q, $J_{A,B}$ 6.5, $J_{A,C}$ 14	4.38 s	5.05 d, $J_{1,2}$ 3.4	5.2–6.5 e	6.53 s	7.23 s
2+3[b]	2.68 e	3.56 q, $J_{A,B}$ 7 3.64 q, $J_{A,C}$ 14	4.54 s 4.57 s	5.20 d, $J_{1,2}$ 3.0	5.25–6.65 e	6.67 s 6.70 s	5.4 e 5.1 e
4[c]	2.80 s	(ethylidene H) 4.91 q, *J* 5	4.54 s	5.06 d, $J_{1,2}$ 3.0	5.8–6.8 e	6.93 s	(ethylidene CH_3) 8.97 d, *J* 5
5[b]	2.63 s		4.39 s	5.00 d, $J_{1,2}$ 3.8	5.8–6.6 e	6.92 s	5.35 d, $J_{2,OH}$ 1.8
6[c]	2.60 s	3.59 q, $J_{A,B}$ 6.5 3.70 q, $J_{A,C}$ 14	4.47 s	5.12 d, $J_{1,2}$ 3.6	5.42–6.5 e	6.58 s	
7[c]	2.56 s		4.45 s	5.20 d, $J_{1,2}$ 3.1	5.68–6.80 e	6.55 s	$2CH_3$ 8.43, 8.47, *J* 7

[a]Chemical shifts are on the τ-scale relative to external Me_4Si (Me_2SO-d_6) or internal Me_4Si ($CDCl_3$); s, singlet; d, doublet; q, quartet; e, envelope; *J* values are in Hz. [b]In Me_2SO-d_6; [c]In $CDCl_3$.

The n.m.r. spectrum of A in chloroform-*d* showed a high-field, methyl doublet (τ 8.98, *J* 5 Hz) in addition to the usual features of methyl 4,6-*O*-benzylidenehexopyranosides[9]. These data support a structural assignment as methyl 4,6-*O*-benzylidene-2,3-*O*-ethylidene-α-D-glucopyranoside (**4**). The stereochemistry of **4** is probably that shown, since models of the alternative structure showed considerable steric crowding between the 1-methoxyl group and the ethylidene methyl groups. Orthoesters[10–12], which form during acyl migration, show similar structural configurations.

Based upon t.l.c. evidence, the vinylation reaction-mixture contained some 30–50% of starting material, together with both monovinyl ethers. No divinyl ether was present, nor did the crude mixture (a) contain any *O*-ethylidene derivative (0.5% could have been detected by n.m.r.). Crystallization and chromatography on Woelm alumina gave only the 3-vinyl ether (**2**), the 2,3-ethylidene acetal (**4**), the starting material **1**, and a mixture of products that, according to the i.r. spectrum, contained no benzylidene group.

Recoveries of 90% indicated that the 3-vinyl ether **2** is reasonably stable to chromatography, and thus did not give rise to the ethylidene acetal **4**. The formation of the latter thus appears to have been due to the cyclization of the 2-vinyl ether **3** on the alumina column.

The *O*-benzylidene di-*O*-vinyl derivative **6** was prepared easily by using *p*-dioxane alone as the solvent for the vinylation reaction. No hydroxyl groups were apparent in the i.r. spectrum of **6**, and both the i.r. and n.m.r. spectra were consistent with the structure assigned (see Table I). Confirmation of the structure was obtained by hydrogenation of **6** to the corresponding diethyl ether (**7**), and comparison of this product with the diethyl ether synthesized independently from **1**.

EXPERIMENTAL

Melting points are uncorrected. T.l.c. was performed on Silica Gel G (E. Merck, Germany), with benzene–ethanol or benzene–butyl alcohol. The plates were developed by spraying the dried chromatogram with a 10% (w/v) solution of phosphomolybdic acid, followed by heating for 5 min at approximately 100°. I.r. spectra were recorded on a Perkin–Elmer Infracord spectrometer, Model 137, and optical rotations were measured on a Bendix Automatic polarimeter or a Rudolph polarimeter Model 80. N.m.r. spectra were measured on a Varian Associates A-60 spectrometer (60 MHz), and tetramethylsilane ($\tau = 10.00$) was used as the internal or external standard. Methyl sulfoxide-d_6 and 1% tetramethylsilane in chloroform-*d* (Silanor) were obtained from Merck, Sharp and Dohme, Ltd., Canada, and were used without purification. Most of the samples for n.m.r. were degassed. All peaks assigned to hydroxyl groups were confirmed by deuterium oxide exchange. Elemental analyses were performed by Schwarzkopf Microanalytical Laboratory, Woodside, New York.

Mono-O-vinylation of methyl 4,6-O-benzylidene-α-D-glucopyranoside (**1**). — A mixture of 20 g (0.07 mole) of methyl 4,6-*O*-benzylidene-α-D-glucopyranoside, 2 g of potassium hydroxide, 100 ml of redistilled *p*-dioxane, and 100 ml of water was placed

in a 300-ml, stirred autoclave. The autoclave was flushed with nitrogen and heated to 150°. Acetylene gas, compressed to 400 p.s.i.g., was admitted and maintained at that pressure for 6 h. The cooled autoclave was then vented, and carbon dioxide was used to carbonate the reaction mixture. After filtration, the filtrate was evaporated to dryness, and the residue was recrystallized twice from ethyl ether. This mixture (a) showed two spots on t.l.c. (0.25 mm silica gel G, 3% BuOH in benzene). The slower-moving spot corresponded to starting material. The faster-moving spot appeared to contain two substances having R_F values close to that of compound **5**.

When a portion of the reaction mixture was recrystallized five times from ether, a mixture (b) was obtained; m.p. 107–109°, $[\alpha]_D^{22}$ +86.8° (*c* 1, chloroform). T.l.c. showed only the faster-moving spot. The n.m.r. spectra of this mixture (b) indicated that it contained methyl 4,6-*O*-benzylidene-3- and -2-*O*-vinyl-α-D-glucopyranosides (**2** and **3**) (see Table I).

Anal. Calc. for $C_{16}H_{20}O_6$: C, 62.33; H, 6.49. Found: C, 62.18; H, 6.59.

Mixture (a) (3.3 g) was dissolved in benzene and chromatographed on Woelm alumina, activity I (60 g) with benzene as the eluent. After the first 50 ml of eluate had been discarded, the next 150 ml was evaporated, to give 2.65 g of crystalline product which showed one spot on t.l.c. After three recrystallizations from ether, the pure monovinyl ether **2** was obtained and dried; m.p. 134–134.5°, $[\alpha]_D^{25}$ + 95.6° (*c* 1, chloroform); n.m.r. and i.r. spectral data are given in Tables I and II.

Anal. Calc. for $C_{16}H_{20}O_6$: C, 62.33; H, 6.49. Found: C, 62.12; H, 6.40.

*Identification of the pure methyl 4,6-*O*-benzylidene-mono-*O*-vinyl-α-D-glucopyranoside.* — Compound **2** (200 mg, 6.5 mmoles) was hydrogenated in 95% ethanol, with 5% Pd/C as catalyst. Three recrystallizations from ethanol gave 150 mg (74%) of crystalline product, m.p. 168–169°, $[\alpha]_D^{22}$ +111.1° (*c* 0.99, chloroform). On admixture with authentic methyl 4,6-*O*-benzylidene-3-*O*-ethyl-α-D-glucopyranoside (**5**), this product showed no depression of m.p. The n.m.r. and i.r. spectra of the product could be superimposed on those of the authentic sample (see Tables I and II).

*Formation and isolation of methyl 4,6-*O*-benzylidene-2,3-*O*-ethylidene-α-D-glucopyranoside* (**4**). — The mother liquors from which mixture (a) had been removed were evaporated, to give 16 g of crude solid. This was dissolved in benzene, and chromatographed over Woelm aluminum oxide, activity I (300 g). Fraction A (2.5 g) was obtained by eluting with 1.8 liters of benzene; and fraction B (5.8 g) with 2 liters of 5% of ether in benzene. The column was then washed with 5% of methanol in benzene to give 7.2 g of fraction C, which was identified as methyl 4,6-*O*-benzylidene-α-D-glucopyranoside (**1**). (Fraction B was a mixture of at least four compounds, and was not characterized further. The i.r. spectrum of B showed hydroxyl bands and a band at 1680 cm^{-1} (PhCHO), but no vinyl bands.) Fraction (a) was identified as methyl 4,6-*O*-benzylidene-2,3-*O*-ethylidene-α-D-glucopyranoside (**4**), m.p. 139-140°. The arguments in favor of this assignment are outlined in the Discussion.

Anal. Calc. for $C_{16}H_{20}O_6$: C, 62.33; H, 6.49. Found: C, 62.21; H, 6.35.

*Methyl 4,6-*O*-benzylidene-2,3-*di*-O-vinyl-α-D-glucopyranoside* (**6**) — A mixture of methyl 4,6-*O*-benzylidene-α-D-glucopyranoside (20 g, 0.07 mole), 10 g of powdered

potassium hydroxide, and 200 ml of redistilled *p*-dioxane was placed in a 300-ml autoclave and stirred. The autoclave was flushed with nitrogen, and acetylene gas was admitted and maintained at 400 p.s.i.g. for 6 h. The autoclave was cooled, and vented, and carbon dioxide was introduced to carbonate the reaction mixture. After filtration, the filtrate was evaporated to dryness, the residue was extracted with ethyl ether, and the extract was dried (anhydrous potassium carbonate), and evaporated. The resulting crude solid was mixed with a minimal volume of Skellysolve B, the suspension was filtered, the filtrate was evaporated, the residue was dissolved in a large volume of ether, and the solution was treated with charcoal. The solid resulting after filtration and evaporation was recrystallized four times from ether; m.p. 92–93°, $[\alpha]_D^{20}$ +87.3° (*c* 1, chloroform), $\nu_{max}^{CCl_4}$ 3050, 2950, 1640, 1625 (sh), 1465, 1410 (sh), 1385 (sh), 1370, 1350 (sh), 1325, 1310, 1280, 1200 (sh), 1190, 1170, 1150 (sh), 1120, 1105, 1090, 1055, 1030, 995, 970, 940, 915, 878, 835, 690 cm^{-1}. N.m.r. spectral data are given in Table I.

Anal. Calc. for $C_{18}H_{22}O_6$: C, 64.67; H, 6.58. Found: C, 64.51; H, 6.39.

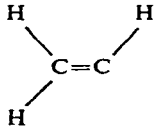
Preparation of methyl 4,6-O-benzylidene-2,3-di-O-ethyl-α-D-glucopyranoside (7) *from* (6). — To a suspension of 0.5 g of 5% Pd/C in 100 ml of 95% ethanol was added 1 g (~ 3 mmoles) of methyl 4,6-*O*-benzylidene-2,3-di-*O*-vinyl-α-D-glucopyranoside; after 3 h, the theoretical amount of hydrogen had been absorbed. The reaction conditions were maintained for a further 2 h, but no more hydrogen was taken up. The product was recrystallized three times from absolute ethanol; m.p. 91–92°, $[\alpha]_D^{25}$ +86.2° (*c* 1, chloroform).

Anal. Calc. for $C_{18}H_{26}O_6$: C, 63.90; H, 7.69. Found: C, 64.08; H, 7.90.

Methyl 4,6-O-benzylidene-2,3-di-O-ethyl-α-D-glucopyranoside (7). — To a solution of 2.82 g (10 mmoles) of **1** and 6.0 ml (75 mmoles) of ethyl iodide in 50 ml of *N*,*N*-dimethylformamide was added 10.5 g (45 mmoles) of dry silver oxide, and the suspension was stirred for 28 h at room temperature. The silver oxide was then removed by filtration, and the filtrate was evaporated to dryness. The crude product was recrystallized to constant optical rotation, yield 2.1 g (62%), m.p. 91–92°, $[\alpha]_D^{25}$

TABLE II

CHARACTERISTIC INFRARED ABSORPTION BANDS[a] OF THE METHYL 4,6-*O*-BENZYLIDENE-*O*-VINYL-α-D-GLUCOPYRANOSIDES

Compound	*OH*		*CH*	*Ring vibrations*	*Type 2a bands*
2+3	3550, 3450	3085, 1640, 1620, (1601)	2930, 2860	913	835
3	3600, 3400	3100, 1645, 1625, (1605)	2950, 2870	919	842
4		(1605)	2950	918	832
6		3080, 1640, 1625 (sh), (1608)	2940, 2850	918	840
7		(1601)	2995, 2950, 2850	917	

[a] In $CHCl_3$ solution (polystyrene calibration); frequencies in cm^{-1}.

+86.2° (*c* 1, chloroform). The product proved to be identical in all respects (m.p., i.r., and n.m.r. spectra, and behavior on t.l.c.) with the hydrogenation product prepared from the divinyl compound.

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A DETERMINATION OF THE STRUCTURE OF 4-(D-*arabino*-TETRAHYDROXYBUTYL)-4-THIAZOLINE-2-THIONE

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ABSTRACT

The structure of 4-(D-*arabino*-tetrahydroxybutyl)-4-thiazoline-2-thione (**1**) was determined by means of synthesis, and was in agreement with the recent structural assignment by Jochims *et al.* Procedures of carbohydrate chemistry afforded typical derivatives of **1**, and provided a total synthesis from D-glucose. The heterocyclic ring of **1** gave reactions characteristic of HS-C=N- and S=C-NH forms of a 4-thiazoline-2-thione. Periodate oxidation of **1** provided a route to the lowest homolog **11**, which was alternatively prepared by a known method from the appropriate α-chloroketone and ammonium dithiocarbamate. An unequivocal preparation by a similar reaction with 3,4,5,6-tetra-*O*-acetyl-1-bromo-1-deoxy-D-*arabino*-hexulose led to **1**. The formation of carbohydrate-derived 4-thiazoline-2-thiones appears to be a general reaction of α-halo ketoses with ammonium dithiocarbamate.

INTRODUCTION

The title compound (**1**) is of particular interest, since it exhibits certain properties both of a carbohydrate and of a thione-substituted heterocycle that is capable of tautomerism. Zemplén *et al.*[1] reported **1** as a substance, melting at 218°, that can be separated in 2% yield from the products of the reaction of D-fructose with thiocyanic acid in hydrochloric acid solution. Jochims *et al.*[2] recently proposed the structure now assigned to **1**. They reported that the reaction of 2-amino-2-deoxy-D-glucose with carbon disulfide yielded a 5-hydroxythiazolidine[3], an example of a new class of compound. Subsequent dehydration and acetylation gave a tetraacetate that was identical with the tetraacetate of the substance reported[1] by Zemplén *et al.* The structures reported by Jochims and co-workers were based on elemental and spectral analyses and determination of molecular weight.

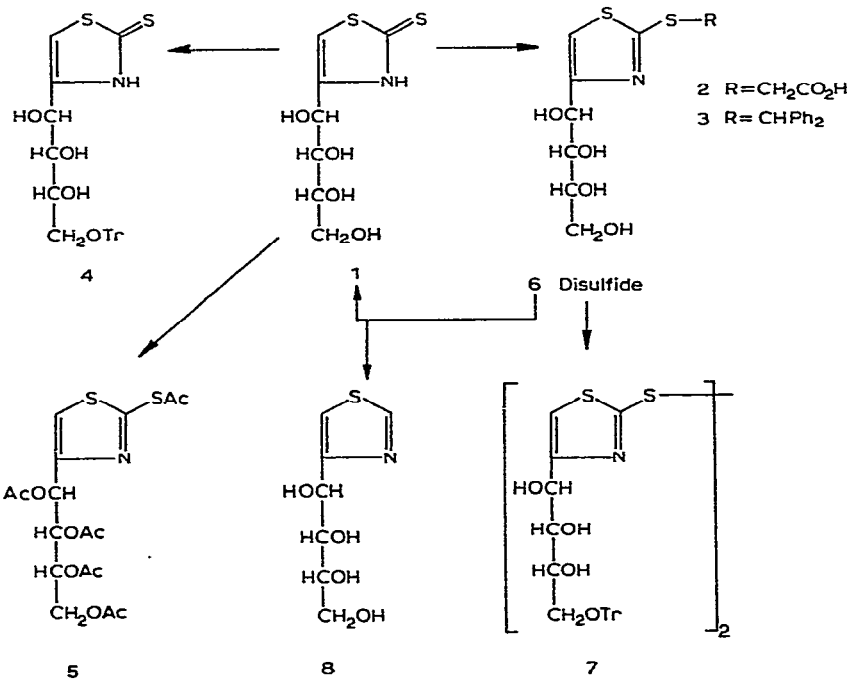
The procedure of Zemplén *et al.* had previously been applied to L-arabinose, D-fructose, D-galactose, D-glucose, D-lyxose, D-mannose, D-ribose, and D-xylose. The products were isolated in good yield, but, in some cases, there was uncertainty as to the structure[4–6]. Subsequent studies revealed these structures to be those of oxazolidine-2-thiones fused with furanoid or pyranoid ring-systems[2,7–9]. The present article reports a structural determination based on the chemical properties

of **1**, and an unequivocal synthesis of **1**. The results obtained support the concurrent characterization[2] by Jochims *et al.*

RESULTS AND DISCUSSION

The reactions of **1** are typical of HS-C=N- and S=C-NH tautomers, and of primary and secondary hydroxyl groups. Although **1** is readily recrystallized from water, it is insoluble in the solvents commonly used in ebullioscopic or cryoscopic methods for determination of molecular weight. However, titration of **1** with standard sodium hydroxide or iodine solution permitted the determination of a neutralization equivalent and of an empirical formula. Treatment of **1** with sodium hydrogen carbonate plus an equivalent of sodium chloroacetate gave the *S*-alkylated derivative 2-(carboxymethyl)thio-4-(D-*arabino*-tetrahydroxybutyl)thiazole (**2**). Use of chlorodiphenylmethane in pyridine also afforded an *S*-derivative (**3**), but similar use of chlorotriphenylmethane in pyridine gave the trityl ether (**4**). Complete acetylation of **1** yielded the pentaacetate (**5**). Oxidation of **1** with iodine or ammonium persulfate afforded 2,2′-dithiobis[(4-D-*arabino*-tetrahydroxybutyl)thiazole] (**6**), and tritylation of this disulfide **6** gave a ditrityl ether (**7**).

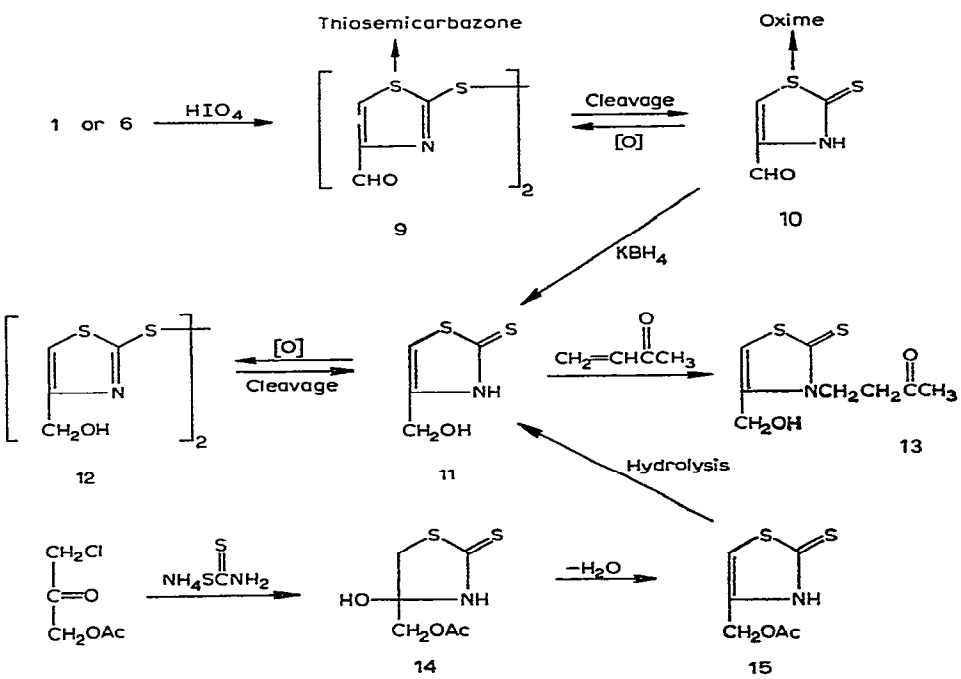
The disulfide **6** was found to be cleaved readily by aqueous sodium hydroxide, carbonate, or hydrogen carbonate, to give **1** in approximately 82% yield; a by-product, 4-(D-*arabino*-tetrahydroxybutyl)thiazole (**8**), was also isolated (*ca.* 13%). Facile cleavage by alkali was also characteristic of the other thiazolyl disulfides encountered



in the study. These reactions suggest that **1** contains three secondary hydroxyl groups, one primary hydroxyl group, and a tautomeric thiol-thione group.

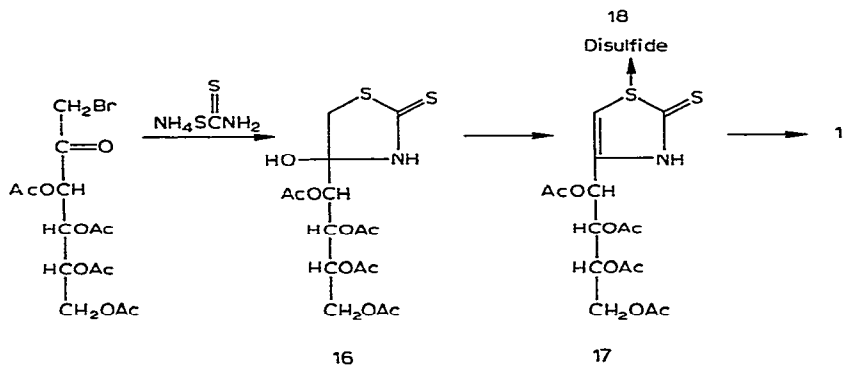
Compound **1** showed a maximum absorption in the u.v. spectrum at 317 nm, comparable to that of 4-thiazoline-2-thione at 314 nm and to that of 4-methyl-4-thiazoline-2-thione at 321 nm, but in contrast to that of the saturated ring of thiazolidine-2-thione at 273 nm. The *S*-derivatives of **1** showed maximum absorption at 262–282 nm.

The type of heterocyclic ring in **1** and the position of substitution were indicated by typical reactions. Preparative, periodate oxidation of **1** or **6** yielded the disulfide (**9**), which showed, in its i.r. spectrum, absorption for an aldehyde group. Alkaline cleavage of the disulfide **9** yielded the thione (**10**). The aldehydes **9** and **10** formed Schiff-base derivatives. Reduction of **10** with potassium borohydride provided 4-(hydroxymethyl)-4-thiazoline-2-thione (**11**), the lowest homolog of **1**. Oxidation of **11** under mild conditions gave the disulfide (**12**). Base-catalyzed, conjugate addition at the nitrogen hetero atom of 4-thiazoline-2-thiones has been shown to be a characteristic reaction of the heterocyclic system[10]. Treatment of **11** with 3-buten-2-one in this way afforded the adduct (**13**), which showed, in its u.v. spectrum, maximum absorption at 321 nm. Alternatively, **11** was prepared by a known, synthetic method[11] for 4-thiazoline-2-thiones. By this procedure, the reaction of 1-acetoxy-3-chloro-2-propanone and ammonium dithiocarbamate gave a typical intermediate[12], 4-(acetoxymethyl)-4-hydroxythiazolidine-2-thione (**14**). Facile, acidic dehydration of **14** gave the 4-thiazoline structure (**15**), and subsequent alkaline hydrolysis of **15** gave **11**. The observed proper-



ties of **11** as obtained from **1** by way of periodate oxidation, and by the synthesis from an α-halo ketone and ammonium dithiocarbamate, were the same.

The configuration of the tetrahydroxybutyl substituent in **1** was confirmed by preparation by known methods of carbohydrate chemistry. D-Glucose in aqueous, methanolic potassium hydroxide was oxidized with oxygen to afford potassium D-arabinonate[13] (76%) which, after acetylation with acetic anhydride–hydrogen chloride, yielded tetra-*O*-acetyl-D-arabinonic acid[14] (48%). Thionyl chloride[15] converted the latter into tetra-*O*-acetyl-D-arabinonoyl chloride[16] (85%) which, on treatment with diazomethane, yielded 3,4,5,6-tetra-*O*-acetyl-1-deoxy-1-diazo-D-*arabino*-hexulose[17] (84%). Subsequent addition of hydrogen bromide to the diazo ketone in ether provided 3,4,5,6-tetra-*O*-acetyl-1-bromo-1-deoxy-D-*arabino*-hexulose[17] (85%). Use of the latter α-bromo ketone permitted synthesis, by the ammonium dithiocarbamate method, of a 4-thiazoline-2-thione bearing a 4-substituent of known configuration.



The intermediate, 4-hydroxy-4-(D-*arabino*-tetraacetoxybutyl)thiazolidine-2-thione (**16**) was isolated, and was readily dehydrated to give 4-(D-*arabino*-tetraacetoxybutyl)-4-thiazoline-2-thione (**17**). The tetraacetate **17** was independently obtained by Jochims and co-workers[2] from the more stable 5-hydroxythiazolidine by boiling the latter for 16 h in pyridine. Oxidation of **17** under mild conditions gave the disulfide **18**. Hydrolysis of either **17** or the precursor **16** gave **1**. The overall yield from D-glucose was 17%; all products were obtained crystalline. The observed properties of **1**, as prepared from D-fructose by the method of Zemplén *et al.*[4] and from the α-bromo ketose derived from D-glucose, were the same.

The preferred method of synthesis of 4-thiazoline-2-thiones derived from carbohydrates depends largely upon the availability of the starting material. Of the required ketoses, only D-fructose has been reported to have been used for this purpose by the method of Zemplén *et al.*[4] The procedure of Jochims *et al.*[2] *via* a 5-hydroxythiazolidine has been reported to be a general reaction of α-amino carbonyl compounds with carbon disulfide. The method described here, *via* a 4-hydroxythiazolidine, has been found to have significant application. Homologs of **1**, prepared from α-halo ketoses by this method, will be described in a subsequent paper.

EXPERIMENTAL

Melting points, determined in thin-walled capillary tubes, are corrected. Infrared spectra were recorded for potassium bromide disks on a Baird Atomic spectrophotometer, Model NK-1, having sodium chloride optics; and ultraviolet spectra on a Cary spectrophotometer, Model 11-M. Specific rotations were determined in a 2-dm polarimeter tube.

4-(D-arabino-Tetrahydroxybutyl)-4-thiazoline-2-thione (**1**). — Modification of the method of Zemplén *et al.*[1] provided **1**. To a stirred solution of D-fructose (1,080 g, 6 moles) and potassium thiocyanate (600 g, 6.18 moles) in water (240 ml) was added hydrochloric acid (7.2 moles), in portions during 30–40 min, with cooling to 20–30°. The mixture was cooled for 30 min longer, nucleated with **1**, and kept overnight at room temperature; solids were precipitated. Crushed ice (500 g) was added, and the mixture was stirred for 2 h in an ice bath. The solids remaining, which were collected and rinsed with cold water, weighed 42 g. (Oxazolidine-2-thiones can be separated by concentration of the filtrate[2].) Excess sodium carbonate was added, with stirring, to a suspension of the crude product in water (400 ml), and the resultant solution was stirred with powdered charcoal, and filtered. Acidification with hydrochloric acid precipitated **1**. Recrystallized from water (650 ml), the product weighed 28 g (2%); m.p. 218° (lit.[1] m.p. 218°); λ_{max}^{MeOH} 317 nm (ε 13,700); λ_{max} (aqueous sodium hydroxide, pH 11) 293 nm (ε 9,600); pK_a^{25} 7.05–7.10 (0.001N solution); solubility 0.25 g/100 ml of water at 25°, and 5 g/100 ml at 100°; $[\alpha]_D^{25}$ −77 ±2° (*c* 1, *N,N*-dimethylformamide) [lit.[2] $[\alpha]_D^{20}$ −75° (*c* 1, *N,N*-dimethylformamide)].

2-(Carboxymethyl)thio-4-(D-arabino-tetrahydroxybutyl)thiazole (**2**). — To a boiling mixture of **1** (23.7 g, 0.1 mole), sodium chloroacetate (12.8 g, 0.11 mole), and water (130 ml) was added sodium hydrogen carbonate (10 g) in portions. The resulting solution was boiled for 5 min, cooled, and acidified with hydrochloric acid to give **2**, yield 27.5 g (93%), m.p. 151°. Recrystallized from water, it had λ_{max}^{MeOH} 277 nm (ε 6,600), $[\alpha]_D^{25}$ −143 ±1° (*c* 1, *N,N*-dimethylformamide).

Anal. Calc. for $C_9H_{13}NO_6S_2$: C, 36.6; H, 4.4; N, 4.7; S, 21.7. Found: C, 36.5; H, 4.5; N, 4.8; S, 21.5.

2-(Diphenylmethyl)thio-4-(D-arabino-tetrahydroxybutyl)thiazole (**3**). — A solution of **1** (23.7 g, 0.1 mole), chlorodiphenylmethane (20.3 g, 0.1 mole), and pyridine (175 ml) was kept for 2 days at room temperature. After removal of most of the pyridine *in vacuo*, with warming, and dilution to 1 liter with an excess of aqueous hydrochloric acid, precipitation occurred, to give 14.2 g of crude **3**. (Concentration of the filtrate yielded 10.7 g of unreacted **1**). Two recrystallizations from ethanol gave pure **3**, yield 11 g (27%), m.p. 157°. λ_{max}^{MeOH} 282 nm (ε 7,500); $[\alpha]_D^{25}$ −52 ±1 (*c* 1, *N,N*-dimethylformamide).

Anal. Calc. for $C_{20}H_{21}NO_4S_2$: C, 59.5; H, 5.2; N, 3.5; S, 15.9. Found: C, 59.8; H, 5.4; N, 3.5; S, 15.6.

4-[D-arabino-2,3,4-Trihydroxy-1-(trityloxy)butyl]-4-thiazoline-2-thione (**4**). — Treatment of **1** with chlorotriphenylmethane for 7 days by the preceding method

gave **4** (25%); recrystallized from chloroform–petroleum ether, it had m.p. (unsharp) *ca.* 100°; $\lambda_{max}^{HCONMe_2}$ 328 nm (ε 12,300); $[\alpha]_D^{25}$ −37 ±2° (*c* 1, *N,N*-dimethyl-formamide).

Anal. Calc. for $C_{26}H_{25}NO_4S_2$: C, 65.1; H, 5.3; N, 2.9; S, 13.4. Found: C, 65.2; H, 5.4; N, 2.7; S, 13.2.

2-Acetylthio-4-(D-arabino-*tetraacetoxybutyl)thiazole* (**5**). — A solution of **1** (10.8 g, 0.046 mole) and potassium acetate (12 g) in acetic anhydride (120 ml) was kept for 1 h at 100°, and, after concentration *in vacuo*, the mixture was poured into water (3 liters) to yield 15 g (81%) of solid, m.p. 94–96°. Several recrystallizations from petroleum ether gave 7 g of **5**, m.p. 97–98°.

Anal. Calc. for $C_{17}H_{21}NO_9S_2$: C, 45.7; H, 4.9; N, 3.2; S, 14.6. Found: C, 45.8; H, 4.6; N, 3.2; S, 14.3.

2,2'-Dithiobis[(*4-*D-arabino-*tetrahydroxybutyl)thiazole*] (**6**). — Oxidation of **1** (30 g, 0.127 mole) in boiling water (2.5 liters) by the iodine method[18] gave **6**, yield 27.7 g (82%), m.p. 218° (alone; but 203–205° if mixed with **1**).

Anal. Calc. for $C_{14}H_{20}N_2O_8S_4$: C, 35.6; H, 4.3; N, 5.9; S, 27.1. Found: C, 35.8; H, 4.3; N, 6.2; S, 27.5.

2,2'Dithiobis[(*4-*D-arabino-*2,3,4-trihydroxy-1-trityloxybutyl)thiazole*] (**7**) — Tritylation of **6** (10.8 g, 0.023 mole) by the method just described gave **7** yield 12.9 g (59%), after repeated recrystallization from benzene–petroleum ether, m.p. (unsharp) at *ca.* 84°; λ_{max}^{MeOH} 268 nm (ε 10,900); $[\alpha]_D^{25}$ −24 ±2° (*c*, 1 *N,N*-dimethylformamide).

Anal. Calc. for $C_{52}H_{48}N_2O_8S_4$: C, 65.2; H, 5.1; N, 2.9; S, 13.4. Found: C, 65.6; H, 5.4; N, 2.8; S, 13.2.

4-(D-arabino-*Tetrahydroxybutyl)thiazole* (**8**). — A mixture of **6** (40 g, 0.085 mole) sodium hydrogen carbonate (22 g), and water (1 liter) was boiled until a solution formed. The solution was cooled, and acidified with hydrochloric acid. Collection of **1** (in two crops) gave 32.7 g (82%). Concentration of the filtrate to dryness, and extraction with hot ethanol gave **8**, yield 4.5 g (13%), m.p. 172–173°; recrystallized from ethanol it had λ_{max}^{MeOH} 242 nm (ε 3,300); $[\alpha]_D^{25}$ −35 ±1° (*c* 1, *N,N*-dimethylformamide).

Anal. Calc. for $C_7H_{11}NO_4S$: C, 41.0; H, 5.4; N, 6.8; S, 15.6. Found: C, 41.2; H, 5.3; N, 6.6; S, 15.2.

2,2'-Dithiobis(4-thiazolecarboxaldehyde) (**9**). — Oxidation of **1** (25 g, 0.11 mole) by the sodium metaperiodate method[19] gave **9**, yield 10.9 g (72%). Recrystallized from xylene, it had m.p. 193°, λ_{max}^{KBr} 5.96 (conjugated CHO), 6.2 μm (C=C).

Anal. Calc. for $C_8H_4N_2O_2S_4$: C, 33.3; H, 1.4; N, 9.7; S, 44.5. Found: C, 33.5; H, 1.8; N, 9.9; S, 44.2.

Treatment of **6** by the same procedure gave similar results.

The thiosemicarbazone of **9** melted at 245° after recrystallization from pyridine–water.

Anal. Calc. for $C_{10}H_{10}N_8S_6$: N, 25.8. Found: N, 26.0.

4-Thiazoline-2-thione-4-carboxaldehyde (**10**). — Treatment of **9** (5.8 g, 0.02 mole) with sodium carbonate (3.5 g) and water (8 ml) gave **10**; yield 2.8 g (48%). Twice

recrystallized from water, it had m.p. 173°, λ_{max}^{KBr} 5.98 (conjugated CHO), 6.2 μm (C=C); λ_{max}^{MeOH} 313 nm (ε 14,700).

Anal. Calc. for $C_4H_3NOS_2$: C, 33.1; H, 2.1; N, 9.6; S, 44.2. Found: C, 33.2; H, 2.1; N, 9.4; S, 43.8.

Oxidation of **10** with iodine[18] gave **9**.

The oxime of **10** melted at 192° after recrystallization from methanol–water.

Anal. Calc. for $C_4H_4N_2OS_2$: N, 17.5. Found: N, 17.3.

4-(Hydroxymethyl)-4-thiazoline-2-thione (**11**). — A solution of **10** (3.2 g, 0.022 mole) in methanol (35 ml) was treated with a solution of potassium borohydride (1.2 g, 0.022 mole) in water (5 ml), and the solution was concentrated *in vacuo*. Treatment with excess hydrochloric acid gave **11**, yield 1.6 g (50%). Twice recrystallized from water, it had m.p. 121°; λ_{max}^{KBr} 2.95–3.6 (OH, NH, hydrogen bonding, CH), 6.23 μm (C=C); λ_{max}^{MeOH} 316 nm (ε 14,800).

Anal. Calc. for $C_4H_5NOS_2$: C, 32.6; H, 3.4; N, 9.5; S, 43.6. Found: C, 33.0; H, 3.6; N, 9.3; S, 44.0.

2,2'Dithiobis[4-(hydroxymethyl)thiazole] (**12**).—Oxidation of **11** (30 g, 0.22 mole) by the ammonium persulfate method[20] gave **12**; yield 19.5 g (61%), m.p. 115° after recrystallization from water; λ_{max}^{KBr} 2.90 μm (OH); λ_{max}^{MeOH} 266 nm (ε 9,400).

Anal. Calc. for $C_8H_8N_2O_2S_4$: C, 32.9; H, 2.8; N, 9.6; S, 43.9. Found: C, 32.6; H, 2.6; N, 9.5; S, 43.9.

Treatment of **12** by the method just given for the alkaline cleavage of disulfides gave **11**.

4-(Hydroxymethyl)-3-(3-oxobutyl)-4-thiazoline-2-thione (**13**). — The conjugate addition[21] of **11** (44.2 g, 0.3 mole) to 3-buten-2-one (51.6 g, 0.6 mole), catalyzed by sodium methoxide, gave **13**, yield 24 g (52%); recrystallized from methanol, m.p. 114°; λ_{max}^{KBr} 3.02 (OH), 5.88 μm (C=O); λ_{max}^{MeOH} 318 nm (ε 13,900).

Anal. Calc. for $C_8H_{11}NO_2S_2$: C, 44.2; H, 5.1; N, 6.4; S, 29.5. Found: C, 44.3; H, 5.2; N, 6.3; S, 29.2.

4-(Acetoxymethyl)-4-hydroxythiazolidine-2-thione (**14**). — Treatment of 1-acetoxy-3-chloro-2-propanone[22] (120.5 g, 0.8 mole) plus ammonium dithiocarbamate (98 g, 0.89 mole) by the method[11] for preparing 4-hydroxythiazolidines gave **14**, yield 152 g (91%), m.p. 114° after recrystallization from methanol; λ_{max}^{KBr} 3.05-3.08 (OH, NH), 5.75 μm (OAc); λ_{max}^{MeOH} 242, 277 nm (ε 6,600; 14,600).

Anal. Calc. for $C_6H_9NO_3S_2$: C, 34.8; H, 4.4; N, 6.8; S, 30.9. Found: C, 35.0; H, 4.1; N, 6.7; S, 30.7.

4-(Acetoxymethyl)-4-thiazoline-2-thione (**15**). — Recrystallization of **14** from water containing a drop of hydrochloric acid gave **15** in 80% yield; m.p. 80°; λ_{max}^{MeOH} 317 nm (ε 14,800).

Anal. Calc. for $C_6H_7NO_2S_2$: C, 38.1; H, 3.7; N, 7.4; S, 33.4. Found: C, 37.7; H, 3.8; N, 7.4; S, 33.3.

Treatment of **15** (114 g, 0.6 mole) with potassium hydroxide (114 g) and water (570 ml) for 5 min at 100°, and subsequent acidification at 5°, gave **11**, yield 71 g (80%).

4-Hydroxy-4-(D-arabino-*tetraacetoxybutyl)thiazolidine-2-thione* (**16**). — Treat-

ment of 3,4,5,6-tetra-*O*-acetyl-1-bromo-1-deoxy-D-*arabino*-hexulose[17] (14.7 g, 0.036 mole) with ammonium dithiocarbamate (4.4 g, 0.04 mole) by the method[11] for preparing 4-hydroxythiazolidines gave **16**, yield 13.1 g (86%), m.p. 145°, unchanged on recrystallization from ether; λ_{max}^{MeOH} 242, 278 nm (ε 5,800; 12,200).

Anal. Calc. for $C_{15}H_{21}NO_9S_2$: C, 42.5; H, 5.0; N, 3.3; S, 15.1. Found: C, 42.8; H, 4.8; N, 3.2; S, 15.2.

Treatment of **16** (8.3 g, 0.02 mole) by the alkaline hydrolytic method gave **11**; yield 4.2 g (90%).

4-(D-arabino-Tetraacetoxybutyl)-4-thiazoline-2-thione (**17**). — Recrystallization of **16** from water containing a drop of hydrochloric acid gave **17**, yield 69%, m.p. 168° after recrystallization from ether (lit.[3] m.p. 169–170°); λ_{max}^{KBr} 3.2–3.6 (NH, CH), 5.78 μm (OAc); λ_{max}^{MeOH} 316 nm (ε 12,200); $[\alpha]_D^{24}$ −79 ±1° (*c* 2, chloroform) [lit.[3] $[\alpha]_D^{20}$ −37° (*c* 1, *N*,*N*-dimethylformamide)].

Anal. Calc. for $C_{15}H_{19}NO_8S_2$: C, 44.4; H, 4.7; N, 3.5; S, 15.8. Found: C, 44.4; H, 4.3; N, 3.4; S, 15.6.

Alternatively, heating **16** to 195° gave **17** in quantitative yield.

Treatment of **17** (8.1 g, 0.02 mole) by the alkaline hydrolytic method gave **11**, yield 3.7 g (78%).

2,2'-Dithiobis [(*4*-D-arabino-*tetraacetoxybutyl*)*thiazole*] (**18**). — Oxidation of **17** (3.9 g, 0.096 mole) by the iodine method[18] gave **18**, yield 3.4 g (88%), m.p. 145° (recrystallized from ether); λ_{max}^{MeOH} 266 nm (ε 9,100); $[\alpha]_D^{24}$ −13° (*c* 4, chloroform).

Anal. Calc. for $C_{30}H_{36}N_2O_{16}S_4$: C, 44.5; H, 4.5; N, 3.5; S, 15.9. Found: C, 44.9; H, 4.8; N, 3.2; S, 15.5.

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O-(ALKYL- AND ARYL-OXYTHIOCARBONYL) SUGAR DERIVATIVES*

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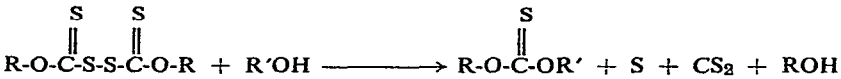
ABSTRACT

Bis(1,2:5,6-di-*O*-isopropylidene-3-*O*-thiocarbonyl-α-D-glucofuranose) disulfide (**1**) in pyridine undergoes a fragmentation reaction when treated with excess methyl, ethyl, propyl, or butyl alcohols, or phenol, to give the corresponding *O*-oxythiocarbonyl derivatives (**2–6**). A faster reaction and higher yield result when iodine is included in the pyridine solution. The oxythiocarbonyl compounds are stable when distilled (near 190°) under diminished pressure. Selective, acid hydrolysis of 3-*O*-(ethoxythiocarbonyl)-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**3**) gave 3-*O*-(ethoxythiocarbonyl)-1,2-*O*-isopropylidene-α-D-glucofuranose (**10**), which rearranged, on standing in triethylamine, to 1,2-*O*-isopropylidene-α-D-glucofuranose 5,6-thionocarbonate (**12**). Oxidation of **3** with lead tetraacetate or silver nitrate gave the corresponding 3-*O*-ethoxycarbonyl derivative (**8**), whereas reduction of **3** with Raney nickel gave 3-*O*-(ethoxymethylene)-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**11**).

INTRODUCTION

Previous studies showed that bis(1,2-*O*-isopropylidene-3-*O*-thiocarbonyl-α-D-glucofuranose) disulfide undergoes intramolecular displacement–fragmentation in pyridine to yield elemental sulfur, carbon disulfide, 1,2-*O*-isopropylidene-α-D-glucofuranose, and 1,2-*O*-isopropylidene-α-D-glucofuranose 5,6-thionocarbonate[1]. Subsequently, other suitably protected sugar derivatives, possessing a hydroxyl group vicinal to a bis(*O*-thiocarbonyl) disulfide group, were shown[2,3] to undergo the same process in pyridine. We now report studies undertaken to ascertain whether the bis(*O*-thiocarbonyl) disulfide group would undergo intermolecular reaction when the hydroxyl group was provided by added alcohols. This reaction, represented as follows,



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would lead to the formation of *O*-(alkyloxythiocarbonyl) compounds, representatives of a new type* of sugar derivative.

When bis(1,2:5,6-di-*O*-isopropylidene-3-*O*-thiocarbonyl-α-D-glucofuranose) disulfide (**1**) was dissolved in pyridine, and treated with methyl, ethyl, propyl, or butyl alcohols or phenol, the corresponding *O*-oxythiocarbonyl derivatives (**2**–**6**) (see Scheme I) were obtained, in addition to elemental sulfur, carbon disulfide, and 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**7**). The same kind of fragmentation occurs when bis(1,2:3,4-di-*O*-isopropylidene-6-*O*-thiocarbonyl-α-D-galactopyranose) disulfide is dissolved in pyridine, and treated with ethanol.

The structure of these new derivatives is consistent with their elemental analyses, molecular weight (in the case of compound **3**), infrared spectra (absorption maxima at 7.8 μm for O(C=S)O), and ultraviolet spectra [A_{230}^{MeOH} about ε 9,500; bis(1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose) thionocarbonate has A_{230}^{MeOH} ε 9,600]. Analysis of the n.m.r. spectra of **2**–**6** supports the proposed structures. For example, the n.m.r. spectrum of **6** shows a low-field doublet (assigned to the anomeric proton) at τ 4.0 with a coupling constant ($J_{1,2}$) of 4.0 Hz; a doublet centered at τ 5.2 with a coupling constant ($J_{1,2}$) of 4.0 Hz is assigned to H-2; a doublet centered at τ 4.3 with a coupling constant of 2.5 Hz is assigned to H-3; a multiplet centered at τ 5.7 is assigned to H-4 and H-5; and a multiplet centered at τ 5.9 is assigned to the two nonequivalent protons on C-6. The four methyl groups of the isopropylidene groups give rise to four partially overlapping singlets centered at τ 8.4, and the signal of the phenyl group appears as a downfield multiplet centered at τ 2.7. These assignments are in general agreement with the positional assignments reported by Horton and Prihar[4] for 3-*O*-(dimethylthiocarbamoyl)-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose.

The course of the displacement–fragmentation reaction was followed by thin-layer chromatography (t.l.c.), which showed that the rate of formation of the alkoxythiocarbonates is considerably less than the rate of formation of intramolecular, cyclic thionocarbonates. Even though an excess of alcohol was used, production of the former derivatives was complete after 16 h for **2**, and 100 h for **3**, **4**, and **5**. Under similar reaction conditions, formation of the cyclic thionocarbonate was complete[2] in 30 min.

The rates of formation of **2**–**6**, and the yields of **2**–**5**, were increased substantially when iodine was added to the reaction mixtures. For example, the conversion of 1 mole of **1** into **2**, in the presence of iodine, was complete in 3 h, to yield 1.5 moles of **2**, as compared to a reaction period of 16 h and a yield of 0.9 mole of **2** without iodine.

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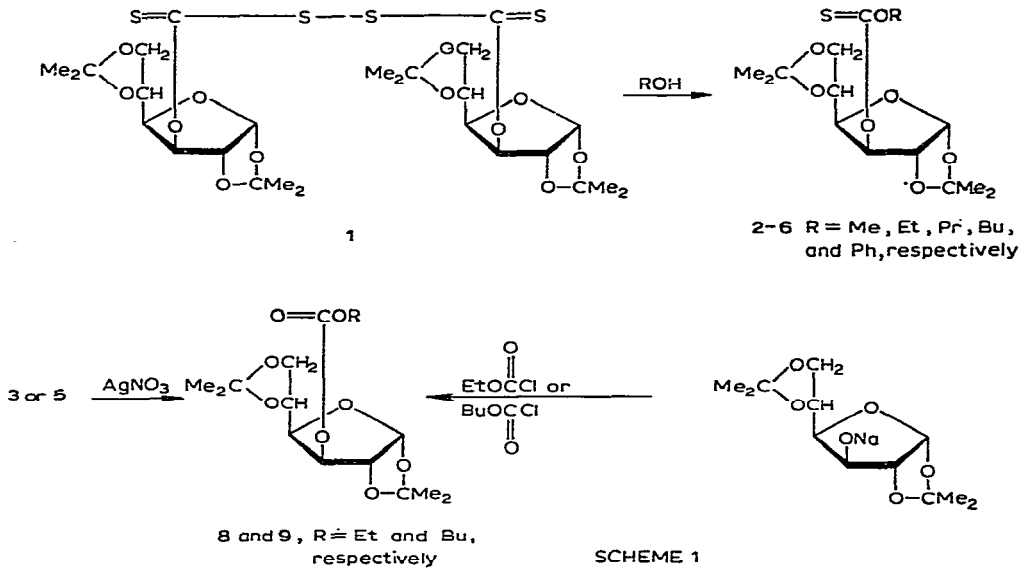
*According to the nomenclature of carbonate and thiocarbonate derivatives proposed by L. Hough, J. E. Priddle, and R. S. Theobald in *Advan. Carbohydrate Chem.*, 15 (1960) 99, sugars containing a thionocarbonate group are those having the group $XO\overset{\overset{S}{\|}}{C}OX'$, where X and X′ are carbohydrate residues. Sugars containing *O*-alkyl(or aryl -oxythiocarbonyl groups are those having the group $XO\overset{\overset{S}{\|}}{C}OR$, where X is a carbohydrate residue and R is an alkyl or aryl group.



In addition to **2–5**, elemental sulfur and pyridine hydroiodide were also isolated. It is possible that the free iodine or pyridine diiodide (which is presumed to be formed by reaction of iodine with pyridine in chloroform solution[5]), or both, reacts with bis(*O*-thiocarbonyl) disulfide derivatives to give a thiocarbonate iodide derivative, which further reacts with the alcohol to give an alkyloxythiocarbonate and pyridine hydriodide. A thiocarbonyl chloride derivative was isolated[6] when bis(di-*O*-ethylthiocarbonyl) disulfide was treated with chlorine or chlorine–binding agents in carbon tetrachloride. The role of iodine in increasing the yields of **2–5** might also be explained by recoupling of any xanthate ion formed during the rearrangement of **1**. Iodine may also be used to increase the yield of the cyclic thionocarbonate formed on rearrangement of sugars that possess the bis(*O*-thiocarbonyl) disulfide group and a vicinal hydroxyl group. For example, one mole of bis(methyl 4,6-*O*-benzylidene-2-*O*-thiocarbonyl-α-D-glucopyranoside) disulfide[3] gives 1.5 moles of methyl 4,6-*O*-benzylidene-α-D-glucopyranoside 2,3-thionocarbonate in the presence of iodine, and 1.0 mole in its absence.

PROPERTIES

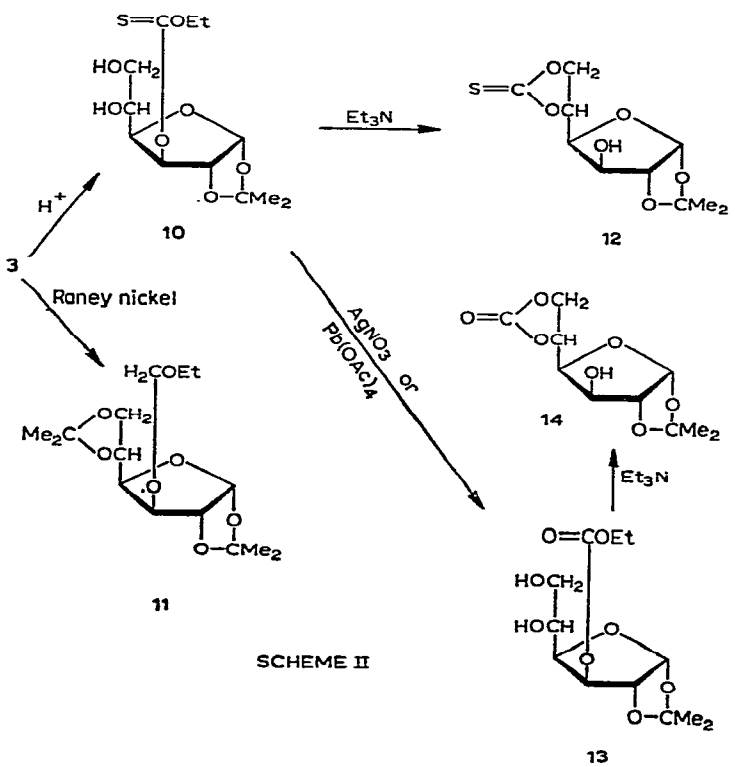
The oxythiocarbonyl compounds (**2–6**) undergo no detectable change when stored for several months at 25°. When heated to 190° under diminished pressure, the compounds distil, and are recovered in high yield.

The thiocarbonyl group in **3** or **5** is readily converted into the corresponding carbonyl group by treatment with lead tetraacetate or silver nitrate (see Scheme I). Compound **3** rapidly consumed 1.9 moles of lead tetraacetate per mole, and gave 3-*O*-(ethoxycarbonyl)-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**8**) in 85% yield.

These results are similar to those reported[7] for the oxidation of bis(1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose) 3,3′-thionocarbonate to the corresponding 3,3′-carbonate. When **3** was treated with silver nitrate, **8** was formed in almost quantitative yield. Similar treatment of **5** gave **9**. Compounds **8** and **9** were readily prepared by reaction of the sodium alcoholate of **7** with ethyl or butyl chloroformate, respectively.

As expected, the oxythiocarbonyl groups are stable to mild hydrolysis by acid. The 5,6-*O*-isopropylidene group was selectively removed from **3** with acid, to give 3-*O*-(ethoxythiocarbonyl)-1,2-*O*-isopropylidene-α-D-glucofuranose (**10**). The latter did not undergo change when treated with pyridine, but decomposed in triethylamine to form 1,2-*O*-isopropylidene-α-D-glucofuranose 5,6-thionocarbonate (**12**) (see Scheme II). Similarly, the corresponding carbonyl derivative (**13**) of **10** decomposed in triethylamine, to give 1,2-*O*-isopropylidene-α-D-glucofuranose 5,6-carbonate (**14**).

Compound **3** underwent reductive desulfurization when refluxed in ethanol containing activated Raney nickel. T.l.c. of the product revealed two components in approximately equal amounts. The slower-moving component was identified as 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**7**), and the other as 3-*O*-(ethoxymethylene)-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**11**). When the reaction was conducted at 25° under hydrogen (500 lb. in^{-2}), **11** was obtained in a higher yield, and only traces of **7** were found.



SCHEME II

The structure proposed for **11** is consistent with its elemental analysis, ethoxyl content, molecular weight, and n.m.r. spectrum. The last shows a singlet centered at τ 5.25 for the two protons of the OCH_2O group, and a quartet centered at τ 6.3 for the two protons of the methylene in the ethoxy group. The ethoxymethylene derivative (**11**) was hydrolyzed with acid, and gave a glucose and formaldehyde; this type of derivative has been shown to be hydrolyzed readily to yield formaldehyde (from the methylene group) and the corresponding alcohol (from the alkoxyl group)[8].

EXPERIMENTAL

Unless otherwise stated, all of the reactions and measurements were conducted at 25°. Melting points were determined in a Thomas–Kofler* melting-point apparatus, and are uncorrected. Optical rotations were measured in chloroform within the concentration range of 2–4%. I.r. spectra were recorded, with a Perkin–Elmer Model 137 spectrophotometer having silver chloride optics, as Nujol mulls or films, and the u. v. spectra were recorded with a Perkin–Elmer Model 202 spectrophotometer, with methanol as the solvent. N. m. r. measurements were made in chloroform by means of a Varian A-60 n.m.r. spectrometer, with tetramethylsilane (τ 10.00) as the internal, reference standard. Pyridine, methanol, ethanol, propyl alcohol, and butyl alcohol were certified grades, and the chloroform was of spectroscopic grade. For t.l.c., Silica Gel G was used as the adsorbent, 9:1 (v/v) carbon disulfide–ethyl acetate as the solvent, and 19:1 (v/v) methanol–sulfuric acid as the spray reagent. Adsorbosil was obtained from Applied Science Laboratories, Inc., State College, Pennsylvania. Fractionations were performed in a 1.8 × 20 cm column. Bis(1,2:5,6-di-*O*-isopropylidene-3-*O*-thiocarbonyl-α-D-glucofuranose) disulfide (**1**) was prepared according to a procedure reported[1] earlier; m.p. 129–130°, λ_{max} 280 (ε 8,100) and 230 nm (ε 17,200). Bis(1,2:3,4-di-*O*-isopropylidene-6-*O*-thiocarbonyl-α-D-galactopyranose) disulfide was also prepared according to a reported[7] procedure; m.p. 133–134°, λ_{max} 288 (ε 8,900) and 240 nm (ε 18,900).

1,2:5,6-Di-O-isopropylidene-3-O-(methoxythiocarbonyl)-α-D-glucofuranose (**2**). — (*a*) A solution of **1** (1.0 g) in pyridine (10 ml) was treated with methanol (10 ml). The reaction, which was monitored by t.l.c., was complete in 16 h. The mixture was then evaporated to a yellow syrup (mixed with crystals identified as sulfur by elemental analysis). The syrup was dissolved in about 2 ml of hexane, and adsorbed onto Adsorbosil for 10 min. Elution with hexane (100 ml) removed the elemental sulfur; elution with hexane containing 5% of acetone (150 ml), followed by evaporation, gave compound **2** (450 mg), as a yellowish syrup that crystallized on being kept for a few days; m.p. 69–71°, λ_{max} 230 nm (ε 9,000), $[\alpha]_D$ −45° (chloroform).

Anal. Calc. for $C_{19}H_{22}O_7S$: C, 50.3; H, 6.6; S, 9.6. Found: C, 50.1; H, 6.5; S, 10.0.

*Mention of firm names or trade products does not imply that they are endorsed or recommended by the U. S. Department of Agriculture over other firms or similar products not named.

Elution with acetone removed 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**7**), which was subsequently crystallized from hexane.

In a separate experiment, carbon disulfide was isolated and identified as reported earlier[1].

(*b*) A solution of **1** (1.0 g) in pyridine (10 ml) was treated with methanol (10 ml) and a saturated solution of iodine in chloroform (8 ml). The reaction was complete in 3 h, after which time the mixture was evaporated to a yellow syrup mixed with a crystalline compound. The syrup, which consisted mainly of **2** (t.l.c.), was extracted with hexane, and purified by adsorption on Adsorbosil as above, to yield **2** (750 mg). The crystalline compound was identified as pyridine hydriodide by comparison with an authentic sample.

3-O-(Ethoxythiocarbonyl)-1,2:5,6-di-O-isopropylidene-α-D-glucofuranose (**3**). — (*a*) A solution of **1** (1.0 g) in pyridine (10 ml) was treated with ethanol (10 ml). After the reaction was complete (100 h), compound **3** was isolated by the procedure used for the preparation of **2**, part (*a*); $[\alpha]_D$ −45° (chloroform), λ_{max} 230 nm (ε 9,000).

Anal. Calc. for $C_{15}H_{24}O_7S$: C, 51.6; H, 6.9; S, 9.2; mol wt., 348. Found: C, 51.0; H, 6.9; S, 9.8; mol wt., 340 (Rast in camphor).

(*b*) A solution of **1** (1.0 g) in pyridine (10 ml) was treated with ethanol (10 ml) and a saturated solution of iodine in chloroform (8 ml). After the reaction was complete (12 h), **3** (665 mg) was isolated by the procedure used for **2**, part (*a*).

1,2:5,6-Di-O-isopropylidene-3-O-(propoxythiocarbonyl)-α-D-glucofuranose (**4**). — (*a*) A solution of **1** (1.0 g) in pyridine (10 ml) was treated with propyl alcohol (10 ml). After the reaction was complete (100 h), compound **4** (345 mg) was isolated by the procedure used for **2**, part (*a*); $[\alpha]_D$ −39° (chloroform), λ_{max} 230 nm (ε 9,200).

Anal. Calc. for $C_{16}H_{26}O_7S$: C, 53.0; H, 7.2; S, 8.9. Found: C, 52.9; H, 7.2; S, 8.9.

(*b*) A solution of **1** (1.0 g) in pyridine (10 ml) was treated with propyl alcohol (10 ml) and a saturated solution of iodine in chloroform (8 ml). After the reaction was complete (12 h), compound **4** (620 mg) was isolated by the procedure used for **2**, part (*a*).

3-O-(Butoxythiocarbonyl)-1,2:5,6-di-O-isopropylidene-α-D-glucofuranose (**5**). — (*a*) A solution of **1** (1.0 g) in pyridine (10 ml) was treated with butyl alcohol (10 ml). After the reaction was complete (100 h), compound **5** (410 mg) was isolated, $[\alpha]_D$ −34° (chloroform), λ_{max} 230 nm (ε 9,150).

Anal. Calc. for $C_{17}H_{28}O_7S$: C, 54.3; H,7.5; S, 8.5. Found: C, 54.3; H, 7.4;S, 8.7.

(*b*) A solution of **1** (1.0 g) in pyridine (10 ml) was treated with butyl alcohol (10 ml) and a saturated solution of iodine in chloroform (8 ml). After the reaction was complete (14 h), compound **5** (610 mg) was isolated by the procedure used for **2**, part (*a*).

1,2:5,6-Di-O-isopropylidene-3-O-(phenoxythiocarbonyl)-α-D-glucofuranose (**6**). — (*a*) A solution of **1** (1.0 g) in pyridine (10 ml) was treated with phenol (10 g). After 16 h, the pyridine and excess of phenol were evaporated by heating the mixture on a steam bath while passing a stream of hot air through the mixture. T.l.c. showed two spots,

one of which corresponded to 7. Compound **6** (200–300 mg) was isolated by the procedure used for **2**, part (*a*); m.p. 103°, $[\alpha]_D$ −43° (chloroform), λ_{max} 237 nm (ε 7,600).

Anal. Calc. for $C_{19}H_{24}O_7S$: C, 57.6; H, 6.1; S, 8.1. Found: C, 57.3; H, 6.2; S, 8.1.

(*b*) A solution of **1** (1.0 g) in pyridine (10 ml) was treated with phenol (10 g) and a saturated solution of iodine in chloroform (8 ml). After 8 h, anhydrous ether (50 ml) was added, and the resultant, fine precipitate was filtered off. The filtrate was concentrated somewhat, and water (200 ml) was added, forming two layers. After the mixture had been warmed on a steam bath for a few min, the upper layer was decanted, and the lower layer was evaporated as in part (*a*), to give crude **6**, which was purified as for **2**, part (*a*); yield 200–300 mg.

6-O-(Ethoxythiocarbonyl)-1,2:3,4-di-O-isopropylidene-α-D-galactopyranose. — (*a*) A solution of bis(1,2:3,4-di-*O*-isopropylidene-6-*O*-thiocarbonyl-α-D-galactopyranose) disulfide (1.0 g) in pyridine (10 ml) was treated with ethanol (10 ml). After the reaction was complete (100 h), the title compound (400 mg) was isolated by the procedure used for **2**, part (*a*), $[\alpha]_D$ −60° (chloroform), λ_{max} 230 nm (ε 9,000).

Anal. Calc. for $C_{15}H_{24}O_7S$: C, 51.6; H, 6.9; S, 9.2. Found: C, 52.0; H, 6.9; S, 8.7.

(*b*) A solution of bis(1,2:3,4-di-*O*-isopropylidene-6-*O*-thiocarbonyl-α-D-galactopyranose) disulfide (1.0 g) in pyridine (10 ml) was treated with ethanol (10 ml) and a saturated solution of iodine in chloroform (8 ml). After 16 h, the title compound (640 mg) was isolated by the procedure used for **2**, part (*a*).

Methyl 4,6-O-benzylidene-α-D-glucopyranoside 2,3-thionocarbonate. — A solution of bis(methyl 4,6-*O*-benzylidene-2-*O*-thiocarbonyl-α-D-glucopyranoside) disulfide[3] (0.76 g) in pyridine (8 ml) was treated with a saturated solution of iodine in chloroform (8 ml). After 2 h, the solution was evaporated and the resultant syrup was adsorbed on Adsorbosil, which was then eluted with chloroform (600 ml). Evaporation of the eluate gave the title compound, which was crystallized from carbon disulfide (yield 0.51 g), and identified by t.l.c. and mixed melting point with an authentic sample[3].

3-O-(Ethoxycarbonyl)-1,2:5,6-di-O-isopropylidene-α-D-glucofuranose (**8**). — (*a*) 1,2:5,6-Di-*O*-isopropylidene-α-D-glucofuranose (5.0 g) was dissolved in 75 ml of anhydrous ether, and an excess of sodium was added. After the mixture had been kept for 16 h, the ether solution was decanted, and treated with ethyl chloroformate (1.6 ml). A fine precipitate, presumably sodium chloride, formed immediately. The suspension was kept for 10 min, and filtered, and the filtrate was evaporated to a syrup that crystallized from aqueous acetone to yield compound **8** (4.0 g); m.p. 73°, $[\alpha]_D$ −38° (chloroform). The compound was homogeneous by t.l.c.

Anal. Calc. for $C_{15}H_{24}O_8$: C, 54.3; H, 7.3. Found: C, 54.2; H, 7.4.

(*b*) The conversion of compound **3** into **8** was effected by treating **3** with silver nitrate in the presence of barium carbonate, according to a procedure reported[3] earlier. The yield of **8** was almost quantitative.

3-O-(Butoxycarbonyl)-1,2:5,6-di-O-isopropylidene-α-D-glucofuranose (**9**). — (*a*)

Compound **9** was prepared in 70% yield by essentially the procedure used for **8**, part (*a*), but with butyl chloroformate instead of ethyl chloroformate; m.p. 37–38°, $[\alpha]_D$ −35° (chloroform).

Anal. Calc. for $C_{17}H_{28}O_8$: C, 56.5; H, 7.9. Found: C, 56.6; H, 7.5.

(*b*) Reaction of **5** under the conditions used for preparing **8** (part (*b*)) gave **9** in almost quantitative yield.

3-O-(Ethoxythiocarbonyl)-1,2-O-isopropylidene-α-D-glucofuranose (**10**). — To a solution of 1 g of **3** in 50 ml of acetone was slowly added, with stirring, 10 ml of 5N hydrochloric acid. After the mixture had been kept for 2.5 h, most of the acetone was evaporated off under diminished pressure (until a slightly turbid solution resulted). The solution was diluted with water (100 ml), and extracted with two 100-ml portions of ethyl ether, and the extract was dried (anhydrous magnesium sulfate), and evaporated to dryness, to yield **10** as a colorless syrup (0.55 g), $[\alpha]_D^{25}$ + 9.5° (chloroform), λ_{max} 230 nm (ε 8,100).

Anal. Calc. for $C_{12}H_{20}O_7S$: C, 46.8; H, 6.5; S, 10.4. Found: C, 46.6; H, 6.8; S, 10,5.

Except for the presence of a hydroxyl band, the i.r. spectrum of **10** was very similar to that of **3**. When a solution of **10** in pyridine was kept for a few days at room temperature, **10** was recovered unchanged, as revealed by its i.r. spectrum.

1,2-O-Isopropylidene-α-D-glucofuranose 5,6-thionocarbonate (**12**). — To a solution of **10** (900 mg) in a mixture of *p*-dioxane (2 ml) and ethanol (2 ml), triethylamine (2 ml) was added. On being kept for 18 h, crystals appeared, and these were filtered off and washed with a small volume of anhydrous ether, to yield **12** (239 mg); m.p. 206–208°. Additional thionocarbonate (260 mg) was obtained on evaporation of the filtrate, and addition of ether. The identity of this product was established by comparison with an authentic sample[1] of **12**.

1,2-O-Isopropylidene-α-D-glucofuranose 5,6-carbonate (**14**). — To a solution of **8** (1 g) in methanol (8 ml), N hydrochloric acid (2 ml) was added. The mixture was kept for 15 min at 60°, neutralized with silver carbonate, the suspension filtered, and the filtrate evaporated to a thick syrup, which was dissolved in a mixture of triethylamine (5 ml), *p*-dioxane (5 ml), and ethanol (5 ml). The title compound, which crystallized on standing for 24 h, was filtered off, and washed with water, to give **14** (350 mg); m.p. 228–229°. The i.r. spectrum was indistinguishable from that of an authentic sample[1] of **14**.

Lead tetraacetate oxidation of **3**. — The oxidation of **3** was performed by a procedure reported previously[7]. One mole of the compound consumed 1.9 moles of oxidant during 10 min, with no additional consumption during the next 3 h. The oxidized product (**8**) was isolated from the reaction mixture in 85% yield; m.p. and mixed m.p. with an authentic sample of **8**, 73–74°.

Reductive desulfurization of **3**. — Fresh, active Raney nickel was prepared according to Dominguez *et al.*[9]. A solution of **3** (1 g) in ethanol (100 ml) was refluxed with an excess of Raney nickel for 3 h. After filtration of the suspension, the filtrate was evaporated to a thin syrup which was dissolved in about 30 ml of hexane. On

slow evaporation of the hexane, a crystalline compound, mixed with some syrup, was obtained. The crystals (265 mg) were filtered off, after addition of a few ml of hexane, and were identified as **7** by comparison with an authentic sample. T.l.c. of the mother liquor showed mainly one component, which was identified as 3-*O*-(ethoxymethylene)-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**11**). The syrup obtained by evaporation of the mother liquor was triturated with water and extracted with hexane, to give pure **11** (405 mg).

Anal. Calc. for $C_{15}H_{26}O_7$: C, 56.5; H, 8.2; OEt, 14.1%; mol. wt., 318. Found: C, 56.3; H, 8.3; OEt, 13.6%; mol. wt., 321 (Rast in camphor).

When the reductive desulfurization reaction was performed for 3 h at 25° under a hydrogen pressure of 500 $lb.in^{-2}$, only traces of **7** were obtained, and pure **11** was isolated in high yield (750 mg).

Acid hydrolysis of **11**. — A suspension of **11** (20 mg) in N hydrochloric acid (10 ml) was heated for 30 min under reflux on a steam bath; a solution was obtained after 10–15 min. A 1-ml portion was withdrawn, diluted to 20 ml with water, and analyzed for formaldehyde with the 2,4-pentanedione reagent. The amount of formaldehyde in repeated experiments was 0.65–0.85 mole per mole of **11**. Paper chromatography of the hydrolyzate, with 10:4:3 (v/v) ethyl acetate–pyridine–water or 40:11:19 (v/v) butyl alcohol–ethanol–water as the solvent and silver nitrate–sodium hydroxide solution as the detecting reagent, revealed only the presence of a glucose.

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THE URONIC ACIDS IN A HYDROLYZATE OF SAPOTE GUM*

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ABSTRACT

The molar ratios of D-xylose, L-arabinose, D-glucuronic acid, and 4-*O*-methyl-D-glucuronic acid in the hydrolyzates of ten nodules of sapote gum obtained from two different commercial batches were very similar and averaged 2.2:1.0:0.42:0.58. A small proportion of nonacidic polysaccharide material was associated with the gum, and accounted for the trace quantities of galactose, glucose, and mannose detected in chromatograms of its hydrolyzate. Partial hydrolysis with acid gave two groups of acidic fragments, containing 4-*O*-methyl-D-glucuronic acid and D-glucuronic acid, respectively. Of these, the aldotriouronic acids, *O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-(1→2)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (**1**) and *O*-α-D-glucopyranosyluronic acid-(1→2)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (**2**), crystallized after isolation by paper chromatography. Compound **1** was identified by comparison with an authentic sample. Compound **2** yielded *O*-α-D-glucopyranosyluronic acid-(1→2)-D-xylopyranose on partial hydrolysis. Reduction of **2**, followed by methylation and methanolysis yielded a 1,2,3,5-tetra-*O*-methylxylitol, as well as other methanolysis products identical to those obtained from reduced **1** after methylation and methanolysis.

INTRODUCTION

In previous studies on the structure of sapote gum, Anderson and Ledbetter[1] found that partial hydrolysis with acid yielded mixtures of aldobiouronic and aldotriouronic acids which contain residues of D-glucopyranosyluronic acid and a monomethyl ether thereof, linked to D-xylopyranose residues. At a later date, White[2] found that (1) the main chain probably consists of (1→4)-linked D-xylopyranose residues, although D-glucopyranosyluronic acid residues and glycosyl (1→2) bonds might also be involved in the main chain; (2) D-glucopyranosyluronic acid, D-xylopyranose, and L-arabinofuranose residues are terminal, and are probably attached to *O*-2 of D-xylo-

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pyranose residues in the main chain; and (3) D-glucopyranosyluronic acid residues are joined by glycosyl (1→2) bonds to D-xylopyranose residues.

The objectives of the present study were the isolation, separation, and identification of some of the acidic oligosaccharides discovered by Anderson and Ledbetter[1]. A preliminary determination of the composition was made because of a report[3] that a sample thought to be sapote gum differed greatly from samples previously investigated[1,2].

Two samples of sapote gum were examined. One sample had been stored at The Institute of Paper Chemistry for some years, and was believed to be a portion of the sample studied by White[2]. A fresh sample of commercial sapote gum was obtained, in 1964, from Exportadora El Sol S. A., Apartado 2404, Lima, Peru. Both samples consisted of brown nodules, which had not been processed in any way since their collection, and were believed to have come from the species *Sapota achras*, which occurs in Peru.

The compositions of five nodules chosen at random from each of the two samples were determined, with the results shown in Table I. It is apparent that there were only small inter- and intra-sample variations in these compositions. Adsorption of a small portion of the gum from aqueous solution onto *O*-[(2-diethylamino)ethyl]-cellulose left in solution a small amount of neutral material, which was then hydrolyzed and the hydrolyzate inspected by paper chromatography; spots corresponding to

TABLE I
COMPOSITION OF THE ACID HYDROLYZATES OF SAPOTE GUM[a]

Nodule	*"Anhydro-D-galactose", %*	*"Anhydro-L-arabinose", %*	*"Anhydro-D-xylose", %*	*Methoxyl (as CH_3), %*	*"Uronic anhydride", %*	*Total, %*
1[b]	1.00	22.0	45.6	1.30	27.6	97.5
2	0.93	25.4	44.4	1.06	26.1	97.9
3	0.82	20.2	47.8	1.23	28.1	98.1
4	1.03	22.2	43.5	1.46	28.6	96.8
5	1.05	24.6	45.1	1.27	26.5	98.6
Average	0.97	22.9	45.3	1.26	27.4	97.8
1[c]	1.07	18.1	46.0	1.56	28.0	94.8
2	0.55	24.6	45.8	1.19	26.8	99.0
3	0.74	21.8	44.2	1.38	27.4	95.5
4	1.04	21.7	45.9	1.25	26.7	96.6
5	1.68	20.0	46.8	0.98	28.1	97.6
Average	1.02	21.2	45.7	1.27	27.4	96.7
Grand average	1.00	22.1	45.5	1.27	27.4	97.3

[a] All values are corrected for moisture and ash. [b]These nodules were obtained from Exportadora El Sol S.A., Apartado 2404, Lima, Peru. [c]These nodules were thought to have been a portion of the sample studied by White[2].

galactose, glucose, and mannose were observed. The quantity of this material was sufficient to suggest that all of the galactose in the hydrolyzates of sapote gum originated from this galactose-containing, polysaccharide contaminant. All additional experiments were conducted on the fresh sample of sapote gum.

Examination of the compounds present in the partial hydrolyzates (with acid) of sapote gum showed D-xylose and L-arabinose to be present. Eight additional spots, which were resolved by paper chromatography in solvent B, could be divided into two groups, depending on whether they yielded D-glucuronic acid or 4-*O*-methyl-D-glucuronic acid on complete hydrolysis with acid. One series of acids (the A series in Table II) corresponded in chromatographic mobility to 4-*O*-methyl-D-glucuronic acid (*A1*), *O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-(1→2)-D-xylopyranose (*A2*), *O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-(1→2)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (*A3*), and *O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-(1→2)-*O*-β-D-xylopyranosyl-(1→4)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (*A4*). Apart from D-glucuronic acid (*B1*), no controls were available for comparison with the other series of acidic fragments (the *B* series), but the comparison of their chromatographic mobilities in solvent B and their color reactions with *p*-anisidine are given in Table II.

TABLE II

ACIDIC FRAGMENTS FROM THE PARTIAL HYDROLYSIS OF SAPOTE GUM BY ACID

Substances yielding 4-O-methyl-D-glucuronic acid[a] after acid hydrolysis			*Substances yielding D-glucuronic acid[a] after acid hydrolysis*		
Acid	*Chromatographic mobility* (R_X)[b]	*Color with p-anisidine spray*	*Acid*	*Chromatographic mobility* (R_X)[b]	*Color with p-anisidine spray*
A1	1.10	pink	*B1*	0.68	pink
A2	0.94	pink-orange	*B2*	0.52	pink-orange
A3 (1)	0.58	orange	*B3* (2)	0.30	orange
A4	0.13	brown	*B4*	0.08	brown

[a]Determined by qualitative paper-chromatography in 9:2:2 ethyl acetate–acetic acid–water. [b]Expressed as the ratio of the distance travelled to the distance travelled by D-xylose.

Acids *A3* (**1**) and *B3* (**2**) crystallized after isolation by preparative paper-chromatography, and **1** was identified as *O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-(1→2)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose by comparison with an authentic sample (**3**). Compounds **1** and **3** had, within experimental accuracy, identical melting points, specific rotations, chromatographic mobilities, and X-ray diffraction patterns, and they also apparently gave the same products on partial hydrolysis with acid. Compound **2** was identified as *O*-α-D-glucopyranosyluronic acid-(1→2)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose by (*a*) identification of *O*-α-D-glucopyranosyluronic acid-(1→2)-D-xylopyranose as a partial-hydrolysis product of **2**; (*b*) identification of a 1,2,3,5-tetra-*O*-methylxylitol as a hydrolysis product of the neutral, methylated, nonreducing derivative of **2**; (*c*) the similarity of the methanolysis products of the neutral, methylated, nonreducing derivatives of **1**, **2**, and **3**; and (*d*) a consideration

of the isorotation rules, which, because of the similarities of the specific rotations of **1** and **2**, indicated that the xylose-to-xylose bond of **2** was in the β-D configuration.

Partial hydrolysis of **1** followed by paper-chromatographic examination of the hydrolyzate revealed spots that corresponded in migration rate and color to *A1*, *A2*, and a xylose. Similarly, **2** gave spots that corresponded to *B1*, *B2*, and a xylose.

Reduction of the reducing group of **1**, **2**, and **3** with sodium borohydride, and subsequent methylation, gave syrups, the i.r. spectra and specific rotations of which appeared, within experimental accuracy, to be identical. Reduction of the ester group of each of these products to a primary hydroxyl group with lithium aluminum hydride gave neutral, methylated, nonreducing derivatives of **1**, **2**, and **3**. A portion of each of these derivatives was hydrolyzed, and another portion of each was methanolyzed. Paper chromatography of the three hydrolyzates gave spots identical to those of 2,3,4-tri-*O*-methyl-D-glucose and 3,4-di-*O*-methyl-D-xylose. Gas–liquid chromatography of the hydrolyzate revealed one peak identical with that given by a 1,2,3,5-tetra-*O*-methylxylitol, and its identity was confirmed by preparation of the crystalline *p*-nitrobenzoate. Five peaks for the methanolyzates were observed by gas chromatography. One of these, for all three samples, corresponded to a 1,2,3,5-tetra-*O*-methylxylitol, and the other four for the derivatives of **1** and **2** were identical to those from the corresponding derivative of **3**. They were tentatively identified as caused by the anomers of methyl 3,4-di-*O*-methyl-D-xylopyranoside and the anomers of methyl 2,3,4-tri-*O*-methyl-D-glucopyranoside.

The identification of **1** establishes the location of a methoxyl group at C-4 of some of the uronic acid residues in the molecule of sapote gum. It seems probable that all of the methoxyl groups are so situated. Identification of **1** and **2** shows that the uronic acid-to-xylose bonds in sapote gum are in the α-D configuration, that at least some of the xylose-to-xylose bonds have the β-D configuration, and that the latter bonds are glycosyl (1→4) bonds. Only future experiments can determine whether one (or both) of these acids occupies a nonterminal position in the molecule of sapote gum.

EXPERIMENTAL

Paper chromatography. — Sugars were separated by descending paper-chromatography on Whatman No. 1 paper for analytical purposes, and on Whatman No. 17 paper (with an attached wick of No. 1 paper) or Whatman 3MM paper for preparative purposes. Solvent A (8:2:1 ethyl acetate–pyridine–water) was used to separate neutral sugars, solvent B (9:2:2 ethyl acetate–acetic acid–water) was used to separate uronic acids, and solvent C (water-saturated butanone) was used to separate partially methylated sugars. Sugars were detected with *p*-anisidine hydrochloride[5] and alkaline silver nitrate[6] sprays.

Purification of sapote gum. — Sapote gum (150 g) was mixed with 10 liters of distilled water, and the suspension was filtered. To the filtrate was added, in two equal parts, 5 h apart, an aqueous solution (125 ml) containing 10 g of sodium chlorite, 5 ml of glacial acetic acid, and 1 ml of formic acid. After 2 days at room temperature,

100 ml of 37% hydrochloric acid was added; the solution was kept for 30 min, poured into 30 liters of 95% ethanol, and the precipitated gum was freed of supernatant liquor by decantation, washed with 95% ethanol, and dissolved in 3 liters of distilled water. To the solution, 30 ml of hydrochloric acid was added, and the solution was kept for 45 min and poured into 9 liters of 95% ethanol. The resulting precipitate was thoroughly washed with anhydrous ethanol and then with anhydrous ether, and air-dried. The yield was 96 g; the moisture content was 9.85, and the ash content was negligible.

Analysis of individual nodules. — Five nodules of gum from the original sample employed by White[2] and five nodules of the fresh sample of sapote gum were purified by the general procedure outlined above. Each of the ten individual samples (50 mg) was dissolved in 17 ml of 8.5% sulfuric acid and heated for 60 min at approximately 115°. After neutralization with barium carbonate, filtration, and evaporation to a syrup, paper chromatography (solvents A and B) gave spots corresponding to xylose, arabinose, galactose (very faint), glucuronic acid, and 4-*O*-methylglucuronic acid. Sugar analyses[7], "uronic anhydride"[8], and methoxyl[9] were determined for each of the ten nodules (see Table I).

Isolation and identification of D-*xylose and* L-*arabinose.* — Four 2.0-g samples of gum were each dissolved in 300 ml of 8% sulfuric acid and heated for 120 min at 115°. The hydrolyzates were isolated by conventional techniques[10], and, after preparative chromatography in solvent A, the areas of the chromatograms containing D-xylose and L-arabinose were cut from the chromatograms and eluted. D-Xylose was identified as the crystalline di-*O*-benzylidene dimethyl acetal [11]; m.p. 208° alone or in admixture with an authentic sample. The arabinose was identified by its i.r. spectrum only, because Anderson and Ledbetter[1] and White[2] had identified it previously.

Isolation of aldotriouronic acids. — In each of eight beakers was placed 12 g of gum; 250 ml of 0.25N sulfuric acid was added, and the samples were heated for 50 min at 115°. The hydrolyzates were rendered neutral with barium carbonate, the suspensions were filtered, and the filtrates were combined, and concentrated to 200 ml. The barium salts of the acids were precipitated by addition of 300 ml of absolute ethanol. The precipitate was dissolved in 300 ml of water, and the solution was acidified with dilute sulfuric acid to remove barium ions, lead acetate was added to remove sulfate ions, hydrogen sulfide was passed in to remove lead ions, and air was passed in to remove hydrogen sulfide. Activated carbon (100 g) was added to the sulfide-free solution, the suspension was stirred for 3 h, and filtered, and the carbon was extracted successively with 1.5 liters of distilled water and 1.5 liters of 50% aqueous ethanol. Chromatographic examination of the aqueous ethanol extract showed the eight acidic fragments described in Table II to be present, together with a small proportion of galactose and arabinose. The chromatographic mobility of these fragments in solvent B was compared with that of D-glucuronic acid and with those of the acidic fragments isolated from the partial (acid) hydrolyzate of a 4-*O*-methyl-glucuronoxylan from a coniferous pulp[10]. This mixture of substances crystallized on being dried over phosphorus pentaoxide.

The mixture of acids was separated by chromatography in solvent B, and the

areas corresponding to each of the acids were cut out of the chromatogram and eluted with water. Chromatographic examination of the complete (acid) hydrolyzates of each of these acidic fragments showed that they could be classified according to whether D-glucuronic or 4-*O*-methyl-D-glucuronic acid was present; these results are given in Table II. The two major components, acids *A3* (**1**) and *B3* (**2**) crystallized on being dried, and weighed 2.81 and 1.209 g, respectively.

Characterization of aldotriouronic acids. — Characteristics of **1** and of authentic *O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-(1→2)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (**3**), respectively, were: equiv. wt., 520 and 523; m. p., 182–184° and[10] 184°, $[\alpha]_D$ +60° (*c* 0.025, water) and[10] +57°; and chromatographic mobility (R_X in solvent B), 0.58 and 0.58. Substances **1** and **3** had identical X-ray diffraction patterns and no significant differences in i.r. absorption spectra, and gave, on hydrolysis, chromatographic spots corresponding to xylose and acids *A1* and *A2*. Substance **2** had equiv. wt. 497; m. p., 194–197°; $[\alpha]_D$ +53° (*c* 0.25, water), and a chromatographic mobility of 0.30 in solvent B. The i.r. spectrum of **2** was similar to that of **1**, and the presence or absence of a methoxyl group could not be determined from the i.r. spectrum. The hydrolysis products of **2** corresponded, by paper chromatography, to xylose and acids *B1* and *B2*.

Identification of O-α-D-*glucopyranosyluronic acid*-(1→2)-D-*xylopyranose.* — A solution of 500 mg of **2** in 50 ml of 1.0N sulfuric acid was sealed in a glass ampoule, heated for 3 h at 105°, neutralized with barium carbonate, filtered, deionized on a column of Amberlite IR-120 (H^+) ion-exchange resin, and evaporated to a syrup. Paper chromatography revealed four spots which corresponded to a xylose, *B1*, *B2*, and **2**. The material corresponding to *B2* was isolated by preparative paper-chromatography, and evaporated to a syrup (226 mg), which represented 68.5% of the theoretical yield of aldobiouronic acid.

The syrup was converted into the methyl ester methyl glycosides, and acetylated by the procedures outlined by Smith *et al.*[12,13]. The colorless, crystalline product (186 mg) melted at 255°, alone or in admixture with an authentic sample of methyl 3,4-di-*O*-acetyl-2-*O*-(methyl 2,3,4-tri-*O*-acetyl-α-D-glucopyranosyluronate)-β-D-xylopyranoside. Melting points previously recorded for this compound are[12] 250° and[13] 255–257°. The specific rotation, $[\alpha]_D^{26}$, was +100° (*c* 0.053, chloroform), compared with[11] +101° and[12] +103°.

Sodium borohydride reduction. — Each of the three aldotriouronic acids **1**, **2**, and **3** (200 mg) was dissolved in 0.1N sodium hydroxide (30 ml) that contained 4% of sodium borohydride. After 3 h at room temperature, the solutions were neutralized with 2N acetic acid, passed through a column of Amberlite-120 (H^+) ion-exchange resin, and evaporated to dryness. The residues were repeatedly dissolved in methanol and evaporated until all of the boric acid had been removed.

Methylation. — The reduced products were each dissolved in 6.25 ml of 4.8% sodium hydrogen carbonate solution, and were methylated with methyl sulfate according to the procedure of Smith *et al.*[12], and then with solid sodium hydroxide and methyl sulfate according to the procedure of Falconer and Adams[14].

Each of the syrups from the methyl sulfate methylation was dissolved in 5 ml of dry *N,N*-dimethylformamide. Methyl iodide (2 ml) and 0.25 g of Drierite (anhydrous calcium sulfate) were added to each solution. The solutions were stirred for 4 h, and 2 g of silver oxide was added during 3 h. The next day, 2 g of silver oxide was added with stirring. The methylated products were then isolated by the procedure described by Kuhn and co-workers[15], as syrups which were stored over phosphorus pentaoxide.

The i.r. spectra of the syrups from **1**, **2** (after remethylation), and **3** were identical and showed no absorption in the hydroxyl-stretching region. Specific rotations $[\alpha]_D^{25-26}$ of these syrups from **1**, **2**, and **3** were: +38° (*c* 0.046, ethanol), +38° (*c* 0.013, ethanol), and +36° (*c* 0.025, ethanol), respectively.

Low yields of these reduced and methylated products from **1**, **2** and **3** (139, 40, and 75 mg, respectively) were attributed to mechanical losses arising from formation of emulsions during the solvent extractions that followed each methylation step.

Reduction with lithium aluminum hydride. — The reduced and methylated syrups from **1**, **2**, and **3** were each dissolved in dry tetrahydrofuran (10 ml), and 150 mg of lithium aluminum hydride was slowly added to each. After the solution had been stirred for 2 h, ethyl acetate was added to decompose unreacted hydride. Cold, distilled water was added, and the suspensions were filtered. The filtrates were deionized on a column of Amberlite MB-3(H^+, OH^-) ion-exchange resin, and the eluates were evaporated to syrups that were used for methanolysis and hydrolysis experiments.

Hydrolysis of reduced, methylated trisaccharides. — Each of the three syrups from the lithium aluminum hydride reductions (20 mg) was dissolved in 5 ml of 4% sulfuric acid, and the solution was sealed in a glass ampoule, heated for 120 min at 100°, cooled, neutralized with barium carbonate, filtered, deionized on a column of Amberlite MB-3(H^+, OH^-) ion-exchange resin, and evaporated to a syrup. Paper chromatography (Solvent C) revealed two spots common to all three syrups (R_{TMG}* 0.57 and 0.78), with no intersample variation in migration rate. The ratio of the area of the faster spot to that of the slower spot was 0.80 for each of the hydrolyzates, indicating that the ratios of the quantities of the materials which produced the spots were probably the same in the three hydrolyzates[16].

The faster spot (R_{TMG} 0.78) corresponded in migration rate to authentic 2,3,4-tri-*O*-methyl-D-glucose and the slower spot (R_{TMG} 0.57) corresponded in migration rate to authentic 2,3-di-*O*-methyl-D-xylose, which, in solvent C, is known to migrate at almost the same rate as 3,4-di-*O*-methyl-D-xylose.

G.l.c. of these three hydrolyzed syrups (on an analytical column packed with acid-washed diatomaceous earth coated with 15% 1,4-butanediol succinate polyester according to the procedure of Bearce[17], at 165° and eluted with nitrogen at 20 lb.in^{-2}) produced one peak, which appeared after 8.1 min for each sample. The retention times of the three peaks were, therefore, identical.

*Mobility relative to that of 2,3,4,6-tetra-*O*-methyl-D-glucose.

Identification of a 1,2,3,5-tetra-O-methylxylitol. — The substances that produced the three peaks were collected by condensation. Each syrup was dissolved in 10 ml of dry pyridine, and 35 mg of recrystallized *p*-nitrobenzoyl chloride was added to each solution. The solutions were sealed in glass ampoules, heated for 50 min at about 65°, and then kept overnight at room temperature. The mixtures were neutralized with saturated sodium carbonate solution, and extracted three times with redistilled chloroform, and the extracts were dried overnight (anhydrous calcium sulfate), and evaporated to dryness. The crystalline products were washed with cold, dry methanol; yield of each, approximately 10 mg. Their melting points, alone or in admixture with one another, were 185–187°, indicating that a 1,2,3,5-tetra-*O*-methylxylitol can be derived from **1**, **2**, and **3**. The melting point of a 1,2,3,5-tetra-*O*-methyl-4-*O*-*p*-nitrobenzoylxylitol has been reported[18] as 187–189°.

Methanolysis of reduced, methylated trisaccharides. — The unhydrolyzed portions of the three syrups obtained from the lithium aluminum hydride reduction were each dissolved in 10 ml of 4% methanolic hydrogen chloride. The solutions were sealed in glass ampoules, heated for 5 h at 105°, neutralized with silver carbonate, and the suspensions filtered. The filtrates were deionized on a column of Amberlite MB-3(H^+, OH^-) ion-exchange resin and evaporated to syrups. G.l.c. of these three syrups revealed, in each case, five peaks, which appeared at 8.1, 15.3, 29.5, and 43.7 min, with no intersample variations in retention time. One of the peaks (8.1 min) corresponded to the single peaks of the 1,2,3,5-tetra-*O*-methylxylitol isolated in the previous experiment. Presumably, the others were due to the anomers of methyl 3,4-di-*O*-methyl-D-xylopyranoside and of methyl 2,3,4-tri-*O*-methyl-D-glucopyranoside. Peaks produced by an authentic sample of a mixture of the anomers of methyl 2,3-di-*O*-methyl-D-xylopyranoside had retention times of 17.5 and 21.2 min, and were different from the five peaks listed above.

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PREPARATION AND CHARACTERIZATION OF 1,2,6,2′,3′,4′,6′-HEPTA-*O*-ACETYL-β-MALTOSE*

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ABSTRACT

Acetylation of a slurry of β-maltose monohydrate in cold toluene with acetylpyridinium chloride gave 1,2,6,2′,3′,4′,6′-hepta-*O*-acetyl-β-maltose (**1**) in 70% yield, with octa-*O*-acetyl-β-maltose as a byproduct. Crystalline 3-*O*-methyl (**2**) and 3-*O*-phenylcarbamoyl (**3**) derivatives of **1** were readily obtained. A deacetylated sample of **2** was shown to yield 3-*O*-methyl-α,β-D-glucose and α,β-D-glucose after aqueous hydrolysis. To discriminate between the O-3 and O-3′ positions, a second portion of deacetylated **2** was reduced with sodium borohydride and the product methanolyzed, to yield 3-*O*-methyl-D-glucitol and methyl α,β-D-glucopyranoside; components of the methanolyzate were identified by g.l.c. Deacetylation and methanolysis of **3** gave methyl 3-*O*-phenylcarbamoyl-α,β-D-glucopyranoside (**5**), from which methyl 2,4,6-tri-*O*-benzoyl-3-*O*-phenylcarbamoyl-β-D-glucopyranoside (**6**) was isolated crystalline; synthesis of **6** from 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose proved its structure.

INTRODUCTION

To increase the possibilities for industrial utilization of maltose (4-*O*-α-D-glucopyranosyl-D-glucopyranose), a current program of this laboratory is designed to expand the chemistry of this readily accessible sugar. In one phase of the work, chemical reactivities of the individual hydroxyl groups of maltose are being investigated. A distinctly lower relative reactivity has been found at the 3-hydroxyl group toward acetylation by acetylpyridinium chloride in toluene. A procedure for slow, heterogeneous acetylation was developed expressly to show reactivity differences between hydroxyl groups. Under identical conditions of reaction, both α- and β-D-glucose were fully acetylated in 95% yields.

Synthesis of the title compound (**1**) in 70% yield provides access to 3-*O*-substituted derivatives of maltose. Other heptaacetates of maltose that are known have free hydroxyl groups at C-1, C-6, and C-6′. These have been obtained by hydrolysis of

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acetylmaltosyl halides[1], by the action of piperidine on octa-*O*-acetyl-β-maltose[2], and by detritylation of the acetylated 6-trityl or 6′-trityl ethers of maltose[3,4].

The use of acetyl halides for the preparation of acetylglycosyl halides has been reviewed by Staněk and his colleagues[5]. Sugihara[6] summarized investigations before 1958, which showed high reactivities of the hydroxyl groups at C-6 and C-2 of D-glucose toward acylation. In 1967, Williams and Richardson[7] established a reactivity sequence for the secondary hydroxyl groups in methyl α-D-glucopyranoside, toward benzoyl chloride in pyridine, but the sequence that they found, namely, 2-OH > 3-OH > 4-OH, may not hold for other acylating systems or other hexosides.

Specificities that depend on the class of acylating agent employed were demonstrated by Jeanloz and Jeanloz[8]. They reported that methyl 4,6-*O*-benzylidene-α-D-glucopyranoside is selectively acylated at O-2 by carboxylic acid chlorides, and at O-3 by the corresponding anhydrides. In most cases, yields were modest, with various degrees of reagent discrimination.

Attempts to rationalize selective acylations in the monosaccharide series have involved a number of as yet incompletely evaluated parameters: the mechanism of acylation, the class of acylating reagent, the catalyst[9], the anomeric configuration, the steric environment of the hydroxyl groups, hydrogen bonding, the ring size, and the conformation of the molecule. Similar specificities doubtless apply to maltose and other disaccharides, but with the possible added complication of intramolecular hydrogen bonding between hydroxyl groups of adjoining saccharide molecules. From 3-dimensional X-ray diffraction data, Hybl *et al.*[10] showed hydrogen bonds between O-2 and O-3 of each contiguous pair of D-glucose residues in the cyclohexaamylose–potassium acetate complex, and the results were extrapolated to embrace helical V-amylose. From n.m.r. and i.r. spectral measurements, Casu *et al.*[11] deduced hydrogen-bonding between the 3-OH and 2′-OH groups in maltose, maltocyclodextrins, and amylose; and Chu and Jeffrey[12] found the same bonding in crystalline methyl β-maltoside monohydrate by X-ray diffraction data. Whether this bonding is responsible for the inhibited acetylation at 3-OH in maltose is being investigated by acetylation of other disaccharides alleged to be intramolecularly hydrogen-bonded.

RESULTS AND DISCUSSION

β-Maltose monohydrate was slurried in cold toluene, and acetylated slowly with acetylpyridinium chloride. Reaction variables of temperature, time, and reagent concentration were evaluated by thin-layer chromatographic estimation of compositional changes in the reaction mixtures. A 70% yield of the new heptaacetate (**1**) was obtained with the optimum procedure. The anomeric configuration remained unchanged during the acetylation. Finely divided maltose was required for maximum conversion. Purification of the product mixture required column chromatography on silica gel. The t.l.c. systems of Wolfrom and de Lederkremer[13] gave excellent separation of **1** and the byproduct, octa-*O*-acetyl-β-maltose.

The purified heptaacetate **1** was readily converted into octa-*O*-acetyl-β-maltose in cold pyridine–acetic anhydride. A control reaction on β-maltose monohydrate in

cold pyridine–acetic anhydride gave the same yield of octaacetate as **1**, and neither reaction product showed any α anomer by t.l.c. examination[14].

In chloroform-*d*, the heptaacetate **1** and octa-*O*-acetyl-β-maltose showed nearly identical low-field doublets for the anomeric proton (τ 4.36 and τ 4.25, respectively, with $J_{1,2}$ 8 Hz). Such deshielding of the anomeric proton is characteristic for a 1-acetate, and the location and large coupling constant indicate an axial orientation (β anomer)[15–17]. The integrated spectrum confirmed the presence of seven *C*-methyl (acetyl) groups in **1**, within the region assigned for equatorial *O*-acetyl groups (τ 7.93–8.01). No signals could be detected at the resonance of τ 7.82 reported[16] for axial *O*-acetyl groups, and since no other *C*-methyl resonances were observed, any ortho-ester forms are ruled out.

These findings agree with the β configuration that was also assigned by use of Hudson's rule and by the isolation of octa-*O*-acetyl-β-maltose after peracetylation of **1** in cold pyridine–acetic anhydride. The i.r. spectrum of **1** in a potassium bromide disc showed the expected strong absorption band (type 2b, 890 cm^{-1}). This band, supposedly specific for a β-D configuration in the D-aldopyranose acetate series[18], was accompanied by a weak band (type 2a, 844 cm^{-1}), allegedly indicating the presence of the α-D-(1→4) interglycose linkage.

The resonance of the hydroxyl proton was observed in methyl sulfoxide-d_6. For 2,3,6,2′,3′,4′,6′-hepta-*O*-acetyl-β-maltose, the 1-OH signal appeared far downfield as a doublet (τ 2.42, $J_{1,OH}$ 6 Hz). Our heptaacetate, however, showed a different doublet for the hydroxyl proton (τ 4.30, *J* 7 Hz). Both doublets were eliminated by exchange with deuterium oxide. Although a position for the free hydroxyl group could not be assigned by n.m.r. spectroscopy alone, similar low-field resonances were found by Casu *et al.*[11] for the C-3 and C-2′ hydroxyl protons of β-maltose in methyl sulfoxide solution.

It was assumed that the hydroxyl group was not at C-2 when it was observed that **1** failed to mutarotate in aqueous pyridine or in acetic acid solution. Lemieux and Morgan[16] have shown that an analogous C-2-hydroxylated compound, 1,3,4,6-tetra-*O*-acetyl-α-D-glucopyranose, readily "mutarotates" in aqueous acetic acid to give the anomers of 2,3,4,6-tetra-*O*-acetyl-D-glucopyranose by acetyl migration.

Although the heptaacetate **1** was not crystallized, crystalline derivatives were obtained in high yield by methylation or by treatment with phenyl isocyanate.

Initial methylations by the procedure of Kuhn *et al.*[19] were complicated by acetyl migration before substitution. Similar problems had been reported by Angyal and Melrose[20]. The 3-methyl ether (**2**) was formed in a modified boron trifluoride–diazomethane[21,22] system. The n.m.r. spectrum of **2** at 100 MHz in chloroform-*d* showed the expected *O*-methyl resonance (τ 6.59), and the anomeric-proton doublet remained unchanged at τ 4.36, $J_{1,2}$ 8 Hz. Examination of the integrated spectrum confirmed the presence of a single *O*-methyl group and of seven *C*-methyl (acetyl) groups (τ 7.93–8.01). A comparison spectrum of the analogous 3-*O*-phenylcarbamoyl derivative (**3**) showed the anomeric proton doublet at τ 4.22 ($J_{1,2}$ 8 Hz), essentially identical with that for octa-*O*-acetyl-β-maltose.

The first step in our proof of structure was identification of the mono-*O*-methyl-D-glucose produced by deacetylation and hydrolysis of **2**. Gas–liquid chromatography (g.l.c.), with a liquid phase of Carbowax* 20M, greatly simplified identification. All samples were converted into their trimethylsilyl ethers[23] before injection. By co-injecting reference compounds with the hydrolyzate, and by referring to relative retention values (see Table I), the presence of 3-*O*-methyl-α,β-D-glucose and of α,β-D-glucose was established. No other products were observed.

TABLE I

RELATIVE RETENTION VALUES OF D-GLUCOSE DERIVATIVES[a]

Sample[b]	*Internal reference standards*[c]		
	α-D-Glucose, 150°	*Methyl β-D-glucoside, 160°*	*D-Glucitol, 160°*
3-*O*-Methyl-D-glucitol	0.85	0.47	0.86 (150°) 0.96 (160°)
D-Glucitol	[d]	0.49	
3-*O*-Methyl-α-D-glucose	0.76	0.44	
4-*O*-Methyl-α-D-glucose	0.90		
2-*O*-Methyl-α-D-glucose	1.08		
3-*O*-Methyl-β-D-glucose	1.25	0.72	
2-*O*-Methyl-β-D-glucose	1.50		
6-*O*-Methyl-α-D-glucose	1.52		
4-*O*-Methyl-β-D-glucose	1.67		
β-D-Glucose	1.82		
6-*O*-Methyl-β-D-glucose	1.85		

[a] t/t_{std} at designated temperature, as pertrimethylsilyl ethers. [b] The order of appearance for the 4-*O*-methyl-D-glucose anomers presumably follows that observed for known anomers available to us. In all, the α-D preceded the β-D. [c] With 19.5% Carbowax 20M on Chromosorb W. [d] Coincides with the standard. Peaks were separable on a column packing of Carbowax 20M on Gas-Chrom Q.

On a larger scale, purification on a Celite column was used to isolate 3-*O*-methyl-α,β-D-glucose in amounts sufficient for conversion into the phenylosazone. A mixed m.p. of the isolated phenylosazone with authentic 3-*O*-methyl-D-*arabino*-hexulose phenylosazone was undepressed.

To distinguish between substitutions at the O-3 and O-3′ positions of maltose, a second sample of the 3-*O*-methylmaltose heptaacetate (**2**) was deacetylated, the product was reduced with aqueous sodium borohydride, and the substituted alditol was refluxed in methanolic hydrogen chloride. After trimethylsilylation of the product, g.l.c. showed a mixture of 3-*O*-methyl-D-glucitol and methyl α,β-D-glucopyranoside, when co-injection of authentic reference compounds was again used.

Additional support for the structure 1,2,6,2′,3′,4′,6′-hepta-*O*-acetyl-β-maltose for **1** was gained by examination of its mono-*O*-phenylcarbamoyl derivative (**3**).

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Although alkaline procedures caused extensive wandering of the phenylcarbamoyl group, deacetylation in 2% methanolic hydrogen chloride[24] produced methyl 3-*O*-phenylcarbamoyl-α,β-D-glucopyranoside (**5**) as the sole monosubstituted product. After column purification, substance **5** could be converted into pure methyl 2,4,6-tri-*O*-benzoyl-3-*O*-phenylcarbamoyl-β-D-glucopyranoside (**6**). The β-D assignment was based on the use of Hudson's rule, and confirmed by n.m.r. (100 MHz in chloroform-*d*). The H-1 doublet (τ 5.29, $J_{1,2}$ 8 Hz) corresponded closely to that observed in a reference spectrum for methyl 2,3,4,6-tetra-*O*-benzoyl-β-D-glucopyranoside (τ 5.21, $J_{1,2}$ 8 Hz). Assignment of **6** as the 3-*O*-phenylcarbamoyl derivative was confirmed by comparison of the m.p., specific rotation, and n.m.r. spectrum with those of an authentic, crystalline specimen prepared from 1,2:5,6-di-*O*-isopropylidene-3-*O*-phenylcarbamoyl-α-D-glucofuranose (**7**) by methanolysis and benzoylation.

EXPERIMENTAL

Melting points were determined with a Thomas–Hoover melting-point apparatus (Arthur H. Thomas Co., Philadelphia, Pa.) and are corrected. Optical rotations were measured with a Rudolph polarimeter in a 1-dm tube. I.r. spectra were recorded with a Perkin–Elmer Model 621 spectrophotometer, by the potassium bromide disc technique. N.m.r. spectra were measured at 100 MHz with a Varian HA-100 spectrometer. Tetramethylsilane was used as an internal standard in chloroform-*d* or methyl sulfoxide-d_6. Chemical shifts are given on the τ scale. An F & M research chromatograph, Model 810, was employed for g.l.c. The column was a 12-ft length of 1/4-in o.d. copper tubing, packed with 19.5% Carbowax 20M on Chromosorb W* (80–100 mesh). Operation was isothermal at 150°, with helium as the carrier gas, and flame ionization detection.

All samples were dissolved in pyridine and converted into their trimethylsilyl ethers approximately 18 h before injection. T.l.c. on Silica Gel G (E. Merck, Darmstadt, Germany) was performed without heat activation of the plates. Solvent proportions are on the v/v basis. For column chromatography, Baker Analyzed Silica Gel (J. T. Baker Chemical Co., Phillipsburg, N. J.) was used without pretreatment. Solutions were concentrated below 40° under diminished pressure. Pyridine was removed from organic phases by alternately washing with water and 5% aqueous cupric sulfate. Calcium hydride was used whenever rigorous drying of organic liquids was needed, and anhydrous sodium sulfate was used for drying solutions.

Acetylation of β-maltose monohydrate. — All solvents were anhydrous. The reaction temperature was kept below 5° at all times. β-Maltose monohydrate[25] (10 g, free from D-glucose and oligosaccharides) was finely powdered in a mortar and then transferred to the reaction vessel with 200 ml of toluene and 30 ml of pyridine. The stirred slurry was treated dropwise with 17.5 ml of acetyl chloride (98%) in 50 ml of toluene during 0.5 h. Maximum reaction required 60 h, at which time the pink slurry

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was filtered. The solids were rinsed twice with fresh toluene, and the combined filtrates were washed, dried, and evaporated to a syrup (17 g). T.l.c. examination with 1:1 ethyl acetate–benzene (A) or 200:7 benzene–methanol (B) disclosed only two components, octa-*O*-acetyl-*β*-maltose and **1**. For solvent A, the R_F values were 0.61 and 0.40, respectively. Unreacted maltose (0.5 g) was recovered from the filtered solids after several washes on the funnel with chloroform.

Any increases in temperature, time, or concentration of acetyl chloride caused a corresponding increase in the amount of octa-*O*-acetyl-*β*-maltose produced. Decrease in the reaction time or in the acetyl chloride or pyridine concentration lowered the conversion into acetylated products, with no increase in the ratio of **1** to octaacetate. Substitution of benzene for toluene gave the same results.

*1,2,6,2′,3′,4′,6′-Hepta-*O*-acetyl-β-maltose* (**1**). — A sample (6.1 g) of the syrup obtained above was purified on a column of silica gel packed, and irrigated, with solvent A. Of 6.0 g of product that was recovered, the octa-*O*-acetyl-*β*-maltose weighed 1.7 g (28%) and **1** weighed 4.3 g (71%). All attempts to crystallize **1** failed. Use of multiple-ascent t.l.c. with solvent B showed **1** to be a single anomer. The only exception noted was for acetylation of a slurry of anomerized maltose, which gave **1** in both anomeric forms. Distillation of **1** under diminished pressure was accompanied by decomposition. Purified **1** had $[\alpha]_D^{20}$ +87.5° (*c* 0.4, chloroform); n.m.r. data (chloroform-*d*): τ 4.36 (doublet, $J_{1,2}$ 8 Hz, H-1), τ 7.93–8.01 (seven acetyl groups); in methyl sulfoxide-d_6: τ 3.98 (doublet, $J_{1,2}$ 8 Hz, H-1), τ 4.30 (doublet, *J* 7 Hz, hydroxyl proton), τ 7.63–7.69 (seven acetyl groups).

Anal. Calc. for $C_{26}H_{36}O_{18}$: C, 49.06; H, 5.70; acetyl, 47.3. Found: C, 48.96; H, 5.95; acetyl[26], 46.6.

Peracetylation of **1**. — A 100-mg portion of **1** was dissolved in a cold solution of 1 ml of acetic anhydride in 3 ml of pyridine. The temperature was kept for 24 h below 5°, and was then allowed to rise to 25°. After a total of 72 h, 5 ml of methanol was added, and the solution was concentrated to a thin syrup. The syrup was dissolved in ethyl acetate, and the solution was freed of pyridine and acetic acid, dried, and reconcentrated. Multiple-ascent t.l.c. with solvent B showed no traces of *α*-maltose octaacetate or of **1**. Comparison with a standard showed **1** to have been completely converted into octa-*O*-acetyl-*β*-maltose.

Essentially the same results were obtained by acetylating *β*-maltose monohydrate in pyridine–acetic anhydride under the same conditions.

*1,2,6,2′,3′,4′,6′-Hepta-*O*-acetyl-3-*O*-methyl-β-maltose* (**2**). — A solution of 2.5 g of purified **1** in 25 ml of dichloromethane was chilled in an acetone–solid CO_2 bath. Prepared solutions of boron trifluoride (1.7 ml of BF_3 etherate diluted to 50 ml with dichloromethane) and diazomethane (0.42M, in dichloromethane) were chilled in a separate bath. Both solutions were kept at the bath temperature at all times. The reaction was initiated by adding 1 ml of the stock solution of BF_3 and 10 ml of the diazomethane solution to the solution of **1**. At 12-min intervals during 2 h, 1-ml portions of BF_3 solution and 20 ml of CH_2N_2 solution were alternately added until a total of 6 ml of BF_3 solution and 110 ml of CH_2N_2 solution had been added; the

mixture was then kept for 30 min, which was usually sufficient for discharge of all residual color. T.l.c. with solvent A showed an approximately 90% conversion into **2**. At this point, 5 ml of pyridine was added, and the solution was rapidly warmed to 25°. Polymethylene was removed by filtration, and the filtrate, washed to remove pyridine and other water-soluble substances, was added to a column of silica gel; elution with solvent A gave 2 g of product, readily crystallized from aqueous methanol. After being dried under vacuum, this hygroscopic compound had m.p. 115–116°, $[\alpha]_D^{20}$ +77.2° (*c* 1.1, chloroform); n.m.r. data (chloroform-*d*): τ 4.36 (doublet, $J_{1,2}$ 8 Hz, H-1), τ 6.59 (one OMe group), τ 7.93–8.01 (seven acetyl groups).

Anal. Calc. for $C_{27}H_{38}O_{18}$: C, 49.85; H, 5.89; OMe, 4.77. Found: C, 49.96; H, 5.85; OMe, 5.05.

Parallel attempts at methylation, essentially by the techniques of Kuhn and co-workers[19], were unsuccessful. Deacetylation and hydrolysis of the methylated products gave (as shown by t.l.c.) several mono-*O*-methyl-D-glucoses.

Gas–liquid chromatography. — Experimentation with standard mono-*O*-methyl-D-glucoses (2-*O*-, 3-*O*-, 4-*O*-, and 6-*O*-methyl isomers) showed that g.l.c. would afford ready identification of the mono-*O*-methyl-D-glucose(s) obtained by deacetylation and hydrolysis of **2**, either as the glycoside or as the reducing sugar. For each sample, a solution of 10–20 mg in 0.5 ml of pyridine was treated with 0.2 ml of hexamethyldisilazane and chlorotrimethylsilane. A reaction period of 18 h was used, to ensure complete trimethylsilylation. The relative retention times are listed in Tables I and II.

TABLE II

RELATIVE RETENTION VALUES OF METHYL D-GLUCOPYRANOSIDES AND DERIVATIVES[a]

Methyl D-*Glucopyranoside*[b]	*Internal reference standards*[c]		
	α-D-*Glucose*, *150°*	*Methyl* β-D-*glucoside*, *160°*	D-*Glucitol*, *160°*
3-*O*-methyl-α-	1.11	0.69	
3-*O*-methyl-β-	1.27		
4-*O*-methyl-α-	1.41		
2-*O*-methyl-α-	1.50		
4-*O*-methyl-β-	1.57		
α-	1.63	0.93	1.90
β-	1.76		2.05
2-*O*-methyl-β-	1.79		
6-*O*-methyl-α-	2.33	1.38 (150°)	
6-*O*-methyl-β-	2.54	1.50 (150°)	

[a] t/t_{std} at designated temperature, as pertrimethylsilyl ethers. [b] The order of anomer appearance for the 2-, 3-, and 6-*O*-methyl-D-glucosides presumably follows that observed for the known anomers available to us. In all, the α-D preceded the β-D. [c] With 19.5% Carbowax 20M on Chromosorb W.

Hydrolysis of **2**. — A 1.0-g portion of **2** in 15 ml of 0.05M barium methoxide was kept for 18 h at 0°. The syrup obtained after neutralization with Amberlite IR-50 resin and evaporation was dissolved in 20 ml of 0.25M hydrochloric acid, and the solution

was kept for 18 h at 100°. T.l.c. with 3:1 ethyl acetate–methanol (C) then showed only one mono-*O*-methyl-D-glucose.

A sample of the hydrolyzate was trimethylsilylated, and co-injected with a reference sample of pertrimethylsilylated *α*-D-glucose. Three product components were observed, having retention values equal to those for 3-*O*-methyl-*α*- and -*β*-D-glucose (0.76 and 1.25) and for *β*-D-glucose (1.82). When pertrimethylsilylated methyl *β*-D-glucopyranoside was used as the internal standard, a fourth component was observed; it was identified as *α*-D-glucose.

By contrast, examination of the hydrolyzate from the product from the silver oxide–*N*,*N*-dimethylformamide–methyl iodide system[19] showed a complex mixture of mono-*O*-methyl D-glucoses, and confirmed the earlier observations made by t.l.c.

Detection of 3-O-methyl-D-glucitol. — A solution of a 50-mg portion of deacetylated **2** in 5 ml of deionized water was chilled to 5°, and treated with 100 mg of sodium borohydride. After 24 h at room temperature, the solution was evaporated to a thick syrup which was mixed with 10 ml of methanol. After 1 h, 1 ml of concentrated hydrochloric acid was added, and the slurry was evaporated to dryness, with four re-treatments with methanol. The residue was mixed with 10 ml of fresh methanol and 0.3 ml of acetyl chloride, and the mixture was refluxed for 1 h, and kept for 24 h at room temperature. After evaporation and four additional retreatments with methanol, the solution was rendered neutral with ammonium hydroxide and evaporated twice more. The final residue was extracted with 2 ml of pyridine, and a 0.5-ml aliquot of the extract was converted into the trimethylsilyl ether as before. G.l.c. at 160° revealed that the hydrolyzate contained only 3-*O*-methyl-D-glucitol and methyl *α*,*β*-D-glucopyranoside. Authentic samples of methyl *β*-D-glucopyranoside, D-glucitol, and 3-*O*-methyl-D-glucitol[27] were prepared for comparison.

3-O-Methyl-D-arabino-hexulose phenylosazone. — The 3-*O*-methyl-*α*,*β*-D-glucose in the deacetylated hydrolyzate from **2** was freed of contaminants on a Celite column[28] irrigated with 2:5:5 (v/v) pyridine–ethyl acetate–water[29]. This column afforded 155 mg of chromatographically pure material, which was converted into the corresponding phenylosazone[30]. A mixed m.p. with authentic material was undepressed (at 172–175°).

1,2,6,2',3',4',6'-Hepta-O-acetyl-3-O-phenylcarbamoyl-β-maltose (**3**). — A crude product (11 g) from the slurry acetylation, containing approximately 70% of **1**, was dissolved in toluene (250 ml) and pyridine (10 ml). Phenyl isocyanate (10 ml) was added, and the solution was kept for 24 h at room temperature. The reaction was completed by heating for 15 min on a steam bath. Excess phenyl isocyanate was decomposed by addition of water, and the mixture was evaporated to dryness. The residue was extracted with 400 ml of chloroform, and the extract was filtered, and washed to remove pyridine. After the solution had been dried, it was again filtered (to remove the last traces of carbanilide). Chloroform was removed completely, and the product was dissolved in the minimal volume of ethyl ether. Crystallization was spontaneous, and **3** readily separated at room temperature; wt., 8.5 g. Recrystallization from abs. ethanol gave pure **3**, m.p. 174.5–175.5, $[\alpha]_D^{20}$ +58.7° (*c* 0.9, chloro-

form); n.m.r. data (chloroform-*d*): τ 4.22 (doublet, $J_{1,2}$ 8 Hz, H-1), τ 7.89–8.11 (seven acetyl groups).

Anal. Calc. for $C_{33}H_{41}NO_{19}$: C, 52.45; H, 5.47; N, 1.85. Found: C, 52.68; H, 5.81; N, 1.88.

2,3,6,2',3',4',6'-Hepta-O-acetyl-1-O-phenylcarbamoyl-β-maltose (**4**). — This compound was prepared for comparison with **3**. A 2.0-g sample of 2,3,6,2',3',4',6'-hepta-*O*-acetyl-β-maltose was dissolved in 10 ml of pyridine, and 1 ml of phenyl isocyanate was added. After 1 h at 100°, the solution was processed as for **3**. After two recrystallizations from ethanol, the compound had m.p. 146–148°, $[\alpha]_D^{20}$ +60.1° (*c* 0.98, chloroform); n.m.r. data (chloroform-*d*): τ 4.21 (doublet, $J_{1,2}$ 8 Hz, H-1), τ 7.93–8.01 (seven acetyl groups).

Anal. Calc. for $C_{33}H_{41}NO_{19}$: C, 52.45; H, 5.47; N, 1.85. Found: C, 52.04; H, 5.55; N, 1.82.

Isolation of methyl 2,4,6-tri-O-benzoyl-3-O-phenylcarbamoyl-β-D-glucopyranoside (**6**). — A 4.0-g sample of **3** was deacetylated in 80 ml of anhydrous methanol mixed with 2.3 ml of 98% acetyl chloride. After 48 h at room temperature, the acid was neutralized with Amberlite IR-45 (OH^-) ion-exchange resin, the suspension was filtered, and the filtrate was evaporated to a syrup (2.3 g). T.l.c. with 9:1 ethyl acetate–ethanol (D) showed the presence of only one methyl glucoside monocarbanilate. Purification was accomplished on a column of silica gel, packed with ethyl acetate and irrigated with solvent D. The syrupy methyl 3-*O*-phenylcarbamoyl-α,β-D-glucopyranoside (**5**), weighing 1.1 g, was not examined further, but was converted directly into the tribenzoate, essentially as described in the next section. After isolation, the β-D anomer (**6**) crystallized readily from ethanol. After two recrystallizations, **6** had m.p. 160–161°, $[\alpha]_D^{20}$ +43.0° (*c* 1.0, in chloroform); n.m.r. data (chloroform-*d*): τ 5.29 (doublet $J_{1,2}$ 8 Hz, H-1), τ 6.51 (OMe).

Anal. Calc. for $C_{35}H_{31}NO_{10}$: C, 67.19; H, 4.99; N, 2.24. Found: C, 67.41; H, 5.22; N, 2.16.

Direct synthesis of methyl 2,4,6-tri-O-benzoyl-3-O-phenylcarbamoyl-β-D-glucopyranoside (**6**). — A solution of 3.5 g of 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose and 2 ml of phenyl isocyanate in 25 ml of pyridine was heated for 45 min at 100°. Excess of isocyanate was decomposed with water, and the solution was evaporated to dryness. The residue was extracted with 250 ml of hot chloroform, and the extract was filtered, washed to remove pyridine, dried, and refiltered. Evaporation gave a syrup that was used without further examination.

The crude 1,2:5,6-di-*O*-isopropylidene-3-*O*-phenylcarbamoyl-α-D-glucofuranose (**7**) was dissolved in 80 ml of warm, 1:1 water–methanol, and the solution was treated with 1 ml of concentrated sulfuric acid. A 90-min period at reflux completed the hydrolysis; t.l.c. with 4:1 ethyl acetate–ethanol (E) was used to determine the progress of the reaction. After being processed, the sample was purified on a Celite column in the way described for 3-*O*-methyl-α,β-D-glucose. Efforts to crystallize 3-*O*-phenylcarbamoyl-α,β-D-glucose (**8**) have not yet been successful. Acetylation in cold pyridine–acetic anhydride gave crystalline 1,2,4,6-tetra-*O*-acetyl-3-*O*-phenylcarbamoyl-α-D-

glucopyranose (**9**); m.p. 199.5–200.5°, $[\alpha]_D^{20}$ + 61.1° (*c* 0.51, chloroform); n.m.r. data (chloroform-*d*): τ 3.65 (doublet, $J_{1,2}$ 4 Hz, H-1 of α-D anomer), τ 7.82 (one axial acetate group), τ 7.94–8.02 (three equatorial acetate groups).

Anal. Calc. for $C_{21}H_{25}NO_{11}$: C, 53.96; H, 5.39; N, 3.00. Found: C, 54.16; H, 5.53; N, 3.02.

A 2.0-g sample of **8** was dissolved in 100 ml of methanol, 1.5 ml of 98% acetyl chloride was added, and the mixture was refluxed for 3 h. T.l.c. with solvent D showed conversion into the methyl 3-*O*-phenylcarbamoyl-α,β-D-glucopyranosides (**5**). After isolation, purification on silica gel with solvent D removed all traces of byproducts.

A solution of pure **5** in 5 ml of pyridine and 30 ml of dichloromethane was cooled to below 5° in an ice bath, and 2.6 ml of benzoyl chloride in 15 ml of dichloromethane was added dropwise during 30 min. Stirring was continued for 18 h below 5° and for 4 h at 25°. Excess reagent was decomposed with water, and the solution was evaporated at 25° to a thin syrup which was dissolved in ethyl acetate; the solution was washed to remove pyridine and benzoic acid, and dried. As before, it crystallized readily from ethanol, to give **6** having m.p. 160–161°. A mixed m.p. with **6** isolated above was undepressed.

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2-DEOXY SUGARS.

PART XII. PYRIMIDINE NUCLEOSIDES DERIVED FROM 2-DEOXY-D-*arabino*-HEXOFURANOSE*

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ABSTRACT

An anomerically pure furanoside was obtained in crystalline form from the methyl glycosidation of 2-deoxy-D-*arabino*-hexose; it was converted into a stable, crystalline *O*-acylglycofuranosyl halide by the following sequence of reactions: methyl 2-deoxy-α-D-*arabino*-hexofuranoside → methyl 5,6-*O*-carbonyl-2-deoxy-α-D-*arabino*-hexoside → methyl 5,6-*O*-carbonyl-2-deoxy-3-*O*-*p*-nitrobenzoyl-α-D-*arabino*-hexoside →5,6-*O*-carbonyl-2-deoxy-3-*O*-*p*-nitrobenzoyl-α-D-*arabino*-hexosyl bromide. The new halide was treated with 2,4-dimethoxypyrimidine by the Hilbert–Johnson procedure to give 1-(5,6-*O*-carbonyl-2-deoxy-3-*O*-*p*-nitrobenzoyl-β-D-*arabino*-hexosyl)-4-methoxy-2(1*H*)-pyrimidinone, which underwent ammonolysis to yield 1-(2-deoxy-α-D-*arabino*-hexofuranosyl)cytosine. Demethylation of the pyrimidinone, followed by deacylation, gave the corresponding uracil nucleoside. 1-(2-Deoxy-β(?)-D-*arabino*-hexofuranosyl)-thymine, the furanoid isomer of 1-(2-deoxy-β-D-*arabino*-hexopyranosyl) thymine (a powerful and specific inhibitor of a pyrimidine phosphorylase obtained from Ehrlich ascites tumor cells) was prepared in a manner similar to that for the uracil nucleoside.

INTRODUCTION

In 1962, we reported[1] on the preparation of 1-(2-deoxy-β-D-*arabino*-hexopyranosyl)thymine, which was subsequently shown[2] to be a powerful and specific inhibitor of a pyrimidine nucleoside phosphorylase obtained from Ehrlich ascites tumor cells. 1-(2-Deoxy-β-D-*arabino*-hexopyranosyl)uracil[3] is likewise effective as an inhibitor of the same enzyme (but to a lesser degree); however, 1-β-D-glucopyranosylthymine[2] and 1-(2-deoxy-β-D-*ribo*-hexopyranosyl)thymine[4] are without effect. It appears, therefore, that, in terms of the carbohydrate component, the structural requirements for inhibition of the enzyme are highly specific. Whether this specificity is also a function

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of the 6-membered, pyranoid ring is an important question, and it is for this reason that we initiated studies with a view to preparing some pyrimidine nucleosides containing, as the carbohydrate residue, the 2-deoxy-D-*arabino*-hexofuranosyl group. The present article reports full details of the preparation of the first stable, *crystalline* *O*-acylglycofuranosyl halide of a 2-deoxyaldohexose[5] and the first known nucleosides containing a 2-deoxyaldohexosyl group in its *furanoid* form.

RESULTS AND DISCUSSION

Methods for converting fully hydroxylated aldohexoses into their furanoid forms *via* di-*O*-alkylidene derivatives are not applicable to 2-deoxyaldohexoses, owing to the absence of a hydroxyl group at C-2. For example, α-D-glucose readily forms 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose on treatment with acetone in the presence of sulfuric acid, and it appears that the driving force for the formation of the furanoid ring is, in this case, concurrent acetal formation at O-1 and O-2.

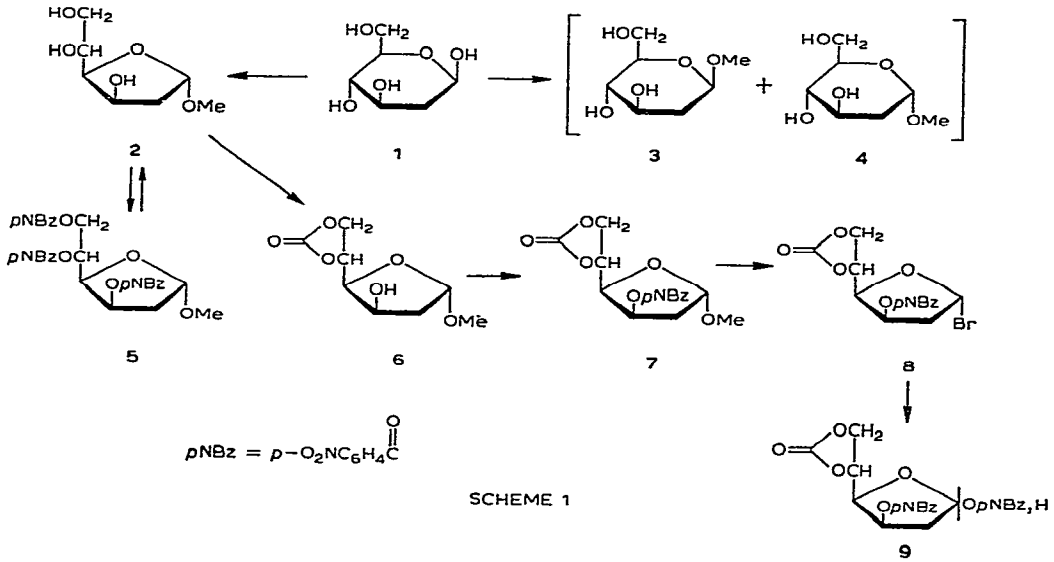
In contrast, when we attempted to prepare 2-deoxy-5,6-*O*-isopropylidene-D-*arabino*-hexose from 2-deoxy-D-*arabino*-hexopyranose ("2-deoxy-D-glucose"; **1**) in an analogous manner, decomposition of the sugar occurred, as evidenced by a darkening of the solution and our failure to isolate a pure product or products. Also, we were unsuccessful in [illegible]ts to prepare 5,6-*O*-carbonyl-2-deoxy-D-*arabino*-hexose by treating **1** with [illegible]. chloride; as shown by thin-layer chromatography, a complex reaction mix[illegible]as obtained and, therefore, this approach was abandoned.

An al[illegible]ive route was considered that involved the direct methyl glycosidation of compound **1**, as reported[6] by Hughes *et al.*, who claimed to have obtained almost exclusively "α,β-methyl-2-deoxy-D-glucofuranoside". Notwithstanding the experimental evidence they gave, their results are at variance with the observations and conclusions of Levene *et al.*[7], who made an exhaustive study of the methyl glycosidation of several sugars. Nevertheless, this method was, because of its simplicity, attractive, despite our conviction that conversion of the sugar into its methyl furanoside(s) would be far from quantitative.

Treatment of 2-deoxy-D-*arabino*-hexose (**1**) with 0.1% methanolic hydrogen chloride resulted in a syrup, the value of the specific rotation of which agreed with that reported[6] for "α,β-methyl-2-deoxy-D-glucofuranoside". In an effort to resolve the mixture, the syrup was *p*-nitrobenzoylated in pyridine, and the resulting mixture of crude, crystalline *p*-nitrobenzoic esters was dissolved in acetone, whereupon a tris-*p*-nitrobenzoate crystallized out in almost pure form, and the quantity obtained accounted for approximately 25% of the original glycoside mixture. Its physical constants (m.p. 142–144°, $[\alpha]_D$ −122°) did not agree with those of the known methyl 2-deoxy-3,4,6-tri-*O*-*p*-nitrobenzoyl-β-D-*arabino*-hexoside[8] (m.p. 159–161°, $[\alpha]_D$ −36°) but, unfortunately, the anomer of this pyranoside was not available, thus precluding a comparison with the latter.

Accordingly, the new tris-*p*-nitrobenzoate was deacylated with methanol containing a trace of methoxide ion, to give a crystalline glycoside having m.p. 80–81° and

$[\alpha]_D$ +117°; these values are *not* concordant with the corresponding values for either of the known anomers of methyl 2-deoxy-D-*arabino*-hexopyranoside[9] and, therefore, the new glycoside must be a furanoside. Because of its strongly positive specific rotation, the glycoside most probably has the α-D configuration as shown in structure **2**, in which the methoxyl group at C-1 and the side chain at C-4 have the *trans* arrangement. This constitutes the first report of a crystalline methyl glycofuranoside of a 2-deoxyaldohexose.



The structure of the original tris-*p*-nitrobenzoate as **5** must, therefore, follow from that of glycofuranoside **2**, although, at first, a clear relationship between the two furanosides was obscured by the dramatic change in rotation that occurred when **5** was deacylated. The molecular rotational difference amounts to 98,000 units, an abnormally large value in light of our experience with *p*-nitrobenzoic esters of sugars, and is especially noteworthy when compared with Δ[M] 15,000 for methyl 2-deoxy-β-D-*arabino*-hexopyranoside and its tris-*p*-nitrobenzoic ester[8]. However, *p*-nitrobenzoylation of crystalline **2** yielded the original triester (**5**), thus removing any doubts concerning the structural relationship between the two compounds.

Paper chromatography of the furanoside **2** in admixture with authentic samples of the pyranosides (**3** and **4**) led to a distinct separation, with **2** moving ahead of **3** and **4**, which appeared together as a "dumb-bell" spot. Accordingly, a direct evaluation of the methyl glycosidation of 2-deoxy-D-*arabino*-hexose (**1**) was possible; additional "α-β-methyl-2-deoxy-D-glucofuranoside" was prepared[6] and chromatographed on paper. From the developed chromatogram, a rough estimate of products was made, disclosing about 35% of a compound subsequently shown to be unreacted 2-deoxy-D-*arabino*-hexose (**1**) (appearing as a slow-moving spot), approximately 30% of a 1:1 mixture of the two pyranosides (**3** and **4**), and about 35% of methyl 2-deoxy-α-D-

arabino-hexofuranoside (**2**). The estimated percentage of **2** is in gross agreement with that calculated from the amount of tris-*p*-nitrobenzoate **5** recovered in our original glycosidation study; and, furthermore, **2** appeared as a single spot, uncontaminated by its anomer. Therefore, the "α,β-methyl-2-deoxy-D-glucofuranoside" of Hughes *et al.*[6] is a mixture containing only 30–35% of anomerically pure methyl 2-deoxy-α-D-*arabino*-hexofuranoside (**2**), and these results are not inconsistent with the observations[7] of Levene *et al.*

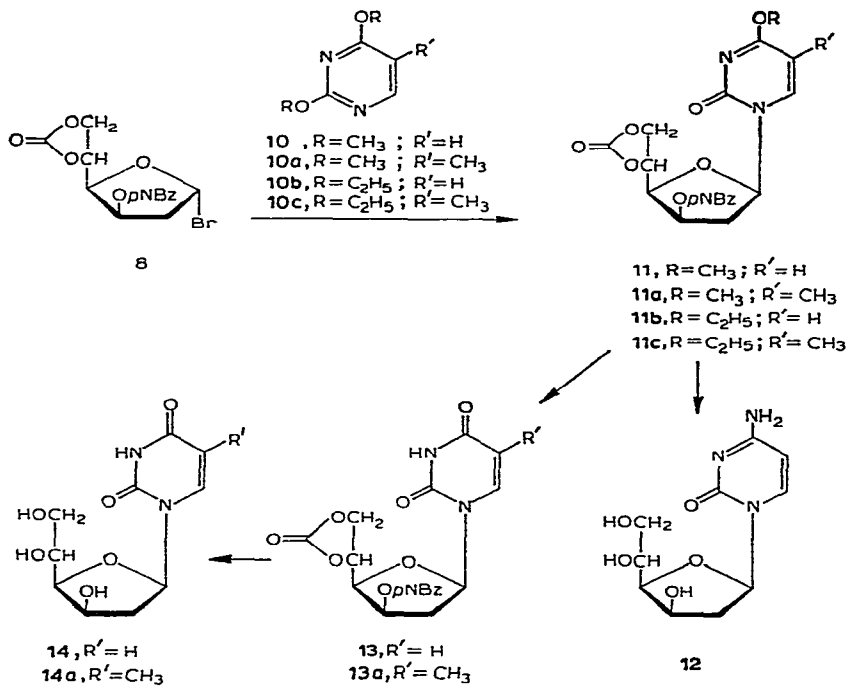
The foregoing chromatographic procedure served as an analytical method for monitoring the glycosidation of **1** during a time–yield study, and it was found that the maximum proportion of furanoside **2** was formed at 12 minutes, after which time, only a slow conversion of **1** into the pyranosides (**3** and **4**) was noted, the percentage of furanoside (**2**) being unaffected. Based on these results, preparative glycosidation studies were performed with 0.1% methanolic hydrogen chloride for a period of 12 minutes, and the purified syrup was chromatographed on a column of cellulose powder with the same solvent system used in the paper-chromatographic experiments. The amount of furanoside **2** obtained in each case by this method agreed closely with that estimated from the paper chromatograms.

Attempts to convert the tris-*p*-nitrobenzoate **5** directly into a tri-*O*-*p*-nitrobenzoylglycosyl halide were performed with hydrogen bromide in dichloromethane. When **5** was treated with one mole-equivalent of hydrogen bromide for short periods of time (15–30 min), only starting material **5** could be recovered; but, after one hour, some *p*-nitrobenzoic acid was liberated. Prolonged treatment (one hour or more) with increased proportions of hydrogen bromide always yielded one mole-equivalent of *p*-nitrobenzoic acid, affording evidence that a displacement had taken place; however, in no case was even a partial conversion into a tri-*O*-*p*-nitrobenzoylglycosyl halide realized. Inspection of a Fisher–Hirschfelder model of the tris-*p*-nitrobenzoate (**5**) showed that, when either the bulky *p*-nitrobenzoyloxy group at C-3 or the *p*-nitrobenzoyloxy group at C-6 was in apposition to C-1, the latter was almost obscured. The failure to replace the methoxyl group of **5** is, therefore, reasonable on steric grounds.

In an effort to protect O-5 and O-6 of the furanoside **2** with a group or groups having the smallest possible bulk, we investigated the preparation of the 5,6-*O*-carbonyl derivative (**6**). In contrast to our experience with the unsubstituted sugar (**1**), treatment of the furanoside (**2**) with carbonyl chloride resulted in an excellent yield of crystalline methyl 5,6-*O*-carbonyl-2-deoxy-α-D-*arabino*-hexofuranoside (**6**); *p*-nitrobenzoylation of **6** gave the high-melting, 3-*O*-*p*-nitrobenzoyl derivative (**7**). The fully protected **7** reacted readily with hydrogen bromide in dichloromethane to yield crystalline 5,6-*O*-carbonyl-2-deoxy-3-*O*-*p*-nitrobenzoyl-D-*arabino*-hexosyl bromide (**8**). The anomeric configuration of **8** has not yet been determined, but is presumed to be α-D, in which the bromine atom occupies a position *trans* to the substituted side-chain at C-4 of the furanoid ring. The new halide has excellent stability, and may be stored for short periods in a desiccator containing phosphorus pentaoxide; it reacted readily with silver *p*-nitrobenzoate to give the di-*O*-*p*-nitrobenzoyl derivative (**9**), indicating

that the halide (**8**) would have utility in the preparation of furanosyl nucleosides containing 2-deoxy-D-*arabino*-hexose (**1**) as the carbohydrate residue.

Because of the cost of the bromide (**8**), and because of the unsatisfactory yields of protected nucleosides in condensations involving the 2,4-diethoxypyrimidines (**10b** and **10c**), careful, small-scale studies were made that included other dialkoxypyrimidines. Results with 2,4-dibenzyloxypyrimidine were disappointing, and 2,4-



SCHEME 2

diallyloxypyrimidine offered no advantages over the corresponding diethoxy derivative. Condensations with 2,4-diethoxypyrimidine (**10b**) were restudied, and it was found that the optimal yield (25%) of pure, protected uracil nucleoside (**11b**) was obtained after 90 minutes at 85°. In contrast, when **8** was heated with 2,4-dimethoxypyrimidine (**10**) for only 5 minutes at 70°, 60% of an anomerically pure 1-(5,6-*O*-carbonyl-2-deoxy-3-*O*-*p*-nitrobenzoyl-D-*arabino*-hexosyl)-4-methoxy-2(1*H*)-pyrimidinone (**11**) could be obtained. Accordingly, the dimethoxy derivatives (**10** and **10a**) were used throughout.

Treatment of compound **11** with methanolic ammonia led directly to 1-(2-deoxy-D-*arabino*-hexofuranosyl)cytosine (**12**), which was secured in crystalline form in 59% yield. The demethylation of **11** was performed with 5% methanolic hydrogen chloride, and the reaction was monitored continuously by thin-layer chromatography. The reaction was complete after 6 h, and the hydrogen chloride was neutralized

with silver carbonate to avoid cleavage of the nucleoside bond during processing*; this afforded, in good yield, crystalline 1-(5,6-*O*-carbonyl-2-deoxy-3-*O*-*p*-nitrobenzoyl-D-*arabino*-hexosyl)uracil (**13**), which underwent methoxide-catalyzed de-esterification to give 85% of 1-(2-deoxy-D-*arabino*-hexofuranosyl)uracil (**14**).

The condensation of the bromide **8** with 2,4-dimethoxy-5-methylpyrimidine (**10a**) was performed under essentially the conditions given for the preparation of **11**, yielding the protected thymine nucleoside (**11a**) as crystalline material. Demethylation of **11a** gave the desired intermediate (**13a**) which, however, could not be obtained in crystalline form, even after an ultimate purification by preparative, thin-layer chromatography. The amorphous **13a**, homogeneous on chromatograms, failed to give a satisfactory elementary analysis; therefore, in subsequent experiments, compound **13a** was deacylated in methanol without regard to its purity, affording 1-(2-deoxy-D-*arabino*-hexofuranosyl)thymine (**14a**) as a crystalline product.

Because 2-deoxyaldohexofuranosyl nucleosides were heretofore unknown, assignment of the anomeric configuration of **12**, **14**, and **14a** through direct comparison of n.m.r. spectra was not possible. There is, however, no valid reason to expect that the splitting patterns for the anomeric protons of the new nucleosides would be different from those containing 2-deoxyaldopentofuranosyl residues. The patterns described by Lemieux[10] for the anomeric proton in thymidine and its anomer were a triplet for the β-D anomer and a quartet for the α-D anomer. Similar patterns for a series of anomer pairs of 2-deoxy-D-*erythro*-pentofuranosyl purine and pyrimidine nucleosides were observed by Goodman and co-workers[11]. A n.m.r. spectrum of the uracil nucleoside **14** showed a triplet pattern centered at δ 6.28 ($J_{1',2'}$ 7 Hz) for the C-1′ proton; therefore, the β-D configuration is tentatively assigned to compound **14**, from which the configuration of the cytosine nucleoside (**12**) must follow. Insufficient quantities of the corresponding thymine nucleoside (**14a**) were available for n.m.r. studies, but, because its synthesis was performed in a manner analogous to that of **14**, **14a** most probably has the β-D configuration.

EXPERIMENTAL

A Kofler hot-stage was used for determining melting points. N.m.r. spectra were measured in deuterium oxide with a Varian Model A-60 spectrometer.

Methyl 2-deoxy-3,5,6-tri-O-p-*nitrobenzoyl*-α-D-arabino-*hexoside* (**5**).—2-Deoxy-D-*arabino*-hexose (**1**) (2.5 g, 15 mmoles) was converted into a crude mixture of methyl glycosides according to the directions given[6] for "α,β-methyl-2-deoxy-D-glucofuranoside". A solution of 400 mg of the resulting syrup in 25 ml of dry pyridine was added to a solution of 3.5 g of *p*-nitrobenzoyl chloride in 20 ml of dry pyridine at 0°. The mixture was stirred for 1 h at 0°, and for 1 h at room temperature, and kept in a

*In our first condensation experiment, involving 2,4-diethoxypyrimidine (**10b**), the protected uracil nucleoside (**11b**) was de-ethylated without neutralization of the hydrogen chloride prior to evaporation of the reaction mixture. Consequently, toward the end of the evaporation, the concentration of hydrogen chloride became sufficiently high to effect cleavage of the nucleoside bond.

refrigerator for 3 days. It was then stirred for 1 h at room temperature, and slowly added, with efficient stirring, to 30 ml of saturated, aqueous sodium hydrogen carbonate. Crushed ice (300 ml) was added, the mixture was stirred until the ice had melted, and the resulting precipitate was filtered off, washed with water, and dried in a vacuum desiccator containing phosphorus pentaoxide. Three recrystallizations from acetone gave 370 mg (27%) of pure **5**, m.p. 142–144° and also 168–169°, $[\alpha]_D^{24}$ −122.3° (*c* 1.0, chloroform).

Anal. Calc. for $C_{28}H_{23}N_3O_{14}$: C, 53.72; H, 3.71; N, 6.72. Found : C, 53.70; H, 3.66; N, 6.49.

*Methyl 2-deoxy-α-*D-arabino-*hexofuranoside* (**2**). — (a) *From the tris-*p-*nitrobenzoate* (**5**). — To a suspension of 3.5 g of compound **5** in 100 ml of anhydrous methanol was added 0.23 ml of 4M methanolic sodium methoxide, the suspension was stirred for 24 h at room temperature, and the resulting solution was evaporated to dryness under diminished pressure at 40°. The residue was suspended in 25 ml of water, the suspension was extracted with three 50-ml portions of ether, the aqueous layer was stirred for 5 min with 3 g of Rexyn 300 (H^+-OH^-) mixed-bed, ion-exchange resin*, and the suspension was filtered. The filtrate was stirred with a little Darco G-60 decolorizing carbon, the suspension filtered, and the filtrate evaporated under diminished pressure at 45°. The resulting syrup (800 mg, 80%) crystallized after storage in a vacuum desiccator containing phosphorus pentaoxide; m.p. 78–79.5°. One recrystallization from ether–ethanol gave pure **2**, m.p. 80–81°, $[\alpha]_D^{24}$ +117.1° (*c* 0.99, ethanol).

Anal. Calc. for $C_7H_{14}O_5$: C, 47.19; H, 7.92. Found: C, 47.20; H, 7.82.

(b) *Direct preparation through chromatography.* To a solution of 2.462 g (15 mmoles) of 2-deoxy-D-*arabino*-hexose (**1**) in 100 ml of anhydrous methanol was added 0.3 ml of a 35% solution of hydrogen chloride in methanol. The mixture was stirred for 12 min, and the acid was neutralized with an excess (2 g) of silver carbonate. The suspension was filtered through a bed of Darco G-60 decolorizing carbon, the filtrate was concentrated to about 30 ml, stirred with 2 g of Rexyn 300 (H^+-OH^-) mixed-bed, ion-exchange resin, and the suspension was filtered. The solvent was removed by evaporation under diminished pressure at 45°, the residual syrup was dissolved in 5 ml of anhydrous methanol, and to this solution was added 100 ml of dry acetone. The resulting solution was kept in a refrigerator for two days, to allow most of the unreacted **1** to crystallize out, dry ether was added to incipient turbidity, and the mixture was kept in a refrigerator for an additional two days. The solution was decanted, the solid in the flask was rinsed with two 10-ml portions of dry methanol, and the decanted liquor and rinses were combined and evaporated to dryness. A solution of the syrupy residue in 10 ml of acetone was placed on a column (5 × 45 cm) of Whatman No. 1 cellulose powder that had been packed with acetone and prewashed with the upper layer of 10:6:1:3 (v/v) ethyl acetate–isobutyl alcohol–toluene–water. Elution was conducted with the same solvent, 3-ml fractions being collected. From the beginning, the fractions were continuously monitored by spotting on Whatman No. 1

*Fisher Scientific Company.

filter paper and testing with a boric acid spray reagent[12]. When a positive test (pink to violet coloration) was obtained, the subsequent fractions were monitored by ascending paper-chromatography with the solvent used for elution of the column. The desired furanoside (**2**) has* R_F 0.511, and the fractions containing it were combined and evaporated to dryness. The resulting syrup crystallized on standing, and recrystallization from ether–absolute ethanol gave 610 mg (23%) of pure **2**.

*Methyl 5,6-O-carbonyl-2-deoxy-α-*D-arabino-*hexofuranoside* (**6**). — A solution of 1.782 g (10 mmoles) of the furanoside **2** in 20 ml of dry pyridine and 15 ml of dry carbon tetrachloride was cooled to −10°, and to the cold solution was slowly added dropwise, with stirring, a 20% (w/w) solution (5 ml) of carbonyl chloride in dry toluene. The mixture was stirred for 1 h at −10°, and for 1 h at room temperature, and was then poured, with stirring, into a mixture of 4 g of freshly prepared barium carbonate and about 150-ml of crushed ice. The mixture was stirred until all of the ice had melted, and was then filtered through a bed of Hyflo Super Cel. The filtrate was extracted with six 150-ml portions of ethyl acetate, and the extracts were combined, dried (anhydrous sodium sulfate), and evaporated to dryness. The residue was dissolved in warm tetrahydrofuran and, on addition of pentane to incipient turbidity, the product (**6**) crystallized out; yield 1.65 g (80%), m.p. 91–92°, $[\alpha]_D^{24}$ +132.4° (*c* 1.0, ethanol).

Anal. Calc. for $C_8H_{12}O_6$: C, 47.06; H, 5.92. Found: C, 47.21; H, 6.15.

*Methyl 5,6-O-carbonyl-2-deoxy-3-*O-p-*nitrobenzoyl-α-*D-arabino-*hexoside* (**7**). — To a solution of 742 mg (4 mmoles) of *p*-nitrobenzoyl chloride in 10 ml of pyridine was added 613 mg (3 mmoles) of compound **6**. The mixture was stirred at room temperature for 1 h, and kept in a refrigerator for 24 h. The mixture was then slowly added, with stirring, to 10 ml of saturated, aqueous sodium hydrogen carbonate, and ice (about 150 ml) was next added, with stirring. When the ice had melted, the solid was filtered off, well washed with water, and dried for 24 h in a vacuum desiccator over phosphorus pentaoxide. The solid was crystallized from acetone–ether; two recrystallizations from this solvent gave 850 mg (80%) of pure **1**, m.p. 213–214°, $[\alpha]_D^{24}$ +22.4° (*c* 1.0, dichloromethane).

Anal. Calc. for $C_{15}H_{15}NO_9$: C, 51.00; H, 4.28; N, 3.96. Found: C, 51.08; H, 4.10; N, 3.89.

*5,6-O-Carbonyl-2-deoxy-3-*O-p-*nitrobenzoyl-α-*D-arabino-*hexosyl bromide* (**8**). — To a solution of 707 mg (2 mmoles) of compound **7** in 15 ml of dry dichloromethane was added 25 ml of a saturated solution of hydrogen bromide in dichloromethane. The mixture was stirred for 25 min at room temperature with rigorous exclusion of moisture, 50 ml of dry ether was added, and the mixture was kept in a refrigerator for 4 h. The crystals that separated were filtered off, and washed with dry ether, to give 650 mg (81%) of pure **8**, m.p. 125° (dec.), $[\alpha]_D^{24}$ −35.4° (*c* 0.436, acetone).

* The furanoside (**2**) is attended by some unreacted starting-material (**1**) (R_F 0.12) (because of incompleteness of precipitation in processing the reaction mixture), and substantial proportions of the α-D-pyranoside (R_F 0.391) and the β-D-pyranoside (R_F 0.325).

5,6-O-Carbonyl-2-deoxy-1,3-di-O-p-nitrobenzoyl-D-arabino-hexose (**9**). — A mixture of 75 mg (0.19 mmole) of the bromide (**8**) and 300 mg of dry silver *p*-nitrobenzoate in 20 ml of dry benzene was stirred for 24 h, and the resulting suspension was filtered through a bed of Hyflo Super Cel, followed by three 15-ml washes with dichloromethane. The filtrate was evaporated under diminished pressure at 40°, and the residue was recrystallized five times from ether–dichloromethane to give 31 mg (33%) of pure **9**, m.p. 204–205° (crystallizes again) and decomposing above 206°, $[\alpha]_D^{24}$ −3.25° (*c* 0.286, dichloromethane).

Anal. Calc. for $C_{21}H_{16}N_2O_{12}$: C, 51.64; H, 3.30; N, 5.74. Found: C, 51.82; H, 3.33; N, 5.65.

1-(5,6-O-Carbonyl-2-deoxy-3-O-p-nitrobenzoyl-β-D-arabino-hexosyl)-4-methoxy-2(1H)-pyrimidinone (**11**). — A mixture of 302 mg (0.75 mmole) of the halide **8** and 2.5 g (17.6 mmoles) of 2,4-dimethoxypyrimidine (**10**) was heated for 5 min at 75°/20 mm Hg, and then kept overnight at room temperature and atmospheric pressure. To the mixture was added 20 ml of ether, and the solid was crushed, repeatedly washed with ether, and filtered off. It was dissolved in 10 ml of dichloromethane, and on addition of ether, a gel-like mass separated; the crystalline mass (with solvent entrapped) was filtered off, washed with ether, and dried by suction. Recrystallization from ether–dichloromethane gave 205 mg (60%) of pure **11**, m.p. 209–211°, $[\alpha]_D^{24}$ −7.35° (*c* 0.55, dichloromethane).

Anal. Calc. for $C_{19}H_{17}N_3O_{10}$: C, 51.01; H, 3.83; N, 9.39. Found: C, 51.27; H, 3.63; N, 9.36.

1-(5,6-O-Carbonyl-2-deoxy-3-O-p-nitrobenzoyl-β-D-arabino-hexosyl)-4-ethoxy-2-(1H)-pyrimidinone (**11b**). — A mixture of 125 mg of the bromide **8** and 800 mg (4.76 mmoles) of 2,4-diethoxypyrimidine (**10b**) was heated (with exclusion of moisture) for 2 h at 85°. The gel-like mixture was kept overnight at room temperature, and then stirred with 25 ml of ether and filtered. The precipitate was washed with three 10-ml portions of ether, dried overnight in a vacuum desiccator containing phosphorus pentaoxide, and dissolved in dry dichloromethane. On addition of ether, a gel formed, with crystals entrapped; these were filtered off by suction, which simultaneously destroyed the gel. Four additional recrystallizations from ether–dichloromethane gave 25 mg (17% based on the bromide **8**) of pure **11b**, m.p. 223–224.5°, $[\alpha]_D^{24}$ −7.4° (*c* 1.54, dichloromethane).

Anal. Calc. for $C_{20}H_{19}N_3O_{10}$: C, 52.07; H, 4.15; N, 9.11. Found: C, 52.21; H, 4.22; N, 9.09.

1-(2-Deoxy-β-D-arabino-hexofuranosyl)cytosine (**12**). — A suspension of 224 mg (0.5 mmole) of the protected nucleoside **11** in 20 ml of dry methanol presaturated with ammonia was placed in a pressure bottle and heated for 9 h at 85°. It was then kept overnight at room temperature, and the solvent was evaporated off under diminished pressure. The residue was mixed with 10 ml of water and 10 ml of chloroform, the mixture was shaken, the chloroform layer was discarded, and the water layer was successively washed with 10 ml of chloroform and two 10-ml portions of ether. The aqueous solution was evaporated to dryness at 45° under diminished pressure, the

residue was dissolved in 1 ml of water, and to the solution were added 2 ml of ethanol and a little Darco G-60 decolorizing carbon . The suspension was then stirred and filtered. The filtrate was evaporated to dryness at 45° under diminished pressure, the residue was dissolved in a few drops of water, and 2 ml of ethanol was added. On addition of ether, the nucleoside crystallized out; two similar recrystallizations gave 76 mg (59%) of pure **12**, m.p. 217–218°, $[\alpha]_D^{24}$ −4.10° (*c* 0.95, water), λ_{max}^{MeOH} 274 nm (log ε 3.91).

Anal. Calc. for $C_{10}H_{15}N_3O_5$: C, 46.69; H, 5.88; N, 16.33. Found: C, 46.96; H, 6.07; N, 16.13.

1-(5,6-O-Carbonyl-2-deoxy-3-O-p-nitrobenzoyl-β-D-arabino-hexosyl)uracil (**13**). — To a solution of 448 mg (1 mmole) of the protected nucleoside **11** in 10 ml of dichloromethane and 10 ml of methanol was added 10 ml of methanol presaturated with hydrogen chloride (34%), and the mixture was stirred for 6 h at room temperature. The acid was neutralized with an excess of silver carbonate (15 g), and the suspension was filtered; the filtrate was evaporated to dryness, and the residue was crystallized from ethanol–water to yield 330 mg* (76%) of **13**, m.p. 138–140°.

Anal. Calc. for $C_{18}H_{15}N_3O_{10}$: C, 49.89; H, 3.49; N, 9.70. Found: C, 49.83; H, 3.70; N, 9.68.

1-(2-Deoxy-β-D-arabino-hexofuranosyl)uracil (**14**). — To a solution of 175 mg (0.4 mmole) of compound **13** in 20 ml of dry methanol was added 0.20 ml of 4M methanolic sodium methoxide and the mixture was stirred for 2 h at room temperature. Acetic acid (0.2 ml) was added, and the solvent was evaporated off under diminished pressure. The residue was mixed with 15 ml of water and 15 ml of ether, and the mixture was shaken; the ether layer was discarded, and the aqueous layer was washed with three 15-ml portions of ether, and stirred with 2 g of Rexyn 300 (H^+-OH^-) mixed-bed, ion-exchange resin for 10 min, and the suspension was filtered. The resin was stirred with 10 ml of water containing 1 ml of acetic acid, the suspension was filtered, and the two filtrates were combined, stirred with a little Darco G-60 decolorizing carbon, and the suspension filtered. The filtrate was evaporated to dryness at 45° under diminished pressure, the residue was dissolved in 5 ml of ethanol, and ether was added to incipient turbidity. The mixture was kept for 4 h at room temperature, pentane (5 ml) was added, and the mixture was kept in a refrigerator overnight. The resulting crystals were filtered off, and washed with ether; yield of **14**, 88 mg (85%), m.p. 180–182°, $[\alpha]_D^{24}$ −16.8° (*c* 1.1, water), λ_{max}^{MeOH} 208 nm (log ε 3.90).

Anal. Calc. for $C_{10}H_{14}N_2O_6$: C, 46.51; H, 5.46; N, 10.85. Found: C, 46.64; H, 5.60; N, 10.78.

1-(5,6-O-Carbonyl-2-deoxy-3-O-p-nitrobenzoyl-β(?)-D-arabino-hexosyl)-4-methoxy-5-methyl-2(1H)-pyrimidinone (**11a**). — To 2.16 g(14 mmole) of molten 2,4-dimethoxy-5-methylpyrimidine (**10a**) was added 525 mg (1.3 mmoles) of the bromide **8**, and the mixture was heated for 10 min at 75° and cooled. It was then crushed, and stirred with 50 ml of ether; the crude product was filtered off, washed with three 30-ml

*The optical rotation of compound **13** was not determined.

portions of ether, and dried overnight in a vacuum desiccator containing phosphorus pentaoxide. It was recrystallized from ether–dichloromethane, to give 150 mg (26%) of the protected nucleoside (**11a**), m.p. 195–197°. An analytical sample was prepared by recrystallization from acetone–ether; m.p. 203–205°, $[\alpha]_D^{24}$ −6.5° (*c* 0.337, dichloromethane).

Anal. Calc. for $C_{20}H_{19}N_3O_{10}$: C, 52.07; H, 4.15; N, 9.11. Found: C, 51.83; H, 4.16; N, 9.26.

*1-(5,6-O-Carbonyl-2-deoxy-3-*O*-p-nitrobenzoyl-β(?)-*D*-arabino-hexosyl)-4-ethoxy-5-methyl-2(1H)-pyrimidinone* (**11c**). — To 912 mg (5 mmoles) of molten 2,4-diethoxy-5-methylpyrimidine (**10c**) was added 125 mg (0.31 mmole) of the bromide **8**, and the mixture was heated, with exclusion of moisture, for 2 h at 85°. The mixture was kept overnight at room temperature, and then crushed and stirred with 30 ml of ether, and the solid was filtered off. It was washed with three 20-ml portions of ether, dried for 2 h in a vacuum desiccator containing phosphorus pentaoxide, and recrystallized four times from ether–dichloromethane to yield 14 mg (10%) of pure **11c**, m.p. 204–205°, $[\alpha]_D^{24}$ −3.3° (*c* 0.16, dichloromethane).

Anal. Calc. for $C_{21}H_{21}N_3O_{10}$: C, 53.06; H, 4.45; N, 8.84. Found: C, 52.82; H, 4.25; N, 8.80.

*1-(2-Deoxy-β(?)-*D*-arabino-hexofuranosyl)thymine* (**14a**). — To a solution of 110 mg (0.24 mmole) of the protected nucleoside **11a** in 8 ml of dichloromethane and 5 ml of anhydrous methanol was added 5 ml of methanolic hydrogen chloride (34%). The mixture was stirred, with exclusion of moisture, for 6 h at room temperature, the acid was neutralized with an excess of silver carbonate (12 g), the mixture was filtered through a bed of decolorizing carbon, and the filtrate was evaporated to dryness. The residue was purified by preparative, thin-layer chromatography on silica gel (Camag DF-5) plates by the ascending technique, with the upper layer of a solvent mixture of 10:6:5:3 (v/v) ethyl acetate–methanol–water–2,2,4-trimethylpentane. The zones containing the product (chief component) were located by means of ultraviolet light, scraped off the plates, and combined. The adsorbent–adsorbate thus collected was thoroughly extracted with dichloromethane, and the extract was filtered and evaporated to dryness. The residue failed to crystallize from a wide variety of solvents and solvent mixtures; nevertheless, the demethylated nucleoside (**13a**) thus purified was homogeneous on thin-layer chromatograms in the foregoing solvent system and in one reported by Goodman and co-workers[11].

The purified **13a** (60 mg, 0.134 mmole) was suspended in 20 ml of anhydrous methanol, 0.2 ml of 4M sodium methoxide in methanol was added, and the mixture was stirred for 6 h at room temperature. Acetic acid (0.2 ml) was added, the solution was evaporated to dryness, and the residue was mixed with 15 ml of water and 15 ml of ether. The ether layer was discarded, and the aqueous layer was washed with three 15-ml portions of ether, and stirred with 2 g of Rexyn 300 (H^+-OH^-) mixed-bed, ion-exchange resin, and the mixture was filtered through a thin bed of Darco G-60 decolorizing carbon. The resin was suspended in 15 ml of water containing 0.5 ml of acetic acid, and the suspension was stirred for 10 min, and filtered off through a bed of Darco

G-60 decolorizing carbon as before. The two filtrates were combined, and evaporated to dryness at 45° under diminished pressure; the residue was dissolved in 3 ml of absolute ethanol, ether was added to incipient turbidity, and the solution was kept for 24 h in a refrigerator. The resulting crystals were filtered off and washed with ether, to give 30 mg (80%) of pure **14a**, m.p. 166–168°, $[\alpha]_D^{24}$ −10.5° (*c* 0.10, water), λ_{max}^{MeOH} 268 nm (log ε 4.11).

Anal. Calc. for $C_{11}H_{16}N_2O_6$: C, 48.53; H, 5.92; N, 10.29. Found: C, 48.65; H, 6.07; N, 9.96.

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STUDIES WITH RADIOACTIVE SUGARS

PART I. ASPECTS OF THE ALCOHOLYSIS OF D-XYLOSE AND D-GLUCOSE; THE ROLE OF THE ACYCLIC ACETALS

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ABSTRACT

Examination of the early stages of the acid-catalysed methanolysis of D-xylose and D-glucose, using radioactive sugars, has shown that the hitherto undetected dimethyl acetals are formed, but that they are not primary products, and their concentrations do not exceed 2.5% at any stage. The possible mechanisms of the first step in glycosidation are discussed, and the suggested retention of anomeric configuration occurring during the ring expansion of furanosides is reconsidered.

INTRODUCTION

The occurrence of multiple products in glycosidations of sugars makes these reactions appreciably more difficult to study than the converse hydrolyses of glycosides, and this is reflected in the attention that the two processes have received. We here describe experiments, using radiochemical techniques, planned to provide a fuller understanding of the alcoholysis of D-xylose and D-glucose.

As has been known for many years[1], furanosides as well as pyranosides are formed during acid-catalysed alcoholysis of sugars. In addition, acyclic acetals and hemiacetals are possible reaction products or intermediates, and although the former were proposed by Fischer[2] as likely precursors of the glycosides, they escaped detection until Heard and Barker[3] recorded the presence of D-arabinose dimethyl acetal in the methanolysis products of this sugar*. We now report that the corresponding derivatives feature in the glycosidation of D-xylose and D-glucose. Hemiacetals have not been detected but cannot, on this evidence, be discounted as possible reaction intermediates.

Following Fischer's early work[1,2], Levene and his associates[4] studied the glycosidation of several aldoses, using analytical methods that depended upon the preferential susceptibility of furanosides to acid hydrolysis, and showed that these five-membered cyclic compounds were formed at an early stage and subsequently underwent ring

*These results will be published in full in J. Org. Chem. The conclusions reached on the role of the D-arabinose acetal are effectively the same as those arrived at for the D-xylose acetal in this paper. We are grateful to Drs. Heard and Barker for allowing us to examine their results prior to publication.

expansions to give the thermodynamically more-stable pyranosides. Anomers could not be differentiated by these means, but the introduction of chromatographic methods made this possible, and Mowery and Ferrante[5], using cellulose-column fractionations, first applied them to gain a fuller understanding of the glycosidation of D-galactose. With the aid of much more sensitive and convenient g.l.c. techniques, Bishop and Cooper[6,7] later carried out detailed, elegant examinations of the methanolyses of the pentoses and were able to analyse the overall processes in terms of four distinct, successive reactions: (*i*) the formation of furanosides, (*ii*) the anomerisation of furanosides, (*iii*) the ring expansion of furanosides, and (*iv*) the anomerisation of pyranosides. They obtained no evidence of acyclic compounds, but these, under the conditions of the chromatography, may not have been detectable, so that arguments based on their apparent absence[6] could be invalidated.

Whereas, in their studies with radioactive L-arabinose, Heard and Barker[3] used dilution techniques to analyse for the acetal, the procedure adopted in this work is one that has found extensive use in biochemistry[8] and involves the resolution of the reaction components on paper chromatograms, their detection by radioautography, and their direct quantitative determination with an end-window Geiger counter.

RESULTS AND DISCUSSION

In Fig. 1 is shown a radioautograph obtained during the study of the methanolysis of D-xylose. Under the chromatographic conditions employed, the two furanosides are separated, but the pyranosides are unresolved, and a fifth product is readily observable. That this was D-xylose dimethyl acetal was shown by carrying out two parallel methanolyses that were identical, except that, in one, ^{14}C was incorporated in the sugar, whereas, in the other, the methanol was labelled. In relative terms, twice the activity was incorporated into the fifth component in the second experiment, showing that it arises from condensation of two molecules of alcohol per D-xylose molecule. Confirmation of the nature of this fifth product was obtained by eluting it from the chromatography paper, mixing it with radio-inactive D-xylose dimethyl acetal (obtained by hydrogenolysis of the 2,4:3,5-di-*O*-benzylidene derivative[9]), carrying out two-dimensional chromatography on the mixture, and observing that the active and inactive samples had indistinguishable mobilities.

From Fig. 2, which illustrates the observed general course of the methanolysis of D-xylose, it can be seen that the two furanosides are formed first, that the β-anomer preponderates over the α-form, and that, latterly, ring expansion occurs to give pyranosides that constitute 95% of the reaction products at equilibrium. These results agree qualitatively with the findings of Bishop and Cooper[6,7] (who used twice the concentration of D-xylose but otherwise the same conditions), but there are quantitative differences between the results obtained by the two methods: the "equilibrium" α, β-furanoside-ratio pertaining during the ring-expansion stage was found to be 1:1.72 by the g.l.c. method[6] but 1:1.2 [confirmed in a duplicate experiment and by carrying out methanolysis of radioactive α- and β-furanosides] in this radio-

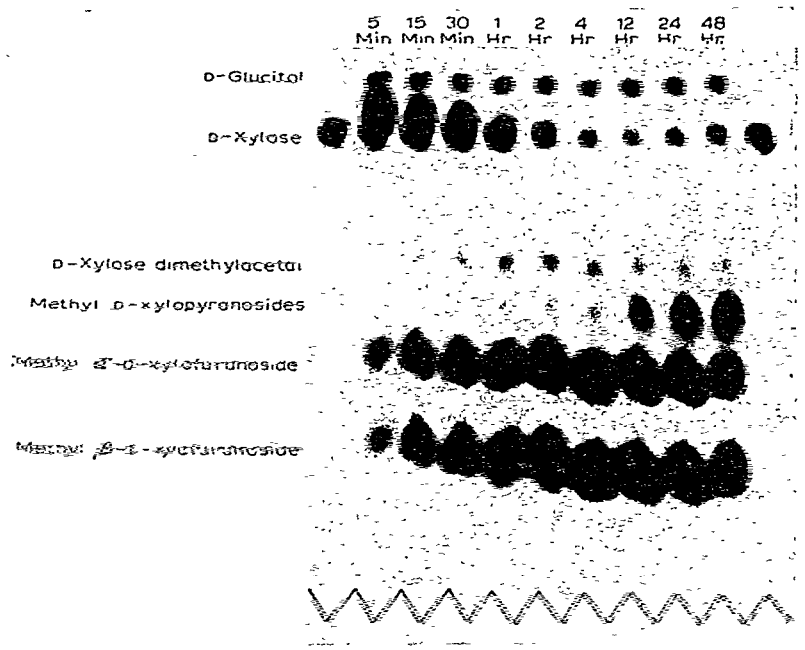


Fig. 1. Radioautograph showing the chromatographically resolved components in the reaction between D-xylose (1%) and methanolic hydrogen chloride (0.5%) at 25°. Radioactive D-glucitol was added as a reference compound.

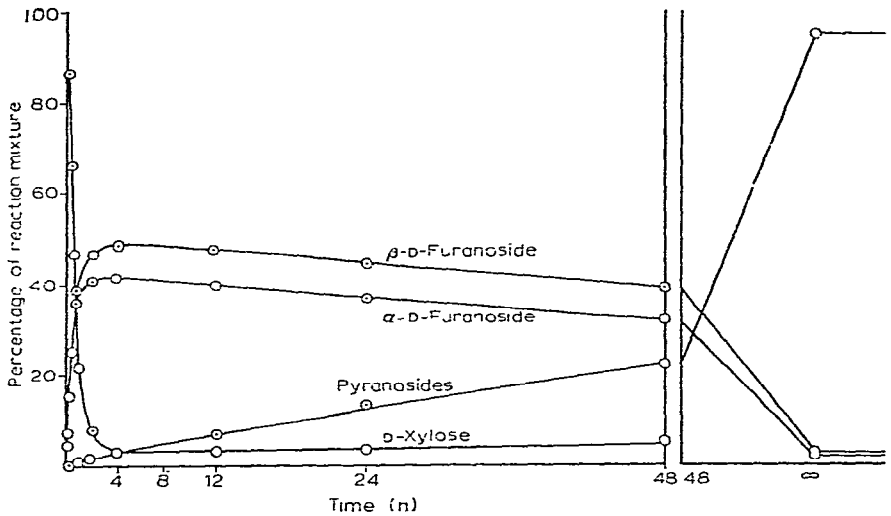


Fig. 2. Components in the reaction between D-xylose (1%) and methanolic hydrogen chloride (0.5%) at 25°.

chemical work, and, whereas we find the maximum percentages of α- and β-furanosides to be 42 and 49, respectively, Bishop and Cooper[6] found these to be *ca.* 35 and 62,

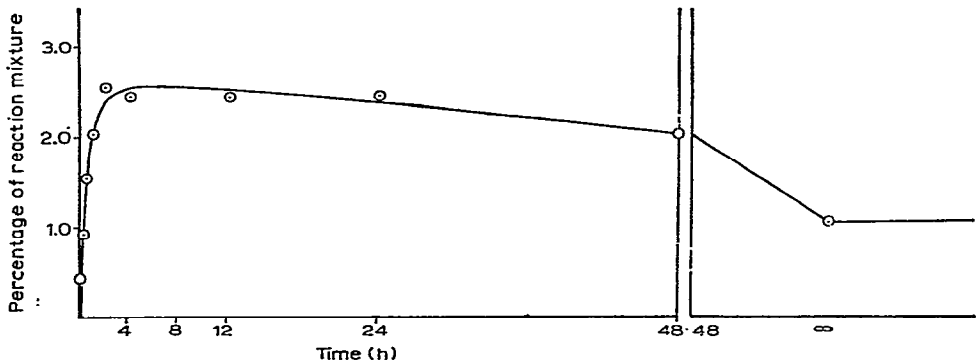


Fig. 3. The concentration of D-xylose dimethyl acetal in the reaction between D-xylose (1%) and methanolic hydrogen chloride (0.5%) at 25°.

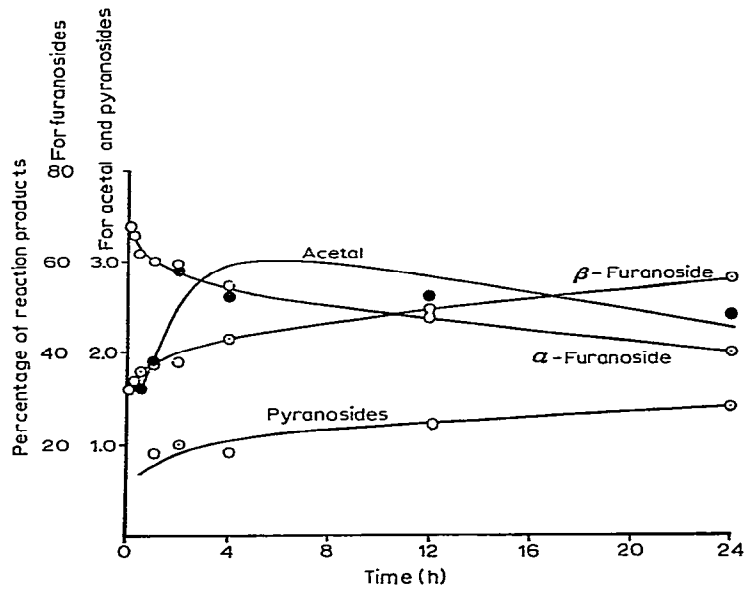


Fig. 4. The first products of the reaction between D-xylose (1%) and methanolic hydrogen chloride (0.01%) at 35°.

respectively. At this stage, the pyranoside and acetal concentrations are both 2.5%, and unreacted D-xylose accounts for the remainder. Likely sources of errors (and their corrections) in the radiochemical procedures are referred to in the Experimental section.

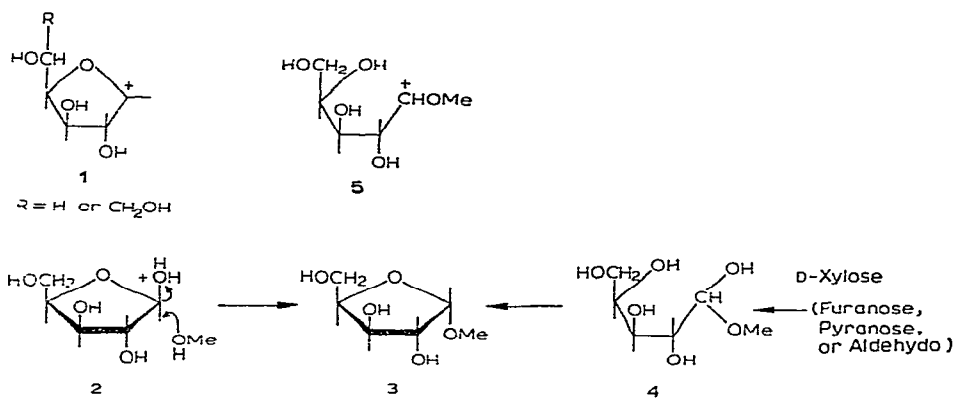
As is shown in Fig. 3, the concentration of the acetal builds up to 2.5% at the time when the furanosides are present in maximum proportions, and, with them, it then subsides as the pyranosides are formed, until, at equilibrium, 0.5% remains.* So that the early stages of the reaction could be examined, the methanolysis was

*It is not clear why this figure is so high; it should be *ca.* 0. 15% if the acetal and the furanosides are equilibrated throughout.

repeated under extremely mild conditions, and the products formed initially were considered in detail. Extrapolations of the proportions of furanosides and acetal in the products (Fig. 4) show that the α-D-glycoside, despite the fact that it is less stable than the β-D form (see Fig. 2), is formed initially in greatest proportions (α:β,2.1:1), and suggest that the acetal is not the primary product from which the furanosides are derived. However, the acetal methanolyses at a rate in excess of that of D-xylose itself, so no large, initial concentration could be expected if it was a key intermediate.

Brief examination of the early stages of the methanolysis of D-glucose revealed that, as with D-xylose, the furanosides are formed first [with the α-isomer preponderating (extrapolated, initial α:β ratio = 2.2:1)], that the acetal is formed in minor proportions, and that, even at very early stages, it is not a major component. Glycosidations of the two sugars were found to be similar in form, and in neither case did the acetal concentration exceed 2.5% at any time.

The extrapolated, initial ratio (68:32) of α- and β-D-xylofuranosides compares well with that (α:β, 65:35) obtained[7] by gas–liquid chromatographic analysis of products formed after 4.8% reaction, and, together with similar observations with D-glucose, suggest that furanosyl carbonium ions (**1**) are not involved in furanoside formation, since the C-2 hydroxyl group would be expected to shield the "α-side" of C-1 and cause preferential solvolytic attack to give β-D-glycosides. Alternatively, the initial step in glycosidation could involve a synchronous process (**2**→**3**) that would satisfy the initial finding of preponderant amounts of α-D-furanoside, since the β-D modification of the furanoid free sugar would be expected to preponderate in solution. Furthermore, the anomerisation of methyl β-D-glucofuranoside in [^{14}C]-methanol leads to labelled furanosides[10], and additional support comes from studies of the ethanethiolysis of D-xylose[11]. However, a referee has kindly pointed out that **2**→**3** is unlikely to represent a major pathway, since the rate of formation of furanosides from D-xylose (which would have a low furanose concentration in methanol) has been found to be similar to the rate of anomerisation of the methyl D-xylofuranosides[7].



A further alternative is that the furanosides could be formed by way of the acyclic hemiacetals (**4**→**3**) which can arise from the aldehydo sugar by alcoholation

or from any of the cyclic modifications by protonation of the ring oxygen and either by a synchronous displacement reaction at C-1 or after a primary ring-opening step. Evidence which lends support to the possibility of an acyclic hemiacetal intermediate was obtained by finding that methanolysis of D-xylose dimethyl acetal (radioactive) occurs with great facility to give, initially, the α- and β-D-furanosides in the same proportions (2.1:1) as does D-xylose itself, and both compounds could react by way of the acyclic ion **5**. However, it has been shown that D-glucose dimethyl acetal reacts during hydrolysis to the methyl furanosides by a concerted process and not by the intermediacy of an acyclic ion[12], so the identity of the ratios may be fortuitous and cannot be taken as providing strong support for the existence of the hemiacetal. If a concerted mechanism operates, it would seem improbable that the acetal on the one hand, and diastereoisomeric hemiacetals on the other, would give the furanosides in the same proportions. The hemiacetals were not detected at any stage in the investigation, although they would have been separable from the other reaction components and would have been observed in concentrations of *ca.* 0.3%, but their expected high reactivity in the methanolysis medium or during subsequent operations could account for their apparent absence.

Capon and Thacker[13] in their studies of the acid-catalysed hydrolysis of furanosides found negative entropies of activation for the reactions and concluded that, as the corresponding functions for pyranoside hydrolyses are positive, the two classes of glycosides react by different mechanisms, and consequently that furanoside hydrolyses do not involve ions of the type **1**. No evidence was obtained which allowed a distinction to be made between the other possible mechanisms and so, as in the present work, no final conclusion could be reached.

Bishop and Cooper[6], on finding that the methyl α- and β-D-xylopyranosides were produced in the ratio 1:1.7 [*i.e.*, the equilibrium ratio determined by them for the furanosides (see above)], concluded that ring expansion occurs with retention of configuration at the anomeric centre. This deduction has been justifiably criticised[10] on the grounds that furanoside anomerisation is rapid compared with the rate of the ring-expansion reaction, but it would be valid in the event of the ring-expansion rates of the two furanosides being identical. The current re-investigation of the methanolysis of D-xylose did not allow a further examination of this point, since the methyl D-xylopyranosides were chromatographically indistinguishable under the conditions used. However, the four ethyl D-xylosides were readily resolvable, so that an examination of the ethanolysis of radioactive D-xylose allowed the ring-expansion reaction to be followed in detail. The overall glycosidation was closely similar to the methanolysis: the α- and β-D-furanosides were formed initially in the ratio 2.6:1, they anomerized to give an "equilibrium" ratio of 1:1.3 (average of 8 measurements; variation, ±0.1), they then underwent ring expansion to α- and β-D-pyranosides formed in the ratio 1:1.9 (average of 8 measurements; variation, ± 0.1), and the pyranosides anomerised to give the final equilibrium mixture. In this reaction, therefore, the α,β ratio changes appreciably during ring expansion, but no information on the stereochemistry of the process can be deduced from this.

No evidence was obtained for the presence of the diethyl acetal in the reaction since, in the chromatographic solvent employed, it was unresolvable from ethyl α-D-xylofuranoside. The four ethyl D-xylosides prepared during this work are described.

In agreement with the results obtained during the g.l.c. studies[6,7], these observations show that in the formation stages of both furanosides and pyranosides of D-xylose it is the thermodynamically unstable anomers which are formed preferentially, and it is therefore surprising that Capon *et al.*[10] should have observed that ring expansions of the methyl D-glucofuranosides resulted initially in the formation of appreciably more α- than β-D-pyranosides.

EXPERIMENTAL

[^{14}C]-D-Xylose (U), [^{14}C]-D-glucose (U), and [^{14}C]-methanol were obtained from the Radiochemical Centre, Amersham, England, at specific activities of 3.4, 8.5, and 9.2 mcuries/mmole, respectively.

Glycosidations of D-xylose were carried out in the presence of known amounts of radioactive D-glucitol which, as an internal inert reference compound, was readily resolvable from reaction components, and could be used to interrelate the activities present in each sample. Aliquot portions of solution were neutralised with Deacidite FF (OH^-) resin and were applied to Whatman No. 1 paper chromatograms developed in butanone saturated with water. Developing times were 12 and 8 h for the methanolysis and ethanolysis of D-xylose, and 30 h for the methanolysis of D-glucose. The papers were carefully dried in a still atmosphere, and the components were located using Kodak standard X-ray film. The reaction products were identified by their chromatographic mobilities relative to those of inactive samples.

Counting was performed on one side of the chromatograms by using a standardised end-window Geiger tube (General Electric Company, type 2B2) which gave a linear response up to 40,000 counts/min and had a reproducibility within one standard deviation for counts less than 2,500 min, and within 2% for counts greater than this value. Chromatographic sections larger than the uniformly sensitive area of the window (3.5 cm in diameter), and those showing activity greater than 40,000 counts/min were counted in portions. Overall counting efficiency was *ca.* 3%, whereas the counter itself had an efficiency of *ca.* 10%, but it was observed from standardisation experiments that the measured activity of a sample diminished slightly as the distance travelled on a chromatogram increased. In five experiments, the mean losses in activity were: 4% over 5 cm (measured from the position of first counting, which was 5 cm from the origin), 4.8% over 12 cm, 6.4% over 20 cm, and 6.5% over 32 cm. Corrections were applied throughout to compensate for these. Further corrections were applied to compensate for "trailing", *i.e.*, for small amounts of activity (*ca.* 1% per area of average spot) remaining on paper over which an active component had passed.

Since it has been shown that isomeric glycosides can be separated on the basis of their selective adsorbtion on anionic resin[14], the errors associated with the use of resin in the neutralisation step were assessed. Alcohol solutions containing mixtures

of methyl α- and β-D-furanosides and ethyl α- and β-D-pyranosides of known radiochemical composition were separately treated with resin as in the neutralisation procedure and were then analysed by chromatographic separation, radioautographic detection, and counting. Slight selectivity was shown for the α-D-furanoside and the β-D-pyranoside, *i.e.*, the chromatographically less-mobile anomers, so that the true equilibrium ratio of α- and β-D-furanosides is 1:1.1–1.2, and that of the α- and β-D-pyranosides is 1:1.9–2.1. Free D-xylose was adsorbed appreciably more selectively than the glycosides (in one competitive experiment, 76% of D-xylose and 25% of glycoside were adsorbed respectively) which adds justification to the corrections applied in Table I. It is concluded that all of the results are subject to small errors rising from this factor, but that these do not alter the findings in any appreciably way.

Methanolysis of D-*xylose.* — (*a*) Conditions similar to those used by Bishop and Cooper[6] were selected; the D-xylose concentration, however, was reduced from 2 to 1% to overcome solubility difficulties. D-Xylose (10 mg, containing 10 μcuries activity) and D-glucitol (0.25 mg, containing 0.5 μcurie activity) were dissolved in methanol (0.5 ml) to which was added methanolic hydrogen chloride (0.5 ml, 1.0%), and the solution was kept at 25°. Samples (0.10 ml) were withdrawn, treated with resin (*ca.* 6 mg), and chromatographed (Fig. 1). Results of radioassay of the components are given in Table I and are represented graphically in Figs. 2 and 3. Equilibrium values were determined in a separate experiment carried out at elevated temperatures.

TABLE I

ACTIVITIES (COUNTS/MIN)[a] OF THE RESOLVED COMPONENTS FROM THE METHANOLYSIS OF D-XYLOSE AT 25 (D-XYLOSE, 1%; HYDROGEN CHLORIDE, 0.50%).

Compound	*Time (h)*							
	0.083	*0.25*	*0.5*	*1*	*2*	*4*	*12*	*24*
D-Xylose	67,510	49,760	36,290	18,760	6,870	2,760	2,750	2,800
D-Xylose (corrected)[b]	74,325	56,235	39,710					
Acetal	305	790	1,280	1,660	2,210	2,050	2,030	1,990
Pyranosides	130	375	450	905	1,620	2,530	5,880	11,020
α-D-Furanoside	6,480	14,600	21,750	29,780	35,850	36,050	33,280	31,300
β-D-Furanoside	3,760	13,000	21,610	32,340	41,000	41,760	40,080	37,720
Total counts (uncorrected)	78,185	78,525	81,380	83,445	87,550	85,150	84,020	84,830

[a]All counts are corrected for paralysis, for the background count, and for distance travelled on the chromatogram (see above), and have been standardised by using factors (between 0.85 and 1.15) obtained from the measured activities of D-glucitol. [b]The deficiencies in the total activities measured in the early samples are assumed to arise as a result of specific adsorptions of free sugar on the resin. Corrections are applied to the D-xylose figures to bring the total count to 85,000, *i.e.*, the average obtained from the later samples.

(*b*) The experiment was repeated at 35° using D-xylose (10 mg, 5 μcuries), D-glucitol (0.25 mg, 0.5 μcurie), and methanolic hydrogen chloride (1 ml, 0.01%). Only small quantities of resin were required (*ca.* 0.2 mg) to neutralise the acid in 0.1-ml

samples, and errors arising from specific adsorptions of free sugar are ignored. The results are summarised in Table II and are illustrated in Fig. 4.

Ethanolysis of D-*xylose.* — (*a*) D-Xylose (2.5 mg, 5 μcuries) and D-glucitol (0.5 mg, 1.0 μcurie) were dissolved in ethanolic hydrogen chloride (1 ml, 0.5%) and

TABLE II

ACTIVITIES (COUNTS/MIN)[a] OF THE RESOLVED COMPONENTS FROM THE METHANOLYSIS OF D-XYLOSE AT 35° (D-XYLOSE, 1%; HYDROGEN CHLORIDE, 0.01%).

Compound	*Time (h)*							
	0.083	*0.25*	*0.5*	*1*	*2*	*4*	*12*	*24*
Acetal	0	0	24	58	160	295	620	1,020
Pyranosides	0	0	0	28	57	97	280	610
α-D-Furanoside	156	500	920	1,800	3,260	5,970	11,260	17,140
β-D-Furanoside	74	255	529	1,140	2,075	4,760	11,710	23,800
Percentage reaction	0.4	1.2	2.3	4.9	8.3	21.6	55.0	82.1

[a]All counts are corrected for coincidence, for background count, and for distance travelled on chromatograms, and have been standardised by using factors (between 0.80 and 1.20) obtained from the measured activities of D-glucitol.

kept at 35°. Mobilities of the products, relative to that of D-xylose were: β-D-pyranoside, 4.9; α-D-pyranoside, 5.6; α-D-furanoside, 6.4; β-D-furanoside, 7.5; (diethyl acetal, 6.2). Results are given in Table III.

TABLE III

PERCENTAGES AND RATIOS OF GLYCOSIDES IN THE PRODUCTS OF ETHANOLYSIS OF D-XYLOSE AT 35° (D-XYLOSE, 0.2%; HYDROGEN CHLORIDE, 0.5%)

Glycoside	*Time (h)*							
	2	*4*	*6*	*12*	*24*	*48*	*72*	*120*
β-D-Pyranoside	2.1	4.7	6.9	13.0	22	41	49	56
α-D-Pyranoside	1.2	2.2	3.6	6.7	13	21	26	32
α-D-Furanoside	43	40	40	34	29	17	11	5
β-D-Furanoside	54	53	50	47	36	22	14	7
β-:α-D-Pyranosides	1.8	2.1	1.9	1.7	1.7	2.0	1.9	1.8
β-:α-D-Furanosides	1.3	1.3	1.25	1.4	1.2	1.3	1.3	1.4

(*b*) In an experiment using D-xylose (2.5 mg, 1 μcurie), D-glucitol (0.25 mg, 0.5 μcurie), and ethanolic hydrogen chloride (1 ml, 0.01%) carried out at 35°, the α-:β-D-furanoside ratio observed when 1% reaction had occurred (5 min) was 2.6:1, and equilibration of the furanosides occurred in 48 h, by which time the β:α ratio was 1.3:1.

Methanolysis of [^{14}C]-*methyl* D-*xylofuranosides.* — Radioactive furanosides were obtained in >98% radiochemical purity by elution from paper chromatograms. Samples (*ca.* 0.5 μcurie) were treated with methanolic hydrogen chloride (0.5 ml, 0.01%) at 35°. "Equilibria" were established within 24 h, and at that time the products

from the α-D-glycoside contained D-xylose, 5% (presumably formed by hydrolysis caused by traces of water); acetal, 3.1%; α-D-furanoside, 41%; and β-D-furanoside, 51%. The corresponding figures obtained in the reaction of the β-D-glycoside were 4.6, 2.4, 41, and 52%. At no time did the acetal concentration exceed 3%.

Methanolysis of [^{14}C]-D-*xylose dimethyl acetal.* — The radioactive acetal (0.001 g, containing 0.5 μcurie activity) and D-glucitol (0.0004 g, 0.5 μcurie) were dissolved in methanolic hydrogen chloride (0.2 ml, 0.005%) at 25°. Samples taken after 1, 5, 15, and 30 min were found to have undergone conversions into furanosides to the extent of 49, 79, 95, and 96%. Th α:β furanoside ratios at these times were 2.1, 2.0, 2.1, and 1.8.

Methanolysis of D-*glucose.* — D-Glucose (4 mg containing 5 μcuries activity) was dissolved in methanol (0.5 ml), methanolic hydrogen chloride (0.5 ml, 0.02%) was added, and the solution was maintained at 35°. The products had the following mobilities on chromatograms relative to D-glucose: α-D-pyranoside, 2.6; β-D-pyranoside, 2,6; acetal, 3.8; α-D-furanoside, 5.8; β-D-furanoside, 6.5. The acetal was identified by two-dimensional cochromatography with a crystalline, authentic sample (see below). Results of the early stage of the reaction are given in Table IV.

TABLE IV

PERCENTAGE OF COMPONENTS IN THE METHANOLYSIS OF D-GLUCOSE AT 35° (D-GLUCOSE, 0.25%; HYDROGEN CHLORIDE, 0.01%).

Compound	*Time (h)*							
	1	*2.5*	*5*	*9*	*24*	*72*	*120*	*144*
D-Glucose	98.1	96.5	92.2	88.3	74	52	48	50
Pyranosides	0.1	0.1	0.3	0.4	0.7	1.4	1.6	1.6
Acetal	<0.05[a]	<0.1[a]	<0.1[a]	0.15	0.5	0.8	0.9	1.0
α-D-Furanoside	1.1	2.0	4.0	5.5	11	18	19	18
β-D-Furanoside	0.6	1.3	3.4	5.7	14	28	30	29

[a]Based on 10–50 counts/min, and consequently unreliable.

Characterisation of D-*xylose dimethyl acetal.* — (*a*) The acetal (*ca.* 0.02 μcurie) was eluted from paper chromatograms with cold water, mixed with inactive acetal (*ca.* 1 mg)[9], and chromatographed on a square paper by using butyl alcohol–ethanol–water (4:1:5, upper phase) in one direction, and butanone saturated with water in a perpendicular direction. The active sample was detected by radioautography, and the inactive with a silver nitrate spray.

(*b*) Two parallel experiments were performed with D-xylose (1%) in methanolic hydrogen chloride (0.4 ml, 0.5%) at 25°. In the first, the D-xylose contained 0.4 μcurie activity, and in the second, the solvent contained 80 μcuries. After 2 h, samples taken from the solutions showed acetal:α-D-furanoside activity ratios of 0.063 and 0.128, respectively. After 4 h, these ratios were 0.058 and 0.108, respectively.

[^{14}C]-D-*Xylose dimethyl acetal.* — D-Xylose (0.04 g) was mixed in aqueous solution with radioactive D-xylose (20 μcuries), the water was removed by freeze-

drying, and the residue was dissolved in methanolic hydrogen chloride (1 ml, 3%). After cooling to 0°, redistilled benzaldehyde (0.2 ml) was added, and the mixture was kept for five days at room temperature. After a further two days at 0°, crystalline 2,4:3,5-di-*O*-benzylidene-D-xylose dimethyl acetal was removed by filtration and washed with methanol (0.066 g, 67%, m.p. 207–209°, lit.[9] 209–210°). A portion (0.015 g) was hydrogenolysed in 2-methoxyethanol (10 ml) in the presence of palladium on charcoal (0.02 g, 10%) and anion-exchange resin [Deacidite FF (OH^-), 0.01 g]. Removal of the solids and solvent gave a syrup (0.008 g, 100%), which, after resolution on a paper chromatogram, was shown to contain acetal (95%), methyl α-D-xylofuranoside (4%), and methyl β-D-xylofuranoside (1%).

Ethyl D-*xylofuranosides.* — D-Xylose (10 g) was heated under reflux in ethanolic hydrogen chloride (500 ml, 0.01%) for 1.5 h, when the solution was neutralised with lead carbonate, filtered, and deionised with anionic and cationic resins. Removal of the solvent gave a syrup (10.8 g, 91%), $[\alpha]_D$ +21° (water), which was shown by paper chromatography to contain two major products having mobilities higher than the ethyl D-xylopyranosides. These were isolated in chromatographically pure form after resolution on a column of cellulose powder. Ethyl β-D-xylofuranoside {$[\alpha]_D$ −68° (*c* 1, water)} was eluted first and was followed by the α-D-anomer {$[\alpha]_D$ +140° (*c* 1, water)}. The ring size was established by the standard periodate method (1 mol. reduced in each case), and the rotations suggested that each was contaminated slightly by the other, since the $[\alpha]_D$ values for the methyl α- and β-D-xylofuranosides are +180° and −86°, respectively[15]. From the unfractionated furanoside mixture (10.5 g), ethyl β-D-xylofuranoside 3,5-benzeneboronate was obtained in 37% yield, m.p. 110–111° (from benzene–light petroleum), $[\alpha]_D$ −146° (*p*-dioxane) (Found: C, 58.9; H, 6.5; B, 4.3. $C_{13}H_{17}BO_5$ calc.: C, 59.1; H, 6.5; B, 4.1%). High-resolution infrared examination of dilute solutions revealed a non-bonded hydroxyl group at C-2 (ν 3622 cm^{-1}). The same compound was prepared in 83% yield from the β-D-glycoside, but no crystalline ester of the α-D-anomer was obtained either from the fractionated or unfractionated glycosides. The hydroxyl-stretching frequency of the syrupy products (ν 3542 cm^{-1}) was, however, consistent with the formation of a 3,5-boronate[15].

Ethyl D-*xylopyranosides.* — D-Xylose (10.2 g) was heated under reflux in ethanolic hydrogen chloride (50 ml, 1%) for 6 h. After neutralisation of the acid (lead carbonate), deionisation, and removal of the solvent, a syrup (11.8 g, 100%), $[\alpha]_D$ +55° (water), was obtained, which slowly solidified. A portion (3.53 g) was resolved on a column of cellulose powder to give the chromatographically pure α-D-pyranoside (1.08 g), m.p. 111–112°, $[\alpha]_D$ +156° (*c* 1, water) (Found: C, 46.9; H, 7.7. $C_7H_{14}O_5$ calc.: C, 47.2; H, 7.9%). This was followed by the chromatographically pure β-D anomer (0.35 g), m.p. 92–93°, $[\alpha]_D$ −68° (*c* 1, water) [lit.[16], 95–96°, $[\alpha]_D$ −38° (water)]. In another preparation, a fraction having m.p. 95–96°, $[\alpha]_D$ −44° (water), was obtained but was shown by chromatography to contain contaminating α-D-glycoside. Both pyranosides reduced sodium periodate (2 mol.). From the unfractionated pyranoside mixture, ethyl α-D-xylopyranoside 2,4-benzeneboronate was obtained in 38% yield, m.p. 137–138°, $[\alpha]_D$ +11° (*p*-dioxane) (Found: C, 59.8; H, 6.6; B, 4.0. $C_{13}H_{17}BO_5$

calc.: C, 59.1; H, 6.5; B, 4.1%). Hydroxyl stretching occurred in dilute carbon tetrachloride solution at 3622 cm^{-1}. No β-D ester was obtained in crystalline form, but its presence was detected by the finding of a strong hydroxyl stretching band at 3508 cm^{-1} in the syrup obtained after removal of the α-D derivative[15].

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STUDIES WITH RADIOACTIVE SUGARS

PART II[1]. THE ETHANETHIOLYSIS OF D-XYLOSE

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ABSTRACT

D-Xylose in ethanethiol–*N*,*N*-dimethylformamide (4:1), and in the presence of an acid catalyst, reacts to give ethyl thiofuranosides which are then converted chiefly into D-xylose diethyl dithioacetal. This then slowly loses water and undergoes migration of an ethylthio group to give 3-ethylthio-2-(ethylthiomethyl)furan. Pyranosides do not comprise more than 6% of the products of ethanethiolysis at any stage.

INTRODUCTION

Whereas the alcoholysis of a small number of free sugars has been subjected to kinetic study[2] and has been shown to proceed in four successive steps [(*i*) the formation of furanosides, (*ii*) the anomerisation of furanosides, (*iii*) the conversion of furanosides into pyranosides, and (*iv*) the anomerisation of pyranosides], no such investigations of the analogous thiolysis reactions have been undertaken. The most-detailed available information has been obtained by semi-quantitative, paper-chromatographic methods[3], which have shown that D-glucose, D-galactose, and D-mannose with ethanethiol and concentrated hydrochloric acid give the diethyl dithioacetals in kinetically controlled reactions, and that these are then converted to a large extent into the corresponding thiopyranosides. The high nucleophilic character of sulphur accounts for the much greater significance of the acyclic acetals in these reactions than in simple alcoholysis of sugars, and dialkyl dithioacetals are normally prepared in high yield by treating free sugars with alkanethiols in the presence of an acid catalyst.

The radiochemical methods described in the preceding paper have now been applied in an investigation of the course of the ethanethiolysis of D-xylose, and particular attention has been paid to the role of the thiofuranosides in the reaction. These are formed first on partial demercaptalation of aldose dialkyl dithioacetals[4,5] and have been shown to be important products of the reaction between D-ribose and methanethiol in the presence of dilute, aqueous hydrochloric acid[6], but their significance in the thiolysis of sugars has not been well defined, although there is evidence that they are formed directly from free sugar[6].

RESULTS AND DISCUSSION

Radioactive D-xylose was allowed to react with ethanethiol containing *N,N*-dimethylformamide (20%, added to facilitate dissolution, but believed ultimately to destroy the catalyst) and hydrochloric acid (2.4%). Samples were withdrawn, the acid was neutralised, and the compositions of the mixtures were determined by direct counting after resolution of the components on paper chromatograms[1]. As in the alcoholysis of D-xylose, the initial products were found to be the thiofuranosides (again the α-D anomer preponderated), which then anomerised and were converted into D-xylose diethyl dithioacetal at comparable rates. In 2–3 h, the thiofuranosides were at their maximum concentration and comprised half of the reaction components, and unreacted sugar and acetal were then present in approximately equal amounts, whereas, after 24 h, the acetal constituted more than 80% of the mixture, and the sugar and the thiofuranosides were absent (Fig. 1). The thiopyranosides did not

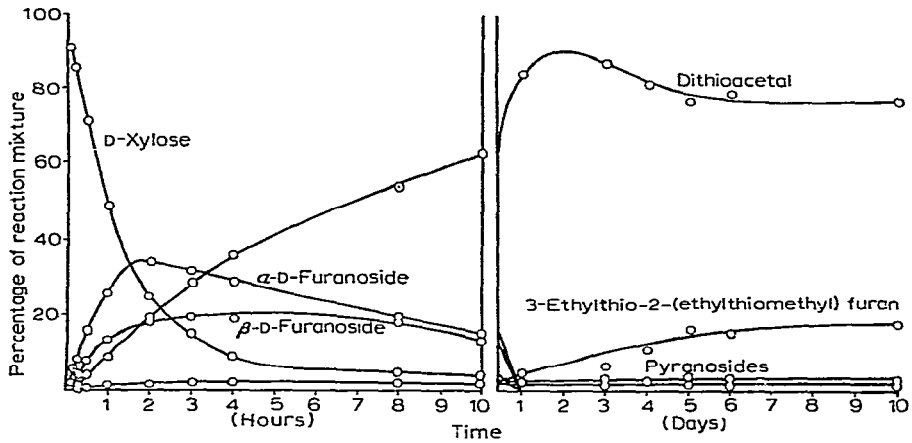
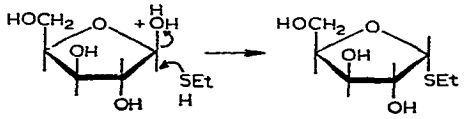


Fig. 1. Components in the reaction between D-xylose (2%) and ethanethiol [containing hydrochloric acid (2.4% initially) and *N,N*-dimethylformamide (20%)] at 25°.

exceed 6% of the products at any stage, but were present in small proportions after the thiofuranosides had been removed, and are evidently components of the equilibrium mixture. Their presence as major final products in normal thiolyses of sugars, in which ethanethiol and concentrated (aqueous) hydrochloric acid are used[3,5], is probably attributable to displacements of equilibria (from the thioacetal in favour of the thioglycosides) caused by the water present in the systems. The apparent absence of thiofuranosides in the final products is to be expected on the grounds of their instability (relative to the thiopyranosides), and the relatively high nucleophilicity of sulphur accounts for the complete conversion of free sugar into sulphur-containing products.

The initial finding of the α- and β-D-thiofuranosides in the approximate ratio 2:1, and as the sole primary products, suggests that the first reaction may be a direct displacement of the C-1 hydroxyl groups of the furanoses (Scheme *A*), but the possibil-

ity also exists that a hemithioacetal is involved. It seems unlikely that the first products are formed by way of a cyclic, furanoid carbonium ion, since the α-D-thiofuranoside preponderates.



SCHEME A

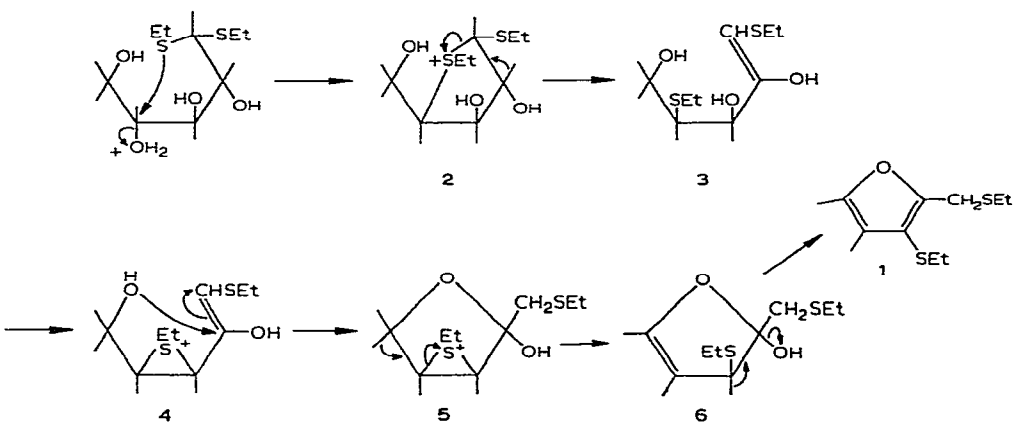
In contrast to the findings with the alkyl glycosides[2], the thiofuranosides anomerise relatively slowly (Fig. 1), and no mixtures containing preponderant amounts of the thermodynamically more-stable β-D anomers were obtained. Furthermore, it was the α- and not the β-D-pyranoside which was found to be formed predominantly, so support is added to the postulate[2a] that ring expansion of furanosides can occur without racemisation at the anomeric centre. This parallels the observation that, in *p*-dioxane in which anomerisation reactions are slow, methyl α-D-xylofuranoside gives methyl α-D-xylopyranoside predominantly[2a].

In addition to the products already discussed, radioautography revealed other compounds having high chromatographic mobilities. One (degradation product *A*) became apparent after comparatively short times (maximum concentration, 1.5%) but was undetectable in later samples; a second product (*B*) built up slowly and was present to the extent of 5% when the carbohydrate products were equilibrated (1 day). Subsequently, it was produced at the expense of the thioacetal until, in five days, it constituted 17% of the reaction products, and then an apparent state of true equilibrium existed (Fig. 1). Although no tests were carried out to establish the point, it is considered that this situation was brought about as a result of neutralisation of the hydrochloric acid following slow hydrolysis of the *N*,*N*-dimethylformamide present in the solvent. In preparative work with inactive material, crude products were obtained in yields appreciably greater than 17%.

In order that product *B* might be examined, inactive D-xylose diethyl dithioacetal was treated for two weeks with ethanethiol saturated with hydrochloric acid in the presence of a desiccant. An optically inactive, oily product, which corresponded in its chromatographic mobility with compound *B*, was obtained after solvent extraction, column adsorption chromatography, and distillation. It reduced potassium permanganate solution, decolourised bromine immediately, and showed one peak on gas–liquid chromatography distinguishable from that given by furfural diethyl dithioacetal, which was considered a possible reaction product, since D-xylose is degraded to furfural (2-furaldehyde) on acid treatment[7]. The n.m.r. spectra revealed that compound *B* and furfural diethyl dithioacetal were different but isomeric, and indicated that the compound derived from D-xylose diethyl dithioacetal was a furan derivative bearing one α- and one β-substituent. Further, the spectrum [τ 2.63, 1-proton doublet (*J* 1.95 Hz); 3.62, 1-proton doublet (*J* 1.95 Hz); 6.22, 2-proton singlet; two *S*-ethyl signals] related closely to that of furfuryl mercaptan[8] [τ 2.67, 1 proton

(H-5); 3.72, 1 proton (H-4); 3.83, 1 proton (H-3); 6.27, 2 protons (methylene); 8.1, 1 proton (SH)]. The compound is therefore assigned an ethylthio-2-(ethylthiomethyl)-furan structure, which was supported on examination of the mass spectra of the compound and that of furfural diethyl dithioacetal. Both showed a molecular-ion peak at 202, a base peak representing the ion $(M\text{-}SEt)^+$, and two other readily detectable ions [$(M\text{-}Et)^+$ and $(M\text{-}SEt\text{-}C_2H_4)^+$], but only in the spectrum of the degradation product was the ion $(M\text{-}CH_2SEt)^+$ observed. Furans show $J_{2,3}$ and $J_{2,4}$ values of 1.85 $\pm$0.15 and 0.85 $\pm$0.15 Hz, respectively[9], and, on this basis, the compound is therefore assigned the structure 3-ethylthio-2-(ethylthiomethyl)furan (**1**). In support of this, in a double-resonance experiment at 100 MHz, coupling of 0.4 Hz was detected between the furfuryl methylene protons and H-4*; had the 4 position been substituted, a value for J_{CH_2-3} of *ca.* 0.8 Hz should have been observed[9]. The ultraviolet spectrum showed absorption bands at 207, 223, and 246 nm (log ε 3.86, 3.94, and 3.63, respectively), whereas furan itself has one band at 208 nm (log ε 3.9), and vinylic and allylic sulphides absorb near 255 and 220 nm, respectively[10]. Furfural diethyl dithioacetal showed λ_{max} at 202 and 225 nm (log ε 3.82 and 3.94, respectively), which supports the assignment of the 246 nm band in the spectrum of compound **1** to a sulphur chromophore attached directly to the furan ring.

That the 2,3-substituted furan was not produced by way of the diethyl dithioacetal of furfural was established by subjecting this compound to ethanethiolysis conditions and finding (by g.l.c.) that none of the disubstituted furan was formed. A possible, alternative route is shown in Scheme *B* which, since it contains an intermediate cyclic sulphonium ion (**2**), requires that the ethylthio group migrates first to the 4 and not the 3 position. However, the thioethyl group could subsequently



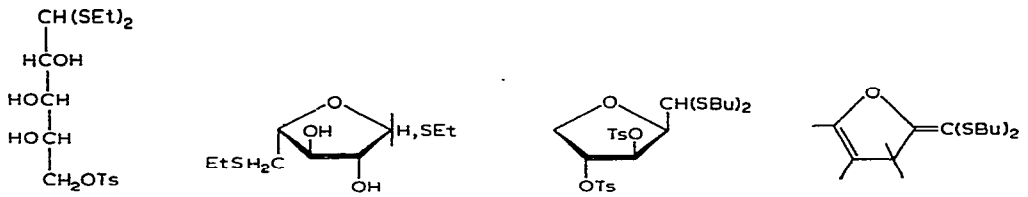
SCHEME *B*

migrate to position 3 by way of an episulphonium ion (**4**), since the allylic hydroxyl in the acyclic intermediate **3** would serve as a suitable leaving group. Analogous migra-

*This value agrees with that previously recorded for couplings between such protons [G. S. Reddy and J. H. Goldstein, *J. Phys. Chem.*, 65 (1961) 1539].

tions of alkylthio groups have been used successfully in nucleoside chemistry to isomerise 3-deoxy-3-thio compounds and afford a route to 2-deoxy derivatives[11]. The episulphonium ion **4** could cyclise and then deprotonate by one route only to give the 3-thio intermediate **6** which, on loss of water, affords product **1**.

Migrations of ethylthio groups from diethyl dithioacetals have been observed previously in carbohydrate chemistry, as when, for example[12], 5-*O*-toluene-*p*-sulphonyl-L-arabinose diethyl dithioacetal (**7**) is heated in aqueous acetone with barium carbonate to give the thioglycoside derivative **8**. Furthermore, analogous displacements of hydroxyl groups have been observed; for example, in the ethanethiolysis of 1,2,3,4-tetra-*O*-acetyl-α-L-arabinose, which reacts by way of 2,3,4-tri-*O*-acetyl-L-arabinose diethyl dithioacetal to give, after deacetylation, 5-*S*-ethyl-5-thio-L-arabinose diethyl dithioacetal[13], or in the corresponding reaction of 3,4,5-tri-*O*-benzoyl-2-*S*-ethyl-2-thio-D-pentose diethyl dithioacetal[14].



7 8 9 10

SCHEME *C*

Alternatively, the formation of compound **1** could have involved an intermediate 4-*S*-ethyl-4-thiopentose diethyl dithioacetal formed by thiol attack at C-1 of the cyclic sulphonium ion, and this could have lost water to give a 2,5-anhydride by attack of O-2 on C-5. Such 2,5-anhydrides are formed from pentose diethyl dithioacetals[15] that contain a good leaving group at C-5, and, in the present case, the leaving properties of the 5-OH group would be enhanced by neighbouring-group participation of the ethylthio group at C-4. Migration of the ethylthio group *via* an episulphonium ion, and acid-catalysed loss of ethanethiol and water could give the 2,3-substituted furan.

In the course of studies of the reaction occurring on treatment of 2,5-anhydro-3,4-di-*O*-toluene-*p*-sulphonyl-D-xylose di-isobutyl dithioacetal (**9**) with sodium iodide in *N*,*N*-dimethylformamide, Defaye and Hildesheim[16] obtained a diene to which they assigned structure **10**, but which had n.m.r. spectral features closely similar to those of compound **1** [τ 2.70 (1 proton), 3.82 (1 proton), and 6.37 (2 protons); all singlets]. Dr. J. C. P. Schwarz[17] has pointed out that a structure in which an ethylthio group was substituted at C-4 would correlate more satisfactorily with the observed spectral features than the allocated structure, and a mechanism having two initial steps analogous to those in Scheme *B* would be consistent with migration of the butylthio group and the eliminations that could result in furan formation. However, in this case, the *cis*-stereochemical relationship at C-3 and C-4 precludes the possibility of episulphonium ion formation, and the product should therefore be a 4-alkylthio-2-alkyl-

thiomethylfuran. Consistent with this is the reported lack of observable splitting in the n.m.r. signals of the ring protons. Such a mechanism, rather than direct eliminations of sulphonic acid as was originally proposed, would account for the lack of reaction when 2,5-anhydro-3,4-di-*O*-toluene-*p*-sulphonyl-D-xylose dimethyl acetal is treated with sodium iodide in *N,N*-dimethylformamide[16].

During this work, the four ethyl D-thioxylosides were prepared, and characterised by conversion into their crystalline benzeneboronic esters which were examined by high-resolution infrared spectroscopy in dilute carbon tetrachloride solution. As with the corresponding oxygenated methyl[18] and ethyl[1] D-xyloside esters, the α-D-pyranoside and β-D-furanoside derivatives contained non-bonded hydroxyl groups, whereas intramolecular O-H---S bonds were detected in the anomers. However, in keeping with sulphur's weak hydrogen-bond acceptor property[19], the thioglycoside derivatives showed appreciably smaller $\Delta\nu$ values than did the corresponding oxygenated D-xyloside boronates. The ring sizes of the thioglycosides were determined by the periodate oxidation method. One and two moles were reduced by the furanosides and pyranosides, respectively, and oxidation of the sulphur did not occur at sufficient speed to invalidate the method. Wolfrom and co-workers[20] have previously found that ring sizes of thioglycosides can be determined by periodate oxidation; other workers[6] have also employed this method to determine ring size of glycosides but used conditions which also caused oxidation of the sulphur atoms.

EXPERIMENTAL

The radiochemical techniques employed are described in the preceding paper. Infrared measurements were made in carbon tetrachloride solution at concentrations in the range 0.003–0.005M by using a Unicam S.P. 700 spectrophotometer. N.m.r. spectra were measured in carbon tetrachloride solution by using a Varian A-60 instrument, but the double-resonance experiment was carried out at 100 MHz, and the mass spectra were obtained with an A.E.I. MS 12 instrument.

Rotations were measured at room temperature within the concentration range 0.8–1.2%, and R_F values were determined by using Whatman No. 1 papers and butanone saturated with water as solvent. Periodate oxidations were carried out by using 0.015M reagent at 25°; consumptions were followed spectrophotometrically[21], and the recorded values were measured after 2 h.

Ethanethiolysis of D-*xylose.* — [^{14}C]-D-Xylose (0.40 g, containing 10 μcuries activity) was dissolved in *N,N*-dimethylformamide (0.40 ml). Ethanethiol (1.60 ml, containing 3.0% hydrogen chloride) was added, and the solution was kept at 25°. Sixteen samples (0.02 ml) were withdrawn at various times, the acid was neutralised with resin [Deacidite FF (OH^-), 5 mg], and the solutions were applied with methanol washings to a paper chromatogram which was developed for 3 h in butanone saturated with water. The five direct products were completely resolved (R_F values: β-D-pyranoside, 0.47; α-D-pyranoside, 0.53; α-D-furanoside, 0.65; β-D-furanoside, 0.71; dialkyl dithioacetal, 0.81), were characterised by comparison of their mobilities with

those of authentic samples and were determined quantitatively by direct counting. The two products formed by degradation of the carbohydrate derivative had R_F values of 0.88 and 0.94. Representative results obtained from the 16 samples are given in Table I, and the results of the complete experiment are illustrated in Fig. 1.

TABLE I

ACTIVITIES (COUNTS/MIN)[a] OF THE RESOLVED COMPONENTS FROM THE ETHANETHIOLYSIS OF D-XYLOSE AT 25° [D-XYLOSE (2%) IN ETHANETHIOL–*N*,*N*-DIMETHYLFORMAMIDE (4:1) CONTAINING (INITIALLY) 2.4% HYDROCHLORIC ACID].

Compound	*Time (h)*							
	0.083	*0.25*	*1*	*4*	*10*	*72*	*120*	*240*
D-Xylose	1640	1730	2040	690	380	0	0	0
D-Xylose (corrected)[b]	7730	6870	3920	690	380	0	0	0
β-D-Pyranoside	0	0	0	0	0	190	180	200
α-D-Pyranoside	0	25	135	195	200	280	330	330
α-D-Furanoside	140	635	2050	2160	1290	0	0	0
β-D-Furanoside	80	310	1050	1420	1130	0	0	0
Acetal	0	80	665	2625	5510	7190	6750	7730
Degradation product *A*	0	35	70	0	0	0	0	0
Degradation product *B*	0	0	60	250	180	590	1500	1820
Total counts (uncorrected)	1860	2815	6070	7340	8690	8250	8760	10,080

[a]All counts are corrected for paralysis, for background count, and for distance travelled[1]. [b]The deficiencies in the total activities measured in the early samples are assumed to result from specific adsorption of D-xylose on the resin[1]. Corrections are consequently applied to the D-xylose figures to bring the total counts/min to 7950, the average obtained from all the later samples (based on a counting efficiency of 3%, the calculated figure is 6600 counts/min). In this experiment, no internal reference compound was employed, and the total activities measured in the different samples were consequently more variable than in the corresponding alcoholysis experiments[1]. On both these accounts, the proportions of compounds determined for the early samples are likely to contain appreciable errors, but these do not influence the deductions which are made.

Ethanethiolysis of D-*xylose diethyl dithioacetal.* — D-Xylose diethyl dithioacetal was prepared by conventional methods and had m.p. 62–63°, $[\alpha]_D$ −31° (water) [lit.[22], m.p. 63–65°, $[\alpha]_D$ −30.8° (water)]. It gave a bis(benzeneboronate) in 88% yield (on treatment with triphenylboroxole), having m.p. 134°, $[\alpha]_D$ +34.5° (*p*-dioxane) (Found: C, 58.9; H, 6.3; B, 5.2. $C_{21}H_{26}B_2O_4S_2$ calc.: C, 58.9; H, 6.1; B, 5.1%). The diethyl dithioacetal (20 g) was added at 0° to ethanethiol saturated with hydrogen chloride (50 ml), the temperature was allowed to rise slowly to 25°, and "Hi-drite" (20 g) was added. After 14 days, the acid was neutralised (lead carbonate), and the solids and solvent were removed to leave a yellow, mobile oil which was dissolved in light petroleum (b.p. 60–80°) and extracted with water. From the organic phase, an oil (5.7 g) was obtained which was shown by t.l.c. [silica gel, light petroleum (b.p. 40–60°) — benzene (1:1)] to contain four components that gave the following R_F values and colours on spraying with an anisaldehyde spray[23]: (*i*) 0.7, bright green; (*ii*) 0.6, yellow; (*iii*) 0.4, brown; (*iv*) 0.2, red. Component (*i*) corresponded to the degradation

product *B* observed during the radiochemical experiment. Resolution of this mixture (5.0 g) on a column of silica gel by using graded elution with light petroleum (b.p. 60–80°)–benzene gave firstly diethyl disulphide [λ_{max} (EtOH) 202, 203, 207, 255 nm; lit.[24], 202, 251 nm], and then a fraction (0.3 g) obtained as a mobile, optically inactive oil, which gave a homogeneous green reaction on thin-layer chromatograms sprayed with anisaldehyde, and reduced potassium permanganate and decolourised bromine immediately. Further purification was effected by distillation [120° (bath temperature), 10^{-4}mm] and gave 3-ethylthio-2-(ethylthiomethyl)furan (0.03 g), homogeneous by g.l.c. (20% Apiezon L on Celite at 170°). The spectral characteristics are described in the discussion section.

The slow moving components were present in smaller proportions and were not obtained in pure form.

Ethanethiolysis of furfural diethyl dithioacetal. — Furfural diethyl dithioacetal (b.p. 77–79°, 0.05 mm, n_D^{23} 1.5450; lit.[25], b.p. 80–82°, 0.07 mm, n_D^{20} 1.5458) gave n.m.r. signals in deuterochloroform at τ 2.58 (H-5), 3.62 (H-3, H-4), and 4.93 (acetal proton). A sample (2.0 g) was dissolved in ethanethiol (10 ml, containing 6% hydrochloric acid) and allowed to stand at 25° in the presence of "Hi-drite" (2 g) for one week. The acid was neutralised with lead carbonate, and the solids and solvent were removed to leave a mobile oil (1.9 g) which was shown by g.l.c. (as above) to contain about 30% of a new component which had a retention time of 1.27 relative to the initial dithioacetal. It was readily distinguishable from 3-ethylthio-2-(ethylthiomethyl)furan (retention time, 1.13) but was not isolated or characterised.

Ethyl 1-thio-α- and -β-D-xylopyranosides. — A modification of Lemieux's method[26] for the synthesis of ethyl tetra-*O*-acetyl-1-thio-β-D-glucopyranoside was used. 1,2,3,4-Tetra-*O*-acetyl-β-D-xylose (20 g) was added to a solution of ethanethiol (100 ml) and zinc chloride (8 g) at 0°. After shaking for 20 min, the solution was kept for 4 h, poured into saturated, aqueous sodium hydrogen carbonate (500 ml), and filtered, and the filtrate was extracted with chloroform. After drying, the solvent was removed to leave ethyl 2,3,4-tri-*O*-acetyl-1-thio-β-D-xylopyranoside (which was crystallised from ethanol) (9.2 g, 46%), m.p. 100–101°, $[\alpha]_D$ −83° (chloroform) [lit.[27], m.p. 101°, $[\alpha]_D$ −83.5° (chloroform)]. Deacetylation with catalytic amounts of sodium methoxide in methanol afforded ethyl 1-thio-β-D-xylopyranoside in 70% yield, m.p. 118–119°, $[\alpha]_D$ −79° (water) [lit.[27], m.p. 117°, $[\alpha]_D$ −78° (water)], R_F 0.47; periodate consumption, 1.96 mol.

A mixture containing 53% of α-D isomer (n.m.r. analysis) was obtained by anomerising ethyl tri-*O*-acetyl-1-thio-β-D-xylopyranoside. The β-D compound (5 g) was dissolved in dry chloroform (120 ml), titanium tetrachloride (2 g) in chloroform (50 ml) was added, and the mixture, which rapidly turned black, was heated under reflux for 45 min and then poured into water. The pale-yellow organic phase was washed with saturated, aqueous sodium hydrogen carbonate and water, dried, and taken to dryness to leave a clear syrup (5 g), $[\alpha]_D$ +59° (chloroform). Deacetylation was carried out catalytically, using sodium methoxide in methanol, and gave a syrup, $[\alpha]_D$ +100° (water), which was shown by paper chromatography to contain the ethyl

thioglycopyranosides in approximately equal proportions, together with a trace of free sugar. Resolution of this mixture (2.1 g) on a column of cellulose eluted with butanone saturated with water afforded ethyl 1-thio-α-D-xylopyranoside [0.5 g; which was recrystallised from ethyl acetate–light petroleum (b.p. 60–80°)], m.p. 108–110°, $[\alpha]_D$ +269° (water), R_F 0.53; periodate consumption, 2.04 mol. (Found: C, 43.4; H, 7.3; S, 16.2. $C_7H_{14}O_4S$ calc.: C, 43.3; H, 7.3; S, 16.5%).

A second fraction gave the crystalline β-D isomer.

Ethyl 1-thio-α- and -β-D-xylofuranosides. — D-Xylose (8 g) was dissolved in *N,N*-dimethylformamide (80 ml) and ethanethiol (320 ml, containing 3% hydrogen chloride). After 1.5 h at 25°, the acid was neutralised with lead carbonate. Removal of the solids and solvents gave a syrup which was shown by paper chromatography to contain mainly furanosides and D-xylose diethyl dithioacetal, together with a small proportion of a faster-moving component which was removed by extraction of an aqueous solution of the syrup with light petroleum. Continuous extraction of the aqueous phase with chloroform then allowed the preferential removal of the diethyl dithioacetal. After 3 h, the chloroform contained the dithioacetal (2.5 g); after a further 3 h, a mixture of dithioacetal and furanoside (1.0 g); and after 9 h, further furanosides and traces of pyranosides (0.35 g). At this stage, the aqueous phase contained furanosides contaminated with a small proportion of pyranosides and, on evaporation, gave a syrup (4.8 g, 46%), a portion of which (4.5 g) was resolved on a column of cellulose by using butanone saturated with water as eluting solvent. Two fractions were obtained, which gave each syrupy thiofuranoside contaminated with only small proportions of the anomer: β-D isomer, 0.13 g, $[\alpha]_D$ −67° (ethanol), R_F 0.71, periodate consumption 0.95 mol.; α-D isomer, 0.06 g, $[\alpha]_D$ +105° (ethanol), R_F 0.65, periodate consumption 1.03 mol. In addition, a central fraction (1.7 g) was obtained, which consisted of a mixture of furanosides alone. This was used in the preparation of the benzeneboronate derivatives which were separated by fractional crystallisation (see below).

Properties of the derived thioglycoside benzeneboronates. — The esters of the four thioglycosides were prepared by using triphenylboroxole[28] in *p*-dioxane. By analogy with the derivatives of the oxygenated methyl D-xylosides[29], it is assumed that those derived from the pyranosides and furanosides have 2,4- and 3,5- cyclic structures, respectively. The properties of the esters are given in Table II.

TABLE II

PROPERTIES OF THE D-THIOXYLOSIDE BORONATES

Ethyl D-*thioxyloside benzeneboronate*	*m.p. (degrees)*	$[\alpha]_D$ (*p-dioxane*) (*degrees*)	ν_{max} (cm^{-1})	*Found* (%)[a] *C*	*H*	*B*
α-D-pyranoside	143–145	+ 79.5	3623[b]	55.2	6.1	3.7
β-D-pyranoside	109–110	−233	3613[b]	55.4	6.0	4.0
α-D-furanoside	102–104	+ 27	3618[b]	56.0	6.2	4.0
β-D-furanoside	157–158	−254	3622[b]	56.0	6.0	4.0

[a] $C_{13}H_{17}BO_4S$ calc.: C, 55.7; H, 6.1; B, 3.9%. [b] The figures[18] for the corresponding methyl D-xyloside benzeneboronates are 3623, 3512, 3543, and 3623 cm^{-1}.

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STUDIES ON URONIC ACID MATERIALS

PART XXIV*. AN ANALYTICAL STUDY OF DIFFERENT FORMS OF THE GUM FROM *Acacia senegal* WILLD.

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ABSTRACT

Some of the analytical parameters for *Acacia senegal* gum have been determined for (*a*) twelve "bulk" samples of tapped gum from three different districts of the Sudan, and (*b*) thirteen, different, single-nodule specimens of "natural exudate" gum. These samples show the same range of analytical variation that has been noted previously for samples of gum from other *Acacia* species. In addition, the following atypical variants of *Acacia senegal* gum have been studied: (*a*) three specimens of "non-nodular "gum, (*b*) a sample from a tree infested with wood-boring beetles, and (*c*) a sample of "Hennawi" gum, a dark-colored, sweet-tasting gum from the main stem of trees.

The viscosity data obtained indicate that values of $\overline{M}_w$ for the samples fall in the range 0.26×10^6 to 1.16×10^6; this explains the lack of agreement for $\overline{M}_w$ of *A. senegal* gum samples studied by earlier investigators.

The atypical, non-nodular and beetle-borer specimens of gum do not show any striking analytical differences from the mean values obtained for the typical samples. The "Hennawi" variant differs from typical, tapped samples in two important respects: the uronic acid residues present in the free acid form are fewer in number, and the rhamnose content is considerably less.

INTRODUCTION

Earlier investigators[2,3] considered that different samples of gum arabic (*Acacia senegal* Willd., syn. *verek*) did not vary in properties to any great extent, but this is not the current industrial view. Botanically, *A. senegal* is very variable[4], and the Sudanese consider that the gum viscosity of a sample is dependent on the age of the tree involved. A sa result of empirical, technological observations, some manufacturers restrict their purchases to gum originating from a certain district; others decline a current season's crop ("green gum"), preferring gum stored in the Sudan since the previous season.

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It is now clearly established that distinct internodule variations occur in the gums from *A. seyal*[5], *A. nilotica*[6], and *A. laeta*[7], but there are no modern analytical values for specimens of *A. senegal* gum. Widely differing values for the molecular weight (both for $\overline{M}_n$ and $\overline{M}_w$) of *A. senegal* gum have been reported, and these have been summarised[8]; the implication that different samples of *A. senegal* gum may vary widely in $\overline{M}_n$ or $\overline{M}_w$ must therefore be considered, and the possible range of variation requires evaluation. Information on the viscosity behaviour of a wide selection of specimens would therefore indicate those likely to display the widest extremes when studied more fundamentally by the light-scattering technique.

Accordingly, authentic specimens of *A. senegal* gum from different geographical locations and different types of soil, from different seasons, and from different times of collection within a season, have been investigated. In addition, the variation from tree to tree has been investigated for normal specimens of the gum obtained (*i*) by "tapping" and (*ii*) by natural exudation. We have also examined three unusual forms of natural-exudate gum from *A. senegal*: (*a*) non-nodular forms of the gum, exuded as thin, spiral filaments or ribbons, rather than as oval-shaped nodules; (*b*) "Hennawi" gum, which is a very dark-colored, sweet-tasting form of *A. senegal* gum exuded from the main stems of trees, as opposed to the upper branches usually tapped; (*c*) the gum exuded by trees infested with wood-boring beetles. This exudate was in the form of hard, dark-brown masses of wood borings and debris, bound together with a gum exudate comprising about 40% of the masses by weight.

EXPERIMENTAL

Analytical methods. — The standard methods involved have been described[9] previously.

Origins of specimens. — All of the specimens, from *A. senegal* trees that could be identified botanically beyond all doubt, were collected by (the late) Mr. M. P. Vidal-Hall, formerly Gum Research Officer to the Republic of the Sudan. Details of their collection are as follows:

1. "*QN*" *samples.* These were bulk samples from many trees, growing on heavy clay soil at Qala en Nahal, Kassala Province, Eastern Sudan: 1960 crop, first (QN1), second (QN2), third (QN3), and final collection (QN4); 1962 crop, first collection (QN5).

2. "*UR*" samples. These were bulk samples from many trees, growing on sandy soil at Umm Ruaba, Kordofan Province, Central Sudan: 1960 crop, representative sample (UR1), final collection (UR2).

3. "*GG*" samples. These were bulk samples from many trees, growing on sandy soil at Goz el Ganzara, Kordofan Province, Central Sudan: 1960 crop, representative sample (GG1); 1962 crop, first (19 January, GG2), second (4 February, GG3), third (19 February, GG4), and final collection (5 March, GG5).

All of the QN, UR, and GG samples were normal samples of top commercial-quality gum from *A. senegal*, pale straw in colour, and virtually free from sand

and bark. Exudation was in response to tapping. Each sample (*ca.* 1500 g) was crushed and sieved to give material representative of the complete sample.

4. Single-nodule samples of natural exudate gum from individual trees of *A. senegal*, collected at Umm Ruaba (sandy soil) on 9 March, 1960:
Tree *A* (age 25 years) gave 3 nodules (samples A1, A2, and A3); *B* (12 years), 2 nodules (B1 andB2); *C* (8 years), 2 nodules (C1 and C2); *D* (15 years), 1 nodule (D); *E* (12 years 1 nodule (E); *F* (17 years), 1 nodule (F); *G* (20 years), 1 nodule (G); *H* (10 years), 1 nodule (H); *I* (15 years), 1 nodule (I). Each of these samples (15–30 g) was crushed to a fine powder and investigated individually.

5. "*Hennawi*" *sample*. This very dark brown variant of *A. senegal* gum has a sweet taste and is exuded naturally at wounds to the lower, main stem of the tree. Such gum is not usually tapped. It is known in the Sudan as "*Hennawi*" gum, eagerly sought by native children as a sweetmeat. The specimen (HW) of *Hennawi* gum studied was collected at Goz el Ganzara on 13 March 1963.

6. "*NN*" *samples*. Occasionally, *A. senegal* trees exude gum that differs from the customary oval nodules in forming long filaments, spirals, and ribbons. Three non-nodular (NN) forms of the gum were recognised: broad, opaque, flat ribbons (NN1); thin, circular, opaque filaments (NN2); and thin, circular, strongly refractive filaments (NN3). These samples were collected at Goz el Ganzara on 11 February 1961.

7. "*BB*" *samples*. Trees infested with wood-boring longhorn (*Cerambycidae*) or jewel (*Buprestidae*) beetles exude hard, dark-brown masses of a gum mixed with wood borings and other debris. The sample (180 g) of beetle-borer gum was collected at Goz el Ganzara in January 1965. One half-portion of the exudate (90 g) was stirred with cold water (750 ml) for 48 h, filtered, dialysed against tap-water, and then exhaustively electrodialysed. The gum polysaccharide, sample BB(*a*), was isolated as the freeze-dried product (33.4 g, 37%). The remainder of the gum exudate (90 g) was stirred with cold water (750 ml) for 24 h, and this extraction was processed in the same way as for BB(*a*) to give sample BB(*b*) (27.7 g, 31%). The residue from the extraction yielding BB(*b*) was then further exhaustively extracted with cold water (500 ml) and, after processing as before, this extraction gave sample BB(*c*) (8.9 g, 10%).

Purification of samples. — All of the QN, UR, GG, HW, and NN samples, and, the single-nodule samples A-I, were purified by dissolution in cold water, filtration dialysis, and exhaustive electrodialysis.

RESULTS

Table I gives the analytical data obtained for the various bulk samples QN, UR, GG, NN, and HW. Table II gives the analytical data for the single-nodule samples from individual trees. Table III gives more-detailed analytical information for samples QN1, HW, BB(*a*), BB(*b*), and BB(*c*). Figure 1 gives the viscosity plots from which the limiting-viscosity numbers, $[\eta]$, recorded in Tables II and III were found.

Since the modified Staudinger constants K' and a are now known[10] to have

TABLE I

ANALYTICAL DATA FOR BULK SAMPLES OF *Acacia senegal* GUM

Sample	*Moisture* %	*Ash* %	*Nitrogen* %	*Insoluble*[a] %	*Flow-time number* ($cm^3\ g^{-1}$) *Crude gum*[b]	*Purified gum*[c,d]
QN1	12.8	3.64	0.33	0.4	57	25 (19.9)
QN2	13.4	3.37	0.34	0.3	47	18
QN3	13.3	3.07	0.36	0.4	43	20
QN4	13.1	2.91	0.35	0.2	37	17
QN5	12.7	3.62	0.34	0.6	62	30
UR1	13.2	3.05	0.39	0.4	35	21
UR2	13.5	3.52	0.39	1.0	34	22
GG1	12.4	3.68	0.39	1.5	54	36
GG2	12.4	4.16	0.42	1.6	64	43 (24.5)
GG3	12.9	3.98	0.41	1.3	67	36
GG4	13.0	3.93	0.38	0.5	79	38
GG5	13.2	3.81	0.38	0.2	71	32
NN1	13.4	3.88	0.40	0.4	83	36
NN2	13.2	3.70	0.34	0.2	62	36
NN3	13.1	3.97	0.39	0.3	70	35
HW	13.6	5.67	0.37	0.5	39	35 (20.6)
Average	13.1	3.75	0.37	0.6	56	30
Range	12.4–13.6	2.91–5.67	0.33–0.42	0.2–1.6	34–83	17–43
Standard deviation	0.36	0.63	0.028	0.47		

[a]Insoluble after 24 h in cold water; [b]0.03 g/ml in water at 25.0°; [c]0.03 g/ml in 4% sodium chloride solution at 25.0°; [d]values in brackets are the corresponding limiting (C→O) flow-time numbers.

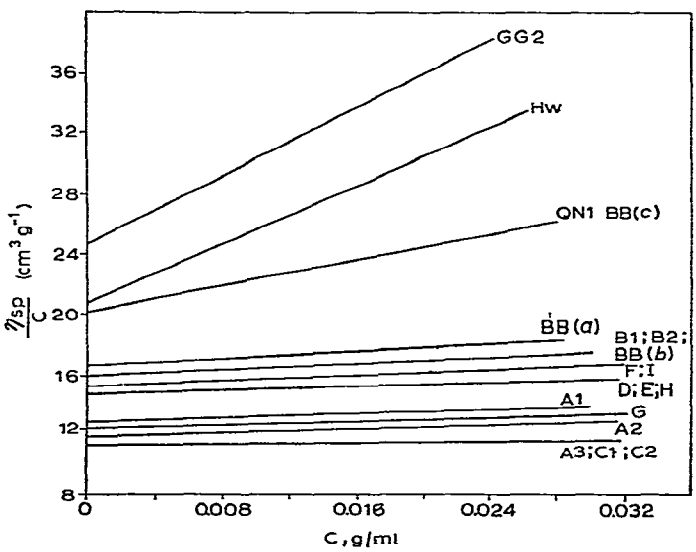

Fig. 1. The viscosity–concentration relationship for different specimens of *Acacia senegal* gum. The following limiting-viscosity numbers and M_w values (from $[\eta]=K'M_w^a$, where[10] $K'=1.3\times10^{-2}$, and $a=0.54$) were obtained for samples GG2, 24.6, 1.16×10^6; HW, 20.6, 0.84×10^6; QN1 and BB(*c*), 19.9, 0.79×10^6; BB(*a*), 16.5, 0.55×10^6; A1, 12.4, 0.33×10^6; A3, C1, C2, 11.0, 0.26×10^6.

TABLE II

ANALYTICAL DATA FOR SINGLE-NODULE NATURAL EXUDATE *Acacia senegal* GUM

Sample	*Age of tree (years)*	*Crude material*		*Electrodialysed material*			$\overline{M}w^a$
		Moisture %	*Ash* %	*Nitrogen* %	$[\alpha]_D$ *(degrees)*	*Limiting-viscosity number* ($cm^3 g^{-1}$)	
A1	25	12.6	3.72	0.24	−29	12.5	334,000
A2		11.6	3.78	0.23	−31	11.6	291,000
A3		11.1	3.80	0.24	−32	11.0	264,000
B1	12	11.7	4.43	0.23	−28	15.6	504,000
B2		12.9	4.44	0.23	−29	15.8	516,000
C1	8	10.4	3.40	0.27	−29	11.0	264,000
C2		9.8	3.66	0.26	−31	11.0	264,000
D	15	15.6	3.88	0.32	−30	14.1	418,000
E	12	12.8	4.64	0.34	−32	14.5	440,000
F	17	13.3	4.01	0.35	−29	15.1	474,000
G	20	15.4	4.15	0.23	−31	12.2	320,000
H	10	14.7	3.97	0.45	−29	14.4	434,000
I	15	12.1	3.27	0.33	−27	15.0	468,000
Average		12.6	3.93	0.29	−30	13.4	
Range		9.8–15.6	3.27–4.64	0.23–0.45	−27–−32	11.0–15.8	
Standard deviation		1.8	0.40	0.06	1.5	1.9	

[a]From $[\eta] = K'M_w^a$, where $K' = 1.3 \times 10^{-2}$ and $a = 0.54$ (see Ref. 10).

values of 1.30×10^{-2} and 0.54, respectively, values for $\overline{M}_w$ can be calculated from the viscosity data by the relationship $[\eta] = K'M_w^a$, and these values are shown in Fig. 1.

DISCUSSION

From Fig. 1 and the viscosity data in Tables I, II, and III, it is clear that $\overline{M}_w$ for *Acacia senegal* gum varies widely from sample to sample; the variation, for the samples studied, is from 1.16×10^6 (for GG2) to 0.26×10^6 (for nodules A3, C1, and C2). This explains the lack of agreement[8] between the values for $\overline{M}_w$ reported by earlier investigators, *viz.* 0.58×10^6 (Deb and Mukherjee[11]) and 1.00×10^6 (Veis and Eggenberger[12]). From our experiments, a value of 0.58×10^6 (Ref. 8) appears to be a more representative value than 1.00×10^6, although values higher than the latter may yet be found. *A. senegal* gum has a broad, skew, molecular-weight distribution, and fractions having $\overline{M}_w$ ranging from 1.18×10^6 to 0.1×10^6 have been obtained[10] for sample QN1. It should be emphasised that the values deduced for $\overline{M}_w$ in this paper are based on the modified Staudinger constants obtained from fractionations[10] of only one *A. senegal* gum sample: these values for K' and a do not hold for other *Acacia* species[10], and, indeed, they may not be generally applicable to all specimens of such a complex polymer system as *A. senegal* gum.

TABLE III

COMPARATIVE ANALYTICAL DATA[a] FOR SOME ELECTRODIALYSED TYPICAL (QN1) AND ATYPICAL (HW, BB) SPECIMENS OF *Acacia senegal* GUM

	QN1	*HW*	*BB(a)*	*BB(b)*	*BB(c)*
Ash, %	0.07	0.01	0.02	0.01	0.02
Nitrogen, %	0.33	0.35	0.25	0.21	0.28
$[\eta]$, cm^3g^{-1}	19.9	20.6	16.4	15.8	20.1
$[\alpha]_D$ (*c* 1.0, water)[b]	−31.0°	−27.6°	−30.5°	−31.3°	−31.5°
Methoxyl[b,c], %	0.25	0.25	0.25	0.25	0.25
Equivalent weight[b]	1085	1047	1066	1065	1060
Hence, uronic anhydride[b,d], %	16.2	16.8	16.5	16.5	16.5
Uronic anhydride [b,c], %	17.2	17.5	17.4	17.3	17.5
Formic acid released on periodate oxidation[b] (mole/g) × 10^3	1.62	1.69	1.76	1.85	1.90
Periodate consumed[b] (mole/g) × 10^3	5.07	5.18	5.23	5.34	5.42
Hence, $\frac{\text{periodate consumption}}{\text{formic acid released}}$	3.13	3.06	2.97	2.89	2.85
Rhamnose[b,c], %	14.0	6.2	13.0	12.6	12.8
Galactose[b], %	40	46	43	43	44
Arabinose[b], %	28	30	27	27	26
Glucuronic acid[b], %	16.0	16.5	15.5	15.5	15.5
4-*O*-methylglucuronic acid, %	1.5	1.5	1.5	1.5	1.5

[a]All data corrected to a dry-weight basis; [b]corrected for all non-carbohydrate material; [c]determined by vapour-phase, infrared methods; [d]assuming that all acidity arises from uronic acid groups; [e]assuming that all methoxyl content arises from 4-*O*-methylglucuronic acid.

Many factors influence[13] the viscosity of *Acacia* gum solutions. We have observed that electrodialysed specimens, after storage in the freeze-dried state for several months, give increased limiting-viscosity numbers when solutions are made up in a standardised way to facilitate comparison; for sample QN1, $[\eta]$ increased from 19.9 to 29.0, and for sample GG2, $[\eta]$ increased from 24.5 to 35.1. Other *Acacia* species give the same effect, and this is under investigation. The viscosity data presented in Tables I and II were obtained for 3% solutions immediately after dissolution. Table II gives some support for the belief that gum viscosity is dependent on the age of the tree; the oldest and youngest trees studied gave gum of low viscosity.

The "bulk" samples, representative of many nodules, show distinct analytical differences, although they must be expected to show "averaged" values. The differences shown in Table II for the single-nodule specimens are perhaps more fundamental in significance. Table I shows that the GG samples are more viscous than the QN or UR samples; it is interesting (*cf.* Ref. 5) that the most-viscous samples have higher than average nitrogen contents, although a high nitrogen content does not necessarily imply high viscosity, as shown by the UR samples. Insufficient data are available for any conclusions regarding seasonal effects to be reached; there is a suggestion, however, that the viscosity of the QN samples decreases as the season continues, whereas, in contrast, the viscosity of the GG samples increases.

The results for trees *A*, *B*, and *C* in Table II indicate that each tree tends to

produce a characteristic form of the gum, the different nodules from a particular tree being strikingly similar to each other in comparison to those from other trees.

The "Hennawi" variant of the gum has two distinctive features. It contains only 6% of rhamnose, *i.e.*, less than 50% of that present normally. A solution of "Hennawi" gum has pH 6.25; all of the other samples studied fell within the range pH 4.49 to 4.66, and the electrodialysed specimens fell within the range pH 2.47 to 2.60. The comparatively sweet taste of "Hennawi" gum is not due to the presence of free sugars; its reducing power is closely similar to that of the other samples. The explanation appears to involve the fact that "Hennawi" gum has very few, if any, free uronic carboxyl groups, with many more than usual being involved in salt formation with cations. The fact that the ash content of clean "Hennawi" gum is much higher than usual [5.67%, compared with an average (Table I) of 3.75%] lends support to this explanation.

When the data given in Tables I and II were obtained, it became apparent that a structural study of the HW and BB samples offered an opportunity to acquire information on the biosynthesis of *A. senegal* gum. The effect of different external stimuli to gum production (*e.g.*, natural exudation, tapping wounds, beetle infestation) is not yet known. Accordingly, it was decided to compare the structural features of samples HW, BB(*a*), BB(*b*), and BB(*c*) with those of a typical tapped-gum sample QN1. Table III shows the analytical differences between these samples; their structural differences will be discussed in a subsequent paper.

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STUDIES ON URONIC ACID MATERIALS

PART XXV*. SOME UNUSUAL FORMS OF THE GUM FROM *Acacia senegal* WILLD.

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ABSTRACT

A comparative study, involving classical methylation and Smith-degradation techniques, has been carried out on samples of normal "tapped" gum from *Acacia senegal*, natural exudate gum ("Hennawi" gum) from the main trunk of the tree, and the gum exuded by a tree infested with wood-boring beetles. The studies have shown that the beetle-borer specimen was virtually identical to the specimen of normal exudate gum; the specimen of "Hennawi" gum was basically similar, but differed in two important aspects concerning some of the peripheral end-group positions involving rhamnose and glucuronic acid residues. Theories of the origin, function, precursors, and mode of biosynthesis of plant gums are examined critically in terms of the results obtained.

INTRODUCTION

In the preceding part of this series, we reported the results of an analytical study of some different forms of the gum exudate from *Acacia senegal* Willd. Two of the specimens were of particular interest; these were (*a*) the gum exuded by a tree infested with wood-boring beetles, and (*b*) "Hennawi" gum, exuded from the lower, main trunk of the tree, and found[1] to contain less than 50% of the customary proportion of rhamnose. There is, at present, a lack of knowledge regarding the biosynthetic mechanism of gum production, and the nature of the external injury or physiological stimulus necessary to cause the formation of gum. It was therefore decided to carry out structural studies of the "Hennawi" and beetle-borer variants of *Acacia senegal* gum, and, for comparison, of a normal specimen of "tapped" gum.

EXPERIMENTAL

Origins of specimens. — These have been described[1]. The sample of "Hennawi" gum is designated HW; the beetle-borer sample is designated BB [extraction into three

*For Part XXIV, see Ref. 1.

fractions BB(*a*), BB(*b*), and BB(*c*) has been described[1]]. These atypical specimens of gum have been compared, in this study, with specimen QN1, which is representative[1] of the best quality, tapped gum from the first collection of the 1960 gum season at Qala en Nahal, Kassala Province, Eastern Sudan.

Analytical methods. — The standard methods involved have been described previously[2]. Paper chromatography was carried out on Whatman No. 1 paper with the following solvent systems (v/v): (*a*) benzene–butyl alcohol–pyridine–water (1:5:3:3, upper layer); (*b*) ethyl acetate–pyridine–water (10:4:3); (*c*) ethyl acetate–acetic acid–formic acid–water (18:3:1:4); (*d*) butyl alcohol–ethanol–water (4:1:5, upper layer); (*e*) butyl alcohol–acetic acid–water (4:1:5, upper layer); (*f*) butanone–water–ammonia (sp. gr. 0.880) (200:17:1). R_G values of methylated sugars refer to distances moved relative to that of 2,3,4,6-tetra-*O*-methyl-D-glucose in solvent (*d*). Chromatograms were developed with aniline hydrogen oxalate, alkaline silver nitrate, or the periodate–permanganate reagent.

Gas–liquid partition chromatography (chromatograph Type S3A, fitted with flame-ionisation detectors, Gas Chromatography Ltd.) was carried out at nitrogen flow-rates of *ca.* 100 ml/min. on columns of (*i*) 15% of poly(butane-1,4-diol succinate) on Celite (120 × 0.5 cm) at 175°, (*ii*) 15% of poly(ethylene glycol adipate) on Celite (75 × 0.5 cm) at 160°. Retention times (T) are quoted relative to that of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside as standard.

Polysaccharides were methylated successively with methyl sulphate and sodium hydroxide solution, and with methyl iodide and silver oxide. Methanolyses were carried out under reflux for 7 h with 5% methanolic hydrogen chloride.

Periodate oxidations were carried out at room temperature in darkness by mixing equal volumes of 4% polysaccharide solutions and 0.25M periodate. The formic acid released was determined by titration with standard sodium hydroxide solution. Periodate consumption was determined by titration with standard arsenite solution[3].

Attempted fractionations. — Samples HW, BB(*a*), BB(*b*), BB(*c*), and QN1 each migrated as a single band on electrophoresis on cellulose acetate film in (*a*) 0.1M ammonium carbonate buffer (pH 8.9), or (*b*) 0.1M acetate buffer (pH 4.7), and all the samples had the same mobility when run under comparable conditions at 18.8 volts/cm.

Samples HW, BB(*a*), BB(*b*), BB(*c*), and QN1 were examined chromatographically on a DEAE-cellulose column (45 × 1.5 cm). On gradient elution with sodium chloride solutions (0.0 → 0.3M) in 0.02M acetate buffer (pH 4.1), each sample gave a slightly asymmetric peak.

RESULTS

The analytical data for samples HW, BB(*a*), BB(*b*), BB(*c*), and QN1 have been reported[1].

Methylation study of the samples. — Each (300 mg) of the five samples HW, BB(*a*), BB(*b*), BB(*c*), and QN1 was methylated with the classical Haworth and Purdie reagents. Yields, specific rotations, and methoxyl contents of the methylated products

are shown in Table I; methoxyl contents were not raised on further attempted methylation. On methanolysis, and examination of the mixture of methyl glycosides by g.l.c., the methylated product of each sample gave identical chromatograms, except

TABLE I

DATA FOR *O*-METHYL DERIVATIVES OF SAMPLES QN1, HW, BB(*a*), BB(*b*), AND BB(*c*)

	QN1	*HW*	*BB*(a)	*BB*(b)	*BB*(c)
Yield (mg)[a]	250	240	250	260	240
$[\alpha]_D$ (*c* 1.0, chloroform)	−46°	−42°	−45°	−46°	−46°
Methoxyl, %	41.5	42.0	42.1	41.6	41.5

[a]Weight of sample methylated = 300 mg.

that the methylated product from HW gave less 2,3,4-tri-*O*-methyl-L-rhamnose and more 2,3,4-tri-*O*-methyl-D-glucuronic acid than did the other methylated products. Table II gives the results of the chromatographic examinations; retention times on columns (*i*) and (*ii*) were comparable with those for methyl glycosides from the authentic *O*-methyl sugars. A portion of each methanolysate was hydrolysed (N sulphuric acid, 4 h) on a boiling water-bath. The cooled solutions were neutralised (barium carbonate), filtered, treated with Amberlite resin IR-120 (H^+), and concentrated. Paper chromatography of each hydrolysate [solvents (*d*) and (*f*)] revealed the presence of some 2-*O*-methylgalactose in addition to those *O*-methyl sugars already identified by g.l.c. of the methyl glycosides (Table II).

TABLE II

CHROMATOGRAPHIC EXAMINATIONS OF METHANOLYSIS PRODUCTS FROM SAMPLES QN1, HW, BB(*a*), BB(*b*), AND BB(*c*)

Relative retention times (T) *on g.l.c.*[a] *Column* (i)	*Column* (ii)	*Paper chromatography*, R_G *in solvent* (d)	*O-Methyl sugars identified*
0.48	(0.51)	1.01	2,3,4-Tri-*O*-methyl-L-rhamnose
0.58; 0.72	(0.51); 0.64	0.97	2,3,5-Tri-*O*-methyl-L-arabinose
1.00	0.85	0.79	2,3,4-Tri-*O*-methyl-L-arabinose
1.79; 3.16	1.29; (2.21)	0.82	2,5-Di-*O*-methyl-L-arabinose
1.68	1.64	0.88	2,3,4,6-Tetra-*O*-methyl-D-galactose
3.77; 4.22	3.01; 3.51	0.74	2,4,6-Tri-*O*-methyl-D-galactose
6.35	5.14	0.74	2,3,4-Tri-*O*-methyl-D-galactose
14.6; 16.4	9.7; 11.0	0.53	2,4-Di-*O*-methyl-D-galactose
2.35; 2.94	(2.21); 2.65	—	2,3,4-Tri-*O*-methyl-D-glucuronic acid[b]
7.9; 9.3	5.8; 6.9	—	2,3-Di-*O*-methyl-D-glucuronic acid[b]
		0.34	2-*O*-Methyl-D-galactose
1.18; 1.44	0.98; 1.13; 2.50	—	Unknown sugars

[a]Incompletely resolved components shown in brackets. [b]As methyl ester methyl glycoside.

Smith degradations[4] *of samples.* — Each of the samples HW, BB(*a*), BB(*b*), BB(*c*), and QN1 was subjected to the Smith degradation process as follows. The sample (2.0 g) was dissolved in water, and 0.25M sodium metaperiodate (50 ml) was added. The oxidation mixtures were left in darkness for 48 h at room temperature. The reactions were stopped by the addition of ethylene glycol (2 ml), and the solutions were dialysed against tap water for 48 h. Sodium borohydride (0.5 g) was added to the solution, and the solutions were kept for 30 h at room temperature and were then dialysed for 48 h. The resulting polyalcohol was hydrolysed at room temperature (N sulphuric acid; 48 h), followed by dialysis against tap water for 48 h to remove sulphuric acid and small carbohydrate fragments. The Smith-degraded polysaccharides were then isolated as the freeze-dried products (yields and analytical data shown in Table III), and the values of $\bar{M}_n$ were determined by molecular-sieve chromatography[2] on a previously calibrated "Bio-Gel P300 " column.

TABLE III

ANALYTICAL DATA[a] FOR THE SMITH-DEGRADED POLYSACCHARIDES

	Sample				
	QN1	*HW*	*BB*(a)	*BB*(b)	*BB*(c)
Yield, %	67	63	67	66	65
Nitrogen, %	0	0	0	0	0
$[\alpha]_D$ (c 1.0, water)	−28.2°	−28.0°	−29.1°	−28.0°	−28.5°
M_n[b]	98,000	96,000	93,000	96,000	93,000
Uronic acid[c], %	3.7	1.6	4.4	3.8	4.3
Galactose, %	70	71	70	70	69
Arabinose, %	26	27	26	26	27
Formic acid released on periodate oxidation (moles/g) × 10^3	1.85	1.80	1.83	1.78	1.75
Periodate consumed (moles/g) × 10^3	5.53	5.45	5.31	5.29	5.42

[a]All data corrected to dry-weight basis. [b]Estimated by molecular-sieve chromatography. [c]By acidic decarboxylation.

Hydrolysis (N sulphuric acid) of the Smith-degraded product from sample HW, followed by paper chromatography of the hydrolysate, showed the presence of galactose and arabinose. Hydrolyses of the Smith-degraded products from samples BB(*a*), BB(*b*), BB(*c*), and QN1, followed by paper chromatography, showed the presence of galactose, arabinose, and a trace of an aldobiouronic acid having the mobility of 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose [R_{Gal} 0.23, solvent (*c*)]. Mild hydrolysis of the Smith-degraded polysaccharides (0.5N sulphuric acid, 1 h, on a boiling water-bath), followed by paper chromatography, showed the presence of galactose, arabinose, and two neutral disaccharides having the chromatographic mobilities of 3-*O*-β-D-galactopyranosyl-D-galactose [R_{Gal} 0.49, solvent (*a*); R_{Gal} 0.53, solvent (*b*)] and 6-*O*-β-D-galactopyranosyl-D-galactose [R_{Gal} 0.29, solvent (*a*); R_{Gal} 0.38, solvent (*b*)].

Methylation of the Smith-degraded polysaccharides. — Each of the Smith-

degraded polysaccharides obtained respectively from samples HW, QN1, BB(*a*), BB(*b*), and BB(*c*) was methylated successively with the Haworth and Purdie reagents. Yields, specific rotations, and methoxyl contents of the methylated products are shown in Table IV; methoxyl contents were not raised on further attempted methylation. On methanolysis and g.l.c. of the resulting mixtures of methyl glycosides, the methylated products from each of the Smith-degraded polysaccharides gave identical traces, with the exception that the methylated product from Smith-degraded sample HW gave no detectable amounts of 2,3,4-tri-*O*-methyl-D-glucuronic acid. The results of the g.l.c. analyses are shown in Table V. A portion of each methanolysate was hydrolysed (N sulphuric acid, 4 h, boiling water-bath). The cooled solutions were neutralised with barium carbonate, filtered, treated with Amberlite resin IR-120 (H^+), and concentrated. Paper-chromatographic examination of the hydrolysates [solvents (*d*) and (*f*)] showed the presence of 2-*O*-methylgalactose, in addition to those *O*-methyl sugars already identified by g.l.c. of their methyl glycosides (Table V).

TABLE IV

DATA FOR *O*-METHYL DERIVATIVES OF THE SMITH-DEGRADED POLYSACCHARIDES FROM SAMPLES QN1 HW, BB(*a*), BB(*b*), AND BB(*c*)

	QN1	*HW*	*BB*(a)	*BB*(b)	*BB*(c)
Yield (mg)[a]	220	212	220	215	225
$[\alpha]_D$ (*c* 1.0, chloroform)	−41°	−40°	−41°	−41°	−42°
Methoxyl, %	40.5	40.9	41.1	40.9	40.5

[a]Weight of sample taken = 300 mg.

TABLE V

CHROMATOGRAPHIC EXAMINATIONS OF METHANOLYSIS PRODUCTS FROM METHYLATED SMITH-DEGRADED POLYSACCHARIDES FROM SAMPLES QN1, BB(*a*), BB(*b*), AND BB(*c*)[a]

Relative retention times (T) *on g.l.c.*[b]		*Paper chromatography*, R_G *in solvent* (d)	*O-Methyl sugars identified*
Column (i)	*Column* (ii)		
0.57; 0.73	0.50; 0.64	0.97	2,3,5-Tri-*O*-methyl-L-arabinose
1.79; 3.15	1.30; (2.21)	0.82	2,5-Di-*O*-methyl-L-arabinose
1.68	1.65	0.88	2,3,4,6-Tetra-*O*-methyl-D-galactose
3.78; 4.22	2.99; 3.50	0.74	2,4,6-Tri-*O*-methyl-D-galactose
6.34	5.14	0.74	2,3,4-Tri-*O*-methyl-D-galactose
9.1	6.5; 6.9; 7.7	0.59	2,6-Di-*O*-methyl-D-galactose
14.6; 16.4	9.7; 11.0	0.53	2,4-Di-*O*-methyl-D-galactose
2.35; 2.95	(2.21); 2.66	—	2,3,4-Tri-*O*-methyl-D-glucuronic acid[c]
—	—	0.34	2-*O*-Methyl-D-galactose
1.08; 1.52	0.86; 2.48	—	Unknown sugars

[a]Identical results for sample HW, except that 2,3,4-tri-*O*-methyl-D-glucuronic acid was absent. [b]Incompletely resolved peaks shown in brackets. [c]As methyl ester methyl glycoside.

DISCUSSION

The results presented in this paper indicate that, structurally, the three fractions from the beetle-borer gum exudate are very similar to normal, tapped gum from *Acacia senegal*. Although the sample of "Hennawi" gum is basically similar as far as the interior structure is concerned, it differs in two important aspects that concern some of the peripheral end-group positions of the gum molecule.

It is now some time since theories[5-9] concerning the origin and function of plant gums were considered, and there has been little agreement on either of these points to date. It has also been debated whether gum exudates are formed at the site of a wound, or whether they are generated elsewhere in the plant and then transported to the site of exudation. It has been suggested that gum exudates may be a product of normal plant metabolism[6,7], that they may arise from a pathological condition of the tree[6], or that they may arise from some infection or invasion[7] by micro-organisms which may be fungal[6] or bacterial [7,10,11] in nature. These theories were advanced much earlier this century; and there has been no modern work to substantiate or refute them. Some of these theories are discussed in non-critical reviews that contain factual errors *e.g. A. verek* is not a variety of *A. arabica*[11,12], and gum arabic does not contain galacturonic acid[11,12]. Misprints and such errors in reviews tend to be perpetuated (*cf.* Ref. 13).

It has been suggested[5,9] that starch may undergo transformation into gum, but the enzyme systems necessary to transform a polyglucan into a highly branched polysaccharide containing galactose, arabinose, rhamnose, glucuronic acid, and 4-*O*-methylglucuronic acid appear to be impossibly complex; starch was not found[14] in the wood tissues of excised branches from *A. senegal* trees. It seems more reasonable that the gum acid has, as its precursor, some highly branched arabino-galactan of a hemi-cellulosic type, to which is added rhamnose-, glucuronic acid-, and 4-*O*-methylglucuronic acid-terminated side-chains in the final stages of gum production. The enzyme systems probably differ at different parts of the tree (*cf.* "Hennawi" gum).

Although modern commercial samples of good quality, Sudanese gum arabic originate almost entirely from *A. senegal*, contamination or adulteration with other genera[15] and with other *Acacia* species is quite possible, since the various nodules cannot be distinguished by colour, shape, or size. Commercial samples should never be used for fundamental studies. Early specimens of gum arabic were undoubtedly mixtures from various *Acacia* species[8,16], which are now known (*cf.* earlier parts of this series) to differ considerably in sugar composition and structure. The reason why no rhamnose was detected in certain samples of gum arabic[17] is now quite clear, if the term "gum arabic" was taken to apply to the exudate from any *Acacia* species[8]; some *Acacia* species contain 12–14% of rhamnose, others 6–7% of rhamnose, and some less than 1% of rhamnose[18]. In addition to this variation between species, our results now show that authentic nodules from *A. senegal* can have different amounts of rhamnose. Much of the evidence to date regarding the chemical heterogeneity of gum arabic was based on the depleted rhamnose content of certain fractions from initially hetero-

geneous, commercial samples; the point was made some time ago[7] that the classical work of Heidelberger and his colleagues should be repeated on specimens from a single *Acacia* tree or at least from one variety of *Acacia*.

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A MODEL SYSTEM FOR THE ENZYMIC FORMATION OF 6-DEOXY-HEXOSES

THE CONVERSION OF METHYL-D-GALACTOPYRANOSIDES INTO METHYL D-FUCOPYRANOSIDES

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ABSTRACT

Catalytic oxidation of methyl α- and β-D-galactopyranoside, followed by catalytic reduction, led to the formation of methyl α- and β-D-fucopyranoside (15% and 35% yield), respectively. This oxidation–reduction sequence with selective oxidation at C-4 as the first step is structurally closely related to the pathway of 6-deoxyhexose biosynthesis. In the model system as well as in the enzymic reaction, a labile 4-hexulose derivative is formed. It appears that it is the intrinsic property of this intermediate to undergo molecular rearrangement, thereby resulting, after subsequent reduction, in the formation of a 6-deoxyhexose.

INTRODUCTION

A nucleotide-bound 6-deoxy-4-hexulose is the only intermediate isolated in the pathway of 6-deoxyhexose biosynthesis, and its formation has been demonstrated in a variety of enzymatic systems[1–4]. In addition, this intermediate has been shown to be a precursor for a number of more complex deoxy sugars such as 4-amino-4,6-dideoxyhexoses[5], 3,6-dideoxyhexoses[6], and 3-amino-3,6-dideoxyhexoses[7].

The events leading to the formation of this intermediate are still hypothetical. According to the proposed reaction sequence[8], the first step is an oxidation of the nucleotide-bound hexopyranose at C-4, followed by an elimination of water between C-5 and C-6, and the resulting formation of 4-hex-5-enulose. Subsequent reduction of this compound yields the 6-deoxy-4-hexulose intermediate (Fig. 1).

The above oxidation–reduction sequence has been studied on a model system with structurally related analogs of the naturally occurring substrate wherein methyl α- and β-D-galactopyranoside were subjected to catalytic oxidation–reduction. The isolation and identification of D-fucose as a major product of the catalytic oxidation–reduction provides indirect support for the validity of the proposed reaction sequence[8].

RESULTS AND DISCUSSION

It is well established that oxygen in the presence of platinum (Adams catalyst) can achieve the specific oxidation of secondary alcohols by a preferential attack upon

the hydrogen atom in an equatorial position[9]. Moreover, when methyl α-L-fucopyranoside was subjected to this catalytic oxidation, the expected methyl 6-deoxy-L-*xylo*-hexopyranosid-4-ulose was obtained[10]. Proof for the selective oxidation at C-4 was provided by recovery of the two isomers, 6-deoxy-L-glucose and 6-deoxy-L-galactose, following reduction of the keto derivative with sodium borohydride.

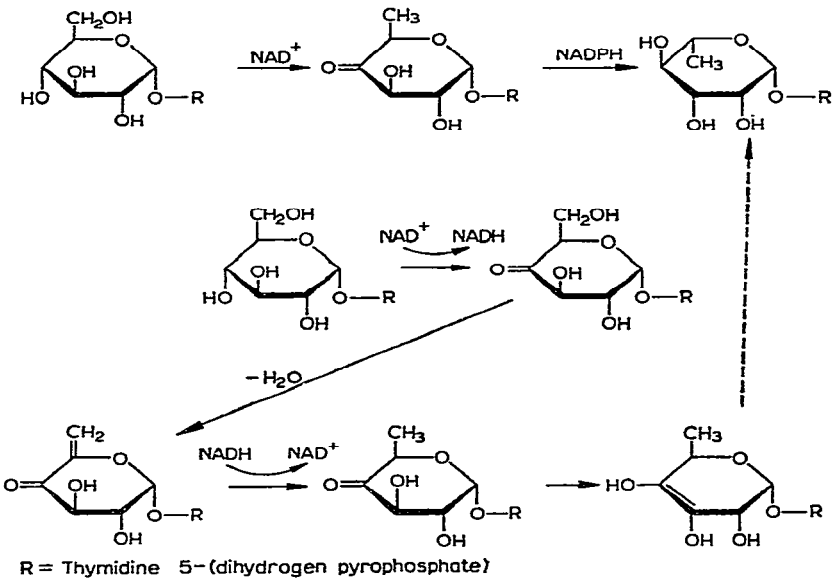

Fig. 1. Intermediates in the proposed pathway of the biosynthesis of 6-deoxy-L-mannose catalyzed by thymidine 5-(D-glucosyl dihydrogen pyrophosphate) oxidoreductase.

The direct application of this selective catalytic oxidation at C-4 to methyl α-D-galactopyranoside is comparable to the proposed oxidation step of C-4 of the hexose moiety in the enzymic reaction. Under the conditions of the metal-catalyzed oxidation, it is known that the primary alcoholic function of methyl α-D-galactopyranoside will be converted into a carboxylic group. However, it was found that in unbuffered solution, this side-reaction proceeds to less than 1% of the starting material.

The formation of keto derivatives during the catalytic oxidation was observed by the determination of reducing substances[11], a positive semicarbazide assay[12], and ultraviolet absorption after addition of alkali[4]. From these data, as well as by estimation of the unchanged methyl α-D-galactopyranoside, it was concluded that about 10% of the total material was oxidized under the conditions of the experiment.

The reaction mixture was examined by electrophoresis in a sodium hydrogen sulfite buffer[13] and by paper chromatography. In addition to the main component, unchanged methyl α-D-galactopyranoside, a complex mixture of reducing substances was observed.

Several of these components proved to be labile derivatives that underwent rearrangement during isolation. In order to minimize artifact formation, the oxidation

mixture was reduced catalytically with platinum–hydrogen without isolation of the intermediary products. The resulting compounds were isolated and identified. Enzymic analysis of the hydrolyzate with glucose oxidase[14] or with hexokinase and glucose-6-phosphate dehydrogenase[15] was negative in all experiments.

When methyl β-D-galactopyranoside, α-D-galactopyranosyl dihydrogen phosphate, lactitol, or methyl α,β-D-galactopyranoside 6-(dihydrogen phosphate) were subjected to catalytic oxidation–reduction, no evidence for the formation of D-glucose or its derivatives could be found.

Further paper chromatographic examination of the reaction products after catalytic oxidation–reduction of methyl α-D-galactopyranoside revealed to presence of a component having properties of a 6-deoxyhexose. The component was isolated by column chromatography[16] on Dowex-1 (OH^-) in about 15% yield, and identified as methyl α-D-fucopyranoside. When the same experiment was performed on methyl β-D-galactopyranoside, 35% of the oxidized material was isolated and identified as methyl β-D-fucopyranoside.

The isolation of both anomeric forms of methyl D-fucopyranoside, and the concomitant absence of D-glucose among the reaction products was interpreted as indicating that the initially formed methyl D-*xylo*-4-hexulopyranoside was unstable and underwent rearrangement. Thus, β-elimination of water between C-5 and C-6 resulted in the formation of a 4-hexulos-5,6-een derivative which, upon reduction, gave D-fucose. Upon reduction, the 4-hexulos-5,6-een can, theoretically give rise to

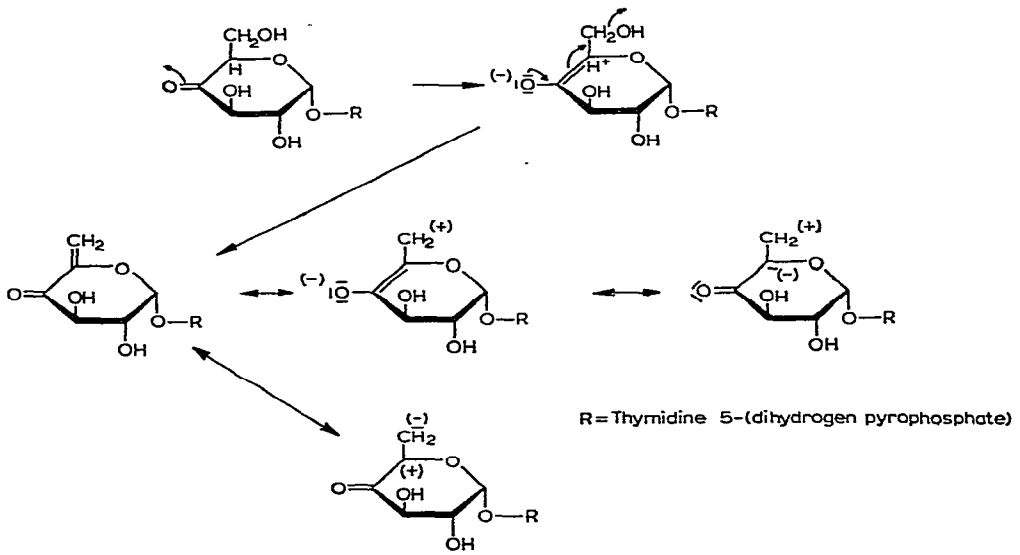

Fig. 2. Proposed formation of a 4-hex-5-enulose derivative by β-elimination between C-5 and C-6.

four isomers: 6-deoxy-D-glucose, 6-deoxy-D-galactose, 6-deoxy-L-idose, and 6-deoxy-L, altrose. However, the preferential formation of one isomer has been demonstrated previously[17] by the catalytic reduction of methyl hexopyranosiduloses, and no attempt

was made here to isolate and identify the minor products of reaction. Stabilization gained by resonance energy, as indicated in Fig. 2, could be the driving force for the β-elimination. Significantly, when sodium borohydride was substituted for $Pt–H_2$ as the reducing agent, no D-fucose was formed. This observation is consistent with the known requirements for catalytic reduction of carbon–carbon double bonds and is in accord with the interpretation that a hex-5-enose is the immediate precursor of the methyl D-fucopyranoside.

The instability of methyl D-*xylo*-hexopyranosid-4-ulose stands in sharp contrast to the properties of the structurally closely related 4-keto derivative of methyl α-L-fucopyranoside. Extensive studies on the stability of anomeric methyl hexopyranosiduloses having the keto group at position 2, 3, or 4, respectively, have been reported by Theander[18], who pointed out their lability in aqueous solution and demonstrated their degradation under mildly acidic or alkaline conditions. Theander also noted that the 3-keto derivative appeared to be the more stable compound as compared to the 2- and 4-keto derivatives. The latter compounds are convertible into the 3-keto compound *via* the corresponding enediols.

The limited number of known dicarbonyl carbohydrates does not yet permit a good understanding of the reasons for the drastically changed properties when compared to the parent hexose derivatives. The introduction of a keto group into the pyranose ring undoubtedly causes considerable changes in the valence angle, distance between atoms, shape of the ring, and dipole moment of the molecule. The differences in stability between methyl *xylo*-hex-4-ulose pyranoside having a primary alcoholic function at C-6 as compared to the compound having a deoxy group in this position of is considerable theoretical interest. It points to the necessity to expand and reconsider some of our concepts of conformational analysis.

The formation of D-fucose as a major component in the model system indicates that this reaction sequence has some features in common with the enzymatic pathway: If the oxidation at C-4 of the hexose moiety is accepted as the primary reaction taking place in the enzymic conversion as well as in the model system, it appears that it is an intrinsic property of the labile hexos-4-ulose to rearrange by β-elimination of water with the resultant formation of a hex-5-enose derivative. This rearrangement precludes the formation of hexoses and leads, upon subsequent reduction, to the formation of a 6-deoxyhexose derivative.

EXPERIMENTAL

Methyl α- and β-D-galactopyranoside. — D-Galactose (50 g) was heated under reflux with a 1% solution of hydrogen chloride in dry methanol (400 ml). After 14 h, the solution was cooled and stirred with silver carbonate (16 g) for several hours. After addition of a small amount of Norit, the solution was filtered through a Büchner funnel in presence of Hyflo filter-aid. The isolation of the pure methyl galactosides was effected by column chromatography on Dowex-1 (OH^-) according to Austin *et al.*[16].

Direct crystallization from the crude mixture* of methyl galactosides by use of seed crystals obtained by the above-described procedure was performed according to Frahn and Mills[19].

Methyl α,β-D-galactopyranoside 6-(dihydrogen phosphate). — Dry D-galactose 6-(disodium phosphate) (Sigma, 68 mg) was heated under reflux for 3 h with methanol (25 ml) in the presence of methanol-washed Amberlite IR-120 (H^+). After this time a 10-μl aliquot spotted on paper showed no reaction with silver nitrate except after treatment with periodic acid. The resin was filtered off, washed with methanol, and the pooled filtrates were evaporated to dryness. The residue was dissolved in methanol (0.5 ml) and applied to Whatman No. 3 MM paper. The sample was then chromatographed in 2-propanol–ammonia–water (7:1:2) for 36 h. The area having a mobility between 9 and 11 cm gave a positive reaction with the phosphate reagent of Hanes and Isherwood[23] and with the silver nitrate dip after periodate oxidation. This area was eluted with water.

Preparation of lactitol. — Lactose (360 mg) and boric acid (120 mg) were dissolved in water (10 ml) and a solution of sodium borohydride (80 mg in 2.0 ml of water) was added slowly with stirring at room temperature. After 1 h at room temperature, the reaction mixture was passed through a small column of Dowex-50 (X-8, H^+), and the eluate was evaporated to dryness. The dry residue was treated several times with 50-ml aliquots of methanol, and evaporated to dryness each time *in vacuo*. The resulting white, crystalline material was chromatographically indistinguishable from authentic lactitol (solvent: pyridine–ethyl acetate–water, 1:3.6:1.15).

Catalytic oxidation–reduction. — Methyl α- or β-D-galactopyranoside (500 mg) was dissolved in water (5 ml), and freshly prepared Adams catalyst (100 mg) was added[9]. Oxygen was bubbled through the solution with a sintered-glass disc for 30 min at 45–50°. The course of oxidation was followed by the appearance of silver nitrate-positive material[11]. Chromatography in butyl alcohol–acetic acid–water (10:1:3.3), as well as electrophoresis in sodium hydrogen sulfite buffer[13], revealed the presence of numerous products. The electrophoresis was performed in carbon tetrachloride on Whatman No. 3 MM paper in sodium hydrogen sulfite buffer for 1 h at pH 4.7 and 600 V. The buffer was prepared[13] by dissolving sodium hydrogen sulfite (9.5 g) and sodium acetate (8.8 g) in water (1000 ml) and adjusting the pH with glacial acetic acid to 4.7.

The major part of the sample, together with the platinum catalyst used in the oxidation, was subjected to hydrogenation at 30 lb/in^2 for 3 h at room temperature. The catalyst was removed by filtration on a sintered-glass funnel, and the filtrate was concentrated *in vacuo*.

The syrup resulting from the catalytic oxidation–reduction of methyl α-D-galactopyranoside was dissolved in not more than 3 ml of water and the solution was poured

*It should be noted that we were not able to isolate the pure compounds by the original procedure described by Dale and Hudson[20]. Similar observations were made by Ault *et al.*[21] and by Sorkin and Reichstein[22].

onto a Dowex-1 column (X-2, 200–400 mesh, 16 × 2 cm, OH^-). The column was eluted with CO_2-free water, and 2.5-ml fractions were collected at 7.5-min intervals. Aliquots (5 ml) of the fractions were applied to Whatman No. 1 paper and examined with the silver nitrate reagent before and after treatment with periodic acid. Pure methyl α-D-fucopyranoside was eluted in fractions 26–30. Additional methyl α-D-fucopyranoside was eluted with small amounts of α-methyl D-galactopyranoside in fractions 31–34. The major portion of unreacted methyl α-D-galactopyranoside was separated and recovered in fractions 35–47.

Fractions 26–30 and 31–34 were combined. The solution was subjected to rechromatography in ethyl acetate–pyridine–water (3.6:1.0:1.15) on Whatman No. 3 MM paper to give the pure methyl α-D-glycoside. Methyl β-D-fucopyranoside was obtained from methyl β-D-galactopyranoside by the same procedure.

A solution of pure methyl α- or β-D-fucopyranoside (about 20 μmoles) was evaporated to dryness *in vacuo* and N H_2SO_4 (1 ml) was added. The solution was heated for 5 h in a boiling water-bath, and then it was cooled and neutralized with 0.3N $Ba(OH)_2$ to the phenolphthalein end point. The resulting $BaSO_4$ was removed by centrifugation, and the supernatant fluid was passed through a small column of Dowex-50 (X-8, 200-400 mesh, 1.0 × 0.5 cm H^+). The combined eluate and water wash were evaporated to dryness *in vacuo*.

Identification of D-*fucose.* — The identification was performed on the intact methyl glycosides and on the free sugar, obtained as described above. The methyl α-D-fucopyranoside showed $[\alpha]_D^{20}$ +200° (*c* 0.68, water) (lit.[24]: methyl α-L-fucopyranoside, $[\alpha]_D^{20}$ − 198.0°; methyl β-L-fucopyranoside, $[\alpha]_D^{20}$ + 16.0°. Paper chromatography of the methyl α-D-glycoside and D-fucose in presence of methyl α-L-fucopyranoside and L-fucose, respectively, in pyridine–ethyl acetate–water (1.0:3.6:1.15): $R_{glucose}$ of D- and L-fucose: 2.62; $R_{glucose}$ of methyl α-L-fucopyranoside: 3.81).

The colorimetric reaction with cysteine–sulfuric acid[25] for 6-deoxyhexose gave the characteristic absorption maximum at 400 nm for the unhydrolyzed as well as for the hydrolyzed sample. The hydrolyzed sample (about 0.1 μmole of free sugar in 180 μl water) was reduced by the addition of two 25-μl aliquots of sodium borotritide (1000 μmoles/ml). The solution was kept for 1 h at room temperature. The excess borohydride was destroyed by addition of glacial acetic acid (20 μl). After evaporation to dryness *in vacuo*, the residue was dissolved in 200 μl of water, and the solution was passed through a small column of Dowex-50 (H^+) and evaporated to dryness. Repeated addition of 1-ml aliquots of methanol, followed by evaporation to dryness, gave a salt-free solution. Authentic L-fucose was subjected to the same procedure. The sample, as well as the standard, was chromatographed on paper in pyridine–ethyl acetate–water. The silver nitrate reagent revealed one spot in both samples with a mobility faster than that of L-fucose ($R_{glucose}$ 2.62) and identical with that of authentic 6-deoxy-D-galactitol $R_{glucose}$ 3.16). In addition, all the tritium activity coincided with the spot corresponding to 6-deoxy-L-galactitol.

About 10 μmoles of the hydrolyzed sample, as well as authentic L-fucose, was converted into tetra-*O*-acetyl-D-fucose diethyl dithioacetal[26]. Thin-layer chromato-

graphy on silica gel G in benzene–methanol (9:1, v/v) showed one single component having identical mobilities for sample and standard. Elution from the thin-layer plate with acetone and colorimetric assay with cysteine–sulfuric acid showed the characteristic absorption maximum for 6-deoxyhexoses. G.l.c. of the methyl glycosides, as well as of the free sugar, was performed, after converting them to the corresponding trimethylsilyl derivatives[27], with the use of a gas chromatograph F. & M., Model 500, with flame ionization attachment and a stainless-steel column 6 ft × 0.25 in, packed with 3% SE-52. The column was operated isothermally at 140°. Identical retention times for the methyl glycosides as well as for the free sugar were found, when compared to the corresponding authentic L-fucose derivatives: fucose 3.56 min (small peak), 4.23 min (major peak), 5.10 min (major peak); methyl α-L-fucopyranoside 3.38 min.

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Notes

Acid-catalyzed hydrolysis of maltose derivatives containing nitrogen

Difficulties in determining the structures of polysaccharides have recently been pointed out[1]. The structures of other biopolymers are commonly deduced by partial hydrolysis, determination of structures of the resultant oligomers, and, by matching overlapping sequences of monomer units, determination of the structure of the polymer. This method is unsuccessful with some polysaccharides, because polysaccharides are usually composed of only one or two different types of monosaccharide unit and their chains are often branched.

Necessary to meaningful study of branched-chain polysaccharides is a method that will remove monosaccharide, disaccharide, or trisaccharide residues sequentially from one end of the chain. Certain enzymes have the ability to split off such units from the nonreducing ends, but they are so specific that the process stops when a different type of linkage is reached. Alkaline degradation has been suggested as a means of removing one unit at a time from the reducing end, but this method suffers from two limitations: (*a*) the "stopping reaction" prevents complete degradation, and (*b*) the individual sugar residues can become so altered that determination of the identity of the original sugar may not be possible[2,3].

BeMiller and Mann[4] recently reported on the acid-catalyzed hydrolysis of selected maltose derivatives, an investigation undertaken in an effort to find a reducing-end derivative that would greatly enhance the rate of hydrolysis, so that only the reducing end-unit of an oligo- or poly-saccharide could be removed upon treatment with acid. Such a process would permit sequence determinations by a stepwise, peeling process from the reducing end of the polysaccharide. Maltose 1-phenylflavazole was found to undergo hydrolysis much more rapidly than maltose (a model for the other linkages in an oligosaccharide), but only at low temperatures where hydrolysis is too slow to be of practical use.

As an alternative to a change of the temperature, control of hydrolysis of derivatives might also be accomplished by a change in the acid used. Painter and Morgan[5] found that basic carbohydrates are hydrolyzed much faster by water-soluble poly(styrenesulfonic acid) than by an equivalent normality of mineral acid. In a series of papers, Painter[6–8] has reported that, in hydrolysis catalyzed by this polymeric acid, glycosidic linkages near a positively charged group are hydrolyzed faster than those more distant from it.

In the present investigation, maltose, maltitol, maltose 1-phenylflavazole, and maltose phenylosotriazole were hydrolyzed with 3.2×10^{-4}N water-soluble poly-

(styrenesulfonic acid), to determine if a change in the acid used would result in a significant difference in reactivity between the nitrogen-containing derivatives and the other derivatives.

Maltose, maltitol, and maltose 1-phenylflavazole showed no hydrolysis at 75, 85, or 95°, even after 48 h. Lack of hydrolysis was indicated by the absence of change in optical rotation and by the absence of hydrolysis products (papergram). However, maltose phenylosotriazole was hydrolyzed at a measurable rate, even at the very low concentration of acid used. Thermodynamic parameters are given in Table I.

TABLE I

HYDROLYTIC RATE DATA FOR MALTOSE PHENYLOSOTRIAZOLE

Temperature, degrees	*Rate* ($K \times 10^5$ *sec.*$^{-1}$)	$\Delta H^{\ddagger}$ (*kcal. mole*$^{-1}$)	$\Delta S^{\ddagger}$ (*cal. mole*$^{-1}$. *deg.*$^{-1}$)
75	2.43		
85	4.53	15.4	−35.9
95	8.61		

Reported values[9] for the hydrolysis of pyranosides are in the following ranges: $\Delta H^{\ddagger}$ 28.1 to 38.2 kcal.mole^{-1} and $\Delta S^{\ddagger}$ +0.8 to +22.9 cal.mole^{-1}. deg.$^{-1}$. The one exception is the acid-catalyzed hydrolysis of maltose 1-phenylflavazole, which also has a low $\Delta H^{\ddagger}$ value and a large, negative $\Delta S^{\ddagger}$ value[4].

It is quite likely that the use of a polymeric acid as a catalyst results in an ordered transition state in which the basic nitrogen atoms of maltose phenylosotriazole bind with the acid and bring other sulfonic acid groups into proximity to the glycosidic linkage, where one of the acid groups then donates a proton for hydrolysis. Such an ordered structure could be the reason for the large, negative entropy of activation noted for this reaction.

These results indicate that the reducing end-unit can be removed specifically from an oligo- or poly-saccharide by first converting it into the phenylosotriazole and then treating the derivative with a dilute solution of water-soluble poly(styrenesulfonic acid). The hydrolysis of maltose phenosotriazole in 3.2×10^{-4}N poly(styrenesulfonic acid) was complete in 12 h at 95°, conditions under which maltose did not react. Although such a method might not permit the identification of each unit in an oligosaccharide in a stepwise manner (because the asymmetry at C-2 is destroyed when this derivative is formed), it might allow of an erosion of a molecule from the reducing end.

It is not yet clear why poly(styrenesulfonic acid) does not also specifically catalyze the hydrolysis of maltose 1-phenylflavazole. Perhaps, the geometry of this molecule is such that a special kind of hydrolysis cannot occur.

EXPERIMENTAL

Sugars. — Maltose[10], maltitol[11], maltose phenylosotriazole[2], and maltose 1-phenylflavazole[12] were prepared by published procedures. The last compound was

recrystallized several times as follows. A solution in the minimal volume of hot, glacial acetic acid was placed in a freezer at $-10°$. The acetic acid froze to colorless crystals, and the flavazole to yellow crystals. The acetic acid was melted at room temperature, and water was added to increase the yield of product. Crystals of maltose 1-phenylflavazole were then collected by filtration, and dried; m.p. 264°, lit.[12] m.p. 262–264°.

Acid. — Water-soluble poly(styrenesulfonic acid) was prepared by the method of Painter[13]. The normality was determined by potentiometric titration with a standard solution of sodium hydroxide. A stock solution (6.4×10^{-4}N) of the acid was used, because the dark color of more concentrated solutions prevented polarimetric measurements.

Sugar solutions. — The following solutions were prepared: 0.8% solutions of maltose and maltitol in water, a 0.8% solution of maltose phenylozotriazole in ethylene glycol, and a 0.4% solution of maltose 1-phenylflavazole in ethylene glycol.

Determination of hydrolysis rates. — Two ml of aqueous acid was added to 2 ml of sugar solution, with stirring, to give a solution that was 3.2×10^{-4}N in acid (pH 3.9). The resulting solution was injected into a preheated, water-jacketed, polarimeter cell of a Bendix ETL-NPL Automatic Polarimeter equipped with a mercury (546 nm) interference filter.

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Carbohyd. Res., 6 (1968) 118–120

The acid hydrolysis of glycosides
VII. Hydrolysis of 1-thio-β-D-glucopyranosides and 1-thio-β-D-glucopyranosiduronic acids

It has been shown in earlier investigations that the rate of acid-catalyzed hydrolysis of glycopyranosides is not affected by the polarity of the aglycon[1], but that this factor has a strong influence on the hydrolysis of glycopyranosiduronic acids[2]. It was therefore of interest to establish whether 1-thio-β-D-glucopyranosides and 1-thio-β-D-glucopyranosiduronic acids behave similarly on acid hydrolysis. Consequently, a few such compounds having different aglycons have now been synthesized, and their hydrolysis has been studied.

RESULTS AND DISCUSSION

Isopropyl (**1**), propyl (**2**), and 2-hydroxyethyl (**3**) 1-thio-β-D-glucopyranosides and the corresponding 1-thio-β-D-glucopyranosiduronic acids (**4**, **5**, and **6**) were prepared by standard methods and were characterized, partly through their crystalline peracetates. Hydrolysis was performed, as described previously[1], at 60, 70, 80, and 90°. Rate coefficients, and energies and entropies of activation, are shown in Table I. In Table II, rates of hydrolysis at 70° are compared with those of corresponding β-D-glucopyranosides and β-D-glucopyranosiduronic acids.

The 1-thio-β-D-glucopyranosides are all hydrolyzed more slowly than the β-D-glucopyranosides, as has been found by others[3-5]; this behavior has been attributed[3] to the lower basicity of the sulfur atom, which results in a lower degree of protonation. The difference is largest for the 2-hydroxyethyl compounds. Compound **6** is hydrolyzed more slowly than **4** and **5**, but faster than 2-hydroxyethyl β-D-glucopyranosiduronic acid. Energies and entropies of activation are lower for the 1-thio-β-D-glucopyranosiduronic acids than for the 1-thio-β-D-glucopyranosides. The 1-thio-β-D-glucopyranosiduronic acids, and especially the 2-hydroxyethyl derivative **6**, are hydrolyzed at rates higher than those of the corresponding 1-thio-β-D-glucopyranosides.

It is evident that the electron affinity of the aglycon influences the rate of hydrolysis, not only of the 1-thio-β-D-glucopyranosiduronic acids but also of the 1-thio-β-D-glucopyranosides. It is not yet clear why 1-thio-β-D-glucopyranosides, but not β-D-glucopyranosides[1,2], are affected. Possibly, the protonation step is rate-determining in the hydrolysis of 1-thioglucopyranosides, because sulfur is less basic than oxygen, and because the carbon–sulfur bond is weaker than the carbon–oxygen bond. A lessening of the electron density at the glycosidic sulfur atom by an electron-attracting group in the aglycon would impede protonation and result in a lower rate of hydrolysis.

It had been expected that the 1-thio-β-D-glucopyranosiduronic acids would be hydrolyzed at a rate lower than that of the analogous β-D-glucopyranosiduronic

TABLE I

RATE COEFFICIENTS AND KINETIC PARAMETERS FOR THE HYDROLYSIS OF 1-THIO-β-D-GLUCOPYRANOSIDES AND 1-THIO-β-D-GLUCOPYRANOSIDURONIC ACIDS IN 0.5M SULFURIC ACID

Glycoside	k × 10^6, sec^{-1}				E (*kcal* $mole^{-1}$)	$\Delta S^{\ddagger}$ (*cal* $deg.^{-1}$ $mole^{-1}$)
	60°	*70°*	*80°*	*90°*		
1-Thio-β-D-glucopyranoside						
isopropyl (1)	—	4.77	25.6	84.9	35.5	+19.0
propyl (2)	—	3.66	14.4	—	33.1	+11.5
2-hydroxyethyl (3)	—	0.668	2.81	11.1	34.8	+13.0
1-Thio-β-D-glucopyranosiduronic acid						
isopropyl (4)	4.97	19.4	66.7	—	30.3	+ 6.6
propyl (5)	4.17	16.4	57.7	—	30.7	+ 7.3
2-hydroxyethyl (6)	1.38	5.37	19.4	—	30.9	+ 5.8

TABLE II

RATIO OF RATE COEFFICIENTS AT 70°

Glycosides compared	*Isopropyl*	*Propyl*	*2-Hydroxyethyl*
β-D-Glucopyranoside/β-D-Glucopyranosiduronic acid	0.18	0.48	3.2
β-D-Glucopyranoside/1-Thio-β-D-glucopyranoside	2.4	2.2	11.3
β-D-Glucopyranosiduronic acid/1-Thio-β-D-glucopyranosiduronic acid	3.3	1.0	0.44
1-Thio-β-D-glucopyranoside/1-Thio-β-D-glucopyranosiduronic acid	0.25	0.22	0.13

acids, but this only occurs with the isopropyl derivative **4**. The propyl derivative **5** is hydrolyzed at the same rate as propyl β-D-glucopyranosiduronic acid, and **6** is cleaved at a rate that is twice that of 2-hydroxyethyl β-D-glucopyranosiduronic acid. It is clear that the basicity of the sulfur atom is less decisive in the 1-thio-β-D-glucopyranosiduronic acid series than in the 1-thio-β-D-glucopyranoside series. The fact that **6** is hydrolyzed faster than the β-D-glucopyranosiduronic acid analog indicates a less effective transmission of the inductive effect of the 2-hydroxyethyl group to sulfur than to oxygen.

EXPERIMENTAL

General experimental conditions were the same as in previous investigations[1,2,6]. Experimental data are presented in Tables II and III.

2-Hydroxyethyl 1-thio-β-D-glucopyranoside[7] (3). — 2,3,4,6-Tetra-*O*-acetyl-α-D-glucosyl bromide (40 g) was added to a solution of potassium (4.0 g) and 2-mercaptoethanol (100 g) in anhydrous methanol (300 ml) at −20°; the temperature was then allowed to rise to room temperature. The mixture was kept for 2 h, and filtered through Celite–carbon, and the filtrate was evaporated to dryness. The resulting syrup was

TABLE III

EXPERIMENTAL DATA FOR THE PERACETYLATED ALKYL 1-THIO-β-D-GLUCOPYRANOSIDES AND PERACETYLATED METHYL (ALKYL 1-THIO-β-D-GLUCOPYRANOSID)URONATES

Glycoside	*Yield, %*	*M.p., degrees*	$[\alpha]_D$, *degrees*
2,3,4,6-Tetra-*O*-acetyl-1-thio-β-D-glucopyranoside			
isopropyl		112	−22.0
propyl	29	90.5–92	−28.0
		79–82[a,b]	−22.2[a,b]
2-acetoxyethyl	26	108–108.5	−31.5
Methyl (2,3,4-tri-*O*-acetyl-1-thio-β-D-glucopyranosid)-uronate			
isopropyl	38	106–107	−39.3
	37[c]	99–101[c]	−36.9[c]
propyl	37	100–100.5	−43.9
	40[c]	95–96	−42.2[c]
2-acetoxyethyl	46	132–132.5	−49.0
	62[c]	132–132.5	−46.4[c]

[a]Ref. 7. [b]Ref. 9. [c]Ref. 8.

TABLE IV

MELTING POINTS AND SPECIFIC ROTATIONS OF 1-THIO-β-D-GLUCOPYRANOSIDES AND 1-THIO-β-D-GLUCOPYRANOSIDURONIC ACIDS

Glycoside	*M.p., degrees*	$[\alpha]_D$, *degrees*
1-Thio-β-D-glucopyranoside		
isopropyl (**1**)		−48.6
propyl (**2**)		−59.7
2-hydroxyethyl (**3**)	114–116	−61.8
1-Thio-β-D-glucopyranosiduronic acid		
isopropyl (**4**)	145–148	−93.0
propyl (**5**)	143.5–144.5	−90.0
	136–139[a]	−84.9[a]
2-hydroxyethyl (**6**)		−90.2

[a]Ref. 8.

dissolved in pyridine (100 ml) at −20°, and acetic anhydride (100 ml) was added. After 15 h at 15°, chloroform (200 ml) was added, and the solution was washed with ice–water; evaporation gave a syrup that was dissolved in anhydrous ethanol, and the solution was treated with decolorizing carbon. Crystallization from the same solvent afforded 11.2 g (26%) of 2-acetoxyethyl 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside.

Anal. Calc. for $C_{18}H_{26}O_{11}S$: C, 47.99; H, 5.82; S, 7.12. Found: C, 47.83; H, 5.99; S, 7.40.

Compound **3** was obtained after deacetylation with sodium methoxide and methanol in the usual way; it crystallized directly from the syrup[7].

Anal. Calc. for $C_8H_{16}O_6S$: C, 39.99; H, 6.71; S, 13.35. Found: C, 39.68; H, 6.70; S, 13.13.

Compounds **1** and **2** were prepared similarly.

2-Hydroxyethyl 1-thio-β-D-glucopyranosiduronic acid[8] (**6**). — A solution of methyl 2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy-α-D-glucopyranuronate (40 g) in benzene (100 ml) was added to a solution of potassium (4.0 g) and 2-mercaptoethanol (100 g) in methanol (300 ml) at −20°. The bromide dissolved when the mixture was allowed to warm slowly to room temperature. After 6 h, the mixture was filtered, and the filtrate was evaporated to dryness; the resulting syrup was dissolved in pyridine (100 ml), and acetic anhydride (100 ml) was added. The product was isolated as described for the peracetate of **3**, and was recrystallized from ethanol (yield 14.5 g, 37%). The acetyl groups were then removed, giving syrupy **6** which was chromatographically pure.

Compounds **4** and **5** were prepared similarly, and were crystallized from ethyl acetate–methanol.

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Book review

The Amino Sugars. The Chemistry and Biology of Compounds Containing Amino Sugars. Vol. IIB. Metabolism and Interactions; edited by E. A. BALAZS and R. W. JEANLOZ, Academic Press Inc., New York and London, 1966, xviii+513 pages, $ 22.00.

This book, which appeared towards the end of 1966, reminds one of a recently discovered, lost manuscript; it may have been up to date when written, but was out of date when found. This is demonstrated by a remark of one of the authors in a footnote: "I have attempted to treat the immunochemistry of amino sugars comprehensively up to and including 1963. The delay in publication of this tome and the rapid progress in many of the areas of research covered have made it impossible to refer to original work (including that from my laboratory) which became known after the summer of 1964".*

Also, the Editors apologize in the preface for the failure of Volume I to appear before Volume II; this situation is disturbing to the reader, because references to Volume I appear in the text of Volume IIB.

The book, which is essentially a collection of review articles, gives extensively documented, authoritative surveys of each subject treated. An unusual feature is the combined bibliography and author index of 87 pages containing 2900 references, which makes location of specific references very convenient. The 14 chapters cover: Metabolism of amino sugars; Metabolism of glycosaminoglycans (the latest name for polysaccharides that contain amino sugars); Metabolism of glycoproteins, glycopeptides, and glycolipids; Effect of steroid hormones on glycosaminoglycans of target connective tissues; Hexosaminidases; Neuraminidases; Enzymes degrading glycosaminoglycans; Sulfatases of glycosaminoglycans; Activation and inhibition of enzymes by polyanions containing amino sugars; Chemical and physical changes of glycosaminoglycans and glycoproteins caused by oxidation–reduction systems and radiation: Interaction of polyanions with blood components; Immunochemistry; Interaction between glycoproteins and viruses; and Interaction of amino sugars and amino sugar-containing macromoleculas with viruses, cells, and tissues.

The structure of the book could be improved, and, indeed, it does not permit the presentation of overall, unifying perspectives. Thus, chapter 13 of Volume I is to

**Editorial note.* At this point in the review we have taken the liberty of deleting a paragraph dealing mainly with the book-publishing industry as, in our opinion, it would not have been helpful to the reader in appraising the book. D. HORTON AND R. S. TIPSON.

deal with blood-group substances, yet a proposed genetic pathway for their biosynthesis is given, with inadequate explanation, in the chapter on the metabolism of glycoproteins and glycolipids. The chapter on immunochemistry deals so thoroughly with blood-group substances that one wonders what will be left for treatment in Volume I; a comprehensive discussion of the entire subject in one place would have been desirable and much more informative.

The book will prove extremely useful for locating work on compounds containing amino sugars, on their biological activities, and on the enzymes that catalyze attack on them, together with relatively limited comments and interpretations.

ELVIN A. KABAT (*New York*)

Carbohyd. Res., 6 (1968) 125–126

Stoffwechsel der Galaktose und ihrer Derivate (Metabolism of Galactose and its Derivatives); by W. FISCHER and H. WEINLAND, Physiologisch-Chemisches Institut der Universität Erlangen-Nürnberg, Germany. Biochemie und Klinik: Monographs in a Random Sequence, edited by G. Weitzel, University of Tübingen, and N. Zöllner, University of München. Georg Thieme Verlag, Stuttgart, 1965, XII + 270 pp., 51 Figures, 82 Tables, 15.5 × 23 cm, paperback. *DM* 59.00; Intercontinental Medical Book Corporation, New York, $ 14.75.

This excellent monograph, which is in three parts, describes the biochemistry of (I) D-galactose, (II) D-galactose derivatives and relatives, and (III) L-galactose and its derivatives and relatives. Part I first discusses (in 32 pages) the occurrence of D-galactose as: the free sugar; esters; ethers; oligosaccharides; plant glycosides; animal and plant glycolipides; animal, plant, and bacterial polysaccharides; and animal glycoproteins. There follows a description of the enzymology of D-galactose (37 pages) which covers its synthesis and degradation, the formation of D-galactosyl derivatives, and the enzymic fission of D-galactosides. Chapters on (*a*) the normal metabolism of D-galactose in mammals (31 pages), invertebrates (5 pages), plants (5 pages), and micro-organisms (11 pages), and (*b*) its pathological metabolism (11 pages) conclude Part I.

In Part II (48 pages) is discussed the biochemistry of 2-amino-2-deoxy-D-galactose; D-galacturonic acid; L-arabinose; D-*glycero*-D-*galacto*-heptose; and related alditols, ketoses, 2-epimers, 6-deoxyhexoses, and dideoxy compounds. Part III (18 pages) presents the occurrence, the chirality in metabolism, and the utility as a source of energy, of L-galactose and its relatives, together with an account of the biosynthesis of L-galactose, L-fucose, D-arabinose, 3,6-anhydro-L-galactose, and 3,6-dideoxy-L-*xylo*-hexose; the breakdown of the first three sugars is also covered. This Part ends with a description (1 page) of relevant hydrolases. An appendix (7 pages) discusses the nomenclature of the enzymes involved, provides the physical constants of the galactoses and their derivatives, and gives an addendum covering recent work. The book concludes with a list of 3,123 references and a Subject Index. An Author Index is not provided.

This book, which is well printed on good paper, is an extremely valuable compendium that should be purchased by all biochemists interested in the field; considering the wealth of information it contains, it is a bargain. Readers should, however, be advised that the names employed for some of the sugars and their derivatives are not in conformity with the Rules of Carbohydrate Nomenclature [*J. Org. Chem.*, 28 (1963) 281].

R. STUART TIPSON (*Washington, D. C.*)

Preliminary announcement

A Gordon Conference on the Chemistry of Carbohydrates will be held during the period June 10–14, 1968, at Tilton School, Tilton, New Hampshire, U.S.A. Details of the Conference and of the theme titles will be published in due course.

Corrigenda

Carbohyd. Res., Vol. 5, No. 3 (November 1967), page 350 ;

- line 3 under EXPERIMENTAL, the word "to" should be deleted ;
- line 11 in same paragraph, for 159-260° read 259-260°

Carbohyd. Res., Vol. 5, No. 4 (December 1967)

page 392, Table II.

The values for Asialo-PS original, and its first degradation product in the fifth column (2.7, 0.4 and 0, 0 respectively) should be moved to the sixth column, so that they are listed under L-Fucose instead of under Sialic acid.

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

HYDROLYSIS OF BENZYL 2-ACETAMIDO-4-*O*-(2-ACETAMIDO-2-DEOXY-β-D-GLUCOPYRANOSYL)-2-DEOXY-β-D-GLUCOPYRANOSIDE BY EGG-WHITE LYSOZYME*

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Received August 11th, 1967; in revised form, October 5th, 1967)

ABSTRACT

The synthesis of methyl and benzyl 2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside, and the action of egg-white lysozyme on the latter compound, as well as on tri-*N*-acetyl-chitotriose, tetra-*N*-acetylchitotetraose, and penta-*N*-acetylchitopentaose, are described.

INTRODUCTION

Chitin, and in particular the oligosaccharides of low molecular weight derived from chitin, serve as substrates for hen's egg-white lysozyme[2-12]. The enzyme catalyzes hydrolysis and also the transglycosylation of these compounds. The *p*-nitrophenyl glycosides of both di-*N*-acetylchitobiose and tri-*N*-acetylchitotriose, namely, *p*-nitrophenyl 2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside and *p*-nitrophenyl *O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside, have also been shown[13,14] to be substrates for lysozyme. The different modes of action of the enzyme toward these two *p*-nitrophenyl glycosides, as well as the low reactivity of these substrates compared with that of the chitin oligosaccharides, made desirable a study of the degradation of benzyl 2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside by lysozyme.

RESULTS AND DISCUSSION

The acetylated glycosyl chloride obtained by treatment of 2-acetamido-4-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-1,3,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranose (octa-*O*-acetylchitobiose, 6) with hydrogen chloride in glacial

*Amino sugars LIV. This is publication No. 442 of the Robert W. Lovett Memorial Group for the Study of Crippling Diseases, Harvard Medical School at the Massachusetts General Hospital, Boston, Massachusetts. This work was supported by a research grant from the National Institute of Allergy and Immunology (Grant AI-06692), National Institutes of Health, United States Public Health Service. A preliminary communication has been presented[1].

**On leave of absence from the Weizmann Institute of Science, Rehovoth, Israel.

acetic acid[14] was treated with benzyl alcohol or with methyl alcohol, in the presence of silver carbonate, to give the benzyl (**7**) and methyl (**8**) β-D-glycosides, respectively. *O*-Deacetylation of **7** and **8** with barium methoxide gave the *N*-acetyl derivatives **9** and **10**, respectively.

Evidence for the β-D anomeric configuration at the glycoside terminus was based on the mode of synthesis and on the negative optical rotation of compounds **7**–**10**.

The benzyl glycoside **9** was treated with lysozyme, and the course of the reaction was followed by a test for reducing sugars[15] (see Fig. 1) and also by chromatography (see Table I). Examination of the incubation mixture by paper chromatography

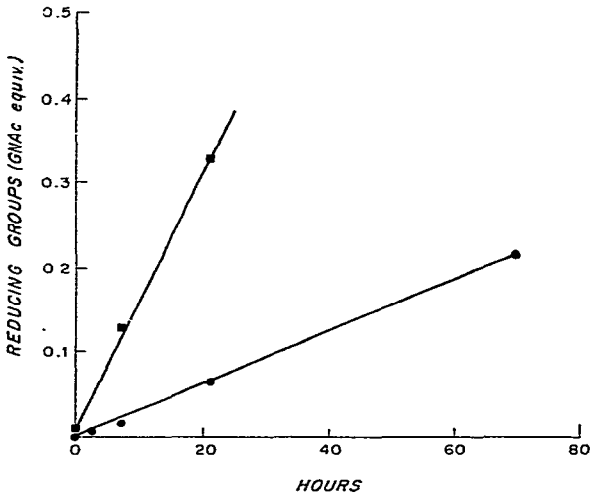


Fig. 1. Increase of reducing groups, expressed as equivalents of 2-acetamido-2-deoxyglucose observed in the lysozyme-catalyzed hydrolysis of benzyl 2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside (**9**). The concentration of enzyme was 0.1 mg (•) and 0.5 mg (□) per ml.

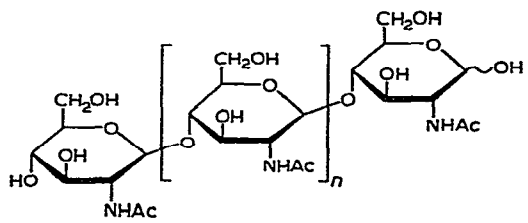
TABLE I

CHROMATOGRAPHIC PROPERTIES OF CHITIN OLIGOSACCHARIDES AND VARIOUS DERIVATIVES

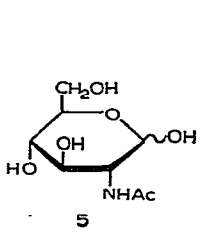
Compounds	*Paper chromatography on Whatman Paper No. 1 in 2-pentanol–pyridine–water (1:1:1)*, R_{GNAc}	*Thin-layer chromatography on silical gel G in 2:1 acetone–methanol* R_{SR}
2-Acetamido-2-deoxy-D-glucose (**5**)	1.0	0.78
Di-*N*-acetylchitobiose (**1**)	0.80	0.46
Tri-*N*-acetylchitotriose (**2**)	0.61	
Tetra-*N*-acetylchitotetraose (**3**)	0.42	
Penta-*N*-acetylchitopentaose (**4**)	0.19	
Benzyl 2-acetamido-2-deoxy-β-D-glucopyranoside		0.93
Benzyl β-glycoside of di-*N*-acetylchitobiose (**9**)		0.66
Benzyl β-glycoside of tri-*N*-acetylchitotriose (**11**)		0.39

showed the formation of monosaccharide **5** and disaccharide **1**, and thin-layer chromatography (t.l.c.) revealed the presence of three additional components, namely the starting material **9**, benzyl 2-acetamido-2-deoxy-β-D-glucopyranoside, and a slow-moving material having R_{SR} 0.39 (SR, Sudan Red). The last material was isolated by preparative t.l.c.

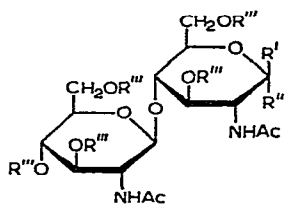
After hydrogenolysis, it gave a product migrating on paper at a rate similar to that of the trisaccharide **2**, and the structure of benzyl *O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (**11**) was tentatively ascribed to it.



1 $n = 0$
2 $n = 1$
3 $n = 2$
4 $n = 3$



5



6 R' = H ; R'' = OAc ; R''' = Ac
7 R' = OCH_2Ph ; R'' = H ; R''' = Ac
8 R' = OMe ; R'' = H ; R''' = Ac
9 R' = OCH_2Ph ; R'' = R''' = H
10 R' = OMe ; R'' = R''' = H

The mixture resulting from the treatment of compound **9** with lysozyme for 74 h had a reducing equivalent of 0.22. The Morgan–Elson test (in the modification of Reissig *et al.*[18]) showed the monosaccharide **5** and the disaccharide **1** to be present in the degradation product in the ratio of 1.2 to 1; this result is based on a color yield of 3% for **1**, as compared to **5**, when heat was applied for 3 min. Whereas the reducing-sugar test measures the formation of **5** and **1**, the Morgan–Elson test determines **5** only. Thus, it may be concluded that both of the glycosidic bonds of **9** are split by the enzyme, to give compounds **5**, **1**, and benzyl 2-acetamido-2-deoxy-β-D-glucopyranoside. The benzyl glycoside (**11**) of tri-*N*-acetylchitotriose results from transglycosylation, and it is most probably formed through transfer of a 2-acetamido-2-deoxy-D-glucose residue to **9**. The glycoside **9** is always present in great excess in the incubation mixture.

The rate of enzymic hydrolysis of **9** was found to be of the same order of magnitude as that of the trisaccharide **2** and of the tetrasaccharide **3** (see Fig. 2)[7]. It should be noted that, under the conditions used in the present work, no hydrolysis of benzyl 2-acetamido-2-deoxy-β-D-glucopyranoside or of di-*N*-acetylchitobiose (**1**) was observed. The enzymic hydrolysis of the trisaccharide **2**, under the same conditions,

was very slow, and it could be followed only by determination of the reducing value or by the Morgan–Elson test (see Figs. 2 and 3); it could not be observed by chromatography.

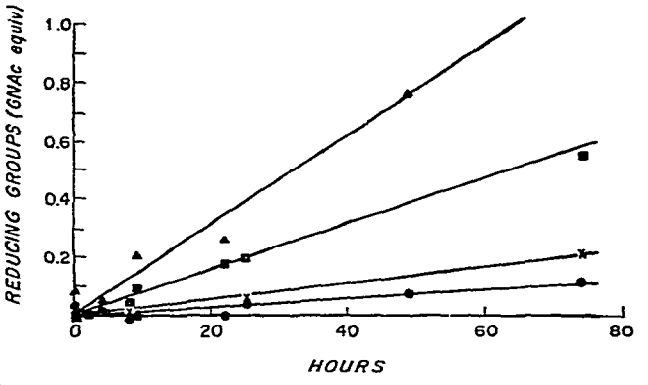


Fig. 2. Increase of reducing groups, expressed as equivalents of 2-acetamido-2-deoxyglucose observed for the lysozyme-catalyzed hydrolysis of tri-*N*-acetylchitotriose (**2**) (•), tetra-*N*-acetylchitotetraose (**3**) (■), penta-*N*-acetylchitopentaose (**4**) (▲), and benzyl 2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside (**9**) (×). The values of the reducing power of the hydrolyzates of compounds **2**, **3**, and **4** were decreased by the reducing value of the compounds (0.880, 0.734, and 0.686 equiv. for **2**, **3**, and **4**, respectively) and by the reducing value of the enzyme solution.

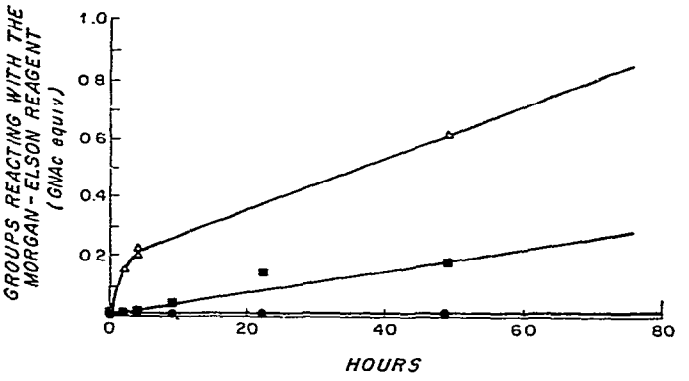


Fig. 3. Increase of groups reacting with the Morgan–Elson test reagent, expressed as equivalents of 2-acetamido-2-deoxyglucose, observed in the lysozyme-catalyzed hydrolysis of tri-*N*-acetylchitotriose (**2**) (•), tetra-*N*-acetylchitotetraose (**3**) (■), and penta-*N*-acetylchitopentaose (**4**) (△).

The trisaccharide **2** and the benzyl glycoside **9** of the disaccharide share the property of having two glycosidic bonds that may be attacked. Whereas, under the present conditions, the trisaccharide **2** seems to form mainly a nonproductive enzyme–substrate complex, the benzyl glycoside **9** forms a productive enzyme–substrate complex. On the basis of the lysozyme model proposed by Phillips[19], it is possible to build two enzyme–substrate complexes in which the units at sites D and E are involved, and

thus to explain the products obtained from the degradation of the benzyl glycoside **9** by lysozyme.

Location of saccharide units in enzyme cleft:	A	B	C	D	E	F
Compound **9**:				X	X	CH_2Ph
Compound **9**:			X	X	CH_2Ph	

The lysozyme degradation of the tetra- and penta-saccharides **3** and **4** was observed by the color reaction (see Figs. 2 and 3), as well as by chromatography (see Table II) of the resulting product. The results of the chromatography confirm

TABLE II

CHROMATOGRAPHIC STUDY OF THE INCUBATION OF TETRA-*N*-ACETYLCHITOTETRAOSE (3) AND PENTA-*N*-ACETYLCHITOPENTAOSE (4) WITH EGG-WHITE LYSOZYME

Substrate	*Time in h*	*Relative amounts of compounds formed*[a]					
		1	2	3	4	5	*Precipitate*[b]
Compound **3**	4.3	0	0	4	0	0	
	9	2	2	4	0	2	2
	26	2	2	4	1	3	2
	50	3	2	3	0	3	2
Compound **4**	0.4	0	2	3	4	0	0
	1.7	2	2	3	4	2	2
	4.3	3	3	3	3	3	4
	9	3	2	2	1	4	4
	26	4	2	3	0	4	4
	50	4	1	2	0	4	4

[a]The paper-chromatographic system is described in the experimental section. The spots were revealed by treatment with alkali[17]. A concentration of 1/50th of the original amount of substrate could be detected, and the relative proportions were estimated visually. The proportions are relative to each other for the same experiment only (same duration of the incubation and same substrate), and are graded from 0 to 4; relative amounts on different lines cannot be compared. [b]The precipitate obtained after incubation of compound **4** for 4 h required 3 min of centrifugation at 3,000 r.p.m. before the sample could be examined by chromatography or color reaction. Intense tailing was observed on the chromatograms of an incubation mixture kept for 1.7 h. Tailing was diminished after 4.3 h, and disappeared after 9 h.

and extend those reported by Powning and Irzykiewicz[10] shortly after the present work had been completed. The high initial rate that was observed for the chitin oligosaccharides[7] by means of a test for reducing sugars does not bear a simple relationship to the results obtained when the products of the hydrolysis are examined by the Morgan–Elson test[18], which measures the formation of 2-acetamido-2-deoxy-D-glucose (**5**). Paper chromatography of the products resulting from a short (25-min) incubation of the pentasaccharide **4** with lysozyme shows only the tetra- and trisaccharides **3** and **2**. Apparently the 2-acetamido-2-deoxy-D-glucose (**5**) and di-*N*-acetylchitobiose (**1**) formed, but undetected, are further transformed by transglyco-

sylation. In the model proposed by Phillips[19] for the action of lysozyme, the glycosidic bond of the substrate is cleaved between units D and E (vertical arrow), where

Location of saccharide units in enzyme cleft:	A B C D↓E F
(A)	X—X—X—X—X→
(B)	X—X—X—X—X→
(C)	X—X—X—X—X→
(D)	X—X—X—X—X→

X = GNAc residue. The reducing end is designated by an arrow.

It is not possible to ascertain which of the glycosidic bonds in **4** is preferentially split, and, consequently, which of the lysozyme–pentasaccharide complexes (A-D) is favored. If cleavage of the first glycosidic bond (from the nonreducing end) and of the second bond (reactions A and B) occur in the early stage of the degradation, one or two monosaccharide units would extend outside the cleft formed by the enzyme. Degradation of the third and fourth glycosidic bonds (reactions C and D) would, however, not require part of the substrate to be located outside the cleft; the latter pattern of cleavage has recently been presented by Rupley and Gates[12].

In a communication published after this work had been completed, Rupley and Gates[12] explained the results of lysozyme action by the formation of enzyme–substrate complexes in a 1:1 or 1:2 molar ratio; such a complex in a 1:2 ratio at the early stage of the hydrolysis of the pentasaccharide **4** would explain the results obtained in the present work. In the hydrolysis of both the tetrasaccharide **3** and the pentasaccharide **4**, surprisingly small proportions of the trisaccharide **2** accumulated, although the hydrolysis of **2**, under the conditions used in this work, could not be observed by chromatography. The complexity of the degradative process is increased by the marked transglycosylation reaction. Oligomers of low molecular weight appeared and disappeared in due course, and a chitin-like polymer was formed (see Table II). In the degradation of the pentasaccharide **4**, the chitin-like, insoluble polymer was formed in a yield of over 17%; the formation of similar polymers has already been reported by Kravchenko and Maksimov[8].

EXPERIMENTAL

General. — Melting points were determined on a hot stage equipped with a microscope, and correspond to corrected melting points. Specific rotations were determined with a Perkin–Elmer polarimeter Model No. 142. Infrared spectra were determined for KBr pellets with a Perkin–Elmer spectrophotometer Model 237. Paper chromatography was performed on Whatman paper No. 1, descending, in 1:1:1 (v/v) 2-pentanol–pyridine–water[16]. The chromatograms were revealed by alkaline treatment[17], by the aniline hydrogen phthalate reagent (examined under u.v. light), and by the silver nitrate reagent. Thin-layer chromatography was performed on silica gel G (E. Merck, Germany), in 2:1 (v/v) acetone–methanol. The spots were revealed by charring with sulfuric acid. R_{SR} refers to the mobility relative to that of Sudan Red, a component of the test mixture supplied by C. Desaga, Heidelberg, Germany.

Colorimetric determinations were made with a Zeiss PMQII spectrophotometer. Evaporations were performed *in vacuo*, with the bath temperature below 45°. Small amounts of volatile solvent were evaporated under a stream of dry nitrogen. Hen egg-white lysozyme (3 times crystallized; *N*-acetylmuramide glycanhydrolase EC 3.21.17) was purchased from Mann Research Laboratories, New York, N. Y. The microanalyses were performed by Dr. M. Manser, Zürich, Switzerland.

N-*Acetylated chitin oligosaccharides.* — These oligosaccharides were obtained essentially according to the method of Rupley[7], and no impurities were detected by paper chromatography with the solvent system used for the study of the enzymic degradation. The oligosaccharides were crystallized from water–methanol, and showed the following physical properties: 2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-α-D-glucose (**1**), m.p. 260–263° (dec.), $[\alpha]_D^{27}$ +32.3 (5 min) → 16.0° (at equilibrium; *c* 0.74, water); *O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-2-deoxy-D-glucose (**2**), dec.p. 282°, $[\alpha]_D^{27}$ +5.4 (10 min) → +3.7° (at equilibrium: *c* 0.43, water); *O*-2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-2-deoxy-D-glucose (**3**), dec.p. 300°, $[\alpha]_D^{27}$ −3.8° (after 15 min and at equilibrium; *c* 1.05, water); *O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-2-deoxy-D-glucose (**4**), precipitated as a gel, dec.p. 210°, $[\alpha]_D^{27}$ − 5.7 (9 min) → −7.2° (at equilibrium; *c* 0.5, water).

2-Acetamido-4-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-1,3,6-tri-*O*-acetyl-2-deoxy-α-D-glucose (**6**), was prepared in 83% yield from **2** by treatment with acetic anhydride in pyridine, and was crystallized from methanol as prisms, dec.p. 308°, $[\alpha]_D^{26}$ +54.1° (*c* 0.72, acetic acid).

*Benzyl 2-acetamido-4-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranoside* (**7**). — A solution of **6** (2 g) in acetic acid (30 ml) presaturated at 0° with hydrochloric acid was kept for 48 h at room temperature. The reaction mixture was extracted with chloroform (330 ml), and the extract was successively washed with a saturated solution of sodium hydrogen carbonate and water, dried (sodium sulfate), and concentrated *in vacuo* to 60 ml. The solution was added to a mixture, preshaken for 30 min, of benzyl alcohol (66 ml), silver carbonate[20] (1.65 g), and crushed Drierite (1.65 g). Shaking was continued in the dark for 7 days at room temperature. The mixture was then filtered through a carbon–Celite pad, the filtrate was partially evaporated *in vacuo*, and a crystalline mass was precipitated by addition of hexane. Pyridine (20 ml) and acetic anhydride (5 ml) were added to the precipitate, and the mixture was kept in a closed vessel overnight at room temperature. Ice was added, and the reaction mixture was kept for 2 h at room temperature, extracted with chloroform (100 ml), and the extract washed successively with water, saturated sodium hydrogen carbonate, and water, dried (sodium sulfate), and evaporated to a light-yellow residue. Two recrystallizations from methanol gave

needles (0.41 g, 19%), m.p. 281–282°, $[\alpha]_D^{26}$ −61° (*c* 0.34, chloroform); t.l.c. in 1:1 (v/v) 1,2-dichloroethane–acetone: R_{SR} 0.62; i.r. data: the 935 cm^{-1} (m) peak present in **6** had disappeared, and two new peaks, at 680 and 730 cm^{-1}, were present.

Anal. Calc. for $C_{33}H_{44}N_2O_{16}$: C, 54.69; H, 6.12; N, 3.87. Found: C, 54.21; H, 5.84; N, 4.00.

Benzyl 2-acetamido-4-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside (**9**). — A solution of **7** (319 mg) in 0.1M methanolic barium methoxide (15 ml) was kept overnight at 4°. Carbon dioxide was then bubbled through the solution, the precipitate was filtered off, and the filtrate was evaporated to give a crystalline residue; t.l.c. in 3:2 (v/v) methanol–acetone, R_{SR} 0.46 (as compared to R_{SR} 0.59 for **5** and 0.30 for **1**). Recrystallization from methanol gave needles (100 mg, 95%), m.p. 270–271°, $[\alpha]_D^{26}$ −37° (*c* 0.16, water). The compound was nonreducing.

Anal. Calc. for $C_{23}H_{34}N_2O_{11}$: N, 5.45. Found: N, 5.32.

Methyl 2-acetamido-4-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-3,6-di-O-acetyl-2-deoxy-β-D-glucopyranoside (**8**). — This compound was prepared from **6** (0.2 g) as described for **7**, but with the use of methanol instead of benzyl alcohol. Crystallization from methanol gave needles (33 mg, 17%), m.p. 285–287°, $[\alpha]_D^{26}$ −43° (*c* 0.17, chloroform); t.l.c. in 2:1 (v/v) ethyl acetate–acetone, R_{SR} 0.20 (as compared to R_{SR} of 0.37 for **6**).

Anal. Calc. for $C_{27}H_{40}N_2O_{16}$: C, 49.98; H, 6.22. Found: C, 49.85; H, 6.14.

Methyl 2-acetamido-4-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside (**10**). — A solution of **8** (19 mg) in 0.1M barium methoxide in methanol was kept overnight at room temperature. The solution was neutralized with carbon dioxide, and evaporated to a residue which was dissolved in water (5 ml); the solution was filtered through a Celite–Dowex-50 (H^+) pad and evaporated. The residue was homogenous on t.l.c. in 2:1 (v/v) acetone–methanol, R_{SR} 0.49 (as compared to R_{SR} of 0.78 for **5**, and 0.46 for **1**). Crystallization from methanol–ethyl acetate gave needles (7 mg, 56%), m.p. 232–236° (dec.), $[\alpha]_D^{26}$ −31° (*c* 0.28, water). The compound was nonreducing.

Anal. Calc. for $C_{17}H_{30}N_2O_{11} \cdot 0.5\ H_2O$: C, 45.63; H, 6.98. Found: C, 45.53; H, 7.69.

Incubation with lysozyme. — Unless otherwise mentioned, the enzymic degradation was performed with a substrate concentration of 3mM and 0.1 mg of enzyme per ml, in 0.04M sodium citrate buffer (pH 5.25) at 37°. A drop of toluene was added to every sample. For the reducing-sugar determination[15] of the degradation of **9**, samples of 0.1 ml were taken, whereas, for the other substrates, 0.1-ml samples of the solutions, diluted fivefold, were used. The color obtained was determined at 690 nm, and the results are reported as equivalents of 2-amino-2-deoxy-D-glucose in Figs. 1, 2, and 3. The reducing values obtained for the hydrolyzates of compounds **2**, **3**, and **4** (see Table II) were decreased by the reducing value of the parent compounds (0.880, 0.734, and 0.686 equiv., respectively), and by that of the enzyme solution. For the Morgan–Elson test[18], 0.1-ml samples were used, the color was determined at 585 nm, and the results are reported in Fig. 2. The samples used for the colorimetric tests were immed-

iately frozen by dipping the test tubes in an acetone–solid carbon dioxide bath, and were kept at −20° until analyzed. For chromatographic examination, samples of 50 μl were applied immediately to paper or to thin-layer silica gel.

A period of less than 15 min elapsed before the chromatograms were placed in the development tank. Two chromatographic systems were used (see Table I).

The amount of precipitate formed after a 49-h incubation of **4** was determined by filtering an aliquot of 0.4 ml of the incubation mixture through a Millipore filter (GS 0.22 μm). The filter was washed three times with water (2 ml), and the nitrogen content of the filter was determined by the Kjeldahl procedure. Since the washings of the unused filter showed a high blank value, the yield of precipitate obtained (19% based on **4**) is only approximate.

Isolation of compound **11**. — The isolation of compound **11**, having R_{SR} 0.39, from the incubation mixture of **9** was performed on a sample (0.2 ml) applied to a t.l.c. plate measuring 20 × 20 cm. The plate was developed with 2:1 (v/v) acetone–methanol until the Sudan Red marker had travelled 14.6 cm. The zone at 4.2–6.2 cm was eluted with water (20 ml), the suspension was filtered, and the filtrate was divided into halves. One half was hydrogenolyzed with hydrogen at atmospheric pressure over 10% palladium-on-charcoal for 24 h, and the other was kept for reference. Both solutions were evaporated to dryness under nitrogen, and water (0.1 ml) was added to each. Examination of the hydrogenolyzed sample by paper chromatography showed the presence of a compound migrating at the same speed as that of **2**, whereas, in the untreated sample, compound **1** was detected by paper chromatography and compound **11** by t.l.c.

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MASS-SPECTROMETRIC STUDY OF CARBOHYDRATES

A NEW FRAGMENTATION REACTION INDUCED BY ELECTRON IMPACT — "*h*-RUPTURE"

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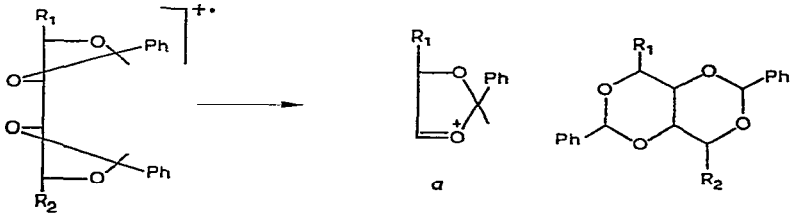
ABSTRACT

Cyclic acetals of alditols that contain 1,3,6,8-tetraoxabicyclo[4.4.0]decane ring systems have been found to undergo a novel type of fragmentation (*h*-rupture) on mass spectrometry. The implications of this finding are discussed.

DISCUSSION

During the course of our study of the mass spectra of the cyclic acetals of alditols**, we have observed an unexpected phenomenon. One might anticipate that the mass spectra of 4,4'-bis-1,3-dioxolane derivatives **1** would be distinguished from those of the isomeric 1,3,6,8-tetraoxabicyclo[4.4.0]decanes **2**, since, in compounds of the type **1**, rupture of the bond connecting the 1,3-dioxolane rings must give rise to the stable ion *a* (when $R_1 = R_2$, the mass of this ion must be equal to one half of the molecular weight). For compounds of type **2**, formation of a similar ion seems improbable.



1 **2**

However, for all of the 1,3,6,8-tetraoxabicyclo[4.4.0]decanes studied [1,3:2,4-di-*O*-benzylidene-L-threitol (**3a**), 1,3:2,4-di-*O*-benzylidene-erythritol (**4a**), 1,3:2,4-di-*O*-benzylideneribitol (**4b**), 1,3:2,4-di-*O*-benzylidenexylitol (**3b**), 2,4:3,5-di-*O*-benzylidene-D-mannitol (**3c**), and 2,4:3,5-di-*O*-benzylidene-D-glucitol (**3d**)], the mass spectra contain very intense peaks having mass numbers equal to one half of the

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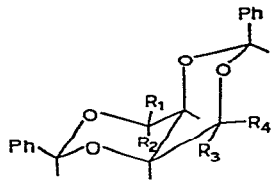
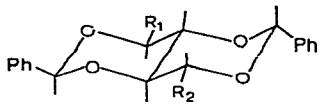
**For experimental details, see the next paper.

molecular weight for symmetrically substituted compounds (**3a**, **3c**, **3d**, and **4a**). For compounds **3b** and **4b**, there are pairs of peaks, for which the sum of the mass numbers is equal to the molecular weight, and the difference is equal to the difference in mass of the groups R_1 and R_2. We designate these ions as "half-ions" ("*h*-ions") and the reaction leading to their formation as "*h*-rupture". The term "*h*-ion" is used conventionally, even when the mass number is not equal to one half of the molecular weight.

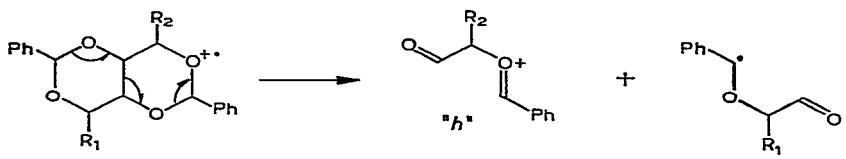




3a $R_1=R_2=R_3=R_4=H_4$
3b $R_1=CH_2OH$; $R_2=R_3=R_4=H$
3c $R_1=R_3=H$; $R_2=R_4=CH_2OH$
3d $R_2=R_3=H$; $R_1=R_4=CH_2OH$

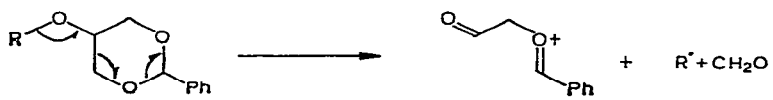
4a $R_1=R_2=H$
4b $R_1=H$; $R_2=CH_2OH$

To explain the formation of "*h*-ions", we assume that the following electronic shifts result in the rupture of three bonds to give, simultaneously, two very stable entities, *viz.* the "*h*-ion" and the "*h*-radical".



$R_2=H$, *m/e* 149
$R_2=CH_2OH$, *m/e* 179

The following arguments favour such a mechanism. In the mass spectra of the *cis*- (**5a**) and *trans*-5-hydroxy-2-phenyl-1,3-dioxanes (**6**), there are peaks at *m/e* 149. The formation of the corresponding ion is in agreement with the mechanism described above and can be represented as follows:

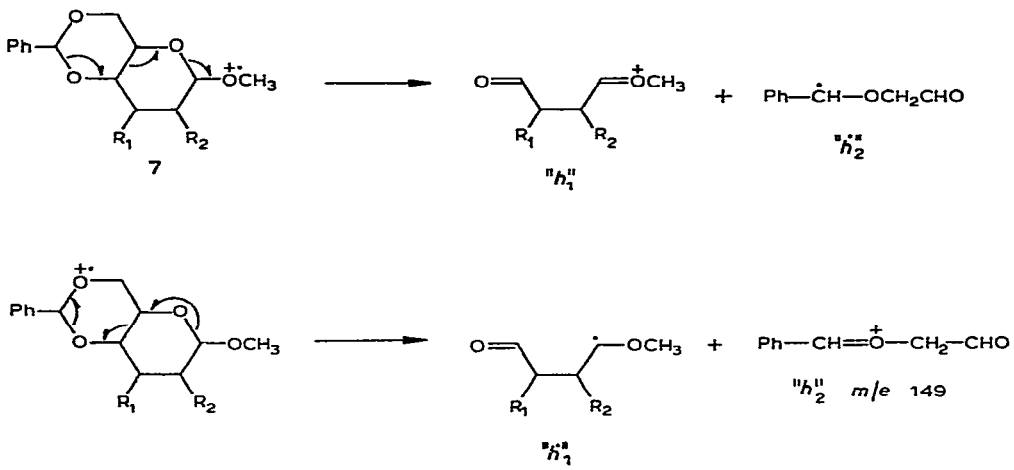


5a R=H
5b R=D
5c $R=PhCH_2$

"*h*", *m/e* 149

Replacement of the hydrogen atom in the hydroxyl group by deuterium (**5b**) does not change the mass number of the "*h*-ion", because the peak at *m/e* 149 does not shift. Hence, the hydrogen atom of the hydroxyl group is actually split off when the ion having *m/e* 149 arises. The abundance of the "*h*-ion" for compounds **5a** and **6**

is relatively small (*ca.* 15% of the M^+-ion). This fact can be explained by the instability of the H·-radical (*cf.* ref. 1), which hinders the formation of the "*h*-ion". In full agreement with such an assumption is the fact that for 5-benzyloxy-2-phenyl-1,3-dioxane (**5c**) the abundance of the "*h*-ion" increases very strongly, because, in this case, "*h*-rupture" leads to the stable benzyl radical (the abundance of the "*h*-ion" is equal to that of the M^+-ion for this compound). There is a metastable peak in the mass spectrum of compound **5c**, which corresponds to the transformation of the M^+-ion to the "*h*-ion", and which indicates the one-step mechanism of the "*h*-rupture" (calc. for 270→149, *m** 82.2; found, 82.2). For methyl 4,6-*O*-benzylidenehexopyranosides (**7**) belonging to the 1,3,6-trioxabicyclo[4.4.0]decane series, "*h*-rupture" is one of the main directions of fragmentation:



The nature of the substituents in the pyranoside ring has a substantial influence on the charge distribution during "*h*-rupture" and, consequently, on the intensity of the peaks of the "*h*-ions". The "h_2-radical" is more stable than the "h_1-radical", and therefore in the mass spectra of compounds **7a-e**, the "h_1-ion" arises preferentially and is more abundant than the "h_2-ion". Replacement of the glycosidic methoxyl group by a phenyl group (*e.g.*, compound **8**, Table I) changes the stability ratio of the "*h*·-radicals" and "*h*-ions": the "h_1-radical", in this case, has approximately the same stability as the "h_2-radical", but the "h_2-oxonium ion" is more stable than the "h_1-carbonium ion". This results in a change of relative abundance; the "h_2-ion" becomes more abundant than the "h_1-ion".

For compounds **7a-c** which have no hydroxyl groups, "h_1-ions" are more abundant than for compounds **7d-f**, which have unprotected hydroxyl groups (see Table I).

The fragmentation reaction induced by electron impact, and described here as "*h*-rupture", is evidently a general one for all compounds of the R-O-C-C-O-C-O-X type:

$$\text{R-O-C-C-O-C-O-X}^{+\cdot} \rightarrow \text{R}^{\cdot} + \text{O=C+C=O+C=O}^{+}\text{-X}$$

where R is a group that splits off as a radical, and X is an electron-donating group.

The formation of some ions for which no satisfactory explanation could previously be given can now be interpreted in terms of "*h*-rupture". For instance, ions having *m/e* 101, 129, and 159, and arising from 1,2:3,5-di-*O*-isopropylidene-α-D-xylofuranose and 2,3:4,6-di-*O*-isopropylidene-L-sorbofuranose[2], are consistent with "*h*-rupture":

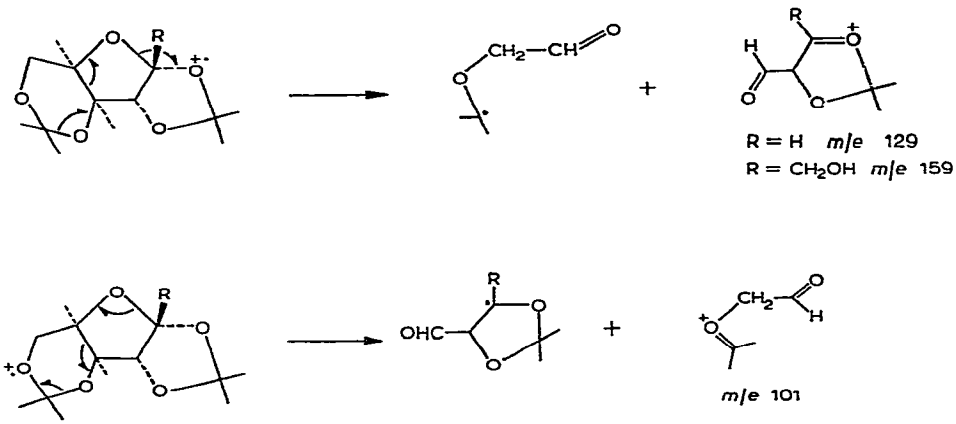


TABLE I

MASS NUMBERS AND RELATIVE ABUNDANCE OF "h_1"- AND "h_2-IONS" FOR METHYL 4,6-*O*-BENZYLIDENE-HEXOPYRANOSIDES

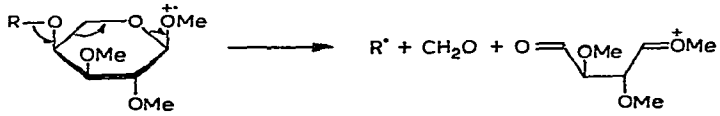
Methyl 4,6-O-benzylidene derivative of		R_1	R_2	h		h_2	
				m/e	*intensity*	m/e	*intensity*
2,3-Di-*O*-methyl-α-D-glucopyranoside	**7a**	OCH_3	OCH_3	161	8.0	149	1.8
2,3-Di-*O*-methyl-β-D-galactopyranoside	**7b**	OCH_3	OCH_3	161	12.0	149	1.2
2,3-Anhydro-α-D-allopyranoside	**7c**		-O-	115	20.0	149	1.6
2-*O*-Methyl-α-D-altropyranoside	**7d**	OCH_3	OH	147	1.4	149	0.6
α-D-Glucopyranoside	**7e**	OH	OH	133	4.3	149	2.2
β-D-Galactopyranoside	**7f**	OH	OH	133	2.4	149	1.8
β-D-Glucopyranosylbenzene	**8**			179	2.8	149	4.8

It has been emphasised that "*h*-rupture" can lead to the formation (from structural isomers) of ions having different structures, but the same mass. It has been mentioned above that compound **1**, and **3c** and **3d**, unexpectedly give the ions *a* and "*h*", respectively having mass numbers corresponding to the peaks of high intensity. If the structures of the di-*O*-benzylidene-D-mannitol **3c** and the di-*O*-benzylidene-D-glucitol **3d** had not been fully proved[3,4], it could have been wrongly concluded from the mass spectra that compounds **3c** and **3d** have structures analogous to that of di-*O*-benzylidenegalactitol and so are derivatives of the 4,4'-bis-1,3-dioxolane. It has been suggested[5] that the mass spectra of furanoses are characterised by the formation of

fragments arising by rupture of the C-4–C-5 bond; for instance, the fragment having *m/e* 161 is characteristic of methyl furanoside methyl ethers:

m/e 161

However, if the substituent R can give a sufficiently stable radical R· a fragment of the same mass is produced from the isomeric pyranose derivative as a result of "*h*-rupture".



m/e 161

Therefore, in mass spectrometric studies of cyclic acetals, it is necessary to take into consideration the possibility of "*h*-rupture", in order to avoid a wrong interpretation of the experimental data, especially when the ring size in derivatives of carbohydrates is detected by the mass spectrometric method.

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MASS-SPECTROMETRIC STUDY OF CARBOHYDRATES

BENZYLIDENE ACETALS OF ALDITOLS

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ABSTRACT

The mass spectra of benzylidene acetals of various alditols have been measured. An interpretation of the fragmentation processes is presented. The molecular weight can readily be obtained, since a molecular ion is formed. It is possible to relate the characteristics of the mass spectra to the structure and configuration of the compounds studied.

INTRODUCTION

The structures of various acetals of alditols have not yet been proved unequivocally, in spite of a number of investigations (for a review, see refs. 1 and 2). Of several methods available for elucidation of structure in this class of compound, some are of limited value. For instance, partial hydrolysis with acid is not sufficiently reliable (because there is the possibility of rearrangement), partial hydrogenolysis[3] is not a general method, and n.m.r. spectroscopy has been applied mainly to benzylidene[4] and isopropylidene[5] derivatives. Thus, it seemed desirable to apply mass spectrometry for a study of the structures of cyclic acetals.

As a rule, the mass spectra of stereoisomeric methyl ethers or acetates of carbohydrates are indistinguishable[6]. Acetals of stereoisomeric alditols are frequently structural isomers, and their mass spectra must be quite different if their stereochemistry is to be elucidated (*cf.* ref. 7). For stereoisomeric alkylidene derivatives, it may be expected that there will be more-definite differences in the mass spectra of fused-ring systems (such as those that occur in the majority of benzylidene acetals of alditols) than are observed for acyclic or monocyclic compounds[8].

We now report on the fragmentation patterns of some cyclic benzylidene acetals of alditols.

EXPERIMENTAL

The mass spectra were determined with an MX-1303 mass spectrometer equipped

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with a glass inlet system for direct introduction of the sample into the ion source operated at 140–160°; an ionizing potential of 30–38 electron volts and an ionizing current of 0.7–1.0 mamp. were used. Relative intensities are expressed as a fraction of the total ion current.

RESULTS AND DISCUSSION

The mass spectra of the following compounds were studied: 2,3:4,5-di-*O*-benzylidenegalactitol (**1**), 2,3:4,5-di-*O*-benzylidene-1,6-dideoxygalactitol (**2**), 1,3:2,4-di-*O*-benzylideneerythritol (**3**), 1,3:2,4-di-*O*-benzylidene-L-threitol (**4**), 1,3:2,4-di-*O*-benzylideneribitol (**5**), 1,3:2,4-di-*O*-benzylidenexylitol (**6**), 2,4:3,5-di-*O*-benzylidene-D-glucitol (**7**), 2,4:3,5-di-*O*-benzylidene-D-mannitol (**8**). These compounds belong to two types of cyclic acetal, involving 4,4′-bis-1,3-dioxolane (compounds **1** and **2**) and 1,3,6,8-tetraoxabicyclo[4.4.0]decane ring systems (compounds **3**–**8**). A simpler fragmentation pattern is characteristic of the first type.

4,4′-Bis-1,3-dioxolanes. — The main directions of fragmentation of the molecular ions of these compounds are shown in Fig. 1.

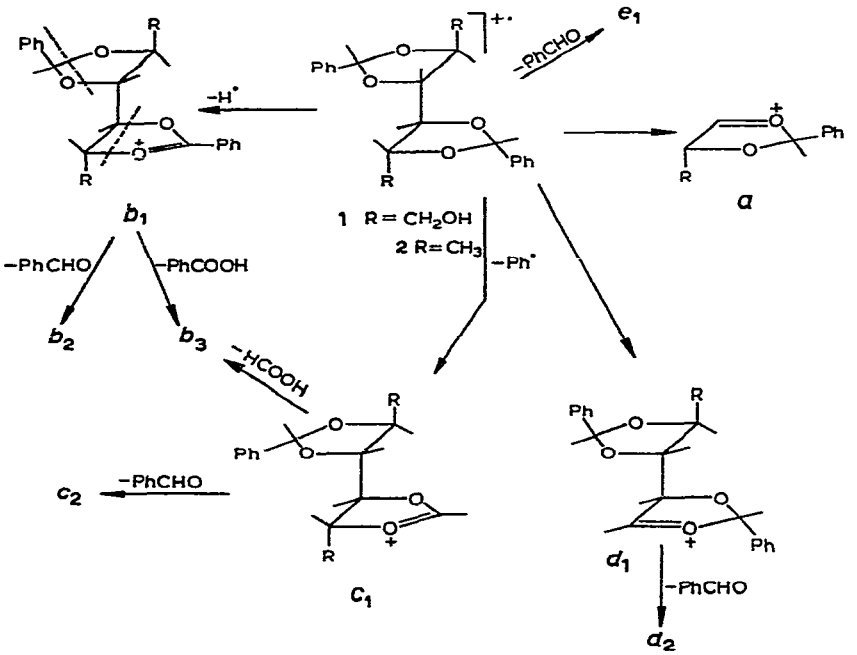


Fig. 1. Fragmentation pattern of the 4,4′-bis-1,3-dioxolanes.

The main fragmentation reaction consists in cleavage of the bond connecting the dioxolane rings. The *a*-ions arising in this way [*m/e* 179 for (**1**), *m/e* 163 for (**2**)] are the most abundant in the mass spectra. All other directions of fragmentation lead to ions and radicals that give peaks of low intensity. As with other 1,3-dioxolanes[9], the molecular ions of compounds **1** and **2** can lose a hydrogen atom (probably that linked

to the acetal carbon atom). The process leads to the b_1-ion. Elimination of a molecule of benzaldehyde or benzoic acid from the b_1-ion leads to fragments b_2 and b_3. Loss of a phenyl radical is very disadvantageous thermodynamically, and the peaks of very low intensity correspond to c_1-ions arising in this way. Expulsion of benzaldehyde or formic acid from fragment c_1 gives the c_2- and b_3-ions. The molecular ions of compounds **1** and **2** can lose the substituent R as a radical, leading to the ion d_1, which loses benzaldehyde and forms the d_2-ion. The direct expulsion of a benzaldehyde molecule from molecular ions is also possible, and leads to the e_1-fragments. The mass numbers of the ions arising from the fragmentation of the molecular ions **1** and **2** are given in Table I, and the corresponding mass spectra are shown in Fig. 2.

TABLE I

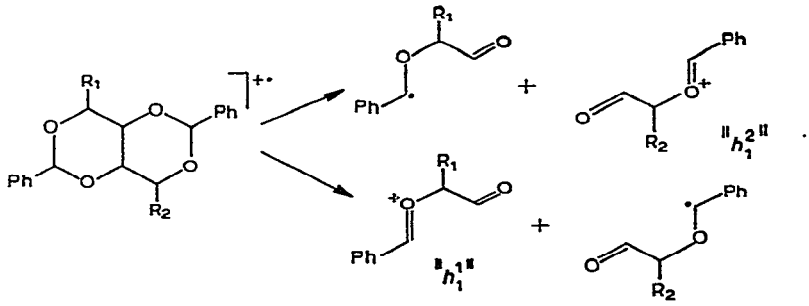
MASS NUMBERS OF THE MAIN IONS ARISING FROM COMPOUNDS **1** AND **2**.

Compound	m/e									
	M^+	a	b_1	b_2	b_3	c_1	c_2	d_1	d_2	e
1	358	179	357	251	235	281	175	327	221	252
2	326	163	325	219	203	249	143	(311)	205	220

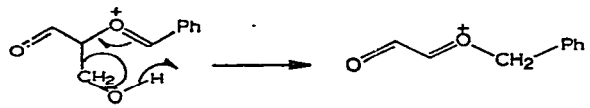
In the mass spectrum of compound **2**, the peaks of the ions (a, d_1, and d_2) containing only one substituent R are shifted 16 m.u. to lower *m/e* values, and the peaks of ions (b_1, b_2, b_3, c_1, and c_2) retaining both the terminal substituents are shifted 32 m.u. to lower *m/e* values. The scheme of fragmentation discussed is similar to that proposed for isopropylidene derivatives of monosaccharides[7] and alditols[10].

In addition to the peaks of the *a–e* series, in the mass spectra of the compounds **1** and **2**, there are several very intense peaks (*m/e* 105, 91, 77) corresponding to the aromatic ions $C_6H_5CO^+$, $C_7H_7^+$, and $C_6H_5^+$. Formation of ions of the *b*, *c*, *d*, and *e* series and of aromatic fragments is characteristic of all of the benzylidene derivatives studied. If a compound contains a hydroxyl group, an intense peak at *m/e* 107 appears in its mass spectrum, and is assigned to the protonated molecule of benzaldehyde, $C_6H_5CH=\overset{+}{O}H$.

1,3,6,8-Tetraoxabicyclo[4.4.0]decanes. — There are several intense peaks in the mass spectra of the compounds of this series, in addition to those belonging to the *b*-, *c*-, *d*-, and *e*-ions. Firstly there are peaks of *h*-ions, formed[11] by "*h*-rupture".

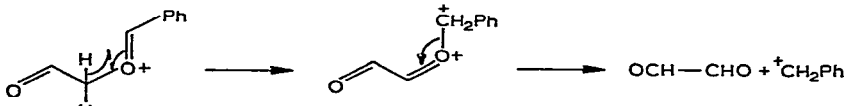


For the compounds (**3**, **4**, **7** and **8**) where $R_1 = R_2$, the mass numbers of the "h_1^1" and "h_1^2-ions" are the same, if $R_1 \neq R_2$ (compounds **5** and **6**), these ions have different *m/e* values. The "h_1-ions" can undergo two types of transformation. When R is CH_2OH, the "h_1-ion" can lose formaldehyde giving rise to the "h_2-ion":



"h_1", *m/e* 179 "h_2", *m/e* 149

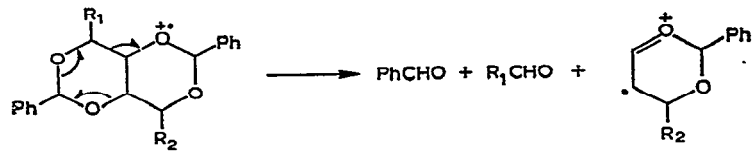
When R = H, the isomerisation "h_1" → "h_2" can proceed as follows:



"h_1" *m/e* 91

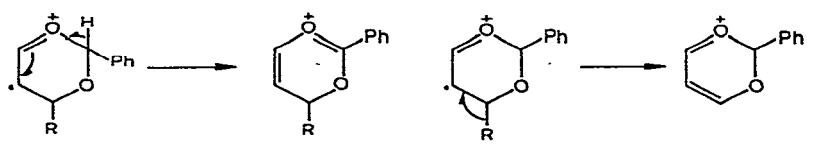
Fragmentation of the "h_2-ion" into a glyoxal molecule and a benzyl ion (*m/e* 91) is supported by a metastable-ion peak at *m/e* 55.6 in the mass spectra of compounds **3** and **4** (calc. for 149 → 91, m^* 55.5).

The formation of the ion *f*, the mass number of which corresponds to one 1,3-dioxane ring, is very characteristic of all of the 1,3,6,8-tetraoxabicyclo[4.4.0]decanes. The following mechanism seems most probable:



f_1

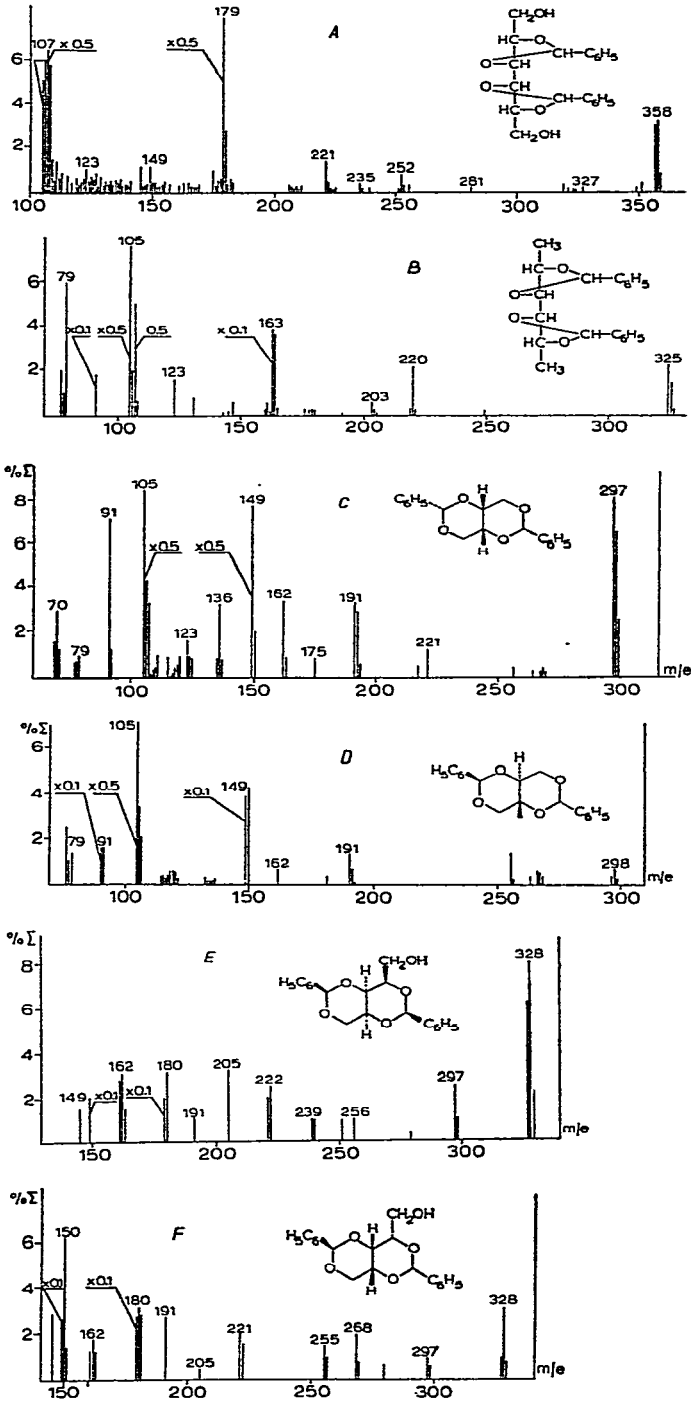
This mechanism is corroborated by the metastable-ion peak at *m/e* 87.8 in the mass spectrum of compound **4** (calc. for 298 → 162, m^* 88.0). The f_1-ion can undergo two further types of transformation. The loss of a hydrogen atom gives the f_2-ion, whereas expulsion of the R radical produces the f_3-fragment.



f_1 f_2 f_3

The presence of the peaks at *m/e* 192, 191, and 161 (f_1, f_2, and f_3) in the mass spectra of the 1,3,6,8-tetraoxabicyclo[4.4.0]decanes **7** and **8** allows these compounds to be distinguished from the isomeric galactitol derivative (**1**), which belongs to the 4,4′-bis-1,3-dioxolane series.

The mass numbers of the main ions arising from the molecular ions of compounds **3–8** are listed in Table II, and the mass spectra are shown in Fig. 2.



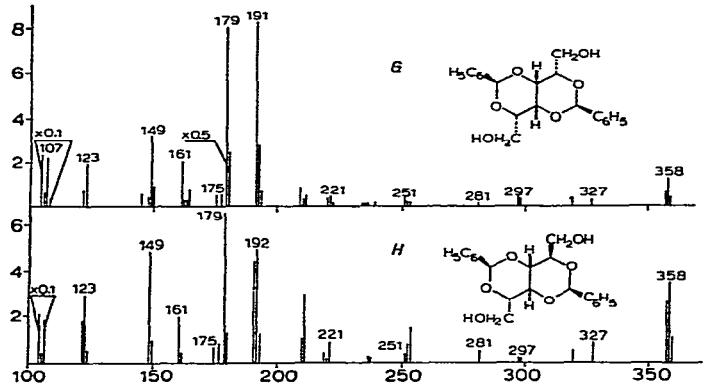


Fig. 2. Mass spectra of *A*, 2,3:4,5-di-*O*-benzylidenegalactitol (**1**); *B*, 2,3:4,5-di-*O*-benzylidene-1,6-dideoxygalactitol (**2**); *C*, 1,3:2,4-di-*O*-benzylidene-L-threitol (**4**); *D*, 1,3:2,4-di-*O*-benzylidene-erythritol (**3**); *E*, 1,3:2,4-di-*O*-benzylidenexylitol (**6**); *F*, 1,3:2,4-di-*O*-benzylideneribitol (**5**); *G*, 2,4:3,5-di-*O*-benzylidene-D-mannitol (**8**); *H*, 2,4:3,5-di-*O*-benzylidene-D-glucitol (**7**).

TABLE II

MASS NUMBERS[a] OF THE MAIN IONS ARISING FROM COMPOUNDS **3–8**

Compound	m/e													
	M^+	b_1	b_2	b_3	c_1	c_2	d_1	d_2	e	"h_1"	"h_2"	f_1	f_2	f_3
3	298	297	191	(175)	(221)	115	—	—	192	149	—	162	(161)	—
4	298	297	191	175	221	115	—	—	192	149	—	162	(161)	—
5	328	327	221	205	(251)	145	297	191	222	149	—	162	161	—
										179	149	192	191	161
6	328	327	221	205	251	145	297	191	222	149	—	162	161	—
										179	149	192	191	161
7	358	357	251	(235)	281	175	327	221	252	179	149	192	191	161
8	358	357	251	(235)	281	175	327	221	252	179	149	192	191	161

[a]Numbers in parenthesis indicate that the corresponding peaks are of very low intensity or are absent from the mass spectrum.

Comparison of the mass spectra of the *cis*, *trans* stereoisomers **3** and **4**, and **5** and **6** reveals substantial differences in the intensities of the main peaks. The molecular ions are more abundant in the mass spectra of the *cis* isomers (**4** and **6**), than in those of the *trans* isomers (**3** and **5**); the "h_1-peaks" are more intense for the *trans* isomers. On the other hand, the peaks of the f_1-ions (formed by cleavage of the bond that is α to the carbon atoms common to both the fused rings) are more intense in the mass spectra of the *cis* compounds **4** and **6**. A similar pattern has been observed for a number of fused carbo- and hetero-cyclic systems[8]. The nature of the substituents strongly affects the fragmentation of the 1,3,6,8-tetraoxabicyclo[4.4.0]decane system. Thus, the above-mentioned differences in the mass spectra are more pronounced for the unsubstituted *cis*, *trans* isomers **3** and **4**, than for the substituted derivatives **5** and **6**. Such substituents as $(EtS)_2CH$ completely change the fragmentation pattern,

as has been demonstrated for the diethyl dithioacetals of 2,4:3,5-di-*O*-benzylidene-D-xylose and D-ribose[12].

For compounds **7** and **8**, a comparison may be made of the influence on the fragmentation processes of substituents in axial or equatorial positions. Both of these compounds have the "*O*-inside" conformation[4], the former having one and the latter having two axial CH_2OH groups. The more sterically strained compound **8** shows a much less-intense molecular-ion peak than isomer **7**. In the mass spectrum of compound **7**, the ions f_1 and f_2 have approximately the same abundance, but, for compound **8**, the f_2-peak is three times more intense than the f_1-peak.

Thus, the characteristic features of the mass spectra of benzylidene derivatives of alditols may be used for detecting the structure and stereochemistry of their cyclic acetals, and, in some cases, for determination of the configuration of the parent alditol.

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The authors thank Professor A. B. Foster, Chester Beatty Research Institute, London, for samples of the benzylidene acetals used in this study.

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STRUCTURE OF THE CELL-WALL GLUCAN OF YEAST (*Saccharomyces cerevisiae*)*

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ABSTRACT

The cell-wall D-glucan ($[\alpha]_D^{25}$ $-10°$ in methyl sulfoxide) of bakers' yeast has been shown by methylation and periodate oxidation studies to be a β-(1→3)-D-glucan having branches at C-6. Hydrolysis of the methylated D-glucan (1 mole) yielded 2,3,4,6-tetra- (1 mole), 2,4,6-tri- (7 moles), and 2,4-di-*O*-methyl-D-glucose (1 mole), and trace proportions of 2,3,4-tri-*O*-methyl-D-glucose, which indicated the presence of a small proportion of (1→6)-linked residues in the molecule. Periodate oxidation of the D-glucan (DP 410), followed by reduction and mild hydrolysis with acid, eliminated one D-glucose residue out of every 9, and yielded the corresponding, degraded D-glucan (DP 150), glycerol, and a small proportion of a 1-*O*-β-D-glucosylglycerol, which would be derived from the sequence of the D-glucose residues in the glucan molecule represented by the system →6)-G*p*-(1→3)-G*p*-(1→6)-G*p*-(1→. Methylation of the degraded glucan gave material which, on hydrolysis, gave, per mole, 2,3,4,6-tetra-(1 mole), 2,4,6-tri-(6 moles), and 2,4-di-*O*-methyl-D-glucose (1 mole). The (1→3)-β-D-glucanase from *Rhizopus arrhizus* acted on the D-glucan and on the degraded D-glucan to give the same products, *viz.*, D-glucose, laminaribiose, gentiobiose, and higher oligosaccharides. The structure of the cell-wall D-glucan is discussed on the basis of these findings.

INTRODUCTION

Yeast cell-wall is reported[1] to consist of a glucan (29%), a mannan (31%), protein (13%), and lipid (8.5%), and more recently it has been suggested[2] that

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2-amino-2-deoxy-D-glucose provides the link between the protein and the polysaccharide components.

The polysaccharide component of the yeast cell-wall that is insoluble in water and in dilute alkali is now known as yeast glucan, although, when first isolated[3] in 1894, it was designated as yeast cellulose. Preparations of yeast glucan, free from glycogen-like material, were later produced[4,5] by boiling the residue from the alkaline treatment of yeast (*Saccharomyces cerevisiae*) with dilute mineral acid.

Early methylation studies[4,5] revealed that hydrolysis of the methylated glucan gave 2,4,6-tri-*O*-methyl-D-glucose, and, because of the low specific optical rotations of the methylated and acetylated derivatives of the glucan, it was deduced[5] that the glycosidic bonds were β-D-(1→3).

Later, a glucan from bakers' yeast was subjected to repeated methylation, and the product was hydrolyzed, to give, per mole, 2,3,4,6-tetra-*O*-methyl-D-glucose (1 mole), 2,4,6-tri-*O*-methyl-D-glucose (7 moles), and 4,6-di-*O*-methyl-D-glucose (1 mole). These findings led to the conclusion that the yeast glucan possesses a highly branched structure of (1→3)-linked main chains with (1→2)-linked side chains. The glycosidic bonds were deduced[6] to be β-D.

Structural studies by the technique of partial hydrolysis, whereby there were obtained gentiobiose, β-D-*Gp*-(1→6)-D-*Gp*; laminaribiose, β-D-*Gp*-(1→3)-D-*Gp*; gentiotriose, β-D-G*p*-(1→6)-β-D-G*p*-(1→6)-D-G*p*; 6-*O*-β-laminaribiosyl-D-glucose, β-D-G*p*-(1→3)-β-D-G*p*-(1→6)-D-G*p*; laminaritriose, β-D-G*p*-(1→3)-β-D-G*p*-(1→3)-D-G*p*; 3-*O*-β-gentiobiosylglucose, β-D-G*p*-(1→6)-β-D-G*p*-(1→3)-D-G*p*; and gentiotetraose, β-D-G*p*-(1→6)-β-D-G*p*-(1→6)-β-D-G*p*-(1→6)-D-G*p*, prompted the suggestion that yeast glucan possesses a linear instead of a branched-chain structure[7]. It was further suggested that the former methylation results[6], indicating a branched-chain structure, might have been due to incomplete methylation and that the formic acid liberated during periodate oxidation of the D-glucan arose, not from terminal, nonreducing D-glucose residues, but from (1→6)-linked residues[7], a conclusion that was said to be substantiated by *p*-toluenesulfonylation experiments[8,9].

The establishment of a new procedure[10] for the controlled degradation of polysaccharides, involving periodate oxidation, reduction, and mild acid hydrolysis[11], seemed to provide an approach that might resolve these conflicting structural concepts. Thus, the relatively high proportion of (1→6)-linked D-glucose residues, if present, should be recognizable not only by the formation of 2,3,4-tri-*O*-methyl-D-glucose on hydrolysis of the methylated D-glucan, but also by the extensive cleavage of the parent D-glucan when it is subjected to oxidation with periodate. Furthermore, it is apparent that any oligosaccharide, or degraded polysaccharide, derived by periodate oxidation of a linear D-glucan containing only (1→3) and (1→6) linkages should contain only (1→3) linkages, and should consequently give rise only to 2,4,6-tri-*O*-methyl-D-glucose on methylation and hydrolysis. The present report is concerned with the results of methylation and periodate-oxidation studies on (a) a D-glucan isolated from bakers' yeast, and (b) the corresponding degraded D-glucan produced by periodate oxidation, reduction, and mild hydrolysis with acid.

RESULTS AND DISCUSSION

The D-glucan used in the investigations reported herein was isolated from fresh and dried yeast, both of which are regarded as belonging to the same strain of bakers' yeast (*Saccharomyces cerevisiae*). Protein, glycogen, and mannan were removed by treatment of the yeast with dilute alkali, and glycogen-like material in the residue was removed by heating the material with dilute acetic acid followed by autoclaving with water. Although the yeast glucan thus obtained was insoluble in water, alkali, and the common organic solvents, it dissolved gradually in 90% formic acid[7] and in methyl sulfoxide, the latter being one of the best solvents for many polysaccharides. The yeast glucan recovered from the methyl sulfoxide solution by precipitation with water showed $[\alpha]_D^{25}$ $-10°$ in methyl sulfoxide; it gave no color with iodine and, on hydrolysis with acid, yielded only D-glucose.

The fully methylated D-glucan {$[\alpha]_D^{25}$ $+6°$ ($CHCl_3$), OMe, 44.4%}, obtained by successive application of the Muskat[12], Haworth[13], and Kuhn[14] methods of methylation, on hydrolysis furnished (per mole) 2,3,4,6-tetra-*O*-methyl-D-glucose (1 mole), 2,4,6-tri-*O*-methyl-D-glucose (7 moles), and 2,4-di-*O*-methyl-D-glucose (1 mole). Only trace proportions of 2,3,4-tri-*O*-methyl-D-glucose were detected by paper chromatography (see Table I); this finding may indicate the presence of a very

TABLE I

HYDROLYSIS OF THE METHYL DERIVATIVE OF YEAST GLUCAN AND OF DEGRADED YEAST-GLUCAN

Cleavage product	*Structural feature indicated*	*Molar proportions*	
		Original D-glucan	*Degraded D-glucan*
O-Methyl-D-glucose			
2,3,4,6-tetra-	G*p*-(1→	1	1
2,4,6-tri-	→3)-G*p*-(1→	7	6
2,3,4-tri-	→6)-G*p*-(1→	trace	0
2,4-di-	→6) →3)-G*p*-(1→	1	1

small proportion of linear (1→6)-linked D-glucose residues, but it is not sufficient to explain the number of (1→6)-linked, nonterminal residues suggested from the proportion of (1→6)-linked oligosaccharides formed by acid hydrolysis[7].

The isolation of 2,3,4,6-tetra- and 2,4-di-*O*-methyl-D-glucose in equimolecular proportions (11%) from a polysaccharide that was essentially fully methylated proved that this yeast glucan is highly branched. Characterization of the nonterminal residues as 2,4,6-tri-*O*-methyl-D-glucose revealed that the linkages are, indeed, of the (1→3) type, a result in agreement with the early methylation studies[4,5]. The isolation of 2,4-di-*O*-methyl-D-glucose revealed the presence of branching residues of D-glucose having linkages at O-1, O-3, and O-6. This observation does not accord with the previous isolation of 4,6-di-*O*-methyl-D-glucose[6], which had indicated the presence of D-glucose

residues joined through O-1, O-2, and O-3, or with the suggestion[7] that yeast glucan possesses a linear structure. No explanation can yet be offered for this observed difference, unless the difference depends on the conditions used for culturing the yeast. Further support for the presence of D-glucose residues triply-linked at O-1, O-3, and O-6 has recently been provided[15] by the observation that 2,4-di-*O*-methyl-D-glucose, and not the 4,6-isomer, is the major di-*O*-methyl-D-glucose formed on methylation and hydrolysis of yeast glucan.

When the yeast glucan was treated with periodate at 10°, two molar proportions of periodate were consumed with the simultaneous liberation of one molar proportion of formic acid, for an average of about eight residues of D-glucose. This finding agrees with previous values[6,7] (*cf* ref. 15), and is in harmony with the structural requirements deduced from the methylation data, namely, that the repeating unit of the yeast glucan consists of one terminal, nonreducing residue, seven (1→3)-linked, nonterminal residues, and one branching residue joined through O-1, O-3, and O-6.

As only trace proportions of 2,3,4-tri-*O*-methyl-D-glucose were produced by hydrolysis of the methylated D-glucan, it is to be expected that (*a*) periodate oxidation would attack only the terminal, nonreducing, D-glucose residues of the parent D-glucan, (*b*) the residual molecule should remain a polysaccharide and not be a simple oligosaccharide, and (*c*) the recovered polysaccharide should amount to about 90% of the weight of the parent D-glucan, because one out of every nine D-glucose residues is removed by periodate oxidation, reduction, and hydrolysis. Some support for these conclusions was derived from the observation that the degree of polymerization of the degraded D-glucan is 150, indicating that (a) it is a polysaccharide (not a simple oligosaccharide), and (b) the degraded D-glucan obtained by the reaction sequence of periodate oxidation, reduction, and hydrolysis corresponded to 90% of the weight of the parent yeast-glucan.

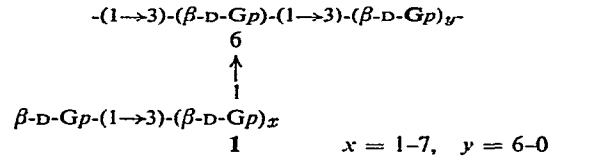
That periodate oxidation mainly attacks the terminal, nonreducing, D-glucose residues is proved by the fact that, on hydrolysis, the fully methylated, degraded D-glucan gave the same products as the permethylated D-glucan, and that, as expected, six molar proportions of 2,4,6-tri-*O*-methyl-D-glucose were produced (instead of the seven obtained from the permethylated, parent D-glucan). Of interest and, perhaps, of structural significance is the observation that no trace of 2,3,4-tri-*O*-methyl-D-glucose was detected amongst the hydrolysis products of the methylated, degraded D-glucan (see Table I).

A careful search of the products formed simultaneously with the degraded D-glucan when the yeast glucan was treated successively with periodate, sodium borohydride, and dilute sulfuric acid[10,11] revealed the presence of glycerol (the major component, which arises from the terminal, nonreducing, D-glucose residues), and a relatively small proportion of a 1-*O*-β-D-glucosylglycerol, recognized by hydrolysis and by paper chromatography. It is believed that this fragment arises from a (1→3)-linked D-glucopyranose residue that is flanked with (1→6)-linked D-glucose residues, thus: →6)-G-(1→3)-G-(1→6)-G-(1→. However, as already stated, the proportion of such a sequence of three D-glucose residues must necessarily be small, because only

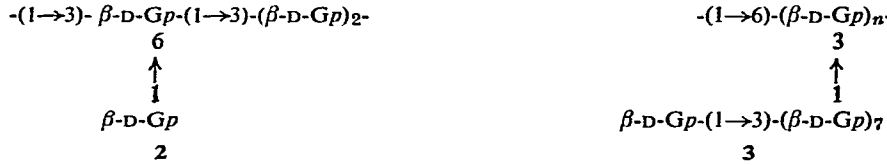
traces of 2,3,4-tri-*O*-methyl-D-glucose are produced by hydrolysis of the methylated, undegraded D-glucan.

Consideration of the findings reported here leads to a structure such as **1**, of which there are clearly, for the repeating unit of the undegraded yeast glucan, seven possible variations, in which the number of residues in the side chain can be 1 to 7. The isolation of a 1-*O*-β-D-glucosylglycerol indicates the presence in the D-glucan molecule of a structural discontinuity consisting of the periodate-susceptible system: →6)-G-(1]$_n$→3)-G-(1→[6)-G-(1]$_m$→, where n and m must of necessity be small number [in order to explain both the high yield of degraded glucan (90%) and its relatively high degree of polymerization (DP) (150) compared with that (410) of the parent D-glucan].

However, a strong argument against such formulations is the fact that the parent D-glucan is insoluble in water and in dilute alkali, and is soluble only in such solvents as formic acid and methyl sulfoxide, which have high dielectric constants and which possess the ability to disrupt hydrogen-bonding. Glucans having such structures as **1** and its related variations would most probably be soluble in water and in alkali. One such polymer, having the formula shown in **2**, has already been encountered[16]. This polysaccharide, having only one D-glucose residue linked by a (1→6) bond to one out of every three units of the main chain of (1→3)-linked D-glucose residues, is readily dispersible in water, as well as in dilute alkali.

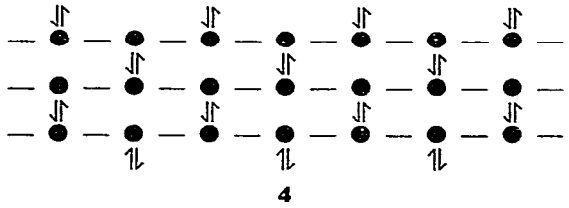


1 $x = 1$–7, $y = 6$–0



If consideration is now given to the previous results of graded hydrolysis by acid, whereby the β-D-(1→6)-linked D-glucose oligosaccharides, gentiobiose, gentiotriose, and gentiotetraose were obtained[7], the structure shown in **3** appears feasible. Such a formulation provides structural regularity in two directions and permits close association of the side chains composed of eight β-D-(1→3)-linked D-glucopyranose residues. Attached to all of the β-D-(1→6)-linked D-glucose residues of the main chain, these side chains would provide the means for the development of strong hydrogen-bonding in many directions; one such system, showing one-dimensional hydrogen-bonding, is shown diagramatically in formula **4**. With numerous side-chains, three-dimensional hydrogen-bonding could readily occur. A structure such as **3**, or a modification of it, would constitute a stable framework upon which the remainder of the components of the yeast cell-wall might well be built.

Additional evidence relating to the structure of the yeast glucan and its degraded product is forthcoming from the use of specific enzymes. Thus the (1→3)-β-D-glucanase enzyme from *Rhizopus arrhizus*[16] acts on the D-glucan and on its de-

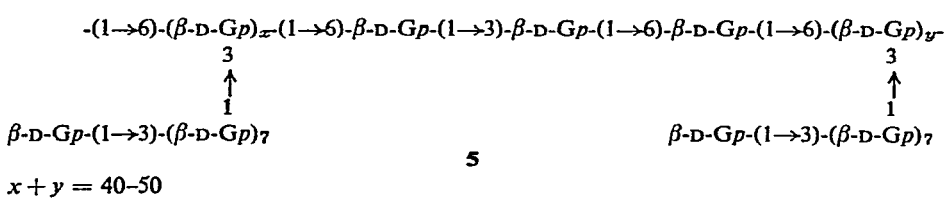


4

● = D-glucopyranose

graded derivative to give the same products, namely, D-glucose, laminaribiose, and gentiobiose, as well as unidentified higher oligosaccharides. It is apparent that the gentiobiose formed from the undegraded D-glucan is not derived from D-glucose residues joined only through O-1 and O-6, because such (1→6)-linked D-glucose residues would be cleaved during the periodate oxidation of the parent D-glucan. The fact that the degraded D-glucan does, in fact, on enzymic breakdown, yield gentiobiose in the same manner as the parent D-glucan clearly demonstrates that the (1→6)-linked D-glucose residues that give rise to the gentiobiose and which are stable to periodate must, therefore, correspond to the D-glucose residues that constitute the branch points in the molecule and which are linked through O-3 as well as O-6. This finding would appear to rule out the linear structure suggested from controlled degradation by acid[7], and to favor some type of branched-chain structure, as formerly proposed[6].

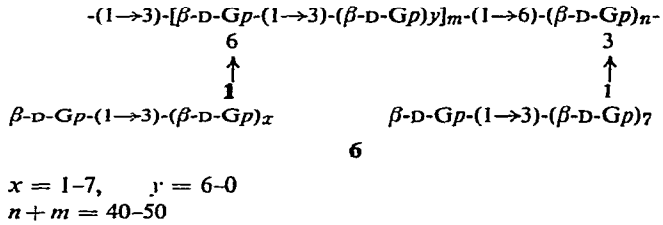
Because periodate oxidation degrades the D-glucan so as to lessen the DP from 410 to 150 (approximately), with concomitant liberation of glycerol (largely from the terminal residues of the side chains) and of 1-*O*-β-D-glucosylglycerol, it is suggested that the latter product is derived from about two locations in the D-glucan molecule where the sequence of the D-glucose residues is represented by the system: →6)-G-(1→3)-G-(1→6)-G-(1→. This conclusion would lead to **5** as one possible structure for yeast glucan. Although structure **5** explains all of the facts known at present, the possiblity of such variations as **6**, which is a combination of **1** and **3**, cannot be disregarded. Indeed, if it were assumed that gentiotetraose is derived from the highest sequence of contiguous (1→6)-linkages in the D-glucan, there is the possibility that the main chain of the D-glucan molecule might consist of an alternating sequence of three (1→6)-linked D-glucose residues, each having a side chain composed of eight D-glucose residues, as in **3** (where $n=3$), and a group of (1→3)-linked residues, as in **6**. If the (1→3)-linked side-chains, rather than the sequences of (1→3)-linked units in



5

$x+y = 40–50$

the main chain, were to play the dominant architectural role, and were capable of forming hydrogen bonds with similar side-chains of neighboring molecules, an explanation for the insoluble character of the D-glucan and its degraded derivative might still be forthcoming. The structural feature that consists of an isolated, (1→3)-linked, D-glucose residue flanked by (1→6)-linked D-glucose residues would also be present as an added structural detail, as in **5**, in such positions (probably two) as to give rise, on



periodate oxidation, to a degraded glucan of DP 150. Until further diagnostic experimental data become available, formula **5** is adopted as a working model for the structure of yeast glucan, and **3** for the structure of the degraded D-glucan.

EXPERIMENTAL

General. — All evaporations were performed under diminished pressure at 40–50°. Paper chromatography was conducted by the descending method on Whatman No. 1 paper with the following solvent systems: (A) 4:1:5 butyl alcohol–ethanol–water (upper phase)[17], (B) 3:2:1 butyl alcohol–ethanol–water, (C) 5:2:7 ethyl acetate–pyridine–water (upper phase)[18], (D) 10:4:3 ethyl acetate–pyridine–water, (E) butanone–water azeotrope[19]. Paper electrophoresis[20] was effected on Whatman No. 1 paper with 0.1M borate buffer, at 600 volts for 3.5 h. Chromatograms were sprayed with ammoniacal silver nitrate or *p*-anisidine hydrochloride in butyl alcohol saturated with water; for paper electropherograms, *p*-anisidine trichloroacetate[21] or lead tetraacetate–*p*-rosaniline reagent[22] was used, the latter being particularly useful for detection of disaccharides.

Isolation of yeast glucan. — Fleischmann's compressed yeast (900 g) (Standard Brands Inc., New York) was dispersed in 6% aqueous sodium hydroxide[6], and the mixture was stirred overnight at room temperature. The insoluble residue, which was recovered by centrifugation, was heated with 3% sodium hydroxide for 3 h at 75°, and the mixture was kept overnight at room temperature. After two such treatments with 3% sodium hydroxide, the alkaline digest was brought to pH 4.5 with hydrochloric acid, and the cell-wall material was collected (centrifuge), washed successively with water (thrice), ethanol, and ether, and dried *in vacuo* (yield 21.0 g).

This cell-wall material was heated with 0.5M acetic acid for 3 h at 90°, and the residue, which showed a slightly positive stain with iodine, was subjected to seven such treatments with 0.5M acetic acid, and then dispersed in water (500 ml), and the dispersion was heated at 15 lb.in^{-2} for 30 min at 100°. After the mixture had been cooled,

the insoluble D-glucan was collected (centrifuge), washed successively with water (thrice), ethanol (thrice), and ether (twice), and was dried *in vacuo* (yield 7.6 g). This preparation of yeast glucan did not give a color with iodine.

For further purification, this D-glucan (3.5 g) was suspended in methyl sulfoxide (200 ml), and the mixture was shaken for 40 h at room temperature. Water (250 ml) was added to the viscous solution of the D-glucan, and the mixture was kept overnight. The precipitated D-glucan was successively washed with water (6 times), 50% aqueous methanol (thrice), and methanol to remove traces of methyl sulfoxide, and dried *in vacuo* (yield 3.2 g).

In another experiment, powdered yeast (150 g) (Red Star Co., Milwaukee, Wisconsin) was treated as described for the fresh yeast. After treatment with 3% alkali, the cell-wall material amounted to 19.7 g. From this, D-glucan was obtained by treatment with dilute acetic acid followed by autoclaving with water (yield 7.2 g).

Properties of yeast glucan. — The D-glucan, either from compressed yeast or powdered yeast, appeared to be free from glycogen-like material, as it gave no color with iodine. Although the polysaccharide was insoluble in water and alkali, it gradually dissolved in formic acid (90%) and in methyl sulfoxide. Yeast glucan showed $[\alpha]_D^{25}$ $-10°$ (*c* 0.6, methyl sulfoxide).

Yeast glucan (50 mg) was heated with 90% formic acid (2 ml) for 3 h, and to the resulting clear solution was added 0.5M sulfuric acid (3 ml), and the solution was heated in a sealed tube for 3 h at 100°. The hydrolyzate was cooled, neutralized with barium carbonate, and filtered, and the filtrate was evaporated to a small volume. Paper chromatography of the solution showed a glucose only. The glucose content of the glucan was determined by the phenol–sulfuric acid method[3] after the D-glucan had been heated with 90% formic acid followed by 0.5M sulfuric acid at 100° to dissolve it. Found: (*a*) 51.5 mg glucan (from fresh yeast) gave 52.0 mg (as a glucose) (*b*) 57.5 mg glucan (from powdered, dry yeast) gave 58.2 mg (as a glucose).

When the product from reduction of yeast glucan (154 mg) with sodium borohydride was oxidized with periodate[24], it gave rise to an amount of formaldehyde (2.3 μmoles) corresponding to a DP of 410 (assuming that the terminal, reducing group was linked through O-3, and would yield two molecules of formaldehyde) or DP 205 (assuming that the terminal reducing group was linked through O-6, and would yield 1 molecule of formaldehyde.)

Periodate oxidation of yeast glucan and preparation of the degraded glucan. — *Experiment (1).* Yeast glucan (1.52 g), prepared from compressed yeast, was dissolved in methyl sulfoxide (250 ml) by shaking for 5 days, and water (250 ml) was added, after which the D-glucan was separated (centrifuge) and dialyzed against water for 5 days to remove traces of methyl sulfoxide. The D-glucan was recovered (centrifuge), and washed 3 times with water. The residual, flocculent D-glucan was oxidized with 0.08M sodium periodate (250 ml) at 25° in the dark with continuous stirring. The periodate consumption was determined by the arsenite method [25], and formic acid was titrated with 0.01M sodium hydroxide[26].

The results are shown in Table II.

TABLE II

PERIODATE OXIDATION OF YEAST GLUCAN

Time (days)	1	2	7	10	20	30	40	50	60	65
Periodate consumption (molecules/glucose residue)	0.18	0.19	0.24	0.32	0.52	0.73	0.84	1.07	1.23	1.30
Formic acid production (molecules/glucose residue)	0.07	—	0.11	0.15	0.24	0.36	0.41	0.51	0.60	0.62

In another experiment, the D-glucan (3.11 g) was oxidized with 0.2M periodic acid (400 ml) for 60 days at room temperature (periodate consumption, 0.95 molecule per D-glucose residue), and the oxidized D-glucan was separated (centrifuge), and washed with water. The product was suspended in water (200 ml) and reduced with sodium borohydride (500 mg) as previously described[27]. The excess of sodium borohydride was decomposed by careful acidification with hydrochloric acid. The concentration of acid was adjusted to 0.1M, and the mixture was kept for 44 h at room temperature. The insoluble polysaccharide remaining was separated (centrifuge), and successively washed with water (thrice) and ethanol (thrice), and dried *in vacuo* (yield of modified D-glucan, 2.55 g). The supernatant liquor from the mild hydrolysis and the washings were combined, and neutralized with sodium hydroxide, and the neutral solution was evaporated to dryness. After removal of borate by evaporation with methanolic hydrogen chloride (1.5%) at room temperature, followed by passage through Amberlite IR-120 (H^+) and Duolite A4 (OH^-), the de-ionized solution was evaporated to a syrup (136 mg). Paper chromatography with solvents A and C revealed the presence of a glucosylglycerol, erythritol (trace), and glycerol (major component).

Experiment (2). A suspension of the preparation of D-glucan (634.5 mg) used above was stirred with water (100 ml) for 24 h, and then cold 0.2M sodium metaperiodate (50 ml) was added. Oxidation was conducted at 10°, with stirring. A slight over-consumption of periodate occurred, but it was much less than at room temperature. By extrapolating to zero time, the periodate consumption was estimated to be 0.25 molecule per D-glucose residue, and the formic acid liberated was 0.11 molecule per D-glucose residue. After oxidation for 20 days, the residual reaction mixture, containing 465 mg of the original D-glucan, was reduced with sodium borohydride, and the product was subjected to mild hydrolysis as previously described; this yielded 333 mg of the modified D-glucan, and 35.3 mg of a syrupy mixture that contained glycerol (main component), erythritol (trace), and a 1-*O*-*β*-D-glucosylglycerol.

Methylation of the degraded yeast-glucan. — For structural studies on the modified D-glucan, which was obtained by periodate oxidation of the original D-glucan followed by reduction and mild hydrolysis by acid, the preparation oxidized at room temperature was used, because complete cleavage of the (1→6)-linked units was deemed necessary. Since the modified D-glucan (1.2 g) was insoluble in water and in alkali, it was dispersed in liquid ammonia (200 ml). Sodium (0.6 g) and methyl iodide (18 ml) were added in portions during 3 h to the stirred solution, and after it had been

stirred for a further 2 h at −80°, the liquid ammonia was allowed to evaporate at room temperature. The residue was dispersed in water (50 ml), and the dispersion was extracted with chloroform (4 × 150 ml). The chloroform extract was washed with water, dried (magnesium sulfate), and evaporated to dryness (yield 730 mg). The partially methylated product was subjected to two methylations with methyl iodide (25 ml) and silver oxide (2.5 g), the mixture being refluxed for 18 h according to the Purdie method (yield 641 mg; OCH_3, 43.2%). To complete the methylation, the product was methylated twice by the Kuhn method[14] with *N*,*N*-dimethylformamide (25 ml), methyl iodide (8 ml), and silver oxide (8 g), the mixture being shaken for 24 h at room temperature. The reaction mixture was extracted with chloroform, and the extract (150 ml) was washed successively with 5% potassium cyanide and water. After being dried (sodium sulfate), the chloroform extract was evaporated to dryness (yield 450 mg; OCH_3, 44.2%).

The fully methylated product thus obtained was dissolved in acetone (25 ml), and an acetone-insoluble fraction was filtered off [Fraction 1, 75 mg, $[\alpha]_D^{25}$ +4.5° (chloroform)]. Petroleum ether (200 ml) was added to the acetone solution to give Fraction 2 (189 mg), ($[\alpha]_D^{25}$ +6.4° in chloroform; OCH_3 44.25%). By addition of more petroleum ether (250 ml), Fraction 3, 167 mg, ($[\alpha]_D^{25}$ +6.5° in chloroform; OCH_3 44.30%) was obtained.

Hydrolysis of the methylated, degraded glucan, and identification of methylated sugar components. — A portion (25 mg) of each of the above fractions of methylated D-glucan was heated in a sealed tube with 2% methanolic hydrogen chloride (5 ml) for 24 h at 100°. After the solution had been cooled, the acid was neutralized with silver carbonate, the suspension was filtered, and the filtrate was evaporated to a syrup. This was hydrolyzed with 0.5M sulfuric acid (5 ml) for 32 h at 100°, the hydrolyzate was rendered neutral (barium carbonate), and filtered, and the filtrate was evaporated to a syrup. On paper chromatograms (solvents A and E), the hydrolyzate (fractions *a*, *b*, and *c*) of fractions *1*, *2*, and *3*, respectively showed the same components, namely, 2,3,4,6-tetra-*O*-methyl- (R_F 0.78 in solvent E), 2,4,6-tri-*O*-methyl (R_F 0.49), and 2,4-di-*O*-methyl-D-glucose (R_F 0.21).

For identification of these components, fraction *2* (155 mg) was hydrolyzed as just described. The syrup (130 mg) thus obtained was fractionated into three components by paper chromatography on Whatman No. 3 paper with solvent E. Each component was eluted with water, the eluate was concentrated to a syrup, and this was purified by extraction with the minimal volume of aqueous ethanol, filtration, and evaporation to dryness (total recovery, 118.4 mg, or 91.2%).

*Identification of 2,3,4,6-tetra-*O*-methyl-*D*-glucose.* — Fraction *a* (16.0 mg), $[\alpha]_D^{25}$ +92° (*c* 0.9, water), was recrystallized from ether–petroleum ether; m.p. and mixed m.p. 92–93° with an authentic specimen of 2,3,4,6-tetra-*O*-methyl-D-glucose. Treatment of fraction *a* with aniline afforded 2,3,4,6-tetra-*O*-methyl-*N*-phenyl-D-glucosylamine, m.p. and mixed m.p. 138–140° (after recrystallization from ethanol–ether).

*Identification of 2,4,6-tri-*O*-methyl-*D*-glucose.* — Fraction *b* (88.4 mg) was recrystallized from ethanol–ether; m.p. and mixed m.p. 115–117°, $[\alpha]_D^{25}$ +120° →

+74.6° (42 h) (*c* 1.5, methanol). The 2,4,6-tri-*O*-methyl-D-glucose was transformed into the aniline derivative[28], m.p. and mixed m.p. 164–165° (recrystallization from ether), and into the 1,3-di-*p*-nitrobenzoate which, on recrystallization from ether–methanol, had m.p. and mixed m.p. 157–157.5° and $[\alpha]_D^{25}$ +173° (*c* 0.6, chloroform).

Identification of 2,4-di-O-methyl-D-glucose. — Fraction *c* (14.0 mg), $[\alpha]_D^{25}$ +75.2° (*c* 1.0, water), contained a trace of 4,6-di-*O*-methyl-D-glucose (M_G 0.23), but the major component (M_G 0.10) corresponded to a 2,4-di-*O*-methylglucose, as revealed by paper electrophoresis. Crystalline 2,4-di-*O*-methyl-D-glucose was obtained after nucleation; m.p. and mixed m.p.[29] 124–126°. Treatment of the mother liquor with *p*-nitroaniline afforded 2,4-di-*O*-methyl-*N*-(*p*-nitrophenyl)-D-glucosylamine[29], which was recrystallized from ethyl acetate; m.p. and mixed m.p. 253–254°, $[\alpha]_D^{25}$ −260° after 1 h (*c* 0.2, pyridine).

Methylation of yeast glucan. — The yeast glucan from compressed yeast was methylated in essentially the manner described for the degraded D-glucan. The D-glucan (1.4 g) was dispersed in liquid ammonia (250 ml), and methylated with methyl iodide (24 ml) and sodium (0.8 g). After completion of the reaction and evaporation of the ammonia, the residue was re-treated in the same way by using methyl iodide (16 ml) and sodium (0.5 g). The residue was stirred with water (50 ml), and the partially methylated product was extracted with chloroform (4 × 200 ml). As the yield (157 mg) from the chloroform extract was low, the aqueous layer was dialyzed against water for 48 h, and then evaporated to dryness. The residue thus obtained was combined with the material from the chloroform extract, and methylation was effected with 30% sodium hydroxide (50 ml) and methyl sulfate (20 ml) at 55° in the usual way[13]. After extraction with chloroform, the rest of the product was recovered by dialyzing and evaporating the solution. The partially methylated product from the chloroform extract was combined with the residue from the dialyzate, dissolved in *p*-dioxane (20 ml), and re-treated with 30% sodium hydroxide (100 ml) and methyl sulfate (40 ml). The reaction mixture was rendered neutral with sulfuric acid, and the methylated product was recovered by extraction with chloroform (3 × 100 ml). After a further methylation in the same way, the product was methylated by the Kuhn technique with *N*,*N*-dimethylformamide (15 ml), silver oxide (8.5 g), and methyl iodide (10 ml), as previously described. After separation in the usual way, the fully methylated product was purified by dissolving it in chloroform (20 ml) and adding petroleum ether (20 ml). The turbid solution was centrifuged, and the supernatant liquor was evaporated to dryness (yield 494 mg).

The methylated D-glucan thus obtained, OCH_3 44.45% and $[\alpha]_D^{25}$ +4.5° (*c* 2.2, chloroform), was separated into two fractions: fraction I (176 mg, acetone-insoluble) and fraction II (318 mg, acetone-soluble). Both fractions showed the same properties: OCH_3 44.45%, $[\alpha]_D^{25}$ +4.6° (chloroform). On hydrolysis, both fractions gave 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,4,6-tri-*O*-methyl-D-glucose, 2,4-di-*O*-methyl-D-glucose, and a trace of 2,3,4-tri-*O*-methyl-D-glucose.

Hydrolysis of the methylated yeast-glucan. — A portion (162 mg) of the acetone–soluble fraction was refluxed with 4% hydrogen chloride in methanol (10 ml) for

25 h, and the solvent was removed; the residue was hydrolyzed with 0.5M sulfuric acid (20 ml) for 30 h as described previously. After neutralization (barium carbonate), the hydrolyzate was evaporated, and the product was examined by paper chromatography. With solvent E, four components were detected, corresponding to 2,3,4,6-tetra-*O*-methyl- (R_F 0.78), 2,4,6-tri-*O*-methyl- (R_F 0.49) and 2,4-di-*O*-methylglucose (R_F 0.21), and a faint spot (R_F 0.58) corresponding to 2,3,4-tri-*O*-methylglucose. For identification of these methylated sugars, the above hydrolyzate (68.5 mg) was fractionated on Whatman No. 3 paper with solvent E, and the following components were characterized as already described. (A) *2,3,4,6-Tetra*-O-*methyl*-D-*glucose* (6.0 mg), $[\alpha]_D^{25}$ +90.5° (*c* 0.5, water), giving 2,3,4,6-tetra-*O*-methyl-*N*-phenyl-D-glucosylamine, m.p. and mixed m.p. 138–139°. (B) *2,4,6-Tri*-O-*methyl*-D-*glucose* (40.3 mg), m.p. and mixed m.p. 115–117°, $[\alpha]_D^{25}$ +120 → +73.5° (*c* 1.5, methanol). (C) *2,3,4-Tri*-O-*methyl*-D-*glucose*. This component was revealed as a faint spot that overlapped the leading edge of the 2,4,6-tri-*O*-methyl-D-glucose on paper chromatograms developed with solvent E. This mixed fraction (9.6 mg) was separated twice on a paper chromatogram (solvent E), to give pure 2,3,4-tri-*O*-methyl-D-glucose (2.1 mg), which had R_F 0.58 and $[\alpha]_D^{25}$ +70.5° (*c* 0.5, water) and was characterized as the 1,6-di-*p*-nitrobenzoate, m.p. and mixed m.p. 134–135° (recrystallized from methanol). (D) *2,4-Di*-O-*methyl*-D-*glucose*. This component (5.1 mg), $[\alpha]_D^{25}$ +75° (*c* 1.0, water), afforded the *p*-nitroaniline derivative, m.p. and mixed m.p. 253–254°. There appeared to be a trace of 4,6-di-*O*-methyl-D-glucose (M_G 0.23) in this fraction.

Isolation of glycerol and of a 1-O-β-D-glucosylglycerol from yeast glucan after periodate oxidation and reduction. — Yeast glucan (465 mg) was oxidized with 0.08M sodium periodate (100 ml) for 20 days at 20°, and the product was reduced with sodium borohydride (500 mg in 20 ml water). The product was hydrolyzed for 24 h with 0.1N hydrochloric acid at 25°, giving the degraded glucan (333 mg) as an insoluble compound. The supernatant liquor and the washing were combined and, after neutralization of the acid with dilute sodium hydroxide, the solution was evaporated to dryness. Borate was removed by treatment of the residue with 1.5% hydrogen chloride in methanol[20]. A solution of the residue in water was de-ionized by passage through Amberlite IR-120 (H^+) and Duolite A4 (OH^-) resins, and the effluent was evaporated to a syrup (yield 35.5 mg). On paper chromatograms (solvents A and C), this syrup showed three spots, corresponding to glycerol, erythritol (trace), and a glucosylglycerol. A portion of the syrup (20 mg) was separated by paper chromatography with solvent C. *Component 1* (9.0 mg), R_G 2.73 (solvent C), 2.46 (solvent B), was identical with glycerol on paper chromatograms and afforded the tri-*p*-nitrobenzoate, m.p. and mixed m.p. 191–192°. *Component 2* (*ca.* 0.5 mg), R_G 2.10 (solvent C), 1.84 (solvent B), corresponded to erythritol. *Component 3* (2.5 mg), $[\alpha]_D^{25}$ −25.5° (*c* 0.5, water), R_G 0.82 (solvent C), 0.94 (solvent B), was chromatographically identical with 1-*O*-β-D-glucopyranosyl-L-glycerol ($[\alpha]_D$ −13.2° in water[30]). On hydrolysis with acid, component *3* yielded equal parts of a glucose (R_G 1.0, solvent C) and glycerol (R_G 2.73).

When 20 mg out of 35.3 mg of the hydrolyzate (treatment with mild acid) was separated by paper chromatography, 9.0 mg of glycerol and 2.5 mg of a 1-*O*-β-D-

glucosylglycerol were recovered. Therefore, the total recoveries of glycerol and the glucosylglycerol were 15.9 mg (9.0 × 35.3/20) and 4.4 mg (2.5 × 35.3/20), respectively.

From the amount of formic acid produced by periodate oxidation of the parent D-glucan and the molecular weight of the resulting, degraded D-glucan (as determined by a chemical method; see next paragraph), the theoretical amounts of glycerol (19.4 mg)* and 1-*O*-β-D-glucosylglycerol (4.46 mg)** are in reasonably good agreement with the amount of glycerol (15.9 mg) and glucosylglycerol (4.4 mg) actually obtained.

Determination of molecular weight by chemical methods. — As these results indicated that one glycerol residue is attached to the reducing end of the degraded glucan, the degree of polymerization of the degraded glucan can be calculated (1) from direct determination of this glycerol by periodate oxidation and (2) by hydrolysis of the degraded glucan, followed by separation and determination of the glycerol.

(*1*) *Direct determination of glycerol*[24]. The degraded D-glucan (142.5 mg) was oxidized with 0.1 M sodium periodate (20 ml) for 20 h at 5°, and to a portion (5 ml) of the reaction mixture was added saturated lead acetate solution (5 ml). The mixture was dialyzed against water (5 ml) until equilibration of formaldehyde had been reached. An aliquot (1 ml) of the dialyzate was used for colorimetric determination with the chromotropic acid reagent (10 ml), the absorbance being determined at 570 nm by the method of Lambert and Neish[31]. The average of triplicate determinations showed the DP of the degraded glucan to be 155, corresponding to an average molecular weight of 25,000.

(*2*) *Hydrolysis and determination of glycerol.* The degraded D-glucan (192 mg) was hydrolyzed by heating it with M sulfuric acid (5 ml) in a sealed tube for 18 h at 100°. The acid was neutralized (barium carbonate), the suspension was filtered, and the filtrate was evaporated to a syrup. Glycerol was separated by paper chromatography, and determined by periodate oxidation and with the chromotropic acid reagent[31]. The amount of glycerol corresponded to a DP of 148 (mol. wt. 24,000) for the degraded D-glucan.

Isolation and identification of gentiobiose and laminaribiose from the action of (1→3)-β-D-glucanase on yeast glucan and on degraded yeast-glucan. — The (1→3)-β-D-glucanase was prepared from a culture of *Rhizopus arrhizus*, with a 25-fold purification[32]. This enzyme preparation, which readily degraded β-D-(1→3)-linked D-glucans, was homogeneous by ultracentrifugation, but was resolved into 3 fractions by Tiselius electrophoresis. Portions of the native D-glucan (10 mg) from compressed yeast and from powdered yeast, and of the degraded D-glucan, were suspended in acetate buffer (pH 4.8, 2 ml), to which the enzyme preparation (1 ml) was then added. The mixture was incubated for 45 min at 37°, the digest was centrifuged, and the

*It is assumed that the glycerol is derived from those residues that are cleaved by periodate to give formic acid, and, hence, that the molar yield of glycerol will be equal to the molar yield of formic acid.
**This figure is arrived at by assuming that the degraded glucan has a DP of 150 and that one mole of the glucosylglycerol is generated for every 150 glucose residues.

supernatant liquor was de-ionized by passage through Amberlite IR-120 (H^+) and Duolite A4 (OH^-) resins, and concentrated to a small volume.

All digests from the above three substrates were found, by paper chromatography with solvent D, to contain the same products; namely, a glucose (R_G 1.0), laminaribiose (0.72), an unidentified disaccharide (0.55), gentiobiose (0.42), and two higher saccharides (0.17 and 0.06).

In order to identify these components, the D-glucan from powdered yeast (700 mg) was treated in the same way with the enzyme preparation at pH 4.8 for 48 h. The digest was then centrifuged at 10,000 r.p.m. to remove insoluble material (D-glucan recovered, 430 mg), ethanol (final concentration, 50%) was added to the supernatant liquor, and the mixture was recentrifuged. After evaporation of the ethanol from the supernatant liquor, the solution was de-ionized by successive passage through Amberlite IR-120 (H^+) and Duolite A4 (OH^-) resins, and the mixture was separated by partition chromatography on Whatman No. 3 paper. The separated sugars were weighed, and characterized as follows. (*a*) D-*Glucose* (57.0 mg), $[\alpha]_D^{25}$ +50° (*c* 1.0, water). (*b*) *Laminaribiose* (5.5 mg), $[\alpha]_D^{25}$ +19° (*c* 1.5, water), m.p. and mixed m.p. 189–190°, after recrystallization from aqueous methanol; β-octaacetate, m.p. and mixed m.p. 161–163°. (*c*) *Unknown disaccharide* (3.5 mg); this resembled cellobiose on paper chromatograms, but, in paper electrophoresis, the migration (M_G 0.40) did not agree with that of maltose (M_G 0.54), cellobiose (M_G 0.48), or kojibiose (M_G 0.53). (*d*) *Gentiobiose* (22.7 mg), $[\alpha]_D^{25}$ +11.5° (*c* 4.0, water), corresponded chromatographically to gentiobiose (R_G 0.42 and M_G 0.66) and afforded β-gentiobiose octaacetate, m.p. and mixed m.p. 190–191°, $[\alpha]_D^{25}$ −6.5° (*c* 1.7, chloroform) (recrystallization from ethanol).

In addition to the above components, two unknown oligosaccharides were obtained: (*1*) (7.0 mg), R_G 0.17 (solvent D), $[\alpha]_D^{25}$ +38.5° (*c* 3.5, water) and (*2*) (9.5 mg), R_G 0.06 (solvent D), $[\alpha]_D^{25}$ −9.5° (*c* 2.0, water). These components will form the subject of a further study.

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PHYSICO-CHEMICAL CHARACTERISTICS OF THE LEVAN PRODUCED BY *Streptococcus salivarius*

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ABSTRACT

The levan from *Streptococcus salivarius* has an intrinsic viscosity of 0.15–0.16 dl/g. It sedimented as a single peak in the ultracentrifuge with a sedimentation constant of 215–217 S. The levan has a molecular weight of 16–23 million. The biological implications of these properties have been discussed.

INTRODUCTION

The polyfructoses of plants are comparatively small polymers having molecular weights lower than 10.000[1,2], whereas bacterial levans are truly macromolecular. It has long been known that bacteria derived from the oral and nasopharyngeal flora are capable of synthesizing polysaccharides[3]. One such organism, *Streptococcus salivarius*, synthesizes levan when grown on a medium containing sucrose. The culture conditions for optimal yield of levan have been studied[4,5], and chemical analyses of this levan reported. The present investigation describes the physico-chemical properties of the levan from *Strep. salivarius* as determined by ultracentrifugation and viscometry.

METHODS

Isolation, purification, and characterization. — The levan was prepared from a culture of *Strep. salivarius* (ATCC 13419) by a modified procedure of Niven *et al.*[4]. Considerable difficulty was experienced in removing Norit from the levan after the decolorization step. Substitution of Darco G for Norit facilitated the procedure. The dialyzed levan was finally dried *in vacuo* for 2 days at 55°. The yield from 1 liter of culture was 3 grams.

The fructose content of the levan was determined directly by the resorcinol method[6]. The moisture content was determined by drying at 100°.

Infrared absorption spectra of fructans were recorded with a Beckman Model IR9 spectrophotometer by both the Nujol mull technique with a sodium chloride prism and by the potassium bromide disc method.

The levan was hydrolyzed with 1% oxalic acid for 1 h at 100°. The oxalic acid

was removed by precipitation with saturated $Ba(OH)_2$. The supernatant solution was chromatographed on paper for 15 h in ethyl acetate–pyridine–water (12:5:4). The spots were detected with naphthoresorcinol and aniline–diphenylamine reagents[7]. The hydrolyzate was also chromatographed on thin-layer plates of MN cellulose powder 300 (Macherey, Nagel Co.), with the same solvent mixture and tests as for paper.

Gel chromatography. — The void volume of columns of Sephadex G-100 and G-200 (Pharmacia) gel was determined with blue dextran, mol. wt. 2×10^6 (Pharmacia). The void volume of a Biogel A 50 m Agarose (BioRad) column was determined with dextran, mol. wt. $5–40 \times 10^6$ (Sigma). The interstitial volume was determined with sodium chloride. A sample of levan was applied to these columns, and its appearance in the eluate was determined colorimetrically by the anthrone method[8].

Molecular-weight determination. — The rates of sedimentation were determined with a Spinco analytical ultracentrifuge, Model E, at rotor speeds of 14,290 revolutions per min at 25°. The migration of the boundary was followed by the Schlieren technique. The magnification of the lens system was 2.167 and the average distance of the reference line from the axis of rotatation was taken as 7.32 cm. Measurements of the rates of sedimentation were made at several concentrations of levan (2–10 mg/ml).

The viscosity was determined with an Ubbelohde type viscometer. The efflux time for the solvent, distilled water, was 100.6 sec at 25.0°. The reduced viscosity (η_{sp}/c) was determined at several concentrations (where $\eta_{sp}=\eta_{rel}{}^{-1}$). The relative viscosity (η_{rel}) was calculated as the ratio of flow time of the solution to that of the solvent, no kinetic energy corrections being made. The intrinsic viscosity $[\eta]$ was obtained from the graph of η_{sp}/c against c, as $[\eta]=\lim_{c\to 0}(\eta_{sp}/c)$. For both sedimentation and viscosity determinations, a sample of the dried levan was dissolved in distilled water, and the solution was filtered through fine sintered glass. A 2% solution was diluted as required and used for all measurements.

The molecular weight of the levan was computed from the equation:

$$M^{2/3} = \frac{S_0\eta_0\sqrt[3]{[\eta]}\,N}{\beta(1-\bar{v}\rho)}$$

assuming a value of $\bar{v}=0.64$ for the partial specific volume (*i.e.* the same as for glycogen), and $\beta=2.12\times10^6$ or 2.23×10^6 (*i.e.* an axial ratio of 1 or 5, respectively).

RESULTS AND DISCUSSION

Table I shows the result of the chemical and physical analyses of two samples of levan obtained from separate cultures of *Strep. salivarius*. The levan is composed of only fructose with a minor proportion of moisture.

The infrared absorption spectra of the two samples of levan prepared from separate *Strep. salivarius* cultures were identical. The absorption peaks of the spectrum obtained by the Nujol-mull technique were similar to those reported for other bacterial levans[10,11].

Chromatography of the hydrolyzed levan on paper and thin-layer cellulose revealed a single spot having R_F 0.42. The substance moved identically with, and gave the same color reaction as a fructose standard, R_F 0.42. The enzymic assay (Glucostat, Worthington Biochem. Corp., New Jersey) of the hydrolyzate did not detect any glucose.

TABLE I

ANALYSIS OF LEVAN FROM *Streptococcus salivarius*

	Sample A	*Sample B*
Fructose (%)	94.3	94.3
Moisture (%)	7.17	4.40
Ash (%)	0.22	0.0
Sedimentation constant, S_{25}	217.4	213.5
Intrinsic viscosity @ 25°	0.15	0.16
Average molecular weight		
β=2.12	23.3×10^6	18.0×10^6
β=2.23	21.6×10^6	15.5×10^6

In gel filtration through Sephadex G-100 and G-200, the levan appeared immediately following the void volume. This indicated a molecular weight in excess of 200,000 (Fig. 1). On an Agarose A 50 m column, the levan also appeared as a single peak shortly after the void volume. Some resolution of the smaller levan molecules could be detected, as shown by tailing of the elution pattern.

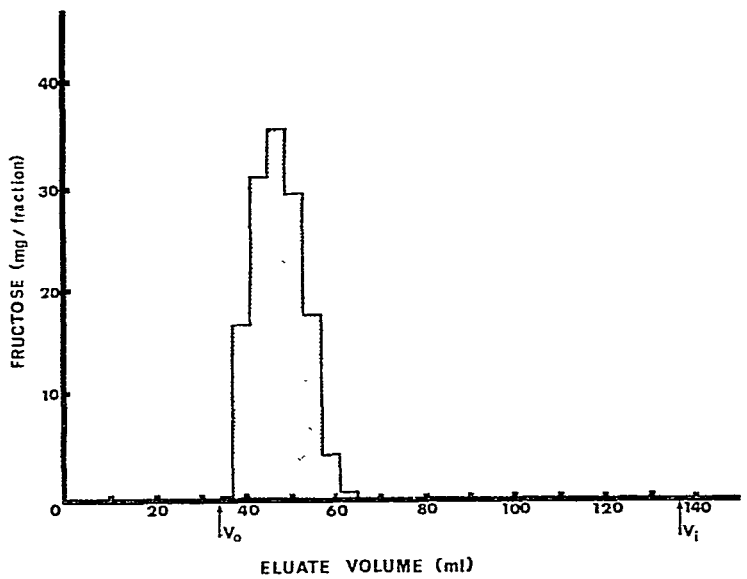

Fig. 1. Filtration of levan through Sephadex G-100 gel. The fractions were scanned with the anthrone method; v_0, void volume; v_i, intestinal volume.

On ultracentrifugation, the levan was found to sediment extremely rapidly as a single peak (Fig. 2). The sedimentation constant was calculated by plotting the

increase of the sedimentation coefficient against the concentration and extrapolating to zero concentration (Fig. 3). A sedimentation constant of 215–217 S_{25} and an intrinsic viscosity [η] of 0.15–1.06 dl/g were obtained for the two samples of *Strep. salivarius* levan. Computation of the molecular weight from these data gave a value of $16–23 \times 10^6$ for this bacterial levan.

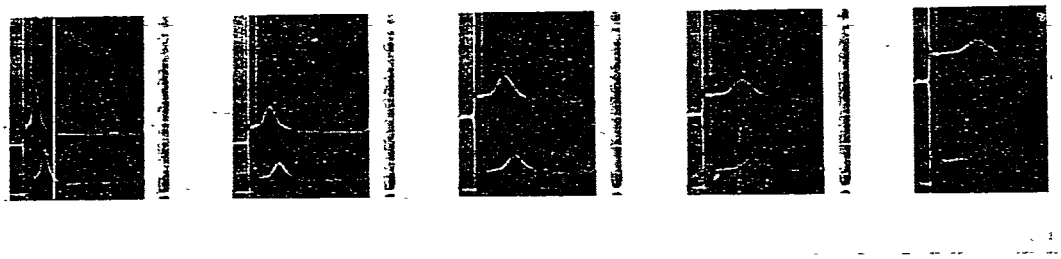

Fig. 2. The sedimentation of levan of *Strep. salivarius*, upper pattern 6.0 mg/ml, lower pattern 4.0 mg/ml. The patterns were obtained with the Spinco model E ultracentrifuge, average temperature 20.0°, rotor speed 27.690 rev/min. The photographs were taken at 2-min intervals.

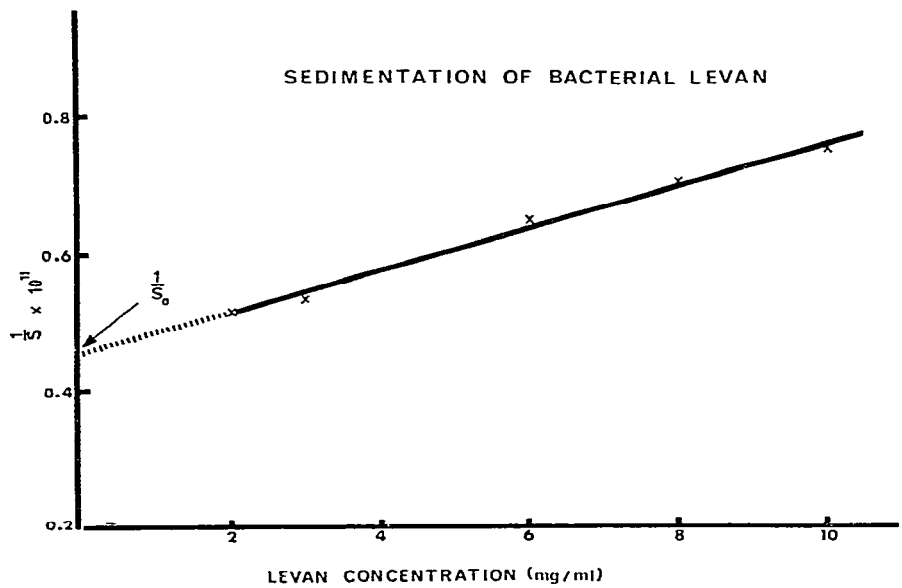

Fig. 3. Plot of the sedimentation of the levan of *Strep. salivarius* at various concentrations extrapolated to infinite dilution. S_0 sedimentation coefficient at infinite dilution.

Because of the solubility of bacterial levans in water, it has been proposed[12] that their molecular weights are extremely small or that the levans are highly branched. The levan of *Bacillus vulgatus*[13] has, however, been found to have a molecular weight

of $50–100 \times 10^6$, that of *Bacillus subtilis*[14] a molecular weight of 25×10^6, and that of *Aerobacter levanicum*[15] a molecular weight of 17×10^6. The levan prepared by the action of an enzyme isolated from *A. levanicum* had a molecular weight, $40–67 \times 10^6$, even higher than that of the levan obtained from cell cultures of the same organism[15,16]. Furthermore, determinations of the chemical structure by methylation have shown branching of the levan of *Bacillus polymyxa*[17], *B. subtilis*, *Pseudomonas prunicola*[18], *A. levanicum*, and *Azobacter chroococcum*[20].

The present investigation indicates that the levan isolated from *Strep. salivarius* has a molecular weight in the same high range as those of the levans isolated from *B. subtilis* and *A. levanicum*. The low intrinsic-viscosity of the levan of *Strep. salivarius* in comparison with its high sedimentation constant suggests that its molecule is compact and symmetrical, but of high molecular weight. Examination[21] of this molecule with the electron microscope revealed the spherical or ellipsoidal appearance that would be expected of a highly branched structure. The actual appearance of the levan molecule varied somewhat depending on the technique used in the preparation for the electron microscopy. For all of the samples examined, however, the axial ratio was in the range of 1 to 5, with most of the molecules showing an axial ratio of 2 or less. The data computed in Table I give the molecular weight for the maximum range of axial ratios. The appearance of most of the levan molecules suggests that the higher molecular-weight value is the correct one.

The biological role of the levan of *Strep. salivarius* is of considerable interest. Although this micro-organism is only a minor component of the flora of the dental plaque[22,23], McDougall has shown that *in vivo* the levan content of plaque can more than double in 30 min after a 40% sucrose rinse[24]. The *Strep. salivarius* levan is susceptible to attack by mixed cultures from saliva, plaque, or tongue[25], and could function as an ideal reservoir of substrate for bacterial metabolism within the plaque. Sucrose can enter the dental plaque, but the high molecular-weight levan, once formed, would not be able to diffuse out so readily.

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PHYSICO-CHEMICAL STUDIES ON STARCHES

PART XXXIV*. THE DISTRIBUTION OF MOLECULAR WEIGHT IN AMYLOSE SAMPLES OBTAINED BY THE LEACHING AND THE DISPERSION OF POTATO STARCH

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(Received August 21st, 1967)

ABSTRACT

Leached and total amylase from potato starch have been subfractioned by fractional precipitation. The former has been shown to have an exponential distribution of molecular weights as expected for a random *A-A* type polymer. The distribution of molecular weight for the total amylose is much broader, compatible with the presence of a branched fraction.

INTRODUCTION

Measurements of sedimentation velocity show that amylose, like most polysaccharides, occurs naturally with a wide distribution of molecular weights. However, little is known of the mathematical form of this distribution. Previously, we have reported distributions of molecular weight in various glycogen samples by an analysis of the patterns of sedimentation velocity[1], but application of this method to amylose is much more difficult, because of the concentration dependence of the sedimentation coefficient in this case. We have therefore applied the technique of fractional precipitation[2], and wish to show here the type of information, regarding the initial distribution of molecular weight, which can be obtained from the resultant data.

We have used two samples of amylose obtained by (*a*) the aqueous leaching, and (*b*) the total dispersion of potato starch[3,4]. *Leached amylose is* only a fraction of the amylose present, and has a low limiting-viscosity number, $[\eta]$, and a β-amylolysis limit of *ca.* 100%. In contrast, the *total amylose*, which is all of the butyl alcohol-complexable material in the granule, has a much higher value of $[\eta]$, but is always incompletely degraded by β-amylase; a residue of high molecular weight remains, which is completely impervious to the enzyme. We have suggested[4-6] that this enzymically resistant portion of amylose is branched, and that this branching is long-chain in nature, *i.e.*, the branch points are separated by many hundreds of D-glucose residues. This type of branching would be expected to alter profoundly the molecular-weight distribution within the amylose sample; the distribution for the total amylose should be very much wider than that for the leached material. This paper describes the results for such a comparison.

*Part XXXIII, Stärke, in the press.

EXPERIMENTAL

Isolation of the amylose samples. — Amylose was leached from potato starch at 62°, as previously described[3]. A small proportion of material that was resistant to β-amylase was removed from solution in methyl sulphoxide by fractional precipitation with butyl alcohol.

Total amylose was isolated from potato starch by aqueous dispersion of the granules[3]. The amylose was purified by re-crystallisation (three times) from hot, aqueous butyl alcohol.

Fractionation. — Dehydrated amylose was dissolved in methyl sulphoxide to give a 0.5% solution. Fractionation of the amylose was carried out by the stepwise addition of (*a*) ethanol[7] at 4° (total amylose), and (*b*) butyl alcohol[8] at 25° (leached amylose). Fractions were stirred with precipitant, dried, and weighed.

Physical measurements. — Weight-average molecular weights ($\bar{M}_w$) were obtained from light-scattering measurements[9].

Enzymic characterisation. — The measurement of β-amylolysis limits was carried out by the standard method[3].

RESULTS AND DISCUSSION

Table I shows the yields, molecular weights, and β-amylolysis limits of the different fractions in order of increasing molecular weight; also shown are the

TABLE I

FRACTIONATION DATA FOR LEACHED AND TOTAL AMYLOSE

Leached amylose					*Total amylose*				
Fraction	*Yield* (% *wt*)	$W(M)^a$	$\bar{M}_w \times 10^{-6}$	$[\beta]^b$	*Fraction*	*Yield* (% *wt*)	$W(M)^a$	$\bar{M}_w \times 10^{-6}$	$[\beta]^b$
1	8.5	0.042	0.084	101	1	7.4	0.037	0.160	100
2	8.5	0.127	0.214	100	2	10.8	0.126	0.350	100
3	7.5	0.207	0.321	100	3	16.8	0.266	0.800	98
4	6.8	0.279	0.382	101	4	14.4	0.423	1.05	67
5	7.5	0.350	0.427	99	5	12.2	0.557	1.70	98
6	7.0	0.423	0.461	98	6	9.8	0.667	2.29	98
7	11.0	0.513	0.489	101	7	6.1	0.747	3.00	75
8	10.1	0.618	0.612	101	8	7.5	0.815	3.51	88
9	10.2	0.720	0.753	101	9	6.8	0.886	6.75	74
10	8.4	0.813	0.944	99	10	8.0	0.960	14.4	60
11	8.2	0.893	1.22	100	Original	—	—	2.57	85
12	6.3	0.968	1.74	99					
Original	—	—	0.610	98					

[a]Cumulative yield (fractional) to the mid point of each fraction; [b][β]=β-amylolysis limit.

cumulative amounts to the mid-point of each fraction. The integral and differential molecular-weight distribution curves, obtained from these data, are shown in Fig. 1*a*

and 1*b* for the *leached amylose* and *total amylose*, respectively. These graphs suggest that the molecular-weight distribution curve for the total amylose has a "tail" of high molecular weight, which is absent from the leached amylose. In order to confirm this difference, the data were fitted to various mathematical models.

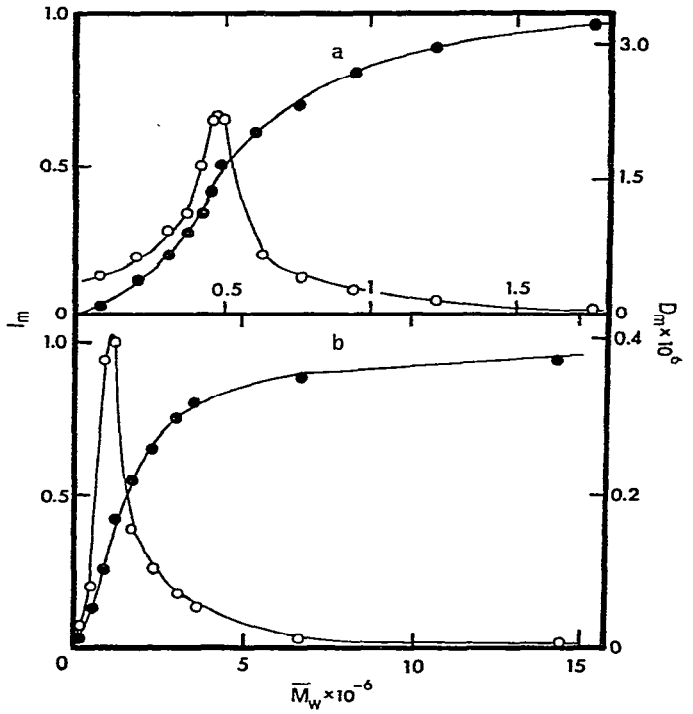

Fig. 1. The integral ($I_m = W(M)$; •) and differential ($D_m = dW(M)/dM$; o) molecular-weight distribution curves for (*a*) *leached amylose*, and (*b*) *total amylose*.

It is likely that leached amylose has an exponential (most probable) distribution of the form[10]

$$dW(M)/dM = (4M/\bar{M}_w^2) \exp(-2M/\bar{M}_w). \quad (1)$$

In this type of distribution, the ratio between successive averages is given by

$$\bar{M}_n:\bar{M}_w:\bar{M}_z = 1:2:3. \quad (2)$$

The integrated form of equation (1) is

$$[1-W(M)]/[(2M/\bar{M}_w)+1] = \exp(-2M/\bar{M}_w), \quad (3)$$

where $W(M)$ is the cumulative weight-fraction up to molecular weight M, and $\bar{M}_w$ is the weight-average molecular weight of the unfractionated starting material. The validity of equation (3) is readily shown by graphing log $\{[1-W(M)]/[2M/M_w)+1]\}$ against $(2M/\bar{M}_w)$; an exponential distribution will give a linear relation. The data for leached and total amylose are graphed in this manner in Figs. 2*a* and 2*b*, respectively.

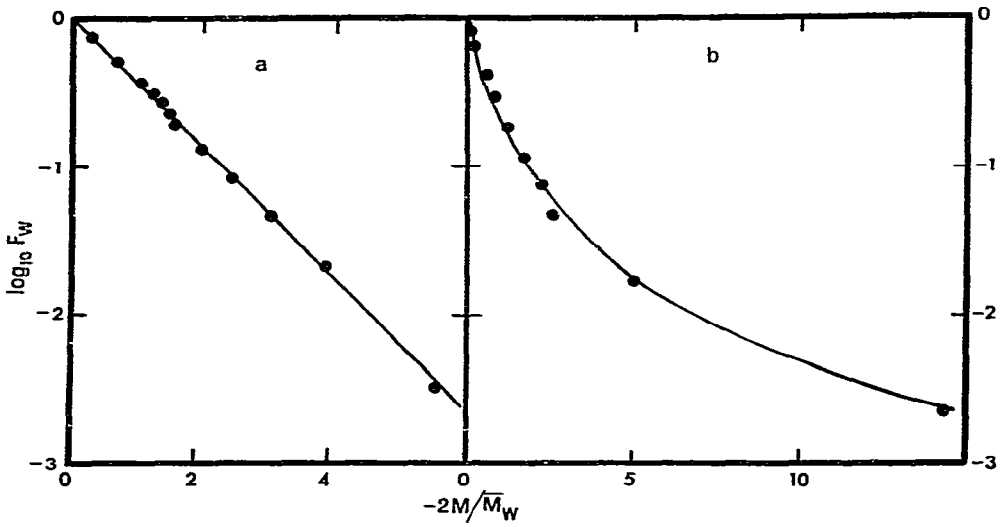

Fig. 2. Exponential distribution; graph of $\log_{10}F_w$ against $2M/M_m$ for (*a*) *leached amylose*, and (*b*) *total amylose*; $F_w=[1-W(M)]/[2M/M_w+1]$; see Text.

The correlation between the experimental results and the theoretical relation is satisfactory for the leached material, but for the total amylose there is no such correlation. It may thus be concluded that leached amylose has a molecular-weight distribution defined by equation (3), whereas total amylose has a much wider distribution. This accords well with the experimentally determined value of $\overline{M}_n$ of the leached sample, which, from an enzymic assay[11], was found to be 0.287×10^6; from equation (2), the calculated value of $\overline{M}_n$ is 0.305×10^6.

A more-generalised form of the exponential distribution has been suggested by Tung[12] for polymers having a rather wide distribution of molecular weight. The integrated form of this is given by

$$W(M)=1-\exp(-aM^b) \tag{4}$$

i.e., a plot of log $1/[1\text{-}W(M)]$ against M on logarithmic graph-paper should yield a linear relation. Such a graph is shown in Fig. 3 for the experimental data for the total amylose. It can be seen that the results cannot be fitted by unique values of a and b; the results in fact are best fitted by two straight line (Tung has also noted similar deviations in the case of some polyethylenes[13]). Thus, equation (4) does not fit the experimental data.

Another, widely applied, distribution function is that due to Wesslau[14]. The integrated form of this equation is given by

$$W(M)=(\beta\pi^{\frac{1}{2}})^{-1}\int_0^M M^{-1}\exp[-\beta^{-2}\ln^2(M/M_0)]\,dM. \tag{5}$$

A test for the applicability of this function is to plot log M *vs* $W(M)$ on probability graph-paper, when a straight line should result if the equation is obeyed. This graph

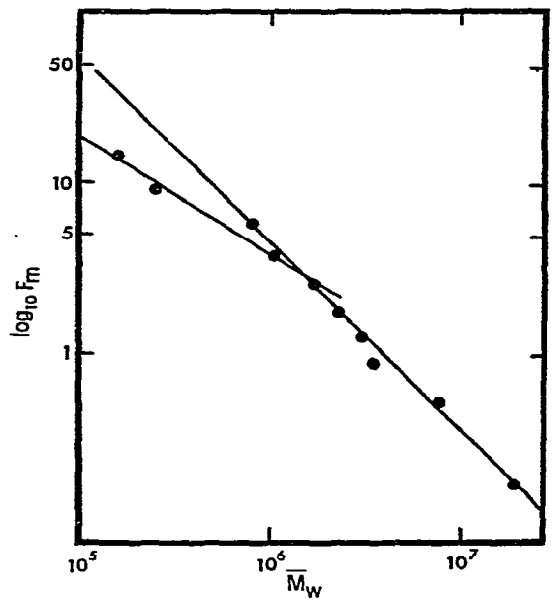

Fig. 3. Tung distribution[12] for *total amylose*; graph of $\log_{10}F_m$ against M_w; $F = \log_{10}1/[1-W(M)]$, where $W(M) = 1-\exp(-aM^b)$; see Text.

for the total amylose is shown in Fig. 4, and the data then show a reasonable correlation with equation (5).

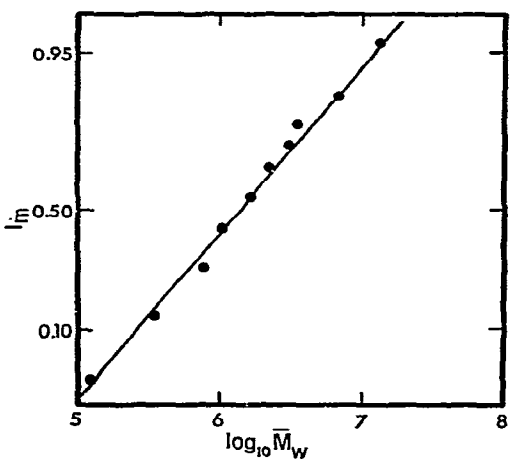

Fig. 4. Wesslau distribution[14] for *total amylose*; I_m as a function of $\log_{10}$ on probability graph-paper; $I_m = W(M)$ see Text.

A quantitative measure of the broadness of this distribution may be obtained by means of the Schulz[15] inhomogeneity factor, U, defined as

$$U = (\overline{M}_w/\overline{M}_n) - 1. \qquad (6)$$

The principal averages $\overline{M}_w$ and $\overline{M}_n$ are obtained from equation (5) as[14]

$$\overline{M}_w = M_0 \exp(\beta^2/4), \tag{7}$$

and

$$M_n = M_0 \exp(-\beta^2/4), \tag{8}$$

which on substitution in (6) gives

$$U = \exp(\beta^2/2) - 1. \tag{9}$$

The parameter, β, is defined[14] by

$$\beta = \{M[\mathrm{d}W(M)/\mathrm{d}M]\}^{-1}_{M=M_0} \cdot \pi^{-\frac{1}{2}},$$

where M_0 is the value of the molecular weight when $W(M) = 0.50$. The factor $M[dW(M)/dM]$ is evaluated from a graph of $W(M)$ against log M. For the total amylose, the values of these parameters were $M_0 = 1.45 \times 10^6$; $\beta = 1.63$. Hence, by means of equation (9), the value of U is calculated to be 2.7. The corresponding value for the leached amylose is, of course, unity.

The above results demonstrate that leached amylose has an exponential, *i.e.*, the "most probable", distribution. Total amylose, on the other hand, has a much wider distribution, which is compatible with the presence of a branched fraction.

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A NOVEL METHOD OF DETERMINING THE NUMBER-AVERAGE DEGREE OF POLYMERIZATION OF LINEAR MALTODEXTRINS AND AMYLOSE*

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ABSTRACT

An enzymic method for determining the number-average degree of polymerization ($\overline{DP}_n$) of maltodextrins and amylose is described. In this technique, the D-glucans are treated with β-amylase under conditions in which enzymic degradation is complete, *i.e.*, molecules having an even number of residues form maltose, and molecules having an odd number of residues yield maltose and one D-glucose unit. The amount of D-glucose is estimated by using glucose oxidase. The total concentration of the D-glucan itself is measured by use of amyloglucosidase. Hence, on the basis that the sample contains equal numbers of molecules having odd and even numbers of D-glucose residues, $\overline{DP}_n$ can be calculated.

Experimental conditions for this assay are discussed, and the accuracy of the technique has been confirmed by using (*a*) pure maltodextrins, and (*b*) synthetic mixtures of two degraded amyloses of $\overline{DP}_n$ *ca.* 400 and 8. The method has been found to be applicable to linear amylose; $\overline{DP}_n$ values greater than 1000 can be obtained to within 5%.

INTRODUCTION

In many investigations of amylose, particularly during α-amylolytic and acid hydrolysis, a knowledge of the number-average degree of polymerization $(\overline{DP})_n$ is extremely valuable. We have now developed a highly accurate, but experimentally simple, enzymic assay. The basis of the method is that crystalline β-amylase will quantitatively degrade any linear α-D-(1→4)-glucan to maltose and maltotriose, the maltotriose arising from molecules having an odd number of D-glucose residues. At high concentrations of the enzyme, maltotriose will itself be degraded quantitatively to D-glucose and maltose. Hence, if the amylosic sample is considered to contain an equal number of molecules having an odd and even number of D-glucose residues, the statistical probability of obtaining D-glucose on complete β-amylolysis is 0.5, and determination of the amount of this D-glucose enables the *DP* to be calculated.

*This is Part XXXV in the series "Physico-chemical Studies on Starches": for Part XXXIV, see preceding paper.

Success of this method depends on the quantitative determination of D-glucose in the presence of very large proportions of maltose. Enzymic assay with glucose oxidase[1-3] satisfies this condition. In this method, the D-glucose is oxidized to D-gluconic acid, with the production of hydrogen peroxide that is then utilised by peroxidase to oxidize *o*-dianisidine to the corresponding imine, which is determined by measuring its absorbance.

The total concentration of D-glucan in solution has also to be determined. In these laboratories, the polysaccharide concentration has been estimated routinely by acid hydrolysis[4], but more recently we have found that this hydrolysis can be carried out enzymically by using amyloglucosidase [α(D)-1,4-glucan-glucohydrolase]. Furthermore, the liberated D-glucose may be determined by using glucose oxidase.

The whole determination of *DP* can thus be performed enzymically, and we shall discuss here the necessary experimental conditions and the accuracy of the technique.

EXPERIMENTAL

Substrates. — Linear amylose was obtained by leaching of potato-starch granules[4]. Oligomers of D-glucose (G_1 = D-glucose; G_2 = maltose; G_3 = maltotriose; and G_4 to G_7) were prepared by the paper-chromatographic separation[5] of an α-amylolytic digest of amylose. The oligomers were pure as shown by paper chromatography.

Enzymes. — β-Amylase (Worthington Biochemical Corporation; *ex.* sweet potato; crystalline); glucose oxidase [Boehringer Corporation (London) Ltd.; grade GOD II]; peroxidase [Boehringer Corporation (London) Ltd.; horseradish, grade POD II]; α(D)-1,4-glucan-glucohydrolase (semi-purified preparation, *ex. Aspergillus niger*, kindly donated by Dr. I. D. Fleming, Glaxo Research Ltd.).

Preliminary experiments. (a) Enzymic impurities in the crystalline β-amylase. — To be applied successfully, our method of determining $\overline{DP}_n$ requires that the maltotriose produced during β-amylolysis of the amylosic sample is quantitatively converted into D-glucose and maltose. This hydrolysis occurs slowly under normal digest conditions, and high concentrations of enzyme have therefore to be used to complete the hydrolysis in a reasonable time. Lee and Whelan[5] have shown that certain samples of crystalline β-amylase may be contaminated with a minute proportion of maltose-splitting impurity, the effect of which can be ignored when the enzyme is used at normal concentrations, but not if employed at high concentrations. Since the effect of such an impurity would be to produce D-glucose from maltose, an anomalously low result would be obtained for the $\overline{DP}_n$. Thus, all samples of crystalline β-amylase must be checked for this impurity. This was carried out by setting up a digest containing 0.1% maltose solution (3.0 ml), 0.1M acetate buffer (pH 4.8; 0.3 ml), 4000 units[6] of crystalline β-amylase (0.4 ml), and water (0.3 ml). A control digest without the enzyme was also set up. After incubation for 48 h at 37°, 1-ml aliquots were withdrawn and assayed with glucose oxidase (see below). In the digest containing β-amylase, some 10% of D-glucose was found for one sample of β-amylase.

It has been shown[2,7] that polyhydroxy compounds act as inhibitors for this maltose-splitting, enzymic impurity. The following digest was therefore prepared:

0.3% maltose solution (1.0 ml), 0.1M acetate buffer (pH 4.8; 0.2 ml), glycerol–waert (2:1 v/v; 2.4 ml), and 4000 units of crystalline β-amylase (0.4 ml). A control digest without the enzyme was also prepared. After incubation for 48 h at 37°, no D-glucose was detected by glucose oxidase. Even after 5 days, there was still no trace of D-glucose, and so if glycerol (40% by volume) is incorporated into the digest, the only D-glucose liberated in an amylose digest will arise from a molecule having an odd number of residues.

(*b*) *Conditions for the quantitative hydrolysis of maltriose by β-amylase.* — A digest was prepared as follows: 0.1% maltotriose solution (1.0 ml), 0.1M acetate buffer (pH 4.8; 1.0 ml), glycerol–water (3:2 v/v; 7.0 ml), and 10,000 units of crystalline β-amylase (1.0 ml). The digest was incubated at 37°, and aliquots (1.0 ml) were withdrawn at different times and their D-glucose contents assayed. The results were as follows:

Time (h)	3.5	8.0	13.5	23.5	29.5	34.0
Fraction of maltotriose hydrolyzed	0.455	0.710	0.875	0.980	0.995	1.00

A quantitative yield of D-glucose was obtained in 30–34 h under these conditions. Determinations carried out after 48, 72, and 96 h gave the same result.

(*c*) *Effect of methyl sulphoxide on enzymic activity.* — The presence of methyl sulphoxide (15% by volume) was shown to have no appreciable effect on the activities of the enzymes.

Assay. (*1*) *Estimation of* D-*glucose.* — The reagent consisted of a mixture of glucose oxidase (60 mg), peroxidase (6 mg), and *o*-dianisidine dihydrochloride (40 mg) dissolved in tris buffer [200 ml, prepared from a solution of 2-amino-2-(hydroxymethyl)propane–1,3-diol (15.25 g) in 5N hydrochloric acid (21.5 ml), which was diluted to 250 ml, followed by the addition of glycerol (165 ml), and adjustment of the pH to 7]. When stored at 4° in a darkened bottle, the reagent had a useful life of 3–4 weeks.

Reagent (2 ml) was added to the sample (1 ml; containing 0–40 μg of D-glucose), and the mixture was kept for 60 min at 35°, and then acidified with 9M sulphuric acid (2 ml). The absorbance of the imine solution produced was measured with an Eel colorimeter (No. 625 filter). A typical calibration curve is shown in Fig. 1.

In our experience, the most-favourable range for using the colorimeter is between readings of 2.0 and 5.0, and when estimating concentrations, dilutions are made so that readings fall between these values. Repeated determinations on samples containing 10–25 μg of D-glucose per ml have shown errors of only ±1%.

This procedure holds for the determinations of G_T (see below). If organic solvents are present, as in the determination of G_β (see below), the calibration must be carried under exactly the same conditions (see Fig. 1).

(*2*) *Production of* D-*glucose* (G_β) *by β-amylolysis of* D-*glucan.* (*a*) *Oligomers.* — Digests were set up as follows: 0.1% oligomer solution (1 ml), 0.1M acetate buffer (pH 4.8; 1.0 ml), glycerol–water (3:2, v/v; 7.0 ml), and 10,000 units of β-amylase (1.0 ml). After incubation for 48 h at 37°, the concentration of D-glucose was assayed.

(*b*) *Amylose.* — Digests were set up as follows, to achieve the necessary high concentrations of polysaccharide: amylose (*ca.* 150 mg) was dissolved in methyl

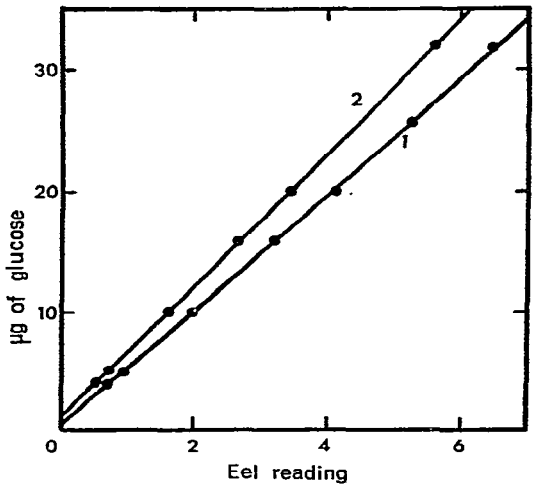

Fig. 1. Calibration curves for the determination of D-glucose by glucose oxidase under conditions for (*1*) G_T and (*2*) G_β (see text for definition of G_T and G_β).

sulphoxide (1 ml) by standing overnight at 35°. A mixture (8 ml) of glycerol (50%) and 0.05M acetate buffer (pH 4.8; 50%) was then added, and the mixture was shaken for 1 h. (It is of interest that the presence of 40% glycerol greatly stabilized this solution.) β-Amylase (1 ml; 10,000 units) was added, the digest was incubated for 48 h at 37°, and the D-glucose content was then assayed.

(*3*) *Production of* D-*glucose* (G_T) *by the total hydrolysis of* D-*glucan.* — Control experiments showed that hydrolysis was complete under the following digest conditions: a solution containing 50–400 μg of D-glucan (*ca.* 8 ml), 0.2M acetate (pH 4.6; 0.5 ml), 0.5 unit* of amyloglucosidase (0.04 ml), and water to 10 ml. After incubation for 2 h at 35°, aliquots (1 ml) were assayed for their contents of D-glucose.

(*4*) *Calculation.* — The number-average degree of polymerisation is given by $\overline{DP}_n = G_T/2G_\beta$, where G_T is the concentration of D-glucose obtained on complete hydrolysis of the sample, and G_β is the concentration of D-glucose produced on β-amylolysis.

Indirect assay of $\overline{DP}_n$ *of amylose by acid hydrolysis.* — Amylose (450 mg) was dissolved in methyl sulphoxide (5 ml) at 35°, and water (15 ml, also at 35°) was added. (For such high concentrations of amylose, a temperature >30° avoids the occurrence of aggregation.) The solution was then equilibrated to 70° and 0.5N hydrochloric acid at 70° was added. Aliquots (3 ml) were removed at different times, and were neutralized (temperature, >35°) by stirring with anion-exchange resin (Amberlite IR-45). Digests were then prepared as follows: neutralized samples (1.5 ml), glycerol (1.6 ml), 0.1M

*1 unit = amount of enzyme which liberates 1 μmole of D-glucose per min from soluble starch at pH 4.6 and 37°.

acetate buffer (pH 4.8; 0.5 ml), and 4,000 units of β-amylase (0.4 ml). After incubation for 48 h, the D-glucose contents were assayed. The total polysaccharide content was also assayed, and the $\overline{DP}_n$-value obtained. $\overline{DP}_n^{-1}$ was then graphed as a function of the hydrolysis time[8].

RESULTS

Estimation of $\overline{DP}_n$ *for maltodextrins and degraded amylose.* — In order to confirm the accuracy of this technique, we measured the $\overline{DP}_n$ of some maltodextrins. Obviously this technique is only applicable to pure oligomers having an odd number of residues. The results are shown in Table I. At this level of $\overline{DP}_n$, the accuracy of thed etermination is *ca.* ±0.5%. As expected, the oligomer having an even number of residues did not yield any D-glucose.

TABLE I

$\overline{DP}_n$ FOR OLIGOMERS OF D-GLUCOSE

Sample	G_β^a (μg/ml)	G_T^a (μg/ml)	*Experimental* $\overline{DP}_n$
G_4	0	96	—
G_5	23.1	117	5.07
G_7	14.1	98	6.95

[a] G_β and G_T are as defined in the Text.

Table II shows a comparison of the experimental and calculated $\overline{DP}_n$ obtained on mixing different amounts of two acid-degraded amylose samples which had an enzymically-determined $\overline{DP}_n$ of 386 (*A*) and 8.0 (*B*). The weight fractions of *A* and *B*

TABLE II

$\overline{DP}_n$ FOR MIXTURES OF ACID-DEGRADED AMYLOSE

W_A[a]	W_B[a]	*Experimental* $\overline{DP}_n$	*Calculated* $\overline{DP}_n$[b]
1.000	0.000	386	—
0.946	0.054	110	109
0.895	0.105	63	65
0.781	0.219	32	34
0.486	0.514	14.5	15.2
0.000	1.000	8.0	—

[a] Weight fraction of component; [b] See Text.

in the mixture are represented by W_A and W_B, respectively. The theoretical value of $\overline{DP}_n$ for each mixture has been calculated from the standard expression for number-average values, *i.e*, $\overline{DP}_n = 1/\Sigma(w_i/DP_i)$, where w_i and DP_i are the weight fraction and

degree of polymerization of any particular species, *i*. In all cases, there is excellent agreement between the experimental and theoretical results.

Estimation of $\overline{DP}_n$ *for undegraded, linear amylose.* — The $\overline{DP}_n$ of a sample of undegraded, linear amylose was determined directly, as described in the Experimental section. Five digests were prepared containing 12–17 mg of amylose per ml. The values of $\overline{DP}_n$ were 1,660, 1,740, 1,700, 1,610, and 1,760, to give an average of 1,690 $\pm$80. We feel that this agreement is better than can be achieved by any other direct method.

No other independent measure of the number-average size of this sample of amylose has been made, but light-scattering measurements have given a weight-average value (DP_W) of 3,500. Since leached amylose has a "most probable" distribution in molecular size[9], the ratio $\overline{DP}_W/\overline{DP}_n$ is 2. Thus, the calculated value of $\overline{DP}_n$ is 1,750, in good agreement with the experimental value.

It is, of course, mandatory with this technique that the amylose samples are *completely* degraded by β-amylase. The presence of branched amylose (as shown by incomplete β-amylolysis) would involve the introduction of an arbitrary correction factor, which would make determinations less meaningful.

The size of a linear amylose can also be obtained indirectly by hydrolysis and measurement of $\overline{DP}_n$ at various times, if there are no weak bonds in the polymer[8]. We have shown elsewhere[8] that a graph of $\overline{DP}_n^{-1}$ against time will then allow extrapolation to be made to zero time, to obtain the initial degree of polymerization. Fig. 2 shows the results of such a determination for the same sample of linear amylose.

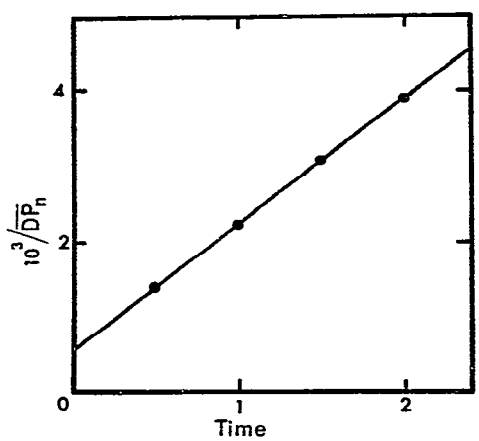

Fig. 2. Graph of $\overline{DP}_n^{-1}$ as a function of time (h) for amylose hydrolyzed by acid.

The extrapolated value of $\overline{DP}_n$ of 1,670 $\pm$140 agrees with that obtained directly; the larger error is inherent in the extrapolation procedure.

It is expected, therefore, that this technique will have many applications in studies of the α-amylolytic and acidic hydrolysis of amylosic materials.

ACKNOWLEDGMENT

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SYNTHESIS OF VARIOUS GLYCOSIDES OF 2-AMINO-3-*O*-(D-1-CARBOXYETHYL)-2-DEOXY-D-GLUCOPYRANOSE (MURAMIC ACID)*

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ABSTRACT

Methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranoside, and methyl, benzyl, and *p*-nitrophenyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside were condensed with DL-2-chloropropionic acid to give, in preponderant yields (64, 46, 68, and 25%, respectively), the respective 3-*O*-(D-1-carboxyethyl) derivatives, separated as their methyl esters. Only from the condensation of the methyl α-D-glucoside was the separation of a significant proportion (11% yield) of the 3-*O*-(L-1-carboxyethyl) derivative achieved. Acetolysis of methyl 2-acetamido-4,6-di-*O*-acetyl-2-deoxy-3-*O*-[(D-1-methoxycarbonyl)ethyl]-α-D-glucopyranoside gave an oxazoline; this was treated with hydrobromic acid in acetic acid, and then with silver oxide and methanol, to give a methyl β-D-glucoside derivative identical with that obtained from methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside. Benzyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-[D-1-(methoxycarbonyl)ethyl]-β-D-glucopyranoside was also obtained by condensation with pure L-chloropropionic acid. Benzyl and *p*-nitrophenyl 2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-β-D-glucopyranoside were resistant to the action of egg-white lysozyme.

INTRODUCTION

The synthesis of 2-amino-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose (muramic acid), a constituent of the cell wall of bacteria[2], is based on the formation of an ether link between D-lactic acid and the hydroxyl group at C-3 of 2-amino-2-deoxy-D-glucose. Most syntheses[3–8] start from the sodium salt (at O-3) of the methyl[3–6], ethyl[4], or benzyl[7,8] glycoside of 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-gluco-

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pyranose. Condensation with a racemic 2-halopropionic acid derivative leads to the formation of both the D- and L-(1-carboxyethyl) derivatives (muramic acid and isomuramic acid, respectively[3,4,8]).

In the present work, we investigated the influence of the chemical constitution of the aglycon group, and of the isomerism at C-1, on the relative proportion of muramic and isomuramic acid derivatives obtained by condensation with racemic 2-chloropropionic acid. The starting materials were methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranoside and methyl, benzyl, and *p*-nitrophenyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside. The synthesis starting from the benzyl α-D-glycoside has been reported previously[8].

An additional motive for the preparation of various glycosides of muramic acid was an attempt to find synthetic, easily prepared substrates for egg-white lysozyme. This enzyme, which has been classified[9] as a "muramidase", was found to split the glycosidic linkage of the 2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-β-D-glucopyranosyl residues of the peptidoglycan that forms the backbone of the bacterial cell-wall[10].

DISCUSSION

Condensation of the sodium salt of methyl 2-acetamido-4,6-*O*-benzylidene-α-D-glucopyranoside (**1**) with a derivative of a DL-2-halopropionic acid was the main step in the original preparation[3] of muramic acid. This method has the advantage of avoiding the tedious separation of the isomers of the 2-halopropionic acid[6], or their respective preparation from the costly D- or L-alanine[5]. Its disadvantage is the low yield of the final product; this probably results from the separation of muramic acid from isomuramic acid on ion-exchange resins at the last stage of the preparation. A better yield of muramic acid has been obtained by separating the glycosides of muramic and isomuramic acid on a charcoal column[4]. Thus, it was of interest to study the separation one step earlier in the preparation, namely, at the stage of the 4,6-*O*-benzylidene derivatives, to take advantage of the separative effect of adsorption chromatography in an organic medium. In this type of separation, amounts of substance larger than those separated by adsorption on charcoal or on ion-exchange resin can be manipulated.

Since 2-chloropropionic acid has been shown[5] to give higher yields than the other 2-halopropionic acids, condensation of DL-2-chloropropionic acid with the sodium salt of **1** was first studied. The condensation was followed by esterification, with diazomethane, of the resulting mixture of 3-*O*-(1-carboxyethyl) derivatives (**3** and **6**). Separation on a silica gel column showed that the 3-*O*-[D-1-(methoxycarbonyl)-ethyl] ester **4** had been formed in preponderant amount, as compared to the 3-*O*-[L-1-(methoxycarbonyl)ethyl] ester **7**. A similar observation has been reported[8] for the condensation of the racemic 2-chloropropionic acid with benzyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranoside (**2**).

Condensation of DL-2-chloropropionic acid with the sodium salt of methyl (**9**), benzyl (**10**), and *p*-nitrophenyl (**11**) 2-acetamido-4,6-*O*-benzylidene-β-D-glucopyrano-

side gave almost exclusively the corresponding 3-*O*-(D-1-carboxyethyl) derivatives, characterized as the methyl esters **12**, **15**, and **16**, respectively. The yield of the benzyl β-D-glycosides (**14** and **15**) was 68%, whereas that of the methyl β-D-glycosides (**12**) was 40%, and that of the *p*-nitrophenyl β-D-glycoside (**16**) only 25% (30%, if the recovered starting material is considered). However, our inability to isolate significant amounts of the 3-*O*-(L-1-carboxyethyl) derivatives of the β-D anomers does not indicate with certainty that these derivatives are formed in very small proportions. Re-investigation, by thin-layer chromatography, of the product of condensation of benzyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranoside (**2**), which had previously[8] been thought to afford mainly the 3-*O*-(D-1-carboxyethyl) derivative (**5**) and traces of the 3-*O*-(L-1-carboxyethyl) derivative, has shown, indeed, that the latter compound may be formed in a proportion up to 20%. It was also found that the respective yields of both isomers were variable, because of the difficulty in exactly reproducing the conditions of the condensation in a two-phase system[11]. In addition, the 3-*O*-(L-1-carboxyethyl) derivatives of the β-D-glycoside series show very low solubilities in most solvents, and it is probable that these derivatives crystallize in the column during chromatographic separation. Finally, the conditions of the condensation are quite drastic, and this may, in part, explain the low yield resulting from the condensation of the (less stable) *p*-nitrophenyl β-D-glucoside **11**. Despite the great variations in the yield of the products obtained, it is nevertheless possible to conclude that the condensation reaction with DL-2-chloropropionic acid is stereoselective for all of the glucosides investigated, and that the 3-*O*-(D-1-carboxyethyl) derivatives are obtained in preponderant yield.

The compounds obtained by condensation of **1** with DL-2-chloropropionic acid were identified by comparison with the compound obtained by condensation of **1** with L-2-chloropropionic acid[5,6] and D-2-chloropropionic acid[5], respectively.

Roth and Pigman[12] have described the preparation of methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (**9**) by methylation of 2-acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-glucose in aqueous solution. Re-investigation of this reaction showed that the product **9** previously described contained as an impurity the α-D anomer; purification could not be achieved by fractional recrystallization, but only by chromatography. The physical constants of the product thus obtained were in good agreement with those of the compound formed by alkaline hydrolysis of methyl 2-acetamido-3-*O*-benzoyl-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside[13]. Condensation of methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (**9**) with DL-2-chloropropionic acid led to the isolation of only one isomer (**12**). This compound gave, after alkaline hydrolysis, the acid (**13**), which had the same properties as those of the compound described by Matsushima and Park[5]. Further proof of the configuration of the lactyl residue of **12** was obtained by acetolysis of methyl 2-acetamido-4, 6-di-*O*-acetyl-2-deoxy-3-*O*-[D-1-(methoxycarbonyl)ethyl]-α-D-glucopyranoside[6] (**22**), followed by treatment with hydrogen bromide in methanol in the presence of silver oxide. Although the crystalline product resulting from the acetolysis was obtained in 67% yield and had an elementary analysis corresponding to that of a

2-acetamido-tri-*O*-acetyl-2-deoxy-3-*O*-[(methoxycarbonyl)ethyl]hexose, its i.r. spectrum was that of an oxazoline structure, and therefore structure **23** was attributed to this product. Oxazolines have been obtained in the past by treatment of *N*-benzoyl derivatives of acetylated hexosamines with hydrobromic acid in glacial acetic acid[14] and with aluminum chloride[15], and, more recently, by treatment of *N*-acetyl derivatives with acetic anhydride and zinc chloride[16], but oxazolines obtained by treatment of *N*-acetyl derivatives with an acetolysis mixture (acetic anhydride, acetic acid, and sulfuric acid) have not hitherto been reported. Treatment of the oxazoline **23** with hydrobromic acid in glacial acetic acid, and then with methanol in the presence of silver oxide gave the acetylated methyl β-D-glucoside **17**. This compound was identical with the compound obtained from **12** by removal of the benzylidene group followed by acetylation. Definite identification of the oxazoline **23** could not be achieved by treatment with hydrated *p*-toluenesulfonic acid in methanol, as described by Pravdić, Inch, and Fletcher[16], because application of these reagents to methyl 2-acetamido-4,6-di-*O*-acetyl-2-deoxy-3-*O*-[D-1-(methoxycarbonyl)ethyl]-α-D-glucopyranoside (**22**) resulted in rapid de-*O*-acetylation, to give a compound moving, on thin-layer chromatograms, similarly to methyl 2-acetamido-4-*O*-acetyl-2-deoxy-3-*O*-[D-1-(methoxycarbonyl)ethyl]-α-D-glucopyranoside[6]. Direct treatment of the α-D-glucoside **22** with hydrobromic acid in glacial acetic acid, followed by condensation with methanol in the presence of silver oxide, gave unchanged starting-material **22**.

For the preparation of benzyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (**10**), the intermediate benzyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside (**21**) was obtained from 2-acetamido-1,3,4,6-tetra-*O*-acetyl-D-glucopyranose *via* the corresponding α-D-glucopyranosyl bromide (which was not isolated). Treatment of this bromide with the Koenigs–Knorr reagent had been reported to give syrupy products, and use of mercuric cyanide as a catalyst was recommended[17]. Modification of the Koenigs–Knorr procedure led to the crystalline glycoside (**21**) previously described[17]. Condensation of the benzyl glycoside (**10**) with DL-2-chloropropionic acid gave, in addition to the expected 3-*O*-(D-1-carboxyethyl) derivative **14**, a compound having a higher m.p. which may be the 3-*O*-(L-1-carboxyethyl) isomer. Identification of the configuration of the lactyl group of **14** was obtained by condensation of **10** with L-2-chloropropionic acid. Removal of the benzylidene group of **14** gave benzyl 2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-β-D-glucopyranoside (**19**), which was characterized by the crystalline 4-(cyclic ester) **24** and tested as a substrate for lysozyme.

p-Nitrophenyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (**11**) was prepared from the known *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside[18]. The yield of product from its condensation with DL-2-chloropropionic acid could not be raised above 25%, and the product was quite unstable. Because the final product **20**, obtained by removal of the benzylidene group from **16**, was shown not to be a substrate for lysozyme, further study of the condensation reaction was not pursued.

Both benzyl (**19**) and *p*-nitrophenyl (**20**) 2-acetamido-3-*O*-(D-1-carboxyethyl)-

2-deoxy-β-D-glucopyranoside were investigated as substrates for egg-white lysozyme. These aglycons were selected because (*a*) numerous studies[19] had shown that aryl aglycons are more reactive with hydrolases than are alkyl aglycons, and (*b*) the products of the reaction, benzyl alcohol and *p*-nitrophenol, can be readily detected on chromatograms. In addition, *p*-nitrophenol can be determined colorimetrically and, thus, the kinetics of the enzyme reaction may be studied. The β-D-glycosides were selected because, for chitin, egg-white lysozyme splits a 2-acetamido-2-deoxy-β-D-glucosyl linkage[20] which is assumed to be of the same type as that of the peptidoglycan of bacterial cell-wall[21]. No reaction of compounds **19** and **20** could be detected with egg-white lysozyme after an incubation period of up to 48 h, under the conditions known to be optimal for the action of the enzyme[22]. Thus, it may be concluded that egg-white lysozyme is not a "muramidase" of wide specificity, but rather an "endo-hexosaminidase" acting on substrates having three or more monosaccharide units. A similar conclusion had been reached by Matsushima and associates, who synthesized phenyl[23] and *p*-aminophenyl[24] 2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-β-D-glucopyranoside and observed their resistance to degradation by lysozyme.

EXPERIMENTAL

Melting points were determined on a hot stage equipped with a microscope, and correspond to "corrected melting points". Rotations were determined with a polarimeter equipped with a Rudolph photoelectric polarimeter attachment Model 200, or with the Perkin–Elmer No. 141 polarimeter. The chloroform used was A. R. grade and contained approximately 0.75% of ethanol. Infrared spectra were recorded, for potassium bromide discs, with a Perkin–Elmer spectrophotometer Model 237. The homogeneity of the compound synthesized was determined by chromatography on plates covered with a thin layer of a 3:1 mixture of silica gel G (Merck) and silica gel GF (Merck). Column chromatography was performed on "Silica Gel Davison", from the Davison Co., Baltimore, Maryland 21201 (grade 950, 60–200 mesh), which was used without pretreatment. When deactivation by contact with moist air occurred, reactivation was conducted by heating to 170–200° (manufacturer's instructions). The sequence of eluents was hexane, benzene (or 1,2-dichloroethane), ether, ethyl acetate, acetone, and methanol, individually or in binary mixtures. The ratio of weight of substance to weight of adsorbent was 1:50 to 1:100. The ratio of weight of substance (in g) to volume of fraction of eluent (in ml) was 1:100. The ratio of diameter to length of the column was 1:20. Evaporations were conducted *in vacuo*, with the bath temperature below 45°. Volumes of volatile solvent smaller than 20 ml were evaporated under a stream of dry nitrogen. The microanalyses were performed by Dr. M. Manser, Zurich, Switzerland.

Condensation of DL-*2-chloropropionic acid with methyl 2-acetamido-4,6-O-benzylidene-2-deoxy-α-*D-*glucopyranoside* (**1**). — A solution of dry **1** (5.0 g)[25] in *p*-dioxane (450 ml, freshly distilled in presence of sodium) was kept at 60–70°. Under vigorous stirring and protection from moisture, a suspension of sodium hydride (2.5 g) in dry *p*-dioxane (25 ml) [prepared from a commercially available suspension of the

hydride in oil (Alfa Inorganics, Beverly, Massachusetts)] was added during 10 min. After 15 min of stirring, a solution of freshly distilled DL-2-chloropropionic acid (16.4 g) in dry *p*-dioxane (50 ml) was slowly added, and the mixture was stirred for one h at 60–70°. A suspension of sodium hydride (10 g) in dry *p*-dioxane (50 ml) was then slowly added at 40°, the mixture was treated with water (75–100 ml) to decompose the excess sodium hydride, and the solution was concentrated to a syrup, which was dissolved in water (100 ml). Unreacted starting material and traces of oil (from the sodium hydride suspension) were removed by washing the solution with chloroform. To the aqueous layer was then added 300 ml of chloroform, ice to maintain the temperature at 0°, and 3M hydrochloric acid (about 75 ml) in small portions with vigorous shaking between additions, until pH 3 was reached. The chloroform layer was washed 3 times with ice-cold water, dried (sodium sulfate), and evaporated to dryness. The residue was dissolved in methanol (300 ml), and the solution was treated with a solution of diazomethane (6 g) in ether–ethanol. After 30 min, the solution was evaporated, and the residue was dissolved in 1,2-dichloroethane and chromatographed on silica gel. Elution with 1,2-dichloroethane–ether (1:1) gave two crystalline compounds. Intermediate fractions containing a mixture of these compounds were rechromatographed. After recrystallization from acetone, the material first eluted gave 4.54 g (71%) of methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-[D-1-(methoxycarbonyl)ethyl]-D-glucopyranoside (**4**); m.p. 211–212°, $[\alpha]_D^{25}$ +115° (*c* 0.58, chloroform), showing no depression of m.p. on admixture with the compound previously described[6], and an identical i.r. spectrum.

The second compound eluted with the same solvent mixture gave, after crystallization from acetone, 0.68 g (11%) of methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-[L-1-(methoxycarbonyl)ethyl]-α-D-glucopyranoside (**7**), as long needles, m.p. 267–270°, $[\alpha]_D^{18}$ +33° (c 0.48, chloroform).

Anal. Calc. for $C_{20}H_{27}NO_8$: C, 58,67; H, 6.65; OCH_3, 15.16. Found: C, 58.65; H, 6.63; OCH_3, 15.36.

Treatment of the L isomer **7** with sodium hydroxide in methanol gave, in 83% yield, methyl 2-acetamido-4,6-*O*-benzylidene-3-*O*-(L-1-carboxyethyl)-2-deoxy-α-D-glucopyranoside (**6**), m.p. 283–286°, $[\alpha]_D^{18}$ +39° (*c* 0.47, ethanol); lit.[5] m.p. 280–3°, $[\alpha]_D^{25}$ +39.1° (*c* 0.20, ethanol).

*Methyl 2-acetamido-2-deoxy-3-*O*-[-L-1-(methoxycarbonyl)ethyl]-α-D-glucopyranoside* (**8**). — Treatment of compound **7** (150 mg) with 60% acetic acid (5 ml) for 30 min at 100° was followed by evaporation to dryness in the presence of abs. toluene. The residue was dissolved in chloroform, and chromatographed on silica gel. Crystalline fractions eluted with 2:1 ethyl acetate–acetone were recrystallized from acetone to give short needles (74% yield), m.p. 165–168°, $[\alpha]_D^{24}$ +36° (*c* 0.45, chloroform).

Anal. Calc. for $C_{13}H_{23}NO_8$: C, 48.59; H, 7.22; OCH_3, 19.32. Found: C, 48.53; H, 7.17; OCH_3, 19.20.

*Methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside* (**9**). — Methyl glycosidation of 2-acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranose

in water was performed as described by Roth and Pigman[12], and gave a product having m.p. 273–6°; $[\alpha]_D^{24}$ −83° (*c* 0.25, methanol), −68° (*c* 0.60, methyl sulfoxide); lit.[12] m.p. 278–9°, $[\alpha]_D^{20}$ −63.8° (*c* 0.5, methyl sulfoxide). Part of this product (6.0 g) was dissolved in chloroform and chromatographed on silica gel. Elution with pure ethyl acetate gave 850 mg of crude methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranoside. Further elution with ethyl acetate gave 4.3 g of compound **9**, with some intermediate fractions containing a mixture of both anomers. Recrystallization of **9** gave 3.27 g of needles, m.p. 298–300°, with sublimation starting at 285°; $[\alpha]_D^{24}$ −95° (*c* 0.33, methanol), $[\alpha]_D^{24}$ −82° (*c* 0.53, methyl sulfoxide). The product did not show a depression in m.p. on admixture with the product described below.

A solution of methyl 2-acetamido-3-*O*-benzoyl-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside[13] (50 mg) in 0.2M barium methoxide (2 ml) was kept overnight at 0°. After neutralization with sulfuric acid and evaporation of the solution, the residue resulting was extracted with methanol. Crystallization from the same solvent gave 23 mg of long needles, m.p. 306–308°, $[\alpha]_D^{27}$ −98° (*c* 0.27, methanol).

Anal. Calc. for $C_{16}H_{21}NO_6$: C, 59.43; H, 6.55. Found: C, 59.43; H, 6.37.

Acetylation of **9** with acetic anhydride and pyridine gave the 3-*O*-acetyl derivative, m.p. 304–305°, $[\alpha]_D^{30}$ −94° (*c* 0.43, chloroform); lit. m.p. 158°, $[\alpha]_D$ −12.9° (*c* 0.9, chloroform)[26]; m.p. 300–301°, $[\alpha]_D$ −95.1° (*c* 0.48, chloroform)[27].

*Methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-[D-1-(methoxycarbonyl)ethyl]-β-D-glucopyranoside* (**12**). — The condensation of methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (**9**) (3.0 g) with DL-2-chloropropionic acid was performed as described for the α-D anomer, with the following modifications. Because of the low solubility of the starting material and final products, the proportions of *p*-dioxane and of chloroform, used for the reaction and for the extraction, were increased by a factor of 1.5–2. A small amount of starting material (45 mg) was recovered from the first chloroform extract. The residue obtained after esterification (2.09 g) was chromatographed on silica gel, and crystalline fractions were eluted with 1,2-dichloroethane–ether (1:1). Recrystallization from acetone gave 1.75 g (46%) of long needles, m.p. 282–285°, $[\alpha]_D^{22}$ −22° (*c* 0.42, chloroform). All of the crystalline fractions eluted from the column were homogeneous, as shown by optical rotation and by t.l.c. on silica gel.

Anal. Calc. for $C_{20}H_{27}NO_8$: C, 58.67; H, 6.65; OCH_3, 15.16. Found: C, 58.72; H, 6.83; OCH_3, 15.01.

Treatment of **12** with sodium hydroxide in methanol gave methyl 2-acetamido-4,6-*O*-benzylidene-3-*O*-(D-1-carboxyethyl)-2-deoxy-β-D-glucopyranoside (**13**) in 80% yield, after two recrystallizations from acetone; small needles, m.p. 277–280°, $[\alpha]_D^{24}$ −8.0° (*c* 0.81, ethanol); lit.[5] m.p. 277–280° (dec.), $[\alpha]_D^{25}$ −79° (*c* 0.17, ethanol).

*Methyl 2-acetamido-4,6-di-*O*-acetyl-2-deoxy-3-*O*-[D-1-(methoxycarbonyl)ethyl]-β-D-glucopyranoside* (**17**) *from* **12**. — Treatment of compound **12** (50 mg) with 60% acetic acid for 30 min at 100°, followed by evaporation, and acetylation with acetic anhydride (2 ml) and pyridine (3 ml) overnight, gave, after crystallization from acetone–ether, needles (32 mg, 65%), m.p. 174–176°, $[\alpha]_D^{23}$ +42° (*c* 0.19, chloroform).

Anal. Calc. for $C_{17}H_{17}NO_{10}$: C, 50.37; H, 6.71. Found: C, 50.29; H, 6.56.

2-Phenyl-4,5-[3,4,6-tri-O-acetyl-D-glucopyrano]-2-oxazoline (**23**). — A solution of methyl 2-acetamido-4,6-di-*O*-acetyl-2-deoxy-3-*O*-[D-1-(methoxylcarbonyl)ethyl]-α-D-glucopyranoside (**22**, 1.0 g)[22] in a mixture of acetic anhydride (15 ml), acetic acid (10 ml), and conc. sulfuric acid (0.175 ml) was kept for 48 h at room temperature, and extracted with ice-cold chloroform (25 ml). The organic layer was washed 3 times with ice-cold, saturated sodium hydrogen carbonate solution and 3 times with ice-cold water, dried (sodium sulfate), and evaporated. After a few days, the residue crystallized, and recrystallization from ether gave needles (302 mg, 67%), m.p. 96–97° $[\alpha]_D^{24}$ +122° (*c* 0.45, chloroform); ν_{max}^{KBr} 1670 (C=N), 1725, and 1740 (OAc) cm^{-1}; no absorption band in the regions 1460–1670 and 3000–3600 (amide) cm^{-1}.

Anal. Calc. for $C_{18}H_{27}NO_{11}$: C, 49.88; H, 6.28; N, 3.23. Found: C, 50.00; H, 6.40; N, 3.30.

Methyl 2-acetamido-4,6-di-O-acetyl-2-deoxy-3-O-[D-1-(methoxycarbonyl)ethyl]-β-D-glucopyranoside (**17**) *from* **23**. — A solution of **23** (150 mg) in glacial acetic acid presaturated at 0° with hydrobromic acid (2 ml) was kept in the dark for 6 h at room temperature, and then diluted with chloroform (10 ml). The solution was rapidly washed twice with ice-cold water, twice with ice-cold, saturated sodium hydrogen carbonate solution, and twice with ice-cold water, and dried (sodium sulfate). To the solution was added anhydrous sodium sulfate (0.8 g), freshly prepared silver oxide (0.75 mg), methanol (1.5 ml), and a crystal of iodine, according to Inouye *et al.*[28], and the suspension was shaken for 2 days in the dark, and filtered. The filtrate was washed twice with water, dried (sodium sulfate), and evaporated, giving a residue which was crystallized from acetone–ether to give 51 mg (38%) of sharp needles, m.p. 174–176°, $[\alpha]_D^{25}$ +42° (*c* 0.19, chloroform), The i.r. spectrum was similar to that of the product previously described, and on admixture, the m.p. was not depressed.

Anal. Calc. for $C_{17}H_{27}NO_{10}$: C, 50.37; H, 6.71; OCH_3, 15.31. Found: C, 50.29; H, 6.85; OCH_3, 15.21.

Benzyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranoside. — 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl bromide was prepared from 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-D-glucopyranose (5.0 g) according to Inouye *et al.*[28]. The resulting chloroform solution (200 ml) was dried with sodium sulfate and filtered, and used immediately. Anhydrous sodium sulfate (15 g), benzyl alcohol (5.0 g), silver oxide (10 g), and iodine (0.2 g) were added, and the mixture was shaken for 60 h at room temperature in the dark, and then filtered. The residue was washed with alcohol-free chloroform, and the filtrate and washings were combined, washed with water, dried (sodium sulfate), and evaporated. The residue was triturated with ether, to give a gel which was filtered off, and washed exhaustively with ether. Recrystallization from boiling water gave 2.25 g (42%) of needles, m.p. 167–8°, $[\alpha]_D^{20}$ −44° (methanol); lit.[17] m.p. 165–7°, $[\alpha]_D$ −43.3° (methanol).

Anal. Calc. for $C_{21}H_{27}NO_9$: C, 57.66; H, 6.22. Found: C, 57.69; H, 6.26.

Benzyl 2-acetamido-4,6-O-benzylidene-2-deoxy-β-D-glucopyranoside (**10**). — Treatment of benzyl 2-acetamido-3,4,6-tri-*O*-acetyl-β-D-glucopyranoside with barium

methoxide in methanol at 0°, followed by removal of the barium ions with Dowex-50, gave, in 80% yield, benzyl 2-acetamido-2-deoxy-β-D-glucopyranoside having physical constants similar to those of the product described by Kuhn and Kirschenlohr[17]. This product (2.0 g) was shaken for 24 h at room temperature with benzaldehyde (20 ml) and freshly fused zinc chloride (200 mg). Hexane (30 ml) and water (30 ml) were added, the mixture was shaken for 1 h, and the supernatant liquor was discarded. The addition and removal of hexane and water was repeated twice, and the resulting precipitate was filtered off, and washed exhaustively with water and then with hexane. It was recrystallized from methanol and from methanol–water to give needles (1.60 g, 62%), m.p. 266–8°, $[\alpha]_D^{20}$ −64° (*c* 0.55, methanol). This product was identical with the product described previously[29].

Anal. Calc. for $C_{22}H_{25}NO_6 \cdot H_2O$: C, 63.30; H, 6.52. Found: 63.87; H, 6.50.

Condensation of benzyl 2-acetamido-4,6-O-benzylidene-2-deoxy-β-D-glucopyranoside (**10**) *with* DL-*2-chloropropionic acid.* — Condensation of compound **10** (1.5 g), prepared as already described or as reported by Gross and Jeanloz[29], with DL-2-chloropropionic acid was performed as described for the preparation of **12**. Extraction of the alkaline phase with chloroform resulted in the recovery of some starting material (55 mg). After acidification of the aqueous solution and extraction with chloroform, the aqueous phase contained, in suspension, an insoluble material which was filtered off, washed with water, and dried (0.60 g). It was recrystallized twice from abs. ethanol to give benzyl 2-acetamido-4,6-*O*-benzylidene-3-*O*-(D-1-carboxyethyl)-2-deoxy-β-D-glucopyranoside (**14**), needles, m.p. 264–5°; $[\alpha]_D^{20}$ −52° (*c* 0.41, ethanol).

Anal. Calc. for $C_{22}H_{22}NO$: C, 63.68; H, 6.20. Found: C, 63.59; H, 6.21.

The chloroform extract of the acid phase was evaporated, and the residue was dissolved in methanol. The solution was treated with diazomethane, and evaporated, and the residue (0.80 g) was dissolved in 1,2-dichloroethane and chromatographed on silica gel. 1,2-Dichloroethane–ether (4:1) eluted crystalline fractions (0.62 g), which were recrystallized from acetone–methanol to give benzyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-[D-1-(methoxycarbonyl)ethyl]-β-D-glucopyranoside (**15**), long needles, m.p. 250–1° (sublimation at 205°); $[\alpha]_D^{18}$ −51° (*c* 0.37, chloroform). The same compound was obtained by esterification of **14** (described above) with diazomethane.

Anal. Calc. for $C_{26}H_{31}NO_8$: C, 64.32; H, 6.44; N, 2.88; OCH_3, 6.39. Found: C, 64.38; H, 6.64; N, 3.08; OCH_3, 6.89.

The crystalline fractions next obtained by elution with 1,2-dichloroethane–ether (4:1) were recrystallized from ethanol–acetone to give 12 mg of small needles, m.p. 281–2° (sublimation at 230°), not further investigated. Pure ether and 9:1 ether–ethyl acetate eluted the crystalline starting material (50 mg). The yield of D-1-carboxyethyl derivatives (**14** and **15**) was 68%, and that of recovered starting material was 7%.

Condensation of benzyl 2-acetamido-4,6-O-benzylidene-2-deoxy-β-D-glucopyranoside (**10**) *with* L-*2-chloropropionic acid.* — The procedure used for this condensation was a modification of that just described for condensation with DL-chloropropionic acid. To a solution of compound **10** (1.6 g) in dry *p*-dioxane (100 ml), at 95°, was added in small portions a suspension (50%) of sodium hydride (0.9 g) in mineral oil.

The mixture was stirred for 7 h at 95°, and was then cooled to 65°. L-2-Chloropropionic acid (prepared by a modification[30] of the method of Fischer and Raske[31] for D-2-bromopropionic acid) (1.75 ml) in dry *p*-dioxane (10 ml) was added dropwise to the mixture; after 1 h, a 50% suspension of sodium hydride (3.7 g) was added in small portions, and the mixture was stirred overnight at 65°. The mixture was cooled to 0°, and treated with water (50 ml), and the upper, organic layer was decanted, filtered, and partially concentrated (about 100 ml was evaporated). Water (25 ml) was added, and the solution was washed with chloroform, cooled to 0°, and acidified with 6M hydrochloric acid (about 1 ml) to pH 3. The resulting precipitate was immediately filtered off, carefully washed with cold water, and dried in a desiccator. Recrystallization from methanol gave 1.1 g (58%), m.p. 264–5°, $[\alpha]_D^{20}$ −76° (*c* 0.40, pyridine), which was identical with compound **14** already described.

*Benzyl 2-acetamido-2-deoxy-3-*O-[D-*1-(methoxycarbonyl)ethyl]-β-*D *glucopyranoside* (**18**). — Treatment of compound **15** with 60% acetic acid for 30 min at 100°, followed by evaporation, gave a crystalline residue which was recrystallized from ethyl acetate to give small needles (80% yield), m.p. 169–170°, $[\alpha]_D^{25}$ −53° (*c* 0.57, chloroform).

Anal. Calc. for $C_{19}H_{27}NO_8$: C, 57.42; H, 6.85; N, 3.52. Found: C, 57.06; H, 6.24; N, 3.23.

*Benzyl 2-acetamido-3-*O-(D-1-*carboxyethyl)-2-deoxy-β-*D-*glucopyranoside* (**19**). — A solution of compound **18** (125 mg) in 0.2M sodium hydroxide (10 ml) was kept overnight at room temperature, and was then passed through a column of Dowex-50 (H^+) ion-exchange resin. Evaporation of the eluate, followed by crystallization of the residue from ethanol–water, gave long needles (86 mg, 79%), m.p. 189–191° and 225–227°, $[\alpha]_D^{20}$ −36 (*c* 2.2, methanol).

Anal. Calc. for $C_{18}H_{25}NO_8$: C, 56.39; H, 6.57; N, 3.65. Found: C, 55.99; H, 6.56; N, 3.49.

The same compound was obtained in 87% yield by treatment of **14** with 60% acetic acid.

Sublimation at 220–220° under high vacuum gave benzyl 2-acetamido-3-*O*-[(*R*)-1-carboxyethyl]-2-deoxy-β-D-glucopyranoside 4-(inner ester) (**24**), m.p. 223–5°, $[\alpha]_D^{20}$ −4° (*c* 0.10, pyridine).

Anal. Calc. for $C_{18}H_{23}NO_7$: C, 59.17; H, 6.34; N, 3.83. Found: C, 59.10; H, 6.40; N, 3.84.

p-*Nitrophenyl 2-acetamido-4,6-*O-*benzylidene-2-deoxy-β-*D-*glucopyranoside* (**11**). — A mixture of *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside[18] (450 mg), freshly fused zinc chloride (350 mg), and freshly distilled benzaldehyde (5 ml) was shaken for 24 h at room temperature, hexane (4 ml) and water (4 ml) were added, the mixture was shaken for 1 h, and the liquid phase was decanted. Hexane and water were added to the solid, and the procedure was repeated three times. Finally, the solid was filtered off, washed thoroughly with water and hexane, dried in a desiccator, and recrystallized from methanol–water to give white needles (460 mg, 81%), m.p. 245–7° (dec.), $[\alpha]_D^{20}$ −52° (*c* 0.57, chloroform).

Anal. Calc. for $C_{21}H_{22}N_2O_8$: C, 58.60; H, 5.15; N, 6.51. Found: C, 58.48; H, 5.02; N, 6.42.

p-*Nitrophenyl 2-acetamido-4,6-O-benzylidene-2-deoxy-3-O-*[D-*1-(methoxycarbonyl)ethyl]-β-*D-*glucopyranoside* (**16**). — To a stirred solution of compound **11** (190 mg) in dry *p*-dioxane (100 ml) at 60–70° was added sodium hydride (150 mg, as a 54% suspension in mineral oil). After the mixture had been stirred for 15 min, DL-2-chloropropionic acid (0.75 ml) and *p*-dioxane (5 ml) were added, and the suspension was stirred for 2 h at 60–70°. Sodium hydride (2 g) in mineral oil and *p*-dioxane were added portionwise, and the mixture became very thick. It was shaken vigorously by hand for 10 min, and then mechanically overnight at 45–50°, and processed by to the method used for the preparation of **12**. Starting material (38 mg, 20%) was recovered from the chloroform extract of the alkaline phase and from the fractions eluted with ether–ethyl acetate (4:1 and 1:1) from the silica gel column. The fractions eluted with (9:1) 1-2-dichloroethane–ether gave, after recrystallization from acetone–ether cream-colored needles (56 mg, 25%), m.p. 254–5°, $[\alpha]_D^{20}$ −16° (*c* 0.21, chloroform).

Anal. Calc. for $C_{25}H_{28}N_2O_{10}\cdot 0.5\ H_2O$: C, 57.14; H, 5.53; N, 5.33. Found: C, 57.16; H, 5.62; N, 5.76.

p-*Nitrophenyl 2-acetamido-3-O-*(D-*1-carboxyethyl)-β-*D-*glucopyranoside* (**20**). — Treatment of compound **16** (40 mg) with 60% acetic acid at 100° and removal of the acetic acid and benzaldehyde, as described for the preparation of **17**, was followed by treatment with 0.5M sodium hydroxide, as described for the preparation of **19**. The residue was purified by dissolution in benzene and chromatography on silica gel. Ether–ethyl acetate (1:1) eluted fractions which were crystallized, and which gave, after recrystallization from ethanol–ether, 16 mg (50%) of small clusters of needles, m.p. 167–170° (turning yellow at 155°), $[\alpha]_D^{20}$ −27° (*c* 0.26, ethanol).

Anal. Calc. for $C_{17}H_{22}N_2O_{10}$: C, 49.28; H, 5.35. Found: C, 49.01; H, 5.99.

Action of egg-white lysozyme on compounds **19** *and* **20**. — To a 4% solution (1 ml) of **19** or **20** in water was added 0.1M ammonium acetate buffer (0.025 ml; adjusted to pH 6.30 with acetic acid) and a 0.0025% solution (0.05 ml) of thrice-recrystallized, egg-white lysozyme (General Biochemicals, Chagrin Falls, Ohio). Control solutions without lysozyme were prepared, and the mixtures were incubated at 37°. Aliquots were examined by descending chromatography on Whatman No. 1 paper with butanol–acetic acid–water (4:1:5, upper phase). 2-Acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose (*N*-acetylmuramic acid), benzyl alcohol, and *p*-nitrophenol, were chromatographed as standards. The spots were revealed by the silver nitrate method and by dipping of the dried chromatograms in a solution of 0.5M sodium hydroxide in 6:4 ethanol–propanol, heating for 5 to 10 min at 120°, and then examining under u.v. light[32].

No product of hydrolysis could be observed with either **19** or **20** for incubation times of up to 48 h.

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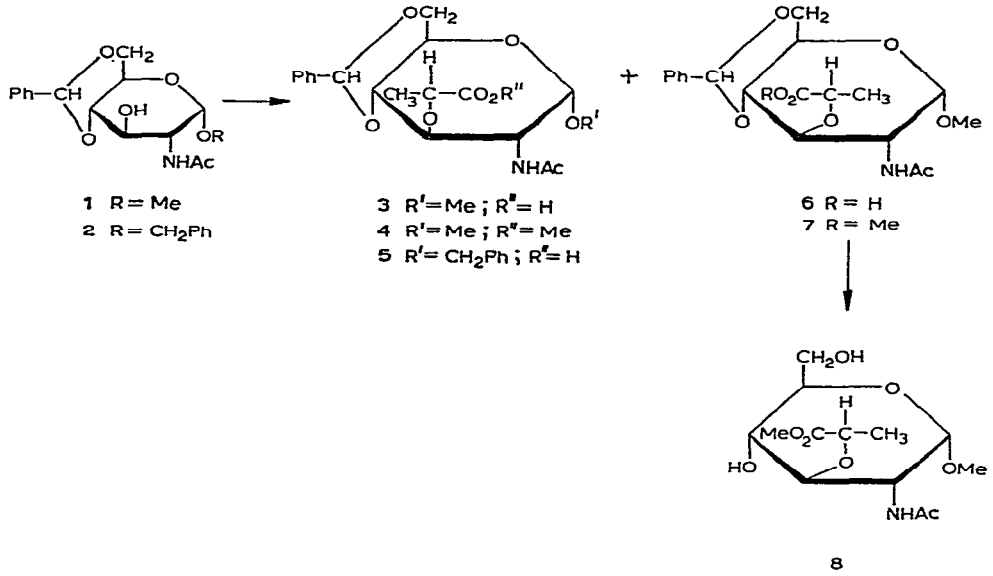
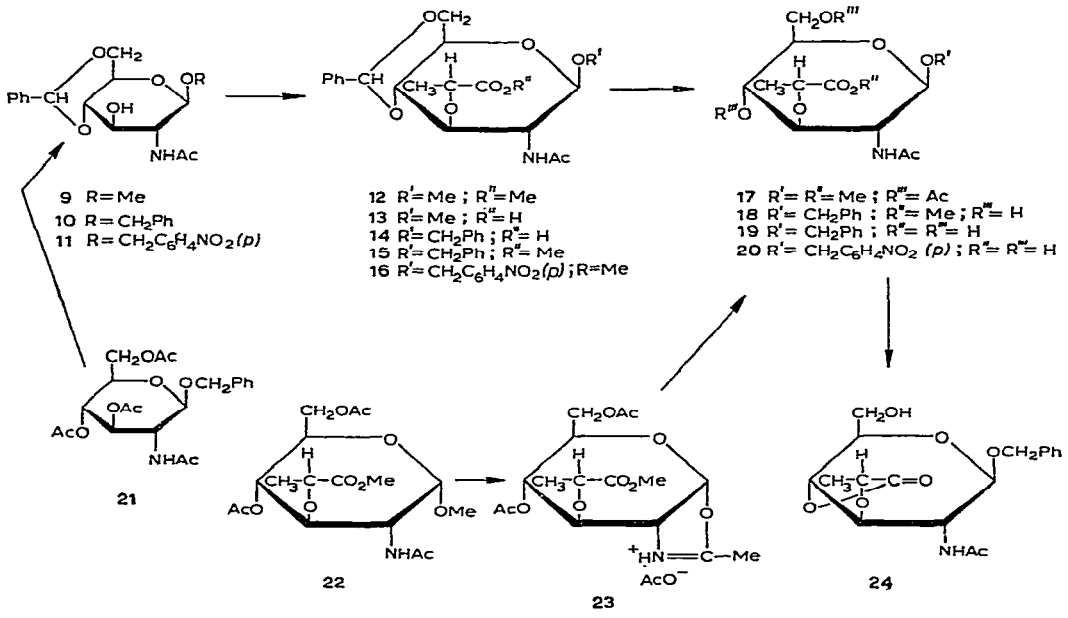


1 R = Me
2 R = CH_2Ph

3 R^{I} = Me ; R^{II} = H
4 R^{I} = Me ; R^{II} = Me
5 R^{I} = CH_2Ph ; R^{II} = H

6 R = H
7 R = Me

8



9 R = Me
10 R = CH_2Ph
11 R = $CH_2C_6H_4NO_2$ *(p)*

12 R^{I} = Me ; R^{II} = Me
13 R^{I} = Me ; R^{II} = H
14 R^{I} = CH_2Ph ; R^{II} = H
15 R^{I} = CH_2Ph ; R^{II} = Me
16 R^{I} = $CH_2C_6H_4NO_2$ *(p)* ; R = Me

17 R^{I} = R^{II} = Me ; R^{III} = Ac
18 R^{I} = CH_2Ph ; R^{II} = Me ; R^{III} = H
19 R^{I} = CH_2Ph ; R^{II} = R^{III} = H
20 R^{I} = $CH_2C_6H_4NO_2$ *(p)* ; R^{II} = R^{III} = H

21

22

23

24

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METHYL TERMINAL-4-*O*-METHYLMALTO-OLIGOSACCHARIDES

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ABSTRACT

Malto-oligosaccharides substituted at only one hydroxyl group of the nonreducing end were prepared by the following sequence of reactions; tri-*O*-benzylamylose was prepared by treating amylose with benzyl chloride and sodium hydroxide in methyl sulfoxide, and was methanolyzed to give perbenzylated methyl malto-oligosaccharides having only the hydroxyl group at C-4 of thenonreducing end unsubstituted. Methylation with methyl iodide and sodium hydride in *N,N*-dimethylformamide yielded perbenzylated methyl terminal-4-*O*-methylmalto-oligosaccharides; debenzylation was effected by hydrogenolysis with the use of large amounts of Raney nickel catalyst, to yield methyl terminal-4-*O*-methylmalto-oligosaccharides having D.P. 1–16. Separation by carbon–Celite column chromatography gave individual components having degrees of polymerization of 1 to 3 which were characterized, by thin-layer and gas–liquid chromatography before and after hydrolysis.

INTRODUCTION

Synthesis of malto-oligosaccharides modified only at C-4 of the nonreducing terminus* was undertaken in order to provide compounds for ascertaining whether *beta*-amylase acts by the template[1,2] or by an induced-fit[3,4]mechanism.

beta-Amylase hydrolyzes only the penultimate linkage of an amylose, amylopectin, or malto-oligosaccharide chain to produce β-maltose. If the linear portions of these molecules are considered to be chains of maltose residues, the enzyme must have a way of distinguishing the terminal maltosyl residue from all other maltosyl residues. The maltosyl residue at the nonreducing terminus differs chemically from the internal maltosyl residues in having an unsubstituted hydroxyl group at C-4 of the terminal D-glucosyl residue.

Thoma and Koshland[5], in a discussion of the induced-fit mechanism of *beta*-amylase action, suggested that the free hydroxyl group at C-4 is necessary to the alignment process. They further suggested that, although this group is necessary for enzyme action, its role may be either active or passive. On the basis of the template theory, the role of the C-4 hydroxyl group could be explained either by assuming that it

*Herein designated terminal-4-*O*-substituted malto-oligosaccharides.

provides a key hydrogen bond, without which no net attraction occurs, or by assuming that the hydroxyl group fits into a small cavity, which is unable to accomodate D-glucosyl or poly-D-glucosyl groups. Hence, determination of the binding affinity of compounds that have maltosyl residues but no C-4 hydroxyl groups could indicate the mechanism by which *beta*-amylase acts.

The title compounds, which are malto-oligosaccharides modified in the terminal C-4 position, might be used for obtaining this information. Their synthesis could be effected in either of two ways: (*a*) addition of a 4-*O*-methyl-D-glucopyranosyl moiety to a malto-oligosaccharide chain, or (*b*) acid-catalyzed methanolysis of completely substituted amylose or cycloamylose, followed by methylation and removal of protecting groups. Use of a completely substituted amylose was chosen as the method for this investigation.

DISCUSSION

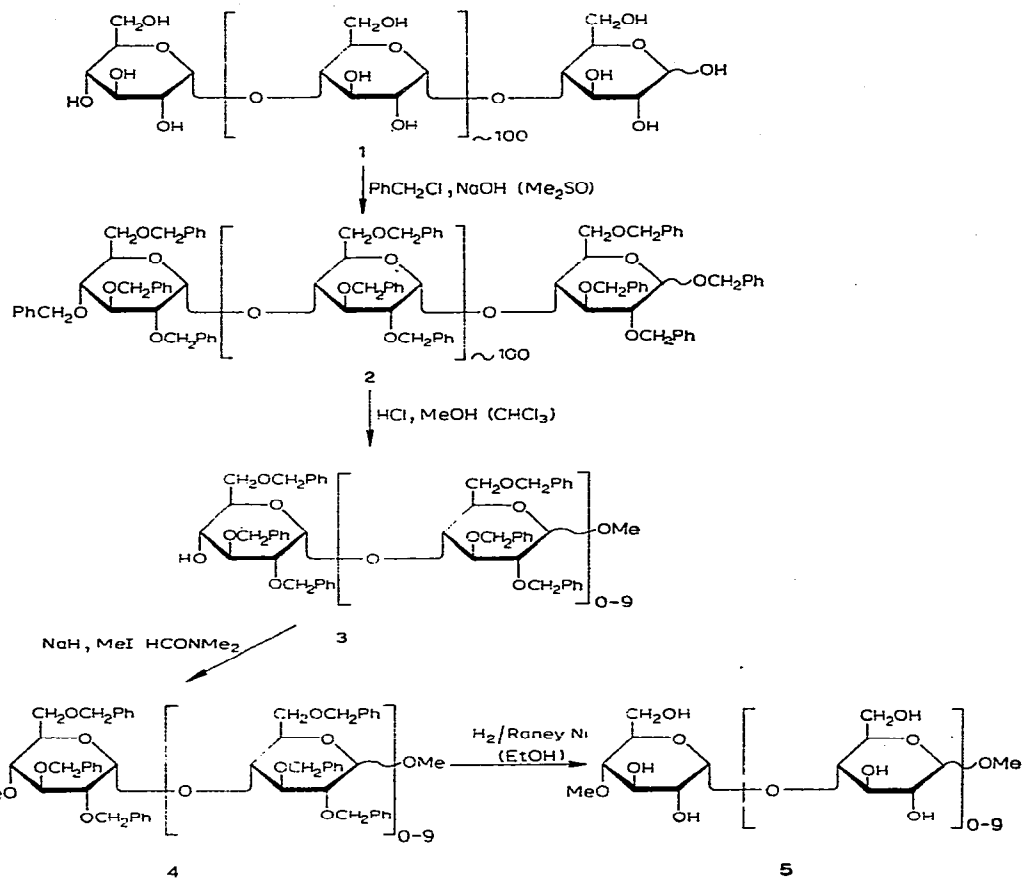
One attempt to synthesize malto-oligosaccharides modified only at C-4 has been reported[6]. The method used was similar to that reported in the present work; cyclohexaamylose was the starting compound. Benzylated terminal-4-*O*-methyl-malto-oligosaccharides were formed, but could be only incompletely debenzylated.

In the present investigation, it was decided to prepare a completely substituted amylose (perester or perether) and subject it to partial solvolysis with acid. If the solvolysis is conducted in methanol, the methyl glycoside is formed, leaving unsubstituted only the terminal C-4 hydroxyl group for subsequent alteration. After alteration (methylation) at O-4, the other hydroxyl groups can be regenerated to produce the desired products.

Esters were investigated first, because of their ease of formation and the mild conditions needed for subsequent regeneration of the hydroxyl groups. Amylose triacetate was prepared, and methanolyzed, and the product was methylated with diazomethane. After removal of acetyl groups and subsequent hydrolysis, g.l.c analysis indicated the presence of 4,6-di-*O*-methyl-D-glucose and 6-*O*-methyl-D-glucose, which could have been formed only if an ester group had been removed or had migrated during the methanolysis[7]. Propionyl, butyryl, and benzoyl groups were also found to be unsatisfactory as protecting groups[7].

Benzyl ethers were then chosen as protecting groups, because they are stable both to acids and bases, but can be removed under mild conditions. Benzylated starch has been reported[8–18], but the methods used for its preparation lead to considerable degradation and a low degree of substitution; these methods involved the reaction of benzyl chloride with alkali-starch in aqueous sodium hydroxide. The preparation of tri-*O*-benzylamylose has not been reported. Benzylation of amylose was effected with benzyl chloride and sodium hydroxide in methyl sulfoxide. One treatment gave slightly less than complete etherification; two treatments gave the desired product. Substitution of sodium hydride for sodium hydroxide, and addition of sodium iodide to the reaction mixture, failed to give faster reactions or higher degrees of substitution.

Methanolysis effects cleavage of a polysaccharide chain with concomitant formation of a methyl glycoside at the new reducing end. In this way, the reducing end is protected. Both anomeric glycosides of each malto-oligosaccharide were formed



during methanolysis. If pure α- or β-D anomers are desired, separation of the per-benzylated compounds can be accomplished by preparative t.l.c. (see, for example, Table I).

Methanolysis of tri-*O*-benzylamylose was investigated as a function of time. A homologous series of oligosaccharides could be detected after methanolysis for 2 h, but 24 h was required in order to convert all of the polymeric material into oligosaccharides having the desired degrees of polymerization (D.P. 1–16). If the methanolysis was continued for 125 h, with further additions of methanolic hydrogen chloride to maintain sufficient acidity, four main products* were obtained, namely, methyl

*In this work, trace amounts (<5%) of methyl 2,3-di-*O*-benzyl-α,β-D-glucopyranoside were formed from the small proportion of amylopectin present in the commercial amylose. This would indicate the presence of a very few 6-*O*-methyl groups in the final products.

TABLE I

THIN-LAYER CHROMATOGRAPHIC DATA FOR TRI-*O*-BENZYLAMYLOSE AFTER METHANOLYSIS FOR 24 H[a]

Compound	R_F *value*[b]	
	α Anomer	*β Anomer*
Methyl tetra-*O*-benzyl-D-glucoside	0.47	0.52
Methyl tri-*O*-benzyl-D-glucoside	0.31	0.36
Methyl hexa-*O*-benzylmaltoside	0.22	0.25
Methyl nona-*O*-benzylmaltotrioside	0.16	0.18
Methyl dodeca-*O*-benzylmaltotetraoside	0.10	0.12
Methyl pentadeca-*O*-benzylmaltopentaoside	0.06	0.09

[a]Silica Gel H, Brinkmann Instruments Co., Westbury, L. I., N. Y. [b]Irrigant[19]: 5:1 petroleum ether (b.p. 30–60°)–acetone

2,3,4-6-tetra-*O*-benzyl-α,β-D-glucopyranoside (obtained from the nonreducing residue at the end of each amylose chain) and methyl 2,3,6-tri-*O*-benzyl-α,β-D-glucopyranoside (obtained from each internal residue). The latter two compounds can be used for preparing 4-*O*-substituted methyl α,β-D-glucopyranosides, which are important model compounds in relation to amylose and cellulose[19].

The sirup obtained after methanolysis could be methylated quantitatively by several methods. As the procedure of Kuhn and coworkers[20] gave the desired products in 15–18 h, it was used most frequently. Occasionally, traces of *N,N*-dimethylformamide remained and had to be removed, because it acts as a poison toward Raney nickel[21]. Removal of *N,N*-dimethylformamide was accomplished by several extractions with methanol. Infrared analysis of the methylated products showed no hydroxyl-group absorption.

Debenzylation of these oligosaccharides by standard methods was incomplete, as previously reported[6]. However, the desired oligosaccharides were obtained by using an amount of Raney nickel far in excess of that usually considered to be catalytic. Since the benzylated oligosaccharides were insoluble in ethanol, and coated the catalyst, these large amounts were used to adsorb all of the sirup. The temperature (60°) and the hydrogen pressure (65 lb. in^{-2}) also appeared to be critical, because higher or lower temperatures or pressures resulted in very little debenzylation. The oligosaccharides of higher molecular weight (D.P. 6–16) were also insoluble in ethanol after debenzylation; they were isolated by washing the Raney nickel with water.

A new chemical method of debenzylation of carbohydrate derivatives was also developed[22]. The procedure involves free-radical bromination of one of the methylene hydrogen atoms of the benzyl ether group to form a 1-bromobenzyl ether. This stable compound undergoes hydrolysis with alkali to give an intermediate hemiacetal (α-hydroxy ether), which spontaneously decomposes into benzaldehyde and carbohydrate, with retention of configuration. Details of this method will be published elsewhere.

The series of oligosaccharides so formed gives a linear relationship in a plot of the log of a function of R_F against D.P. (see Fig. 1). This observation is one indication

of a homologous series[23]. The mixture is rapidly hydrolyzed, but to only a small extent, by *beta*-amylase[24], indicating a small percentage of molecules that are unmethylated on the terminal O-4; these molecules (methyl malto-oligosaccharides)

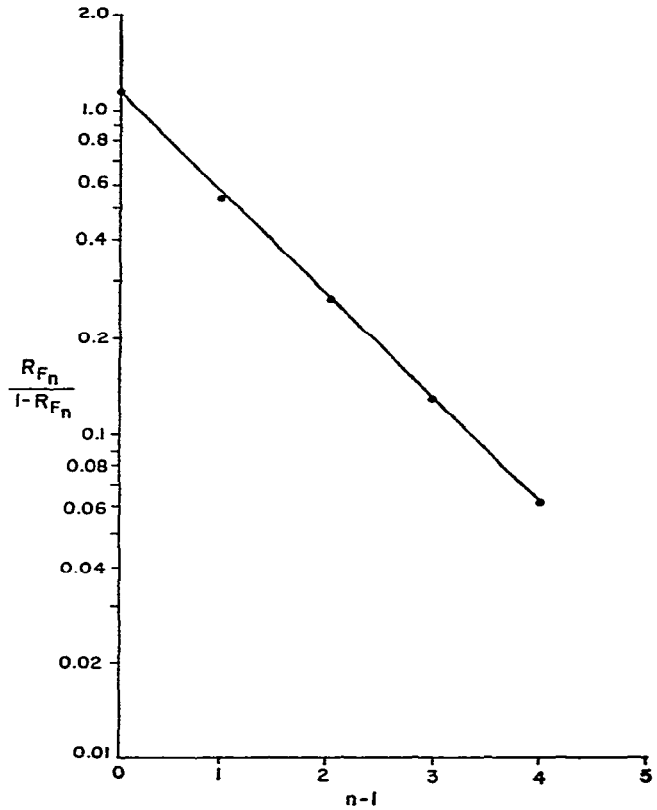


Fig. 1. Degree of polymerization *vs.* a function of R_F for the methyl terminal-4-*O*-methylmalto oligosaccharides.

arise from the original, nonreducing end-units of the amylose, and are removed by such enzyme treatment. Also, potato phosphorylase has very little, if any, action on these compounds. The first three members of the desired series have been separated by carbon–Celite column chromatography, and have been shown by t.l.c. and g.l.c. to be homogeneous.

Characterization was accomplished by acid hydrolysis followed by analysis of the products by g.l.c. Analysis of the gas–liquid chromatograms showed only three peaks for the hydrolysis products from the dimer and trimer. Further investigation of the R_T values of pertrimethylsilylated D-glucose and 4-*O*-methyl-D-glucose showed that there is an overlap of the peak for α-D-glucose with that for 4-*O*-methyl-β-D-glucose.

Samples of D-glucose, which contained a drop of ammonium hydroxide to ensure rapid establishment of anomeric equilibrium, were also analyzed. It was found

that the equilibrium composition is almost constant for D-glucose, and corresponds to the ratios reported by Sweeley and coworkers[25]. Similar analysis of the hydrolysis products from methyl 4-*O*-methyl-α,β-D-glucopyranoside gave results for the anomers of 4-*O*-methyl-D-glucose which were also constant. When average values of 55.65% for β-D-glucose and 41.18% for α-D-glucose (3.17% furanose) were used, the amounts of α-D-glucose and 4-*O*-methyl-β-D-glucose in the peak containing both could be calculated. The values of α:β ratios of 4-*O*-methyl-D-glucose from the hydrolysis of dimer and trimer were found to be essentially the same as those for the monomer.

Ratios of D-glucose:4-*O*-methyl-D-glucose were then calculated (see Table II). They agree with the calculated ratios of 0:1, 1:1, and 2:1 for the hydrolysis products from **4** having degrees of polymerization of 1, 2, and 3, respectively.

TABLE II

RATIO OF D-GLUCOSE: 4-*O*-METHYL-D-GLUCOSE IN HYDROLYSIS PRODUCTS OF OLIGOSACCHARIDES

Degree of polymerization	D-*Glucose*	4-*O*-*Methyl*-D-*glucose*
1	0 0	1.00, 1.00
2	1.00, 1.00	1.01, 1.02
3	2.00, 2.00	0.97, 0.99

The synthetic method described can, no doubt, be used as a general method with other polysaccharides, to give oligosaccharides modified, in the nonreducing terminus, at the hydroxyl group that is normally involved in the glycosidic linkage. Such modified oligosaccharides might be used for testing the enzyme specificity of other exoenzymes, such as β-D-glucosidases. The modified oligosaccharides could also be used as model compounds in study of the mechanism of acid-catalyzed hydrolysis or methanolysis. Finally, this synthetic approach can be used for the synthesis of modified monomers that are difficult to obtain by other means. For all of these uses, reactions other than methylation could be effected at the free hydroxyl group. Some of these reactions will be reported in subsequent publications.

EXPERIMENTAL

Tri-O-benzylamylose (**2**). — Commercial "Nepol" amylose (**1**, 5 g) (A. E. Staley Mfg. Co., Decatur, Illinois, U. S. A.) and 200 ml of methyl sulfoxide were placed in a 3-necked, 1-liter, round-bottomed flask. The flask was placed in a water bath at 60°, and the contents were stirred to dissolution (1 h). At this time, 33.6 g of finely powdered sodium hydroxide (10 moles per mole of hydroxyl groups) and another 100 ml of methyl sulfoxide were added. This mixture was stirred for 1 h under nitrogen. Over the next 30 min, 39 ml of benzyl chloride (4 moles per mole of hydroxyl groups) was added dropwise. After all of the benzyl chloride had been added, the temperature

was raised, and kept for 16 h at 70°. After 1, 2, and 16 h, an additional 10 ml of benzyl chloride was added dropwise. After 16 h, the temperature was raised, and kept for 2 h at 85°.

The reaction mixture was cooled to room temperature, and 500 ml of water and 500 ml of chloroform were added in that order. The chloroform layer was removed, and the aqueous layer was extracted with three 200-ml portions of chloroform. The chloroform extracts were combined, washed successively with 1.5 l of water, 1 l of 0.5M sulfuric acid, and 2 l of water, and evaporated under diminished pressure at 60° to a sirup. Tri-*O*-benzylamylose was precipitated from the syrup with 500 ml of 95% ethanol in a Waring Blendor. After the solid had been washed once with 500 ml of 95% ethanol, it was stirred for 24 h with several 250-ml portions of petroleum ether (b.p. 30–60°) to remove the final traces of methyl sulfoxide and benzyl chloride. The product was filtered off and air-dried; yield 11.8 g (87%), slight hydroxyl absorption at 3800–3600 cm^{-1}. The solid was rebenzylated by the same procedure to give a white powder, yield 10.0 g (74%), no hydroxyl absorption, m.p. 164–165°, $[\alpha]_D^{25}$ +102° (*c* 1.0, chloroform)

Anal. Calc. for $C_{27}H_{28}O_5$; C, 75.00; H, 6.48. Found: C, 74.25; H, 6.33.

Perbenzylated methyl terminal 4-hydroxymalto-oligosaccharides (**3**). — Tri-*O*-benzylamylose (**2**, 2 g) was dissolved in 200 ml of anhydrous chloroform, and an anhydrous 0.8M methanolic solution of hydrogen chloride (prepared from 100 ml of cold methanol and 18 ml of acetyl chloride) was added. Various periods of refluxing (1 to 125 h) were used, depending on the series of products desired. Reactions were monitored by t.l.c.[19]. The R_F values of the products of methanolysis for 24 h are given in Table I. The solutions were cooled to room temperature, and neutralized with Amberlite IR-45(OH^-) ion-exchange resin (Rohm and Haas Co., Philadelphia, Pa.). After filtration, the solvent was removed under diminished pressure; yield 2.05 g (100%)*.

Perbenzylated methyl terminal-4-O-methylmalto-oligosaccharides (**4**). — Substance **3** (2.05 g) was dissolved in 40 ml of *N,N*-dimethylformamide in a 100-ml, round-bottomed flask. Sodium hydride (1.1 g) was added, the reaction was allowed to proceed for 1 h, 20 ml of methyl iodide was then added, and the mixture was stirred overnight. Sodium iodide was then removed by filtration, and the solid was washed with chloroform. Chloroform and *N,N*-dimethylformamide were removed from the filtrate under diminished pressure at 100°, and then trace amounts of *N,N*-dimethylformamide were removed by washing with methanol; yield 2.07 g (100%) of a sirup. T.l.c. of this sirup gave the data shown in Table III.

Methyl terminal-4-O-methylmalto-oligosaccharides (**5**). — A solution of compound **4** (1.7 g) in 50 ml of chloroform was placed in a hydrogenation flask, the chloroform was removed under diminished pressure, absolute ethanol (300 ml) was added, and the mixture was heated to reflux. Approximately 5 g of freshly activated Raney

*All calculations of yield in this paper were made on the assumption that the product(s) was a trisaccharide.

TABLE III

THIN-LAYER CHROMATOGRAPHIC DATA[a] FOR THE PERBENZYLATED METHYL TERMINAL-4-*O*-METHYLMALTO-OLIGOSACCHARIDES

Degree of polymerization	R_F *value*[b]	
	α Anomer	*β Anomer*
1	0.47	0.52
2	0.41	0.44
3	0.33	0.37
4	0.25	0.29
5	0.18	0.22

[a]Silica Gel H, Brinkmann Instruments Co., Westbury, L. I., N. Y. [b]Irrigant[19], 5:1 petroleum ether (b.p. 30–60°)–acetone.

nickel was carefully added to the hot solution, and the insoluble material was scraped off the inside of the flask so that it could be adsorbed onto the catalyst. The flask was then attached to a Parr hydrogenator, and flushed three times with hydrogen, and hydrogenolysis was accomplished at 60° under 65 lb. in^{-2} of hydrogen for 48 h. The Raney nickel was filtered off, and the filtrate was evaporated to dryness under diminished pressure. Partially benzylated material was removed from the resulting syrup by extraction with chloroform. The sirup remaining (0.259 g) was analyzed by t.l.c. (see Table IV).

TABLE IV

THIN-LAYER CHROMATOGRAPHIC DATA FOR THE METHYL TERMINAL-4-*O*-METHYLMALTO-OLIGOSACCHARIDES[a]

Degree of polymerization	R_F *value*[b]	
	Irrigant A	*Irrigant*[c] B
1	0.54	0.94
2	0.35	0.91
3	0.21	0.87
4	0.12	0.83
5	0.06	0.78
6	0.00	0.73
7		0.69
8		0.66
9		0.63
10		0.59
11		0.55
12		0.52
13		0.49
14		0.45
15		0.42
16		0.37

[a]Silica Gel H, Brinkmann Instruments Co., Westbury, L. I., N. Y. [b]Irrigant A[26]: 9:6:3:1 BuOH–HOAc–Et_2O–H_2O; irrigant B[27]: 5:2:3 PrOH–$MeNO_2$–H_2O [c]Three ascents.

The catalyst was then washed with 100 ml of chloroform to remove any benzylated compounds. After filtration, the Raney nickel was stirred with 200 ml of water to remove oligosaccharides of higher molecular weight. The Raney nickel was filtered off, and the filtrate was stirred with a mixed, anion–cation exchange resin. Evaporation gave 0.232 g of compounds having degrees of polymerization of 6–16 (see Table IV). The total yield of completely debenzylated malto-oligosaccharides was 0.491 g (80%).

Separation. — Methyl terminal-4-*O*-methylmalto-oligosaccharides were separated by graded elution from a 1:1 carbon–Celite 545 (Johns-Manville Co., New York, N. Y.) column. The carbon–Celite mixture had been deactivated as follows: it was treated for 0.5 h with hot, conc. hydrochloric acid, and successively washed with water (until the washings were neutral), 0.5M sodium cyanide solution, and water (until the washings were free from cyanide ion by the silver-ion test).

To a 450 × 30-mm carbon–Celite column was added 0.66 g of a mixture of methyl terminal-4-*O*-methylmalto-oligosaccharides (D.P. 1–5) in 2 ml of water, and the compounds were eluted with water–ethanol mixtures (see Table V). Slight overlap was obtained just before each component of higher D.P. was eluted.

TABLE V

SEPARATION OF METHYL TERMINAL-4-*O*-METHYLMALTO-OLIGOSACCHARIDES ON CARBON–CELITE COLUMNS

Me D-*glycosides*	*Percent of ethanol in eluant*	*Liters of eluant*	*Yields of pure components (g)*
Me α,β-D-glucoside	1	3	0.05
D.P. 1	2.5	20	0.20
D.P. 2	5.0	9	
D.P. 2	7.5	9	0.08
D.P. 2	10	20	
D.P. 3	15	20	0.03

Hydrolysis. — The title compounds were hydrolyzed in 30 ml of M sulfuric acid for 36 h at 80° by using 93 mg of monomer, 19 mg of dimer, and 8.5 mg of trimer. After hydrolysis, the solutions were neutralized with Amberlite IR-45(OH^-) ion-exchange resin, and evaporated to dryness under diminished pressure. The only products observed, by paper-chromatographic, t.l.c., and g.l.c. analysis, were D-glucose and 4-*O*-methyl-D-glucose. G.l.c. analysis was performed by the method of Brobst and Lott[28].

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SYNTHESIS OF SOME AROMATIC ESTERS OF THE METHYL D-GLUCOPYRANOSIDES, AND THEIR STABILITY TO HIGH-ENERGY RADIATION

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ABSTRACT

The esters obtained by complete esterification of methyl α-D-glucopyranoside with each of eight differently substituted aromatic acids were subjected to irradiation (7.3×10^{19} ev/g/h) in the solid state. Only the *p*-toluenesulfonic esters were altered; the substituted benzoic esters, regardless of the substitution on the benzene ring, were stable. Radiation change in the two *p*-toluenesulfonic esters occurred at the glycosidic bond, as shown by the i.r. spectra of the products. The stability of the other substituted D-glucosides was so high that, even after dosages as high as 5.2×10^{21} ev/g, no reducing power was measurable. The presence of either electropositive or electronegative substituents on the aromatic ring did not significantly affect the radiation change of the glycosidic bond, and it was suggested that protection arises mainly from intramolecular transfer of energy to the aromatic group, followed by dissipation of the energy as heat or light.

INTRODUCTION

Cleavage of the glycosidic bond is the predominant reaction when methyl α-D-glucopyranoside[1,2] and di-, tri-, and poly-saccharides[3] are exposed to high-energy radiation, both in the solid state and in aqueous solution. Phillips, Blouin, and Arthur[4] showed that a considerable amount of protection of the glycosidic bond can be achieved, over distances greater than one D-glucose residue, when aromatic esters of D-glucosides are subjected to γ-radiation. Protection appeared to be due to preferential transfer of energy to the aromatic group, with dissipation of the energy as heat or light.

The effects of the substitution of electropositive and electronegative groups (on the aromatic rings) on the stability of some of the properties of aromatic esters of the methyl D-glucopyranosides to high-energy radiation are now reported. Also, the synthesis and characterization of eight of these esters are given.

RESULTS

The glycosidic group in all of the compounds was found to be highly resistant

*Resident Postdoctoral Research Associate.

to radiation cleavage. No measurable amount of reducing power, as determined by the method of Somogyi[5], was found in any of the D-glucosides irradiated, even at dosages as high as 5.2×10^{21} ev/g. Moreover, after debenzoylation of the irradiated D-glucosides by alkaline hydrolysis[6], no reducing power was detectable. For methyl 2,3,4,6-tetra-*O*-*p*-tolylsulfonyl-β-D-glucopyranoside (**1**) and methyl 4-chloro-2,3,6-tri-*O*-*p*-tolylsulfonyl-β-D-glucopyranoside (**2**) under mild conditions of alkaline hydrolysis, desulfonylation was not accomplished, and complete dissolution was not possible. With methanolic sodium hydroxide or with sodium methoxide in methanol[7] under reflux, ring opening occurred, as indicated from the analysis of the unirradiated control.

In the i.r. spectra of irradiated **1** and **2**, a new band appeared at 1725 cm^{-1}, indicating the formation of carbonyl groups. For all of the other compounds, the i.r. and n.m.r. spectra were the same before and after irradiation. With the exception of **1** and **2**, the irradiated D-glucosides showed no component other than the starting material when examined by t.l.c.; irradiated **1** and **2**, showed an extra component (t.l.c.). Radiation-induced cleavage of the sulfonic ester linkage was not observed.

An estimation of the radiation decomposition of the glycosidic bond was made from the changes in optical rotation of the various compounds on irradiation. A sample of each irradiated D-glucoside was debenzoylated[6], and the optical rotation was measured; the value was compared with that for the debenzoylated control. The radiation decomposition for the D-glucosides at a dosage of 5.2×10^{21} ev/g was estimated to be as follows: methyl 2,3,4,6-tetra-*O*-nicotinoyl-α-D-glucopyranoside (**3**), 5.5%; methyl 2,3,4,6-tetra-*O*-(phenylcarbamoyl)-α-D-glucopyranoside (**4**), 1.0%; methyl 2,3,4,6-tetra-*O*-(*p*-methoxybenzoyl)-α-D-glucopyranoside (**5**), 2.2%; methyl 2,3,4,6-tetra-*O*-(*p*-nitrobenzoyl)-α-D-glucopyranoside (**6**), 2.2%; methyl 2,3,4,6-tetra-*O*-(*p*-ethoxycarbonylbenzoyl)-α-D-glucopyranoside (**7**), 5.5%; and methyl 2,3,4,6-tetra-*O*-(*o*-chlorobenzoyl)-α-D-glucopyranoside (**8**), 1.0%. The irradiated **1** had $[\alpha]_D^{25}$ −2.47° (*c* 0.8, acetone) as compared with $[\alpha]_D^{25}$ −9.7° (*c* 0.8, acetone) for the unirradiated sample. If the other D-glucosides irradiated were not debenzoylated prior to the determination, the changes in the optical rotation were not significant.

For all of the D-glucosides, irradiation induced long-lived free-radicals which were detected by the single line in their electron-spin resonance (e.s.r.) spectra; this indicated that the free radicals were, most probably, formed by dehydrogenation at the C-1–H bond. When the irradiated D-glucosides were stored under vacuum, the free radicals were still detectable several weeks after irradiation. When they were stored in air, the concentration of the radicals decreased; however, the shape of the e.s.r. spectrum did not change. The formation of stable free-radicals in carbohydrates on irradiation has been reported by other workers[8–10]. The color of the D-glucosides did not change on irradiation, except for **3**, which turned green; the green color increased in intensity with the dosage, and disappeared completely on storage in the air.

The fluorescence spectra of the D-glucosides changed very little on irradiation, as shown in Table I. In most cases, a new peak appeared in the longer-wavelength region

of the spectra. The intensity of the new peak increased with increase in radiation dosage.

TABLE I

FLUORESCENCE SPECTRA OF ESTERS OF METHYL D-GLUCOPYRANOSIDES[a]

Ester		*Before irradiation*		*After irradiation*	
		Activation peak, nm	*Fluorescent peak, nm*	*Activation peak, nm*	*Fluorescent peak, nm*
2,3,4,6-Tetra-*O*-(phenylcarbamoyl)-α-D-	(4)	277 (w)	302.5 (w)	341	405
2,3,4,6-Tetra-*O*-*p*-tolylsulfonyl-β-D-	(1)	306.6	370	309, 325	370, 450
4-Chloro-2,3,6-tri-*O*-*p*-tolylsulfonyl-β-D-	(2)	266.3	288.5	322	446
2,3,4,6-Tetra-*O*-(*p*-methoxybenzoyl)-α-D-	(5)	287	363.6	290	344
2,3,4,6-Tetra-*O*-(*p*-nitrobenzoyl)-α-D-	(6)	390 (vw)	442.6 (vw)	—	—
2,3,4,6-Tetra-*O*-(*p*-ethoxycarbonyl-benzoyl)-α-D-	(7)	300	375	310, 335	370, 400, 450
2,3,4,6-Tetra-*O*-(*o*-chlorobenzoyl)-α-D-	(8)	268 (vw)		264(vw)	
		297.7	331, 376	300, 365	333, 415
2,3,4,6-Tetra-*O*-nicotinoyl-α-D-	(3)	310	340	310 (vw)	336 (vw)
				339 (vw)	398 (vs)

[a]All of the spectra were measured in chloroform. [b]The spectra were determined 2–3 weeks after irradiation; key: s, strong; v, very; w, weak.

DISCUSSION

When carbohydrates in the solid state are irradiated by ionizing radiation, the mechanism of the loss of energy by the incident radiation to the carbohydrate molecule is probably an initial, random, nonlocalized acceptance of the energy, followed by dissipation of the energy as high-energy electrons within the molecule. The localization of the energy of these electrons is influenced by the presence of aromatic groups. This localization of energy could be effected through intramolecular transfer of energy to the aromatic group or selective absorption of energy by the aromatic group, or both. An aromatic system is able to accept the energy, to form well-defined, excited states; the energy could then be dissipated as heat or light without causing bond cleavage in the carbohydrate molecule. One simple way to deactivate the excited state of the aromatic group is by fluorescence. All of the substituted D-glucosides reported here exhibited fluorescence when excited by high-energy radiation. The intensity of the new fluorescence peak, which appeared after irradiation of the D-glucosides, increased with increase in radiation dosage.

For the *p*-toluenesulfonylated D-glucosides, **1** and **2**, the new band at 1725 cm^{-1} in the i.r. spectra was assigned to the carbonyl group, an intermediate formed during radiation damage of the glycosidic bond. The formation of this reducing group could be explained by slight delocalization of the aromatic, π-electron system by the *d*-orbital participation of the sulfur atom. This energy was

apparently insufficient to break the carbon–oxygen bond so as to cleave the *p*-toluenesulfonic group from the D-glucose residue.

Although intramolecular transfer of energy is the most probable path, intermolecular transfer of energy to the aromatic group is also a possibility. It has been shown that intermolecular transfer of energy occurs during the irradiation of carbohydrates[11].

EXPERIMENTAL

Melting points were determined in a Fisher–Johns apparatus, and were not corrected. Infrared (i.r.) spectra were recorded with a Perkin–Elmer Infracord spectrophotometer. Nuclear magnetic resonance (n.m.r.) spectra were determined for solutions in deuteriochloroform at room temperature, unless otherwise stated, with tetramethylsilane as the internal standard, by using a Varian A-60 spectrometer equipped with a V-6040 variable-temperature probe. The purity of the compounds was examined by thin-layer chromatography (t.l.c.) on Silica Gel G, with 1:1:3 (v/v) acetic acid–water–ethyl acetate for unsubstituted D-glucosides and 1:1 (v/v) ethyl acetate–petroleum ether (b.p. 30–40°) for the substituted D-glucosides. The spray reagent used was 5% sulfuric acid in ethanol (with subsequent charring for 10 min at 110°).

Methyl 2,3,4,6-tetra-O-(*phenylcarbamoyl*)-α-D-*glucopyranoside* (**4**). — This compound was prepared by a method described in the literature[12]. After recrystallization five to six times from alcohol–acetone, the product had m.p. 248–250° (dec)., $[\alpha]_D^{23}$ +72.9° (*c* 2.0, acetone); lit.[12] m.p. 227° (dec.), $[\alpha]_D^{23}$ +73.0° (*c* 3, acetone). ν_{max}^{KBr} 3390 (NH), a doublet at 1770 and 1730 (CO), 1618, 1466 (aryl C=C), 1548 cm^{-1} (Amide II); n.m.r. data (Me_2SO-d_6 at 52°): τ 0.24–0.9 (4-proton multiplet, NH); τ 2.49–3.57 (20-proton multiplet, aromatic); τ 4.32–5.49 (4-proton multiplet, pyranoside ring protons); τ 5.60–6.40 (3-proton multiplet, other ring proton and C-6-methylene protons); τ 6.75 (3-proton singlet, OMe).

Anal. Calc. for $C_{35}H_{34}N_4O_{10}$: C, 62.68; H, 5.07. Found: C, 63.25; H, 5.38.

Methyl 2,3,4,6-tetra-O-p-*tolylsulfonyl*-β-D-*glucopyranoside* (**1**). — This compound was prepared by the method described by Hess and Stenzel[13]; it had m.p. 183–4°, $[\alpha]_D^{23}$ −9.7° (*c* 0.7, acetone); lit.[13] m.p. 183–4°, $[\alpha]_D^{19}$ −9.8° (acetone). ν_{max}^{KBr} 1613, 1515, 1471 (aryl C=C), 1404, 1385 (-O-SO_2-), 1208, 1193 cm^{-1} (-O-SO_2-); n.m.r. data (Me_2SO-d_6 at 64°): τ 1.98–2.79 (16-proton multiplet, aromatic); τ 6.8 (3-proton singlet, OMe); τ 7.53 (singlet, aromatic methyl). Other protons could not be identified, because a poor spectrum was obtained owing to the insolubility of the compound.

Methyl 4-chloro-2,3,6-tri-O-p-*tolylsulfonyl*-β-D-*glucopyranoside* (**2**). — This compound was prepared by the method described by Hess and Stenzel[13]; it had m.p. 186–7°, $[\alpha]_D^{21}$ −18.7° (*c* 0.84, acetone); lit.[13] m.p.186–7°, $[\alpha]_D^{20}$ −18.9° (*c* 0.94, acetone). ν_{max}^{KBr} 1616, 1515, 1471 (aryl C=C), 1404, 1389 (-O-SO_2-), 1208, 1190 cm^{-1} (-O-SO_2-); n.m.r. data (Me_2SO-d_6 at 50°): τ 1.92–2.71 (12-proton multiplet, aromatic); τ 6.71 (3-proton singlet, OMe); τ 7.49 (singlet, aromatic methyl). Other protons could not

be identified, because a poor spectrum was obtained owing to the insolubility of the compound.

Methyl 2,3,4,6-tetra-O-(p-*methoxybenzoyl*)-α-D-*glucopyranoside* (5). — A solution of methyl α-D-glucopyranoside (4.8 g, 0.025 mole) in chloroform (20 ml) was cooled in ice, and a precooled solution (0°) of anisoyl chloride (25.5 g, 0.15 mole) and anhydrous pyridine (24 g, 0.3 mole) in chloroform (20 ml) was added, with stirring, at such a rate that the temperature did not rise above 0°. After the D-glucoside had dissolved, the mixture was kept in the ice-bath for 24 h, and then poured over ice-water, and the mixture was extracted thoroughly with chloroform. The extract was successively washed with ice-cold, dilute sulfuric acid, aqueous sodium carbonate, and water, dried (anhydrous sodium sulfate), and evaporated to a thick syrup which was dissolved in ether–chloroform. On cooling the solution, compound 5 crystallized (yield 80–85%). After two recrystallizations from the same solvent mixture, it had m.p. 144°; $[\alpha]_D^{25}$ +79.2° (*c* 2.0, acetone); ν_{max}^{KBr} 2857 (OMe), 1754 (CO), 1629, 1538 (aryl C=C), 1274 (O-CO-R) cm^{-1}; n.m.r. data: two sets of multiplets of a total of 16 protons (due to aromatic ring) centered at τ 2.01 (8 protons) and τ 3.11 (8 protons); τ 3.56–4.84 (4-proton multiplet, pyranoside ring protons); τ 5.17–5.75 (3-proton multiplet due to 1 ring-proton and 2 C-6-methylene protons); 4 singlets centered at τ 6.18 (12 protons, aromatic OMe); τ 6.48 (3-proton singlet, OMe).

Anal. Calc. for $C_{39}H_{38}O_{14}$: C, 64.10; H, 5.20. Found: C, 63.74; H, 5.10.

Methyl 2,3,4,6-tetra-O-(p-*nitrobenzoyl*)-α-D-*glucopyranoside* (6). — A solution of *p*-nitrobenzoyl chloride (12 g, 0.065 mole) in anhydrous pyridine (15 g, 0.19 mole) was added, with stirring, to a solution of methyl α-D-glucopyranoside (2 g, 0.010 mole) in anhydrous chloroform at 0°. The mixture was kept in an ice-bath overnight, and then processed as for 5. The solid product was recrystallized from alcohol–chloroform; (yield 80%); m.p. 181–2°; $[\alpha]_D^{25}$ +92.2° (*c* 1.0, acetone); ν_{max}^{KBr} 1761 (CO), 1626, 1550 (aryl C=C), 1366 (NO_2), 1290 (O-CO-R) cm^{-1}; n.m.r. data: τ 1.47–2.31 (16-proton multiplet, aromatic); τ 3.52–4.8 (4-proton multiplet, pyranoside ring protons); τ 5.22–5.6 (3-proton multiplet, other ring proton and C-6-methylene protons); τ 6.44 (3-proton singlet, OMe).

Anal. Calc. for $C_{35}H_{26}N_4O_{18}$: C, 53.16; H, 3.29. Found: C, 52.90; H, 3.41.

Methyl 2,3,4,6-tetra-O-(p-*ethoxycarbonylbenzoyl*)-α-D-*glucopyranoside* (7). — A solution of 10 ml of *p*-ethoxycarbonylbenzoyl chloride (prepared from the half-acid ester of terephthalic acid by the thionyl chloride method) was added, with stirring, to a solution of methyl α-D-glucopyranoside (1.5 g, 0.077 mole) in anhydrous pyridine (12 ml) at 0°, and the mixture was kept overnight at room temperature. The mixture, which had become dark red, was processed in the usual way, affording a dark-brown gum which did not crystallize, and was therefore purified by chromatography on neutral alumina. This treatment gave a pale-yellow oil which solidified on trituration with ether–petroleum ether (b.p. 30–40°). Several recrystallizations from alcohol gave colorless needles (yield 50–60%); m.p. 125°; $[\alpha]_D^{23}$ +69.3 °(*c* 2.0, acetone); ν_{max}^{KBr} 1761 (CO), 1592, 1520, 1471 (w, aryl C=C), 1290 (broad, -CO_2R-) cm^{-1}; n.m.r. data: τ 1.93, 1.98, 2.03, 2.09 (multiplets, 16 protons, aromatic); τ 3.67–4.88 (4-proton multiplet,

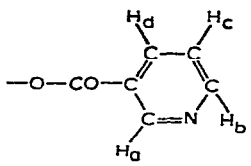
pyranoside ring protons); τ 5.28–5.88 (11-proton multiplet, 1 ring proton, 2 C-6-methylene protons and $8CO_2C\underline{H_2}CH_3$ protons); τ 6.46 (3-proton singlet, -OMe); τ 8.33–8.83 (12-proton multiplet, $-OCOCH_2C\underline{H_3}$).

Anal. Calc. for $C_{47}H_{46}O_{18}$: C, 62.80; H, 5.12. Found: C, 62.94; H, 5.17.

Methyl 2,3,4,6-tetra-O-(*o-chlorobenzoyl*)-α-D-*glucopyranoside* (**8**). — This compound was prepared from methyl α-D-glucopyranoside and *o*-chlorobenzoyl chloride by the method used for **7**, and the product was purified by chromatography on alumina. The resulting solid was recrystallized from alcohol–chloroform, giving a colorless, granular solid (yield 70%); m.p. 118–9°; $[\alpha]_D^{23}$ +76.6° (*c* 2.1, acetone); ν_{max}^{KBr} 1770 (CO), 1608, 1488, 1449 (aryl C=C), 1266 (CO_2R) cm^{-1}; n.m.r. data: τ 1.93–3.1 (16-proton multiplet, aromatic); τ 3.5–4.87 (4-proton multiplet, pyranoside ring protons); τ 5.16–5.8 (3-proton multiplet, other ring protons, and C-6-methylene protons); τ 6.55 (3-proton singlet, OMe).

Anal. Calc. for $C_{35}H_{26}Cl_4O_{10}$: C, 56.15; H, 3.47. Found: C, 56.45; H, 3.46.

Methyl 2,3,4,6-tetra-O-*nicotinoyl*-α-D-*glucopyranoside* (**3**). — Nicotinoyl chloride (9.0 g, 0.064 mole, prepared by the method of Wingfield *et al.*[14]) was added, with stirring, to a solution of methyl α-D-glucopyranoside (2.0 g, 0.010 mole) in a mixture of pyridine (10 ml) and chloroform (20 ml) at 0°. The mixture was kept overnight at room temperature, and then poured over ice, and the mixture was extracted with chloroform. The extract was extracted several times with cold, dilute sulfuric acid, and the aqueous extracts were combined and neutralized with dilute sodium hydroxide solution. An oil separated, and was collected by extraction with chloroform; the extract was washed with water, dried, and evaporated to dryness, giving a residue which was crystallized from alcohol–ether; fine needles (yield 60%); m.p. 142° (lit.[15] m.p. 137°); $[\alpha]_D^{23}$ +96.1° (*c* 2.0, acetone); ν_{max}^{KBr} 1754 (CO), 1613, 1497, 1441 (C=C and C=N, pyridine ring), 1307 ($-CO_2R$) cm^{-1}; n.m.r. data: four sets of multiplets of a total of 16 protons (due to the pyridine ring) centered at τ 0.84 (4 protons, H_a); τ 1.265 (4 protons, H_b); τ 1.74 (4 protons, H_d); τ 2.63 (4 protons, H_c); τ 3.47–4.84 (4-proton multiplet, pyranoside ring protons); τ 5.1–5.74 (3-proton multiplet, other ring protons, and C-6-methylene protons); τ 6.44 (3-proton singlet, OMe).



Anal. Calc. for $C_{31}H_{26}N_4O_{10}$: C, 60.59; H, 4.23. Found: C, 60.93; H, 4.53.

Irradiation. — The esters of methyl α-D-glucopyranoside in the solid state were irradiated in air at room temperature in the SRRL ^{60}Co radiation source[16] to the desired dosage. The dose rate, determined by ferrous–ferric dosimetry[17], was 7.3×10^{19} ev/g/h. The irradiated samples were analyzed immediately after irradiation.

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STUDIES ON URONIC ACID MATERIALS

PART XXVI*. THE ALDOBIOURONIC ACIDS IN GUMS FROM *Acacia* SPECIES

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ABSTRACT

Acacia seyal gum contains four aldobiouronic acids that have been characterised as 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose (*A*), 4-*O*-(α-D-glucopyranosyluronic acid)-D-galactose (*B*), 6-*O*-(4-*O*-methyl-β-D-glucopyranosyluronic acid)-D-galactose (*C*), and 4-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-galactose (*D*). With these acids as chromatographic standards, the aldobiouronic acids present in a further seventeen species of *Acacia* gum have been investigated. Gums having positive rotations and significant methoxyl contents give aldobiouronic acids *A*–*D*, whereas gums having positive rotations and low methoxyl contents do not give significant proportions of acids *C* and *D*. Gums having negative rotations and significant methoxyl contents give acids *A* and *C*; one gum having a negative rotation and a low methoxyl content did not give a significant proportion of acid *C*.

INTRODUCTION

Although *Acacia* gums have been studied for many years, the possibility of the presence of methoxyl groups was largely ignored in early work. Recently, however, it was suggested[2] that a re-examination of the aldobiouronic acid fraction of species studied earlier (*e.g.*, *A. pycnantha*) might reveal structurally significant features, and it was reported[3] that *A. nilotica* gum contains four aldobiouronic acids. We have therefore investigated the nature of the aldobiouronic acids present in eighteen different species of *Acacia* gum, in order to ascertain whether the presence of four aldobiouronic acids is a general feature within the *Acacia* group. Structural investigations undertaken simultaneously with the present study have shown that *A. senegal*[4] gum and *A. laeta*[5] gum each contain two aldobiouronic acids and that *A. arabica*[6] gum contains four aldobiouronic acids. Stephen and Vogt[7] have also reported the result of a re-investigation of *A. karroo* gum, which contains two aldobiouronic acids.

EXPERIMENTAL

Origins of specimens. — The origins of the following specimens of gum have

*For Part XXV, see Ref. 1.

been given elsewhere: *Acacia nilotica*[3,8], *A. arabica*[6,8], *A. campylacantha*[8], *A. fistula*[8], *A. nubica*[8], *A. senegal*[4], *A. laeta*[5], *A. drepanolobium*[8], *A. dealbata*[8], and *A. seyal*[9]. The following specimens were collected by the Sudanese Gum Research Officer: *A. multijuga, A. mellifera, A. tortilis, and A. adansoniana.* The following specimens were collected by officers of the Botanical Research Institute, Pretoria: *A. giraffae* Burch., *A. karroo* Hayne, *A. mearnsii* De Willd. (syn. *A. mollissima* and *A. decurrens* of some authors). The gum from *A. pycnantha* was collected by Mr. Bednall, Conservator of Forests, Adelaide.

Analytical methods. — The standard methods involved have been described[5]. Gas–liquid chromatography was performed as described previously[4]. Paper chromatography was performed as described[3], except that solvent *E* was ethyl acetate–acetic acid–formic acid–water (18:8:3:9), and solvent *G* was butanone–water–conc. ammonia (200:17:1).

RESULTS

Location of methoxyl groups. — A report[10] that *A. seyal* gum probably contained a mono-*O*-methylpentose (R_G 0.55; solvent *A*) could not be confirmed, and a report[11] that *A. mollissima* gum contained 4-*O*-methylglucuronic acid led to the following experiments to discover if the methoxyl content of *A. senegal* gum is associated with its acidic components. A sample of *A. senegal* gum (3% aqueous solution) was autohydrolysed at 100°. Samples were taken at intervals, and dialysed, and the non-dialysable material was freeze-dried. The results of analyses of the uronic anhydride and methoxyl content are shown in Table I.

TABLE I

AUTOHYDROLYSIS OF *A. senegal* GUM

Time of hydrolysis, h	*Uronic anhydride (UA), %*	*Methoxyl (OMe), %*	*OMe × 100/UA*
0	15.5	0.24	1.6
10	18.0	0.29	1.6
24	20.2	0.31	1.5
36	21.5	0.34	1.5
50	21.7	0.37	1.6

To ascertain whether the methoxyl content was present to any extent as the methyl ester group of a uronic acid, a 5% solution of the sodium salt of *A. seyal* gum (preferred to *A. senegal* gum because of its higher methoxyl content[2]) in 0.05N sodium hydroxide was kept at 60°, and samples were taken at intervals. After neutralisation and dialysis, the freeze-dried samples were analysed as shown in Table II. A similar constancy in the ratio of methoxyl to uronic anhydride content was also obtained for samples of the gum from *A. senegal, A. nilotica,* and *A. tortilis.*

The aldobiouronic acids of A. seyal. — A sample of the gum was hydrolyzed (N sulphuric acid, 100°, 8 h). Paper chromatography (solvent *D*) showed four acidic

components *A*, *B*, *C*, and *D* having R_{Gal} values of 0.26, 0.32, 0.60, and 0.69, respectively. Components *A* and *B* were separated from *C* and *D* by chromatography on Whatman No. 3MM paper (solvent *D*). Hydrolysis (2N sulphuric acid, 6 h) of components *A* and

TABLE II

SAPONIFICATION OF *A. seyal* GUM

Time of saponification, h	*Methoxyl (OMe), %*	*Uronic anhydride (UA), %*	*OMe×100/UA*
0	0.86	9.9	8.7
12	0.82	9.7	8.2
24	0.84	9.3	9.0
32	0.71	8.8	8.1
48	0.72	7.8	9.2
56	0.65	8.0	8.1
72	0.66	7.8	8.5

B gave, on paper-chromatographic fractionation, D-galactose {$[\alpha]_D$ +81° (*c*, 1.0); m.p. and mixed m.p. 169°} and D-glucurono-6,3-lactone {$[\alpha]_D$ +18° (*c*, 1.0); m.p. and mixed m.p. 177°}. A portion (*ca.* 40 mg) of the mixture of acids *A* and *B* was dissolved in methyl sulphoxide (10 ml), and methylated[12] with sodium hydride (500 mg) and methyl iodide (1.4 ml). After methanolysis, the products were identified (g.l.c.) as 2,3,4-tri-*O*-methyl-D-glucuronic acid, 2,3,4-, 2,3,5-, and 2,3,6-tri-*O*-methyl-D-galactose. A portion of the mixture of methyl glycosides was hydrolysed to the free sugars; paper chromatography (solvents *F* and *G*) indicated the presence of 2,3,4-tri-*O*-methyl-D-glucuronic acid, and 2,3,4-tri- and 2,3,6-tri-*O*-methyl-D-galactose.

Hydrolysis of the mixture of acids *C* and *D* gave galactose and 4-*O*-methylglucuronic acid (examination in solvents *D* and *E*). A portion (60 mg) of the mixture was methanolysed and neutralised (Ag_2CO_3). The filtered solution was evaporated to give a syrup that was dissolved in ammoniacal methanol (10%) and kept at 0°. After some months, crystals of the amide of methyl 4-*O*-methyl-α-D-glucopyranosiduronic acid were obtained, which, after recrystallisation from ethanol, had m.p. 231–233° (lit.[13], 234–236°). After removal of the crystalline amide, the solution was concentrated, and the residue was hydrolysed. Paper chromatography showed the presence of galactose and 4-*O*-methylglucuronic acid; D-galactose {m.p. and mixed m.p. 169–170°; $[\alpha]_D$ +80° (*c* 1.0)} was isolated by chromatography on 3MM paper.

A portion of the mixture of acids *C* and *D* was methylated in methyl sulphoxide[12], methanolysed, and examined by g.l.c. and by paper chromatography, as described for the mixture of acids *A* and *B*. Acids *C* and *D* gave the same products as acids *A* and *B*.

The acids *A*, *B*, *C*, and *D* were then separated by chromatography on thick paper with solvent *D*. As the zones moved closely together, they were detected by dipping the chromatograms (after careful drying to remove all of the acetic and formic acids) in a mixed-indicator system containing Thymol Blue (50 mg), Methyl Red

(250 mg), and Bromothymol Blue (600 mg) in ethanol (1 litre), to which N sodium hydroxide solution had been added until the colour became blue–green; acidic sugars appeared as red spots on a yellow–green background. The sugar acids were eluted from the paper with water, and the aqueous solutions (at mildly acidic pH) were extracted with chloroform to remove the indicators.

Acid *A* {R_{Gal} 0.26 (solvent *D*), $[\alpha]_D$ −3° (*c* 2.5)} was chromatographically identical with authentic 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose in solvents *D* and *E*. Formaldehyde was not evolved on attempted periodate oxidation. A portion (200 mg) of the acid was methylated by four additions of the Haworth reagents, the methylated sugars were extracted with chloroform, the extract was concentrated, and the residue was hydrolysed. Paper chromatography (solvents *A* and *E*) revealed the presence of 2,3,4-tri-*O*-methyl-D-glucuronic acid and 2,3,4-tri-*O*-methyl-D-galactose. The uronic acid had already been characterised in the unmethylated, acidic disaccharide, and was not investigated further. The 2,3,4-tri-*O*-methyl-D-galactose (80 mg) was isolated by chromatography on thick paper (solvent *A*), and had R_G 0.63 (solvent *A*); $[\alpha]_D$ +110° (*c* 1.0); the aniline derivative had m.p. 168°.

Acid *B* {R_{Gal} 0.32 (solvent *D*), $[\alpha]_D$ +101° (*c* 0.2)} was chromatographically identical with authentic 4-*O*-(α-D-glucopyranosyluronic acid)-D-galactose in solvents *D* and *E*. Hydrolysis gave only galactose and glucuronic acid; periodate oxidation at pH 8 for 24 h gave formaldehyde. A portion (150 mg) of the acid was methylated as described for acid *A*, and the methyl glycosides produced on methanolysis of the product had the same retention times in g.l.c. as the glycosides of 2,3,4-tri-*O*-methyl-D-glucuronic acid and 2,3,6-tri-*O*-methyl-D-galactose. The latter compound was isolated by chromatography on thick paper with solvent *A* {yield, 50 mg; R_G 0.70 (solvent *A*); $[\alpha]_D$ +84° (*c* 0.5)}; the product was oxidised with bromine to give 2,3,6-tri-*O*-methyl-D-galactonolactone, m.p. 96°.

Acid *C* {R_{Gal} 0.60 (solvent *D*); $[\alpha]_D$ +4° (*c* 0.2)} was chromatographically identical with authentic 6-*O*-(4-*O*-methyl-β-D-glucopyranosyluronic acid)-D-galactose in solvents *D* and *E*. Hydrolysis gave *ca.* equal amounts of 4-*O*-methylglucuronic acid and galactose. Formaldehyde was not produced on attempted oxidation with periodate A portion (200 mg) of the acid was methylated and then hydrolysed, and the resulting methylated sugars were the same as those given by acid *A*. The 2,3,4-tri-*O*-methyl-D-galactose was characterised as 2,3,4-tri-*O*-methyl-*N*-phenyl-D-galactosylamine, m.p. 164°.

Acid *D* {R_{Gal} 0.69 (solvent D), $[\alpha]_D$ +95° (*c* 0.1)} was chromatographically identical with authentic 4-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-galactose. Hydrolysis gave *ca.* equal amounts of galactose and 4-*O*-methylglucuronic acid. Formaldehyde was released on periodate oxidation. A portion (120 mg) of the acid was methylated, and the methyl glycosides produced on methanolysis of the product were the same as those given by acid *B*; the 2,3,6-tri-*O*-methyl-D-galactose was characterised as 2,3,6-tri-*O*-methyl-D-galactonolactone, m.p. 94°.

Identification of the aldobiouronic acids in other Acacia *species.* — Each of the remaining seventeen species of *Acacia* gum was subjected to partial hydrolysis (N sul-

phuric acid, 100°, 8 h), and the hydrolysates were examined by paper chromatography in solvents *D* and *E*, with acids *A–D* as reference standards. The results are given below.

Specific rotations of the Acacia *species containing aldobiouronic acids* A, B, C, *and* D. — *A. adansoniana* (+83°); *A. arabica* (+100°); *A. drepanolobium* (+75°); *A. fistula* (+61°); *A. multijuga* (+40°); *A. nilotica* (+108°); *A. seyal* (+58°); *A. tortilis* (+96°); and *A. giraffae* (water-soluble portion, positive rotation). Independent investigations, carried out simultaneously with this study, have confirmed the results for *A. nilotica*[3], *A. arabica*[6], *A. drepanolobium*[14], and *A. seyal*[15]. *A. karroo* (+54°) and *A. nubica* (+100°) gave isolable amounts of acids *A* and *B*, but only chromatographic traces of acids *C* and *D*; the methoxyl content of these species is low, and the proportions of acids *C* and *D*, if present, must be very small.

Specific rotations of the Acacia *species containing aldobiouronic acids* A *and* B *only.* — *A. campylacantha* (−3°); *A. dealbata* (−25°); *A. laeta* (−36°); *A. mearnsii* (−49°); *A. mellifera* (−51°); *A. senegal* (−31°); and *A. pycnantha* (− 8°; this species gave only a chromatographic trace of acid *C*). These conclusions have been verified independently for *A. laeta*[5] and *A. senegal*[4].

DISCUSSION

The results in Tables I and II indicate that the methoxyl content in an *Acacia* gum polysaccharide is associated with the uronic acid residues, but is not present as methyl ester. Searches to date have failed to confirm the presence of any methylated sugars in the gums studied.

The four aldobiouronic acids present in *A. seyal* gum are 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose (*A*), 4-*O*-(α-D-glucopyranosyluronic acid)-D-galactose (*B*), 6-*O*-(4-*O*-methyl-β-D-glucopyranosyluronic acid)-D-galactose (*C*), and 4-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-galactose (*D*). These acids have been used as reference standards in a chromatographic examination of the aldobiouronic acids in seventeen further *Acacia* species of gum.

Of the species studied to date, those having positive specific rotations contain the four aldobiouronic acids (*A–D*); the exceptions are *A. karroo* and *A. nubica* gums, which have low methoxyl contents and consequently small proportions (if any) of the 4-*O*-methyl acids *C* and *D*. The *Acacia* species having negative specific rotations contain only aldobiouronic acids (*A* and *C*) that have β-D linkages; the presence of acid *C* in *A. pycnantha* gum is doubtful, however, since this species has a low methoxyl content.

From the species studied to date, it therefore appears that, provided the species in question has a significant methoxyl content, *Acacia* gums having positive specific rotations contain aldobiouronic acids *A–D*, whereas *Acacia* species having negative specific rotations contain only acids *A* and *C*.

The uronic acid content of the majority of *Acacia* gums is only of the order of 10%, and it is therefore unlikely that the presence of the aldobiouronic acids having α-D linkages (acids *B* and *D*) is alone sufficient to convert a negative rotation into a

strongly positive one. The explanation is more likely to be found in fundamental structural differences involving the arabinose side-chains and/or the branched framework of galactose residues.

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TWO *aldehydo*-D-ERYTHROSE DERIVATIVES

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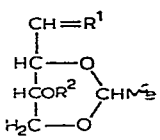
ABSTRACT

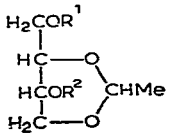
The *aldehydo*-D-erythrose derivatives 3-*O*-benzyl-2,4-*O*-ethylidene-*aldehydo*-D-erythrose and 3-*O*-benzoyl-2,4-*O*-ethylidene-*aldehydo*-D-erythrose have been prepared. Both sugar derivatives have been prepared by oxidation of a partially protected D-erythritol, and by degradation of a protected D-erythrose hydrazone with nitrous acid.

INTRODUCTION

A spontaneous dimerization, polymerization, or aldolization of tetroses and of partially protected tetroses has earlier been reported to occur under very mild conditions[1-9]. To avoid side reactions in an aldol condensation of erythrose, an *aldehydo* derivative of the tetrose was required. This paper describes the preparation of 3-*O*-benzyl-2,4-*O*-ethylidene-*aldehydo*-D-erythrose (**4**) and of 3-*O*-benzoyl-2,4-*O*-ethylidene-*aldehydo*-D-erythrose (**6**). Both *aldehydo*-D-erythrose derivatives **4** and **6**, which have not been obtained in crystalline form, have been prepared by two methods, namely, by oxidation of a partially protected D-erythritol, and by degradation of a protected D-erythrose hydrazone. The preparation of **4** by a different way has recently been reported[10].



1 $R^1 = O$; $R^2 = H$
2 $R^1 = NN(Me)Ph$; $R^2 = H$
3 $R^1 = NN(Me)Ph$; $R^2 = CH_2Ph$
4 $R^1 = O$; $R^2 = CH_2Ph$
5 $R^1 = NOH$; $R^2 = CH_2Ph$
6 $R^1 = O$; $R^2 = Bz$
7 $R^1 = NOH$; $R^2 = Bz$



8 $R^1 = H$; $R^2 = H$
9 $R^1 = Tr$; $R^2 = H$
10 $R^1 = Tr$; $R^2 = CH_2Ph$
11 $R^1 = Tr$; $R^2 = CH_2C_6H_4Br(p)$
12 $R^1 = Tr$; $R^2 = Bz$
13 $R^1 = H$; $R^2 = CH_2Ph$
14 $R^1 = Ts$; $R^2 = CH_2Ph$
15 $R^1 = COC_6H_4NO_2(p)$; $R^2 = CH_2Ph$
16 $R^1 = H$; $R^2 = COC_6H_4Br(p)$
17 $R^1 = H$; $R^2 = Bz$
18 $R^1 = Ts$; $R^2 = Bz$

2,4-*O*-Ethylidene-1-*O*-trityl-D-erythritol (**9**)[11] was etherified with benzyl chloride or with *p*-bromobenzyl bromide in the presence of potassium hydroxide to give 3-*O*-benzyl-2,4-ethylidene-1-*O*-trityl-D-erythritol (**10**) and 3-*O*-*p*-bromobenzyl-2,4-*O*-ethylidene-1-*O*-trityl-D-erythritol (**11**) respectively. Both reactions are exothermic. Benzoylation of **9** in pyridine solution gave 3-*O*-benzoyl-2,4-*O*-ethylidene-1-*O*-trityl-D-erythritol (**12**).

Detritylation of **10**, **11**, and **12**, with no noticeable hydrolysis of the acetal or ester bonds, was achieved in aqueous acetic acid at boiling water temperature in a few minutes. 3-*O*-Benzyl-2,4-*O*-ethylidene-D-erythritol (**13**), was identified as its *p*-toluenesulfonic (**14**) and *p*-nitrobenzoic (**15**) esters. 3-*O*-Benzoyl-2,4-*O*-ethylidene-D-erythritol (**17**) was also identified as the *p*-toluenesulfonic ester (**18**).

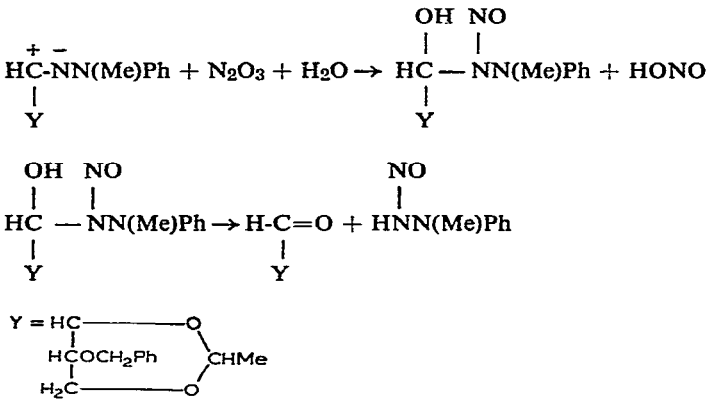
Attempts to oxidize the primary hydroxyl groups of **13** and **17** with methyl sulfoxide and acetic anhydride[12] or to oxidize their *p*-toluenesulfonic esters **14** and **18** with methyl sulfoxide and sodium hydrogen carbonate[13] to the respective *aldehydo* sugar derivatives **4** and **6** were unsuccessful. Also, heterogeneous oxidation of **13** in benzene solution with manganese dioxide[14] did not give the required carbonyl compound. The *aldehydo*-D-erythrose derivatives **4** and **6** were formed, however, by oxidation of **13** and **17** with *N*,*N*′-dicyclohexylcarbodiimide in methyl sulfoxide according to Pfitzner and Moffatt[15,16].

2,4-*O*-Ethylidene-D-erythrose (**1**)[7] isolated as its methyl phenylhydrazone (**2**) was etherified to 3-*O*-benzyl-2,4-*O*-ethylidene-D-erythrose 2-methyl-2-phenylhydrazone (**3**). Sugar phenylosazones have been shown to react with nitrous acid to produce, depending on the amount of reagent used, aldosuloses ("osones") or aldosulose 1-phenylhydrazones[17]. Ohle *et al.*[17] suggested that the free phenylhydrazine, formed from the hydrazone by hydrolysis, reacted with nitrous acid to form phenyl azide. Wolfrom *et al.*[18] found that acetylated hexose phenylosazones treated with nitrous acid give phenylosotriazole tetraacetates in about 80% yield. For the phenylosazone reaction they suggested a mechanism involving formation of N^1-nitroso derivatives of the cyclic hydrazino hydrazone, followed by hydrolytic cleavage of the C–N bond; for the acyclic acetylated phenylosazone reaction they postulated N^2-nitroso-intermediates which were cleaved at the N^1–N^2 bonds with subsequent cyclization to the triazole ring.

We report herein the application of the nitrous acid reaction to two protected, di-N^2-substituted hydrazones of D-erythrose. Thus, the *aldehydo*-D-erythrose derivatives **4** and **6** were obtained in good yields from the respective hydrazones (**3** and 2-benzoyl-2-phenylhydrazone of **6**) in dilute acetic acid at room temperature. Assuming that no hydrolysis of the hydrazone occurs, the mechanism shown at the top of the next page is suggested for the degradation of **3** by nitrous acid.

The 1-methyl-2-nitroso-1-phenylhydrazine presumably formed in this reaction is spontaneously decomposed[19] to form nitrous oxide and *N*-methylamine which is immediately nitrosated to *N*-methyl-*N*-nitrosoaniline. The latter amine has been isolated from the reaction mixture.

Ohle *et al.*[17] isolated D-*arabino*-hexosulose-1-(2-methyl-2-phenylhydrazone)



from the reaction of D-fructose-2-methyl-2-phenylosazone with two equivalents of nitrous acid. When **3** was treated with two equivalents of nitrous acid, in dilute acetic acid, the reaction mixture contained, as shown by t.l.c., the *aldehydo* sugar **4** and large proportions of unchanged **3**. The latter compound disappeared, however, from the reaction mixture when an excess of nitrous acid was used. Degradation of 3-*O*-benzoyl-2,4-*O*-ethylidene-D-erythrose 2-benzoyl-2-phenylhydrazone[7] with nitrous acid gave **6** and benzanilide. The latter compound is not nitrosated with nitrous acid[20] in aqueous acetic acid solution.

EXPERIMENTAL

General methods. — Etherifications and esterifications were performed with magnetic stirring. Etherifications with benzyl chloride or *p*-bromobenzyl bromide were performed with technical potassium hydroxide (B.D.H., flake). Thin-layer chromatography (t.l.c.) was performed at room temperature on silica gel (Kieselgel D-5, Camag, Muttenz, Switzerland) activated at 110°, using solvents: (a) butyl-alcohol–ethyl alcohol–water[21] (45:5:50), (b) ethyl acetate–cyclohexane[22] (1:1), (c) diisopropyl ether[23]. The compounds were detected by spraying with naphthoresorcinol–phosphoric acid[24]. Solvents were evaporated under vacuum at 45° (bath temperature). Unless otherwise stated, organic solutions were dried with sodium sulfate. Melting points were determined with a Büchi melting-point apparatus (Tottoli) and were not corrected. Infrared spectra were recorded with a Perkin–Elmer grating spectrophotometer model 337. Ultraviolet spectra were recorded with a Beckmann DB spectrophotometer.

2,4-O-Ethylidene-D-erythritol (**8**). — 4,6-*O*-Ethylidene-D-glucose[25] (103.0 g, 0.5 mole) in water (250 ml) was oxidized with sodium metaperiodate (224.7 g, 1.05 mole)[8]. The oxidation mixture was kept at room temperature for 1 h, then neutralized with barium carbonate, and the neutral filtrate was reduced with sodium borohydride (15.0 g), according to the procedure of Barker and MacDonald[25]. The crude product was crystallized from chloroform or from butyl acetate, m.p. 97° (58.5 g, 79%); R_F 0.63 in (a).

3-O-Benzyl-2,4-O-ethylidene-1-O-trityl-D-erythritol (**10**). — Compound[11] **9** (11.7 g, 0.03 mole), benzyl chloride (38.0 g, 0.3 mole), and potassium hydroxide (16.8 g, 0.3 mole) were stirred for 6 h at 100–110°. The cooled solution was filtered by suction (Celite), and the residue was washed with hot benzene. The filtrates were combined, washed with water, and dried. The solution was concentrated, the benzyl chloride was removed under vacuum (0.2 mm, boiling-water bath), and petroleum ether (60–80°) was added to the residue. Crystallization occurred usually overnight in the refrigerator. The product, m.p. 108–109° (13.5 g, 93.7%), was recrystallized from ethyl acetate and methanol or from benzene and methanol to a constant m.p. of 110–111°, $[\alpha]_D^{27}$ −24.0° (*c* 1.0, chloroform); R_F 0.77 in (b), 0.68 in (c).

Anal. Calc. for $C_{30}H_{32}O_4$: C, 79.97; H, 6.71. Found: C, 79.89; H, 6.66.

3-O-p-Bromobenzyl-2,4-O-ethylidene-1-O-trityl-D-erythritol (**11**). — Compound **9** (3.9 g, 0.01 mole), *p*-bromobenzyl bromide (3.25 g, 0.013 mole), xylene (10 ml), and potassium hydroxide (5.6 g, 0.1 mole) were stirred for 1.5 h at 135–140°. Benzene was added to the cooled reaction mixture, the solution was filtered by suction, and the residue was extracted with hot benzene. The combined filtrates were washed with water, dried, and the solvents evaporated. Residual amounts of xylene were removed at boiling-water temperature and 0.1 mm pressure. The residue was crystallized from methanol (100 ml), m.p. 92.5–93.5° (4.4 g, 78%). Recrystallization from the same solvent did not raise the m.p., $[\alpha]_D^{27}$ −18.8° (*c* 0.81, chloroform); R_F 0.77 in (b), 0.65 in (c).

Anal. Calc. for $C_{32}H_{31}BrO_4$: C, 68.71; H, 5.58; Br, 14.28. Found: C, 68.56; H, 5.63; Br, 14.41.

3-O-Benzoyl-2,4-O-ethylidene-1-O-trityl-D-erythritol (**12**). — A solution of compound **9** (11.7 g, 0.03 mole) in pyridine (100 ml) was benzoylated at 0° with benzoyl chloride (8.4 g, 0.06 mole) added in two portions. The reaction mixture was kept overnight at room temperature. Then most of the pyridine was evaporated, crushed ice was added to the residue and the mixture was kept overnight. The oily or solid ester that separated was dissolved in benzene, and the solution was washed with alkali, with acid, and then dried. The solid left after evaporation of the solvent was crystallized from dilute ethanol, m.p. 86° (13 g, 88%). The product was recrystallized from the same solvent to a constant m.p. of 88–90°, $[\alpha]_D^{27}$ −90.0° (*c* 1.5, chloroform); R_F 0.71 in (b).

Anal. Calc. for $C_{32}H_{30}O_5$: C, 77.71; H, 6.11. Found: C, 77.66; H, 6.11.

3-O-Benzyl-2,4-O-ethylidene-D-erythritol (**13**). — Compound **10** (14.4 g, 0.03 mole) was dissolved in hot acetic acid (90% v/v, 60 ml) and heated in a boiling-water bath. After 3 min at this temperature, water (120 ml) was added to the cooled solution. The reaction mixture was kept for a few hours in the refrigerator, and the quantitatively precipitated triphenylmethanol was removed by suction. The filtrate was extracted with chloroform (3 × 60 ml), and the combined extracts were washed with water and dried. The solvent was evaporated, and the residue (6.6–6.8 g, 92–95%), which distilled at 125° (0.3 mm), solidified on cooling at −30°

and was crystallized from petroleum ether (80–100°), white needles, m.p. 55°, $[\alpha]_D^{27}$ −52.5° (*c* 2.0, chloroform); R_F 0.49 in (b), 0.25 in (c).

Anal. Calc. for $C_{13}H_{18}O_4$: C, 65.53; H, 7.61. Found: C, 65.43; H, 7.39.

3-O-Benzyl-2,4-O-ethylidene-1-O-p-tolylsulfonyl-D-erythritol (**14**). — The oily compound **13**, obtained from **10** (0.03 mole) as described above, was dissolved in pyridine (30 ml) and *p*-toluenesulfonyl chloride (6.3 g, 0.33 mole) was added in portions at room temperature. The reaction mixture was kept at this temperature for 24 h. Water (3 ml) was then added, and the solution was concentrated. The residue was dissolved in benzene, and the solution was washed with acid, alkali, and dried. The residue, left after removal of the solvent, was crystallized from benzene–petroleum ether (60–80°), m.p. 70–71° (10.1 g, 85% based on **10**). Recrystallization from the same solvents raised the melting point to 71–72°, $[\alpha]_D^{27}$ −38.0° (*c* 2.0, chloroform); R_F 0.65 in (b), 0.39 in (c).

Anal. Calc. for $C_{20}H_{24}O_6S$: C, 61.20; H, 6.16; S, 8.16. Found: C, 61.15; H, 6.22; S, 8.26.

3-O-Benzyl-2,4-O-ethylidene-1-O-p-nitrobenzoyl-D-erythritol (**15**). — *p*-Nitrobenzoyl chloride (2.78 g, 0.015 mole) dissolved in pyridine (40 ml) was added, at room temperature, to a solution of **13** (2.38 g, 0.01 mole) in pyridine (10 ml). The crude product, obtained as described above for **14**, was crystallized from benzene–petroleum ether (60–80°) in long needles, m.p. 81–83° (3.4 g, 87%). Recrystallization raised the melting point to 84°, $[\alpha]_D^{27}$ −56.5° (*c* 1.5, chloroform); R_F 0.66 in (b), 0.48 in (c).

Anal. Calc. for $C_{20}H_{21}NO_7$: C, 62.01; H, 5.46; N, 3.61. Found: C, 62.00; H, 5.28; N, 3.40.

3-O-p-Bromobenzyl-2,4-O-ethylidene-D-erythritol (**16**). — The trityl ether **11** (16.8 g, 0.03 mole) was dissolved in hot aqueous acetic acid (90% v/v, 60 ml) maintained in a boiling-water bath. The solution was kept in the boiling bath for 3 min. The solution was cooled, 60 ml water were added, and the crystallization of the triphenylmethanol was completed overnight in the refrigerator. After filtration, water (120 ml) was added to the filtrate with stirring and the ether **16** crystallized. After the suspension had been kept overnight in the refrigerator, the crystals were filtered off, m.p. 55° (7.35 g, 77.2%). T.l.c. showed that the product was contaminated with triphenylmethanol: R_F 0.87 in (b), 0.83 in (c).

Three recrystallizations from petroleum ether (80–100°) produced the chromatographically pure ether, m.p. 56–57°, $[\alpha]_D^{27}$ −52.5° (*c* 0.4, chloroform); R_F 0.49 in (b), 0.25 in (c).

Anal. Calc. for $C_{13}H_{17}BrO_4$: C, 49.22; H, 5.40; Found: C, 49.27; H, 5.31.

3-O-Benzoyl-2,4-O-ethylidene-D-erythritol (**17**). — Detritylation of compound **12** (14.8 g, 0.03 mole) was performed in aqueous acetic acid (90% v/v, 60 ml) as described above. The cooled hydrolyzate was diluted with water (60 ml) and refrigerated overnight. The triphenylmethanol was removed by suction, and the filtrate was diluted with water (50 ml) and extracted with chloroform (3 × 50 ml). The chloroform solution was washed, dried, and concentrated. The syrupy residue was crystallized from cyclohexane–petroleum ether (b.p. 40–60°), m.p. 62–63° (6.8 g, 90%). Recrystallization

from the same solvent did not change the m.p., $[\alpha]_D^{27}$ −62.0° (*c* 2.0, chloroform); R_F 0.34 in (b).

Anal. Calc. for $C_{13}H_{16}O_5$: C, 61.89; H, 6.39; Found: C, 62.14; H, 6.33.

3-O-Benzoyl-2,4-O-ethylidene-1-O-p-tolylsulfonyl-D-erythritol (**18**). — A solution of compound **17** (2.52 g, 0.01 mole) in pyridine (5 ml) was *p*-toluenesulfonylated with 2.11 g *p*-toluenesulfonyl chloride in 20 ml pyridine as described above. The crude product left after removal of the solvent was crystallized from ethyl acetate–petroleum ether (40–60°), m.p. 138–139° (3.5 g, 86%). Recrystallization raised the m.p. to 139–140°, $[\alpha]_D^{27}$ −93.0° (*c* 2.0, chloroform); R_F 0.49 in (b).

Anal. Calc. for $C_{20}H_{22}O_7S$: C, 59.10; H, 5.45. Found: C, 59.16; H, 5.39.

2,4-O-Ethylidene-D-erythrose 2-methyl-2-phenylhydrazone (**2**). — The filtered solution of the crude **1**, prepared from 0.3 mole of 4,6-*O*-ethylidene-D-glucose (see above, **8**), was stirred in an ice bath, and a solution of 1-methyl-1-phenylhydrazine (0.33 mole, 39 ml) in aqueous acetic acid (1:1, 40 ml) was added dropwise. Crystals appeared after a short time. The reaction mixture was stirred for 1 h after all the hydrazine had been added. The hydrazone was collected by suction (yield practically quantitative). Crystallization from acetone or acetone–water formed plates, m.p. 151–153° (90–96%). Recrystallization from benzene gave needles, m.p. 152–153°, $[\alpha]_D^{27}$ +43.5° (*c* 2.0, chloroform) [lit.[10]: m.p. 152–153°, $[\alpha]_D^{20}$ −9.1° (absolute ethanol)]; R_F 0.29 in (b); $\nu_{max}^{chloroform}$: 3500 (OH); 2980, 2850 (HC=), 1700 (C=N), and 1600-1500 cm^{-1} (phenyl); $\lambda_{max}^{ethanol}$ 280 nm (ε 16700).

Anal. Calc. for $C_{13}H_{18}N_2O_3$: C, 62.38; H, 7.24; N, 11.19. Found: C, 62.34; H, 7.40; N, 11.36.

3-O-Benzyl-2,4-O-ethylidene-D-erythrose 2-methyl-2-phenyl-hydrazone (**3**). — A stirred solution of **2** (15.0 g, 0.06 mole) in benzene (50 ml) was heated under reflux with potassium hydroxide (13.4 g, 0.24 mole) while benzyl chloride (30.4 g, 27.4 ml, 0.24 mole) was added dropwise in 60–90 min. The heating was then continued for 6.5 h. The cooled reaction mixture was filtered by suction through Celite, and the residue was washed with benzene. The filtrate was washed with water and dried. The residue, left after the distillation of the benzene and the benzyl chloride (see above), solidified on cooling, and was crystallized from ethanol as pale-yellow needles, m.p. 105–106° (15.1 g, 74%). From the mother liquor, 1.5 g more of the product was obtained (yield 81%). Recrystallization from ethanol did not change the m.p., $[\alpha]_D^{27}$ + 72.5° (*c* 2.0, chloroform) [lit.[10]: m.p. 107–108°, $[\alpha]_D^{20}$ −30.1° (benzene)]; R_F 0.53 in (b). The infrared spectrum in chloroform was identical in the 3000–1400 cm^{-1} region with that of **2**.

Anal. Calc. for $C_{20}H_{24}N_2O_3$: C, 70.56; H, 7.10; N, 8.22; Found: C, 70.45; H, 6.95; N, 8.32.

3-O-Benzyl-2,4-O-ethylidene-aldehydo-D-*erythrose* (**4**) (*a*). — To a solution of **13** (7.14 g, 0.03 mole) in methyl sulfoxide (45 ml) and benzene (45 ml) were added pyridine (2.4 ml, 0.03 mole), trifluoroacetic acid (1.2 ml, 0.016 mole), and dicyclohexylcarbodiimide (18.6 g, 0.09 mole). The reaction mixture was kept at room temperature for 20 h, and then ether (750 ml), followed by a solution of oxalic acid dihydrate

(11.34 g, 0.09 mole) in methanol (75 ml), was added. The resulting suspension was stirred for 30 min and filtered. The filtrate was successively washed with water, a sodium hydrogen carbonate solution (8%), again with water, and then dried. The syrup obtained after removal of the solvent was dissolved in ethanol (25 ml), and a fresh solution of sodium hydrogen sulfite (15 g) in water (28 ml) was added. The solution was kept for 4 h at room temperature, and then evaporated to dryness. Benzene was added to the residue, and the solution was evaporated. The dry residue was thoroughly extracted with ether, and the residue was dissolved in a sodium carbonate solution (10%, 120 ml). The *aldehydo* sugar derivative was extracted from the aqueous solution with ethyl acetate or chloroform (5 × 20 ml). The extracts were dried and concentrated and the residual syrup was dissolved in acetone. The solution was filtered to remove some crystals of dicyclohexylurea and concentrated. The residual syrup (6.0 g, 84.6%) was shown by chromatography to be pure **4**.

(*b*). — The hydrazone **3** (13.6 g, 0.04 mole) was dissolved in acetic acid (240 ml) containing water (40 ml). Small portions of sodium nitrite (27.6 g, 0.4 mole) were added in 4 h to the stirred solution at room temperature. The solution was concentrated and the residue was partitioned between chloroform and water. The aqueous layer was thoroughly extracted with chloroform, and the combined extracts were washed with water, with a sodium hydrogen carbonate solution (8%), again with water, and then dried and concentrated. The residue, dissolved in ethanol (30 ml), was treated with a solution of sodium hydrogen sulfite (20.0 g, in 35 ml water) for 4 h at room temperature. The reaction mixture was concentrated to dryness. Ethanol (30 ml) and then ether (150 ml) were added to the residue. The sodium bisulfite addition compound was collected by suction, and dissolved in sodium carbonate solution (150 ml, 10%), and the solution was extracted with chloroform (3 × 50 ml). The extract was washed with water, dried, and the solvent evaporated. The oily residue (7.56 g, 80%) was shown by chromatography to be pure **4**, R_F 0.34 in (b), $[\alpha]_D^{27}$ −45.8° (*c* 0.98, chloroform); ν_{max} (sandwich cell) 1730 cm^{-1} (C=O). The derivative decomposed slightly on attempted distillation (125°/0.65 mm). The 2-methyl-2-phenylhydrazone prepared from **4** obtained by methods (*a*) or (*b*) was identical with authentic **3**.

*3-O-Benzyl-2,4-O-ethylidene-*D*-erythrose oxime* (**5**). — A solution of hydroxylamine hydrochloride (0.78 g, 11.4 mmoles) in water (5 ml) and pyridine (1 ml) was added to a solution of **4** (obtained by method (*a*) or (*b*), 2.7 g, 11.4 mmoles) in methanol (20 ml). The solution was kept overnight at room temperature, and then concentrated, and the syrupy residue was dissolved in benzene. The solution was washed with water, alkali, acid, and water again, and then dried and concentrated. The residue was crystallized from chloroform–cyclohexane as needles, m.p. 60°. Recrystallization from the same solvent raised the m.p. to 61–63°, $[\alpha]_D^{27}$ −5.9° (*c* 1.0, chloroform); R_F 0.42 in (b).

Anal. Calc. for $C_{13}H_{17}NO_4$: C, 62.14; H, 6.81; N, 5.57. Found: C, 62.16; H, 6.69; N, 5.41.

Isolation and identification of N-*nitroso*-N-*methylaniline*. — The hydrazone **3**

(18.7 g, 55 mmoles) was treated with sodium nitrite, and the sodium bisulfite addition compound of the *aldehydo* sugar derivative was isolated, as described above. The filtrate of the bisulfite compound was concentrated, and the residue was distilled under *vacuum*. The fraction distilling at 84–85°/0.5 mm (3.55 g, 47%) solidified in the refrigerator. The straw-yellow nitroso compound (positive Liebermann test) showed $\nu_{max}^{chloroform}$ 1470, 1500, 1600 (phenyl); 1440 (NO); and 2930, 3040 cm^{-1} (CH_3). It was identified by its reduction to 1-methyl-1-phenylhydrazine, characterized as the 2-hydroxy-5-nitrobenzaldehyde 2-methyl-2-phenylhydrazone[26], m.p. 178°, $\lambda_{max}^{methanol}$ 343 nm (ε 25880), $\lambda_{max}^{methanol}$ 303 (ε 23430). The compound showed no depression of the m.p. on admixture with an authentic sample of this hydrazone.

3-O-Benzoyl-2,4-O-ethylidene-aldehydo-D-*erythrose* (**6**). (*a*). — To a solution of **17** (3.78 g, 15 mmoles) in methyl sulfoxide (45 ml) and benzene (45 ml) were added pyridine (1.2 ml, 15 mmoles), trifluoroacetic acid (0.6 ml, 7.5 mmoles) and *N,N'*-dicyclohexylcarbodiimide (9.3 g, 45 mmoles). The reaction mixture was kept overnight at room temperature. Ether (375 ml) and then a solution of oxalic acid dihydrate (5.67 g, 45 mmoles) in methanol (35 ml) were added. The bisulfite addition compound of **6** was isolated from the ethereal solution, and the *aldehydo* sugar was obtained from it (yield 6–10%), as described above.

(*b*). — Sodium nitrite (19.3 g, 0.28 mole) was added, in portions, at room temperature, to a solution of 3-*O*-benzoyl-2,4-*O*-ethylidene-D-erythrose 2-benzoyl-2-phenylhydrazone[7] (17.76 g, 40 mmoles) in acetic acid (240 ml) and water (20 ml). After 4 h the solution was cooled in an ice–salt bath, and neutralized with sodium hydroxide (152 g dissolved in 400 ml of water). The aqueous solution was extracted with benzene (6 × 100 ml), and the combined extracts were washed, dried, and concentrated. The residue was dissolved in ethyl acetate, and petroleum ether (b.p. 40–60°) was added to turbidity. The crystals that separated were identified as benzanilide by their m.p. (163°) and the m.p. of an admixture with authentic benzanilide. The filtrate was concentrated, and the *aldehydo* sugar derivative **6** was separated through its sodium bisulfite addition compound, as described above, to yield a syrup (1.6 g, 16%). The product was dissolved in methanol, the solution was treated with carbon (Norit A), and the solvent evaporated. The residual syrup was shown by t.l.c. to be pure[6]; R_F 0.29 in (b), ν_{max} (sandwich cell) 1730 (C=O), and 2850, 2980 cm^{-1} (HC=). The sugar derivative was characterized as its oxime.

3-O-Benzoyl-2,4-O-ethylidene-D-*erythrose oxime* (**7**). — A solution of **6** obtained by method (*a*) or (*b*) (0.36 g, 1.44 mmoles) in methanol (6 ml) containing hydroxylamine hydrochloride (100 mg, 1.44 mmoles), pyridine (0.12 ml, 1.44 mmoles), and water (0.5 ml) was kept at room temperature for 24 h. The solution was concentrated, and the residue was dissolved in benzene. The solution was washed with water, acid, alkali, again with water, dried and evaporated. The syrupy residue was crystallized from cyclohexane as needles, m.p. 106° (0.266 g, 69.7%), $[\alpha]_D^{27}$ −84.0° (*c* 2.0, chloroform); R_F 0.44 in (b).

Anal. Calc. for $C_{13}H_{15}NO_5$: C, 58.86; H, 5.69; N, 5.28. Found: C, 59.02; H, 5.57; N, 5.18.

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Notes

Reaction of methyl sulfoxide–acetic anhydride with 1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose*†

The Pfitzner–Moffatt reagent[2] (methyl sulfoxide–*N*,*N*′-dicyclohexylcarbodiimide–pyridinium phosphate) provides a facile route for converting "isolated" secondary alcohols into ketones, and primary alcohols into aldehydes, under mild conditions. The reagent has been used in this laboratory[3] for converting 1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose (**1**) into 1,2:3,4-di-*O*-isopropylidene-6-*aldehydo*-α-D-*galacto*-hexodialdo-1,5-pyranose (**4**), an intermediate required for chain-extension reactions leading, by means of Grignard reagents, to C_8 sugar derivatives related to lincomycin. A disadvantage of Pfitzner–Moffatt[2] and similar[4] oxidation procedures is that removal of the nonvolatile byproducts of the oxidation may be experimentally inconvenient. Methyl sulfoxide–acetic anhydride, a reagent also very effective for converting "isolated" secondary alcohols into ketones[5], is particularly useful in the carbohydrate field[6,7], because all of the excess reagents and byproducts can be removed by evaporation at low temperature[7]. It was of interest to determine whether the methyl sulfoxide–acetic anhydride reagent could be used to prepare aldehydes from "isolated" primary alcohol groups in carbohydrate derivatives. The present report shows that treatment of the "isolated" primary alcohol **1** with methyl sulfoxide–acetic anhydride does *not* provide a practical, preparative route to the aldehyde **4**. Instead, the reaction gives the 6-(methylthio)methyl ether (**2**) of **1** as the principal product, together with small proportions of the 6-acetate (**3**) of **1** and, apparently, the aldehyde **4**.

Treatment of 1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose[8] (**1**) with methyl sulfoxide–acetic anhydride at room temperature caused conversion of **1** into products that migrated more rapidly than **1** on thin-layer chromatograms. After 48 h, when all of the starting material (**1**) had reacted, the product was isolated as a distilled syrup, which showed carbonyl absorption in the i.r. spectrum and which gave positive Tollens and Schiff tests. T.l.c. indicated the presence of a major, fast-moving component, together with small proportions of two slower-moving components. Chromatographic purification of the main component revealed that it was nonreducing, and its i.r. spectrum showed no absorption for a carbonyl group. One of the side-products

*Part VI in the series "Extension of Sugar Chains Through Acetylenic Intermediates". For Part V, see ref. 1.

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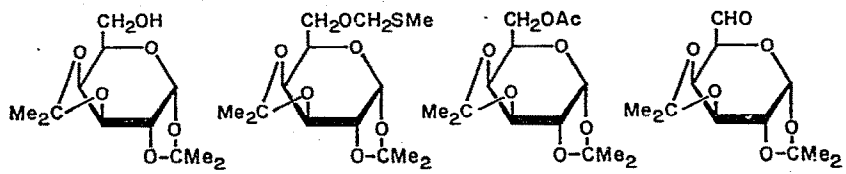
was isolated crystalline, and was identified as 6-*O*-acetyl-1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose[9,10] (**3**) by comparison with an authentic sample. The presence of this compound would account, in part, for the carbonyl absorption observed in the crude reaction product, and the presence of a small proportion of the aldehyde **4** can be inferred from the positive Schiff test observed with the crude product.



1 **2** **3** **4**

The major component of the reaction product was formulated as 1,2:3,4-di-*O*-isopropylidene-6-*O*-(methylthio)methyl-α-D-galactopyranose (**2**) on the basis of analytical and spectroscopic evidence. Microanalytical data supported the molecular formula assigned, and the n.m.r. spectrum revealed a pattern of signals almost identical to those given by known[10,11] 6-*O*-substituted derivatives of 1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose. The only signals in the spectrum not thus accounted for were a 3-proton singlet at τ 7.86 that may be assigned to an *S*-methyl group, and a 2-proton singlet at τ 5.29 that may be assigned to the protons of an -OCH_2S- group. No alternative formulation of the side-chain on C-5 that is compatible with the n.m.r. spectral data is possible, and the absence of carbonyl absorption from the i.r. spectrum rules out the possibility that aldehyde, ketone, or ester groups are present. The mass spectrum of the product **2** (see Figure 1) showed the molecular-ion peak at *m/e* 320, and a strong peak at *m/e* 305 corresponding to loss of a methyl group from the molecular ion. A series of weak peaks at *m/e* 247 (M-15-58), *m/e* 245 (M-15-60), and *m/e*

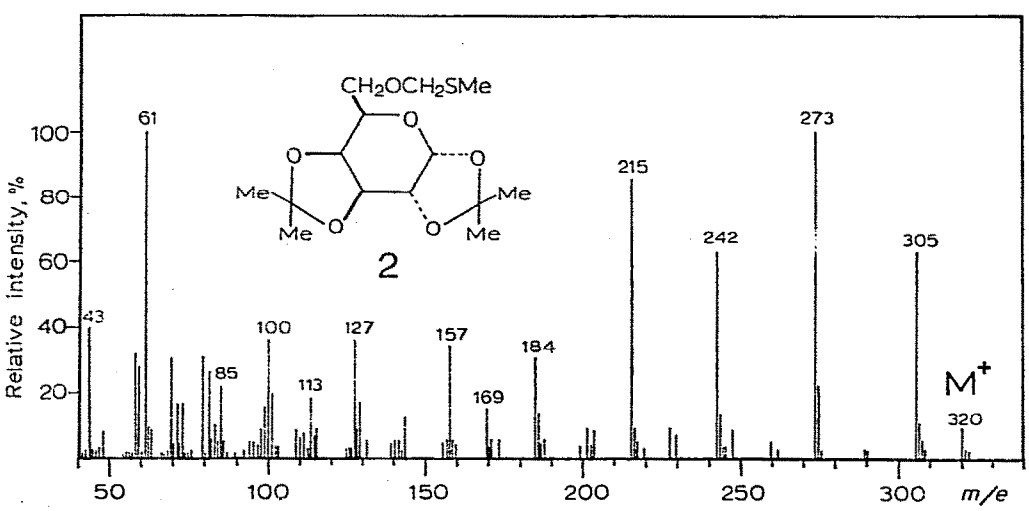


Fig. 1. The mass spectrum of 1,2:3,4-di-*O*-isopropylidene-6-*O*-(methylthio)methyl-α-D-galactopyranose (**2**).

187 (M-15-58-60) correspond to successive loss of acetone, acetic acid, or both, from the M-15 ion, as has been observed[12] in the mass spectrum of **1**. Strong peaks at *m/e* 100, 85, 59, and 43 may be assigned to the same fragments as those observed[12] from **1**. Strong peaks of high mass-number, not corresponding to fragmentations reported[12] for **1**, are observed in the spectrum of **2**. The fragment having *m/e* 273 results most probably from cleavage of the MeS· radical from the molecular ion; and that having *m/e* 215, by loss of the elements of acetone from this fragment. A strong peak at *m/e* 242 most probably arises from loss of the C-6 substituent and a rearranged hydrogen atom. A strong peak is observed at *m/e* 61; this probably corresponds to the $Me\text{-}\overset{+}{S}=CH_2$ ion.

It has recently been shown[13] that treatment of various noncarbohydrate alcohols with methyl sulfoxide–acetic anhydride may lead to (methylthio)methyl ethers in a side reaction that competes with oxidation to carbonyl derivatives.

EXPERIMENTAL

*Treatment of 1,2:3,4-di-*O*-isopropylidene-α-*D*-galactopyranose* (**1**) *with methyl sulfoxide and acetic anhydride.* — A slow stream of nitrogen was passed through a solution of **1** (6.83 g) in a mixture of dry methyl sulfoxide (27 ml) and acetic anhydride (18 ml) that was kept for 48 h at room temperature. The solution was poured into aqueous sodium hydrogen carbonate (1 liter) and, after 30 min, the mixture was extracted with three 100-ml portions of ether. The extract was washed with aqueous sodium hydrogen carbonate (100 ml), and with six 100-ml portions of water. The dried (magnesium sulfate) extract was evaporated, and the residue was distilled to give a yellow syrup, yield 4.86 g (71%), b.p. 130°/0.5 torr, that gave positive Schiff and Tollens tests and showed carbonyl absorption in its i.r. spectrum. T.l.c. on Silica Gel G (E. Merck, Darmstadt, Germany), with 19:1 benzene–methanol as developer and indication with sulfuric acid, showed the product to contain a major component, R_F 0.8 together with minor components having R_F 0.4 and 0.7. The starting material **1**, R_F 0.3, was absent. A 1-g portion of the syrup was resolved by chromatography on a column (25 × 2.5 cm) of Silica Gel Davison (grade 950, 60–200 mesh, Davison Division of the W. R. Grace Co., Inc., Baltimore, Maryland), with 9:1 benzene–ether as the eluant. The first 100 ml of effluent was discarded. The next 25 ml contained, as the sole component, the product having R_F 0.8 (392 mg). Fractions eluted in the next 100 ml (425 mg) contained mainly the component having R_F 0.8, in admixture with some of the component having R_F 0.7. A further 50 ml of effluent was collected, and was found to contain the product having R_F 0.7 as the sole component (50 mg). Crystallization of the latter from ethanol gave 6-*O*-acetyl-1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose (**3**), m.p. 106–107° (lit.[9] m.p. 108°), identical with an authentic sample[10] of **3** by mixed m.p., t.l.c., comparative i.r. and n.m.r. spectra,[10] and X-ray powder diffraction pattern.

*1,2:3,4-Di-*O*-isopropylidene-6-O-(methylthio)methyl-α-*D*-galactopyranose* (**2**). — The homogenous fraction (392 mg) having R_F 0.8, from the preceding experiment, was

distilled[14] to give **2** as a colorless syrup, b.p. 145–160° (bath)/0.1 torr, $[\alpha]_D^{25}$ −85 ±5° (*c* 0.9, chloroform); λ_{max}^{film} 7.25, 7.30 μm (CMe_2), hydroxyl and carbonyl absorption absent; n.m.r. data (chloroform-*d*, tetramethylsilane internal standard, Varian A-60 spectrometer): τ 4.46 (1-proton doublet, $J_{1,2}$ 4.8 Hz, H-1), τ 5.29 (2-proton singlet, OCH_2S), τ 5.38 (1 proton, $J_{2,3}$ 2.3 Hz, H-3), τ 5.60–6.36 (multiplets, 5 protons, H-2,4,-5,6,6'), τ 7.86 (3-proton singlet, SMe), τ 8.45, 8.54, 8.65 (3-, 3-, and 6-proton singlets, CMe_2).

Anal. Calc. for $C_{14}H_{24}O_6S$: C, 52.50; H, 7.56; O, 29.97; S, 10.00. Found: C, 52.46; H, 7.55; O, 30.10; S, 9.95.

Mass spectrum of **2**. — The sample was introduced* by a direct-inlet system, the temperature of the ion source was 250°, and the ionizing energy was 12 eV. The observed *m/e* values and relative intensities of the principal peaks are shown in Figure 1.

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Electron-spin resonance spectra of sodium–hydroxide cellulose system

Electron-spin resonance (e.s.r.) spectra of irradiated, frozen water and aqueous, alkaline solutions have attracted much attention[1–4], and efforts have been made to obtain evidence for the polarons or other free radicals postulated as being produced during irradiation. Although it had been considered that measurement of e.s.r. absorption of electrons in aqueous solutions is not feasible[5], attempts to overcome these difficulties have been made, and g-values have been assigned to trapped electrons in aqueous systems[1,6]. No report has, as yet, been made on the presence of free radicals in similar nonirradiated systems. We now report a multiple-signal, e.s.r. spectrum that indicates the existence of unexpected, and previously undetected, electron paramagnetic species in sodium hydroxide–cellulose systems. These species seem to be in equilibrium, and are relatively stable.

Purified cotton-cellulose was soaked in a sodium hydroxide solution (1 to 50% concentration) for 10–25 sec at about 25°, and then frozen by immersing it in liquid nitrogen. After it had been warmed to −100°, a six-line e.s.r. spectrum (see Fig. 1

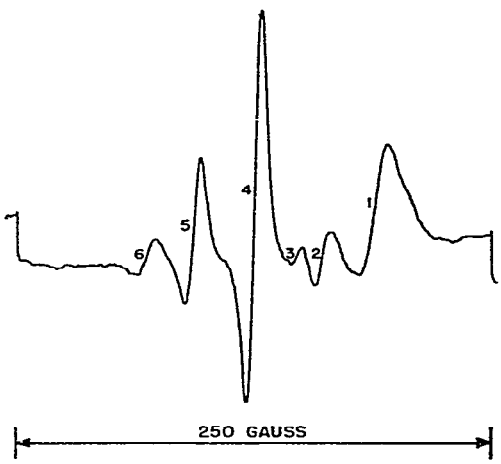

Fig. 1. Electron-spin resonance spectrum generated by sodium hydroxide (18%)–cellulose system at −100°. (Magnetic field increases from left to right.)

was obtained. With increasing concentration of the sodium hydroxide solution, the intensity of the e.s.r. spectrum increased to a maximum (at about 15–20% concentration) and then decreased. Neither the sodium hydroxide solution nor the purified cotton-cellulose (separately) generated an e.s.r. spectrum. When the sodium hydroxide was removed from the cellulose by washing the sodium hydroxide–cellulose with water and drying the cellulose, no e.s.r. spectrum was generated by the cellulose. On freezing, water-soaked cotton-cellulose did not give an e.s.r. signal; however, when treated with sodium hydroxide as described above, this cellulose generated an e.s.r. spectrum. De-aeration of the system did not change the nature of the e.s.r. spectrum.

Under various experimental conditions, the e.r.s. spectra generated by systems of cellulose plus (*a*) alkali metal, (*b*) alkaline-earth metal, or (*c*) tetraalkylammonium hydroxides were similar. At about 25°, the intensity of the e.s.r. spectra decayed, with a half-life of about one h. At higher temperatures, the intensity of the spectra decreased sharply. g-Values and line-widths for various absorption lines in the e.s.r. spectrum, as numbered in Fig. 1, are recorded in Table I. No simple relationships existed between the g-values, line-widths, and signal intensities.

TABLE I

ANALYSIS OF E.S.R. SPECTRA GENERATED BY SODIUM HYDROXIDE–CELLULOSE AND SODIUM HYDROXIDE–Cu^{2+} SYSTEMS AT −100°

	0.5M NaOH on cellulose		*10^{-4} M Cu^{2+} in 0.5M NaOH*		*10^{-4} M Cu^{2+} in 0.5M NaOH on cellulose*		*10^{-4}M Cu^{2+} in 18% NaOH on cellulose after 1.5 h at room temp.*	
Line Number	*g-Value*	*Line-width (gauss)*	*g-Value*	*Line-width (gauss)*	*g-Value*	*Line-width (gauss)*	*g-Value*	*Line-width (gauss)*
1	2.0045	13.3	2.0115	14.4	2.0052	17.8	2.0045	13.2
2	2.0206	8.0	2.0238	9.1	2.0204	10.6	2.0203	8.5
3	2.0294	5.4	2.0326	4.4	2.0289	—	2.0289	6.0
4	2.0427	7.4	2.0464	8.6	2.0432	9.5	2.0426	8.0
5	2.0630	7.8	2.0656	8.8	2.0633	9.5	2.0630	7.7
6	2.0778	9.0	2.0802	10.5	2.0787	13.0	2.0783	13.4

Copper(II) in sodium hydroxide solution generated an e.s.r. spectrum (see Fig. 2) similar to that generated by the sodium hydroxide–cellulose system (see Fig. 1). The line-widths of the spectrum generated by the Cu^{2+}–sodium hydroxide system (see Fig. 2) were slightly greater than those of the spectrum generated by the sodium hydroxide–cellulose system. Also, the g-values for the spectrum of the system containing Cu^{2+} had a tendency to shift to a magnetic field lower than those of the spectrum of the sodium hydroxide–cellulose system. The spectrum generated by the Cu^{2+}–sodium hydroxide–cellulose system was almost identical with those generated by the sodium hydroxide–cellulose system and the Cu^{2+}–alkali system, except that the line-widths were greater for the spectrum generated by the Cu^{2+}–sodium hydroxide–cellulose system. Part of the e.s.r. spectrum of the sodium hydroxide–cellulose system could be generated by a trace of copper, which, on addition of sodium hydroxide, was released from a natural cellulose–copper chelate. However, repeated extraction of the purified cellulose with solutions of alkali hydroxides and hydrazine did not diminish the species generating the e.s.r. spectrum.

When deuterated water was used instead of water in these systems, the resolution of the lines of the e.s.r. spectra was increased, and the line-widths of the more intense lines were decreased by about 30% of their normal magnitude; this result indicated that hydrogen and deuterium both interacted with the species generating the e.s.r.

signal. This interaction was mainly responsible for determining the line-widths of the signals. As the line-widths for the Cu^{2+}–NaOH and Cu^{2+}–NaOH–cellulose systems are slightly greater than those for the cellulose–NaOH system, Cu^{2+} seems to affect the line-widths.

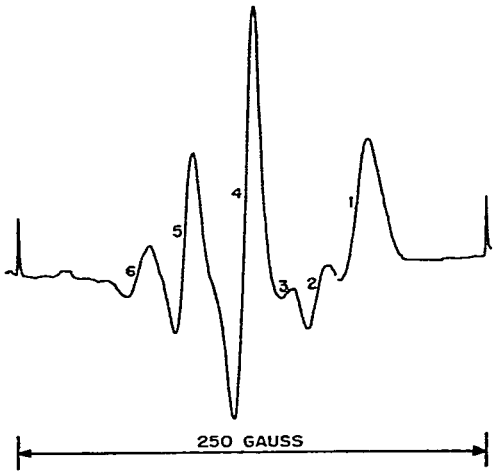

Fig. 2. Electron-spin resonance spectrum generated by 10^{-4} M Cu^{2+} in sodium hydroxide (18%) at −100°. (Magnetic field increases from left to right.)

The identity of these e.s.r. spectra, together with evidence that there is coupling with the protons of the aqueous system, suggests that these signal-generating species are trapped in the alkaline matrix instead of on the pyranoside ring or by the cupric ion. Furthermore, it suggests that these species are of the same nature, although the mechanism of their production may be different. This conclusion necessitates re-interpretation of some of the e.s.r. spectra of copper complexes[7].

It is tentatively suggested that the spin-free electron in the complex cupric ion in sodium hydroxide solution is delocalized by a strong ligand-field provided by OH^- or O^{2-} anions, and that it may, under certain conditions, become aquated, or spin-paired with another electron from the system. This would result in Cu^{3+} ions which, being powerful oxidants, may abstract an electron from the surrounding OH^- ions and thus produce ·OH radicals. The aquated or spin-paired electrons may produce other radicals. That this type of phenomenon occurs is indicated by the decreased interaction of the Cu nuclear spin with free electrons, resulting in very weak absorption attributed[7] to $g_{\parallel}$. Correspondingly, if the $g_{\perp}$ absorption lines are also weak, these must be masked by the new, unidentified lines now reported.

The ordered chains of the pyranoside rings in crystalline cellulose may, by their electrophilic character, also be reponsible for the stabilization of hydroxyl radicals and e_{aq} during the process of swelling or mercerization, or both. In this regard, potential gradients existing between the cellulose chains would be significant. During the soaking process and the reaction of sodium hydroxide with celllulose, ·OH radicals and free electrons could be produced; these radicals would react with the medium to

produce more-stable radicals. By lessening the rate of diffusion, cotton cellulose would increase the half-lives of the species generating the e.s.r. spectrum.

It has further been observed that the intensity of the lines in the e.s.r. spectrum of cupric ion dissolved in supercooled alkali glasses is much greater (by a factor of about 4) than when the glass crystallizes. This result indicates that the number of radicals is diminished on crystallization (as a result of recombination and destruction processes). Evidence is also available[8] that, on crystallization of these solutions, the e.s.r. spectrum shows absorptions, due mainly to cupric ion, having lines of equal intensity for $g_{\parallel}$ and $g_{\perp}$.

Work in progress is expected to elucidate these phenomena.

EXPERIMENTAL

The e.s.r. spectra were recorded with a Varian 4502-15 EPR spectrometer. The system was equipped with a dual sample-cavity, cell accessories for aqueous solutions and solid samples, and a variable-temperature device that permitted operation from about −185 to +300°. All of the spectra were recorded at —100°.

Cotton cellulose was purified as previously described[9]. The purified cellulose had the type I crystalline lattice, and a viscosity-average molecular weight of about 700,000.

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Acetic anhydride–phosphoric acid as an acetylating agent*

For some time, there has been a need for an effective agent for acetylating enolic compounds. For example, all known methods of acetylation, including the recently developed Fritz–Schenk[1] reagent (acetic anhydride and perchloric acid in ethyl acetate), have failed to provide an acetate from croconic acid[2]. It has now been found that the acetate of croconic acid can be obtained with a reagent prepared from acetic anhydride and anhydrous phosphoric acid.

The new reagent has proved to be effective for acetylating carbohydrates, cyclitols, enols, phenols, sterically hindered secondary alcohols, and tertiary alcohols; non-isomerized acetates are usually produced. The mildness of the acetylating agent is attributable to the non-oxidizing character of phosphoric acid; moreover, the latter is a good solvent, and, because of its chelate-forming ability, a stabilizer.

The active acetylating species in the acetic anhydride–phosphoric acid reagent is believed to be the monoacetic phosphoric anhydride (**1**), which was isolated as the disilver salt in a low yield. No diacetic or triacetic phosphoric anhydrides were isolable from the acetylating reagent.

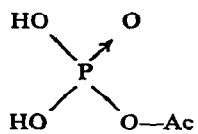

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Table I summarizes the conditions employed and the results obtained in the application of the acetylating agent to a variety of organic compounds. Table II summarizes the experiments that provide other acyl derivatives of *myo*-inositol. Tables I and II show the time used in each esterification and the yields of the esters produced; it is presumed that longer reaction times will afford higher yields.

EXPERIMENTAL

Anhydrous phosphoric acid. — Phosphorus pentaoxide (113 g) is dissolved in commercial orthophosphoric acid (150 g) at 60–70°. The resulting product is liquid, but crystallizes on standing (lit.[3] m.p. 42°). The crystalline material can be liquefied by heating it in an oven at 60° (prolonged heating at higher temperatures leads to formation of polyphosphoric acid[4,5]; polyphosphoric acid is not miscible with acetic anhydride under the conditions described). Instead of anhydrous phosphoric acid, superphosphoric acid, 105% (FMC Corporation, New York) may be used; anhydrous phosphoric acid is also available from Matheson Co., Chicago.

The acetylating reagent. — The acetylating reagent is prepared just before use by adding anhydrous phosphoric acid (1 ml) to acetic anhydride (4 ml) (mole ratio,

*Part II in the series *Methods in Inositol Chemistry*; for Part I, see Ref. 12 (b).

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TABLE I

ACETYLATION OF CERTAIN ORGANIC COMPOUNDS WITH ACETIC ANHYDRIDE IN PHOSPHORIC ACID

Starting material[a]	*Volume of reagent (ml)*	*Temp. of reaction (degrees)*	*Time of reaction (min)*	*product*	*Yield (%)*	*Melting point (degrees)*	$[\alpha]_D$ *(degrees)*	*Solvent*	*References*
α-D-Glucopyranose	10	55–60	5	α-pentaacetate[b,c]	65	114–115	+101	$CHCl_3$	9
Lactose	10	55–60	5	α-octaacetate	75	96–98	+53	$CHCl_3$	11
DL-*epi*-Inosose-2	25	55	30	pentaacetate	65	138–140			12
D-Glucitol	10	55	5	hexaacetate	90	99	+10	$CHCl_3$	13
myo-Inositol	25	60	5	hexaacetate	92	215–216			14
Kojic acid	10	25	5	diacetate	68	103			15
Croconic acid[2]	25	50	15	diacetate	70[b,d]	144–145			
p-Benzoquinone	10	60	5	1,2,4-triacetoxybenzene	55	95–97			16
2,5-Di-*tert*-amylhydroquinone	10	60	5	diacetate	90[e]	109–110			

[a]Wt., 1 g. [b]Combined solid and extracts. [c]Treatment of D-glucose with phosphoric propionic anhydride yielded a syrupy propionate (80% yield); D-glucose pentapropionate has been reported to be syrupy[10]. Similarly, D-glucose isobutyrate[10], m.p. 102–104°, was obtained. In the same way, 0.6 g of dextran was treated with phosphoric acetic anhydride (30 ml for 5 min at 85°); a solid acetate (0.9 g) was obtained. [d]A sample of the diacetate, recrystallized from chloroform (by concentration), yielded white prisms; the sample was dried for 2 h at 25°/0.05 mm and analyzed (Calc. for $C_9H_6O_7$: C, 47.79; H, 2.69. Found: C, 47.85; H, 2.77). On being kept at room temperature, the product underwent slow hydrolysis to the parent compound. [e]A sample of diacetate, recrystallized from ethanol, was dried for 2 h at 78°/0.05 mm (Calc. for $C_{20}H_{30}O_4$: C, 71.82; H, 9.04. Found: C, 71.94; H, 9.15).

TABLE II

PREPARATION OF PROPIONIC, BUTYRIC, AND ISOBUTYRIC ESTERS[14] OF *myo*-INOSITOL[a]

Reagent[b]	*Temperature of reaction (degrees)*	*Product*	*Yield (%)*	*Melting point (degrees)*
A	85–90	hexapropionate	97	115–116
B	90–95	hexabutyrate[c]	92	93–95
C	90–95	hexaisobutyrate[c,d]	90	180–181

[a]Treatment of 1 g of *myo*-inositol with 25 ml of the reagent for 15 min. [b]A, Phosphoric propionic anhydride; B, butyric phosphoric anhydride; C, isobutyric phosphoric anhydride. [c]During decomposition of the reaction mixture with ice–water, a small quantity of 5% aqueous sodium hydrogen carbonate was added to facilitate the dissolution of the acid. [d]No complete dissolution of the solid was observed.

1:1.7) in a graduated cylinder and stirring with a glass rod until a homogeneous solution is obtained. The temperature of the mixture usually rises to 50–55°.

Examination of the acetylating reagent. — The acetylating reagent (25 ml), prepared at 5°, was stirred with cold, dry 1:1 (v/v) benzene–chloroform) (200 ml) and the benzene–chloroform layer was separated from the syrupy residue (*A*), dried (magnesium sulfate and molecular sieves), and concentrated under diminished pressure to a syrup. The latter was kept in a deep-freeze for 100 h, and then at room temperature for 120 h, whereupon crystallization started. A white, waxy solid was isolated (3.2 g); when fresh, this material was soluble in benzene, but upon storage, its solubility in benzene diminished. A sample recrystallized from benzene–pentane began to decompose at 144–146° (effervescence) and melted completely at 300°; λ_{max}^{Nujol} triplet at 5.62, 5.72, and 5.88 μm (C=O); other prominent bands were observed at 2.88 (OH), 7.80 (P-CH_2), 8.20 (sh) (acetate); and 8.90 μm (acetate). Analysis showed C, 5.23; H, 1.8; P, 25.8. It was concluded, therefore, that the crystalline material corresponds most closely to an acetyl derivative of polyphosphoric acid[6].

The syrup (*A*) remaining after the extraction was sensitive to moisture; it was quickly dried *in vacuo* in the cold; $\lambda_{max}^{Nujol\ or\ film}$ 2.90 (OH), 5.65 (C=O), and 8.0–8.3 μm (acetate); the band at 5.65 μm disappeared after exposure of the product to the air for 3 min. Careful processing of syrup *A* at 0° afforded compound **1** as the sparingly soluble disilver salt[7,8]. The steps employed in its isolation were (*a*) partial neutralization (to pH 3–3.5) by shaking the syrup with ice-cold, saturated, aqueous potassium carbonate, (*b*) washing the syrup with water, (*c*) complete neutralization (to the phenolphthalein end-point) with barium carbonate and then with a saturated solution of barium hydroxide, and (*d*) treatment of the ice-cold, filtered solution with 25% aqueous silver nitrate. The yellowish white product which was precipitated (8–10 g), containing some silver acetate, was purified by a published procedure[8] in which the crude silver salt was converted into the sodium salt and then the latter salt was reconverted into the silver salt, isolated as colorless needles: λ_{max}^{Nujol} 6.88 (C=O) and 8.10 μm (acetate); other prominent bands were observed at 8.96, 9.22, 9.65, 10.10, 10.75, 10.90, 13.25, and 15.00 μm.

Acetylation procedure. — Acetylation was usually performed by stirring the reactants at 50–60° until dissolution occurred, although acetylation at room temperature was also found to be effective. Generally, a finely powdered reactant (1 g) was treated with 10 ml (or 25 ml) of the reagent, and the product was recovered either by treatment with ice–water or by extraction with (*a*) dichloromethane containing 5 to 25% of ethyl ether or (*b*) chloroform. Acetylation is readily performed on a large scale. For example, by stirring *myo*-inositol (20 g) with the acetylating mixture (200–250 ml) at 85° for 15 min, followed by treatment with ice–water, 47 g (a 97% yield) of almost pure *myo*-inositol hexaacetate (m.p. 214–215°) was isolated.

This procedure may readily be extended to the preparation of esters of other fatty acids, as indicated in Table II for the preparation of the butyrate, isobutyrate, and propionate of *myo*-inositol. Usually, the higher temperature was employed to effect dissolution of the solid; this is because the solubility of *myo*-inositol in the various mixed anhydrides diminishes with increase in the length of the carbon chain (acetic > propionic > butyric > isobutyric).

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Studies on starches of high amylose-content
Part IX. The detection of linear material in the anomalous amylopectin from amylomaize starch

The controversial studies on the nature of the components of maize starches of high amylose-content have been reviewed elsewhere[1]. There is, however, general agreement that when amylomaize starch is subjected to conventional fractionation involving dispersion of the granules and precipitation of the amylose by addition of butyl alcohol, the resulting amylopectin has three anomalous properties: (*a*) the β-amylolysis limit of *ca.* 65% conversion into maltose is much greater than the value (54–56%) observed for amylopectin from most normal starches; (*b*) the chain length (*CL*) of *ca.* 35 D-glucose residues is greater than that (20–25) usually found for amylopectin; (*c*) large amounts of iodine (1.5 mg of iodine/100 mg of polysaccharide) are bound relative to normal amylopectin (*ca.* 0.2 mg of iodine/100 mg of polysaccharide). The iodine uptake at the temperature (20°) normally employed for this measurement is very different in form to that observed for samples of normal amylose or amylopectin (or any mixture of the two), and, in fact, reasonable values for the iodine-binding capacities of these amylopectins from starches of high amylose-content can be obtained only if measurements are carried out at low temperature (2°).

Two very different explanations have been offered for the anomalous properties of these amylopectin fractions. The first[2–4] is that, in the starches of high amylose-content, the balance of the synthesising enzymes has been upset in such a way that a structurally homogeneous amylopectin is produced, which differs from normal amylopectin *only* in having a greater external chain-length than usual (25 D-glucose residues as opposed to *ca.* 15). This, of course, *must* lead to the anomalous amylopectin having a greater overall chain-length and higher β-limit than the normal material. It *may* also explain the peculiar iodine-binding characteristics of the anomalous fraction.

A second explanation[5–7] is that the anomalous material is a mixture of normal amylopectin and degraded amylose [degree of polymerization ($\overline{DP_n}$) of the order of 100 D-glucose residues]; the size of the contaminating amylose is so small that the normal complexing agents (butyl alcohol, thymol, etc.) cannot combine with it to form stable complexes, and hence separation by this technique is not possible. Again, such an explanation would account for the higher chain-length and β-amylolysis limit. Additionally, it has been shown that the iodine-binding characteristics of degraded amyloses are similar in form to those of anomalous amylopectins[8]. We have previously reported a separation process for this degraded amylose, based on differential ultracentrifugation[5,6], but this method has been criticised[4]. We now wish to present an enzymic method that can show the presence of linear D-glucan in admixture with an amylopectin.

The basis of this method is that the β-amylolysis of a branched α-D-(1→4)-glucan (glycogen or amylopectin) can yield only maltose and a residue of high molecular weight (β-limit dextrin), as this exoamylase hydrolyses alternate α-D-(1→4)

linkages, and cannot by-pass α-D-(1→6) branch points. β-Amylolysis of a linear amylose, on the other hand, results in the complete hydrolysis of the polysaccharide. Furthermore, whilst the complete β-amylolysis of an amylose molecule containing an even number of D-glucose residues leads only to the production of maltose, that of a polymer chain containing an odd number of residues (which statistically would be expected to be half of the total number of amylose molecules) produces, in addition to maltose, one molecule of maltotriose. The maltotriose undergoes slow β-amylolysis to maltose and D-glucose. We have shown[9] that, by increasing the concentration of enzyme sufficiently, this process can be made quantitative in a period of *ca.* 30 h. Small amounts of D-glucose (2–30 μg) can be easily and accurately determined in the presence of a large excess of maltose, using the glucose-oxidase technique of Dahlqvist[10]. Thus, if, after β-amylolysis of an anomalous amylopectin, D-glucose can be detected in the digest, it shows unambiguously that the polysaccharide is a mixture of amylopectin and amylose. Conversely, the absence of D-glucose from the digest shows that the starting material contained only a branched polysaccharide.

EXPERIMENTAL

The samples of anomalous amylopectins used were those isolated from amylomaize[6]. The preparation of the control samples of rabbit-liver glycogen and potato amylopectin has also been described[11,12].

To achieve the necessary high concentration of polysaccharide, digests were set up as follows. Polysaccharide (*ca.* 150 mg) was dissolved in methyl sulphoxide (1 ml) by standing overnight at 35°. A mixture (8 ml) of glycerol (50%) and 0.05M acetate buffer (pH 4.8) (50%) was added and the mixture shaken for 1 h. β-Amylase (1 ml; 10,000 units[13]) was then added, and the digest incubated for 48 h at 37°. The D-glucose content was then assayed, using the glucose-oxidase technique of Dahlqvist[10].

The total concentration of polysaccharide was obtained by hydrolysing an aliquot of the digest, using α(D)-1,4-glucan-glucohydrolase (semi-purified preparation, *ex Aspergillus niger*, kindly donated by Dr. I. D. Fleming, Glaxo Research Ltd.) The resulting D-glucose was again estimated by the glucose-oxidase method.

RESULTS AND DISCUSSION

The amount of D-glucose obtained on the β-amylolysis of various polysaccharides is shown in Table I, as a function of the total D-glucose (polysaccharide concentration) present. Only in the case of the samples from the starches of high amylose-content is an appreciable proportion of D-glucose obtained on β-amylolysis. Thus, there is no doubt that these samples contain a considerable proportion of linear material, which must explain, to some extent, the high values observed for their chain lengths.

Assuming that the $\overline{DP_n}$ of the contaminating amylose is about 100 D-glucose

residues, then, using the values in Table I, it can easily be shown that the "Amylon 50" and "Amylon 70" amylopectins contain 31 and 38%, respectively, of this linear material. The measured chain-length of the anomalous amylopectins is a number-average value, with contributions from both linear and branched fractions. Thus

$$1/x = (a/y) + (1-a)/z,$$

where x is the chain length of the mixture, a is the fraction of linear material of chain length (degree of polymerisation) y, and z is the chain length of the "true" amylopectin (= 25 D-glucose residues in this case). Use of the figures derived above for a and y gives values for x of 33 and 35 D-glucose residues for the chain length of the anomalous amylopectins from "Amylon 50" and "Amylon 70" starches, respectively. These calculated values are in good agreement with the experimental values shown in Table I.

TABLE I

D-GLUCOSE PRODUCED ON β-AMYLOLYSIS OF BRANCHED α-D-(1→4)-GLUCANS

Sample	*Chain length*	D-*Glucose produced on β-amylolysis* (%)
Rabbit-liver glycogen	12	0
Potato amylopectin 1	23	0.01
Potato amylopectin 2	24	0.01
"Amylon 50" amylopectin	35	0.16
"Amylon 70" amylopectin	36	0.19

We therefore conclude that our previous postulate[1] is essentially correct — the anomalous characteristics of amylopectin fractions from starches of high amylose-content are due to the presence of contaminating, short-chain, linear material.

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Carbohyd. Res., 6 (1968) 241–244

A Diels–Alder reaction: the dimerisation of (2*R*,3*R*)-5-benzoyl-2,3-dihydro-2,3-*O*-isopropylidenefuran-2,3-diol

This communication reports the facile dimerisation of (*2R,3R*)-5-benzoyl-2,3-dihydro-2,3-*O*-isopropylidenefuran-2,3-diol* (**2**) by a reaction that has a formal similarity to the Diels–Alder reaction[1], in that the conjugated α,β-unsaturated carbonyl compound functions both as a diene (1,4 addition) and as a dienophile (1,2 addition). The unsaturated ketone (**2**) was prepared by treating the toluene-*p*-sulphonate **1** with sodium carbonate in *N,N*-dimethylformamide, when both the furan derivative (**2**; 52%) and the dimer (**3**; 17%) were isolated. On storage for 48 h at room temperature, compound **2** dimerized completely. The furan derivative (**2**), which rapidly decolourised dilute, aqueous potassium permanganate and bromine water, was identified by its n.m.r.spectrum (Table I). The doublet at 6.18 p.p.m. was assigned to

TABLE I

N.M.R. ASSIGNMENTS[a] FOR COMPOUNDS **2** AND **3**

Compound	*H-2*	*H-2′*	*H-3*	*H-3′*	*H-4*	*H-4′*	$J_{2,3}$	$J_{2',3'}$	$J_{3,4}$	$J_{3',4'}$	$J_{4',4'}$
2[b]	6.18	—	5.38	—	5.92	—	5.2	—	2.6	—	—
3[b]	6.31	6.18	4.91	4.82	2.34	3.68	4.6	3.6	1.9	<0.5	12.3

[a]Chemical shifts in parts per million; coupling constants in Hz. [b]Compound **2** showed methyl signals at 1.42 and 1.46 p.p.m., and compound **3** showed methyl signals at 1.18, 1.27, 1.48, and 1.56 p.p.m. Both compounds showed a quartet at 8.15 and 8.35 p.p.m., respectively, characteristic of two equivalent aromatic protons *ortho* to a carbonyl function.

H-2 by analogy with derivatives of 1,2-*O*-isopropylidene-α-D-xylofuranose[2]. The signals at 5.38 and 5.92 p.p.m. were then assigned to H-3 and H-4, respectively, and have values consistent with the proposed structure. A dimeric structure for com-

*Compound **2** may be alternatively named 3-deoxy-1,2-*O*-isopropylidene-5-*C*-phenyl-α-D-*glycero*-pent-3-enofuranos-5-ulose.

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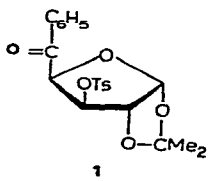
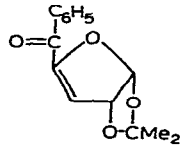
*Compound **2** may be alternatively named 3-deoxy-1,2-*O*-isopropylidene-5-*C*-phenyl-α-D-*glycero*-pent-3-enofuranos-5-ulose.

pound **3** was suggested by the presence of ten aromatic protons, six ring protons, and four methyl groups in its n.m.r. spectrum.

By analogy with dimerisations of α,β-unsaturated aldehydes, and of reactions between α,β-unsaturated aldehydes and vinyl ethers[3], it was expected that dimerisation of compound **2** would result in the formation of a carbon–carbon bond between C-4 of the two molecules and a carbon–oxygen bond between C-5 of one molecule and the carbonyl oxygen of the other, rather than the reverse process. Eight structures are possible for the dimer, since three new asymmetric centres are formed during the dimerisation process. If the most probable transition states are considered and the Woodward–Katz principle[4] is invoked, it is possible to predict the dimer structure.



1



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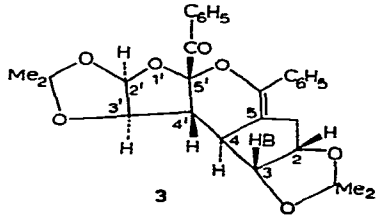
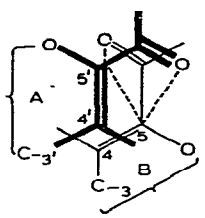


3

The Woodward–Katz principle states that the diene assumes a *quasi-cis* conformation and that the diene and dineophile approach one another initially in parallel planes orthogonal to the direction of the bond to be formed. Further, conformational specificity about the newly forming bond is determined by secondary attractive forces not directly associated with the primary bonding forces. An inspection of molecular models reveals that the transition state (represented diagrammatically as **4**) which meets the Woodward–Katz requirements is that which is also most favoured in terms of "bulk" steric effects. The "*A*" molecule takes up a situation immediately "above" the "*B*" molecule, prior to dimerisation. Such a transition state requires that H-4 should be below the plane of the pyran ring in the dimeric molecule, and that H-4′ and the 5′-*C*-benzoyl substituent should be above the plane. The parameters obtained from a detailed first-order analysis of the n.m.r. spectrum of compound **3** were in accord with the predicted configurations of the three new asymmetric centres.



4

The coupling constants for the "*A*" ring protons $J_{2',3'}$ 3.6 Hz and $J_{3',4'} < 0.5$ Hz are consistent with the coupling constants observed in a large number of α-D-xylofuranose derivatives[2]. In ring "*B*", the presence of the exocyclic double-bond

causes a flattening of the ring which is reflected by the increase in the values of $J_{2,3}$ (4.6 Hz) and $J_{3,4}$ (1.9 Hz). The large $J_{4,4'}$ coupling of 12.3 Hz is only possible if H-4 and H-4′ have a *trans*-diaxial relationship[5]. The observed coupling constants and chemical shifts are consistent with no other structure and unequivocally establish that the dimer of compound **2** has structure **3**.

EXPERIMENTAL

Thin-layer chromatography (t.l.c.) was performed on microscope slides coated with Silica Gel G, and column chromatography was performed with Silica Gel of particle size 0.05–0.2 mm (both adsorbents were manufactured by E. Merck, Darmstadt, Germany). N.m.r. spectra were measured with a JEOL JNM-4H-100 n.m.r. spectrometer at 100 MHz, with deuteriochloroform as solvent and with tetramethylsilane as internal standard.

1,2-O-Isopropylidene-5-C-phenyl-3-O-toluene-p-sulphonyl-α-D-xylopentofuranos-5-ulose (**1**). — A solution of 1,2-*O*-isopropylidene-5-*C*-phenyl-α-D-xylopentofuranos-5-ulose[6] (10 g) in dry pyridine (30 ml) was added to a solution of toluene-*p*-sulphonyl chloride (9.5 g) in dry pyridine (25 ml) at 0°, and the solution was stored for 24 h at room temperature. The solution was poured into water (250 ml), and extracted with chloroform, and the chloroform extract was washed with dilute hydrochloric acid, aqueous sodium hydrogen carbonate, and water, dried ($MgSO_4$), and concentrated. The residue was recrystallised from ethanol to yield compound **1** (13 g, 80%), m.p. 101–102°, $[\alpha]_D$ −75° (*c* 6.3, chloroform) (Found: C, 60.2; H, 5.1. $C_{21}H_{22}O_7S$ calc.: C, 60.3; H, 5.3%).

Treatment of compound **1** *with sodium carbonate.* — A mixture of compound **1** (10 g) and anhydrous sodium carbonate (10 g) in *N,N*-dimethylformamide (75 ml) was heated for 72 h at 50°. At intervals, samples (1 ml) were removed and partitioned between water and ether, and then the ether solution was examined by t.l.c. with light petroleum (b.p. 40–60°)–isopropyl ether (3:2). The unsaturated products were detected with dilute, aqueous potassium permanganate, and compound **1** was detected with sulphuric acid. The solution was poured into water (200 ml) and extracted with ether, and the ether extract was washed with water, dried, and concentrated. Chromatographic resolution of the residue on silica gel with light petroleum (b.p. 40–60°)–isopropyl ether (3:2) yielded compound **2** (3 g, 52%) and **3** (1 g, 17%). The α,β-unsaturated ketone **2** was unstable, and dimerised during 48 h to compound **3**. On crystallisation from ethanol or light petroleum (b.p. 60–80°), compound **3** had m.p. 162–164°, $[\alpha]_D$ +340° (*c* 1.2, chloroform) (Found: C, 68.3; H, 5.6. $C_{28}H_{28}O_8$ calc.: C, 68.3; H, 5.7%).

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Carbohyd. Res., 6 (1968) 244–247

A convenient synthesis of D-glucopyranose 1,6-diphosphate

The importance of α-D-glucopyranose 1,6-diphosphate as a coenzyme is well established[1]. More recently, its possible role in the biosynthesis of glycogen has been suggested[2]. In order to test this hypothesis appreciable quantities were required. Posternak has described two fairly long syntheses[3] of this compound from D-glucose *via* 2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl bromide 6-(diphenyl phosphate). Ray and Roscelli[4] have modified this procedure by separating the α- and β-D anomers by ion-exchange chromatography. A short and convenient synthesis from D-glucose 6-phosphate is now described.

Treatment of D-glucose 6-(disodium phosphate) with acetic anhydride and sodium acetate, followed by cation exchange with Amberlite IR-120 resin (H^+), gave 1,2,3,4-tetra-*O*-acetyl-β-D-glucopyranose 6-(dihydrogen phosphate), isolated in yields of 50–55% as the crystalline methanolate. This compound was identical with authentic material[5] prepared by hydrogenolysis of 1,2,3,4-tetra-*O*-acetyl-β-D-glucopyranose 6-(diphenyl phosphate).

Treatment of 1,2,3,4-tetra-*O*-acetyl-β-D-glucopyranose 6-(dihydrogen phosphate) methanolate with crystalline phosphoric acid[6], followed by deacetylation, gave D-glucopyranose 1,6-diphosphate, conveniently isolated in yields of 30–35% as the crystalline tetracyclohexylammonium salt tetrahydrate. The molecular rotation of this product ($[M]_D$ +19,560°) indicated, from calculations based on Posternak's optical rotational data[3], it to contain the α- and β-D anomers in the ratio 2.85:1. After three further recrystallisations from aqueous ethanol, the molecular rotation had risen to +25,070° (ratio of α- and β-D anomers, 9:1). Acidic hydrolysis of this compound gave only D-glucose 6-phosphate and orthophosphate, as revealed by paper electrophoresis.

Alternatively, the compound could be isolated in yields of 30–35% by direct precipitation as the dibarium salt. The molecular rotation ($[M]_D$ +18,020°, after

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conversion into the tetrasodium salt) indicated that this product contained the α- and β-D anomers in the ratio 7:3*.

EXPERIMENTAL

1,2,3,4-Tetra-O-acetyl-β-D-glucopyranose 6-(dihydrogen phosphate) methanolate. — D-Glucose 6-(disodium phosphate) (2.1 g) was added in portions to boiling acetic anhydride (25 ml) containing anhydrous sodium acetate (1.25 g). Boiling was continued for 0.5 h, and the cooled solution was then poured into ice–water (70 ml) and stirred for 3 h at 0°. Amberlite IR-120 (H^+, 75 g) was added, and, after being stirred for a further 0.5 h, the solution was filtered, and the resin was washed with water until the washings were free from acid. The combined filtrate and washings were freeze-dried, and the residue was recrystallised from a mixture of methanol (70 ml) and light petroleum (b.p. 60–80°, 70 ml) to give 1,2,3,4-tetra-*O*-acetyl-β-D-glucopyranose 6-(dihydrogen phosphate) methanolate (1.82 g, 48.1%), m.p. 125–128°, identical (mixed m.p. and infrared spectrum) with an authentic specimen, prepared by hydrogenolysis of 1,2,3,4-tetra-*O*-acetyl-β-D-glucopyranose 6-(diphenyl phosphate)[5].

D-Glucopyranose 1,6-(tetracyclohexylammonium diphosphate) tetrahydrate. — 1,2,3,4-Tetra-*O*-acetyl-β-D-glucopyranose 6-(dihydrogen phosphate) methanolate (98.5 mg) was stirred for 2 h at 50° *in vacuo* with anhydrous phosphoric acid (197 mg). The cooled syrup was shaken with ice-cold 2M lithium hydroxide (4 ml), and the precipitated lithium phosphate was filtered off, and washed with 0.01M lithium hydroxide (10 ml). The combined filtrate and washings were passed down a column (1 × 10 cm) of Amberlite IR-120 (H^+) at 4°, and the column was washed with water. The acidic effluent was passed, with stirring, into a solution of cyclohexylamine (0.5 ml) in water (10 ml), and the solution was evaporated to dryness. The residue was recrystallised by dissolving it in water (0.1 ml) and adding ethanol (3 ml) at 4°. After storage for 18 h at 4°, there was obtained D-glucopyranose 1,6-(tetracyclohexylammonium diphosphate) tetrahydrate** (55.7 mg, 32.2%), $[\alpha]_D^{20}$ +24.2° (*c* 0.5, water), $[M]_D$ +19,560° (Found: C, 44.4; H, 8.5; N, 6.65; P, 8.0. $C_{30}H_{74}N_4O_{16}P_2$ calc.: C, 44.6; H, 9.2; N, 6.9; P, 7.7%). This product had an R_F value of 0.18 when examined by t.l.c. (Whatman CC41 cellulose; propyl alcohol–ammonia–water, 6:3:1; molybdate detection[8]), and an M_G value of 1.43 when examined by paper electrophoresis (Whatman 3MM paper; 0.05M borate; 4000 volts, 40 min; molybdate detection[8]). After a further three recrystallisations by the method described above, the product had $[\alpha]_D^{20}$ +31.0° (*c* 0.5, water), $[M]_D$ +25,070°.

*Since the completion of this work, Khan *et al.*[7] have described a synthesis of D-glucose 1,6-diphosphate, by reaction of an uncharacterised tetra-*O*-acetyl-D-glucose 6-(disodium phosphate) with anhydrous phosphoric acid, in which they claim that only the pure α-D anomer is obtained. However, no optical rotational data for their product were recorded.

**Although, to the author's knowledge, this salt is not described in the literature, it is marketed by The Boehringer Corporation (London) Ltd. A specimen obtained from this source had $[\alpha]_D^{20}$ +30.9° (*c* 0.5, water); $[M]_D$ +25,010°.

D-*Glucopyranose 1,6-(dibarium diphosphate)*. — 1,2,3,4-Tetra-*O*-acetyl-β-D-glucopyranose 6-(dihydrogen phosphate) methanolate (0.6 g) was stirred for 2 h *in vacuo* at 50° with anhydrous phosphoric acid (1.6 g). The cooled syrup was shaken with ice-cold 2M lithium hydroxide (25 ml), and the precipitated lithium phosphate was filtered off and washed with 0.01M lithium hydroxide (25 ml). To the combined filtrate and washings was added barium acetate (800 mg), followed by ethanol (50 ml). The precipitate was centrifuged, washed with boiling water (2 × 1 ml), ethanol, and ether, and dried to give D-glucopyranose 1,6-(dibarium diphosphate) (0.32 g, 35%) (Found: P, 9.5. $C_6H_{12}O_{13}P_2Ba_2$ calc.: P, 9.85%). After conversion into the sodium salt by Posternak's method[3], this product had $[M]_D$ +18,020°.

Hydrolysis of D-*glucopyranose 1,6-diphosphate*. — A solution of D-glucopyranose 1,6-(tetracyclohexylammonium diphosphate) tetrahydrate (10 mg) in N hydrochloric acid (1 ml) was stored for 0.5 h at 100°, cooled, and neutralised with N sodium hydroxide. Examination of the solution by paper electrophoresis (conditions as above) indicated complete disappearance of the starting material (M_G 1.43) and appearance of spots having the M_G values of D-glucose 6-phosphate (1.26) and orthophosphate (1.65).

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Centose; a branched trisaccharide containing α-D-(1→2) and α-D-(1→4) glycosidic linkages*

Few trisaccharides are known which contain the α-D-(1→2) glycosidic bond. Kojitriose [two α-D-(1→2) links] is a reported transglucosylation product of *Schizosaccharomyces pombe*[1,2], and *O*-β-D-glucopyranosyl-(1→4)-*O*-[α-D-glucopyranosyl-(1→2)]-D-glucose and *O*-β-D-galactopyranosyl-(1→4)-*O*-[α-D-glucopyranosyl-(1→2)]-D-glucose have been characterised[3].

During the fractionation of honey by column chromatography on charcoal–Celite, a trisaccharide fraction *K* was eluted with 15% ethanol, which gave [4,5], *inter alia*, a reducing trisaccharide K_3, now tentatively identified as *O*-α-D-glucopyranosyl-(1→4)-*O*-[α-D-glucopyranosyl-(1→2)]-D-glucose. This new trisaccharide has been named centose, after Canada's centennial year.

Centose showed $[\alpha]_D^{27}$ +140° (*c* 1.15, water), and moved as a single spot on paper electrophoresis[6] in borate buffer and on paper chromatography in three different solvent systems, although the methylation data indicated the presence of a contaminant. Total hydrolysis with acid produced glucose only, and partial hydrolysis yielded the unhydrolysed trisaccharide, kojibiose, maltose, and glucose.

These results suggest that centose contains α-D-glycosidic bonds, and support for this conclusion was provided by the resistance towards emulsin and the ease of hydrolysis with amyloglucosidase. The low mobility (M_G 0.22) of centose on electrophoresis in borate buffer was also indicative of the absence of (1→6) or (1→3) links at the reducing glucose residue.

Methylation of centose, followed by methanolysis and hydrolysis, gave two major methyl sugars identical with 2,3,4,6-tetra-*O*-methyl-D-glucose (paper chromatography, i.r. spectrum) and 3,6-di-*O*-methyl-D-glucose (paper chromatography and electrophoresis, i.r. spectrum). Small proportions of 3,4,6-tri-*O*-methyl-D-glucose were also detected (paper chromatography and electrophoresis). Quantitative determination of the *O*-methyl sugars by g.l.c.[7] of their methyl glycosides revealed ratios of methyl 2,3,4,6-tetra-*O*-methyl-D-glucosides to methyl 3,4,6-tri-*O*-methyl-D-glucosides to methyl 3,6-di-*O*-methyl-D-glucosides of 2.2:0.4:1.0.

The methylation analysis showed that centose had the following branched structure *O*-α-D-glucopyranosyl-(1→4)-*O*-[α-D-glucopyranosyl-(1→2)]-D-glucose, and that it was probably contaminated by another trisaccharide, presumably kojitriose.

EXPERIMENTAL

Paper chromatography was performed by the descending method on Whatman No. 1 and 3 MM papers with the organic phases of (*A*) butyl alcohol–pyridine–water (10:3:3); (*B*) ethyl acetate–pyridine–water (8:2:1); (*C*) ethyl acetate–water–pyridine

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(2.5:2.5:1); (*D*) butanone, saturated with water containing 2% of ammonia. Paper electrophoresis was performed on Whatman No. 3 MM paper with 0.2M borate buffer (pH 10), at 800 volts for 2–3 h. Detection was effected with (*A*) aniline hydrogen phthalate[8]; (*B*) naphthoresorcinol[9]; (*C*) alkaline silver nitrate. T.l.c. was performed on Silica Gel G, with freshly made mixtures of methanol (4–10%) in benzene. Sugar acetates were located in t.l.c. by (*A*) spraying with water, or (*B*) spraying with 5% sulphuric acid in ethanol and charring. Evaporations were carried out at 35° with a rotary evaporator. Rotations are equilibrium values.

Isolation and purification of centose. — Centose was found as a component of a trisaccharide fraction eluted with 15% aqueous ethanol during the fractionation of a large batch of honey by column chromatography on charcoal–Celite. It was separated[4] from other trisaccharides by preparative paper-chromatography with solvent *A*. Further purification was effected by treating the trisaccharides with an equal weight of freshly fused sodium acetate in acetic anhydride for 3 h at 120°; the hendecaacetate was purified by t.l.c. After deacetylation, the trisaccharide was purified by paper chromatography with solvent *B*. The approximate proportion[5] of the trisaccharide in the oligosaccharide fraction (3.65% of honey) was 0.05%.

Characterization of centose. — (*a*) The purified trisaccharide had $[\alpha]_D^{27}$ +140° (*c* 1.15, water). Paper chromatography in solvents *A* and *B* showed a single component having R_G 0.2 and 0.08, respectively. Paper electrophoresis revealed a single component (M_G 0.22). The trisaccharide had $R_{sucrose}$ 0.29 in solvent *A*; *cf.* isomaltotriose (0.18), panose (0.25), isopanose (0.21), and maltotriose (0.35).

(*b*) Complete hydrolysis of centose (2 mg) in 2N sulphuric acid (0.25 ml) for 4 h at 100° gave glucose as the only detectable sugar on paper chromatography and electrophoresis. Paper chromatography of a partial hydrolysate (2 mg of centose in 0.1N sulphuric acid for 45 min at 100°) in solvent *A*, and paper electrophoresis, showed 3 components corresponding to glucose, maltose or kojibiose (or both), and the unhydrolysed trisaccharide. However, paper chromatography of the partial hydrolysate in solvent *B* revealed 4 components having colour reactions and R_G values identical with those of D-glucose, maltose, kojibiose, and the unhydrolysed trisaccharide.

(*c*) Centose (2 mg) in water (0.2 ml) was incubated for 24 h with emulsin (2 mg) at 35°. A similar portion was similarly incubated with amyloglucosidase. Paper chromatography in solvent *B* revealed the absence of glucose in the former hydrolysate, and the presence of large proportions of glucose in the latter. Maltose and kojibiose were hydrolysed by amyloglucosidase under the same conditions.

(*d*) The trisaccharide (30 mg) in water (1 ml) was stirred with methyl sulphate (0.25 ml) at 0°. Sodium hydroxide (30%, 0.5 ml) was added dropwise over 5 h, and the reaction mixture was stirred overnight. Methylation was continued by treating the reaction mixture with 30% sodium hydroxide (0.5 ml) followed by methyl sulphate (0.3 ml), added dropwise over 7 h, and the reaction mixture was then stirred overnight at 0°. A third addition of methylating agents was similarly made. The methylated trisaccharide, recovered by chloroform partition, was methylated six times with the Purdie reagents and then purified on one t.l.c. plate with solvent *D* to yield a pro-

duct (15 mg), $[\alpha]_D^{25}$ +113° (*c* 1.5, chloroform), which showed no hydroxyl absorption in its infrared spectrum.

Methanolysis and hydrolysis of methylated centose.—The methylated trisaccharide (15 mg) was dissolved in 2% methanolic hydrogen chloride (1.5 ml), and the solution was refluxed for 18 h. After neutralization (Ag_2CO_3), filtration, and evaporation, the syrupy glucosides were hydrolysed with N sulphuric acid (1 ml) for 12 h at 100°. The solution was neutralized ($BaCO_3$), and the filtrate was concentrated to a syrup (9 mg).

A portion (2 mg) of this syrup was refluxed with 2% methanolic hydrogen chloride for 18 h, and neutralized (Ag_2CO_3), and the resulting glucosides were examined by g.l.c. with a 4-ft column of 15% polyphenyl ether on Gas Chrom A at 166°. The areas under the peaks gave the following quantitative molar ratios: 2,3,4,6-tetra-*O*-methyl-D-glucoside (α,β) 2.2; 3,6-di-*O*-methyl-D-glucosides (α,β) 1; 3,4,6-tri-*O*-methyl-D-glucosides (α,β), 0.4.

Paper chromatography of a portion of the syrupy free sugars in solvent *D* showed three components having R_F values (0.78, 0.47, and 0.18), and colour reactions identical with those of authentic samples of 2,3,4,6-tetra-*O*-methyl-D-glucose, 3,4,6-tri-*O*-methyl-D-glucose, and 3,6-di-*O*-methyl-D-glucose. Paper electrophoresis revealed three components having M_G values 0.00, 0.20, and 0.55. The M_G values for the dimethyl ethers of glucose[10] are M_G 0.00 (2,4-); 0.09 (2,6-), 0.135 (2,3-), 0.185 (4,6-), 0.28 (3,4-), and 0.546 (3,6).

The remaining mixture of sugars was separated on one paper chromatogram (7 × 22 in) with solvent *D*. The two major components, thus recovered, had infrared spectra that were respectively identical with those of authentic samples of 2,3,4,6-tetra-*O*-methyl-D-glucose and 3,6-di-*O*-methyl-D-glucose, also recovered from the paper under similar conditions.

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A convenient synthesis of D-glucopyranose 1,6-diphosphate

The importance of α-D-glucopyranose 1,6-diphosphate as a coenzyme is well established[1]. More recently, its possible role in the biosynthesis of glycogen has been suggested[2]. In order to test this hypothesis appreciable quantities were required. Posternak has described two fairly long syntheses[3] of this compound from D-glucose *via* 2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl bromide 6-(diphenyl phosphate). Ray and Roscelli[4] have modified this procedure by separating the α- and β-D anomers by ion-exchange chromatography. A short and convenient synthesis from D-glucose 6-phosphate is now described.

Treatment of D-glucose 6-(disodium phosphate) with acetic anhydride and sodium acetate, followed by cation exchange with Amberlite IR-120 resin (H^+), gave 1,2,3,4-tetra-*O*-acetyl-β-D-glucopyranose 6-(dihydrogen phosphate), isolated in yields of 50–55% as the crystalline methanolate. This compound was identical with authentic material[5] prepared by hydrogenolysis of 1,2,3,4-tetra-*O*-acetyl-β-D-glucopyranose 6-(diphenyl phosphate).

Treatment of 1,2,3,4-tetra-*O*-acetyl-β-D-glucopyranose 6-(dihydrogen phosphate) methanolate with crystalline phosphoric acid[6], followed by deacetylation, gave D-glucopyranose 1,6-diphosphate, conveniently isolated in yields of 30–35% as the crystalline tetracyclohexylammonium salt tetrahydrate. The molecular rotation of this product ($[M]_D$ +19,560°) indicated, from calculations based on Posternak's optical rotational data[3], it to contain the α- and β-D anomers in the ratio 2.85:1. After three further recrystallisations from aqueous ethanol, the molecular rotation had risen to +25,070° (ratio of α- and β-D anomers, 9:1). Acidic hydrolysis of this compound gave only D-glucose 6-phosphate and orthophosphate, as revealed by paper electrophoresis.

Alternatively, the compound could be isolated in yields of 30–35% by direct precipitation as the dibarium salt. The molecular rotation ($[M]_D$ +18,020°, after

conversion into the tetrasodium salt) indicated that this product contained the α- and β-D anomers in the ratio 7:3*.

EXPERIMENTAL

1,2,3,4-Tetra-O-acetyl-β-D-glucopyranose 6-(dihydrogen phosphate) methanolate. — D-Glucose 6-(disodium phosphate) (2.1 g) was added in portions to boiling acetic anhydride (25 ml) containing anhydrous sodium acetate (1.25 g). Boiling was continued for 0.5 h, and the cooled solution was then poured into ice–water (70 ml) and stirred for 3 h at 0°. Amberlite IR-120 (H^+, 75 g) was added, and, after being stirred for a further 0.5 h, the solution was filtered, and the resin was washed with water until the washings were free from acid. The combined filtrate and washings were freeze-dried, and the residue was recrystallised from a mixture of methanol (70 ml) and light petroleum (b.p. 60–80°, 70 ml) to give 1,2,3,4-tetra-*O*-acetyl-β-D-glucopyranose 6-(dihydrogen phosphate) methanolate (1.82 g, 48.1%), m.p. 125–128°, identical (mixed m.p. and infrared spectrum) with an authentic specimen, prepared by hydrogenolysis of 1,2,3,4-tetra-*O*-acetyl-β-D-glucopyranose 6-(diphenyl phosphate)[5].

D-Glucopyranose 1,6-(tetracyclohexylammonium diphosphate) tetrahydrate. — 1,2,3,4-Tetra-*O*-acetyl-β-D-glucopyranose 6-(dihydrogen phosphate) methanolate (98.5 mg) was stirred for 2 h at 50° *in vacuo* with anhydrous phosphoric acid (197 mg). The cooled syrup was shaken with ice-cold 2M lithium hydroxide (4 ml), and the precipitated lithium phosphate was filtered off, and washed with 0.01M lithium hydroxide (10 ml). The combined filtrate and washings were passed down a column (1 × 10 cm) of Amberlite IR-120 (H^+) at 4°, and the column was washed with water. The acidic effluent was passed, with stirring, into a solution of cyclohexylamine (0.5 ml) in water (10 ml), and the solution was evaporated to dryness. The residue was recrystallised by dissolving it in water (0.1 ml) and adding ethanol (3 ml) at 4°. After storage for 18 h at 4°, there was obtained D-glucopyranose 1,6-(tetracyclohexylammonium diphosphate) tetrahydrate** (55.7 mg, 32.2%), $[\alpha]_D^{20}$ +24.2° (*c* 0.5, water), $[M]_D$ +19,560° (Found: C, 44.4; H, 8.5; N, 6.65; P, 8.0. $C_{30}H_{74}N_4O_{16}P_2$ calc.: C, 44.6; H, 9.2; N, 6.9; P, 7.7%). This product had an R_F value of 0.18 when examined by t.l.c. (Whatman CC41 cellulose; propyl alcohol–ammonia–water, 6:3:1; molybdate detection[8]), and an M_G value of 1.43 when examined by paper electrophoresis (Whatman 3MM paper; 0.05M borate; 4000 volts, 40 min; molybdate detection[8]). After a further three recrystallisations by the method described above, the product had $[\alpha]_D^{20}$ +31.0° (*c* 0.5, water), $[M]_D$ +25,070°.

*Since the completion of this work, Khan *et al.*[7] have described a synthesis of D-glucose 1,6-diphosphate, by reaction of an uncharacterised tetra-*O*-acetyl-D-glucose 6-(disodium phosphate) with anhydrous phosphoric acid, in which they claim that only the pure α-D anomer is obtained. However, no optical rotational data for their product were recorded.

**Although, to the author's knowledge, this salt is not described in the literature, it is marketed by The Boehringer Corporation (London) Ltd. A specimen obtained from this source had $[\alpha]_D^{20}$ +30.9° (*c* 0.5, water); $[M]_D$ +25,010°.

D-*Glucopyranose 1,6-(dibarium diphosphate)*. — 1,2,3,4-Tetra-*O*-acetyl-β-D-glucopyranose 6-(dihydrogen phosphate) methanolate (0.6 g) was stirred for 2 h *in vacuo* at 50° with anhydrous phosphoric acid (1.6 g). The cooled syrup was shaken with ice-cold 2M lithium hydroxide (25 ml), and the precipitated lithium phosphate was filtered off and washed with 0.01M lithium hydroxide (25 ml). To the combined filtrate and washings was added barium acetate (800 mg), followed by ethanol (50 ml). The precipitate was centrifuged, washed with boiling water (2 × 1 ml), ethanol, and ether, and dried to give D-glucopyranose 1,6-(dibarium diphosphate) (0.32 g, 35%) (Found: P, 9.5. $C_6H_{12}O_{13}P_2Ba_2$ calc.: P, 9.85%). After conversion into the sodium salt by Posternak's method[3], this product had $[M]_D$ +18,020°.

Hydrolysis of D-*glucopyranose 1,6-diphosphate*. — A solution of D-glucopyranose 1,6-(tetracyclohexylammonium diphosphate) tetrahydrate (10 mg) in N hydrochloric acid (1 ml) was stored for 0.5 h at 100°, cooled, and neutralised with N sodium hydroxide. Examination of the solution by paper electrophoresis (conditions as above) indicated complete disappearance of the starting material (M_G 1.43) and appearance of spots having the M_G values of D-glucose 6-phosphate (1.26) and orthophosphate (1.65).

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MULTIPLE FORMS OF SIALIC ACIDS*

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ABSTRACT

Incremental titrations and paper chromatography have shown the presence of multiple forms of sialic acids. Additional evidence was obtained by X-ray diffraction, n.m.r. spectroscopy, and determination of the mutarotation. It was found that the different forms of sialic acids may represent an equilibrium mixture of the open chain and of the anomers. At lower pH values, a fraction of the *N*-acetyl groups has been cleaved from *N*-acetylneuraminic acid by the action of the acidic medium.

INTRODUCTION

Sialic acids are unique among the carboxylic acids occurring in biological polymers because of their high dissociation constants. Svennerholm[1] has reported a pK value of 2.60 for *N*-acetylneuraminic acid (5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-nonulosonic acid), whereas Bettelheim[2] has found two pK values: 2.60 and 2.85. Similarly, Berggård and Odin[3] found multiple spots when *N*-acetylneuraminic acid, *N*,*O*-diacetylneuraminic acid, and *N*-glycolylneuraminic acid were chromatographed on paper. It was also reported[2] that the corresponding pK values of bovine submaxillary mucin were 2.46 and 2.60. No explanation has been advanced as to the nature of the multiple forms of sialic acids. Hence this investigation was undertaken to study, with different physical chemical techniques, the multiple forms of free sialic acids as well as the forms of those bound in bovine and porcine submaxillary mucins and the enzymatic hydrolyzates of bovine and porcine submaxillary mucins.

EXPERIMENTAL

N-Acetylneuraminic acid, type III, lot A 11B-216 (isolated from eggs), and type IV, lot 85B-1680 (synthetic) were obtained from Sigma Chemical Company, St. Louis; grade A, lot 30165 (synthetic) from Calbiochem, Los Angeles. *N*,*O*-Diacetylneura-

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minic acid, grade II, lot D 33B-276 (isolated from bovine submaxillary glands) was obtained from Sigma Chemical Company. *N*-Glycolylneuraminic acid, lot G 22B-097 (isolated from porcine submaxillary mucin) was obtained from Sigma Chemical Company.

Bovine submaxillary mucin was prepared by the procedure of S. Tsuiki, Y. Hashimoto, and W. Pigman[4], using method B. The mucin was found to contain 40.1% protein and 23.9% sialic acid. The mucin migrated as a single boundary in free electrophoresis at pH 11.2. Porcine submaxillary mucin was prepared as reported earlier[5]. The bovine and porcine submaxillary glands were kindly supplied by Dr. Pigman of New York Medical College.

The calcium salt of bovine submaxillary mucin was treated with neuraminidase (Sigma Chemical Company, lot N 91B-064) at 37° in a 0.1M phosphate buffer (pH 6.4). After two days this solution was dialyzed against distilled water, and the residual degraded mucin was lyophilized and subsequently used in the titration study. The dialyzate was concentrated and then lyophilized. The sialic acid was then extracted with methanol[6] and finally recrystallized. The sialic acid of porcine submaxillary mucin was prepared in a similar manner for titration.

Inositol was obtained from General Biochemical, Inc., Chagrin Falls, Ohio, and D-mannitol (C. P.) from Fisher Scientific Company, Pittsburgh, Pennsylvania. 2-Amino-2-deoxy-D-galactose, lot 172389, was obtained from Mann Research Laboratories, New York, and 2-acetamido-2-deoxy-D-galactose from Nutritional Biochemicals Corp., Cleveland, Ohio. *i*-Inositol hexaacetate and D-mannitol hexaacetate were prepared by the method of Wolfrom and Thompson[7].

The titration curves were determined with a Radiometer pH-meter (type PHM 4C) having a glass electrode and a saturated calomel electrode as a reference. A Gilmont micropipet (buret with a 1.0 ml capacity which could deliver 0.0100 $\pm$ 0.0005 ml) was used in adding small increments of 0.1M HCl. The titration vessel was kept in a Faraday cage, and the solution was stirred with a magnetic stirrer which was switched off during each measurement. The solution was also temperature controlled at 25.0 $\pm$ 0.1° during each titration. A typical sample consisted of 10 mg of sialic acid in 10 ml of solution which was neutralized to pH 3.5. The freshly prepared solutions were titrated with HCl. The duration of a titration was about 2 h.

The optical rotation was measured with a Schmidt and Haentsch polarimeter, with a sodium vapor lamp. The polarimeter tube was a 4-dm capillary tube with a capacity of 2.5 ml.

Paper chromatography was performed on Whatman No. 1 paper. The solvent systems employed were butyl alcohol–pyridine–water (6:4:3) and butyl alcohol–acetic acid–water (4:1:5). The spray reagents employed for staining the chromatograms were Congo Red, Ehrlich's reagent[8], alkaline silver nitrate[9], and ninhydrin[10].

X-Ray diffraction was performed with a Norelco unit using CuKα radiation and a 57.3-mm powder camera. The samples were ground and sealed in capillary tubes having 0.0007-mm wall thickness. Only forward scattering was obtained, and the interplanar distances were evaluated by the Wilson technique[11].

RESULTS

The pK values for free sialic acids and for those bound in mucins were obtained from the inverse buffer-capacity curves and are given in Table I. A typical plot of the

TABLE I

pK VALUES OF SIALIC ACIDS

Compound	*Medium*	*pK*							
N-Acetylneuraminic acid	H_2O				2.2	2.3		2.6	2.8
N-Acetylneuraminic acid	0.1M KCl				2.1	2.3			2.8
N-Acetylneuraminic acid[a]	H_2O			2.0		2.3	2.5		2.7
N,O-Diacetylneuraminic acid	H_2O			2.0		2.3	2.5	2.6	
N-Glycolylneuraminic acid	H_2O			2.0	2.2		2.4		2.7
Calcium salt of bovine submaxillary mucin	H_2O				2.2		2.4	2.6	2.8
Calcium salt of bovine submaxillary mucin	0.1M KCl			2.0		2.3			2.7
Calcium salt of bovine submaxillary mucin[b]									
a. dialyzable fraction	H_2O				2.2	2.3			2.7
b. nondialyzable fraction	H_2O			2.0		2.3		2.6	
Calcium salt of porcine submaxillary mucin	H_2O	1.6	1.9		2.1		2.5		2.7
Calcium salt of porcine submaxillary mucin[b]									
a. dialyzable fraction	H_2O	1.7	1.9	2.0	2.2				
b. nondialyzable fraction	H_2O			2.0	2.2		2.4	2.5	

[a]Treated with HCl for 48 h at room temperature and lyophilized; [b]Treated with neuraminidase.

inverse buffer-capacity curve is given in Fig. 1 for *N*-acetylneuraminic acid. On the ordinate, $\Delta Q/\Delta pH$ represents the increment of acid titrant necessary to cause a unit change in pH, and the abscissa represents the final pH. This inverse buffer-capacity

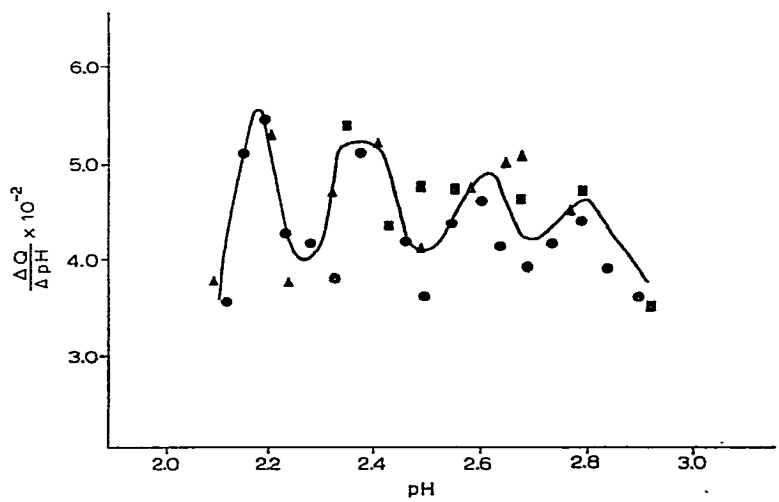

Fig. 1 — Inverse buffer-capacity curve of three samples of *N*-acetylneuraminic acid; ● synthetic (Sigma), ■ synthetic (Calbiochem), ▲ isolated from eggs (Sigma). The line represents a computer-calculated average of the three samples.

curve represents the determination on three *N*-acetylneuraminic acid samples having been corrected for the buffer capacity of water, both experimentally (by titration of water) and theoretically as was given in equation 5 of our previous communication[2]. The buffer capacity of water, as found experimentally, was in good agreement with that calculated from the above-mentioned equation within the pH range of our investigation.

When $\Delta Q/\Delta pH$ is plotted against pH theoretically, one is able to obtain the pK of acidic groups, since at the maxima of the curve the pK equals the pH. However, the theoretical maxima of such buffer-capacity curves are relatively broad and, therefore, closely spaced pK values should yield either a broad combined maximum or small shoulders of individual maxima[12]. On the individual curves of one sample we obtained variations in $\Delta Q/\Delta pH$ with pH which were larger than predicted theoretically. This may depend on the inaccuracies involved in the incremental titration. With this reservation in mind, a combined buffer-capacity curve of three different samples is presented in Fig. 1. This demonstrates that, in spite of the numerical variation of the $\Delta Q/\Delta pH$ value from sample to sample, the positions of the maxima in the curves are well defined. Therefore the pH at these maxima can be taken as representing multiple pK values.

In order to see whether the different pK values of the sialic acids may be due to the different forms of open and cyclic structures, the optical rotatory power of *N*-acetylneuraminic acid was measured in water and in a 0.01M HCl solution. The mutarotation observed is represented in Fig. 2. In water the mutarotation between the two anomeric forms is a rapid process, whereas in acid solution the anomer having the greater negative optical rotation is more stable than in water.

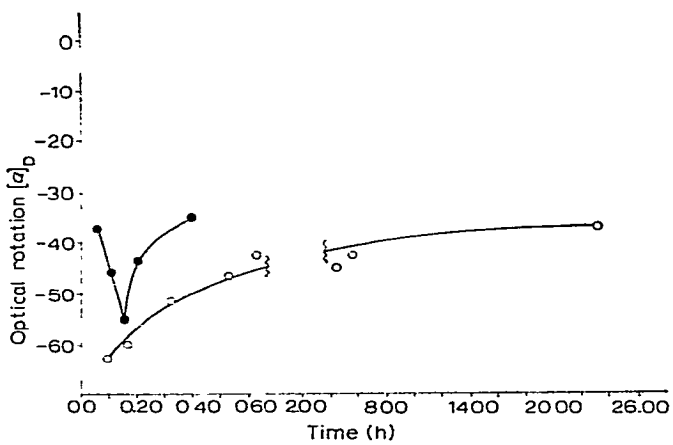

Fig. 2 — Mutarotation of *N*-acetylneuraminic acid in water (●————●) and in 0.01M HC solution (○————○).

X-Ray diffraction patterns of *N*-acetylneuraminic acid were measured on commercial samples and on samples recrystallized from water and from 0.01M HCl. The

data are presented in Table II. The interplanar spacings, as calculated from the powder diagrams, are given together with the visually estimated intensities of the corresponding reflections. The data from columns B and C indicate that the crystal struc-

TABLE II

INTERPLANAR SPACINGS AND ESTIMATED INTENSITIES OF X-RAY POWDER DIAGRAMS OF (A) COMMERCIAL *N*-ACETYLNEURAMINIC ACID, (B) RECRYSTALLIZED FROM AN AQUEOUS SOLUTION, AND (C) RECRYSTALLIZED FROM 0.01M HCl

A		*B*		*C*	
d^a *in Å*	I^b	d^a *in Å*	I^b	d^a *in Å*	I^b
8.01	s				
7.24	s			7.41	w
				6.43	s
		6.27	w		
5.21	s	5.33	w		
		4.77	w		
		4.58	s		
				4.32	s
		4.22	m		
4.16	vs	4.13	s		
		3.99	m	3.96	vs
3.64	vs	3.58	m	3.58	s
		3.41	m	3.47	m
3.30	m				
3.07	w	3.06	w		
		2.96	m	2.97	w
2.89	m				
2.68	vw	2.62	w	2.67	w
2.51	s	2.51	m	2.55	w
				2.41	vw
2.28	vw	2.30	m		
2.15	m			2.20	m
2.02	w			2.07	m
1.92	w			1.90	vw
				1.77	vw

[a]d, interplanar spacing. [b]I, relative intensity; vs, very strong; s, strong; m, medium; w, weak; vw, very weak.

ture of *N*-acetylneuraminic acid is quite different when the samples are recrystallized from aqueous or acidic solutions. Although the X-ray diffraction pattern of the commercial sample contains interplanar spacings which are different from those observed for any of the pure crystals, most of the spacings of the original material correspond to those of the material recrystallized from water. One may notice that the low-order reflections presented in column A have their corresponding second-order reflection appearing in column B (8.01→3.99; 7.24→3.58). Some of the broader peaks observed in the pattern of the commercial sample may be a combination of two peaks shown by the crystallized material, for example 4.16 Å in column A being a combination of 4.22 Å and 4.13 Å (column B). In general, there are more dissimilarities between

the results presented in column C and column B than between the results of column A and column B. A difference in the X-ray diffraction patterns does not by itself indicate different chemical forms and may simply represent polymorphism. Nevertheless, these differences considered in connection with the data of optical rotation and the pK values reinforce the suggestion that the two different crystal structures may be the result of two or more different chemical forms.

Since the previous measurements indicated that sialic acid preparations in solutions may be mixtures of different forms at equilibrium, it was decided to try to separate these forms by paper chromatography and identify them with different spotting reagents. *N*-Acetyl- and *N*-glycolylneuraminic acids were chromatographed before titration (pH 3.0) and after titration (pH 1.6). The results, reported in Table III, indicate that both sialic acids yield multiple spots with different reagents. In general, the number of components separated by chromatography increased with the acid treatment. The R_F values were compared to those reported earlier in the literature. In butyl alcohol–acetic acid–water (4:1:5), Whitehouse and Zilliken[13] observed an R_F value of 0.09 for *N*-acetylneuraminic acid compared to our values of 0.08 and 0.10; and for *N*-glycolylneuraminic acid 0.19 as compared to our values of 0.05, 0.08, and 0.14. For determinations in butyl alcohol–pyridine–water (6:4:3), they reported an R_F of 0.02 for *N*-acetylneuraminic acid compared to our values of 0.07 and 0.12; and for *N*-glycolylneuraminic acid a value of 0.14 compared to our findings of 0.04, 0.07, and 0.12.

DISCUSSION

The results of the various physical chemical measurements on free sialic acids and on sialic acids bound in mucins suggest the presence of multiple forms. An interesting feature of the inverse, buffer-capacity, titration curves was the disappearance of the pK value at 2.6 by the addition of a neutral electrolyte, KCl. If the effect of the neutral electrolyte would have been strictly a swamping of the charges, one would expect that the pK at 2.8 would disappear first. If, however, the different pK values represent equilibrium states between the different forms of *N*-acetylneuraminic acid, then an added electrolyte may affect the equilibrium between these different forms. The same phenomenon has been observed for bovine submaxillary mucin. An attempt was made, on the basis of comparisons, to assign to the different pK values found certain specific equilibrium structures. The sialic acids hydrolyzed by neuraminidase have been identified as *N*-acetylneuraminic acid (major) and mono-*O*-acetylneuraminic acid (minor), whereas the *N*,*O*-diacetylneuraminic acid seems to be nonhydrolyzable by neuraminidase[14]. Our study showed that neuraminidase removes a large part of the sialic acids present which become dialyzable and had three pK values, but not the value at 2.6. Therefore, we assume that the pK value at 2.6 represents *N*,*O*-diacetylneuraminic acid. The pK values at 2.8, 2.3, and 2.2 may correspond to an equilibrium mixture between the different ring forms and open chain structure of *N*-acetylneuraminic acid. In general, one can see also that the *N*-glycolylneuraminic

TABLE III

CHROMATOGRAPHY OF SIALIC ACIDS

Compound	*Reagent* [a]					*Solvent* [b]
	R_F	*A*	*B*	*C*	*D*	
N-Acetylneuraminic acid	.08			+		E
	.10	+		+		
	.15	+		+		
N-Acetylneuraminic acid titrated with HCl	.06			+		E
	.10	+		+		
	.15	+		+ ?		
	.22		+			
	.30				+	
N-Acetylneuraminic acid	.07	+	+	+		F
	.12	+	+	+		
N-Acetylneuraminic acid titrated with HCl	.03	+		+		F
	.06	+	+			
	.10		+			
	.18				+	
	.22				+	
N-Acetylneuraminic acid	.04			+		G
N-Acetylneuraminic acid titrated with HCl	.04			+		G
	.06			+		
N-Glycolylneuraminic acid	.05	+		+		E
	.08	+		+		
	.14	+				
N-Glycolylneuraminic acid titrated with HCl	.05			+		E
	.07	+		+		
	.14	+				
	.21	+				
N-Glycolylneuraminic acid	.04			+		F
	.07	+	+ ?	+		
	.12	+				
N-Glycolylneuraminic acid titrated with HCl	.04	+		+		
	.07		+ ?	+		F
	.11	+	+ ?	+		
	.29	+				

[a] A, alkaline silver nitrate; B, ninhydrin; C, Ehrlich reagent; and D, Congo Red. [b] E, butyl alcohol–acetic acid–water (4:1:5); F, butyl alcohol–pyridine–water (6:4:3); and G, butyl alcohol–pyridine–water (4:6:3).

acid has lower pK values than *N*-acetylneuraminic acid or *N,O*-diacetylneuraminic acid, especially in regard to the first two pK values. From the porcine submaxillary mucin, the neuraminidase removed the sialic acids having the lowest pK values, whereas the nondialyzable fraction contained the sialic acids having the highest pK values. One may tentatively ascribe the pK values at 1.7 and 1.9 to *N*-glycolylneuraminic acid.

The optical rotatory power of *N*-acetylneuraminic acid showed (Fig. 2) that the

anomer with the greatest negative rotation is more stable in acidic medium than in water and, therefore, at any point during the acidic titration there may be an equilibrium mixture of at least three different forms; namely, two ring-form anomers and the open-chain form. Gottschalk also indicated that the open-chain form is part of the equilibrium mixture[15]. The fact that the crystal structure of *N*-acetylneuraminic acid isolated from 0.01M HCl produced an X-ray diffraction pattern different from that of *N*-acetylneuraminic acid recrystallized from water is in agreement with the optical rotatory power data presented here. The crystal pattern obtained from acidic solution may represent the packing arrangement of *N*-acetylneuraminic acid in one of its more predominant anomeric forms.

The multiple forms of sialic acids in the equilibrium mixture have been shown also by paper chromatography. Sialic acids in aqueous solutions yielded 2 to 3 spots, whereas, after acidic titration, 4 to 5 spots were obtained. Similar observations on *N*-acetyl, *N*-glycolyl-, and *N,O*-diacetylneuraminic acids were reported by Berggård and Odin[3]. Both in the present study and in that of Berggård and Odin[3], recrystallized sialic acids were examined and, therefore, the multiple spots are not an indication of impurities.

Using paper chromatography, Berggård and Odin observed that heating *N*-acetylneuraminic acid with mild acid or alkali led to several degradation products. In the present study more chromatographic spots were also obtained after acid treatment. Since some of these spots were stained with ninhydrin, some cleavage of the *N*-acetyl bond may have occurred in acidic media, thus increasing the number of species in the equilibrium mixtures. This was substantiated with the n.m.r. spectra obtained on acid treated *N*-acetylneuraminic acid as compared to *N*-acetylneuraminic acid and other reference compounds. The number of pK values also increased from two to four when the titration was carried to lower pH values, thus indicating that the deacetylated form of the sialic acid may have its own pK value.

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ALKALINE-REDUCTIVE CLEAVAGE OF OVINE SUBMAXILLARY MUCIN

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ABSTRACT

Alkaline-reductive cleavage of ovine submaxillary mucin released 80% of the sialic acid from the mucin. Seventy-six percent of the released sialic acid was recovered linked to 2-acetamido-2-deoxy-D-galactitol in a compound which was shown to be *O*-(*N*-acetylneuraminyl)-(2→6)-2-acetamido-2-deoxy-D-galactitol by elementary analysis, quantitative sugar analysis, periodate oxidation, and Smith degradation. Isolation of this compound was achieved by chromatography on carbon and diethylaminoethyl Sephadex A-25.

INTRODUCTION

Graham and Gottschalk[1] presented evidence that the principal carbohydrate side chain of ovine submaxillary mucin is the disaccharide *O*-(*N*-acetylneuraminyl)-(2→6)-2-acetamido-2-deoxygalactose (**1**). This evidence was based upon periodate oxidation, enzymatic cleavage, chromatographic, electrophoretic, and elementary analysis of the dialyzable fragments obtained by hydrolysis with dilute aqueous barium hydroxide. This hydrolysis procedure split off 39% of the sialic acid of the mucin in dialyzable form, but only about half of the dialyzable sialic acid was attached to 2-acetamido-2-deoxygalactose. The remainder of the dialyzable sialic acid was present as free *N*-acetylneuraminic acid and as *N*-acetylneuraminic acid attached to chromogens presumably formed by the action of alkali upon the 2-acetamido-2-deoxygalactose residue.

The present work was undertaken to obtain evidence by an independent route that the disaccharide unit **1** constitutes the principal side-chain of ovine submaxillary mucin. Alkaline-reductive cleavage with sodium borohydride was used to obtain a high yield of the disaccharide *O*-(*N*-acetylneuraminyl)-(2→6)-2-acetamido-2-deoxygalactitol (**2**).

EXPERIMENTAL

Degradation of ovine submaxillary mucin. — The mucin was isolated as the calcium salt by the method of Tsuiki *et al.*[2] as modified by Tettamanti *et al.*[3]. Analyses

of three lyophilized preparations of ovine submaxillary mucin (isolated by the same procedure from one large batch of ovine submaxillary glands and designated as Preparations I, II, and III, respectively) showed a content of 35–41% protein, 26–29% *N*-acetylneuraminic acid, 18.3–19.5% hexosamine, 1.3–2.2% hexose (including fucose), and 6.0–8.2% water. Moving boundary electrophoresis of these preparations in phosphate buffer, $\Gamma/2=0.1$, pH 7.58 with the Perkin Elmer model 238 gave a single peak of mobility -6.9×10^{-5} cm^{-2} V^{-5} sec^{-1}.

Alkaline-reductive cleavage was performed by incubating the mucin for 6 h at 37° with 0.3M $NaBH_4$ in a 0.1M NaOH aqueous solution; this mixture was kept with constant stirring in a vessel equipped with a guard tube containing NaOH pellets. The reaction was terminated by acidification of the chilled solution with N acetic acid until pH 5.0 was reached, and then the reaction mixture was dialyzed 3 times against distilled water. A second alkaline-reductive cleavage of the nondialyzable fraction was sometimes performed. The dialyzate was lyophilized, and the residue was dissolved in a minimum volume of water and acidified to pH 4.5 by dropwise addition of N acetic acid. Methanol was added, and the borate ions were removed as volatile methyl borate in a flash evaporator at 35°. Acetamido-2-deoxy-D-glucitol was added, as an internal standard, at the end of the reductive cleavage, prior to dialysis, for the quantitative determination of 2-acetamido-2-deoxy-D-galactitol by gas–liquid chromatography (g.l.c.); otherwise the addition of 2-acetamido-2-deoxy-D-glucitol was omitted.

In an experiment performed to determine whether the conditions of exposure to alkaline borohydride caused extensive modification of the 2-acetamido-2-deoxy-D-galactose other than its conversion into the corresponding *N*-acetylated amino sugar alcohol, 2-acetamido-2-deoxy-D-galactose (10.0 mg) was treated with borohydride as described above; 2-acetamido-2-deoxy-D-glucitol (10.4 mg) was added as an internal standard. The residue was trimethylsilylated and submitted to g.l.c. Only two peaks were observed (Fig. 1), one which corresponded to the derivative of the internal standard, and one (18 min after the first pyridine peak) to 2-acetamido-2-deoxy-D-galactitol (9.9–10.1 mg) indicating lack of degradation.

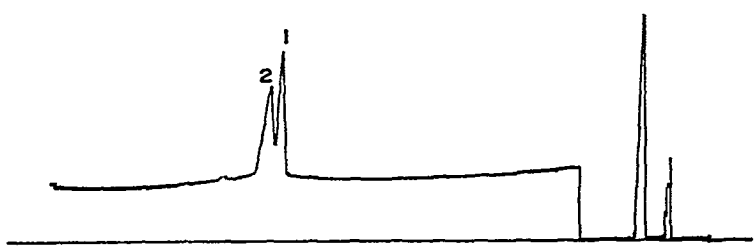

Fig. 1. Separation of trimethylsilyl derivatives by gas–liquid chromatography on a 12-foot butanediol succinate column. Peak 1, 2-acetamido-2-deoxy-D-glucitol (internal standard) eluted at 207°. Peak 2, 2-acetamido-2-deoxy-D-galactitol eluted at 210°.

Preparative chromatography. — After removal of the borate ions, the dialyzates were fractionated by carbon chromatography[4]. A 3:2 mixture (w/w) of Darco G-60

carbon and Celite was extracted with conc. HCl, washed with distilled water, and then further washed with 60% ethanol and distilled water; the carbon was deactivated[5] further by treatment with 1% stearic acid in 95% ethanol. A 10 × 1.2 cm column of this mixture could fractionate material containing up to 3 mg of sialic acid; overloading of the column resulted in extensive tailing of the eluted fraction containing sialic acid.

More rapid processing of larger amounts of the dialyzate was effected by adsorption chromatography on carbon pads, rather than on tall carbon columns. The Darco G-60–Celite mixture was poured into a glass funnel containing a "fine" sintered glass disc, 3 cm in diameter, upon which was layered an 0.8-cm thick pad of Celite. The height of the carbon pad was 5 cm. A sample of dialyzate (30 mg) dissolved in water (1 ml) was added to the pad, and desorption was effected by sequential addition of water (100 ml), 5% aq. ethanol (100 ml), and 15% aq. ethanol (400 ml). The filtrates were collected under a mild vacuum at the rate of 18 ml/h. The filtrates were analyzed for sialic acid and examined by high-voltage electrophoresis.

Further purification of the eluates from the carbon chromatography was achieved by chromatography on a column of diethylaminoethyl Sephadex A-25 (Pharmacia, New Market, N.J., lot T 01601). The ion exchanger swelled in an excess of water, and the fine particles were removed by decantation. The remaining beads were converted into the chloride form with 0.5M HCl, and then washed with water; they were then converted into the hydroxide form with 0.5M NaOH and washed again with water. The beads were extracted with M pyridinium acetate, pH 5.1, followed by washing with an excess of water, and finally they were equilibrated with 0.05M pyridinium acetate, pH 5.1. They formed a column bed (19 cm × 0.9 cm), and a flow of 12 ml/h was maintained with a polystaltic pump (Buchler Instruments, New Jersey). Fractions of 10 ml were collected with a refrigerated fraction collector (Buchler) kept at 6°. The effluent fractions were analyzed as described before, and they were concentrated to dryness by flash evaporation at 35°. The dry residues were dissolved in water and the solution was evaporated; this procedure was repeated until no more pyridinium acetate distilled. The samples were finally lyophilized and then dried for 24 h at 55° under vacuum. The elementary analyses and the acetyl determination were performed by Schwarzkopf Microanalytical Laboratory, Woodside, New York.

Colorimetric analysis. — Quantitative analysis of amino sugars was performed by the Elson–Morgan[6] method, as modified by Boas[7]; the samples were hydrolyzed in 2M HCl for 6 h at 100°. *N*-Acetylneuraminic acid was determined with the resorcinol reagent, as described by Miettinen and Takki-Luukkainen[8]. Bound sialic acid was determined with the thiobarbituric acid reagent, by the method of Warren[9], after acid hydrolysis in 0.1N H_2SO_4 for 1 h at 80°. Hexose was determined with the orcinol–H_2SO_4 reagent as described by Vasseur[10], and fucose was determined with the cysteine–H_2SO_4 reagent and the 10-minute heating modification as described by Dische and Shettles[11]. Nitrogen was determined by a micro-Kjeldahl procedure with a ninhydrin reagent[12,13] and 2-acetamido-2-deoxy-D-galactitol and *N*-acetylneuraminic acid as standards.

Paper chromatography, thin-layer chromatography, and high-voltage electro-

phoresis. — Paper chromatography was performed on Whatman No. 1 paper and by the descending technique at 25°. The tanks were equilibrated for 6 h with the aqueous phase for two-phase systems or with the moving phase for single-phase systems. Glass plates coated to a thickness of 250 μ with Silica Gel G or with Avicel (Analtech Inc., Wilmington, Delaware) were used for t.l.c. Plates coated with Silica Gel G were activated by heating for 1 h at 100°.

High-voltage electrophoresis was performed on Whatman 3 MM paper at 30 V/cm for 1½ h with a water-cooled, flat-plate instrument (Savant Instruments, Hicksville, N.Y.); the mobility was expressed relative to N-acetylneuraminic acid.

The solvent systems A-F were employed for chromatography and G-H for high-voltage electrophoresis: (A) butyl alcohol–pyridine–water (6:4:3); (B) ethyl acetate–pyridine–acetic acid–water (5:5:1:3); (C) ethyl acetate–pyridine–acetic acid–water (5:5:1:5); (D) chloroform–acetone–5M ammonia (8:1:1); (E) butyl alcohol–acetone–diethylamine–water (10:10:2:5); (F) phenol–water (75:25 w/w); (G) pyridine–acetic acid–water (200:8:1200 v/v), pH 6.4; (H) aqueous 1% sodium tetraborate, pH 9.2.

The spots on paper chromatograms and electrophoretograms were detected by benzidine–periodate[14] and by 0.5% ninhydrin in acetone. The amino acids on Avicel plates were semiquantitatively evaluated by visual comparison of the intensity of the ninhydrin color in the sample spots with the color intensity of spots from graded concentrations of standard amino acids. Spots on Silica Gel G plates were detected by charring in the presence of conc. H_2SO_4 and with the benzidine–periodate reagent.

Gas chromatography. — Analysis for hexosaminitols was performed by g.l.c. with the Perkin–Elmer Model 801 gas chromatograph. 2-Acetamido-2-deoxy-D-galactitol and 2-acetamido-2-deoxy-D-glucitol have been separated from one another and from amino sugars by g.l.c. on 6-foot columns of butanediol succinate[15], but improved separation for quantitative analysis was achieved in this work with glass columns (12 foot × ¼ inch OD) containing butanediol succinate at a coating weight of 10% on acid-washed Celite 545 (Perkin–Elmer Corp., Norwalk, Connecticut). The conditions of operation were: Helium pressure 80 lbs/sq. inch, flow 38 ml/min, and 4°/min linear program from 150–225°. 2-Acetamido-2-deoxy-D-galactitol, m.p. 175–176°, $[\alpha]_D^{25}$ −40° (*c* 0.35, water) and 2-acetamido-2-deoxy-D-glucitol, m.p. 153–154°, $[\alpha]_D^{20}$ −8.5° (*c* 2.37, water), prepared by the method of Crimmin[16], served as reference compounds for qualitative analysis by g.l.c., t.l.c., and by high-voltage electrophoresis. The dialyzates and the purified fractions isolated from the dialyzates of the alkaline-borohydride reaction mixtures were hydrolyzed with 2M HCl for 6 h at 100°. After removal of HCl *in vacuo*, the hydrolyzates were *N*-acetylated by the method of White[17]. The resulting solutions were filtered, evaporated *in vacuo*, and the residues were dissolved in pyridine. The trimethylsilyl ethers were prepared as described by Sweeley *et al.*[18].

For quantitative analysis by g.l.c., a known amount of 2-acetamido-2-deoxy-D-glucitol was added as an internal standard to the sample (reaction products before dialysis, or to purified fractions) prior to acid hydrolysis. The chromatogram areas

of the trimethylsilyl derivatives of the *N*-acetylated alditols were measured, and the amount of 2-acetamido-2-deoxygalactitol was estimated from a calibration curve; this latter curve was established by plotting the ratio of the area of 1,3,4,5,6-penta-*O*-trimethylsilyl-2-acetamido-2-deoxy-D-galactitol to the area of 1,3,4,5,6-penta-*O*-trimethylsilyl-2-acetamido-2-deoxy-D-glucitol against the ratio of the amount of 2-acetamido-2-deoxy-D-galactitol to the amount of 2-acetamido-2-deoxy-D-glucitol.

Analysis of mixtures of the amino sugar alcohols by g.l.c. showed a precision of ± 7% expressed as average deviation from the mean. Precision better than this could not be obtained because of the need for estimating the areas of curves which overlapped, despite the improvement obtained by the use of the 12-foot columns (Fig. 1) instead of the 6-foot columns.

Approximately 14% of the two *N*-acetylated amino sugar alcohols were modified during acid hydrolysis and acetylation as judged by examination of the standards similarly treated. Corrections were not made for these changes, since both *N*-acetylated amino sugar alcohols showed these effects to an approximately equal extent.

Preparative g.l.c. was performed with a neopentylglycol succinate column (3 ft. × ¼ in. O.D.)[19], coating weight 10%, on acid-washed and silanized Gas Chrom. P 80/100. The trimethylsilyl derivative was collected in a glass capillary tube attached to the exit port[20]. A small piece of dry ice was passed over the capillary to cool the tube. The trimethylsilyl derivative was dissolved in $CHCl_3$ and deposited on 10 mg of KBr. A semi-micro pellet, 1½ mm in diameter, was pressed with a micro die (Perkin–Elmer Corp., Norwalk, Connecticut). The spectra were recorded with the Perkin–Elmer model 137 Infrared Spectrophotometer equipped with a beam condenser (1 × 4) and a reference beam attenuator.

Periodate oxidation. — A sample containing 6.55 mg of *N*-acetylneuraminic acid and 4.7 mg of 2-acetamido-2-deoxy-D-galactitol obtained from the diethylaminoethyl Sephadex A-25 eluate (D_2) was treated with a three-fold excess of 0.06M $NaIO_4$ in acetate buffer, pH 4.5, in the dark at 3°. The periodate consumption was determined by the method of Fleury and Lange[21], and the production of formaldehyde with the chromotropic acid reagent[22]. DL-Serine served as a standard for the formaldehyde determination.

In the application of the Smith degradation[23], the excess of periodate was destroyed after 18 h by addition of 2,3-butanediol. The reaction mixture was adjusted to pH 7–8 by dropwise addition of M NaOH, treated with an amount of $NaBH_4$ which was three-fold in excess of the theoretical number of aldehyde groups generated, kept 4 h at room temperature, and then overnight at 3°. The excess of borohydride was destroyed with M HCl; methanol was added, and methyl borate was removed by flash evaporation. The product was dissolved in 0.05N H_2SO_4 and was hydrolyzed for 1 h at 80°, and then for 18 h at 25°. The hydrolyzate was neutralized with $BaCO_3$, and the resulting precipitate of $BaSO_4$ was removed by filtration on Whatman No. 42 filter paper. The filtrate was passed through a column of the mixed bed resin Bio Rad AG 501-X8, and the effluent was concentrated by flash evaporation. The concentrate was examined for the presence of 1,2-ethanediol by t.l.c.[24].

The oxidation of hexosamine residues in ovine submaxillary mucin also was investigated. An aqueous solution (5 ml) containing ovine submaxillary mucin (5.3 mg) was mixed with a solution containing 6 mmoles of $NaIO_4$ dissolved in 0.3M acetate buffer of pH 4.0 (20 ml), and incubated in the dark at 20°. At the end of 20 h, a 10 ml-aliquot was removed, and 1,2-ethanediol (10 mmoles) was added with chilling to the withdrawn aliquot. At the end of 27 h, 1,2-ethanediol (10 mmoles) was added with chilling to the remaining 15-ml portion. These solutions and a solution of mucin not exposed to periodate (control) were concentrated to near dryness, dissolved in a minimum of water, and dialyzed for 3 days. The contents of the bags were lyophilized, and then hydrolyzed with 2M HCl and analyzed for hexosamine content.

Enzymic cleavage of the disaccharide. — An aliquot of D_2 (25.1 mg) was dissolved in 0.2M sodium acetate buffer, pH 5.5, containing 1% NaCl and 0.1% $CaCl_2$ (15 ml). To this solution was added a neuraminidase solution (0.5 ml) (General Biochemical, Chagrin Falls, Ohio; Vibrio enzyme, 500 units/ml), and the solution was incubated for 26 h at 37°. Enzyme and buffer controls were also established. The amount of *N*-acetylneuraminic acid liberated was determined, and then the incubation mixture was filtered through a pad (4 cm h. × 5 cm w.) of stearate-treated charcoal, and the pad was washed with distilled water (300 ml). The filtrate was concentrated *in vacuo* in a flash evaporator, and the concentrate was passed through a column of mixed-bed resin (AG-501 X8, 20-50 mesh, Bio-Rad). The column was washed with distilled water (200 ml), and the effluents and washings were pooled and lyophilized, to give a white powder (MBD_2N).

RESULTS AND DISCUSSION

Isolation of O-(N-*acetylneuraminyl*)-(*2→6*)-*2-acetamido-2-deoxy*-D-*galactitol* (**2**). — Table I lists the amount of *N*-acetylneuraminic acid and 2-acetamido-2-deoxy-D-galactose in the nondialyzable fraction and the corresponding amounts of these two compounds and 2-acetamido-2-deoxygalactitol in the dialyzate after one and two alkaline-reductive cleavages of ovine submaxillary mucin. The results show that one treatment cleaved 62% and two treatments 80% of the *N*-acetylneuraminic acid resi-

TABLE I

LIBERATION OF DIALYZABLE SIALIC ACID AND 2-ACETAMIDO-2-DEOXY-D-GALACTITOL FOLLOWING ALKALINE-REDUCTIVE CLEAVAGE OF OVINE SUBMAXILLARY MUCIN

Number of treatments	*N-Acetylneuraminic acid, mmoles*		*2-Acetamido-2-deoxy-D-galactose, mmoles*		*2-Acetamido-2-deoxy-D-galactitol, mmoles*	*Ratio of N-acetylneuraminic acid to 2-acetamido-2-deoxy-D-galactitol*
	Bag	Dialyzate	Bag	Dialyzate	Dialyzate	
1	16.4	26	13.6	nil[a]	24	1.08
2	52.2	218	42.2	nil[a]	228	0.96

[a]Not detectable in acid hydrolyzates.

dues of Preparation I and II, respectively, releasing *N*-acetylneuraminic acid in dialyzable, but bound form. The ratio of *N*-acetylneuraminic acid to 2-acetamido-2-deoxygalactitol in the dialyzates was essentially 1:1. In other single-treatment cleavage experiments (for which complete analyses were not performed), 70% of the *N*-acetylneuraminic acid of Preparation I was released in bound, dialyzable form, and 59% of the *N*-acetylneuraminic acid from Preparation III.

Free *N*-acetylated amino sugar alcohols (*i.e.*, not bound to sialic acid or to glycopeptides) were not detected by g.l.c. in unhydrolyzed dialyzates. Paper chromatographic examination (system A) of the aqueous and 5% ethanolic eluates from the carbon pads showed trace amounts of 2-acetamido-2-deoxygalactitol in these fractions obtained from Preparation I, but none in the corresponding fractions from Preparations II and III.

Since small amounts of peptides and other decomposition products were present in the dialyzates, it was necessary to fractionate the dialyzates on charcoal–Celite and characterize the purified fraction containing sialic acid to show that the sialic acid was linked to the 2-acetamido-2-deoxygalactitol. Sialic acid (yield 80–90%) was found only in the 15% eluate (C-15). Examination by high-voltage electrophoresis in systems G and H of three C-15 preparations isolated from freshly prepared carbon columns revealed a band which was benzidine–periodate positive, ninhydrin negative, and exhibited the following mobilities: $M_{\text{N-acetylneuraminic acid}} = 0.75$ in system G and 1.1 in system H. Qualitative analysis of C-15 and of the electrophoretogram eluate showed no amino sugars by g.l.c. or by the Elson–Morgan method, no free hexoses or hexitols (solvent C), and no free *N*-acetylated amino sugar alcohol by g.l.c. and by high-voltage electrophoresis. Quantitative t.l.c. on Avicel of an acid hydrolyzate of C-15 (6M HCl, 18 h, 100°) showed less than 0.7 μg of amino acid per 40 μg of *N*-acetylneuraminic acid. Quantitative analysis of C-15 and of the electrophoretogram eluate showed 2-acetamido-2-deoxygalactitol to be in a molar ratio of essentially 1 relative to *N*-acetylneuraminic acid; the eluate of the band given by high-voltage electrophoresis of C-15 contained *N*-acetylneuraminic acid and 2-acetamido-2-deoxygalactitol in the ratio 1.3:1.0 by weight.

Anal.: Calc. for $C_{19}H_{34}N_2O_{14}$: N, 5.45% Found: N, 5.50%.

Further fractionation on diethylaminoethyl Sephadex A-25 of a sample (11 mg) gave resorcinol-positive fractions in the first 10–28 ml fraction (D_1) and in the 48–80 ml fraction (D_2) as shown in Fig. 2. Ninety-six percent of the applied sialic acid was recovered in the eluate. Fraction D_1 contained 4% of the eluted sialic acid. High-voltage electrophoresis of fraction D_1 in system H showed three benzidine-positive components of $M_{\text{N-acetylneuraminic acid}}$ 1.0, 0.8, and 0.45 respectively. Fraction D_2 contained 96% of the eluted *N*-acetylneuraminic acid and gave a single benzidine–periodate positive spot R_{lactose} 0.41 in solvent A and $R_{\text{N-acetylneuraminic acid}}$ 1 in solvent B; fraction D_2 exhibited the same mobilities on high-voltage electrophoresis as did fraction C-15, *i.e.*, $M_{\text{N-acetylneuraminic acid}}$ 0.75 in G and 1.1 in H. Lyophilization of fraction D_1 gave a barely visible, buff-colored film, and lyophilization of fraction D_2 a white powder (Compound **2**).

Anal.: Calc. for $C_{19}H_{34}N_2O_{14}$: C, 44.05; H, 6.67; N, 5.45; acetyl, 16.73%. Found: C, 43.23; H, 6.61; N, 5.27; acetyl, 17.37%.

The deviation of the analytical data from the theoretical values may have resulted from the uptake of water by the sample which was hygroscopic; use of a lyophilized rather than a crystalline preparation also could have contributed to the discrepancies.

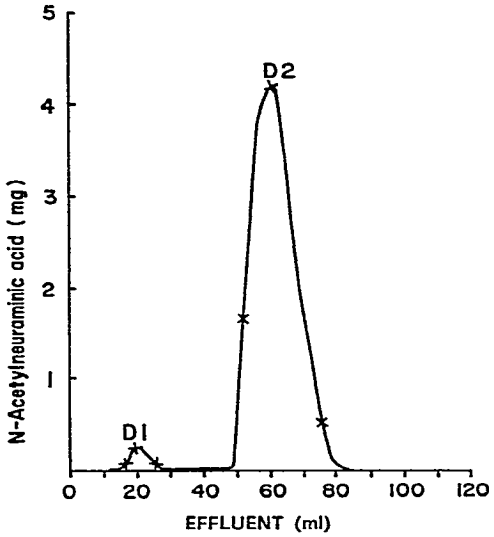

Fig. 2. Column chromatography on diethylaminoethyl Sephadex A-25 of a sample (11 mg) from the carbon-pad eluate. The fractions were analyzed with the resorcinol assay for sialic acid. Two peaks, D_1 and D_2, containing *N*-acetylneuraminic acid were obtained.

Free *N*-acetylneuraminic acid was detected in one sample after prolonged storage (for 5 months at 0°) of the carbon-pad eluate. Free *N*-acetylneuraminic acid was eluted 10 ml after fraction D_2, and it was identified by chromatography in systems A and B, electrophoresis in G and H and by g.l.c. of the trimethylsilyl derivative on a column of SE-30[18].

Properties of O-(N-*acetylneuraminyl*)-(*2→6*)-*2-acetamido-2-deoxy*-D-*galactitol* (2). — Cleavage of fraction D_2 by neuraminidase gave in quantitative yield *N*-acetylneuraminic acid. Paper chromatography of MBD_2N showed one benzidine–periodate positive spot which was ninhydrin negative and had the same $R_{lactose}$ in solvent A as that given by 2-acetamido-2-deoxy-D-galactitol. The trimethylsilyl derivative of MBD_2N gave a single peak on g.l.c.; the retention time and i.r. spectrum of the sample isolated by g.l.c. were identical with those of authentic 1,3,4,5,6-penta-*O*-trimethylsilyl-2-acetamido-2-deoxy-D-galactitol.

Periodate oxidation of fraction D_2 showed by extrapolation to the ordinate a consumption of 4.2 moles of periodate per mole of disaccharide in agreement with the theoretical value (4.0 moles) for **2**. Production of 1.1 mole of formaldehyde per mole of disaccharide in 24 h indicates a C-6 substituted disaccharide. Examination of the

products of the Smith degradation by t.l.c. on Silica Gel G plates with system D[24] showed a benzidine–periodate positive spot having the same R_F (0.32) as ethylene glycol, thus confirming structure **2**.

Periodate oxidation of ovine submaxillary mucin resulted in the destruction of 93% of the hexosamine after 20 h and 94.4% after 27 h. This result confirms the earlier report by Graham and Gottschalk[1] and provides additional support that **1** is the principal side chain in ovine submaxillary mucin.

The results reported here, namely the release of 80% of the *N*-acetylneuraminic acid residues in the form of bound, yet dialyzable fragments, and recovery in fraction D_2 of 78% of the released *N*-acetylneuraminic acid residues show that we have accounted for 62% of the *N*-acetylneuraminic acid residues in ovine submaxillary mucin. The losses on carbon chromatography averaged 15% and on diethylaminoethyl Sephadex chromatography 4%. Repeated treatment or increased time of exposure to alkaline borohydride resulted in increased cleavage of the peptide core with release of dialyzable glycopeptides rather than release of dialyzable oligosaccharides. Whether this indicates that some of the remaining oligosaccharides are attached by linkages other than glycosidic linkages of amino sugar to serine or threonine or that the peptide core becomes more labile as a consequence of the removal of the side chains remains to be ascertained.

A disaccharide reported to be *O*-(*N*-glycolylneuraminyl)-2-acetamido-2-deoxy-D-galactitol, similar to the one reported in the present study, was isolated by Carlson[25] and by Katzman and Eylar[26] from the alkaline-reductive cleavage products of porcine submaxillary mucin.

Kabat *et al.*[27] reported the destruction during alkaline-reductive cleavage of some terminal and internal galactosaminyl residues present in the side chains of blood-group substances; they commented that C-3 linkages probably contributed to the destruction of this sugar and to the peeling reaction. The high recovery of **2** from ovine submaxillary mucin indicates the absence of any appreciable peeling reaction or destruction of amino sugar. The absence of substitution at C-3 of the amino sugar residue and the occurrence of short side-chains in ovine submaxillary mucin (in contrast to the longer side chains present in the blood group A, B, and H substances) may account in large measure for the observed differences in destruction and peeling. It should also be noted that Kabat and coworkers[27] employed 0.2M alkali and 1% $NaBH_4$ for one week at room temperature, whereas 0.1M alkali and 1.1% $NaBH_4$ for 6 h at 37° were used in the present study.

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isolated from ovine submaxillary mucin during investigations into the biosynthesis of side chains of ovine submaxillary mucin[28].

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OXIDATION OF CARBOHYDRATES WITH METHYL SULFOXIDE CONTAINING PHOSPHORUS PENTAOXIDE

I. SYNTHESIS OF SOME ALDOSULOSES AND ALDOSIDULOSES*

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(Received August 1st, 1967; in revised form, September 7th, 1967)

ABSTRACT

Methyl sulfoxide containing phosphorus pentaoxide oxidizes secondary alcohol groups of carbohydrates to ketones. Oxidation proceeds most efficiently with *N,N*-dimethylformamide as solvent and with 3–4 molar equivalents of methyl sulfoxide and 1.5–2.0 molar equivalents of phosphorus pentaoxide.

The following carbohydrates were oxidized to afford the corresponding aldosuloses and aldosiduloses in good or moderate yields: methyl 4,6-*O*-benzylidene-2-*O*-(*p*-tolylsulfonyl)-α-D-gluco- and allo-pyranoside (**1** and **18**), methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-gluco- and -allo-pyranoside (**3** and **19**), 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**6**), 1,2-*O*-isopropylidene-5-*O*-(*p*-tolylsulfonyl)-α-D-xylo- and -ribo-furanose (**11** and **13**), 1,2-*O*-isopropylidene-5-*O*-(di-*O*-phenylphosphono)-α-D-xylofuranose (**14**), and 1,2-*O*-isopropylidene-α-D-glucofuranurono-6,3-lactone (**16**).

INTRODUCTION

In recent years, oxidation of "isolated" hydroxyl groups of carbohydrates has been achieved by different methods. The synthetic utility and biological significance of dicarbonyl carbohydrates has prompted a search for more effective and less expensive reagents for oxidation. Many new aldosuloses and aldosiduloses have been synthesized with such oxidants as chromium trioxide, platinum oxide, and ruthenium tetroxide, and have been used successfully in the preparation of amino, branched-chain, and rare sugars[1,2].

Since Kornblum *et al.*[3] reported the oxidation of simple alkyl toluene-*p*-sulfonates and halides with sulfoxides, several systems using sulfoxides, especially methyl sulfoxide, have been developed for oxidation of alcohols[4]. In 1963, an effective oxidation of alcohols to aldehydes and ketones under very mild conditions was described by Pfitzner and Moffatt[5], who used the methyl sulfoxide–*N,N*-dicyclohexylcarbodiimide (DCC) system. This system has proved to be of potential value in the carbohydrate field, as well as with other complex alcohols, such as steroids and alkaloids[6].

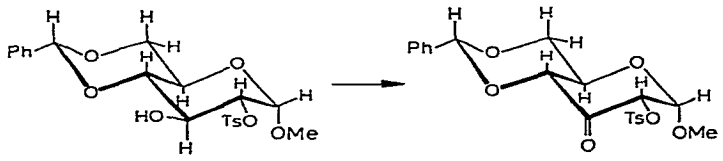
* A preliminary report of this work was given in Ref. 10.

In the course of our study on a polymerization reaction of reducing mono- and di-saccharides[7], we have found that methyl sulfoxide containing phosphorus pentaoxide rapidly oxidises the alcoholic groups of sugars at room temperature to give aldehydes, ketones, or carboxylic acids. The reaction of sulfoxides with phosphorus pentaoxide has been little investigated. Sekera *et al.*[8] used phosphorus pentaoxide in a reaction of sulfonamides with methyl sulfoxide, and Micheel *et al.*[9] suggested that phosphorus pentaoxide in methyl sulfoxide could be a polymerization reagent for reducing sugars, but no comment was made on the oxidation reaction of methyl sulfoxide. At about the time when the preliminary report of our results appeared[10], Albright and Goldman[11] reported that methyl sulfoxide–acid anhydride mixtures were effective for oxidation of alcohols. Since then, several papers have been published[12] on the application of the methyl sulfoxide–acid anhydride system to carbohydrates. In this paper, the synthesis of some aldosuloses and aldosiduloses, using methyl sulfoxide and phosphorus pentaoxide, is described.

RESULTS AND DISCUSSION

The stability of various protective groups and linkages commonly used in carbohydrate chemistry towards the present oxidant was first investigated, because of the previous report[13] that some new reactions of carboxylic acids and other organic compounds proceeded under the reaction conditions used for oxidation. The following, fully substituted carbohydrates were treated with methyl sulfoxide–phosphorus pentaoxide at room temperature: 7-(tetra-*O*-acetyl-β-D-glucopyranosyl)theophylline, 9-(tetra-*O*-acetyl-β-D-glucopyranosyl)-6-benzamidopurine, 2,4:3,5-di-*O*-benzylidene-D-xylose diethyl dithioacetal, phenyl tetra-*O*-acetyl-α-D-glucopyranoside, tri-*O*-acetyl-1,6-anhydro-β-D-glucopyranose, octa-*O*-acetylsucrose, tri-*O*-acetyl-4,6-*O*-benzylidene-D-glucopyranose, 1,2,5-tri-*O*-acetyl-D-glucurono-6,3-lactone, methyl 1,2,3,4-tetra-*O*-acetyl-D-glucuronate, penta-*O*-acetyl-α-D-glucopyranose, tetra-*O*-acetyl-6-*O*-trityl-β-D-glucopyranose, 1,2:3,4-di-*O*-isopropylidene-6-*O*-(*p*-tolylsulfonyl)-α-D-galactopyranose, 5-*O*-acetyl-1,2-*O*-isopropylidene-α-D-glucofuranurono-6,3-lactone, methyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranoside, 1,2,3-tri-*O*-acetyl-4,6-*O*-ethylidene-D-glucopyranose, and methyl 4,6-*O*-benzylidene-α-D-glucopyranoside 2,3-dinitrate. The reactions were followed by thin-layer chromatography and by the change of optical rotation. It was found that all of these substances were stable to the oxidation conditions. However, two glycosylamines, *N*-*p*-tolyl-2,3,4-tri-*O*-benzoyl-L-arabinosylamine and *N*-*p*-tolyl-2,3,4,6-tetra-*O*-acetyl-D-glucopyranosylamine, underwent reaction. For example, the reaction mixture containing the latter compound became brown after 15 h at room temperature, and only 10% of the starting material was recovered; no further study of this reaction was made in the present work. From these results, it appears that sulfonyloxy, acetoxy, benzoyloxy, isopropylidene, benzylidene, ethylidene, methoxyl, nitrate, and acetamido groups, and glycosidic bonds involving nucleosidic, phenolic, alkyl, and thioacetal substituents are stable towards the oxidant. Consequently, carbohydrates having these protective groups can be safely employed in the synthesis of dicarbonyl sugars.

The optimal conditions for the oxidation of "isolated" secondary alcoholic groups of carbohydrates were evaluated by using methyl 4,6-*O*-benzylidene-2-*O*-(*p*-tolylsulfonyl)-α-D-glucopyranoside (**1**) as a model compound. Oxidation of this compound was first accomplished by Baker *et al.*[6c] with the methyl sulfoxide–DCC reagent. The oxidation was examined at different temperatures, with different proportions of phosphorus pentaoxide and methyl sulfoxide, and with different solvents and



1 2

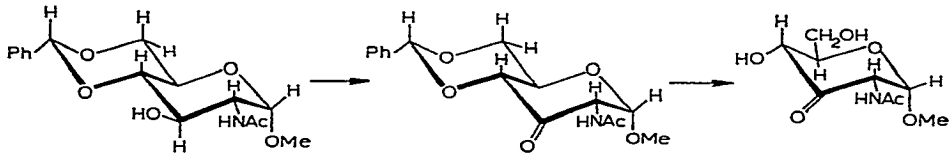
catalysts. The reaction products were easily isolated, and analyzed by n.m.r. spectroscopy and by thin-layer chromatography for the determination of the yield of the keto sugar. It was found that the crude reaction products contained only compounds **1** and **2**; no side reactions were observed. The maximal yield (85–92%) of compound **2** was obtained by treating one mole of reactant **1** with 3–4 moles of methyl sulfoxide and 1–1.5 moles of phosphorus pentaoxide (as P_4O_{10}) in *N*,*N*-dimethylformamide (DMF) for 1.5–2.0 h at 65–70°.

With methyl sulfoxide as solvent, oxidations were best performed at room temperature, since decomposition occurred at higher temperatures, resulting in a decreased yield. The use of too large an excess of phosphorus pentaoxide also decreased the yield of product. A catalytic amount of phosphorus pentaoxide was also ineffective. These results suggest that one mole of phosphorus pentaoxide per mole of alcohol and methyl sulfoxide participate in the oxidation. Other catalysts, such as zinc chloride, aluminium chloride, sulfur trioxide, arsenic oxides (As_2O_3, As_2O_5), antimony oxides (Sb_2O_3, Sb_2O_5), and *p*-toluenesulfonic acid, were all ineffective. Solvents other than methyl sulfoxide and DMF were not effective, and partial replacement of methyl sulfoxide and DMF by benzene or chloroform resulted in a marked decrease in the yield. After the completion of this work, Brimacombe *et al.*[12g] reported that oxidation of compound **1** with methyl sulfoxide–phosphorus pentaoxide gave a 49% yield of compound **2**.

The introduction of a ketonic function into amino sugars is of considerable synthetic utility, since it provides a route to isomeric, branched-chain, and di-amino sugars. Oxidation of compound **3** with methyl sulfoxide–phosphorus pentaoxide in DMF afforded (71% yield) the corresponding ketose (**4**), previously prepared from compound **3** by Baker *et al.*[6c] with the methyl sulfoxide–DCC reagent. Debenzylidenation of compound **4** with 60% acetic acid gave methyl 2-acetamido-2-deoxy-α-D-*ribo*-hexopyranosid-3-ulose (**5**).

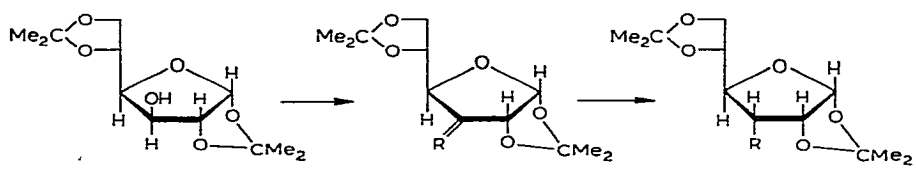
Isopropylidene acetals of carbohydrates have frequently been used in the synthesis of aldofuranosuloses, and typical examples are 1,2:5,6-di-*O*-isopropylidene-α-D-xylofuranose (**6**) and 1,2-*O*-isopropylidene-5-*O*-substituted-α-D-xylofuranose

(**11** and **14**), both of which have a dioxolane ring *cis*-fused to a furanoid ring, and an *exo*-hydroxyl group. The lability of the isopropylidene group towards acid, and the steric hindrance caused by the dioxolane ring have considerable effects on the reactivity of the hydroxyl groups. For example, attempts to oxidize



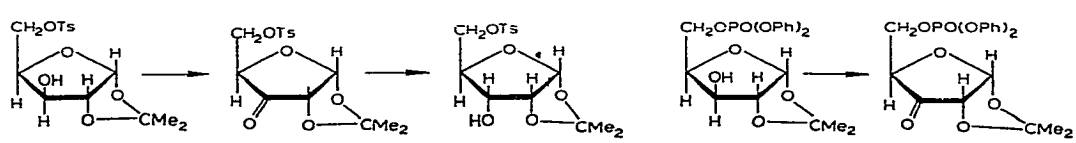
3 **4** **5**

compound **6** with chromium trioxide–pyridine, the Oppenauer reagent, and lead tetraacetate were reported to be ineffective[14]. Since D-*ribo*-hexos-3-ulose derivatives have been found as components of some microbial disaccharides[15], and have much synthetic value[16], the effective oxidation of compounds **6**, **11**, and **14** is of interest.



6 **7** R = O; **8** R = N—OH; **9** R = NH_2; **10** R = NHAc

Oxidation of compound **6** with two molar equivalents of phosphorus pentaoxide in methyl sulfoxide for 1.5–2 h at 60–65° or for 15 h at room temperature afforded 1,2:5,6-di-*O*-isopropylidene-α-D-*ribo*-hexofuranos-3-ulose (**7**) in 65% yield; the latter oxidation conditions usually gave a better yield. The oxidation in DMF gave a 50–60% yield of product. The ketose, isolated as the hydrate, reacted with hydroxylamine, thiosemicarbazide, or semicarbazide. The *allo*-amine (**9**) was obtained by reduction of the crystalline oxime **8** with lithium aluminium hydride; the *gluco* isomer was not detected on paper chromatograms of the acid hydrolyzate of the crude amine. This result is in accordance with the published behaviour of 1,2-*O*-isopropylidene-α-D-*ribo*- or -*erythro*-aldofuranos-3-ulose on reduction with metal hydrides, which give exclusively, or mainly, α-D-*allo* or *ribo* derivatives[17–19]. Acetylation of the amine **9** gave 3-acetamido-3-deoxy-1,2:5,6-di-*O*-isopropylidene-α-D-allofuranose (**10**). The facile oxidation of compound **6** is remarkable, since it is inert to the methyl sulfoxide–DCC reagent[6c], and no other oxidant, except ruthenium tetroxide[2], had been reported to be effective. The C-3 epimer of compound **6**, 1,2:5,6-di-*O*-isopropylidene-α-D-allo-

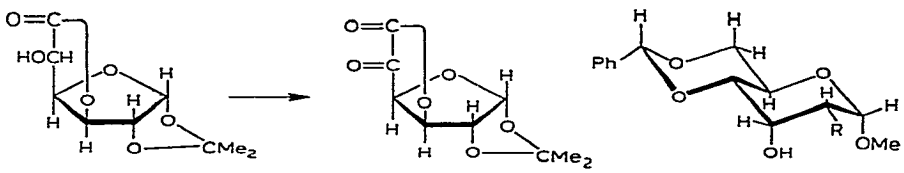


11 **12** **13** **14** **15**

furanose, is easily oxidized[19] catalytically over platinum oxide and with chromium trioxide–pyridine. The oxidation of compound **6** with acetic anhydride and methyl sulfoxide has recently been accomplished by Sowa and Thomas[12a].

Oxidation of 1,2-*O*-isopropylidene-5-*O*-(*p*-tolylsulfonyl)-α-D-xylofuranose (**11**) and 1,2-*O*-isopropylidene-5-*O*-(di-*O*-phenylphosphono)-α-D-xylofuranose (**14**) was performed in DMF with three molar equivalents of methyl sulfoxide and one molar equivalent of phosphorus pentaoxide for 1–1.5 h at 60–65°. 1,2-*O*-Isopropylidene-5-*O*-(*p*-tolylsulfonyl)-α-D-*erythro*-pentofuranos-3-ulose (**12**) was obtained in 35% yield. Reduction of compound **12** with lithium aluminium hydride gave 1,2-*O*-isopropylidene-5-*O*-(*p*-tolylsulfonyl)-α-D-ribofuranose (**13**). Similarly, 1,2-*O*-isopropylidene-5-*O*-(di-*O*-phenylphosphono)-α-D-*erythro*-pentofuranos-3-ulose (**15**) was obtained from compound **14** in 35% yield, and was characterized as the monohydrate and as the semicarbazone.

Oxidation of 1,2-*O*-isopropylidene-α-D-glucofuranurono-6,3-lactone (**16**) with active manganese dioxide[20] and chromium trioxide[21] has recently been reported. Oxidation of lactone **16** in methyl sulfoxide with phosphorus pentaoxide produced 1,2-*O*-isopropylidene-α-D-*xylo*-hexofuranurono-6,3-lactone-5-ulose (**17**) in 40% yield.



16 17 18, R = OTs
19, R = NHAc

The yield of ketoses in oxidation reactions is usually markedly influenced by the orientation of hydroxyl groups in the pyranoid (axial–equatorial) or fused bicyclic systems (*exo–endo*). As demonstrated above, the methyl sulfoxide–phosphorus pentaoxide reagent oxidizes sterically hindered secondary alcohols. This fact, together with other results[12e,22], may be a reflection of the small influence of steric factors in this oxidation reaction. In the present study, three epimeric pairs of sugar alcohols were treated with methyl sulfoxide–phosphorus pentaoxide. The following compounds, having axial or *endo* secondary hydroxyl groups, were synthesized from the corresponding ketoses: (*a*) methyl 4,6-*O*-benzylidene-2-*O*-(*p*-tolylsulfonyl)-α-D-allopyranoside (**18**), (*b*) methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-allopyranoside (**19**), and (*c*) 1,2-*O*-isopropylidene-5-*O*-(*p*-tolylsulfonyl)-α-D-ribofuranose (**13**). Oxidation of these compounds was performed under conditions similar to those employed for oxidation of the epimeric derivatives. The yield of ketose from compound **18** was 80% (*cf.* 92% for the *gluco* isomer). For compound **19** and its *gluco* isomer, the yields were 58 and 71%, respectively. In the case of compound **13** and its *xylo* isomer, the yields of ketose were 31 and 28%, respectively. These results show that the orientation of hydroxyl groups does not affect the rate of the oxidation by methyl sulfoxide and phosphorus pentaoxide.

EXPERIMENTAL

General. — Melting points were measured on a hot stage. Specific rotations were determined with a Yanagimoto direct-reading polarimeter. Nuclear magnetic resonance (n.m.r.) spectra were recorded at 60 MHz with a Varian A-60 spectrometer, and chemical shifts are expressed on the δ scale in parts per million (p.p.m.) downfield displacement from tetramethylsilane or sodium 2,2-dimethyl-2-silapentane-5-sulfonate as internal standard. Infrared (i.r.) spectra were measured on a Shimazu AR-7 spectrometer, using a sodium chloride prism. Paper chromatography was carried out by the descending technique with Toyo Roshi No. 51 filter paper. Thin-layer chromatography (t.l.c.) was performed on Silica Gel G (RSCO).

Methyl sulfoxide was freshly distilled under decreased pressure from Linde Molecular Sieves. Phosphorus pentaoxide (P_4O_{10}) of commercial grade was used for oxidation. Sodium sulfate was used to dry organic solutions.

Fully substituted derivatives of carbohydrates listed in the Discussion were prepared by the literature methods[23,24].

Treatment of fully substituted carbohydrates with methyl sulfoxide–phosphorus pentaoxide. — Each derivative and 1 molar equivalent of phosphorus pentaoxide were dissolved in methyl sulfoxide, and the solution was allowed to stand at room temperature with occasional shaking. The reaction mixtures were examined by t.l.c. (benzene–methanol, 98:2), and for change in optical rotation.

Methyl 4,6-O-benzylidene-2-O-(p-tolylsulfonyl)-α-D-ribo-hexopyranosid-3-ulose (**2**). — A mixture of 7.2 g of methyl 4,6-*O*-benzylidene-2-*O*-(*p*-tolylsulfonyl)-α-D-glucopyranoside[25] [**1**; n.m.r. ($CDCl_3$): δ 4.84 (H-1, $J_{1,2}$ 3.2 Hz), 3.34 (OCH_3)], 5 g of methyl sulfoxide, 8 g of phosphorus pentaoxide, and 200 ml of DMF was heated for 2 h at 65–70° with stirring. The reaction mixture was poured into ice–water, and the solution was kept in a refrigerator overnight. The crystals (6.7 g, 92%) were collected by filtration and washed thoroughly with water. This preparation was found to contain no starting material and no by-products on examination by t.l.c. (benzene–methanol, 98:2) and n.m.r. spectroscopy. Crystallization from ethanol gave white crystals, m.p. 162–164°; $[\alpha]_D^{28}$ +44.6° (*c* 1.0, chloroform) [lit.[6c] m.p. 165–167°, $[\alpha]_D^{25}$ +44.9° (DMF)]; ν_{max}^{Nujol} 1775 cm^{-1} (C=O); n.m.r. ($CDCl_3$): δ 5.26 (H-1, doublet, $J_{1,2}$ 4.1 Hz), 5.12 (H-2, doublet), 3.42 (OCH_3).

For the study of the reaction conditions, 0.3 g of compound **1** was dissolved in 6 ml of solvent. The oxidation was performed on a water bath with occasional shaking, and exclusion of moisture. The reaction mixture was poured into ice–water to effect precipitation. When small proportions of precipitate were obtained, extraction with chloroform was also performed. The white, throughly washed product was subjected to t.l.c. and n.m.r. spectroscopy to determine the yield of the ketose.

A solution of methyl 4,6-*O*-benzylidene-2-*O*-(*p*-tolylsulfonyl)-α-D-allopyranoside[6c] (**18**; 0.3 g), methyl sulfoxide (0.162 g), and phosphorus pentaoxide (0.190 g) in DMF (6 ml) was heated for 2 h at 65–70°. Isolation of compound **2** (80%) was performed as described above.

*Methyl 2-acetamido-4,6-O-benzylidene-2-deoxy-α-*D*-ribo-hexopyranosid-3-ulose* (**4**). — A mixture of 1.8 g of methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranoside [**3**; n.m.r. (pyridine-d_5): δ 5.26 (H-1, doublet, $J_{1,2}$ 4.0 Hz)], 2 g of phosphorus pentaoxide, and 30 ml of methyl sulfoxide was heated for 15 h at 65–70°. The reaction mixture was diluted with 30 ml of chloroform, and then 30 ml of cold water was added. Upon vigorous shaking, the mixture separated into two phases. The chloroform layer was washed with a small portion of ice–water until neutral, and then dried. Evaporation of the chloroform *in vacuo* at 30–40° afforded 0.8 g (44% yield) of compound **4**. Oxidation with 4 molar equivalents of methyl sulfoxide and 1 molar equivalent of phosphorus pentaoxide in DMF gave a 71% yield of compound **4**. Recrystallization from methanol gave white crystals, m.p. 222°; $[\alpha]_D^{22}$ +128° (*c* 1.0, DMF) [lit.[6c] m.p. 227–228°, $[\alpha]_D^{25}$ +110°]; ν_{max}^{Nujol} 1735 (C=O) cm^{-1}; n.m.r. (pyridine-d_5): δ 4.60 (H-1, doublet, $J_{1,2}$ 4.3 Hz).

A solution of methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-allopyranoside[6c] (**19**; 0.3 g), methyl sulfoxide (0.290 g), and phosphorus pentaoxide (0.27 g) in DMF (6 ml) was heated for 2 h at 65–70°. Isolation of compound **4** (58%) was performed as described above.

*Methyl 2-acetamido-2-deoxy-α-*D*-ribo-hexopyranosid-3-ulose* (**5**). — Debenzylidenation of compound **4** by the method of Jeanloz[26] gave ketone **5** in 80% yield. Recrystallization from methanol afforded white crystals, m.p. 174–176°; $[\alpha]_D^{22}$ +118° (*c* 1.0, water); ν_{max}^{Nujol} 1735 (C=O), 1650, 1555, 955, 850 cm^{-1}; n.m.r. (D_2O): δ 5.20 (H-1, doublet, $J_{1,2}$ 4 Hz), 4.95 (H-2, quartet, $J_{2,4}$ 1.0 Hz), 4.50 (H-4, quartet, $J_{4,5}$ 10.0 Hz).

Anal. Calc. for $C_9H_{15}NO_6$: C, 46.35; H, 6.48; N, 6.01. Found: C, 46.33; H, 6.54; N, 5.99.

*1,2:5,6-Di-O-isopropylidene-α-*D*-ribo-hexofuranos-3-ulose* (**7**). — To a stirred solution of 15 g of 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose [**6**; n.m.r. ($CDCl_3$): δ 5.93 (H-1, doublet, $J_{1,2}$ 3.7 Hz)] in 150 ml of methyl sulfoxide was carefully added 15 g of phosphorus pentaoxide whilst the temperature was maintained at 25–30° with cooling. After being stirred for 20 h at room temperature, the brownish red mixture was diluted with 150 ml of chloroform, and then water (150 ml) was added with cooling. After vigorous shaking, the mixture separated into two phases. The chloroform layer was washed with a small portion of ice–water until neutral, and then dried. Evaporation of the chloroform *in vacuo* at 30–40° afforded a syrup which crystallized upon standing at room temperature. Recrystallization from light petroleum gave white crystals, yield 9.7 g (65%); m.p. 108–112°; $[\alpha]_D^{28}$ +40.0° (*c* 2.0, chloroform). This compound was recrystallized three times from light petroleum to give the monohydrate, m.p. 118–119°, $[\alpha]_D^{28}$ +110° (*c* 1.0, chloroform) {lit.[2] b.p. 97° (0.01 mm), $[\alpha]_D$ +107°; monohydrate[2], m.p. 109–113°, $[\alpha]_D$ +45° in chloroform; monohydrate[19], m.p. 108–110°, $[\alpha]_D^{22}$ +40.2° (*c* 0.5, water)}; n.m.r. ($CDCl_3$): δ 5.87 (H-1, doublet, $J_{1,2}$ 3.8 Hz), 4.28 (H-2, doublet, $J_{2,1}$ 3.8 Hz). A strong ketone absorption was observed at 1770 cm^{-1} in the i.r. spectrum (Nujol) of the syrup that was obtained by heating the monohydrate for 1 h at 100° *in vacuo*.

Compound **7** (3 g) and hydroxylamine hydrochloride (3 g) were dissolved in 20 ml of ethanol and 20 ml of pyridine. The solution was refluxed for 2 h. A syrup obtained on evaporation of solvents was washed with a small portion of cold water, and crystallised from ether to give oxime **8** (2 g). Recrystallization from ether afforded material having m.p. 103–104°; $[\alpha]_D^{28}$ +187° (*c* 1.5, chloroform) {lit.[2] m.p. 103–104°, $[\alpha]_D$ +180°}.

3-Amino-3-deoxy-1,2:5,6-di-O-*isopropylidene*-α-D-*allofuranose*(**9**). — To a stirred solution of compound **8** (250 mg) in 30 ml of anhydrous tetrahydrofuran was added 200 mg of lithium aluminum hydride with cooling. The reaction mixture was refluxed for 3 h with exclusion of moisture. After the addition of 30 ml of ethyl acetate, the mixture was poured into ice–water and extracted with chloroform. The extract was concentrated to give a crystalline residue (120 mg), which was recrystallized from ether to give compound **9**, m.p. 88–90°; $[\alpha]_D^{28}$ +41.3° (*c* 1.2, chloroform) {lit.[27,28] m.p. 88–89°, 92–93°, $[\alpha]_D$ +41.0°, +40.5°}; ν_{max}^{KBr} 1590, 1505, 775, 880, 860, 830 cm^{-1}; n.m.r. ($CDCl_3$): δ 5.77 (H-1, doublet, $J_{1,2}$ 3.5 Hz.), 4.56 (H-2, quartet, $J_{2,3}$ 5.0 Hz), 3.13 (H-3, quartet, $J_{3,4}$ 8.5 Hz), 3.65 (H-4, quartet, $J_{4,5}$ 6.5 Hz), 4.2–3.9 (H-5 protons, multiplet).

Acetylation of amine **9** with acetic anhydride in pyridine gave the *N*-acetyl derivative; m.p. 128–129°; $[\alpha]_D^{28}$ +71.8° (*c* 0.8, chloroform) {lit.[29] m.p. 127–128°, $[\alpha]_D$ +71.3°}; ν_{max}^{KBr} 3320, 1680, 1540, 880, 845, 800 cm^{-1}.

1,2-O-*Isopropylidene-5*-O-(p-*tolylsulfonyl*)-α-D-erythro-*pentofuranos-3-ulose*(**12**). — 1,2-*O*-Isopropylidene-5-*O*-(*p*-tolylsulfonyl)-α-D-xylofuranose [**11**, n.m.r. ($CDCl_3$): δ 5.88 (H-1, doublet, $J_{1,2}$ 4 Hz)] (5 g), phosphorus pentaoxide (8 g), and methyl sulfoxide (0.86 g) were dissolved in DMF (120 ml). The solution was heated for 4 h at 60–65° with stirring, and then extracted with chloroform (50 ml) three times. The extract was washed with cold water, dried, and concentrated *in vacuo*, and the syrupy residue was dissolved in methanol and decolorized with charcoal. After evaporation of the solvent, recrystallization of the residue was effected from methanol–ether–light petroleum to give white needles (1.7 g). Further recrystallization gave the methanolate of the title compound, m.p. 105–106°; $[\alpha]_D^{18}$ +70.5° (*c* 1.0, chloroform); ν_{max}^{Nujol} 3430, 1600, 890, 870, 840 cm^{-1}; n.m.r. ($CDCl_3$): δ 5.87 (H-1, doublet, $J_{1,2}$ 4.0 Hz), 4.37 (H-2, doublet, $J_{2,1}$ 4.0 Hz), 3.27 (OCH_3), 2.45 (C-CH_3, 6 H), 7.81 and 7.35 (aromatic protons, 4 H).

Anal. Calc. for $C_{16}H_{21}O_8S$: C, 51.46; H, 5.76; S, 8.58. Found: C, 51.45; H, 5.93; S, 8.64.

A solution of compound **13** (0.473 g), phosphorus pentaoxide (0.25 g), and methyl sulfoxide (0.25 g) in DMF (8 ml) was heated for 1 h at 65–70°. Isolation of compound **12** (31%) was performed as described above.

1,2-O-*Isopropylidene-5*-O-(p-*tolylsulfonyl*)-α-D-*ribofuranose* (**13**). — Reduction of compound **12** (200 mg) with lithium aluminium hydride for 2 h at 35–40° in tetrahydrofuran gave a white solid (m.p. 99–102°,150 mg). Recrystallization from ether–light petroleum gave compound **13**; m.p. 105–106.5°; $[\alpha]_D^{20}$ +23.6° (*c* 1.4, chloroform); ν_{max}^{Nujol} 3400 (OH), 1590, 890, 870, 850, 815, 810 cm^{-1}; n.m.r. ($CDCl_3$):

δ 5.77 (H-1, doublet, $J_{1,2}$ 4 Hz), 4.6–3.8 (H-2,3,4,5,5', multiplet, 5 H), 2.47 (C-CH_3, 3 H), 1.53, 1.37 (isopropylidene group, 6 H).

Anal. Calc. for $C_{15}H_{20}O_7S$: C, 52.32; H, 5.86; S, 9.32. Found: C, 52.24; H, 6.03; S, 9.11.

1,2-O-Isopropylidene-5-O-(di-O-phenylphosphono)-α-D-erythro-pentofuranos-3-ulose (**15**). — 1,2-*O*-Isopropylidene-5-*O*-(di-*O*-phenylphosphono)-α-D-xylofuranose [**14**; n.m.r. ($CDCl_3$): δ 5.88 (H-1, doublet, $J_{1,2}$ 3.5 Hz)] (1 g) was oxidized with three molar equivalents of methyl sulfoxide and two molar equivalents of phosphorus pentaoxide in 30 ml of DMF for 2 h at 60–65°. Isolation was performed as for compound **12**. The syrup obtained was dissolved in the minimal amount of ethanol, and water was added to produce turbidity. After storage in a refrigerator overnight, white crystals formed, m.p. 65–67°. Recrystallization from water–ethanol gave the title compound (35%), m.p. 75–78°; $[\alpha]_D^{23}$ +44.5° (*c* 1.0, chloroform); ν_{max}^{Nujol} 3350, 1600, 1500, 880, 840 cm^{-1}; n.m.r. ($CDCl_3$): δ 5.77 (H-1, doublet, $J_{1,2}$ 3.5 Hz).

Anal. Calc. for $C_{20}H_{21}O_8P \cdot H_2O$: C, 54.79; H, 5.28. Found: C, 54.31; H, 5.10.

The semicarbazone, prepared by the usual procedure, had m.p. 170–171°; $[\alpha]_D^{20}$ +284° (*c* 1.0, chloroform); ν_{max}^{Nujol} 1780, 1720, 1600, 1500, 890, 880, 840 cm^{-1}.

Anal. Calc. for $C_{21}H_{26}N_3O_8P$: C, 52.61; H, 5.47. Found: C, 52.72; H, 5.60.

1,2-O-Isopropylidene-α-D-xylo-hexofuranurono-6,3-lactone-5-ulose (**17**). — 1,2-*O*-Isopropylidene-α-D-glucofuranurono-6,3-lactone [**16**; n.m.r. ($CDCl_3$): 6.02 (H-1, doublet, $J_{1,2}$ 3.5 Hz)] (10 g) and phosphorus pentaoxide (10 g) were stirred in 115 ml of methyl sulfoxide for 10 min at 35–40° and then for 20 h at 15–20°. The chloroform extract obtained as described for the preparation of compound **7** was concentrated *in vacuo*. Recrystallization of the residue (4.7 g) from hot water gave needles of compound **17**, m.p. 146–148°; $[\alpha]_D^{26}$ +88° (*c* 1.0, methyl sulfoxide) {lit.[20,21] m.p. 146–149°, 145–148°, $[\alpha]_D^{25}$ +76° (water), $[\alpha]_D$ +73° (water)}; ν_{max}^{Nujol} 3350 (OH), 1790 (γ-lactone) cm^{-1}; n.m.r. (pyridine-d_5): δ 6.18 (H-1, doublet, $J_{1,2}$ 3.75 Hz), 5.0 (H-2, doublet, $J_{2,3}$ 0.5 Hz); 5.37 (H-3, $J_{3,4}$ 3.5 Hz), 5.11 (H-4, doublet); (methyl sulfoxide-d_6): δ 5.96 (H-1, doublet, $J_{1,2}$ 3.5 Hz), 4.42 (H-4, doublet), 4.87 (H-3, quartet, $J_{3,4}$ 2.8 Hz), 4.83 (H-2, quartet, $J_{2,3} < 0.1$ Hz), 7.45 and 7.28 (singlets, *gem*-OH).

Compound **17** was treated with cold barium hydroxide solution for 2 h. The solution was poured into a large volume of ethanol to give a white precipitate of the barium salt which was collected by centrifugation and dried: ν_{max}^{Nujol} 1725 (C=O), 1640 cm^{-1} (broad peak, COO^-).

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STEREOSELECTIVE RING-OPENING OF β-D-MANNOPYRANOSE 1,2-(ALKYL ORTHOACETATES)*

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ABSTRACT

The stability towards alkali of the 3,4,6-tri-*O*-acetyl-β-D-mannose 1,2-(alkyl orthoacetates) provides a route to the corresponding 3,4,6-tri-*O*-benzyl-β-D-mannose 1,2-(alkyl orthoacetates). Alkyl groups that have been incorporated onto the orthoacetate ring include methyl, isopropyl, and cyclohexyl. 3,4,6-Tri-*O*-benzyl-β-D-mannose 1,2-(methyl orthoacetate) (**2**) was hydrolyzed to 3,4,6-tri-*O*-benzyl-D-mannose; periodate oxidation converted this compound into 2,3,5-tri-*O*-benzyl-D-arabinose. Methanolysis of **2** led to methyl 3,4,6-tri-*O*-benzyl-α-D-mannoside (**5**) in high yield; methylation of **5**, followed by debenzylation and acetylation, afforded crystalline methyl 2-*O*-methyl-3,4,6-tri-*O*-acetyl-α-D-mannoside. An acid-catalyzed, stereoselective rearrangement of the 3,4,6-tri-*O*-benzyl-β-D-mannose 1,2-(alkyl orthoacetates) was observed. The resulting products were demonstrated to be the corresponding alkyl 3,4,6-tri-*O*-benzyl-α-D-mannosides.

INTRODUCTION

In the light of unusual rates of periodate oxidation of the carbohydrate group in ovalbumin, attributed to a 2-*O*-substituted D-mannose residue[1], it was of interest to synthesize model D-mannopyranosides that are substituted at O-2. Recent disclosures of simple synthetic routes to 3,4,6-tri-*O*-acetylhexose 1,2-(alkyl orthoacetates)[2,3], and the marked stability of the 1,2-(alkyl orthoacetate) group to base, provided a suitable derivative of D-mannose for the synthesis required. However, the known tendency of *O*-acetyl groups to migrate, particularly the group at O-3 in 3,4,6-tri-*O*-acetyl-D-mannose[4], indicated that a more stable protecting group would be required. *O*-Methyl groups have been used in the synthesis of the 3,4,6-tri-*O*-methyl derivatives of D-glucose[5] and D-mannose[6] 1,2-(alkyl orthoacetates), and this work suggested that benzyl ethers might be used for our purpose.

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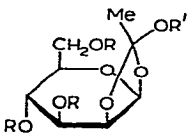
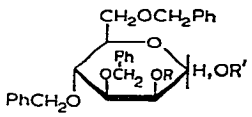
RESULTS AND DISCUSSION

The present report describes the synthesis of methyl 2-*O*-methyl-α-D-mannopyranoside from 3,4,6-tri-*O*-benzyl-β-D-mannose 1,2-(methyl orthoacetate). It is also shown that ring-opening of the 1,2-(alkyl orthoacetates) occurs rapidly with an acid catalyst, and in the absence of an alcohol, to give an almost stereospecific conversion into the corresponding alkyl α-D-mannopyranoside. The benzylation of 3,4,6-tri-*O*-acetyl-β-D-mannose 1,2-(methyl orthoacetate) (**1**) in tetrahydrofuran proceeded smoothly to yield the corresponding 3,4,6-tribenzyl ether (**2**) crystalline, in good yield. The n.m.r. signals of the orthoacetate C-Me and C-OMe protons in **2** had chemical shifts similar to those reported[7] for **1**. A solution of **2** in absolute methanol containing 2% of hydrogen chloride was refluxed to give a syrupy mixture containing more than 90% of methyl 3,4,6-tri-*O*-benzyl-α-D-mannoside (**5**). Compound **5** was obtained as a pure syrup by column chromatography on silica gel. Acetylation of **5** provided syrupy methyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α-D-mannoside (**6**), and the corresponding benzoic ester was also obtained as a syrup.





1 R = Ac; R′ = Me
2 R = $PhCH_2$; R′ = Me
7 R = Ac; R′ = Me_2CH
8 R = $PhCH_2$; R′ = Me_2CH
11 R = Ac; R′ = C_6H_{11}
12 R = $PhCH_2$; R′ = C_6H_{11}

3 R = H; R′ = H
5 R = H; R′ = Me (α-D)
6 R = Ac; R′ = Me (α-D)
9 R = Ac; R′ = Me_2CH (α-D)
10 R = H; R′ = Me_2CH (α-D)
13 R = Me; R′ = Me (α-D)
15 R = Ac; R′ = C_6H_{11}
16 R = H; R′ = C_6H_{11}

Attempts to hydrolyze **2** in acidic methanol–water mixtures gave low yields of crystalline 3,4,6-tri-*O*-benzyl-D-mannose (**3**), contaminated with the corresponding methyl glycosides. Similar difficulties had been reported earlier by Barker and Fletcher[8] in the synthesis of 2,3,5-tri-*O*-benzyl-L-arabinose. Almost quantitative yields of **3** were obtained by heating a solution of **2** in 60% aqueous acetic acid, followed by deacetylation of the resulting syrup.

Support for the structure assigned to **3** was obtained by its oxidation with periodate to form 2,3,5-tri-*O*-benzyl-D-arabinose. The mixture from the oxidation reaction was examined by t.l.c., and a compound, presumably 2,3,5-tri-*O*-benzyl-4-*O*-formyl-D-arabinose, was present which, after catalytic deacylation, was converted into 2,3,5-tri-*O*-benzyl-D-arabinose. Compound **3** was converted into **5** by refluxing it in anhydrous methanol containing 2% of hydrogen chloride.

A previous synthesis of 2-*O*-methyl-D-mannose had been accomplished through 3,4:5,6-di-*O*-isopropylidene-D-mannose dimethyl dithioacetal[9], or 1,3,4,6-tetra-*O*-acetyl-β-D-mannose[10]. Compounds **5** or **6** provided other intermediates for the

synthesis of derivatives of methyl 2-*O*-methyl-α-D-mannopyranoside. Methylation of **5** with a 1.5 molar excess of sodium methanesulfinyl corbonium[11] and methyl iodide provided syrupy methyl 3,4,6-tri-*O*-benzyl-2-*O*-methyl-α-D-mannoside (**13**) in less than 60% yield, whereas the reaction of **5** with methyl sulfate and potassium hydroxide in tetrahydrofuran gave **13** almost exclusively. Catalytic hydrogenolysis of **13** produced methyl 2-*O*-methyl-α-D-mannopyranoside, from which crystalline methyl 3,4,6-tri-*O*-acetyl-2-*O*-methyl-α-D-mannoside was obtained by acetylation. The n.m.r. signals assigned to the OMe protons in this compound were observed at τ 6.52 and 6.55.

Ring opening of the 1,2-(alkyl orthoacetates) has been studied by using various catalysts. The action of acids on **1** in the presence of alcohols has been investigated by Perlin[4]. The results were in keeping with a mechanism which had been proposed by Pacsu[12] for the acid-catalyzed ring-opening in an excess of methanol. Alternatively, the rearrangement of 3,4,6-tri-*O*-benzoyl-β-D-glucose 1,2-(methyl orthobenzoate), with somewhat more than catalytic amounts of mercuric bromide and anhydrous hydrogen chloride in nitromethane[13], gave crystalline methyl 2,3,4,6-tetra-*O*-benzoyl-β-D-glucoside in 57% yield. More recently, the catalyzed alcoholysis of 3,4,6-tri-*O*-acetyl-α-D-glucose 1,2-(alkyl orthoacetates) with *p*-toluenesulfonic acid (*p*-TsOH) in dichloromethane has been reported[14], the alcohol being introduced to form the corresponding glycoside, mainly with retention of configuration at the anomeric center. Concomitant loss of the 2-*O*-acetyl group was observed, leading to the postulation of a 1,2-oxirane intermediate product. Kochetkov and co-workers[15–17] have treated 3,4,6-tri-*O*-acetyl-α-D-glucose and -D-galactose 1,2-(ethyl orthoacetates) with a properly protected carbohydrate in the presence of mercuric bromide and *p*-TsOH, or mercuric bromide alone, to yield an oligosaccharide in which the original anomeric configuration in the orthoacetate had been inverted. These reactions proceeded with retention of the 2-*O*-acetyl group.

In the present investigation, the ring opening of **2** in dichloromethane in the presence of *p*-TsOH and methanol was followed in an n.m.r. spectrometer tube by observing the decrease of the C-Me signals at τ 8.35 and 8.56 or the appearance of the *O*-acetyl peak at τ 7.91. A similar technique had been used in a study of the hydrolysis of acyclic orthoacetates[18]. The methanolysis of **2** was complete within 5 min. Chromatography of the resulting syrup on silica gel demonstrated an unexpected stereoselectivity in the opening of the orthoacetate ring, with isolation of 65% of **6** and 18% of **5**. The reaction proceeded similarly, but much less rapidly, when catalyzed with mercuric bromide in nitromethane, with isolation of 66% of **6** and 20% of **5**. Molar ratios of alcohol:orthoacetate of 1:1 and 5:1 effected little change in the stereoselectivity of the reaction; slightly more **5** was recovered with the higher proportion of alcohol, ostensibly due to an acid-catalyzed transacetylation after methanolysis.

Attempts to use anhydrous methanolic hydrogen chloride in dichloromethane for the methanolysis were unsuccessful. The changes in n.m.r. spectrum indicated an increase in the intensity of the *O*-acetyl peak corresponding to the concentration of hydrogen chloride, probably relating to the formation of the corresponding glycosyl chloride.

It was observed that storage of crystalline **2** in the laboratory atmosphere transformed the material into a syrup composed of **5** and **6**, as determined by t.l.c. Compound **2** was stable when stored over sodium hydroxide. This behavior caused speculation as to the ease of cleavage of the orthoacetate ring by acid in the absence of any alcohol. Lemieux[14] had made brief mention of such an experiment, but the result was inconclusive. A solution of **2** in dichloromethane, containing a catalytic amount of *p*-TsOH, was observed by n.m.r. spectroscopy to lose the C-Me signals at τ 8.35 and 8.56 completely in 20–22 min. Chromatography of the reaction product on silica gel gave 83% of **6** and 7% of **5**. The use of mercuric bromide in nitromethane as catalyst led to a slower reaction, but a similar distribution of products was noted.

The stereoselectivity and high yield of product observed in the ring-opening of **2** suggested that this rearrangement might be generally applicable for the synthesis of oligosaccharides, a possibility recognized long ago by Isbell[19]. Replacement of the methoxyl group in the orthoacetate by a suitably protected sugar would be required. As further models for this proposal, 3,4,6-tri-*O*-acetyl-β-D-mannose 1,2-(isopropyl orthoacetate) (**7**) and 1,2-(cyclohexyl orthoacetate) (**11**) were synthesized by the method of Mazurek and Perlin[3] and were obtained crystalline. The signal of the C-Me protons in **7** and **11** occurred at τ 8.22 and 8.26, respectively, in the region associated with the *endo* *C*-alkyl groups observed earlier[3]. Conversion of **7** and **11** into the corresponding crystalline benzyl ethers, **8** and **12**, was straightforward. The n.m.r. spectrum of **8** in the τ 8.5–9.0 region showed that each methyl group of the isopropyl group gave rise to a doublet having $J_{1,2} = 1.8$ Hz.

Compounds **8** and **12** both rearranged in dichloromethane containing *p*-TsOH, under the conditions used for **2**. In the case of **8**, the rearrangement required approximately 70 min, and gave isopropyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α-D-mannoside (**9**) in 74% yield. In addition, 11% of isopropyl 3,4,6-tri-*O*-benzyl-α-D-mannoside was also obtained. Compound **12** required approximately 100 minutes to rearrange; 79% of cyclohexyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α-D-mannoside was isolated, together with 8% of cyclohexyl 3,4,6-tri-*O*-benzyl-α-D-mannoside.

It is interesting to note that the ring-opening of **1** proceeded at a much lower rate, and n.m.r. spectroscopy showed a greater diversity of products, indicating that the benzyl residues have a special role. The difference is being studied further.

This rearrangement appears to be general for the benzyl ethers of D-mannose 1,2-(alkyl orthoacetates). It provides a high degree of stereoselectivity, especially where the OR group of the orthoacetate is that of a secondary alcohol, in which case, no β-D anomer was detected in the products. The ease of rearrangement would demand stringent choice of conditions in using these orthoacetate derivatives as precursors for oligosaccharides by glycosidation reactions.

The mechanism of the ring-opening rearrangement appears to be essentially that proposed by Kochetkov and co-workers[17]. In the case of the methyl orthoacetate (**2**), which is principally in the *endo*-CMe form (see Fig. 1), it was observed that, although the total concentration of **2** quickly decreased, with over 90% disappearing in 15 min at 46.5°, there was an initial increase in the concentration of the *exo*-CMe

form (**2a**) to a maximum at about 6 min. There was, therefore, a preferential reaction of the *endo*-CMe form (**2b**) to give the methyl α-D-glycoside (**6**). It would seem that, by the mechanism shown, the rate of attack of methanol at the anomeric carbon atom is more rapid than at the carbon atom of the ortho ester group, but that, in the latter

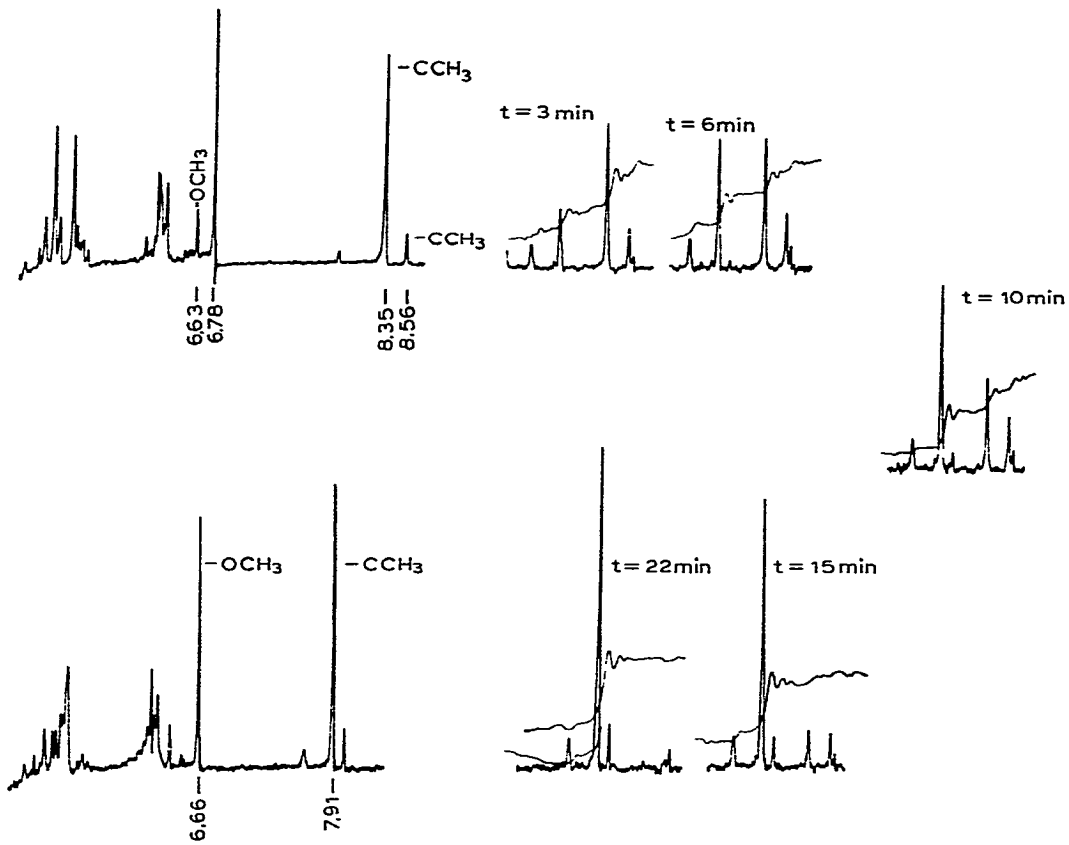


Fig. 1. Acid-catalyzed rearrangement (0.037M *p*-toluenesulfonic acid) of tri-*O*-benzyl-β-D-mannose 1,2-(methyl orthoacetate). The n.m.r. spectra (τ in p.p.m.) in dichloromethane at 60 MHz are shown, to be read clockwise from top to bottom.

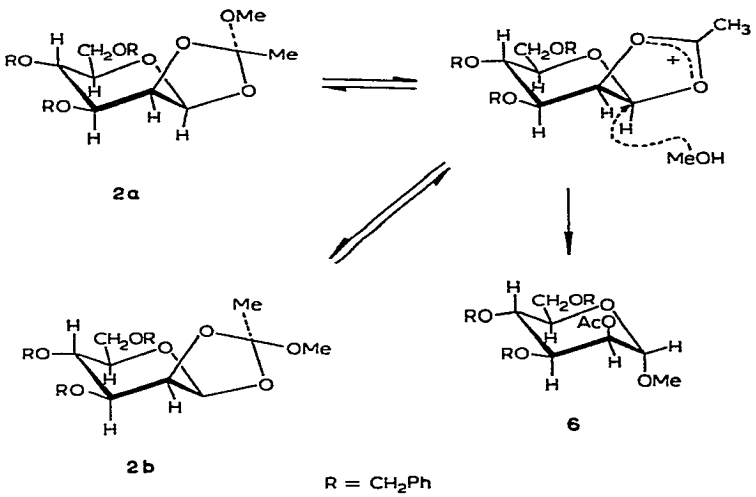
event, the favored approach of the entering methoxyl group is from the side of the ring-oxygen atom. Alternative mechanisms that would account for these results are being considered further.

EXPERIMENTAL

General. — Reagents were used without further purification, except for lutidine and benzyl chloride. Lutidine was distilled from barium oxide, and benzyl chloride was distilled to remove extraneous color. The powdered potassium hydroxide was

commercially available*. *p*-Toluenesulfonic acid monohydrate was dried overnight at 100° *in vacuo*.

Solutions were concentrated *in vacuo* with bath temperatures generally no higher than 50°. Melting points (Fisher-Johns apparatus) are uncorrected. All optical rotations were measured in dichloromethane.



The n.m.r. spectra were obtained with a Varian A-60 spectrometer. The solvent was chloroform and tetramethylsilane was used as an external standard. The proton resonance of chloroform, obscured the signal of the phenyl group. For rearrangement studies, the n.m.r. sample-tubes were cleaned with chromic acid, rinsed successively with water and dilute ammonium hydroxide, and dried at 120°.

T.l.c. was effected on Silica Gel G (E. Merck, Darmstadt, Germany) with 1:1 chloroform–ethyl acetate as eluent; zones were detected by spraying with sulfuric acid and heating the plates for 20 min at 110–120°. Dry-column chromatography[20] was used, with silica gel (0.05–0.20 mm) (E. Merck, Darmstadt, Germany) in a column loaded with no more than 1 g of compound per 50 g of adsorbent. The absorbent was poured into the column in a continuous stream and was then packed by light tapping of the column with a cork ring. Better separations were achieved if the sample to be chromatographed was evaporated onto a small amount of adsorbent and this was placed at the top of an already packed column. Elution was with dichloromethane, followed by dichloromethane–ethyl acetate (3:1 v/v), and finally with dichloromethane–ethyl acetate (1:1 v/v). Microanalyses were performed by Galbraith Laboratories, Knoxville, Tennessee.

*Preparation of 3,4,6-tri-*O*-acetyl-β-*D*-mannose 1,2-(methyl orthoacetate)* (**1**). — The preparation was essentially that described by Mazurek and Perlin[3]. Syrupy tetra-*O*-acetyl-α-D-mannosyl bromide, prepared from 10 g of D-mannose, was dissolved in chloroform (88 ml). To this solution was added 2,6-lutidine (11.5 ml) in

*Hooker Chemical Company, Buffalo, New York.

absolute methanol (88 ml). A slight warming was observed during the addition. After the solution had been kept overnight at room temperature, chloroform (100 ml) was added, and the solution was washed with ice-cold, 3% aqueous sodium hydrogen carbonate. The aqueous solution was extracted with chloroform (50 ml) and this extract was combined with the chloroform extract. The combined chloroform extracts were washed once with ice–water and dried with anhydrous sodium sulfate. After addition of several grams of Darco G-60, the mixture was filtered, and the filtrate was evaporated *in vacuo*; yield after azeotropic distillation with toluene (20 ml), 16.3 g. The product was crystallized from methanol–water (some excess lutidine present is desirable) to yield 14 g of crystalline **1**, m.p. 109–110° (lit.[3] m.p. 111–113°).

Synthesis of 3,4,6-tri-O-benzyl-β-D-mannose 1,2-(methyl orthoacetate) (**2**). — A solution of **1** (25 g) in benzyl chloride (140 ml) and tetrahydrofuran (50 ml) in a 3-necked, round-bottomed flask (equipped with a stirrer and a reflux condenser) was heated under anhydrous conditions to reflux. External heat was removed and potassium hydroxide (50 g) was added in portions such that the exothermic reaction maintained the solution at reflux. Efficient stirring was mandatory, since the reaction mixture became quite viscous after the initial addition.

When all of the potassium hydroxide had been added, the reaction mixture was stirred for an additional 3–4 h at reflux, and then cooled to room temperature. Water (200 ml) was added, and the mixture was extracted with dichloromethane (150 ml). The extract was washed several times with concentrated, aqueous sodium hydrogen carbonate, dried with sodium sulfate, decolorized with Darco G-60, filtered, and concentrated *in vacuo* at $\leq 50°$. The residual syrup was then heated slowly to 100° at 0.1 torr to remove dibenzyl ether. To the residue was added ethyl ether (20 ml) followed by hexane (50 ml). Crystallization, induced by seeding, was continued overnight at 5°, and the crystals, separated by decantation of the mother liquors, were triturated with hexane and filtered off. The product was dried *in vacuo* over paraffin wax and sodium hydroxide; yield 29.3 g, m.p. 76–78°, $[\alpha]_D^{23.5}$ +12.1° (*c* 1.65).

Anal. Calc. for $C_{30}H_{34}O_7$: C, 71.13; H, 6.77. Found: C, 71.10; H, 6.81.

3,4,6-Tri-O-benzyl-D-mannose (**3**) *from 3,4,6-tri-O-benzyl-β-D-mannose 1,2-(methyl orthoacetate)*. — A solution of **2** (3.43 g) in glacial acetic acid (41 ml) and water (27 ml) was heated for 2 h on a steam bath, and then concentrated *in vacuo*. Residual acetic acid was removed by dissolving the syrup in dichloromethane and washing several times with saturated, aqueous sodium hydrogen carbonate. The dichloromethane solution was dried (sodium sulfate), filtered, and concentrated *in vacuo* to a syrup, which was deacetylated overnight with a catalytic amount of sodium methoxide in methanol. The solution was neutralized with glacial acetic acid, and the methanol was removed *in vacuo*. The product was kept for several h in a vacuum desiccator over sodium hydroxide, and it was then extracted with ethyl ether (25 ml). The ether extract was treated with Darco G-60, and filtered. Cyclohexane was added to the filtrate to incipient opalescence, and the solution was then refrigerated, to give a total of 2.9 g of **3**, m.p. 98–99°, $[\alpha]_D^{24}$ +22.7° (*c* 1.92).

Anal. Calc. for $C_{27}H_{30}O_6$: C, 71.98; H, 6.71. Found: C, 71.91; H, 6.73.

Periodate oxidation of 3,4,6-tri-O-benzyl-D-mannose to form 2,3,5-tri-O-benzyl-D-arabinose. — 3,4,6-Tri-*O*-benzyl-D-mannose (**3**) (0.7 g) was added to a well-stirred mixture of methanol (70 ml) and 0.769M sodium periodate (17 ml) at room temperature. Sodium iodate began to crystallize from the solution almost immediately. Progress of the reaction could be followed by t.l.c. Starting material (R_F 0.19) was, for the most part, converted into a second compound (R_F 0.68), demonstrably different from authentic 2,3,5-tri-*O*-benzyl-D-arabinofuranose (R_F 0.56).

After 22 h, saturated aqueous sodium hydrogen carbonate (5 ml) was added to the mixture; this was filtered, and the precipitate was washed with additional methanol. The methanol was removed *in vacuo*, and the product was extracted with dichloromethane. The combined extracts were washed twice with equal volumes of water, dried (sodium sulfate), and concentrated to a syrup (R_F 0.68) which was probably 2,3,5-tri-*O*-benzyl-4-*O*-formyl-D-arabinose. Attempts to crystallize this material were unsuccessful. The syrup was deacylated, as usual, with methanolic sodium methoxide to give **4** (R_F 0.56) as a syrup, which was extracted with dichloromethane. The extract was washed with water, dried (sodium sulfate), and concentrated *in vacuo* to a syrup, which crystallized on being kept in a vacuum desiccator over sodium hydroxide. Recrystallization from ether–hexane yielded crystalline **4**, 299 mg, m.p. 72–85°, undepressed on admixture with an authentic sample. A further amount of **4** (167 mg) was obtained by recrystallizing the residual mother liquor from isopropyl ether–petroleum ether[8].

Anal. Calc. for $C_{26}H_{28}O_5$: C, 74.26; H, 6.71. Found: C, 74.55; H, 6.88.

Synthesis of 3,4,6-tri-O-acetyl-β-D-mannose 1,2-(isopropyl orthoacetate) (**7**). — To syrupy tetra-*O*-acetyl-α-D-mannopyranosyl bromide (from 25 g of D-mannose) in dichloromethane (175 ml) was added, with shaking, lutidine (40 ml), and 2-propanol (30 ml). After 2 days at room temperature, the reaction mixture was treated in the same manner as for **1**, with crystallization from methanol–water (buffered with small amounts of lutidine), yield 31.5 g, m.p. 104.5–106°, $[\alpha]_D^{21}$ −13° (*c* 2.38). One preparation, crystallized from ethyl ether–petroleum ether, exhibited a melting point from 87–89°, followed by solidification, and remelting at 104.5–106°.

Anal. Calc. for $C_{17}H_{26}H_{10}$: C, 52.30; H, 6.71. Found: C, 51.82; H, 6.71.

3,4,6-Tri-O-benzyl-β-D-mannose 1,2-(isopropyl orthoacetate) (**8**). — A solution of benzyl chloride (28 ml), benzene (25 ml), and **7** (5 g) was heated to reflux with vigorous stirring. Powdered potassium hydroxide (10 g) was added in portions, and the source of external heat was removed. The potassium hydroxide was added at such a rate that reflux was maintained. Additional benzene (25 ml) was added when the solution became extremely viscous. After the addition of base was complete, heating was resumed, and the reaction mixture was refluxed with continuous stirring for an additional 3 h. The mixture was then allowed to cool, and water (50 ml) was added to dissolve the solids. The aqueous layer was removed, and extracted with benzene (20 ml), and the extracts were combined, washed with saturated, aqueous sodium hydrogen carbonate (3 × 100 ml), and dried (sodium sulfate). Concentration of the solution was conducted as in the preparation of **2**. The resulting syrup was dissolved

in ethyl ether (25 ml), and the solution was treated with Darco G-60, the suspension filtered, and the residue washed with additional ether (25 ml). The product began to crystallize out at this point. The solution was, therefore, warmed, petroleum ether (50 ml) was added, and the mixture was kept overnight in the refrigerator; yield 3.5 g, m.p. 97–100°, $[\alpha]_D^{29}$ +13° (*c* 1.73).

Anal. Calc. for $C_{32}H_{38}O_7$: C, 71.89; H, 7.16. Found: C, 72.07 H, 7.29.

3,4,6-Tri-O-acetyl-β-D-mannose 1,2-(cyclohexyl orthoacetate) (**11**). — A solution of syrupy tetra-*O*-acetyl-α-D-mannopyranosyl bromide (from 25 g of D-mannose) in dichloromethane (175 ml) containing lutidine (40 ml) and cyclohexanol (30 ml) was agitated briefly, and kept at room temperature for 2 days. The isolation and crystallization procedure was that described for **7**; yield 23.3 g, m.p. 129–141°, $[\alpha]_D^{24}$ −11.4° (*c* 1.97).

Anal. Calc. for $C_{20}H_{30}O_{10}$: C, 55.80; H, 7.03. Found: C, 55.10; H, 7.00,

Microanalysis of material that had been dried *in vacuo* over phosphorus pentaoxide at 100° provided analytical figures quite different from those above (Found: C, 51.62; H, 6.39).

3,4,6-Tri-O-benzyl-β-D-mannose 1,2-(cyclohexyl orthoacetate) (**12**). — The benzylation of **11** was performed exactly as described for the synthesis of **8**; yield 1.83 g, m.p. 87.5–90°, $[\alpha]_D^{29}$ +13.6° (*c* 1.84).

Anal. Calc. for $C_{35}H_{42}O_7$: C, 73.14; H, 7.37. Found: C, 73.28; H, 7.38.

Methyl 3,4,6-tri-O-benzyl-α-D-mannoside (**5**). — (*a*) Freshly prepared **2** (19.65 g) was dissolved in absolute methanol (400 ml) with stirring, and the solution was heated almost to reflux. Acetyl chloride (12 ml) was added dropwise, with continued stirring, and the solution was refluxed under anhydrous conditions for 18 h; t.l.c. then indicated that over 90% of the material present had R_F 0.47, the next most-intense zone (probably methyl 3,4,6-tri-*O*-benzyl-β-D-mannoside) having R_F 0.42. The methanol was removed *in vacuo*, the resulting syrup was dissolved in chloroform, and the solution was washed several times with equal volumes of saturated aqueous sodium hydrogen carbonate, dried (sodium sulfate), and concentrated *in vacuo*. The residual syrup was kept for an additional 24 h in a vacuum desiccator over sodium hydroxide; yield 17.7 g. Some of this syrup was subjected to dry-column chromatography, and the resulting, pure (by t.l.c.) material had $[\alpha]_D^{21}$ +59.7° (*c* 1.85).

Anal. Calc. for $C_{28}H_{32}O_6$: C, 72.39; H, 6.94. Found: C, 71.90; H, 6.94.

(*b*) Acetyl chloride (0.6 ml) was added dropwise to a solution of **3** (1.0 g) in absolute methanol (50 ml), and the solution was refluxed for 19 h under anhydrous conditions; t.l.c. then indicated that virtually all of the starting material (R_F 0.2) had been converted, principally into **5** (R_F 0.46). Refluxing was continued for an additional 24 h, the methanol was removed *in vacuo*, and the product was treated as in (*a*). The yield of syrupy material was 0.97 g, $[\alpha]_D^{25}$ +53.5° (*c* 1.94), which amounts to a purity of over 90%, based on Hudson's rules of isorotation. This syrup was chromatographed on silica gel by the dry-column method, to yield **5**, identical (by t.l.c.) with that prepared by method (*a*). A small amount of transesterification evidently catalyzed by the

silica gel, occurred between **5** and the ethyl acetate used as the eluent, to give the corresponding 2-*O*-acetyl derivative (**6**).

Methyl 2-O-acetyl-3,4,6-tri-O-benzyl-α-D-mannoside (**6**). — Several grams of the crude methanolysis product containing **5** was acetylated with acetic anhydride–pyridine in the usual way. A portion of this product (0.7 g) was chromatographed on silica gel by the dry-column technique, to provide **6** (0.55 g) as a pure syrup (n.m.r. and t.l.c.); $[\alpha]_D^{27}$ +27.9° (*c* 2.24).

Anal. Calc. for $C_{30}H_{34}O_7$: C, 71.13; H, 6.77. Found: C, 70.57; H, 6.97.

Methyl 3,4,6-tri-O-acetyl-2-O-methyl-α-D-mannoside (**14**). — Syrupy **5** (approx. 10.5 g, from the methanolysis with hydrogen chloride) was dissolved in tetrahydrofuran, and powdered potassium hydroxide (7.4 g) was added, with stirring, to this solution. Stirring was continued, and methyl sulfate (4.1 ml) was added dropwise. The slurry initially became warm, and then it cooled. Stirring was continued at room temperature for another 18 h, the mixture being protected from atmospheric moisture. Water (60 ml) was added to dissolve the solids, and the resulting mixture was heated at 60° with stirring. The tetrahydrofuran was evaporated off in a stream of nitrogen, and heating was continued for 30 min after completion of the evaporation. The mixture was cooled, and adjusted to pH 8 with sulfuric acid. The product was extracted with chloroform, and the extract was washed with saturated aqueous sodium hydrogen carbonate, dried (sodium sulfate), decolorized, and concentrated *in vacuo* to a syrup (10.4 g). T.l.c. showed one major zone, at R_F 0.56. A small amount of this methylated material was chromatographed on silica gel (dry column), and the resulting syrup had $[\alpha]_D^{25}$ +50.3° (*c* 2.11).

The crude syrupy material (**13**, 10.3 g) was dissolved in absolute methanol (100 ml), and the solution was refluxed with several g of decolorizing carbon for 20 min. The suspension was filtered, and the filtrate was shaken overnight in an atmosphere of hydrogen (Parr apparatus) with a catalyst (1 g) of 10% palladium chloride on carbon. The catalyst was filtered off, and the methanol was removed *in vacuo*. The syrup was acetylated overnight with pyridine (10 ml) and acetic anhydride (20 ml).

Excess of acetic anhydride was destroyed by pouring the mixture over cracked ice, and, after 30 min, the product was extracted from the mixture with chloroform. The extract was washed with saturated, aqueous hydrogen carbonate, dried (sodium sulfate), filtered, and concentrated *in vacuo* to yield a syrup (6.53 g). Some difficulty was encountered in crystallizing this material; seed crystals were obtained by dry-column chromatography of a small amount of the syrup. Once nucleated, the substance crystallized readily from ethyl ether–petroleum ether. The yield from 5.5 g of syrupy material was 3.36 g, m.p. 59–61°, $[\alpha]_D^{27}$ +53° (*c* 2.09). The n.m.r. spectrum was in accord with the values expected for **14**.

Anal. Calc. for $C_{14}H_{22}O_9$: C, 50.27; H, 6.63. Found: C, 50.24; H, 6.51.

Methanolysis of **2**. — A solution of **2** (0.202 g, 0.4 mmole), anhydrous methanol (0.013 g, 0.4 mmole), and *p*-toluenesulfonic acid (0.014 g, 0.008 mmole) in dichloromethane (1.2 ml), contained in an n.m.r. spectrometer tube, was observed at 46.5°. A scan made 5 minutes after addition of the *p*-toluenesulfonic acid indicated that the

characteristic, orthoacetate C-Me peaks at τ 8.35 and 8.56 had disappeared, and, in their place, an *O*-acetyl peak had appeared at τ 7.91. A small peak at τ 8.03 also appeared, and was assigned to a small amount of acetic acid that had evidently been formed by hydrolysis during the reaction. No further change was observed after an additional hour.

The resulting syrup was placed on a column (1.2 × 42 cm) of dry silica gel, the column was developed as described earlier, and 2-ml fractions were collected. Three distinct zones were found. The most abundant (132 mg, 65%) and fastest-moving material was determined to be **6**, on the basis of n.m.r. and t.l.c. data. Deacetylation with methanol and sodium methoxide yielded material having an R_F value identical with that of **5**. The next largest amount (36 mg, 18%) was **5**, and the slowest-moving material (11 mg, 5%) had a t.l.c. mobility slightly less than that of **5**. On this basis, this compound was tentatively assumed to be methyl 3,4,6-tri-*O*-benzyl-β-D-mannoside.

A solution of 0.202 g (0.4 mmole) of **2**, 0.013 g (0.4 mmole) of anhydrous methanol, and 0.0043 g (0.012 mmole) of mercuric bromide in nitromethane (1 ml) in an n.m.r. spectrometer tube was observed as before. The n.m.r. spectrum was much more complicated than that observed in dichloromethane. It was observed that, after the sample had been kept overnight at 40°, there was no further reaction. Removal of the nitromethane *in vacuo*, dissolution of the sample in dichloromethane, and observation of the n.m.r. spectrum, demonstrated that the product had a composition quite similar to that of the crude mixture obtained in (*a*) above. Silica-column chromatography yielded 134 mg (66%) of **6**, and 40 mg (20%) of **5**.

Experiments with an orthoacetate: alcohol ratio of 1:5 gave comparable results.

Acid-catalyzed rearrangement of **2**. — (*a*) A solution of **2** (202 mg, 0.4 mmole) and 7 mg (0.04 mmole) of *p*-toluenesulfonic acid (7 mg, 0.04 mmole) in dichloromethane (1 ml), contained in an n.m.r. spectrometer tube, was observed at 46.5° at intervals, as described for the preceding experiment. Disappearance of the orthoacetate C-Me signals at τ 8.35 and 8.56, and the concomitant appearrance of the *O*-acetyl peak at τ 7.91, were used as guides to indicate the progress of the reaction. Less than 10% of the orthoacetate remained after 15 min. During the rearrangement, the n.m.r. peak at τ 8.56 (representing the *exo*-orthoacetate C-Me) appeared to increase with respect to the peak τ 8.35 representing the *endo*-orthoacetate C-Me group. After 6 min, the *exo* C-Me peak still represented only 12–13% of the total acetyl orthoacetate present.

The reaction mixture was chromatographed on silica gel, and three components were isolated. The first compound eluted was **6**, $[\alpha]_D^{21.5}$ +28° (*c* 3.36); a recovery of 168 mg (83%) was obtained. The second component (14 mg, 7%) was assumed to be **5** on the basis of its t.l.c. mobility. A small amount (2 mg) of a compound (R_F 0.42), presumably methyl 3,4,6-tri-*O*-benzyl-β-D-mannoside, was eluted as the third component.

(*b*) A solution of **2** (202 mg, 0.4 mmole) and mercuric bromide (4.3 mg, 0.012 mmole) in nitromethane (1 ml) was observed at 46.5° in a spectrometer tube. The rearrangement was much less rapid than in (*a*). After 48 h, there was no further

change in the spectrum observed. The nitromethane was removed *in vacuo*, and the sample was kept overnight in a vacuum desiccator over sodium hydroxide. The n.m.r. spectrum of this mixture, in dichloromethane, demonstrated that the orthoacetate C-Me peaks had been converted into an *O*-acetyl peak at τ 7.91.

Column chromatography of this mixture yielded 146 mg (72%) of **6**. Compound **5** (31 mg, 15%) was also recovered.

Rearrangement of 3,4,6-tri-O-benzyl-β-D-mannose 1,2-(isopropyl orthoacetate) (**8**).—A solution of **8** (214 mg, 0.4 mmole) in 0.037M *p*-toluenesulfonic acid in dichloromethane (1 ml), contained in a spectrometer tube at 46.5°, was monitored by observing the disappearance of the orthoacetate C-Me peak at τ 8.32 and the appearance of the *O*-acetyl peak at τ 7.91. The reaction was complete in 65–70 min.

Column chromatography demonstrated the presence of two components, obtained as syrups. The faster-moving product (159 mg, 74%) was isopropyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α-D-mannoside (**9**), $[\alpha]_D^{26}$ +24.5° (*c* 1.59). A 3-proton singlet at τ 7.96 (in $CHCl_3$) was still observed in the n.m.r. spectrum of this compound after silica-gel chromatography.

Anal. Calc. for $C_{32}H_{38}O_7$: C, 71.89; H, 7.16. Found: C, 72.16; H, 7.45.

The second, slower-moving product (24 mg, 11%) was isopropyl 3,4,6-tri-*O*-benzyl-α-D-mannoside (**10**). Deacetylation of **9** with a catalytic amount of sodium methoxide in methanol yielded **10**, $[\alpha]_D^{22}$ +49.9° (*c* 2.02).

Anal. Calc. for $C_{30}H_{26}O_6$: C, 73.14; H, 7.37. Found: C, 72.42; H, 7.51.

Rearrangement of 3,4,6-tri-O-benzyl-β-D-mannose 1,2-(cyclohexyl orthoacetate) (**12**). — A solution of **12** (230 mg, 0.4 mmole) in 0.037M *p*-toluenesulfonic acid in dichloromethane (1 ml) was treated as described for **8**. The reaction was complete in 95–100 min, as judged by change in the *O*-acetyl peak at τ 7.91.

T.l.c. demonstrated the presence of two compounds, R_F 0.72 and 0.62; these materials were separated by column chromatography. A syrup (132 mg, 79%) appeared as the faster-moving zone, $[\alpha]_D^{20}$ +28.2° (*c* 1.77). This compound was cyclohexyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α-D-mannoside (**15**).

Anal. Calc. for $C_{35}H_{42}O_7$: C, 73.14; H, 7.37. Found: C, 73.33; H, 7.28.

The second syrupy material (19 mg, 8%) was identified as cyclohexyl 3,4,6-tri-*O*-benzyl-α-D-mannoside (**16**), since this compound could also be obtained by deacetylation of **15** with methanolic sodium methoxide; $[\alpha]_D^{23}$ +52.8° (*c* 1.83).

Anal. Calc. for $C_{33}H_{40}O_6$: C, 74.40; H, 7.51. Found: C, 74.53; H, 7.68.

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CONVERSION OF 2-AMINO-2-DEOXY-D-GLUCOSE DIETHYL DITHIOACETAL HYDROCHLORIDE INTO 2-*S*-ETHYL-2-THIO-D-GLUCOSE*†

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ABSTRACT

Treatment of 2-amino-2-deoxy-D-glucose diethyl dithioacetal hydrochloride (**1**) with nitrous acid in aqueous hydrochloric acid gives 2-*S*-ethyl-2-thio-D-glucose (**2**) as the principal product, and an anhydro-D-hexose diethyl dithioacetal is not formed. Substance **2** was characterized by conversion into the phenylhydrazone (**3**) and further into D-*arabino*-hexulose phenylosazone (**6**); n.m.r. spectroscopy confirmed that **2** was an aldose having the D-*gluco* configuration. Acetylation of **2** gave the anomeric pyranose tetraacetates (**4** and **5**), and the n.m.r. spectrum of the β-D tetraacetate (**4**) further confirmed the structure assigned to **2**. Nitrous acid in aqueous acetic acid converted **1** into a mixture of **2** and an anhydro-D-hexose diethyl dithioacetal.

INTRODUCTION

Primary alkylamines react with nitrous acid by carbonium ion-type processes[2]. The reaction possesses great driving force under mild conditions. The net reaction path observed may vary widely as the structure and stereochemistry of the starting amine are varied. In derivatives of the 2-amino-2-deoxyhexoses the course of the reaction[3] is profoundly influenced by the structure of the group at C-1. For example, 2-amino-2-deoxy-D-glucose[4,5], or its methyl α-D or β-D-glycoside[6], react with aqueous nitrous acid to give 2,5-anhydro-D-mannose[5], whereas 2-amino-2-deoxy-D-gluconic acid reacts to give 2,5-anhydro-D-gluconic acid[7]. The corresponding alditol, 2-amino-2-deoxy-D-glucitol, reacts to give 2-deoxy-D-*arabino*-hexose[8]. In the first example, inversion at C-2 takes place, presumably by rearside attack at C-2 by O-5 as a nitrogen molecule leaves from C-2. A net double inversion, by initial formation of an α-lactone,

*Part of a series "Action of Nitrous Acid on Derivatives of Amino Sugars". For a preliminary report, see ref. 1.

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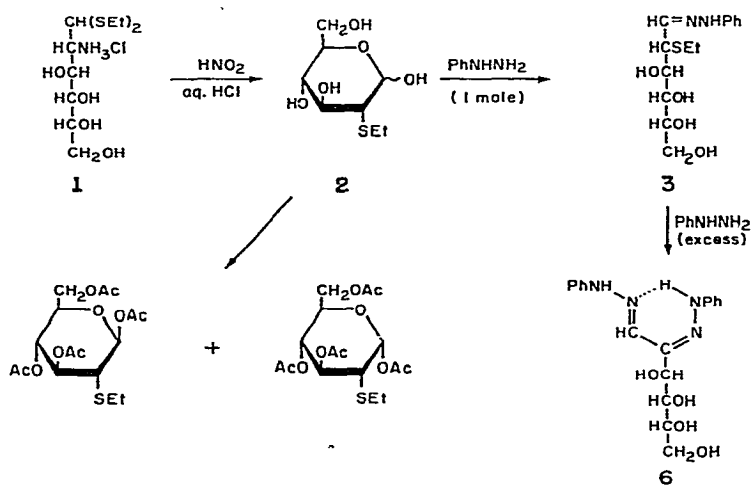
has been proposed[9] as the mechanism for the reaction observed with the aldonic acid. The reaction observed with the alditol can be interpreted[9] in terms of a 1→2 hydride shift, with concomitant loss of a proton from the hydroxyl group at C-1.

The present report describes the reaction of 2-amino-2-deoxy-D-glucose diethyl dithioacetal hydrochloride (**1**) with nitrous acid. In aqueous solution the principal product is shown to be 2-*S*-ethyl-2-thio-D-glucose (**2**). In aqueous acetic acid the reaction leads to **2** together with a larger proportion of an anhydrohexose diethyl dithioacetal; the latter product is not formed when acetic acid is absent.

DISCUSSION

An aqueous solution of 2-amino-2-deoxy-D-glucose diethyl dithioacetal hydrochloride[10] (**1**) was treated at 0° with a 5-molar excess of nitrous acid. The nitrous acid was generated from sodium nitrite and a slight excess of hydrochloric acid. The starting material was completely composed after 6–8 h and ethanethiol was released. The reaction mixture was found to contain a major component that crystallized slowly from ethanol or methanol as needles, m.p. 158–160°. Elemental analysis gave the formula $C_8H_{16}O_5S$. The product reduced Fehling solution and showed rapid upward mutarotation, $[\alpha]_D$ +30→+63° (water). A crystalline phenylhydrazone, $C_{14}H_{22}N_2O_4S$, was obtained by treatment with one equivalent of phenylhydrazine. An excess of phenylhydrazine caused liberation of ethanethiol and D-*arabino*-hexulose phenylosazone (**6**) was formed. These data indicate that the product was either 2-*S*-ethyl-2-thio-D-glucose (**2**), 2-*S*-ethyl-2-thio-D-mannose, or possibly 1-*S*-ethyl-1-thio-D-fructose.



The n.m.r. spectrum of the product in deuterium oxide established that it was 2-*S*-ethyl-2-thio-D-glucose (**2**). The signals anticipated for the ethylthio group were observed. Two doublets at low field could readily be assigned as the anomeric proton signals of a mutarotated aldose[11–13]. The large (~8 Hz) splitting of the higher

field of these two signals was consistent only with the 1,2-diaxial arrangement of protons in the β-D-pyranose tautomer of the D-glucose derivative (**2**). Further, supporting data were provided by n.m.r. spectroscopy of the β-D-pyranose tetraacetate (**4**).

Direct crystallization of **2** from the reaction mixture did not lead to high recoveries, but yields of **2** up to 54% were obtained by preparative, paper chromatography, and the total yield of **2** was estimated to be ~70% of the products. A side-product having a lower chromatographic mobility than **2**, comprising 15–20% of the product, and several minor products (<10% of the total) were also formed; these were not characterized. It was established that an anhydro-D-hexose diethyl dithioacetal* was not present to a detectable extent (papergram) in the reaction product.

The n.m.r. spectrum of **2** at mutarotational equilibrium in deuterium oxide showed, at lowest field, a narrow doublet, τ 4.67, $J_{1,2}$ 3.2 Hz, and a wide doublet τ 5.26, $J_{1,2}$ 8.8 Hz, in approximately 1:1 proportion. These signals were assigned to H-1 of the α-D and β-D anomers of **2**, respectively. It was necessary to heat the solution[11–13] to observe the signal at τ 5.26 because of interference by the HOD signal at room temperature. In Table I the chemical shifts of H-1, and the $J_{1,2}$ coupling constants are compared for **2**, D-glucose, 2-acetamido-2-deoxy-D-glucose, and 2-amino-2-deoxy-D-glucose hydrochloride, together with the values for the corresponding D-mannose analogs. Close correlation between the coupling constants and the H-1 chemical shifts is observed for the D-glucose derivatives, including **2**. The values for the corresponding D-mannose derivatives are in sharp contrast, especially with regard to the $J_{1a,2}$ coupling constant. It is noteworthy that the methyl protons of the ethyl group give a sharp triplet in the mutarotated mixture, but the methylene protons of this group are not equivalent in the two anomers, so that two quartets are observed for the CH_2S protons.

In methyl sulfoxide-d_6 containing a small proportion of deuterium oxide and hydrogen chloride, compound **2** showed rapid mutarotation to give an equilibrated mixture containing 60% of the α-D pyranose anomer and 40% of the β-D pyranose anomer; the respective H-1 signals were observed at τ 4.89 and τ 5.50. The crystalline sugar **2** was shown to be the pure β-D pyranose tautomer by its spectrum in dry methyl sulfoxide-d_6. Exchange of hydroxyl protons is extremely slow in this solvent[14], so that H,OH proton–proton coupling can be observed[15,16]. The spectrum showed no H-1e signal at τ 4.89, indicating that the α-D pyranose anomer was absent. A doublet at τ 3.28 was assigned[15] to the 1-OH proton, and the signals for the other three hydroxyl protons were observed at higher field. Addition of a small proportion of deuterium oxide caused the individual hydroxyl-proton signals to disappear, leaving initially only the H-1a signal at low field; the H-1e signal subsequently appeared as mutarotation took place.

Acetylation of the crystalline β-D anomer of **2** with acetic anhydride–pyridine gave crystalline 1,3,4,6-tetra-*O*-acetyl-2-*S*-ethyl-2-thio-β-D-glucopyranose (**4**), together

*A sample of an anhydrohexose diethyl dithioacetal prepared by the action of nitrous acid on **1** in aqueous acetic acid was kindly furnished for comparison by Dr. J. Defaye.

Carbohyd. Res., 6 (1968) 299–309

TABLE I

CHEMICAL SHIFTS, $J_{1,2}$ COUPLING CONSTANTS, AND ANOMERIC COMPOSITIONS OF ALDOSE DERIVATIVES AT MUTAROTATIONAL EQUILIBRIUM IN DEUTERIUM OXIDE

Compound	*Chemical shift of H-1*			*Coupling constants, Hz*		*Anomeric Composition*
	α-D *Anomer*, τ	β-D *Anomer*, τ	*Difference, p.p.m.*	α-D *Anomer*	β-D *Anomer*	α-D:β-D
Derivatives of D-glucopyranose						
Unsubstituted[a]	4.72	5.30	0.58	3.5	7.5	36:64
2-*S*-Ethyl-2-thio[b] (2)	4.67	5.26	0.57	3.5	9	50:50
2-Amino-2-deoxy, hydrochloride[a]	4.54	5.03	0.49	3.5	8.3	63:37
2-Acetamido-2-deoxy[a]	4.82	5.30	0.48	2.8	7.4	68:32
Derivatives of D-mannopyranose						
Unsubstituted[a]	4.80	5.08	0.28	1.2	1.1	67:33
2-*S*-Ethyl-2-thio[c]	4.60	4.93	0.33	1.6	1.6	60:40
2-Amino-2-deoxy, hydrochloride[a]	4.60	4.78	0.18	1.1	1.5	43:57
2-Acetamido-2-deoxy[a]	4.89	4.99	0.10	1.5	1.4	57:43

[a]Data from ref. 13. [b]This work. [c]Data from ref. 1.

with some of the α-D anomer (**5**). The proportion of **5** obtained was greater if non-crystallized **2** was acetylated. The n.m.r. spectrum of **4** in chloroform-*d* (Fig. 1) showed the H-1 signal as a wide doublet, τ 4.33, $J_{1,2}$ 9.0 Hz, clearly establishing the *β*-D-*gluco* configuration of **4**. A comparison of H-1 chemical shifts and $J_{1,2}$ coupling constants, for **4**, its anomer **5**, and the corresponding peracetates of D-glucopyranose[17], is shown in Table II. The H-3 and H-4 signals of **4** had closely similar chemical shifts and were strongly coupled, so that second-order effects due to virtual[18] 2,4 coupling are observed in the H-2 signal. Similarly, the octet anticipated[19] for H-5 in a first-order spectrum is perturbed because of virtual 3,5-coupling. In benzene-d_6 the H-3 and H-4 signals are further separated and the effects of virtual coupling are less. All vicinal ring-protons show large (8–9 Hz) vicinal couplings, as anticipated for the *β*-D-glucopyranose structure. In either solvent the H-2 signal is observed at high field, as compared with the other ring protons. This accords with the well known smaller deshielding effect of sulfur in comparison with oxygen[19,20]. The H-5 signal is observed at

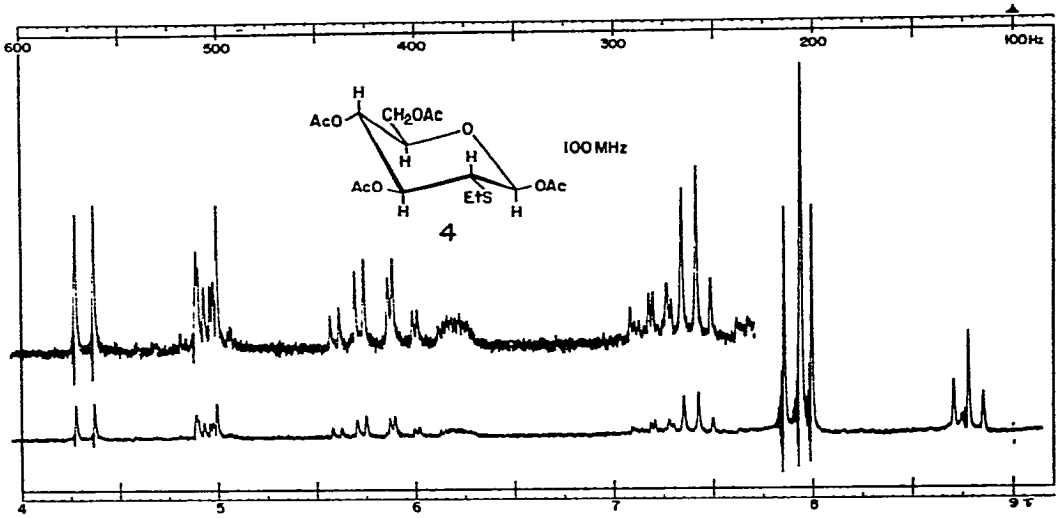


Fig. 1. The 100-MHz n.m.r. spectrum of 1,3,4,6-tetra-*O*-acetyl-2-*S*-ethyl-2-thio-*β*-D-glucopyranose (**4**) in chloroform-*d*.

TABLE II

CHEMICAL SHIFTS OF H-1 AND $J_{1,2}$ COUPLING CONSTANTS FOR ACETYLATED DERIVATIVES OF D-GLUCOPYRANOSE

Peracetate of	α-D *Anomer*		*β*-D *Anomer*	
	H-1, τ	$J_{1,2}$ (Hz)	H-1, τ	$J_{1,2}$ (Hz)
D-Glucopyranose[a]	3.66	3.3	4.24	6.9
2-*S*-Ethyl-2-thio-D-glucopyranose[b]	3.68	4.0	4.33	9.0

[a]Data from ref. 17. [b]This work.

relatively high field in benzene-d_6. A similar effect has been noted[19] with related aldopyranose acetates.

The n.m.r. spectrum of the phenylhydrazone **3** in methyl sulfoxide-d_6 indicates that **3** adopts the acyclic structure shown. A multiplet at τ 5.33–5.81 is observed for the four hydroxyl-group protons, and a singlet at τ 0.38 is assigned to the resonance of the NH proton. All of these signals disappear when the sample is deuterated; the hydroxyl protons underwent exchange more rapidly than the NH-proton. Three hydroxyl-group protons and two NH protons would have been detected if **3** had an intramolecularly cyclized structure.

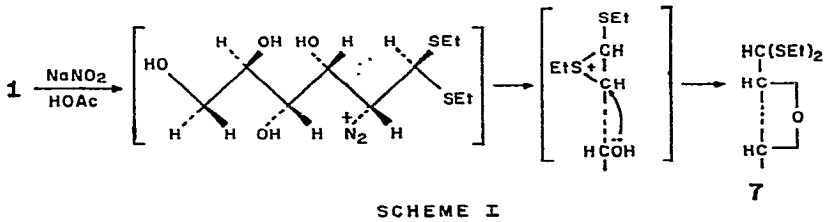
In 1932 Brigl and Schinle[21] prepared a 2-*S*-ethyl-2-thio-D-hexose having m.p. 158° and $[\alpha]_D$ +64.4° (in water). From the evidence presented by these authors, their product could have had the D-*gluco* or the D-*manno* configuration[22]. The close similarity between their constants and those reported herein for **2** establish that the product of Brigl and Schinle has the D-*gluco* configuration, as assumed without definitive evidence by those authors.

Recently, Defaye[23] has reported that deamination of **1** with nitrous acid in aqueous acetic acid gives a mixture of products different from those observed[1] when the reaction is conducted in water. The principal product isolated by Defaye was identified as an anhydrohexose diethyl dithioacetal, and three minor, unidentified products were also observed. Direct evidence for the structure of the major product was not presented, but it was formulated as 2,5-anhydro-D-glucose diethyl dithioacetal because of the fact that it differed from the known[5] D-*manno* analog. Since it is not conceivable that **2** could arise by way of such an anhydro derivative, it is evident that the course of the reaction is critically dependent on the nature of the solvent medium. In this laboratory it was found that treatment of **1** in water with 5 moles of sodium nitrite and 5.3 moles of acetic acid gave a major product, R_F 0.79, that was absent when the reaction was conducted in water alone. A minor product from the reaction in aqueous acetic acid, approximately one-third of the amount of major product, was isolated and characterized as 2-*S*-ethyl-2-thio-D-glucose (**2**). The major product was found to be identical to the anhydrohexose diethyl dithioacetal of Defaye; the n.m.r. parameters of the triacetate of this derivative corresponded closely with values reported by Defaye.

The conversion of **1** into the ethylthio derivative **2**, in aqueous nitrous acid containing some hydrochloric acid, takes place more slowly than the reaction of **1** with sodium nitrite in aqueous acetic acid, which gives mainly the anhydrohexose diethyl dithioacetal. It is known[2] that deamination of amines by nitrous acid is retarded at low pH. The conversion of **1** into the anhydro derivative[23] may follow a course resembling that postulated[9] for the deamination of 2-amino-2-deoxy-D-gluconic acid, by way of a 1,2-episulfonium ion intermediate which subsequently suffers attack at C-2 by one of the hydroxyl groups on the chain to give the anhydroaldose diethyl dithioacetal **7** (Scheme I).

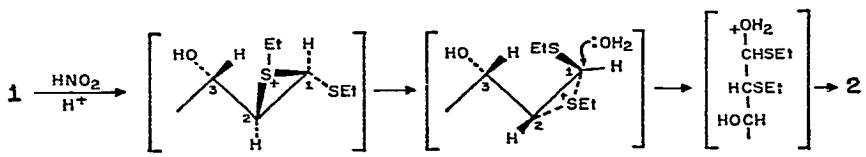
There is at present no experimental evidence to establish the mechanism of the conversion of **1** into **2**. At the low pH of this reaction, the oxygen atoms of the hydroxyl groups can be expected to be less nucleophilic than at the higher pH of the

reaction in which conversion of **1** into **7** is observed. Possibly the conversion into **2** proceeds by a double inversion at C-2, involving attack on an intermediate 1,2-episul-



SCHEME I

fonium ion by the second ethylthio group, with subsequent attack by water at C-1 to give a protonated hemithioacetal. The latter would then undergo conventional hydrolysis to the aldose (Scheme II). A water molecule approaching C-1 of the first 1,2-episulfonium ion would be hindered by the C-3 hydroxyl group, whereas there would be no such hindrance to attack by solvent on the second 1,2-episulfonium ion.



SCHEME II

EXPERIMENTAL

General methods. — Solutions were evaporated below 30°. Melting points were determined with a Thomas–Hoover "Unimelt" apparatus and are uncorrected. Optical rotations were determined in a 2-dm polarimeter tube. Infrared spectra were measured by the potassium bromide disc technique with a Perkin–Elmer Model 137 infrared spectrophotometer. N.m.r. spectra were recorded with Varian A-60 or HA-100 n.m.r. spectrometers, with tetramethylsilane ($\tau = 10.00$) as the internal standard for spectra measured in chloroform-*d*, benzene-d_6, or methyl sulfoxide-d_6, and sodium 4,4-dimethyl-4-silapentane-1-sulfonate ($\tau = 10.00$) as the internal standard for spectra measured in deuterium oxide. Microanalyses were performed by W.N. Rond. X-ray powder diffraction data give interplanar spacings, Å, for CuKα radiation. The camera diameter was 114.59 mm. Relative intensities were estimated visually: s, strong; m, moderate; w, weak; v, very. The strongest lines are numbered in order (1, strongest); double numbers indicate approximately equal intensities. The recorded R_F values refer to paper chromatograms on Whatman No. 1 paper, with 3:1:1 butyl alcohol–ethanol–water as the developer and indication with alkaline silver nitrate. Preparative paper-chromatography was effected with 46 × 57 cm sheets of Whatman No. 3 MM paper with the same developer; zones were located by means of guide strips and components were eluted from the paper with methanol.

2-S-Ethyl-2-thio-D-glucose (**2**). — A solution of 2-amino-2-deoxy-D-glucose

diethyl dithioacetal hydrochloride (**1**, 20 g) in water (150 ml) and conc. hydrochloric acid (26 ml) was cooled to 0°, sodium nitrite (21.5 g) was added, and the pink solution was kept for 10 h at 0°. A stream of nitrogen was then passed through the solution for 1 h, and the residual acid was neutralized by adding small portions of Dowex-1 (CO_3^{-2}) ion-exchange resin. The filtered solution was evaporated by lyophilization, the residue was extracted with methanol (200 ml), and the mixture was filtered to remove undissolved sodium chloride. The filtrate was concentrated, whereupon more sodium chloride separated and was removed by filtration. The filtrate was concentrated to a syrup that crystallized partially after it had been kept for several days at 0°. Methanol (20 ml) was added and the crystals were filtered and washed with methanol, yield 1.48 g (10.6%). After recrystallization from ethanol the product had m.p. 158–160°, $[\alpha]_D^{21}$ +30 (initial, extrapolated) → +51 (10 min) → +63° (45 min, equil.; unchanged after 24 h) (*c* 1.0, water); R_F 0.58; n.m.r. data (100 MHz, deuterium oxide) τ 4.67 (~0.5-proton doublet, $J_{1,2}$ 3.2 Hz, H-1 of α-D anomer), τ 5.26 (~0.5-proton doublet, $J_{1,2}$ 8.8 Hz, axial H-1 of β-D anomer), τ 6.15–6.65 (5-proton multiplet, H-3,4,5,6,6'), τ 7.01–7.60 (3-proton multiplet, H-2 and methylene of EtS), τ 8.76 (3-proton triplet, *J* 7.5 Hz, CH_3 of Et); X-ray powder diffraction data: 7.55 m, 6.23 m, 5,60 vw, 5.15 m, 4.64 s, 4.04 s, 3.50 w.

Anal. Calc. for $C_8H_{16}O_5S$: C, 42.83; H, 7.19; S, 14.28. Found: C, 43.03; H, 7.14; S, 13.94.

A form of **2** having m.p. 143–144°, was encountered in some of the early preparations[1].

The residual, uncrystallized syrup (10.6 g, 76%) was found (papergram) to contain a principal component, R_F 0.58 (approximately 70% of the total by densitometric scanning), whose chromatographic characteristics were indistinguishable from those of an authentic sample of **2**. In addition, a minor product having R_F 0.43 (approximately 20%) was present, in addition to trace components having R_F 0.08, 0.13, and 0.26. No trace of a component having R_F 0.79, the mobility of a reference sample of the anhydro-D-hexose diethyl dithioacetal prepared according to Defaye[23], was detected.

Resolution of a sample of the syrup (0.97 g) on 8 sheets of Whatman No. 3 MM paper gave the component, R_F 0.58, as crystals, yield 0.55 g (57% of the syrupy mixture), m.p. 158–160°, indistinguishable from **2** by X-ray powder diffraction pattern and n.m.r. spectrum; this corresponds to a yield of 54% of crystalline **2** from **1**. The component having R_F 0.43 was eluted from the paper, yield 0.19 g. Rechromatography of this substance indicated that it was a complex mixture of at least three components having very similar R_F values.

N.m.r. spectrum of 2-S-ethyl-2-thio-β-D-glucopyranose in methyl sulfoxide-d_6. — A solution (*ca.* 10%) prepared by dissolving crystalline **2**, m.p. 158–160°, in methyl sulfoxide-d_6 showed the following n.m.r. data (60 MHz): τ 3.28 (1-proton doublet, $J_{1a,OH}$ 6.5 Hz, 1-OH of β-D-pyranose anomer), τ 5.02–5.18 (2-proton multiplet, 3-OH, 4-OH), τ 5.40–5.67 (2-proton multiplet, H-1a, 6-OH), τ 8.82 (3-proton triplet, CH_3 of Et). No signal was observed near τ 4.9.

These data, measured ~2 min after dissolution, were essentially unchanged after 25 min. A small proportion of deuterium oxide was then added. The signals assigned to OH protons disappeared and were replaced by a signal at τ 6.08 (HOD). A one-proton, wide doublet, $J_{1a,2}$ 8.6 Hz, remained at τ 5.50 (H-1a of β-D pyranose anomer). After 2 min a signal near τ 4.9 began to appear. A trace of hydrogen chloride was then added and the spectrum was measured 6 min after the addition. A narrow doublet, assigned to H-1e of the α-D pyranose anomer was observed at τ 4.89, and gave $J_{1e,2}$ 3.1 Hz. Integration[13] indicated that the α:β ratio of pyranose anomers was 60:40. After 22 min the observed ratio was 61:39.

2-S-Ethyl-2-thio-D-glucose phenylhydrazone (**3**). — To a solution of **2** (150 mg) in water (2 ml) was added phenylhydrazine (108 mg, 1.5 molar equiv.) and a drop of acetic acid. The mixture was kept for 4 h at room temperature. The hydrazone (**3**) separated as light-yellow crystals, yield 178 mg (84%). Recrystallization from ethanol gave pure **3** as white, lustrous crystals, m.p. 180–181° (dec.), $[\alpha]_D^{20}$ −157 ±1° (*c* 1, pyridine); R_F 0.80; λ_{max}^{KBr} 3.00 (NH), 3.06 (OH), 6.23, 6.55, 6.70, 6.90 (aryl C=C), 14.40 μm (substituted benzene); n.m.r. data (100 MHz, methyl sulfoxide-d_6): τ 0.38 (1-proton singlet, disappears slowly on deuteration, NH), τ 2.80–3.40 (5-proton multiplet, Ph), τ 5.33-5.81 (4-proton multiplet, disappears rapidly on deuteration, 3,4,5,6-OH), τ 6.05–6.65 (6-proton multiplet, H-1,3,4,5,6,6'), τ 7.27–7.66 (3-proton multiplet, H-2, CH_2 of ethyl), τ 8.78 (3-proton triplet, *J* 7 Hz, CH_3 of ethyl); X-ray powder diffraction data: 8.37 m, 6.23 m, 4.96 s (1), 4.71 s (2,2), 4.46 vw, 4.24 s (2,2), 3.66 s (2,2), 3.52 vw, 3.37 s (2,2).

Anal. Calc. for $C_{14}H_{22}N_2O_4S$: C, 53.50; H, 7.01; N, 8.91; S, 10.19. Found: C, 53.31; H, 6.87; N, 9.20; S, 10.19.

For the phenylhydrazone of a 2-*S*-ethyl-2-thio-D-hexose having either the D-*gluco* or D-*manno* configuration, Brigl and Schinle[21] reported m.p. 187°, $[\alpha]_D^{20}$ −157° (pyridine).

Conversion of 2-S-ethyl-2-thio-D-glucose (**2**) *into* D-arabino-*hexulose phenylosazone* (**6**). — Treatment of **2** (427 mg) with phenylhydrazine (1 g, ~5 molar equiv.) and acetic acid (1.25 ml) as in the preceding experiment, but with heating for 1 h at 100°, gave the crystalline phenylosazone **6**, identical by X-ray powder diffraction pattern with an authentic sample.

1,3,4,6-Tetra-O-acetyl-2-S-ethyl-2-thio-β-D-glucopyranose (**4**). — To a solution of **2** (300 mg) in pyridine (5 ml) was added acetic anhydride (4 ml) and the mixture was kept for 18 h at room temperature. The mixture was poured into ice–water and the crystalline product that separated was filtered off, washed with water, dried, and recrystallized from ether; yield 295 mg (56%), m.p. 78–79°, $[\alpha]_D^{21}$ +43° (*c* 1, chloroform); λ_{max}^{KBr} 5.74 μm (OAc); n.m.r. data (100 MHz, chloroform-*d*, see Fig. 1): τ 4.33 (1-proton doublet, $J_{1,2}$ 9.4 Hz, H-1), τ 4.90–5.07 (2-proton multiplet, H-3,4), τ 5.67 (1-proton quartet, $J_{5,6}$ 4.5 Hz, $J_{6,6'}$ 12.5 Hz, H-6), τ 5.94 (1-proton quartet, $J_{5,6'}$ 2.2 Hz, H-6'), τ 6.20 (1-proton multiplet, $J_{4,5}$ ~10 Hz, H-5), τ 7.20 (1-proton multiplet, H-2), τ 7.40 (2-proton quartet, *J* 7.3 Hz, CH_2 of Et), τ 7.84, 7.93, 7.98 (3-, 6-, 3-proton singlets, acetyl), τ 8.77 (3-proton triplet, CH_3 of Et); in benzene-d_6

(100 MHz): τ 4.30 ($J_{1,2}$ 9.4 Hz, H-1), τ 4.70–5.00 (7-peak, symmetrical multiplet, $J_{3,4}$ ~10 Hz, H-3,4), τ 5.70 ($J_{5,6}$ 4.2 Hz, $J_{6,6'}$ 12.5 Hz, H-6), τ 6.05 ($J_{5,6'}$ 2.2 Hz, H-6'), τ 6.79 ($J_{4,5}$ ~10 Hz, H-5), τ 7.30 (triplet, $J_{2,3}$ 10 Hz, H-2), τ 7.62 (CH_2 of Et), τ 8.20, 8.28, 8.30, 8.37 (acetyls), τ 9.01 (CH_3 of Et); X-ray powder diffraction data: 10.64 s (1,1), 8.34 s (1,1), 6.19 vw, 5.29 s (2,2), 4.91 w, 4.62 s (1,1), 4.37 w, 4.07 s (2,2), 3.83 s (2,2).

Anal. Calc. for $C_{16}H_{24}O_9S$: C, 48.98; H, 6.16; S, 8.16. Found: C, 49.00; H, 6.22; S, 8.28.

Acetylation by the above procedure of syrupy **2** that had been obtained by evaporation of an aqueous solution at mutarotational equilibrium, and isolation of the product by extraction with chloroform, gave syrupy 1,3,4,6-tetra-*O*-acetyl-2-*S*-ethyl-2-thio-α,β-D-glucopyranose in essentially quantitative yield. The n.m.r. spectrum of this product in chloroform-*d* indicated that it was an approximately 1:1 mixture of the β-D anomer (**4**) and the α-D anomer (**5**). The H-1 signal of the latter was observed at τ 3.68 as a doublet, $J_{1,2}$ 4.0 Hz.

Treatment of 2-amino-2-deoxy-D-glucose diethyl dithioacetal hydrochloride (**1**) *with nitrous acid in aqueous acetic acid.* — A solution of **1** (2.0 g) in a mixture of acetic acid (2 ml) and water (50 ml) was cooled to 0°, and sodium nitrite (2.15 g) was added. The solution was kept for 2 h at 0° and then for 15 min at room temperature. Nitrogen was passed through the solution for 45 min, the solution was evaporated and the solid residue was extracted with methanol (20 ml). The residue was dissolved in water, deionized by passing through a column of Amberlite MB-3 mixed-bed, ion-exchange resin, and the effluent was lyophilized to give a syrup (0.8 g). Paper chromatography revealed the presence of two principal components, R_F 0.79 and R_F 0.58, of approximate intensity 3:1, together with minor components having R_F 0.37, 0.33, and 0.13. The component having R_F 0.79 was chromatographically indistinguishable from a reference sample of the anhydro-D-hexose diethyl dithioacetal of Defaye[23]. Refrigeration of a methanolic solution of the syrup gave a low yield of a crystalline product, m.p. 155–157°, R_F 0.58, which gave an X-ray powder diffraction pattern identical to that of 2-*S*-ethyl-2-thio-D-glucose (**2**).

Resolution of the residual syrup (720 mg) by preparative, paper chromatography on 7 sheets of Whatman 3 MM paper gave the component having R_F 0.58, yield 145 mg (20%), which on treatment with phenylhydrazine (1 equiv.) gave 2-*S*-ethyl-2-thio-D-glucose phenylhydrazone (**3**), identical by mixed m.p., i.r. spectrum, and paper-chromatographic mobility to an authentic sample. The fast-moving zone was extracted from the paper with methanol; it gave 440 mg (61% of the mixture) of the component having R_F 0.79. Acetylation of this product by the procedure used for preparation of **4** gave a syrup whose n.m.r. spectrum (100 MHz, chloroform-*d*) gave the following data: τ 4.44 (1-proton quartet, width 8 Hz), τ 4.78 (1-proton multiplet, width 10 Hz), τ 4.83 (1-proton doublet, $J_{1,2}$ 8 Hz, H-1), τ 5.42–5.95 (3-proton ABX system), τ 6.80 (1-proton quartet, spacings 8 Hz and 4.5 Hz), τ 7.18–7.50 (4-proton multiplet, CH_2S of dithioacetal), τ 7.90, 7.95, 8.00 (3-proton singlets, acetyls), τ 8.70 (6-proton multiplet, CH_3 of dithioacetal). For a compound formulated as 2,5-anhydro-

D-glucose diethyl dithioacetal triacetate, Defaye[23] reported (60 MHz, chloroform-*d*) τ 8.7 (CH_3 of Et), τ 7.43 (CH_2 of ethyl), and τ 7.92 (acetyls). For the *O*-deacetylated analog in chloroform-*d*, Defaye[23] reported τ 4.78 (doublet, $J_{1,2}$ 8 Hz, H-1), τ 6.79 (quartet, $J_{2,3}$ 4.5 Hz, H-2).

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CELL-BOUND EXODEXTRANASE OF *Bacillus* SPECIES

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ABSTRACT

Inoculation of a synthetic medium containing dextran with soil resulted in the accumulation of several *Bacillus* species, which were isolated and characterized as *B. subtilis* and *B. megatherium*. These dextran-splitting bacilli did not produce an extracellular dextranase, in contrast to several other micro-organisms reported in the literature, which break down dextran outside the cell into comparatively large fragments (oligosaccharides of the isomaltose series). The *Bacillus* enzyme was found in the cell extract after disruption of the cells. The action of this enzyme on dextran liberated D-glucose as the sole product of hydrolysis. Isomaltodextrins were likewise rapidly hydrolyzed to give only D-glucose. The mechanism of action of the *Bacillus* dextranase involves endwise cleavage of D-glucose residues from the terminal groups, leaving the rest of the dextran molecule intact.

INTRODUCTION

Dextrans are extracellular polysaccharides formed by several bacterial species, for example, *Leuconostoc dextranicum* and *L. mesenteroides*, when they grow on sucrose as a substrate. They are built up from α-D-(1→6)-linked chains of D-glucose residues. Dextrans have frequently been found to contain variable proportions of α-D-(1→4)-, α-D-(1→3)-, and α-D-(1→2)-linked residues of D-glucopyranose[1,2]. These non-(1→6)-linked residues are generally considered to be branch points in the molecule, but they may also occur in linear portions of the polysaccharide.

Dextranases (systematic name: α-D-(1→6)-glucan 6-D-glucanohydrolase, EC 3.2.1.11) are enzymes capable of hydrolyzing the α-D-(1→6)-glucosidic linkages of dextrans, if non-(1→6)-linkages do not interfere. Dextranases occur in a variety of mammalian tissues[3], and may be produced adaptively by numerous molds[4,5] and certain bacteria[6–9], but they are not found in dextran-producing bacteria.

Mammalian tissues contain exodextranases[3], which degrade dextran by endwise removal of D-glucose residues up to the branch points[10]. Dextranases produced adaptively by molds have usually been found free in the culture fluids (that is, extracellularly)[5,11–13], although some have been associated with the mycelia[4]. Intracellular-enzyme preparations from *Penicillium lilacinum* and *P. funiculosum*, grown

on dextran-containing media, were found active on dextran-derived oligosaccharides, but have not been tested on dextran[13]. The fungal dextranases most frequently studied are the extracellular endohydrolases from *P. lilacinum* and *P. funiculosum*[5,12–14], which attack internally situated, α-D-(1→6)-links. Macromolecular dextrans are degraded to polymeric fragments of successively decreasing size; the ultimate products from dextrans that are essentially unbranched are, almost exclusively, oligosaccharides of the isomaltose series[12,14]. Branched dextrans also give rise to oligosaccharides carrying the branch points[13].

Dextranases, produced adaptively by certain bacteria when cultured on dextran-containing media, have been found in the cell-free culture fluids[7,15] or associated with the cells[6,9]. In addition to the extracellular dextranase from *Lactobacillus bifidus*, an intracellular enzyme has been obtained that hydrolyzes isomaltodextrins but not dextran[16]. Bacterial dextranases show endohydrolase action, apparently exclusively[9,17,18], but show distinctive patterns of action for each organism. An enzyme extract from cultures of the cellulose-degrading bacterium *Cellvibrio fulva*[6] split dextran chiefly into comparatively large fragments; apparently no D-glucose or disaccharides from the ends of the molecule were formed. An aerobic bacterium of the genus *Cytophaga* adaptively produced an endodextranase which was bound to the cell-wall surface[9]. Strictly anaerobic, asporogen rods (genus *Bacteroides*), producing extracellular endodextranase, have been isolated from the human intestine[7] and also (*Lactobacillus bifidus*) from the rumen of cows[8].

In the present investigation, several species of aerobic, spore-forming rods (genus *Bacillus*) were isolated from soil, which adaptively produced a cell-bound dextranase that shows exohydrolase action.

EXPERIMENTAL

Materials. — The dextrans used in this investigation were commercial products having the following specifications: dextran 5-40-F (Sigma), mol. wt. $5–40 \times 10^6$; dextran, Type 2000 (Sigma), average mol. wt. 2×10^6; dextran (Nutritional Biochemicals Corporation), mol. wt. $2–3 \times 10^5$; dextran, Type 100C (Sigma), average mol. wt. 186,000. Further samples used were: amylopectin (amylose-free, Calbiochem); glycogen (Calbiochem); D-glucose oxidase, Type II (fungal, Sigma); and catalase from bovine liver, crystallized (Sigma). Isomaltodextrins were prepared by partial hydrolysis of dextran with acid, and were chromatographed on carbon–Celite[19].

Accumulation and isolation of dextran-degrading micro-organisms from soil. — Garden soil from the laboratory was inoculated into a medium of the following composition: $(NH_4)_2SO_4$, 0.3%; K_2HPO_4, 0.1%; $MgCl_2$, 0.02%; $CaCO_3$, 0.25%; dextran, 1% (mol. wt. $2–3 \times 10^5$) dissolved in tap water. Erlenmeyer flasks of 300-ml capacity containing 100 ml of the above medium were incubated for several days at 30° on a rotary shaker. At the end of this period, good growth had occurred. The bacteria were isolated in pure form by plating them on an agar medium of the same composition.

Identification of the organisms isolated[20]. — By the above procedure, a number of spore-forming rods were obtained. Two of them were identified, and were maintained on agar slants of the same medium. Strain D_2 had the following properties: dimensions of vegetative rods, 1.0–1.2 μm by 4–5 μm; sporangia not distinctly swollen; spores, 1.2 μm by 1.5 μm, oval, central; colonies smooth, glistening, round, convex; growth on D-glucose–nutrient agar slants more abundant than on nutrient agar; good growth on D-glucose–nitrate[20] agar; starch hydrolysis, positive; no formation of 3-hydroxy-2-butanone (acetylmethylcarbinol); D-glucose, sucrose, and D-mannitol were fermented, with production of acid and gas. Formation of nitrite from nitrate; no anaerobic growth in D-glucose–nutrient broth. Conclusion: *Bacillus megatherium.*

Strain D_5 had swollen sporangia; spores ellipsoidal; growth in both 5% NaCl and 7% NaCl solution; no gas from nitrate under anaerobic conditions; anaerobic growth in D-glucose–nutrient broth, positive; reduction of nitrate to nitrite, positive; Voges–Proskauer reaction (acetylmethylcarbinol), positive; starch hydrolysis, positive; vegetative rods, 0.8 μm by 3–4 μm, motile. Fermentation tests: acid without gas on L-arabinose, D-xylose, D-glucose, sucrose, and D-mannitol. Conclusion *Bacillus subtilis.*

Preparation of cell extracts. — Dextran-degrading bacilli were cultivated in dextran medium (1 liter) in a Kluyver flask that was aerated by passing sterile air through the medium. Cells were harvested by centrifugation in an MSE (Measuring and Scientific Equipment) refrigerated centrifuge at 20,000 *g*, and were washed with distilled water, and suspended in water. The cells were disrupted for 15 min with an MSE ultrasonic disintegrator while being cooled in ice. The crude extract was centrifuged at 20,000 *g* to remove cell debris and non-disrupted cells.

Dextranase activity was assayed by incubating 0.2 ml of dextran (1% of mol. wt. $2–3 \times 10^5$), 0.2 ml of citrate buffer (0.2 ml, pH 5.45), and 0.1 ml of bacterial extract for 1 h at 30°. The increase in reducing-sugar content was measured by the Somogyi–Nelson method, and is expressed as mg of D-glucose liberated per h.

Chemical and enzymic analysis of sugars. — Total carbohydrate was measured by the anthrone method[21]; reducing sugars were determined by the method of Somogyi and Nelson[22]. The enzymic assay of D-glucose was performed by use of the D-glucose oxidase–catalase system. The sample (0.1 ml), 0.2M citrate buffer (0.4 ml, pH 5.45), D-glucose oxidase (0.3 ml, 1%), and 10:4:3 (v/v) catalase (0.1 ml) were combined in a Warburg vessel, and the oxygen uptake of the system was measured manometrically. This system was also used for determining dextranase activity, by continuous measurement of the D-glucose liberated. Chromatography of sugars was effected on Whatman No. 1 paper, with 10:4:3 (v/v) ethyl acetate–pyridine–water as developer, and aniline hydrogen phthalate as detecting spray.

Structural studies on dextrans. — The percentage of α-D-(1→6)-glucosidic bonds in samples of dextrans was determined by periodate oxidation: 1 ml of sodium periodate solution (8 g per 100 ml of water) was added to 1 ml of 1% dextran solution, and the mixture was incubated for 24 h at 4° in the dark. The excess of sodium periodate

was then decomposed by adding 0.2 ml of 1,2-ethanediol, and the formic acid liberated was titrated with 0.01M sodium hydroxide, with Bromocresol Purple as the indicator.

Fractionation of dextrans[23] was effected by gel filtration on Sephadex columns, in order to separate the hydrolyzed dextrans according to molecular size. Samples (10 ml, 1–2%) of the dextran solution were applied to a column (bed volume 150 ml, "void" volume 50 ml) packed with a mixed bed of Sephadex G-100 and G-25, and the column was eluted with water. Fractions (6 ml) of the effluent were collected.

The average degree of polymerization ($\overline{DP}$) of the dextran fractions was determined by end-group analysis (Somogyi method). The carbohydrate content of the fractions were determined by the anthrone method.

The specific viscosity (η_{sp}) of dextran solutions was determined at 30° in water by means of an Ubbelohde viscometer.

RESULTS

Action of Bacillus *dextranase on a dextran having molecular weight* $2–3 \times 10^5$. — Dextran (0.4 ml, 1%), 0.2M citrate buffer (0.4 ml, pH 5.45), and bacterial extract (0.2 ml, *Bacillus* D_5) were incubated at 30°. The increase of reducing-sugar content in the incubation mixture, as measured by the Somogyi–Nelson method, closely paralleled the release of D-glucose as judged by D-glucose oxidase assay (see Fig. 1). The

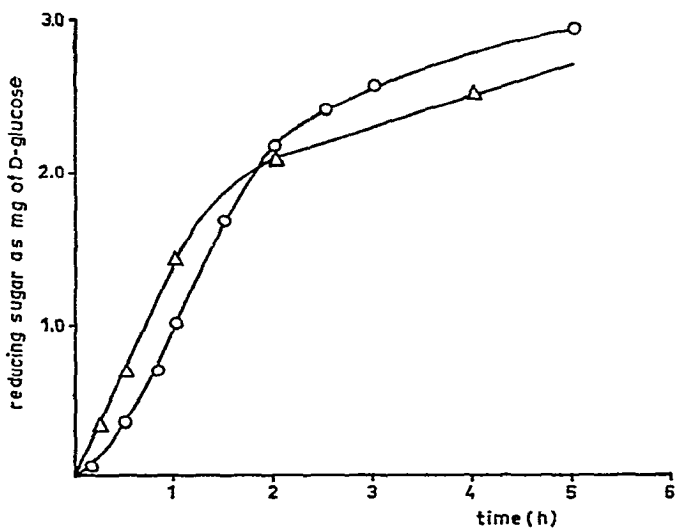

Fig. 1. Action of *Bacillus* dextranase on dextran having mol. wt. $2–3 \times 10^5$. △—△ Increase of reducing-sugar content of incubation mixture, as determined by the Somogyi–Nelson method. ○—○ Release of D-glucose, as determined by the D-glucose oxidase–catalase system.

results suggested that the dextranase action was limited to the liberation of D-glucose. Chromatographic analysis of the hydrolyzates also confirmed that D-glucose was the only reducing sugar liberated during the process.

Action of Bacillus *dextranase on isomaltodextrins.* — Isomaltodextrins (0.4 ml, 1%), 0.2M citrate buffer (0.4 ml, pH 6.4), and bacterial extract (0.1 ml, *Bacillus* D_5) were incubated at 30°. Samples (0.1 ml) were withdrawn at different times, and the reducing-sugar content of the samples was determined by the Somogyi–Nelson method. Chromatography revealed that D-glucose was the only sugar component released during the course of the reaction. Liberation of D-glucose was measured by the increase in reducing-sugar content of the incubation mixture (see Fig. 2). Isomaltodextrins were hydrolyzed at a higher rate than dextrans 100C and 2000 under the same conditions.

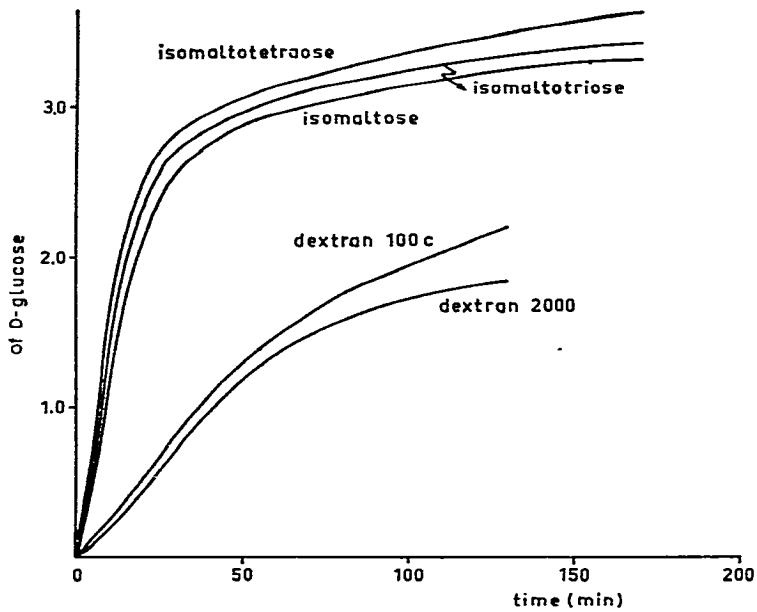

Fig. 2. Action of *Bacillus* dextranase on isomaltodextrins and some dextrans.

Mechanism of degradation of dextran by Bacillus *dextranase.* — To prove that the mechanism of the breakdown of dextran consists of a stepwise degradation of the dextran molecule from the terminal groups, the following experiment was performed. Dextran 100C (50 ml, 2.5%), 0.2M phosphate buffer (5 ml, pH 6.4), and bacterial extract (*Bacillus* D_5, 5 ml) were incubated at 30°. Samples (10 ml) were withdrawn at 0 h, 2 h, and 12.5 h, and were pipetted into 1 ml of 3% perchloric acid. After the dextran samples had been neutralized with solid sodium hydrogen carbonate, they were fractionated by gel filtration through Sephadex (see Fig. 3). D-Glucose was liberated during the course of the reaction, and appeared as a separate peak in the last fractions of the effluent. Although the carbohydrate content of the dextran fractions was lowered by the release of D-glucose, the molecular-weight distribution of the residual dextran remained virtually unaltered. This result indicated an endwise breakdown of the dextran by the exodextranase activity of the *Bacillus* extract during the first stages

of the degradation. This mechanism is in contrast to the random cleavage of the α-D-(1→6)-glucosidic bonds that takes place when dextrans are subjected to hydrolysis

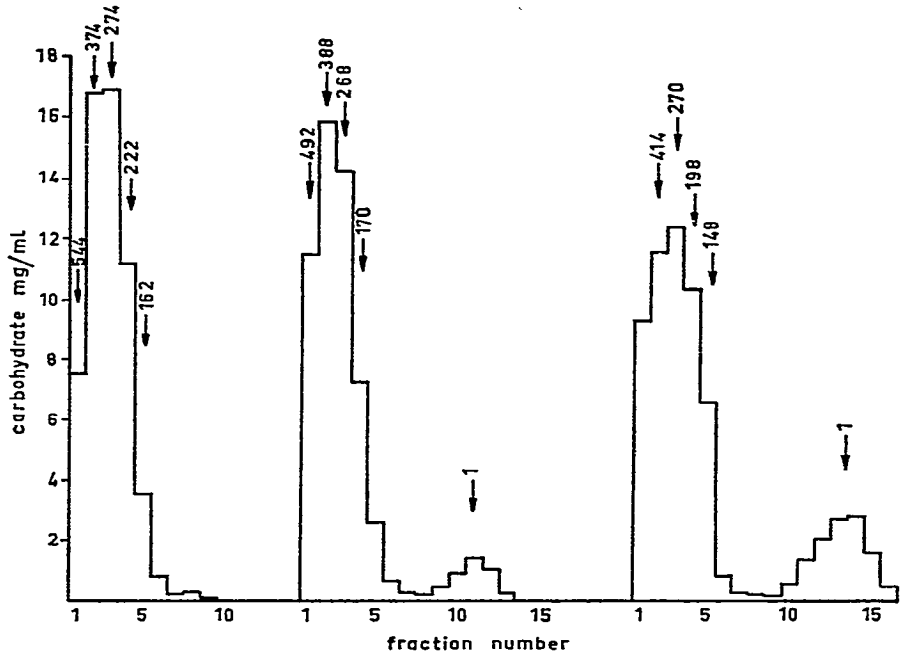

Fig. 3. Fractionation diagrams of enzymically hydrolyzed dextran 100C from a column of a mixed-bed Sephadex (G-25+G-100) at 0 h, 2 h, and 12.5 h. The arrowed figures indicate measured $\overline{DP}$ values of the fractions.

by endodextranase or by acid. The latter mode of action is exemplified by the hydrolysis of dextran 100C by hydrochloric acid (see Fig. 4).

Effect of pH on dextranase activity.—Dextran having mol. wt. 2–3 × 10^5 (0.2 ml, 1%), 0.2M buffer (0.2 ml, various pH values), and bacterial extract (0.1 ml, *Bacillus* D_5, protein concentration 9.2 mg/ml) were incubated at 30°. The rate of release of reducing sugar was determined by the Somogyi–Nelson method.

The rate of increase of specific fluidity ($\psi_{sp} = 1/\eta_{sp}$) of enzyme–dextran solutions was determined by incubation of dextran 5-40-F (15 ml, 1%), 0.2M buffer (5 ml, various pH values), and bacterial extract (0.5 ml, *Bacillus* D_5) at 30° (see Fig. 5). From these experiments, it was concluded that the liberation of reducing sugar and the increase of specific fluidity had the same optimal pH value of 5.8–6.0, suggesting that both effects (saccharifying and liquefying) are caused by the same enzyme system (see also, Discussion).

DISCUSSION

Among micro-organisms that have been screened for their ability to produce extracellular dextranase, a large number of molds[5] have been found to produce the

enzyme. A number of bacterial species[6–9] that adaptively produce endodextranases have also been isolated.

Dextran-splitting bacteria readily accumulate in a synthetic medium containing dextran, after inoculation with soil. In the present investigation, several species of

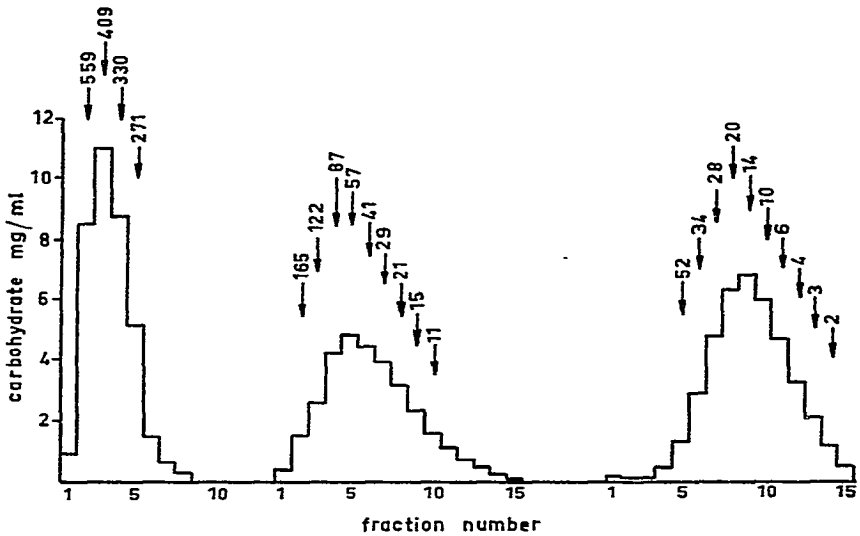

Fig. 4. Fractionation diagrams of acid-hydrolyzed dextran 100C from a Sephadex (G-25+G-100) column at 0 h, 1 h, and 4 h. Dextran 100C (50 ml, 2.5%) and 0.66M hydrochloric acid (7.5 ml) were heated at 100°; after being cooled, samples (10 ml) were neutralized with 0.5M sodium hydroxide, and fractionated.

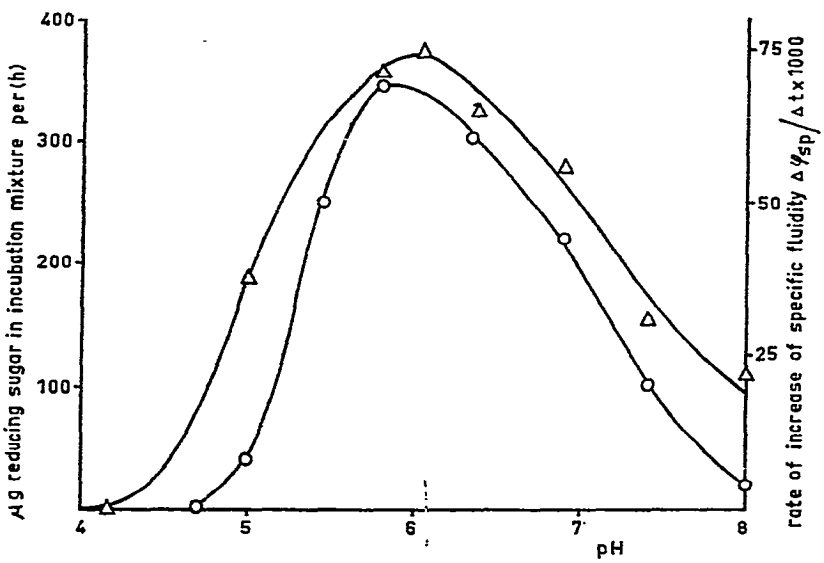

Fig. 5. Effect of pH on dextranase activity. ○—○ Rate of release of reducing sugar, as determined by the Somogyi–Nelson method (mol. wt. of dextran, 2–3 × 10^5). △—△ Rate of increase of specific fluidity of enzyme–dextran solutions (mol. wt. of dextran, 5–40 × 10^6).

Bacillus were isolated that were capable of utilizing dextran as their sole source of carbon. Experiments were conducted with one strain (D_5) of *Bacillus subtilis*, although the properties of the dextranase of a strain (D_2) of *Bacillus megatherium* were found to be similar.

Whole, washed cells were active in breaking dextran down. These cells were active only when they were grown in a dextran-containing medium. The mechanism by which whole cells attack dextran, and the site of enzymic action, are not yet clear, and the investigation is being continued.

The cell-free, culture fluid had no dextranase activity; the highest activity was obtained in the soluble extract obtained after disruption of the cells. The activity of the insoluble cell-particles was lower. Degradation of dextran by these cells take place either inside the cell or in the cell wall, probably without the prior action of an extracellular dextranase. It could not be decided whether the enzyme was intracellular or was localized at the cell wall. If the enzyme is associated with the cell wall, it must have been solubilized during disruption. If the enzyme is intracytoplasmic, the way in which the dextran molecule penetrates the permeability barrier remains to be proved.

The dextranase of *Bacillus* acts by endwise cleavage of the α-D-(1→6)-glucosidic bonds of the dextran molecule, liberating D-glucose as the sole product of hydrolysis. In addition to whole dextrans, isomaltodextrins are also rapidly hydrolyzed by the *Bacillus* extract, at an even higher rate. *Bacillus* dextranase was without effect on polysaccharides of the glycogen–starch group (glycogen, amylopectin, and maltose were not attacked); therefore, the enzyme preparation did not have α-D-(1→4)-glucosidase activity. Not only do α-D-(1→4)-glucosidic bonds of the glycogen–starch class of polysaccharides remain unattacked, but the α-D-(1→6)-glucosidic branch points of these carbohydrates were also not hydrolyzed. *beta*-Amylase limit-dextrin from glycogen, containing a high proportion (15%) of branch points, remains unaltered after subjection to the action of *Bacillus* dextranase. These branches need a specific α-D-(1→6)-glucosidase (debranching enzyme) for cleavage.

The dextrans used in this investigation contained a high proportion of α-D-(1→6)-glucosidic bonds. Analysis by periodate oxidation indicated that 93–96% of α-D-(1→6)-glucosidic linkages was present. These almost unbranched dextrans were fully hydrolyzed by the *Bacillus* dextranase after 24 h under the experimental conditions. The extent to which highly branched dextrans may be degraded by the *Bacillus* dextranase has yet to be investigated.

A soluble-enzyme system, capable of hydrolyzing some dextrans to D-glucose as the sole or major product, has been obtained from an intestinal bacterium of the genus *Bacteroides*[18]. This system evidently contained two different dextranases, as it could either "liquefy" or "saccharify" dextrans. At pH 5.0–5.5, the principal effect was a rapid lowering of the viscosity; at pH 7.0–7.5, the major effect was a rapid release of D-glucose. At pH 7.4, the *Bacteroides* system hydrolyzed both α-D-(1→6)- and α-D-(1→4)-glucosidic linkages in oligosaccharides and dextrans. The enzymic attack generally appeared to be limited to the outer portions of the molecules. In this respect, the *Bacteroides* enzyme resembles the *Bacillus* dextranase.

The attack on dextran by *Lactobacillus bifidus* is initiated by an extracellular endodextranase[15]. The cell extract of *Lactobacillus bifidus* contains an α-D-(1→6)-glucosidase that readily hydrolyzes isomaltodextrins having degrees of polymerization of 2–9 to D-glucose; the hydrolysis proceeds by cleavage of single D-glucose residues from the nonreducing end of the molecule[16]. Dextrans were not hydrolyzed by this extract. The same pattern of action was found in the dextranase system of molds (*Penicillium lilacinum, P. funiculosum*). After the hydrolysis of unbranched dextrans to D-glucose, isomaltose, and isomaltotriose by the extracellular endodextranase of these organisms[12], the oligosaccharides of the isomaltose series were broken down by an intracellular hydrolase prepared from cell extracts of the molds[13].

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A FACILE SYNTHESIS OF TRITIATED D-GLUCOSE AND D-GALACTOSE LABELED UNIQUELY AT CARBON 4

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ABSTRACT

A method is described for the simple synthesis of D-glucose and D-galactose specifically labeled at C-4 with tritium. Methyl α-D-galactopyranoside (**1**) was converted into methyl 2,3,6-tri-*O*-benzoyl-α-D-galactopyranoside (**2**). Oxidation of **2** with methyl sulfoxide gave methyl 2,3,6-tri-*O*-benzoyl-α-D-*xylo*-hexopyranosid-4-ulose (**3**). Reduction of **3** with sodium borotritiide resulted in the formation of the expected 4-tritiated methyl 2,3,6-tri-*O*-benzoyl-α-D-glucopyranoside and α-D-galactopyranoside respectively. On debenzoylation with sodium methoxide, and acid hydrolysis, the free sugars D-glucose-*4-t* and D-galactose-*4-t* were obtained. At least 99% of the tritium activity was associated with C-4.

INTRODUCTION

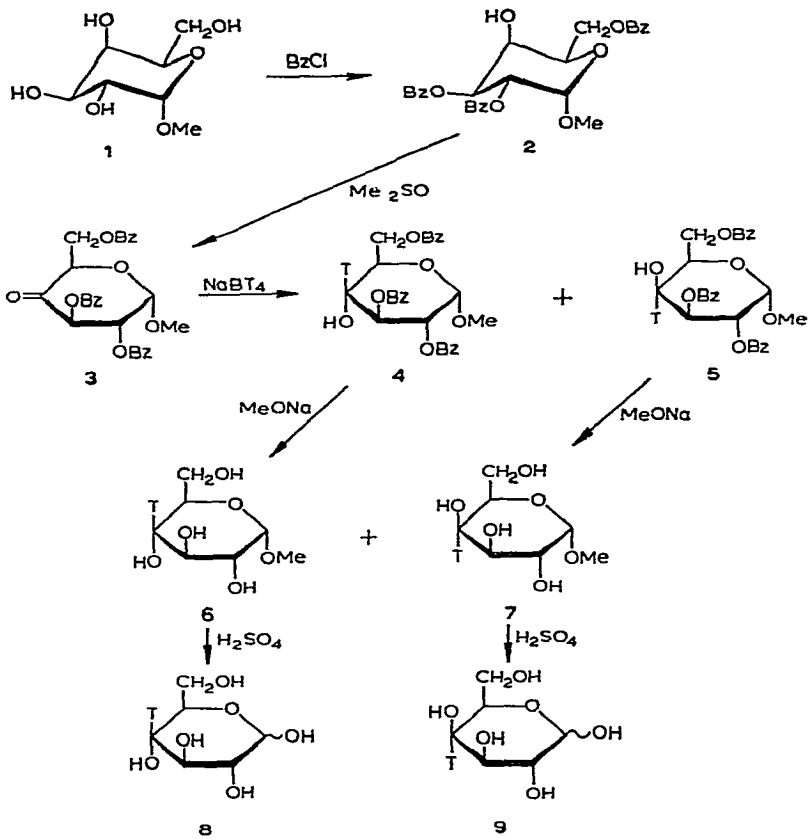
The use of specifically tritiated sugars in metabolic experiments and in studies on the mechanism of enzymic reactions has increased as such sugars become more easily available. For studies on the mechanism of the biosynthesis of 6-deoxy sugars carried out in this laboratory, it was important to have, in addition to the previously prepared D-glucose-*3-t*[1], a specifically 4-tritiated D-glucose.

Although several synthetic preparations of this sugar have been reported in the literature, the procedures employed have been laborious[2,3] and, in some cases, the labeling imprecise[4]. Several 5-tritiated hexoses as well as D-galactose-*4-t* were prepared recently by hydroboration of enolic derivatives with tritiated diborane[5]. An enzymic method for the preparation of D-glucose-*4-t* has been reported wherein the products obtained were of low specific activity and indeterminate radiopurity[6]. A review on the synthesis and metabolism of these compounds has been published[7]. The present paper describes a simplified procedure for the synthesis of D-glucose and D-galactose specifically labeled with tritium at C-4. A summary of the reaction sequence is illustrated in Scheme 1.

DISCUSSION

The synthesis described here takes advantage of the reduced reactivity of the axial hydroxyl groups as compared to those in the equatorial position. In its most

stable C-1 chair conformation D-galactose possesses a single axial hydroxyl function at C-4. Therefore, benzoylation of methyl α-D-galactopyranoside (**1**) proceeds faster at positions 2, 3, and 6, respectively, than on C-4, resulting in the formation of the

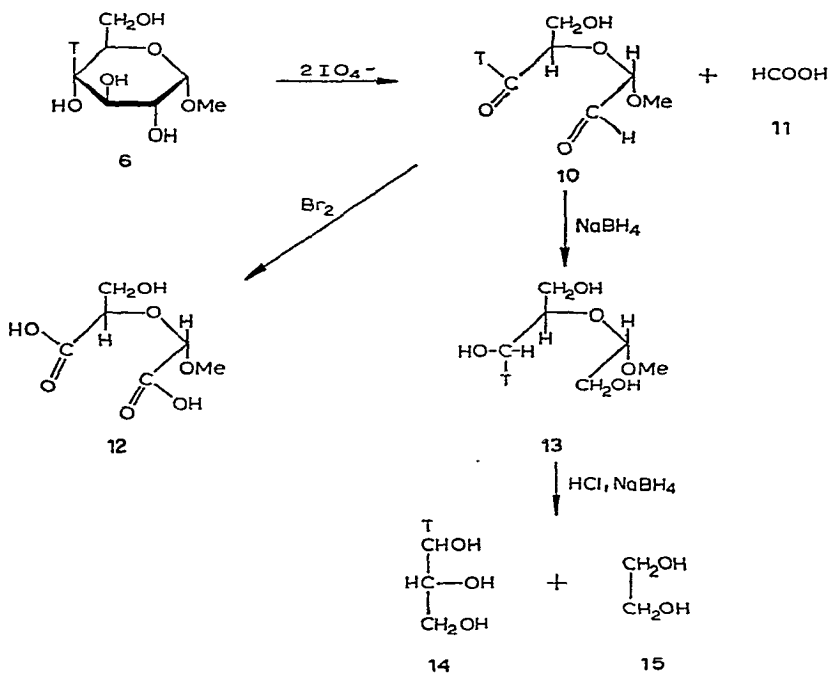


Scheme 1

2,3,6-tri-*O*-benzoyl derivative **2** in good yield[8]. The mild conditions of oxidation employing methyl sulfoxide[9] led to the complete utilization of **2** and resulted in the formation of the expected 4-keto derivative **3** and a second component, presumably methyl 4-*O*-acetyl-2,3,6-tri-*O*-benzoyl-α-D-galactopyranoside. The 4-keto derivative is unstable and readily undergoes molecular rearrangement. Consequently, chromatographic purification and reduction of the compound should be carried out without delay after completion of the oxidation. In order to avoid reductive cleavage of the protecting benzoyl groups, the reduction with sodium borotritiide is carried out for a short time (10 min) and at low temperature (0°). Indeed, even under these conditions several unidentified components were demonstrated by thin-layer chromatography. Consequently, the reaction mixture was subjected to preparative thin-layer chromatography in order to remove the partially debenzoylated products. Only material with mobility identical to that of compound **2** was eluted; no differentiation between the

benzoylated derivatives of glucose **4** and galactose **5** was made at this step. No attempts have been made so far to examine some of the partially debenzoylated compounds for their tritium content. Following debenzoylation of the mixture of **4** and **5** with sodium methoxide to the corresponding methyl glycosides **6** and **7** and subsequent acid hydrolysis, the free sugars D-glucose-*4-t* (**8**) and D-galactose-*4-t* (**9**) were obtained. The total yield of the mixture of the methyl α-D-glycosides of glucose (**6**) and galactose (**7**) varied from 6 to 10%, based on the oxidation of 500 μmoles of **2**.

The radiopurity of the specifically tritiated sugars was determined by periodate degradation of methyl α-D-glucopyranoside-4-*t* (**6**) (Scheme 2) wherein C-3 was



Scheme 2

liberated as formic acid and isolated as ammonium formate (**11**) free from radioactivity. The absence of tritium from C-1, 5 and 6, respectively, was demonstrated by the isolation of the crystalline diglycolate (**12**) which was similarly free from radioactivity. Of the remaining possibilities, C-2 was excluded on the basis of the absence of radioactivity in the isolated ethylene glycol (**15**). Finally, the tritium was shown to be present uniquely in C-4 by the recovery of glycerol (**14**) exhibiting a specific activity identical to that of the original methyl α-D-glucopyranoside (**6**) (Table I).

EXPERIMENTAL

General. — Paper chromatography was performed on Whatman No. 1 paper, descending, with pyridine–ethyl acetate–water (20:72:23). Thin-layer chromatography on silica gel G (Merck) was performed in the following solvents: (a) Ethyl acetate–pentane

TABLE I

ANALYSIS OF ETHYLENE GLYCOL (15) AND GLYCEROL (14); RESULTS OF PERIODATE OXIDATION AND DETERMINATION OF TRITIUM CONTENT

	Compound *Ethylene glycol* (15)	*glycerol* (14)
R_F	0.47	0.29
IO_4^- consumed, μmoles	0.137	0.166
Formaldehyde formed, μmoles	0.244	0.155
Molar ratio		
IO_4^- consumed	1.0	2.0
formaldehyde formed	1.8	1.9
Radioactivity		
μmoles	0.59	0.80
counts/min	37	7.670
counts/min/μmole	63	9.600

(3:1 v/v); (b) benzene–methanol (99:1 v/v), (c) benzene–methanol (95:5 v/v), and (d) benzene–methanol (90:10 v/v). Solvent systems a to d were used with plates spread with a suspension of 25 g of silica gel G in 62 ml of water and activated for 30 min at 110°. The time of development was about 1 hour. Microscope slides (75 × 25 mm) coated with silica gel G were used for analytical screening. Compounds were routinely detected on all thin-layer plates with one of the following methods: (a) examination with u.v. light. (b) spray with 10% sulfuric acid in methanol, followed by heating on a hot plate: brown to black spots are formed. (c) treatment with 2,4-dinitrophenylhydrazine to detect keto derivatives[10]. (d) Spray, with a lead tetraacetate solution to detect glycerol and 1,2-ethanediol. The spray consisted of a 1% solution of lead tetraacetate in benzene, prepared each time immediately before application. The spray should be applied heavily and for an extended period of time: white spots on a dark background are formed. (e) Detection of benzoylated sugar derivatives with water[11].

Melting points were determined on a Kofler hot stage, except for ammonium formate, where the m.p. was determined with an open capillary, since partial decomposition of this material has been observed during determination[12,13].

*Methyl 2,3,6-tri-*O*-benzoyl-α-*D*-galactopyranoside* (2). — The benzoylated methyl galactoside (2) was synthesized from methyl α-D-galactopyranoside (1)[14] by the procedure of Reist *et al.*[8] with a 50% yield, m.p. 132–136°; t.l.c. in solvents a–d showed only a single component.

Oxidation of (2) *to methyl 2,3,6-tri-*O*-benzoyl-α-*D*-xylo-hexopyranosid-4-ulose* (3). — Compound 2 (250 mg, 500 μmoles) was dissolved in dimethylsulfoxide (1.5 ml, 15 mmoles) and acetic anhydride (1.0 ml) in a glass-stoppered test tube and kept for 19 h at room temperature[9]. T.l.c. (solvent b) revealed that all of the starting material (R_F 0.47) was converted into two new components: one component (R_F 0.62) reacted with 2,4-dinitrophenylhydrazine, whereas the second component (R_F 0.70) was made

visible only after heating with sulfuric acid. The reaction mixture was fractionated by column chromatography on a silicic acid (California Biochemical Corporation) column (3 × 19 cm, 1 ml fractions per 10 min) with 99:1 benzene–metanol. Aliquots (5 μl) of each fraction were spotted on a silica gel plate and the compounds present were detected by treatment with sulfuric acid. Positive tests were obtained with fractions 35–38 and 40–43. After combining fractions 35–38 and 40–43, t.l.c. (solvent b) revealed the presence of the expected phenylhydrazine-reactive derivative in the 40–43 group.

Reduction of **3** *to methyl 2,3,6-tri-*O*-benzoyl-α-*D*-glucopyranoside-*4-t (**4**) *and to methyl 2,3,6-tri-*O*-benzoyl-α-*D*-galactopyranoside-*4-t (**5**). — The combined fractions 40–43 were concentrated *in vacuo* at room temperature to dryness and the residue was dissolved in ethanol (5 ml). The stirred solution was cooled with an ice bath, and a solution of sodium borotritiide (1000 μmoles/ml, about 10 mCi, New England Nuclear Corporation) was added (200 μl). After 10 min, the pH of the heterogeneous reaction mixture, which contained some crystalline material, was adjusted to 4.0 with glacial acetic acid. The suspension was evaporated to dryness *in vacuo*, acetone was added (1 ml), and the suspension was again evaporated to dryness. The residue was suspended in methylene chloride (2 ml), and the organic layer was washed 3 times with water (1 ml); the aqueous phases were discarded. The methylene chloride solution was evaporated to dryness and dissolved in acetone (1 ml). Examination by t.l.c. (solvent c) revealed three major components: The first spot (R_F 0.68) was radioactive and had the same R_F as (**2**). The second component (R_F 0.79) also contained tritium, whereas the third component (R_F 0.86) was free from radioactivity.

The reaction mixture was streaked onto five 20 × 20 cm thin-layer plates and subjected to chromatography in solvent c. A reference standard of methyl 2,3,6-tri-*O*-benzoyl-α-D-galactopyranoside (**2**) was included on each plate. The compounds were located by examination in u.v. light, and the area corresponding to **2**, **4**, and **5** was eluted with acetone. The eluate was concentrated to dryness *in vacuo* and stored over silica gel under vacuum in a desiccator overnight.

Debenzoylation of **4** *to methyl* α-D-*glucopyranoside-*4-t (**6**) *and* **5** *to methyl* α-D-*galactopyranoside-*4-t (**7**). — The dry residue containing the mixture of the benzoylated derivatives **4** and **5** was dissolved in dry methanol (1 ml) and a freshly prepared solution of 0.5% sodium methoxide (60 μl) was added. After 30 min at room temperature, an aliquot was examined on t.l.c. (solvent c). All of the product remained at the origin, thereby indicating quantitative debenzoylation.

Identification of methyl α-D-*glucopyranoside-*4-t (**6**) *and methyl* α-D-*galactopyranoside-*4-t (**7**). — Compounds **6** and **7** were examined by descending chromatography on Whatman No. 1 paper in pyridine–ethyl acetate–water (1.0:3.6:1.15). A single radioactive and silver nitrate-positive area was located in each instance which coincided exactly with that of authentic methyl α-D-glucopyranoside and methyl α-D-galactopyranoside, respectively. About two-thirds of the total material corresponded to the methyl glucoside, one-third to the methyl galactoside. The entire mixture containing compounds **6** and **7** was then subjected to paper chromatography

in pyridine for 48 h, and the well-separated methyl glycosides were located by their radioactivity and eluted with water.

An aliquot, which contained 3 μmoles of pure methyl α-D-glucopyranoside-4-*t* (**6**) having a specific activity of 3.18×10^6 counts/min/μmole (about 30% counting efficiency), was diluted with 1008 μmoles of carrier methyl α-D-glucopyranoside and dissolved in water (25 ml). The resulting specific activity was determined to be 9530 c/min/μmole theory: 9436 c/min/μmole and it remained constant on recrystallization of the product from ethanol.

Acid hydrolysis of methyl α-D-glucopyranoside-4-t (**6**) *and methyl α-D-galactopyranoside-4-t* (**7**). — Aliquots of the eluted pure methyl glycosides (**6**) and (**7**) (250 μl) containing about 3–5 μmoles each were evaporated to dryness. The residues were dissolved in 0.5M sulfuric acid (0.5 ml), and the solutions heated for 1 h in a boiling water bath. The samples were chilled, neutralized with 0.15M $Ba(OH)_2$ in presence of phenolphthalein as indicator. The $BaSO_4$ was removed by centrifugation and washed with a small volume of water. The supernatant and the water wash were passed through a 1.0×0.5 cm column of Dowex-50 (H^+).

The eluates were examined by paper chromatography. The hydrolysate of **6** gave D-glucose-*4-t* (**8**), that of (**7**) D-galactose-*4-t* (**9**). In each instance, all the radioactivity coincided with the mobility of the expected monosaccharides D-glucose and D-galactose, respectively.

Proof of the identity of the free sugars was provided by enzymatic assays: D-glucose (**8**) was a substrate for hexokinase and glucose 6-phosphate dehydrogenase[15]. Similarly, D-galactose (**9**) was oxidized with galactose oxidase[16].

Proof of radiopurity of methyl α-D-glucopyranoside-4-t (**6**). — In order to provide experimental evidence for the selective tritiation at C-4, a modification of the degradation procedure described by Bevill *et al.*[17] was elaborated. A schematic representation of this method is presented in figure 2.

The carrier-diluted solution (20 ml) of methyl α-D-glucopyranose-*4-t* (40.4 μmoles/ml; 9530 counts/min/μmole) was mixed with a solution (11.0 ml) containing 415 mg of periodic acid, and the reaction mixture was kept overnight in the dark. The pH of the solution was adjusted to 7.0 by addition of $BaCO_3$ (0.6 g) with stirring. The suspension of precipitated barium salts was filtered off on a Buchner funnel, washed with water, and the combined filtrate and washing concentrated *in vacuo* to dryness. The white residue was extracted with 3 portions of ethanol (50 ml each), the remaining insoluble material being filtered off each time. The combined clear filtrates were evaporated *in vacuo* to give syrupy D'-methoxy-D-hydroxymethyl diglycolic aldehyde (**10**) which was then dissolved in water (20 ml). The aqueous solution was immediately processed according to procedures B and C.

A. *Tritium linked to C-3; isolation of ammonium formate* (**11**). — The white residue of the ethanol extract containing barium formate was dissolved in water (3 ml) and M $(NH_4)_2SO_4$ (about 0.5 ml) was added. The barium sulfate precipitate was removed by centrifugation, and the clear supernatant contained ammonium formate and excess ammonium sulfate. The aqueous solution was concentrated to dryness at

room temperature, and the residue was extracted 5 times with 1-ml portions of hot methanol. The extract was evaporated and the residue was purified by sublimation *in vacuo* at 40°. The sublimation was complete in several min. The freshly sublimed ammonium formate, m.p. (open capillary) 116°, (21.00 mg, 333 μmoles) was dissolved in water (3.33 ml). Aliquots corresponding to 5 and 10 μmoles, respectively, were assayed for radioactivity. No detectable amount of radioactivity could be demonstrated where 0.1% of the original activity would have indicated 100 counts/min above background.

B. *Tritium linked to C-1, 5, and 6; oxidation of* D′*-methoxy-*D*-hydroxymethyl diglycolic aldehyde* (**10**) *to strontium* D′*-methoxy-*D*-hydroxymethyl diglycolate* (**12**). — To the aqueous solution (10 ml, one-half of total) containing the dialdehyde **10**, water (40 ml), $SrCO_3$ (1.0 g), and bromine (0.2 ml) were added in a 500-ml round-bottom flask. The reaction mixture was stirred with a magnetic bar at room temperature for 30 min, and then aerated with nitrogen for another 30 min. After complete disappearance of the brown color of excess bromine, Ag_2CO_3 (0.5 g) was added to the white suspension, the stirring was continued for 10 min, and the reaction mixture was then kept overnight at room temperature. The suspension was filtered on a Buchner funnel, and the precipitate washed with water. The combined filtrate and washing were treated with H_2S for 5 min, and then aerated with nitrogen for 30 min. The black suspension of silver sulfide was filtered through a sintered-glass funnel, and the clear, colorless solution was concentrated *in vacuo* to 1-2 ml. The solution was warmed on a water bath, and warm methanol (6.0 ml) was added with stirring. Upon cooling, strontium D′-methoxy-D-hydroxymethyl diglycolate (**12**) formed a white crystalline precipitate. It was collected on a micro funnel and washed with a small volume of cold absolute methanol. The crystalline material was redissolved in hot CO_2-free water (2.0 ml), the solution was filtered, and the filter was rinsed with water (1.0 ml). To the warm solution, warm methanol (5.0 ml) was carefully added, mixed well, and the solution was kept at room temperature. The resulting fine, white needles were filtered off, washed with cold methanol and ether, and finally dried in a vacuum desiccator overnight; $[\alpha]_D^{20}$ 51.8°[18,19]. A solution of strontium D′-methoxy-D-hydroxymethyl diglycolate (40 μmoles/ml) in CO_2-free water was prepared and aliquots corresponding to 5 and 10 μmoles, respectively, were assayed and found to have no radioactivity, *i.e.*, less than 0.1% of the original radioactivity was present.

C. *Reduction of* D′*-methoxy-*D*-hydroxymethyl diglycolic aldehyde* (**10**) *with sodium borohydride; hydrolysis and reduction to ethylene glycol* (**15**) *and glycerol* (**14**). — Sodium borohydride (280 mg) was dissolved in water (10 ml). To this solution the other half of the aqueous solution containing D′-methoxy-D-hydroxymethyl diglycolic aldehyde (**10**) was added slowly with stirring. The reaction mixture was kept overnight at room temperature to give D′-methoxy-D-hydroxymethyl diethyleneglycol (**13**). To this solution conc. HCl (2.2 ml) was added to bring the concentration of hydrochloric acid to about M. The reaction mixture was kept for 1 h at room temperature to hydrolyze D′-methoxy-D-hydroxymethyl diethylene glycol (**13**) into glycolaldehyde (**15**) (C-1 and 2) and glycerol (**14**) (C-4, 5, and 6). The pH of the solution was adjusted to

7.0 by addition of Na_2CO_3 (1 mmole). To this solution sodium borohydride (140 mg), dissolved in water (5 ml), was added slowly. After careful mixing the solution was kept for 3 h at room temperature. The pH of the solution was adjusted to 7.0 with M HCl, and the solution was evaporated to dryness *in vacuo*. The large residue of white salts was extracted 3 times with 50-ml portions of ethanol. The filtered, combined extracts were again evaporated to dryness, and the residue was extracted 3 times with 1-ml portions. The ethanol extract was cooled with an ice-bath, the precipitate removed by centrifugation, and the supernatant was passed through a small column (1 × 0.5 cm) of a mixed resin (Amberlite MB-3). The eluate containing ethylene glycol (**15**) (C-1 and 2) and glycerol (**14**) (C-4, 5, and 6) was concentrated to a volume of 500 µl.

D. *Separation and isolation of ethylene glycol* (**15**) *and glycerol* (**14**). — The separation of ethylene glycol from glycerol was achieved by t.l.c. on silica gel G–boric acid plates in butyl alcohol–water (90:10).

The plates were prepared in the following way: silica gel G (30 g) was suspended in 0.033M boric acid (65 ml) and activated for 30 min at 110°. The time of development was about 2 h. A qualitative test was performed by streaking an aliquot of the mixture of ethylene glycol and glycerol onto a 5 × 20 cm thin-layer plate together with a sample of reference standards. After development in the solvent, the plate was first scanned for radioactivity, and then sprayed with the lead tetraacetate reagent. All of the radioactivity was located in a spot with the mobility of glycerol, and none of the radioactivity could be found in the area corresponding to the mobility of ethylene glycol. The rest of the mixture containing ethylene glycol (**15**) and glycerol (**14**) was streaked onto 4 thin-layer plates, developed, and located by protecting the middle part of the chromatograms with a glass plate and spraying guide strips on each side. The areas corresponding to ethylene glycol (R_F 0.47) and glycerol (R_F 0.29) were eluted with three 1-ml portions of water. In order to remove the boric acid, the eluate was passed through a small column of Dowex-50 (H^+), which was washed with water. The eluate and washing were evaporated to dryness, and the residue was repeatedly dissolved in

TABLE II

LOCALIZATION OF TRITIUM ACTIVITY IN METHYL α-D-GLUCOPYRANOSE-*4-t* (**6**)

Tritium activity associated with carbon atom	*Isolated as*	*Activity*	
		counts/min/µmole	%
Before degradation	Methyl α-D-glucopyranoside (**6**)	9,530	100
3	Ammonium formate (**11**)	0	0
1, 5, 6	Strontium D′-methoxy-D-hydroxymethyl diglycolate (**12**)	0	0
1, 2	Ethylene glycol (**15**)	63	0.7
4, 5, 6	Glycerol (**14**)	9,600	101

methanol (1 ml), and the solution subsequently evaporated to dryness. The salt-free residues of the eluates of the separated components were assayed by periodic acid uptake[20] and by determination of formaldehyde[21].

E. *Determination of tritium associated with ethylene glycol* (**15**), *(C-1 and 2) and glycerol* (**14**), *(C-4, 5, and 6).*—The results of quantitative determinations of ethylene glycol and glycerol obtained by t.l.c. as well as the tritium activity associated with them are reported in Table I.

The results of the degradation of methyl α-D-glucopyranose *4-t* and the tritium activity found at each carbon atom in the hexose moiety are reported in Table II.

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THE PECTIC SUBSTANCES OF *Zosteraceae*

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ABSTRACT

The polysaccharide composition of several *Zosteraceae* plants has been studied and found to be nearly identical. Zosterine isolated from various *Zosteraceae* species appeared to be a homogeneous, pectic polysaccharide containing D-galacturonic acid, D-galactose, D-xylose, L-arabinose, D-apiose, and a mono-*O*-methyl-D-xylose as components of its carbohydrate chain.

INTRODUCTION

Seaweeds of the *Zosteraceae* family are widely distributed in Nature. The peculiar systematism and oecology of these plants suggests that there may be interesting features of chemical composition. Indeed, a polysaccharide containing residues of 6-deoxyhexoses and hexuronic (mainly galacturonic) acids was isolated in 1940 from White Sea *Zostera marina* and named zosterine[1]. The jelly-forming properties of zosterine have been studied and suggested for industrial utilization. Later[2], D-apiose was found in the seaweed *Posidonia australis* which is closely related to *Z. marina*. The resistance to natural decomposition, so characteristic of *Zosteraceae* plants, was thought to be due to the presence of derivatives of D-apiose[2]. In 1964, this monosaccharide, D-xylose, and 2-*O*-methyl-D-xylose were isolated[3] from *Z. marina*. In addition, a polysaccharide containing residues of D-apiose, D-xylose, and an unidentified uronic acid was also found[3] in *P. australis*. Analysis[4] of the uronic acids in a crude *Zosteraceae* polysaccharide by gas–liquid chromatography revealed galacturonic, mannuronic, and glucuronic acids.

At present, there is no detailed information about *Zosteraceae* polysaccharides, and it is therefore of interest to investigate their chemical nature. This paper is concerned with the isolation and characterization of an unusual pectic polysaccharide (zosterine) from *Zosteraceae* plants.

EXPERIMENTAL AND RESULTS

Partition chromatography was performed on Whatman 3 MM or Leningrad factory "Goznak" paper with the following solvent systems (v/v): *A* butyl alcohol–

acetic acid–water (4:1:5, upper layer); *B* butyl alcohol–benzene–pyridine–water (5:1:3:3; upper layer); *C* ethyl acetate–acetic acid–pyridine–water (5:1:5:3). Monosaccharides were detected with aniline hydrogen phthalate. Thin-layer chromatography (t.l.c.) was performed as described elsewhere[5]. Pretreated[6] DEAE-cellulose (phosphate form) was used for ion-exchange chromatography. Gel filtration was carried out on Biogel columns[7]. Biogels P-20, P-30, and P-60 ("BIO-RAD Laboratories", Richmond, California) were employed. Void volumes (V_o) were determined by using dextran sulphate (Mol. wt. 500,000). Free-boundary electrophoresis was carried out in a Tiselius apparatus "Elektrophöreseren", Model 35 (Carl Zeiss, Jena). All solutions were concentrated *in vacuo* at 30–40°. Uronic acid was determined by a modified carbazole reaction[8]. Ash was determined by heating polysaccharide samples to constant weight at 600°.

Origin of specimens. — *Zosteraceae* plants were collected from the Japan Sea in August 1966.

Extraction and purification of zosterine. — Air-dried, powdered, plant material (1 kg) was extracted with methanol, and then treated with 1% aqueous formaldehyde for 12–16 h at 20°. The residual material was treated with dilute hydrochloric acid (pH 4–5) for 3–4 h at 50°, and then separated and washed with cold water. Zosterine was extracted three times with 1% aqueous ammonium oxalate for 3–5 h at 70° and precipitated by adding the extract to 4 volumes of methanol. A white precipitate was obtained (yield, 100–120 g; 10–12% of the air-dried material). Crude zosterine was reprecipitated from 80% ethanol, and redissolved in a small volume of water, and the solution was dialyzed for 3 days against several changes of water and freeze-dried. The colorless product, had $[\alpha]_D^{20}$ +230° (*c* 0.5, water), molecular weight of *ca.* 40,000–45,000. Analytical data for zosterine samples are listed in Table I.

TABLE I

ANALYTICAL DATA FOR PURIFIED ZOSTERINE FROM *Zosteraceae* SPECIES

	Z. marina	Z. pacifica	Phyllospadix
Carbon, %	38.8	39.1	39.45
Hydrogen, %	6.21	5.90	5.85
Nitrogen, %	2.3	2.45	2.68
Methoxyl, %	0.97	0.80	0.78
Ash, %	1.12	1.57	1.87
"Uronic anhydride", %	38	36	40
Amino acids[a]	trace	trace	trace

[a]Determined by paper chromatography.

Acid hydrolysis of zosterine. — Zosterine (2.0 g) was hydrolysed with 2N sulphuric acid (200 ml) for 16 h at 75°. After neutralization (barium carbonate), de-ionization [Amberlite IR-120 (H^+)], and concentration of the hydrolysate, the syrupy residue was examined by paper chromatography (solvents *A*-*C*) and t.l.c. Galacturonic acid, galactose, arabinose, xylose, apiose, and *O*-methyl-xylose were

detected. The syrup (1.4 g) was applied to a column (3.5×46 cm) of cellulose and eluted with solvent *A* or *C*. Fractions were collected, and tested by paper and thin-layer chromatography, and appropriate fractions were combined and concentrated. Crude monosaccharides were purified by preparative paper-chromatography (solvents *A*, *B*). The following monosaccharides were obtained by elution from the paper with 50% ethanol: D-galacturonic acid (500 mg), $[\alpha]_D^{20}$ +58° (*c* 2.0, water), which was oxidized with bromine to give galactaric acid, m.p. and mixed m.p. 219° (the i.r. spectrum was identical with that of an authentic sample); D-galactose (100 mg), $[\alpha]_D^{20}$ +82° (*c* 3.0, water) (phenylosazone, m.p. and mixed m.p. 182–184°); L-arabinose (50 mg) (phenylosazone, m.p. and mixed m.p. 166–168°); D-xylose (200 mg), m.p. 146°, $[\alpha]_D^{20}$ +19° (*c* 3.0, water) (phenylosazone, m.p. and mixed m.p. 153–154°).

All of the above sugars were also identified by paper and thin-layer chromatography on impregnated silica gel[5]. A monosaccharide (20 mg) having the highest R_F value was also isolated, and had m.p. 110° (uncorrected). Permethylation of the sugar with methyl sulphate in the presence of 30% alkali gave a compound that was chromatographically identical with 2,3,4-tri-*O*-methyl-D-xylose. The R_F value suggests that the substance may be a mono-*O*-methylxylose, but rigorous identification was impossible because of the limited amount of material.

Preparation of D-*apiose*[2]. — D-Apiose (mainly) and D-xylose, contaminated with traces of other sugars, were obtained as a result of hydrolysis of zosterine or the plant itself with 0.5–2% sulphuric acid for 4 h at 70°. Additional purification by preparative chromatography (solvents *A* and *C*) gave crystalline D-apiose (250 mg), m.p. 167° (from ethanol), $[\alpha]_D^{20}$ +5° (*c* 3.0, water); phenylosazone, m.p. 158° (*cf.* L-rhamnose phenylosazone, m.p. 182°). D-Apiose and L-rhamnose have the same R_F value on paper chromatograms[2]. Periodate oxidation afforded no acetaldehyde, and the formation of formaldehyde (more than 1.5 moles per mole) suggested the presence of a branched chain. On thin layers of impregnated silica gel, the product possessed a higher R_F value than that of L-rhamnose. T.l.c. of the products of partial methylation of the sugar and L-rhamnose also revealed that they were not identical.

Partial hydrolysis of zosterine with acid. — Zosterine (50 mg) was hydrolyzed with N sulphuric acid (5 ml) for 5 h at 70°. The precipitate formed was separated, washed with water, and completely hydrolysed as above. Paper and thin-layer chromatography revealed the presence of galacturonic acid as the only product.

Free-boundary electrophoresis of zosterine. — A solution of zosterine (0.2%) in phosphate buffer (pH 7.0) was subjected to electrophoresis (12.4 volts, 7.1 mamp). A single peak of the acidic polysaccharide was observed (Fig. 1).

Chromatography of zosterine on DEAE-cellulose. — Zosterine (0.1 g) in water (5 ml) was applied to a DEAE-cellulose column (3.5×41 cm) and eluted with a continuous gradient involving water → 0.5M NaH_2PO_4 and then 0.5M NaH_2PO_4 → 0.3M NaOH. Fractions (20 ml) were collected. The elution curve is shown in Figure 2. Fractions corresponding to the peak of the elution curve were combined and hydrolysed. Paper chromatography of the hydrolysate revealed the presence of all of the above-mentioned monosaccharides.

Gel-filtration of zosterine on Biogels P-20, P-30, and P-60. — Zosterine (10 mg) in 0.05M NaH_2PO_4 (2 ml) was applied to a column (2-cm diameter) with 2.5 g of Biogel P-20, P-30, or P-60, and eluted with water or 0.05M NaH_2PO_4; 2.0-ml fractions were collected. Elution curves are shown in Figure 3. Zosterine (10 ml) was treated with 5% alkali for 1 h at 70°. After neutralization with hydrochloric acid, the solution was subjected to gel-filtration on Biogel P-60. The elution curve (Fig. 3*d*) revealed considerable degradation of zosterine.

DISCUSSION

The three principal Far-Eastern *Zosteraceae* plants investigated were *Z. marina*, *Z. pacifica*, and *Phyllospadix*. Preliminary, chromatographic analysis of

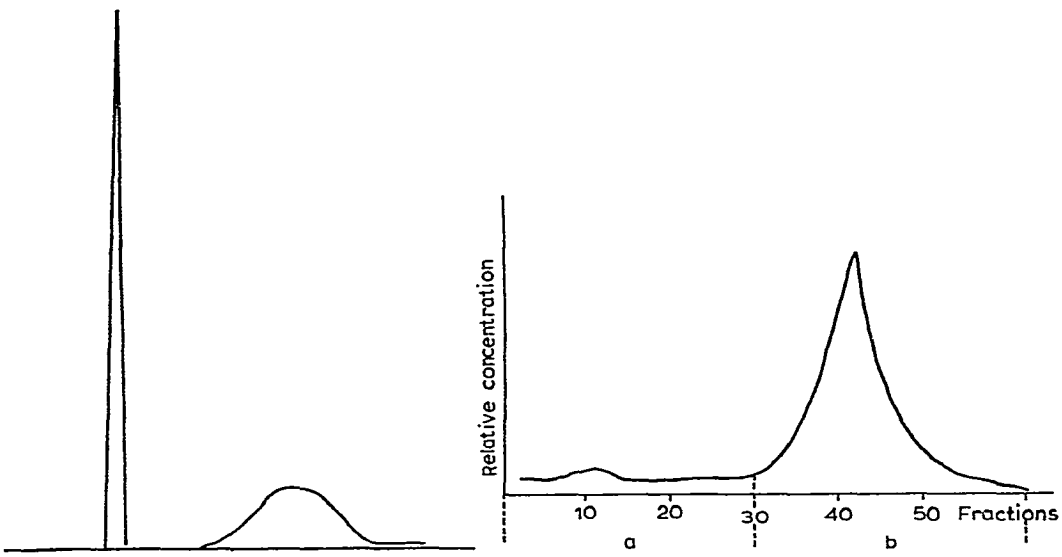

Fig. 1. Free-boundary electrophoresis of zosterine.

Fig. 2. Chromatography of zosterine on a DEAE-cellulose column. Elution: *a*) $H_2O \rightarrow$ 0.5M NaH_2PO_4; *b*) 0.5M $NaH_2PO_4 \rightarrow$ 0.1M NaOH.

the acid hydrolysates of these plants demonstrated identical monosaccharide composition. Zosterine was isolated from each plant. The inability of the polysaccharide fraction to react with iodine indicated the absence of contaminating starch. Analytical data for the zosterine samples are presented in Table I. All attempts to further purify zosterine, using Cetavlon, molecular-sieve chromatography, ion-exchange resins, and fractionation of calcium salts, failed. Considerable losses of zosterine were observed, but the quality of the polysaccharide was practically unchanged. Complete hydrolysis of zosterine with acid afforded D-galacturonic acid, D-galactose, D-xylose, D-apiose, and a mono-*O*-methylxylose. Partial hydrolysis with acid afforded a polygalacturonan, thus suggesting that zosterine is a pectic polysaccharide.

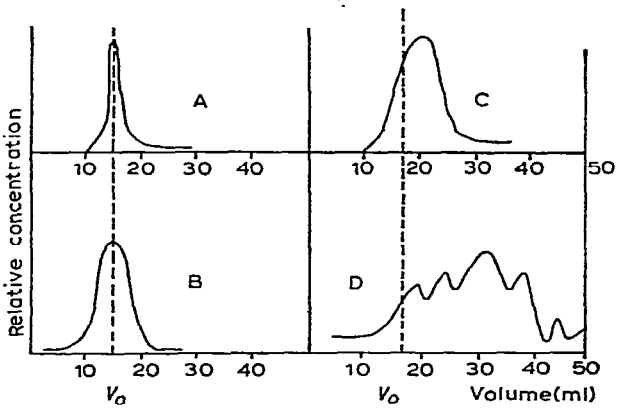

Fig. 3. Molecular-sieve chromatography of zosterine on Biogels. A, Biogel P-20, $V_0 = V_e = 15$ ml; B, Biogel P-30, $V_0 = V_e = 15$ ml; C, Biogel P-60, $V_0 = 17$ ml, $V_e = 22$ ml; D, Alkali-treated zosterine on Biogel P-60, $V_0 = 17$ ml.

Apparently, pectic substances have not previously been isolated from marine organisms[10]. The presence of D-apiose in *Zosteraceae* pectin is a unique feature, and the low methoxyl content, which is also characteristic of pectic polysaccharides of the fresh-water alga *Nitella translucens*[10], is noteworthy.

The homogeneity of zosterine has been proved by a number of methods. In free-boundary electrophoresis, a single peak of acidic polysaccharide was obtained (Fig. 1). The absence of neutral polysaccharides was demonstrated by chromatography on a DEAE-cellulose column (Fig. 2). Molecular-sieve chromatography on Biogels also confirmed the homogeneity of zosterine (Fig. 3) and indicated that the molecular weight is *ca.* 50,000. Like other pectic polysaccharides, zosterine is alkali-sensitive[11].

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ELECTRON-SPIN RESONANCE STUDY OF SOME IRRADIATED D-GLUCOPYRANOSIDES

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ABSTRACT

The e.s.r. spectra generated in methyl α-D-glucopyranoside (**1**) and some selected derivatives thereof by high-energy radiation have been examined to determine the nature of the radiation damage. Evaluations of the e.s.r. spectra of irradiated methyl tetra-*O*-acetyl-α-D-glucopyranoside (**2**), methyl tetra-*O*-[(*p*-ethoxycarbonyl)benzoyl]-α-D-glucopyranoside (**3**), methyl tetra-*O*-(*p*-methoxybenzoyl)-α-D-glucopyranoside (**4**), methyl tetra-*O*-(*p*-nitrobenzoyl)-α-D-glucopyranoside (**5**), methyl tetra-*O*-(*o*-chlorobenzoyl)-α-D-glucopyranoside (**6**), methyl tetra-*O*-*p*-tolylsulfonyl-β-D-glucopyranoside (**7**), methyl tetra-*O*-nicotinoyl-α-D-glucopyranoside (**8**), and methyl tetra-*O*-(phenylcarbamoyl)-α-D-glucopyranoside (**9**) contained mainly one type of stable free-radical, together with some secondary radical in low concentration. The e.s.r. spectrum of compound **1** consisted of one doublet and one singlet; this observation was explained by assuming that the free electron position is at C-1, so that the cleavage of the C-1–*O* glycosidic bond would give rise to the doublet, and the dehydrogenation of the C-1–H bond would give rise to the singlet. For most of the substituted D-glucopyranosides studied, irradiation gave singlet spectra which were explained by assuming the radical site to be C-1 (by the dehydrogenation of the C-1–H bond). The aromatic groups present in the substituted D-glucopyranosides were known to protect the glycosides from radiation damage[1]. In the aromatically substituted D-glucopyranosides, no reducing power was measurable, even when the compounds were irradiated to dosages as high as 5.2×10^{21} e*V*/g; this was additional evidence that no cleavage of the C-1–*O* glycosidic bond occurs during irradiation of these compounds. The absence of hyperfine splitting of the free electron at C-1 with H-2 was explained by a change in the conformation of the molecule after H-1 had been cleaved. Displacement of the hydrogen atoms would then occur to remove any deformation of the ring caused thereby; this would remove H-2 from the vicinity of the unpaired electron at C-1. No thermally induced free-radicals could be detected in these compounds, unless they were heated to charring.

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INTRODUCTION

During an investigation of the effects of high-energy radiation on certain cellulose derivatives, a series of aromatically substituted carbohydrates had been studied as model compounds[1]. The extent of radiation damage of these substituted carbohydrates was estimated by determining the extent of hydrolytic cleavage of the glycosidic bond as measured by increases in reducing power and by changes in the i.r. and n.m.r. spectra. All of the aromatically substituted carbohydrates examined were found to be highly resistant to radiation damage[1]. The nature of the radiation-induced, stable free-radicals in sugars has been determined by use of electron-spin resonance (e.s.r.) spectroscopy[2-8].

We have now applied these techniques to elucidation of the nature of the radiation damage of aromatically substituted D-glucopyranosides, and the nature of the radiation-induced, stable free-radicals in these derivatives is described.

RESULTS

For all of the irradiated D-glucopyranosides, the g-values, calculated for the center of the respective e.s.r. spectrum and recorded in Table I, were almost the same as the free-electron value. The nature of the e.s.r. spectra of samples irradiated and

TABLE I

ELECTRON-SPIN RESONANCE SPECTRA OF IRRADIATED METHYL D-GLUCOPYRANOSIDES[a]

Methyl D-*glucopyranoside*	*No. of lines* (in vacuo)[b]	*Line width, gauss*[c]	*g-Value*[d]
Tetra-*O*-(phenylcarbamoyl-)α- (9)	1 (s)	12.5	2.0047
Tetra-*O*-*p*-tolylsulfonyl-β- (7)	1 (w)	14.7	2.0075
Tetra-*O*-(*p*-methoxybenzoyl)-α- (4)	3 (w)	15.3 (m.l.)	2.0053
Tetra-*O*-(*p*-nitrobenzoyl)-α- (5)	5 (s)	[e]	2.0067
Tetra-*O*-[(*p*-ethoxycarbonyl)benzoyl]-α- (3)	5 (w)	16.0 (m.l.)	2.0049
Tetra-*O*-(*o*-chlorobenzoyl)-α- (6)	2 (w)	11.6 (m.l.)	2.0059
Tetra-*O*-nicotinoyl-α- (8)	1 (s)	12.3	2.0036
Tetra-*O*-acetyl-α- (2)	2 (w)	13.4 (m.l.)	2.0088
α- (1)	3 (vs)	[e]	2.0047

[a]Irradiated in the solid state by ^{60}Co γ-radiation to a dosage of 2.6×10^{20} e*V*/g, except for compound 1, which was irradiated to a dosage of 5.2×10^{19} e*V*/g. [b]Key: s, strong; v, very; w, weak. [c]m.l., main line. [d]Calculated for center of spectrum. [e]Lines overlapped.

recorded in air did not differ from those irradiated and recorded *in vacuo*. If the irradiated D-glucopyranosides were stored *in vacuo*, the e.s.r. spectra of the materials were stable for several weeks after irradiation. In the presence of air, however, the

concentration of free radicals in the irradiated D-glucosides decreased. The rate of decrease in the concentration of free radicals depended on the nature of the compound. When the spectra were recorded at a low temperature (*e.g.*, −100°), the peak-to-peak distance of the e.s.r. signal was less than that at 25°; however, the nature of the e.s.r. spectra was not changed. The e.s.r. spectra of the D-glucopyranosides, irradiated in the solid state, are shown in Fig. 1.

The e.s.r. spectrum of irradiated methyl α-D-glucopyranoside (**1**) was very strong. The spectrum contained a clearly resolved doublet having a separation of about 12 gauss, and a singlet, suggesting the formation of more than one radical. This spectrum was very similar to that of irradiated sucrose, reported by Ueda[8].

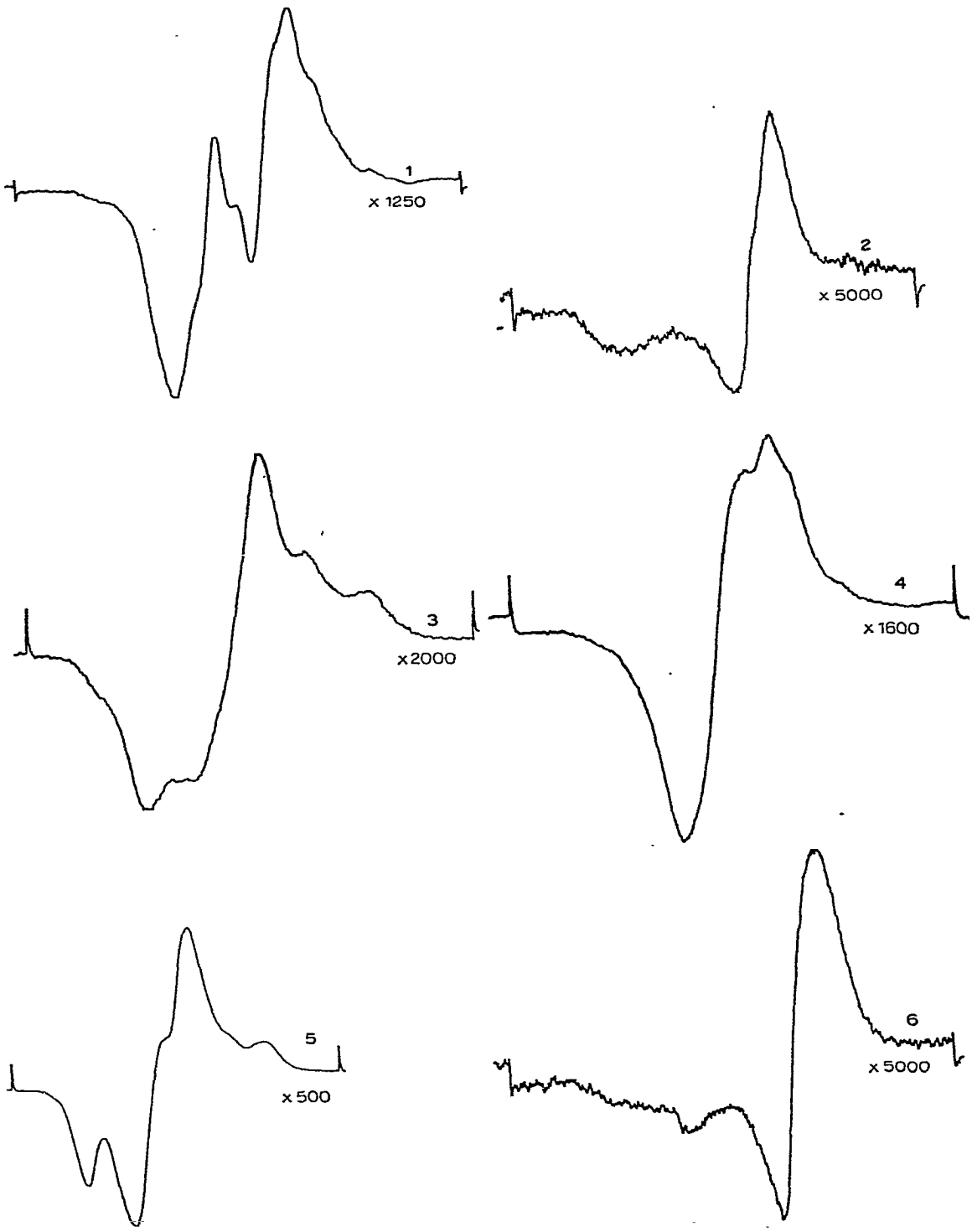
Irradiated methyl tetra-*O*-acetyl-α-D-glucopyranoside (**2**) gave a two-line spectrum having a line separation of 25 gauss. The weak line did not appear to be a part of a hyperfine structure associated with the main resonance-line, and probably indicated the presence of more than one radical. Irradiated methyl tetra-*O*-[(*p*-ethoxycarbonyl)benzoyl]-α-D-glucopyranoside (**3**) gave a spectrum having a strong line, together with several weak lines that indicated the presence of more than one radical.

Irradiated methyl tetra-*O*-(*p*-methoxybenzoyl)-α-D-glucopyranoside (**4**) gave one main line, together with two weak lines that, also, could be due to some other secondary radicals. No signal could be detected for compounds **3** and **4** irradiated to dosages of less than 2.6×10^{20} e*V*/g. When irradiated in air to a dosage of 3.6×10^{20} e*V*/g, compound **4** gave only one line, having a width of 11.87 gauss; under the same conditions, compound **3** gave no signal.

Except for methyl tetra-*O*-(*p*-nitrobenzoyl)-α-D-glucopyranoside (**5**), all of the glycosides examined gave, on irradiation, mainly a singlet spectrum. For methyl tetra-*O*-(*o*-chlorobenzoyl)-α-D-glucopyranoside (**6**), which initially gave a two-line spectrum having a separation of about 19 gauss, the spectrum changed to a sharp singlet on storage in air. The same type of change was observed in the case of methyl tetra-*O*-*p*-tolylsulfonyl-β-D-glucopyranoside (**7**). At low dosage (*e.g.*, 5.2×10^{19} e*V*/g), compound **7** gave a two-line spectrum in which the lines were of equal intensity, with hyperfine splitting of about 19 gauss. With an increase of dosage to 1.6×10^{20} e*V*/g, the symmetrical, two-line spectrum became unsymmetrical, and finally changed to a singlet at a dosage of 2.6×10^{20} e*V*/g.

No changes in the shape of the e.s.r. spectra for the irradiated carbohydrates were observed within the dosage range of 5.2×10^{19} to 5.2×10^{21} e*V*/g. The shape of the spectra also remained unchanged when the irradiated carbohydrates were heated to temperatures just below their melting points; during this heating, only a slow decay of the radicals was observed. The radicals disappeared completely when the carbohydrates were melted. If the compounds were heated above their melting points, a single-line spectrum having a line-width of 7–8 gauss was observed, as reported for charred cellulose and cellobiose[10].

On being heated, the tetrakis(*p*-nitrobenzoate) **5**, m.p. 181–2°, behaved differently from the other esters. When compound **5** was heated to 225°, a four-line spectrum developed. It had approximately equal line-spacing (9.0 gauss), with a main resonance-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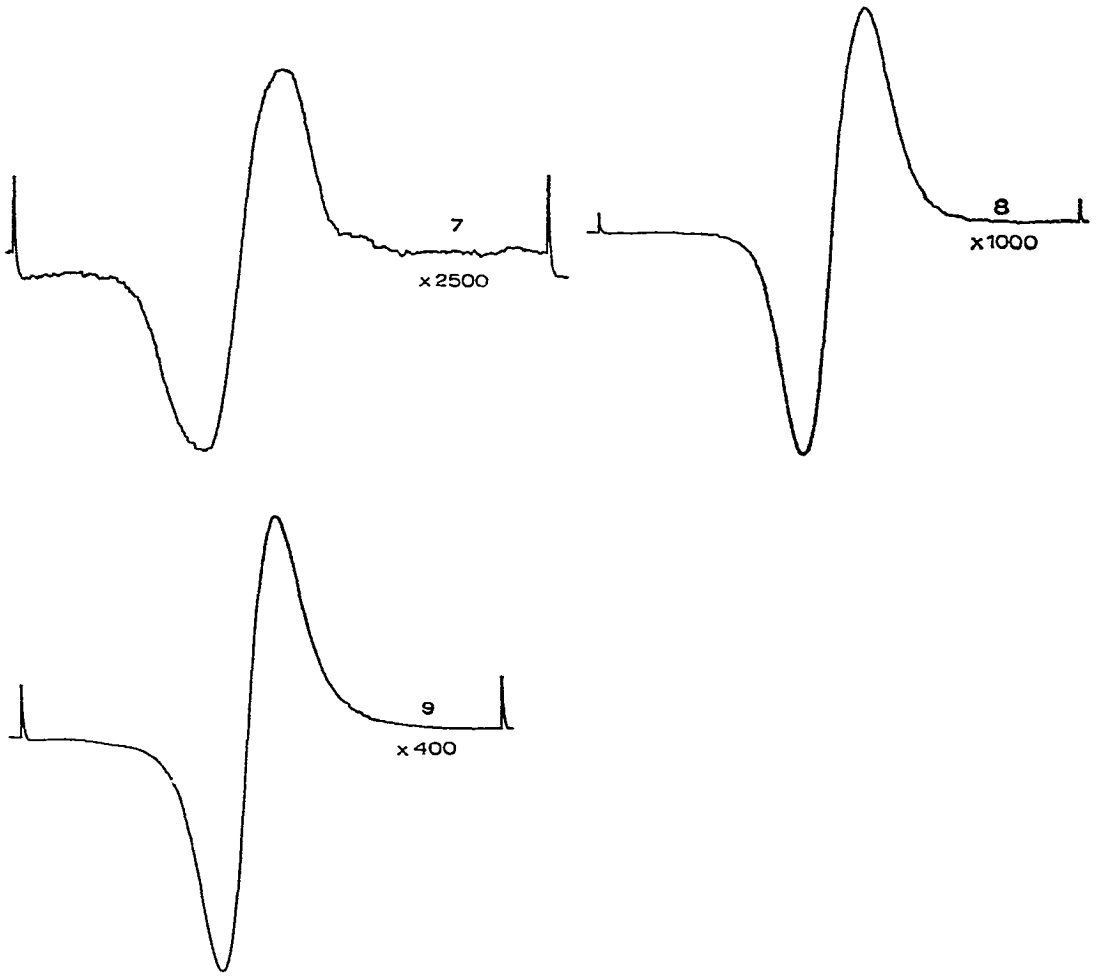


Fig. 1 Electron-spin resonance spectra of irradiated D-glucopyranosides. The number gives the relative amplification of the signal; the magnetic field increases from left to right; sweep, 100 gauss for 5 min.

line of width about 6–7 gauss, as shown in Fig. 2. On heating **5** to 250°, its e.s.r. spectrum changed to a sharp singlet having a line-width of about 7 gauss, probably due to the formation of charred material.

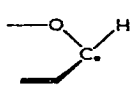
DISCUSSION

High-energy irradiation both of substituted and unsubstituted methyl α-D-glucopyranosides gave stable free-radicals. There have been several suggestions as to the structure of radicals in irradiated carbohydrates. Thus, it has been postulated that the free-radical site is at either C-1 or C-5, because of the strong, delocalization

effect of the oxygen atom present in the pyranoid ring[7]. The spectrum of methyl α-D-glucopyranoside (1) is a combination of a doublet and a singlet, whereas the formation of a radical at C-5 should give rise to a triplet. Hence, the radicals here are

Fig. 2. Electron-spin resonance spectrum of methyl tetra-*O*-(*p*-nitrobenzoyl)-α-D-glucopyranoside (5) heated to 225°. The magnetic field increases from left to right; sweep, 100 gauss for 5 min.

probably formed both through (*a*) the rupture of the C-1–O glycosidic bond (I) and the dehydrogenation of the C-1–H bond (II). This would give rise to two different radicals, namely, I and II, and account for the spectrum observed. For all of the other substituted D-glucopyranosides examined, the formation of a radiation-induced



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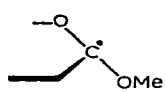

II

stable free-radical could be assigned to a sharp-singlet spectrum, accompanied by some other free radical whose concentration was very low compared with that of the radical recorded by the main resonance-line.

The most likely position for the damage is at C-1, by the scission of the C-1–H bond, because it is the only carbon atom in these compounds that is bonded to two oxygen atoms. A doublet spectrum might be expected from such a radical by weak interaction with H-2. Now, it has been shown by X-ray studies that the dispositions of the hydroxyl groups in α- and β-(D or L)-glucopyranose are, respectively, 1*a*2*e*3*e*4*e* and 1*e*2*e*3*e*4*e*, and that the molecule is in the less-strained chair conformation[11]. The pyranoid ring is not so stable to radiation damage as the cyclohexane ring. The instability of the pyranoid ring structure is not unusual in substituted D-glucose or substituted D-glucopyranosides[9], because of the interaction of all of the bulkier groups, which are equatorially disposed. The formation of a singlet spectrum from the radical at C-1, as we suggest, can be explained only by a change in

the conformation of the molecule such that the interaction of the unpaired electron with H-2 would be diminished; this would decrease the hyperfine splitting. The transformation of the doublet spectrum into a singlet spectrum in the case of the tetra-*p*-toluenesulfonate (**7**) and the tetrakis-*o*-chlorobenzoate (**6**) can be explained on the above grounds.

The change in the dispositions of the hydrogen atoms might occur in order to compensate for the ring deformation occurring after the scission of the C-1–H bond. The doublet spectrum, observed only for compounds **6** and **7**, may be explained on the assumption of a time lag for the change in the conformation of the molecule. Such observations have been made by Ueda[12] for D-fructose and L-sorbose. Other possibilities, such as migration of the radical site or cleavage of the pyranoid ring, are excluded, because none of them would yield the simple, singlet spectrum observed.

The tetrakis-*p*-nitrobenzoate (**5**) behaved rather differently. The spectrum was identical with those of γ-irradiated *p*-nitrobenzoic acid and methyl *p*-nitrobenzoate. The origin of the complex spectrum might be the ejection of hydrogen from the carbon atom, with subsequent delocalization of the free spin and coupling with the aromatic protons and the nitrogen atom. It should be mentioned that the spectra of other model esters (*e.g.*, methyl *p*-methoxybenzoate, dimethyl terephthalate, methyl *p*-toluenesulfonate, and ethyl nicotinate) and of *o*-chlorobenzoic acid were different from those of the tetrakis-*p*-methoxybenzoate (**4**), the tetrakis (*p*-ethoxycarbonyl)-benzoate (**3**), the tetrakis-*o*-chlorobenzoate (**6**), the tetrakis-*p*-toluenesulfonate (**7**), and methyl tetra-*O*-nicotinoyl-α-D-glucopyranoside (**8**), respectively, when these were irradiated under similar conditions. The four-line spectrum of the tetrakis-*p*-nitrobenzoate (**5**) at 225° could be due to the breakdown of the pyranoid ring.

It may be concluded that the e.s.r. spectra recorded on irradiation of substituted D-glucopyranosides differ from those of irradiated D-glucopyranosides. For cellulose, the e.s.r. spectra obtained on irradiation both of cellulose and substituted cellulose (*e. g.*, *O*-benzoylcellulose) are similar, except that the relative concentration of free-radicals is greatly lowered for the benzoylated cellulose[13]. The similarity suggests that, although, on exposure to radiation, benzoyl groups protect cellulose from degradation to a considerable extent, the localization of the nondissipated, radiation-induced, electron-spin resonance in the cellulose molecule is not substantially altered.

EXPERIMENTAL

The preparation and properties of the aromatically substituted D-glucopyranosides have already been described[1]. The substituted D-glucopyranosides were chemically pure and in the form of crystalline powders when irradiated. Irradiations were conducted in the SRRL ^{60}Co radiation source[14], which had a dose rate, determined by ferrous–ferric dosimetry[15], of 7.3×10^{19} e*V*/g/h. The samples were irradiated *in vacuo* or in air, as indicated, to a total dosage of 2.6×10^{20} e*V*/g. For irradiation *in vacuo*, the samples, in Tygon tubing connected to a quartz tube, were dried *in vacuo* at about 25°. After irradiation, the quartz tubes were heat-treated

to remove thermoluminescent, damage centers (which give rise to a characteristic absorption in e.s.r. spectra). The samples were then transferred from the Tygon tubes to the quartz tubes, and the spectra were recorded.

The e.s.r. spectra were determined in a Varian 4502-15 EPR spectrometer system*. The system was equipped with a variable temperature accessory permitting operation from about -185 to $+300°$. Spectra were determined at about 25°, and were recorded in the form of the first derivative of the absorption line, unless otherwise noted.

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*Trade names are given as part of the exact experimental conditions, and not as an endorsement of the products over those of other manufacturers.

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LINKAGE ANALYSIS OF OLIGOSACCHARIDES BY THIN-LAYER CHROMATOGRAPHY OF THEIR ALKALINE-DEGRADATION PRODUCTS

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ABSTRACT

Based on the formation of saccharinic acids, a method for linkage analysis of oligosaccharides has been devised, which utilises thin-layer chromatography on cellulose and silica gel, and specific sprays for the detection and identification of the various types of saccharinic acid.

INTRODUCTION

Specific, sensitive methods[1] of assaying isosaccharinic (2-*C*-hydroxymethyl-3-deoxypentonic) and metasaccharinic (3-deoxyhexonic) acids have been used in the linkage analysis of oligosaccharides containing hexoses and hexuloses. In such studies, a visual picture of the mixture of oligosaccharides and their alkaline-degradation products, the saccharinic acids, is desirable, since, after a short interval, the erosion of a trisaccharide may be occurring simultaneously with the degradation of di- and mono-saccharides derived from it. To monitor such reactions, thin-layer chromatography offers distinct advantages of speed and sensitivity over paper chromatography. Further advantages would accrue if the detection reagent for the saccharinic acids were modelled on the specific methods used for their assay in solution. Additionally, the type of saccharinic acids might be indicated by the ease of lactone formation. Progress in these studies is now reported, based on the use of thin-layer chromatography on cellulose and silica gel, together with thin-layer ionophoresis.

METHODS

Thin-layer chromatography on cellulose. — Chromatoplates were prepared by stirring 15 g of MN-cellulose powder 300 (E. Stahl; average particle size, 10 μm) in 80 ml of water in a top-drive macerator (Townson and Mercer, Croydon) and spreading the smooth slurry on glass plates ($10 \times 10 \times 0.2$ cm) by means of a Desaga applicator. Plates were dried for 30 min at 100° and stored without special precautions.

For separation of oligosaccharides, 3 μg of a single oligosaccharide or 5 μg of a mixture were applied to the plates with a micropipette, and separated by using either

butyl alcohol–pyridine–water (6:4:3) or butyl alcohol–ethanol–water (5:3:2) as developer for 1 h. Multiple development was effected as appropriate to complete the separation, and the plates were sprayed with aniline hydrogen phthalate[2].

For separation of saccharinic acids or their lactones, 2 μg of isosaccharinic acid or 6 μg of metasaccharinic acid were applied to the plates. Alkaline degradations of oligosaccharides were effected in 0.04N barium hydroxide at 25°, with a sugar concentration of 2 mg/ml. Aliquots (0.01 ml; 20 μg of original oligosaccharide) were suitable for analysis after removal of barium ions with Amberlite IR-120 (H^+) to give the free saccharinic acid. Lactonization was effected by evaporation of another aliquot to dryness after removal of barium ions and heating for 30 min at 60°. Developing solvents were either butyl alcohol–pyridine–water (6:4:3) or butyl alcohol–ethanol–water (10:3:5). After 1 h, the plates were air dried for 15–20 min and then sprayed with 0.05M sodium periodate in 2M phosphoric acid. After 20 min at room temperature, or, preferably, 10 min at 60°, the excess of periodate can be removed by spraying with ethylene glycol–acetone–conc. sulphuric acid (50:50:0.3) and leaving for 10 min at room temperature. In our experience, this step is unnecessary, and the plate can be sprayed directly with 6% sodium 2-thiobarbiturate, heated for 5 min at 100°, sprayed again with the thiobarbiturate reagent, and heated for a further 10 min at 100° to produce coloured spots that are more intense for acids than for lactones (isosaccharinic acid, purple; lactone, purple; metasaccharinic acid, pink). Depending on concentration, mono-, di-, and oligo-saccharides usually react with this spray reagent to give yellowish brown spots. It is known[3] that formic acid, which would be produced during the periodate-oxidation step, gives a chromogen (λ_{max} 450 nm) with 2-thiobarbituric acid. Fortunately, all of the lactones (see Table I) have higher R_F values than those of the saccharides. In addition, the chromogen from a lactone or acid can be eluted with a few drops of water, and its absorption maximum determined in cyclohexanone. Iso- and meta-saccharinic acids gave chromogens having λ_{max} 549 and 532 nm, respectively.

A second modification of the thiobarbiturate spray, when used on cellulose plates, was found to give a positive reaction only with isosaccharinic acid. After the plates had been sprayed with 0.02M sodium periodate, kept for 15 min at room temperature, sprayed with 6% sodium thiobarbiturate, and heated for 5 min at 100°, only the isosaccharinic acid gave a characteristic, pale-yellow spot. If the spray was used on silica plates, metasaccharinic acid, which gave a purple spot, could also be detected.

Alkaline degradation of oligosaccharides.—By the analytical procedure described above, the alkaline degradation of maltose (100 h) was shown to give isosaccharinic acid, with no metasaccharinic acid; turanose (48 h) and laminaribiose (48 h) gave metasaccharinic acid, with no isosaccharinic acid. Melibiose gave no iso- or meta-saccharinic acid, but gave a component reacting to give a yellow colour with the periodate-thiobarbiturate spray, and having mobility slightly different from that of melibiose. The mixture obtained by alkaline degradation of melibiose for 100 h was treated with Amberlite IR-120 (H^+), hydrolysed with 2N sulphuric acid for 3 h

TABLE I

SEPARATION OF SACCHARINIC ACIDS BY THIN-LAYER CHROMATOGRAPHY

Compound	*Colour with periodate–thiobarbituric acid in 0.025N H_2SO_4[b] 2M H_3PO_4[c]*		*R_F values[a] Cellulose Solvent I*	*Silica gel Solvent I*	*Cellulose Solvent II*
α-D-Isosaccharino-lactone	Yellow initially	Red–purple	0.83	0.85	0.66
β-D-Metasacc-harinolactone	Purple	Pink–Purple	0.75	0.80	0.63
α-D-Metasacc-harinolactone	Purple	Pink–purple	0.73	0.78	0.61
α-D-Isosaccharinic acid	Yellow Initially	Red–purple	0.36	0.32	0.40
β-D-Metasaccharinic acid	Purple	Pink–purple	0.32	0.36	0.39
α-D-Metasaccharinic acid	Purple	Pink–purple	0.30	0.46	0.37
Maltose	Negligible	Pale-yellow	0.36	0.69	0.24
Melibiose	Negligible	Yellow	0.25	0.65	0.12
Turanose	Negligible	Pale-yellow	0.45	0.68	0.31

[a]Solvent I, butyl alcohol–pyridine–water. Solvent II, butyl alcohol–ethanol–water. [b]For silica gel; slightly fainter colours on cellulose. [c]For silica gel; see text for slightly different colours on cellulose.

at 100°, neutralized with barium hydroxide, and treated again with Amberlite IR-120 (H^+), and a freeze-dried aliquot was analysed before and after being heated for 30 min at 60°. Components were detected which had different mobilities from those of iso- or meta-saccharinic acid, or lactone. Alkaline degradation of panose (72 h) gave isosaccharinic acid, together with a second product that gave a yellow colour with the spray and had a lower R_F value. After acid hydrolysis, as above, this second product disappeared, and two components, one having the same mobility as D-glucose, were detected.

The products of alkaline degradation of panose have also been investigated by using thin-layer ionophoresis. The products of alkaline degradation were applied directly to a cellulose plate (19 × 11 cm), which was afterwards sprayed with electrolyte. The products were separated by ionophoresis at 20 volts/cm for 30 min in an electrolyte (pH 9.5) containing sodium hydrogen carbonate (3.36 g/l) and sodium carbonate (0.36 g/l). After being dried, the products were detected as described above. The products of alkaline degradation of panose were isosaccharinic acid and a second component, which had half the mobility of isosaccharinic acid and gave a yellow colour with the spray reagent. After hydrolysis, the latter component was absent from the mixture, and spots having the mobility of glucose and an unsubstituted acid were present.

Thin-layer chromatography on silica gel. — Chromoplates (20 × 20 × 0.2 cm) were prepared by using Silica Gel G (25 g) in 50 ml of water. Saccharinic acids and

their lactones were separated for 3 h in butyl alcohol–pyridine–water (6:4:3). After being sprayed with 0.05M sodium periodate in 2M phosphoric acid, the plates were kept for 10 min at 60°. They were then sprayed with 6% sodium 2-thiobarbiturate, heated for 5 min at 100°, and sprayed and heated again to reveal both types of saccharinic acid. If 0.025M sodium periodate in 0.025M sulphuric acid was substituted in this procedure, and only one spray–heat cycle with thiobarbiturate was performed, the meta- and iso-acids were again detected. The colour quality and the characteristic changes with time (see Discussion) were superior to those obtained with cellulose plates. Oligosaccharides were also detectable with the phosphoric acid spray but not with the modification involving dilute sulphuric acid.

Results similar to those reported above for cellulose plates were obtained when the products of alkaline degradation of maltose and turanose were examined on silica-gel plates. A nigeran trisaccharide [α-D-G*p*-(1 → 4)-α-D-G*p*-(1 → 3)-D-G*p*] was degraded under the standard conditions, and alkaline hydrolysates (2 and 4 h) were examined on cellulose. Only metasaccharinic acid was detectable after 2 h, but meta- and iso-saccharinic acids were detectable after 4 h. A better separation of these acids was achieved on silica gel. With the periodate–phosphoric acid–thiobarbiturate spray, both acids could be distinguished clearly, and by using the selective periodate–sulphuric acid–thiobarbiturate spray, the characteristic yellow colour of the iso-acid, and the purple of the meta-acid, made the identification of each acid unambiguous.

DISCUSSION

Wolfrom and his co-workers[4] demonstrated the separation of a wide range of sugar derivatives by thin-layer chromatography on micro-crystalline cellulose, and recommended this procedure for preparative purposes. Paper-chromatographic studies of oligosaccharides and saccharinic acids had been reported[5], but previous spray reagents[6] for the saccharinic acids were not specific.

Periodate oxidation of isosaccharinic acid gives formylpyruvic acid, which is also produced from *N*-acetylneuraminic acid under the Warren conditions of assay[7]. Formylpyruvic acid condenses with 2-thiobarbituric acid to give a chromophore having λ_{max} 549 nm. Malonaldehyde also reacts in the same assay (chromophore, λ_{max} 532 nm) and is the product of periodate oxidation of metasaccharinic acid under acid conditions. A spray modelled on this reagent was devised and detects both of these types of saccharinic acid.

A spray, based on the Warren method[7], for detecting *N*-acetylneuraminic acid has been previously reported[8]. This uses 0.05N sulphuric acid, instead of 2M phosphoric acid, during the periodate oxidation, and the excess of periodate is removed before the spraying with thiobarbituric acid. We have found that removal of the excess of periodate is unnecessary. Isosaccharinic acid and its lactone show a distinctive yellow colour, changing to red–purple after several hours, whereas metasaccharinic acid and its lactone always give purple spots. The colour change, from yellow to red in the case of the iso-acid, is due to changes in pH; the yellow colour is stable if the

cellulose plate is kept over a desiccant, and, when the coloured compound is eluted from the plate with water, it is stable at pH 8, but changes into the red compound (λ_{max} 550 nm) at more acidic pH values. The colour change is more rapid when silica-gel plates are used, due to the acidic nature of this type of silica[9].

The differences in colour formation were also checked by elution of the coloured substances from the chromatogram and determination of the values of λ_{max}. The colour differences proved to be useful, since the solvents employed on cellulose did not adequately separate a mixture of iso- and meta-saccharinic acids. This problem was overcome by effecting the separation on silica gel, which also had the advantage of providing brighter colours and less background colour, but had the disadvantage of giving more-fragile plates.

The value of the technique was demonstrated by its application to melibiose. Corbett and Kenner[10] reported that treatment of melibiose with 0.033N calcium hydroxide for 19 days at 21–22° produced a mixture of 6-*O*-α-D-galactopyranosyl-saccharinic acids, which, on hydrolysis, gave β-metasaccharinic acid, together with some isosaccharinic acid. After treatment of melibiose with 0.05N barium hydroxide for 100 h at 25°, no free meta- or iso-saccharinic acid could be detected on thin-layer plates. However, a component was present that had the mobility of a disaccharide. Acid hydrolysis gave free glucose, but no meta- or iso-saccharinic acid. These results agree with those obtained[1] by the assay methods used in solution, since no formaldehyde was liberated after periodate oxidation of the mixture obtained by alkaline degradation of melibiose.

The superiority of silica gel for the separation of the two types of acid enabled elucidation of the sequence of acid liberation during the alkaline degradation of a nigeran trisaccharide [α-D-G*p*-(1→4)-α-D-G*p*-(1→3)-D-G*p*]. As would be expected theoretically, the order was meta- and iso-saccharinic acid, parallelling the results reported for analyses in solution[1].

It has been possible to show the presence of bound saccharinic acids in the degradation products of several oligosaccharides by using thin-layer ionophoresis. Compounds having similar ionophoretic mobilities have been observed in the alkaline degradation products of panose and gentiobiose; isomaltotriose yields a compound of lower mobility, and both saccharinic acids have an identical and higher mobility.

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FLUOROCARBOHYDRATES

PART XVIII. 9-(3-DEOXY-3-FLUORO-β-D-XYLOFURANOSYL)ADENINE AND 9-(3-DEOXY-3-FLUORO-α-D-ARABINOFURANOSYL)ADENINE

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ABSTRACT

An alternative synthesis of 3-deoxy-3-fluoro-α-D-xylose (**4a**) from methyl 2,3-anhydro-5-*O*-benzyl-β-D-ribofuranoside (**1**) is described. Methyl 5-*O*-benzyl-3-deoxy-3-fluoro-β-D-xylofuranoside (**3**) and methyl 5-*O*-benzyl-3-deoxy-3-fluoro-α-D-arabinofuranoside (**11**) were converted, *via* the 2,5-di-*O*-benzoyl derivatives (**6**) and (**12**), into the corresponding αβ-D-glycosyl bromides (**7**) and (**13**). The latter compounds were then condensed with 6-benzamidopurine to yield the fluorinated nucleosides, 6-benzamido-9-(2,5-di-*O*-benzoyl-3-deoxy-3-fluoro-β-D-xylofuranosyl)purine (**8**) and 6-benzamido-9-(2,5-di-*O*-benzoyl-3-deoxy-3-fluoro-α-D-arabinofuranosyl)purine (**14**), respectively. Structural assignments of the fluoronucleosides (**8**) and (**14**) were based upon u.v. comparison with known 9-(3-deoxy-β-D-pentofuranosyl)adenines, and the fact that, on alkaline hydrolysis, compounds **8** and **14** yielded crystalline fluoronucleosides (**9**) and (**15**) which gave the 5′-toluene-*p*-sulphonates **10** and **16**; on heating, compound **10** formed a 3,5′-cyclic *p*-toluenesulphonate, whereas compound **16** did not. These results are consistent with the anomeric configuration assigned to 9-(3-deoxy-3-fluoro-β-D-xylofuranosyl)adenine (**9**) and 9-(3-deoxy-3-fluoro-α-D-arabinofuranosyl)adenine (**15**).

This synthesis of deoxyfluoronucleosides is considered to be less limited in application than those so far reported.

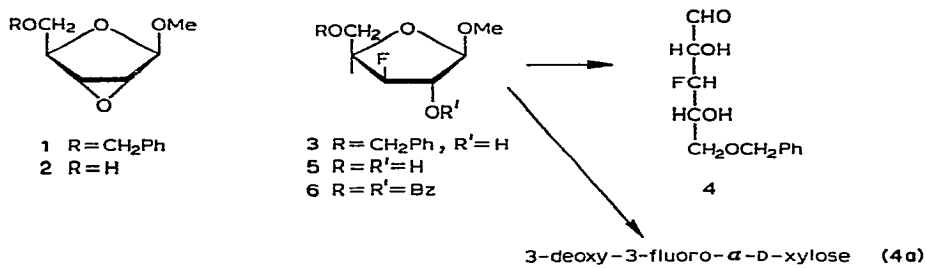
INTRODUCTION

Work so far reported on the introduction of fluorine into nucleosides has been based on (*i*) replacement of hydrogen by fluorine in the heterocyclic moiety (*e.g.*, 2-fluoroadenosine[1] and 5-fluorouridine[2]), or (*ii*) the replacement of the 2′-hydroxyl group of the nucleoside directly by fluorine (*e.g.*, 2′-deoxy-2′-fluorouridine[3]). As part of a synthetic programme directed towards the replacement of hydroxyl groups by fluorine, we now report the synthesis of 9-(3-deoxy-3-fluoro-β-D-xylofuranosyl)-adenine (**9**) and 9-(3-deoxy-3-fluoro-α-D-arabinofuranosyl)adenine (**15**).

RESULTS AND DISCUSSION

The method of introducing fluorine into the 2′-position of uridine by Fox *et al.*[3] appears to be limited to pyrimidines having an oxygen function at the 2-position

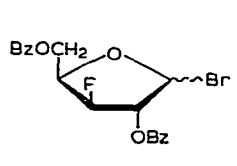
of the heterocycle. The use of suitably substituted deoxyfluoroglycosyl halides and their subsequent conversion into the nucleoside by established procedures, however, does not possess this limitation. In the pentose series, both 3-deoxy-3-fluoro-α-D-xylose and 3-deoxy-3-fluoro-β-D-arabinose are now available[4a], and we have, therefore, used these compounds as models for fluoronucleoside synthesis. 3-Deoxy-3-fluoro-αβ-D-xylose was previously synthesised by us from methyl 2,3-anhydro-4-*O*-benzyl-β-D-ribopyranoside[4a]. In order to utilise the furanose form of the carbohydrate, we have now synthesised this fluoro sugar by the action of potassium hydrogen fluoride (KHF_2) on methyl 2,3-anhydro-5-*O*-benzyl-β-D-ribofuranoside (**1**), obtained by benzylation of methyl 2,3-anhydro-β-D-ribofuranoside[5] (**2**). Acid hydrolysis of the resulting fluorohydrin **3** produced from compound **1** gave a reducing sugar **4** which consumed periodate (1 mole/mole) and liberated formic acid (1 mole/mole). These results are consistent with the 3-deoxy-3-fluoro-D-xylofuranose structure for compound **3**. Moreover, catalytic hydrogenation of compound **3**, followed by acid hydrolysis, gave a free deoxyfluoro sugar identical with crystalline[4b] 3-deoxy-3-fluoro-α-D-xylose (**4a**).



Catalytic hydrogenation of compound **3** gave the glycoside **5** which, on benzoyl ation, yielded methyl 2,5-di-*O*-benzoyl-3-deoxy-3-fluoro-β-D-xylofuranoside (**6**). Treatment of compound **6** with hydrogen bromide in glacial acetic acid gave the αβ-glycosyl bromides (**7**) which were immediately condensed with 6-benzamidopurine[6]. This reaction was carried out by two established methods: (*a*) with the 6-benzamidopurine as a chloromercuri salt[6], and (*b*) using nitromethane in the presence of mercuric cyanide[7]. The latter method gave better yields of a nucleoside which was assigned the 9-(3-deoxy-3-fluoro-β-D-xylofuranosyl) configuration (**8**), on the basis of ultraviolet comparison with 9-(3-deoxy-β-D-pentofuranosyl)adenines[8], the Trans Rule for nucleoside formation[9], and the fact that alkaline hydrolysis of **8** yielded a crystalline nucleoside (**9**) which gave a 5′-toluene-*p*-sulphonate (**10**) (this exchanged with sodium iodide in acetone in accordance with Oldham and Rutherford's rule[10]) that could be quaternized at N-3 of the purine on heating. The formation of this 3,5′-cyclo salt is in agreement with results reported by various workers[11] and indicates a β-D-configuration at the anomeric centres of compounds **8** and **9**.

A similar sequence of reactions was then applied to methyl 5-*O*-benzyl-3-deoxy-3-fluoro-α-D-arabinofuranoside[4a] (**11**), which was converted, *via* the 3,5-di-*O*-benzoyl derivative (**12**), into the αβ-glycosyl bromides (**13**). Condensation of **13** with

6-benzamidopurine by methods (*a*) or (*b*) gave a major nucleoside component which was assigned the 9-(3-deoxy-3-fluoro-α-D-arabinofuranosyl) configuration (**14**). This configuration was based on criteria already described for compound **8**. Confirmation



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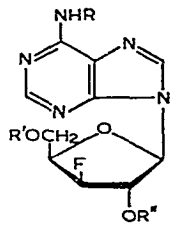
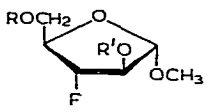
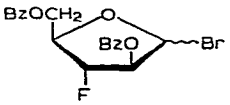


8 R = R' = R" = Bz
9 R = R' = R" = H
10 R = H, R' = Ts, R" = H



11 R = CH_2Ph, R' = H
12 R = R' = Bz



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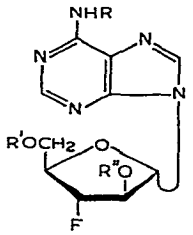

14 R = R' = R" = Bz
15 R = R' = R" = H
16 R = H, R' = Ts, R" = H

of the α-D-configuration of compound **14** was obtained by its alkaline hydrolysis to yield the crystalline nucleoside **15** which gave a 5′-toluene-*p*-sulphonate (**16**) that did not quaternize to a 3,5′-cyclic compound on heating.

EXPERIMENTAL

Melting points were determined with an Electrothermal apparatus and are uncorrected. Paper chromatography was performed as previously described[4a]. Thin-layer chromatography (t.l.c.) was carried out with Silica-gel G for carbohydrates and Silica Gel PF254 for nucleosides and purine bases. Carbohydrates were detected by spraying with sulphuric acid–ethanol (1:1) and heating for 10 min at 120°, and nucleosides and bases by viewing the plates under u.v. illumination. Preparative t.l.c. (p.l.c.) was performed as before[4a]. The solvent systems used were light petroleum (b.p. 40–60°)–ethyl acetate, 2:1 (*A*), 1:1 (*B*), acetone–ethyl acetate[8], 1:1 (*C*), and chloroform–methanol[12], 4:1 (*D*).

Microanalyses and fluorine determinations were performed by Drs. Weiler and Strauss, Oxford.

Methyl 2,3-anhydro-5-O-benzyl-β-D-ribofuranoside (**1**). — To methyl 2,3-anhydro-β-D-ribofuranoside[5] (**2**) (5.0 g) in anhydrous *N,N*-dimethylformamide (25 ml) were added silver oxide (7.0 g) and benzyl bromide (6 ml). The mixture was shaken for 24 h at room temperature and then diluted with chloroform (250 ml) and water (250 ml). The chloroform layer was separated and filtered, pyridine (30 ml)

was added, and the solution was washed successively with water (6 × 100 ml), 2M hydrochloric acid (3 × 100 ml), saturated aqueous sodium hydrogen carbonate (100 ml), and water (100 ml). The chloroform extract was dried ($MgSO_4$), and evaporated to dryness, and the resulting light-brown oil was distilled to give the product as a colourless oil (6.4 g), b.p. 130° (bath)/0.01 mm, $[\alpha]_D^{24}$ −90.8° (*c* 1.4, chloroform) (Found C, 66.7; H, 6.9. $C_{13}H_{16}O_4$ calc.: C, 66.1; H, 6.8%).

Methyl 5-O-benzyl-3-deoxy-3-fluoro-β-D-xylofuranoside (**3**). — A solution of methyl 2,3-anhydro-5-*O*-benzyl-β-D-ribofuranoside (**1**) (3.0 g) and KHF_2 (6.0 g) in ethane-1,2-diol (60 ml) was refluxed gently for 1.5 h. When cool, the solution was poured into saturated aqueous sodium hydrogen carbonate (500 ml) with stirring, and extracted with chloroform (3 × 100 ml); the extract was then dried ($MgSO_4$), and evaporated to dryness *in vacuo*. The resulting syrup on t.l.c. (solvent *B*) contained two major components, R_F 0.4 and 0.6 (starting material). The mixture was resolved by p.l.c. (2 elutions with solvent *A*) to yield a fluorine-containing syrup (**3**) (1.1 g), $[\alpha]_D^{20}$ −62.0° (*c* 1.0, chloroform), which had i.r. absorption bands at 3440 (OH) and 1100–1000 cm^{-1} (C–F). (Found: C, 60.8; H, 6.9; F, 7.1. $C_{13}H_{17}FO_4$ calc.: C, 61.0; H, 6.6; F, 7.4%).

5-O-Benzyl-3-deoxy-3-fluoro-αβ-D-xylose (**4**). — Methyl 5-*O*-benzyl-3-deoxy-3-fluoro-β-D-xylofuranoside (**3**) (1.0 g) was dissolved in a mixture of *p*-dioxane (50 ml) and 0.25M sulphuric acid (50 ml), and the solution was refluxed for 3 h. The cooled solution was neutralized with barium carbonate, filtered, and evaporated to dryness *in vacuo*. The residue was taken up in absolute ethanol and filtered, and the filtrate was evaporated to dryness *in vacuo*, to yield a viscous, colourless, reducing syrup (**4**) (740 mg), R_F 0.26 (solvent *B*), $[\alpha]_D^{20}$ −2.5° (*c* 1.1, ethanol) (Found: C, 59.2; H, 6.3; F, 7.5. $C_{12}H_{15}FO_4$ calc.: C, 59.5; H, 6.2; F, 7.8%).

Periodate oxidation of 5-O-benzyl-3-deoxy-3-fluoro-αβ-D-xylose. — 5-*O*-Benzyl-3-deoxy-3-fluoro-αβ-D-xylose (**4**) (102.1 mg) was dissolved in water (20 ml) and 0.05M sodium metaperiodate (20 ml). At intervals, 2-ml portions were withdrawn, and the periodate consumed and acid liberated were determined as previously described[4a]. The results were as follows:

Time (min)	2	15	30	45	24 h
Periodate consumed (moles/mole)	0.97	0.94	0.98	1.01	1.01
Acid liberated (moles/mole)	0.80	0.85	0.90	0.90	0.93

The acid liberated was identified as formic acid by reduction to formaldehyde and characterisation with chromotropic acid[13].

Methyl 3-deoxy-3-fluoro-β-D-xyloside (**5**). — Methyl 5-*O*-benzyl-3-deoxy-3-fluoro-β-D-xylofuranoside (**3**) (1.0 g) in ethanol (25 ml) containing 5% palladium-on-charcoal (1.0 g) was hydrogenated at room temperature and atmospheric pressure until the uptake of hydrogen ceased (1 mole, 30 min). After filtration, the solution was evaporated to yield compound **5** as a viscous, non-reducing syrup, R_F 0.12 (solvent *B*), $[\alpha]_D^{20}$ −63.0° (*c* 1.6, ethanol) (Found: C, 43.2; H, 6.8; F, 11.2. $C_6H_{11}FO_4$ calc.: C, 43.4; H, 6.6; F, 11.4%). The glycoside was characterised as the

2,5-dibenzoate (**6**), m.p. 67°, $[\alpha]_D^{22}$ −22.0° (*c* 1.1, ethanol) (Found: C, 64.4; H, 5.2; F, 5.4. $C_{20}H_{19}FO_6$ calc.: C, 64.1; H, 5.1; F, 5.1%).

*3-Deoxy-3-fluoro-α-*D*-xylose* (**4a**). — Methyl 3-deoxy-3-fluoro-β-D-xylofuranoside (**5**) (0.50 g) was treated with refluxing 0.05M sulphuric acid (50 ml) for 1 h. The cooled solution was neutralized with barium carbonate, and the filtered solution was evaporated to dryness *in vacuo*. The residue was taken up in absolute ethanol, and the filtered solution was evaporated to dryness, yielding 3-deoxy-3-fluoro-αβ-D-xylose as a colourless, reducing syrup (0.401 g). This crystallized upon seeding with authentic compound **4a**, and was recrystallized from ethanol; m.p. 126–128°, $[\alpha]_D^{25}$ +75.3 → +25.7° (1 h) (*c* 1.7, water) (Found: C, 39.6; H, 6.2; F, 12.0. $C_5H_9FO_4$ calc.: C, 39.5; H, 5.9; F, 12.5%).

6-*Benzamido-9-(2,5-di-*O*-benzoyl-3-deoxy-3-fluoro-β-*D*-xylofuranosyl)purine* (**8**). — Methyl 2,5-di-*O*-benzoyl-3-deoxy-3-fluoro-β-D-xylofuranoside (**6**) (1.0 g) was dissolved in glacial acetic acid (10 ml) containing 2% of acetic anhydride. A solution of hydrogen bromide in glacial acetic acid (10 ml, 45% w/v) was added, and the resulting yellow solution was kept for 3 h at room temperature, and then evaporated to dryness *in vacuo* (< 30°). Residual hydrogen bromide and acetic acid were removed by three evaporations with anhydrous benzene. 2,5-Di-*O*-benzoyl-3-deoxy-3-fluoro-αβ-D-xylofuranosyl bromide (**7**) was obtained as a yellow syrup that was used immediately for the next step.

Method (*a*). A suspension of chloromercuri-6-benzamidopurine[6] (1.270 g) in xylene (100 ml, A.R.) was dried by slow azeotropic distillation of *ca.* 25 ml. The suspension was cooled to near room temperature, and a solution of compound **7** (prepared from 1.0 g of the glycoside **6**), in xylene (10 ml) was added. The suspension was refluxed under anhydrous conditions for 2 h. While hot, the suspension was rapidly filtered, and light petroleum (200 ml; b.p. 40–60°) was added to the filtrate. After being cooled to room temperature and storage for 1 h, the white flocculent precipitate was collected by filtration, and washed with light petroleum. The dried solid was dissolved in chloroform (50 ml) and the solution was washed with 30% aqueous potassium iodide (3 × 30 ml) and water (30 ml), and then dried ($MgSO_4$). Evaporation of the solution to dryness *in vacuo* gave a yellow, glassy solid, shown by t.l.c. (solvent *C*) to be a mixture of four components, including the two starting materials. Resolution of the mixture was accomplished by using p.l.c (solvent *C*), and the major product (R_F 0.68) was recovered by extraction with ethyl acetate. Evaporation of the extract to dryness gave compound **8** (0.80 g) as a chromatographically pure, pale-yellow glass, $[\alpha]_D^{21}$ −46.6° (*c* 0.8, chloroform), λ_{max} 231 (ε 35,200) and 279.5 nm (ε 18,800). (Found: C, 63.4; H, 4.5; N, 11.6; F, 3.4. $C_{31}H_{24}FN_5O_6$ calc.: C, 64.0; H, 4.1; N, 12.0; F, 3.3).

Method (*b*)[7]. A suspension of 6-benzamidopurine[6] (0.640 g) in nitromethane (40 ml) was dried by azeotropic distillation of *ca.* 10 ml of the solvent, during which most of the solid dissolved. To the warm suspension was added a solution of compound **7** (prepared from 1.0 g of the glycoside **6**) in nitromethane (5 ml), and finely powdered mercuric cyanide (1.0 g). The suspension was refluxed for 1 h,

dissolution being complete in this time. The solution was evaporated to dryness *in vacuo*, and the residue was suspended in chloroform (100 ml). After being washed with 30% aqueous potassium iodide (2 × 30 ml) and water (30 ml), the solution was dried ($MgSO_4$) and evaporated to dryness *in vacuo*. The product, a colourless glass, was identical on t.l.c. to the mixture obtained by procedure (*a*), and was similarly resolved, to give compound **8** (0.85 g), which was identical in all respects to that obtained by procedure (*a*).

6-Benzamido-9-(2,5-di-O-benzoyl-3-deoxy-3-fluoro-α-D-arabinofuranosyl)purine (**14**). — Methyl 5-*O*-benzyl-3-deoxy-3-fluoro-α-D-arabinofuranoside (**11**) was converted into methyl 2,5-di-*O*-benzoyl-3-deoxy-3-fluoro-α-D-arabinofuranoside (**12**) as previously described[4a]. Treatment of compound **12** with hydrogen bromide in acetic acid, as detailed above, gave 2,5-di-*O*-benzoyl-3-deoxy-3-fluoro-αβ-D-arabinofuranosyl bromide (**13**) as a yellow gum that was used without further characterisation.

When procedures (*a*) and (*b*) were applied to compound **13**, compound **14**, in yields of 60% and 65%, respectively, was obtained as a glass, $[\alpha]_D^{21}$ +58.2° (*c* 0.8, chloroform), R_F 0.66 (solvent *C*), λ_{max} 230.5 (ε 28,800) and 279.5 nm (ε 16,700) (Found: C, 63.2; H, 4.6; N, 11.8; F, 3.2. $C_{31}H_{24}FN_5O_6$ calc.: C, 64.0; H, 4.1; N, 12.0; F, 3.3%).

9-(3-Deoxy-3-fluoro-β-D-xylofuranosyl)adenine (**9**). — A solution of 6-benzamido-9-(2,5-di-*O*-benzoyl-3-deoxy-3-fluoro-β-D-xylofuranosyl)purine (**8**) (58 mg) in 0.05N methanolic sodium methoxide (2 ml) was refluxed for 1 h, and then evaporated to dryness *in vacuo*. The residue was dissolved in water (2 ml) and neutralized with 2N acetic acid. Methyl benzoate was removed by washing with chloroform (3 × 1 ml), and the aqueous layer was evaporated to dryness *in vacuo*. The brown, gummy residue was readily crystallized from water to give compound **9** as colourless needles, m.p. 218–220°, R_F 0.22 (solvent *D*), $[\alpha]_D^{21}$ −40.1° (*c* 0.5, water), $\lambda_{max}^{H_2O,\ pH7}$ 259.5 nm (ε 13,300) (Found: C, 37.3; H, 5.7; N, 21.4; F, 6.3. $C_{10}H_{12}FN_5O_3 \cdot 3H_2O$ calc. C, 37.2; H, 5.6; N, 21.6; F, 5.9%). Karl Fischer analysis confirmed the presence of water of crystallization.

9-(3-Deoxy-3-fluoro-α-D-arabinofuranosyl)adenine (**15**). — By a similar procedure to that described above, 6-benzamido-9-(2,5-di-*O*-benzoyl-3-deoxy-3-fluoro-α-D-arabinofuranosyl)purine (**14**) was hydrolysed, giving compound **15** as colourless needles, m.p. 133–135°, R_F 0.25 (solvent *D*), $[\alpha]_D^{22}$ +64.2° (*c* 0.5, water), $\lambda_{max}^{H_2O,\ pH7}$ 259 nm (ε 14,500) (Found: C, 42.5; H, 4.8; N, 24.5; F, 7.0. $C_{10}H_{12}FN_5O_3.H_2O$ calc.: C, 41.8; H, 4.9; N, 24.4; F, 6.6%). Karl Fischer analysis confirmed the presence of water of crystallization.

9-(3-Deoxy-3-fluoro-5-O-tosyl-β-D-xylofuranosyl)adenine* (**10**). — 9-(3-Deoxy-3-fluoro-β-D-xylofuranosyl)adenine (**9**) (101 mg) was dissolved in anhydrous pyridine (2 ml) and cooled in ice. Toluene-*p*-sulphonyl chloride (165 mg) was added, and the solution was kept for 24 h at room temperature and then poured into water (20 ml). The product was extracted with chloroform (3 × 15 ml), and the extract was washed

*Tosyl = toluene-*p*-sulphonyl.

with saturated aqueous sodium hydrogen carbonate (2 × 20 ml) and water (20 ml), dried ($MgSO_4$), and evaporated to dryness *in vacuo*. On cooling, the residue solidified. T.l.c. (solvent *D*) showed the presence of one major component, R_F 0.60, and small proportions of compounds having R_F 0.68 and 0.75. The major component, an amorphous solid (51 mg), which proved to be the title compound **10**, was isolated by p.l.c. (solvent *D*). It had λ_{max}^{EtOH} 261 nm (ε 12,800) (Found: C, 48.4; H, 4.6. $C_{17}H_{18}FN_5O_5S$ calc.: C, 48.2; H, 4.3%). Bands in the i.r. spectrum at 1175 and 1365 cm^{-1} indicated the presence of the sulphonyloxy residue. On refluxing sulphonate **10** (47 mg) with sodium iodide (33 mg) in anhydrous acetone (0.35 ml), sodium toluene-*p*-sulphonate was deposited.

9-(3-Deoxy-3-fluoro-5-O-tosyl-α-D-arabinofuranosyl)adenine (**16**). — Toluene-*p*-sulphonylation of compound **15**, as described above, gave the title compound as an amorphous solid, R_F 0.60 (solvent *D*), λ_{max}^{EtOH} 261 nm (ε 7550). The i.r. spectrum contained bands at 1180 and 1365 cm^{-1} ($-SO_2R$), and the sulphonyloxy group of compound **16** exchanged with sodium iodide–acetone in accordance with Oldham and Rutherford's rule[10].

3,5'-Cyclo-9-(3-deoxy-3-fluoro-β-D-xylofuranosyl)adenine toluene-p-*sulphonate.* — 9-(3-Deoxy-3-fluoro-5-*O*-tosyl-β-D-xylofuranosyl)adenine (37 mg) was dissolved in anhydrous *p*-dioxane[11b] (3 ml) and the solution was refluxed for 3 h. A white solid separated. The solvent was evaporated, and the residue was re-suspended in acetone. The solid was filtered off, and washed with acetone. The acetone-soluble material was shown (t.l.c.) to be starting material, and the insoluble portion (15 mg), on the basis of t.l.c. [2 spots, R_F 0.02, 0.21 (toluene-*p*-sulphonate anion), solvent *D*] and u.v. spectrum [$\lambda_{max}^{H_2O\,(pH7)}$ 274 nm (ε 10,900)], was assigned the structure of the title compound. The appearance of intense bands in the i.r. spectrum at 1215 and 684 cm^{-1}, assigned to the toluene-*p*-sulphonate anion, provided further evidence for this structure.

9-(3-Deoxy-3-fluoro-5-*O*-tosyl-α-D-arabinofuranosyl)adenine underwent no change on similar treatment, and therefore possessed the α-D-configuration at the anomeric centre.

ACKNOWLEDGMENTS

A sample of crystalline 3-deoxy-3-fluoro-α-D-xylose was kindly provided by Professor A. B. Foster, Chester Beatty Research Institute, London. The authors thank the Medical Research Council for a grant for equipment (N.F.T.), and the Science Research Council for the award of a Studentship (J.A.W.).

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A CHROMATOGRAPHIC AND MASS-SPECTROMETRIC STUDY OF 6-*O*-(2-HYDROXYETHYL)-D-GLUCOSE AND ITS ETHYLENE OXIDE ADDUCTS

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ABSTRACT

6-*O*-(2-Hydroxyethyl)-D-glucose, and its ethylene oxide adducts having 2–5 ethylene oxide units in the 6-position, were synthesised, and separated by partition chromatography on an anion-exchange resin. Paper chromatography, gas–liquid chromatography, and mass spectrometry were used in the characterisation of these compounds.

INTRODUCTION

As shown by Brownell and Purves[1], 6-*O*-(2-hydroxyethyl)-D-glucose (s_6) can be prepared by treating 1,2:3,5-di-*O*-methylene-D-glucofuranose with ethylene oxide in alkaline medium. The hydroxyl groups present in the substituent can react further and give rise to 6-*O*-[2-(2-hydroxyethoxy)ethyl]-D-glucose (s_{66}). This substance was isolated by Croon and Lindberg[2] from hydrolysates of *O*-(2-hydroxyethyl)-cellulose. Further reaction would give rise to higher members of the series $RO(CH_2CH_2O)_{n-1}CH_2CH_2OH$, and these will be denoted as s_{666}, s_{6666}, etc.

The purpose of this paper is to describe the isolation of the derivatives s_6–s_{66666} of this series by partition chromatography on an anion-exchange resin. Paper chromatography, gas–liquid chromatography of the trimethylsilyl (TMS) derivatives, and the mass spectra of these derivatives were used to characterize the substances.

EXPERIMENTAL

1,2:3,5-Di-*O*-methylene-D-glucofuranose 6-acetate was saponified in alkali and a 20-fold excess of ethylene oxide was bubbled through the solution for 1.5 h at 70°. The solution was allowed to stand at room temperature for 2 h and was then extracted with chloroform. After being dried under vacuum, the product was hydrolysed in dilute hydrochloric acid under conditions given by Brownell and Purves[1]. The solution was deionized, and evaporated under diminished pressure to give a syrup.

Partition chromatography on a preparative scale on an anion-exchange resin was used for the fractionation of the reaction mixture. The resin was a carefully fractionated anion-exchanger, Dowex-1X8, 17–24 μm, in its sulfate form. The

elution was carried out at 87° with mixtures of ethanol and water. The equipment was of the usual type with a jacketed glass-column and piston-type pump of stainless steel for feeding in the eluant. The eluate was analyzed continuously with a Technicon AutoAnalyzer, using the orcinol method[3]. The main part (95%) of the eluate was collected in a time-actuated fraction collector. Appropriate fractions were evaporated under diminished pressure. All derivatives were obtained as syrups.

The TMS-derivatives were prepared according to Sweeley *et al.*[4], and applied to a LKB 9000 gas chromatograph–mass spectrometer installed at the Swedish Institute for Food Preservation Research. The g.l.c. unit contained a glass column (300 × 0.3 cm i.d.) filled with silanized and acid-washed Chromosorb P (100–120 mesh), with 1% SE 30 as stationary phase. All experiments were performed at constant temperature, in the range 210–250°.

An electron energy of 70 eV was employed in the mass spectrometer. The temperature of the molecule separator was 205–255°. A linear correction for column bleeding was applied.

The g.l.c. experiments were performed on a Perkin–Elmer 800 Gas Chromatograph equipped with a dual differential flame-ionization detector.

Paper-chromatographic separations were made on Whatman No. 1 filter paper, with *p*-anisidine phthalate as spraying reagent.

DISCUSSION

Chromatographic separation on a preparative scale. — In Fig. 1, the separation of 410 mg of the synthesis mixture is shown. The derivatives were eluted with 92.4%

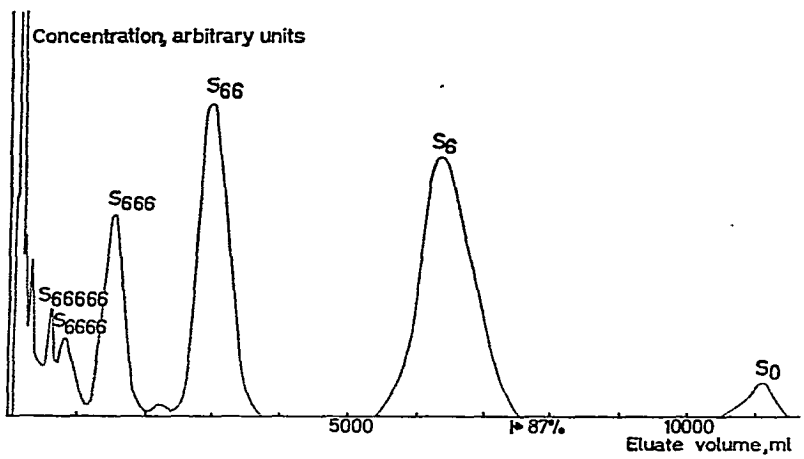

Fig. 1. Fractionation of 410 mg of the synthesis mixture in ethanol–water at 87°. Resin bed: 20 × 840 mm. Flow rate: 1.66 ml.cm^{-2}.min^{-1}.

aqueous ethanol (w/w), and then unreacted D-glucose was stripped off with 87% ethanol. Of the amount applied to the column, 87% was recovered, and 78% was isolated and identified as s_6–s_{66666}.

The identity of the compounds could be established by mass spectra of their TMS-derivatives, since the mass spectra of the higher members of the series could be predicted from spectra recorded with D-glucose and an authentic sample of 6-*O*-(2-hydroxyethyl)-D-glucose.

It is seen that the derivatives were eluted in the order of decreasing molecular weight, which is expected with derivatives containing the same number of free hydroxyl groups[3]. The two compounds (s_{66666} and s_{6666}) which appeared first in the eluate were probably better separated from each other than would appear from Fig. 1. There was a high background absorption in this part of the chromatogram due to tailing of the huge band at the beginning. However, with appropriately cut fractions, pure derivatives were obtained. In Table I, the amounts of the individual derivatives and their distribution coefficients[5] (D_v) are listed.

TABLE I

AMOUNTS OF ISOLATED DERIVATIVES AND THEIR CHROMATOGRAPHIC BEHAVIOR

Compound	*Isolated*		D_V	R_G[a]	
	mg	*mol.%*		*A*	*B*
D-Glucose (s_0)	22.8	9.3	—	1.00	1.00
s_6	127.0	41.5	24.8	1.29	1.26
s_{66}	122.1	33.4	11.5	1.30	1.39
s_{666}	53.0	12.4	5.8	1.42	1.59
s_{6666}	12.7	2.6	3.0	1.46	1.64
s_{66666}	4.3	0.8	2.1	1.33	1.58
sum	341.9				

[a]Paper-chromatographic migration relative to that of glucose; solvent *A*: butyl alcohol, saturated with water; solvent *B*: ethyl acetate–acetic acid–water, 9:2:2.

Paper chromatography. — The mobilities relative to that of glucose (R_G) of the five derivatives in two systems are given in Table I. The results are as expected, and in agreement with literature values[1,2,6] for s_6 and s_{66}. It is interesting to note that, in both solvent systems, the mobilities increase with an increased number of ethylene oxide units, within the range 1–4, but decrease when one more ethylene oxide unit is present.

Gas–liquid chromatography. — Lott and Brobst[7] earlier used g.l.c. for the analysis of *O*-(2-hydroxyethyl)amylose hydrolysates, in which they found 6-*O*-(2-hydroxyethyl)-D-glucose. In the present work, the most-satisfactory separations were achieved with the fluorinated silicone fluid QF-1. A chromatogram from a temperature-programmed run is reproduced in Fig. 2. Other stationary phases used were the nonpolar silicon elastomers, SE-52 and SE-30. The separations of the higher homologues were not quite satisfactory on these phases. As can be seen from Fig. 2, two peaks were recorded for each compound. They represent, as shown by mass spectrometry, the α- and β-D anomers of the pyranose form. From peak areas, it has been calculated that the α:β ratio for all of the derivatives was *ca.* 1:1.

Mass spectrometry. — The mass spectra of the fully trimethylsilylated D-glucose derivatives were very similar to that of fully trimethylsilylated D-glucose.

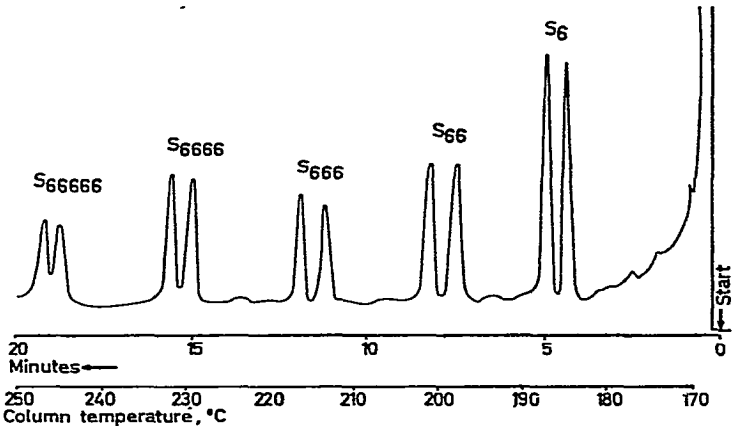

Fig. 2. Separation of 6-*O*-(2-hydroxyethyl)-D-glucose and its ethylene oxide adducts as trimethylsilyl-ethers. Column: 200 × 0.2 cm i.d. stainless steel. Column packing: 3% DC QF-1 on Gas Chrom Q 100/120 mesh. Column temperature: programmed at 4.2°/min from 170 to 250°. Carrier gas: N_2. Flow rate: 30 ml/min.

As can be seen from Table II, the most-prominent peaks have the same mass number and are of about the same relative intensity, which shows that the fragmentation patterns must be very similar.

TABLE II

MASS SPECTRA: RELATIVE INTENSITY OF THE MOST-PROMINENT IONS AND IONS OF HIGH MASS-NUMBER

Compound	m/e										
	73	*103*	*117*	*129*	*147*	*191*	*204*	*217*	*M-105*	*M-90*	*M-15*
D-Glucose (s_0) α	89	6	6	8	24	40	100	17	2.1	—	—
β	79	6	5	6	22	40	100	18	1.5	—	—
s_6 I	69	4	11	6	17	38	100	18	0.9	0.03	0.06
II	67	4	10	6	15	41	100	18	1.0	—	0.09
s_{66} I	66	5	11	7	13	35	100	17	0.3	0.03	0.02
II	72	6	11	8	13	37	100	16	0.5	0.05	0.02
s_{666} I	64	6	14	9	12	35	100	20	0.7	0.04	—
II	62	6	13	8	11	36	100	18	0.6	0.06	—
s_{6666} I	68	7	17	10	12	32	100	22	0.3	0.05	—
II	63	6	16	9	10	34	100	18	0.4	0.05	—
s_{66666} I	—										
II	82	11	22	12	14	32	100	27	0.2	0.10	—

The molecular ion (M) was not recorded for any derivative, whereas peaks corresponding to M-15 were recorded for those with lower molecular weight and

M-90 in low intensity for all. The most-suitable ion for determination of the molecular weight was M-105 (M-90-15).

The intense peaks at m/e = 191, 204, and 217 are analogues of ions having m/e = 75, 88, and 101 in the mass spectra of fully methylated monosaccharides[8,9]. They have also been recorded for TMS-derivatives of monosaccharides[10]. The mass number of the base peak was equal to 204 in all spectra. In the lower part of the spectrum, several prominent ions (m/e = 73, 103, 147) were found, which were earlier shown to be characteristic of TMS-derivatives[10,11].

Unfortunately, few ions of high intensity characteristic of 6-*O*-substituted monosaccharides are formed. Ions which were recorded for ethylene oxide adducts carrying the substituent in the 6-position, but which did not appear in the spectra of 2-*O*-(2-hydroxyethyl)-D-glucose, 3-*O*-(2-hydroxyethyl)-D-glucose, and higher members of these series, are listed in Table III.

TABLE III

MASS SPECTRA: RELATIVE INTENSITY OF IONS CHARACTERISTIC OF 6-SUBSTITUTION

Compound	*n*	m/e				
		249+(n-1)44	*363+(n-1)44*	*377+(n-1)44*	*389+(n-1)44*	*405+(n-1)44*
*s*6	1	0.4	0.33	0.04	0.30	0.14
*s*66	2	2.2	0.30	0.04	0.17	0.09
*s*666	3	4.5	0.28	0.07	0.25	0.08
*s*6666	4	4.7	0.35	0.03	0.21	0.06
*s*66666	5	5.0	0.34	—	0.30	—

The peak at $m/e = 377+(n-1)44$ probably represents the ion C_2 defined by Kochetkov and Chizhov[8]. The peaks at $m/e = 389+(n-1)44$ and $405+(n-1)44$ can be explained as arising from splitting off of trimethylsilanol, trimethylsiloxyl groups, and methyl groups, M-90-90-15 and M-89-90. The relative intensity of the ion $m/e = 249+(n-1)44$ increases with increasing number of ethylene oxide units.

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A GALACTOMANNAN FROM THE SEEDS OF *Sesbania grandiflora pers*

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Received October 9th, 1967)

ABSTRACT

A galactomannan, having $[\alpha]_D$ +50° and a D-galactose–D-mannose ratio of 1:2, has been isolated from the seeds of *Sesbania grandiflora pers.* Methylation of the polysaccharide, followed by hydrolysis, affords 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,3,6-tri-*O*-methyl-D-mannose, and 2,3-di-*O*-methyl-D-mannose in equimolecular proportions. Periodate oxidation of the polysaccharide, followed by reduction and hydrolysis, gives glycerol (1 mole) and erythritol (1.8 mole). The structural significance of these results is discussed.

INTRODUCTION AND DISCUSSION

Galactomannans occur as water-soluble polysaccharides in the seed endosperms of legumes[1]. The two most well-known and industrially important galactomannans are from guar (*Cyamopsis tetragonolobus*) seeds[2] and from the locust bean (*Cerotonia siliqua L.*). Many other galactomannans, such as those isolated from the seeds of *Trifolium pratense*[4], *Trifolium repens L.*[5], *Trigonella foenum graecum*[6], and *Medicago sativa*[7] have been studied. Although the galactose to mannose ratio varies in different galactomannans, the main structural features are similar and involve branched structures comprising a backbone of β-(1 → 4)-linked D-mannopyranose residues which carry, through position 6, α-D-galactopyranose residues. The physical properties of the galactomannans are dependent upon the degree of branching and the degree of polymerization (*D.P.*) of the polymer.

This communication is concerned with the structure of a galactomannan isolated from the seed tegmen (inner seed-coat) of *Sesbania grandiflora pers.* Recently, Rao and Rao[8] have studied this galactomannan, but the structural aspects were not investigated in any detail.

The polysaccharide, purified by fractionation with Fehling's solution, had $[\alpha]_D^{28}$ +50° in water. Hydrolysis with acid produced galactose and mannose in the

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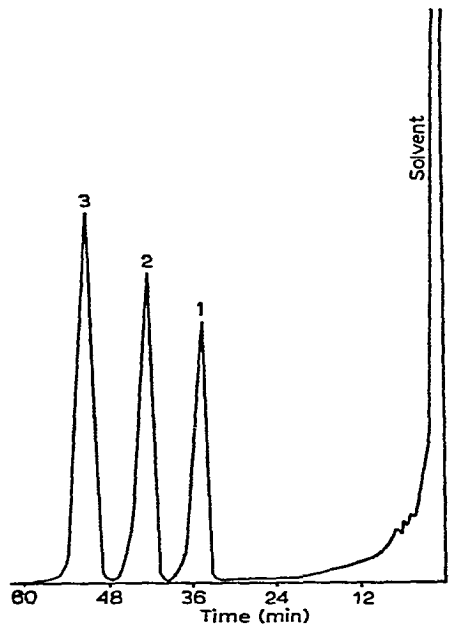
molar ratio of 1:2, as determined by the phenol–sulphuric acid method[9] and by a g.l.c. method (Fig. 1).

The galactomannan was exhaustively methylated, and the permethylated polysaccharide, which had $[\alpha]_D^{28}$ +42° (chloroform), was hydrolysed with sulphuric acid[10] to afford 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,3,6-tri-*O*-methyl-D-mannose, and 2,3-di-*O*-methyl-D-mannose. Quantitative determination of the methylated sugars was carried out by (1) separation of the methylated sugars on paper, followed by extraction and weighing, and (2) conversion of the methylated sugars into the corresponding methyl glycosides, trimethylsilylation, and analysis of the derivatives by g.l.c. (Fig. 2). Both these methods showed that the three methylated sugars were present in equimolar proportions.



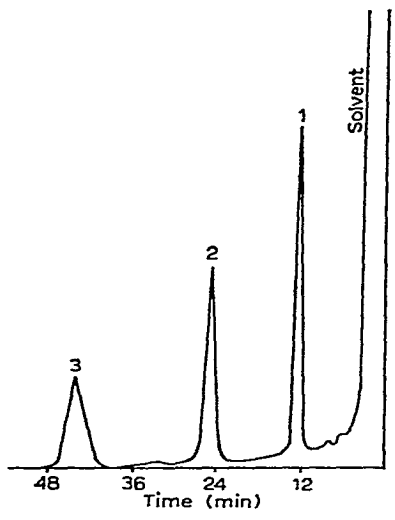
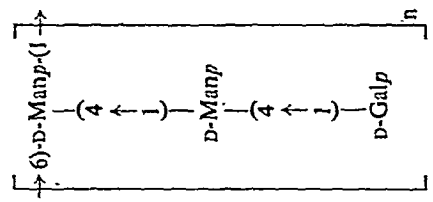


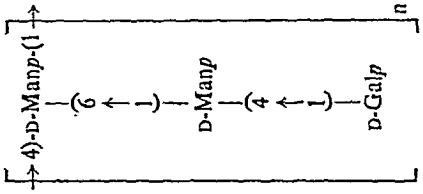
Fig. 1. Gas–liquid chromatogram of methylated galactomannan after hydrolysis and remethylation: 1, methyl 2,3,4,6-tetra-*O*-methyl-α-D-mannoside; 2, methyl 2,3,4,6-tetra-*O*-methyl-α,β-D-galactoside; 3, methyl 2,3,4,6-tetra-*O*-methyl-β-D-mannoside.

Fig. 2. Gas–liquid chromatogram of methylated galactomannan after hydrolysis, glycosidation and trimethyl silylation: 1, methyl 2,3-di-*O*-methyl-4,6-di-*O*-trimethyl silyl-α-D-mannoside; 2, methyl 2,3,6-tri-*O*-methyl-4-*O*-trimethyl silyl-α-D-mannoside; 3, methyl 2,3,4,6-tetra-*O*-methyl-α,β-D-galactoside.

The galactomannan consumed 1.2 moles of sodium metaperiodate, with concomitant liberation of 0.33 mole of formic acid, per "anhydrohexose" unit. The oxopolysaccharide was reduced with sodium borohydride, and the derived polyalcohol was hydrolysed with acid (Smith degradation[11]) to produce glycerol and erythritol in a molar ratio of 1:1.8. None of the sugar units in the galactomannan survived periodate oxidation.


→6)-D-Manp-(1→
(4←1)
D-Manp
(4←1)
D-Galp
n

4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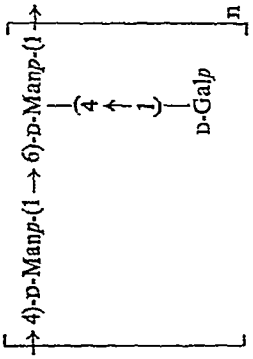
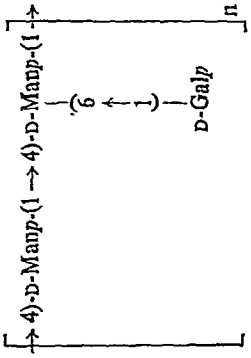
(6←1)
D-Manp
(4←1)
D-Galp
n

3


→4)-D-Manp-(1→6)-D-Manp-(1→
(4←1)
D-Galp
n

2


→4)-D-Manp-(1→4)-D-Manp-(1→
(6←1)
D-Galp
n

1

Based on the results of methylation and periodate oxidation of the galactomannan from *Sesbania* seeds, four structures (**1–4**) may be written for the polysaccharide. In order to decide which of the structures **1–4** represents the galactomannan under investigation, it would be necessary to degrade the polysaccharide by acid and/or an enzyme to obtain oligosaccharides of constitutional significance. However, inasmuch as the specific rotation and the galactose–mannose ratio of this galactomannan correspond very closely with similar data from guar galactomanann[2], the two polysaccharides may have the same chemical structure (**1**).

EXPERIMENTAL

Paper chromatography was carried out on Whatman No. 1 paper with the following solvent systems (all v/v): (*a*) butyl alcohol–pyridine–water (6:4:3), (*b*) butanone–water azeotrope, (*c*) benzene–ethanol–water–ammonia (200:47:14:1). Gas–liquid chromatography (g.l.c.) was carried out on a Pye-Argon instrument with β-ionisation detection, using a four-foot column of 20% poly(butane-1,4-diol succinate) on Celite 545 at 175°; flow rate, 40 ml/min. Demethylation was carried out with boron trichloride.

Isolation and purification of the polysaccharide. — The tegmen portion (10 g) of *Sesbania grandiflora* seeds in the form of a dry powder was extracted four times with 10% sodium hydroxide, and the extract, after cooling, was precipitated with ethanol. The crude polysaccharide was fractionated twice *via* its copper complex to give a white, amorphous powder (3.3 g), $[\alpha]_D^{28}$ +50.0° (*c* 0.5, water).

Hydrolysis of the polysaccharide (10 mg) with N sulfuric acid (1 ml) for 16 h at 100°, followed by neutralization and paper chromatography, showed the presence of galactose and mannose. Quantitative determination of the sugars in the hydrolysate was carried out by the phenol–sulphuric acid method[9]. The galactose:mannose ratio was found to be 1:1.8.

Methylation of the galactomannan. — The galactomannan (4.5 g) was methylated successively with methyl sulphate and alkali[15], methyl iodide–barium oxide–methyl sulphoxide[16], and with methyl iodide and silver oxide[14], to yield a thick, clear-yellow syrup (2.2 g). The low yield of the methylated product was due to the formation of an oily complex (during methylation with the second reagent) from which the polysaccharide derivative could not be recovered completely. The syrupy product was fractionated with acetone–ethyl ether to afford the galactomannan methyl ether {2 g; $-OCH_3$, 42%; $[\alpha]_D^{28}$ +42° (*c* 0.5, chloroform)}. It showed no hydroxyl absorption in the i.r. spectrum, and further methylations with the Purdie reagents did not increase its methoxyl content.

Determination of sugar ratio by g.l.c. — The methylated polysaccharide was hydrolysed, and the hydrolysate (50 mg) was permethylated by one methylation each with (*i*) methyl iodide–barium oxide–barium hydroxide–*N*,*N*-dimethylformamide[13] and (*ii*) methyl iodide and silver oxide[14]. The mixture of fully methylated methyl glycosides of mannose and galactose was examined by g.l.c. (Fig. 1). The ratio of

galactose to mannose, on the basis of the peak areas of their methylated methyl glycosides, was found to be 1:2.

Hydrolysis and identification of the methylated sugars. — The methylated polysaccharide (1.8 g) was hydrolysed according to the method of Croon *et al.*[10]. Paper chromatography of the hydrolysate in solvents (*b*) and (*c*) showed the presence of methylated sugars which had mobilities identical with those of 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,3,6-tri-*O*-methyl-D-mannose, and 2,3-di-*O*-methyl-D-mannose. The methylated sugars were separated quantitatively on paper and were found to be present in equimolar proportions.

Quantitative determination of the methylated sugars was also carried out by another method. The mixture (50 mg) was treated with 3% methanolic hydrogen chloride, and the resulting methyl glycosides were trimethylsilylated[17]. The product was examined by g.l.c. (Fig. 2), and, on the basis of the peak areas of their derivatives, the three methylated sugars were found to be present in equimolar proportions.

Characterization of methylated sugars. — *2,3,4,6-Tetra*-O-*methyl*-D-*galactose.* The syrup, $[\alpha]_D$ +109° (*c* 0.5, water)[18], on demethylation produced primarily galactose. The derived "anilide" had m.p. and mixed m.p.[19] 196°. *2,3,6-Tri*-O-*methyl*-D-*mannose.* The syrup, $[\alpha]_D$ −9° (*c* 0.5, methanol)[20], on demethylation produced mainly mannose. It was treated with *p*-nitrobenzoyl chloride, in the usual manner, to afford 2,3,6-tri-*O*-methyl-1,4-di-*O*-(*p*-nitrobenzoyl)-D-mannose, m.p. and mixed m.p.[21] 186°. *2,3-Di*-O-*methyl*-D-*mannose.* The syrup, $[\alpha]_D^{28}$ +6° (*c* 0.5, methanol)[22], had the same mobilities on paper chromatograms and electrophoretograms as an authentic sample of 2,3-di-*O*-methyl-D-mannose. Upon demethylation, it gave preponderantly mannose. Methyl glycosidation, followed by complete methylation, afforded methyl 2,3,4,6-tetra-*O*-methyl-α-D-mannoside which was identified by g.l.c.

Periodate oxidation. — Galactomannan (1 g) was oxidized with sodium metaperiodate (0.05M, 500 ml) at 10°. Aliquots were withdrawn at different time intervals and analysed for periodate consumption[23] and formic acid liberation[24]. The results are given in Table I.

TABLE I

PERIODATE OXIDATION OF GALACTOMANNAN

Time (h)	*7*	*24*	*48*	*72*	*96*
Periodate consumed (mole/mole)	0.87	1.05	1.1	1.18	1.2
Formic acid liberated (mole/mole)	0.11	0.22	0.26	0.34	0.34

Smith degradation. — After the periodate oxidation was complete, the solution was treated with barium carbonate and filtered. To the filtrate, sodium borohydride (0.5 g) was added, and the mixture was stored for 4 h at room temperature and then acidified with acetic acid. The resulting solution was treated with Amberlite IR-120

(H^+) resin and evaporated to dryness, and boric acid was removed from the residue by repeated distillations with methanol. The residue was hydrolysed with N sulphuric acid in a sealed tube for 16 h at 95°. The hydrolysate was neutralised ($BaCO_3$), and then deionised with Amberlite resins IR-120 and IR-45. Analysis by paper chromatography showed the presence of glycerol and erythritol; no sugars were detected. Glycerol and erythritol were separated quantitatively on paper and estimated by the chromotropic acid method[25]. The ratio of these alcohols was found to be 1:1.8.

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THE ACID HYDROLYSIS OF ALKYL β-D-XYLOPYRANOSIDES

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ABSTRACT

Rate coefficients and kinetic parameters were determined for the hydrochloric acid-catalysed hydrolysis of twenty-seven alkyl β-D-xylopyranosides. Application of the Hammett and Bunnett criteria indicates a unimolecular (A-1) mechanism, as does the high, positive entropy of activation. A linear relation between activation entropy and energy in this series was proved by the Exner method, and related to a similar relation for alkyl β-D-glucopyranosides.

INTRODUCTION

Considerable interest has been shown in the acid-catalysed hydrolysis of glycopyranosides. The mechanism now accepted was suggested by Edward[1] and confirmed by several investigators[2-10]. It involves a rapid, equilibrium-controlled protonation of the glycosidic oxygen atom. The slow, rate-determining step involves unimolecular heterolysis of the glycoside conjugate acid to form a cyclic carbonium–oxonium ion (probably in the half-chair conformation) which then reacts rapidly with water to yield the glycopyranose. The mechanism is thus analogous to the A-1 mechanism proposed for the acid-catalysed hydrolysis of acetals[11-12]. The present investigation is concerned with the influence on the rate parameters of alkyl aglycon groups in the acid-catalysed hydrolysis of several alkyl β-D-xylopyranosides.

RESULTS AND DISCUSSION

Twenty-seven alkyl β-D-xylopyranosides were hydrolysed in 0.5M aqueous hydrochloric acid at different temperatures. The kinetic parameters and estimated standard deviations are presented in Table I. All of the reactions are first order, and $\ln k$ is a linear function of $1/T$.

Influence of the acid concentration. — According to the Zucker–Hammett[13] hypothesis, an acid-catalysed reaction, whose transition state behaves like the conjugate acid of the uncharged reactant, will show a linear correlation with unit slope between the logarithm of the rate constant and the Hammett acidity function H_o. Accordingly, k_1 was determined, at constant temperature and various concentrations of hydrochloric acid, for a number of β-D-xylosides (Table II).

TABLE I

RATE COEFFICIENTS AND KINETIC PARAMETERS FOR THE HYDROLYSIS OF ALKYL β-D-XYLOPYRANOSIDES IN 0.5M HYDROCHLORIC ACID

No.	*Aglycon group*	$10^5 k_1$ *sec*$^{-1}$		E	*log* A	ΔS‡ *at 60°*	ΔG‡
		60°	*80°*	*kcal. mole*$^{-1}$		*cal. deg.*$^{-1}$ *mole*$^{-1}$	*kcal. mole*$^{-1}$
1	Methyl	0.61	10.8	33.21±0.19	16.6	+16.1	27.49
2	Ethyl	0.91	14.4	32.33±0.46	16.2	+14.1	26.95
3	Propyl	0.97	15.1	32.11±0.33	16.1	+13.6	27.21
4	Butyl	0.95	14.4	31.79±0.22	15.8	+12.6	27.11
5	Pentyl	0.91	14.7	32.48±0.60	16.3	+14.6	26.94
6	Hexyl	1.03	15.5	31.67±0.60	15.8	+12.4	26.86
7	Heptyl	0.99	15.6	32.22±0.51	16.1	+14.0	26.89
8	2-Methyl-1-propyl	1.02	14.9	31.40±0.20	15.6	+11.5	26.87
9	3-Methyl-1-butyl	0.94	13.3	30.97±0.13	14.0	+10.1	26.92
10	2,2-Dimethyl-1-butyl	1.13	16.6	31.40±0.10	16.7	+11.8	26.80
11	2,2-Dimethyl-1-propyl	1.18	17.9	31.70±0.20	15.9	+12.9	26.77
12	Benzyl	0.88	14.4	32.70±0.11	15.5	+15.1	26.96
13	2-Phenethyl	1.05	16.0	31.82±0.50	15.9	+12.9	26.85
14	3-Phenylpropyl	0.72	11.5	32.43±0.32	16.1	+14.0	27.10
15	Isopropyl	2.00	30.3	31.76±0.26	16.1	+14.0	26.42
16	3-Pentyl	3.32	48.7	31.38±0.22	16.1	+13.9	25.76
17	*tert*-Butyl	77.0	1000	30.26±0.16	16.7	+16.7	24.01
18	2-Methyl-2-butyl	266	3240	29.21±0.17	16.6	+16.0	23.19
19	Cyclopentyl	1.93	26.7	30.72±0.31	15.6	+10.8	26.44
20	Cyclohexyl	2.44	34.1	30.78±0.22	15.6	+11.6	26.29
21	4-Methylcyclohexyl	2.05	27.1	30.20±0.32	15.0	+ 9.4	26.40
22	3-Methylcyclohexyl	2.07	93.3	30.96±0.21	15.6	+11.6	26.40
23	2-Methylcyclohexyl	2.03	28.1	30.77±0.23	15.5	+11.0	26.41
24	2-Chloroethyl	1.38	21.0	31.80±0.16	16.0	+13.4	26.76
25	3-Bromopropyl	0.73	12.6	33.20±0.26	16.6	+16.3	27.09
26	2-Methoxyethyl	0.97	15.8	32.68±0.14	16.4	+15.3	26.91
27	2-Hydroxyethyl	1.01	15.4	31.81±0.19	15.9	+13.2	26.87

In all cases, $\log k_1$ showed a linear dependence on H_o, but the slopes were greater than unity. A least-squares fit of the data yielded the values given in Table III, where b represents the slope, s_b the estimated standard-deviation, and n the number of points. A t-test indicates the deviation to be significant, at better than the 0.99 level of significance in all cases, except for the 2-chloroethyl derivative where the deviation is not significant. The requirement of unit slope is thus only approximately fulfilled. These cases have been reviewed by Bunnett[14] who suggested that plots of $\log k + H_o$ *versus* the logarithm of the activity of water ($\log a_{H_2O}$) should be linear, and the slopes should define a new parameter (w), characteristic of the reaction. The slope of the line $\log k - \log (HCl)$ *versus* $\log a_{H_2O}$ defines the parameter w^*. Both plots are distinctively curved, however, and the slopes are dependent on the acid concentration. It is thus impossible to calculate the w and w^* parameters. The most-extreme values, estimated graphically, are given in Table III.

TABLE II

INFLUENCE OF THE ACID CONCENTRATION

	$10^5 k_1$ sec^{-1} at 60.2°									
HCl, M	0.1	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	5.0
H^a_o	+0.98	+0.20	−0.20	−0.47	−0.69	−0.87	−1.05	−1.23	−1.40	−1.76
Aglycon group										
Methyl	—	0.66	1.75	3.48	5.94	—	15.9	—	37.3	88.6
Ethyl	—	0.95	2.47	4.94	8.53	—	23.6	—	57.5	140
2-Methyl-1-propyl	—	0.98	2.57	—	9.45	—	33.2	—	79.5	194
Cyclohexyl	—	2.44	6.74	—	24.6	—	68.4	—	181	421
Isopropyl	—	2.04	6.07	12.3	21.3	37.3	59.3	—	148	—
3-Pentyl	—	3.29	9.20	18.9	31.8	—	91.1	147	233	—
tert-Butyl[b]	2.87	19.5	—	—	205	—	498	—	—	—
2-Methyl-2-butyl[c]	—	7.73	21.6	42.6	—	115	225	—	—	—
2-Chloroethyl	—	—	3.64	—	12.4	—	30.2	—	64.3	149

[a]From Ref. 18. [b]At 50.2°. [c]At 35.0°.

TABLE III

SLOPES OF THE HAMMETT PLOTS; w AND w^* PARAMETERS

Aglycon group	b	s_b	n	w		w*	
Methyl	−1.09	0.01	7	−0.5 to −2.0		−5.7 to −12	
Ethyl	−1.12	0.01	7	−0.5	−2.4	−6.8	−12
2-Methyl-1-propyl	−1.22	0.03	6	−0.7	−5.3	−6.0	−17
Cyclohexyl	−1.16	0.01	6	0	−4.0	−5.8	−16
Isopropyl	−1.16	0.01	6	−1.0	−3.8	−7.5	−14
3-Pentyl	−1.16	0.03	7	−0.1	−5.8	−13.0	−81
tert-Butyl	−1.11	0.01	4	−0.7	−7.2	−6.2	−13
2-Methyl-2-butyl	−1.09	0.004	5	−1.0	−6.7	−7.3	−17
2-Chloroethyl	−1.03	0.02	5	−0.1	−1.8	−5.0	−10

In Hammett plots, slopes that are greater than unity correspond to negative w parameters, which are within the range of w values for reactions classified by Bunnett as proceeding by the unimolecular mechanism. Although this criterion thus supports the unimolecular mechanism in the case of β-D-xylosides, it remains to be seen if it is of general application in the hydrolysis of glycosides.

From the data of Timell (Ref. 7; Table XIII) for the hydrolysis of methyl α-D-glucoside in three mineral acids, the slopes (b) in the Hammett plot and the w and w^* values were calculated:

	b	w	w^*
sulphuric acid	−0.79	+1 to +5	−3 to −7
perchloric acid	−0.91	+1.6 (?)	−5 to −8
hydrochloric acid	−1.06	−0.8 to −2	−5 to −14

Values for the hydrochloric acid-catalysed reaction are in accordance with our results and with the unimolecular mechanism. The values for sulphuric and perchloric acid, however, lead to the conclusion that the hydrolysis should proceed by an S_N2 mechanism, entailing a nucleophilic attack of water on the conjugate acid. A difference in mechanism seems, however, highly improbable. It is noteworthy that all reactions for glycoside hydrolysis, classified by Bunnett as proceeding by an S_N2 mechanism, were performed in perchloric acid. If these reactions had been carried out in hydrochloric acid, w would probably have been negative (as can be seen from the data for methyl α-D-glucoside) and the reactions classified as S_N1. It is clear that, in these cases, conclusions about the mechanism are rather doubtful and cannot invalidate the evidence from other criteria.

As suggested by Timell[7], the conversion of the glycoside into its conjugate acid should be more complete in a strongly acidic solution, and an electron-attracting (repelling) group in the aglycon might increase (decrease) the rate of hydrolysis. Timell compared (at 60°) the rates of hydrolysis of 2-methoxyethyl and methyl β-D-glucoside in 0.5M and 5M sulphuric acid and found the ratios 2-methoxyethyl–

methyl to be 1.33 (0.5M) and 1.23 (5M), respectively. There was accordingly no increase in the relative rates, rather a decrease.

We have compared the rates of hydrolysis of several β-D-xylosides (having electron-repelling aglycon groups), in increasing concentrations of hydrochloric acid, with the rate of hydrolysis of methyl β-D-xyloside. As can be seen from Table IV, the relative rate increases slightly, but steadily, with increasing strength of acid. On the other hand, with an electron-attracting group (2-chloroethyl), the rate decreases, in agreement with the ratio reported by Timell. The polar effect of the aglycon group is thus more pronounced at high concentrations of acid.

TABLE IV

RELATIVE RATE (k_1 ALKYL/k_1 METHYL) AT 60°

HCl, M	*Isopropyl*	*3-Pentyl*	*2-Methyl-1-propyl*	*Cyclohexyl*	*Ethyl*	*2-Chloroethyl*
0.5	—	5.00	1.29	3.63	1.44	—
1.0	3.48	5.26	1.47	3.83	1.43	2.08
1.5	3.51	5.40	—	—	1.40	—
2.0	3.61	5.39	1.60	4.17	1.44	2.08
3.0	3.73	5.73	2.09	4.30	1.48	1.90
4.0	3.97	6.26	2.13	4.85	1.54	1.72
5.0	—	—	2.19	4.75	1.58	1.68

Isokinetic relationship. — According to Exner[15], a linear relation between activation enthalpy and entropy in a series of similar reactions can be proved by plotting two values of log k, obtained at two different temperatures, against each other. Using the k_1 values from Table I, a plot of log 10^7 k at 60° *versus* log 10^7 k at 80° indeed gives a linear relationship for β-D-xylosides **1** to **27**, with the exception of **17** and **18**. Regression analysis leads to the equation:

log 10^7 k (80°) = 1.424 + 0.883 log 10^7 k (60°), with slope b = 0.883 ±0.021, β = 646°K, T_2/T_1 = 333/353 = 0.943, n = 25, the correlation coefficient r = 0.993, and the standard error of the estimate $s_{y/x}$ = 0.02.

According to the Exner classification, these hydrolyses represent the case 3/*a* or compensation, where both activation parameters are variable, in the sense that their effects partially compensate each other. The isokinetic temperature β was calculated to be 646°K. The points for tertiary alkyl derivatives (**17** and **18**) do not fit the isokinetic line. The deviations are small, but statistically significant. It is known[10] that glycosides of tertiary alcohols are hydrolysed by a unimolecular mechanism in which the oxygen–aglycon bond is cleaved.

The same reaction series was analysed by the Leffler[16] method, in which E was plotted *versus* $\Delta S^{\ddagger}$. In this plot, secondary and tertiary alkyl derivatives deviate from the isokinetic line. Regression analysis leads to the equations:

(*A*) With secondary, but without tertiary derivatives,
$E = 26.412 + 0.411\Delta S^{\ddagger}$,
with $\beta = 411 \pm 29$°K,
$s_{y/x} = 0.254$, $n = 23$, and $r = 0.947$.

(*B*) Without secondary and tertiary derivatives,
$E = 26.252 + 0.427\Delta S^{\ddagger}$,
with $\beta = 427 \pm 20$°K,
$s_{y/x} = 0.175$, $n = 21$, and $r = 0.977$.

The correlation is thus better without secondary alcohols. It seems that, in this case, a Leffler plot is not merely an error slope but reflects a real isokinetic relationship. There are, however, some significant differences with the Exner plot. The Exner method gives a higher isokinetic temperature, does not differentiate between primary and secondary aglycons, reduces the deviations for tertiary aglycons, but gives a better correlation. From the data of Timell (Ref. 7, Tables VI and VIII; Ref. 8, Table III) for the hydrolysis of alkyl β-D-glucopyranosides, the Exner equation, calculated by regression analysis, yields:

$\log 10^7 k\ (80°) = 1.391 + 0.881 \log 10^7 k\ (60°)$,
with slope $b = 0.881 \pm 0.021$, $\beta = 637$°K, $T_2/T_1 = 0.943$, $s_{y/x} = 0.015$, $n = 19$, and $r = 0.995$.

This equation also fits the data of Timell (Ref. 17; Table I) for the hydrolysis of methyl mono-*O*-methyl-β-D-glucopyranosides and 6-*O*-(mono-*O*-methyl-β-D-glucopyranosyl)-D-glucoses. The data for all of these glycosides lead thus to function lines having the same slope, which results in the same isokinetic temperature for all groups. If these data of Timell are used to construct a Leffler plot, one finds the equation:

$E = 28.46 + 0.353\Delta S^{\ddagger}$, with $\beta = 353$°K.

Several points, however, do not fit the line, *e.g.*, those for neopentyl, carboxymethyl, and propyl β-D-glucosides. Methyl mono-*O*-methyl-β-D-glucosides and methyl mono-*O*-methyl-β-D-gentiobiosides fit the line, except for the 3-*O*-methylated and 4-*O*-methylated compounds of both groups, which strongly deviate.

From the data of Timell (Ref. 8; Table I) for the hydrolysis of alkyl α-D-glucosides in 0.5M sulphuric acid, we calculated the equation:

$\log 10^8 k\ (80°) = 1.24 + 1.02 \log 10^8 k\ (60°)$,
with slope $b = 1.02 \pm 0.03$, $\beta = +90$°K, $r = 0.998$, $n = 6$, $s_{y/x} = 0.04$, $T_2/T_1 = 0.943$, and $(T_1 + T_2)/2 = 343$°K.

For these α-D-glucosides, a classification according to Exner is difficult, considering the uncertainty in the β value. The most-probable value $\beta = 90$°K ($b = 1.02$) represents case 3/*b* or compensation with $b > 1$ and $\beta < 333$°K. If *b* is taken as 0.99, the corresponding β value is -50°K. This represents case 4, the reverse of compensation, with $T_2/T_1 < b$ and $\beta < 0$. A Leffler plot is impossible because of the high scattering of the experimental points.

It follows from the above results that, in all cases, there exists an isokinetic relationship. The isokinetic temperature is different for β- and α-D-glycosides but does not equal the mean temperature of the experiments. Although log k is approximately constant within the whole series, the relationship between $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ seems to be real in these cases. According to Exner[15], who cites a theory of Palm, the isokinetic temperature should be the same in various reaction series if the substituent affects the reaction centre in the same and simple manner. This is highly probable in the case of β-D-glucosides and β-D-xylosides, which have, indeed, the same β value. On the other hand, the axial position of the alkyl groups in α-D-glucosides should result in a different influence on the reaction centre and hence in a different isokinetic temperature. Because of the lack of data and the close resemblance of the compounds, it remains to be proven that this is more than a coincidence.

Effect of the aglycon. — The effect of the aglycon group can be seen from Table I. The fact that the size of the aglycon is not important is in accordance with the unimolecular reaction mechanism and the equatorial position of the aglycon. Even bulky groups, if separated by at least one methylene group from the D-xylose molecule, as in the case of aromatic and branched-chain primary alcohols, have no effect. Secondary and cyclic alkyl groups produce a slightly higher rate. This can be explained by the steric strain imposed on the initial state of the molecule by the bulky groups which, in this case, are not separated from the glycon by a methylene group. A possible explanation would be the polar influence of the electron-repelling isopropyl and 3-pentyl group (σ^* −0.19 and σ^* −0.225, respectively). This effect increases the equilibrium concentration of the conjugate acid, but decreases the rate of glycosyl–oxygen bond heterolysis. The higher rate indicates that the effect on protonation should predominate. In this case, however, 2-chloroethyl (σ^* +0.38) should decrease the reaction rate. Actually, the rate slightly increases. Furthermore, it can be seen from Table I that introduction of polar groups in the aglycon has no influence on the reaction rate, probably because the two opposing effects will tend to cancel out. It is thus doubtful if, in the case of secondary alkyl groups, the increased rate can be explained by the cancelling polar effects.

A third possibility is that the mechanism for secondary alkyl β-D-xylosides stands somewhere in a gradual series ranging from the mechanism for primary alkyl compounds to that for tertiary alkyl compounds, and proceeds to a certain extent *via* a secondary carbonium ion. In this case, electron-repelling groups will promote both protonation and heterolysis. It may be recalled that the points for secondary alkyl β-D-xylosides represent an intermediary case between primary and tertiary compounds in the Leffler plots. The values for entropy and energy of activation are rather constant within this series. The large, positive entropies of activation are characteristic of acid-catalyzed cleavage of carbon–oxygen bonds where the slow heterolysis step is unimolecular, and thus support the A-1 mechanism.

EXPERIMENTAL

The synthesis of the alkyl β-D-xylopyranosides was performed as described

previously[19–21]. The polarimetric measurements were carried out at 436 nm, with a Perkin–Elmer model 141 photoelectric polarimeter, in jacketed polarimeter tubes connected to an ultrathermostat bath, accurate to within 0.05°.

A solution of the D-xyloside (0.05 to 0.025M) in hydrochloric acid was placed in the tube, and readings were started after the temperature equilibration was complete. The first-order rate coefficients ($\ln e$, sec^{-1}) were calculated from least-squares, straight-line fits of plots: $\log(\alpha_t \pm \alpha_\infty) = \log(\alpha_0 \pm \alpha_\infty) - kt$. Because α_∞ is a function of the temperature and the acid concentration, these values were determined experimentally for corresponding D-xylose solutions. The expected, infinite-time rotations were checked by actual measurements, and agreed within experimental error. As a check, some rate coefficients were determined by the Guggenheim[22] method. These duplicate runs agreed within the estimated error.

The energy of activation (E), $\log A$, and the estimated standard-deviation were calculated from the least-squares, straight-line fits of Arrhenius plots. A minimum of five rate constants was determined, usually at 60 to 80°. Calculations of other thermodynamic activation-functions were based on absolute-reaction rate theory. The entropy of activation ($\Delta S^\ddagger$) was calculated for 60° from the relationship:

$$T\Delta S^\ddagger = E - RT - RT \ln (kT/h) + RT \ln (k_1/h_0),$$

where k_1 represents the first-order rate coefficient (sec^{-1}), and $h_0 = 0.631$ for 0.5M hydrochloric acid. The other terms have their usual significance. The uncertainty in $\Delta S^\ddagger$ is ± 1 e.u., corresponding to a standard deviation of 2% in the k_1 values.

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Notes

Separation of the anomers of glycosyl phosphates

The separation of one anomer of a glycosyl phosphate from the other by chromatographic or ion-exchange techniques is made very difficult by the close similarity in behavior, in such systems, of the members of any pair of anomers. As yet, no uniformly successful method is available for such a separation. Attention has recently been turned in this laboratory to the problem, which had arisen in connection with the synthesis of such phosphates by the fusion procedure[1]. Although some measure of success has been achieved, it is apparent from the work now reported that the search for a separation method applicable to any anomeric pair of glycosyl phosphates is still hampered by a number of as-yet-unidentified factors.

The paper or thin-layer chromatographic separation of a pair of anomers of a glycosyl phosphate from each other has met with very limited success. Although a two-dimensional system of paper chromatography has been developed for the separation from each other of a wide variety of phosphorylated carbohydrates[2], the system does not provide good separation of anomeric pairs and, in addition, some of the more labile glycosyl phosphates decompose in the alcoholic picric acid used as the solvent. Recently, the anomers of D-glucopyranosyl phosphate have been separated from each other on paper in an acid solvent[3], and this system may be applicable to other pairs of anomers. A separation (on paper) having no practical importance has been described, for the anomers of 2-deoxy-D-*erythro*-pentofuranosyl phosphate[4], that uses ammoniacal solvents and excessively long development times. Appropriate derivatives of a number of phosphorylated sugars have been separated by gas–liquid chromatography[5,6], and the selectivity of this method should be ideally suited to the separation of anomeric glycosyl phosphates. However, such phosphates are presumably too labile to withstand the conditions of gas–liquid chromatography. The trimethylsilyl derivative of α-D-glucopyranosyl phosphate decomposes to give three peaks, only one of which, that due to inorganic phosphate, was identified[6].

Ion-exchange chromatography has been used successfully for certain separations. The anomeric 2-acetamido-2-deoxy-D-glucopyranosyl phosphates were resolved satisfactorily on Dowex-1 (Cl^-) by use of dilute hydrochloric acid as the eluant[7]. Similarly, the anomers of D-glucopyranose 1,6-diphosphate are separable from each other on a column of Dowex-1 ($HCOO^-$) by use of a linear pyridine–formic acid gradient[8] at pH 3. Each of these separations involved relatively stable glycosyl phosphates, and the acidic conditions used would not be ideal for those of normal lability to acid. Adequate separations under neutral conditions that have been reported include (*a*) the anomers of D-galactopyranosyl phosphate by use of Dowex-1 (Cl^-) and

elution with ammonium chloride[9], and (*b*) the anomers of 2-acetamido-2-deoxy-D-glucopyranosyl phosphate by use of gradients of lithium chloride or ammonium chloride on[10] Dowex-1 (Cl^-).

In the present paper are recorded efforts to achieve an ion-exchange separation of the anomers of glycosyl phosphates from each other under neutral or slightly basic conditions. The separations were all made on Dowex-1-X8 (analytical grade, 100–200 mesh) converted into the appropriate salt form, and the readily available anomers of D-glucopyranosyl phosphate were chosen as model compounds. By use of various gradients of lithium chloride, satisfactory separations were not obtained, the first compound to emerge (the α-D anomer) appearing as a very broad peak. Better results were obtained with dilute calcium chloride and with gradients of calcium acetate, but the products separated were accompanied by vast proportions of the salt. This result suggested the use of volatile salts, and triethylammonium hydrogen carbonate, introduced by Porath[11], was chosen. A satisfactory separation was achieved by use of gradient elution with 0.05M→0.3M salt, pH 7.5. This technique was directly applicable to the separation of the anomers of D-galactopyranosyl phosphate (see Figs. 1 and 2). In each instance, the α-D anomer was the first to emerge. The elution pattern with the anomers of 2-deoxy-D-*erythro*-pentofuranosyl phosphate[4] is devoid of any suggestion of separation (see Fig. 3). When the method was applied to the anomers of D-ribofuranosyl phosphate or D-ribopyranosyl phosphate, only a slight shoulder appeared, in each instance, on the trailing edge of an otherwise symmetrical peak.

Thin-layer chromatography can be used to differentiate between the members of certain pairs of glycosyl phosphates (as their cyclohexylammonium, or other amine, salts). By use of Silica Gel G (Merck) and 5:3:1 ethanol–conc. ammonium hydroxide–water, the best separation was obtained with the 2-deoxy-D-*erythro*-pentofuranosyl phosphates, the α-D anomer having R_F *ca.* 0.22 and the β-D anomer, *ca.* 0.28. The anomers of D-ribopyranosyl, D-ribofuranosyl, and D-xylopyranosyl phosphate were resolved in a less satisfactory manner (in each instance, the β-D anomer was the faster of a given pair). The anomers of D-glucopyranosyl, D-galactopyranosyl, and L-arabino-pyranosyl phosphate were inadequately resolved. The spots were visualized by spraying with anisaldehyde spray and heating[12].

EXPERIMENTAL

The ion-exchange chromatograms were made with a column (3.6 cm diam. × 43 cm) of Dowex-1-X8 (analytical grade, 100–200 mesh) ion-exchange resin, which was converted into the hydrogen carbonate form by washing it with potassium hydrogen carbonate. Triethylammonium hydrogen carbonate of a desired molarity was prepared by making a mixture of the appropriate quantity of triethylamine and water, and saturating it in the cold with carbon dioxide; this solution, prior to use, was brought to room temperature, and excess carbon dioxide was removed by agitation. Prior to use, the ion-exchange column was washed with 0.05M triethylammonium

hydrogen carbonate solution, and then 250 μmoles of each anomer to be separated, or 500 μmoles of a mixture of anomers, in 0.05M triethylammonium hydrogen carbonate was added. The column was developed with a linear gradient of triethylammonium hydrogen carbonate, starting with 2 liters of 0.05M solution in the mixing vessel and 2 liters of 0.3M solution in the reservoir. The flow rate was 1 ml per min, and 15-ml fractions were collected. Samples (0.1 ml) were diluted with Fisk–SubbaRow reagent and acid molybdate to a final volume of 5 ml, and were read at 830 nm after they had been heated[13] for 7 min. The results of the separations are shown in the Figures.

The D-*glucopyranosyl phosphates.* — The mixture to be separated contained 93 mg (250 μmoles) of α-D-glucopyranosyl (dipotassium phosphate) dihydrate and 114 mg (250 μmoles) of β-D-glucopyranosyl (dicyclohexylammonium phosphate). The tubes containing a particular component (see Fig. 1) were combined, and the three

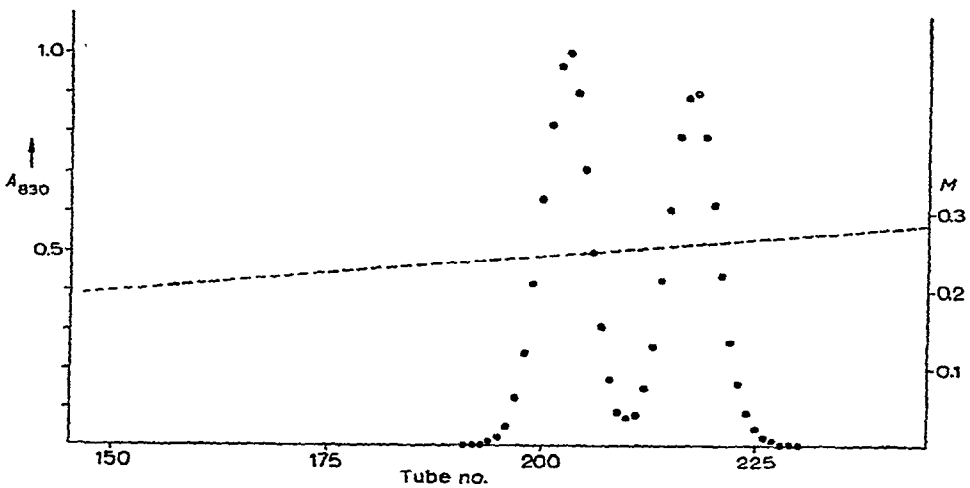

Fig. 1. Ion-exchange chromatography of the anomers of D-glucopyranosyl phosphate. The tube number is plotted against the A_{830} of a sample (0.1 ml) in a total volume of 5 ml. The dotted line represents the gradient of the concentration of triethylammonium hydrogen carbonate.

tubes separating the two components were discarded. Each of the two fractions was concentrated *in vacuo* at *ca.* 15 torr, and the residue was made up to 10 ml with water. Determination of optical rotation and of phosphate indicated 238 μmoles of α-D-glucopyranosyl phosphate (95% recovery) in the first fraction, $[M]_D$ +29,000, and 222 μmoles of β-D-glucopyranosyl phosphate (89% recovery) in the second fraction, $[M]_D$ +2,600. From optical rotations previously recorded for salts of these phosphates, the molecular rotations calculated are +29,000 and +2,700, respectively. The phosphates can be converted into appropriate salts, and these can be crystallized. In this way were obtained α-D-glucopyranosyl (dipotassium phosphate) dihydrate (73.6 mg; 79%), $[\alpha]_D^{23}$ +77.6° (lit.[14] +78°) and β-D-glucopyranosyl (dicyclohexylammonium phosphate) (76.3 mg; 66%), $[\alpha]_D^{23}$ +5.3° (lit.[15] +5.9°).

The D-*galactopyranosyl phosphates.* — A sample of β-D-galactose pentaacetate gave, on short (5 min)[15] fusion with anhydrous phosphoric acid, a 47% yield of acid-labile phosphate. On the basis of the optical rotation of the barium salt isolated, the material was an almost equimolar mixture of the anomers. The mixture was converted into the cyclohexylammonium salt, and some of the α-D anomer was removed by crystallization. A sample of the mother liquors, containing 500 μmoles of phosphate, was applied to the column, and separated as already described. The first fraction (see Fig. 2) contained 199 μmoles of α-D-galactopyranosyl phosphate, $[M]_D$ +36,000, and the second fraction, 304 μmoles of β-D-galactopyranosyl phosphate, $[M]_D$ +9,700. Putman and Hassid[16] reported +36,000 and +10,000 for the respective molecular rotations of these compounds.

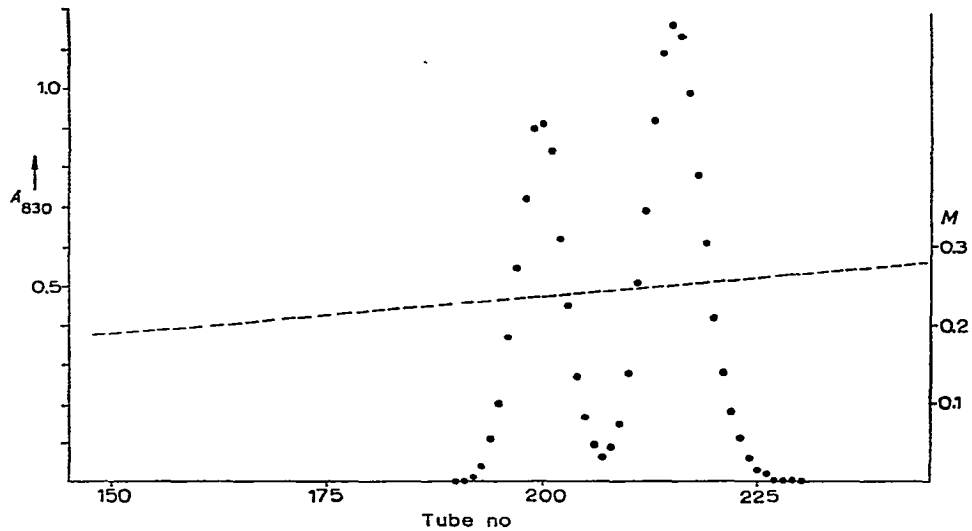

Fig. 2. Ion-exchange chromatography of the anomers of D-galactopyranosyl phosphate. (Depiction as in Fig. 1.)

*The 2-deoxy-*D-erythro-*pentofuranosyl phosphates.* — A sample (500 μmoles) of the cyclohexylammonium salt of 2-deoxy-D-*erythro*-pentofuranosyl phosphate[4] was applied to the column. On the basis of its optical rotation (+2.4°), this material consisted of one part of α-D anomer to two parts of β-D anomer. As may be seen from Fig. 3, no separation occurred.

The D-*ribofuranosyl phosphates.* — A mixture of β-D-ribofuranose tetraacetate[17] (1 g) and anhydrous phosphoric acid (1 g) in 0.8 ml of dry tetrahydrofuran was heated for 2 h at 50°. The solution was cooled, and basified by the addition of 22 ml of cold 2M lithium hydroxide. After 4 h, the precipitated lithium phosphate was removed, and washed with dilute lithium hydroxide; the resulting solution contained labile phosphate in a yield of 42%. Both anomers were present, as indicated by thin-layer chromatography. A sample of this solution, containing 500 μmoles of phosphate, was brought to pH 7 by addition of Dowex 50 (H^+) ion-exchange resin, and was fraction-

ated on the column. The results were similar to those shown in Figure 3, but there was a slight shoulder on the trailing edge of the peak.

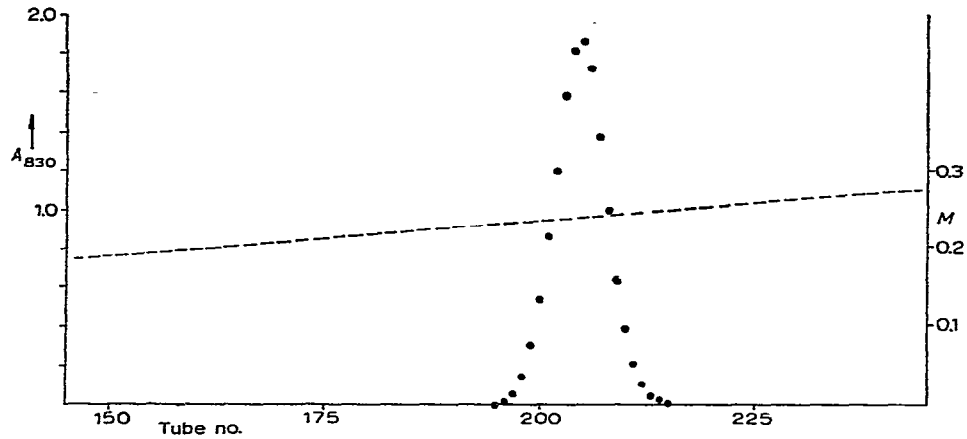

Fig. 3. Ion-exchange chromatography of the anomers of 2-deoxy-D-*erythro*-pentofuranosyl phosphate. (Depiction as in Fig. 1.)

The D-*ribopyranosyl phosphates.* — A sample (0.8 g) of β-D-ribopyranose tetraacetate[17,18] was heated with 1 g of anhydrous phosphoric acid for 2 h at 50°, and processed in the usual way. A mixture of glycosyl phosphates was formed, as shown by t.l.c., in 55% yield. Attempted separation of the anomers by ion-exchange chromatography (500 μmoles, neutralized solution) was likewise unsuccessful.

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Preparation of β-D-mannopyranose and some derivatives*†

In the past, both α-D-mannopyranose and β-D-mannopyranose could be obtained. The β-D anomer was isolated from such traditional sources as ivory nut. However, after crystallization of the α-D anomer had been accomplished, the β-D anomer could not be obtained in the same laboratory[1]. This situation has probably been occasioned by the presence of micro seed-crystals of the α-D anomer which have become common in preparatory laboratories. As a result of the seed-crystal problem, only the α-D anomer of D-mannopyranose is now commercially available.

It has become desirable to synthesize α-D-mannopyranosyl phosphate. The chemically useful routes to the glycosyl phosphates have basically involved reactions of analogous types. Two of the more common procedures are (*a*) the replacement of the halogen atom of the tetra-*O*-acetyl-α-D-aldohexopyranosyl halide by treatment with silver orthophosphate[2,3] or a diester thereof, and (*b*) the direct displacement of the 1-*O*-acetyl group of the fully acetylated sugar by treatment with anhydrous orthophosphoric acid[4]. In both cases, the β-D-pentaacetate is the more synthetically useful intermediate. For this reason, it has been important to obtain crystalline β-D-mannose prior to the acetylation reaction.

By the procedures now described, it has been found possible to (1) obtain β-D-mannopyranose in good yield and purity from ivory nut, (2) convert commercially available α-D-mannose into β-D-mannopyranose, and (3) acetylate the β-D anomer, to form the highly useful β-D-pentaacetate.

EXPERIMENTAL

Preparation of β-D-mannopyranose[5–8]. — Four liters of 1% sodium hydroxide solution in a 6-liter Erlenmeyer flask was heated to boiling on a hot plate equipped with a magnetic stirrer, and 400 g of ivory-nut powder‡ was gradually added with continuous stirring. The flask was removed from the hot plate, and the mixture was stirred intermittently with a glass rod for 30 min, filtered through several layers of cheesecloth, and the residue washed with distilled water until the filtrate was clear. The filter cake was transferred to a Pyrex dish, and dried at 50°.

The dried meal (200 g) was hydrolyzed by adding it slowly, with constant stirring, to a beaker containing 65% (w/w) sulfuric acid (165 ml), while keeping the temperature below 50°. The brownish, dough-like material was kept overnight at room temperature to permit continuation of the hydrolysis. The material was then dissolved in 2 liters of distilled water, and the solution was transferred to a 5-liter flask and refluxed (heating mantle) for 3 h. Barium carbonate (336 g) was slowly added to the hot hydrolyzate during 1.5 h with continuous heating, and finally until

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‡Obtained from Pfanstiehl Laboratories, Inc., Waukegan, Illinois.

the mixture was neutral to Congo Red paper. The hot suspension was filtered by suction, and the cake was washed with hot, distilled water. Excess barium ions in the filtrate were removed by dropwise addition of 1:20 sulfuric acid–water until no further precipitate of barium sulfate was formed and the solution was not acid to Congo Red paper. Barium sulfate was removed by filtration, and a few ml of acetic acid were added to the filtrate. The solution was cooled to room temperature, decolorized twice with Norit (10 g), the suspension was filtered, and the solution was concentrated at 30° (rotary, flash evaporator) to 60–65% of solids as determined by a refractometer. The resulting, light-yellow solution (100 ml) was added to 200 ml of 95% ethanol, with stirring, and the suspension was kept overnight at room temperature, and filtered. The filtrate was concentrated in a 1-liter, round-bottomed flask to 87–88% of solids, an equal volume of acetic acid was added, and the mixture was warmed and shaken until thoroughly mixed. The solution was allowed to cool to room temperature, and the flask was stoppered and placed in a refrigerator. After several days, with intermittent scratching of the inside walls of the flask, crystallization occurred (crystallization may be hastened by nucleation with crystals of pure β-D-mannose). After 2 to 3 weeks, the crystals were filtered off, and washed twice with acetic acid, several times with 95% ethanol, and once with absolute ethanol. The crystals, protected from dust, were dried in the air, and then in a vacuum desiccator over calcium chloride at room temperature; yield 50 g (25% of the weight of the dried, extracted meal) of β-D-mannopyranose, m.p. 132° (dec.), $[\alpha]_{546}^{25}$ −17° (*c* 4.0, water).

Conversion of α-D-mannopyranose into β-D-mannopyranose. — α-D-Mannose* (10 g) was dissolved in warm, de-ionized, distilled water (*ca.* 10 ml), and the solution was filtered through Whatman No. 1 paper into a well-rinsed, previously unused, 50-ml, round-bottomed flask, and the solution was evaporated to dryness. After 2 h at 30° (rotary, flash evaporator), 2.0 ml of acetic acid was added, a few seed crystals of β-D-mannose were added, and the mixture was kept for 10 days at 4°, during which time, crystallization occurred. With the aid of ice-cold acetic acid, the crystals were filtered with suction; they were washed with a small volume of cold absolute ethanol, and dried *in vacuo* over phosphorus pentaoxide (Abderhalden dryer) at 65°; yield 6.35 g of β-D-mannose, $[\alpha]_{578}^{25}$ −15.7 → +14.6° (*c* 4.0, water); lit.[9] −17 → +14.6°.

Penta-O-acetyl-β-D-mannopyranose[10]. — Powdered β-D-mannose (10 g; dried as described) was mixed with pyridine (67 g) and acetic anhydride (50 g)†. The flask was stoppered and kept in an ice bath, and the suspension was stirred magnetically until complete dissolution occurred (2 h). To complete the acetylation, the reaction mixture was kept for two days at 4°; the clear, straw-colored solution was then poured, with vigorous stirring, onto finely cracked ice (250 g). The crystals were collected by filtration, air-dried for 2 h, recrystallized from 96% ethanol (30 ml), and washed with cold, absolute ethanol; yield of penta-*O*-acetyl-β-D-mannose, 11.4 g; m.p. 116–117°; $[\alpha]_{578}^{25}$ −24° (*c* 4.0, chloroform); lit.[10] m.p. 117–118°; $[\alpha]_{D}^{25}$ −24.9° (chloroform).

*Obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.

†Pyridine was dried by refluxing it over calcium hydride chips, and both the pyridine and the acetic anhydride were freshly distilled before use.

The acetylation procedure was repeated with 6.4 g of β-D-mannose-^{14}C, and the corresponding proportions of the acetylating agents. The β-D-mannose-^{14}C was prepared, as already described, from a mixture of α-D-mannose-^{14}C and α-D-mannose; yield of the ^{14}C-labeled penta-*O*-acetyl-β-D-mannose, 8.4 g; m.p. 117–118°.

DISCUSSION

Before the isolation and characterization of α-D-mannose, conditions for crystallization of D-mannose from biological sources favored the β-D anomer. Later, however, as with D-glucose, if seeds of the α-D anomer were present, the α-D anomer crystallized.

The present procedure describes the isolation of β-D-mannose from ivory nut. Commercially available α-D-mannose may be converted into the β-D anomer by essentially the same crystallization steps. The product so isolated has a strong negative rotation which reaches the correct equilibrium value for the α,β mixture. Further characterization of the β-D anomer was afforded by direct acetylation to crystalline penta-*O*-acetyl-β-D-mannopyranose; the properties of this compound were the same as those previously reported. These values are quite different from those reported for penta-*O*-acetyl-α-D-mannopyranose, and direct acetylation to yield the crystalline α-D anomer by this procedure has proved impossible.

By employing crystalline penta-*O*-acetyl-β-D-mannopyranose, α-D-mannopyranosyl phosphate has been prepared by displacement, by use of silver phosphate, of the halogen atom from tetra-*O*-acetyl-α-D-mannopyranosyl halides. Alternatively, this sugar phosphate has been prepared by the MacDonald procedure, by direct displacement of the 1-*O*-acetyl group of the β-D-pentaacetate by treatment with crystalline orthophosphoric acid.

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STUDIES ON URONIC ACID MATERIALS

PART XXVII*. THE STRUCTURE OF THE GUM FROM *Acacia nubica* BENTH.

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ABSTRACT

The polysaccharide exuded by *Acacia nubica* trees has a high, positive specific rotation, has low methoxyl and L-rhamnose contents, and contains D-galactose, L-arabinose, and D-glucuronic acid, which is present in two aldobiouronic acids, 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose and 4-*O*-(α-D-glucopyranosyluronic acid-D-galactose. Autohydrolysis experiments gave 3-*O*-β-L-arabinofuranosyl-L-arabinose, 3-*O*-β-L-arabinopyranosyl-L-arabinose, β-(1 → 3)-linked-L-arabinose trisaccharides, and a degraded gum *A* of molecular weight 5,730, which was studied by linkage and methylation analysis. Partial hydrolysis with acid gave 3-*O*-β-D-galactopyranosyl-D-galactose and 6-*O*-β-D-galactopyranosyl-D-galactose. An examination of the *O*-methyl derivative of degraded gum *A* gave 2,3,4,6-tetra-, 2,3,4-, 2,3,6-, and 2,4,6-tri-, and 2,4-di-*O*-methyl-D-galactose; 2,3,4-tri-*O*-methyl-L-arabinose; and 2,3,4-tri-*O*-methyl-D-glucuronic acid. Degraded gum *A* was subjected to a Smith degradation, and the product was examined by linkage and methylation analysis.

The *O*-methyl derivative of the whole gum gave 2,3,4- and 2,3,5-tri-, and 2,5- and 3,5-di-*O*-methyl-L-arabinose; 2,3,4,6-tetra-, 2,4,6-, 2,3,6-, and 2,3,4-tri-, 2,6- and 2,4-di-, and 2-*O*-methyl-D-galactose; and 2,3,4-tri-*O*-methyl-D-glucuronic acid. The whole gum was subjected to five successive Smith-degradations, and the Smith-degraded polysaccharides *S1–S5* were each examined by linkage and methylation analysis.

The structural evidence suggests that *A. nubica* gum molecules possess highly branched D-galactan frameworks, to which are attached D-glucuronic acid residues and L-arabinose-containing side-chains, some of which are at least six units long. The gum contains the largest proportion of L-arabinose in any of the *Acacia* gum exudates studied to date.

INTRODUCTION

In a preliminary study of the gum exudates from several *Acacia* species, *Acacia*

*For Part XXVI, see Ref. 1.

nubica Benth. was found[2] to differ in several interesting respects from the *Acacia* species studied previously. Thus, *A. nubica* gum gave a high, positive specific rotation (+100°), very low methoxyl and rhamnose contents, and the highest arabinose content of any *Acacia* species examined to date. A structural study of the gum from *A. nubica* was therefore undertaken, since these analytical parameters indicated that the gum might provide an example of one extreme structure-type within the genus *Acacia*. Recent studies[1,3] have indicated that structural variations between gums of different *Acacia* species are more pronounced than was at one time supposed.

RESULTS

The crude gum from *Acacia nubica* is dextrorotatory ($[\alpha]_D$ +100°) and is composed (see Table I) of D-galactose, L-arabinose, D-glucuronic acid, and L-rhamnose in the proportions 33:59:7:1. There is a very small methoxyl content (0.05–0.1% for

TABLE I

ANALYTICAL DATA[a] FOR THREE SAMPLES OF *Acacia nubica* GUM

	Sample A		*Sample B*		*Sample C*	
	Crude	*Purified*	*Crude*	*Purified*	*Crude*	*Purified*
Moisture, %	10.4	5.2	11.1	5.0	9.2	5.1
Ash, %	1.54	0.02	1.52	0.01	1.54	0.01
Nitrogen, %	0.20	0.21	0.23	0.21	0.21	0.16
Uronic anhydride, %[b]	6.4	7.3	7.5	7.6	7.6	7.2
Cold-water insoluble, %	1.4	—	1.5	—	1.9	—
pH, 3% solution	4.70	2.5	4.74	—	4.85	—
Free, titratable acidity[c]	0.49	—	0.64	—	0.60	—
$[\alpha]_D^{20}$ (*c* 3.0)	+98°	+101°	+100°	+100°	+99°	+100°
Methoxyl, %[b]	—	0.05	—	0.05	—	0.06
D-Galactose, %	33	29	—	28	—	28
L-Arabinose, %	59	63	—	64	—	64
L-Rhamnose, %[b]	1	0.6	—	trace	—	trace

[a]All data corrected to dry-weight basis. [b]By vapour-phase infrared methods. [c]As ml of 0.02N NaOH per 10 ml of 3% solution.

different specimens), and a small proportion (0.3%) of the uronic acid is probably present as 4-*O*-methyl-D-glucuronic acid. The gum contains nitrogenous material (N, 0.21%) of which some, at least, was shown to be proteinaceous. The crude gum gave a positive test for peroxidase. Paper-chromatographic examination of a solution of crude gum showed that material of low molecular weight was present; after isolation by dialysis, it was shown that free arabinose, galactose, rhamnose, and 3-*O*-*β*-L-arabinopyranosyl-L-arabinose were present. The proportion of rhamnose appeared (visual examination of chromatograms) to be greater than that obtained on complete hydrolysis of the purified gum polysaccharide.

Autohydrolysis experiments. — Reasonably complete autohydrolysis of the gum (5% solution) required 120 h at 98° [*cf.*, autohydrolysis of *A. senegal* gum (uronic acid, 16%) which requires only 50 h at 98°]. During autohydrolysis, the pH decreased from 2.5 to 1.9, and the specific rotation increased from +100 to +108° (24 h) and then decreased to +87° (144 h) as shown in Table II, which also indicates the nature of the reducing sugars liberated. Three arabinose oligomers were detected: 3-*O*-β-L-arabinopyranosyl-L-arabinose(*X*),3-*O*-β-L-arabinofuranosyl-L-arabinose(*Y*), and a trisaccharide (*Z*), identified tentatively as *O*-β-L-arabinopyranosyl-(1 → 3)-*O*-β-L-arabinofuranosyl-(1 → 3)-L-arabinose. Disaccharide *X* has been detected previously in several *Acacia* gums, and disaccharide *Y* has been detected in the gums from *A. pycnantha* and *A. senegal*; trisaccharide *Z* has not been reported previously. The disaccharide, 3-*O*-α-D-galactopyranosyl-L-arabinose, was not detected; it has been reported to be present in the gums from *A. senegal*, *A. cyanophylla*, and *A. karroo*.

Degraded gum *A*, isolated after autohydrolysis (yield 24%; methoxyl, 0.1%; $[\alpha]_D$ +44°), contained glucuronic acid (12%), galactose (83%), and arabinose (5%); its reducing end-group was shown to be a galactose unit, and its degree of polymerisation[4] was found to be 33 (M_w = 5,730). This value must be compared with the value of 4,800 obtained for a sample of *A. senegal* gum degraded by autohydrolysis[5]. Mild hydrolysis of degraded gum *A* gave 3-*O*-β-D-galactopyranosyl-D-galactose, 6-*O*-β-D-galactopyranosyl-D-galactose, the β-D-(1 → 3)- and β-D-(1 → 6)-linked galactose trisaccharides, and a fraction which was probably a mixture of tri- and tetra-saccharides. The β-D-(1 → 3)-linked trisaccharide has been detected in *A. senegal* gum, but this is the first report of the β-D-(1 → 6)-linked trisaccharide in an *Acacia* gum. In contrast to *A. senegal* gum, the *A. nubica* degraded gum *A* gave more of the β-D-(1 → 6)- than of the β-D-(1 → 3)-linked disaccharides.

Graded hydrolysis experiments. — Hydrolysis of the whole gum (N-sulphuric acid, 100°, 8 h), followed by cellulose-column chromatography, gave L-rhamnose, L-arabinose, D-galactose, D-glucurono-6,3-lactone, and 4-*O*-methylglucuronic acid. The aldobiouronic acids were shown to be 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose and (in minor proportion) 4-*O*-(α-D-glucopyranosyluronic acid)-D-galactose.

Methylation studies. — Degraded gum *A* was methylated[6] by the sodium hydride–methyl iodide–methyl sulphoxide system, and cellulose-column chromatography of the methylated products showed the presence of 2,3,4,6-tetra-*O*-methyl-D-galactose; 2,3,4-tri-*O*-methyl-L-arabinose; 2,3,4-, 2,4,6-, and 2,3,6-tri-*O*-methyl-D-galactose; 2,4-di-*O*-methyl-D-galactose; and 2,3,4-tri-*O*-methyl-D-glucuronic acid.

After methylation[6] of the whole gum, cellulose-column chromatography showed the presence of 2,3,5-tri-*O*-methyl-L-arabinose; 2,3,4,6-tetra-*O*-methyl-D-galactose; 2,5- and 3,5-di-*O*-methyl-L-arabinose; 2,3,4-tri-*O*-methyl-L-arabinose; 2,3,4-, 2,4,6-, and 2,3,6-tri-*O*-methyl-D-galactose; 2,6- and 2-4-di-*O*-methyl-D-galactose; 2-*O*-methyl-D-galactose; and 2,3,4-tri-*O*-methyl-D-glucuronic acid. About 50% of the arabinose was present as 2,3,5-tri-*O*-methyl-L-arabinose, with a major proportion of di-*O*-methyl-L-arabinose, and a small proportion of 2,3,4-tri-*O*-methyl-

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TABLE II

THE CHANGES OCCURRING ON AUTOHYDROLYSIS

Period of autohydrolysis (h)	$[\alpha]_D$	*pH*	*Reducing sugars*[a]	*Reducing sugars liberated*[b]				*Galactose disaccharides*	
				Arabinose	*Galactose*	*Arabinose oligomers*[c]	*Arabinose disaccharide*[d]	*β-D-(1→6)*[e]	*β-D-(1→3)*[f]
0	+100°	2.5	–	–	–	–	–	–	–
6	+104°	2.3	10.1	++	–	+	–	–	–
12	+105°	2.2	16.4	+++	–	+	–	–	–
24	+108°	2.2	27.7	+++	trace	+	trace	–	–
48	+98°	2.1	46.2	+++	+	+	+	–	–
72	+90°	2.1	55.8	+++	+	+	+	trace	–
96	+89°	2.0	63.7	+++	++	+	+	+	trace
120	+88°	1.9	65.2	+++	++	trace	+	+	trace
144	+87°	1.9	65.9	+++	++	trace	++	+	+
177	–	–	–	+++	++	trace	++	+	+

[a]Expressed as mg of free arabinose per ml. [b]Chromatographic solvent (*b*); aniline oxalate spray. [c]Oligomers, pink streak R_{Gal}-0.4 to 0.65. [d]R_{Gal} 0.79: 3-*O*-β-L-arabinopyranosyl-L-arabinose. [e]R_{Gal} 0.32. [f]R_{Gal} 0.47.

L-arabinose. The di-*O*-methylarabinose fraction consisted of 2,5-di-*O*-methyl-L-arabinose (one part) and the less-common 3,5-di-*O*-methyl-L-arabinose (four parts). L-Arabinofuranosyl-(1 → 2)-L-arabinose units must therefore be present in the gum, and arabinopyranose units appear to occur only as non-reducing end-groups. The major galactose-containing fraction from the methylated whole gum (about 60% of the total galactose) was present as a mixture of 2,4,6-tri-*O*-methyl-D-galactose (5 parts), 2,3,4-tri- (one part), and 2,3,6-tri-*O*-methyl-D-galactose (trace). Most of the remaining galactose occurred as 2,4-di-*O*-methyl-D-galactose, with relatively little 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,6-di-*O*-methyl-D-galactose, or 2-*O*-methyl-D-galactose. Under-methylation of O-4 of galactose units has been reported[5] in *A. senegal* gum; the occurrence of 2,6-di- and 2-*O*-methylgalactose in *A. nubica* gum may also arise from under-methylation. The acidic residues in the gum are present as end groups. When the methylation results are compared, the most striking feature is the very small proportion of galactopyranose end-groups in the whole gum, when compared with the degraded gum. The whole gum contains proportionately more 2,4,6-tri-*O*-methyl-D-galactose and 2,4-di-*O*-methyl-D-galactose. It therefore appears that the point of attachment of the arabinose-containing units in the whole gum is to C-3 of (1 → 6)-linked galactose units and otherwise unsubstituted galactose units. Some arabinose may be linked to C-6 of galactose residues, but this cannot occur to any great extent as the most abundant, methylated galactose from the methylated whole gum would then be 2,3,4-tri-*O*-methyl-D-galactose.

Smith degradations. — Five successive Smith-degradations were carried out on the whole gum, and the five degraded polysaccharides (*S1–S5*) isolated were examined by hydrolysis and methylation. Every stage of the sequence of Smith degradations gave the same methylated sugars, *viz.*, 2,3,5-tri-*O*-methyl-L-arabinose; 2,5- and 3,5-di-*O*-methyl-L-arabinose; 2,3,4,6-tetra-*O*-methyl-D-galactose; 2,3,4- and 2,4,6-tri-*O*-methyl-D-galactose; and 2,4-di-*O*-methyl-D-galactose. Although it is unlikely that complete reaction is obtained at each stage of such a sequence of Smith degradations, the following conclusions appear to be reasonable. (*a*) Product *S1* did not contain rhamnose, glucuronic acid, or arabinopyranose units. This confirms the evidence from the methylation study, which indicated that acidic and arabinopyranose units are present exclusively as end groups. The rhamnose residues are oxidised by periodate and are probably present in peripheral, end-group positions. (*b*) Only the first Smith-degradation gave ethylene glycol and threitol as low molecular-weight products. Thus, all of the arabinopyranose end-groups, and oxidisable galactose residues linked through C-4, are eliminated at the first stage. (*c*) Yields from the degradations were low at every stage, compared with the yields obtained[5] in a study of *A. senegal* gum. This indicates a higher proportion of end groups in *A. nubica* and suggests a higher degree of branching than in *A. senegal*, which is itself extensively branched[5]. (*d*) Arabinose was the major sugar present in all the Smith-degraded polysaccharides *S1–S5*: this suggests that most of the arabinose-containing side-chains are at least 6 arabinose residues in length, and some may be considerably longer.

DISCUSSION

The core of *A. nubica* gum appears to be a highly branched galactan framework; some branches are terminated by D-glucuronic acid (and a very small proportion of its 4-*O*-methyl derivative) linked mainly β-(1 → 6) to D-galactose, but with some α-(1 → 4) links also. The only linkages detected between the D-galactose residues were β-(1 → 3)- and β-(1 → 6), with the latter type preponderating. Blocks of three contiguous β-(1 → 6)-linked D-galactose residues occur. There is no evidence for a "backbone" of (1 → 3)-linked D-galactose residues; this is at variance with early studies of *Acacia* gum exudates, but is in agreement with recent investigations of gums from *A. senegal*[5] and *A. arabica*[3].

Chains of L-arabinose residues are attached to the branched galactan framework, mainly at C-3 of the D-galactose residues; the L-arabinose chains are, on average, at least six units long, and some are terminated by L-arabinofuranose and L-arabinopyranose residues linked β-(1 → 3) to L-arabinose. The chains contain only (1 → 2)-linked L-arabinofuranose residues, with a smaller proportion of (1 → 3)-linkages. It is not known whether chains occur which are exclusively (1 → 2)- or (1 → 3)-linked, or whether both types of linkage occur in one chain.

A. nubica and *A. arabica*[3] exudates are the first strongly dextrorotatory *Acacia* gums to be studied structurally, and it is of interest to consider the nature of the major structural differences between these species and the *Acacia* species that give laevorotatory gums, *e.g.*, *A. senegal*[5]. Although the (1 → 4)-linked aldobiouronic acid present has a high positive rotation, it is not sufficiently abundant to make a decisive contribution to the overall rotation. Indeed, when the (1 → 4)-aldobiouronic acid linkage was destroyed by periodate oxidation, the residual polysaccharide was even more strongly dextrorotatory, and this effect persisted for Smith-degradation products *S*2 and *S*3. The origin of the highly positive rotation is clearly a deep-seated feature of the gum molecule.

A. nubica gum resembles other *Acacia* gums in giving a degraded gum consisting mainly of D-galactose residues linked β-(1 → 3) and β-(1 → 6). Unlike the gums from *A. senegal*[5] and *A. pycnantha*[7], β-D-(1 → 6)-linkages preponderate in *A. nubica* gum. It has been deduced[5] that *A. senegal* gum has a branched, tree-like core of D-galactose residues; there is evidence that the core of *A. nubica* gum is even more highly branched. As in *A. senegal*[5] gum, the L-arabinose chains are attached to C-3 of D-galactose; in *A. pycnantha*[7], they are attached to C-6 of D-galactose, and in *A. seyal*[8] they are attached to both C-3 and C-6 of D-galactose. The L-arabinose chains in *A. nubica* gum are much longer, however, than in *A. senegal*[5] gum (where four Smith-degradations eliminated all of the L-arabinose). The L-arabinose chains also differ in the respect that they consist largely of (1 → 2)-linked L-arabinofuranose units. Until recently, 3,5-di-*O*-methyl-L-arabinose had been reported to occur in only one species, *A. pycnantha*[7], but from studies carried out at the same time as this work[9], this sugar is now known to occur in *A. arabica* gum[3], and also in gums from several other *Acacia* species[10].

It therefore appears that *A. nubica* gum has a markedly different structure to that of *A. senegal* gum. It is not known whether *A. nubica* and *A. arabica* are typical of the dextrorotatory species that have low contents of L-rhamnose, or whether *A. senegal* is typical of the laevorotatory species that have relatively high contents of L-rhamnose; further studies of a wider range of *Acacia* gum exudates are required.

EXPERIMENTAL

The standard analytical methods have been described[5,11]. Paper chromatography was carried out on Whatman No. 1 and 3MM papers with the following solvent systems (v/v): (*a*) butyl alcohol–ethanol–water (4:1:5, upper layer); (*b*) butyl alcohol–pyridine–water–benzene (5:3:3:1, upper layer); (*c*) ethyl acetate–pyridine–water (10:4:3); (*d*) ethyl acetate–acetic acid–formic acid–water (18:3:1:4); (*e*) ethyl acetate–acetic acid–formic acid–water (18:8:3:9); (*f*) butyl alcohol–acetic acid–water (4:1:5, upper layer); (*g*) acetone–ethanol–propan-2-ol–borate buffer (0.05M, pH 10) (3:1:1:2); (*h*) butanone–acetic acid–water, saturated with boric acid (9:1:1); (*i*) butanone–water–conc. ammonia (200:17:1). R_{Gal} values refer to distances moved relative to galactose; R_G values of *O*-methyl sugars refer to distances moved relative to 2,3,4,6-tetra-*O*-methyl-D-glucose. Zone electrophoresis of sugars on Whatman No. 1 paper was carried out in 0.05M borate buffer (pH 10); M_G values refer to the true migration of the sugar relative to that of glucose. G.l.c. [chromatograph Type S3A, fitted with flame-ionisation detectors (Gas Chromatography Ltd.)] of mixtures of *O*-methyl sugars was carried out at nitrogen flow-rates of *ca.* 100 ml/min on columns of (*i*) 15% by weight of poly(butane-1,4-diol succinate) on 60–70 mesh Celite (5 ft × 0.25 in) at 175°, and (*ii*) 15% by weight of poly(ethylene glycol adipate) on 60–70 mesh Celite (3 ft × 0.25 in) at 160°. Retention times (*T*) are quoted relative to that of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside. Unless otherwise stated, methylations were carried out by the sodium hydride–methyl iodide–methyl sulphoxide system[6].

Origins of specimens. — Three samples of the exudate from *A. nubica* Benth. were available; they were collected by (the late) M.P. Vidal-Hall, formerly Gum Research Officer, El Obeid, from a single tree at Goz el Ganzara, Kordofan Province, Republic of the Sudan, on 15 December 1962 (sample *A*), 30 December 1962 (sample *B*), and 25 January 1963 (sample *C*).

Analytical data for the crude samples. — The results of analyses of samples *A*, *B*, and *C* are shown in Table I.

Purification and analysis of the purified gum. — Aqueous solutions of samples *A*, *B*, and *C* were filtered, dialysed, and then exhaustively electrodialysed. The results of analyses are shown in Table I. All further studies were made on Sample *A*.

Separation and characterisation of neutral sugars. — A series of experiments established that hydrolysis with N sulphuric acid for 8 h at 100° cleaved all glycosidic linkages (excepting those of aldobiouronic acids), without causing significant degradation of the sugars released. Gum sample *A* (5 g) was hydrolysed, and the hydrolysate

was neutralised ($BaCO_3$), deionised [IR-120(H^+) resin], and concentrated to a syrup that was applied to a cellulose column (60 × 4 cm), which was then developed with solvent (*b*). Fractions (25 ml) were collected; arabinose first appeared in tube 50. *Fraction 1:* the contents of tubes 11 to 47 were combined and evaporated to a syrup (15 mg). Chromatography [solvents (*a*), (*b*), (*c*), and (*d*)] revealed the presence of rhamnose. The syrup was taken up in ethanol–water. On standing, crystals were obtained: these gave an X-ray diffractogram identical to that of authentic rhamnose. *Fraction 2* had m.p. 158°, $[\alpha]_D^{20}$ +105° (*c* 0.5, water), and was identical chromatographically to L-arabinose in solvents (*a*), (*b*), and (*c*). The sugar was characterised as its phenylosazone, m.p. 163–164°; the X-ray diffractogram was identical to that of authentic L-arabinose. *Fraction 3* had m.p. 171°, $[\alpha]_D^{20}$ +81° (*c* 0.5, water) and was identical chromatographically to D-galactose in solvents (*a*), (*b*), and (*c*). This sugar was characterised as its phenylosazone, m.p. 200°; the X-ray diffractogram was identical to that of authentic D-galactose.

Separation and characterisation of acidic sugars. — The acidic sugars, virtually immobile in solvent (*b*), were eluted with water from the cellulose column described above, and isolated as a syrup (600 mg): chromatography [solvents (*b*) and (*d*)] revealed the presence of galactose, glucuronic acid, and glucurono-6,3-lactone. The syrup (200 mg) was hydrolysed with sulphuric acid (2N, 100°, 8 h), and the neutralised hydrolysate ($BaCO_3$) was filtered and deionised. The products were separated into neutral and acidic components on a column (40 × 3 cm) of Duolite A4 resin. The neutral sugars, with traces of glucurono-6,3-lactone, were eluted with water and not investigated further. The acidic fractions were eluted with formic acid (5%), and isolated as a syrup (95 mg) that was chromatographed [solvent (*d*)] on thick paper; five fractions were obtained. *Fraction 1* was D-glucurono-6,3-lactone, R_{Gal} 3.49, $[\alpha]_D$ +19° (*c* 2.0, water); re-crystallisation from water gave crystals, m.p. and mixed m.p. 177°. *Fraction 2* (3 mg) was syrupy 4-*O*-methyl-D-glucuronic acid, R_{Gal} 2.58, $[\alpha]_D$ +33° (*c* 0.1, water), and was chromatographically identical to authentic material [solvents (*d*) and (*e*)]. Reduction (borohydride) of the methyl ester methyl glycoside gave a trace of material that was chromatographically identical [solvent (*a*)] to 4-*O*-methyl-D-glucose. *Fraction 3* was chromatographically identical to D-glucuronic acid, in solvents (*d*) and (*e*), and since glucurono-6,3-lactone had been characterised from Fraction 1, this material was not examined further. *Fraction 4* was chromatographically identical to D-galactose in solvents (*a*), (*b*), and (*c*). *Fraction 5* consisted of a trace of unhydrolysed aldobiouronic acid, R_{Gal} 0.23, identical chromatographically in solvent (*d*) to 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose.

The aldobiouronic acids. — The aldobiouronic acid fraction was obtained by partial hydrolysis of the gum with N sulphuric acid for 8 h. The reaction product was neutralised ($BaCO_3$), filtered, concentrated to a syrup, and added to ethanol. The precipitated barium aldobiouronates were removed at the centrifuge, deionised, and purified by paper chromatography on thick paper [solvent (*d*)]. Traces of material [R_{Gal} 0.61, solvent (*d*)] could not be isolated in sufficient quantity for characterisation. The major component was a syrup [R_{Gal} 0.28, solvent (*d*)], $[\alpha]_D$ + 11° (*c* 0.5, water).

Since this did not correspond to any of the aldobiouronic acids commonly found[1] in the *Acacia* group, the product was re-examined; prolonged paper chromatography [solvent (*d*)] resolved two components having R_{Gal} 0.26 (major) and R_{Gal} 0.32 (minor component). The syrupy major component was chromatographically identical to authentic 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose, and had $[\alpha]_D$ −4° (*c* 1.0, water); the minor component was identical chromatographically to 4-*O*-(α-D-glucopyranosyluronic acid)-D-galactose, $[\alpha]_D$ +87° (*c* 1.0, water). These two aldobiouronic acids were then rigorously characterised by the same sequences of reactions already described[1] for the identification of the aldobiouronic acids in *A. seyal* gum.

Autohydrolysis experiments. — Solutions (5%) of purified samples *A* and *B* were heated at 98°; portions (5 ml) were withdrawn at various intervals and analysed. The results for solution *A* are shown in Table II; solution *B* gave virtually identical results.

Degraded gum *A* was obtained by autohydrolysis of a solution of sample *A* (29.14 g in 600 ml of water) for 100 h at 98°. The solution was cooled, filtered, and dialysed against distilled water for 5 days; the dialysates were concentrated, and retained for further study. The solution of degraded gum *A* was freeze-dried (yield, dry weight basis, 24.5%); $[\alpha]_D$ +44° (*c* 1.9, water); methoxyl, 0.10; uronic acid, 12; galactose, 83; arabinose, 5%.

Examination of degraded gum A. — Degraded gum *A* was hydrolysed at 100° in sulphuric acid (0.5N); aliquots were removed at 0.5, 1, 1.5, 2, 3, 4, and 5 h, neutralised, and examined chromatographically in solvents (*a*) and (*b*). The yield of disaccharides, estimated visually, was greatest after 1 h. Accordingly, degraded gum *A* (1.793 g) was dissolved in sulphuric acid (0.5N, 100 ml) and hydrolysed for 1 h. The solution was neutralised, filtered, concentrated, and examined by paper chromatography in solvents (*a*), (*b*), and (*c*). Five fractions were obtained. *Fraction 1*, R_{Gal} 0.54 [solvent (*a*)], 0.47 (*b*), 0.51 (*c*), was chromatographically identical to 3-*O*-β-D-galactopyranosyl-D-galactose. Crystallisation from acetone–water gave a product having $[\alpha]_D$ +61° (*c* 1.1, water); m.p. and mixed m.p. 161°. *Fraction 2*, R_{Gal} 0.36 (*a*), 0.31 (*b*), and 0.44 (*c*), did not crystallise; it had $[\alpha]_D^{17}$ +33° (*c* 0.12, water), and the phenylosazone had m.p. 198–199°. The fraction was chromatographically identical to 6-*O*-β-D-galactopyranosyl-D-galactose; it was methylated[6], and, after methanolysis, the methyl glycosides of 2,3,4,6-tetra-*O*-methyl-D-galactose [T = 1.67 (*i*); 1.66 (*ii*)]; 2,3,4-tri-*O*-methyl-D-galactose [T = 5.06 (*i*); 6.22 (*ii*)]; and 2,3,5-tri-*O*-methyl-D-galactose [T = 3.16, 4.12 (*i*); 3.92, 5.30 (*ii*)] were identified by g.l.c. Fraction 2 was about three times more abundant than Fraction 1. *Fraction 3*, R_{Gal} 0.22, (*a*) and (*b*), present in trace amount only, was chromatographically identical to *O*-β-D-galactopyranosyl-[1 → 3)-*O*-β-D-galactopyranosyl-(1 → 3)-D-galactose, with a degree of polymerisation[12] = 2.91. Partial hydrolysis gave only galactose and 3-*O*-β-D-galactopyranosyl-D-galactose. *Fraction 4*, R_{Gal} 0.14 (*a*), 0.16 (*b*); degree of polymerisation[12] 2.80; was a syrup having $[\alpha]_D$ +18° (*c* 0.11, water). Partial hydrolysis with acid gave galactose and 6-*O*-β-D-galactopyranosyl-D-galactose. After methylation[6] and methanolysis, the methyl glycosides of 2,3,4,6-tetra-*O*-methyl-D-galactose, and 2,3,4- and 2,3,5-tri-*O*-methyl-D-galactose, were identified by g.l.c. The mixture of methyl glycosides

was hydrolysed, and double development of the resulting sugars in solvent (*j*) gave spots corresponding to 2,3,4,6-tetra-*O*-methyl-D-galactose and 2,3,4-tri-*O*-methyl-D-galactose. It was concluded that the trisaccharide was *O*-β-D-galactopyranosyl-(1 → 6)-*O*-β-D-galactopyranosyl-(1 → 6)-D-galactose. *Fraction 5* had R_{Gal} 0.08 (*a*), 0.09 (*b*); degree of polymerisation[12] = 3.6. Partial hydrolysis by acid, with and without prior reduction of the reducing end-group, showed the presence of 3-*O*-β-D-galactopyranosyl-D-galactose and 6-*O*-β-D-galactopyranosyl-D-galactose. It was concluded that this fraction was a mixture of tetra- and tri-saccharides.

Degraded gum *A* (415 mg) was dissolved in water (100 ml), and sodium borohydride (400 mg) was added. After 1 day, further sodium borohydride (100 mg) was added. After dialysis, the reduced, degraded gum was freeze-dried; yield, 91%. A portion of the reduced, degraded gum was hydrolysed (N sulphuric acid, 100°, 7 h). After neutralisation and concentration, chromatographic analysis (solvent *h*) showed galactose, traces of arabinose, and galactitol. A portion of the reduced, degraded gum (200 mg) was hydrolysed, neutralised, filtered, deionised, and concentrated to *ca.* 25 ml. The solution was made 0.1N with respect to ammonia, and IRA-400 resin (OH^-) was added[13]. The solution was heated (100°, 6 h), cooled, filtered, and taken to dryness. Paper chromatography [solvent (*b*)] of a solution of the residue revealed galactitol and immobile material. With solvent (*d*), galactitol and slow-moving material were found: galactose was not detected with either solvent. On evaporation, a semi-crystalline mass was obtained; this was treated with acetic anhydride containing a trace of sulphuric acid. After 24 h, water was added; hexa-*O*-acetylgalactitol (6 mg) was precipitated, and recrystallisation from ethanol gave crystals, m.p. 168°.

Molecular weight of degraded gum A. — Reduced, degraded gum *A* (41.83 mg) was dissolved in *p*-hydroxybenzaldehyde solution (0.1%, 10 ml), and degraded gum *A* (44.23 mg) was likewise dissolved. After complete oxidation with sodium meta-periodate, 9.946 μg of formaldehyde was liberated from each mg of the degraded gum, and 16.734 μg/mg was obtained from the reduced, degraded gum, corresponding to a molecular weight of 5,730 for the degraded gum *A*.

Investigation of other autohydrolysis products. — (*a*) *Proteinaceous material.* Filtration of the solution of autohydrolysed gum gave a brown residue which was suspended in hydrochloric acid (6N) and hydrolysed for 12 h (sealed tube). Concentration under diminished pressure gave a brown syrup which was chromatographed in solvent (*a*). On detection with ninhydrin, blue spots were revealed, indicative of the proteinaceous nature of the residue. (*b*) *Carbohydrate material.* The dialysate of the autohydrolysate contained arabinose and galactose (in the ratio 4:1), a mixture of oligosaccharides, and some 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose.

The yield of arabinose oligomers was, however, very low, and attempts were made to improve the yield by carrying out the autohydrolysis in a dialysis sac so that material of low molecular weight was not, in turn, subjected to continued hydrolytic conditions. After trial kinetic experiments to establish the conditions that gave the maximum yield of oligosaccharides, a 5% solution of the purified whole gum was

autohydrolysed for 16 h at 95°. The dialysate was concentrated, and four fractions were obtained by chromatography in solvent (*d*). *Fraction 1* was identical to arabinose in solvents (*a*), (*b*), (*c*), and (*d*), and was not examined further. *Fraction 2*; R_{Gal} 1.23 (*d*), 1.28 (*b*) (*cf.*, L-arabinose, 1.39), 1.24 (*c*) (*cf.*, L-arabinose, 1.38); was a syrup having $[\alpha]_D$ +88° (*c* 0.1, water). Hydrolysis gave only arabinose, and the phenylosazone had m.p. 201–203°. It was suspected that this disaccharide was 3-*O*-β-L-arabinofuranosyl-L-arabinose (lit.[14], $[\alpha]_D$ +89° and +94°; phenylosazone, m.p. 200°). After methylation[6] and methanolysis, the products were found (g.l.c.) to be the methyl glycosides of 2,3,5-tri-*O*-methyl-L-arabinose [*T* = 0.52, 0.67 (*i*); 0.54, 0.73 (*ii*)]; 2,3,4-tri-*O*-methyl-L-arabinose (trace) [*T* = 0.82 (*i*); 0.96 (*ii*)]; 2,5-di-*O*-methyl-L-arabinose [*T* = 1.30, 2.23 (*i*); 1.77, 3.16 (*ii*)]; and a trace of 2,4-di-*O*-methylarabinose [*T* = 1.47, 1.60 (*i*); 2.19, 2.29 (*ii*)]. *Fraction 3* had R_{Gal} 0.70 [solvent (*d*)], 0.88 (*c*), and was chromatographically identical to authentic 3-*O*-β-L-arabinopyranosyl-L-arabinose. Hydrolysis gave only arabinose, and the phenylosazone had m.p. 229–230° and mixed m.p. 230° (with an authentic sample[14] of m.p. 233°). After methylation[6] and methanolysis, the products were found (g.l.c.) to be the methyl glycosides of 2,3,4-tri-*O*-methyl-L-arabinose [*T* = 0.83 (*i*); 0.98 (*ii*)]; 2,5-di-*O*-methyl-L-arabinose [*T* = 1.30, 2.21 (*i*); 1.78, 3.17 (*ii*)]; and a trace of 2,4-di-*O*-methyl-L-arabinose [*T* = 1.47, 1.61 (*i*); 2.20, 2.28 (*ii*)]. *Fraction 4* had R_{Gal} 0.54 [solvent (*d*)], and hydrolysis gave only arabinose. Mild hydrolysis (sulphuric acid, 0.01N; 100°, 2 h), followed by neutralisation (Deacidite FF resin), gave arabinose and 3-*O*-β-L-arabinopyranosyl-L-arabinose as the major products, with traces of 3-*O*-β-L-arabinofuranosyl-L-arabinose. Methylation[6], methanolysis, and g.l.c. examination of the products showed the following glycosides to be present: 2,3,4- and 2,3,5(trace only)-tri-*O*-methylarabinose; 2,5- and 2,4(trace only)-di-*O*-methylarabinose. This fraction was therefore identified as *O*-β-L-arabinopyranosyl-(1 → 3)-*O*-β-L-arabinofuranosyl-(1 → 3)-L-arabinose, although the presence of the homologous β-(1 → 3)-linked L-arabinofuranosyl trisaccharide cannot be excluded.

Methylation studies. — The methylations of *A. nubica* gum and of degraded *A. nubica* gum A have been described[6].

Examination of methylated, degraded gum A. — The methylated, degraded gum had $[\alpha]_D$ +10° (*c* 0.1, chloroform); methoxyl, 41.8%. Methanolysis, followed by g.l.c. examination of a portion of the mixture of methyl glycosides, gave the results shown in Table III. Hydrolysis of the major portion, with examination of the free *O*-methyl sugars by paper chromatography in solvents (*a*), (*f*), and (*j*), gave the results in Table III. The mixture of methylated sugars was then fractionated on a cellulose column [65 × 4 cm, solvent (*a*)]; when necessary, sub-fractions were obtained by thick-paper chromatography [solvent (*j*)]. When all of the neutral sugars had been eluted, acidic components were eluted with water. *Fraction 1* (193 mg), which had R_{Gal} 0.91 [solvent (*a*)], $[\alpha]_D$ +111° (*c* 1.9, water), was chromatographically identical to 2,3,4,6-tetra-*O*-methyl-D-galactose in solvents (*a*), (*f*), and (*j*). It was characterised by conversion into 2,3,4,6-tetra-*O*-methyl-*N*-phenyl-D-galactosylamine, m.p. 195–196° (from ethyl acetate). *Fraction 2* (12 mg), R_G 0.84 [solvent (*a*)], was chromatographically

TABLE III

EXAMINATION OF METHANOLYSIS AND HYDROLYSIS PRODUCTS FROM METHYLATED, DEGRADED GUM *A*

Relative retention times (T) *of methyl glycosides*[a]		R_G *values in*			*O-Methyl sugars identified*
Column (i)	*Column* (ii)	*Solvent* (a)	*Solvent* (f)	*Solvent* (j)[b]	
0.82	1.04	0.85	—	0.73	2,3,4-tri-*O*-methyl-L-arabinose
1.65	1.65	0.92	0.91	0.87	2,3,4,6-tetra-*O*-methyl-D-galactose
2.03, 2.61	2.31, (2.89)	0.10	0.80	0	2,3,4-tri-*O*-methyl-D-glucuronic acid[c]
2.40, (2.93), (3.35)	(2.89), (3.71), (4.18),	0.73	0.72	0.67	2,3,6-tri-*O*-methyl-D-galactose
(2.93), (3.35)	(3.71), (4.18)	0.73	0.72	0.56	2,4,6-tri-*O*-methyl-D-galactose
4.99	6.39	0.73	0.72	0.51	2,3,4-tri-*O*-methyl-D-galactose
9.4, 10.7	14.6, 16.4	0.49	0.50	0.21	2,4-di-*O*-methyl-D-galactose

[a]Values in parentheses indicate incompletely resolved components. [b]Using double development. [c]As methyl ester.

identical, in solvents (*a*) and (*j*), to 2,3,4-tri-*O*-methyl-L-arabinose. *Fraction 3* (341 mg) was a mixture [solvent (*j*)] of 2,3,4-, 2,4,6-, and 2,3,6-tri-*O*-methyl-D-galactose. Since the 2,3,4- and 2,4,6-tri-*O*-methyl-D-galactose could not be separated satisfactorily, the procedure of Dutton and Unrau[15] was applied to Fraction 3. As a result, galactitol, arabinitol, and threitol were obtained in the proportions 16:9:1, and hence the proportions of 2,4,6-, 2,3,4-, and 2,3,6-tri-*O*-methyl-D-galactose must have been 11:7:1. *Fraction 4* (266 mg), R_G 0.48 [solvent (*a*)], $[\alpha]_D$ +84° (*c* 2.5, water), was chromatographically identical to 2,4-di-*O*-methyl-D-galactose in solvents (*a*) and (*j*). Demethylation gave D-galactose, and the sugar was characterised as 2,4-di-*O*-methyl-*N*-phenyl-D-galactosylamine, m.p. 214–215° (from ethyl acetate). *Fraction 5* (130 mg), R_G 0.80 [solvent (*f*)], $[\alpha]_D$ +55° (*c* 2.9, water), was identical chromatographically to 2,3,4-tri-*O*-methyl-D-glucuronic acid in solvent (*f*). The methyl ester methyl glycoside was reduced (lithium aluminium hydride) to give 2,3,4-tri-*O*-methyl-D-glucose, which was purified by thick-paper chromatography [solvent (*a*)]; yield, 52 mg. This sugar was characterised as 2,3,4-tri-*O*-methyl-*N*-phenyl-D-glucosylamine, m.p. 147°.

Examination of methylated, whole gum. — The methylated[6] gum (methoxyl, 41.0%) was methanolysed, and g.l.c. examination of a portion of the mixture of methyl glycosides gave the results shown in Table IV. The major portion was hydrolysed, and the free *O*-methyl sugars were examined by paper chromatography in solvent (*a*) (results in Table IV). The mixture of methylated sugars was then fractionated on a cellulose column (65 × 4 cm); the initial eluant was light petroleum–butyl alcohol (7:3), saturated with water. When most of the tri-*O*-methylgalactoses had been eluted, the eluant was changed to light petroleum–butyl alcohol (1:1), saturated with water. When necessary, sub-fractions were obtained by thick-paper chromatography in solvents (*a*) and (*j*). After the neutral sugars had been eluted from the column, the acidic components were eluted with water to give the following fractions. *Fraction 1* (942 mg, syrup) had $[\alpha]_D$ −29° (*c* 1.0, water), and was identical chromatographically to 2,3,5-tri-*O*-methyl-L-arabinose. Demethylation gave arabinose only. The sugar was characterised by conversion into 2,3,5-tri-*O*-methyl-L-arabinonamide, m.p. 136° (from ethyl acetate). *Fraction 2* (33 mg) had $[\alpha]_D$ +110° (*c* 0.7, water), and was identical chromatographically to 2,3,4,6-tetra-*O*-methyl-D-galactose. The sugar was characterised by conversion into 2,3,4,6-tetra-*O*-methyl-*N*-phenyl-D-galactosylamine, m.p. and mixed m.p. 195–196°. *Fraction 3* (750 mg, syrup) was a mixture of 2,5- and 3,5-di-*O*-methyl-L-arabinose. Paper-electrophoretic examination (7.3 volts/cm, 0.6 mamps/cm, Whatman 3 MM paper, 6 h) in 0.05M borate buffer (pH 10) confirmed the presence of 2,5-(M_G 0.00) and 3,5-di-*O*-methylarabinose (M_G 0.70). Continuous electrophoresis on a paper curtain at 600 volts allowed the two components to be separated, the 3,5-di-*O*-methylarabinose migrating towards the anode as the borate complex. After elution of the sugars with water, the solutions were deionised and taken to dryness, and borate was removed by several distillations of 1% methanolic hydrogen chloride from the residue. The resulting methyl glycosides were hydrolysed to the free sugars as subfractions (*a*) and (*b*). Subfraction (*a*) (101 mg), $[\alpha]_D$ −37° (*c* 0.10, water), had the same mobility as 2,5-di-*O*-methyl-L-arabinose,

TABLE IV

EXAMINATION OF METHANOLYSIS AND HYDROLYSIS PRODUCTS FROM METHYLATED *A. nubica* GUM

Relative retention times (T) *of methyl glycosides*[a]		R_G *value in solvent* (a)	*Colour with aniline oxalate*	O-*Methyl sugars identified*
Column (i)	*Column* (ii)			
0.51, 0.64	0.58, 0.74	0.97	black	2,3,5-tri-*O*-methyl-L-arabinose
(0.78)	(1.03)	0.78	pink	2,3,4-tri-*O*-methyl-L-arabinose
(0.78), (1.67)	(1.03), (2.39)	0.81	dark brown	3,5-di-*O*-methyl-L-arabinose
1.29, (2.14)	1.77, 3.16	0.84	black	2,5-di-*O*-methyl-L-arabinose
(1.67)	1.66	0.91	red–brown	2,3,4,6-tetra-*O*-methyl-D-galactose
(2.14), 2.65	(2.39), (2.93)	*ca.* 0.1	red	2,3,4-tri-*O*-methyl-D-glucuronic acid[b]
2.39, (3.02), (3.39)	(2.93), (3.81), (4.23)	0.71	red–brown	2,3,6-tri-*O*-methyl-D-galactose
(3.02), (3.39)	(3.81), (4.23)	0.71	red–brown	2,4,6-tri-*O*-methyl-D-galactose
5.09	6.52	0.71	red–brown	2,3,4-tri-*O*-methyl-D-galactose
6.13	9.35	0.54	red–brown	2,6-di-*O*-methyl-D-galactose
9.15, 10.18	15.0, 17.0	0.52	red–brown	2,4-di-*O*-methyl-D-galactose
—	—	0.36	red–brown	2-*O*-methyl-D-galactose

[a]Figures in parentheses denote unresolved components. [b]As methyl ester methyl glycoside.

and gave the same colour with the aniline oxalate spray: it was immobile on paper electrophoresis in borate buffer. The sugar was characterised by conversion into 2,5-di-*O*-methyl-L-arabinonamide, m.p. 129°, (from ethyl acetate). Subfraction (*b*) (392 mh), $[\alpha]_D$ −29°, was identical to 3,5-di-*O*-methyl-L-arabinose on paper chromatography[16], and paper electrophoresis[17] in borate buffer. The sugar was characterised by conversion into 3,5-di-*O*-methyl-L-arabinonamide, m.p. 143° (from ethyl acetate). *Fraction 4* (70 mg), $[\alpha]_D$ +112° (*c* 1.3, water), was identical chromatographically to 2,3,4-tri-*O*-methyl-L-arabinose, and was characterised by conversion into 2,3,4-tri-*O*-methyl-L-arabinonamide, m.p. 103°. *Fraction 5* (505 mg) was shown by paper chromatography and g.l.c. to be a mixture of 2,4,6-, 2,3,4-, and 2,3,6-tri-*O*-methyl-D-galactose in the proportions 5:1:trace. These sugars had already been characterised during the examination of the degraded gum, and they were not investigated further. *Fraction 6* (10 mg) gave galactose on demethylation, and was chromatographically identical to 2,6-di-*O*-methyl-D-galactose in solvents (*a*) and (*j*). *Fraction 7* (254 mg) had $[\alpha]_D$ +86° (*c* 2.5, water), and crystallised spontaneously, m.p. 86–87°. The sugar was identical chromatographically to 2,4-di-*O*-methyl-D-galactose, and was characterised as 2,4-di-*O*-methyl-*N*-phenyl-D-galactosylamine, m.p. and mixed m.p. 214°. *Fraction 8* (14 mg), $[\alpha]_D$ +77°, gave only galactose on demethylation, and was identical chromatographically to 2-*O*-methyl-D-galactose in solvents (*a*) and (*j*). *Fraction 9* (173 mg) had $[\alpha]_D$ +53° (*c* 1.0, water), and was identical in solvent (*f*) to 2,3,4-tri-*O*-methyl-D-glucuronic acid. Reduction of the methyl ester methyl glycoside gave 2,3,4-tri-*O*-methyl-D-glucose. This sugar had already been characterised in the degraded gum and was not examined further.

Smith degradation of degraded gum A. — Degraded gum *A* (1.782 g) was dissolved in water (100 ml), and 0.25M sodium metaperiodate solution (100 ml) was added. The amount of formic acid released (mmoles/g) was 6.93 (23 h), 7.14 (29 h), and 7.19 (33 h). After 36 h, the reaction was stopped by addition of ethylene glycol. The solution was dialysed (36 h), and then sodium borohydride (2 g) was added. After 48 h, the solution was dialysed for 100 h, and the dialysate was shown [solvent (*a*)] to contain glycolic acid, glycerol, and threitol. The polyalcohol was hydrolysed in N sulphuric acid for 48 h at room temperature. After neutralisation ($BaCO_3$), and concentration to 15 ml, the solution was added to ethanol (1 litre). The precipitate was re-dissolved, and the solution was neutralised, filtered, and freeze-dried to give degraded gum *B* (yield, 16.2%).

Partial acid hydrolysis and methylation of degraded gum B. — Degraded gum *B* (10 mg) was hydrolysed (0.5N sulphuric acid, 1 h), and the products were examined chromatographically in solvents (*b*), (*c*), and (*d*). Galactose, traces of arabinose and glycerol, and three oligosaccharides, *viz.*, 6-*O*-β-D-galactopyranosyl-D-galactose, 3-*O*-β-D-galactopyranosyl-D-galactose, and the β-(1 → 3)-linked D-galactose trisaccharide were identified by comparison with authentic samples; the trisaccharide was tentatively identified from its reported chromatographic mobility.

Degraded gum *B* (82 mg) was methylated[6]; methanolysis of the product, followed by g.l.c. examination of the mixture of glycosides, gave the results shown

in Table V. A portion of the mixture of methylated glycosides was hydrolysed, and the free sugars were examined in solvents (*a*) and (*j*); the sugars listed in Table V were identified.

TABLE V

EXAMINATION OF METHANOLYSIS PRODUCTS FROM METHYLATED DEGRADED GUM *B*

Relative retention times (T) *of methyl glycosides*		*Approx. relative proportions*	O-*Methyl sugar identified*
Column (i)	*Column* (ii)		
1.67	1.67	+ + +	2,3,4,6-tetra-*O*-methyl-D-galactose
2.97, 3.40	3.74, 4.21	+ + +	2,4,6-tri-*O*-methyl-D-galactose
5.06	6.49	+	2,3,4-tri-*O*-methyl-D-galactose
9.59, 10.9	14.6, 16.4	+	2,4-di-*O*-methyl-D-galactose
0.54, 0.67	0.57, 0.73	trace	2,3,5-tri-*O*-methyl-L-arabinose

Successive Smith-degradations of the whole gum. — Borohydride reduction of periodate-oxidised whole gum (20.6 g), followed by controlled acid hydrolysis of the polyalcohol for 48 h at room temperature, gave polysaccharide *S1* (yield, 54%; analytical data as shown in Tables VI and VII). The dialysate from the controlled

TABLE VI

FORMIC ACID RELEASED[a] ON PERIODATE OXIDATIONS

Time (h)	*3*	*6*	*9*	*12*	*24*	*27*	*30*	*48*	*54*	*96*	*142*
A. nubica gum	0.39	0.50	0.58	0.66	0.83		0.89	1.0			
Polysaccharide *S1*	0.63	0.66	0.68		0.74	0.74					
Polysaccharide *S2*	0.46	0.47	0.48		0.48						
Polysaccharide *S3*	0.32	0.34		0.37	0.41		0.42	0.48			
Polysaccharide *S4*					0.10		0.14			0.40	0.46

[a]As mmoles of formic acid per g of polysaccharide.

acid hydrolysis of the polyalcohol was concentrated to a syrup; chromatographic examination in solvent (*b*) revealed the presence of glycolaldehyde (R_F 0.67, major product); glycerol (R_F 0.53, major product); threitol (R_F 0.40, minor product), and arabinose (R_F 0.34, trace). Examinations were also made in solvents (*a*), (*c*), (*d*), and (*h*), with similar conclusions.

Examination of Smith-degraded polysaccharide S1. — Hydrolysis, followed by chromatographic examination [solvents (*b*), (*c*), and (*d*)], showed only galactose and arabinose (35:65). Partial hydrolysis (0.5N sulphuric acid, 2 h), followed by chromatographic examination [solvents (*b*) and (*c*)], showed arabinose, galactose, 3-*O*-β-D-galactopyranosyl-D-galactose, and 6-*O*-β-D-galactopyranosyl-D-galactose to be present.

TABLE VII

RESULTS OF SMITH DEGRADATIONS

	Whole gum	*Smith-degraded polysaccharides*				
		S1	*S2*	*S3*	*S4*	*S5*
Yield from preceding polysaccharide, %	—	54	37	29	25	11
$[\alpha]_D$	+100°	+102°	+117°	+112°	+98°	+89°
Formic acid released on periodate oxidation[a]	1.01	0.74	0.48	0.49	0.46	—
Sugar ratios, %: galactose	33	35	35	30	26	31
arabinose	59	65	65	70	74	69
rhamnose	0.7	—	—	—	—	—
glucuronic acid	7	—	—	—	—	—

[a]As mmoles of formic acid per g of polysaccharide.

Polysaccharide *S1* (350 mg) was methylated[6] (yield, 300 mg; methoxyl, 39.7%). A portion of the methylation product was methanolysed, and half of the product was examined by g.l.c.; the remainder was hydrolysed to the free sugars and examined by paper chromatography [solvents (*a*) and (*j*)]. The methyl glycosides and methylated sugars listed in Table VIII were identified. The 2,3,4- and 2,4,6-tri-*O*-methyl-D-galactose were resolved in solvent (*j*); by g.l.c., the ratio of 2,3,4- to 2,4,6-tri-*O*-methyl-D-galactose was 1:2.

TABLE VIII

EXAMINATION OF METHANOLYSIS AND HYDROLYSIS PRODUCTS FROM METHYLATED, DEGRADED GUMS *S1–S5*.

Relative retention times (T) *of methyl glycosides*[a]		R_G *in solvent* (*a*)	*O-Methyl sugars identified*
Column (i)	*Column* (ii)		
0.51, 0.64	0.58, 0.74	0.97	2,3,5-tri-*O*-methyl-L-arabinose
0.78, (1.67)	1.05, 2.40	0.81	3,5-di-*O*-methyl-L-arabinose
1.29, 2.18	(1.80), 3.19	0.84	2,5-di-*O*-methyl-L-arabinose
(1.67)	(1.67)	0.92	2,3,4,6-tetra-*O*-methyl-D-galactose
3.00, 3.45	3.78, 4.26	0.73	2,4,6-tri-*O*-methyl-D-galactose
5.11	6.53	0.73	2,3,4-tri-*O*-methyl-D-galactose
9.62, 11.01	14.9, 16.9	0.48	2,4-di-*O*-methyl-D-galactose

[a]Parentheses denote sugars that were incompletely resolved.

Preparation, partial and complete hydrolyses, and methylation of degraded polysaccharide S2. — The second Smith-degradation product, *S2*, was obtained from *S1* (10.6 g) in a manner similar to that described above for the preparation of polysaccharide *S1* from the whole gum. The yield and analytical data for polysaccharide *S2* are shown in Tables VI and VII. Hydrolysis, and partial hydrolysis, gave the same sugars, in similar proportions to those identified in polysaccharide *S1*. A portion

(326 mg) of polysaccharide *S2* was methylated[6] (yield, 120 mg; methoxyl, 40.0%). Paper-chromatographic and g.l.c. examination of the methanolysis and hydrolysis products from methylated polysaccharide *S2* gave the methyl glycosides and *O*-methyl sugars shown in Table VIII. In this instance, however, the ratio of 2,3,4- to 2,3,6-tri-*O*-methyl-D-galactose was 2:5.

Preparation, partial and complete hydrolysis, and methylation of degraded polysaccharide S3. — The third Smith-degradation product, *S3*, was obtained from *S2* (3.26 g) by the method outlined above for *S1*. The yields and analytical data for polysaccharide *S3* are shown in Tables VI and VII. Partial and complete hydrolyses showed the same sugars as identified in polysaccharides *S1* and *S2*. A portion (346 mg) of polysaccharide *S3* was methylated (methoxyl, 40.1%). Methanolysis and hydrolysis of the product was followed by g.l.c. and paper chromatography; the methyl glycosides and *O*-methyl sugars shown in Table VIII were identified. The proportion of 2,3,4,6-tetra-*O*-methyl-D-galactose was, however, much smaller, and the ratio of 2,3,4- to 2,4,6-tri-*O*-methyl-D-galactose was 1:5.

Preparation, hydrolyses, and methylation of degraded polysaccharide S4. — In view of the low yield of *S3* (0.856 g from 20 g of *A. nubica* gum), the first three Smith-degradations were repeated on a larger scale (90 g of *A. nubica* gum). These were carried out serially as described for the preparation of polysaccharides *S1*, *S2*, and *S3*, with the exception that *S1* and *S2* were not isolated. The product (3.34 g), *S3*, from the third degradation, was freeze-dried and analysed: it had the same ratio of galactose to arabinose as *S3* described above, and this preparation of *S3* (3.05 g) was used to prepare *S4*.

The percentage yield and analytical data for *S4* are shown in Tables VI and VII. Partial and complete hydrolyses showed arabinose, galactose, 6-*O*-β-D-galactopyranosyl-D-galactose, 3-*O*-β-D-galactopyranosyl-D-galactose (and its homologous trisaccharide) to be present. A portion of *S4* (61 mg) was methylated: the product was shown by g.l.c. and paper chromatography to contain the methylated sugars shown in Table VIII. The ratio of 2,3,4- to 2,4,6-tri-*O*-methyl-D-galactose was 1:12.

Preparation, hydrolysis, and methylation of degraded polysaccharide S5. — The fifth Smith-degraded product, *S5*, was obtained from *S4* (487 mg). The yield and analytical data are shown in Tables VI and VII. Total hydrolysis showed only galactose and arabinose to be present. The remaining material (30 mg) was methylated[6]. The product was methanolysed, and 50% of the solution obtained was retained for g.l.c. examination; the remainder was hydrolysed to the free sugars and examined by paper chromatography. The glycosides and sugars shown in Table VIII were identified although only a trace of 2,3,4,6-tetra-*O*-methyl-D-galactose was present; the ratio of 2,3,4- to 2,4,6-tri-*O*-methyl-D-galactose was 1:5. In view of the small amount of, *S5* obtained (48 mg), the results must be considered with caution. Perhaps the most significant result from the preparation of this degraded polysaccharide is the small yield (11%). Considering the Smith-degradation sequence overall, the significant factors are: (*a*) the progressively smaller yields at each stage, (*b*) the steady decrease in the amount of 2,3,4-6-tetra-*O*-methyl-D-galactose detected, and (*c*) the preponder-

ance of 3,5-di-*O*-methyl-L-arabinose as a major product in all of the polysaccharides; the proportion of 2,5- relative to 3,5-di-*O*-methyl-L-arabinose decreased as the degradation sequence proceeded from *S1* to *S5*.

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REAKTION VON 2,3,4-TRI-*O*-ACETYL-1,6-ANHYDRO-β-D-GLUCOPYRANOSE MIT *as.*-DIHALOGENMETHYLÄTHER

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ABSTRACT

2,3,4-Tri-*O*-acetyl-1,6-anhydro-β-D-glucopyranose (**1**) reacts with excess of dichloromethyl methyl ether to give 2,3,4-tri-*O*-acetyl-6-*O*-(dichloromethyl)-α-D-glucopyranosyl chloride (**4**); with 1.2–1.3 moles/mole of the ether, compound **1** gives 2,3,4-tri-*O*-acetyl-6-*O*-formyl-α-D-glucopyranosyl chloride (**3**). Compound **4** was very readily hydrolysed by thin-layer chromatography on silica gel to give compound **3** and 2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl chloride (**6**). With silver acetate–acetic acid, compounds **3** and **4** give 1,2,3,4-tetra-*O*-acetyl-6-*O*-formyl-β-D-glucopyranose (**8**) which may also be obtained by formylation of 1,2,3,4-tetra-*O*-acetyl-β-D-glucopyranose with the *N,N*-dimethylformamide–phosgene reagent. With 1.2 moles/mole of dibromomethyl methyl ether, compound **1** is converted into 2,3,4-tri-*O*-acetyl-6-*O*-formyl-α-D-glucopyranosyl bromide (**10**). Treatment of compounds **3** and **10** with ethanol–silver carbonate gives the ethyl β-D-glucoside derivative.

ZUSAMMENFASSUNG

2,3,4-Tri-*O*-acetyl-1,6-anhydro-β-D-glucopyranose (**1**) reagiert mit überschüssigem *as.*-Dichlormethyläther zu 2,3,4-Tri-*O*-acetyl-6-*O*-(dichlormethyl)-α-D-glucopyranosylchlorid (**4**), mit 1.2–1.3 Mol *as.*-Dichlormethyläther zu 2,3,4-Tri-*O*-acetyl-6-*O*-formyl-α-D-glucopyranosylchlorid (**3**). Außerordentlich leicht schon bei Dünnschichtchromatographie auf Kieselgel wird **4** in **3** bzw. 2,3,4-Tri-*O*-acetyl-α-D-glucopyranosylchlorid (**6**) hydrolytisch gespalten. Mit Silberacetat–Eisessig konnten **3** und **4** in 1,2,3,4-Tetra-*O*-acetyl-6-*O*-formyl-β-D-glucopyranose (**8**) umgewandelt werden, welche unabhängig auch durch Formylierung von 1,2,3,4-Tetra-*O*-acetyl-β-D-glucopyranose mit Dimethylformamid-dichlorid erhältlich ist. Mit 1.2 Mol *as.*-Dibrommethyläther kann **1** in 2,3,4-Tri-*O*-acetyl-6-*O*-formyl-α-D-glucopyranosylbromid (**10**) übergeführt werden. Reaktion von **3** und **10** mit Äthanol–Silbercarbonat liefert das Äthyl-β-D-glucosid-Derivat (**5**).

EINFÜHRUNG

In früheren Untersuchungen haben wir gefunden, daß bei der Reaktion von vollacetylierten Zuckern und Glycosiden mit *as.*-Dichlor- und Dibromäthern eine selektive Spaltung der glycosidischen C-O-Bindung eintritt. Hierbei bilden sich die stabilen Anomeren der Acetohalogenzucker[1-2]. Von Interesse sind in diesem Zusammenhang die 1,6-Anhydro-aldohexosen, wie z.B. die 1,6-Anhydro-β-D-glucopyranose. Diese Verbindungen können formal als „innere Glycoside" angesehen werden und es war die Frage zu überprüfen, ob und in welcher Weise *as.*-Dihalogenäther hier die glycosidische C-O-Bindung angreifen. Wir haben daher die Reaktionen von 2,3,4-Tri-*O*-acetyl-1,6-anhydro-β-D-glucopyranose (**1**) mit *as.*-Dihalogenäther untersucht.

DISKUSSION

Die Spaltung des Anhydroringes von **1** mit Titantetrahalogenid führt zu 2,3,4-Tri-*O*-acetyl-α-D-glucopyranosylhalogenid[3]. Spaltung mit Bromwasserstoff–Eisessig gibt in glatter Reaktion Tetra-*O*-acetyl-α-D-glucopyranosylbromid[4]. Acylbromide reagieren analog zu 2,3,4-Tri-*O*-acetyl-6-*O*-acyl-α-D-glucopyranosylbromid[5]. Auch ist seit längerem die Reaktion mit flüssigem Bromwasserstoff oder mit Phosphorpentabromid bekannt[6]. Diese etwas energischeren Reagentien führen zur Bildung von 1,6-Dibromderivaten.

Erwärmt man 2,3,4-Tri-*O*-acetyl-1,6-anhydro-β-D-glucopyranose (**1**) mit überschüssigem *as.*-Dichloräther in Gegenwart von wasserfreiem Zinkchlorid, so kann man nach der üblichen Aufarbeitung in *ca.* 70%iger Ausbeute ein kristallines Produkt (*A*) isolieren, das eine stark positive Drehung aufweist. Daraus läßt sich auf eine Aufspaltung des Anhydroringes schließen. Das Produkt enthält jedoch an Stelle des für ein Monochlorprodukt erwarteten Chlorgehalts von *ca.* 10% durchschnittlich 23–24% Chlor.

Das Dünnschichtchromatogramm von *A* auf Kieselgel (abs. Äther–abs. Toluol) zeigte zwei Flecken. Bei chromatographischer Trennung von 1 g *A* ließen sich beide Verbindungen nach Eluieren und Umkristallisieren in reiner, kristalliner Form isolieren, wobei die Verbindung mit kleinerem R_F-Wert (*B*) zu 2%, die andere Verbindung (*C*) zu 70% erhalten wurde. Der Chlorgehalt der beiden Verbindungen betrug *ca.* 10%. Dieser gegenüber dem Ausgangsprodukt *A* viel niedrigere Chlorgehalt zeigt, daß *B* und *C* Monochlorderivate darstellen. Im Verlauf der Dünnschichtchromatographie war offenbar eine Solvolysereaktion eingetreten. Daraus kann geschlossen werden, daß *A* neben einem C-1-Halogenatom noch weitere Chloratome in reaktiver Form enthalten muß.

Die Substanz *B* konnten wir mittels Dünnschichtchromatographie und Mischschmelzpunkt als 2,3,4-Tri-*O*-acetyl-α-D-glucopyranosylchlorid (**6**) identifizieren.

Im NMR-Spektrum von *C* sind, wie Tab. I zeigt, die chemischen Verschiebungen und Kopplungskonstanten für die Protonen H-1, H-2, H-3 und H-4 nahezu identisch

mit den entsprechenden Werten des NMR-Spektrums von **6**. Dies deutet darauf hin, daß die Substituenten am Ringsystem in *C* mit denen in **6** übereinstimmen. Die OH-Gruppe am C-6 in *C* sollte acyliert sein, da die H_2-6-Protonen gegenüber **6** zu niedrigerem Feld verschoben sind. Bei einer Acylierung von OH-Gruppen wird stets eine geringere Abschirmung beobachtet. Bei niedrigstem Feld wird ein Singulett für ein Proton bei τ 2.00 gefunden, welches auf Grund der chemischen Verschiebung einem Formylproton zukommen kann. Das Spektrum ist am besten mit der Struktur eines 2,3,4-Tri-*O*-acetyl-6-*O*-formyl-α-D-glucopyranosylchlorids (**3**) vereinbar.

Zum Beweis dieser Struktur wurde *C* mit Silberacetat–Eisessig zu einem bei 136–7° schmelzenden Acetat umgesetzt. Die Verbindung war nicht identisch mit den bekannten einfachen Zuckeracetaten; im IR-Spektrum zeigten sich zwei Carbonylbanden (bei 1754 und 1712 cm^{-1}) von unterschiedlicher Intensität, was auf das Vorhanden[sein] einer anderen Carbonylgruppe hinwies. Nach Hydrolyse mit Lauge konnte [mit] atometrischem Wege Ameisensäure nachgewiesen werden. Danach sollte [das Ace]tat die Struktur einer 1,2,3,4-Tetra-*O*-acetyl-6-*O*-formyl-β-D-glucopyra[nose] (**8**) besitzen, was durch eine unabhängige Synthese von **8** bestätigt werden kon[nte]: 1,2,3,4-Tetra-*O*-acetyl-β-D-glucopyranose (**7**) ließ sich mit Dimethylformamid-dichlorid analog einem Verfahren von Arnold[7] in 6-Stellung in guter Ausbeute zu **8** formylieren. Die experimentellen Befunde bestätigen demnach die aus dem NMR-Spektrum abgeleitete Struktur von *C*. Schließlich konnte **8** durch

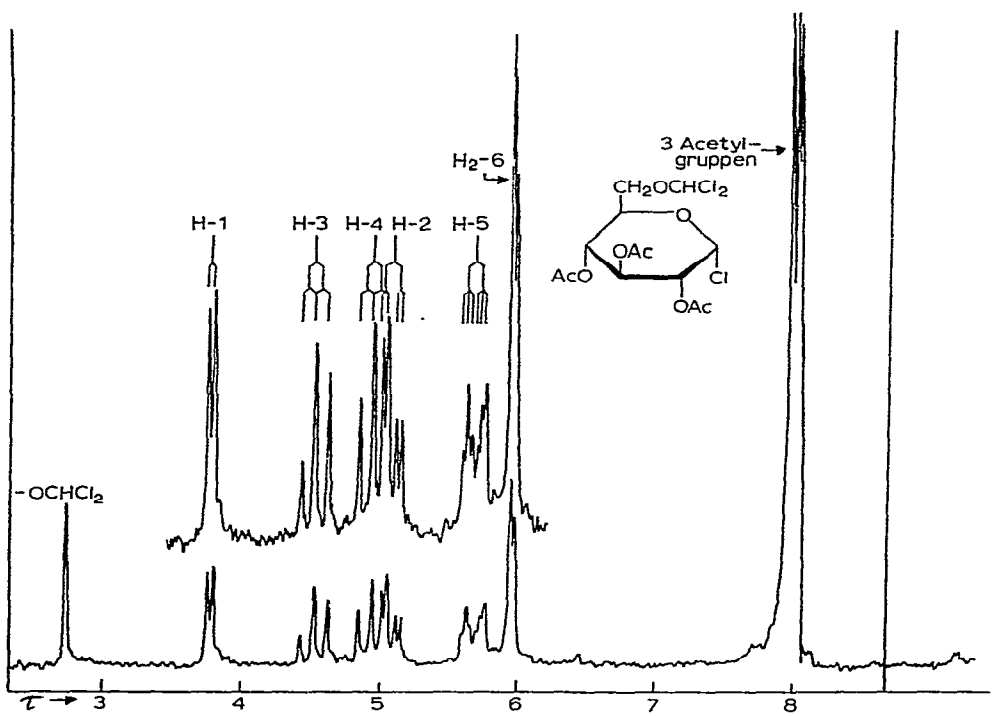


Abb. 1. 100 MHz-NMR-Spektrum von 2,3,4-Tri-*O*-acetyl-6-*O*-(dichlormethyl)-α-D-glucopyranosylchlorid (**4**) in $CDCl_3$. Innerer Standard TMS.

Umsetzung mit $TiCl_4$ nach Pacsu[8] wieder in **3** zurückverwandelt werden (**3** = Verbindung *C*). Die Substanz **3** ließ sich nach den üblichen Verfahren in das Äthyl-β-D-glucosid **5** überführen.

Wie erwähnt war **3** zunächst durch eine Solvolysereaktion im Verlauf der dünnschichtchromatographischen Trennung von *A* entstanden. Es war demnach zu erwarten, daß **3** auch aus **1** durch Umsetzung mit einem geringen Überschuß des Chlorüberträgers zugänglich sein müßte. Bei Reaktion von **1** mit 1.2–1.3 Mol Dichloräther–$ZnCl_2$ konnte in 71%iger Ausbeute **3** erhalten werden. Die Verbindung wurde nach Dünnschichtchromatographie auf Kieselgel in 60%iger Ausbeute rein erhalten. Auf dem Dünnschichtchromatogramm ist vor der chromatographischen Trennung in geringen Mengen auch **6** nachweisbar.

Im NMR-Spektrum von *A* (Tab. I und Abb. 1) sind wiederum chemische Verschiebung und Kopplungskonstanten der Protonen H-1, H-2, H-3 und H-4

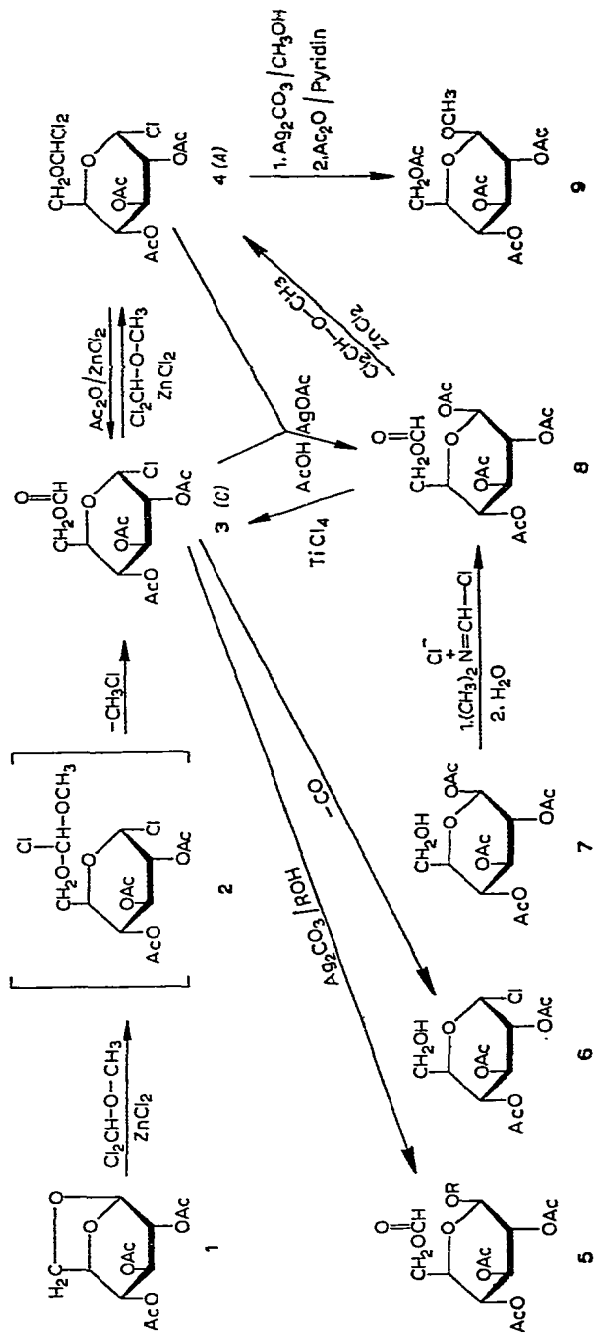
TABELLE I

CHEMISCHE VERSCHIEBUNG IN τ-WERTEN. KOPPLUNGSKONSTANTEN IN Hz NACH EINER ANALYSE ERSTER ORDNUNG. SPEKTREN BEI 100 MHz IN $CDCl_3$, TETRAMETHYLSILAN ALS INNERER STANDARD

Verbindung	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H_2-6*	*-OCHO*	*-$OCHCl_2$*
B (6)	3,76	5,10	4,45	4,96	5,9	6,4	—	—
C (3)	3,80	5,09	4,52	4,96	5,8	5,8	2,00	—
A (4)	3,78	5,09	4,52	4,94	5,69	5,95	—	2,72
Verbindung	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$				
B (6)	4,0	9,8	9,8	9,9				
C (3)	4,0	9,8	9,8	9,9				
A (4)	4,0	9,9	9,8	9,8				

nahezu identisch mit denen im Spektrum von **3** und **6**. Die H_2-6-Protonen liegen bei niedrigem Feld. Bei τ 2.72 tritt ein Singulett für ein Proton auf, das einem Dichloräther-Proton zukommen könnte. Für CH_3-O-$CHCl_2$ findet man das Dichloräther-Proton bei τ 2.65. Auf Grund dieser Daten sollte *A* ein 2,3,4-Tri-*O*-acetyl-6-*O*-(dichlormethyl)-α-D-glucopyranosylchlorid (**4**) darstellen. Diese Struktur stimmt mit dem hohen Chlorgehalt überein und erklärt auch die außerordentlich leichte Solvolysierbarkeit der Verbindung bei der Chromatographie.

Zum Beweis dieser Struktur wurden die folgenden Reaktionen durchgeführt: mit Silberacetat–Eisessig konnten wir **4** in **8** überführen, dessen Struktur wie erwähnt durch eine unabhängige Synthese gesichert war. Schließlich konnten wir aus **4** mit Methanol–Silbercarbonat und anschließender Acetylierung das bereits bekannte Methyl-β-D-glucosidtetraacetat (**9**) erhalten. Bei dieser Reaktion bildet sich wahrscheinlich zunächst ein C-6-Orthoformylderivat, das in nicht genau bekannter Weise (z.B. durch Methanolyse) den Formylrest abspaltet. Das so erhaltene Triacetylderivat geht bei der Acylierung in **9** über.



Der Austausch der beiden Chloratome in Dichlormethylalkyläthern gegen Sauerstoff verläuft ohne Schwierigkeiten z.B. bei Umsetzung mit Acetanhydrid, wobei neben dem entsprechenden Alkylformiat Acetylchlorid entsteht[9]:

$$CH_3\text{-}O\text{-}CHCl_2 + (CH_3CO)_2O \rightarrow CH_3OCHO + 2CH_3COCl$$

Analog war es möglich, **4** mit 1 Mol Acetanhydrid unter Abspaltung von Acetylchlorid in **3** umzuwandeln (Ausb. 68%).

Schließlich gelang die Synthese von **4** auch durch Reaktion von Dichloräther–$ZnCl_2$ sowohl mit **3** (41% Ausbeute) als auch mit dem auf unabhängigen Wegen synthetisierten **8**. Im letzteren Falle wird nicht nur die Formylgruppe in die Dichlormethylgruppe übergeführt, sondern gleichzeitig der Acetoxyrest am C-1 durch Chlor ausgetauscht. Mit diesen Befunden kann die aus dem NMR-Spektrum abgeleitete Struktur von **4** als gesichert angesehen werden.

Die Reaktion der 2,3,4-tri-*O*-Acetyl-1,6-anhydro-*β*-D-glucopyranose mit asymm. Dichloräther* verläuft demnach vermutlich primär unter Aufspaltung des 1,6-Anhydroringes zu einem unstabilen Zwischenprodukt (**2**), das dann entweder mit asymm. Dichloräther direkt in **4** übergeführt wird oder aber unter Methylchloridabspaltung in **3** übergeht. Wie oben gezeigt kann **3** mit überschüssigem Dichloräther zu **4** chloriert werden.

Mit *as.*-Dibromäther reagiert **1** ganz analog: mit 1.2 Mol Dibromäther entsteht das **3** entsprechende Bromderivat. Mit überschüssigem Dibromäther wurde eine **4** entsprechende Verbindung in sirupöser Form erhalten. Das Monobromderivat reagiert wie erwartet mit Silberacetat–Essigsäure ebenfalls zu **8** und kann wie üblich in das Glucosid **5** übergeführt werden.

EXPERIMENTELLER TEIL

2,3,4-Tri-O-acetyl-6-O-(dichlormethyl)-α-D-glucopyranosylchlorid (**4**). — 2,3,4-Tri-*O*-acetyl-1,6-anhydro-*β*-D-glucopyranose (**1**, 2 g) wird mit 6 ccm *as.*-Dichlordimethyläther und einer katalytischen Menge (0.1–0.2 g, 5–10%) von wasserfreiem $ZnCl_2$ 1 Stunde am Wasserbad auf 75–80° erwärmt. Dann wird i.Vak. zur Trockne eingedampft und in *ca.* 20 ccm $CHCl_3$ aufgenommen. Die Chloroform-Lösung wurde mit Eiswasser, mit $NaHCO_3$-Lösung dann wieder mit Eiswasser gewaschen, getrocknet und i.Vak. völlig eingedampft. Der sirupöse Rückstand kristallisiert aus Äther–Petroläther in schönen Nadeln. Ausbeute 2.8 g (70% d.Th.), Schmp. 96–98°, $[\alpha]_D$ +164° (*c* 0.5, Chloroform). $C_{13}H_{17}O_8Cl_3$ (407.6) Ber.: Cl, 26.1. Gef.: Cl, 23.5%.

Die Verbindung enthält Verunreinigungen und kann durch Umkristallisieren nicht weiter gereinigt werden.

Dünnschichtchromatographische Trennung von **4** *unter Solvolyse.* — Die Verbindung **4** wurde über Kieselgel G mit einem Gemisch von abs. Äther–abs. Toluol

*Untersuchungen über den Ablauf entsprechender Spaltungsreaktionen siehe *Acta. Chim. Acad. Sci. Hung.*, im Druck.

(1:2) chromatographiert. Zur Entwicklung wurde wie üblich mit 5%iger alkoholischer Schwefelsäure gesprüht. Die Verbindung **4** gab 3 Flecke, der Fleck mit dem größten R_F-Wert war sehr schwach. Die anderen beiden Verbindungen wurden (von einer anderen Platte) mit abs. Äther eluiert und die so gewonnenen kristallinen Substanzen aus Äther–Petroläther umkristallisiert. Wir erhielten in 2%iger Ausbeute 2,3,4-Tri-*O*-acetyl-α-D-glucopyranosylchlorid (**6**), Schmp. 124° (lit. Schmp. 125°)[10]. Die Substanz gab keine Schmelzpunktdepression mit einem authentischen Muster. $C_{12}H_{17}O_8Cl$ (324.7) Ber.: Cl, 10.9. Gef.: Cl, 10.7%.

Die andere Verbindung mit größerem R_F-Wert (Ausbeute: 70% d.Th.) schmolz bei 75–76°; $[\alpha]_D$ +176° (*c* 0.6, Chloroform) und war identisch mit dem auf anderem Wege hergestellten 2,3,4-Tri-*O*-acetyl-6-*O*-formyl-α-D-glucopyranosylchlorid (**3**). $C_{13}H_{17}O_9Cl$ (352.7) Ber.: Cl, 10.07. Gef.: Cl, 10.2%.

2,3,4-Tri-O-acetyl-6-O-formyl-α-D-glucopyranosylchlorid (**3**). — (*a*) *Aus* **1** *mit* as.-*Dichlordimethyläther.* Die Substanz **1** (2 g) wird mit 1 ccm *as.*-Dichlordimethyläther und einer katalytischen Menge (5-10%) wasserfreiem $ZnCl_2$ auf dem Wasserbad 1 Std. auf 75° erwärmt und das Reaktionsgemisch wie bei **4** aufgearbeitet.

Die aus Äther–Petroläther gewonnenen nadelförmigen Kristalle (1.75 g; Schmp. 65–66°) sind uneinheitlich. Das Produkt enthält nach den Angaben des Dünnschichtchromatogramms (Einzelheiten siehe vorstehend) als Verunreinigung **6** und kann davon durch Umkristallisieren nicht gereinigt werden. Nach dünnschichtchromatographischer Trennung (die Bedingungen sind identisch mit dem für die Verbindung **4** angewandten Verfahren) und nachfolgendem Eluieren mit abs. Äther gewannen wir die Verbindung in 60%iger Ausbeute, Schmp. 76°, $[\alpha]_D$ +178° (*c* 0.4, Chloroform). Gef.: Cl, 10.05%. Nach Eluieren mit trockenem $CHCl_3$ (Ausbeute 20% d.Th.) schmolz die Substanz bei 77–78°.

(*b*) *Aus* **8** *mit* $TiCl_4$. 1,2,3,4-Tetra-*O*-acetyl-6-*O*-formyl-β-D-glucopyranose (**8**, 1.2 g) wird in 25 ccm wasserfreiem $CHCl_3$ gelöst und mit 2 ccm $TiCl_4$ auf dem siedenden Wasserbad 45 Min. lang erhitzt. Nach dem Abkühlen wird die Lösung mit Eiswasser säurefrei gewaschen, mit $CaCl_2$ getrocknet und i.Vak. bis zur Trockne eingedampft. Der sirupöse Rückstand kristallisiert aus Äther–Petroläther. Ausbeute 0.4 g (36% d.Th.), Schmp. 76–77°, $[\alpha]_D$ +177° (*c* 0.43, Chloroform). Gef.: Cl, 9.84%. Die Verbindung zeigte mit der nach (*a*) hergestellten Substanz keine Schmelzpunkterniedrigung.

(*c*) *Aus* **4** *mit Acetanhydrid.* Die Substanz **4** (2 g) wird in 3 ccm Chloroform mit 0.5 g Acetanhydrid (geringer Überschuß) und mit einer Spur $ZnCl_2$ *ca.* 10 Min. unter Durchleiten von trockenem Stickstoff auf 50° erwärmt. Eine gekühlte Vorlage enthielt eine Mischung von $CHCl_3$ und Acetylchlorid. Durch Umsetzung mit einer ätherischen Anilinlösung konnte Anilinhydrochlorid (Schmp. 195°) und Acetanilid (Schmp. 114°) erhalten werden.

Die Reaktionsmischung wurde nach Verdünnen mit $CHCl_3$ mit Eiswasser geschüttelt, mit Bicarbonatlösung säurefrei gewaschen und die getrocknete Lösung i.Vak. eingedampft. Der sirupöse Rückstand wurde aus Äther–Petroläther umkristallisiert. Ausbeute 1.2 g (68% d.Th.), $[\alpha]_D$ +175° (*c* 0.78, Chloroform). Die Substanz

schmolz nach nochmaligem Umkristallisieren bei 73–74°; keine Schmelzpunktdepression mit dem nach (*a*) hergestellten Präparat.

Überführung von **3** *in* **4**. — 2,3,4-Tri-*O*-acetyl-6-*O*-formyl-α-D-glucopyranosylchlorid (**3**, 1 g) wird mit 3 ccm *as.*-Dichlordimethyläther und katalytischen Mengen (5-10%) von wasserfreiem $ZnCl_2$ 1 Std. auf 75–80° erwärmt. Das Reaktionsgemisch wurde dann wie bei **4** aufgearbeitet. Ausbeute 0.47 g (41% d.Th.), Schmp. 95°, $[\alpha]_D$ +163° (*c* 0.42, Chloroform). Gef.: Cl, 23.2%. Die Substanz gibt keine Schmelzpunktdepression mit dem aus 2,3,4-tri-*O*-Acetyl-1,6-anhydro-β-D-glucopyranose hergestellten Produkt.

*1,2,3,4-Tetra-*O*-acetyl-6-*O*-formyl-β-D-glucopyranose* (**8**). — (*a*) 2,3,4-Tri-*O*-acetyl-6-*O*-(dichlormethyl)-α-D-glucopyranosylchlorid (**4**, 1 g) wird in 10 ccm Eisessig gelöst und mit 1.9 g Silberacetat unter Rühren auf dem siedenden Wasserbad 1.5 Stdn. erhitzt. Das Reaktionsgemisch wird abgesaugt, die Lösung i.Vak. eingedampft und der Rückstand mit Wasser verrieben. Das kristalline Produkt (0.6 g, 67% d.Th.) schmolz nach mehrmaligem Umkristallisieren aus Alkohol bei 136–137°; $[\alpha]_D$ +10.5° (*c* 3.7, Chloroform). $C_{15}H_{20}O_{11}$ (376.0) Ber.: C, 47.87; H, 5.31; OAc, 45.74. Gef.: C, 47.72; H, 5.21; OAc[11], 44.64%. Die Substanz gibt mit Penta-*O*-acetyl-β-D-glucopyranose (Schmp. 136°) *ca.* 30° Schmelzpunktdepression.

(*b*) 2,3,4-Tri-*O*-acetyl-6-*O*-formyl-α-D-glucopyranosylchlorid (**3**, 0.35 g) (die wir vorher mittels Dünnschichtchromatographie gereinigt hatten) wurde in 6 ccm Eisessig mit 0.8 g Silberacetat analog (*a*) umgesetzt. Ausbeute 0.14 g (38% d.Th.), Schmp. 136°. Gibt mit dem nach (*a*) hergestellten Formylderivat keine Schmelzpunktdepression. Gef.: C, 47.92; H, 5.19; OAc[11], 44.03%.

(*c*) 1,2,3,4-Tetra-*O*-acetyl-β-D-glucopyranose[12] (**7**, 2.33 g) wird zu einer eisgekühlten Lösung von Dimethylformamid-dichlorid gegeben, die wir nach Arnold[7] aus 2.9 g abs. Dimethylformamid in 16 ccm trockenem $CHCl_3$ und 3.9 ccm 4 M Phosgenlösung in $CHCl_3$ gewonnen haben. Die klare Lösung wird 1 Std. bei Raumtemperatur stehengelassen, dann mit Eiswasser geschüttelt und in der wäßrigen Phase 5.82 g Natriumformiat gelöst. Die Chloroformschicht wird mit $KHCO_3$-Lösung ausgeschüttelt, getrocknet und i.Vak. eingedampft. Der kristalline Rückstand schmolz, aus Alkohol umkristallisiert, (1.97 g, 81% d.Th.) bei 136°, $[\alpha]_D$ +11.3° (*c* 2.7, Chloroform). Die Substanz zeigte keine Schmelzpunktdepression mit den nach (*a*) und (*b*) hergestellten Verbindungen.

Überführung von **8** *in* **4**. — 1,2,3,4-Tetra-*O*-acetyl-6-*O*-formyl-β-D-glucopyranose (**8**, 0.5 g) wurde mit 2 ccm *as.*-Dichlordimethyläther und katalytischen Mengen (5-10%) $ZnCl_2$ nach dem bei **4** angegebenen Verfahren umgesetzt und aufgearbeitet. Nach zweimaligem Umkristallisieren aus Äther–Petroläther erhielten wir 0.25 g (46% d.Th.) Substanz, die bei 94–96° schmolz, $[\alpha]_D$ +166° (*c* 0.61, Chloroform). Die Verbindung gab keine Schmelzpunktdepression mit einem authentischen Präparat.

*Äthyl-2,3,4-tri-*O*-acetyl-6-*O*-formyl-β-D-glucopyranosid* (**5**) *aus* **3**. — Die Substanz **3** (2.4 g) wurde in 45 ccm abs. Äthanol gelöst und mit 4.5 g frisch hergestelltem, trockenem Silbercarbonat 16 Stdn. bei Raumtemperatur geschüttelt. Nach dem Absaugen wird die alkoholische Lösung geklärt und i.Vak. bis zur beginnenden

Kristallisation eingedampft. Die Substanz wird dann aus Äthanol umkristallisiert. Ausbeute 1.2 g (50% d.Th.), Schmp. 138°, $[\alpha]_D$ −11.2° (*c* 0.53, Chloroform). $C_{15}H_{22}O_{10}$ (362.3) Ber.: OC_2H_5, 12.43. Gef.: OC_2H_5, 12.30%.

*Methyl-tetra-*O*-acetyl-β-*D*-glucopyranosid* (**9**) *aus* **4**. — Die Substanz **4** (1.3 g) wurde in 20 ccm abs. Methanol gelöst und mit 2.7 g Silbercarbonat 25 Stdn. geschüttelt. Nach Filtrieren wird die Lösung i.Vak. völlig eingedampft, der sirupöse Rückstand in 5 ccm abs. Pyridin und 5 ccm Acetanhydrid gelöst und 18 Stdn. bei Zimmertemperatur stehengelassen. Nach dem Eindampfen der Reaktionsmischung i.Vak. wurde der ölige Rückstand aus Alkohol umkristallisiert. Ausbeute 0.5 g (43% d.Th.), Schmp. 105°, $[\alpha]_D$ −17.6° (*c* 1.02, Chloroform). [Lit.[13] Schmp. 104–105°, $[\alpha]_D^{25}$ −18.2° (Chloroform)]. Mischschmelzpunkt mit authentischer Verbindung keine Depression.

Umsetzung von **1** *mit überschüssigem asymm. Dibromdimethyläther.* — Die Substanz **1** (1 g) wurde in 1.5 ccm Chloroform mit 2.5 ccm *as.*-Dibromdimethyläther und einer Spur wasserfreiem $ZnCl_2$ bei 65–70° erwärmt und wie bei dem analogen Chlorderivat aufgearbeitet. Es wurden 1.4 g Sirup erhalten, der sich nicht umkristallisieren ließ. Die sirupöse Substanz erhielt 48.4% Brom (für das Tribromderivat berechneter Wert 44.3%).

*2,3,4-Tri-*O*-acetyl-6-*O*-formyl-α-*D*-glucopyranosylbromid* (**10**). — Die Substanz **1** (3 g) wird in 5 ccm abs. $CHCl_3$ aufgelöst und mit 1.29 ccm *as.*-Dibromdimethyläther und einer Spur wasserfreiem $ZnCl_2$ 50 Min. auf dem Wasserbad auf 60° erwärmt. Das Reaktionsgemisch wird dann wie das entsprechende Chlorderivat aufgearbeitet. Die Substanz kristallisiert aus Chloroform–Petroläther in schönen Nadeln. Ausbeute (2.28 g) (41% d.Th.), Schmp. 96–97°, $[\alpha]_D$ +237° (*c* 0.43, Chloroform). $C_{13}H_{17}BrO_9$ 397.2 Ber.: Br, 20.1. Gef.: Br, 21.2%. Die Verbindung enthielt nach dünnschichtchromatographischen Daten (Einzelheiten über die Durchführung der Dünnschichtchromatographie siehe früher) in geringen Mengen 2,3,4-Tri-*O*-acetyl-α-D-glucopyranosylbromid. Die Substanz **10** läßt sich durch Umkristallisieren nicht weiter reinigen.

*1,2,3,4-Tetra-*O*-acetyl-6-*O*-formyl-β-*D*-glucopyranose* (**8**) *aus* **10**. — Die Substanz **10** (1.83 g) wird in 20 ccm Eisessig mit 2.2 g Silberacetat umgesetzt. Die Reaktion wurde unter den früher angegebenen Bedingungen durchgeführt. Es wurde 1 g (58% d.Th.) reines **8** erhalten, Schmp. 135–136°, $[\alpha]_D$ +9.8° (*c* 2.3, Chloroform), identisch mit authentischem **8**.

*Äthyl-2,3,4-tri-*O*-acetyl-6-*O*-formyl-β-*D*-glucopyranosid* (**5**) *aus* **10**. — Die Substanz **10** (2.4 g) wurde in 50 ccm abs. Äthanol gelöst und mit 5 g frisch hergestelltem trockenem Silbercarbonat 16 Stdn. bei 20° geschüttelt. Das Reaktionsgemisch wurde nach der bei der analogen Umsetzung von **3** angegebenen Weise aufgearbeitet. Ausbeute 1.0 g (48% d.Th.), Schmp. 138–139°, $[\alpha]_D$ −12.0° (*c* 0.75, Chloroform). Die Verbindung gab keine Schmelzpunkterniedrigung mit dem aus **3** gewonnenen Glucosid.

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SYNTHESIS OF β-CARBOLINYL SUGARS

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ABSTRACT

Decomposition of penta-*O*-acetyl-1-deoxy-1-diazo-*keto*-D-*gluco*-heptulose, in the presence of *N*-methylindole and copper powder as catalyst, results in the formation of penta-*O*-acetyl-1-deoxy-1-(*N*-methylindol-3-yl)-*keto*-D-*gluco*-heptulose (**1**). Compound **1** is acylated by carboxylic anhydrides at C-2 of the *N*-methylindole moiety, and cyclisation of the intermediate diketone, in the presence of 70% perchloric acid, gives indolo-[2,3-*c*]-pyrylium perchlorates.

With aqueous ammonia, these perchlorates are easily transformed into β-carbolinyl sugars that are close structural analogues of the indole alkaloids harmane, harmine, and melinonine.

INTRODUCTION

Although there exists a great variety of methods[1] for the synthesis of sugars having heterocyclic *C*-substituents, similar compounds containing the alkaloid-like system of β-carboline were hitherto unknown. Lobry de Bruyn and Alberda van Ekenstein[2] failed to synthesise β-carbolinyl derivatives by condensing D-glucose with tryptamine according to the Pictet–Spengler method[3,4].

We thought it worthwhile to synthesize β-carbolinyl sugars, which are near structural analogues of the indole alkaloids, harmane, harmine, melinonine, and others[5].

EXPERIMENTAL

Infrared spectra (mineral oil) were obtained with a ИКС-14 spectrophotometer. Ultraviolet spectra were recorded for methanolic solutions (10^{-5}M) with a Сф-4 spectrophotometer. Polarimetry of the products was precluded by their intense colours.

Penta-O-*acetyl-1-deoxy-1*-(N-*methylindol-3-yl*)-keto-D-gluco-*heptulose* (**1**). — To a mixture of *N*-methylindole (3 g, 0.02 mole), dry toluene (5 ml), and a catalytic quantity of copper powder at 95–100°, was added dropwise a solution of penta-*O*-acetyl-1-deoxy-1-diazo-*keto*-D-*gluco*-heptulose[6] (4.3 g, 0.01 mole) in toluene (50 ml).

Liberation of nitrogen began at once, and when this was complete, the catalyst was filtered off, and toluene was removed *in vacuo*. The residual syrup was treated 5 or 6 times with light petroleum and then dissolved in benzene (50 ml), and the solution was gradually poured into light petroleum (500 ml). The product separated as a dark syrup that was dissolved in chloroform–methanol (19:1), and the solution was shaken with a small quantity of alumina for several hours. The alumina was filtered off, and the filtrate was evaporated *in vacuo*. The residue was dissolved in benzene (50 ml), and the solution was poured into light petroleum (500 ml). The crude product was precipitated as an amorphous, yellow powder, and was purified by repeated reprecipation from light petroleum; the material precipitated initially was discarded. The title compound (1.8 g, 29%) was finally obtained as an amorphous, yellow powder that was soluble in most organic solvents, but insoluble in water and light petroleum, and had ν_{max}^{NaCl} 1756 (OAc), 1723 (C=O), 1640, 1510 (heterocycle), and 1222 cm^{-1} (C-O-C); ν_{max}^{LiF} 2812 cm^{-1} (>N–CH_3); λ_{max} 222 (ε 26,500) and 282 nm (6,200) (Found: C, 58.20; H, 5.88; N, 2.80. $C_{26}H_{31}NO_{11}$ calc.: C, 58.53; H, 5.81; N, 2.62%).

1,9-Dimethyl-3-(D-gluco-*pentaacetoxypentyl*)-*β-carboline* (**4a**). — Perchloric acid (70%, 0.8 ml) was added dropwise to compound **1** (4 g, 0.007 mole) dissolved in 10 ml of acetic anhydride. After 15–20 min, the highly resinous mixture was diluted with 200 ml of ether. The resulting, dark-brown residue of the perchlorate **3** was carefully ground with ether, filtered off, and washed several times with ether in order to remove anhydride. The product was suspended in ether and treated with an excess of 20% aqueous ammonia. The product was extracted 5–10 times with ether, and the dried (Na_2SO_4) extract was purified with charcoal. The solvent was distilled off, leaving compound **4a** (1 g, 25%) as an amorphous, yellow powder. Further purification of the product was effected by repeated reprecipitation from chloroform (with light petroleum); the first material to be precipitated was discarded in each case. The product was soluble in most organic solvents, but not in water and light petroleum, and had ν_{max}^{NaCl} 1736 (OAc), 1624, 1603, 1535, 1485 (heterocycle), and 1218 cm^{-1} (C–O–C); λ_{max} 239 (ε 24,200), 278 nm (14,000) (Found: C, 60.11; H, 5.96; N, 4.78. $C_{28}H_{32}N_2O_{10}$ calc.: C, 60.43; H, 5.75; N, 5.03%).

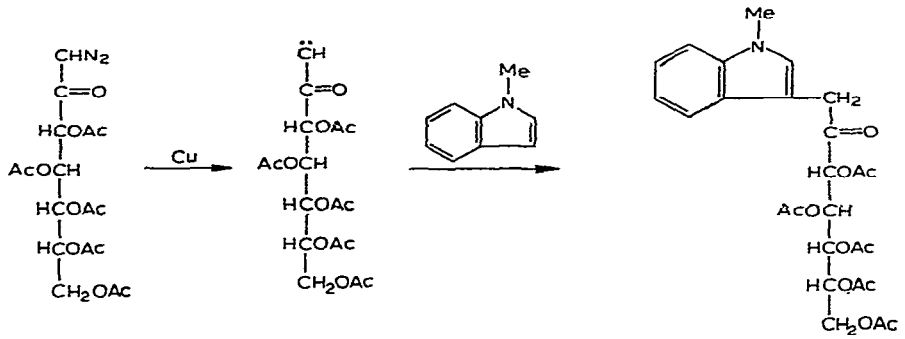
1-Ethyl-9-methyl-3-(D-gluco-*pentaacetoxypentyl*)-*β-carboline* (**4b**). — According to the directions given for obtaining compound **4a**, compound **1** (2 g, 0.003 mole) was treated with propionic anhydride (5 ml) and perchloric acid (0.4 ml) to give compound **4b** as an amorphous, yellow powder (0.61 g, 29%); ν_{max}^{NaCl} 1748 (OAc), 1630, 1604, 1538, 1489 (heterocycle), and 1215 cm^{-1} (C–O–C); λ_{max}238 (ε 24,200), 279 nm (14,000) (Found: C, 61.31; H, 5.97; N, 5.21. $C_{29}H_{34}N_2O_{10}$ calc.: C, 61.05; H, 5.96; N, 4.91%).

9-Methyl-3-(D-gluco-*pentaacetoxypentyl*)-*1-propyl-β-carboline* (**4c**). — According to the procedure described for the preparation of compound **4a**, compound **1** (2 g, 0.004 mole) was treated with butyric anhydride (5 ml) and perchloric acid (70%, 0.4 ml) to give compound **4c** (31%) as an amorphous, yellow powder that was soluble in most organic solvents, insoluble in water and light petroleum, and had ν_{max}^{NaCl} 1745 (OAc), 1624, 1610, 1530, 1499 (heterocycle), and 1217 cm^{-1} (C–O–C);

λ_{max} 238 (ε 24,200), 278 nm (14,000) (Found: C, 62.70; H, 5.30; N, 4.81. $C_{31}H_{36}N_2O_{10}$ calc.: C, 62.41; H, 5.36; N, 4.69%).

DISCUSSION

The oxocarbenes formed by the decomposition of diazoketoses, in the presence of copper powder, attack[7,8] C–3 of *N*-methylindole (Scheme 1) to give 1-deoxy-1-(*N*-methylindol-3-yl)-ketose acetates. Penta-*O*-acetyl-1-deoxy-(*N*-methylindol-3-yl)-

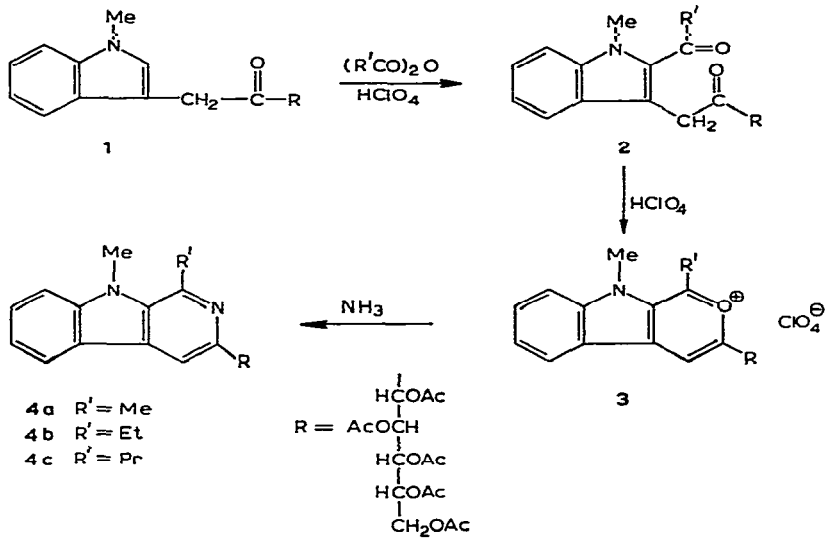


Scheme 1

keto-D-*gluco*-heptulose (**1**) is an amorphous, coloured powder. Its study by polarimetry is impossible because of the intense colour of its solutions. The intense bands at 1756 and 1222 cm^{-1} in the i.r. spectrum are characteristic of the acetyl groups, and the weak bands at 2812, 1640, and 1510 cm^{-1} may be assigned to the *N*-methylindole moiety[9]. The u.v. spectrum, having λ_{max} 222 (ε 26,500) and 282 nm (6,200), was similar to those of indole derivatives[8].

Indolylacetone and alkylindolylacetones are acylated[10] at C–2 by carboxylic anhydrides in the presence of 70% perchloric acid to yield indolo-[2,3-*c*]-pyrylium perchlorates. In order to obtain *C*-substituted indolopyrylium salts and carbolines, we investigated the acylation of compound **1** with acetic, propionic, and butyric anhydrides in the presence of 70% perchloric acid. The acylating agents in these reactions are acyl perchlorates[11]. The diketone **2**, formed as an intermediate in the presence of perchloric acid, cyclises to give the pyrylium salt **3**. The reaction is accompanied by resin formation and a consequent lowering of the yield of the final product. The acylation of indol-3-ylketoses having an unsubstituted nitrogen atom have not been studied, since, in this case, acylation of indole, and a number of other processes connected with the lability of the indole system in strongly acid media are possible. In the acylations of indol-3-ylacetone, the yield of products does not exceed 10%. The ease with which a pyrylium oxygen atom is replaced by nitrogen makes it possible to convert indolo-[2,3-*c*]-pyrylium salts into derivatives of β-carboline. The transformation of pyrylium salts **3** into β-carbolines **4** took place directly at room temperature. The i.r. spectra of the β-carbolinyl sugars (**4**) show strong bands at

1750–1736 and 1220–1214 cm^{-1}, which are regions that are characteristic of the acetylated sugar moiety. The bands at 1630, 1600, 1530, and 1500 cm^{-1} are consistent



Scheme 2

with the heterocyclic moiety. There was no band characteristic of a free carbonyl group[8], and the band in the region 1750–1730 cm^{-1} was narrow.

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OPTICAL ROTARY DISPERSION STUDIES. CORRELATION BETWEEN THE STRUCTURE AND O.R.D. CURVES OF ACYCLIC KETOSES

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ABSTRACT

The o.r.d.curves of some tetruloses and 2- and 3-pentuloses have been measured, and a relation has been found between the structure of the ketoses and their Cotton effects.

INTRODUCTION

In studying the o.r.d. curves of organic compounds, most attention has been devoted to substances possessing a carbonyl group, which is a suitable chromophore for generating the Cotton effect. Since sugars generally exist as cyclic hemiacetals, they exhibit plain o.r.d. curves[1,2] in the visible and near-ultraviolet regions. In spite of recent studies[3–5] of the relation between the structure of sugars and the magnitude and sign of optical rotation at *ca.* 200 nm, these correlations are of small practical importance. Because of this, various sugar derivatives containing optically active chromophores have been examined, *e.g.*, xanthates[6,7], disulphides[8], nitro derivatives[9–13], and compounds[14–19] having azo, acetyl, lactone, azido, or carbonyl groups.

The first systematic study of acyclic compounds was carried out by Levene and Rothen[20]. Acyclic aldehydes and ketones[21] may be compared with saccharide derivatives (peracetates) possessing a free carbonyl group. The prediction that, in solution, suitable pentuloses and tetruloses would contain free carbonyl groups has been verified experimentally by the generation of a Cotton effect. Since intramolecular, cyclic hemiacetals of tetruloses and 3-pentuloses cannot be formed, only hydration influences the proportion of free carbonyl groups. In the case of pentuloses, formation of a cyclic hemiacetal can take place, resulting in a further lowering of the proportion of free carbonyl groups. These effects are reflected in the amplitudes of the measured Cotton effects.

RESULTS AND DISCUSSION

The ketoses studied in this work are shown in Table I, together with the signs and amplitudes of the Cotton effects. The ultraviolet absorption spectra of these compounds show maxima or shoulders at *ca.* 280 nm ($\varepsilon < 100$), corresponding to

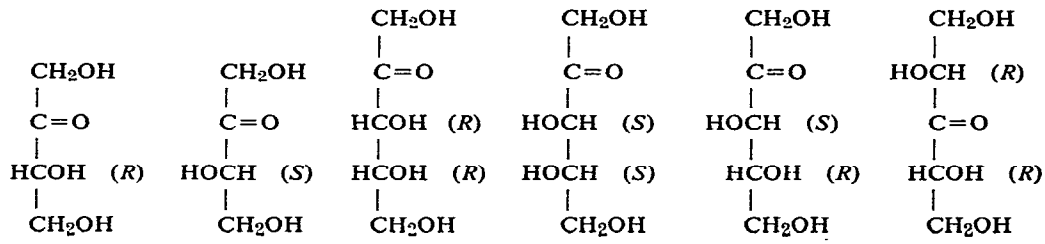
TABLE I

SIGNS AND AMPLITUDES OF THE COTTON EFFECTS FOR VARIOUS KETOSES

Compound	*Cotton effect*[a]
D-*glycero*-tetrulose (**1**)	−(22.1)
L-*glycero*-tetrulose (**2**)	+(22.0)
D-*erythro*-pentulose (**3**)	−(4.2)
L-*erythro*-pentulose (**4**)	+(5.8)
D-*threo*-pentulose (**5**)	+(5.9)
D-*threo*-3-pentulose(**6**)	−(83.1)
erythro-3-pentulose (**7**)	

[a]The figures given parenthetically are the amplitudes, ([ø] first extremum — [ø] second extremum)/100.

the n → π* transition of a carbonyl chromophore. L-*glycero*-Tetrulose (**2**), L-*erythro*-pentulose (**4**), and D-*threo*-pentulose (**5**) exhibit a positive Cotton effect in their o.r.d. curves; whereas D-*glycero*-tetrulose (**1**), D-*erythro*-pentulose (**3**), and D-*threo*-3-pentulose (**6**) exhibit negative effects (Fig. 1). The first extremum in the o.r.d. curves of these substances appears at *ca.* 295 nm, and the second is found at *ca.* 255 nm. The single exception is D-*threo*-3-pentulose (**6**) for which both extrema are shifted 10 and 5 nm, respectively, to higher wavelengths. This observation is in complete



1 **2** **3** **4** **5** **6**

agreement with the bathochromic shift of the u.v. absorption maxima [*cf.* also *erythro*-3-pentulose (**7**)] in comparison with the other compounds.

The occurrence of Cotton effects for these saccharides indicates that a free carbonyl group is present. D-*glycero*-Tetrulose (**1**) has the centre of chirality at C-3, and its absolute configuration[22] is *R*; the o.r.d. curve exhibits a negative Cotton effect. L-*glycero*-Tetrulose (**2**), having an absolute *S* configuration at C-3, shows a positive Cotton effect. The amplitudes of the Cotton effects are of the same magnitude (see Fig. 1*a* and Table I).

The signs of the Cotton effects of the pentuloses are influenced by two centres of chirality. D-*erythro*-Pentulose (**3**) has the *R* configuration at both C-3 and C-4, and shows a negative Cotton effect. Its enantiomer, L-*erythro*-pentulose (**4**) shows a positive Cotton effect. The amplitudes of the Cotton effects are, as expected, nearly identical (see Fig. 1*b* and Table II). D-*threo*-Pentulose, which possesses two centres

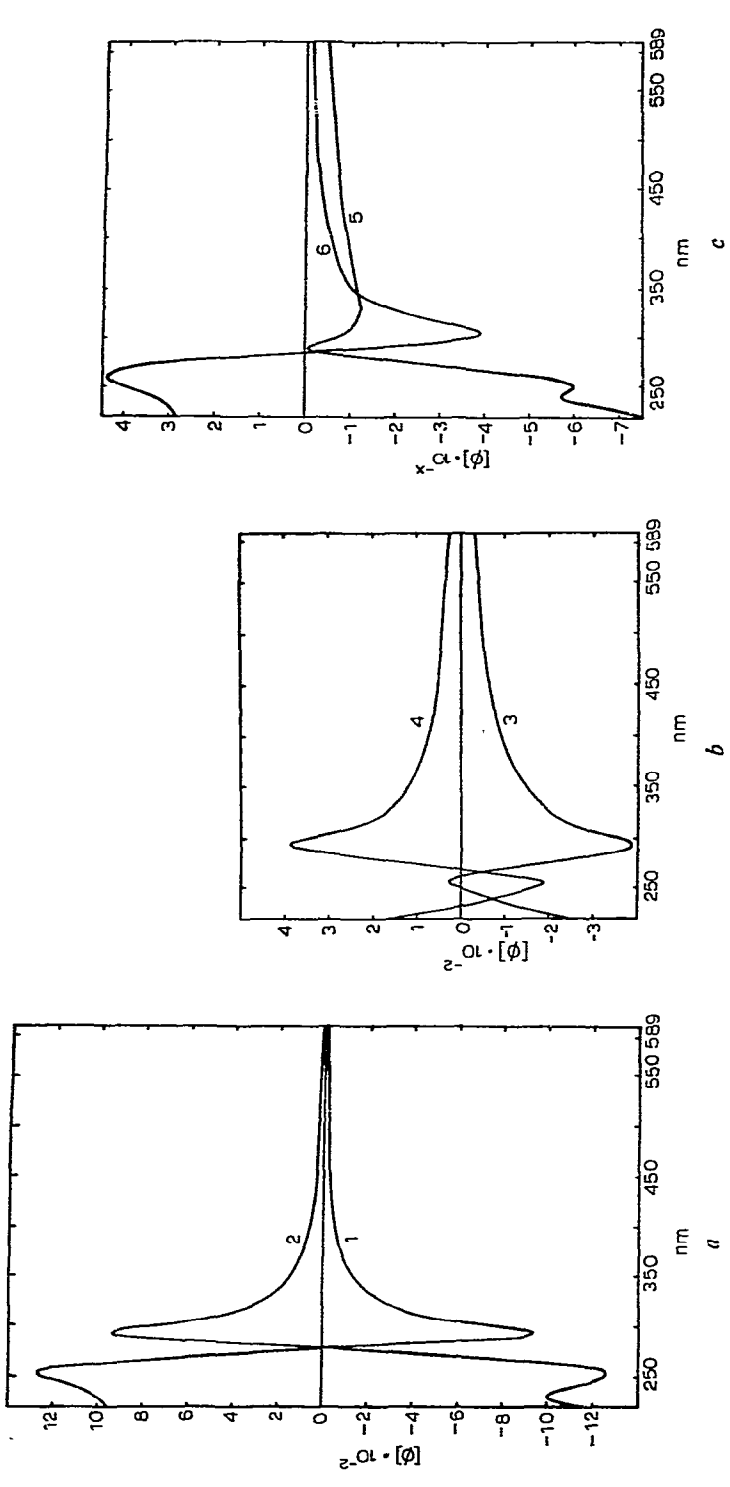


Fig. 1*a*, *b*, *c*. O.r.d. curves for D-*glycero*-tetrulose (1), L-*glycero*-tetrulose (2), D-*erythro*-pentulose (3), L-*erythro*-pentulose (4), D-*threo*-pentulose (5)[a], and D-*threo*-3-pentulose (6)[a].

[a]The index x in the expression $[\emptyset] \cdot 10^{-x}$ is 2 for D-*threo*-pentulose, and 3 for D-*threo*-3-pentulose.

of chirality having different absolute configuration (*S* at C-3 and *R* at C-4), shows a positive Cotton effect. The sign of the Cotton effect is therefore influenced by the absolute configuration at the closest centre of chirality. If two such centres having the same absolute configuration are attached to the chromophore, as in D-*threo*-3-pentulose (**6**), both centres contribute equally to the sign of the Cotton effect, as shown in Fig. 1*c*. More-distant centres of chirality do not influence the sign of the Cotton effect, but the overall shape of the o.r.d. curve is affected in a manner that depends on the absolute configuration. On this basis, the character of the o.r.d. curve of D-*threo*-pentulose may be explained; for this compound, the entire curve, including the positive Cotton effect, is situated in the range of negative values of rotation. *erythro*-3-Pentulose is optically inactive in the whole of the measured region. D-*glycero*-Tetrulose (**1**), L-*glycero*-tetrulose (**2**), and D-*threo*-3-pentulose (**6**) exhibit substantially bigger amplitudes than those for the other compounds (Table I), which corresponds with the fact that these compounds, in contrast to the pentuloses, cannot cyclise.

For the acyclic derivatives, penta-*O*-acetyl-D-fructose[23], tetra-*O*-acetyl-L-fucose[23], and D-fructose 1,6-diphosphate[24], positive Cotton effects were found, in agreement with the *S* configuration at the centre of chirality adjacent to the carbonyl group.

It seems reasonable to assume that, in the case of acyclic saccharides, the centre of chirality adjacent to a carbonyl group determines the sign of the Cotton effect. If the absolute configuration of the neighbouring centre of chirality is *R*, the induced Cotton effect will be negative, whereas it will be positive when the configuration is *S*.

EXPERIMENTAL

U.v. and o.r.d. spectra were measured on a JASCO UV/ORD–5 Spectrophotometer in the region from 600 to 220 nm at 24–27° in 1–5 cm cells. Concentrations are expressed in g/100 ml in redistilled water. Molecular rotations are given according to Djerassi[25]. All of the substances studied were chromatographically pure; D- and L-*glycero*-tetruloses were purified on a cellulose (Whatman) column, and an ionex column[26] was used for the other compounds.

D-*glycero*-Tetrulose (**1**), prepared[27] from D-erythrose, had $[\alpha]_D^{25}$ −10.7° (*c* 2.0), λ_{sh} 278 nm (1.67), and gave an *o*-nitrophenylhydrazone, $[\alpha]_D^{26}$ −51° (*c* 0.45, abs. ethanol), m.p. 152–153°. O.r.d. data (Fig. 1*a*) (*c* 0.0959): $[\emptyset]_{600}$ −10°, $[\emptyset]_{589}$ −15°, $[\emptyset]_{295}$ −940° (min), $[\emptyset]_{255}$ +1270° (max), $[\emptyset]_{220}$ +960°.

L-*glycero*-Tetrulose[28] (**2**), $[\alpha]_D^{22}$ +10.6° (*c* 1.0) {lit.[28], +11.4 ±1° (*c* 2.37)}, λ_{max} 274 nm (1.40), was characterised as the *o*-nitrophenylhydrazone, $[\alpha]_D^{25}$ +49° (*c* 0.44, abs. ethanol), m.p. 152–153°; [lit.[28], $[\alpha]_D^{18}$ +48 ±2° (*c* 1.0, abs. ethanol), m.p. 152–153°], o.r.d. data (Fig. 1*a*) (*c* 0.0945): $[\emptyset]_{600}$ −10°, $[\emptyset]_{589}$ −15°, $[\emptyset]_{294}$ +940° (max), $[\emptyset]_{254}$ −1260° (min), $[\emptyset]_{230}$ −1000° (max), $[\emptyset]_{220}$ −1150°.

D-*erythro*-Pentulose (**3**), $[\alpha]_D^{22}$ −21° (*c* 0.20), λ_{max} 279 nm (1.24), was prepared[29] from D-arabinose and identified as the *o*-nitrophenylhydrazone, $[\alpha]_D^{25}$ −48°, m.p. 168–169°; lit.[29], m.p. 168–169.5°. O.r.d. data (Fig. 1*b*) (*c* 0.1118): $[\emptyset]_{600}$ −30°, $[\emptyset]_{589}$ −30°, $[\emptyset]_{294}$ −390° (min), $[\emptyset]_{257}$ +30° (max), $[\emptyset]_{220}$ −240°.

L-*erythro*-Pentulose (**4**), $[\alpha]_D^{24}$ +21° (*c* 0.10), {lit.[31], $[\alpha]_D^{21}$ +15 → +16.3° (*c* 2.08)}, λ_{max} 279 nm (1.16), was prepared by biochemical oxidation of ribitol[30]. O.r.d. data (Fig. 1*b*) (*c* 0.10): $[\emptyset]_{600}$ −30°, $[\emptyset]_{589}$ −30°, $[\emptyset]_{295}$ +390° (max), $[\emptyset]_{256}$ −190° (min), $[\emptyset]_{220}$ +150°.

D-*threo*-Pentulose (**5**), $[\alpha]_D^{23}$ −32.8° (*c* 0.10), {lit.[32], $[\alpha]_D^{18}$ −33.2 ±0.4° (*c* 0.253)}, λ_{sh} 275 nm (1.52), was prepared[32] from D-xylose. O.r.d. data (Fig. 1*c*) (*c* 0.106): $[\emptyset]_{600}$ −45°, $[\emptyset]_{589}$ −50°, $[\emptyset]_{290}$ −10° (max), $[\emptyset]_{250}$ −600° (min), $[\emptyset]_{240}$ −570° (max), $[\emptyset]_{220}$ −740°.

D-*threo*-3-Pentulose (**6**), $[\alpha]_D^{22}$ −85° (*c* 1.0), λ_{max} 284 nm (1.74), was prepared[26] by oxidation of D-arabinitol with mercuric acetate[33]. O.r.d. data (Fig. 1*c*) (*c* 0.10): $[\emptyset]_{600}$ −120°, $[\emptyset]_{589}$ −130°, $[\emptyset]_{305}$ −3930° (min), $[\emptyset]_{260}$ +4380° (max), $[\emptyset]_{220}$ +2780°.

erythro-3-Pentulose (**7**) was prepared by oxidation of ribitol with mercuric acetate[33], and had λ_{max} 287 nm (1.95).

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NUCLEOPHILIC DISPLACEMENT REACTIONS IN CARBOHYDRATES

PART IV*. THE SOLVOLYSIS OF 3-ACETAMIDO-3-DEOXY-1,2-*O*-ISOPROPYLIDENE-5,6-DI-*O*-METHANESULPHONYL-α-D-GLUCOFURANOSE**

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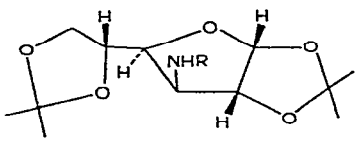
(Received October 24th, 1967)

ABSTRACT

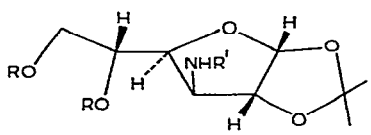
Solvolysis of 3-acetamido-3-deoxy-1,2-*O*-isopropylidene-5,6-di-*O*-methanesulphonyl-α-D-glucofuranose (**3**) in either 95% 2-methoxyethanol or *N*,*N*-dimethylformamide, in the presence of sodium acetate, gives 3,6-(acetylepimino)-3,6-dideoxy-1,2-*O*-isopropylidene-β-L-idofuranose (**6**) as the product. The structure is assigned on the basis of chemical and spectroscopic evidence. The mechanism of the solvolysis, which involves neighbouring-group participation by the amide group, is discussed.

INTRODUCTION

We have recently reported[1] on a facile synthesis of 3-acetamido-3-deoxy-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**1**) and its conversion into 3-acetamido-3-deoxy-D-glucose and 3-amino-3-deoxy-D-xylose. Graded, acid hydrolysis of compound **1** removed the 5,6-*O*-isopropylidene group[1], and the resulting diol (**2**) was converted into 3-acetamido-3-deoxy-1,2-*O*-isopropylidene-5,6-di-*O*-methanesulphonyl-α-D-glucofuranose (**3**) on methanesulphonylation. Disulphonate **3** is of interest, since it contains sulphonic ester groups that are two and three carbon atoms removed from an acetamido group, which could conceivably participate[2] in their removal; **3** is, therefore, of potential value in the synthesis of diamino sugars of biological interest.



1 R = Ac
5 R = H



2 R = H ; R' = $COCH_3$
3 R = Ms; R' = $COCH_3$
4 R = Ms; R' = $COCD_3$

*Part III: J. S. Brimacombe, (Miss) P. A. Gent, and M. Stacey, *J. Chem. Soc. C*, (1968) 567.
**This work was presented, in part, at the 4th International Symposium on Carbohydrate Chemistry, Kingston, Ontario, July, 1967.

Amides are ambident nucleophiles, and examples of nucleophilic participation involving attack by the oxygen and nitrogen atoms of the amide group are known[2b]. The carbohydrate field is particularly rich in examples of neighbouring amide-group participation, which have proved of immense value in amino-sugar syntheses[3]. In most of the cases examined, the participating and departing groups have been located on vicinal carbon atoms, and there are comparatively few reports on amide-group participation where this relationship is not found. One example was recorded by Meyer zu Reckendorf[4], who observed that solvolysis of methyl 2,6-dibenzamido-2,6-dideoxy-4-*O*-methanesulphonyl-3-*O*-methyl-β-D-glucopyranoside, with ethanolic sodium ethoxide, occurred with inversion of configuration at C-4 to give a six-membered dihydro-oxazine derivative. A second case was described by Hanessian[5] during the course of this investigation. Thus, treatment of 5-acetamido-5-deoxy-1,2-*O*-isopropylidene-3-*O*-methanesulphonyl-β-D-arabinofuranose with sodium benzoate in *N*,*N*-dimethylformamide afforded 5-acetamido-5-deoxy-1,2-*O*-isopropylidene-β-D-lyxofuranose, by breakdown of the intermediate dihydro-oxazinium ion.

We now report on the solvolysis of disulphonate **3**.

DISCUSSION

Solvolysis of dimethanesulphonate **3**, with boiling 95% 2-methoxyethanol (Methyl Cellosolve) in the presence of sodium acetate, for 24 h gave a crystalline product (*A*), in moderate yield, following chromatography of the reaction mixture on silica gel. Attempts to follow the course of the reaction by thin-layer chromatography were made difficult by the fact that the various components were not well-separated, and the presence of compound *A* in the reaction mixture was best judged by the reddish spot produced on spraying the chromatogram with the vanillin–sulphuric acid reagent[6]. Product *A* was also formed when the solvolysis was carried out in *N*,*N*-dimethylformamide–sodium acetate.

Compound *A* had a molecular weight of 243 (by mass spectrometry) and elemental analyses corresponding to the molecular formula $C_{11}H_{17}NO_5$. The absence of sulphonic ester groups in *A* was confirmed by infrared spectroscopy, which also indicated the presence of an isopropylidene group and a hydroxyl group. The spectrum showed only one absorption band in the region 1500–1700 cm^{-1}, signifying that the acetamido group had been modified in some way during the solvolysis.

The n.m.r. spectrum of compound *A* (Fig. 1) established the presence of an isopropylidene group, but the most significant feature of the spectrum was the appearance of a pair of doublets at τ 4.16 and 4.27 (J 3.5 Hz), corresponding to one proton, which could be ascribed to the anomeric proton. Integration showed that these signals were in an approximate ratio of 1:2.5, and the pair of singlets at *ca.* τ 8.00, corresponding to three protons, had the same ratio. The spectrum simplified when the temperature was raised to 100° and, noticeably, the doublets at low field and the pair of singlets at τ *ca.* 8.00 each coalesced; the process was reversed on cooling. This behaviour is characteristic of sugars in which the ring oxygen atom is replaced

by the $>NCOCH_3$ group and is attributable to hindered internal rotation about the nitrogen–acetyl bond, which allows the rotamers to be distinguished by n.m.r. spectroscopy[7].

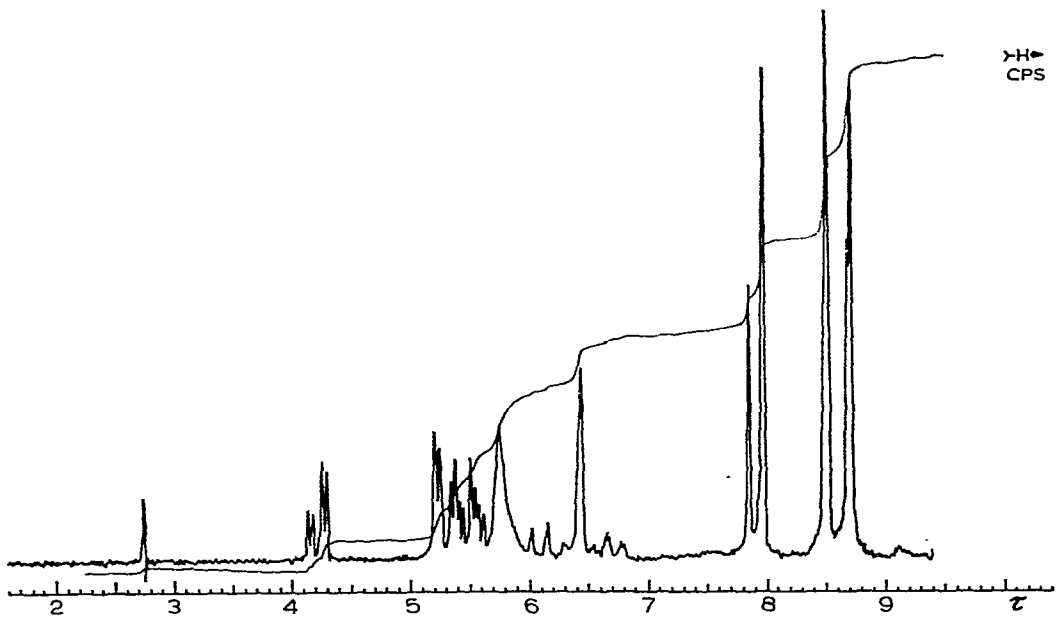


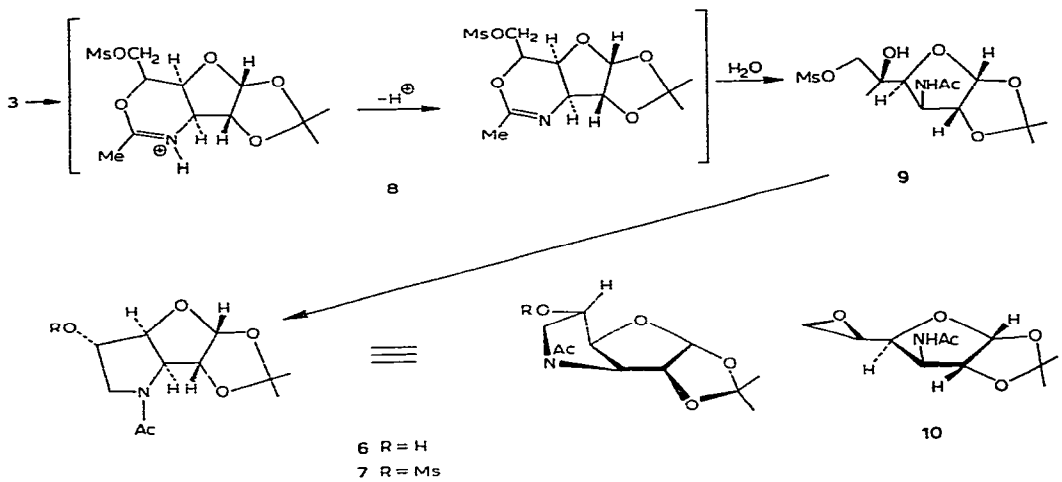
Fig. 1. N.m.r. spectrum (100 MHz) of compound *A*, subsequently identified as 3,6-(acetylepimino)-3,6-dideoxy-1,2-*O*-isopropylidene-β-L-idofuranose (6).

The presence of the $>NCOCH_3$ group in compound *A* was also inferred from mass spectrometry. Fragmentation of acetamido sugars invariably gives rise[8] to a peak in the spectrum at *m/e* 43, attributable to the $MeC{\equiv}\overset{+}{O}$ ion. With *A*, however, this ion will also result from fragmentation of the isopropylidene group[9]. These fragmentation modes were distinguished by examining the product resulting from the solvolysis of 3-deoxy-1,2-*O*-isopropylidene-5,6-di-*O*-methanesulphonyl-3-(trideuterioacetamido)-α-D-glucofuranose (**4**). Compound **4** was obtained by *N*-trideuterioacetylation of 3-amino-3-deoxy-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**5**), followed by selective removal of the α-acetal group with acid, and methanesulphonylation. The mass spectrum of the product from the solvolysis of compound **4** showed peaks, *inter alia*, at *m/e* 43 ($MeC{\equiv}\overset{+}{O}$) and 46 ($CD_3C{\equiv}\overset{+}{O}$), which were considered to arise by fragmentation of an isopropylidene and an $>NCOCD_3$ group, respectively.

Acetylation and methanesulphonylation of compound *A* gave a mono-ester, in each case, signifying the presence of a single hydroxyl group. The methanesulphonyloxy group was not exchanged on treatment with sodium iodide in either butanone or *N*,*N*-dimethylformamide, so that it is unlikely[10] to be present at a primary position.

The foregoing evidence points to a 3,6-(acetylepimino)-3,6-dideoxy-1,2-*O*-isopropylideneglycofuranose as the basic structure for compound *A*, leaving the stereochemistry at C-5 undecided for the moment.

Although mechanistic considerations imply an L-*ido* configuration (**6**) for compound *A*, it seemed desirable to confirm the stereochemistry at C-5 by independent means, particularly as desulphonylation, resulting from O–S bond cleavage, has been observed[5] under conditions comparable to those used for the solvolysis. The methanesulphonate, derived from compound *A*, was also found to be resistant to nucleophilic attack by either benzoate or azide ions, although thin-layer chromatography indicated that other reactions were occurring in both cases. The unreactivity of the sulphonic ester towards bimolecular nucleophilic displacement signified that it had an *exo* (*i.e.*, L-*ido*) configuration (**7**) with respect to the oxa-azabicyclo[3.3.0]-octane ring system. This argument is enhanced by the knowledge that the *endo*-sulphonyloxy group of 3,6-anhydro-1,2-*O*-isopropylidene-5-*O*-(toluene-*p*-sulphonyl)-α-D-glucofuranose is smoothly displaced by azide[11] and benzoate[12] ions. Moreover, with the closely related, bicyclic ring-system present in the 1,4:3,6-dianhydrohexitols, it has been established[13] that S_N2 displacement of *exo*-sulphonates is sterically hindered, whereas displacement of an *endo*-substituent is facile. On this evidence, compound *A* can be assigned as 3,6-(acetylepimino)-3,6-dideoxy-1,2-*O*-isopropylidene-β-L-idofuranose (**6**).



Carbohydrate sulphonic esters are relatively stable under acidic and neutral conditions[10], but are readily solvolysed in the presence of a group capable of rendering anchimeric assistance. Of relevance to the present discussion, it has been observed[14] that 3-*O*-acetyl-1,2-*O*-isopropylidene-5,6-di-*O*-(toluene-*p*-sulphonyl)-α-D-glucofuranose is solvolysed, in 95% 2-methoxyethanol–sodium acetate, to give 3,6-anhydro-1,2-*O*-isopropylidene-5-*O*-(toluene-*p*-sulphonyl)-α-D-glucofuranose, with deacetylation but *without* cleavage of the sulphonic ester group at C-5. Thus, the first step in

the conversion **3** → **6** is likely to involve the acetamido group in O^-–6 participation*, followed by attack of water on the intermediate **8** to give 3-acetamido-3-deoxy-1,2-*O*-isopropylidene-6-*O*-methanesulphonyl-β-L-idofuranose (**9**) as the first product (*cf.* Ref. 5). Subsequent loss of the C-6 substituent from compound **9** is best attributed to N^-–5 participation by the amide group, presumably by way of a small amount of the conjugate base existing in equilibrium under the basic reaction conditions. The overriding importance of *N*-attack in the solvolysis of the C-6 sulphonic ester is clearly governed by the size of the ring formed in the transition state. This route from compound **9** appears to be more likely than one involving *N*-attack on a 5,6-epoxide (**10**), which might arise under the reaction conditions. The acetamido group has already indicated a preference to participate in nucleophilic attack at C-5; hence, an assisted opening of the epoxide ring might be anticipated by analogy with that observed under acidic conditions, with a neighbouring acetoxyl group[15].

EXPERIMENTAL

Thin-layer chromatography (t.l.c.) was performed on Kieselgel G, and detection was effected with the vanillin–sulphuric acid reagent[6]. Infrared spectra were obtained on a Perkin–Elmer 125 spectrometer and were in agreement with the structures assigned. Mass spectra were measured with an A.E.I. MS9 mass spectrometer, and n.m.r. spectra were normally obtained with a Varian A–60 spectrometer for *ca.* 10% solutions in deuteriochloroform with tetramethylsilane as internal reference; spectra obtained with a Varian HA–100 spectrometer were measured by Dr. L. D. Hall. Light petroleum refers to the fraction having b.p. 60–80°.

*3-Acetamido-3-deoxy-1,2-*O*-isopropylidene-5,6-di-*O*-methanesulphonyl-α-D-glucofuranose* (**3**). — A solution of 3-acetamido-3-deoxy-1,2-*O*-isopropylidene-α-D-glucofuranose[1] (**2**) (1.5 g) and methanesulphonyl chloride (1.8 ml) in pyridine (10.5 ml) was set aside for 72 h at room temperature, water (3 ml) was then added, and the solvents were removed. The residue was taken up in ethanol (10 ml) and chromatographed on silica gel by elution with acetone. Combination and concentration of the appropriate fractions gave disulphonate **3** (1.55 g), m.p. 123–124°, $[\alpha]_D$ +5° (*c* 2.1, chloroform), on recrystallisation from chloroform–light petroleum (Found: C, 37.7; H, 5.6; N, 3.2; S, 15.3. $C_{13}H_{23}NO_{10}S$ calc.: C, 37.4; H, 5.6; N, 3.4; S, 15.4%). N.m.r. data: τ 4.10 (doublet, $J_{1,2}$ 3.5 Hz, anomeric proton); 6.82, 6.85 (3-proton singlets, sulphonic ester groups); 7.94 (3-proton singlet, NAc); 8.46, 8.67 (3-proton singlets, CMe_2).

*Solvolyses of 3-acetamido-3-deoxy-1,2-*O*-isopropylidene-5,6-di-*O*-methanesulphonyl-α-D-glucofuranose* (**3**). — (*a*) *Using sodium acetate in 95% 2-methoxyethanol.* A solution of compound **3** (1 g) and sodium acetate (1.3 g) in 95% 2-methoxyethanol (48 ml) was heated under reflux for 24 h. The solvents were removed, and water was evaporated several times from the residue, which was then dissolved in water (20 ml).

*In describing nucleophilic participation, it is frequently convenient to use the symbol "G-*n*", where G is the participating group and *n* the size of the ring formed in the transition state [2b].

The solution was extracted with chloroform (6 × 100 ml), and the combined and dried ($MgSO_4$) extracts were concentrated to *ca.* 2 ml, and chromatographed on silica gel by elution with chloroform–ethanol (20:1). Combination and evaporation of the appropriate fractions gave 3,6-(acetylepimino)-3,6-dideoxy-1,2-*O*-isopropylidene-β-L-idofuranose (**6**) (0.285 g, 45%), m.p. 169–170° (from acetone–light petroleum), $[\alpha]_D$ −75° (*c* 0.4, chloroform) (Found: C, 54.6; H, 7.0; N, 5.9. $C_{11}H_{17}NO_5$ calc.: C, 54.3; H, 7.0; N, 5.8%). The mass spectrum of the product showed the highest peak at *m/e* 228 (M–15)[9], corresponding to a molecular weight of 243; it also contained a peak at *m/e* 43. Its infrared spectrum exhibited absorptions at 3500 (OH) and 1380 cm^{-1} (isopropylidene group); the amide II band, present in the spectrum of compound **3**, had disappeared while the amide I band (1650 cm^{-1}) persisted. The n.m.r. spectrum of compound **6** is shown in Fig. 1.

(*b*) *Using sodium acetate in* N,N-*dimethylformamide.* A solution of compound **3** (0.2 g) and sodium acetate (0.13 g) in dry *N,N*-dimethylformamide (16.8 ml) was heated under reflux for 3 h. The solvent was then removed, the residue was dissolved in water (20 ml), and the solution was extracted with chloroform (6 × 40 ml). The combined and dried ($MgSO_4$) extracts were concentrated to *ca.* 2 ml; t.l.c. (chloroform–ethanol, 20:1) revealed the presence of two components (R_F 0.1 and 0.6). Chromatography, as in (*a*), gave the faster-moving component (40 mg, 32%) which proved to be identical with compound **6**, m.p. and mixed m.p. 169–170°. The chromatographic properties and infrared spectra of the two compounds were indistinguishable.

*3-Deoxy-1,2-*O*-isopropylidene-5,6-di-*O*-methanesulphonyl-3-(trideuterioacetamido)-α-*D*-glucofuranose* (**4**). — This compound, m.p. 123–124°, mixed m.p. 123–124° with compound **3**, was obtained by methanesulphonylation of 3-deoxy-1,2-*O*-isopropylidene-3-(trideuterioacetamido)-α-D-glucofuranose, essentially as described above. The latter compound was prepared by trideuterioacetylation of 3-amino-3-deoxy-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**5**), followed by graded hydrolysis with acid, as described elsewhere[1].

Solvolysis of compound **4**, as detailed in (*a*), gave 3,6-dideoxy-1,2-*O*-isopropylidene-3,6-[(trideuterioacetyl)epimino]-β-L-idofuranose, m.p. 169–170°, $[\alpha]_D$ −75° (*c* 0.45, chloroform); no depression of melting point was observed on admixture with the product from (*a*). The mass spectrum of the deuterated product exhibited, *inter alia*, the following peaks, *m/e* 231 (M–15), 46 ($CD_3C{\equiv}\overset{+}{O}$), and 43 ($CH_3C{\equiv}\overset{+}{O}$).

*5-*O*-Acetyl-3,6-(acetylepimino)-3,6-dideoxy-1,2-*O*-isopropylidene-β-L-idofuranose.* — This compound, m.p. 126–127° (from chloroform–light petroleum), was obtained by acetylation of compound **6**, with acetic anhydride in pyridine, in the normal way (Found: C, 54.1; H, 6.7. $C_{13}H_{19}NO_6$ calc.: C, 54.2; H, 6.6%). N.m.r. data: τ 4.13, 4.25 (doublets, $J_{1,2}$ 3.5 Hz, anomeric proton); 7.85, 7.96 (singlets, 3-proton, integrated ratio *ca.* 1:2.5, NAc); 7.95 (3-proton singlet, OAc); 8.50, 8.70 (3-proton singlets, CMe_2).

*3,6-(Acetylepimino)-3,6-dideoxy-1,2-*O*-isopropylidene-5-*O*-methanesulphonyl-β-L-*

idofuranose (7). — A solution of compound **6** (0.1 g) and methanesulphonyl chloride (0.05 ml) in pyridine (0.5 ml) and benzene (0.2 ml) was set aside overnight, water was then added, and the solution was concentrated. The syrupy residue was dissolved in chloroform (25 ml), and the solution was washed with dilute, aqueous cadmium chloride, dried ($MgSO_4$), and concentrated. Chromatography of the residue on silica gel by elution with ethanol–chloroform (1:20) gave sulphonate **7** (0.11 g), m.p. 144–145° (from chloroform–light petroleum) (Found: C, 44.7; H, 5.7; N, 4.0; S, 10.2. $C_{12}H_{19}NO_7S$ calc.: C, 44.9; H, 5.9; N, 4.4; S, 10.0%).

Attempted displacement reactions with compound **7**. — (*a*) *With sodium iodide.* A solution of compound **7** (20 mg) and sodium iodide (30 mg) in dry *N*,*N*-dimethylformamide (1 ml) was heated in a sealed tube overnight at 95–100°. The solvent was removed, the residue was dissolved in water (10 ml), and the solution was extracted with chloroform (5 × 20 ml). Starting material (13 mg), m.p. and mixed m.p. 144–145° (from chloroform–light petroleum), was recovered on removal of the solvent. T.l.c. indicated that no other components were present.

Starting material was also recovered when the displacement was attempted in butanone.

(*b*) *With sodium benzoate.* A solution of compound **7** (50 mg) and sodium benzoate (0.1 g) in *N*,*N*-dimethylformamide (3.75 ml) was heated under reflux for 5 h. The solution was processed in the usual manner, and the residue was chromatographed on silica gel with ethanol–chloroform (1:20). This gave two unidentified components, neither of which showed an appreciable absorption attributable to benzoic ester in its infrared spectrum.

(*c*) *With sodium azide.* A solution of compound **7** (0.1 g), sodium azide (23 mg), and urea (3 mg) in water (0.05 ml), contained in a sealed tube, was heated for 36 h at 115° and then processed in the usual way. The residue was shown by t.l.c. (ethyl acetate) to contain at least five components, but its infrared spectrum showed insignificant absorption at *ca.* 2100 cm^{-1} attributable to azide groups.

In a parallel experiment, 3,6-anhydro-1,2-*O*-isopropylidene-5-*O*-(toluene-*p*-sulphonyl)-α-D-glucofuranose was smoothly converted into the azido derivative in high yield (*cf.* Ref. 11).

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A STUDY OF RUTHENIUM TETROXIDE AS AN OXIDANT FOR ALCOHOLS

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ABSTRACT

The oxidation of benzhydrol and partially protected sugars with ruthenium tetroxide has been studied and the stoichiometry of the reaction verified. Axial and equatorial hydroxyl groups on an otherwise protected pyranoid ring are oxidized with equal ease. The generation of the tetroxide with periodate and ruthenium dioxide has been confirmed to be an easy reaction, providing that the dioxide has been prepared by the precipitation process.

INTRODUCTION

Recently, we introduced ruthenium tetroxide as an oxidant for partially protected carbohydrates[1]. This oxidant represented a considerable improvement on the methods then available for oxidizing alcoholic groups in partially protected pyranoid or furanoid rings. Although another method is now available[2], which uses less expensive reagents, we still find ruthenium tetroxide to be the reagent of choice, when a good yield of clean product is quickly required. We were surprised, therefore, to learn that some workers[3] had found difficulties in preparing the tetroxide from the dioxide, until we experienced the same trouble[4].

This problem has now been resolved. Ruthenium dioxide is available commercially in an anhydrous form, prepared by direct oxidation of ruthenium metal, and a hydrated form, with the probable composition $RuO_2 \cdot 2H_2O$, obtained by a precipitation process. The form required must be specified when purchasing, since the chemical catalogues list them both under one heading. Only the hydrated form is oxidizable under the mild conditions that we used[4,5]. It is noteworthy that the dioxide recovered from the oxidations described below was always easy to reoxidize.

We now report on a detailed procedure for these oxidations and an examination of the stoichiometry of the reactions involved.

RESULTS AND DISCUSSION

Ruthenium tetroxide was formed by shaking a suspension of hydrated ruthenium dioxide with an aqueous solution of sodium periodate and extracting the tetroxide

as a yellow solution into carbon tetrachloride. The stoichiometry of this conversion was determined by treating a weighed quantity of $RuO_2 \cdot 2H_2O$ with a measured excess of sodium periodate. Shaking was continued until all of the insoluble, black dioxide had been consumed. The RuO_4 was extracted by several washings with carbon tetrachloride. The residual aqueous solution was then examined spectroscopically[6] in order to determine the amount of remaining sodium periodate. The results reported in the experimental section show the expected stoichiometry, depicted by equation (*i*).

$$RuO_2 \cdot 2H_2O + 2NaIO_4 \rightarrow RuO_4 + 2NaIO_3 + 2H_2O \qquad (i)$$

Ammonium persulphate, which has been shown[7] to oxidize ruthenium salts, did not effect this conversion. Although RuO_4 can be obtained from RuO_2 by treatment with sodium hypochlorite[8], the process is not efficient.

The stoichiometry of the conversion of alcohols into ketones was then investigated because it is known[9] that Ru^{VIII} can, in certain circumstances, be reduced to Ru^{III} or to Ru^{VI} rather than the more common[10] Ru^{IV}. The benzhydrol–benzophenone conversion was selected for this study because the yield, based on the alcohol, is high[11]. Also, the amount of ketone formed in the crude product could be measured easily by ultraviolet spectroscopy. The method adopted was to treat a measured excess of the alcohol with carbon tetrachloride containing a known amount of ruthenium tetroxide. This was obtained by oxidizing a weighed sample of $RuO_2 \cdot 2H_2O$ with an excess of aqueous sodium periodate solution followed by extraction of the RuO_4 into carbon tetrachloride. The results recorded in Table I clearly show that the stoichiometry for this oxidation is that given by equation (*ii*).

$$RuO_4 + 2RR'CHOH \rightarrow RuO_2 + 2H_2O + 2RR'CO \qquad (ii)$$

These results also show (see Entries 1 and 2 in Table I) that carbon tetrachloride is the solvent of choice for dissolving the alcohol. Entry 6 shows that acetone is a poor

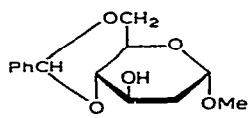
TABLE I

Entry[a]	*Alcohol oxidized (mmoles)*	*Ketone (mmoles) formed by 1 mmole of RuO_4*	*Solvent, 25 ml*
1	Benzhydrol (4.08)	1.90	CCl_4
2	Benzhydrol (4.63)	1.88	CCl_4
3	Benzhydrol (3.85)	1.78	CH_2Cl_2
4	Benzhydrol (3.84)	1.62	CH_2Cl_2
5	Benzhydrol (3.95)	1.50	CH_2Cl_2
6	Benzhydrol (3.93)	1.22	$(CH_3)_2CO$
7	**1** (3.92)	1.53	CH_2Cl_2
8	**2** (3.99)	1.54	CH_2Cl_2

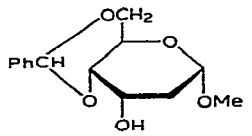
[a]Entry 2 and 4: the extracts of ruthenium tetroxide were run into solvent, and the mixture was stirred for 5 min before addition of solid benzhydrol. Entry 5: as 2 and 4, but stirring was continued for 30 min before addition of benzhydrol.

solvent; dichloromethane is quite good, providing that the oxidant is not in contact with it for more than a few minutes (*cf.* Entries 3, 4, and 5).

The behaviour of the oxidant towards methyl 4,6-*O*-benzylidene-2-deoxy-α-D-*arabino*-hexopyranoside (**1**) was then examined. Ultraviolet spectroscopy was not suitable for estimating the amount of glycopyranosidulose in the product, but n.m.r. spectroscopy was applicable, since the signals for the anomeric protons of the "oxo sugar" and the parent alcohol were well separated. The benzylidene, one-proton singlet was used to represent 100%, since its chemical shift was the same in both starting material and product. Compound **1** was picked because it is crystalline and therefore obtainable in a high state of purity. Its solubility in carbon tetrachloride is low, and dichloromethane was therefore used. This drawback is aggravated by the relatively slow oxidation of compound **1** compared to benzhydrol. Thus, the result given in Entry 7, that only 1.53 moles of compound **1** are consumed by 1.0 mole of oxidant, must be compared with Entries 4 or 5 (probably Entry 5, since the reaction time for the oxidation of compound **1**, under the conditions used, approaches 30 min). We conclude, therefore, that the stoichiometry for the oxidation of the hydroxyl group in partially protected sugars is that depicted in equation (*ii*), and that the loss of 0.47 mole of oxidant in the case of compound **1** is due mainly to oxidation of the dichloromethane.



1



2

Since equations (*i*) and (*ii*) indicate that no acid is produced, it appears that the necessity to add base in the catalytic RuO_4/IO_4^- oxidation[3b] must arise from carboxylic acids produced through oxidation and hydrolytic breakdown of some sugar molecules. Possibly, they could be produced partly from hydrolysis of lactones of the type described by Nutt *et al.*[12] as arising from over oxidation by prolonged treatment with an excess of ruthenium tetroxide. We find[1] that formation of such lactones proceeds slowly, but consecutively with simple oxidation, from the initial stages of the reaction.

The oxidation of methyl 4,6-*O*-benzylidene-2-deoxy-α-D-*ribo*-hexopyranoside (**2**) is also noteworthy. The efficiency of this oxidation is again lowered by the use of dichloromethane, but the amount of carbonyl-group formation is virtually identical to that produced from compound **1**. Thus, ruthenium tetroxide appears to oxidize axial and equatorial hydroxyl groups with equal ease, and, in other work, we have shown that it oxidizes equally well *endo* and *exo* hydroxyl groups in 1,4:3,6-dianhydrides[13].

EXPERIMENTAL

Conversion of ruthenium dioxide into ruthenium tetroxide. — Precipitated

ruthenium dioxide ($RuO_2 \cdot 2H_2O$) (0.1691 g, 0.001 mole) was added to an aqueous solution (50 ml) of sodium periodate (2.14 g, 0.01 mole). The mixture was shaken until all of the insoluble, black dioxide had been converted into tetroxide. The tetroxide was then removed by washing with carbon tetrachloride (25 ml × 3). The residual, aqueous solution was made up to a standard volume, and the residual periodate was found to be 0.008 mole by spectroscopic measurement.

Oxidation of alcohols with ruthenium tetroxide. — (*a*) *Benzhydrol.* Precipitated ruthenium dioxide (0.1691 g) was oxidized with an aqueous solution containing an excess of sodium periodate. The known amount of tetroxide so formed was quantitatively extracted into carbon tetrachloride (25 ml × 3) (the partition of RuO_4 between CCl_4 and H_2O is 60:1). This solution of oxidant was added to a stirred solution of benzhydrol (0.004 mole) in carbon tetrachloride, dichloromethane, or acetone. After 10 min, the oxidation was complete, and the precipitated ruthenium dioxide was filtered off, and washed with solvent (25 ml × 2). The combined colourless filtrates and washings were evaporated to small volume, transferred to a 250-ml volumetric flask, and diluted to standard volume with ethanol. The benzophenone in the crude product was estimated by measuring the absorbance at 333 nm, with a Unicam SP.500 spectrophotometer. In calibration experiments, benzophenone had λ_{max} 333 nm (ε 155). Only a small correction for benzhydrol at this wavelength was necessary.

(*b*) *Sugar derivatives* **1** *or* **2**. The method was similar to that used in (*a*), except that dichloromethane was used as solvent for these compounds and the oxidation period was longer, *i.e.*, 30 min. A portion of the filtrate from this reaction was removed, and evaporated to give about 50 mg of crude product. This was dissolved in deuteriochloroform and examined with a Varian A-60 n.m.r. spectrometer over the range 250–350 Hz from tetramethylsilane at sweep width of 100 Hz. The benzylidene signal which appeared at τ 4.42 for both starting material and oxidation product, and the anomeric protons of compound **1** at τ 5.20 (quartet) and oxidation product at τ 4.86 (quartet) were integrated several times. The composition of the crude product was then estimated by comparing the size of the signals at τ 5.20 and 4.86 with the one-proton signal at τ 4.42.

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BRÄUNUNGSREAKTIONEN UND FRAGMENTIERUNGEN VON KOHLENHYDRATEN

TEIL IV. VERGLEICH DER FLÜCHTIGEN ABBAUPRODUKTE BEI DER PYROLYSE VON MONO-, OLIGO- UND POLYSACCHARIDEN

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ABSTRACT

On pyrolysis at 300–500° for a short period, D-erythrose, D-xylose, D-ribose, D-arabinose, D-glucose, L-sorbose, D-fructose, D-glucurono-6,3-lactone, cellobiose, maltose, lactose, sucrose, raffinose, amylose, amylopectin, and cellulose give the same volatile products of degradation. This finding suggests that, by degradation, dehydration, and condensation reactions, all of these compounds form similar polymeric intermediates that then undergo a secondary thermal degradation. A different distribution of pyrolysis products was found with DL-glyceraldehyde, hexitols, pentitols, and 1,6-anhydrohexoses. Addition of acid salts has a small effect on the composition of the pyrolysis products; neutral salts have no effect. Addition of basic salts suppresses the formation of furans and facilitates the formation of carbonyl compounds. Analysis of the products of pyrolysis was effected by a combination of gas–liquid chromatography and mass spectrometry.

ZUSAMMENFASSUNG

D-Erythrose, D-Xylose, D-Ribose, D-Arabinose, D-Glucose, L-Sorbose, D-Fructose, D-Glucuronsäurelacton, Cellobiose, Maltose, Lactose, Saccharose, Raffinose, Amylose, Amylopektin und Cellulose liefern bei der Kurzzeitpyrolyse im Bereich von 300–500° die gleichen flüchtigen Zersetzungsprodukte. Dieser Befund legt die Annahme nahe, daß durch Spaltungs-, Dehydrierungs- und Kondensationsreaktionen von allen Verbindungen ähnliche Polymerkörper gebildet werden, die einer sekundären thermischen Spaltung unterliegen. Eine abweichende Verteilung der Pyrolyseprodukte wurde bei DL-Glycerinaldehyd, bei Hexiten, Pentiten sowie bei 1,6-Anhydrohexosen gefunden. Neutralsalzzusätze haben keinen, saure Zusätze einen geringen Einfluß auf die Zusammensetzung der Pyrolyseprodukte. Basische Zusätze unterdrücken die Bildung von Furankörpern und erleichtern die Bildung von Carbonylverbindungen. Die Analyse der Pyrolyseprodukte erfolgte durch Kombination von Kapillarchromatographie und Massenspektrometrie.

EINLEITUNG

In unserer vorhergehenden Arbeit[1] haben wir die beim Erhitzen von D-Glucose auf 300° gebildeten flüchtigen Pyrolyseprodukte untersucht und zum größten Teil identifizieren können. Unter diesen Produkten war eine Gruppe von 23 verschieden substituierten Furanen von besonderem Interesse. Diese Furane werden sogar in größerer Ausbeute gebildet, wenn man den bei der Pyrolyse von D-Glucose gebildeten schwarzen Polymerkörper einer Nacherhitzung bei 500° unterwirft. Dieser Befund wai ein erster experimenteller Hinweis dafür, daß im schwarzen Polymerkörper Furanringe als Bauelemente vorgebildet sind, die bei der Pyrolyse teilweise abgespalten werden und dann als verschieden substituierte Furane im Pyrolysat auftreten.

In der vorliegenden Untersuchung haben wir die flüchtigen Pyrolyseprodukte und das Verhalten der gebildeten Polymerkörper einer Reihe von Monosacchariden, wie Triosen, Tetrosen, Pentosen, Hexosen, Hexuronsäure, 1,6-Anhydrohexosen, Zuckeralkoholen und von Oligo- und Polysacchariden mit verschiedener Verknüpfungsart untersucht. Ferner wurde der Einfluß von anorganischen Salzen, Basen und Säuren auf die pyrolytische Zersetzung studiert.

Um eine große Anzahl von Proben untersuchen zu können, mußte ein Verfahren ausgearbeitet werden, bei dem schon mit wenigen Milligrammen einer Substanz eine Pyrolyse und schnelle Analyse der gebildeten Produkte möglich ist. Wir entwickelten eine Pyrolyse-Zelle, mit der Kurzzeit-Pyrolysen mit Substanzmengen von 5 mg ausgeführt und eine Reaktionsdauer von 10 Sek. erreicht wurde. Unter diesen Bedingungen werden Sekundärreaktionen der gebildeten Pyrolyseprodukte weitgehend verhindert, da sie rasch aus der heißen Zone herausgeführt und sofort analysiert werden können. Zur Analyse wurde die Pyrolysezelle direkt in den Trägergasstrom eines Kapillargaschromatographen geschaltet. Die aufgetrennten Substanzen wurden über ein Direkteinlaßsystem unmittelbar in das Massenspektrometer geleitet. Da von unseren Pyrolyseuntersuchungen an der D-Glucose[1] die gaschromatographischen Retentionszeiten und die Massenspektren der meisten Pyrolyseprodukte bekannt waren, gestaltete sich die Identifizierung relativ einfach.

ERGEBNISSE

1. Pyrolyse von Mono-, Oligo- und Polysacchariden

Folgende Kohlenhydrate wurden unter Standardbedingungen, gemäß den Angaben im experimentellen Teil pyrolysiert: DL-Glycerinaldehyd (dimer), D-Erythrose, D-Xylose, D-Ribose, D-Arabinose, D-Glucose, L-Sorbose, D-Fructose, D-Glucuronsäurelacton, Cellobiose, Maltose, Lactose, Saccharose, Raffinose, Amylose, Amylopektin und Cellulose. Es ist zweckmäßig, die Ergebnisse der Pyrolysen im Temperaturbereich (*a*) 300–450°, (*b*) 500–600°, (*c*) 700–900° getrennt zu betrachten.

Überraschend zeigte sich, daß bei den verschiedenen Temperaturen alle untersuchten Kohlenhydratverbindungen keine wesentlichen Unterschiede in der qualitativen Zusammensetzung der Pyrolysate aufwiesen. Unterschiede traten in

einigen Fällen nur in der quantitativen Verteilung der Produkte auf. Allerdings erhält man bei niedrigen Zersetzungstemperaturen (300–350°) aus den untersuchten Polysacchariden auf Grund ihrer höheren thermischen Beständigkeit keine flüchtigen Zersetzungsprodukte, während Mono- und Oligosaccharide bereits in merklichem Umfang flüchtige Stoffe bilden. Wird die Pyrolysetemperatur aber um 30–40° erhöht, erhält man aus den Polysacchariden die gleichen Fragmentierungsprodukte, die aus Monosacchariden schon bei tieferen Temperaturen entstehen.

(*a*) *Temperaturbereich 300–450°*. — Die Pyrogramme sind einfach und enthalten wenige Komponenten als Hauptbestandteile. Neben- und Spurenkomponenten werden nur in geringem Maße gebildet, s. Abb. 1. Die Pyrolyseprodukte der D-Glucose bei 350° werden auch aus Pentosen, Hexoaldosen, Hexoketosen, Disacchariden und aus Raffinose erhalten. Glycerinaldehyd bildet bei 350° lediglich kleine Mengen Acetaldehyd. Aus D-Erythrose entstehen infolge ihrer geringen thermischen Stabilität bereits bei 350° die Produkte, die z.B. aus Hexosen erst bei 400° gebildet werden. Polysaccharide liefern bei 350° noch keine flüchtigen Produkte. In Tabelle I wird ein Überblick über die bei 400° und 450° entstehenden Pyrolyseprodukte gegeben. CO_2, CO, H_2 und H_2O sind als wesentliche Fragmentierungsprodukte nicht mit aufgeführt, da sie von dem Detektor (F.I.D.) nicht erfaßt werden. D-Erythrose bildet

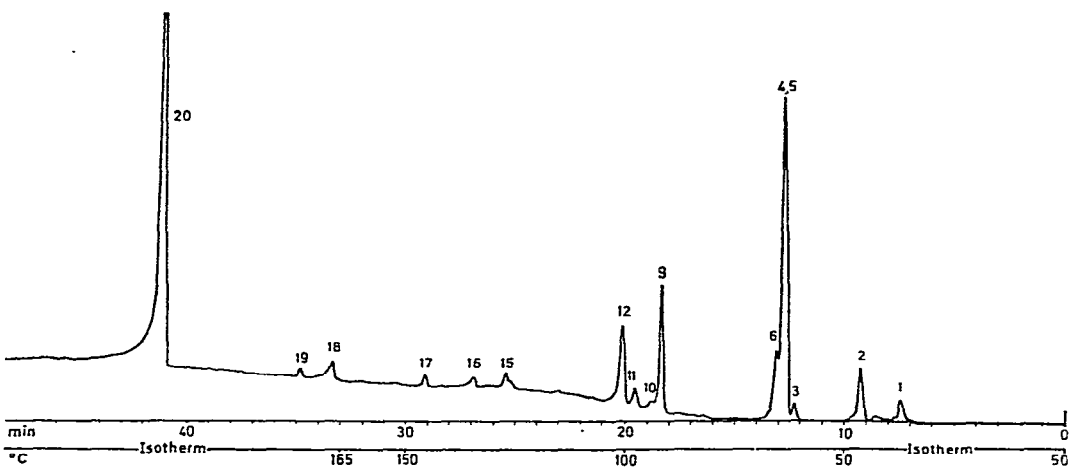

Abb. 1. Flüchtige Pyrolyseprodukte aus 5 mg D-Glucose, Pyrolyse-Temperatur 400°, Säule (*a*).

bei 450° noch etwa 50 weitere Nebenkomponenten, deren Konzentration jeweils < 0.2% des Gesamtpyrolysates ist. Diese Stoffe ließen sich zum Teil identifizieren (s. Abschnitt b). Außerdem entsteht aus D-Erythrose noch eine weitere, nicht identifizierte Komponente mit geschätztem Sdp. 155°, Molgew. 84, in einer Konzentration von etwa 1%. In den Pyrogrammen von Pentosen ist der Anteil von 2-Methylfuran und 2,5-Dimethylfuran kleiner, der von Furfurol größer als bei den Hexosen, Disacchariden und Raffinose.

TABELLE I

PYROLYSEPRODUKTE VERSCHIEDENER REINER KOHLENHYDRATE BEI 400 UND 450°

Peak-Nr. in Abb. 1	*Pyrolyseprodukte*[a]	*Glycerinaldehyd*		*D-Erythrose*		*Pentosen, Hexosen, Disacch., Raffinose*		*Polysaccharide*	
		400°	*450°*	*400°*	*450°*	*400°*	*450°*	*400°*	*450°*
1	Gasfraktion[b]	+++	+++	+	+++	+	+++	+	+++
2	Acetaldehyd	+++!	+++!	++	+++	++	+++	+	+++
3	Propionaldehyd	+	++	+	+	+	+	+	++
4	Furan	+	++	+++!	+++	++	+++	++	+++
5	Aceton	+	++	+	++	+	++	+	++
6	Acrolein	++	++	+	++	+	++	+	++
	Methacrolein	+	+	+	+	−	+	−	+
9	2-Methylfuran	−	+	+	++	++	+++	++	++
10	Butanon	−	++	+	++	+	+	−	+
11	Butenon-2	−	++	+	++	+	+	+	+
12	Biacetyl	+	+++	+++	+++!	++	+++	++	++
	Benzol	−	−	−	+	−	+	−	+
	Crotonaldehyd	−	−	−	+	−	+	−	+
15	2,5-Dimethylfuran	−	−	−	+	+	++	−	+
16	2,3-Pentandion	−	+	−	++	+	+	−	+
17	2-Vinylfuran	−	−	++	+	+	+	+	+
18	Pentadienal	−	−	+	+	+	+	−	+
19	2-Methyl-5-vinylfuran	−	−	+	+	+	+	−	+
20	Furfurol	−	+	+++	+++	+++!	+++!	+++!	+++!
	5-Methylfurfurol	−	−	+	+	+	+	+	+

[a] +++! = Hauptprodukt; +++ = > 10% des Gesamtpyrolysates; ++ = 1–10% des Gesamtpyrolysates; + = < 1% des Ges.-Pyr. [b] Gasfraktion = Gemisch von niedrig siedenden, einfachen Kohlenwasserstoffen, s. unten.

(*b*) *Temperaturbereich 500–600°*. — Bei Pyrolysetemperaturen zwischen 500 und 600° nehmen Anzahl und Menge der flüchtigen Zersetzungsprodukte bei allen untersuchten Kohlenhydraten stark zu. In den Pyrogrammen lassen sich bis zu 150 Substanzen erkennen. Eine Ausnahme bildet Glycerinaldehyd, bei dessen Pyrolyse nur die Stoffe in größerer Menge auftreten, die auch bei 450° erhalten werden. Die Hauptkomponenten der übrigen Pyrogramme entsprechen den bei einer Zersetzungstemperatur von 450° gefundenen Stoffen (s. Tabelle I), ihre Menge steigt jeweils um das 3–4fache an. Besonders stark tritt der Anteil der Gasfraktion hervor. Ihre Konzentration ist deutlich größer als die des Furfurols. Die von uns bei der langsamen Pyrolyse von D-Glucose bei 300° nachgewiesenen Produkte treten hier in den bei 550° erhaltenen Pyrogrammen gleichfalls auf. Zusätzlich wurden Cyclopentadien, Methylcyclopentadien, Cyclohexadien, Äthyl-vinyl-keton und 2-Methyl-cyclopentanon gefunden. Die Gasfraktionen, die bei 550° aus D-Glucose, D-Fructose und L-Sorbose entstehen, wurden auf einer Dimethylsulfolan-Säule gesondert verglichen. Sie sind bei den drei Monosacchariden qualitativ ebenfalls gleich, die quantitative Zusammensetzung zeigt äußerst geringe Unterschiede. Folgende Verbindungen wurden massenspektrometrisch und durch Vergleich der Retentionswerte gefunden: Methan, Äthan, Propan, *n*-Butan, *n*-Pentan, Äthylen, Propen, *i*-Buten, 1-Buten, 2-Buten (*cis*- und *trans*-), 1-Penten, 2-Penten (*cis*- und *trans*-), Cyclopenten, 1,3-Butadien, Isopren und Acetylen.

Die Zusammensetzung des Gesamtpyrolysates von D-Glucose, D-Fructose und L-Sorbose bei 600° wurde sehr genau quantitativ bestimmt. Sie ist bei diesen drei Kohlenhydraten innerhalb der Fehlergrenze (±5% bezogen auf den Einzelwert) identisch. Die Hauptkomponenten der Pyrolysate (einschließlich Gasfraktionen) sind in Gewichtsprozenten: Furfurol 21%, Acetaldehyd 15%, Methan 12%, Acrolein 11%, Äthan+Äthylen 9%, Furan 6%, Propen 5%, Aceton 2.5%, Biacetyl 2.5%, 5-Methylfurfurol 2.5%, Propionaldehyd 2%, Butadien-1,3 1.5%. D-Glucuronsäurelacton wurde bei 550° pyrolysiert. Das Pyrogramm zeigt qualitativ und quantitativ die gleichen Produkte, die auch aus D-Glucose entstehen.

(*c*) *Temperaturbereich 700–900°*. — Wird die Pyrolysetemperatur von 600° auf 700° erhöht, beobachtet man eine sehr starke Verschiebung der Konzentrationsverhältnisse der Pyrolyseprodukte. Die sauerstoffhaltigen Verbindungen nehmen ab, die aliphatischen Kohlenwasserstoffe (Gasfraktion) und die aromatischen Kohlenwasserstoffe nehmen zu. Bei 800° und 900° erhält man zunehmend einfache Pyrogramme mit geringerer Komponentenzahl.

Die quantitative Analyse der Pyrolyseprodukte von D-Glucose bei 900° liefert als Hauptkomponenten (in Gewichtsprozenten): Methan 44%, Äthan+Äthylen 25%, Acetylen 7.5%, Benzol 7%, Acetaldehyd 3.5%, Propen 1.8%, Naphthalin 1.5% und Furan 1.5%. Ferner enthält das Pyrolysat jeweils 0.5–1.0% Toluol, Styrol, Inden, Phenylacetylen und Butadien. Daneben entstehen als Spurenkomponenten noch einige weitere Kohlenwasserstoffe sowie einfachere Carbonylverbindungen. Furfurol und andere Furankörper (außer Furan) fehlen ganz. Qualitativ und quantitativ gleiche Pyrolysate erhält man auch hier aus D-Fructose und L-Sorbose.

Bei diesen Temperaturen zwischen 700 und 900° treten zunehmend für Kohlenhydrate uncharakteristische Spaltprodukte auf.

2. Pyrolyse von Zuckeralkoholen und 1,6-Anhydroaldosen

Xylit, D-Mannit, 1,4-Anhydro-D-mannit, D-Glucit und *myo*-Inosit wurden bei 450° und 550° pyrolysiert. Bei 450° verdampft die Hauptmenge der eingesetzten Zuckeralkohole unzersetzt. Xylit ist thermisch am wenigsten beständig und liefert als einziger der eingesetzten Stoffe geringe Mengen einfacher Kohlenwasserstoffe (Gasfraktion), Acetaldehyd, Aceton und Acrolein. Bei 550° lassen sich von allen pyrolysierten Zuckeralkoholen leichtflüchtige Pyrolyseprodukte in größerer Menge nachweisen, obgleich auch hier ein Teil des Materials unzersetzt verdampft. Die Pyrogramme sind einfach; sie enthalten wenige Komponenten und sind qualitativ gleich. Als Hauptprodukte entstehen, unabhängig vom eingesetzten Material, je etwa 30% Acetaldehyd, Acrolein und Gasfraktion. Als Nebenkomponenten treten auf (Reihenfolge in abnehmender Konzentration) Furan, Propionaldehyd, Biacetyl, Methacrolein; 2-Methylfuran und Furfurol sind nur in Spuren vorhanden, andere Furankörper lassen sich nicht nachweisen.

Im Pyrogramm des 1,4-Anhydro-D-mannits erscheint Furan als Hauptkomponente. Diese Besonderheit beruht wahrscheinlich auf dem im 1,4-Anhydro-D-mannit bereits vorgebildeten Furanring.

Bei 550° bilden 1,6-Anhydro-β-D-glucopyranose und 1,6-Anhydro-β-D-galactopyranose die gleichen flüchtigen Pyrolyseprodukte wie die Zuckeralkohole; auch im quantitativen Verhältnis treten keine besonderen Unterschiede auf.

3. Pyrolyse von Kohlenhydraten mit anorganischen Zusätzen

(*a*) *Neutralsalzzusätze.* — Frisch bereitete Lösungen von D-Glucose mit 0.5%, 5%, 10% und 20% KCl sowie von D-Glucose mit 5% und 10% $NaHCO_3$ wurden nach Verdunsten des Lösungsmittels bei 300°, 350°, 400° und 450° pyrolysiert. Bei allen Versuchen wurde festgestellt, daß diese Salzzusätze weder die quantitative noch die qualitative Zusammensetzung der Pyrogramme – verglichen mit jenen von reiner D-Glucose bei gleichen Temperaturen – beeinflussen. Die Konzentration des Salzzusatzes ist gleichfalls ohne Einfluß. Der Zusatz von Neutralsalzen erleichtert oder beschleunigt die thermische Zersetzung von D-Glucose nicht.

(*b*) *Alkalische Zusätze.* — Folgende Gemische wurden bei 250°, 300°, 350°, 400°, 450° und 550° pyrolysiert: D-Glucose, D-Fructose, D-Xylose, Glycerinaldehyd mit 0.5%, 5%, 10%, 20% NaOH, Na_2CO_3, Na_2SO_3. Der Zusatz von Basen bewirkt eine sehr starke Verschiebung der relativen Konzentrationen der Pyrolyseprodukte. Im gesamten untersuchten Temperaturbereich zeigen die Pyrogramme folgende Veränderungen: (*i*) eine sehr ausgeprägte Abnahme der Menge aller Furanderivate, besonders des Furfurols, (*ii*) die bevorzugte Bildung der α-Dicarbonylverbindungen Biacetyl und 2,3-Pentandion, (*iii*) eine hohe Konzentration der einfacheren Monocarbonylverbindungen (C_2-C_4).

Die Zusammensetzung der Pyrogramme hängt ab von der Konzentration der

zugesetzten Base, ist aber unabhängig von der Art der Base. Werden nur 0.5% Base zugesetzt, weichen die Pyrogramme nur unwesentlich von denen reiner Kohlenhydrate ab. Bei 5% Basezusatz sind Abweichungen stärker, nehmen bei 10% Zusatz noch zu, bleiben aber bei 20% Basezusatz unverändert. Die Pyrogramme von D-Xylose,

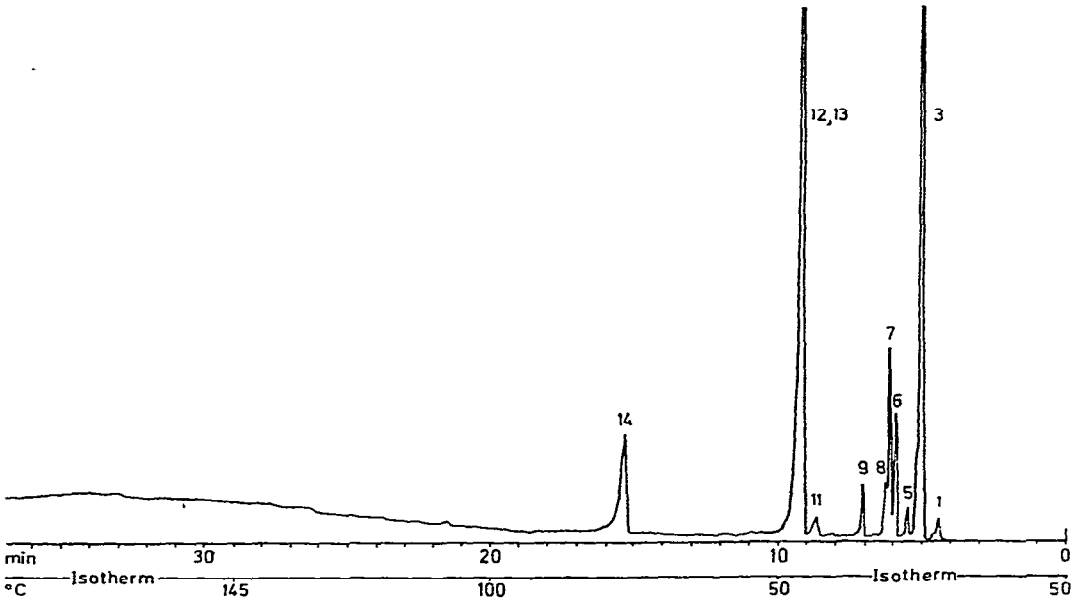

Abb. 2. Flüchtige Pyrolyseprodukte aus 5 mg D-Glucose + NaOH (9:1). Pyrolyse-Temperatur 300°, Säule (*b*).

D-Fructose oder Glycerinaldehyd mit Basen stimmen qualitativ und quantitativ mit den aus D-Glucose und Base erhaltenen überein. Die Tabelle II zeigt die flüchtigen Pyrolyseprodukte, die aus D-Glucose mit 10% Basezusatz bei Pyrolysen zwischen 250° und 450° erhalten werden. Bei 550° treten qualitativ die gleichen Produkte auf wie in den Pyrogrammen reiner Kohlenhydrate. Die oben erwähnten Verschiebungen in der quantitativen Verteilung der Komponenten bleiben aber bestehen.

(*c*) *Saure Zusätze.* — Folgende Gemische wurden bei 300°, 350°, 450° und 550° pyrolysiert: D-Glucose, D-Fructose, Glycerinaldehyd mit 0.5%, 5%, 10% und 20% Borsäure. Borsäure wurde wegen ihrer Schwerflüchtigkeit gewählt.

Im gesamten untersuchten Temperaturbereich zeigen die Pyrogramme von D-Glucose- bzw. D-Fructose–Borsäure–Gemischen einige Besonderheiten: (*i*) Die Furankörper treten stark hervor, besonders Furfurol, Furan, 2-Methylfuran, 2-Vinylfuran, weniger ausgeprägt auch 2,5-Dimethylfuran und 2-Methyl-5-vinylfuran. (*ii*) Die Carbonylverbindungen werden in geringerer Menge gebildet, besonders Biacetyl, 2,3-Pentandion, Aceton; bei Acetaldehyd ist der Unterschied weniger auffällig.

Der Zusatz von 0.5% Borsäure beeinflußt die Zusammensetzung der Pyrogramme nur wenig, Borsäurezusatz von 5–20% führt zu gleichen Ergebnissen: Im Temperaturbereich 300° bis 350° treten die Abweichungen weniger deutlich als bei

TABELLE II

PYROLYSEPRODUKTE VON D-GLUCOSE UND 10% BASE ZWISCHEN 250 UND 450°

Peak-Nr. in Abb. 2	*Pyrolyseprodukte*[a]	*Pyrolysetemperatur*				
		250°	*300°*	*350°*	*400°*	*450°*
1	Gasfraktion[b]	−	+	++	++	+++
3	Acetaldehyd	+++	+++	+++	+++	+++
5	Furan	−	+	+	+	+
6	Propionaldehyd	−	++	++	+++	+++
7	Aceton	−	++	+++	+++	+++
8	Acrolein	−	+	++	++	+++
9	2-Methylfuran	−	+	+	+	+
11	Butanon	−	+	+	+	++
12	Butenon-2	−	++	++	++	++
13	Biacetyl	+++!	+++!	+++!	+++!	+++!
	Benzol	−	−	−	−	+
	2,5-Dimethylfuran	−	−	−	−	+
	Crotonaldehyd	−	−	+	+	+
14	2,3-Pentandion	−	++	++	++	++
	2-Vinylfuran	−	−	−	−	+
	Pentadienal	−	−	−	−	+
	Furfurol	−	−	−	+	+
	5-Methylfurfurol	−	−	−	−	+

[a] +++! = Hauptprodukt; +++ = > 10% des Gesamtpyrolysates; ++ = 1–10% des Gesamt pyrolysates; + = < 1% des Gesamtpyrolysates. [b] Tabelle I.

450° und 550° auf. Pyrolysen von Glycerinaldehyd und Borsäure liefern die gleichen flüchtigen Produkte wie bei der Pyrolyse reinen Glycerinaldehyds. Eine bevorzugte Bildung von Furanderivaten erfolgt hier nicht.

EXPERIMENTELLER TEIL

Die Abb. 3 gibt einen schematischen Überblick über die Versuchsanordnung, bestehend aus Gaschromatograph F6/4HF, Perkin–Elmer, und Pyrolysezelle. Das Trägergas kann durch den Verteiler A wahlweise über den Einspeisblock E zur Kapillarsäule K (Weg 1) oder über die Pyrolysezelle P und die Vorsäule V zur Säule K (Weg 2) geleitet werden. Nach Einführen der Probe in die Pyrolysezelle werden P und V 10 sec vom Trägergas durchströmt (Weg 2), danach wird, bis zur Beendigung der GC-Analyse, das Trägergas über den Weg 1 zur Trennsäule geleitet.

Vor Beginn der nächsten Pyrolyse werden die in der Vorsäule zurückgehaltenen schwerer flüchtigen Produkte aus der Apparatur gespült: nach Öffnen der Pyrolysezelle bei B wird der Gasstrom über E, V und P geführt und bei B ins Freie gelassen. Die

Vorsäule besteht aus V2A Stahl (100 × 3 mm), ihre Füllung aus 5% Silicongummi SE 30 auf Diatoport S (60–80 mesh). Sie wird während der Pyrolyse und während

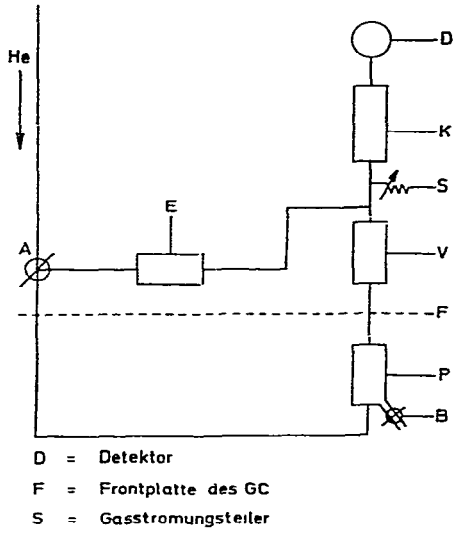

Abb. 3.

des Rückspülens auf 180° gehalten, durch eine Widerstandsheizung, die von der Heizung des Säulenofens unabhängig ist. Die Abb. 4 zeigt die Pyrolyseeinheit.

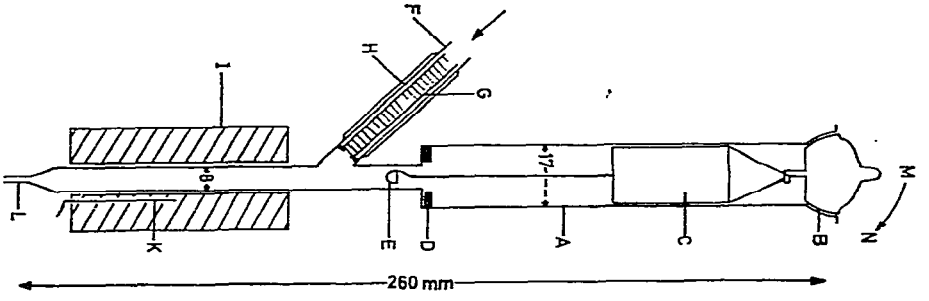

Abb. 4.
A, Pyrolyserohr aus Quarzglas. B, Kugelschliff mit Haken. C, Führungskörper des Probengebers, hohler Messingzylinder (35 × 16 mm). D, Teflonring (Stoßdämpfer). E, Probengeber, zylindrischer Pt-Drahtnetzkorb; Drahtstärke 0.06 mm, Siebweite 1024 Maschen/cm². F, Trägergaszuführung zur Pyrolysezelle. G, Füllung mit Cu-Drahtwendeln. H, Heizmantel. J, Zylindrischer Ofen mit Widerstandsheizung ("Kurzbrenner" d. Fa. Heraeus, Hanau). K, Thermoelement. L, Anschlußstück zur Vorsäule.

Ausführung der Pyrolyse (vergl. Abb. 4). — Der Pyrolyseofen wird auf die gewünschte Arbeitstemperatur gebracht, der seitliche Arm F des Quarzrohres A auf 250°, die Vorsäule auf 180°. In den Probengeber, den Platinkorb E, werden aus wäßriger Lösung (bzw. Suspension), nach Verdunsten des Lösungsmittels, etwa 5 mg

Substanz als gleichmäßiger Film aufgebracht. Probengeber und Führungszylinder werden dann an dem Kugelschliff des Pyrolyserohres befestigt, die Zelle wird durch eine Kugelschliffkammer gasdicht abgeschlossen. Durch das Rohr F wird die Pyrolysezelle mit vorgeheiztem Trägergas gespeist. Zur Einführung der Probe in die heiße Ofenzone wird der Kern B des Kugelschliffes seitlich bewegt (von Stellung M nach Stellung N). Nach Durchlaufen der Fallstrecke bis zum Teflonring D, gelangt die Probe mit reproduzierbarer Geschwindigkeit stets an die gleiche Stelle der Heizzone. Durch diese Art der Dosierung läßt sich ein reproduzierbares Aufheizen der Probe erreichen. 10 Sek. nach der Dosierung wird der Trägergasstrom umgestellt (Weg 1, Abb. 3). Nun gelangen aus Pyrolysator und Vorsäule keine Substanzen mehr in die Kapillarsäule.

Gaschromatographische Trennung der Pyrolysate. — Trennsäulen: (*a*) Polypropylenglycol, 50 m × 0.25 mm, Stahlrohr; (*b*) 2,4-Xylenylphosphat, 50 m × 0.25 mm, Stahlrohr; (*c*) Marlophen 87 (CWH), 25 m × 0.25 mm, Stahlrohr; (*d*) Dimethylsulfolan 25% auf Chromosorb W, 4.5 m × 6 mm, Kupferrohr (zur Analyse der Gasfraktion). Säuleneinlaßdruck: 0.85 atü, Trägergas He (99.9%) Linde. Gasströmungsteilung bei Säule: (*a*) und (*b*) 1:80, 1.0 ml/min durch die Kapillarsäule; (*c*) 1:50, 1.5 ml/min. Detektor: F.I.D.; H_2 = 25 ml/min; Luft = 250 ml/min. Detektortemperatur, 250°. Säulentemperatur siehe Gaschromatogr. 1 und 2.

Kombination Gaschromatographie und Massenspektrometrie. — Der Ausgang der Kapillarsäule wurde durch eine auf 150° geheizte Stahlkapillare und einen Einlaßteil EC2 mit einem Massenspektrometer CH4 ATLAS verbunden. Bei schnellem Massendurchlauf wurden die Massenspektren über einen UV-Lichtpunktschreiber auf Photopapier registriert. Ein parallel geschalteter Oszillograph diente als Detektor.

Identifizierung der Pyrolyseprodukte. — Die Identifizierung der flüchtigen Abbauprodukte erfolgte durch ihre Massenspektren und durch Vergleich der gaschromatographischen Retentionswerte mit authentischen Vergleichssubstanzen auf den Säulen (*a*), (*b*) und (*c*).

Quantitative Bestimmung der Komponenten der Pyrogramme. — Da die Identität aller Substanzen in den Pyrogrammen, deren Konzentration etwa >0.1% (Flächen-%) beträgt, bekannt war, konnte eine quantitative Analyse durchgeführt werden. Zur Bestimmung der Bandenflächen der Substanzen wurde der elektronische Integrator D_2, Perkin–Elmer, mit Digitaldrucker Kienzle verwendet. Aus den Integralwerten (Flächenwerten) wurde unter Berücksichtigung der stoffspezifischen Korrekturfaktoren f_i für die F.I.D.-Anzeige die Zusammensetzung der Pyrolysate in Gewichtsprozenten ermittelt. Die Faktoren f_i verschiedener Carbonylverbindungen und Furane wurden unter Verwendung von Benzol (f_{Benzol} = 1.000) als Bezugssubstanz bestimmt, die Faktoren niedrig siedender Kohlenwasserstoffe aus der Literatur[2] entnommen.

DISKUSSION

Die untersuchten Mono-, Oligo- und Polysaccharide liefern beim Erhitzen unter Standardbedingungen die gleichen Pyrolyseprodukte. Eine Ausnahme bildet

nur der Glycerinaldehyd. Dadurch wird die Vermutung nahegelegt, daß alle diese Kohlenhydratverbindungen im Verlauf der thermischen Fragmentierung analoge Zwischenstufen durchlaufen, die zur Entstehung der gleichen flüchtigen Pyrolyseprodukte führen. Man könnte vermuten, daß dies schon höhermolekulare Vorstufen zum schwarzen Polymerkörper sind, dessen thermische Fragmentierung die beobachteten flüchtigen Produkte liefert. Es ist bemerkenswert, daß dabei innerhalb gewisser Grenzen die Länge der Kohlenstoffkette des Zuckers keine Rolle spielt; ob ein Mono-, ein Oligo- oder ein Polysaccharid eingesetzt wird, ist gleichfalls ohne Einfluß. Glycerinaldehyd zeigt ein von den anderen Kohlenhydraten abweichendes Verhalten insofern, als bei der Pyrolyse entstehende Furankörper nur noch in geringer Menge auftreten.

Bei der Kurzzeitpyrolyse von D-Glucose und anderen Kohlenhydraten werden bei 550–600° die gleichen flüchtigen Spaltprodukte gebildet wie bei der langsamen Pyrolyse[1] von D-Glucose bei 300°. Die Ergebnisse sind somit durchaus vergleichbar. Die Bildung von Sekundärprodukten, die man beim langsamen Erhitzen und langsamer Entfernung der Pyrolyseprodukte aus der Heizzone erwarten könnte, dürfte nicht sehr erheblich sein. Bei dem schnellen Aufheizen und kurzzeitiger Erhitzung der D-Glucose auf die vorgegebene Temperatur sollten derartige Reaktionen auf ein Minimum beschränkt bleiben. Andererseits tritt bei der Kurzzeitpyrolyse bei 300° nur geringfügige Fragmentierung ein, so daß flüchtige Produkte kaum entstehen. Erst bei 400° und höheren Temperaturen werden flüchtige Produkte in ausreichender Menge gebildet, um eine Analyse durchführen zu können.

Uronsäuren neigen auch ohne Einwirkung von Aminoverbindungen leicht zu Bräunungsreaktionen[3]. D-Glucuronsäurelacton liefert jedoch die gleichen Pyrogramme wie D-Glucose. Wahrscheinlich erfolgt bei der Pyrolyse des Säurelactons zunächst eine Decarboxylierung[4] zur Pentose, aus der dann die entsprechenden Spaltstücke entstehen.

Die Zuckeralkohole verhalten sich bei der Pyrolyse abweichend, sie hinterlassen keinen Rückstand und liefern keine Furanderivate. Die fehlende Carbonylfunktion sollte für dieses Verhalten maßgeblich sein[5].

Glassner und Pierce[6] pyrolysierten Cellulose und 1,6-Anhydro-β-D-glucose (170–320°, Pyrolysedauer 6 Min.) und fanden bei der gaschromatographischen Untersuchung eine qualitative Übereinstimmung der flüchtigen Reaktionsprodukte. Sie vertreten die Ansicht, daß Laevoglucosan das entscheidende Zwischenprodukt beim thermischen Abbau von Cellulose ist. Wir konnten dagegen keine Übereinstimmung in der Zusammensetzung der Pyrolyseprodukte von Polysacchariden oder anderen Kohlenhydraten mit denen von 1,6-Anhydro-β-D-glucose bzw. 1,6-Anhydro-β-D-galactose finden. Die beiden Anhydrozucker bilden die gleichen flüchtigen Produkte wie die Zuckeralkohole; Furfurol und andere für die Pyrogramme von Kohlenhydraten typischen Furanderivate werden nur in Spuren gebildet. Wir nehmen daher nicht an, daß den Glycosanen eine zentrale Bedeutung beim thermischen Abbau der Kohlenhydrate zu flüchtigen Pyrolyseprodukten und dem schwarzen Polymerkörper zukommt.

Die bevorzugte Bildung von Furankörpern bei der Pyrolyse von D-Glucose mit Borsäurezusatz (5–20%) entspricht dem Befund, daß beim Erhitzen wäßriger Hexoselösungen in saurem Milieu die Furfurolbildung sehr stark zunimmt[7]. Interessant ist ein Vergleich der Pyrogramme von D-Glucose–Borsäure–Gemischen mit denen von unlöslichen Zuckerhuminen, die durch Behandlung von L-Sorbose, D-Fructose und D-Tagatose mit konzentrierten Halogenwasserstoffsäuren bei 75° hergestellt wurden[8]. Bei dem Vergleich der bei 500–550° erhaltenen Pyrogramme findet man qualitativ eine gute Übereinstimmung und eine große Ähnlichkeit der quantitativen Zusammensetzung. Dies deutet wiederum darauf hin, daß aus dem Zucker bei der Kurzzeitpyrolyse intermediär höhermolekulare Zwischenprodukte entstehen, bei deren Fragmentierung dann die flüchtigen Produkte gebildet werden.

Bei der Pyrolyse von D-Glucose mit Basenzusatz (10–20%) erhält man vorwiegend offenkettige Carbonylverbindungen geringer Kohlenstoffzahl (C_2-C_4), Furankörper treten nur als Neben- bzw. Spurenkomponenten auf (s. Abb. 2). Diese Befunde entsprechen der Beobachtung, daß beim Erhitzen von D-Glucoselösungen in basischem Milieu eine Aufspaltung der Kohlenstoffkette des Zuckers erfolgt[7].

Der Basenzusatz bewirkt eine deutliche Beschleunigung der pyrolytischen Zersetzung von D-Glucose. Das ergibt sich daraus, daß unter den gewählten Arbeitsbedingungen D-Glucose–Base–Gemische bereits bei 250° merklich zersetzt werden und bei 300° Pyrogramme entstehen, deren Banden steile Flanken und gute Auflösung zeigen (Abb. 2). Durch die schnelle Zersetzung der Probe in der Pyrolysezelle tritt hier keine Verzögerung bei der Speisung der Kapillarsäule ein. Reine D-Glucose liefert selbst bei 350 und 400° weniger scharf aufgelöste Banden (Abb. 1). Ein Basezusatz von 0.5% hat einen sehr geringen Einfluß auf die Pyrogramme von D-Glucose, da anzunehmen ist, daß die bei der thermischen Zersetzung des Zuckers freiwerdenden Carbonsäuren den geringfügigen Basezusatz schnell unwirksam machen.

Eine Beschleunigung der thermischen Zersetzung von Cellulose durch Na_2CO_3 wurde von Madorsky und Mitarb.[9] gefunden; eine beschleunigte Zersetzung erfolgt auch durch Borax–Borsäure (7:3)[10].

Die katalytische Wirkung der Zusätze kann man beurteilen, indem man die untere Pyrolyse-Temperaturgrenze bestimmt, bei der mit bestimmten Zusätzen ein Zucker unter Standardbedingungen noch eine merkliche und hinreichend schnelle Zersetzung zeigt. Diese Temperaturgrenze beträgt für D-Glucose mit 10-20% NaOH, Na_2CO_3, Na_2SO_3: 300°; D-Glucose mit 5-20% Borsäure: 350°; D-Glucose mit 0,5-20% KCl, $NaHCO_3$: 420–450°; Reine D-Glucose: 420–450°.

Ein Vergleich der Wirkung zeigt, daß die Zusammensetzung der Pyrolysate von Basen am stärksten, von Säuren weniger stark und von Neutralsalzen nicht beeinflußt wird. Diese Reihenfolge entspricht der katalytischen Wirkung der zugesetzten Stoffe auf die Zucker-Pyrolyse.

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PRECIPITIN-LIKE REACTIONS OF POLYSACCHARIDES WITH GELATIN IN AQUEOUS SOLUTION

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ABSTRACT

The following polysaccharides and polysaccharide derivatives give precipitin-like reactions with gelatin in distilled, de-ionized water: glycogen, soluble starch, lichenan; phosphates of soluble starch, glycogen, and dextran; phosphono-D-mannan, and phosphono-D-galactan. Uronic acid-containing, and sulfated polysaccharides which elicited precipitin reactions were: dextran sulfate (mol. wt. 2×10^6), agar, poly-D-galacturonic acid, gum karaya, L-arabinan, pectin, gum tragacanth, and gum arabic. The following acidic polysaccharides and phosphorus-containing biopolymers elicited precipitin reactions, but only at high concentrations of gelatin (10–30 mg of gelatin per mg of polymer): dextran sulfates (mol. wt. 1.8×10^4 and 5×10^5), fucan, carrageenans, chondroitin sulfate, hyaluronic acid, sodium pectate, D-xylan, RNA, DNA, poly(adenylic acid), phosvitin, and ovalbumin. With the exception of dextran sulfates and fucan–gelatin interactions, all polymer–gelatin precipitin reactions were inhibited, either partially or totally, in the presence of 0.145M sodium chloride. In contrast, the dextran sulfates and fucan showed enhanced precipitation with gelatin in the presence of 0.145M sodium chloride. All polymer–gelatin precipitin reactions were inhibited by 1.0M urea or by 0.3M guanidine. The complex, phenol-extracted, bacterial lipopolysaccharides of *Salmonella* and *Escherichia* genera also elicited precipitin reactions with gelatin in distilled, de-ionized water, whereas the corresponding lipopolysaccharide preparations extracted with 5% trichloroacetic acid (TCA) failed to elicit precipitin reactions. Both trypsin-digested and 5% TCA-extracted lipopolysaccharides from *Serratia marcescens* elicited precipitin curves similar to the acidic polysaccharide–gelatin precipitin curves. Soluble starch, glycogen, and lipopolysaccharides of *E. coli* 0111:B4 gave rise to characteristic u.v.-scattering spectra when complexed with gelatin. The i.r. absorption spectra of gelatin and gelatin–D-glucan complexes indicated spectral shifts on complexation in the regions of 1650–1450 cm^{-1} and 1100–900 cm^{-1}. Optical rotatory dispersion spectra were used to detect formation of a complex between gelatin and neutral D-glucans. The effects of sodium chloride, urea, and guanidine hydrochloride, in conjunction with u.v. difference spectra, on various biopolymeric, gelatin complexes are discussed in terms of the electrostatic and hydrogen-bonding forces most probably responsible for specific polymer–gelatin, precipitin-reactions.

INTRODUCTION

The results of previous studies[1–3] have shown that gelatin interacts with polysaccharides to form highly stable complexes that are soluble in such organic solvent systems as 5% trichloroacetic acid (TCA), 83% ethanol, or 0.83% TCA–83% ethanol. These solvents are generally considered to be protein or polysaccharide precipitants, or both. It has also been recently noted that phenol-extracted lipopolysaccharides (LPS) of Gram-negative bacteria can be partitioned by TCA–ethanol into as many as three hexose-containing, macromolecular subfractions: **1**, insoluble in 5% TCA; **2**, TCA-soluble, ethanol-insoluble; and **3**, TCA-soluble, ethanol-soluble[4]. Therefore, in addition to soluble complexes, precipitin-like reactions between gelatin and LPS have been observed in organic solvent systems.

Other investigators have studied polysaccharide–protein (concanavalin A) precipitin reactions in aqueous environments, and have used such model systems as a means of investigating the functional groups in the polysaccharide moieties of antigens[5–9]. Precipitin-like reactions between specific enzymes and their parent (or related) polysaccharide substrates have also been shown to occur[10–14]. The present study was designed to investigate interactions between gelatin and a number of polysaccharides in terms of the precipitation of biopolymeric complexes from aqueous solution.

EXPERIMENTAL

Materials. — An acid-precursor, pigskin gelatin (isoelectric point 9.1, batch No. 70-3998) was supplied by Eastman Organic Chemicals, Rochester, N.Y. Prior to its use as the binding protein in these studies, a 5% solution of gelatin was dialyzed against cold, distilled, de-ionized water, and then lyophilized.

The source of numerous polysaccharides and the preparation of D-glucan derivatives have been previously noted[2,3]. Solutions of all polysaccharides and their derivatives were dialyzed and lyophilized prior to use in these studies. Shellfish glycogen (lot N 1605), soluble starch (lot P 1043), and dextran (mol. wt. 80,000, lot G 2431) were obtained from Mann Research Laboratories, New York, N.Y. The degree of substitution (D.S.) of the phosphated D-glucans was 0.048, 0.033, and 0.079 for phosphates of glycogen, soluble starch, and dextran, respectively. In addition to the dextrans having known linkages, and the bacterial levans previously used[3], Dr. Allene R. Jeanes, Northern Regional Research Laboratories, Peoria, Illinois, also kindly supplied phosphono-D-mannan (Y-411), phosphono-D-galactan, and an acidic polysaccharide (Y-1401) from *Cryptococcus laurentii* var. *flavescens*. The following, water-soluble polysaccharides were gifts from Stein, Hall and Co., Inc., New York, N.Y.: carrageenans (Genulacta L-100, Genuvisco "J", and Genulacta K-100), D-galacto-D-mannans of guar gum (Jaguar J25-1 non-ionic, Jaguar 808 anionic, and Jaguar Plus-cationic), a D-galacto-D-mannan from locust-bean gum, gum karaya, gum arabic, gum tragacanth, and L-arabino-D-galactans (Stractan, and

Stractan AF-2). Dextran sulfates, mol. wt. 5×10^5 (lot No. 263) and mol. wt. 2×10^6 (lot No. 6668), and Ficoll (lot No. 4975), a polysucrose polymer of mol. wt. 4×10^5, were obtained from Pharmacia Fine Chemicals, Piscataway, N.J. Dextran sulfate (lot No. 93-B-1550), average mol. wt. 1.8×10^4, was obtained from Sigma Chemical Co., St. Louis, Missouri. Heparin, sodium salt (lot No. 2031), hyaluronic acid (lot No. 7082), and phosvitin (lot No. 4615) were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Chondroitin sulfate (lot No. 102) and highly polymerized, salmon-sperm 2′-deoxyribonucleic acid (lot No. 50072) were obtained from Calbiochem, Los Angeles, California. Poly(D-galacturonic acid) (lot No. C2623), D-xylan (lot No. N2236), pectin (lot No. M2281), sodium polypectate (lot No. N2542), agarose (special grade), ovalbumin, 2 × cryst. (lot No. R3237), highly polymerized yeast ribonucleic acid (lot No. P1194), and poly(adenylic acid), potassium salt (lot No. P2457) were supplied by Mann Research Laboratories, Inc., New York, N.Y. D-Mannan, L-arabinan, and fucan were supplied by Pierce Chemical Co., Rockford, Illinois. Agar, purified (control 464028), and the following Boivin-extracted (B), Westphal-extracted (W), and trypsinized (TS) bacterial lipopolysaccharides (LPS) were purchased from Difco Laboratories, Detroit, Michigan: *Salmonella typhimurium*, (W, lot No. 137085) and (B, lot No. 462074); *Salmonella abortus equi* (W, lot No. 46601D) and (B, lot No. 466006); *Escherichia coli* 0111:B4 (W, lot No. 471289) and (B, lot No. 462737); *Escherichia coli* 0128:B12 (W, lot No. 134454) and (B, lot No. 140431); and *Serratia marcescens* (B, lot No. 134455) and (TS, lot No. 462038).

Turbidimetric assay. — The absorbancies (A) of suspensions of the various polymers and biopolymeric complexes, in distilled, de-ionized water, were measured in a Coleman Jr. spectrophotometer at 450 nm. With respect to bacterial LPS–gelatin complexes, the total reaction-mixture (2.0 ml), which contained 1.0 mg of LPS and 0.5–10 mg of gelatin, was incubated for 30 min at 25° in spectrophotometer tubes (8 mm in diameter) and A was then read against the appropriate gelatin blank. For neutral D-glucans (erythrodextrin-free soluble starch, glycogen, and dextran) and their derivatives (formates, acetates, and phosphates of D-glucan, partially and fully methylated, and periodate-oxidized), the total reaction-mixture (7.0 ml), which contained 2.0 mg of the D-glucan or its derivative in addition to 0.5–30 mg of gelatin, was incubated for 30 min at 25° in spectrophotometer tubes (19 mm in diameter) before A was read against the respective blank of D-glucan. For all other biopolymers (RNA, DNA, poly(adenylic acid), and phosphonoproteins), homo- and heteropolysaccharides, or polysaccharide mixtures (pectin, gum karaya, and gum arabic), the total reaction-mixtures (7.0 ml), containing 1.0 mg of the biopolymers, were assayed in a manner similar to that outlined for the D-glucans. The ratio of the quantity of gelatin required to produce maximal turbidimetric readings with 1.0 mg of the respective polysaccharide is defined as the equivalence binding-ratio.

The effect of sodium chloride on the turbidities of biopolymer–gelatin complexes was determined by adding a 0.05 vol. of 2.9M sodium chloride to the reaction mixtures in distilled, de-ionized water (final concentration of sodium chloride, 0.145M) and, after incubating for 30 min at 25°, reading A at 450 nm (A_{450}).

For testing the effect of specific antiserum on formation of the LPS–gelatin complex, the total reaction mixtures (2.0 ml) contained 1.0 mg of LPS from *E. coli* 0111:B4 (W), 1.0 mg of dialyzed Bacto, *E. coli* OB antiserum, 0111:B4, control 489522, (Difco Laboratories, Detroit, Michigan), and weights of gelatin that ranged from 0.5–30 mg. Following incubation for 30 min at 25°, turbidimetric readings at 450 nm were read against a blank of distilled water containing 1.0 mg of antiserum. Other proteins substituted for antiserum were 1.0-mg samples of the phosphonoproteins, ovalbumin, and phosvitin.

The effects of guanidine hydrochloride (lot No. 44A, Eastman Organic Chemicals, Rochester, N.Y.) and urea (ultrapure, Mann Research Labs., Inc., New York, N.Y.), on the turbidities produced by macromolecular interactions in distilled, de-ionized water were also determined by adding either 0.1 vol. of 10M urea or 0.05 vol. of 6M guanidine hydrochloride to the reaction mixtures. After the reaction mixture had been incubated for 30 min at 25°, A_{450} was recorded. In some instances, the combined effects of 0.145M sodium chloride and either 1.0M urea or 0.3M guanidine hydrochloride on the dissolution of the macromolecular, gelatin precipitate were also assayed.

Spectral analyses — U.v. absorption spectra for gelatin and gelatin–polysaccharide mixtures were determined in a Cary Model 14 recording spectrophotometer. "Scattering spectra" for gelatin–polysaccharide complexes were obtained according to the procedure of Holladay *et al.*[15]. In essence, spectra of aqueous, gelatin solutions were obtained, with distilled, de-ionized water as a blank in the reference beam. In order to eliminate effectively any absorption due to the polysaccharide, the spectra of gelatin–polysaccharide complexes were obtained with the respective polysaccharide or lipopolysaccharide in the reference beam.

I.r. spectra of gelatin, D-glucans, and gelatin–D-glucan mixtures were obtained with a Beckman Model IR-7 infrared spectrophotometer equipped with an attenuated total reflectance (ATR) attachment and a thallous iodide-bromide (KRS-5 Barnes Engineering Co.), internal-reflecting crystal. Thin films cast from 4% gelatin, D-glucan, or gelatin–D-glucan solutions served as the samples. All films were prepared simultaneously, to minimize possible spectral differences induced by atmospheric conditions. However, no dectable spectral changes were observed in samples prepared on different days.

Optical rotatory dispersion curves for gelatin, D-glucans, or gelatin–D-glucan mixtures were determined by use of a Jasco–Durrum (Model ORD/UV 5) recording spectropolarimeter and a 1-cm silica cuvette.

RESULTS AND DISCUSSION

During the course of investigations on the formation of polysaccharide–gelatin complexes in acidified ethanol solutions[3], it was observed that gelatin–D-glucan (soluble starch or glycogen) complexes, in distilled, de-ionized water formed turbid suspensions. However, upon the addition of TCA, the solutions became clear. These

observations suggested that polysaccharide–gelatin interactions may also be characterized by their differential solubility characteristics in distilled, de-ionized water. The data in Fig. 1-A show that interactions of glycogen and soluble starch with gelatin resemble

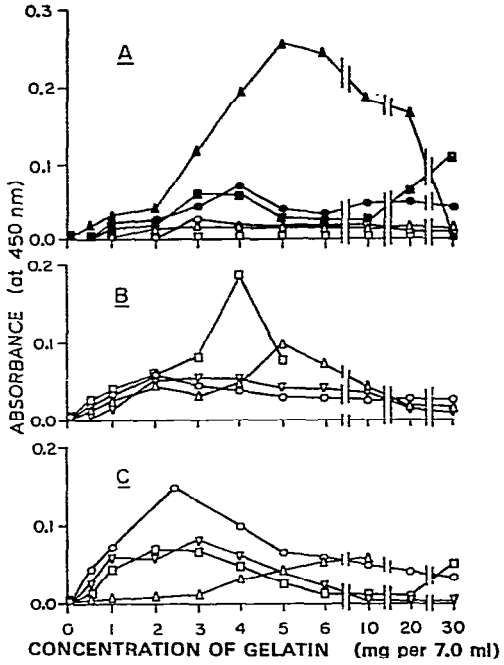

Fig. 1. Turbidimetric changes accompanying formation of complexes between neutral (and substituted) polysaccharides and gelatin in distilled, de-ionized water. *A*: 2.0 mg of soluble starch (○), dextran (□), and glycogen (△); 2.0 mg of soluble-starch phosphate, D.S. = 0.033, (●), dextran phosphate, D.S. = 0.079, (■), and glycogen phosphate, D.S. = 0.048, (▲). *B*: 1.0 mg of lichenan (○), phosphonogalactan (□), phosphonomannan (△), and agar (▽). *C*: 1.0 mg of D-galacturonan (○), gum karaya (□), L-arabinan (▽), and dextran (NRRL-Y1401) (△).

the classical, antigen–antibody, precipitin curves. The gelatin–soluble starch and gelatin–glycogen equivalence binding-ratios were 1.5 and 1.0, respectively. These binding ratios closely paralleled the stoichiometric binding-ratios previously noted for the solubilization of D-glucan–gelatin complexes in acidified ethanol solutions[2,3]. On the other hand, dextran–gelatin solutions failed to exhibit a precipitin reaction throughout a range of weight of gelatin of 0.5–30 mg.

The data in Fig. 1-A also show that (phosphorylated D-glucan)–gelatin mixtures not only elicit an increase in precipitin formation, when compared to the respective neutral D-glucans, but also show "anomalous" precipitin curves at high concentrations of gelatin (10–30 mg of gelatin per 7 ml). Whereas the (glycogen phosphate)–gelatin precipitin curve exhibited the typical, antigen–antibody, precipitin curve, both dextran phosphate and soluble-starch phosphate showed inflection points at low concentrations of gelatin and increased precipitate formation at high concentrations of gelatin. A similar increase in precipitable gelatin—but not the biopolymeric

complexes—at gelatin to glucan ratios of 20:1 or higher, has previously been noted for the glucan–gelatin solubilization reaction in acidified ethanol solutions[16]. Consequently, increased precipitation at high concentrations of gelatin may only be a reflection of the aggregation of gelatin. Data presented in this study do not permit differentiation between such nonspecific, protein-exclusion phenomena by polysaccharides[17–19] and enhancement of macromolecular complex-formation in the presence of high concentrations of protein.

The addition of 0.145M sodium chloride to either neutral D-glucan– or (D-glucan phosphate)–gelatin complexes inhibited precipitin formation throughout the concentration range of 0.5–30 mg of gelatin per 2 ml. The inhibition of (D-glucan phosphate)–gelatin precipitation by 0.145M sodium chloride suggests that electrostatic interactions may be the primary mechanism of reaction involved in precipitation of these macromolecular complexes. On the other hand, it is difficult to attribute inhibition, by sodium chloride, of neutral, D-glucan–gelatin, precipitin formation to electrostatic interactions, as neither glycogen nor soluble starch possesses the requisite ionic groups necessary for the formation of salt-like complexes.

The effect of glycogen on the u.v. absorption spectrum of gelatin (see Fig. 2) provides evidence for the occurrence of macromolecular interactions in distilled, de-ionized water. Similar spectra for trypsin–agar complexes have been recorded[14]. (Soluble starch)–gelatin complexes had a higher u.v. absorbance (not shown) than glycogen, whereas dextran was ineffective in inducing a "scattering spectrum".

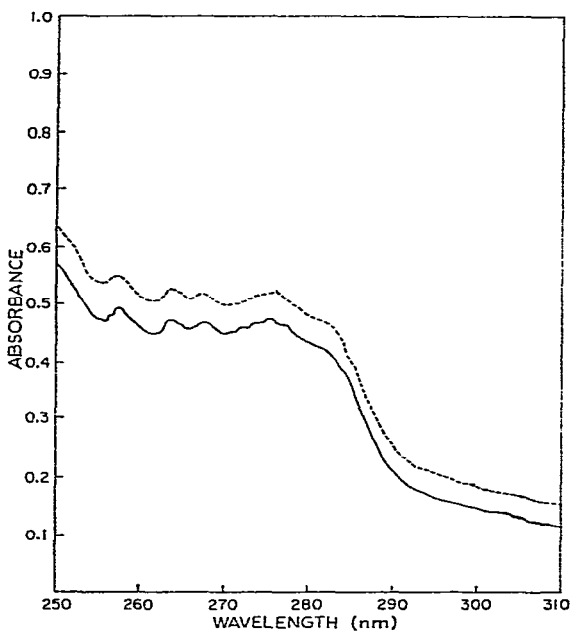

Fig. 2. U.v. spectra of gelatin and the gelatin–glycogen complex. Gelatin (6.0 mg/ml) against a blank of distilled, de-ionized water (—), and 6.0 mg of gelatin/ml + 2.0 mg of glycogen/ml against a blank of 2.0 mg of glycogen/ml (— — —).

These results are consistent with the data shown in Fig. 1-A. Addition of 5M urea or 3M guanidine hydrochloride inhibited formation of the gelatin–D-glucan complex when measurements were made at either 280 nm or 450 nm. Decreasing the concentrations of urea and guanidine hydrochloride to 1.0 and 0.3M, respectively, yielded identical decreases in turbidimetric measurements at 450 nm. Thus, the gelatin–D-glucan complexes resemble the concanavalin A–D-glucan complexes, in that agents breaking hydrogen bonds interfere with the interaction properties of the polymers[20]. These differences in absorbance at 280 and 450 nm, for the complexes of soluble starch and also of glycogen, in conjunction with the inability of dextran–gelatin complexes to produce any differential alterations in absorbance, most probably indicate that specific, differential binding-sites exist in the protein macromolecule for these three D-glucans. Addition of D-glucose to gelatin had no effect on the u.v. spectra of gelatin solutions; consequently, it is suggested that the altered u.v. absorption curves of D-glucan–gelatin complexes not only confirm the existence of biopolymeric complex-formation in distilled, de-ionized water, but also afford a means of differentiating between the D-glucans by way of their interactions with gelatin.

Although dextran–gelatin interactions in distilled, de-ionized water could not be detected by either u.v. or visible spectral measurements, ATR-i.r. studies indicated that specific solute-interactions did exist (see Fig. 3). Thus, dextran–gelatin complexes induced shifts to higher frequencies in the i.r. region of 1650–1450 cm^{-1}. Similar shifts were obtained with soluble starch and glycogen. In the case of gelatin, the two absorption maxima at 1620 and 1530 cm^{-1} correspond to the Amide I and Amide II bands, respectively, which are characteristic of proteins and polypeptides[21]. D-Glucan (soluble starch, dextran, or glycogen)–gelatin complexes also induced shifts in the 1100–900 cm^{-1} region of the i.r. spectrum. The data in Fig. 3 show that the absorption maximum for dextran (990 cm^{-1}) is shifted to a higher frequency (1003 cm^{-1}) when

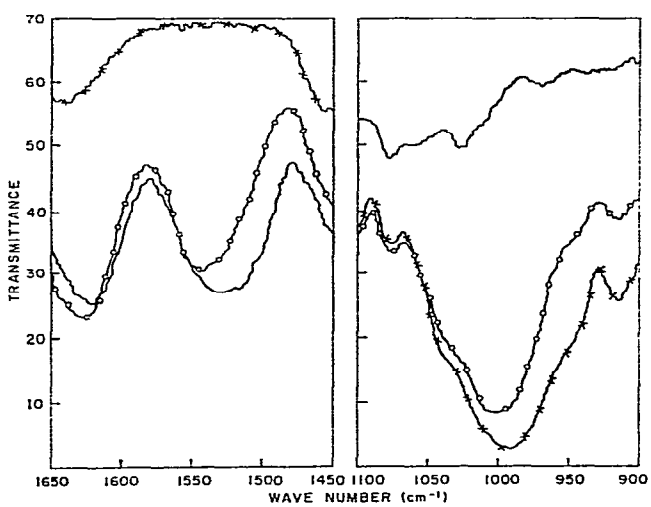

Fig. 3. ATR-i.r. spectra of gelatin, dextran, and the gelatin–dextran complex in the 1100–900 cm^{-1} and 1650–1450 cm^{-1} regions. Gelatin (—); dextran (×—×); gelatin–dextran complex (○—○).

dextran is complexed with gelatin. The band assignments found in this i.r. region are numerous[22], and interpretation of shifts for polysaccharides is extremely complex. However, in conjunction with shifts in the Amide I and II regions, the concomitant shifts from 990 to 1003 cm^{-1} most probably arise from alterations in the O–H bending modes. These results suggest that immunologically important, protein–polysaccharide interactions that do not elicit precipitin reactions may be detected by similar analysis of their i.r. spectra.

Other regions studied (1650–300 cm^{-1} and 1450–1100 cm^{-1}) were devoid of shifts. The region 3600–2900 cm^{-1}, widely used in studying hydrogen bonding between molecules, was also scanned. However, strong, intramolecular hydrogen-bonding in both gelatin and the D-glucans tended to give rise to wide absorption areas that masked possible shifts in gelatin–glucan mixtures. These data are difficult to interpret, in view of the fact that hydrogen bonding would tend to lower the absorption maxima in gelatin. It could be supposed that the D-glucans alter the normal, internal hydrogen-bonding in gelatin to give rise to the spectral shifts. In this connexion, however, D-glucans do not influence the gelation of 4% gelatin solutions[2]. Moreover, crystallinity would not appear to be a factor here, as all three of these D-glucans gave rise to essentially identical shifts.

D-Glucan–gelatin interactions also cause marked changes in rotatory dispersion properties when compared with those of the individual polymers (see Table I). Thus, the specific rotations of glycogen–gelatin complexes are not equivalent to the sum of those of the two polymers. Similar differences were noted for dextran and soluble starch when these were mixed with gelatin. Although these data do not reveal possible sites or mechanisms of interaction, they are useful in detecting formation of a complex. In contrast other results showed that D-glucose did not markedly influence the rotatory properties of concanavalin A in its Cotton-effect region[23]. Similar differences in o.r.d. spectra between nucleotides of low molecular weight and the sum of their monomers have been reported by others[24,25], and have been utilized to detect interactions between solutes.

TABLE I

ROTATIONS[a] OF GELATIN–GLYCOGEN COMPLEXES

Wavelength nm	*Gelatin*[b]	*Glycogen*[b]	*Gelatin + glycogen*	
			Calculated	*Observed*
420	−339	+310	−29	−71
400	−369	+350	−19	−92
370	−441	+429	−12	−100
350	−510	+490	−20	−119

[a]Values reported as degrees of rotation. [b]Concentrations of gelatin and glycogen were each 1 mg/ml.

The data in Fig. 4 show that precipitin formation with dextran sulfates and gelatin is dependent on the molecular weight of the substituted dextran. Whereas

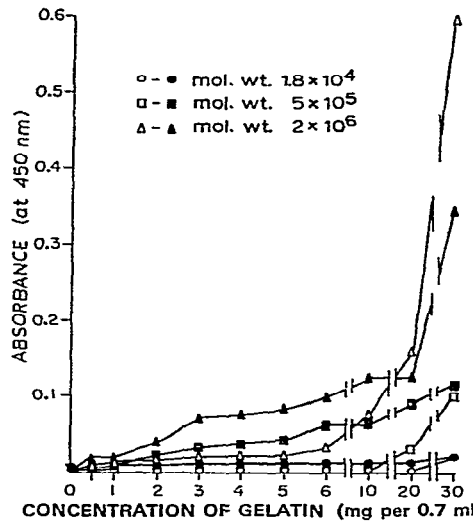

Fig. 4. Turbidimetric changes accompanying (dextran sulfate)–gelatin interactions. Dextran sulfate (1.0 mg) in distilled, de-ionized water (○, □, △), and in 0.145M sodium chloride (●, ■, ▲).

the dextran sulfate of mol. wt. 2×10^6 elicited an atypical precipitin curve, interactions of gelatin with either dextran sulfate of mol. wt. 1.8×10^4 or dextran sulfate of mol. wt. 5×10^5 did not yield a precipitate except at high concentrations of gelatin (10–30 mg of gelatin per mg of dextran sulfate). The sulfated polysaccharide fucan reacted with gelatin in a manner similar to that of the dextran of mol. wt. 5×10^5 within the range of concentration of 0.5–6.0 mg of gelatin per experiment. At higher concentrations of gelatin, the fucan–gelatin precipitin curve parallels the precipitin curve for the dextran sulfate of mol. wt. 2×10^6.

An unexpected finding was the enhancement of precipitin formation of all (dextran sulfate)–gelatin and fucan–gelatin complexes in the presence of 0.145M sodium chloride within the concentration range of 0.5–6.0 mg of gelatin per experiment. In contrast, 1.0M urea or 0.3M guanidine hydrochloride not only inhibited the precipitin reaction of the high mol. wt. (dextran sulfate)–gelatin complex, but also solubilized the precipitate induced by 0.145M sodium chloride of both (dextran sulfate)– and fucan–gelatin complexes.

It is possible that the salt aided formation of the gelatin–fucan and (dextran sulfate)–gelatin complexes by the interposition of cations (Na^+) between ionic sites (such as sulfate) on the polymers, thereby forming bridged complexes. Although divalent cations were not examined, univalent cations would not be expected to be so effective as multivalent cations.

The data in Table II further characterize the specificity of polysaccharide–gelatin precipitin reactions in distilled, de-ionized water. Of the numerous neutral homo- and hetero-polysaccharides tested, only soluble starch, glycogen, and lichenan were capable of forming a precipitate with gelatin. Precipitin formation with substituted homopolysaccharides was limited to the phosphoric esters of soluble starch,

glycogen, dextran, D-mannan, and D-galactan, as well as a sulfated derivative of agarose and a dextran of high molecular weight. The precipitin curves in Fig. 1-B reveal the stoichiometry of gelatin interactions with lichenan, phosphonogalactan, phosphonomannan, and purified agar. Both 1.0M urea and 0.3M guanidine hydrochloride, as well as 0.145M sodium chloride, inhibited precipitin formation with purified agar and gelatin. Since only limited amounts of the other polysaccharides in Fig. 1-B were available, the effects on these macromolecular interactions of the respective monomers, sodium chloride, and agents for breaking hydrogen bonds, were not investigated.

Although the sulfated polymer agar elicited the precipitin reaction, its corresponding neutral homopolymer, agarose (Table II) did not form a precipitate with gelatin. Other notable differences between agar–gelatin and (sulfate-containing polysaccharide)–gelatin interactions were the lack of any nonspecific increase of precipitin formation at high concentrations of gelatin (10–30 mg of gelatin per 7 ml) and the lack of enhancement of precipitin formation by 0.145M sodium chloride. The carrageenans, chondroitin sulfate, and hyaluronic acid exhibited precipitin curves similar to that of fucan. In contrast to the enhanced precipitin reactions of fucan and dextran sulfate in the presence of 0.145M sodium chloride, the carrageenans, chondroitin sulfate, and hyaluronic acid did not exhibit corresponding precipitin reactions. The sulfated polymer heparin failed to yield precipitin-like reactions with gelatin under any of the varied experimental conditions.

These differential solubility-characteristics of sulfated polysaccharides with gelatin in distilled, de-ionized water, and in the presence of either 0.145M sodium chloride or agents breaking hydrogen bonds, not only provide an additional means for preliminary identification of sulfated polysaccharides isolated, but also reveal the complex nature of the bonding forces most probably involved in complex-formation. It is evident that charged groups on a polysaccharide do not necessarily induce precipitation with gelatin, because neutral formates and acetates of D-glucan resemble heparin in not forming precipitates with gelatin.

On the other hand, numerous uronic acid-containing polysaccharides listed in Table II gave precipitin reactions (see Fig. 1-C). Polysaccharide mixtures containing uronic acid residues (such as gum karaya, pectin, and gum arabic) also exhibited precipitin reactions with gelatin. Precipitin formation between all glycuronans and gelatin, within a range of concentration of gelatin of 0.5–6.0 mg per experiment, was inhibited by 0.145M sodium chloride, by 1.0M urea, and by 0.3M guanidine hydrochloride. These results re-emphasize the complex nature of the bonding forces involved in polysaccharide–gelatin precipitin reactions.

The inhibitory effects of sodium chloride on precipitin formation suggest that the polyionic polysaccharides that elicit precipitin reactions may react with the positively charged gelatin to form salt-like complexes. However, the inhibitory effects of both urea and guanidine hydrochloride on these same acidic polysaccharide–gelatin complexes is not explained by this postulate.

The mechanisms whereby urea and guanidine hydrochloride denature proteins

TABLE II

SPECIFICITY OF POLYSACCHARIDE–GELATIN PRECIPITIN FORMATION IN DISTILLED, DE-IONIZED WATER[a]

Neutral polysaccharides		*Substituted polysaccharides*		*Acidic polysaccharides*	
No reaction	*Precipitation*	*No reaction*	*Precipitation*	*No reaction*	*Precipitation*
Amylopectin	soluble starch	soluble starch	soluble starch	fucan[b]	poly(galacturonic
Nigeran	glycogen	formate	phosphate	carrageenans[b]	acid)
Laminaran	lichenan	acetate	glycogen	chondroitin	gum karaya
Dextran		partially	phosphate	sulfate[b]	NRRL-Y1401
Agarose		methylated	dextran	hyaluronic acid[b]	L-arabinan
Inulin (linear)		periodate-	phosphate	Na polypectate[b]	pectin
Levans (branched)		oxidized	sulfate	D-xylan[b]	gum tragacanth
Ficoll		*O*-(2-hydroxyethyl)	2×10^6	heparin	gum arabic
D-Galactan		glycogen	mol. wt.		
L-Arabino-D-galactans		formate	phosphonomannan		
D-Galacto-D-mannans		acetate	phosphonogalactan		
locust bean		partially and fully	agar, purified		
and guar gums		methylated			
D-Mannan (yeast)		*periodate*			
		oxidized			
		dextran			
		formate			
		acetate			
		partially and fully			
		methylated			
		periodate-			
		oxidized			
		sulfates[b]			
		1.8×10^4 and			
		5×10^5 mol. wt.			
		D-galacto-D-mannans			
		guar gum			
		anionic and			
		cationic			

[a]Conditions for detecting precipitin formation are the same as described in Fig. 1. [b]Nonspecific precipitation occurred only in the presence of high concentrations of gelatin (10–30 mg of gelatin per mg of polysaccharide).

still remain obscure. Based on thermodynamic measurements of urea–peptide bond-interactions in water, Kresheck and Benjamin[26] have shown that urea frees water molecules from peptide groups through hydrogen bonding. Robinson and Jencks[27] have implicated both hydrophobic and non-hydrophobic effects of these compounds on peptides of low molecular weight, and have suggested that these denaturing agents react directly with synthetic tetrapeptides to form complexes. With respect to (macromolecular protein)–urea interactions, Olson and Liener[28] have shown that 8M urea dissociates concanavalin A into protein subunits that are not capable of eliciting a precipitin reaction with glycogen. Other studies have shown that both urea and guanidine hydrochloride competitively inhibit precipitin formation[20] between polysaccharide and concanavalin A. From the data presented, dissociation of gelatin into nonreactive protein subunits cannot be ascertained.

Although hydrogen bonds between tetrapeptides in aqueous solution are stated to be unstable[27], in such macromolecular interactions as gelatin–polysaccharide complex-formation, wherein polyfunctional hydrogen-bonding can occur, a favorable entropy of formation could be realized[29]. Therefore, the possibility of hydrogen bonding between proteins and polysaccharides in aqueous solution cannot be excluded, even though such complexes may have only a low stability. In this respect, the ability of 3M urea or 0.6M guanidine hydrochloride to inhibit precipitin formation between gelatin and nonionic D-glucans (soluble starch and glycogen) suggests that hydrogen bonding may be the primary force operative in these biopolymeric interactions.

With the exception of DNA, the data in Fig. 5 show that precipitin reactions

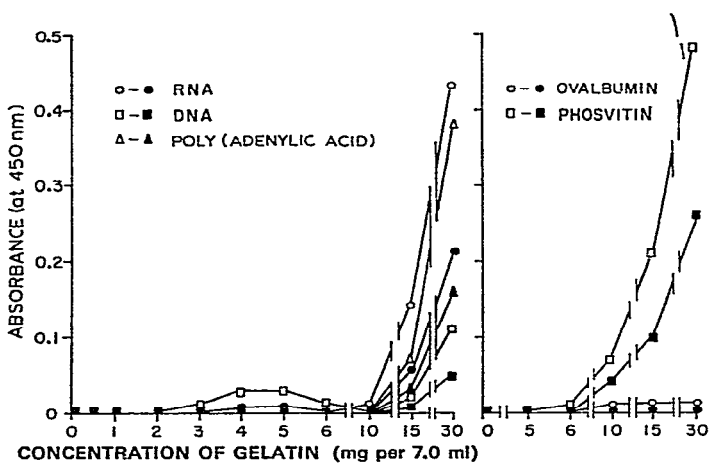

Fig. 5. Turbidimetric changes accompanying gelatin–(phosphorus-containing biopolymer) interactions in aqueous solution. Phosphorus-containing polymer (1.0 mg) in distilled, de-ionized water (○, □, △) and in 0.145M sodium chloride (●, ■, ▲).

of phosphorus-containing biopolymers [RNA, DNA, poly(adenylic acid), phosvitin, and ovalbumin] did not occur within a concentration range of 0.5–6.0 mg of gelatin per experiment. Interactions of DNA with gelatin gave rise to highly viscous, gel-like

solutions that could not be measured accurately by the turbidimetric assay procedure. Consequently, the turbidimetric readings of gelatin–DNA mixtures within the range of concentration of gelatin of 0.5–6.0 per experiment (see Fig. 5) cannot be attributed to a specific, precipitin-like reaction. Because numerous polysaccharides (phosphorylated soluble-starch and dextran, dextran sulfates, fucan, carrageenans, chondroitin sulfate, hyaluronic acid, and D-xylan), as well as the phosphorus-containing biopolymers noted in Fig. 5, all elicited enhanced precipitin formation at high concentrations of gelatin (10–30 mg per experiment), the specificity of gelatin–polysaccharide precipitin formation is limited mainly to ratios of gelatin to polysaccharide of 0.5:1 to 6:1.

The data in Fig. 6A and 6B, show that phenol-extracted preparations of bacterial endotoxins (Westphal lipopolysaccharides) invariably exhibited precipitin-like reactions with gelatin, whereas the corresponding TCA-extracted endotoxins (Boivin endotoxins) did not produce increased turbidities under identical experimental conditions. The differences in the slopes of the precipitin curves, in conjunction with differences in equivalence binding-ratios, indicate that specificity is also involved

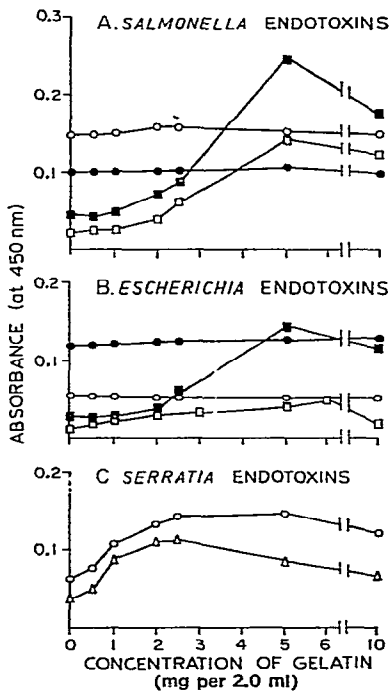

Fig. 6. Turbidimetric changes accompanying formation of (*Enterobacteriaceae* endotoxin)–gelatin complexes in distilled, de-ionized water. Boivin-extracted endotoxin (1.0 mg) (○, ●), Wesphal-extracted endotoxin (○, ■), and trypsin-digested endotoxin (△). *A*: *S. typhimurium* (○, □), and *S. abortus equi* (●, ■). *B*: *E. coli* 0111:B4 (○, □), and *E. coli* 0128:B12 (●, ■). *C*: *S. marcescens* (○, △). All endotoxin–gelatin complexes were measured against blanks containing the appropriate concentration of gelatin.

with gelatin–lipopolysaccharide precipitin reactions. These interactions are more difficult to interpret than those discussed in the foregoing paragraphs, because of contamination with such other macromolecules as protein and RNA[4]. However, as RNA alone did not elicit a precipitin reaction within the concentration range of 0.5–6.0 mg of gelatin per experiment (see Fig. 5), the RNA contaminants, which are normally associated with crude, phenol-extracted, bacterial LPS, most probably do not contribute to the turbidimetric values of phenol-extracted LPS–gelatin complexes.

With the exception of the Boivin-extracted LPS from *Serratia marcescens*, TCA-extracted preparations of LPS invariably exhibited initially higher turbidimetric values, which were not altered on subsequent addition of gelatin. Since Boivin-extracted endotoxins contain 6–72 times as much protein as the respective phenol-extracted LPS[4], it is suggested that the inherent protein may not only account for the initially high turbidimetric values, but may also occupy or mask the gelatin binding-sites in the Boivin preparation.

Since both the Boivin (14.5% of protein) and trypsin-digested (10.9% of protein) LPS preparations of *S. marcescens* were capable of eliciting precipitin reactions, it appears that innate protein contaminants do not invariably inhibit precipitin reactions; however, LPS preparations from *S. marcescens* are unique, in that they contain uronic acid (11.8% of the dry weight) which is absent from the LPS preparations[4] from *E. coli* and *Salmonella*. The similarity in the precipitin curves of (*S. marcescens* LPS)–gelatin complexes (see Fig. 6-C) to those of the glycuronan–gelatin complexes (see Fig. 1-C) suggests that the uronic acid moiety within the *S. marcescens* LPS may be the major monomeric constituent effecting precipitin formation with gelatin.

Although the Boivin-extracted LPS of *Escherichia coli* 0111:B4 did not elicit a precipitin reaction (see Fig. 6-A), alterations in the u.v. absorption spectrum of gelatin by LPS (see Fig. 7) suggest that complex-formation occurred between these biopolymers in distilled, de-ionized water. The data in Fig. 7 also show that the Westphal-extracted LPS produced greater changes in the u.v. absorption spectrum of gelatin than did the corresponding Boivin preparation at a gelatin: LPS ratio of 6:1 (2.0 mg of gelatin +0.33 mg of the respective LPS preparations). In addition, alterations in the u.v. spectra of (Westphal-extracted LPS)–gelatin complexes in distilled, de-ionized water are dependent on the LPS concentration. It is inferred from these results that the Westphal preparation most probably possesses functional groups that are more accessible, and that can react more readily with gelatin, than those in the corresponding Boivin preparation. Owing to the presence of numerous functional groups within these LPS preparations, meaningful interpretations of the u.v. scattering spectra are difficult.

The present results also show that the interaction of LPS with gelatin is not suppressed in the presence of specific anti-LPS serum (see Fig. 8). However, precipitin formation was inhibited by 0.145M sodium chloride. These observations suggest that the reactive components of LPS–gelatin and LPS–antibody are different.

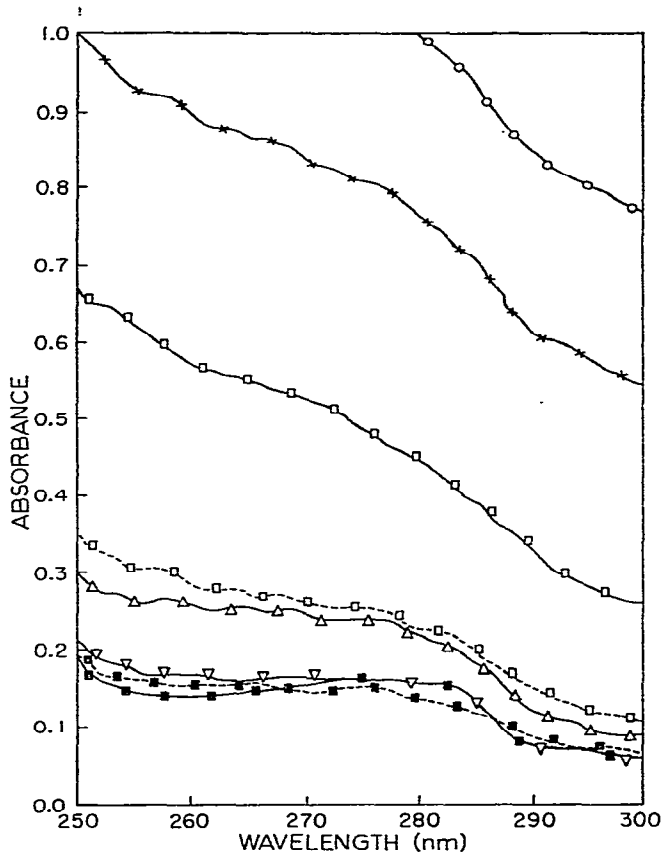

Fig. 7. U.v. spectral changes associated with *Escherichia coli* 0111:B4 lipopolysaccharide (LPS)–gelatin complexes in aqueous solution. All samples contained 2.0 mg of gelatin/ml and were measured against blanks containing the respective concentration of LPS. LPS concentrations used were: 0.5 mg/ml (○); 0.33 mg/ml (□); 0.25 mg/ml (×); and 0.125 mg/ml (△) in distilled, de-ionized water and in 0.145M sodium chloride (■). Absorption curve of gelatin (2 mg/ml) in distilled, de-ionized water or 0.145M sodium chloride (▽). Dotted lines represent Boivin-extracted LPS, and solid lines, the Westphal-extracted LPS, respectively.

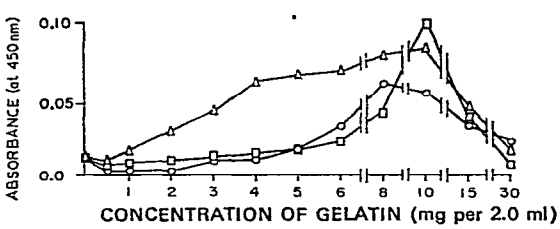

Fig. 8. Turbidimetric changes accompanying the interactions of Westphal-extracted *E. coli* 0111:B4 LPS with binary protein solutes in distilled, de-ionized water. In addition to the concentrations of gelatin listed, the reaction mixtures contained 1.0 mg of LPS and 1.0 mg of one of the following proteins: *E. coli* 0111:B4 antiserum (—○—), phosvitin (—□—), or ovalbumin (—△—). The blank contained 1.0 mg of LPS and 1.0 mg of antiserum, ovalbumin, or phosvitin, respectively.

The data in Fig. 8 also show that tertiary polymeric mixtures containing phosphoproteins (instead of specific antiserum) induced alterations in both the shape of the precipitin curve and the equivalence binding-ratio of LPS–gelatin complexes. In the presence of 1 mg of either phosvitin or ovalbumin, the equivalence binding-ratio was increased to 10. The complexity of the resultant interactions in tertiary biopolymeric mixtures is further evidenced by the observation that the high turbidities of gelatin–phosvitin mixtures in the presence of 10–30 mg of gelatin per 2 ml (see Fig. 5) were inhibited by the presence of 1 mg of LPS (see Fig. 8). Since simple, concentration effects cannot account for these decreased absorbances, it is suggested that both nonprecipitating (LPS–phosvitin) and precipitating (LPS–gelatin) interactions were present.

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MIXED ACYLARYLOSAZONES

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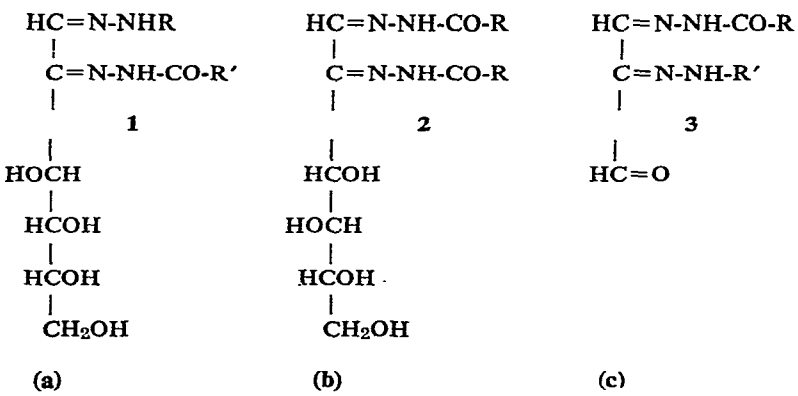
ABSTRACT

D-*arabino*- and D-*lyxo*-Hexosulose 1-acylhydrazone 2-arylhydrazones and 2-acylhydrazone 1-arylhydrazones have been prepared from the respective hexosulose 1-hydrazone and the corresponding hydrazine. Their properties are compared with those of saccharide aryl- and acyl-osazones. Also prepared were a number of mesoxalaldehyde 1,2-bis(acylhydrazones), 1-acylhydrazone 2-arylhydrazones, and 2-acylhydrazone 1-arylhydrazones.

INTRODUCTION

In a previous communication[1], we described the preparation of a number of hexosulose 2-acylhydrazone 1-arylhydrazones (**1**) and compared their properties and reactions with those of aryl- and acyl-osazones (**2**). To complete this comparative study, we have now prepared a number of 1-acylhydrazone 2-arylhydrazones (**3a**) and 2-acylhydrazone 1-arylhydrazones (**1a**) and (**1b**).



Starting with D-*arabino*-hexosulose 1-benzoylhydrazone[2] and phenyl- or *p*-tolyl-hydrazine, we prepared D-*arabino*-hexosulose 1-benzoylhydrazone 2-phenylhydrazone and 1-benzoylhydrazone 2-*p*-tolylhydrazone. Like the osazones of type **1**, these osazones are yellow, and their electronic spectra show three maxima, at 256,

306, and 390 nm. Their infrared absorption spectra show a band at 1600 (C=N) and at 1660 cm^{-1} (CONH). They give colors with ferric chloride (indicating that their acylhydrazone residues are enolized), and form stable copper complexes, and thus resemble, in these respects, both the bis(acylhydrazones)[3] and the 2-acylhydrazone 1-arylhydrazones[1].

Of the 2-acylhydrazone 1-arylhydrazones, the following compounds were prepared: of D-*arabino*-hexosulose, the 2-(*p*-iodobenzoyl)hydrazone 1-phenylhydrazone, the 2-benzoylhydrazone 1-*p*-tolylhydrazone, and the 2-nicotinoylhydrazone 1-*p*-tolylhydrazone; and of D-*lyxo*-hexosulose, the 2-benzoylhydrazone 1-phenylhydrazone and the 2-(*p*-chlorobenzoyl)hydrazone 1-phenylhydrazone.

For preparation of mesoxalaldehyde 1,2-bishydrazones, the bis(acylhydrazones), 2-acylhydrazone 1-arylhydrazones, and 1-acylhydrazone 2-arylhydrazones of D-*arabino*-hexosulose were oxidized with periodic acid. Mesoxalaldehyde 1,2-bis-(acylhydrazones) (**2c**) were colorless, whereas the mixed types (**1c**) and (**3c**) were orange. Their infrared absorption spectra showed, in addition to the (C=N) and (C=O) bands, a CONH band at 1650–1680 cm^{-1}. Unlike mesoxalaldehyde 1,2-bis-(arylhydrazones)[4], which cyclize to 1-aryl-4-arylazo-2-pyrazolin-5-one on treatment with acid reagents, all of the mesoxalaldehyde 1,2-bis(hydrazones) studied failed to cyclize.

EXPERIMENTAL

I.r. spectra were recorded with a Unicam SP-200 spectrophotometer, and u.v. absorption spectra with a Unicam SP-800 spectrophotometer.

D-arabino-*Hexosulose 1-benzoylhydrazone 2-phenylhydrazone* (**3a**; $R = R' = Ph$). — A solution of D-*arabino*-hexosulose 1-benzoylhydrazone (0.3 g) in ethanol (10 ml) was treated with a solution of phenylhydrazine (0.11 g) in ethanol (5 ml) and a few drops of acetic acid. The mixture was gently warmed on the water-bath for 2 min, and then kept for 24 h at room temperature. The product (0.11 g) crystallized from methanol in yellow plates, m.p. 207–212°; ν_{max}^{KBr} 1600 (C=N), 1660 (CONH), 3350 cm^{-1} (OH); λ_{max}^{EtOH} 256, 306, 390 nm (log ε 1.00, 0.70, 2.30), λ_{min}^{EtOH} 279, 327 nm (log ε 0.50, 0.70).

Anal. Calc. for $C_{19}H_{22}N_4O_5$: C, 59.1; H, 5.7; N, 14.5. Found: C, 59.5; H, 6.0; N, 14.4.

D-arabino-*Hexosulose 1-benzoylhydrazone 2*-p-*tolylhydrazone* (**3a**; $R = Ph$, $R' = C_6H_4Me$-p). — A solution of D-*arabino*-hexosulose 1-benzoylhydrazone (0.4 g) was treated with *p*-tolylhydrazine (0.17 g), and the mixture was warmed on the water-bath for 2 min and then allowed to cool. The product (0.25 g) was filtered off, and washed with and crystallized from methanol, in yellow plates, m.p. 215–220°.

Anal. Calc. for $C_{20}H_{24}N_4O_5$: C, 60.0; H, 6.0; N, 14.0. Found: C, 60.4; H, 6.5; N, 14.0.

D-arabino-*Hexosulose 2-*(p-*iodobenzoyl)hydrazone 1-phenylhydrazone* (**1a**; $R = Ph$, $R' = C_6H_4I$-p). — A solution of D-*arabino*-hexosulose 1-phenylhydrazone[5] (1 g) in water (15 ml) was treated with a solution of *p*-iodobenzoylhydrazine (0.6 g)

in methanol (10 ml) and a few drops of acetic acid. After being warmed on the water-bath for 5 min, it was allowed to cool, and the product that separated was filtered off, washed, and dried (1.1 g). It crystallized from *p*-dioxane in yellow needles, m.p. 198–200° (dec.); ν_{max}^{KBr} 1600 (C=N), 1665 (CONH), 3350 cm^{-1} (OH).

Anal. Calc. for $C_{19}H_{21}N_4IO_5$: C, 44.6; H, 4.1. Found: C, 44.7; H, 4.2.

D-arabino-*Hexosulose 2-benzoylhydrazone 1*-p-*tolylhydrazone* (**1a**; *R* = *C_6H_4Me*-p, *R'* = *Ph*). — A solution of D-*arabino*-hexosulose 1-*p*-tolylhydrazone (0.5 g) in ethanol (10 ml) was treated with benzoylhydrazine (0.7 g) as above. The product (0.5 g) was filtered off, and crystallized from ethanol in yellow plates, m.p. 218–219° (dec.); ν_{max}^{KBr} 1600 (C=N), 1660 (CONH), 3350 cm^{-1} (OH); λ_{max}^{EtOH} 242, 282, 384 nm (log ε 1.00, 0.90, 1.20), λ_{max}^{EtOH} 265, 323 nm (log ε 0.30, 0.80).

Anal. Calc. for $C_{20}H_{24}N_4O_5$: C, 60.0; H, 6.0; N, 14.0. Found: C, 59.9; H, 6.1; N, 14.2.

D-arabino-*Hexosulose 2-nicotinoylhydrazone 1*-p-*tolylhydrazone* (**1a**; *R* = *C_6H_4Me*-p, *R'* = *3-pyridyl*). — The title compound was prepared as above from D-*arabino*-hexosulose 1-*p*-tolylhydrazone (0.5 g) and nicotinoylhydrazine (0.7 g). It crystallized from methanol in yellow plates, wt. 0.4 g, m.p. 206–208° (dec.); ν_{max}^{KBr} 1595 (C=N), 1670 (CONH), 3350 cm^{-1} (OH).

Anal. Calc. for $C_{19}H_{23}N_5O_5$: C, 56.8; H, 5.8; N, 17.5. Found: C, 56.8; H, 6.3; N, 17.8.

D-lyxo-*Hexosulose 2-benzoylhydrazone 1-phenylhydrazone* (**1b**; *R* = *R'* = *Ph*). — A solution of D-*lyxo*-hexosulose 1-phenylhydrazone (1 g) in water (10 ml) was treated with benzoylhydrazine (0.6 g) in methanol (5 ml) and a few drops of acetic acid. After being warmed for 10 min on the water-bath, the mixture was allowed to cool, and the product (1 g) that separated was filtered off, washed, and dried. It crystallized from methanol–water in yellow needles, m.p. 221–222° (dec.); ν_{max}^{KBr} 1603 (C=N), 1660 (CONH), 3400 cm^{-1} (OH).

Anal. Calc. for $C_{19}H_{22}N_4O_5 \cdot H_2O$: C, 56.5; H, 6.0; N, 13.9. Found: C, 56.5; H, 5.8; N, 14.4.

D-lyxo-*Hexosulose 2-(p-chlorobenzoyl)hydrazone 1-phenylhydrazone* (**1b**; *R* = *Ph*, *R'* = *C_6H_4Cl*-p). — D-*lyxo*-Hexosulose 1-phenylhydrazone (1 g) was dissolved in water (10 ml) and treated with *p*-chlorobenzoylhydrazine (2.7 g) as above. The product (1.1 g) was filtered off, and crystallized from methanol–water in yellow needles, m.p. 206–208° (dec.); ν_{max}^{KBr} 1605 (C=N), 1665 (CONH), 3450 cm^{-1} (OH).

Anal. Calc. for $C_{19}H_{21}ClN_4O_5 \cdot 0.5\ H_2O$: C, 53.1; H, 5.1; N, 13.0. Found: C, 53.1; H, 5.4; N, 13.1.

Mesoxalaldehyde 1,2-bis(benzoylhydrazone) (**2c**; *R* = *Ph*). — A suspension of D-*arabino*-hexosulose bis(benzoylhydrazone)[3] (1 g) in water (10 ml) was treated with a solution of metaperiodic acid (2 g) in water (20 ml), and the mixture was kept for 24 h at room temperature, with occasional shaking. The aldehyde (0.7 g) was filtered off, washed, and crystallized from ethanol, giving needles, m.p. 220–222° (dec.); ν_{max}^{KBr} 1605 (C=N), 1650 (CONH), 1690 cm^{-1} (C=O); λ_{max}^{EtOH} 259, 332 nm (log ε 4.44, 4.24), λ_{min}^{EtOH} 218, 298 nm (log ε 4.13, 4.06).

Anal. Calc. for $C_{17}H_{14}N_4O_3$: C, 63.3; H, 4.3; N, 17.4. Found: C, 63.7; H, 4.8; N, 17.3.

Mesoxalaldehyde 1,2-bis(m-*toluoylhydrazone*) (**2c**; $R = C_6H_4Me$-m)*. — A suspension of D-*arabino*-hexosulose bis(*m*-toluoylhydrazone)[3] (1 g) in water (10 ml) was oxidized with metaperiodic acid (2 g) as before. The aldehyde (0.6 g) crystallized from ethanol in needles, m.p. 215–216° (dec.); ν_{max}^{KBr} 1610 (C = N), 1680 (CONH), 1710 cm^{-1} (C=O).

Anal. Calc. for $C_{19}H_{18}N_4O_3$: C, 65.1; H, 5.2; N, 16.0. Found: C, 64.6; H, 5.0; N, 16.2.

Mesoxalaldehyde 1,2-bis(p-*toluoylhydrazone*) (**2c**; $R = C_6H_4Me$-p)*. — D-*arabino*-Hexosulose bis(*p*-toluoylhydrazone)[3] (1 g) in water (10 ml) was oxidized as before with metaperiodic acid (2 g). Mesoxalaldehyde 1,2-bis(*p*-toluoylhydrazone) (0.6 g) crystallized from methanol in needles, m.p. 240–242° (dec.); ν_{max}^{KBr} 1615 (C=N), 1680 (CONH), 1690 cm^{-1} (C=O).

Anal. Calc. for $C_{19}H_{18}N_4O_3$: C, 65.1; H, 5.2; N, 16.0. Found: C, 65.5; H, 5.2; N, 16.2.

Mesoxalaldehyde 1,2-bis(m-*chlorobenzoylhydrazone*) (**2c**; $R = C_6H_4Cl$-m)*. — D-*arabino*-Hexosulose bis(*m*-chlorobenzoylhydrazone)[2] (1 g) suspended in water (30 ml) was treated with metaperiodic acid (2 g) as above. The product (0.55 g) crystallized from methanol in needles, m.p. 225–226° (dec.); ν_{max}^{KBr} 1600 (C=N), 1670 (CONH), 1690 cm^{-1} (C=O).

Anal. Calc. for $C_{17}H_{12}Cl_2N_4O_3$: C, 52.1; H, 3.5; N, 14.3. Found: C, 51.5; H, 3.7; N, 14.8.

Mesoxalaldehyde 1,2-bis(p-*chlorobenzoylhydrazone*) (**2c**; $R = C_6H_4Cl$-p)*. — A suspension of D-*arabino*-hexosulose bis(*p*-chlorobenzoylhydrazone)[3] (1 g) in water (30 ml) was treated with metaperiodic acid (2 g) and kept for 24 h at room temperature. The product (0.6 g) crystallized from ethanol in plates, m.p. 235° (dec.); ν_{max}^{KBr} 1595 (C=N), 1680 (CONH), 1700 cm^{-1} (C=O).

Anal. Calc. for $C_{17}H_{12}Cl_2N_4O_3$: C, 52.1; H, 3.5; N, 14.3. Found: C, 52.0; H, 3.1; N, 14.5.

Mesoxalaldehyde 2-benzoylhydrazone 1-phenylhydrazone (**1c**; $R = R' = Ph$). — A suspension of D-*arabino*-hexosulose 2-benzoylhydrazone 1-phenylhydrazone[1] in water (10 ml) was oxidized with metaperiodic acid (0.5 g) as before. The aldehyde crystallized from methanol in orange needles, m.p. 175–177° (dec.); ν_{max}^{KBr} 1605 (C=N), 1665 (CONH), 1700 cm^{-1} (C=O).

Anal. Calc. for $C_{16}H_{14}N_4O_2$: C, 65.3; H, 4.8; N, 19.1. Found: C, 65.7; H, 5.0; N, 19.5.

*Mesoxalaldehyde 1-phenylhydrazone 2-*p-*toluoylhydrazone* (**1c**; $R = Ph$, $R' = C_6H_4Me$-p). — D-*arabino*-Hexosulose 1-phenylhydrazone 2-*p*-toluoylhydrazone[1] (0.5 g) was oxidized with metaperiodic acid (0.7 g) as above. The product (0.2 g) crystallized from ethanol in orange, prismatic plates, m.p. 178–181° (dec.).

*These experiments were performed by M. Nassr.

Anal. Calc. for $C_{17}H_{16}N_4O_2$: C, 66.3; H, 5.2; N, 18.2. Found: C, 66.6; H, 5.5; N, 18.6.

Mesoxalaldehyde 2-(p-*chlorobenzoyl)hydrazone 1-phenylhydrazone* (**1c**; *R* = *Ph*, *R'* = *C_6H_4Cl*-p). — D-*arabino*-Hexosulose 2-(*p*-chlorobenzoyl)hydrazone 1-phenylhydrazone[1] (0.3 g) was oxidized with metaperiodic acid (0.5 g) as before. The aldehyde (0.15 g) crystallized from ethanol in yellow-orange plates, m.p. 195–196° (dec.).

Anal. Calc. for $C_{16}H_{13}Cl_2N_4O_2$: C, 58.5; H, 4.0; N, 17.1. Found: C, 59.0; H, 4.1; N, 17.4.

Mesoxalaldehyde 1-benzoylhydrazone 2-phenylhydrazone (**3c**; *R* = *R'* = *Ph*). — A suspension of D-*arabino*-hexosulose 1-benzoylhydrazone 2-phenylhydrazone (0.2 g) in water (10 ml) was treated with metaperiodic acid (0.4 g) in water (5 ml), and kept for 24 h at room temperature, with occasional shaking. The product (0.1 g) crystallized from methanol in orange needles, m.p. 186–190° (dec.).

Anal. Calc. for $C_{16}H_{14}N_4O_2$: C, 65.3; H, 4.8; N, 19.1. Found; C, 65.7; H, 5.0; N, 19.0.

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ANHYDRO ARYLOSAZONES

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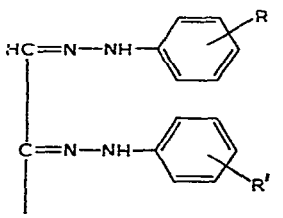
ABSTRACT

A number of mixed arylosazones were prepared and converted into dianhydro derivatives of the Percival type (2) by deacetylation of their tetra-*O*-acetyl derivatives, and into dianhydrides of the pyrazole type (3 and 4) by boiling with acetic anhydride.

INTRODUCTION

In this paper, we describe the preparation of some new, mixed arylosazones and their conversion (together with some other known arylosazones) into (*a*) dianhydro-osazones of the Percival type, by deacetylation of their acetates[1], and (*b*) dianhydro-osazones of the pyrazole type, by boiling with acetic anhydride[2].

The mixed osazones prepared, of type (1) (see Table I), were: D-*arabino*-hexulose 2-(*p*-chlorophenyl)-1-phenyl-, 2-(*p*-bromophenyl)-, and 2-(*p*-iodophenyl)-osazones; and 2-(*p*-chlorophenyl)-1-*p*-tolyl-, 2-(*p*-bromophenyl)-, and (2-*p*-iodophenyl)-osazones. Crystalline tetra-*O*-acetyl derivatives were obtained from the mixed (2-*p*-bromophenyl)-1-phenylosazone and the 2-(*p*-iodophenyl)-1-phenylosazone, as well as from simple arylosazones of D-*arabino*-hexulose and D- or L-*erythro*-pentulose (see Table I). Deacetylation of the hexose derivatives yielded dianhydro-osazones of the Percival type (2) (see Table II).



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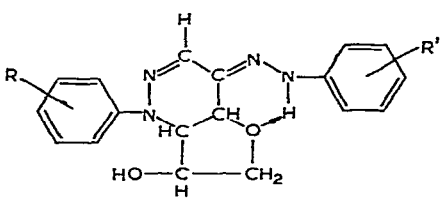

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On boiling D-*arabino*-hexulose *o*-tolylosazone with acetic anhydride, and hydrolyzing the product, we obtained 5-(D-*glycero*-1,2-dihydroxyethyl)-3-formyl-1-*o*-tolylpyrazole *N*-acetyl-*o*-tolylosazone (3, R = *o*-Me). Similar treatment of D-*arabino*-hexulose *m*-tolylosazone and *p*-tolylosazone yielded 5-(D-*glycero*-1,2-

TABLE I

OSAZONES AND THEIR *O*-ACETYL DERIVATIVES (1)

Structure[a] 1	*R*	*R'*	*m.p.*, *degrees*[b]	$[\alpha]_D^{20}$ *degrees*[c]	*Formula*	*Calc.* C	H	N	*Found* C	H	N	ν_{max}^{KBr} C=N	OH	λ_{max}^{EtOH}	*log ε*	λ_{min}^{EtOH}	*log ε*
2	H	*p*-Cl	200–203(d.)	+98.5	$C_{18}H_{21}ClN_4O_4 \cdot 0.5H_2O$	53.8	5.5	13.9	54.3	6.0	13.9	—					
	H	*p*-Br	197–198(d.)	+33.3	$C_{18}H_{21}BrN_4O_4$	49.4	4.8	12.8	49.6	4.8	12.4	—		392,311,259	3.6,3.0,2.9	333,285	1.7,0.9
	H	*p*-I	184–187(d.)	—	$C_{18}H_{21}IN_4O_4 \cdot H_2O$	—	—	11.1			10.9	—					
	p-Me	*p*-Cl	204–208(d.)	—	$C_{19}H_{23}ClN_4O_4 \cdot H_2O$	53.8	5.9	13.2	54.1	5.4	13.6	—					
	p-Me	*p*-Br	200–203(d.)	—	$C_{19}H_{23}BrN_4O_4 \cdot 0.5H_2O$	49.5	5.2	12.2	49.0	5.3	11.9	—					
	p-Me	*p*-I	222–224(d.)	—	$C_{19}H_{23}IN_4O_4 \cdot H_2O$	44.2	4.9	10.9	44.4	5.0	10.8	—					
O-Acetyl derivatives																	
2	H	*p*-Br	138–139	+70.3	$C_{26}H_{29}BrN_4O_8$	51.6	4.8	9.3	51.1	4.6	9.0			*C=N OAc*			
	H	*p*-I	144–146	+84.9	$C_{26}H_{29}IN_4O_8$	47.9	4.4	8.6	47.4	4.4	8.1						
	R = R'																
	4-Br-2-Me		172–174	−32.6	$C_{28}H_{32}Br_2N_4O_8$	47.2	4.5	7.9	47.3	4.5	7.6	1580	1740	406,335,265	4.2,0.6,5.2	351,322	0.3,0.5
3	*p*-Br		173–174	—	$C_{26}H_{28}Br_2N_4O_8$	45.6	4.1	8.2	45.8	4.4	7.7	1600	1745	394,316,264	3.3,0.9,2.6	343,287	1.0,0.8
4	*o*-Me		155–157	+80	$C_{25}H_{30}N_4O_6$	62.2	6.2	11.6	62.0	6.6	11.6	1595	1740	400,305,256	2.8,2.8,1.7	346,280	0.9,0.8
	p-Me		146–148	—	$C_{25}H_{30}N_4O_6$	62.2	6.2	11.6	62.6	6.5	11.8	1595	1740	406,317,259	3.5,1.5,3.5	356,284	0.9,0.6
5	*o*-Me		156–158	−78.3	$C_{25}H_{30}N_4O_6$	62.2	6.2	11.6	62.6	6.6	12.0	1615	1740	400,305,256	3.2,3.3,1.8	347,284	0.9,0.8

[a] 1, *Parent sugar*; 2, D-Glucose; — 3, D-Galactose; — 4, D-Xylose; — 5, L-Xylose.
[b] The symbol d. indicates decomposition. [c] Specific rotations were determined in methanol.

dihydroxyethyl)-3-formyl-1-*m*-tolylpyrazole *N*-acetyl-*m*-tolylhydrazone (3, R = *m*-Me) and 5-(D-*glycero*-1,2-dihydroxyethyl)-3-formyl-1-*p*-tolylpyrazole *N*-acetyl-*p*-tolylhydrazone (3, R = *p*-Me). Acetylation of the last compound afforded 5-(D-*glycero*-1,2-diacetoxyethyl)-3-formyl-1-*p*-tolylpyrazole *N*-acetyl-*p*-tolylhydrazone.

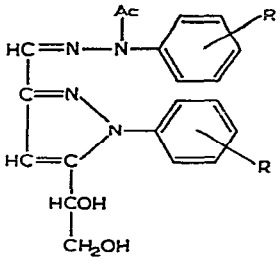
TABLE II

DIANHYDRO-OSAZONES OF THE PERCIVAL TYPE (2)

Parent sugar	*R*	*R'*	*m.p., degrees*[a]	*Formula*	*Calc.*			*Found*			ν_{max}^{KBr}	
					C	*H*	*N*	*C*	*H*	*N*	*C=N*	*OH*
D-Glucose	H	*p*-Br	251–255 (d.)	$C_{18}H_{17}BrN_4O_2$	53.8	4.2	14.0	53.4	4.1	13.6		
	p-Me	H	224–227 (d.)	$C_{19}H_{20}N_4O_2$	67.8	6.0	—	67.5	6.3	—		
	R = R'											
	4-Br-2-Me		204–206 (d.)	$C_{20}H_{20}Br_2N_4O_2$	47.2	3.9	—	46.9	4.0	—		
D-Galactose	*p*-Br		268–271 (d.)	$C_{18}H_{16}Br_2N_4O_2$	45.0	3.3	11.7	45.4	3.8	11.3		
L-Sorbose	*p*-Br		270–272 (d.)	$C_{18}H_{16}Br_2N_4O_2$	45.0	3.3	11.7	45.1	3.6	11.5	1595	3500
	o-Me		208–210[5]	$C_{20}H_{22}N_4O_2$	68.6	6.3	16.0	68.5	6.8	15.8	1595	3350
	p-Me		264–266[5]	$C_{20}H_{22}N_4O_2$	68.6	6.3	16.0	68.8	6.8	16.0	1615	3550

[a]The symbol d. indicates decomposition.

Similar treatment of D-*threo*-pentulose *p*-tolylosazone with boiling acetic anhydride yielded 5-(acetoxymethyl)-3-formyl-1-*p*-tolylpyrazole *N*-acetyl-*p*-tolylhydrazone, which, on hydrolysis, afforded 3-formyl-5-(hydroxymethyl)-1-*p*-tolylpyrazole *N*-acetyl-*p*-tolylhydrazone (4).



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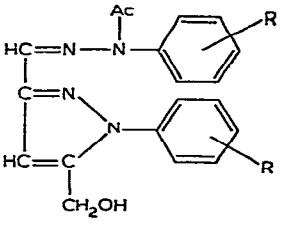

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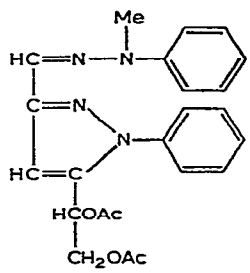
The *N*-acetylated derivatives (3 and 4) (see Table III) showed infrared spectra absorption characteristic of the *N*-acetyl group at 1690–1660 cm^{-1}, whereas the fully acetylated compound showed the ester band at 1740, the amide band at 1690, and the C=N band at 1610 cm^{-1}. Similar treatment, with acetic anhydride, of D-*arabino*-hexulose 1-(2-methyl-2-phenyl)-2-phenylosazone yielded 5-(D-*glycero*-1,2-diacetoxyethyl)-3-formyl-1-phenylpyrazole (2-methyl-2-phenyl)hydrazone (5), which is further proof that closure of the pyrazole ring involves the phenylhydrazone residue on C-2 of the osazone, and not that on C-1.

TABLE III

DIANHYDRO-OSAZONES OF THE PYRAZOLE TYPE (3 AND 4) AND THEIR *O*-ACETYL DERIVATIVES

Parent sugar	*R*	*m.p., degrees*	*Formula*	*Calc.*			*Found*			ν_{max}^{KBr}		
				C	*H*	*N*	*C*	*H*	*N*	*C=NNAc*		*OH*
D-Xylose	*p*-Me	129–131	$C_{21}H_{22}N_4O_2$	—	—	15.5			15.5	1610	1660	3450
D-Glucose	*o*-Me	164–166	$C_{22}H_{24}N_4O_3$	67.3	6.1	14.3	67.0	6.2	14.3	1610	1690	3400
	m-Me	173–175	$C_{22}H_{24}N_4O_3$	67.3	6.1	14.3	67.1	6.2	14.5	1610	1660	3400
	p-Me	185–188	$C_{22}H_{24}N_4O_3$	67.3	6.1	14.3	67.4	6.6	14.5	1610	1685	3420
	p-OMe	164–165	$C_{22}H_{24}N_4O_5$	62.3	5.5	13.2	62.1	5.4	13.4			
L-Sorbose	*p*-Me	186	$C_{22}H_{24}N_4O_3$	67.3	6.1	14.3	67.5	6.3	14.6	1610	1680	3400
***O*-Acetyl derivatives**												*ν*OAc
D-Xylose	*p*-Me	88–90	$C_{23}H_{24}N_4O_3$	68.3	5.9	13.9	68.5	6.2	14.3			
D-Glucose	*p*-Me	128–130	$C_{26}H_{28}N_4O_5$	65.5	5.9	11.8	65.5	5.9	11.6	1610	1690	1740
	p-I	166–168	$C_{20}H_{22}I_2N_4O_5$	41.1	3.1	8.0	41.1	3.4	7.8	1610	1690	1740



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EXPERIMENTAL

Infrared spectra were recorded on a Unicam SP-200 spectrophotometer, and ultraviolet spectra on a Unicam SP-800 spectrophotometer. Microanalyses were performed by A. Bernhardt, Mulheim, Germany.

Mixed osazones. — A solution of D-*arabino*-hexosulose 1-phenylhydrazone[3] (0.7 g), or 1-*p*-tolylhydrazone[4] (0.6 g) in ethanol (10 ml) was treated with (*p*-chlorophenyl)hydrazine (0.6 g), (*p*-bromophenyl)hydrazine (0.6 g), or (*p*-iodophenyl)-hydrazine (0.5 g) in ethanol. A few drops of acetic acid were added, and the mixture was warmed on a hot-water bath for 10 min, and cooled. The osazone obtained was collected, washed with dilute ethanol, and dried. The mixed osazones (see Table I) were recrystallized from dilute ethanol, giving yellow needles, soluble in methanol, ethanol, or acetone, and insoluble in water.

O-Acetyl derivatives of osazones. — A solution of the osazone (0.5 g) in pyridine (10 ml) was treated with acetic anhydride (10 ml), and the mixture was kept overnight at room temperature. It was then poured onto crushed ice, and the acetate obtained (see Table I) was filtered off, and recrystallized from dilute ethanol, to give yellow needles, soluble in methanol, ethanol, or ether, and insoluble in water.

Dianhydro-osazones of the Percival type. — A solution of the osazone acetate (0.3 g) in acetone (25 ml) was deacetylated with 1.5% aqueous sodium hydroxide (30 ml) overnight at room temperature. The dianhydro derivative that separated was filtered off, and recrystallized from ethanol, to give yellow needles, soluble in methanol, ethanol, or ether, and insoluble in water (see Table II).

Dianhydro-osazones of the pyrazole type. — A solution of the osazone (5 g) in acetic anhydride (50 ml) was refluxed for 2 h, and then poured onto crushed ice. After 24 h, the aqueous layer was decanted and discarded, and the residual oil was washed with water. This product was then hydrolyzed for 24 h at room temperature with ethanolic ammonia (20%, 30 ml). The solution was evaporated almost to dryness on a hot-water bath, whereupon the dianhydro-osazone of the pyrazole type (see Table III) separated. It was recrystallized from dilute ethanol, to give colorless plates, soluble in methanol, ethanol, or ether, and insoluble in water.

O-*Acetyl derivatives of the dianhydro-osazones of the pyrazole type.* — A solution of the dianhydro-osazone (0.4 g) in pyridine (10 ml) was treated with acetic anhydride (5 ml), and the mixture was kept overnight at room temperature. It was then poured onto crushed ice, and the *O*-acetyl derivative that separated was recrystallized from dilute ethanol, to give colorless plates, soluble in methanol, ethanol, or ether, and insoluble in water (see Table III).

5-(D-glycero-*1,2-Diacetoxyethyl)-3-formyl-1-phenylpyrazole (2-methyl-2-phenyl)-hydrazone.* — D-*arabino*-Hexulose 1-(2-methyl-2-phenyl)-2-phenylosazone (0.5 g) was refluxed with acetic anhydride (5 ml) for 30 min, and the mixture was poured onto crushed ice. The residue obtained (0.1 g) was washed, and recrystallized from ethanol, to give colorless, prismatic needles, m.p. 155–156°; soluble in methanol, ethanol, or chloroform, and insoluble in water.

Anal. Calc. for $C_{23}H_{24}N_4O_4$: C, 65.7; H, 5.7; N, 13.3. Found: C, 65.3; H, 6.0; N, 13.2.

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THE ACID HYDROLYSIS OF GLYCOSIDES

VIII. SYNTHESIS AND HYDROLYSIS OF THREE ALDOTRIOURONIC ACIDS

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ABSTRACT

2-*O*-(β-D-Glucopyranosyluronic acid)cellobiose, 6-*O*-(β-D-glucopyranosyluronic acid)cellobiose, and 6′-*O*-(β-D-glucopyranosyluronic acid)cellobiose have been synthesized and characterized, and kinetic data have been determined for the acid-catalyzed hydrolysis of the D-glucuronide and D-glucoside linkages in each compound. The D-glucosidic bonds are all hydrolyzed at a rate lower than that for cellobiose, the stabilizing effect of the D-glucuronic acid residue being most pronounced in the last two compounds. The results are discussed with reference to recent theories concerning the acid hydrolysis of acidic polysaccharides.

INTRODUCTION

Previous studies[1] in the present series have dealt with factors influencing the acid-catalyzed hydrolysis of the glycoside (glycuronide) bonds of glycosiduronic acids. Many polysaccharides carry residues of hexuronic acid attached directly to their main chain. For some time, the effect of the uronic acid residue on the adjacent glycosidic bonds in the polymer backbone has been a moot question. Various predictions have been made as to the nature and magnitude of this effect[2], and attempts have been made to estimate it qualitatively[3,4]. The object of the present investigation was to adduce quantitative information on this point. The first approach involved synthesis of three aldotriouronic acids, and a study of their rate of hydrolysis.

RESULTS

The three aldotriouronic acids were 2-*O*-(β-D-glucopyranosyluronic acid)-cellobiose (**1**), 6-*O*-(β-D-glucopyranosyluronic acid)cellobiose (**2**), and 6′-*O*-(β-D-glucopyranosyluronic acid)cellobiose (**3**). Compound **1** was synthesized by condensation of 1,3,6,2′,3′,4′,6′-hepta-*O*-acetyl-α-cellobiose[5] with methyl 2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy-α-D-glucopyranuronate[6] in the presence of active silver carbonate and silver perchlorate[7]. The resulting, crystalline compound (10% yield), after

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deacetylation and saponification, afforded **1** as a chromatographically pure syrup. Permethylated **1**, on methanolysis, gave the expected *O*-methylated compounds, both before and after reduction of the carboxyl group. Partial, acid hydrolysis yielded 2-*O*-(β-D-glucopyranosyluronic acid)-D-glucose (**4**).

1,2,3,2′,3′,4′,6′-Hepta-*O*-acetyl-β-cellobiose[8] was condensed with methyl 2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy-α-D-glucopyranuronate to give a syrupy compound (40%) which was characterized *via* the crystalline 1,2,3,2′,3′,4′,6′-hepta-*O*-acetyl-6-*O*-[methyl (2,3,4-tri-*O*-acetyl-β-D-glucopyranosyl)uronate]-β-cellobiose (**5**). Deacetylation and saponification afforded chromatographically pure **2**, which was identified in the same way as for **1**. Partial, acid hydrolysis gave 6-*O*-(β-D-glucopyranosyluronic acid)-D-glucose (**6**).

Compound **3** was prepared by condensation of 2,3,2′,3′,4′-penta-*O*-acetyl-1,6-anhydrocellobiose[9] with methyl 2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy-α-D-glucopyranuronate (85%), followed by deacetylation, saponification, and hydrolysis of the crystalline intermediate. The chromatographically pure syrup was characterized as for the compounds already discussed.

Hydrolysis was effected in 0.5M sulfuric acid, as described previously[10]. In compounds **1**, **2**, and **3**, the glucuronide bond is much more stable towards acid than is the glucoside linkage. Since formation of cellobiose was not observed in the course of the hydrolyses, it may be assumed that the aldotriouronic acids are hydrolyzed largely to aldobiouronic acid and D-glucose, and to only a minor extent to D-glucuronic acid and D-glucose. A minor proportion of the aldobiouronic acid is also hydrolyzed, presumably at the same rate as for the D-glucuronide bond in the aldotriouronic acid. If α_1 is the optical rotation of the reaction mixture at any instant, and $(\alpha_2 - \alpha_2^\circ)$ is the change in optical rotation caused by hydrolysis of the aldobiouronic acid, $\alpha = [\alpha_1 - (\alpha_2 - \alpha_2^\circ)]$ is the corrected value of the optical rotation attained on hydrolysis of the aldotriouronic acid to the aldobiouronic acid and D-glucose. The rate of hydrolysis of the D-glucosidic bond was, accordingly, determined by following polarimetrically the hydrolysis of the aldotriouronic and corresponding aldobiouronic acid, and correcting for the contribution of the latter, as shown in Fig. 1. The values

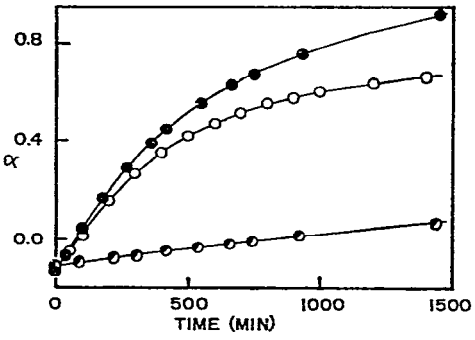


Fig. 1. Acid hydrolysis at 80°. Optical rotation (α) as a function of time.
[—●—, 2-*O*-(β-D-Glucopyranosyluronic acid)cellobiose (uncorrected plot);
—○—, 2-*O*-(β-D-glucopyranosyluronic acid)cellobiose (corrected plot); and
—◑—, 2-*O*-(β-D-glucopyranosyluronic acid)-D-glucose.]

thus obtained, when plotted as shown in Fig. 2, gave linear relationships throughout, as required for a pseudo-first-order reaction. Kinetic data for the di- and tri-saccharides

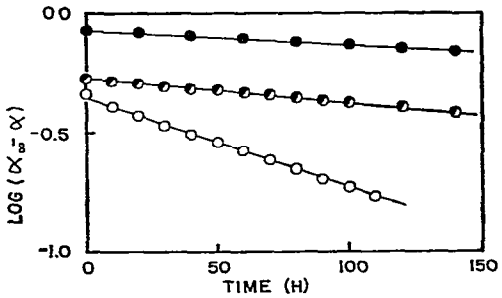


Fig. 2. Hydrolysis of aldotriouronic acids at 60°.
[—O—, 2-*O*-(β-D-Glucopyranosyluronic acid)cellobiose;
—◑—, 6-*O*-(β-D-glucopyranosyluronic acid)cellobiose; and
—●—, 6′-*O*-(β-D-glucopyranosyluronic acid)cellobiose.]

are summarized in Table I. Application of the Guggenheim method resulted in rate coefficients very close to the above values.

TABLE I

KINETIC DATA FOR THE HYDROLYSIS OF DI- AND TRI-SACCHARIDES IN 0.5M SULFURIC ACID

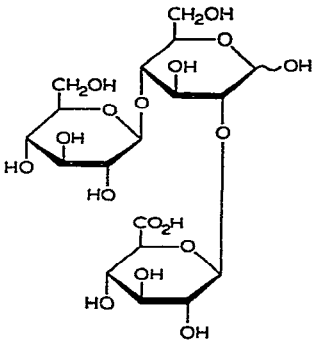
Compound	k × 10^6, sec^{-1}			E, *kcal* $mole^{-1}$	$\Delta S^{\ddagger}$, *at 60°, cal* deg^{-1} $mole^{-1}$
	60°	*70°*	*80°*		
Disaccharides					
Cellobiose[10]	2.72	9.63	39.5	31.5	+9.0
2-*O*-(β-D-Glucopyranosyluronic acid)-D-glucose (4)	0.12	0.52	1.98	32.2	+5.1
6-*O*-(β-D-Glucopyranosyluronic acid)-D-glucose (6)	0.25	0.69	2.57	31.3	+3.7
Trisaccharides (glucosidic bond)					
2-*O*(β-D-Glucopyranosyluronic acid)cellobiose (1)	2.38	8.64	34.4	32.7	+12.3
6-*O*-(β-D-Glucopyranosyluronic acid)cellobiose (2)	0.63	4.45	15.4	37.4	+24.1
6′-*O*-(β-D-Glucopyranosyluronic acid)cellobiose (3)	0.43	2.23	7.54	33.7	+11.8

DISCUSSION

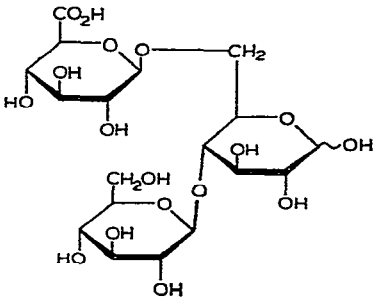
Cellobiose is hydrolyzed 23 times as fast as **4**, and 11 times as fast as **6**. It had previously been found[11] that (1 → 6)-linked aldobiouronic acids are hydrolyzed more rapidly than those linked (1 → 2) or (1 → 4).

The D-glucosidic linkages in the three aldotriouronic acids are all more stable towards acid than is the linkage in cellobiose. For **1**, the difference is slight, but for **2** and **3**, the bond is 3–4 and 5–6 times as stable, respectively. The lower rate of hydrolysis of **2**, as compared to that of **1**, is probably attributable to greater steric

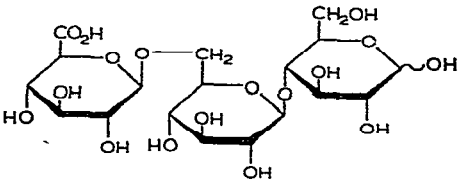
hindrance to protonation at the D-glucosidic oxygen atom in **2**. From the results of McKee and Dickey[3], it is evident that the carboxyl group in the D-glucuronic acid residue is not involved in the case of compound **3**. Instead, these investigators suggested



1



2



3

the operation of a conformational effect[12]. Bearce[4], who adduced indirect evidence for the stabilization of glycosidic linkages, such as those in **1** and **2**, proposed that the D-glucuronic acid residue interferes with the protonation of the glycosidic oxygen atom.

It seems likely that steric and conformational factors are both responsible for the stabilization of the D-glucosidic bonds noted here. Polar effects are, however, probably also involved, as indicated by the recent results by Höök and Lindberg, who found that *O*-alkylated methyl D-glucopyranosides are rendered more labile towards acid by *O*-isopropyl[13], but are stabilized by *O*-(2-hydroxyethyl)[14], substituents. In agreement with the present data, Klemer[15] found that the (1 → 4)- and (1 → 6)-linkages in 6-*O*-*β*-D-glucopyranosylmaltose are both hydrolyzed at somewhat lower rates than are corresponding bonds in maltose and gentiobiose.

Marchessault and Rånby[2,16,17] have claimed that, in polysaccharides having the general structure

$$\rightarrow A \rightarrow B \rightarrow C \rightarrow D \rightarrow E \rightarrow$$
$$\uparrow$$
$$GA$$

where A to E represent neutral sugar residues and GA is a residue of D-glucuronic acid, linkage C → D should be stabilized towards acids, whereas linkage B → C should be destabilized, by the D-glucuronic acid residue. The present results offer direct evidence for the non-existence of any such[2] "activating inductive effect"; on

the contrary, the B → C bond, is stabilized by the acid side chain. This conclusion is in agreement with theoretical considerations of other investigators[3,4,9,10].

Recently, Feather and Harris[18] have shown that the bond at the nonreducing end of cellotriose is hydrolyzed 1.5 times as fast as that at the reducing end. Interestingly, compound **2** in the present study is also hydrolyzed 1.5 times as fast as **3**. Of course, the reason for this behavior is, the same in both cases—namely, the fact that, for compound **2**, the D-glucose residue undergoing hydrolysis is unsubstituted, whereas, in compound **3**, it carries a bulky substituent that impedes the transition from a chair to a half-chair conformation[12].

EXPERIMENTAL

Melting points are corrected. Thin-layer chromatography (t.l.c.) was performed with Silicagel H (E. Merck) with 9:1 (v/v) benzene–methanol; R_F values refer to α-cellobiose octaacetate. Paper chromatography (p.c.) was conducted with 18:7:8 (v/v) ethyl acetate–acetic acid–water; R_F values refer to cellobiose. Gas–liquid chromatography (g.l.c.) was performed with an F and M Model 810 instrument, with 10% diethylene glycol succinate (column A) or 10% Apiezon M (column B) on Chromosorb W. Kinetic measurements were made as described previously[1].

2-O-(-β-D-Glucopyranosyluronic acid)cellobiose (**1**). — α-Cellobiose octaacetate (100 g) was converted in the usual way[18] into 2,3,6,2′,3′,4′,6′-hepta-*O*-acetyl-α-cellobiosyl bromide (88 g, 83%), m.p. 184–185°, $[\alpha]_D^{25}$ +93° (*c* 2.0, chloroform); lit.[18] m.p. 185°, $[\alpha]_D$ +94°. A portion of this bromide (50 g) was added to tetrahydrofuran (500 ml) containing water (1.2 ml). Active silver nitrate (17.5 g) was added, and the mixture was boiled. After 5 min, calcium carbonate (20 g) was added, and boiling was continued for 30 min. The suspension was filtered, and the filtrate was poured into ethyl ether (1500 ml), giving a precipitate which was removed by filtration. The filtrate was concentrated to a syrup which was dissolved in ethyl ether (1200 ml); crystals (16 g) formed immediately. The crude product was recrystallized from 4:1 (v/v) ethanol–ether, giving needles (6.1 g, 13.4%) of 1,3,6,2′,3′,4′,6′-hepta-*O*-acetyl-α-cellobiose[5], m.p. 178–180°, $[\alpha]_D^{25}$ +66° (*c* 2.0, chloroform), $R_{T.L.C.}$ 0.39; lit.[5], m.p. 178–180°, $[\alpha]_D$ +67.6°.

Anal. Calc. for $C_{26}H_{36}O_{18}$: C, 49.1; H, 5.70. Found: C, 49.1; H, 6.10.

The compound (10 g) was dissolved in ethanol-free, dry chloroform (50 ml), active silver carbonate (10 g) and anhydrous calcium sulfate (15 g) were added, and the mixture was stirred for 2 h. Iodine (0.5 g) and silver perchlorate[7] (0.5 g) were introduced, and then a solution of methyl 2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy-α-D-glucopyranuronate[6] (7.5 g) [m.p. 106–107°, $[\alpha]_D^{25}$ +196° (*c* 2.0, chloroform)], in chloroform (20 ml) was added during 16 h. After 12 h, silver carbonate (5 g), anhydrous calcium sulfate (5 g), and iodine (0.5 g) were added, and a solution of the bromide (3 g) in chloroform (20 ml) was introduced during 8 h. The condensation was repeated with the same amounts of reagents. The filtrate was evaporated to dryness, and the residue was dissolved in anhydrous ethanol (100 ml), giving needles (1.2 g) which were recrystallized twice from methanol to yield 1,3,6,2′,3′,4′,6′-hepta-*O*-acetyl-

6-*O*-[methyl (2,3,4-tri-*O*-acetyl-β-D-glucopyranosyl)uronate]-α-cellobiose (0.7 g), m.p. 272–276°, $[\alpha]_D^{25}$ +11.4° (*c* 2.0, chloroform), $R_{T.L.C.}$ 0.64.

Anal. Calc. for $C_{39}H_{52}O_{24}$: C, 49.15; H, 5.46. Found: C, 49.07; H, 5.44.

No condensation occurred in the absence of silver perchlorate. Deacetylation and saponification gave **1** in an overall yield of 10%, $[\alpha]_D^{25}$ −3.2° (*c* 2.0, water).

Anal. Calc. for $C_{18}H_{30}O_{17}$: equiv. wt., 518. Found, equiv. wt., 526.

On prolonged hydrolysis, compound **1** afforded D-glucose and a small proportion of D-glucuronic acid. After methanolysis, the fully methylated product gave peaks for the anomers of methyl 2,3,4,6-tetra-*O*-methyl-D-glucopyranoside and methyl (methyl 2,3,4-tri-*O*-methyl-D-glucopyranosid)uronate on g.l.c. (column A). After reduction with lithium aluminum hydride and further methanolysis, peaks were obtained for the anomers of methyl 3,6-di-, 2,3,4-tri-, and 2,3,4,6-tetra-*O*-methyl-D-glucopyranoside (column B).

Partial, acid hydrolysis of **1** yielded pure 2-*O*-(β-D-glucopyranosyluronic acid)-D-glucose, $[\alpha]_D^{25}$ −1.6° (*c* 2.0, water), which, on further hydrolysis, afforded D-glucose and D-glucuronic acid.

6-O-(β-D-Glucopyranosyluronic acid)cellobiose (**2**). — 1,2,3,2′,3′,4′,6′-Hepta-*O*-acetyl-β-cellobiose[8] [m.p. 198–200°, $[\alpha]_D^{25}$ −20.4° (*c* 2.0, chloroform)] (8 g) was condensed with methyl 2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy-α-D-glucopyranuronate (12 g) as described for the corresponding derivative of **1**. Silver perchlorate had no effect on the yield. After deacetylation and saponification, the reaction mixture contained equal amounts of cellobiose and a trisaccharide. The trisaccharide was isolated by preparative, paper chromatography; yield 2.3 g (34%). After further purification with active carbon, compound **2** (1.7 g) had $[\alpha]_D^{25}$ +3.3° (*c* 2.0, water).

Anal. Calc. for $C_{18}H_{30}O_{17}$: equiv. wt., 518. Found: equiv. wt., 539.

After permethylation, methanolysis, reduction, and renewed methanolysis, compound **2** gave the 2,3-di-, 2,3,4-tri-, and 2,3,4,6-tetra-methyl ethers of methyl α,β-D-glucopyranoside. Partial, acid hydrolysis yielded D-glucose and 6-*O*-(β-D-glucopyranosyluronic acid)-D-glucose which, on further hydrolysis, afforded D-glucose and D-glucuronic acid.

Compound **2** (200 mg) was dissolved in water (10 ml), and methanol (60 ml) was added. A slight excess of diazomethane was added at −77°, the solvents were removed by evaporation, and the resulting methyl ester was boiled for 5 h with acetic anhydride (10 ml) in the presence of anhydrous sodium acetate (1 g). The mixture was poured into ice–water (150 ml), and the mixture was extracted with three 50-ml portions of chloroform. The extracts were combined, dried, and evaporated, and the product was crystallized twice from methanol, giving 105 mg (31%) of **4**, m.p. 217–220°, $[\alpha]_D^{25}$ −2.5° (*c* 2.0, chloroform).

Anal. Calc. for $C_{39}H_{52}O_{24}$: C, 49.15; H, 5.46. Found C, 49.60; H, 5.44.

6′-O-(β-D-Glucopyranosyluronic acid)cellobiose (**3**). — 2,3,2′,3′,4′-Penta-*O*-acetyl-1,6-anhydrocellobiose, [m.p. 156–157°, $[\alpha]_D^{25}$ −59° (*c* 2.0, chloroform)] was prepared according to Lindberg and Selleby[9].

Anal. Calc. for $C_{22}H_{30}O_{15}$: C, 49.40; H, 5.66. Found: C, 49.37; H, 5.63.

This compound (10 g) was condensed, as already described, with methyl 2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy-α-D-glucopyranuronate (15 g), to give 2,3,2′,3′,4′-penta-*O*-acetyl-1,6-anhydro-6′-*O*-(methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosyluronate)cellobiose (12.5 g, 85%), which was recrystallized twice from methanol, m.p. 210–211°, $[\alpha]_D^{25}$ −54.2° (*c* 2.0, chloroform), $R_{T.L.C.}$ 0.75.

Anal. Calc. for $C_{35}H_{46}O_{24}$: C, 49.90; H, 5.42. Found: C, 49.15; H, 5.57.

After deacetylation and saponification, the resulting 1,6-anhydro-6′-*O*-(β-D-glucopyranosyluronic acid)cellobiose was boiled under reflux with 0.5M sulfuric acid (25 ml) for 5.5 h. The reaction product was resolved into two components by preparative, paper chromatography. One component ($R_{P.C.}$ 0.84) (160 mg) was 6-*O*-(β-D-glucopyranosyluronic acid)D-glucose and the other ($R_{P.C.}$ 0.60) (330 mg, 45%) was compound **3**, $[\alpha]_D^{25}$ −4.8° (*c* 2.0, water).

Anal. Calc. for $C_{18}H_{30}O_{17}$: equiv. wt., 518. Found: equiv. wt., 530.

On partial, acid hydrolysis, compound **3** afforded D-glucose, 6-*O*-(β-D-glucopyranosyluronic acid)-D-glucose, and a small proportion of D-glucuronic acid. On methanolysis, permethylated **3** gave the anomers of methyl (methyl 2,3,4-tri-*O*-methyl-D-glucopyranosid)uronate and methyl 2,3,4,6-tetra-*O*-methyl-D-glucopyranoside. Reduction and further methanolysis yielded the anomers of methyl 2,3,4-tri-*O*-methyl-D-glucopyranoside and methyl 2,3,6-tri-*O*-methyl-D-glucopyranoside.

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THE ACID HYDROLYSIS OF GLYCOSIDES

IX. HYDROLYSIS OF TWO ALDOTRIOURONIC ACIDS DERIVED FROM A (4-*O*-METHYLGLUCURONO)XYLAN

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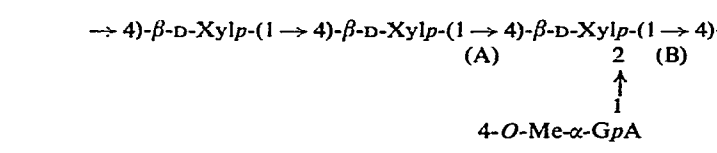
ABSTRACT

Kinetic data have been determined for the acid-catalyzed hydrolysis of the xylosidic bond in 2-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)xylobiose, 2′-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)xylobiose, and 2′-*O*-(4-*O*-methyl-α-D-glucopyranosyl)xylobiose. Xylobiose is hydrolyzed 1.2, 7.0, and 3.4 times as fast as the above compounds, respectively, indicating the stabilizing effect of the 4-*O*-methyl-D-glucuronic acid residue. The lower rate of hydrolysis of the xylosidic bond in the trisaccharides is attributed to conformational and steric effects. There is no evidence for the existence of any "activating inductive effect", postulated by earlier investigators.

INTRODUCTION

The influence of the acid side-chains on the acid-catalyzed hydrolysis of (4-*O*-methylglucurono)xylans has been the subject of considerable controversy in recent years. Marchessault and Rånby[1] have suggested that, in such xylans, linkage A is destabilized towards acids by an "activating inductive effect", whereas linkage B is



subjected to a "stabilizing inductive effect", both effects being exerted by the carboxyl group in the 4-*O*-methyl-D-glucuronic acid residue. Some investigators have supported this theory[2] or have suggested similar ones[3]. In contrast, the existence of an "activating inductive effect" has been doubted by other investigators[4,5], and has been conclusively shown to be of no consequence in the acid hydrolysis of pseudoaldobiouronic acid[6] and two aldotriouronic acids[7]. It was the objective of the present investigation to acquire additional evidence on this point by establishing the kinetic

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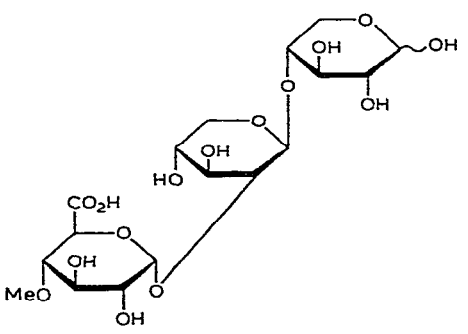
parameters for the hydrolysis of linkages A and B in a (4-*O*-methylglucurono)xylan, and also to evaluate the effect of the carboxyl group attached to C-5.

RESULTS

On alkaline extraction, delignified aspen wood gave a pure (4-*O*-methylglucurono)xylan in a yield of 18.3%. When this compound was subjected to partial, acid hydrolysis with 45% formic acid, 2-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-xylose (**1**), a mixture of two aldotriouronic acids, and a mixture of aldotetraouronic acids were obtained in yields of 4.5, 7.6, and 4.0% respectively; these yields are considerably higher than those reported by previous investigators, who used mineral acids, which are non-swelling.

The two aldotriouronic acids could not be separated from each other by paper chromatography or by paper electrophoresis[5]. A portion of the mixture of these two acids was permethylated, and the product subjected to methanolysis, giving a neutral and an acid fraction. The former consisted of the methyl glycosides of 2,3-di- and 2,3,4-tri-*O*-methyl-D-xylose in the molar ratio of 28.9:1, as estimated by gas–liquid chromatography (g.l.c.). The acid fraction contained two partly methylated aldobiouronic acids, which were reduced and subjected to methanolysis, affording the methyl glycosides of (*a*) 3-*O*-methyl-D-xylose, 3,4-di-*O*-methyl-D-xylose, and (*b*) 2,3,4-tri-*O*-methyl-D-glucose. The ratio (*b*-*a*)/*b* was 30.3. These results show that the aldotriouronic acid mixture consisted of 2′-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)xylobiose (**2**), and 2-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)xylobiose (**3**) in the ratio of approximately 30:1. The possible occurrence of both aldotriouronic acids in a partial hydrolyzate of acidic xylans has been mentioned[8] previously, but never proved. The fact that compound **3** constituted only a minor proportion of the mixture suggests that linkage A is less stable towards acids than is B.

Repeated recrystallization removed most of compound **2** from an aqueous solution of the mixture of **2** and **3**, leaving a residue which could not be further resolved by recrystallization, and which contained **2** and **3** in the ratio of 0.87:1. Attempts to isolate compound **3** in the pure state were unsuccessful.



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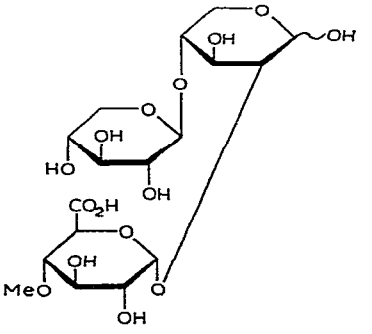

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Crystalline **2** was permethylated, and the product was subjected to methanolysis; methyl 2,3-di-*O*-methyl-D-xyloside was the only neutral glycoside formed. The physical properties of **2** were those observed by previous investigators[8,9] for the same compound. Reduction with diborane of the peracetylated aldotriouronic acid gave 2-*O*-(4-*O*-methyl-α-D-glucopyranosyl)xylobiose[5] (**4**). Compound **1** was similarly reduced to 2-*O*-(4-*O*-methyl-α-D-glucopyranosyl)-D-xylose[10] (**5**).

The acid-catalyzed hydrolysis of **1**, **2**, **4**, and **5** was followed polarimetrically, and the rate of hydrolysis of the xylosidic bond in **2** and **4** was calculated as described previously[7]. Hydrolysis curves are shown in Figs. 1 and 2. The rate of hydrolysis of

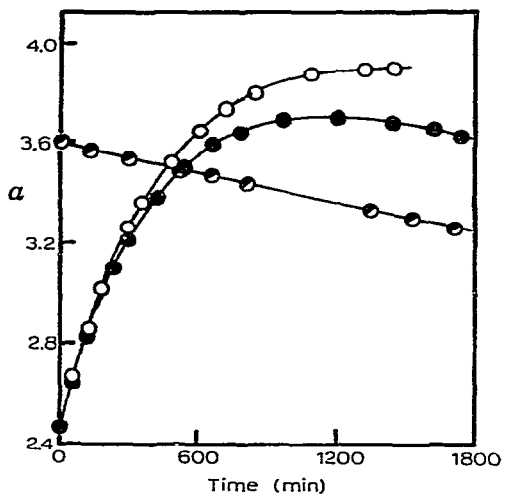

Fig. 1. Acid hydrolysis at 80°; optical rotation (α) as a function of time. [–●–, 2′-*O*-(4-*O*-Methyl-α-D-glucopyranosyluronic acid)xylobiose (**2**) (uncorrected plot); –o–, 2′-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)xylobiose (**2**) (corrected plot); –◑–, 2-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-xylose (**1**).]

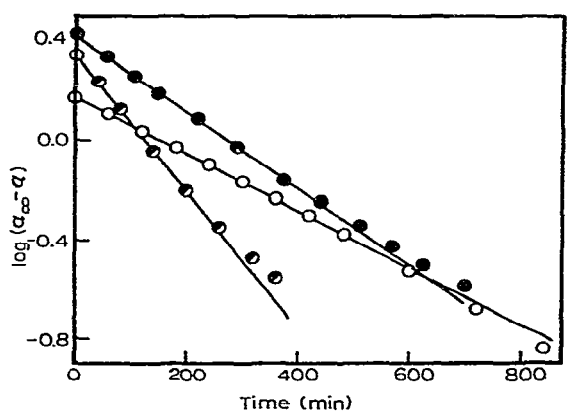

Fig. 2. Hydrolysis of three compounds at 80°. [–o–, 2′-*O*-(4-*O*-Methyl-α-D-glucopyranosyluronic acid)xylobiose (**2**); –●–, 2-*O*-(4-*O*-methyl-α-D-glucopyranosyl)-D-xylose (**5**); –◑–, 2′-*O*-(4-*O*-methyl-α-D-glucopyranosyl)xylobiose (**4**).]

the xylosidic bond in **3** was determined from the hydrolysis of the 0.87:1 mixture of **2** and **3**, by use of the method of Brown and Fletcher[11] for two parallel, first-order reactions producing common products. The curve for log $(\alpha_\infty - \alpha)$ *versus* time for the mixture became linear after 40 h at 60°, indicating that compound **3** had been completely hydrolyzed at that time. When the known, extrapolated value of $(\alpha_\infty - \alpha)$ for **2** was subtracted from that observed for the mixture, $(\alpha_\infty - \alpha)$ for the pseudo-first-order hydrolysis of **3** was obtained. The kinetic data are summarized in Table I.

TABLE I

KINETIC DATA FOR THE HYDROLYSIS OF THE XYLOSIDIC BOND IN XYLOBIOSE AND IN THREE TRISACCHARIDES

Compound	$k \times 10^6$, sec^{-1}			E, *kcal. mole*$^{-1}$	$\Delta S^\ddagger$, *at 60° cal.deg.*$^{-1}$ *mole.*^{1}
	60°	*70°*	*80°*		
Xylobiose[4]	19.0	69.8	274	32.7	+16.5
2′-*O*-(4-*O*-Methyl-α-D-glucopyranosyluronic acid)xylobiose (**2**)	2.71	10.1	43.4	32.4	+11.8
2-*O*-(4-*O*-Methyl-α-D-glucopyranosyluronic acid)xylobiose (**3**)	15.4				
2′-*O*-(4-*O*-Methyl-α-D-glucopyranosyl)-xylobiose (**4**)	5.6	27.9	105	34.3	+19.0

DISCUSSION

Xylobiose is hydrolyzed 7.0 times as fast as the xylosidic bond of **2**, 1.2 times as fast as that of **3**, and 3.4 times as fast as that of **4**. Substitution at C-6′ in cellobiose had previously been found[7] to stabilize the D-glucosidic bond to approximately the same extent (6.4 times) as in **2**. In both cases, the bulky substituent would hinder the chair to half-chair transformation[12]. In addition, the protonation of the xylosidic oxygen atom in **2** is probably impeded by the 4-*O*-methyl-D-glucuronic acid residue. Comparison of the data for **2** and **4** shows that the carboxyl group also lowers the rate of hydrolysis of the xylosidic bond, an effect that probably involves the polarity of the carboxyl group. McKee and Dickey[5] have postulated the operation of a conformational effect only, but this is evidently not the only factor involved.

The reason why the xylosidic bond in compound **3** is slightly more stable than in xylobiose is probably that the bulky 4-*O*-methyl-D-glucuronic acid residue at C-2 offers hindrance to the protonation of the xylosidic bond. A D-glucuronic acid residue attached to C-6 in cellobiose had previously been found[7] to render the D-glucosidic bond 4 times as stable as that in cellobiose. This difference can probably be attributed to the greater proximity and steric availability of the substituent on C-6 as compared to that on C-2.

It is evident that the 4-*O*-methyl-D-glucuronic acid side-chain in acidic xylans stabilizes linkage A to some extent, and linkage B to a considerable extent. A destab-

ilization of A has not been observed, and the "activating inductive effect" proposed by Marchessault and Rånby[1] has neither experimental nor theoretical[5,6] foundations. This conclusion is not unexpected, because this alleged effect does not take cognizance of the fact that the hydrolysis is influenced, not only by the rate of heterolysis, but also by the extent of protonation of the glycosidic oxygen atom.

EXPERIMENTAL

General experimental conditions and kinetic measurements were those given in the previous paper[7]. R_{Xyl} refers to the rate of movement on the paper chromatogram relative to D-xylose, with 18:7:8 ethyl acetate–acetic acid–water as the developer.

Isolation and partial, acid hydrolysis of (4-O-methylglucurono)xylan. — Extractive-free sawdust of aspen (*Populus tremuloides*) was delignified with chlorous acid. The resulting holocellulose (73% yield) was exhaustively extracted with 24% (w/w) aqueous, potassium hydroxide, giving pure (4-*O*-methylglucurono)xylan in a yield of 18.3% (based on the wood). The polysaccharide (80 g) was stirred for 30 min at room temperature with 90% formic acid (800 ml), water (800 ml) was added, the mixture was stirred for 255 min at 100°, and cooled, and formic acid was removed by repeated addition and evaporation of water. The aqueous solution was treated with Amberlite IR-120 (H^+) ion-exchange resin (500 ml), and the suspension was filtered through a bed of Celite and Darco G-60 carbon. The filtrate and washings were combined, concentrated to 500 ml, and added to the top of a column (5 × 55 cm), containing Dowex-1 X4 (OAc^-) ion-exchange resin. The neutral sugars were eluted with water, and the acid sugars were removed with 30% aqueous acetic acid (5 liters), giving 18.5 g of an acid fraction (23%).

The mixture of sugar acids was partly resolved by preparative paper-chromatography, affording an aldobiouronic acid (**1**) (3.6 g, 4.5%, R_{Xyl} 1.02), aldotriouronic acids (6.1 g, 7.6%, R_{Xyl} 0.75), and aldotetraouronic acids (3.2 g, 4.0%, R_{Xyl} 0.52).

Characterization of the aldotriouronic acid **2**. — Compound **2** (5.0 g) was recrystallized three times from water, giving large, needle-shaped crystals (2.6 g), m.p. 180–183° (dec.). An anhydrous preparation had $[\alpha]_D$ +53.0° (*c* 2.0, water).

Anal. Calc. for $C_{17}H_{28}O_{15}$: OCH_3, 6.56; equiv. wt., 472. Found: OCH_3, 6.53; equiv. wt., 478.

Partial, acid hydrolysis gave D-xylose and 2-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-xylose.

Compound **2** (100 mg) was permethylated by the methods of Haworth[13] and Kuhn, and co-workers[14], to yield a product (60 mg, 49%) that did not show any hydroxyl band in its i.r. spectrum. G.l.c. (column A) of the methanolyzate showed the presence of only methyl 2,3-di-*O*-methyl-D-xylosides in the neutral fraction.

Analysis of the aldotriouronic acid mixture. — (*a*) The mixture of aldotriouronic acids obtained by paper chromatography (1.6 g) was permethylated, giving a product (1.4 g, 71%) that was boiled for 11 h under reflux with 0.9M methanolic hydrogen chloride. G.l.c. showed the presence of methyl 2,3-di-*O*-methyl-D-xylosides and methyl

2,3,4-tri-*O*-methyl-D-xylosides in the ratio of 29:1. Traces of methyl 3,4-di-*O*-methyl-D-xylosides were also noted. After saponification and acidification, the acid part of the methanolyzate was recovered with an ion-exchange resin, and esterified, the ester was reduced with lithium aluminum hydride, and the product subjected to methanolysis, followed by trimethylsilylation in the usual way. G.l.c. (3% SE 52 on Chromosorb W) indicated the presence of trimethylsilyl ethers of methyl 3-*O*-methyl-D-xylose, methyl 3,4-di-*O*-methyl-D-xylose, and methyl 2,3,4-tri-*O*-methyl-D-glucose.

(*b*) The mother liquor remaining after most of **2** had been removed from a mixture of **2** and **3** (by recrystallization) was permethylated, and the product methanolyzed. G.l.c. (column A) showed the presence of methyl 2,3-di-*O*-methyl-D-xylosides and methyl 2,3,4-tri-*O*-methyl-D-xylosides in the molar ratio of 47:53.

2'-O-(*4*-O-*Methyl*-α-D-*glucopyranosyl*)*xylobiose* (**4**). — Compound **2** (1.0 g) was boiled for 4 h under reflux with acetic anhydride (20 ml) and sodium acetate (1.3 g). The peracetylated product (1.6 g) was dissolved in tetrahydrofuran (150 ml), and reduced with diborane as described previously[10]. Deacetylation of the product gave a neutral trisaccharide (**4**) (0.54 g, 56%), $[\alpha]_D$ +49° (*c* 2.0, water), R_{Xyl} 0.23.

Anal. Calc. for $C_{17}H_{30}O_{16}$: OCH_3, 6.78. Found: OCH_3, 6.25.

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Notes

The acid hydrolysis of glycosides
X. Hydrolysis of 2-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-xylose and related disaccharides

The great resistance to hydrolysis of the 4-*O*-methyl-D-glucuronic acid residues in (4-*O*-methylglucurono)xylans is well known. It has been found that, under conditions of hydrolysis that cause cleavage of all xylopyranosidic bonds, two-thirds of the 4-*O*-methyl-D-glucopyranuronide linkages remain intact[1,2]. Several investigators have tried to determine the rate of hydrolysis of the acid side-chains in various xylans[3,4], but the results do not agree, even after application of correction factors[5]. Whistler and Richards[3] found that 2-*O*-(4-*O*-methyl-α-D-glucopyranosyl)-D-xylitol is hydrolyzed at the same rate as maltose, and 18 to 19 times as fast as 2-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-xylose. The rate coefficients, however, decreased notably during the course of the hydrolysis. It was the object of the present investigation to adduce more-exact information on this point.

RESULTS AND DISCUSSION

2-*O*-(4-*O*-Methyl-α-D-glucopyranosyluronic acid)-D-xylose (**1**) was obtained by partial, acid hydrolysis of an aspen (4-*O*-methylglucurono)xylan, as described previously[9]. Reduction of peracetylated **1** with diborane afforded[6] 2-*O*-(4-*O*-methyl-α-D-glucopyranosyl)-D-xylose (**2**). When treated with borohydride, compound **1** gave 2-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-xylitol (**3**). Kinetic data for the three disaccharides were determined as described earlier[7-9]. The results are presented in Table I, together with values for maltose[7] and for 2-*O*-(β-D-glucopyranosyluronic acid)-D-glucose[8] (**4**).

TABLE I

KINETIC DATA FOR THE HYDROLYSIS OF DISACCHARIDES

Compound	$k \times 10^6$, *sec*$^{-1}$			E, *kcal. mole*$^{-1}$	$\Delta S^{\ddagger}$ *at 60° cal. deg.*$^{-1}$ *mole*$^{-1}$
	60°	*70°*	*80°*		
2-*O*-(4-*O*-Methyl-α-D-glucopyranosyluronic acid)-D-xylose (1)	0.062	0.275	1.22	32.4	+ 4.6
2-*O*-(4-*O*-Methyl-α-D-glucopyranosyl)-D-xylose (2)	3.62	14.1	59.5	33.0	+14.0
2-*O*-(4-*O*-Methyl-α-D-glucopyranosyluronic acid)-D-xylitol (3)			6.95		
Xylobiose[7]	19.0	69.8	274	32.7	+16.5
Maltose[7]	5.56	23.6	91.0	32.7	+14.0
2-*O*-(β-D-Glucopyranosyluronic acid)-D-glucose[8] (4)	0.12	0.52	1.98	32.2	+ 5.1

Despite its α-D-glycosidic bond, compound **1** is hydrolyzed half as fast as **4**, possibly owing to its C-4 methoxyl group. The pronounced influence of the carboxyl group on the rate of hydrolysis is shown by the fact that compound **2** is hydrolyzed 60 times as fast as **1**. The largest ratio so far reported between the rates of hydrolysis of an aldobiouronic acid and the corresponding, neutral disaccharide is[10] 37, recorded for cellobiouronic acid and cellobiose[11]. Maltose is hydrolyzed 90 times as fast as **1**. The factor of 19 previously reported[3] is probably incorrect.

Compound **3** is hydrolyzed almost 6 times as fast as **1**, presumably because of the greater mobility of the acyclic xylitol end-group present in **3**. Both isomaltose[12] and maltose[13], on the other hand, have been reported to be hydrolyzed at a somewhat lower rate than isomaltitol and maltitol, respectively. Obviously, care has to be exercised when comparison is made between rates of hydrolysis of aldose residues and those of alditol residues, a fact not always realized by previous investigators[3].

Xylobiose is hydrolyzed 250 to 300 times as fast as compound **1**. Even when allowance is made for the fact that the two linkages on either side of the branch point are somewhat more stable[9], the overall difference in rate of hydrolysis of the glucuronide and the xyloside linkages in (4-*O*-methylglucurono)xylans is very large, thus confirming previous, qualitative observations[1,2].

EXPERIMENTAL

General experimental conditions were the same as in previous investigations[7-9].

Preparation and characterization of disaccharides. — Compound **1** had $[\alpha]_D^{25}$ +110.6° (*c* 2, water) and gave D-xylose and 4-*O*-methyl-D-glucuronic acid on hydrolysis. The latter had $[\alpha]_D^{25}$ +45° (*c* 2, water); the derived amide[14] had m.p. 231–232° and $[\alpha]_D^{25}$ +135° (*c* 2, water).

Compound **1** was reduced to **2** as described previously[6].

For preparation of **3**, compound **1** (380 mg) was reduced in the usual way with potassium borohydride (800 mg), giving a product which was purified by preparative, paper chromatography to give **3** (205 mg, 54%), $[\alpha]_D^{25}$ +82.9° (*c* 2, water).

Anal. Calc. for $C_{12}H_{22}O_{11}$: OCH_3, 9.10. Found: OCH_3, 10.0.

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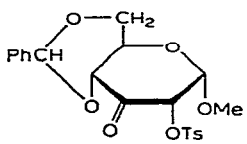
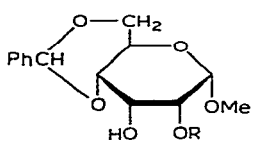
Synthesis of the methyl D-allopyranosides

Although a synthesis of D(L)-allose from D(L)-ribose has been known[1] for some time, surprisingly little is known of the chemistry of this sugar. The introduction of new oxidation reagents[2,3] has now made D-allose even more accessible from D-glucose derivatives[4] and should encourage interest in its reactions. In this context, the protection of position 1 of D-allose by glycosidation is likely to be of importance in future synthetic work.

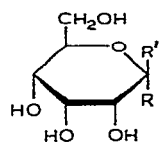
Methyl β-D-allopyranoside (**5**) was prepared[5] initially, in small amount, by reduction of methyl β-D-*ribo*-hexopyranosid-3-ulose with Raney nickel. This procedure also yielded methyl β-D-glucopyranoside, and subsequent studies[6] indicated that the proportion of alloside **5** was increased when reduction was carried out with hydrogen and a platinum catalyst. Methyl α-D-allopyranoside (**4**) is formed on reduction of methyl α-D-*ribo*-hexopyranosid-3-ulose but was not isolated[6]. Recently, allosides **4** and **5** have been prepared *via* benzoate displacement reactions on the appropriate methyl 2,4,6-tri-*O*-benzoyl-3-*O*-(toluene-*p*-sulphonyl)-D-glucopyranoside, followed by deacylation[7]. In connexion with the synthesis of some naturally occurring 6-deoxy-D-allose derivatives[8], we devised the following, convenient synthesis of allosides **4** and **5** from the readily available[9] methyl 4,6-*O*-benzylidene-2-*O*-(toluene-*p*-sulphonyl)-α-D-*ribo*-hexopyranosid-3-ulose (**1**).



(1)



(2) R = Ts
(3) R = H



(4) R=OMe, R'=H
(5) R = H, R'= OMe

Ts = $SO_2 \cdot C_6H_4Me$-*p*

Compound **1** was reduced stereospecifically, with sodium borohydride in methanol, to give methyl 4,6-*O*-benzylidene-2-*O*-(toluene-*p*-sulphonyl)-α-D-allopyranoside (**2**), which afforded methyl 4,6-*O*-benzylidene-α-D-allopyranoside (**3**) on treatment with methanolic sodium methoxide[9]. Removal of the benzylidene group from compound **3** was best accomplished by using a hot, aqueous suspension of Amberlite IR-120 (H^+) to give methyl α-D-allopyranoside (**4**), which, in our hands, was obtained as an amorphous solid, $[\alpha]_D$ +154° (water); this value is somewhat higher than that recorded (+134°) for a syrupy material[7]. The molecular rotation of our compound is +299°, in good agreement with the value (+309°) calculated[7] by Whiffen's method[10] for the *CI*(D) conformation. The n.m.r. spectrum of alloside **4** and the physical constants of the derived tetraacetate were in close agreement with reported data[7].

Treatment of the α-D anomer **4** with boiling, 2% methanolic hydrogen chloride

gave a mixture consisting mainly of the methyl D-allopyranosides **4** and **5**, which were judged (by n.m.r. spectroscopy) to be present in approximately equal amounts. Chromatography of the mixture on silica gel afforded crystalline methyl β-D-allopyranoside (**5**)[5,7]. In subsequent experiments, the β-D anomer was obtained, without resort to chromatography, by seeding of the glycoside mixture.

EXPERIMENTAL

Thin-layer chromatography was performed with Kieselgel G (Merck, 7731) and detection with vanillin–sulphuric acid[11]. N.m.r. spectra were measured with a Perkin–Elmer R-10 spectrometer for *ca.* 10% solutions in deuterium oxide, with sodium 2,2-dimethyl-2-silapentane-5-sulphonate as internal reference. Where possible, compounds were identified by mixed m.p. and by infrared spectroscopy.

Methyl 4,6-O-benzylidene-2-O-(toluene-p-sulphonyl)-α-D-ribo-hexopyranosid-3-ulose (**1**). — This compound was prepared from methyl 4,6-*O*-benzylidene-2-*O*-(toluene-*p*-sulphonyl)-α-D-glucopyranoside by using either the literature procedure[9] or the following method.

A solution of the glucoside (0.81 g) in methyl sulphoxide (6 ml) containing acetic anhydride (4 ml) was kept overnight at room temperature, chloroform (75 ml) and water (75 ml) were then added, and the solution was adjusted to pH 8 with 2.5M aqueous potassium carbonate. The aqueous layer was separated, and washed with chloroform (4 × 50 ml), and the combined organic layers were dried ($MgSO_4$) and evaporated. Recrystallisation of the residue from ethanol gave compound **1** (0.62 g), m.p. 165–166°; lit.[9], m.p. 165–167°.

Methyl 4,6-O-benzylidene-α-D-allopyranoside (**3**). — This compound, m.p. 175–176°, $[\alpha]_D$ +128 ±1° (*c* 0.5, chloroform), was prepared from compound **1** by borohydride reduction, and desulphonylation with sodium methoxide–methanol, essentially as described by Baker and Buss[9], who gave m.p. 175–177°, $[\alpha]_D$ +117 ±2° (*N,N*-dimethylformamide).

Methyl α-D-allopyranoside (**4**). — A solution of compound **3** (1.3 g) in water (30 ml), containing sufficient ethanol to effect solution, was treated with Amberlite IR-120 (H^+) (20 ml) for 1 h at 60°. The resin was filtered off, and washed thoroughly with water, and the filtrate and washings were evaporated to give alloside **4** (0.8 g, 89%), $[\alpha]_D$ +154 ±1° (*c* 0.5, water); lit.[7], $[\alpha]_D$ +134° (water). The product was initially obtained as a clear syrup that solidified to a readily manageable material after storage *in vacuo* over silica gel. The amorphous nature of the solid was demonstrated by X-ray diffraction (kindly obtained by Miss P.A. Gent). (Found: C, 42.8; H, 7.05. $C_7H_{14}O_6$ calc.: C, 43.3; H, 7.3%). N.m.r. data: τ 5.23 (pair of doublets, $J_{1,2}$ 4, $J_{1,3}$ *ca.* 1 Hz, H-1); 5.92 (triplet, $J_{2,3} = J_{3,4}$ *ca.* 3 Hz, H-3); 6.17-6.45 (multiplet, H-2,4,5,6); 6.59 (3-proton singlet, OMe).

Methyl 2,3,4,6-tetra-*O*-acetyl-α-D-allopyranoside, prepared in the usual way, had m.p. 115–116°, $[\alpha]_D$ +103° (*c* 1.2, chloroform); lit.[7], m.p. 115–116°, $[\alpha]_D$ +97.7° (*c* 1.3, chloroform).

Methyl β-D-allopyranoside (**5**). — A solution of the α-D anomer **4** (0.5 g) in 2% methanolic hydrogen chloride (12 ml) was heated under reflux for 2 h, whereupon t.l.c. (ethyl acetate–methanol, 1:1) showed the presence of two principal components. The cooled solution was neutralised ($PbCO_3$) and filtered, and the solvents were removed under diminished pressure to afford a syrup, which was chromatographed over silica gel (200 g). Elution with ethyl acetate–methanol (1:1) gave first methyl β-D-allopyranoside (**5**) (0.18 g, 36%), m.p. 150–151° (from ethanol), $[\alpha]_D$ −55° (*c* 0.6, water); lit., m.p. 150–151°, $[\alpha]_D$ −53° (*c* 2, water)[5]; m.p. 154–155°, $[\alpha]_D$ −50.8° (*c* 5.7, water)[7]. N.m.r. data: τ 5.42 (doublet, $J_{1,2}$ 8 Hz, H-1); 5.85 (triplet, $J_{2,3}$ *ca.* 3, $J_{3,4}$ *ca.* 2.5 Hz, H-3); 6.16-6.50 (multiplets, H-4,5,6); 6.45 (3-proton singlet, OMe); 6.60 (pair of doublets, $J_{1,2}$ 8, $J_{2,3}$ *ca.* 3 Hz, H-2). Continued elution with the same solvent gave a mixture of allosides **4** and **5** from which a further quantity (20 mg, 4%) of the β-D anomer was obtained upon the addition of ethanol, seeding, and cooling.

In a second experiment, β-D-alloside **5** was obtained directly, in 34% yield, by seeding and cooling an ethanolic solution of the glycosides. Integration over the methoxyl proton signals of the n.m.r. spectrum of the original mixture indicated that allosides **4** and **5** were present in roughly equal amounts.

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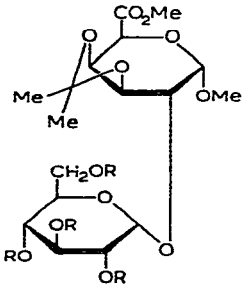
The synthesis of a pseudoaldobiouronic acid having an α-D-(1→2)-linkage

Recently[1], the direct synthesis of a derivative of a pseudoaldobiouronic acid having an α-D-(1 → 2)-linkage, *viz.*, 2-*O*-α-D-glucopyranosyl-D-galacturonic acid (**1**), was reported. This derivative, which was synthesized by a modified Koenigs–Knorr condensation, was methyl [methyl 3,4-*O*-isopropylidene-2-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl)-α-D-galactopyranosid]uronate (**2**). In a similar experiment, we have obtained, in addition to disaccharide **2**, the corresponding β-D-(1 → 2)-linked disaccharide **3** by treatment of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (**7**) with methyl (methyl 3,4-*O*-isopropylidene-α-D-galactopyranosid)uronate (**6**) in nitromethane in the presence of mercuric cyanide. Compounds **2** and **3** were fractionated by chromatography, and, on deacetylation, yielded methyl [methyl 3,4-*O*-isopropylidene-2-*O*-(α-D-glucopyranosyl)-α-D-galactopyranosid]uronate (**4**) and methyl [methyl 3,4-*O*-isopropylidene-2-*O*-(β-D-glucopyranosyl)-α-D-galactopyranosid]uronate (**5**), respectively.



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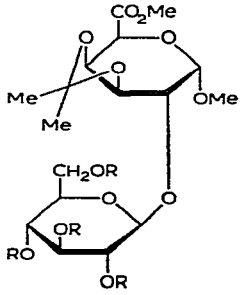
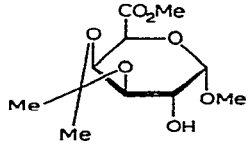
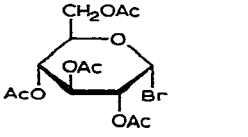
4 R=H



3 R=Ac
5 R=H



6



7

Although mercuric cyanide is reported[2] to specifically catalyse the formation of α-D linkages in the Koenigs–Knorr reaction, in our first experiment, the reaction mixture contained mainly the β-D-linked disaccharide. For further investigation of this situation, the series of experiments listed in Table I was performed. In each case, the β-D-linked disaccharide was formed in an amount 2–3 times larger than that of the α-D-linked disaccharide. The change in the molar ratio of α- and β-D-linked disaccharides with change of reactants, concentration, or molar excess of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (**7**) was not very great. The presence of a desiccating agent in the reaction mixture did not affect the α:β ratio, except that use of Drierite gave almost exclusive formation of the β-D-linked disaccharide.

It is often difficult to distinguish between S_N1 and S_N2 mechanisms in substitution reactions. The Koenigs–Knorr reaction is generally considered to involve an S_N1 mechanism, similar to that of the substitution reactions of alkyl halides which are catalysed[3] by silver or mercury salts. The formation of both disaccharides **2** and **3** would be expected for an S_N1 mechanism. A continuous change from an S_N1

TABLE I

SYNTHESIS OF DISACCHARIDES IN NITROMETHANE

Expt.	*Concentration of* **6** *(moles/ml)*[a]	*Molar excess of* **7**[b]	*Molar excess of* $Hg(CN)_2$	*Desiccant (g/ml)*	*Yield of disaccharides, %*			*Molar ratio,* **2:3**
					α-D-(1 → 2) (**2**)	β-D-(1 → 2) (**3**)	2+3	
1	7.6×10^{-5}	1.0	1.0	Na_2SO_4 (0.03)	8.0[c]	20.0[c]	28.0[c]	1:2.5
2	7.6×10^{-5}	1.0	1.0	Na_2SO_4 (0.03)	7.0	18.0	25.0	1:2.6
3	2.2×10^{-5}	1.4	1.6	—	13.0	41.0	54.0	1:3.2
4	7.6×10^{-5}	1.0	1.0	—	10.0	14.5	24.5	1:1.5
5	2.2×10^{-5}	1.4	1.6	Na_2SO_4(0.03)	6.0	22.0	28.0	1:3.8
6	7.6×10^{-5}	1.0	1.0	Drierite (0.1)	2.5	26.0	28.5	1:10.4

[a]**6** = Methyl (methyl 3,4-*O*-isopropylidene-α-D-galactopyranosid)uronate. [b]**7** = Tetra-*O*-acetyl-α-D-glucopyranosyl bromide. [c]Determined by direct weighing of isolated fractions from chromatography.

to an S_N2 mechanism may occur on variation of reaction conditions, although the "point" or "region" of change in mechanism is sometimes difficult to establish. The almost exclusive formation of disaccharide **3** in the experiment with Drierite indicates that such a change to an S_N2 mechanism may have occurred. It was found that, under the conditions of reaction, 0.04 g of Drierite and 0.01 g of sodium sulphate were soluble in 100 ml of anhydrous nitromethane. This larger solubility of Drierite may be responsible for the change in mechanism, but further experiments are required to elucidate this point.

EXPERIMENTAL

Melting points (uncorrected) were determined on a micro hot-stage Type HMK 61214 (Franz Küstner, Dresden). Optical rotations were measured on a Bendix-Ericcson ETL-NPL Automatic Polarimeter Type 143a. T.l.c. was performed with Silica Gel G, buffered with 0.02M sodium acetate, and benzene–methanol (95:5). Preparative column-chromatography was effected on silica gel (Merck, 75–250 US mesh) with benzene–acetone (8:2) and the "dry-column" method[4].

Synthesis of the disaccharide derivatives **2** *and* **3**. — A solution of methyl (methyl 3,4-*O*-isopropylidene-α-D-galactopyranosid)uronate[5] (**6**) (1 g) in 50 ml of absolute nitromethane was treated with a 1-molar excess of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide[6] (**7**), mercuric cyanide was added, and the reaction mixture was shaken for 21 days with the addition of 1.5 g of anhydrous sodium sulphate. The solid phase was filtered off, the filtrate was diluted with chloroform, and the solution was then washed with aqueous sodium hydrogen carbonate and water. The chloroform solution was dried (Na_2SO_4), and evaporated *in vacuo*, and the residue was subjected to column chromatography to give the β-D anomer **3** (0.45 g, 20%) as a glass; the α-D isomer **2** was obtained as a yellowish syrup (0.18 g, 8%).

Compound **3** crystallized from methanol, yielding white plates, m.p. 151–152°; $[\alpha]_D^{23}$ +62.7° (*c* 1.0, chloroform) (using Hudson's rules, a calculated value of ~ +50° was obtained); R_F (t.l.c.) 0.53. The i.r. spectrum of compound **3** was closely similar to that[1] of compound **2**, as was the n.m.r. spectrum, except for small differences in the region of the anomeric proton (τ 4.5–5.5).

Anal. Calc. for $C_{25}H_{36}O_{16}$: C, 50.67; H 6.12; OCH_3, 10.48. Found: C, 50.61; H, 6.12; OCH_3, 10.87.

The physical constants of compound **2** have been reported[1]. Deacetylation of compound **2** by the Zemplén method[7] yielded compound **4** as white needles, m.p. 137–139° (from isopropyl alcohol–ethanol), $[\alpha]_D^{25}$ 134.3° (*c* 1.0, methanol).

Anal. Calc. for $C_{17}H_{28}O_{12}$: C, 48.08; H, 6.65; OCH_3, 14.79. Found: C, 47.81; H, 6.62; OCH_3, 14.53.

Deacetylation[7] of compound **3** yielded compound **5** as a colourless syrup, $[\alpha]_D^{25}$ +54.4° (*c* 1.0, methanol).

Syntheses of disaccharides under various reaction conditions. — A series of experiments performed by the above general procedure is shown in Table I. The chromatographic separation was omitted, and the yields of disaccharides were

determined by t.l.c., in comparison with known amounts of reference substances. The t.l.c. plates were sprayed with sulphuric acid and then heated in an oven for 20 min at 110°. The developed spots were scanned with a densitometer ERI 10 (C. Zeiss, Jena); the calibration curves for reference substances and for each experiment were separately constructed.

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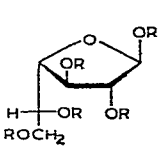
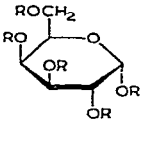
Mutarotation of D-galactose. Tautomeric composition of an equilibrium solution in pyridine

Such sugars as D-glucose, D-mannose, and D-xylose, which exhibit simple, pseudo-first-order kinetics of mutarotation, are expected to have only two major components present at equilibrium (*i.e.* α- and β-pyranoses). Such sugars as D-fructose, D-arabinose, and D-galactose, which exhibit complex mutarotation, are expected to have more than two tautomers present in significant proportions at equilibrium. Smith and Lowry[1] examined the complex kinetics observed for the mutarotation of D-galactose, and postulated a mechanism involving two consecutive and reversible first-order reactions in which "γ-D-galactose" was an intermediate in the α-D- $\rightleftarrows$ β-D-pyranose transformation. The "γ-form" was initially presumed to be an acyclic modification. Later, however, these authors[2] recognized that two furanose modifications, yielding a total of five tautomers, were probably present at equilibrium. Although polarographic and spectral[3] techniques show only trace proportions of an acyclic tautomer of D-galactose to be present at equilibrium, there is evidence to indicate that an initial rapid phase in the mutarotation of D-galactose is associated with pyranose $\rightleftarrows$ furanose interconversion[4]. However, examination of solutions of D-galactose in deuterium oxide by p.m.r. spectroscopy has failed to reveal signals of anomeric protons that can be assigned to furanose tautomers[5].

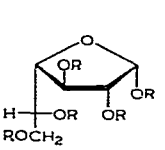
It seems unlikely that furanose forms are intermediates in pyranose–pyranose anomeric interconversion. This leads us to believe that the initial mechanism proposed



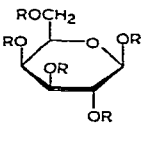
1, R = H
2, R = Me_3Si



3, R = H
4, R = Me_3Si



5, R = H
6, R = Me_3Si



7, R = H
8, R = Me_3Si

by Smith and Lowry[1] is but a partial description of the complete phenomenon. A prerequisite to the formulation of a complete mechanism for the mutarotation of D-galactose is the identification of all tautomers present at equilibrium in solution. This report describes the ring tautomers that prevail at mutarotational equilibrium in pyridine.

RESULTS AND DISCUSSION

In a preliminary communication[6], the per-*O*-trimethylsilyl derivatives of two additional tautomers formed from α-D-galactopyranose during mutarotation in pyridine were reported. Together with β-D-galactopyranose, a third component,

associated with a distinct, rapid phase of the mutarotation, was observed. After isolation by preparative g.l.c., the third component was identified as the per-*O*-trimethylsilyl derivative of β-D-galactofuranose.

In a personal communication, Sörensen[7] showed that calculations from the optical rotatory data of Smith and Lowry[1] and of Sörensen[8] indicate that, at equilibrium in water, the so-called "γ-D-galactose" is probably α-D-galactofuranose ($[\alpha]_D^{20}$ +46.3°), and that it probably accounts for 92–95% of the total furanose forms present at equilibrium. Careful re-examination of our data and techniques confirmed the original assignment for the third component. However, the shape of the chromatographic peak assigned to the β-D-galactopyranose derivative did not, at that time, exclude the possibility that it contained an unknown amount of a fourth, or even a fifth, component. Mathematical analysis of that peak, with a computer programmed for a slightly skewed Gaussian distribution, indicated that a fourth component was probably present. The fourth component was finally isolated from a mutarotated solution of D-galactose that had been per(trimethylsilyl)ated and the product resolved on a g.l.c. column having a stationary phase of (ethylcyano)silicone. More than 100 mg of each component was isolated, and a typical preparative chromatogram is shown in Fig. 1.

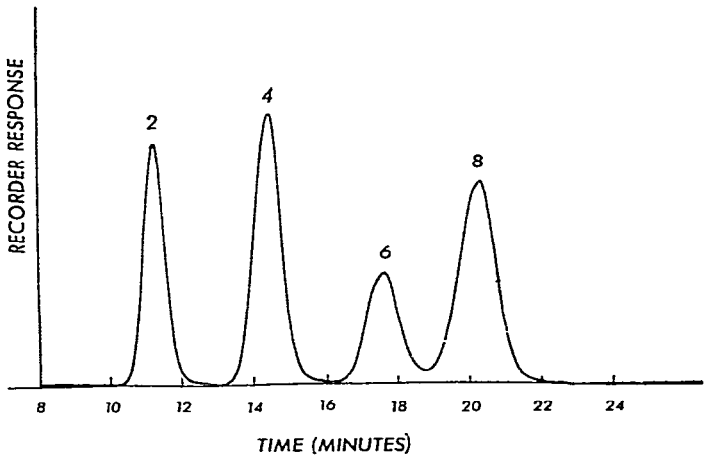


Fig. 1. Gas–liquid chromatogram of per-*O*-trimethylsilylated tautomers of D-galactose formed in pyridine at 80°.

Data used to establish the identity of each peak, in descending order of elution from the columns, are shown in Table I. The molecular rotations found are consistent with those reported for the peracetylated tautomers of D-galactose[9], and indicate that the fourth component isolated is the per-*O*-trimethylsilyl derivative of α-D-galactofuranose (6).

The p.m.r. spectra of the four individual per-*O*-trimethylsilyl derivatives of D-galactose yielded three sets of signals for protons. One set occurred at τ 5, and is assigned to the anomeric protons. A second set was found at τ 5.7–6.9, and is assigned

to the remaining methine and methylene protons. The third set, resonating at τ 10, is assigned to protons of the *O*-trimethylsilyl substituent groups. The integrated areas of these three sets of proton signals were in the ratios of 1:6:45. These ratios are

TABLE I

DATA CHARACTERIZING THE TRIMETHYLSILYL TETRA-*O*-(TRIMETHYLSILYL)-D-GALACTOSIDES

Compound	$[\alpha]_D^{23}$, *degrees*	$[M]_D$	*P.m.r. data at 60 MHz*		*Elemental analysis**		
			Chemical shift of H-1 signal, τ	$J_{1,2}$ *Coupling constant, Hz*	*C*	*H*	*Si*
2	−36.2	−15,950	5.00 (doublet)	2.5	46.83	9.43	24.41
4	+66.2	+35,750	5.10 (singlet)	0.0	46.79	9.64	24.41
6	+52.3	+28,200	5.02 (doublet)	4.5	46.74	9.33	24.38
8	+2.2	+ 1,190	5.63 (doublet)	6.0	46.95	9.51	24.23

*Calc. for these derivatives: C, 46.66; H, 9.63; Si, 25.92%.

consistent with the view that the four compounds isolated are, in fact, the per-*O*-trimethylsilylated derivatives of D-galactose, and the possibility that one of the compounds is the hexakis(trimethylsilyl) derivative of an open-chain, hydrated aldehyde can be excluded. Moreover, i.r. analysis failed to reveal any bands arising from hydroxyl or carbonyl absorption.

Coupling constants (Hz) shown in Table I for the four derivatives of D-galactose further indicate that the third and fourth components are **2** and **6**. With reference to the Karplus equation, however, these coupling constants are found to be about 2 Hz lower than the values predicted; this difference is probably due to difficulties in defining suitable parameters for use with the Karplus equation as applied to these compounds[10].

The data on chemical shift for the anomeric protons, shown in Table I, suggest that it would not be possible at 60 Hz to resolve three of the H-1 signals of D-galactose at mutarotational equilibrium. At 100 MHz, Perlin[11] observed at least three anomeric hydroxyl-proton signals in methyl sulfoxide solution, and two of these signals were partly superimposed. We examined the p.m.r. spectrum of a mutarotated solution of D-galactose (15%, in deuterium oxide) and found at τ 5.57 a sharp doublet having a coupling constant of 6 Hz (assigned to H-1 of **7**). A second broad (20 Hz wide), unsymmetrical signal occurred at about τ 4.90, and this might comprise the H-1 signal for **1**, **3**, and **5**. Apparently, the furanosides, and possibly the furanoses also, have an electronic environment about the anomeric proton that is very similar to that of the anomeric protons of the α-D-pyranose and the pyranosides of D-galactose.

The distribution of tautomers of D-galactose, after 8 h at 80° in pyridine, as revealed by g.l.c. of the per-*O*-trimethylsilyl derivatives, is: **2**, 23.4%; **4**, 31.7%; **6**, 13.7%; and **8**, 31.2%. After several weeks at 25°, a constant $[\alpha]_D^{23}$ value of +50.5° was observed, and the distribution of tautomers at equilibrium was found to be 12.1%, 33.8%, 5.1%, and 49.0%, respectively. Mathematical analysis of these peaks

for deviation from Gaussian distribution suggests that the presence of additional, unresolved compounds is unlikely.

In our studies, we have found that the equilibrium composition of aldose tautomers in pyridine differs markedly from that found in water. Changes in the position of the mutarotation equilibrium with changes in the polarity of the solvent is currently being studied, as well as the effect of the silylation reaction on the position of the equilibrium.

EXPERIMENTAL

*Preparation of per-*O*-trimethylsilyl derivatives of* D*-galactose.* — A solution of anhydrous α-D-galactopyranose (5 g) in 500 ml of redistilled and dried pyridine (b.p. 114–114.5°) was kept for 8 h at 80°. To this solution was added 100 ml of hexamethyldisilazane followed by 50 ml of chlorotrimethylsilane. After one h, precipitated salts were removed by decantation and centrifugation. The solution was then concentrated *in vacuo* to a thick, yellow oil, which was filtered through Whatman No. 52 paper (by gravity) to remove silicone polymers of high molecular weight, yielding 15 g of a mixture of per-*O*-trimethylsilyl derivatives of D-galactose.

*Preparative g.l.c. separation of the per-*O*-trimethylsilyl derivatives of* D*-galactose.* — The four isomers were isolated in greater than 99.9% purity from center cuts of the respective peaks by using a Varian Aerograph, Model A-700, preparative gas-chromatographic unit equipped with a variable effluent-splitter and a flame-ionization detector. Samples (30 μl) were injected repeatedly. The vaporization chamber was maintained at 270° to ensure complete volatilization. The tautomers were separated on an aluminum column (6 m × 0.94 mm i.d.) containing 30% (w/w) XF-1150* stationary phase on a 60–80 mesh support of Chromosorb A. The column oven was maintained isothermally at 155°. Nitrogen was used as the carrier gas, at a flow rate of 100 ml per min. The detector and collector temperatures were maintained at 270° and 210°, respectively. After 20 days of continuous operation, about 500 mg of each component was collected. In order to remove stationary phase that had been eluted with the initial samples, all compounds isolated were rechromatographed, to yield 140–350 mg of each individual tautomer.

Mathematical analysis of chromatograms. — Deviations from Gaussian distribution in the g.l.c. data were studied with a Dupont Model No. 310 modified analog computer (Curve Resolver).

Determination of optical rotation. — Optical rotatory data were obtained with a Rudolph Model No. 80 polarimeter equipped with an oscillating polarizer and a photoelectric read-out attachment. Rotations in chloroform were observed with a 1-dm tube. The concentrations of the D-galactosides used were: **2**, 2.4%; **4**, 3.7%; **6**, 1.5%; and **8**, 1.4%.

P.m.r. and i.r. spec... Th ...m.r. data reported were measured with a

*An (ethylcyano)silicone column supplied by Varian Aerograph, Walnut Creek, California (U.S.A.).

Varian A-60 n.m.r. spectrometer with chloroform solutions. The chloroform served as the internal standard instead of tetramethylsilane, so that interference with substituent trimethylsilyl groups was avoided. Chemical-shift data are expressed relative to τ 7.27 for the chloroform proton signal. The i.r. spectra were recorded for films by using a Beckman Model IR-7 instrument equipped with a sodium chloride prism.

ACKNOWLEDGMENTS

We thank R.M. Butts for assistance with the g.l.c. separations, and Gorton Wood (Baker Laboratory of Chemistry, Cornell University) for assistance with the p.m.r. analyses. This work was supported, in part, by grants from the Public Health Service (to R.S.S.) and from the Campbell Institute for Food Research (to T.E.A.).

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Carbohyd Res., 6 (1968) 498–502

Preliminary communication

Formation of chlorodeoxy glycosides during sulfonylation of methyl D-glucopyranosides

Displacement of *p*-toluenesulfonic ester groups by pyridinium chloride during *p*-toluenesulfonylation of methyl α- and β-D-glucopyranoside in pyridine at 75° was demonstrated by Hess and Stenzel[1]. The chlorinated products were described[1] as methyl 4-chloro-4-deoxy-2,3,6-tri-*O*-*p*-tolylsulfonyl-α- (**1**) and -β-D-glucopyranoside (**2**) and methyl 4,6-dichloro-4,6-dideoxy-2,3-di-*O*-*p*-tolylsulfonyl-α- (**3**) and -β-D-glucopyranoside (**4**), but the correctness of these designations has been questioned by Tipson[2].

Recently, we described[3] an improved preparation of some methyl 6-chloro-6-deoxy-D-hexopyranosides, including methyl 6-chloro-6-deoxy-α- (**5**) and -β-D-glucopyranoside[4,5] (**6**); *p*-toluenesulfonylation of **5** and **6** at 30° gives derivatives having physical properties identical with those reported[1] for I and II. By repeating the experiments described by Hess and Stenzel[1], we have shown by direct comparison that the compounds assigned[1] the structures **1** and **2** are, in fact, methyl 6-chloro-6-deoxy-2,3,4-tri-*O*-*p*-tolylsulfonyl-α- and -β-D-glucopyranoside. These new structural assignments are based on analysis of n.m.r. spectra at 60 and 100 MHz.

When *p*-toluenesulfonylation of methyl α-D-glucopyranoside or **5** was performed at 75°, we obtained methyl 4,6-dichloro-4,6-dideoxy-2,3-di-*O*-*p*-tolylsulfonyl-α-D-galactopyranoside having physical properties identical with those reported[1] for the compound described as **3**. The n.m.r. spectrum showed $J_{1,2}$ 3.5 Hz, $J_{2,3}$ 10.0 Hz, $J_{3,4}$ 3.7 Hz, and $J_{4,5}$ 1.3 Hz, and H-5 gave a triplet having an average splitting of 6.5 Hz; this pattern due to H-5 has been observed with several D-galactose derivatives[6].

Similar treatment of methyl β-D-glucopyranoside or **6** afforded methyl 4,6-dichloro-4,6-dideoxy-2,3-di-*O*-*p*-tolylsulfonyl-β-D-galactopyranoside having the same physical properties as the compound described by Hess and Stenzel[1] as **4**. The n.m.r. spectrum showed $J_{1,2}$ 7.7 Hz, $J_{2,3}$ 9.5 Hz, $J_{3,4}$ 3.6 Hz, and $J_{4,5}$ 1.5 Hz, together with a triplet due to H-5 having an average splitting of 6.5 Hz. Another product obtained from this reaction in 23% yield is thought to be **4**; for this compound, the coupling constant of $J_{1,2}$ 7.5 Hz is consistent with the D-*gluco* or D-*allo* configuration, but not with a D-*manno* configuration. Stevens and co-workers[7] have obtained deoxyhalogeno compounds that are epimeric at C-4 by treating methyl 6-deoxy-2,3-di-*O*-benzyl-4-*O*-(methylsulfonyl)-α-D-gluco- or -D-galactopyranoside with sodium iodide in 2,4-pentanedione at 125°.

When *p*-toluenesulfonylation of methyl α- or β-D-glucopyranoside was performed for 16 days at 27°, the introduction of chlorine was again observed.

The α-D anomer afforded tetra-*O*-*p*-tolylsulfonyl and 6-chloro-6-deoxy-2,3,4-tri-*O*-*p*-tolylsulfonyl derivatives in yields of 59% and 37%, respectively, whereas the β-D anomer gave 90% of tetra-*O*-*p*-tolylsulfonyl derivative, but only 4% of 6-chloro-6-deoxy-2,3,4-tri-*O*-*p*-tolylsulfonyl derivative. Chlorination at a primary hydroxyl group during *p*-toluenesulfonylation at room temperature has previously been observed[8] with uridine, resulting in formation of 3-(5-chloro-5-deoxy-2,3-di-*O*-*p*-tolylsulfonyl-β-D-ribofuranosyl)uracil.

We have obtained mass spectra of the above compounds at 200° and 70 eV by introducing the sample on a probe into a Consolidated Electrodynamics Corporation CEC 21-110 mass spectrometer. A feature common to these mass spectra is that the ion of greatest mass is that corresponding to loss of a *p*-tolylsulfonyl group from the parent molecule. Previous mass spectra of methyl glycoside derivatives[9] lacking *p*-tolylsulfonyl groups have shown the ion of greatest mass to be that corresponding to loss of the glycosidic methoxyl group. The number of chlorine atoms in the above compounds was readily ascertained[10] from the isotopic clusters derived from fragments containing ^{35}Cl and ^{37}Cl.

Methanesulfonylation of methyl α-D-glucopyranoside at 75° has also been performed, and leads to the formation of methyl 6-chloro-6-deoxy-2,3,4-tri-*O*-(methylsulfonyl)-α-D-glucopyranoside and methyl 4,6-dichloro-4,6-dideoxy-2,3-di-*O*-(methylsulfonyl)-α-D-galactopyranoside. Similar reaction of methyl β-D-glucopyranoside gives methyl 6-chloro-6-deoxy-2,3,4-tri-*O*-(methylsulfonyl)-β-D-glucopyranoside and methyl 4,6-dichloro-4,6-dideoxy-2,3-di-*O*-(methylsulfonyl)-β-D-glucopyranoside, but no methyl 4,6-dichloro-4,6-dideoxy-2,3-di-*O*-(methylsulfonyl)-β-D-galactopyranoside was found.

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Book review

Carbohydrate Chemistry: by EUGENE A. DAVIDSON, Duke University, Durham, N.C., U.S.A. Holt, Rinehart, and Winston, Inc., New York, 1967, iii + 441 pp., 15.5 × 23.5 cm, cloth. $ 11.95.

The theory and practice of carbohydrate chemistry have undergone profound changes in the past twenty years, and the established information on all aspects of the subject has grown by several orders of magnitude. As a result, our general theoretical insight has developed to such a degree as to make possible a highly condensed treatment which could serve as the working basis for research in carbohydrate chemistry at a level of effectivity that could not have been dreamed of only twenty years ago. This strength has developed mainly through advances in technique and instrumentation (chromatography, nuclear magnetic resonance and infrared spectroscopy, polarimetry, and electrophoresis) and in general theory based on electronic, stereoelectronic, and conformational considerations. For these reasons, the time has, for the past few years, been ripe for the appearance of an authoritative book that would give the new generation of carbohydrate chemists the advantage of ready access to these developments, and thereby catalyze their efforts on the rapidly receding frontiers of research in carbohydrate chemistry. Furthermore, carbohydrate chemistry is in need of a new image if it is to regain, as a field of competence, the respect of the general body of organic chemists, and thereby attract to its challenges its proper share of our best developing minds. Holding these views, I accepted with enthusiasm the invitation to review this book, which was advertised, in part, "to correlate modern theory with carbohydrate reactions", and as "a discussion of methods (n.m.r., g.l.c., o.r.d., etc.) currently used in determining the three-dimensional structure of molecules". I was soon to regret this decision.

The author obviously possesses some competence for assessing developments in carbohydrate chemistry in the early 1950's. However, and most unfortunately, the major breakthroughs in the past fifteen years are beyond his ken. The result of this is that the author has contributed to the perpetuation of a lot of the bad and obsolete chemistry that almost inevitably exists as a prelude to genuine advance. Furthermore, and more seriously, the book grossly misrepresents the inherent value of some of the major advances made in recent years, through unbelievably wrong and badly documented statements. However, the book probably does not represent so serious a threat to carbohydrate chemistry as one might imagine, since it is so replete with errors at the most elementary level that no-one of competence could possibly maintain any interest past page 23, where basically wrong definitions of axial and equatorial bonds are given. A detailed criticism of this book, which is replete with

errors that, charitably, may be described as typographical, and theory that is either erroneous or inadequate, is beyond reasonable expectation. The only conceivable use for the book that occurs to this reviewer would be as source material for "true or false" type examinations.

This book should not have been published. Have publishers no responsibility in this regard? Certainly, a publishing firm must be expected to provide a far more acceptable format than is represented by this thoroughly badly produced book.

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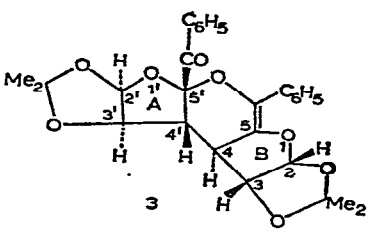
Carbohyd. Res., 5 (1968) 505–506

Corrigendum

Carbohyd. Res., Vol. 6, No. 2 page 245;

Formula 3 should read:



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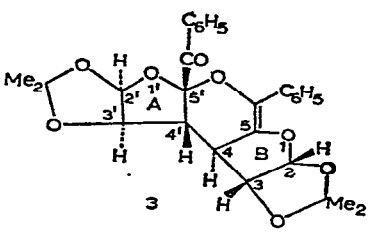

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