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SELECTIVE CLEAVAGE OF GLYCOSIDIC LINKAGES: STUDIES WITH THE POLYSACCHARIDE COMPONENT OF *Shigella dysenteriae* TYPE 6 LIPOPOLYSACCHARIDE

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ABSTRACT

The polysaccharide component obtained from the lipopolysaccharide of *Shigella dysenteriae* type 6 was subjected to mild hydrolysis with acid, and the products were fractionated on Sephadex G-50. An acidic hexosaminoglycan and a core oligosaccharide fraction were obtained, the former containing D-glucose, D-galactose, 2-acetamido-2-deoxy-D-galactose (in the ratios 1:1:1), and an unidentified acidic component (*X*). The hexosaminoglycan was *N*-deacetylated and then hydrolysed and deaminated to give 3-*O*-(2-amino-2-deoxy-β-D-galactopyranosyl)-D-galactose (**1**), identified as the *N*-acetyl derivative (**2**), and 2,5-anhydro-3-*O*-(6-*O*-α-D-galactopyranosyl-α-D-glucopyranosyl)talitol (**3**). On the basis of the structure of **2** and the methylation-analysis data for the polysaccharide and **3**, together with that for the determination of linkage configurations by chromic anhydride oxidation, the hexosaminoglycan is considered to have the repeating structure

$$\rightarrow 3)\text{-}\beta\text{-D-GalNAc}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow 6)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow \quad .$$
$$\begin{array}{c} 4 \\ \uparrow \\ X \end{array}$$

INTRODUCTION

The polysaccharide chains of the antigenic lipopolysaccharides from the majority of *Sh. boydii*[1] and *Sh. dysenteriae*[2] serotypes appear to be complex hexosaminoglycans. In seeking a general approach to the structural investigation of hexosaminoglycans, we are investigating the selective cleavage[3] of *N*-deacetylated derivatives by acid hydrolysis and by deamination with nitrous acid, following studies with the model compounds benzyl 2-acetamido-2-deoxy-*O*-hexosyl-D-glucosides[4–6]. This approach has been used successfully in a structural investigation of *Sh. dysenteriae* type 3 polysaccharide[7], and we now describe its application to the polysaccharide component of *Sh. dysenteriae* type 6 lipopolysaccharide.

RESULTS AND DISCUSSION

The lipopolysaccharide of *Sh. dysenteriae* type 6 was isolated in 4% yield from dry bacterial cells by extraction with hot, aqueous phenol followed by precipitation of nucleic acids with Cetavlon[8]. The high type-6 specificity of the material was confirmed in passive haemagglutination reactions with O-antisera prepared by immunization of rabbits with the autoclaved bacterial cells. The lipopolysaccharide was hydrolysed with dilute acetic acid to split off lipid A, and the resulting degraded polysaccharide, when subjected to chromatography on Sephadex G-50, gave a polysaccharide of high molecular weight eluted in the void volume, and an oligosaccharide (core) fraction. The polysaccharide was considered to be attached by its reducing end to the core residue, and the yield of polysaccharide was 25% based on the lipopolysaccharide.

On paper electrophoresis, the polysaccharide migrated as a sharp zone towards the anode. It had i.r. bands at 1730 (carbonyl), 1650, and 1560 cm^{-1} (amide), and the p.m.r. spectrum contained signals for carbohydrate protons and the NAc group (δ, 1.96 p.p.m.). The absence of other signals indicated that the acidic component (*X*) of the polysaccharide was neither pyruvic nor an aliphatic acid. After hydrolysis of the polysaccharide, paper electrophoresis revealed the presence of neutral sugars and hexosamine. The absence of a negatively charged compound after hydrolysis with 2M or 0.01M hydrochloric acid indicated that the acidic component was neither a uronic acid nor the unidentified acidic substance present in the *Sh. dysenteriae* type 3 polysaccharide[9], nor the 2-amino-2-deoxyhexuronic acid found[10] in the lipopolysaccharide of *Sh. sonnei* phase I. The last statement is also confirmed by analysis data for amino sugar obtained with an amino acid analyser. The acidic component apparently was not the 3-deoxypentulosonic acid discovered recently in the *Klebsiella* type 38 polysaccharide[11], as it survived cleavage of the parent lipopolysaccharide with dilute acetic acid. Thus, the acidic component *X* may be a new component of bacterial lipopolysaccharides.

Neutral monosaccharides were identified as D-glucose and D-galactose, both by chromatographic and enzymatic methods; the content of D-glucose was 20%. Hexosamine was identified as 2-amino-2-deoxy-D-galactose on the basis of behaviour in the amino acid analyser and the reaction with D-galactose oxidase; the content in the hydrolysate obtained with 4M hydrochloric acid (4 h, 100°) was 22.5%. After deamination of the hydrolysate with nitrous acid, g.l.c.[12] revealed 2,5-anhydrotalose, galactose, and glucose in the ratios 1.1:1.0:1.0. Thus, the *Sh. dysenteriae* type 6 polysaccharide contains D-glucose, D-galactose, and 2-acetamido-2-deoxy-D-galactose (in the ratios 1:1:1), together with an unidentified acidic component (*X*).

After reduction with sodium borohydride, the polysaccharide was *N*-deacetylated with anhydrous hydrazine in the presence of hydrazine sulphate at 105°. The extent of *N*-deacetylation was evaluated on the basis of acid-hydrolysis data, as described below. After 10 h, the content of free amino groups was ~70%, whereas, after similar treatment, the *N*-deacetylation of model biosides[4–6] and *Sh. dysenteriae* type 3 polysaccharide[7] was quantitative. More-prolonged treatment (20 h) caused

90–95% *N*-deacetylation and partial destruction of polymer; the yield of material recovered from the void volume of the Sephadex G-50 column had decreased to 75%. The polysaccharide having the higher content of free amino groups was chosen for a structural investigation.

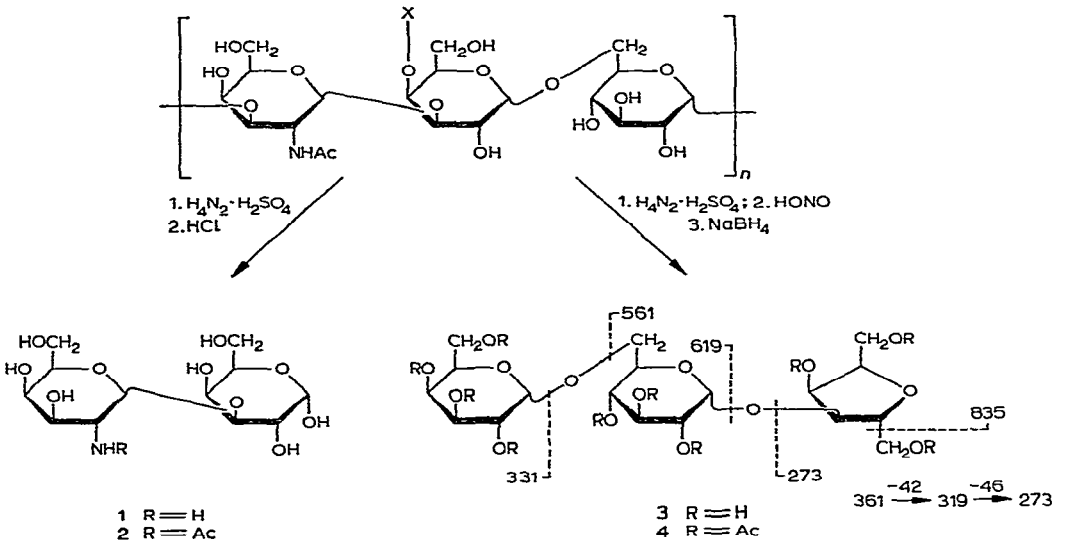
The monosaccharide composition of the modified polysaccharide was identical with that of the starting material, as shown by g.l.c. data for the alditol acetates obtained by application in sequence of *N*-deacetylation, deamination with nitrous acid, acid hydrolysis, reduction with borohydride, and acetylation. When the deamination step was omitted, the only monosaccharide detected by g.l.c. was glucose. Therefore, the 2-amino-2-deoxy-D-galactose residue in the polysaccharide is attached to galactose, and the latter sugar is retained in a 2-amino-2-deoxygalactosylgalactose disaccharide under the conditions of acid hydrolysis. The hydrolysate contained (amino acid analyser) 2-amino-2-deoxygalactose (small amount), 2-amino-2-deoxygalactosylgalactose (**1**), and an unknown product tentatively considered as a modified acidic component.

Since the ratio of **1** and 2-amino-2-deoxygalactose reflected the content of the latter and 2-acetamido-2-deoxygalactose residues in the *N*-deacetylated polysaccharide, then assuming the acid hydrolysis to be complete, this ratio was used to evaluate the extent of *N*-deacetylation.

The *N*-deacetylated polysaccharide was treated with 2M hydrochloric acid under optimal conditions (100°, 3 h) and the hydrolysate was fractionated on a Dowex-50W x8 cation-exchange resin. The neutral sugars and **1** were eluted with 25mM and 0.1M pyridine acetate buffer, respectively, whereas the third component was retained on the column. Deamination with nitrous acid cleaved **1** into galactose and 2,5-anhydrotalose (in the ratio 1:1), which were identified by g.l.c. of their alditol acetates. Thus, **1** was 2-amino-2-deoxygalactosylgalactose and was identical, (amino acid analyser data) with 3-*O*-(2-amino-2-deoxy-β-D-galactopyranosyl)-D-galactose isolated from *Sh. dysenteriae* type 3 polysaccharide by an analogous procedure. *N*-Acetylation of **1** gave a neutral compound (**2**) which was homogeneous and identical (p.c.) with the corresponding *N*-acetyl derivative isolated from *Sh. dysenteriae* type 3 polysaccharide[7], and gave a strong, positive reaction with the Morgan–Elson reagent[13]. Acid hydrolysis of **2** gave 2-amino-2-deoxygalactose and galactose in the ratio 1:1, and treatment with β-D-hexosaminidase[14] from pig epididymis gave 2-acetamido-2-deoxygalactose and galactose. Thus, **2** was 3-*O*-(2-acetamido-2-deoxy-β-D-galactopyranosyl)-D-galactose. The presence of a (1→3)-linkage between the 2-acetamido-2-deoxyglactose and galactose residues in the polysaccharide was confirmed by methylation analysis of the fragment obtained after deamination of the *N*-deacetylated polysaccharide with nitrous acid, as described below.

Further, when the parent and the *N*-deacetylated polysaccharides were oxidised with sodium periodate, the 2-amino-2-deoxygalactose residues survived, proving that the 2-acetamido-2-deoxygalactopyranose residue was substituted at position 3.

Deamination of the *N*-deacetylated polysaccharide with sodium nitrite in dilute acetic acid, followed by reduction with sodium borohydride, and chromatography



on Sephadex G-50 showed that the polysaccharide had been completely degraded into oligosaccharide fragments. P.c. revealed two oligosaccharides [$R_{LACTOSE}$ 0.76 (major) and 1.10] which were isolated by preparative p.c.; the major component was identified as the trisaccharide **3**. Acid hydrolysis of the more-mobile oligosaccharide gave glucose and galactose in the ratio 1:1 (g.l.c.). This oligosaccharide may arise by β-elimination, as this phenomenon occurs with 3-*O*-substituted 2,5-anhydrohexoses[4,7] under the alkaline conditions of reduction with borohydride. It was not further investigated because of the very low yield.

Acid hydrolysis of **3** gave equimolar amounts of 2,5-anhydrotalitol, galactose, and glucose, *i.e.*, the same monosaccharides which were identified in the hydrolysate of the starting polysaccharide. Thus, **3** may represent the modified chemical repeating-unit of the specific polysaccharide from *Sh. dysenteriae* type 6. The structure of **3** was established on the basis of mass-spectral data for its peracetate (**4**) and by methylation analysis. The fragmentation pattern of **4** under electron impact was similar to that of acetylated 2,5-anhydro-3-*O*-β-D-galactopyranosyl-D-mannitol[4] (see **4**). Comparative methylation analysis of **3** and the polysaccharide, by the Hakomori procedure[15], followed by acid hydrolysis and identification, by g.l.c.-m.s.[16], of the resulting partially methylated sugars gave the data presented in Table I. Two major, partially methylated monosaccharides were identified as 2,3,4-tri-*O*-methylglucose and 2,6-di-*O*-methylgalactose, proving that the polysaccharide was branched. Peaks of the minor components were due to monosaccharides originating from the core oligosaccharide, to which the O-specific polysaccharide chain is known to be attached in the lipopolysaccharide, and were not considered. The absence of any fully methylated monosaccharide indicated that side chains were terminated with residues of the acidic component (*X*).

TABLE I

METHYLATION ANALYSIS OF POLYSACCHARIDE (PS) AND OLIGOSACCHARIDE 3

Peaks	*Identity*	T[a]	*Molar ratios*	
			PS	3
A	2,5-Anhydro-1,4,6-tri-*O*-methyltalitol	0.5	—	1.0
B	2,3,4,6-Tetra-*O*-methylgalactitol	1.0	0.06	0.9
C	2,4,6-Tri-*O*-methylglucitol	1.56	0.13	—
D	2,3,4-Tri-*O*-methylglucitol	2.00	1.0	1.0
E	3,4,6-Tri-*O*-methylgalactitol	2.00	0.1	—
F	2,6-Di-*O*-methylgalactitol	3.14	1.12	—
G	4,6-Di-*O*-methylglucitol	3.24	0.1	—

[a]*T* relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol at 155°.

Methylation analysis of **3** gave 2,5-anhydro-1,4,6-tri-*O*-methyltalitol, 2,3,4,6-tetra-*O*-methylgalactopyranose, and 2,3,4-tri-*O*-methylglucopyranose in equimolar ratios. The appearance of tetra-*O*-methyl- instead of di-*O*-methyl-galactose, which was identified in the hydrolysate of the methylated polysaccharide, was due to elimination of 2-amino-2-deoxygalactose and acidic component residues which occurred as a result of deamination of the *N*-deacetylated polysaccharide. Thus, **3** is galactopyranosyl-(1→6)-glucopyranosyl-(1→3)-2,5-anhydrotalitol, and it provides important structural information about the polysaccharide. Firstly, **3** appeared to be practically the sole product of selective cleavage of the polysaccharide, thus proving that the polysaccharide was built up of regularly repeated oligosaccharide units. Secondly, the residues of 2-amino-2-deoxygalactose and glucose are in the linear chain of the polysaccharide, whereas the acidic component is attached as a branch to a galactose residue. Thirdly, the presence of galactopyranose at the non-reducing terminus of **3** substantiates the existence of galactopyranose in the polysaccharide, which did not follow from the methylation analysis of the polysaccharide.

For the determination of the configurations of the glycosidic linkages, the acetylated trisaccharide derivative **4** was oxidised with chromic anhydride[17,18]. The glucose and galactose residues survived the oxidation and, thus, were joined by α-linkages. The almost complete oxidation of the 2,5-anhydrotalitol moiety was unexpected, but confirmed by oxidation of 2,5-anhydrotalitol acetate. The β-D-configuration of the 2-acetamido-2-deoxygalactopyranosidic linkage followed from the results of enzymatic cleavage of **2**. The $[\alpha]_D^{20}$ values (+56.5° and +74°, respectively) of the polysaccharide and **3** were consistent with the determined configurations of the glycosidic linkages.

The foregoing results establish the chemical repeating-unit shown in the scheme for the polysaccharide of *Sh. dysenteriae* type 6.

EXPERIMENTAL

General. — Paper chromatography (p.c.) was effected by the descending method on FN-11 paper, using *A* 1-butanol–pyridine–water (6:4:3) and *B* ethyl acetate–acetic acid–water–formic acid (18:3:4:1). Electrophoresis was carried out on FN-11 paper in 25mM pyridine acetate buffer (pH 4.5) at 28 V/cm for 90 min. Detection was effected with alkaline silver nitrate, aniline hydrogen phthalate, and potassium periodate–benzidine.

Gel filtration was performed on Sephadex G-50 (column, 55 × 3.7 cm) and G-25 (column, 35 × 2.3 cm), using pyridine acetate buffer (10 ml of acetic acid, 4 ml of pyridine, and 1 litre of water); elution was monitored by the phenol–sulphuric acid procedure[19]. Amino sugars and hexoses were determined by g.l.c., as previously described[11]. G.l.c. was carried out on a Pye Unicam Series 104 (Model 64) instrument with a dual flame-ionization detector, and a glass column (90 × 0.4 cm) packed with 3% of ECNSS-M on Gaschrom Q (100–120 mesh). G.l.c.–m.s. was performed on a Varian instrument (Gnom MAT 111), using the column described above. M.s. was performed on a Varian CH-6 instrument. P.m.r. spectra were recorded for solutions in deuterium oxide at 90° on a Varian XL-100 instrument. Optical rotations were measured with a Perkin–Elmer polarimeter, Model 141. Solvents were evaporated *in vacuo* at <40°.

Isolation of lipopolysaccharide and polysaccharide. — The lipopolysaccharide (LPS) of *Sh. dysenteriae* type 6, strain 458-679, was isolated from dry bacterial cells by the standard procedure[8]. After sedimentation at 105,000 *g* for 4 h and lyophilization, LPS was obtained in a yield of 4% based on dry cells. The LPS (500 mg) was hydrolysed with dilute acetic acid (60 ml, pH 3.4, 100°) for 1.5 h. The precipitate of lipid A was removed by centrifugation at 105,000 *g* for 1 h, and degraded polysaccharide was separated by chromatography on Sephadex G-50. The appropriate fractions were combined and freeze-dried to give 125 mg of polysaccharide having $[\alpha]_D^{20}$ +56.5° (*c* 1, water), and 140 mg of oligosaccharide (core) material.

N-*Deacetylation of the polysaccharide.* — A portion (100 mg) of the polysaccharide was reduced with sodium borohydride (25 mg) in water (3 ml) for 2 h. The excess of reagent was destroyed by addition of acetic acid, and the solution was desalted by passing through a column of Sephadex G-25, and freeze-dried. The reduced polysaccharide was dried *in vacuo* over P_2O_5 at 70° and then heated with anhydrous hydrazine (2 ml) containing hydrazine sulphate (100 mg) in a sealed tube for 20 h at 105°. Hydrazine was removed by evaporation, and the residue was dried over conc. sulphuric acid *in vacuo* and then purified by gel filtration on Sephadex G-50. Fractions eluted within the void volume of the column were combined and freeze-dried to give the *N*-deacetylated polysaccharide (72 mg). The material obtained moved on paper electrophoresis towards the cathode as a sharp zone.

Periodate oxidation of the N-*deacetylated polysaccharide.* — A solution of the *N*-deacetylated polysaccharide (10 mg) in 0.1M sodium periodate (2 ml) was kept in the dark at room temperature for 48 h, then reduced with sodium borohydride

(80 mg) for 2 h, and acidified with acetic acid. The solution was passed through a column of Sephadex G-25, fractions eluted within the void volume were concentrated, and the residue was hydrolysed with 2M hydrochloric acid (2.5 ml) for 4 h at 100°. G.l.c. of the hydrolysate revealed the presence of galactose, 2-amino-2-deoxygalactose, and glucose in the ratios 1:1:0.1.

Selective cleavage of the N-*deacetylated polysaccharide with acid.* — Six portions (0.5 mg) of the *N*-deacetylated polysaccharide were hydrolysed severally with 2M hydrochloric acid (0.5 ml) at 100° in sealed tubes. After cooling, the solutions were concentrated over sodium hydroxide in a vacuum desiccator, and the residues were investigated with an amino acid analyser. The results were as follows:

Time (h)	1	2	3	4	6	8
Disaccharide **1** (%)	63	84	100	98	98	96
2-Amino-2-deoxygalactose (%)	4	5	7	8	9	11

The elution time of **1** was 0.62, and that of modified component *X* was 1.17, relative to that of 2-amino-2-deoxygalactose; the yield of **1** after hydrolysis for 3 h was taken as 100%.

After defining the optimal conditions of acid hydrolysis, the *N*-deacetylated polysaccharide (50 mg) was hydrolysed with 2M hydrochloric acid (2 ml) for 3 h, and the product mixture was fractionated on a column (11 × 0.6 cm) of Dowex-50W x8(H^+) resin. Neutral sugars and disaccharide **1** were eluted, respectively, with 25mM and 0.1M pyridine acetate buffer, the separation being monitored by electrophoresis. Fractions containing **1** (R_{GalN} 0.65) were combined and freeze-dried.

A portion of the residue was deaminated and reduced. G.l.c. of the products revealed 2,5-anhydrotalitol and galactitol in the ratio 1:1. A second portion of **1** was *N*-acetylated with acetic anhydride in the presence of Amberlite IRA-401(CO_3^{2-}) resin in aqueous methanol for 2 h. The solution was concentrated and the residue was subjected to p.c. (solvent *A*). After staining the guiding strips, the zone with R_{Glc} 0.58 was eluted to give **2**. A solution of **2** in 0.1M sodium phosphate–citrate buffer (pH 3.8, 0.2 ml) was incubated with a β-D-hexosaminidase preparation[13] from pig epididymis, and enzymatic hydrolysis was monitored by p.c. (solvent *A*). Disaccharide **2** was completely cleaved into galactose and 2-acetamido-2-deoxygalactose within 5 h.

Selective cleavage of the N-*deacetylated polysaccharide by deamination.* — A solution of *N*-deacetylated polysaccharide (25 mg) in water (1 ml) was treated in succession with 5% aqueous sodium nitrite (1.5 ml) and 33% acetic acid, and then kept for 40 min at room temperature. The mixture was passed through a column of KU-2(H^+) resin and then freeze-dried, and the residue was reduced with potassium borohydride. After routine treatment, the product was separated by preparative p.c. (solvent *B*) into two oligosaccharides having mobilities $R_{LACTOSE}$ 0.76 (**3**, 5.2 mg, major) and 1.10; **3** had $[\alpha]_D^{20}$ +74° (*c* 0.4, water). The oligosaccharide (0.6 mg) of higher mobility was composed of glucose and galactose in the ratio 1:1 (g.l.c.), and

was not further investigated. A portion of **3** was hydrolysed with 2M hydrochloric acid for 4 h at 100° to give 2,5-anhydrotalitol, galactose, and glucose in the ratios 1:1:1, identified by g.l.c. With acetic anhydride in pyridine, **3** gave the acetate **4**. Mass spectrum: *m/e* 848 (M−AcOH), 835 (M−CH_2OAc), 788 (M−2AcOH), 619, 561, 331, 319, and 273.

Methylation analysis of the polysaccharide and **3**. — Methylation was performed by the Hakomori procedure[14]. The methylated polysaccharide was purified by dialysis against distilled water, and the methylated oligosaccharide by extraction with chloroform from the reaction mixture diluted with water. The extract was washed with water, dried (Na_2SO_4), and concentrated. The methylated preparations were treated in succession with 85% formic acid for 24 h at 100° and 0.3M hydrochloric acid for 16 h at 100°, and then reduced with sodium borohydride. The resulting partially methylated alditols were investigated as acetates by g.l.c.–m.s. (see Fig. 1). The results of methylation analysis are presented in Table I. Mass spectrum for 3-*O*-acetyl-2,5-anhydro-1,4,6-tri-*O*-methyltalitol: *m/e* 203, 143, 129, 115, 111, 103, 101, 87, 75, 71, 59, 55, 45, and 43. The mass spectra of other partially methylated alditol acetates were interpreted according to Lindberg *et al.*[15].

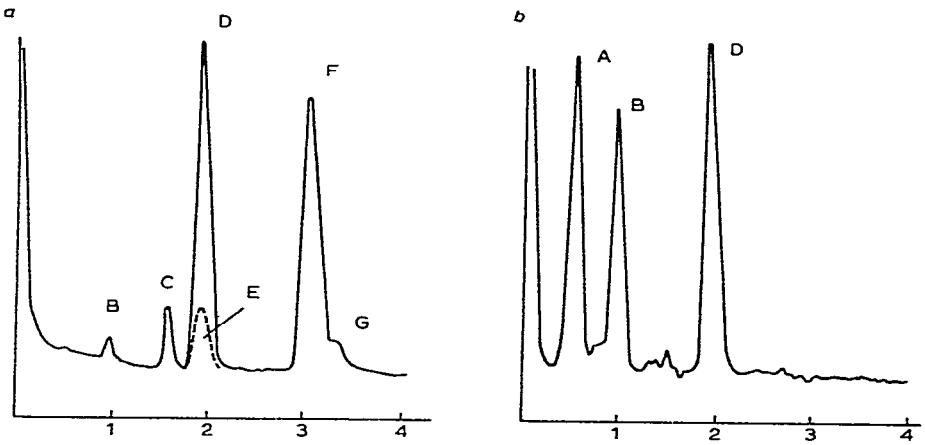


Fig. 1. Gas chromatograms of partially methylated alditol acetates obtained from methylated polysaccharide (*a*) and methylated oligosaccharide **3** (*b*). For key, see Table I.

Configuration of the glycosidic linkages. — Acetate **4** (2 mg) was dissolved in acetic acid (0.2 ml) and treated with chromic anhydride (20 mg) at 50° for 2 h. The solution was diluted with water and then extracted with chloroform. The extract was washed with water, dried (Na_2SO_4), and concentrated, and the residue was hydrolysed with 2M hydrochloric acid. G.l.c. of the product revealed galactose, glucose, and 2,5-anhydrotalitol in the ratios 1:1:0.15.

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