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DETERMINATION, BY METHYLATION ANALYSIS, OF THE GLYCOSYL-LINKAGE COMPOSITIONS OF MICROGRAM QUANTITIES OF COMPLEX CARBOHYDRATES*,†

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ABSTRACT

A methylation-analysis procedure has been developed by which the glycosyl-linkage compositions of microgram quantities of complex carbohydrates, including those containing hexosyluronic acid residues, can be determined. The effectiveness of the procedure was demonstrated by correctly determining the glycosyl-linkage compositions of 1 μg of a disaccharide and 5 μg of an acidic polysaccharide whose structures were unknown to the analyst. The development of a new technique, namely, reversed-phase chromatography on Sep-Pak C_{18} cartridges, to recover and purify microgram quantities of per-*O*-methylated complex carbohydrates from methylation-reaction mixtures, was critical to the success of the microscale procedure. The use of gas–liquid chromatography–mass spectrometry with multiple, selected-ion monitoring was also essential for identification and semiquantitation of the partially *O*-methylated alditol acetates derived from 1 to 5 μg of a complex carbohydrate.

INTRODUCTION

Glycosyl-linkage compositions of complex carbohydrates are usually determined by methylation analysis[1–4]. However, no reports exist of the use of methylation analysis to determine the glycosyl-linkage composition-analysis of less than 100 to 200 μg of a complex carbohydrate. Complex carbohydrates of biological origin are frequently available in only small amounts. A procedure has now been developed by which the glycosyl-linkage compositions of 1 to 5 μg of complex carbohydrates can be determined by using methylation analysis.

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EXPERIMENTAL

General. — Anthrone, β-cellobiose, β-gentiobiose, laminaran (cv *Laminaria hyperborea*), maltotriose (95%), and *myo*-inositol were purchased from Sigma Chemical Company, and used without purification. Lichenan (cv *Cetraria islandica*), also purchased from Sigma, was purified by three dissolution–freeze–thaw cycles[5], then dissolved in distilled water, and the solution lyophilized. *myo*-Inositol hexaacetate was obtained from Supelco, Inc. Dichlorodimethylsilane (99%) and decane (99 + %) were purchased from Aldrich Chemical Company. Dowex 50W-X12 (H^+) cation-exchange resin (2.35 meq/mL; 200–400 mesh), purchased from the J. T. Baker Chemical Company, was washed extensively with 2M hydrochloric acid, and then with de-ionized water until the pH and conductivity of the wash matched that of the de-ionized water. Dimethyl sulfoxide (Fisher Scientific Co.) was distilled at 1.33–3.99 kPa (10–30 mm Hg) from calcium hydride onto 4A molecular sieves. "HPLC"-grade water and acetonitrile, and spectrometric-grade methanol and dichloromethane were also purchased from Fisher.

A mixture of oligo-β-D-glucopyranosides (mainly of d.p. 8) that had been isolated from fungal cell-walls (*Phytophthora megasperm* f. sp. *glycinea*) was the gift of J. K. Sharp. Samples of the acidic polysaccharide secreted by *Rhizobium phaseoli* strain 127 K36 and of the per-*O*-methylated polysaccharide were donated by W. F. Dudman. The partially *O*-methylated alditol acetates resulting from methylation analysis of "rhamnogalacturonan I, a pectic polysaccharide isolated from sycamore cell-walls, were the gift of J. M. Lau.

Sep-Pak C_{18} cartridges were obtained from Waters Associates, Inc., and Baker-10 Octadecylsilica (ODS) extraction columns (1 mL) were purchased from J. T. Baker Chemical Company. Reactivials (0.3-mL size), Tuf-Bond Teflon-silicone discs, open-top Reactivial screw-caps, Hypovials (1-mL size), silicone-rubber septa, and aluminum seals were obtained from the Pierce Chemical Company.

Per-*O*-methylated cellobiitol (per-*O*-methylated 4-*O*-β-D-glucopyranosyl-D-glucitol) served as a model compound for optimizing several steps in the micro-scale, methylation-analysis procedure. This compound was prepared by reducing β-cellobiose with sodium borohydride, and permethylating the resulting disaccharide-alditol.

Silanization of glassware. — All glassware used for microscale methylation analysis was silanized, in order to minimize losses of native and per-*O*-methylated carbohydrates through adsorption, and to facilitate the transfer of carbohydrate-containing solutions. The glassware was silanized in a solution of 2% (v/v) dichlorodimethylsilane in toluene for at least 15 min. After the silanization solution had been decanted, the glassware was successively rinsed with methanol (technical grade), hot tap-water, and distilled water, and then allowed to dry.

Colorimetric estimation of hexoses. — The anthrone method[7] was used in method-development experiments in order to determine the amount of native or per-*O*-methylated carbohydrate present.

Per-O-methylation of carbohydrates. — Carbohydrate samples were per-*O*-methylated according to the procedure developed by Hakomori[8] as adapted by Sandford and Conrad[9]. Modifications of this procedure for per-*O*-methylation of microgram quantities of carbohydrates are presented in Results and Discussion.

Deuterio-reduction of the carboxyl groups of hexosyluronic acid residues. — Deuterio-reduction of the carboxyl groups of hexosyluronic acid residues was accomplished by using sodium borodeuteride[10], with the modifications for microscale analysis described (see Results and Discussion).

Gas–liquid chromatography. — A Hewlett–Packard model 5880 gas chromatograph equipped with dual flame-ionization detectors (here termed f.i.d., for "flame-ionization detection") was used for all analyses. Helium was employed both as the carrier and the make-up gas. Injection port and f.i.d. temperatures were 250°.

All g.l.c. separations were performed in a Hewlett–Packard methylsilicone–fused silica, capillary column (either 12 or 25 m × 0.20 mm i.d.).

Injections of partially *O*-methylated alditol acetates and per-*O*-methylated disaccharide-alditols were made from dichloromethane solution, using the split mode (split ratio 10:1), unless stated otherwise. The following oven-temperature program, unless stated otherwise, was used for split-mode analyses: 3 min at an initial temperature of 140°, increase to 240° at the rate of 6°/min, and hold for 5 min at 240°.

Splitless injections were often necessary for g.l.c. analysis of per-*O*-methylated alditol acetates derived from samples containing <25 μg of complex carbohydrate. In these instances, decane was added to an acetone or dichloromethane solution of the partially *O*-methylated alditol acetates, in order to produce a solution containing 50% (v/v) of decane before injection. The following program was used for splitless-injection, g.l.c. analyses: 5 min at an initial temperature of 120°, increase to 150° at 15°/min, and then to 240° at 6°/min, and hold for 5 min at 240°.

The f.i.d.–g.l.c. peak areas of the per-*O*-methylated alditol acetates and of the internal standard, *myo*-inositol hexaacetate, were converted into molar values by using their effective carbon-response factors[11].

Gas–liquid chromatography–mass spectrometry (g.l.c.–m.s.). — A Hewlett–Packard model 5985 g.l.c.–m.s. system that included an H-P model 5840 gas chromatograph and an H-P model 1000 data system was used for g.l.c.–m.s. analyses. Partially per-*O*-methylated alditol acetates and per-*O*-methylated oligosaccharide-alditols were separated on a wide-bore (15 m × 0.33 mm i.d.) fused-silica capillary column (DB-1; J and W Scientific Co.).

Samples were injected in the on-column mode by using a Hewlett–Packard on-column injector. Dichloromethane was added to a sample, and diluted with sufficient decane to produce a 1:1 (v/v) decane–dichloromethane solution before injection. The gas-chromatographic effluent was ionized by electron impact-mass spectrometry (e.i.-m.s.) at 70 eV, with a source temperature of 200°. Scan rates for g.l.c.–m.s. analyses were either 267 or 400 amu.s^{-1}. Dwell times of 50 ms were

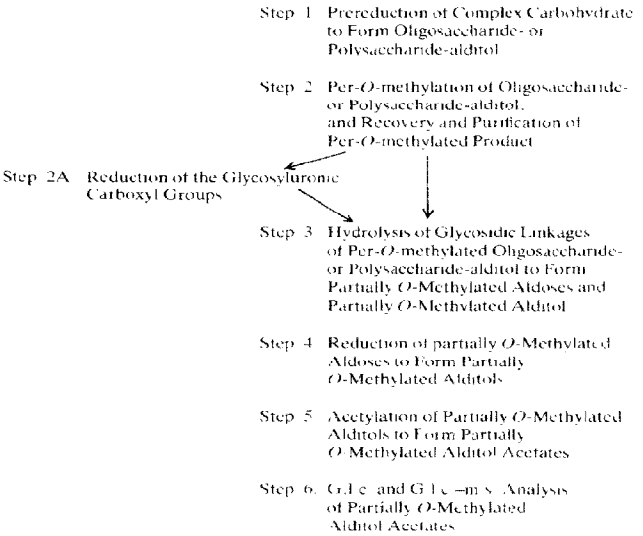
used for all ions during g.l.c.–m.s. analyses, with multiple, selected-ion monitorings.

G.l.c.–m.s. of per-*O*-methylated oligosaccharide-alditols was programmed at 150° for 2 min, then to 220° at 30°/min, and finally, to 340° at 6°/min.

RESULTS AND DISCUSSION

Development of the microscale, methylation-analysis procedure. — The methylation-analysis method of Hakomori[8] as used by Lindberg and co-workers[2,3] has been used effectively to determine the glycosyl-linkage compositions of samples containing >0.2 mg of complex carbohydrates. Glycosyl-linkage-composition analyses of samples containing as little as 1 μg required development of a scaled-down and refined procedure. Each step of the procedure was carefully examined in an attempt to identify those steps and reaction conditions that, when improved, would probably result in an effective, microscale procedure. Experiments were then performed to optimize the recovery of reaction products from each step.

The procedure developed for methylation analysis of microgram quantities of complex carbohydrates, summarized in Scheme 1, is described in detail in the fol-



Scheme 1. A flow-chart of the microscale procedure for the methylation analysis of a complex carbohydrate.

lowing section. It should be pointed out that the development of this procedure did not follow the sequence of steps as given next. In fact, the hydrolysis, reduction, and acetylation steps (Steps 3, 4, and 5) of the microscale procedure were studied first, so that they could be used to analyze the reaction products from the per-*O*-methylation step (Step 2). The reaction conditions and procedures for Steps 3, 4, and 5 were optimized by using per-*O*-methylated cellobiitol as the model carbohydrate. Microgram amounts of per-*O*-methylated cellobiitol were hydrolyzed, the products reduced, the alditols acetylated, and the resulting, partially *O*-methylated alditol acetates analyzed by f.i.d.–g.l.c., using *myo*-inositol hexaacetate as the internal standard.

Step 1. Prereduction of the complex carbohydrate

Oligosaccharides are reduced with sodium borodeuteride[5] before they are per-*O*-methylated. Polysaccharides are generally not reduced before they are per-*O*-methylated, except to prevent degradation[12]. However, before per-*O*-methylation, polysaccharides containing glycosyluronic acid residues should be passed through a cation-exchange resin (H^+ form) in order to convert all of the carboxyl groups into the protonated form; this treatment permits more-complete *O*-methylation of such polysaccharides[13].

Quantities of a complex carbohydrate amounting to <100 μg were prereduced in the following way. A solution of sodium borodeuteride in M ammonium hydroxide (100 μL; 10 μg of sodium borodeuteride/μL) was added to the complex carbohydrate in a test tube (13 × 100 mm). The contents of the tube were mixed by vortexing, and the mixture was kept for 2 h at room temperature. The excess of borodeuteride was converted into borate by adding glacial acetic acid (5–10 μL), and this was removed as trimethyl borate by evaporations with acidified methanol[14]. Acetic acid in methanol (0.5 mL, 10% [v/v]) was added to the tube containing the prereduced carbohydrate, the tube contents mixed, and the solvents evaporated to dryness. This evaporation procedure was repeated three times with 10% (v/v) acetic acid in methanol, and then four times with methanol (0.5 mL). The evaporations were accelerated by blowing gently with filtered air, while the test tube was heated to ~50° in a water bath.

The prereduced, borate-free complex carbohydrate was desalted by passing it through a column of cation-exchange resin (H^+ form). The sodium acetate formed during the evaporations of the trimethyl borate was thus converted into acetic acid, which was subsequently removed during lyophilization of the carbohydrate. In this procedure, the prereduced carbohydrate was dissolved in deionized water (0.2 mL), and transferred to a cation-exchange column with a pipet. The cation-exchange column, consisting of a 0.25-mL Luer-Lok syringe, contained ~0.1 mL of Dowex 50W-X12 resin (2.35 meq/mL) packed on 5 mm of silanized glass wool. (This quantity of resin can exchange approximately 100 times the amount of sodium ions present.) The sample solution containing the prereduced carbohydrate was gently pushed through the column with air pressure until the liq-

uid level reached the top of the resin bed. Four 0.2-mL portions of de-ionized water were then used to rinse the test tube and to elute the carbohydrate from the cation-exchange column. The first three portions were pushed through the column until the liquid level reached the top of the bed; the last 0.2-mL portion was pushed completely through the bed. The cation-exchange column effluent (~1 mL) containing the desalted, borate-free, prereduced complex carbohydrate was collected in a Hypovial (1-mL size), and the volume of the sample solution diminished to ~0.3 mL by evaporation, by blowing gently on the solution with a stream of filtered air at room temperature. The solution of the prereduced complex carbohydrate was then lyophilized to dryness. Following lyophilization, a Teflon-coated, magnetic stirring-bar (3 × 10 mm) was added to the Hypovial, and the prereduced carbohydrate was dried under diminished pressure over phosphorus pentaoxide for at least 12 h at 45° before per-*O*-methylation.

Step 2. Per-O-methylation of the prereduced complex carbohydrate, and recovery and purification of the per-O-methylated product

The procedure developed to per-*O*-methylate microgram quantities of prereduced complex carbohydrates was modified from the Hakomori method[5] as adapted by Sandford and Conrad[9]. It was observed that lessening the quantities of dimethyl sulfoxide, sodium dimethylsulfinyl anion, and methyl iodide used decreased the amounts of the contaminants that were introduced into the sample relative to the amounts of partially *O*-methylated alditol acetates being synthesized.

Microgram quantities of a prereduced complex carbohydrate were per-*O*-methylated in the following manner. A Hypovial containing the dried, desalted, prereduced complex carbohydrate was sealed with a silicone-rubber septum and an aluminum crimp-on cap. Dry dimethyl sulfoxide (0.25 mL) was added to the Hypovial through the septum cap. The mixture was then magnetically stirred, with precautions taken to minimize heating, until the prereduced complex carbohydrate dissolved. A period of 2 to 4 h was usually sufficient, although some polysaccharides were stirred overnight. (Note: microgram quantities of complex carbohydrates should be solubilized and deprotonated by stirring rather than by sonicating. Many spurious peaks were detected during g.l.c. analysis of partially *O*-methylated alditol acetates derived from sonicated carbohydrates. The presence of such contaminants could result from increased leaching of the septum of the Hypovial at the temperatures, ~50°, attained during sonication.) Sodium dimethylsulfinyl-anion solution (30 μL, 4M) was added to the Hypovial, and the mixture was stirred for 2 to 4 h at room temperature. An aliquot (*e.g.*, 5 μL) of the reaction mixture was tested at this time with triphenylmethane[5]. If the triphenylmethane test was negative, another 30-μL aliquot of the anion solution was added, and the mixture was stirred for an additional 2 h and retested with triphenylmethane. Finally, methyl iodide (35 μL) was slowly added during 10–15 s to the stirred mixture, which was maintained at 20 to 25° by cooling the Hypovial in water. The Hypovial was removed from the water, and the reaction mixture was stirred overnight at room tem-

perature. The per-*O*-methylated, prereduced complex carbohydrate was isolated from the mixture, and purified by reversed-phase chromatography as described next.

It was determined that, for successful methylation analysis, the per-*O*-methylated carbohydrate must be recovered in good yield and be completely separated from the other components of the methylation-reaction mixture, *i.e.*, dimethyl sulfoxide, sodium iodide, reaction side-products, and reagent and solvent impurities. Each of the purification procedures in use up to the time of this work (extraction, gel-permeation chromatography, and dialysis) posed serious drawbacks to the recovery and purification of microgram amounts of per-*O*-methylated complex carbohydrates. Therefore, a purification technique was developed to utilize reversed-phase chromatography on Sep-Pak C_{18} cartridges.

Microgram quantities of a per-*O*-methylated, prereduced complex carbohydrate were recovered and purified by reversed-phase chromatography in the following way. A Sep-Pak C_{18} cartridge (preflushed with 40 mL of 100% ethanol to remove contaminants from the cartridge and to increase the recovery of the per-*O*-methylated carbohydrate) was preconditioned by passing through the cartridge 2 mL of 100% acetonitrile (HPLC-grade) followed by 4 mL of water (HPLC-grade). The methylation-reaction mixture containing the per-*O*-methylated carbohydrate was diluted with an equal volume of HPLC-grade water to produce a 50% (v/v) dimethyl sulfoxide–water solution. This solution was slowly (1–2 drops per second) pushed, using the syringe plunger, through the cartridge bed until the liquid level was just above the resin bed. The Hypovial was rinsed with 1:1 (v/v) dimethyl sulfoxide–water (0.5 mL). This rinse was then loaded onto the cartridge in the same manner as the sample solution.

The more-polar contaminants in the methylation-reaction mixture, including the dimethyl sulfoxide and sodium iodide, were eluted from the sample-containing cartridge with four 2-mL flushes of water (HPLC-grade). The first three portions were pushed through the cartridge with the syringe plunger until the liquid level was just above the resin bed; the fourth portion was pushed completely through the cartridge. The less-polar contaminants from the methylation-reaction mixture were eluted from the cartridge by using 2-mL flushes of (*a*) 3:17 (v/v) and (*b*) 1:4 (v/v) acetonitrile–water. Generally, four 2-mL flushes of the former removed contaminants from per-*O*-methylated disaccharide-alditols, more of the latter solvent being used; three 2-mL flushes of solvent *a*, followed by one 2-mL flush of solvent *b*, removed contaminants from intermediate-sized, per-*O*-methylated oligosaccharide-alditols (d.p. 3–10); and two 2-mL flushes of solvent *a*, followed by two 2-mL flushes of solvent *b*, removed contaminants from larger per-*O*-methylated oligosaccharide-alditols (d.p. >10) and from per-*O*-methylated polysaccharides (or polysaccharide-alditols).

Per-*O*-methylated oligosaccharide-alditols (d.p. 2–10) were eluted from the Sep-Pak C_{18} cartridge with 100% acetonitrile (2 mL). The elution of larger per-*O*-methylated oligosaccharide-alditols (d.p. >10), and of per-*O*-methylated polysac-

charides (or polysaccharide-alditols), required 2 mL of 100% acetonitrile, followed by 4 mL of 100% ethanol. The effluent from the Sep-Pak C_{18} cartridge containing the per-*O*-methylated, prereduced complex carbohydrate was collected in a test tube (13 × 100 mm), and the solvents were evaporated to dryness by blowing with filtered air at room temperature. Dichloromethane (1 mL) was added to the test tube, the contents mixed by vortexing, and the solvent gently evaporated to dryness with filtered air. (The addition and evaporation of dichloromethane increased the proportion of per-*O*-methylated carbohydrate situated at the bottom of the test tube.) The per-*O*-methylated, prereduced complex carbohydrate was then trans-

TABLE I

RECOVERIES OF SEVERAL PER-*O*-METHYLATED OLIGOSACCHARIDE-ALDITOL PREPARATIONS FROM SEP-PAK C_{18} CARTRIDGES, USING VARIOUS ELUTION SOLVENTS

Sample[a]	*D.p.*[b]	*Recovery (%)*					*Total recovery (%)*
		Acetonitrile–water [3:1 (v/v)]	*100% Acetonitrile*	*100% Methanol*	*100% Ethanol*	*Chloroform*	
Per-*O*-methylated maltotritol (40 μg)	3	101 98	0 0	— —	— —	— —	101 98
Per-*O*-methylated, prereduced β-oligoglucopyranoside mixture (46 μg)	7–9	70 77	31 24	— —	— —	— —	101 101
Per-*O*-methylated, prereduced lichenan (42 μg)	60–250	— —	20 16	19 20	29 28	4 7	72 71

[a]Quantity loaded, in μg of glucose equivalents. [b]Degree of polymerization.

TABLE II

RECOVERIES OF MICROGRAM QUANTITIES OF PER-*O*-METHYLATED COMPLEX CARBOHYDRATES FROM SEP-PAK C_{18} CARTRIDGES

Sample	*Quantity loaded*[a] *(μg)*	*Recovery (%)*
Per-*O*-methylated cellobiitol	17	104
Per-*O*-methylated, prereduced laminaran	24	79
Per-*O*-methylated, prereduced lichenan	30	66
Per-*O*-methylated, *R. phaseoli* strain 127 K36 polysaccharide	31	97

[a]In glucose equivalents of the per-*O*-methylated carbohydrate.

ferred to a Reactivial (0.3-mL size) with three ~0.2-mL aliquots of dichloromethane, and the dichloromethane was gently evaporated with filtered air. The per-*O*-methylated, prereduced complex carbohydrate in the Reactival was then ready for further analysis.

The use of reversed-phase chromatography on Sep-Pak C_{18} cartridges enabled recovery in good yields of per-*O*-methylated products derived from complex carbohydrates as diverse in size as disaccharide-alditols and large polysaccharides from methylation-reaction mixtures (see Tables I and II). Moreover, reversed-phase chromatography on Sep-Pak C_{18} cartridges, which can be used to recover and purify from 1 μg to 5 mg of per-*O*-methylated complex carbohydrates, required only 15 to 20 min per sample (excluding solvent-evaporation time).

Step 2A. Reduction of the glycosyluronic carboxyl groups

It was found to be easier to reduce the carboxyl groups of methyl-esterified hexosyluronic acid residues in microgram quantities of a per-*O*-methylated polysaccharide than it would be to carboxyl-reduce, recover, and per-*O*-methylate such quantities of the native polysaccharide. The procedure for reducing the carboxyl groups of hexosyluronic acid residues in microgram quantities of complex carbohydrates was adapted from that of Dutton *et al.*[10], and involved the reaction of the per-*O*-methylated carbohydrate with a solution of sodium borodeuteride in 27:73 (v/v) 95% ethanol–oxolane. Methyl-esterified hexosyluronic acid residues reduced by this procedure were converted into the corresponding 6,6-dideuterio-labeled hexosyl residues.

The reduction of glycosyluronic carboxyl groups was accomplished as follows. A solution of sodium borodeuteride in 27:73 (v/v) 95% ethanol–oxolane was prepared by dissolving 1.8 mg of sodium borodeuteride in 0.21 mL of 95% ethanol. Oxolane (0.51 mL) was added to the solution of sodium borodeuteride in 95% ethanol, and the resulting solution mixed. An aliquot (75 μL) of the solution of sodium borodeuteride in 27:73 (v/v) 95% ethanol–oxolane was added to the Reactivial containing the methylated, prereduced complex carbohydrate. The vial was sealed, its contents mixed, and the sample kept for 18 h at room temperature. The vial was heated for 1 h at 70°, and then allowed to cool to room temperature. The excess of borodeuteride was converted into borate by adding acetic aid (5 μL) to the vial. The borate was removed as its volatile trimethyl ester by evaporating the sample with 10% (v/v) acetic acid in methanol and with methanol, as described in Step 4.

The sodium acetate formed during the evaporations of the trimethyl borate would, if not removed, interfere with the hydrolysis of the deuterio-carboxyl-reduced, partially *O*-methylated polysaccharide. The residual sodium acetate was removed as described (Step 1), with the following changes. The Dowex 50W-X12 resin (0.1 mL) was rinsed with several bed volumes of HPLC-grade water, followed by several bed volumes of 1:1 (v/v) ethanol–HPLC-grade water, before sample loading. The carboxyl-reduced, partially *O*-methylated polysaccharide was sol-

ubilized in 1:1 (v/v) ethanol–HPLC-grade water (0.2 mL) by vibrating the sample in this solvent mixture for 15 min in a small, cleaning sonicator. The resulting solution was loaded onto the cation exchange column, and two 0.2-mL portions of 1:1 (v/v) ethanol–HPLC-grade water were used to rinse the Reactivial and to elute the carboxyl-reduced, partially *O*-methylated polysaccharide. The effluent (~0.6 mL) from the cation-exchange column was collected in a clean Reactivial and the solvents were evaporated with filtered air at room temperature.

Step 3. Hydrolysis of the glycosidic linkages of the per-O-methylated, prereduced complex carbohydrate, to form partially O-methylated aldoses and alditol

Per-*O*-methylated, prereduced complex carbohydrate (derived from 100 μg or less of complex carbohydrate) was hydrolyzed into its partially *O*-methylated aldoses and partially *O*-methylated alditol in the following way. Trifluoroacetic acid (TFA) (75 μL; 2M) was added to the per-*O*-methylated carbohydrate in a Reactivial. The vial was sealed, its contents mixed, and the vial heated for 1 h at 121°. The TFA was evaporated with filtered air at room temperature, until the sample was just dry. Methanol (75 μL) was added to the vial, its contents mixed, and the methanol gently evaporated to dryness with filtered air at room temperature. The addition of methanol to the Reactivial rinsed traces of TFA from the walls of the vial to the bottom of the vial cone, evaporation of this solution decreased the amount of residual TFA.

Step 4. Reduction of the partially O-methylated aldoses to form partially O-methylated alditols

Partially *O*-methylated aldoses (derived from 100 μg or less of complex carbohydrate) were reduced to the corresponding partially *O*-methylated alditols in the following way. A 50-μL aliquot of a solution of sodium borodeuteride in 95% ethanol that was 1 M in ammonia (10 μg of sodium borodeuteride/μL) was added to the Reactivial containing the partially *O*-methylated aldoses, and the contents of the vial were thoroughly mixed. The Reactivial was sealed and kept for at least 2 h at room temperature. Acetic acid (5 μL) was then added to the vial to convert the excess of borodeuteride into borate. 1:9 (v/v) Acetic acid–methanol (50 μL) was added to the vial, its contents were mixed, and the solvents blown dry with filtered air at room temperature (to volatilize trimethyl borate). Three additional evaporations were performed with 1:9 (v/v) acetic acid–methanol (50 μL each), followed by one evaporation to dryness with methanol (75 μL).

Step 5. Acetylation of the partially O-methylated alditols to form partially O-methylated alditol acetates

The partially *O*-methylated alditols (derived from 100 μg or less of a complex carbohydrate) were acetylated in the following way. Acetic anhydride (50 μL) was added to the Reactivial containing the partially *O*-methylated alditols, the vial sealed, its contents mixed, and the vial heated for 3 h at 121°. The vial was then at

lowed to cool to room temperature, and toluene (50 μL) was added. The contents of the vial were mixed, and the solvents evaporated *just to dryness*. (Note: great care must be taken during evaporation of the acetic anhydride–toluene to prevent the loss of some of the more volatile, partially *O*-methylated alditol acetates.) One additional evaporation *just to dryness* with toluene (50 μL) was then performed. Dichloromethane (25 μL) was added to the vial, its contents were mixed, and the dichloromethane was allowed to evaporate at room temperature. This addition and evaporation, which tended to concentrate the partially *O*-methylated alditol acetates at the bottom of the vial cone, permitted a smaller volume of injection solvent(s) to be used in subsequent g.l.c. and g.l.c.–m.s. analyses.

Step 6. Gl.c. and g.l.c.–m.s. analyses of the partially O-methylated alditol acetates

G.l.c. analysis of partially O-methylated alditol acetates. — The relative molar percentages of the partially *O*-methylated alditol acetates and the identities of the derivatives (as ascertained by g.l.c.–m.s., and by their g.l.c. retention-times relative to those of authentic derivatives or of internal standards) were used to determine the glycosyl-linkage composition of complex carbohydrates. The relative molar percentages of the partially *O*-methylated alditol acetates resulting from methylation analysis of a complex carbohydrate could be determined by g.l.c. *if* a sufficient quantity of sample (greater than 10 μg) was available for analysis. If lesser amounts of sample were available, the analysis was performed by g.l.c.–m.s. (see later).

Microgram quantities of partially *O*-methylated alditol acetates were analyzed by g.l.c. in the following way. Dichloromethane (50–100 μL) was added to samples containing a complex carbohydrate (25–100 μg), and f.i.d.-g.l.c. analysis of such samples was achieved by using split injection (10:1 split ratio). Partially *O*-methylated alditol acetates derived from samples containing 10 to 25 μg of a complex carbohydrate were dissolved in 20 to 50 μL of 1:1 (v/v) decane–dichloromethane; they were analyzed by f.i.d.-g.l.c. by using splitless or on-column injections. Under these circumstances, decane was added to the dichloromethane solution to permit the "solvent effect"[16,17] to take place at injection temperatures above the boiling point of the dichloromethane. This mixed-solvent system allowed injections to be made at an initial oven-temperature of 120°, which significantly shortened the analyses by decreasing the oven-temperature recycle-times. The relative areas of the peaks resulting from f.i.d.-g.l.c. analysis in the split, splitless, or on-column mode were converted into relative mole percentages by using the effective-carbon-response factors[11] of the derivatives.

The recoveries of partially *O*-methylated alditol acetates slowly increased (~10 to 15%) after the addition of the solvent(s) to the Reactivials, and then stabilized after 2 to 3 h. This phenomenon was probably due to slow solubilization of the derivatives from the interstices of the crystalline sodium acetate that remained in the Reactivial cone. Therefore, the partially *O*-methylated alditol acetates were equilibrated with the injection solvent(s) for at least 3 h before f.i.d.-

g.l.c. or g.l.c.–m.s. analysis, in order to ensure maximum sensitivity, accuracy, and reproducibility.

G.l.c.-m.s. analysis of partially O-methylated alditol acetates derived from 10 μg or more of complex carbohydrate. — Partially *O*-methylated alditol acetates derived from more than 10 μg of complex carbohydrate can be identified by standard g.l.c.–m.s., for example, by obtaining e.i. mass spectra scanning from *m/z* 85 to 350. Quantitation of the partially *O*-methylated alditol acetates derived from such samples is readily achieved by f.i.d.-g.l.c. analysis (see earlier).

G.l.c.–m.s. analysis of partially O-methylated alditol acetates derived from less than 10 μg of complex carbohydrate by using multiple, selected-ion monitoring. — Partially *O*-methylated alditol acetates derived from 10 μg or less of complex carbohydrate require a more sensitive and selective means of identification and quantitation, because of the small amount of sample and the presence of contaminants (plasticizers, reagent and solvent impurities, and reaction side-products), in some instances at concentrations comparable to those of the partially *O*-methylated alditol acetates themselves. Therefore, partially *O*-methylated alditol acetates derived from 10 μg or less of carbohydrate must be identified, and their relative molar percentages estimated by g.l.c.–m.s. in the multiple, selected-ion mode ("stacked") electron-impact, mass chromatography[18].

A multiple, selected-ion-monitoring (m.s.i.m.) computer program (Hewlett Packard, program 3200) was used for g.l.c.–m.s. (m.s.i.m.) analysis of partially *O*-methylated alditol acetates. This program was used to acquire data with two groups of up to 20 ions each. The mass spectrometer computer switched from the first ion group to the second ion group at a predetermined run time.

The diagnostic fragment-ions for all possible partially *O*-methylated alditol acetates were predicted by applying the fragmentation rules described by Björndal

TABLE IIIA

ION GROUP I[a] OF THE ION-GROUP FILE FOR G.L.C.–M.S. (M.S.I.M.) ANALYSIS OF PARTIALLY *O*-METHYLATED ALDITOL ACETATES DERIVED FROM PREREDUCED HEXOSE AND FROM HEXOSYL RESIDUES[b]

Derivatives of prereduced hexose and terminal hexosyl residues	*Diagnostic and abundant e.i.-m.s. fragment-ions (m/z)*
Prereduced 2-linked	130, 162, 206, 250
Prereduced 3-linked	90, 206, 249, 250
Prereduced 4-linked	89, 205, 249, 250
Prereduced 6-linked	117, 161, 178, 249
Terminal	118, 161, 162, 205

[a]Group I ions: derivatives of prereduced hexose and terminal hexosyl residues, *m/z*: 89, 90, 117, 118, 130, 161, 162, 178, 205, 206, 249, and 250. [b]This Table includes the diagnostic and abundant e.i.-m.s. fragment-ions for g.l.c.–m.s. (m.s.i.m.) analysis of partially *O*-methylated alditol acetates derived from prereduced hexopyranose and terminal hexopyranosyl residues, but not those of the derivatives of prereduced hexofuranose and terminal hexofuranosyl residues.

TABLE IIIB

ION GROUP II[a] OF THE ION-GROUP FILE FOR G.L.C.–M.S. (M.S.I.M.) ANALYSIS OF PARTIALLY *O*-METHYLATED ALDITOL ACETATES DERIVED FROM PREREDUCED HEXOSE AND FROM HEXOSYL RESIDUES[b]

Derivatives of hexosyl residues	*Diagnostic and abundant e.i.-m.s. fragment-ions (m/z)*
Terminal	118, 161, 162, 205
2-Linked	161, 190
3-Linked	118, 161, 234, 277
4-Linked	118, 233
6-Linked	118, 162, 189, 233
2,3-Linked	161, 262
2,4-Linked	130, 190, 233
2,6-Linked	129[c], 130, 189, 190
3,4-Linked	118, 305
3,6-Linked	118, 189, 234, 305
4,6-Linked	118, 261
2,3,4-Linked	129[c], 185
2,3,6-Linked	189, 262
2,4,6-Linked	190, 261
3,4,6-Linked	118, 333

[a]Group II ions: Derivatives of terminal, linear, and branched hexosyl residues; *m/z*: 118, 129[c], 130, 161, 162, 185, 189, 190, 205, 233, 234, 261, 262, 277, 305, and 333. [b]This Table includes the diagnostic and abundant e.i.-m.s. fragment-ions for g.l.c.–m.s. (m.s.i.m.) analysis of partially *O*-methylated alditol acetates derived from terminal, linear, and branched hexopyranosyl residues, but not those of the derivatives of terminal, linear, and branched hexofuranosyl residues. [c]The fragment-ion *m/z* 129 was not included in ion-group II for g.l.c.–m.s. (m.s.i.m.) analyses of partially *O*-methylated alditol acetates derived from hexosyl residues in the data presented herein, but its inclusion in the ion-group file makes the identification of such derivatives more conclusive.

et al.[2]. Prudent choice of these diagnostic fragment-ions allowed the partially *O*-methylated alditol acetates to be identified, and their relative molar percentages to be determined semiquantitatively from the relative peak-areas of the fragment ions in the "total-m.s.i.m.-ion" mass chromatogram, that is, the mass chromatogram that is the sum of all selected-ion, mass chromatograms obtained during g.l.c.–m.s. (m.s.i.m.) analysis. In general, the relative abundances of only a few, diagnostic fragment-ions were needed in order to determine the arrangement of *O*-methyl and *O*-acetyl groups on a particular alditol, whereas a greater number of fragment-ions was generally required for semiquantitation.

An ion-group file was constructed for g.l.c.–m.s. (m.s.i.m.) analysis of all of the partially *O*-methylated alditol acetates that could be derived both from prereduced hexose* (see Table IIIA) and hexosyl residues (see Table IIIB)[2]. Several fragment-ions of the partially *O*-methylated hexitol acetates derived from pre-

*Only those derivatives of prereduced hexose residues having one glycosyl residue attached to them could be analyzed by the e.i.-m.s. fragment-ions listed in Table IIIA. Ion group I can be modified to include branched, prereduced hexose residues by addition of the ions at *m/z* 146, 173, 174, 217, 218, 233, 234, 277, and 288.

reduced hexosyl residues, which were also abundant fragment-ions of commonly detected contaminants (especially, *m/z* 101, 102, and 103), are not included in the ion-group files. The omission of such fragment-ions diminished the areas of the "total m.s.i.m.-ion" mass chromatography of these derivatives, but did not interfere with their identification.

The ion-group file was divided into groups in such a way that the diagnostic and abundant fragment-ions of the earlier-eluted, partially *O*-methylated hexitol acetates (derived from prereduced hexose[*] and terminal hexosyl residues) were included in ion-group I, and the diagnostic and abundant fragment-ions of the later-eluted derivatives (derived from terminal, linear, and branched hexosyl residues) were included in ion-group II. The analysis of ion-group I (see Table IIIA) was begun after the elution of the decane peak of the solvent mixture, but before the elution of partially *O*-methylated alditol acetates derived from any of the prereduced hexose residues. The analysis of ion-group I was terminated after the elution of all of the derivatives of prereduced hexose residues[*] and, when possible, before the elution of partially *O*-methylated alditol acetates derived from any of the terminal hexosyl residues, although, when necessary, these could be analyzed by ion-group I. The analysis of ion-group II (see Table IIIB) was begun immediately upon termination of the analysis of ion-group I, and was continued until the end of the g.l.c.–m.s. (m.s.i.m.) analysis.

Similar ion-group files for g.l.c.–m.s. (m.s.i.m.) analysis of partially *O*-methylated alditol acetates derived from prereduced pentose, pentosyl, prereduced deoxyhexose, and deoxyhexosyl residues may be constructed. When a sample contains hexosyl, pentosyl, and deoxyhexosyl residues, two analyses by g.l.c.–m.s. (m.s.i.m.), using different ion-group files for each analysis, may be necessary for a complete analysis of the sample.

Partially *O*-methylated alditol acetates were introduced into the g.l.c.–m.s. (m.s.i.m.) in the following way. Dichloromethane (10 μl) was added to the Reactivial containing the partially *O*-methylated alditol acetates. The vial contents were mixed, the dichloromethane solution was diluted with decane (10 μl), and the contents were remixed. An aliquot (1 to 2 μl) of this solution was analyzed, using either splitless or on-column injections.

An example of a "total-m.s.i.m.-ion" mass chromatogram obtained from g.l.c.–m.s. (m.s.i.m.) analysis of the partially *O*-methylated alditol acetates derived from 1 μg of maltotriose is presented in Fig. 1.

Evaluation of microscale methylation procedures by methylation analysis of microgram quantities of complex carbohydrates having structures unknown to the analyst

The effectiveness of the procedures just described for microscale methylation analysis and for g.l.c.–m.s. (m.s.i.m.) analysis was tested by analyzing microgram quantities of two complex carbohydrates whose structures were unknown to the analyst. A 1-μg sample of a nonacidic oligosaccharide (designated "X") and a 5-μg

[*]See footnote on previous page.

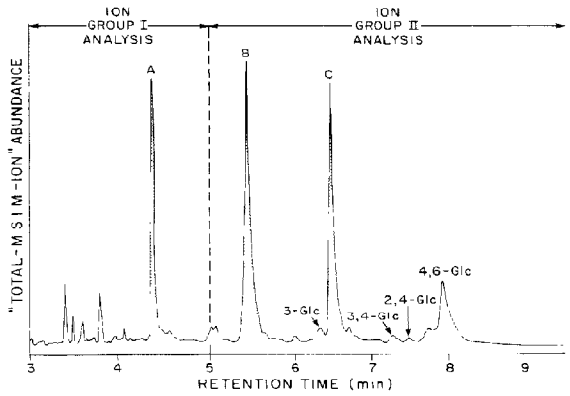


Fig. 1. The "total-m.s.i.m.-ion" mass chromatogram obtained from g.l.c.–m.s. (m.s.i.m.) analysis of the partially *O*-methylated alditol acetates derived from 1 μg of maltotriose. [The analysis of ion group I (see Table IIIA) was begun 3 min after sample injection, and was continued for 2 min. The analysis of ion group II (see Table IIIB) was begun upon termination of the ion-group I analysis, and was continued for 5 min. Peak A corresponds to the partially *O*-methylated alditol acetate (4-*O*-acetyl-1-deuterio-1,2,3,5,6-penta-*O*-methyl-D-glucitol) derived from the prereduced, 4-linked glucose residue (PR 4-Glc), peak B to the partially *O*-methylated alditol acetate (1,5-di-*O*-acetyl-1-deuterio-2,3,4,6-tetra-*O*-methyl-D-glucitol) derived from the terminal glucosyl group (T-Glc), and peak C to the partially *O*-methylated alditol acetate (1,4,5-tri-*O*-acetyl-1-deuterio-2,3,6-tri-*O*-methyl-D-glucitol) derived from the 4-linked glucosyl residue (4-Glc). The relative mole ratios of the three derivatives as obtained from the relative areas of the corresponding peaks in the chromatogram were the following: PR 4-Glc, 0.63; T-Glc, 0.93; and 4-Glc, 1.00. The partially *O*-methylated alditol acetates derived from 3-linked, 3,4-linked, 2,4-linked, and 4,6-linked glucosyl residues result from incomplete *O*-methylation of the trisaccharide-alditol.]

sample of an acidic polysaccharide (designated "z") were analyzed.

Analysis of oligosaccharide "x". — Oligosaccharide "x" was prereduced and per-*O*-methylated, and the resulting per-*O*-methylated oligosaccharide-alditols recovered and purified as described (Steps 1 and 2). Four 2-mL portions of 3:17 (v/v) acetonitrile–water were used in the polar-contaminant-cleanup procedure (Step 2), to ensure that the per-*O*-methylated oligosaccharide-alditols would be retained on the Sep-Pak C_{18} cartridges if oligosaccharide "x" was a disaccharide. The per-*O*-methylated oligosaccharide-alditol was removed from the Sep-Pak cartridge by eluting with 100% acetonitrile (2 mL). The per-*O*-methylated oligosaccharide-alditol derived from oligosaccharide "x" was fully hydrolyzed, reduced, and acetylated (Steps 3–5). The resulting, partially *O*-methylated alditol acetates were then analyzed by g.l.c.–m.s. (m.s.i.m.), using the ion-group file for the derivatives of prereduced hexose and hexosyl residues (see Tables IIIA and IIIB).

The "total-m.s.i.m.-ion" mass chromatogram obtained from g.l.c.–m.s. (m.s.i.m.) analysis of the partially *O*-methylated alditol acetates derived from 1 μg

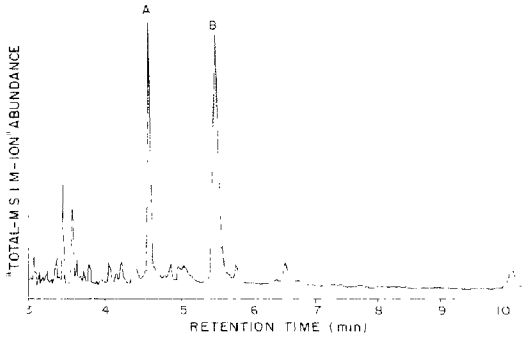


Fig. 2. The "total-m.s.i.m.-ion" mass chromatogram obtained from g.l.c.–m.s. (m.s.i.m.) analysis of the partially *O*-methylated alditol acetates resulting from methylation analysis of 1 μg of oligosaccharide "x". [The analysis of ion group I (see Table IIIA) was begun 3 min after sample injection, and was continued for 2.5 min. The analysis of ion group II (see Table IIIB) was begun upon termination of the ion-group I analysis and was continued for 5 min. The shaded peaks correspond to the partially *O*-methylated alditol acetates, and unshaded peaks, to contaminants. Peak A corresponds to a partially *O*-methylated alditol acetate (6-*O*-acetyl-1-deuterio-1,2,3,4,5-penta-*O*-methylhexitol) derived from a prereduced, 6-linked hexose residue, and peak B to a partially *O*-methylated alditol acetate (1,5-di-*O*-acetyl-1-deuterio-2,3,4,6-tetra-*O*-methylhexitol) derived from a terminal hexosyl group.]

of oligosaccharide "x" is shown in Fig. 2. The two principal peaks, A and B, in the mass chromatogram were identified from their background-subtracted, multiple selected-ion mass spectra (see Table IV). Peak A corresponded to a partially *O*-methylated alditol acetate derived from a prereduced, 6-linked hexose residue, and peak B to a partially *O*-methylated alditol acetate derived from a terminal hexosyl residue. The molar percentage of the partially *O*-methylated alditol acetate derived from the prereduced 6-linked hexose residue, relative to the molar percentage of the partially *O*-methylated alditol acetate derived from the terminal hexosyl residue, was, as determined from the relative peak areas, 0.63 (theoretical molar ratio, 1.0). Such a relative molar ratio for these derivatives was expected. (Partially *O*-methylated alditol acetates derived from prereduced hexose residues are particularly volatile, and subject to losses during sample workup, because they are substituted with five *O*-methyl groups and only one *O*-acetyl group.) The 6-linked hexose and terminal hexosyl residues were both shown to be derived from glucose by comparison of their g.l.c. retention-times to those of standards. Thus, oligosaccharide "x" was correctly found to be gentiobiose.

Analysis of polysaccharide "z". — The 5-μg sample of acidic polysaccharide "z" was passed through 0.1 mL of Dowex 50W-X12 cation-exchange resin in order to ensure that any carboxyl groups formed would be in the protonated form (Step 1). The sample was per-*O*-methylated, and the per-*O*-methylated polysaccharide recovered and purified as described (Step 2). The carboxyl groups of the glyco-

TABLE IV

THE BACKGROUND-SUBTRACTED, MULTIPLE SELECTED-ION, MASS SPECTRA OF THE PARTIALLY *O*-METHYLATED ALDITOL ACETATES DERIVED FROM 1 μg OF OLIGOSACCHARIDE "x"

Complex carbohydrate	*Glycose or glycosyl residue*	*Relative ion abundance*[a,b]											
		m/z											
		89	90	117	118	130	161	162	178	205	206	249	250
Oligosaccharide "x" (1 μg)	*Peak A* (PR 6-Hex)	11	40	**100**	5.9	0	**47**	6.6	**23**	0.5	<0.1	**1.0**	0.2
	Peak B (T-Hex)	16	2.6	100[c]	**89**	0	55	**67**	1.6	**30**	3.4	0.1	0

[a]Both compounds were detected with ion group I (see Table IIIA), because peak B was eluted before the start of the analysis of ion-group II. [b]The relative ion abundances of the diagnostic fragment-ions of the partially *O*-methylated alditol acetates (see Table IIIA) are shown in bold type. [c]The fragment ion *m/z* 117 is the base peak of this multiple selected-ion mass spectrum, because of a co-eluting contaminant. Contaminants that are present in mixtures of partially *O*-methylated alditol acetates often yield fragment ions at this *m/z* value[4].

syluronic acid residues of the per-*O*-methylated polysaccharide were reduced, and the product desalted (Step 2A).

The desalted, deuterio-carboxyl-reduced, partially *O*-methylated, polysaccharide sample was fully hydrolyzed, its hydrolysis products reduced, and the alditols acetylated (Steps 3–5). The resulting, partially *O*-methylated alditol acetates were analyzed by g.l.c.–m.s. (m.s.i.m.) as described (Step 6). The e.i. mass spectra of the partially *O*-methylated alditol acetates derived from the dideuterio-carboxyl-reduced hexosyluronic acid residues include diagnostic fragment-ions that are two *m/z* units greater than the same derivatives of hexosyl residues. An ion-group file for g.l.c.–m.s. (m.s.i.m.) analysis of partially *O*-methylated alditol acetates derived from deuterio-carboxyl-reduced hexosyluronic acid residues was constructed in the following way. Diagnostic and abundant e.i.-m.s. fragment-ions of partially *O*-methylated alditol acetates derived from terminal, linear, and branched carboxyl-reduced hexosyluronic acid residues were selected according to the fragmentation

TABLE V

THE ION-GROUP FILE FOR G.L.C.–M.S. (M.S.I.M.) ANALYSIS OF PARTIALLY *O*-METHYLATED ALDITOL ACETATES DERIVED FROM CARBOXYL-DIDEUTERIO-REDUCED HEXOSYLURONIC ACID RESIDUES AND FROM HEXOSYL RESIDUES[a]

Derivatives of carboxyl-dideuterio-reduced hexosyluronic acid residues and of hexosyl residues	*Ion group*[b] *Diagnostic and abundant e.i.-m.s. fragment-ions*
Terminal hexosyl	118, 161, 162, 205
2-Linked hexosyl	161, 190
3-Linked hexosyl	118, 161, 234, 277
4-Linked hexosyl	118, 162, 233
6-Linked hexosyl	118, 162, 189, 233
Terminal hexosyluronic acid	118, 162, 191, 235
2,3-Linked hexosyl	161, 262
2,4-Linked hexosyl	190, 233
2,6-Linked hexosyl	189, 190
2-Linked hexosyluronic acid	190, 191
3,4-Linked hexosyl	118, 305
3,6-Linked hexosyl	118, 189, 234, 305
3-Linked hexosyluronic acid	118, 191, 234, 307
4,6-Linked hexosyl	118, 261
4-Linked hexosyluronic acid	118, 263
2,3,6-Linked hexosyl	189, 262
2,3-Linked hexosyluronic acid	191, 262
2,4,6-Linked hexosyl	190, 261
2,4-Linked hexosyluronic acid	190, 263
3,4,6-Linked hexosyl	118, 273, 333
3,4-Linked hexosyluronic acid	118, 275, 335

[a]This Table does not include the diagnostic and abundant e.i.-m.s. fragment-ions of partially *O*-methylated alditol acetates derived from 2,3,4-linked hexosyl and from carboxyl-dideuterio-reduced 2,3,4-linked hexosyluronic acid residues. [b]Group I ions: Derivatives of carboxyl-dideuterio-reduced hexosyluronic acid residues and of hexosyl residues, *m/z* 118, 161, 162, 189, 190, 191, 205, 233, 234, 235, 261, 262, 263, 273, 275, 277, 305, 307, 333, and 335.

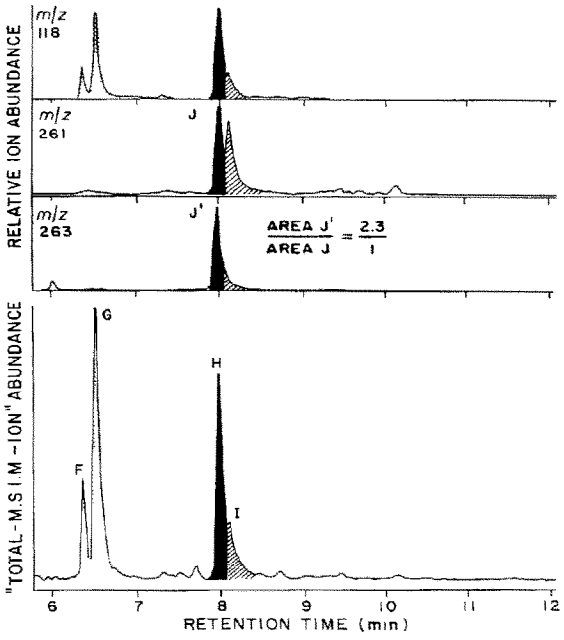


Fig. 3. The selected-ion, mass chromatograms for *m/z* 118, 261, and 263, and the "total-m.s.i.m.-ion" mass chromatogram obtained from g.l.c.–m.s. (m.s.i.m.) analysis of the partially *O*-methylated alditol acetates resulting from per-*O*-methylation, and carboxyl reduction with sodium borodeuteride, of 5 μg of polysaccharide "z", followed by hydrolysis, reduction, and acetylation of the deuterio-carboxyl-reduced, partially *O*-methylated polysaccharide. [The ion-group file used for this g.l.c.–m.s. (m.s.i.m.) analysis is listed in Table V. Peak F corresponds to a partially *O*-methylated alditol acetate (1,3,5-tri-*O*-acetyl-1-deuterio-2,4,6-tri-*O*-methylhexitol) derived from a 3-linked hexosyl residue; peak G to a partially *O*-methylated alditol acetate (1,4,5-tri-*O*-acetyl-1-deuterio-2,3,6-tri-*O*-methylhexitol) derived from a 4-linked hexosyl residue; peak H to partially *O*-methylated alditol acetates (1,4,5,6-tetra-*O*-acetyl-1-deuterio-2,3-di-*O*-methylhexitol and 1,4,5,6-tetra-*O*-acetyl-1,6,6-trideuterio-2,3-di-*O*-methylhexitol) derived from 4,6-linked hexosyl and carboxyl-dideuterio-reduced 4-linked hexosyluronic acid residues, respectively; and peak I to a partially *O*-methylated alditol acetate (1,4,5,6-tetra-*O*-acetyl-1-deuterio-2,3-di-*O*-methylhexitol) derived from another 4,6-linked hexosyl residue. The glycosyl-linkage composition of the polysaccharide as determined from the relative areas of the peaks in this chromatogram is given in Table VII.]

rules of Björndal *et al.*[2] (see Table V). To this ion-group file were added the diagnostic and abundant e.i.-m.s. fragment-ions of partially *O*-methylated alditol acetates derived from terminal, linear, and branched hexosyl residues. The diagnostic and abundant fragment-ions of partially *O*-methylated alditol acetates de-

TABLE VI

THE BACKGROUND-SUBTRACTED, MULTIPLE SELECTED-ION MASS SPECTRA OF THE PARTIALLY *O*-METHYLATED ALDITOL ACETATES DERIVED FROM 5 μg OF POLYSACCHARIDE "z"[a]

Complex carbohydrate	*Glycose or glycosyl residue*	*Relative ion abundance*[b]																			
		m/z																			
		118	120	131	161	162	175	189	190	191	203	233	234	261	262	263	275	277	305	333	335
Polysaccharide "z" (5 μg)	*Peak F* (3-Hex)	**100**	8.8	0	**47**	1.0	2.2	0.1	1.2	0	4.2	0	**19**	0	0.2	**0**	**0**	1.3	**0**	0	0
	Peak G (4-Hex)	**100**	3.1	17	0	9.8	0	0	0	0	0.1	**41**	3.6	0	0	0	0	0.1	0	0	0
	Peak H (4,6-Hex and 4-Hex A[c])	**100**	2.9	1.0	3.7	5.4	0	1.3	0	0	3.6	0.1	0	**3.2**	1.5	**6.4**	0	0	0.1	0	0
	Peak I (4,6-Hex)	**100**	3.5	2.2	0	5.3	0	0.2	0	0	0	0	0.1	**18**	2.3	0	0	0	0.1	0	0.1

[a]Polysaccharide "z" was per-*O*-methylated, and the methyl-esterified carboxyl groups of hexosyluronic acid residues were reduced with sodium borodeuteride. The carboxyl-deuterio-reduced, partially *O*-methylated polysaccharide was fully hydrolyzed, to give partially *O*-methylated aldoses, the partially *O*-methylated aldoses were reduced, to give partially *O*-methylated alditols, and the partially *O*-methylated alditols were acetylated. [b]The relative abundances of all of the e.i.-m.s. fragment-ions that were analyzed are given; those of the diagnostic fragment-ions of the partially *O*-methylated alditol acetates (see Table V) are shown in bold type. [c]4-Hex A refers to the partially *O*-methylated alditol acetate (1,4,5,6-tetra-*O*-acetyl-1,6,6-trideuterio-2,3-di-*O*-methyl-D-glucitol) that was derived from a carboxyl-dideuterio-reduced, hexosyluronic acid residue.

rived from prereduced hexose residues were not included, because these derivatives are not important in methylation analyses of polysaccharides. The ion-group file consisted of only one ion group, because all of the known, partially *O*-methylated alditol acetates derived from dideuterio-carboxyl-reduced hexosyluronic acid residues and from hexosyl residues could be analyzed by using 20 e.i.-m.s. fragment-ions.

The "total-m.s.i.m.-ion" mass chromatogram obtained from g.l.c.–m.s. (m.s.i.m.) analysis of the partially *O*-methylated alditol acetates derived from 5 μg of polysaccharide "z" is shown in Fig. 3. Partially *O*-methylated alditol acetates derived from 3-linked hexosyl (peak F), 4-linked hexosyl (peak G), and two different 4,6-linked hexosyl residues (H and I) were identified from their respective, background-subtracted, multiple selected-ion mass spectra (see Table VI). The partially *O*-methylated alditol acetates corresponding to peaks H and I were identified, from their g.l.c. retention-times relative to those of authentic derivatives, as being de-

TABLE VII

THE GLYCOSYL-LINKAGE COMPOSITION OF THE ACIDIC POLYSACCHARIDE SECRETED BY *R. phaseoli* STRAIN 127 K44

Glycosyl residue	*Position of O-methyl groups*	*Glycosyl linkage deduced*	*Glycosyl-linkage composition (mol %)*		
			I[a] *Theoretical*	*II*[b] *5 μg*	*III*[c] *1 mg*
Glucosyl	2,4,6	3	11	11	11
Glucosyl	2,3,6	4	33	44	39
Glucosyl and 6,6-dideuterio-labeled glucosyl[d]	2,3	4,6	44	33	41
Galactosyl[e]	2,3	4,6	11	11	9

[a]The theoretical glycosyl-linkage composition of the polysaccharide as calculated from its repeating unit[20]. [b]The polysaccharide was per-*O*-methylated, and the methyl-esterified carboxyl groups of the hexosyluronic acid residues were reduced with sodium borodeuteride. The carboxyl-deuterio-reduced, partially *O*-methylated polysaccharide was hydrolyzed, to give partially *O*-methylated aldoses, the partially *O*-methylated aldoses were reduced, to give partially *O*-methylated alditols, and the partially *O*-methylated alditols were acetylated. The resulting, partially *O*-methylated alditol acetates were identified, and their relative mole ratios determined, by g.l.c.–m.s. (m.s.i.m.) analysis, using the ion-group file listed in Table V. [c]The glycosyl-linkage composition of the polysaccharide as obtained by Franzén *et al.*[20]. The polysaccharide was per-*O*-methylated and the product carboxyl-reduced with lithium aluminum deuteride. The carboxyl-deuterio-reduced, partially *O*-methylated polysaccharide was fully hydrolyzed, and its hydrolysis products were reduced, and the alditols acetylated. The resulting, partially *O*-methylated alditol acetates were quantitated by f.i.d.-g.l.c. [d]The 6,6-dideuterio-labeled, 4,6-linked glucosyl residues are derived from 4-linked glucosyluronic acid residues. The mole percentages of the partially *O*-methylated alditol acetates derived from the 4,6-linked glucosyl and 6,6-dideuterio-labeled, 4,6-linked glucosyl residues were combined, to make it easier to compare the results of Franzén and co-workers with the results obtained by using g.l.c.–m.s. (m.s.i.m.). [e]The partially *O*-methylated alditol acetates derived from 4,6-linked galactosyl residues originate from the terminal galactosyl residues that had pyruvic acetal groups attached to them at O-4 and O-6 prior to hydrolysis of the per-*O*-methylated polysaccharide.

rived from 4,6-linked glucosyl and 4,6-linked galactosyl residues, respectively. Furthermore, a partially *O*-methylated alditol acetate derived from a 6,6-dideuterio-labeled, 4,6-linked glucosyl residue was detected in peak H (see Fig. 4, selected-ion mass chromatograms for *m/z* 118 and 263), indicating that polysaccharide "z" also contained 4-linked glucosyluronic acid residues.

Polysaccharide "z" was later revealed to be the acidic polysaccharide secreted by *R. phaseoli* strain 127 K44, whose nine-glycosyl-residue repeating-unit was determined by Franzén *et al.*[19]. The glycosyl-linkage composition of this polysaccharide, determined by using 5 μg of the sample, is compared in Table VII with the theoretical glycosyl-linkage composition of the polysaccharide calculated from its repeating unit and with that found by Franzén and his co-workers by using milligram amounts of the polysaccharide. In both analyses, the molar percentage of the sum of the 4,6-linked glucosyl residues and the 6,6-dideuterio-labeled, 4,6-linked glucosyl residues (derived from carboxyl-reduced, 4-linked glucosyluronic acid residues) is lower than that predicted from the repeating unit of the polysaccharide. This is partly due to loss of glycosyluronic acid residue degraded by base-catalyzed elimination reactions during per-*O*-methylation of some types of polysaccharide[19,20]. However, the amount of 6,6-dideuterio-labeled, 4,6-linked glucosyl residues reported in this analysis was lower than that obtained by Franzén *et al.*[19]. This underreduction may have occurred because of differences in the methods of reduction. Franzén *et al.* used lithium aluminum deuteride rather than sodium borodeuteride. However, for some polysaccharides, sodium borodeuteride has been shown to give complete reduction[5,10].

In summary, the glycosyl-linkage compositions of samples containing 1 to 5 μg of two complex carbohydrates whose structures were not known *a priori* by the analyst have been determined, by using the microscale, methylation-analysis procedure. The success of microscale, methylation analysis in detecting and identifying the hexosyluronic acid residues in 5 μg of an acidic polysaccharide has also been demonstrated.

GENERAL DISCUSSION

The effectiveness of microscale, methylation analysis as an analytical technique has been demonstrated by determining the glycosyl-linkage compositions of 1- and 5-μg samples of a representative oligo- and poly-saccharide, respectively. Such quantities represent a 20- to 100-fold decrease in the amount of complex carbohydrate previously needed for methylation analysis.

The use of g.l.c.–m.s. (m.s.i.m.) was important for the analysis of partially *O*-methylated alditol acetates derived from samples containing 1 to 10 μg of a complex carbohydrate. G.l.c.–m.s. (m.s.i.m.) was found to be about seven times as sensitive as standard g.l.c.–m.s. in the analysis of such samples. However, for the analysis of partially *O*-methylated alditol acetates derived from samples containing *more* than 10 μg of carbohydrate, standard g.l.c.–m.s. is preferable to g.l.c.–m.s.

(m.s.i.m.), because more fragment-ions can be used to identify the various derivatives. Quantitation of the relative molar percentages of the partially *O*-methylated alditol acetates from such samples is best accomplished by f.i.d.-g.l.c. analysis.

Glycosyl-linkage composition-analysis of samples containing less than 1 μg of a complex carbohydrate was not possible by using the methylation-analysis procedure described. The amount of a complex carbohydrate that can be analyzed is limited by the ability of the g.l.c.–m.s. (m.s.i.m.) techniques to identify and semi-quantitate partially *O*-methylated alditol acetates in the presence of relatively high concentrations of noncarbohydrate components. The amounts of such "contaminants" must be decreased in order to permit the analysis of lesser quantities of partially *O*-methylated alditol acetates.

Small amounts of a per-*O*-alkylated oligosaccharide-alditol prepared by this procedure can be analyzed directly by g.l.c.–m.s.[3,21]. In some cases, direct g.l.c.–m.s. will give all the structural information necessary to determine the structure of the per-*O*-alkylated oligosaccharide-alditol. A 1-μg sample of gentiobiose (unknown "x") was prereduced, methylated (Steps 1 and 2, and Fig. 1), and analyzed directly by g.l.c.–m.s., yielding a mass spectrum comparable to those obtained on larger amounts of per-*O*-alkylated disaccharide-alditols[21].

The procedures described herein for microscale methylation analysis can also be readily adapted for glycosyl-sequence analysis of microgram quantities of complex carbohydrates. In a method for glycosyl-sequence analysis[21], a complex carbohydrate was converted into partially *O*-ethylated, partially *O*-methylated oligosaccharide-alditols (d.p. 2–4) by reduction and *O*-ethylation of the partially *O*-methylated oligosaccharides produced by partial depolymerization of the per-*O*-methylated carbohydrate. The methods used to per-*O*-methylate the carbohydrate, to recover and purify the per-*O*-methylated product, to depolymerize partially the per-*O*-methylated carbohydrate, to reduce the partially *O*-methylated carbohydrate, to reduce the partially *O*-methylated oligosaccharides formed, and to ethylate the resulting partially *O*-methylated oligosaccharide-alditols, can be adapted directly from the microscale, methylation-analysis procedure here described. The partially *O*-ethylated, partially *O*-methylated oligosaccharide-alditols can then be recovered and purified by reversed-phase chromatography on Sep-Pak C_{18} cartridges. Thus, the microscale, methylation-analysis procedure will not only decrease the amount of sample needed for glycosyl-linkage analysis of a complex carbohydrate, but will lessen the amount required for glycosyl-sequence analysis as well.

REFERENCES

1 N. SHARON, *Complex Carbohydrates, Their Chemistry, Biosynthesis and Functions*, Addison-Wesley, Boston, 1975.
2 H. BJORNDAL, C. G. HELLERQVIST, B. LINDBERG, AND S. SVENSSON, *Angew. Chem., Int. Ed. Engl.*, 9 (1970) 610–619.
3 B. LINDBERG, *Methods Enzymol.*, 28 (1972) 178–195.

4 H. RAUVALA, J. FINNE, T. KRUSIUS, J. KARKKAINEN, AND J. JARNEFELT, *Adv. Carbohydr. Chem. Biochem.*, 38 (1981) 389–415
5 B. S. VALENT, A. G. DARVILL, M. McNEIL, B. K. ROBERTSEN, AND P. ALBERSHEIM, *Carbohydr. Res.*, 79 (1980) 165–192.
6 M. McNEIL, A. G. DARVILL, AND P. ALBERSHEIM, *Plant Physiol.*, 70 (1982) 1586–1591
7 Z. DISCHE, *Methods Carbohydr. Chem.*, 1 (1962) 477–514
8 S. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205–208
9 P. A. SANDFORD AND H. E. CONRAD, *Biochemistry*, 5 (1966) 1508–1517
10 G. G. S. DUTTON, K. L. MACKIE, A. V. SAVAGE, AND M. D. STEPHENSON, *Carbohydr. Res.*, 66 (1978) 125–131.
11 D. P. SWEET, R. H. SHAPIRO, AND P. ALBERSHEIM, *Carbohydr. Res.*, 40 (1975) 217–225
12 B. LINDBERG, J. LONNGREN, AND S. SVENSSON, *Adv. Carbohydr. Chem. Biochem.*, 31 (1975) 185–240
13 A. S. CHAUDHARI, C. T. BISHOP, AND W. F. DUDMAN, *Carbohydr. Res.*, 28 (1973) 221–231
14 P. ALBERSHEIM, D. J. NEVINS, P. D. ENGLISH, AND A. KARR, *Carbohydr. Res.*, 5 (1967) 340–345
15 H. RAUVALA, *Carbohydr. Res.*, 72 (1979) 257–260
16 K. GROB AND G. GROB, *J. Chromatogr. Sci.*, 7 (1969) 584 and 587
17 K. GROB AND K. GROB JR., *J. HRC. CC*, 1 (1978) 57.
18 G. C. TSAY, G. DAWSON, AND S.-S. J. SUNG, *J. Biol. Chem.*, 251 (1976) 5852
19 L.-E. FRANZEN, W. F. DUDMAN, M. McNEIL, A. G. DARVILL, AND P. ALBERSHEIM, *Carbohydr. Res.*, 117 (1983) 157–167
20 K. SHIMIZU, *Carbohydr. Res.*, 92 (1981) 65–74.
21 M. McNEIL, A. DARVILL, P. AMAN, L.-E. FRANZEN, AND P. ALBERSHEIM, *Methods Enzymol.*, 83 (1982) 3–45