Carbohydrate Research, 168 (1987) 219–243
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

SELECTIVE DEGRADATION OF THE GLYCOSYLURONIC ACID RESIDUES OF COMPLEX CARBOHYDRATES BY LITHIUM DISSOLVED IN ETHYLENEDIAMINE*

JAMES M. LAU†, MICHAEL MCNEIL‡, ALAN G. DARVILL**, AND PETER ALBERSHEIM**,§
Department of Chemistry, University of Colorado, Boulder, and Complex Carbohydrate Research Center and School of Chemical Sciences, University of Georgia, Athens, GA, (U.S.A.)
(Received October 20th, 1986, accepted for publication in revised form, April 28th, 1984)

ABSTRACT

Lithium metal dissolved in ethylenediamine had been demonstrated to cleave a 3-linked glycosyluronic acid-containing polysaccharide [A. J. Mort and W. D. Bauer, *J. Biol. Chem.*, 257 (1982) 1870–1875]. The present study with model compounds has established that, by lithium treatment, carbohydrates are cleaved at the sites of the glycosyluronic acid residues, regardless of the point at which other glycosyl residues are attached to the glycosyluronic acid residues. Treatment of carbohydrates with lithium metal dissolved in ethylenediamine also results in cleavage of methyl glycosides, reduction of aldoses, and cleavage of methyl ethers and pyruvic acetals of glycosyl residues. Model compounds were used to demonstrate that oligosaccharides containing only neutral glycosyl residues are largely stable to the reaction conditions (except for the reduction of the glycose residue of each oligosaccharide). Thus, a general procedure for the selective cleavage of underivatized carbohydrates at the glycosyluronic acid residues is described.

INTRODUCTION

Elucidating the structure of a complex carbohydrate requires that the sequence of its glycosyl residues be determined. The available techniques for determining the sequence of the glycosyl residues of carbohydrates include ^{1}H-n.m.r.[1–3] and ^{13}C-n.m.r.[1–3] spectroscopy and mass spectrometry[3–5]. These techniques usually require that large oligo- and poly-saccharides be cleaved into a mixture of smaller oligosaccharides. A number of methods for forming oligosaccharide

*Supported by U.S. Department of Energy Grant No. 09-85ER13424.000 and 09-85ER13426.000.
†Current address: Hewlett–Packard Company, West 120 Century Road, Paramus, NJ 07653, U.S.A.
‡Current address: Department of Microbiology, Colorado State University, Fort Collins, CO 80523, U.S.A.
**Current address: Carbohydrate Center, Russell Laboratory, P.O. Box 5677, Athens, GA 30613, U.S.A.
§To whom correspondence should be addressed.

0008-6215/87/$ 03.50

fragments from larger carbohydrates have been described[6-20]. Methods that cleave only selected glycosyl residues of large carbohydrates lessen the effort subsequently needed to separate, characterize structurally, and piece together the fragment oligosaccharides, in order to deduce the structure of the intact complex carbohydrate.

A new method for cleaving underivatized glycosyluronic acid-containing carbohydrates by use of lithium dissolved in ethylenediamine was reported by Mort and Bauer[21]. Lithium and other metals of group I dissolved in amine solvents have been used for a variety of chemical transformations of complex carbohydrates[22-29]. Lithium dissolved in an amine solvent is a powerful reducing reagent. The Birch reduction[22] and reduction of aldehydes, ketones, carboxylic acids, and conjugated alkenes[23-27] are examples of the wide-ranging effects of this reagent. Lithium in ethylenediamine has also been used to remove *O*-methyl substituents from glycosyl residues[28]. Indeed, Mort and Bauer[21] were attempting to remove an *O*-methyl substituent from a polysaccharide when they discovered that lithium in ethylenediamine effects degradation of glycosyluronic acid residues. Although they reported that this reagent cleaved a polysaccharide secreted by *Rhizobium japonicum* 3I1b 138 at the site of the glycosyluronic acid residues, the products of the cleavage were not fully characterized by them.

We attempted to use lithium in ethylenediamine to fragment underivatized rhamnogalacturonan I, a pectic polysaccharide[29] from plant cell-walls. The reagent fragmented the acidic polysaccharide, but yielded products that were not easily characterized. Because we required a well defined reaction, we decided to attempt to develop lithium treatment as a general method for the selective cleavage of underivatized glycosyluronic acid-containing carbohydrates. Our goals were to (*1*) develop a procedure for the isolation of the products of the cleavage reaction, (*2*) identify the products of the cleavage reaction, and (*3*) study the effects of the treatment on the other glycosyl residues and on the non-glycosyl substituents of carbohydrates. We now report the effect of lithium–ethylenediamine treatment on a variety of structurally defined, neutral and acidic, complex carbohydrates.

EXPERIMENTAL

Materials. — L-Arabinose, D-galactose, D-glucose, L-rhamnose, D-galacturonic acid, methyl α-D-galactopyranoside, methyl α-D-glucopyranoside, cellobiose, gentiobiose, laminarabiose, 2-deoxy-D-*lyxo*-hexose, methyl 2,3-di-*O*-methyl-α-D-glucopyranoside, and ethylenediamine were obtained from Sigma Chemical Co. Lithium wire (45 mg/cm), hexanal, hexanol, hexanoic acid, 2-hydroxyhexanoic acid, sodium hydride, and Dowex 50W X-12-200 (H^+) cation-exchange resin were obtained from Aldrich Chemical Co. Acetic acid, acetic anhydride, pyridine, toluene, dimethyl sulfoxide, trifluoroacetic acid, and methyl iodide were obtained from Fisher Scientific Co. Sodium borohydride and sodium borodeuteride were obtained from Alfa Products. Bio-Gel P-2 (100–200 mesh) was obtained from Bio-

Rad Laboratories. Trisil Z was obtained from Pierce Chemical Co. The extracellular polysaccharides respectively secreted by *Klebsiella* strains K-17, K-44, and K-62 were the gift of Dr. G. G. S. Dutton (Univ. of British Columbia). The trisaccharide obtained by treatment of the polysaccharide secreted by *R. japonicum* strain 3I1b 138 with HF was the gift of Dr. Andrew Mort (Oklahoma State Univ.). The extracellular polysaccharide secreted by *R. phaseoli* strain K-87 was the gift of Jan Darvill of this laboratory.

Preparation of methyl α-D-galactosiduronic acid. — D-Galacturonic acid (50 mg) was treated overnight in boiling M methanolic HCl (20 mL) under reflux. The solution was evaporated to dryness under a stream of filtered air, the residue dissolved in the minimal volume of hot 5:5:2 ethyl acetate–ethanol–water and the solution kept until crystals formed. The crystals were collected, recrystallized from the same solvent, washed with acetone (5 mL), dissolved in D_2O, and shown by ^{1}H-n.m.r. spectroscopy to be methyl (methyl α-D-galactopyranosid)uronate[30,31]. A 1-mg sample of this methyl ester was de-esterified in M NaOH solution at room temperature, the reaction being monitored by thin-layer chromatography; all starting material had been converted into the de-esterified product after 1 h. The mixture was applied to a column of Dowex 50W X-12-200 (H^+) ion-exchange resin, to remove the Na^+ ions, and the eluate was lyophilized. The ^{1}H-n.m.r. spectrum of a solution of the resulting white powder in D_2O confirmed the conversion of methyl (methyl α-D-galactosid)uronate into methyl α-D-galactosiduronic acid.

Colorimetric analysis of hexoses and uronic acids. — The anthrone[32] and Blumenkrantz–Asboe-Hansen[33] methods were used to determine the content of hexosyl and glycosyluronic acid residues, respectively, in samples of complex carbohydrates.

Per-O-acetylation of carbohydrates. — Samples were per-*O*-acetylated[34] with 1:1 acetic anhydride–pyridine for 25 min at 120°.

Per-O-methylation of carbohydrates. — Samples were per-*O*-methylated according to the method of Harris and co-workers[35], which is a modification of the Hakomori procedure[36]. Per-*O*-methylated carbohydrates were purified on Sep-Pak C-18 cartridges (Waters and Associates) as described[34].

Gas–liquid chromatography. — A Hewlett–Packard (HP) 5880 g.l. chromatograph with flame-ionization detection was used throughout. G.l.c. separations of partially-*O*-acetylated, partially-*O*-methylated methyl glycosides and per-*O*-acetylated monosaccharides were performed on a capillary column (either 10 m or 30 m × 0.25 mm i.d.) of SP-2330 (Supelco, Inc.), respectively operated isothermally at 220° and 235°. Separations of per-*O*-acetylated mono-*O*-glycosylalditols and per-*O*-acetylated disaccharides were performed on a capillary column (15 m × 0.32 mm i.d.) of DB-1 (J & W Scientific Co.). For such compounds, the oven temperature was maintained for 3 min at an initial temperature of 140°, and increased at 6°/min to 250°, where it was maintained for 10 min.

Combined g.l.c.–m.s. — An HP 5985 g.l.c.–m.s. system (HP 5840 g.c., HP-1000 series-E data system) was used for all g.l.c.–m.s. analyses. Analyses of per-*O*-

acetylated alditols and per-*O*-acetylated monosaccharides were performed on a capillary column (30 m × 0.25 mm i.d.) of SP-2330 (Supelco, Inc.). For such compounds, the oven temperature was maintained for 2 min at an initial temperature of 80°, increased at 30°/min to 220°, and then at 6°/min to 240°. Ionization for electron impact-mass spectrometry (e.i.-m.s.) was performed at 70 eV and at a source temperature of 200°. Chemical ionization-mass spectrometry (c.i.-m.s.) was performed with the source temperature at 150°; isobutane was the reagent gas. G.l.c.–m.s. of per-*O*-acetylated mono-*O*-glycosylalditols, per-*O*-acetylated disaccharides, per-*O*-methylated disaccharides, and per-*O*-methylated oligoglycosylalditols was performed on a capillary column (25 mm × 0.32 mm i.d.) of DB-1 (J & W Scientific Co.) using on-column injection. Separation was achieved by maintaining the oven for 3 min at 140°, then increasing at 30°/min to 220°, and then 6°/min to 340°.

Gel-permeation chromatography. — Gel-permeation chromatography was conducted on a column (71 × 1.6 cm) of Bio-Gel P-2 eluted with H_2O. Fractions (1.5 mL) were collected.

Fragmentation of complex carbohydrates with lithium in ethylenediamine. — The published procedure[21] for fragmenting complex carbohydrates by treatment with lithium in ethylenediamine was modified for use with the small amounts of carbohydrate that were available. The carbohydrate (1–5 mg) was added to a screw-capped tube (13 × 100 mm) that contained a small, Teflon-coated, stirring bar. The sample was dried *in vacuo* overnight at 40°. Ethylenediamine (1 mL) that had been dried over 4A molecular sieves was added, and the mixture was stirred until the carbohydrate had dissolved; dissolution of some of the carbohydrates required sonication. In cases where larger amounts of carbohydrate were available (*e.g.*, 5–10 mg), a larger screw-capped tube (20 × 120 mm) was used, and additional ethylenediamine (1 mL/5 mg of carbohydrate) was added. Upon dissolution of the sample, small (2–5 mm) pieces of lithium wire (45 mg/cm) were added to the solution. After the solution turned deep blue (normally, 5–20 min), additional small pieces of lithium wire were added as needed to maintain the deep blue color. The relationship between the amount of lithium added and the amount of carbohydrate in the sample was variable due to the different constituents of the carbohydrate samples. The amount of lithium added was determined by that amount required to maintain the deep blue color for a predetermined duration. A 1-h treatment was used for the fragmentation of most of the carbohydrates.

The lithium reaction can be quenched by adding a protic solvent to consume the excess of lithium metal and solvated electrons. Quenching the lithium reaction with methanol as described[21] resulted in the formation of a white precipitate that complicated subsequent separation of carbohydrate from reaction by-product (presumably lithium methoxide), ethylenediamine, and methanol. This side effect was unacceptable when evaluating small quantities of carbohydrates (0.1–0.5 mg), owing to the losses incurred when handling solid precipitates. Therefore, a modified procedure for quenching the reaction was developed.

Water was selected to quench the lithium reaction because it solubilized all of the reaction products and by-products. The procedure developed was as follows. Samples that had been lithium-treated were cooled in an ice bath (to solidify the mixture) and H_2O (5 mL) was slowly added. The mixture was vortexed, with intermittent cooling in the ice bath, until dissolution was complete. The resulting clear solution was transferred to a 50-mL round-bottomed flask, and toluene (25 mL) was added in order to form an azeotrope with the ethylenediamine and the H_2O. The flask was shaken and the mixture rotoevaporated to dryness; toluene (25 mL) was added, and the mixture rotoevaporated to dryness twice more. This procedure is faster and more efficient than that published[21], involving removal of the ethylenediamine and methanol under diminished pressure in the presence of H_2SO_4.

The resulting powdery, white residue (LiOH and carbohydrate) was cooled in an ice bath and dissolved in H_2O (5 mL). The solution was cooled in an ice bath, titrated to pH 4.5 with glacial acetic acid, and passed through a 10-mL column of Dowex AG-50W X12 (H^+) ion-exchange resin to remove the lithium ions, and the resin washed with H_2O. A large excess of the ion-exchange resin is needed owing to the low affinity of the Dowex AG-50W X12 resin for lithium ions. The eluate from this column was lyophilized to yield the "lithium-treated" carbohydrate products.

Lithium treatment of monosaccharides and monosaccharide methyl glycosides. — Monosaccharides and monosaccharide methyl glycosides were treated with lithium and the products isolated as follows. Duplicate samples of L-arabinose (2.0 mg), D-galactose (2.0 mg), D-glucose (2.0 mg), L-rhamnose (2.1 mg), methyl β-L-arabinoside (2.0 mg), methyl α-D-galactoside (2.0 mg), methyl α-D-glucoside (2.1 mg), methyl α-L-rhamnoside (2.0 mg), and methyl α-D-galactosiduronic acid (0.5 mg) were dried overnight *in vacuo* at 40°, dissolved in ethylenediamine (1 mL), and treated with lithium for 1 h as already described. The reactions were quenched with 5-mL portions of H_2O and the mixtures transferred to 50-mL, round-bottomed flasks, and rotoevaporated three times with toluene (25 mL). The residues were dissolved in H_2O (5 mL), titrated to pH 4.5 with glacial acetic acid, washed through columns of Dowex AG-50W X12 with H_2O, and lyophilized. Each lyophilizate was dissolved in H_2O (5 mL), a 0.5-mL aliquot of the solution was evaporated under a stream of filtered air in a screw-capped tube (13 × 100 mm). *myo*-Inositol (0.1 mL of a 0.1 mg/mL solution) was added to each of the aliquots as a g.l.c. standard. The aliquots were evaporated to dryness, the residues per-*O*-acetylated, and the per-*O*-acetylated alditols or per-*O*-acetylated monosaccharides analyzed by g.l.c. and g.l.c.–m.s.

For g.l.c. retention time and g.l.c.–mass-spectral comparison, standard per-*O*-acetylated alditols of each sugar were prepared by sodium borohydride reduction (0.2 mL of a 10 mg/mL solution in M NH_4OH for 1 h), the borate removed by four co-evaporations with a solution of 10% acetic acid in methanol under a stream of filtered air, and the resulting alditols per-*O*-acetylated. Standard per-*O*-acetylated

monosaccharides and monosaccharide methyl glycosides were prepared. An additional 0.5-mL aliquot of the solution containing the products of lithium-treated methyl α-D-galactosiduronic acid was evaporated to dryness and per-*O*-(trimethylsilyl)ated with Trisil Z for 20 min at 80°. The mixture was evaporated to dryness, the residue dissolved in hexane, and the resulting per-*O*-(trimethylsilyl)ated derivative analyzed by g.l.c.–m.s.

Lithium treatment of selected disaccharides. — Cellobiose (2.0 mg), 4-*O*-β-D-galactosyl-D-mannose (0.5 mg), laminarabiose (2.0 mg), 3-*O*-β-D-galactosyl-L-arabinose (0.5 mg), sophorose (0.5 mg), and gentiobiose (2.0 mg) were treated with lithium, and the products isolated as for the monosaccharides. The lyophilizates from each sample were dissolved in H_2O (5 mL). Two 0.5-mL aliquots were taken from each solution, and analyzed by different methods.

In one method, the 0.5-mL aliquot was evaporated, *myo*-inositol (0.1 mL of a 0.1 mg/mL solution) was added, and the sample was per-*O*-acetylated as described. The products were analyzed as the per-*O*-acetylated mono-*O*-glycosylalditols and per-*O*-acetylated disaccharides by g.l.c. and g.l.c.–m.s. The standard per-*O*-acetylated mono-*O*-glycosylalditol of each disaccharide tested was prepared (for retention-time and mass-spectral comparison) by reduction with sodium borohydride (0.2 mL of a 10 mg/mL solution in M NH_4OH for 1 h); the borate was removed by four co-evaporations with 10% acetic acid in methanol under a stream of filtered air, and the samples were per-*O*-acetylated as described.

In the second method, the 0.5-mL aliquot was evaporated to dryness, the residue reduced with sodium borohydride (0.2 mL of a 10 mg/mL solution in M NH_4OH for 1 h), and the borate removed by four co-evaporations with a solution of 10% acetic acid in methanol under a stream of filtered air. The resulting mono-*O*-glycosylalditols were hydrolyzed with 2M TFA for 1 h at 120°, the sugars reduced with sodium borodeuteride (0.2 mL of a 10 mg/mL solution in M NH_4OH for 1 h), and the alditols per-*O*-acetylated. The products were analyzed by g.l.c. and g.l.c.–m.s.

*Lithium treatment of methyl 2,3-di-*O*-methyl-*D*-glucoside.* — Methyl 2,3-di-*O*-methyl-D-glucoside (0.64 mg) was treated with lithium, and the products were isolated as for the monosaccharides except that the lithium treatment lasted only 0.5 h. The partially *O*-methylated, partially-*O*-acetylated alditols and partially-*O*-methylated, partially-*O*-acetylated monosaccharide products were analyzed by g.l.c.–m.s.

Lithium treatment of the acidic polysaccharides secreted by R. phaseoli *K-87 and* Klebsiella *K-44, K-17, and K-62.* — The polysaccharides secreted by *R. phaseoli* K-87 (2.9 mg), *Klebsiella* K-44 (10.6 mg), *Klebsiella* K-17 (8.1 mg), and *Klebsiella* K-62 (12.5 mg) were separately placed in screw-capped tubes (20 × 125 mm), and dried *in vacuo* overnight at 40°. The polysaccharides secreted by *Klebsiella* K-44 and K-17 were each solubilized in ethylenediamine (2 mL) after stirring for 0.5 h, treated with lithium for 1 h as already described, and, after lyophilization, the products were isolated just as for the monosaccharides. The polysaccharides secreted by *R. phaseoli* K-87 and *Klebsiella* K-62 were each suspended in

ethylenediamine (2 mL), and the suspensions stirred for 1 h, and then sonicated for 1 h. The partially solubilized polysaccharides were treated with lithium for 1 h.

The products from treatment of the polysaccharides with lithium were dissolved in H_2O (2 mL) and the solutions chromatographed on a column (71 cm × 1.6 cm) of Bio-Gel P-2 that was eluted with H_2O. The eluate was collected in 1.5-mL fractions and the hexosyl and glycosyluronic acid residues in the fractions were assayed colorimetrically.

The fractions containing the material eluted in the void volume and in the partially included volumes were pooled, lyophilized, and weighed. One mg of the material in each sample eluted in the partially included volume was reduced with sodium borohydride (0.2 mL of a 10 mg/mL solution in M NH_4OH for 1 h). The borate was removed by four co-evaporations with a solution of 10% acetic acid in methanol under a stream of filtered air. The reduction products were washed through a column of Dowex AG-50W X12 (H^+) ion-exchange resin to remove the Na^+ ions, and evaporated under a stream of filtered air. The residues were dissolved in dimethyl sulfoxide (1 mL) and per-*O*-methylated[35], and the products were isolated by chromatography on a column[34] of Sep-Pak C-18, and analyzed for per-*O*-methylated oligoglycosylalditols[37] by g.l.c.–m.s.

Isolation of the trisaccharide resulting from HF treatment of the polysaccharide secreted by R. japonicum *(3I1b 138).* — A triglycosyl fluoride accounted for ~50% of the product of HF-treated *R. japonicum* polysaccharide[37a]; the rest of the product was the corresponding trisaccharide. A mixture (0.3 mg) of the trisaccharide and the triglycosyl fluoride was hydrolyzed in 0.1 mL 0.1M $CaCO_3$ for 30 min at 100° followed by 0.1M NaOH (0.1 mL) for 1 h at 25°, to remove the fluorine substituent from C-1 of the mannosyl residue and the acetyl substituent from O-4 of the galactosyluronic acid residue[38]. The base-hydrolyzed trisaccharide was purified by means of Dowex AG-50W X12 resin (to remove the Ca^{2+} and Na^+ ions) and the eluate lyophilized. This material was estimated to weigh 0.2 mg by quantitation of the products of hydrolysis with 2M TFA for 1 h at 120°, sodium borohydride reduction (0.2 mL of a 10 mg/mL solution in M NH_4OH for 1 h), and per-*O*-acetylation.

Treatment of the R. japonicum *3I1b 138 trisaccharide with lithium.* — The trisaccharide isolated from HF-treated *R. japonicum* 3I1b 138 polysaccharide was treated with lithium for 1 h, and the products isolated as described for the lithium-treated monosaccharides. The lyophilizate was dissolved in H_2O (2 mL) and the solution analyzed by three methods. In the first, a 1-mL aliquot was transferred to a screw-capped tube (13 × 100 mm) and evaporated to dryness. The residue was dried *in vacuo* overnight at 40°, dissolved in dimethyl sulfoxide (1 mL), and per-*O*-methylated. The products were purified by chromatography on a column of Sep-Pak C-18 and analyzed for per-*O*-methylated oligoglycosylalditols by g.l.c.–m.s. (e.i. and c.i.).

In the second method, a 0.4-mL aliquot was evaporated to dryness, and the residue reduced overnight with sodium borohydride (0.2 mL of a 10 mg/mL solution in 1:1 ethanol–H_2O. This procedure reduced any lactones formed during

lyophilization of the effluent from the Dowex AG-50W X12 column and any free reducing sugars. The borate was removed by four co-evaporations with a solution of 10% acetic acid in methanol and the Na^+ ions by a column of Dowex AG-50W X12 (H^+) ion-exchange resin eluted with H_2O. The reduction products were hydrolyzed with 2M TFA for 1 h at 120°, and the sugars reduced with sodium borodeuteride (0.2 mL of a 10 mg/mL solution in 1:1 ethanol–H_2O) for 2 h. The borate was removed as already described and the residue was per-*O*-acetylated and the per-*O*-acetylalditols analyzed by e.i.- and c.i.-g.l.c.–m.s.

In the third method, a 0.4-mL aliquot was evaporated to dryness, and the residue reduced overnight with sodium borodeuteride (0.2 mL of a 10 mg/mL solution in 1:1 ethanol–H_2O). This reduction labeled with a ^2H atom any lactones and free reducing sugars in the sample. The borate and Na^+ ions were removed as already described, and the reduction products were hydrolyzed with 2M TFA for 1 h at 120°, reduced again with sodium borodeuteride (0.2 mL of a 10 mg/mL solution in 1:1 ethanol–H_2O) for 2 h, the borate removed, and the reduction products per-*O*-acetylated. The products were analyzed for per-*O*-acetylated alditols by e.i.- and c.i.-g.l.c.–m.s.

The per-*O*-acetylated D-enantiomer of 2-deoxy-L-*lyxo*-hexitol, was prepared by sodium borohydride reduction (0.2 mL of a 10 mg/mL solution in H_2O for 1 h) of 2-deoxy-D-*lyxo*-hexose (0.3 mg, Sigma Chemical Co.). The borate was removed by co-evaporation with a 10% solution of acetic acid in methanol, and the hexitol was per-*O*-acetylated.

Treatment of 2-hydroxyhexanoic acid with lithium. — A solution of 2-hydroxyhexanoic acid (10 mg) in ethylenediamine (1 mL) was treated with lithium for 1 h. The products were isolated as follows. The reactions were quenched with H_2O (5 mL), and the solutions titrated to pH 5 with conc. HCl, and extracted with CH_2Cl_2 (3 x 10 mL); the extracts were combined and evaporated under a stream of filtered air to an oil, which was dissolved in CH_2Cl_2 (5 mL) and analyzed by g.l.c.–m.s. The spectra and retention times of selected products of the reaction were compared to the spectra and retention times of hexanol, hexanal, hexanoic acid, and 2-hydroxyhexanoic acid.

RESULTS AND DISCUSSION

Treatment of rhamnogalacturonan I with lithium in ethylenediamine. — Rhamnogalacturonan I, a pectic polysaccharide isolated from the walls of suspension-cultured sycamore cells, was treated with lithium in ethylenediamine in order to effect the fragmentation described in a preliminary report by Mort and Bauer[21]. The fragmentation resulted in a complex mixture of structurally undefined products that were isolated in <30% yield. Despite the low yield, the need for a method of selectively fragmenting the glycosyluronic acid residues of underivatized carbohydrates led us to undertake the following investigation in order to optimize the reaction conditions and product-isolation procedures, and to characterize structur-

TABLE I

PRODUCTS OF LITHIUM-TREATED MONOSACCHARIDES AND MONOSACCHARIDE METHYL GLYCOSIDES[a]

Material treated with lithium–ethylenediamine	*Products recovered*	
	Starting material (%)	*Alditol (%)*
L-Arabinose	trace	74
L-Rhamnose	2	77
D-Galactose	trace	84
D-Glucose	trace	88
D-Galacturonic acid	trace	0
Methyl β-L-arabinoside	6	80
Methyl α-L-rhamnoside	12	71
Methyl α-D-galactoside	8	80
Methyl α-D-glucoside	7	73
Methyl α-D-galactosiduronic acid	trace	0

[a]The recoveries of products listed are reported relative to *myo*-inositol, which was added as an internal standard after the lithium treatment. The products were analyzed as per-*O*-acetylated derivatives by g.l.c. and g.l.c.–m.s., except in the cases of α-D-galacturonic acid and methyl α-D-galactosiduronic acid, which were analyzed as the per-*O*-(trimethylsilyl)ated derivatives.

ally the products obtained by treatment of a variety of substrates with lithium.

The effect of lithium on selected monosaccharides and methyl glycosides of monosaccharides. — Samples (2 mg each) of L-arabinose, D-galactose, D-glucose, and L-rhamnose were treated with lithium in ethylenediamine for 1 h, and the products isolated as already described. *myo*-Inositol was added to each sample as a standard to assist in the determination of yields. An aliquot of each sample was per-*O*-acetylated and the products analyzed by g.l.c. and g.l.c.–m.s. Lithium treatment converted each sugar into its corresponding alditol (as shown by comparison with an authentic standard), which was isolated in 73–88% yield (see Table I).

D-Galacturonic acid (2 mg) was treated with lithium and the products were isolated as for the neutral sugars. Analysis of the residue by attempting to form the per-*O*-acetylated and per-*O*-(trimethylsilyl)ated derivatives failed to yield any detectable product other than a trace of starting material.

Methyl β-L-arabinoside, methyl α-D-galactoside, methyl α-D-glucoside, and methyl α-L-rhamnoside (2 mg each) were treated with lithium, and the products were isolated and characterized. In each case the major product was the alditol of the corresponding sugar; minor amounts of starting material were also recovered (see Table I). The formation of alditols from the methyl glycosides was unexpected, as lithium in ethylamine had been reported to cleave methyl ethers but not methyl glycosides[28]. Thus, lithium in ethylenediamine must be a more potent reagent than lithium in ethylamine.

Lithium treatment of methyl α-D-galactosiduronic acid (2 mg) gave no isolable product when the residue was analyzed as either the per-*O*-acetylated or

TABLE II

COMPARISON OF THE EFFECT OF LITHIUM TREATMENT ON THE METHYL ETHERS AND METHYL GLYCOSIDE OF METHYL-2,3-DI-*O*-METHYL-D-GLUCOSIDE[a]

Residue isolated after lithium treatment	*Positions of O-acetyl groups*	*Positions of O-methyl groups*	*Relative recovery (%)*
Glucose	4,6	1,2,3	4
Glucose	2,3,4,6	—	30
Glucitol	1,4,5,6	2,3	4
Glucitol	1,3,4,5,6	2	trace
Glucitol	1,2,4,5,6	3	trace
Glucitol	1,2,3,4,5,6	—	61

[a]Material was analyzed as the partially *O*-methylated, partially *O*-acetylated alditols after reduction with sodium borohydride and acetylation with acetic anhydride and pyridine.

per-*O*-(trimethylsilyl)ated derivative (see Table I).

Methyl 2,3-di-*O*-methyl-α-D-glucoside (0.64 mg) was treated with lithium in ethylenediamine to compare the rate of cleavage of methyl ether and methyl glycoside substituents. The lithium treatment was allowed to proceed for 30 (rather than 60) min in an attempt to effect only partial cleavage of the methyl ether and methyl glycoside substituents. Despite the shortened reaction time, all of the methyl ethers were cleaved. However, only two-thirds of the methyl glycoside was cleaved. The major products were glucitol (~61%) and the unsubstituted methyl glucoside (~30%). This result confirmed that lithium in ethylenediamine cleaves both methyl glycoside and methyl ether substituents, and that, as with lithium in ethylamine, methyl ether substituents are cleaved the more rapidly (see Table II).

Effect of lithium on the glycosidic linkages of neutral sugar residues. — Gentiobiose (2 mg) was treated with lithium in ethylenediamine to test the stability of the glucosidic linkage to O-6 of glucose. Cellobiose (2 mg) and 4-*O*-β-D-galactosyl-D-mannose (0.5 mg) were treated to test the stability of the glucosidic and galactosidic linkages to O-4 of glucose and mannose, respectively; laminarabiose (2 mg) and 3-*O*-β-D-galactosyl-L-arabinose (0.5 mg) were treated to test the stability of glucosidic and galactosidic linkages to O-3 of glucose and arabinose, respectively; sophorose (0.5 mg) was treated to test the stability of the glucosidic linkage to O-2 of glucose.

The products of lithium treatment were isolated, divided into two portions, and the extent of cleavage of the respective glycosidic linkages determined by two methods. The first involved g.l.c.–m.s. analysis of the per-*O*-acetylated derivatives. If the disaccharides were cleaved, the expected products would be per-*O*-acetylated monosaccharides, or alditols if the glycoses were reduced by the lithium. If the disaccharides were not cleaved, the expected products would be per-*O*-acetylated mono-*O*-glycosylalditols (if the glycose residue at the reducing end of the disaccharide was reduced by the lithium) or per-*O*-acetylated disaccharides (if the glycose

TABLE III

RECOVERY OF PRODUCTS OF LITHIUM–ETHYLENEDIAMINE TREATMENT OF MODEL DISACCHARIDES

Disaccharide	*Analysis method I*[a] *Recovery of material (%)*			
	Monoglycosyl alditol	*Disaccharide*	*Alditol*	*Monosaccharide*
Gentiobiose	93	—	7	—
Cellobiose	79	12	7	4
Laminarabiose	82	14	—	2
Sophorose	30	60	5	6
	Analysis method II[b,c] *Recovery of material (%)*			
	Alditol	*^{1}H-reduced*	*^{2}H-reduced*	
Gentiobiose	glucitol	54	46	
Cellobiose	glucitol	49	51	
Laminarabiose	glucitol	59	41	
Sophorose	glucitol	52	48	
β-Gal-(1→4)-Man	mannitol	92	8	
	galactitol	4	95	
β-Gal-(1→3)-Ara	arabinitol	99	1	
	galactitol	9	91	

[a]In method I, samples were derivatized by per-*O*-acetylation; this was followed by analysis using g.l.c. and g.l.c.–m.s. [b]In method II, samples were derivatized by per-*O*-acetylation after reduction with sodium borohydride, hydrolysis, and reduction with sodium borodeuteride. This was followed by analysis using g.l.c. and g.l.c.–m.s. [c]The values for the ^{1}H- and ^{2}H-reduced alditols are corrected for the amount of ^{1}H and ^{2}H reduction observed after sodium borohydride prereduction, hydrolysis, sodium borodeuteride reduction, and acetylation of the untreated disaccharides.

residue at the reducing end of the disaccharide was not reduced by the lithium). The only characterizable product from treatment of each of the six disaccharides was the per-*O*-acetylated mono-*O*-glycosylalditol. Thus, lithium in ethylenediamine does not cleave the glycosidic linkage between a neutral glycosyl group and a glycose residue during one-hour treatment (see Table III).

The products of treatment of the disaccharides with lithium in ethylenediamine were examined by a second method, wherein the reaction products were reduced with sodium borohydride, hydrolyzed, reduced with sodium borodeuteride, and acetylated. The resulting per-*O*-acetylated alditols were analyzed by g.l.c.–c.i.-m.s. For the disaccharides derived from glucose substituted with a glucosyl group (gentiobiose, cellobiose, laminarabiose, and sophorose), a 1:1 ratio for ^{1}H relative to ^{2}H introduced at C-1 of the per-*O*-acetylated glucitol would indicate that no cleavage of the glycosidic linkage had occurred, and an increase in the proportion of ^{1}H relative to ^{2}H would indicate that cleavage had occurred (the glycose released by cleavage would be reduced either by the lithium or by the

TABLE IV

REPEATING UNITS OF THE POLYSACCHARIDES USED AS MODELS

Source of polysaccharide	*Structure of polysaccharide repeating unit*
Klebsiella K-62 (ref. 39)	→4)-α-Glc-(1→2)-β-GlcA-(1→2)-α-Man-(1→3)-β-Gal-(1→ 3 ↑ 1 α-Man
Klebsiella K-17 (ref. 40)	→4)-β-Glc-(1→2)-α-Rha-(1→4)-α-GlcA-(1→3)-α-Rha-(1→ 3 ↑ 1 α-Rha
Rhizobium phaseoli K-87 (ref. 41)	→4)-α-Glc-(1→4)-β-GlcA-(1→4)-β-GlcA-(1→4)-β-Glc-(1→ 6 ↑ 1 β-Glc 4 ↑ 1 β-Glc 4 ↑ 1 β-GlcA 4 * 1 α-Gal 6 ↑ 1 β-Glc 6 ↑ 1 β-Gal 6 ↑ 1 β-Gal-3,4-pyruvic acetal
Klebsiella K-44 (ref. 42)	→3)-β-Glc-(1→4)-α-Glc-(1→4)-β-GlcA-(1→2)-α-Rha-(1→3)-α-Rha-(1→
Rhizobium japonicum 311b 138 (ref. 21)	β-Glc-(1→3)-α-GalA-(1→3)-Man[a]

[a]The polysaccharide secreted by *Rhizobium japonicum* 311b 138 was not available; however, the portion of the repeating unit released by treatment of the polysaccharide with anhydrous HF was available and its structure is as shown.

$NaBH_4$). For the disaccharides containing a galactosyl group attached to either arabinose (3-*O*-β-D-galactosyl-L-arabinose) or mannose (4-*O*-β-D-galactosyl-D-mannose), the galactosyl group would be reduced with ^2H in the absence of cleavage; any reduction of the galactosyl group with ^1H would indicate that cleavage had occurred. In each of these experiments, treatment of the disaccharides with lithium

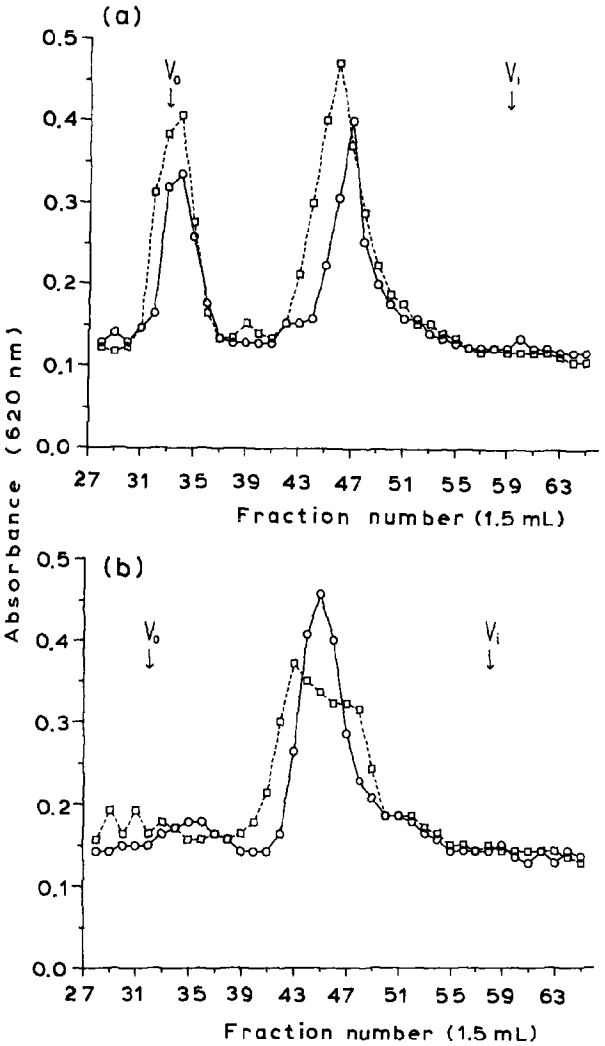


Fig. 1. (a) Gel-permeation chromatography on Bio-Gel P-2 of the products of lithium treatment of the polysaccharides secreted by *R. phaseoli* K-87 (circles) and *Klebsiella* K-62 (squares). Neither of these polysaccharides was completely solubilized by the ethylenediamine before the addition of lithium. The carbohydrate content in each fraction was determined by the anthrone colorimetric method[32]. (b) Gel-permeation chromatography on Bio-Gel P-2 of the products of lithium treatment of the polysaccharides secreted by *Klebsiella* K-44 (circles) and *Klebsiella* K-17 (squares). Both of these polysaccharides were completely solubilized by the ethylenediamine before the addition of lithium. The carbohydrate content in each fraction was determined by the anthrone colorimetric method[32].

in ethylenediamine for the standard time of 1 h resulted in little (<10%) cleavage. These experiments established that various neutral disaccharides can be treated with lithium–ethylenediamine without unacceptable levels of cleavage, and without conversion into other products, except for conversion of reducing glycoses into their corresponding alditols (see Table III).

Effect of lithium on glycosyluronic acid-containing polysaccharides. — The lithium–ethylenediamine cleavage of glycosyluronic acid residues in polysaccharides was examined with polysaccharides of defined structure that contained glucosyluronic acid residues in either α- or β-glycosidic linkages, and galactosyluronic acids in α-glycosidic linkages. The polysaccharides selected for these studies possessed glycosyluronic acid residues having a neutral glycosyl group attached at either O-2 or O-4. The polysaccharide secreted by *Klebsiella* K-62 contains a 2-linked glycosyluronic acid. The polysaccharides secreted by *Klebsiella* K-44 and K-17, and *R. phaseoli* K-87 contain 4-linked glycosyluronic acids. A structurally defined trisaccharide obtained by HF treatment of a polysaccharide secreted by *R. japonicum* 3I1b 138 contains a 3-linked glycosyluronic acid residue; the effect of lithium treatment on this trisaccharide will be discussed in the next section. In addition, the polysaccharide secreted by *R. phaseoli* K-87 is substituted with a pyruvic acetal attached at O-3 and O-4 of a terminal galactosyl group, allowing the effect of lithium treatment on this derivative to be determined. The structures of the repeating units of the polysaccharides examined, and of the trisaccharide derived from HF treatment of the *R. japonicum* 3I1b 138 polysaccharide are listed in Table IV.

The polysaccharides secreted by *R. phaseoli* K-87 (2.9 mg), *Klebsiella* K-44 (10.6 mg), *Klebsiella* K-17 (8.1 mg), and *Klebsiella* K-62 (12.5 mg) were treated with lithium and the products were isolated by the procedure described in the Experimental section. The polysaccharides secreted by *R. phaseoli* K-87 and *Klebsiella* K-62 could not be completely solubilized in ethylenediamine prior to the

TABLE V

PRODUCTS OF THE LITHIUM TREATMENT OF *Rhizobium* AND *Klebsiella* EXTRACELLULAR POLYSACCHARIDES

Polysaccharide secreted by	*Weight treated*[a] *(mg)*	*Percentage recovered*[b]	
		Uncleaved	*Cleaved*
R. phaseoli K-87	2.9	28	52
Klebsiella K-17	10.6	—	83
Klebsiella K-44	8.1	—	50
Klebsiella K-62	12.5	22	54

[a]Amount treated was determined by dry weight. [b]Amount of uncleaved polysaccharide recovered was determined by dry weight of the material eluted at the void volume of a Bio-Gel P-2 column. Amount of cleaved carbohydrate was determined by dry weight of the material eluted at the partially included volume of a Bio-Gel P-2 column.

addition of the lithium. Nevertheless, the effect of lithium on these polysaccharides could be ascertained, as is demonstrated by the following analysis of the products of the treatment.

The residue isolated from the treatment of each polysaccharide with lithium was dissolved in H_2O (2 mL) and chromatographed in H_2O on a column of Bio-Gel P-2. The effluent for each reaction was analyzed for carbohydrate by the anthrone colorimetric method[32]; the profile of each column is shown in Figs. 1a and 1b. The eluted carbohydrates were isolated, weighed, and analyzed. The results are presented in Table V.

The products recovered from the lithium treatment of the polysaccharides secreted by *R. phaseoli* K-87 and *Klebsiella* K-62 were distributed between the void volume and the partially included volumes of the Bio-Gel P-2 column. The column profiles indicated that the reaction cleaved some of the polysaccharide in each sample. However, due to the incomplete solubilization in ethylenediamine some uncleaved polysaccharide was recovered. The polysaccharides secreted by *Klebsiella* K-44 and *Klebsiella* K-17 were fully soluble in ethylenediamine. In these cases, lithium treatment gave only the cleaved product, which was present in the partially included volume of the Bio-Gel P-2 column.

TABLE VI

STRUCTURALLY CHARACTERIZED PER-*O*-METHYLATED OLIGOGLYCOSYLALDITOLS OBTAINED FOLLOWING LITHIUM TREATMENT OF THE POLYSACCHARIDE

Bacterial source of polysaccharide	*Designation herein*	*Per-O-methylated oligosaccharide*[a]
R. phaseoli K-87	A	β-Gal-(1→6)-β-Gal-(1→6)-β-Glc-(1→6)-Galol
R. phaseoli K-87	B	β-Glc-(1→4)-Glcol 6 ↑ 1 β-Glc 4 ↑ 1 β-Glc
Klebsiella K-44	C	α-Rha-(1→3)-α-Rha-(1→3)-β-Glc-(1→4)-Glcol
Klebsiella K-17	D	β-Rha-(1→3)-β-Glc-(1→2)-Rhaol 3 ↑ 1 α-Rha
Klebsiella K-62	E	α-Man-(1→3)-α-Man-(1→3)-β-Gal-(1→4)-Glcol

[a]All oxygen atoms not involved in glycosyl bonds or ring formation were substituted with methyl groups.

TABLE VII

DIAGNOSTIC EI MS IONS FOR THE PER-*O*-METHYLATED OLIGOSYL ALDITOLS OBTAINED BY FRAGMENTATION OF *Rhizobium* AND *Klebsiella* EXTRACELLULAR POLYSACCHARIDES

Per-O-methylated oligosyl alditol	*R.t.*[b] *in g.l.c.*	*Electron impact-m.s. fragment-ions*[a]									
		aJ_1'	aJ_2	abJ_1	abJ_2	$abcJ_1$	$abcJ_2$	dA_1	dA_2	dcA_1	dcA_2
A	27.88	295 (0.5)	235 (31.1)	479 (0.3)	439 (1.0)	—	643 (0.3)	219 (12.2)	187 (32.9)	423 (0.5)	391 (0.5)
		aJ_1	aJ_2	$ab'J_1$	$ab'J_2$	$abcJ_1$	$abcJ_2$	cA_1	cA_2	cbA_1	cbA_2
B	26.88	—	—	499 (2.4)	439 (0.3)	— —	643 (0.3)	219 (4.6)	187 (59.9)	423 (0.2)	391 —
		aJ_0	aJ_2	abJ_0	abJ_2	dA_1	dA_2	dcA_1	dcA_2	$dcbA_1$	$dcbA_2$
C	17.10	281 (26.4)	235 (31.3)	485 (2.9)	439 (T)	189 (100)	157 (51.5)	363 (6.2)	331 (0.8)	567 (3.6)	535 (0.3)
		$ab'J_1$	$ab'J_2$	$abb'J_1$	$abb'J_2$	cA_1	cA_2	cbA_1	cbA_2		
D	15.92	— —	379 (40.3)	643 (0.9)	583 (0.7)	189 (100)	157 (27.4)	393 (2.0)	361 (1.1)		
		aJ_0	aJ_2	abJ_0	abJ_2	$abcJ_1$	$abcJ_2$	dA_1	dA_2	dcA_1	dcA_2
E	18.70	281 (30.9)	235 (47.2)	— —	— —	— —	— —	219 (28.0)	187 (77.7)	423 (2.0)	391 (1.0)

[a]Figures in parentheses show intensity of peak relative to base peak (100). The base peak was often of low mass and not diagnostic, and therefore not included in the Table. [b]Retention time (min) in the DB-1 capillary g.l.c. column under conditions described in the Experimental section. [c]The J and A fragment-ion nomenclature has been accepted by *Carbohydrate Research*[8] and is shown in Fig. 2.

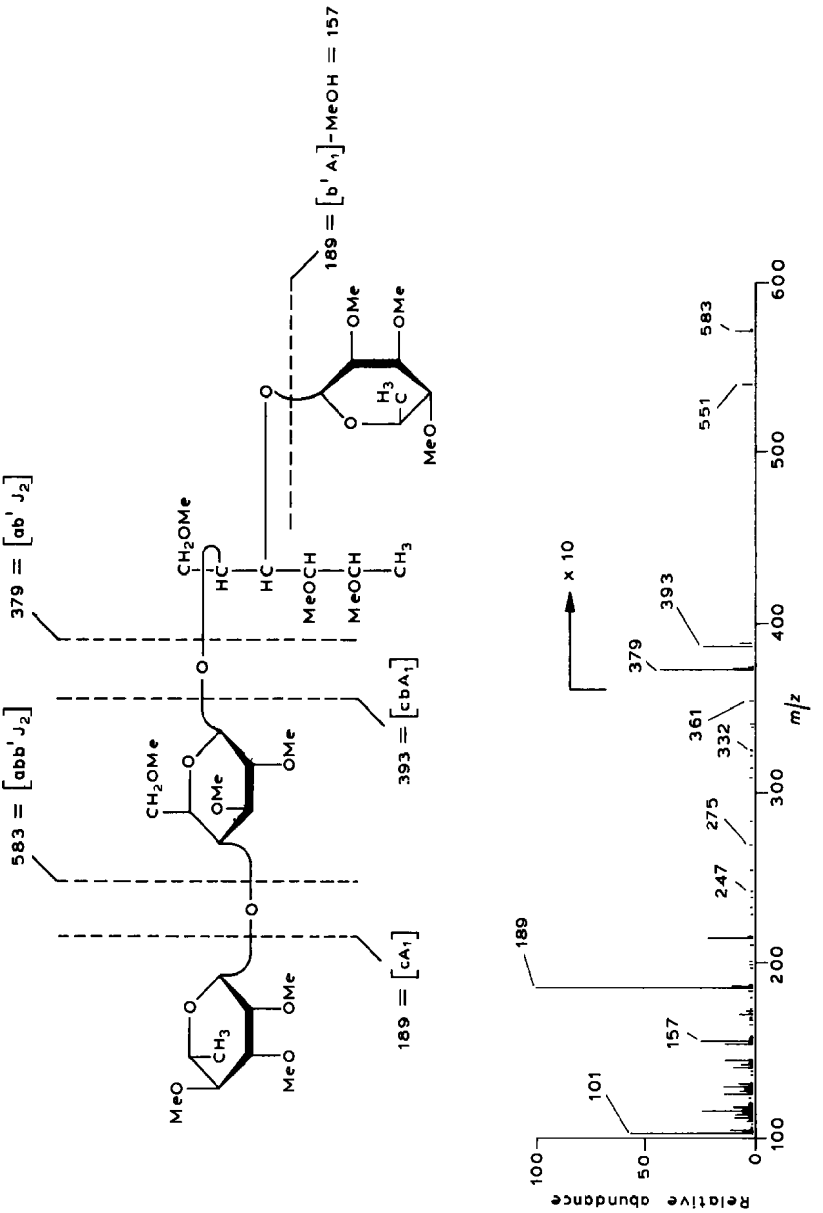


Fig. 2. Sample fragmentation pattern from the e.i.-m.s. analysis of the per-*O*-methylated oligoglycosylalditol derived from lithium-treated, extracellular polysaccharide from *Klebsiella* K-17.

The lithium-treated polysaccharide fragments present in the fractions of the partially included volumes of Bio-Gel P-2 were per-*O*-methylated, and the products analyzed by g.l.c.–m.s. For the polysaccharides secreted by K-17, K-44, and K-62, the product of the lithium treatment was found to be a single triglycosylalditol. For the polysaccharide secreted by K-87, two triglycosylalditols were found. The structures of the per-*O*-methylated oligoglycosylalditols, shown in Table VI, were derived from diagnostic fragment-ions obtained during e.i.-m.s. (see Table VII).

The fragmentation pattern obtained for one such per-*O*-methylated triglycosylalditol is presented in Fig. 2. An alditol derived from the glycosyl residue attached to the glucosyluronic acid residue in the intact polysaccharide was found at the reducing terminus of each per-*O*-methylated triglycosylalditol. This indicated that degradation of the glycosyluronic acid residues resulted in the release and reduction of the glycosyl residue attached to either O-4 or O-2 of the glycosyluronic acid. The glycosyl residues to which the degraded glucosyluronic acid residues were attached appeared as terminal (nonreducing) glycosyl groups in each of the triglycosylalditol products. This established that the glycosidic linkage at C-1 of the hexosyluronic acid residues had been cleaved, and that no portion of the glycosyluronic acid residue remained attached to the triglycosylalditol products.

Lithium treatment removed the pyruvic acetal group from the terminal galactosyl residue of triglycosylalditol A (see Table VI) obtained from the polysaccharide secreted by *R. phaseoli* K-87 (see Table IV). The galactosyl group of triglycosylalditol A was present as a terminal nonreducing group, and was therefore not further altered by the loss of the pyruvic acid residue. Thus, lithium treatment of each of the three polysaccharides containing 4-linked glycosyluronic acid residues effected polysaccharide cleavage at the glycosyluronic acid residues, releasing the glycosyl groups that had been attached to O-4 and O-1 of the glycosyluronic acid residue. The glycosyl residue attached to O-4 of the glycosyluronic acid residue was recovered largely as the alditol and to a lesser extent as the glycose. The glycosyl residue to which the glycosyluronic acid residue had been attached was recovered free of any portion of the glycosyluronic acid residue.

The polysaccharide containing 2-linked glycosyluronic acid residues was cleaved in a similar manner. The glycosyl residue to which the 2-linked glycosyluronic acid residue had been attached was intact in the fragment, whereas the glycosyl group that had been attached to O-2 of the glycosyluronic acid residue was largely recovered as the corresponding alditol.

The effect of lithium on a 3-linked glycosyluronic acid residue. — Isolation of a trisaccharide containing a 3-linked glycosyluronic acid residue. Although no polysaccharide that possesses a 3-linked glycosyluronic acid residue was available, we did obtain a mixture of trisaccharides that contained a 3-linked galactosyluronic acid residue (the gift of Dr. Andrew J. Mort).

The mixture containing a 3-linked galactosyluronic acid trisaccharide was prepared by partial HF-catalyzed hydrolysis of the polysaccharide secreted[37a] by *R. japonicum* 3I1b 138. Analysis of the trisaccharide mixture by ^{1}H-n.m.r. spectros-

copy showed that it was composed of the trisaccharide illustrated in Table IV, and, in ~50% of the trisaccharides, the mannosyl residue (the "reducing" glycose of the trisaccharide) contained a fluorine atom on C-1. In addition, some of the 3-linked galactosyluronic acid residues of the trisaccharides were substituted with an acetyl group on O-4. The trisaccharide mixture (0.3 mg) was hydrolyzed in 0.1 mL of 0.1M $CaCO_3$ for 30 min at 100° and 0.1 mL of 0.1M NaOH for 1 h at 25°, in order to convert all of the trisaccharide mixture into the single unsubstituted trisaccharide by removing the fluorine and acetyl substituents. Lithium treatment of the trisaccharide substituted with fluorine at C-1 of the mannosyl residue, followed by hydrolysis and acetylation, resulted in isolation of the 1,5-anhydroglycitol derivative from mannose, presumably due to reductive cleavage of the fluorine atom at C-1. The resulting unsubstituted trisaccharide (fluorine- and acetyl-free as shown by ^{1}H-n.m.r. spectroscopy) was used for the experiments described next.

Analysis of the products obtained by lithium treatment of a trisaccharide containing a 3-linked galactosyluronic acid residue. — The products of lithium treatment of the 3-linked galactosyluronic acid-containing trisaccharide were isolated in the usual way, and analyzed by three methods. These methods and the products derived from each are illustrated in formulas **1–11**.

Method 1. The first method was similar to that used to analyze the lithium-treatment products of the *Klebsiella* and *Rhizobium* polysaccharides; it involved per-*O*-methylation of the products isolated and analysis by g.l.c.–m.s. The products were hexa-*O*-methylmannitol **8** (recovered in low yield due to its volatility) and three compounds (**5, 6,** and **7**), each the approximate size of a per-*O*-methylated disaccharide. Each of these three was shown by e.i.-m.s. fragment-ions [strong A_1 (*m/z* 219), A_2 (*m/z* 187), and A_2 − MeOH (*m/z* 155)] to contain a 2,3,4,6-tetra-*O*-

TABLE VIII

DIAGNOSTIC E.I.-M.S. IONS FOR THE PER-*O*-METHYLATED DISACCHARIDE AND MONOGLYCOSYLALDITOLS RECOVERED FROM LITHIUM TREATMENT OF THE HF-DERIVED TRISACCHARIDE FROM THE *Rhizobium japonicum* 3I1b 138 EXTRACELLULAR POLYSACCHARIDE

Per-O-methylated oligoglycosylalditol or oligosaccharide	*R.t.*[b] *in g.l.c.*	*Electron-impact-m.s. fragment-ions*[a]							
		aJ_1	aJ_2	bA_1	bA_2	*Alditol cleavages*			
5	14.85	249 (64.6)	189 (46.7)	219 (2.3)	187 (10)	aJ_2-MeOH = 157 (33.2)		Me J_1[c] = 75 (29.0)	
6	11.93	279 (2.4)	219 (0.3)	219 (62.6)	187 (68.4)	45, (55.5)	59, (33.4)	89, (29.5)	117 (12.9)
7	9.97	265 (40.9)	205 (31.4)	219 (8.3)	187 (43.4)	45, (100)	89, (30.7)	103, (26.7)	

[a]Figures in parentheses show intensity of peak relative to base peak (100). The base peak was often of low mass and not diagnostic, and therefore not included in the Table. [b]Retention time (min) in the DB-1 capillary g.l.c. column under conditions described in the Experimental section. [c]J_1 fragment-ion containing the methyl glycoside.

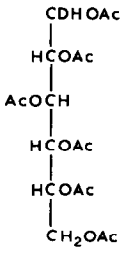
CH2OH
HOCH
HOCH
HCOH
HCOH
CH2OH
4
CH2OH
HCOH
OCH
CH2
CH2OH
CH2OH
OH
OH
HO
3
CH2OH
HCOH
OCH
HOCH
CH2
C=O
OH
CH2OH
OH
OH
HO
2
CH2OH
OH
CH2OH
OH
O
OH
O
CH2OH
OH
OH
HO
1
CH2OMe
MeOCH
MeOCH
HCOMe
HCOMe
CH2OMe
8
CH2OMe
HCOMe
OCH
MeOCH
CH2
CH2OMe
CH2OMe
OMe
OMe
MeO
7
CH2OMe
HCOMe
OCH
MeOCH
CH2
C=O
OH
CH2OMe
OMe
OMe
MeO
6
CH2OMe
OMe
CH2OMe
OMe
O
OMe
O
CH2OMe
OMe
OMe
MeO
5

methylhexosyl group at the nonreducing terminus (see Table VIII). In each case, this group was attached to a modified galactosyluronic acid residue, demonstrating that the glycosidic linkage of the glucosyl residue to O-3 of what had been the 3-linked galactosyluronic acid residue had not been cleaved. The recovery of the disaccharide and mannosyl derivatives established that it was the glycosidic linkage between the galactosyluronic acid and mannosyl residues that had been cleaved. Thus, the effect of the lithium treatment on the 3-linked glycosyluronic acid residue differed from its effect on the 2- or 4-linked glycosyluronic acid residues in which the glycosidic linkages both to and from the glycosyluronic acid residue were cleaved.

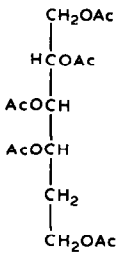
The exact structures of the per-*O*-methylated oligomeric products **5, 6,** and **7**, and, in particular, the portions consisting of the modified galactosyluronic acid residue, could not be determined from their mass spectra. Thus, the products of the lithium reaction on the 3-linked galactosyluronic acid-containing trisaccharide were converted, in Methods 2 and 3, into their corresponding per-*O*-acetylated alditols. Two different ^{2}H-labeling patterns, together with the mass spectra of **5, 6,** and **7**, allowed their structures to be determined.

Method 2. In Method 2, the products of the lithium-treated 3-linked galactosyluronic acid-containing trisaccharide were reduced overnight with sodium borohydride in 1:1 (v/v) ethanol–H_2O, in order to assure that any functional group made susceptible to reduction by the lithium reaction was "labeled" with hydrogen. The sample was then hydrolyzed and reduced again, this time for 2 h with sodium borodeuteride in 1:1 (v/v) ethanol–H_2O, in order to label with deuterium any reducible group formed during the hydrolysis. The products of this reduction were then per-*O*-acetylated, and the acetates analyzed by e.i.- and c.i.-g.l.c.–m.s.

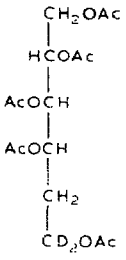
Three products were isolated from the reaction sequence of Method 2. One was glucitol (**9**) containing one deuterium atom at C-1. This was the expected product from the glucosyl residue present on the non-reducing terminus of disaccharides (see Method 1). The isolation of the glucitol derivative confirmed that the glucosyl residue was not cleaved from the modified galactosyluronic acid residue by the lithium treatment. Mannitol (**11**) was a second product; it contained only hydrogen on C-1. Thus, as expected, it was reduced by the lithium reaction or by the treatment with sodium borohydride before acid hydrolysis. The third product isolated



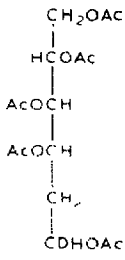
9



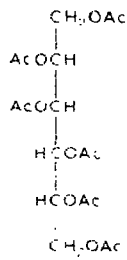
10



10a



10b



11

was the modified galactosyluronic acid residue; it was shown to be 2-deoxy-L-*lyxo*-hexitol (**10**). This was confirmed by comparing the g.l.c. retention time and e.i.- and c.i.-g.l.c.–mass spectra with those of its enantiomer, 2-deoxy-D-*lyxo*-hexitol. Penta-*O*-acetyl-2-deoxy-D-*lyxo*-hexitol was prepared by sodium borohydride reduction of 2-deoxy-D-*lyxo*-hexose and per-*O*-acetylation of this alditol.

The 2-deoxy-L-*lyxo*-hexitol obtained from the trisaccharide by Method 2 was reduced with hydrogen at both C-1 and C-6. This established that both the carboxyl group at C-5 and the aldehyde at position 1 of the 3-linked galactosyluronic acid residue were reduced by either the lithium treatment or the sodium borohydride reduction, that is, before acid hydrolysis. This confirmed that the glycosidic linkage between the 3-linked galactosyluronic acid residue and the mannosyl residue was cleaved by the lithium treatment. The oxygen atom at C-5 of the galactosyluronic acid was lost during the lithium reaction. The oxygen atom on C-5 may have moved to C-1 *via* an alpha elimination concomitant with the loss of the oxygen atom at C-1 to its aglycon. This may be the mechanism by which all complex carbohydrates containing glycosyluronic acid residues are cleaved by lithium treatment. The loss of O-5 converted the galactosyluronic acid residue into a 2-deoxy-L-hexose.

Method 3. The third method used for analyzing the lithium products of the *R. japonicum* trisaccharide consisted of an initial, overnight reduction with sodium borodeuteride in 1:1 (v/v) ethanol–H_2O, followed by hydrolysis and a 2-h reduction with sodium borodeuteride in 1:1 (v/v) ethanol–H_2O, in order to determine the extent of reduction of the carboxyl group at C-5 of the galactosyluronic acid that occurred during the lithium treatment. Comparison of the deuterium-labeling pattern in the products of Method 3 with those of Method 2 allowed us to determine the extent to which C-1 of the various glycosyl residues was reduced by the lithium treatment or by sodium borohydride (before acid hydrolysis). The products of this analysis were per-*O*-acetylated, and the acetates analyzed by both e.i.- and c.i.–g.l.c.–m.s.

Again, three products were isolated. As in Method 2, glucitol (**9**) containing a deuterium atom at C-1 was isolated, confirming that it was not reduced by the lithium treatment. Mannitol (**11**) containing a hydrogen atom on C-1 was isolated, indicating that reduction of the mannosyl residue of the trisaccharide occurred during the lithium treatment.

The third product was again 2-deoxy-L-*lyxo*-hexitol containing two hydrogen atoms at C-1, indicating that the reduction of the aldehyde at position 1 of the galactosyluronic acid occurred during the lithium reaction. However, this time the 2-deoxy-L-*lyxo*-hexitol was a mixture, containing 0 (**10**), 1 (**10a**), or 2 (**10b**) deuterium atoms at C-6. This indicated that the carboxyl group at C-5 of the galactosyluronic acid had been reduced to various extents during the lithium treatment. C.i.-mass-spectral analysis of the isotopic ratios established that, following the lithium treatment, ~29% of the carboxyl groups of the galactosyluronic acid (now deoxy at O-5 because of the lithium reaction, but retaining the glucosyl residue at O-3) remained unreduced. This 2-deoxyaldonic acid derivative (**2**) presumably formed a lactone when evaporated to dryness after isolation *via* the Dowex column. The lactone would have been reduced with sodium borohydride in Method 2 and with the initial sodium borodeuteride reduction in Method 3.

Mass-spectral analysis showed that ~43% of the carboxyl groups were reduced to the aldehyde oxidation state (**1**) by the lithium treatment. This led to the sodium borohydride reduction of the aldehyde in Method 2 and reduction with sodium borodeuteride in Method 3.

Some 28% of the 2-deoxy-L-*lyxo*-hexitol was found to be reduced with two hydrogen atoms at C-6, indicating that it had been completely reduced to the alcohol oxidation state (**3**) during the lithium reaction.

The structure of the products of lithium treatment. — The complete structures of **5, 6,** and **7** were determined from the deuterium-labeling experiments (Methods 2 and 3) and from their mass spectra. The structure of **7** was determined by its e.i.-m.s. fragment ions [J_1 (*m/z* 265), J_2 (*m/z* 205), and alditol (*m/z* 45, 89, and 103)] to be 2-deoxy-L-*lyxo*-hexitol substituted at O-4 with a glucosyl group (see Table VIII). This conclusion is supported by the fact that some of the 2-deoxy-L-*lyxo*-hexitol was completely reduced to the alditol (**10**) by the lithium reaction and contained no deuterium atoms on C-6.

The structure of **6** was determined from its e.i.-m.s. fragment-ions [J_1 (*m/z* 279), J_2 (*m/z* 219), and alditol (*m/z* 45, 59, 89, and 117)] to be the methyl ester of 2-deoxy-*lyxo*-hexonic acid substituted at O-4 with a glucosyl residue (see Table VIII). This assignment was supported by the fact that some of the 2-deoxy-L-*lyxo*-hexitol characterized in Method 3 contained two deuterium atoms on C-6 (**10a**). It is likely that, after lithium treatment, **6** was present as a lactone. Moisture present during methylation probably resulted in formation of the methyl ester.

The structure of **5** was determined from e.i.-m.s. fragment-ions [J_1 (*m/z* 249), J_2 (*m/z* 189), and intense ions resulting from the successive loss of methanol moieties from the J_2 ion] to be a mixture of the methyl α- and β-glycosides of 2-deoxy-*lyxo*-hexose substituted at O-4 with a glucosyl group (see Table VIII). This product was probably formed when the carboxyl group of the galactosyluronic acid was converted into an aldehyde, which formed a hemiacetal with the former O-2 of the galactosyluronic acid residue. This possibility is supported by the observation (in Method 3) that some of the 2-deoxy-L-*lyxo*-hexitol contained one deuterium

atom on C-1 (**10b**) (formerly C-6 of galactosyluronic acid). These results established that the polymer was selectively cleaved even though the derivative of the glycosyluronic acid residue remained at the reducing terminus of oligosaccharides.

As in the cases of the 2-linked and 4-linked glycosyluronic acid-containing polymers, reduction with sodium borohydride prior to acid hydrolysis is recommended in order to simplify analysis of the products.

Lithium treatment of 2-hydroxyhexanoic acid. — The 3-linked galactosyluronic acid residue examined lost O-5 during lithium treatment. In order to investigate the possibility that α-elimination of the oxygen atom on C-5 is the mechanism by which glycosyluronic acid-containing complex carbohydrates are cleaved, 2-hydroxyhexanoic acid (10 mg) was treated with lithium in ethylenediamine for 1 h. Products isolated by acidification of the reaction mixture and extraction with dichloromethane were analyzed by g.l.c.–m.s. Comparison of the retention times and mass spectra of the products of lithium treatment of 2-hydroxyhexanoic acid with those of hexanol, hexanal, and hexanoic acid showed that all were present. This result supports the hypothesis that, as expected, an oxygen atom alpha to a carboxyl group can be cleaved by treatment with lithium.

GENERAL DISCUSSION

The studies described herein demonstrate the utility of lithium–ethylenediamine treatment for selective cleavage of complex carbohydrates that contain glycosyluronic acid residues. The stability of neutral glycosyl residues in this reaction was demonstrated through the use of model compounds. The site of cleavage in complex carbohydrates containing glycosyluronic acid was demonstrated through the use of substrates of defined structures. The procedure for performing the reaction and isolating products has been improved and, under the reaction conditions used, relatively few side products are formed.

The lithium reaction is particularly valuable because of its ability to cleave underivatized complex carbohydrates selectively at the site of glycosyluronic acid residues. The cleavage of underivatized carbohydrates will allow the products to be used for studies of both structure and biological activity.

REFERENCES

1 K. BOCK AND H. THØGERSEN, *Annu. Rep. NMR Spectrosc.*, 13 (1982) 1–57.
2 A. BAX, W. EGAN, AND P. KOVAČ, *J. Carbohydr. Chem.*, 3 (1984) 593–611.
3 C. C. SWEELEY AND H. A. NUNEZ, *Annu. Rev. Biochem.*, 54 (1985) 765–801.
4 J. LÖNNGREN AND S. SVENSSON, *Adv. Carbohydr. Chem. Biochem.*, 29 (1974) 41–106.
5 V. N. REINHOLD AND S. A. CARR, *Mass Spectrom. Rev.*, 2 (1983) 153–221.
6 B. LINDBERG, J. LÖNNGREN, AND S. SVENSSON, *Adv. Carbohydr Chem. Biochem.*, 31 (1973) 185–240.
7 G. O. ASPINALL, *Pure Appl. Chem.*, 49 (1977) 1105–1134.
8 M. W. SPELLMAN, M. MCNEIL, A. G. DARVILL, AND P. ALBERSHEIM, *Carbohydr. Res.*, 122 (1983) 131–153.
9 M. MCNEIL, A. G. DARVILL, P. ÅMAN, L.-E. FRANZEN, AND P. ALBERSHEIM, *Methods Enzymol.*, 83 (1982) 3–45.

10 B. S. VALENT, A. G. DARVILL, M. MCNEIL, B. K. ROBERTSON, AND P. ALBERSHEIM, *Carbohydr. Res.*, 79 (1980) 165–192.
11 L. ROSENFIELD AND C. E. BALLOU, *Carbohydr. Res.*, 32 (1974) 287–298.
12 B. NILSSON AND S. SVENSSON, *Carbohydr. Res.*, 65 (1978) 169–171.
13 A. J. MORT, *Carbohydr. Res.*, 122 (1983) 315–321.
14 I. J. GOLDSTEIN, G. W. HAY, B. A. LEWIS, AND F. SMITH, *Methods Carbohydr. Chem.*, 5 (1965) 361–370.
15 S. J. ANGYAL AND K. JAMES, *Aust. J. Chem.*, 23 (1970) 1209–1215.
16 D. ROLF AND G. R. GRAY, *J. Am. Chem. Soc.*, 104 (1982) 3539–3541.
17 N. K. KOCHETKOV, O. S. CHIZHOV, AND A. SUIRDOV, *Carbohydr. Res.*, 14 (1970) 277–285.
18 G. O. ASPINALL AND K.-G. ROSELL, *Can. J. Chem.*, 56 (1978) 685–690.
19 G. O. ASPINALL, H. K. FANOUS, N. S. KUMAR, AND V. PANESARAJAH, *Can. J. Chem.*, 59 (1981) 935–940.
20 G.– O. ASPINALL AND K.-G. ROSELL, *Can. J. Chem.*, 56 (1978) 680–684.
21 A. J. MORT AND W. D. BAUER, *J. Biol. Chem.*, 257 (1982) 1870–1875.
22 A. J. BIRCH, *Q. Rev. Chem. Soc.*, 4 (1950) 69–93.
23 A. W. BURGSTAHLER, L. R. WORDEN, AND T. B. LEWIS, *J. Org. Chem.*, 28 (1963) 2918–2919.
24 A. O. BEDENBAUGH, J. H. BEDENBAUGH, W. A. BERGIN, AND J. D. ADKINS, *J. Am. Chem. Soc.*, 92 (1970) 5774–5775.
25 J. W. HUFFMAN AND J. T. CHARLES, *J. Am. Chem. Soc.*, 90 (1968) 6486–6492.
26 H. ADKINS AND R. GILLESPIE, *Org. Syn. Coll. Vol.*, 3 (1955) 671–673.
27 H. A. SMITH, B. J. L. HUFF, W. J. POWERS III, AND D. CAINE, *J. Org. Chem.*, 32 (1967) 2851–2856.
28 C. MONNERET, J. FLORENT, I. KABORÉ, AND Q. KHUONG-HUU, *J. Carbohydr. Nucleos. Nucleot.*, 1 (1974) 161–168.
29 M. MCNEIL, A. G. DARVILL, AND P. ALBERSHEIM, *Plant Physiol.*, 66 (1980) 1128–1134.
30 S. B. TJAN, A. G. J. VORAGEN, AND W. PILNIK, *Carbohydr. Res.*, 34 (1974) 15–23.
31 B. MATSUHIRO, A. B. ZANLUNGO, AND G. G. S. DUTTON, *Carbohydr. Res.*, 97 (1981) 11–18.
32 Z. DISCHE, *J. Biol. Chem.*, 204 (1953) 983–997.
33 N. BLUMENKRANTZ AND G. ASBOE-HANSEN, *Anal. Biochem.*, 54 (1973) 484–489.
34 T. J. WAEGHE, A. G. DARVILL, M. MCNEIL, AND P. ALBERSHEIM, *Carbohydr. Res.*, 123 (1983) 281–304.
35 P. J. HARRIS, R. J. HENRY, A. B. BLAKENEY, AND B. A. STONE, *Carbohydr. Res.*, 127 (1984) 59–73.
36 S. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
37 N. K. KOCHETKOV AND O. S. CHIZHOV, *Adv. Carbohydr. Chem.*, 21 (1966) 39–93.
37a A. J. MORT, personal communication.
38 F. MICHEEL AND A. KLEMER, *Adv. Carbohydr. Chem.*, 16 (1961) 85–104.
39 G. G. S. DUTTON AND M.-T. YANG, *Carbohydr. Res.*, 59 (1977) 179–192.
40 G. G. S. DUTTON AND T. E. FOLKMAN, *Carbohydr. Res.*, 80 (1980) 147–161.
41 W. F. DUDMAN, L.-E. FRANZÉN , M. MCNEIL, A. G. DARVILL, AND P. ALBERSHEIM, *Carbohydr. Res.*, 117 (1983) 169–183.
42 G. G. S. DUTTON AND T. E. FOLKMAN, *Carbohydr. Res.*, 78 (1980) 305–315.