The 6-O-Methylglucose-containing Lipopolysaccharide of Mycobacterium phlei

IDENTIFICATION OF D-GLYCERIC ACID AND 3-O-METHYL-D-GLUCOSE IN THE POLYSACCHARIDE*

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SUMMARY

The polysaccharide containing 6-O-methylglucose, from Mycobacterium phlei, is an acidic molecule with at least seven 6-O-methyl-D-glucopyranosyl residues linked α-1,4', and with a side chain of at least three α-1,4'-D-glucopyranosyl units. A simplified procedure is described for isolating the polysaccharide (MGP) from acetone-dried M. phlei cells with improved yield.

Exhaustive α-amylase digestion of the polysaccharide released glucose, maltose, and a disaccharide. The last yielded D-glucose and 3-O-methyl-D-glucose after acid hydrolysis; while hydrolysis, after reduction with sodium borohydride, gave glucitol and 3-O-methylglucose. Methylation analysis established that the disaccharide was 3-O-methylα-D-glucopyranosyl-(1→4)-D-glucose.

Limited α-amylolysis of MGP yielded a trisaccharide and a tetrasaccharide, each containing 1 mole of 3-O-methylglucose at the nonreducing end. The trisaccharide was digested by α-amylase to the disaccharide 3-O-methylglucosyl-(1→4)-glucose and glucose, while α-amylolysis of the tetrasaccharide gave these products plus maltose. A glucoamylase preparation converted the tetrasaccharide to the trisaccharide and glucose. That 3-O-methylglucose was at the nonreducing terminus of each saccharide was shown by partial acid hydrolysis studies and by the products of exhaustive propylation, which yielded 3-O-methyl-2,4,6-tri-O-propylglucose.

Propylation of the intact polysaccharide and analysis of the fully alkylated methylglucosides by gas-liquid chromatography established that 3-O-methylglucose and glucose were in the nonreducing terminal positions. After α-amylase digestion, glucose was the only terminal residue. Glucoamylase removed an additional glucosyl residue, which had been linked α-1,4' to a 6-O-methylglucosyl residue.

An organic acid having the chromatographic and electrophoretic properties of glycemic acid was isolated from acid hydrolysates of MGP. The glycemic acid was oxidized by 1 mole of sodium periodate, with the release of 1 mole of formaldehyde and 1 mole of glyoxalate. It was shown to have the D-configuration. 3-O-Methylglyceric acid was obtained by hydrolysis of methylated MGP, showing that the linkage to the polysaccharide involved the 2-hydroxyl group. MGP was converted to the hydroxamate, and a Lossen rearrangement was performed in water at neutral pH. The reducing sugar thus formed was reduced with sodium borotritide, and the radioactive polyol obtained by acid hydrolysis was identified as glucitol. Thus, the glycemic acid must be attached glycosidically to a glucose residue.

The intact lipopolysaccharide, MGLP, was not associated with purified M. phlei cell wall or particulate preparations. The material was recovered quantitatively from the high speed supernatant, apparently in free form. An attempt to degrade the main chain enzymatically with extracts from M. phlei was unsuccessful, and a search both for low molecular weight oligosaccharides containing 6-O-methylglucose and for large polysaccharides into which MGLP might have been incorporated was unproductive. Thus, our investigations have failed to suggest a structural or metabolic function for the lipopolysaccharide.

MGLP, isolated from the cultural filtrate of M. phlei cells in the stationary phase, was indistinguishable from the intracellular molecule. It was released into the medium during all stages of the growth cycle, the kinetics following those for the appearance of low molecular weight carbohydrates and of mannose- and arabinose-containing polysaccharides.

Previous publications from this laboratory (1, 2) have described the isolation and partial characterization of a lipopolysaccharide, from Mycobacterium phlei, which contains D-glucose and 6-O-methyl-D-glucose. The "methylglucose polysaccharide" obtained by deacetylation of the lipopolysaccharide contains a chain of at least seven 6-O-methylglucosyl units that are linked α-1,3', to which is attached, by an α-1,3' linkage, a chain of...
several glucose units also linked α-1, 4'. The polysaccharide, which is nonreducing, has a molecular weight of about 3000, equivalent to 18 hexose units, and contains one acid dissociation, pK 3.7. The glucose chain can be removed, in part, by the action of α-amylase.

In this paper we report further studies on the polysaccharide which establish that the acidic component is α-glyceric acid that is linked glycosidically, through its 2-position, to a glucose unit at what would otherwise be the reducing end of the polysaccharide. We have also found that the glucose-containing fragment released by α-amylase digestion of the polysaccharide is at least as long as a tetrasaccharide, and that it is terminated at the nonreducing end by a molecule of 3-O-methyl-D-glucose. The 6-O-methylglucose-containing polysaccharide from Mycobacterium tuberculosis also yields glyceric acid on hydrolysis and gives the same 3-O-methylglucose-containing oligosaccharides by the action of α-amylase. Thus, its structure must be essentially the same as that of the M. phlei polysaccharide.

EXPERIMENTAL PROCEDURE

Materials—Sephadex G-25 (fine), Sephadex G-50 (fine), and DEAE-Sephadex (medium) were obtained from Pharmacia. Silica Gels G and H were purchased from Merck.

Porcine pancreas α-amylase, bovine heart lactate dehydrogenase, and Glucostat Special, a glucose oxidase preparation for specific enzymatic assay of α-glucose, were purchased from Worthington. Yeast hexokinase (Type III) was obtained from Sigma and was dialyzed before use. Glucosaminidase I from Aspergillus niger (3) was generously supplied by Drs. John Panaur and D. R. Linebach. The rabbit muscle preparation, used for the characterization of α-glycerate, was an ammonium sulfate fraction (taken between 50 and 75% of saturation) of a rabbit muscle extract (4). This was dialyzed several times against neutral phosphate buffer before use.

The procedures for the syntheses of 6-deoxyglucose (5, 6), 2-O-methylglucose (7), 3-O-methylglucose (8), 4-O-methylglucose (9), and 6-O-methylglucose (10) have been described. A mixture of 4-O-methyl terminal maltoligosaccharides (degree of polymerization, 1 to 6) (11) was kindly supplied by Dr. R. E. Wing. A crystalline sample of the rhamnolipid from Pseudo- monas aeruginosa (12) was a gift from Dr. John Law.

A mixture of disaccharides, containing the two isomers of 6-O-methylmaltose, was synthesized by Dr. R. Oetgen by benzylation partially tritylated maltose, removing the trityl groups, methylating with methyl iodide and silver oxide, and removing the benzoyl groups with barium methoxide in dry methanol. Chromatography for 4 days in Solvent A (see "Chromatography and Electrophoresis") revealed the presence of two compounds with Rf values of 0.89 and 0.78. The compounds were separated by preparative paper chromatography and passed through a Sephadex G-25 column. Both contained equimolar amounts of glucose and 6-O-methylglucose. Sodium borohydride reduction, followed by acid hydrolysis and paper chromatography in Solvent B, showed that glucose was at the reducing end of the faster compound, and 6-O-methylglucose was at the reducing end of the slower compound.

Calcium α-glycerate and methyl α-glycerate were synthetic samples. Methyl 2-O-methylα-glycerate and methyl 3-O-methylglycerate were prepared from 3-O-benzylglycerate (13) and 2-O-benzylglycerate (14), respectively, by methylation of the methyl esters with silver oxide and methyl iodide in dimethylformamide, followed by reductive removal of the benzyl groups (13). Standard monobenzylethyleneglycol was synthesized by periodate oxidation followed by sodium borohydride reduction of α-benzylglycerol, which had been synthesized by benzylation of isopropylidene glycerol. Glycronic acids were synthesized from the corresponding free sugars by oxidation with bromine in ammonium carbonate buffer. Salts were then removed by repeated evaporation from water. Standard polysaccharides were prepared from the corresponding sugars by sodium borohydride reduction in water at room temperature. After reduction was complete, excess sodium borohydride was destroyed by addition of Dowex 50-H+ resin, and basic acid was removed as methyl borate by repeated evaporation from methanol. Remaining salts were removed by treatment with the carbonate form of a mixed bed resin (Dowex AG-50).

Boron trimethide was purchased from Matheson, Coleman, and Bell. 1-Ethyl-3-(3'-dimethylaminopropyl)carbodiimide hydrochloride was obtained from the Ott Chemical Company. Hexamethyldisilazane and trimethylchlorosilane were obtained from K and K Laboratories, and sodium borohydride-H2 (specific activity, 26 mCi per mg) was purchased from New England Nuclear. Other materials were of the highest purity available commercially.

Analytical Procedures—Total carbohydrate was determined by the phenol-sulfuric acid method with glucose as standard (15), and reducing sugar by a modification of the Nelson-Somogyi method (16). Total phosphate and inorganic phosphate were determined as described by Bartlett (17). Formaldehyde was estimated by the chromatropic acid method of Hanahan and Olley (18). The hydroxyamic acid method for the determination of ester content was that of Steen and Shapiro (19). N-Hydroxysuccinimide, prepared from crystalline succinimide anhydride, was used as the standard.

Hydroxamates were prepared from the corresponding esters by treatment with a solution composed of equal volumes of 0.1 M hydroxylamine hydrochloride and 0.7 M NaOH. After 10 min, Dowex 50-H+ resin was added to remove cations. The resin was removed by filtration and washed exhaustively with water and methanol.

Total acid hydrolysis of oligosaccharides and polysaccharides was carried out in a sealed tube at 100°C; either in 1 N HCl for 4 to 6 hours or in 2 N HCl for 2 to 3 hours. Volatile solvents were removed by rotary evaporation at 35-45° under vacuum. The effluent from columns was analyzed for carbohydrate by the phenol-sulfuric acid assay, followed by analysis of the constituent monosaccharides by paper chromatography. For non-specific analysis of compounds containing adjacent hydroxyl groups, but giving no fufural with sulfuric acid, a periodate assay was used. An aliquot from each tube was added to 1 ml of 10-4 M sodium periodate, and the absorbance at 225 mµ was determined after a 4-hour oxidation period. Excess glyceric acid totally reduced the periodate to iodide, causing a decrease in the absorbance of about 0.7 optical density unit.

Methylation of oligosaccharides was carried out as follows: To 2 mg of the oligosaccharide in 0.5 ml of dimethylformamide were added 0.2 ml of methyl iodide and 0.2 g of silver oxide. The reaction mixture was shaken at room temperature for 24 hours; then 0.1 ml of methyl iodide and 0.1 g of silver oxide were added, and shaking was continued for 24 hours. Benzene (10 ml) was added; salts were removed by centrifugation, and the benzene solution was extracted with water three times, dried over sodium.
sulfate, and evaporated to dryness. The methylated product was methanolized in 6% HCl at 100° for 12 hours in a sealed tube; after removal of solvent, the product was dissolved in 0.1 ml of carbon disulfide and chromatographed. Methylation of MGP and standard compounds was carried out in a similar fashion. Ethylation with ethyl iodide and silver oxide in dimethylformamide, and propylation with propyl iodide and silver oxide in dimethylformamide, were found to proceed satisfactorily under the conditions used for methylation.

**Chromatography and Electrophoresis**—Descending paper chromatography was carried out on Whatman No. 1 filter paper. Whatman No. 3MM filter paper, previously washed for 24 hours with 2% aqueous ammonia, was used for preparative separations. The following solvents (composition in volume ratios) were used: A, 1-butanol-pyridine-water, 10:3:3; B, ethyl acetate-acetic acid-formic acid-water, 18:3:1:4; C, ethyl acetate-pyridine-water, 10:6:4; D, 1-butanol saturated with water; E, benzene-ethanol-water, 170:47:15 (organic phase); F, 1-butanol-pyridine-0.05 M morpholine borate, pH 8.6, 7:5:2 (20); G, 1-butanol-ethanol-water, 3:1:1; H, 2-propanol-ammonium-water, 7:1:2; I, butanol-ethanol-water, 5:1:4; J, 1-butanol-acetic acid-diethyl ether-water, 9:6:3:1; K, diethyl ether-hexane, 1:1; L, 2-propanol-ammonia, 2:1.

Chromatographic analysis of monosaccharide mixtures was usually done with Solvent A. Rp values in this solvent were not affected by the presence of salts and were very reproducible. Solvent B, an acidic solvent in which monosaccharides and carbohydrate acids migrate relatively rapidly, was used for the same purpose, but it was particularly valuable for separating sugars from the corresponding polyols. Solvents C and D were useful for analysis of oligosaccharide mixtures. Separations in Solvent C were achieved in 12 to 15 hours, while it was sometimes necessary to develop chromatograms for 2 weeks in Solvent D. Solvents E and L were used for the separation of short chain fatty acylhydroxamates; and Solvents A, D, and E were used for separations of hydroxamates of carbohydrate acids. Phosphate esters of sugars were analyzed with Solvents H and L.

For detection of sugars on paper chromatograms, aniline-hydrochloric acid used for the detection of neutral sugars (21). alkaline AgNO₃ (22), and periodate-benzidine reagents (23) were used. The periodate-benzidine dip reagent was preferred for detection of polyols and glyconic acids. A 10% aqueous solution of ferric chloride was sprayed onto chromatograms for the detection of hydroxamates (24), while a 0.5% ninhydrin spray was used for detection of amino acids and amino sugars (25).

Thin layer chromatograms of monosaccharides were developed with the ascending technique on Silica Gel H with Solvent B; for quantitative analyses, development was performed twice. Members of a homologous series of oligosaccharides could easily be separated on Silica Gel H plates with Solvent J. Sugars were detected by spraying the plates with 50% aqueous sulfuric acid and heating them at 125° for 1 hour. Methyl esters of fatty acids were separated with Solvent K on Silica Gel G plates.

Quantitative determination of sugars, after separation by paper chromatography in Solvent B, was carried out by the procedure of Pridham (26), except that the concentration of p-anisidine hydrochloride was increased to 4%. The same method was applied to the quantitative determination of sugars after separation by thin layer chromatography. After color development for 15 min at 105°, the colored areas were scraped from the glass plates with a razor blade and eluted from the powder with a methanolic stannous chloride solution (26). The absorbance at 400 nm was linear in the range from 25 to 150 μg of sugar applied to the chromatogram. Standard curves for each sugar were made at the time of determination, since the color yield was dependent both on the nature of the sugar and on the conditions chosen. The absorbance was stable for hours, and accuracy was within 5%. Reported values are the averages of triplicate determinations.

Gas-liquid chromatography of trimethylsilylated sugars (27), alkylated methylglycosides, and methyl esters was performed with an Aerograph Hy-Fi model 600-B or a Varian Aerograph series 1200 gas chromatogram attached to a hydrogen flame ionization detector (Wilkins Instruments and Research). Stainless steel columns (5 ft. x 0.125 inches) packed with 10% Carbopack B (Column I), or 13% diethylene glycol succinate on Anakrom ABS (Column II), were used, with a nitrogen gas flow rate of 15 to 20 ml per min. Other conditions were as described under "Results."

**Instrumentation**—Nuclear magnetic resonance spectra were determined at 23° with a Varian model A-60 spectrometer. Optical rotations were measured with a Rudolph polarimeter. A Cary 15 recording spectrophotometer was used for measuring spectra, and a Zeiss PMQ spectrophotometer for determinations of absorbance in enzymatic and colorimetric assays. The distribution of radioactivity on chromatographic paper strips was determined with a Nuclear-Chicago thin layer plate conveyor system, model 1066. Titrations were made with a Radiometer PHM4 pH meter with 0.05 N NaOH.

**RESULTS**

**Polysaccharide from Acetone-dried M. phlei Cells**

**Isolation**—M. phlei cells were grown on a complex medium (28) in a New Brunswick fermenter, harvested, washed twice with acetone, and air-dried. MGP was not present in the acetone wash. The dry cells were extracted and deacylated as before (29). The water-soluble material was dialyzed twice against 4 liters of water in the cold for 4 hours. About 25% of the MGP and 90% of the total carbohydrate passed through the tubing. The material inside the tubing was further purified on columns of DEAE-Sephadex (borate form) (2), Sephadex G-50, and DEAE-Sephadex (carbonate form), in that order. 6-O-Methylglucose-containing material was eluted from the first two columns as a single compound, but only about half of this material adhered to the carbonate form of DEAE Sephadex, giving a neutral MGP fraction. The acidic fraction was eluted as a single peak with a linear gradient of ammonium bicarbonate.

**Properties of Neutral and Acidic MGP**—The ratio of hexose to monomethylhexose, as determined by quantitative thin layer chromatography, was 2:3 for both the neutral and acidic fractions. These fractions were eluted together from a Sephadex G-50 column. Neither fraction contained detectable phosphate or amino acids. Titration of acidic MGP showed 1 mole of acid per mole of polysaccharide (pK 3.8; equivalent weight, 3200), but no inflection point was observed for the neutral material.
MGLP, purified under mild conditions (2), is completely adsorbed to DEAE-Sephadex. Therefore, it appeared that neutral MGP was an artifact formed from acidic MGP during the deacylation step. In the following experiments, acidic and neutral MGP were estimated by applying the material to a DEAE-Sephadex column (1 x 5 cm, carbonate form). Neutral material passed through, while acidic polysaccharide adhered to the resin and could be eluted with dilute ammonium carbonate. The amounts of carbohydrate in the two fractions were determined by the phenol-sulfuric acid assay. Treatment of acidic MGP with 0.1 N NaOH at room temperature for 48 hours, 0.4 N NaOH at room temperature for 48 hours, or aqueous pyridine at 120° for 48 hours did not convert the acidic molecule to a neutral form.

Treatment of neutral MGP with 0.4 N HCl for 24 hours at room temperature resulted in 45% conversion to the acidic form, while treatment with 0.1 N NaOH for 20 min resulted in complete conversion. These results suggested that neutral MGP was an ester or a lactone. This conclusion was confirmed by converting the neutral polysaccharide to a hydroxamate (0.9 μ mole formed per 3.0 mg of carbohydrate). Passage of this material through a Sephadex G-25 column established that the hydroxamate was associated with the polysaccharide.

If neutral MGP were a lactone, it should be formed from acidic MGP by dehydration under acidic conditions. Acidic MGP was dissolved in 4 N HCl, evaporated to dryness, evaporated twice from benzene, and then left under vacuum at 60°. After 4 hours, 15% of the carbohydrate was neutral; after 12 hours, 25% was neutral. This rate of conversion is much slower than expected for lactone formation. Subsequent treatment of this neutral carbohydrate with base did not reconvert it to an acidic form, suggesting that neutral material resulted from degradation of the polysaccharide rather than from lactone formation. Treatment of acidic MGP with methanolic HCl resulted in rapid esterification, and formation of neutral material. Therefore, it is probable that neutral MGP was an ester which formed after acylation of the methanolic solution in which deacylation was carried out.

**Improved Isolation Procedure**—The following simplified purification procedure, based on the above results, gave an increased yield of MGP in the same high degree of purity. Acetone-dried cells (200 g) were twice extracted with 3 liters of refluxing 70% ethanol for 2 hours, and solvents were removed. About 400 ml of 0.2 N NaOH was added to the residue, and the solution was shaken for 30 min at room temperature. The solution was acidified by addition of Dowex 50 H⁺ resin; the resin was removed by filtration, insoluble material was removed by centrifugation, and the clear, aqueous solution was washed once with chloroform. The volume was reduced, and the solution was applied to a Sephadex G-25 column (4 x 150 cm) in 50-ml portions. Void volume material was pooled, concentrated to 10 ml, centrifuged to remove insoluble material, and passed through a Sephadex G-50 column (2 x 190 cm) in 0.1 M acetic acid in two portions (2). Material which was eluted in the peak corresponding to MGP (see Fig. 1, Peak B) was pooled, concentrated to dryness, taken up in water, and applied to a DEAE-Sephadex column (carbonate form, 2 x 15 cm). Some neutral carbohydrate passed through, while all of the 6-O-methylglucose-containing material adhered to the resin and was eluted as a single, symmetrical peak with a linear gradient of 0.0 to 0.1 M ammonium bicarbonate. About 2 mg of MGP were obtained per g of acetone-dried cells.

**Lipopolysaccharide from Cultural Filtrate**

*Isolation*—Preliminary experiments showed that 6-O-methylglucose-containing material was present in the cultural filtrate of *M. phlei* and a purification of the material was attempted. Forty liters of medium, from which the bacteria (in stationary phase) had been removed by centrifugation, were concentrated to about 3 liters with a steam-heated, continuous flow evaporator. Protein was removed by shaking the solution with chloroform, centrifuging, and removing the chloroform layer and interfacial material. The aqueous phase was dialyzed twice for 4 hours, in dialysis tubing with a diameter of 5 cm, against 40 liters of water at room temperature to remove glycerol and salts. About one-third of the total carbohydrate passed through the tubing. Material inside the tubing was applied to a Dowex 1-formate column (8 x 50 cm). Most of the carbohydrate, including the 6-O-methylglucose-containing material, passed through. The material which adhered to the resin was eluted with a linear gradient of ammonium formate, 0 to 0.6 M. Carbohydrate (1.5 g) was eluted at about 0.15 M ammonium formate as a symmetrical peak, and several small peaks were eluted at higher salt concentrations. The latter peaks contained nucleotides, probably arising by degradation of nucleic acids. The compound displaced by 0.15 M ammonium formate was eluted from a Sephadex G-25 column like a disaccharide. Chromatography in Solvent H showed a single spot. The molar ratio of sugar to phosphate was 2:1, and mild acid hydrolysis gave two spots, one of which chromatographed with glucose and the other with glucose 6-phosphate. Therefore, this compound was probably trehalose 6-phosphate (30).

An aliquot of the material which passed through the Dowex 1-formate column was applied to a Sephadex G-50 column (2 x 190 cm). The elution pattern is reproduced in Fig. 1. Acid hydrolysis of Peak A yielded mannose and arabinose, with smaller amounts of glucose and galactose. Peak B contained the 6-O-methylglucose polysaccharide. Peak C was heterogeneous, but one compound predominated which chromatographed with trehalose (Solvent A) and yielded glucose on acid hydrolysis.
Further purification was effected on the assumption that
the 6-O-methylglucose polysaccharide was MGLP. The
material which had passed through the Dowex 1-formate
column was applied to a Sephadex G-25 column (4 x 150 cm)
in several 50-ml aliquots. Carbohydrate, eluted in the
void volume, was pooled and applied to a DEAE-Sephadex
column (carbonate form, 3 x 20 cm). Acidic material was
eluted by a linear gradient of ammonium bicarbonate.
Essentially all of the methylglucose lipopolysaccharide
was eluted as a single, symmetrical peak at low salt
concentration. This fraction was finally passed through
a Sephadex G-50 column (2 x 190 cm). Acid hydrolysates
of the peak corresponding to MGLP contained primarily glucose
and 6-O-methylglucose, but mannose and arabinose, amounting
to about 5% of the total carbohydrate, were also present. The
yield was 500 mg of carbohydrate.

Characterization of Extracellular MGLP—The properties of extracellular MGLP on DEAE-Sephadex and Sephadex columns
were those of intracellular MGLP. The ratio of glucose to mono-
methylglucose was 2:3. The deacylated polysaccharide was
digested by α-amylase, and the resultant fragments gave the
same elution pattern from a Sephadex G-25 column as had been
observed for intracellular MGLP (see Fig. 8 of Reference 2).
An acidic component (Acid X; see below) was detected in acid
hydrolysates of the polysaccharide. The acyl content was 2.9
µmoles of ester per 3.0 mg of carbohydrate. The monocar-
boxylic acyl hydroxamates from extracellular MGLP, prepared
and chromatographed as described in “Experimental Pro-
cedure,” corresponded to those which have been obtained from
intracellular MGLP. Thus, the lipopolysaccharide isolated
from the cultural filtrate is very similar to the intracellular
MGLP.

Structure of 3-O-Methyl-α-glucose-containing
Oligosaccharides from MGP

Identification of 3-O-Methyl-α-glucose from MGP—Ten milli-
grams of MGP were digested with pancreatic α-amylase until no
further increase in reducing power was observed, and the prod-
ucts were separated by gel filtration. The elution pattern from
Sephadex G-25 was similar to that shown in Fig. 8a of Reference
2. The first peak, containing 80% of the total carbohydrate,
was nonreducing and acidic, and it had a ratio of monomethyl-

\[ R_{MGLC} \] of Sugar Z (Solvent A) corresponded exactly with
that reported for 6-deoxyglucose (31), and Sugar Z was found t,o
chromatograph with standard 6-deoxyglucose in Solvents A and
B. The acid hydrolysate of Peak 1 was tested for the presence
of a 6-deoxy sugar by the procedure of Dische (32). A mixture
of glucose and 6-deoxyglucose, 2:1, gave a positive test, but the
sample containing Sugar Z was negative and gave the same result
as pure glucose.

MGP, 100 mg, was digested with 3-α-amylase, and after acid
hydrolysis of Peak 1 Sugar Z was isolated by preparative paper
chromatography. Impurities from the paper were removed by
gel filtration. The yield of sugar (phenol-sulfuric acid assay)
was 4.2 mg, corresponding to about 1 mole of sugar per mole of
polysaccharide subjected to α-amylase digestion. The chromo-
phore produced by the phenol-sulfuric acid assay had a maximum
absorbance at 488 mp, the expected value for a hexose. The
NMR spectrum of Sugar Z in D2O showed a sharp peak, 63 cps
upfield from the HDO peak, suggestive of a methyl ether. Re-
sonance lines characteristic of a 6-deoxyhexose were absent.

For demethylation, the dry sugar (0.25 mg) was dissolved in

\[ J. M. Keller and C. E. Ballou, unpublished results. \]
0.5 ml of pyridine and 0.5 ml of acetic anhydride and heated on an oil bath at 100° for 1 hour. Solvents were removed, and the fully acetylated sugar was taken up in 0.4 ml of dichloromethane. Fifty microliters of boron tribromide were added, and after 2 min the mixture was evaporated to dryness. The product was deacetylated by adding 4 drops of barium methoxide in methanol to a methanolic solution of the sugar. After 15 min, the mixture was acidified by addition of Dowex 50-H⁺, water was added, and the resin was removed by filtration. The product was established as α-glucose by chromatography in Solvents A and B and by determination with the Glucostat assay.

The single methyl ether peak observed in the NMR spectrum of Sugar Z suggested that it was a monomethyl ether. Further evidence for this was obtained by thin layer chromatography on an activated Silica Gel H plate (Solvent B). The rate of migration of methylated hexoses in this system depends on the number of methyl groups present. The RF values of hexoses center around 0.10; monomethylhexoses, 0.22; dimethylhexoses, 0.35; trimethylhexoses, 0.60; and tetramethylhexoses, 0.65. Sugar Z had the RF of a monomethyl ether.

Sugar Z was oxidized with 0.02 M NaIO₄ in 0.4 M H₂SO₄ for 45 min. After this period of time, 0.6 mole of formaldehyde had formed per mole of sugar, which indicated that the methyl group was not attached to the 5- or 6-hydroxyl. Sugar Z was converted to the methylglycoside by heating in methanolic HCl. The methylglycoside was dissolved in 0.1 ml of 0.4 M acetate buffer, pH 4.0, and 0.1 ml of 0.1 M NaIO₄ was added. After 2 hours at room temperature, mixed bed resin (carbonate form) was added to remove salts, and the product was hydrolyzed at 100° for 1 hour in 1 N HCl. Chromatography of the product showed that Sugar Z remained intact, while 4-O-methylglucoside and arabinose, treated similarly, were completely degraded. Of the monomethyl ethers of methylglucoside, only methyl 3-O-methylglucoside would be expected to escape periodate oxidation.

Sugar Z was compared directly with all of the monomethyl ethers of glucose. The chromatographic, electrophoretic, and NMR data which prove that it is 3-O-methyl glucose are presented in Table I. The specific optical rotation was +52° (60% in water), in agreement with the value of +55° reported for 3-O-methyl glucose.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Rf₁₉₅ (Solvent A)</th>
<th>Rf₂₅₄⁺</th>
<th>Chemical shift</th>
<th>α</th>
<th>Value</th>
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<td>Glucose</td>
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<td>1.0</td>
<td>3.22 4.80</td>
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<tr>
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<tr>
<td>Sugar Z</td>
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<td>0.79</td>
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<td>0.87</td>
<td>3.76 76</td>
<td>65</td>
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</tbody>
</table>

*α* Retention times of the trimethylsilyl derivatives (β and α anomers) on Column I at 170°.

The chemical shift of the methyl ether peak, cycles per second upfield from HDO, at 24° (60 Mc).

**Properties of monomethyl ethers of glucose**

The reduced disaccharide was methanolyzed and examined by gas chromatography on Column I. Four peaks were observed, corresponding to the β and α anomers of methyl 2,3,4,6-tetramethylglucoside and methyl 2,3,6-trimethylglucoside. The relative areas under these peaks were the same as those observed for the methanolysis product of methylated maltose. The NMR lines for the anomeric protons of Disaccharide Z were consistent with the α configuration. Therefore, we conclude that the substance is 3-O-methyl-α-D-glucopyranosyl-(1 → 4)-D-glucose.

**Structures of Triosaccharide Z and Tetrasaccharide Z.** Limited α-amylase digestion of MGP was carried out as described previously (2). The resulting oligosaccharides were purified by gel filtration and by preparative paper chromatography in Solvent A. The elution pattern of the oligosaccharides in the digest was similar to that shown in Fig. 8b of Reference 2. The four peaks corresponded to glucose, maltose, and two larger oligosaccharides, which were eluted as a triosaccharide (Triosaccharide Z) and a tetrasaccharide (Tetrasaccharide Z). Acid hydrolysis of both oligosaccharides gave glucose and 3-O-methylglucosyl. The two oligosaccharides, together with Disaccharide Z, 3-O-methylglucosyl, and oligosaccharides of the maltose series, were chromatographed in Solvent A, and the relative mobilities of these compounds were plotted (Fig. 3). A straight line was obtained for the oligosaccharides containing 3-O-methylglucosyl, which indicated that they were members of a homologous series. That this line was parallel to the one resulting from a plot of the maltose series suggested that each of the 3-O-methylglucosyl-containing oligosaccharides differed from the next lower homologue by the addition of an α (1 → 4) linked glucoseyl residue. NaBH₄ reduction decreased the color yields in the phenol-sulfuric acid assay in amounts confirming the proposed degrees of polymerization (Table II). On acid hydrolysis, reduced Tri- and Tetrasaccharide Z yielded glucitol, glucose, and 3-O-methylglucosyl. Methylolation of the two oligosaccharides confirmed that they had only 1,4' linkages (Table II).

Both Tri- and Tetrasaccharide Z were attacked by α-amylase. The products were identified by paper chromatography in Solvents C, D, and A, with which all possible products could be unambiguously distinguished (Table II). Triosaccharide Z yielded Disaccharide Z and glucose, while Tetrasaccharide Z gave malt-
Properties of oligosaccharides from MGP

<table>
<thead>
<tr>
<th>Compound</th>
<th>Decrease in molar ratio of tri- to tetramethyl glucose</th>
<th>R&lt;sub&gt;F&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-O-Methylglucose</td>
<td>45</td>
<td>1.28</td>
</tr>
<tr>
<td>Disaccharide Z</td>
<td>30</td>
<td>1.02</td>
</tr>
<tr>
<td>Trisaccharide Z</td>
<td>21</td>
<td>0.82</td>
</tr>
<tr>
<td>Glucose</td>
<td>98</td>
<td>1.00</td>
</tr>
<tr>
<td>Maltose</td>
<td>48</td>
<td>0.81</td>
</tr>
<tr>
<td>Maltotriose</td>
<td></td>
<td>0.69</td>
</tr>
</tbody>
</table>

* Percentage decrease in the absorbance at 490 nm observed with the phenol-sulfuric acid assay for sugar after sodium borohydride reduction. The color yield was standardized with a known amount of DL-glyceric acid.

* The ratio of the areas of the methyl 2,3,6-trimethylglucose peaks to those of the methyl 2,3,4,6-tetramethylglucose peaks for each oligosaccharide were determined and compared with those observed for standard methanolyzed methylated maltose. Methylation and methanolysis of the oligosaccharides were carried out as described in "Experimental Procedure." Column 1 at 210° was used for gas-liquid chromatography.

ose, glucose, and Disaccharide Z. Prolonged α-amylase digestion of maltotriose and maltotetraose yielded only glucose and maltose. A mixture of malto-oligosaccharides terminated at the nonreducing end with a 4-O-methylglucosyl residue (II) was hydrolyzed by α-amylase to glucose and disaccharides, 4-O-methyl-α-D-glucosyl-(1→4)-D-glucose being among the products. Since 4-O-methyl-terminal maltotriose and maltotetraose were degraded by α-amylase to give products corresponding to those obtained from Trisaccharide Z and Tetrasaccharide Z, the methylated residues in the latter two oligosaccharides were also probably in the nonreducing terminal positions. The glucoamylase preparation from A. niger did not attack Trisaccharide Z, but Tetrasaccharide Z was degraded to glucose and a compound with the chromatographic properties of Trisaccharide Z. Further confirmation of the structure of the latter two oligosaccharides was obtained by gas-liquid chromatography of fully alkylated methylglycosides. After propylation and purification of the products, the glycosides were hydrolyzed in 90% formic acid at 100° for 4 hours and converted to the methylglucosides at room temperature in dry methanolic HCl. Column II was used for chromatographic analysis at 174°. The retention times of the α and β anomers of methyl 3-O-methyl-2,4,6-tri-O-propylglucoside (I), methyl 6-O-methyl-2,3,4-tri-O-propylglucoside (II), and methyl-2,3,4,6-tetra-O-propylglucoside (III) were 3.79 and 4.58, 3.90 and 4.70, and 4.20 and 5.15 min, respectively. A, a mixture of standard I and II; B, the fully alkylated methylglucosides from propylated Tetrasaccharide Z; C, the fully alkylated methylglucosides from propylated intact MGP; D, a mixture of standard II and III; E, the fully alkylated methyl glucosides from propylated, α-amylase-digested MGP; F, the fully alkylated methylglucosides from propylated, α-amylase- and glucoamylase-digested MGP.

Fig. 4. Gas-liquid chromatographic elution patterns of fully alkylated methylglucosides. After propylation and purification of the products, the glycosides were hydrolyzed in 90% formic acid at 100° for 4 hours and converted to the methylglucosides at room temperature in dry methanolic HCl. Column II was used for chromatographic analysis at 174°. The retention times of the α and β anomers of methyl 3-O-methyl-2,4,6-tri-O-propylglucoside (I), methyl 6-O-methyl-2,3,4-tri-O-propylglucoside (II), and methyl-2,3,4,6-tetra-O-propylglucoside (III) were 3.79 and 4.58, 3.90 and 4.70, and 4.20 and 5.15 min, respectively. A, a mixture of standard I and II; B, the fully alkylated methylglucosides from propylated Tetrasaccharide Z; C, the fully alkylated methylglucosides from propylated intact MGP; D, a mixture of standard II and III; E, the fully alkylated methyl glucosides from propylated, α-amylase-digested MGP; F, the fully alkylated methylglucosides from propylated, α-amylase- and glucoamylase-digested MGP.
glucose is conclusive evidence that the 3-O-methylglucose is at the nonreducing end of the saccharides.

**Identification of Nonreducing Terminal Residues in MGP**

Total alkylation of a polysaccharide followed by complete methanolysis results in the formation of fully alkylated methylglycosides only from the nonreducing terminal residues. The methylglycosides from internal residues have one or more free hydroxyl groups. The former compounds chromatograph in gas-liquid systems with much shorter retention times than the latter compounds, and they also migrate more rapidly on thin layer plates. Preliminary experiments showed that ethylation of MGP with ethyl iodide and silver oxide in dimethylformamide proceeded as readily as did methylation. However, these derivatives were unsatisfactory, since the \( \alpha \) and \( \beta \) anomers of methyl 6-O-methyl-2,3,4-tri-O-ethylglucoside, methyl 3-O-methyl-2,4,6-tri-O-ethylglycoside, and methyl 2,3,4,6-tetra-O-ethylglucoside could not be separated by gas-liquid chromatography. Partial separation of the free sugars of these derivatives was obtained on thin layer plates of activated Silica Gel H (40 cm in length), developing twice with Solvent E; the relative rates of migration of 2,3,4,6-tetra-O-ethylglucose, 3-O-methyl-2,4,6-tri-O-ethylglucoside, and 6-O-methyl-2,3,4-tri-O-ethylglucose were 1.00:0.97:0.88.

More satisfactory results were obtained with propylated derivatives. On Column II, the retention times of the anomers of methyl 3-O-methyl-2,4,6-tri-O-propylglucoside and methyl 6-O-methyl-2,3,4-tri-O-propylglucoside differed sufficiently for positive identification when only one of these two glucosides was present, and the anomers of methyl 2,3,4,6-tetra-O-propylglucoside were well separated (Fig. 4, A and D). Interestingly, methanolysis at 100° resulted in a preponderance of the \( \alpha \) anomer, while hydrolysis of the alkylated derivatives in 90% formic acid at 100° followed by conversion to the methylglycosides by dry methanolic HCl at room temperature resulted in the formation of about equal amounts of the \( \alpha \) and \( \beta \) anomers. All standards and unknowns were prepared by both methods in order to ensure that peak assignments were correct.

Fig. 4C shows the pattern of the fully alkylated methylglucosides obtained from the hydrolysate of protoployglucosides. The retention time corresponds to those of the \( \alpha \) and \( \beta \) anomers of 3-O-methyl-2,4,6-tri-O-propylglucoside and methyl 6-O-methyl-2,3,4-tri-O-propylglucoside differed sufficiently for positive identification when only one of these two glucosides was present, and the anomers of methyl 2,3,4,6-tetra-O-propylglucoside were well separated. Fig. 4A and B). Interestingly, methanolysis at 100° resulted in a preponderance of the \( \alpha \) anomer, while hydrolysis of the alkylated derivatives in 90% formic acid at 100° followed by conversion to the methylglycosides in dry methanolic HCl at room temperature resulted in the formation of about equal amounts of the \( \alpha \) and \( \beta \) anomers. All standards and unknowns were prepared by both methods in order to ensure that peak assignments were correct.

The retention times (Column II, 200°) for 2,3,4,6-tetra-O-propylglucitol, 6-O-methyl-2,3,4-tri-O-propylglucitol, and 3-O-methyl-2,4,6-tri-O-propylglucitol were 3.00, 2.80, and 3.40 min, respectively. Analysis of the reduced methylpropyl sugars obtained from intact MGP showed that only 2,3,4,6-tetra-O-propylglucitol and 3-O-methyl-2,4,6-tri-O-propylglucitol were present. Only peak corresponding to that of 6-O-methyl-2,3,4-tri-O-propylglucitol. With the same column at 170°, the ratio of the areas under the peaks corresponding to 2,3,4,6-tetra-O-propylglucitol (retention time, 8.85 min) and 3-O-methyl-2,4,6-tri-O-propylglucitol (retention time, 10.05 min) was 0.85. When this is corrected for the different detector responses based on carbon content, the molar ratio is 0.95, in good agreement with the expected value if the two end groups are present in equal amounts.

Samples of \( \alpha \)-D-glucopyranosyl-(1 \( \rightarrow \) 4)-D-methyl-D-glucose were treated separately with \( \alpha \)-amylase and glucoamylase. Conditions were the same as for digestion of the polysaccharide. The products were analysed by paper chromatography and thin layer chromatography. No 6-O-methyl-D-glucose or glucose was released by \( \alpha \)-amylase, but glucoamylase completely digested the disaccharide to the constituent monosaccharides. Both enzymes were inactive on 6-O-methyl-\( \alpha \)-D-glucopyranosyl-(1 \( \rightarrow \) 4)-D-glucose and on maltoligosaccharides composed entirely of 6-O-methyl-D-glucose.

An attempt was made to define more precisely the activity of glucoamylase on \( \alpha \)-amylase-digested MGP. If the enzyme is a true exoglucosidase, it should not attack the 3-O-methylglucose terminal side chain in intact MGP. However, incubation of MGP with glucoamylase resulted in the release of Trisaccharide \( \mathcal{Z} \) as well as glucose, and a kinetic study revealed that the trisaccharide was released more readily than free glucose. The rate at which MGP was digested was only a small fraction of that at which maltoligosaccharides were converted to glucose (34).

**\( \alpha \)-D-Glyceraldehyde, the Aglycon of MGP**

\( \alpha \)-D-Glyceraldehyde from MGP. MGP is a nonreducing polysaccharide with a single acidic function (2). A reasonable hypothesis is that the acid (Acid X) is an \( \alpha \)-glycon linked to what would be the reducing end of the polysaccharide.

The stability of the bound acid to strong mineral acid and base was studied as follows. MGP (1 \( \mu \) mole) was heated at 100° for 30 min in Fehling's solution (4 \( \times \) NaOH). No reaction was detected. Similar treatment of 1 \( \mu \) mole of a reducing hexasaccharide, or a non-reducing rhamnooligosaccharide in which the \( \alpha \)-glycon was a \( \beta \)-hydroxy fatty acid (12, 35), produced a heavy precipitate. MGP was left for 1 week in 1 \( \times \) NaOH at room temperature. The solution was passed through a Dowex 50-H\(^+\) column and applied to a small DEAE-Sephadex column (carbonate form). Most of the carbohydrate (95%) was retained, and was therefore still acidic. The rate of formation of neutral carbohydrate by partial acid hydrolysis of MGP (50%) after 40 hours in 0.1 \( \times \) HCl at 60° corresponded to that expected for cleavage of glycosidic bonds. Gel filtration of the neutral carbohydrate showed that extensive breakage of glycosidic bonds had occurred. Acid X, therefore, was stable to strong mineral acid and base, and the linkage between the polysaccharide and the acid was not appreciably less stable than the other glycosidic linkages.

MGP (20 \( \mu \)g) in water was desalted, extracted with ether to remove possible ether-soluble contaminants, and titrated (pK =
3.9; equivalent weight = 3000). The polysaccharide was then hydrolyzed in HCl at 100°; the solvent was evaporated, the residue was distributed between water and ether and the ether extract was refluxed in 4% methanolic sulfuric acid for 4 hours to convert any acids to esters, and the product was chromatographed on a Silica Gel G thin layer plate with Solvent K. No hydroxy fatty acid ester could be detected with the dichlorofluorescein spray reagent (36) or the basic hydroxylamine-ferric chloride procedure (37). Titration of the aqueous layer, after removal of cations, revealed that Acid X had remained in the water extract.

Glyonic acids give intense, light yellow spots on chromatograms developed with the periodate-benzidine reagent, while sugars give faint, white spots. In basic solvents, acids migrate slowly; in acidic solvents, their RF values are much increased. Chromatography of 0.2 mg of an acid hydrolysate of MGP in these two solvents, followed by detection with the periodate-benzidine reagent, revealed a component with RF values of Acid A = 0.14, and RF values of Acid B = 1.7. This component was present in acid hydrolysates both of the intracellular MGP preparations and of the MGLP preparation from the cultural filtrate. The compound migrated faster than gluconic acid, 6-O-methylglucuronic acid, erythronic acid, and threonitic acid, but it migrated with glyceric acid in both solvent systems. The RF values of Acid X in Solvents F and G differed from those of standard glyceric acid, but they co-chromatographed in both systems, indicating that the differences were due to the presence of different salt forms of the same acid.

Acid X was isolated by preparative paper chromatography in Solvents B and A, successively, followed by gel filtration to remove paper impurities. The electrophoretic mobility of Acid X in 0.1 m sodium borate was the same as that for glycerate. Gas-liquid chromatography of the methyl ester of Acid X and of standard methyl glycerate (Column I at 170°) showed that they had identical retention times. The esters were converted to the hydroxamates, and these were shown to co-chromatograph in Solvent A. Thus, all chromatographic and electrophoretic evidence indicated that Acid X was glyceric acid. In addition, Acid X reacted with naphthoresorcinol in concentrated sulfuric acid at 100° (38) to give the same chromophore as was observed for authentic glyceric acid (absorption maxima at 620 and 660 mp).

Periodate oxidation of Acid X in 10⁻² m NaIO₄ gave approximately 1 mole of formaldehyde per mole of periodate consumed (Fig. 5A). Glyoxalate should also be produced by mild periodate oxidation of glycerate. The broad substrate specificity of bovine heart lactate dehydrogenase allowed this enzyme to be utilized for the estimation of the glyoxalate. The rates of the enzyme-catalyzed oxidation of NADH by sodium glyoxalate, periodate-oxidized glycerate, and periodate-oxidized Acid X were the same. No oxidation of NADH occurred in control experiments in which glycerate, periodate, or enzyme was omitted (Fig. 5B). The glyoxalate produced by mild periodate oxidation of Acid X was also identified chromatographically by means of the periodate-benzidine reagent.

The configuration of Acid X was established enzymatically, with the use of yeast hexokinase to phosphorylate the glycerate and a crude rabbit muscle preparation for the reduction of d-glycerate 3-phosphate by NADH. This system has been shown to be specific for d-glycerate (39, 40). In the absence of added substrate, slow NADH oxidation occurred, and this could not be eliminated by dialysis of the enzyme preparations. Since this oxidation was linear, it did not interfere with the assay (Fig. 6). The quantitative data on which the characterization of Acid X is based are summarized in Table III.

Linkage of d-Glycerate to Reducing End of Polysaccharide—MGP, 20 mg, was exhaustively methylated. After methanalysis of the product, the mixture was examined by gas-liquid chromatography. Distinct peaks corresponding to synthetic methyl 2-O-methylglycerate or methyl 3-O-methylglycerate were not observed because of the presence of impurities. Assay for ester showed that about 1 μmole of ester was present per 3 mg of carbohydrate. The methanalysis mixture was treated with

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**Fig. 5.** A, kinetics of the oxidation of 0.1 μmole of standard DL-glycerate (O) and of Acid X (Δ) in 2.0 ml of 10⁻⁴ m sodium periodate. The oxidation was followed by observing the decrease in absorbance at 244 mp. The control (■) lacked glycerate. After oxidation was complete, ethylene glycol was added to reduce excess periodate to iodate, which did not interfere with the enzymatic oxidation of NADH. B, kinetics of the lactate dehydrogenase-catalyzed oxidation of NADH by the glyoxalate produced during the periodate oxidation of standard DL-glycerate (O) and of Acid X (Δ) in A. The reaction was carried out in 0.1 m phosphate buffer, pH 6.0. The control, in which no glycerate was added to the solution of sodium periodate, is indicated by ●. The absorbance at 340 mp was used to determine the extent of reaction.

**Fig. 6.** Determination of the configuration of Acid X. The reaction mixtures contained 0.05 ml of 0.05 m phosphate buffer (pH 7.3), 50 μl of 0.05 m ATP, 0.25 mg of NADH, 0.50 mg of yeast hexokinase, and the rabbit muscle preparation, 1.0 mg of protein. Additions of 0.1 μmole of glycerate were made as indicated in the figure. The change in absorbance at 340 mp was used as a measure of the NADH present.
TABLE III
Quantitative characterization of Acid X

<table>
<thead>
<tr>
<th>Compound</th>
<th>Naphthoresorcinol color</th>
<th>Periodate oxidation</th>
<th>Formaldehyde produced (μl)</th>
<th>Glyoxalate produced (μl)</th>
<th>NADH oxidized (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glyceraldehyde</td>
<td></td>
<td>X 10 5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acid X</td>
<td></td>
<td>X 10 5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-Glyceraldehyde</td>
<td></td>
<td>X 10 5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* After color development as described in Reference 38, the solutions were diluted 1:5 with concentrated sulfuric acid before determination of the absorbance at 660 μm, which is expressed as molar extinction.

** See Fig. 5 for conditions.

*** See Fig. 6 for conditions. The consumption of NADH was calculated from its reaction with a known amount of 3-glyceraldehyde acid.

TABLE IV
Paper chromatography of monomethyl ethers of glyceric hydroxamate

<table>
<thead>
<tr>
<th>Compound</th>
<th>Distance migrated (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-O-Methylglyceric hydroxamate</td>
<td>12.7</td>
</tr>
<tr>
<td>3-O-Methylglyceric hydroxamate</td>
<td>45.5</td>
</tr>
<tr>
<td>Hydroxamate from methylated MGP</td>
<td>44.8</td>
</tr>
</tbody>
</table>

basic hydroxylamine and chromatographed in Solvent A, which showed that a single hydroxamate was present, with an Rf value intermediate between those of glyceric hydroxamate and dimethylglyceric hydroxamate. It was identical with that of 3-O-methylglyceric hydroxamate in Solvents D and E (Table IV).

The hydroxamate was purified by preparative paper chromatography (Solvent D), detected on the paper with the ferric chloride spray, eluted from the paper with water as the ferric complex, and finally treated with Dowex 50-H⁺ in order to remove ferric ions. The hydroxamate was hydrolyzed in 1 N HCl at 100°C for 1 hour, and converted to the methyl ester in methanolic HCl. Gas-liquid chromatography showed that methyl 3-O-methylglycerate was the only ester present (Fig. 7). The above experiment was repeated, reducing a larger amount of ferric ion had been lost. After 5 hours, acetate buffer, pH 5.0, was added to 0.1 M to decompose the reagent. The pH was adjusted to 8.0, and 20 mg of radioactive NaBH₄ (specific activity, 0.52 μC per mg) were added. After another 5 hours, excess NaBH₄ was destroyed by addition of acetic acid, and the mixture was evaporated to dryness. Boric acid was removed by repeated evaporation from methanol, and the product was passed through a Sephadex G-25 column (2 x 200 cm) to remove salts and radioactive impurities. The polysaccharide was hydrolyzed in acid, and the products were chromatographed on paper (Solvent B), 0.1 μg of sugar being applied to the chromatogram. The polypeptide, product, which proved to be glycerol, was detected with the periodate-benzidine reagent. The distribution of radioactivity on the paper strip showed that glycerol was the only radioactive product (Fig. 9). Similar treatment of intact MGP with radioactive NaBH₄ did not result in the formation of a radioactive product (Fig. 9).

The above experiment was repeated, reducing a larger amount of radioactive NaBH₄.
of Lossen-rearranged MGP with sodium borotritide that had a specific activity of 2.6 mC per mg. After acid hydrolysis and paper chromatography in Solvent B, the peak of radioactive glucitol was 25 times above the background level, and again no radioactivity migrated with standard 6-O-methylglucitol. Very little glyceric acid was present in the acid hydrolysate. The radioactive polyol was isolated by preparative chromatography, rechromatographed in Solvents A and B, and subjected to electrophoresis in sodium tetraborate buffer. In each case all of the radioactivity migrated with authentic glucitol.

The same conditions were used to carry out Lossen rearrangements on model compounds. 2-O-Benzylglyceric hydroxamate rearranged with the release of free benzyl alcohol (detected by gas-liquid chromatography on Column II at 170°). Rearrangement of 3-O-benzylglyceric hydroxamate, followed by reduction with NaBH4 and extraction of the product into ether, resulted in the formation of a compound with the retention time of monobenzylethylene glycol. In this case no benzyl alcohol could be detected. Therefore, formation of radioactive glucitol from MGP following the Lossen rearrangement confirmed the conclusion that the polysaccharide is linked to the 2 hydroxyl of glycercate.

6-O-Methylglucose-containing Polysaccharide from *M. tuberculosis*

Lee (2) has shown that a 6-O-methylglucose-containing polysaccharide could be isolated from *M. tuberculosis* by a procedure similar to the one followed for MGP from *M. phlei*. The molecule had a similar size and the same molar ratio of 6-O-methylglucose to glucose. This comparison is extended by our observations that the *M. tuberculosis* polysaccharide contains both glyceric acid and 3-O-methylglucose (identified by paper chromatography), and that it is digested by \(\alpha\)-amylase to give a large resistant fragment, glucose, maltose, and Disaccharide Z in the same relative amounts.

Physiological Investigations on MGLP

Metabolism of MGLP by *M. phlei*—It was considered that MGLP either might be incorporated into a larger molecule or might be metabolized as an energy source. Acetone-dried *M. phlei* cells, which had been harvested in the stationary phase, were extracted successively with boiling 70% aqueous ethanol, boiling chloroform-methanol-water, and hot aqueous pyridine. The combined extract was deacetylated in an organic solvent, and a search was made for 6-O-methylglucose-containing compounds other than MGP. Carbohydrate was fractionated first on a Dowex I-formate column, eluting batchwise, and then by gel filtration. Acid hydrolysates of all fractions were examined by paper chromatography in Solvent A. 6-O-Methylglucose could be detected only in the fraction containing MGP. A similar examination of the cultural filtrate of cells in the stationary phase revealed that MGLP was the only compound there which yielded 6-O-methylglucose on acid hydrolysis.

*M. phlei* cells, harvested in early log phase, were broken in a French press in neutral phosphate buffer. The broken cell preparation (1 g of wet cells) was incubated with 100 mg of \(\alpha\)-amylase-digested MGP for 2 days at 37°. To remove protein and particulate material, the digest was boiled for 10 min and centrifuged. All of the 6-O-methylglucose-containing material was eluted from a Sephadex G-25 column just after the void volume, showing that the molecule had not been degraded.

Subcellular Localization of MGLP—*M. phlei* cells in the stationary phase were broken in a French press in a phosphate-EDTA-sucrose medium, and the bacterial cell walls and a ribosomal fraction were prepared by differential centrifugation (42). Examination of these two preparations in the electron microscope showed that they were essentially free of contaminating material. Extraction of the cell walls with boiling chloroform-methanol-water (80:32:5), followed by acid hydrolysis of the soluble material, failed to reveal the presence of 6-O-methylglucose. Nor could it be detected after acid hydrolysis of the whole cell walls. The sodium chloride wash of the cell walls was also free of 6-O-methylglucose-containing material, suggesting that ionic association of MGLP with the cell walls did not occur. A similar examination of the particles showed that detectable amounts of 6-O-methylglucose were absent from this fraction.

The high speed supernatant was shaken with chloroform, and the chloroform layer and interfacial material were discarded. The aqueous layer was passed through a Sephadex G-25 column. Material which was eluted in the void volume was passed through a Sephadex G-50 column. A peak corresponding in position to MGLP (see Fig. 1) yielded 6-O-methylglucose after acid hydrolysis (characterized by paper chromatography and gas-liquid chromatography of the trimethylsilyl derivative). The amount of 6-O-methylglucose present corresponded approximately to the amount obtained by extraction of an equivalent amount of dry *M. phlei* cells.

Physical State of MGLP in Broken Cell Preparations—MGLP passes slowly through dialysis tubing. After dialysis of the high speed supernatant against dilute neutral phosphate buffer for 2 days at 4°, about half of the MGLP had passed through the tubing. Therefore, intracellular MGLP does not appear to be tightly associated with a high molecular weight molecule, such as a protein. Nor does it appear to aggregate into micelles.

The possibility remained that MGLP was esterified through the.
The supernatant, or a known fraction of it, was passed through a after addition of trichloracetic acid to a concentration of 1%. 

Cellosolve was determined by quantitative paper chromatography in Solvent B. This value was multiplied by 1.67 to obtain the amount of MCLP present. The results in B are corrected for evaporation of the medium during growth.

The working hypothesis upon which we have based the above discussion is that the 3-O-methylglucose-containing fragments released by \( \alpha \)-amylase and glucoamylase are derived from a side chain of the polysaccharide attached to the glucopyranosyl residue which is linked \( \alpha-1\,3' \) to the main chain. Our experiments have not excluded the alternative possibility that Tetrasccharide \( Z \) terminates the 6-O-methylglucose main chain, and that the side chain consists of only 1 or 2 glucose units. If glucose had been released from the intact MGP by glucoamylase, leaving 6-O-methylglucose and 3-O-methylglucose in the non-reducing terminal positions, this possibility would have been eliminated. However, this enzyme cleaved Trisaccharide \( Z \) from the polysaccharide more readily than glucose. We do not know whether this result means that the enzyme attacks endo linkages more readily than it cleaves a glucopyranosyl unit linked \( \alpha-1\,4' \) to a poly 6 O methylglucose chain, or whether Tetrasccharide \( Z \) in fact terminates the 6-O-methylglucose-containing main chain. Further experiments are needed to clarify this point. Fig. 11 represents alternative structures for the polysaccharide part of the lipopolysaccharide. The lipid components will be used for degradative purposes. Since the structures of the sub-

When the gross composition of the 6-O-methylglucose-containing lipopolysaccharide of \( M. \) phlei was reported (1, 2) it was emphasized that this was the first and only record of the natural occurrence of this glucose methyl ether. With the present finding that the polysaccharide contains \( D \)-glyceric acid, we note that this is to our knowledge the first report of the presence of this substance in a polysaccharide. This unusual natural polymer takes on a new structural complexity with our observation that the glucose chain is terminated by a 3-O-methyl-

Lee (2) has demonstrated that the polymer contains at least seven 6-O-methyl-D-glucose units in a chain, although the molecular weight of the polysaccharide suggests that the actual number may be closer to 10. We now find that a glucose unit, glycosidically linked to position 2 of the glyceric acid, terminates the reducing end of this chain. It is not yet known to which 6-O-

Because the exact structures of the substrates were not defined (47, 48). In our study on the structure of MGP, two amylases were used for degradative purposes. Since the structures of the sub-

The sugars in the acid hydrolysates of the various intracellular and extracellular fractions are listed in Table V. The finding that myoinositol, mannose, and glycerol are in the cell walls and the particles confirms the report (43) that the mannophosphoinositides (44) are associated with these structures.

**Table V**

**Sugars found in acid hydrolysates of fractions from**

**M. phlei** **in stationary phase**

<table>
<thead>
<tr>
<th>Cellular fraction</th>
<th>Components in acid hydrolysate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Myo-inositol</td>
</tr>
<tr>
<td><em>M. phlei</em> cell walls</td>
<td>+</td>
</tr>
<tr>
<td>Particles</td>
<td>+</td>
</tr>
<tr>
<td>Cell sap</td>
<td>+</td>
</tr>
<tr>
<td>Total cultural filtrate</td>
<td>+</td>
</tr>
<tr>
<td>Polysaccharides in cell sap and in cultural filtrate</td>
<td>+</td>
</tr>
</tbody>
</table>

"carboxyl group of glyceric acid to an alcohol. Therefore, acetone-dried *M. phlei* cells were extracted with basic hydroxylamine, and the extracted polysaccharide was purified on Sephadex G-25 and G-50 columns.\(^4\) No hydroxamate was associated with MGP, although less than 0.1 mole per mole of the polysaccharide would have been detected.

\(^4\) This experiment was performed by Dr. J. M. Keller.
Substrate specificities of pancreatic a-amylase and A. niger glucoamylase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGP</td>
<td>Tetrasaccharide Z, Trisaccharide Z, Disaccharide Z, maltose, glucose</td>
</tr>
<tr>
<td>Tetrasaccharide Z</td>
<td>Disaccharide Z, maltose, glucose</td>
</tr>
<tr>
<td>Trisaccharide Z</td>
<td>No reaction</td>
</tr>
<tr>
<td>Disaccharide Z, 4-O-Methyl-terminal malto-oligosaccharides</td>
<td>No reaction</td>
</tr>
<tr>
<td>Malto-oligosaccharides</td>
<td>Maltose, glucose</td>
</tr>
<tr>
<td>Fully 6-O-methylated malto-oligosaccharides</td>
<td>No reaction</td>
</tr>
<tr>
<td>6-O-Methyl-α-D-glucopyranosyl-(1 → 4)-α-D-methyl-D-glucose</td>
<td>No reaction</td>
</tr>
<tr>
<td>6-O-Methyl-α-D-glucopyranosyl-(1 → 4)-α-D-glucose</td>
<td>No reaction</td>
</tr>
</tbody>
</table>

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REFERENCES

The 6-O-Methylglucose-containing Lipopolysaccharide of Mycobacterium phlei: IDENTIFICATION OF d-GLYCERIC ACID AND 3-O-METHYL-d-GLUCOSE IN THE POLYSACCHARIDE

Milton H. Saier, Jr. and Clinton E. Ballou


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