Biosynthesis of the Mycobacterial \( \text{O}-\text{Methylglucose Lipopolysaccharide} \)

CHARACTERIZATION OF PUTATIVE INTERMEDIATES IN THE INITIATION, ELONGATION, AND TERMINATION REACTIONS* 

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From the 70% ethanol extract of \textit{Mycobacterium smegmatis} cells, we isolated a mixture of weakly acidic oligosaccharides composed mainly of glucose and \( \text{6-} \text{O-} \text{methylglucose} \). The elution pattern from a Bio-Gel P-4 column suggested that the oligosaccharides were smaller than the \( \text{O}-\text{methylglucose polysaccharide} \) (MGP) and could be biosynthetic precursors. Analysis by fast-atom-bombardment mass spectrometry revealed that the oligosaccharides fit into a pattern for polysaccharide synthesis based on an alternate glucosylation-methylation mechanism. The expected intermediates accumulate in very small amounts and because the putative precursors of the methylglucose lipopolysaccharide has, until now, been less fruitful because the expected intermediates accumulate in very small amounts and because cell extracts contain other unrelated acidic partially methylated glucans. In the present report, however, we describe the isolation of weakly acidic and partially methylated glucans of the size predicted for the putative precursors of the methylglucose polysaccharide, and analysis by fast-atom-bombardment mass spectrometry reveals that the oligosaccharides fit a pattern of sequential glucosylation-methylation for biosynthesis of the \( \text{6-} \text{O-} \text{methylglucose}-\text{containing} \) portion of the polymer. The data also support a novel mechanism for termination of the elongation reaction.

*This work was supported in part by United States Public Health Service Grant AI-12522 and by National Science Foundation Grant PCM84-00251 (to C. E. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Received support from the Medical Research Council.

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EXPERIMENTAL PROCEDURES

Materials and Methods—Sources for most of the materials used in this study are listed in earlier publications (13, 14). Affi-Gel 601, for binding sugars with cis-diols, was from Bio-Rad. Mycobacterium \textit{smegmatis} ATCC 356 was grown in a 200-liter fermentor on a glycerol medium (15), and the cells were harvested with a large steam-driven Sharples centrifuge to give about 2 kg of wet cell paste. The cells were frozen until required. The methodologies for gas, thin layer, and ion exchange chromatography, for gel filtration, for mass spectrometry, and for colorimetric determination of carbohydrate have been described (13).

Isolation of Putative MGP Precursors—\textit{M. smegmatis} cell paste (500 g) was stirred in acetone (2 liters) at 20–23 °C for 18 h, and the insoluble cell debris was collected on a Buchner funnel with glass filter paper, washed with acetone, and dried in air. The acetonewashed cells were extracted twice for 2 h each time with refluxing 70% ethanol (2 liters), and the combined ethanolic filtrates were concentrated to a small volume and saponified with 1 N NaOH (100 ml) at 100 °C for 8 h. After the cooled solution was neutralized with...
Dowex AG 50W-X8 resin, it was extracted with diethyl ether (200 ml) twice to remove any lipids and concentrated to dryness. To remove strongly acidic material, the residue was dissolved in water (50 ml) and applied to a column of DEAE-Sephadex A-25 (HCO₃⁻) (150 ml), which was washed with 40 mM ammonium bicarbonate (500 ml). The effluent which contained neutral and weakly acidic materials was lyophilized to remove most of the salt, the residue was treated with 1 N NaOH (25 ml) at room temperature for 5 h to saponify any glyceric acid lactone that may have formed during lyophilization, and the cations were again removed with Dowex AG 50W-X8. The solution was applied to a column of DEAE-Sephadex A-25 (HCO₃⁻) (150 ml), which was washed with water to remove a neutral fraction and then eluted stepwise with 10, 20, and 40 mM ammonium bicarbonate (400 ml each) to yield acidic fractions. The yield of carbohydrate as glucose was 18.1 mmol (neutral fraction), 3.3 mmol (10 mM acidic fraction), 0.43 mmol (20 mM acidic fraction), and 0.69 mmol (40 mM acidic fraction). Following elution of the first DEAE column with 40 mM salt, the strongly acidic fraction was eluted with 80 mM salt to give 0.43 mmol of carbohydrate.

The 10 mM acidic fraction (200 mg of carbohydrate) was dissolved in 2 ml of 0.25 M ammonium acetate, pH 8.8, and the solution was passed through a 5-ml column of Affi-Gel 601 to remove mannose-containing materials. The column was washed with three 5-ml portions of the same buffer to recover unbound material, and the effluent was concentrated and lyophilized several times after addition of water to remove the salt. The residue was dissolved in 2 ml of 0.1 M acetic acid and fractionated on a Bio-Gel P-4 (400 mesh) column (2 x 190 cm) by elution with the same solvent. The other acidic fractions were treated in a similar manner.

RESULTS

Isolation of MGP Precursors—The 70% ethanol extract of acetone-dried M. smegmatis cells contains many oligosaccharide components, some of which are acetylated. To remove the acyl groups completely, the material had to be treated in hot alkaline solution for several hours. Because MGP and its precursors are weak acids, it was important to decationize the resulting solution completely to ensure efficient binding of the oligosaccharides to the DEAE-Sephadex column. The unbound material yielded mostly mannose, arabinose, glucose, and 3-O-methylmannose on acid hydrolysis, whereas oligosaccharides rich in 6-O-methylglucose were eluted with 10 mM salt. The 20 mM salt fraction also gave some 6-O-methylglycosic acid on hydrolysis, but the materials eluted at higher salt were composed mainly of glucose, mannose, arabinose, and other sugars.

Because arabinose and mannose related to a known acidic arabinomannan (16) contaminated hydrolysates of most fractions, the MGP precursors were purified by passing the solutions through a phenyl boronate column, which bound most of the former but not the latter. The unbound material from the 10 mM salt elution was then fractionated according to size on a Bio-Gel P-4 column. The vast majority of material was eluted in the position of mature MGP, which peak was separated into 3 fractions, while the trailing end of the MGP peak was separated arbitrarily into several fractions as indicated on Fig. 1. The monosaccharide compositions of the fractions revealed that many of the samples were still contaminated with small amounts of sugar-containing materials unrelated to MGP, but these did not interfere with the mass spectral analysis. Estimates of the degree of polymerization of oligosaccharides in these fractions must take into account the facts that each methyl ether group contributes the equivalent of 1.6 hexose units (17) and that the glyceric acid group contributes about 2 hexoses to the apparent size because of its charge.

The material isolated from the DEAE column by elution with 20 mM salt was treated in a similar manner, and the distribution of carbohydrate in the Bio-Gel P-4 column effluent is shown in Fig. 2. The large peak in the void volume is related to the acidic arabinomannan (16) mentioned above, whereas the other two larger peaks between fractions 180 and 200 are related to a glycolipid (13) and a phospholipid (18, 19) described previously.

The 40 mM salt eluate of the DEAE-Sephadex column gave a single carbohydrate-containing peak by gel filtration on a Bio-Gel P-4 column that appeared at the position of a trisaccharide (not shown). This material proved to be identical to fraction 15 in Fig. 2, and it was characterized as glucosylglyceric acid (20, 21) (see below). Elution of the DEAE column with 80 mM salt gave more carbohydrate-containing material that was rich in glucose, arabinose, mannose, and phosphate, which suggested that this fraction contained trehalose phosphate (10) and glucolipid (13) and also was related to the acidic arabinomannan (16). Preliminary analyses confirmed this conclusion, but further characterization was not attempted.

Analysis of the Acidic MGP Precursor Fraction Eluted by 10 mM Salt—The majority of carbohydrate in the putative precursor fraction, eluted from DEAE-Sephadex with 10 mM salt and fractionated by gel filtration on a Bio-Gel P-4 column (Fig. 1), appeared at a position characteristic of MGP, but a small amount trailed after the main peak to a position at
which an eicosasaccharide (Glcto) would appear. From other work (17), we expect an oligosaccharide composed of 5 O-methylglucoses, 5 glucoses, and a glyceric acid unit to be eluted at the GlcM position, whereas MGP is eluted near the void volume of a Bio-Gel P-4 column.

The fractions from the Bio-Gel P-4 column were combined arbitrarily as indicated in the figure and the FAB mass spectrum of each was determined in the positive mode. The major ions in the spectra of all 8 fractions (Table I) corresponded to the sodium-cationized forms of MGP-related fragments [M + Na]+, although all spectra also showed ions for [M + H]+ and some had ions corresponding to [M + K]+. Compositions and plausible structures are indicated in the table.

The FAB mass spectrum of MGP (19) previously demonstrated that about 20% of the molecules have an extra methyl group, which must be near the glyceric acid end of the chain because it is also present in AGMGP (22). This minor homolog is enriched in fraction 1 of Fig. 1, and we have used this material to determine the exact location of the methyl group. The sample was digested with Rhizopus amylase to remove most of the 6-O-methylglucopyranoside chain (14), and the acidic limit product was isolated by ion exchange and fractionated on a Bio-Gel P-4 column (Fig. 3). Peaks A and C correspond to fractions A-1 (MeGlc3Glc6Gla) and A-2 (MeGlc4Glc5Gla) from a previous study (14) and are derived from the major MGP component. Peaks B and D are displaced on the gel filtration pattern by an amount suggesting that they differ from peaks A and C, respectively, by one methyl group (apparent volume = 1.6 hexoses/methyl). The compositions (MeGlc3Glc6Gla and MeGlc4Glc5Gla) confirm this and demonstrate that the extra methyl group is on position 6 of one of the 5 glucose units in A-1 and A-2 (Table II).

An attempt was made to determine the location of the extra methyl group from the anomeric proton NMR spectra (Table III). The signals for the 4 glucoses nearest the glyceric acid end (A, B, C, and D in Fig. 4) are shifted no more than 0.003 ppm on addition of the methyl group, whereas 3 of the other 4 hexoses are significantly affected. The anomeric proton of the α(1→3)-linked glucose is shifted upfield 0.024-0.027 ppm, whereas the methylglucose to which it is attached is slightly shielded and the other two 6-O-methylglucoses are deshielded about 0.025 ppm. We found previously that methylation at position 6 of glucose has a relatively small effect on the anomeric proton of that hexose, but it can affect the shifts of neighboring glucoses (14). Therefore, we concluded that the NMR spectra do not allow a definitive assignment.

Convincing evidence that the extra methyl group was on glucose D (Fig. 4) came from study of the fragments in the FAB mass spectrum of peak B. The parent ion is at m/z 1457 [M – H]−, whereas fragment ions were observed at m/z 1295 [M – 162] and at m/z 1133 [M – 324]. The presence of ions for loss of two hexoses means that both side chain glucoses are unmethylated. An ion at m/z 591 corresponds to GlcGla, which could be derived from glucoses A, B, C or A, B, D (Fig. 4), but in either instance it suggests that glucose B is not a site of methylation. Because glucose A is already substituted at position 6, it also can be ruled out as a site of methylation. In the fragment series corresponding to ions of the type hexose-O-CH=CHO−, we observed Hex2MeHex−O-CH=CHO− (m/z 1087) and Hex,MeHex−O-CH=CHO− (m/z 749). Taken together, all of these ions are fully consistent with methylation of glucose D, which terminates the α(1→4)-linked polymer chain and is itself linked β(1→3).

Fractions 5–8 from Fig. 1 show an interesting and suggestive pattern in which the ions correspond to molecules that contain, alternately, 4 and 5 glucoses along with varying numbers of O-methylglucoses (Table I). The 5th glucose in each instance is represented as occurring at the glyceric acid end of the chain (MeGlc3Glc6Gla). Support for this conclusion was obtained by analysis of the products formed by Rhizopus amylase digestion of the oligosaccharide mixture. Such digestion of mature MGP yields MeGlc3Glc6Gla and MeGlc4Glc5Gla (see above (14)), and digestion of the combined precursor fractions 5–8 gave ions on FABMS at m/z 1443 and 1457, which correspond to these same fragments.

Fraction 4 from Fig. 1 gave a complex assortment of ions that extends the series found in fractions 5–8 up to the homologs with 11 and 12 methylglucoses, at which point ions are observed corresponding to the additions of 1 and 2 glucose units without further methylation. This is consistent with a biosynthetic pathway in which, following construction of the

| Table I |
|-----------------|-----------------|-----------------|
| Fractions and ions observed in positive mode [M + Na]+ | Proposed compositions |
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| 3561a | 3551 | 3537 | 3537 | 3213 | 3213 | 3037 | 3037 |
| 2889 | 2787 | 2713a | 2699a | 2699a | 2537 | 2537 | 2537 |
| 2523 | 2523 | 2523 | 2361 | 2347a | 2347a | 2185b | 2185b |
| 2099 | 2099 | 2099 | 2099 | 2099 | 2099 | 2099 | 2099 |
| 1995a | 1995a | 1995a | 1995a | 1995a | 1995a | 1995a | 1995a |
| 1833 | 1833 | 1833 | 1833 | 1833 | 1833 | 1833 | 1833 |

*a To compare with ions in Table IV, subtract 24 mass units.  
*b Preponderant ion. Most fractions also showed ions for [M + H] and some for [M + K].
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Fig. 3. Gel filtration of the Rhizopus amylase digest of a mixture of MGP homologs. The material in fraction 1 of Fig. 1 was treated as described under "Experimental Procedures," and the acidic product was fractionated on a Bio-Gel P-4 column (1 x 190 cm) (top panel). Characteristic data for each peak are given in Table II. Peaks A and C are derived from the major MGP homolog with 11 6-O-methylglucoses, and peaks B and D are from the minor homolog with 12 6-O-methylglucoses. Similar processing of the total MGP component (fractions 1, 2, and 3) gave the pattern in the bottom panel (14).

Table II
Molecular size and composition of acidic amylase fragments from MGP

| Peak* | Apparent size by gel filtration | Composition | FABMS molecular ion [M - H]+ |
|-------|--------------------------------|-------------|------------------------------|
| A     | 15 (14.6)                      | Glc 6-MeGlc | 1443                         |
| B     | 16.4 (16.1)                    | Glc 6-MeGlc | 1457                         |
| C     | 17.4 (17.4)                    | Glc 6-MeGlc | 1619                         |
| D     | 18.7 (19.0)                    | Glc 6-MeGlc | 1633                         |

*From Fig. 3.

†In hexose units on a Bio-Gel P-4 column calibrat ed with maltodextrins. The numbers in parentheses are the apparent sizes calculated assuming that each glucose has a value of 1, each 6-O-methylglucose has a value of 2.6, and the glyceric acid has a value of 2.

‡Determined by gas chromatography on an OV-225 column, by which method all monomethylglucitol pentasaccharides have characteristic retention times. No peak for a dimethyl derivative was observed.

Table III
Anomeric proton NMR data for acidic amylase fragments of MGP

| Hexose unit | Peak A* | Peak B* | Peak C* | Peak D* |
|-------------|---------|---------|---------|---------|
| δ ppm       | ω 1,3   | ω 1,2   | δ ppm   | ω 1,3   |
| H2          |         |         | H2      |         |
| A           | 5.022   | 3.6     | 5.019   | 3.6     | 5.022   | 3.6     | 5.022   | 3.6     |
| B           | 4.936   | 3.6     | 4.936   | 3.5     | 4.936   | 3.6     | 4.935   | 3.6     |
| C           | 4.906   | 8.0     | 4.903   | 7.7     | 4.906   | 8.0     | 4.902   | 7.9     |
| D           | 4.897   | 7.9     | 4.897   | 7.7     | 4.897   | 7.8     | 4.895   | 8.2     |
| E           | 5.342   | 3.8     | 5.333   | 3.9     | 5.342   | 3.6     | 5.336   | 3.8     |
| F           | 5.437   | 3.8     | 5.413   | 4.0     | 5.456   | 3.8     | 5.432   | 3.9     |
| G-1*        | 5.578   | 3.8     | 5.408   | 3.8     | 5.387   | 4.0     | 5.416   | 3.9     |
| G-2*        | 5.361   | 3.9     | 5.383   | 3.9     | 5.378   | 3.9     | 5.391   | 3.9     |
| G-3*        | 5.370   | 5.363   | 4.0     |

*Refer to Fig. 4.
†Refer to Fig. 3.
‡Assignments arbitrary. Other assignments based on comparison to reference compounds (14).
§Overlapping signals prevented measuring coupling constants.

Analysis of the Acidic MGP Precursor Fraction Eluted by 20 mM Salt—Similar analysis of the Bio-Gel P-4 fractions from the material eluted from DEAE-Sephadex with 20 mM salt (Fig. 2) demonstrated the existence of related smaller precursors. In this series, the FAB mass spectra were determined in the negative mode, and the preponderant ions correspond to [M-H]- (Table IV). To compare the ions of the two tables, one need only add or subtract 24 mass units as appropriate. Fractions 1–3 of Fig. 2 gave ions for several intermediates that overlap with those in fractions 7 and 8 of Fig. 1, whereas fractions 4–12 of Fig. 2 gave the expected ions extending the homologous series down to MeGlc,Glc,Glu (where m/z 1091). Two ions were seen (m/z 1957 and 2133), with intensities about 10% of the major ions in the spectra, that correspond to intermediates with an extra unmethylated glucose we have placed at the nonreducing end and are presumed to be the methyl acceptors for synthesis of the next higher homologs (m/z 1971 and 2147). Another ion (m/z 943) corresponded to MeGlc,Glc,Glu and could be a precursor of the more highly methylated form of MGP (m/z 3551). An ion at m/z 735 and one at m/z 557 reveal that fractions 11 and 12 are contaminated with non-MGP fragments related to a glycolipid (13) and a phospholipid (18) described previously.

The assignments of structures to the ions listed in Table IV are supported by the FAB mass spectrum of the Rhizopus amylase limit digest of the combined fractions 1–5. The acidic fraction from the digest gave ions for MeGlc,Glc,Glu (where x = 4–6) and MeGlc,Glc,Glu (where x = 3–5), and no ion for a compound with 6 glucoses was observed, as expected if the 6th glucose in fractions 1 and 3 was in a terminal position where it would be removed by amylase.

Finally, fractions 14 and 15 of Fig. 2 gave single ions...
corresponding, respectively, to Glc4Gla and Glc6Gla, that could represent the first two intermediates in MGP synthesis. The assignments of these two ions are consistent with their elution positions on the Bio-Gel P-4 column because the glyceric acid moiety contributes the equivalent of 2 hexoses to the apparent volume of a glycoside. It is notable that ions for intermediates between Glc2Gla and MeGlc6Glc4Gla were not observed. By gel filtration of the material eluted from DEAE-Sephadex with 40 mM salt, a single carbohydrate-containing peak was obtained that corresponded to Glc6Gla and gave a single ion at m/z 267. This sample was characterized in detail (following section).

**Characterization of Glucosyl-Glyceric Acid**—From the precursor fraction eluted from the DEAE-Sephadex column with 40 mM salt, a major component was obtained by gel filtration on a Bio-Gel P-4 column at the elution volume of a trisaccharide. Thin layer chromatography of the material on Silica Gel G-60 in ethanol/acetic acid/water (41:2, v/v) revealed a single component after charring with sulfuric acid (Rf = 0.56), whereas glucose was the only sugar detected in an acid hydrolysate of the sample. The 13C NMR spectrum showed the 9 signals expected for glucosyl-glyceric acid, and the chemical shifts were similar to those reported for α-D-glucopyranosyl-(1→2)-D-glyceric acid (Table V) (20), although the shifts for the glyceric acid part were between those of the free acid and the ionized form, indicating that our sample was a mixture of the two species. The specific optical rotation, [α]D = +146° (C, 0.47, water), confirmed the anomic configuration, and the negative FAB mass spectrum gave a pseudomolecular ion at m/z 267 [M – H]– in agreement with the calculated molecular weight (Mw = 268). A specific rotation of +140° (water) has been reported for the free acid (21).

**Characterization of Diglucosyl-Glyceric Acid**—The FAB mass spectra of fractions 13 and 14 from Fig. 2 gave single ions at m/z 429, which agrees with the composition Glc6Gla, and analysis of the acid hydrolysate of each revealed glucose as the only sugar component. The anomic proton NMR spectrum of the combined fractions showed four signals of about equal intensities assignable to α-linked glucose units at δ4.94, 4.95, 5.02, and 5.39, each with a coupling constant of 3.8–3.9 ppm. We assign the set at δ4.94 and 5.02 to α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→2)-D-glyceric acid, an expected MGP precursor, whereas the other set at δ5.39 and 4.95 suggests the presence of a related compound with the structure α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→2)-D-glyceric acid. The assignment for the first compound is supported by the chemical shifts we observed for a diglucosyl-glyceric acid obtained by partial acid hydrolysis of MGP (δ4.94 and 5.02). We are unable to account for the second diglucosyl-glyceric acid, but it could be a precursor of some other unreported oligosaccharide.

**DISCUSSION**

The biosynthesis of the O-methylglucose lipopolysaccharide is interesting because the polymer has a precisely defined length and sequence, which is unusual for polysaccharides of this size. Since the structure of polysaccharides is determined solely by enzyme specificity, this implies that the α(1→4)-glucosyltransferase(s) can recognize when the 15th in a chain of similarly linked sugars has been added, and the 6-O-methyltransferase(s) can sense when the 11th glucose unit in the otherwise monotonous α(1→4)-linked chain has been methylated. Second, the polysaccharide chain is acylated in 8.
specific positions (23) by 5 different kinds of acyl groups (24), which indicates the existence of several highly specific acyltransferases (25). In previous studies in which maltooligosaccharides, α(1→4)-linked glucans, were used as exogenous acceptors for the putative methyl- and acyltransferases, it was found that the acceptor ability in both reactions was dependent on partial acetylation (17) implying that methylation and acylation may proceed together.

The present report documents the existence in *M. smegmatis* cell extracts of smaller oligosaccharides related in their structures to the methylglucose polysaccharide and of such a composition as to support a mechanism for elongation of the chain based on sequential glucosylation-methylation. Weakly acidic oligosaccharides with the composition MeGlc,Glc,Gla, where \( x = 1-11 \), were detected by mass spectrometry, along with a parallel series with the composition MeGlc,Glc,Gla, where \( x = 2-12 \). These structures reveal that the precursors have 4 or 5 glucose units at the glycric acid end of the chain in agreement with the fact that mature MGP is a mixture of isomers that possesses 4 or 5 glucose units in this region.

The mass spectral data further show that the addition of glucose units to the oligosaccharide MeGlc,Glc,Gla then occurs without further methylation to yield the presumed intermediate Glc,MeGlc,Glc,Gla. Conversion of this oligosaccharide to mature MGP requires the addition of two more glucoses to the terminal Glc2 unit and methylation of the last one. The expected intermediates for these steps were not observed, however, which suggests that the reactions may occur rapidly and in concert to give mature MGP. We expect that the oligosaccharide with the presumed structure Glc2,MeGlc,Glc,Gla will associate readily with long-chain fatty acid to form an inclusion complex in which the polymer assumes a tightly coiled conformation (9), and it is possible that such a complex is presented to the transferases that catalyze the termination reactions. Such postulated coiling might inhibit further O-methylation and promote 3-O-methylation. If such a mechanism is involved, it would parallel in some ways that which appears to regulate termination of methylmannose polysaccharide elongation (12).

In Fig. 5, we have selected representative structures detected by FABMS and have ordered them into a plausible pathway for biosynthesis of both forms of MGP. It is apparent that the extra methyl group in the minor homolog is introduced at an early stage in the process, but we cannot confute that it might also be added to some molecules at later times. That most of the methylated intermediates are terminated by O-methylhexose suggests that the O-methyltransferase must have a lower \( K_e \) for the acceptor than the glucosyltransferase. Two intermediates possessing 6 unmethylated glucose units were detected, however, and our structural analysis indicates that the sixth glucose is at the nonreducing end of the chain where it could serve as the methyl acceptor in the next step.

Support for the conclusion that the MGP precursors consist of homologs with 4 or 5 glucose units at the glycric acid end of the chain came from analysis of the limit product resulting from digestion of the oligosaccharides with a mold amylase that catalyzes hydrolysis of 6-O-methyl-α-D-glucosyl (1→4) linkages (14). The product had the composition MeGlc,Glc,Gla, MeGlc,Glc,Gla, MeGlc,Glc,Gla, and MeGlc,Glc,Gla, which also are obtained when a mixture of mature MGP-I and MGP-II is digested in a similar manner.

Our analysis of the MGP fraction enriched in the homolog with one extra methyl suggests that the methyl group is located on position 6 of the β(1→3)-linked glucose unit. It seems unlikely that this methyl group is introduced by the same enzyme that catalyzes methylation of the α(1→4)-linked glucose units of the terminal Glc2 unit of mature MGP (17). From the FAB mass spectra, however, the ion related to this glycolipid (m/z 735) was easily recognized as such.

Although the evidence is limited, we believe it supports our conclusion that the presumed precursors are not degradation products of MGP. First, previous attempts to demonstrate the existence of enzymic activities in *M. smegmatis* cells that could degrade radiolabeled MGP gave negative results (27). Second, the pattern of oligosaccharide structures we have found is not that expected for the amylolytic degradation of MGP as observed with the *Rhizopus* amylase (14), because intermediates at m/z 1957 and 2133 should not be formed.

Since most of the fractions we collected from the Bio-Gel P-4 column encompassed a size range of several hexose units, we would expect multiple ions in the mass spectra and that the smaller ions of one fraction should overlap with the larger ions of a neighboring fraction, which was observed. Although FAB mass spectra can yield fragment ions of the parent compounds, the multiple ions we observe in each spectrum reflect the presence of real components. This conclusion is
supported by the relative intensities of the various ions and the fact that some could not be derived from larger ions in the spectrum by the expected fragmentation mechanisms. Finally, some fractions gave single ions, and the molecular range of the ions we observed in each fraction agreed with the Bio-Gel P-4 column elution volumes we calculate from their sizes and compositions (17).

Jordan (28) has investigated the mechanism of 6-O-methylglucose lipopolysaccharide biosynthesis by analyzing the gradient of radioactivity in the methyl groups of MGP formed in a short incubation of the cells with t-[methy]-H]methionine. The labeled 6-O-methylglucose lipopolysaccharide was isolated, deacetylated, and chemically permethylated. After hydrolysis, the specific activities of derivatives corresponding to the branched 6-O-methylglucose, the ten internal unbranched 6-O-methylglucoses, and the terminal 3-O-methylglucose were determined, while the activity of the 6-O-methylglucose nearest the nonreducing end of the chain was obtained after enzymic removal of the 3-O-methylglucose and the neighboring 3 glucose units (29). The gradient of H radioactivity increased from the branch point through the chain of 6-O-methylglucoses to the terminal 3-O-methylglucose, a result that is consistent with the pathway we have inferred from the structures of the putative oligosaccharide precursors. Such a study, of course, does not distinguish between a pathway in which the polymer is formed and then methylated and one in which the polymerization and methylation occur hand in hand, as our results suggest.

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