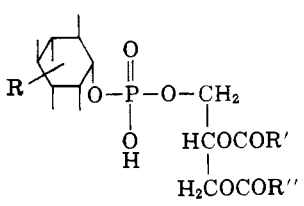
Complete Structures of the Glycophospholipids of Mycobacteria*

Yuan Chuan Lee and Clinton E. Ballou

ABSTRACT: 1-Glycerylphosphoryl-L-myoinositol di-, tri-, tetra-, and pentamannosides were isolated from the deacylated chloroform-methanol extracts of *Mycobacterium phlei* and *M. tuberculosis* cells by a combination of DEAE-Sephadex column chromatography and paper chromatography. The structures of the carbohydrate portion of these glycophospholipids were studied after removal of the glycerylphosphoryl group by ammoniacal hydrolysis. The methanolysates of the permethylated myoinositol tri- and tetramannosides contained 1,3,4,5-tetra-*O*-methylmyoinositol as well as the methyl glycosides of 2,3,4,6-tetra-*O*-methyl-D-mannose and 2,3,4-tri-*O*-methyl-D-mannose, in ratios of 1:2:1 and 1:2:2, respectively. The results indicate that the tri- and tetramannosides have the same branched structure as the pentamannoside (Y. C. Lee and C. E. Ballou, *J. Biol. Chem.* *239*, 1316 [1964]), in which the mannoses are attached to positions 2 and 6 of the myoinositol ring. Partial acid hydrolysis of the myoinositol pentamannoside with Dowex 50 yielded a pair of myoinositol tetramannoside isomers and a pair of myoinositol trimannoside isomers. Upon methylation, one of the tetramannoside isomers gave 1,2,3,4,5-penta-*O*-methylmyoinositol, 2,3,4,6-tetra-*O*-methyl-D-mannose, 2,3,4-tri-*O*-methyl-D-mannose, and 3,4,6-tri-*O*-methyl-D mannose in a ratio of 1:1:2:1, thus establishing a linear structure in which the myoinositol ring is substituted at the 6 position by a mannotetraose chain. The other isomer was shown to have the same branched structure as the pentamannoside, but it contained only 1,6′ linkages. One of the trimannoside isomers had a similar linear structure, while the other had a branched structure. A linear mannotetraose was also isolated from the hydrolysate. Enzymatic removal of the nonreducing terminal mannose residue caused disappearance of the 1,2′ linkage in the pentamannoside, confirming that this linkage was located at the end of the mannotetraose unit. On the basis of these results, the complete structure of phosphatidyl myoinositol pentamannoside can be described as 1-phosphatidyl-2-*O*-α-D-mannopyranosyl-6-[*O*-α-D-mannopyranosyl-(1-2)-*O*-α-D-mannopyranosyl-(1-6)-O-α-D-mannopyranosyl-(1-6)-*O*-α-D-mannopyranosyl]-L-myoinositol. Analysis of the anomeric hydrogen signals by proton magnetic resonance confirmed the above-mentioned structure for the pentamannoside. An improved method for gas-chromatographic separation of methyl *O*-methylmannosides and *O*-methylmyoinositols by means of trimethylsilylation is described.

Mycobacterium tuberculosis and *Mycobacterium phlei* contain a family of myoinositol phospholipids in which the myoinositol ring is substituted with varying amounts of D-mannose, and which correspond to the following general structure:



R′, R″ = fatty acids
(I) R = H
(II) R = mannosyl
(III) R = dimannosyl
(IV) R = trimannosyl
(V) R = tetramannosyl
(VI) R = pentamannosyl

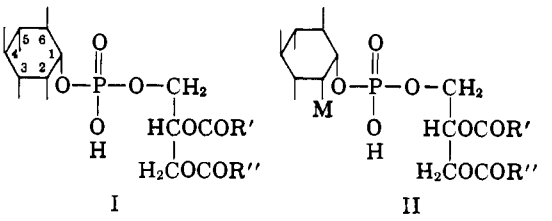
Anderson (1940) first demonstrated the presence of myoinositol and myoinositol dimannoside ("manninositose") as parts of a *Mycobacterium* phospholipid. This was confirmed by Vilkas (1960), who also isolated myoinositol monomannoside as a degradation product of the lipid mixture. Nojima (1959) has described the isolation of myoinositol tetramannoside and pentamannoside from *M. tuberculosis* phospholipid, while Lederer (1961) has reported a myoinositol trimannoside obtained from the same source. Thus there is suggestive evidence for a complete spectrum of compounds with structures I to VI.

The position of substitution by the phosphatidyl group on the myoinositol ring has been established (Ballou *et al.*, 1963). It is the same in all of the analogs investigated (I, III, and VI), and is that illustrated in the foregoing structure, namely, the L-1 position.

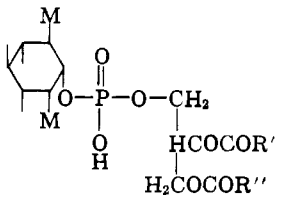
The mannose present in these phospholipids has the D configuration. The optical rotation of the myoinositol monomannoside isolated by Vilkas indicates that the glycosidic linkage is α. A sample of the monomannoside isolated by Ballou *et al.* (1963) has been methylated (Ballou and Lee, 1964) and shown to have the mannose attached to position 2 of the myoinositol ring. In other studies (Lee and Ballou, 1964) it has been established that the myoinositol dimannoside, "manninositose," has the mannoses attached separately to positions 2 and 6 of the myoinositol. It is possible, therefore, to write structures for compounds I, II, and III (where M = mannose) (see below).

Some preliminary studies on the structure of the myoinositol pentamannoside component of *Mycobacterium* phospholipid have been reported (Lee and Ballou, 1964). It was shown that the five mannoses are attached

* From the Department of Biochemistry, University of California, Berkeley. *Received March 15, 1965.*

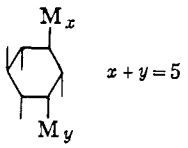


I II



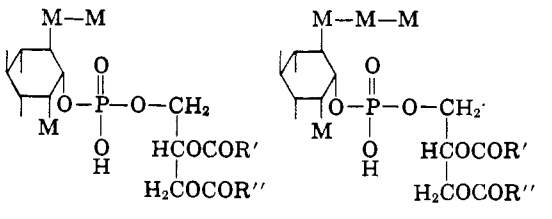
III

as short chains to positions 2 and 6 of the myoinositol ring, and that two 1,6′ linkages and one 1,2′ linkage are involved in connecting the mannose units to each other. These results indicated that the myoinositol pentamannoside had the following general structure, which could exist in twelve isomeric forms differing in

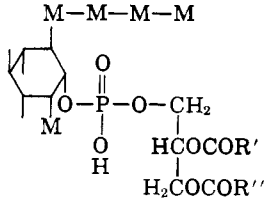


the values of x and y, in the order of the 1,2′ and 1,6′ linkages and in the arrangements of the oligosaccharide units on positions 2 and 6 of the myoinositol ring.

In this paper we are reporting investigations which show that the myoinositol pentamannoside has one mannose on the 2 position and the other four mannoses are on the 6 position, and that the single 1,2′ linkage occupies the terminal position of the mannotetraose side chain. We have also obtained small amounts of myoinositol tetramannoside and myoinositol trimannoside, and have found that they differ from the pentamannoside in lacking, respectively, one and two of the terminal mannoses in the mannotetraose chain. The results indicate that compounds IV, V, and VI have the following structures:



IV V



VI

M. phlei also contains a phosphatidyl myoinositol hexamannoside which has a structure analogous to compound VI, with an additional α-1,2′-linked mannose at the end of the mannotetraose chain.

Materials and Methods

The sources of the glycolipids from *Mycobacterium tuberculosis* and *Mycobacterium phlei* and the procedures for determination of glycerol, phosphorus, myoinositol, carbohydrate, and reducing sugar have been described previously (Lee and Ballou, 1964). Sweet almond emulsin with α-mannosidase activity was purchased from Worthington Biochemical Corp., under the name of "β-glucosidase," and was used without further purification.

Paper chromatography was carried out at room temperature by the descending technique using Whatman No. 1 filter paper. The solvent systems used were: (A) 2-propanol–concentrated ammonia, 2:1 (B) 1-butanol-ethanol-water, 2:1:1; (C) 1-butanol-pyridine-water, 10:3:3.

General procedures for gas chromatography were the same as described in the previous report. In addition to neopentylglycol succinate, QF-1 (Fluorosilicone, supplied by the Dow Corning Corp.) was used as a stationary phase.

Proton magnetic resonance spectra were measured on a Varian Model A-60 spectrometer in deuterium oxide solution. Exchangeable hydrogens were replaced by evaporating the deuterium oxide solution once. As an internal standard, a drop of glacial acetic acid was included in the sample solution, and the signal of the acetoxy protons was set at 2.10 ppm downfield from tetramethylsilane.

Determination of Ratios of Mannose to Myoinositol by Gas Chromatography. The myoinositol oligomannoside, about 1.0 mg, was hydrolyzed in 1 ml of 1 N sulfuric acid for 5 hours at 100°. The hydrolysate was neutralized with Dowex 1-X8 (carbonate form) and evaporated to dryness. The residue was dissolved in 0.1 ml of dry pyridine with slight warming. To the solution were added 0.05 ml of hexamethyldisilazane and 0.05 ml of trimethylsilyl chloride. The mixture was shaken vigorously and allowed to stand at room temperature for 20 minutes with occasional shaking.

Direct injection of the reaction mixture (Sweeley *et al.*, 1963), though satisfactory for qualitative analysis, was not suitable for quantitative determination owing to tailing of the pyridine peak and the high detector noise caused by inorganic substances in the reaction mixture. For quantitative analysis, the white precipitate formed upon addition of the reagents to the pyridine solution was removed by centrifugation and the clear supernatant was evaporated to dryness at room temperature. The residue was dissolved in 0.2 ml of carbon disulfide, and an appropriate amount of the solution was injected onto the 5% QF-1 column (Lee and Ballou, 1965).

The ratio of mannose to myoinositol was obtained from the peak areas of the individual compounds. A correction was applied for the relative response of the

TABLE I: Gas Chromatography of Methyl and Trimethylsilyl Derivatives of Mannose and Myoinositol.

Derivatives		Column and Temperature				
		1.3% QF-1	2.3% Neopentylglycol Succinate			
		130°	128°	137°	140°	142°
		Retention Times (min)				
Mannose						
CH_3O-	$(CH_3)_3SiO$-					
1,2,3,4,6-		4.1	10.8		6.6	
1,3,4,6-		5.8	23		13.5	
1,2,3,4-		6.0	23		13.5	
2,3,4-	1,6-	5.8	7.8		4.6	
3,4,6-	1,2-	4.7	4.9		3.2	
1,2,3,4-	6-		7.8			
1,3,4,6-	2-		5.6			
Myoinositol						
CH_3O-	$(CH_3)_3SiO$-					
1,2,3,4,5,6-				4.3		
1,2,4,5,6-	3-		8.4	5.6		4.9
1,3,4,5,6-	2-			3.6		
1,2,3,4,5-	6-			6.4		
1,2,3,4,6-	5-			7.7		
1,3,4,5-	2,6-		6.8	5.3		3.3
1,4,5,6-	2,3-					3.8

compounds, which was determined by analyzing under the same conditions a series of synthetic mixtures of mannose and myoinositol. At 175°, the respective retention times for the trimethylsilyl derivatives of α-D-mannose, β-D-mannose, and myoinositol were 2.5, 3.7, and 7.4 minutes. A solution of mannose, which had equilibrated between α and β forms, was evaporated to dryness and analyzed as before. The ratio of α and β anomers was 74:26, in good agreement with the reported value (Sweeley *et al.*, 1963).

*Gas Chromatography of Trimethylsilyl Derivatives of Methyl O-Methyl-*D*-mannosides and O-Methylmyoinositols.* Under the conditions for gas chromatography described in the previous work (Lee and Ballou, 1964), methyl 2,3,4-tri-*O*-methyl-D-mannoside could not be separated from methyl 3,4,6-tri-*O*-methyl-D-mannoside. An improved procedure has been developed for qualitative analysis of these sugars. The methanolysate, after removal of methanol and hydrochloric acid, was trimethylsilylated according to the procedure described. The reaction mixture was centrifuged and a small portion of the supernatant was injected onto a column of 2% neopentylglycol succinate operated at about 140°. The retention times are shown in Table I.

It was also possible to separate the trimethylsilyl derivatives of 2,3,4-tri-*O*-methyl- and 3,4,6-tri-*O*-methyl-D-mannose (rather than their methyl glycosides) on either the 5% QF-1 column or the 2% neopentylglycol succinate column (Table I). By the same technique, all possible penta-*O*-methylmyoinositols could be separated from each other and from hexa-*O*-methylmyoinositol (Table I) more effectively than as the unsubstituted ethers (Ballou and Lee, 1964).

Isolation of Glycerylphosphorylmyoinositol Tri-, Tetra-, and Pentamannosides. In the water-soluble fraction of the deacylated glycophospholipid extracted from *M. phlei*, there were several oligomannosides other than the dimannoside (Figure 1). These could be separated only partially by DEAE-Sephadex column chromatography, but could be resolved completely by paper chromatography in solvent A. The compounds were isolated by preparative chromatography on Whatman No. 3 paper in the same solvent. The rates of migration, relative to 1-glycerylphosphoryl-2,6-bis-α-mannopyranosyl-L-myoinositol, were 0.45, 0.18, and 0.05, respectively.

The aqueous eluate of each component was concentrated and added to a DEAE-Sephadex (A-25, carbonate form) column, 10 × 1 cm, and the column was washed with water to remove carbohydrate material derived from the filter paper. The glycerylphosphorylmyoinositol oligomannosides were then displaced from the column with 0.1 M ammonium carbonate, *p*H 8.6, and the eluates were evaporated to dryness. Group analyses of these compounds showed that they were glycerylphosphorylmyoinositol tri-, tetra-, and pentamannosides (Table II). The trimannosides from the two species of mycobacteria, as well as the tetramannosides, could not be distinguished by paper chromatography in solvent A.

Methylation Analysis of the Myoinositol Oligomannosides. Attempts to methylate glycerylphosphorylmyoinositol oligomannosides were not successful owing to their insolubility in dimethylformamide. Therefore,

TABLE II: Analyses of Myoinositol Oligomannosides from *M. phlei* and *M. tuberculosis*.

	Molar Ratios				
	P	Glycerol[a]	Myoinositol[b]	Mannose[c]	R[d]
M. phlei					
Trimannoside	1	0.93	0.93	2.9	0.45
Tetramannoside	1	0.96	0.92	4.2	0.18
Pentamannoside	1	0.94	0.90	5.1	0.05
M. tuberculosis					
Trimannoside	1	0.97	1.01[e]	3.0	0.45
Tetramannoside	1	0.98	0.94[e]	4.3	0.18

[a] As α-linked glycerol. [b] By gas chromatography. [c] By phenol–sulfuric acid method. [d] Migration distance relative to 1-glycerylphosphoryl-2,6-bis-α-D-mannopyranosyl-L-myoinositol (glycerylphosphoryl myoinositol dimannoside) in solvent A. [e] By microbiological assay.

TABLE III: Methylation Analysis of Myoinositol Mannosides.

	Methyl *O*-Methylmannosides			*O*-Methylmyoinositols	
	2,3,4,6-	2,3,4-	3,4,6-	1,3,4,5-	1,3,4,5,6-
Hexamannoside	2	2	2	1	
Pentamannoside	2	2	1	1	
Tetramannoside	2	2		1	
Trimannoside	2	1		1	
Dimannoside	2			1	
Monomannoside	1				1

the compounds were dephosphorylated with 10% ammonia in a sealed tube at 160° for 24 hours (Ballou *et al.*, 1963), and the neutral oligosaccharides were obtained by treatment of the dephosphorylation mixture with DEAE-Sephadex (A-25, carbonate form). Glycerol, which was formed during the dephosphorylation reaction and was present in the neutral oligosaccharide fraction, was not removed, since its methyl ether did not interfere with the analysis by gas chromatography.

The oligomannoside, 1 mg, was dissolved in 0.1 ml of dimethylformamide, and 0.1 ml of methyl iodide and 0.1 g of silver oxide were added in that order. If turbidity developed upon addition of methyl iodide to the dimethylformamide solution, more dimethylformamide was added dropwise until a clear solution was obtained. The mixture was shaken for 24 hours at room temperature. A second batch of the reagents was added and the mixture again was shaken for 24 hours. It was then centrifuged, and the precipitate was washed with 2 ml of methanol several times. The washings and the supernatant were combined and evaporated to dryness, and the methylation was repeated in the same manner. The final product was extracted with chloroform. The extract was washed repeatedly with 1% potassium cyanide solution and water, dried over sodium sulfate, and evaporated to dryness. The residue was methanolyzed with 3% hydrogen chloride in dry methanol at 100° for 12 hours, and dried *in vacuo* over sodium hydroxide at room temperature.

Analysis of the methanolysate of the methylated tri-, tetra-, and pentamannosides of *M. phlei* by gas chromatography on the 2% neopentylglycol succinate column at 190° revealed the presence of 1,3,4,5-tetra-*O*-methylmyoinositol in all cases, indicating disubstitution on the myoinositol moiety at positions 2 and 6. Each compound gave about 2 moles of methyl 2,3,4,6-tetra-*O*-methylmannoside, and, respectively, about 1, 2, and 3 moles of methyl tri-*O*-methylmannoside (Table III).

After trimethylsilylation of the methanolysates, gas chromatography on the neopentylglycol succinate column at 128° confirmed in each case the identity of the tetra-*O*-methylmyoinositol. Moreover, the methyl tri-*O*-methylmannoside produced from the tri- and tetramannosides contained only the 2,3,4 isomer. On the other hand, the pentamannoside yielded both the 2,3,4- and 3,4,6-trimethyl derivatives.

The results of methylation analysis of the tri- and tetramannosides from *M. tuberculosis* were essentially the same as those from *M. phlei*.

Emulsin Digestion of Myoinositol Pentamannoside. Myoinositol pentamannoside, 64 mg, was digested with 90 mg of sweet almond emulsin in 25 ml of 0.1 M sodium acetate buffer, *p*H 4.1, at 40°. The course of digestion was followed by the increase in reducing power. After

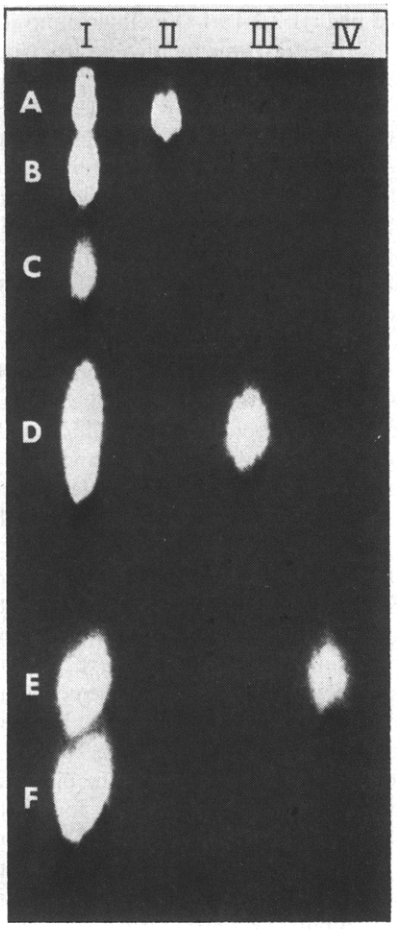


FIGURE 1: Paper chromatogram, developed with the periodate-benzidine reagent, of the deacylated glycolipids of *M. phlei* (left-hand column). Standards from left to right, glycerylphosphoryl myoinositol pentamannoside, glycerylphosphoryl myoinositol dimannoside, and glycerylphosphoryl myoinositol.

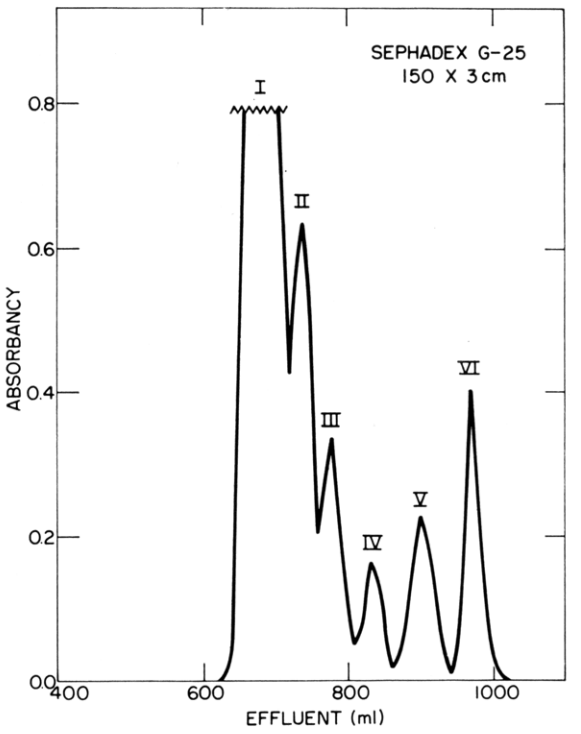


FIGURE 2: Separation of partial acid hydrolysate of myoinositol pentamannoside on Sephadex G-25. Mannose-containing components were detected by the phenol–sulfuric acid reagent. Peak I is starting material, II is tetramannosides, III trimannosides.

15 hours, when about 1 mole of reducing sugar per mole of the pentamannoside was liberated, the digestion mixture was treated with a large excess of Dowex 50-X2 (H^+ form) to remove the bulk of proteins as well as cations, and the filtrate was evaporated to dryness. Paper chromatography in solvent B indicated that two new oligosaccharides, having migration rates of 0.58 and 0.34 relative to the myoinositol dimannoside, were formed as the main products. These were isolated by paper chromatography on Whatman 3MM paper, after development for 10 days in the above mentioned solvent. They were designated as E-IM_3 (emulsin inositol trimannoside) (about 1 mg) and E-IM_4 (emulsin inositol tetramannoside) (15 mg). In both solvents A and B, E-IM_3 and the myoinositol trimannoside from *M. phlei* had the same R_F, as did also E-IM_4 and the tetramannoside from *M. phlei*.

On methylation analysis of E-IM_4, 1 mole of 1,3,4,5-tetra-*O*-methylmyoinositol, 2 moles of methyl 2,3,4,6-tetra-*O*-methylmannoside, and 2 moles of methyl 2,3,4-tri-*O*-methylmannoside were formed. The identities of these products were confirmed by gas chromatography after trimethylsilylation.

Partial Acid Hydrolysis of Myoinositol Pentamannoside. Myoinositol pentamannoside, 170 mg, was dissolved in 10 ml of water and heated with 2 g of Dowex 50-X2 (200–400 mesh, H^+ form) at 100° for 1 hour. The resin was removed by filtration and thoroughly washed with water. The hydrolysate was subjected to gel filtration on a column of Sephadex G-25 (150 × 3 cm), with water as eluent. Thereby the lower oligomannosides produced by the mild acid hydrolysis were separated according to their size (Figure 2). The pentamannoside (peak I) was recovered and subjected to a second hydrolysis and fractionation. The pentamannoside remaining after the second treatment was again hydrolyzed and fractionated. The myoinositol tetra- and trimannoside fractions (peaks II and III), from each of three runs of the gel filtration, were combined and designated as H-II (28 mg) and H-III (21 mg). The H-II and H-III fractions were fractionated by paper chromatography on Whatman No. 1 paper, developing with solvent B for 12 days. The relative positions on the paper chromatogram of the major subfractions, with reference to the myoinositol trimannoside and myoinositol tetramannoside from *M. phlei*, are represented schematically in Figure 3. Each component was eluted from the paper, and the

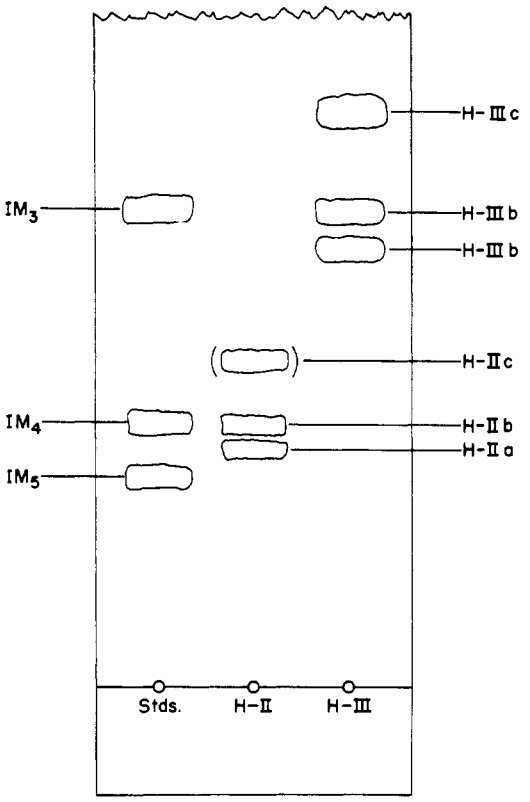


FIGURE 3: Paper chromatogram illustrating the further resolution of myoinositol tri- and tetramannosides obtained by partial acid hydrolysis of myoinositol pentamannoside. Compound H-II corresponds to peak II and compound H-III corresponds to peak III from Figure 2. Compound H-IIc is a mannopentaose which appeared in some chromatograms owing to contamination of the pentamannoside with myoinositol hexamannoside.

eluate was analyzed for mannose and evaporated to dryness *in vacuo*. The amounts of the components, based on the mannose analyses, were as follows: H-IIa (7.2 mg), H-IIb (9.6 mg), H-IIIa (4.2 mg), H-IIIb (3.6 mg). and H-IIIc (3 mg).

The subfractions thus obtained were studied by methylation using the techniques described. The results of gas chromatographic analysis of the methanolysate of the permethylated compounds H-IIa and H-IIb are shown in Figure 4. Compound H-IIa (Figure 4A) produced 1 mole of methyl 2,3,4,6-tetra-*O*-methylmannoside (peak I), 3 moles of methyl tri-*O*-methylmannoside (peak II), and 1 mole of penta-*O*-methylmyoinositol (peak III). The last was identified as 1,2,3,4,5-penta-*O*-methylmyoinositol by gas chromatography at 127°. After trimethylsilylation of the methanolysate the identity of the pentamethylmyoinositol was confirmed, and the methyl trimethylmannoside was found to contain both the 2,3,4- and the 3,4,6-trimethyl derivatives.

Methylation analysis of compound H-IIb produced 2 moles of methyl 2,3,4,6-tetra-*O*-methylmannoside, 2 moles of methyl 2,3,4-tri-*O*-methylmannoside, and 1 mole of 1,3,4,5-tetra-*O*-methylmyoinositol (Figure 4B), a result similar to that produced by the myoinositol tetramannoside of *M. phlei* and by E-IM$_4$. These results show that compound H-IIa was a linear oligosaccharide, whereas compound H-IIb was a branched oligosaccharide. A trace of 1,3,4,5-tetra-*O*-methylmyoinositol (peak IV) found in the H-IIa product and a small amount of 1,2,3,4,5-penta-*O*-methylmyoinositol (peak III) in the H-IIb product is due to a slight mutual contamination, since the two myoinositol tetramannosides were not separated completely.

By a similar investigation, compound H-IIIa was found to produce tetra- and tri-*O*-methylmannosides in a molar ratio of about 1:2, in addition to 1,2,3,4,5-penta-*O*-methylmyoinositol. Compound H-IIIb was similar to the myoinositol trimannoside obtained from *M. phlei*, and it yielded 1,3,4,5-tetra-*O*-methylmyoinositol.

Isolation and Characterization of Myoinositol Hexamannoside. In some preparations of myoinositol pentamannoside, a component was found having a rate of migration about 0.75 relative to the pentamannoside. This substance was separated from the pentamannoside by chromatography on Whatman No. 1 paper, developing with solvent system B for 2–3 weeks.

On complete acid hydrolysis of the pure substance, only mannose and myoinositol were found, the ratio being 5.5-6.0:1. Methylation analysis of this compound revealed the presence of 1,3,4,5-tetra-*O*-methylmyoinositol, trimethylmannose, and tetramethylmannose in a ratio of about 1:4:2.

The myoinositol hexamannoside was subjected to partial hydrolysis with Dowex 50 (H^+ form) under conditions similar to those applied to the pentamannoside, and the myoinositol tetramannoside fraction was isolated by gel filtration. Paper chromatography of this fraction in solvent B showed a new spot (H-IIc) in addition to the spots corresponding to compound H-IIa and H-IIb. Component H-IIc had a migration rate in the above solvent of 0.60 relative to an authentic mannotetraose isolated from the yeast mannan (Lee and Ballou, 1965), and the yield was about 3% of the starting material. It did not contain myoinositol.

A portion of compound H-IIc was reduced with sodium borohydride in the cold for 48 hours, and the remaining mannose was determined by the phenol–sulfuric acid method (Dubois *et al.*, 1956) and resorcinol-sulfuric acid method (Devor *et al.*, 1958). The color value was 75–80% of that of the starting material. Methylation analysis of this oligosaccharide gave tetramethylmannose and trimethylmannose in a ratio of 1:4. Neither tetramethylmyoinositol nor pentamethylmyoinositol was found. The trimethylmannose consisted of 3,4,6- and 2,3,4-tri-*O*-methylmannose in approximately equal amounts.

Proton Magnetic Resonance Studies. The signals in the proton magnetic spectra due to the anomeric hydrogens of the naturally occurring myoinositol mannosides are shown in Figure 5. The measurements were made at 3–5% concentrations of the oligomannosides in deute-

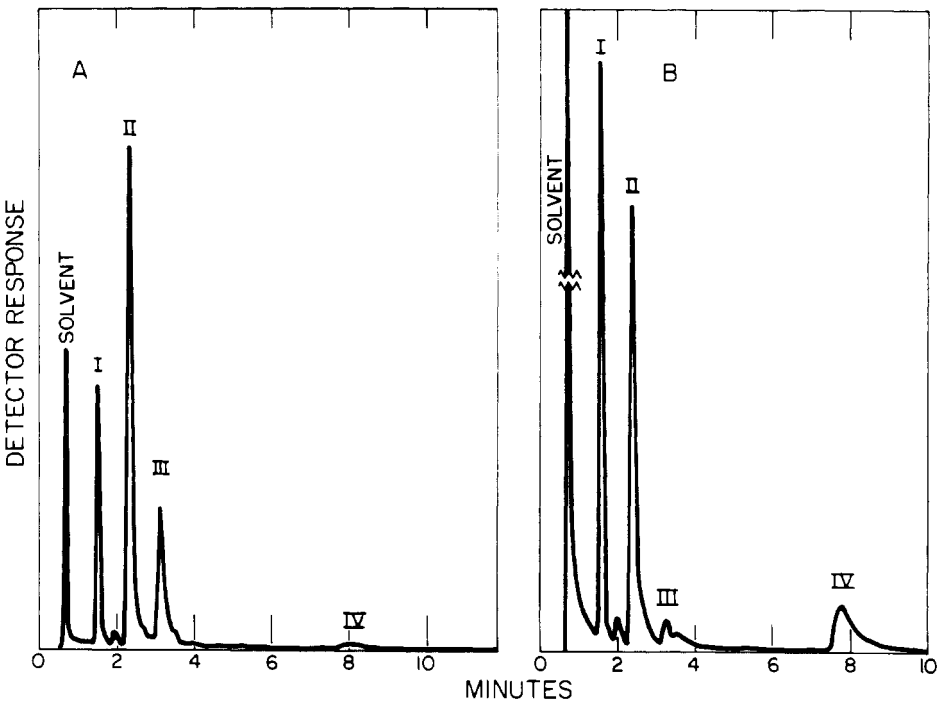


FIGURE 4: Gas chromatographic tracings of the methanolyzed permethylated myoinositol tetramannosides obtained by partial acid hydrolysis of myoinositol pentamannoside. Figure 4A is from compound H-IIa and Figure 4B is from compound H-IIb shown in Figure 3.

rium oxide. No difference was observed between the same oligomannosides produced from the two different species of *Mycobacteria*. The spectrum of the myoinositol tetramannoside (E-IM_4), obtained from the emulsin digestion of the pentamannoside, was the same as that of the naturally occurring tetramannoside. The PMR[1] spectrum of compound H-IIc showed four anomeric hydrogen signals, at 4.96, 5.10, 5.20, and 5.35 ppm.

Discussion

Gas Chromatography of Trimethylsilyl Derivatives. The procedure of trimethylsilylation of sugars (Sweeley *et al.*, 1963) has broadened the scope of gas chromatography as a tool in carbohydrate chemistry. In this way, sugars as large as tetrasaccharides can be made "volatile" enough for gas chromatography. We have used the procedure also for determination of mannose-to-myoinositol ratios. The method has an advantage of speed over the microbiological assay, but more material is needed for satisfactory results.

The usefulness of trimethylsilylation is not restricted to the effect of "volatilization." Introduction of trimethylsilyl groups into partially methylated mannoses and myoinositols reduced their retention times, but also resulted in improved separations. Thus, methyl 2,3,4-tri-*O*-methyl-D-mannoside and methyl 3,4,6-tri-*O*-methyl-D-mannoside, which were inseparable on several columns we tested, could be separated after trimethylsilylation. In the gas-chromatographic procedure we have reported previously (Ballou and Lee, 1964), all the possible penta-*O*-methylmyoinositols could be separated and identified, but the retention times were

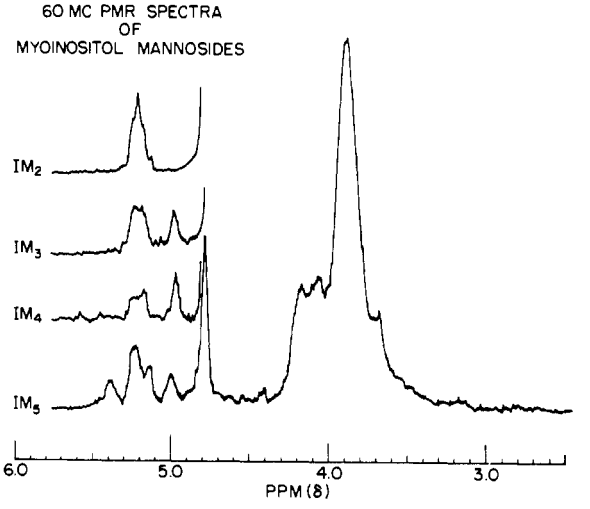


FIGURE 5: PMR spectra in deuterium oxide of myoinositol dimannoside (IM_2), trimannoside (IM_3), tetramannoside (IM_4), and pentamannoside (IM_5), showing the resonance lines due to the anomeric protons.

[1] Abbreviation used in this work: PMR, proton magnetic resonance.

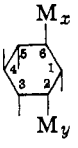
40–50 minutes. Trimethylsilylation of these derivatives resulted in better resolution and reduced the time of operation to less than 15 minutes.

No general rule can be drawn from the available data as to the change in the chromatographic pattern after addition of the trimethylsilyl group. However, the substitution of a free axial hydroxyl group seemed to decrease the retention time more than the substitution of a free equatorial hydroxyl group. For example, 1,3,4,5,6-penta-*O*-methylmyoinositol is only slightly faster than 1,2,3,4,5-penta-*O*-methylmyoinositol. After trimethylsilylation, the former compound emerged not only before the rest of the pentamethylmyoinositols, but ahead of the fully methylated derivative (Table I).

Isolation and Characterization of Myoinositol Oligomannosides. Myoinositol dimannoside was predominant in the glycophospholipid of *M. phlei*, but the tri-, tetra-, and pentamannosides could be isolated, as well as a trace of a hexamannoside. By paper chromatography, each pair of the corresponding oligomannosides from *M. phlei* and *M. tuberculosis* was indistinguishable, suggesting the close resemblance or identity of their structures.

An intriguing aspect of these compounds, revealed by the methylation studies, is that they all have the same branched structure, in which the mannoses are attached to positions 2 and 6 of the myoinositol ring. This mode of substitution was reported previously in the di- and pentamannosides (Lee and Ballou, 1964), and seems common to all the myoinositol mannosides obtained from either species of *Mycobacterium*.

Methylation studies showed that the tri- and tetramannosides from both species contained only 1,6′-mannosyl linkages. Like the pentamannoside from *M. tuberculosis*, the pentamannoside of *M. phlei* possessed in addition a 1,2′-mannosyl linkage. Thus the methylation analyses suggest structures IV′, V′, and VI′ for the tri-, tetra- and pentamannosides. The hexamannoside has the analogous structure, where $x + y = 6$.



(IV′) $x+y=3$ (IV′) $x + y = 3$
(V′) $x+y=4$ (V′) $x + y = 4$
(VI′) $x+y=5$ (VI′) $x + y = 5$

Emulsin Digestion of the Pentamannoside. The 1,2′-mannosyl linkage occurs in the pentamannoside but not in the lower homologs. Therefore it may be speculated that this linkage occupies a nonreducing terminal position in the pentamannoside. This speculation was tested by producing artificially the lower oligomannosides from the pure pentamannoside.

Sweet almond emulsin is known to contain an α-mannosidase. When the pentamannoside was digested with emulsin, myoinositol tetramannoside (E-IM$_4$) and trimannoside (E-IM$_3$) were formed with liberation of mannose. On a paper chromatogram, these oligomannosides appeared to be identical to the corresponding oligomannosides of natural origin. Methylation of E-IM$_4$ showed that it had only 1,6′-mannosyl linkages and the branched 2,6-disubstituted myoinositol structure. This reduced the number of possible structures for the myoinositol pentamannoside to those in which the 1,2′-mannosyl linkage is in a terminal position.

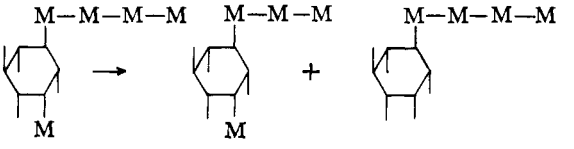
The α-mannosidase in emulsin is known to attack from nonreducing ends, and, theoretically, two myoinositol tetramannosides should be obtained by the removal of 1 mole of mannose from either end of the branched pentamannoside. Although little is known about the specificity of this enzyme, the fact that only one tetramannoside was produced suggested that there was a distinctive difference between the two mannose chains.

Partial Acid Hydrolysis of the Pentamannoside. To achieve less specific degradation, hydrolysis by an acidic ion-exchange resin was employed. The products were separated into groups according to size by gel filtration on a Sephadex G-25 column (Figure 2). Each fraction was resolved further by paper chromatography to yield a pair of myoinositol tetramannosides and a pair of myoinositol trimannosides. The results of the methylation studies on these compounds are given in Table IV.

TABLE IV: Methylation Analysis of Fragments Produced by Partial Acid Hydrolysis of Myoinositol Pentamannoside.

Fraction	Methyl *O*-Methylmannosides			*O*-Methylmyoinositols	
	2,3,4,6-	2,3,4-	3,4,6-	1,3,4,5-	1,2,3,4,5-
H-IIa	1	2	1		1
H-IIb	2	2		1	
H-IIIa	1	2			1
H-IIIb	2	1		1	
H-IIIc	1	2	1		

One of the myoinositol tetramannosides had the same properties as the compound obtained by emulsin digestion and as the natural myoinositol tetramannoside. Methylation confirmed the branched structure. The other tetramannoside, however, yielded methylation products indicative of a linear structure with a mannotetraose chain attached to the myoinositol ring. The pentamethylmyoinositol was identified as the 1,2,3,4,5 isomer, which established that the oligomannoside chain was on position 6. The isolation of a linear mannose tetrasaccharide confirmed this result. This reaction is outlined in the following scheme.



Methylation studies on the myoinositol trimannosides obtained by acid hydrolysis were consistent with

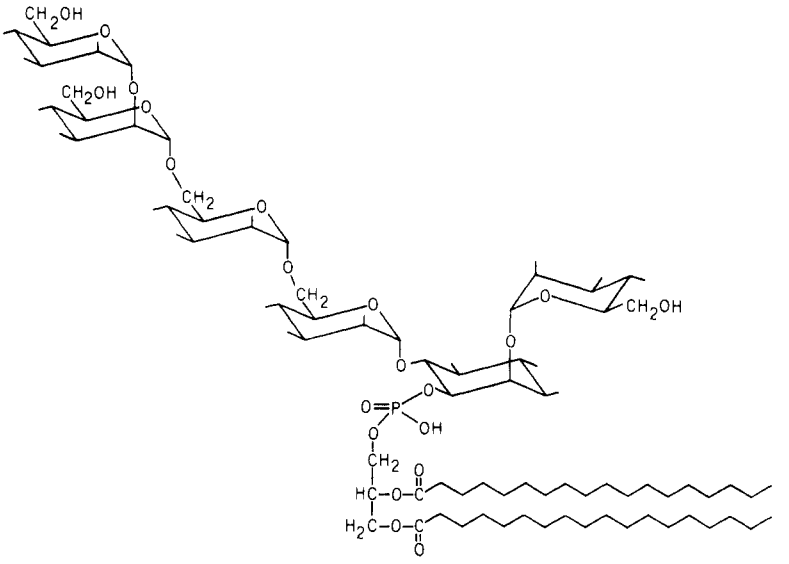
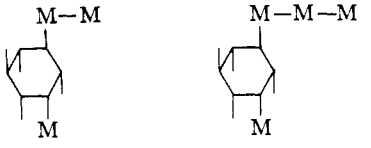


FIGURE 6: Complete structure of phosphatidyl myoinositol pentamannoside.

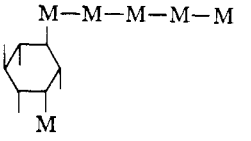
these same structures. Since the 1,2′-linked mannose was present in the linear myoinositol tetramannoside and in the free mannotetraose, but absent in the branched myoinositol tetramannoside, it is clear that this linkage is at the end of the mannotetraose side chain.

Having established the structure of the myoinositol pentamannoside, and having obtained from it by partial hydrolysis myoinositol tri- and tetramannosides with branched structures which appear identical to the natural myoinositol tri- and tetramannosides, it is reasonable to conclude that the latter substances have the following structures.



The complete structure of phosphatidyl myoinositol pentamannoside can be represented as shown in Figure 6, for which the definitive name 1-phosphatidyl-2-*O*-α-D-mannopyranosyl-6-[*O*-α-D-mannopyranosyl-(1-2)-*O*-α-D-mannopyranosyl-(1-6)-*O*-α-D-mannopyranosyl-(1-6)-*O*-α-D-mannopyranosyl]-L-myoinositol may be proposed. The lower homologs differ from this in lacking one or more of the mannose residues.

Structure of the Hexamannoside. A myoinositol polymannoside, with chromatographic properties slightly different from those of the myoinositol pentamannoside, was isolated from the alkaline degradation products of some preparations of the mycobacterial glycophospholipids. This substance gave a mannose-to-myoinositol ratio of 6, indicating it to be a hexamannoside. Methylation analysis established the branched structure. Partial acid hydrolysis yielded a mannopentaose which contained two 1,2′ linkages and two 1,6′ linkages. We presume that the hexamannoside has a structure analogous to the pentamannoside, with an additional mannose attached to the 2 position of the terminal mannose of the mannotetraose side chain as shown here.



PMR Spectra of the Myoinositol Oligosaccharides. We have cited previously (Lee and Ballou, 1964) the manner in which the line positions of the anomeric hydrogens in the myoinositol mannosides can be used to confirm the structures arrived at by other methods. Based on correlations made with reference compounds, it was clear that myoinositol di- and pentamannosides contained only α-mannosidic linkages.

The line positions for anomeric hydrogens of α-mannosides, however, are affected also by the nature of the aglycon. The signal due to the anomeric hydrogens in myoinositol dimannoside occurs at 5.20 ppm, characteristic of α-glycopyranosides of myoinositol. The spectrum of the trimannoside (Figure 5) has, like the dimannoside, a signal at 5.20 ppm equivalent to two hydrogens; but it has an additional signal at 4.96 ppm equivalent to one hydrogen, and in a position characteristic of α-1,6′-linked oligosaccharides. Since the trimannoside is known to have a mannose residue in α-1,6′ linkage to one of the original mannoses on the myoinositol ring, this new signal can be assigned to the anomeric hydrogen of that terminal group.

The spectrum of the tetramannoside differs from that of the trimannoside only in the intensity of the signal at 4.96 ppm, which is now equivalent to two hydrogens. Since the fourth mannose residue is also α-1,6′ linked, the assignment of the additional signal at 4.96 ppm to this group is internally consistent.

The signals due to the anomeric hydrogens in the pentamannoside have been assigned tentatively (Lee and Ballou, 1964), but the spectrum is considerably more complex. From the structural studies on the pentamannoside, it is known that the last mannose residue is attached by an α-1,2′ linkage. The new signal appearing in the spectrum of the pentamannoside at 5.10 ppm must be due to the anomeric hydrogen of this mannose, since this is the kind of shift we have noted for other α-1,2′-linked mannoses (Lee and Ballou, 1965). The signal equivalent to two hydrogens remains in the position characteristic of mannoses attached to the myoinositol ring, while the signal at 4.96 ppm has only half the intensity observed in the tetramannoside. This indicates that the anomeric hydrogen of one of the α-1,6′-linked mannoses has been deshielded, and we assign the signal at 5.37 ppm to this group. Such deshielding is known to occur owing to substitution on the 2 position of a sugar (Dedonder and Hassid, 1964; Lee and Ballou, 1965). Table V gives our interpretations of the PMR spectra of the various myoinositol mannosides.

TABLE V: Assignments of Anomeric PMR Signals (ppm).

			M | −5.10
		M | −4.96	M | −5.37
	M | −4.96	M | −4.96	M | −4.96
M | −5.20	M | −5.20	M | −5.20	M | −5.20
I | −5.20	I | −5.20	I | −5.20	I | −5.20
M	M	M	M

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