Structural Studies on the Myo-inositol Phospholipids of Mycobacterium tuberculosis (var. bovis, strain BCG) *

CLINTON E. BALLOU, ERNA VILKAS, AND EDGAR LEDERER

From the Institut de Chimie des Substances Naturelles, Gif-sur-Yvette, France, and the Department of Biochemistry, University of California, Berkeley, California

(Received for publication, August 7, 1962)

During the last few years, the myo-inositol-containing phospholipids have elicited considerable interest from biochemists because of their possible dynamic functions in cell membranes. Parallel with this interest in function has been a concerted attack on the problem of elucidating the detailed structures of these complex substances. In general, the more definitive results have come from the latter investigations.

Myo-inositol is present in nature most usually in conjugated form in phospholipids, substances which have the descriptive name of inositides or phosphoinositides (1). Many tissues of plant and animal origin contain phosphatidyl-myo-inositol (I), structurally the simplest member of the group, and a compound that has an obvious relationship to phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine. Brain tissue contains a complex of phosphatidyl-myo-inositol phosphates (2-6), and Mycobacterium contains phosphatidyl-myo-inositol glycosides (7). Other less well defined myo-inositol phospholipids have been found in plant seeds (8).

An interesting and important structural feature of all of these phospholipids concerns the position on the myo-inositol ring to which the phosphatidyl group is substituted. Recent investigations have indicated that this feature is under rigid stereochemical control, since the position of substitution is the same in samples of phosphatidyl-myo-inositol phosphates (2-6), and Mycobacterium contains phosphatidyl-myo-inositol glycosides (7). Other less well defined myo-inositol phospholipids have been found in plant seeds (8).

The general nature of the intergroup linkages of phosphatidyl-myo-inositol dimannoside has been established (20). The fatty acids are attached to glycerol, which is fixed in a phosphodiester linkage to myo-inositol. The n-mannose is in α-n-glycosidic linkage to myo-inositol, and the linkage between the two mannoses has been shown to be α-1,6. The suggestion was made previously (20) that the phosphatidyl group was on position 1 of myo-inositol since acid hydrolysis of the lipid had yielded a mixture of myo-inositol 1- and 2-phosphate. However, the ready migration of phosphate groups under acid conditions invalidates this interpretation of the results.

The work described in this paper was undertaken to define in greater detail the structure of the phospholipids of Mycobac-
**terium tuberculosis**, and in particular to ascertain the positions of substitution on the myo-inositol ring. By studies on the deacylated phospholipid complex, we have established more firmly the existence of a family of myo-inositol-containing phospholipids with the following structures: phosphatidyl-myoinositol, phosphatidyl-myoinositol mannoside, phosphatidyl-myoinositol dimannoside, and phosphatidyl-myoinositol pentamannoside. In agreement with the structures already assigned to the inositides from all other sources, the phosphatidyl-myoinositol of *M. tuberculosis* have the phosphatidyl group on the L-1-position of the myo-inositol ring.

**EXPERIMENTAL PROCEDURE**

**Methods**

Phosphorus was determined by the method of King (23), mannose by the anthrone method (24) with methyl α-D-mannoside as the standard, ε-linked glycerol by the method of Hancock and Olles (25) with erythritol as the standard, and reducing sugar with the Nelson-Somogyi reagent (26). Myo-inositol was determined by periodate oxidation after its isolation by paper chromatography, and microbiologically with Kleoccera brevis as the test organism (27). The phospholipids were deacylated by a modification of the procedure of Dawson (28).

Periodate oxidations were followed by the change in absorbancy at 290 mg (29), the sodium periodate stock solution first being standardized by titration with sodium thiosulfate. For the quantitative determination of myo-inositol, the periodate being standardized by titration with sodium thiosulfate. For isolation of the components, the solution was stripped on sheets of Whatman No. 3 paper, after approximately 1 mole of sugar per mole of P, was deacylated as described in the preceding paragraph. The water-soluble component was stripped on four sheets of Whatman No. 3 paper (46 x 57 cm), material equivalent to 10 to 40 mg of P being applied to each sheet. The papers were chromatographed descending with isopropanol-ammonia solvents in the following proportions: 1:1, 1 to 2 days; 2:1, 2 to 3 days; 3:1, 3 to 5 days; and 4:1, 5 days or longer, the choice depending on the nature of the mixture. Increasing the proportion of isopropanol markedly increased the resolution of the various components.

After the papers were removed from the chromatographic cabinets and dried, narrow guide strips were cut from each side and developed with the silver nitrate-sodium hydroxide dip reagents or with the sodium periodate-benzidine dip reagents. The latter was more sensitive for the oligomannosides, while the former was better for the components with little or no mannose. The bands, corresponding to each component on the guide strips, were cut out and eluted with water. The water eluates were analyzed for total phosphorus, sugar, and ε-linked glycerol, and for further study they were then evaporated to dryness on a rotary evaporator to a small volume for chromatographic analysis. The yield of water-soluble P was 98 to 100%.

For isolation of the components, the solution was stripped on sheets of Whatman No. 3 paper (46 x 57 cm), material equivalent to 10 to 40 mg of P being applied to each sheet. The papers were chromatographed descending with isopropanol-ammonia solvents in the following proportions: 1:1, 1 to 2 days; 2:1, 2 to 3 days; 3:1, 3 to 5 days; and 4:1, 5 days or longer, the choice depending on the nature of the mixture. Increasing the proportion of isopropanol markedly increased the resolution of the various components.

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**Isolation of d-Glycerol 1-(α-Myo-inositol 1-Phosphate)—** One gram of a phospholipid fraction, with mannose content approximately 1 mole of sugar per mole of P, was deacylated as described in the preceding paragraph. The water-soluble component was stripped on four sheets of Whatman No. 3 paper, after which the sheets were subjected to descending chromatography with isopropanol-ammonia (9:1) for 3 days. Guide strips developed with silver nitrate-sodium hydroxide indicated four major components with \( R_{myo-inositol} \) 0.45, 0.87, 1.0, and 1.3. The component with \( R_{myo-inositol} \) 1.0 was eluted with water and...
found to contain 6.3 mg of P, no sugar by the anthrone test, and an α-glycerol to P ratio of 0.85.

The solution was passed through a small column containing Dowex 50-H (200 to 400 mesh) to remove the cations, and the eluate was adjusted to pH 8 by the addition of dilute aqueous cyclohexyloxamine. The solution was concentrated to approximately 1 ml on a rotary evaporator, and 5 ml of ethanol were added followed by acetone until the solution became turbid. This solution was then left at −5° for several days during which crystals formed. These were collected by filtration, washed on the funnel with acetone, and then dried in a desiccator over phosphorus pentoxide. The material weighed 80 mg and was chromatographically homogeneous. The specific rotation at 589 nm for n-glycerol 1-(l-myo-inositol 1-phosphate) under similar conditions is −13.1° (c 0.6, water). The rotation reported for n-glycerol 1-(l-myo-inositol 1-phosphate) was −13.5° (12) and −13.2° (37).

C₉H₁₉O₉P·C₆H₈NH₂ (433.4)

Calculated: N 3.23, P 7.16

Found: N 3.28, P 6.85

A sample of the material heated in 1 N sodium hydroxide at 100° for 30 minutes showed on chromatography (isopropanol-ammonia, 3:1) the characteristic pattern of myo-inositol 1- and 2-phosphate along with glycerol 1- and 2-phosphate.

Characterization of Glycerol Myo-inositol Phosphate Monomannoside—Phospholipid, 1 g, was chromatographed on a column containing 30 g of silica gel and 15 g of Celite, the elution being effected with chloroform containing increasing amounts of methanol. Nothing was eluted with chloroform containing 10% methanol. With chloroform containing 20% methanol, 0.34 g of material was obtained in seven 100-ml fractions. The material was combined and deacylated as described above. A chromatogram showed that it consisted mainly of the component in the crude phospholipid mixture that had Rₓ myo-inositol 0.87.

Analysis of the material showed that it had a mannose to phosphorus ratio of 1.1 to 1.0 and that it contained 0.82 mole of α-linked glycerol per mole of P. The molecular rotation was +20,700° (water, sodium D-line).

A sample of the substance was hydrolyzed in 2 N sulfuric acid for 4 hours. After treatment of the solution with Amberlite MB3 resin to remove all ions, a chromatogram of the residue showed that it contained three components corresponding to glycerol, myo-inositol, and mannose.

Another sample of the substance, equivalent to 4.2 mg of P, was sealed in a glass tube with 5 ml of 10% ammonium and heated at 150° for 10 hours to dephosphorylate it. The tube was opened, the solution was concentrated to dryness, the residue was dissolved in water, and the solution was treated with Amberlite MB3 resin. On evaporation of the aqueous filtrate, 35 mg of sirup were obtained. This material was acetylated in a mixture of 0.5 ml of pyridine and 0.5 ml of acetic anhydride. The reaction mixture was then evaporated to dryness on a rotary evaporator, and on addition of water to the residue, it crystallized. The product was recrystallized from ethanol to give 10 mg of pure α-mannosyl-my o-inositol nonaacetate. It melted at 176-178°, and the melting point of a mixture with the previously characterized myo-inositol nonaacetate (22) (m.p., 178-180°) was not depressed.

Barry Degradation of Monomannoside—A 1-ml aliquot of a solution (1.5 mg of P per ml) of glycerol myo inositol phosphatase monomannoside isolated above was mixed with 1.5 ml of 0.1 N sodium periodate, and the reaction was allowed to proceed for 40 minutes. A drop of ethylene glycol was added to destroy the excess periodate; then 6 ml of ethanol were introduced to precipitate the sodium iodate. After the mixture was chilled in ice-water for 15 minutes, the salt was removed by filtration.

To the filtrate were added 0.5 ml of glacial acetic acid and 0.2 ml of phenylhydrazine. The solution was then refluxed for 1 hour, cooled to room temperature, and concentrated under vacuum to remove the ethanol. The water solution was extracted five times with ether and then was treated batchwise with Dowex 50-H (200 to 400 mesh) to remove all cations. The filtrate from the resin was concentrated to a small volume on a rotary evaporator with a bath temperature of 30°. The resulting solution was chromatographed on Whatman No. 3 filter paper (15 cm wide) with the solvent isopropanol-ammonia (1:1) for 24 hours. The band corresponding to myo-inositol monophosphate was cut out and eluted. Rechromatography of the material on Whatman No. 1 filter paper with the solvent isopropanol-ammonia (3:1) for 4 days descending, along with standards of myo-inositol 1-, 2-, and 4-phosphate, showed only one component in the reaction mixture, which corresponded exactly with myo-inositol 1-phosphate and was distinctly different from the other two isomers.

For further characterization, the reaction product was refluxed for 30 minutes in glacial acetic acid, conditions which are known to cause migration of myo-inositol 1- and 2-phosphate (38), but not the other isomers. A chromatogram of the acid-treated substance now showed two spots corresponding to myo-inositol 1-phosphate and myo-inositol 2-phosphate.

Partial Characterization of Glycerol Myo-inositol Phosphate Dimannoside—The deacylated products from 1 g of phospholipid were subjected to preparative paper chromatography on four sheets of Whatman No. 3 filter paper with isopropanol-ammonia (4:1). In addition to glycerol myo-inositol phosphate and glycerol myo-inositol phosphate monomannoside, there was a third component with Rₓ myo-inositol 0.7. This latter band was cut out and eluted with water to give a solution containing 5.75 mg of organic phosphorus. The ratio of phosphorus to mannose to α-linked glycerol was 1.0:1.93:0.88. After acid hydrolysis, the presence of glycerol, mannose, and myo-inositol were detected chromatographically.

A Barry degradation performed as described for the monomannoside yielded myo-inositol 1-phosphate as the only phosphate-containing product. Since only a trace of the dimannoside was obtained in these experiments, its further characterization was not possible.

Isolation and Characterization of Glycerol Myo-inositol Phosphate Pentamannoside—Decacylated phospholipid was separated by chromatography on sheets of Whatman No. 3 filter paper with isopropanol-ammonia (3:1), and the fraction with Rₓ myo-inositol 0.43 was eluted with water. On evaporation of the water, a thick sirup was obtained that turned to a brittle glass. The substance contained 2.2% P. The ratio of mannose to P was 5.2, the ratio of mannose to myo-inositol was 5.5, and that of α-linked glycerol to P was 0.8. After hydrolysis in 1 N hydrochloric acid for 10 hours, a chromatogram of the solution showed the presence of components corresponding to glycerol, mannose, and myo-inositol.

A solution of 100 mg of the substance in 50 ml of 1 N hydrochloric acid was refluxed for 5 hours. The solution was then...
concentrated to dryness on a rotary evaporator. The syrup was dissolved in water, and the solution was deionized with Amberlite MB3 resin. The filtrate from the resin was evaporated to 2 ml, and 100 mg each of phenylhydrazone and 98% acetic acid were added, followed by 2 ml of ethanol. After 2 hours, the precipitate of mannose phenylhydrazone was filtered off, washed with ethanol, and dried. The weight was 84 mg, or 76% of the calculated yield. An identical preparation starting with authentic D-mannose gave a yield of 80%. The substance had m.p. 100-101°C, and the melting point of a mixture with authentic D-mannose phenylhydrazone (m.p. 190-192°C) was not depressed. The specific rotation in pyridine was +26° (sodium D-line). The reported value for D-mannose phenylhydrazone is +26° under the same conditions (39).

Barry Degradation of Glycerol Myo-inositol Phosphate Pentamannoside—A sample of chromatographically homogeneous glycerol myo-inositol phosphate pentamannoside (26.3 mg of P; mannose to P ratio, 3:1; α-glycerol to P, ratio 0.7) in 150 ml of water was mixed with 150 ml of 0.1 M sodium periodate. The reaction was followed by removing 0.06 ml of the solution and adding it to 3.0 ml of water in a quartz cuvette for determination of the absorbancy at 260 nm against a water blank. The zero time reading was determined to give a value of 0.980. The reaction gave the following readings: 1 minute, 0.840; 5 minutes, 0.730; 15 minutes, 0.940; 30 minutes, 0.580; 45 minutes, 0.580. The consumption of periodate was 7.2 moles per mole of P. A control oxidation carried out on methyl α-mannopyranoside showed that in the starting material.

After 5 minutes, the absorbancy determined as previously mentioned had decreased to 0.130. To the solution were added 700 ml of ethanol, and the mixture was chilled in ice to precipitate the sodium iodate. After 30 minutes at this temperature, the salt was removed by filtration on a Buchner funnel. After drying in air, it weighed 3.0 g (theoretical yield is 3.0 g).

To the filtrate were added 50 ml of glacial acetic acid and 20 ml of phenylhydrazine. The mixture was refluxed for 1 hour (during which it turned dark red); then it was cooled and concentrated on a rotary evaporator until the ethanol was removed and a milky water solution remained. This water layer was extracted in a separatory funnel with an equal volume of ethyl ether three times. The ether extracts were combined and set aside (see the following). A total phosphorus analysis on the water layer showed that it contained 14.2 mg of P, or 45% of that in the starting material.

The water solution was treated batchwise with an excess of Dowex 50-H (200 to 400 mesh) to remove the phenylhydrazine, and the strongly acidic filtrate from the resin was adjusted to pH 3 by the addition of a few drops of concentrated ammonia, the intention being to neutralize only the strong acid but not the acetic acid present in the solution. The solution was then concentrated to dryness at 30°C bath temperature on a rotary evaporator. A chromatogram of the residue (isopropanol-ammonia, 1:1) revealed only myo-inositol monophosphate and a yellow, salt-like component that ran near the solvent front. The material was dissolved in 2 ml of water, and the solution was stripped on two large sheets of Whatman No. 3 filter paper. After chromatography for 3 days with the solvent isopropanol-ammonia (2:1), the myo-inositol phosphate was located on guide strips cut from each side of the sheets by the use of the silver nitrate-sodium hydroxide dip reagent. The bands were cut out and eluted with water. The water eluate was treated with Dowex 50-H to remove all cations, and cyclohexylamine was added to the filtrate from the resin to make the solution basic. The solution was evaporated under vacuum to approximately 1 ml, and crystallization of the myo-inositol phosphate cyclohexylamine salt was initiated by the addition of acetone to cause turbidity. After an hour, the crystals were collected by filtration and dried to give 31 mg of pure material. The specific rotation was +3.7° (water at approximately pH 9), and -10.4° (water at approximately pH 2), both values determined at the sodium D-line. The recorded values for L-myo-inositol 1-phosphate cyclohexylamine salt are +3.4° (water, pH 9) and -9.8° (water, pH 2) (9).

The material used for determination of the rotation was reconverted to the cyclohexylamine salt and recrystallized in the same fashion to give 26 mg. This was dried at 25°C and 0.1 mm for an hour for analysis.

\[ \text{C}_{62}H_{12}O_{15}P \cdot 2\text{C}_{4}H_{2}N\text{H}_{3} \text{ (485)} \]

Calculated: N 6.11, P 6.77

Found: N 5.35, P 6.90

In an attempt to improve the yield in the phenylhydrazinolysis reaction, the ether extract from the preceding paragraph was concentrated to approximately 50 ml, and 100 ml of ethanol and 4 ml of phenylhydrazine were added. This mixture was refluxed for 1 hour; then it was worked up as described previously to give a water extract that contained an additional 3.8 mg of organic phosphorus. Paper chromatography of this solution showed that it contained more myo-inositol 1-phosphate.

Alkaline Degradation of Glycerol Myo-inositol Phosphate Pentamannoside—A solution of 40 mg of the pentamannoside in 10 ml of 1 N sodium hydroxide was heated in a boiling water bath in a polyethylene tube. Two-milliliter aliquots were removed and neutralized by the addition of Dowex 50-H, and approximately 1 ml of the solution was stripped on 15-cm wide pieces of Whatman No. 3 filter paper. After descending chromatography for 5 days with isopropanol-ammonia (3:1), the papers were sprayed with molybdate reagent, and the bands containing phosphate were revealed by use of an ultraviolet lamp. The bands were cut out and the phosphate content was determined after digestion of the paper strips with perchloric acid. After 30 minutes of heating, only approximately 5% of the diester was cleaved, as indicated by the formation of glycerol phosphate and myo-inositol phosphate pentamannoside (Rmyo-inositol 0.1). After 2 hours, 33% of the diester had been cleaved, and the ratio of myo-inositol phosphate pentamannoside to glycerol phosphate was 45:55. After 5 hours of heating, the cleavage of the diester was 66% complete.

Isolation and Characterization of Myo-inositol Pentamannoside—A sample of phospholipid was desalted as described previously, and the water-soluble material was dephosphorylated by heating at 150°C in a sealed tube with 10 N ammonium hydroxide for 12 hours. The tube was opened, the contents were evaporated to dryness under vacuum, and the residue was dissolved in water. After deionization with Amberlite MB3 resin, the solution was evaporated to a small volume and the material...
was stripped on sheets of Whatman No. 3 paper for chromatography 5 days descending with isopropanol-ammonia (3:1). The bands were detected on guide strips with the periodate-benzidine reagents, the slowest major component with \( R_{myo-inositol} \) 0.4 was collected by elution, and the eluate was evaporated to dryness.

The dry material formed a transparent brittle glass, and after further drying in a high vacuum at 100°C for several hours, the myo-inositol pentamannoside gave the following analysis.

\[
\text{C}_{46}\text{H}_{82}\text{O}_{37}\cdot\text{H}_2\text{O} \quad (1008)
\]

Calculated: C 42.7, H 6.35  
Found: C 42.7, H 6.04

A 10.0-mg sample of the material was refluxed in 100 ml of 1 N hydrochloric acid, and aliquots were removed at appropriate times for determination of the reducing sugar with the Nelson-Somogyi copper reagent and with mannose as the standard. Hydrolysis was complete in 3 hours, and the half-time was approximately 50 minutes. The remaining solution was evaporated to dryness under vacuum to remove the acid, and the residue was redissolved in the same volume of water. Aliquots were taken for determination of total sugar as mannose, by the anthrone method, and of myo-inositol by microbiological assay. This gave values of 8.36 mg of mannose and 1.6 mg of myo-inositol in the original solution, and a mannose to myo-inositol ratio of 5.2.

**Myo-inositol pentamannoside**

Calculated: Mannose 82.0, myo-inositol 16.3  
Found: Mannose 83.5, myo-inositol 16.9

A chromatogram of the previously mentioned acid hydrolysate on Whatman No. 1 filter paper descending for 18 hours with the solvent system ethyl acetate-pyridine-water (7:3:2) showed only two spots, corresponding to myo-inositol and mannose, on revelation with the silver nitrate-sodium hydroxide reagents. No trace of glucose or any other sugar was detected.

A determination of the optical rotation of myo-inositol pentamannoside gave \( [\alpha]_D +78.5^\circ \) (c 1, water). The molecular rotation, +78.5 times 900, is +77,700°. Since there are five glycerol linkages in the molecule, this gives a value of 15,500° as the molecular rotation per mannooligosaccharide linkage. Methyl α-D-mannopyranoside has \( [\alpha]_D +79.2^\circ \) (water) and a molecular rotation of +15,200° (40).  

**Conversion of Glycerol Myo-inositol Phosphate Pentamannoside to Myo-inositol Phosphate Pentamannoside**—A solution of 100 mg of glycerol myo-inositol phosphate pentamannoside in 100 ml of water was analyzed for \( \alpha \)-glycerol, which gave a value of 70.2 µmoles. To this solution was added 0.71 ml of 0.1 M sodium periodate, or just sufficient to oxidize the glycerol moiety. After 30 minutes, most of the periodate had been consumed, and the solution was concentrated on a rotary evaporator to 20 ml. Approximately 50 mg of phenylhydrazine and 50 mg of acetic acid were added, and the mixture was left at room temperature for 18 hours. The solution was then heated to boiling for 5 minutes, cooled to room temperature, and extracted with ether. The water layer was treated batchwise with Dowex 50-H, and the filtrate from the resin was neutralized with ammonia and concentrated to a small volume. The resulting solution was stripped on a piece of Whatman No. 3 filter paper (46 × 57 cm), which was subjected to descending chromatography for 6 days with isopropanol-ammonia (2:1). Guide strips from the sheet were developed with the phosphate spray, revealing two components, one corresponding to the starting material and a second with very low \( R_p \) corresponding to the myo-inositol phosphate pentamannoside formed in the alkaline degradation of glycerol myo-inositol phosphate pentamannoside. The slower of the bands was cut out and eluted. A determination of total phosphate content indicated that it contained approximately 40% of the phosphate of the starting material.

The new substance was chromatographically homogeneous, and had \( R_{myo-inositol} \) 0.1 (isopropanol-ammonia, 2:1). It contained 2.2% P (calculated for the bisammonium salt 2.8% P), and had a mannose to P ratio of 5.3.

**Attempts to Isolate Diglyceride from Phosphatidyl-myo-inositol Pentamannoside**—Approximately 100 mg of a sample of the pentamannoside containing a small amount of dimannoside were dissolved in 5 ml of warm glacial acetic acid, and the mixture was refluxed for 2 hours. On cooling, a precipitate formed which was collected by centrifugation and dried. The material, approximately 60 mg, was readily soluble in chloroform. The chloroform solution was treated with methanolic sodium hydroxide for decylation. The water-soluble product, obtained as described in the previous section on decylation, was chromatographed with the solvent isopropanol-ammonia (3:1). Glycerol myo-inositol phosphate pentamannoside and glycerol myo-inositol phosphate dimannoside were the only substances detected.

To a good emulsion of 200 mg of the same lipid in 50 ml of water were added 10 ml of concentrated hydrochloric acid. The mixture was heated under reflux. For the first minute, it foamed, then it changed to a milky solution. After 5 minutes, a lipid layer suddenly came out of solution on the surface as a gelatinous mass. The solution was cooled and extracted with ether. The ether extract was concentrated to dryness, and the residue was chromatographed on thin layer silicic acid. The plates were sprayed with 5% sulfuric acid and heated on a hot plate. A single spot corresponding to fatty acid (palmitic acid reference) was detected. No diglyceride component corresponding to the reference dipalmitin was present.

**RESULTS AND DISCUSSION**

The materials used in this study were phospholipid preparations of *Mycobacterium tuberculosis*, var. bovis (strain DCG) isolated by methanol extraction, and which had been partially fractionated on the basis of solubility in methanol. Therefore, a quantitative value for each of the components in the original extract could not be obtained. Fractionation into pure components by solvent precipitation or by silicic acid chromatography was unsuccessful, although it was easy to obtain material that contained only those components of high sugar content by precipitation from chloroform solution by the addition of methanol.

The crude phospholipid mixture was most easily fractionated after decylation. The water-soluble products (glycerol myo-inositol phosphate diesters) were separated by preparative filter paper chromatography. In this manner, four components were isolated for which structures were assigned as follows: glycerol myo-inositol phosphate, glycerol myo-inositol phosphate mannoside, glycerol myo-inositol phosphate dimannoside, and glycerol myo-inositol phosphate pentamannoside. Presum-
ably, each of these substances represents the deacylated form of a phosphatidyl-type inositol.

The glycerol myo-inositol phosphate was isolated as the crystalline cyclohexylamine salt. It was identical with \( \delta \)-glycerol 1-\( \delta \)-myo-inositol 1-phosphate (V) previously obtained by deacylation of phosphatidylinositol from beef (12) and horse liver (37). Thus, the microorganism contains a \( \delta \)-phosphatidyl-\( \delta \)-myo-inositol (I) configurationally identical with that which occurs generally in plants and animals.

The three glycerol myo-inositol phosphate mannosides were not obtained crystalline, although they form white powders that might be easily mistaken for crystals. Their general characterization was based on determinations of the ratios of \( \alpha \)-linked glyceroi to phosphorus to myo-inositol to mannose. These corresponded to a monomannoside, a dimannoside, and a pentamannoside, respectively. To determine the position of the phosphate group on the myo-inositol ring in each of the compounds, a modification of the Barry degradation (30) was combined with the degradation reaction described by Brown et al. (10). The substance under investigation (for example, III, \( R = \delta \) H) was first subjected to a controlled oxidation with sodium periodate such that only the glycerol portion and the mannoseyl ring were cleaved to give the intermediate IV. This was followed by a phenylhydrazinolysis reaction to remove the oxidized crystalline cyclohexylamine salt, the identity and configuration of which were established by elemental analysis and determination of the optical rotation.

The results established that this strain of *Mycobacterium*, cultured as mentioned in the experimental part, contains a family of at least four \( \delta \)-phosphatidyl-\( \delta \)-myo-inositols, in each of which the phosphatidyl group occupies the same position on the myo-inositol ring, and in which this aspect of the structure is identical with that found in related compounds from all other sources so far investigated.

This uniformity of structure suggests that a common pathway exists for the biosynthesis of all phosphatidyl-myoinositols. Phosphatidylinositol in animal tissue is formed by the reaction of cytidine diphosphate diglyceride with free myo-inositol (41). The biosynthesis of the brain polyphosphoinositides involves the stepwise phosphorylation of preformed phosphatidylinositol (42). By analogy, one might expect that the phospholipids of mycobacteria are formed by glycosylation of phosphatidylinositol. Thus, it is probable that the initial step in the biosynthesis of all complex phosphatidyl myo-inositols is the coupling of the phosphatidyl group to free myo-inositol, and the structural similarity now shown indicates that the enzymes from all tissues show the same stereospecificity for this reaction.

The glycerol myo-inositol phosphate monomannoside was hydrolyzed in dilute ammonium to yield a myo-inositol mannoside that was isolated as the crystalline acetate. This substance was identical with the \( \alpha \)-\( \beta \)-mannosyl-myo-inositol nonacetate previously described by Vilakas (22). Angyal has recently synthesized 1-\( \beta \)-\( \alpha \)-mannosyl-\( \beta \)-myo-inositol, a glycoside that differs from the naturally occurring galactinol (43, 44) in containing mannose in place of galactose. This synthetic mannosylmyo-inositol is identical with the glycoside obtained from the phospholipid of the mycobacteria, which result indicates that the mannose is on the L-3-position in the phosphatidylinositol mannoside. Therefore, the complete structure of this inositol can be represented as shown in III, in which case the R-groups stand for fatty acids.

The major component in the methanol-insoluble fractions of the tubercle bacillus phospholipid corresponded to a phosphatidylinositol pentamannoside. Such a compound has been described by Nojima (21), who has also isolated the deacylated glycerol myo-inositol phosphate pentamannoside. We have characterized the substance by group analysis, by partial degradation to myo-inositol phosphate pentamannoside, to myo-inositol pentamannoside, to myo-inositol 1-phosphate and glycerol phosphate.

The phosphodiester bridge in the phosphatidylinositol pentamannoside is surprisingly stable. Whereas phosphatidylinositol can be cleaved by heating in 98% acetic acid at 100° for 30 minutes with the formation of dicarboxylic acid and myo-inositol phosphate (11), the pentamannoside is unchanged by refluxing in 98% acetic acid for 2 hours. Only by heating under acid conditions adequate to hydrolyze the glycosidic linkages (1 to 2 N hydrochloric acid) can the dicarboxylic acid be cleaved from the molecule, and under these conditions, the major lipid product was free fatty acid. Since this cleavage reaction has been shown to involve neighboring group participation by a free hydroxyl on the myo-inositol ring, with intermediates...
ate formation of a myo-inositol cyclic phosphate ester (45), the unreactivity of the phosphatidyl-myoinositol pentamannoside suggests that the oligosaccharide substituent introduces considerable steric hindrance. This would be plausible in a molecule in which the oligomannose chain was substituted on position 2 or 3 of the myo-inositol ring, positions which are adjacent and cis to one that substituted by the phosphatidyl group.

The glycerol myo-inositol phosphate pentamannoside obtained by deacylation of the lipid is also unusually resistant to alkaline hydrolysis. This makes it easier to understand that Nojima (21) could have obtained intact glycerol myo-inositol phosphate pentamannoside in spite of the strenuous alkaline conditions under which he conducted the deacylation. We find that the phosphodiester bridge can be cleaved by heating the substance in 1 N sodium hydroxide at 100°, but the reaction is only 96% completed in 5 hours, whereas glycerol myo-inositol phosphate is completely degraded in 30 minutes under the same conditions (46). This general reaction also involves neighboring group participation (47) and can yield either myo-inositol phosphate or glycerol phosphate, depending on which group initiates attack on the phosphorus atom. When the neighboring free hydroxyl on the cyclohexane ring is cis to the one involved in the phosphodiester linkage with the glycerol, the ratio of products is slightly in favor of formation of cyclohexanolic phosphate (48). When the neighboring free hydroxyl on the ring is trans, the formation of glycerol phosphate is greatly favored. Whereas glycerol myo-inositol phosphate has been found to give 40% myo-inositol phosphate and 60% glycerol phosphate, the glycerol myo-inositol phosphate pentamannoside gave 48% myo-inositol phosphate pentamannoside and 55% glycerol phosphate. These ratios suggest that the adjacent cis hydroxyl on position 2 of the myo-inositol ring is still free to participate in attack on the phosphorus atom, and that steric hindrance from the oligosaccharide chain (probably on position 3 of the ring) does not shift the balance in favor of attack by either group. Were the oligosaccharide on position 2, one could expect almost exclusive formation of glycerol phosphate. However, it is also apparent that the oligosaccharide markedly decreases the rate of attack at the phosphate bridge by glycerol as well as by myo-inositol.

Selective removal of glycerol from the glycerol myo-inositol phosphate pentamannoside by the periodate-phenylhydrazine method (10) yielded myo-inositol phosphate pentamannoside. The greatly decreased chromatographic mobility of this product relative to the parent compound containing glycerol (R$_{m,yo-inositol}$ 0.1 and 0.4, respectively) is consistent with the formation of an additional charge on the phosphate group as a result of removal of the glycerol. Thus, myo-inositol phosphate and glycerol myo-inositol phosphate are related in a similar fashion and show comparable differences in chromatographic properties (R$_{m,yo-inositol}$ 0.24 and 1.0, respectively).

By complete dephosphorylation in 10% ammonia at 150°, glycerol myo-inositol phosphate pentamannoside was converted to myo-inositol pentamannoside. It is therefore clear that all of the mannose is attached to the myo-inositol, and the following evidence suggests that all of the hexose units have the $\alpha$-$d$-pyranosyl configuration. First, the half-time for acid hydrolysis is that expected for an $\alpha$-mannopyranoside. Secondly, the partial molecular rotation per n-mannosyl unit in the pentamannoside (+15,500°) is very close to the molecular rotation of methyl $\alpha$-d-mannopyranoside (+15,200°). This calculation assumes a minor contribution to the rotation from an asymmetrically substituted myo-inositol, an assumption that is reasonable in view of the small rotations of the asymmetrical methyl ethers of myo-inositol (49).

A phosphatidyl-myoinositol trimannoside has been mentioned recently (50). From a chromatographic study of the deacylated product from a sample of this material, it has been found that the presumed trimannoside was actually a mixture of the pentamannoside and mono- or dimannoside in such a proportion that the mannose to myoinositol ratio was 3:1. No evidence has been obtained in the present chromatographic study of the deacylation products of crude phospholipid fractions for a complete series of lipids with from 1 to 5 molecules of mannose, the missing members being the tri- and tetramannoside. However, Nojima (21) has described briefly a phosphatidyl-myoinositol tetramannoside isolated from BCG, and it may be that, under the right conditions of culture, a trimannoside would also be formed in recognizable amount. The variability in the composition of different preparations of the inositides from Mycobacterium has been emphasized by Anderson (19) and can be attributed to differences in strain, method of culturing, and procedure of extraction. When the phospholipids are extracted by the procedure of Anderson (51) with first ethanol-ether and then chloroform, the ethanol-ether extract contains inositides with not more than 2 mannose molecules; the pentamannoside is found in the chloroform extract accompanying the 'wax D' fraction (21). If the mycobacteria are first defatted by acetone and then extracted with boiling methanol, as in the preparation of the "antigène méthylque" (52), the methanol contains a mixture of all types of inositides mentioned above. If the methanol extract is filtered hot and left standing at room temperature, a precipitate is formed containing most of the phosphatidyl-myoinositol pentamannoside. The inositides with lower sugar content remain in solution and are precipitated by cooling of the methanolic filtrate. The chromatographic study of the deacylated phospholipid mixture provides a relatively rapid and simple procedure by which to determine the inositol composition of the crude phospholipid fractions of mycobacteria.

The presence of glucose in some phospholipid fractions of mycobacteria has been reported (see reference 50), and, in one instance, a fully acetylated compound was obtained, m.p. 79-80° (increasing to 104° on drying under vacuum), which was reported to be a myo-inositol pentaglucoside (22). Having noted the presence of trehalose in some of the deacylated glycerol myoinositol phosphate preparations studied herein, we have reinvestigated this compound and have found that it is in reality trehalose octaacetate (no depression of the melting point on admixture with the authentic compound, and identity of infrared spectra). This finding is in agreement with the fact that cord factor (6,6-di-O-mycoloyl-$\alpha$, $\alpha$-trehalose) (53) can be iso-

2 J. Asselineau and A. M. Miquel, unpublished experiments.
INOSITOL: GLYCEROL MYO-INOSITOL PHOSPHATE, GLYCEROL MYO-INOSITOL PHOSPHATE MANNOSE, GLYCEROL MYO-INOSITOL PHOSPHATE DIMANNOSE, AND GLYCEROL MYO-INOSITOL PHOSPHATE PENTAMANNOSE.

REFERNCEs

Structural Studies on the Myo-inositol Phospholipids of Mycobacterium tuberculosis (var. bovis, strain BCG)
Clinton E. Ballou, Erna Vilkas and Edgar Lederer


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