# Purification of serologically active phosphoinositides of *Mycobacterium tuberculosis*

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ABSTRACT Glycolipids extracted with pyridine from three strains of *Mycobacterium tuberculosis* were fractionated. Phosphatidyl inositol, two phosphatidyl inositol dimannosides (A and M), and two phosphatidyl inositol pentamannosides (G and K) were separated and purified by a combination of solvent fractionations and chromatography on silicic acid. In each of these lipids, palmitic and tuberculostearic acids accounted for more than 90% of the total fatty acids. Mole ratios of fatty acid to P were: for phosphatidyl inositol, 2; for G and M, 3; for A and K, 4. Certain mixtures of A or M with G fixed complement with human sera from cases of tuberculosis. Two serologically inactive dimannosides, each containing two fatty acid ester groups per atom of P, were also present.

KEY WORDS *Mycobacterium tuberculosis* · phosphatidyl inositol · di- and pentamannosides · phosphoinositides · complement fixation · glycolipids · tuberculosis

THE ORIGINAL PURPOSE of this work was a search for an improved antigen for use in complement fixation tests in tuberculosis. Wadsworth, Maltaner, and Maltaner (1) employed boiled aqueous extracts of tubercle bacilli as an antigen, recognizing that the active component of these extracts was probably a lipid; Witebsky, Klingenstein, and Kuhn (2) described an antigen prepared by pyridine extraction. In the initial studies of this series, carried out by the senior author with the assistance of Miss Consuelo Padron, the active fraction of boiled water extracts was found to be a mixture of glycolipids; it was readily soluble in pyridine, hence the same fraction was probably responsible for the activity of the Witebsky antigen. Serologic studies of our earlier preparations were carried out by Dr. J. O. de Almeida (3), who subsequently reported an application of the glycolipid antigens in the control of treatment for leprosy (4, 5).

The chemistry of mycobacterial lipids has been reviewed by Anderson (6) and by Lederer (7) and the glycolipids have been the subject of recent reviews by Lederer (8, 9). The fraction originally described as "phosphatide" by Anderson is now known to be a mixture of phosphatidyl inositol mannosides (10–12) and a polyglycerophosphatidic acid analogous to cardiolipin (13). Recently the structure of the water-soluble compounds resulting from deacylation of the native glycolipids of BCG has been established by Ballou and his associates (14–17). Their findings indicate that the original mixture contained phosphatidyl inositol and at least three phosphatidyl inositol mannosides having 1, 2, and 5 mannose units, respectively.

The object of our work was to separate and purify the original intact phospholipids.

## MATERIALS

Three strains of *Mycobacterium tuberculosis* were studied: BCG, H37Rv, and a second human strain identified by this laboratory's culture number 48189. No differences were found among corresponding lipid fractions from different strains. The examples reported are representative of numerous experiments on all three strains. The BCG cultures were grown for 8–12 weeks on Sauton's medium; the human strains for 8 weeks on Long's medium. The culture fluid was decanted and to the cell mass in each culture bottle was added at least 200 ml of acetone. The cells were thoroughly dispersed in the acetone and the mixture was kept at room temperature for at least 24 hr, usually longer. After the bacteria had been 24 hr under acetone, no living cells could be detected by cultural methods or animal inoculation. Sev-

Abbreviations: BCG, Bacillus Calmette-Guérin; TLC, thin-layer chromatography; PI, phosphatidyl inositol; NIH, National Institutes of Health; "Folch solvent," chloroform–methanol–water 86:14:1. A and M, arbitrary designations of two phosphatidyl dimannosides; G and K, arbitrary designations of two phosphatidyl inositol pentamannosides.

eral additional acetone extractions were made in order to complete the dehydration and to remove most of the acetone-soluble lipid. The cells in the last acetone suspension were ground in a Waring Blendor, then filtered and air-dried.

## METHODS

### *Serologic Activity*

The complement fixation technique used was that described by Kent and Fife (18). Human serum was obtained from persons with tuberculosis; "normal" control sera were from laboratory personnel. Antigens were prepared from methanolic stock solutions of the lipids containing 1 mg of lipid per ml. A 1:20 dilution was prepared by adding 3.8 ml of triethanolamine-buffered saline (18) to 0.2 ml of the stock solution. From the 1:20 dilution, further dilutions in triethanolamine-buffered saline were prepared as required. When mixed lipids were tested, the mixtures were made in the methanolic stock solution.

### *Analysis*

*Phosphorus* was determined gravimetrically as ammonium phosphomolybdate (19) and colorimetrically by King's (20) modification of the method of Fiske and Subbarow, or by the method of Bartlett (21). *Sugars* were determined by Seibert's (22) modification of the carbazole method, with mannose as a standard. The brown color given by mannose in the carbazole reaction is readily differentiated from the rose-red tints given by other monosaccharides. Mannose was also identified chromatographically in acid hydrolysates by the method of Adachi (23), with the use of ethyl acetate–acetic acid–methanol–water 120:3:3:2 for development of the chromatogram and phenol–sulfuric acid–ethanol spray to reveal the spots. No monosaccharides other than mannose were detected. *Inositol* and *glycerol* were identified by paper chromatography after the samples were subjected to the procedure of Böhm and Richarz (24) modified by the use of Whatman No. 1 filter paper and *n*-butanol–ethanol–water 40:11:19 (25) as the developing solvent. When glycerol was to be identified, the samples were hydrolyzed in 3 N HCl instead of 6 N. The method of Böhm and Richarz was also used for the quantitative determination of inositol. *Glycerol* was determined by the method of Blix (26); *fatty acid ester* groups by the method of Rapport and Alonzo (27). Methyl esters of fatty acids were prepared by the method of Hendrickson and Ballou (28) and dissolved in *n*-hexane for gas–liquid chromatography on a Barber-Colman model No. 10 gas chromatograph equipped with an argon ionization detector. Separations were obtained on a column of diethylene glycol succinate polyester, 15.4% by weight, on 80–100 mesh, acid-washed Chromosorb W, supplied by Applied Science Laboratories Inc., State College, Pa. The column was maintained at 170°C and the flow rate of argon was 2.5 ml/sec. The instrument was standardized with NIH standard methyl ester mixtures. Reproducible results within 1.5% of actual composition were obtained with mixture D. Where possible, each fatty acid peak was identified by comparison of retention times with methyl ester standards. The NIH standard mixture and the individual methyl esters were obtained from Applied Science Laboratories. Branched-chain fatty acids were separated from straight-chain fatty acids by the urea inclusion method (29, 30). Fatty acids (200 mg) were dissolved in petroleum ether containing 2% methanol and the solution was poured onto 1 g of finely ground urea moistened with methanol. The mixture was stirred gently, the supernatant solution decanted, and the urea complex washed several times by decantation. The petroleum ether solution was washed once with 3 N HCl, then six times with distilled water. The urea complex was dissolved in water and petroleum ether, and the petroleum ether layer was washed in the same way as the uncomplexed fraction.

### *Cation Exchange and Washing*

Although our earlier experiments were carried out on the native mixed salts of the phospholipids, much cleaner separations resulted when these mixed salts were converted to sodium salts with EDTA. A residue of this reagent could not be tolerated, as it would interfere with the complement fixation test. Dialysis proved inadequate to remove it. A rigorous washing procedure was essential for this purpose and also for the elimination of water-soluble impurities, largely polysaccharide, which traveled with the lipids during column chromatography. Both requirements were satisfied by the following method.

The lipids were dissolved in the minimal volume of chloroform and transferred to a separatory funnel with sufficient methanol–water 1:1 to dissolve all the chloroform. The solution then had approximately the composition of the upper phase of the separation mixture described by Folch, Lees, and Sloane Stanley (31). If the lipid sample was to be treated with EDTA, the reagent was added at this point: for each gram of lipid, 5 ml of a 0.1 M solution of the disodium salt of EDTA, brought with NaOH to pH 7.2–7.5, was used. After a few minutes, 1 ml of saturated NaCl per 100 ml of the solution was added and the mixture was twice extracted by vigorous shaking with about $^1/_5$ of its volume of the lower phase of the Folch mixture. This lower phase, chloroform–methanol–water 86:14:1, is hereafter referred to as "Folch solvent."

The lipids recovered by evaporating the chloroform

extracts were emulsified in water (50 ml/g), an equal volume of acetone was added, and the mixture was stirred until all lumps were dispersed. One milliliter of saturated NaCl for each 40 ml of water was then added and the mixture vigorously shaken; the lipids flocculated and were separated by centrifugation. After five or six repetitions of this procedure, the lipids were once more partitioned in the two-phase Folch mixture and recovered by evaporation of the lower phase extracts.

*Chromatography*

Thin-layer chromatography was carried out on 20 × 20 cm glass plates that had been coated with 0.2-cm thick layers of Silica Gel G (Brinkmann Instruments Inc., Westbury, N.Y.) and activated at 100°C for 90 min. Test samples contained 1–2 mg of lipid per ml of chloroform. To the plates were applied 20-$\mu$l portions of such solutions; the plates were developed for 90 min in chloroform–acetic acid–methanol–water 40:25:3:7, dried, sprayed with 40% $H_2SO_4$ v/v, and heated in a 100°C oven until charred spots were visible. The glycolipid spots could be observed after 1 hr of heating, whereas the phosphatidyl inositol spot required 4–18 hr.

For column chromatography a mixture of 3 parts by weight of silicic acid to 2 parts of Celite[1] was used. Columns were usually prepared from a slurry of the adsorbent mixture in chloroform–methanol–water 180:19:1 and the same solvent was used for application of the sample. Addition of water was necessary because of the unexpected behavior of the pentamannoside K, which was not completely adsorbed from anhydrous chloroform. Mixtures that did not contain K could be chromatographed on the column prepared with the above solvent or on one prepared from chloroform alone. In either case, the sample, 5–10 mg/g of adsorbent mixture, was applied in a volume of 3–4 ml and washed down with at least 1 displacement volume of chloroform–methanol 9:1. Elution was carried out with methanol–chloroform mixtures, the methanol content being increased stepwise. After elution with methanol–chloroform 1:1, 90–100% of the material applied to the column was recovered.

Columns of 18 and 22 mm I.D. were used, the amount of mixed adsorbent in the smaller column being 30–50 g and in the larger column 60–100 g, depending on the size of the sample to be applied. Flow rate in the smaller column was 10–12 ml/20 min and in the larger column 13–15 ml/20 min. Fractions collected at 20-min intervals were examined on the thin-layer plate.

Certain persistent impurities were not efficiently removed by chromatography. After the first chromatographic separation, the several fractions, pooled according to their TLC patterns, were again washed successively in the two-phase Folch mixture and by precipitation from 50% acetone. A chloroform solution of the washed lipids, 30 mg/ml, was mixed with 10 volumes of methanol, heated to boiling, and cooled to room temperature. If an appreciable precipitate separated, it was reprecipitated from chloroform with methanol as before and the final insoluble residue was discarded. The lipid fractions were recovered by evaporation of the pooled chloroform–methanol solutions.

Each fraction identified by a TLC spot was rechromatographed until a limit of purity according to this criterion was reached: if less than 10% of a sample recovered from a column occurred in tubes showing secondary TLC spots, while all tubes of the major portion of the eluate gave the same single spot on the plate, the material collected from this central portion of the eluate was judged "chromatographically pure."

*Separation of Phosphoinositides*

Dried cells were extracted 5 times with pyridine (5 ml/g): the mixture was warmed to 50°C for 1 hr, allowed to cool, and filtered by suction. Pyridine extracts were concentrated at least 20-fold in vacuo, then mixed with 3 volumes of methanol. The flocculent "wax" precipitate was separated, redissolved in the minimal volume of pyridine, and reprecipitated with 3 volumes of methanol. The combined pyridine–methanol solutions were evaporated to dryness in vacuo and the residue was dissolved in chloroform, 8 ml/g; to this solution were added 4 ml/g of methanol and 3 ml/g of 0.4% NaCl (31) and the mixture was shaken vigorously. The lower phase was evaporated to dryness and the residue was washed twice with acetone, then triturated with absolute ethanol (100 ml/g), the mixture being stirred overnight. The ethanol-insoluble portion was the glycolipid fraction, while the ethanol extract contained the cardiolipin-like phosphatidic acid. A typical batch of 200 g of dried cells of strain 48189 yielded 5.2 g of crude glycolipid.

The glycolipid was dissolved in Folch solvent (50 ml/g) and mixed with an equal volume of methanol; the precipitate was redissolved and reprecipitated 4 times more in the same way, then 3 times by addition of $^1/_2$ volume of methanol. The last precipitate was designated *fraction I;* all the chloroform–methanol supernatant fractions were combined as *fraction II*. The amounts of the two fractions in most preparations were nearly equal.

Resolution of the various components on the thin-layer plate is illustrated in Fig. 1. Fraction I consisted chiefly of the pentamannosides which we have referred to as G and K. Fraction II contained two dimannosides which we designate A and M, and phosphatidyl inositol.

[1] Silicic acid: $H_2SiO_3 \cdot nH_2O$, "precipitated," Fisher Scientific Company, New York, N.Y. Celite 545: (Johns-Manville) washed 6× with $H_2O$, 3× with methanol, 3× with acetone, and dried in vacuo.

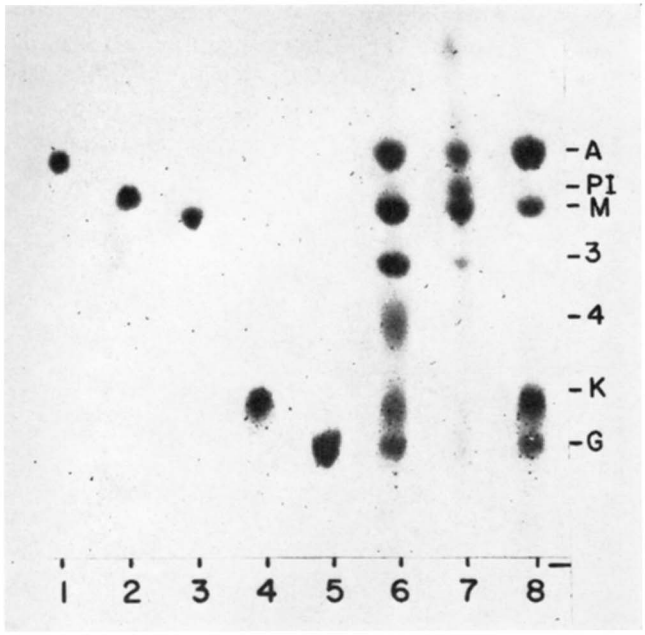


FIG. 1. Thin-layer chromatogram of glycolipids. Lanes 1–5, purified components (arbitrarily named) as designated at right of figure. Lane 6, crude fraction I, strain H37Rv; lane 7, crude fraction II, strain 48189; lane 8, original glycolipid mixture, strain 48189.

Other components, e.g., numbers 3 and 4 in Fig. 1, are present in smaller amounts and may not be visible on the thin-layer chromatogram until they have been concentrated by preliminary fractionation.

*Fraction I* was treated with EDTA, washed thoroughly, placed on the silicic acid column, and eluted with at least 4 displacement volumes each of 20, 30, 40, and 50% methanol in chloroform. The first two solvents washed out A and M. The 40% mixture eluted components 3, 4, and K, in that order, and the 50% mixture removed G. In the first separation there was usually considerable overlapping of adjacent components. At the end of the 50% eluate there was a trace of material which moved still more slowly on the TLC plate than G. Eluates of like TLC pattern were pooled for further purification.

*Fraction K.* Any tubes of the K fraction that also showed spots 3 and 4 were pooled and the material was reprecipitated 2 or 3 times from Folch solvent by 1/2 volume of methanol; components 3 and 4 were largely removed in the supernatant solutions. The reprecipitated material was then pooled with any eluate fractions that consisted chiefly of K, and the whole was rechromatographed. The column was first eluted with 30% methanol in chloroform, then with 9–10 displacement volumes of 40% mixture, and finally with 50% mixture.

*Fraction G.* The column was eluted with 1 displacement volume of 30% methanol in chloroform, then with at least 5 displacement volumes of the 40% mixture to remove K, and finally with the 50% mixture until all material was recovered. Fractions showing single TLC spots were pooled and rechromatographed if necessary until "pure." Three or four column runs, including the first separation of crude fraction I, were usually sufficient to yield chromatographically pure G and K.

*Fraction II* was precipitated twice from chloroform, 30 mg/ml, by addition of 5 volumes of ethanol. The precipitate was treated with EDTA and the washed sodium salt mixture was chromatographed. Separation of a typical batch is illustrated in Table 1.

*Fraction A* was rechromatographed. The column was washed with 2 displacement volumes each of 10 and 15% methanol in chloroform, at least 4 volumes of the 20% mixture, and finally with the 25% mixture until no more material was eluted. The 10% eluate contained a trace of pigment; the 15% eluate, a little A together with an unidentified component moving faster than A on the plate. In the 20% eluate, 70% of the total material was recovered, all tubes showing the A spot only. The final portion of material eluted by the 25% mixture was a mixture of A and M, accounting for about 10% of the original.

*Phosphatidyl Inositol.* Fractions containing a large proportion of PI admixed with M were difficult to resolve by column chromatography but could be separated by crystallizing out PI. Eluate fractions showing no TLC spots other than M and PI were first clarified to remove traces of amorphous methanol-precipitable substances; the dried material was dissolved in chloroform (20 mg/ml) and mixed with 2 volumes of methanol, and the mixture was warmed slightly and immediately centrifuged. The soluble portion was evaporated to dryness and dissolved in pyridine (50 mg/ml). An equal volume of methanol was added and the mixture was first warmed to effect complete solution, then allowed to stand at room temperature for at least 24 hr. The PI typically separated in dense rosettes. The specified concentration was critical: from more concentrated pyridine solutions, fraction

TABLE 1 CHROMATOGRAPHY OF FRACTION II

| Eluting Solvent $CH_3OH$ in $CHCl_3$ | Volume | Weight | TLC Spot Pattern |
|---|---|---|---|
| % | *ml* | *mg* | |
| 15 | 100 | 53 | Yellow resin<br>Fast spots, unidentified |
| 15 | 50 | 48.6 | A |
| 20 | 35 | 97.4 | A |
| 20 | 630 | 344 | A |
| 20 | 180 | 47.7 | A and M |
| 25 | 400 | 160 | M and PI* |
| 50 | 400 | 167 | 3, 4, and G |

980 mg of fraction II, derived from *M. tuberculosis* strain 48189, was applied to 100 g of silicic acid–Celite 3:2.

* Individual tubes of the 25% eluate were tested on the plate but all were mixtures of M and PI.

TABLE 2 PROPERTIES OF PHOSPHOINOSITIDES

| Fraction | $[\alpha]_D$ $CHCl_3$ | Phosphorus | | No. of Moles Per G-Atom P | | | |
|---|---|---|---|---|---|---|---|
| | | Found | Theory* | Inositol | Glycerol† | Mannose | Carboxyl Esters |
| | | % | | | | | |
| A | +33.5° | 1.88 | 1.83 | 0.9 | 1.3 | 2.0–2.2 | 3.9–4.0 |
| M | +39.5° | 2.11 | 2.16 | 1.0 | 1.2 | 1.9–2.3 | 2.9–3.2 |
| 3‡ | — | 2.41 | — | — | — | 2.2 | 2.0 |
| 4‡ | — | 2.33 | — | — | — | 2.1 | 1.9 |
| G | +56.0° | 1.56 | 1.62 | 0.9 | 1.2 | 5.0–5.5 | 3.1–3.3 |
| K | +55.5° | 1.47 | 1.42 | 0.9 | 1.2 | 4.7–5.4 | 3.9–4.3 |
| PI | +9.7° | 3.60 | 3.64 | 1.0 | 1.1 | None | 2.0 |

* Calculated from whole number ratios deduced from observed data, assuming the molecular weight of the fatty acids to be the mean of the weights of palmitic and tuberculostearic, 256 + 298/2 = 277.

† The Blix method for glycerol gives high results in the presence of sugars, but we consider that these figures taken in conjunction with the other analyses confirm the presence of 1 mole of glycerol per atom of P.

‡ These samples were not rechromatographed.

TABLE 3 FATTY ACID DISTRIBUTION IN PHOSPHOINOSITIDES

| Fraction | Palmitic Acid | Tuberculostearic Acid | Stearic Acid |
|---|---|---|---|
| | *% of total fatty acids* | | |
| A | 68 | 28 | 4 |
| M | 63 | 33 | 4 |
| G | 62 | 34 | 4 |
| K | 49 | 43 | 8 |
| PI | 56 | 44 | Absent |

M also separated. When precipitation appeared to have ceased, the precipitate was separated and washed with acetone, and the pyridine–methanol supernatant solution was mixed with 4× its volume of acetone to precipitate M.

The behavior of PI in mixtures illustrates the marked effect of the glycolipids on the solubility of accompanying compounds. Thus, while the above procedure was consistently successful when only M and PI were present, it could not be applied to mixtures containing appreciable amounts of fractions A, G, or K. After one separation from pyridine–methanol, the PI could be recrystallized from chloroform–methanol mixtures, although it did not separate from these same mixtures in the presence of M. It was dissolved in a mixture of equal parts of Folch solvent and methanol, and filtered while hot to remove traces of amorphous impurities, then allowed to crystallize at room temperature overnight. The precipitate was redissolved in chloroform (20 mg/ml), mixed with 2 volumes of methanol, heated until it dissolved completely, and again allowed to crystallize. The pure PI was not appreciably soluble in pyridine. Under the microscope it showed rosettes of needle crystals, strongly birefringent in polarized light. No sugar was detected in the recrystallized substance.

*Fraction M* was rechromatographed after the PI had been separated by crystallization. Elution with 2 displacement volumes of 20% methanol in chloroform removed any remaining A. A little PI was usually found in the first few tubes of the 25% eluate, while 70–80% of the total substance was recovered in the central portion of the 25% eluate, showing only the M spot on the plate. A final elution with 50% mixture yielded small amounts of components 3 and 4.

## RESULTS

### *Composition of Phosphoinositides*

The results of analyses of the purified fractions are summarized in Table 2. Preliminary data on components 3 and 4 are also included, although these fractions were not rigorously purified. To determine whether the "chromatographically pure" fractions were actually substances of definite composition, we determined ester:P and mannose:P ratios in individual tubes of eluate. The range of values found in the major peak of the material in each case is given in the last two columns of Table 2.

The two pentamannosides G and K differed characteristically in the appearance of their aqueous solutions, which were examined by dissolving 1–2 mg of glycolipid in 5 ml of distilled water and observing the turbidity in a Klett-Summerson colorimeter with blue filter No. 42, before and after addition of 3 drops of saturated NaCl. The G fraction swelled to transparent lumps that dissolved only on boiling, and yielded a highly viscous solution that showed no detectable turbidity before or after addition of NaCl. The K fraction dissolved readily at room temperature, without noticeable viscosity, the turbidity readings being 12–15 in distilled water and 50–65 in 0.9% NaCl. Incompletely purified K preparations showed still greater turbidity in NaCl solutions, with rapid sedimentation.

### *Fatty Acids*

No unsaturation could be detected by iodine number

determinations in any of the glycolipid fractions. Gas chromatograms of fatty acid esters from A, M, G, K, and PI showed the same two major peaks in each instance. The first peak was identified as methyl palmitate by comparison with the reference standard. The second did not correspond to any of the available reference esters; it occurred between the peak positions of methyl stearate and methyl oleate, consistently with the assumption that this peak represents the ester of tuberculostearic (10-methyl octadecanoic) acid. Fatty acids from phosphatidyl inositol, strain 48189, were separated by the urea inclusion method. The liquid branched-chain fraction had an equivalent weight by titration of 300; theory for tuberculostearic acid, 298.

The fatty acid distribution calculated from areas under the chromatogram peaks is shown in Table 3. In addition, the four mannosides showed traces of oleic and at least one unidentified acid, the amounts in each case being less than 1% of the total acids. These trace components were not found in the acids from phosphatidyl inositol.

*Serologic Activity.* Fractions A and M gave marked nonspecific reactions with complement. However, by mixing either of these fractions with G, we were able to eliminate the anticomplementary reaction while retaining reactivity with tuberculous serum. The demonstration of "specific" reactivity depended on the use of a balanced mixture of the di- and pentamannosides, the optimal ratios being approximately two parts of A or M to one of G. The M fraction appeared to be somewhat more active than A.

Fraction G alone did not fix complement; this substance was, however, the major component of the "G-1" fraction previously studied by de Almeida (3), who was able to demonstrate by indirect methods that the G-1 fraction did combine with antibody. Fraction K alone was weakly active.

Phosphatidyl inositol reacted nonspecifically with complement and admixture with G had no selective effect: when enough G was added to eliminate the anticomplementary reaction there was no longer any reaction with tuberculous serum.

Components 3 and 4 showed no reactivity in the complement fixation test, whether tested alone or in admixture with G.

## DISCUSSION

The only difference so far discovered between the members of the pairs A and M and G and K is in the number of fatty acid residues. It seems probable that in each case two of the fatty acids are esterified on glycerol, forming a structure analogous to that of phosphatidyl inositol; all the free hydroxyls of the inositol and mannose are then possible sites for esterification of additional fatty acids, two in the case of A and K, one in G and in M. Isomers differing in the position of attachment of these "extra" fatty acids might exist. The pentamannosides recently described by Pigretti, Vilkas, Lederer, and Bloch (32) apparently contained fewer fatty acid residues than G or K.

Lee and Ballou (15, 17) showed that all the mycobacterial mannosides they studied had the same branched structure, the mannose units being attached to the 2- and 6-positions of the myoinositol ring, and that, of the two possible groupings of mannose units in the pentamannosides, only one was found, having four mannoses in one side-chain and one in the other. These workers also established the configuration of the mannose–mannose linkages. The possibility of isomerism in the mannose–inositol structure seems therefore to be excluded.

The serologic activity of the several purified fractions is to be the subject of further studies. We mention our preliminary findings at this time only in order to indicate that the complement-fixing activity of the crude water-soluble (1) and pyridine-soluble (2) antigens is in fact a property of certain of the glycolipids present in such extracts.

Animal inoculations and cultural tests of the viability of bacterial cells were carried out by Miss Mary Clark. For the gas–liquid chromatography of fatty acid esters we are indebted to Dr. John Glenn and Mr. Edward Opalka of the Albany Medical College. Serologic tests were performed by Mrs. Wilhelmine B. Lawson under the direction of the late Dr. John F. Kent.

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