Biosynthesis of Mycobacterial Lipoarabinomannan*

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The mycobacterial lipoglycans, lipomannan (LM) and lipoarabinomannan (LAM), are potent immunomodulators in tuberculosis and leprosy. Little is known of their biosynthesis, other than being based on phosphatidyl-ositol (PI), and they probably originate in the phosphatidylinositol mannosides (PIMs; PIMans). A novel form of cell-free incubation involving in vitro and in situ labeling with GDP-[14C]Man of the polyprenyl-P-mannose (C15/C25-P-Man) and the simpler PIMs of mycobacterial membranes, reisolation of the [14C]Man-labeled membranes, and in situ chase demonstrated the synthesis of a novel α(1→6)-linked linear form of LM at the expense of the C15/C25-P-Man. There was little or no synthesis under these conditions of PIMan, with its terminal α(1→2)Man unit or the mature LM or LAM with copious α(1→2)Man branching. Synthesis of the linear LM, but not of the simpler PIMan, was susceptible to amphotericin, a lipopeptide antibiotic that specifically inhibits polyprenyl-P-requiring translocases. A mixture of P[3H]II and P[3H]Man was incorporated into the linear LM, supporting other evidence that, like the PIMs, LM and LAM, it is a lipid-linked mannoooligosaccharide and a new member of the mycobacterial glycosylphosphatidylinositol lipoglycan/glycolipid class. Hence, the simpler PIMs originate in PI and GDP-Man, but further growth of the linear backbone emanates from C15/C25-P-Man and is amphotericin-sensitive. The origin of the α(1→2)Man branches of mature PIMan, LM, and LAM is not known at this time but is probably GDP-Man.

The cell wall of Mycobacterium spp. consists of a core composed of peptidoglycan linked to the heteropolysaccharide arabinogalactan which, in turn, is attached to the mycolic acids (1). Complementing the mycolyl residues is a variety of free lipids, and interspersed in this framework are lipoarabinomannan (LAM),¹ lipomannan (LM), and various proteins (1). LAM has emerged as a major modulator of the host immune response in the course of the tuberculous infection, and synergizes with the other cell wall constituents to promote mycobacterial survival. LAM may also act as a key ligand in the phagocytosis of Mycobacterium tuberculosis (5).

The biological importance of LAM dictates an understanding of its biosynthesis. Both LM and LAM are based on phosphatidylinositol (PI) (6, 7), specifically on monoacyl phosphatidylinositol dimannoside (Ac1PIMan2) (8, 9), a member of the PIM family with a characteristic mycoinositol (Ino) 2,6-dimannosyl unit (10, 11). In the case of LM, a linear α1→6-linked mannann with extensive α1→2 Man branches emanates from position 6 of the Ino residue (8, 12). LAM contains an additional arabinan, similar but not identical to the arabinan of arabinogalactan (13, 14).

The structural progression from PI to PIMan2 to Ac1PIMan2 to LM and LAM has suggested a similar biosynthetic order (8); however, to date, this was mere speculation. In this present study, we have defined the origins of the Man units of the PIMs and LM and, in the course of the work, identified an α1→6-linked linear form of LM, the apparent precursor of mature LM and LAM.

EXPERIMENTAL PROCEDURES

Preparation of Enzymatically Active Membranes and Cell Envelope—The transformable strain of Mycobacterium smegmatis, mc155 (15), was grown in Bacto Nutrient broth (Difco Labs, Detroit, MI) to mid-log phase (about 24 h), harvested, washed with physiological saline, and stored at −20 °C. M. tuberculosis H37Ra (ATCC 25177) was grown in a liquid medium containing glycerol, alanine, and salts (16) for 14–16 days before harvesting. Mycobacteria (20 g, wet weight) were first washed and then resuspended at 4 °C in a buffer (20 ml) containing 50 mM MOPS (adjusted to pH 7.9 with KOH), 5 mM β-mercaptoethanol, 10 mM MgCl₂, (buffer A), 150 μg of DNase I (type IV, Sigma), and 250 μg of RNase (microsomal nuclease (Sigma)) and subjected to five passes through a French pressure cell (Amino, Silver Spring, MD) at 10,000 p.s.i. The preparation was centrifuged initially at 600 × g for 1 h at 4 °C. These (i.e. membranes arising from 20 g wet weight of cells) were resuspended in 1 ml of buffer A or buffer B (0.1 M Tris-HCl (pH 8.0), containing 0.25 mM sucrose and 1 mM EDTA-Na₂) (17) to yield a total of −20 mg of protein that was frozen in small aliquots; the enzyme activity in membranes was slightly diminished (~20%) by prolonged (2 months) storage at −20 °C. The pellet from the 27,000 × g centrifugation was resuspended in 7 ml of buffer A (20 mg protein/ml) or occasionally in the same volume of buffer B and used directly as an enzymatically active cell envelope fraction containing both cell walls and some membranes; the enzyme activity of this preparation was unaffected by prolonged storage (2 months) in small aliquots at −20 °C. Alternatively, the 27,000 × g pellet was resuspended in 40 ml of buffer A divided equally among four 40-ml centrifuge tubes to which were added 15 ml of Percoll (Pharmacia, Sweden) and centrifuged at 27,000 × g for 60 min at 4 °C (18). The precipitate, upper diffuse band, containing both cell walls and membranes, was removed, collected by centrifugation, washed three times in buffer A, and finally resuspended in 4 ml of buffer A. The final concentration of this Percoll-60-purified cell envelope fraction (P-60) was 8–10 mg of protein/ml. Over 80% of the

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enzyme activity was lost on freezing and thawing, and hence, this fraction was freshly prepared.

Pulse Incorporation of Radiolabeled Man from GDP-[3H]Man or GDP-[14C]Man into Membrane Lipids—Early assays involved incubation of membranes (50 μl, 0.5 mg of protein) or cell envelope (50 μl, 1 mg of protein) with buffer A or buffer B, which were incubated for 1 h, extracted with CHCl3/CH3OH (2:1) followed by CHCl3/CH3OH/H2O (10:10:3). Conditions: Suspension of the [14C]Man-labeled membranes were washed in place several times with buffer A or buffer B, followed by centrifugation at 10,000 × g, for 30 min. The supernatants from these centrifugations were removed, and the [14C]Man-labeled membranes were then carefully resuspended in buffer A (e.g. 1.0 ml for the proceeds from 10 replicate reactions), and 100-μl aliquots (2 mg of protein) were then reconstituted into 10 fresh tubes, each containing 62.5 μM ATP and usually 2 mg (200 μl) of the Percoll-purified cell envelope (P-60) in a total volume of 320 μl and incubated for 1 min.

Pretreatment of Membranes with Amphotycin—Amphotycin (calcium salt) was a gift to C. J. Wachter from Bristol Laboratories and R. Bedensky, Case Western Reserve University, Cleveland, OH. The lipoprotein (up to 2 mg) was dissolved in 500 μl of 0.1 N acetic acid, and the solution was adjusted to 0.05 M sodium acetate (pH 7.0) with 0.1 N NaOH for a final concentration of 2 mg/ml (19). Membranes with or without the in situ labeled [14C]Man lipids were preincubated with amphotycin (10 μg/100 μl of reaction mixture) at 37 °C for 10 min prior to various manipulations such as the addition of the P-60-purified cell envelope fraction and further incubation.

Extraction of [3H][14C]Man-labeled Products from Reaction Mixtures—At the end of incubations, the reactions were terminated by the addition of CHCl3/CH3OH (2:1) (2.5 ml per 100 μl of reaction mixture) followed by centrifugation to separate the pellet (17, 20). The pellet was extracted once more with one-half the volume of CHCl3/CH3OH (2:1). The combined CHCl3/CH3OH (2:1) extracts were washed with 0.9% NaCl followed by CHCl3/CH3OH/H2O (3:4:8:7) to yield the washed CHCl3/CH3OH (2:1) lipids. The resulting insoluble pellet, in the case of reactions involving membranes with in situ labeled [14C]Man lipids, was directly extracted twice with CHCl3/CH3OH/H2O (9:1) and the insoluble pellets derived from membranes that were directly labeled with GDP-[3H]Man or GDP-[14C]Man were first washed with 0.9% NaCl in CH3OH, H2O/CH3OH (1:1) and pure CH3OH (17) to remove residual GDP-[14C]Man before extracting with CHCl3/CH3OH/H2O (10:10:3). In an effort to determine whether the insoluble [14C]Man-containing products associated with the final insoluble residue were LM/LAM, we found that these lipoglycans could be extracted with refluxing 50% ethanol and 88% phenol, as described (7).

Preparation of Mycobacterial PIMan-s, P[3H]I, and P[14C]Man-s—Characterization of the various PIMs followed earlier work (9, 11, 21) and also by comparison with well defined, two-dimensional TLC maps of the full spectrum of PIMs (6–8). Specifically, it was shown that the family of PIMan-s (PIMan1–6) (6–8). Conditions: Suspension of the [3H][14C]Man-labeled membranes from 50 mg of mycobacterial cell envelope fraction and further incubation.

The Specific Question—The immediate question was the manner of biosynthesis of LM with a view to ultimate LAM synthesis. Work in the late 1960s provided possible clues. Brennan and Ballou (23, 24) and Ballou (35) demonstrated that mycobacterial PIMan-s catalyzed the transfer of [14C]Man from GDP-[14C]Man to PI to produce PI[14C]Man-s with the further addition of palmitate residues from palmitoyl-CoA to yield a mixture of PIMan-s, the monoacyl (Ac-PIMan-s), and the diacyl (Ac2-PIMan-s) derivatives. It was later recognized that LAM, in its various forms, i.e. ManLAM and Ara-LAM (22), and LM contain a mannann core linked to PI similar to that in the family of PIMs (PIMan-s, PIMan-s) (6–8). Specifically, it was shown that these lipoglycans contain the d-myo-inositol 2,6-bis-α-
TABLE I

Incorporation of \(^{3}H\)/Man from GDP-\(^{3}H\)/Man into mycobacterial membrane or envelope mannolipids

| Fraction                  | Membranes | Envelope | Nature of products |
|---------------------------|-----------|----------|-------------------|
| CHCl\(_{3}\)/CH\(_{2}\)OH (2:1)-soluble lipids | 0.41 \times 10^6 (89%) | 0.172 \times 10^6 (83%) | C\(_{50}\)-P-Man; PIMan\(_{2}\) |
| CHCl\(_{3}\)/CH\(_{2}\)OH/H\(_{2}\)O (10:10:3)-soluble lipids | 0.01 \times 10^6 (2%) | 0.017 \times 10^6 (8%) | Nondescript |
| Insoluble residue         | 0.04 \times 10^6 (9%) | 0.019 \times 10^6 (9%) | — |

Manp unit and thus to be based on PIMan\(_{2}\), and hence, it was assumed that PIMan\(_{2}\) (and probably PIMan\(_{3}\)) was the immediate precursor of LM/LAM. Moreover, since Kho and co-workers (9) demonstrated the presence of a palmitate substituent on C-6 of the Manp unit that is linked directly to C-2 of the Ino within LM/LAM, it was further assumed that Ac\(_{6}\)PIMan\(_{2}\) was the precise precursor. Throughout, it was assumed that GDP-Man was the immediate donor of all of the Manp units of LM/LAM simply because it was the demonstrated precursor of the Manp units of the PIMan\(_{2}\) family (23, 24). However, shortly after this initial work, Takayama et al. (36, 37) and Schultz and Elbein (38) described two alkali-stable mannophospholipids in M. tuberculosis and M. smegmatis, a mannolysyl-1-phosphoryl-decaprenol (C\(_{50}\)-P-Man) and a mannosyl-1-phosphoryl heptaprenol (C\(_{35}\)-P-Man), which, in light of their group transfer potential and known role in mannolipid synthesis from other organisms (39), could be donors of polymerized Man in mycobacterial cell walls. Indeed, Schultz and co-workers (38, 40) demonstrated indirectly that C\(_{50}\)/C\(_{35}\)-P-Man was the Man donor of undefined polymers. In addition, more recently, Yokoyama and Ballou (41) demonstrated that the Manphosphoryldecaprenol (C\(_{50}\)-P-Man) was the direct Man donor of a series of \(\alpha\)(1→6)-linked oligosaccharides, clearly not LM or LAM with their copious \(\alpha\)(1→2) branches. Hence, the questions posed at the outset of this work concerned the metabolic relationships among GDP-Man, C\(_{35}/C_{50}\)-P-Man, the PIMs, LM/LAM, and the connection, if any, between the \(\alpha\)(1→6)-linked manno-oligosaccharides and LM/LAM.

Incorporation of GDP-\(^{3}H\)/\(^{14}C\)/Man by M. smegmatis Membranes and Cell Wall—A variety of incubation conditions was applied as follows: those developed in the context of PIM biosynthesis (23, 24); those (17) designed to examine the specificity of dolichyl-P-Man-dependent mannosyltransferase activity in mammalian glycoprotein synthesis; those for the incorporation of Man from GDP-Man and C\(_{35}/C_{50}\)-P-Man into the \(\alpha\)(1→6)-linked oligosaccharides of M. smegmatis (41); and a more universal assay appropriate for the incorporation of \(^{14}C\)/GlcNAc, \(^{14}C\)/Rha, \(^{14}C\)/Gal, and \(^{14}C\)/Ara from their corresponding donors into the mycobacterial cell wall “core” (15, 18). Incorporation in all cases was quantitatively and qualitatively comparable. However, replacement of 10 mM MgCl\(_{2}\) (17) with 10 mM MnCl\(_{2}\) or 10 mM CaCl\(_{2}\) resulted in about 35% reduction of activity but no change in product profile; inclusion of 5 mM EDTA reduced incorporation by about 90%, pointing to the need for divalent cations for these mannosyltransferase activities. Under all of these conditions, the majority (~92%; \(\sim 4 \times 10^6\) cpm/mg protein/reaction mixture) of the incorporated \(^{3}H\)/Man was present in the CHCl\(_{3}\)/CH\(_{2}\)OH (2:1)-soluble membrane lipids with only about 10% in other material (Table I). Substitution of membranes with active cell envelope preparations resulted in lower specific activity, but still ~80% of the incorporated \(^{3}H\)/Man appeared in the membrane lipids. Obviously, under these conditions, there was little or no synthesis of LM/LAM or large manno-oligosaccharides which are not extracted by those solvents and would be in the insoluble residue. TLC autoradiography (see below) of the lipids synthesized by membranes and cell envelope preparations showed no significant differences in profile; about 75% of the total radioactivity was distributed between C\(_{50}\)-P-Man and C\(_{35}\)-P-Man, with the remainder in the PIMan\(_{2}\). Short pulses (10 min) of membranes with GDP-\(^{3}H\)/\(^{14}C\)/Man showed lesser synthesis of the PIMan\(_{2}\); the C\(_{35}\)-P-Man and C\(_{50}\)-P-Man predominated. Longer incubations showed increased synthesis of the PIMan\(_{2}\).

Effects of Amphomycin on Incorporation of \(^{14}C\)/Man from GDP-\(^{14}C\)/Man into Membrane Lipids—Amphomycin is one of several families of compounds that specifically disrupt the action of a variety of translocase enzymes, by chelating poly-prenyl monophosphates in the presence of Ca\(^{2+}\) ion and thus inhibiting the transfer of a range of monomeric units to poly-prenyl-P carriers (19, 42, 43). It was applied in the present context to determine (a) whether it would specifically inhibit the incorporation of \(^{14}C\)/Man from GDP-\(^{14}C\)/Man into C\(_{35}/C_{50}\)-P-Man, and (b) its effects on the synthesis of the PIMan\(_{2}\) family, i.e. to determine whether the Man units of PIMan\(_{2}\) arose in C\(_{35}/C_{50}\)-P-Man or GDP-Man. Preincubation of membranes with 10 \(\mu\)g of amphomycin prior to the addition of GDP-\(^{14}C\)/Man had a profound effect on the synthesis of the membrane mannophospholipids. First, there was a dramatic overall reduction in the synthesis of total membrane mannolipids (~60%). Second, inhibition was specific for the C\(_{35}/C_{50}\)-P-Man pair of lipids, and synthesis of the PIMan\(_{2}\) was unaffected (Fig. 1). Therefore, the immediate donor of the Man residues of PIMan\(_{2}\) is not C\(_{35}/C_{50}\)-P-Man, but GDP-Man, obviously reacting with PI as demonstrated previously (23, 24).

Chase of in Situ Labeled C\(_{35}/C_{50}\)-P-\(^{14}C\)/Man—To determine whether GDP-Man or C\(_{35}/C_{50}\)-P-Man was the donor for further mannosylation (e.g. in LM/LAM biosynthesis), a novel assay system was designed. Membranes were pulsed with GDP-\(^{14}C\)/Man during a short (10 min) incubation period, but instead of extracting with CHCl\(_{3}\)/CH\(_{2}\)OH, the \(^{14}C\)/Man-labeled membranes were re-harvested by centrifugation at 100,000 \(\times\) g, washed by suspending in MOPS buffer, and again harvested. The \(^{14}C\)/Man-labeled membranes, shown to be devoid of GDP-\(^{14}C\)/Man, were then further incubated for various times with or without the cell envelope (P-60) prior to extraction with CHCl\(_{3}\)/CH\(_{2}\)OH (4:2:1) to form a biphasic and provide the CHCl\(_{3}\)/CH\(_{2}\)OH (2:1)-soluble lipids for TLC (Fig. 2). The results were striking. At the zero chase time in the presence of membranes only, practically all of the radioactivity was associated with the CHCl\(_{3}\)/CH\(_{2}\)OH (2:1)-soluble lipids (310,800 cpm/mg protein), i.e. the C\(_{35}/C_{50}\)-P-Man combination and Ac\(_{6}\)PIMan\(_{2}\) (Fig. 2, lane 1). Further incubation of membranes for 10 min resulted in little change in lipid radioactivity (Fig. 2, lane 2), whereas additional incubation for 60 min resulted in a significant loss of radioactivity (201,700 cpm/mg protein/reaction mixture) (Fig. 2, lane 3). However, the addition of the cell envelope (P-60) fraction to the reaction mixture in addition to the 60 min chase resulted in dramatic loss of lipid radioactivity (68,400 cpm/mg protein/reaction mixture), and TLC indicated...
that this loss was from the C_{35}/C_{50}-P-Man population, not the PIMan\textsubscript{n} family (Fig. 2, lane 5). Thus, this form of in situ chase of the radioactivity demonstrated that C_{35}/C_{50}-P-Man was the source of the CHCl\textsubscript{3}/CH\textsubscript{3}OH (2:1)-insoluble Man-containing products.

Properties of the End Products of the C_{35}/C_{50}-P-[\textsuperscript{14}C]Man Chase—Efforts to identify the end products of the chase were based on this new assay in which these lipids were generated in situ through short (10 min) pulse labeling of membranes with GDP-[\textsuperscript{14}C]Man to preferentially generate C_{35}/C_{50}-P-[\textsuperscript{14}C]Man which were then further incubated for 1 h in the presence of the cell envelope (P-60) preparation. Twenty of these basic reactions were conducted, but this time, after extraction with CHCl\textsubscript{3}/CH\textsubscript{3}OH (2:1) and washing of the pellet to remove possible residual GDP-[\textsuperscript{14}C]Man, the pellet was further extracted with CHCl\textsubscript{3}/CH\textsubscript{3}OH/H\textsubscript{2}O (10:10:3) in search of lipid-linked oligosaccharides, as possible intermediates on the pathway to LM/LAM. The results of this extraction are shown in Table II. A surprisingly large proportion of the incorporated radioactivity was solubilized by extraction with CHCl\textsubscript{3}/CH\textsubscript{3}OH/H\textsubscript{2}O (10:10:3).

The results of a comparison of the TLC radioautography profiles of the CHCl\textsubscript{3}/CH\textsubscript{3}OH (2:1) and the CHCl\textsubscript{3}/CH\textsubscript{3}OH/H\textsubscript{2}O (10:10:3)-soluble lipids (Fig. 3) demonstrated the occurrence in the latter fraction of a family of graded highly polar [\textsuperscript{14}C]Man-containing glycolipids. This population was susceptible to alkali treatment but resistant to the mild acid conditions, indicating that they were possibly a family of PIMs intermediate in length between PIMan\textsubscript{n} and LM. Since the chromatographic components of the CHCl\textsubscript{3}/CH\textsubscript{3}OH/H\textsubscript{2}O (10:10:3)-soluble lipids were minor, they were not further characterized. Clearly, the bulk of the CHCl\textsubscript{3}/CH\textsubscript{3}OH/H\textsubscript{2}O (10:10:3)-soluble material synthesized at the expense of C_{35}/C_{50}-P-[\textsuperscript{14}C]Man remained at the origin in this solvent. Synthesis of this material also proved to be sensitive to amphotycin. When membranes were pretreated with amphotycin as described in Fig. 1 and then incubated with GDP-[\textsuperscript{14}C]Man, there was a 90% reduction (compared with non-treated membranes) in incorporation of [\textsuperscript{14}C]Man into the CHCl\textsubscript{3}/CH\textsubscript{3}OH/H\textsubscript{2}O (10:10:3)-soluble material remaining at the origin.

**PIMan\textsubscript{n} Is Not Appreciably Synthesized during the C_{35}/C_{50}-P-[\textsuperscript{14}C]Man Chase—**Although the PIMan\textsubscript{n} and PIMan\textsubscript{sa} (21, 44) represent an appreciable proportion of the PIM population of mycobacteria, there was little evidence for their synthesis under the conditions of C_{35}/C_{50}-P-[\textsuperscript{14}C]Man chase (Fig. 3). To establish categorically whether or not the higher PIMs were synthesized under these conditions, M. smegmatis was grown in the presence of [\textsuperscript{14}C]Glc (45) or [\textsuperscript{3}H]Ins, and the CHCl\textsubscript{3}/CH\textsubscript{3}OH/H\textsubscript{2}O (10:10:3)-soluble lipids from each harvest were compared with the lipids likewise extracted from the cell-free C_{35}/C_{50}-P-[\textsuperscript{14}C]Man chase assays. Clearly, under standard growth conditions, the two PIMan\textsubscript{n} (PIMan\textsubscript{sa} and Ac\textsubscript{c}PIMan\textsubscript{sa}) were the only higher PIMs appreciably synthesized (Fig. 4A and B) by M. smegmatis. However, there was no synthesis of these PIMan\textsubscript{n} under the cell-free C_{35}/C_{50}-P-Man chase conditions (Fig. 4C). The implications were that C_{35}/C_{50}-P-Man is not a precursor of the C_{11}—C_{12}Man unit that differentiates PIMan\textsubscript{n} from the PIMan\textsubscript{sa} and PIMan\textsubscript{sa} (21). The results of the experiments described in Fig. 4 also demonstrated the presence of [\textsuperscript{3}H]Ins in the material at the origin (Fig. 4B), indicating for the first time that the major CHCl\textsubscript{3}/CH\textsubscript{3}OH/H\textsubscript{2}O (10:10:3)-soluble product of the C_{35}/C_{50}-P-Man chase experiments may contain PI and may be a linear (\alpha\textsubscript{1}—\textsubscript{6})-linked form of LM.

Characterization of CHCl\textsubscript{3}/CH\textsubscript{3}OH/H\textsubscript{2}O (10:10:3)-Soluble Polymer as an \alpha(1—6) Linear Mannooligosaccharide Attached to PI—To characterize the CHCl\textsubscript{3}/CH\textsubscript{3}OH/H\textsubscript{2}O (10:10:3)-soluble [\textsuperscript{14}C]Man lipids that remain at the origin in a variety of TLC solvents, the material was subjected to gel filtration before and after treatment with alkali and weak acid (Fig. 5). Gel filtration of the CHCl\textsubscript{3}/CH\textsubscript{3}OH/H\textsubscript{2}O (10:10:3), even on Bio-Gel A.
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P100 in 0.1 M CH₃COONa, demonstrated that all of the radioactivity was excluded (Fig. 5A), possibly more a reflection of micelle formation than size. The deacylated fraction was included in Bio-Gel P100 (Fig. 5B) and Bio-Gel P10 (Fig. 5C) and, barely, in Bio-Gel P-2, generally demonstrating the retention volumes of eicosasaccharides. Clearly, its size was smaller than that of deacylated LM and deacylated LAM (Fig. 5, B and C).

A large scale, 14-fold reaction mixture was prepared in which the membranes from 20 g of cells were preincubated with 14 µCi of GDP-[14C]Man, harvested, washed, and then further incubated with the cell envelope fraction from the same batch of cells for 1 h. The CHCl3/CH3OH/H2O (10:10:3)-soluble lipids were obtained after pre-extraction with CHCl3/CH3OH (2:1). About 1 x 10⁶ cpm were recovered. SDS-polyacrylamide gel electrophoresis and subsequent autoradiography of the dried gels showed that this material had mobility properties intermediate between that of the family of PIMs and LM, pointing again to a product intermediate between the higher PIMs and LM.

To directly demonstrate the presence of PI in the CHCl3/CH3OH/H2O-soluble polymer, the membranes containing the in situ labeled C₆₀/C₅₀-P-[14C]Man (from five reaction mixtures) were further incubated with [3H]IMan₂ (5 x 500,000 cpm) for 1 h and extracted with CHCl3/CH2OH/H2O (10:10:3). The latter extract was treated with alkali and applied to Bio-Gel P-2 (Fig. 6). The [3H]Ino and the [14C]Man counts were coincident, suggesting the presence of P[3H]IMan₂ in the de novo synthesized product.

All of the evidence pointed to the synthesis of a new form of LM (linear LM) under the in vitro conditions. To examine the linkage pattern of Man in the newly synthesized product, the CHCl3/CH2OH/H2O (10:10:3)-soluble material was subjected to a number of methylation attempts. Only the dimethylsulfonium carbononium-catalyzed method (33) was successful, with about 50% conversion of the original 1 x 10⁶ cpm into a full permethylated product. This was purified on a small column of SepPak, hydrolyzed with 2 M CF₃COOH, reduced with NaBH₄, acetylated, and the alditol acetates analyzed by gas chromatography/mass spectrometry with simultaneous counting of radioactivity (15). Only two products were obtained, the terminal Manp derivative (1,5-di-O-Ac-2,3,4,6-tetra-O-Me-mannitol) and the 6-Linked Manp derivative (1,5,6-tri-O-Ac-2,3,4-tri-O-Me-mannitol); there was no evidence of any 2-Linked Manp derivative. Thus, the combined evidence points to the synthesis of an α(1→6) linked mammalipoooligosaccharides linked to PI.

**Fig. 3. TLC autoradiography of the [14C]Man glycolipids from a large scale GDP-[14C]Man pulse and C₆₀/C₅₀-P-[14C]Man chase experiment.** The standard pulse-chase reaction was replicated 20 times, and approximately 50,000 cpm were applied to each lane to demonstrate the minor reaction products. Lane 1, the CHCl3/CH2OH (2:1)-soluble lipids. Lane 2, the CHCl3/CH2OH/H2O (10:10:3)-soluble lipids.

**Fig. 4. Synthesis of PIMan₅ during growth but lack of synthesis under conditions of cell-free C₆₀/C₅₀-P-[14C]Man chase.** A. M. smegmatis (100 ml) at mid-log phase was grown in the presence of 1 µCi/ml [U-14C]Glc (250 mCi/mmol) for 2 h. The cells were harvested, washed with 0.9% NaCl, and extracted with CHCl3/CH2OH (2:1), 0.9% NaCl in CH2OH, and 50% CH2OH with probe sonication before extraction with CHCl3/CH2OH/H2O (10:10:3). The CHCl3/CH2OH/H2O (10:10:3) extract (10,000 cpm) was applied to TLC. Inset, two-dimensional TLC. B. M. smegmatis at early log phase was incubated with 1.8 µCi/ml [2-3H]Inositol (210 Ci/mmol) for 12 h before harvesting. The cells were extracted as described above, and 100,000 cpm of the CHCl3/CH2OH/H2O (10:10:3) extract were applied to TLC. C, membranes with in situ C₆₀/C₅₀-P-[14C]Man were prepared by incubating 100 µl of membranes (2.5 mg/protein), 10 mM CaCl₂, 1.22 mM CH₃COONa buffer, and 0.0625 mM ATP for 10 min at 37 °C and then adding 1 µCi of [14C]GDP-Man (321.4 mCi/mmol) in a total volume of 320 µl/reaction mixture (total of 8 such reaction mixtures) and incubating for an additional 10 min. The reaction mixtures were combined and centrifuged at 100,000 x g for 1 h, washed, and resuspended to 800 µl. Eight new reaction mixtures were prepared containing 100 µl of the radiolabeled membranes, 200 µl of the cell envelope P-60 fraction (4 mg/protein), and 0.08 mM ATP in a total volume of 320 µl. The reaction mixtures were incubated at 37 °C for 1 h and then extracted with CHCl3/CH2OH (2:1) followed by CHCl3/CH2OH/H2O (10:10:3). The CHCl3/CH2OH/H2O (10:10:3) extract (25,000 cpm) was applied to TLC. One-dimensional TLC plates were run in the CHCl3/CH2OH, 1.8 CH3COONa/NaOH/H2O (180:140:9:23) and analyzed for radioactivity using a Bio-Scan System 200 Imaging Scanner. Two-dimensional TLC was conducted, first in CHCl3/CH3OH/H2O (60:30:6) and then in CHCl3/CH3COOH/CH3OH/H2O (40:25:3:6).

**Synthesis of the Linear LM in M. tuberculosis—In situ labeling of the C₆₀/C₅₀-P-[14C]Man population of M. tuberculosis membranes was accomplished in like fashion. However, the C₆₀-C₅₀-P-[14C]Man product represented the majority, ~90%, of the material synthesized. Likewise, further incubation of these [14C]Man-labeled membranes with a corresponding P-60 fraction from M. tuberculosis demonstrated incorporation into**
CHCl₃/CH₃OH/H₂O-soluble material with all of the characteristics of linear LM, i.e. 6-linked Manₖ only, the presence of [³H]Ins, and in vitro incorporation of P[³H]IMan₂.

Conclusions—This present body of research solves several questions left in abeyance for many years but now of importance in light of the role of LAM in phagocytosis of M. tuberculosis and generalized immunosuppression. The structural relationship between LAM/LM and the PIMs was first recognized with the discovery of the presence of basic PI (7) and PIMan₂ (8) units in the molecules. However, the biosynthetic origins of the PIMs had always been in doubt and that of LM/LAM had never been explored. Ballou and colleagues (23, 24, 35, 46) had clearly demonstrated that GDP-Man was the source of the Man units of PIMan₂ and PI was a suitable acceptor of [¹⁴C]Man from GDP-[¹⁴C]Man. However, this evidence emerged prior to the recognition of the existence of a heptaprenyl (3,7,11,15,19,23,27-heptamethyl-2,6,10-octacosatriene-1-ol)-P-Man and a decaprenyl-P-Man (the structure of the decaprenol is probably similar to that in the decaprenyl-P-Araf (47)) in mycobacterial membranes (37) and their possible roles in polymer synthesis (38, 40). The use, in this present work, of amphotycin, a member of the mureidomycin family known to inhibit a variety of translocases by chelating to a variety of polypropenyl monophosphates (19), resulted in complete inhibition of the synthesis of the polyprenyl-P-Man, but allowed continuing synthesis of the PIMan₂ₙ, demonstrating that the pathway proposed by Brennan and Ballou (23) prevails and that suggested by others (48) is incorrect.

The first definitive evidence of the precursor role of a polypropenyl-P-Man, the decaprenyl-P-Man, in mycobacterial mannan synthesis came from the work of Yoykoyama and Ballou (41), definitive in that it involved the isolation of pure C₅₀-P-

![Fig. 5](image)

**FIG. 5.** Gel filtration chromatography of the CHCl₃–CH₃OH–H₂O (10:10:3)-soluble lipids from the membranous C₅₀–P-[¹⁴C]Man chase experiment. Experimental conditions are described in Fig. 3. A, the intact CHCl₃–CH₃OH–H₂O (10:10:3) extracted [¹⁴C]Man lipids were suspended in 0.1 M CH₃COONa with sonication and applied to a column (1 × 120 cm) of Bio-Gel P-100 in 0.1 M CH₃COONa. For each of the subsequent columns, 20,000 cpm of the [¹⁴C]Man lipid fractions were treated with 1 N NaOH at 40 °C for 1 h, neutralized, counted (typical recovery >80%), and applied to columns (1 × 130 cm) of Bio-Gel P-100 (B) and Bio-Gel P-10 (C).

![Fig. 6](image)

**FIG. 6.** Simultaneous incorporation of in situ C₅₀–P-[¹⁴C]Man and exogenous P[³H]IMan₂ into linear LM. The membranes containing in situ C₅₀–P-[¹⁴C]Man were obtained from five of the standard incubations. These were distributed into five new reaction mixtures to each of which was added 500,000 cpm of the P[³H]IMan₂ preparation. Incubation was for 1 h. The reaction mixture was extracted with CHCl₃–CH₃OH–H₂O (10:10:3), treated with alkali, and applied to a column (1 × 123 cm) of Bio-Gel P-2 and fractions counted for [¹⁴C]Man and [³H]Ins.

![Fig. 7](image)

**FIG. 7.** Postulated pathways for the biosynthesis of the PIMs, linear LM, native LM, and LAM of mycobacteria. R₁ = tuberculostearic acid
R₂ = palmitic acid

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[14C]Man, its incubation with membrane preparations, and characterization of end products. Clearly, the newly synthesized products were α(1→6)-linked mannooligosaccharides, and there was no synthesis of α(1→2)-Man branches. However, the relationship of these mannooligosaccharides to the present linear LAM, or LAM proper is not clear. Whether these mannooligosaccharides were lipid-linked or even reducing is also not clear. However, it would seem from earlier work (49) that at least one of these is an α(1→6)-linked eicosasaccharide, non-reducing, and hence with no apparent direct relationship to LM or LAM. These mannooligosaccharides may be natural autolysis products of linear LM; mycobacteria contain natural autolysis products of linear LM; mycobacteria contain a non-lipidated, reducing mannan (50), apparently identical to the mannan within LM. Regardless of any relationship, the present body of work and that of Yokoyama and Ballou (41) demonstrate that the α(1→6)-linked Man backbone of the mannooligosaccharides, the linear LM, and presumably mature LM and LAM arise in C50-P-Man, and the α(1→2)-linked branches of PIMan2s, LM, LAM, and other mannooligosaccharides arise elsewhere. Present tentative evidence indicates that GDP-Man is the source of these external Man residues as in the case of the PIMan8s. Further incubation of GDP-[14C]Man-pulsed, washed and chased membranes with fresh GDP-[14C]Man resulted in modest incorporation of [14C]Man into products indistinguishable from LM and LAM (results not shown). Thus, the combined evidence from the past and the present points to the pathway outlined in Fig. 7 for the biosynthesis of the native branched LM. Separately, we have shown that the arabinan of LAM arises by the single transfer of Araβ units from C50-P-Araβ (47) to an endogenous acceptor (18). Whether or not this acceptor is endogenous LM or linear LM has not been established.

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