The "core" structure of the cell wall of Mycobacterium and related genera is unique among prokaryotes, consisting of a covalently linked complex of mycolic acids, \( \alpha \)-arabinan and \( \delta \)-galactan (mycolylarabinogalactan, mAG), which, in turn, is linked to peptidoglycan via a special linkage unit, \( \beta-\delta-Rha\{1\rightarrow3\}\beta-GlcNAc-P \). Little is known of the biosynthesis of this complex, although it is the site of action of several common anti-tuberculosis drugs. Isolated cell membranes of Mycobacterium smegmatis catalyzed the incorporation of \([^{14}C]\)GlcNAc from UDP-\([^{14}C]\)GlcNAc into two glycolipids (1 and 2) and of \([^{14}C]\)Rha from TDP-\([^{14}C]\)Rha into glycolipid 2. These products were characterized as polypropenyl-P-GlcNAc (glycolipid 1) and polypropenyl-P-P-GlcNAc-Rha (glycolipid 2) based on sensitivity of synthesis to tunicamycin, chromatographic characterization of the products of mild acid hydrolysis, and mass spectral analysis of the glycosyl and propenyl units. Glycolipids 1 and 2 were shown to be precursors of the linkage unit in polymerized cell wall. The inclusion in the assays of UDP-\([^{14}C]\)Galp and a preparation of cell walls allowed the incorporation of \([^{14}C]\)Gal into two further glycolipids (3 and 4). Preliminary evidence indicates a precursor-product relationship among glycolipids 1, 2, 3, and 4. Thus, the first steps in the biosynthesis of the mycobacterial cell wall involve synthesis of the linkage disaccharide on a polypropenyl-P carrier followed by growth of the galactan unit. Assays are thus defined for the screening of new anti-tuberculosis drugs active against cell wall synthesis.

The core or skeletal cell wall of members of the Mycobacterium genus consists of extensively cross-linked peptidoglycan to which is attached the linear \( \delta \)-galactan composed of alternative 5- and 6-linked \( \alpha \)-O-Gal units (1). Attached in turn to the \( \delta \)-galactan are extensively branched chains of \( \delta \)-araf-containing arabinan, the distal ends of which are almost completely esterified with mycolic acids (2). We have described this vast macromolecular structure as the mycolylarabinogalactan (mAGP) \(^*\) complex (3). The biogenesis of the mAG portion of this complex is under current investigation because several of the widely used anti-tuberculosis drugs affect aspects of its synthesis and resistance to these drugs is a serious world-wide public health problem (4). For instance, a target for isoniazid (INH) is a NADH-requiring 2-trans-enoyl fatty acyl reductase apparently on the pathway to mycolic acid biosynthesis and some of the resistance to isoniazid is due to a point mutation in the inhA gene and/or overexpression of the target (5). Ethambutol specifically inhibits the synthesis of the arabinan of AG and of liparabinomannan, apparently through its action on a family of arabinosyltransferases (6).

Some dues about the initiation of mAG biosynthesis have arisen from earlier structural work. It has long been known that the AG heteropolysaccharide chains are attached through phosphodiester linkages to C-6 of a proportion of the muramic acid residues of mycobacterial cell walls (7). More recently, chemical analysis of degradation fragments arising from the reducing end of AG obtained from the cell walls of Mycobacterium tuberculosis, Mycobacterium bovis BCG, and Mycobacterium leprae demonstrated the existence of the terminal sequence \(-5\;\beta-Galf\{1\rightarrow6\}\beta-Galf\{1\rightarrow5\}\beta-Galf\{1\rightarrow4\}L-Rhap\{1\rightarrow3\}\beta-GlcNAc\) (1, 8). Based on the acid lability of the 3-linked GlcNAc unit, the presence of about equal amounts of L-Rhap\{1\rightarrow3\}\beta-GlcNAc and muramyl-6-P in an isolated cell wall fragment and \(\text{\[^{31}P\]\ NMR analysis}, it was concluded that the terminal GlcNAc residue is in phosphoryl linkage to the 6-position of some of the muramyl residues of mycobacterial peptidoglycan (8, 9). Thus, this aspect of mycobacterial cell wall structure, and, presumably, biosynthesis, shares similarity with the teichoic acid-peptidoglycan complex of many Gram-positive bacteria (10). In view of the role of the mycobacterial linkage unit as the fulcrum of cell wall integrity and as a potential singular site for target-directed chemotherapy against tuberculosis, we set about elucidating its biosynthesis in the belief that such information will give rise to assays amenable to high-throughput screening for new growth inhibitors of M. tuberculosis.

**EXPERIMENTAL PROCEDURES**

* M. smegmatis mc2155 (a gift from Dr. W. J. Jacobs) was grown in a glycerol/alanine salts medium (11). Cells were grown to mid-log phase (about 24 h), harvested, washed with physiologically buffered saline, and stored at \(-70^\circ\text{C}\) until required.

**Particulate Enzyme Preparations**—M. smegmatis (10 g wet weight) was washed and resuspended in a buffer containing 50 mM MOPS (adjusted to pH 8.0 with KOH), 5 mM 2-mercaptoethanol, and 10 mM MgCl\(_2\) (buffer A) (30 ml) at 4 °C and subjected to probe sonication (Soniprep 150; MSE Ltd., Crawley, Sussex, United Kingdom; 1-cm probe) at 4 °C for a total time of 10 min in 10 60-s pulses with 90-s cooling intervals between pulses. The whole sonicate was centrifuged at 27,000 × g for 12 min at 4 °C. The cell wall-containing pellet was resuspended in buffer B to a final volume of 20 ml and divided between two centrifuge tubes. Percoll (Pharmacia, Sweden) was added to each tube to achieve a 60% suspension, and the mixture was centrifuged at 27,000 × g for 60 min at 4 °C (12). The particulate, upper, diffuse, cell wall-containing band was collected and washed three times in buffer B and resuspended in this buffer (5 ml) to give the Percoll-60, enzymatically active (12) cell wall fraction, which had a protein concentration of 8–10 mg/ml.

Membranes of M. smegmatis were obtained by centrifugation of the fraction at 100,000 × g. Membranes of M. smegmatis were then solubilized by boiling in a solution containing 1% sodium dodecyl sulfate (SDS) and the solubilized membranes were centrifuged through 50% Percoll in buffer B. Membranes were washed in buffer B and then resuspended in the buffer at a concentration of 5–10 mg/ml.
Enzymatic Synthesis of dTDP-Rha and dTDP-[U-14C]Rha—The enzyme mixture (1 ml) of UDP-[U-14C]Rha from Oka- zaki et al. (13) and Escherichia coli B ATCC 23848 was grown to mid-log phase and harvested after 2 h of growth, washed twice in cold 20 mM Tris-HCl buffer, pH 7.7, at 4 °C, disrupted by sonication as described above for M. smegmatis, centrifuged, and the supernatant used as a source of enzymes. The reaction mixture contained 2 mM UDP-[U-14C]Gal (specific activity 50 mCi/mmol; ICN Biomedicals), 0.5 mM Na2EDTA, 5 mM MgCl2, 10 mM NADPH, and 250 μM of the soluble E. coli enzyme extract (13) in a total volume of 500 μl which was incubated at 37 °C while monitoring NADPH utilization at 340 nm. For the conversion of dTDP-[U-14C]Gal to [14C]Gal, described under "Experimental Procedures" was followed by the addition of 0.1 ml of 0.2 M NaOH in MeOH and incubation for 20 min at 100 °C. The HCl was then added to reach a concentration of 2 M HCl at 100 °C for 10 min. The HCl was removed through biphasic washings (18). TLC of glycolipids was conducted on platelets of silica gel in CHCl3/CH3OH/H2O (5:3) and returned to the second dimension in isobutyric acid, 0.5 M aqueous NH4OH (5:3). The polymer remained at the origin and was counted. The unreacted nucleotide sugar, degraded sugar phosphate, and glycolipid intermediates migrated down the paper (15).

Other Preparatory and Analytical Procedures—The preparation of the mAGP complex from M. bovis BCG cell wall has been described (1, 16). The isolation of the linkage unit (isobutyril acid-NH2O) (5,3). The polymer from mAGP has also been described (3). The electron impact mass spectrum (EI-MS) of the disaccharide showed characteristic A-series fragments (17): Aα (m/z 189); Aβ (m/z 157); Aα (m/z 125); ald (m/z 276); and the aldol degradation ions 89, 130, 348, 392, and 436 (3, 8). Hydrolysis, reduction, and per-O-acetylation yielded 1,5-di-O-acetyl-3,4,6-tri-O-methylmannitol (m/z 215, 118, 131, 162, and 175) and 2-N-Me-2-N-Ac-3-O-Ac, 4,5,6,7-tetra-O-methylglucosaminitol (m/z 130, 246, and 290).

Akkal treatment of the [14C]-labeled glycolipids served to demonstrate their alkaline stability and to provide a first step in their purification. Excellent recovery (about 85%) was obtained by dissolving the lipid fraction (30% in methanol) in 0.1 M NaOH followed by the addition of 0.1 ml of 0.2 M NaOH in MeOH and incubation at 37 °C for 20 min. Mixtures were neutralized with 2.5 μl of glacial CH3COOH, dried, and suspended in 1.5 ml of CHCl3/CH2OH (2.1), and 0.25 ml of H2O, centrifuged, and the lower (CHCl3) phase retained. First steps in the purification of the glycolipids involved application of the alkali-stable lipids to a column (7 × 0.5 cm) of DEAE-cellulose (acetate form) poured in CH2OH and equilibrated in CHCl3/CH2OH (2.1). The lipid fraction was applied in CHCl3/CH2OH (2.1) and the column developed with 3 column volumes each of CHCl3/CH2OH (2.1), CH2OH, and 50, 100, 200 mM, and 1 mM ammonium formate in CH2OH. Salt was removed by biphasic washings (18). TLC of glycolipids was conducted on plates of silica gel in CHCl3/CH2OH/NH4OH/CH3OH/H2O (65:25:0.5:3) which were exposed to Kodak X-Omat AR film at −70 °C and subsequently sprayed for the presence of phosphorus with a molybdenum reagent (19) or for polypeptides with a reagent containing p-anisaldehyde (20). Mild acid hydrolysis of glycolipids (21) was conducted on radiolabeled preparations in 100 mM HCl in CHCl3/CH2OH (2.1) (1 ml) at 20 °C for 2 h or 10 mM HCl at 100 °C for 10 min. The HCl was neutralized with 0.5 mM NaOH before adding H2O (150 μl) to form a biphasic, each phase of which was counted.

Hydrolysis of glycolipids and polymer for neutral sugar content was conducted in 2 M CH3COOH at 120 °C for 2 h. Hydrolysis of glycolipid preparations for amino sugar content was conducted in 4 mM HCl at 100 °C for 4 h. Sugars were analyzed in a variety of ways. Radioactive sugar preparations were generally applied to a P-lam column (250 × 4.6 mm) with a linear gradient of ammonium formate (15–0 mM) at a flow rate of 2 ml/min. Fractions were collected, and retention time compared to sugar standards. Fractions were counted, collected, and retention time compared to standards.

Other chromatographic systems have been described (14). For analysis of radioactive products (alditol acetates; partially permethylated oligosaccharides) a Durabond (DB)-1 fused silica column (J&W Scientific, Rancho Cordova, CA) was used as described (24, 25) as part of the Hewlett-Packard 5890 Series II Plus Gas Chromatograph, coupled to the Lablogic GC-RAM radioactive counter (INUS Systems, Tampa, FL).

RESULTS

Recognition of Two Novel [14C]GlcnAc-containing Glycolipid Intermediates—The basic assay mixture containing UDP-[U-14C]GlcnAc described under “Experimental Procedures” was scaled up 4-fold and the reaction mixture subjected to a biphasic organic extraction. About 5% of the radioactivity was incor-

27,000 × g supernatant (from the previous step) at 100,000 × g for 1 h at 4 °C. The supernatant was carefully removed, and the yellow-pigmented opalescent membranes were gently and superficially washed with buffer A and finally suspended in 0.4–0.5 ml of this buffer (protein concentration 15–20 mg/ml).

Incubation Conditions—The initial, standard reaction mixture for incorporation of [14C]GlcnAc from UDP-[U-14C]GlcnAc into glycolipid precursors and polymer were as follows: UDP-[U-14C]GlcnAc (ammonium salt; Amersham, Bucks, United Kingdom) (1 μCi; specific activity, 214 μCi/mmol), 0.06 mM ATP, 10 mM MgCl2, 5 mM 2-mercaptoethanol, 50 mM MOPS adjusted to pH 8.0 with KOH (buffer A), and 100 μl of the membrane preparation (1.5–2.0 mg of protein) in a total volume of 320 μl. Standard conditions for the incorporation of [U-14C]Rha from dTDP-[U-14C]Rha were as follows: dTDP-[U-14C]Rha (0.01 μCi/mmol), 0.02 mM UDP-GlcNac, 0.06 mM ATP, buffer A, and 100 μl of membranes in a total volume of 320 μl. Similar conditions were used initially to examine the incorporation of [14C]Gal from UDP-[U-14C]Gal (Amersham) (1 μCi; 257 μCi/mmol) into glycolipids. At other times, the Percoll-20% wall enzyme fraction (160 μl; 1.3–1.6 mg of protein) and cold UDP-[U-14C]Gal and UDP-glucose were added (final concentrations as described in the text). Other variations to these reaction mixtures are described in the text. To study the effect of tunicamycin (Sigma), the antibiotic was prepared as a sonicated suspension in buffer A (2 mg/ml), and 5 μl were added to the reaction mixture to achieve a final concentration of 50 μg/ml. Reactions were incubated at 37 °C for 1 h, followed by the addition of 6 ml of CHCl3/CH2OH (2:1). Reactions were shaken at room temperature for 20 min, followed by the addition of 680 μl of water and centrifuged. The lower organic phase was backwashed with CHCl3/CH2OH/H2O (1:47:48) before application to TLC plates. Distilled and Al2O3-treated CHCl3 (to remove traces of HCl in commercial CHCl3) was used throughout.

For purposes of estimating incorporation of radioactivity into polymer, the approach described by McArthur et al. (15) was used. The entire reaction mixture, or a portion of it, was applied to Whatman 3MM chromatography paper which was developed in a descending fashion with isobutyric acid-water/ethyl acetate (1:1:1). The polymer remained at the origin and was counted. The unreacted nucleotide sugar, degraded sugar phosphate, and glycolipid intermediates migrated down the paper (15).
porated into the lipid fraction under these conditions. The omission of ATP from the assay had no appreciable effect on incorporation. Addition of decaprenol-P did not increase incorporation. Addition of various non-ionic detergents, such as n-octylglucopyranoside or higher concentrations of ATP, substantially inhibited incorporation. Other major changes in the basic reaction and the consequences are described below.

Samples of the radioactive lipids were applied to silica gel TLC plates which were developed in CHCl3/CH3OH/NH4OH/basic reaction and the consequences are described below. Surprisingly clean products were obtained consisting of two closely migrating glycolipids (GL 1 and GL 2). Conditions that resulted in partial inhibition of [14C]GlcNAc incorporation into the lipid fraction (i.e., prolonged storage of membranes, some detergents) consistently resulted in a more marked inhibition of synthesis of GL 2 (Fig. 1A), suggesting that the formation of GL 2 involved an additional enzymatic step beyond GL 1. Treatment of the GL 1/GL 2 mixture with 0.1 M HCl in CHCl3/CH3OH (2:1) at 20°C (21) resulted in over 50% loss of lipid radioactivity after 5 min; and ~80% loss after 20 min. Treatment of the glycolipids with 0.1 M NaOH at 37°C resulted in 94 and 95% recovery of radioactivity in two experiments, supporting the evidence that these products were polyprenyl-P based glycolipids (24–27). To confirm that the doublet was glycolipid in nature rather than residual nucleotide sugar or degraded sugar-P, synthesis of GL 1/GL 2 was shown to increase over time (Fig. 1B), and when these products were eluted from the gel, the lipid solubility was confirmed in the two-phase system. Attempts to better resolve the doublet were unsuccessful; most solvents gave the false impression that the product was homogeneous. Thus the evidence pointed to the synthesis of two novel polyprenol-containing glycolipids, much more polar than those described previously in mycobacteria, all of which contained neutral sugars and decaprenol.

Effect of Tunicamycin on Synthesis of the Glycolipid Intermediates—The antibiotic tunicamycin inhibits the transfer of GlcNAc-1-P from UDP-GlcNAc to polyenyl monophosphates catalyzed by membrane preparations from a variety of organisms including Gram-positive bacteria (28–30). Thus, in the present instance, inhibition by tunicamycin would implicate a polyprenyl-P-P rather than a polyprenyl-P linkage. Tunicamycin was added to the standard reaction mixture as described under “Experimental Procedures” at a concentration of 50 μg/ml. It had a dramatic inhibitory effect on [14C]GlcNAc incorporation into GL 1/GL 2 (Table I), indicating that the initial step in mycobacterial cell wall synthesis involves formation of a polyprenyl-P-P GlcNAc unit.

Incorporation of [14C]Rha from dTDP-[14C]Rha into GL 2 of the Glycolipid Doublet—The dTDP-[14C]Rha was enzymatically synthesized from commercially available dTDP-[14C]Glc using a crude extract of E. coli (13) obviously containing the active enzymes rfb B, C, and D of the rfbABCD gene cluster responsible for dTDP-Rha synthesis (31). The synthetic dTDP-[14C]Rha and cold dTDP-Rha were finally purified by open column chromatography and preparative HPLC. Analytical HPLC on Partisol SAX 10 with a linear gradient of monobasic acid at pH 7.0 gave two peaks of radioactivity; dTDP-[14C]Rha and cold dTDP-Rha.

### Table I

| Reaction | Total cpm in lipid fraction |
|----------|-----------------------------|
| Completea | 30,890 | 41,330 |
| + Tunicamycinb | 1,210 | 970 |
| + dTDP-Rhac | 40,000 | 50,830 |
| Completeb | 4,380 |

---

a The reaction mixture was the standard one described under “Experimental Procedures” and contained 100 μg of membranes (1.3 mg of protein); 1 μCi of UDP-[14C]GlcNAc; 0.06 mM ATP; and buffer A to a final volume of 320 μl.

b 50 μg/ml of tunicamycin, i.e. 16 μg/reaction, prepared as described under “Experimental Procedures.”

c 0.02 mM final concentration in experiment 1; 0.01 mM in experiment 2.

d dTDP-Rha/dTDP-[14C]Rha, 0.01 mM, 250,000 cpm; UDP-GlcNAc, 0.02 mM.
ammonium phosphate showed only one peak. Acid hydrolysis,
TLC, and autoradiography of the \([14C]\)sugars showed only
\([14C]\)Rha. GC-MS of the alditol acetates demonstrated the presence
of pure rhamnitol acetate (m/z 99, 129, 171, 201, and 231)
(14). The specific activity of the working solution was low, only
1915 mCi/mmol, due to low recovery of both dTDP-[14C]Rha and
dTDP-Rha, and the need to extend the supply of dTDP-
[14C]Rha, since dTDP-[14C]Glc is no longer commercially avail-
able. The synthetic, pure dTDP-[14C]Rha was included in the
standard reaction mixture. On account of its lesser specific
activity, incorporation was considerably less than in the case of
UDP-[14C]GlcNAc (Table I). However, it was clear from auto-
radiography that the incorporation of [14C]Rha took place only
into GL 2 of the glycolipid doublet (Fig. 2). Incorporation of
[14C]Rha into the apolar glycopeptidolipids (GPLs) was also
evident (Fig. 2). The GPLs are composed of a lipopeptide con-
taining among others, l-alaninol and d-allothreonine, which
provide attachment points for a variety of glycosyl units, among them, invariably, O-methylrhamnosides (32).

Characterization of GL 1 and 2—The lipid phases arising
from a 10-fold scale-up of the standard UDP-[14C]GlcNAc and
dTDP-[14C]Rha incubations were washed repeatedly to remove
any residual nucleotide sugars and the products applied to a
column of DEAE-cellulose (acetate) which was irrigated with
CHCl₃/CH₃OH (2:1), CH₃OH, and increasing concentrationsof
HCOONH₄ in CH₃OH. The majority of the [14C]GlcNAc-con-
taining GL 1 and GL 2 from the UDP-[14C]GlcNAc experi-
ments, together, but in a highly pure state, emerged in the 40
and 100 mM HCOONH₄ eluates. In the case of the dTDP-
[14C]Rha experiments, the neutral [14C]Rha-labeled GPLs ap-
ppeared in the CHCl₃/CH₃OH (2:1) and the CH₃OH eluates,
whereas GL 2 was in the 100 mM HCOONH₄ eluate. The
[14C]Rha from dTDP-[14C]Rha was incorporated about equally
into the neutral GPLs and the acidic GL 2.
The $[^{14}C]$GlcNAc-labeled GL 1/GL 2 mixture was subjected to strong acid hydrolysis (4 N HCl, 100 °C, 4 h), partitioned between CHCl$_3$ and H$_2$O, and the aqueous phase dried repeatedly and applied to cellulose TLC plates and developed in CHCl$_3$/CH$_3$OH/H$_2$O (4:2:1), and the lipid in the organic phase subjected to strong acid hydrolysis (4N HCl, 100°C, 4 h), partitioned within a mixture of 1-butanol/pyridine/0.1 N HCl (5:3:2). Autoradiography showed the presence of just one radioactive sugar, corresponding to GlcNH$_2$. Hydrolysis with 2M CF$_3$COOH (2 h, 120°C) of the acidic $[^{14}C]$Rha-labeled GL 2, followed by cellulose TLC in t-butanol/ethylmethylketone/formic acid/water (40:30:15:15) and autoradiography, showed only $[^{14}C]$Rha (results not shown).

It was obvious throughout that the quantities of GL 1 and 2 generated in scale-ups of the standard reaction mixtures were too small for adequate chemical characterization. Accordingly, the standard reaction mixture was increased 158 times (that containing UDP-$[^{14}C]$GlcNAc was increased 46 times; the corresponding UDP-GlcNAc-cold reaction was increased 112 times). A total of 1.142 g of membrane preparation (1.5 mg of protein), 74 ml (0.7 mg of protein) of Percoll-60, ATP (0.06 mM), UDP-Gal (0.02 mM), TDP-Rha (0.01 mM), UDP-$[^{14}C]$GlcNAc (0.5 μCi), Total volume, 160 ml. Ten such tubes were incubated, quenched at 37 °C for the indicated times, stopped by the addition of 1 ml of C$_2$H$_5$OH, and the entire reaction mixture applied to sheets of Whatman 3MM paper which were developed overnight in isobutyric acid, 0.5 M ammonium hydroxide (5:3) overnight. The origins of the paper strips, containing the polymerized cell wall, were cut out and counted.

The $[^{14}C]$GlcNAc-labeled GL 1/GL 2 mixture was subjected to mild acid hydrolysis (4 N HCl, 100 °C, 10 min) partitioned within a mixture of CHCl$_3$/CH$_3$OH/H$_2$O (4:2:1), and the lipid in the organic phase subjected to MS analysis as described (3) (Fig. 3B, inset), seeking chemical evidence for the presence of the α-L-Rhap-(1→3)-α-L-GlcNAc unit. The neutral, dried, combined hydrolysates were reduced with NaBD$_4$ and subjected to the NaOH methylation procedure of Ciucanu and Kerek (35). The methylation reaction was quenched with H$_2$O and the per-O-methylated products extracted with CHCl$_3$, O-acetylated, and subjected to GC on the Durabond DB-1 fused silica column and compared to the authentic, derivatized α-L-Rhap-(1→3)-α-L-GlcNAc linkage unit (3) (Fig. 3). Singly and by co-chromatography, both showed identical retention times of 12.95 min. The per-O-methylated glucosaminol derivative arising from GL 1 was not obvious in any of these work-ups; presumably, it was lost in the initial purification of the per-O-methylated products.

**TABLE II**

| Reaction mixture | Total cpm in polymer |
|------------------|----------------------|
| Complete$^a$     | 3,800                |
| Complete + tunicamycin | 3,500            |

$^a$ A mixture of GL 1 and GL 2 (the 40–100 ml ammonium formate in methanol fraction from DEAE cellulose) was dissolved in 2% Nonidet P-40 (35 μl) and 15 μl of this mixture (42,800 cpm) was added to the reaction mixture (containing 100 μl of membrane preparation (1.5 mg of protein); 143 μl of the P-60 cell wall preparation (1.1 mg of protein); 0.1 ml UDP-Gal and buffer A to a final volume of 320 μl; 0.1 ml UDP-GlcNAc and 0.05 ml TDP-Rha were included, although apparently not required). Incubation was for 1 h at 37 °C. The whole reaction mixture was applied on Whatman 3MM paper, which was developed in isobutyric acid, 0.5 M ammonium hydroxide (5:3) overnight. The origins of the paper strips, containing the polymerized cell wall, were cut out and counted.

**TABLE III**

| Experiment | cpm in lipid fraction |
|------------|-----------------------|
| Standard$^a$ | 30,890                |
| +P60       | 24,500                |
| +P60 + tunicamycin$^c$ | 1,210          |
| +P60 + dTDP-Rha (0.54 mM) | 40,000       |

$^a$ As described in Table I.
$^c$ 160 μl containing 1.4 mg of protein.

**FIG. 4.** Time course of incorporation of $[^{14}C]$GlcNAc from UDP-$[^{14}C]$GlcNAc into polymerizer linkage region and the GL-containing lipid fraction. The incubation conditions were a variation of those described under “Experimental Procedures,” i.e. 50 μl (0.9 mg of protein) of membrane, 74 μl (0.7 mg of protein) of Percoll-60, ATP (0.06 mM), UDP-Gal (0.02 mM), TDP-Rha (0.01 mM), UDP-$[^{14}C]$GlcNAc (0.5 μCi), Total volume, 160 ml. Ten such tubes were incubated, incubated at 37 °C for the indicated times, stopped by the addition of 1 ml of C$_2$H$_5$OH, and the entire reaction mixture applied to sheets of Whatman 3MM paper which were developed overnight in isobutyric acid/NH$_4$OH (5:3), cut into strips and counted. The origin represented the polymer, while the solvent front contained the glycolipids (8).

**TABLE IV**

| Reaction | cpm in lipid fraction |
|----------|-----------------------|
| Experiment 1 | 40,560                |
| Experiment 2 | 28,020                |
| Experiment 3 | 26,910                |

**FIG. 5.** Reaction mixture Total cpm in polymer

| Experiment | Total cpm in polymer |
|------------|----------------------|
| Complete$^a$ | 3,800                |
| Complete + tunicamycin | 3,500            |

$^a$ As described in Table I.
acid/0.5 M NH₄OH (5:3). The areas around the solvent front, Whatman 3MM and chromatographed overnight in isobutyric times. The entire reaction mixtures were applied to strips of dTDP-Rha and the Percoll-60 cell wall fraction for various 

GL[14C]GlcNAc in the presence of 0.1 mM UDP-Gal and 0.05 mM under these conditions, membranes were incubated with UDP-

NAc present in GL 1/GL 2 into this polymer was observed (Table II).

In order to examine the nature of the polymer synthesized under these conditions, membranes were incubated with UDP-[14C]GlcNAc in the presence of 0.1 mM UDP-Gal and 0.05 mM dTDP-Rha and the Percoll-60 cell wall fraction for various times. The entire reaction mixtures were applied to strips of Whatman 3MM and chromatographed overnight in isobutyric acid/0.5 M NH₄OH (5:3). The areas around the solvent front, corresponding to GL 2/GL 3 were excised and counted. Likewise, the material at the origin, the cell wall polymer, was counted. Incorporation into both populations was linear over the course of the experiment (Fig. 4). Thus, the kinetics were more reminiscent of the relationship between the dolichol-bound oligosaccharide precursors and the core region of yeast mannoproteins (36), which also involves a GlcNAc-containing (chitobiose) linkage (37), than that of the simpler mycobacterial polyprenol-P-Man precursors and mycobacterial mannan (24, 34), indicative of a greater similarity to yeast mannoprotein synthesis (37). The labeled polymer was hydrolyzed, subjected to cellulose TLC and autoradiography as described for GL 1/GL 2. Only GlcNH₂ was present; there was no evidence for synthesis of muramic acid and hence of peptidoglycan, under these conditions. Application of the approach (Fig. 3 and Ref. 3) used to identify the Rha(1–3)-[14C]GlcNAc linkage region in the GL 1/GL 2 mixture produced the radiolabeled disaccharide from the polymer.

Higher Glycolipid Intermediates—The addition of the cell wall enzyme preparation (Percoll-60) to the standard reaction mixture resulted in a slight inhibition of incorporation of [14C]GlcNAc into lipids; certainly there was no enhancement of activity as was expected (Table III). However, the presence of Percoll-60 in the assays had a dramatic qualitative effect on the profile of glycolipids synthesized in that TLC showed the emergence of other new, more polar glycolipids, GL 3 and GL 4 (Fig. 5). These new products were similar to GL 1 and GL 2 in terms of acid lability and alkaline stability, and thus it seemed likely that the higher GL 3 and GL 4 were more glycosylated, specifically galactosylated, versions of GL 1 and GL 2. To further prove the point and identify the nature of the new glycosyl substituents, a series of reactions were installed con-

taining membranes, the Percoll-60 cell wall fraction, cold nucleotide sugars, and UDP-[14C]GlcNAc, TDP-[14C]Rha, or UDP-[14C]Gal. The lipids were extracted, treated with alkali, and subjected to TLC and autoradiography (Fig. 6). The effects of the new adducts (Percoll-60 and higher concentrations of all likely nucleotide sugar precursors) to this reaction mixture were decidedly obvious in the UDP-[14C]GlcNAc-containing assay with the clear-cut emergence of the higher glycolipid homologs, GL 3 and GL 4 (Fig. 6, lane 1). These higher homologs were also faintly evident in the [14C]Rha labeling experiment (lane 2).

However, the inclusion of UDP-[14C]Galp in the assay had the most dramatic effect: Only GL 3 and GL 4 and material at the origin, perhaps higher homologs, became labeled, indicating growth of the galactan chain on the polyprenol-P-P-Glc-

Nac-Rha unit. The lipid products from these three reactions were subjected to mild acid hydrolysis and the water soluble products chromatographed on paper against the Rha(1–3)-GlcNH₂ standard. Analysis of the derivatized linkage disaccharide (Fig. 3) confirmed the dominance of products with retention times indicative of [14C]Gal-containing tri- and tetrasaccharide.

In order to demonstrate that the [14C]Gal transferred from the UDP-[14C]Galp precursor appeared as [14C]Gal in GL 3 and GL 4, the NaBH₄ reduced, methylated and acetylated [14C]Gal oligosaccharide preparation, as described for the derivatized linkage disaccharide (Fig. 3), confirmed the dominance of products with retention times indicative of [14C]Gal-containing tri- and tetrasaccharide.
biosynthesis of mycobacterial linkage unit

In these experiments, no direct evidence was provided that GL 3 and GL 4 were derived from GL 1/GL 2, i.e., a precursor-product relationship was not demonstrated. In order to generate preliminary evidence to this effect, tubes containing UDP-[\(^{14}\)C]GlcNAc and the standard reaction mixture were incubated at 37 °C for 30 min, followed by the addition of "cold" UDP-Gal as a substrate for further synthesis and more UDP-GlcNAc as a chase. Tubes were then incubated further for variable times (Fig. 8). The emergence of the [\(^{14}\)C]GlcNAc-containing GL 3 and GL 4 and also, apparently, a GL-5 was evident, particularly after the longer incubation periods. The other most distinctive quantitative effect of this form of chase was a steady loss of radioactivity from the total lipid fraction (175,000 cpm/reaction mixture/0.8 mg of protein at 0 chase time (tube 1), compared to 70,000 cpm after the 90 min chase). Over this period, the GL 1/2 combination lost over half of its radioactivity (70,000 compared to 35,000 cpm after 90 min chase). This form of chase was not effective in demonstrating the existence of distinct but analogous linkage units in mycobacteria. Thus, current evidence indicates that the whole mycolylarabinogalactan (mAG) complex of mycobacterial cell walls is covalently linked to peptidoglycan through a crucial structural unit, the linkage unit.

In addition, hydrolysis (2 m CF\(_3\)COOH) of the [\(^{14}\)C]Gal-labeled lipids and analysis by thin-layer chromatography on the Dionex CarboPac PA1 column (a categorical means of distinguishing Gal and Glc) established that all of the lipid radioactivity was in Gal and not in Glc.

In these experiments, no direct evidence was provided that GL 3 and GL 4 were derived from GL 1/GL 2, i.e., a precursor-product relationship was not demonstrated. In order to generate preliminary evidence to this effect, tubes containing UDP-\([^{14}\text{C}]\text{GlcNAc}\) and the standard reaction mixture were incubated at 37 °C for 30 min, followed by the addition of "cold" UDP-Gal as a substrate for further synthesis and more UDP-GlcNAc as a chase. Tubes were then incubated further for variable times (Fig. 8). The emergence of the \([^{14}\text{C}]\text{GlcNAc}\)-containing GL 3 and GL 4 and also, apparently, a GL-5 was evident, particularly after the longer incubation periods. The other most distinctive quantitative effect of this form of chase was a steady loss of radioactivity from the total lipid fraction (175,000 cpm/reaction mixture/0.8 mg of protein at 0 chase time (tube 1), compared to 70,000 cpm after the 90 min chase). Over this period, the GL 1/2 combination lost over half of its radioactivity, and incorporation into GL 3 and GL 4 increased 4-fold and 15-fold, respectively.

**DISCUSSION**

Present success in defining the early stages of mycobacterial cell wall synthesis arose from the realization of chemical, and hence biosynthetic, similarities with the cell walls of Gram-positive bacteria (38). Early chemical studies of Gram-positive cell walls had revealed that teichoic acids released from cell walls by treatment with dilute acid contained a phosphate group esterified at C6 of some of the muramic acid residues in the wall peptidoglycan (7). Subsequent investigation of the ribitol teichoic acid of Staphylococcus aureus demonstrated an attachment to peptidoglycan by a discrete "linkage unit" containing GlcNac-1-P and 2 or 3 glycerol-P residues (39). From detailed analysis of teichoic acid attachments in a wide range of species, it is now clear that linkage units consist of a disaccharide-1-phosphate (N-acetylmannosaminyl-N-acetylglucosamine-1-phosphate) unit with a small number (1–3 depending on the species) of glycerol-P residues attached to the N-acetylmannosamine (ManNac) (10). This unit is attached, in turn, to muramic acid in peptidoglycan through the GlcNac-1-P, while the teichoic acid chain proper, composed of ribitol-P units, is linked through a phosphodiester to the terminal glycerol-P residue of the linkage unit. The structure is thus: \(\text{ribitol-P}_n\cdot\text{glycerol-P}_m\cdot\text{ManNac-GlcNac-1-P-MurNac} \ldots\) (40). Of more direct relevance to this study, linkage units are also involved in cell wall attachment of a polysaccharide in Micrococcus luteus, and of teichoic acids in actinomycete and related bacteria (10). The GlcNac-1-P link is highly susceptible to acid hydrolysis, accounting for the ease of extraction of teichoic acid from the cell wall under acidic conditions and the retention of a phosphate group on muramic acid. The similar susceptibility of the mycobacterial arabinogalactan-peptidoglycan linkage, and the isolation of (Gal\(_f\))\(_3\) Rha-GlcNac units (8) demonstrated the existence of distinct but analogous linkage units in mycobacteria. Thus, current evidence indicates that the whole of the mycolylarabinogalactan (mAG) complex of mycobacterial cell wall is covalently linked to peptidoglycan through a crucial...
disaccharide linker unit attached to the nonreducing terminus of the galactan of mAG in the following arrangement: -α-Gal(α1→4)-α-Rha(1→3)-β-GlcNAc(1→P-6)Mur-N-glycolyl (8). Presumably only the occasional uronic acid residue is so occupied (8). Yet to be resolved is the old evidence that attachments not involving phosphorus also exist (41). The special version of linkage unit formed in mycobacteria also extends to a broad range of Mycobacterium, Rhodococcus, and Nocardia spp. (42, 43).

The type of linkage unit that attaches teichoic acid to peptidoglycan in the Gram-positive cell wall is highly conserved among a wide range of only distantly related species indicating that it confers a significant advantage in either the synthesis or the properties of the cell wall (10). Likewise, we have attributed comparable significance to the mycobacterial linkage unit (38). The initial studies of teichoic acid synthesis in S. aureus demonstrated the key role played by the linkage unit in initiation of new teichoic acid polymer chains (10). A membrane fraction from mechanically disrupted bacteria catalyzed the synthesis of a trace of ribitol teichoic acid from the precursor CDP-ribitol. However, addition of the precursors of the linkage unit, UDP-GlcNAc, and CDP-glycerol, dramatically stimulated teichoic acid synthesis. More detailed investigations, including pulse-radiolabeling experiments, showed that the biosynthetic system catalyzed the incorporation of GlcNAc-1-P from UDP-GlcNAc into a lipid molecule which in turn gave rise to other lipids in the presence of CDP-glycerol. When CDP-ribitol was added, radioactivity from these lipids appeared in the newly synthesized teichoic acid (44). The lipophilic part of the lipids was shown to be an undecaprenol phosphate of the type involved in peptidoglycan synthesis, and the initial transfer to it of GlcNAc-1-P was very sensitive to inhibition by the antibiotic tunicamycin (44). In the case of the M. luteus polysaccharide, the initial biosynthetic reaction is also the tunicamycin-sensitive transfer of GlcNAc-1-P to a polyphosphate carrier lipid (45). Thus, these experiments demonstrated that the first stage in teichoic acid and M. luteus polysaccharide synthesis was the assembly of linkage unit on the polysaccharide-phosphate carrier lipid, and that this linkage unit-lipid then acted as the primer on which the polymer was assembled from CDP-ribitol.

This pioneering work on the biosynthesis of the cell wall of Gram-positive bacteria provided the framework for the present experiments. Based solely on the precedents of teichoic acid and M. luteus polysaccharide synthesis, it was possible to propose the following initial steps for the first stages of mAG synthesis in Mycobacterium: polyisoprenyl-P + UDP-GlcNAc → polyisoprenyl-P-P-GlcNAc + UMP; polyisoprenyl-P-P-GlcNAc + dTDP-Rha → polyisoprenyl-P-P-GlcNAc-Rha + dTDP; polyisoprenyl-P-P-GlcNAc-Rha + UDP-Gal → polyisoprenyl-P-P-GlcNAc-Rha-Gal + UDP; and polyisoprenyl-P-P-GlcNAc-Rha-Gal + UDP-Gal → polyisoprenyl-P-P-GlcNAc-Rha-Gal-Gal + UDP.

The evidence presented in this report that these steps represent the initial events in mycobacterial cell wall biosynthesis is firm, although mostly on comparative radiolabeling experiments, and sensitivity of products to acid, base, and tunicamycin. The paucity of tangible quantities of polyisoprenyl-P-P-GlcNAc (the proposed structure for glycolipid 1) and polyisoprenyl-P-P-GlcNAc-Rha (glycolipid 2) precluded characterization of the polyisoprenyl component. The answer to this question may lie in the isolation of glycolipid 2, which appears present in relatively large, steady state levels. The question of the nature of the polyisoprenyl carrier is an important one, since mycobacteria to date have yielded only decaprenols (24, 25, 27) and heptaprenols (23, 33) as their version of the bactoprenols, but never the common undecaprenol. Subsequent steps in cell wall biogenesis are less well established. UDP-Galp is a very effective substrate for the Galf-containing galactan component of mAG and, according to present results of the apparent glycolipid intermediates. In Salmonella enterica, UDP-Galp is a precursor of the Galf in T1 polysaccharides (46). In Penicillium charlsonii, the Galf of UDP-Galp can be transferred to the polysaccharide galactocarboxyllose (47). Stevenson et al. (48) based on genetic analysis concluded that a single enzyme can convert UDP-Galp to UDP-Galf through a 2-keto intermediate and that or6 is the gene involved. Present results would indicate that this enzyme is membrane-bound. Recently, we described the presence of the rfb (rhamnose biosynthetic) genes close to a new insertion-like element (ISL445) in the genome of M. tuberculosis, which included the 3′-region of the rfbB gene, the whole rfbC gene, and the 5′-region of the rfbA gene; however, the rest of the rfbA gene and the following rfbD gene were not obvious in this region. Accordingly, the genetics and enzymology of mAG-linkage unit synthesis show the promise of unique molecular principles to match the novelty of the biochemical pathway described herein. Thus, this work represents a return to the topic of mycobacterial cell wall biogenesis which proved an intractable problem in previous times, and the assays and intermediates described pave the way for screens for new anti-tuberculosis drugs to counteract the serious problem of drug resistance.

Acknowledgments—We thank Caroline Morehouse for conducting some of the experiments necessary for final acceptance of this manuscript, Marilyn Hein for preparing the manuscript, and Carol Marander for the graphics.

REFERENCES

1. Daffe, M., Brennan, P. J., and McNeil, M. (1990) J. Biol. Chem. 265, 6734–6743.

2. McNeil, M., Daffe, M., and Brennan, P. J. (1991) J. Biol. Chem. 266, 13217–13223.

3. Besra, G. S., Khoo, K.-H., McNeil, M. R., Deli, A., Morris, H. R., and Brennan, P. J. (1995) Biochemistry 34, 4257–4266.

4. Bloch, A. B., Cauthen, G. M., Onorato, I. M., Dansbury, K. G., Kelly, G. D., Driver, C. R., and Snider, D. E., Jr. (1994) Am. Med. Assoc. 271, 665–671.

5. Dessen, A., Quémard, A., Blanchard, J. S., Jacobs, W. R., Jr., and Sacchettini, J. C. (1995) Science 267, 1638–1641.

6. Mikulášová, K., Slavík, R., Besra, G. S., and Brennan, P. J. (1995) Antimicrob. Agents Chemother. 39, 2484–2489.

7. Liu, Y.-Y., and Gotschlich, E. C. (1967) J. Biol. Chem. 242, 471–476.

8. McNeil, M., Brennan, D. A., and Brennan, P. J. (1990) J. Biol. Chem. 265, 18200–18206.

9. Brennan, P. J., and Nikaido, H. (1995) Annu. Rev. Biochem. 64, 29–63.

10. Archibald, A. R., Hancock, I. C., and Harwood, C. R. (1993) in Bacillus subtilis and Other Gram-Positive Bacteria: Biochemistry, Physiology, and Molecular Genetics (Sonenshein, A. L., Hoch, J., and Losick, R., eds) pp. 381–410, American Society for Microbiology, Washington, D.C.

11. Takayama, K., Slavík, R., Besra, G. S., Armstrong, E. L., and Boyle, R. W. (1975) J. Lipid Res. 16, 308–317.

12. Wheeler, P. R., Besra, G. S., Minnikin, D. E., and Ratledge, C. (1993) Biochim. Biophys. Acts 1167, 182–188.

13. Okazaki, R., Okazaki, T., Strominger, J. L., and Michelson, A. M. (1962) J. Biol. Chem. 237, 3014–3026.

14. McNeil, M., Chattarjee, D., Hunter, S. W., and Brennan, P. J. (1989) Methods Enzymol. 179, 215–242.

15. McArthur, H. A. I., Hancock, I. C., Roberts, F. M., and Baddiley, J. (1980) FEBS Lett. 111, 317–323.

16. Herschfield, G. R., McNeil, M., and Brennan, P. J. (1990) J. Bacteriol. 172, 1005–1013.

17. Lundblad, A., Masson, P. K., Norden, N. E., Svensson, S., and Oederman, P. A. (1975) Biochem. Mass Spectrom. 2, 289–287.

18. Felch, J., Lees, M., and Stanlie-Stanley, G. H. (1957) J. Biol. Chem. 226, 497–509.

19. Dittmer, J. C., and Lester, R. L. (1964) J. Lipid Res. 5, 126–130.

20. Dunphy, P. T., Kerr, T. D., Pennock, T. F., and Whittle, K. T. (1966) J. Chem. Ind. 1549–1550.

21. Yamamori, S., Murazumi, N., Araki, Y., and Ito, E. (1978) J. Bacteriol. 131, 616–622.

22. Dawson, R. M. C., Elliott, D. C., Elliott, W. H., and Jones, K. M. (1959) in Data for Biochemical Research pp. 470–475, Clarendon Press, Oxford, UK.

23. McNeil, M., Tsang, A. Y., and Brennan, P. J. (1987) J. Biol. Chem. 262, 2630–2635.

24. Takayama, K., and Goldman, D. S. (1970) J. Biol. Chem. 245, 6251–6257.

25. P. J. Brennan and G. S. Besra, unpublished data.

26. T.-Y. Lee, T.-J. Lee, J. T. Belisle, P. J. Brennan, and S.-K. Kim, unpublished data.
25. Takayama, K., Schnoes, H. K., and Semmler, E. J. (1973) Biochim. Biophys. Acta 163, 212–221
26. Schultz, J., and Elben, A. D. (1974) Arch. Biochem. Biophys. 160, 311–322
27. Wolucka, B. A., McNeil, M. R., de Hoffmann, E., Chojnacki, T., and Brennan, P. J. (1994) J. Biol. Chem. 269, 23328–23335
28. Takatsuni, A., Kono, K., and Tamura, G. (1975) Agric. Biol. Chem. 39, 2089–2091
29. Kno, S. C., and Lampden, J. O. (1974) Biochem. Biophys. Res. Commun. 58, 287–295
30. Ward, J. B. (1977) FEBS Lett. 78, 151–154
31. Schnaitman, C. A., and Klena, J. D. (1993) Microbiol. Rev. 57, 655–682
32. Aspinall, G. O., Chatterjee, D., and Brennan, P. J. (1995) Adv. Carbohydr. Chem. Biochem. 51, 169–242
33. Beira, G. S., Sievert, T., Lee, R. E., Slayden, R. A., Brennan, P. J., and Takayama, K. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12735–12739
34. Schultz, J. C., and Takayama, K. (1975) Biochim. Biophys. Acta 381, 175–184
35. Ciucanu, I., and Kerek, F. (1984) Carbohydr. Res. 131, 209–217
36. Lehle, L. (1980) Eur. J. Biochem. 109, 589–601
37. Ballou, C. E. (1976) Adv. Microbial Phys. 14, 93–158
38. McNeil, M. R., and Brennan, P. J. (1991) Res. Microbiol. 142, 451–463
39. Coley, J., Tarelli, E., Archibald, A. R., and Baddiley, J. (1978) FEBS Lett. 88, 1–9
40. Kojima, N., Araki, Y., and Ito, E. (1983) J. Biol. Chem. 258, 9043–9045
41. Kanetsuna, F., and San Blas, G. (1970) Biochim. Biophys. Acta 208, 434–443
42. Fujioka, M., Koda, S., and Morimoto, Y. (1985) J. Gen. Microbiol. 131, 1323–1329
43. Daffe, M., McNeil, M., and Brennan, P. J. (1993) Carbohydr. Res. 249, 383–398
44. Hancock, I. C., Wiseman, G., and Baddiley, J. (1976) FEBS Lett. 69, 75–80
45. Kojima, N., Araki, Y., and Ito, E. (1983) J. Bacteriol. 161, 299–306
46. Sarvas, M., and Nikaido, H. (1971) J. Bacteriol. 105, 1063–1072
47. Trejo, A., Chittenden, G. J. F., Buchanan, J. G., and Baddiley, J. (1970) Biochem. J. 117, 637–639
48. Stevenson, G., Neal, B., Liu, D., Hobbs, M., Packer, N. H., Batley M., Redmond, J. W., Lindquist, L., and Reeves, P. (1994) J. Bacteriol. 176, 4144–4156
