Recognition of Multiple Effects of Ethambutol on Metabolism of Mycobacterial Cell Envelope

LINGYI DENG,[†] KATARÍNA MIKUŠOVÁ, KIMBERLY G. ROBUCK, MICHAEL SCHERMAN,
PATRICK J. BRENNAN, AND MICHAEL R. MCNEIL*

Department of Microbiology, Colorado State University, Fort Collins, Colorado 80523

Received 12 October 1994/Returned for modification 21 November 1994/Accepted 3 January 1995

Ethambutol is known to rapidly inhibit biosynthesis of the arabinan component of the mycobacterial cell wall core polymer, arabinogalactan (K. Takayama and J. O. Kilburn, *Antimicrob. Agents Chemother.* 33:1493–1499, 1989). This effect was confirmed, and it was also shown that ethambutol inhibits biosynthesis of the arabinan of lipoarabinomannan, a lipopolysaccharide noncovalently associated with the cell wall core. In contrast to cell wall core arabinan, which is completely inhibited by ethambutol, synthesis of the arabinan of lipoarabinomannan was only partially affected, demonstrating a differential effect on arabinan synthesis in the two locales. Further studies of the effect of ethambutol on cell wall biosynthesis revealed that the synthesis of galactan in the cell wall core is strongly inhibited by the drug. In addition, ethambutol treatment resulted in the cleavage of arabinosyl residues present in the mycobacterial cell wall; more than 50% of the arabinan in the cell wall core was removed from the wall 1 h after addition of the drug to growing mycobacterial cultures. In contrast, galactan was not released from the cell wall during ethambutol treatment. The natural function of the arabinosyl-releasing enzyme remains unknown, but its action in combination with inhibition of synthesis during ethambutol treatment results in severe disruption of the mycobacterial cell wall. Accordingly, ethambutol-induced damage to the cell wall provides a ready molecular explanation for the known synergetic effects of ethambutol with other chemotherapeutic agents. Nevertheless, the initial direct effect of ethambutol remains to be elucidated.

Ethambutol [(S,S)-2,2'-(ethylenediimino)di-1-butanol] (1, 2) is an important antimycobacterial drug. It is effective against tuberculosis, and it is often administered in combination with isoniazid for the treatment of tuberculosis (32, 33). This drug also shows some efficacy against *Mycobacterium avium*, especially when it is used in combination with other drugs (11, 13, 14, 18, 19, 27, 37).

The direct site of action of ethambutol is unknown. Ethambutol is specific to the mycobacteria, and thus, all other bacteria, fungi, and yeasts are insusceptible to the drug (33). Early mode-of-action studies addressed the issue of inhibition of nucleic acid metabolism (9), but the lack of early effects on [³H]thymidine incorporation into DNA (20) ruled out DNA replication as the primary target. The data with respect to RNA metabolism remain more confusing (33).

In an elegant series of experiments, Takayama and Kilburn (30) suggested that the drug inhibits arabinan biosynthesis in the cell wall. Since mycobacteria have a unique cell wall structure, this conclusion is consistent with the specificity of the drug for mycobacteria. Initially, Kilburn and Greenberg (20) showed that ethambutol treatment caused rapid bacterial de-clumping and parallel morphological changes in *Mycobacterium smegmatis*. These results were consistent with some type of cell wall change. Suspecting a reduction in the amount of lipids in the cell wall, Takayama et al. (29) demonstrated that ethambutol inhibited the transfer of mycolic acids to the cell wall. Consistent with this result was the observation that ethambutol induced accumulation of trehalose monomycolate, trehalose dimycolate, and free mycolic acids in the culture

medium (21). Suspecting that the mycolate was not transferred into the wall because of a lack of arabinan acceptor, Takayama and Kilburn (30) demonstrated that the in vivo conversion of [¹⁴C]glucose into cell wall arabinan was inhibited almost immediately after addition of the drug. They thus concluded that the primary mode of action of ethambutol was inhibition of arabinan synthesis in the cell wall.

Recent experiments by Silve et al. (28) showed that the effects of ethambutol could be overcome with specific monosaccharides. These experiments were interpreted (28) to suggest that the drug might inhibit synthetic reactions early in the pathway of arabinan or other glycan biosynthesis, although, as discussed below, other evidence suggests that the inhibition is later in the pathway.

The cell wall of mycobacteria consists (23) of an outer lipid layer of tightly associated mycolic acids covalently connected to an inner peptidoglycan (12) layer via the arabinan-containing polysaccharide arabinogalactan (AG). It is reasonable that a molecule that interferes with the synthesis of the connecting polysaccharide, AG, should function as a drug against the bacteria, and as an inhibitor of arabinan synthesis, ethambutol does just that. Much of the primary structure of the arabinan component of AG has recently been elucidated in our laboratory (7, 24, 25). The structure is summarized in Fig. 1. The arabinan is a complex branched polysaccharide with mycolic acids attached as illustrated in Fig. 1 to the nonreducing end pentaarabinofuranoside [β -D-Araf-(1 $\rightarrow$ 2)- α -D-Araf-(1 $\rightarrow$)]₂-3&5)- α -D-Araf units (25). Arabinan is attached at its reducing end to a galactofuranose-containing galactan, which is finally attached to the peptidoglycan via the actinomycete-specific linker disaccharide [$\rightarrow$ 4-Rha-(1 $\rightarrow$ 3)-GlcNAc-(1 $\rightarrow$ phosphate)] (24). Mycobacteria contain a second arabinan-containing polymer, lipoarabinomannan (LAM) (3, 5, 6, 16), which is believed to be primarily anchored in the cell membrane through its

* Corresponding author. Phone: (303) 491-1784. Fax: (303) 491-1815.

[†] Present address: Channing Laboratory, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115.

FIG. 1. Structure of the mycobacterial cell wall AG. The reducing end (the innermost part) of AG is attached to peptidoglycan via the reducing disaccharide (Rha→GlcNAc); only a single residue of the peptidoglycan, a muramic acid (Mur) unit, is illustrated. On the other end of the AG are the mycolic acids. The figure is not meant to illustrate three-dimensional relationships, and thus, in the actual cell wall the mycolic acids are strongly associated with each other to form an outer lipid layer, the peptidoglycan forms an inner layer, and the two layers are connected by AG. The site of action of isoniazid (INH) on mycolic acids and of ethambutol on arabinan and the product shown to build up after ethambutol treatment, trehalose monomycolate (21), are illustrated.

phosphatidylinositol substituent and therefore is not covalently associated with the cell wall core (17, 23). The structure of the arabinan of LAM (Fig. 2) is similar to that of the arabinan found in cell wall core AG (4).

Results on the effects of ethambutol on the biosynthesis of the two arabinans and of galactan are presented here. Ethambutol shows a differential effect on the synthesis of the two forms of arabinan and strongly inhibits galactan biosynthesis. Unexpectedly, ethambutol also stimulates the enzymatic release of arabinan from the cell wall core, pointing to widespread, rapid, and dramatic effects on general cell wall integrity.

MATERIALS AND METHODS

Materials and microorganisms. Radiolabelled compounds were obtained as follows: [U-¹⁴C]glycerol (44 mCi/mmol) was from New England Nuclear (Boston, Mass.), [U-¹⁴C]glucose (242 mCi/mmol) was from ICN Radiochemicals (Irvine, Calif.), and [1-¹⁴C]ribose (50 mCi/mmol) was from American Radiolabelled Chemicals, Inc. (St. Louis, Mo.). Ethambutol was obtained from Sigma Chemical Company (St. Louis, Mo.). All other chemicals used were of reagent grade. *M. smegmatis* mc²155, which was obtained from William R. Jacobs, Albert Einstein College of Medicine of Yeshiva University, Bronx, N.Y., was used, except that in the glycerol labeling experiment (see Table 2), which was done early in the study, the parent of this strain, namely, *M. smegmatis* 607 (American Type Culture Collection, Rockville, Md.), was used; this slight difference in strains should not affect the results. *M. avium* serovar 2 strain 2151 (clinical isolate from sputum obtained from Anna Tsang, National Jewish Center for Immunology and Respiratory Disease, Denver, Colo.) was also used.

Analytical techniques. The phenol-sulfuric acid assay for carbohydrate was performed as described previously (8). To determine radioactive glycosyl com-

positions, samples were hydrolyzed for 1 h at 120°C in 2 M trifluoroacetic acid. Cell wall hydrolysates were partitioned between CHCl₃ and H₂O (1:1); mycolic acids appear in the CHCl₃ phase, and monosaccharides appear in the water phase. The monosaccharides were separated by thin-layer chromatography (TLC) on Baker-flex cellulose plastic plates (J. T. Baker, Phillipsburg, N.J.), which were developed four times in formic acid-water-tertiary butanol-methyl ethyl ketone (15:15:40:30). Autoradiograms were produced by exposure of the resulting TLC plates to Kodak X-Omat AR film at -70°C. Radioactive bands were scraped from the TLC plate and were counted by liquid scintillation. Standards of 200 nmol each of ribose, arabinose, mannose, glucose, and galactose were run on each plate and were visualized by spraying with phthalic acid-*n*-butanol-aniline reagent (15) and subsequent heating at 120°C for 10 min. Alternatively, the radioactive monosaccharides released by the acid treatment were assayed by high-pressure liquid chromatography (HPLC; Dionex, Sunnyvale, Calif.) with an advanced-gradient pump with pulsed amperometric detection and a Dionex CarboPac PA1 column (4 by 250 mm) eluted with 15 mM NaOH. Fractions were collected and counted by the scintillation procedure, and retention times were compared with those of the standards. Nonradioactive glycosyl compositions were determined by gas chromatography of alditol acetate derivatives as described previously (36). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (22) and electrophoretic transfer of lipomannan (LM) and LAM to nitrocellulose were performed as described previously (17). Autoradiograms of LAM and LM were obtained by exposing the nitrocellulose to Kodak X-Omat AR film at -70°C.

Bacterial growth and ethambutol treatments. For the representative growth curve, *M. smegmatis* mc²155 was grown in multiple 50-ml Erlenmeyer flasks containing 10 ml of glycerol-alanine-salts (GAS) medium (31) at 37°C on a rotary shaker. Ethambutol at 10 µg/ml was added to all of the drug-treated cultures at the early log phase (10 h after inoculation). At various times, flasks containing control cells or ethambutol-treated cells were harvested by centrifugation, lyophilized, and weighed.

Long-term effects of ethambutol on cell wall and LM and LAM. The long-term effects of ethambutol on AG and LAM biosynthesis in the cell wall were both determined in a single labelling experiment. Cultures of *M. smegmatis* mc²155

FIG. 2. Structure of the mycobacterial polysaccharide LAM. The arabinan region is similar but not identical to that found in AG, and in LAM the arabinan is attached to a mannan chain which is in turn attached to a phosphatidylinositol lipid anchor. The polysaccharide LM is essentially the mannan and phosphatidylinositol lipid anchor part of the molecule without the arabinan.

were inoculated into 50-ml Erlenmeyer flasks containing 10 ml of GAS medium. At the time (10 h) of ethambutol addition (10 μ g/ml), 10 μ Ci of [14 C]glucose was added to both control and drug-treated cells. They were further incubated for 20 h, harvested, washed twice with 0.9% (wt/vol) aqueous NaCl and once with distilled water, and lyophilized. Lyophilized cells were suspended in distilled water (10 mg/ml) and were subjected to probe sonication. Six volumes of CHCl_3 - CH_3OH (2:1) was added to the sonicate, resulting in an organic phase, an aqueous phase, and insoluble material at the interphase. The insoluble material and the aqueous phase were combined and refluxed in 50% (vol/vol) aqueous ethanol (1 ml/10 mg of starting cell dry weight) for 2 h three times, resulting in a 50% aqueous ethanol-soluble fraction containing LM and LAM and an insoluble fraction containing the cell wall. The LM-LAM fraction was dried and partitioned between hot phenol and water as described previously (17), resulting in a partially purified LM-LAM fraction. The insoluble cell wall fraction was further extracted with SDS as described previously (17).

Short-term effects of ethambutol on cell wall AG. To determine the short-term effects of ethambutol on cell wall AG biosynthesis, *M. smegmatis* 607 was grown to the mid-log phase (A_{600} , ~0.4) in 10 ml of GAS medium (31). The bacteria were harvested by centrifugation and were washed two times in carbon-free medium (GAS medium without glycerol, casein enzyme hydrolysate, alanine, or MgCl_2). The cells were then incubated for 15 min in 2 ml of carbon-free medium, after which 5 μ Ci of [14 C]glycerol was added alone to control cells and along with 10 μ g of ethambutol per ml to the drug-treated cells. The reaction was terminated at appropriate times by the addition of 10 μ l of nonradioactive glycerol and chilling to 4°C in an ice bath. The tubes were then centrifuged, the supernatants were discarded, and the cell pellets were washed with water three times. The cell pellets were taken up in 0.5 ml of buffer (10 mM Tris-HCl [pH 8.4] containing 0.2 M NaCl, 1 mM EDTA, 2 mg of NaN_3 per ml, and 2.5 mg of deoxycholate per ml) and were sonicated with a microprobe sonicator (Heat Systems-Ultrasonic, Farmington, N.Y.). The cell wall pellets were recovered by centrifugation and were washed two times with buffer at room temperature and two times with water to remove LAM and other soluble arabinose- or galactose-containing products. The pellets were then hydrolyzed with 250 μ l of 2 M TFA at 120°C for 0.5 h. The resulting monosaccharides were separated and counted by using the Dionex system as described above.

Effects of ethambutol on glycosyl composition of the cell wall. Two flasks containing 100 ml of medium (GAS medium plus 0.05% Tween 80) were inoculated with *M. smegmatis* 607, and the organisms were allowed to grow to an A_{600} of 0.45 (mid-log phase) at 37°C. Ethambutol at 10 μ g/ml was added to one of the flasks, and the contents of both flasks were harvested after 7 h of incubation. In the case of *M. avium*, flasks containing 7H11 medium (Difco Laboratories, Detroit, Mich.) were inoculated with strain 2151, and the organisms were allowed

to grow to an A_{600} of 0.45 (48 h). Ethambutol was then added to one of the flasks, and the contents of both flasks were harvested after a further 7 h of incubation. Cell walls were isolated, and arabinose and galactose were analyzed as described previously (7, 10).

Analysis of material released into the culture medium. *M. smegmatis* mc²155 cells were grown (A_{600} , 0.50) in GAS medium, and 1 ml of this culture was added to 10 ml of GAS medium without glycerol (resulting in a net 10-fold reduction in the glycerol content). [14 C]D-ribose (1 μ Ci) was added, and the cells were grown for 4 h. Nonradioactive D-ribose was added to a concentration of 5 mM. The cells were allowed to grow for 2 h, recovered by centrifugation, washed four times with phosphate-buffered saline (PBS), resuspended in PBS with and without ethambutol (20 μ g/ml), and incubated at 37°C for 4 h, and the medium was recovered by centrifugation and assayed for radioactivity. For further analysis of the released products, the concentrated culture media were treated with 4 volumes of CHCl_3 - CH_3OH (2:1), and the aqueous and organic fractions were analyzed for their radioactivities and radioactive sugar compositions.

Preparation of radioactive cell walls. *M. smegmatis* mc²155 was grown in GAS medium (A_{600} , 0.50), and 10 ml of this culture was transferred into 100 ml of glycerol-free GAS medium. After 1 h, 100 μ Ci of [14 C]glucose was added and the cells were grown for 13 h. Nonradioactive glucose was added to a concentration of 40 mM. The cells were allowed to grow for 4 h, harvested, autoclaved, centrifuged, and washed with PBS containing 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). Cell walls were prepared by sonication in PBS containing 0.5% CHAPS; this was followed by extensive washing in PBS containing 0.5% CHAPS until the supernatants were free of radioactivity (~20 washes were necessary).

Preparation of crude mycobacterial enzymes. Cells (*M. smegmatis* mc²155) in the logarithmic phase of growth were harvested, frozen in liquid nitrogen, and ground in a prechilled mortar and pestle in a buffer composed of 20 mM Tris-HCl (pH 7.6) containing 20 mM magnesium acetate, 0.05% CHAPS (a detergent), 0.1% Thesit (a detergent), 5% glycerol, 0.5 mM EDTA, 1 mM 2-mercaptoethanol, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM ATP, 0.1 mM NADPH, and 0.2 mM (each) UTP, GTP, CTP, and TTP. The preparation (7 mg of protein per ml) was divided into aliquots and was kept frozen at -70°C, thawed, and used directly or centrifuged at 28,000 $\times$ g to provide pelleted cell wall protein and cytosol-membrane preparations.

Assay of arabinolytic activity. Radioactive cell walls (~400,000 cpm) were incubated at 30°C with 1 ml of the appropriate mycobacterial enzyme or boiled enzyme preparations sometimes supplemented with 50 or 100 μ g of ethambutol per ml. After 95 min, the reaction mixtures were centrifuged for approximately 5 min at 5,000 $\times$ g and were washed with buffer. The original supernatant, wash, and remaining pellet were counted, and the amount of material released by the

FIG. 3. Effect of ethambutol on the growth of *M. smegmatis*. The weight at each time point was determined from an individual flask. Ethambutol was added at 10 μ g/ml at 10 h to flasks used for the drug-treated cells as indicated. At the indicated times, the cells were harvested, lyophilized, and weighed.

enzyme was calculated. In an experiment performed to analyze the released material for its radioactive glycosyl composition, 400,000 cpm of cell walls was treated with 1 ml of unfractionated mycobacterial enzyme and 100 μ g of ethambutol per ml for 1 h at 30°C. The reaction mixture was centrifuged, and the supernatant was desalted on a Sephadex G-10 column, hydrolyzed with 2 M TFA, and analyzed for radioactive sugars by HPLC on a Dionex column.

RESULTS

Selection of experimental conditions. The effect of ethambutol on mycobacteria is dependent not only on the concentration of drug but also on the density of the bacterial population (20). Much of the work of Takayama and colleagues (20, 21, 29, 30) was done at a low bacterial density (A_{600} , 0.025) (20) to try to represent published MIC conditions. However, since many of the studies presented here involved the actual isolation of various components, most experiments were conducted with larger amounts of bacteria and thus necessarily higher drug concentrations. A typical growth curve of *M. smegmatis* mc²155 with and without ethambutol (10 μ g/ml) is presented in Fig. 3. The point of addition of ethambutol represents culture absorption at an A_{600} of about 0.20, although clumping makes an accurate measurement difficult. Thus, ethambutol strongly inhibited growth under the conditions used, but a small increase in the bacterial dry weight still occurred during drug treatment.

Ethambutol treatment inhibits the biosynthesis of the entire AG. Takayama and Kilburn (30) showed that ethambutol inhibits cell wall core arabinan synthesis in less than a few minutes. Radiolabel supplied as [¹⁴C]glucose, under their conditions, was readily incorporated into cell wall arabinan but not into cell wall galactan. Using a different labelling protocol in which the ¹⁴C label from [¹⁴C]glucose was incorporated into both arabinan and galactan, we first investigated the long-term effects of ethambutol treatment on the biosynthesis of both arabinan and galactan. Thus, bacteria were grown in the presence of [¹⁴C]glucose (Fig. 3), the cells were harvested 20 h

TABLE 1. Incorporation of ¹⁴C from [¹⁴C]glucose into cell wall fractions in the presence and absence of ethambutol

Treatment	cpm/10 ml of culture		
	Total cell wall ^a	Mycolic acid fraction ^b	Carbohydrate fraction ^b
Control	1,148,000	252,000	896,000
Ethambutol	68,060	12,600	55,460

^a Cells (*M. smegmatis* mc²155) were harvested and cell walls were purified after cell disruption by sonication as described in Materials and Methods. An aliquot of the cell walls was counted, and the total radioactivity present in the cell walls present in the entire flask was calculated.

^b The mycolic acid fraction is defined as the organic soluble radioactivity present after partitioning a 2 M TFA hydrolysate of the cell wall in CHCl₃-H₂O (1:1). The carbohydrate fraction is defined as the aqueous soluble radioactivity.

later, and cell walls were prepared. The radioactivity present in the total cell wall was reduced to 6% of that of the control (Table 1), indicating that ethambutol resulted in almost the complete shutdown of the biosynthesis of the entire cell wall, thus including peptidoglycan, mycolic acids, and AG. An aliquot of the cell wall was hydrolyzed and separated into organic (mycolic acid-containing) and aqueous (AG and peptidoglycan-containing) fractions. The results (Table 1) reflected those for the entire wall; biosynthesis of both lipid and nonlipid components was strongly inhibited by the drug. When equal amounts of aqueous radioactivity from acid hydrolysis of ethambutol-treated and control cell walls were analyzed by TLC (Fig. 4B), it was seen that very little arabinan was synthesized. Calculations (Table 2) revealed that ethambutol caused an inhibition of the galactan biosynthesis to 5% of that of the control, whereas arabinan synthesis was inhibited to 1% of that of the control. Thus, after drug treatment essentially no arabinan and very little galactan were synthesized.

Similar results were obtained in an experiment to measure the immediate effects of ethambutol on AG biosynthesis. In

FIG. 4. (A) Autoradiogram of TLC analysis of the radioactive sugars present in the hydrolysate of the LAM-LM fraction prepared from control (lane 1) and ethambutol-treated (lane 2) *M. smegmatis*. (B) Autoradiogram of TLC analysis of the radioactive sugars present in the hydrolysate of the cell walls from control (lane 3) and ethambutol-treated (lane 4) *M. smegmatis*. In all cases (lanes 1 to 4), 50,000 cpm was applied to the cellulose TLC plate. The TLC plate was developed as described in Materials and Methods. The migration positions of the sugar standards under the conditions used are indicated with arrows. Rib, ribose; Ara, arabinose; Man, mannose; Glc, glucose; Gal, galactose.

TABLE 2. Incorporation of ^{14}C from $[^{14}\text{C}]$ glucose or $[^{14}\text{C}]$ glycerol into the galactose and arabinose components of the cell wall of ethambutol-treated and control cells^a

Treatment	Treatment time (min)	Source of ^{14}C	cpm/10-ml flask	
			Galactose-glucose ^b	Arabinose
Control	5	$[^{14}\text{C}]$ gro	52	122
	20	$[^{14}\text{C}]$ gro	311	771
	1,200	$[^{14}\text{C}]$ glucose	168,860	134,632
Ethambutol	5	$[^{14}\text{C}]$ gro	ND ^c	ND
	20	$[^{14}\text{C}]$ gro	38	ND
	1,200	$[^{14}\text{C}]$ glucose	8,790	1,694

^a Cells were *M. smegmatis* 607 for 5 and 20 min and mc²155 for 1,200 min.

^b Galactose and glucose were not well separated by TLC (Fig. 4B), and thus, the radioactivity represents the sum of both sugars in the case of the 1,200-min treatment time. Cell walls do not contain glucose but may be contaminated with small amounts of glucan. Examination of Fig. 4B suggests that most of the radioactivity is in galactose.

^c ND, no radioactivity above the background was detected.

that experiment, $[^{14}\text{C}]$ glycerol was added simultaneously with ethambutol and incorporation was terminated by the addition of nonradioactive glycerol and chilling of the cells. Cells were then harvested, cell walls were prepared and hydrolyzed, and the radioactivity present in arabinose and galactose was determined by HPLC on a Dionex column. The data presented in Table 2 indicate that arabinan biosynthesis was completely inhibited by 5 min, supporting the data of Kilburn and Takayama (21). At 5 min after drug treatment there was no evidence of galactan biosynthesis, but under the experimental conditions used, small amounts of galactan synthesis would be missed because of the low level of incorporation even into control cells. At 20 min, a clear although not complete inhibition of galactan biosynthesis was seen, consistent with the long-term results reported in Table 2. These cell wall-labeling results are most readily interpreted in terms of arabinan biosynthesis in the cell wall being the initial target of ethambutol and the biosynthesis of AG is controlled in such a way that when arabinan biosynthesis is inhibited the formation of the entire mycolylarabinogalactan is unable to proceed efficiently.

Inhibition of biosynthesis of the arabinan component of LAM. Mycobacteria contain two closely related phosphatidyl-inositol-anchored polysaccharides, LAM and LM (5, 16, 17); LAM is an arabinosylated version of LM (Fig. 2). Therefore, if ethambutol inhibits the synthesis of cell wall-bound arabinan, that in LAM should also be affected. Thus, a fraction rich in LAM-LM was extracted from control and ethambutol-treated cells 20 h after the addition of label. The total radioactivity in the fraction from control cells was 2.45×10^6 (10 ml of culture), whereas it was 1.15×10^6 in ethambutol-treated cells, a decrease of 53% in the biosynthesis of polysaccharides present in the LAM-LM fraction compared with a decrease of 94% in mycolylarabinogalactan synthesis (Table 1). Equal amounts of radioactive LAM-LM were applied to polyacrylamide gels, and autoradiography was used to examine the differential effects on LAM and LM (Fig. 5). Ethambutol caused a marked, although not complete, decrease in the amount of newly synthesized LAM, while the synthesis of LM was not strongly affected. Hydrolysis of the $[^{14}\text{C}]$ LAM- $[^{14}\text{C}]$ LM fraction and then TLC showed radioactivity in inositol, mannose, arabinose, glucose, and ribose (Fig. 4A). The counts in glucose and ribose arise from glucosyl- and ribosyl-containing polymers, respectively, which contaminate the LAM-LM preparation. As expected from the results presented in Fig. 5, the amount of label in

FIG. 5. Autoradiogram of SDS-PAGE analysis of the LAM-LM fractions of $[^{14}\text{C}]$ glucose-labeled *M. smegmatis* from the control experiment (lane 1) or ethambutol-treated *M. smegmatis* (lane 2). A total of 50,000 cpm was loaded onto each lane. After electrophoresis, the gels were electrotransferred to nitrocellulose and autoradiography was performed.

arabinose was substantially decreased after drug treatment (Fig. 4A). The radioactive spots of the thin-layer chromatograms shown in Fig. 4A were cut out and counted. The amounts of mannose and arabinose synthesized per 10 ml of culture in the LAM-LM fraction were then calculated and were 42,078 and 57,660 cpm, respectively, for control treatments and 22,205 and 13,655 cpm, respectively, for ethambutol treatments. These results show that ethambutol inhibits mannan synthesis to about 50% of that of the control, in contrast to arabinan synthesis, which was slowed to about 23% of that of the control. The results were surprising in that they indicated a differential effect of ethambutol on the synthesis of the arabinan components of AG and LAM.

Effect of ethambutol on arabinan synthesized before drug treatment. In the experiments described above (e.g., Fig. 3), *M. smegmatis* underwent about three generations of replication prior to the addition of ethambutol. Accordingly, cell wall composition, as distinct from de novo synthesis, should not be drastically altered. Yet it was found that the ratio of arabinose to galactose was shifted from 1.90 to 0.83 after 20 h of ethambutol treatment. In *M. avium* the relationship changed from 1.80 to 0.70 after 30 h of ethambutol treatment. Since no synthesis of galactan occurred (Table 2), these results suggest that ethambutol, at least under conditions of prolonged treatment, stimulates the degradation of arabinan. Further analysis of this relationship (Fig. 6) showed that the ratio of arabinose to galactose stayed fairly constant for control cells over time, but in the case of ethambutol-treated cells, it dropped quite rapidly, to near unity in 1 h and to 0.5 at the later time points.

Examination of the carbohydrate contents of culture media from control and ethambutol-treated cells showed a 2.4-fold increase in soluble and released carbohydrate in ethambutol-treated cells over that in control cells. To determine the identity of this excess carbohydrate, *M. smegmatis* was labeled primarily in the arabinosyl residues by metabolic labelling with $[^{14}\text{C}]$ ribose, and the release of radioactivity by these cells in the presence and absence of ethambutol was determined 4 h after

FIG. 6. Glycosyl compositions of the cell walls of *M. smegmatis* (normalized mole percent) at different times after treatment with and without drug. Cells were grown as described in the legend to Fig. 3. The bacteria were harvested, and the cell walls were isolated and analyzed for carbohydrates by gas chromatography at the indicated times. (A) Group in which no ethambutol was added. (B) As for panel A, except that at 10 h ethambutol was added at 50 µg/ml. ▨, arabinose; □, galactose; ▤, N-acetylglucosamine (a constituent of peptidoglycan).

transfer to PBS. The release of radioactivity was found to be stimulated by ethambutol; 13,500 cpm was released by the control cells (10 ml of culture), whereas 35,000 cpm was released by the drug-treated cells. The released radioactivity was further fractionated by CHCl_3 - CH_3OH -water (8:4:1) into organic and aqueous fractions. About 25% of the radioactivity from the drug-treated cells was found in the organic fraction, and upon hydrolysis, drug-treated cells yielded mostly radioactive arabinose and, to a lesser extent, glucose, pointing to the preferential release of mycolylarabinan from ethambutol-treated cells. The material in the aqueous fraction in the culture filtrate from ethambutol-treated cells mostly contained arabinose.

The evidence that ethambutol stimulates the release of both lipidated and nonlipidated arabinose-containing products from *M. smegmatis* prompted a search for the enzymatic activity. Uniformly labeled cell walls were prepared by labeling *M. smegmatis* with [^{14}C]glucose, which, when treated with a soluble enzyme extract of *M. smegmatis*, allowed the release of 6 to 10% of the cell wall radioactivity in a soluble form. Gel filtration chromatography on Bio-Gel P2 showed that all of the radioactivity was excluded, and hydrolysis and HPLC analysis on a Dionex column (Fig. 7) demonstrated that the arabinose-containing polymer was specifically released by the mycobacterial enzymes.

DISCUSSION

The results shown in Table 2 confirmed the observation of an immediate inhibition of cell wall arabinan biosynthesis first shown by Takayama and Kilburn (30). Furthermore, inhibition

FIG. 7. Analysis by HPLC on a Dionex column of the radioactive sugars released by acid hydrolysis of the radioactive cell walls (A) and the material released from the radioactive cell walls by mycobacterial enzymes (B). The enzymatic release was performed as described in Materials and Methods. The elution times of arabinose (Ara), galactose (Gal), glucose (Glc), and mannose (Man) are indicated. The small amounts of glucose and mannose in the cell wall hydrolysate are from the contaminating mannan and glucan present in the cell walls.

of the biosynthesis of the arabinan component of LAM (Fig. 5; see Results) was also inhibited, although the effect was not as decisive as that in the case of arabinogalactan-associated arabinan. The differential effect of ethambutol on the two polymers is not immediately explicable. Some type of separation, presumably topographic, of the biosynthesis of the two polymers may occur or different enzymes with differing susceptibilities to ethambutol may be involved in the synthesis of each, or both. Beyond the effects of arabinan biosynthesis, the data presented in Tables 1 and 2 are consistent with the concept that the biosynthesis of the entire mycolylarabinogalactan-peptidoglycan cell wall core is inhibited by ethambutol. It is unlikely that ethambutol independently inhibits the synthesis of all of these macromolecules. The best explanation of the pleiotropic effects is to assume that the synthesis of the entire cell wall core is dependent on the proper formation of arabinan. Yet ethambutol does not (under the conditions used) cause the termination of all cell wall anabolism, since despite the appreciable inhibition of synthesis of LAM-associated arabinan, LM biosynthesis proceeds almost normally.

The change in cell wall composition (Fig. 6) in the absence of biosynthetic activity (Tables 1 and 2) requires that arabinosyl residues be actively removed from the cell wall in the presence of the drug. Thus, an important finding of the present study was that a potent arabinase activity is apparent in the presence of ethambutol. It is not clear if the arabinase is actually stimulated by ethambutol or whether its activity merely becomes apparent because of the ethambutol-mediated inhibition of synthetic enzymes. The arabinase was shown to be present in both *M. smegmatis* and *M. avium*. We have shown that at least a portion of the arabinosyl residues that are re-

moved end up in the culture medium and, furthermore, that some of them are lipidated, presumably with mycolic acids, as judged from their solubilities in organic solvents. The arabinolytic activity was present in crude mycobacterial enzyme extracts and was surprisingly specific for arabinan (Fig. 7), indicating an important physiological role. Furthermore, given the elution of the released arabinan in the Bio-Gel P-2 void volume it must contain more than 13 arabinosyl residues (35).

A possible target for the action of ethambutol is one or several of the arabinosyl transferases involved in the formation of the diverse motifs of arabinan. It appears unlikely that ethambutol inhibits enzymes catalyzing the formation of arabinan precursors such as the β -D-Araf-monophosphodecaprenol (34) or a sugar nucleotide of arabinose because of the rapidity of this effect and the differences between the effects on AG and LAM. However, if an arabinosyl transferase is the primary target of ethambutol, an explanation for the observed arabinolytic activity is still required. It might be that the arabinosyl cleaving activity is always present and that the lack of offsetting synthetic activity in the presence of ethambutol results in enhanced activity. In contrast, it is possible that the drug does not actually inhibit arabinan biosynthesis but instead just stimulates the arabinase activity. This would amount to shutting down arabinan biosynthesis because any newly synthesized material would be removed from the wall as quickly as it was inserted. It must be emphasized, however, that the important goal of identifying the initial target of ethambutol remains to be accomplished.

The arabinase enzyme must have a normal biological function. It is interesting to speculate on the normal biological role of the enzyme which cleaves arabinosyl linkages. One possibility is that the enzyme is a hydrolase necessary for mycobacterial growth. It is reasonable to expect that cleavage of the polymer between the rigid peptidoglycan inner layer and the rigid mycolic acid outer layer needs to occur for cell wall elongation. Alternatively, an attractive hypothesis would be that the arabinase is normally an enzyme that not only cleaves but also rejoins arabinan polymers so that cell elongation can occur. An enzyme capable of cleaving and rejoining the polymer xyloglucan has recently been characterized in plants (26), in which an analogous slippage between macromolecules (cellulose fibers in the case of plants) held together by a polysaccharide (xyloglucan in the case of plants) is necessary for growth. Any number of perturbations could result in the continuation of cleavage of arabinan but disruption of the rejoining reaction. Stated differently, the transfer of cleaved arabinan from the enzyme to the hydroxyl group of water might compete favorably with its transfer to the hydroxyl group of an arabinan chain, depending on the physical and biochemical conditions. Purification of the arabinase activity and further studies are necessary before the natural role of this enzyme can be assessed.

The synergy between ethambutol and other drugs is well documented (19, 27) and is presumed to occur because ethambutol enables the other drugs to gain better access to the inside of the mycobacterium. The actual removal of the cell arabinan and possibly cell wall mycolylarabinan provides a ready molecular explanation for this postulated increased permeability (19, 27). It is logical to envision that the badly damaged cell wall would be substantially less of a barrier to antibiotics that need to gain access to the inner regions of the mycobacteria to exert their effects. Physical evidence for this damage to the cell wall goes back to early studies by Kilburn and Greenberg (20), who showed by electron microscopy that ethambutol causes a change in cell shape to a more spherical shape with a reduced surface-to-volume ratio. This morphological change in cell

shape could well be caused by the rapid removal of part of the cell wall reported herein.

ACKNOWLEDGMENTS

This work was supported by funds provided through the National Cooperative Drug Discovery Group program, National Institute of Allergy and Infectious Diseases, National Institutes of Health (AI-30189), and by Public Health Service grant NIAID, NIH (AI-33706).

We gratefully acknowledge the invaluable assistance of Delphi Chatterjee and Gurdyal Besra in designing many of the experimental protocols, and the constructive scientific discussions with both of these scientists.

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