Inhibition of Synthesis of Arabinogalactan by Ethambutol in *Mycobacterium smegmatis*

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Ethambutol at 3.0 µg/ml inhibited the transfer of label from D-[¹⁴C]glucose into the D-arabinose residue of arabinogalactan in whole cells of a drug-susceptible strain of *Mycobacterium smegmatis*. This inhibition began almost immediately after exposure of the cells to the drug. When drug-resistant *M. smegmatis* was used in a similar experiment, no such drug inhibition was detected. A much higher concentration of ethambutol (>50 µg/ml) was required to show this inhibition. The drug also inhibited synthesis of arabinose-containing oligosaccharides when a cell-free enzyme system was used. These results suggest that the site of action of ethambutol is somewhere on the pathway between the conversion of D-glucose to D-arabinose and the transfer of arabinose into arabinogalactan. The primary mode of action of ethambutol appears to be inhibition of arabinogalactan synthesis.

Ethambutol is an effective and very specific drug used in combination with isoniazid to treat tuberculosis (8, 9, 17, 18). The mechanism of action of this valuable chemotherapeutic agent is not known (4). Forbes et al. (6, 7) first proposed that ethambutol interferes with the role of polyamines and divalent cations in RNA metabolism. Studies by Beggs and Andrews (5) on ethambutol binding by *Mycobacterium smegmatis* did not support the existence of a divalent cation-requiring system as a specific ethambutol target site. More recent studies by Kilburn and Greenberg (10) showed that the apparent number of viable *M. smegmatis* cells rose sharply within the first 4 h of exposure to ethambutol. They theorized that the normally large clumps of cells disaggregated into smaller clusters, thus increasing their apparent viability. A reduction in the lipid content of the cell envelope was inferred to cause this disaggregation. Takayama et al. (15) showed that ethambutol simultaneously inhibited transfer of mycolic acid into the cell wall and stimulated synthesis of trehalose dimycolate in *M. smegmatis*. They reported that this inhibition began as early as 15 min after exposure to the drug.

Further studies by Kilburn and Takayama (11) on the early effects of ethambutol showed that the drug causes an even earlier accumulation of trehalose monomycolate (within about 1 min), trehalose dimycolate, and free mycolic acid (the latter two within 8 to 12 min). Our continued search for the earliest point of drug inhibition in the metabolic pathways of mycobacteria has taken us to the synthesis of arabinogalactan. We now report that ethambutol inhibits the synthesis of cell wall arabinogalactan.

MATERIALS AND METHODS

Materials. Distilled-in-glass methanol and chloroform were purchased from Burdick and Jackson Laboratories, Inc., Muskegon, Mich. For high-performance liquid chromatography (HPLC), HPLC-grade water was obtained from Alltech Associates, Inc., Deerfield, Ill. D-[U-¹⁴C]glucose, D-[U-¹⁴C]arabinose, D-[U-¹⁴C]galactose, and D-[U-¹⁴C]man-

nose were obtained from New England Nuclear Corp., Boston, Mass. All other chemicals used were reagent grade.

Growth of microorganisms. Both ethambutol-susceptible and ethambutol-resistant mutants (resistant to 3 µg/ml) of *M. smegmatis* were used in this study (10). The ethambutol-susceptible *M. smegmatis* was an in-house strain of the Mycobacteriology Laboratory, Centers for Disease Control, Atlanta, Ga., and the ethambutol-resistant mutant was derived from this parent susceptible strain. The cells used to prepare cell extracts and to isolate the arabinogalactan and arabinomannan were grown in glycerol-alanine-salts medium (16) in a 28-liter New Brunswick fermentor (New Brunswick Scientific Co., Inc., Edison, N.J.) at 37°C for 24 h. Cells of *M. smegmatis* used to determine the in vivo synthesis of polysaccharides were grown in supplemented Middlebrook 7H9 broth (Difco Laboratories, Detroit, Mich.) at 37°C with shaking (11) to an A_{650} of 0.05 (ca. 2.5×10^6 CFU/ml). The albumin-dextrose enrichment was omitted, and 0.2% glycerol and 0.05% Tween 80 (Sigma Chemical Co., St. Louis, Mo.) were substituted.

Preparation of cell-free enzyme system for synthesis of arabinose-containing oligosaccharides. Harvested cells of drug-susceptible *M. smegmatis* (25 g, wet weight) were suspended in 25 ml of 0.05 M potassium phosphate buffer, pH 7.0, and sonicated at full power in a Branson sonifier at 0 to 10°C for 7 min. The suspension was centrifuged at $10,000 \times g$ for 30 min, and the turbid supernatant was recovered. This supernatant was recentrifuged under the same conditions as before to yield the $10,000 \times g$ supernatant fraction. This preparation, containing about 43 mg of protein per ml as determined by the biuret method, was used to study the effects of ethambutol on the cell-free synthesis of arabinose-containing oligosaccharides.

Purification of arabinogalactan from commercial source. To a solution of plant arabinogalactan (1.0 g; K and K Laboratories, Inc., Plainview, N.Y.) in 20 ml of water, 24.4 ml of ethanol was added, and the mixture was centrifuged at $500 \times g$ for 10 min. The dark brown residue was discarded; 100 ml of ethanol was added to the supernatant, which was then mixed well and centrifuged. The resulting residue, which

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TABLE 1. Sugar content of arabinomannan and arabinogalactan isolated from cell wall of *M. smegmatis*

Fraction	Insoluble fraction (% ethanol)	Molar ratio of sugar ^a				
		Arab-inose	Galac-tose	Man-nose	Glu-cose	Inosi-tol
Arabinomannan	55-65	0.72	0.21	1.00	0.10	0.04
Arabinogalactan	65-85	0.88	1.00	0.14	0.04	0.00

^a Based on gas-liquid chromatography of the alditol acetates of the acid-hydrolyzed polysaccharides.

represented the 55 to 85% ethanol-insoluble fraction, was suspended in ethanol, filtered, washed once with acetone, and air-dried to yield 303 mg of a white powder. This preparation represented the purified arabinogalactan, and it was used as the unlabeled carrier in the assay for synthesis of arabinogalactan by *M. smegmatis*. Sugar analysis showed such a preparation to contain only arabinose and galactose in a molar ratio of 0.30:1.00.

Preparation of arabinogalactan and arabinomannan from cell walls of *M. smegmatis*. Cells of *M. smegmatis* (74.9 g, wet weight) were suspended in 75 ml of water, sonicated at full power for 15 min at <15°C, and centrifuged at 10,000 × *g* for 30 min to yield a residue fraction that represented the crude cell wall. This residue was washed four times with 0.05 M potassium phosphate buffer, pH 7.0, suspended in 175 ml of potassium phosphate buffer, pH 7.6, and incubated with stirring at 22°C in 0.03% (wt/vol) trypsin (Sigma Chemical Co.) for 3 h. The suspension was centrifuged at 10,000 × *g* for 30 min; the residue was washed four times with water, suspended in acetone, and filtered. The acetone-dried sample (3.45 g) was extracted three times with 150-ml portions of chloroform-methanol (2:1, vol/vol) by stirring overnight for each extraction. This procedure yielded 2.89 g of organic solvent-extracted cell wall. This preparation was suspended in 1.0 M NaOH at 50 mg/ml and incubated with mixing at 37°C for 48 h. The suspension was then neutralized with glacial acetic acid, dialyzed against water, and centrifuged at 10,000 × *g* for 30 min. The supernatant was recovered, and its volume was reduced to about 20 ml on a rotary evaporator. The polysaccharides were fractionated by ethanol precipitation as previously described by Misaki and Yukawa (14). Two desired fractions were obtained: arabinomannan (55 to 65% ethanol insoluble), 3.5 mg, and arabinogalactan (65 to 85% ethanol insoluble), 100.2 mg. The results of sugar analysis of these two fractions are given in Table 1. The

TABLE 2. Effectiveness of incorporation of label from various monosaccharides into the arabinogalactan plus arabinomannan fraction in growing cells of *M. smegmatis*^a

U- ¹⁴ C-labeled metabolite	Sp act (μCi/μmol)	Label in AG + AM fraction (cpm)	Distribution of label (%)			
			Arab-inose	Glu-cose	Galac-tose	Man-nose
D-Arabinose	8	844	100	0	0	0
D-Glucose	87	38,922	37	23	2	37
D-Galactose	32	1,588	57	23	0	20
D-Mannose	50	3,154	17	0	0	83

^a To 3.0 ml of an ethambutol-susceptible *M. smegmatis* culture (A_{600} , 0.05), 2.5 μCi of labeled sugar was added and incubated at 37°C for 60 min. The arabinogalactan plus arabinomannan fraction was prepared and analyzed for sugar content by HPLC. Abbreviations: AG, arabinogalactan; AM, arabinomannan.

FIG. 1. Effect of ethambutol (EMB) on the time course of synthesis of arabinomannan and arabinogalactan in cells of ethambutol-susceptible *M. smegmatis*. The assay measured the incorporation of label from [¹⁴C]glucose into the 55 to 85% ethanol-insoluble fraction. The drug (3.0 μg/ml) was added to the culture at 2.5 min (arrow).

lower arabinose content of our preparation compared with that of the preparations of Misaki et al. (13, 14) could be due to the more drastic conditions of our hydrolysis. Nevertheless, it is clear that we have isolated both arabinomannan and arabinogalactan in the 55 to 65% and 65 to 85% ethanol-insoluble fractions, respectively.

Hydrolysis of polysaccharide and preparation of alditol acetate derivative of the monosaccharides. The method of Albersheim et al. (1), with modifications, was used to determine the sugar content of the polysaccharides. The sample (2 to 3 mg) was dissolved in 200 μl of 2 M trifluoroacetic acid, sealed, and hydrolyzed at 100°C for 3 h to yield the monosaccharides. The acid was removed in a warm water bath with a stream of nitrogen; 50 μl of 1 M NH₄OH and 1 mg of sodium borohydride were added, and the mixture was incubated overnight in the dark at 22°C. One drop of glacial acetic acid was then added to destroy excess borohydride. The borate was converted to the methyl derivative by addition of methanol and repeated drying of the

FIG. 2. Effect of ethambutol (EMB) on the time course of incorporation of label from [¹⁴C]glucose into the arabinose residue of arabinomannan and arabinogalactan in cells of ethambutol-susceptible *M. smegmatis*. The drug (3.0 μg/ml) was added to the culture at 2.5 min (arrow).

FIG. 3. Radio-HPLC analysis of ^{14}C -alditol acetates derived from the polysaccharides obtained from control (A) and drug-treated (B) cells of ethambutol-susceptible *M. smegmatis*. This pair represents the 30-min points of a time course assay for the synthesis of arabinomannan and arabinogalactan (Fig. 1). One-fifth of each sample (control, 14,350 cpm; drug-treated, 11,106 cpm) was analyzed for distribution of radioactivity. The conditions of chromatography were: column, C_{18} -bonded silica; solvent, methanol-water (35:65, vol/vol); detector, radioactive flow with full scale of 10^2 cpm. The radioactivity of the alditol acetate derivatives is given in parentheses. Abbreviations: Ara, arabinose; Glc, glucose; Gal, galactose; Man, mannose.

methanol solutions. To the dried alditol derivative, 100 μl of acetic anhydride was added. This was sealed and incubated at 121°C for 3 h. Excess acetic anhydride was destroyed by adding 400 μl of water, and the alditol acetates were purified on a Sep-Pak cartridge (Waters Associates, Inc., Milford, Mass.) by the method described by Alpenfels (2). The purified sample was analyzed by either gas-liquid chromatography or HPLC.

Gas-liquid chromatography. Gas-liquid chromatography of the alditol acetates was performed on a Packard model 428 gas chromatograph (Packard Instrument Co., Inc., Downers Grove, Ill.) with a glass column (3.4 m by 2 mm) of 10% SP-2330 on 100/120 Chromosorb W AW (Supelco, Inc., Bellefonte, Pa.). The sample was dissolved in chloroform, and 0.50 μl was injected into the gas chromatograph. A temperature gradient of 200 to 270°C increasing at a rate of $4^\circ\text{C}/\text{min}$ was used. For quantitation, an internal standard of inositol was added to the sample before acid hydrolysis.

HPLC analysis. HPLC of the alditol acetates was performed with a Waters 6000A solvent delivery system (Waters Associates, Inc.), a Waters U6K universal liquid chromatograph injector, a variable-wavelength detector (model LC-85B; Perkin-Elmer Corp., Norwalk, Conn.), a Flo-One radioactivity flow detector connected to a stream splitter (models HS and ES, respectively; Radiomatic Instruments and Chemical Co., Inc., Tampa, Fla.), and a radial compression module (model RCM-100; Waters Associates, Inc.). A radial pak A cartridge (8 mm by 10 cm; C_{18} -bonded silica, 10

FIG. 4. Effects of ethambutol (EMB) concentration on inhibition of incorporation of radiolabel from $[^{14}\text{C}]$ glucose into the D-arabinose residue of the arabinomannan plus arabinogalactan fraction (AM/AG) of the ethambutol-susceptible (○) and ethambutol-resistant (●) strains of *M. smegmatis*. Growing cells of *M. smegmatis* were incubated at 37°C for 20 min at various concentrations of ethambutol in the presence of $[^{14}\text{C}]$ glucose. The amount of radiolabel going into the D-arabinose residue of the arabinomannan plus arabinogalactan fraction was determined by radio-HPLC analysis of the alditol acetate derivative. The amount of radiolabeled D-arabinose (Ara) in the arabinomannan plus arabinogalactan fraction of control cells (no ethambutol) was set at 100%. Each point on the two curves represents the average of duplicate samples.

FIG. 5. Fractionation of the products of control and drug-treated cell-free reactions for synthesis of the [^{14}C]arabinose-containing oligosaccharides by gel filtration. The sample was applied to a Bio-Gel P-2 column (2 by 24 cm) and eluted with water. Two-milliliter fractions were collected for determination of radioactivity. The drug-treated reaction mixture contained 15 μg of ethambutol per ml. Only fraction II contained [^{14}C]arabinose. Glc, Glucose.

μm ; Waters Associates, Inc.) was used. Carrier alditol acetates of arabinose, galactose, glucose, mannose, and inositol (Supelco, Inc.) were added to the radiolabeled samples to identify the labeled peaks, and mass peaks were detected at 210 nm. The solvent system for fractionation of the alditol acetates was methanol-water (35:65, vol/vol) at a flow rate of 2.0 ml/min.

Assay for in vivo synthesis of arabinogalactan and arabinomannan. To a 3.0-ml culture of *M. smegmatis*, 1.0 μCi of [^{14}C]glucose (87 mCi/mmol) was added, and the culture was incubated at 37°C for the desired time. Cells were killed by adding 30 μl of 1.0 M KCN. A suspension of unlabeled *M. smegmatis* cells (32 mg, dry weight) was added, mixed, and centrifuged at $5,000 \times g$ for 10 min. The supernatant was discarded, and 1.0 ml of 1.0 M NaOH was added to the recovered pellet. This was incubated at 70 to 75°C for 2 h. The suspension was acidified with 200 μl of 6 M HCl, and

carrier arabinogalactan (5.0 mg, plant source) was added. To this mixture, 1.7 ml of ethanol was added, mixed, and centrifuged for 10 min. The residue containing glucan (14) was discarded, and 8.0 ml of ethanol was added to the supernatant. This mixture was cooled to 0°C for 10 min and centrifuged. The residue represented the arabinogalactan plus arabinomannan fraction (55 to 85% ethanol-insoluble fraction) (13, 14). This residue was washed three times with 3-ml portions of ethanol and finally dissolved in 1.00 ml of water. A portion of this solution was taken for radioactivity determination. Each experiment was performed at least twice, and duplicate samples were analyzed. The average of the duplicates reported was within the 5% standard deviation.

Assay for cell-free enzymatic synthesis of arabinose-containing oligosaccharides. The cell-free reaction mixture for synthesis of arabinose-containing oligosaccharides consisted

FIG. 6. Radio-HPLC analysis of ^{14}C -alditol acetates derived from the oligosaccharides (Bio-Gel fraction II) synthesized by control (A) and drug-treated (B) cell-free enzyme systems. Fraction sizes analyzed were 2.88×10^5 cpm (A) and 2.60×10^5 cpm (B). Other conditions of analysis and abbreviations are described in the legend to Fig. 3.

of the following, in a final volume of 1 ml: potassium phosphate, pH 7.0, 75 mM; MgCl_2 , 2.5 mM; GTP, 750 μM ; $[\text{U-}^{14}\text{C}]\text{glucose}$, 2.5 μCi (38 mCi/mmol); and 10,000 $\times g$ supernatant fraction from ethambutol-susceptible *M. smegmatis*, 21.7 mg. The reaction mixture was incubated at 37°C for 30 min; the reaction was terminated by adding 110 μl of 10 M NaOH and incubation at 70 to 75°C for 2 h. Then, 200 μl of 6 M HCl was added and centrifuged, and a clear supernatant was recovered. The supernatant was applied to a Bio-Gel P-2 column (2 by 24 cm; 200/400 mesh; Bio-Rad Laboratories, Richmond, Calif.) and eluted with water. Two-milliliter fractions were collected, and the radioactivity was determined. A fast-moving minor peak (fraction I; effluent volume, 40 to 69 ml) and a slow-moving major peak (fraction II; effluent volume, 70 to 90 ml) were obtained. Fraction II contained the $[\text{U-}^{14}\text{C}]\text{arabinose}$ in the form of oligosaccharides (data not presented). This fraction was hydrolyzed with acid and derivatized to the alditol acetates. The $[\text{U-}^{14}\text{C}]\text{arabinose}$ content was quantitated by reverse-phase HPLC of the alditol acetates.

RESULTS

Preliminary studies on synthesis of arabinomannan and arabinogalactan. Studies were initiated to determine the appropriate substrate to use in following the synthesis of arabinomannan and arabinogalactan in growing cells of *M.*

smegmatis. Radiolabeled D-arabinose, D-glucose, D-mannose, and D-galactose were tested, and D-glucose was found to be the most effective (Table 2). Surprisingly, D-arabinose was not very effective. When $[\text{U-}^{14}\text{C}]\text{glucose}$ (<80 mCi/mmol) was used as a metabolite to follow synthesis of the polysaccharides in *M. smegmatis*, there was a linear increase in the synthesis of arabinomannan plus arabinogalactan over a 45-min period (Fig. 1). The labeled product (in the 55 to 85% ethanol-insoluble fraction of the 30-min incubation sample) was fractionated on a Sephadex G-75 column (15), hydrolyzed with acid, and identified by C_{18} -bonded silica HPLC as the $[\text{U-}^{14}\text{C}]\text{alditol}$ acetates by using a radioflow detector. The relative radioactivity distribution of arabinose, galactose, mannose, and glucose in the polysaccharide fraction was 2.4:0.5:2.5:3.0. These results suggested the presence of residual glucan and low conversion of D-glucose to D-galactose. It was clear that D-glucose was readily converted to D-arabinose. The presence of significant amounts of labeled mannose suggested, as expected, that $[\text{U-}^{14}\text{C}]\text{arabinomannan}$ was also present in this preparation.

Effect of ethambutol on synthesis of arabinomannan and arabinogalactan in *M. smegmatis*. The time course of synthesis of arabinomannan plus arabinogalactan by control and drug-treated (3.0 $\mu\text{g}/\text{ml}$) cells of ethambutol-susceptible *M. smegmatis* indicated that inhibition began about 10 min after exposure to the drug (Fig. 1), based on the total counts per

FIG. 7. Speculation on how D-arabinose might be formed from D-glucose and incorporated into arabinomannan and arabinogalactan of the cell wall in *M. smegmatis*. Reaction a represents the pentose phosphate pathway. Possible enzymes involved are as follows: 1, glucokinase; 2, glucose-6-phosphate dehydrogenase; 3, 6-phosphogluconic acid dehydrogenase; 4, ribulose-5-phosphate isomerase; 5, phosphopentomutase; 6, guanidyl transferase; and 7, transglycosidase. Abbreviations: Glc, glucose; Ara, arabinose; GlcoA, gluconic acid; Ribul, ribulose; Rib, ribose; P, phosphate.

minute entering the 55 to 85% ethanol-insoluble fraction from [^{14}C]glucose. When the same two sets of samples were analyzed for [^{14}C]arabinose content by radio-HPLC of the alditol acetate derivative, a different time course plot emerged (Fig. 2). The plot showed an immediate inhibition in the transfer of label from [^{14}C]glucose into the arabinose residue of arabinomannan and arabinogalactan. Similar results were obtained when the [^{14}C]arabinose content was analyzed by paper chromatography (data not presented). A direct comparison of the total difference between control and drug-treated cells after the 30-min incubation time is shown in Fig. 3. This analysis showed 90 and 53% inhibition of labeling in arabinose and mannose, respectively, whereas there was essentially no change in the labeled galactose content. A slight difference in levels of labeled glucose was observed, but this may not be significant.

We then compared the ethambutol-susceptible and ethambutol-resistant strains of *M. smegmatis* for the concentration-dependent inhibition by ethambutol of incorporation of radiolabel from [^{14}C]glucose into the D-arabinose residue of the arabinomannan plus arabinogalactan fraction. Striking differences were noted (Fig. 4). With the ethambutol-susceptible strain, maximum inhibition occurred at about 5 μg of ethambutol per ml, whereas with the ethambutol-resistant strain, this value was 12-fold higher (60 $\mu\text{g}/\text{ml}$).

Effect of ethambutol on cell-free transfer of label from [^{14}C]glucose into the arabinose residue of oligosaccharides. A cell-free enzyme system was developed to follow the synthesis of arabinose-containing oligosaccharides in mycobacteria. This might represent an early sequence of reactions in the biosynthesis of cell wall arabinomannan and arabinogalactan. In a phosphate buffer, [^{14}C]glucose, GTP, and MgCl_2 were added to the $10,000 \times g$ supernatant fraction from ethambutol-susceptible *M. smegmatis*, in the presence and

absence of ethambutol (15 $\mu\text{g}/\text{ml}$). After incubation at 37°C for 30 min, each of the two reaction mixtures was saponified, then acidified, and separated on a Bio-Gel P-2 column into two fractions: control I and II, and drug-treated I and II (Fig. 5). Each fraction was acid-hydrolyzed, converted to the alditol acetates, and assayed for radioactivity. Control I had 9.36×10^5 cpm; control II had 2.88×10^6 cpm; drug-treated I had 9.07×10^5 cpm; and drug-treated II had 2.61×10^6 cpm. One-tenth of each sample was analyzed by reverse-phase HPLC. Fractions control I and drug-treated I were devoid of labeled arabinose (data not shown). HPLC comparison of control II with drug-treated II showed a 74% drug inhibition of transfer of label from [^{14}C]glucose into the arabinose residue of the oligosaccharides (Fig. 6). This kind of inhibition is similar to that observed previously with ethambutol in the intact cell. Unknown-1 (Unk-1), which was abundant (7,676 cpm) in the control sample, was virtually absent in the drug-treated sample. The drug caused a large accumulation of Unk-2. Although the significance of these changes is not known, the two unknown compounds might represent precursors of D-arabinose.

DISCUSSION

Early studies by Forbes et al. (6, 7) suggested that the synthesis of RNA was affected by ethambutol. However, further work, especially by Beggs and co-workers (4, 5), showed that this was not the case and that the mechanism of action of this important antituberculous drug was still unknown. More recent studies by Kilburn and Greenberg (10), Takayama et al. (15), and Kilburn et al. (11, 12) were centered on the question of how ethambutol affects lipid metabolism. These studies revealed that the early effect of ethambutol (between 1 and 12 min after exposure) was gross

accumulation of trehalose monomycolate, trehalose dimycolate, and free mycolic acid. This soon followed by cellular release of certain phospholipids.

The study reported here is a result of our search for a point of early drug inhibition (rather than accumulation) in the metabolic pathways of ethambutol-susceptible *M. smegmatis*. The only early drug inhibition observed to date is the inhibition of incorporation of mycolic acid into the cell wall of *M. smegmatis* (15). Ethambutol did not inhibit synthesis of mycolic acid. We now report that ethambutol inhibits incorporation of D-arabinose into cell wall arabinogalactan. This occurs almost immediately upon exposure to the drug. We suggest that the inhibition depletes the sites for transfer of mycolic acid to the cell wall. Cell wall mycolic acids are known to be specifically attached to the 5-position of the distal D-arabinose residues (3). With this depletion, the newly synthesized mycolic acids must find alternative sites for transfer. Because of this, the transfer is thought to be diverted to the trehalose molecule, causing overproduction of trehalose monomycolate and trehalose dimycolate. We suggest that this initial effect of the drug triggers a cascade of changes in the lipid metabolism of mycobacteria that eventually leads to cell death. Early gross morphological changes have been described by Kilburn and Greenberg (10), primarily swelling of the cells and disaggregation of the clumps. Fine-structure changes associated with this drug action on ethambutol-susceptible *M. smegmatis* have been examined (unpublished results), but the physical changes were so subtle that we could not distinguish between drug-treated and control cells by either scanning, thin-section, or negative-staining electron microscopy. The primary mode of action of ethambutol would then be inhibition of synthesis of cell wall arabinogalactan.

The site of action of ethambutol must lie somewhere between conversion of D-glucose to D-arabinose and transfer of the arabinose into arabinogalactan (Fig. 7). Since arabinose metabolism in mycobacteria is not known, we have speculated (by analogy with studies of other biological systems) that the pentose phosphate pathway is involved in this sugar conversion (reaction a). A reaction in this pathway might be drug sensitive. In the synthesis of the polysaccharides, sugars in the form of nucleotide derivatives are usually transferred to a primer molecule (reaction b). This transfer reaction might be ethambutol sensitive. We have evidence that the activated sugar is guanosine diphospho-D-arabinose (unpublished data). This evidence is based on the direct comparison of ATP, GTP, and CTP as cofactors and the use of guanosine diphospho-[¹⁴C]-D-arabinose as a substrate in the cell-free synthesis of the arabinose-containing oligosaccharide. As indicated in reaction b (Fig. 7), further addition of sugar units yields the final arabinogalactan molecule, which is presumed to be the acceptor of mycolic acid residues. Since we have now isolated a drug-sensitive enzyme system in the 10,000 × g supernatant of ethambutol-susceptible *M. smegmatis* that transfers label from D-glucose to an arabinose residue in oligosaccharide products, further study of this system is expected to reveal the exact site of action of ethambutol.

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