Effect of Ethambutol on Nucleic Acid Metabolism in *Mycobacterium smegmatis* and Its Reversal by Polyamines and Divalent Cations

M. FORBES, N. A. KUCK, AND E. A. PEETS

Experimental Therapeutics Research, Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York

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**Abstract**

FORBES, M. (Lederle Laboratories Division, Pearl River, N.Y.), N. A. KUCK, AND E. A. PEETS. Effect of ethambutol on nucleic acid metabolism in *Mycobacterium smegmatis* and its reversal by polyamines and divalent cations. J. Bacteriol. 89:1299-1305. 1965.—*Mycobacterium smegmatis*, harvested from cultures inhibited by ethambutol and then suspended in drug-free medium, exhibited a prolonged lag before growth resumed. Polyamines and magnesium ions shortened this lag. Polyamines and magnesium added to the culture increased the minimal inhibitory concentration of the drug and reversed the inhibitory effect of the drug, even when added after the drug had already inhibited growth. When ethambutol was added to a culture in its exponential phase of growth, synthesis of protein and deoxyribonucleic acid (DNA), as measured by incorporation of S35 and P32, continued for 3 hr at a rate slightly less than in the control cells and then essentially ceased. Synthesis of ribonucleic acid (RNA) was depressed, but it proceeded even after protein synthesis had ceased. Even though the synthesis of RNA continued, the net RNA decreased, and inhibited cells became deficient in RNA. Polyamines and divalent cations, which reverse the inhibitory effect of the drug, have been reported to be involved in nucleic acid turnover. These considerations suggested that ethambutol may exert its inhibitory effect by interfering with a function of cellular polyamines and divalent cations in RNA metabolism.

Ethambutol (dextro-2,2'-[ethylenediiminoo]di-1-butanol) has specific antimycobacterial activity and is therapeutically effective in tuberculosis in animals (Thomas et al., 1961; Wilkinson et al., 1961) and in man (Bobrowitz, Garber, and Sukumalantra, 1963). Reports on its in vivo activity were reviewed by Robson and Sullivan (1963).

Studies on the mode of action of ethambutol showed that, even though the drug was taken up rapidly by both proliferating and nonproliferating mycobacteria, it had no effect on the viability and metabolism of nonproliferating cells. When added to growing cultures, ethambutol inhibited growth. The inhibitory effect of the drug became apparent only several hours after addition of the drug to the culture when the population had at least doubled (Forbes, Kuck, and Peets, 1962; Kuck, Peets, and Forbes, 1963). Gale and McIain (1963) in studying the effect of ethambutol on the cytolgy of *Mycobacterium smegmatis* found that "absence of probable nuclear substance was the most evident alteration incurred by the cells." The authors postulated that the drug may block one or more steps in the synthesis of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA), ultimately causing cessation of cellular division.

Preliminary observations (Forbes, Kuck, and Peets, 1964) showed that *M. smegmatis*, harvested from cultures inhibited by ethambutol and then suspended in drug-free medium, exhibited a prolonged lag before growth resumed. In a continuation of these studies, it was found that polyamines and divalent cations reversed the inhibitory effect of ethambutol. The present report describes the reversal of the inhibitory effect of ethambutol and the effect of the drug on nucleic acid and protein metabolism in *M. smegmatis*.

**Materials and Methods**

*M. smegmatis* ATCC 607 cultured in Sauton's medium containing 0.02% Tween 80 (polyoxyethylene sorbitan monoleate; Atlas Powder Co., Wilmington, Del.) was used throughout these studies. The cultures were incubated at 37 C on a reciprocal shaker, and growth was followed by measuring optical density at 600 mu in colorimetric tubes (2.54 cm) with a Bausch & Lomb Spectronic-20 colorimeter. In some experiments,
the cells were washed and suspended in a nitrogen-free "resting" medium made with the same ingredients as those of Sauton's medium, except that asparagine was omitted, and the ferric ammonium citrate was replaced with ferric citrate.

The number of viable units was estimated by plating dilutions of culture in Blood Agar Base (BBL). Colony counts were made after 4 to 5 days of incubation to allow for the slower growth of cells from ethambutol-inhibited cultures.

For the determination of protein and nucleic acid content, cells were harvested by centrifugation at 4°C, washed with water, and processed according to the method of Winder and O'Hara (1962). In this procedure, the cells were successively extracted with cold acetone, cold trichloroacetic acid, ethyl alcohol, and hot ethyl alcohol-ether. The residue was digested at room temperature with 1 N KOH, and a sample of the digest was removed for determination of protein by the method of Lowry et al. (1951). The remainder of the digest was precipitated with perchloric acid, and cold trichloroacetic acid was then added to a concentration of 5%. RNA was determined from the ultraviolet absorption of the supernatant fluid and washes of the precipitate. DNA in the precipitate was solubilized with hot trichloroacetic acid and determined by its ultraviolet absorption. Absorption was measured at 268.5 nm in a Beckman model DU spectrophotometer. RNA and DNA (both obtained from Calbiochem) were used as standards. Human albumin, fraction V (Mann Research Laboratories, New York, N.Y.) was the standard for the protein determination.

Ethambutol was determined by an agar diffusion technique with modified Sauton's agar seeded with M. smegmatis (Place and Thomas, 1963).

Protein synthesis was followed by measuring the S35 (added to the cultures as H35PO4) in the KOH digest of the trichloroacetic acid-insoluble fraction of the cells. Nucleic acid synthesis was followed by adding P32 (as H32PO4) to the cultures. The P32 incorporated into the RNA and DNA was determined from the difference between the radioactivity of the appropriate cell fractions before and after adsorption of the nucleotides onto Northcell charcoal (Crane, 1958; Harold, 1960). Ultraviolet-absorption measurements on the charcoal filtrates indicated that 95 to 100% of the nucleotides were adsorbed onto the charcoal. The S35 radioactivity in neutralized protein fractions was determined in a Packard Tri-Carb liquid scintillation spectrometer by use of a naphthalene phosphor solvent system (Werbin, Chaikoff, and Imada, 1959). The S35 radioactivity was expressed in absolute units (disintegrations per minute, dpm), the absolute activity being determined by means of an internal standard technique (Davidson and Feigelson, 1957). The P32 radioactivity was determined in a scintillation well counter (Baird-Atomic Inc., Cambridge, Mass.).

All materials added to the cultures were dissolved in Sauton's medium. The pH of the stock solutions was adjusted to 7.2 to 7.4. To test the effect of cations, the following salts were used: NaCl, Na2SO4, MgSO4, MnSO4·4H2O, ZnSO4·7H2O, CaSO4, CaHPO4, CaCl2, and FeCl3. These stock solutions were autoclaved, and all other materials were sterilized by filtration through ultrafine sintered glass. Spermidine PO4·6H2O and spermine PO4·6H2O were obtained from Mann Research Laboratories, Inc.; 1,2-diaminoethane, from Matheson Co., Inc., East Rutherford, N.J.; 1,3-diaminopropane hydrochloride, from Aldrich Chemical Co., Inc., Milwaukee, Wis.; 1,4-diaminobutane-2HCl (putrescine), from Distillation Product Industries, Division of Eastman Kodak Co., Rochester, N.Y.; 1,5-diaminopentane-2HCl (cadaverine) and the amino acids, from Nutritional Biochemicals Corp., Cleveland, Ohio. Purines, pyrimidines, and nucleosides were obtained from Schwarz BioResearch Inc., Mount Vernon, N.Y. S25 and P32, carrier-free, were obtained from Oak Ridge National Laboratories, Oak Ridge, Tenn.

RESULTS

Reversal of ethambutol effect by polyamines and divalent cations. When growing cells of M. smegmatis were exposed to ethambutol for 3 to 6 hr...
and then harvested and suspended in drug-free medium, there was a 20-hr lag before growth resumed (Fig. 1, curve F). In contrast, cells from cultures to which ethambutol had also been added, but which were held at 4 C, exhibited no prolonged lag before growing when they were suspended in the drug-free medium (Fig. 1, curve A). Isotopic measurements showed that both proliferating (37 C) and nonproliferating (4 C) cells bound C14-ethambutol to the same extent. The prolonged lag, therefore, appeared not to be related to residual ethambutol in the cells, but rather to the time required for the cells to recover from the damage incurred during growth in presence of the drug.

A number of compounds, including amino acids, purines, pyrimidines, and nucleosides, were tested for their ability to shorten the lag phase of cells harvested from ethambutol-inhibited cultures and suspended in drug-free medium. Of the various materials tested, only certain diamines and polyamines and magnesium ions had such an effect. The effect of spermidine, magnesium ions, or both, on the recovery of ethambutol-inhibited cells suspended in drug-free medium is shown in Fig. 1 (curves C, D, and E). These substances had no stimulatory effect on the growth in drug-free medium of cells harvested from cultures to which ethambutol had been added, but which had been held at 4 C (Fig. 1, curve B). Other amines which aided the recovery of ethambutol-inhibited cells were 1,3-diaminopropane, putrescine, cadaverine, and spermine. No concentration of spermidine and magnesium was found which reduced the extent of the lag phase to that occurring in normal cells.

Spermidine and magnesium ions also counteracted the inhibitory effect of ethambutol in the presence of the drug. Thus, when spermidine and magnesium, or both, were added to the medium, the minimal inhibitory concentration (MIC) of ethambutol needed to inhibit growth of M. smegmatis was increased (Table 1). Assay of the ethambutol concentration at the termination of the test showed that the culture tubes with added spermidine and magnesium contained as much ethambutol as the corresponding control tubes to which no additions had been made. Thus, the blocking of the inhibitory action of ethambutol was not a result of the inactivation of ethambutol by spermidine and magnesium.

That the increase in the MIC of the drug was, indeed, due to the added magnesium ions was demonstrated by the fact that both MgCl2 and MgSO4 were effective, and equivalent molar concentrations of NaCl and Na2SO4 had no effect. Addition to the medium of CaCl2 (10 mM) also increased the MIC of ethambutol, but CuSO4,

### Table 1. Effect of added spermidine and magnesium on the MIC of ethambutol for Mycobacterium smegmatis in Sauton's medium

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<tr>
<th>Addition to Sauton's medium</th>
<th>MIC of ethambutol*</th>
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<tr>
<td>Spermidine</td>
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* Minimal concentration of ethambutol preventing pellicle formation for 72 hr in stationary culture tubes containing twofold dilutions of ethambutol. In control cultures without drug, pellicle growth was apparent in 24 hr. The concentration of magnesium in Sauton's medium was 4 μmoles/ml.

ZnSO4, and MnSO4 at concentrations of 1 mM were ineffective. Ferric chloride was ineffective at 10 mM. Higher concentrations of these substances either inhibited growth or caused a precipitate. Cadaverine and putrescine, at concentrations of 1 and 5 mM, had little effect in increasing the MIC. Spermine, at 1 and 0.2 mM, had an effect similar to spermidine at 1 mM. Spermidine and magnesium reversed the inhibitory effect of the drug, even when added after the drug had already inhibited growth. When spermidine and magnesium were added to cultures 6 hr after ethambutol, they allowed growth to resume in presence of the drug (Fig. 2). Spermidine added to the medium at a concentration of 0.5 mM gave optimal effects, and magnesium, in concentrations of 20 to 40 mM, gave maximal effects in reversing an inhibition of growth by 0.05 mM ethambutol. Combinations of spermidine and magnesium were more effective than either of the substances alone. In other experiments, it was shown that the ability of these concentrations of spermidine and magnesium to reverse the inhibitory effect of ethambutol decreased as the concentration of the drug was increased.

**Effect of ethambutol on nucleic acids and protein**. When ethambutol was added to a culture of M. smegmatis in its exponential phase of growth, the net protein and DNA continued to increase for the first 3 hr. Thereafter, the protein remained constant, and the DNA continued to increase, but at a sharply reduced rate. The net RNA also increased for the first 3 hr after addition of the
Incorporation of DNA and RNA in Mycobacterium smegmatis. $P^{32}$ (final concentration, 0.33 $\mu$Ci/ml) or $S^{35}$ (final concentration, 0.018 $\mu$Ci/ml) was added to a culture in the early exponential phase (OD$_{600}$ = 0.20) of growth. The culture was then immediately divided into two parts; ethambutol (10 $\mu$g/ml) was added to one and both parts were reincubated. Samples of culture were collected at intervals, and protein and nucleic acid contents of the cells and radioactivity in the cell fractions were determined. At 0 hr, the cultures contained per 100 ml; protein, 2,500 $\mu$g; DNA, 940 $\mu$g; RNA, 270 $\mu$g. Symbols: solid lines, control; broken lines, ethambutol.

A decrease in the net RNA indicates that the rate of degradation exceeds the rate of synthesis. To test whether the drug blocked steps in the synthesis of RNA, precursors of nucleic acids were added to the cultures. Adenine, guanine, uracil, cytosine, thymine, and nucleosides, in concentrations of 10 or 100 $\mu$g/ml, did not counteract the inhibitory effect of ethambutol. To test whether the drug accelerated enzymes degrading RNA, cells were suspended in a nitrogen-free "resting" medium. In this medium, the RNA of...
EFFECT OF ETHAMBUTOL IN M. SMEGMATIS

**Fig. 4.** Effect of ethambutol on the protein and nucleic acid composition of Mycobacterium smegmatis. A culture in the early exponential phase of growth (OD$_{600}$ = 0.20) was divided into two parts; ethambutol (10 µg/ml) was added to one, and both parts were reincubated. Cells were collected at intervals and freeze-dried. Analyses were performed on weighed samples of dried cells. The vertical bars represent the range of values of duplicate determinations in three replicate experiments. Symbols: solid lines, control; broken lines, ethambutol.

The cells gradually decreased and then remained at a constant level. Ethambutol did not affect the rate of degradation (Fig. 5).

**DISCUSSION**

Inhibition of growth of *M. smegmatis* by ethambutol was accompanied by a decrease in the RNA content of the cells. During the first 3 hr after addition of the drug to the culture, RNA metabolism was depressed more than DNA or protein metabolism, and the fall in the RNA–protein ratio preceded the arrest of growth (Fig. 3 and 4). A deficiency in RNA would result in cessation of protein synthesis and growth. The experiments do not permit a conclusion as to whether the RNA deficiency is due to a depression of the synthesis of RNA coupled with a normal rate of breakdown, or to instability of the RNA formed in presence of the drug. No indication was found that ethambutol accelerated the degradation of RNA by enzymes (Fig. 5).

Precursors of the nucleic acids did not relieve the growth inhibition by ethambutol, but poly-
amines and metal cations did. These substances did not inactivate the drug. One possible explanation of their effect may be that ethambutol acts by interfering with a function of cellular polyamines and metal cations. The polyamines and metal cations added to the medium would then counteract this action of the drug. This possibility is supported by the fact that ethambutol is a substituted ethylenediamine with structural similarity to polyamines, and that alkanol-substituted ethylenediamines, such as ethambutol, are known to form metal chelates (Hall, Dean, and Pacosky, 1960). Polyamines and divalent cations have been reported to play a role in nucleic acid turnover in bacteria (Tabor and Tabor, 1964). They stabilize ribosomes (Cohen and Lichtenstein, 1960; Martin and Ames, 1962), inhibit RNA degradation in whole cells (Herbst and Doctor, 1959), and are required for the maximal synthesis of RNA by RNA polymerase (Doerfler et al., 1962; Krakow, 1963; Fox and Weiss, 1964). In these reactions, it is often found that there is a concentration of the polyamine that is optimal, higher concentrations being less effective. Metal cations, specifically magnesium or calcium, may often replace polyamines in their functional role in nucleic acid turnover. It is characteristic that higher concentrations of the metal ions than of the polyamines are needed to accomplish the same effect (Tabor and Tabor, 1964). The pattern of the effect of spermidine and magnesium in counter-acting ethambutol, thus, shows analogies with the effect of these substances in nucleic acid metabolism.

The data show that ethambutol has no effect on nonproliferating cells of M. smegmatis. The drug, after a delay, arrests the growth of actively proliferating cells. Cytological examinations (Gale and McLain, 1963) of cells inhibited by ethambutol revealed no alteration of the integrity of the cell wall and membrane. Chemical determinations showed that inhibited cells became deficient in RNA. The early effects of the drug are reversible. Cells harvested from cultures inhibited by ethambutol and then suspended in drug-free medium eventually resume growth. Polyamines and divalent cations, which are known to be involved in nucleic acid turnover and the maintenance of the integrity of ribosomes, aid the recovery of these cells. These substances in the presence of the drug also counteract the inhibitory effect of the drug. All these considerations are consistent with the view that ethambutol exerts its inhibitory effect on growth by interfering with a function of cellular polyamines and divalent cations in the synthesis or stabilization of RNA.

LITERATURE CITED


