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Nonspecific Ionic Inhibition of Ethambutol Binding by *Mycobacterium smegmatis*

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Magnesium sulfate and spermidine were tested for their effects on binding of ^{14}C-ethambutol by *Mycobacterium smegmatis*. Concentrations were used that protected the organism from ethambutol inhibition. Sodium salts were examined as possible ethambutol antagonists to test the previously reported specificity of the divalent cation salt effect. Consistent with growth-protection experiments, 20 mM $MgSO_4$ or 2.0 mM spermidine prevented and reversed ^{14}C binding by cells shaken with 0.2 μg of ^{14}C-ethambutol per ml of Sauton medium at 37 C. Sodium salts were not effective ethambutol antagonists when tested at 20 mM, but at concentrations equivalent in ionic strength (μ) to that provided by 20 mM $MgSO_4$ they were effective. Thus, 20 mM $MgSO_4$, 80 mM NaCl, or 27 mM Na_2SO_4 (μ = 0.08) all gave similar results in growth protection and binding experiments, suggesting that $MgSO_4$ antagonism is a nonspecific ionic effect. Because spermidine ($\mu \leq 0.012$) antagonized ethambutol at an ionic strength substantially less than that required for the metal salts, its effect may hinge on structural similarity to ethambutol rather than its cationic character. Drug and polyamine may compete for one site or a heterogeneous group of binding sites involving adsorption, transport, and intracellular target reactions. Until we know at which of these levels spermidine antagonizes ethambutol binding, the relationship between polyamines and ethambutol action will remain obscure. However, these studies have weakened the earlier argument for a divalent cation-requiring system as a specific ethambutol target site.

Forbes and co-workers (7) showed that $MgSO_4$, $MgCl_2$, $CaCl_2$, and certain polyamines can protect *Mycobacterium smegmatis* cells from ethambutol growth inhibition. No such effect was observed when equimolar concentrations of NaCl or Na_2SO_4 were tested. We confirmed these results (3), and more recently the $MgSO_4$ effect was shown with *M. tuberculosis* BCG (J. K. McClatchy, personal communication). Based largely on their growth protection studies, Forbes et al. (7) hypothesized that ethambutol interferes with normal metabolic functions of divalent cations and polyamines. Presumably, this interference involves competition for specific target sites within the cell, leading to metabolic disturbances. However, it is equally possible that $MgSO_4$ antagonism of ethambutol action results from nonspecific ionic interactions unrelated to the primary mode of action of the drug. Growth protection might be due to general ionic effects at either specific or nonspecific sites, resulting in inhibition of drug binding. Spermidine, by virtue of its structural similarity to ethambutol, might compete for a transport site, in which case the drug would be inaccessible to the organism. These possibilities are important to consider, particularly in view of reports that metal salts and polyamines can antagonize certain antimicrobial agents unrelated to ethambutol, such as streptomycin (4, 5, 8, 9). In a recent report from our laboratory, it was shown that $MgSO_4$ prevented streptomycin inhibition of *M. smegmatis* and antagonized drug binding by the organism (3). Clearly, then, one important step in a proper test of the Forbes hypothesis is an assessment of ethambutol binding in the presence of growth-protective concentrations of metal salts and polyamines.

In a preliminary study from this laboratory (W. H. Beggs and N. E. Auran, Abstr. Annu. Meeting Amer. Soc. Microbiol., p. 116, 1972), it was reported that binding of ^{14}C activity by *M. smegmatis* cells exposed to 0.2 μg of labeled ethambutol per ml of Sauton medium was not affected by addition of 20 mM $MgSO_4$ or 2.0

mM spermidine. In these earlier studies, small samples of treated cells were washed with drug-free Sauton medium prior to assay of cell-bound radioactivity. It was subsequently found, however, that one Sauton wash followed by two deionized-water washes reduced the level of cell-bound ^{14}C activity by 60 to 65% in relation to cells washed three times with deionized water only (unpublished data). In the present study, we greatly increased the size of individual samples and assayed deionized water-washed cells. With this system, we were able to demonstrate marked effects of inorganic salts and spermidine on ethambutol binding by *M. smegmatis*.

MATERIALS AND METHODS

Organism. *M. smegmatis* H-607 (3) was grown routinely in 200-ml volumes of modified Sauton synthetic liquid medium (2) at 37 C with aeration (rotary shaking at 150 rpm). Under these conditions, the organism was markedly inhibited by as little as 0.2 μg of ethambutol (free base) per ml. Growth was estimated turbidimetrically. Optical densities were measured at 600 nm in 18 by 150 mm tubes with a Coleman Junior spectrophotometer. An optical density of 0.2 was equivalent to about 0.3 mg (dry weight) of cells per ml.

Ethambutol. Ethambutol dihydrochloride (Myambutol) was a gift from Lederle Laboratories, Pearl River, N.Y. The preparation of ^{14}C-labeled ethambutol was described earlier (1). This material demonstrated biological activity equivalent to that of unlabeled drug, and it had a specific activity of 5.12 μCi per mg (6.92 μCi per mg of free base).

^{14}C-ethambutol uptake experiments. Overnight log-phase cultures were combined and adjusted to an optical density of 0.2 with fresh Sauton medium. Either 75- or 100-ml portions of adjusted suspension were placed in 500-ml Erlenmeyer flasks. ^{14}C-ethambutol, metal salts, and spermidine were added from aqueous stock solutions. At selected time intervals during aerated incubation at 37 C, the cells contained in single flasks were collected on bacteriological filter membranes (diameter, 47 mm; pore size, 0.8 μm) and washed three times with decreasing volumes of deionized water (30, 15, and 2 ml). To avoid corrections of data for filter-pad adsorption of ^{14}C activity, each washed cell mass was scraped from the filter and transferred to a second tared membrane 2.54 cm in diameter. The samples were then dried, reweighed, and placed in scintillation vials containing 10-ml volumes of liquid scintillation counting cocktail. The cocktail consisted of 1 liter of toluene, 42 ml of Liquifluor (Pilot Chemicals, Inc., Watertown, Mass.), and 40 g of Cab-o-sil (Packard Instrument Co., Downers Grove, Ill.). After brief vigorous shaking of the vials, ^{14}C activity was assayed with a Beckman LS-100C liquid scintillation system. The samples were counted either for 20 min or until 4,000 counts had been collected, whichever came first. From 23 to 33 mg (dry weight) of cells was assayed in individual samples, and counting efficiencies were not significantly affected over this range. The data were corrected for background (about 50 counts/min) and normalized to 25 mg of cells.

Growth-protection experiments. Overnight log-phase cultures were combined and diluted with fresh medium to an optical density of about 0.05. Portions of 75 ml were placed in 500-ml nephelo-culture flasks equipped with 19 by 130 mm side arms (Bellco Glass, Inc., Vineland N.J.). Additions of drug and antagonists were made from aqueous stock solutions. Optical densities were measured at time zero and periodically thereafter during aerated incubation at 37 C.

RESULTS

In our system, either 20 mM $MgSO_4$ (plus 4 mM normally present in Sauton medium) or 2.0 mM spermidine trihydrochloride prevented the growth-inhibitory effects of 0.2 μg of ethambutol per ml (3). These represent antagonist-drug molar ratios of 24,000:1 and 2,000:1, respectively. No protective effect was observed when ethambutol was raised to 0.5 μg per ml and the $MgSO_4$ concentration was unchanged. Therefore, all of the binding and growth protection studies reported here were done with drug at the 0.2-μg level.

Binding of ^{14}C activity by growing cells exposed to labeled ethambutol and the effect of 20 mM $MgSO_4$ and 2.0 mM spermidine on the system are shown in Fig. 1. Similar to data reported by Forbes et al. (6), the control cells

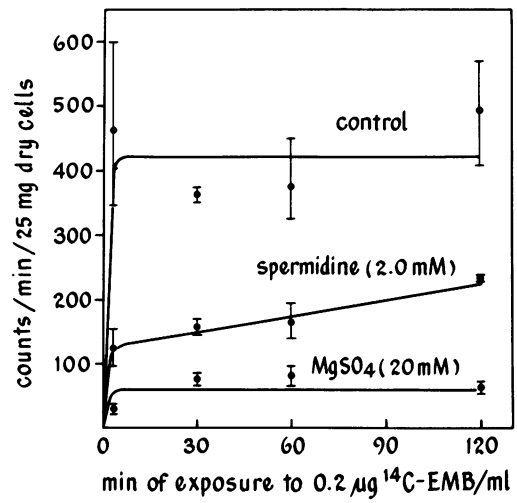

FIG. 1. *Inhibitory effects of 20 mM $MgSO_4$ or 2.0 mM spermidine trihydrochloride on binding of ^{14}C activity by M. smegmatis cells exposed to 0.2 μg of ^{14}C-ethambutol (^{14}C-EMB) per ml. Points shown for the control represent average values of three separate experiments. Those shown for $MgSO_4$ and spermidine are averages of two separate experiments.*

quickly bound a level of radioactivity that did not appear to change significantly during the remainder of the 2-h period examined. When added with labeled drug at time zero, $MgSO_4$ or spermidine exerted pronounced inhibitory effects on cellular binding of ^{14}C activity.

Sodium chloride and Na_2SO_4 did not prevent ethambutol growth inhibition when tested at the same molar concentration as $MgSO_4$ and $MgCl_2$ (7). However, if magnesium salts do, in fact, protect by a nonspecific ionic effect, NaCl and Na_2SO_4 should also be protective if tested at molar concentrations equivalent in ionic strength to 20 mM $MgSO_4$. From the equation $\mu = \sum mZ^2/2$, where μ is ionic strength, m represents the ion molarities, and Z is the ion charges, it was calculated that $\mu = 0.08$ for 20 mM $MgSO_4$. The growth experiment presented in Fig. 2 shows that NaCl (80 mM, $\mu = 0.08$) and Na_2SO_4 (27 mM, $\mu = 0.08$) exerted protective effects that were identical to each other and very similar to that of $MgSO_4$. NaCl (80 mM) also prevented binding of ^{14}C activity when cells were exposed to ^{14}C-ethambutol under the conditions of Fig. 1 (not shown). However, inhibition in the range of 50% was seen rather than the 80 to 85% observed with $MgSO_4$.

Magnesium sulfate and spermidine reversed ethambutol bacteriostasis after a substantial period of previous drug exposure (7). Data presented in Fig. 3 show that addition of 80 mM NaCl to a culture after 90 min of previous ethambutol exposure was about as effective as addition at time zero, and the protected growth responses were very close to those obtained with $MgSO_4$. Also included in Fig. 3 are data showing that 2.0 mM spermidine trihydrochloride ($\mu \leq 0.012$) protected the organism from ethambutol action whether incorporated at time zero or after 90 min of previous drug exposure. Addition of 20 mM $MgSO_4$, 80 mM NaCl, or 2.0 mM spermidine trihydrochloride to cultures after 90 min of exposure to ^{14}C-ethambutol resulted in rapid dramatic reductions in cell-bound ^{14}C activity (Fig. 4). These data are consistent with the growth-protection studies just described (Fig. 3).

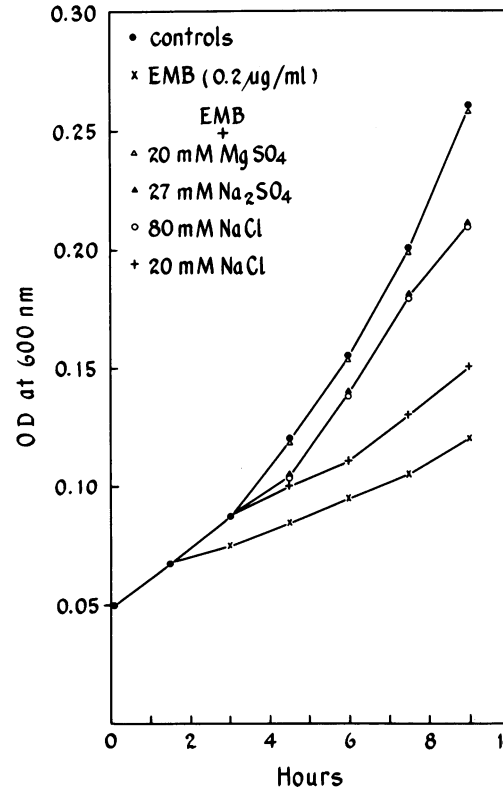

FIG. 2. *Protection of M. smegmatis from ethambutol (EMB, 0.2 μg per ml) growth inhibition by 20 mM $MgSO_4$, 27 mM Na_2SO_4, or 80 mM NaCl. Each of these salt concentrations represents an ionic strength of 0.08. In the absence of drug, the salts neither inhibited nor stimulated growth significantly.*

DISCUSSION

Results from this investigation suggest that $MgSO_4$ and spermidine antagonize ethambutol growth inhibition of *M. smegmatis* by a mechanism(s) involving inhibition of drug binding. The data showed that either antagonist prevented binding and displaced or replaced previously bound drug. Although these studies by themselves explain growth protection, they do not answer the question of whether ethambutol directly and specifically interacts at a cellular target site requiring a divalent cation, a polyamine, or possibly both.

Of considerable importance was our observation that NaCl and Na_2SO_4 behaved almost identically to $MgSO_4$ in the growth-protection studies when tested on the basis of ionic strength rather than molarity. Although drug binding was both blocked and reversed more effectively with 20 mM $MgSO_4$ ($\mu = 0.08$) than with 80 mM NaCl ($\mu = 0.08$), both salts apparently kept cell-bound drug below a critical level. Their capacities to prevent growth inhibition were indistinguishable. These results, in conjunction with studies showing antagonism of streptomycin by inorganic salts (3-5, 8, 9), indicate that binding inhibition and growth protection by $MgSO_4$ are nonspecific. Protection appears to involve a general ionic effect that may operate at one or more levels including primary adsorption, transport, or a specific target site of ethambutol action. Forbes' argu-

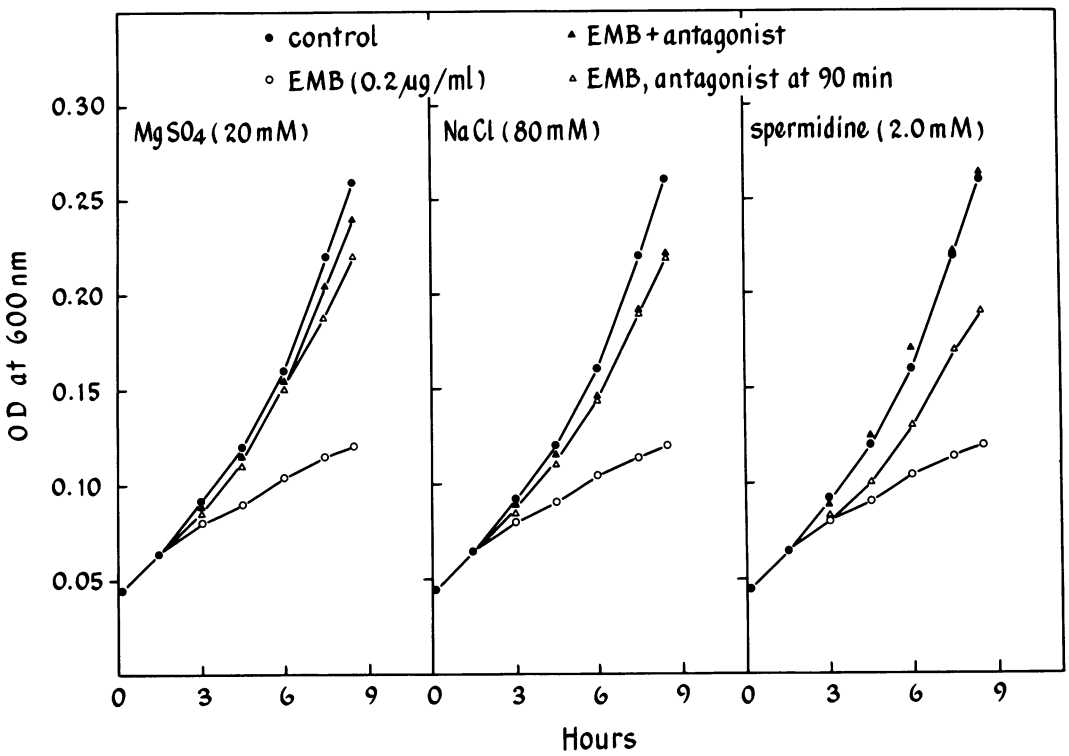

FIG. 3. *Protection of M. smegmatis from ethambutol (EMB, 0.2 μg per ml) growth inhibition by 20 mM $MgSO_4$, 80 mM NaCl, or 2.0 mM spermidine trihydrochloride added either with drug at time zero or after 90 min of drug exposure.*

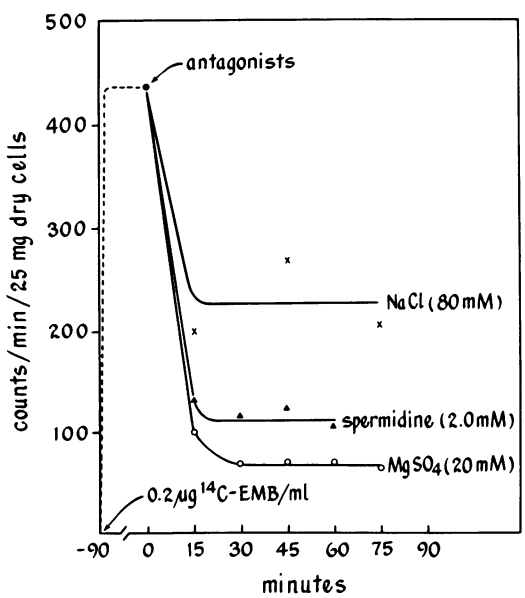

FIG. 4. *Reversal of ^{14}C binding to M. smegmatis by 20 mM $MgSO_4$, 80 mM NaCl, or 2.0 mM spermidine added after 90 min of exposure to 0.2 μg of ^{14}C-ethambutol (^{14}C-EMB) per ml.*

ment for a divalent cation-requiring system as a specific target of ethambutol action is, therefore, considerably weakened.

Spermidine (like NaCl) did not block ethambutol binding as effectively as $MgSO_4$ at the concentration tested. Based on the growth-protection studies, however, one must conclude that the modest binding inhibition observed was sufficient to keep the cellular level below that necessary for growth-inhibitory activity. Since polyamines often can partially replace Mg^{2+} in physiological processes (10), spermidine trihydrochloride may antagonize ethambutol by virtue of its cationic character. However, spermidine was effective at a molarity and an ionic strength substantially less than that required for the metal salts ($\mu = \leq 0.012$ versus $\mu = 0.08$). It seems likely, therefore, that the effectiveness of spermidine rests more on its structural similarity to ethambutol than on its ionic nature. Drug and polyamine may compete for one specific site or for a heterogenous group of binding sites involving initial adsorption, transport, and inhibition of particular intracellular biochemical reactions. At present, we do not know at which of these possible levels

spermidine antagonizes ethambutol binding. Until such information is available, nothing definite can be concluded regarding the possible relationship between polyamines and ethambutol action.

LITERATURE CITED

1. Beggs, W. H., and N. E. Auran. 1972. Uptake and binding of ^{14}C-ethambutol by tubercle bacilli and the relation of binding to growth inhibition. Antimicrob. Ag. Chemother. **2**:390-394.
2. Beggs, W. H., and J. W. Jenne. 1967. Mechanism for the pyridoxal neutralization of isoniazid action on *Mycobacterium tuberculosis*. J. Bacteriol. **94**:793-797.
3. Beggs, W. H., and N. E. Williams. 1971. Protection of *Mycobacterium smegmatis* from ethambutol and streptomycin inhibition by $MgSO_4$ and polyamines. Infect. Immunity **3**:496-497.
4. Bragg, P. D., and W. J. Polglase. 1963. Action of dihydrostreptomycin and antagonism by cations. J. Bacteriol. **85**:590-594.
5. Donovick, R., A. P. Bayan, P. Canales, and F. Pansy. 1948. The influence of certain substances on the activity of streptomycin. III. Differential effects of various electrolytes on the action of streptomycin. J. Bacteriol. **56**:125-137.
6. Forbes, M., N. A. Kuck, and E. A. Peets. 1962. Mode of action of ethambutol. J. Bacteriol. **84**:1099-1103.
7. Forbes, M., N. A. Kuck, and E. A. Peets. 1965. Effect of ethambutol on nucleic acid metabolism in *Mycobacterium smegmatis* and its reversal by polyamines and divalent cations. J. Bacteriol. **89**:1299-1305.
8. Mager, J., M. Benedict, and M. Artman. 1962. A common site of action for polyamines and streptomycin. Biochim. Biophys. Acta **62**:202-204.
9. Plotz, P. H., D. T. Dubin, and B. D. Davis. 1961. Influence of salts on the uptake of streptomycin by *Escherichia coli*. Nature (London) **191**:1324-1325.
10. Tabor, H., and C. W. Tabor. 1964. Spermidine, spermine, and related amines. Pharmacol. Rev. **16**:245-300.