Inhibition of Dihydrostreptomycin Action on Mycobacterium smegmatis by Monovalent and Divalent Cation Salts

WILLIAM H. BEGGS* and FRED A. ANDREWS

Bacteriology Research Laboratory, General Medical Research Service, Veterans Administration Hospital, Minneapolis, Minnesota 55417

Received for publication 20 January 1975

We have examined and compared the effects of monovalent and divalent cation salts on dihydrostreptomycin (DSM) action against Mycobacterium smegmatis. The Sauton synthetic liquid medium used was supplemented with test salts on the basis of ionic strength (\(\mu\)). Turbidimetric growth experiments showed that 0.02 M MgSO\(_4\) (\(\mu = 0.08\)) prevented growth inhibition by 0.1 \(\mu\)g of dihydrostreptomycin per ml, but 0.02 M NaCl (\(\mu = 0.02\)) did not. However, at molarities equivalent to \(\mu = 0.08\), four monovalent cation salts, including NaCl, Na\(_2\)SO\(_4\), NH\(_4\)Cl, and (NH\(_4\))\(_2\)SO\(_4\), all prevented inhibition by dihydrostreptomycin. When magnesium and sodium salts were compared at \(\mu = 0.02\), 0.04, and 0.05, two distinct growth protective patterns were seen. These data were indicative of two different mechanisms of dihydrostreptomycin antagonism by salts; the first being divalent cation and concentration dependent, and the second being nonspecific and ionic strength dependent. Viability studies supported the existence of two mechanisms.

Inorganic salts can reduce or neutralize the antimicrobial activity of streptomycin (3, 5, 9, 12) and related antibiotics (8, 11, 15, 17-19). Both monovalent (5, 9, 12, 15, 17) and divalent (3, 8, 9, 11, 15, 18, 19) cation salts have been implicated as aminoglycoside antagonists, but the latter have generally appeared to be the most effective (9, 11, 15). The double positively charged metal-ion component of these divalent cation salts apparently plays the primary role in this inhibitory activity (9, 11, 15). Experiments with \(^{14}\)C-labeled streptomycin have shown that salts interfere with drug uptake or binding by susceptible organisms (3, 6, 16).

Since tuberculosis chemotherapy has relied heavily upon streptomycin, it is surprising that the influence of salts on aminoglycoside action against the mycobacteria has received little attention. In 1971, we reported that MgSO\(_4\) neutralized the action of streptomycin on Mycobacterium smegmatis (3). Further studies with dihydrostreptomycin indicated that this antagonism was a divalent cation-dependent effect (2). However, several recent preliminary experiments showed that under the proper conditions NaCl could inhibit dihydrostreptomycin (DSM) action as effectively as MgSO\(_4\). In the present study, we have examined and compared monovalent and divalent cation salt antagonism of DSM action on M. smegmatis. The results suggested that the two classes of salts interfere with drug action by different mechanisms.

**MATERIALS AND METHODS**

Organism and growth conditions. M. smegmatis (H-607) was provided by the United States-Japan Cooperative Medical Science Program, National Institute of Allergy and Infectious Diseases. It was grown at 37 C with rotary shaking (150 rpm) in Sauton synthetic liquid medium comprised of 2.0 g of citric acid, 0.5 g of MgSO\(_4\), 50 mg of ferric ammonium citrate, 0.5 g of K\(_2\)HPO\(_4\), 4.0 g of L-asparagine, 35 ml of glycerol, 0.02% Tween 80, and deionized water to 1 liter. The medium was adjusted to pH 7.4 with 5 N NaOH and autoclaved.

**Test salts.** Sauton medium, with a measured ionic strength of approximately 0.03 (2), was supplemented with various test salts at molarities calculated to give desired added increments of ionic strength. These concentrations were determined from the equation \(\mu = \sum mZ^2/2\), where \(\mu\) is ionic strength, \(m\) is ion molarity, and \(Z\) is ion charge. The salts studied included MgSO\(_4\), MgCl\(_2\), NaCl, Na\(_2\)SO\(_4\), NH\(_4\)Cl, and (NH\(_4\))\(_2\)SO\(_4\). These compounds were either added as dry salts during the preparation of Sauton medium or added from sterile stock solutions after sterilization of the medium. Changes in medium pH resulting from these additions were negligible.

**Drugs.** DSM sulfate was obtained from Calbiochem Co., La Jolla, Calif., and kanamycin sulfate from Bristol Laboratories, Syracus, N.Y. Stock solutions were prepared in distilled water and filter sterilized. All concentrations are expressed in terms of free-base drug.
Turbidimetric growth experiments. Salts and drugs were added to 50-ml portions of Sauton medium contained in 125-ml Erlenmeyer flasks. Each flask was inoculated from a log-phase culture, incubated for 20 to 22 h at 37 C with rotary shaking, and assayed for growth with a Coleman Junior spectrophotometer. Optical densities were measured at 600 nm in tubes (18 by 150 mm). Time zero optical densities were approximately 0.008.

Viability studies. The experimental conditions employed in the viability studies were identical to those described above. At selected time intervals over a 12-h incubation period, 1.0-ml portions of each culture were serially diluted in 0.9% NaCl solution containing 0.02% Tween 80. Triplicate samples of appropriate dilutions were pipetted into petri dishes and pour plates were prepared with tryptose blood agar base (Difco Laboratories, Detroit, Mich.). Colonies were counted after 3 days of incubation at 37 C.

RESULTS

In our earlier studies, the addition of 0.02 M MgSO₄ (µ = 0.08) to Sauton medium protected M. smegmatis from inhibition by 0.1 µg of streptomycin/ml (3). At 0.02 M, NaCl (µ = 0.02) was ineffective in this respect (unpublished observation). Data presented in Fig. 1 showed that when NaCl was added to Sauton medium at 0.08 M (µ = 0.08), the organism was protected from 0.1-µg concentrations of either DSM or kanamycin per ml. Both drugs were highly inhibitory at this level in unsupplemented medium. Further studies showed that NaCl, Na₂SO₄, NH₄Cl, and (NH₄)₂SO₄ were essentially equivalent to magnesium salts as DSM antagonists when all were tested at µ = 0.08 (Fig. 2).

Two distinctly different patterns of DSM antagonism were seen when the chloride and sulfate salts of sodium and magnesium were compared at several ionic strengths less than µ = 0.08 (Fig. 3). The inhibitory effect of 0.1 µg of DSM/ml was 90 to 100% neutralized by the incorporation of either magnesium salt at µ = 0.04 (0.01 M MgSO₄ and 0.013 M MgCl₂). In contrast, the sodium salts were only 35 to 40% protective at µ = 0.04 (0.04 M NaCl and 0.013 M Na₂SO₄). As ionic strength was increased with the sodium salts, the degree of growth protection also increased. All four salts were 90 to 100% protective at µ = 0.08, as expected.

Viability data presented in Fig. 4 showed that both MgCl₂ and NaCl inhibited the lethal action of 0.2 µg of DSM/ml when tested at µ = 0.03. However, the two salts demonstrated markedly different capacities for antagonism of drug. At 0.01 M, MgCl₂ completely prevented bactericidal activity for at least 12 h. Although 0.03 M NaCl delayed significantly the onset of drug kill, there was over a 2-log reduction in viable organisms between 6 and 12 h.

DISCUSSION

When Sauton medium was supplemented with 0.02 M MgSO₄, inhibition of M. smegmatis by 0.1 µg of DSM/ml was prevented. In contrast, drug action was not affected significantly by 0.02 M NaCl. However, four monovalent cation salts including NaCl, Na₂SO₄, NH₄Cl, and (NH₄)₂SO₄ prevented DSM activity when tested at molarities calculated to give the same ionic strength as 0.02 M MgSO₄ (i.e., µ = 0.08). Kanamycin action on the organism was also inhibited by NaCl at µ = 0.08, indicating that the NaCl effect was not peculiar to DSM. One might conclude from these data that salt antagonism of aminoglycoside action simply involves a nonspecific ionic strength effect. Although this conclusion apparently is at least partially correct, further experiments were indicative of a more complex situation.

The two distinctly different patterns of antagonism seen in Fig. 3 suggested that monovalent and divalent cation salts inhibit drug action by different mechanisms. When µ was increased from 0.02 to 0.04 with either magnesium salt, there was a sudden shift from very little effect to complete neutralization of DSM action. Since the anion components of the sodium and magnesium salts compared were identical, this single-step pattern was very likely an effect produced by the double positive-charged mag-
Bactericidal activity of DSM against *M. smegmatis* was markedly reduced by NaCl, thus confirming the turbidometric growth experiments which showed that monovalent cation salts can antagonize DSM activity. However, at a fixed ionic strength of $\mu = 0.03$, MgCl$_2$ was clearly superior to NaCl in this respect. These data provided further evidence that antagonism of drug action by divalent cation salts involves more than simply an ionic strength effect.

We have presented evidence that salt antagonism of DSM action on *M. smegmatis* involves both a divalent cation concentration-dependent mechanism and a nonspecific ionic strength-dependent effect elicited by monovalent cation salts. The nature of these salt-drug interactions and the cellular and subcellular sites that could be involved remain for further study. With regard to the divalent cation-dependent mechanism, there are several interesting possibilities. A double-charged metal ion might simply compete effectively with positive-charged drug molecules for binding sites at or near the cell surface. Such binding sites could be associated with either initial electrostatic physicochemical adsorption of drug (4, 10, 13) or with a streptomycin transport protein (13). Another possibility is that divalent cations protect or maintain the integrity of the bacterial cell wall as

![Graph](image)

**Fig. 2.** Protection of *M. smegmatis* from DSM growth inhibition by MgSO$_4$, MgCl$_2$, and several monovalent cation salts. The test salts were compared for antagonism of DSM action on the basis of ionic strength ($\mu = 0.08$).

![Graph](image)

**Fig. 3.** Protection of *M. smegmatis* from DSM as a function of ionic strength ($\mu$) increases with either magnesium or sodium salts. At each ionic strength of each salt tested, growth was assayed in the presence and absence of DSM. Percentage of growth protection was calculated from these pairs of values.
suggested by Zimelis and Jackson (19). Since the primary target of streptomycin action exists at one or more sites on the bacterial ribosome, antagonism of drug by divalent cations might occur at this subcellular level. Studies with cell-free systems prepared from Escherichia coli have strengthened this possibility. For example, the work of Mager et al. (14) suggested that divalent cations can compete with streptomycin for ribosomal binding sites. A second example is the recent work of Chang and Flaks (7). These workers showed that although optimal binding of DSM to E. coli ribosomes required about 10 mM magnesium ion, higher concentrations of the cation resulted in reduced levels of ribosomal-bound drug. Whether these interactions observed in cell-free systems occur to any significant degree within the intact cell is, of course, not known.

LITERATURE CITED