Inhibition of Dihydrostreptomycin Binding to *Mycobacterium smegmatis* by Monovalent and Divalent Cation Salts

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To better understand salt antagonism of dihydrostreptomycin (DSM) action on *Mycobacterium smegmatis*, the effects of monovalent and divalent cation salts on drug uptake were studied in relation to the lethal activity of DSM. In Sauton liquid medium NaCl, MgCl₂, and SrCl₂ inhibited the initial instantaneous binding of [³H]DSM to the organism and suppressed secondary uptake. These data correlated well with the capacity of each salt to prevent the lethal activity of DSM. It was concluded that monovalent and divalent cation salt antagonism of DSM action on *M. smegmatis* involves nonspecific interference with drug uptake.

Previous studies in our laboratory showed that both monovalent and divalent cation salts can antagonize the inhibitory and bactericidal effects of dihydrostreptomycin (DSM) on *Mycobacterium smegmatis* (3, 4). The mechanisms involved, however, appeared to be different for the two classes of salts (3). Results suggested that antagonism by monovalent cation salts is a nonspecific ionic strength effect. In contrast, neutralization of DSM action by MgSO₄ and MgCl₂ appeared to involve a divalent cation concentration-dependent mechanism. Since chloride salts of the nutritionally nonessential cations Sr²⁺ and Ba²⁺ inhibited DSM action as effectively as the chlorides of Mg²⁺ and Ca²⁺ (4), it appeared that divalent cation antagonism is dependent more upon a double positive charge than on a particular metal ion species. We postulated, therefore, that the divalent cation effect, like the ionic strength effect, probably involves nonspecific interactions that suppress cellular uptake of the aminoglycoside. Streptomycin uptake by susceptible organisms appears to be a diphasic process: an initial instantaneous binding followed by a secondary period of time-dependent uptake (1, 7, 11, 14). In the present study, evidence is presented that monovalent and divalent cation salts can interfere with both of these events. Uptake data correlated well with viability studies, showing that each salt prevented or retarded the bactericidal action of DSM.

MATERIALS AND METHODS

General. The source of *M. smegmatis*, the composition of Sauton medium, growth conditions, preparation of DSM solutions, additions of test salts, and

methods used in viability studies were previously described in detail (3, 5). It should be stated again, however, that Sauton medium was supplemented with test salts on the basis of ionic strength (μ). From the equation $\mu = \Sigma mZ^2/2$, where m is the ion molarity and Z is the ion charge, molarities were calculated that gave desired μ values.

[³H]DSM binding and uptake experiments. Labeled drug ([³H]DSM sesquisulfate) was purchased from Amersham/Searle, Arlington Heights, Ill. The material had a specific activity of 3.0 Ci/mmol (2.1 mCi/mg). Aqueous solutions of labeled and unlabeled DSM sesquisulfate were mixed to give a working stock solution that contained 2.0 μ g of [³H]DSM-free base plus 18 μ g of DSM-free base per ml (total of 20 μ g of DSM, and 5.0 μ Ci/ml). Overnight 16- to 18-h Sauton cultures of *M. smegmatis* grown at 37 C with rotary shaking (150 rpm) were diluted with fresh medium to an optical density (600 nm) of 0.05 (0.07 mg [dry weight] of cells per ml). Desired concentrations of salts were added to 100-ml volumes of this suspension in 250-ml Erlenmeyer flasks. Labeled drug was then added at a concentration of 0.2 μ g of [³H]DSM-free base and 0.05 μ Ci/ml. These cultures were incubated at either 4 or 37 C with rotary shaking. At selected time intervals, the cells contained in 8.0-ml samples were collected on bacteriological filter membranes (diameter, 2.54 cm; pore size, 0.8 μ m) under negative pressure. The cell-containing filter membranes were then washed three times with 2-ml volumes of either deionized water or drug-free Sauton medium, dried, and assayed for radioactivity as previously described (2). In the 37 C experiments, optical densities at 600 nm were determined at appropriate sampling times and converted to milligrams (dry weight) of cells per milliliter from a standard curve. Counting data were corrected for filter membrane adsorption of radioactivity in the following manner. Labeled drug was added at 0.2 μ g/ml to portions of Sauton medium with and without appropriate concentrations of the various test

salts. An 8.0-ml volume of each solution was passed through a separate filter membrane, followed by an 8.0-ml volume of unlabeled cell suspension (optical density at 600 nm = 0.05). These samples were then washed twice with either deionized water or Sauton medium, dried, and counted. Counts obtained in these blank samples were subtracted from values obtained in the corresponding experimental samples.

RESULTS

Both MgCl_2 and NaCl protected *M. smegmatis* from the lethal action of 0.2 μg of DSM per ml (Fig. 1). It is apparent, however, that MgCl_2 was more effective than NaCl . Note that a high level of NaCl (0.08 M, $\mu = 0.08$) mimicked the protection provided by MgCl_2 (0.01 M, $\mu = 0.03$). The complete protection from lethal activity provided by SrCl_2 (0.01 M, $\mu = 0.03$) was consistent with earlier findings (4) and made it increasingly doubtful that divalent cation salt antagonism of DSM involves a specific physiological or metabolic effect. Therefore, further experiments were designed to test the possibility that each of the salts inhibits DSM action against *M. smegmatis* by nonspecific interference with drug uptake.

The kinetics of $[^3\text{H}]\text{DSM}$ uptake by *M. smegmatis* are shown in Fig. 2. A diphasic curve similar to that shown in streptomycin uptake studies with other organisms (1, 7, 11, 14) was seen in the results with water-washed cells.

FIG. 1. Protection of *M. smegmatis* from the lethal action of DSM by monovalent and divalent cation salts. μ , Calculated ionic strengths of added salts.

FIG. 2. Kinetics of $[^3\text{H}]\text{DSM}$ uptake by *M. smegmatis* at 37°C. Comparison of cells washed three times with deionized water (water wash) to cells washed three times with Sauton medium (Sauton wash).

After an apparent instantaneous binding of the drug, there was a 60-min plateau followed by a rapid linear period of drug uptake lasting 2 h. The ease with which 90% of the drug bound during the first minute was removed by Sauton (salt solution) washing indicated a weak drug-cell interaction that may primarily involve electrostatic attraction (11). Approximately 10% of the $[^3\text{H}]\text{DSM}$ taken up during the first minute was rather firmly bound, as evidenced by its resistance to Sauton washing. Note in Fig. 2 that for all of the time periods selected for sampling, subtraction of Sauton-wash from water-wash values gave counts approximating instantaneous binding that plotted as a horizontal line. This shows that essentially all drug taken up after initial instantaneous binding was rather firmly bound.

NaCl (0.08 M, $\mu = 0.08$) protected *M. smegmatis* from the lethal action of 0.2 μg of DSM per ml when both were added at time zero (Fig. 1). When NaCl was added 45 min after DSM (i.e., the late plateau period of the uptake curve in Fig. 2), the same high level of protection was observed (Fig. 3). Although addition of NaCl after 90 min of previous DSM exposure delayed the onset of measurable bactericidal activity, it did not prevent such activity. At 90 min, secondary uptake of DSM was well under way (Fig. 2). These data suggested that binding to critical sites of DSM action is associated with the Sauton-stable or firmly bound fraction.

The experiment presented in Fig. 4 shows the inhibitory effects of the various salts on

[^3H]DSM uptake by *M. smegmatis* (as measured in Sauton-washed cells). The rate and extent of uptake was strongly inhibited in each case. Low-level NaCl was the least suppressive, followed in increasing order of effectiveness by MgCl_2 , high-level NaCl, and SrCl_2 . These data correlated well with the viability studies seen in Fig. 1. Note that between 3 and 4 h uptake in the presence of 0.03 M NaCl approached the

4,000- to 5,000-counts/min level measured at 90 min in the control culture containing no added salt. It is interesting that measurable bactericidal activity began in the control and in the 0.03 M NaCl-containing cultures after 90 min and 3 h, respectively (Fig. 1). Previous reports by other workers showed that cellular streptomycin can reach levels in considerable excess of that required for inhibitory activity (14, 15). The 2×10^4 -counts/min level reached in the control (no salt) culture at 3 h (Fig. 4) supports these earlier findings.

The effects of salts on instantaneous binding of DSM to *M. smegmatis* cells are shown in Fig. 5. To minimize complications imposed by secondary uptake, these studies were performed at 4 C. The salts markedly blocked instantaneous binding, and the degree of inhibition correlated well with uptake studies (Fig. 4) and with the ability of each salt to prevent the lethal action of DSM (Fig. 1). Instantaneous binding was measured with water-washed cells. As a control in the 4 C experiment (Fig. 5), duplicate samples were taken from the culture containing no added salt. One sample was water washed, and the second was Sauton washed. The negligible levels of bound radioactivity measured in the

FIG. 3. Protection of *M. smegmatis* from the lethal action of DSM by 0.08 M NaCl (ionic strength, 0.08) added at time zero and at 45 and 90 min after DSM.

FIG. 4. Effects of various salts on [^3H]DSM uptake by *M. smegmatis* at 37 C. MgCl_2 and SrCl_2 tested at 0.01 M (ionic strength, 0.03). NaCl tested at (asterisk) 0.03 M (ionic strength, 0.03)* and at 0.08 M (ionic strength, 0.08). Uptake measured in Sauton-washed cells.

FIG. 5. Effects of various salts on initial binding of [^3H]DSM by *M. smegmatis* at 4 C. μ , Calculated ionic strengths of added salts. Binding measured in water-washed cells, except where Sauton wash is indicated.

latter indicated that over 95% of the cell-associated drug was Sauton labile and therefore weakly bound.

DISCUSSION

It has been clearly established that inorganic salts can adversely affect the action of aminoglycoside antibiotics on various bacterial species (3, 6, 8, 10, 12, 13, 16, 19). As shown in earlier work from our laboratory (3, 4) and confirmed in the present report, both monovalent and divalent cation salts can inhibit DSM activity against *M. smegmatis*. Although differences in the characteristics of DSM antagonism by the two different types of salts were observed (3), we felt that both effects probably involved interference with drug uptake. The main purpose of the present investigation was to test this hypothesis. Salt antagonism of [^{14}C]streptomycin uptake by bacteria has been reported (6, 9, 17), and recently Ramirez-Ronda et al. (18) showed that Mg^{2+} and Ca^{2+} inhibited initial rapid binding [^3H]gentamicin to *Pseudomonas aeruginosa*. In the present study, we examined and compared the effects of monovalent and divalent cation salts on the different phases of [^3H]DSM uptake by *M. smegmatis*.

Consistent with capacities to prevent or retard the lethal action of DSM, NaCl , MgCl_2 , and SrCl_2 all markedly inhibited both the rate and extent of Sauton-stable drug uptake. Even more interesting, and perhaps of greater significance, was the inhibitory effect that each salt had on primary or instantaneous Sauton-labile binding of DSM. Each salt blocked this reaction to a degree that roughly coincided with ability to prevent lethality. These data indicated clearly that both types of salts interfere with DSM uptake. On both molar- and ionic-strength bases, MgCl_2 and SrCl_2 were more effective DSM antagonists than NaCl . This may be not only due to the presence of a double positive charge, but also to the particular distribution of these charges on the cations.

Studies on the kinetics of [^3H]DSM uptake indicated that weak binding precedes appreciable Sauton-stable binding, but we did not show conclusively that weak binding is a prerequisite for secondary uptake. In the presence of salts, however, decreases in Sauton-labile binding correlated well with decreases in secondary uptake. If we can assume that weak binding is a prerequisite for secondary uptake, it is probable that inhibition of the initial rapid weak-binding reaction is the key event responsible for monovalent and divalent cation salt antagonism of DSM action on *M. smegmatis*.

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