Nature of the Bactericidal Action of Antimycin A for Bacillus megaterium

ROBERT E. MARQUIS

Department of Microbiology, The University of Rochester School of Medicine and Dentistry, Rochester, New York

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ABSTRACT

Marquis, Robert E. (University of Rochester, Rochester, N.Y.). Nature of the bactericidal action of antimycin A for Bacillus megaterium. J. Bacteriol. 89:1453-1459. 1965.—Antimycin A, a fungicidal antibiotic which specifically inhibits metabolic reduction of cytochrome c, was found to be lethal for Bacillus megaterium. However, the bactericidal action was correlated with a capacity of antimycin to hinder plasma-membrane functions other than cytochrome-mediated respiration. With conditions under which oxygen consumption was not appreciably depressed, antimycin almost completely inhibited concentrative uptake of both \( \alpha \)-aminoisobutyrate and \( \alpha \)-methylglucoside, and also caused death of cells. When present in amounts greater than those required for killing or for inhibition of nutrient uptake, antimycin also induced extensive loss of inorganic phosphate and other substances from whole cells, inhibited aerobic respiration, and acted as a lytic agent for isolated protoplasts. The lytic potency of antimycin was greater, on a molar basis, than that of digitonin, hexachlorophene, polymyxin B, and all but one of a number of test detergents. Protoplasts concentrated antimycin primarily in or on the plasma membrane, and the refractive index of isolated protoplast membranes rose sharply as a result of antimycin binding. In all, antimycin-induced lysis appeared not to include dissolution of the protoplast membrane similar to that produced by dodecyl sulfate. Rather, the lytic process seemed more akin to that induced by cationic detergents or by polymyxin.

Antimycin A is a fungicidal agent which has been found (Leben and Keitt, 1948; Lockwood, Leben, and Keitt, 1954; Strong, 1958) to inhibit the growth of many molds and yeasts when present in low concentrations, about \( 10^{-8} \) to \( 10^{-6} \) m. The mechanism of inhibition appears to be related to an interference with electron transfer between cytochromes \( b \) and \( c \) (Strong, 1958; Gottlieb and Ramachandran, 1961). Antimycin A is also a powerful respiratory inhibitor for mammalian cells and for cells of higher plants (Strong, 1958), but it is generally considered to have little or no antibacterial action. The original papers of Leben and Keitt (1948) and Lockwood et al. (1954) did, however, indicate that relatively large amounts of antimycin were toxic for a number of bacterial species, including members of the genus Bacillus. Szulmajster (1964) recently reported inhibition of electron transport in reduced nicotinamide adenine dinucleotide-oxidizing particles of \( B. subtilis \) exposed to \( 10^{-5} \) to \( 10^{-4} \) mmoles of antimycin A per ml. I have found that antimycin is lethal for \( B. megaterium \) when employed in these relatively large amounts. However, as will be described in this report, the bactericidal action of antimycin can be more closely correlated with its capacity to affect membrane-centered transport processes than with its role as an inhibitor of electron transport.

MATERIALS AND METHODS

Organism. \( B. megaterium \) KM was grown at 25 C in 2\% peptone (Oxoid) broth with aeration and agitation. This strain is asporogenous; the species is strictly aerobic and possesses a functional cytochrome system (Weibull and Bergström, 1958; Vernon and Mangum, 1960). Cells were harvested by centrifugation in the cold at the phase of declining growth rate; growth was measured turbidimetrically.

Chemicals. Antimycin A (B grade, Calbiochem) was dissolved in absolute ethyl alcohol for use; it is almost insoluble in water. \( \alpha \)-Aminoisobutyric acid (\( \text{AIB} \))-\( -\text{C}^{14} \) was obtained from Nuclear Chicago Corp., Des Plaines, Ill., and \( \alpha \)-methyl-\( \text{n} \)-glucoside-\( \text{U} \)-\( \text{C}^{14} \), from Calbiochem.

Experiments with whole cells. Harvested cells were washed once with cold distilled water and suspended in cold water. Portions of the resulting suspension were added to Warburg flasks containing appropriate solutions for the final suspending medium. The Warburg flasks were quickly put into
a water bath at 25 C, and, after temperature equilibration with agitation, 0.1 ml of ethyl alcohol or of an ethanolic solution of antimycin per 3.0 ml of final incubation mixture was tipped in from a side arm. This amount of ethyl alcohol slightly stimulated oxygen consumption, but did not affect viability. In experiments involving measurement of aminoisobutyrate or methylglucoside uptake, solutions of these compounds were also tipped in from the side arms at the same time as antimycin.

Methods for measuring uptake of aminoisobutyrate were described previously (Marquis and Gerhardt, 1964), and the same methods were used for methylglucoside.

For measurements of viability, 1.0-ml samples of control or antimycin-treated suspensions were pipetted from the Warburg flasks into 99 ml of 1% peptone broth. Further dilutions were made with peptone broth, and triplicate 0.1-ml samples of diluted suspensions were spread over the surface of plates of Trypticase Soy Agar (BBL). The plates were incubated at 25 to 30 C for 36 hr before colonies were counted.

Orcinol-positive substances in supernatant fluids were measured according to the method of Ceriotti (1955); inorganic phosphate was measured by the method of Fiske and SubbaRow (1925), and the biuret reaction was carried out according to the method described by Gornall, Bardawill, and David (1949).

The dry weight of cells was determined by weighing the residue of suspensions of cells which had been washed in cold water and then dried overnight at 100 C.

Experiments with protoplasts. Harvested cells were suspended in cold molal sucrose solution containing 0.1 mg of lysozyme per ml and 2 mg phosphate buffer (pH 7). They were essentially all converted to protoplasts during overnight incubation at 4 C. The suspensions were warmed to room temperature (about 25 C), and diluted to the desired concentration (about 2 X 10^8 protoplasts per milliliter) with sucrose solution. A 0.1-ml amount of ethyl alcohol or ethanolic antimycin solution was added to 3.0 ml of the suspension at zero-time. Optical density changes at 700 m,u were then determined by use of a Beckman DU spectrophotometer. Protoplast concentrations were measured with a Petroff-Haussier counting chamber and, if possible, at least 100 protoplasts were counted per sample.

Experiments with protoplast membranes. Protoplast membranes were prepared by first lyzing centrifuged protoplasts by suspending them in cold MgCl solution (2 X 10^{-3} M) containing 0.04 M phosphate buffer (pH 7), and then centrifuging the lysate for 25 min at 65,900 X g in a refrigerated Spinco ultracentrifuge (model L). The resulting pellet was washed four times with cold MgCl-phosphate solution before being used in experiments similar to those described above for intact protoplasts. The membrane preparation appeared, microscopically, to be homogeneous and devoid of both whole cells and unbroken protoplasts.

Protoplast membranes were suspended in water by repeatedly expelling a mixture of membranes and water through a 21-gauge needle attached to a 10-ml syringe. Before being used, the resulting suspension was centrifuged for about 1 min at 3,020 X g in a Servall centrifuge (model RC-2) to remove large clumps of material.

Assay of antimycin. Antimycin was assayed by measuring its absorbancy at 325 m,u (Strong, 1958).

**RESULTS**

Experiments with whole cells. The effects of antimycin A on viability and oxygen consumption of nonmultiplying cells of _B. megaterium_ are depicted in Fig. 1. This antifungal agent was highly bactericidal in amounts which stimulated rather than depressed oxygen consumption, and it appeared that death was due to some cause other than respiratory arrest. Time, temperature, aeration, and the amount of antimycin present were all found to be variables in the killing process. The dose of antimycin required for 50% death of cells under aerobic conditions within 90 min was 3.8 mmoles per mg (dry weight) of cells.
Comparable values for the detergents Triton X-100 and cetyltrimethylbenzylammonium chloride were, respectively, 23 and 25 μmoles per mg of cells.

A number of fungicides have been found to hinder plasma membrane functions, and a similar action was evident for antimycin. Cells exposed to the antibiotic released compounds of small molecular weight (Table 1). Relatively large amounts of antimycin were required to induce these permeability defects, which were accompanied by a drop in oxygen consumption. Indeed, 68 μmoles of antimycin per mg (dry weight) of cells induced essentially no loss of solutes of small molecular weight nor any decline in respiration, but was found to cause almost 90% death. Hence, major breakdown of the cell's osmotic barriers did not appear to constitute the primary lethal action of antimycin.

The proposition that antimycin impairs normal functions of the plasma membrane without causing membrane lysis was explored by studying the effects of this compound on uptake of AIB, a nonmetabolized analogue of alanine. AIB is taken up via two processes by B. megaterium (Marquis and Gerhardt, 1964). One is obligatorily coupled to aerobic respiration and leads to concentration of AIB in the cell protoplast. The other is independent of respiration and not concentrative. As shown in Fig. 2, respiration-coupled AIB uptake was strongly inhibited by antimycin [50% inhibition at 6.2 μmoles of antimycin per mg (dry weight) of cells], even though respiration was largely unaffected. AIB uptake was not depressed entirely to the passive level, but this result is probably interpretable in terms of membrane damage and subsequent exposure of AIB-binding sites within the protoplast. In this experiment, respiration was solely endogenous and independent of uptake of exogenous respiratory substrates. Antimycin also hindered AIB uptake by cells exposed to exogenous glucose, but did not depress their oxygen consumption. Further, antimycin did not seem specifically to inhibit cytochrome-linked oxidations, since respiration was equally sensitive to cyanide in antimycin-poisoned and control cells. Endogenous oxygen uptake was depressed some 60% by 4.6 × 10⁻⁴ M HCN in either case.

![Figure 2. Effects of antimycin A on endogenous respiration and uptake of α-aminoisobutyrate (AIB).](http://jb.asm.org/)

Table 1. Antimycin-induced loss of intracellular materials from Bacillus megaterium

<table>
<thead>
<tr>
<th>Treatment of cells</th>
<th>Substance found in suspending fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Orcinol-positive</td>
</tr>
<tr>
<td>Antimycin, μmoles/mg (dry wt) of cells</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>5.8</td>
</tr>
<tr>
<td>68</td>
<td>5.8</td>
</tr>
<tr>
<td>136</td>
<td>12.4</td>
</tr>
<tr>
<td>Trichloroacetic acid, 5%</td>
<td>25.8</td>
</tr>
</tbody>
</table>

* Experimental conditions: 13.6 mg (dry weight) of cells were suspended for 30 min at 25°C in 3 ml of 0.03 M NaCl solution containing 0.1 ml of ethyl alcohol or ethanolic solution of antimycin. QO₂ values were: 48.1 for control cells, 46.6 and 34.4 for cells exposed to 68 and 136 μmoles of antimycin, respectively.

† Orcinol-positive substances and inorganic phosphate are expressed as millimicromoles per milligram (dry weight) of cells. Ribose was used as a standard for measuring orcinol-positive substances.

‡ Biuret-positive substances are expressed in terms of amounts per milligram (dry weight) of cells, equivalent in reactivity to micromgram quantities of bovine serum albumin.

Fig. 2. Effects of antimycin A on endogenous respiration and uptake of α-aminoisobutyrate (AIB). Cells were incubated in shaken Warburg flasks for 90 min at 25°C. Each flask contained 28.4 mg (dry weight) of cells, in a final volume of 3 ml. The suspending fluid contained 50 mM phosphate buffer (pH 7.0) and 80 mM labeled aminoisobutyrate. The dashed line in the figure indicates the level of passive uptake at 4°C by intact cells. Control values were: QO₂ = 35.0, aminoisobutyrate uptake = 313 μmoles per g (dry weight) of cells.
Antimycin also inhibited uptake of α-methyl-glucoside, a compound which enters this organism under anaerobic conditions, but uptake of the sugar was somewhat less sensitive to antimycin than was AIB uptake.

In all, it appeared that uptake of nutrients was hindered at antimycin levels lower than those required to cause extensive loss of cellular solutes of small molecular weight. A similar response of yeasts to nystatin was reported by Stachiewicz and Quastel (1963).

Experiments with protoplasts. Protoplasts of B. megaterium, suspended in molal sucrose solution, were rapidly lysed by the action of antimycin (Fig. 3): this finding strongly supported the view that antimycin affects the plasma membrane. The antimycin level required for 50% lysis of protoplasts was found to be 37 μmole per mg of cells. (The cell weight given for protoplasts is the dry weight of cells before removal of walls.)

The curves of Fig. 3 show an initial rise in optical density of protoplast suspensions after addition of antimycin. This rise was followed by a drop in turbidity associated with protoplast lysis. Part of the initial rise was caused by precipitation of antimycin, but, from turbidity measurements of antimycin suspensions in aqueous sucrose solution, it appeared that only a minor part of the rise could be accounted for in this manner. Control suspensions showed a steady rise in optical density owing to protoplast shrinkage, which could be measured microscopically. This shrinkage was a consequence of utilization of intracellular solutes for endogenous respiration, and it did not occur in chilled or anaerobic suspensions. However, processes other than protoplast shrinkage seemed to be involved in the antimycin-induced effect. Similar rises in optical density were measured after addition of antimycin to suspensions of protoplast membranes or chilled whole protoplasts, whereas untreated suspensions of these materials showed no rise in optical density. Thus, it seemed that the initial binding of antimycin to protoplasts increased their refractive index and that this reaction preceded lysis.

The binding of nonlytic, but lethal, amounts of antimycin to protoplasts did not appear to alter their inherent permeability. In a typical experiment, the extracellular sucrose concentrations required for 50 and 100% osmotic stabilization of protoplasts were 0.61 and 0.83 molal, respectively; these concentrations were unchanged when protoplasts were treated with 11 μmole of antimycin per mg of cells before dilution of the suspending medium with water.

Protoplasts exposed to antimycin concentrated the antibiotic in the lipid-rich membrane fraction (Table 2), especially when induced lysis was not extensive. The addition of antimycin to aqueous suspensions of isolated protoplast membranes led to large increases in optical density of the suspensions (Fig. 4), an effect which may be related to the prelytic reaction of antimycin with intact protoplasts. However, the rise was not followed by a drop in turbidity. In contrast, sodium lauryl sulfate caused the optical density of membrane suspensions to drop precipitously, and, as described by Salton (1957), the membranes seemed to disappear when viewed under a phase-contrast microscope. Thus, dissolution of the protoplast membrane did not appear to accompany antimycin-induced lysis; this conclusion was further supported by phase microscopic observations. The antimycin-induced process was grossly similar to that induced by cationic detergents (Salton, 1957; Gilby and Few, 1960) or by polymyxin (Newton, 1956), and it
Table 2. Concentration of antimycin in the membrane fraction of protoplasts*

<table>
<thead>
<tr>
<th>Added antimycin</th>
<th>Lysis induced by antimycin</th>
<th>Per cent antimycin recovered†</th>
<th>In membrane fraction</th>
<th>In suspending fluid and soluble fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>μmoles/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.023</td>
<td>22</td>
<td>83</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>0.230</td>
<td>98</td>
<td>68</td>
<td>23</td>
<td></td>
</tr>
</tbody>
</table>

* Protoplasts (2.2 X 10^8 per milliliter) suspended in molal sucrose solution (4 °C) were treated with antimycin for 20 min before being shocked osmotically by a threefold dilution of the original suspending fluid with cold distilled water. The membrane fraction, obtained by differential centrifugation, was extracted with absolute ethyl alcohol to recover antimycin.
† Antimycin was assayed spectrophotometrically.

could be prevented by exposing protoplasts to uranyl nitrate solutions before antimycin.

Table 3 presents comparative data on the extent of lysis by agents known to disrupt protoplasts. Under the test conditions, antimycin was more potent than digitonin, polymyxin, and all but one of the test detergents, which included cationic, anionic, and nonionic representatives. Antimycin appeared to be of about the same potency as hexachlorophene, but the latter induced a much slower lytic process. The action of hexachlorophene on whole cells and protoplasts of this organism has been described by Joswick and Gerhardt (1960).

Degradation of the antimycin molecule by mild alkaline hydrolysis (Liu, van Tamelen, and Strong, 1960) or by reaction with ferricyanide (Walter and Lardy, 1964) completely abolished its lytic capacity. The products of the reaction with ferricyanide are not known, but it has been reported that they fail to inhibit cytochrome-mediated electron transport. The hydrolysis products have been found to be a mixture of

Table 3. Comparative potencies of a number of agents that induce protoplast lysis*

<table>
<thead>
<tr>
<th>Agent</th>
<th>Amt (μmoles/ml) required for 50% lysis</th>
<th>90% lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cetyltrimethylbenzylammonium chloride</td>
<td>0.016</td>
<td>0.030</td>
</tr>
<tr>
<td>Antimycin A</td>
<td>0.037</td>
<td>0.047</td>
</tr>
<tr>
<td>Hexachlorophene</td>
<td>0.049</td>
<td>0.12</td>
</tr>
<tr>
<td>Polymyxin B sulfate</td>
<td>0.054</td>
<td>—</td>
</tr>
<tr>
<td>Triton X-100*</td>
<td>0.094</td>
<td>0.14</td>
</tr>
<tr>
<td>Sodium dodecyl sulfate</td>
<td>0.25</td>
<td>0.41</td>
</tr>
<tr>
<td>Hexadecyltrimethylammonium bromide</td>
<td>0.25</td>
<td>—</td>
</tr>
<tr>
<td>Digitonin</td>
<td>0.53</td>
<td>1.17</td>
</tr>
</tbody>
</table>

* Experimental conditions: protoplast concentrations at start of lytic process were approximately 2 X 10^6 per milliliter of molal sucrose solution; temperature was room temperature (about 25 °C); agents were added as concentrated aqueous solutions or, for antimycin and hexachlorophene, concentrated ethanolic solutions.

† In this instance, 90% lysis did not occur owing to clumping of protoplasts in the presence of 0.075 mM polymyxin; 82% lysis occurred in the presence of 0.070 mM polymyxin.

* Triton X-100 is the trade name (Rohm and Haas Co., Philadelphia, Pa.) for alkyl phenoxyl polyethoxethanol. The molecular weight used for calculating concentrations was 647.5.

* With this concentration of protoplasts, 90% lysis did not occur below the critical detergent concentration for micelle formation.
formic acid, antimycin acid, N-formylantimycin acid, and antimycin lactone. It appears that the lytic potency of antimycin depends on a large portion of the whole molecule, and that it is much greater than the potencies that may be associated with either the phenolic or alkyl groups in the structure.

**Discussion**

From the above results, it appears that the following amounts of antimycin per milligram (dry weight) of cells were required for 50% end points with respect to each of the indicated effects: 3.8 mmoles for death, within 90 min, of cells in aerobic suspension; 6.2 mmoles for inhibition of AIB uptake; 37 mmoles for lysis of protoplasts; and somewhat more than 125 mmoles for release of inorganic phosphate from whole cells, or for inhibition of their oxygen consumption. In constructing a hypothesis based on these results as to mode of action, one might envisage an association of lipophilic antimycin molecules with the lipid-rich plasma membrane in a lethal reaction which occludes reactive sites for nutritite uptake and for other transport processes but does not damage the osmotic barrier. Binding to protein as well as lipid could be involved, for Reif (1953) described the binding of antimycin to serum albumin. The aggregation of more antimycin at the membrane surface must then lead to lesions in the osmotic barrier which permit an outflow of small molecules from whole cells or cause lysis of protoplasts. The hindrance of vital processes other than transport reactions by relatively low levels of antimycin was not ruled out in this study; thus, although interference with transport reactions or plasma membrane lysis may be sufficient to cause death, the primary bactericidal action of antimycin may depend on other metabolic impairments.

The lytic action of antimycin for *B. megaterium* is similar to that of polyene antibiotics for yeasts and molds. However, Kinsky (1963) found that protoplasts of this organism were unaffected by amphotericin B or filipin when these agents were present at concentrations well above those required for lysis of sensitive fungal protoplasts. Further, nystatin has been reported (Kinsky, 1962; Shockman and Lampen, 1962) to be without effect on *B. megaterium* protoplasts and to be bound by them in only small amounts. I have found that ascosin also is ineffective. Thus, the lytic mechanism induced by antimycin appears distinct from polyene-induced processes, and is probably more closely related to detergent-induced lysis.

**Acknowledgments**

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**Literature Cited**


