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Mechanism of Action of Rifampin on *Mycobacterium smegmatis*

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Deoxyribonucleic acid (DNA)-dependent ribonucleic acid (RNA) polymerase (EC 2.7.7.6) isolated from a rifampin-sensitive strain of *Mycobacterium smegmatis* was 90% inhibited by 1 μg of rifampin per ml; enzyme from a rifampin-resistant mutant was not affected by this concentration of antibiotic. Inhibition of phenylalanine-*1*-^{14}C incorporation by rifampin in growing cultures was complete about 6 min after addition of antibiotic. Under the same conditions, uracil-*2*-^{14}C incorporated was blocked after 1.5 to 2 min. Rifampin kills *M. smegmatis* very slowly. When rifampin-inhibited cultures were transferred to a rifampin-free medium, there was a partial resumption of uracil-*2*-^{14}C incorporation, even in the presence of chloramphenicol. We conclude that a primary event in the inhibition of *M. smegmatis* by rifampin is the block of DNA-dependent RNA polymerase.

Rifampin (2), formerly known as rifampicin also, is a semisynthetic antibiotic derived from rifamycin B, a fermentation product of *Streptomyces mediterranei* (16). It is an orally active, broad-spectrum antibiotic exhibiting no cross-resistance with other antibiotics in current clinical use. In vitro experiments have shown that this antibiotic is a specific inhibitor of bacterial deoxyribonucleic acid (DNA)-dependent ribonucleic acid (RNA) polymerase (EC 2.7.7.6), blocking the initiation of RNA chain synthesis but not affecting chain elongation (8, 11). Rifampin-resistant mutants of *Escherichia coli* (5, 18), *Staphylococcus aureus* (20), and *Bacillus subtilis* (4, 17) have all been shown to contain altered polymerases that are more resistant to the antibiotic in vitro. Recently, Zillig (13) reported that a modified β subunit of this enzyme is responsible for resistance to rifampin in an *E. coli* mutant.

Although the most important clinical application of rifampin is in the treatment of tuberculosis (1), little information is available regarding its mechanism of action on *Mycobacterium tuberculosis*. Recently, rifampin has shown promise against leprosy (14), another mycobacterial disease. In the present report, we present the results of our research into the mechanism of rifampin action on mycobacteria, with *M. smegmatis* used as a model.

MATERIALS AND METHODS

Radiochemicals. Uracil-*2*-^{14}C (52.5 mCi/mM), phenylalanine-*1*-^{14}C (52 mCi/mM), and adenosine triphosphate-*8*-^{14}C (1 mCi/mM) were all purchased from the Radiochemical Centre, Amersham, England.

Chemicals. Ammonium sulfate (enzyme grade) was purchased from Mann Research Laboratories, New York, N.Y. Bovine serum albumin, dithiothreitol, and highly polymerized calf thymus DNA were purchased from the Sigma Chemical Co., St. Louis, Mo. Rifampin and streptomycin sulfate were made by Lepetit, Milan, Italy.

Bacterial strains and growth conditions. *M. smegmatis* ATCC 20, or a spontaneous rifampin-resistant derivative of this strain, was used in all experiments. The minimal inhibitory concentration of rifampin was 20 μg/ml for the parent strain and more than 200 μg/ml for the resistant mutant (determined in shaken cultures with an inoculum of 10^5 bacteria/ml). For incorporation experiments, the bacteria were grown in a minimal medium containing: $Na_2HPO_4 \cdot 12H_2O$, 19 g; KH_2PO_4, 2.5 g; $MgSO_4 \cdot 7H_2O$, 0.6 g; sodium citrate $\cdot 2H_2O$, 2.5 g; L-asparagine, 5 g; glycerol, 30 ml; polyoxyethylene sorbitan monooleate (Tween 80), 40 g; distilled water, 1,000 ml. The unusually high content of Tween 80 in this medium was necessary to prevent the bacteria from clumping. After sterilization, the *p*H was 7.4. Larger quantities of cells for extraction of RNA polymerase were prepared by growth in TN medium (Difco). For the preparation of enzyme, it was important that the culture not be allowed to exceed a cell density equivalent to 10 g (wet weight)/liter. Stationary-phase cultures (about 25 g (wet weight)/liter) gave a markedly diminished polymerase yield. Growth in both media was at 37 C with shaking.

Preparation of RNA polymerase. Enzyme from the parent strain and the rifampin-resistant derivative was partially purified by the following procedure. Cells (10 g, wet weight) were suspended in 10 ml of buffer G (3) and disrupted by passage through a French pressure cell (American Instruments Co.); this and all subse-

quent operations were carried out at 4 C. Large cellular debris was then removed by centrifuging at 23,500 × *g* for 20 min. The supernatant fraction was stirred, and a 40% (w/v) solution of streptomycin sulfate was added dropwise to give a final concentration of 4%. After the preparation was stirred for an additional 30 min, precipitated DNA and remaining particulate matter were sedimented by centrifuging at 200,000 × *g* for 1.5 hr. The resulting high-speed supernatant fluid was fractionated with ammonium sulfate by using essentially the procedure described by Burgess (3), with the minor modification that the first fraction was 0 to 30%, not 0 to 33%. Most of the enzyme activity was found in the fraction insoluble in 30 to 42% saturated ammonium sulfate. This precipitate was dissolved in storage buffer (3) and stored at −20 C. Enzyme prepared in this way was 90 to 99% DNA-dependent and had an activity of 7 to 13 nmoles of adenosine monophosphate (AMP) incorporated per mg of protein per 10 min at 37 C. (This represents a fourfold purification when compared with the 200,000 × *g* supernatant fraction.) Protein was measured by the biuret method (6) with bovine serum albumin used as a standard.

Assay of RNA polymerase. Enzyme was assayed by using the procedure described by Burgess (3). When the sensitivity of RNA polymerase to rifamycins was tested, antibiotic and enzyme were preincubated together for 3 min at 37 C, and the reaction was started by adding a solution containing nucleoside triphosphates and DNA.

Assay of RNA and protein synthesis in whole cells. RNA synthesis in whole cells was followed by measuring the incorporation of uracil-*2*-^{14}C (2.5 μg/ml, 0.15 μCi/ml) into the cold 5% trichloroacetic acid-insoluble fraction. Protein synthesis by whole cells was determined by measuring incorporation of phenylalanine-*1*-^{14}C (1 μg/ml, 0.15 μCi/ml) into the cold 5% trichloroacetic acid-insoluble fraction. All incorporation experiments were performed with growing cultures in minimal medium adjusted to an absorbance of 0.07 at 650 nm immediately before addition of isotope.

Assay of radioactivity. Labeled RNA and protein samples were collected on glass fiber filters (Whatman GF/C) and washed with 10 ml of 2% trichloroacetic acid and then 10 ml of 95% ethanol. Washed filters were dried and counted in a liquid scintillation analyzer (Philips) using a scintillation solution containing 2.5 g of Premix M (Packard Instrument Co.) per liter of toluene.

RESULTS

Bactericidal effect of rifampin. Addition of antibiotic at 100 μg/ml to a growing culture of *M. smegmatis* caused a rapid bacteriostasis followed by a much slower decrease in the viable count (Fig. 1). Higher concentrations of rifampin resulted in an increased bactericidal effect. Thus 800 μg/ml gave six times fewer survivors than 100 μg/ml after 48 hr of contact with the antibiotic.

Inhibition of uracil-2-^{14}C and phenylalanine-1-^{14}C incorporation in whole cells. Incorporation of uracil-*2*-^{14}C into the acid-precipitable fraction by a growing culture of *M. smegmatis* was blocked by the addition of 200 μg/ml rifampin. Inhibition was complete after 1.5 to 2 min of contact with antibiotic (Fig. 2). Under the same conditions, incorporation of phenylalanine-*1*-^{14}C into the acid-precipitable fraction continued at a decreasing rate for about 6 min after the addition of rifampin (Fig. 3).

Resumption of incorporation after removal of antibiotic. Preliminary experiments indicated that, when inhibited cultures were harvested and suspended in fresh rifampin-free medium, there was a partial recovery of uracil-*2*-^{14}C and, subsequently, phenylalanine-*1*-^{14}C incorporation. This recovery suggested that the slowness of killing (Fig. 1) might be caused by reversibility of the binding of rifampin to RNA polymerase; some caution must be exercised when comparing the recovery of incorporation and killing experiments, as they were performed in different media (minimal and TB, respectively). To exclude the possibility that resumption of uracil incorporation was simply dependent on the de novo synthesis of polymerase, an experiment was carried out in which protein synthesis after the removal of rifampin was inhibited by the addition of chloramphenicol (Fig. 4). Chloramphenicol did not prevent the resumption of uracil-*2*-^{14}C incorporation; the control culture containing rifampin and chloramphenicol showed no recovery. A parallel culture containing phenylalanine-*1*-^{14}C and chloramphenicol gave no resumption of incorporation.

Effect of rifampin on DNA-dependent RNA polymerase. Partially purified RNA polymerase from *M. smegmatis* was 90% inhibited by 1.0 μg/ml rifampin, whereas enzyme from a resistant mutant was not affected by this concentration of antibiotic (Fig. 5). A mixture of the two enzymes gave an intermediate result. Higher concentrations did not give complete inhibition of the sensitive polymerase. This resistant fraction probably resulted from the presence of endogenous DNA, as RNA polymerase prepared in this way varied from 90 to 99% in its DNA dependence. It was previously reported that a preformed complex of DNA and enzyme is resistant to rifampin (11).

DISCUSSION

The effect of rifampin on partially purified *M. smegmatis* RNA polymerase and the kinetics of rifampin inhibition of uracil and phenylalanine incorporation by *M. smegmatis* support the suggestion that inhibition of RNA synthesis is a primary lesion in bacteriostasis caused by this antibiotic. Inhibition of RNA synthesis begins 1 to 1.5 min after addition of rifampin and is com-

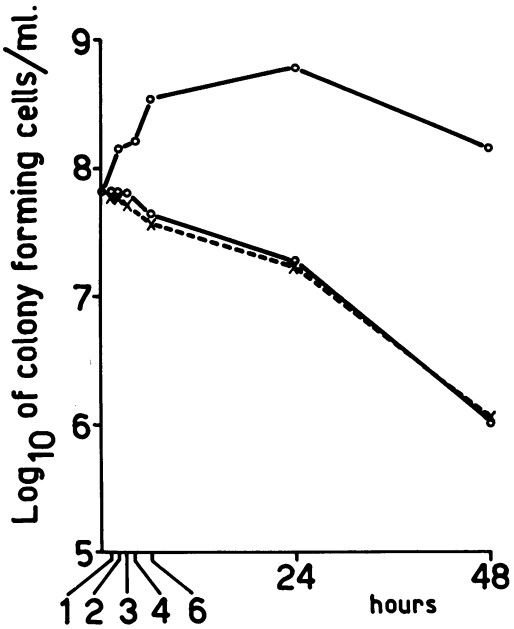

FIG. 1. *Effect of rifampin on viable counts. Exponentially growing cultures of Mycobacterium smegmatis (TB medium) were treated with indicated concentration of rifampin at zero time. Viable counts were determined by plating quadruplicate samples on Dubos Oleic agar after suitable dilution in sterile saline. Untreated control, O; 100 μg/ml rifampin, ×; 200 μg/ml rifampin, ●.*

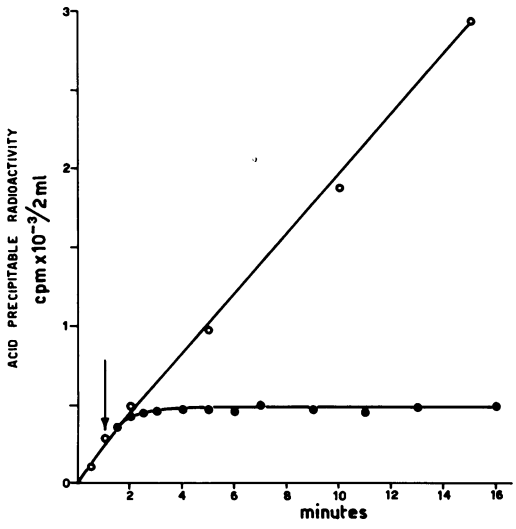

FIG. 2. *Inhibition of uracil-2-^{14}C incorporation by rifampin. Uracil-2-^{14}C (0.15 μCi/ml, 2.5 μg/ml) was added to growing culture of Mycobacterium smegmatis (absorbance 0.07 at 650 nm). After 1 min, rifampin was added to give a final concentration of 200 μg/ml. At indicated times, 2-ml samples were withdrawn and ejected into 2 ml of 10% trichloracetic acid, and insoluble material was collected on glass fiber filters. After samples were washed and dried, the amount of radioactivity on the filters was determined with a scintillation counter. Control culture, O; 200 μg/ml rifampin added at 1 min, ● (see arrow).*

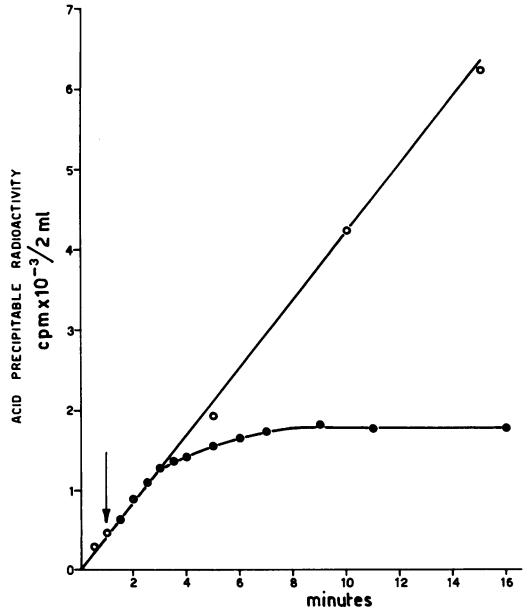

FIG. 3. *Inhibition of phenylalanine-1-^{14}C incorporation by rifampin. Conditions are those described for Fig. 2, except that phenylalanine-1-^{14}C (0.15 μCi/ml, 1 μg/ml) was added instead of uracil-2-^{14}C. Control culture, O; 200 μg/ml rifampin added at 1 min, ● (see arrow).*

plete by 3 min, whereas protein synthesis is not affected at all until 3 to 3.5 min. Further support for the effect of rifampin on transcription comes from the data on RNA polymerase in a resistant mutant, because the mutant contains an altered enzyme that has a markedly decreased sensitivity to the antibiotic in vitro. The fact that a mixture of sensitive and resistant RNA enzymes gave a plateau of rifampin-resistant RNA polymerase activity at about 50% (Fig. 5) excludes the possibility that the antibiotic is inactivated by the resistant extract. The length of lag before complete inhibition of RNA and protein synthesis, in whole cells, is not substantially different from that reported for other microorganisms (7, 10, 15). Presumably, in the case of uracil incorporation, this lag was due to the completion of RNA chains already initiated before addition of rifampin (11), and, for phenylalanine, represented the maximum life of messenger RNA. Trnka and Smith (19) reported that rifampin causes a 50% inhibition of polyphenylalanine synthesis by

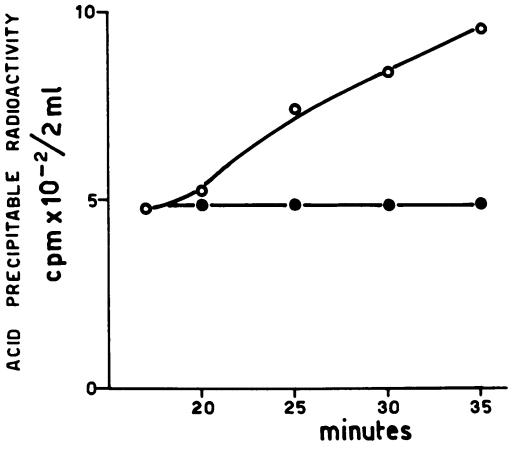

FIG. 4. *Resumption of uracil-2-^{14}C incorporation on removal of rifampin. A culture of Myocobacterium smegmatis, growing in the presence of uracil-2-^{14}C and inhibited by 200 μg/ml rifampin as described in Fig. 2, was harvested by membrane filtration after 15 min of contact with antibiotic and suspended in fresh rifampin-free medium containing 500 μg of chloramphenicol per ml. The culture was then divided into two parts, and rifampin was added to one to give a final concentration of 200 μg/ml. Graph shows incorporation of uracil-2-^{14}C into acid-precipitable material after suspension of the rifampin-inhibited culture in fresh medium containing chloramphenicol with and without rifampin. Culture containing chloramphenicol, ○; culture containing chloramphenicol and rifampin, ●.*

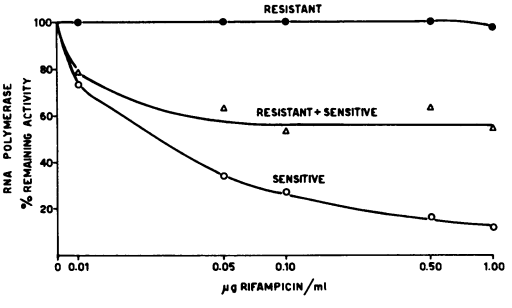

FIG. 5. *Effect of rifampin (rifampicin) on DNA-dependent RNA polymerase isolated from M. smegmatis. Enzyme was isolated and partially purified from M. smegmatis and a rifampin-resistant mutant. Polymerase activity was measured by estimating the incorporation of ^{14}C-adenosine monophosphate (AMP) into acid-precipitable material (3). For the sensitive enzyme, 20 μg of protein was used per incubation (○); for the resistant enzyme, 26 μg of protein (●); for the mixture, sensitive enzyme at 10 μg of protein and resistant enzyme at 13 μg of protein (▲). Specific activity of RNA polymerase in the absence of rifampin was 12.65 nmoles of AMP incorporated per 10 min per mg of protein for the sensitive enzyme, and 7.62 for the resistant one.*

M. tuberculosis ribosomes in vitro; our results indicate that, with *M. smegmatis*, high concentrations of rifampin (200 μg/ml) inhibit RNA synthesis earlier than protein synthesis, and therefore, we doubt that a direct inhibition of translation is of primary importance in bacteriostasis.

The slow bactericidal effect of rifampin for *M. smegmatis* is similar to that obtained for *M. tuberculosis* (12). Our results are consistent with the theory that growth of *M. smegmatis* on removal of rifampin is due to the recovery of preexisting molecules of polymerase and not to the synthesis of enzyme de novo. If this is true, it implies that the ability of cells to recover depends on the stability of the enzyme-rifampin complex. At present, it is not known whether there is any difference in stability of the complex between those bacteria which are killed very rapidly, e.g., *E. coli* (9), and those which die more slowly, like mycobacteria.

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