Mechanism of Action of Isoniazid on Mycobacterium bovis Strain BCG

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The mechanism of action of isoniazid (INH) on Mycobacterium bovis strain BCG was studied. The rates of synthesis of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein after the addition of INH to growing cultures were followed by measuring the incorporation of \(^{3}H\)-thymidine, \(^{3}H\)-uridine, and \(^{14}C\)-L-valine, respectively. After the addition of INH, the rate of DNA synthesis began to decrease and was abolished within 4 hr. RNA synthesis ceased after 6 hr, and protein synthesis was inhibited after 7 hr. Thus, it appears that inhibition of the synthesis of DNA is one of the earliest events after INH addition. The inhibition of the synthesis of DNA was further found to correspond to losses in viability of treated cultures. Degradation of preexisting DNA in INH-treated strain BCG was not detected.

Isoniazid (INH), the most commonly used of antituberculosis drugs, has been reported by a number of investigators to affect a variety of metabolic processes in susceptible mycobacteria. Youatt (9) has recently reviewed the literature concerning the mechanism of action of INH and suggested that many of the contradictions and discrepancies in the literature are the result of the variety of experimental conditions (aeration, temperature, growth phase of bacteria, drug concentrations, etc.) used in the investigations. However, a number of investigators found that INH affects only growing susceptible mycobacteria. These observations suggest that the drug, or one of its metabolites, has a primary effect on a vital metabolic process or on the synthesis of an essential macromolecule.

This paper reports the results of studies on the effects of INH on the incorporation of radioactive precursors of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein by Mycobacterium bovis strain BCG.

MATERIALS AND METHODS

Bacteria and conditions of growth. An INH-susceptible, nonpathogenic mycobacterium, M. bovis BCG, was grown in liquid medium at 37°C with vigorous shaking for aeration. The mineral salts medium of Sauton (6) containing 0.05% Tween 80 (SMT; Hill Top Laboratories, Cincinnati, Ohio) was employed. Transfers were made into fresh medium at frequent intervals to avoid bacterial clumping.

Bacterial growth was measured by determining the optical density (OD) of cultures in a Coleman Junior II spectrophotometer (Coleman Instrument Corp., Maywood, Ill.) at 600 nm. An OD of 0.1 corresponds to approximately \(7 \times 10^6\) colony-forming units per ml of SMT or 25 \(\mu\)g of bacterial protein per ml. The numbers of viable bacteria were determined by serial dilution plate counting on \(7\)H11 agar (1). The plates were incubated at 37°C in an atmosphere of 5% CO\(_2\) for 3 weeks prior to counting. Bacterial protein was measured by the method of Lowry et al. (3).

Measurement of the synthesis of cellular macromolecules. The distribution of radioactivity in macromolecular fractions of strain BCG growing in the presence of various radioactive precursors was determined by using a modification of the membrane-filter fractionation technique of Roodyn and Mandel (5). Thymidine-methyl-\(^3\)H (5 \(\mu\)Ci/ml; 16.2 Ci/mm, Amer sham-Searle, Des Plaines, Ill.), \(^3\)H-uridine (5 \(\mu\)Ci/ml; 2 Ci/mm, New England Nuclear Corp., Boston, Mass.), and \(^14\)C-L-valine (uniformly labeled; 5 \(\mu\)Ci/ml; 209 mCi/mm, New England Nuclear Corp.) were added separately to growing cultures of strain BCG (OD = 0.2), and samples were taken after 8 hr. Incorporation into DNA was determined by pipetting the samples into equal volumes of 5.5 N NaOH and incubating at 37°C for 2 hr. After cooling in ice, the samples were acidified by the addition of 0.5 volume of 6 N HCl, and the DNA was precipitated with 1 volume of 10% trichloroacetic acid. The precipitates were collected on membrane filters (0.2 \(\mu\)m pore size, Millipore Corp., Bedford, Mass.) and washed with 10 ml of cold 5% trichloroacetic acid and followed by 5 ml of cold 1% trichloroacetic acid. After air drying the filters were placed in vials containing 10 ml of scintillation solution [100 mg of 1, 4-bis-2-(5-phenyl oxazolyl)-benzene per liter and 4 g of 2,5-diphenyl oxazole per liter in toluene], and the radioactivity was determined in a Mark I liquid scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.).
In some experiments, the effects of 0.05 μg of INH/ml of SMT on rates of synthesis of DNA, RNA, and protein were studied by adding the drug to growing cultures of strain BCG (O.D. ≥ 0.3) at the same time as 3H-thymidine, 3H-uridine, and 14C-valine (2 μCi/ml each), respectively. At intervals, samples were pipetted into equal volumes of ice-cold 10% trichloroacetic acid. After 20 min, the acid-insoluble precipitates were collected on membrane filters, washed, dried, and counted as described above.

**Measurement of uptake of labeled compounds.** To study the uptake of 14C-INH (1 μg/ml; 9.6 mCi/mm, Amersham-Searle, Des Plaines, Ill.) and 3H-thymidine (5 μCi/ml), respectively, the compounds were added to cultures of strain BCG growing in SMT. At intervals, samples were pipetted directly onto membrane filters, washed with 20 volumes of chilled SMT, and dried and, the radioactivities were determined.

**Measurement of the effect of INH on the stability of preexisting DNA in strain BCG.** Strain BCG (OD = 0.2) was grown for 4 hr in SMT plus 2 μCi of 3H-thymidine per ml to label the DNA. The bacteria were then washed free of exogenous 3H-thymidine by repeated centrifugations and resuspensions in fresh SMT. Half of the washed bacteria were then resuspended in SMT and half in SMT plus 5 μg of INH per ml, incubated at 37°C, and pipetted into cold 10% trichloroacetic acid. The radioactivities were determined as previously described.

**RESULTS**

Effects of INH on growth and viability. The addition of 0.05 μg of INH per ml to growing cultures of strain BCG caused a decrease in viability beginning after 1 hr of incubation. After 12 hr, a 97% loss in viability was evident (Fig. 1). Even though a reduction in viable count occurred, a slight increase in turbidity for about 12 hr was detected (Fig. 2). The generation time of strain BCG growing under these conditions was about 30 hr.

**Uptake of 14C-INH.** Strain BCG rapidly takes up radioactive INH. In fact, the bacteria appear to be saturated with the drug within 2 or 3 hr after addition of 1 μg of 14C-INH to a growing culture (Fig. 3). The uptake of 14C-INH by an INH-resistant mutant of strain BCG (BCG-INH\(^R\)) is shown for comparison. This result confirms the observation of a number of other workers that resistant organisms are deficient in INH uptake. The isolation and characterization of the INH-resistant mutant are described elsewhere (4).

**Distribution of radioactivity in 3H-thymidine, 3H-uridine, and 14C-valine-labeled bacteria.** Prior
TABLE 1. Distribution of radioactivity in chemical fractions of strain BCG

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Radioactive tracer &amp;</th>
<th>Radioactive tracer &amp;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[H]-thymidine</td>
<td>[H]-uridine</td>
</tr>
<tr>
<td>Lipid</td>
<td>2.4</td>
<td>1.7</td>
</tr>
<tr>
<td>Protein</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Deoxyribonucleic acid</td>
<td>89.7</td>
<td>2.7</td>
</tr>
<tr>
<td>Ribonucleic acid</td>
<td>5.3</td>
<td>99.6</td>
</tr>
</tbody>
</table>

* Results expressed as a percentage of the radioactivity in the cold trichloroacetic acid residue.

Fig. 5. Effect of isoniazid on the synthesis of RNA as measured by the incorporation of [H]-uridine. The bacterial protein content of each sample was normalized to 1 mg, and the data are expressed as counts/minute of [H]-uridine incorporated into RNA per milligram of bacterial protein.

Fig. 6. Effect of isoniazid on the synthesis of protein as measured by the incorporation of [C]-L-valine. The bacterial protein content of each sample was normalized to 1 mg. The data are expressed as counts/minute of [C]-L-valine incorporated per milligram of protein.

The synthesis of cellular proteins, as measured by the incorporation of 2 μCi of [C]-L-valine/ml, was unaffected until 6 to 7 hr after addition of INH (Fig. 6).

Therefore, the earliest event, if not the primary event, seen after addition of INH to a growing culture of strain BCG, was an inhibition of DNA synthesis. RNA and protein synthesis were affected only later. The time of inhibition of
DNA synthesis and loss of viability coincide approximately.

Uptake of thymidine. Since incorporation of 3H-thymidine was used as an index of DNA synthesis, the effect of INH on the uptake of 3H-thymidine was measured. The transport of 3H-thymidine was not significantly affected by INH until 3 to 4 hr and then began to decrease gradually (Fig. 7). The initial uptake represents entry of the exogenous thymidine into the metabolic pool. The later slowdown probably results from a lack of incorporation from the intracellular pool into DNA.

Effects on preexisting DNA. The possibility that INH inhibits DNA synthesis by degrading pre-existing DNA was also considered. Table 2 indicates that no loss of acid-insoluble radioactivity after exposure to the drug was detected.

**DISCUSSION**

The experiments reported here indicate that one of the early events after the addition of isoniazid to a growing culture of strain BCG is inhibition of DNA synthesis; this is followed later by inhibition of RNA synthesis and even later by inhibition of protein synthesis. The inhibition of DNA synthesis coincides with the loss of viability; DNA synthesis is inhibited by 99% within 3.5 to 4 hr; a 90% loss of viability is evident within about 4 hr. The continued synthesis of protein and the increase in absorbancy in treated cultures indicate that INH does not have a primary effect on energy metabolism.

The mechanism of the inhibition of DNA synthesis is not yet clear. However, several possibilities have been eliminated. The drug does not seem to affect the uptake of nucleic acid precursors; neither the uptake of thymidine nor the incorporation of uridine was inhibited early. In addition, since RNA synthesis continued for a time after the cessation of DNA synthesis, it appears that INH does not affect the formation of the purine and pyrimidine bases and their conversion to the corresponding ribonucleotides. However, a specific inhibition of a step in the conversion of thymidine to thymidine triphosphate has not been eliminated. Degradation of preexisting DNA by INH was not detected.

The above observations lead to a consideration of the possibility that INH had a direct effect on the synthesis of DNA. The lack of an immediate and complete inhibition of DNA synthesis by INH might also suggest that only initiation of DNA synthesis is affected and not the polymerization. However, it should be remembered that 4 hr is a relatively short period in an organism with a generation time of 30 hr. The inhibition of DNA synthesis, which is complete 4 hr after addition of the drug, might also be explained if this time period were required for the cells to take up the amount of INH required for bacterial killing. This does not seem to be the case since 14C-INH uptake is maximal within 2 to 3 hr. There is a possibility that the lag period preceding the complete inhibition of DNA synthesis is required for conversion of isoniazid to a biologically active metabolite. It has not been established whether INH per se is the bactericidal compound or whether an INH-metabolite is the effector (9).

Other workers (2, 7) have also reported the inhibition of nucleic acid synthesis in sensitive mycobacteria by INH. However, in both reports, nucleic acid synthesis was determined by measuring the incorporation of 32P into cellular fractions. Youatt (8) has suggested that INH might have an effect on general phosphate metabolism.

Experiments are now in progress to study the effects of INH on several enzymes which catalyze steps in the synthesis of DNA. The metabolic fate of INH is also being investigated.
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LITERATURE CITED


