Effect of Isoniazid on the Protoplasmic Viscosity in *Mycobacterium tuberculosis*

KUNI TAKAYAMA,* ALEC D. KEITH, AND WALLACE SNIPES

*Institute for Enzyme Research,* †University of Wisconsin, and Veterans Administration Hospital, Madison, Wisconsin 53705, and Department of Biophysics, Pennsylvania State University, University Park, Pennsylvania 16802

The effect of isoniazid on the protoplasmic viscosity in the H37Ra strain of *Mycobacterium tuberculosis* was determined by using electron spin resonance spectroscopy and a small spin label tempone (2,2,6,6-tetramethylpiperidine-N-oxyl radical). Isoniazid (0.5 μg/ml) caused the internal cellular viscosity to increase gradually over the first 15 h of exposure from a rotational correlation time value (Tc) of 2.4 × 10⁻¹⁰ to 3.4 × 10⁻¹⁰ s and then decrease linearly to the control level after 27 h. These results could be interpreted to mean that isoniazid allows a continued and normal synthesis of the protoplasmic components while the rate of increase in the cell volume is reduced. A degradative process may begin after the initial 15-h exposure time, which would cause the reduction in the internal viscosity.

Isoniazid (INH) is known to inhibit the synthesis of mycolic acids early in its bacteriocidal action against tubercle bacilli (8). Although the inhibition precedes the loss of viability, it has not been directly established whether it is responsible for the lethal action of the drug. Since the cells exposed to INH continued to develop for a single generation time (1, 5) in the absence of mycolic acid synthesis, an imbalance in the composition of the cell envelope would occur. This should result in early physical change (within the first few hours) that may be crucial to the viability of the microorganism. Early physical change in tubercle bacilli caused by this drug has always been difficult to detect and it probably requires the use of an extremely sensitive technique. A highly sensitive method was recently developed by Keith and Snipes (3) to measure the protoplasmic microviscosity in living cells. This spin label technique has been applied to bacterial, plant, and mammalian cells.

We have applied this new technique to measure the change in the protoplasmic viscosity in *Mycobacterium tuberculosis* H37Ra exposed to INH for various time periods. We report that within the first 15 h of exposure to 0.5 μg of INH per ml there is a gradual rise in the protoplasmic viscosity.

**MATERIALS AND METHODS**

**Growth of bacterial culture.** *M. tuberculosis* H37Ra was grown in Middlebrook 7H9 medium enriched with Tween 80 and ADC (Difco) on a benchtop environmental shaker model G-25 (New Brunswick Scientific Co., New Brunswick, N.J.) at 37 C and 150 rpm. Growth of the bacteria was followed in Nephlo flasks by measuring absorbance at 650 nm with a Coleman model 44 spectrophotometer. A 5.0-ml inoculum of a culture of H37Ra having an absorbance value of 0.33 was used to initiate growth in 100 ml of 7H9-supplemented medium. When the growth had reached an absorbance of 0.20 (4 days), INH was added to a final concentration of 0.5 μg/ml and incubated at 37 C for 6, 12, 18, and 24 h. Then sterile tempone (2,2,5,5-tetramethylpipieridine-N-oxyl radical) was added to a final concentration of 10⁻² M and further incubated at 37 C for 3 h and cooled in an ice bucket, and the cells were harvested by centrifugation. The cells were suspended in a small volume of 10⁻¹ M tempone and stored overnight in an ice bucket. Growth curves were obtained for both control and INH-treated cells.

**Measurement of protoplasmic viscosity.** Cells of *M. tuberculosis* treated with INH were suspended in 0.3 M NiCl₂ and 5 mM tempone, mixed in a Vortex mixer, and centrifuged to a pellet, and a portion of the pellet was sealed in a capillary for electron spin resonance analysis. Three sets of spectra, using expanded line widths, were carried out on each sample. A few crystals of K₃Fe(CN)₆ were added to each sample to prevent bioreduction of the N-oxyl group.

An X-Band Japan Electron Optics Laboratory spectrometer model JES-ME-1X operated at room temperature (24 C) was used for all measurements. The equation for rotational correlation time (Tc), Tc = KWₗ[(h₀/hₙ)ⁿ – 1], was used as described previously (2). The theoretical treatment which justifies the use of this equation was presented by Kivelson (4). Wₗ is the width of the first-derivative mid-field line, h₀ and hₙ are the heights of the mid- and high-field first-derivative lines, and K is a constant that depends on the spectral parameters of the spin label and on the instrument settings. The Tc values
shown are within the range of motion where spectral measurements are accurate.

**Chemicals.** INH was purchased from Calbiochem, Los Angeles, Calif. Uniformly labeled L-[14C]-amino acid mix was obtained from New England Nuclear Corp., Boston, Mass. Tempone was obtained from Aldrich Chemical Co., Milwaukee, Wis. All other chemicals and reagents were reagent grade.

**RESULTS AND DISCUSSION**

Under the condition of drug treatment used (culture of H37Ra with absorbance at 650 nm of 0.20 and 0.5 μg of INH per ml), the growth of INH-treated cells leveled off after about 3 days of exposure (see Fig. 1). The control cells continued to grow rapidly for an additional 5 days. Previous work on cells of H37Ra exposed to this concentration of INH showed that the cellular mycolate synthetase activity was completely inhibited after 60 min of exposure (8). Thus a continued growth occurred, producing cells whose walls are deficient in mycolic acids (6).

The protoplasmic microviscosity of INH-treated cells was determined by utilizing electron spin resonance and a small spin label tempone. The data are shown in Fig. 2, relating the $T_c$ measurement to the treatment duration with the drug INH. Cells of H37Ra were shown to have a $T_c$ value of $2.4 \times 10^{-10}$ s, which is slightly lower than the value obtained for *Escherichia coli* but comparable to anaerobic yeast (3). The resulting smooth curve in Fig. 2 indicated that INH causes the internal cell viscosity to increase over the first 15 h, reaching a maximal $T_c$ value of $3.4 \times 10^{-10}$ s, and then to linearly decrease back to the control level after 27 h.

*M. tuberculosis* exposed to INH continues to grow for an additional generation time (1, 5). During this period, protein synthesis continues at a near normal rate (Fig. 3), whereas the synthesis of mycolic acid is inhibited (8). Past studies have shown that INH does not significantly affect protein synthesis (10). One likely way in which the protoplasmic viscosity could

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**Fig. 1.** Inhibition of growth of *M. tuberculosis* H37Ra by INH. INH was added on the fourth day of growth to 100 ml cultures of H37Ra to a final concentration of 0.5 μg/ml. Growth was followed by measuring absorbance at 650 nm. Symbols: O, control cells; ●, INH-treated cells.

**Fig. 2.** Effect of exposure time to INH on the rotational correlation time ($T_c$) for tempone in cells of *M. tuberculosis* H37Ra. Cells of H37Ra were exposed to INH at a concentration of 0.5 μg/ml for various time periods, and their $T_c$ values for tempone were determined. The plot shows actual data points and their averages.

**Fig. 3.** Effect of INH on protein synthesis in *M. tuberculosis*. Protein synthesis was measured by the rate of incorporation of L-[14C]-labeled amino acid mix into the boiling trichloroacetic acid-insoluble material (7). After 15.5 h of incubation, the control sample had 73,500 counts/min, whereas the INH-exposed sample had 71,680 counts/min. Symbols: ●, control; ×, plus 0.5 μg of INH per ml.
increase with length of drug treatment is for the cell volume to increase slowly while the protein synthesis in the protoplasm continues at a normal rate. Under such a condition, the cells would tend to become packed with protoplasmic components in a smaller volume. This is consistent with the expected and observed higher $T_c$ values in drug-treated cells than in the normal cells. The decrease in the $T_c$ value after 15 h of incubation in the INH could be due to the degradation of the cells and the subsequent loss of membrane permeability. The change in morphology of cells of M. tuberculosis exposed to INH has already been examined by utilizing scanning electron microscopy (9). These studies showed that cellular degradation and fragmentation are detectable after 24 h of exposure to the drug.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AI-11297 from the National Institute of Allergy and Infectious Diseases and by the U.S. Atomic Energy Commission.

We thank Richard W. Boyle for his excellent technical assistance.

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