Relationship Between the Uptake of Isoniazid and Its Action on In Vivo Mycolic Acid Synthesis in *Mycobacterium tuberculosis*

LYNN WANG¹ AND KUNI TAKAYAMA

Tuberculosis Research Laboratory, Veterans Administration Hospital, Madison, Wisconsin 53705, and Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin 53706

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A direct relationship was established between the rate of uptake of isoniazid and the action of this drug on in vivo mycolic acid synthesis in *Mycobacterium tuberculosis* H37Ra. The rate of uptake of isoniazid increased linearly with its external concentration and appeared to reach a maximal value of 52 pmoles per hr per 10^9 cells at an external concentration of about $13 \mu\text{M}$. Correspondingly, the rate of inhibition of mycolic acid synthesis increased with the rise in the rate of uptake of the drug. A 50% inhibition of mycolic acid synthesis occurred when the uptake of isoniazid reached 5.2 pmoles per 10^9 cells. Calculations showed that this level of drug uptake represents an internal cellular concentration of $9 \mu\text{M}$. These results show clearly that the action of isoniazid on the mycolate synthetase system of *M. tuberculosis* is rapid and that this enzyme system is highly sensitive to the drug.

Isoniazid (INH) is one of the most effective and widely used drugs today in the treatment of tuberculosis. Since its discovery some two decades ago, much work has been done to determine the nature of its tuberculocidal action. Despite these efforts, little progress has been made. Recently, an important clue has emerged on how INH may act on the tubercle bacilli. Winder and Collins (11) observed that INH inhibited the incorporation of ^{14}C -glycerol into the mycolic acids after a 6-hr exposure of *Mycobacterium bovis* BCG to the drug. Using ^{14}C -acetate as the precursor, we have shown that INH inhibits the in vivo synthesis of mycolic acids very early in *M. tuberculosis* (10). At a drug concentration of $3.65 \mu\text{M}$, the synthesis of mycolic acids was completely inhibited in about 60 min. The loss in the viability of the cells followed this event very closely, with a time lag of about 30 min. Thus, it was demonstrated that INH acts on a specific metabolic pathway, the mycolate synthetase (MS) enzyme system, that is unique for the mycobacteria.

More experimental data are needed to evaluate the relationship of this INH inhibition of MS activity to the drug's tuberculocidal action. *M. tuberculosis* has been shown to be highly susceptible to INH (2). Thus, if the primary mechanism of action of INH is on the MS system, then one

would expect a low concentration of INH to act rapidly on this enzyme system. In this report we show a direct relationship between the rate of uptake of INH and the time course of the INH inhibition of mycolic acid synthesis in *M. tuberculosis* H37Ra. Careful quantitation of our results reveals that a very low uptake of the drug is sufficient to cause a rapid inhibition of the synthesis of the mycolic acids.

MATERIALS AND METHODS

Bacterial culture. *M. tuberculosis* H37Ra was grown in Middlebrook 7H9 medium enriched with Tween 80 and OADC (Difco) in a bench-top environmental shaker, model G-25 (New Brunswick Scientific Co., New Brunswick, N.J.) at 37°C and 150 rev/min. Frequent transfers were made to assure well-dispersed cultures. The number of viable cells was determined by plate count as reported earlier (10). A standard curve of cell numbers versus absorbancy was obtained, and the formula of $\log_{10}$ number of cells = $7.04 + (9.8 \times \text{absorbancy at } 650 \text{ nm})$ was used. A cell count of 3.95×10^9 corresponded to 1.0 mg (dry weight).

Assay for mycolic acid synthesis. INH was added aseptically to a log-phase culture to a specific final drug concentration. Samples were taken to assay for the in vivo MS activity as previously described (10).

Determination of uptake of ^{14}C -INH. Cultures of *M. tuberculosis* H37Ra with an absorbancy of 0.16 to 0.21 were used for all INH uptake experiments. Labeled INH (sterile) was added, and the cultures

¹ Present address: Department of Bacteriology, Hadassah Medical School, The Hebrew University, Jerusalem, Israel.

were incubated at 37 C on a rotary shaker. Appropriate samples (duplicates) of 1.0 to 5.0 ml were taken aseptically at various time intervals and carefully applied to a wetted membrane filter (0.45 μ m pore size, 25 mm in diameter, Millipore Corp., Bedford, Mass.) connected to an aspirator vacuum. The filtered samples were washed with 10 ml of water, then with 100 ml of 0.9% NaCl, and finally with 10 ml of water. The filters were dried and monitored for radioactivity by use of a scintillation spectrometer in a scintillator gel containing 10 ml of Aquasol (New England Nuclear Corp., Boston, Mass.) and 2.50 ml of water. Internal standards were used to determine the counting efficiency.

Chemicals. Acetic acid-1- 14 C was obtained from New England Nuclear Corp. 14 C-INH (isonicotinic hydrazide, 14 C-carbonyl) with a specific radioactivity of 11.1 μ Ci per μ mole was purchased from Amersham/Searle Corp., Arlington Heights, Ill. INH was purchased from Calbiochem, Los Angeles, Calif. All other chemicals were reagent grade.

RESULTS AND DISCUSSION

The rate of uptake of INH by the H37Ra strain of *M. tuberculosis* was initially studied. A typical curve is presented in Fig. 1, which shows that the uptake of INH by the tubercle bacilli increased with time. The linear uptake rate occurred only during the early period of exposure to the drug. Somewhat similar uptake curves have been reported by McClatchy (6) for *M. bovis* BCG and by Beggs et al. (1) for *M. tuberculosis* H37Ra. We performed a number of uptake experiments at various concentrations of INH and determined

FIG. 1. Rate of uptake of INH by a growing culture of *M. tuberculosis*. External concentration of 14 C-INH, 3.65 μ M.

the initial linear INH uptake rates. The rate of uptake increased linearly with the rise in the external INH concentration and became constant when the rate reached 52 pmoles per hour per 10^9 cells at about 13 μ M INH (Fig. 3a). Beggs et al. (1) reported a time course of the INH uptake for the H37Ra strain of *M. tuberculosis* in Sauton's medium at 0.73 μ M INH. Their reported rate of uptake is calculated to be 4.6 pmoles per hr per 10^9 cells, which compares favorably with our value of 2.4 pmoles per hr per 10^9 cells at the same concentration.

The kinetics of the inhibition of the MS activity by INH (Fig. 2) indicates that the rate of inhibition of MS activity by INH is concentration-dependent. Using various external concentrations of INH, we obtained a family of curves similar to those shown in Fig. 2. The time of drug exposure at which a 50% inhibition of MS activity occurs was then determined and is presented in Fig. 3b as a function of the external drug concentration. This plot shows that as we increased the external concentration of INH the rate of inhibition of the MS activity increased, and this effect became constant at about 9 μ M INH. At this point, a 50% inhibition of the MS activity occurred in 6 min. A definite relationship can be seen between the rate of uptake of the drug (Fig. 3a) and the time required for a 50% inhibition of MS activity (Fig. 3b) when they are plotted simultaneously against the external INH concentration. Within the concentration range tested (up to 36.5 μ M INH), a 50% inhibition of the MS activity occurred only after the cellular

FIG. 2. Inhibition of the mycolate synthetase (MS) activity with time of exposure of *M. tuberculosis* to INH. Two different INH concentrations were tested: X, 2.2 μ M; O, 7.1 μ M. The amount of 14 C-mycolic acids synthesized in the zero time sample was set at 100% activity.

FIG. 3. Relationship of the external INH concentration to (a) the rate of uptake of INH by *M. tuberculosis* and (b) the time of exposure to INH which causes a 50% inhibition of the mycolate synthetase (MS) activity.

TABLE 1. Quantitative determination of the sensitivity of the mycolate synthetase (MS) activity of *M. tuberculosis* to INH

External INH concn (μM)	Rate of MS inhibition (min at 50% inhibition) ^a	INH uptake at 50% inhibition of MS (pmoles per 10^9 cells) ^b
0.73	160	5.5
1.46	60	5.2
3.65	25	5.3
5.11	18	5.1
12.9	6	4.7
36.5	6	5.3

^a Obtained directly from the plot of Fig. 3b.

^b Calculated with values obtained from Fig. 3. The average value was 5.2 ± 0.2 (SD).

uptake had reached a value of 5.2 pmoles per 10^9 cells (Table 1). If we assume the average tubercle bacillus to be 0.5 μm in diameter and 3 μm long (8), the calculated internal INH concentration at 50% inhibition of the MS activity is 9 μM . A more direct study on the kinetics of this INH inhibition is not possible because a cell-free MS system is presently unavailable. This value is comparable in magnitude to the inhibitory concentrations of a few potent inhibitors of biochemical reactions: 1 μM for 2-alkyl-4-hydroxy quinoline-*N*-oxide to complex III of the electron transport system in mitochondria (7); a K_i of 30 μM for D-cycloserine to D-alanine-D-alanine synthetase in *M. tuberculosis* (3); and a K_i of 4.2 μM for hadacidin for the *Escherichia coli* adenylosuccinate synthetase (4).

The fact that we can obtain a constant INH uptake value (5.2 pmoles per 10^9 cells) at 50% inhibition of MS activity which is independent of the uptake rate means that the action of the drug on the MS activity is immediate. If the drug acted at the transcription, translation, or protein synthesis level, a lag period would be expected. Such a possibility was considered in view of reported early inhibition of deoxyribonucleic acid synthesis by INH in *M. bovis* BCG (5, 6). Our results, however, support the proposition that INH acts to inhibit the *in vivo* MS activity at the enzymatic level.

The demonstrated action of INH on *M. tuberculosis* as presented here and in our previous publication (10) satisfies two important criteria for explaining the mechanism of action of INH (9): (i) the drug is specific in its action against the mycobacteria by virtue of its direct action on the MS enzyme system (a system that is unique to mycobacteria), and (ii) the inhibitory concentration of INH against the MS activity is very low, just as the growth inhibitory and the bactericidal concentrations are also low. A close examination of the relationship between the INH inhibition of MS activity and cell viability is now crucial in deciding whether this is indeed the primary mode of action of INH on the tubercle bacilli.

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LITERATURE CITED

1. Beggs, W. H., J. W. Jenne, and W. H. Hall. 1968. Isoniazid uptake in relationship to growth inhibition of *Mycobacterium tuberculosis*. *J. Bacteriol.* 96:293-297.

2. Bernstein, J., W. A. Lott, B. A. Steinberg, and H. L. Yale. 1952. Chemotherapy of experimental tuberculosis. V. Isonicotinic acid hydrazide (nydrazid) and related compounds. *Amer. Rev. Tuberc.* 65:357-364.
3. David, H. L., K. Takayama, and D. S. Goldman. 1969. Susceptibility of mycobacterial D-alanyl-D-alanine synthetase to D-cycloserine. *Amer. Rev. Resp. Dis.* 100:579-581.
4. Franklin, T. J., and G. A. Snow. 1971. Biochemistry of antimicrobial action. Academic Press Inc, New York.
5. Gangadharam, P. R. J., F. M. Harold, and W. B. Schaefer. 1963. Selective inhibition of nucleic acid synthesis in *Mycobacterium tuberculosis* by isoniazid. *Nature (London)* 198:712-714.
6. McClatchy, J. K. 1971. Mechanism of action of isoniazid on *Mycobacterium bovis* strain BCG. *Infect. Immunity* 3:530-534.
7. Mahler, H. R., and E. H. Cordes. 1966. Biological chemistry. Harper & Row Publishers, New York.
8. Middlebrook, G., and J. Freund. 1952. The mycobacteria, p. 324-357. In R. J. Dubos (ed.), Bacterial and mycotic infections of man. J. B. Lippincott Co., Philadelphia.
9. Ramakrishnan, T., P. S. Murthy, and K. P. Gopinathan. 1972. Intermediary metabolism of mycobacteria. *Bacteriol. Rev.* 36:65-108.
10. Takayama, K., L. Wang, and H. L. David. 1972. Effect of isoniazid on the in vivo mycolic acid synthesis, cell growth, and viability of *Mycobacterium tuberculosis*. *Antimicrob. Ag. Chemother.* 2:29-35.
11. Winder, F. G., and P. B. Collins. 1970. Inhibition by isoniazid of synthesis of mycolic acids in *Mycobacterium tuberculosis*. *J. Gen. Microbiol.* 63:41-48.