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Isoniazid Inhibition of Mycolic Acid Synthesis by Cell Extracts of Sensitive and Resistant Strains of *Mycobacterium aurum*

ANNAÏK QUÉMARD, CHARLOTTE LACAVE, AND GILBERT LANÉELLE*

Centre de Biochimie et de Génétique Cellulaires du Centre National de la Recherche Scientifique and Université P. Sabatier, 31062 Toulouse Cedex, France

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Isonicotinic acid hydrazide (isoniazid; INH) inhibition of mycolic acid synthesis was studied by using cell extracts from both INH-sensitive and -resistant strains of *Mycobacterium aurum*. The cell extract of the INH-sensitive strain was inhibited by INH, while the preparation from the INH-resistant strain was not. This showed that the INH resistance of mycolic acid synthesis was not due to a difference in drug uptake or the level of peroxidase activity (similar in both extracts). As INH did not induce accumulation of any labeled intermediates, it is postulated that the drug acts either on production of labeled chain elongation precursors of mycolic acids or an early step of this elongation. The level of inhibition was not changed by addition of NAD or nicotinamide; thus, INH does not act on mycolic acid synthesis as an NAD antimetabolite. Benzoic or acetic acid hydrazides and known or postulated metabolites of INH (i.e., the corresponding acid, aldehyde, or alcohol) were not inhibitors of cell-free mycolic acid synthesis; the complete structure of INH was required, as already known for inhibition of mycobacterial culture growth. Extracts prepared from INH-treated cells showed reduced mycolic acid synthesis, and the inhibition level was not modified by either extensive dialysis or pyridoxal phosphate. This latter molecule efficiently antagonized INH action by reacting rapidly with INH, as shown by differential spectroscopy. Moreover, pyridoxal phosphate did not release inhibition of INH-treated extracts. It is proposed that INH may covalently react with an essential component of the mycolic acid synthesis system.

Isonicotinic acid hydrazide (isoniazid; INH) very specifically inhibits growth in some mycobacteria and is one of the most efficient antituberculous agents. It has been used since 1952, but despite the great number of studies and reviews devoted to this drug its mode of action is not unequivocally known (for reviews, see references 5, 8, 14, 17, and 20).

Three main hypotheses have been proposed to explain its activity. Two are based on the structural features and chemical reactivity of the molecule and state that INH may act as an antimetabolite of two major coenzymes, NAD (10; see also reference 5) and pyridoxal phosphate (14). The third hypothesis postulates that INH inhibits a metabolism specific to mycobacteria, namely, mycolic acid synthesis (18, 21). These compounds are very long-chain α-branched, β-hydroxy fatty acids characteristic of mycobacterial cell walls. Production of isonicotinic acid and detection of iso-NAD (after 8 days of incubation) in treated mycobacteria supported the hypothesis of INH anti-NAD activity (5). It was confirmed that INH inhibits various pyridoxal phosphate-requiring enzymes (8).

The loss of acid fastness of INH-treated cells (7) suggested the hypothesis of inhibition of mycolic acid synthesis. It is based on early inhibition of this synthesis by INH in cells (17, 21), on inhibition of C_{24}-C_{26} monounsaturated fatty acid synthesis in cells (3), and on inhibition of cell-free systems synthesizing very long-chain fatty acids (6). However, there was no direct experimental evidence that these fatty acids were precursors of mycolic acids. Moreover, with intact cells it is impossible to discriminate between direct effects on mycolic acid synthesis and indirect effects due to general metabolism inhibition by INH acting as an antimetabolite.

We recently obtained the first cell-free system able to produce labeled mycolic acids from [^{14}C]acetate as the substrate (12, 13). It is a particulate system associated with the cell wall. Use of a cell-free system eliminates unanswered questions about uptake, transport, and cytoplasmic metabolism of the tested drug. We determined whether inhibition of mycolic acid synthesis was due only to antimetabolite effects of INH against NAD or pyridoxal phosphate or whether this synthesis was one of the direct targets of the drug. We also determined whether INH acted in its intact form or after modification, for instance, transformed into isonicotinic acid by a peroxidase-catalase enzyme (4, 17). Cell-free systems from strains sensitive or resistant to INH were used and compared.

MATERIALS AND METHODS

Cultures and cell-free system preparation. Two strains of *Mycobacterium aurum* were used: ATCC 23366 (INH resistant) and Institut Pasteur Collection strain A$^+$ (INH sensitive). Cultures were performed as previously described (11).

Cell extracts were obtained by a procedure adapted from reference 12. About 6 g (wet weight) of cells from mid-log growth phase cultures were washed by centrifugation (0.01 M potassium phosphate, pH 7.0). The cells were suspended in 30 ml of cold buffer A (0.05 M potassium phosphate, 3 mM mercaptoethanol, pH 7.0) and broken twice in a cold French pressure cell (Aminco). Intact cells were removed by centrifugation (15 min, 3,000 × *g*), and the active cell-free preparation was recovered in the supernatant "fluffy layer." In some experiments, this fraction was sedimented twice (30 min, 40,000 × *g*) to eliminate soluble compounds. Under routine conditions, the fluffy layer was homogenized after addition of 4 to 6 ml of buffer A. This preparation is hereafter called the cell extract or cell-free system. The above-described operations were performed at 4°C.

When INH-treated bacteria (5 μg of INH per ml 1 h before harvesting) were used to prepare the cell-free system, the

* Corresponding author.

above-described conditions were used, except that the preparation was washed twice by centrifugation (buffer A, 30 min at 40,000 × *g*) or dialyzed (buffer A, overnight, 2 × 200 volumes) to eliminate free INH or derivatives. A control preparation of nontreated cells was submitted to the same treatment.

Cell-free mycolic acid synthesis. Incubation conditions were as follows, unless otherwise stated. A cell extract sample containing 2 to 3 mg of protein (as determined by the Lowry procedure) was used in a final volume of 1.5 ml of buffer B containing 70 μmol of potassium phosphate (pH 7.0), 5 μmol of $MgCl_2$, 10 μmol of $HKCO_3$, and 10 μmol of glucose (for *M. aurum* A^+ preparations only). A 30-min preincubation time (at 37°C) was used before the start of labeling by addition of [1-^{14}C]acetate (70 nmol; specific activity, 1.9 GBq/mmol; CEA, Gif-sur-Yvette, France). Incubation times of up to 90 min were used (37°C on a rotary shaker at 160 rpm). The molecules and drugs tested were added at the beginning of the preincubation period. The reaction was stopped by addition of 1.5 ml of KOH (10%, wt/vol) in methanol-benzene (8:2, vol/vol). Fatty acids were obtained by overnight saponification in the above-described medium (at 70°C). Free fatty acids were extracted, and methyl esters were prepared by diazomethane.

The total radioactivity of each sample was determined by scintillation counting of samples. Esters were analyzed by thin-layer chromatography (Silica Gel G60 [Merck]; solvent, dichloromethane), and relative proportions of esters were determined by surface radioactivity counting on a linear automatic analyzer (LB 2832; Berthold). Spots were visualized by spraying with 20% sulfuric acid, followed by charring. The average activity of A^+ cell extracts was $5.2 \times 10^3 \pm 0.8 \times 10^3$ cpm/mg of protein per min of incubation (110 pmol of acetate incorporated per mg of protein per min).

Peroxidase activity. Peroxidase activity was determined with a final volume of 1 ml containing 2.6 μmol of phenol, 0.8 μmol of amino 2-antipyrine, 1 μmol of $MgCl_2$, 2 μmol of $HKCO_3$, 1.0 μl of 10 M H_2O_2, cell extract (around 1 mg of protein), and 0.1 M Tris-HCl buffer (pH 8.0). Incubations were performed at 37°C on a rotary shaker and absorbance was measured at 500 nm (the dye formed has an E_{500} of 12,000 M^{-1} cm^{-1}; two dye molecules are formed per H_2O_2 molecule consumed).

Chemicals. INH, isonicotinic acid, 4-pyridine carboxaldehyde, nicotinamide, NAD, isonicotinamide, pyridoxal, and pyridoxal phosphate were obtained from Sigma. Acetic acid hydrazide was from Merck, and benzoic and nicotinic acid hydrazides were from Aldrich. 4-Hydroxymethylpyridine was prepared by reducing 4-pyridinecarboxaldehyde with $NaBH_4$.

RESULTS AND DISCUSSION

Effects of certain factors on cell-free fatty acid synthesis. The activities of cell extracts of the INH-sensitive (A^+) and -resistant (ATCC 23366) strains of *M. aurum* were compared (Table 1). Both strains synthesize the same types of mycolic acids (unsaturated and oxo- and dicarboxymycolic acids), with differences in relative abundance. It appeared that 7 mM glucose greatly increased [^{14}C]acetate incorporation into the three types of mycolic acids by strain A^+ cell extract but not in the strain 23366 preparation, while trehalose, an activator of cell extracts from *Corynebacterium diphtheriae* (19), and ATP were without significant effect (Table 1). Thus, 7 mM glucose was routinely added to A^+ preparations.

TABLE 1. Effects of certain factors on [^{14}C]acetate labeling of fatty acids by cell-free systems from *M. aurum* ATCC 23366 (INH resistant) and A^+ (INH sensitive)

Medium	Acetate incorporation (%)	
	23366	A^+
SIM[a]	100[b]	100[b]
SIM + 7 mM glucose	60	165
SIM + 7 mM trehalose	95	85
SIM + 3 mM ATP	110	90

[a] SIM, standard inorganic medium.
[b] The 100% standard refers to average labeled acetate incorporation (counts per minute per milligram of protein) under standard conditions (see Materials and Methods) for cell-free systems of each strain.

Labeling of fatty acids from intact A^+ cells and from the cell-free system was determined by radioscanning (Fig. 1A and Table 2). With the cell-free system, it appeared that 63% ± 4% of the radioactivity was in mycolic acids (peaks 3, 4, and 5) and 11% ± 3% was in nonhydroxylated fatty acids (peak 6). Other labeled compounds were unidentified polar (peaks 1 and 2) and nonpolar molecules (peak 7), representing 20% ± 3% and 8% ± 2% of the total radioactivity, respectively. It is worth noting that the ratio of mycolic acids to nonhydroxylated fatty acids was significantly higher with the cell-free system than in intact cells (ratios of 6 and 2,

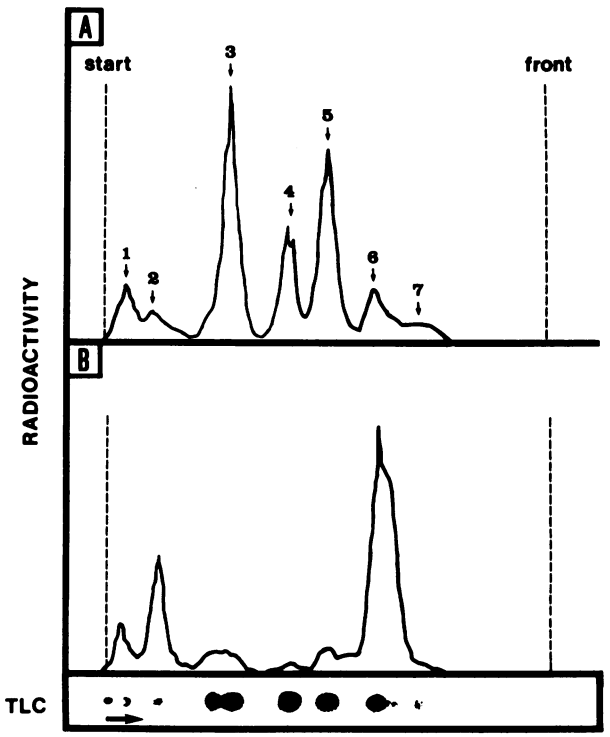

FIG. 1. Labeling profiles of saponification products from cell-free synthesis (*M. aurum* A^+). Saponified lipids were esterified by diazomethane and analyzed by thin-layer chromatography (TLC) (solvent, CH_2Cl_2), and chromatograms were radioscanned. (A) Without INH. (B) With 15 μg of INH per ml (109 μM). Peaks: 1 and 2, unknown polar compounds; 3, dicarboxymycolic methyl esters; 4, oxomycolic methyl esters; 5, unsaturated mycolic methyl esters; 6, medium-length chain nonhydroxylated fatty methyl esters; 7, very long-chain nonhydroxylated fatty methyl esters.

TABLE 2. Labeling distribution in mycolic acids from *M. aurum* A+[a]

Condition	Relative labeling (% of total)			Ratio of mycolic to nonhydroxylated acids
	Unsaturated mycolic acid	Oxomycolic acid	Dicarboxymycolic acid	
Cell-free system	37 ± 5	17 ± 6	46 ± 5	6 ± 2
Cells in growth medium	32	5	63	2
Cells in medium for cell-free system	29	21	50	1

[a] All experiments were conducted with [^{14}C]acetate labeling for 90 min. The labeling distribution in mycolic acids from growing cells is identical to the known mycolic acid composition of the cells (16).

respectively). The relative proportions of labeling in the three types of mycolic acids produced by the cell-free system (Table 2) were close to the actual proportions of mycolic acids in this strain (16), with a higher oxomycolic and a lower dicarboxymycolic acid content. These differences could be due to the absence of regulation or the incubation conditions (as shown by the relative labeling of mycolic acids in intact cells incubated in the medium used for the cell-free system [Table 2]).

INH effect on cell-free mycolic acid synthesis by the A+ strain. Cell extracts from the A+ strain were used to study INH effects on mycolic acid synthesis, since this strain is INH sensitive.

The MIC of INH under the growth conditions used was 1 μg/ml (7.3 μM). After we tested several INH concentrations with the cell-free system, it appeared that 15 μg/ml (109.5 μM) repeatedly produced strong and specific inhibition of mycolic acid labeling (Fig. 1B). Thus, mycolic acid synthesis was INH sensitive in the cell extracts, as it was in whole cells. This strongly supports the hypothesis of a direct effect of INH on mycolic acid synthesis.

The 15/1 ratio between a concentration that acts efficiently on cell-free synthesis and the MIC is compatible with the expected inhibitory concentration inside living cells, since it is accepted that INH uptake by intact cells is energy dependent (17) and it has been shown that *M. tuberculosis* was able to concentrate INH 20 to 50 times inside cells, depending on the outside concentration (1).

Syntheses of the three types of mycolic acid were inhibited equally; consequently, only total mycolic acid synthesis inhibition is reported.

The kinetics of the INH (15 μg/ml) effect was examined first by adding the drug at the same time as acetate (Fig. 2). As seen from the slopes of the curves in Fig. 2, there was constant strong inhibition (80%) of acetate incorporation rate between 15 and 60 min. After this time, there was only a nonsignificant increase in inhibition of labeling (up to 87%). Thus, 30 min of preincubation of the cell-free system with 15 μg of INH per ml (109 μM) before addition of [^{14}C]acetate was used routinely, except when otherwise stated. Under these experimental conditions, there was almost 90% inhibition of mycolic acid labeling. Glucose addition did not influence the inhibition level.

It is worth noting that INH inhibition of mycolic acid labeling did not cause accumulation of any radioactive intermediates. There was just a slight increase of nonhydroxylated fatty acid radioactivity which could be due to the absence of competition for the added acetate. We have shown (13) that the cell-free system elongates nonlabeled

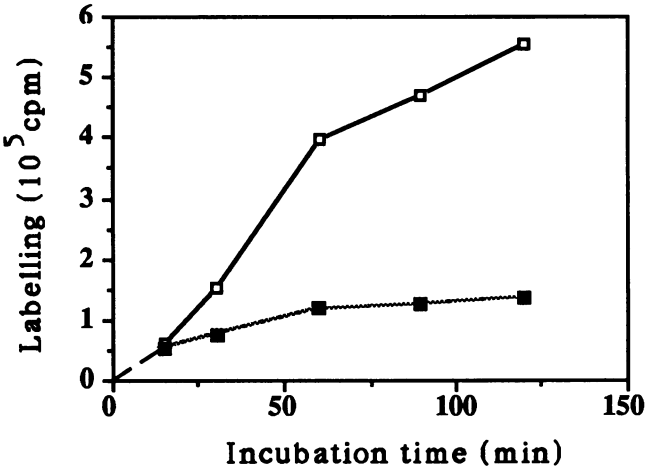

FIG. 2. INH inhibition kinetics of cell-free mycolic acid synthesis. INH and [^{14}C]acetate were added at time 0 to the cell-free system from *M. aurum* A+ (INH-sensitive strain). Each point corresponds to two independent assays, stopped at the indicated time. Symbols: □, control (without INH); ■, with 109 μM INH.

precursors already present in the preparation by adding carbon atoms from acetate (under an unknown intermediate metabolic form). Thus, the INH target could be either synthesis of the acetate metabolite or some early step of its condensation on preformed precursors.

INH resistance of the cell-free *M. aurum* ATCC 23366 system. *M. aurum* ATCC 23366 is known to be an INH-resistant strain (it is unaffected by 200 μg/ml). Comparison of the effects on cell extracts from *M. aurum* A+ and 23366 (Table 3) showed that the preparation from 23366 can be considered resistant to INH, since 1,000 μg of INH per ml caused no significant inhibition.

Intact cells can resist drugs through low uptake, but a cell-free system cannot. It is also frequently postulated that INH-sensitive mycobacteria have a peroxidase activity necessary for INH sensitivity (17). This activity probably relies on a peroxidase that, in addition, has a catalase activity and the ability to produce yellow pigments (Youatt's enzyme) (4). Both of the *M. aurum* strains used here are known to be catalase positive, and peroxidase activity was determined in cell extracts of the two strains. Identical low activities were found in both extracts (about 13 nmol of substrate transformed per h/mg of protein). It can thus be concluded that resistance of strain ATCC 23366 was due to neither low permeability to INH nor absence of peroxidase activity, as far as mycolic acid synthesis is concerned. It has been

TABLE 3. Inhibition by INH of cell-free mycolic acid labeling[a]

Strain	INH concn, μg/ml (μM)	Mean % inhibition ± SEM (no. of samples)
A+	5 (36.4)	51 ± 13 (3)
	15 (109.5)	86 ± 5 (6)
23366	50 (364)	0
	1,000 (7,300)	10

[a] INH was added at the beginning of a 30-min preincubation period, and then [^{14}C]acetate was added to start mycolic acid labeling (90-min incubation). Noninhibited controls were treated under the same conditions without INH.

TABLE 4. Effects of known and potential INH degradation products and hydrazides on mycolic acid labeling by cell extracts of *M. aurum*

Strain	Relative labeling (%) with the following added molecules[a]:							
	None (control)	INH metabolites				Hydrazides		
		Amide	Acid	Aldehyde	Alcohol	Acetic	Benzoic	Nicotinic
A^+	100	105	112	87	96	94	94	88
23366	100	97	103	96	95	ND[b]	ND	ND

[a] Compounds (220 μM) were added for 30 min of preincubation.
[b] ND, not determined.

shown that horseradish peroxidase presents a Youatt's enzyme activity close to that of *M. tuberculosis* peroxidase (4). Addition of horseradish peroxidase to INH-resistant cell extract (ATCC 23366) did not induce INH sensitivity (data not shown).

Thus, it can be said that the mycolic acid synthesis system of *M. aurum* is itself either sensitive or resistant to INH and also that the INH sensitivity of the cell-free system corresponds to the sensitivity of the strain. In addition, INH resistance of cell-free mycolic acid synthesis is not related to the level of peroxidase activity.

Can INH inhibition of mycolic acid synthesis be related to NAD depletion? As mentioned in the Introduction, it has been proposed that INH may block cell growth by leading to synthesis of metabolically inactive isomers of nicotinamide and NAD (10; see also reference 5). INH (0.1 mM) was added to the A^+ cell-free system in the presence of a 1 mM concentration of either nicotinamide, nicotinic acid hydrazide (known to antagonize INH activity), NAD^+, or NADH. There was no significant release of INH inhibition in the presence of these compounds (data not shown). INH did not seem to act on this system as an antimetabolite of nicotinamide or NAD.

INH metabolites are not active against mycolic acid synthesis. It is known that INH can be transformed by sensitive mycobacteria into isonicotinic acid and the corresponding alcohol (4-hydroxymethylpyridine), with an aldehyde as the postulated intermediate, and then possibly into isonicotinamide (10; see also reference 5). These four molecules were tested on both cell-free systems at twice the molar concentration used routinely for INH. None of the INH metabolites had a significant effect on mycolic acid labeling (Table 4); the hydrazide moeity was required for inhibition. Thus, other hydrazides were tested and it can be concluded from the results in Table 4 and from the beginning of this paragraph that acetic, benzoic, and nicotinic acid hydrazides had no effect on cell-free mycolic acid synthesis. These results are in agreement with the known structural requirements for INH, i.e., that both the isonicotinic structure and the hydrazide moiety are required to obtain significant inhibition of mycobacterial culture growth (9).

Effect of INH in the presence of pyridoxal or pyridoxal phosphate. It was shown in early studies on *M. tuberculosis* growth inhibition by INH that compounds with a carbonyl group antagonize INH action, pyridoxal being the most active one (2, 15; see also references 8 and 14). These compounds react nonenzymatically with INH. Pyridoxal and pyridoxal phosphate were tested by adding them (independently at 1,090 μM) with INH (109 μM) at the beginning of the preincubation period of the cell-free system (see Materials and Methods). Pyridoxal had no effect (nearly 90% mycolic acid labeling inhibition), while pyridoxal phosphate nearly abolished the INH effect (10% inhibition). Such a difference between these two compounds was explained by their respective chemical reactivities. Differential spectroscopy (Fig. 3) showed that equimolar concentrations (150 μM) of INH and pyridoxal phosphate, mixed in the buffer used for cell-free mycolic acid labeling, reacted fast enough at room temperature to allow detection, within less than 0.5 h, of a newly formed molecular species with an absorption band at 290 nm (Fig. 3C), while INH (Fig. 3A) and pyridoxal phosphate (Fig. 3B) had quite different absorption maxima. The reaction was apparently complete after 2 days (Fig. 3D), while under the same conditions pyridoxal gave no detectable reaction with INH (data not shown). Since the carbonyl group must be protonated to react, it is likely that the presence of the phosphate moiety is responsible for the high

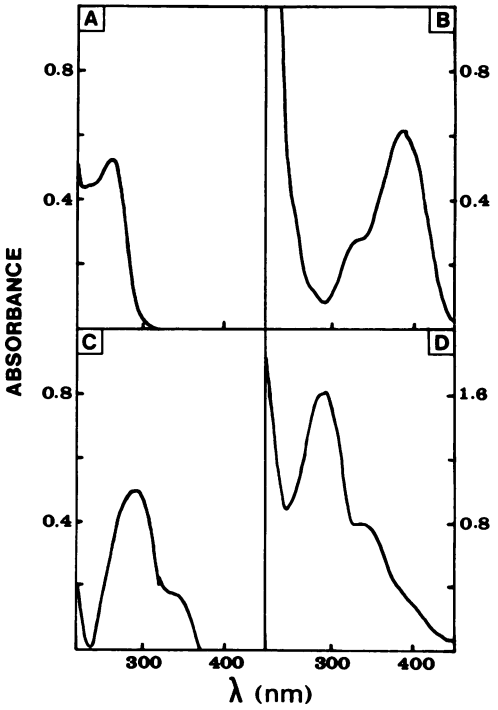

FIG. 3. UV spectra of INH, pyridoxal phosphate, and their reaction products. INH and pyridoxal phosphate were at a final concentration of 150 μM in buffer at pH 7.0. (A) INH UV spectrum. (B) Pyridoxal phosphate UV spectrum. (C) INH plus pyridoxal phosphate differential UV spectrum (after a 30-min reaction in a cuvette; reference beam with both compounds at 150 μM in separate cuvettes). (D) INH plus pyridoxal phosphate UV spectrum (after a 2-day reaction).

pyridoxal phosphate reactivity. Thus, it can be postulated that INH is able to react quickly with pyridoxal phosphate on enzymes bearing this coenzyme. However, it is very unlikely that the effect of INH on the cell-free system was due to participation of pyridoxal phosphate in the synthesis, since the other hydrazides tested (at twice the concentration of INH) did not inhibit this synthesis (Table 4), despite their high reactivities toward pyridoxal phosphate (data not shown).

Implication of a covalent bond between INH and the cell-free system. Since the hydrazide function is highly reactive, it may react chemically and form a covalent bond with a constituent of the cell-free system. To test this hypothesis, a mid-log-phase *M. aurum* A^+ culture was treated for 1 h with 5 μg of INH per ml, and then the cell-free system was prepared and either washed twice by centrifugation (40,000 × *g*) or dialyzed overnight to eliminate free INH or derivatives. Mycolic acid labeling was then determined. The cell-free system from INH-treated cells showed 52% inhibition (data not shown). This result supports the notion of covalent modification of the cell-free system.

Further evidence was brought by using the high reactivity of pyridoxal phosphate toward INH. Pyridoxal phosphate (1,090 μM) was added to the cell-free system prepared from INH-treated bacteria for 30 min of preincubation, and there was no release of labeling inhibition (data not shown).

Conclusion. Use of a cell-free system produced several new data on INH activity in mycobacteria that could not be firmly established by using intact cells. INH-resistant and -sensitive strains yielded resistant and sensitive mycolic acid synthesis systems, respectively; thus, it can be concluded that INH resistance of mycolic acid synthesis was due to neither deficient INH uptake nor the peroxidase activity levels, since these activities were low and very similar in both strains.

It appeared that mycolic acid synthesis was a direct target for INH and not, according to a proposed model, a secondary target of NAD depletion. This synthesis is very specific to mycobacteria and related organisms and may explain why INH is active only against mycobacteria. Inhibition of mycolic acid synthesis can greatly disturb wall building, and this should facilitate the attack on infecting mycobacteria by the host immune system.

Inhibition of cell-free mycolic acid synthesis did not result in accumulation of any labeled intermediate. This suggests that the target of INH is either synthesis of acetate metabolites used to elongate the fatty chains or some early steps of chain elongation, since we know that the cell-free system uses certain long-chain precursors present in the preparation (13). In addition, with a powerful antagonist that reacts with INH (pyridoxal phosphate), it appeared that INH could act on mycolic acid synthesis by covalently reacting with some essential constituent of the system.

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