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Effect of Isoniazid on the In Vivo Mycolic Acid Synthesis, Cell Growth, and Viability of *Mycobacterium tuberculosis*

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When an actively growing culture of the H37Ra strain of *Mycobacterium tuberculosis* was exposed to isoniazid at a concentration of 0.5 μg/ml, the cells began to lose their ability to synthesize mycolic acids immediately. After 60 min, the cells had completely lost this ability. The synthesis of the three mycolate components—α-mycolate, methoxymycolate, and β-mycolate—was inhibited. The viability of the isoniazid-treated cells was unaffected up to about 60 min of exposure, after which time there was a gradual decline in the viability to about 18% after 180 min. Correspondingly, growth of the drug-treated cells slowed down and stopped after 24 hr. The inhibition of the synthesis of mycolic acids was reversible if the drug was removed before the loss of viability set in. Incubation of the viable cells in the absence of the drug for 24 hr restored the mycolate synthesis. These results strongly suggest that the inhibition of the synthesis of the mycolic acids is closely associated with the primary mechanism of action of isoniazid on the tubercle bacilli. The sequence of events which leads to the loss of viability of cells exposed to isoniazid is described.

Isoniazid is the mainstay of antituberculosis therapy. Since its discovery as a tuberculostatic agent (12), much work has been done to elucidate its mechanism of action (32). The precise nature of the primary action of isoniazid on the tubercle bacilli is unknown today.

One of the more significant early observations was that tubercle bacilli lose their acid-fastness when exposed to isoniazid (14), suggesting that the composition of a certain lipid might be somewhat altered. The altered component could be the mycolic acids which have been implicated in the acid-fastness of this organism (11). Studies of several workers suggested an intimate relationship between the effect of the drug and the synthesis of specific lipid components unique to the cell envelope of the tubercle bacilli (4, 10, 25, 26). Recently, Winder and associates found evidence that isoniazid interfered with the formation of the cell envelope, specifically the synthesis of mycolic acids (30, 31).

We have investigated the effect of isoniazid on the in vivo synthesis of mycolic acids in *M. tuberculosis* H37Ra using ^{14}C-acetate as the metabolite. We found that isonizaid at a concentration of 0.5 μg/ml immediately began to reduce the cells' ability to synthesize mycolic acids. The gradual decline in viability of the cells began only after complete inhibition of the synthesis of mycolic acids had occurred. In addition, the effect of the drug on the synthesis of mycolates was reversible if the drug was removed before the cells lost their viability. These findings are used to describe the possible mechanism of the action of isoniazid.

MATERIALS AND METHODS

Bacterial strain and growth. *M. tuberculosis* H37Ra was grown in either (A) glycerol-alanine-salts medium or (B) the Middlebrook 7H9 medium (Difco) with Tween 80 and ADC enrichment in a rotary shaker at 37 C. Medium B was used when quantitative sampling of the culture was required. Because of the dispersed growth in the latter medium, growth could be followed by measuring the absorbancy at 650 nm with a Coleman spectrophotometer model 6/20.

Determination of viable cells. The number of viable cells was determined by plating appropriately diluted samples onto Middlebrook 7H10 agar medium (Difco). The plates were incubated for 3 weeks in an atmosphere of 5 to 10% CO_2, and the colonies were counted. Seven samples of each point were plated to insure statistical significance.

Thin-layer chromatography. Methyl mycolates were

chromatographed on Silica Gel G (0.5 mm) thin-layer plates with the use of one of the following solvent systems: I, petroleum ether-diethyl ether (9:1, v/v); II, benzene; and III, petroleum ether-diethyl ether (8:2, v/v). The methyl mycolate spots (or bands) were located either by the 1% methanolic iodine spray or by the dichromate-sulfuric acid spray (24). When preparative-scale thin-layer chromatography was used, distilled water was sprayed heavily on the plates to locate the white lipid bands in a dark wetted background. Two-dimensional thin-layer chromatography was performed with solvent I in the first dimension and solvent II in the second dimension. When radioactive methyl mycolates were chromatographed, the mycolate bands were located with iodine spray; 1-cm strips were scraped directly into counting vials and were counted in 15 ml of toluene-BBOT (2,5-bis-[2-5-*tert*-butylbenzoxazolyl]-thiophene) by use of a Packard Tri-Carb scintillation spectrometer.

Isolation and purification of mycolic acids from M. tuberculosis. Mycolic acids were isolated from either the washed cells or the crude cell wall fraction of *M. tuberculosis* H37Ra by a procedure described previously (1). In addition, the crude mycolic acid was dissolved in one volume of diethyl ether and precipitated out with two volumes of ethanol prior to the methylation step. The methyl mycolates were then fractionated on a Sephadex LH-20 column (28). The methyl mycolates were then separated into the three major components by performing preparative-scale thin-layer chromatography, first with solvent I and then with solvent II (developed twice). When fractionation of a small amount of sample was desired, two-dimensional thin-layer chromatography was utilized.

Assay for in vivo mycolate synthesis. A 2.5-μCi amount of [*1*-^{14}*C*]-acetate (2.88 μCi/μmole) was added to 5 ml of actively growing culture of *M. tuberculosis* with an optical density at 650 nm of approximately 0.1, and the culture was incubated for 15 min. Preliminary experiments showed that under the condition of assay the incorporation of labeled acetate into the mycolic acid fraction was linear up to 40 min. Growth was then terminated by adding 1 ml of 12% perchloric acid. Cells recovered after centrifugation were saponified in 5 ml of 5% KOH in 50% ethanol at 85 C for 4 hr. The saponified lipid was acidified with 1.2 ml of 6 N HCl and was extracted with a total of 12 ml of diethyl ether. The ether extract was evaporated to dryness, and one-tenth of the sample was used to assay for total radioactivity. Carrier mycolic acids (100 μg) were added to the remaining extract, and were methylated with diazomethane. The entire methylated mycolate sample was applied to a Silica Gel G thin-layer plate and developed with solvent III. The methyl mycolate spots were located with iodine spray and analyzed for radioactivity as described.

Chemicals. [*1*-^{14}*C*]-acetic acid, [*2*-^{14}*C*]-acetic acid, [*2*-^{14}*C*]-malonic acid, [*1*-^{14}*C*]-propionic acid, [*1*-^{14}*C*]-palmitic acid, [*1*-^{14}*C*]-stearic acid, and L-[methyl-^{3}H] methionine were obtained from New England Nuclear Corp., Boston, Mass. Isoniazid was purchased from Calbiochem, Los Angeles, Calif. The ethanol-diethyl ether-insoluble fraction of the whole cell lipid prepared as described above represented the crude mycolic acid that was used as the carrier lipid in the assay for mycolate synthesis.

RESULTS

Isolation and characterization of the mycolic acids of M. tuberculosis H37Ra. To investigate the effects of isoniazid on the synthesis of mycolic acid in the H37Ra strain, several points had to be established. The results of Acharya and Goldman (1) on the structure of the mycolic acids of the H37Ra strain of *M. tuberculosis* differ somewhat from those of Etamadi (8), Etamadi and Lederer (9), and Minnikin and Polgar (19–22) for the mycolic acids of the tubercle bacilli. The first group concluded that there were two homologue series of mycolic acids (A and B). The point of controversy is the homologue series A, which appears to be related to β-mycolate, in that both show a keto functional group. However, the homologues of series A have neither the methylene side chain nor the cyclopropane ring present in β-mycolate. In place of the cyclopropane ring, the series A compounds contain a double bond. For this reason, we have reinvestigated the structure of the mycolic acids in the H37Ra strain. In addition, we desired to separate each of the several mycolate components and identify them so that we could measure the effects of isoniazid on each mycolic acid component.

The bound lipids were extracted from 7.8 g of crude cell wall preparation of the H37Ra strain, methylated, and then fractionated on a silicic acid column as previously described (1) to obtain the crude methyl mycolate fraction. The Sephadex LH-20 column fractionation yielded 132.5 mg of partially purified methyl mycolate. This sample was then further fractionated into three major mycolate components by Silica Gel G thin-layer chromatography in solvents I and II (developed twice). By infrared, nuclear magnetic resonance, and mass spectral analysis, the three components were identified as follows (in the order of decreasing R_F values): α-mycolate, 70.2 mg (melting point, 49 to 50 C); methoxymycolate, 5.9 mg (melting point, 42.5 C); and β-mycolate, 1.6 mg (melting point, 45 C). The total recovery of the methyl mycolates was 59%. These results correspond to the published data on the identity of major mycolate components of tubercle bacilli (8, 9, 19–22). The difference between our results and those obtained previously (1) on the structure of mycolic acids in the H37Ra strain can probably be attributed to the fact that other investigators attempted to determine the structural components by interpret-

TABLE 1. *Percent distribution of radioactivity in the three components of ^{14}C-mycolic acids synthesized in vivo by Mycobacterium tuberculosis*[a]

Labeled metabolite used	Yield of ^{14}C-mycolic acid (counts/min)	Distribution of radioactivity (%)		
		α-Mycolate	Methoxymycolate	β-Mycolate
[1-^{14}C]-propionate (9.78 μCi/μmole)	1.45×10^5	72	14	14
[2-^{14}C]-acetate (1.95 μCi/μmole)	6.25×10^5	77	13	10
[1-^{14}C]-stearate (10.10 μCi/μmole)	9.20×10^5	79	12	9

[a] To three flasks (80 ml) of 9-day-old cells of *M. tuberculosis* grown in medium A, 5 μCi of radioactive metabolite per flask was added and incubated for 17 hr on a shaker at 37 C. The cells were harvested by filtration and washed with water. The ether extracts from the saponified and acidified cells were further fractionated by an ethanol-ether extraction to obtain the ^{14}C-mycolic acids. A sample of the labeled mycolic acids (4,500 counts/min plus 100 μg of carrier mycolic acids) was methylated and then fractionated by two-dimensional thin-layer chromatography to yield the three components. Radioactivity in each component was determined and the percent distribution was calculated.

ing the mass spectra of a partially purified mixture of the methyl mycolates.

Determination of a suitable labeled metabolite to use in measuring mycolate synthesis. For our studies into the effects of isoniazid on the synthesis of mycolic acids in the tubercle bacilli, we needed to determine the most appropriate labeled metabolite to use in measuring the in vivo synthesis. We found labeled glycerol to be unsatisfactory. The ^{14}C-labeled precursors tested were acetate, malonate, propionate, palmitate, and stearate. Our results showed that labeled acetate was the most suitable, because it was rapidly taken up by the growing cells and gave the highest incorporation of the radioactivity into the mycolic acid fraction.

Incorporation of labeled metabolites into the three components of mycolic acids and the inhibition by isoniazid. The radioactive methyl mycolates obtained from the in vivo incorporation of specific metabolites, namely, ^{14}C-propionate, ^{14}C-acetate, and ^{14}C-stearate, were fractionated into each of the three components, and their distribution of radioactivity was examined (Table 1). The radioactivity was incorporated into all three major mycolic acid components, and the ratios of the radioactivity incorporated into the three components of mycolates were similar, yielding a ratio of α-mycolate-methoxymycolate-β-mycolate of about 75:15:10. These results reflect the relative abundance of the three components of mycolic acids in *M. tuberculosis* H37Ra.

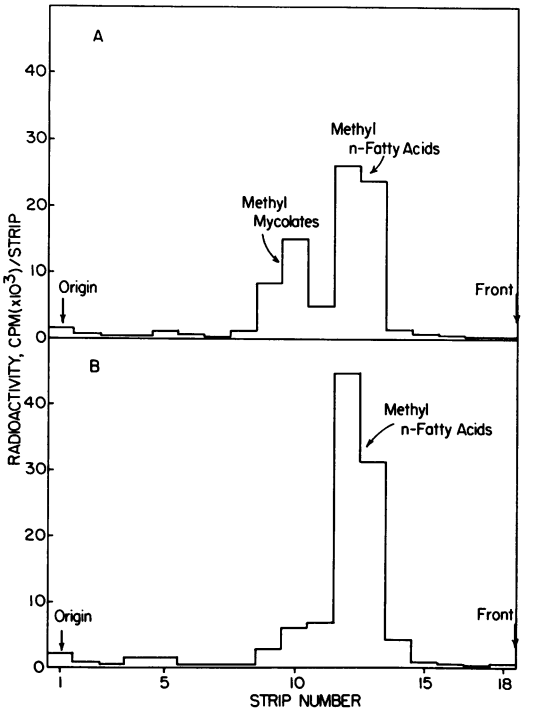

FIG. 1. *Comparison of the thin-layer chromatograms of the methylated ^{14}C-lipids obtained from M. tuberculosis incubated in the absence (A) and in the presence (B) of isoniazid. To two flasks (80 ml) of 9-day-old cells of M. tuberculosis grown in medium A, isoniazid was added to a final concentration of 0.5 μg/ml followed by the addition of 5 μCi of [2-^{14}C]-acetate (specific radioactivity, 1.95 μCi/μmole) per flask. Control cultures contained only the added labeled acetate. The cells were incubated at 37 C for 30 min, harvested at 0 C, washed with cold water, and saponified. The saponification mixture was acidified and extracted with diethyl ether. The radioactivity recovered in the ether extracts was as follows: control cells, 1.20×10^6 counts/min; isoniazid-treated cells, 1.18×10^6 counts/min. A portion of each sample (10^5 counts/min) was methylated and subjected to thin-layer chromatography with solvent III. Strips (1 cm) were scraped into counting vials and radioactivity was determined. Standards of oleyl alcohol, methyl mycolate, and methyl oleate were run simultaneously.*

TABLE 2. *Effect of isoniazid on the incorporation of [2-^{14}C]-acetate into mycolic acids of Mycobacterium tuberculosis*[a]

Methyl ester	Specific radioactivity (disintegrations per min per mg)		Inhibition (%)
	Control	Isoniazid-treated	
α-Mycolate.....	53,965	11,576	78.5
β-Mycolate plus methoxymycolate...	19,719	4,384	78.0

[a] The methyl mycolates obtained from both the control and the isoniazid-treated cultures as described in Fig. 1 were isolated and purified by thin-layer chromatography. They were separated into the three components and their specific radioactivities were determined.

An experiment was performed to determine whether isoniazid inhibits the synthesis of the mycolic acids. Figure 1 shows that, when isoniazid was added at a concentration of 0.5 μg/ml, the incorporation of ^{14}C-acetate into the total mycolate fraction was drastically inhibited. This figure also shows that the drug did not inhibit the incorporation of ^{14}C-acetate into the normal fatty acids. Table 2 shows that the synthesis of all three components of mycolic acids was inhibited to the extent of 78%.

A similar experiment was performed to determine the effect of isoniazid on the incorporation of the tritiated methyl group from L-[methyl-^{3}H]-methionine into the mycolic acids. Whereas the label from ^{14}C-acetate would be expected to become evenly distributed throughout the mycolate molecules, the tritium in the CH_3 group of L-methionine can be incorporated into the methylene side chain (2, 16) and the cyclopropane ring (5, 33) of the mycolates. Since the points of entry of these two metabolites into the pathway for the synthesis of mycolic acids differ, they represent two types of assays for mycolate synthesis. The results of such an experiment showed that again isoniazid inhibited the synthesis of the mycolic acids (Fig. 2).

Effect of isoniazid concentration on mycolic acid synthesis. To 10-ml tube cultures of *M. tuberculosis* grown in medium B with shaking, different amounts of isoniazid were added, and the cultures were incubated for 1 hr. The cultures were assayed immediately for their ability to synthesize the mycolates. Figure 3 shows that mycolic acid synthesis decreased nonlinearly with increasing concentration of isoniazid. Under the assay conditions used, the inhibition approached completion at a drug concentration of about 0.5 μg/ml. We thus chose this threshold concentration for all subsequent experiments. Isoniazid has been reported to inhibit the incorporation of ^{14}C-acetate into mycobacteria (7, 29). A close examination of Wimpenney's data (29) reveals that the uptake of ^{14}C-acetate in a 14- to 21-day surface culture of *M. tuberculosis* BCG containing isoniazid at a concentration of 10 μg/ml is slightly lower than in the control after 15 min. In our assay for mycolate synthesis, we detected no inhibition of the uptake and the incorporation of ^{14}C-acetate into the total lipids of *M. tuberculosis* in the presence of isoniazid in the concentration range of 0.10 to 1.00 μg/ml.

Effect of time of exposure of M. tuberculosis cells to isoniazid on their ability to synthesize mycolic acids and their viability. Isoniazid was added to a final concentration of 0.5 μg/ml to an actively growing culture of *M. tuberculosis*.

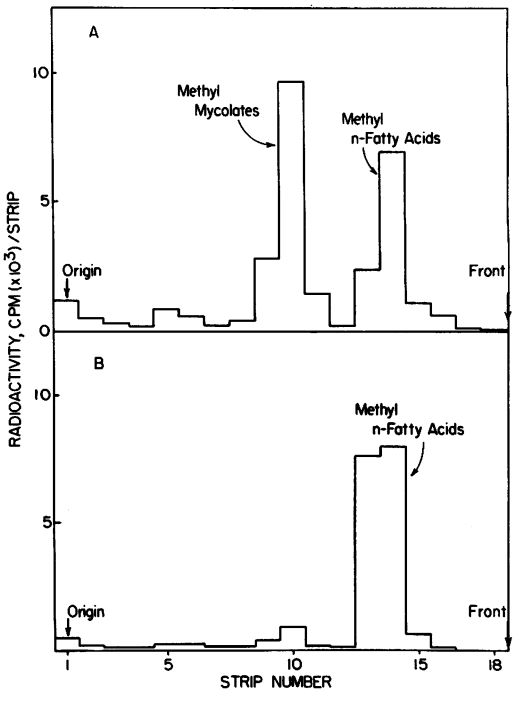

FIG. 2. *Comparison of the thin-layer chromatograms of the methylated ^{3}H-CH_3-lipids obtained from M. tuberculosis incubated in the absence (A) and presence (B) of isoniazid. The detailed procedure for this experiment is identical to that in Fig. 1 except that 20 μCi of L-[^{3}H-CH_3]-methionine (3.27 Ci/mmole) was added to each flask instead of ^{14}C-acetate. The radioactive lipids recovered in the acidic diethyl ether extracts were as follows: control cells, 3.00 × 10^{5} counts/min; isoniazid-treated cells, 1.76 × 10^{5} counts/min. One-tenth of each sample was used for thin-layer chromatographic analysis.*

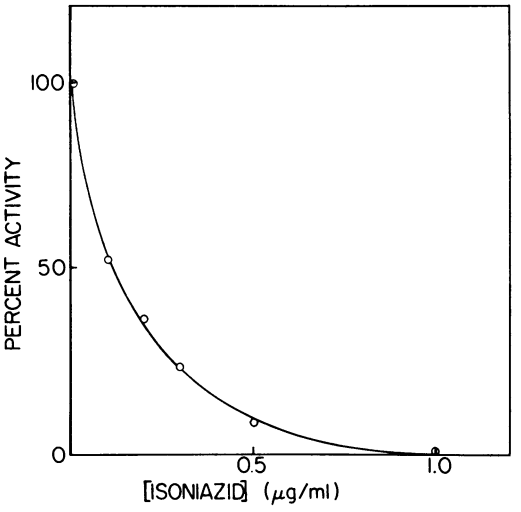

FIG. 3. *Effect of isoniazid concentration on the inhibition of mycolic acid synthesis. To 10 ml of actively growing tube cultures of M. tuberculosis in medium B having an absorbancy at 650 nm of 0.21, various amounts of isoniazid were added and incubated for 1 hr. [1-^{14}C]-acetate (2.5 μCi) was used in the assay for mycolic acid synthesis. Approximately 15,000 counts/min were incorporated into the total ether extract in each assay. The amount of ^{14}C-mycolate synthesized in the control culture without isoniazid addition was set at 100% activity.*

Samples were taken out to measure the cells' ability to synthesize the mycolic acids at different time intervals. As shown in Fig. 4, the ability of the cells to incorporate labeled acetate into the mycolic acids decreased linearly with time of exposure; after 60 min, the activity was measured to be 1%. The growth of both the isoniazid-treated and the control cultures was followed simultaneously by measuring their absorbancy at 650 nm. We found that isoniazid-treated cultures continued to increase in absorbancy for 24 hr before leveling off. This is consistent with published observations (3, 17, 18). After the loss of mycolate synthesis, the viability of these isoniazid-treated cells remained unaffected for about 30 min (Fig. 4). A statistically significant drop in the number of viable cells occurred after 90 min of exposure to the drug. After 180 min, the population of viable cells was reduced to 18%.

Restoration of mycolic acid synthesis in washed cells of M. tuberculosis after exposure to isoniazid. Since there seemed to be an apparent lag between the time of complete inhibition of mycolate synthesis (60 min) and the beginning of the statistically significant decrease in cell viability (90 min) when the tubercle bacillus was exposed to 0.5 μg of isoniazid/ml, we proceeded to investigate the possibility of reversing the inhibition of mycolate synthesis. Table 3 shows that, as before, the cells exposed to isoniazid for 60 min had a greatly reduced ability to synthesize mycolates. In a separate experiment, we found that when such isoniazid-treated cells were washed free from the drug and assayed immediately, the cells were unable to synthesize the mycolic acids. However, when these washed cells were reincubated in a fresh medium for 24 or 48 hr, as shown in Table 3, they recovered their ability to synthesize the mycolates. In these experiments, the washed and reincubated cells continued to grow normally for at least three generation times, showing a typical growth curve for *M. tuberculosis*. The cells that were left to grow in the original culture medium containing isoniazid showed the usual growth inhibition after 24 hr.

DISCUSSION

Our experiments verify the report of Winder and Collins (30) that isoniazid does indeed inhibit the synthesis of mycolic acids in the tubercle bacilli. Using labeled acetate as the most appropriate metabolite in measuring the in vivo synthesis of mycolic acids in *M. tuberculosis* H37Ra, we were able to detect an immediate

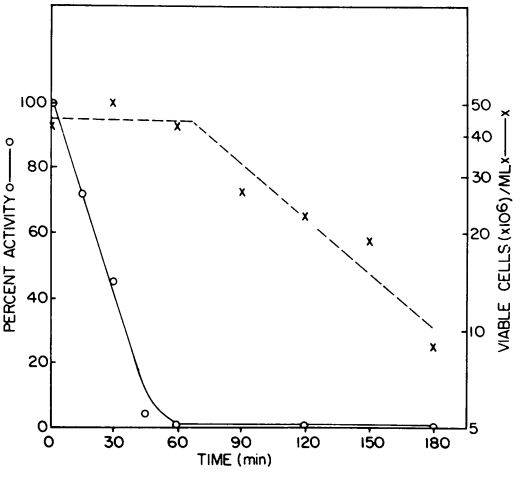

FIG. 4. *Effect of time of exposure to isoniazid on the in vivo mycolate synthesis and cell viability of M. tuberculosis H37Ra. To young cultures of M. tuberculosis grown in medium B having an absorbancy at 650 nm of 0.10, isoniazid was added to a final concentration of 0.5 μg/ml. Samples (5 ml) were pipetted out aseptically, and their ability to incorporate labeled acetate into mycolic acid was measured as described in Materials and Methods. In each case, 2.5 μCi of [1-^{14}C]-acetate was used and about 20,000 counts/min were recovered in the ether extract. The zero time control before isoniazid addition was set at 100% activity.*

TABLE 3. *Restoration of mycolate synthesis by washed cells of Mycobacterium tuberculosis after isoniazid treatment*[a]

Expt. no.	Condition	Specific activity of mycolic acid synthesis[b]
I	Before isoniazid addition	830
	After 1-hr exposure to isoniazid	78
	Isoniazid-pretreated cells, washed and reincubated for 24 hr	690
	Isoniazid-pretreated cells, washed and reincubated for 48 hr	650
II	Control cells	580
	Control cells, washed and reincubated for 24 hr	690

[a] *M. tuberculosis* cells were grown in medium B to an absorbancy of approximately 0.1 at 650 nm. In experiment I, the cells were exposed to isoniazid at 0.5 μg/ml for 60 min. Samples (10 ml) were transferred into sterile tubes, centrifuged, and washed three times with sterile medium at 0 C under aseptic conditions. The cells were then resuspended in 5 ml of prewarmed medium and incubated at 37 C for 24 and 48 hr. These washed and reincubated cells were assayed for mycolate synthesis. Control samples (5 ml) from the original culture before and after the 1 hr of isoniazid exposure were also measured for their ability to synthesize mycolate. [*1*-^{14}C]-acetate was pulsed in, and the total radioactivity recovered in the ether extract was approximately 35,000 counts/min in all cases. Optical density of all cultures was measured, and the number of cells was calculated from a standard curve. In experiment II, a control culture was assayed under identical conditions as in experiment I without the addition of isoniazid.

[b] Specific activity = counts per minute of ^{14}C-mycolate synthesized per 10^9 cells per 15 min.

inhibition of the synthesis of these lipids after exposing an actively growing culture to 0.5 μg of the drug per ml. All three components of the mycolic acids were similarly inhibited. It is interesting to note that the time required for the cells' complete loss of mycolate synthesis (60 min) coincided with the extrapolated beginning of the decline in the viability of the cells. The level of the drug used (0.5 μg/ml) approaches the minimal inhibitory concentration for growth, viability, and mycolate synthesis. These observations, together with the facts that the *M. tuberculosis* cells contain their own unique forms of mycolic acids (15) and are highly sensitive to isoniazid (23, 27), suggest that a close relationship exists between the synthesis of these specific lipids and the viability and growth of the cells.

Our observation of the immediate inhibition of the synthesis of mycolic acid is, of course, the earliest event ever reported. Previously, the effect of the drug was examined several hours and even days after its addition. Other effects of isoniazid reported to occur within 60 min were the uptake of the drug, its metabolism, and the formation of pigments (32). In the recent work of Winder and Collins (30), in which they used ^{14}C-glycerol as the metabolite, the minimal exposure time was 3 hr. Virtually all other studies done on the tubercle bacilli at threshold levels of the drug used exposure times of at least 24 hr (32).

The order of action of isoniazid on *M. tuberculosis* can be described as follows. Cells exposed to 0.5 μg of isoniazid/ml gradually lose their ability to synthesize mycolic acids, until a complete loss occurs after about 60 min. At this point, the cells slowly begin to lose their viability so that, after an additional 120 min, 80% of the cells are nonviable. Thus, there is a certain period of time (up to about 2 hr) when the cells continue to grow in the absence of mycolate synthesis and still maintain their viability. Also within this period, it is apparent that the complete inhibition in the in vivo synthesis of mycolic acid is slowly reversible by simply removing the drug and reincubating in fresh medium. The action of the isoniazid on the mycolate synthesis mechanism is immediate. The reaction of the mycolate-synthesizing system to the drug in vivo is a gradual response. The lethal event appears to develop even more gradually.

One can perhaps describe the lethal action of the drug in the following manner. A certain critical point is reached in the growth of a bacillus when, owing to the lack of mycolic acids in the newly developed cell wall and envelope, the cells are irreversibly damaged and cannot perform the function of dividing. Perhaps we can call this a form of "unbalanced growth," which eventually becomes lethal after a definite exposure time that we can predict to be a fraction of a generation time. A similar conclusion was reached by Winder and Collins (30). The concept that the bactericidal effects of a drug are produced by an imbalance in the synthesis of cellular constituents has considerable experimental support (26).

The key question to be asked is, how does isoniazid inhibit mycolate synthesis? Since the action is immediate, we would expect the drug to act directly on the system at the transcription or translation level, or on the enzyme(s) of the pathway of mycolate synthesis. A clue may be provided by the work of Gangadharam et al.

(13). When one extrapolates their data to less than 3 hr of exposure to isoniazid, it shows that both total protein and ribonucleic acid synthesis are unaffected, whereas deoxyribonucleic acid synthesis may be inhibited by isoniazid. They suggested this to be analogous to the lethal "unbalanced growth" of the thymineless mutants of *Escherichia coli* during thymine starvation (6). We are presently investigating this possibility.

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