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Mechanism for the Pyridoxal Neutralization of Isoniazid Action on *Mycobacterium tuberculosis*

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In Sauton's synthetic liquid medium, 10 μg of pyridoxal per ml completely protected *Mycobacterium tuberculosis* ($H37R_a$) from the effects of a minimal inhibitory concentration of isoniazid (0.01 μg/ml). ^{14}C-labeled isoniazid was employed to study the nature of this protective effect. Uptake of the drug by cells in a Sauton environment containing 0.01 μg of ^{14}C-isoniazid per ml was inhibited 20 to 40% by 10 μg of pyridoxal per ml during the early hours of drug exposure. A stronger inhibition of uptake resulted when labeled isoniazid and pyridoxal were increased to 0.1 μg/ml and 50 to 100 μg/ml, respectively. Further studies revealed that certain Sauton nutrients are required to achieve this effect. When L-asparagine or salts ($MgSO_4$ and ferric ammonium citrate) or both were deleted from the menstruum, pyridoxal did not inhibit isoniazid incorporation by the tubercle bacilli. Pyridoxal also failed to inhibit uptake when $(NH_4)_2SO_4$ was substituted for L-asparagine. Growth experiments in Sauton's medium modified to contain $(NH_4)_2SO_4$ instead of L-asparagine were consistent with the latter finding. Pyridoxal did not prevent isoniazid growth inhibition in this medium. It is postulated that a large excess of pyridoxal in Sauton's medium protects tubercle bacilli from the effects of isoniazid through formation of an extracellular complex involving drug, vitamin, and certain medium constituents, thereby reducing the level of isoniazid available to the cells.

At extremely low concentrations, isoniazid (INH) exerts a profound and highly specific inhibitory effect on the growth of *Mycobacterium tuberculosis*. Although numerous attempts have been made to identify the primary target or site of INH action, no one hypothesis is generally accepted. The high degree of specificity exhibited in the INH-tubercle bacillus relationship can be explained in part on the basis of drug uptake. INH-susceptible tubercle bacilli take up the drug much more readily than do resistant strains or other microbial species (1, 2, 11, 16). This finding indicates that the primary site of action need not be a metabolic function peculiar to the tubercle bacillus.

Numerous reports suggest that INH interferes with vitamin B_6 metabolism (5, 7-10, 12, 13, 15). One of the arguments used to support this hypothesis is based on the observation that the presence of excess pyridoxal in culture media protects *M. tuberculosis* from growth inhibition by isoniazid (3, 9, 10). Experiments reported by Boone et al. (3) and by Youatt (16) indicated that pyridoxal stimulates ^{14}C-INH uptake by the tubercle bacillus. The uptake studies were particularly intriguing since they seemed to be inconsistent with the growth inhibition experiments.

The results reported in this communication indicate that a large excess of pyridoxal in growth media protects the tubercle bacillus from INH by preventing cellular accumulation of toxic drug levels. It is postulated that an extracellular complex is formed involving pyridoxal, INH and certain medium constituents.

MATERIALS AND METHODS

Organism. M. tuberculosis strain $H37R_a$ was used exclusively in these studies. The stock strain carried in this laboratory was obtained originally from the National Communicable Disease Center, Atlanta, Ga. It was grown routinely in the synthetic liquid medium described below.

Medium. The growth medium employed in these studies was essentially the same as that described by Sauton (14). It consisted of citric acid, 2.0 g; $MgSO_4$, 0.5 g; ferric ammonium citrate, 50 mg; K_2HPO_4, 0.5 g; L-asparagine, 4.0 g; glycerol, 35 ml; and distilled-deionized water to 1 liter. Tween 80 was then added to 0.02%, and the medium was adjusted to *p*H 7.4 with 6 N NaOH. Portions of 200 ml were distributed into 500-ml Erlenmeyer flasks, and sterilization was accomplished by autoclaving at 121 C for 20 to 30 min.

Pyridoxal and INH. Pyridoxal monohydrochloride (B_6al) was obtained from Calbiochem, Los Angeles, Calif. INH, supplied as Rimifon powder, was a gift

from Hoffmann-La Roche, Inc., Nutley, N.J. Labeled isoniazid was supplied by Nuclear-Chicago Corp., Des Plaines, Ill., as isonicotinic hydrazide (^{14}C-carbonyl) with a specific activity of 9.8 mc/mmole. The biological activity of the labeled drug was confirmed. When required, solutions of drug and vitamin were sterilized by filtration.

Phosphate buffer-glycerol. A menstruum designated as phosphate buffer-glycerol (PBG) was used in certain of the ^{14}C-INH uptake studies. To 1 liter of 0.1 M potassium phosphate buffer (*p*H 7.4) were added citric acid, 2.0 g; glycerol, 35 ml; and Tween 80, 0.02%. The menstruum was readjusted to *p*H 7.4 with 6 N NaOH.

Experimental cultures. Sauton's medium was inoculated to an optical density (600 mμ) of 0.005 to 0.015 and incubated for 3 to 8 days at 37 C with aeration (rotary shaking at 160 rev/min). Log-phase cells obtained in this manner were used for both the growth and drug-uptake experiments.

Growth experiments. Portions of 200 ml of the appropriate experimental medium were inoculated and incubated in the dark at 37 C with aeration. At appropriate time intervals, 8-ml samples were removed aseptically, homogenized with a Teflon tissue grinder, and read at 600 mμ in a Coleman Junior Spectrophotometer by use of matched 18 by 150 mm tubes.

^{14}C-INH uptake experiments. Either 25- or 50-ml portions of cells suspended in the appropriate menstruum were distributed into 125-ml Erlenmeyer flasks and exposed to labeled isoniazid at 29 or 37 C with aeration. At various time intervals, 5.0-ml samples were removed, and the cells were collected on 2.54-cm filter membranes (pore size, 0.8 μ; Gelman Instrument Co., Ann Arbor, Mich.). The membranes were washed with 5 ml of fresh menstruum, glued to planchets with rubber cement, and dried. ^{14}C activity was measured with a gas flow counter, and results are expressed as millimicrograms of ^{14}C-INH per milligram (dry weight) of cells. Dry weights were determined routinely from standard curves relating optical density to milligrams of dry weight of cells per milliliter.

RESULTS

The inhibitory effect of INH on the growth of *M. tuberculosis* and the prevention of this inhibition by pyridoxal (B_6al) are shown in Fig. 1. A concentration of 10 μg of pyridoxal per ml of culture completely protected the organism from a minimal inhibitory concentration of drug (0.01 μg of INH per ml).

Tubercle bacilli in Sauton's medium readily incorporated ^{14}C-labeled INH and this process was inhibited by pyridoxal (Fig. 2). The results presented in Fig. 2A were obtained by use of the same initial concentrations of drug and vitamin as were used in the growth experiment shown in Fig. 1. When labeled INH was employed at 0.1 μg/ml and pyridoxal at 50 or 100 μg/ml, inhibition of drug incorporation was more severe than at the lower concentrations (Fig. 2B). Uptake was determined only during the early hours of isoniazid exposure to minimize errors which could result from such factors as drug metabolism (17), chemical alteration of the drug (3), and cell death.

The uptake data presented in Fig. 2 do not agree with those of previous reports (3, 16) which

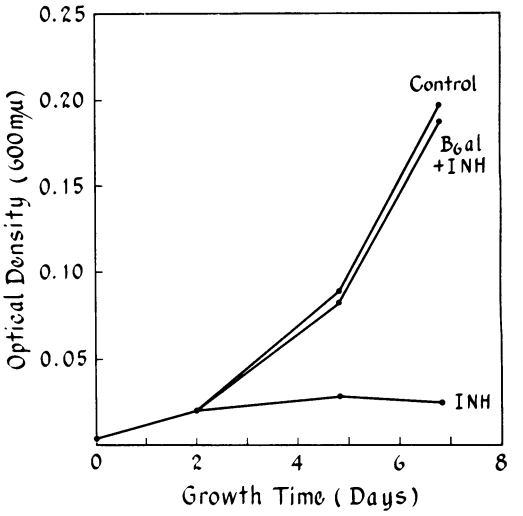

FIG. 1. *Effect of pyridoxal on INH growth inhibition of tubercle bacilli in Sauton's medium. Each flask was inoculated with 8.0 ml of a log-phase Sauton culture. Pyridoxal concentration, 10 μg/ml; INH concentration, 0.01 μg/ml. The control culture contained no drug or vitamin.*

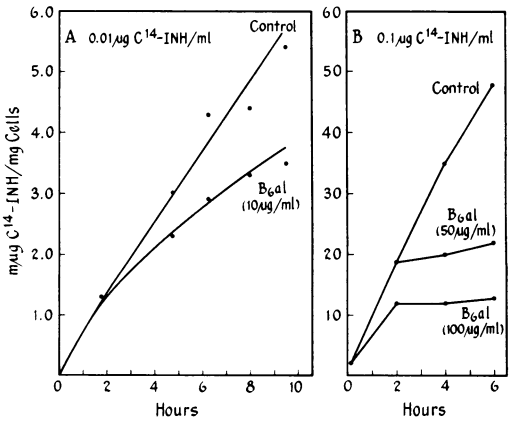

FIG. 2. *Effect of pyridoxal on ^{14}C-INH uptake by tubercle bacilli in Sauton's medium. For each of the experiments, a log-phase Sauton culture containing 0.09 mg of cells per ml was used. Samples of the cultures were distributed into separate flasks, and pyridoxal and ^{14}C-INH were added as indicated. Incubation was at 37 C with aeration.*

suggested that pyridoxal stimulates cellular incorporation of labeled INH. Since pyridoxal can form complexes with various amino compounds (4), it seemed possible that the L-asparagine in Sauton's medium might play a role in the pyridoxal effect reported here (Table 1). Cells were harvested from a Sauton culture and suspended to a concentration of 0.13 mg/ml in fresh medium lacking asparagine. Portions of 25 ml of the suspension were placed in flasks containing weighed samples of the appropriate nitrogen compounds such that the final concentration in each case was 0.03 M (the concentration of asparagine ordinarily employed in Sauton's medium). Pyridoxal and ^{14}C-INH were then added, and the flasks were incubated at 37 C with aeration for 16 hr. Growth during this period amounted to less than one doubling of the cell mass. Although there were slight differences in growth depending on the nitrogen source, pyridoxal did not appreciably affect the final cell density for any particular nitrogen source. From the data presented in Table 1, it is clear that, in addition to pyridoxal, L-asparagine was required to inhibit cellular accumulation of labeled INH. Inhibition was also achieved when glutamate was substituted for asparagine. However, in the absence of a nitrogen compound or in the presence of inorganic nitrogen, pyridoxal failed to inhibit drug uptake.

From the findings reported thus far, it was reasoned that pyridoxal might not exert a protective effect on INH growth inhibition of cells in a medium containing $(NH_4)_2SO_4$ as the sole source of nitrogen. The data presented in Fig. 3 clearly show that pyridoxal did not protect the organism from INH in such a medium.

Pyridoxal, amino compounds, and cations can react together to form complexes of varying composition (4). In view of this, it seemed worthwhile to examine the effect of two Sauton salts, $MgSO_4$ and ferric ammonium citrate, on the pyridoxal inhibition of INH accumulation (Table 2). Log-phase cells were harvested from Sauton's medium, washed once in water, and homogenized in approximately 10 ml of PBG. Additional PBG was then added to give a final cell concentra-

TABLE 1. *Effect of nitrogen source on the pyridoxal inhibition of ^{14}C-INH uptake*

Nitrogen source (0.03 M)	Amt (mμg) of ^{14}C-INH/mg of cells[a]	
	No Bεal	With Bεal (10 μg/ml)
None	6.3	6.4
Asparagine	5.6	3.2
$(NH_4)_2SO_4$	6.6	6.1
Glutamate	5.4	3.7

[a] Cells were incubated with 0.01μg of ^{14}C-INH per ml for 16 hr at 37 C with aeration.

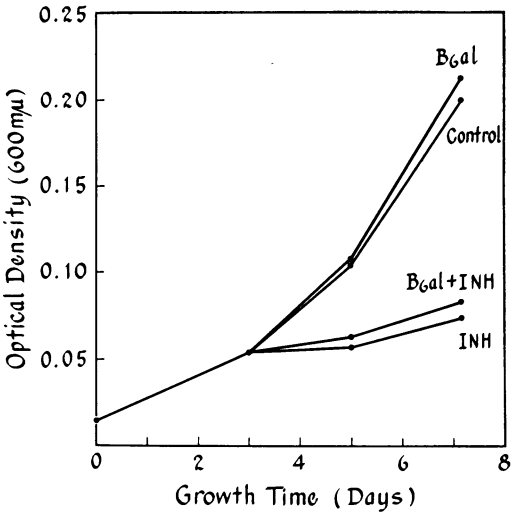

FIG. 3. *Effect of pyridoxal on INH growth inhibition of cells in an $(NH_4)_2SO_4$ medium. Cells from a log-phase Sauton culture were harvested and homogenized in a small volume of Sauton's medium containing 4.0 g of $(NH_4)_2SO_4$ per liter instead of asparagine. Flasks containing 200-ml portions of this same medium with drug and vitamin where appropriate were inoculated with 1.5 ml of homogenized suspension. Pyridoxal concentration, 10 μg/ml; INH concentration 0.01 μg/ml. The control culture contained no drug or vitamin.*

TABLE 2. *Effect of pyridoxal on ^{14}C-INH uptake by tubercle bacilli in various nutrient environments*

System no.	Cells in PBG (0.06 mg/ml) plus				Amt (mμg) of ^{14}C-INH /mg of cells[a]
	Asparagine (4 mg/ml)	Salts[b]	Bεal (50 μg/ml)	C^{14}-INH (0.1μg/ml)	
1	−[c]	−	−	+	22.2
2	−	−	+	+	26.2
3	+	−	−	+	22.2
4	+	−	+	+	33.5
5	−	+	−	+	21.2
6	−	+	+	+	26.1
7	+	+	−	+	22.2
8	+	+	+	+	11.6

[a] Cells incubated for 6 hr at 29 C with aeration.
[b] $MgSO_4$ and ferric ammonium citrate at concentrations used in Sauton's medium.
[c] Minus and plus signs indicate absence or presence of constituents.

tion of 0.06 mg/ml. Portions of 25 ml of this suspension were added to each of eight 125-ml flasks, four of which contained 100 mg of powdered L-asparagine. Further additions were made as indicated. Cells were exposed to ^{14}C-INH in these various nutrient environments for 6 hr at 29 C with aeration. Pyridoxal retarded the accumulation of drug only when both asparagine and salts were present (uptake systems 7 and 8). The data reveal an inhibition of approximately 50% in this case. When either asparagine or salts or both were absent, pyridoxal stimulated ^{14}C uptake from labeled INH. Note that in the absence of pyridoxal the amount of labeled drug accumulated was the same regardless of the combinations of other nutrients (uptake systems 1, 3, 5, and 7).

DISCUSSION

It has been reported elsewhere that growth inhibition of *M. tuberculosis* by INH can be prevented by a large excess of pyridoxal in the culture medium (3, 9, 10). Although this observation was confirmed, it no longer offers a sound argument in favor of the hypothesis that isoniazid action is involved with vitamin B_6 metabolism. The results of this investigation indicate that exogenously supplied pyridoxal protects at an extracellular rather than an intracellular level. It is possible, however, that, in the absence of pyridoxal, INH enters the cell and interferes with endogenous vitamin B_6 metabolism. This concept is supported by studies with cell-free vitamin B_6-requiring enzyme systems (5, 7, 8, 13).

Tubercle bacilli in either Sauton's medium or buffer containing all Sauton nutrients readily accumulated ^{14}C-labeled INH. However, when pyridoxal was added to these systems in concentrations ranging from 500 to 1,000 times that of INH, incorporation of the label was inhibited. It is interesting to compare the growth and uptake experiments in Sauton's medium employing pyridoxal and INH at concentrations of 10 and 0.01 μg/ml, respectively. The uptake data indicate that INH accumulation was inhibited only 20 to 40% by pyridoxal during the early hours of drug exposure, and yet the growth experiments reveal that the tubercle bacilli were not adversely affected in such an environment. In preliminary studies, it was found that 0.01 μg of INH per ml was indeed a minimal inhibitory concentration in this system. Therefore, a modest inhibition of drug accumulation was apparently sufficient to keep intracellular INH below a toxic level.

Further studies revealed that certain Sauton nutrients are necessary for pyridoxal to inhibit INH incorporation by *M. tuberculosis*. Pyridoxal inhibited drug uptake only in the presence of both asparagine (or glutamate) and salts. Uptake of labeled drug was not inhibited by pyridoxal in the absence of a nitrogen compound or in the presence of $(NH_4)_2SO_4$. Growth experiments in Sauton's medium containing $(NH_4)_2SO_4$ instead of asparagine were consistent with the latter finding. Pyridoxal did not protect the tubercle bacilli from INH growth inhibition in this environment. The absence of asparagine or salts or both from an uptake menstruum containing 0.1 μg of ^{14}C-INH and 50 μg of pyridoxal per ml resulted in a stimulation of ^{14}C uptake by the organism. A 20 to 50% increase was noted, and these results agree with those reported by Youatt (16), who used saline or a dilute solution of Tween 80 as a menstruum to study INH uptake over short time intervals. Youatt attributed this stimulation to the formation of pyridoxal isonicotinoyl hydrazone which was more readily taken up than INH alone. Boone et al. (3) reported that pyridoxal stimulated uptake of ^{14}C activity from labeled INH by as much as 100-fold. In these experiments, tubercle bacilli were grown in Cohn's medium for 1 week prior to the addition of pyridoxal and labeled INH. Incubation was then continued for an additional week at which time ^{14}C activity accumulated by the cells was determined. It is difficult to correlate this study by Boone et al. with that reported by Youatt and with the experiments presented here.

From the results of this investigation, it is concluded that a large excess of pyridoxal in Sauton's medium protects the tubercle bacillus from INH growth inhibition by preventing cellular accumulation of toxic drug levels. As indicated earlier, pyridoxal, amino compounds, and cations can combine to form complexes (4). Hawkins and Steenken (6) found that pyridoxal reversed the inhibitory effect of INH on mycobacterial catalase and suggested that a complex was formed involving pyridoxal, cupric ions, and INH rather than an amino compound. In the present study, it was found that L-asparagine and certain Sauton salts were required to demonstrate a pyridoxal inhibition of ^{14}C-isoniazid uptake by *M. tuberculosis*. It is postulated, therefore, that INH, pyridoxal, metal ions, and asparagine combine to form an extracellular complex which renders the drug less available to the cells. Direct evidence to support the existence of such a complex is lacking, and alternate models can be visualized which would also explain inhibition of INH uptake by pyridoxal.

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