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Tryptophan Uptake By *Mycobacterium tuberculosis* $H_{37}Rv$: Effect of Rifampin and Ethambutol

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Uptake of radioactive tryptophan by *Mycobacterium tuberculosis* $H_{37}Rv$ grown in vitro and in vivo was investigated. K_m values indicated low affinity, and sodium azide inhibited uptake. Rifampin at the minimal inhibitory concentration had no effect, whereas ethambutol inhibited uptake only in the bacilli grown under in vitro conditions. The significance of these results is discussed.

Since the initial work of Gale (5, 6), it is well known that the transport of amino acids into the bacterial cell occurs by physical diffusion or by an active process requiring specific membrane proteins and the generation of energy. Although many workers (17-19, 24) have studied the transport of amino acids in mycobacteria, no report on the effect of antituberculous drugs on such transport appears to be available. Some of these drugs may inhibit amino acid transport by a primary or a secondary effect. D-Cycloserine, for example, owes its bactericidal action to its ability to inhibit the incorporation of alanine into the bacterial cell wall (16). The present investigation was undertaken in view of the paucity of essential information on the effect of antituberculous drugs on the uptake of amino acids.

Because differences exist between various species of mycobacteria, the pathogenic $H_{37}Rv$ strain of *Mycobacterium tuberculosis*, which causes the disease in man, was selected for uptake studies. Here again, the in vitro-grown bacillus has been shown to be different in chemical composition and staining properties from the in vivo-grown bacillus (12, 20, 21). Hence, in addition to in vitro-grown tubercle bacilli, bacilli isolated from tuberculous mouse lung in vivo were also employed in the present investigation. If the endogenous level of the amino acid is very low because of rapid metabolic conversion to other products, determination of its initial rate of upake and a study of the kinetics involved pose difficulties. Therefore, it is not possible to study the kinetics of transport of all amino acids. In the present investigation, tryptophan, one of the aromatic amino acids, which is not attacked by any strain of mycobacteria (7), was selected. Kinetics of tryptophan uptake and the effect of rifampin and ethambutol are discussed in this paper.

MATERIALS AND METHODS

Preparation of *in vitro*-grown bacilli. *M. tuberculosis* $H_{37}Rv$ was grown at 37°C by shake culture in Kirchner synthetic medium (pH 7.0) containing Tween 80 (0.05%) and bovine serum albumin fraction V (0.1%) (15). Growth was measured turbidimetrically at 580 nm in a Bausch and Lomb Spectronic 20 colorimeter. Standard curve was established by the use of barium sulphate standards (22), and by this curve it was possible to know the dry weight of the bacilli present. This was again verified gravimetrically by filtering a known amount of the culture through a filter (Millipore Corp.), drying, and weighing. Cells were harvested after 10 days while at the exponential phase of growth and washing three times in ice-cold Kirchner medium (pH 7.0) containing 0.05% Tween 80, without asparagine and albumin. Cells were then suspended in the same medium, and cell density was adjusted to 1 mg/ml (dry weight). Tween 80 was always kept in the suspending medium to avoid clumping of cells. These stock suspensions were maintained in the cold (2 to 4°C) and used for tryptophan uptake experiments within 2 to 3 h of preparation.

Isolation of *in vivo*-grown bacilli. Adult, bacteria-susceptible albino mice of either sex were injected intraperitoneally with 5 mg of virulent *M. tuberculosis* $H_{37}Rv$ grown in a modified Sauton medium containing Tween 80 and bovine serum albumin fraction V (22). The culture was shaken continuously and was 10 days old. After 3 weeks of infection, the mice were sacrificed, and the tuberculous lungs were removed and stored at 0°C until a sufficient amount of the tissue was available for the isolation of in vivo bacilli. In vivo-grown bacilli were isolated by the method of Kanai (9) with a slight modification. The third step of centrifugation of the supernatant was repeated for 10 min again at 1,000 rpm (International Equipment Co., Refrigerated centrifuge model B-20). This gave a purer collection of acid-fast bacilli without any blue-stained tissue debris, although the yield was reduced from 1 to 0.8 mg of bacilli per infected mouse lung. The bacilli were suspended in the same nitrogen-free medium containing Tween 80 as for in vitro-grown bacilli, and the cell density was adjusted to 1 mg/ml (dry weight).

Amino acid transport. A cell suspension (10 ml) of 10-mg (dry weight) bacilli was pipetted into a 50-ml Erlenmeyer flask and aerated by shaking in a Dubnoff metabolic shaker at 37°C throughout the experiment. After 5 min, 1 μmol of DL-[^{3}H]tryptophan (2 μCi/μmol) was added, and 1-ml portions of the cell suspension were removed at various time intervals and filtered through filters (Millipore Corp.; pore size, 0.45 μm). Filter disks containing bacilli were washed with 10 ml of the same medium in which they were suspended and dried. Scintillation fluid was added to glass vials containing dried samples, and radioactivity was assayed with a model LS-233 Beckman liquid scintillation counter. The scintillation fluid contained 4 g of 2,5-diphenyloxazole and 0.20 g of 1,4-bis-2-(5-phenoxazolyl)benzene per liter of toluene.

For kinetic studies, different concentrations of labeled tryptophan were used. Minimal inhibitory concentrations of Rifampin and ethambutol were added along with tryptophan, and uptake was studied. After 10 min of preincubation with sodium azide (2 mmol), labeled tryptophan was added, and uptake was studied in the same way.

RESULTS AND DISCUSSION

Figures 1 and 2 clearly indicate that a transport system existed for tryptophan in both in vitro- and in vivo-grown tubercle bacilli. The K_m was 10 times higher than that reported for *M. phlei* (19), indicating low affinity. Bacilli grown under in vivo conditions had more affinity for tryptophan than those grown in vitro (Fig. 3 and 4). Sodium azide effectively prevented tryptophan uptake in both the cases (Fig. 5 and 6), implying that generation of energy is necessary for the process. However, the possibility of a small amount of amino acid entering the cells by simple diffusion cannot be ruled out. In *M. phlei* sodium azide also has been shown to inhibit tryptophan uptake (19).

The minimal inhibitory concentration of rifampin (0.5 μg/ml) has no effect on the uptake, although a slight fall was seen after 10 min (Fig. 7 and 8). Rifampin is a specific inhibitor of the

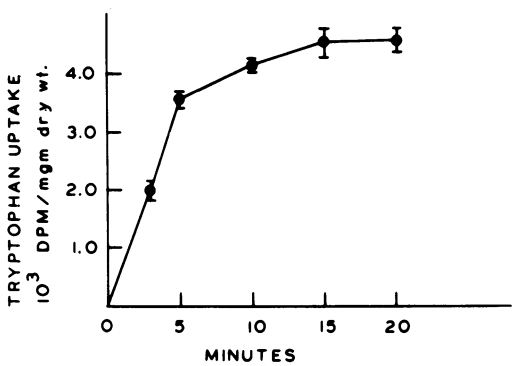

FIG. 1. *Time course of uptake of labeled tryptophan by in vitro-grown tubercle bacilli. Incubation mixture (10 ml) containing prewarmed cells (10 mg) was maintained at 37°C with shaking for 5 min, after which 1 μmol of [^{3}H]tryptophan (2 μCi/μmol) was added. At indicated times, portions (1 ml) were withdrawn, filtered immediately through filters (Millipore Corp.), and washed, and radioactivity was measured in a liquid scintillation counter as described in the text. The data plotted are obtained by subtraction of radioactivity measured at zero time. In this and subsequent figures, the values represent means ± standard error for three separate experiments.*

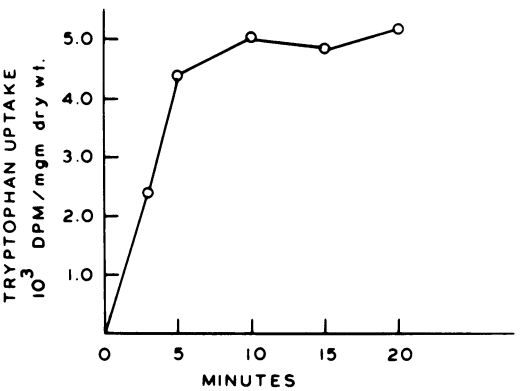

FIG. 2. *Time course of uptake of labeled tryptophan by in vivo-grown tubercle bacilli. A 10-mg amount of in-vivo-grown M. tuberculosis $H_{37}Rv$ cells separated from tuberculous mouse lungs as described in the text were used. Other details were the same as given in the legend to Fig. 1. Here and in Fig. 4, 6, 8, and 10, the values represent the average of two separate experiments.*

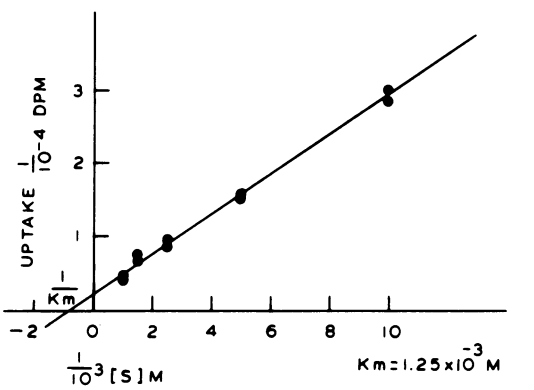

FIG. 3. *Kinetics of DL-[^{3}H]tryptophan uptake by in vitro-grown tubercle bacilli. Details of the experiment were the same as given in the legend to Fig. 1. Different concentrations of labeled tryptophan were used, and portions were withdrawn at 0, 3, and 5 min of incubation.*

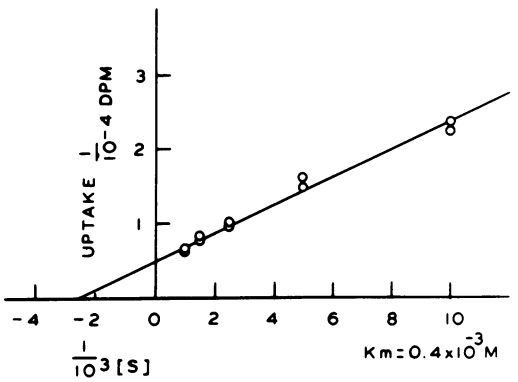

FIG. 4. *Kinetics of DL-[^{3}H]tryptophan uptake by in vivo-grown tubercle bacilli. Details were the same as given in the legend to Fig. 3.*

deoxyribonucleic acid-dependent ribonucleic acid polymerases (23). Electron microscopic studies (4) have shown that in cultures containing inhibitory concentrations of rifampin, lysis of the bacterial body starts within 96 h. Konno et al. (13) have published a series of ultrathin sections of $H_{37}Ra$ subjected to 10 μg of rifampin per ml for periods of 6, 12, 24, and 48 h. Breakdown of cellular organization was evident at 12 h. It is difficult to explain the slight inhibition of uptake seen after 10 min in the present experiment. As protein biosynthesis is inhibited at the transcription level because of rifampin (2, 3), these data further show that tryptophan is not incorporated into the protein. Because tryptophan is also not metabolized inside the cell (7), the uptake represents the true K_m for the transport of tryptophan.

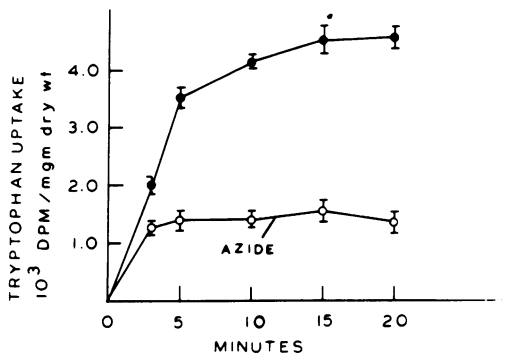

FIG. 5. *Effect of sodium azide on [^{3}H]tryptophan uptake by in vitro-grown tubercle bacilli. Tryptophan uptake in the presence of sodium azide was carried out with cells preincubated for 10 min with 2 mmol of sodium azide. Other details were the same as given in the legend to Fig. 1.*

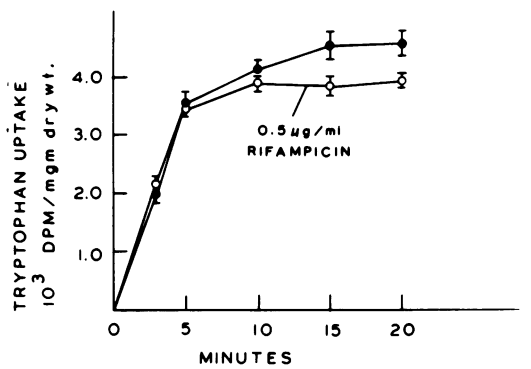

FIG. 7. *Effect of rifampin on tryptophan uptake by in vitro-grown tubercle bacilli. A minimal inhibitory concentration (0.5 μg/ml) of rifampin was added along with tryptophan, and incorporation was studied in the same way as given in the legend to Fig. 1.*

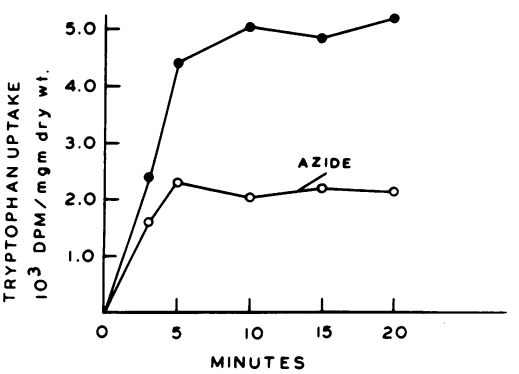

FIG. 6. *Effect of sodium azide on labeled tryptophan uptake by in vivo-grown tubercle bacilli. Details were the same as given in the legend to Fig. 5.*

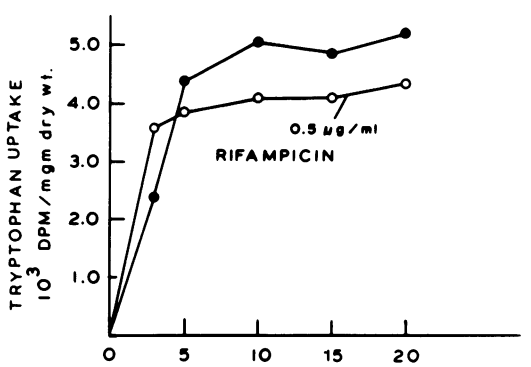

FIG. 8. *Effect of rifampin on tryptophan uptake by in vivo-grown tubercle bacilli. Details were same as given in the legend to Fig. 7.*

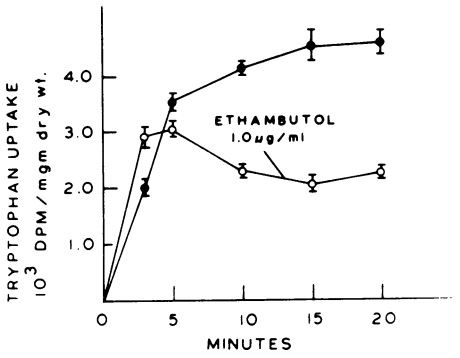

FIG. 9. *Effect of ethambutol on tryptophan uptake by in vitro-grown tubercle bacilli. A minimal inhibitory concentration (1.0 µg/ml) of ethambutol was added along with tryptophan, and incorproation was studied in the same way as given in the legend to Fig. 1.*

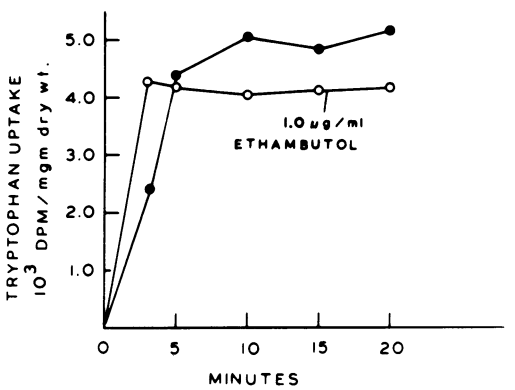

FIG. 10. *Effect of ethambutol on tryptophan uptake by in vivo-grown tubercle bacilli. Details were same as given in the legend to Fig. 9.*

With the minimal inhibitory concentration of ethambutol (1.0 µg/ml), however, interesting results were observed. Ethambutol inhibited 50% of uptake only in in vitro-grown bacilli and had no significant effect on in vivo-grown bacilli (Fig. 9 and 10). Reduction of the respiratory chain between D-lactic dehydrogenase and cytochrome b_1 is responsible for the efflux of preformed amino acids and sugar pool. Compounds that act before the point of energy coupling block the uptake and do not induce efflux (8, 11). Ethambutol may be blocking the uptake like the latter compounds. Earlier studies with *M. tuberculosis* $H_{37}Ra$ (1) indicated that the total binding of [^{14}C]ethambutol by growing cells is both time and concentration dependent. It may be because of this time dependence that inhibition started only after 5 min (Fig 9). Ethambutol probably has no effect on in vivo-grown bacilli because of the lack of cytochrome components (13). The chemical composition of in vivo- and in vitro-grown *M. tuberculosis* have been reviewed recently (10). The results of the present investigation suggest that ethambutol acts differently in both of the bacilli and also raises the doubt as to how far it is justifiable to use in vitro-grown tubercle bacilli for drug interaction studies.

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LITERATURE CITED

1. **Beggs, W. H., and N. E. Auran.** 1972. Uptake and binding of ^{14}C-ethambutol by tubercle bacilli and the relation of binding to growth inhibition. Antimicrob. Agents Chemother. **2**:390–394.
2. **David, H. L.** 1974. Biogenesis of β-carotene in *Mycobacterium kansasii*. J. Bacteriol. **119**:527–533.
3. **Dickinson, J. M., P. S. Jackett, and D. A. Mitchison.** 1972. The effects of pulsed exposures to rifampin on the uptake of uridine ^{14}C by *Mycobacterium tuberculosis*. Am. Rev. Respir. Dis. **105**:519–527.
4. **Frontali, L., and G. Tecce.** 1967. Rifamycins, p. 415–426. *In* D. Gottlieb and P. D. Shaw (ed.), Antibiotics, vol. 1. Springer-Verlag, New York.
5. **Gale, E. F.** 1947. The assimilation of amino acids by bacteria. 1. The passage of certain amino acids across the cell wall and their concentration in the internal environment of *Streptococcus faecalis*. J. Gen. Microbiol. **1**:53–76.
6. **Gale, E. F.** 1951. The assimilation of amino acids by bacteria. 10. Action of inhibitors on the accumulation of free glutamic acid in *Streptococcus aureus* and *Streptococcus faecalis*. Biochem. J. **48**:286–290.
7. **Grange, J. M.** 1976. Enzymatic breakdown of amino acids and related compounds by suspensions of washed mycobacteria. J. Appl. Bacteriol. **41**:425–431.
8. **Kaback, H. R., and E. M. Barnes, Jr.** 1971. Mechanism of active transport in isolated membrane vesicles. II. The mechanism of energy coupling between D-lactic dehydrogenase and beta-galactoside transport in membrane preparations from *Escherichia coli*. J. Biol. Chem. **246**:5523–5531.
9. **Kanai, K.** 1967. Detection of host-originated acid phosphatase on the surface of in vivo grown tubercle bacilli. Jpn. J. Med. Sci. Biol. **20**:73–90.
10. **Kanai, K., and K. Kondo.** 1974. Chemistry and biology of mycobacteria grown in vivo. Jpn. J. Med. Sci. Biol. **27**:135–160.
11. **Kerwar, G. K., A. S. Gordon, and H. R. Kaback.** 1972. Mechanism of active transport in isolated membrane vesicles. IV. Galactose transport in isolated membrane vesicles from *Escherichia coli*. J. Biol. Chem. **247**:291–297.
12. **Kondo, E., and K. Kanai.** 1972. Further demonstration of bacterial lipids in *Mycobacterium bovis* harvested from infected mouse lungs. Jpn. J. Med. Sci. Biol. **25**:249–257.
13. **Konno, K., K. Oizumi, F. Ariji, J. Yamaguchi, and S. Oka.** 1973. Mode of action of rifampin on mycobacteria. 1. Electron microscopic study of the effect of rifampin

on *Mycobacterium tuberculosis*. Am. Rev. Respir. Dis. **107**:1002-1005.

14. **Kusaka, T., R. Sato, and K. Shoji.** 1964. Comparison of cytochromes of mycobacteria grown in vivo and in vitro. J. Bacteriol. **87**:1383-1388.
15. **McKee, C. M., G. Rake, R. Donovick, and W. P. Jambor.** 1949. The use of mouse in a standardized test for antituberculous activity of compounds of natural or synthetic origin. I. Choice and standardization of culture. Am. Rev. Tuberc. **60**:90-108.
16. **Neuhaus, F. C.** 1967 D-Cycloserine and *O*-carbamyl-D-serine. p. 40-83. *In* D. Gottlieb and P. D. Shaw (ed.), Antibiotics, vol. 1. Springer-Verlag, New York.
17. **Prasad, R., V. K. Kalra, and A. F. Brodie.** 1975. Effect of phospholipase A on active transport of amino acids with membrane vesicles of *Mycobacterium phlei*. J. Biol. Chem. **250**:3699-3703.
18. **Prasad, R., V. K. Kalra, and A. F. Brodie.** 1975. Active transport of glutamine and glutamic acid in membrane vesicles from *Mycobacterium pheli*. Biochem. Biophys. Res. Commun. **63**:50-56.
19. **Prasad, R., V. K. Kalra, and A. F. Brodie.** 1976. Different mechanisms of energy coupling for transport of various amino acids in cells of *Mycobacterium phlei*. J. Biol. Chem. **251**:2493-2498.
20. **Segal, W.** 1965. Comparative study of *Mycobacterium* grown in vivo and in vitro. V. Differences in staining properties. Am. Rev. Respir. Dis. **91**:285-287.
21. **Segal, W., and W. T. Miller.** 1965. Comparative study of in vivo and in vitro grown *Mycobacterium tuberculosis*. III. Lipid composition. Proc. Soc. Exp. Biol. Med. **118**:613-616.
22. **Wayne, L. G., and G. A. Diaz.** 1967. Autolysis and secondary growth of *Mycobacterium tuberculosis* in submerged culture. J. Bacteriol. **93**:1374-1381.
23. **White, R. J., G. C. Lancini, and L. G. Silvestri.** 1973. Mechanism of action of rifampin in *Mycobacterium smegmatis*. J. Bacteriol. **108**:737-741.
24. **Yabu, K.** 1967. The uptake of D-glutamic acid by *Mycobacterium avium*. Biochim. Biophys. Acta **135**:181-183.