TRYPTOPHAN UPTAKE BY MYCOBACTERIUM TUBERCULOSIS $H_{37}Rv$ - INHIBITION BY ISONIAZID *

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SUMMARY The effect of various sub-inhibitory concentrations of isoniazid on tryptophan uptake by Mycobacterium tuberculosis $H_{37}Rv$ grown in vitro and in vivo was studied. Uptake, measured after 3 minutes of drug exposure was inhibited mildly by 0.1 µg/ml and 0.2 µg/ml concentration and completely by 0.3 µg/ml. However, with the minimal inhibitory concentration (MIC)** of 0.5 µg/ml, not only inhibition but also a strong efflux of the preformed tryptophan pool were observed. The results are discussed in the light of the theory that isoniazid interferes with the cell wall mycolate synthesis.

A voluminous amount of literature has accumulated on the mode of action of isoniazid (INH) and the subject has been reviewed periodically (1-4). However, the precise mechanism of action of INH on the tubercle bacilli still remains obscure because of inherent limitations in the studies undertaken. Most investigators used different strains of non-pathogenic mycobacteria grown in vitro. The presence of a large number of species in the genus mycobacterium (5), and the differences observed in their growth rate, culture conditions, pathogenicity and pigment production suggest that all of them are unlikely to possess similar biochemical mechanisms for the utilization of nutrients. Even the two saprophytes, M. phlei and M. smegmatis show variations in their cofactor requirements for malate dehydrogenase activity as well as in malate oxidation and subsequent phosphoryla-

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* Isoniazid - INH, Isonicotinic acid hydrazide. $C_6 H_7 N_3 O$

** MIC - The minimal inhibitory concentration of a drug is the lowest concentration per ml that will stop the growth of the bacilli in a culture.

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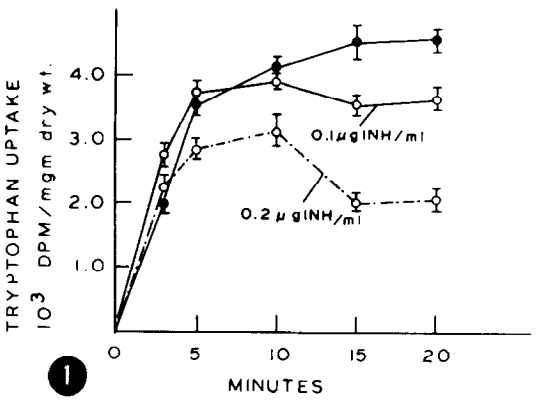
tion (6). Again, bacilli grown *in vitro* are not the same as those encountered *in vivo* as regards chemical composition and staining properties (7-9). Another flaw in earlier work as pointed out by Takayama *et al.* (10) is that the effect of the drug was examined several hours and even days after the addition.

Preliminary studies revealed the existence of a kinetic system for tryptophan transport in both *in vitro* and *in vivo* grown *M. tuberculosis* $H_{37}Rv$. This paper describes the effect of various sub-inhibitory concentrations of INH on this transport.

MATERIALS AND METHODS

Preparation of in vitro grown bacilli: *M. tuberculosis* $H_{37}Rv$ was grown at 37°C by shake culture in Kirchner's synthetic medium (pH 7.0) containing Tween 80 (0.05%) and bovine serum albumin fraction V (0.1%) (11). 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 (12) and by this curve it was possible to know the dry wt. of the bacilli present. This was again verified gravimetrically by filtering a known amount of the culture through a Millipore filter, drying and weighing. Cells were harvested after 10 days, while at the exponential phase of growth and washing three times in ice-cold Kirchner's medium (pH 7.0) containing 0.05% Tween 80, without asparagine and albumin. Cells were then suspended in the same medium and cell density adjusted to one mg per ml (dry wt.). 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 hrs. 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 (12). After three weeks of infection, the mice were sacrificed and the tuberculous lungs were removed and stored at 0°C until sufficient amount of the tissue was available for the isolation of *in vivo* bacilli. *In vivo* grown bacilli were isolated according to the method of Kanai (13) with a slight modification. The step III centrifugation of the supernatant was repeated for 10 minutes again at 1000 r.p.m. (IEC-refrigerated centrifuge, model-B-20). This gave a more pure collection of acid-fast bacilli without any blue-stained tissue debris though the yield was reduced from 1 mg to 0.8 mg 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 one mg per ml (dry wt.).



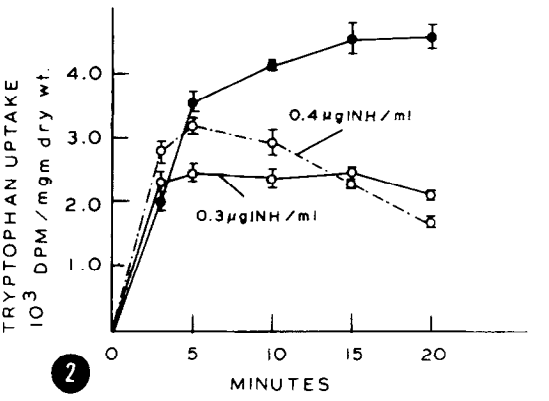


Fig. 1 and 2. Effect of various sub-inhibitory concentrations of Isoniazid on labelled tryptophan uptake by in vitro grown tubercle bacilli. Incubation mixture (10 ml) containing pre-warmed cells (10 mg) was maintained at 37°C with shaking for 5 minutes after which 1 µmole of (^{3}H)-tryptophan (2 µCi/µmole) was added. At indicated times, aliquots (one ml) were withdrawn and filtered immediately through millipore filters, washed and radioactivity measured in a liquid scintillation counter as described under "Materials and Methods". Various sub-inhibitory concentrations of Isoniazid (0.1 µg/ml to 0.4 µg/ml) were added along with labelled tryptophan and uptake studied in the same way. Values represent means ± S.E. for three separate experiments.

Amino acid transport: A cell suspension (10 ml) of 10 mg bacilli (dry wt.) was pipetted into a 50 ml-Erlenmeyer flask and aerated by shaking in a Dubnoff metabolic shaker at 37°C throughout the experiment. After five minutes 1 µmole of (^{3}H)-DL-tryptophan (2 µCi/umole) was added, and one ml portions of the cell suspensions were removed at various time intervals and filtered through Millipore filters (pore size 0.45 µm). Filter discs 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 0.2 g of 1,4-bis-2-(5-phenoxazolyl) benzene per litre of toluene.

Different sub-inhibitory concentrations of INH (0.1 µg/ml to 0.5 µg/ml) were added along with labelled tryptophan and uptake was studied at various time intervals. However, only the minimal inhibitory concentration, 0.5 µg/ml was tested with in vivo grown bacilli, because of their limited availability.

RESULTS AND DISCUSSION

The results presented here, to our knowledge appear to be the first report on the mode of action of INH in M. tuberculosis $H_{37}Rv$

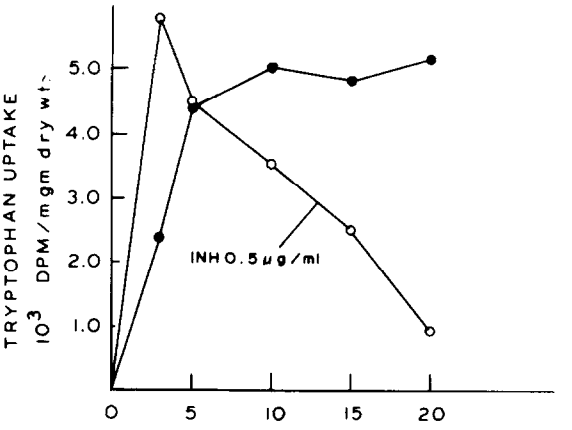


Fig. 3. Effect of Isoniazid on tryptophan uptake by *in vitro* grown tubercle bacilli. 0.5 µg/ml Isoniazid was added along with tryptophan and incorporation studied in the same way as given in Fig. 1 & 2. Values represent means ± S.E. for three separate experiments.

grown *in vivo*. Unlike in studies by earlier investigators, the effect of INH was studied within short time (3 to 20 minutes) of exposure. Sub-inhibitory concentrations, 0.1 µg/ml and 0.2 µg/ml, blocked uptake after 10 minutes (Fig. 1); with 0.3 µg/ml uptake was blocked after 5 minutes and thereafter kept constant throughout the experiment (Fig. 2); 0.4 µg/ml, however caused an efflux of the tryptophan pool after 5 minutes (Fig. 2). With the minimal inhibitory concentration of 0.5 µg/ml, however, interesting results were observed both in *in vitro* and *in vivo* grown bacilli. Uptake which rose in both types immediately after 3 minutes following addition, was more than doubled with *in vivo* grown bacilli. Thereafter, a strong efflux of tryptophan was seen and the curve fell steeply. This efflux was very significant in *in vivo* bacilli (Fig. 4).

Compounds which block respiration beyond the point of energy coupling (D-lactate dehydrogenase in *E. coli*) cause efflux of pre-

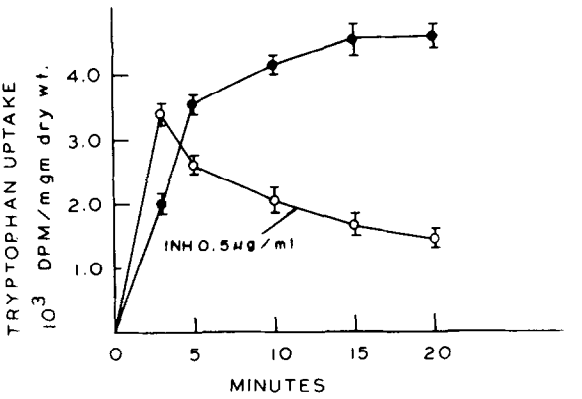


Fig. 4. Effect of Isoniazid on tryptophan uptake by *in vivo* grown tubercle bacilli. 0.5 µg/ml Isoniazid was added along with tryptophan and incorporation studied in the same way as given in Fig. 1 & 2. The values represent average of two separate experiments.

formed amino acid and sugar pools, but compounds which act before the point of energy coupling block uptake and do not induce efflux (14, 15). The present study reveals clearly that INH resembles the former.

Holden *et al.* (16) have demonstrated that in mutants showing lipid overproduction, alterations in membrane lipid composition change the characteristics of transport systems for certain amino-acids while those for others remain unaffected. Esfahani *et al.* (17) have shown that the lipid composition of membrane vesicles determine the transition temperature for proline transport and succinic dehydrogenase activity. Studies with physical probes provide irrefutable evidence that transport transitions arise from a physical alteration of the bulk lipid phase (18). It is not unreasonable to conclude in the light of these observations, that INH by interacting with lipids in the cell wall, has a dis-ordering effect on lipid packing, which disturbs the normal transport of tryptophan.

These results strongly support the theory of Winder and Collins (19) and that of Takayama et al. (10) that the primary mechanism of action of INH on the tubercle bacillus is the inhibition of cell wall mycolic acids (5). Takayama et al. (10) have shown that when the MIC of INH is added to growing cultures of M. tuberculosis $H_{37}Ra$, the ability of the cells to synthesize mycolic acids decreases linearly from the time of exposure and after 60 minutes it is completely inhibited. Our results are in agreement with this observation as the efflux of the pre-formed tryptophan pool starts immediately after the third minute of addition.

Acknowledgements

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REFERENCES

1. Long, E.R. (1958). The Chemistry and Chemotherapy of tuberculosis. pp 362-388, Williams & Wilkins Co., Baltimore, U.S.A.
2. Goldman, D.S. (1961). Advan . Tuberc. Res. (Karger, Basel, New York), 11, 1-44.
3. Winder, F. (1964). In " Chemotherapy of Tuberculosis ". pp 111-149, Butterworths, London.
4. Youatt, J. (1969). Amer. Rev. Resp. Dis. 99, 729-749.
5. Barksdale, L. and Kim, K.S. (1977). Bacteriol. Rev. 41, 217-372.
6. Reddy, T.L.P., Suryanarayana Murthy, P. and Venkitasubramanian, T.A. (1975). Biochim. Biophys. Acta 376, 210-218.
7. Segal, W. and Miller, W.T. (1965). Proc. Soc. Exp. Biol. Med. 118, 613-616.
8. Kondo, E. and Kanai, K. (1972). Japan J. Med. Sci. Biol. 25, 249-257.
9. Segal, W. (1965). Amer. Rev. Resp. Dis. 91, 285-287.
10. Takayama, K., Wang, L. and David, H.L. (1972). Antimicrob. Agents Chemother. 2, 29-35.
11. McKee, C.M., Rake, G., Donovick, R. and Jambor, W.P. (1949). Amer. Rev. Tuberc 60, 90-108.
12. Wayne, L.G. and Diaz, G.A. (1967). J. Bacteriol. 93, 1374-1381.
13. Kanai, K.(1967). Japan J. Med. Sci. Biol. 20, 73-90.
14. Kaback, H.R. and Barnes, E.M. Jr. (1971). J. Biol. Chem. 246, 5523-5531.

15. Kerwar, G.K., Gordon, A.S. and Kaback, H.R. (1972). J. Biol. Chem. 247, 291-297.
16. Holden, J.T., Utech, N.M., Hegeman, G.D. and Kenyon, C.N. (1973). Biochem. Biophys. Res. Commun. 50, 266-279.
17. Esfahani, M., Limbrock, A.R., Knutton, S., Oka, T. and Wakil, S.J. (1971). Proc. Nat. Acad. Sci. U.S. 68, 3180-3184.
18. Tsukagoshi, N. and Fox, C.F. (1973). Biochemistry. 12, 2816-2822.
19. Winder, F.G. and Collins, P.B. (1970). J. Gen. Microbiol. 63, 41-48.