STUDIES ON THE MODE OF ACTION OF ISO-NICOTINIC ACID HYDRAZIDE (INH)

BY P. R. J. GANGADHARAM AND M. SIRSI
(Pharmacology Laboratory, Indian Institute of Science, Bangalore-3)

SUMMARY

The action of iso-nicotinic acid hydrazide (INH) on the respiration of *Mycobacterium tuberculosis* $H_{37}R_v$ strain as also on some enzymes has been studied.

Based on these results and in consideration with the previous literature on the subject a hypothetical postulation regarding its mode of action has been advanced.

Soon after iso-nicotinic acid hydrazide (INH) was established as a powerful anti-tubercular drug, several people have attempted to study its mode of action. Thus Zeller[1] studied the action of INH on bacterial and mammalian diamine oxidase and found that the drug inhibited these enzyme systems. The action of the drug on the tryptophanase and amino-acid decarboxylases of *E. coli* was studied by Yoneda and co-workers[2, 3] who found that the drug inhibited these enzymes and that pyridoxine hydrochloride counteracted this inhibition. They also mentioned that the inhibition is competetive. However Boone and Woodward[4] studying the relation of pyridoxine derivatives to the mechanism of action of INH against *E. coli* and Mycobacteria, mentioned that the antagonism by pyridoxine hydrochloride is not competetive. Newberg and Forrest[5] found that the drug competes with the nicotinamide utilisation by the bacteria. Aronson *et al.*[6] found that INH inhibited the catalase activity of the tubercle bacilli, but did not modify the succinic dehydrogenase activity. They also repeated these studies with the attenuated B.C.G. strain. However Polster[7] could not find any inhibition of the catalase activity in the tubercle bacilli. Barclay *et al.*[8] studying the bacteriostatic action of INH using radio-active isotopes, suggested that it acts by interfering with the formation of an essential metabolite. Zatman and co-workers[9] studied the action of INH on diphospho-pyridine nucleotidase (DPNase) from various sources and suggested that the drug might act through the formation of an INH-analogue of DPN. The inhibition of succinic oxidase by this drug has also been reported recently by Arora and Krishnamurthy.[10]

In this communication the authors have presented the results of their studies on the action of this drug on the respiration of *Mycobacterium tuberculosis*, $H_{37}R_v$ strain, as also on some enzyme systems and proposed a view, in consideration with the results of the studies of previous workers, regarding the mode of action of this drug. The enzymes studied include succinic oxidase, cytochrome oxidase, catalase, fumarase, glutamic acid decarboxylase, transaminase and tryptophanase.

EXPERIMENTAL

Enzyme preparations

Bacilli preparations.—Six to eight loops of the tubercle bacilli are taken from the surface pellicle of a Youmans' tube (12–14 days old) into a clean, dry mortar and ground uniformly with the pestle until the whole mass turned to be a paste. About 10 ml. of distilled water is then added and mixed well. The [illegible] suspension is then carefully transferred to a 50 ml. centrifuge tube, tightly plugged with non-absorbent cotton and centrifuged at 4000 RPM for 20 minutes.

The supernatant is then carefully decanted into boiling phenol solution (1%) and the washing repeated once again with some more water. Finally sufficient amount of the suitable buffer is added and the solution thoroughly stirred with a glass rod. It is then transferred to a thick-walled pyrex test-tube which is kept in the cold storage.

Succinic oxidase and cytochrome oxidase.—These enzymes have been prepared from sheep heart according to the method of Ball *et al.*[11] Cytochrome C is prepared from sheep heart by the method of Keilin and Hartree[12] and standardised spectrophotometrically.

Catalase.—This enzyme has been prepared from rat liver by the method of Gordon and Quastel.[13]

Fumarase.—This enzyme has been prepared from sheep heart, according to the method of Massey[14] with slight modifications.

Glutamic acid decarboxylase.—A suspension of *Clostridium welchii* SR 12, grown and harvested according to the method of Gale[15] has been used as the enzyme source.

Transaminase.—This enzyme has been prepared from rat liver by the method of Green *et al.*[16]

Tryptophanase.—A suspension of *E. coli*, harvested after washing, from nutrient broth containing *dl*-tryptophane has been used as the enzyme.

Estimation of enzyme activities

Warburg manometric techniques.—The action of the drug on the respiration of the tubercle bacilli ($H_{37}R_v$ strain) and on the enzymes succinic oxidase, cytochrome oxidase, catalase, glutamic acid decarboxylase, transaminase have been studied using the standard Warburg manometric technique. In all these cases, the drug has been taken in the side limb and is tipped into the main compartment after the contents have attained equilibrium with the bath.

Other methods.—The action of the drug on the enzyme fumarase has been studied by the titrimetric method of Laki and Laki,[17] and on the tryptophanase activity by the colorimetric method of Wood *et al.*[18]

Results

The results of the action of the drug on the respiration of the tubercle bacilli, succinic oxidase, cytochrome oxidase, glutamic acid decarboxylase, transaminase and tryptophanase are all represented graphically in Figs. 1–6, while the results of

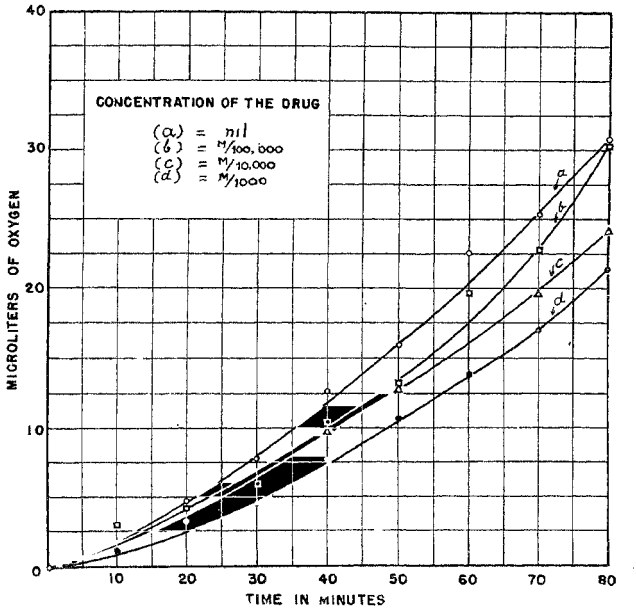


Fig. 1. Effect of Iso-nicotinic Acid Hydrazide on Oxygen Consumption of Tubercle Bacilli.

Table I

Flask No.	Concentration of INH (Final)	Microlitres of O_2 liberated (after 15 minutes)	Percentage inhibition
1	..	31·8	..
2	M/1,000	12·68	60·12
3	M/10,000	21·82	31·38
4	M/100.000	30·9	2·83

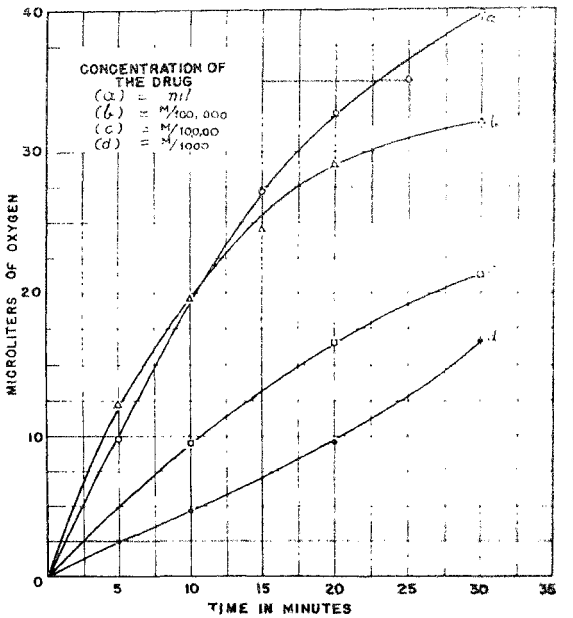
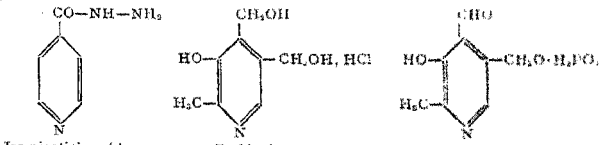


FIG. 2. Effect of Iso-nicotinic Acid Hydrazide on Succinic Oxidase

the action of the drug on catalase are presented in Table I. The drug, however, does not show any action on the enzyme fumarase.

DISCUSSION

The enzymes glutamic acid decarboxylase, transaminase and tryptophanase occupy important positions in the protein metabolism of the bacteria. They all require pyridoxal phosphate as the co-enzyme. Yoneda and co-workers[2, 3] reporting similar observations with tryptophanase, proposed that this drug possesses a competetive action with the co-enzyme, probably due to the slight structural similarity.



Iso-nicotinic acid hydrazide (INH) | Pyridoxine hydrochloride | Pyridoxal phosphate

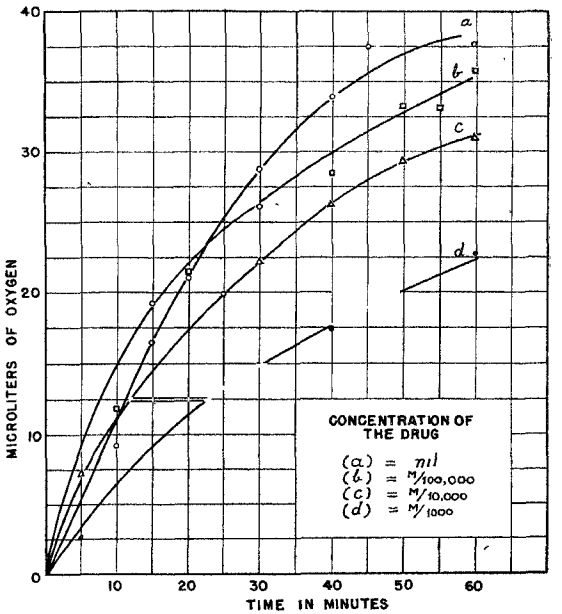


FIG. 3. Effect of Iso-nicotinic Acid Hydrazide on Cytochrome Oxidase.

This view is further strengthened by the recent observations that the toxemic and neuritic after effects produced during INH therapy[19] resemble those caused by pyridoxine deficiency, and that treatment with pyridoxine in such patients has definite beneficial results.

To verify the above postulation, the authors have carried out some *in vitro* studies using the H_{37} R_v strain of *Mycobacterium tuberculosis* in Youmans' medium with various amounts of INH and pyridoxine hydrochloride. (Pyridoxal phosphate could not be used because of its unstable nature for long periods at incubator temperature.) Various concentrations of INH, ranging from 1/1000 to 1/100,000,000 are arranged and the antagonistic effects, if any, of various concentrations of pyridoxine hydrochloride (ranging from 0·1 mg. to 4·0 mg. per ml.) have been tried.

It has been found that even the highest concentrations of pyridoxine hydrochloride could not exert any reversing effect on the activity of INH. Of course,

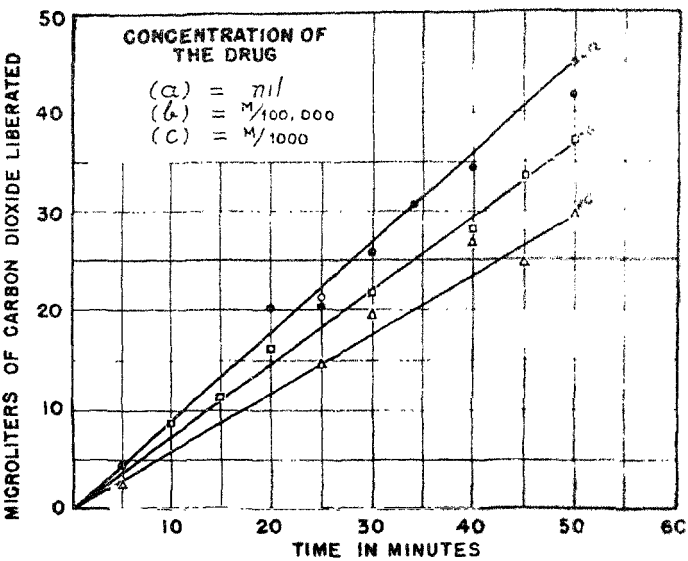


FIG. 4. Effect of Iso-nicotinic Acid Hydrazide on the Glutamic Decarboxylase. (*Cl. welchi* S.R. 12)

INH has been found to inhibit the growth of the bacilli at 1 [illegible] concentration, while pyridoxine hydrochloride could not exert any bacteriostatic action even at 4 mg. per ml. concentration.

These results show, that, if permeability factors are exempted, pyridoxine hydrochloride cannot compete with the drug or *vice versa*. Further, the ability of the bacteria to grow well, even without the extraneous supply of this vitamin indicates that they may not require exogenous supply for growth.

Similar results are also reported recently by Boone and Woodward,[3] Biehl and Vilter[20] and Ungar and co-workers.[21] Even desoxy-pyridoxine, tested by Tirunarayanan[22] (of this laboratory) for its anti-tubercular activity, indicated that this anti-vitamin does not possess any tuberculostatic action.

All these points warn us to some extent that we should view with some reservation this possibility of INH-pyridoxine hydrochloride antagonism as the principal mechanism of action.

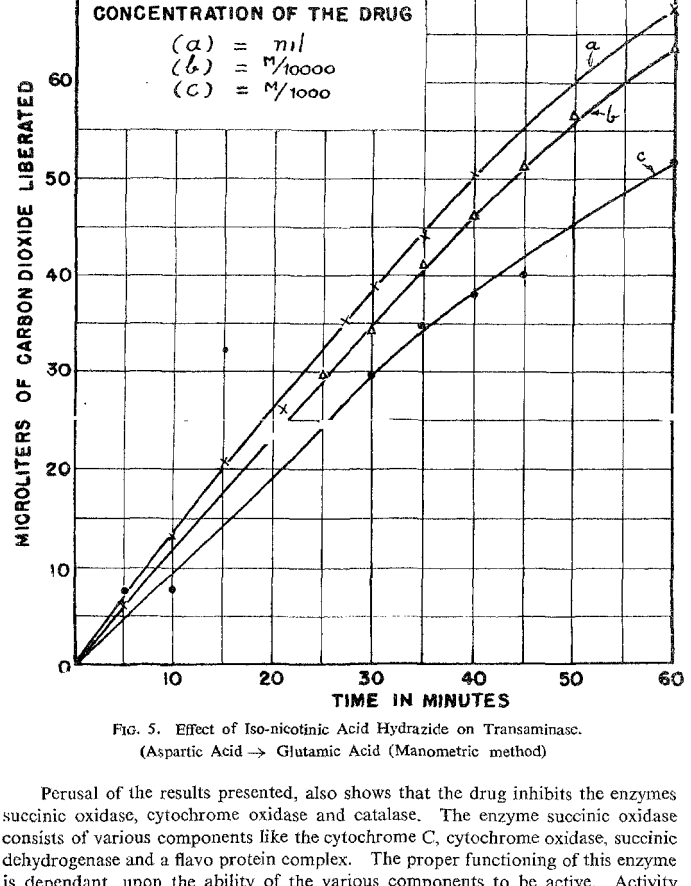


FIG. 5. Effect of Iso-nicotinic Acid Hydrazide on Transaminase.
(Aspartic Acid → Glutamic Acid (Manometric method)

Perusal of the results presented, also shows that the drug inhibits the enzymes succinic oxidase, cytochrome oxidase and catalase. The enzyme succinic oxidase consists of various components like the cytochrome C, cytochrome oxidase, succinic dehydrogenase and a flavo protein complex. The proper functioning of this enzyme is dependant upon the ability of the various components to be active. Activity will be lost even if one of the components is inactivated. The inhibition of the cytochrome oxidase by the drug is therefore responsible for the inactivation of

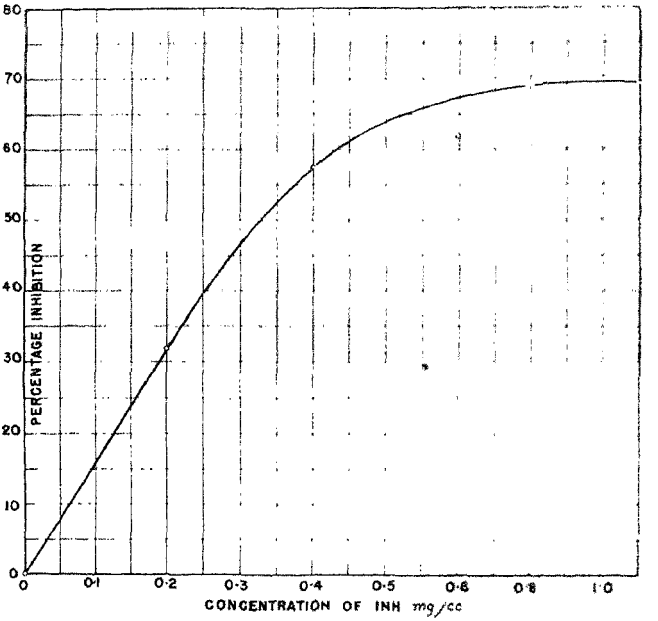
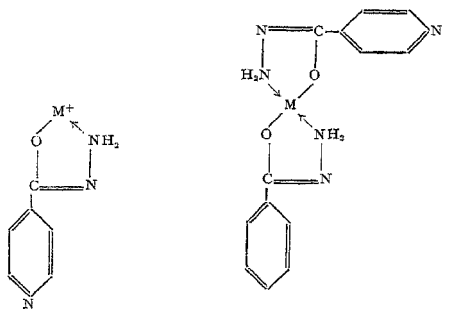


FIG. 6. Effect of Iso-nicotinic Acid Hydrazide on Tryptophanase (*E. coli*).

succinic oxidase. The lack of inactivation of succinic dehydrogenase by this drug has already been reported.[6]

The existence of a well-defined cytochrome system in the mycobacteria is well established.[23] The inhibitory action of INH on the enzymes cytochrome oxidase and catalase indicate its paralysing action of the iron-porphyrin type of enzymes. The mechanism of inactivation of these enzymes by INH can be explained in the following manner.

It has been found that INH and its allied drugs form chelating compounds with heavy metals like iron and copper. These complexes have been prepared and their physico-chemical properties studied recently by Albert.[24]



1 : 1 and 2: 1 metallic complexes with INH

In view of these, the mode of action of INH can be interpreted in two ways: either it " fixes " up the iron in the porphyrin nuclei, thereby inactivating the iron-porphyrin system of enzymes, or it forms a metallic complex with these, in which state it exerts its bacteriostatic activity. The first possibility fits well in the above results explaining the inactivation of the iron-porphyrin enzymes cytochrome oxidase and catalase. This view is further supported by the recent findings that hæmin counteracts the action of INH[25] and that INH is not active in alkaline Dubos medium wherein iron-ammonium citrate is present.[26]

The second possibility of the complex formation hypothesis cannot also be ruled out, due to the fact[27] that copper ions are found to increase the *in vitro* activity of INH. The drug may form a complex with the metal, exerting its bacteriostatic action in that form. Such theories of complex formation, in relation to mode of action, have also been proposed before, for the anti-bacterial activity of 8-hydroxy quinoline[28] and the anti-tubercular activity of thiosemicarbazones[29] and *p*-amino salicylic acid.[30]

At the present state, with the paucity of knowledge of the trace element requirements of *Mycobacterium tuberculosis*, it can only be said that the drug exerts its action by interfering with the heavy metals in the cells either by " fixing " them up or by forming active complexes.

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