

# STUDIES ON AN ENZYME REACTING WITH ISONIAZID FROM *MYCOBACTERIUM TUBERCULOSIS* H37Rv

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## ABSTRACT

*Mycobacterium tuberculosis* H37Rv possesses an enzyme (referred to as 'Y enzyme') which catalyses in the presence of INH and NAD, the formation of a product, which turns yellow on acidification. The requirements for the reaction, such as enzyme concentration, INH concentration, etc., have been standardized. The substrate specificity of the enzyme with respect to INH and NAD has been determined. The reaction is specific for the INH-sensitive strain and is totally absent in INH-resistant strains. Furthermore, the 'Y enzyme' shows some characteristic features of a peroxidase in its requirement for oxygen and sensitivity to inhibition by various reagents. The requirements of this enzyme which is involved in the action of isoniazid in *M. tuberculosis* H37Rv is described for the first time.

## LIST OF ABBREVIATIONS USED

- INH : isonicotinic acid hydrazide (isoniazid)  
NAD : nicotinamide-adenine dinucleotide  
NADH : reduced form of NAD  
NADP : nicotinamide-adenine dinucleotide phosphate  
NADPH : reduced form of NADP  
NMN : nicotinamide mononucleotide

## INTRODUCTION

ISONIAZID has been recognized as the most effective antitubercular drug available ever since it was introduced for the chemotherapy of tuberculosis. The drug is specific in its action against mycobacteria and several authors have attempted to elucidate the mechanism of action of this drug. The subject has been reviewed periodically (Long 1958; Goldman 1961; Winder 1964; Youatt and Tham 1969 *b*; Ramakrishnan *et al* 1972).

One of the models to explain the mechanism of action of INH is by Youatt and Tham (1969 *a*) and is based on an enzymatic reaction between isoniazid and NAD, observed in whole cells and cell-free extracts of BCG. The reaction leads to the formation of a product, which turns yellow on acidification. The nature of the product or the coloured compound (after acidification), referred to as "pigment" by these authors, is not established. The pigment production and isoniazid uptake run parallel (Youatt and Tham 1969 *b*). Both whole cells and cell-free extracts of isoniazid-resistant strains of BCG failed to carry out the reaction.

In this communication, evidence is presented for the existence of the enzyme in whole cells and cell-free extracts of the virulent strain of *Mycobacterium tuberculosis* H37Rv. A brief description of the general properties and requirements of the enzymatic reaction is also presented. Hereafter, the enzyme will be referred to as "Y enzyme", denoting Youatt's enzyme. A preliminary report on this enzyme has already been published (Gayathri Devi *et al* 1972).

#### MATERIALS AND METHODS

*Chemicals.*—NAD was from V.P. Chest Institute, New Delhi, India; NADP, NADH, NADPH and NMN were from Sigma Chemical Company, St. Louis, Missouri, USA; pyridine-3-sulphonic acid, acetyl pyridine adenine dinucleotide and acetyl hypoxanthine dinucleotide were from Nutritional Biochemicals Corporation, Cleveland, Ohio, USA; isoniazid was from Dumex India (Private) Ltd., Bombay, India; N-methyl nicotinamide was from Eli Lilly Co., Indianapolis, USA; ethionamide and pyrazinamide were gift samples from the Tuberculosis Chemotherapy Centre, Madras, India. Isoniazid analogues were synthesized at Organic Chemistry Department, Indian Institute of Science, Bangalore, India, for routine antimycobacterial drug screening purposes.

Nicotinic acid adenine dinucleotide was prepared according to the method of Honjo *et al* (1964). The procedure is based on the observation that beef spleen NAD (P) nucleosidase can catalyse a direct exchange reaction of nicotinamide of NAD with nicotinate. The over-all yield of deamido-NAD was 40–45% based on the NAD employed as starting material.

Picolinic acid hydrazide and nicotinic acid hydrazide were prepared according to Yale *et al* (1953).

*Organism.*—*Mycobacterium tuberculosis* var. *hominis* H37Rv (strain No. 7416) was obtained from National Collection of Type Cultures (England).



out at 37° and was terminated by the addition of 0.2 ml of 60% perchloric acid. After removing the precipitated protein by centrifugation, the yellow colour in the supernatant was measured at 420 nm. A zero time control was always included. 100  $\mu$ g of chloramphenicol was included in long time incubations.

*Enzyme assay with whole cells.*—The enzyme assays with whole cells were carried out in the same way as described for cell-free extracts except that a suspension of whole cells (100 mg wet weight of washed cells/ml of the reaction mixture) was used in place of cell-free extracts.

### RESULTS

Whole cells and cell-free extracts of *M. tuberculosis* H37Rv, when incubated with INH and NAD at 37°, led to the formation of a compound which turned yellow on acidification. Trichloroacetic acid, perchloric acid, sulphuric acid or hydrochloric acid function equally well in producing the colour intensity of the yellow compound. The enzyme activity was present in 100,000 g supernatant of the crude extracts and was not associated with the particulate fraction.

The two INH-resistant mutants of this strain, *viz.*, *inh-r-4* and *inh-r-6* failed to show the reaction under similar experimental conditions.

*Rate of reaction.*—The formation of the yellow product as a function of time is shown in figure 1. The rate of formation of the product increased with time, reaching the maximum at 6 hr and decreased thereafter.

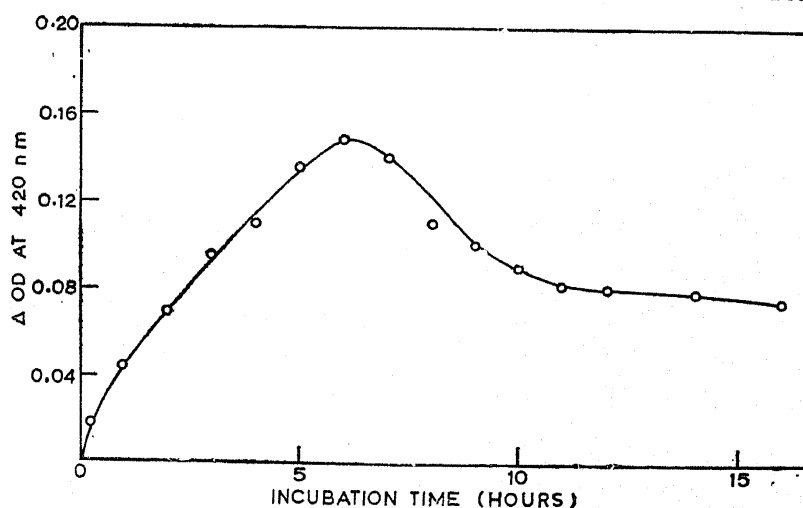


FIG. 1. Time course of Y enzyme reaction in cell-free extract. The assay procedure is as described under 'Methods'.

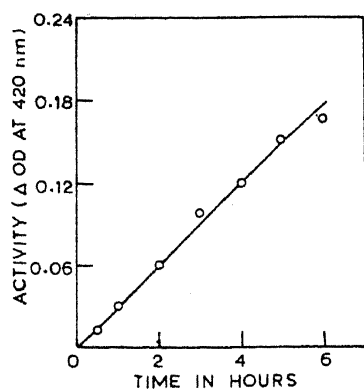


FIG. 2

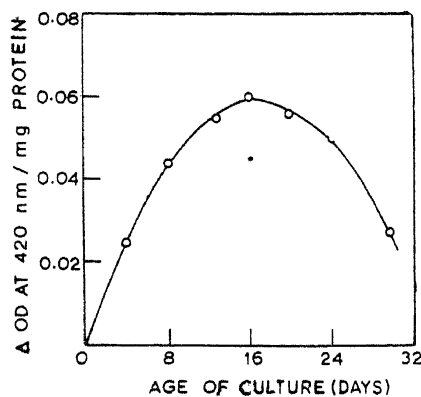


FIG. 3

FIG. 2. Time course of Y enzyme reaction in whole cells of *M. tuberculosis* H37Rv.

FIG. 3. Effect of age of culture on Y enzyme reaction. Standard assay conditions were used. Enzymatic incubations were carried out for 2 hr at 37° C.

As can be seen from figure 2, the formation of the product with whole cells also was linear with time, at least for 6 hr.

*Effect of age of culture on the enzyme reaction.*—Starting from 4th day after inoculation, the cells were harvested every four days and the enzyme activity in the extracts was estimated. The results are presented in figure 3.

The product formation increased with age and the activity was found to be maximum on the sixteenth day and decreased thereafter. The specific activity of the enzyme derived from cells older than 4 weeks was less than 50% of the activity found in actively growing cells.

*Effect of varying enzyme concentrations.*—The effect of increasing enzyme concentration on the product formation is shown in figure 4. The reaction was linear upto 3–4 mg protein/ml under the given conditions.

*Effect of varying INH concentration.*—The concentration of INH added was varied in the standard enzyme assay mixture, at a fixed high concentration of NAD (0.45  $\mu$  moles). The results obtained (figure 5) showed that the product formation is linear with increasing concentration of INH and reaches the maximum at about 2  $\mu$  moles of INH per ml.

*Effect of varying NAD concentration.*—The effect of NAD concentration on the enzyme activity was tested in the range 0.0075–0.525  $\mu$  moles per ml. The product formation increased with increase in concentration of NAD and the activity reached a maximum at a concentration of 0.45  $\mu$  moles (figure 6).

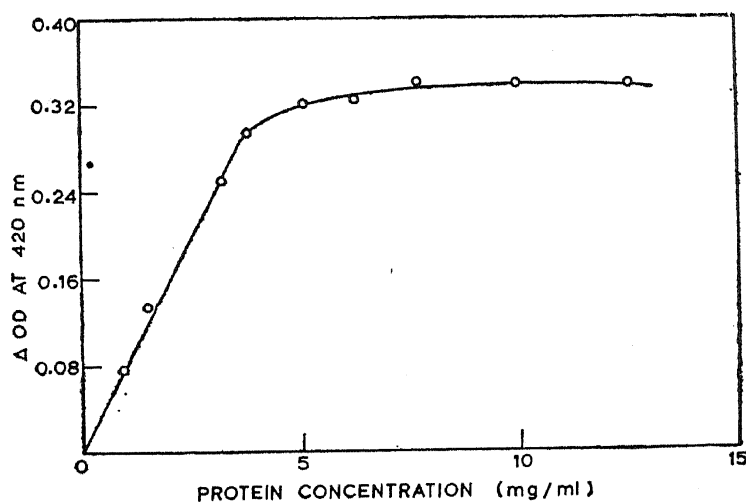


FIG. 4. Effect of enzyme concentration on Y enzyme activity. The reaction mixture contained varying concentrations of enzyme, and the rest of the conditions were the same as given under 'Methods'. Time of enzymatic incubation was 90 min.

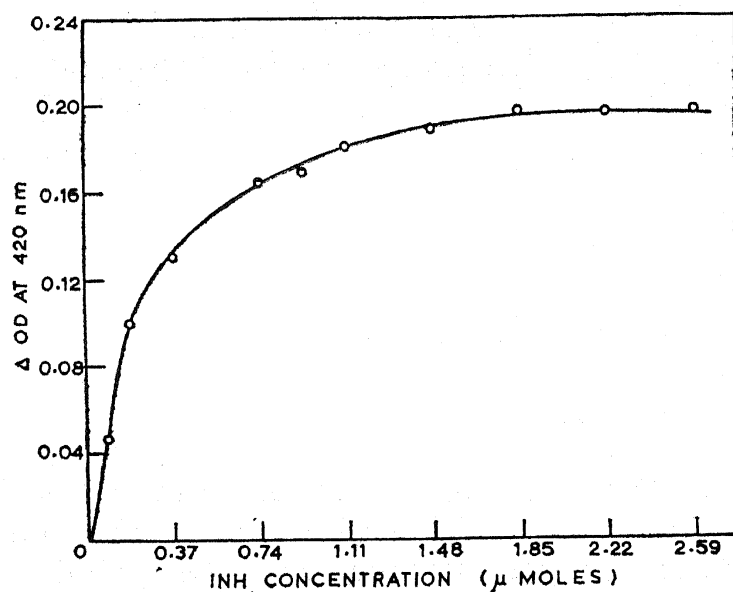


FIG. 5. Effect of varying INH concentration. The concentration of INH was varied in the standard assay mixture, at a fixed NAD concentration ( $0.45 \mu\text{mole}$ ). Time of enzymatic incubation, 90 min; protein used, 2.4 mg.

*Effect of dialysis.*—On dialysis of the crude extract against distilled water or against  $0.02 M$  potassium phosphate buffer,  $pH 7.0$  for 18–20 hr (with three changes), more than 50% of the activity was lost. Addition of either heated crude extract or cations like  $Mg^{2+}$ ,  $Na^+$ ,  $K^+$  or  $NH_4^+$  did not restore the activity.

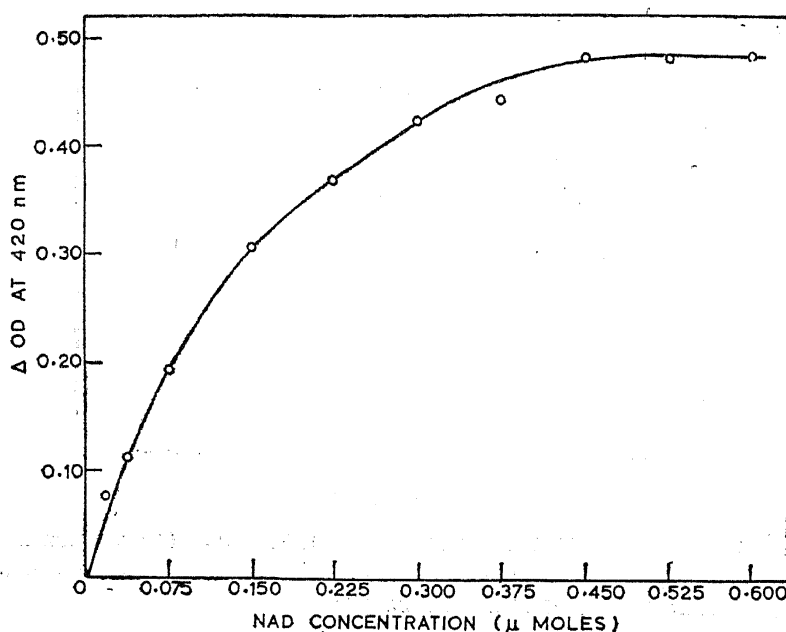


FIG. 6. Effect of varying NAD concentration. The concentration of NAD was varied in the standard assay mixture, at a fixed INH concentration ( $2 \mu$ moles). Time of enzymatic incubation, 90 min; protein used, 2.4 mg.

The enzyme was found to be stable for several weeks when kept frozen at  $-20^{\circ}$ .

#### *Substrate specificity*

(a) *INH and related compounds.*—The effect of various compounds related to INH, as substrate in the enzyme reaction, was studied. The results are presented in table 1.

As can be seen from the table, neither isoniazid analogues (nicotinic acid hydrazide and picolinic acid hydrazide) nor-substituted derivatives of isoniazid (isonicotinyl thiosemicarbazide, isonicotinyl alanine hydrazide, isonicotinyl alanine amide and isonicotinyl methionine) showed the reaction. Isonicotinic acid, a metabolite of INH, also failed to give this reaction. Therefore, any substitution or alteration on the hydrazide group of isoniazid appears to result in the loss of enzyme activity. These data suggest that the reaction is specific for INH.

Other antitubercular drugs such as ethionamide (2-ethyl thioisonicotinamide), pyrazinamide and streptomycin do not substitute for INH in the enzyme reaction.

**Table 1.** Effect of INH and related compounds on the enzyme reaction

Substrate	% activity	Substrate	% activity
INH	100·0	Isonicotinyl methionine	0·0
Picolinic acid hydrazide	0·0	Isonicotinyl thiosemicarbazide	0·0
Nicotinic acid hydrazide	0·0	Isonicotinic acid	0·0
Isonicotinyl alaninamide	0·0	Pyrazinamide	0·0
Isonicotinyl alanine hydrazide	0·0	Ethionamide	0·0

The assay was carried out as described in the text, except that INH was replaced with the appropriate compounds. The compounds used were tested at two different concentrations: 100 µg/ml and 200 µg/ml. The incubation was carried out for 2 hours at 37°.

(b) *Effect of NAD and related compounds.*—Table 2 shows the effect of various compounds related to NAD on the enzyme reaction when used in place of NAD. NADP, NADH, NADPH and nicotinamide hypoxanthine dinucleotide showed 100% activity, whereas NMN showed 50% activity. Nicotinic acid adenine dinucleotide, acetyl pyridine adenine dinucleotide and acetyl pyridine hypoxanthine dinucleotide were found to be inactive. N-methyl nicotinamide, nicotinamide, nicotinic acid and pyridine sulphonic acid also failed to give this reaction.

*Effect of oxygen and nitrogen on the enzyme reaction.*—As can be seen from table 3, product formation was stimulated by bubbling oxygen through the reaction mixture, whereas with nitrogen, the rate of formation reduced to more than 50%.

*Effect of some compounds on the enzyme reaction.*—Since the reaction was stimulated by oxygen, the effect of some reducing agents like glutathione, β-mercaptoethanol and dithiothreitol was studied. The effect of some compounds like cyanide, hydroxylamine, sulphide, sulphite, azide, fluoride and sulphate was also studied on the enzyme reaction (table 4). β-mercaptoethanol inhibited the reaction to 100% at 1 mM concentration. The inhibition with glutathione and dithiothreitol was 36% and 21% respectively. Of the other compounds tested, cyanide, hydroxylamine, azide, sulphide and sulphite inhibited the reaction, even at 0·1 mM and 0·01 mM concentrations. Sulphate and fluoride had no effect on the enzyme.



**Table 2.** Effect of NAD and related compounds on the enzyme reaction

Substrate	% activity	Substrate	% activity
NAD	100	3-acetylpyridine adenine dinucleotide	0
NADP	100	3-acetylpyridine hypoxanthine dinucleotide	0
NADH	100	N-methyl nicotinamide	0
NADPH	100	Nicotinamide	0
NMN	50	Nicotinic acid	0
Nicotinic acid adenine dinucleotide	0	Pyridine sulphonic acid	0
Nicotinamide hypoxanthine dinucleotide	100		

The assay was carried out as described in the text, except that NAD was replaced with the appropriate compounds. The incubation was carried out for 2 hours at 37°.

**Table 3.** Effect of oxygen and nitrogen on the enzyme reaction

Sample	OD 420 nm	
Control	0.145	The reaction mixture was as described in the text, oxygen and nitrogen were bubbled through the reaction mixture during incubation. The enzymatic incubation was carried out for 2 hours at 37°.
Oxygen	0.190	
Nitrogen	0.063	

#### DISCUSSION

An enzymatic reaction between INH and NAD leading to the formation of an yellow compound on acidification, similar to the one described by Youatt (1969 *a*) in BCG, has been observed in *M. tuberculosis* H37Rv (referred to as Y enzyme reaction). Since this enzyme reaction was strongly implicated in the mechanism of action of INH (Youatt and Tham 1969 *a, b*), the biochemical characteristics of the reaction have been elucidated in detail,

Table 4. Effect of various inhibitors on the enzyme reaction

Compounds	per cent inhibition		
	$10^{-3} M$	$10^{-4} M$	$10^{-5} M$
Cyanide	100.0	89.3	58.4
Hydroxylamine	100.0	74.6	21.8
Sulphite	100.0	63.0	23.1
Sulphide	56.0	23.4	12.8
Azide	100.0	67.1	34.6
Sulphate	0.0	0.0	0.0
Fluoride	0.0	0.0	0.0
Glutathione	35.84	..	..
$\beta$ -mercaptoethanol	100.0	..	..
Dithiothreitol	20.84	..	..

The reaction mixture was as described in the text, but in addition contained the inhibitors at the concentrations mentioned. The enzyme was preincubated with the inhibitors for 10 min at room temperature before the addition of INH and NAD.

in the present study. The reaction was absent in the two different types of single step INH-resistant mutants (inh-r-4 and inh-r-6) used. In these INH-resistant mutants, in addition to the loss of Y-enzyme reaction, peroxidase activity was also found to be lost. Since these resistant strains were apparently single step mutants, they may be expected to have a single biochemical alteration each, which is responsible for all these changes. Hence, it is reasonable to conclude, that these properties are due to a single protein.

The idea that the Y-enzyme may be peroxidase, is further strengthened by the observation that this enzyme reaction showed some characteristics of a peroxidase enzyme. Inhibitors like cyanide, azide, hydroxylamine, sulphite and sulphide which are known inhibitors or peroxidase inhibited the Y-enzyme reaction at low concentration. The reaction also showed a requirement for oxygen and was completely inhibited under anaerobic conditions. Furthermore, pure preparations of horse radish peroxidase can also catalyse the Y-enzyme reaction (unpublished observations),

The reaction was specific for INH and the other anti-tubercular drugs like ethionamide, pyrazinamide and streptomycin and analogues of INH (picolinic acid hydrazide and nicotinic acid hydrazide) failed to give this reaction. When the hydrazide group of INH was substituted by an amino acid (methionine) or amino acid amide (alaninamide) or substituted by thiosemicarbazide, the reaction was not observed. Isonicotinyl alanine hydrazide which possesses a hydrazide group after the alanine moiety was also found to be not active. Isonicotinic acid, which is considered to be an active metabolite of INH, did not give the reaction. The free hydrazide group ( $-\text{CO}-\text{NH}-\text{NH}_2$ ) directly attached to the pyridine ring seemed to be necessary for the enzyme reaction. Any substitution or alteration on the hydrazide group led to the loss of enzyme activity. The specificity of INH in this reaction was similar to growth inhibitory conditions of mycobacteria by this drug. The hydrazide of isonicotinic acid (isoniazid) is the most active one against *M. tuberculosis*; many other hydrazides, are comparatively inactive. The introduction of substituents into the pyridine nucleus was disadvantageous. When the hydrogen on the nitrogen adjacent to the carbonyl group in isoniazid was replaced, activity was lost (Cymerman-Craig *et al.* 1955). The two free hydrogen atoms on the nitrogen were not required for the growth inhibition because 1-isonicotinyl-2-isopropyl hydrazine (iproniazid), where the hydrogen of  $-\text{NH}_2$  is replaced by an isopropyl group, had high activity *in vitro* (Bernstein *et al.* 1953). Hence, the essential grouping for the activity may be the CONHNH.

The conditions and the requirements for the Y enzyme reaction have been standardized. The product formation reached a maximum at 6 hr and the reaction was linear up to 3-4 mg protein/ml. The presence of NAD was very essential and the reaction was not observed in the absence of NAD. NADP, NADH and NADPH were all equally active when used in place of NAD. It is possible that the activity of the reduced nicotinamide nucleotides may be due to the presence of powerful NADH- and NADPH- oxidases present in the crude extracts (Gopinathan *et al.* 1963). Nicotinamide hypoxanthine dinucleotide, where the adenine part of NAD is replaced by hypoxanthine, was found to be an efficient substrate, similar to NAD. Nicotinamide mononucleotide (NMN) was only 50% active. The activity observed with NMN may be due to the 50% contamination with NAD, which was detected later. Compounds like nicotinic acid adenine dinucleotide and acetyl pyridine adenine dinucleotide in which the amide ( $-\text{CONH}_2$ ) group of NAD was substituted by a carbonyl group and acetyl group respectively, were not active. Likewise, compounds lacking pyridine-ribose glycosidic bond, such as, N-methyl nicotinamide, nicotinic acid, nicotinamide and

pyridine sulphonic acid, also failed to show the reaction. Thus, it appears that both the free amide group and the pyridine-ribose glycosidic linkage are very essential for the enzyme reaction.

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