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Isoniazid-resistant Mutants of *Mycobacterium tuberculosis* H 37 Rv: Uptake of Isoniazid and the Properties of NADase Inhibitor

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SUMMARY

Six independent isoniazid-resistant mutants of *Mycobacterium tuberculosis* were isolated under conditions which largely ensured the selection of one-step mutants. The properties of these mutants with respect to the nicotinamide adenine dinucleotide nucleosidase (NADase) inhibitor, isoniazid uptake and peroxidase activity were studied. The uptake of isoniazid by the mutants and their sensitivity to isoniazid were increased by ethylenediaminetetra-acetic acid (EDTA) and sodium dodecylsulphate. It is suggested that EDTA and sodium dodecylsulphate bring about these effects by altering the cell permeability.

INTRODUCTION

Isoniazid (INH) is specific in its inhibitory action on the growth of mycobacteria; its mechanism of action on this group of organisms has been examined by many workers. Gopinathan *et al.* (1963, 1964, 1966) showed the presence of a heat-labile inhibitor of nicotinamide adenine dinucleotide nucleosidase (NADase) in *Mycobacterium tuberculosis* H37Rv and purified the enzyme and the inhibitor. Bekierkunst (1966) noticed that INH, which had no effect on mycobacterial NADase (Gopinathan *et al.* 1964), caused a decrease in the NAD content of the organism and suggested that the drug might inactivate the inhibitor, thereby enhancing the activity of NADase. Sriprakash & Ramakrishnan (1966) confirmed that INH *in vitro* interacted with NADase-inhibitor complex and thereby enhanced the activity of NADase. It can be argued on the basis of these findings that if the primary mechanism of action of INH is through the inactivation of the inhibitor of NADase, then the inhibitor from INH-resistant mutants might have lost its sensitivity to INH. The results presented in this paper show that resistance to INH is accompanied by an alteration in the permeability of the organism to the drug, while only in a few of these mutants the inhibitor also lost the sensitivity to INH. It is also shown that in both types of mutants, the organism is sensitized to INH by treating it with ethylenediaminetetra-acetic acid (EDTA) or sodium dodecylsulphate. A preliminary communication has been published (Sriprakash & Ramakrishnan, 1968).

METHODS

Chemicals. NAD was obtained from Sigma Chemical Co., St. Louis, U.S.A.; INH from Dumex (India) Private Ltd. Bombay; EDTA from E. Merck, Darmstadt, Germany; phenylethylalcohol from Distillation Products Industries, Rochester, U.S.A.; labelled 7-[^{14}C]INH (10 mc./m-mole) was purchased from the Radiochemical Centre, Buckinghamshire, England. Other reagents were of Analar grade.

Organisms. Mycobacterium tuberculosis H37Rv, originally obtained from National Collection of Type Cultures, London (NCTC 7416) and maintained on Petrik medium, was used in the present studies. To obtain inocula for growth experiments Sauton liquid medium was used. For enzyme studies the organism was grown in the liquid medium of Youmans & Karlson (1947) with glycerol as carbon source and asparagine as nitrogen source. All incubations were at 37°.

Preparation of cell-free extracts. This was done by ultrasonic treatment as described by Murthy, Sirsi & Ramakrishnan (1962).

Enzyme assays. NADase activity was measured by the cyanide addition method (Zatman, Kaplan & Colowick, 1953). The purification and assay of NADase inhibitor were described by Gopinathan, Ramakrishnan & Vaidyanathan (1966). One unit of NADase is defined as that amount of enzyme which breaks down 1 nmole NAD/min. at 37° under the conditions of assay. One unit of NADase inhibitor is defined as that amount of inhibitor which inhibits 1 unit of enzyme by 50% at 37°.

Peroxidase assay was done as follows. One ml. of the reaction mixture contained potassium phosphate buffer (pH 7·0) 100 μmole; hydrogen peroxide 0·1 μmole; pyrogallol, 5 μmole; and enzyme. The reaction was stopped after 10 min. with 3 ml. 5N-H_2SO_4 and the solution extracted three times with ether. The volume of the ethereal extract was made to 5·0 ml. with ethanol (95%, v/v in water) and the colour measured in a Klett–Summerson Colorimeter with filter no. 42.

Other assays. Total oxidized pyridine nucleotides were estimated by the ethylmethylketone method of Carpenter & Kodicek (1950) by using a Carl Zeiss Spekol Fluorimeter. Radioactive measurements of the bacteria were done by drying them on tared aluminium planchets and counting in a gas-flow proportional counter. Other radioactivity measurements were made with a Beckman liquid scintillation counter, Model LS-100.

Isolation of INH-resistant mutants. Six tubes each containing 1 ml. Sauton medium were inoculated with about 10^3 *Mycobacterium tuberculosis* H37Rv organisms and incubated till the colony count was 10^7. Samples (0·1 ml.) from each of the tubes were streaked on to Petrik medium containing 0·1 μg. INH/ml. and incubated till the growth was visible. The isolated colonies were subcultured and maintained on Youmans & Karlson medium containing the same concentration of INH.

RESULTS

INH-resistant mutants. Six independent INH-resistant mutants were isolated. They were all found to be resistant to 1·0 μg. INH/ml., when tested in Youmans medium, in spite of their selection of INH resistance at 0·1 μg./ml. All these mutants were inhibited by INH at 1·5 μg./ml. and above. These mutants are referred to as *inh-r*-1, *inh-r*-2, *inh-r*-3, *inh-r*-4, *inh-r*5, *inh-r*-6.

The effect of INH on NAD concentrations. The results presented in Table 1 indicate that in the INH-resistant strains INH did not cause the depletion of NAD, up to 1 μg. INH/ml., to which concentration these bacilli were resistant. On the other hand, in the wild type NAD was decreased to the extent of 50% with 0·1 μg. INH/ml. These results are consistent with those reported by Bekierkunst & Bricker (1967) for *Mycobacterium tuberculosis* H37Ra.

Effect of INH on NADase. NADase was purified from *Mycobacterium tuberculosis* H37Rv and the mutants *inh-r*-4 and *inh-r*-6, and the effect of INH was studied on the

enzyme. INH had no direct activating effect on NADase of wild type or on either type of INH-resistant bacilli (Table 2).

Table 1. *Effect of INH on NAD concentration in sensitive and resistant tubercle bacilli*

Different concentrations of INH were aseptically added to a 15-day culture which was then further incubated for 24 hr. The bacilli were harvested, washed with cold water, suspended in cold 5 % trichloracetic acid and treated ultrasonically for 5 min. at 0 to 3° in 10 kc. Raytheon Sonic Oscillator. The suspensions were then centrifuged at 13,000 *g* in cold for 30 min. and the supernatant fluid after neutralization with N-NaOH was used for determination of NAD.

Culture	INH (μg./ml.)	μg. NAD(P)/g. wet bacteria
M. tuberculosis H 37 RV	0	220
	0·1	115
	0·5	4
Mutant *inh-r*-4	0	220
	0·1	231
	0·5	217
	1·0	222
	1·5	132
Mutant *inh-r*-6	0	175
	0·1	177
	0·5	168
	1·0	170
	1·5	122

Table 2. *Effect of INH on NADase*

The reaction mixture contained 100 μmole potassium phosphate buffer (pH 6·7); NAD, 0·2 μmole; enzyme, 1 unit; total volume 0·6 ml. The mixture was incubated for 1 hr. INH 1·0 μmole was included in the reaction mixture.

Cultures	NADase activity (Extinction/hr/unit enzyme)	
	No INH	INH 1·7 μmole/ml.
M. tuberculosis H 37 RV	0·105	0·106
inh-r-4	0·105	0·103
inh-r-6	0·105	0·101

Effect of INH on NADase-inhibitor complex. It can be seen (Fig. 1) that the organisms were of two types. The wild type and the mutants *inh-r*-1, *inh-r*-3, *inh-r*-4 and *inh-r*-5 belonged to the first type, where the inhibitor was sensitive to INH. The mutants *inh-r*-2 and *inh-r*-6 belonged to the second type, where the inhibitor was not sensitive to INH. The effect of INH on NADase-inhibitor heterocomplexes was studied to find whether the change had occurred in the inhibitor or the enzyme in mutants *inh-r*-2 and *inh-r*-6.The heterocomplexes were obtained by mixing purified NADase of one strain with the purified inhibitor of a different strain. The results (Table 3) indicated that INH annulment of inhibitor activity was not observed with the heterocomplexes which were composed of the inhibitor of mutant *inh-r*-2 or *inh-r*-6. It was therefore concluded that the inhibitor of these two strains alone was altered.

To investigate whether the alteration of the inhibitors of *inh-r*-2 and *inh-r*-6 resulted in their inability to bind INH, equilibrium dialysis was done with these inhibitors and

labelled INH. Mutants *inh-r*-4 and *inh-r*-6 were chosen as the representatives of two types of mutants. The results (Table 4) showed that the binding of INH by the inhibitor of *inh-r*-6 was insignificant as compared to that by the inhibitors of *inh-r*-4 and wild-type organisms.

Peroxidase activity of Mycobacterium tuberculosis. While the wild type was peroxidase-positive all the mutants were peroxidase-negative. To find whether peroxidase and the NADase inhibitor were one and the same protein the inhibitor was purified up to the alumina Cγ-gel elution stage and both activities were determined at every stage. The ratio of peroxidase activity to the inhibitor activity was almost constant up to the alumina Cγ-gel elution stage of purification (Table 5).

INH uptake by Mycobacterium tuberculosis

Since a change in the sensitivity of NADase inhibitor to INH could explain the resistance to INH of only two of six resistant strains, the uptake of INH by these

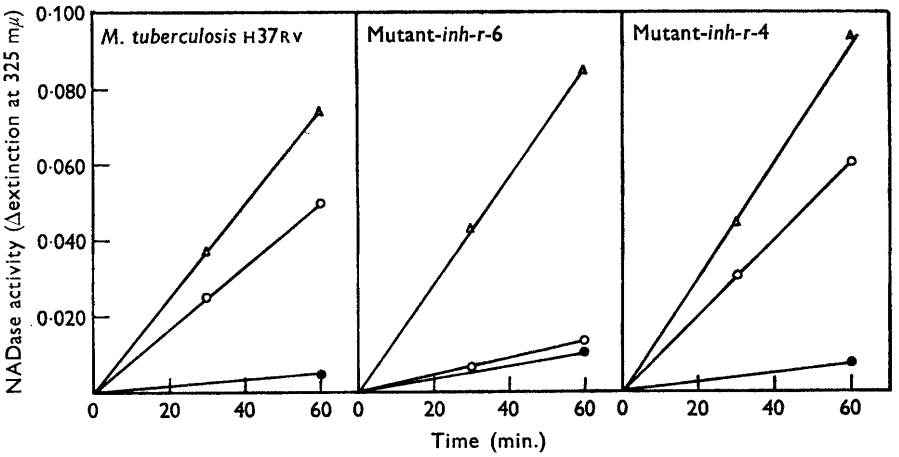

Fig. 1. *The effect of INH on NADase-inhibitor complex.* Incubation mixture (0·6 ml.) contained 100 μmole phosphate buffer (pH 6·7), with or without 1 μmole INH and crude cell-free extract. The reaction mixture was incubated for 20 min. at 37°. NAD (0·25 μmole) was added and further incubated for 1 hr. The total NADase activity was determined by heating the cell-free extract at 80° for 1 min. before adding NAD. Incubation temperature 37°. △, NADase activity of heat-treated cell-free extract; ○, NADase activity of INH-treated cell-free extract; ●, NADase activity of cell-free extract.

Table 3. *Effect of INH on the heterocomplexes*

Purified NADase and inhibitor from different sources were mixed and incubated for 15 min. at 37°. The effect of INH on the heterocomplex was studied by further incubation of this mixture with 1·0 μmole INH for 20 min. at 37° and the NADase activity assayed.

	Source of the inhibitor				
Source of NADase	H 37 Rv	*inh-r*-1	*inh-r*-2	*inh-r*-4	*inh-r*-6
H 37 Rv	+	+	−	+	−
inh-r-1	+	+	−	+	−
inh-r-2	+	+	−	+	−
inh-r-4	+	+	−	+	−
inh-r-6	+	+	−	+	−

+ = Annulment of inhibitor activity; − = no annulment of inhibitor activity.

strains was examined. INH uptake was found to be decreased in all the mutants as compared with the wild type. The results with two of the mutants (*inh-r*-4, *inh-r*-6) are illustrated in Table 6. The effect of certain compounds which have been reported to alter cell permeability of other bacteria, on the uptake of INH was examined. These compounds were incorporated into the Youmans & Karlson medium along with [^{14}C]INH. The results (Table 6) showed that ethylenediaminetetra-acetate and sodium dodecylsulphate increased the uptake of both types of resistant mutants, while phenylethylalcohol inhibited this uptake. These effects were consistent when the experiments were repeated a number of times.

Table 4. *Equilibrium dialysis of NADase inhibitor*

The purified inhibitor was concentrated by lyophilization to about 500 μg. protein/ml. The concentrated inhibitor was dialysed against 0·05 M-potassium phosphate buffer (pH 7·0) containing 0·01 μC. 7-[^{14}C]INH/ml. for 24 hr at 0 to 5° by using Visking tubing, pretreated by boiling in 10^{-3}M-EDTA thrice. The radioactivity inside and outside the Visking tubing was measured.

Source of inhibitor	Counts/min.*	Protein content (μg.)	[^{14}C]INH bound/mg. inhibitor protein
M. tuberculosis H 37 RV	625	500	1250
inh-r-4	725	550	1320
inh-r-6	180	580	310

* Represents the difference in counts between the outside and inside of the dialysis tubing.

Table 5. *Ratio of inhibitor activity to peroxidase activity*

Stage of purification	Units of inhibitor/ml.	Units of peroxidase/ml.	Ratio of inhibitor activity to peroxidase activity
DEAE-eluate	36·0	2·2	16·0
DEAE-eluate dialysed	32·0	1·9	17·0
Alumina Cγ-gel eluate I	27·0	1·8	15·0
Alumina Cγ-gel eluate II	44·0	2·9	15·0
Alumina Cγ supernatant	26·5	1·5	18·0

The effect of increased INH uptake on INH-resistant tubercle bacilli

Experiments were made to determine whether these compounds increased the bacteriostatic effect of INH. Various concentrations of these compounds and INH were incorporated into Youmans & Karlson medium and inoculated with INH-resistant bacilli. The results are given in Table 7. EDTA at 5×10^{-3}M when included in the medium with INH 0·5 μg./ml. completely inhibited the growth of both types of resistant mutant. However, the concentration of EDTA used by itself slightly retarded growth. On the other hand, sodium dodecylsulphate at 0·01 % + INH 0·5 μg./ml. inhibited the growth of both kinds of INH-resistant mutant; sodium dodecylsulphate alone was not inhibitory. Phenylethylalcohol did not supplement the effect of INH on any of the INH-resistant mutants and was itself inhibitory to growth at 0·1 %.

Table 6. *The effect of ethylenediaminetetra-acetate, phenylethylalcohol and sodium dodecylsulphate on the uptake of INH by INH-sensitive and INH-resistant mycobacteria*

The 15-day cultures were inoculated into Youmans & Karlson medium containing 0·05 μc. INH/ml. and incubated for 40 hr. The organisms were then washed with Youmans & Karlson medium containing 10 μg. unlabelled INH/ml., and finally with distilled water.

	M. tuberculosis H 37 Rv		Mutant *inh-r*-4		Mutant *inh-r*-6	
Treatment	Counts*/ 5 min./ 10 mg. dry wt	Relative % of [^{14}C]INH uptake	Counts*/ 5 min./ 10 mg. dry wt	Relative % of [^{14}C] uptake	Counts*/ 5 min./ 10 mg. dry wt	Relative % of [^{14}C] uptake
—	420	100·0	70	17	140	33
5×10^{-3}M-EDTA	360	85·0	110	26	220	52
0·01 % Sodium dodecylsulphate	440	105·0	120	29	230	55
0·1 % Phenylethylalcohol	240	57·0	30	7	100	24

* Represents the total counts from which the background (110 counts/5 min.) counts have been subtracted.

Table 7. *Effect of ethylenediaminetetra-acetate, sodium dodecylsulphate and phenylethylalcohol on the growth of Mycobacterium tuberculosis inh-r-6 in the presence of subinhibitory concentration of INH*

The growth was measured by determining the dry weight of organisms after 15 days of incubation. The pattern for mutant *inh-r*-4 was essentially the same.

		Concentration of INH (μg./ml.)			
Compounds used	Concentration	0	0·1	0·5	1·0
		Growth (mg. dry wt)			
—	—	16	17	17	16
Ethylenediamine-tetra-acetate	5×10^{-3}M	15	10	0	0
	10^{-2}M	9	11	0	0
Sodium dodecyl-sulphate	0·005 %	16	15	17	17
	0·01 %	17	17	12	0
Phenylethyl-alcohol	0·01 %	18	16	16	17
	0·05 %	12	10	11	9
	0·1 %	0	0	0	0

DISCUSSION

In this work the mechanism of INH resistance in *Mycobacterium tuberculosis* H 37 Rv has been investigated by using independent mutants of the organism selected under conditions that largely ensured the isolation of single-step mutants, by exposing the organism to low growth-inhibitory concentration of INH. Such single-step mutants, unlike resistant strains selected by exposure to graded concentrations of INH, may be expected to have a single biochemical alteration which is solely responsible for resistance. By the above procedure two types of INH-resistant strains were isolated, one in which the NADase inhibitor had lost its sensitivity to INH accompanied by decreased uptake of INH and a loss of peroxidase activity, while in the second type only the uptake of INH and the peroxidase activity were lost. Apparently both types are the results of mutations in the same locus, since the same method of isolation had been used and they were resistant to the same maximum dose of INH. Though the mutants were isolated on media containing 0·1 μg.INH/ml. they were

all resistant to a maximum concentration of 1·0 μg. INH/ml. Evidence for one-step mutation being capable of causing the highest degree of resistance of *M. tuberculosis* to isoniazid was presented by Middlebrook (1957).

If a single mutational step is involved in producing strains which are altered in isoniazid uptake, in the NADase inhibitor and in peroxidase activity, this suggests that all these are functions of the same protein. In partial support of this we have shown that the NADase inhibitor and peroxidase activities go together when the protein is purified. The involvement of peroxidase in INH uptake was suggested by Wimpenny (1967). The existence of mutants altered only in two functions, namely, isoniazid uptake and peroxidase activity, indicates that this protein can be altered so as to change two of its functions, but not the third.

There appears to be direct correlation between the sensitivity of *Mycobacterium tuberculosis* to INH and the depletion of NAD in the organism in the presence of isoniazid. The mechanism of this relationship is not clear. The hypothesis of Bekierkunst (1966) that depletion of NAD is due to the indirect activation of NADase does not appear to be wholly valid since the effect of isoniazid on the NAD content of the resistant mutants is the same whether or not the NADase inhibitor is altered. Two other possible explanations for the difference in the depletion of NAD in the presence of INH between INH-sensitive and INH-resistant strains were disproved by appropriate experiments. INH at 1·5 μmole/ml. had no direct activating effect on the NADase of wild type or INH-resistant mutants of *M. tuberculosis*. On the other hand, INH has inhibitory action on NAD biosynthesis in the extracts of *M. tuberculosis*, but this inhibition is exerted to the same extent in INH-sensitive and INH-resistant strains (Sriprakash & Ramakrishnan, 1969). The possibility that INH brings about the leakage of intracellular NAD is being investigated. However, a simple explanation of this difference may be that the decrease in NAD is a secondary consequence of other damage to the cell by INH, which would be expected to occur only in resistant strains at concentrations of INH higher than those to which these strains are resistant.

The results from the [^{14}C]INH experiments show that alteration in permeability to INH is a major factor in the resistance of *Mycobacterium tuberculosis* to this drug. Evidence of the decreased uptake of INH by resistant tubercle bacilli has been given by many workers (Barclay, Ebert & Koch-weser, 1953; Barclay, Koch-weser & Ebert 1954; Boone, Strang & Rogers, 1957; Youatt, 1958, 1960*a*, *b*; Wimpenny, 1967). It was observed in the present work that compounds such as ethylenediaminetetraacetate and sodium dodecylsulphate which have been shown to act on membranes of other bacteria (Lieve, 1965; Bayer & Anderson, 1965) increased the uptake of INH by the resistant organism. Phenylethylalcohol, another membrane-active agent (Silver & Wendt, 1967), on the other hand, had no such effect. Once INH had accumulated in the resistant organisms, it exerted its bacteriostatic effect irrespective of whether or not the NADase inhibitor was sensitive to INH. This appears to confirm the conclusion that in the mechanism of INH resistance, permeability plays a more important role than the sensitivity of the inhibitor to INH. The emergence of resistance to isoniazid by *M. tuberculosis* appears to be more frequent than that to streptomycin, which has been reported to be 10^{-9} (Szybalski & Bryson, 1952). The frequency of resistance to isoniazid is reported by the above authors to be (1 to 3) $\times 10^{-6}$ by using the statistical method of Delbruck & Luria.

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Note added in proof

The first step INH-resistant mutants were all found to be resistant to 4·0 μg INH/ml. when tested on Petrik medium, though in Youman's medium they were resistant only to 1·0 μg INH/ml.