SPECIFICITY OF ISONIAZID ACTION AGAINST MYCOBACTERIA AND A POSSIBLE MECHANISM FOR THE DEVELOPMENT OF DRUG RESISTANCE

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ABSTRACT

Analysing the genetic and biochemical aspects associated with the development of isoniazid resistance by mycobacteria, a unified model invoking peroxidase in the uptake of the drug by the sensitive organism has been proposed; the inability of the organism to take up isoniazid renders it resistant to the drug. Further, the specificity of isoniazid action against mycobacteria has been attributed to the presence of this enzyme (a peroxidase, capable of interacting with the drug and thereby transporting it into the cell).

ISONIAZID, the powerful antituberculous agent, has occupied a unique position in the treatment of tuberculosis, since its introduction in 1952. The special features about the action of this molecule are: (i) it is extremely specific in its action against mycobacteria, and (ii) it is very effective at low concentrations (0·01-0·1 μg/ml being the *in vitro* concentration to bring about the growth inhibition of *M. tuberculosis* and 0·1-10 μg/ml for the saprophytic mycobacteria; under similar conditions, more than 600 μg/ml of the drug is needed to inhibit the growth of other bacteria).

The mechanism of action of isoniazid (INH) has been studied by several workers and the following modes have been suggested (for details see references 1 and 2): (*a*) INH acts through its chelation properties; (*b*) It antagonizes the function of pyridoxal (or its related coenzymes) and therefore interferes with metabolic reactions such as transaminases, deaminases, etc; (*c*) Isoniazid due to its structural similarity to nicotinamide, antagonizes the function of nicotinamide coenzymes (NAD, NADP) and interferes in the oxidation-reduction reactions; (*d*) The drug inactivates the proteinaceous inhibitor of the enzyme NADase leading to a reduction in the levels of the nicotinamide nucleotide coenzymes in the cell; (*e*) Hydrogen peroxide and the free radicals produced during the metabolism of the drug exert the toxic effect on the organism; (*f*) Isoniazid interferes in the synthesis of hemoproteins (catalase and perioxidase); (*g*) The drug inhibits protein and nucleic acid synthesis; (*h*) It interferes with the synthesis of fatty acids, resulting in the production of a defective envelope and ultimately cell death by the leakage of cellular components; (*i*) INH undergoes a specific reaction (referred to hereinafter as the Y enzyme reaction, see ref. 3) in presence of NAD and an enzyme from drug-sensitive bacteria, which somehow results in the death of the cells.

None of these observations, however, explains the specificity of INH action against mycobacteria. Further it raises the question, 'if indeed INH was acting through all these mechanisms, how does the bacterium so readily develop resistance to the drug?' Here evidence is presented to answer some of these questions.

We had shown earlier that a single protein (purified to homogeneity) from *M. tuberculosis* $H_{37}R_0$ possessed catalase, peroxidase and Y enzyme activities[3]. Moreover, pure preparations of horseradish peroxidase or lactoperoxidase could catalyse the Y enzyme reaction whereas pure preparations of catalase (beef liver catalase) could not do so. It was, therefore, concluded that Y enzyme reaction is a peroxidatic oxidation of INH and this reaction was presumably involved in the uptake of the drug. Further support for this conclusion came from two different types of studies. (1) The single step INH resistant mutants derived from INH sensitive cells (spontaneous mutants isolated independently or mutants isolated after treatment with N-methyl-N-nitro-N' nitrosoguanidine and purified through colony isolation) had lost catalase/peroxidase and Y enzyme activities. All these mutants were defective in the uptake of ^{14}C-labelled INH. When INH was forced into these cells under conditions of altered permeability the cells were still susceptible to the killing action of isoniazid. (2) Earlier workers have shown that when radioactive INH is added to cells of *M. tuberculosis*, no free INH was detectable within the cells; most of the material taken up was present as isonicotinic acid and isonicotinic alcohol[4]; these products might have been formed from the intermediate (unstable) product of the peroxidase oxidation of INH during transport.

The development of resistance to INH can therefore be explained by a unified model in which the enzyme peroxidase is invoked in the uptake of the drug and the inability of the organism to take up isoniazid renders it resistant to the drug. The resistance/sensitivity to INH by mycobacteria is presumably determined by one genetic locus, which may code for the

uptake of the drug; once inside the cell, the drug exerts the antibacterial action by any one of the proposed biochemical steps.

To verify the above model, the genetics of INH resistance was examined employing the fast growng saprophytic species *M. smegmatis* and the transducing mycobacteriophage I3. The catalase, peroxidase and Y enzyme activities of wild type (INH^s), INH^r mutant, INH^s and INH^r transductants are presented in Table I. It is evident that the development of INH resistance either by mutation or transduction led to a simultaneous loss of catalase, peroxidase and Y enzyme activities; corversely, the transduction of drug sensitivity to resistant cell resulted in a concomitant gain of all these activities. These results are in total agreement with our earlier biochemical observations[3] that a single species of protein possessed all the three properties.

The specific activities of peroxidase and Y enzyme reactions in *M. smegmatis* were 1/3–1/5 campared to *M. tuberculosis*, whereas the specific catalase activiies of these organisms were almost identical. There is thus a striking correlation of the peroxidatic activity (measured with the dye as substrate or as the Y enzyme reaction with INH as the substrate) and the INH susceptibility of the two different strains, *M. smegmatis* and *M. tuberculosis*. The specificity of INH action against mycobacteria has been attributed to the presence of this peroxidase, which is necessary for the transport of the drug into the organism.

The results presented can be summarised as shown in Table II.

TABLE I

Catalnge, peroxidose and enzyme activities

Strain	Enzyme specific activity (Δ absorbancy per min per mg protein)		
	Catalase (240 nm)	Peroxidase (460 nm)	Y enzyme (420 nm)
M. smegmatis Parental (INH sensitive)	0·650	0·55	0·018
INH resistant (Parental or transductant)	*n.d.**	*n.d.*	n.d.
INH sensitive (transductant)	0·650	0·65	0·025
M. tuberculosis H 37R, (INH sensitive)	0·653	1·57	0·126

*n.d., not detectable, *i.e.*, abosrbancy less than 0·005 under the conditions of the assay.

TABLE II

Organism	Property examined			
	Catalase	Peroxidase	Y enzyme	INH uptake
INH sensitive	+ve	+ve	+ve	+ve
↓ Mutation				
INH resistant	−ve	−ve	−ve	−ve
Transduction of INH susceptibility				
↓				
INH sensitive	+ve	+ve	+ve	+ve

All the 3 enzyme activities were concomitantly lost in INH resistant mutants and transductants. If they were due to separate (independent) loci carried by a single phage, at least in some strains tested, we should have seen the absence/presence of one character. These results taken together with the biochemical data that a single protein possessed all the 3 enzyme activities[3], suggest that the observed loss/gain in character is due to a single genetic locus.

It is evident that a single mutation from INH sensitivity to resistance has knocked off 4 biochemical functions. The loss of activities seen in the resistant strain can be concomitantly regained by introducing the drug sensitivity marker by phage-mediated transduction. Hence genetic evidence is also provided for our earlier conclusion[3] that the peroxidase is responsible for the uptake of the drug. The present results also forecast that all organisms possessing peroxidase activity (capable of utilizing INH in the reaction) will be susceptible to the action of INH, but this possibility has not been examined.

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L-ASPARAGINASE AND GLUTAMINASE ACTIVITIES IN THE CULTURE FILTRATES OF *ASPERGILLUS NIDULANS*

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A LARGE number of fungal strains of ascomycetes and *fungi imperfecti* produce L-asparaginase and glutaminase in their culture filtrates or within the mycelium. These enzymes, extracted from the mycelia of *Aspergillus terreus*[1] and *Fusarium trincitum*[2], have been found to have antitumour activities. Recently, the properties of L-asparaginase have been studied in the mycelial extracts of *Aspergillus nidulans*[3,4]. *The present investigation reports the conditions for* the optimal expressions of L-asparaginase and glutaminase in the culture filtrates of *Aspergillus nidulans.*

A riboflavin and biotin requiring green conidial (*ribo A*1, *bi A*1) strain from the Departmental stock (FGSC No. 158) was used during the course of the present investigation. Composition of complete (CM) and minimal media (MM) and culture conditions have been described earlier[5,6].

Cultures of the desired strain were raised by inoculating approximately 5×10^7 conidia/50 ml of the complete or minimal media or in media containing L-asparagine or glutamine at a final concentration of 0·2 to 1·0%. Flasks were incubated at 37° C and 150 *rpm in a New Brunswick Gyrotory G25* incubator shaker. Test samples were withdrawn at 24 hr intervals upto 96 hr. Culture filtrates were centrifuged at 12,000 × g for 30 min, before estimating the enzyme activities. Imada's procedure[7] was followed for estimating the activities of these enzymes.

A unit of the enzyme catalyzes the formation of 1 μ mole of ammonia/min, under the conditions of the assay. Standards were prepared by using ammonium sulphate.

L-Asparaginase and glutaminase activities of the *ribo A*1, *bi A*1 strain were estimated in the culture filtrates *in* phosphate, citrate and Tris buffers at 5·5 through 8·0 pH values. Maximum activities were obtained in phosphate buffer at pH 7·0.

Enzyme activities were determined in the culture filtrates at a regular interval of 24 hr up to 96 hr in *cultures grown in complete media* (CM), minimal media (MM) and media containing different percentages of L-asparagine (*Fig. 1*) or glutamine (*Fig. 2*) as the sole source of nitrogen. Results showed that in general, both the enzymes follow a similar pattern. However, glutaminase activity was higher at any particular stage of the submerged growth as compared to the L-asparaginase activity.

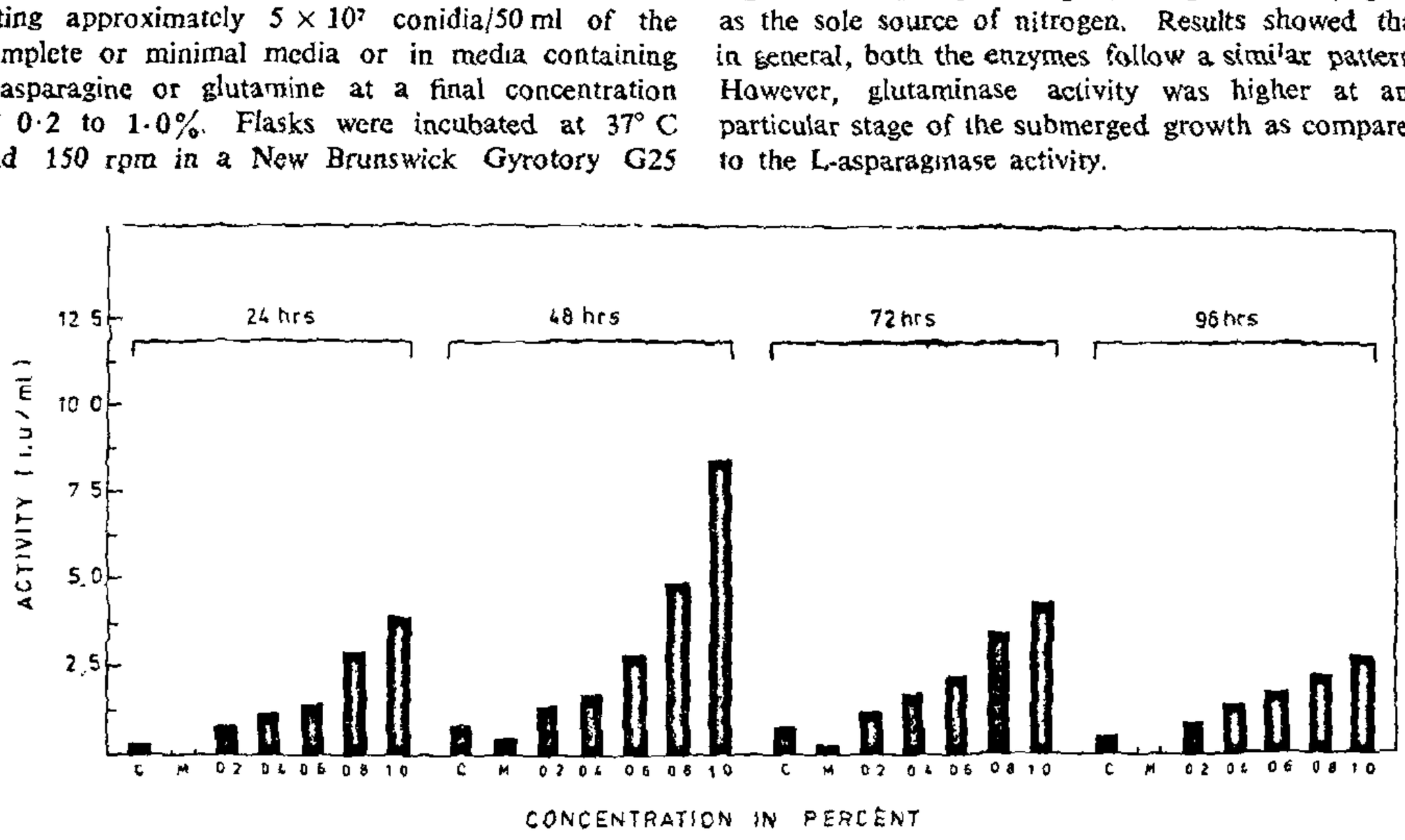

FIG. 1. L-Asparaginase activity (in international units) of the *ribo A*1, *bi A*1 strain of *A. nidulans* in media containing different concentrations of L-asparagine (used as the sole source of nitrogen) as a function *of time. C: complete media, M: minimal media.*

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