Inhibition of Catalase Activity by Isoniazid and the Effect of Vitamin B_6.* (27941)

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Knox and associates(1) reported that preformed catalase, from both bacterial and mammalian sources, was unaffected by isoniazid (INH). They concluded that the relation between INH and catalase, or some peroxidase possibly specific for mycobacteria, involves the synthesis rather than the activity of the enzyme. Subsequently, however, Maher *et al.*(2,3) found that INH in the presence of certain metal ions significantly inhibited the catalase activity of beef liver and of BCG (Bacillus of Calmette and Guérin) intact cells and sonic extracts. Winder(4) demonstrated that copper was also very effective in enhancing the action of INH on the catalase of *Mycobacterium smegamatis* as well as of BCG, but he stated that bovine liver catalase was comparatively insensitive to inhibition by INH.

The present study was undertaken to determine if INH in the presence of divalent copper is generally inhibitory of catalase activity in representative strains of mycobacteria and if this effect is antagonized by Vit. B_6. In view of the discrepancy in the results of Winder and Maher *et al.*, observations on bovine liver catalase are also reported.

Materials and methods. The mycobacteria studied were the human strains H37Rv and its avirulent and catalase negative, INH-resistant variants; the avian strain Kirchberg; bovine strain Ravenel; unclassified mycobacteria Brownell (Group I) and Cole (Group II); and the saprophytes *M. phlei* and H607. Additional strains were included in the tests on the effect of temperature on catalase activity of cell-free preparations. The organisms were grown in a chemically defined medium containing Tween 80(5).

The cells were harvested near the termination of logarithmic growth, washed in saline, then suspended in 0.1 M phosphate buffer, pH 7.0, and exposed to sonic energy for 1 hour in a 9 KC Raytheon Sonic Oscillator. The extracts were freed from intact cells and sterilized by filtration through membrane filters. They were tested for catalase immediately or stored at −20°C. Nitrogen content was determined by the method of Miller and Miller(6).

Reaction mixtures usually contained 5 ml 0.1 M H_2O_2, 5 ml buffer, and 1 ml each of 5 $\times$ 10^{-3} M INH, metal ion and Vit. B_6 form† (neutralized) or distilled water to volume in the combinations indicated in the results. Mammalian catalase, obtained as crude beef liver catalase from Nutritional Biochemicals Corp., was adjusted in concentration so that 0.5 ml would decompose 20-25% of the substrate during the reaction period in the absence of inhibitors. The sonic extracts were used in 1 or 2 ml amounts of known nitrogen content in a total volume of 14 or 15 ml. In tests involving INH, metal ion and Vit. B_6, a preincubation period of at least 1 hour at 37°C was employed during which time the enzyme was in contact with the test substances before addition of H_2O_2. Flasks containing the vitamin forms were protected from light. Unless otherwise stated, all experiments were carried out at 4°C and the reactions stopped by addition of H_2SO_4 at zero minute and at set intervals, generally 1 minute for mammalian catalase and 10 minutes for mycobacterial catalase.

The unused substrate was determined titrimetrically with 0.05 N $KMnO_4$. Since $KMnO_4$ reacts with pyridoxine, pyridoxal and pyridoxamine and to a very slight extent with INH, ample controls were included and corrections made for these effects on the titrations. They were always consistent and predictable for a given concentration at the low concentrations employed. The results are ex-

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† Pyridoxine·HCl, pyridoxal·HCl and pyridoxamine·diHCl were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio.

TABLE I. Effect of INH on Calalase Activity in Presence and Absence of Copper and Pyridoxal.

Additions to calalase	Source of Enzyme: Bovine liver	H37Rv	Ravenel	*M. phlei*	Brownell	Cole
	% inhibition					
INH	22.3	44.8	43.8	20.4	14.0	10.8
Cu^{++}	64.0	39.9	10.9	6.9	12.1	4.3
Pyridoxal	7.4	4.9	–8.0*	–9.1*	–.6*	.9
INH + Cu^{++}	88.4	95.1	94.9	70.5	69.0	77.3
INH + Cu^{++} + pyridoxal	14.9	35.0	43.8	16.0	17.1	10.9
INH + pyridoxal	27.6	25.2	35.0	11.3	6.9	10.8
Cu^{++} + pyridoxal	71.1	39.9	16.8	13.5	9.5	4.3

* Stimulation.

pressed as % inhibition and are based upon the activity of the enzyme preparation in absence of INH, metal ion and Vit. B_6 forms.

Results. The catalase activity of buffered cell suspensions of both H37Rv and *M. phlei* was increased approximately 3-fold during sonic oscillation for 1 hour, but was little affected in H607. Increasing the exposure time to 2 hours gave only slightly more active preparations.

At 4°C mycobacterial catalase was found to be almost linear in rate of activity for 10 minutes, after which the % H_2O_2 broken down per unit time gradually declined. Catalase activity increased with a rise in temperature, but at 37°C the enzyme was rapidly inactivated by the substrate. Cell-free extracts of the human strains H37Rv, H37Ra, Campbell and Erdman and the bovine strain Ravenel were negative for catalase by the permanganate titration method after 30 minutes at 55°C. The avian strains Sheard and Kirchberg were partially inactivated. The saprophyte *M. phlei* and the unclassified organisms Brownell and Coffey (Group I) and Cole (Group II) retained their activity. The optimal pH was 7.0. There was no detectable catalase in the cell-free culture supernatant solutions of the organisms or in the cellular extracts of the INH-resistant H37Rv strain.

The effect of INH on activity of mammalian catalase and mycobacterial catalase from several of the representative strains may be seen in Table I. The inhibitory action of the drug on catalase from both sources was markedly enhanced by addition of divalent copper as copper sulfate. Beef liver catalase was considerably more sensitive to the copper ions alone than was mycobacterial catalase. The inhibitory effect which INH showed, independent of exogenous metal ions, may have been due to trace metal impurities in the reagents since no effort was made to control them other than to use demineralized water. INH is probably innocuous for catalase in the absence of metal ions as has been previously reported(3,4). The less active H37Rv and Ravenel strains were more susceptible to INH alone than the other strains shown. However, the enhancement in INH inhibitory action brought about by Cu^{++} was nearly equivalent for all the strains, amounting to 50.3, 51.1, 50.1, 55.0 and 58.0% for H37Rv, Ravenel, *M. phlei,* Brownell and Cole, respectively.

Pyridoxal, as pyridoxal hydrochloride, reversed the inhibitory effect of the INH-copper complex (Table I). However, as may be seen in Table II, the other forms of the vitamin, pyridoxine and pyridoxamine, failed to neutralize the INH-Cu^{++} suppression and even contributed slightly to it although aliquots of the same H37Rv and *M. phlei* extracts were used and the tests run simultaneously with pyridoxal.

The interaction of the $-NH_2$ radical of pyridoxal with INH and copper was evident in these experiments.

At the concentrations employed in the catalase tests, INH and pyridoxal, in presence or absence of buffer, H_2O_2 and enzyme, reacted to give a bright yellow solution. Mixing INH, pyridoxal and Cu^{++}, regardless of order, resulted in formation of a flocculent brownish yellow precipitate, which dissolved and became a colorless solution when H_2SO_4 was added, as did INH-pyridoxal. Under the

TABLE II. Effect of Pyridoxine and Pyridoxamine on Inhibition of Calalase Activity by INH-Cu^{++}.

Additions to catalase	Strain H37Rv	Strain *M. phlei*
	% inhibition	
INH + Cu^{++}	95.1	70.5
INH + Cu^{++} + pyridoxine	100.0	72.7
INH + pyridoxine	39.9	22.9
Pyridoxine + Cu^{++}	15.4	11.0
Pyridoxine	4.9	1.8
INH + Cu^{++} + pyridoxamine	96.4	77.4
INH + pyridoxamine	35.0	20.4
Pyridoxamine + Cu^{++}	40.0	25.0
Pyridoxamine	9.8	6.9

same conditions, however, combinations of pyridoxine, pyridoxamine and copper with INH either underwent no grossly visible reaction or took on a very slight yellow-green tint.

In addition to copper, zinc and magnesium were also tested. These ions did not significantly inhibit catalase activity or enhance the effect of INH and did not precipitate with drug and pyridoxal.

An important incidental observation in this study was the antimicrobial activity of the bright orange precipitate which is formed as a reaction product of INH and pyridoxal if they are dissolved at concentrations of 10 mg per ml or more. When this precipitate was dried and a known weight put back into solution and diluted in culture media to the theoretical minimal inhibitory concentration for H37Rv, the INH was fully active.

Discussion. A possible explanation for the inhibitory effect of INH for mycobacterial catalase in the presence of certain metal ions has been discussed by Winder(4). Contrary to his findings, however, bovine liver catalase was also found susceptible to INH-Cu^{++}, as was shown by Maher *et al.*(3), but the enhancing effect of 2 other divalent metal ions, Mg^{++} and Zn^{++}, reported by the latter investigators, was not confirmed.

Pyridoxal hydrochloride, the form of Vit. B_6 which, as the 5-phosphate, is known to serve as a coenzyme for a number of enzymic reactions apparently blocked by INH, reversed completely the inhibition of mammalian and mycobacterial catalase (regardless of strain) by INH in combination with Cu^{++}. The antagonism appeared due to a removal of the inhibitor complex by precipitation leaving the enzyme free and active. INH may substitute for amino acids in copper chelate compounds of pyridoxal and certain α-amino acids, such as were described by Baddiley(7).

At higher concentrations of INH and pyridoxal than used in the catalase tests, an orange precipitate is formed by the interaction of the two. This reaction is reversible and does not impair the *in vitro* antituberculosis activity of INH in the least. Apparently, the ratio of pyridoxal to INH required to demonstrate reversal of *in vitro* growth inhibition is quite high and limited to a narrow drug concentration range(8). Pyridoxine and pyridoxamine, the other 2 known forms of Vit. B_6 which are believed to be interchangeable with pyridoxal in mammals, failed to interfere with the inhibition of catalase at the concentrations employed and did not react with INH-Cu^{++} to form a precipitate as did pyridoxal.

While the present study was in progress Woods(9) reported that INH, "in concentrations comparable to those reported in human serum," inhibited mycobacterial catalase activity of cell-free extracts. This investigator was mistakenly referring to *mg* concentrations as μg concentrations; 0.05 M, the lowest concentration he employed, is 6.9 mg/ml, a level not attainable in sera. At the highest INH preincubation concentration which Woods used, 1 M or 137 mg/ml, the amount of inhibition demonstrated was no greater than shown previously by Winder(4) and in the present study with a preincubation concentration of 76 μg or less per ml in the presence of copper.

Mycobacterial catalase is inactivated by H_2O_2 as are catalases from other sources. Sonic, cell-free extracts of mycobacteria showed a pattern of susceptibility to temperature similar to that reported by Kubica and Pool(10) for intact cells. Human and bovine strains were most susceptible, the avian tubercle bacillus was partially inactivated and the saprophytic and unclassified mycobacteria studied were resistant to inactivation by 55°C for 30 minutes.

Summary. The inhibition by INH of mammalian catalase and mycobacterial catalase of representative strains was markedly enhanced by divalent copper ions. Pyridoxal hydrochloride completely reversed the inhibitory action of the INH-Cu^{++} complex. This antagonism apparently was due to chemical removal of the inhibitor by precipitation. Pyridoxamine and pyridoxine, which respectively have slight and no effect *in vitro* against the antituberculosis activity of INH, did not significantly affect the INH-metal ion inhibition of mycobacterial catalase.

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Effect of Goldthioglucose Injections on Survival, Organ Damage and Obesity in the Rat.* (27942)

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Brecher and Waxler(1) reported that injection of goldthioglucose (GTG) in some mice would result in hyperphagia and obesity; they found no lesions in the brain or other organs of these animals. Marshall *et al.*(2) described a similar GTG-induced obesity in mice; on autopsy they found extensive ventromedial hypothalamic (VMH) lesions. Mayer and Marshall(3) investigated the specific nature of various gold compounds in production of VMH damage and obesity; they suggested that the glucose component of the GTG molecule accounted for the selective accumulation of gold in the hypothalamus. In the same study it was stated briefly that goldthioglucose administration did not lead to obesity in the rat. It is the purpose of this report to relate the effects of GTG injections to survival, to production of lesions (brain and other organs), and to the incidence of obesity in the rat.

Materials and methods. Male and female rats of the Long-Evans (L-E) or Sprague-Dawley (S-D) strains were used; animals ranged in size from 50 g to 400 g. Freshly prepared solutions of goldthioglucose (Solganol B)§ dissolved in 0.9% saline were injected IP in doses ranging from 0.1 to 0.75 mg/g. Some rats were fasted prior to injection.

Following injection, animals were placed in metabolic cages and given free access to food and water. If the injected dose appeared to be lethal, the rats were perfused just prior to death with 10% formolacacia in saline, their brains removed and stored in the same fixative; kidneys, pancreas, stomach and testes were also removed and stored in Bouin's fixative. Frozen sections of brain were cut at 50 μ and stained with thionin blue; other tissues were paraffin embedded, cut serially at 5-7 micra and stained with he-

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