THE METABOLISM OF ISONIAZID

IN

MYCOBACTERIUM SMEGMATIS

BY

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Les efforts que font les hommes pour découvrir la vérité sont donc une espèce de jeu de hazard, dans lequel la probabilité de tomber dans l'erreur est très grande, et celle de trouver la vérité est très petite. Ceux qui viennent plus tard sont moins sujets à erreur, parce qu'ils profitent des erreurs des autres, en trouvant diminué le nombre des cas, qui conduisent à l'erreur. De là, l'impossibilité d'avoir un ouvrage achevé, et original dans toutes ses parties de la part d'un seul homme, dans un seul siècle.

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Abstract

The biochemical mechanism of resistance to isoniazid (I.N.H.) has been investigated in an isoniazid resistant strain designated M. smegmatis 503/R. This strain was obtained by serial passage of the parent, isoniazid sensitive M. smegmatis 503 through progressively increasing concentrations of INH. The uptake of radioactive INH was compared in the two strains and it was found that the resistant strain took up much less INH than the sensitive strain.

The effect of CN, glycerol and cloromphenicol on isoniazid $-C^{14}$ uptake was examined individually and it was found that each agent decreased isoniazid uptake by sensitive and resistant cells.

Experiments were then performed where it was noted that the degree of inhibition of growth of sensitive cells by INH was dependent on the starting cell number. The latter results indicated that INH breakdown might be taking place. As a result of the previous observation, experiments were performed where the following results were obtained:

1- Both sensitive and resistant cells broke down INH to isonicotinic acid (INA) and hydrazine (NH_2-NH_2)

2- The enzyme for INH breakdown was induced in both sensitive and resistant cells in response to the introduction of INH into the buffer. This induction could be inhibited by cloromphenicol added to the buffer at zero time.

3- A second enzyme might possibly be induced in response to the hydrazine production from INH breakdown. Its presence however was never

conclusively shown as the product of the hydrazine breakdown, was never identified. Only hydrazine disappearance could be shown. Hydroxylamine and NH_3 were possibly ruled out as products of this breakdown.

4- Points one, two and three occurred in both sensitive and resistant cells but resistant cells broke down INH at a much greater rate than sensitive cells with very little or no hydrazine production.

5- Cells grown with INH also broke down INH at a much greater rate.

The effect of Ouabain, CN and Azide on the breakdown of INH by sensitive and resistant cells was examined individually. It was found that all the above agents decreased the breakdown of INH by both sensitve and resistant cells. Glycerol seemed to increase the rate of breakdown of INH by sensitive and resistant cells but little hydrazine could be detected. Experiments to test the effect of temperature on INH uptake and breakdown by sensitive and resistant cells showed that 25°C decreased both the uptake and breakdown of INH by sensitive and resistant cells while a temperature of 4°C decreased breakdown but seemed to increase uptake of INH.

Other experiments tested the effects of INH, INA, hydrazine, and hydroxylamine on the growth of sensitive and resistant cells. It was found that INA was inhibitory to neither sensitive nor resistant cells while hydrazine and hydroxylamine were inhibitory to both sensitive and resistant cells. The latter results plus the above observations on enzyme induction in response to INH suggest a possible theory of the mechanism of resistance to INH.

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The metabolism of Isoniazid in Mycobacterium Smegmatis

Introduction

Isoniazid Historical:

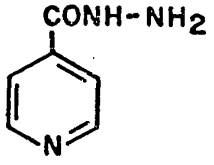
Isoniazid (INH) or iso nicotinic acid hydrazide (Fig. 1) is a potent anti-tuberculous agent whose discovery resulted from the active research of several drug firms in the early 1950's. It was developed as an intermediate to another drug called amithiazone and in this case the intermediate proved more active than the drug itself. The group responsible for this work, headed by Bernstein (16) coined the name isoniazid.

The discovery of isoniazid as an anti-tuberculosis agent proved to be a very important one and the drug became one of the major tools in the fight against tuberculosis. Thus isoniazid has been in existence for sixteen years and for that same period of time scientists have tried to deduce the mode of action of the drug. Their efforts have been unsuccessful and although a number of interesting theories have been advanced to account for the mode of action of INH, it is still under debate.

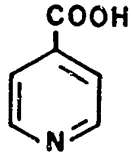
Mode of Action of Isoniazid:

What is meant by the mode of action of any drug? In reality this is a statement of where or at what point in the cell's metabolism the drug inhibits or interferes with normal processes of the cell to affect its growth or structure.

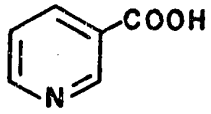
ISONIAZID AND RELATED COMPOUNDS (FIG. 1)



ISONIAZID (INH)



ISO NICOTINIC ACID (INA)



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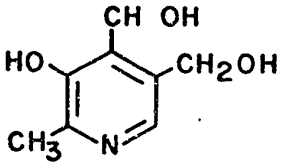
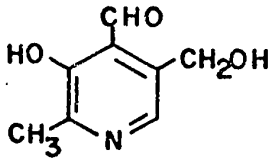
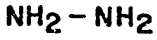


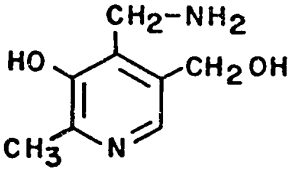
PYRIDOXINE



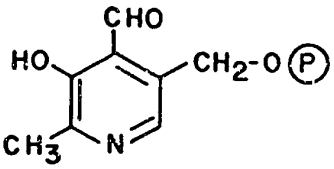
PYRIDOXAL



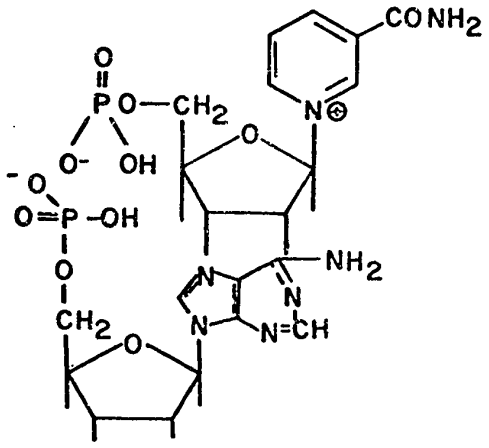
HYDRAZINE



PYRIDOXAMINE



PYRIDOXAL 5 PHOSPHATE



NICOTINE ADENINE DINUCLEOTIDE (NAD)

This interference could be at different levels in the cell such as:

1. Inhibition of isolated bacterial enzymes via competitive inhibition (by being a substrate analogue), chelation or steric hindrance.
2. Inhibition of an organized enzyme system to result in lethal synthesis.
3. Inhibition of the synthesis of structural components of the bacterial cell.
4. Inhibition of the process of transcription.
5. Inhibition of the process of translation.
6. Inhibition of the process of normal permeability of the cell to certain metabolites.

The possibilities of the mode of action of each new drug are therefore as numerous as the steps in metabolism.

Despite this, the mode of action of such drugs as penicillin (83, 84) sulfanilamide (85) and streptomycin (86) has been elucidated. This has not been the case however with INH. The mode of action of isoniazid has remained an enigma ever since its discovery and the situation has remained confused.

Isoniazid Action:

The first and most obvious fact about isoniazid is the specificity of its action. Against tubercle bacilli of human and bovine type isoniazid is effective in very low concentrations, the minimum inhibiting concentration (M.I.C.) being 0.1 ug/ml. Yet against other mycobacteria it is much less effective (M.I.C. 2-8 ug/ml) and against nearly all other species of microorganisms it has little or no inhibitory action even in concentrations as high as 100-500 ug/ml.(2)

Isoniazid is specific also in the sense that tubercle bacilli which have developed resistance to streptomycin and other anti-tuberculosis drugs still remain fully sensitive to isoniazid, while strains which have developed resistance to INH retain their sensitivity to other drugs (3). This shows that no cross-resistance occurs as it does for chemically related compounds and this fact suggests that there must be a unique metabolic step, in **tubercle bacilli only, where** INH acts.

Schaeffer in 1954 (4) found that INH is bactericidal only after a latent period, that the bacteria have to be in an active state to be killed by the drug and as the bacteria are killed by INH they lose their acid fastness. "Acid Fastness" is a property typical, to varying degrees, of the genus Mycobacteria. Thus when bacteria are stained with fuchsin in phenol the mycobacteria subsequently retain the stain against washing with acid and are therefore called acid fast in contrast to other bacteria, which lose the dye. The composition of mycobacteria is unusual, in that the cells are very rich in fats and waxes. M. tuberculosis may contain up to 40% of its dry weight in the form of ether- or acetone-soluble material (87). The property of acid fastness of mycobacteria is attributed to this high lipid content. It is thought that the phenol-dye complex has a much greater solubility in the lipids than in the acid, this being the reason why the acid does not remove the dye (88). Thus the loss of acid fastness under the influence of INH suggests that INH acts at a metabolic step associated with the elaboration of the waxy envelope around the mycobacterium (4).

INH Uptake:

Other workers have investigated what tubercle bacilli do to INH. Barclay (5) studied the uptake of radioactive INH (carbonyl C^{14}-labelled) by strains of tubercle bacilli both sensitive and resistant to it. The sensitive strain took up isoniazid whereas the resistant strain did not. He also investigated the uptake of C^{14}-labelled isonicotinic acid, nicotinamide and nicotinic acid by resistant and sensitive strains. Here it was found that very small amounts of each compound were taken up and no significant difference in uptake was found between the sensitive and resistant strains. Organisms killed by heat or formalin did not take up INH. Uptake was greater at 5°C than at 37°C.

Youatt (6) confirmed these findings using M. tuberculosis B.C.G. She also found that only small amounts of INH were bound if the cells were treated with CN or Azide or killed by heat. On the other hand, washed suspensions or isoniazid resistant strains did take up isoniazid in certain conditions. Youatt suggested that this was due to changes in permeability of the cells. She also showed that uptake increased with increasing temperature which contradicted the results of Barclay (5), and found that oxidizable substrates in the buffer depressed uptake. In a paper published in 1961 Youatt (7) found that nicotinic and benzoic acid hydrazides inhibited INH uptake and were themselves taken up by the cell but at a lower rate which was O_2 dependent.

Wimpenny (8) using B.C.G. found with C^{14}-labelled INH that only sensitive strains took up INH and that uptake was greatest at 4°C,

which contradicted the results of Youatt. In the same paper Wimpenny also stated that the activation energy of binding was of the same order as the activation energy of an enzyme catalyzed reaction.

The general conclusion seems to be that:

1. INH is taken up by sensitive tubercle bacilli.
2. INH is not taken up or very little is taken up by resistant tubercle bacilli.
3. INH related compounds such as INA, NA and Nicotinamide are not taken up or are taken up equally well by sensitive and resistant strains.
4. INH uptake is a process involving some active metabolic step although the fact that according to some reports it is more rapid at low temperatures than at high suggests that there may be some element of physical absorption or that the cell loses its permeability barrier at lower temperatures.

Thus it can be seen that an obvious theory of the mechanism of resistance to isoniazid can be postulated from the above results: changes in permeability to INH in the resistant strain.

INH and Nucleic Acids

Tsuchamura and Mizuno (9) working with M. smegmatis investigated the effect of INH on the incorporation of P^{32} into the fractions of cells obtained by the Schneider procedure (92) which extracts four different types of phosphorus compounds from tissue homogenates: acid soluble phosphorus compounds, phospholipids, nucleic acids and phosphoproteins. They found that P^{32} incorporation

into each of the four fractions was reduced under the influence of INH, but concluded that INH was inhibiting nucleic acid synthesis.

Gangadharam et al (10) in 1963 measured the relative increase in the concentrations of nucleic acids and proteins in equal volumes of cultures of B.C.G. with and without INH, over a period of 24 hours. Their results showed that in the presence of INH total nucleic acid synthesis was inhibited while protein synthesis continued. These results were based on P^{32} and S^{35} incorporation studies which showed a decreased incorporation of P^{32} into the nucleic acid fraction under the influence of INH but no effect on the S^{35} incorporation into protein. Gangadharam et al used the Schmidt - Thannhauser procedure (93) to fractionate their bacteria.

Winder (11) in 1964 found that cells of B.C.G. which had been treated with INH had higher concentrations of phosphate and carbohydrate in the acid soluble fraction and a decreased concentration of phospholipids. He observed no reduction in the nucleic acid content and in a later paper (12) characterized the carbohydrate in the acid soluble fraction as free trehalose. Winder chose to use his own fractionation procedure (94). Winder's later work (13, 14,17) showed that no matter how high the INH content was in the suspension medium there was no accumulation of carbohydrates in the isoniazid resistant strain of B.C.G. These results suggested that accumulation of the carbohydrate was related to bactericidal action of INH and from these results he postulated that INH inhibits a reaction at a central level in metabolism such as glycolysis.

Youatt (15) using a fractionation procedure designed by Harold (95) found that cultures of B.C.G. inhibited by INH showed a reduction in the acid labile phosphorus fraction without an increase in dry weight. She tentatively identified the fraction as inorganic polyphosphate. From these results she suggested that a degradative process rather than a blocked synthesis occurs under the influence of INH. Previous authors (9, 10) had used as controls growing cells which were not exposed to INH. As INH stops growth Youatt argued that it is impossible to compare the phosphate content of the INH exposed cells to the normal cells as Gangadharam (10) had done. Youatt therefore compared the phosphate content of INH exposed cells to the zero time sample cells and found that polyphosphates were reduced under the influence of INH. She suggested that this showed a degradative process instead of inhibition of polyphosphate synthesis. However her interpretation may not be entirely correct, as previous authors (4, 5) had shown that cells exposed to INH double their mass before complete inhibition of growth.

Wimpenny (8) also used the Schmidt - Thannhauser fractions procedure (93) on B.C.G. which were exposed to INH, S^{35} and P^{32} at zero time. He found that INH had no measurable effect on the incorporation of S^{35} and P^{32} into whole cells, yet the incorporation of P^{32} into the T.C.A. insoluble components was inhibited soon after the cells were exposed to INH. On the other hand, incorporation of S^{35} sulphate into T.C.A. - insoluble cytoplasmic components of B.C.G. was not affected by INH over the first 30 to 40 hours; but then ceased completely. From these results Wimpenny postulated

that INH inhibits some aspect of nucleic acid synthesis (reduced P^{32} incorporation) with the result that after a day protein synthesis ceases (reduced S^{35} incorporation).

In summary it can be easily seen that a conflicting series of results exist concerning INH action at the DNA level. As pointed out previously it is impossible to measure DNA against a control whose DNA is doubling and at the same time one cannot but wonder why Youatt did not repeat her experiment in buffer to see if there was DNA breakdown under the influence of INH.

Isoniazid Destruction:

Knox (18) and Pansy (19) observed that in the presence of molecular oxygen in the absence of bacteria and with hemin as a catalyst isoniazid is broken down to isonicotinic acid and di-iso nicotinoyl hydrazine (20,21). The speed of breakdown varies with the composition of the medium, with temperature, concentration of INH and other conditions (22,23). The study of this breakdown reaction is complicated by the fact that hemin combines with INH to form a purple hemochromogen and it may be that this hemochromogen is the real catalyst in the further breakdown of INH (24). If hemin is absent then INH in the presence of oxygen is oxidized, apparently with the liberation of hydrogen peroxide which does not appear in the presence of hemin (25). Winder in 1957 (26) found that the breakdown of INH in uninoculated media (in absence of bacteria) which is known to be catalyzed by some metal ions (27) can be greatly retarded by the addition of chelating agents such as Versene.

Winder (26) suggested that peroxidase greatly accelerates the breakdown of INH whereas catalase has little or no effect upon it and claimed that pure peroxidase did not catalyze the breakdown of INH unless phenol and other substances were present. But though the evidence is conflicting there does seem to be evidence of a peroxidase like destruction of isoniazid occuring in uninoculated media.

At the same time, in 1955, it was shown by Youmans (28) that tubercle bacilli themselves may be able to inactivate isoniazid. Youmans described a heat labile isoniazid inactivating factor present in isoniazid sensitive but not in isoniazid resistant strains (57, 58). Youatt in 1958 (29) showed that living cells of an isoniazid sensitive strain of B.C.G. could rapidly break down INH to isonicotinic acid. However Youatt found that very little INA entered the cells as most could be found in the supernatant and that there was no lag in INA production (to be referred to later). In a later paper in 1960 (30) Youatt showed that INH breakdown was a direct oxidation as it was inhibited by CN and Azide and required O_2. Further work by Youatt (31, 32) showed that besides isonicotinic acid one of the by-product of isonizid metabolism was 4-pyridine methanol. The intermediate in this reaction was pyridine aldehyde and Youatt postulated that the formation of the alcohol occured only in sensitive cells while the formation of

isonicotinic acid occurred in both the sensitive and resistant cells. Youatt did not explain what the later point had to do with the mode of action of INH and this line of work was apparently abandoned as no further papers appeared on the subject.

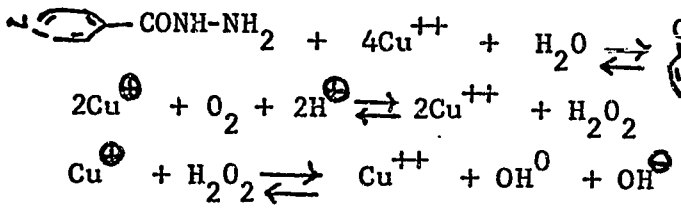
The question of the possible destruction of isoniazid by tubercle bacilli is intimately related to the difference between isoniazid sensitive and isoniazid resistant strains of tubercle bacilli. Middlebrook (33) observed that INH resistant strains were lacking in catalase whereas isoniazid sensitive strains possessed it. These observations were further extended by Tirunarayanon (34, 42, 43) who showed that isoniazid resistant mutants are both catalase and peroxidase negative while sensitive mycobacteria are both catalase and peroxidase positive.

All the above observations on isoniazid destruction led to 3 major theories on isoniazid mode of action.

1. Kruger-Thiemer (35) postulated that as an excellent example of lethal synthesis isoniazid is acted upon by sensitive tubercle bacilli and converted by peroxidase-like action to isonicitonic acid, which is then used for the synthesis of an isonicotinamide analog of coenzyme 1 (NAD) in which the nicotinamide portion is replaced by iso nicotinamide. Kruger suggests that if this occurs, then the metabolism of the tubercle bacillus is diverted from NAD-cytochrome pathway to a flavin pathway and that ultimately hydrogen peroxide is produced which now cannot be destroyed by peroxidase or a catalase-like enzyme and is lethal. There is no good evidence for this hypothesis

and experiments with M. smegmatis that challenge this theory will be discussed later.

2. Another possible mode of action of INH was proposed by Winder in 1960 (36). Winder proposed that isoniazid works through the formation of free radicals (OH^0) from its metal catalysed autoxidation. This process occurred as follows:



This hypothesis was shown to be false and will be referred to later.

3. Wimpenny (8) proposed that INH uptake is an active process, i.e. is enzymatic where the uptake enzyme is the peroxidase mentioned in the literature (34). This enzyme according to Wimpenny does not exist in the resistant strain and it converts INH to an unnamed product A which is not toxic and is released into the cell. In the cell a second enzyme converts A to a product B which interferes with nucleic acid synthesis. This hypothesis will also be discussed later as a possible sequence of isoniazid destruction.

Isolated Enzyme Systems and INH

Little is known about the precise enzymes which are inhibited by the majority of antibacterial drugs whereas with some known enzyme inhibitors the point at which the drug interferes with the enzyme is known. A good example is iodoacetic acid which is a specific inhibitor which reacts with SH groups of sulfhydryl group-containing

enzymes. Yet the inhibition of a specific enzyme by an inhibitor is not necessarily relevant to the action of that inhibitor on intact cells. Isoniazid shows this clearly.

INH is an inhibitor of tryptophanase in E. coli extracts yet whole cells of E. coli are quite resistant to INH (37,38). Other workers have also shown that isoniazid inhibits diamine oxidase and decarboxylase (39,40,44,48,49,59) and Youatt (41) has shown that isoniazid inhibits a transaminase of M. tuberculosis. As a consequence of the latter results the author postulated that the mode of action of isoniazid was that of a competitive inhibitor of enzymes which require pyridoxal phosphate or Vit.B_6 as a co-factor. This is possible as isoniazid and Vit.B_6 have very similar structures (Fig. 1).

This theory is however rather limited as it is difficult to extrapolate work done on cell free extracts to whole cells. This is because whole cells present a permeability barrier to the drug and the drug before entering the cytoplasm can be metabolically changed. Youatt (47) showed that transaminase extracts of sensitive and resistant B.C.G. are equally inhibited by INH.

The first indication that pyridoxal phosphate might antagonize the action of INH was found with tuberculosis patients. These patients were treated with INH and as a result of the INH treatment the patients developed neuropathies i.e. degenerative changes in peripheral nerves. Under pyridoxal phosphate treatment the neuropathies were reversed (45, 46, 47). Other studies showed that growth

inhibition of M. tuberculosis by INH can be prevented by a large excess of pyridoxal in the culture media (50,51,52). On the other hand Youatt (6) had shown that pyridoxal stimulates the uptake of radioactive INH by B.C.G. in buffer. The latter results were challenged by Beggs in 1967(53) who showed a 50% decrease in radioactive INH entry into B.C.G. in the presence of Vit.B_6. Beggs thus postulated that exogenously supplied pyridoxal phosphate protects the organism at an extracellular site rather than at an intracellular level by preventing the INH from entering the cell.

Hemin has the most striking antagonion for INH yet shown. Fisher (54,55) showed that; hemin antagonizes the action of INH against sensitive tubercle bacilli and that INH resistant strains require hemin as a growth factor in certain media. Fisher suggested that hemin was required for the synthesis of porphyrin-containing enzymes. Later, however, it became clear that the apparent requirement of resistant strains for hemin was not a true nutritional requirement but that the hemin was acting by destroying peroxides in the media as INH resistant mutants are peroxidase negative. The antagonistic action of hemin on sensitive strains was explained later (20,21) by showing that the hemin acted as a catalyst in the breakdown of INH. Thus hemin is no longer considered a true antagonist.

A notable step in elucidating the mode of action of INH was taken in 1961. Toida (56) discovered that acetone-dried cell free extracts of avian mycobacteria could break down isoniazid to hydrazine (NH_2-NH_2). He also found that unlike the enzyme that breaks down INH described by Youmans this enzyme was present in greater concentration in resistant mycobacteria.

As a summary it can be seen that rarely can results obtained in cell free extracts be applied to whole cell systems. One reason for this is that cells can chemically change the drug when it enters the cell or the cells might be impermeable to the drug altogether.

Isoniazid and NADase.

Zatman (60) discovered in 1954 that isoniazid inhibits the activity of mammalian NADase. This enzyme is able to bring about an exchange between INH and nicotinamide moiety of NAD and to form an analogue of NAD which has no effect on NAD-dependent dehydrogenase reactions. This paper was followed by that of Gopinathan (61,62) who showed that some representatives of genus mycobacteria had the NADase, that this enzyme did not form NAD analogues with INH and that there was no inhibition by INH of the purified enzyme. Bekierkunst (63) showed that tubercle bacilli incubated with INH showed a clear-cut decrease in their NAD content (this fact will be referred to later). He also showed that this decrease in NAD was not due to the formation of an isoniazid analogue of NAD and showed that there was increased NADase activity in presence of INH, i.e. the enzyme whose activity is nil or very small becomes active after prior incubation with INH. From the above results and the fact that INH has no effect on the purified bacterial enzyme (61,62) Bekierkunst postulated that the observed increase in the activity of the enzyme is due to an inactivation of an endogenous inhibitor of the enzyme by INH.

Thus Bekierkunst postulated that INH somehow reacts with the endogenous inhibitor of NADase to result in greater activation of

the enzyme which in turn results in a decrease in NAD content in the cell.

Further papers in 1967 by Bekierkunst (64), (65) showed that:

1. There is a clear cut decrease in NAD content of cells incubated with INH, the rate of decrease being dependent upon the concentration and time of exposure to the drug.
2. The more sensitive the mycobacterial strain was to INH the less of the drug was needed to cause approximately a 50% depletion in NAD content.

As a result Bekierkunst concluded that the depletion of NAD under the influence of INH via the activation of NADase is the primary mode of action of INH in tubercle bacilli which later results in all the other observable changes such as a decrease in nucleic acids.

Lipids and INH:

Barclay (5) found that under the influence of INH M. tuberculosis organisms lost their acid fastness. As mentioned previously acid fastness is correlated with the high lipid content of M. tuberculosis and it was therefore postulated that INH somehow inhibited normal lipid metabolism.

In 1955 Russe (66) demonstrated that tubercle bacilli exposed to INH in vitro show a decrease in the amount of lipids which may be extracted with organic solvents. This observation showed that somehow lipid metabolism is indeed affected by INH.

Schaeffer (69) showed that INH causes a marked stimulation of the oxido-reduction reactions of tubercle bacilli. As stimulation of the oxido-reduction reactions, increased metabolic rate, increased cell

permeability and increased osmotic fragility are characteristic symptoms of fatty acid deficiency in animals (70), Schaeffer postulated that fatty acid metabolism was affected by INH in tubercle bacilli.

Winder in 1964 (72) working with M. tuberculosis showed that under the influence of INH soluble compounds containing sugar and phosphate accumulate in the cell. At the same time he observed a decrease in the phospholipids of the organism and therefore postulated that INH inhibits the incorporation of sugar and phosphate compounds into phospholipids.

A later paper by Winder (73) showed that cell-free extracts of M. tuberculosis are not affected by INH in the synthesis of fatty acids from acetate.

Another paper by Winder in 1965 (74) indicated that the decreased lipid content of M. tuberculosis under the influence of INH was due to decreased lipid synthesis. In this paper Winder showed that there was decreased incorporation of C^{14} glycerol into lipid and also showed that the lipid fraction whose synthesis was inhibited by INH had a high glycerol content. As a result Winder postulated that INH stimulates one of the reactions in the conversion of glycerol to hexose phosphate with a resulting reduction in the concentration of intracellular glycerol available for lipid synthesis.

It is known that acetate is incorporated into long chain fatty acids by M. tuberculosis with hexa-cosanoic acid (26) being the main product (71). Thus somehow acetate is involved in lipid synthesis in M. tuberculosis. INH in turn somehow influences acetate metabolism as shown by Meadows with B.C.G. (67,68) who showed that INH causes a

30% inhibition of oxidation of acetate.

As a summary it can be said that lipids are definitely affected in M. tuberculosis on exposure to INH. In what way this comes about, whether through inhibition of glycerol metabolism, or acetate metabolism is still an open question. However the fact remains that INH decreases the lipid content of cells exposed to the drug.

Proteins and INH

The first report that INH affects protein synthesis appeared in 1964. Tsukamuro (75) found that INH inhibits the incorporation of α-ketogentarate-$5C^{14}$ and DL-glutamic acid $7C^{14}$ into the protein fraction of M. jucho. From these results Tsukamuro postulated that INH inhibits protein synthesis in mycobacteria at the step of amino acid incorporation into protein.

A later paper by Tsukamuro (76) showed that INH inhibits the incorporation of S^{35} into proteins of M. jucho.

However other authors found different results. Wimpenny (8) had shown that the incorporation of S^{35} sulphate into TCS-insoluble cytoplasmic components of B.C.G. was not affected over the first 30-40 hour period, then stopped completely. From these results (and results discussed previously in the section of INH and Nucleic Acids) Wimpenny postulated that protein synthesis stopped as a result of inhibition of nucleic acid synthesis by INH. Gangadharam et al (10) working with M. tuberculosis showed via chemical estimation of protein and nucleic acids that protein synthesis continued for 24 hours despite the presence of INH while accumulation of DNA and RNA was inhibited. Thus it can be seen that Gangadharam and Wimpenny obtained

essentially the same results but both disagreed with Tsukamuro as to the time when protein synthesis stopped under the influence of INH. The difference in results may be due to the fact that two different bacterial species were used.

Genetics and Isoniazid

As soon as INH came into general use against tuberculosis it was realized that in some cases it had very little effect. It was soon discovered that this was not because the mycobacteria were resistant to the drug but because the human hosts inactivated the drug (89). Soon it was discovered that the inactivation of INH in humans was a genetic trait and that there was not only individual differences but also racial difference concerning INH metabolism (90). Sunahara (97) showed that three phenotypes existed within the population - rapid inactivators, intermediate inactivators and slow inactivators controlled by two allelic genes ("slow" allele and "rapid" allele). The same paper showed that isoniazid is inactivated mainly by acetylation in intermediate inactivators but the biochemical process involved in "slow" allele and "rapid" allele inactivation remains unexplained.

Thus INH is biochemically changed in humans as well as bacteria.

The Mode of Action of INH - A Summary

A number of contradictory theories have been postulated for INH action and the mode of action of INH is therefore under vigorous debate.

The difficulty with INH is the fact that the authors cannot even agree as to the locus of INH attack. The locus of attack by the drug has to be known before the mode of action of the drug is discovered.

To take an example of a known locus cloromphenicol inhibits the protein fraction of E. coli (101). Thus its locus of attack is proteins, later it was discovered that it inhibits protein synthesis (102) and now in what manner it inhibits synthesis is under debate. No such general locus, however, can be assigned to INH which has been attributed with inhibiting the synthesis of proteins, lipids and nucleic acids. The theory proposed for INH action would somehow have to incorporate these three widely differing loci of INH inhibition. As can be imagined this is very difficult to do.

Other ideas of INH action come from the studies of the resistant mutants to INH. The resistant mutant seems to have different properties from the sensitive. The former is impermeable or is partially impermeable to INH and is catalase and peroxidase negative. The latter observation suggests that the sensitive strain destroys itself by acting metabolically on INH via its peroxidase system to create a toxic metabolite.

The nature of this metabolite is also under vigorous debate. Thus Winder believes that it is a free radical (36) while Kruger (35) believes it is INA, which is incorporated into NAD as a lethal synthesis.

The reconciliation of these results is difficult but not impossible if one remembers that these authors used different mycobacteria namely B.C.G., tubercle bacillus, and saphrophytic mycobacteria like M. smegmatis. Sometimes inhibition studies were performed on growing cells and at other times were performed on cells suspended in buffer. These reasons may account for the conflicting results.

In the following work efforts were made to perform the experiment in a well defined system. Thus the experiment were rarely performed with growing cells but incubations were usually performed on cells harvested in an active state of growth and suspended in buffer. The general approach was to see in what way INH influenced these cells and efforts were made to determine how INH is changed by sensitive and resistant strains.

MATERIALS AND METHODS

The Organism

Mycobacterium Smegmatis 503 was obtained from The National Research Council Stock Culture Collection. M. smegmatis is a saphrophytic strain of mycobacteria which under the microscope appears as a slender rod of varying lengths with deep staining ovoid bodies. The strain was acid fast after incubation for five days on glycerol agar. Stock cultures of this organism were maintained on Sauton Agar and subcultured bi-weekly.

Culture Media

A number of liquid synthetic media were tried to see which media provided the best growth within the shortest period of time. The two best media were those of Sauton and Albridge and it was decided to use Sauton (77) media as it was the simplest to prepare. It is prepared as follows:

Asparagine	4 gms
$MgSO_4$	.5 gms
K_2HPO_4	.5 gms
Citric Acid	2 gms
Glycerol	40 gms
Ferric Ammonium Citrate	.05 gms

Adjust pH to 7.0 via NH_4OH. Add CaSein Hydrolyzate 3 gms

Tween 80 .5 gms

+ 950 ml. H_2O

Stir

Culture Conditions

Unless otherwise specified bacteria were grown at 37°C with aeration in screw-cap Erlenmeyer flasks containing 1/5 volume of growth medium and fitted with 18 mm. internal diameter side arms for turbidimetric measurements. Cultures were shaken vigorously either on an Eberbach reciprocating shaker at 106 strokes / minute or on a New Brunswick gyratory shaker at 115 revolutions per minute. Bacterial growth was always measured at 660 mu in a Coleman Junior Spectrophotometer.

Buffers

A number of incubation experiments were also performed. In these experiments the cells were incubated in phosphate buffer (M/100) at pH 7.02. The buffer was made up by mixing M/100 KH_2PO_4 and K_2HPO_4 in the ratio of 3 to 7.

Reagents and Chemicals

Isoniazid was purchased from Fisher Scientific Company. Solutions of isoniazid were not stored in any special containers but were refrigerated. As it was known that isoniazid undergoes a little spontaneous degeneration these solutions were changed approximately every month.

Radioactive isoniazid Carbonyl C^{14} was purchased from the Radiochemical Centre, Amersham England. The specific activity of the sample was 70 uc/mg and the sample had the activity of .1 mc or 100 u curies/vial.

The liquid scintillation mixture used in the scintillation counter was that of G.A. Bray (78) which was quite successful and had the following formula:

Naphthalene	60 g
PPO	4 g
POPOP	.2 g
Methanol (Absolute)	100 ml
Ethylene Glycol	20 ml
p-dioxane up to 1 liter	

All other reagents and chemicals used in the study were either "Analar" grade (British Drug Houses) or Fisher Certified (Fisher Scientific Co.).

Analytical Methods

Free INH was estimated according to the method of Eidus (79) developed in 1962 to estimate the amount of free INH in the serum of patients suffering from tuberculosis to whom INH was administered. The INH was extracted from serum with n-butanol-chloroform mixture and re-extracted from organic solvent with dilute HCl. The hydrazine was then reacted with alcoholic heliothropin to form a hydrazone, the latter compound absorbing at 365 mu. For our purpose this procedure was adapted to buffer which was acidified before the addition of heliothropin.

Nicotinic acid and isonitocinic acid were measured according to the method of Rubin (80). This method was developed to measure INH in blood and urine by hydrolyzing it to INA. The method was modified for use in buffer and a procedure was worked out where the addition of ammonia and 10% cyanogen bromide gave a colour absorbing at 430 mu.

The nicotinic acid was measured via a reaction of metol (114) and 3% cyanogen bromide absorbing maximaly at 410 mu.

Ammonia was measured according to the method of Modzac (87). This method is enzymatic and was used because of its great specificity. It involves the use of glutamic dehydrogenase in the following stoichiometric reaction:

$$\alpha \text{ ketoglutaric acid} + H^+ + NADH + NH_3 \rightleftharpoons \text{L-glutamic acid} + NAD + H_2O$$

of which the equilibrium is far to the right.

Thus the decline in Optical Density at 340 mu gives an indication of how much NH_3 is present in the solution. This method had the inherent difficulty that the activity of the enzyme changed with age.

Hydrazine was measured according to the method of Toida (86). In this method hydrazine was reacted with modified Ehrlich's reagent and the resulting yellow colour was measured at 458 mu.

Hydroxylamine was measured according to the method of Osajima (96) where 1% alcoholic-8 hydroxyl-quinoline and 2N Na_2CO_3 are added to the solution and the resulting colour is read at 700 mu.

All spectrophotometric measurements were done on a Beckman DU Quartz Spectrophotometer.

Sterility Precautions

All growth media and glassware used in the growth experiments were sterilized by autoclaving, and samples were withdrawn using sterile pipettes. All innoculations were done using accepted sterile procedures. Before each experiment the cells were checked under the phase contrast micros-

cope for any contaminants which might be present. If any doubt existed as to the nature of the organism an acid-fast staining procedure was carried out.

Radioactive Counting of INH Uptake of Sensitive M. Smegmatis

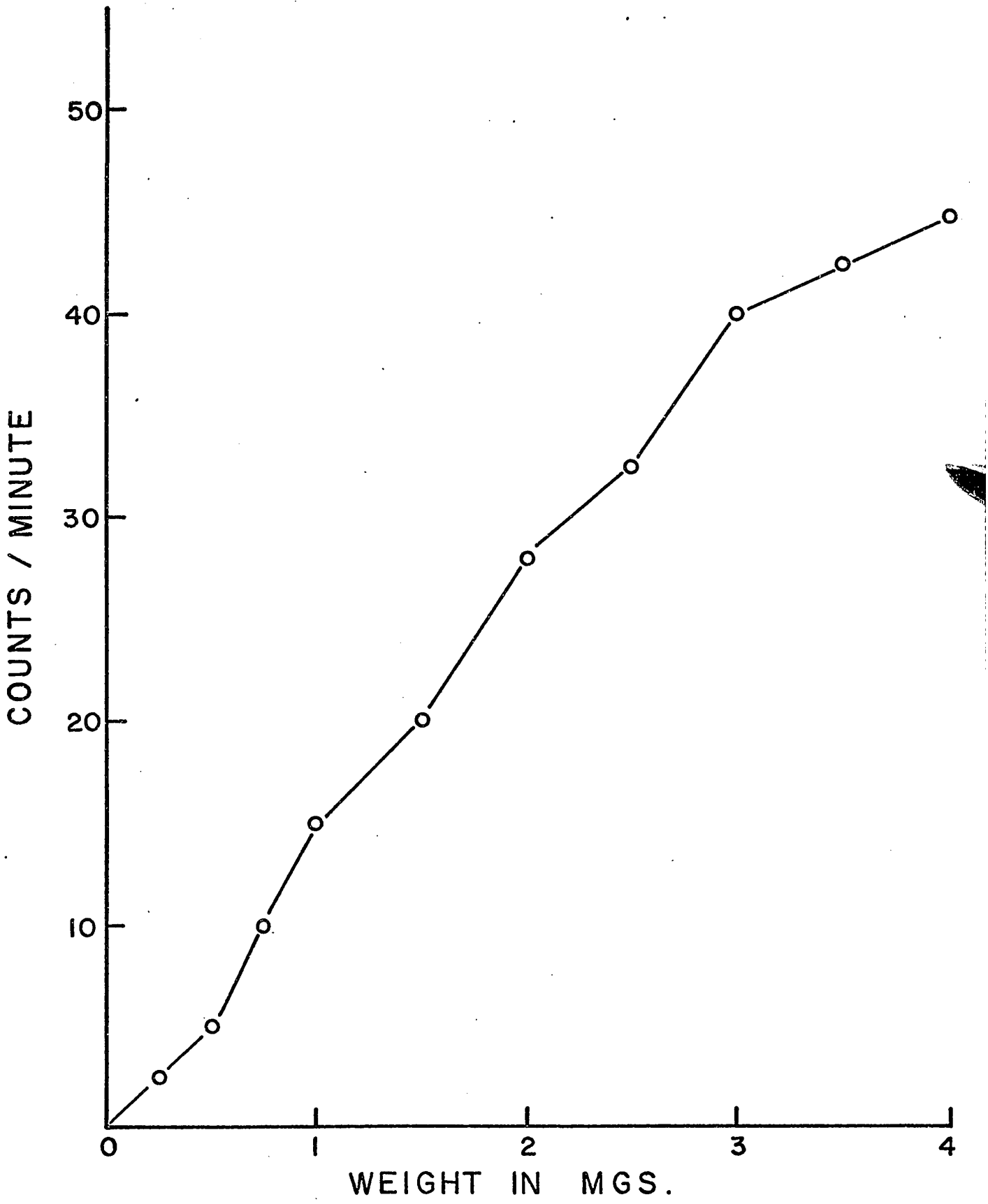
The INH-C^{14} (100 u curies) was dissolved in 10 ml distilled H_2O giving a concentration of 10u curies/ml. Cells were usually grown up into the active phase of growth, washed twice in buffer and suspended in buffer to an optical density of .90 (2.75 mgs cells/ml.). Isoniazid C^{14} was then added to a concentration of .04 u curies/ml. Samples of the cells were then taken at various times, washed twice in cold INH by centrifugation and placed in scintillation vials where they were dried in vacuo and scintillation mixture was then added (25 ml). The cells were then counted in a Nuclear Chicago Scintillation Counter Model 8 401.

In order to determine the error due to self-adsorption cells were incubated with radioactive INH (.04u curies/ml) for 24 hours. Then various concentrations or weights of the cells were placed in scintillation vials, scintillation mixture was added (25 ml) and the mixture was counted. The resultant curve showed that no appreciable self-absorption resulted until the weight of the cells in the scintillation vial reached 3mgs (Fig. 2). Therefore in all future experiments less than 3mgs of cells were placed in each scintillation vial.

Another problem encountered was that the cells would not remain suspended in the scintillation mixture. Efforts were made to solve this problem by dissolving the cells in hymine (p-diisobutyl-

FIG. 2

THE SELF-ABSORPTION
OF
RADIOACTIVITY
BY CELLS IN THE
LIQUID SCINTILLATION MIXTURE


50
40
30
20
10
COUNTS / MINUTE
0
1
2
3
4
WEIGHT IN MGS.

cresoxethoxyethyl dimethylbenzyl-ammonium chloride) obtained from Nuclear Chicago, which is a very strong base that dissolves lipid and protein. However with its use on Mycobacterium smegmatis a darkly coloured solution resulted which gave rise to colour quenching. No successful method could be found for getting the bacteria into solution. An experiment was then performed where the change in the count of the cells was measured as the cells sedimented out with time. It was found that no appreciable change in the count appeared until half an hour after shaking the vials (Table 1). A procedure was then adopted in which the vials were shaken before being counted and the counting time was set for ten minutes on the counter. Standard corrections were made for background.

Gas Flow Counting

As many problems were encountered with liquid scintillation it was decided to try Gas Flow Counting. A procedure was adopted where samples were taken from the incubation buffer and filtered onto a metricel filter which had been shown in separate experiment not to absorb any INH. The filtered bacteria were then washed 3 times in non-radioactive INH and the filter paper was removed and pasted on stainless steel planchets which were then dried at 110°C and then counted in a Nuclear Chicago Gas Flow Counter, Model C-110A.

It was found that no corrections had to be made for the self-absorption of the sample if the sample was below 3 mgs in weight. This figure was arrived at by exposing the cells for 24 hours to radioactive INH then, filtering certain weights of the cells and

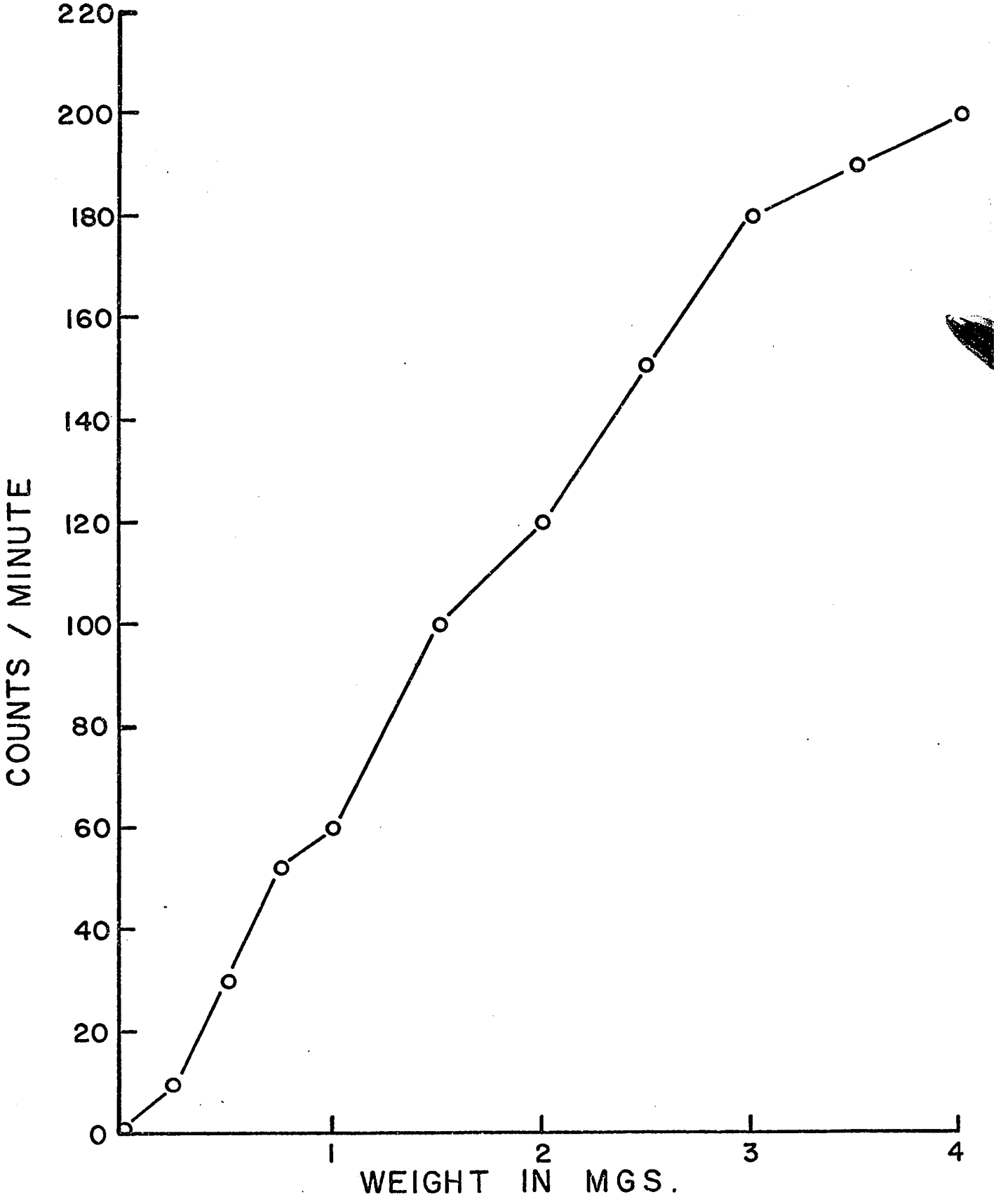
Table I

Changes in the count rate of sensitive cells (3mgs) suspended in liquid scintillation mixture with time.

Approximate time after suspension and shaking	Counts/min.
1 minute	35
5 "	33
10 "	36
15 "	35
20 "	32
25 "	33
30 "	31
35 "	28
40 "	25

FIG. 3

THE SELF-ABSORPTION
OF
RADIOACTIVITY
BY CELLS COUNTED
VIA GAS FLOW


220
200
180
160
140
120
100
80
60
40
20
0
COUNTS / MINUTE
1
2
3
4
WEIGHT IN MGS.

counting these. Weight vs counts was then plotted and where the curve levelled off was the point of where self-absorption began to take place. This point was 3 mgs (Fig. 3).

In the series of experiments carried out with gas flow counting a high concentration of isotope was used in the incubation media (.42uc/ml of C^{14} INH which is approximately 10x that used in the scintillation experiments).

Viable Counts

The viable count was determined by plating on Sauton media with agar and incubating for twenty-four hours at 37°C. The plates were dried overnight at 37°C prior to use. The spot plate technique for viable-cell counts was used as developed by Gandy (82).

Isolation of INH Resistant Strain

The resistant strain, which has been designated as M. smegmatis 503 R was obtained by serial passage through increasingly higher concentrations of INH. Namely 5ug/ml, 10ug/ml, 50ug/ml to 100ug/ml. An isoniazid concentration of 5 ug/ml inhibited the growth of the sensitive or parent strain (Fig. 6). Upon prolonged incubation, however, some cells did grow after a long lag in the presence of 5 ug/ml INH. These cells were exposed to progressively increasing concentrations of INH as above.

Resistance was characterized by the fact that the resistant cells showed abundant growth in the presence of a concentration of INH which completely suppressed the growth of the parent sensitive strain. The stability of the resistant mutant was tested by sub-culturing the

resistant strain repeatedly on Sauton agar without isoniazid then subculturing the strain to agar with INH (100 ug/ml). The ability of the resistant mutant to grow on agar with INH was not impaired even after growth on agar without INH. The resistant strain was then maintained and subcultured bi-weekly on agar containing 100/ug/ml INH and had all the morphological characteristics of the sensitive strain but grew slower. When resistant cells were required for experiments they were grown up, as were sensitive cells with no INH in the media.

Preparation of a Standard Curve for the Measurement of Mycobacterium Smegmatis Concentration.

It was necessary for later experiments to measure the amount of bacteria present in suspensions. A standard curve for turbidometric measurements was constructed in the following manner:

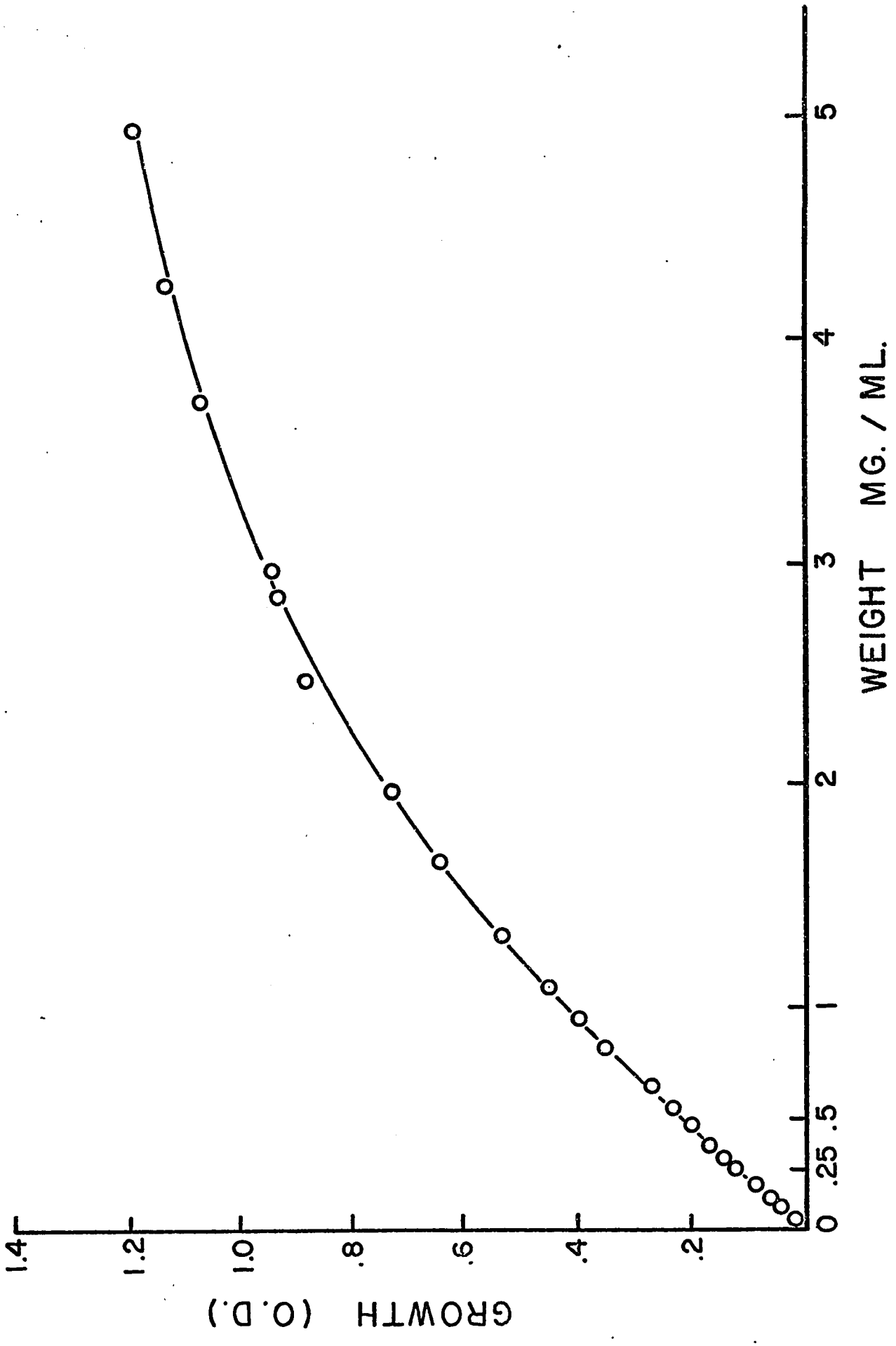
Cells were grown up to an optical density of .49 which is in the active state of growth. They were then centrifuged down, washed three times in buffer and suspended in buffer to an optical density of 1.39. Six one ml. samples of the suspension were placed in weighted vials and dried in vacuo. The 1.39 O.D. suspension was then diluted in series and the resulting O.D. was recorded. A standard curve was then plotted of O.D. vs weight of cells (mg/ml) (Fig.4).

Preparation of the Standard Growth Curve for Mycobacterium Smegmatis 503.

It was found desirable for future experiments to determine approximately how long it would take the culture to reach the active state of growth if the culture was innoculated with a certain concentration of cells. An experiment was performed in which media was innoculated from a preculture in active state of growth with four concentrations of cells,

FIG. 4

DETERMINATION OF WEIGHT
OF CELLS/ML
OF BUFFER FROM THE OPTICAL DENSITY
OF
THE SOLUTION


GROWTH (O.D.)
1.4
1.2
1.0
.8
.6
.4
.2
0
.25
.5
1
2
3
4
5
WEIGHT MG. / ML.

(.002mg/ml, .005mg/ml, .01mg/ml, and .025mg/ml). The growth of each of these cultures was followed (Fig. 5).

Whole Cell Incubation Experiments.

To study INH breakdown, cells from the active growth phase were washed twice in buffer and suspended in buffer to an optical density of .9 or 2.15 mg/ml. INH was then added to the incubation media at zero time and at certain intervals, samples were removed, spun down at 6000g for 10 minutes and the cells discarded. The supernatant was then tested for INH, INA, NH_2-NH_2, NH_3, and NH_2OH. In some experiments the effect of agents such as glycerol, on INH breakdown were tested. Controls were set up in each experiment to determine if the production of each metabolite is spontaneous or under cellular influence.

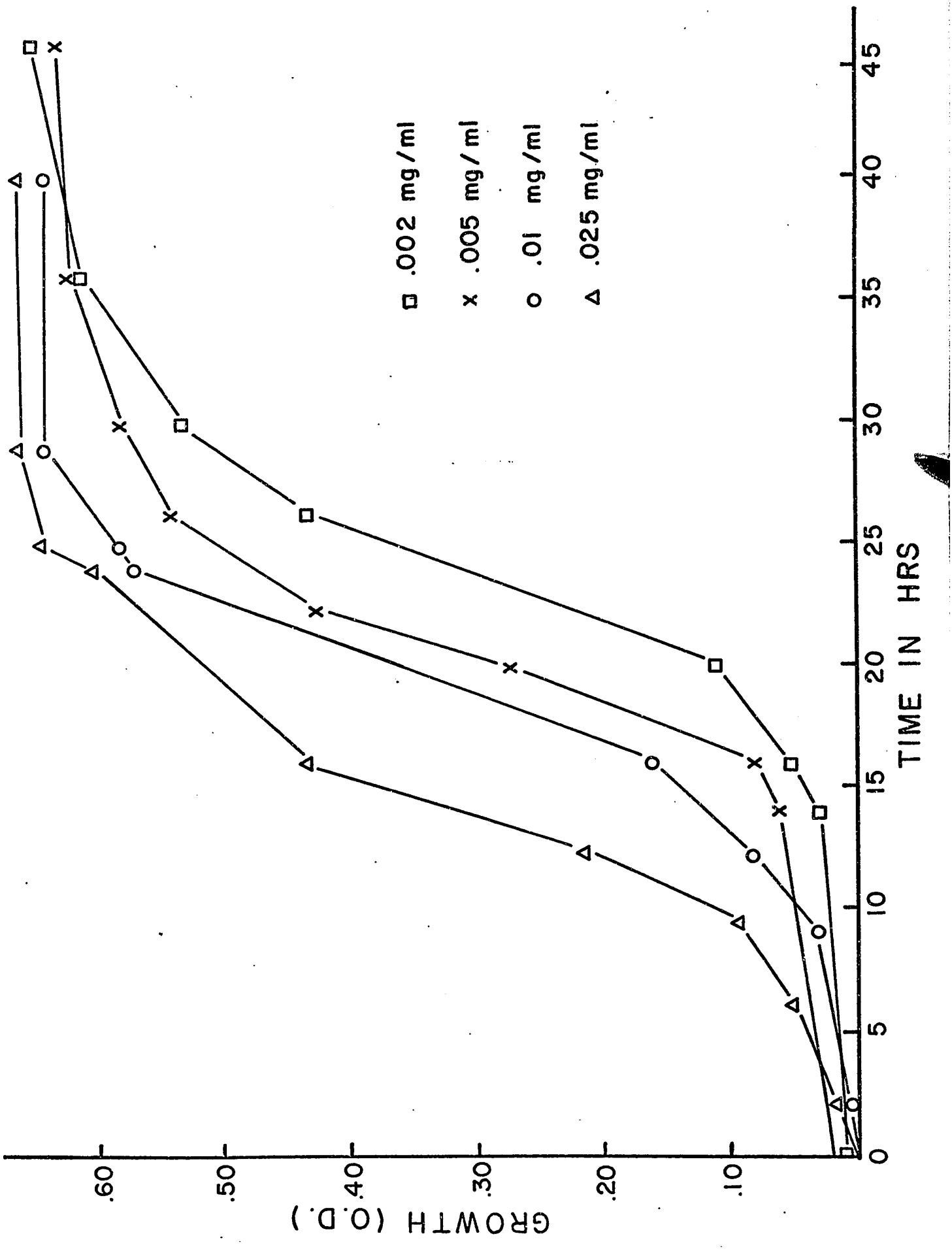
These controls involved the introduction of INH into a flask with buffer only and no cells. The control was then tested at certain time intervals for spontaneous non-cellular production of the above possible breakdown products of INH.

Another control involved the measurement of all the above agents (INH, INA, NH_2-NH_2, NH_3 and NH_2OH) in the supernatant solution of cells suspended without INH. None of these agents could be detected over a twenty-four hour period.

Two sets of experiments were performed. In one set 40ug/ml INH or 0.292u moles/ml was introduced into the culture media at zero time and in the second set 100ug/ml INH or 0.730u moles/ml was introduced. The cells were always incubated at 37°C on the gyratory shaker.

FIG. 5

THE GROWTH CURVES OF
M. SMEGMATIS 503 INOCULATED
WITH VARIOUS CONCENTRATIONS OF CELLS
FROM A PRE-CULTURE (.490D)


□ .002 mg/ml
x .005 mg/ml
o .01 mg/ml
Δ .025 mg/ml
GROWTH (O.D.)
.10
.20
.30
.40
.50
.60
TIME IN HRS
0
5
10
15
20
25
30
35
40
45

EXPERIMENTAL RESULTS

The Change in the Acid Fastness of M. smegmatis 503 With Age.

Various authors (4,97) had observed that under the influence of INH, tubercle bacilli lose their acid fastness within a short period of time. The same experiment was attempted with sensitive M. smegmatis. It was found that the organism was not acid-fast through all its phases of growth. It was only about 70% acid-fast in the active growth phase, this being the phase in which INH was added. In the stationary phase the cells were 100% acid-fast. When INH was added (10 ug/ml) the proportion of acid-fast cells decreased to approximately 20% after a ten-hour interval under INH influence.

Effect of INH on the Growth of the Sensitive and Resistant Strains.

The effect of progressively increasing concentrations of INH on the growth of the sensitive strain is shown in Fig. 6. Complete cessation of growth is observed at 10 ug/ml but only partial cessation of growth at 5 ug/ml where the cells approximately double their optical density.

The resistant strain is completely impervious to the action of INH (Fig. 7). Here growth comparable to that of the control takes place even at 100 ug/ml.

The Effect of INH on the Viable Cell Count of M. smegmatis 503.

As growth still resulted at 5ug/ml INH (Fig. 6) it was decided to measure the viable count of cells at different concentrations of

FIG. 6

THE INHIBITION OF GROWTH

OF

SENSITIVE CELLS BY INH

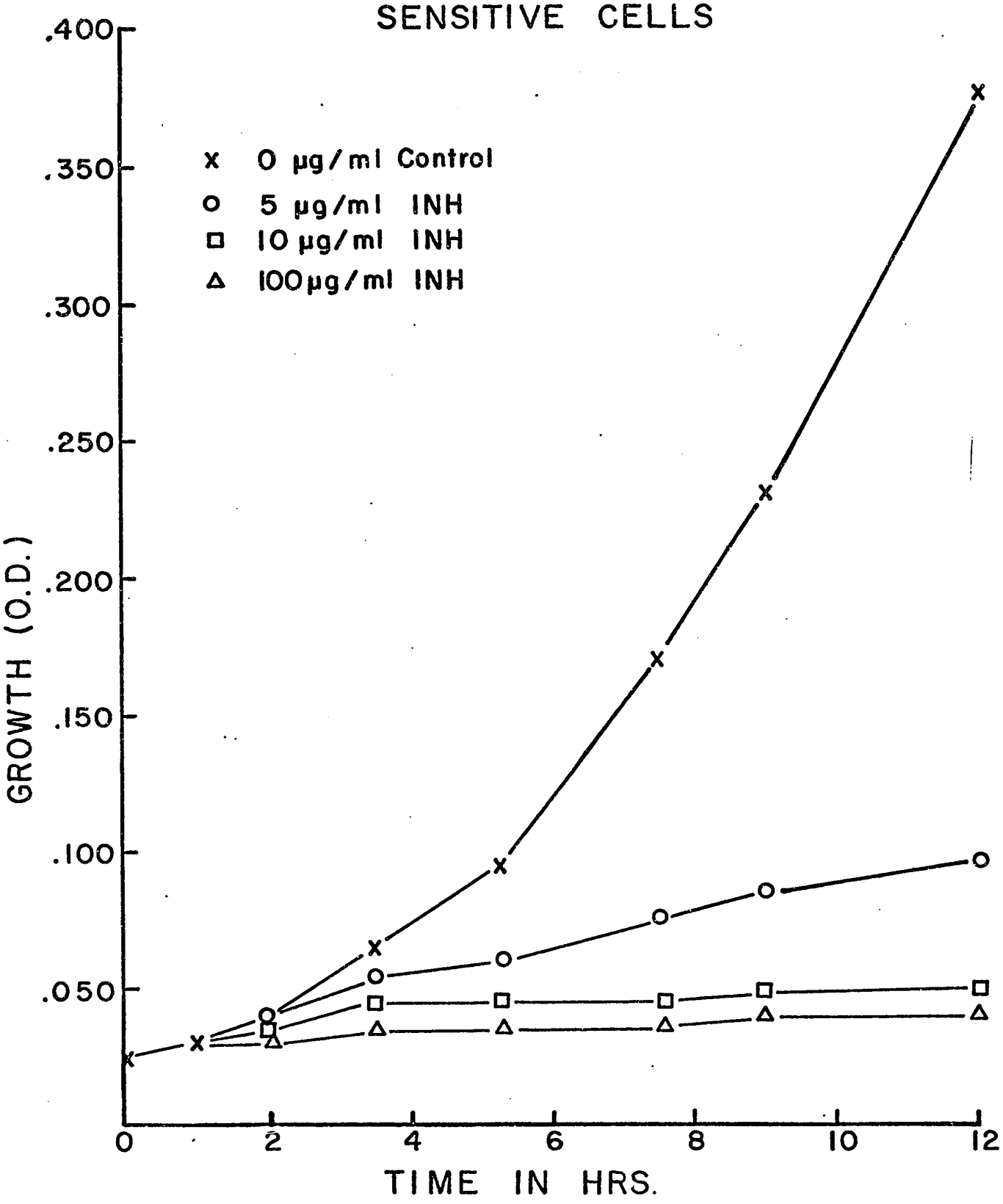
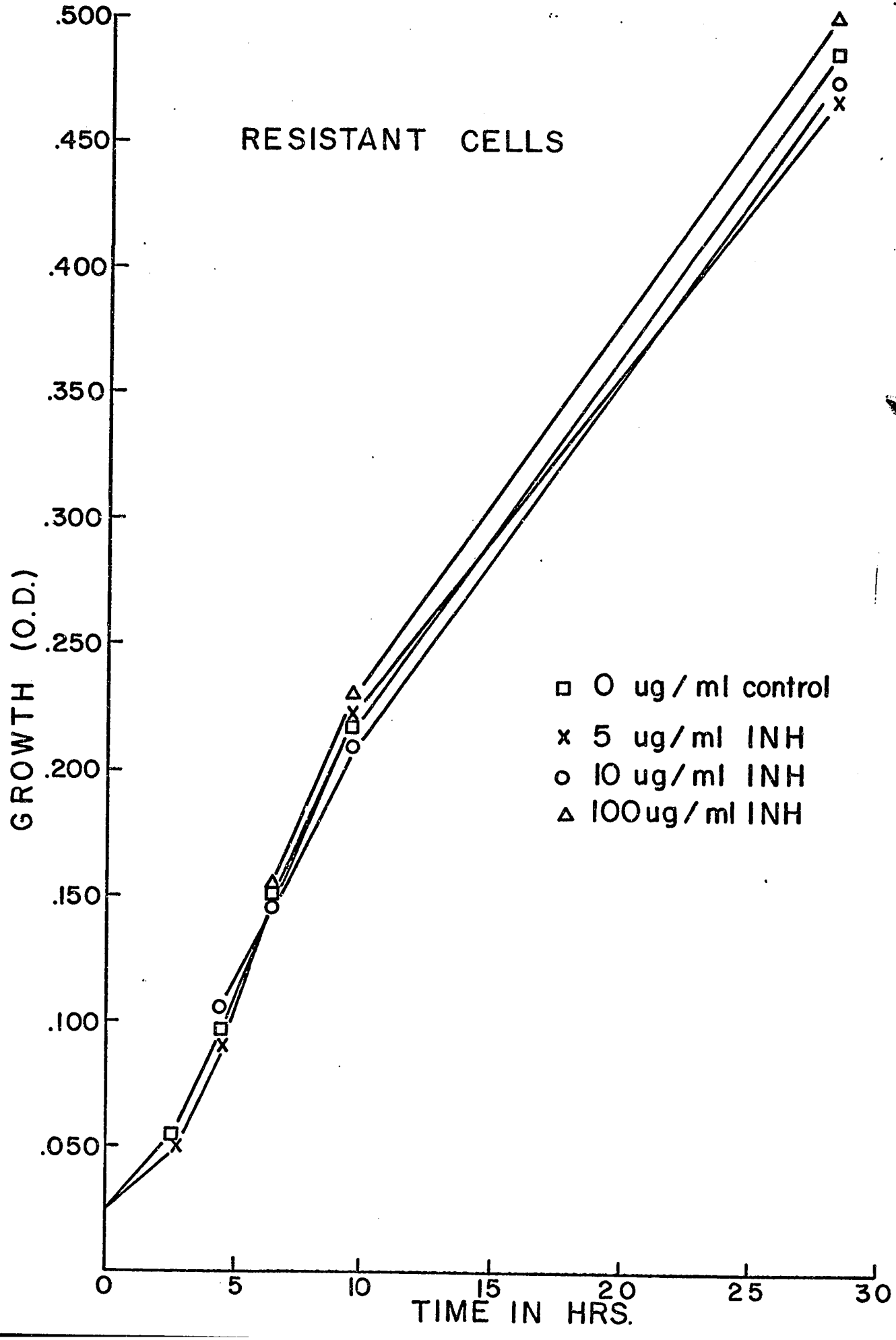

SENSITIVE CELLS
x 0 µg/ml Control
o 5 µg/ml INH
□ 10 µg/ml INH
△ 100µg/ml INH
.400
.350
.300
.250
.200
.150
.100
.050
GROWTH (O.D.)
0
2
4
6
8
10
12
TIME IN HRS.

FIG. 7

THE GROWTH OF THE
RESISTANT STRAIN
UNDER THE INFLUENCE OF INH


RESISTANT CELLS
GROWTH (O.D.)
.500
.450
.400
.350
.300
.250
.200
.150
.100
.050
0
5
10
15
20
25
30
TIME IN HRS.
□ 0 ug/ml control
x 5 ug/ml INH
o 10 ug/ml INH
△ 100ug/ml INH

INH. Sensitive cells were therefore grown up to an optical density of .8 or 2.25 mg/ml. Then a 1 ml sample of the bacterial suspension was taken and suitably diluted. One ml aliquots of the dilutions were then placed on agar plates which contained differing amounts of INH. The plates were then incubated at 37°C for a few days and the colonies that developed were counted.

The results are seen in Fig. 8. It can be immediately observed that most of the cells are killed at 5 ug/ml INH. These results partly explain why very little growth was observed at 5 ug/ml INH (Fig. 6). However even at 100 ug/ml INH some cells survive and these are probably resistant mutants to INH.

The Effect of INH on the Growth of M. Smegmatis 503 at a Concentration of .25 mg/ml cells.

One of the first observations on INH inhibition of growth was that isoniazid was very rarely completely bacteriostatic (Fig. 6 being an exception). The second observation was that the degree of bacteriostasis seemed to be dependent on the INH concentration. This last purely non-numerical observation was shown by the following experiment. Cells were grown up to an optical density of .12 or 25 mgs/ml. At this point the cells were divided into 5 batches to which INH was added in concentrations shown in Fig. 9 and the growth was recorded with time.

Here it is seen that as mentioned above 5 ug/ml causes no inhibition which is contrary to the results shown in Fig. 6. This difference could be attributed to the starting concentration of cells which is 2x that in Fig. 6. Thus comparing Fig. 6 and Fig. 9 it can be seen

FIG. 8

THE EFFECT OF INH
ON THE VIABLE CELL COUNT OF
SENSITIVE CELLS

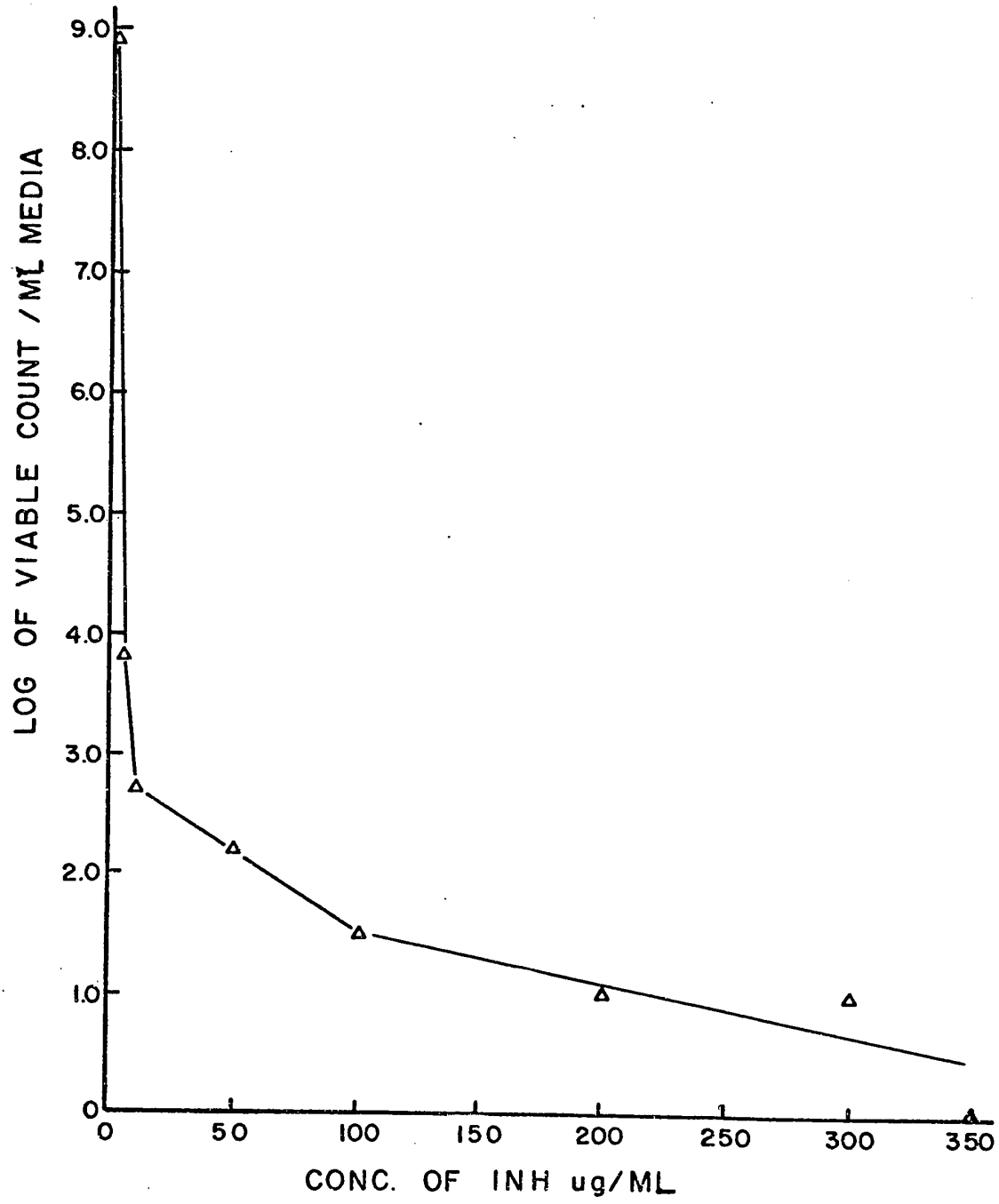
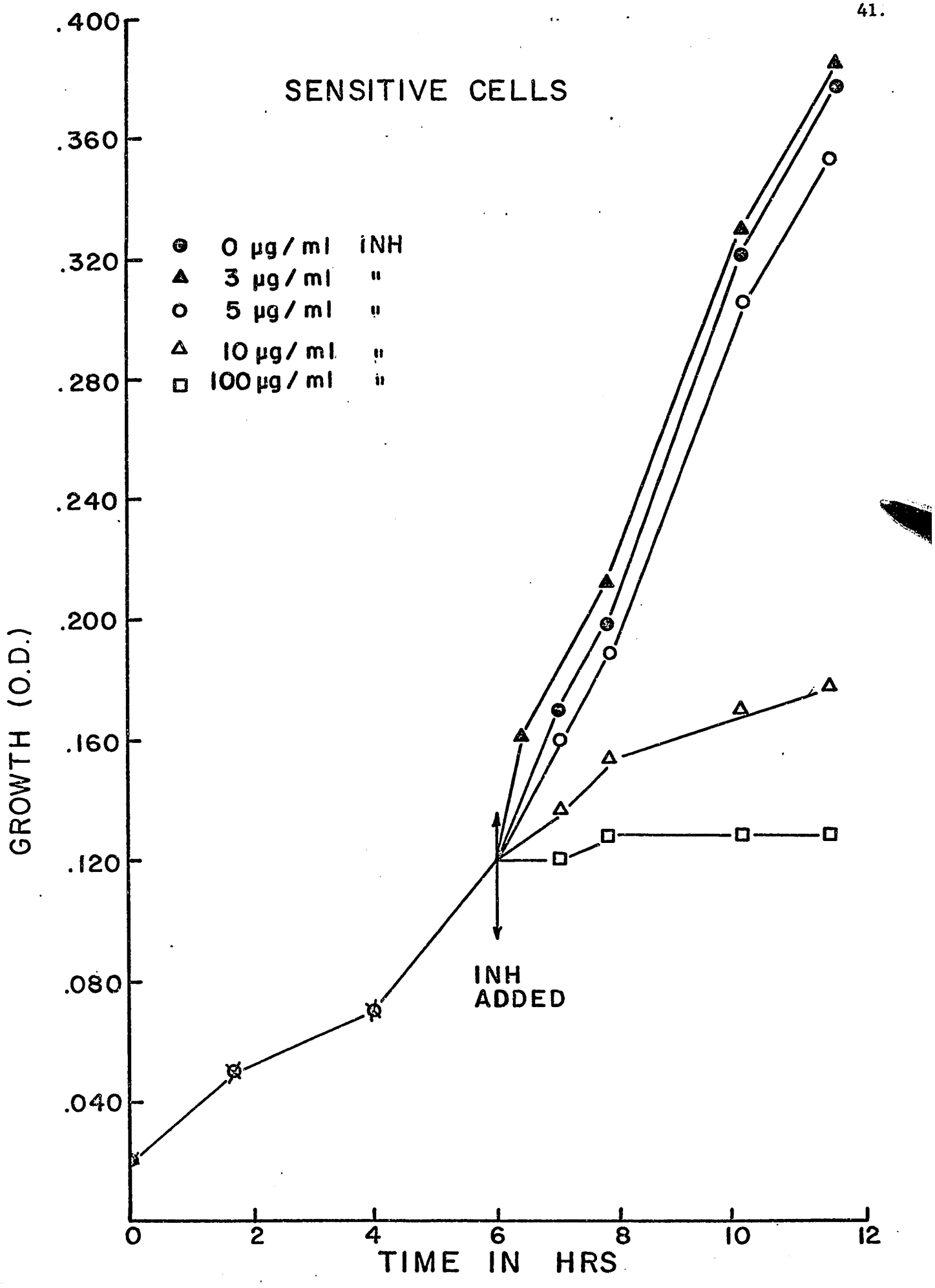

LOG OF VIABLE COUNT / ML MEDIA
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
0
50
100
150
200
250
300
350
CONC. OF INH ug/ML

FIG. 9

THE EFFECT OF
VARIOUS CONCENTRATIONS OF INH
ON THE GROWTH OF SENSITIVE CELLS
AT A HIGH O.D.


SENSITIVE CELLS
0 µg/ml INH
3 µg/ml "
5 µg/ml "
10 µg/ml "
100 µg/ml "
INH ADDED
GROWTH (O.D.)
.400
.360
.320
.280
.240
.200
.160
.120
.080
.040
0
2
4
6
8
10
12
TIME IN HRS

that the cells in Fig. 9 are not as affected by the same INH concentrations as the cells in Fig. 6.

The Variation In Inhibition By INH On Various Cell Numbers Of Sensitive Cells.

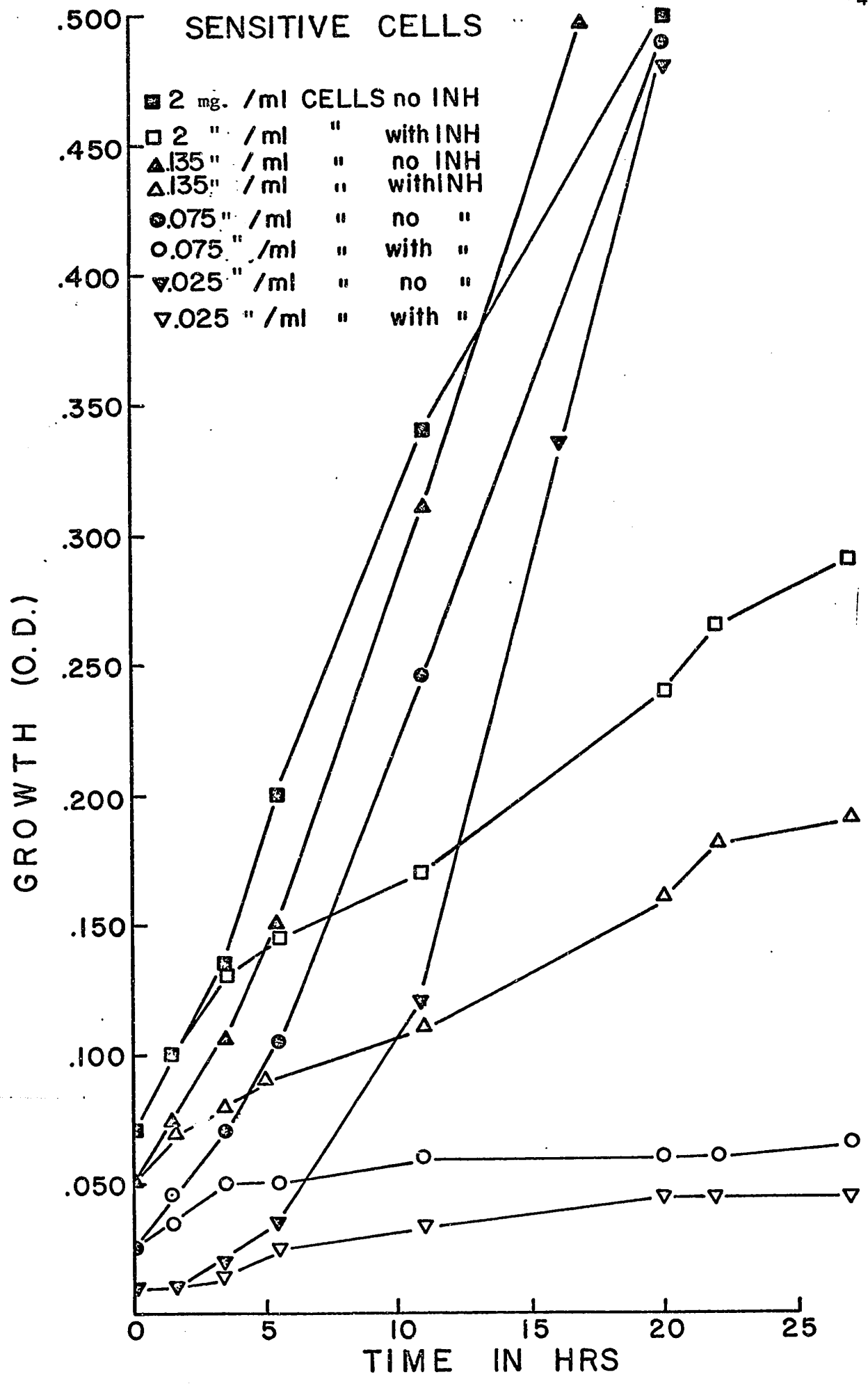
Meadows (68) was the first to show that the concentration of INH required to inhibit the growth of mycobacteria depends on inoculum size and the particular strain used. Her work was done on B.C.G. and the results were arrived at by measuring the effect of INH inhibition on acetate oxidation. Our results obtained in Fig. 6 and Fig. 9 also suggested that the degree of inhibition was dependent on the size of the innoculum. This idea was tested further by the following experiment.

Cells were grown up to an optical density of .5 (1.25 mg/ml). The cells were then split up into a number of batches which were diluted with fresh sterile media to .2, .135, .075 and .025 mg cells/ml. The same concentration of INH (10 ug/ml) was then added to each culture and the growth was recorded. The results in Fig. 10 show that the degree of inhibition is dependent on the original cell number or the size of the innoculum and that complete inhibition is obtained only if the starting size of the innoculum is very small.

The most obvious explanation for the above results would be that the number of mutants resistant to INH would be greater in the larger sized innoculums. However this is unlikely as the differences in the sizes of the innoculums are not great enough. Another explanation has to be attached to the above phenomena and it will be discussed later.

FIG. 10

THE VARIATION IN INHIBITION
BY
INH ON VARIOUS CELL NUMBERS
OF SENSITIVE CELLS


SENSITIVE CELLS
2 mg. /ml CELLS no INH
2 " /ml " with INH
.135 " /ml " no INH
.135 " /ml " with INH
.075 " /ml " no "
.075 " /ml " with "
.025 " /ml " no "
.025 " /ml " with "
GROWTH (O.D.)
.500
.450
.400
.350
.300
.250
.200
.150
.100
.050
0
5
10
15
20
25
TIME IN HRS

The Uptake of Radioactive INH by Sensitive and Resistant M. smegmatis.

Previous work (5, 6, 7, 8) had indicated that a profound difference existed between sensitive and resistant mycobacteria in that sensitive organisms took up INH (C^{14}), while resistant organisms did not take up the drug. The above results led to the theory that resistant organisms were impermeable to INH and this theory was tested as follows.

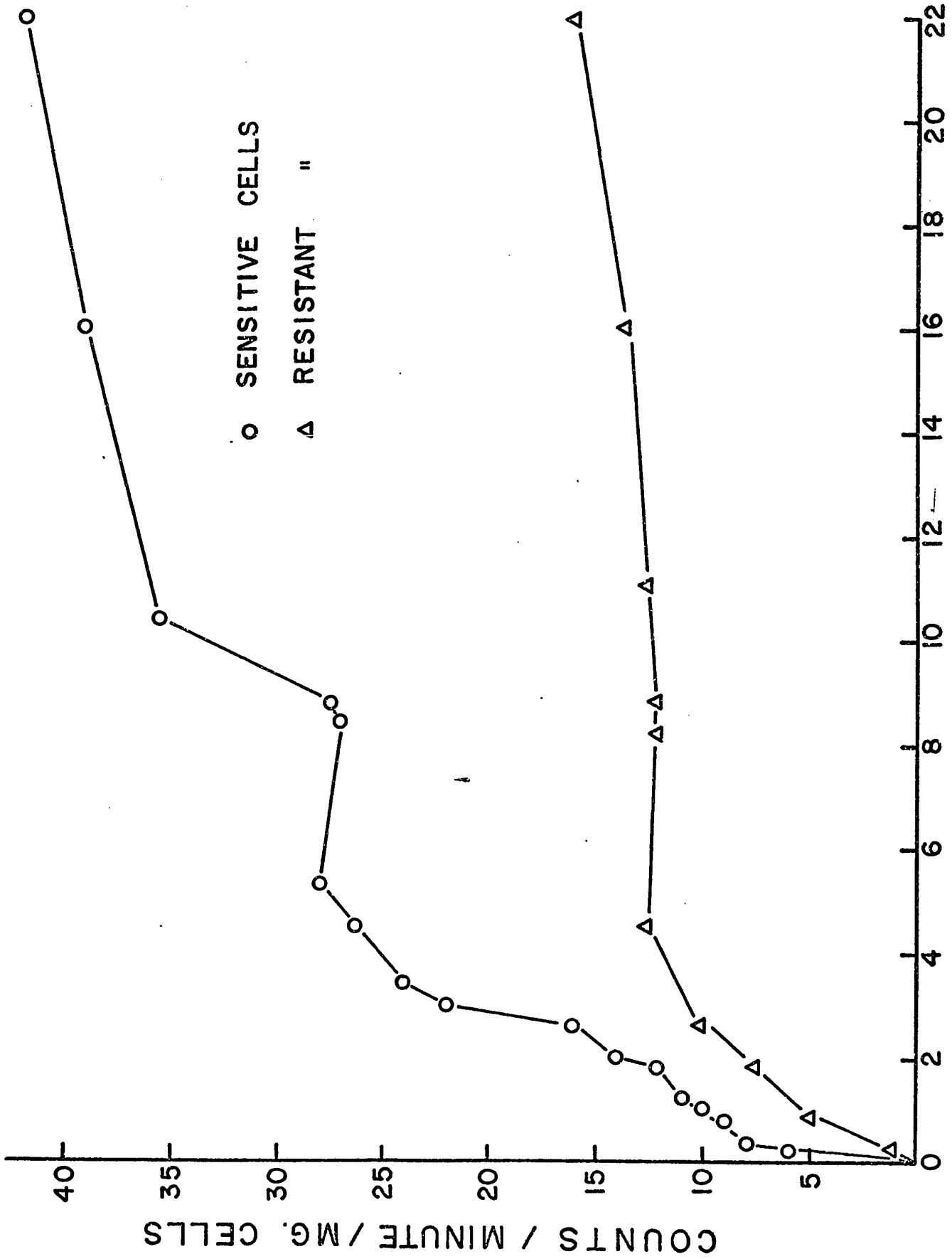
The first part of this work was done using liquid scintillation as the method of counting but the results were of limited accuracy due to the technical reasons described in methods. Nevertheless a curve of INH uptake was obtained (Fig. 11). In this figure it can be seen that the uptake of the sensitive organism is greater than that of the resistant and that most of the uptake takes place within a period of 5 hours. These results substantiated those of Barclay (5) and Youatt (6) although it was difficult to compare our results to those of Barclay's as he worked with growing cells in Dubos media (98). Both authors also worked with B.C.G. which are very slow growing organisms and their results are therefore over a period of a week.

The second part of the uptake studies were performed using gas flow counting and uptake was studied under different conditions of temperature (37°C, 25°C, 4°C) and at 37°C in the presence of glycerol, cyanide and clorophenicol. The results are seen in the following figures.

1. The uptake of INH at 37°C (Fig. 11A)
2. The uptake of INH at 25°C (Fig. 11B)
3. The uptake of INH at 4°C (Fig. 11C)
4. The uptake of INH at 37°C with CN (1u mole/ml) (Fig. 11D)

FIG. 11

THE UPTAKE OF INH-C^{14}
BY
SENSITIVE CELLS COUNTED
VIA
LIQUID SCINTILLATION



5. The uptake of INH at 37°C with 10% glycerol (Fig. 11E)
6. The uptake of INH at 37°C with Cloromphenicol (1u mole/ml) (Fig. 11A)

Essentially the same results were obtained with gas flow counting (11A) as with liquid scintillation (Fig. 11) at 37°C. Thus the uptake of the sensitive cells at 37°C is much greater than that of the resistant cells and the peak in uptake is reached within a period of eight hours after which uptake decreases.

The uptake at 25°C (Fig. 11B) is less than the uptake at 37°C for both the sensitive and resistant organisms. These results seem to agree with those of Youatt (6) who showed that the process of INH binding by B.C.G. was temperature dependent but disagree with those of Wimpenny (7) who showed that the uptake is larger at 25°C than at 37°C. Again it is difficult to compare Wimpenny's results to ours as he worked with growing B.C.G. in Sauton medium (methods).

The uptake at 4°C (Fig. 11C) is initially (first four hours) greater than the uptake at 37°C for both the sensitive and resistant cells. These results agree with those of Barclay (5) and Wimpenny (7) but are very difficult to explain, after approximately fourteen hours the uptake of the sensitive and resistant cells become equal. It could be postulated that the cold somehow damages the permeability barrier of the cell and a leakage inwards results. At higher temperatures the uptake of INH would be temperature dependent as postulated by Youatt (6).

Cyanide greatly reduces INH uptake by the sensitive bacteria and slightly reduces uptake by the resistant bacteria so that both take up the same amount of radioactivity. These results agree with those

FIG. 11A

THE UPTAKE OF INH-C^{14}
BY
SENSITIVE AND RESISTANT CELLS
COUNTED VIA GAS FLOW
AND THE INFLUENCE
OF
CLOROMPHENICOL ON UPTAKE

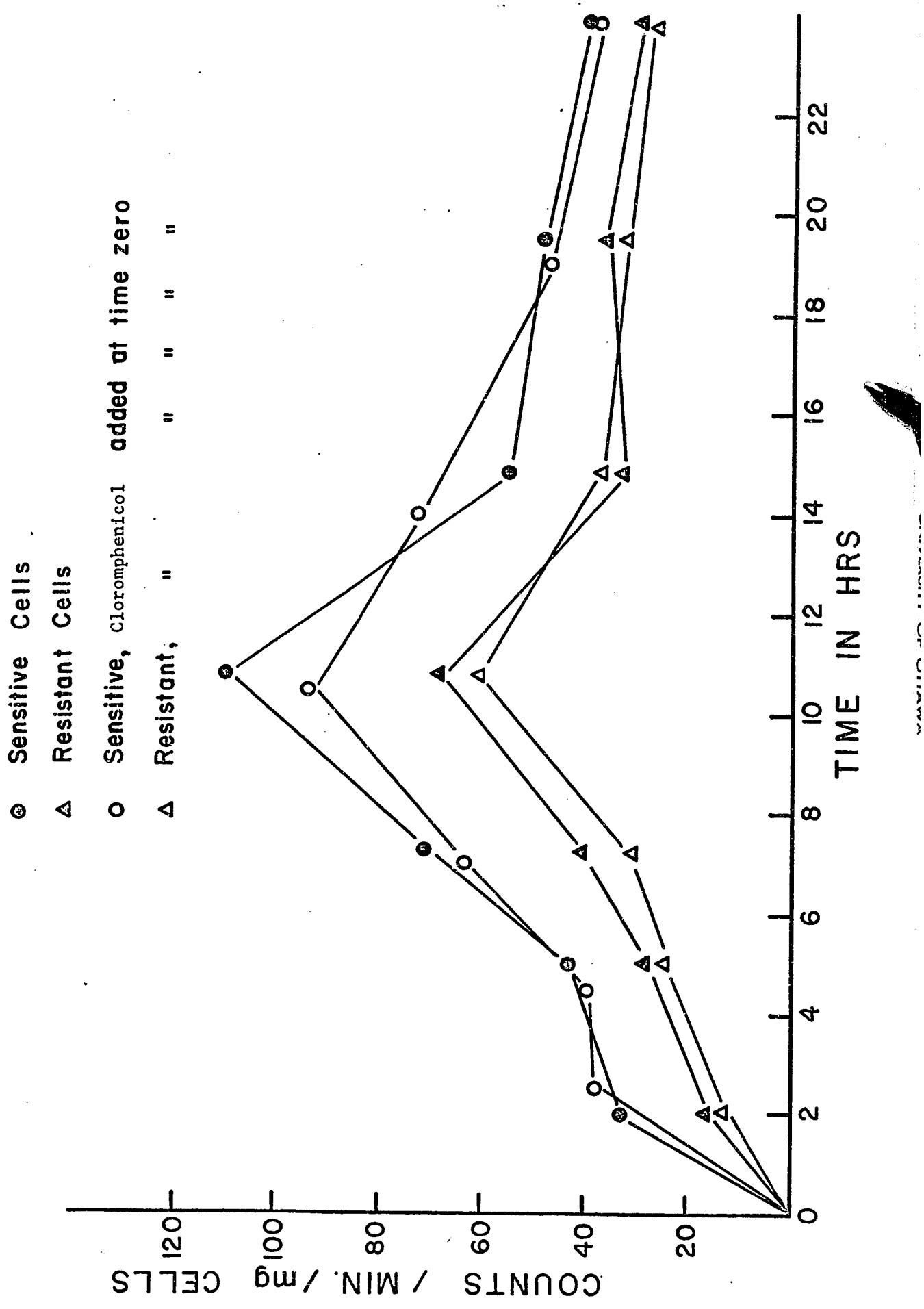

Sensitive Cells
Resistant Cells
Sensitive, Cloromphenicol added at time zero
" " " "
Resistant, " " " "
COUNTS / MIN. / mg CELLS
20
40
60
80
100
120
0
2
4
6
8
10
12
14
16
18
20
22
TIME IN HRS

FIG. 11B

THE UPTAKE OF INH-C^{14}
AT 25°C
BY SENSITIVE AND RESISTANT CELLS
COUNTED VIA GAS FLOW

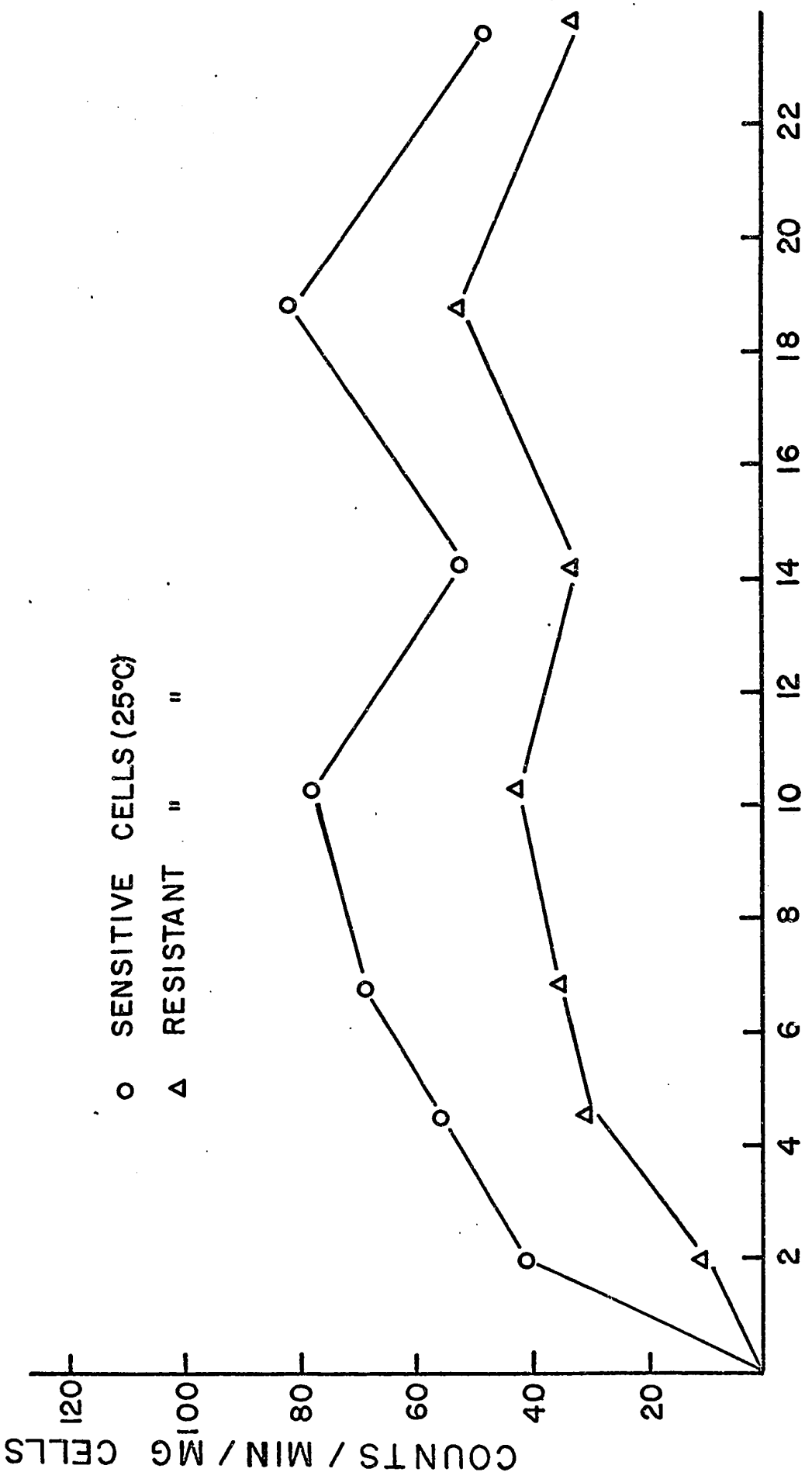

COUNTS / MIN / MG CELLS
120
100
80
60
40
20
2
4
6
8
10
12
14
16
18
20
22
TIME IN HRS
o SENSITIVE CELLS (25°C)
Δ RESISTANT " "

FIG. 11C

THE UPTAKE OF INH-C^{14} AT 4°C

BY

SENSITIVE AND RESISTANT CELLS

COUNTED VIA GAS FLOW

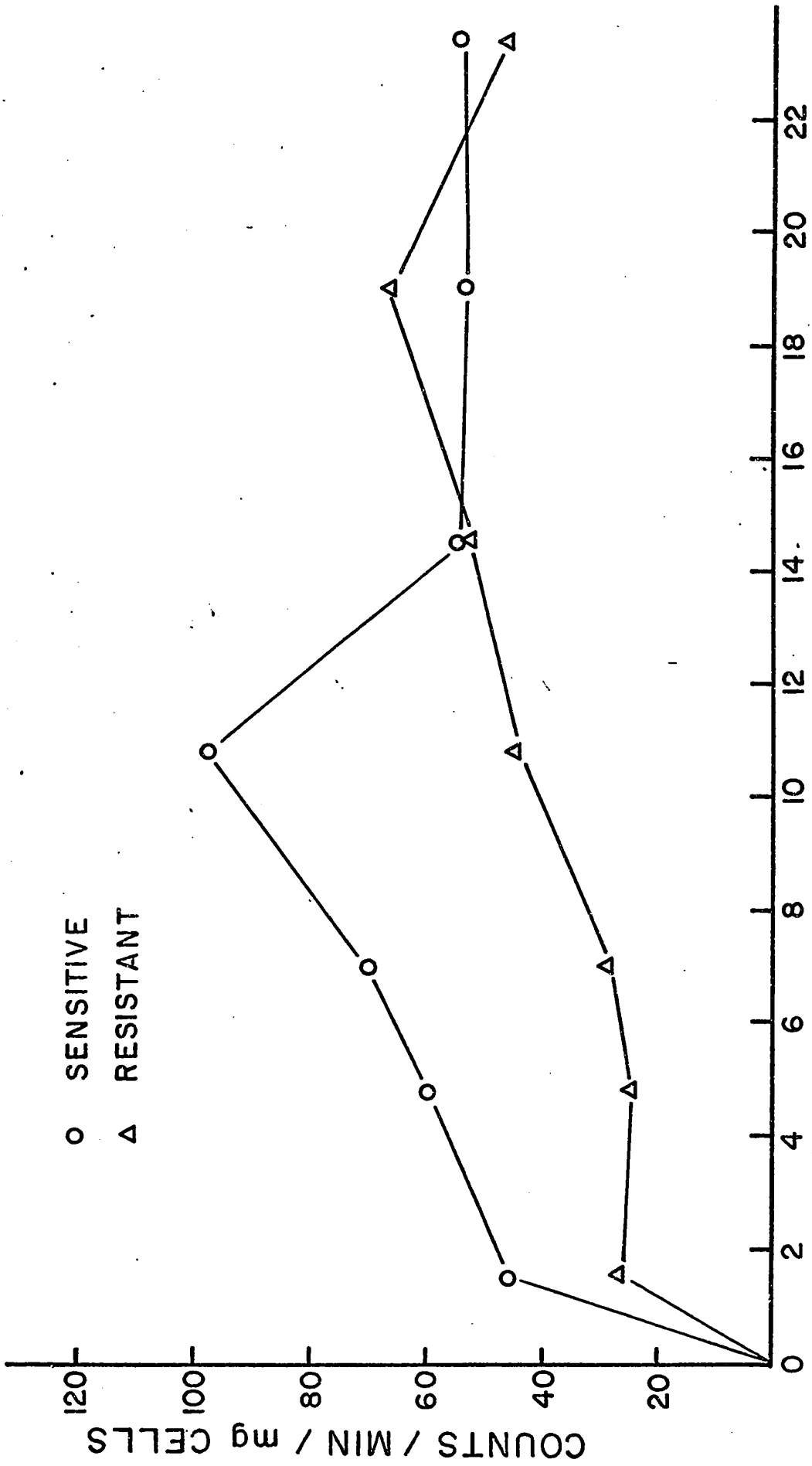


FIG. 11D

THE INFLUENCE OF CYANIDE ON
THE UPTAKE OF INH-C^{14}
BY
SENSITIVE AND RESISTANT CELLS
COUNTED VIA GAS FLOW

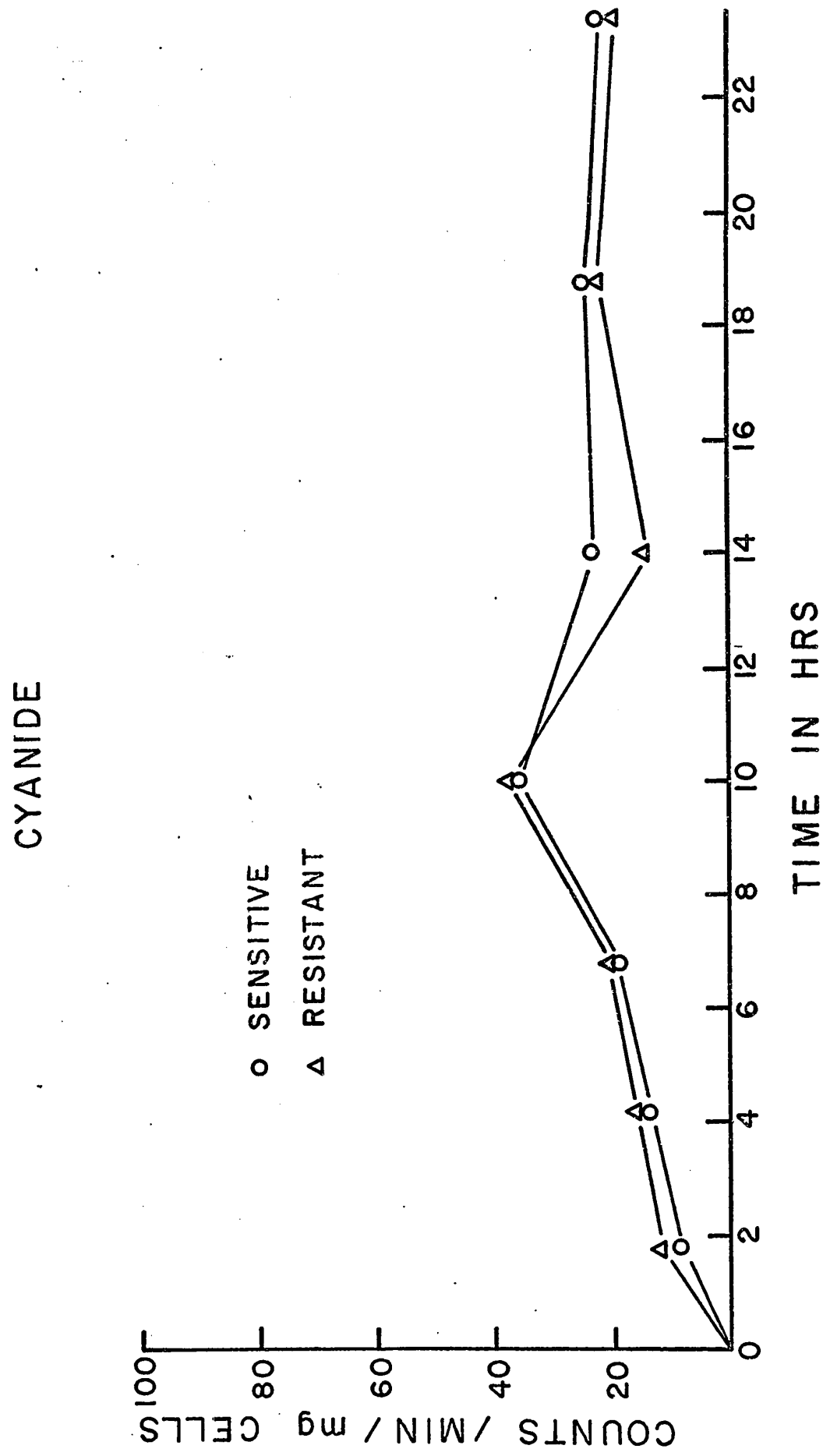
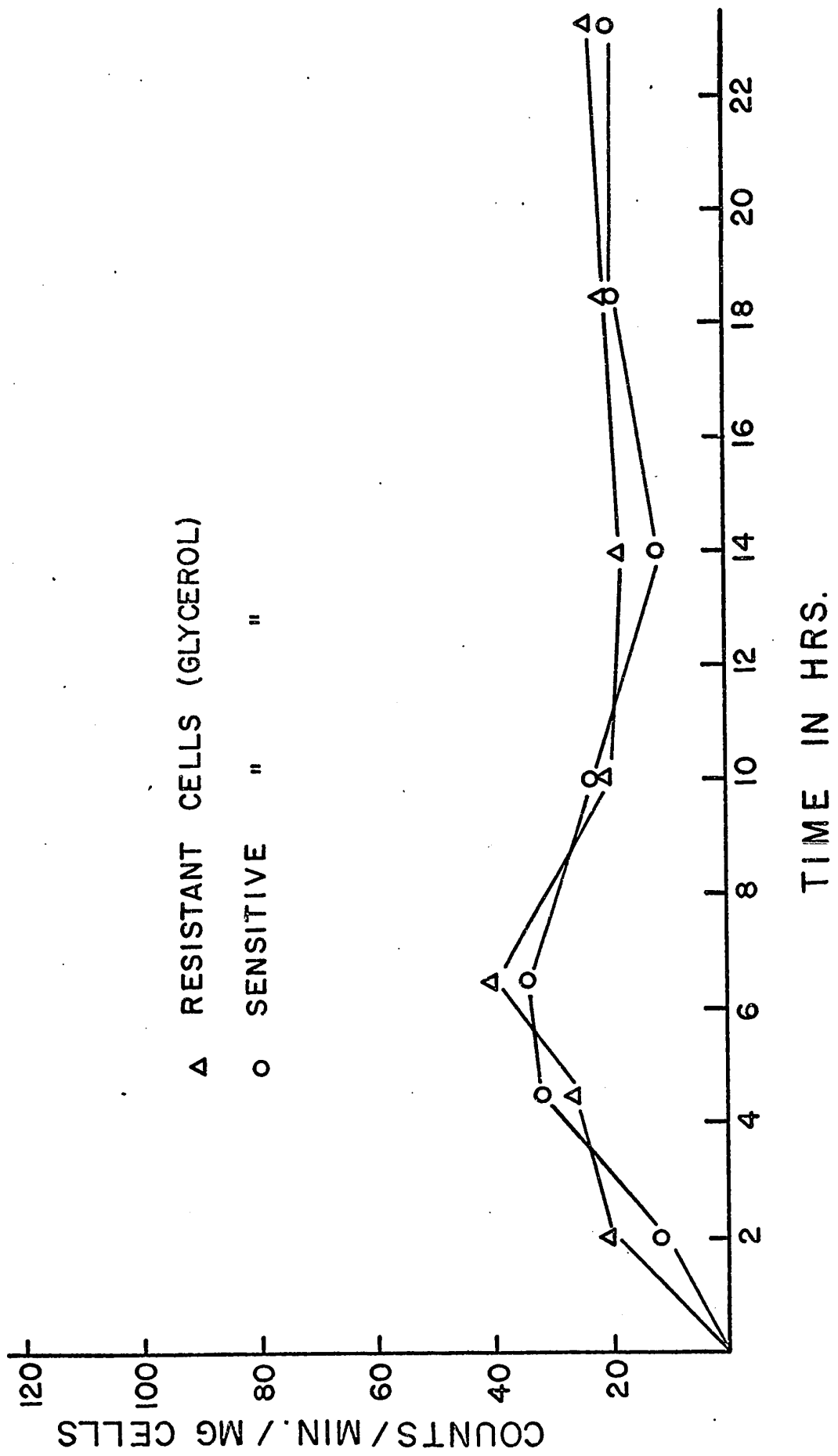

CYANIDE
O SENSITIVE
Δ RESISTANT
COUNTS /MIN/ mg CELLS
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
18
20
22
TIME IN HRS

FIG. 11E

THE INFLUENCE OF 10% GLYCEROL
ON THE UPTAKE OF INH-C^{14}
BY
SENSITIVE AND RESISTANT CELLS
COUNTED VIA GAS FLOW


COUNTS / MIN. / MG CELLS
120
100
80
60
40
20
2
4
6
8
10
12
14
16
18
20
22
TIME IN HRS.
Δ RESISTANT CELLS (GLYCEROL)
O SENSITIVE "

of Wimpenny (7) and Youatt (6).

Cyanide is an inhibitor of energy metabolism acting directly on cytochrome oxidase (99). Sometimes uptake of solutes is linked to energy metabolism, as shown by the uptake of phosphate by yeast (100), which requires nutrients and is inhibited by cyanide, dinitrophenol and azide. Thus it could be postulated that INH uptake is energy linked but Youatt (6) found that the uptake of INH is increased in the presence of dinitrophenol and is decreased in the presence of glycerol.

We also found that Glycerol drastically decreased the uptake of INH (C^{14}) in the sensitive organism and decreased the uptake of the resistant cells only slightly (Fig. 11E). What is more important is that glycerol seemed to equalize the uptake of the resistant and the sensitive cells. These results are very difficult to explain via any theory that states that INH uptake is energy dependent.

Cloromphenicol, well known as an inhibitor of protein synthesis (101,102) did not affect C^{14}-INH uptake by either sensitive or resistant cells (Fig. 11A).

The Correlation of INH Disappearance and Hydrazine Production and the Possible Induction of INH Hydrolyzing Enzyme in M. Smegmatis 503 Sensitive.

Early in the work it was realized that uptake studies of radioactive INH provided limited information especially if the drug is destroyed or metabolically changed as suggested by such authors as Youmans (57). It was therefore decided to trace the metabolic fate of the drug beginning with whole cell experiments.

The most obvious by-product if breakdown did occur was thought to be hydrazine. A preliminary incubation experiment was therefore performed in which INH and hydrazine were measured simultaneously (Fig. 12). This experiment showed that:

1. There is a lag of two hours before hydrazine is produced.
2. INH disappears from the medium at a fairly steady rate from the time of its first contact with the cells.
3. Hydrazine levels are never as great as the amounts of INH which has disappeared.
4. Eventually hydrazine levels level off and then start to decrease.

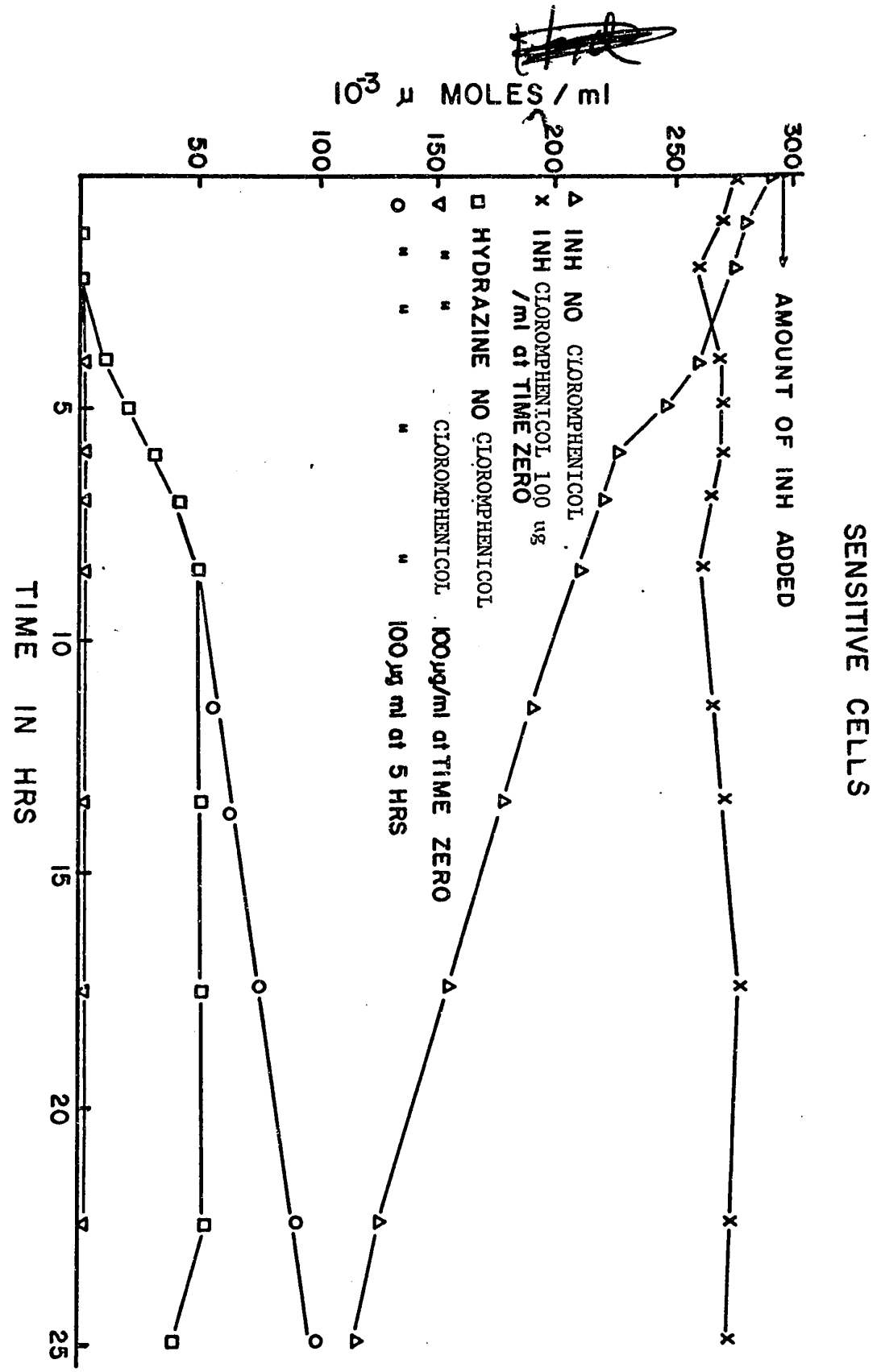
The lag of two hours before hydrazine production became noticeable immediately suggested that this time was required for enzyme induction. A sister experiment was therefore performed which is also shown in Fig. 12. Here clorompheniçol which inhibits protein synthesis (as discussed previously) was added in a concentration of 100ug/ml either at time zero or five hours after the INH was added. Samples of the supernatant were taken at various times and analyzed for the presence of INH and hydrazine.

As can be seen from Fig. 12 adding clorompheniçol at time zero produced no hydrazine and very little INH dissappeared. However if clorompheniçol was added at five hours the hydrazine production seemed to increase above the level of the control and approach that of the INH taken up in the absence of clorompheniçol.

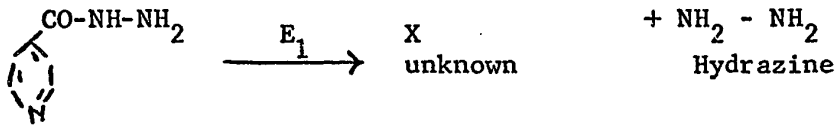
As clorompheniçol is known to inhibit induced enzyme formation (101,102) it was thought the clorompheniçol added at time zero inhibited

FIG. 12

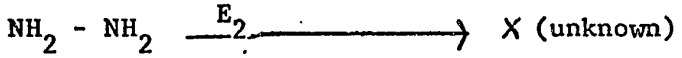
THE CORRELATION OF INH DISAPPEARANCE
AND
HYDRAZINE APPEARANCE
UNDER THE INFLUENCE OF SENSITIVE CELLS
AND
THE POSSIBLE INDUCTION OF AN ENZYME FOR
INH BREAKDOWN


SENSITIVE CELLS
AMOUNT OF INH ADDED
10^3 µ MOLES / ml
300
250
200
150
100
50
TIME IN HRS
5
10
15
20
25
Δ INH NO CLOROMPHENICOL
x INH CLOROMPHENICOL 100 ug /ml at TIME ZERO
□ HYDRAZINE NO CLOROMPHENICOL
▽ " " CLOROMPHENICOL 100µg/ml at TIME ZERO
o " " " 100µg ml at 5 HRS

the induction of the enzyme responsible for the breakdown of INH to hydrazine (as shown):



If cloromphenicol was added at 5 hours more hydrazine was produced than without cloromphenicol. This was thought due to inhibition of formation of a second induced enzyme that breaks down hydrazine. (as shown)



The idea that INH induces an enzyme in M. Smegmatis 503 (S) for its own breakdown to hydrazine and that hydrazine in turn induces an enzyme for its own breakdown to hydrazine and that hydrazine in turn induces an enzyme for its own breakdown was the hypothesis put forward and most of the following experiments were performed in an effort to prove or disprove this hypothesis.

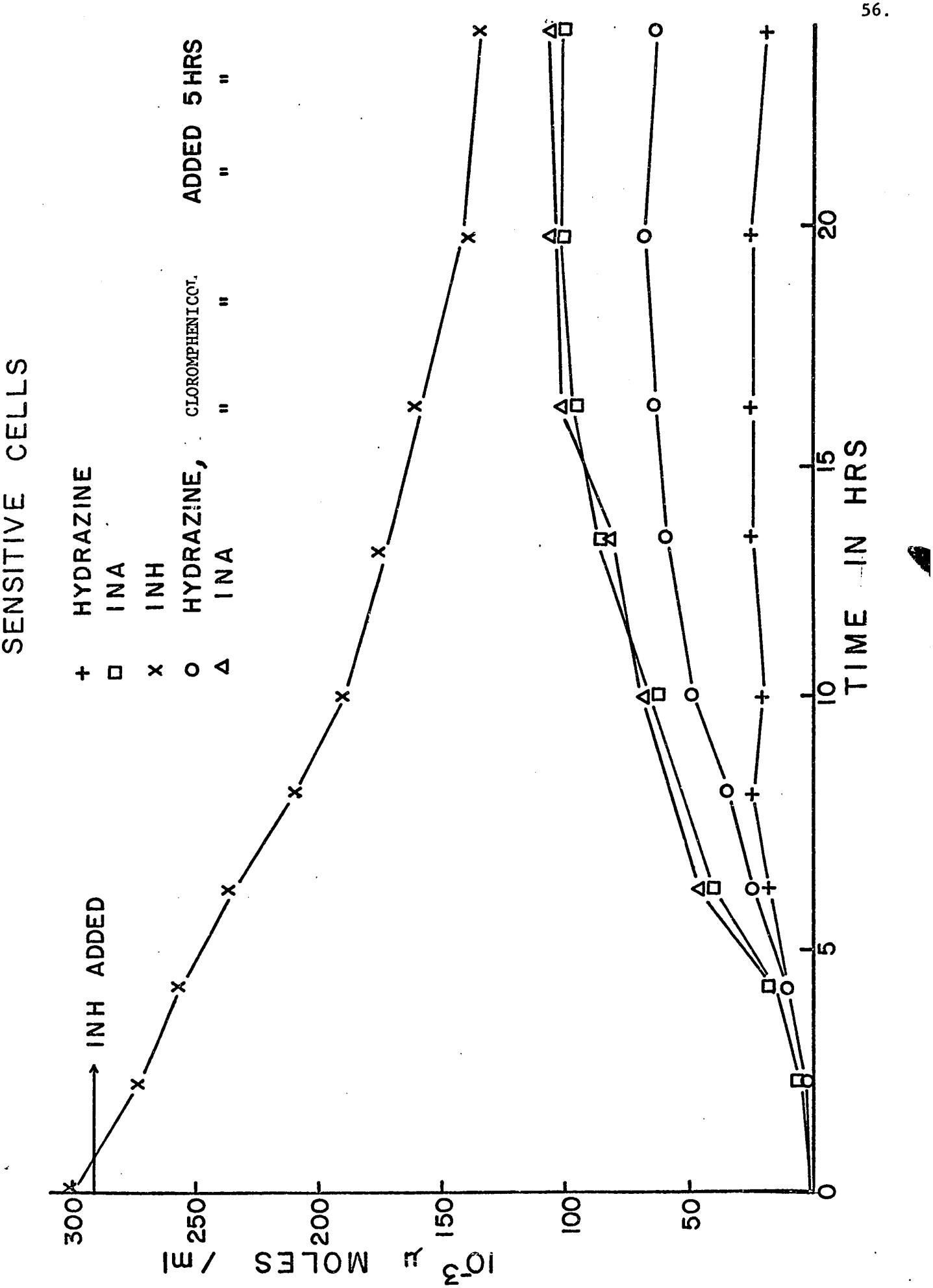
The Correlation of INH Disappearance and INA and Hydrazine Appearance Produced by Sensitive Whole Cells of M. Smegmatis.

The previous experiment had shown that whole sensitive cells breakdown INH to hydrazine via an apparent enzymatic reaction which is stopped if cloromphenicol is added at time zero. It was decided that the reaction itself should be studied further so that the second by-product of INH breakdown besides the hydrazine would be investigated.

Previous authors had shown that cells of B.C.G. broke down INH to isonicotinic acid (INA) (30). The latter compound seemed to be a

FIG. 13

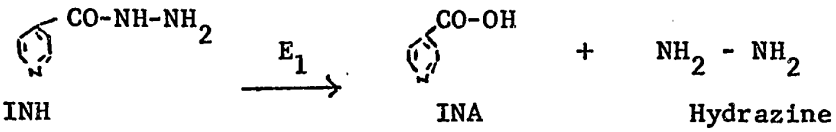
THE CORRELATION OF INA,
AND HYDRAZINE PRODUCTION FROM INH
BY SENSITIVE CELLS
AND THE EFFECT OF CLOROMPHENICOL
ADDED AT 5 HRS.
ON INA PRODUCTION


SENSITIVE CELLS
+ HYDRAZINE
□ INA
x INH
o HYDRAZINE, CLOROMPHENICOL. ADDED 5HRS
Δ INA " "
INH ADDED
300
250
200
150
100
50
0
5
10
15
20
TIME IN HRS
10⁻³ MOLES /ml

logical by-product of the reaction that produced hydrazine.

An experiment was therefore performed where whole sensitive cells were incubated with INH (.292u moles/ml) and the production of INA and hydrazine measured simultaneously. The results of this experiment (Fig. 13) showed that:

1. As INH disappears from the media, INA and hydrazine are indeed produced together so that the reaction can be postulated to go as follows:



2. The production of hydrazine and INA is by no means on a mole per mole basis but much more INA is produced than hydrazine. This may be explained by the hypothesis that the hydrazine induces an enzyme for its own destruction and therefore never reaches INA levels.

<u>The Effect of Cloromphenicol (100ug/ml) on the INA Production by Sensitive Cells</u>.

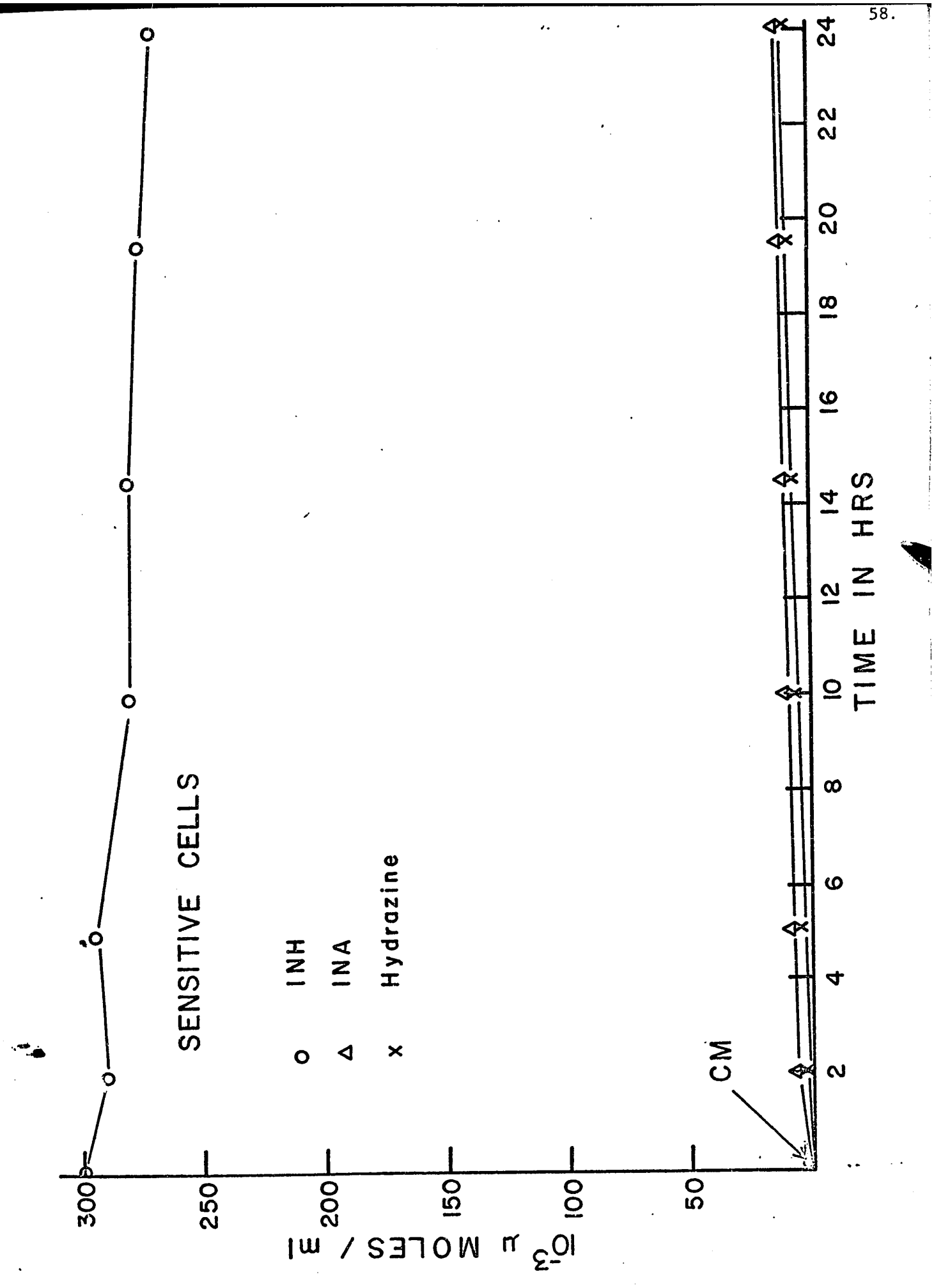
The effect of cloromphenicol on the INA production was then studied. It was found that:

1. If cloromphenicol is put in at time zero, there is a very slight INH disappearance but no hydrazine or INA is produced. (Fig. 14)

2. If cloromphenicol is put in at time five hours INH disappearance continues and hydrazine production is increased approaching INA production the latter remaining the same as in the absence of cloromphenicol (Fig. 13).

FIG. 14

THE EFFECT OF CLOROMPHENICOL
ADDED AT TIME ZERO
ON THE INH DISAPPEARNACE AND HYDRAZINE - INA APPEARANCE
BY SENSITIVE CELLS


SENSITIVE CELLS
INH
INA
Hydrazine
CM
TIME IN HRS
2
4
6
8
10
12
14
16
18
20
22
24
300
250
200
150
100
50
10^{-3} μ MOLES / ml

The Breakdown of INH by Resistant Cells.

As the ultimate object of the study was the discovery of the mode of action of INH, the metabolism of INH in resistant cells was compared to that of the sensitive cells. It was found (Fig. 15) that:

1. The resistant mutant caused INH to disappear much faster from the buffer than did the sensitive strain. This greater disappearance was not due to greater uptake but is probably due to greater breakdown of INH by resistant cells.

2. The resistant cells produced greater quantities of INA than the sensitive cells within the same period of time and INH was destroyed more quickly.

3. Curiously however the resistant cells produces very little hydrazine. This indicates that either the resistant cells do not produce hydrazine from INH or that the hydrazine produced is broken down so fast that it is virtually undetectable. The second possibility suggests that resistant cells may already possess enzymes for INH and hydrazine breakdown, enzymes which, as shown above may have to be induced in sensitive cells.

The Metabolism of INH in Resistant Cells Exposed to Cloromphenicol (100 ug/ml) at Time Zero.

The possibility that resistant cells might have enzyme system for INH and hydrazine breakdown already induced was investigated as follows:

Resistant cells were incubated in the presence of 100 ug/ml cloromphenicol at time zero to see if the enzyme system was already present in the resistant cells.

FIG. 15

THE BREAKDOWN OF INH

BY

RESISTANT CELLS

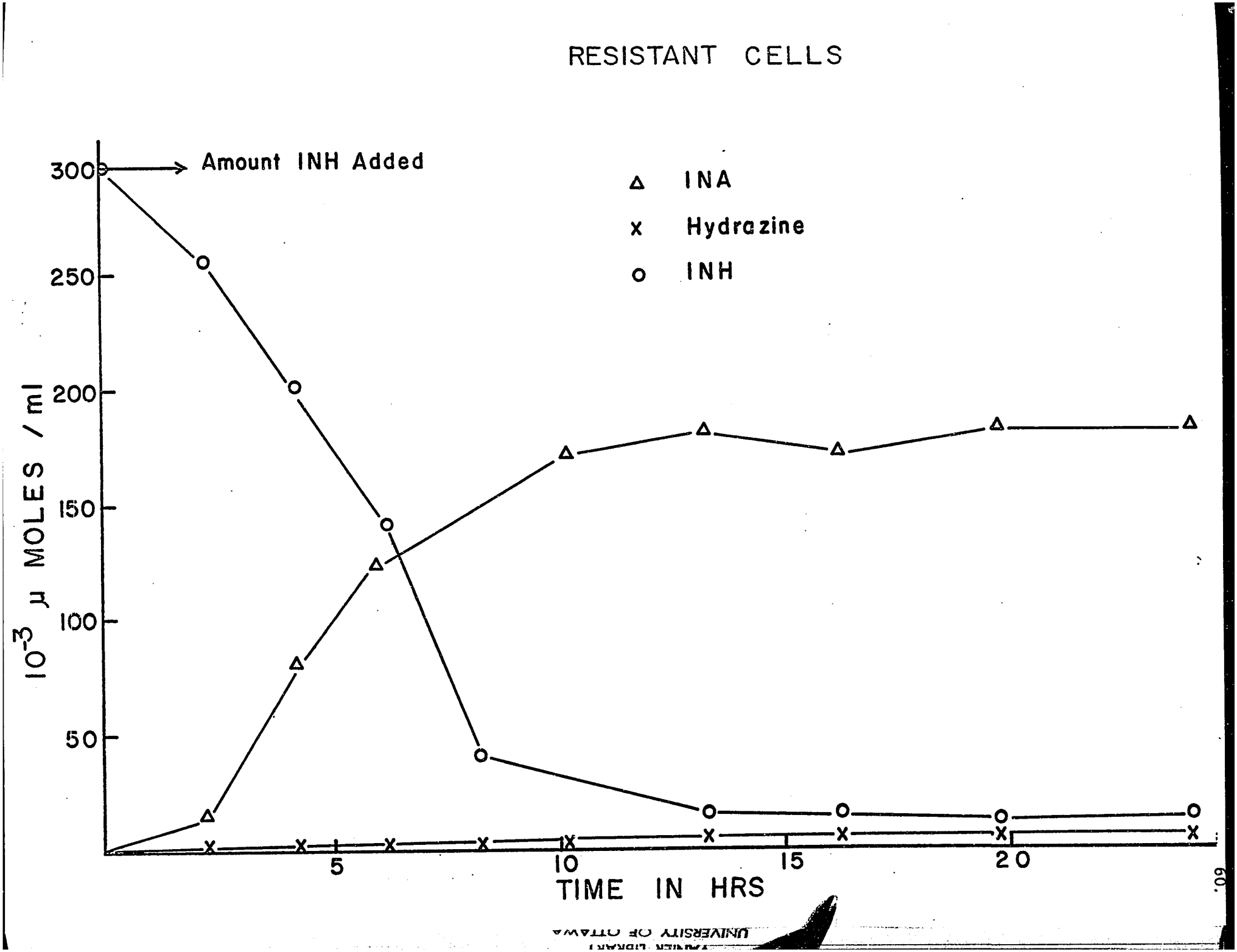
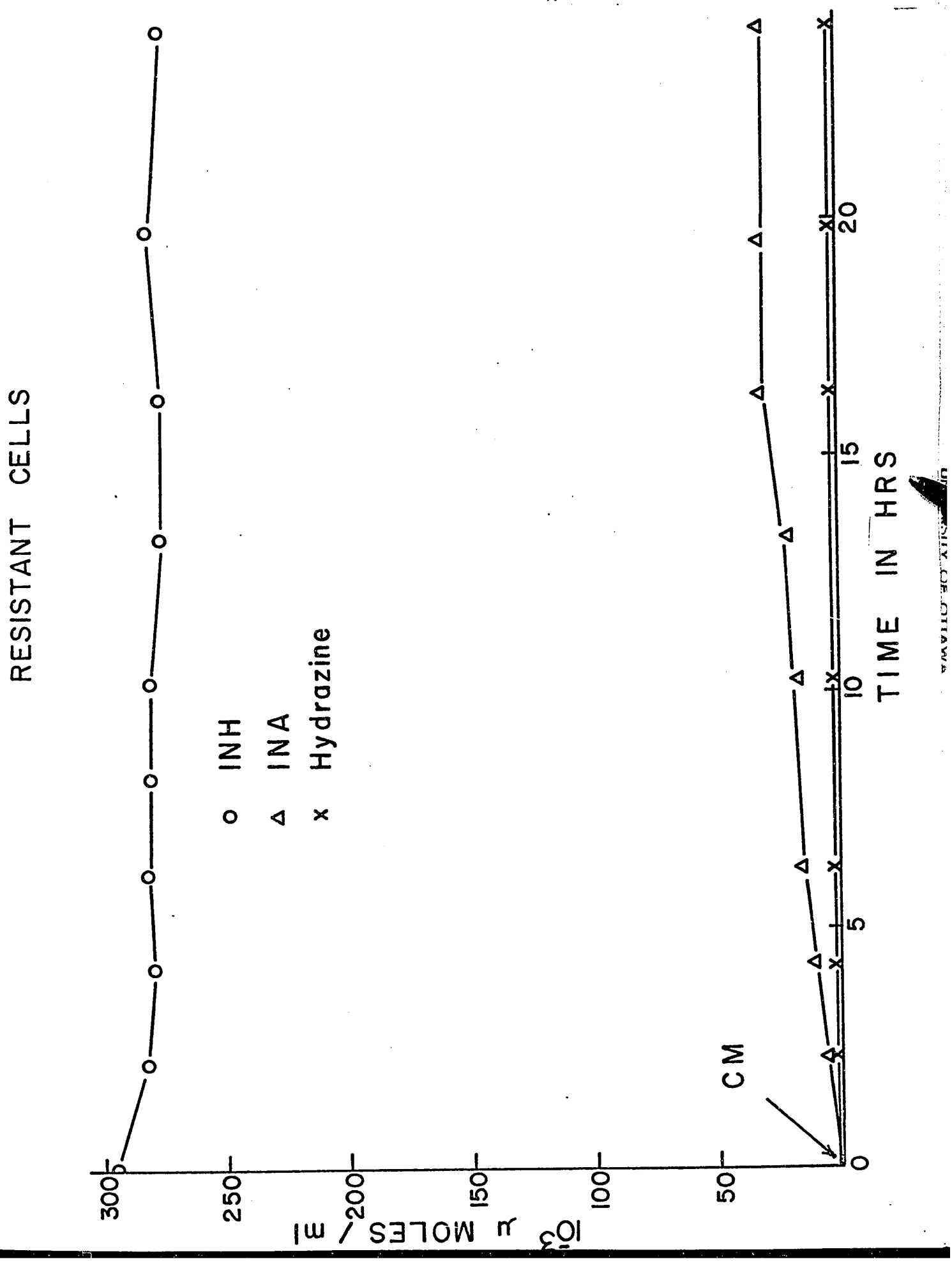

RESISTANT CELLS
Amount INH Added
INA
Hydrazine
INH
300
250
200
150
100
50
10^{-3} μ MOLES / ml
5
10
15
20
TIME IN HRS

FIG. 16

THE METABOLISM OF INH IN RESISTANT CELLS
UNDER THE INFLUENCE OF CLOROMPHENICOL
ADDED AT TIME ZERO


RESISTANT CELLS
o INH
Δ INA
x Hydrazine
CM
TIME IN HRS
0
5
10
15
20
50
100
150
200
250
300
10^{-3} μ MOLES / ml

The results of the experiment (Fig. 16) showed that clorom-phenicol inhibits both the disappearance of INH from the buffer and the production of INA and hydrazine although there is more INA produced in this case than when sensitive cells were incubated with clorompheni col at time zero.

These results indicated that clorompheni col also inhibited the induction of the enzyme system in the resistant cells as well as the sensitive cells. The hypothesis that the enzyme system is inherent in resistant cells but not the sensitive cells was therefore abandoned.

The Metabolism Sensitive Cells Grown in the Presence of lug/ml INH.

Previous experiments (Fig. 12, Fig. 13) led us to believe that when M. Smegmatis 503 sensitive was exposed to INH (.292u moles/ml) an enzyme was induced in the organism to break the INH down to INA and hydrazine. To further test this hypothesis the following experiment was performed.

Sensitive cells were grown up in the normal manner as in methods but lug/ml INH a sub-inhibitory concentration, was added to the Sauton Media. The cells were harvested, washed twice in equal volume of buffer and suspended in buffer to the usual concentration. INH was then added to a concentration of .292u moles/ml. INH, INA and hydrazine were then measured in the usual manner. The results of the experiment are shown in Fig. 17.

Comparing Fig. 17 to Fig. 13 it can be easily seen that the cells grown in the presence of INH broke down INH at a greater rate as more INA was produced and also a greater concentration of hydrazine which however reached a plateau. These results seemed to indicate that cells grown in the presence of INH would have the enzyme system for break-

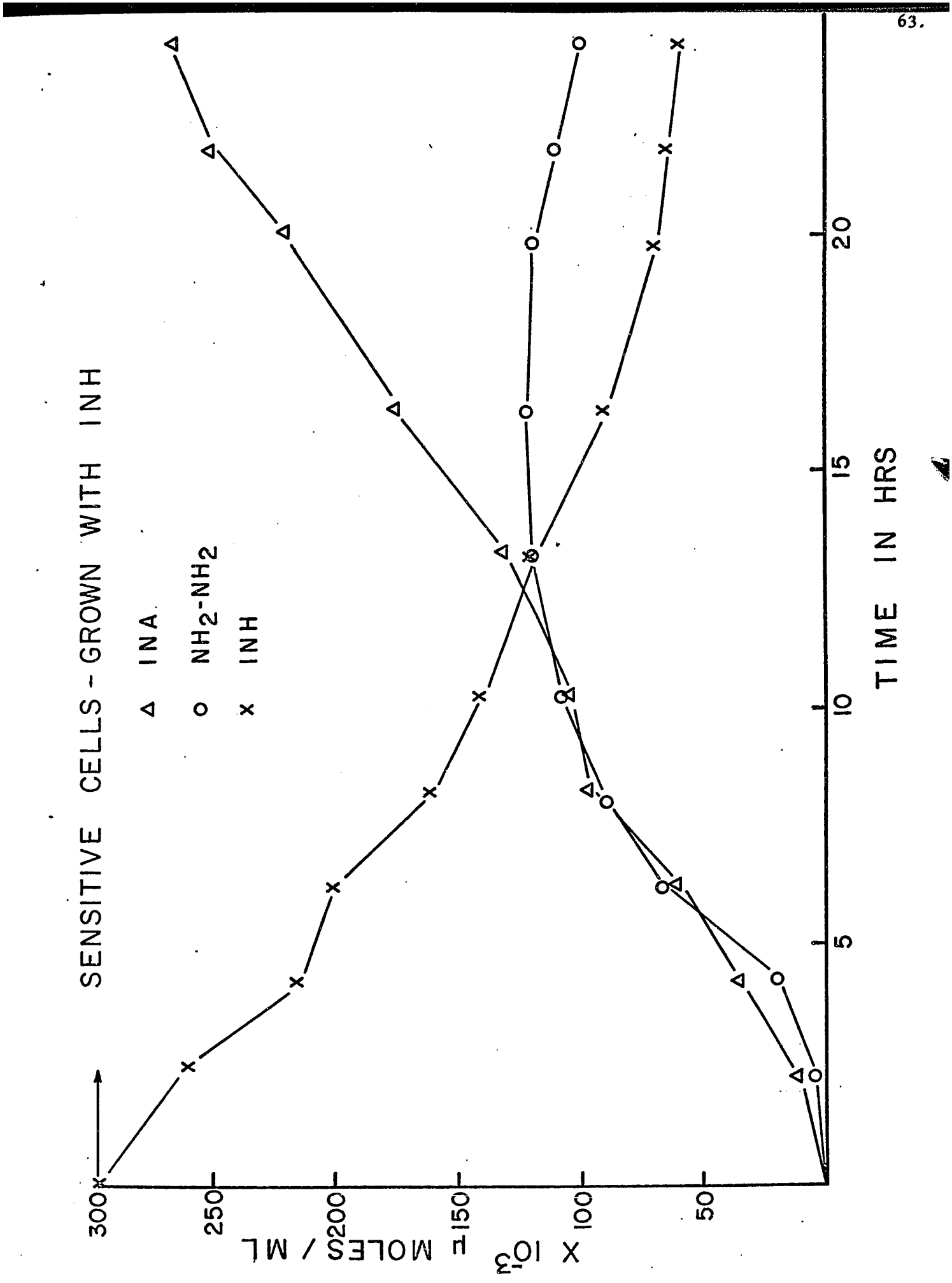
FIG. 17

THE BREAKDOWN OF INH

BY

SENSITIVE CELLS GROWN IN THE PRESENCE

OF A SUB-INHIBITATORY CONCENTRATION OF INH (1 ug/ml.)


SENSITIVE CELLS - GROWN WITH INH
Δ INA
o NH_2-NH_2
x INH
300
250
200
150
100
50
X 10^3 = MOLES / ML
5
10
15
20
TIME IN HRS

down of INH already induced and therefore breakdown INH at a greater rate.

The Metabolism of INH in Sensitive Cells Grown in the Presence of INH (1 ug/ml) with Clorompheniçol Added at Time Zero After Suspension in Buffer.

To test the hypothesis that cells grown in the presence of 1 ug/ml INH have the INH breakdown system already induced the following experiment was performed.

Cells underwent the same treatment as in Fig. 17 but before the cells were incubated in the buffer with .292u moles/ml INH clorom-phenicol was added at 100 ug/ml.

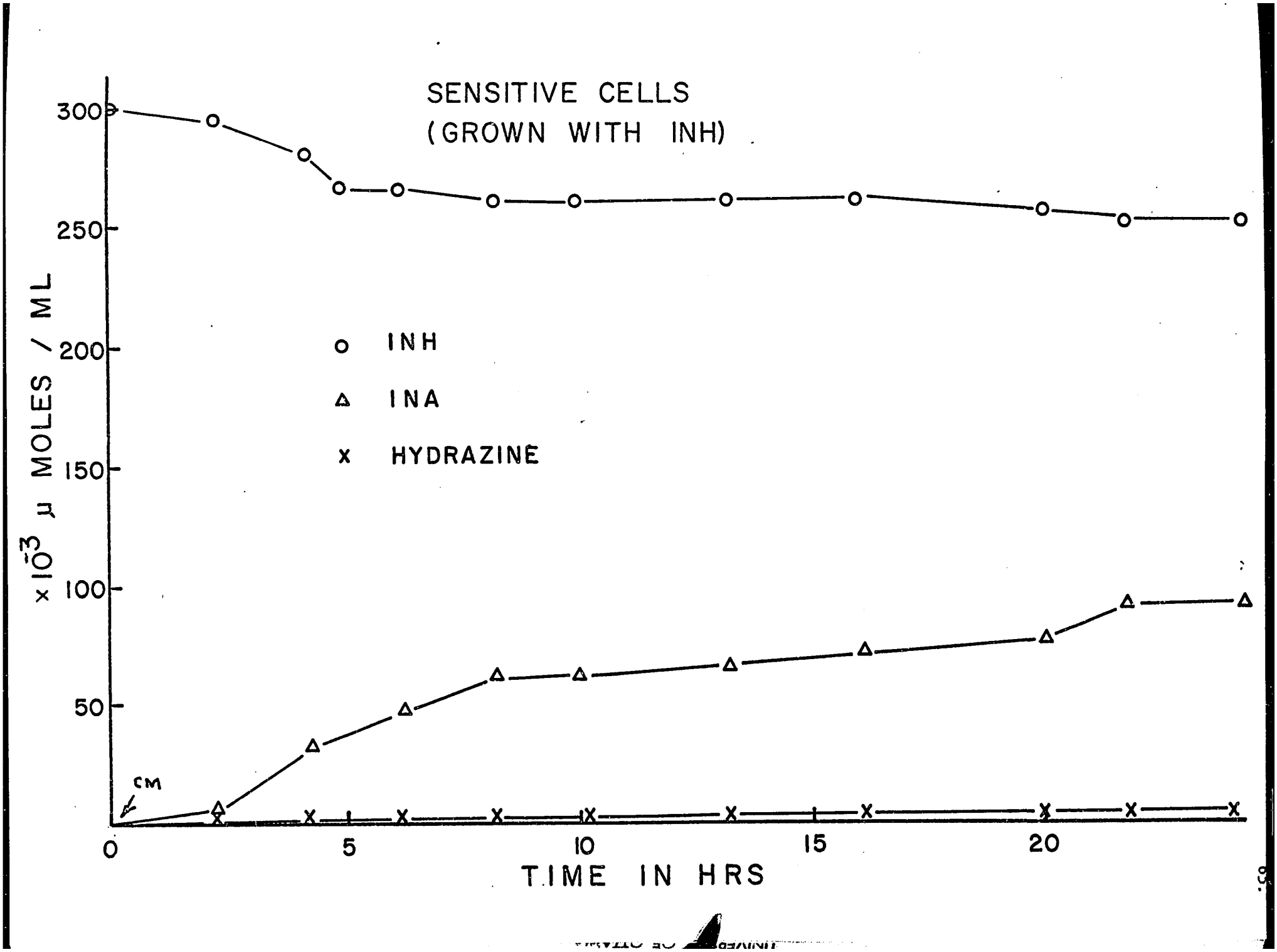
The results of the experiment (Fig. 18) show that very little INH is broken down and very little hydrazine is produced. These results seemed to contradict the interpretation given to the previous experiment.

The Metabolism of INH in the Resistant Cells Grown in the Presence of INH (1 ug/ml).

The same experiment as in Fig. 17 was repeated with the resistant mutant. The results of this experiment are seen in Fig. 19 (b). If these results are compared to Fig. 15 (metabolism of INH in the resistant) it can be seen that more hydrazine was produced by cells grown with INH but less INH is broken down. The significance of these results is unknown.

FIG. 18

THE METABOLISM OF INH IN SENSITIVE CELLS
GROWN WITH INH(1 ug/ml)
WITH CLOROMPHENICOL ADDED AT TIME ZERO

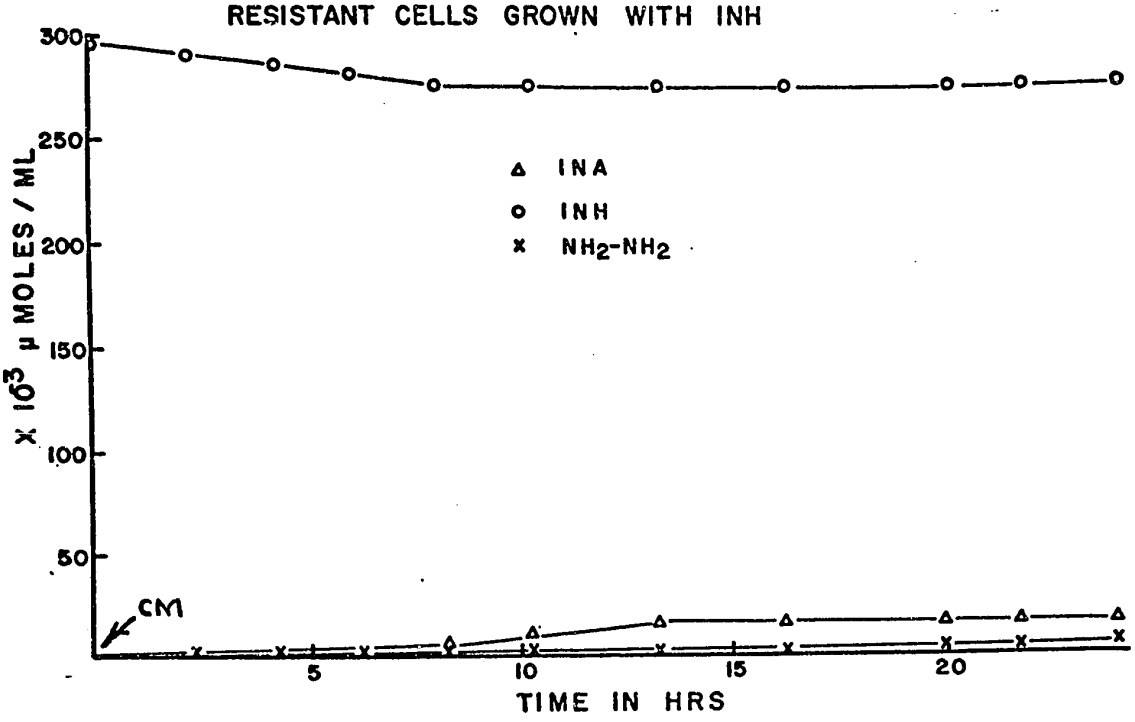

SENSITIVE CELLS
(GROWN WITH INH)
300
250
200
150
100
50
0
5
10
15
20
TIME IN HRS
x 10⁻³ μ MOLES / ML
INH
INA
HYDRAZINE
CM

FIGS. 19A AND 19B

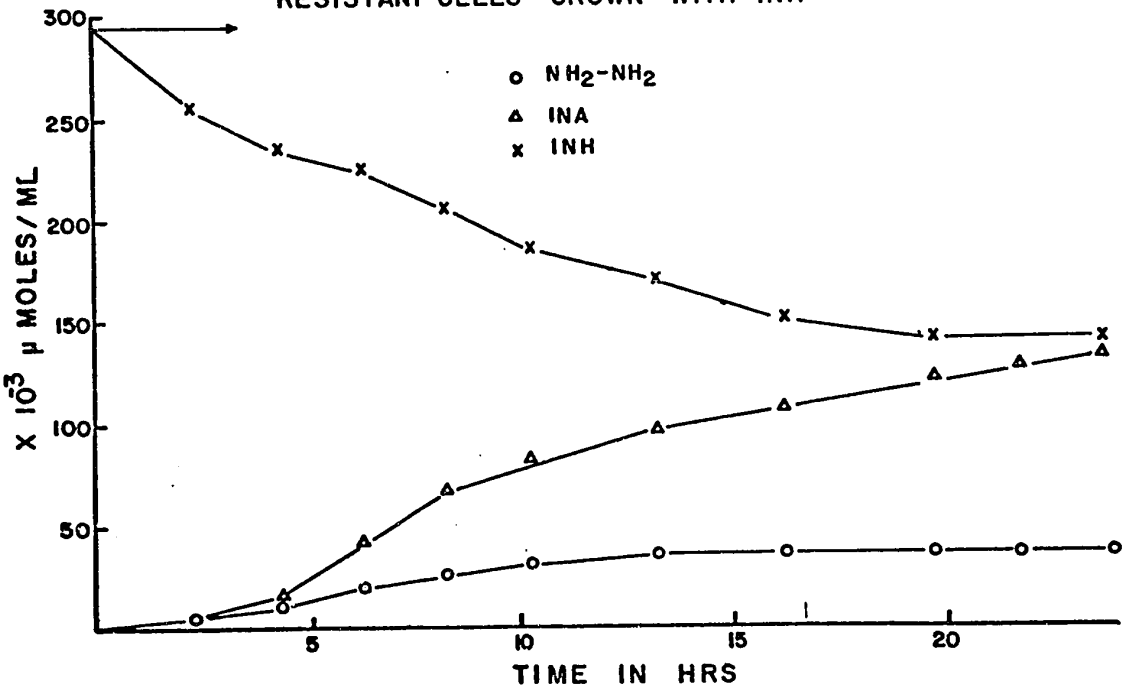
THE BREAKDOWN OF INH BY RESISTANT CELLS
GROWN WITH INH(1 ug/ml)
AND THE BREAKDOWN OF INH
BY THESE CELLS WITH CLOROMPHENICOL
ADDED AT TIME ZERO

CLOROMPHENICOL T_0

RESISTANT CELLS GROWN WITH INH



RESISTANT CELLS - GROWN WITH INH



The Metabolism of INH by Resistant Cells Grown With INH (1ug/ml), Clorompheniçol Added at Time Zero.

Resistant cells grown in the presence of 1ug/ml INH were subjected to 100ug/ml clorompheniçol at time zero. The results show (Fig.19A) that under the influence of clorompheniçol no INH was broken down and no hydrazine was produced. The significance of these results will be discussed later.

The INH Breakdown by Sensitive and Resistant Cells at the Concentration of 0.73u moles/ml or 100ug/ml INH Added at Zero Time.

Since the resistant mutant was resistant to 100ug/ml or .730 u moles/ml INH it was decided to test the effect of a higher concentration of INH (.73 u moles/ml instead of 0.29 u moles/ml) on the INH breakdown of sensitive and resistant cells. The results are seen in Figs. 20A and 20B INH only

21A and 21B INH + clorompheniçol (100ug/ml) zero time

20A and 20B INH + clorompheniçol (100ug/ml) 5 hours

22A and 22B INH only at 25° C

23A and 23B INH only at 4° C

24A and 24B INH + glycerol (10%)

25A and 25B INH + ouabain (1u mole/ml)

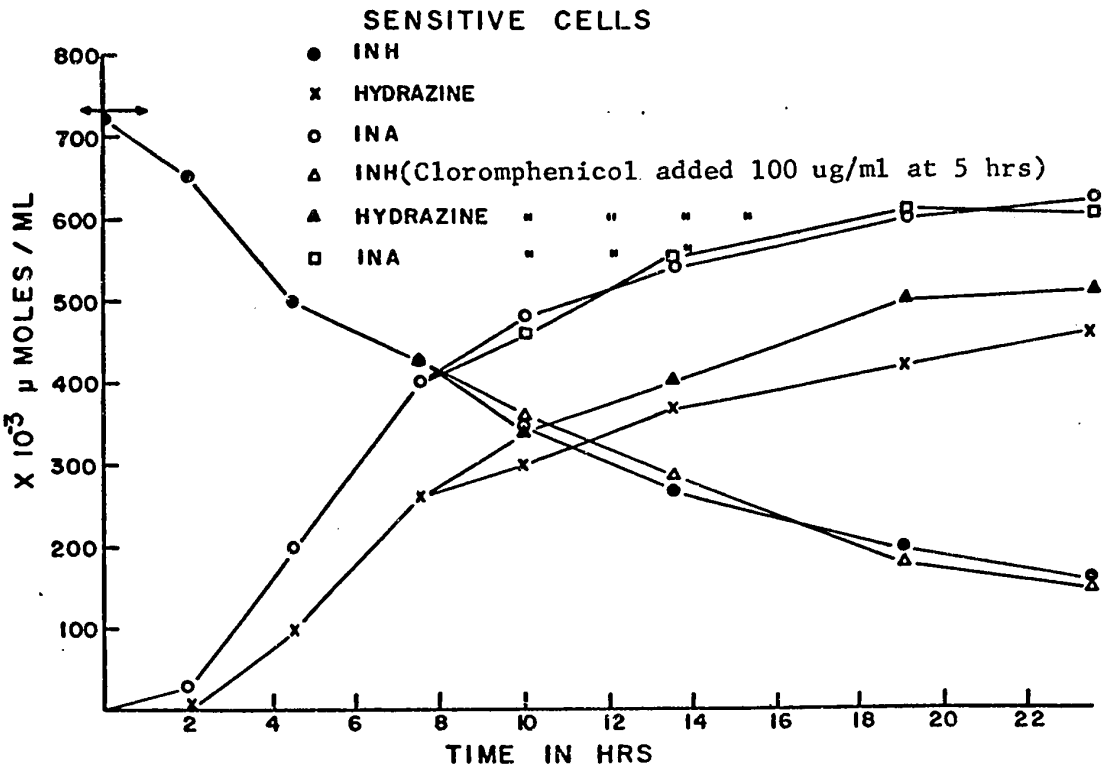
26A and 26B INH + CN (1u mole/ml)

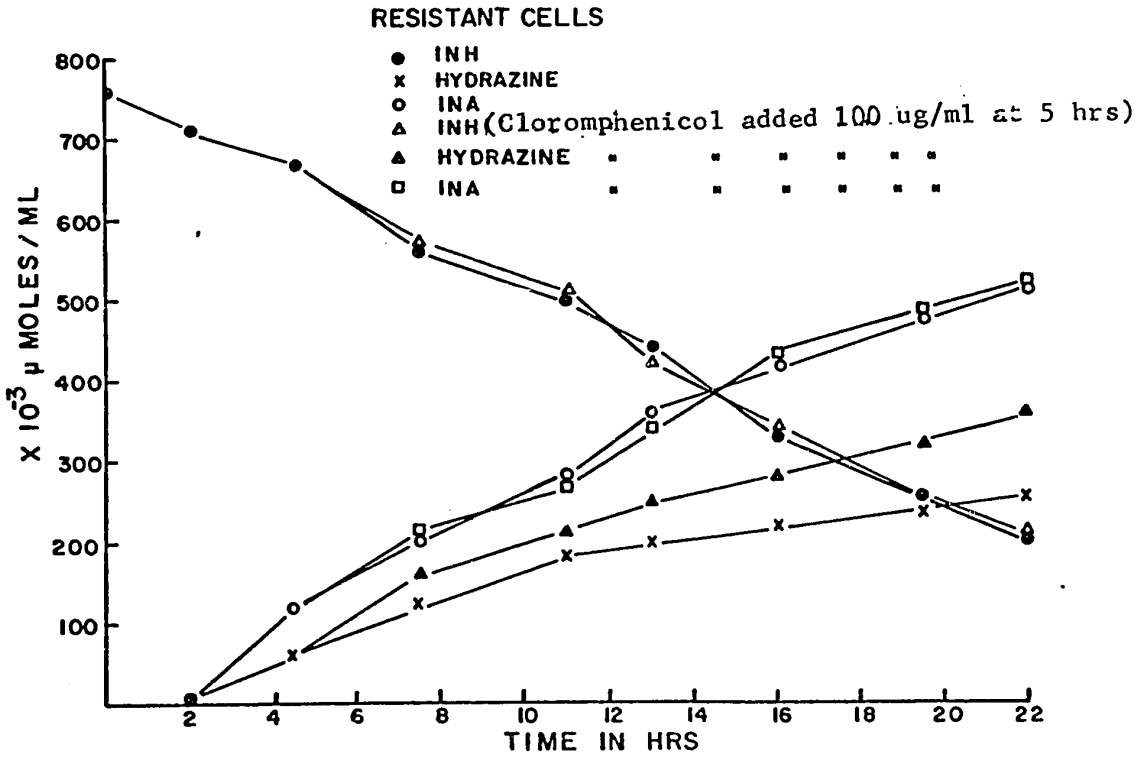
27A and 27B INH + Azide (1u mole/ml)

The above figures represent one experiment which was done by growing up large quantities of sensitive and resistant cells, splitting them up, washing them and suspending the cells in buffer. Then the

FIGS. 20A AND 20B

THE METABOLISM OF INH
BY
SENSITIVE AND RESISTANT CELLS
AND THE METABOLISM OF INH
BY
SENSITIVE AND RESISTANT CELLS
WITH CLOROMPHENICOL
ADDED AT 5 HRS.


SENSITIVE CELLS
INH
HYDRAZINE
INA
INH(Cloromphenicol added 100 ug/ml at 5 hrs)
HYDRAZINE " " " "
INA " " " "
X 10⁻³ μ MOLES / ML
800
700
600
500
400
300
200
100
2
4
6
8
10
12
14
16
18
20
22
TIME IN HRS


RESISTANT CELLS
INH
HYDRAZINE
INA
INH(Cloromphenicol added 100 ug/ml at 5 hrs)
HYDRAZINE " " " " " "
INA " " " " " "
X 10⁻³ μ MOLES / ML
800
700
600
500
400
300
200
100
2
4
6
8
10
12
14
16
18
20
22
TIME IN HRS

above chemicals were added as in the figures and INH, INA and hydrazine production was measured. In the text for purposes of clearer presentation this experiment was split up into a series of experiments as represented by the above figures and this is why comparisons are made between the various figures within the experiment. Comparisons between values obtained at .292u moles/ml INH and .730 u moles/ml INH are also made.

If Fig. 20A is compared to Fig. 13 (also sensitive cells but at .292u moles/ml) it can be seen that the same type of curve is obtained. A characteristic lag phase is observed in hydrazine and INA production and less hydrazine is produced than INA.

Comparing Fig. 20B (resistant) and Fig. 15 (resistant cells at .292u moles/ml) it can easily be seen that the two curves differ widely. At lower concentration (.292u moles/ml INH) the resistant cells produce very little hydrazine while at higher concentration INH (.730u moles/ml) the resistant cells produce substantial amounts of hydrazine.

If Fig. 20A and 20B are compared it can be easily seen that at higher INH concentration the resistant cells also produce less hydrazine than the sensitive cells. This correlates with results obtained at a lower INH concentration.

The above results are very difficult to interpret. The fact that resistant cells at higher concentration of INH produce hydrazine but not at lower concentrations is significant to a theory of the mechanism of resistance to INH which will be discussed later.

The Effect of Cloromphenicol (100ug/ml Added at Five Hours) on the INH Breakdown (.730u moles/ml) by Sensitive and Resistant Cells.

As seen in Fig. 12 if cloromphenicol (100ug/ml) is added to the incubation media at 5 hours with INH added at time zero the hydrazine production will increase and go above the control value (no cloromphenicol). These results seemed to indicate that the cloromphenicol was inhibiting the formation of an enzyme responsible for the breakdown of hydrazine. These experiments were repeated with a higher concentration of INH (.730u moles/ml). The results are included in Figs. 20A and 20B.

Comparing Fig. 12 and Fig. 20A it can be seen that cloromphenicol added at five hours has the same effect at .292u moles/ml INH as at .730u moles/ml INH. That is: 1.) The concentration of hydrazine in the incubation media rises above the control value. 2.) Cloromphenicol seems to have no effect on the INH disappearance and INA appearance in the incubation media.

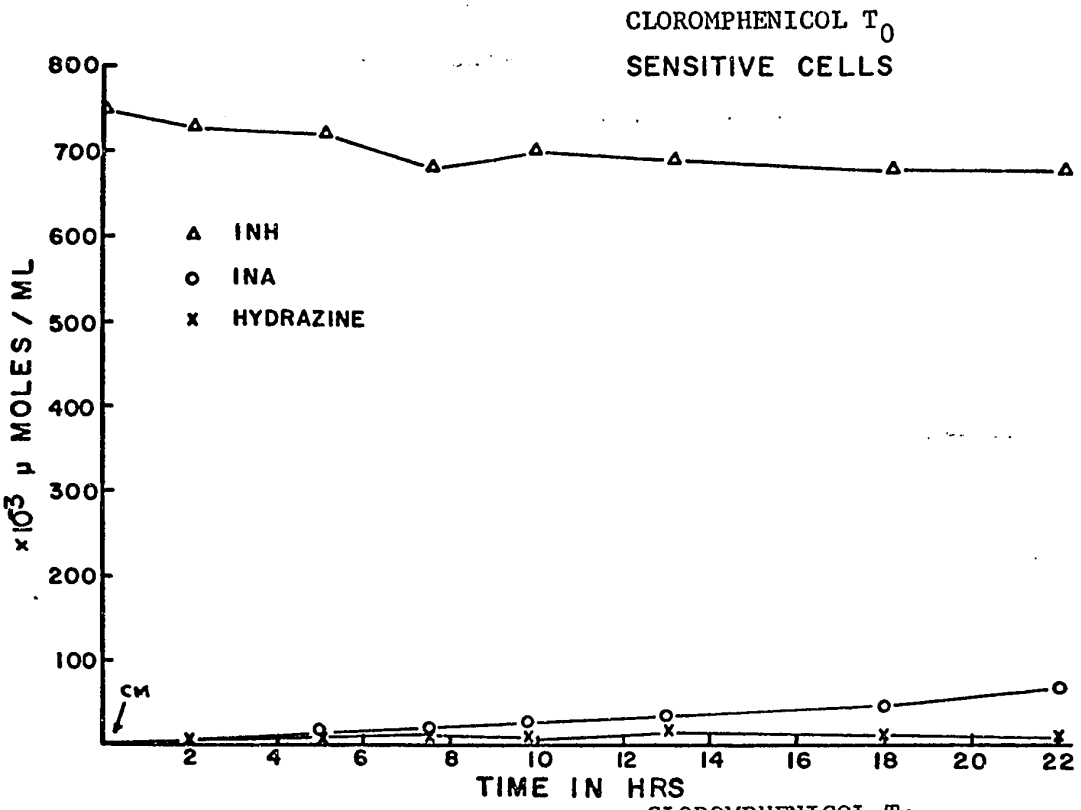
Cloromphenicol added to resistant cells five hours after .730u moles/ml INH had the same effect as on sensitive cells (Fig. 20A and Fig. 20B).

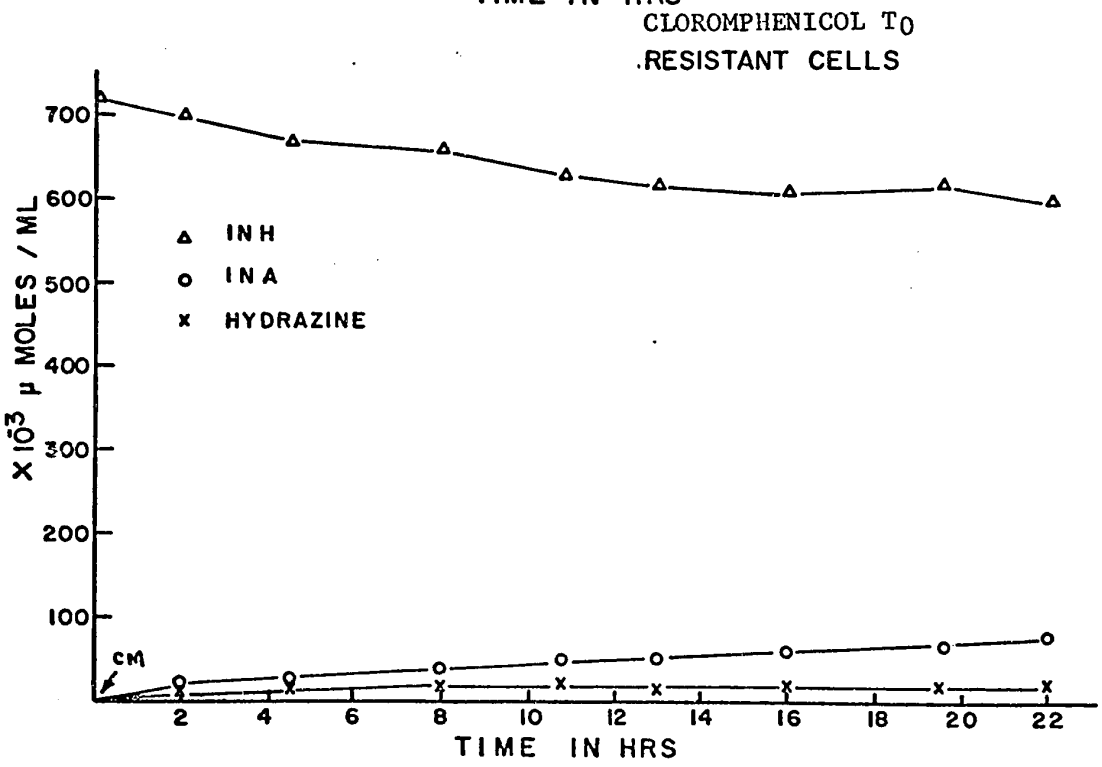
The Breakdown of INH (.730u moles/ml) by Sensitive and Resistant Cells Under the Influence of Cloromphenicol (100ug/ml) Added at Time Zero.

The effect of cloromphenicol (100 ug/ml) on INH (.292u moles /ml) breakdown was previously tested upon sensitive cells (Fig. 12) and resistant cells (Fig. 16). It was therefore decided that the effect of cloromphenicol (100ug/ml) at zero time should be tested at a higher initial concentration of INH (.730u moles/ml (Fig. 21A and 21B).

FIGS. 21A AND 21B

THE BREAKDOWN OF INH BY SENSITIVE AND RESISTANT CELLS
UNDER THE INFLUENCE OF
CLOROMPHENICOL ADDED AT TIME ZERO


CLOROMPHENICOL T_0
SENSITIVE CELLS
800
700
600
500
400
300
200
100
$\times 10^3$ μ MOLES / ML
INH
INA
HYDRAZINE
CM
2
4
6
8
10
12
14
16
18
20
22
TIME IN HRS


CLOROMPHENICOL T_0
RESISTANT CELLS
700
600
500
400
300
200
100
$\times 10^3$ μ MOLES / ML
INH
INA
HYDRAZINE
CM
2
4
6
8
10
12
14
16
18
20
22
TIME IN HRS

Comparing Fig. 12 sensitive cells and Fig. 21A (sensitive cells) it can be seen that cloromphenicol has the same effect at low and high concentrations of INH — very little INH breakdown resulting in little hydrazine and INA produced. Looking at Figs. 16 (resistant cells) and 21B the same effect can also be seen due to the cloromphenicol -- little INH breakdown and little INA and hydrazine production. Comparing Figs. 21A (sensitive cells) and 21B (resistant cells) it can be seen that cloromphenicol has the same effect on the sensitve and resistant cells ie. inhibition of INH breakdown.

The INH Breakdown by Sensitive and Resistant Cells at 25°C

Previous experiments (Figs. 12, 13) had indicated that the breakdown of INH was an enzymatic reaction. The fact that cloromphenicol did not impede uptake of radioactive INH (Fig. 11A) but inhibited INH breakdown (Fig. 12) suggested that the two processes might be distinct. However uptake was inhibited slightly by lowering the temperature to 25°C (Fig. 11B). It was therefore decided to measure the effect of an incubation temperature of 25°C on the INH breakdown of sensitive and resistant cells (Figs. 22A and 22B).

Comparing Fig. 20A (sensitive cells at 37°C) to Fig. 22A (sensitive cells at 25°C) it can be seen that both the production of hydrazine and INA is lower at 25°C than at 37°C. If Fig. 20B (resistant cells at 37°C) and 22B (resistant cells at 25°C) are compared essentially the same results are obtained. The breakdown of INH at 37°C by resistant cells is larger than the breakdown at 25°C. Finally if Fig. 22A (sensitive cells) and 22B (resistant cells) are compared it can be seen that approximately the same amount of INH is

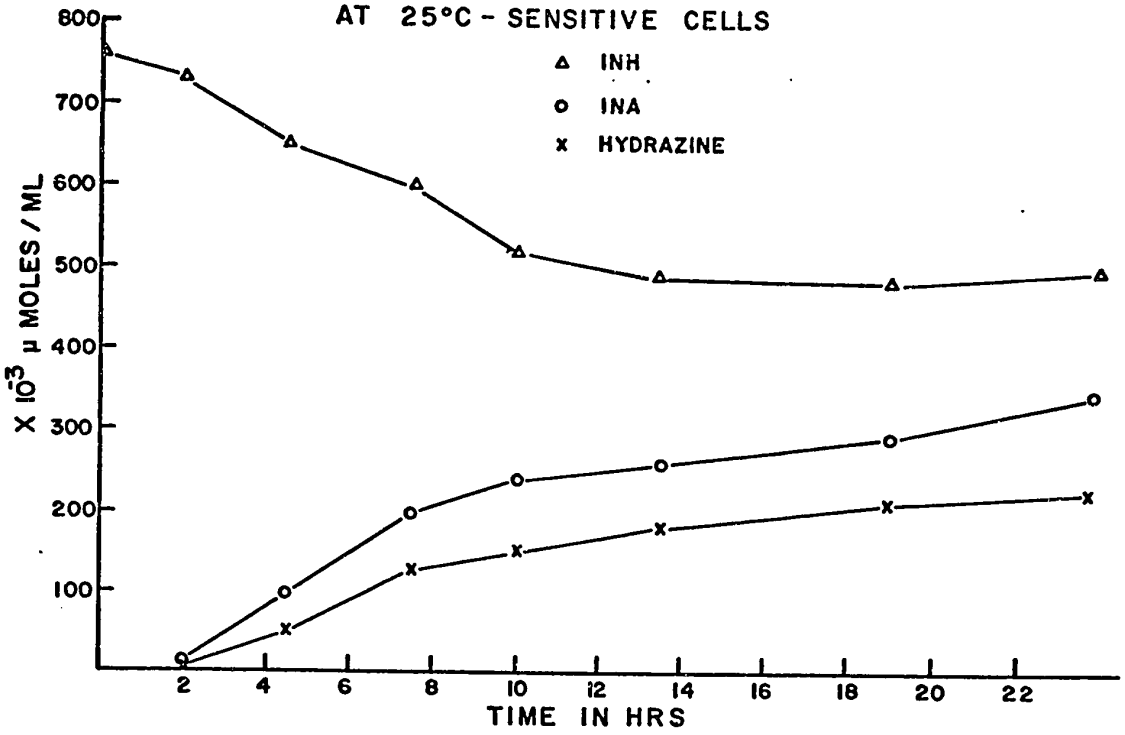
FIGS. 22A AND 22B

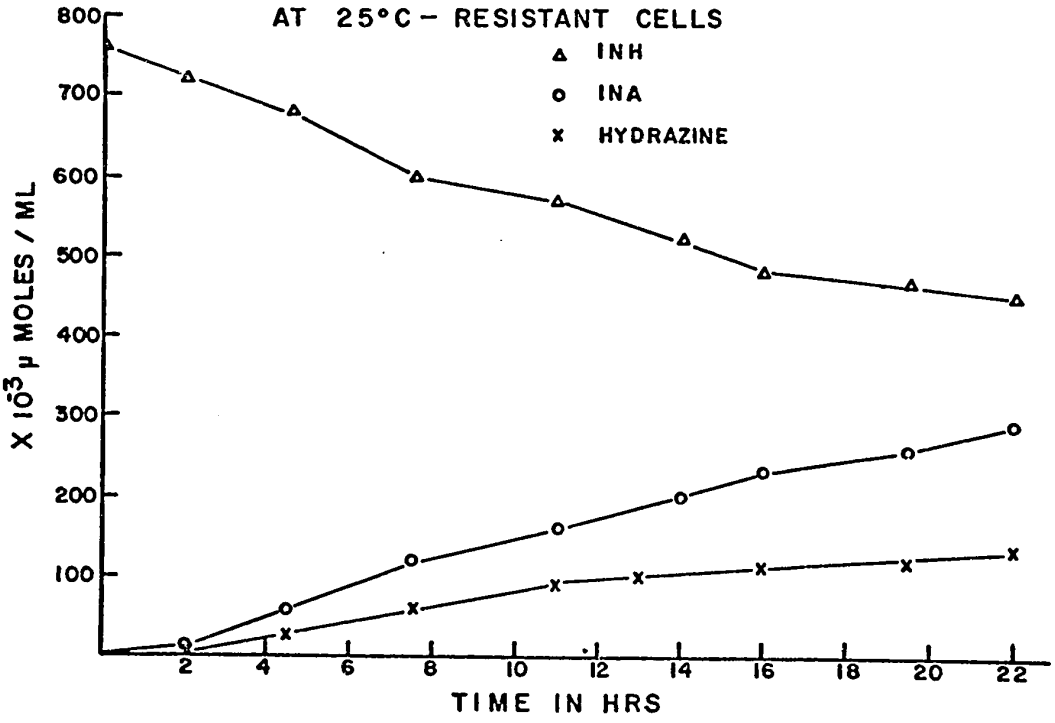
THE BREAKDOWN OF INH

BY

SENSITIVE AND RESISTANT CELLS

AT 25°C


AT 25°C - SENSITIVE CELLS
Δ INH
o INA
x HYDRAZINE
X 10⁻³ μ MOLES / ML
800
700
600
500
400
300
200
100
2
4
6
8
10
12
14
16
18
20
22
TIME IN HRS


AT 25°C - RESISTANT CELLS
Δ INH
o INA
x HYDRAZINE
X 10⁻³ μ MOLES / ML
800
700
600
500
400
300
200
100
2
4
6
8
10
12
14
16
18
20
22
TIME IN HRS

broken down but that the resistant produces less hydrazine.

These results are to be expected if the INH breakdown reaction is enzymatic and they do not conflict with the results obtained at 37°C as the resistant cells still produce less hydrazine than the sensitive cells at 25°C.

The INH Breakdown of Sensitive and Resistant Cells at 4°C.

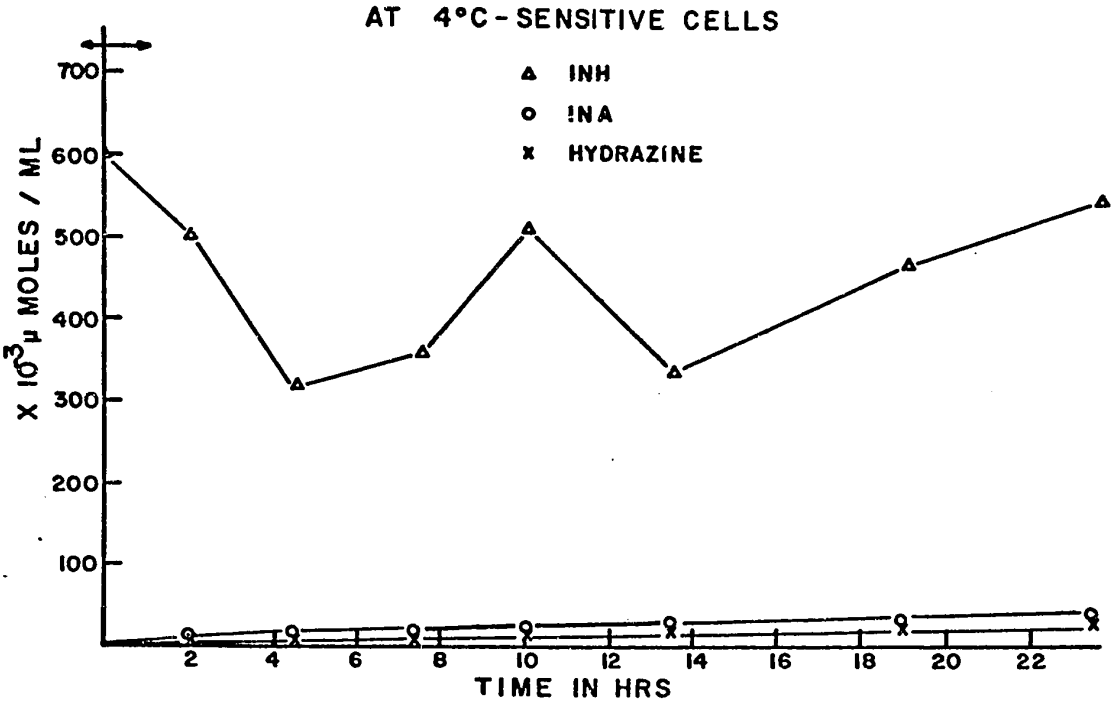
When the uptake of the drug (INH) was measured at 4°C (Fig. 11C) it was found that the uptake of both the sensitive and resistant cells was above the value at 37°C. These results contradicted the results obtained at 25°C (Fig. 11B) as one would expect uptake to be temperature dependent if the uptake reaction involved an enzymatic process. It was therefore decided that the breakdown of INH would be studied at 4°C.

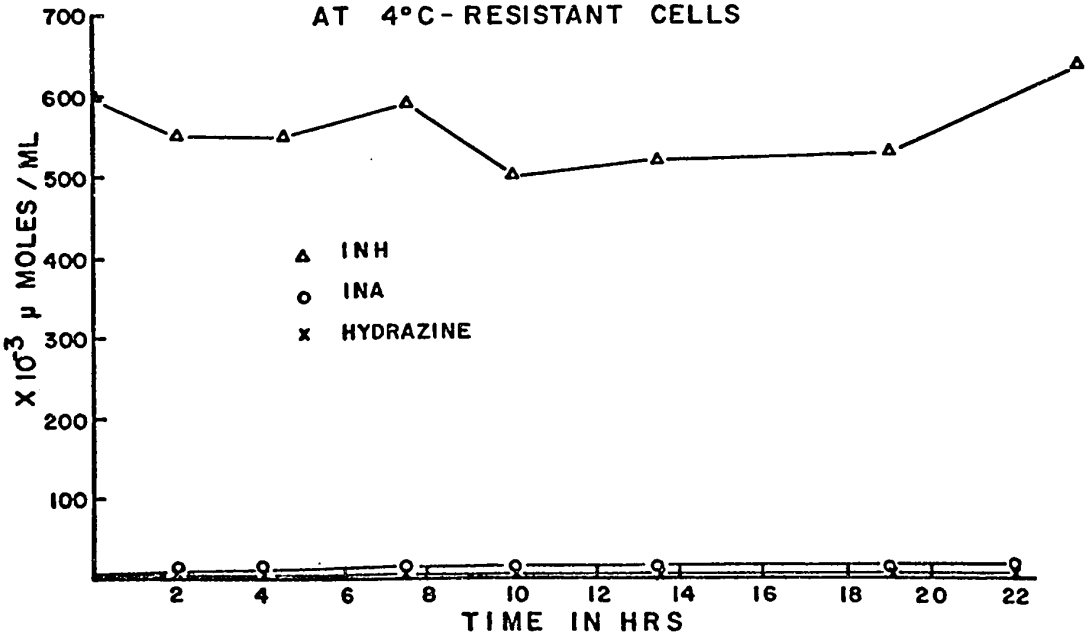
The results (Figs. 23A and 23B) showed that there is no significant hydrazine or INA production for either the sensitive or resistant strain at 4°C. The sensitive cells seem to produce slightly more hydrazine than the resistant cells but the difference is small.

However if one looks at the INH disappearance it can be easily seen that INH is taken up by sensitive and resistant cells with most of the uptake taking place at zero time. The amount of INH disappearing in the case of the sensitive cells seems to be greater than in the case of resistant cells but it is difficult to tell as the results fluctuated. It can also be seen (Figs. 23A and 23B) that the disappearance of INH is not connected with the breakdown to INA and hydrazine as neither is produced. The INH disappearance at 4°C with no breakdown substantiates the uptake measured with isotopes (C^{14}-INH) (Fig. 11C).

FIGS. 23A AND 23B

THE BREAKDOWN OF INH
BY
SENSITIVE AND RESISTANT CELLS
AT 4°C


AT 4°C - SENSITIVE CELLS
INH
INA
HYDRAZINE
X 10^-3 μ MOLES / ML
700
600
500
400
300
200
100
2
4
6
8
10
12
14
16
18
20
22
TIME IN HRS


AT 4°C - RESISTANT CELLS
INH
INA
HYDRAZINE
X 10^-3 μ MOLES / ML
700
600
500
400
300
200
100
2
4
6
8
10
12
14
16
18
20
22
TIME IN HRS

The significance of these results is hard to define. Possibly they show that enzymes involved in uptake are different from those that breakdown INH. However, Wimpenny (8) has postulated that the uptake of radioactive INH by B.C.G. at 4°C is a process involving an element of absorption. This last possibility was guarded against in our experiments by washing our cells in non-radioactive INH and yet uptake was still present at 4°C (Fig. 11C). The latter results indicate that INH C^{14} was therefore very tightly bound or that it was in a location which was not reached by the non-radioactive INH. This location could be postulated to be inside the cell as other scientists have shown that the cell's permeability barrier is disrupted at lower temperatures.

The Effect of 10% Glycerol in the INH Breakdown by Sensitive and Resistant Cells at 37°C.

In the uptake experiments using C^{14} INH it was found that glycerol reduced INH uptake of sensitive cells to the level which was found in the resistant cells (Fig. 11E).

These curious results prompted us to test the effect of 10% glycerol on INH breakdown by the resistant and sensitive cells.

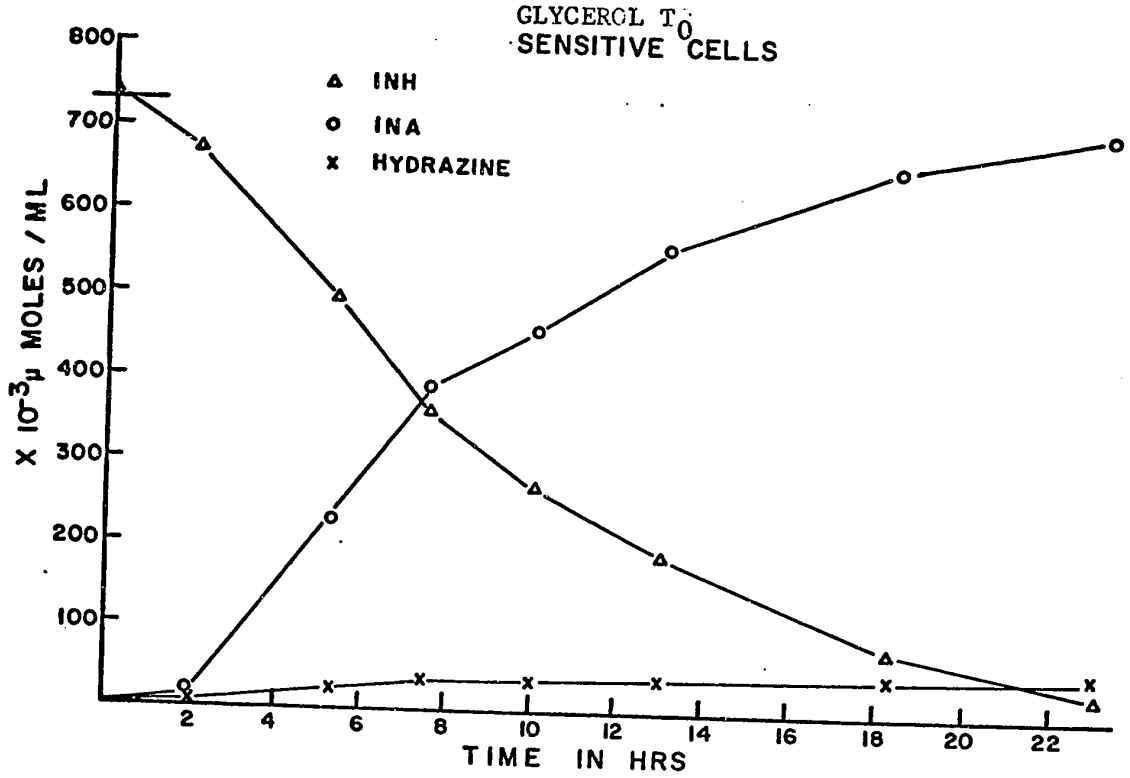
The following results were obtained (Figs. 24A and 24B).

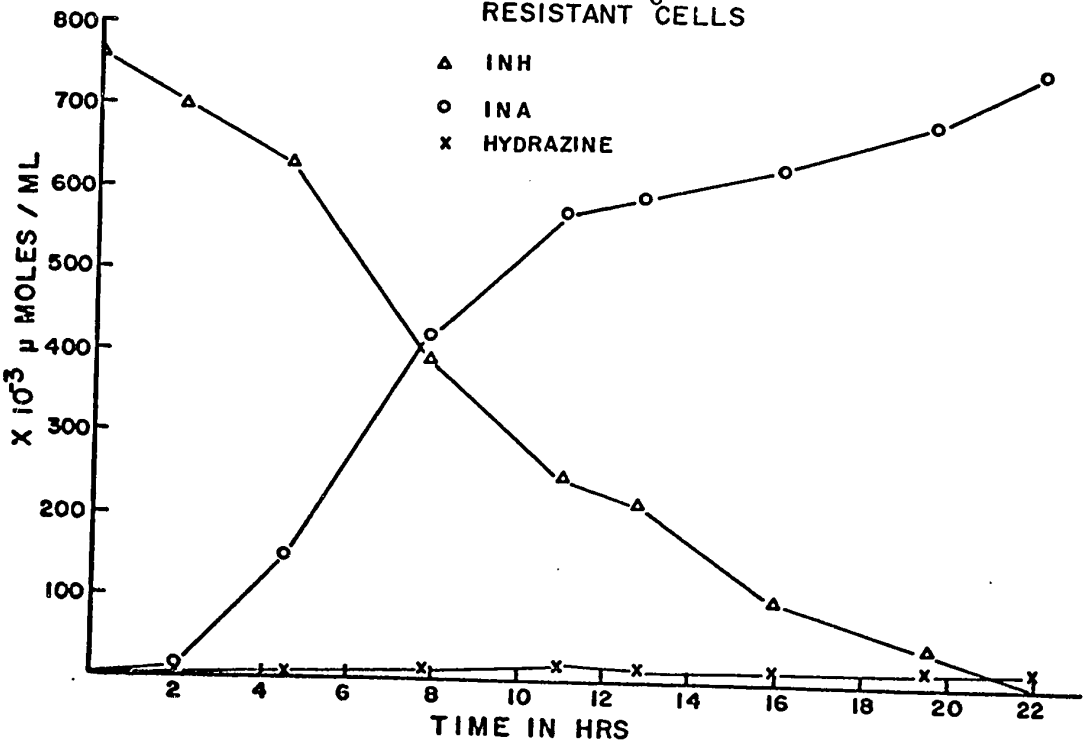
1- Comparing Fig. 20A and Fig. 24A it can be seen that under the influence of glycerol much more INH is broken down within a shorter period of time by the sensitive organism.

2- This breakdown is accomplished with very little hydrazine production whereas where no glycerol is added hydrazine production is substantial.

FIGS. 24A AND 24B

THE EFFECT OF GLYCEROL (10%)
ON THE INH BREAKDOWN
BY
SENSITIVE AND RESISTANT CELLS


GLYCEROL T0
SENSITIVE CELLS
INH
INA
HYDRAZINE
X 10-3μ MOLES / ML
800
700
600
500
400
300
200
100
2
4
6
8
10
12
14
16
18
20
22
TIME IN HRS


GLYCEROL T0
RESISTANT CELLS
INH
INA
HYDRAZINE
X 10-3 μ MOLES / ML
800
700
600
500
400
300
200
100
2
4
6
8
10
12
14
16
18
20
22
TIME IN HRS

3- The resistant cells breakdown INH more vigorously in the presence of glycerol.

4- The resistant cells produce much less hydrazine under the influence of glycerol than without glycerol (Fig. 20B).

5- The breakdown of INH is approximately equal in sensitive and resistant cells in presence of glycerol.

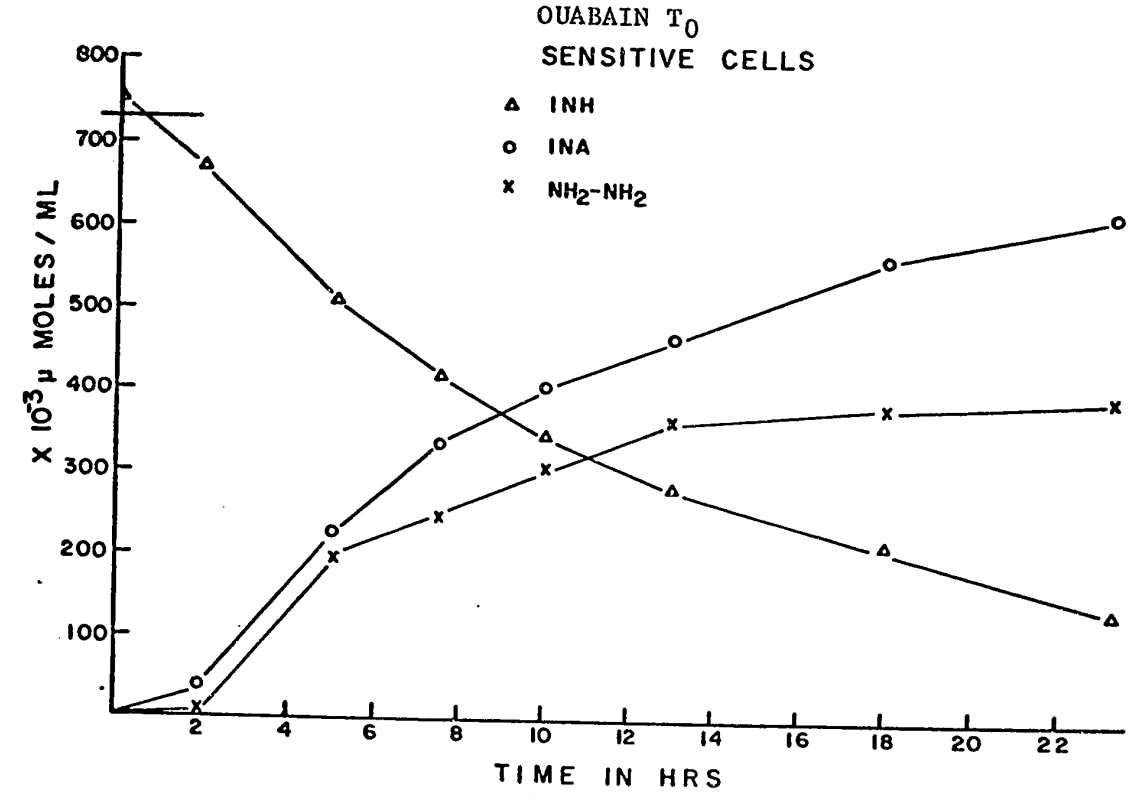
Mycobacteria oxidize glycerol completely into H_2O and CO_2 (106) and glycerol is therefore included in most growth media of Mycobacteria. If INH uptake is somehow energy dependent it should be increased in the presence of glycerol. This however is not the case as seen in Fig. 11E but is decreased in sensitive cells to the uptake value of the resistant cells. Yet glycerol seems to increase the breakdown rate of INH by sensitive and resistant strains without hydrazine production. Possible reasons for these results will be discussed later.

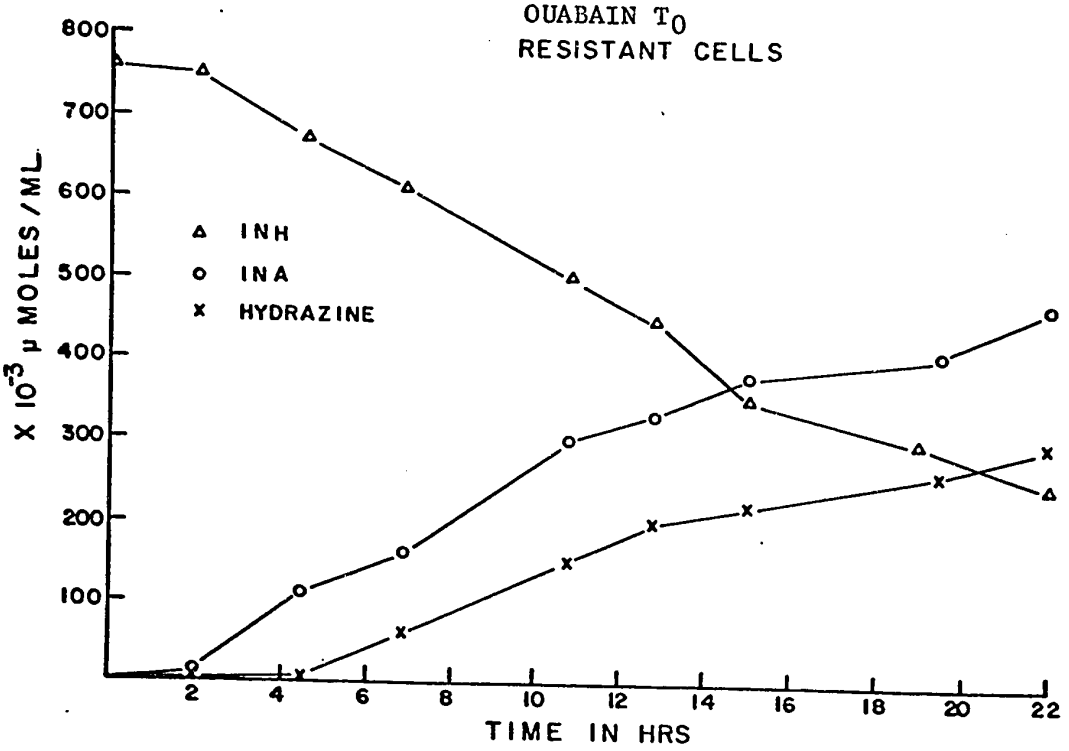
<u>The Influence of 1u mole/ml Ouabain on the INH Breakdown of Resistant Cells at 37°C.</u>

A very valuable finding for studies of active transport of K^+ and Na^+ occured in 1953 when Schatzmann (107) found that a cardiac glycoside (ouabain) completely inhibited the extrusion of Na^+ and the accumulation of K^+ by cold stored erythrocytes without interfering with glycolysis which supplies metabolic energy to the erythrocyte. Whittam (108) showed that as ouabain inhibited active transport it also spared the ATP supply of the cell. Post (109, 110) showed with erythrocyte ghosts that their membrances exhibited ATPase activity which was blocked by cardiac glycosides. Thus it was shown that ouabain

FIGS. 25A AND 25B

THE EFFECT OF OUABAIN (1 ug/ml)
ON THE BREAKDOWN OF INH
BY
SENSITIVE AND RESISTANT CELLS


OUABAIN T_0
SENSITIVE CELLS
INH
INA
NH_2-NH_2
X 10^{-3} μ MOLES / ML
800
700
600
500
400
300
200
100
2
4
6
8
10
12
14
16
18
20
22
TIME IN HRS


OUABAIN T_0
RESISTANT CELLS
INH
INA
HYDRAZINE
X 10^{-3} μ MOLES / ML
800
700
600
500
400
300
200
100
2
4
6
8
10
12
14
16
18
20
22
TIME IN HRS

inhibits the active transport of K^+ - Na^+ in mammalian tissues via inhibition of ATPase located on the cell surface responsible for the obtainment of energy for the process.

It seemed interesting to test the effect of ouabain on the transport and breakdown of INH by the mycobacterial cell. The idea was that an ATPase might be involved in INH transport into the mycobacterial cell as uptake of INH-C^{14} in the presence of cyanide (Fig. 11D) had indicated that the uptake might be energy dependent.

Ouabain (1u moles/ml) was added at time zero and the results are seen in Figs. 25A and 25B.

If the necessary comparisons are made within the experiment it can be seen that ouabain does not affect INH breakdown in either the sensitive or resistant cells.

The Influence of Cyanide (CN) (1u mole/ml) on the INH Breakdown By Sensitive and Resistant Cells at 37°C.

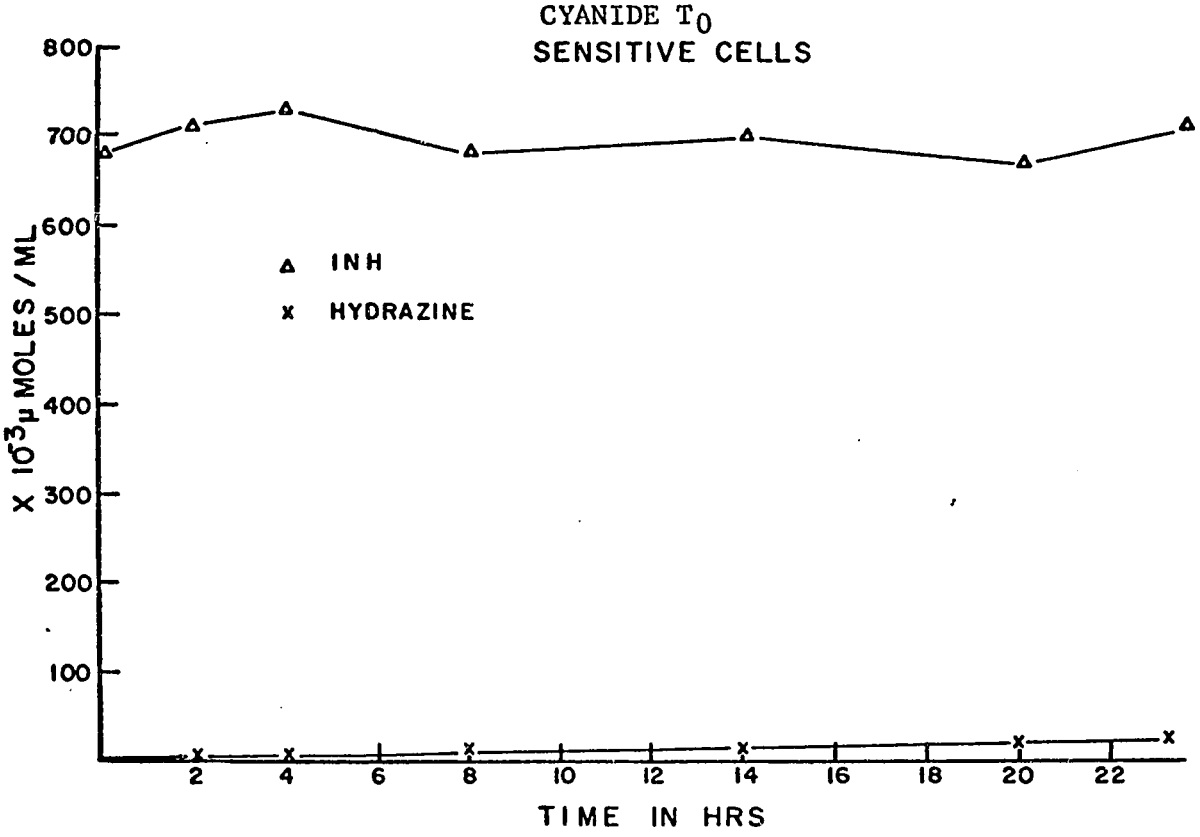
Previous radioactivity experiments (Fig. 11D) using C^{14} INH and CN had suggested that INH uptake might be energy linked. The effect of cyanide on INH breakdown in sensitive and resistant cells was therefore investigated.

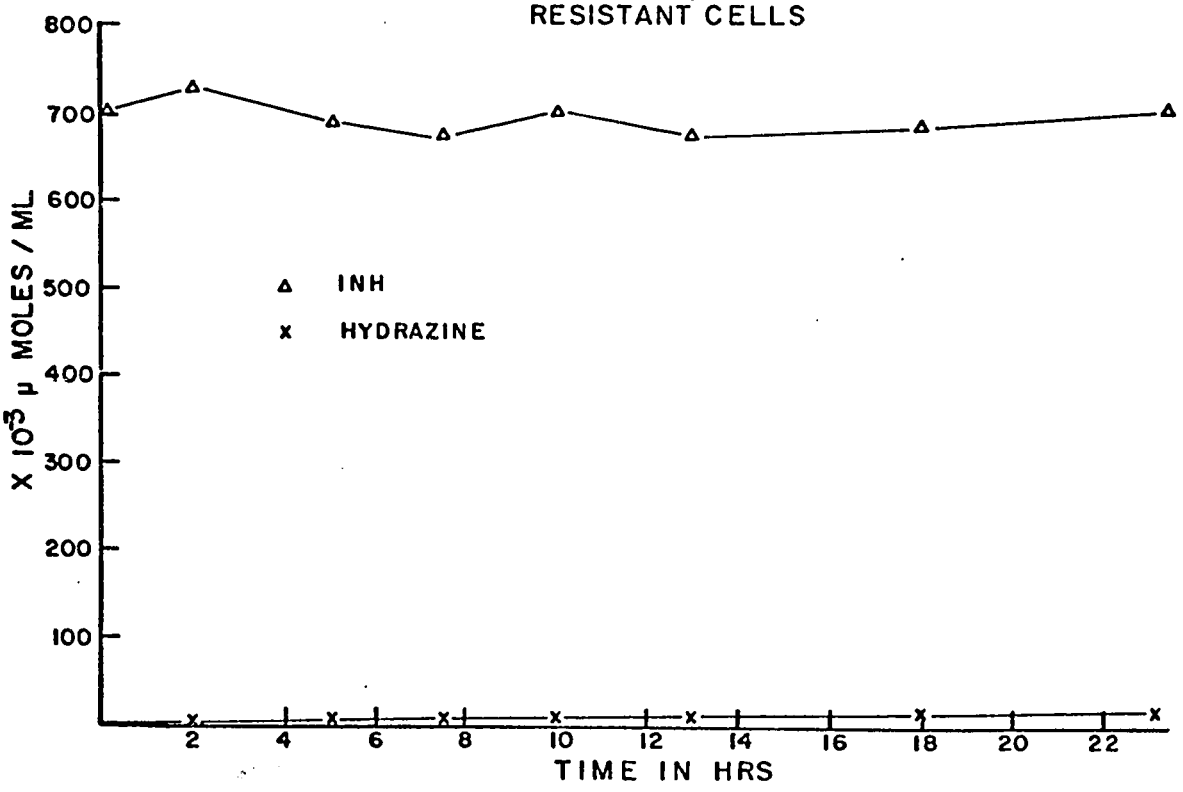
Cyanide (1u mole/ml) was added at time zero and the results are seen in Figs. 26A and 26B. The results only show INH and hydrazine as the CN interfered with INA measurement due to the fact that one of the reagents in the determination of INA contained CN.

If the necessary comparisons are made within the experiment it can be easily seen that in the presence of cyanide there is no INH disappearance or hyrazine appearance either in sensitive or resistant cells.

FIGS. 26A AND 26B

THE INFLUENCE OF CYANIDE (1 U MOLE/ML)
ON THE INH BREAKDOWN
BY
SENSITIVE AND RESISTANT CELLS
AT 37° C


CYANIDE T_0
SENSITIVE CELLS
800
700
600
500
400
300
200
100
X 10^{-3} μ MOLES / ML
Δ INH
x HYDRAZINE
2
4
6
8
10
12
14
16
18
20
22
TIME IN HRS


CYANIDE T_0
RESISTANT CELLS
800
700
600
500
400
300
200
100
X 10^{-3} μ MOLES / ML
Δ INH
x HYDRAZINE
2
4
6
8
10
12
14
16
18
20
22
TIME IN HRS

These results seem to indicate that INH breakdown is energy dependent and entail three possibilities for CN inhibition of the process:

1- The synthesis of the enzyme for INH breakdown would obviously be energy dependent and so would be inhibited by CN.

2- If the uptake of the drug is energy dependent as indicated by Fig.11D the INH would not be able to enter the cell to be broken down and so no hydrazine would be produced.

3- The possibility exists that the breakdown process is part of the uptake process, i.e. the two are linked on the cell membrane so that inhibition of one (uptake) would inhibit the other also.

The Effect of Azide (N_2 - NH) (1u mole/ml) on INH Breakdown By Sensitive and Resistant Cells at 37°C.

Azide, along with dinitrophenol and cyanide is an inhibitor of energy metabolism of the cell (100). It was decided to test the effect of Azide (1u mole/ml) added at time zero on INH breakdown. (Figs. 27A and 27B).

If the necessary comparisons are made between the various figures it can be seen that:

1- Azide inhibits the INH disappearance and breakdown in the sensitive cells.

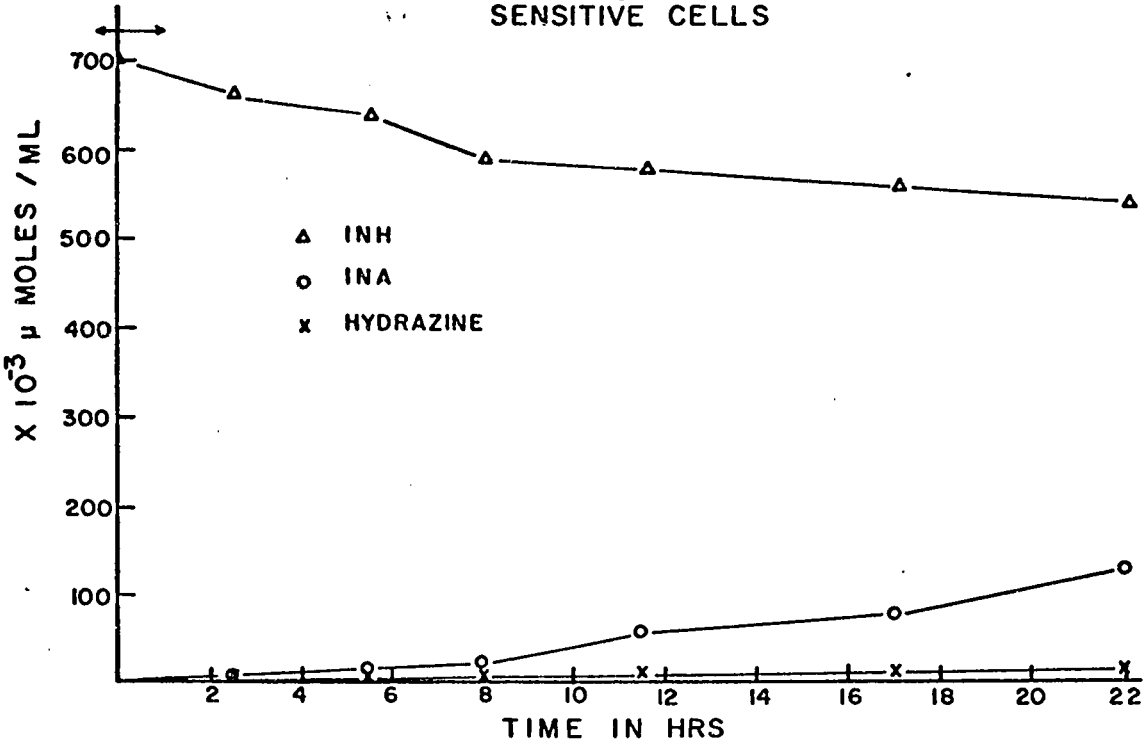
2- Azide does not inhibit INH disappearance and breakdown in the resistant cells as a lot of INA and hydrazine are produced.

A clue as to the reason why Azide does not affect the resistant cells but only the sensitive cells is given by the formula for Azide (HN = N - N = NH) which closely resembles that of Hydrazine (NH_2 - NH_2).

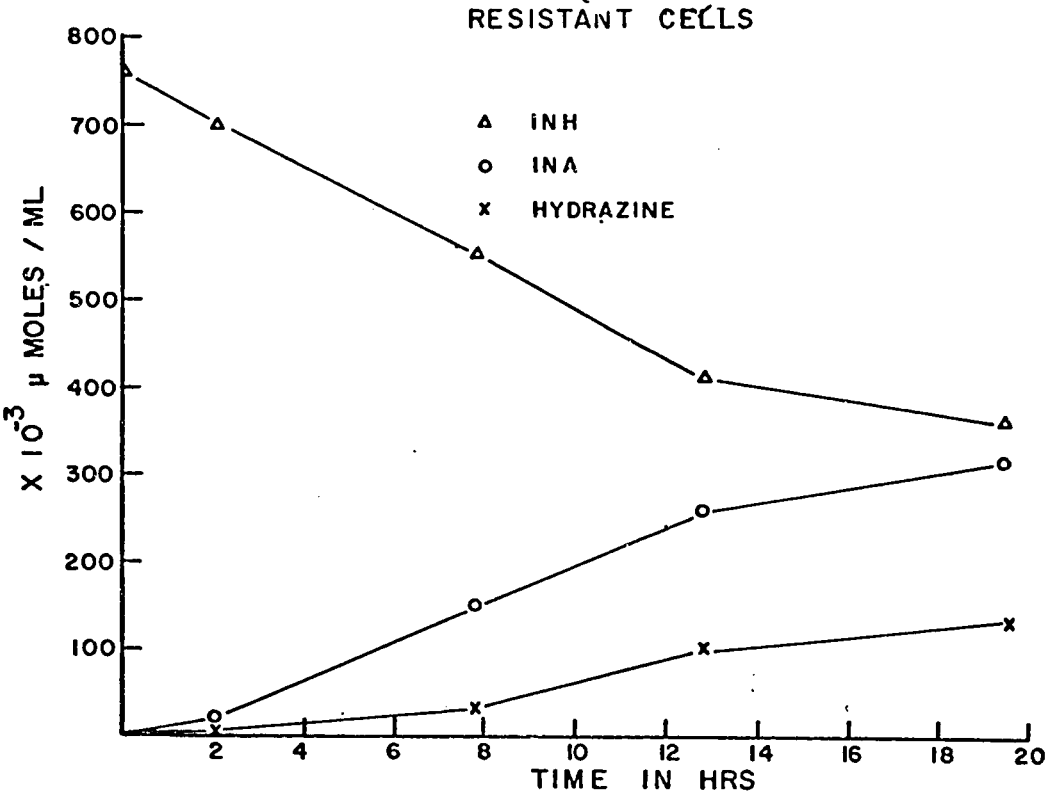
FIGS. 27A AND 27B

THE EFFECT OF AZIDE (1 u mole/ml)
ON INH BREAKDOWN
BY
SENSITIVE AND RESISTANT CELLS

AZIDE T_0
SENSITIVE CELLS



AZIDE T_0
RESISTANT CELLS



As later experiments indicated that the resistant cells do not take up as much hydrazine as sensitive cells it could be postulated that resistant cells are not affected by Azide because they do not take the compound up.

Extraction of Hydrazine, INA and INH from Sensitive and Resistant Cells.

Fig. 13 had shown that the amount of INH which had disappeared did not equal the amount of INA produced. As the reaction INH $\rightarrow$ INA + Hydrazine probably proceeds on a mole/mole basis, various possibilities arise. Either INA is taken up by the cells, like INH is taken up or both are taken up. The possibility that INH, hydrazine or INA might enter the cells was tested as follows.

A normal incubation experiment was performed on sensitive and resistant cells with samples being taken at various times. Supernatant was analyzed for INA, hydrazine and INH and cells were taken up in the original amount of buffer and held at 100°C for 1 hour. The cells were then spun down at 20,000 rpm for 10 minutes and the supernatant was analyzed for INA, INH and hydrazine. Negative results were obtained for each time sample as none of the three compounds could be detected in the boiled buffer for both sensitive and resistant cells. These results indicate that:

1- As no INH was released by the boiled cells therefore INH as such does not enter the cell but only its metabolized product. This is because radioactive studies showed the C^{14} did enter the cell (Fig. 11) but as the label is on the carbonyl atom it is hard to say whether INH itself enters the cell or a metabolized product of INH. Another

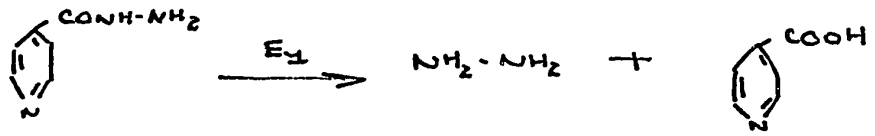
possibility would be that INH is bound to some locus in the cell and is therefore not released.

2- Since no INA is released by boiling, it can be postulated that either INA is changed enzymatically within the cell or it is bound to a cell constituent.

3- The fact that no hydrazine is released by boiling partially substantiates the theory the hydrazine is destroyed by the cells to an unknown product. The latter possibility however does not exclude the idea that hydrazine could be bound to some cell constituent and would therefore not be released.

Attempt to Measure the Production of NH_3 by Sensitive and Resistant Cells Incubated with INH and Hydrazine.

It was shown by previous experiment that INH is broken down by sensitive and resistant bacteria as follows (Figs. 12, 13)



However other evidence (Fig. 13) indicated that a second enzyme was induced which broke down the hydrazine to an unknown product. This product was thought to be NH_3 and this possibility was tested.

Sensitive and resistant cells were washed and suspended in buffer. At zero time INH (.730 u moles/ml) was added to a batch of sensitive and a batch of resistant cells. Hydrazine (.730 u moles/ml) was added to another batch of sensitive and another batch of resistant

cells. The four flasks were incubated at 37°C and time samples were analyzed at 0, 2, 4, 6 and 8 hours. Cells were spun down and the supernatant was analyzed for NH_3.

The results showed that no NH_3 was produced by either the sensitive or resistant cells from INH or Hydrazine.

A sister experiment was then performed where sensitive and resistant cells were incubated with .730 u moles/ml NH_3. Analysis of the results of this experiment showed that at zero time virtually no NH_3 could be detected in the buffer.

This last result is difficult to understand until one remembers that zero is not actually zero time. That is before the cells are actually out of contact with the buffer, a period of approximately fifteen minutes passes.

We can therefore postulate that if NH_3 is the end product of INH metabolism it is not measurable or detectable as it is taken up very quickly from the incubation media.

Attempt to Measure the Production of Hydroxylamine (NH_2-OH) by Sensitive and Resistant Cells Incubated with INH and Hydrazine.

The previous experiment had shown that NH_3 might not be the product of hydrazine breakdown in either the sensitive or resistant strains. The same types of experiments were performed to see if INH

and hydrazine were broken down to hydroxylamine. No hydroxylamine was produced in either the sensitive or resistant cells exposed to INH and hydrazine at time zero. The complete metabolic pathway of INH therefore remained unclear.

The Disappearance of Hydrazine, Nicotinic Acid (NA), INA and Hydroxylamine from the Incubation Media of Resistant and Sensitive Cells.

Early experiments (Figs. 12, 13, etc.) had shown that most of the INH in the media is broken down to INA and hydrazine. It was therefore postulated that INH might not be the agent which produces the inhibition on the bacteria but that one of its breakdown products might cause the inhibition. As a preliminary step in testing this hypothesis it was decided that the uptake of the breakdown products and possible breakdown products should be tested.

These incubation experiments were performed as in methods and at zero time, .730u moles/ml of INA, NA, hydrazine and hydroxylamine were added to separate batches of sensitive and resistant cells. The disappearance of each compound was then measured over twenty-four hours. The results (Figs. 28A and 28B) show that:

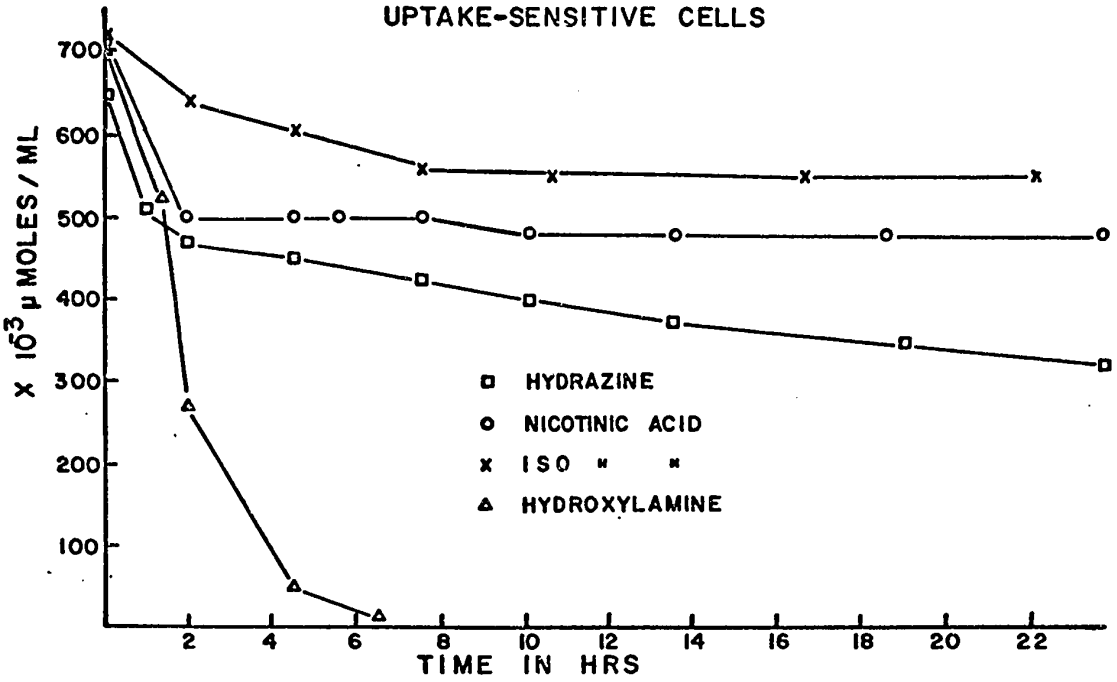
1- Sensitive cells do not take up INA to a great extent with most of the uptake being within the first seven hours.

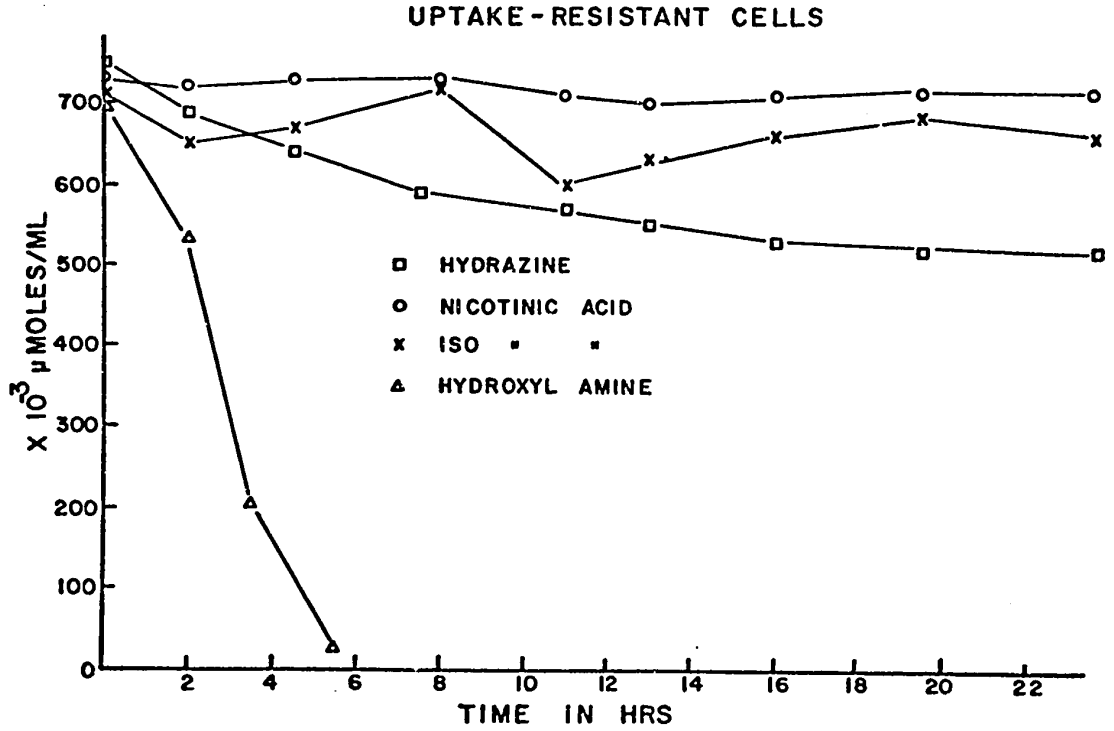
2- Sensitive cells take up more NA than INA with most of the uptake taking place within the first two hours.

3- Sensitive cells take up hydrazine to a greater extent than either INA or NA with most of the uptake taking place within the first two hours.

FIGS. 28A AND 28B

THE UPTAKE OF INH, INA; NICOTINIC ACID(NA)
HYDRAZINE AND HYDROXYLAMINE
BY
SENSITIVE AND RESISTANT CELLS


UPTAKE-SENSITIVE CELLS
X 10^-3 µ MOLES / ML
700
600
500
400
300
200
100
HYDRAZINE
NICOTINIC ACID
ISO " "
HYDROXYLAMINE
2
4
6
8
10
12
14
16
18
20
22
TIME IN HRS


UPTAKE - RESISTANT CELLS
X 10^-3 µ MOLES/ML
700
600
500
400
300
200
100
0
HYDRAZINE
NICOTINIC ACID
ISO " "
HYDROXYL AMINE
2
4
6
8
10
12
14
16
18
20
22
TIME IN HRS

4- Hyroxylamine is almost totally taken up within the first six hours by sensitive cells.

An entirely different picture is obtained with resistant cells (Fig. 28B) where it can be seen that:

1- Nicotinic acid does not disappear in the resistant cells.

2- INA like NA does not seem to disappear from the media in the resistant cells.

3- Hyrazine disappears from the media to a lesser extent in the case of the resistant cells.

4- Under the influence of the resistant cells the hydroxylamine seems to disappear even faster than in the sensitive cells. These results suggest a reason why the resistant cells do not take up radioactive INH (Fig. 11A). Resistant cells break down almost all the INH to INA and at the same time do not take up any INH-C^{14} (Fig. 11A). Therefore the reason these cells do not become as radioactive as sensitive cells is because they do not take up INA (Fig. 28B).

Another interesting point is that resistant cells remove much less hydrazine from the media than sensitive cells. However the results indicate that neither the sensitive nor resistant cells destroy hyrazine to any great extent.

<u>The Inhibition of Growth of Sensitive and Resistant Cells by INA, INH, Hydrazine and Hydroxylamine.</u>

The hypothesis that INH was not the inhibitory agent but that one of its breakdown products inhibited the growth of sensitive cells was further tested. This seemed possible as it was known that hyrazine is toxic to bacterial cells, its probable locus of

action being DNA. As it is sometimes used as a mutagenic agent (111).

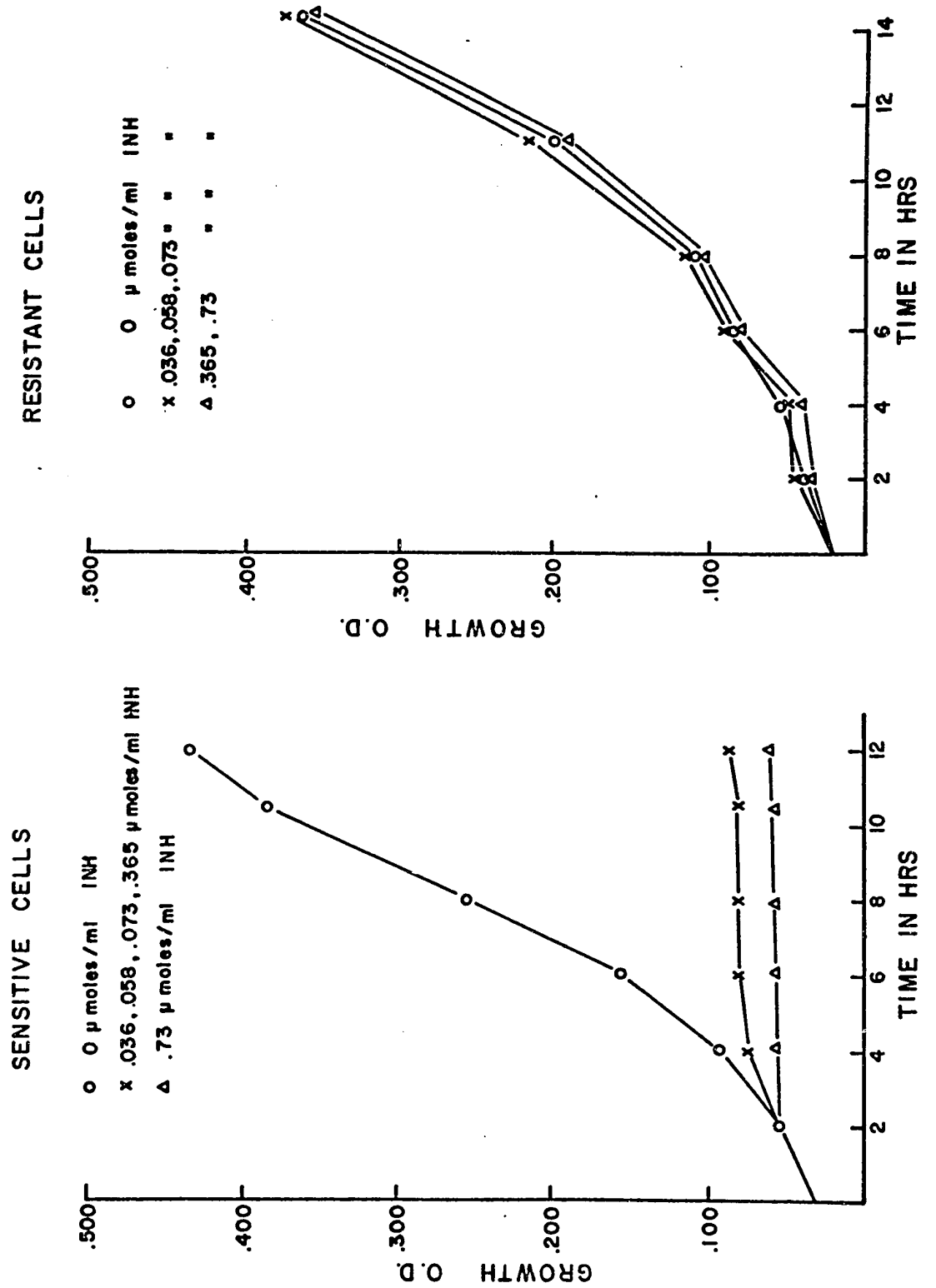
Tsukamura (113) has suggested that hydroxylamine inhibition should be used to differentiate different species of mycobacteria. Thus he showed that M. tuberculosis seems to be inhibited most strongly by hydroxylamine while the saphrophytic mycobacteria were less sensitive to hydroxylamine. The sensitivity of mycobacteria to hydroxylamine strongly parallels the sensitivity of mycobacteria to INH (2).

The experiment was performed as follows. Twenty-one side arm flasks of sensitive and twenty-one side arm flasks of resistant cells were grown up in Sauton media to an optical density of .035 (.13 mgs/ml cells). One flask of each group was left as control and to the other twenty flasks INA, hydrazine, hydroxylamine and INH were added in the following concentrations: .036u moles/ml, .058u moles/ml, .073u moles/ml, .365u moles/ml, and .73u moles/ml. The growth of each flask with a certain concentration of one of the above compounds was carefully recorded with time. The results are shown in the following figures:

Figs 29A	INH-added-sensitive cells
29B	INH-added-resistant cells
30A	hydrazine-added-sensitive cells
30B	hydrazine-added-resistant cells
31A	INA-added-sensitive cells
31B	INA-added-resistant cells
32A	hydroxylamine-added-sensitive cells
32B	hydroxylamine-added-resistant cells

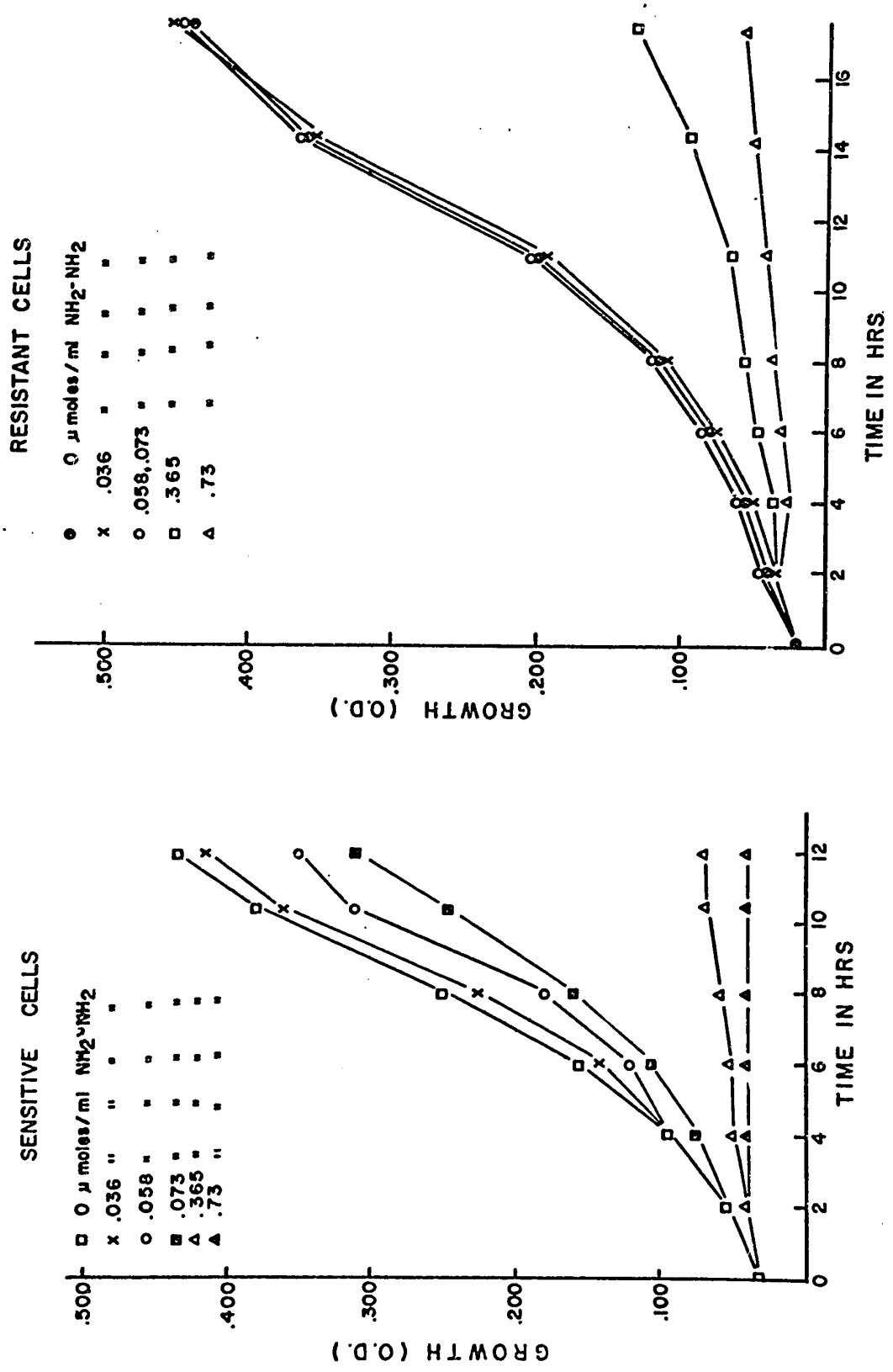
FIGS. 29A AND 29B

THE EFFECT OF INH
ON THE GROWTH OF SENSITIVE
AND RESISTANT CELLS


RESISTANT CELLS
o 0 μ moles/ml INH
x .036, .058, .073 " " "
Δ .365, .73 " " "
GROWTH O.D.
.500
.400
.300
.200
.100
2
4
6
8
10
12
14
TIME IN HRS
SENSITIVE CELLS
o 0 μ moles/ml INH
x .036, .058, .073, .365 μ moles/ml INH
Δ .73 μ moles/ml INH
GROWTH O.D.
.500
.400
.300
.200
.100
2
4
6
8
10
12
TIME IN HRS

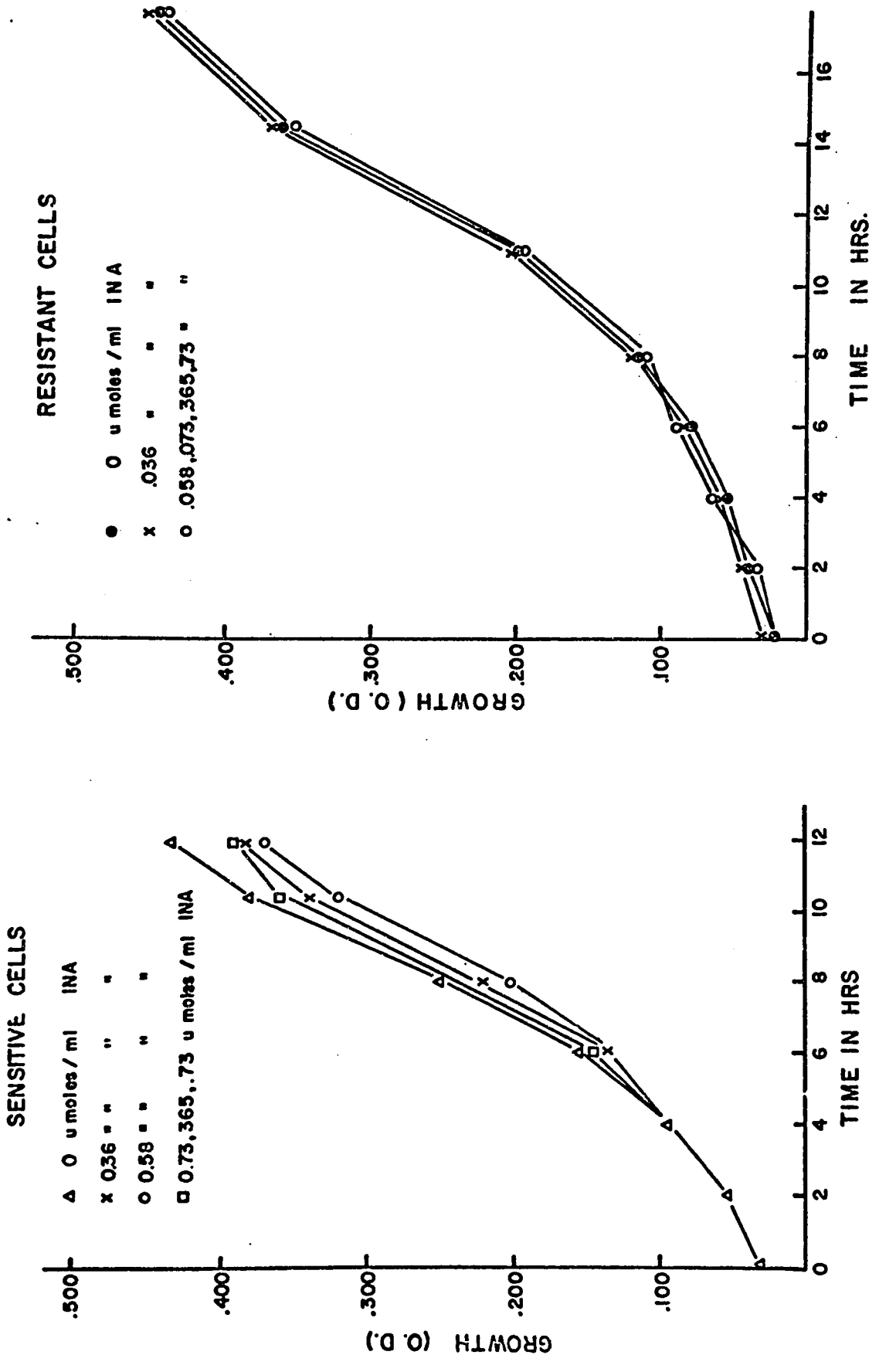
FIGS. 30A AND 30B

THE EFFECT OF HYDRAZINE
ON THE GROWTH OF
SENSITIVE AND RESISTANT CELLS


RESISTANT CELLS
● 0 μ moles/ml NH2-NH2
× .036 " " "
o .038,.073 " " "
□ .365 " " "
△ .73 " " "
GROWTH (O.D.)
.500
.400
.300
.200
.100
0
2
4
6
8
10
12
14
16
TIME IN HRS
SENSITIVE CELLS
□ 0 μ moles/ml NH2-NH2
× .036 " " "
o .058 " " "
■ .073 " " "
△ .365 " " "
▲ .73 " " "
GROWTH (O.D.)
.500
.400
.300
.200
.100
0
2
4
6
8
10
12
TIME IN HRS

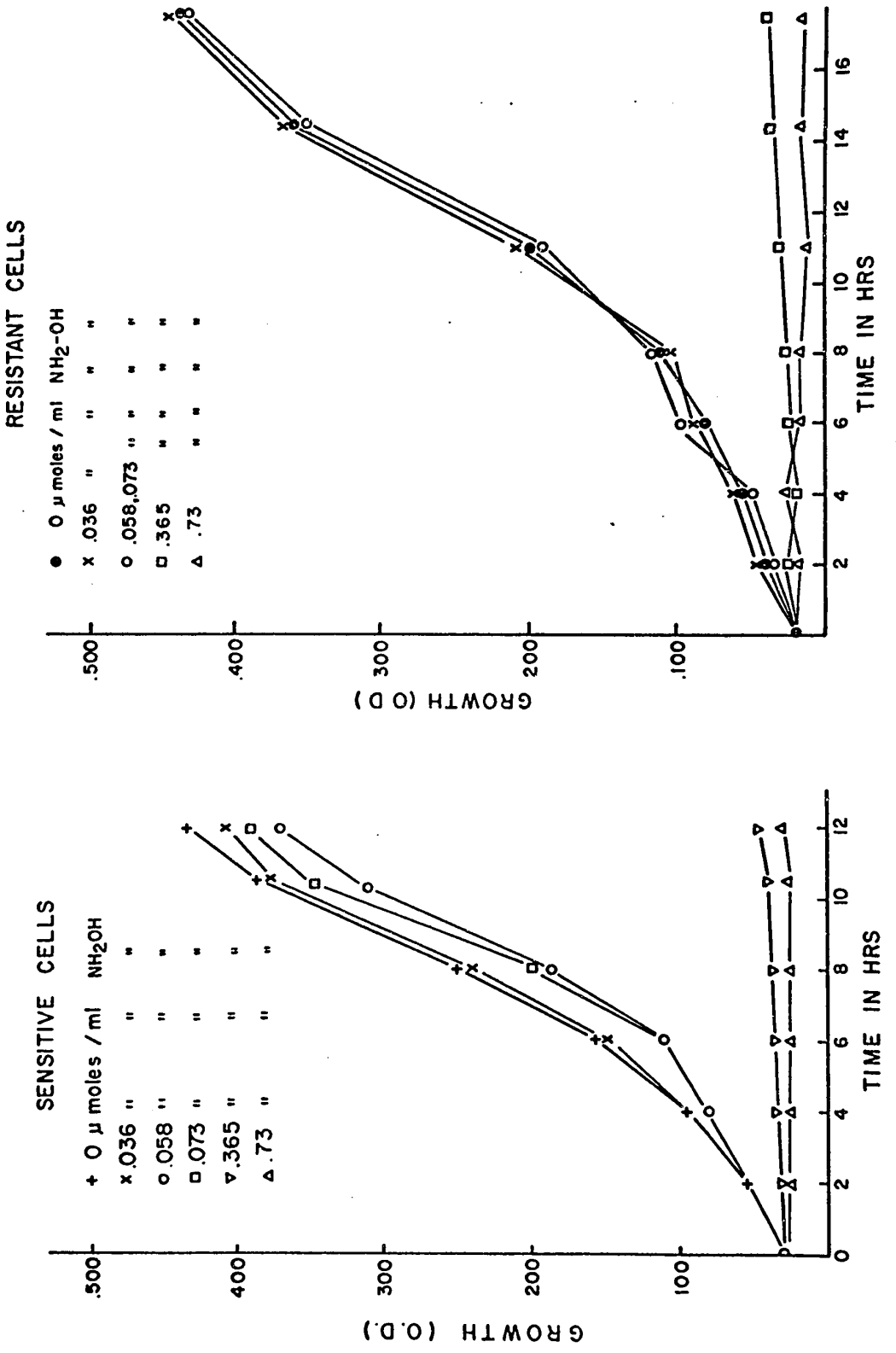
FIGS. 31A AND 31B

THE EFFECT OF INA ON THE GROWTH
OF
SENSITIVE AND RESISTANT CELLS


RESISTANT CELLS
● 0 u moles / ml INA
x .036 " " "
o .058,.073,.365,.73 " "
GROWTH (O. D.)
.500
.400
.300
.200
.100
0
2
4
6
8
10
12
14
16
TIME IN HRS.
SENSITIVE CELLS
△ 0 u moles / ml INA
x 0.36 " " "
o 0.58 " " "
□ 0.73,.365,.73 u moles / ml INA
GROWTH (O. D.)
.500
.400
.300
.200
.100
0
2
4
6
8
10
12
TIME IN HRS

FIGS. 32A AND 32B

THE EFFECT OF HYDROXYLAMINE
ON THE GROWTH OF
SENSITIVE AND RESISTANT CELLS


RESISTANT CELLS
● 0 μ moles / ml NH2-OH
x .036 " " " "
o .058,.073 " " " "
□ .365 " " " "
△ .73 " " " "
GROWTH (O D)
.500
.400
.300
.200
.100
2
4
6
8
10
12
14
16
TIME IN HRS
SENSITIVE CELLS
+ 0 μ moles / ml NH2OH
x .036 " "
o .058 " "
□ .073 " "
▽ .365 " "
△ .73 " "
GROWTH (O.D.)
.500
400
300
200
100
0
2
4
6
8
10
12
TIME IN HRS

The following observations can be made:

1- All the concentrations of INH inhibit the growth of sensitive cells but none inhibit the growth of resistant cells.

2- Hydrazine inhibits the growth of both sensitive and resistant cells at higher concentrations and at lower concentrations hydrazine inhibits the growth of sensitive cells slightly but does not affect the growth of resistant cells.

3- INA does not inhibit the growth of either sensitive or resistant cells.

4- Hydroxylamine affects the growth of the sensitive more than the growth of the resistant at lower concentrations but at higher concentrations it inhibits the growth of both sensitive and resistant cells completely.

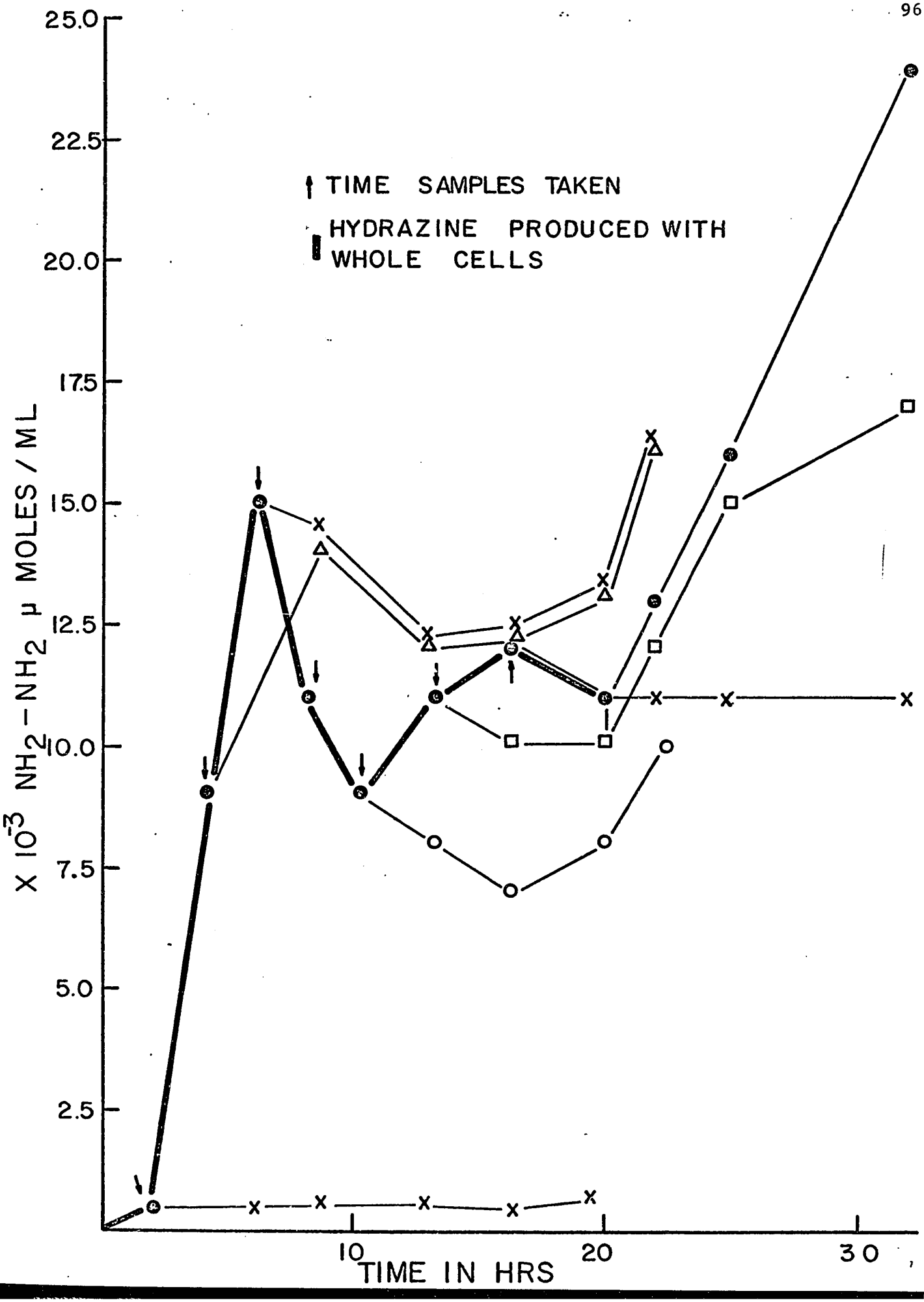
Attempt to Determine if the INH Hydrolysing Enzyme is Released Into the Incubation Media by Whole Cells (sensitive).

Enzymes in bacteria can be located intracellularly, on the cell wall, or released into the media (called exoenzymes). The best studied example of the possibilities involved in the location of enzymes in the bacterial cell is penicillinase.

Penicillinase is formed adaptively in response to penicillin, breaks down the 4 member penicillin ring and is the basis in many organisms for penicillin resistance (103). The enzyme can occur in three forms in Bacillus cereus, one extracellular (exoenzyme), one internal but readily washed out and one tightly bound (104).

FIG. 33

THE CHANGE IN THE CONCENTRATION OF HYDRZINE
IN THE INCUBATION BUFFER
WITH TIME (POSSIBILITY OF EXOENZYME PRODUCTION)


25.0
22.5
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
TIME SAMPLES TAKEN
HYDRAZINE PRODUCED WITH WHOLE CELLS
X 10^{-3} NH_2-NH_2 μ MOLES / ML
10
20
30
TIME IN HRS

The possibility that the enzyme responsible for INH breakdown (INHase) is an exoenzyme was investigated in the following manner. Sensitive cells were incubated with .292u moles/ml INH in buffer. At certain time intervals samples were taken, spun down and part of the supernatant analyzed for hydrazine. The other part of the supernatant was then reincubated at 37°C and at different times samples were analyzed for hydrazine. The results are shown in Fig. 33.

The expected results if the enzyme is extracellular was that earlier samples would show little further production of hydrazine. Later samples on the other hand would show increased activity in the production of hydrazine. As can be seen early samples showed little activity while later samples showed a characteristic decrease in hydrazine followed by an increase in hydrazine production.

These results indicated that there is definite enzymatic activity in later samples. However the expected increase in hydrazine did not at first appear, but rather a decrease followed by an increase. The significance of this was hard to pin-point but it could have been that both the enzyme for INH breakdown and hydrazine breakdown were released in later samples. The fact that something was released is certain as the hydrazine concentration did not remain constant. However even if the results did indicate that an enzyme was released it was difficult to call this enzyme an exoenzyme as the release could have been due to lysis of the cells. This possibility was later investigated.

Attempt to Isolate any Enzymes Released into the Supernatant by Sensitive and Resistant Whole Cells.

The results in the previous experiment (Fig. 33) indicated that the enzyme for INH breakdown might be released into the incubation buffer. This possibility was investigated as follows.

Cells were grown up in the normal manner, washed, suspended in buffer and INH was added (.292u moles/ml). The cells were suspended in this way for ten hours after which time the cells were centrifuged down and the supernatant removed. The supernatant was then dialyzed for twenty-four hours against cold running tap water. The dialyzed supernatant was then split into two equal halves and to one was added INH (.730u moles/ml) and to the other was added hydrazine (.730u moles/ml). The two solutions were then incubated at 37°C and samples were taken at 2,4,8 and 16 hours and analyzed for INH, hydrazine and INA. The results showed that in the presence of INH (.730u moles/ml) there was no appreciable INH disappearance or hydrazine and INA appearance. At the same time in the dialyzed supernatant to which hydrazine was added there was no hydrazine disappearance.

These results indicated either that there was no enzyme released into the incubation media or that such enzymes were inactivated by dialysis.

The Effect of INH, Hydrazine, Hydroxylamine and INA on the Optical Density of Sensitive and Resistant Cells in Buffer.

Fig. 33 had indicated that lysis might be going on in the bacterial suspension under the influence of INH. To test this, washed cells were suspended in buffer to an optical density of .9 (sensitive cells) and

.75 (resistant cells). To separate batches of cells INH, INA, hydrazine and hydroxylamine were added. The optical density of the cells under the influence of each of these agents was then followed for approximately twenty-four hours. [Figs. 34A (sensitive cells) and 34B (resistant cells)]. As can be seen:

1- The control cells (no agents) undergo a slight decrease in optical density.

2- Under the influence of INH, cells undergo a decrease in optical density during the first five hours. This fact is important as it correlates with Fig. 33 where it can be seen that the change in hydrazine concentration without cellular action begins at approximately five hours with the two hour sample undergoing no change. Thus the hydrazine change in the five hour sample (Fig. 33) could be due to simple lysis of the cells and not due to exoenzyme release.

3- Hydrazine, hydroxylamine and INA all cause a decrease in OD larger than that caused by INH. Most of the decrease in OD occurs within the first five hours but eventually hydrazine has the greatest effect.

Looking at Fig. 34B it can be seen that:

1- Resistant cells show a slight decrease in OD as compared to sensitive cells under the influence of INH.

2- Resistant cells show a slight decrease in OD as compared to sensitive cells under the influence of INA.

3- Resistant cells show the largest decrease in OD under the influence of hydrazine and hydroxylamine but this decrease is smaller than that of sensitive cells.

Interpretation of the above results depends on what physiological phenomena underlies the change in OD of the cells. Thus a decrease in OD could signify that the cells are lysing. However this is probably not the case as the changes in OD are not drastic over a short period of time. Another interpretation which could be applied to a decrease in OD of the cells is that the cells had swollen under the influence of the various agents. It has been found that water washed cells of a strain of Pseudomonas aeruginosa do not change in size, i.e. the OD remains constant (115). Yet when salt is added to the suspension the cells shrink rapidly (increase in OD) then swell but much more slowly (116). The swelling is the result of the influx of ions because isomolar sucrose to which the cells are impermeable causes shrinking but little or no subsequent swelling unless ions are added (115).

If one looks at the curve of the OD of the control cells an increase in OD (shrinking) then a decrease in OD (swelling) can be seen. This might be the phenomena which takes place and if it is we can then conclude that:

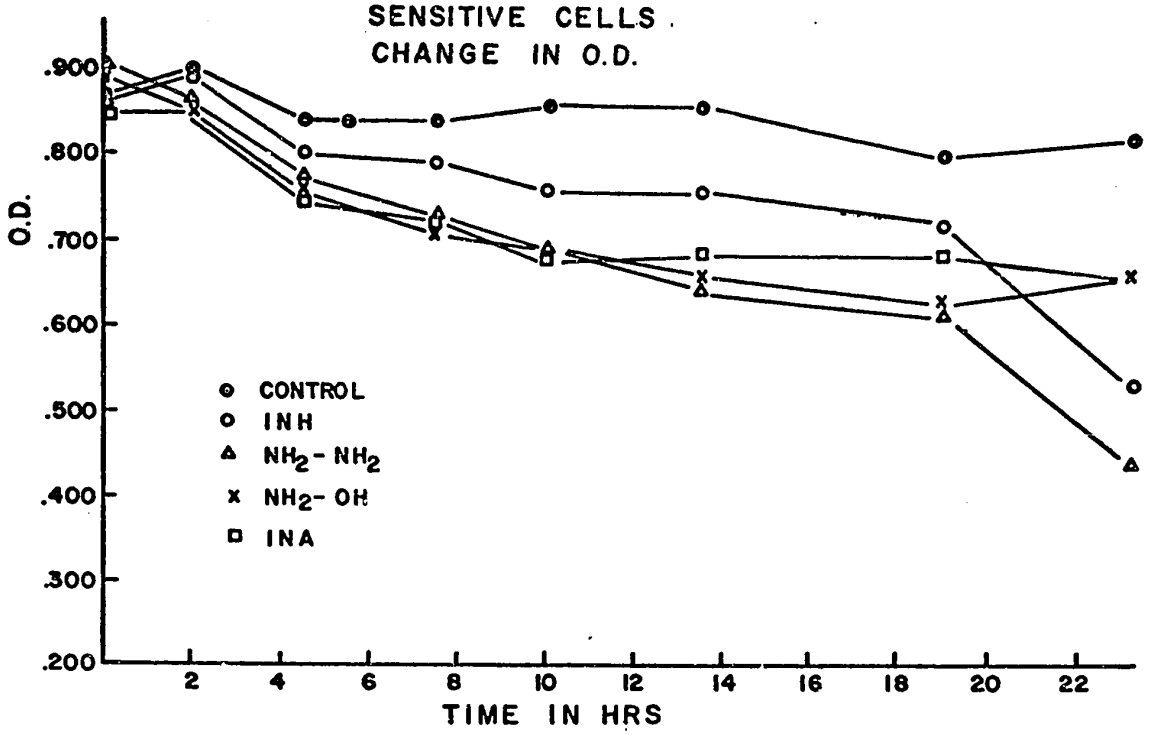
1- Sensitive cells undergo more swelling under the influence of INH than resistant cells. Thus INH seems to affect sensitive cells to a greater extent in this manner.

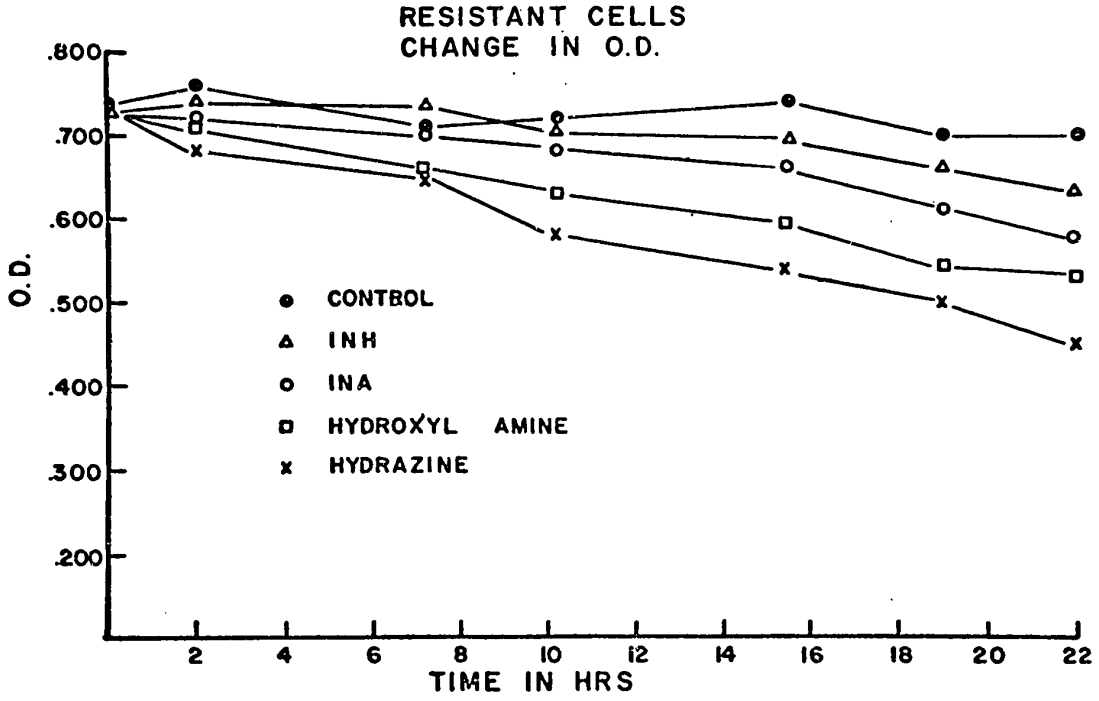
2- Sensitive cells undergo drastic swelling under the influence of INA which has little affect on resistant cells.

3- Sensitive cells undergo swelling under the influence of hydrazine and hydroxylamine which is comparable to that under the influence of INA. Resistant cells undergo swelling under hydrazine and hydroxylamine which is smaller than that of sensitive cells.

FIG. 34A AND 34B

THE EFFECT OF INH, INA, HYDRAZINE
AND HYDROXYLAMINE
ON THE OPTICAL DENSITY
OF
SENSITIVE AND RESISTANT CELLS


SENSITIVE CELLS.
CHANGE IN O.D.
O.D.
.900
.800
.700
.600
.500
.400
.300
.200
CONTROL
INH
NH2 - NH2
NH2 - OH
INA
2
4
6
8
10
12
14
16
18
20
22
TIME IN HRS


RESISTANT CELLS
CHANGE IN O.D.
O.D.
.800
.700
.600
.500
.400
.300
.200
CONTBOL
INH
INA
HYDROXYL AMINE
HYDRAZINE
2
4
6
8
10
12
14
16
18
20
22
TIME IN HRS

If swelling of the cells is correlated to the lethality of INH then it can be seen that sensitive cells swell under the influence of INH and all its metabolic products. Resistant cells do not swell under the influence of INH or INA but only under the influence of hydrazine and hydroxylamine. As very little hydrazine is produced by resistant cells in their breakdown of INH to INA (Fig. 15) one can postulate that the breakdown product of INH in resistant cells is not hydrazine or hydroxylamine. Thus hydrazine and hydroxylamine cause both sensitive and resistant cells to swell. However, sensitive cells swell less than resistant cells under the influence of hydroxylamine. This might be because hydroxylamine could be the breakdown product of hydrazine in sensitive cells and it would then seem logical that sensitive cells would be able to deal better with hydroxylamine than resistant cells (to be referred to later).

Changes in OD of Sensitive and Resistant Cells Under the Influence of INH Plus Various Other Agents (CN, Azide, Glycerol, etc.)

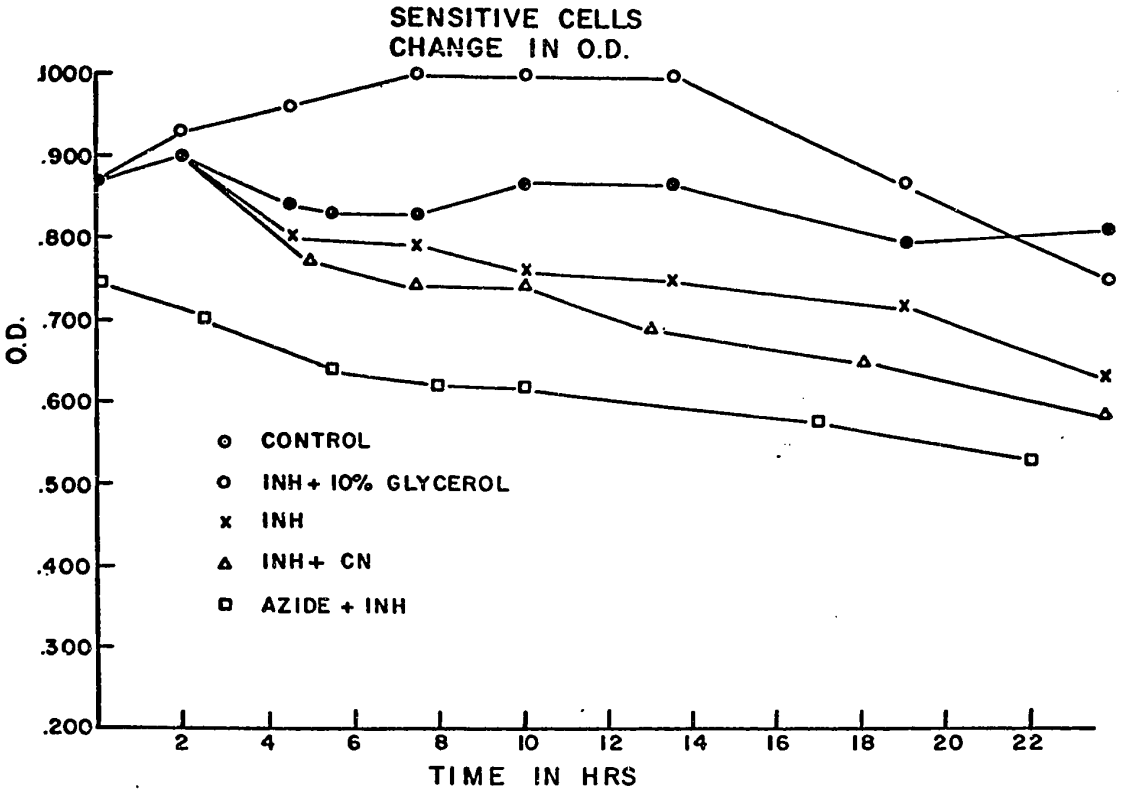
Previous experiments had tested the effect of such agents as CN on the cellular breakdown of INH. It was therefore decided that the effects of these agents should be tested on optical density changes of sensitive and resistant cells. The results (Fig. 35A sensitive cells and Fig. 35B resistant cells) showed that:

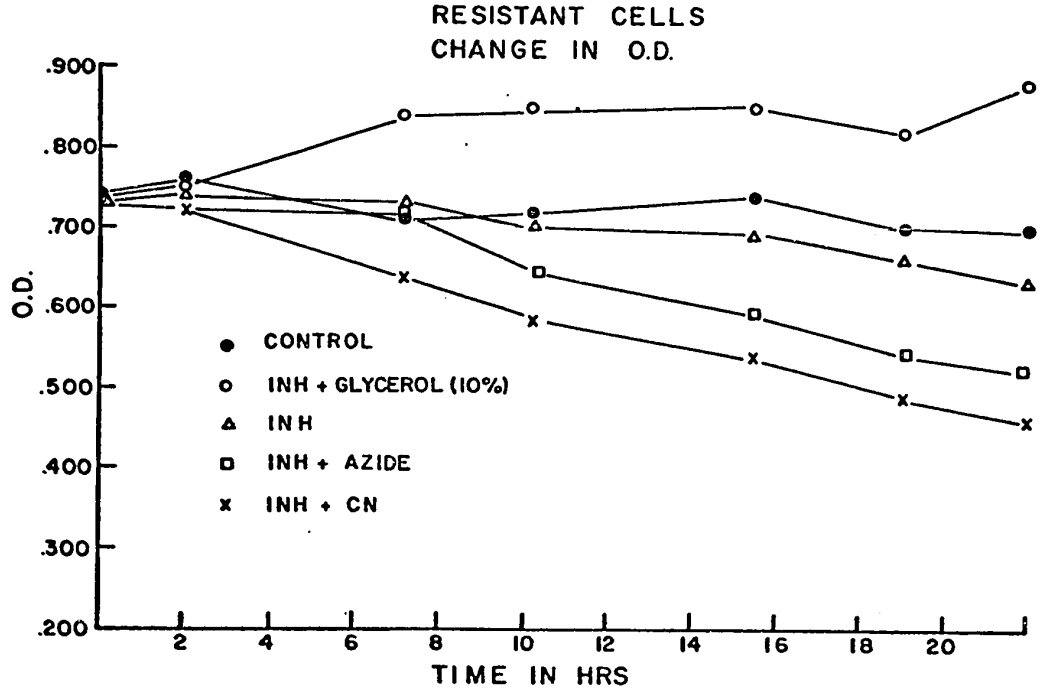
1- Glycerol caused an increase in O.D. in both sensitive and resistant cells.

2- INH caused a greater decrease in O.D. in sensitive cells than in resistant cells.

FIGS. 35A AND 35B

THE EFFECT OF INH ⊕ EITHER, CN, AZIDE OR GLYCEROL
ON THE OPTICAL DENSITY
OF
SENSITIVE AND RESISTANT CELLS


SENSITIVE CELLS
CHANGE IN O.D.
1000
.900
.800
.700
.600
.500
.400
.300
.200
O.D.
CONTROL
INH + 10% GLYCEROL
INH
INH + CN
AZIDE + INH
2
4
6
8
10
12
14
16
18
20
22
TIME IN HRS


RESISTANT CELLS
CHANGE IN O.D.
.900
.800
.700
.600
.500
.400
.300
.200
O.D.
CONTROL
INH + GLYCEROL (10%)
INH
INH + AZIDE
INH + CN
2
4
6
8
10
12
14
16
18
20
TIME IN HRS

3- If cyanide and INH are combined there is a drastic decrease in OD which is approximately the same in sensitive and resistant cells.

4- If Azide and INH are combined there was an instantaneous decrease of OD in sensitive cells and a smaller non-instantaneous decrease of OD in resistant cells.

These results are difficult to interpret but a few general conclusions may be postulated.

1- Previous experiments (Figs. 23A, 23B) had indicated that the metabolism of INH differs if there is a carbon source in the media and this last experiment shows that indeed something different is happening to the cells in the presence of glycerol. The cells could be dividing under the influence of glycerol but this is highly unlikely as there is no nitrogen source in the buffer. Another possibility is that the cells shrink but no definite proof of this possibility was obtained. Whatever is happening, a definite phenomina is however observed.

2- Both CN and Azide cause greater decreases in optical density than INH alone on both sensitive and resistant cells. This is probably because they are metabolic inhibitors and their introduction into the cell causes a decrease in the amount of energy available for ionic concentration control so that the cells no longer pump out ions and thereby swell. Azide seems to act differently as it has a greater affect on sensitive cells. As mentioned previously Azide structure resembles that of hydrazine, the latter disappearing to a greater extent under the influence of sensitive cells than resistant cells (Figs. 28A and 28B). Azide could also be taken up by sensitive cells but not by resistant cells and this could then be the reason why it would have a greater affect on sensitive cells.

Metabolism of INH by Sensitive and Resistant Cells Exposed to INH for Ten Hours in Buffer.

Previous experiments (Fig. 12,13) had indicated that the enzyme for the breakdown of INH is induced in M. Smegmatis mycobacteria on exposure to INH. It was therefore decided to test this hypothesis by comparing the production of hydrazine from INH cells not previously exposed to INH and cells exposed to INH for ten hours.

Washed sensitive and resistant cells were suspended in buffer with .292u moles INH/ml. The hydrazine production of each batch was then measured. At ten hours a large sample of cells was withdrawn in each case spun down, washed 3 times in buffer and suspended in buffer with .292u moles INH/ml. The hydrazine production of these cells was compared against cells incubated without INH for 10 hours but treated in the same manner (Fig. 36).

Looking at Fig. 36 and comparing the production of hydrazine by cells not previously exposed to INH and cells exposed for ten hours to INH it can be seen that:

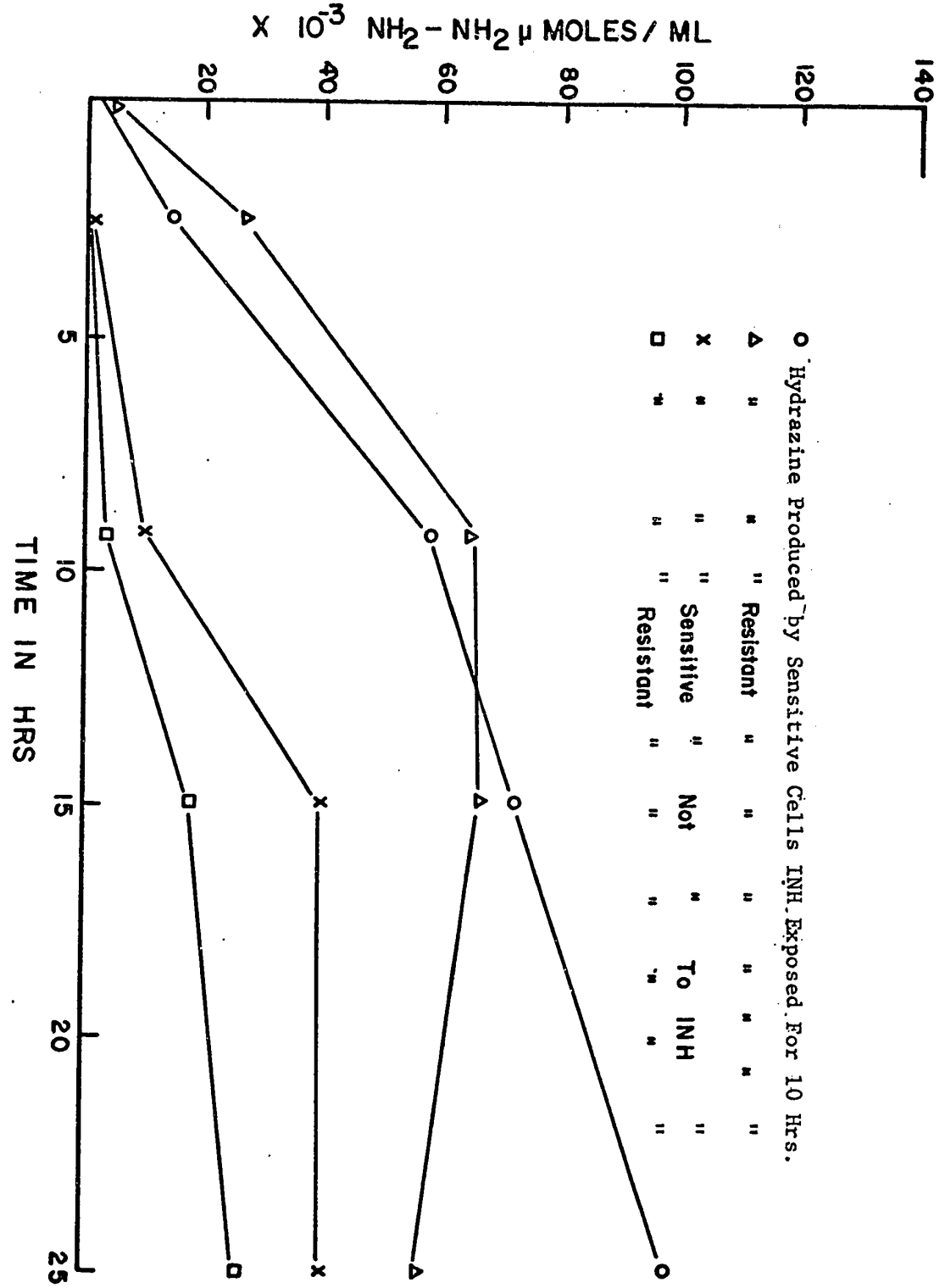
1- Both sensitive and resistant cells previously not exposed to INH breakdown INH to hydrazine much slower than the ten-hour cells.

2- The ten-hour cells (sensitive and resistant) break down INH to hydrazine without a lag while the unexposed cells do so with a lag.

3- Resistant cells produce less hydrazine than senstive cells both ten-hour cell and unexposed cells. These results agree with previous results (Fig. 15).

FIG. 36

THE BREAKDOWN OF INH
BY
SENSITIVE AND RESISTANT CELLS
PREVIOUSLY EXPOSED TO INH
FOR TEN HOURS


X 10⁻³ NH₂ – NH₂ μ MOLES / ML
20
40
60
80
100
120
140
TIME IN HRS
5
10
15
20
25
O Hydrazine Produced by Sensitive Cells INH Exposed For 10 Hrs.
Δ " " Resistant " " " " " " "
X " " " Sensitive " Not " To INH "
□ " " " Resistant " " " " " "

The fact that ten-hour cells produce greater amounts of hydrazine than cells not previously exposed to INH indicated that enzyme induction might indeed be going on, as shown in previous experiments (Fig. 12 and 13).

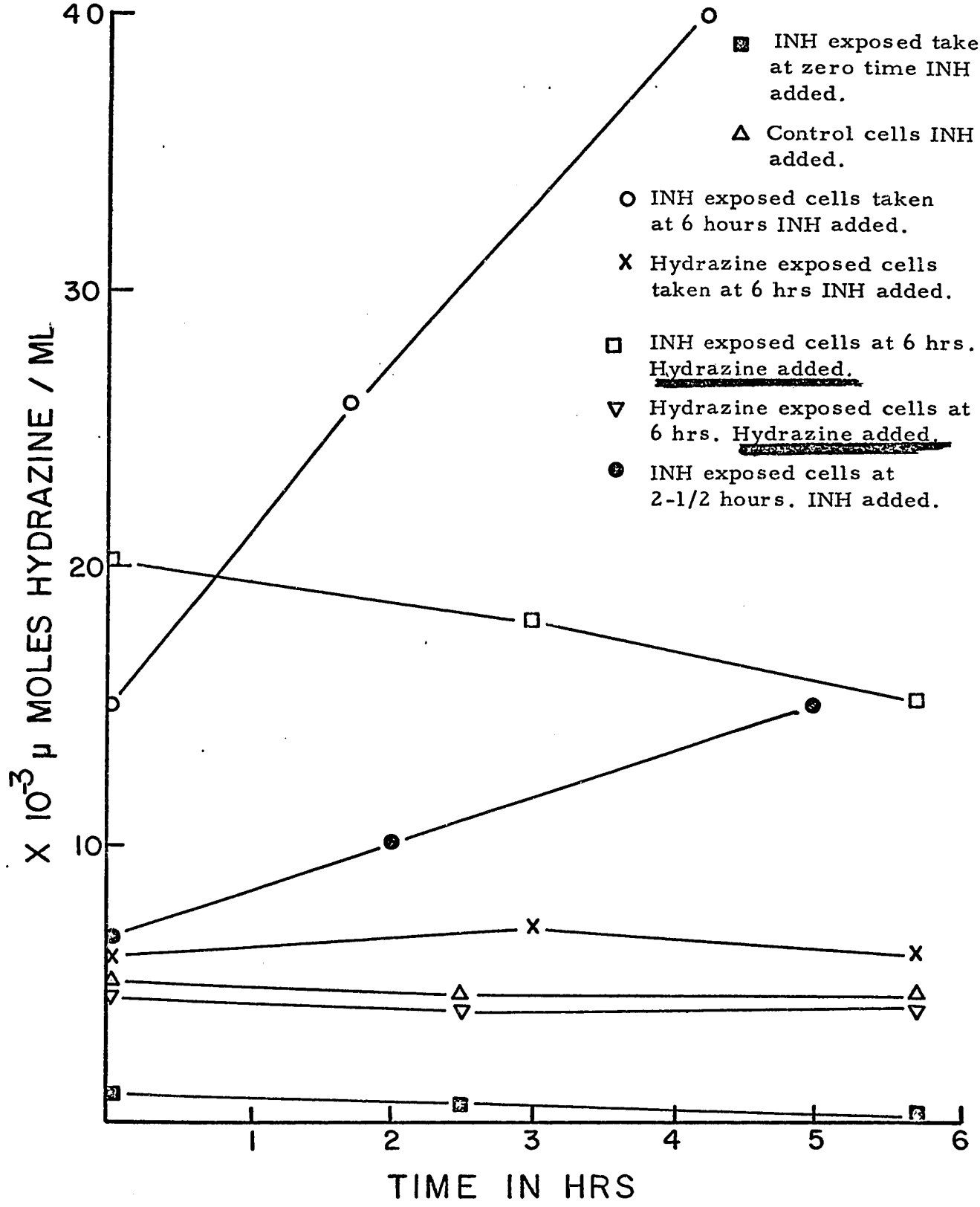
The Production and Uptake of Hydrazine by Sensitive Toluene Lysed Cells Previously Exposed to INH and Hydrazine.

Apart from the previous experiment other efforts were made to show that in cells exposed to INH hydrazine was produced more readily. Thus the following experiment was performed.

Sensitive cells were washed 3 times in buffer, split into 5 equal quantities and suspended in buffer. Then to 1 batch hydrazine was added at zero time (.1u moles/ml), INH to another batch to .73u moles/ml, NH_3 to another batch to .73u moles/ml and the final batch served as a control. At zero time, 2½ hours and 6 hours, samples were taken from the incubation media and centrifuged down at 10,000 g. for 10 minutes. They were then washed once in buffer, centrifuged down again and suspended in the same volume of buffer as previously. One tenth volume toluene was then added to the cells in the buffer and the cells were then put on a wrist action shaker and shaken for 10 minutes at 30 strokes/minute. The toluene was then removed via a separatory funnel and the lysed cells were then reincubated at 37°C. Before incubation each time, sample was split into two parts and to one part INH was added at .73u moles/ml and to the other part hydrazine was added at .1u moles/ml. Samples were then taken at certain time intervals and 1/10 volume of 5% TCA was added to stop the reaction. The cellular debris was then spun down at 10,000 rpm.

FIG. 37

THE PRODUCTION AND POSSIBLE UPTAKE OF HYDRAZINE
BY SENSITIVE TOLUENE LYSED CELLS
PREVIOUSLY EXPOSED TO INH


40
30
20
10
X 10⁻³ μ MOLES HYDRAZINE / ML
1
2
3
4
5
6
TIME IN HRS
INH exposed taken at zero time INH added.
Control cells INH added.
INH exposed cells taken at 6 hours INH added.
Hydrazine exposed cells taken at 6 hrs INH added.
INH exposed cells at 6 hrs. Hydrazine added.
Hydrazine exposed cells at 6 hrs. Hydrazine added.
INH exposed cells at 2-1/2 hours. INH added.

for ten minutes and the supernatant was analyzed for hydrazine as shown in Fig. 37.

As can be seen from Fig. 37 only certain results were plotted as there was no hydrazine production, when INH was added to cells previously exposed to hydrazine, INA, NH_3 and control. Thus the only cells which produce hydrazine from INH were the cells previously exposed to INH for 2 1/2 hours and 6 hours.

When the uptake of hydrazine was measured it was found that only cells previously exposed to hydrazine or INH for 6 hours showed decreases in hydrazine concentration (almost no hydrazine at zero time). Cells exposed to INA, NH_3 and control did not show any hydrazine uptake and the results were therefore not plotted.

Thus it can be seen that sensitive cells previously exposed to INH and then lysed seemed to break down INH more readily than lysed cells not exposed to INH.

Discussion

Isoniazid is bactericidal to tubercle bacilli in very low concentrations (2). Neither the mode of action of the drug or the mechanism of resistance to the drug in tubercle bacilli has been yet discovered.

The object of the above study was therefore to clarify the mode of action of INH and to determine the mechanism of resistance to INH in M. smegmatis. In this work two lines of inquiry were stressed. The first dealt with the uptake of INH (C^{14}) by sensitive and resistant cells and the second dealt with the metabolism of INH by sensitive and resistant cells.

Some of the main observations and conclusions drawn from the above study regarding the metabolism of INH in sensitive and resistant strains of M. smegmatis may be summarized as follows:

1- An enzyme for the breakdown of INH to INA and hydrazine is induced in sensitive cells on exposure to INH in buffer. A second enzyme for the breakdown of hydrazine to an unknown product may also be induced by hydrazine, (Figs. 12, 13, 36, 37).

The above sequence of INH breakdown conflicts with Winder's free radical hypothesis of INH action (36, p. 12 thesis). Winder who worked with M. tuberculosis postulated that INH is broken down by Cu^{++} in the media to N_2 and that eventually free radicals were formed which inhibited the growth of the organism. However our work with M. smegmatis showed that INH is broken down to hydrazine and INA.

2- The above study also showed that INA does not inhibit the growth of sensitive or resistant cells (Figs. 31A and 31B). Sensitive cells were shown to take up INA (Fig. 28A). These results thus challenged Kruger's hypothesis of INH action (35, p. 11)Thesis). Kruger working with M. tuberculosis postulated that INH action is brought about through the lethality of INA (lethal synthesis - p. 2 Thesis).

3- Resistant cells seemed to breakdown INH more vigorously than sensitive cells yet they produced very little hydrazine (Fig. 15). These results had indicated that the enzyme system for INH breakdown might already be induced in resistant cells. However when cloromphenicol was added at zero time and INH breakdown by the resistant cells was measured it was found that no hydrazine was produced (Fig. 16). These results were interpreted to signify that the enzyme system for INH breakdown was not already induced in resistant cells and this theory for the mechanism of resistance in resistant cells was abandoned.

4- It was found that resistant cells take up very little radioactive INH, break INH down vigorously and do not take up INA (Figs. 11, 15, 28B). Sensitive cells do take up INH, break INH down and take up INA. These results indicate that the radioactivity found in sensitive cells might not all be INH but might also include INA activity. If INA is taken up by sensitive cells which break down INH to INA, any studies on INH uptake will therefore have a source of error. This probably also happened in experiments on INH uptake by B.C.G. (6,-p.5 Thesis).

5- It was found that the growth of both sensitive and resistant cells is inhibited by hydrazine and hydroxylamine (Figs. 30A, 30B, 32A and 32B). Both sensitive and resistant cells break down INH but the resistant cells produce very little hydrazine from INH breakdown (Fig. 15). Thus it could be postulated that cells are resistant because they produce little hydrazine when they break down INH or the destructive mechanism for hydrazine in resistant cells is more efficient.

6- Efforts were made to determine the product of hydrazine breakdown in sensitive cells. The possible products were postulated to be either NH_3, or NH_2OH, but neither compound could be detected after incubation of both INH and NH_2-NH_2 with sensitive and resistant cells. It was shown however that NH_3 and NH_2OH introduced into the media containing sensitive cells disappeared very quickly and this may explain why they were not detected. These results therefore do not eliminate NH_3 or NH_2OH as possible products of INH breakdown.

7- It was found that if cloromphenicol is introduced into the media at zero time the enzyme responsible for INH breakdown will not be produced (Fig. 12). Yet our uptake experiments had shown that cloromphenicol does not inhibit the uptake of INH (C^{14}), (Fig. 11A). These results indicate the disassociation of breakdown from uptake and seem to contradict a theory put forward by Wimpenny (8,-p. 12 Thesis) with B.C.G. Wimpenny postulated that INH is broken down by a peroxidase like enzyme before it enters the cell or as it enters the cell i.e.

that the uptake is enzymatic. Some of our results had also indicated that the uptake process is enzymatic namely:

1) The decrease in the uptake of radioactive INH at 25°C as compared to the uptake of 37°C (Fig. 11B).

2) The inhibition of uptake of C^{14} INH in the presence of cyanide (Fig. 11D).

These results therefore indicate that the uptake of INH might be enzymatic but if it is enzymatic the enzyme system involved in the uptake is not the one which breaks down INH to INA and hydrazine.

8- Cyanide depresses the uptake of radioactive INH in both sensitive and resistant cells (Fig. 11D). Glycerol also reduces the uptake of radioactive INH by sensitive and resistant cells (Fig. 11E). Depression of uptake by cyanide seems to indicate that the uptake process is energy dependent. The effect of glycerol, an energy source is difficult to explain but it could be postulated that under the influence of glycerol INH is pumped out of the cell. This was the case found with Kushner and Khan (119) when they studied proflavine uptake by E. coli. They found that cells which had taken up proflavine in buffer released it when an energy source (glucose) was added to the buffer. Whatever the explanation for these results, they seem to agree with those of Youatt (6,-p. 5 Thesis) obtained with B.C.G.

9- At one stage it was thought that an exoenzyme was responsible for INH breakdown (Fig. 33). However, efforts to isolate the enzyme

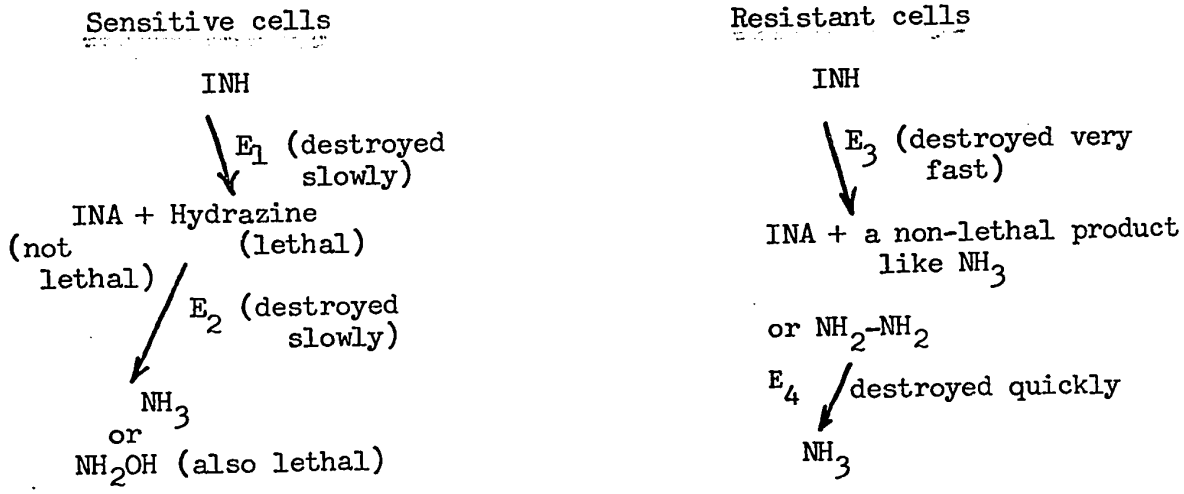
via dialysis failed. Later experiments indicated that the previous results could be due to lysis or swelling of the cells (Fig. 34A and 34B).

10- In the presence of glycerol INH is broken down much more vigorously by sensitive and resistant cells than in its absence (Figs. 24D and 24B). This observation supports the idea that the breakdown process is energy dependent in that energy is needed for enzyme synthesis. Under the influence of cyanide no breakdown of INH occurs (Figs. 26A and 26B). This is also to be expected as cyanide would block enzyme systems which produce energy for protein synthesis.

11- Schaffer (4,-p. 3 Thesis) found that INH was bactericidal to B.C.G. only after a latent period and that the bacteria had to be in an active state to be killed. These previous observations fitted in with our results when it was found that the metabolized products of INH, hydrazine and possibly NH_2-OH were lethal to both sensitive and resistant cells. Thus it was easy to postulate that sensitive cells killed themselves by metabolizing INH to hydrazine while resistant cells produced no hydrazine yet broke INH down to harmless INA. In other words the mechanism of resistance to INH would be the metabolic breakdown of INH without the production of hydrazine. Our experiments had also shown that the degree of inhibition of growth by INH depends on cell number (Figs. 9 and 10) which indicates that the more the INH is broken down the more the cells can grow. This suggests that INH itself is lethal to the cells. The question of whether INH, INA, hydrazine or a product of hydrazine causes the inhibition is central to the problem

and any theory on the mode of action of INH would have to include both INH and hydrazine inhibition of growth.

The previous eleven points suggest a possible theory for the mechanism of resistance to INH. In this, both INH and the metabolic products of its breakdown would be lethal. Sensitive cells would take up INH, begin to synthesize an enzyme for its breakdown and would die because of the action of INH and hydrazine. Resistant cells would break down INH and hydrazine more quickly or breakdown INH to a non-lethal product like NH_3. This scheme can be summarized as follows:



Thus the difference between sensitive and resistant cells would be a genetic change so that the resistant organisms destroyed INH in a different manner from the sensitive strain. This enzyme would also be induced by INH in the resistant strain to a much greater extent than in the sensitive strain.

Resistant cells do produce hydrazine as indicated by some experiments and this is probably because the enzyme for normal INH breakdown as in sensitive cells might still be present in the resistant cells. However, whatever the mechanism of resistance is to INH it has to involve the destruction of hydrazine as this metabolite is toxic to both sensitive and resistant cells.

The previous study although it answers a few questions on the mechanism of resistance to INH does not shed any light on the mode of action of INH. The only definite conclusion that can be drawn is that INH is somehow lethal to sensitive cells but that in their attempt to destroy INH they create a metabolite which is also lethal. This metabolite is hydrazine.

In the future, it is to be expected that more specific properties of the mechanism of resistance to INH will be studied, and eventually even the mode of action of INH may be uncovered. Further work will have to proceed in a different direction. I think the following lines of research will have to be opened:

1- Most of these studies were performed on whole cells suspended in buffer. Another part of the work included growth experiments in Sauton media and most of the previous work by various other authors was done with growing cells. The next step in the elucidation of the mode of action of INH should be a study of INH metabolism in <u>growing</u> cells.

2- Efforts should also be made with cells suspended in buffer to elucidate the possible breakdown product of hydrazine. This is very important as this might be where the mechanism of resistance lies.

3- Another approach should include the isolation of enzymes which break down INH. In this way a true understanding of the enzymatic system under discussion could be obtained.

4- The possible release of isoniazid-hydrolyzing enzymes into the incubation media should be studied further.

5- The possible swelling of cells under the influence of INH should be further investigated.

As a summary it can be seen that the preceeding work has opened many interesting avenues for further research. Many questions were left unanswered but vital clues to a better understanding of the problem of INH resistance in M. smegmatis were discovered.

CLAIMS TO ORIGINAL RESEARCH

1) Proof that isoniazid (INH) inhibition of cell division of M. Smegmatis 503 is dependent upon the number of cells originally present.

2) The induction of an INH hydrolyzing enzyme and proof of induction.

3) The correlation of INH breakdown with isonicotinic acid (INA) and hydrazine production.

4) The demonstration of a more active breakdown of INH by resistant cells with no hydrazine production by the resistant cells.

5) The possible induction of a hydrazine hydrolyzing enzyme in both sensitive and resistant cells.

6) The demonstration of the lethality of hydrazine and hydroxylamine to both sensitive and resistant cells.

7) A demonstration of the fact that INA is not lethal to either sensitive or resistant cells.

8) The possible demonstration that the INH hydrolyzing enzyme might be an exoenzyme.

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