EFFECT OF ISONIAZID ON THE DEHYDROGENASE ACTIVITY OF *MYCOBACTERIUM TUBERCULOSIS*

WERNER B. SCHAEFER

Department of Research and Laboratories, National Jewish Hospital, and Department of Microbiology, University of Colorado School of Medicine, Denver, Colorado

During studies on the effect of isoniazid on the dehydrogenase activity of tubercle bacilli, the surprising observation was made that the bacteria which had been exposed to the drug reduced methylene blue or triphenyltetrazolium chloride at a much faster rate than did the untreated bacteria. The present report describes this phenomenon in detail and our attempts to elucidate its mechanism and significance for the mode of action of isoniazid against tubercle bacilli.

EXPERIMENTAL METHODS

The method used for studying the effect of isoniazid on the dehydrogenase activity of tubercle bacilli was as follows. A young culture of tubercle bacilli in Tween 80 (Hilltop Laboratories, Inc., Cincinnati, Ohio) -albumin liquid medium and of an optical density of 0.15 to 0.2 (measured at 525 mμ in a Coleman Junior spectrophotometer) was distributed in 5.4-ml portions into 15 by 125 mm screw-capped tubes. Stepwise dilutions of isoniazid were added to these tubes to complete the volume to 6 ml and to achieve different concentrations of isoniazid in different culture tubes. Control tubes received water instead of the drug. All cultures were incubated at 37 C in a slanted position on a roller tube apparatus turning at 16 rpm, to facilitate aeration. After various times, a series of cultures exposed to different drug concentrations was removed from the incubator; the bacteria were separated by centrifugation, washed twice in 0.066 M phosphate buffer of pH 6.8 and containing 0.02 per cent Tween 80; and the suspensions were adjusted to a standard optical density of 0.22. Of these suspensions, 4.5 ml were transferred to sterile cotton-plugged Thunberg tubes (large size) and incubated for 10 min in a water bath at 37 C for equilibration of temperature. Then 0.5 ml of an autoclaved 0.5 per cent solution of triphenyltetrazolium chloride in phosphate buffer of pH 6.8 was added, the

cotton plugs were replaced by sterilized and greased glass tops, and all tubes were attached to a manifold connected with a vacuum oil pump. After 5 min of evacuation to about 30 mm Hg the tubes were sealed and then incubated at 37 C for 3 hr. Thereafter, the tubes were opened, 0.3 ml of a 10 per cent formaldehyde solution was added, and the tubes were kept at 4 C for at least 1 hr. Then 3 ml of the bacterial suspensions were transferred to screw-cap tubes, 6-ml aliquots of acetone were added, and the mixtures were kept in the closed tubes in the dark at 4 C overnight. The bacteria, and a precipitate of phosphate which had formed, were separated by centrifugation, the supernatant was decanted into colorimetric cuvettes, and the intensity of the formazan color was measured at 525 mμ. If the color intensity of the extracts was beyond the range in which the optical density was linear with the concentration of the reduced triphenyltetrazolium chloride, the extract was diluted with an appropriate amount of acetone and put in the cold. When a new precipitate had formed, the solution was centrifuged and the color in the supernatant was measured. All operations were performed under subdued light because of the bleaching effect of light on the formazan color.

The bactericidal effect of the drug was measured by plating serial dilutions of the identical suspensions used for the triphenyltetrazolium chloride reduction test onto oleic acid albumin agar plates. The colonies were counted after 3 weeks of incubation under air with increased CO_2 content (Schaefer *et al.*, 1955). The effect of isoniazid on the acid-fast staining property of the bacteria was followed in smears prepared from the cultures and stained by a carbol fuchsin Tween cold stain (Schaefer, 1954).

RESULTS

The effect of isoniazid on the triphenyltetrazolium chloride reducing property is illustrated
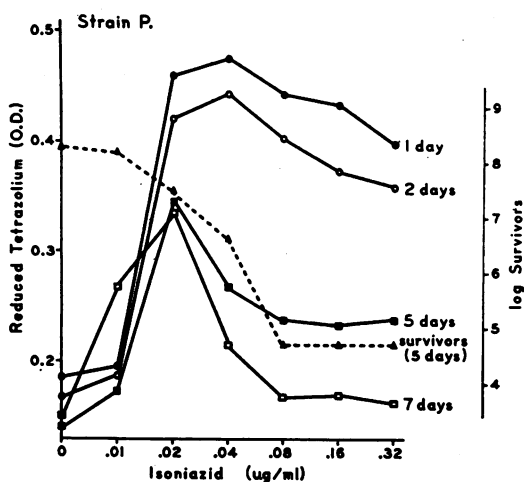
Figure 1. Tetrazolium reduction (optical density) and viable cell counts in suspensions of Mycobacterium tuberculosis strain P after exposure to isoniazid.

in figure 1. The horizontal axis of the graph indicates the concentrations of isoniazid to which the bacteria had been exposed before their dehydrogenase activity was tested. The vertical axis indicates the amounts of triphenyltetrazolium chloride reduced by the various suspensions of isoniazid treated or untreated bacteria. The points connected by solid lines represent the results obtained with cultures incubated for the same length of time. This time is indicated. It can be seen that the bacteria which had been incubated in the presence of 0.02 μg per ml or greater amounts of isoniazid for 24 hr reduced much larger amounts of triphenyltetrazolium chloride than the bacteria incubated in the presence of lower concentrations or in the absence of the drug. The dehydrogenase activity decreased after longer incubation, especially in the bacteria exposed to higher concentrations of the drug, but remained at a relatively high level in the bacteria incubated in the presence of 0.02 μg per ml, the minimal active concentration of drug. On the 7th day of incubation an increase of the dehydrogenase activity was observed in the bacteria incubated in the presence of only 0.01 μg per ml, a subbactericidal concentration of isoniazid. The bactericidal effect of the drug is illustrated by the broken line indicating, on a logarithmic scale, the results of the viable cell counts in the suspensions of the bacteria exposed to isoniazid for 5 days. It can

be seen that the bacterial suspension exposed to 0.02 μg isoniazid per ml, and having the highest dehydrogenase activity, contained about 10 per cent viable bacteria, whereas practically no survivors were found in the suspensions of bacteria exposed to higher drug concentrations. These results suggest that the stimulation of the dehydrogenase activity occurs in an early phase of the drug action and that this initial phase is followed by another phase which begins when the bactericidal effect is completed, and during which the dehydrogenase activity is progressively lost. It is this latter phase which was studied by Koch-Weser et al. (1955). The hypothesis that the stimulation of dehydrogenase activity is a symptom of the beginning drug action is also supported by the observation of the delayed appearance of this effect in the bacteria exposed to a slightly subbactericidal drug concentration. This effect is particularly pronounced in the experiment represented in figure 2 which was performed with another strain of Mycobacterium tuberculosis.

To obtain more insight into the kinetics of the triphenyltetrazolium chloride reduction reaction and in the difference of this reaction in normal and in isoniazid-treated tubercle bacilli, the relationship between the time allowed for the anaerobic incubation of the suspension and the total amount of triphenyltetrazolium chloride reduced was investigated. The result of
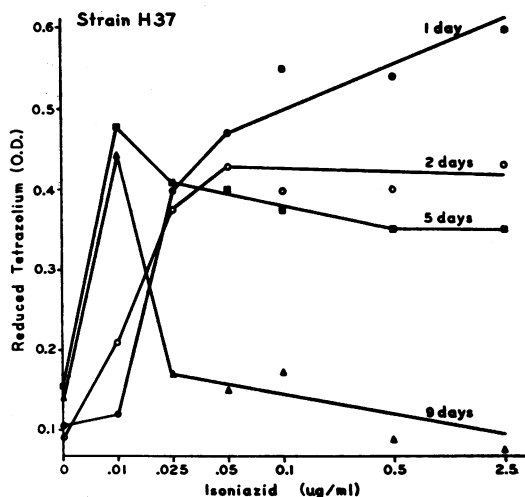

Figure 2. Tetrazolium reduction (optical density) by suspensions of Mycobacterium tuberculosis strain H37Rv after exposure to isoniazid.
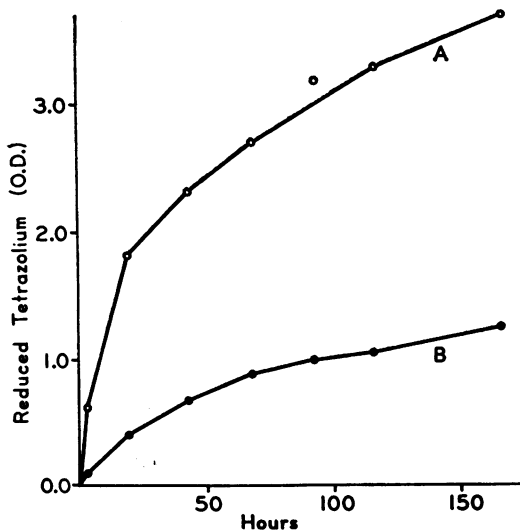
Figure 3. Tetrazolium reduction (optical density) as a function of time. Bacteria from culture incubated for 20 hr in the presence of 0.5 μg isoniazid per ml (A). Untreated bacteria (B).

such an experiment is represented in figure 3. It shows that the amount reduced by the isoniazid-treated bacteria considerably exceeds that reduced by the untreated bacteria even after anaerobic incubation for 7 days.

The reduction of triphenyltetrazolium chloride being an irreversible process, this reaction has often been used for the measurement of dehydrogenase activity without excluding the presence of air. To study the effect of various degrees of aeration on the amounts of triphenyltetrazolium chloride reduced by isoniazid-treated and -untreated tubercle bacilli, the reduction was measured in identical suspensions (a) under exclusion of air (evacuated Thunberg tubes), (b) in open, but unshaken tubes, and (c) in tubes continuously aerated on a roller tube apparatus. The results of such an experiment are represented in figure 4. The amounts of triphenyltetrazolium chloride reduced by suspensions in open, unshaken tubes were less than those reduced under strictly anaerobic conditions and there was no triphenyltetrazolium chloride reduction at all in the continuously aerated suspensions. These results confirm the observations of Brodie and Gots (1951) and Hanks (1951) on the antagonistic effect of atmospheric oxygen on the enzymatic reduction of triphenyltetrazolium chloride, and emphasize

further the necessity to measure dehydrogenase activity in the absence of air if quantitatively significant and reproducible results are desired.

Isoniazid has been shown to be bactericidal only in an environment favorable for growth (Schaefer, 1954). It was, therefore, of interest to determine whether such an environment was also required for the dehydrogenase enhancing effect of the drug. Table 1 summarizes some experiments in which the effect of the absence or presence of a carbon source in a medium with or without isoniazid on the dehydrogenase activity of tubercle bacilli was investigated. The medium used was a simple phosphate buffer solution with 0.01 per cent Triton WR 1339 (Winthrop-Stearns, Inc., New York, New York) as a wetting agent. In the absence of a carbon source the triphenyltetrazolium chloride reduction of the isoniazid-treated bacteria did not significantly differ from that of the untreated bacteria. On the contrary, in the presence of a carbon source and apparently independently of its chemical nature, the dehydrogenase activity of the isoniazid-treated bacteria was strongly enhanced. The presence of a carbon and energy source in the medium, therefore, was necessary for the dehydrogenase enhancing effect of isoniazid as it was also required for the bactericidal effect of the drug.

In other experiments the influence of the intensity of aeration on the dehydrogenase activity was investigated. Identical cultures were incubated under continuous aeration on the roller tube apparatus or in upright standing tubes without shaking. The stimulating effect of isoniazid was much weaker in the poorly aerated cultures and the bactericidal effect was delayed. Isoniazid had no effect on the dehydrogenase activity of tubercle bacilli incubated in a complete medium under strict anaerobiosis or at 4 C. All these observations indicate that the conditions required for the bactericidal effect of isoniazid are also required for stimulation of dehydrogenase activity of the bacteria.

To determine whether the stimulation of the dehydrogenase activity was specific for isoniazid or could be produced also by other drugs, various related and unrelated antituberculous drugs were investigated. Figure 5 shows the effect of Salizid, a salicylaldehyde hydrazone of isoniazid. This drug in a freshly-prepared aqueous solution enhanced the triphenyltetrazolium chloride reduction by tubercle bacilli only after
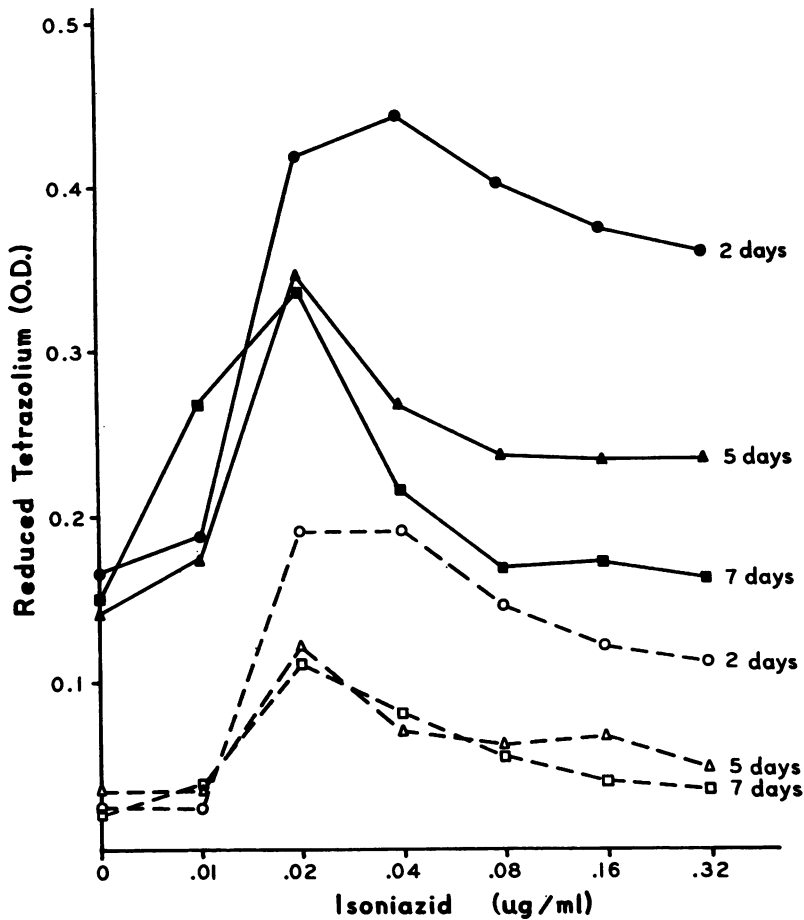
Figure 4. Comparative measurement (optical density) of tetrazolium reduction in the absence and presence of atmospheric air. Suspensions were prepared from cultures exposed to increasing concentrations of isoniazid for 2, 5, and 7 days. *Solid lines,* measurement in absence of air; *broken lines,* measurement in presence of air.

4 to 5 days of incubation. On the contrary, a 3-day-old solution of the drug caused the enhancement within 1 or 2 days of incubation. This result indicated that this hydrazone of isoniazid spontaneously hydrolyzed in aqueous solution and owed its antibacterial activity to the liberated, free isoniazid. Similar results were obtained in the study of Verazide (Rubbo *et al.*, 1958), 3,4-dimethoxybenzaldehyde hydrazone of isoniazid, with the only difference that the hydrolysis into free isoniazid in aqueous solution was more rapid in this instance. These observations support similar conclusions by Cavallini *et al.* (1952) and by Libermann *et al.* (1954), based on consideration of the minimal molar activity of the hydrazone derivatives of

isoniazid. Another drug investigated was α-ethyl-thioisonicotinamide recently introduced by Rist *et al.* (1959) into the chemotherapy of tuberculosis. This drug is bactericidal and causes loss of acid-fastness like isoniazid, but is active also against isoniazid-resistant strains. It enhanced the dehydrogenase activity of tubercle bacilli in the same way as isoniazid. Of particular interest also was the study of two pentaheterocyclic hydrazides (thiophene-2-carboxylic acid hydrazide and furan-2-carboxylic acid hydrazide) which inhibit the growth of bovine tubercle bacilli in a concentration of 0.1 to 0.8 μg per ml (Roth *et al.*, 1953, Bönicke, 1958); but have no effect on human tubercle bacilli. In contrast to the hydrazide of isonicotinic

acid these hydrazides did not cause loss of acid fastness of bovine tubercle bacilli but they induced the formation of long filamentous cells. These compounds had an inhibiting effect on the dehydrogenase activity in bacteriostatic concentrations. Of other drugs tested streptomycin, viomycin and cycloserine had no effect or an inhibiting effect on the dehydrogenase activity at all concentrations tested. p-Amino-salicylic acid, in concentrations of 0.03 to 0.1

TABLE 1

Effect of incubation in phosphate buffer with and without carbon source and with and without isoniazid on tetrazolium reduction by Mycobacterium tuberculosis

Carbon Source	Ison-iazid	Tetrazolium Reduced* by Bacteria Incubated with and without Isoniazid for:			
		16 hr	39 hr	6 days	11 days
	μg/ml				
None	0	0.092	0.085	0.114	0.108
	0.2	0.097	0.081	0.115	0.119
Glucose, 1%	0	0.128	0.114	0.030	0.023
	0.2	0.255	0.398	0.190	0.153
Glycerol, 1%	0	0.144	0.149	0.071	0.071
	0.2	0.215	0.337	0.398	0.432
Oleic acid-albu-	0	0.138	0.114	0.103	0.125
min complex	0.2	0.333	0.444	0.417	0.244

* Optical density.

μg per ml, caused a rather late enhancement of the dehydrogenase activity. 2,4-Dinitrophenol, which enhanced respiration and oxidation of substrates in *Escherichia coli* (Clifton and Logan, 1939) and is an inhibitor of oxidative phosphorylation, had no effect on the dehydrogenase activity in either subinhibitory (5 to 10 μg per ml) or inhibitory (50 to 500 μg per ml) concentrations. It appears from these observations that the dehydrogenase stimulating effect is quite specific for the two antibacterial derivatives of isonicotinic acid, isoniazid and α-ethyl-thioisonicotinamide and is independent of the hydrazide function of isoniazid.

It has been reported by Bloch (1950) and was confirmed by Segal and Bloch (1955) that the dehydrogenase activity of tubercle bacilli, as measured by their ability to reduce methylene blue or triphenyltetrazolium chloride, is increased in the attenuated variants (BCG and H37Ra). These variants are also characterized by a diminution or loss of the ability to grow in the form of serpentine cords (Middlebrook et al., 1947), and it was suggested by Bloch (1950) that the increased dehydrogenase activity of these variants may be due to a greater permeability or alteration of their surface properties. It seemed of interest to repeat these experiments and to study the effect of isoniazid on the dehydrogenase activity of these variants. The results of such experiments are summarized in
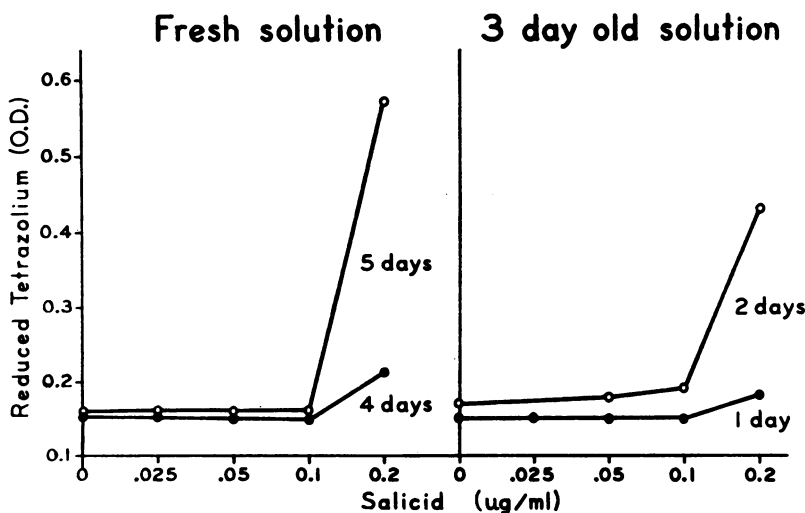

Figure 5. Tetrazolium reduction of Mycobacterium tuberculosis by a fresh and a 3-day-old solution of Salizid.

TABLE 2

Tetrazolium reduction by strains of Mycobacterium tuberculosis of different virulence and cordforming properties incubated with and without isoniazid

Strain	Cord	Iso-niazid	Tetrazolium Reduced* by Bacteria Incubated with or without Isoniazid for:		
			2 days	5 days	7 days
		μg/ml			
H37Rv (virulent)	++	0	0.152	0.174	0.125
		0.2	0.614	0.481	0.310
BCG (attenuated)	+	0	0.328	0.462	0.357
		0.2	0.399	0.292	0.138
H37Ra (avirulent)	0	0	0.509	0.538	0.538
		0.2	0.577	0.450	0.314

* Optical density.

table 2. It can be seen that the dehydrogenase activity of the bacteria varied in the three strains in an inverse relationship to their virulence and cordforming properties. Isoniazid stimulated this dehydrogenase activity in the cells of all three strains, but relatively less in the attenuated strains which already had a high dehydrogenase activity in the absence of the drug. These results strengthen the hypothesis that the dehydrogenase activity is related to the surface and permeability properties of the bacteria. The hypothesis that isoniazid alters the permeability and surface property of tubercle bacilli is further supported by the phenomenon of loss of the acid-fast staining property of the bacilli and our observation that the virulent, cordforming strains of tubercle bacilli, under the effect of isoniazid, lost the ability to bind neutral red in its ionic red form when suspended in an alkaline solution of the dye (Dubos and Middlebrook, 1948).

In addition to human and bovine isoniazid-sensitive strains of tubercle bacilli, isoniazid-resistant variants of such strains were also studied and it was found that the minimal concentration of isoniazid causing stimulation of the dehydrogenase activity of these strains corresponded to the level of their drug susceptibility. Among other mycobacterial species only some photochromogenic mycobacteria were investigated because of their relatively high susceptibility to isoniazid. The result of such an experiment is represented in figure 6. It can be seen that the primary dehydrogenase activity of the strain was rather high and that isoniazid

in a minimal bactericidal concentration caused only a slight and short-lived enhancement of dehydrogenase activity. On the contrary, larger concentrations of the drug or prolonged exposure to the drug caused a rapidly progressing inactivation of this enzymatic activity.

In recent years evidence has been accumulating that bactericidal effects are produced by an unbalance in the synthesis of various cell constituents (Cohen and Barner, 1954). Certain auxotrophic bacterial mutants were found to lose their viability rapidly when supplied with all required growth factors but one. It was shown that under these conditions the bacteria started growing and dividing but formed abnormal nonviable cells because a particular cell component could not be synthesized (Cohen and Barner, 1954; Bauman and Davis, 1957; Ridgway and Douglas, 1958). A similar process of unbalanced and lethal growth was observed in wild strains under the effect of sulfonamide and certain nutritional conditions. The medium had to contain all but one of the metabolites, the synthesis of which was inhibited by the drug. If more than one of these growth factors was missing, the bacteria did not grow and remained viable (Cohen and Barner, 1956). Another instance in which the bactericidal effect could be explained by unbalanced growth is illustrated by penicillin. This antibiotic inhibits
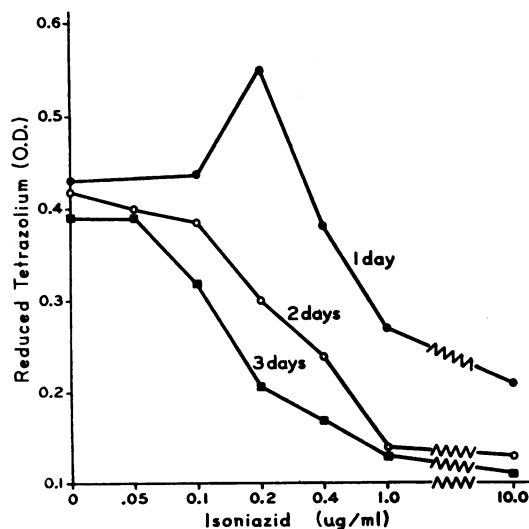

Figure 6. Tetrazolium reduction (optical density) by suspensions of a photochromogenic mycobacterium after exposure to isoniazid.
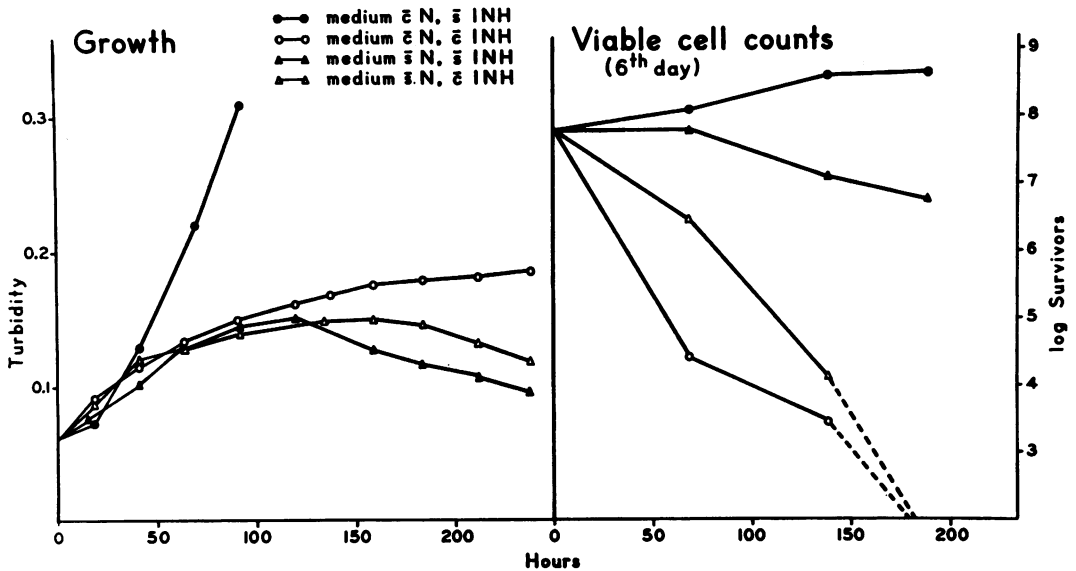
Figure 7. Effect of isoniazid (INH) on growth and viability of *Mycobacterium tuberculosis* in a medium with and without a nitrogen source.

the synthesis of a metabolite required for the cell wall formation (Park and Strominger, 1957) and forms cells without cell wall (protoplasts) (Lederberg, 1956) which are not viable except under very special conditions. To investigate whether or not unbalanced growth may be the cause of the bactericidal effect of isoniazid, experiments were made in which the effects of isoniazid on growth and viability of tubercle bacilli in a complete and in a nitrogen-deficient medium were compared. Both media had a carbon source to induce active metabolism in the bacteria and uptake of the drug. One medium had no nitrogen source, in order to prevent or restrict cell division and growth. The reasoning was that if growth was responsible for the bactericidal effect on the drug, this effect should be smaller in the nitrogen-deficient medium than in the complete medium. The media contained the usual mineral solution, glucose (10 mg per ml), 0.02 per cent Tween, and 0.1 per cent bovine albumin fraction V. The complete medium contained, in addition, ammonium sulfate (0.5 mg per ml) as the nitrogen source. Both media were inoculated with equal amounts of carefully washed bacteria cultivated in a complete medium. The cultures were incubated on the roller tube apparatus in the presence of 0.2 μg isoniazid per ml or in the

absence of the drug. The results of such an experiment are represented in figure 7. It can be seen that isoniazid had a bactericidal effect in both media, but this effect occurred more rapidly in the complete medium. The bacteria multiplied freely in the complete medium but achieved only one division in the nitrogen-deficient medium. This small amount of growth, as shown previously (Schaefer *et al.*, 1949; Marshak and Schaefer, 1952) occurred at the expense of a nitrogen store accumulated in the bacteria during their preceding growth in a complete medium. The addition of isoniazid did not prevent this growth and conditions, therefore, were such that the drug could be bactericidal by unbalanced growth. However, the bacteria were growing slower and to a minor extent in the nitrogen-deficient medium and this accounts for the slower bactericidal effect of the drug. It was also remarkable that the bacteria exposed to the drug in the nitrogen-deficient medium did not lose their acid-fastness, indicating that the action of the drug was incomplete.

DISCUSSION

Isoniazid and α-ethyl-thioisonicotinamide are apparently the only antituberculous drugs causing a pronounced loss of acid-fastness and

a marked stimulation of the oxidoreduction reactions of tubercle bacilli. Evidence has been brought forth that these effects are due to damage of the permeability barrier of tubercle bacilli and the hypothesis was proposed that these two drugs inhibit the synthesis of a substance concerned with this property. In view of the phenomenon of loss of acid-fastness and the preponderance of lipid metabolism in tubercle bacilli, the hypothesis is considered that the substance may be a lipid. The report of Russe and Barclay (1955) of a decrease of the lipid content in isoniazid-treated tubercle bacilli would lend support to this hypothesis. Phenomena of increase in oxidoreduction reactions similar to those described here have been observed in various cell species under the effect of anesthetics, 2,4-dinitrophenol, and gramicidin. These effects have been attributed to an uncoupling of oxidation from phosphorylation, resulting in inhibition of assimilation and synthesis (for review of the literature see McElroy, 1947). The hypothesis that isoniazid may act in this way, however, was not supported in the experiments of Brodie and Gray (1956). Stimulation of oxidoreduction reactions and metabolic rate and increased cell permeability and osmotic fragility are characteristic symptoms of fatty acid deficiency in animals (MacMillan and Sinclair, 1958). Burr and Burr (1930) attributed these phenomena to an alteration of the membranes and MacMillan and Sinclair (1958) more specifically to an alteration of the mitochondrial membranes. The latter authors pointed out that mitochondria are rich in firmly bound phospholipids containing highly unsaturated fatty acids and they suggested that essential fatty acids are concerned in the orientation of respiratory enzymes in mitochondria. It is conceivable that isoniazid inhibits the synthesis of a lipid with a similar function.

The fact that the two antituberculous drugs discussed here have the same ring structure suggests that their antimicrobial effects may be related to this structure. The resemblance of this structure to nicotinamide and to pyridoxal suggested that the drugs may act as antagonists of diphosphorpyridine nucleotide (Zatman et al., 1954; Krueger-Thiemer, 1957) or pyridoxal coenzymes (Yoneda et al., 1952; Cymerman-Craig et al., 1958; Youatt, 1958a) but the experimental evidence in favor of one or the other mechanism is still insufficient. The

relationship of such an inhibition to the assumed disturbance in the lipid metabolism of tubercle bacilli would also have to be elucidated.

The fact that isoniazid manifests its metabolic and bactericidal effects only on growing cells suggested that the process of growth itself plays a role as a cause of this effect. The relationship of drug action and bacterial growth has been clarified by the studies of Cohen and Barner (1954) on the phenomenon of unbalanced growth with formation of abnormal and nonviable cells. The same mechanism seems to apply to the action of isoniazid, and our observations on the retarding effect of nitrogen starvation of the bacteria on the bactericidal effect lend further support to this explanation. The hypothesis of Krueger-Thiemer (1958) that isoniazid causes the accumulation of a bactericidal metabolite (hydrogen peroxide) has found no experimental support so far (Holmes and Rubbo, 1958; Youatt, 1958b).

SUMMARY

Tubercle bacilli exposed to bactericidal concentrations of isoniazid or α-ethyl-thioisonicotinamide in an environment favorable for growth showed an early stimulation of their dehydrogenase activity followed by a decrease and loss of this property. It is suggested that these drugs inhibit the synthesis of a lipid component in the membranes of tubercle bacilli and that the process of unbalanced growth resulting from this inhibition is the cause of irreversible injury to the bacterial cells. Evidence in favor of this hypothesis is discussed.

REFERENCES

BAUMAN, N. AND DAVIS, B. D. 1957 Selection of auxotrophic bacterial mutants through diaminopimelic acid or thymine deprival. Science, 126, 170.

BLOCH, H. 1950 Enzymatic characteristics of

suspensions of different mycobacteria. Am. Rev. Tuberc., 61, 270–271.

Bönicke, R. 1958 Ueber die tuberkulostatische Wirksamkeit pentaheterocyclischer Carbonsäurehydrazide. Z. Hyg. Infektionskrankh., 145, 263–275.

Brodie, A. F. and Clarke, T. G. 1956 Phosphorylation coupled to oxidation in bacterial extracts. J. Biol. Chem., 219, 853–862.

Brodie, A. F. and Gots, J. S. 1951 Effects of an isolated dehydrogenase eyzyme and flavoprotein on the reduction of triphenyltetrazolium chloride. Science, 114, 40–41.

Burr, G. O. and Burr, M. M. 1930 On the nature and role of the fatty acids essential in nutrition. J. Biol. Chem., 86, 578–621.

Cavallini, G. B., Mantegazza, E., Massarani, E., Mazzucchi, F., Ravenna, F., and Tommassini. 1952 Attivita antitubercolare "in vitro" di alcuni idrazidi et isonicotinil idrazoni. Atti. soc. lombarda sci. med. e biol., 7, 161–164.

Clifton, C. E. and Logan, W. A. 1939 On the relation between assimilation and respiration in suspensions and in cultures of Escherichia coli. J. Bacteriol., 37, 523–540.

Cohen, S. S. and Barner, H. D. 1954 Studies on unbalanced growth in E. coli. Proc. Natl. Acad. Sci. U. S., 40, 885–893.

Cohen, S. S. and Barner, H. D. 1956 Studies on the induction of thymine deficiency and on the effects of thymine and thymidine analogues. J. Bacteriol., 71, 588–597.

Cymerman-Craig, J., Willis, D., Rubbo, S. D., and Edgar, J. 1958 Mode of action of isonicotinic acid hydrazide. Nature, 176, 34–35.

Dubos, R. J. and Middlebrook, G. 1948 Cytochemical reaction of virulent tubercle bacilli. Am. Rev. Tuberc., 58, 698–699.

Hanks, J. H. 1951 Measurement of the hydrogen transfer capacity of mycobacteria. J. Bacteriol., 62, 521–528.

Holmes, J. H. and Rubbo, S. D. 1958 Rejection of the peroxide accumulation hypothesis of isoniazid action. Nature, 181, 1203.

Koch-Weser, D. and Ebert, R. H. 1955 Tetrazolium reduction by tubercle bacilli. J. Lab. Clin. Med., 46, 608–614.

Koch-Weser, D., Barclay, W. R., and Ebert, R. H. 1955 The influence of isoniazid and streptomycin on acid-fastness, tetrazolium reduction, growth and survival of tubercle bacilli. Am. Rev. Tuberc. Pulmonary Diseases, 71, 556–565.

Krueger-Thiemer, E. 1957 Biochemie des Isoniazids. Jahresber., 1956/57, Borstel, 298–504.

Krueger-Thiemer, E. 1958 Isonicotinic acid hypothesis of the antituberculous action of isoniazid. Am. Rev. Tuberc. Pulmonary Diseases, 77, 364–367.

Lederberg, J. 1956 Bacterial protoplasts induced by penicillin. Proc. Natl. Acad. Sci. U. S., 42, 574–577.

Libermann, D., Rist, N., Grumbach, F., Moyoux, M., Gauthier, B., Rouaix, A., Maillard, J., Himpert, J., and Cals, S. 1954 Études dans le domaine de la chimiothérapie antituberculeuse. III. Hydrazides et hydrazones. Bull. soc. chim. France, 1430–1443.

MacMillan, A. L. and Sinclair, H. M. 1958 The structural function of essential fatty acids. In Essential fatty acid pp. 208–215 (Fourth international conference on Biochem. Problems of Lipids). Edited by H. M. Sinclair. Academic Press, Inc., New York, and Butterworths Scientific Publications, London.

Marshak, A. and Schaefer, W. B. 1952 Comparison of growth and autolysis of two strains of tubercle bacilli. Am. Rev. Tuberc., 65, 75–82.

McElroy, W. 1947 The mechanism of inhibition of cellular activity by narcotics. Quart. Rev. Biol., 22, 25–28.

Middlebrook, G., Dubos, R. J., and Pierce, C. A. 1947 Virulence and morphological characteristics of mammalian tubercle bacilli. J. Exptl. Med., 86, 175–183.

Park, J. T. and Strominger, J. L. 1957 Mode of action of penicillin. Biochemical basis for the mechanism of action of penicillin and for its selective toxicity. Science, 125, 99–101.

Ridgway, G. T. and Douglas, H. C. 1958 Unbalanced growth of yeast due to inositol deficiency. J. Bacteriol., 76, 163–166.

Rist, N., Grumbach, F., and Libermann, D. 1959 Experiments on the antituberculous activity of alpha-ethyl-thiosonicotinamide. Am. Rev. Tuberc. Pulmonary Diseases, 79, 1–5.

Roth, W., Carrara, G., and Erlenmyer, H. 1953 Über die tuberkulostatischen Eigenschaften des Thiophen-2-carbonsäurehydrazids. Helvet. chim. Acta, 36, 1004–1007.

Rubbo, S., Rouch, L. C., Egan, T. B., Waddington, A. L., and Tellesan, W. G. 1958 III. Verazide in the treatment of pulmonary tuberculosis. Am. Rev. Tuberc. Pulmonary Diseases, 78, 250–258.

Russe, H. P. and Barclay, W. R. 1955 The effect of isoniazid on lipids of the tubercle bacillus. Am. Rev. Tuberc. Pulmonary Diseases, 72, 713–718.

SCHAEFER, W. B. 1954 The effect of isoniazid on growing and resting tubercle bacilli. Am. Rev. Tuberc., **69,** 125–127.

SCHAEFER, W. B., MARSHAK, A., AND BURKHART, B. 1949 The growth of *Mycobacterium tuberculosis* as a function of its nutrients. J. Bacteriol., **58,** 549–563.

SCHAEFER, W. B., COHN, M. L., AND MIDDLEBROOK, G. 1955 The role of biotin and carbon dioxide in the cultivation of *Myocobacterium tuberculosis*. J. Bacteriol., **69,** 706–712.

SEGAL, W. S. AND BLOCH, H. 1955 Effects of sunlamp irradiation on the viability, virulence and biochemical activity of *Mycobacterium tuberculosis*. Am. Rev. Tuberc. Pulmonary Diseases, **71,** 112–125.

YONEDA, M., KATO, N., AND OKAZIMA, M. 1952 Competitive action of isonicotinic acid hydrazide and vitamin B_6 in the formation of indole by *E. coli*. Nature, **170,** 803.

YOUATT, J. 1958a Metabolism of isoniazid by *Mycobacterium tuberculosis* (BCG) with reference to current theories of the mode of action. Am. Rev. Tuberc. Pulmonary Diseases, **78,** 806–809.

YOUATT, J. 1958b The action of isoniazid on the transaminase of *Mycobacterium tuberculosis* (BCG). Biochem. J., **68,** 193–197.

ZATMAN, L. T., KAPLAN, N. O., COLOWICK, S. P., AND PIOTTI, M. M. 1954 Effect of isonicotinic acid hydrazide on diphosphopyridine nucleotidases. J. Biol. Chem., **209,** 453–466.