Revival of Tubercle Bacilli After Prolonged *in vitro* Exposure to Isoniazid. (20943)

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Although there have been several publications(1-3) in which evidence was presented that isoniazid is apparently bactericidal for tubercle bacilli *in vitro,* experiments performed in these laboratories and elsewhere(4) have indicated that under certain conditions isoniazid is not bactericidal. Since it has been demonstrated(5) that tubercle bacilli in a resting as well as a growing state will rapidly and strongly absorb and bind isoniazid, this uptake and associated cellular damage may not be easily reversible under the usual test conditions. In such an event, isoniazid-treated mycobacteria would be called "dead". However, it is the purpose of this communication to describe experiments which have demonstrated that when a suitable isoniazid antagonist is employed, revival of tubercle bacilli can be accomplished even after exposure for 5 weeks to amounts of isoniazid 100,000 times greater than the minimal growth inhibitory concentration. These revived cells were not isoniazid-resistant, nor, as far as could be determined, did they represent a small fraction of the original population.

Methods. All experiments were performed in liquid media and with submerged suspensions of tubercle bacilli. Resting cell suspensions of the H37Rv strain of *Mycobacterium tuberculosis* var. *hominis* were obtained by taking a 14-day growth in Dubos liquid Tween-albumin medium which was washed and suspended in phosphate buffer containing 1000 γ of isoniazid/ml. This buffer consisted of 0.63% $Na_2HPO_4 \cdot 12H_2O$ and 0.1% KH_2PO_4, adjusted to pH 6.8. Replicate volumes of the isoniazid-buffer mixture were inoculated with H37Rv so that about 2 x 10^8 cells/ml were present. These were held at room temperature for 5 weeks, and at frequent intervals nephelometric readings were made to determine whether an appreciable amount of growth or lysis had occurred(6). At weekly intervals, an aliquot of isoniazid-treated cells was washed twice in the buffer and then inoculated in a liquid basal synthetic medium* (BM)(7), BM plus 10% (v/v) bovine serum, and BM plus various concentrations of some compounds which were known to antagonize growth inhibition by isoniazid of the H37Rv strain. These compounds were hemin, potassium ferricyanide, sodium pyruvate, and manganous chloride(7). The various types of media inoculated with isoniazid-treated cells were incubated at 37°C for a maximum of 35 days, with inspections made at 7-day intervals for the detection of growth. Any growth which occurred in revival media after 5 weeks exposure to 1000 γ isoniazid was subcultured in Dubos liquid Tween-albumin medium and tested for isoniazid sensitivity in the basal medium and a serum-enriched medium.

Results. Turbidimetric measurements indicated that there was neither growth nor lysis of cells during the 5-week isoniazid treatment. As shown in Table I, reversal of isoniazid inhibition throughout the 5-week period was accomplished in media containing hemin, ferricyanide, or pyruvate. The least concentrations which effected this reversal were: hemin, 0.63 γ/ml; ferricyanide, 25.0 γ/ml; and pyruvate, 500.0 γ/ml. No reversal was apparent in the basal medium or in media containing $MnCl_2$ in concentrations ranging from 10.0 through 0.02 γ/ml. After 2 weeks exposure to INH, growth did not occur in the serum medium. Although precise quantitative methods were not applied, there appeared to be no significant effect on the growth rate or total growth of control H37Rv cultures by the addition to the basal medium of the aforementioned concentrations of hemin, ferricyanide, or pyruvate. On the other hand, as indicated in Table I, the growth of "revived" cells was progressively slower and scantier following subculture from isoniazid exposure.

* In per cent: asparagine, 0.1; $Na_2HPO_4 \cdot 12H_2O$, 0.63; KH_2PO_4, 0.1; sodium citrate, 0.15; $MgSO_4 \cdot 7H_2O$, 0.15; glycerol, 2.0. pH 6.8.

TABLE I. Growth of *M. tuberculosis* after INH Exposure.

Wks exposed to INH	Growth* in: Basal medium (BM)	BM + serum	BM + hemin	BM + $K_3Fe(CN)_6$	BM + pyruvate	BM + $MnCl_2$
1	0	4+	4+	3+	4+	0
2	0	4+	4+	3+	4+	0
3	0	0	3+	2+	+	0
4	0	0	3+	2+	+	0
5	0	0	3+†	+†	+†	0

* Approximated by visual inspection.
† Tested for INH sensitivity.

This was particularly the case with media containing ferricyanide or pyruvate.

As indicated in Table I, growth which occurred after 5 weeks of isoniazid treatment was tested for isoniazid sensitivity. The minimal inhibitory concentration of isoniazid for all such cultures was from 0.006 to 0.024 γ/ml, which is identical with that obtained for the "parent" H37Rv strain(7).

Discussion. A salient feature of this study, in addition to the failure to detect a pronounced bactericidal action of isoniazid, is the illustration of one fallibility of any experiment for the detection of a bactericidal action. It was demonstrated long ago by Chick(8), in the case of heavy metals and sulfides, that a pseudo-bactericidal effect may be observed if one does not employ an antagonist. In this instance, had the revival tests been conducted with only the basal medium or in the serum-enriched medium, it would have been concluded that isoniazid had "killed" all cells within 2 weeks.

It may be argued that isoniazid, like penicillin, is bactericidal only for growing cells, and that these experiments merely confirm this hypothesis. However, it should be pointed out that: (a) resting cells will firmly bind isoniazid(5) and it may be assumed that this bound isoniazid will contribute largely if not entirely to subsequent growth inhibition; (b) by the criteria previously employed, *i.e.*, without an antagonist in the revival medium, the "resting" isoniazid-treated cells in this study would be classed as "dead"; and (c) additional experiments, not fully described here, were performed with growing H37Rv cells with essentially similar results. The latter experiments were performed only with hemin as a reversing agent, which was added directly to H37Rv cultures held at 37°C for 3 weeks in basal media and containing from 0.02 through 10.0 γ isoniazid/ml. The final hemin concentration was 100 γ/ml, as used here, and the highest isoniazid concentration reversed was 2.5 γ/ml. It should also be mentioned that if as little as 0.01% "Tween 80" was added to the menstruum in which tubercle bacilli were exposed to isoniazid, no reversal (revival) was accomplished by any of the antagonists previously named for either resting or growing cells.

The nature or mechanism of isoniazid reversal is not yet clear. The failure of Mn^{++} to reverse isoniazid inhibition may be a matter of cell impermeability. It is likely, as indicated by chemical studies, that the effect of ferricyanide may be ascribed to a direct destruction of isoniazid through oxidation. On the other hand, since hemin and pyruvate are important metabolites, their reversal of isoniazid activity may have been achieved through some reactivation or by-pass of the metabolic site which was damaged or blocked by isoniazid. Because it has been shown by the author(9) that certain isoniazid-resistant strains require hemin as a growth factor, and, more recently, that pyruvate or α-ketoglutarate may also have this function, the hypothesis is strengthened that the site of action of isoniazid may be directly related to the porphyrin metabolism of tubercle bacilli(7), and indirectly to their carbohydrate metabolism.

Summary. Tubercle bacilli exposed to 1000 γ isoniazid/ml of a buffer medium for 5 weeks at room temperature may be revived by appropriate concentrations of hemin, potassium

ferricyanide, or sodium pyruvate. It was concluded that isoniazid possesses only a bacteriostatic effect under these conditions.

The technical assistance of Misses Noraldeen Palecek and Katherine Byrnes is gratefully acknowledged.

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Received February 26, 1954. P.S.E.B.M., 1954, v85.

Exogenous Androgen and Non-Directed Hyperexcitability in Castrated Male Guinea Pig.* (20944)

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In the course of an investigation of the effects of castration and subsequent therapy with testosterone propionate‡ on the sexual behavior of the male guinea pig(1,2), a type of excited non-sexual behavior became increasingly evident. This behavior, called *non-directed hyperexcitability*, could be best described as a sudden straightening of the limbs, jumping into the air, and frequently, by turning of the head. These actions occurred suddenly and did not fit into any sequence of behavior usually seen. The excited movements seemed to occur without reference to the female or to the other behavior seen during the test.

Since this phenomenon is seen periodically in the intact guinea pig, little attention was given to it at the start of the experiment. However, as the investigation progressed it became evident that an increase in the frequency of this behavior had taken place following castration. As a result, systematic observations were not begun until after the main experiment was well underway.

Method. The procedures used in this experiment have been described(1,2). Briefly each of a group of young, mature male guinea pigs 80 to 150 days of age was isolated in an individual cage and given weekly tests in order to determine the strength of sex drive(3). Following the tenth test, all but the intact controls were castrated. After 16 weeks, during which time sexual activity fell off to a low level, all of the castrates except those used as controls were divided into evenly matched groups and given varying amounts of testosterone propionate for the remaining 15 weeks of the experiment. As indicated above, a portion of the experiment had already been completed before the present work was begun. Twenty-eight male guinea pigs of the larger group used in the original experiments contributed data to the present study; data were collected after the third week following castration. These 28 males were divided in the following manner: 2 were intact controls; 7 were given 12.5 γ testosterone propionate/

* This investigation was supported by research grants from the National Institutes of Health, U. S. Public Health Service; the Committee for Research in Problems of Sex, National Research Council, administered by Dr. William C. Young of the Department of Anatomy, University of Kansas School of Medicine; and the University of Kansas Research Fund.

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‡ Testosterone propionate was supplied by the courtesy of Ciba Pharmaceutical Products, Inc.