The Susceptibility to Hydrogen Peroxide of Indian and British Isoniazid-Sensitive and Isoniazid-Resistant Tubercle Bacilli

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The Susceptibility to Hydrogen Peroxide of Indian and British Isoniazid-Sensitive and Isoniazid- Resistant Tubercle Bacilli

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Cohn and others (1954), Bonicke (1954) and Knox, Meadow and Worssam (1956) have shown that isoniazid-resistant, catalase-negative strains of tubercle bacilli, whether obtained by *in vitro* selection or from treated patients, were inhibited by hydrogen peroxide at lower concentrations than their isoniazid-sensitive, catalase-positive parent strains. These authors also demonstrated that hydrogen peroxide was more rapidly bactericidal to the catalase-negative than to the catalase-positive strains.

Kreis and Le Joubioux (1957a) described a quantitative bactericidal test, in which a large inoculum (0.8 mg./ml.) of a dispersed culture was exposed to 0.03 per cent w/v hydrogen peroxide for 3 hrs. at 37°C., and a viable count of the surviving bacterial population was then made. Strains composed of catalase-negative organisms were completely killed, whereas there was little fall in the counts of those with high catalase activity. Such a test should provide an estimate of the proportion of catalase-positive organisms in a strain composed of a mixture of catalase-positive and catalase-negative bacilli. However, the authors showed that a *small* inoculum of catalase-positive organisms was killed by the prolonged contact with the peroxide employed in the test: the reason for the survival of the bacterial populations in the *large* inocula of these organisms normally used was that they were capable of destroying much of the peroxide itself during the period of exposure. Thus the method could not be relied upon to detect the presence of small numbers of catalase-positive bacilli in a predominantly catalase-negative strain, nor was it certain to what extent any of the estimated proportions of these populations would correspond accurately with the true proportions. No experiments were described in which the size of the inoculum, the concentration of peroxide or the exposure period were varied to obtain optimal conditions.

The present work describes an attempt to modify the method of Kreis and Le Joubioux (1957a) so that it would accurately estimate the relative proportions of catalase-positive and catalase-negative organisms in strains containing mixtures of the two types. A bactericidal test was chosen in preference to a bacteriostatic test, since it is difficult to obtain quantitative measurement with the latter technique. In performing a bactericidal test residual peroxide must be inactivated or removed by dilution so that it does not inhibit the growth of surviving organisms. Knox, Meadow and Worssam (1956) removed peroxide by centrifugation and washing, but this method was considered impracticable if this test were to be used on a large

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scale, and likely to produce inaccurate counts on the surviving organisms. In the present work the method of removal of peroxide was studied as well as the determination of the optimal peroxide concentration and period of exposure which would kill all catalase-negative organisms, but would leave catalase-positive organisms unaffected. In addition, the method of Kreis & Le Joubioux (1957a) was modified by reducing the inoculum of organisms exposed to peroxide so that catalase-positive bacilli would not be able to destroy peroxide during the test itself. The standardised bactericidal test was then employed in comparing the susceptibility to peroxide of isoniazid-sensitive strains from British and Indian patients, and in investigating the relationship between the peroxide susceptibility and the catalase activity of their isoniazid-resistant mutant strains.

Materials and Methods

Strains of tubercle bacilli

Strain H37Rv had been maintained at the Postgraduate Medical School of London on Löwenstein-Jensen medium for over five years. This strain had a degree of catalase activity normally encountered among other isoniazid-sensitive strains.

Strain B, resistant to 50 µg/ml. isoniazid and catalase-negative, was obtained from strain H37Rv by *in vitro* selection in medium containing isoniazid (Barnett, Bushby and Mitchison, 1953). It had been maintained on isoniazid-free Löwenstein-Jensen medium for the past five years. Strain BI was obtained by passaging strain B in 7H10 medium containing 50 µg/ml. isoniazid at the time of the experiments to be described.

A total of 17 isoniazid-sensitive strains was obtained from 9 Indian and 8 British patients who had not received anti-tuberculosis chemotherapy, and heavy suspensions of these strains were inoculated on to slopes of Löwenstein-Jensen medium containing 0.2 and 5 µg/ml. of isoniazid to obtain resistant, mutant colonies. The colonies were subcultured and their catalase activities measured. The most catalase-positive of the mutant clones from 0.2 µg/ml. isoniazid and the most catalase-negative of the mutant clones from 5 µg/ml. isoniazid were selected for further study.

Media

Löwenstein-Jensen medium without potato starch (Jensen and Kiaer, 1959) was used for the maintenance of all cultures and isoniazid sensitivity tests (Medical Research Council, 1953 : Tuberculosis Chemotherapy Centre, 1959). 7H10 Tween-albumin medium, as described by Cohn, Middlebrook and Russell (1959), but with the omission of glycerol, was used for subcultivation of strains in liquid medium. 7H10 oleic acid-albumin medium (double strength), a modification of 7H10 medium (Cohn, Middlebrook & Russell, 1959) was used for viable counts. The composition was: KH_2PO_4, 2.0 g; $Na_2HPO_4.12H_2O$, 12.6 g; 1-Na glutamate, 1.0 g; $(NH_4)_2SO_4$, 1.0 g; Na citrate, 0.8 g; ferrous ammonium citrate (green), 160 mg; $MgSO_4.7H_2O$, 100 mg; $CaCl_2.2H_20$, 1.0 mg; $ZnSO_4.7H_2O$, 20 mg; $CuSO_4.5H_2O$, 2.0 mg; pyridoxine HCl, 2.0 mg; biotin, 1.0 mg; glycerol, 4.0 ml; distilled water to 1 litre. The medium was autoclaved at 15 lb./sq. in. for 20 min. and to it was added, with aseptic precautions, (a) 200 ml. of a solution containing 5 per cent bovine albumin (Fraction V, Armour Ltd.), 2 % glucose and 0.06 % oleic acid, which had been sterilised by Seitz filtration and (b) 0.6 ml. of catalase 1 mg./ml., sterilised by filtration through a sintered glass filter (U.F. grade, Pyrex brand).

Reagents

(1) *Hydrogen peroxide:* 30 **%** w/v. 'Analar' (British Drug House). Solutions were standardised by titration with permanganate.

(2) *Catalase:* Crude beef liver extract (Nutritional Biochemical Inc., Ohio, U.S.A.).

(3) *M/15 phosphate buffer:* pH 7.0, prepared according to Sorensens standard tables, and autoclaved at 15 lbs. for 20 min.

Viable Count

The silica gel method of Selkon and Mitchison (1957) was employed for viable counts, modified by the use of double strength 7H10 medium.

Catalase estimations

Estimates of qualitative catalase activity (Tuberculosis Chemotherapy Centre, 1959) and semi-quantitative catalase activity (Kreis, Le Joubioux and Pariente, 1956; Kreis & Le Joubioux, 1957b) were carried out on all the strains.

The standard bactericidal test

The strain to be examined was grown for 8 days in 7H10 Tween-albumin liquid medium and 0.2 ml. volumes were added to 1 oz. screw capped bottles containing (a) 3.8 ml. of 0.02 w/v hydrogen peroxide in pH7 phosphate buffer and (b) 3.8 ml. of buffer alone. The bottles were incubated for 90 min. at 37ºC, and viable counts were then set up from each bottle, 0.2 ml. volumes of the reaction suspensions, and of 1:40 and 1: 1600 dilutions being added to the viable count bottles, which contained 3.1 ml. double strength 7H10 medium. After addition of 3.3 ml. silica sol, the bottles were incubated at 37°C. for 4 weeks before the counts were read.

Experiments Delineating the Standard Bactericidal Test

(1) *Termination of the activity of hydrogen peroxide by the addition of catalase*

Preliminary experiments were carried out with the aim of developing a very simple method in which the organisms could be exposed to hydrogen peroxide in viable-count bottles. Strains H37Rv or B were added to viable-count bottles containing

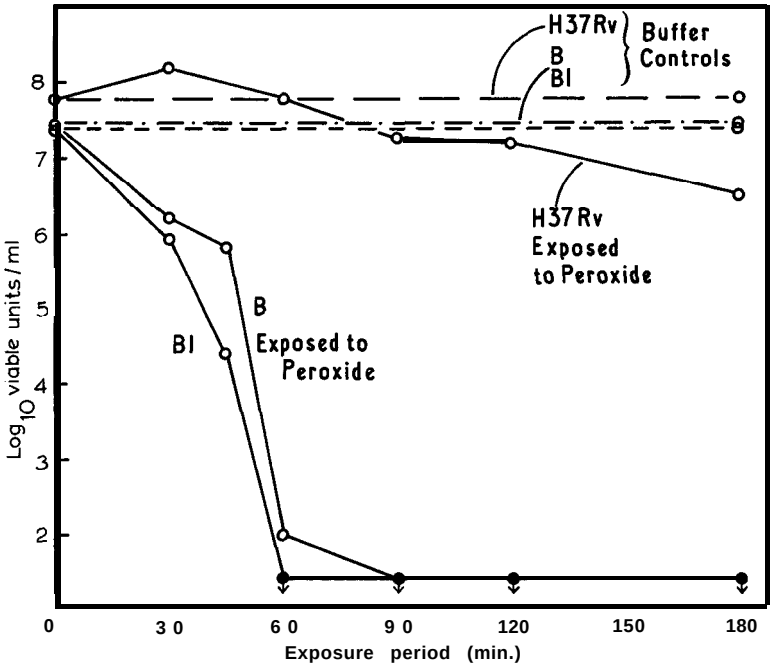

FIG. I. - Viable counts on strain H37Rv and its catalase-negative variants, B and BI, during exposure to 0.02 per cent hydrogen peroxide. ↓ No growth.

various concentrations of peroxide in 7H10 double strength medium and, after incubation, catalase was added to destroy it. A concentration of 0.005 per cent H_2O_2 had no bactericidal action on strain B, while, even after exposure for 80 min., a small proportion of this catalase-negative strain survived exposure to 0.01 per cent H_2O_2 On the other hand strain H37Rv failed to survive exposure to 0.05 per cent H_2O_2 for five min. With the intermediate concentration of 0.02 per cent H_2O_2, strain B was completely killed, while viable counts on H37Rv were almost unaffected. The termination of the activity of the peroxide by catalase was, however, unreliable, since addition of catalase 30 min. *before* the inoculum of strain B to medium containing as low a concentration as 0.02 per cent H_2O_2 did not always allow growth of the organisms.

(2) Termination of the activity of hydrogen peroxide by dilution

In the remaining experiments the test strains were incubated with 0.02 per cent H_2O_2 in pH7 buffer solution as described for the standard bactericidal test, and the activity of the peroxide was terminated by dilution (1: 15 or more) when 0.2 ml. of the suspension was added to the medium in the viable count bottles or to dilution tubes. The results of incubating strains H37Rv, B and BI with peroxide for various periods are shown in Fig. 1. There was no change in the viable counts on strain H37Rv during 60 min. exposure to peroxide, though a fall from 7.8 to 6.5 log $_{10}$ viable units/ml. occurred between 60 and 180 min. On the other hand no surviving organisms of strain BI were detected at or after 60 min. exposure to peroxide.

In an earlier experiment, performed in a similar manner, strain B continued to yield a small number of colonies after exposure to 0.02 per cent peroxide for as long as 90 and 120 min. Sub-cultures from these colonies showed definite and sometimes high catalase activity. Reverse mutation from catalase-negativity to catalase-positivity may have occurred in strain B during the 5 year period of maintenance on drug-free medium since its initial production from strain H37Rv. In view of this possibility, it was passaged through Tween-albumin liquid medium containing 50 µg/ml. isoniazid and the resulting strain (BI) was employed in further experiments. The slower killing of strain B than of strain BI, illustrated in Fig. 1, suggests that catalase-positive reverse-mutants were probably eliminated during the passage.

(3) Reconstruction experiments

Mixtures containing known proportions of isoniazid-sensitive, catalase-positive strains and their resistant, catalase-negative, variant strains were exposed to 0.02 per cent peroxide for various lengths of time to see how accurately the bactericidal test would estimate the proportion of catalase-positive organisms in the mixture. The results of two experiments with strain H37Rv and its catalase-negative variant BI, and with an Indian isoniazid-sensitive strain 15221-S and its catalase-negative variant 15221-R are set out in Tables I and II. In column (a) of these tables are shown the control viable counts on the strains exposed only to buffer, and in columns (b), (c) and (d) are the counts found after exposure to peroxide. In both experiments little change had occurred in the counts on the parent catalase-positive strains after 90 min. exposure to peroxide, whereas the counts on the catalase-negative variants had been reduced from over 10^7 to less than 10^2 viable units/ml. in the same period. The number of catalase-positive organisms to be expected in the mixtures, calculated from the counts on the buffer controls on the assumption that all catalase-positive organisms would survive and all catalase negative organisms would be killed, are given in column (e). Comparison of the values in column (e) with those in columns (b), (c) and (d) shows that the expected counts agree well with those found after exposure of the mixtures to peroxide for 90 min., though they are on average 36 per cent lower. Exposure for 120 min. yielded estimates that were even lower, presumably due to greater killing of the catalase-positive organisms by the peroxide.

TABLE I. - RECONSTRUCTION EXPERIMENT WITH STRAIN H37Rv AND ITS CATALASE-NEGATIVE VARIANT BI

composition of strain tested (volume per cent)		*Viable units per ml.*					
		Found				*Expected (from count on H37Rv)*	
		Buffer only 90 min.	*Period of exposure to H_2O_2*			*Exposed to buffer only*	*After exposure to H_2O_2 for 90 min.*
H37Rv	*BI*	*(a)*	*45 min. (b)*	*60 min. (c)*	*90 min. (d)*	*(column (a)) (e)*	*(column (d)) (f)*
100	0	$1{\cdot}3\times10^{8}$	$1{\cdot}2\times10^{8}$	$1{\cdot}0\times10^{8}$	$1{\cdot}0\times10^{3}$	—	
0	100	$6{\cdot}6\times10^{7}$	$2{\cdot}2\times10^{5}$	$3{\cdot}2\times10^{3}$	$<10^{2}$*	—	
10	90	$6{\cdot}4\times10^{7}$	$1{\cdot}3\times10^{7}$	$1{\cdot}1\times10^{7}$	$7{\cdot}7\times10^{6}$	$1{\cdot}3\times10^{7}$	$1{\cdot}0\times10^{7}$
1	99	$5{\cdot}8\times10^{7}$	$1{\cdot}1\times10^{6}$	$9{\cdot}7\times10^{5}$	$7{\cdot}8\times10^{5}$	$1{\cdot}3\times10^{6}$	$1{\cdot}0\times10^{6}$
0·1	99·9	$6{\cdot}7\times10^{7}$	$2{\cdot}7\times10^{6}$	$1{\cdot}9\times10^{5}$	$1{\cdot}6\times10^{5}$	$1{\cdot}3\times10^{5}$	$1{\cdot}0\times10^{5}$
0·01	99·99	$7{\cdot}0\times10^{7}$	$2{\cdot}0\times10^{5}$	$1{\cdot}8\times10^{4}$	$8{\cdot}2\times10^{3}$	$1{\cdot}3\times10^{4}$	$1{\cdot}0\times10^{4}$
0·001	99·999	$5{\cdot}8\times10^{7}$	$2{\cdot}4\times10^{4}$	$2{\cdot}9\times10^{3}$	$8{\cdot}0\times10^{3}$	$1{\cdot}3\times10^{3}$	$1{\cdot}0\times10^{3}$

* No growth.

Periods of 45 min. or 60 min. exposure yielded high estimates when the proportion of catalase-positive organisms in the mixture was very low (0.001 per cent), presumably due to survival of a small proportion of the catalase-negative bacilli. The optimal period of exposure therefore appeared to be 90 min. and this was adopted for further work.

In column (f) is set out the counts to be expected in the mixtures after exposure to peroxide for 90 min. calculated from the count on the pure catalase-positive strain after exposure to peroxide for the same time and on the assumption that all catalase-negative organisms had been killed. The counts in column (f) agree very closely with those found on the mixtures (Table I, column (d), and Table II, column (c)). It is clear that, even when the mixtures contained as high a proportion as 10 per cent of catalase-positive organisms, the bactericidal activity of the peroxide was not diminished. Thus, the test can be relied upon to estimate accurately the proportion of catalase-positive organisms in a mixed population over a very wide range of mixtures. It is also capable of detecting very small numbers (1 in 10^{5}) of catalase-positive organisms in a predominantly catalase-negative culture.

TABLE II. - RECONSTRUCTION EXPERIMENT WITH MIXTURES OF CATALASE-POSITIVE AND CATALASE-NEGATIVE INDIAN STRAINS

Composition of strain tested (volume per cent)		*Viable units per ml.*					
		Found				*Expected (from count on 15221-S)*	
		Buffer only 90 min.	*Period of exposure to H_2O_2*			*Exposed to buffer only*	*After exposure to H_2O_2 for 90 m.n.*
15221-s	*15221-R*	*(a)*	*60 min. (b)*	*90 min. (c)*	*120 min. (d)*	*(column (a)) (e)*	*(column (d)) (f)*
100	0	$7{\cdot}2\times10^{7}$	$7{\cdot}8\times10^{7}$	$7{\cdot}1\times10^{7}$	$4{\cdot}8\times10^{7}$	—	
0	100	$3{\cdot}1\times10^{7}$	$2{\cdot}4\times10^{4}$	$5{\cdot}0\times10^{1}$*	$1{\cdot}0\times10^{2}$		
10	90	$3{\cdot}8\times10^{7}$	$8{\cdot}5\times10^{6}$	$5{\cdot}1\times10^{6}$	$5{\cdot}8\times10^{6}$	$7{\cdot}2\times10^{6}$	$4{\cdot}8\times10^{6}$
0·1	99·9	$4{\cdot}0\times10^{7}$	$1{\cdot}0\times10^{6}$	$8{\cdot}4\times10^{4}$	$4{\cdot}8\times10^{4}$	$7{\cdot}2\times10^{4}$	$4{\cdot}8\times10^{4}$
0·001	99·999	$4{\cdot}0\times10^{7}$	$8{\cdot}3\times10^{3}$	$3{\cdot}5\times10^{2}$	$2{\cdot}0\times10^{2}$	$7{\cdot}2\times10^{2}$	$4{\cdot}8\times10^{2}$

* 1 colony only.

TABLE III. - BACTERICIDAL ACTION OF HYDROGEN-PEROXIDE ON ISONIAZID SENSITIVE STRAINS OF TUBERCLE BACILLI

Strain No.	*Origin of strain*	*Catalase activity*		*Log_{10} viable units*		*Peroxide resistant organisms (per cent)*
		Qualitative	*Semi-quantitative (per cent)*	*exposed to H_2O,*	*Buffer control*	
I 837	British	++	90	7·53	7·54	98
I 841	,,	++	100	7·66	7·56	126
I 907	,,	++	100	7·84	8·16	48
I 919	,,	++	100	7·28	7·71	37
I 959	,,	++	90	7·39	7·66	54
I 952	,,	++	90	7·76	7·94	66
I 1129	,,	++	100	7·33	7·52	65
I 1107	,,	++	100	7·23	7·72	32
28539	Indian	++	100	7·58	7·47	129
30510	,,	++	80	6·50	6·70	63
31370	,,	++	100	4·90	7·88	0·10
34024	,,	++	90	4·90	7·51	0·25
34254	,,	++	80	7·46	7·80	46
39804	,,	++	60	5·80	7·47	2·1
40038	,,	++	100	5·80	7·70	1·3

Results

ISONIAZID-SENSITIVE STRAINS

In Table III are set out the results of the standard, bactericidal, peroxide sensitivity test on 8 British and 7 Indian isoniazid-sensitive, catalase-positive strains. The percentage of the organisms in the strain that survived exposure to 0.02 per cent peroxide for 90 min. is shown in the right hand column. Among the British strains the average percentage was 67 (range of 32 % to 126 %), a value that agrees well with the percentages of survivors (64 %) in similar isoniazid-sensitive strains obtained in the reconstruction experiments. In contrast, 4 of the Indian strains appeared rather more susceptible to peroxide. The average percentage of surviving organisms was 35, and the range of 0.10 % to 129 % was wider. The difference in susceptibility between British and Indian strains attains significance at the 5% level (Fisher-Behrens test on logarithmic averages).

ISONIAZID-RESISTANT STRAINS

The results of the standard hydrogen peroxide sensitivity test on 24 isoniazid-resistant strains, all of which were clones derived from single colonies, are set out in Table IV, in which the strains are arranged in order of their catalase activity.

British Strains

Considering first the results on the 13 British strains, 6 of these (1952b to 1919b), with semi-quantitative catalase activity of 0% to 10% and no activity in the qualitative test, all contained populations of bacilli apparently entirely susceptible to peroxide, the percentages of surviving organisms being less than 0.001%. The remaining 7 strains with relatively high degrees of catalase activity in the semi-quantitative test (10% to 50%) and in the qualitative test (1 -plus or more) were all moderately resistant to peroxide; the percentages of surviving organisms ranged from 0.11 % to 23 %. These values were lower than any of those of their parent isoniazid-sensitive strains (Table III).

The degree of resistance to isoniazid of the strains has been expressed in two ways. The first of these was the minimal concentration of isoniazid inhibiting the growth of 20 or more colonies (Table IV, column (b)); the results showed only a weak

TABLE IV. - BACTERICIDAL ACTIVITY OF HYDROGEN PEROXIDE ON STRAINS RESISTANT TO ISONIAZID, RELATED TO THEIR CATALASE ACTIVITY AND DEGREE OF ISONIAZID,-RESISTANCE

Strain No.	*Origin of strain*	*Degree of isoniazid-resistance*		*Catalase activity on drug-free control slopes*		*Log$_{10}$ viable units*		*Peroxide resistant organisms (per cent)*
		Highest isoniazid concentration allowing growth of innumerable discrete colonies (μg/ml.) (*a*)	*Minimal isoniazid concentration inhibiting growth of 20 or more colonies (μg/ml.)* (*b*)	*Qualitative*	*Semi-quantitative (per cent)*	*Exposed to H_2O_2*	*Buffer control*	
I 952b	British	50	> 50	0	0	< 2·00	7·20	< 0·0006
I 959 b	,,	50	> 50	0	0	< 1·70	7·40	< 0·0002
I 1129 b	,,	50	> 50	0	0	< 2·00	7·00	< 0·001
I 837 a	,,	50	> 50	0	10	< 2·00	7·51	< 0·0003
I 907 b	,,	50	> 50	0	10	< 2·00	7·60	< 0·0003
I 919 b	,,	50	> 50	0	10	< 2·00	7·80	< 0·0002
I 919 a	,,	1	5	+	10	6·07	7·51	3·6
I 907 a	,,	1	50	+	40	6·26	7·93	2·1
I 959 a	,,	5	50	+	40	4·58	7·52	0·11
I 1107 b	,,	5	> 50	+	40	6·14	7·02	13
I 1107 a	,,	5	50	++*	40	6·44	7·08	23
I 1129 a	,,	1	5	+	50	6·06	7·06	10
I 952 a	,,	0·2	50	++	50	6·10	7·72	2·4
28539 a	Indian	50	> 50	0	0	< 2·00	6·20	< 0·0063
31370 b	,,	5	50	0	0	< 2·00	7·54	< 0·0003
34254 b	,,	50	> 50	0	0	< 2·00	7·53	< 0·0003
30510 a	,,	5	50	+	30	3·95	6·38	0·37
39804 a	,,	50	> 50	++	30	4·91	6·74	1·5
31648 a	,,	1	5	+	40	< 2·00	8·30	< 0·00005
34024 a	,,	1	5	+	40	4·75	7·48	0·19
34254 a	,,	1	5	+	40	6·83	7·48	22
31648 b	,,	5	50	+	50	< 2·00	7·30	< 0·0005
31370 a	,,	0·2	1	++	60	4·20	7·95	0·018
40038 a		0·2	1	+	90	5·11	7·12	0·98

* activity as found with isoniazid-sensitive strains

association with the catalase activity or hydrogen peroxide susceptibility of the strain. The second way (Table IV, column (a)) was the highest concentration of isoniazid which allowed growth of at least innumerable discrete colonies (the control drug-free slopes yielded confluent growth). Such results give a better measure of the degree of resistance of the majority of the bacterial populations in the resistant clones and are less affected by the presence of rare organisms with an exceptionally high degree of resistance. As measured in this way, all of the 7 British strains with moderate resistance to hydrogen peroxide and relatively high catalase activity had low degrees of resistance with growth on slopes containing up to but not more than 5 µg./ml. isoniazid, whereas the remaining 6 strains with peroxide-susceptible populations and low catalase activity all grew on 50 µg./ml. isoniazid.

Indian Strains

Considering the 11 Indian isoniazid-resistant strains, 3 had no demonstrable catalase activity in both the semi-quantitative and the qualitative tests and their bacterial populations appeared entirely susceptible to peroxide. The other 8 strains had from 30 % to 90 % activity in the semi-quantitative test and at least l-plus activity in the qualitative test. Among these 8 strains, 5 had a peroxide susceptibility comparable with the British isoniazid-resistant strains which retained a relatively high catalase activity; the percentages of peroxide-resistant organisms ranged from 0.19 % to 22 %. However, the remaining 3 strains (31648a, 31648b and 31370a) appeared considerably more susceptible to peroxide; the percentages of peroxide-resistant organisms were < 0.00005 %, < 0.005 % and 0.018 %, respectively.

Table IV also shows an association between the degree of isoniazid resistance and the catalase activity of Indian strains, but this is. somewhat less marked than among the British strains. The isoniazid-sensitivity of strains 31648a, 31648b and 31370a did not differ from that of the remaining strains with similar degrees of catalase activity.

The peroxide susceptibilities of pairs of the parent isoniazid-sensitive strains and their isoniazid-resistant mutant strains are compared in Table V for those isoniazid-

TABLE V. - THE HYDROGEN PEROXIDE SUSCEPTIBILITY OF ISONIAZID-SENSITIVE PARENT STRAINS AND THEIR CATALASE-POSITIVE, ISONIAZID-RESISTANT, MUTANT STRAINS

Strain No.	*Origin of strain*	*Peroxide resistant organisms (per cent)*	
		Isoniazid-sensitive parent strain	*Isoniazid-resistant mutant strain*
I 952	British	66	2·4
I 1129	,,	65	10
I 959	,,	54	0·11
I 907	,,	48	2·1
I 1107	,,	32	23
			13
30510	Indian	63	0·37
34254	,,	46	22
39804	,,	2·1	1·5
40038	,,	1·3	0·98
34024	,,	0·25	0·19
31370	,,	0·10	0·018

resistant strains that still retained at least 10% catalase activity in the semi-quantitative test and l-plus activity in the qualitative test, and where tests on both strains of the pair were available. It will be appreciated that these results are selected from Tables III and IV. For the 5 pairs of British strains no association is apparent between the peroxide susceptibilities of the isoniazid-sensitive and resistant strains. Among the 6 pairs of Indian strains there is a suggestion that a low percentage of peroxide-resistant organisms in the parent strain may be associated with a low percentage in the isoniazid-resistant mutant strain. This association fails to attain statistical significance (P=0.1-0.05), but it is noteworthy that the isoniazid-sensitive parent strain 31370 had the lowest percentage of peroxide-resistant organisms among all of the isoniazid-sensitive strains, and its isoniazid-resistant mutant strain also had a percentage of peroxide resistant organisms lower than was found in any of the British strains or of the other Indian strains.

Discussion

The hydrogen peroxide bactericidal test described above has overcome the main defects of the method of Kreis and Le Joubioux (1957a), namely the use of a very large inoculum in which catalase-positive organisms were responsible for destruction of the peroxide during the test exposure, and the consequent use of a prolonged exposure period. In the present test both the size of the inoculum and the exposure period have been reduced, so that it has been possible to estimate accurately the proportion of isoniazid-sensitive, catalase-positive bacilli in a mixture of catalase-positive and catalase-negative organisms. The accuracy was maintained over a wide range of proportions of the two types of organisms and the test was capable of detecting catalase-positive bacilli, even when these were present as only 1 in 10^5 of the bacterial population.

The dilution of the hydrogen peroxide in the viable count procedure appeared to be sufficient to allow subsequent growth of any organisms which had survived the period of exposure in the test. The counts in the different dilutions from a single test showed good proportionality. On the other hand, preliminary attempts to terminate the activity of peroxide by addition of catalase were unsuccessful, when the exposure to peroxide and the viable count were carried out in the same bottle. Subsequent inhibition of growth may have been due to the high concentration of nascent oxygen in the culture medium or to the inactivation of the catalase by the peroxide before it had reduced the peroxide concentration to a sufficiently low value to allow growth.

British and Indian strains showed differences in their susceptibility to hydrogen peroxide. Among the isoniazid-sensitive strains, all of which were inhibited by 0.2 µg/ml. isoniazid, the 8 British strains had a fairly uniform high degree of resistance to 0.02 per cent peroxide, whereas 4 of the 7 Indian strains were more susceptible. No difference was apparent in the catalase activity of the strains from the two countries. A similar difference was also apparent among those isoniazid-resistant strains which retained catalase activity. The 7 British strains contained at least 0.11 per cent peroxide-resistant organisms, whereas 3 of the 8 Indian strains contained 0.018 per cent or less of these organisms. The Indian strains had a degree of catalase activity at least as high as that of the British strains. There was a suggestion that isoniazid-sensitive Indian strains with increased susceptibility to peroxide yielded catalase-positive, isoniazid-resistant, mutant strains also with increased susceptibility. A peroxide susceptibility lower than would be expected from the catalase activity in a proportion of Indian strains can be explained in various ways. The permeability of the cell wall of these organisms to hydrogen peroxide might be lower than in the remaining Indian strains and in those from Britain. Winder (1960)

has shown that the cells of BCG are less permeable to hydrogen peroxide than those of *Mycobacterium smegmatis*. Alternatively, the Indian strains might differ in their proportions of intracellular and extracellular catalase. Finally there could be differences in the biological systems affected by hydrogen peroxide within the cells.

The relationship between peroxide susceptibility and animal virulence of Indian strains is being investigated. This is of particular interest, since Mitchison and others (1960) have shown that isoniazid-sensitive Indian strains of tubercle bacilli, of similar origin to those reported on here, had a wide range of virulence in the guinea-pig and were frequently less virulent than British strains. The composition of the surface of the bacterial cell is likely to play a major role in determining virulence (Bloch, 1950; Middlebrook, Coleman and Schaefer, 1959), and may also control the permeability of the cell wall to peroxide or to bacterial catalase. Furthermore, Coleman and Middlebrook (1956) have postulated that the diminished virulence of *isoniazid-resistant* tubercle bacilli may be directly due to their increased susceptibility to low concentrations of hydrogen peroxide produced from glutathione in host macrophages. Such a mechanism could also explain the attenuation of a proportion of *isoniazid-sensitive* Indian strains.

The occurence in a catalase-negative strain of reverse-mutation *in vitro* to catalase positivity appears to have been demonstrated during the investigations carried out on strain B. This strain was selected from strain H37Rv in 1953, when it was passaged 5 times through liquid medium containing 50 µg/ml. isoniazid. It must then have been composed entirely of organisms resistant to 50 µg/ml. isoniazid and catalase-negative. During the next 5 years it was maintained by serial subcultivation on drug-free Löwenstein-Jensen medium. In the experiments reported here, colonies with full catalase activity were obtained from strain B after exposure to peroxide for 60 or more minutes. It seems reasonable to suggest that these colonies arose from catalase-positive organisms which had emerged by reverse-mutation during sub-cultivation of strain B on drug-free medium. Such reverse-mutants cannot have recovered the full biological potential of the parent H37Rv organisms, since they were present as only a very small proportion of a population that was still predominantly catalase-negative. Evidence for reverse-mutation in stabilized isoniazid-resistant, catalase-negative strains of tubercle bacilli *in vitro* has been presented by Middlebrook (1958) and in monkeys by Schimdt and others (1958).

Summary

A standard, bactericidal, hydrogen peroxide sensitivity test was developed, in which a viable count was done on strains that had been exposed to 0.02 per cent hydrogen peroxide for 90 min. at 37°C. This test has been found to estimate accurately the proportion of catalase-positive, isoniazid-sensitive organisms in a mixture with catalase-negative, isoniazid-resistant organisms.

Among strains from British patients, 8 with full sensitivity to isoniazid were uniformily resistant to peroxide and 7, which were isoniazid-resistant but still retained some catalase activity, were more susceptible to peroxide, but contained at least 0.11 % peroxide-resistant organisms. In contrast, among strains from Indian patients, 4 of the 7 isoniazid-sensitive, catalase-positive strains were more susceptible to peroxide than any of the British strains and 3 of the 8 isoniazid-resistant, catalase-positive strains contained 0.018 % or less peroxide-resistant organisms. All of the 9 catalase-negative, isoniazid-resistant strains from both British and Indian patients were completely susceptible to peroxide.

An association was found between low catalase activity, high susceptibility to peroxide and a high degree of resistance to isoniazid. This association was more clear cut among British than among Indian strains.

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