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TITLE **Studies Of The Catalase Activity Of Hemophilus Pertussis And Antigenically Related Organisms**

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Abstract

STUDIES OF THE CATALASE ACTIVITY OF HEMOPHILUS PERTUSSIS AND ANTIGENICALLY RELATED ORGANISMS

By Lucile M. Portwood

Fifty-five strains of smooth Hemophilus pertussis, four strains of rough H. pertussis, 10 strains of the parapertussis bacillus and 10 strains of Brucella bronchiseptica were examined for catalase activity.

In developing a technic for quantitative measurement of catalase activity, it was found that in a given initial concentration of H_2O_2, the catalase activity of a culture was proportional to the bacterial concentration within a range of one to seven billion organisms per ml., and that the measured catalase activity of a given suspension varied with the initial concentration of H_2O_2.

The conditions of growth that were studied in relation to their effect on catalase activity were culture media, temperature of incubation, and age of culture. When cultures were compared on different media, the highest activity of the parapertussis bacillus and Br. bronchiseptica was obtained in Tryptose-glucose broth and the lowest on veal infusion agar. Strains cultured on Bordet Gengou agar gave intermediate values. The incubation temperature for optimum activity of H. pertussis was 33°C. There was a greater decrease in values at higher temperatures than at lower ones. The age of the culture at which maximum activity was reached and the duration of maximum activity were not the same for all cultures. Cultures of each of the three species usually showed maximum activity after incubation for two days.

Sixteen per cent of the strains of H. pertussis examined showed no catalase activity but maintained all the characteristics of smooth strains.

From agglutination tests performed with adsorbed sera, there was no indication of any difference in the antigenic structure of the groups of strains with and without catalase activity. The strains which showed no catalase activity were killed more readily by H_2O_2 than were those with catalase activity. Strains of H. pertussis which retained all characteristics of smooth strains through a number of subcultures had no significant variation in catalase activity. Three cultures which lost the ability to decompose H_2O_2 showed concurrently a loss in characteristics which typify smooth strains.

Except for the 16 per cent of smooth strains of H. pertussis which showed no activity, smooth strains had a higher catalase activity than rough ones. Detection of dissociation by agglutination in the presence of trypaflavine was not applicable to H. pertussis since both smooth and rough strains agglutinated.

For retaining catalase activity of cells during a period of storage, a low temperature and a high concentration of cells offered the best conditions. Phenyl mercuric borate was the most satisfactory preservative.

The catalase activity of smooth strains of these three species was not significantly different. There was some variation in catalase activity within each species but these differences were not correlated with any other characteristic.

[illegible] CATALASE ACTIVITY OF [illegible]

[illegible]

BY

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THESIS

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Studies on the Catalase Activity of Hemophilus pertussis and Antigenically Related Organisms

STUDIES OF THE CATALASE ACTIVITY OF HEMOPHILUS PERTUSSIS AND ANTIGENICALLY RELATED ORGANISMS

Hemophilus pertussis is a relatively inactive species. For growth it requires a high concentration of blood or enriching substances. This organism does not produce indol, does not reduce nitrates and shows no action in media containing carbohydrates. It does hemolyse red cells and it does show catalase activity. A smooth strain of H. pertussis produces a typical hemorrhagic necrosis in the skin of normal rabbits and is lethal for mice when suspended in mucin and injected intraperitoneally in large doses, such as three billion organisms. As the organism dissociates it shows a decrease in agglutination titer, a loss in toxic properties which produce skin necrosis, a drop in virulence, and it becomes adapted to a blood-free medium.

Included in this study of H. pertussis are the antigenically related species, Brucella bronchiseptica and the parapertussis bacillus. The relationship of the antigenic structure of H. pertussis and Br. bronchiseptica was shown by Perry and Noble (11) with the agglutination and agglutinin-absorption reactions, and by Perry and Felix (12) with complement fixation tests. Like H. pertussis, Br. bronchiseptica is inactive in carbohydrate media and demonstrates catalase activity. Differing from H. pertussis, it is motile, reduces nitrates, and is lethal for mice in smaller doses, about 50 million organisms on intraperitoneal injection.

The parapertussis bacillus was described by Alderin and Kendrick (9). They showed by cultural characteristics and by agglutinin-absorption tests that the organism is related to both H. pertussis and

Br. bronchisepticus but identical with neither. It appears to be an intermediate species since it resembles both of the other two species more than they resemble each other. The parapertussis bacillus does not attack carbohydrates, produce indol nor reduce nitrates. Like the other two species it shows catalase activity.

The catalase activity of B. pertussis was shown to be directly proportional to the virulence of the strains by Huddleson and Stahl (71). It would be of value to know if this relationship exists for other pathogenic organisms and if there is any association with immunological phenomena. It is necessary to show whether the catalase activity of B. pertussis remains constant during a series of transfers cultured under the same conditions. If there are variations in catalase activity, it is important to determine under what conditions they occur and whether they are associated with any changes in cultural, morphological, serological, biochemical, immunological or pathological characteristics. If the transition from smooth to rough forms is accompanied by any alteration in catalase activity, there would be available an additional simple and useful tool for the biological characterization of cultures.

LITERATURE

In the 1860's Schönbein observed the ability of extracts of animal and vegetable tissues to decomposed H_2O_2 into molecular oxygen and water. He thought that this was a property of all enzymes. Hart and Jensen (5) in 1903 noted that microbes as well as other organic substances possessed this characteristic but lost it when heated to 70°C. Battistein (14) in 1895 observed under the microscope the evolution of oxygen bubbles when H_2O_2 was added to Bacillus megatherium and Escherichia coli. He was not sure whether the bacteria were alive, or killed by boiling or by antiseptics. Loewenbeck (8) stated that all living bacteria did not have this property even after the acid was neutralized. Bacillus subtilis, Kim (15) demonstrated that this ability was inhibited in the presence of cyanide.

In 1901, Loew (7) studied the tobacco leaf separated an enzyme into the proteolytic and peptolytic enzymes, and named it catalase. Löwenstein (16) studied the catalase activity in filtrates of Corynebacterium diphtheriae and Staphylococcus sp.; he found none present in filtrates of Clostridium tetani. Calisti (17) observed that Bacillus anthracis showed little catalase activity either in the virulent state or attenuated as vaccine. Bacteria, yeast and a mold isolated from milk were examined by Orla-Jensen (21) for enzyme activities. This investigation included a wide variety of strains of non-pathogenic organisms. The facultative or obligate anaerobes showed no catalase activity. He concluded that the activity was due to an endo-enzyme and was a property of the living cell.

The first quantitative determinations of the catalase activity of bacteria were made by Tyrosch and Lubrosch (74) in 1907. They measured the oxygen liberated when H_2O_2 was added to a definite weight of bacteria. In descending order of their catalase activity, some of the organisms they listed were: "Bacillus prodigiosus, Micrococcus pyogenes aureus, Micrococcus Friedländer, Staphylococcus aureus, Micrococcus prodigiosus, Bacterium pyocyaneus, Bacterium coli, Vibrio Metschnikowii, Bacillus cuniculicida Pfeiffer, Bacterium typhi" and four species of Vibrio. The anaerobes Cl. botulinum and Cl. tetani were the only species which failed to show catalase activity.

Of 20 species of bacteria which Jorns (38) examined, Serratia marcescens showed the highest activity. Bordi (10) studied the catalase activity of a number of animal pathogens. He reported that a strain of Pasteurella bubalseptica increased in virulence when passed through rabbits, but showed no corresponding increase in catalase activity. Jacoby (31) studied the catalase activity of Proteus vulgaris from an enteritis case. Using 22 cultures of Proteus vulgaris and 7 cultures of the X-strain, he found that the latter were of less catalase activity (26). Lauenie (46) examined aerobic spore-forming bacteria for enzymes. All cultures showing more spores than rods evolved gas when H_2O_2 was added. When the spore suspensions were boiled, the catalase activity was reduced though not destroyed entirely.

Collon (8) examined 9 anaerobes and 17 aerobes. All the aerobes demonstrated catalase activity except the 3 species of Streptococcus; none of the anaerobes showed this property. Broth containing catalase did not support growth of anaerobes cultured under aerobic conditions. She noted that aerobes grown anaerobically produced less catalase than when grown aerobically. Sherman (66) called attention to the fact

that not all anaerobes were devoid of catalase since some strains of propionic acid bacteria which were strict anaerobes showed high activity.

H. pertussis showed strong catalase activity as compared to a number of human pathogens according to Shibuno (40). When homologous human serum was added to the culture media in which *Eb. typhosa* and *Vibrio comma* were grown, their catalase activity was not influenced. Stapp (47) noted no effect on the catalase or the peroxidase activities of a group of organisms when treated with hydrogen, nitrogen, or oxygen.

Virtanen and Laurström (50) used the direct cell count as a standard of bacterial density and expressed the catalase activity by the formula

$$\text{Kat. F.} = \frac{\text{reaction velocity constant } k}{\text{number of cells}}$$

They showed that the decomposition of H_2O_2 by organisms is a first order reaction. Virtanen and Linter (51) reported that the decrease in catalase activity which occurred with a drop in pH of the medium was due to death of the cells rather than to the acid produced. Virtanen (52) listed the Kat. F. of a number of species of bacteria. The values varied between 5.7 x 10^{-9} for *L. acidi propionici* to 0.004 -0.02 x 10^{-9} for strains of *B. coli*. Employing the formula of Virtanen, Kirchner and Nagell (18) found the catalase activity of *Staph. aureus* less than that of *Neisseria gonorrhoeae*, the latter than that of *E. coli*. Hartdegen et al (17) compared this property with other physiological characteristics of strains of *Shigella* but found it of little value in differentiating these organisms.

Hernández and Del Índia (10) investigated the effect of combining various sugars and amino acids in the medium and found wide variety in catalase activity depending upon these constituents.

Itano and Aranare (13) studied the catalase activity of Cl. thermocellum and another thermophilic cellulose-fermenting organism. They noted that the catalase activity of both thermophiles showed an optimum activity at 60°C.

Wolfson (38) studied the influence of dyes on catalase activity of Pseudomonas pyocyanea. Of the five dyes studied, the greatest effect was produced by methylene blue which, when present in the culture medium in a concentration of 0.01 per cent, decreased the catalase activity 4.5 fold. No decrease in activity was detected when the organisms were suspended in a solution of the dye and tested.

Burk et al. (7) found that the catalase of Photobacter was active at a pH as low as 2-4 but a temperature of 97°C. for several hours was required for its destruction. Katagiri (35) increased the catalase activity of yeast by aeration; the maximum was attained with the first subculture.

Pitcher (19) examined 11 strains of propionic acid bacteria and concluded that variations in catalase activity were inherent properties of the culture and not associated with any other physiological characteristics.

The catalase activity and the ammonia oxidizing properties of the nitrifying bacteria were considered by Krishnan (20). Nitrosomonas washed free of nitrites showed no catalase activity. Horn (27) suggested the use of catalase activity as a means of differentiating the species of Brucella. Schoenenberg (45) demonstrated that the Warburg apparatus was useful in studying the catalase activity of Lactobacillus bacteria.

Swenson and Rumfeld (49) used a Proteus vulgaris culture of high activity to study active and inactive catalase. Using the viable cell count as the basis of bacterial standardization, they compared the

activity of catalase before and after addition of calf-heart extract or Bolton antigen. Both increased the activity. Eleven species of Acetobacter were examined by Walker and Tošić (34) who confirmed the observation of Moore (20) that in this genus only A. peroxydans failed to show catalase activity.

Bucco (3) studied the effect of eight germicides on the respiratory enzymes of A. aerogenes. Only $HgCl_2$ decreased catalase activity; the latter concentration caused 50 per cent inhibition.

In studying the effect of sodium azide on bacterial growth and respiration, Lichstein and Soule (22) included a study of the action of this disinfectant on catalase activity. With the exception of Strep. citrovus, organisms which showed catalase activity were more sensitive to sodium azide than cultures which produce no catalase. Catalase added to the medium did not affect the resistance to sodium azide. None of the technics employed were sufficiently sensitive to detect H_2O_2 in aerobic cultures.

Madsen and Stahl (21) correlated catalase activity with the virulence of each of the three species of Brucella. These species fell into three zones of activity, Br. suis in the highest and Br. abortus in the lowest. The amount of catalase activity of any given culture decreased as the degree of dissociation increased; some rough cultures of Br. melitensis showed the same activity as did smooth cultures of Br. abortus, but it was possible to differentiate Br. suis from the other two species regardless of type. The catalase activity of smooth cultures of Brucella was directly proportional to their virulence. These authors found that growing the organisms under an increased CO_2 tension

did not alter the catalase activity, but growing them on different media did result in significant differences.

The purpose of this investigation is to study the catalase activity of *B. pertussis*, and the antigenically related organisms, the *parapertussis* bacillus and *B. bronchisepticus*.

METHOD

Bacteriological

Media.

The cultures were grown on Bordet Gengou medium (4) as modified by the Michigan Department of Health for vaccine production. The formula:

Pealed sliced potatoes	150.0 g.
Glycerine, Merck's reagent	10.0 ml.
Agar	30.0 g.
Proteose-peptone	10.0 g.
NaCl	5.4 g.
Distilled water	1000.0 ml.
Sterile defibrinated sheep blood	200.0 ml.

Potatoes were boiled in one-half the volume of distilled water and glycerine until soft, strained through a sterile filter and allowed to settle. To the extract was added salt, peptone, agar and distilled water to volume. The pH was adjusted to 7.2 - 7.4. The medium was autoclaved and blood added aseptically. This medium containing peptone will be referred to as Bordet Gengou vaccine medium and that without peptone as Bordet Gengou diagnostic medium. Since the isolated cells grow better on the medium without the peptone, Bordet Gengou diagnostic medium was used for viability counts and other purposes when the number of cells present was low.

Cultures.

Fifty-five strains of smooth *B. pertussis*, 4 strains of rough *B. pertussis*, 10 strains of *parapertussis*, and 10 strains of *Br. bronchiseptica* were included in the study. The *B. pertussis* strains which are prefixed by the letter L were isolated in Lansing and strain 1405 was received from New York State Department of Health. Dr. Hendrick and Dr. Mooring, of Western Michigan Division Laboratories of the Michigan

Department of Health, supplied all other cultures and also made the initial characterizations.

B. pertussis: Cultures isolated from whooping cough patients had characteristics of smooth strains as described by Kendrick, Lawson and Miller (27). Colonies on Bordet Gengou diagnostic agar were typically smooth, hemolytic and not over 1 mm. in diameter. On microscopic examination the morphology of the organisms was typical. The agglutination titer with specific antiserum determined by the rapid technic as used by Kendrick (26) approximated the titer of the antiserum, i.e., 1:8000. The cultures were virulent for mice. Three billion organisms suspended in mucin and injected intraperitoneally killed 80 per cent of five mice within five days. 0.1 ml. of a 1 billion suspension of the cultures produced the typical skin necrosis in normal rabbits within 48 to 72 hours. Smooth strains did not grow on veal infusion agar slants. Inability to grow on this blood free medium was accepted as an indication of roughness.

The rough strains of B. pertussis, phase IV of Leslie and Gardner (30), which were included were No. 84 isolated by Krumwiede in 1933, No. 10 isolated by Lovitsky in 1916, No. 17 isolated by Bordet in 1911 and No. 61 received from Connaught Laboratories.

Para-pertussis cultures: The 10 strains of para-pertussis bacillus had been isolated from whooping cough patients and were all smooth strains as shown by morphology, colony appearance, hemolysis on Bordet Gengou medium and agglutination with specific antiserum.

Br. bronchisepticus: The 10 strains of Br. bronchisepticus were isolated from guinea pigs and rats and one strain, No. 113, from a child.

All strains showed typical morphology and staining characteristics and were pathogenic for mice as demonstrated by intraperitoneal injection of 50 million organisms in saline. The hemolytic properties, motility and colony characteristics under deflected light (18) varied. With the exception of strain V-89, they were considered intermediate between smooth and rough strains. Strain V-89, recently recovered from a mouse brain following intracerebral injection, maintained motility, hemolytic properties and a typically smooth colony appearance through the period of examination.

Agglutination in the presence of trypaflavine: Dissociation of some Gram negative coccilli may be detected by their agglutination in the presence of a trypaflavine solution (4). Suspensions of B. pertussis cells were emulsified in a drop of saline on a slide, a drop of 1:500 trypaflavine added and the slide gently rocked. With some organisms agglutination indicates a rough culture. This test is apparently not applicable to B. pertussis since all suspensions agglutinated. Both smooth and rough strains agglutinated to the same degree. Colonies, taken from a plate three days after exposure to a whooping cough patient, agglutinated in both neutral and acid trypaflavine.

Method of preparing cells for extract test.

The growth of a culture was scraped from the surface of the medium and suspended in sterile diluent containing 0.03 per cent tryptose and 0.5 per cent NaCl. The pH of the diluting fluid was 6.9 to 7.0. Bits of agar and clumps of bacteria were removed by filtration through a gauze and cotton filter (76) or by allowing particles to settle before withdrawing the top portion of the suspension. To ascertain that no activity was due to blood carried over from the medium, a number of suspensions were centrifuged and the supernatants tested for their

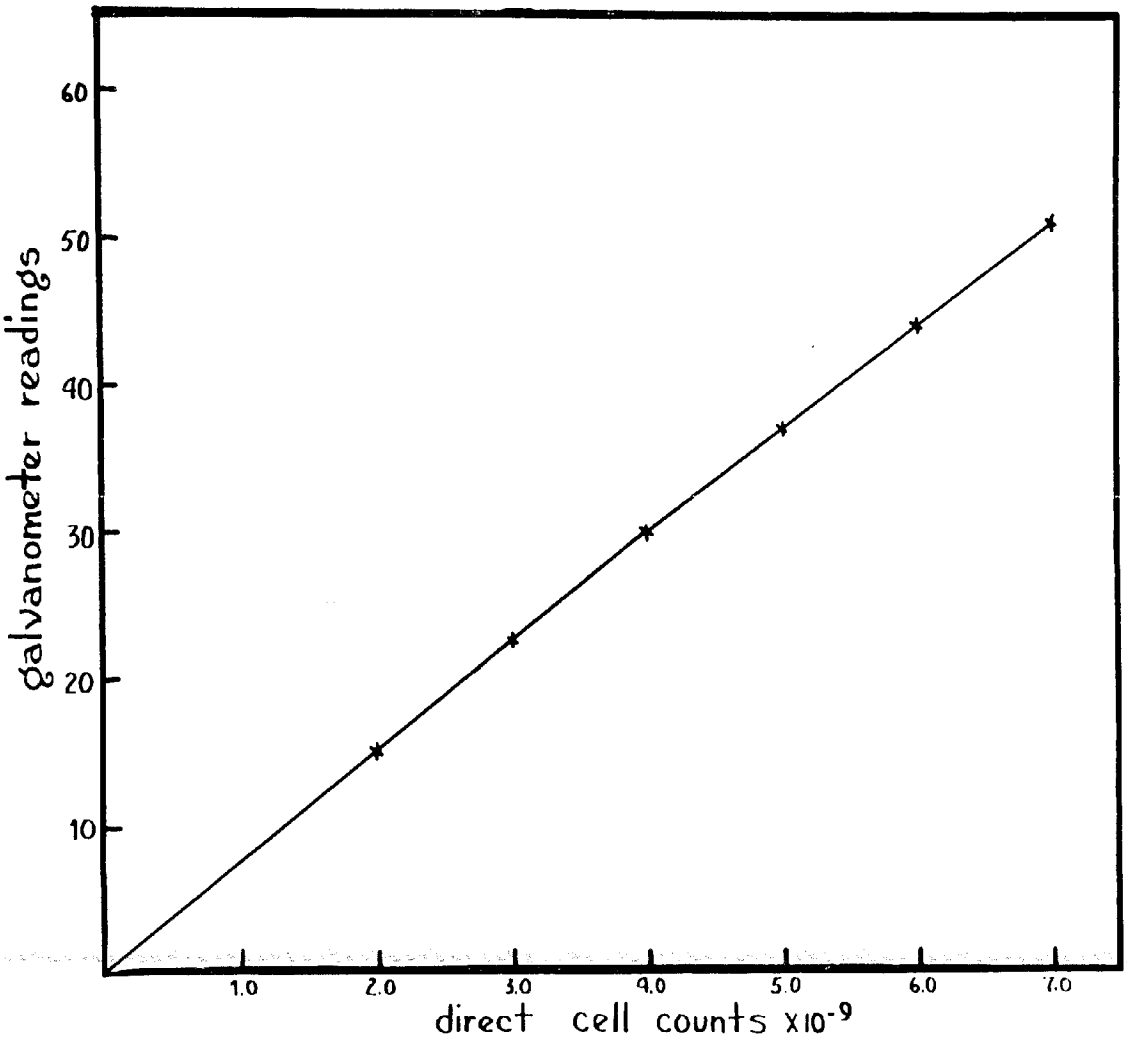


Fig. 1. Standardization of turbidity of bacterial suspensions by direct cell count.

action on H_2O_2 by the method employed for catalase activity. Since no activity due to the medium was ever observed, centrifugation of the suspension was eliminated. With the liquid media used, it was not necessary to centrifuge the cells from the medium.

Standardization of bacterial suspensions.

Bacterial concentration has been determined by other workers from 1) dry weight of the bacterial suspension, 2) direct cell count of the total number of cells, 3) viable count on plate cultures and 4) by nephelometry of cultures. The last was found suitable for comparing factors influencing the reaction.

In this work, the suspensions were standardized with a Libby Photronreflectometer (21) to a turbidity equivalent to 0.3 billion cells per ml. as determined by direct cell count. The cell count of a given turbidity was the same for all three species. Expressed as the count of viable cells, this represented for a two day culture approximately one billion organisms per ml. of *B. pertussis* and parapertussis Bacillus and two billion of *B. bronchisepticus*.

Figure 1 shows the accuracy of density determinations with the Libby photronreflectometer. The calculated error in standardization was ± 25 million organisms.

Chemical

Methods which may be used for quantitative measurements of catalase activity are: 1) collection of oxygen evolved after H_2O_2 is added to the enzyme, 2) titration of the residual H_2O_2 after periods of reaction and 3) measurement by polarographic analysis (35). The first method, which allows the calculation of oxygen evolved at any number of time intervals, has been refined in the Warburg apparatus (15). The unused

H_2O_2 may be measured by the iodometric method (48) or by titration with $KMnO_4$. Since both titrations require the presence of H_2SO_4, the action of the catalase may be halted at desirable time intervals by adding the acid to aliquots.

The method of determination of catalase activity used in this survey was essentially that of Goldacre and Stahl (21). Five ml. of the standardized bacterial suspension were diluted with 10 ml. of the Tryptone-salt diluent and 10 ml. of H_2O_2. The peroxide reagent was prepared by diluting 10 per cent H_2O_2 to a 1 N. solution with the tryptone-salt diluent. The temperature of both reagents was 1°C. when added to the bacterial suspension. The concentration of H_2O_2 in the bacterial suspension was 0.5 N. A stop watch was used to note the time of adding H_2O_2. During the period of reaction the flasks were held on a rotating shaker moving at the rate of 100 rotations per minute.

At the desired time intervals, 5 ml. aliquots were removed and added to 5 ml. of a 1:3 solution of H_2SO_4, which destroyed the enzyme and also supplied sufficient acid for the titration with $KMnO_4$. Unless otherwise indicated the samples were taken after 20 minutes since the reaction was complete by that time. Titrations were made at room temperature. Each aliquot was diluted with distilled water and titrated with 0.1 N $KMnO_4$. A titration requiring less than 3 ml. of $KMnO_4$ was shown to be unreliable, i.e., the decomposition of H_2O_2 was greater if the ratio of cells to peroxide was increased. Any low titration values obtained were rejected and the determinations repeated with half the original concentration of cells.

A control titration was made each day to determine the amount of $KMnO_4$ equivalent to the total amount of H_2O_2 added. From this value

was subtracted the amount necessary to titrate the aliquot removed after the enzyme had acted upon the hydrogen peroxide. Thus the catalase activity is expressed in terms of mls. of 0.1 N $KMnO_4$ equivalent to the H_2O_2 decomposed.

A constant temperature was not maintained throughout the test. The initial temperature was 14 - 15°C. and it rose during the 30 minute period to approximately 25°C.

EXPERIMENTAL

A. Concentration of reactants.

It is beyond the scope of this paper to discuss the dynamics of the interactions of catalase and H_2O_2. It was necessary, however, to determine that within the range of cell concentrations used, the measured activity of the enzyme was proportional to the bacterial concentration, and also to determine any variation in activity that might be due to the concentration of the H_2O_2.

Bacterial concentration: A series of determinations was made with quantities of bacteria ranging from one million to seven billion cells per ml. The concentration of H_2O_2 was 0.3 M in order to provide a sufficient excess for the highest concentrations of bacteria. *B. pertussis*, strain 1063, two phase culture (Kanon vaccine) grown at 37°C. for three days. Figure 1 shows that the catalase activity is directly proportional to the bacterial concentration within the range of one to seven billion organisms per ml.

Concentration of H_2O_2: To determine the effect of the concentration of H_2O_2 on measured catalase activity, volumes of bacterial suspensions were equally divided into three flasks. To each was added a different concentration of H_2O_2. The reaction mixtures were made up to the same total volume by addition of chilled dilution fluid. The bacterial concentration was 5.3 billion per ml. and the concentration of H_2O_2 in the reaction mixtures was 0.1 M, 0.3 M, or 0.5 M. Table 1 expresses the catalase activity in ml. of 0.1 N H_2O_2 then used.

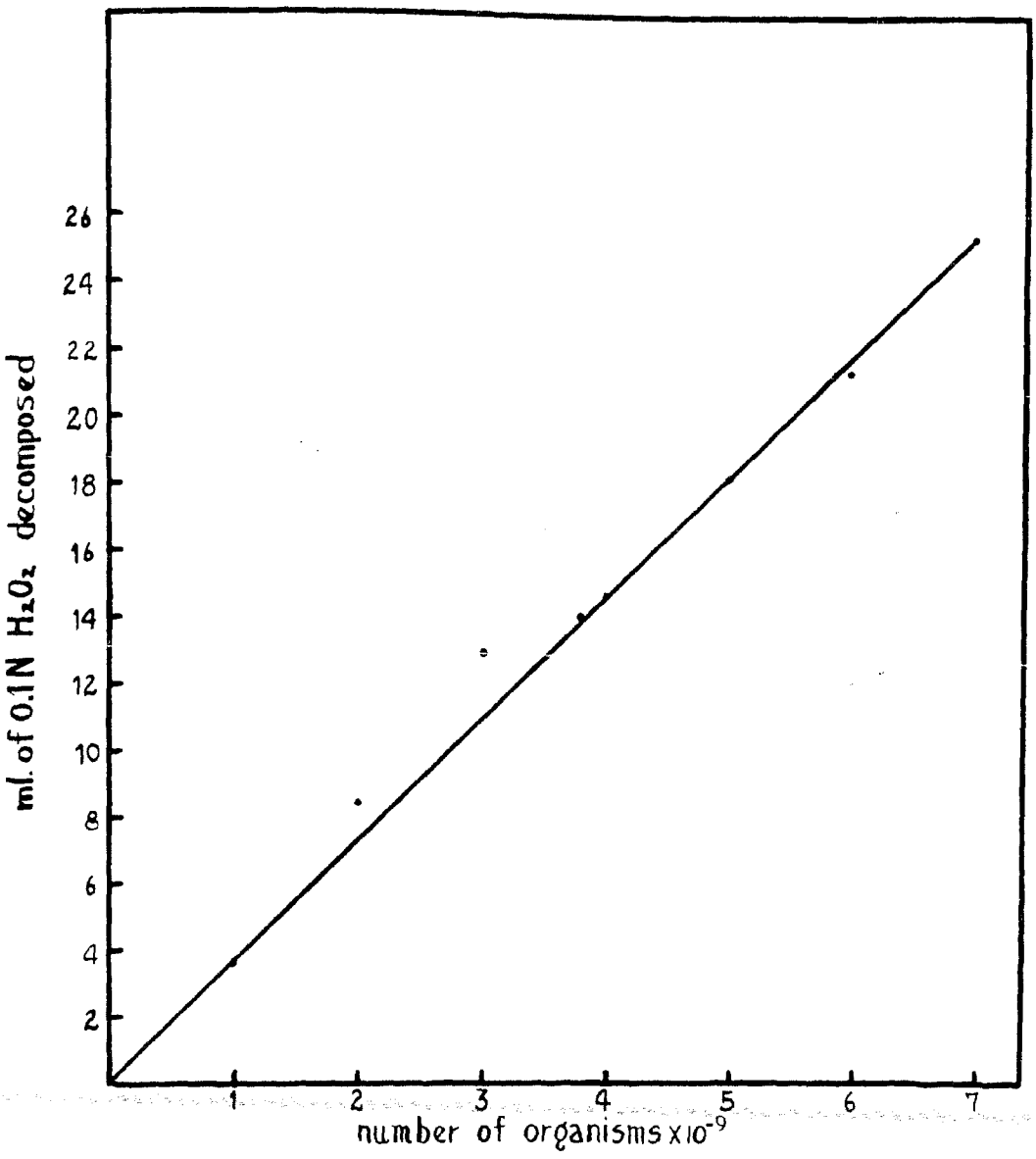

ml. of 0.1N H_2O_2 decomposed
26
24
22
20
18
16
14
12
10
8
6
4
2
1
2
3
4
5
6
7
number of organisms x 10^{-9}

Table 1

Effect of concentration of H_2O_2 in the reaction mixture on measured catalase activity

Culture No.	Catalase activity		
	Concentration of H_2O_2 in reaction mixture		
	0.4 M	0.3 M	0.2 M
10813	17.5	13.1	13.4
10842	10.9	11.1	11.9
10917	13.3	14.4	16.5
11522	11.3		14.2
10934	15.9		15.3

The results in Table 1 show that within the range of concentrations of H_2O_2, used, the measured catalase activity increases as the concentration of H_2O_2 is increased.

The results presented in Table 1 on cultures which were examined with two concentrations of H_2O_2. The suspensions were made from different transfers and at different times, but all conditions of growth of the cultures were the same. The following figures are averages of two to eight determinations.

Table 2

Comparison of values of catalase activity with 0.4 M and 0.3 M H_2O_2 in the reaction mixture

Culture No.	Catalase activity	
	Concentration of H_2O_2 in the reaction mixture	
	0.4 M	0.3 M
10956	14.5	15.1
10904	13.2	13.3
11181	15.3	15.0
11199	13.7	17.3
11403	14.3	16.7
11430	14.9	17.1
12677	10.5	12.5
12833	12.3	14.7
L 39	15.5	17.6
L 40	15.1	15.1
L 41	12.4	15.3

these data indicate that concentrations of 0.4 ml and 0.8 ml H_2O_2 in the reaction mixtures do not give comparable titration values. The initial concentration of H_2O_2 must be the same when comparing activity of cultures.

f. Optimum pH of the reaction mixture.

Though values around a pH of 7.0 have been most frequently used in determinations of bacterial catalase, variations in the optimum pH of the reaction have been reported. Evans (31) observed an optimum pH of 6.8 with a phosphate buffering system. He records that phosphate buffers were unsatisfactory for suspending the organisms when titrations with permanganate were used for determination of residual H_2O_2. Virtanen and Linser (81) obtained no difference in catalase activity of E. coli between pH 7.2 and 8.5. Stapp (67) found a steady rise in activity by changing the pH from 5.0 to 8.7. Neutral and dilute alkaline solutions were of little benefit, but as alkalinity increased there was a decrease in the activity.

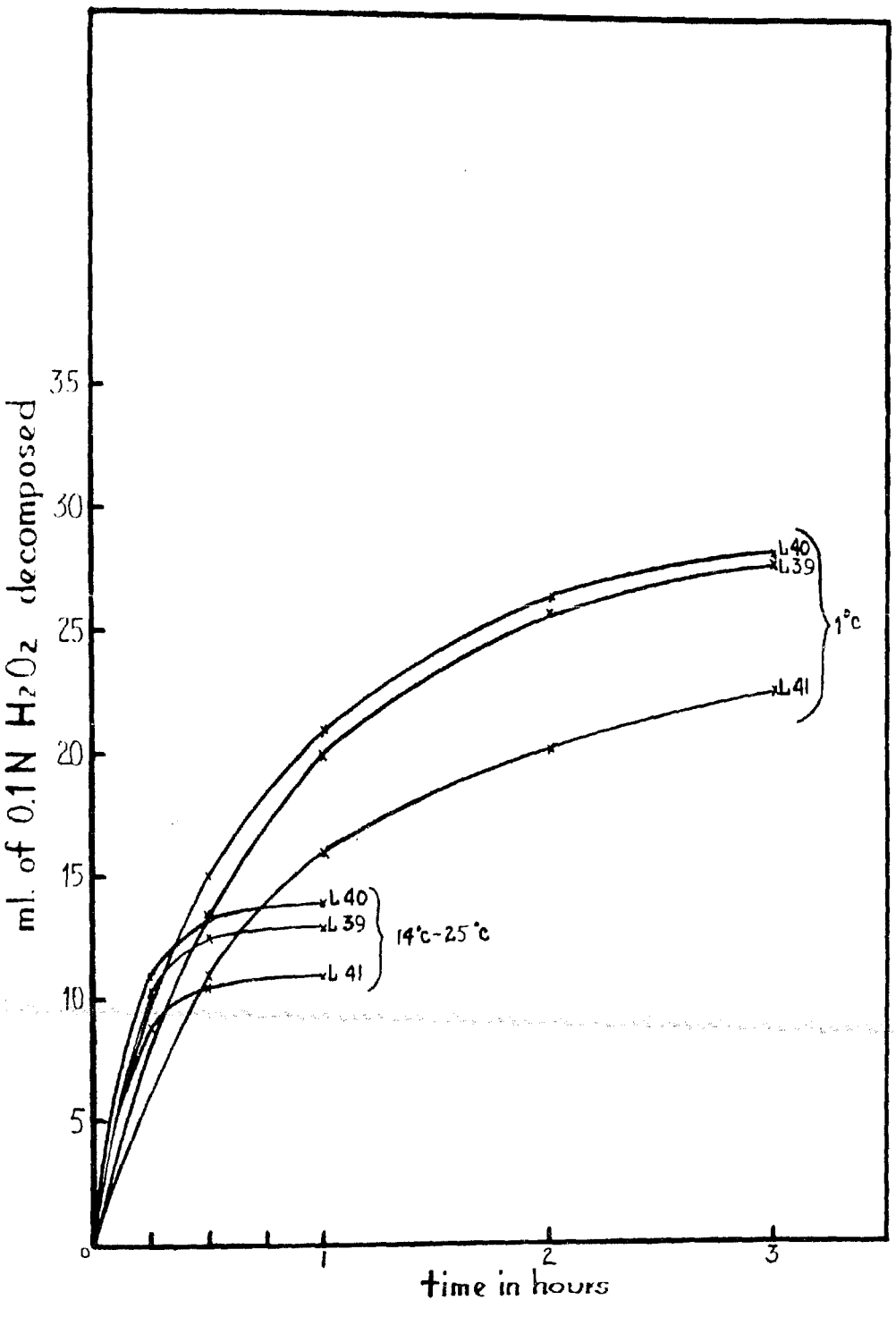
E. perfringis cultures were suspended in dilution fluid of phosphate buffers. The ionic strength of each buffer solution was 0.1 and the pH ranged from 4.85 to 9.35. The H_2O_2 was diluted with the buffer used in preparing the bottle dilution and peroxide reagents were chilled to 1°C. before addition to the suspension of cells.

Table 8

Effect of pH of the reaction mixture on catalase activity

Culture No.	Control (Cil. 21.)	Catalase activity % of the dilution fluid					
		4.85	4.95	5.0	7.0	8.0	9.35
16544	10.7	6.5	7.7		10.5		9.4
17991	11.8	8.5	9.5	9.3	10.9	10.7	10.3
13216	7.8	5.2	5.6	6.1	7.1	6.9	7.5

The optimum is a pH of 7.0 though acid dilutions cause a greater decrease than alkaline.


ml. of 0.1N H_2O_2 decomposed
35
30
25
20
15
10
5
0
1
2
3
time in hours
L40
L39
L41
1°c
L40
L39
L41
14°c-25°c

C. Temperature of determination.

The optimum temperature of the enzyme catalase is near 0°C. Suspensions of _A. aerogenes_ were tested at room temperature (14°C. initial to 18°C. in 1) and at 1°C. Figure 5 shows the comparison of maximum activity and speed of reaction at these temperatures. Aliquots were removed from the mixture at room temperature after reaction of the enzyme on H_2O_2 for 5, 15, 30 and 60 minutes, and from the reaction mixture at 1°C. after 1 hour, 4, 7, and 9 hours.

The amount of H_2O_2 decomposed when the determinations were made at 1°C. were slightly more than twice as much as at room temperature. The quantitative differences in the activity of strains were evident regardless of the temperature. The reactions were complete at room temperature in 30 minutes, at 1°C. only after 4 hours. The shorter time period was adopted for routine examinations and since the results obtained from either temperature proved equally reliable, room temperature was adopted for the technic. The second variation in temperature was not sufficient to alter the results. A suspension required the same amount of $KMnO_4$ for titration whether the temperature during the reaction rose to 25°C. or was brought to 25°C.

D. Influence of culture media on catalase activity.

The influence of the culture medium on the catalase activity of microorganisms has been noted by a number of workers. Jacoby (15) compared essentiality of constituents for catalase activity of _Proteus vulgaris_. Fernández and Guimêndia (10) obtained varied results for catalase activity of _B. coli_ by using combinations of amino acids and

sugars in synthetic media. The combination of asparagine and levulose gave the highest values. Levulose when combined with alanine, tyrosine or ammonium lactate offered a better medium for catalase activity than the combination of these amino acids with any of the other sugars tested. When glutamic acid was used as the source of nitrogen, sucrose was the best source of carbohydrate. Itano (38) made the observation that the vigorous activity demonstrated with thermophilic organisms grown at 65°C. in a medium containing cellulose was absent if glucose was substituted. This difference was accounted for by an accumulation of acid in a medium containing the sugar. Similar activity to that obtained with cellulose was demonstrated with glucose by lowering the incubation temperature to 55°C. Addition of ferrosulfate to the medium did not increase the activity according to Virtanen and Winter (81). Middleton and Abell (71) called attention to the fact that the activities obtained from two different media cannot be compared because of variations in results.

Hemophilus pertussis: Only broth semisolid media were used for growing H. pertussis. Since there is unavoidable variation in preparing any medium containing blood, a record was made of each lot of medium used. There was no appreciable fluctuation in the catalase activity of cultures grown on lots of media made at different times.

Media were dispensed in both tube slants and screw-capped flask bottles. Although there were differences in the oxygen supply, moisture and surfaces in these two containers, there was no difference in the catalase activity of cultures grown on the same medium in the two containers.

Growths from diagnostic and vaccine media were compared to observe the influence of peptone on the catalase activity. Growth on the vaccine

medium was considerably heavier than on diagnostic medium. Cultures were incubated at 37°C. for 4 days, the bacterial concentration in the reaction mixtures was 3.8 billion per c.c. and the concentration of H_2O_2 was 0.4 M. The results are given in Table 4.

Table 4

Influence of peptone in the culture medium on the catalase activity of B. pertussis

Culture No.	Diagnostic medium (no peptone)		Vaccine medium (peptone)	
	Catalase activity	No. tests	Catalase activity	No. tests
1754	10.5	4	11.2	8
16807	11.9	8	11.2	4
16816	8.7	4	7.7	4

Table 4 shows no more variation in the catalase activity of B. pertussis grown with and without peptone than is within the range of experimental error.

The effect of the pH of the medium on catalase activity was studied by using media in which the pH had been lowered by phosphoric acid. Because these media were rather soft, harvesting of the scanty growth was difficult. The cultures used included 3 smooth strains of B. pertussis, one of which was smooth except for a low agglutination titer (17921), and one rough strain.

The results given in Table 5 indicate no significant variation in bacterial catalase of B. pertussis grown on SG media from a pH of 6.4 to 7.4.

Table 5

Effect of pH of the culture medium on catalase activity

Culture No.	Catalase activity			
	pH 7.4	pH 6.8	pH 6.6	pH 6.4
16544 smooth	10.2	8.9	8.0	11.9
16816 "	8.0	7.6	8.4	8.9
17921	11.1	9.3	11.6	11.9
61 rough	4.0	5.7	5.0	6.7

Parapertussis bacillus: Since the known forms of the parapertussis bacillus grow readily on media without blood, a study of this organism offered an opportunity for some variation in culture media. The catalase activity of the organisms was carried on Bordet Gengou vaccine agar, the same medium without blood and veal infusion agar. All cultures were incubated at 36°C. for 72-96 hours. The first medium yielded better growth than the other two. Table 6 gives the catalase activity of the parapertussis bacilli grown on each of the three media; the average deviations are included to indicate the sensitivity of the test.

Table 6

Influence of culture media on catalase activity of the parapertussis bacillus

Culture No.	Bordet Gengou Vaccine			Vaccine without blood			Veal infusion agar		
	Cat. act.	No. tests	Average deviation	Cat. act.	No. tests	Average deviation	Cat. act.	No. tests	Average deviation
501	13.8	6	0.4	11.3	6	0.6	7.0	6	0.3
515	15.7	6	0.6	11.4	4	0.05	5.9	4	0.5
532	16.7	4	0.8	11.9	4	0.3	6.3	4	0.3
7047	17.6	4	0.3	12.6	4	0.4	7.3	4	0.5
10700	15.0	4	1.0	11.6	4	0.7	6.6	4	0.4
15286	14.9	4	1.0	10.1	4	0.7	6.0	4	0.4
14031	14.7	4	1.3	11.1	4	0.6	6.5	4	0.5
11	15.4	4	0.1	10.4	4	0.3	7.4	4	0.2
10357	15.4	4	0.4	10.9	4	0.4	6.2	4	0.3

The presence of blood in the culture medium increased the catalase activity of all the strains. The difference was more marked however between the activity of cultures grown on potato extract agar, (Bordet Gengou without blood) and veal infusion agar than those grown on potato extract agar with and without blood.

Br. bronchisepticus: In further study of the variation in enzyme activity of organisms on different media, cultures of Br. bronchisepticus were grown on Bordet Gengou vaccine agar and on veal infusion agar.

Table 7 presents the catalase activity of the cultures after incubation for two days at 37°C.

Table 7

Influence of culture media on catalase activity of Br. bronchisepticus

Culture No.	Loeffler-Bengou broth			Veal infusion agar		
	Catalase activity	No. tests	Average deviation	Catalase activity	No. tests	Average deviation
P-830	8.1	5	0.4	3.9	5	0.5
P-831	10.5	5	0.03	3.5	4	0.9
[illegible]	8.9	5	0.8	3.7	5	0.8
V-10	8.8	5	0.4	3.9	5	0.6
V-2	8.9	5	0.7	3.9	5	0.1
V-6	7.9	5	0.8	3.6	5	0.4
V-831	9.1	1	0.8	2.8	1	-
888	8.6	5	0.8	3.7	4	0.8
V-879	7.4	5	0.7	1.8	2	0.05

The catalase activity of Br. bronchisepticus grown on Loeffler-Bengou medium was two to three times higher than when grown on veal infusion agar.

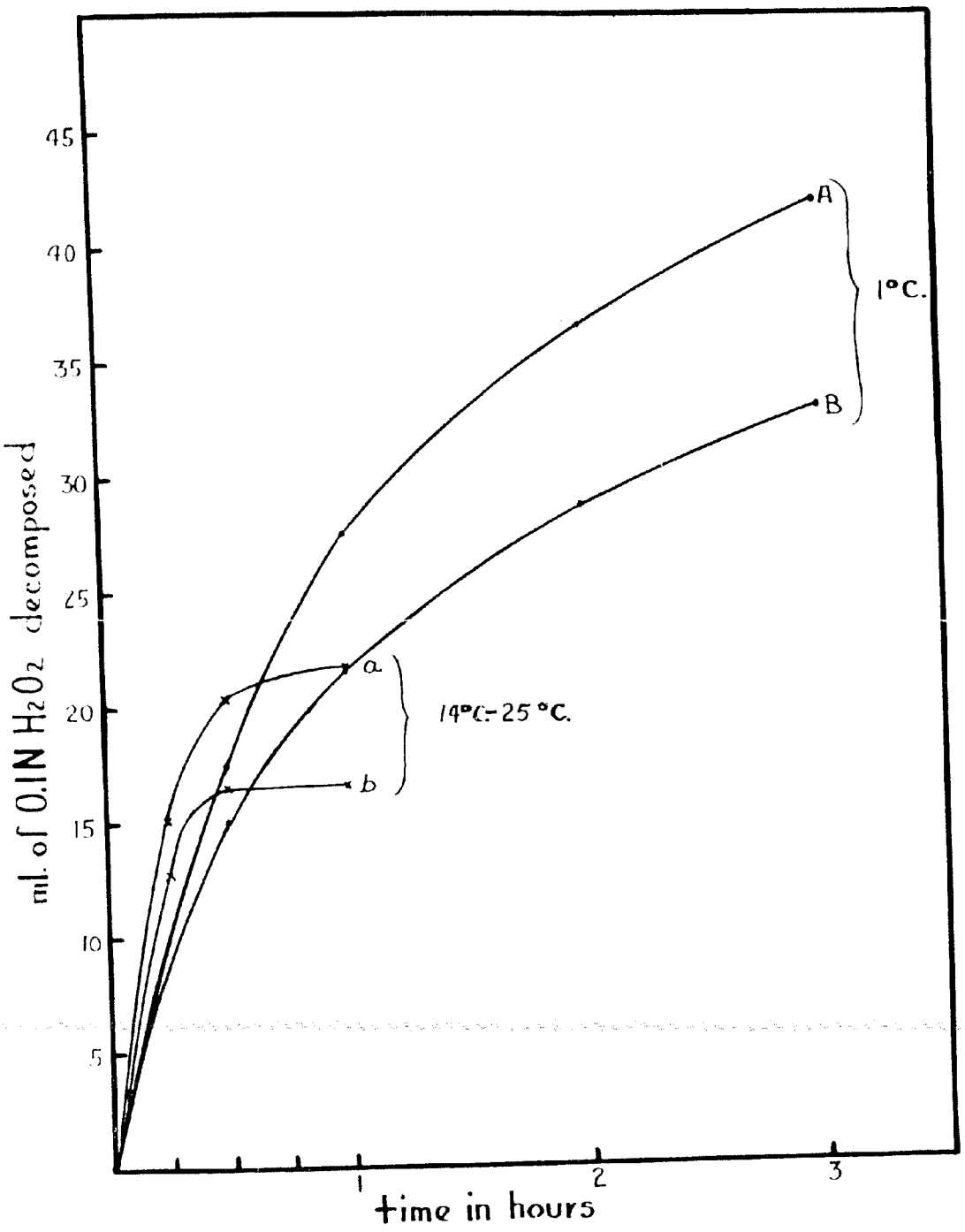
Br. bronchisepticus cultures normally dissociate faster on a veal infusion medium than on one containing blood. Andreason and Stahl (31) have indicated the relationship of the extent of dissociation and the catalase activity of bacilli. It seems reasonable that a medium which tends to cause dissociation would also yield cells with a lower catalase activity than one which maintains cells with smooth characteristics. At the termination of this study no difference in the extent of dissociation of cultures grown on the two media could be detected; they were considered in an intermediate stage of variation.

Culture V-39, which retained hemolytic properties and motility, had a catalase activity of 15 when grown on Loeffler-Bengou vaccine agar. The activity of this smooth strain was higher than that of any of the intermediate strains of Br. bronchisepticus listed in Table 7.

Tryptose-glucose broth: The catalase activity of the parapertussis bacillus and of B. bronchisepticus was appreciably increased when these organisms were grown on tryptose broth containing glucose. There was no additional increase in catalase activity with subsequent subcultures. Strains of B. bronchisepticus were transferred from Bordet Gengou plants and from veal infusion agar to Tryptose broth.

B. bronchisepticus, strain 888, was grown at 37°C. in Tryptose broth for maximum concentrity during the two day incubation period. The catalase activity was determined at room temperature and at 1°C.; H_2O_2 in the reaction mixtures was 0.3 %. Figure 1 shows the effect of media on the maximum activity of the culture and the effect of temperature on the maximum activity and the rate of reaction. The same quantitative differences in determinations were evident as were found for B. pertussis examined at the two temperatures. The catalase activity of each suspension was nearly twice as high when determinations were made at 1°C. as they were when examined at room temperature. The reaction was virtually complete at room temperature in 20 minutes while that determined at 1°C. required three hours.

Cultures planted from veal infusion agar did not attain the activity observed in those transferred from the blood medium. Transplants from Bordet Gengou agar to Tryptose broth are represented by curves A and a and those from veal infusion agar by curves a and b. The activity of the culture had previously been determined as 8.3 on Bordet Gengou agar. In Tryptose broth its activity was 81.7 when tested under the same conditions. The culture, which had an activity of 7.7 on veal agar, showed an increase to 16.5 when transferred to Tryptose broth. The


45
40
35
30
25
20
15
10
5
ml. of 0.1N H_2O_2 decomposed
A
B
1°C.
a
b
14°C.-25°C.
1
2
3
time in hours

activity of the culture cultivated on veal agar and then transferred to Tryptose broth was appreciably lower than the activity of the same culture which had been transferred to Tryptose broth from Borchet Gengou agar. Cultivation on veal infusion agar seemed to modify the catalase activity of the strain. These data pertaining to Tryptose-glucose broth are representative of results obtained with six strains of B. bronchiseptica.

3. Effect of temperature of incubation on catalase activity of B. pertussis.

Strains of B. pertussis were examined for catalase activity after incubation at 35° and 37°C. for three days. The determinations were made with 0.3 N H_2O_2 in the reaction mixture and the results are given in Table 8.

Table 8

Comparison of catalase activity of cultures of B. pertussis incubated at 35°C. and at 37°C.

Culture No.	Catalase activity	
	Temperature of incubation	
	35°C.	37°C.
10056	15.1	13.6
10404	15.3	13.7
10409	14.7	13.5
10952	17.5	14.3
11161	15.0	13.2
11169	17.3	14.7
11200	14.9	13.8
11210	15.8	11.6
15815	10.7	10.9
19812	14.2	14.2
13844	13.5	11.3
18720	14.7	13.3
L 79	17.5	13.7
L 80	13.1	13.5
L 81	13.2	13.5

Most of the strains showed a higher catalase activity after incubation at 35°C. than at 37°C. A broader range of temperature of incubation was used to determine the temperature at which cells have the highest activity. Cultures were seeded on several Borchet Gengou vaccine agar slants with two billion B. pertussis organisms in 0.5 ml., distributed

over the entire surface of the slants and incubated at 25°, 30°, 35°, 37°, and 40°C. All the growth was removed from the slants and the turbidity of the suspension determined to approximate the total amount of growth. Incubation at 40°C. gave an average yield of 13 billion organisms per ml.; 37°C., 170 billion; 35°C., 150 billion; and 25°C., 15 billion. Table 9 shows the catalase activity.

Table 9

Comparison of catalase activity of cultures of *B. pertussis* incubated at temperatures ranging from 25° to 40°C.

Culture No.	Catalase activity Temperature of incubation				
	40°C.	37°C.	35°C.	30°C.	25°C.
17813	17.0	17.3	16.4	17.6	
17918	No growth	17.3	15.4	16.8	
18132	10.7	11.3	11.3	13.0	
11181	10.0		16.1		15.4
11199	17.7		18.4		13.3
11300	9.0		15.8		13.9
11320	7.8		16.5		13.2

Cells grown at 35°C. have the highest catalase activity. At higher temperatures there was a proportionate drop in values even at lower temperatures. This was generally true of the amount of growth, though at temperatures of 42°C. and 37°C. there was no apparent correlation of catalase activity and growth.

c. Age of culture and catalase activity.

Materials: Cultures of *B. pertussis* were planted on order Bordon plates and examined for catalase activity after 48 and 72 hours incubation at 37°C. Table 10 indicates that the activity was slightly higher at 48 hours than when held for 72 hours.

Table 10

Comparison of catalase activity of B. pertussis after 48 and 72 hours of incubation

Culture No.	Catalase activity time of incubation	
	48 hours	72 hours
16544	11.5	11.2
13343	12.5	11.7
16556	10.2	10.4
13567	13.7	12.7
17991	11.6	11.3
13916	9.9	8.7
14231	12.7	12.4

The effect of [illegible] culture over a long period of time was followed in [illegible] cultures grown on Lake Articles. A suspension was made from a one day old culture and inoculated in [illegible]. The tubes were held at 37°C. for the length of the test.

Table 11

Period of incubation and catalase activity of B. pertussis

Culture No.	Catalase activity No. of days incubation at 37°C.					
	2	5	4	6	8	16
16544	12.8	9.6	8.6	8.5	7.4	2.9
13567	9.7	8.8	7.2	6.9	6.7	2.6
13916	8.8	7.0	5.2	1.8	1.2	1.4
17991	9.9	10.2	10.3	10.5	8.2	1.6

The activity of strain 16544 dropped rapidly after two days incubation while that of strain 13916 increased slightly. Strain 17991 showed no drop in activity until after six days of incubation. After eight days the decrease in activity was quite marked in all the strains.

There was a variation from strains in the length of incubation before the maximum value was reached and also in the period of time before an appreciable drop in activity occurred.

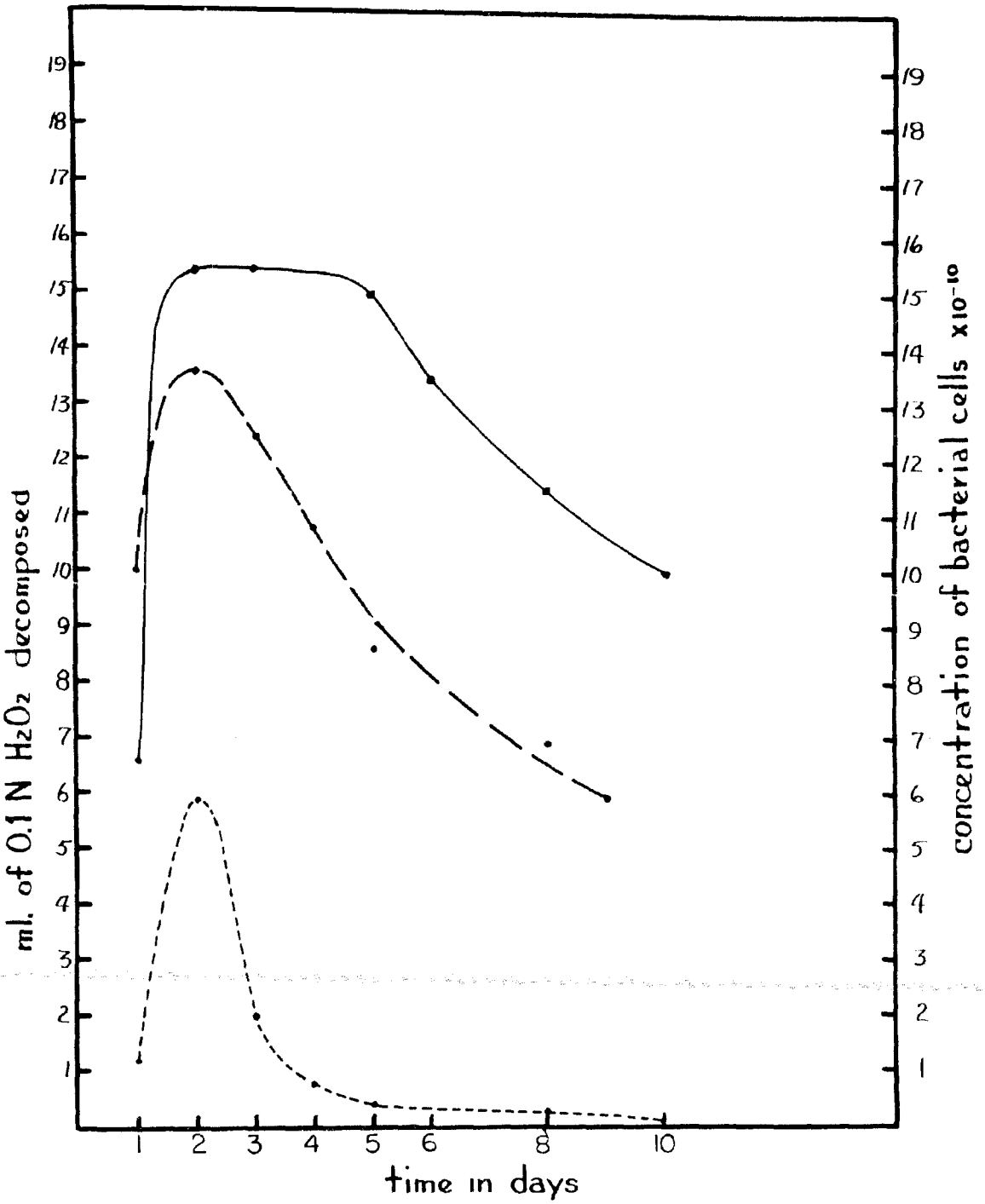
The B. pertussis bacillus and Br. bronchisepticus: In general,

[illegible] activity [illegible] one [illegible] two day old culture of the [illegible] pertussis [illegible] cillus [illegible] ared to the [illegible] tivity of [illegible] two day old [illegible] two day old culture of [illegible] pertussis. [illegible] tivity of [illegible] bronchi-[illegible]tica and the [illegible]pertussis [illegible] cillus [illegible] ed organisms in cultures [illegible]ovided by the [illegible] type. With [illegible] bronchiseptica, [illegible] minum [illegible]erion for [illegible] longer [illegible]eriod of time than that of either [illegible] pertussis [illegible] or [illegible] pertussis [illegible] cillus.

[illegible] cell counts, living cell counts and complete activity.

[illegible] three [illegible]ecies differ in their [illegible] activit[illegible] depending [illegible] on the [illegible]e of the culture and they also differ in their rate and [illegible]nt of [illegible]rowth. [illegible]easurement of total [illegible]ount of cells, number of [illegible] cells, and [illegible]ase activity were made over a [illegible]eriod of 10 [illegible] to determine if any [illegible]ential [illegible]ation.

[illegible]et [illegible] or [illegible]ccine [illegible] ants were each [illegible]ed with 0.2 ml. of a [illegible]ension containing two billion [illegible] pertussis [illegible]anisms, another [illegible] of [illegible]ants with the [illegible] number of the [illegible] pertussis [illegible] cillus [illegible] and with [illegible]00 million [illegible] bronchiseptica. After incubation at [illegible]0°C. at the [illegible]ected time [illegible]ervals, the entire [illegible]rowth of three or more [illegible]ants of each culture was removed and each [illegible]ended in a measured volume of saline. Turbidity [illegible]dings were used as a measure of the total [illegible]crobial [illegible]owth on each [illegible]ant. To determine the number of [illegible]ible cells, ten-fold dilutions were made of a suspension containing five billion organisms per ml., one ml. or one-half ml. of the [illegible]propriate dilutions planted on three or more plates of [illegible]ret [illegible]ou diagnostic [illegible]er and distributed over the entire [illegible]rface. Colonies were counted after incubation for five days in


ml. of 0.1 N H_2O_2 decomposed
concentration of bacterial cells x10⁻¹⁰
time in days
1
2
3
4
5
6
8
10
1
2
3
4
5
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17
18
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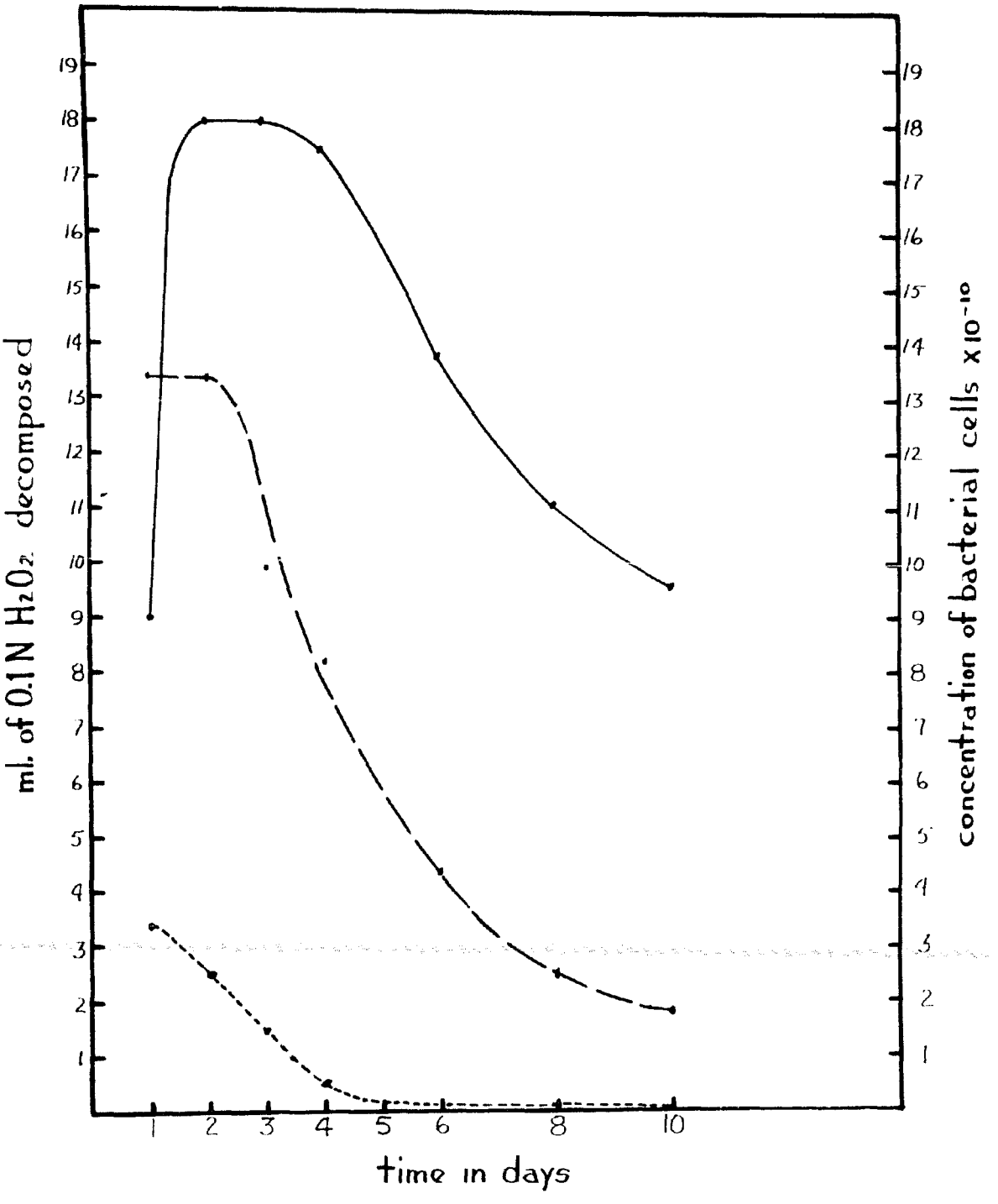
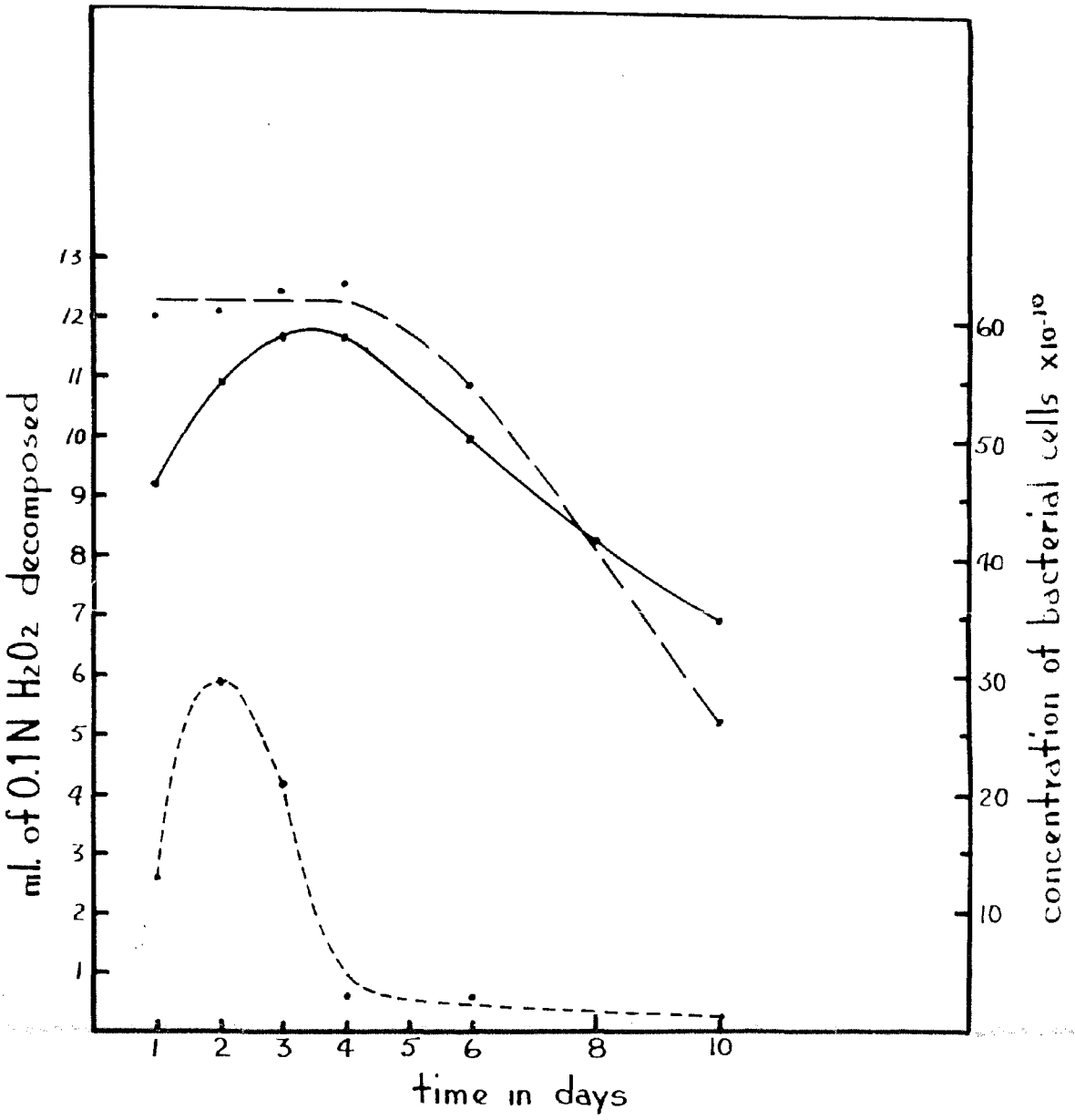

ml. of 0.1 N H_2O_2 decomposed
concentration of bacterial cells x 10^{-10}
time in days
1
2
3
4
5
6
8
10

Fig. 6. [illegible]

[illegible]


ml. of 0.1N H_2O_2 decomposed
concentration of bacterial cells x10-10
time in days
1
2
3
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5
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1
2
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20
30
40
50
60

the curves of *B. pertussis* and parapertussis organisms, and two days for *Br. bronchiseptica*.

Figures 5, 6, and 7 show the total number of cells, viable cell count and catalase activity of *B. pertussis*, the parapertussis bacillus and *Br. bronchiseptica* plotted against time.

The amount of growth, the time required to reach maximum growth, and the rate of autolysis were not the same for the three cultures. *Br. bronchiseptica* grew better and its maximum growth was three times greater than that of the other two species. The amount of growth of the parapertussis bacillus was only slightly higher than that of *B. pertussis*, but the former attained a higher per cent of its growth in the first 24 hours. The rate of autolysis of the parapertussis bacillus was the most marked.

The curves shown in figures 5, 6, and 7 indicate that there are also differences between species in catalase activity. The maximum catalase activity of both *Br. bronchiseptica* and the parapertussis bacillus was reached in the first 24 hours but that of the parapertussis bacillus declined by the third day while that of *Br. bronchiseptica* showed no decline in four days. The number of viable cells and the catalase activity of *B. pertussis* showed a definite rise on the second day after growth reached its maximum. The catalase activity of the parapertussis bacillus as well as the cell count decreased more rapidly than those of *B. pertussis* or *Br. bronchiseptica*. Within 10 days the catalase activity of the parapertussis bacillus had dropped to 15 per cent of its maximum activity compared to 40 per cent for the other two.

The maximum viability count of the non-sporulating oscillus occurred before that of the other two species. B. bronchisepticus had a higher percentage viability after 10 days than either of the others.

For all three organisms the time to maximum catalase activity followed the growth curve. There was a loss of catalase activity although viable cells remained. The decrease in viable count preceded the decrease in catalase activity and loss of [illegible].

The maximum catalase activity was reached in the log growth phase approached a maximum and the decrease in catalase activity was associated with autolysis of the cells.

3. Number of subculture transfers and catalase activity of B. pertussis.

In some instances when B. pertussis is grown on any artificial medium, an irreversible dissociation occurs. However, the number of transfers which may be made before a loss of smooth characteristics can be detected varies. Cultures were studied to correlate any change in catalase activity with the number of times cultured on Bordet Gengou medium. Determinations were made on two strains through 55 subcultures, and two strains through 60 subcultures. These cultures maintained agglutination titer, morphology, colony and skin test characteristics of smooth strains and showed no significant change in catalase activity over this period.

Three other strains showed a loss in activity after a number of subcultures. These were not followed through any period of transition. Strain 18817 had been transferred at weekly intervals and was held at 40C. between transfers. On the ninth subculture, it demonstrated no catalase activity. Strain 18309 had been transferred at three day

intervals and on the 19th subculture showed no catalase activity. Strain 16567, which had catalase activity comparable to other strains through the 38th subculture, was examined on the 39th subculture and showed no catalase activity. None of these strains showed any catalase activity through 10 subsequent subcultures, nor was any demonstrated when determinations were made at 1°C.

To detect any changes accompanying the loss of ability to decompose H_2O_2, these three strains were examined for characteristics which typify smooth strains. Table 12 shows the cultural, morphological, serological and biological characteristics of the cultures which lost catalase activity.

Table 12

Characteristics of cultures which lost ability to decompose H_2O_2

Culture No.	Morphology	Hemolysis on Bordet Gengou agar	Growth on veal infusion agar	Agglutination with Phase I antiserum	* Virulence for mice i.p. inj. .5 billion	** Skin reactions .5E	1L	100%	5%	Catalase activity
18609	Smooth	+	-	1:100	5-5-5-5-5	7	1	-	-	0.0
13317	"	+	+	1:200	1-5-5-5-5	12	5	-	-	0.0
16567	"	+	-	1:100	3-3-4-5-5	10	5	-	-	0.0

* Each figure represents the day of death of one of the five mice challenged; 5 indicates survival on the 5th day after intraperitoneal injection of the challenging dose.

** Diameter of the area of hemorrhagic necrosis in mm. after 48 hours.

Of changes in the characteristics, the drop in agglutination titer was the most marked. Previously those cultures had agglutinated in a 1:3000 dilution of the antiserum. This drop in agglutination titer is an indication of dissociation. Smooth strains give a hemorrhagic necrotic area of about 10 mm. in diameter when 0.1 ml. of a 1 billion

ilution is injected. These cultures in the same dilution produced a smaller necrotic area. Three months later the intradermal injections were repeated and strain 10867 produced no reaction in the one billion dilution but the other strains produced small areas. All three strains showed a decrease in virulence for mice. Strain 10867 grew on a blood-free tera infusion medium and pleomorphic forms appeared in subsequent cultures of all three strains.

In these three cultures there were definite alterations in the characteristics which are regarded as criteria of smooth strains and these changes occurred simultaneously with a loss in catalase activity. These facts raise the question, in the instance of a B. pertussis culture which was capable at some time of showing catalase activity, whether a decrease of this activity may be correlated with dissociation of that culture.

f. Smooth strains of B. pertussis without catalase activity.

Cultures which first showed catalase activity and then lost the property must be differentiated from some smooth strains of B. pertussis which could not be demonstrated to possess catalase activity even though they had all the characteristics of smooth strains. Of the 92 strains of B. pertussis included in this study, there were nine which possessed all the characteristics of smooth cultures but did not exhibit any catalase activity. One of these strains, 10856, was cultured from four separate lyophilized preparations, and tested 55 times by the usual procedure. Conditions of the determination and growth of cultures were varied; the concentration of H_2O_2 from 0.3 M to 0.8 M, pH of the diluent from 4.35 to 9.8, the temperature of incubation from 30°C. to 40°C., the pH of media from 6.4 to 8.0, subculture transfers from 3 to 21. It

was also tested after addition of liver extract, which will be discussed later. Under no conditions was there any indication of catalase activity.

In order to establish optimum conditions for catalase activity, cultures were incubated at 35°C. for two days, bacterial concentrations for the test increased to 6, 12, and 24 billion per c.c. and determinations made at 1°C. No H_2O_2 was decomposed after five hours.

Three cultures in this group were examined before they were lyophilized and two of these on the fourth subculture after isolation. The absence of any catalase activity was found not only in strains isolated in Michigan but also in the one obtained from New York.

The significance of the lack of catalase activity of smooth strains of *H. pertussis* was not apparent from these studies. Despite any effort that probably there is an individual difference in physiological properties of strains.

Antigenic structure: To determine any difference in antigenic structure of these two groups, agglutination tests with antisera were compared. One strain which showed catalase activity and one which failed to show any were used as antigens in producing antisera in rabbits. Each serum was completely absorbed of all antibodies of the other antigen, i.e., the antiserum to "catalase-negative" strain 10336 was absorbed with cells of "catalase-positive" strain 16567, and vice versa. Several cultures from each group were then tested for agglutination with the absorbed antisera. Table II records the results of agglutination tests performed with cultures from each group as antigens against each unabsorbed and absorbed antiserum.

All cell suspensions, irrespective of catalase activity, were agglutinated in a serum dilution of 1:5000 by either of the unabsorbed

Table 13

Agglutination tests with strains of *B. pertussis*

Culture No.	Antiserum from antigen 10556 No catalase activity		Antiserum from antigen 16567 Catalase activity	
	Unadsorbed serum	Adsorbed with 16567 Catalase activity	Unadsorbed serum	Adsorbed with 10556 No catalase activity
	Serum dilution	Serum dilution	Serum dilution	Serum dilution
	50 100 200 1000 2000 3000 4000 5000	50 100 200 1000 2000 3000 4000 5000	50 100 200 1000 2000 3000 4000 5000	50 100 200 1000 2000 3000 4000 5000
No catalase activity				
10556	+ + + + + + + +	- - - - - - - -	+ + + + + + + +	- - - - - - - -
16946	+ + + + + + + +	- - - - - - - -	+ + + + + + + +	- - - - - - - -
16948	+ + + + + + + +	- - - - - - - -	+ + + + + + + +	- - - - - - - -
18295	+ + + + + + + +	- - - - - - - -	+ + + + + + + +	- - - - - - - -
Catalase activity				
15813	+ + + + + + + +	- - - - - - - -	+ + + + + + + +	- - - - - - - -
15842	+ + + + + + + +	- - - - - - - -	+ + + + + + + +	- - - - - - - -
16544	+ + + + + + + +	- - - - - - - -	+ + + + + + + +	- - - - - - - -
16567	+ + + + + + + +	- - - - - - - -	+ + + + + + + +	- - - - - - - -
18216	+ + + + + + + +	- - - - - - - -	+ + + + + + + +	- - - - - - - -
18231	+ + + + + + + +	- - - - - - - -	+ + + + + + + +	- - - - - - - -
18297	+ + + + + + + +	- - - - - - - -	+ + + + + + + +	- - - - - - - -

antiserum. All the antibodies to a "catalase-negative" strain were absorbed by a "catalase-positive" strain. The antiserum to the "catalase-positive" strain absorbed with a "catalase-negative" antigen, partially agglutinated "catalase-positive" cultures in the low dilution of 1:50. There was no significant difference in the antigenic structure of cultures which show catalase activity and those which do not.

Sensitivity to H_2O_2: Cowan and Morton (36) classified species of bacteria according to their catalase activity and noted an inverse relation of catalase activity of the species to its sensitivity to H_2O_2. *B. pertussis* cultures were examined for the effect of H_2O_2 on the viability of the strains. Suspensions were prepared and H_2O_2 added in the same manner and under the same conditions as reaction mixtures for determination of catalase activity.

At the time intervals indicated in Table 14, aliquots were removed from the reaction mixture and placed on Bordet Gengou diagnostic agar. The blood in the medium decomposed the residual H_2O_2 immediately so that exposure of the organisms to peroxide was not prolonged.

Table 14

Viability of *B. pertussis* after exposure to H_2O_2

Culture No.	No. of days in which growth appeared					
	0.4 M			0.8 M		
	Time of removal of samples from the reaction mixture					
	5 mins.	15 mins.	30 mins.	5 mins.	15 mins.	30 mins.
No catalase activity						
10150	3*	-**	-	6	-	
10330	3	-	-	3	-	-
10556	3	6	-	3	-	-
10893	3	-	-	3	-	-
Catalase activity						
10777	3	3	4	3	4	-
11480	3	3	4	3	4	-
11490	3	3	4	3	4	-
13528	3	3	4	3	4	-

* Numbers indicate day of incubation on which growth appeared on transplants.
** No growth after 7 days incubation.

The cultures which had no activity were killed in a shorter time and in a lower concentration of H_2O_2 than the ones which were capable of decomposing it. All strains were killed after 30 minutes exposure to a 0.3 % solution of H_2O_2.

The smooth strains of B. pertussis with and without catalase activity were shown to differ only in their ability to decompose H_2O_2. There was no significant difference in the antigenic structure of the two groups. The strains devoid of catalase activity were more readily killed by H_2O_2.

F. Catalase activity of rough cultures of B. pertussis.

The four strains of rough cultures were not lethal for mice when 2 billion or 6 billion mucin-suspended organisms were injected intraperitoneally. They produced no necrosis when 0.1 ml. quantities of suspensions containing one, five, or ten billion were injected into rabbits intradermally. Strains were tested with an antiserum produced by a strain of B. pertussis typical of those described as Phase IV by Leslie and Gardner (10). The catalase activity and agglutination titers are listed in Table 15.

Table 15

Catalase activity of rough strains of B. pertussis

Culture	Catalase activity on AG Medium	Agglutination titer
24 Minnesota	3.3	1:30
36 Povitsky	0.0	1:10
37 Lordet	1.9	1:10
61 Connaught	3.2	1:40

With the exception of strain 36, the rough cultures of B. pertussis possess some catalase activity though appreciably less than that demonstrated by smooth strains.

K. Effect of liver extract on pneumococcal catalase activity.

Since Swanson and Hartsfield (19) encountered an increase in the catalase activity of a strain of Proteus vulgaris when an extract of liver was added, cultures of H. pertussis were tested for "inactive catalase." The liver extract was prepared according to the method of Wilson and Miles (1) and five ml. quantities were allowed to act on an equal volume of a suitably diluted suspension of cells for five minutes prior to the addition of peroxide. Ten cultures were tested. The cultures showed no catalase activity with or without the liver extract; there was no change in the activity of eight other strains.

L. Effect of preservatives on catalase activity.

Since the catalase activity decreases as incubation is prolonged, the following work was done to determine what conditions could maintain maximum activity of a cell suspension. Growth of H. pertussis was harvested from agar plates of Vaccine I or after three days incubation and the cells suspended in saline containing preservatives. These suspensions were studied to determine effects of various preservatives, the concentration of the preservative, temperature and time of storage, and concentration of cells. Formalin, merthiolate and phenyl mercuric acetate were the preservatives used; phenol and tricresol were not included because they interfere with the $KMnO_4$ titration. Table 16 states the preservative used, the approximate concentration of cells during storage, the period and temperature of storage. The catalase activity retained after storage is expressed as per cent of activity at time of harvest.

Table 16

Effect of preservatives on catalase activity

Preservative	Per cent preservative	Concentration of cells x 10^{-9}	Period of storage	Temperature of storage	Per cent catalase activity retained
Formalin	0.4	4	3 hours	25°C.	24
Merthiolate	0.01	"	"	"	84
"	"	1000	3 days	"	30
"	"	"	7 "	4°C.	32
"	"	"	14 "	"	78
"	"	"	40 "	"	34
"	"	"	4 months	"	25
"	"	100	5 "	"	0
"	0.01	10	40 days	"	35
Phenyl mercuric borate	0.004	1000	40 days	"	27
"	0.004	10	"	"	38

Higher per cent of the catalase activity was retained when suspensions containing preservatives were stored at 4°C. than at 25°C. Under any conditions the catalase activity decreased as the period of storage was prolonged. Results of the study indicate that cells in low concentrations lost their activity faster than in higher concentrations.

The presence of formalin in relatively dilute cell suspensions caused a rapid decrease in the catalase activity. Heavy cellular concentrations in the presence of merthiolate stored at room temperature retained only 30 per cent of the activity after eight days. With the same concentrations of merthiolate and cells, the catalase activity retained was 84 per cent when stored at 4°C. for essentially the same period. However, only one-fourth of the original activity of this suspension remained after four months of storage in the cold. Pertussis vaccine containing 10 billion cells per ml. in 0.01 per cent merthiolate was examined for catalase activity. These lots had been stored in this concentration for one, three, four, five or six months. None of them

showed any activity.

The action of phenyl mercuric borate was least destructive to the enzyme. With a concentration of 0.004 per cent in a bacterial suspension of about 1000 billion per ml., the catalase activity was 97 per cent of its original value after a period of 40 days at 4°C. This compared to 5% per cent in a duplicate suspension containing 0.02 per cent merthiolate. Under the same conditions, suspensions diluted to 10 billion cells per ml. retained 38 per cent of the original activity in the presence of phenyl mercuric borate and only 5% per cent in merthiolate.

The cells suspended in a solution of phenyl mercuric borate were shown to be no longer viable after 40 days. A sample of the cell suspension was diluted into a tube of Brewer's thioglycollate broth (5, 33) to neutralize the preservative; transfers from this were made to Loeffler dengou diagnostic medium and incubated for seven days. There was no growth.

. Reaction velocity constant for catalase activity.

To calculate k, the velocity constant of the first order reaction, and Kat. f. (30), catalase activity expressed as the amount of H_2O_2 decomposed per cell per second, determinations were made at 1°C. The bacterial suspensions were made from 48 hour growth on Loeffler dengou vaccine agar incubated at 35°C. and diluted to a concentration of 5.8 billion per ml. The H_2O_2 concentration was 0.3 M. Aliquots were removed from the reaction mixture at 10 minute intervals and titrated with $KMnO_4$. Figure 8 gives the curves produced by plotting the logarithm of the concentration of H_2O_2 against time. The curves indicate that the reaction is first order.

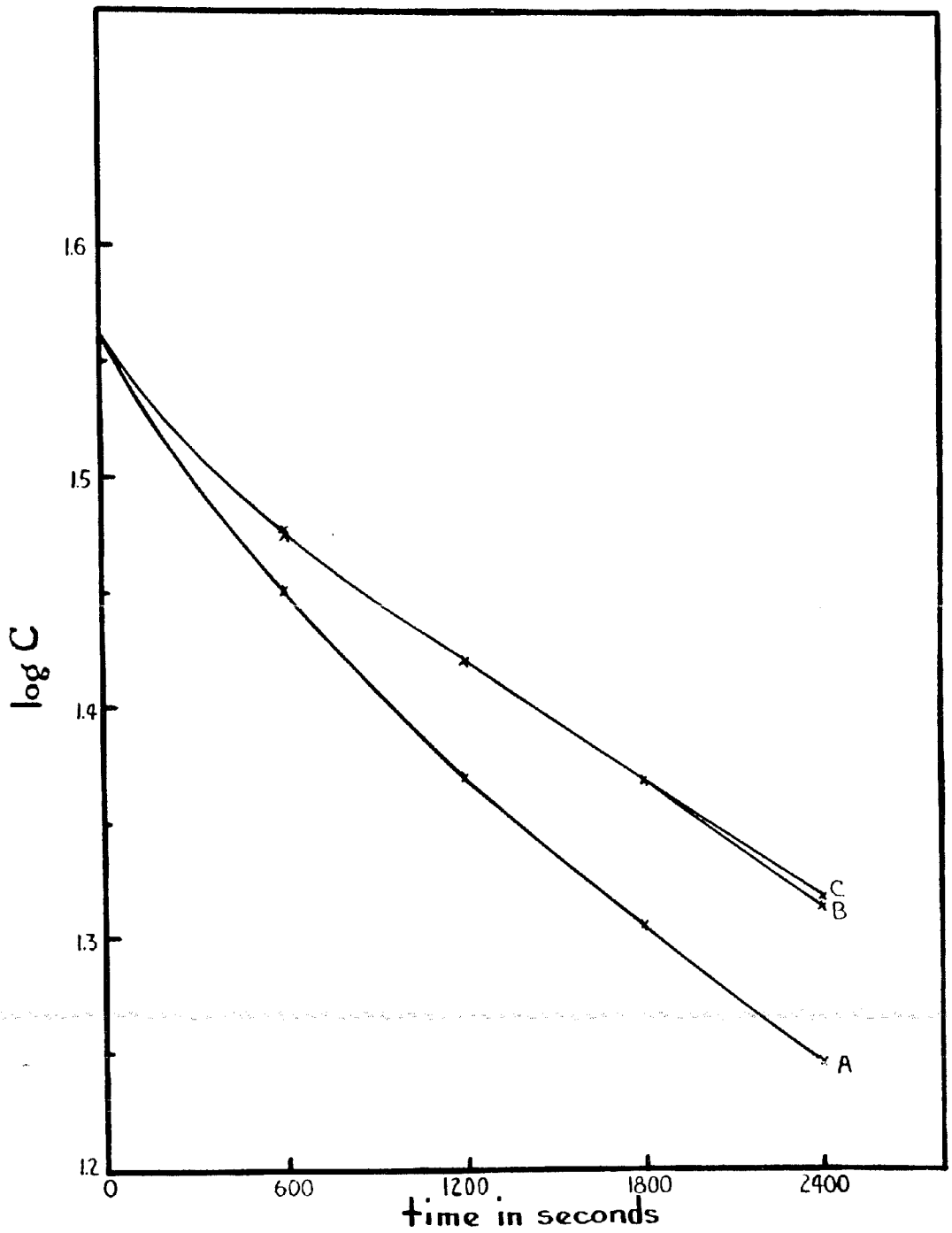
1.6
1.5
1.4
1.3
1.2
log C
0
600
1200
1800
2400
time in seconds
A
B
C

Table 17

Reaction velocity constant K and the Kotelsen'sche Wirksamkeit

Time of removal of aliquot. Seconds	Reaction velocity constant K		
	B. pertussis	Bordetella-pertussis bacillus	B. bronchisepticus
300	.00042	.00033	.00033
1200	.00036	.00027	.00027
1800	.00033	.00024	.00024
2400	.00030	.00024	.00025
Average	.00037	.00027	.00027
C. E. K.	1.87×10^{-14}	1.37×10^{-14}	1.37×10^{-14}

Table 17 gives the reaction velocity constants calculated according to the formula for first order reaction:

$$K = \frac{2.303}{t} \log \frac{C_0}{C}$$

The average of these values was used to calculate the activity based on the number of cells according to the formula proposed by Virtanen and Laurström (30):

$$\text{Cat. F.} = \frac{\text{Number of cells}}{K}$$

DISCUSSION

A technic for quantitative measurement of catalase activity of _B. pertussis_, the non-hemolytic bacillus and _Br. bronchisepticus_ was established by studying the effect of different conditions of the determinations. Within a range of one to seven billion organisms per ml. the number of bacterial cells in the reacting mixture was directly proportional to the catalase activity, but the specific catalase activity of a given cell suspension increased as the initial concentration of H_2O_2 was increased. The optimum pH for the test was 7.0, though increasing the acidity showed less effect on results than decreasing it. Enzyme activity was much less when the temperature of the reaction mixture was 1°C. than at room temperature, but the rate of reaction of the latter was faster.

For these studies was evolved a procedure which is satisfactory for comparing the activity of the three species when grown on various media. Cultures were incubated for two days at 37°C., suspended in diluent at pH 7, and standardized to four billion organisms per ml. To five ml. of the suspension were added 10 ml. of cold 1 % H_2O_2. The reaction is allowed to proceed on a rotating machine at room temperature. After 30 minutes, a five ml. sample is withdrawn and added to H_2SO_4. The residual H_2O_2 is titrated with 0.1 N $KMnO_4$ and the catalase activity expressed as the amount of 0.1 N H_2O_2 decomposed.

The conditions for growth were studied with reference to their influence on bacterial catalase activity. The presence or absence of peptone in Bordet Gengou medium had no effect on the catalase activity of _B. pertussis_ even though there were appreciable differences

in amounts of growth. There were significant differences in catalase activity when cultures were grown on various media. With E. pronucleoptica and the parapertussis bacillus, the highest values were obtained from growth in tryptone-glucose broth and the lowest on veal infusion agar. Activity of the parapertussis bacillus was less when a culture was grown on potato extract medium without blood than on this medium with blood, but there was a more marked decrease in the activity when the cells were grown on veal infusion medium.

B. pertussis showed the highest activity when incubated at 35°C. Varying from this optimum, an increase in temperature of incubation above 37°C. resulted in both lower activity and less growth than did a decrease in temperature below 37°C. The optimum temperature for growth was not always the optimum temperature for catalase activity.

The age of the culture at which maximum activity was reached and the duration of maximum activity were not the same for all cultures. For all three species the maximum catalase activity was attained in a culture as growth approached a maximum and the decrease in catalase activity was associated with autolysis of the cells. Cultures of each of the three species usually showed maximum activity after incubation for two days.

It is difficult to kill bacterial cells and retain catalase activity. When cells are harvested at the time of maximum activity and stored, the rate of decrease in enzyme activity depends on the preservative and on conditions of storage. It was noted that the higher the concentration of cells during storage, the higher the

per cent of activity retained. The catalase activity of a cell suspension decreased faster at 25°C. than at 4°C. There is a difference in the rate of decrease in activity with different preservatives. Phenyl mercuric borate was the most satisfactory preservative tested.

Of two strains of H. pertussis which were examined, 16 per cent did not show any catalase activity though they possessed all the characteristics of smooth strains. The 84 per cent of the strains which did decompose H_2O_2 had constant activity as long as the strains retained their smooth characteristics. Three cultures which once had catalase activity lost this ability and also showed alterations in the characteristics which typify smooth strains. All had a drop in agglutination titer and one of the cultures grew on blood-free veal infusion agar. After further transfers another failed to produce keratogenic necrosis when injected intradermally and all showed an increasing number of pleomorphic forms. The loss of characteristics which are criteria of smooth strains occurred at the same time as the decrease in catalase activity of these three strains.

The 16 per cent of smooth strains of H. pertussis which showed no catalase activity were not antigenically different from the smooth strains which possessed this property. Antiserum produced against each type was adsorbed with cells of the other type and in both cases the antibodies to H. pertussis were removed. A difference was noted in the two groups in their sensitivity to H_2O_2. The strains without catalase activity were killed in less time and in a lower concentration of H_2O_2 than the strains which had the ability to decompose

this chemical. Although no conclusion can be drawn at present as to the significance of those cultures which have all characteristics of smooth strains but do not possess the ability to decompose H_2O_2, results suggest physiological differences in strains.

The catalase activity of smooth strains of *L. pertussis*, the *H. pertussis* reiling and *B. bronchisepticus* when grown on casein agar were not significantly different. Apart from the strains of *L. pertussis* which showed no catalase activity, the smooth organisms had a higher catalase activity than rough ones. There was some variation in catalase activity of strains within each species but these differences were not correlated with any other characteristic.

SUMMARY

1. Fifty-five strains of smooth _B. pertussis_, four strains of rough _B. pertussis_, 10 strains of the parapertussis bacillus and 10 strains of _B. bronchisepticus_ were examined for catalase activity.

2. In a given initial concentration of H_2O_2, catalase activity of a culture was proportional to the bacterial concentration within a range of one to seven billion organisms per c.c. The measured catalase activity of a given cell suspension varied with the initial concentration of H_2O_2.

3. When cultures were compared on different media, the highest activity of the parapertussis bacillus and _B. bronchisepticus_ was obtained in tryptone-glucose broth and the lowest on veal infusion agar.

4. The incubation temperature for optimum activity of _B. pertussis_ was 37°C. There was a greater decrease in values at higher temperatures than at lower ones.

5. There was variation in the age of the culture at which the activity was highest. After two days incubation cultures of all three species most frequently reached the maximum activity.

6. Strains of _B. pertussis_ which retained all characteristics of smooth strains had no significant variation in catalase activity. Three cultures which lost the ability to decompose H_2O_2 showed concurrently a loss in characteristics which typify smooth strains.

7. Sixteen per cent of the strains of _B. pertussis_ examined had no catalase activity but maintained all the characteristics of smooth strains. From agglutination tests performed with adsorbed sera,

there was no indication of a difference in the enzymic structure. Other strains were killed more readily by H_2O_2 than were those with catalase activity.

7. Except for the strains of *L. acidophilus* which showed no catalase activity, cocci strains had a higher catalase activity than rod strains.

8. Detection of dissociation of the bacillus in the presence of oxygen was not applicable to *L. acidophilus*.

10. Addition of extract of liver did not increase the catalase activity.

11. No definite correlation of catalase activity to cells during a period of storage, or to apparent hydrogen-ion concentration of cells of various conditions. Strain variation between the four acidophilus cultures conservative.

12. No catalase activity of broth cultures of these species was of sufficient interest. There was some variation in catalase activity of various strains and species but these differences were not correlated with any other characteristic.

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