PEROXIDE AND PEROXIDOGENIC BACTERIA IN HUMAN SALIVA[1]

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Among the metabolites held responsible for the equilibrium among microbial species found in human saliva, hydrogen peroxide has occupied a prominent position. McLeod and Gordon (1922) first reported on bacterial production of peroxide and studied the inhibition of heterologous organisms by the peroxide formed by pneumococcus. Thompson and Johnson (1951) concluded "that the inhibitory properties of most salivas are due to the hydrogen peroxide produced by the mitis type streptococci."

While the bacterial production of peroxide *in vitro* has been well established, only two studies deal with its occurrence *in vivo*. Wurster (1886, 1889) claimed to have detected hydrogen peroxide in every saliva. We may discount his claim for his reagent, tetramethylparaphenylendiamine, demonstrates indophenol oxidase rather than hydrogen peroxide (Berger, 1953). More recently Tschesche *et al.* (1951) reported that they detected hydrogen peroxide in saliva. They estimated the concentration as 0.03 per cent (300 μg/ml). If saliva were to contain such a bactericidal concentration (Berger, 1953) of hydrogen peroxide, one should expect that other investigators could substantiate the finding.

To contribute to the understanding of the *in vivo* significance of peroxide in saliva the following types of experiments were undertaken: The determination of hydrogen peroxide in saliva, the estimation of the number of peroxidogenic bacteria, and the study of peroxide decomposition in saliva.

MATERIALS AND METHODS

Peroxide determination. Tschesche *et al.* (1951) claimed that titanium sulfate did not react with saliva directly but gave the yellow color reaction characteristic of hydrogen peroxide if saliva was extracted with ether. The titanium reagent was prepared according to the prescription of Bonet-Maury (1944) and the technique of measurement followed the method of Eisenberg (1943). By using the same Klett-Summerson photoelectric colorimeter, a closely similar filter (KS 42 covering a range of 400–465 mμ) and a cell of the same diameter, a standard curve identical with the one published by Eisenberg (1943) was obtained. This standard curve permitted direct conversion of Klett units to H_2O_2 concentration. The lowest concentration of H_2O_2 accurately measurable was 1 μg/ml at a scale reading of 10.

All saliva used was obtained by paraffin stimulation. The size of the test samples varied from 20 to 100 ml, some of the material was pooled and some left in individual samples. The original description (Tschesche *et al.*, 1951) mentioned that saliva was collected daily and stored in the icebox for 3 to 4 days before testing. Because of the scant details specified, a number of variations were tried. The period of storage was varied from 0 to 7 days at either 5 C or 37 C. The shaking with ether was varied from 6 to 60 min and performed either at room temperature or in the ice bath. Controls were employed without ether extraction. Evaporation *in vacuo* was performed at −20 C, at 0 C and at +10 C. Aqueous and ether phases were tested separately with titanium sulfate. The results remained the same throughout these ranges of treatment.

Bacterial estimates. (1) Growth medium:—Penfold (1922) described a benzidine blood agar medium on which peroxide producing bacteria grew in black colonies while the colonies of other bacteria were whitish. Berger (1951) succeeded in modifying the medium for the use of poured plates by substituting lysed erythrocytes for whole blood.

The benzidine-erythrocyte-brain heart (BEB) agar used in this study was prepared as follows: dehydrated brain heart infusion broth (Difco), 37 g and agar, 20 g were dissolved in 1 L of deion-

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TABLE 1

Colony growth of 48 stock cultures in poured benzidine-erythrocyte-brain agar plates after 96 hr aerobic incubation at 37 C

Black
Diplococcus pneumoniae (2)*
Streptococcus mitis (11)
Streptococcus sanguis (1)
Brownish
Bacillus subtilis (1)
Lactobacillus casei (2)
Serratia marcescens (1)
Streptococcus bovis (1)
Streptococcus pyogenes (1)
Streptococcus salivarius (1)
Streptococcus uberis (1)
White
Aerobacter aerogenes (1)
Bacillus cereus var. *mycoides* (1)
Bacillus mycoides (1)
Candida albicans (1)
Corynebacterium diphtheriae (1)
Corynebacterium hoffmanii (2)
Corynebacterium xerose (1)
Escherichia coli (1)
Klebsiella pneumoniae (2)
Lactobacillus sp. (1)
Micrococcus pyogenes var. *albus* (7)
Micrococcus pyogenes var. *aureus* (2)
Neisseria catarrhalis (1)
Pseudomonas aeruginosa (1)
Saccharomyces sp. (2)
Streptococcus salivarius (1)

* Numbers in parentheses denote number of strains tested.

ized water. After dispensing in 100 ml portions, autoclaving (pH 7.4) and cooling to 50 C, an erythrocyte solution (10 ml) and benzidine solution (5 ml) were added to each 100-ml portion. After mixing, plates were poured immediately.

The erythrocyte solution was prepared as follows: defibrinated horse blood was centrifuged (blood must be used fairly fresh, old cells are too fragile for washing), the sediment was washed three times with sterile 0.9 per cent NaCl. To the packed cells a 9-fold volume of distilled water was added to obtain a 10 per cent solution of lysed cell content. Sterilization and separation of stromata was achieved by Seitz filtration. The solution withstood storage in the refrigerator for over one month.

The benzidine solution consisted of 0.25 g of benzidine, 0.35 ml of 25 per cent HCl and 50 ml of deionized water filtered through Corning U.F. fritted glass. The solution could be stored in the dark for over one month.

The growth supporting qualities of BEB agar were tested to eliminate any selective bias exerted by the medium. Strains belonging to 25 microbial species (table 1) were plated in pure culture and all of them found adequate conditions for growth. Occasionally a *Corynebacterium hoffmanii* or a *Lactobacillus casei* showed scanty growth in pure culture but grew perfectly well in company of *Streptococcus mitis*.

(2) Sampling:—Two ml of fresh unstimulated whole saliva were collected from each of 8 subjects at 3 pm. The subjects, 5 females and 3 males, ranged from 24 to 50 years of age; 7 had natural dentitions, one wore full dentures. The sampling was repeated until an average of 21 samples per subject had been analyzed.

The fresh samples were diluted 1,000,000-fold with buffered water (American Public Health Association, 1953*a*) by means of proper pipetting technique (American Public Health Association, 1953*b*). One ml portions of the 1×10^6 dilutions were plated in triplicate with 15 ml of BEB agar. The plates were poured immediately so that refrigeration of saliva and its dilutions became unnecessary.

(3) Incubation and counting:—While pneumococci (Avery and Morgan, 1924; Annear and Dorman, 1952) start producing peroxide during the logarithmic growth phase (7–10 hr), the peroxide from streptococci (Avery and Morgan, 1924; Hadley *et al.*, 1941) occasionally may not be detectable for three or four days. It also frequently takes four days for all lactobacillus colonies to grow. For these reasons all samples were incubated at 37 C under aerobic conditions for four days, but the results would not differ appreciably with 24 hr incubation.

The total number of colonies and the number of black colonies were estimated in each of the triplicate plates with the aid of a Quebec colony counter. The triplicate estimates were averaged arithmetically. All tan or brown colonies were called non-black although some of the brownish appearing colonies were deep colonies of peroxide negative bacteria (figure 1) while others may have differed from the black colonies only in the concentration of peroxide produced.

(4) Statistics:—In a previous study (Kraus and Gaston, 1956) concerned with numbers of

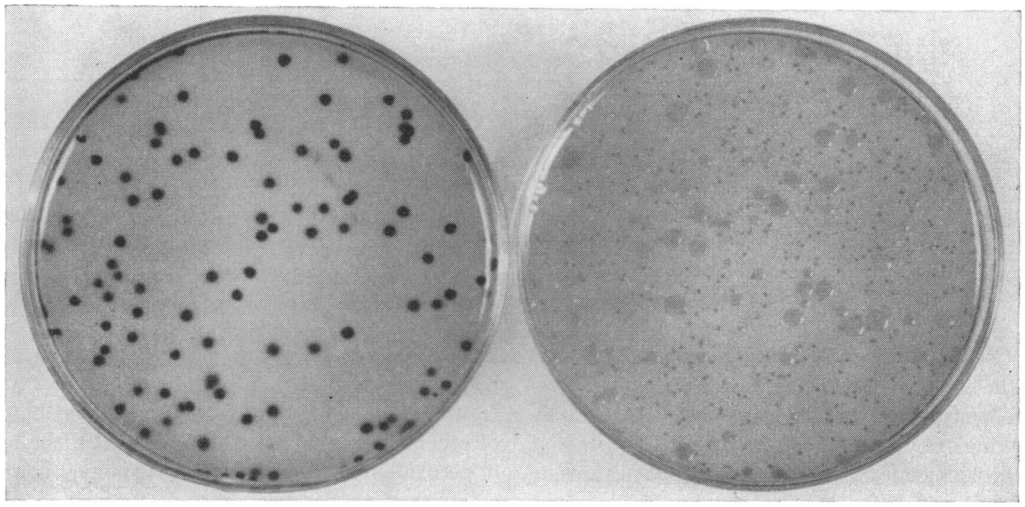

Figure 1. Black color of peroxide producing (*Streptococcus mitis*) and white or tan color of other (*Micrococcus pyogenes* var. *albus*) colonies in poured benzidine-erythrocyte-brain agar plates.

bacterial colonies, the criteria of normality and homogeneity of variance were not met and nonparametric statistical analyses had to be employed. Contrary to our expectation, the present data were found to be normally distributed and the variances homogeneous so that we could proceed with the classical analysis of variance (Snedecor, 1955*a*). A tentative explanation for this discrepancy between two similar studies is: In the present study identical dilutions were used for all specimens and no limitation was placed upon the number of colonies to be counted. This is a much simpler procedure than the one used in the previous study (Kraus and Gaston, 1956) in which a whole battery of dilutions was used simultaneously so as to obtain plates with no fewer than 30 nor more than 300 colonies (American Public Health Association, 1953*b*). The latter selection may be thought to introduce a bias affecting the statistical distribution.

RESULTS

Peroxide in saliva. A number of specimens were collected directly in flasks which contained titanium sulfate or horseradish peroxidase with benzidine or starch iodide, all of which are color indicators of free hydrogen peroxide. Not one sample developed a color and this eliminated the likelihood of free hydrogen peroxide at concentrations in excess of 1 μg/ml of saliva.

Eight specimens were tested using the ether extraction method of Tschesche *et al.* (1951) which reportedly detects bound hydrogen peroxide. The tests were negative. There was a possibility that these particular subjects' saliva might contain exceedingly low numbers of peroxidogenic bacteria or high concentrations of catalase. Therefore, an additional 5 specimens were collected from subjects No. III, IV, V, VII and VIII (table 3) whose mean percentages of salivary bacteria producing peroxide were known to be high. The catalase activities in the saliva of all 13 subjects were determined and found to be within the normal range (Kraus *et al.*, 1957). If any ether extractable hydrogen peroxide was present in these samples, it was below the detectable threshold of 1 μg/ml.

Any hydrogen peroxide naturally present in saliva would presumably be of bacterial origin. In an attempt at increasing its production, some saliva samples were enriched by 0.1 per cent glucose, others by varying proportions of brain heart infusion broth (Difco) and incubated at

TABLE 2

Color of benzidine-erythrocyte-brain agar produced by various concentrations of H_2O_2 reagent

Hydrogen Peroxide		Color of Agar
μg/ml	Normality	
25	0.0015	None
50	0.003	Very light
100	0.006	Light tan
200	0.012	Brownish
300	0.018	Black

TABLE 3

Mean percentages of salivary bacteria producing peroxide

	Subjects								
	I	II	III	IV	V	VI	VII	VIII	Total
No. of triplicate counts	23	27	21	22	20	21	21	13	168
Mean % black colonies	68.9	69.9	67.3	52.2	46.7	44.1	61.4	55.3	58.2*
Std. deviation	9.55	9.49	9.75	10.7	13.28	9.17	8.45	12.07	10.14

*The standard error of the mean is 3.57 and the 95% fiducial limits are 49.8 to 66.6%.

37 C for 1–3 days. Tests for peroxide performed at the end of incubation were uniformly negative. The picture did not change under aeration by shaking or by the addition of a heavy inoculum of a peroxidogenic *S. mitis* to the saliva cultures.

On the offchance that traces of peroxide might be present in the mouth but disappear on collection, saliva was caught in a beaker containing sodium azide. After ether extraction and addition of titanium sulfate, the colorimetric reading of this sample was identical with that of a water control.

Another series of experiments was performed in an attempt at obtaining a color reaction *in vivo*. A mixture of potassium iodide and starch in agar was placed into a perforated inert plastic capsule of about 2-ml volume. This was held in the mouth for 1 hr. The indicator system did not become blue while in the oral cavity and neither did saliva collected during this hour show any blue color. A control capsule kept during this time in 1 μg/ml aqueous H_2O_2 turned blue along the periphery of the agar and imparted its color to the solution. The fact that other substances are capable of producing free iodine from potassium iodide does not invalidate this test for hydrogen peroxide.

In a similar experiment, a small cloth bag containing ground Irish potato (which is rich in peroxidase) was kept in the mouth for an hour and subsequently immersed in a benzidine solution. It did not change color, while a control bag in 5 μg/ml H_2O_2 turned blue under subsequent benzidine treatment.

In the course of experiments with the ether extraction method, three positive tests were encountered. All three were proved to be false. Test #1 was due to lipstick in the sample: The yellow color appeared not only upon addition of titanium sulfate reagent, but also on adding sulfuric acid alone. The same subject's specimens were always negative when the lips were wiped before collection. The false positive test #2 was traced to peroxide contamination of one batch of ether. Tschesche *et al.* (1951) stressed the use of peroxide free ether. However, "The fact is well established that, when ether is exposed to air, moisture, light or certain chemical substances which may act as catalysts, it undergoes oxidation with the formation chiefly of toxic aldehydes and peroxides" (Gold and Gold, 1934). The false positive test #3 was due to prolonged aeration of ether in the presence of water: The control, too, was positive for peroxide after shaking 80 ml of ether with 20 ml of deionized water for 3½ hr. This experience suggests that Tschesche *et al.* (1951) may have produced rather than extracted peroxide.

Peroxidogenic bacteria. Historically the first among the aerobic bacteria known to produce peroxide were pneumococci and some streptococci (McLeod and Gordon, 1922). Later McLeod *et al.* (1923) included *Bacillus bulgaricus* and *Bacillus acidophilus* and Berger (1951) added *Micrococcus antibioticus* and (1952) one strain each of *Alkaligenes faecalis* and of *Pseudomonas fluorescens* to the known aerobic peroxidogenic bacteria. This list of organisms may give an approximate idea as to the identity of black colonies cultured from saliva.

The blackening of the colonies suggested uptake of benzidine and hemoglobin by the bacterial cells. This assumption was supported by the very dark appearance of the sediment of a culture of peroxidogenic *S. mitis* after incubation in broth containing benzidine and erythrocyte solutions. The bacteria from this sediment were observed unstained under oil immersion and appeared to contain a black or opaque core in contrast to the translucent streptococcal cells from ordinary broth. The viability of blackened colonies and the gram staining of black cells were unimpaired. Once a colony had blackened, it remained black

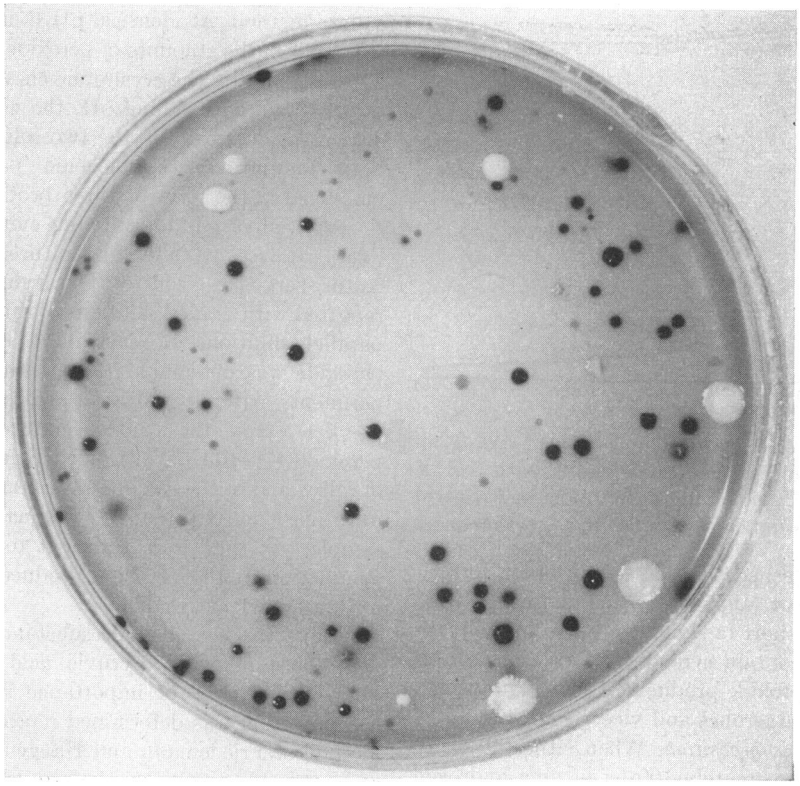

Figure 2. Appearance of a millionfold dilution of saliva in benzidine-erythrocyte-brain agar after 96 hr at 37 C.

permanently and did not change its color even with autoclaving.

The color intensity seemed proportional to the concentration of peroxide. Graded concentrations of peroxide reagent applied to wells in BEB agar plates usually developed the full color within 2 hr. The addition of fresh solution at 4 hr (simulating the assumed continuous peroxide production by microorganisms) exerted no apparent effect. A concentration of 300 μg/ml of H_2O_2 reagent produced a darkening equivalent to that of a *S. mitis* or a pneumococcus colony (table 2).

A culture of saliva containing an approximately even distribution of black and non-black colonies is shown in figure 2. The number of black colonies was exceeded by the number of non-black colonies in fewer than 25 per cent of the average of triplicate estimates. In general the peroxidogenic colonies prevailed. The peroxide producing bacteria may be expected to constitute on an average between ½ and ⅔ of all aerobic colonies grown from saliva of any comparable group of subjects (table 3).

TABLE 4

Analysis of variance of mean percentages of salivary bacteria producing peroxide

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square
Between subjects	7	15,913	2,273
Within subjects	160	16,868	105
Total	167	32,781	

$F = 2{,}273/105 = 21.6$; $P < .01$.

An analysis of variance (table 4) revealed that the difference among individuals was highly significant. In other words, the mean per cent of black colonies from saliva was characteristic of the individual.

The question whether the subject with larger numbers of aerobic salivary bacteria would have a larger proportion of black colonies was an-

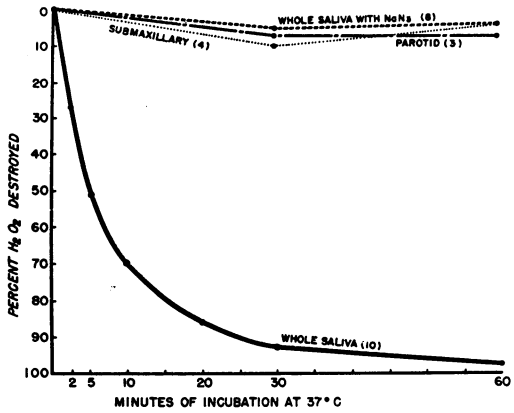

Figure 3. Average decomposition of 500 μg/ml H_2O_2 by whole saliva, whole saliva with 0.01 M sodium azide, submaxillary secretion, and parotid secretion. (Number of samples in parentheses.)

swered in the negative. The rank order correlation (Snedecor, 1955*b*) was found to be +.143 which is far short of significance. An individual's saliva may contain a relatively large mean proportion of peroxide-producing bacteria in a small mean total of aerobes and vice versa.

Peroxide decomposition. When either 40 μg of H_2O_2 or approximately 100 μg of urea-hydrogen peroxide[3] were added to 1 ml of saliva, the reagents were detectable only if tested immediately. Within 10 min they were broken down with the formation of bubbles and at the end of the reaction period no peroxide could be detected. Pretreatment of saliva with potassium cyanide or sodium azide inhibited the breakdown of peroxide.

The effect of the enzyme inhibitors suggested that the breakdown of peroxide by saliva is of enzymatic nature. To study this problem the catalase activity of a saliva sample was determined (Nickerson *et al.*, 1957) and an aliquot of peroxidase free crystalline beef liver catalase (Worthington) was diluted to an equivalent degree of activity.

In one series of experiments identical quantities of H_2O_2 reagent were added to a buffered aliquot of saliva and to a similarly buffered aliquot of beef liver catalase. After 4 min the peroxide concentrations were determined by the titanium sulfate method. At identical pH (buffered to 6.8, 6.0 and 4.1) the amounts of peroxide decomposed by saliva and by the crystalline enzyme were the same. (Also, with falling pH, the catalase titers decreased identically in the two solutions.)

In another series, biogenic peroxide was employed in the form of three broth cultures of *S. mitis*. Saliva was added to one culture and beef liver catalase to another culture. The third culture served as a control. This arrangement was repeated with graded dilutions of saliva and with parallel dilutions of crystalline catalase. No peroxide was detectable at the start of the experiments. After 48 and 72 hr aerated incubation at 37 C, only the control cultures contained peroxide (70-100 μg/ml). The highest dilutions of saliva and of the enzyme were 50 times weaker in catalase activity than the original fresh saliva sample, yet they were sufficient to prevent accumulation of the peroxide produced by a bacterium prevalent in saliva.

Among the non-enzymic substances known to decompose peroxide, pyruvic acid seemed the most likely to be of importance in saliva. Its concentration was determined repeatedly by the method of Friedemann and Haugen (1943). The amount of pyruvate per ml of saliva ranged from 2.5–5.0 μg. In saliva, as in the blood, pyruvate concentration appeared to be higher in the presence of muscular activity. When twice the physiologic concentrations of sodium pyruvate were added to 0.05 per cent (500 μg/ml) H_2O_2 reagent, only about 7 per cent of the peroxide were decomposed and mainly during the first 5 min. When pyruvate was added to beef liver catalase, no effect in addition to that of catalase could be measured.

The last series of experiments compared whole saliva, parotid secretion and submaxillary secretion for their ability to decompose H_2O_2 reagent. The reaction mixture consisted of: 10 ml of 0.1 per cent H_2O_2, 5 ml of M/15 phosphate buffer pH 6.9, 4 ml of whole saliva or glandular secretion, and 1 ml of either water or 0.2 M NaN_3. The latter was used in conjunction with whole saliva only. The number of specimens is indicated in figure 3. After specified times of incubation at 37 C, 1-ml aliquots of the reaction mixture were blown out into flasks containing 0.5 ml of titanium sulfate in 30 ml of deionized water. This stopped any enzymatic reaction and developed the yellow color of the titanium-hydrogen peroxide complex. The solution was then brought up

[3] The use of a peroxide complex was suggested by the insistence of Tschesche *et al.* (1951) that peroxide occurs in saliva in bound form. The crystals of urea-hydrogen peroxide were prepared according to the method of Lu *et al.* (1941).

to 50 ml with deionized water and allowed to stand for 20 min which was adequate for precipitation of the phosphates. After filtration through Whatman #2 paper the colorimetric determination was performed. A control containing 4 ml of deionized water instead of saliva was included with each determination and was taken to represent 0 per cent peroxide decomposition. The decomposition in each test sample was compared with its control and the arithmetic means of the per cent H_2O_2 destroyed were plotted.

Five of the 10 whole saliva specimens decomposed more than 90 per cent of the peroxide in 20 min and 6 specimens destroyed it completely in 30 min. The decomposition was almost entirely inhibited by sodium azide.

DISCUSSION

Several factors may account for the absence of detectable peroxide in saliva which contains a multitude of bacteria capable of producing peroxide *in vitro*. The available methods measure peroxide in concentrations upward of 1 μg/ml. The concentration of peroxide produced intracellularly in bacteria is unknown. The extracellular accumulation of peroxide would depend in part on the rate of formation and the rate of decomposition.

The rate of formation of hydrogen peroxide by bacteria varies in proportion with aeration (McLeod and Gordon, 1922; Avery and Morgan, 1924; Hewitt, 1931; Annear and Dorman, 1952), but some peroxide is formed, according to Wieland, Warburg and Keilin, wherever oxygen is reduced without activation. Only activated oxygen can be hydrogenated to form water. The lactic acid bacteria of the mouth as well as the strict anaerobes are devoid of the cytochrome system activating oxygen and they are rich in flavins which serve as intermediaries in peroxide formation. It may, therefore, be postulated that hydrogen peroxide is formed regardless of the oxidation-reduction potential prevailing at any given anatomic location of the oral cavity.

The redox conditions at the loci at which bacteria multiply are unknown. The average oxidation-reduction potential of fresh saliva is reported to be highly positive (Eisenbrandt, 1945; Eggers-Lura, 1955) so that peroxide might be thought to be produced at a high rate. However, it is open to question to what extent bacteria actually metabolize in the vehicle with which they are expectorated.

Among the substances which prevent the extracellular accumulation of peroxide, though not necessarily its intracellular formation, are catalases, peroxidases, proteins with free thiol groups, peptones, pyruvic acid, etc. All of these are present in saliva and, therefore, the absence of detectable peroxide does not militate against the likelihood of its production.

The experiments on the decomposition of hydrogen peroxide in saliva have led to the conclusion that the main agencies responsible for the breakdown of peroxide are the enzymes catalase and peroxidase. Stern and Bird (1951) have shown that in the presence of both enzymes, hydrogen peroxide is used preferentially by peroxidase in the oxidation of its substrate, provided a suitable substrate is available. "Catalase activity was largely suppressed while the oxidation of the substrate proceeded." If the necessary oxidizable substrates as, for instance, free thiol groups, are available, the decomposition in saliva may be assumed to proceed according to a peroxidatic reaction ($AH_2 + H_2O_2 \rightarrow A + 2H_2O$). In the absence of appropriate hydrogen donors the catalatic reaction ($2H_2O_2 \rightarrow 2H_2O + O_2$) alone would account for the molecular destruction of peroxide.

According to Keilin and Hartree (1945) catalase may act in a peroxidatic fashion if hydrogen peroxide is nascent rather than accumulated and if certain substrates such as ethanol are available. The substrate specificity of this reaction has not been defined but one may speculate that nascent biogenic peroxide may react differently from accumulated biogenic peroxide or from preformed H_2O_2. Preformed (reagent) hydrogen peroxide was the substrate in most decomposition experiments of the present study and this condition may have favored the catalatic reaction.

One other circumstance possibly favoring the catalatic reaction in these decomposition experiments was the concentration of the substrate. The final concentration of 500 μg/ml H_2O_2 (figure 3) was an expedient magnification of the assumed physiologic level of less than 1 μg/ml. The optimum substrate concentration for peroxidase (Nickerson *et al.*, 1957) is 50 μg/ml H_2O_2 and the peroxidase is only about 15 per cent as active at the tenfold substrate concentration. This may partially explain why the parotid and

submaxillary secretions which are devoid of catalase but rich in peroxidase hardly affected the peroxide reagent (figure 3). Catalase has the broader latitude of effectiveness, but this does not solve the question of whether the catalatic or the peroxidatic reaction or both are actually utilized for the *in vivo* decomposition of biogenic peroxide.

It may be of importance for the ecologic effects of peroxide that decomposition of peroxide takes time. It took 30 min for 1 ml of undiluted saliva to destroy 2.5 mg of H_2O_2 (see last paragraph of Results). Lesser amounts would be decomposed in shorter time, but even the minutest quantities might still be biologically effective while they last. After testing the mutual effects of peroxidogenic and of catalase producing bacteria in broth culture, Annear (1951) concluded that "it is by no means certain that H_2O_2 is inactive in the inhibition of the diphtheroid and of the staphylococcus which occurs in the presence of the streptococcus." It may be inferred from Annear's work and from the study by Hewitt (1931) that the relative quantities of peroxide and of catalase available for reaction at any one time are important in determining the resultant effect on interacting microbial species. The effect is complicated by numerous other factors among which environmental or host factors may be predominant (Annear, 1951; Kraus and Gaston, 1956).

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SUMMARY

The majority (58.2 ± 3.57 per cent) of aerobic salivary bacteria form hydrogen peroxide *in vitro*. The average proportion of peroxidogenic bacteria depends on the individual subject rather than on the total number of microorganisms in saliva. Theoretically, the metabolic pathway leading to peroxide production should obtain *in vivo*. The lack of detectable peroxide in saliva is explainable by the high threshold (1 μg/ml) of the indicators and by the destructive agencies available. Chief among the latter are catalases and peroxidases. They are present in saliva in more than sufficient concentrations to prevent extracellular accumulation of peroxide. Because of this, the inhibitory effect of biogenic peroxide *in vivo* is probably slight or absent.

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