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Studies on the Antibacterial Factors of Human Saliva

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Although the effect of saliva on different kinds of microorganisms had earlier been studied, it was not until 1934—1936 that Dold and collaborators [1-3] definitely showed that human saliva inhibits the growth of a great number of bacteria, *e. g. Corynebacterium diphtheriae*, staphylococci, streptococci, *Escherichia coli*, *Eberthella typhosa*, *Salmonella paratyphi B*, *Vibrio cholerae*, *Bacillus pyocyaneum*, and *Bacillus anthracis*. The antibacterial factor proved to be thermolabile, and sensitive to drying and ultraviolet radiation. It was also clear that it could not be identical with Fleming's lysozyme, present in small amounts in saliva and acting principally against a few airborn microorganisms, *viz. Micrococcus lysodeicticus*, and *Sarcina lutea*. According to Dold the antibacterial effect of saliva is due to a substance, possibly of enzymatic character, which is secreted with the saliva, and which he called inhibin. Moreover, Dold was of the opinion that microbial antagonism had no importance with respect to the antibiotic effect, although certain bacteria from saliva occasionally exhibit antagonistic action against other bacteria.

Other workers have ascribed greater importance to possible factors produced by the bacteria in the saliva. In 1937 Prica [5] asserted that the salivary bacteria had an antagonistic effect against at least the capsular bacteria *(Bacterium rhinoscleromatis*, *Bacterium Friedländer*, and *Bacterium ozaenae)*. He showed that culturemedia of staphylococci from saliva, which had been passed through Seitz filters, inhibited their growth, and assumed that the antibacterial effect was due to a secretory or metabolic product from the staphylococci. This product he found to be thermostabile. Mühlenbach [6] considered the antibacterial activity of saliva principally to be due to Dold's inhibin, but he pointed out that under certain conditions streptococci from saliva could antagonize staphylococci and *Corynebacterium diphtheriae*. Hölzl [7] made further studies of the antibiotic effect of salivary streptococci against *Corynebacterium diphtheriae*. By ether extraction of bouillon cultures

of salivary streptococci, he obtained an extract active against *Corynebacterium diphtheriae* both in vitro and in vivo. However, he was of the opinion that under natural conditions microbial antagonism was of slight importance. Thompson and Shibuya [8] found in 1946 that of 49 strains of streptococci cultivated from human saliva, 27 had an antagonistic effect against *Corynebacterium diphtheriae*, 24 of these being of the mitis type, 1 of the salivarius type, and 2 of the enterococcus type.

Other investigations of the antibacterial effect of saliva, sterilized by filtration have produced conflicting results. Dold, Lächele, and Hsing [1], and Weigmann and Noeske [9] found no antibacterial activity in saliva which had been passed through Seitz filters. On the other hand, Weigmann and Koehn [10] and Casassa [11] reported that the antibacterial factor passed through both Seitz and Berkefeld filters. Knorr [12] found antibacterial effect in saliva filtered through Jena glass filters "G 5 on 3". Kesteren, Bibby, and Berry [13] reported that saliva filtered through Berkefeld N filters had antibacterial activity, though it was low in comparison with the activity before filtration.

Dold, Lächele, and Hsing [1], and Weigmann and Noeske [9] were of the opinion that the antibacterial effect of saliva was not due to microbial antagonism, because there was no correlation between the number of salivary bacteria and antibacterial activity. And furthermore, Dold [14] reported that saliva collected directly from ductus submaxillaris in dogs had antibacterial action.

Therefore, it seemed probable from the literature that saliva contained a substance with antibacterial effect. In 1942 Kesteren, Bibby, and Berry [13] made resultless attempts to concentrate and purify it.

Most of the earlier investigators, who had been interested in these problems, found both interindividual and intraindividual variations in the antibacterial power of saliva. The present investigation was begun in order to ascertain the nature of the antibacterial factor, and possible causes of the variation, which has been reported. Were these problems solved, it might be possible to find means of increasing the natural antibacterial activity of the saliva.

EXPERIMENTAL METHODS

Agar plate method according to Dold [1]: Saliva, used directly, or after dilution with distilled water, was mixed with agar at a temperature of 45° C in 1 : 1 proportions (1 ml saliva: 1 ml agar). The mixtures were poured into small Petri dishes, 3 cm in diameter, the final concentration of saliva in the plates varying between 1 per cent and 50 per cent. The agar medium had the following composition: 2 g of agar, 0.4 g Bactopeptone (Parke Davis & Co.), 0.1 g KH_2PO_4, 0.1 g K_2HPO_4, and 0.5 g NaCl per 100 ml of medium at pH 6.8. When streptococci and pneumococci were used as test bacteria, the medium was fortified with 10 ml of human serum, and the pH adjusted to 7.2. The plates were inoculated with 0.05 ml of a suspension of the test bacterium. By cautious rotations of the

plates, the suspension was equally dispersed over the surface, and the plates were then incubated at 37° C for 16 hours. The growth of the test bacterium was classified as +, + +, or + + +. A + indicated slight, but macroscopically visible growth; a + + indicated intermediate growth, and a + + + indicated the same growth as in the controls. Two types of controls were used; plates with the agar medium, and plates with the saliva-agar mixture. In the latter, the postulated antibacterial factor of the saliva had been destroyed by heating at 100° C for 2 min.

Agar diffusion method [15]: A 0.5 ml layer of the same agar medium used in the agar plate method was poured into Petri dishes of 10 cm diameter. Inoculation with the test bacterium was accomplished by mixing 1 ml of a 24 hour old culture with 50 ml agar at 45° C, and pouring 2 ml of this mixture on the surface of each plate. Plates prepared in this way could be kept for use at a temperature of 4° C for about 2 weeks. 0.1 ml of the saliva to be tested was placed upon the agar plate in small, sterile glass cylinders (height 1 cm, inner diameter 0.5 cm) from which the saliva diffused into the medium; 6—8 cylinders could be placed on each plate. The plates were then incubated at 37° C for 16 hours. In this method antibacterial activity is indicated by zones of growth inhibition around the cylinders.

The following test bacteria were used: in the agar plate method *Staphylococcus H* *, *Staphylococcus* 209, *Pseudomonas aeruginosa*, one strain of an alpha and one of a beta streptococcus, *B. coli commune*, *B. coli beta polare*, and a pneumococcus strain, type 19; in the agar diffusion method only *Staphylococcus H*. Inocula for assays were prepared by centrifuging the cells from 1 ml of the bouillon media, washing once with water or 0.9 per cent NaCl, and diluting to a volume of 200 ml with 0.9 per cent NaCl. The two strains of streptococci, and the pneumococcus strain were cultivated for 16 hours in ascites bouillon, and the other test bacteria in peptone bouillon for 24 hours.

EXPERIMENTAL RESULTS

The effect of saliva on its own bacteria

According to Dold [1], the salivary bacteria did not show any growth in the plates for the first two days, owing to the presence of inhibin, which was also supposed to inhibit the common bacteria of the saliva. This hypothesis could not be confirmed in this investigation. After incubation at 37° C for 16 hours, the uninoculated agar-saliva plates assumed a turbid appearance which was shown, at a magnification of ten, to be caused by the formation of a great number of small colonies of bacteria from the saliva. Microscopical control of slides from these plates taken both immediately after pouring, and after incubation at 37° C for 16 hours showed that a great increase in the number of salivary bacteria had definitely occured. Another manifestation of the activity of the salivary bacteria was the change in pH value of the uninoculated plates during the incubation. From the initial value of 6.8 the pH changed

* *B. coli commune* and *B. coli beta polare* were received from professor Ragnar Nilsson, Ultuna, the other test bacteria from the Institute of Hygiene and Bacteriology at the University of Uppsala.

Table 1. Inhibitory effect on Staphylococcus H of saliva from 25 healthy individual 19—50 years of age. Normal diet. Dold's agar plate method.

Saliva no.	Growth of the test bacterium (*Staphylococcus H*)							Number of viable salivary bacteria per mm³
	Controls		Concentration of saliva in the plates per cent					
	agar medium	agar+ heated saliva	50	25	10	5	1	
1	+++	+++	—	—	—	+	++	
17	+++	+++	—	—	—	+	+	
18	+++	+++	—	—	—	—	+	
19	+++	+++	—	—	—	+	++	
20	+++	+++	—	—	—	+	++	
21	+++	+++	—	—	+	++	+++	
22	+++	+++	—	—	—	+	+	
23	+++	+++	—	—	+	++	++	
24	+++	+++	—	—	—	+	++	
25	+++	+++	—	+	+	++	+++	
26	+++	+++	—	—	+	++	+++	
33	+++	+++	—	—	—	+	+++	4 000
34	+++	+++	—	—	+	+	++	6 000
35	+++	+++	—	—	+	+	++	20 000
36	+++	+++	—	—	+	+	++	7 000
37	+++	+++	—	—	+	+	++	12 500
38	+++	+++	—	—	+	+	++	20 000
39	+++	+++	—	+	++	+++	+++	7 000
40	+++	+++	—	—	+	++	+++	10 000
42	+++	+++	—	+	++	+++	+++	500
44	+++	+++	—	—	—	++	+++	15 000
45	+++	+++	—	—	—	+	+++	20 000
46	+++	+++	—	—	—	—	++	25 000
47	+++	+++	—	—	—	++	+++	1 250
48	+++	+++	—	—	—	++	+++	7 500

+ indicates slight growth, ++ intermediate growth, +++ the same growth as in the controls, — indicates, no macroscopic growth of the test bacterium.

to values varying between 6 and 8, depending on the proportions of acid and alkali producing bacteria. If 0.5—1 per cent glucose was added to the agar medium, the lactic acid production changed the pH to about 4.5, in spite of the addition of buffer substances to the agar medium. If glucose was not added, however, the alterations in pH were not great enough to cause a non specific inhibition of the test bacteria.

Table 2. Inhibitory effect on Staphylococcus H of saliva from 10 healthy vegetarians 19—35 years of age. Dold's agar plate method.

Saliva no.	Growth of the test bacterium (*Staphylococcus H*)							Number of viable salivary bacteria per mm³
	Controls		Concentration of saliva in the plates per cent					
	agar medium	agar+ heated saliva	50	25	10	5	1	
27	+++	+++	−	+	++	+++	+++	1 000
28	+++	+++	+	++	+++	+++	+++	375
29	+++	+++	++	++	+++	+++	+++	100
30	+++	+++	++	+++	+++	+++	+++	100
31	+++	+++	−	−	−	+	++	2 500
32	+++	+++	−	−	−	+	++	6 750
41	+++	+++	−	−	−	−	+	100 000
43	+++	+++	−	+	++	+++	+++	500
49	+++	+++	−	+	++	+++	+++	5 000
50	+++	+++	−	−	−	+	++	12 500

+ indicates slight growth, ++ intermediate growth, +++ the same growth as in the controls, − indicates, no macroscopic growth of the test bacterium.

The rapid growth of the bacteria in fresh saliva could also be shown in the following way: Saliva, freed from heavy suspended particles such as epithelial cells and leucocytes by centrifuging at a low rate (1500 turns per minute), was, except for a slight opalescence, clear. After incubation for 24 hours at 37° C, however, it grew turbid due to the growth of salivary bacteria.

The effect of saliva on different test bacteria

Unstimulated saliva, collected 1 to 3 hours after meals, was used in experiments employing Dold's agar plate method. Two groups were investigated, one consisting of 25 healthy persons, 19 to 50 years of age, with a normal diet; the other, of 10 healthy vegetarians, 19 to 35 years of age. The results of these experiments are shown in Tables 1 and 2.

They agree with Dold's experiments to the extent that, with a few exceptions, all the tested salivas showed a strong antibacterial activity against *Staphylococcus H*. Furthermore, if the salivas were heated to 100° C for 2 minutes, or to 56° C for 30 minutes, the activity was completely destroyed. Of the other tested bacteria, active saliva inhibited *Staphylococcus 209*, a strain of an alpha, and a strain of a beta type streptococcus, a pneumococcus strain

of type 19, and two coli strains; *Coli beta polare*, and *Coli commune*. Against *Pseudomonas aeruginosa* the effect was slight, and sometimes completely absent.

The significance of the salivary bacteria for the inhibitory effect

In order to explain the relation of the salivary bacteria to the inhibitory effect of the saliva, a number of experiments were made with salivas, freed from bacteria by filtration through Seitz filters, Jena glass filters "G 5 on 3", and Pyrex filters U. F. In all cases, the salivas lost all their antibacterial activity, when tested with Dold's agar plate method. It seemed possible that the active factor might not have passed through the filters. Larger amounts of saliva were freed from bacteria by centrifugation at a rate of 10 000 turns per minute for two successive periods of 20 minutes each. Furthermore, the sterility of the centrifuged saliva was tested by putting a drop of saliva on an agar plate. As a rule, either no colonies, or at most a few, had developed after 24 hours' incubation at 37° C. Centrifuged saliva from 50 healthy individuals, 19 to 50 years of age, was tested by Dold's agar plate method, and the agar diffusion method. An antibacterial effect could not be shown against *Staphylococcus H*, *Staphylococcus 209*, the alpha and beta streptococci, *B. coli commune*, *B. coli beta polare*, *Pseudomonas aeruginosa*, or the type 19 pneumococcus strain . (In the agar diffusion method, only *Staphylococcus H* was used as test bacterium, as was previously stated). When the centrifuged sediments containing the salivary bacteria were added to the clear salivas, the whole inhibitory effect returned.

In another experiment, 5 uncentrifuged salivas, showing a low antibacterial activity, were tested with Dold's agar plate method before, and after incubation at 37° C for 16 hours. The results are seen in Table 3.

As might be expected, the inhibitory effect was stronger after incubation, owing to the increase in the number of salivary bacteria.

In 24 of the tested salivas the number of viable bacteria was determined in the following way: the salivas were diluted with 0.9 per cent NaCl in the proportions 1 : 100, 1 : 1000, 1 : 10 000, and 1 : 100 000. 0.05 ml of each of these solutions was dropped on the surface of agar plates. After incubation at 37° C for 24 hours, the number of colonies was counted, and the number of bacteria per mm^3 saliva calculated. The number of bacteria in the different salivas is indicated in Tables 1 and 2. There seemed to be a definite correlation between the number of bacteria and the antibacterial effect, for the salivas richest in bacteria had the strongest antibacterial effect, while the salivas with

Table 3. Inhibitory effect of different salivas before and after incubation at 37° C for 24 hours. Dold's agar plate method. Test bacterium Staphylococcus H.

Saliva no.	Growth of the test bacterium (*Staphylococcus H*)											
	Fresh saliva						The same salivas after incubation at 37° C for 24 hours					
	Controls	Concentration of saliva in the plates per cent					Controls	Concentration of saliva in the plates per cent				
		50	25	10	5	1		50	25	10	5	1
51	+++	+	++	+++	+++	+++	+++	−	−	−	+	++
52	+++	+	++	+++	+++	+++	+++	−	−	+	++	+++
53	+++	++	++	+++	+++	+++	+++	−	−	−	++	+++
54	+++	++	+++	+++	+++	+++	+++	−	−	−	+	+++
55	+++	−	+	++	+++	+++	+++	−	−	−	++	+++

\+ indicates slight growth, ++ intermediate growth, +++ the same growth as in the controls;
− indicates, no macroscopic growth of the test bacterium.

the smallest amount of bacteria had the weakest activity. There were, however, many salivas which had a weak inhibitory action, even though they contained a rather large number of bacteria. This fact might be due to a slight rate of growth of the bacteria in these salivas.

Since the great importance of the salivary bacteria for the inhibitory effect of saliva had been shown, it remained to find out how this effect is produced. As mentioned earlier, Prica [5], and Hölzl [7] have, among others, shown that certain bacteria from saliva, cultivated under special conditions, are able to produce substances with an antibiotic effect against other bacteria. To determine whether salivary bacteria under natural conditions can produce such substances, salivas from 50 healthy individuals, 19 to 50 years of age, were examined after incubation at 37° C for 24 hours. The bacteria were removed by centrifuging, as above, and the antibacterial effect was tested by the agar plate method, and the agar diffusion method. Here, as before, all antibacterial effect was removed from the salivas by the centrifugation of the bacteria.

To find out if the antagonistic effect of the salivary bacteria toward the testbacteria was due to competition for growth factors, present in insufficient quantities in saliva, the following experiments were performed: Saliva was sterilized by heating at 100° C for 5 minutes, or by filtration through Jena glass filters "G 5 on 3". 5 ml of the sterile saliva was placed into each of several

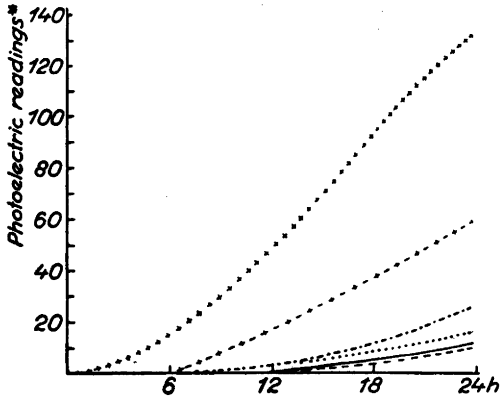

Fig. 1. Growth of different bacteria in sterile saliva at pH 7.

\+ + + + *Miscellaneous culture of salivary bacteria*
\+ — + — *Pseudomonas aeruginosa*
—·—·— *Staphylococcus H*
····· *B. coli commune*
——— *beta type streptococcus*
— — — — *Pneumococcus type 19*

* *Given as scale readings on the Klett-Summerson photoelectric colorimeter. Mean values from two tubes corrected for blank readings.*

sterile glass tubes, and the tubes were then inoculated with 0.05 ml of equally turbid suspensions of different bacteria (the inocula contained approximative three millions of bacteria). The growth of the bacteria was measured by turbidimetric analysis in a Klett-Summerson photoelectric colorimeter, after 6, 12, 18, and 24 hours. The growth rate varied somewhat in the different salivas. In all cases, however, the tubes inoculated with suspensions of miscellaneous salivary bacteria showed a considerably stronger growth than the tubes, which had been inoculated with the different test bacteria. A difference could not be observed between the growth rates in saliva sterilized by heating and in that sterilized by filtration; the growth factors, therefore, did not seem to be destroyed by short heating (100° C for 5 minutes). Fig. 1 shows typical curves of the growth of different bacteria in sterile saliva at pH 7. Streptococci, pneumococci, staphylococci, and *Escherichia coli* grew very slowly, apparently because of insufficient amount of essential growth factors in the salivas. By adding 10 per cent of blood serum as a source of growth factor, the growth rates of streptococci and pneumococci were considerably increased. The adding of 0.2 per cent peptone to the filtered saliva was sufficient to promote a strong growth of staphylococci and *Escherichia coli.*

By adding peptone to unfiltered saliva a similar result could be obtained. Saliva used directly or after dilution with distilled water was mixed in 1 : 1 proportions with agar solution containing 0.4 or 4 per cent of peptone as source of growth factor. The final concentration of saliva in the plates varied between 1 and 50 per cent. For each dilution of saliva two samples were used, one containing 0.2 per cent and the other 2 per cent of peptone. As usual the plates were inoculated with 0.05 ml of a suspension of the test bacterium (*Staphylococcus H*). The result is shown in Table 4.

Table 4. Inhibitory effect of saliva on Staphylococcus H in saliva agar plates with 2 and 0.2 per cent peptone.

Saliva agar plates with different amounts of peptone	Growth of the test bacterium (*Staphylococcus* H)					
	Controls	Concentration of saliva in the plates per cent				
		50	25	10	5	1
2 per cent peptone	+++	−	+	++	+++	+++
0.2 » » »	+++	−	−	−	+	++

+ indicates slight growth, ++ intermediate growth, +++ the same growth as in the controls. − indicates, no macroscopic growth of the test bacterium.

The addition of peptone to the saliva agar plates facilitated the growth of the test bacteria in spite of the presence of salivary bacteria.

In view of the above results, it is surprizing that some authors have found antibacterial effect in saliva sterilized by filtration. However, many of these workers have used as controls agar plates containing only a mixture of agar and unfiltered saliva, in which the antibacterial activity has been destroyed by heating. Since the filtration causes a marked decrease in the amount of organic substances available as nutriment for the test bacteria, the growth of test bacteria in agar plates containing filtered saliva will be less than in the controls with heated, unfiltered saliva. This fact may have been erroneously interpreted as due to the presence in the filtered saliva of substances with antibacterial effect.

The loss of organic substances in filtered saliva can be illustrated by a saliva, in which nitrogen determination was made before and after filtration through a Seitz filter. The quantity of nitrogen diminished from 0.46 to 0.18 mg per ml. By filtration through Jena glass filters "G 5 on 3" the loss of nitrogen was about 20 per cent.

DISCUSSION

The lack of antibacterial activity of saliva, from which the bacteria have been removed by centrifuging, indicates that the antibacterial effect of fresh saliva is due to bacterial antagonism of the salivary bacteria. Although certain bacteria isolated from the saliva are capable, under favorable conditions, of producing substances with an antibiotic effect toward other bacteria, the antagonistic effect of the salivary bacteria does not seem to be caused in this

way under natural conditions. The microorganisms normally found in human saliva seem to grow rather rapidly there. Most pathogenic bacteria, however, grow only very slowly in sterile saliva because of a deficiency of factors essential for their growth. When bacteria with relatively low growth rates as compared with the salivary bacteria enter the saliva, their growth will be inhibited by the lack of growth factors. When these factors are added, however, the inhibitory effect of the salivary bacteria is considerably weakened. Of the bacteria tested, Pseudomonas aeruginosa is least inhibited by the salivary bacteria. This agrees with the observation, that *Pseudomonas aeruginosa*, next to the salivary bacteria themselves, shows the most rapid growth in sterile saliva.

Furthermore, under physiological conditions, the pH of the saliva may also be of importance in the determination of the inhibitory effect toward pathogenic bacteria. The pH of human saliva normally varies between 6 and 7. If it is below 6.5 the growth conditions for many of the pathogenic bacteria is further reduced, since the optimal growth for most of them occurs at pH 7.0—7.4, and they grow very slowly if at all at a pH of less than 6.5. Many of the salivary bacteria still show a rather rapid growth at this pH.

Since the common salivary bacteria inhibit the growth of pathogenic bacteria, the therapeutical use of antibiotics may, by a simultaneous action on sensible microorganisms in the normal bacterial flora of the mouth, increase the possibility of an oral infection by those pathogens, which are to a lesser extent affected by the drug in question. Lipman, Coss and Boots [16] and Long [17] have recently studied the influence of penicillin therapy on the bacteria of the mouth. Before the treatment there were principally grampositive bacteria present. During the treatment, however, the number of these bacteria rapidly diminished and was followed by a flora of gram-negative, penicillin-resistant microorganisms. The changes were the same whether the penicillin was given orally, or by intramuscular injections, provided that in the latter case the drug was given in so high a dose, that it was secreted in the saliva ($>$ 500 000 units a day).

Louis Weinstein [18] has recently described five cases of new bacterial infections during the course of treatment with streptomycin or penicillin. In one patient, who probably had atypical pneumonia, administration of penicillin was followed by some apparent improvement, but on the fourth day of treatment *Hemophilus influenzae* was isolated from the throat and blood for the first time, and the condition became worse. In a second patient, who had faucial diphtheria, pneumonia developed. *Klebsiella pneumoniae*, not found at the beginning of treatment, was seen in throat and sputum after the fourth day of penicillin therapy, and was the only organism in the pulmonary tissues

revealed be postmortem culture. The other three patients were infected with *Staphylococcus aureus* during treatment with streptomycin for infections caused by *Hemophilus influenzae.*

SUMMARY

1. The antibacterial effect of human saliva toward staphylococci, streptococci, pneumococci, *Escherichia coli* and *Pseudomonas aeruginosa* has been investigated. All of these bacteria are inhibited by the saliva, *Pseudomonas aeruginosa* only slightly and inconstantly.

2. The antibacterial effect toward the above bacteria is due to the antagonistic action of the salivary bacteria.

3. This antagonistic effect seems to be connected with a competition between the salivary bacteria and pathogens for essential growth factors in the saliva. The normal bacterial flora require less of such factors, and will accordingly constitute an important defense mechanism against oral infections.

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