THE INHIBITORY ACTION OF SALIVA ON THE DIPHTHERIA BACILLUS: THE ANTIBIOTIC EFFECT OF SALIVARY STREPTOCOCCI

RICHARD THOMPSON AND MADOKA SHIBUYA
(With the technical assistance of Chiye Nao)
Department of Bacteriology, University of Colorado, School of Medicine, Denver, Colorado

Received for publication February 1, 1946

The resistance to infection of the tissues of the mouth greatly impressed early workers, who suspected an antibacterial action of saliva and devised experiments to investigate this possibility. (For complete references see Thompson, 1940.) Up to 1934 the results obtained were indecisive, and there was disagreement as to the presence or importance of such antibacterial action. In 1934 and succeeding years Dold and Weigmann (1934), Dold (1935), Dold, Lachele, and Hsing (1936), Weigmann and Koehn (1936), and Weigmann and Noeske (1937) introduced plating methods and showed quite conclusively that saliva had an inhibitory action against the diphtheria bacillus. The Rochester school, Clough (1934), Taylor and Bibby (1935), Hine (1936), Bibby and Ball (1937), Bibby (1938), Clough, Bibby, and Berry (1938), Bibby, Hine, and Clough (1938), and Kesteren, Bibby, and Berry (1942), demonstrated a definite action against lactobacilli and other organisms. In this paper we are concerned only with the inhibitory action of saliva against the diphtheria bacillus. Dold and his co-workers arrived at no definite knowledge as to the nature of the agent active against the diphtheria bacillus but did present considerable evidence that the salivary bacteria were not concerned. In a previous report Thompson (1941) showed that the agent could not be lysozyme since it could be destroyed or removed from the saliva under conditions in which all the lysozyme was retained or even concentrated.

In view of the findings reported in this paper it seemed desirable to review in some detail the evidence presented by the earlier workers as to the role of salivary bacteria in the inhibitory action of saliva. The results of attempts to destroy or remove the bacteria of saliva while retaining the inhibitory action have been conflicting. Dold, Lachele, and Hsing (1936) and Weigmann and Noeske (1937) found that the inhibitory agent was removed by filtration, but Weigmann and Koehn (1936) and Casassa (1937) reported that the agent passed both Seitz and Berkefeld filters. Weigmann and Noeske (1937) reported that prolonged centrifugation removed the agent but assumed that the loss was due to the temperature (64 C) reached in the centrifuge. The same authors found that metallic copper kept in the saliva for long periods at 37 C killed off most of the bacteria but left the antibacterial power unaffected. The fact that this power could later be destroyed by heat indicated to them that they were not dealing with the oligodynamic effect of copper ions transferred in the saliva. Experiments attempting to show whether or not salivary bacteria have an inhibitory effect on

the diphtheria bacillus have also given contradictory results. Weigmann and Noeske (1937) found that some salivary streptococci and staphylococci had a slight inhibitory action on the diphtheria bacillus but stated that many more organisms than were present in saliva were necessary to produce this action. Besta and Kuhn (1934–35) reported that viridans and hemolytic streptococci from saliva inhibited the diphtheria bacillus. Weigmann and Holzl (1940) and Holzl (1941) more recently studied the antagonistic action of mouth bacteria against the diphtheria bacillus. They found strains of hemolytic and viridans streptococci to be inhibitory but concluded that these organisms could not be mainly responsible for the antibacterial power of saliva since very large numbers of organisms were necessary to produce the action; the property was quickly lost in culture and could be abolished by the presence of other bacteria. Indirect evidence against the role of bacteria was given by Dold, Lachele, and Hsing (1936) and by Weigmann and Noeske (1937), neither group finding any correlation between the potency of the inhibitory action of saliva and the numbers of bacteria present.

METHODS

Except where otherwise noted, all tests for inhibition were made by placing standard drops of the material to be tested on the surfaces of agar pour plates containing various concentrations of diphtheria bacilli and by observing the zones of inhibited growth around these drops after a suitable incubation period. The *Corynebacterium diphtheriae* used as the test organism is a strain isolated several years ago from a typical case in this hospital and maintained since then by frequent transfers on rabbits' blood agar. It has the cultural characteristics of an intermedius type and produces a toxin with characteristic action on guinea pigs. The toxin is neutralized specifically by commercial diphtheria antitoxin. Sixteen- to nineteen-hour cultures of the organism on rabbits' blood tryptose agar slants were emulsified in 3 ml of 2 per cent tryptose (Difco) solution, and several serial dilutions were made in the same medium. As noted below, certain optimal concentrations of the organisms were necessary for the demonstration of maximal inhibition. Since optimal dilutions varied from time to time, two different concentrations were used in all experiments. The desired dilutions of the organisms were added to the agar medium at 45 C and thoroughly mixed; plates containing exactly 13 ml of the agar were poured. The agar medium used had the following composition: 1.5 per cent agar, 0.3 per cent meat extract (Difco), 0.5 per cent NaCl, and 0.3 to 0.5 per cent tryptose (Difco), adjusted to pH 7.2. As noted below, the concentration of tryptose was important and the optimal concentration varied somewhat with each new bottle. After the plates hardened they were inverted and placed in the refrigerator for 1 hour.

The saliva or other material to be tested was dropped onto the agar surface from a 27-gauge needle on a syringe held vertically about 2 inches above the plate. It was found that 5 drops could be satisfactorily tested on one plate. All tests were done in duplicate and frequently in triplicate. The drops were allowed to dry at room temperature with the plate covers slightly displaced.

The plates were then inverted and placed in a 37 C incubator. After incubation for 18 to 48 hours they were carefully examined in an indirect, strong light with a dark background. The widths of the zones around the drops, in which no growth of the diphtheria bacillus occurred, were carefully measured and recorded (figures 1 and 2).

Other details of the method depended on the nature of the particular experiment and will be described in the appropriate sections.

PRELIMINARY OBSERVATIONS ON FACTORS AFFECTING THE INHIBITORY ACTION OF SALIVA ON C. DIPHTHERIAE

The work on the inhibitory action of saliva reported previously was done in New York using infusion agar. On returning to the problem here, some months later, it was very difficult, at first, to show any action at all with most salivas. In view of the possibility that the 2 per cent tryptose agar which we were now using might be too rich a medium, experiments were done in which various con-

TABLE 1

Effect of variations in the numbers of diphtheria bacilli on the inhibitory action of saliva.

DILUTIONS OF BACILLARY SUSPENSIONS IN PLATES		SALIVA DILUTIONS											
		Undiluted			1:10			1:100			1:1,000		
1:1,000	Widths in mm of zones of	0	0	0	3	3	2	5	2	2	2	1	1
1:10,000	inhibition around drops	0	0	0	12	12	15	12	10	10	10	10	10
1:100,000	of saliva dilutions	0	0	0	20	15		20	15		15	8	

centrations of tryptose were used, the other ingredients remaining the same. No inhibition could be detected on the plates containing the usual amount (2 per cent) of tryptose. The largest zones and inhibition in the greatest saliva dilutions were found in the plates containing 0.2 per cent tryptose. In lower concentrations the growth of the diphtheria bacilli was too irregular. Numerous experiments of this type gave similar results (table 7), with the exception that the optimal concentrations of tryptose varied somewhat with each lot of this material. It was more often 0.3 or 0.4 per cent than 0.2 per cent as in this particular experiment. Variations in the concentration of beef extract in the medium had a very much smaller effect on the inhibition than had the variations in tryptose.

Variation in the numbers of *C. diphtheriae* inoculated into the agar medium produced considerable differences in the inhibitory power of the saliva drops. With fewer organisms wider zones of inhibition were obtained and inhibitory action was demonstrated with more dilute saliva. A typical experiment is shown in table 1.

With a hundredfold dilution of the organisms in the plates the widths of the inhibition zones were increased three and four times. With excessive dilutions of the suspensions of bacilli the demarcation between growth area and inhibition

area became indistinct and accurate measurements impossible. In table 1 it is noted that the undiluted saliva had no inhibitory action, but the same saliva when diluted was active (figure 1). This phenomenon has been noted frequently with many different salivas and, as will be discussed in detail below, is due to the presence in the saliva of certain organisms which antagonize the inhibitory action.

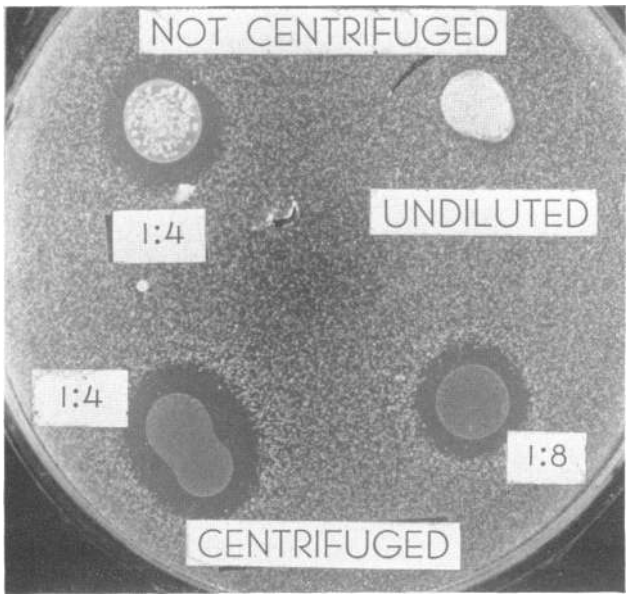

FIG. 1. EFFECT OF DILUTION AND CENTRIFUGATION ON THE INHIBITORY POWER OF SALIVA
Upper left—uncentrifuged saliva diluted 1:4.
Upper right—uncentrifuged undiluted saliva.
Lower right—centrifuged saliva diluted 1:8.
Lower left—centrifuged saliva diluted 1:4.

THE INHIBITION OF C. DIPHTHERIAE BY SALIVARY STREPTOCOCCI

During a long series of unsuccessful attempts to concentrate and purify the active agent in saliva, the possibility of a bacterial factor was ignored. We were not at that time aware of the reports of Weigmann and Holzl (1940) and Holzl (1941), and probably attached too much weight to the negative evidence of the previous workers. It was finally forced on our attention that occasionally very definite zones of inhibition occurred around certain colonies growing within the area of the saliva drops. Pure cultures in broth of these organisms produced marked inhibition when dropped on the plates in the same manner as the saliva dilutions (figure 2).

One hundred and fifteen strains of organisms were isolated in pure cultures at different times from the actively inhibitory salivas of 12 individuals. Eighteen- to twenty-four-hour cultures in 2 per cent tryptose broth were tested for inhibitory action on the diphtheria bacillus. The cultures were also studied on rab-

bits' blood tryptose agar plates, and gram-stained preparations were examined. The results of these studies are summarized in table 2.

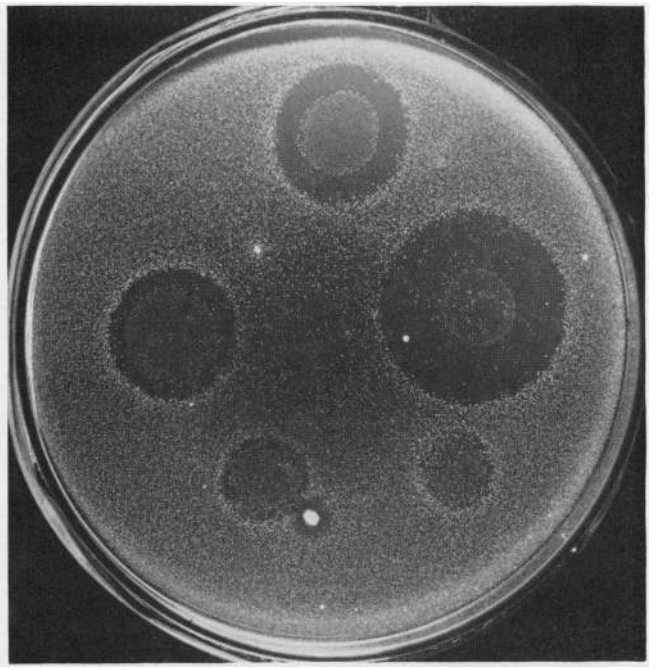

FIG. 2. INHIBITION OF DIPHTHERIA BACILLI BY DILUTIONS OF PURE CULTURE OF MITIS TYPE VIRIDANS STREPTOCOCCUS
The opaque colony showing inhibition is an aerial contaminant

TABLE 2

Inhibition of C. diphtheriae by salivary organisms

TYPE OF ORGANISM	NUMBER OF STRAINS TESTED	NUMBER OF STRAINS PRODUCING DEFINITE INHIBITION
Streptococci showing definite green zones on blood agar	55	48*
Indifferent streptococci	28	3†
Streptococci showing beta hemolysis on blood agar	7	1
Coagulase-negative staphylococci and sarcinae	16	0
Coagulase-positive staphylococci	1	0
Diphtheroids	8	0

* Some negative strains became positive on later transfer.
† One strain showed green zones on later transfer.

These tests were on organisms isolated, more or less at random, from the growth occurring on saliva drops which showed active inhibition, and the distribution or numbers of organisms have of course no necessary relation to the distribution or numbers of organisms in saliva.

Since all the active organisms were streptococci, further studies were made on these streptococci using the methods suggested by Sherman (1937) and Sherman, Niven, and Smiley (1943). Twenty-seven inhibitory strains and twenty-two noninhibitory strains were tested as to the following properties: growth in 6.5 per cent sodium chloride; growth on 30 per cent bile; growth at 45 C; the production of mucoid colonies on 5 per cent sucrose agar; and the amount of acidity produced in glucose broth. The results of these tests are given in table 3. The organisms are grouped according to the types indicated by the results of the tests.

The great majority of actively inhibitory strains were of Sherman's mitis type; only 4 of the 22 inactive strains were of this type. Only one of the sali-

TABLE 3

Properties of inhibitory and noninhibitory streptococci

	INHIBITORY STRAINS	NONINHIBITORY STRAINS
Number of strains studied	27	22
Number of strains with "mitis" properties: No growth in 30% bile No growth in 6.5% salt No mucoid colony on 5% sucrose pH in glucose above 4.4 Definite green zones on blood agar	24	4
Number of strains with "salivarius" properties: No growth in 6.5% salt Mucoid colonies on 5% sucrose pH in glucose 4.4 or below	1 (showed definite green zones on blood agar)	16
Number of strains with "enterococcus"* properties: Growth in 6.5% salt Growth on 30% bile	2 (1 showed definite green zones on blood agar)	2 (1 showed beta hemolysis)

* Many enterococci isolated from feces inhibited the diphtheria bacilli, but we are concerned here with organisms isolated from saliva. Several pneumococcal strains of different types studied have shown marked inhibition.

varius type strains inhibited the diphtheria bacillus, and it was atypical in producing marked green zones on blood agar.

ATTEMPTS TO REMOVE THE STREPTOCOCCI WHILE PRESERVING THE INHIBITORY ACTION OF SALIVA

Filtration. Thompson (1941) showed that the antidiphtheria agent in saliva did not pass filters when the saliva was acidified, although the lysozyme of saliva was filterable under these conditions. To determine whether the inhibitory agent would pass filters without acidification of the saliva, 25- to 50-ml volumes of saliva were diluted with equal parts of 2 per cent tryptose solution, centrifuged briefly to remove coarse particles, and filtered through Seitz, Mandler, and Berke-

feld W and N filters. Various fractions of the filtrates were tested for antibacterial activity by the drop-on-plate method described above. The unfiltered, diluted salivas served as controls. In no case was any inhibition of the diphtheria bacillus produced by the filtered salivas in spite of the high activity of the unfiltered materials.

It was possible that the plate method was not sensitive enough to detect small amounts of the agent which might pass the filters. Since the sterility of the filtrates made a tube method of testing for inhibition possible, several experiments were done in which filtrates were inoculated with various numbers of diphtheria bacilli and the presence or absence of growth was observed. A typical experiment follows: 25 ml of fresh saliva were diluted with equal parts of 2 per cent tryptose broth and centrifuged at 1,800 rpm for ½ hour. Two ml of the supernatant were preserved for control tests, and the remainder was filtered through a Seitz disk at 19 pounds' pressure. The first and second portions of the filtrate were kept separate. The filtrates were distributed in small tubes in 1.5-ml

TABLE 4

No inhibition of diphtheria bacilli by Seitz filtrates of saliva

MEDIUM IN TUBES	RELATIVE GROWTHS OF BACILLI IN VARIOUS TUBES						WIDTH IN MM OF INHIBITION ZONES ON PLATES					
Saline + 2% tryptose	3+	3+	3+	3+	3+	2+						
Saliva filtrate 1st half + 2% tryptose	4+	4+	4+	4+	4+	4+	0	0	0	0	0	0
Saliva filtrate 2nd half + 2% tryptose	4+	4+	4+	4+	4+	4+	0	0	0	0	0	0
Saliva unfiltered							2.5	3	2	5	6.5	5
Dilution of diphtheria bacilli inoculated	10^2	10^3	10^4	10^5	10^6	10^7	10^5			10^6		

amounts. Similar tubes of the broth diluted with equal parts of saline were prepared. The tubes were then inoculated with standard drops (from a 27-gauge needle) of various dilutions of a suspension of an 18-hour culture of the diphtheria bacillus. They were incubated for 18 hours, and the relative amounts of growth were determined by inspection and by microscopic examination of stained preparations. The filtrates and the unfiltered saliva dilutions were also tested by the standard "drop plate" method. The results are shown in table 4.

Similar results were obtained in a duplicate experiment with saliva from a different person and in other experiments using 0.4 per cent tryptose as the diluting medium to eliminate the possibility of the 2 per cent tryptose antagonizing the inhibitory action. Filtered saliva-saline mixtures with no added tryptose also supplied adequate nutrition for the diphtheria bacilli and supplied good growth, indicating clearly that any inhibitory agent had been removed by filtration. Filtration through Mandler filters with tryptose or saline diluents gave similar results. Up to 90-ml volumes of diluted saliva were filtered with no evidence of any inhibitory activity in the filtrate. The saliva filtrate invariably tended to produce better growth than similar broth controls.

Bactericidal effect of copper. The report of Weigmann and Noeske (1937) that they succeeded in killing most of the bacteria of saliva without affecting the antibacterial power has already been referred to. Several unsuccessful attempts were made to repeat this work. Fresh saliva was diluted with equal parts of saline and centrifuged at 1,800 rpm for $\frac{1}{2}$ hour. The supernatant was placed in a tube so as just to cover a coiled 20-gauge copper wire. The coils were such as to get 26 inches of wire into $1\frac{1}{4}$ inches of a tube $\frac{1}{2}$ inch in diameter. A small portion of the centrifuged diluted saliva was kept in the refrigerator to be used to control the original inhibitory activity. The saliva containing the copper was incubated at 37 C, and samples were removed after 24 and 72 hours. They were compared with the refrigerated material as to inhibitory potency by the usual drop plate method. The samples had no antibacterial activity demonstrable by this method although the refrigerated material was very active. Cultures of the treated saliva showed no growth of any organisms.

TABLE 5

Effect of centrifugation on inhibitory activity of saliva supernatants

SPEED AND TIME OF CENTRIFUGATION OF SALIVA	WIDTHS OF ZONES OF INHIBITION IN MILLIMETERS	
Uncentrifuged control	5	5
700 rpm 5 minutes	6	6
700 rpm 15 minutes (in addition to above)	8	8
1,400 rpm 30 minutes (in addition to above)	4	4
1,400 rpm 45 minutes (in addition to above)	2	2
1,700 rpm 90 minutes (in addition to above)	0	0
1,700 rpm 120 minutes (in addition to above)	0	0

Centrifugation experiments. Many experiments using centrifugation for different time periods at various speeds were done with salivas from several different individuals. With the exception of certain apparently anomalous reactions, described and explained below, the results of all the centrifugation experiments were entirely in accord with the concept that the inhibitory action of saliva is due to certain of the bacteria contained in it. With the exception noted, the more rapid and more prolonged the centrifugation the more inhibitory activity was lost from the supernatant fluids. In several instances 2,000 rpm for $1\frac{1}{2}$ hours completely removed all activity from the supernatants, but the sediments were very active in considerable dilutions. Since the sediments were still active, the temperature reached in the centrifuge could not have been responsible for the loss.

In table 5 are shown the results of an experiment in which only the supernatants were tested. Ten ml of saliva were diluted with equal parts of saline and centrifuged at the speeds and for the periods indicated. Samples of supernatant were removed at the various times and tested by the drop plate method without further dilution.

An increased inhibitory action of the saliva after brief centrifugation was evi-

dent. The phenomenon was demonstrable in practically all cases when saliva, undiluted or moderately diluted, was centrifuged at relatively slow speeds for short periods (800 to 1,000 rpm for 5 to 20 minutes). (See figure 1.) The differences, in many experiments, were more marked than in the one charted. In one experiment, for example, the centrifuged saliva produced zones of inhibition 8, 9, and 10 mm in width, whereas the corresponding dilution of the uncentrifuged material showed 2-, 3-, and 2-mm zones. This phenomenon of increased action of the supernatant after brief centrifugation is comparable to the increase with moderate dilution noted previously, and, as will be shown later, is likewise due to the elimination of certain other bacteria which antagonize the inhibitory power of the streptococci. The sediments from the centrifuged salivas showed increased inhibitory activity on moderate dilution in the same manner as the untreated salivas, but the supernatants which retained any activity lost it progressively on dilution.

Effect of heat. It was previously shown (Thompson, 1941) that heating saliva to 100 C at an acid pH destroyed the inhibitory action against the diphtheria bacillus but did not affect the lysozyme. To determine whether the inhibitory action of saliva could be preserved while the streptococci were destroyed, or vice versa, a number of experiments were done to determine the effect of 56 C for various periods. Dilutions of saliva and pure cultures of active streptococci were heated in a water bath at 56 C; samples were removed at various periods, serially diluted, and tested by the standard technique on plates containing diptheria bacilli. In both cases all inhibitory action was destroyed in from 5 to 7 minutes. In both cases also the active streptococci were destroyed in approximately the same periods, slight differences being explained by the survival of a small number of organisms insufficient to produce visible inhibition.

ANTAGONISM OF INHIBITORY ACTION OF SALIVA BY OTHER MOUTH BACTERIA

The increase of inhibitory action on moderate dilution or centrifugation of saliva was always associated with a diminution in the number of raised, opaque bacterial colonies growing from the drops on the test plates (figure 1). The active streptococci formed small, grayish, flat, translucent colonies on the medium used. This suggested that the organisms forming the opaque colonies in some way antagonized the inhibitory action of the green streptococci. Being greatly in the minority as compared to the streptococci, the antagonistic organisms were reduced by dilution or centrifugation below a critical concentration before the inhibitory streptococci were so reduced. When isolated in pure culture the opaque colonies were shown to be staphylococci, in most cases white coagulase-negative strains but occasionally orange and coagulase-positive ones. It was shown that pure cultures of these staphylococci did antagonize the inhibitory action of saliva. Tests of saliva dilutions on the drop plates were prepared in the usual way, and the pure cultures of staphylococci were inoculated directly onto the drops on the plates and the plates dried and incubated. The results of one such experiment are shown in table 6.

Additional similar experiments gave similar results. Many strains of staphylococci and sarcinae produced such antagonism of the inhibitory activity of saliva, but diphtheroids[1] and salivarius type streptococci did not antagonize the inhibition. Both pathogenic and nonpathogenic staphylococci were effective antagonists. Pure cultures of inhibitory streptococci were antagonized in the same manner by the same organisms as was saliva. The mechanism of the

TABLE 6

Antagonism by pure cultures of staphylococci of salivary inhibition of diphtheria bacilli

TYPE OF STAPHYLOCOCCUS CULTURE INOCULATED ONTO DROPS	DILUTIONS OF SALIVA USED IN TEST DROP	WIDTH OF ZONES OF INHIBITION
None	1:8	15 mm
None	1:16	13 mm
None	1:32	12 mm
White, coagulase-negative	1:8	4 mm
White, coagulase-negative	1:16	0
White, coagulase-negative	1:32	0
Orange, coagulase-positive	1:8	0
Orange, coagulase-positive	1:16	0
Orange, coagulase-positive	1:32	0

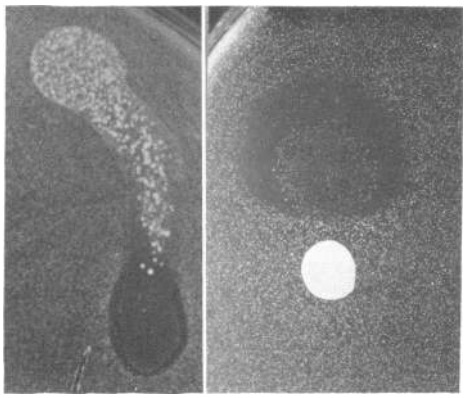

FIG. 3. NO. 1. (LEFT) EFFECT OF ALLOWING DROP OF UNDILUTED SALIVA TO RUN DOWN TEST PLATE
NO. 2. (RIGHT) ANTAGONISM OF INHIBITORY EFFECT OF PURE CULTURE OF STREPTOCOCCUS BY STAPHYLOCOCCUS (OPAQUE WHITE GROWTH)

antagonism has not been adequately studied but the available evidence indicates that it is not the result of an inhibition of the growth of the active streptococci. When the antagonizing organisms were inoculated close to a drop of the inhibitory streptococci, the zone of inhibition of the diphtheria bacilli was abolished in the area but the growth of the streptococci was not apparently

[1] Several diphtheroid strains isolated from saliva were themselves susceptible to inhibition by the streptococci.

affected (figure 3, no. 2). No evidence of inhibition of the active streptococci by the antagonistic organisms was obtained using the drop plate technique with the streptococci in the plates and the antagonizing organisms in the drops. It would appear that the antagonizing organisms either prevented the formation of the inhibitory agent by the streptococci, or neutralized it after it was formed. The relationship between the inhibiting and antagonizing organisms was quantitative: if too many inhibitory streptococci were present no antagonism could be demonstrated. In some instances when the concentration of tryptose in the medium was just below that necessary for good growth of the diphtheria bacilli, it was noted that these organisms grew well around colonies of staphylococci which were antagonistic to inhibition. It is possible but not proved that the staphylococci antagonized the inhibition by supplying some growth factor the absence of which made inhibition by the streptococci possible.

Other phenomena apparently very peculiar when first observed were explained by the antagonistic action of the salivary staphylococci. In many instances when drop plate tests of saliva were done and the plates were not kept level, the saliva drops ran across the plate for some distance. Frequently when this occurred, the zones of growth inhibition were very much wider at the end of the run than around the original area of the drop. The opaque staphylococcal colonies in the drop were almost always concentrated on the original area and obviously antagonized the inhibition in that area, whereas the active streptococci were present in the "run down" position in more or less pure culture (figure 3, no. 1). Another observation was made during some early attempts to determine whether the supposed chemical inhibitory agent was of a dual nature. When fresh, active salivas were added to saliva heated to 56 C (5 to 15 minutes), the fresh salivas frequently were inactivated by the heated saliva. The active streptococci in the heated saliva had been killed, but the more resistant staphylococci were still present and antagonized the inhibitory action of the streptococci in the fresh saliva. When the heated saliva was centrifuged at 1,000 rpm for 10 minutes, the supernatant lost both its power to antagonize and its staphylococci, which were then in the sediment.

INHIBITORY PROPERTY OF SALIVA AND OF STREPTOCOCCAL CULTURES AFFECTED BY THE SAME FACTORS

Indirect evidence that the inhibitory property of saliva was due to the streptococci present in it was given by the fact that a number of different conditions affected fresh saliva and pure cultures of active streptococci in the same fashion.

It was shown above that the tryptose content of the medium used in the tests had a marked influence on the inhibition of diphtheria bacilli by saliva. Variation in the tryptose concentration affected the inhibitory action of pure cultures of streptococci in the same manner. Table 7 shows an experiment in which various dilutions of saliva and of a pure culture of a mitis streptococcus were tested at the same time on plates containing various concentrations of tryptose.

It has been indicated above that the inhibitory powers of cultures of streptococci and of saliva are both antagonized by certain other salivary bacteria.

During some early attempts to determine whether the supposed chemical inhibitory agent in saliva was of a dual nature, it was noted that dilution of the active, fresh saliva in saliva heated to 95 C for 15 minutes, rather than in saline, definitely increased its inhibitory action. Dilution in 2 per cent tryptose had a similar effect. Likewise, the inhibitory action of pure cultures of mitis streptococci was greater when dilutions were made in saliva heated to 95 C or in 2 per cent tryptose solution. The increased inhibitory action with both saliva and streptococcal cultures was associated with a greater growth of streptococci in the nutrient diluents, particularly in the more dilute preparations.

TABLE 7

Parallel effect of variation in tryptose content of medium on inhibitory action of saliva and of streptococcal culture

DILUTION OF AGENT		TRYPTOSE IN MEDIUM			
		0.3%	0.5%	1%	2%
Streptococcus					
1–160	Widths of zones of inhibition in millimeters	Diphtheria bacilli too sparse for accurate measurements	8	6	1
1–320			8	5	0
1–640			11	3	0
1–1,280			9	3	0
1–2,560			9	1	0
1–5,120			5	+	0
Saliva					
1–16			12	6½	0
1–32			10	7	0
1–64			5	1	0
1–128			3	0	0
1–256			2½	0	0
1–512			+	0	0

DISCUSSION

The demonstration that pure cultures of salivary streptococci of the mitis type inhibited the growth of diphtheria bacilli, together with the failure of all attempts to separate the inhibitory action from the streptococci and the indirect evidence presented (both streptococci and inhibitory action of saliva being affected by the same factors), would seem to be fairly adequate proof that the ability of saliva to inhibit diphtheria bacilli, demonstrable by the technique used in these experiments, is entirely due to the inhibitory organisms present in it. The failure of previous workers to find any relationship between the numbers of salivary bacteria and the inhibitory power of the saliva can readily be explained by the number of factors involved, including the numbers and relative power of the active organisms and the numbers and relative power of the antagonizing organisms. The few reports of successful filtration of the agent cannot be explained on the knowledge available. We cannot agree with the reports of Weigmann and Holzl (1940) that extremely large numbers of streptococci are always

necessary to demonstrate the action. Occasionally, zones of inhibition could be demonstrated around single colonies of the streptococci, and the numbers of streptococci required to produce inhibition by pure cultures was not greater than those present in the active saliva dilutions.

SUMMARY

Pure cultures of the *Streptococcus mitis* type of viridans streptococci isolated from saliva inhibited the growth of diphtheria bacilli in the same manner as fresh saliva. The inhibitory action of saliva could not be demonstrated after the streptococci had been removed from it by filtration, centrifugation, heat, or the bactericidal effect of copper. The inhibitory actions of saliva and of pure cultures of mitis streptococci were affected similarly by several factors: the tryptose content of the medium, the antagonistic action of staphylococci, heat, and the use of nutrient materials as diluents.

The inhibitory action of salivary streptococci was best demonstrated when the tryptose content of the medium was between 0.2 per cent and 0.5 per cent, and was abolished in the presence of the normal tryptose content (2 per cent).

Staphylococci antagonized the inhibitory action of salivary streptococci without affecting the growth of these organisms.

REFERENCES

BESTA, B., AND KUHN, H. 1934–35 Untersuchungen über Antagonismus zwischen Diphtheriebacillen und anderen Bakterien. Z. Hyg. Infektionskrankh., **116**, 520–536.

BIBBY, BASIL G., AND BALL, RITA P. Effect of saliva on growth of bacteria. J. Dental Research, **16**, 325.

BIBBY, BASIL G. 1938 Antibacterial agents in human saliva. J. Dental Research, **17**, 308.

BIBBY, BASIL G., HINE, MAYNARD K., AND CLOUGH, O. W. 1938 The antibacterial action of human saliva. J. Am. Dental Assoc., **25**, 1290–1302.

CASASSA, M. T. 1937 Contributo allo studio del potere battericida della saliva umana. Ricerche su salive di saggetti sani ed ammalati di defterite. Giorn. batteriol. immunol. **18**, 381–387. (Abst. *in* Bull. Hyg., 1937, **12**, 524–525.)

CLOUGH, O. W. 1934 Inhibition of bacterial growth by human saliva. J. Dental Research, **14**, 164.

CLOUGH, O. W., BIBBY, B. G., AND BERRY, G. P. 1938 Effect of saliva on *Lactobacillus acidophilus*. J. Dental Research, **17**, 493–498.

DOLD, H. 1935 Beitrage zur Epidemiologie der Diphtherie. Zentr. Bakt. Parasitenk., I, Orig., **135**, 69–75.

DOLD, H., LACHELE, W., AND HSING, DU DSCHENG. 1936 Über die Eigenschaften, Wirkungsbreite und Wirkungsart der antibakteriellen Hemmungstoffe (Inhibine) des menschilichen Speichels. Z. Hyg. Infektionskrankh., **118**, 369–395.

DOLD, H., AND WEIGMANN, FR. 1934 Über die Wirkung des menschlichen Speichels auf Diphtheriebacillen. Z. Hyg. Infektionskrankh., **116**, 158–170.

HINE, MAYNARD K. 1936 Daily observations of bacterial inhibition by saliva. J. Dental Research, **15**, 305–306.

HOLZL, H. 1941 Weitere Untersuchungen ueber die antagonistische Wirkung der Mundstreptokokken auf Diphtheriebacillen. Z. Hyg. Infektionskrankh., **123**, 500–510. (Abst. *in* Bull. Hyg., 1945, **18**, 598.)

KESTEREN, MARY VAN, BIBBY, BASIL G., AND BERRY, GEORGE PACKER. 1942 Studies on the antibacterial factors of human saliva. J. Bact., **43**, 573–583.

SHERMAN, J. M. 1937 The streptococci. Bact. Rev., **1**, 3–97.

SHERMAN, J. M., NIVEN, C. F., JR., AND SMILEY, K. L. 1943 *Streptococcus salivarius* and other non-hemolytic streptococci of the human throat. J. Bact., **45**, 249–263.

TAYLOR, WALTER E., AND BIBBY, BASIL G. 1935 Bactericidal properties of human saliva. J. Dental Research, **14**, 164.

THOMPSON, RICHARD. 1940 Lysozyme and its relation to the antibacterial properties of various tissues and secretions. Arch. Path., **30**, 1096–1134.

THOMPSON, RICHARD. 1941 Certain antibacterial properties of saliva and tears not due to lysozyme. J. Bact., **41**, 77.

WEIGMANN, FR., AND HOLZL, H. 1940 Untersuchungen ueber die antagonistische Wirkung von Streptokokken gegenuber diphtherie und anderen Bakterien. Z. Hyg. Infektionskrankh., **122**, 673–683. (Abst. *in* Bull. Hyg., 1941, **16**, 664.)

WEIGMANN, FR., AND KOEHN, A. 1936 Weitere Untersuchungen über die Wirkung des menschilichen Speichels auf Diphtheriebacillen. Z. Hyg. Infektionskrankh., **118**, 507–515.

WEIGMANN, FR., AND NOESKE, H. 1937 Untersuchungen über den antibakteriell wirksamen Faktor in menschlichen Speichel. Z. Hyg. Infektionskrankh., **119**, 413–424.