Effect of Sonic Treatment on Pure Cultures and Aggregates of Bacteria

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Pure cultures of a variety of bacteria were treated with ultrasonic energy using a sonic probe. Fractions of organisms killed at different sonic energies were calculated, and *Streptococcus mutans* was 600 times more resistant than *Fusobacterium nucleatum*, the most sensitive organism tested. The effects of sonic treatment on aggregates of bacteria were examined, and the results were interpreted as a model of the events that probably occur during the sonic dispersion of dental plaque.

Sonic treatment is the most common method used to disperse dental plaque before isolation and enumeration of the constituent organisms. Increased yields in numbers and varieties of microorganisms have accompanied refinement of this procedure. The use of an anaerobic gas during treatment to protect the sample from oxidation and to maintain a low oxidation-reduction potential has enhanced the isolation of anaerobes (A. D. Manganiello, S. S. Socransky, C. Smith, D. Propas, V. Oram, and I. L. Dogan, Abstr. Annu. Meet. Int. Assoc. Dent. Res. 1971, p. 178). These workers recovered an average of 75% of the potentially viable organisms observed in the direct count of plaque samples after suitable corrections were made for the variation in the plate counts and the number of organisms adhering to the glassware. In their study they used strict anaerobic methods and determined the duration of sonic treatment necessary for the release of the maximum viable count from the plaque samples.

Little attention has been given to the fact that bacteria vary in sensitivity to sonic treatment, with the gram-negative rods being the most sensitive and the gram-positive rods and cocci being the most resistant (3). Recently, similar phenomena have been observed when dispersing plaque by using sonic treatment (E. R. Leadbetter and S. C. Holt, Abstr. Annu. Meet. Int. Assoc. Dent. Res. 1974, p. 106). In the present study it will be shown that a variety of bacteria found in dental plaque differs very widely in sensitivity to sonic breakage and that sonic treatment, although useful in dispersing plaque, may also be a highly effective enrichment method for gram-positive rods and cocci.

MATERIALS AND METHODS

Organisms. The organisms used in this study and their sources were as follows: *Escherichia coli* (K-12) from Jamie Bulkacz; *E. coli* (B. Lilly), William Hook, National Institute for Dental Research; *Streptococcus mutans* 10449, 6715, and SL1, laboratory strains; *Actinomyces viscosus* M-100 and *Lactobacillus casei* (H32-I), George Hageage, National Institute for Dental Research; *Veillonella alcalescens*, Alfred Beaman, National Institute for Dental Research; *Bacteroides fragilis* and *Fusobacterium nucleatum* strains, human isolates supplied by Vee Brenner, National Institutes of Health; and *Neisseria perflava*, ATTC 10555. Cultures of the *F. nucleatum* were maintained under anaerobic conditions by passing a gas mixture of nitrogen, carbon dioxide, and hydrogen (80:10:10) through the tubes during transfer and cultured on prereduced chopped-meat liquid medium (Robbin reduced medium, Scott Laboratories, Inc., Fiskville, R. I.).

Media. Blood agar was prepared by adding 5% sterile defibrinated horse blood to Trypticase soy agar. Levine eosin methylene blue, Mitis Salivarius, and Rogosa SL solid media were used for viable counts. Brain heart infusion broth was used to grow batches of organisms before treatment. All prepared media were obtained from Difco Laboratories, Detroit, Mich., or BBL, Cockeysville, Md.

Sonic devices. A Branson sonifier model W185D was the instrument used to obtain most of the data in this study. A second instrument, described in detail below, was also used. The energy dissipated by the sonifier probe was quantitated by calorimetry. The rise in temperature of 500 g of water was measured after treatment with the probe for discreet time periods at selected outputs. The number of joules was calculated from this, and the energy transmitted to the mass of water was expressed in units of watt-seconds. Temperature measurements were made to the nearest 0.001 C with the aid of a

Beckman differential thermometer. The mass of water was weighed in a glass beaker surrounded by a Styrofoam jacket, and the water was stirred slowly by the use of a magnetic stirrer with a Magna-Stir bar inside the beaker. The temperature change measured due to the combined effect of the mechanical equivalent of heat of the stirring and loss of heat due to surface evaporation was -0.0008 C/min and was thus omitted from subsequent calculations. The arbitrary settings of the sonifier, 3, 4, 5, 6, and 7, correspond to an output of 125, 276, 533, 815, and 1,035 W-s/min, respectively, when using the microprobe. The power output of the probe proved to be an exponential function because a graphic plot of the logarithm of the heat produced against the power setting yielded a straight line over most of the range of the instrument settings. The power transferred to the bulk solution deviated from the exponential relation only at the highest permissible instrument setting for the microprobe. When the macroprobe was used with a Branson sonifier at an instrument setting of 8, the energy dissipated was 4,015 W-s/min. Similar thermal measurements with an MSE 100-W sonic device (M.S.E. Instruments, Cleveland, Ohio) run at a probe displacement of 6 μ m showed the energy output of the instrument to be 418 W-s/min.

Treatment of samples. Organisms were harvested during exponential growth on brain heart infusion broth, and 6-ml aliquots were dispensed into 12-ml conical centrifuge tubes. Air was replaced by passing a gas mixture of nitrogen, carbon dioxide, and hydrogen (80:10:10) over the sample for 1 min before treatment. The flow of anaerobic gas was continued during the sonic treatment, and the tubes were immersed in ice to avoid exposing the sample to excessive heat. When prolonged treatment was necessary to break the more resistant cells, the sample was treated in sequential bursts to ensure that the temperature did not exceed 18 C in the sample tube. After treatment, serial 10-fold dilutions were made in phosphate-buffered (0.05 M, pH 6.8) saline or in prerduced fluid thioglycolate medium. Aliquots (0.1 ml) were dispensed on the appropriate solid medium with an Eppendorf pipette with sterile plastic tips and spread with a bent glass rod. After incubation at 37 C in an anaerobic jar with GasPak (BBL, Cockeysville, Md.) or in an incubator with an atmosphere of 10% carbon dioxide, the plates were removed, and those having between 30 and 300 colonies were counted with the aid of a New Brunswick colony counter. The number of organisms in a given dilution was calculated from the arithmetic mean of the number of colonies counted on at least three replicate plates. Total microscopic counts of organisms were made using a Neubauer bright-line hemacytometer and a Leitz phase-contrast microscope.

Formation of extracellular polysaccharide. Cultures (6 ml, final volume) were invested in a highly tenacious polysaccharide by incubating exponentially growing cells in brain heart infusion medium in the presence of 5% sucrose for 3 h at 37 C along with 1 ml of a crude preparation of *S. mutans* glucosyl transferase containing 0.3 IU. After incubation,

the culture tubes were placed in an ice bath before sonic treatment.

When *E. coli* (K-12) and *S. mutans* (6715) were invested in the polysaccharide together, they were first grown separately overnight in brain heart infusion broth at 37 C. Aliquots of the cultures were added to sterile tubes, and sufficient fresh sterile brain heart infusion broth containing 20% sucrose was added to bring the concentration of the final 6-ml volume to 5% with respect to sucrose. The cultures were incubated at 37 C for an additional 4 h, during which time both organisms grew and became enmeshed in the polysaccharide formed from the sucrose by the extracellular glucosyl transferase. The tubes were removed from the incubator and placed in an ice bath, and each tube was treated for a specific time interval with the sonifier under the conditions described above. The viable units of *E. coli* were enumerated on Levine eosin methylene blue medium, and the viable units of *S. mutans* were enumerated on Mitis Salivarius medium after suitable dilution, plating, and incubation as described above.

Erythrocyte destruction was determined by the release of pigment as the cells were broken. Erythrocytes from defibrinated horse blood were suspended in buffered saline (0.05 M phosphate buffer, pH 6.8, in 0.9% NaCl) and sedimented in an International clinical centrifuge at 3,000 rpm for 10 min. The erythrocytes were washed by repeating this procedure three times. A suspension of the erythrocytes was prepared to ensure that the absorbance of the pigment released on complete lysis by exhaustive sonic treatment of the sample did not exceed an absorbance of 0.800 when read at 550 nm in a Gilford 2400 spectrophotometer using a 1-cm cuvette. The relation of the absorbance at 550 nm and the volume of totally broken washed erythrocytes was linear over this range of absorbance. Triplicate samples of the suspension of washed erythrocytes were treated at several of the arbitrary power output settings for discreet intervals of time up to 60 s. Unbroken cells were sedimented in a clinical centrifuge, and the absorbance of the supernatant liquid was read at 550 nm. The fraction of intact cells remaining at each interval could be estimated from the difference between the absorbance of the sample and the absorbance of an equal amount of erythrocytes that had been completely broken by exhaustive sonic treatment.

RESULTS

The breakage of erythrocytes provides a convenient means to illustrate the properties of the sonic device. Figure 1 shows the results of the disruption of horse erythrocytes and that the breakage of the population of cells is exponential because a plot of the logarithm of the cells remaining yields a straight line when plotted against the duration of treatment. These plots are the same as those used by Marr and Cota-Robles (5) to describe the rate of killing of a population of bacteria. The energy necessary to

FIG. 1. Sonic breakage of washed horse erythrocytes as described in the text. The initial fraction is 100% the log (10) of which = 2. The data shown are from identical suspensions of erythrocytes treated at an energy output of 533 W-s/min (○) and the second at 815 W-s/min (△), using a Branson sonifier with a microtip.

break 50% of the erythrocytes was 40 W-s when calculated from either of the two power settings of the instrument. Because the killing is exponential, the sensitivity of various organisms to the treatment can be expressed by calculating the time required for 50% of the cells to be broken when assayed by viable count. The power output of the probe in watt-seconds can be calculated by the use of the thermal data from the time required to break 50% of the cells.

Table 1 shows a comparison of the sonic energies required to break 50% of a population of some organisms, most of which are commonly found in plaque. The organisms varied in their sensitivity from 28,400 W-s to kill 50% of a population of *S. mutans* to 47 W-s to kill 50% of a population of *F. nucleatum*. Most of the gram-negative organisms were much more sensitive to sonic treatment than the gram-positive organisms. *Veillonella* was the most resistant gram-negative organism tested and required 3,000 W-s of sonic treatment to kill 50% of the population, in contrast to an average of 352 W-s for the two strains of *E. coli* and the *B. fragilis* strain. The thickness of the cell wall of *Veillonella*, as demonstrated by Bladen and Mergenhagen (1), probably accounts for the resistance of this organism to sonic treatment. The *Acti-*

nomyces and *Streptococcus* strains were highly resistant to sonic breakage and easily survived a level of treatment that substantially reduced the numbers of the other organisms tested.

The effect of sonic treatment on a mixture of a moderately sensitive organism, *E. coli* (K-12), and a highly resistant one, *S. mutans* (10449), is shown in Table 2. Starting with a large excess of *E. coli* and treating the mixture at an energy level of about that used to disperse plaque, the numbers of the sensitive organisms were markedly reduced. In contrast, the colony-forming units of the resistant coccus increased because chains were broken up into individual cells and no substantial fraction of the cells was killed. Referring again to Table 1, the *S. mutans* is about 100 times more resistant to sonic treatment than is the K-12 strain of *E. coli*.

A graphic illustration of differential killing by ultrasound of two organisms that differ by only about fourfold in sensitivity is seen in Fig. 2. The data for two independent killing curves

TABLE 1. Energy required to kill half of the cells from pure cultures of a variety of bacteria^a

Culture	W-s for 50% kill
<i>F. nucleatum</i>	47
<i>E. coli</i> (K-12)	204
<i>B. fragilis</i>	374
<i>E. coli</i> (B. Lilly)	478
<i>N. perflava</i>	1,060
<i>V. alcalescens</i>	3,000
<i>L. casei</i>	2,000
<i>A. viscosus</i>	9,636
<i>S. mutans</i>	28,400

^a The data are computed from viable counts of the survivors at various time intervals.

TABLE 2. Sonic treatment of *E. coli* (K-12) and *S. mutans* (10449) with the microprobe of a Branson sonifier at a power output of 1,035 W-s/min^a

Time (s)	Energy (W-s)	Colony-forming units/ml ($\times 10^7$)			<i>S. mutans</i> / <i>E. coli</i> ratio
		<i>E. coli</i>	<i>S. mutans</i>	<i>E. coli</i> / <i>S. mutans</i> ratio	
0	0	7.0	0.15	46.7	0.02
30	516	2.9	2.6	1.13	0.88
90	1,553	0.08	1.86	0.04	22.96

^a Pure cultures of the two organisms were mixed together and treated. The data are calculated from viable counts of the *E. coli* on Levine eosin methylene blue medium and the *S. mutans* on MS medium. The viable counts of each strain are shown, and the ratios, of the two strains are calculated from these viable counts.

FIG. 2. Sonic treatment of pure cultures of *E. coli* (K-12) and *L. casei* at 1,035 W-s/min. Viable counts of two independent experiments are shown in which the killing rate of a pure culture of each organism was determined. The viable counts of the *E. coli* (Δ) were determined on Levine eosin methylene blue media, and the viable counts of the *L. casei* ($\circ$) were determined using Rogosa SL agar.

determined for *E. coli* (B. Lilly) and *L. casei* treated at the same energy level are plotted on the same graph. The counts of the *E. coli* dropped exponentially from the start of the treatment, whereas the number of *L. casei* rose initially as the chains were broken into colony-forming units before reduction in count became apparent.

The events that occur in the sonic treatment of plaque differ from those in the sonic treatment of a pure culture. When plaque is treated with ultrasound, the organisms must be released from a matrix before exposure to the full energy of the sonic field. Figure 3 shows the effect of sonic treatment on a culture of *E. coli* that was invested in a polysaccharide matrix by incubating an exponentially growing culture of *E. coli* (K-12) in the presence of glucosyl transferase and sucrose. The broken line shows the expected behavior of a culture of the organisms suspended without the polysaccharide matrix. A transient rise in the number of viable organisms occurred as the cells were released from the matrix, followed by an exponential killing of the organisms at a rate expected to occur in a culture not containing the polysaccharide.

The study of two organisms bound together in a polysaccharide matrix as a model system provides an explanation of some of the effects

observed when sonic energy is used in the dispersion of dental plaque. Figure 4 shows events that occurred during the sonic treatment of two parallel mixed cultures of *S. mutans* (6715) and *E. coli* (K-12): one mixture was grown in the presence of glucose and one was grown in the presence of sucrose. The effects are readily demonstrated by a plot of the logarithm of the initial fraction versus the time of sonic treatment at this level of energy. The absolute numbers of organisms at the start and finish of the experiment are given in the legend to the figure. In the mixture grown in the presence of glucose, hence minimum polysaccharide production and aggregation, the *E. coli* was killed exponentially during the course of treatment. In contrast, in the sucrose-grown culture, *E. coli* was recovered at a relatively constant level throughout the course of the treatment although the absolute numbers of organisms in the suspension were low. The numbers of *E. coli* recovered then declined substantially towards the end of the treatment. The initial numbers of *S. mutans* recovered from suspensions in the glucose-grown mixture were about 80 times that of the sucrose-grown mixture. The number of colony-forming units of *S. mu-*

FIG. 3. Sonic treatment of *E. coli* (K-12) in a polysaccharide matrix treated at a power output of 1,035 W-s/min. The broken line is the expected rate of killing of a pure culture of the same organism without the polysaccharide and assuming an initial count of 2×10^8 /ml of culture.

FIG. 4. Viable counts from mixtures of *E. coli* (K-12) and *S. mutans* (6715) incubated in the presence of glucose or sucrose and treated with an MSE probe at 418 W-s/min. The starting count is 100%, and the surviving fractions of the starting count are calculated at each time interval. The viable counts in colony-forming units per milliliter for the cultures were as follows: *S. mutans* (glucose, ○), zero = 5.8×10^7 , 30 s = 6.9×10^7 ; *S. mutans* (sucrose, ●), zero = 7.0×10^5 , 30 s = 2.45×10^7 ; *E. coli* (glucose, △), zero = 2.0×10^6 , 30 s = 2.2×10^5 ; *E. coli* (sucrose, ▲), zero = 4.2×10^5 , 30 s = 4.0×10^4 .

tans in the glucose-grown culture increased by almost 20% during sonic treatment. Figure 4 illustrates the large increase in the numbers of colony-forming units of *S. mutans* recovered from the suspension as the organisms were released from the polysaccharide matrix in the sucrose-grown culture.

DISCUSSION

Some of the factors involved in recovering a particular microorganism from a plaque sample are the following: the organism must be separated from the native plaque aggregate, it must survive the dispersing procedure, and it must be cultured on a medium with the adequate nutrients and oxidation-reduction potential to allow it to grow. The ideal method of plaque treatment would be one that would completely disperse the aggregate while providing total recovery of all of the individual components. Physical methods have been popular because they can effectively disperse the aggregate and theoretically should result in mini-

mum damage to the individual microbial components if the proper conditions are used. The most popular physical method used to disperse plaque has been the treatment of the sample with ultrasound. Williams and Eickenberg (8) first used this method to demonstrate an increase in total viable count of bacteria when samples of saliva were treated with sonic energy for varying periods of time. They demonstrated an increase in the number of lactobacilli and streptococci obtained during treatment, which is consistent with our observations that these bacteria are relatively resistant to sonic treatment.

The differences in sensitivity of organisms to lethal damage during sonic treatment may not have been sufficiently appreciated by individuals working in the field of oral microbiology. Although data are available concerning this effect, they are scattered (2-4) and, as far as can be determined, there has been no comprehensive and systematic treatment of the phenomenon as applied to plaque microorganisms. When organisms are released from the plaque aggregate, they will be killed exponentially at a specific rate, which is characteristic of the toughness of the cell wall or envelope of that particular group. Gram-positive organisms are very much more resistant to sonic treatment than the gram-negative microorganisms; therefore, a much larger fraction of the gram-positive organisms will survive and contribute to the final count. Thus, when determining the energy to be used to disperse a plaque sample with a sonic device, the assessment of maximum numbers is illusory. The interpretations of studies of the predominant cultivable flora from a particular site have been discussed by Socransky (6), who concluded that the assumption of maximum numbers was not always valid. These cautions are emphasized as a result of the present study.

Direct-light and electron microscope observations of smears or in situ plaque support the concept that the gram-positive rods and cocci are among the most numerous organisms in supragingival plaque. The cultural estimates of gram-negative organisms in plaque, however, are probably low. An important concern is the recovery of organisms from subgingival sites, particularly pathological subgingival sites such as a periodontal pocket. The organisms sensitive to sonic treatment that are cultured have been exposed to the full intensity of the sonic field for a brief period of time by virtue of being released from the plaque aggregate towards the end of the treatment. Recently, some gram-negative organisms that may be pathogens (A.

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