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THE EFFECTS OF EDTA AND SONICATION ON THE DISAGGREGATION OF ORA--ETC(U)
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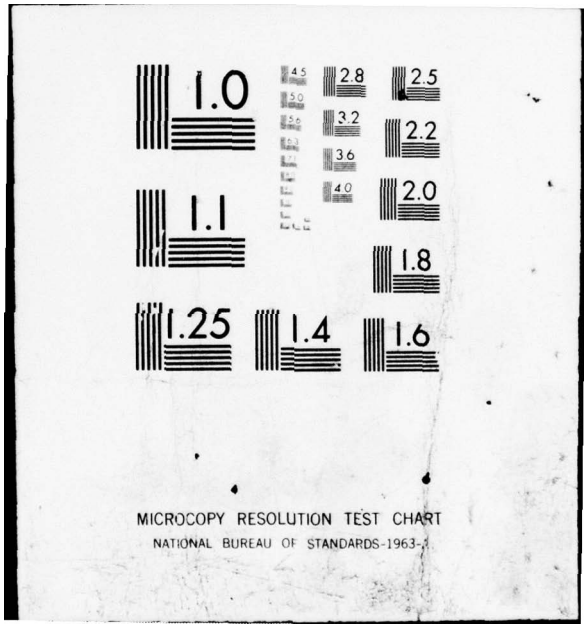
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REPORT DOCUMENTATION PAGE

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1. REPORT NUMBER	2. GOVT ACCESSION NO. / 3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle): The Effects of EDTA and Sonication on the Disaggregation of Oral Bacteria.	5. TYPE OF REPORT & PERIOD COVERED: Manuscript for Publication, 13 Feb 1977 - 15 May 1979
	6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s): Michael B. Dayoub and Charles E. Hawley	8. CONTRACT OR GRANT NUMBER(s)
9. PERFORMING ORGANIZATION NAME AND ADDRESS: U. S. Army Institute of Dental Research, Walter Reed Army Medical Center, Washington, DC 20012	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS: 61102A, 3S161102BS06 04, 012
11. CONTROLLING OFFICE NAME AND ADDRESS: U. S. Army Medical Research & Development Command, HQDA-IS, Fort Detrick, Maryland 21701	12. REPORT DATE: 14 June 1979
	13. NUMBER OF PAGES: 15
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)	15. SECURITY CLASS. (of this report): UNCLASSIFIED
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

LEVEL

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(9) Rept. Sep 13 Feb 77-15 May 79,

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

None

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The dispersive effects of EDTA and ultrasound on bacterial aggregates in an anaerobic environment was studied. Pooled supragingival plaque samples were placed into reduced transport fluid containing 0.0M, .001M, .01M, or 0.1M EDTA and colony forming units were dispersed and cultured at 37C for 72 h. Mean colony forming units were increased 42.7% and 54.4% respectively, in 1 mM and 10 mM concentrations of EDTA and decreased when plaque was dispersed in 100 mM concentrations of EDTA. Fusobacterium nucleatum was grown in broth culture, placed into reduced transport fluid containing no chelating agent, and sonicated for 0, 5, 15, or 30 seconds.

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20. continued
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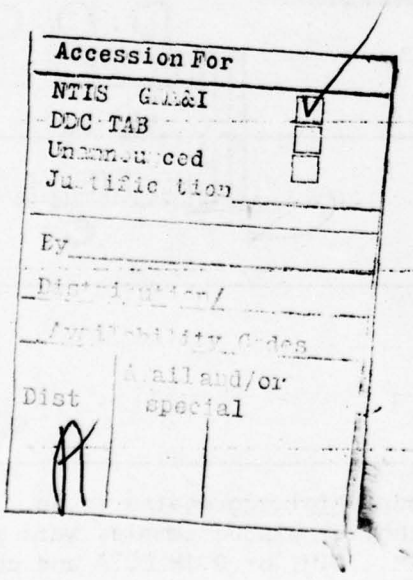

THE EFFECTS OF EDTA AND SONICATION ON THE DISAGGREGATION OF ORAL BACTERIA.

SHORT TITLE: BACTERIAL PLAQUE DISAGGREGATION

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ABSTRACT

The dispersive effects of EDTA and ultrasound on bacterial aggregates in an anaerobic environment was studied. Pooled supragingival plaque samples were placed into reduced transport fluid containing 0.0M, .001M, .01M, or 0.1M EDTA and colony forming units were dispersed and cultured at 37C for 72 hours. Mean colony forming units were increased 42.7% and 54.4% respectively in 1 mM and 10 mM concentrations of EDTA and decreased when plaque was dispersed in 100 mM concentrations of EDTA. *Fusobacterium nucleatum* was grown in broth culture, placed into reduced transport fluid containing no chelating agent, and sonicated for 0, 5, 15, or 30 seconds. Sonication at the lowest power setting produced a statistically significant ($p < .01$) increase in CFU after 5 seconds but did not cause significant changes in CFU up to 15 seconds. The effect of sonication at medium or high power settings was variable, and factors affecting variability are discussed.

The microbiota of dental plaque has been classified and quantitated by a variety of methods (ROSEBURY, MacDONALD & CLARK 1950, LOESCHE, HOCKETT & SYED 1972, LOESCHE & SYED 1973, WILLIAMS, PANTALONE & SHERRIS 1976, WEINER, KAPLAN & NEWMAN 1978). In all instances, one essential step was the disaggregation and dispersion of sample microorganisms. But, because of the tendency of plaque bacteria to adhere to each other and to eucaryotic cells (GIBBONS & VanHOUTE 1973), the identification of some bacterial species and the accurate quantitation of others has been difficult.

In the past, dispersive techniques have involved the mechanical agitation of plaque samples in a suspending medium. Other methods have provided dispersion through the use of a hypodermic syringe (WILLIAMS *et al.* 1976) or a blender (LOESCHE & SYED 1973). Recent studies have shown the value of the divalent cation chelator, disodium ethylenediaminetetraacetate (EDTA) (LOESCHE *et al.* 1972) or sonic oscillation (LOESCHE & SYED 1973, ROBRISH, GROVE, BERNSTEIN, MARUCHA, SOCRANSKY & AMDUR 1976). The purpose of this study was to show the effects of various concentrations of EDTA and modifications of sonic techniques on the dispersion and cultivability of plaque bacteria.

MATERIAL AND METHODS

EDTA As A Bacterial Disaggregant. -

Supragingival dental plaque was obtained from six patients with a spoon-shaped curette. Figure 1 shows the scheme used in processing

samples. Each plaque sample was immediately placed into a tube of chelator-free Reduced Transport Fluid (RTF) (LOESCHE *et al.* 1972). The samples were then immediately placed into an anaerobic chamber (Coy Laboratory Products, Inc.) with a controlled atmosphere of 10% H_2 and 5% CO_2 in nitrogen (ARANKI, SYED, KENNY & FRETER 1969). The reduced environment in the anaerobic chamber was monitored with a platinum electrode (Orion Research). Serial tenfold dilutions of the pooled plaque were then carried out in RTF (< -400 mv) which contained either 0.0M, .001M, .01M, or 0.1M EDTA (Sigma Chemical Co.). Aliquots (0.1 ml) of each dilution were spread in triplicate on pre-reduced (< -140 mv) Schaedler agar plates (Becton, Dickinson & Co.) supplemented with 50 ml/L defibrinated sheep blood (Flow Laboratories) and 0.5 µg/ml menadione, and then cultured anaerobically for 72 hours at 37C. The number of colony forming units (CFU) per aliquot was determined at the end of the incubation period. Six trials were performed.

Sonication As A Bacterial Disaggregant.

The effect of sonication on the disaggregation and viability of microorganisms was examined in a controlled anaerobic environment. The gram negative anaerobic bacterium, *Fusobacterium nucleatum* (ATCC #23726), an organism shown to be sensitive to the effects of sonication (WILLIAMS & ELCKENBERG 1952, ROBRISH *et al.* 1976) was grown in Brewer Thioglycollate Medium (Becton, Dickinson & Co.) for 48 hours at 37C. Aliquots (2.0 ml) were placed into 12 x 75 mm test tubes (Falcon Plastics) for dispersion testing.

A sonic oscillator (Kontes Cell Disruptor) with a 4½ inch titanium magnetostrictive probe was used at low (#1), medium (#4), and High (#10) power settings. The power output for these settings was given by the manufacturer as 292, 541, and 1200 Watts/sq.in., respectively, and the tip frequency for this unit was rated at 23,500 cps with a displacement of 100 micron double amplitude. Two sets of trials were performed. In the first experiment sonication was performed for 0, 5, 15, and 30 seconds in three trials at each of the designated power settings. In a second experiment sonication was performed for 0, 5, and 15 seconds, in five trials, using only the lowest power setting. (After sonication, 1.0 ml aliquots of each test were subjected to tenfold dilutions, spread on duplicate Schaedler supplemented agar plates, incubated at 37C in the anaerobic chamber, and counted 72 hours later for CFU.)

Throughout the study all sampling or dilution procedures were preceded by 30 seconds of vortexing (Vortex-Genie, Scientific Industries) in order to obtain a uniform distribution of CFU.

Statistics

Statistical methods consisted of the analysis of variance of plate counts in square root transform as usually applied to stabilize the variance in Poisson variates. Treatment comparisons were made as sensitive as possible by utilizing the principle of blocking to remove trial-to-trial variation. The principle is analogous to the paired comparisons concept in the t-test, generalized in the analysis of variance.

RESULTS

EDTA

Three different concentrations of EDTA in RTF were used to test the disaggregation of plaque bacteria. The resulting CFU were compared to CFU obtained from RTF which contained no EDTA (Table 1). Mean CFU were averaged for all trials and are shown graphically in long transform (Figure 2). The mean CFU increased 42.7% and 54.4%, respectively, in 1 mM and 10 mM concentrations of EDTA and decreased when plaque was dispersed in 100 mM concentrations of EDTA. Statistical analysis of these differences or an analysis of variance met the .08 level of probability for all groups. When the paired t-test was used, mean CFU for .01 M EDTA and the control group showed differences which were statistically significant at the .02 level of probability.

Sonication

In the first set of trials ($n = 3$), the effect of instrument power setting, and the duration of sonication on the number of cultivable *F. nucleatum*, was determined (Figure 3). Mean CFU from trials were transformed to square roots, in order to stabilize variances, and the transformed values were then tested for significance. Analysis of variance showed no significant differences between mean CFU produced at different sonication times, or between mean CFU obtained from three different instrument power settings at any sonication time. A pattern difference between the low power and the two other power settings was found to be statistically significant at the .05 level of probability. Further trials ($n = 5$) were then performed

at the low power setting. Plate counts were again transformed to square roots and analyzed in a randomized block design by the analysis of variance. A graphic illustration of the means of the CFU is shown in Figure 4. In this experiment, five seconds sonic disaggregation of *F. nucleatum* was effective in producing a statistically significant 66% increase in the number of CFU ($p < .01$). Sonication beyond five seconds and up to 15 seconds did not cause statistically significant changes in CFU.

When the data obtained from the use of the low power setting during the initial trials (Figure 3) was combined with the data of the following trials (Figure 4), the use of the low power setting caused a 73.3% increase in recovery of viable bacteria after five seconds (Figure 5) ($p < .01$). The differences in CFU obtained between 5 and 15 seconds of sonication were statistically indistinguishable.

DISCUSSION

A previous study (LOESCHE *et al.* 1972) showed that a 1 mM solution of EDTA caused a twofold increase in the microscopic count of plaque bacteria. In the present study, CFU counts from supragingival plaque were used to evaluate the disaggregating effects of EDTA. At 1 mM and 10 mM concentrations of EDTA, there appeared to be increased disaggregation of plaque bacteria. One mM solutions of EDTA showed a mean increase of 42.7% in CFU over controls without EDTA, and 10 mM concentrations of EDTA yielded CFU counts 57.4% higher than the same

controls. At 100 mM concentrations, CFU counts were decreased below control levels, which suggested that EDTA had a bactericidal effect, or growth inhibiting effect, on plaque organisms. This growth inhibiting effect may have been due to EDTA disruption of cell wall components and disassociation of cell wall associated enzyme systems (SCHLESINGER 1968, TORRIANI 1968, REYNOLDS and PRUUL 1971). Therefore, the results reported here are probably a measure of both disaggregation at low EDTA concentrations and bactericidal effects at high concentrations of EDTA. EDTA in 10 mM solutions appeared to have a highly significant ($p < .02$) dispersive effect on plaque, and it is not believed that the potentially lethal effects of this agent contraindicate its use at this concentration. Even though divalent cations are not likely to be responsible for all cell to cell attachments (WILLIAMS & GIBBONS 1975), these results suggest the usefulness of 1 mM ($p < .08$) and 10 mM ($p < .02$) EDTA concentrations as plaque disaggregation agents.

The variables involved in sonication procedures are frequently unrecognized or ignored, and should be given greater consideration in current and future studies of microbiology. Cavitation intensity is affected by the volume and viscosity of the sample, the size and rigidity of the container, the area of air interface, and the ultrasonic probe depth within the medium. In addition, the use of ultrasonic probes with high end velocities, or the use of power settings which are excessively high in relation to the volume sonicated, can

result in the production of turbulence and reduced cavitation called cavitation unloading (HUGHES 1971). In this study, every attempt was made to hold these factors constant, except that a consistent probe depth could not always be maintained. It is believed that the turbulence caused by cavitation unloading produced the rapid changes in surface levels and in relative probe depths observed during our high energy sonication tests. The production of turbulence was particularly evident when sonicating at medium and high power settings, and was observed to be dependent upon the level of the probe within the bacterial suspension.

The effects of sonication on CFU depended upon the duration and intensity of sonic energy employed. The tendency of counts to decrease up to five seconds at medium or high power settings may well have been the result of the lethal effects of sonic energy on previously unaggregated bacteria (Figure 3). As the duration of sonication increased to 15 and 30 seconds, the same high power settings generated a rapid degassing of samples. It is thought that this degassing probably reduced the bactericidal sonic effects, thereby increasing CFU after five seconds at both medium and high power settings. However, turbulence was not evident during trials at low power setting. Cavitation unloading did not occur and a 66% increase in CFU was observed after five seconds ($p < .01$). Under the conditions of this experiment, sonication exceeding five seconds at low power settings did not significantly affect changes in bacterial counts.

It is believed that sonication caused molecular bond breakage, physical damage by shock waves, heat denaturation, and reactions of

free radicals (BOUCHER 1972). These effects can be lethal to certain microorganisms. ROBRISH *et al* (1976) has demonstrated the differences in sensitivity of organisms to lethal damage during sonic treatment and the greater susceptibility of gram negative organisms to sonication effects. Some studies have reported that less than 30 seconds sonication may adversely affect cultivable numbers of bacteria (SPIEGEL 1978, LEADBETTER & HOLT 1975). This study shows that with the instrument employed, counts of gram negative organisms may increase with up to five seconds of sonic treatment at low power settings. These results also indicate that the presumed lethal sonic effect on *F. nucleatum* at the low energy setting of the sonicator was not observed after 15 seconds. This suggests that differences reported in the literature regarding the lethal effects of sonic energy may reflect manifestations of any number of the variables listed earlier (volume, viscosity, etc.). However, under the controlled conditions of this investigation, low energy sonication can be employed to disperse gram negative anaerobic bacteria. The use of sonic energy as a disaggregating agent in dental microbiology must be tempered by the flexibility of the ultrasonic instrument as well as the cultivability of the most sonically susceptible organism in the sample.

* * * * * *

Commercial materials and equipment are identified in this report to specify the investigative procedures. Such identification does not imply recommendation or endorsement or that the materials and equipment

are necessarily the best available for the purpose. Furthermore, the opinions expressed herein are those of the authors and are not to be construed as those of the U. S. Army Medical Department.

ACKNOWLEDGEMENTS

The authors wish to express their appreciation to Colonel John M. Brady, DC, USA for his advice and assistance, to Walter Foster, Ph.D. of the Armed Forces Institute of Pathology for his data evaluation and statistical analyses, and to Colonel Arthur Gross, DC, USA for his encouragement and direction of this project.

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TABLE 1. Mean Counts of Colony Forming Units in 0.1 ml Aliquots From Supragingival Plaque Treated With EDTA.

Trial	Control	.001M EDTA	.01M EDTA	0.1M EDTA
1	235	411	316	205
2	198	300	243	111
3	72	50	156	31
4	106	152	253	35
5	133	163	204	251
6	35	35	54	36
Mean	129.8	185.2	204.3	111.5
Standard Error	30.9	59.6	37.0	39.0

LEGENDS

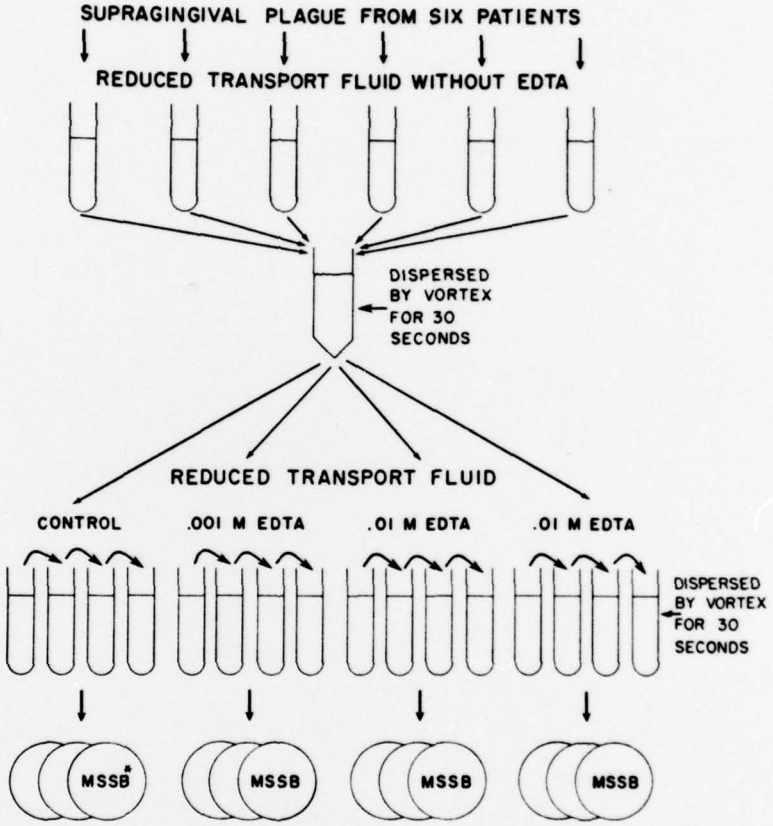
FIGURE 1. METHOD OF PROCESSING PLAQUE SAMPLES IN VARIOUS CHELATOR CONCENTRATIONS TO TEST FOR BACTERIAL DISPERSION.

FIGURE 2. THE EFFECT OF EDTA CONCENTRATION ON BACTERIAL DISAGGREGATION (MEAN ± STANDARD ERROR).

FIGURE 3. THE EFFECT OF TIME AND SONICATOR POWER SETTING ON CULTIVABLE NUMBERS OF *FUSOBACTERIUM NUCLEATUM*.

FIGURE 4. THE EFFECT OF SONICATION AT LOW POWER SETTING ON CULTIVABLE NUMBERS OF *FUSOBACTERIUM NUCLEATUM*. MEAN ± STANDARD ERROR.

FIGURE 5. THE EFFECT OF TIME AT LOW SONICATOR POWER SETTING ON CULTIVABLE NUMBERS OF *FUSOBACTERIUM NUCLEATUM* DURING ALL ($n = 8$) TRIALS. MEAN ± STANDARD ERROR.



*MENADIONE SUPPLEMENTED SHAEDLER BLOOD AGAR PLATES

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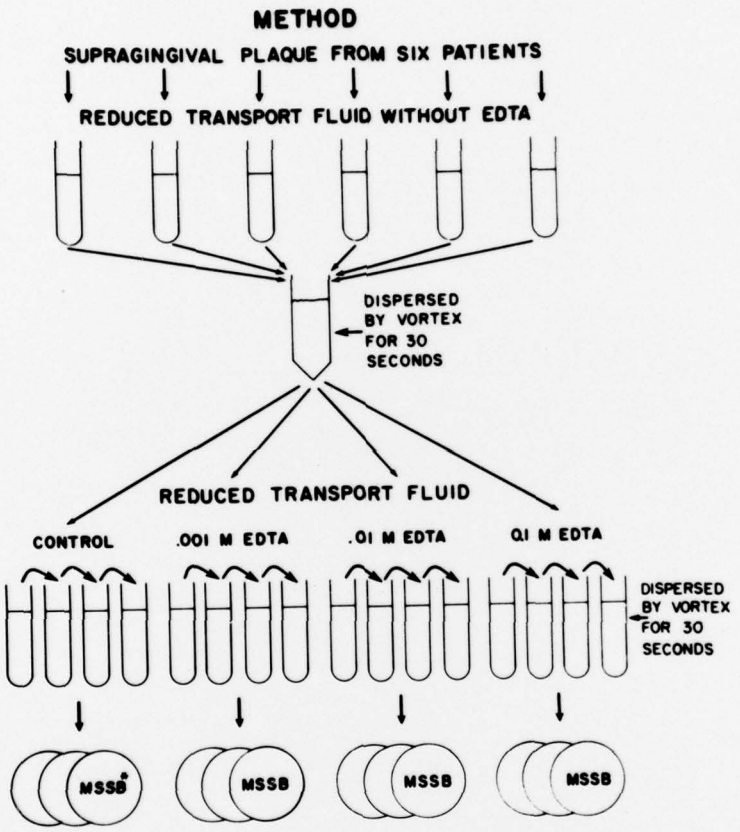

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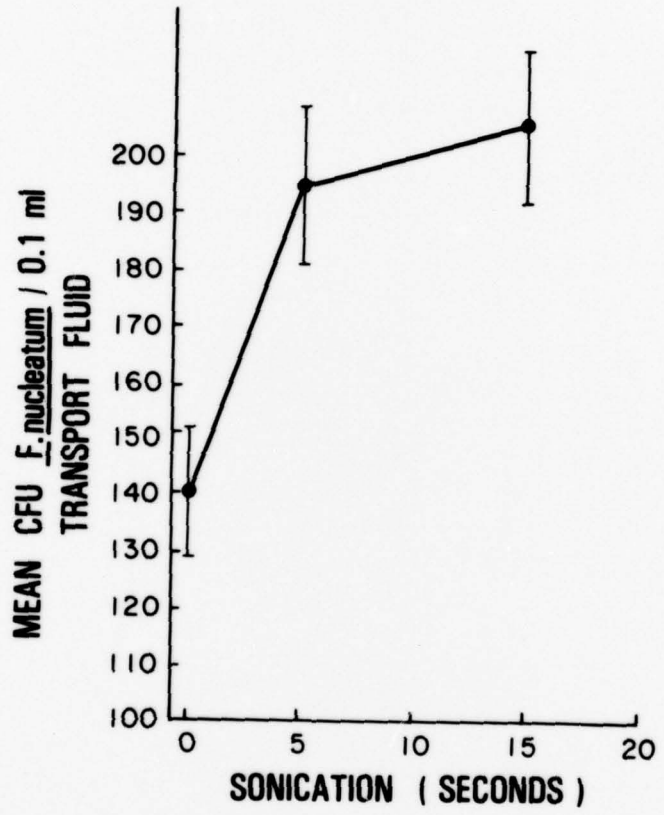
MEAN CFU F. nucleatum / 0.1 ml
TRANSPORT FLUID
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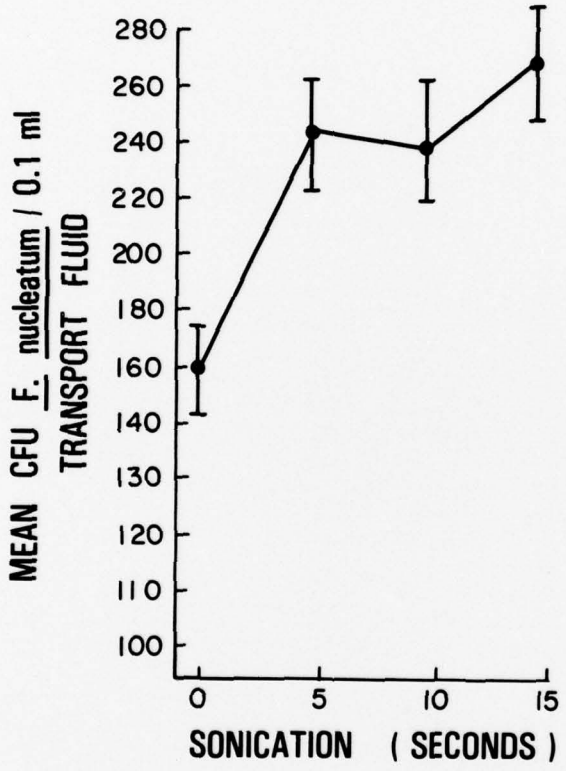
190
180
170
160
150
140
130
120
110
100
0
5
10
15
20
SONICATION (SECONDS)


MEAN CFU F. nucleatum / 0.1 ml
TRANSPORT FLUID
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200
180
160
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130
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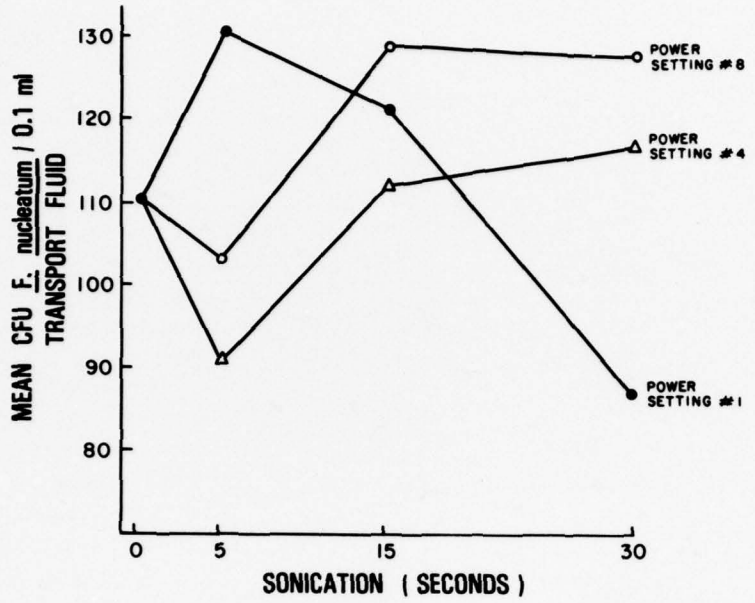
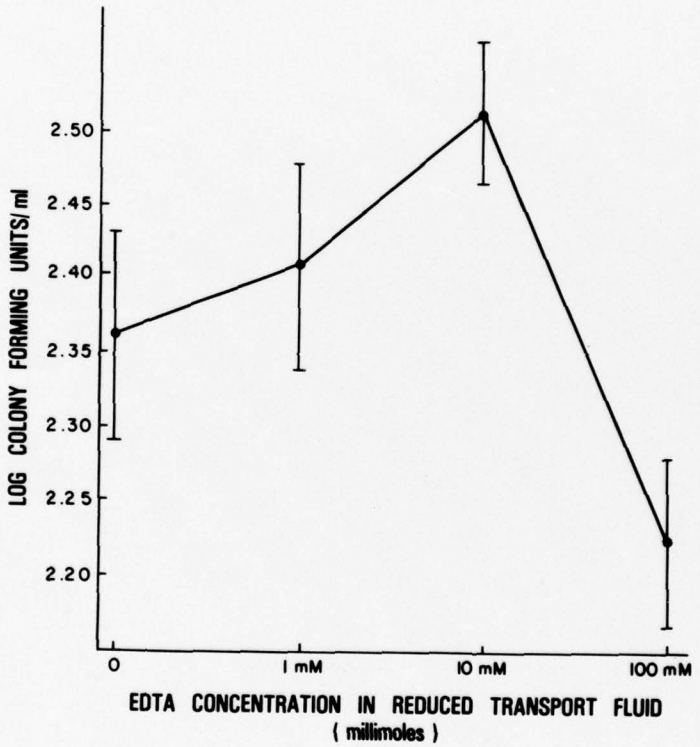
110
100
0
5
10
15
SONICATION (SECONDS)


MEAN CFU F. nucleatum / 0.1 ml
TRANSPORT FLUID
130
120
110
100
90
80
0
5
15
30
SONICATION (SECONDS)
POWER SETTING #8
POWER SETTING #4
POWER SETTING #1


LOG COLONY FORMING UNITS/ml
2.50
2.45
2.40
2.35
2.30
2.25
2.20
0
1 mM
10 mM
100 mM
EDTA CONCENTRATION IN REDUCED TRANSPORT FLUID
(millimoles)

ARTHUR GROSS, Colonel, DC. Received D.M.D. from University of Munich, Munich, Germany; D.D.S. from New York University, M.S. in Microbiology from Georgetown University. Lecturer in Microbiology Georgetown University from 1967-1972. Professorial Lecturer in Oral Biology, Graduate School of Arts and Sciences, George Washington University since 1972. Course Director, Microbiology and Immunology Course, Advanced Theory and Science of Dental Practice (M.S.), U. S. Army Institute of Dental Research, WRAMC, Washington, DC. Serves as lecturer and consultant to numerous Army and civilian groups. Author of 42 articles published in scientific journals and of 31 abstracts published and presented at scientific meetings. Co-inventor of Pulsating Hydrojet Lavage Device and a Tongue Cleaning Device (both patented).