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Quantitative Relationships between Ultrasonic Cavitation and Effects upon Amoebae at 1 MHz

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An amoeba, *Hartmannella castellanii*, which possesses many features typical of higher-order animal cells, was irradiated with 1-MHz ultrasound while suspended in ordinary growth medium and in one with increased viscosity. The ultrasonically produced cavitation was monitored and a strong correlation is found between the number of discrete cavitation events occurring and the decrease in cell numbers, on irradiating at 515 W/cm^2 for 10 min. The growth of treated cells was also examined.

INTRODUCTION

Various interactions between megahertz ultrasound and mammalian tissues have been reported which have been claimed to have arisen from causes other than heating due to acoustic absorption or to cavitation.[1–4] This paper reports on the investigation of growth and structural alterations, sought in ultrasonically irradiated amoebae, as an indication of fundamental interactions between the acoustic field parameters and cells in suspension. Microorganisms possess the following advantages as experimental material for such studies: (1) The short generation, or doubling time, ranging from 30 min for some bacteria to about 20 h for cultured mammalian cells, enables post-irradiation growth to be examined in a much shorter time than for multicellular animals. (2) A continuous supply of material may be produced under rigorously controlled conditions at low cost. (3) Since all cells treated are of the same type, it is possible to apply specific tests of cellular activity to the entire population.

Numerous reports have appeared regarding the effect of ultrasound on microorganisms and viruses, mostly concerned with release of intracellular components by acoustically induced cavitation.[5]

Electron micrographs of *Acanthamoeba* (*Hartmannella castellanii*) exhibit intracellular structures such as the nucleus and nucleolus, Golgi apparatus, "smooth" and "rough" endoplasmic reticula, endocytotic vacuoles, numerous mitochondria, and water expulsion vesicles.[6] Its outer boundary is a double layer of lipoprotein with some suggestion of areas of mucopolysaccharide on the outer surface of the membrane. Thus, in its ultrastructure and in its diameter of 20–30 μ, this amoeba is typical of many animal cells and was considered an appropriate choice for this study. The most obvious features of the cell distinguishable with the light microscope are the nucleus, vacuoles, and pseudopodia.

I. MATERIALS AND METHODS

The cells were grown in 50-ml volumes of 4% mycological peptone (Oxoid) in 250-ml flasks in a reciprocal shaking water bath at 30°C and irradiated ultrasonically in midlog phase after 30-h growth. For growth studies on treated samples, benzyl penicillin (Crystapen Glaxo Ltd.) was added to the cooled autoclaved medium to a final concentration of 400 units/ml. The ratio of flask volume to culture volume (5:1) and the ratio of innoculum to culture volume (1:10) were kept constant in the growth experiments.

The cells were irradiated in two types of containers, viz., a thin-walled aluminum cylinder of 13 mm i.d., 5 mm long and having a volume of 0.65 ml, and a lucite cylinder of 18 mm i.d., 12 mm long, with a volume of 3.3 ml. Rubber O rings were used to attach 0.0005-in.-

thick "Saran" (Dow Corning) foil windows to the cylinders. The cells were loaded into the container with a Pasteur pipet. A drop was removed and the cells were counted in a Fuchs Rosenthal haemacytometer, to serve as a control. The container was then sealed without trapping air bubbles, and placed carefully in the focal region of the acoustic field. The cells were counted again after the irradiation procedure.

Cells examined in the haemacytometer after irradiation showed three stages of damage: (1) At high intensities all cells were completely destroyed, leaving only tiny visible fragments. (2) At intermediate intensities, intact whole cells, cells with apparently intact cytoplasmic membranes with much of their interior organization destroyed, "ghost" cells apparently emptied of their organelles, large fragments of outer membrane, and many free organelles were visible. (3) At lower intensities most of the cells were apparently intact, though a few "ghosts" and cells with their internal organization affected were visible. Only those cells judged to be intact without visible damage were counted when estimating cell survival.

When growth of irradiated cells was planned, the container and its windows were sterilized in ethanol before loading and control cells were placed in a second container for the duration of the irradiation procedure. After irradiation, the number of cells in the treated and control samples were counted, and a 2-ml sample from each container was loaded into 18 ml of sterile medium in 100-ml flasks with added penicillin. Care was taken, by thorough mixing, to minimize counting errors due to cell sedimentation at all stages. Nevertheless, when the cell counting technique was checked by comparing pairs of counts from four containers loaded as above, the variance was 1036 when the average count was 408. This indicated that sources of variance greater than those expected from the Poisson distribution of cell counts were present and also suggested that little was to be gained by counting more cells to reduce the "Poisson distribution" contribution to the variance. Routinely, 500 to 1000 cells were counted.

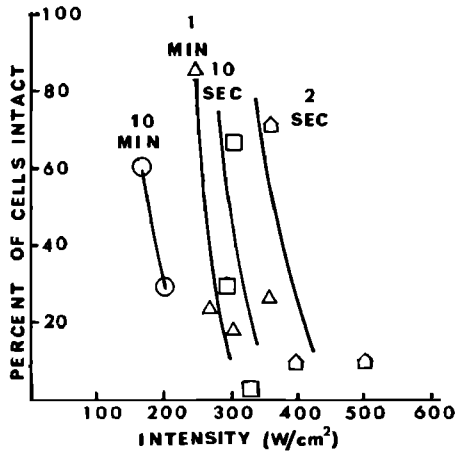

FIG. 1. Percentage of cells intact after irradiation in the aluminum cylinder at different intensities and exposure times.

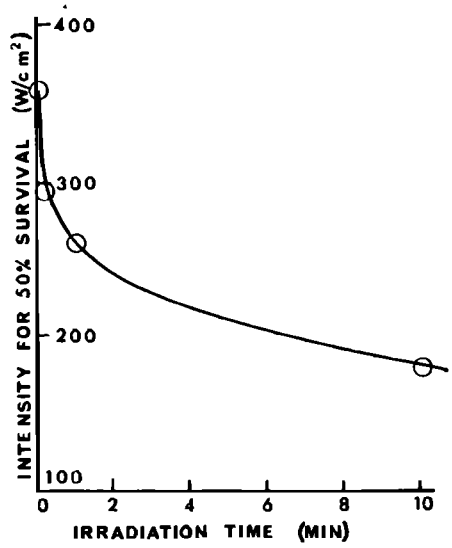

FIG. 2. Acoustic intensity for which 50% of the cells remained intact after treatment for various times (derived from Fig. 1).

Cells were also treated utilizing a viscosity increasing agent, viz., Methocel HG 400 (Dow Corning). Amoebae from a culture in midlog phase were sedimented in a sterilized centrifuge tube and resuspended in a Methocel-mycological peptone–penicillin mixture. Irradiation and subsequent culturing were as described. Because heat sterilization of the Methocel was not possible, only 50% of samples taken through the above procedure grew without subsequent contamination.

The 1-MHz 48-mm radius of curvature 48-mm chord diameter PZT4 ceramic transducer, the irradiation tank, the preparation of degassed water, sound-intensity measurement procedure, transient cavitation, and first-order subharmonic detecting systems are described elsewhere.[7] All are established procedures except for the cavitation detection scheme. Briefly, when the rf voltage applied to the transmitting transducer is rectified and filtered, transient signals may be detected when isolated transient cavitation events occur at the focal region of the field.

II. RESULTS

A. Intensity and Time Dependence of Cell Breakage

Intact cells from the one stock culture were counted, after irradiation at different intensities and exposure times in the aluminum container, and expressed as a percentage of the control cells in Fig. 1, from which Fig. 2 was derived. The latter figure shows that as the intensity decreased, the effect of the destructive mechanisms became much less severe. It was observed that more noticeable cell clumping and sedimentation occurred in samples irradiated for some minutes than

TABLE I. Correlation of cell damage with transient cavitation events at 515 W/cm² for 10 min.

Batch	Medium[a]	Percent of cells surviving (microscope)	Percent of cells surviving (growth)	Number of transient events greater than 0.5 V	Number of transient events between 0.1 and 0.5 V
1	m. pep.	69		90	
1	m. pep. & Meth.	86		8	21
1	m. pep. & Meth.	100		3	22
2	m. pep.	26		162	
2	m. pep. & Meth.	87		0	14
2	m. pep. & Meth.	112		0	29
3	m. pep.	91	60	20	
3	m. pep. & Meth.	103	91	0	31
3	m. pep. & Meth.	115	90	0	15
4	m. pep.	9		212	
5	m. pep.	83	50	21	
5	m. pep. (degassed)	62	21	99	
5	m. pep. (degassed)	67	30	63	
6	m. pep.	99	88	4	
6	m. pep.	75		27	
6	m. pep. (degassed)	71		33	
7	m. pep.	1		greater than 360	
8	m. pep.	34	28	152	
8	m. pep.	83	62	25	
8	m. pep.	88	64	6	
8	m. pep.	58	41	86	

[a] m. pep.—4% mycological peptone, $\eta = 0.9$ cp; m. pep. & Meth.—0.4% Methocel in 4% mycological peptone, $\eta = 6.0$ cp.

in controls. On occasions, the clumps formed a ring on the front container window approximately in the position of the first off-axis zero of the sound beam. While not many of the cells were involved in this clumping, for long time treatments, the irradiation was interrupted after 2 min and the container was shaken to resuspend the cells.

The intensity required to destroy more than 97% of the cells in a 2-sec pulse in the aluminum container varied from 390 to 740 W/cm² in samples treated on ten different days, but was repeatable in cells from the same stock irradiated during any one day. Exhaustive checks on the sound-intensity calibrations, and monitoring of the voltage produced at the terminals of a small hydrophone placed at the end of the irradiation tank farthest from the transducer, showed that the wide range of intensities required for cell destruction was not due to any misbehavior of the sound-generating systems.

A number of bubbles of approximately 0.5-mm diameter were observed in some of the irradiated samples but, while they indicated that the medium had cavitated, they were not a reliable guide to the degree of cell damage inflicted.

When cells in growth medium were centrifuged and resuspended in degassed growth medium, the intensity required to damage 50% of the cells in 2 sec increased from 430 to 660 W/cm². The cells in the degassed medium became spherical, probably changing the mechanical properties of the cell boundary, so it could not be concluded that more cells survived because of any reduction in cavitation resulting from degassing.

Pulsing the sound increased the intensity required to break 50% of the cells from 390 W/cm² in a single 2-sec pulse to 790 W/cm² when the sound was delivered in 90-μsec pulses, mark:space 1:9 (duty cycle) for 20 sec. While pulsing the sound increases the cavitation threshold,[8] the above effect might also have been due to a time-dependent response of the cell to an ultrasonic stress.

Attempts to use the chemical starch–iodine test to detect cavitation in the irradiated medium[9] were unsuccessful because the chemical complex formed between starch and released iodine was interfered with by reaction with some growth medium components, as demonstrated by adding growth medium to an iodine–starch solution, which immediately loses its blue color.

B. Correlation of Cell Damage with Cavitation

The results which follow were obtained by irradiating samples in the lucite cylinder of volume 3.3 ml. An extensive study was carried out at an irradiation intensity of 515 W/cm², since a reasonable number of cells remained intact for subsequent growth studies, and yet this intensity was as high as practicable to identify any direct action between the sound field and the cells.

The cells were irradiated for a total exposure time of 10 min, in 2-min pulses, separated by 1 min to resuspend the cells by shaking the container. The electroacoustic system for detecting and counting single cavitation events was employed for all of the results reported from this point on.[7] However, the electronic counter for the single events had not been incorporated when the following initial experiments were performed. Those transient cavitation events which produced an amplitude greater than 0.5 V were counted by eye from the screen of the oscilloscope which monitored the filter section of the cavitation detecting circuit. The threshold of 0.5 V was chosen, as it was the only way of counting accurately by eye from the oscilloscope screen when profuse cavitation was present. The number of events

TABLE II. Total number of transient events greater than 0.5 V counted in the second half of each of the five 2-min pulses, in 14 samples.

Pulse number	Number of transient events greater than 0.5 V
1	399
2	156
3	125
4	92
5	100

occurring in the second half-minute of each of the five 2-min pulses was noted and totalled (Table I). Degassing of the solutions was affected in this instance by transferring 25 ml of cell suspension containing penicillin into a sterile 100-ml round-bottomed flask and degassing at a pressure of approximately 3 cm Hg. Controls were drawn from the same degassed stock solution.

The comparison of the results for cells irradiated in Methocel and in mycological peptone on the same day (Table I) showed that the protective effect of the former was one of reduction in the generation of transient events. However, Methocel is used in laboratory tissue culture apparatus to protect mammalian cells from shear stresses produced by mixing propellors.[10] Nevertheless, the stresses on erythrocytes suspended in a Methocel solution and sheared in a cone and plate viscometer have been shown to be greater than the stresses produced in isotonic saline.[11] It was possible to count the cavitation transients with amplitudes between 0.1 and 0.5 V in the Methocel samples. It can be seen also from Table I, that degassing, as described above, was not a very efficient way of reducing cavitation since the number of cavitation events increased on the two occasions on which degassing was attempted. The variation of number of transient events from day to day probably had its origins in the amount of dissolved gas and the distribution of nuclei in the suspensions.

The number of transient cavitation events produced decreased as the irradiation continued (Table II). This effect was not observed when irradiating tap water in the open tank,[7] where fresh gas and nuclei were constantly streaming through the focal region, probably because of the limited number of nuclei available in the 3.3-ml volume of the sample container.[12]

Figure 3 shows the result of plotting column 3 of Table I against column 5, i.e., a very strong relationship between the amount of damage and the number of cavitation events. Since the curve may reasonably be drawn through the 100% survival point when no cavitation was detected, it leads to the conclusion that there is no detectable damage to cells, observed under 100× magnification, in the absence of transient cavitation events greater than 0.5 V on irradiation at 515 W/cm² for 10 min at 1 MHz.

TABLE III. Number of transient events and cell survival at different acoustic intensities.

I (W/cm²)	No. of transient events in 10 min	Maximum amplitude	Mean subharmonic amplitude	Percent of cells surviving
580	21 720	4.0	6.8	3.5
450	11 500		7.4	33
340	8196	2.0	6.0	60
268	5620	0.4	4.3	89

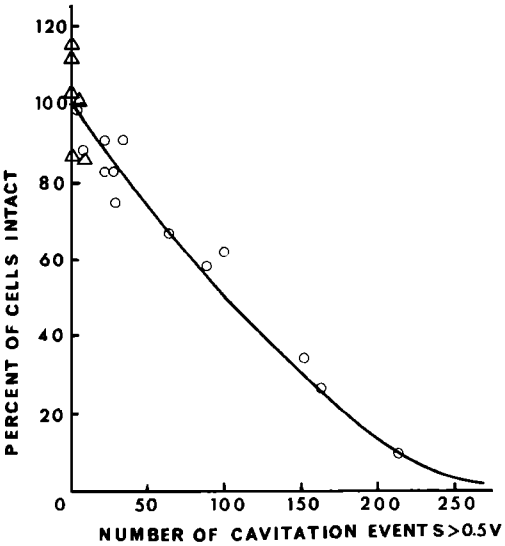

FIG. 3. Percentage of cells intact after irradiation at 515 W/cm² for 10 min versus number of events greater than 0.5 V, counted by eye in the second half-minute of each 2-min acoustic pulse. ○—Mycological peptone; △—mycological peptone and Methocel.

A series of experiments was performed at different sound intensities with five 2-min sound pulses for which cavitation activity was monitored with the complete detecting system. The subharmonic activity from the sample was monitored on the bowl hydrophone. Cavitation transients were counted over the full 10-min period, with the counter threshold set at 45 mV, and the total count was corrected for the counter dead time. The amplitude of the largest events recorded was noted from the oscilloscope monitoring the filter system. The relationship between cavitation event amplitude and duration was the same as that for irradiation in tap water.[7]

The mean subharmonic amplitude was averaged from the readings of the subharmonic at the end of each minute of the 10-min exposure period. Table III shows that, as the sound intensity decreased by a factor of 2, the number of cells surviving irradiation increased by a factor of 25. The number of transient cavitation events decreased by a factor of 4, but there was a much more marked decrease in the maximum amplitude of the transients recorded, by a factor of more than 10. The distribution of amplitude of the transient events at any one intensity was continuous, but was not measured. Subsequent measurement of the amplitude distribution of transients in tap water showed it to be very positively skew.

At 515 W/cm², 33% of the cells survive 150 cavitation events greater than 0.5 V (Fig. 3). Since these events were counted over a quarter of the 10-min irradiation period, about 600 events greater than 0.5 V would occur in 10 min. A total of 11 500 events greater than 45 mV were required to produce the same survival figure at 450 W/cm² (Table III), showing that the

amplitude distribution of the transients is very positively skew. Since 89% of the cells survive 5620 events less than 0.4 V (Table III), it seems likely that the high-amplitude events are those mainly contributing to the damage. Correlation between cell damage and subharmonic activity previously reported[13] for low intensities (ca. 5 W/cm^2) is not evident in the results presented in Table III obtained at intensities of the order of 500 W/cm^2.

Since 33% of the cells survive 11 500 transients (Table III), then 67% of the cells must have been affected at least once by a cavitation event. The fact that some cells may have been affected more than once is ignored in the following calculation of an "effective volume" for a cavitation event. Since two-thirds of the cells were altered, about 2.2 ml of the suspension was affected by 11 500 events, giving a value of 2×10^{-4} ml for the "average effective volume" of a transient event.

An approximate value of the streaming velocities v_f in the cell suspension along the axis of the sound beam is given by[14]

$$v_f=\alpha\rho r^2u^2/2\eta, \tag{1}$$

when

$$2\alpha L\leq 3, \tag{2}$$

where α is the linear acoustic pressure absorption coefficient, ρ is the density, η is the shear viscosity of the suspension, u is the fluid particle velocity, r is the radius of the sound beam, and L is its length. When applied to the focused field with r taken as the half-intensity radius in the center of the focal region, i.e., 0.75 mm in this case, then $v_f=4$ cm/sec. This can only be an order-of-magnitude estimate, owing to the uncertainty in choosing an appropriate value for r and to the nonuniform intensity distribution of the sound field. It is more informative to use the results of Willard[15] in deriving the velocity of the cavitation event in the field. He irradiated at a maximum intensity of 1800 W/cm^2 in a focused field at 2.5 MHz where the first zero was 1.3 mm from the beam axis. His cavitation bursts moved at a speed of 10 m/sec at the focus, presumably at 1800 W/cm^2. Utilizing the dependence of streaming on intensity, absorption, and beam radius (Eq. 1) converts Willard's value of 10 m/sec to one of 1 m/sec for our conditions of intensity of 515 W/cm^2, beam axis to first zero distance of 2.0 cm, and ratio of absorption coefficients[14] of $1/(2.5)^2$.

Two cases are now considered, one in which the cells move in the suspension at the same speed as the cavitation event, and the second in which the cavitation event moves much faster than the liquid under second-order forces. For the first case, consider the average effective volume v_{ef} is $4\pi R_{ef}^3/3$, and since it has been shown above that $v_{ef}=2\times10^{-4}$ ml, this leads to a value of R_{ef}, the average effective radius of 0.36 mm. For the second case, if the event travels along the focus at a speed of 1 m/sec, it would take 13 msec to travel the 13-mm length of the container. The time duration of transients varied from 0.6 to 3.0 msec, mostly about 1 msec. The effective volume would then be a cylinder of approximate volume $\pi R_{ef}^2\times1$ mm^3, leading to a value of 0.25 mm for R_{ef}. If the 600 events greater than 0.5 V of average duration 2 msec (Fig. 3) are producing most of the damage, the two estimates for R_{ef} become 0.9 and 0.7 mm. Thus, all of the above estimates of R_{ef} are approximately two orders of magnitude greater than the 3.3-μ radius of a resonant bubble at 1 MHz.[16]

Measurements from Willard's[15] photographs of cavitation events show that the radius of the microbubble region is about 0.9 mm. Examination of his movie films indicates that these bubbles were moving away from the beam axis and did not persist for longer than 120 μsec. It is not possible to say whether the bubbles were produced in a very narrow cylinder along the beam axis and were then shot rapidly away from the axial zone, or whether they were produced by shock waves in a wider cylinder and then moved slowly away from this region. Whether the microbubbles and the cavitation damage are both products of the shock waves from the unique cavitating center or whether these microbubbles are acoustically active during their short lifetime and are contributing to the biological damage, must remain a matter for conjecture at the moment.

Since 11 500 events of average duration 1 msec were required at 450 W/cm^2 to damage 67% of the cells, this corresponds to one event every 52 msec. Thus, for 98% of the time of irradiation not even one event was occurring in the container. This situation is in marked contrast to the commonly observed drastic breakdown of a liquid, with the production of visible bubbles and audible noise at 20 kHz, when using total acoustic powers similar to those employed in this work.

The fact that irradiations were performed in a container of volume 3.3 ml with a limited supply of cavitation nuclei has already been advanced to explain the decrease in cavitation rate as irradiation continued. This effect may also explain the small number, and in some cases the complete absence of visible bubbles in the container even though cavitation had occurred. Elsewhere[7] it has been reported that only one in 15 bubbles emitting a strong subharmonic signal in tap water subsequently grew to a visible size. The limited supply of gassy water in the container would further decrease the possibility of this occurring.

The cyclic form of cavitation claimed to indicate the breakdown of subharmonic emitting bubbles[7] was absent in the samples treated in this work. It was found that it required 1350 W/cm^2 to produce the cyclic behavior in a sample of tap water in the container compared with about 180 W/cm^2 in tap water in the open tank.

On decreasing the sound intensity from 270 W/cm^2 and increasing the magnification of the microscope to 400×, damage was observed down to 17 W/cm^2.

Because of the variance in the counts of cells surviving treatment, it was more useful, in this region of little damage, to count only damaged cells, i.e., cells whose outer membranes were largely intact, but which had lost a large amount of intracellular material. At least 50 of these cell "ghosts" were counted. The average number of ghosts per hemacytometer square was then expressed as a percentage of the number of intact and ghost cells per square. The variation in cell damage correlates better with transient cavitation activity than with sound intensity in the range 270–127 W/cm² (Table IV). The three differing results at 127 W/cm² are believed to be due to the fact that another mechanism, possibly related to the activity of subharmonic-emitting bubbles, may be making a slight contribution.

The results of irradiating cells in 1% mycological peptone compared with irradiations in a cell suspension which also contained 0.5% Methocel (Table V) again demonstrate the ability of Methocel to suppress transient cavitation and cell damage. Cells which were not as badly damaged as the "ghosts" (Table IV), but which had an over-all granular appearance, no noticeable loss of intracellular organelles, and vacuoles that were not as refractile as in normal cells, were included in the count of damaged cells here.

C. The Growth of Cells Irradiated at 515 W/cm² for 10 min at 1 MHz

Figure 4 shows that the optical estimate of cell survival is greater than the growth estimate (columns 3 and 4, Table I). If the optical method had been capable of detecting all cavitation damage, and if a direct effect of ultrasound on cell metabolism were present so that, say, 50% of the cells surviving obvious damage were incapable of further growth, then it would be expected that the growth curve would intersect the ordinate at 50% and would have a slope of one-half that of the optical-estimate curve at all other points. If, on the other hand, the optical examination was not capable of detecting all of the damage inflicted by cavitation, both estimates should have been, for no cavitation events, 100% ($N=0$), and the growth estimate of survival should have decreased more rapidly than the optical estimate as N increased. Figure 4 supports the latter rather than the former view. The two curves do not intersect at (0,100), but since the slope of growth estimate curve is large as N approaches zero, there is uncertainty as to the point of

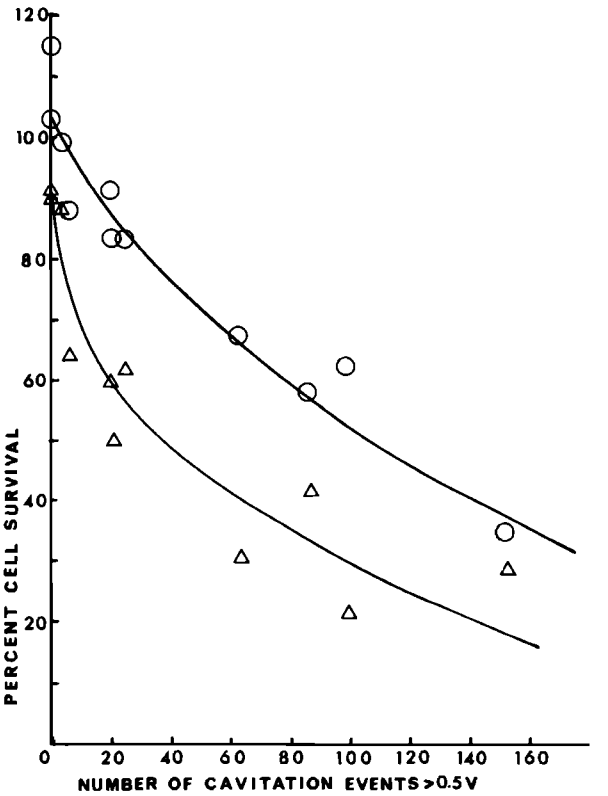

FIG. 4. Percentage of cells estimated to be intact by light microscopy after irradiation at 515 W/cm² for 10 min and the cell survival estimate from growth studies of these cells versus number of events greater than 0.5 V. ○—Microscope; △—growth.

TABLE IV. Percentage of "cell ghosts" produced in the intensity range 32–270 W/cm² as a function of mean subharmonic activity, number of transient cavitation events counted electronically, and the amplitude of the largest event recorded during the 10-min irradiation.

I (W/cm²)	Mean subharmonic amplitude (mV)	Number of transients	Maximum amplitude (V)	Percent "ghost cells"
270	4.7	7532	1.0	9.4
200	4.9	4160	0.8	4.5
185	4.9	10 000	0.4	7.8
127	3.8	4700	0.25	3.5
127	4.2	1687	0.25	2.8
127	3.4	868	0.25	1.6
72	2.5	1650	0.15	1.9
72	2.1	570	0.10	1.6
32	1.7	776[a]	0.04	0.7

[a] Counted by eye.

TABLE V. Percentage of cells damaged in mycological peptone and in mycological peptone +0.5% Methocel.

Medium[a]	I (W/cm²)	Subharmonic amplitude (mV)	Number of transients	Maximum amplitude (mV)	Percent of cells damaged
m. pep.	200	3.5	6920	2500	20
m. pep. & Meth.	200	4.6	873	200	3
m. pep.	128	2.7	3542	320	7.4
m. pep. & Meth.	128	1.6	330	150	3
m. pep.	72	0.72	1906	200	10.8
m. pep. & Meth.	72	1.4	80	100	0

[a] m. pep.—Mycological peptone; m. pep. & Meth.—mycological peptone and Methocel.

intersection with the ordinate. Further data in the range of N less than 20 would improve the estimate of the position of the intercept. This information would place an upper limit on the amount of cell damage not explained in terms of cavitation events with an amplitude greater than 0.5 V. However, the effects of transients of lesser amplitude would also need to be considered since Table V shows significant damage to cells when the amplitude of transients was low. Column 7 of Table I shows that events with amplitudes between 0.1 and 0.5 V were observed when no event greater then 0.5 V was observed. To summarize, the failure of both curves to intercept at (0,100) in this work could not be taken as conclusive evidence for a noncavitational interaction between ultrasound and the cells in suspension.

III. DISCUSSION

It seems appropriate, in view of the increased use of ultrasound as a clinical diagnostic tool, to compare the results of this study with selected reports of the effects of ultrasound on tissues, though it must be recognized that significant differences exist between the situations where cells are irradiated in suspension and in the more highly organized structural arrangements. Cells in suspension are heated to a much lesser degree than are cells in tissue, owing to the higher absorption coefficient of tissue, and to the fact that water streaming past a container of cells in the irradiation tank acts as a heat transfer system. Cells in suspension are free to rotate and change their shapes to accommodate any ultrasonic stress to which they are subjected, while cells in tissue are more constrained by the established architecture. Finally, cells in suspension are free to stream out of the high-intensity region of the field, depending upon the sample container size and shape.

The following calculation is made in order to compare the results of this study with those of whole animal irradiations studies. The half-intensity beamwidth of the focused field employed herein was 1.5 mm. The first zeros were 2 mm normal to the beam axis, so the intensity in a cylinder of radius about 1 mm was equal to, or greater than, 100 W/cm^2 when the peak intensity was 515 W/cm^2. The radius of the cylindrical container was 9 mm, so that 1/81 of the volume of cells was being treated at an intensity greater than 100 W/cm^2 at any one time, if it is assumed that acoustic streaming in the container produced complete mixing. The fraction of 1/81 can be considered equivalent to pulsed radiation with a mark space of 1:80; as the total radiation time was 10 min, the cells were exposed on the average to sound for 600/81 or 7.4 sec. It has been estimated that the speed at which a transient event moves along the axis was 1 mm/msec, and if it is assumed that the cells move at approximately this same speed, a cell would traverse the 13-mm container length in 13 msec, if it were on the beam axis, and at slower speeds for distances away from the axis. It may then be considered that these cells have been irradiated with 13-msec sound pulses at an intensity greater than 100 W/cm^2 with a mark space ratio of 1:80 for 7.4 sec.

An early study, concerned with the roles of heating and cavitation in the production of irreversible effects on living systems, showed that repeated pulses of subthreshold intensities could be integrated to produce suprathreshold effects (with doses similar to that of the above calculation), but in so doing the total irradiation time increased, depending upon pulse width, mark:space, etc.[1] At the risk of appearing specious, it can be argued that the increased irradiation time increased the probability for the requisite number of cavitation events to occur, although these investigators also showed that increasing the hydrostatic pressure of the irradiated system, to values greater than the acoustic pressure amplitudes employed, merely made manifest the pressure coefficient for the observed process, viz., production of paralysis of the hind legs of frogs.[17] A more recent investigation, in which threshold doses to produce structural changes were studied by detailed histological examinations of the treated tissue, appeared to show that cavitation occurs beyond a critical high acoustic intensity.[18] These investigators, however, determined the presence of cavitation by the unique appearance of the lesions and did not monitor for discrete events.

Suspending cells in a gel, so that cavitation would be suppressed and the cells less free to move out of the field, produces conditions more comparable with irradiation in tissue. Mouse lymphoma cells have been irradiated in a gel for 5 min at 15 W/cm^2 without significant ill effects.[19] The temperature rise in this work was small, indicating that the technique may be used at greater sound intensities. Further such studies at higher intensities and frequencies may well be necessary to elucidate the physical mechanisms involved in the acoustic alteration in tissues.

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