SESSION 2:3 INTERACTION OF ULTRASOUND AND MICROORGANISMS IN SUSPENSION

F. DUNN
Bioacoustics Research Laboratory, University of Illinois, Urbana

W. T. COAKLEY
Department of Microbiology, University College, Cardiff

The fundamental interactions between the acoustic field parameters and cells in suspension have been investigated largely because the latter provide a model system for the more highly organized animals; for example, mammals which are generally of greater interest but less convenient for study. As such they exhibit the following advantages: (*1*) the short generation, or doubling time, ranging from 30 minutes for some bacteria to about 20 hours for cultered 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; and (*3*) since all cells treated are of the same type, it is possible to apply specific tests of cellular activity to the entire population. The limitations of such experimental material as model systems for mammalian structures seem to be less widely appreciated. It must, however, be recognized that significant differences exist between the situations where cells are irradiated in suspension and in more highly organized structural arrangements. Cells in suspension may be heated to a much lesser degree than are cells in tissue, owing to the higher absorption coefficient of tissue, and to the fact that fluid streaming about 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 the ultrasonic stress to which they are subjected, while cells in tissue are more constrained by the established architecture. Further, cells in suspension are free to stream out of the high intensity region of the field, dpending upon the sample container size and shape. Thus considerable caution must be exercized in the reporting of results of investigations of these model systems and the ensuing interpretations (and extrapolations) relative to mammalian systems must take account of the kinds of limitations listed above.

Nevertheless considerable attention has been given to the effects of ultrasound on microorganisms and viruses. A detailed compilation lists 198 references dealing with such studies, most of which are concerned with the release of intracellular components because of exposures to cavitating ultrasound at frequencies in the neighborhood of 20 kHz. (*1*). The disintegration of gram-positive, gram-negative, aerobic and anaerobic, pathogenic and nonpathogenic, sporing and nonsporing bacilli form, and actinomycetes has been reported. The sensitivity of cells to rupture by cavitation appears to be a function of the structure of the cell envelope, and of the size of the microorganism, large cells being easier to break.

Yeast breakage has been found to be independent of the cavitation-produced free radicals at 20 kHz and it was concluded that attack on the cells was mechanical rather than chemical (*2*). Microstreaming about non-collapsing bubbles resonant at 20 kHz has been shown to break open *Escherichia coli* and it has been suggested that transient collapse cavitation during cell breakage coincides with the increased activity of resonant bubbles. (*3*). Suspensions of rat bone marrow cells treated at 800 kHz, 1.5 W/cm^2 for 60 seconds show gross damage under electron microscopy (*4*). The high particle acceleration forces associated with the sound wave are considered important, though the type of damage reported is typical of cavitation damage. The death of mouse lymphoma cells has been correlated with cavitation activity at 1 MHz using a technique of container rotation (*5*). Microstreaming about stable bubbles was suggested as the mechanism of breakage. Two reviewers, (*1, 6*)

while noting some claims for noncavitating mechanisms, conclude that most recorded effects are due to cavitation. Comparatively few studies have been aimed at determining whether damage can be produced in the absence of cavitation. The effects of sound at 400, 700 and 1,000 kHz on erythrocytes and plant cells have been studied using a standing wave system (*7*) where haemolysis and cavitation showed maximum activity at the pressure antinodes. Degassing the solutions of erythrocytes, or increasing the pressure on them, reduced damage and also cavitation. The work on plants showed an effect which appeared first at the pressure nodes in a degassed solution. Degassed suspensions of erythrocytes have been irradiated over the frequency range 1-6 MHz with an unfocused field at intensities of hundreds of watts per square centimeter using millisecond pulses, Mark; space ratio of a 1:1000 for 5 minutes (*8*). It was found necessary to raise the temperature of the cells to the range 45° - 55° C. in order for membrane breakage and haemoglobin release to occur. More recently it has been shown that cells maintained at 49° C. for 10 minutes spontaneously shed microspheres and release 1 - 2 percent of their haemoglobin into solution, while at 56° C. half the haemoglobin is released into solution in 10 minutes (*9*). An expression derived for the unidirectional stress produced by microstreaming on the surface of the cell, which was assumed to be a rigid sphere, showed the forces to be independent of frequency above 3.5 MHz, in fair agreement with the experimental results (*8*). Chemical tests for the presence of cavitation were performed and it was found that 350 W/cm^2 were required to give a positive result. The treating intensities were seldom higher than 250 W/cm^2. A search for cavitation bubbles using ultrasonic absorption failed to detect any. If the cavitation threshold continues to rise, in the neighborhood of 6 MHz at the high rate at which it is known to increase in the vicinity of 1 MHz (*10*), then the frequency dependence of cavitation lysis would be expected to be considerably different from that observed.

At least one report (*11*) seems to show that cavitation, and thermal processes, do not account for the observed effects on rotifers. Here, very high frequency sound, 200 - 600 MHz, was employed at extremely low intensities, 10^{-3} W/cm^2, with the observed affect occurring at the discrete frequencies of 270 MHz and 510 MHz. As these frequencies are nearly harmonically related, a resonance phenomena is suggested, though no other studies are available to support this contention.

Since streaming motions of subcellular particles were reported by early workers (*12-14*), it was inevitable that this mechanism would be considered as the cause of nonthermal, noncavitational, damage. Unidirectional forces as a possible mechanism have been strongly implicated in studies involving intracellular motions and rotations of cell contents, many of which are explainable in terms of radiation pressure and acoustic streaming (*15*). The motions have been produced by applying needle-like probes with rounded tips of dimensions no greater than the smallest radius of curvature of the cell to the cell surface and driving them ultrasonically at 25 and 85 kHz. In one such study (*16*) utilizing eggs of sea urchins, clams, and starfish (using the 85 kHz probe) reported rotations and movements of the nucleolus within the nucleus and pinching off of smaller bodies from the nucleolus leading sometimes to disintegration of the whole nucleolus into a large number of small bodies. In plant cells chloroplasts can be released from the cytoplasmic structure and circulate freely in the vacuoles.

Some observations of the biological response of cells to these motions have been made. Changes have been observed in the growth pattern in moss cells derived from single treated apical cells (*17*). Changes in the calcium permeability of muscle cells have been reported (*18*). Irradiation of fertilized Arbacia eggs interferes with the normal course of cell division (*19*). If treatment is begun sometime in the first mitotic cycle the first cleavage is delayed and may be abnormal. The magnitude of the delay and degree of abnormality depend on sonic amplitude, time after fertilization, and duration of treatment. If only one of the daughter cells is treated in the second mitotic cycle, cleavage of the treated cell occurs later than that of the untreated cell. It is to be hoped that more sophisticated biological tests will be performed with these systems producing unidirectional stresses in cells so that impairment of biological function can be associated with degrees of structural change in the cells.

Microstreaming about bubbles and cylinders resonant at 20 kHz has been shown to be capable of breaking erythrocyte membranes and causing DNA back-bone scission (*3, 9, 20, 21*). It has been

suggested (*15*) that since small scale nonuniformities must exist in the sound field to produce unidirectional stresses, small gas bubbles would be particularly effective in producing the local period-to-volume changes necessary to produce membrane vibration in tissue.

Evidence exists to support the view that unidirectional streaming contributes to damage in irradiated plants (*1*), viz., the chloroplasts of fern treated for 5-10 minutes at 2 W/cm² lose starch granuoles, become ovoid in shape and agglutimated. It is believed that the rapid and pronounced changes induced immediately in the physicochemical state of the cell contents are partially caused by an enhancement of the activity of certain enzymes due to loosening of the submicroscopic structures of the chloroplasts.

In a more recent study (*23*), an amoeba possessing many features typical of higher order animal cells was irradiated with 1 MHz ultrasound while suspended in ordinary growth medium and in growth medium with increased viscosity. The ultrasonically produced cavitation was monitored and a strong correlation found between the number of discrete cavitation events occurring and the decrease in cell numbers, on treating the suspensions at 515 W/cm² for 10 minutes. Evidence was also found such that a noncavitational contribution to cell death could not be ruled out. Increasing the viscosity of the suspension by the addition of 0.5 percent Methocel (to the 1 percent mycological peptone) produced a situation wherein transient cavitation was suppressed, as well as, cell damage. 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 minutes at 15 W/cm² without significant ill effects (*24*). The temperature rise in this work was small indicating that the technique may be used at greater sound intensities.

Alleged mutagenic effects of ultrasound understandably produce extreme concern among those in the medical profession who find it of importance to use this agent in the treatment of human disorders. However, the published literature is virtually devoid of reports of an alarming nature. The ability of ultrasound to interact with the genetic apparatus seems to be restricted largely to plant cells. Even here it is considered to be a very inefficient mutagenic agent (*25-27*)/, indeed if it can at all be held responsible for the meagre results reported. Ultrasound can produce chromosomal aberrations, but at such high intensities that the treated material does not survive. Further, the mutagenic responses illicited appear to be lethal as the inherited characteristics are passed on to at most one generation.

Thus, the recent reports (*28*) of chromosomal aberrations produced among human blood cultures exposed to ultrasound from a fetal heart detector need not have attracted wide spread attention because other explanations (*29*) for these findings are quite likely and because these results, in themselves, do not presage pernicious effects of clinical ultrasound. Further, it must be pointed out again (*30*) it is an important piece of data that physicians using ultrasonic instrumentation have *not* reported undesirable effects resulting from their clinical practice.

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