A STUDY OF THE EFFECTS OF ULTRASONIC WAVES ON THE REPRODUCTIVE INTEGRITY OF MAMMALIAN CELLS CULTURED *IN VITRO*

Bambino Isidonio Martins

Lawrence Berkeley Laboratory
University of California
Donner Laboratory
Berkeley, California 94720

ABSTRACT

The effects of monochromatic ultrasonic waves of 0.1, 0.5, 1.0, 2.0 and, 3.3 MHz frequency on the colony-forming ability of mammalian cells (M3-1, V79, Chang's and T-1) cultured *in vitro* have been studied to determine the nature of the action of ultrasonic energy on biological systems at the cellular level. The combined effect of ultrasound and X-rays has also been studied.

The following observations have been made:

(1) The survival curves, in contrast to those for ionizing radiations, are nearly logarithmic; but the slope of the line decreases with increasing dose.

(2) The lethal effects are dose-rate dependent and have a threshold dose rate.

(3) Different cell lines show slightly different sensitivities.

(4) The shape of the survival curve for different frequencies is similar.

(5) Microscopic examination shows that cells exposed to 0.5, 1.0 or 2.0 MHz appear similar. Microscopic appearance of cells exposed to 0.1 or 3.3 MHz is also similar; however, it is different from that of cells exposed to 0.5, 1.0 or 2.0 MHz. Scanning electron micrographs of M3-1 cells exposed to 1.0 MHz show characteristic bumpy outer surface compared with the smooth outer surface of unexposed cells.

(6) For M3-1 cells, 0.5 MHz is found to be the most effective of 0.5, 1.0, 2.0, and 3.3 MHz frequencies.

(7) Dose fractionation shows that exposure to ultrasound sensitizes the cells to subsequent treatment, in contrast to the effect of X-rays.

(8) Cells in M and G1 phase are more resistant than those in S phase, in contrast to the effect of X-rays.

(9) There is a small synergistic effect between ultrasound and X-rays. The degree of synergism depends on X-ray dose and the time interval between treatments, and is greater when ultrasound follows than when it precedes X-rays.

It is concluded that:

(1) Ultrasonic irradiation causes both lethal and sublethal damage.

(2) There is a threshold dose rate for lethal effects.

(3) The effectiveness of ultrasonic waves in causing cell death probably depends on the frequency and the amplitude of the waves for a given cell line, indicating a possible resonance phenomenon.

(4) The lethal effects are not due to cavitation because the intensities used are much lower than those required to produce cavitation, nor are they primarily due to temperature because they are observed even when the temperature does not exceed 37°C.

(5) There seem to be two mechanisms responsible for cell death: at 0.1 and 3.3 MHz, cell death probably results from coagulation of protoplasm; but for 0.5, 1.0, and 2.0 MHz, the primary cause of cell death seems to be damage to the cell membrane.

(6) Synergism between ultrasound and X-rays may be due to an interaction between the nuclear damage caused by X-rays and the damage to the cell membrane caused by ultrasound.

Table of Contents

ABSTRACT

I. INTRODUCTION 1
A. General Remarks 1
B. Literature Review 2
C. Statement of the Problem 12

II. PHYSICS OF ULTRASONIC WAVES 14
A. General Principles 14
B. Generation of Ultrasound 22
C. Measurement of Ultrasonic Energy 25
D. Production of Audiofrequencies 28
E. Dosimetry for Audiofrequencies 28

III. EXPERIMENTAL METHODS AND MATERIALS 29
A. Cell Culture 29
B. Ultrasonic Equipment 33
C. Audiofrequency Equipment 37
D. X-ray Equipment and Dosimetry 37
E. Experimental Technique 38

IV. OBSERVATIONS AND RESULTS 41
A. Reproducibility of Data 41
B. Physical Parameters. 44
C. Dose-Rate Effect 47
D. Cell Line Sensitivity 50
E. Frequency Effect 54
F. Audiofrequencies 54

G. Dose Fractionation 60

H. Synergism 62

I. Cell Cycle 68

J. Growth Curve 68

K. Microscopic Observations 71

L. Electron Spin Resonance: Free Radical Production 74

M. Eosin Y Exclusion Test 75

N. Cell Size Distribution 78

V. DISCUSSION 80

A. Introduction 80

B. Possible Mechanisms of Cell Death 81

1. Cavitation 81

2. Temperature 83

3. Direct Effects 85

C. Explanation of Experimental Results 86

1. Cell Death 86

2. Dose-Rate Effect 89

3. Cell Line Sensitivity 91

4. Frequency Effect 91

5. Dose Fractionation 91

6. Synergism 92

7. Cell Cycle 93

8. Growth Curve 94

VI. SUMMARY AND CONCLUSIONS 95

ACKNOWLEDGMENTS

REFERENCES

I. INTRODUCTION

A. General Remarks

Studying the effects of environmental factors on biological systems has become increasingly important in this day and age, as the very existence of life on this planet--some would have us believe--is threatened by man's interference with the ecosystem of our earth. Two such environmental factors are sonic vibrations and ionizing radiations. The effects of ionizing radiations have been extensively studied, but little work has been done on the effects of sonic vibrations on living systems.

Sonic vibrations of various intensities and frequencies are produced all around us, either deliberately for a specific desirable effect or as "pollutants"--an undesirable byproduct of an industrial society. Using audibility by the human ear and frequency (the number of complete oscillations executed by a particle in unit time) as the criteria, we can classify sonic vibrations into three categories:[1]

(1) Audiofrequencies. This is the range of frequencies that can be heard by man. There are individual variations regarding the lower and upper limits on the range of audiofrequencies, but commonly the range is taken to be from 16 Hz to 16,000 Hz. (Hertz, which is abbreviated Hz, is the unit of frequency and is defined as 1 cycle per second; KHz $\equiv 10^3$ Hz and MHz $\equiv 10^6$ Hz.)

(2) Subsonic frequencies. These are frequencies from 0 to 16 Hz.

(3) Ultrasonic frequencies. These embrace all frequencies above 16,000 Hz. The upper limit for ultrasonic frequencies is set at around 500 MHz by practical limitations of the generators. Frequencies above 15 MHz are sometimes referred to as microwave ultrasonic frequencies because at those high frequencies, even though particle vibration is still being produced, the very short wavelengths tend to make the waves behave like electromagnetic microwaves.

B. Literature Review

Langevin, around 1920, was one of the first to observe the lethal effects of ultrasound on living organisms.[2] During his investigations on the use of ultrasound for submarine location, he noticed that small marine organisms that wandered into the path of his beam were killed rapidly. Because of this, the idea of using ultrasound as death-rays was considered; but it was not seriously explored.

Since then many studies have been made on the biological effects of ultrasound; and today ultrasound is used extensively in biology, medicine, and dentistry.

Ultrasound has been used for such diverse purposes as the production of holograms,[3,4] the removal of bacteria contained in a milk film fixed on metal surfaces;[5] the determination of body tissue composition in slaughter animals,[6-14] the treatment of wine to improve its bouquet and taste,[15] the treatment of farm crop seeds to obtain better yield,[16] and the treatment of menstrual disorders in women.[17] But the primary application of ultrasound in biology has been for the breaking down of cells and the preparation of cell fractions, such as enzymes, to study metabolic pathways and to investigate localization of cell components within the cell.[18-20]

In medicine, the use of ultrasound parallels the use of X-rays.[21] Therapeutic uses of ultrasound, as in treatment of Mèniere's and Parkinson's diseases, generally require high intensities, while low-intensity ultrasound is used in diagnosis to obtain "echograms", i.e., visual images of objects arising from inhomogeneties in acoustic absorption. At intermediate intensities, ultrasound is used in physiotherapy (diathermy), mainly as a source of heat.

In dentistry, two frequency ranges are used: the low-frequency range (20 to 30 KHz) is used mainly for mechanical processes such as cleaning and drilling of teeth, peridontal prophylaxis, and amalgam packing; the high-frequency range (500 to 1,000 KHz) is used therapeutically in the treatment of clinical conditions such as pulpitis, post-extraction edema, and neuralgia.[22]

The biological, medical, and dental applications of ultrasound are reviewed by Newell,[21] Kelly,[23,24] Fry and Dunn,[25] Grossman et al,[26] Goldberg and Sarim,[27] Brown and Gordon,[28] and Hill.[29] The physical, chemical and biological effects of ultrasound are described in detail by El'piner,[30] who has also reviewed the literature extensively. The work relevant to the biological effects of ultrasound at the cellular level will be descirbed here.

Following the initial observations of Langevin, Wood and Loomis[31] in 1927 reported the rupture of Paramecium and Spirogyra in an ultrasonic field; and in 1932 Harvey and Loomis[32] took cinephotomicrographic pictures of sea urchin eggs contained in a drop of liquid and exposed to ultrasonic waves; they observed that cell rupture required less than 1/1200 sec.

These early reports described mainly the almost total disintegration of a large variety of unicellular and invertebrate organisms. More subtle effects have been observed since then. Schmitt[33] in 1929 observed rotation and fragmentation of the nucleolus of Asterias eggs. Feindt[34] reported that Paramecium exposed to low ultrasonic intensities performed rapid circular motion which slowly stopped, the outer membrane becoming detached and bursting, releasing the cytoplasm into the outside medium.

Dyer and Nyborg[35,36] observed complex patterns of ordered motion of intracellular inclusions in individual cells of plants when high-frequency vibrations were applied to selected regions of the cell wall. Hughes and Nyborg[37] exposed suspensions of fresh erythrocytes in 0.9% saline to vibrations of 85 KHz frequency and 0.05 to 5 μ amplitude by dipping a vibrating needle into the suspension, and observed the cells in a microscope. They found that under a variety of conditions the cells were readily damaged, the number of damaged cells increasing with increasing amplitude. Suspensions of the protozoon Tetrahymena pyriformis were exposed under the same conditions; and, depending mainly on the amplitude, effects ranging from temporary inhibition of motility to complete disruption were found. By cinephotomicrography the experimenters noticed that the cells became violently distorted as they entered the region of highest streaming speeds near the tip of the needle, and the contents of the cell moved in a circular manner relative to the cell motion. When suspensions of E. coli in water or in 0.9% NaCl were treated similarly, Hughes and Nyborg observed many empty cells with significant amounts of protein released from the cells.

However, they found that bacteria are more resistant than erythrocytes or protozoa. They ascribed the damage to shearing due to streaming motions and not to cavitation, which is the formation of vaculoles in a liquid exposed to sonic vibrations.

Wilson et al.[38] reported that ultrasound of 85,000 Hz applied to the surface of egg cells of marine invertebrates produces rotation, translation, deformation, and fragmentation of the nucleoli; rotation and deformation of nuclei; acoustic streaming of nucleoplasm and cytoplasm; deformation of the cellular surface; and fragmentation of the cell.

According to El'piner[30] the action of ultrasound on microorganisms is of a complex and diverse nature. He rules out temperature as a primary factor in many of the effects of ultrasound that have been observed in microorganisms and says that the bactericidal effect of ultrasound is due primarily to cavitation in an aqueous medium. Different bacteria differ from one another in their sensitivity to ultrasound. It is believed that the size and the shape of the bacterium is a significant factor in the differential sensitivity of microorganisms to ultrasound.

For viruses, too, the sensitivity to ultrasound depends on the structure and size of the virus particle. El'piner et al.[39] have shown that ultrasound produces distinctive disturbances in the structure of phage T-2, from which they were able to judge features of the morphological structure of this phage. Of the seven T-phages active against E. coli, T_2, T_4, T_6 and T_8 are more sensitive to ultrasound and also to X-rays, ultraviolet light, and radioactive phosphorous as compared with T_1, T_3 and T_7 which are more resistant to ultrasound and also to the other physical factors mentioned above.[30]

Sudo and Dworkin[40] studied the resistance of vegetative cells and microcysts of Myxococcus xanthus. They found that the microcysts were 19.3 times as resistant as the vegetative cells and that the resistance developed during the conversion of rods to refractile spheres.

Ultrasound does not seem to cause coarse mechanical breaks in yeast cells even after prolonged irradiation at high intensities (El'piner[30]; Martins et al.[41]). Lependin and Ustinova[42] report that Bel'kevich et al. found that the greatest destruction for yeast cells occurs at a frequency of 400 KHz; but Martins et al.[41] found that for inhibiting colony formation in S. cerevisiae (strains BZ34 and X841) 1.0 MHz is the most effective of the following frequencies that were tested: 0.5, 1.0, 2.0, and 3.3 MHz.

The role of ultrasound as a mutagen is discussed by Gordon.[43] He says that ultrasound produces both structural and gene mutations and that a number of authors have found that ultrasound is able to cause breaks in chromosomes and in single chromatids, but chromosomal rearrangement or recombination has not been extensively reported. A doubling of the chromosomes to form polyploids has also been reported. Gordon considers three possible mechanisms for the mutagenic effect of ultrasound: high pressures and accelerations causing movements within cells, generation of heat at points, and cavitation. He concludes that although there is much evidence to the contrary cavitation is probably the cause of the mutagenic action of ultrasound. However, the observations of Martins et al.[41] on the ultrasonic production of "-Arg reverse mutants" in S. cerevisiae indicate that the increase in reversion mutation frequency was not caused by cavitation.

Studies on the abnormal embryonic development in Drosophila melanogaster induced by ultrasonic treatment of the eggs at the syncytial blastoderm stage at 1 MHz with 0.3 to 0.5 W/cm^2 for 30 sec are reported by Selman and Counce.[44] Kirsten, Zinsser and Ried[45] reported that whole-body irradiation of LaF_1 mice at 1 MHz frequency for 5 min both continuously and in pulses (intensity 1/7 to 4 W/cm^2) did not produce genetic damage (average litter size being the criterion used) in those mice that were unaffected by the sonication (no skin burn) when these were bred brother-cross-sister for six litters.

Stimulatory effects of ultrasonic waves were first reported by Istomina and Ostrosvkii.[46] They found that ultrasonic treatment of potato seeds increased the weight of the tubers and reduced the weight of the leaves. Stimulatory effects of ultrasound on the germination of seeds and on productivity of fodder beans have been reported by Rubtsova,[16] while Watmough et al.[47] found that ultrasound caused a reduction in the growth rate of seedlings of Vicia faba.

Observations on the effects of ultrasonic waves on metabolism and on submicroscopic structures of cells and tissues in animal organisms have also been reported. Jankowiak et al.[48] found an increase in the RNA content of ultrasonically irradiated rat liver cells and a reduction of the DNA content of the same cells.

Burns,[49,50] in his work on Saccaromyces cerevisiae at sublethal doses of ultrasound (frequency 10 KHz), found inhibition of biosynthesis during sonication, synthesis being resumed immediately after the sonicator was turned off. Ultrasound also inhibited protein synthesis and caused leakage of free histidine, but these effects were not immediately reversed when sonication was stopped. Uptake of

ammonium ion and adenine or purine nucleotides is unaffected, but uptake and/or retention of aspartate is reduced. He believes that ultrasound causes disruption of the supramolecular organization of the cell, particularly the cell membrane, making it more permeable to certain kinds of small molecules but not to other kinds of small molecules or to large molecules.

Mityushin and El'piner[51] have observed very subtle changes in the ultrastructural organization of the ergastoplasmic reticulum, the mitochondria, and the nucleus of the cells of Erlich ascites carcinoma exposed to ultrasound of 800 KHz frequency for 10 min at 15 W/cm^2 intensity.

Slawinski[52] studied the effect on the iodine metabolism in guinea pigs exposed to ultrasound of 800 KHz frequency at an intensity of 0.5 to 1 W/cm^2 for 1 to 30 times with each exposure of 10 min. He concludes that ultrasound stimulated or depressed thyroid function depending on the dose. He also observed morphological changes such as diminished nuclear volume and a lowering of the height of the alveolar epithelium in the exposed thyroid glands.[53] Functional and morphological changes in the thyroid gland are reported also by Hrazdira et al.[54] who exposed rabbits to ultrasound of 800 KHz frequency at 1 and 2 W/cm^2 intensity.

Valtonen[55] studied the effect of ultrasound of 1 MHz frequency on the fine structure of the liver parenchymal cells of mice when the ultrasound was applied to the upper abdomen. The appearance of the liver cells ranged from nearly normal (intensity 1 W/cm^2 for 1 min) to complete loss (coagulation) of the fine structure of the cell organelles (intensity 3 W/cm^2 for 3 min). He also describes a histological method

for measuring the influence of ultrasonic energy on living tissue under experimental conditions. It depends on the observation that the number of injured mast cells in the mesentery of the rat is proportional to the applied ultrasonic dose.[56]

Basurmanova et al.[57] noticed changes in the fine structure of the brain nerve cells of Hyalophora cecropia (a caterpillar) when the whole body of the larva was exposed to ultrasound (760 KHz, at 2 to 3 W/cm^2).

Ultrasonic treatment of rabbit testes at 2.25 MHz frequency and 1 mW/cm^2 intensity for 2 to 10 min produced no noticeable structural or functional changes as stated by Hahn and Foote[58], but Andrianov[59] noticed significant histological, histochemical, and ultrastructural changes in the testes of albino rats exposed to ultrasound of 800 $\pm$ 10 KHz frequency and 0.2 W/cm^2 intensity. These changes were most pronounced 24 to 72 hours after the ultrasonic treatment, and the testicular structures tended to return to normal a month after the treatment.

Covich and Tsukada[60] found that ultrasonication (at 27 KHz) of oosporangial walls of Chara zeylanica separates the inner and outer wall but does not alter the internal cell morphology; however, a 30-min exposure erodes the papillae.

Kovalev[61(a,b)] has studied the effect of ultrasound on Brucella and reports temporary changes in the physiological properties following a single exposure; but continued exposure produces progeny with altered properties, the properties of the original culture being restored following repeated culturing.

Kleimenov[62] has studied the effect of local and general ultrasonic treatment on antibody titers and interrelations between serum proteins in rabbits. He observed inhibition of production of precipitins but no effect on agglutinin synthesis in whole-body irradiation. Local ultrasonic irradiation of the lymph nodes resulted in a marked decrease of the titer of agglutins to E. coli when the latter were injected below the site of the ultrasonic application.

The effect of ultrasound on muscle fibers has been studied by a number of investigators following the pioneering work of Chambers and Harvey.[63] Ultrasonic waves affect the morphology as well as cause subtle changes in the biocatalytic function.

Shtrankfel'd et al.[64] have studied the effect of high-intensity ultrasonic waves (800 KHz, at 10 to 12 W/cm^2 for 30 to 300 min) on G- and F-actin proteins in solution. Exposure to ultrasonic waves leads not only to a "loosening" of the polymer structures of F-actin but also to more significant changes affecting G-actin which is involved in the formation of the polymer molecule of F-actin.

Ravitz and Schnitzler[65] observed changes in the fine structure, particularly the mitochondrial cristae and the components of the sarcotubular system, in frog semitendinosus muscle fibers following highly localized ultrasonic treatment (85 KHz) at intensities that ruled out thermal effects and cavitation.

The effect of ultrasound on cultured mammalian cells has been reported recently by Clarke and Hill.[66] They report that the intensity threshold and intensity optimum for cell disintegration occur at about 1 and 5 W/cm^2. They observed an anomalous dose-rate effect and found

that the medium exerts a chemiprotective effect on the cells. They have also studied the effect of ultrasound as a function of the cell cycle and report that cells in M-phase are more sensitive than the average population.[67]

The combined effects of ultrasound and other physical and chemical factors have been studied in several systems.

Zapf[68] observed that ultrasound enhances the morphological changes produced by penicillin in E. coli. El'piner[30] reports that E. coli becomes more sensitive to ultraviolet light if it is exposed to ultrasound beforehand or simultaneously. Garina[69] reports that preliminary ultrasonic treatment of spores from various Actinomycete strains increased the lethal and mutagenic effects of U.V. radiation. Avakyan[15] reports that combined treatment with ultraviolet and ultrasound is better than either separate treatment in improving the bouquet and taste of wine. According to Spring,[70] radiosensitivity of biological material (seeds of grass: Lollum italicum) may be increased by simultaneous ultrasonic and γ-radiation.

Woeber[71] has observed a synergistic effect between ultrasound and X-rays in the regression of Walker carcinoma in rats. Martins[72] has observed a synergistic effect between ultrasound and X-rays on the colony-forming ability of cultured mammalian cells.

Clarke et al.,[73] however, did not observe any synergistic effect between ultrasound and X-rays in lymphoma cells. Also, Repacholi[74] states that the effect of combined ultrasonic and X-ray treatment on the electrophoretic mobility of Ehrlich ascites tumor cells was additive, rather than synergistic.

C. Statement of the Problem

Ever since Puck[75] and his co-workers developed a technique for culturing single mammalian cells *in vitro*, that technique has been used extensively to study the biological effects of various environmental factors, particularly ionizing radiations.[76] These studies give valuable information about the nature and the mechanism of the action of such factors at the cellular level and help in the evaluation of the effects at the organismal level. Although ultrasound is used extensively for various purposes in industry, biology, medicine, and dentistry, only limited and rather controversial work has been done to study the nature of its effects and the mechanism of its action at the cellular level.

This investigation deals with the effects of ultrasonic waves on the colony-forming ability of mammalian cells cultured *in vitro*. The study was done to obtain the following information:

(1) The nature of the dose-response curve for mammalian cells cultured *in vitro*.

(2) The dose-rate effect and the occurrence of threshold.

(3) The effect of dose fractionation.

(4) The sensitivity of different cell lines to a given frequency.

(5) The sensitivity of a given cell line to different frequencies.

(6) The influence of certain physical and biological factors (such as temperature, presence or absence of medium different stages in cell cycle) on the sensitivity to ultrasound.

(7) The combined effect of ultrasound and X-rays.

(8) The mechanism of the action of ultrasound.

Most of the experiments were done with Chinese hamster bone-marrow cells (M3-1) at 1.0 MHz frequency. The experiments with other cell lines [human kidney (T-1), human liver (Chang's) and Chinese hamster lung (V79)], were done to compare the sensitivity of different cell lines to a given frequency. The experiments at 0.1, 0.5, 2.0, and 3.3 MHz, and at the audio-frequencies, were done to compare the sensitivity of a given cell line to different frequencies.

II. PHYSICS OF ULTRASONIC WAVES

A. General Principles

The physics of ultrasound is treated extensively and thoroughly by Beyer and Letcher,[84] Blitz,[82] Crawford,[1] Richardson and Brown,[81] Bergmann,[77] Kittel,[79] Carlin,[2] Krasil'nikov,[83] and others.[78,80] These references also describe the methods for the generation of ultrasound, and techniques for its measurement. The latter are also described by Fry and Fry,[86] Newell,[87] and by Hill.[88] The physical aspects of ultrasound which are of importance to biological studies are discussed by Fry and Dunn,[25] by Brown and Gordon,[28] and by Peacocke and Prichard.[85]

Sound is a wave motion. It is caused by the vibrations of the particles of a medium which has been disturbed. Sound waves differ from electromagnetic waves in that the latter can be propagated in vacuum. Wave motion may be classified in different ways depending on the criteria one chooses; thus it may be:

(1) Pure if it is produced by a source vibrating with a single frequency, or Complex when the wave motion is the resultant of a number of frequencies.

(2) Continuous when the wave motion is regular as in a musical note, or Discontinuous as in an explosion.

(3) Longitudinal (L-wave) when the vibrations of the particles are in the direction of propagation of the wave; Transverse or Shear (S-wave) when particle vibration is perpendicular to the direction of wave motion; or Surface (Rayleigh wave) when the wave is propagated

over a surface without influencing the bulk of the medium below the surface. Only longitudinal waves can be propagated in fluids, while transverse and Rayleigh waves can travel only in solids.

(4) On the basis of the nature of the wave front (the leading surface of the advancing wave), there are plane, spherical, and cylindrical waves. For plane waves the source of sound has a plane surface and the wave front is planar. When the sound source is a point source, the wave front close to the source is spherical and spherical waves are propagated. If the source of sound is a rod, the wave front is cylindrical, giving rise to cylindrical waves.

The sonic waves used in this study are pure planar longitudinal waves described on the model of a simple sinusoidal harmonic motion (Fig. 1). This is defined as that motion along a line for which the acceleration of a body towards some fixed point on that line varies in proportion to the displacement of the body away from that point.

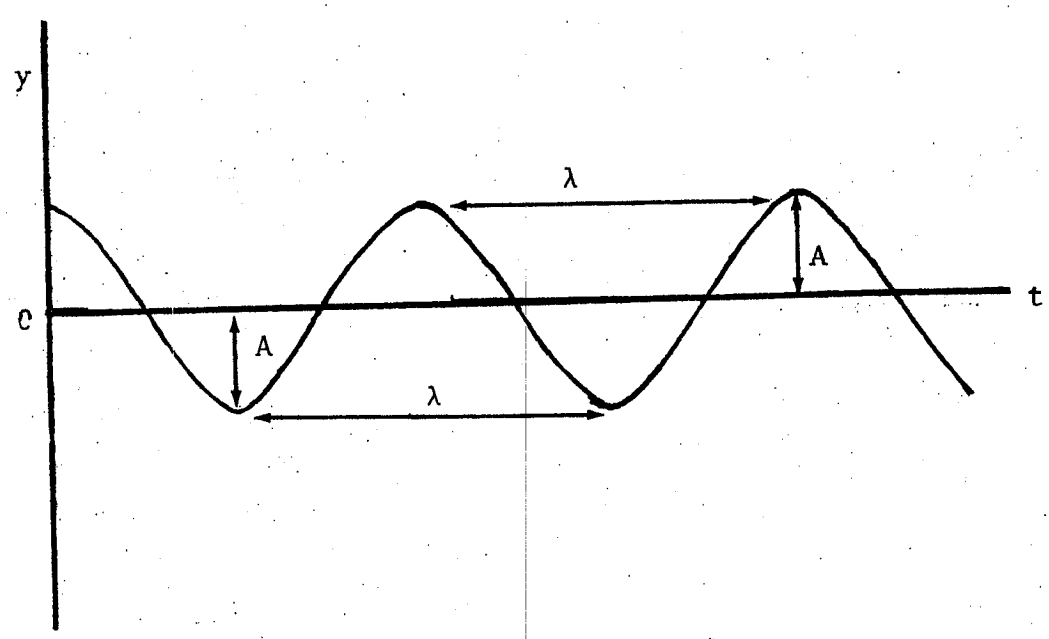


Figure 1. Displacement y of a body executing SHM from the position of rest against time t.

Simple harmonic motion is characterized by the following parameters:

(1) Time period (T): the time taken by the particle to complete a single vibration. The unit for T is sec.

(2) Frequency (f): the number of vibrations completed in one second. It is obvious from the definitions that $f = 1/T$. The unit of frequency is the Hertz which is defined as one cycle per sec.

(3) Displacement amplitude (A): the maximum displacement of the particle from its position of rest. The displacement of a particle in relation to time t obeys a sine law $y = A \sin 2\pi ft$.

(4) Velocity amplitude (v_o): the maximum velocity of the particle, observed as the particle passes through its initial position of rest. The vibrational velocity of a particle is given by

$$v = dy/dt = 2\pi fA\cos 2\pi ft \quad \text{if } A = \text{Constant}$$

$$\therefore v_o = 2\pi fA \quad (\because \cos 2\pi ft = 2n\pi = 1)$$

(5) Sound velocity (c): the distance traveled by the wave in unit time. For gases $c = \sqrt{\gamma P/\rho} = \sqrt{\frac{\gamma}{M} RT}$ where P = pressure, ρ = density, $\gamma = c_p/c_v$ is the ratio of the specific heat at constant pressure (c_p) to the specific heat at constant voluce (c_v), M is the molecular weight, R = gas constant, T = abst. temperature. For liquids $c = \sqrt{1/\beta_{ad}\rho} = \sqrt{\gamma/\beta_{is}\rho}$, where β_{ad} = adiabatic compressibility, β_{is} = isothermic compressibility, $\beta = -1/V\, dv/d\rho$. For solids $c = \sqrt{E/\rho}$, where E is the Young's modulus.

(6) Wave length (λ): is the distance between two consecutive troughs or crests and is defined by the relation $c = fx$. The unit for wavelength is the cm. Since the frequency of sound waves is determined by the source of the vibrations and because the velocity of

sound has a characteristic value for a given medium, it follows that when sound travels from one medium into another only the wavelength will change if the adjoining media differ from one another as regards the velocity of sound.

(7) Intensity or Energy: when a sound wave travels through a medium, there is no actual movement of a particle in the medium away from the source; the motion is entirely vibrational about a fixed point, but there is transfer of energy away from the source. The amount of energy carried by sound vibrations in 1 sec through an area 1 cm^2 perpendicular to the direction of propagation determines the strength or intensity of the sound. For a plane progressive sine wave the sound intensity $I = P^2/2\rho c$ where P is the acoustic pressure or the excess pressure created by the propagation of the wave, over and above the mean pressure in the medium. Acoustic pressure is related to the vibrational velocity (v) being equal to $v\rho c$ where ρc is the specific or characteristic acoustic impedance of the medium.

Sound intensity is measured in Watt/cm^2 or erg/sec-cm^2 (1 W/cm^2 = 10^7 erg/sec-cm^2). Another unit commonly used to express intensity of acoustic energy is the decibel which is ten times the logarithm (to the base 10) of the ratio of a given sound intensity to a reference (threshold) intensity. The threshold intensity usually chosen is 10^{-16} W/cm^2 which is the intensity of sound close to the threshold of audibility of the human ear. A sound intensity of 10^{-2} W/cm^2 (140 db) produces a strong sensation of pain. The intensity of normal conversational speech is 60 db (10^{-10} W/cm^2).[30]

The displacement amplitude (A) of a particle is related to the intensity of sound as follows:[30]

$$v = 2\pi fA$$

$$P = v\rho c = 2\pi fA\rho c .$$

But $$I = P^2/2\rho c; \quad I = \frac{4\pi^2 f^2 A^2 (\rho c)^2}{2\rho c} = 2\pi^2 f^2 A^2 \rho c .$$

Sound does not pass through a vacuum, so for the propagation of sound waves it is essential to have a medium. In discussing the interaction of sound waves with the medium we will confine ourselves to plane longitudinal sine waves incident at normal angle, such being the case in our experimental set up.

A sound wave may undergo any one or more of the following types of interactions with the medium: absorption, reflection and transmission, standing or stationary waves, resonance, diffraction, scattering.

(1) Absorption. When sound waves travel through a medium, there is a conversion of some of the acoustic energy into other forms of energy, primarily heat energy. The loss of acoustic energy is expressed in terms of the absorption or attenuation coefficient α given by the following relationship:

$$A = A_o e^{-\alpha x} \quad \text{or}$$

$$I = I_o e^{-2\alpha x},$$

where A_o and I_o are the amplitude and intensity at any given point and A and I are the amplitude and intensity at a further distance x.

The various mechanisms responsible for absorption of sound may be classified into two general categories: a) viscosity or frictional lag and b) relaxation processes which refer to the thermal and structural reorientation of the molecules. The exact mechanisms leading to absorption of a sound wave in a medium, and particularly in complex biological media, are not fully understood.

(2) Reflection and Transmission

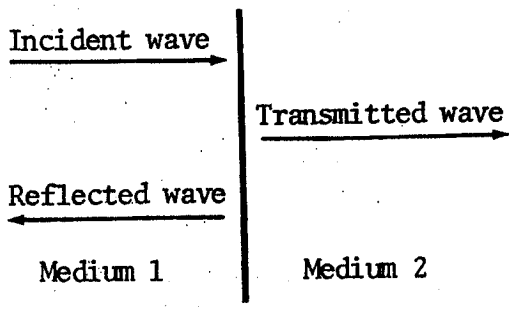


Figure 2

When a plane wave is incident at a normal angle to a plane boundary separating two media, (1) and (2) in Fig. 2, some of the energy of the incident beam is reflected and the remainder is transmitted into the second medium. The relative amounts that are reflected and transmitted are determined by the reflection and transmission coefficients, which depend on the specific acoustic impedances of the two media.[28]

The transmission coefficient

$$\alpha_t = \frac{4\rho_1 c_1 \rho_2 c_2}{\left(\rho_1 c_1 + \rho_2 c_2\right)^2} = \frac{\text{Intensity of transmitted wave}}{\text{Intensity of incident wave}}$$

The reflection coefficient

$$\alpha_r = \left(\frac{\rho_2 c_2 - \rho_1 c_1}{\rho_2 c_2 + \rho_1 c_1}\right)^2 = \frac{\text{Intensity of reflected wave}}{\text{Intensity of incident wave}}$$

for a given boundary,

$$\alpha_t + \alpha_r = 1.$$

From the above equations one can see that since gases have very low characteristic impedances, almost 100% reflection and practically no transmission will take place at a boundary between a gas and a solid or a liquid. This property is utilized in the detection of bubbles in various biological systems.

The efficiency of the transfer of sound energy from one material to another, as expressed by the transmission coefficient, is known as the degree of acoustic matching or coupling. Where α_t is very low the two media are poorly matched; but where α_t is sufficiently high for there to be a loss of only a few decibels (for example, a value of about 10%), the media are said to be well matched. This property is used to obtain "echograms" and detect tumors.

The presence of intervening media creates difficulties since much depends on the thickness of the intervening layers as well as on their characteristic impedances. The resultant value of the transmission coefficient may not necessarily be obtained by multiplying together the coefficient for each of the boundaries.

(3) Stationary or standing waves. When the reflected waves form a continuous beam, they will interfere with the incident waves and give rise to stationary or standing waves. In practice, for any finite sized medium we always have stationary waves. In contrast to standing waves, we have progressive waves, for which wave motion is in one direction only.

(4) Resonance. This occurs when the length of the medium has a certain value related to the wavelength of the sound within that medium. Thus we have half-wavelength or quarter-wavelength resonance.

(5) Diffraction. A parallel beam of sound waves leaving a plane surface will remain parallel up to a certain distance and then diverge. This phenomenon is known as diffraction. It can be shown that within an approximate distance of $D^2/4\lambda$ from the radiating surface (D being the diameter of the source and λ the wavelength) the beam will be approximately parallel. The region in which the beam is parallel is known as the Fresnel region or near field, and the region beyond that where the beam becomes divergent is known as the Fraunhofer region or far field.[28]

(6) Scattering. When the boundary has dimensions which are comparable with or less than one wavelength, scattering takes place, i.e., the beam is reflected in all directions. Scattering, for example, may be caused by cells in suspension in a medium.

B. Generation of Ultrasound

There are four principal ways of producing ultrasonic waves: mechanical generators, thermal generators, magnetostrictive generators, and piezoelectric generators.

(1) Mechanical generators.

a) Tuning forks. These have been used to produce ultrasound of frequency up to 90 KHz, but they are unsuitable because the waves are easily damped and energy output is low.

b) Galton whistle. Sound is produced by letting a current of air escape through a narrow slit and fall upon the sharp edge of an object facing the slit. Frequencies up to 100 KHz have been obtained.

c) Hartmann generator. A jet of compressed air escaping from a hole impinges on a coaxial ring-shaped edge which may be the mouth of a small bottle resonator. Though high-energy outputs may be obtained from these sources, both the amplitude and the frequency are difficult to control.

d) Holtzmann's generator. Sound waves are produced by getting a glass or metal rod to vibrate lengthwise. Holtzmann produced a frequency as high as 33 KHz.

(2) Thermal generators. Alterb generated sound waves up to a frequency of 300 KHz by means of a spark gap fed by a damped oscillator circuit. This generates a mixture of frequencies from which the desired frequency may be selected using a diffraction grating.

(3) Magnetostrictive generators. The principle on which these generators are based is the magnetostrictive or Joule effect, which states that if a rod or tube of ferromagnetic material is brought into a magnetic field parallel to its length, the length is changed. This

change of length is independent of the sign of the direction of the field. If an alternating magnetic field is applied along the direction of the axis of a rod of ferromagnetic material, the rod oscillates at twice the frequency of the applied field. Maximum output is obtained by operating at the fundamental resonant frequency (f_r) of the rod given by

$$f_r = (1/2L)\ (E/\rho)^{1/2},$$

where E is the adiabatic Young's modulus for the material of the rod, ρ its density, and L its length.

(4) Piezoelectric generators. The Curie brothers discovered that if certain crystals having axes of nonsymmetry were subjected to mechanical stress, then electrical charges developed on the surfaces. This is known as the piezoelectric effect. The Curie brothers also observed the converse piezoelectric effect, i.e., when an electric field is applied in the direction of an axis of nonsymmetry, the crystal is mechanically strained, the amount of strain being proportional to the intensity of the applied field. It is this converse piezoelectric effect which is used in piezoelectric generators. A closely related effect is the electrostrictive effect, the significant difference being that for the electrostrictive effect the magnitude of the mechanical strain produced is proportional to the square of the applied field strength. The piezoelectric effect is observed in quartz, Rochelle salt, tourmaline, and similar crystals. The electrostrictive effect is pronounced in ferroelectric materials such as barium titanate and lead zirconate. The ferroelectric materials have an advantage over piezoelectric crystals in that they are polycrystaline and it is

possible to cut them into almost any desired shape; so they can be used to obtain focused ultrasonic beams. A polarized ferroelectric transducer acts like a piezoelectric transducer. For the experiments described herein, X-cut quartz crystals were used to produce ultrasonic waves of 0.5, 1.0, 2.0, and 3.3 MHz frequency; and a barium titanate crystal was used to produce 0.1 MHz frequency.

For an X-cut quartz crystal the natural frequency is given by

$$f_r = nc/2L,$$

where c is the velocity of sound in the crystal, L is the thickness of the plate, and n is order of the harmonic.

Since c = 4500 m/sec, the first order or fundamental frequency is given by

$$f = 2700/L \text{ kc/min}$$

The experimental value is 2880/L kc/min. The discrepancy may be due to the presence of transverse waves as well as longitudinal.[83] If a piezoelectric crystal is placed in an alternating electric field so that a polar axis is in the direction of the field, then the crystal will alternately expand and compress, producing longitudinal oscillations in the surrounding medium.

There are two main types of cricuits used to drive piezoelectric crystals:

a) Self-maintaining type (Pierce)

b) Resonant drive type (Hartley).

In the first type the oscillator controls the frequency of the circuit, and in the second the circuit is precisely tuned to the fundamental or a harmonic of the quartz crystal. The Hartely circuit is preferable to the Pierce circuit for radiating ultrasound into liquids.

C. Measurement of Ultrasonic Energy

Various methods are available for the measurement of ultrasound:

(1) Calorimetric method. The temperature increase in an absorber of known mass and specific heat placed in the ultrasonic beam is taken as a measure of the acoustic energy dissipated in the absorber.

(2) Radiation balance method. At a boundary between acoustically dissimilar materials a radiation pressure is developed and the force exerted on the interface is a direct measure of the ultrasonic intensity in that region.

(3) Thermoelectric probe. This technique developed by Fry and Fry records the temperature increase using a thermocouple probe etched down to between 0.0003 and 0.0005 inches in diameter in the neighborhood of the junction and embedded in a small quantity of acoustic absorbing material.

(4) Piezoelectric receivers. The voltage generated in a transducer placed in an ultrasonic field is proportional to the intensity of the ultrasonic beam and may be used to measure the latter.

(5) Optical methods. These methods are based on the diffraction or refraction of a beam of light by ultrasonic waves. The diffraction method measures the relative light intensity in the various orders of a diffraction pattern whereas the refraction method is based on observing the periodic variation in the gradient of the refractive index.

(6) Film method. Various types of photographic and other films are sensitive to ultrasonic radiation and have been used for measuring sound intensity.

All the above methods were found to be unsuitable for measurement of the ultrasonic intensity for the experimental set-up used. (1) The measurement of the rate of increase of temperature did not give reproducible results even though the monitored electrical energy was constant. (2) Radiation balance methods were unsuitable because of the hydrodynamic flow of the medium, which was evident even at fairly low intensities. (3) The thermoelectric probe can be used only with progressive waves and not in the presence of standing waves. (4) Piezoelectric receivers are very sensitive to orientation, especially in the Fresnel field. A hydrophone produced by Gulton Industries, Inc. (Model: Glennite VP800C) was tried, but it's unsuitable for even qualitative measurements. (5) The optical methods are not suitable for measurements of intensities, but are mainly used for getting pictures of wave forms. (6) The film method is not very reliable and was not tried.

Thus the measurement of the intensity of ultrasonic energy absorbed in the medium could not be made satisfactorily; but it is possible to measure the power absorbed by the transducer from the electrical energy input.

The output of acoustic intensity from a resonant piezoelectric transducer backed by air and radiating into a medium of acoustic impedance ρc is

$$I = 4V^2e^2/L^2\rho c,$$

where V = rms voltage applied, L = thickness, e = appropriate piezoelectric stress constant (e of quartz = 0.17).[21,25,83] Since L is inversely proportional to the resonant frequency, the intensity for a given exciting voltage is proportional to the square of the frequency.

If the oscillator frequency is adjusted to be at resonance with the fundamental frequency of the quartz crystal, the amplitude of the oscillations will be a maximum and the intensity of the ultrasound generated will vary inversely as the acoustic impedance of the medium to which the crystal is coupled, as the square of the applied voltage and as the square of the frequency.

Since all the quantities in the equation are known, one can get a value for the intensity. Also, since the exposure conditions are constant for the various frequencies (except 0.1 MHz), one can easily obtain the intensities at different frequencies.

The intensity values plotted in the graphs were obtained from the equation

$$I = 4V^2e^2/L\rho c$$

For 0.1 MHz, only qualitative data are given because the physical conditions are different and also because the frequency decreased with time as the temperature of the crystal increased during operation.

The nature of the intensity distribution over the surface of the petri dish can be obtained from the knowledge that within an approximate distance of $D^2/4\lambda$ from the radiating surface of a plane circular disc, the beam will be approximately parallel (D being the diameter of the disc and λ the wavelength).[28]

The crystals used in the experiments (except for 0.1 MHz) have a diameter of 35 mm; since the wavelength is 3 mm for 0.5 MHz, 1.5 mm for 1.0 MHz, 0.7 mm for 2.0 MHz, and ~0.3 mm for 3.3 MHz,[50] the beam will be parallel for distances from ~100 mm for 0.5 MHz to 1000 mm for 3.3 MHz.

D. Production of Audiofrequencies

To produce audiofrequency vibrations, electrical signals of desired frequency produced by an audiofrequency oscillator are converted into mechanical motion using a transducer or vibration exciter, which consists of a moveable coil suspended in a strong dc magnetic field. When an alternating current is passed through a coil of wire located in a magnetic field, a force of alternating direction is produced. This causes mechanical motion of the coil. If a sine wave signal is applied to the coil, sinusoidal motion results. By controlling the frequency and amplitude of the signal applied to the moving coil, the force and magnitude of vibration can be controlled. Parts mounted on the exciter table, which is rigidly attached to the coil, can therefore be forced to experience controlled vibration.

E. Dosimetry for Audiofrequencies

The intensity of audiofrequency sound was measured using an accelerometer (Columbia Research Laboratories Model 650-1). An accelerometer is a transducer which developes an electrical signal proportional to acceleration when placed on a vibrating object. These are calibrated to give the acceleration in 'g' units where

$$g = 0.0512f^2.2A,$$

(f being the frequency and A the displacement amplitude)[89] from which one can calculate the intensity in W/cm^2, knowing that

$$I = 2\pi^2 f^2 A^2 \rho c$$

(see page 18).

III. EXPERIMENTAL METHODS AND MATERIALS

A. Cell Culture

Puck and his associates[75] were the first to introduce the technique of growing single mammalian cells *in vitro*. The general principles and techniques have since been described extensively by a number of authors.[90,91,22] The materials and techniques, as used in this laboratory, have been described in detail by Todd[93] and by Siegel[94], so only what is specific to this study will be described here.

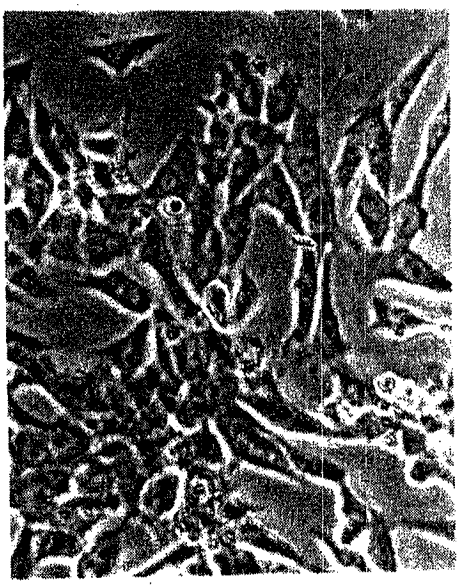
The cultures are maintained in a water-jacketed CO_2 incubator (National Appliance Company Model 3221), which is supplied with a mixture of air and CO_2 so that the final concentration of CO_2 is 5%. The air passes through two filters to remove any moisture which may carry contamination. The mixture of gases is bubbled through water kept in a tray at the bottom of the incubator. This keeps the incubator humid and prevents evaporation of medium from the dishes. The flow rate of CO_2 is adjusted to maintain the pH between 7.2 and 7.4 as indicated by phenol red which is present in the medium. All work requiring sterility is done in a Lab Con Co. hood fitted with an ultraviolet lamp which is turned on for about a minute before the start of work.

Stock cultures of the following cell lines are maintained routinely in the laboratory by subculturing at intervals of 4 to 5 days:

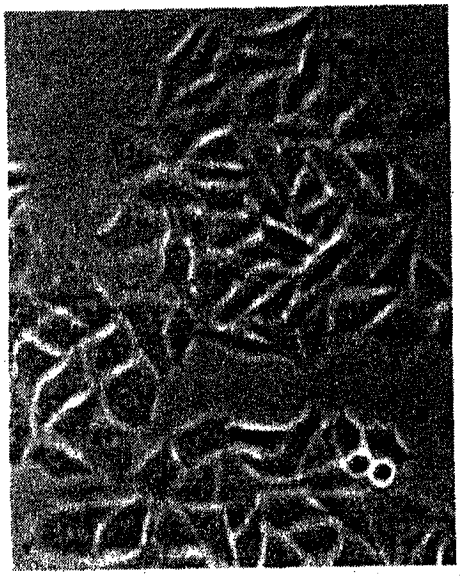
(1) M3-1 cells. These cells are derived from the bone marrow of a male adult Chinese (striped back) hamster (*Cricetulus griseus*) (Fig. 3a).

(2) T-1 cells. A subline derived originally from a normal human kidney (Fig. 3b).

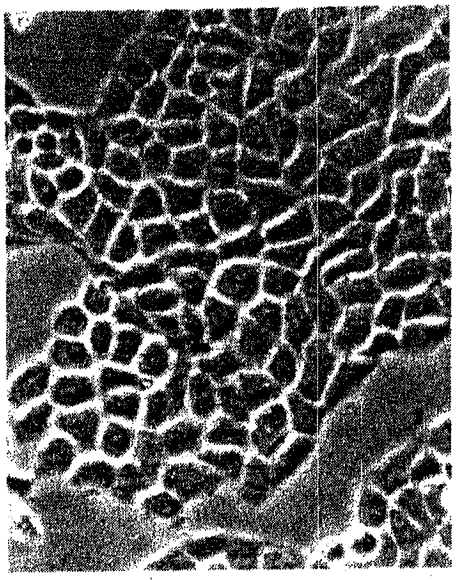
(a)



(b)



(c)



(d)

Figure 3. 2 to 3-day old cultures of (a) M3-1, (b) V79, (c) T-1 and (d) Chang's cells

(3) V79 Cells. Derived from a subline of V79-1 Chinese hamster lung cells (Fig. 3c).

(4) Chang's. Derived from human liver (Fig. 3d).

Stock cultures are grown in 100-mm Falcon plastic dishes (Cat. No. 3003). For sub-culturing, a dish having 1/2 to 2/3 of its surface covered with cells is selected and trypsinized as described below. After resuspension in fresh medium serial dilutions are made into 100-mm Falcon plastic dishes containing 10 ml fresh medium to give 1/10, 1/100, and 1/1000 of the original cell concentration for M3-1 and V79 cells, and 1/5, 1/10, and 1/100 for T-1 and Chang's.

Trypsinization is done as follows:

(1) Aspirate medium out of the dish and rinse with 5 ml of 0.03% trypsin solution.

(2) Add 3 ml of the trypsin solution and incubate for about six minutes at 37°C with gentle agitation after three minutes.

(3) Add 7 ml of fresh medium and pipette repeatedly with strong blowing to break up clumps of cells.

The composition of the solutions used is:

(1) H.U.-15 medium (for M3-1 and V79 cells)

500 ml Eagle's MEM

22 ml NCTC 109

6 ml Penicillin - Streptomycin (5000 units each/ml)

75 ml Fetal Calf Serum

6 ml L-glutamine

(2) T-1 medium (for T-1 cells)

500 ml Eagle's MEM

50 ml Fetal Calf Serum

6 ml Penicillin and Streptumycin (5000 units each/ml)

6 ml L-glutamine

(3) Chang's Medium (for Chang's cells)

BME, Based Med. (Eagle's) with Hank's Salt Solution (IX)..... 500 ml

BME, amino acids (100x)..... 7 ml

MEM non-essential amino-acid solution (100x)..... 12 ml

Calf serum (heat inactivated)..... 60 ml

7.5% Na-Bicarbonate..... 13 ml

L-glutamine - 200 mM (100x)..... 6 ml

Adjust pH to ~7.2 with 1N NaOH

(4) Trypsin. Dissolve 30 mg trypsin (lyophilized trypsin 220 μ/mg Worthington Biochemical Corp.) in 100 ml Puck's saline A (1x); filter through 45 μNalgen filter and add 1 ml pencillin and streptomycin.

B. Ultrasonic Equipment

One of the major difficulties in the analysis of the results of the experiments in ultrasonics that are reported in the literature is the variety of equipment used by different investigators. More often than not details are lacking regarding the method of exposure and the physical parameters of ultrasound.

At the beginning of this investigation we had a commercially available ultrasonic generator (Tomac, Model 1700) made by the American Hospital Supply Corporation and meant for diathermic treatment. It consisted of an X-cut quartz crystal transducer connected to a Hartley oscillator circuit. A timer, a meter to indicate ultrasonic energy in W/cm^2, and a knob to control the intensity were incorporated into the circuit. It was meant to be a 1-MHz frequency generator, but initial investigations soon showed that the intensity-control knob was in fact a frequency-control knob and that the intensity was controlled by detuning.

The electronic circuitry (Fig. 4) was modified by A. Windsor of Donner Laboratory. It is now possible to change the intensity by varying the plate voltage. The plate voltage and the plate current are indicated on meters on the front panel and the frequency is measured on a digital frequency meter made by Beckman Instruments, Incorporated (Eput meter model 8170R). The transducer is made by H. G. Fischer and Company. It is fabricated by cementing a 10-cm^2 X-cut quartz crystal to the inner surface of a cup-shaped metal transducer assembly. The voltage is fed through a coaxial cable and connects to the surface of the crystal by means of a spring loaded round aluminum plate.

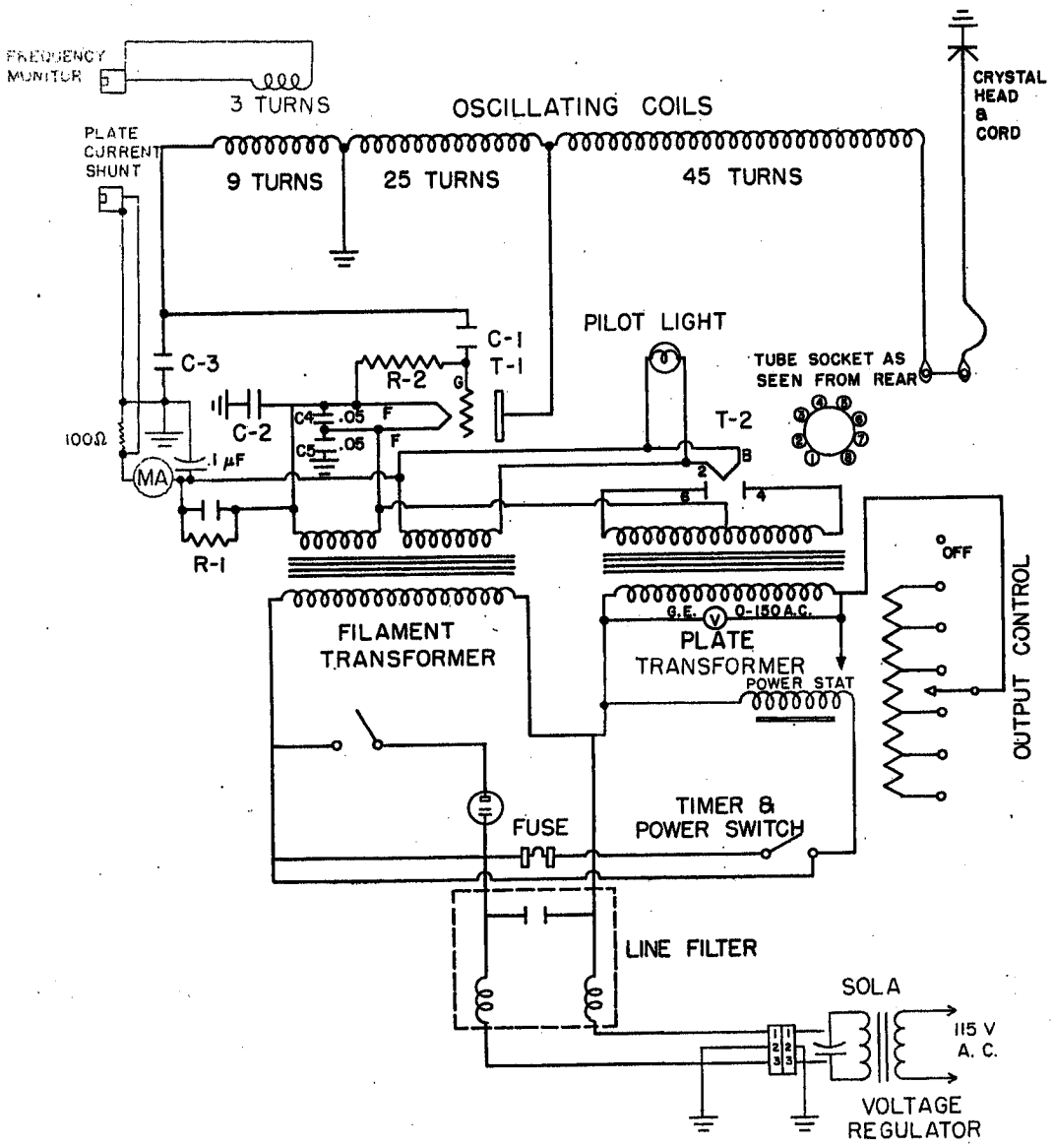


Figure 4. Modified electronic circuitry for the Tomac 1.0 MHz frequency ultrasonic generator. The modifications are shown in light shade.

A second ultrasonic generator (Fig. 5) was designed by A. Windsor to provide 0.1, 0.5, 1.0, 2.0, and 3.3 MHz frequencies. It consists of an oscillator, described below, connected to a transducer with a Hewlett-Packard digital frequency meter to measure the frequency.

The transducers are fabricated as described above except that for 0.1 MHz frequency a 80-cm^2 barium titanate ceramic crystal is used. The oscillator consists of an Eimac 4-400A power tetrode connected in a Hartley oscillator circuit. The coil which resonates to provide the frequency of oscillation plugs into a socket. This permits plugging in of a coil corresponding to the transducer frequency.

The coil and vacuum tube plate are operated at ground potential while the filament circuit can be raised to minus 2,000 volts dc at 250 ma. Plate voltage and plate current are metered on the front panel.

Tests show the output to be about 5000 V peak to peak maximum to a series of quartz transducers. The amplitude is constant to about 1% because of the stability of the load and the line voltage regulator mounted at the bottom of the rack. The output frequency holds to approximately 0.1%. Air dielectric capacitors and a quartz transducer at resonance combine to give a stable frequency with a simple and flexible circuit.

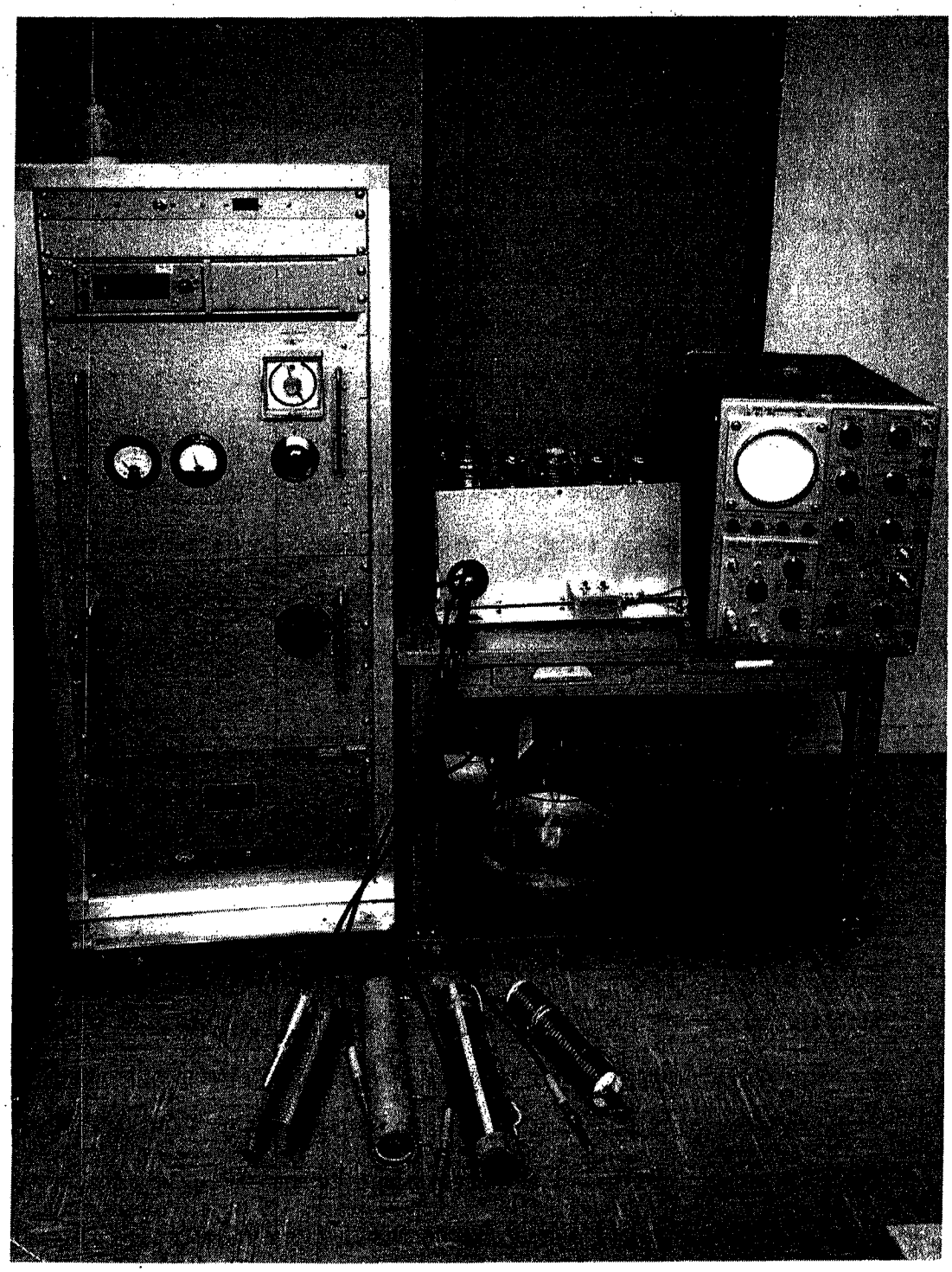


Figure 5. Ultrasonic equipment for 0.1, 0.5, 1.0, 2.0, and 3.3 MHz frequencies.

C. Audiofrequency Equipment

It consists of the following:

(1) Audiofrequency oscillator. Hewlett-Packard low-frequency oscillator (Model 202C) which generates sine waves over the 1 to 100,000 cycles per sec range. Special circuitry insures a wave form of high stability and low distortion that is independent of the load connected to the instrument.

(2) MB Vibramate vibration exciter (Model EA1250) and electronic power amplifier (Model 2120 MB). Mechanical motion is produced by the passage of electric current through a magnetic field. The exciter uses ceramic permanent magnets which offer many advantages. It is usable to 20,000 cps with a maximum 0.5" displacement amplitude limit. The amplifier consists basically of three stages: a differential amplifier type preamp stage, a push-pull voltage amplifier drive stage, and a push-pull parallel power output stage. It is capable of delivering 125 μA in the frequency range 35 to 10,000 cps. It drives the vibration exciter.

D. X-ray Equipment and Dosimetry

X-ray irradiations were performed using Norelco MG 150 Industrial X-ray unit operated at 150 kV and 12 mA and filtered through 1 mm Al. The unit uses Norelco 150 kV beryllium-window tube which has an inherent filtration value of 3 mm Be. The emergent beam angle is 40°.

Exposures were carried out with the cells attached to the petri dishes which contained 2 ml medium and were covered. Samples were exposed, one at a time, at a distance of 26 cm from the focal spot.

The dose was measured with a Victoreen condenser r-meter, with its sensitive volume at the position occupied by the cells. At the energy used, one r corresponds to 0.9 rad in tissue. The dose rate used for all the synergistic experiments was 600 rad/min.

E. Experimental Technique

About 16 hours before the start of an experiment, a 3 to 4 day old culture dish having a desired number of cells (10^5 to 10^6 cells/ml) is selected, the cells trypsinized, resuspended in fresh medium and incubated. This is done to have cells in log phase as well as a cell suspension with low multiplicity for the experiment. At the time of the experiment the same culture dish is retrypsinized, and the cells are suspended in fresh medium; a cell count is made with a hemocytometer or a Coulter counter. Serial dilutions of 10^5, 10^4, 10^3 cells per ml are made from the original suspension.

Aliquots (0.2, 0.1, or 0.05 ml) from the dilutions are plated into 35-mm Falcon plastic petri dishes (catalog number 3001). The number of cells plated per dish is such that at the end of the experiment one has approximately 100 colonies per dish. This requires doing preliminary experiments to determine the expected fractional survival. (The total volume of medium per dish is 2 ml as made up by adding the necessary amount of cell suspension and fresh medium).

The dishes are incubated for 4 to 6 hours at 37°C. This permits the cells to attach themselves to the bottom of the petri dish, recover from any trypsinization damage, and enter the log phase. The dishes are then exposed to the ultrasound. To have maximum transfer of acoustic energy from the crystal to the petri dish, glycerine is used

as a coupling agent. If the petri dish and the transducer are placed in direct contact, they will be in contact only in a few places unless they are both optically flat; in practice a layer of air will separate them. Very little sound energy will then be transmitted; however, a layer of glycerine greatly improves the degree of coupling because of the very large increase in the characteristic impedance of the intervening layer.[28]

After treatment the dishes are reincubated at 37°C for seven days (M3-1, V79 cells) or 12 days (T-1, Chang's cells). After the incubation period the colonies are stained for about 30 min by adding about three drops of 1% aqueous methylene blue directly to medium in each dish. Then the medium is decanted and the dishes rinsed gently with water and inverted to dry overnight. Only the colonies visible to the naked eye are scored. For each experiment four dishes are used for each dose point, and the data points plotted in the graphs are the mean of at least three experiments.

$$\text{Fractional survival} = \frac{\text{number of colonies in treated dish}}{\text{number of cells plated}} \times \frac{1}{\text{P.E.}}$$

$$\text{Plating efficiency (P.E.)} = \frac{\text{number of coloines in control dish}}{\text{number of cells plated}},$$

results are reported only from those experiments where the P.E. was more than 60%. The dose is the dose rate times the time of exposure.

Method of ultrasonic exposure is shown in Fig. 6. The ultrasonic equipment was turned on for about one hour before the start of exposures. About 5 to 6 drops of glycerine were placed on the transducer; a 35-mm plastic petri with 2 ml of medium was placed on the transducer and pressed lightly to squeeze out the excess glycerine. The voltage was turned up and the frequency knob adjusted to give maximum turbulence

in the petri dish. The frequency knob was then fixed at that position and the voltage set to give the desired intensity.

Everytime the dish was changed, fresh glycerine was added onto the transducer; but there was no need for retuning. The frequency, voltage, and current were constantly monitored. The variations in voltage and frequency were mostly insignificant, but occasionally there would be about a 10% change in the current; this resulted in an unusually different survival in those dishes for which the current had changed as compared with the dishes for which the current had not varied. Except for these obvious variations, the reproducibility of the physical parameters, as well as the biological results in terms of fractional survival, were very good.

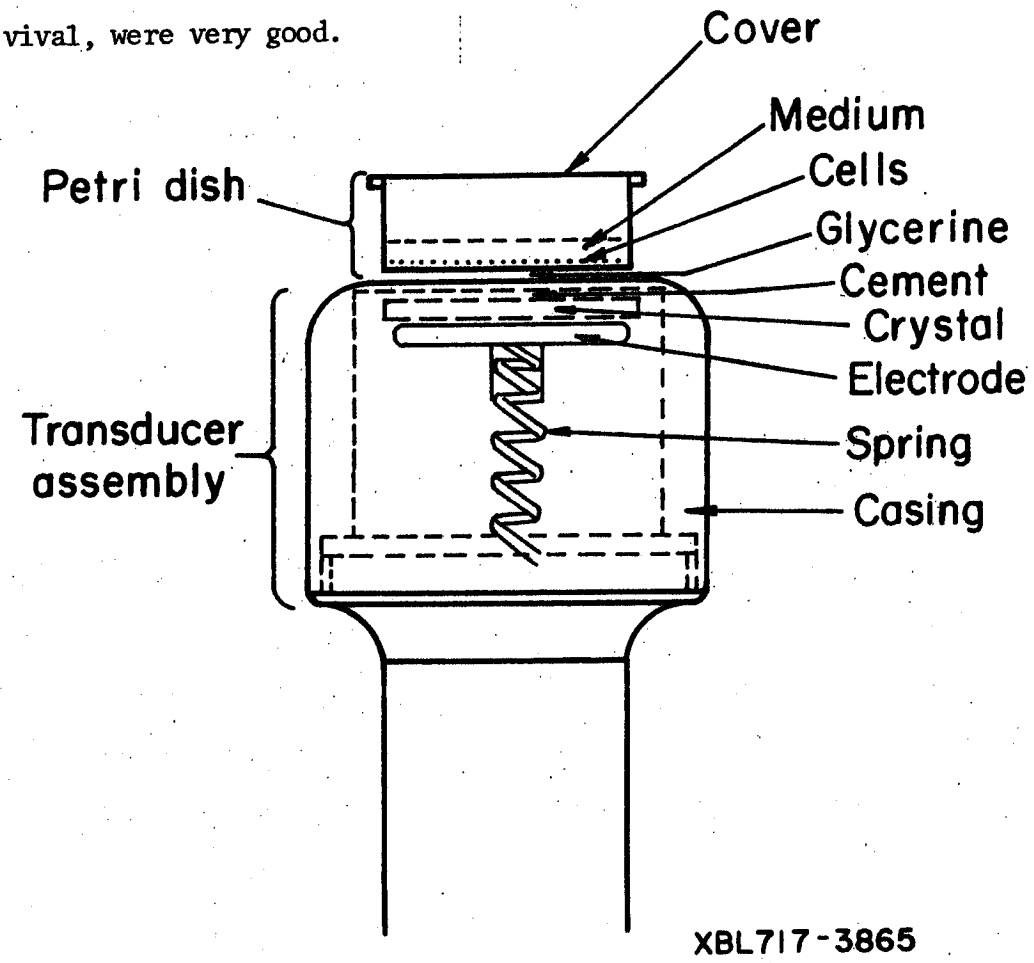


Figure 6. Schematic representation of the exposure setup.

IV. OBSERVATIONS AND RESULTS

A. Reproducibility of Data

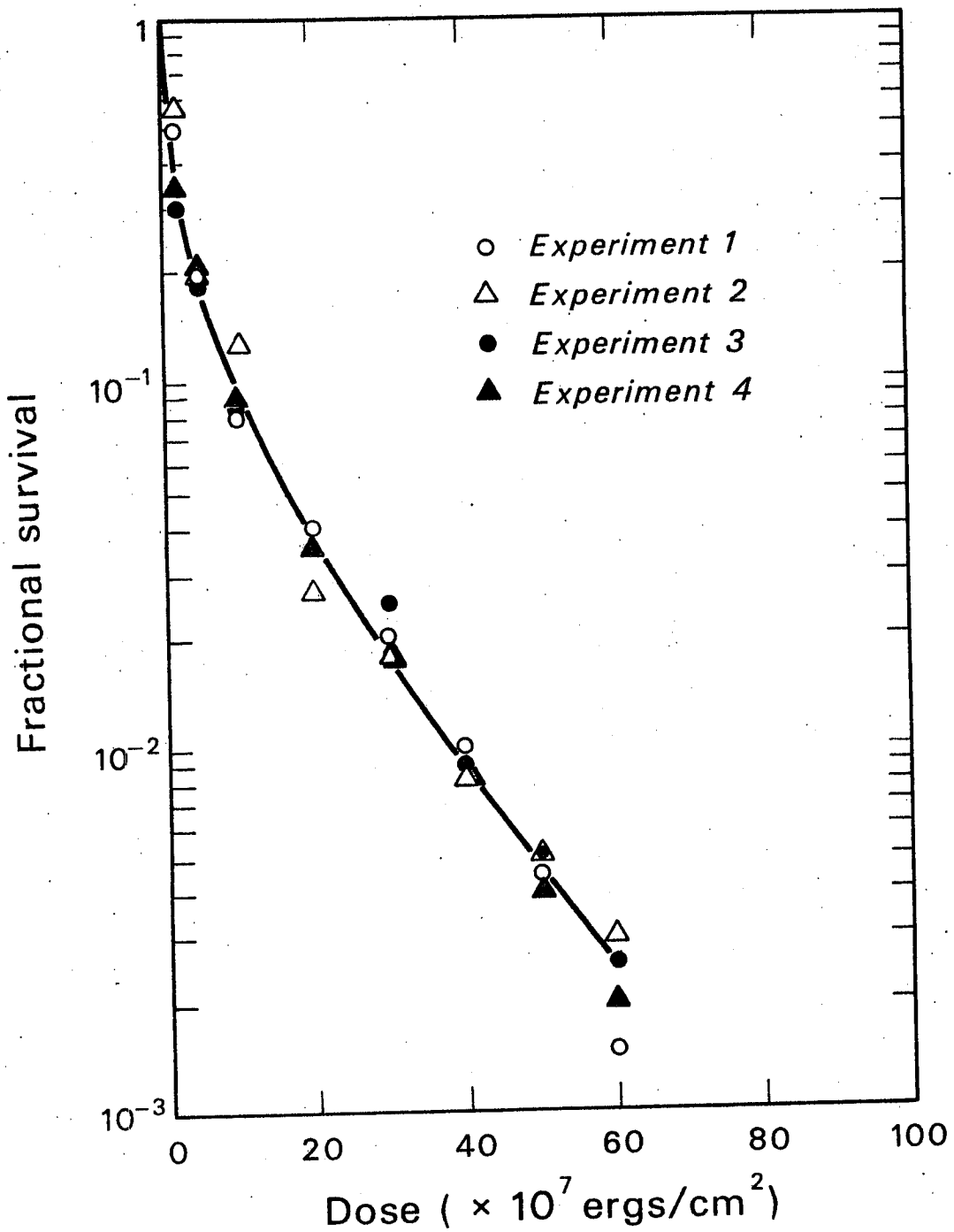
Because of the nature of this experimental setup and the fact that the dose measurements refer only to the monitored dose rather than to the absorbed dose, which may have differed for a given monitored dose because of the possibility of variable loss of acoustic energy due to reflection between the crystal and the cells, it was felt that the data might not be reproducible.

To check the reproducibility of the data, four dishes were used for each dose point and each experiment was done at least three times. Table 1 shows the fractional survival as a function of dose in four experiments involving the exposure of M3-1 cells to 1.0 MHz frequency ultrasound at 1.0 W/cm^2. The data are plotted in Fig. 7.

It is seen that the data are highly reproducible. Also, the results of experiments in which several physical parameters were varied over a range which might be encountered in any experiment, show that such factors as temperature at the time of sonication, the amount of medium (both within the limits studied), and whether the cells are attached or suspended do not significantly affect the results.

TABLE 1

SAMPLE NUMBER	DOSE ($\times 10^7$ erg/cm^2)	FRACTIONAL SURVIVAL EXPT. 1	EXPT. 2	EXPT. 3	EXPT. 4
1	2	5×10^{-1}	5.6×10^{-1}	3.3×10^{-1}	3×10^{-1}
2	5	2×10^{-1}	2×10^{-1}	2.2×10^{-1}	1.9×10^{-1}
3	10	8.2×10^{-2}	1.3×10^{-1}	9×10^{-2}	8.5×10^{-2}
4	20	4.0×10^{-2}	2.8×10^{-2}	3.5×10^{-2}	3.5×10^{-2}
5	30	2×10^{-2}	1.8×10^{-2}	1.8×10^{-2}	2.5×10^{-2}
6	40	1×10^{-2}	8×10^{-3}	8×10^{-3}	9×10^{-3}
7	50	4.5×10^{-3}	5×10^{-3}	4×10^{-3}	5×10^{-3}
8	60	1.5×10^{-3}	3×10^{-3}	2×10^{-3}	2.5×10^{-3}



DBL 717-5911

Figure 7. Reproducibility of data. Fractional survival, for four different experiments, of M3-1 cells exposed to 1.0 MHz at 1.0 W/cm^2.

B. Physical Parameters

A series of experiments was done to determine how experimental conditions may influrence the effects of sonication. The following physical parameters were tested:

(1) To determine whether attachment of cells to the petri dish had any influence on the fractional survival, M3-1 cells were exposed immediately after plating when the cells are rounded and in suspension and four hours later when the cells have flattened out and are attached to the bottom of the petri dish. The results are shown in Fig. 8; it is concluded that it makes no significant difference in the fractional survival whether the cells are exposed in suspension or attached to the petri dish. This observation is confirmed by the results of the experiment with asynchronous V79 cells (Fig. 24) in which it is seen that the fractional survival does not change significantly with time after plating. One would expect cells in suspension to receive a higher absorbed dose than cells attached to the bottom of the petri dish when both are exposed to the same monitored dose. This is because suspended particles act as inhomogeneous objects, causing scattering and thereby increasing absorption of ultrasonic energy. Hence cells in suspension should be more sensitive than attached cells; but our experimental data show no significant difference in sensitivity. This is explicable by assuming that cells in suspension have a greater resistance than attached cells, possibly because cells in suspension are spherical and have a smaller membrane per unit volume than attached cells.

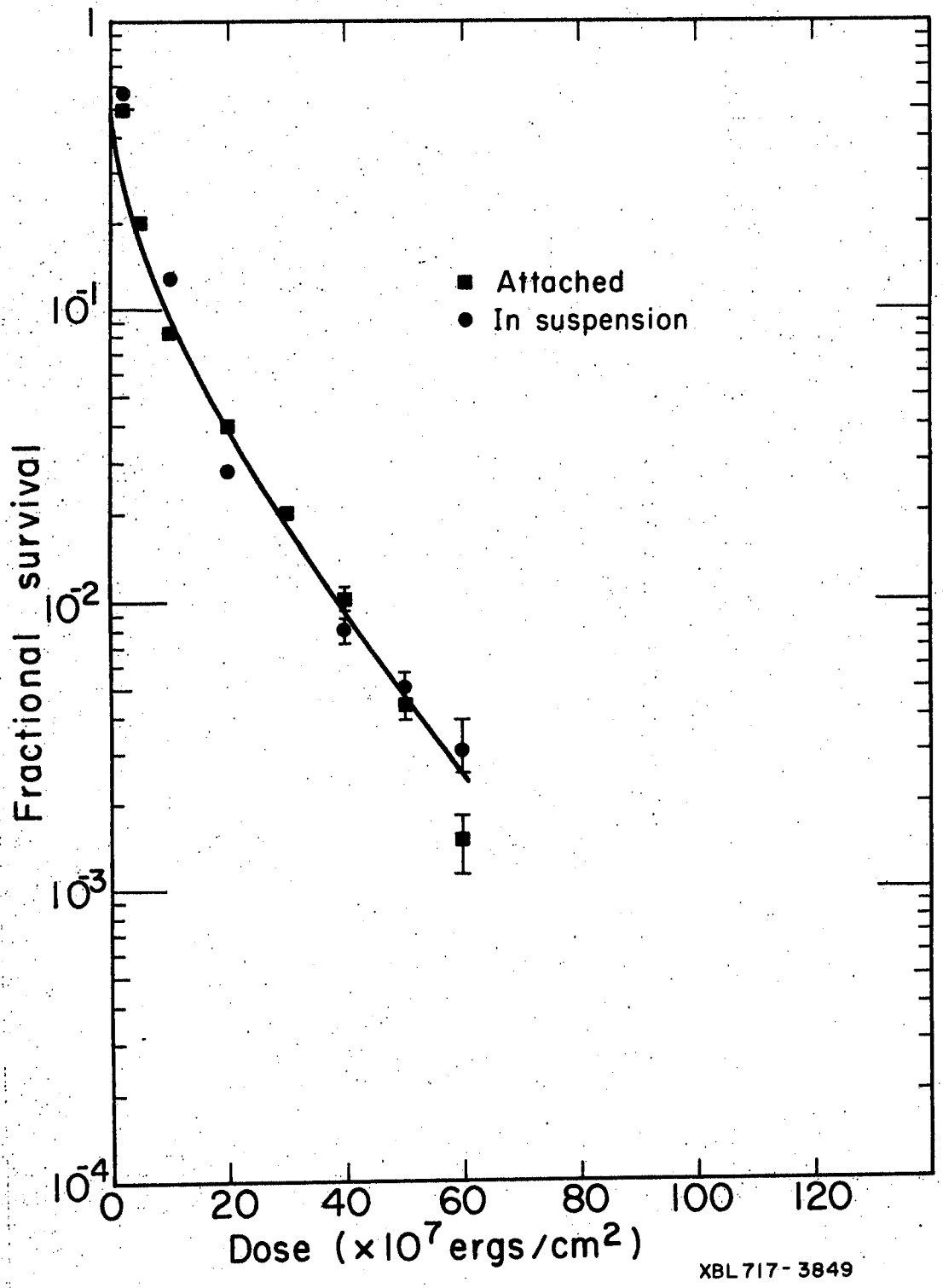


Figure 8. Survival curve of M3-1 cells exposed to 1.0 MHz at 1.0 W/cm^2 in suspension or attached to the bottom of the petri dish.

(2) M3-1 cells were exposed to 1.0 MHz at 1.0 W/cm^2 for five and ten sec with different amounts of medium in the petri dish (0.5, 1.0, 2.1, 2.2, 2.5, and 3.0 ml) to see if that would have any effect on the fractional surrival. There was no effect, probably because exposures were under conditions of stationary waves, which condition would not be affected by the variation in the amount of medium within the limits tested.

(3) To test the possibility that ultrasound may affect the medium and thus damage the cells only indirectly, 2 ml of H.U.-15 medium were exposed to 1.0 MHz frequency at 6.0 W/cm^2 for ten min and an aliquot of M3-1 cells added within five sec of exposure. There was no difference in the fractional survival as compared with controls (although cells exposed to that dose would have a fractional survival of less than 10^{-3}). Sonicating the medium by itself apparently does not produce appreciable amounts of stable toxic materials; and free radicals, if formed, are short-lived and low in amount (less than 10^{11}).

(4) M3-1 cells were plated onto the petri dishes; and after four hours when the cells had attached themselves firmly to the dishes, the medium was sucked out and the dishes were exposed to 1.0 MHz at 6.0 W/cm^2 for ten sec. In another set of experiments, M3-1 cells were plated into petri dishes, incubated for four hours, and then placed in a refrigerator at 0 to -5°C for between 10 to 15 min, freezing the medium. The cells were then exposed in this frozen condition to 1.0 MHz at 6.0 W/cm^2 for ten sec. In neither case was there a significant difference in the fractional survival as compared with controls, though the fractional survival for cells exposed to this intensity with the medium is less than 10^{-3} (the P.E. for the frozen cells was only 45%). It would seem

from these experiments that a liquid medium is required for ultrasound to be effective.

(5) To see how the temperature of the medium at the time of exposure may affect the fractional survival, M3-1 cells were exposed to 1.0 MHz frequency and 1.0 W/cm^2 at the following temperatures: 0 to 5°C, 10 to 15°C, 20 to 25°C. The results are shown in Fig. 9, and it is concluded that the temperature of the medium at the time of exposure, within the limits studied, does not affect the survival. This is because the temperature of the medium did not exceed 37°C in any case.

C. Dose-Rate Effect

M3-1 cells were exposed at the following dose rates: 0.125, 0.25, 0.5, 1.0, and 4.0 W/cm^2 for varying times. The results are given in Fig. 10 which shows the fractional survival of M3-1 cells, exposed to 1.0 MHz frequency ultrasonic waves, as a function of dose for different dose rates. The dose is the product of the dose rate and the exposure time.

It is found that at a dose rate of 0.125 W/cm^2 the ultrasound is ineffective in causing reproductive cell death. At 0.25 W/cm^2 and above the higher dose rates are more effective than the lower although the effect seems to reach a plateau.

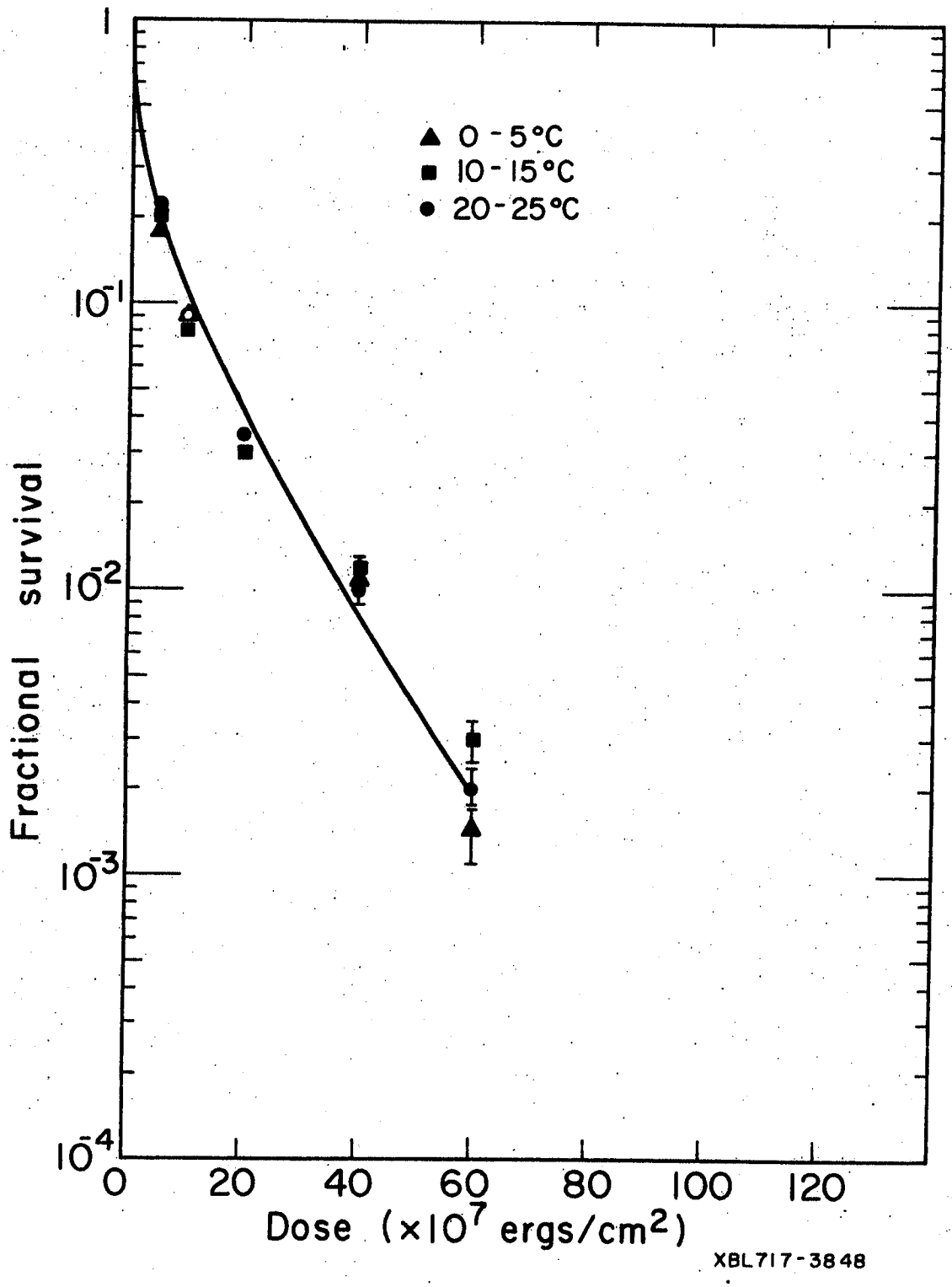


Figure 9. Survival curve of M3-1 cells exposed to 1.0 MHz at 1.0 W/cm^2 at 0-5°C, 10-15°C, and 20-25°C.

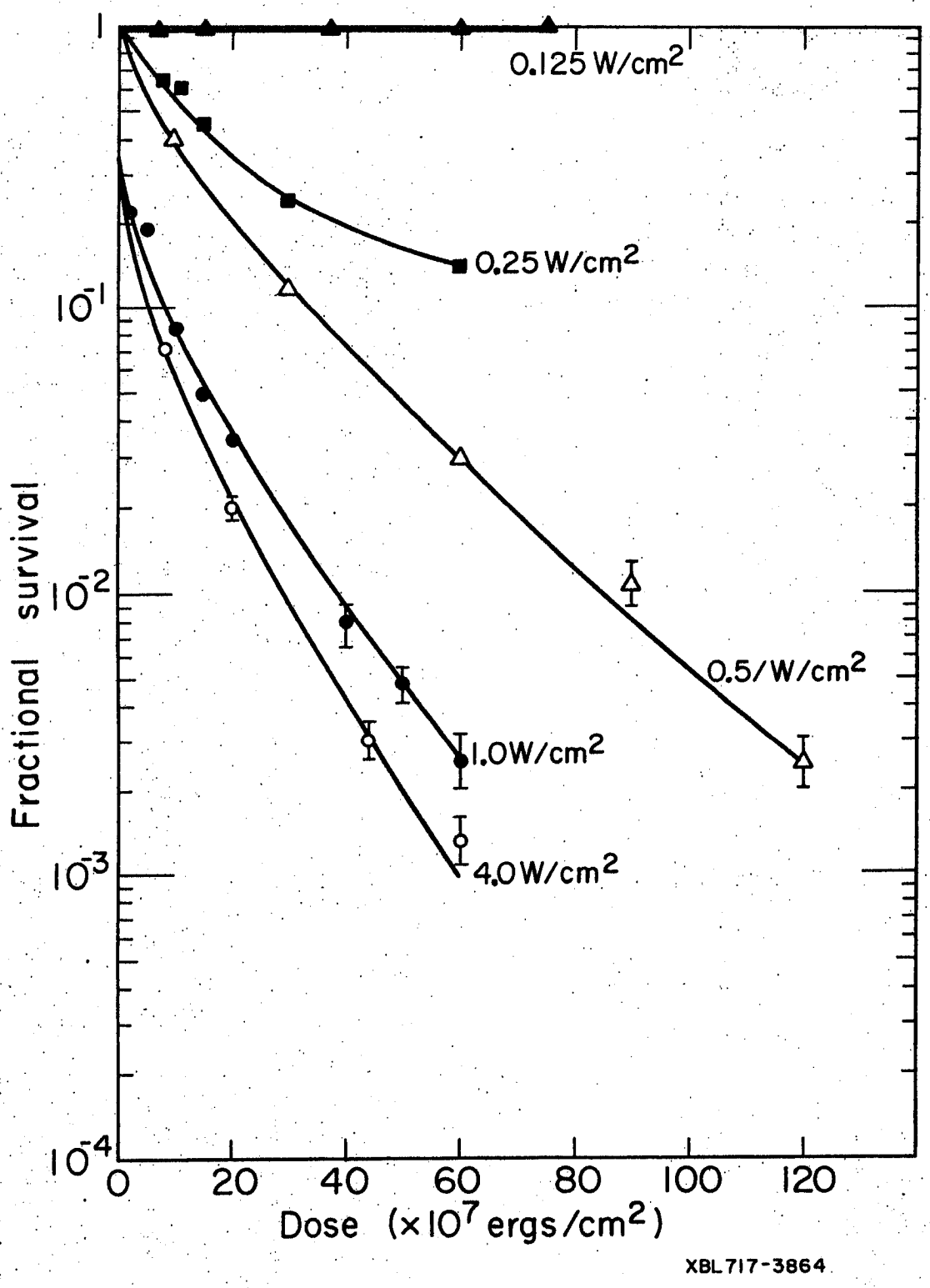


Figure 10. Dose rate effect in M3-1 cells exposed to 1.0 MHz frequency ultrasound.

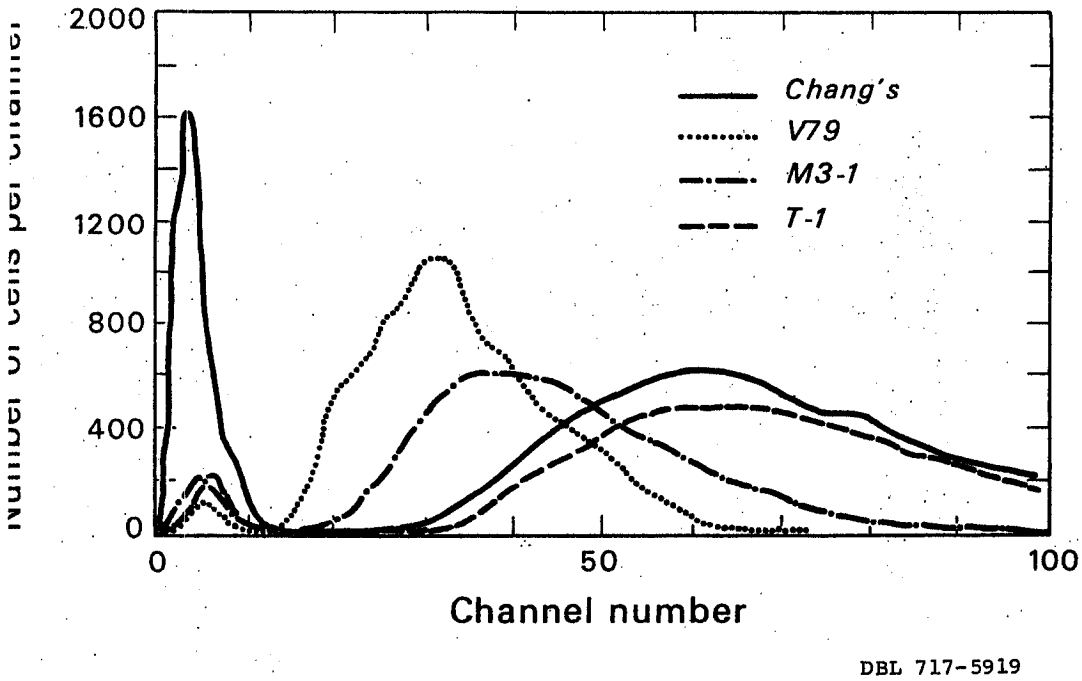


Figure 11. Cell size distribution, in isotone, of cells derived from different mammalian cell lines.

Figure 12 shows the survival curves for the four cell lines at 0.5 W/cm^2 and Fig. 13 shows the survival curves at 1.0 W/cm^2. The shape of the survival curves for the four cell lines is similar and the sensitivities for the four cell lines are not very different. The sensitivity of a cell line to ultrasound does not seem to be related to origin of the cell line, the chromosome number, the doubling time, or the cell-size distribution.

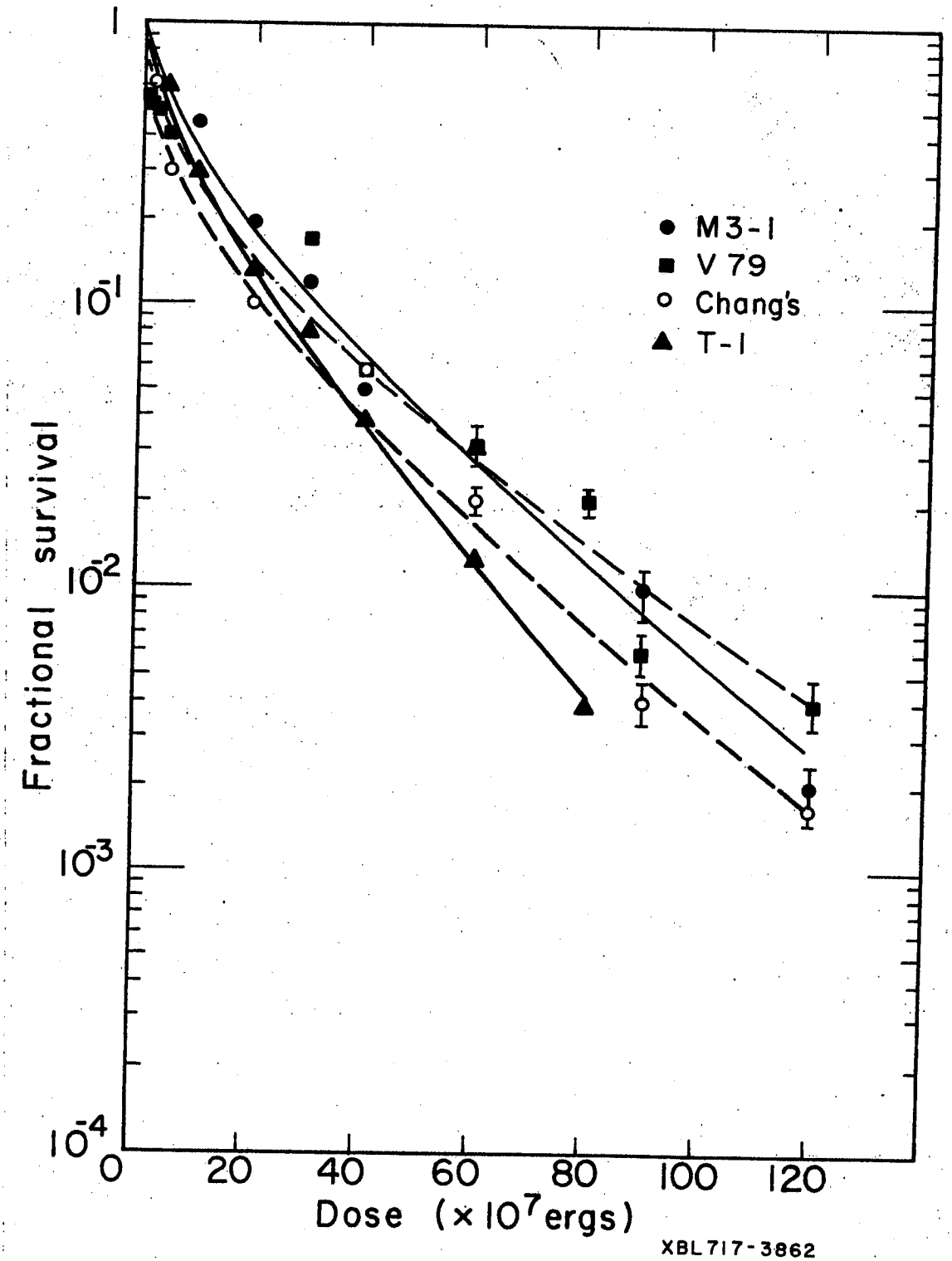


Figure 12. Survival curves for M3-1, V79, Chang's and T-1 cells exposed to 1.0 MHz at 0.5 W/cm^2.

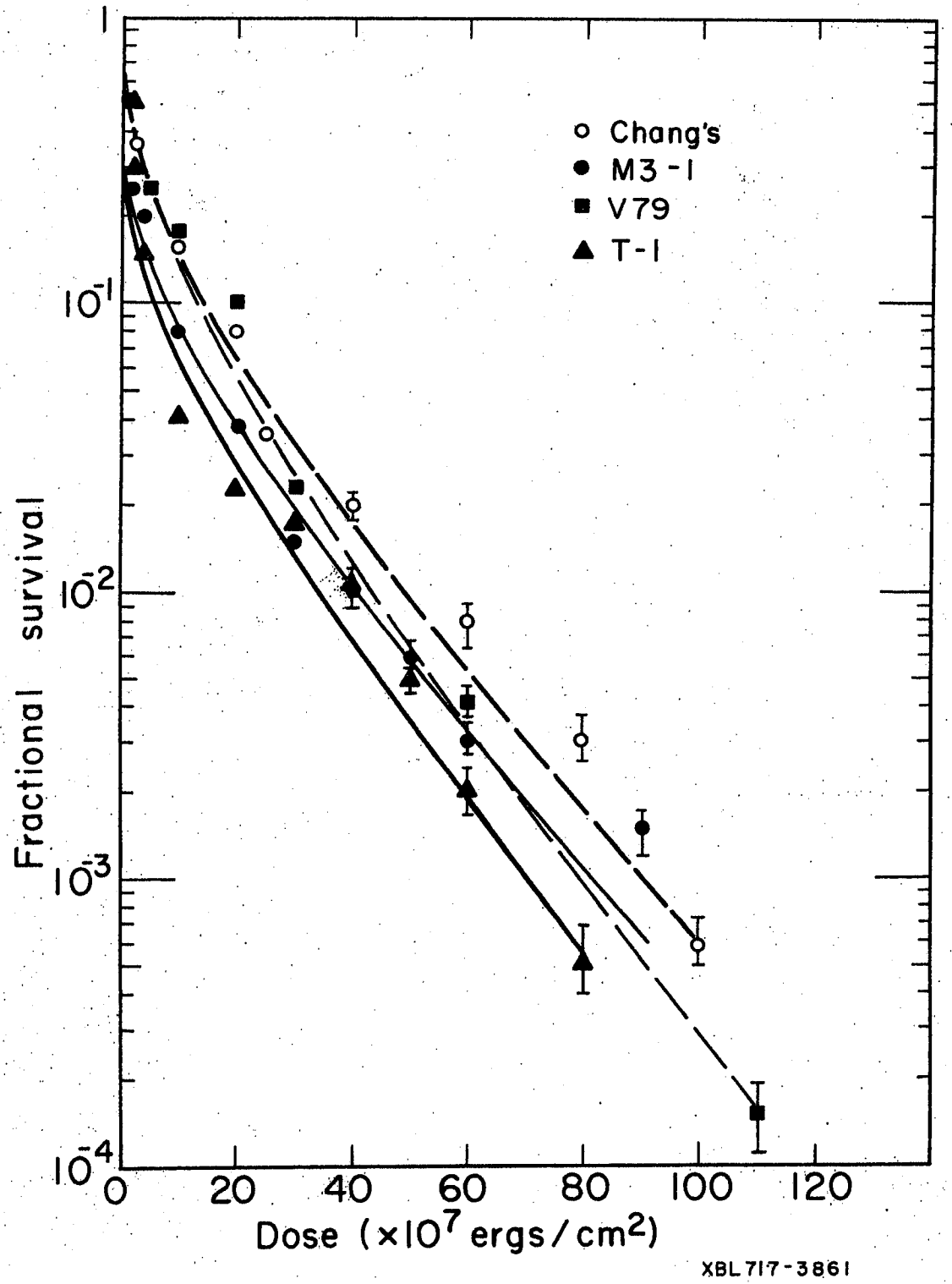


Figure 13. Survival curves for M3-1, V79, Chang's and T-1 cells exposed to 1.0 MHz at 1.0 W/cm^2.

E. Frequency Effect

M3-1 cells were exposed to 0.1, 0.5, 1.0, 2.0, and 3.3 MHz frequencies at two different dose rates. Experimental conditions did not permit exposures to be made at the same dose rate for the different frequencies; but since the exposure conditions are the same in every case except for 0.1 MHz, the dose rate (I) in W/cm^2 for the various frequencies can be obtained from the following relation:

$$I = kV^2f^2$$

where k is a constant and V and f are the voltage and frequency, respectively. For 0.1 MHz, the transducer is barium titanate crystal instead of quartz; the results obtained with that frequency are presented in Fig. 14 simply to show the dose-rate effect and the similarity of the survival curves.

Figures 15 to 18 show the survival curves for 0.5, 1.0, 2.0, and 3.3 MHz frequencies. By comparing the results, it is seen that 0.5 MHz is the most effective of the frequencies tested; but it appears that the effectiveness further increases at still lower frequencies.

F. Aduiofrequencies

There are many reports on the biological effects of audiofrequencies.[96,97,98] To study the effect of audiofrequencies on the reporductive integrity of mammalian cells, M3-1 cells were exposed to different frequencies over the audiofrequency range. The frequencies used were arbitrarily chosen to include the whole range of audiofrequencies. For a given frequency, the maximum intensity used was fixed by the maximum output from the equipment and was kept low enough so

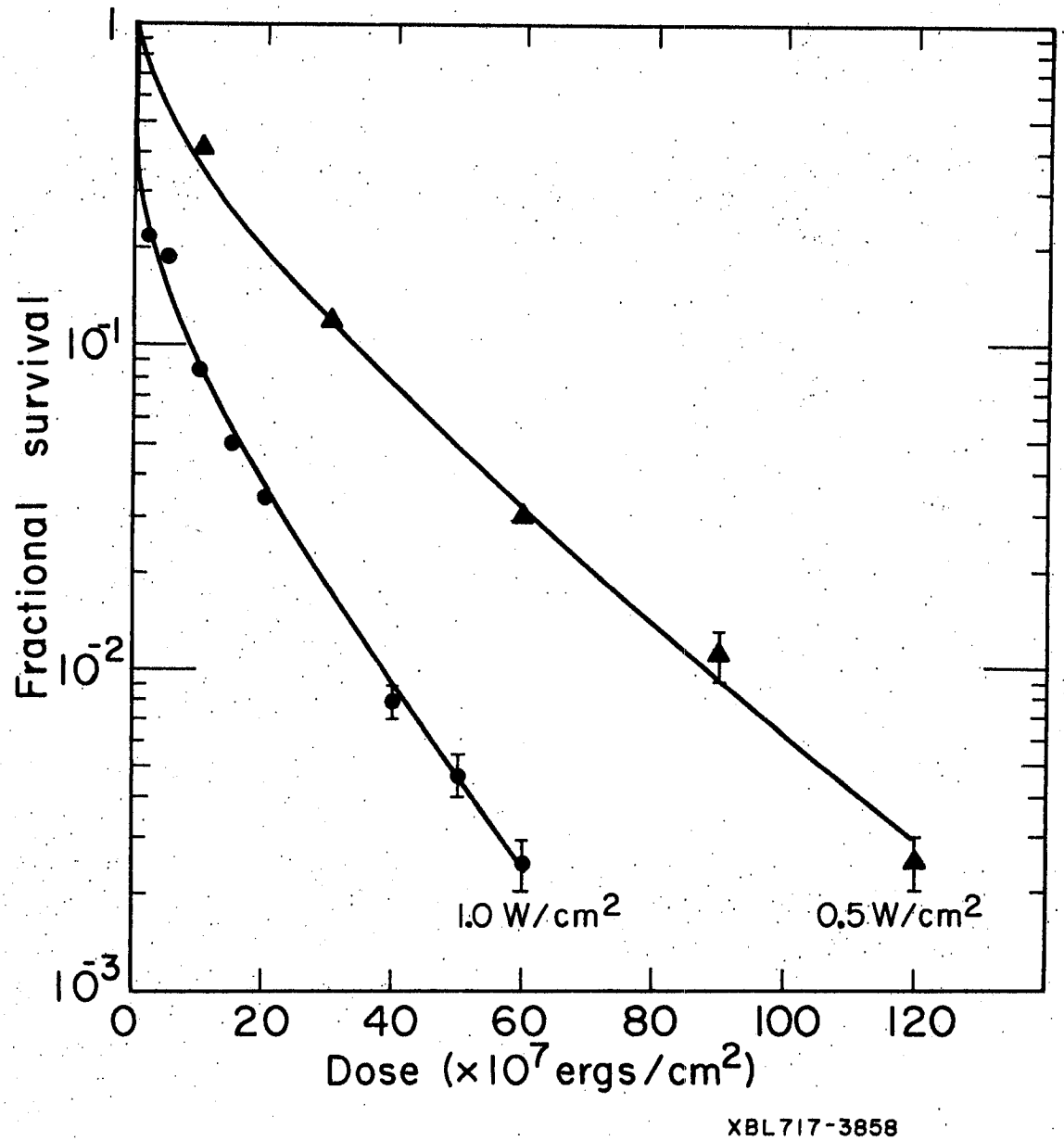


Figure 16. Survival curves for M3-1 cells exposed to 1.0 MHz at 0.5 and 1.0 W/cm^2.

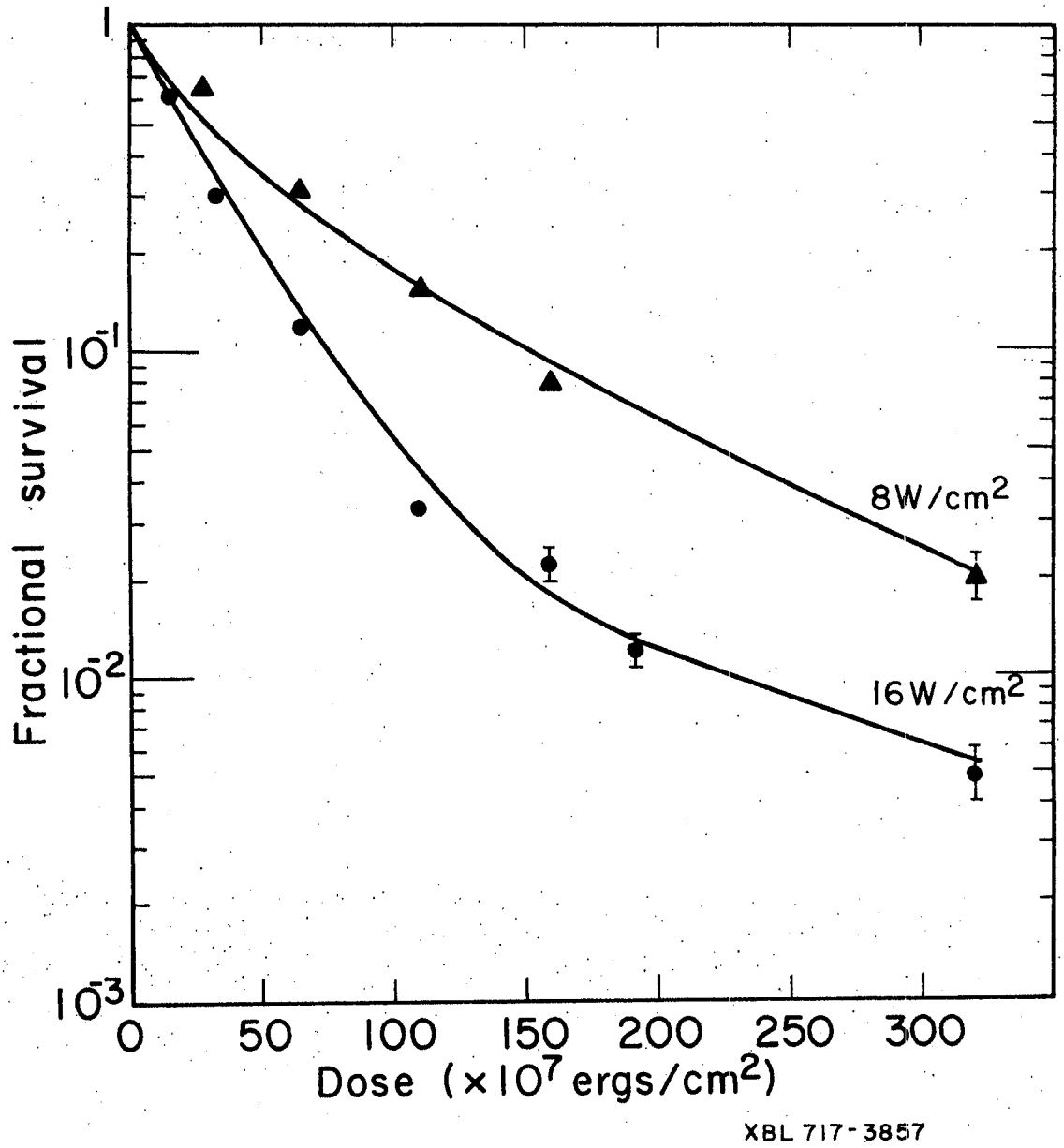


Figure 17. Survival curves for M3-1 cells exposed to 2.0 MHz at 8.0 and 16.0 W/cm^2.

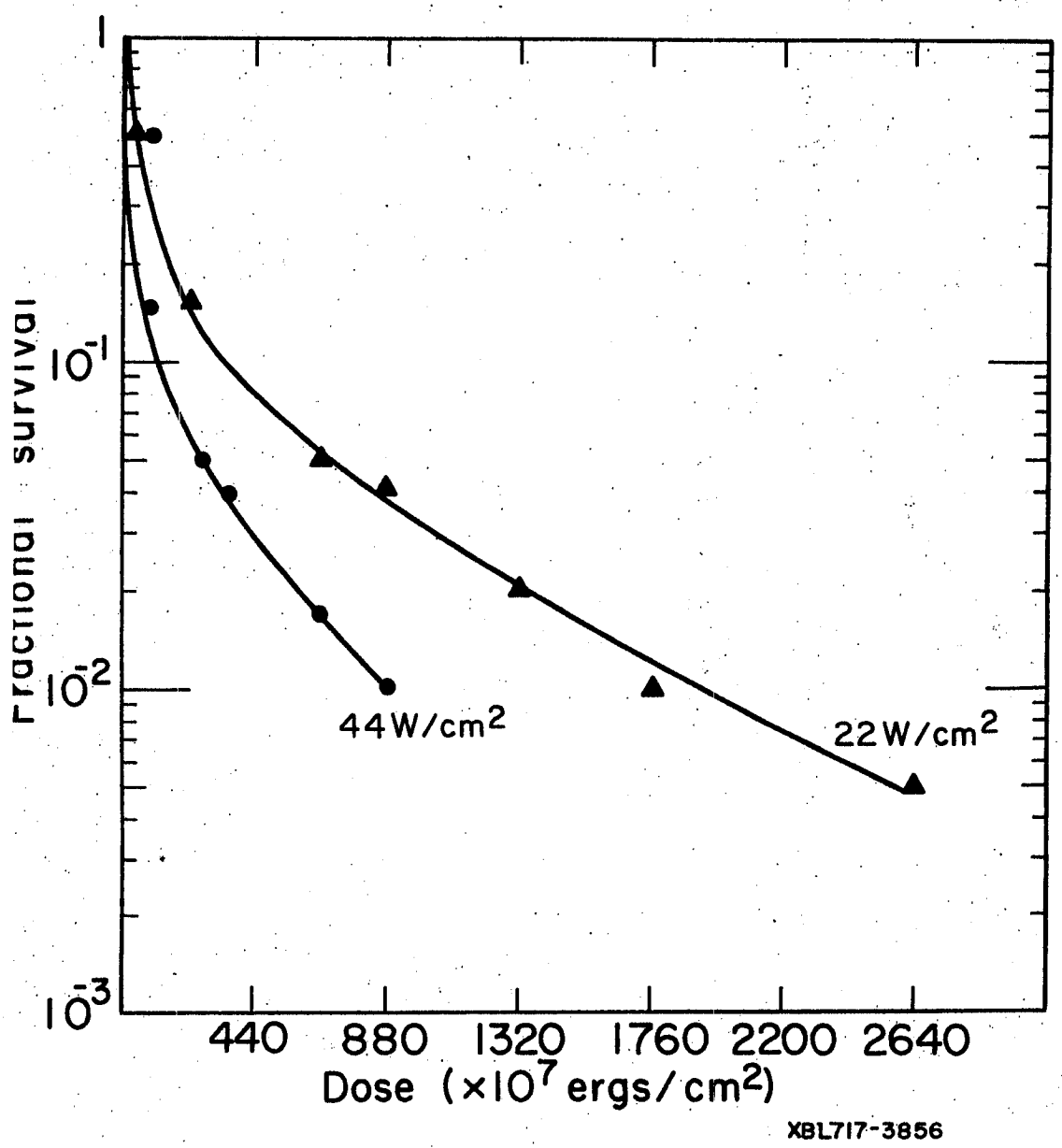


Figure 18. Survival curves for M3-1 cells exposed to 3.3 MHz at 22.0 and 44.0 W/cm^2.

that vibrations did not dislodge the petri dish from the transducer. The maximum intensity for the different frequencies was of the order of 10^{-2} W/cm^2, which is the threshold for pain. The maximum doses used were also much lower than those used for the ultrasonic frequencies. The following frequencies were tried: 20, 50, 60, 80, 100, and 500 Hz; 1, 5, 7, 9, 10, 12, 15, and 20 kHz. In every case the exposures were for two minutes.

The fractional survival varied between 1.0 and 0.95 as compared with that of the control cells. It is concluded that audiofrequencies at the doses used are ineffective in causing reproductive cell death in M3-1 cells. This is not surprising since the doses used are much lower than for ultrasonic frequencies.

G. Dose Fractionation

The split-dose technique has been used extensively in radiobiology to demonstrate "recovery" phenomena in cells exposed to X-rays;[76] we did similar split-dose experiments with ultrasonic radiation. A given dose was split into two equal parts. The first half was administered four hours after plating, and the second half was given at approximately 15, 30, 45, 60 or 120 minutes after the first exposure. Between exposures the cells were incubated at 37°C. The results are presented in Fig. 19. The experiments were done with M3-1 cells exposed to 1.0 MHz frequency at 0.5 W/cm^2 for 45 sec + 45 sec (22.5×10^7 ergs/cm^2 + 22.5×10^7 ergs/cm^2) and 1.0 W/cm^2 for 5 sec + 5 sec (5×10^7 ergs/cm^2 + 5×10^7 ergs/cm^2).

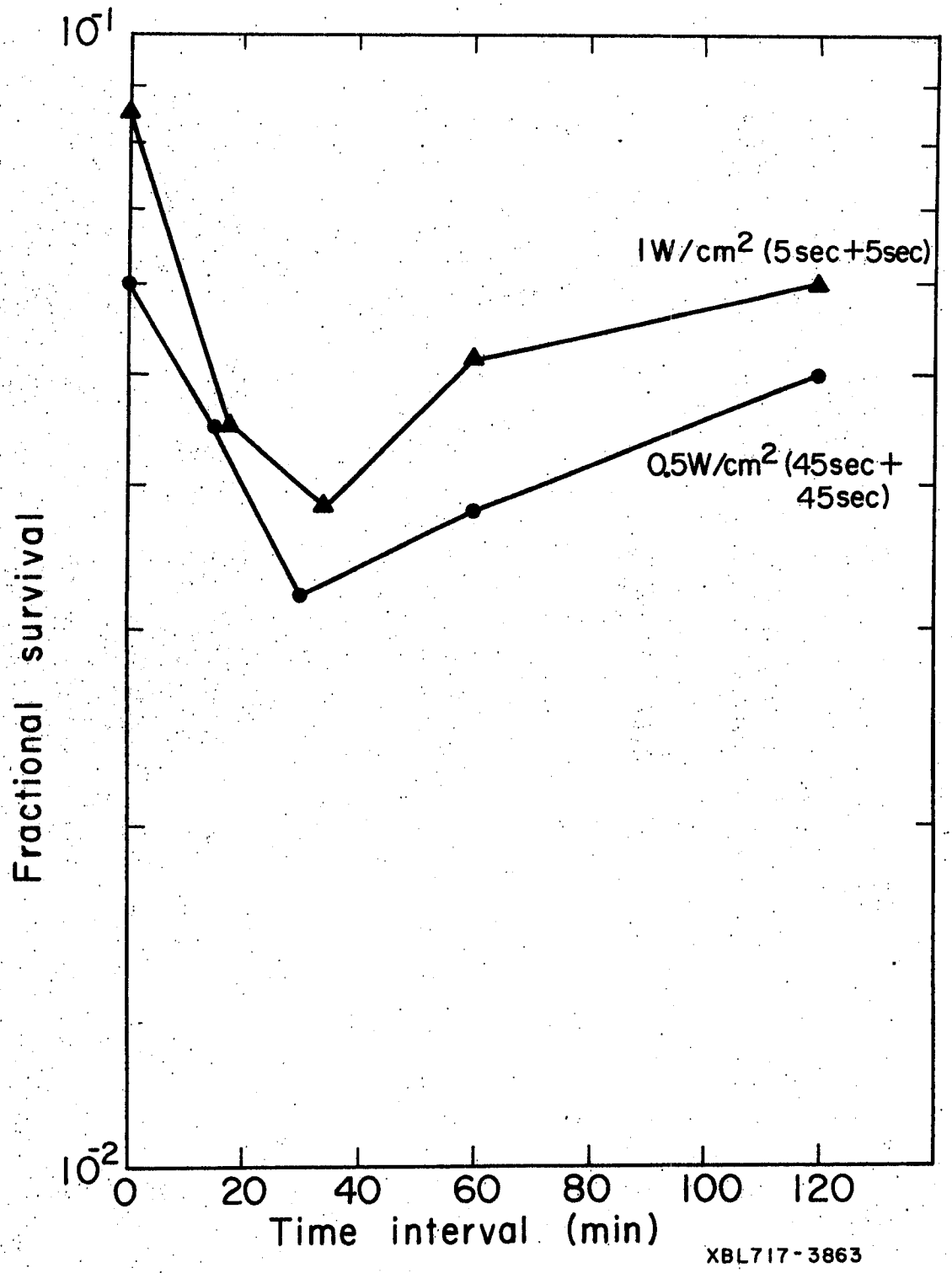


Figure 19. Dose fractionation in M3-1 cells exposed to 1.0 MHz frequency ultrasound.

It is seen that cells exposed to ultrasound are "sensitized" to a subsequent exposure. The maximum sensitivity, in both the experiments, was reached at about 30 min after the first exposure; and the survival was about half of what it would have been if the total dose was given in one exposure. There is some recovery from sensitization, but even at the end of two hours the recovery is not complete. This "sensitization" observed with ultrasonic energy is in contrast to the recovery that is observed in split-dose experiments with ionizing radiations.

H. Synergism

The combined effect of ultrasound and other physical and chemical factors have been studied in several systems. There are many reports of a synergistic effect between ultrasound and other environmental factors in microorganisms.[30] Woeber was the first to report a synergistic effect between X-rays and ultrasound in the regression of Walker carcinoma in rats; Clarke et al.,[73] however, reported that there is no synergistic effect between ultrasound and X-rays in cultured lymphoma cells.

To study the combined effect of treatment with X-rays and ultrasound, M3-1 cells were exposed to either X-rays or ultrasound first and then either immediately (within two minutes) or 30 min later were exposed to the other radiation. The ultrasonic exposures were to a threshold dose or to a dose that resulted in about 40% survival by itself. The frequency was 1.0 MHz. The X-ray doses were such as to cover a survival range from 1.0 to 0.1.

The results are given in Figs. 20 to 23. Figure 20 shows the X-ray survival curve and the survival curves obtained when the cells were exposed to X-rays and within 2 to 5 min to ultrasound, or were first exposed to ultrasound and then to X-rays within 2 to 5 min. Figure 21 is similar to Fig. 20 except that the time interval between treatments was 30 min. In both cases a threshold ultrasonic dose was used.

Figures 22 and 23 are similar to Figs. 20 and 21, respectively, except that in the former the ultrasound dose was such that by itself it gave a 40% survival. In Figs. 22 and 23, the expected survival curve is obtained from the X-ray curve by multiplying the X-ray survival at each dose point by a factor of 0.4.

It is seen that there is a synergistic effect between ultrasound and X-rays even when a threshold ultrasonic dose is used. The degree of synergism depends on 1) the X-ray dose, being greater at higher doses; 2) the time interval between treatments, being more evident when treatments follow each other by a half hour than when treatments are almost simultaneous; 3) whether ultrasound follows or preceeds X-rays (X-rays followed by ultrasound are more effective than ultrasound followed by X-rays).

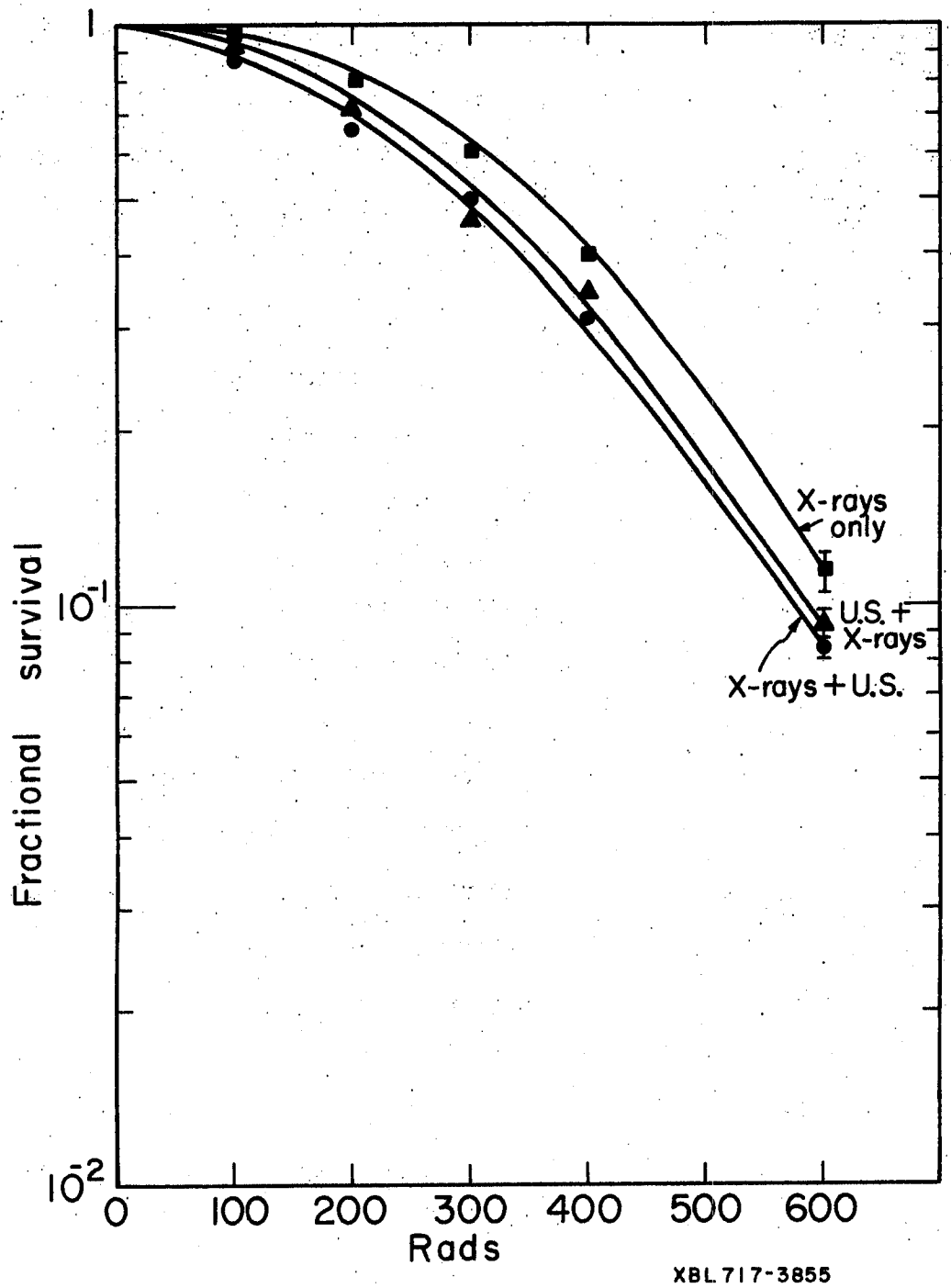


Figure 20. Synergistic effect in M3-1 cells exposed to 150 kV X-rays and 1.0 MHz frequency ultrasound at 0.125 W/cm^2 for 60 sec. The time interval between treatments was 2 to 5 min.

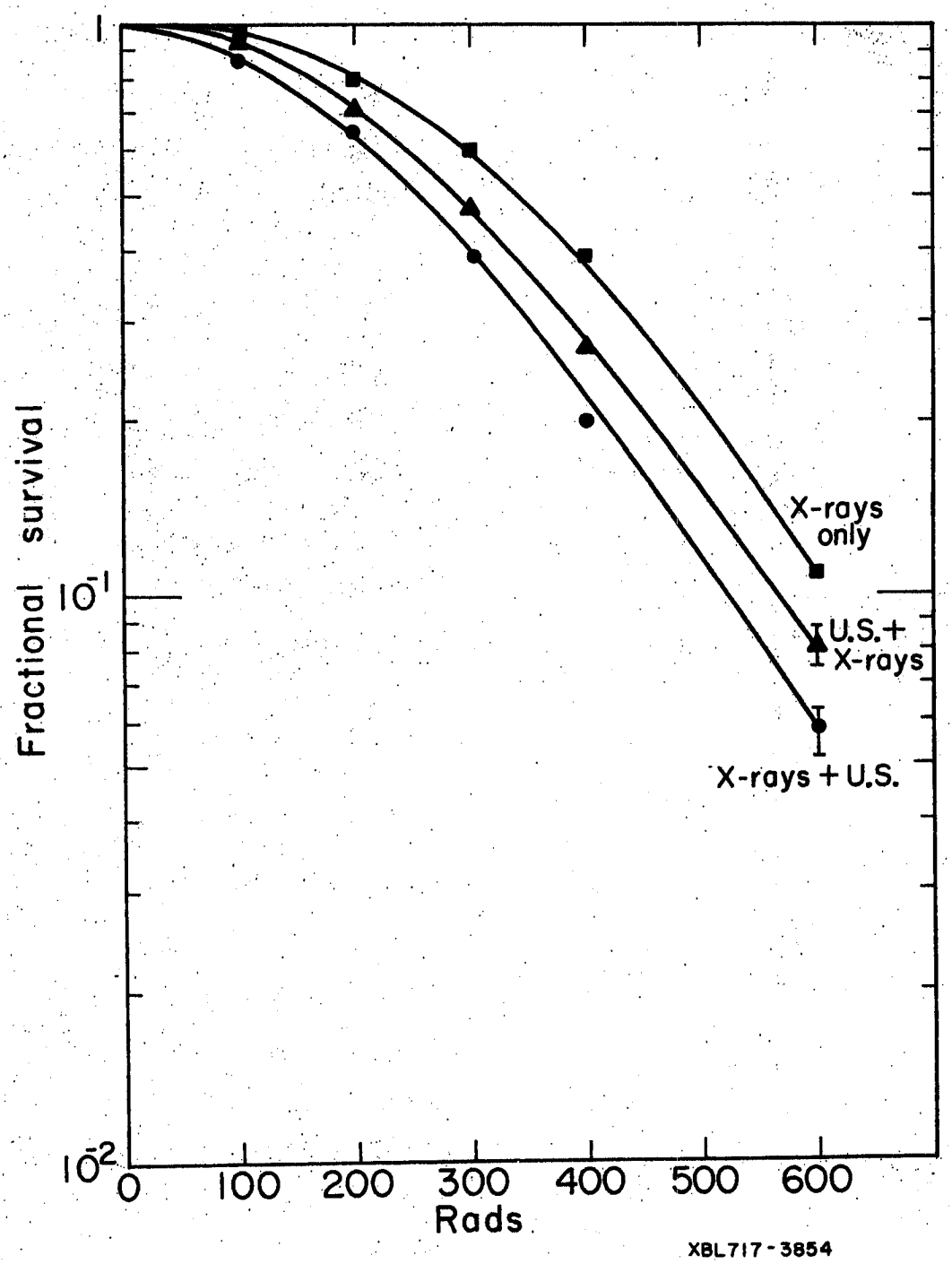


Figure 21. Synergistic effect in M3-1 cells exposed to 150 kV X-rays and 1.0 MHz frequency ultrasound at 0.125 W/cm^2 for 60 sec. The time interval between treatments was 30 min.

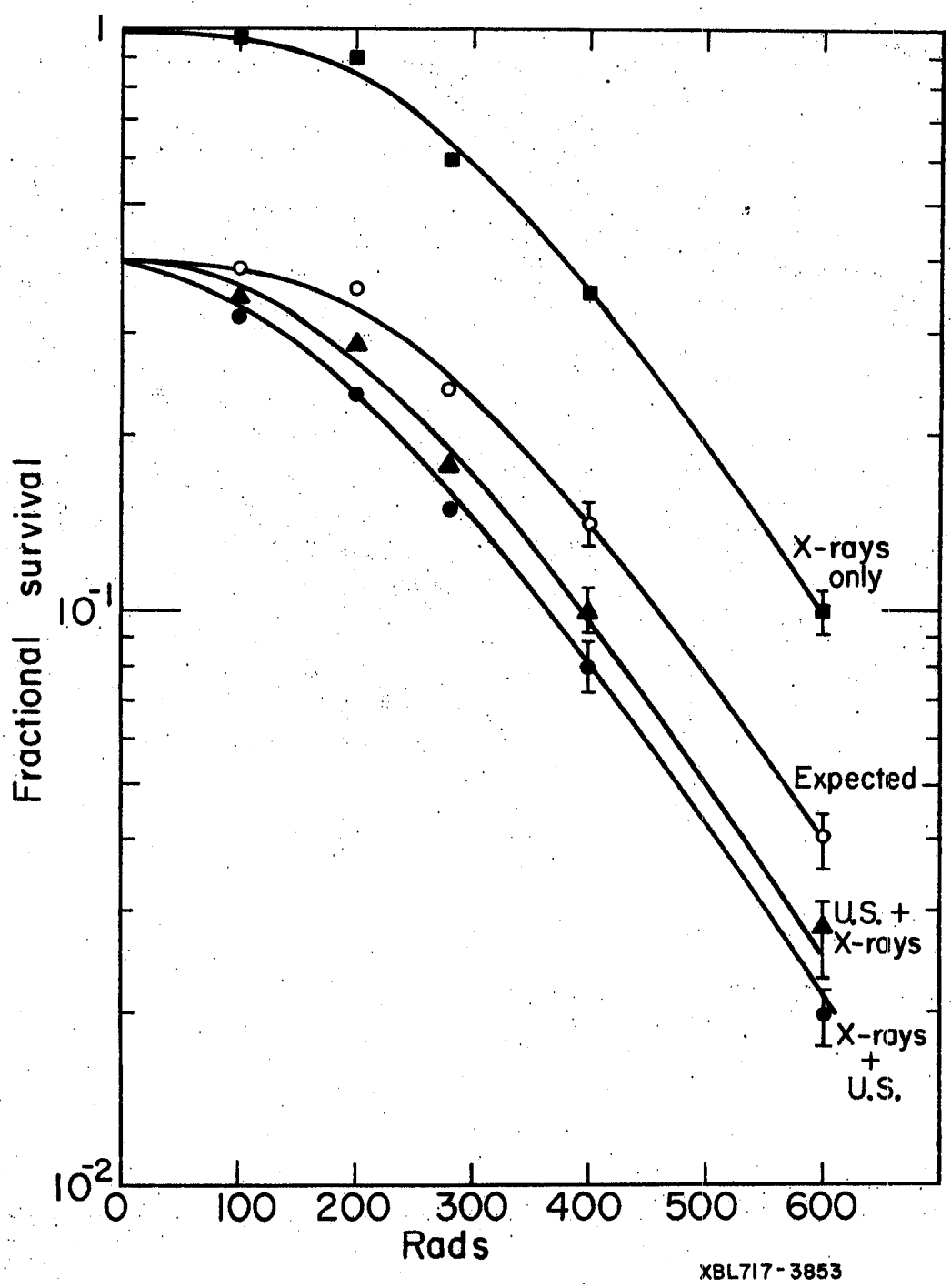


Figure 22. Synergistic effect in M3-1 cells exposed to 150 kV X-rays and 1.0 MHz frequency ultrasound at 0.5 W/cm^2 for 20 sec. The time interval between treatments was 2 to 5 min.

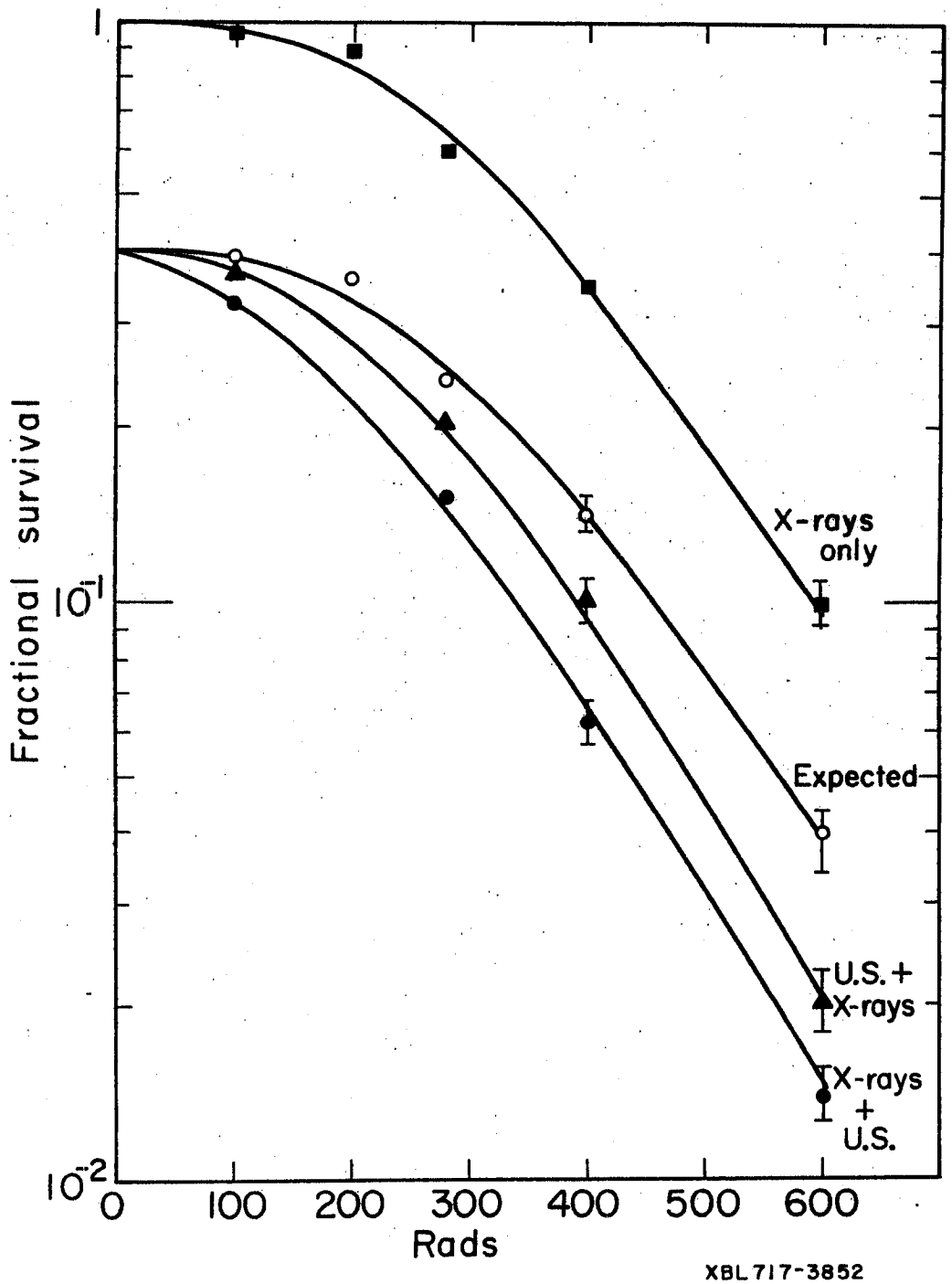


Figure 23. Synergistic effect in M3-1 cells exposed to 150 kV X-rays and 1.0 MHz frequency ultrasound at 0.5 W/cm^2 for 20 sec. The time interval between treatments was 30 min.

I. Cell Cycle

Synchronized V79 cells were obtained through the courtesy of R. Bird by selectively harvesting mitotic cells; these synchronized cells were exposed to a given dose of ultrasound at various stages in the cell cycle. At the same time a control experiment was done wherein an asynchronous V79 cell population was similarly exposed. The ultrasonic frequency was 1.0 MHz and the doses used were 1 W/cm^2 for 10 sec and 1 W/cm^2 for 15 sec for synchronous cells, and 1 W/cm^2 for 10 sec for asynchronous cells.

Figure 24 shows the results. It is seen that the fractional survival for the asynchronous population does not change significantly with time after plating. But for synchronous cell populations, it is found that almost twice as many cells survive a given dose when exposed in M and early G-1 phases as compared with cells exposed in S and G-2 phases to the same dose.

J. Growth Curve

To study the effect of ultrasound on the growth of cultured mammalian cells, M3-1 cells were exposed to 1.0 MHz frequency at 1.0 W/cm^2 for 60 sec and the growth curves of the exposed cells compared with those of control cells. The results are given in Fig. 25. The only effect seems to be an extension of the lag phase by about eight hours in the exposed cells, the doubling time being unaffected.

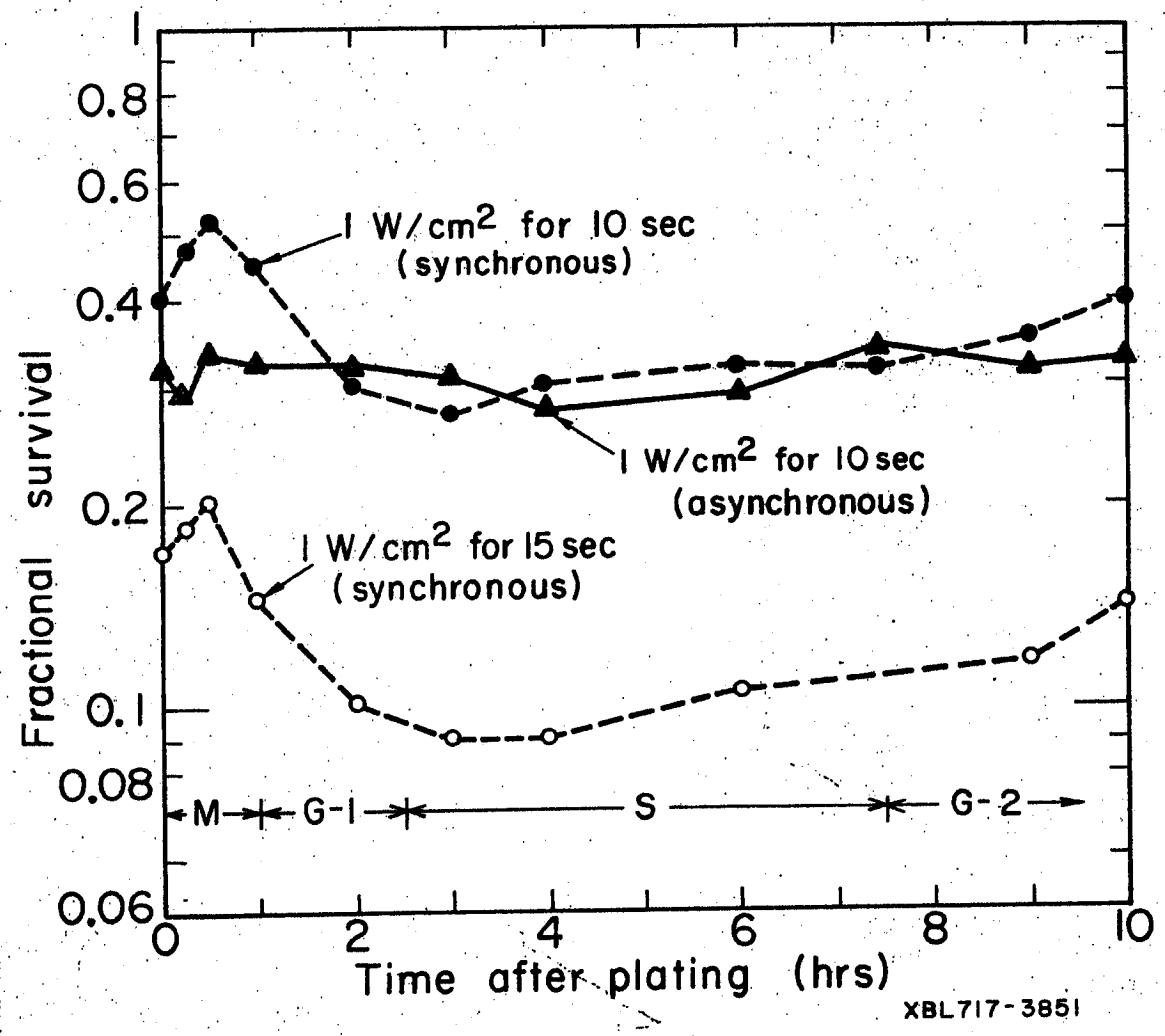


Figure 24. Cell sensitivity as a function of cell cycle in V79 cells exposed to 1.0 MHz frequency ultrasound.

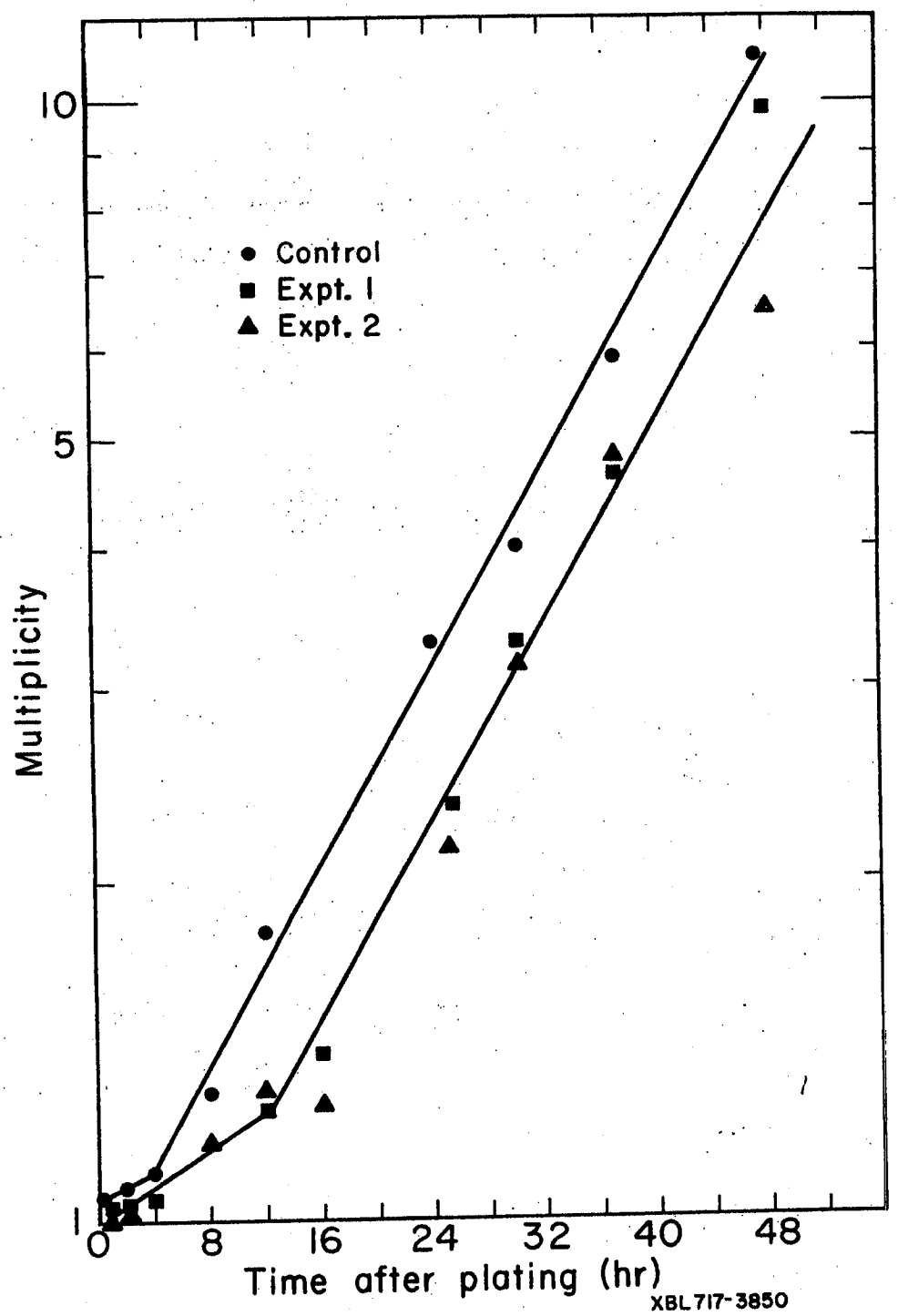


Figure 25. Effect of 1.0 MHz frequency ultrasound on the growth curve of M3-1 cells exposed to 1.0 W/cm^2 for 60 sec.

K. Microscopic Observations

When M3-1 cells are exposed to ultrasound and observed in a phase-contrast microscope at 250X within 2 min after exposure, it is seen that cells exposed to 0.5, 1.0, or 2.0 MHz frequency have a similar appearance. At very low doses the cells remain attached to the petri dish; at intermediate doses, they detach from the dish and round off; many cells have characteristic vescicles around them. At very high intensities, mostly only cell debris is left. Cells exposed to 0.1 or 3.3 MHz resemble each other, though their appearance is different from that of cells exposed to the other frequencies. Cells exposed to 0.1 and 3.3 MHz remain attached to the dish and maintain their shape, although the protoplasm appears coagulated in a manner very similar to that of cells exposed to a chemical fixative or to heat. Figure 26 shows phase-contrast pictures of exposed and control cells.

When M3-1 cells were exposed to 1.0 MHz at 1.0 W/cm^2 for 7.5 sec or at 0.25 W/cm^2 for 30 sec were examined in a scanning electron microscope and compared with controls; it was found that for the exposed cells the outer surface had a characteristic "bumpy" structure, whereas for the controls the outer surface appeared smooth. This is shown in Fig. 27.

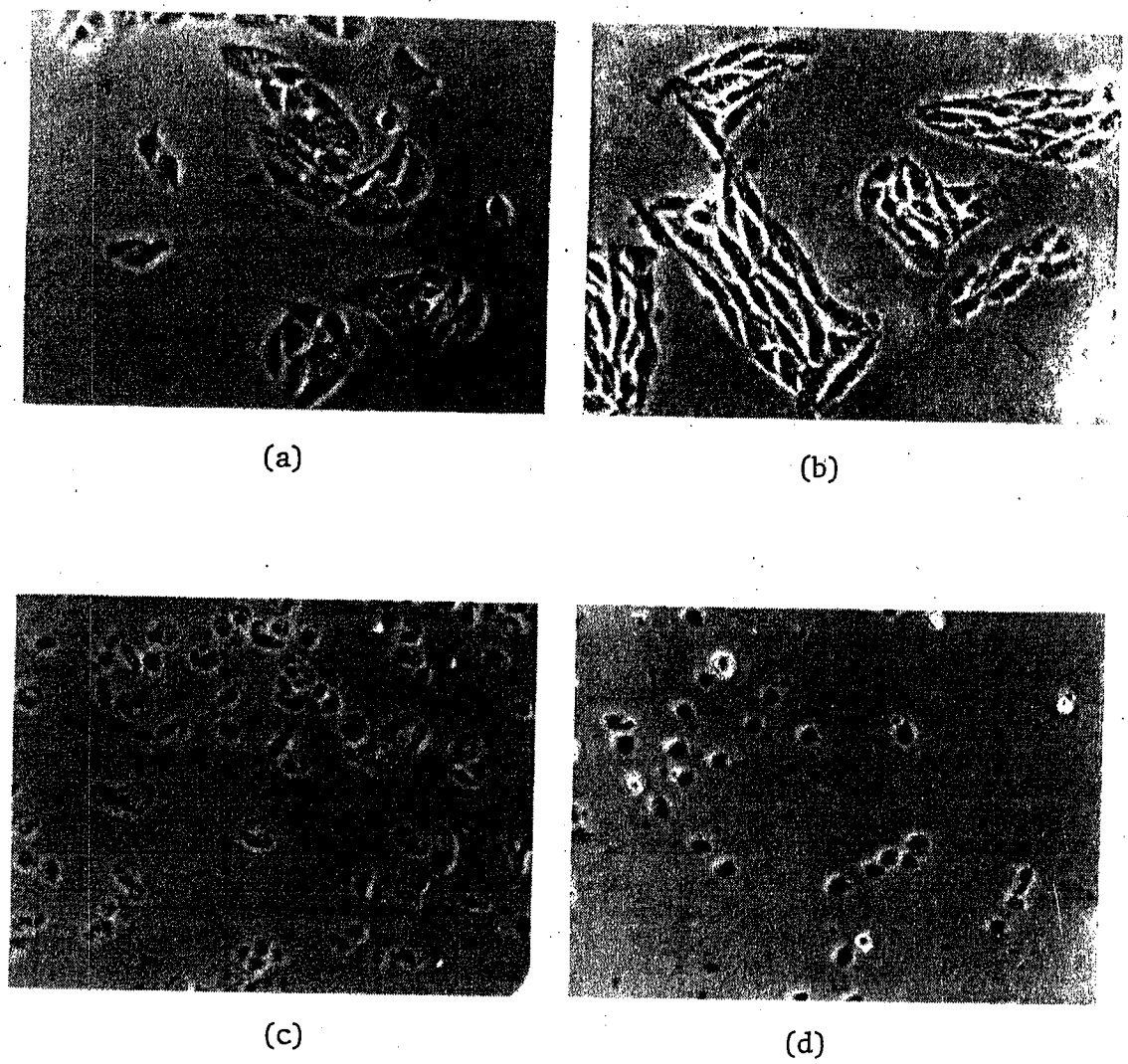
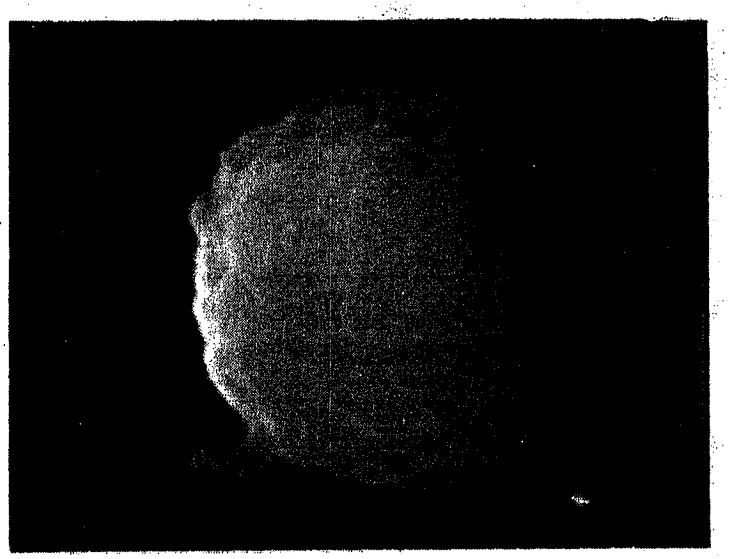
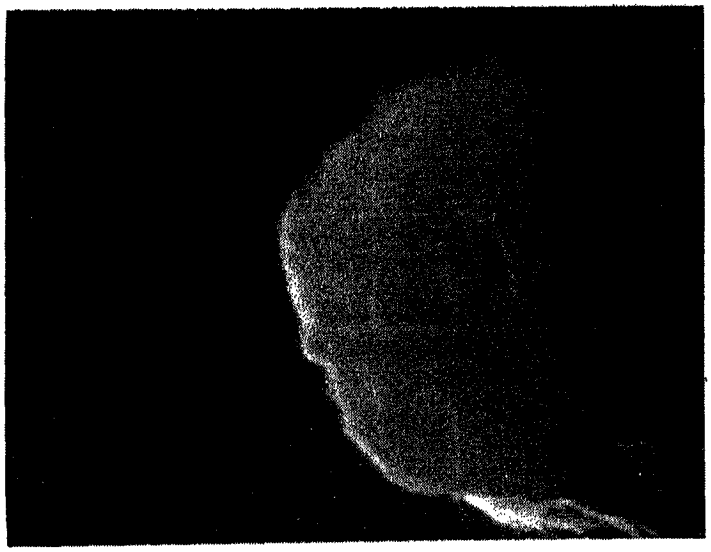


Figure 26. Phase contrast micrographs (X250) of M3-1 cells (a) Control (b) 2 min after exposure to ultrasound of 3.3 MHz frequency at 44 W/cm^2 for 60 sec. Cells exposed to 0.1 MHz frequency look similar. Note the "fixed" appearance of the protoplasm. (c) 2 min after exposure to 1.0 MHz at 8 W/cm^2 for 15 sec. Note the characteristic vesciles around the cells. (d) 2 min after exposure to 1.0 MHz at 8 W/cm^2 for 60 sec. Note the total disintegration of the cells. Cells exposed to 0.5 or 2.0 MHz appear similar to (c) or (d) depending on the dose.

(a) Control



(b) Exposed to 1.0 W/cm^2 for 7.5 sec.



(c) Exposed to 0.25 W/cm^2 for 30 sec.

Figure 27.

Scanning electron microscope pictures (X10,000) of M3-1 cells before and after exposure to 1.0 MHz frequency ultrasound

XBB 719-4381

To prepare the samples for scanning electron microscopy, M3-1 cells were exposed in suspension, i.e., immediately after plating. Two drops of the suspension were placed on a slide and incubated at 37°C for two hours to let the cells attache to the slide. The cells were then fixed in formaldehyde [1 part neutral formalin (40% formaldehyde) and 9 parts saline] for 30 min, rinsed in distilled water, and dehydrated through graded series of methanol. The slides were then air dried, coated with gold, and observed in a scanning microscope at up to 10,000 X.

L. Electron Spin Resonance: Free-Radical Production

To study the ultrasonic production of free radicals, an experiment was done in collaboration with Dr. D. E. Holmes of Donner Laboratory. A known concentration of a stable free radical (10^{-4}M, 4-hydroxy-2,2,6,6, - tetramethyl piperidine-N-oxyl) in water or in H.U.-15 medium was exposed to ultrasonic waves of 1.0 MHz frequency at different intensities (6.0 W/cm^2 for 1,3,4,6 min, and 0.25 W/cm^2 for 2,5,7,10 min in water; 6.0 W/cm^2 for 6 min, and 25 W/cm^2 for 2 min in H.U.-15 medium; and 6.0 W/cm^2 for 6 min in water after passing N_2 gas through the water for 2 min). Two milliliters of the solution were exposed in a petri dish in a manner similar to that used with mammalian cells. The production of free radicals was tested by comparing the concentration of the stable, free radical before and after ultrasonic exposure by electron spin resonance analysis. Induced free radicals are known to destroy the stable free radicals.

No appreciable amount of free-radical production was detected in this experiment. By this technique the production of as few as 10^{11} free radicals would have been detected, i.e., the technique would detect the free radicals produced by exposure of water to 1 rad of X-rays.

M. Eosin Y Exclusion Test

Eosin staining is a well known technique used for the detection of dead or dying cells. To determine the time of cell death, aliquots of sonicated cell suspensions were mixed with eosin Y immediately after exposure and at intervals thereafter; and the percentage of stained cells was counted in the nonsonicated control cells and in the irradiated suspensions. These experiments were done with M3-1 cells, the sonication being carried out within 20 min after plating.

For staining, one drop of 2% eosin Y (Allied Chemical Corporation, N.Y.) in 0.9% NaCl was added to two drops of cell suspension on a slide. The preparation was then examined under a phase-contrast microscope at 250 X, and the eosin-stained as well as the nonstained cells were counted between 2 and 10 min after staining (Fig. 28).

In the nonsonicated controls the percentage of nonstained cells remained constant at approximately 95%. Figure 29 shows the results for the sonicated cells. For comparison, the seven-day survival curve is also included. It is clear that cells begin to die within 15 min after sonication and that most of the cells that will be reproductively dead are dead within two hours after sonication. (After two hours the cells attach to the petri dish; hence the observations were not carried out beyond two hours.) The shape of the survival curves obtained by the eosin Y exclusion test is similar to the seven day "reproductive" death survival curve.

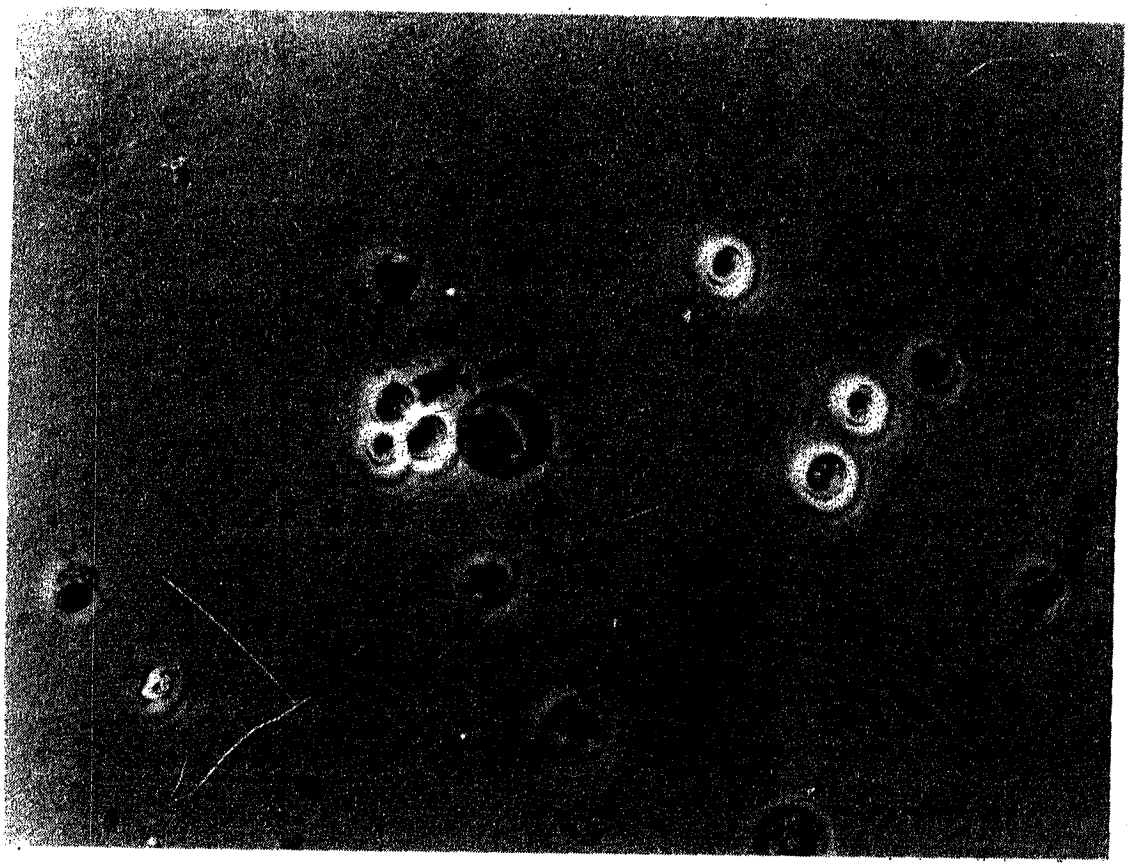
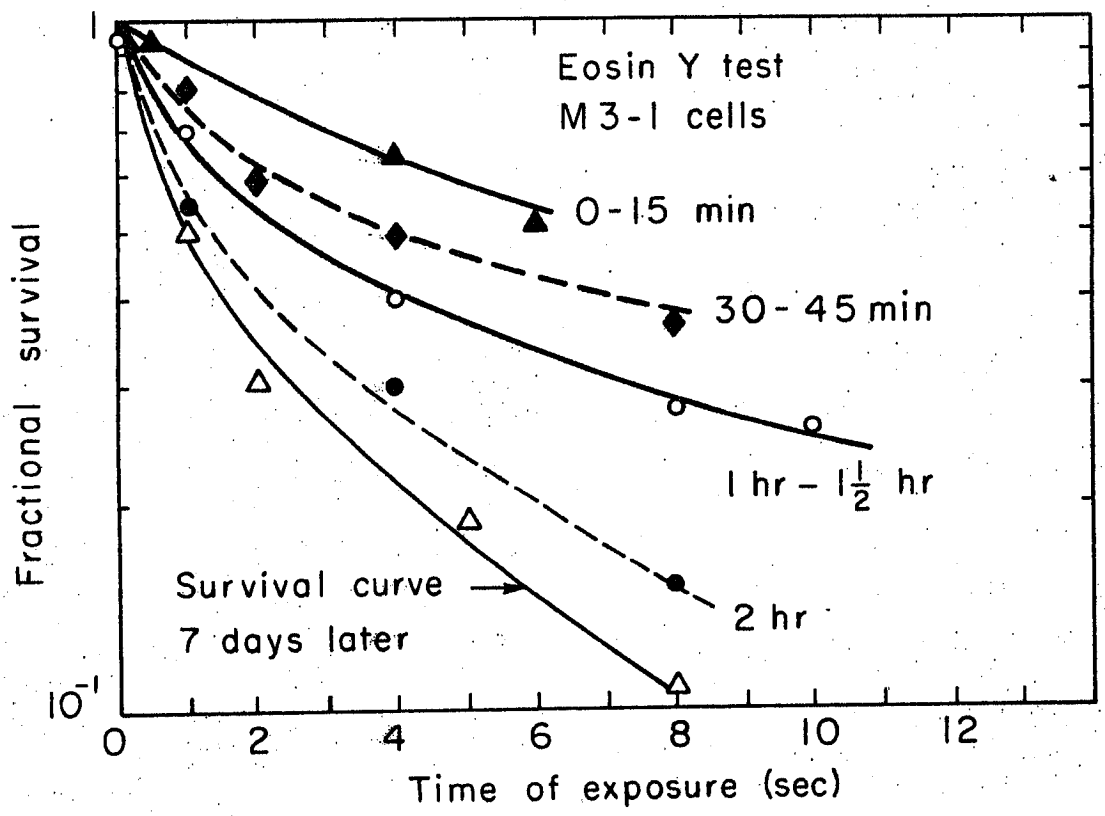


Figure 28. Eosin Y staining test: The cell preparation (M3-1 cells) was exposed to 1.0 MHz at 1.0 W/cm^2 for 10 sec. The living cells do not pick up the stain and appear bright with a halo around them in this phase contrast micrograph (X250)



XBL712-2826

Figure 29. "Survival curves" obtained by eosin Y staining method at various times after exposure of M3-1 cells to 1.0 MHz at 1.0 W/cm^2.

N. Cell-Size Distribution

The cell-size distribution apparatus and technique described by Madhvanath[95] were used to study the effect of ultrasound on cell-size distribution to investigate the possibility that membrane damage may play a part in cell death through sonication.

To obtain the cell-size distribution, 0.5 ml of the cell suspension was added to 4.5 ml of isotone in a vial. The cells were mixed by inverting the vial a few times and were counted in a Coulter counter (Model N with a 70-μ diameter aperture). The cell concentration was kept around 4×10^4 cells/ml. Pulses from the counter were fed through a wide-range linear amplifier (LRL Model 11X1980 P-1) to a 400-channel pulse-height analyzer (RIDL Model 34-12). Connected to the analyzer was a Moseley 2D-2X-Y plotter to plot the distribution.

For the experiment, M3-1 cells were exposed, immediately after plating into 35-mm petri dishes, to 1.0 MHz at 0.5 W/cm^2 for 10 sec. An aliquot of the cell suspension was mixed with either isotone or isotone + distilled water to give a final concentration of 50% isotone. The cell-size distributions of these suspensions were then obtained as described above. The whole analysis was completed 10 to 15 min after exposure.

The results are shown in Fig. 30. The size distribution probably does not represent the exact cell sizes; however, there is a qualitative difference indicating that treating cells with ultrasound is like exposing them to a hypotonic medium.

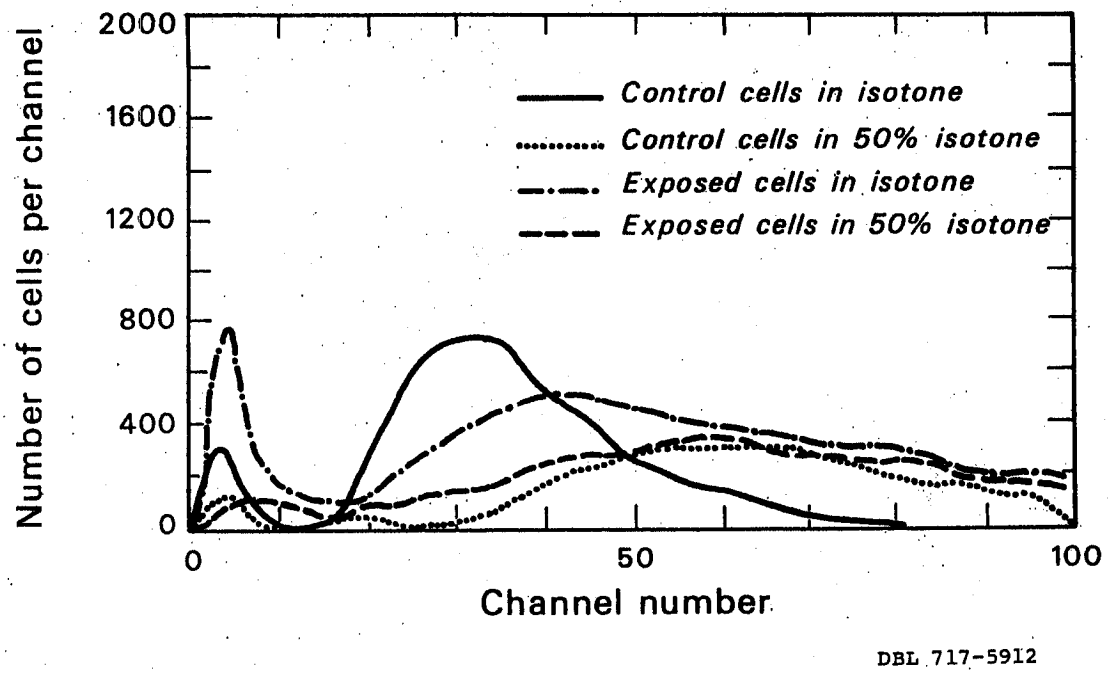


Figure 30. Effect of ultrasound on cell size distribution in M3-1 cells exposed to 1.0 MHz at 0.5 W/cm^2 for 10 sec.

V. DISCUSSION

A. Introduction

The effects of ultrasonic irradiation have been studied by numerous authors in various physical, chemical, and biological systems and attemps made to relate them to parameters of the ultrasonic beam.

The biological effects of ultrasound have been studied in microorganisms, viruses, bacteriophages, and various unicellular and multicellular organisms. A variety of effects ranging all the way from complete destruction of living organisms to stimulatory effects in plants have been reported. Subtle effects on the metabolism and ultrastructure of ultrasonicated living organisms have also been described.

Inspite of all the work done in this field the nature and the extent of the biological effects of ultrasonic energy are still uncertain. One of the problems in interpreting the results of investigations in the field of ultrasonics has been that experiments carried out so far have used widely varying apparatus and have often been only qualitative in nature, making it difficult to compare observations of various researchers because of variations in the techniques of exposure, the dosimetry, the frequency and the biological systems used.

Before attempting to explain our experimental results, some of the mechanisms that have been put forward to understand the biological effects of ultrasonic waves will be discussed.

B. Possible Mechanisms of Cell Death

The various mechanisms by which ultrasonication causes death of cells can be grouped into three principal categories:

(1) Caviation. When liquids are subjected to ultrasonic waves at sufficiently high intensities, the tensile strength of the liquid will be overcome by the large local variations of pressure created by the propagation of the ultrasonic vibrations in the liquid and cavities will be formed. The term caviation is applied to these cavities or vacuoles formed in a liquid exposed to ultrasound. These cavities are formed in the negative half of the pressure cycle of the sound vibration and collapse in the positive half of the cycle. This process is called vaporous or transient cavitation.[85] If the liquid contains dissolved gas, there will be a diffusion of the gas into the cavity with the formation of a bubble which will grow in size and eventually escape to the surface of the liquid. This spontaneous growth of unstable gas bubbles in a sound field is called gaseous cavitation.[85]

The minimum pressure amplitude that will induce cavitation is called the cavitation threshold. It depends on the amount of dissolved gas, the temperature, the viscosity, the hydrostatic pressure, and the frequency. No systematic studies have been done on how these various factors affect the cavitation threshold, but it is known that the threshold increases rapidly in the range from 100 KHz to 1 MHz and that presence of dissolved gas reduces the threshold considerably.[25] The onset of ultrasonic cavitation is accompanied by a characteristic audible hissing noise and the appearance of bubbles in the irradiated liquid.[25,85]

Even at intensities below the cavitation threshold, in a liquid with dissolved gas there is a steady growth of a population of microbubbles from pre-existing nuclei under the action of ultrasonic energy. This is referred to as stable cavitation.[100]

Various physical, chemical, and biological effects have been ascribed to cavitation. The erosion of the propeller screws of ships and the reduction of the flow rate in pipelines are some of the physically destructive effects of cavitation.[30,85] Intense hydraulic shocks resulting from the collapse of the cavities have a strong destructive action; pressures of several tens of thousands of atmospheres can be developed close to a collapsing bubble in a liquid.[30]

Ultrasound induces such diverse chemical reactions as oxidation, reduction, degradation, and synthesis of inorganic and organic substances, polymerization and depolymerization, intramolecular regrouping, and free-radical formation.[30] According to El'piner, no chemical reactions are observed in an ultrasonic field at intensities below the cavitation threshold irrespective of the duration of irradiation. Also, all factors which inhibit the formation of cavitation bubbles also prevent the occurrence of chemical processes.[30]

The destructive effects of ultrasound on unicellular organisms have also been ascribed to cavitation, and some of the more subtle effects of ultrasound such as the acoustic streaming observed in various systems have been ascribed to stable cavitation. Each microbubble as it grows passes through a size at which it is resonant in the applied field. In this condition, with relatively large vibration amplitudes, it effectively converts acoustic energy into energy of both unidirectional microstreaming fields and altered chemical bonds.[100]

Disintegration of bacterial and other cells is usually observed when the intensity of sonic vibrations is sufficient to produce cavitation bubbles in the irradiated medium.[30] The higher the dose rate, the more pronounced are the cavitation effects and the biological action. Thus, an increase in dose rate is more effective than an increase in the duration of exposure. Also, the effectiveness of ultrasonic action on microorganisms depends in a definite way on the concentration of microbial cells; it is less effective against highly concentrated solutions, and this has been explained by assuming that cavitation is suppresed in the highly concentrated solution because of the increase in viscosity.

(2) Temperature. In discussing the role of temperature in the biological effects of ultrasonic waves, there are two important points to bear in mind. The first is the temperature increase of the medium as a whole resulting from the conversion of absorbed acoustic energy into heat energy; the other factor is the localized temperature increase resulting from the adiabatic collapse of cavitation bubbles.

A liquid in an ultrasonic field is heated by absorption of acoustic energy which is partially transformed into heat energy. The temperature of a liquid rises sharply in the first few moments of ultrasonic irradiation. The subsequent temperature rise is extremely slow, or the heating of the medium ceases altogether--probably due to the establishment of equilibrium between the amount of energy delivered and the amount given up to the ambient medium. The physiotherapeutic application of ultrasound, such as in diathermy, is based on this temperature increase produced by ultrasound.

Besides this general temperature increase, high temperatures of the order of 2000°K are created in pulsating resonance cavitation bubbles.[85] However, there are certain objections to the hypothesis of a local, point increase in temperature to sevaral thousands of degrees.[30]

Although many authors have attributed the biological effects of ultrasound to heat, there are significant differences in the nature of the damage produced by ultrasound as compared with that produced by heat. Morphological changes produced in bacteria by ultrasound usually involve breakage of walls and membranes; such damage is not observed in the case of heat injury to bacterial cells.[30]

Sudo and Dworkin[40] report that the resistance of Myxococcus xanthus to sonic vibration developed during conversion of rods to refractile spheres whereas resistance to heat did not appear until after the conversion was complete.

Also, biological effects of ultrasound have been reported at intensities insufficient to cause an increase in temperature which could explain the effects. Newcomer and Wallace[101] in studying mutations induced by ultrasound recorded increases in the temperature of the treatment medium, water, and were of the opinion that the rise in temperature of 25°C from 10°C to 35°C was not sufficient to cause such damage. They did not rule out the possibility that local hot spots had developed; however, Newcomer[102] does not think that heat is a likely cause, as no coagulation of protoplasm was observed.

Watmough, et al.,[47] studied the effects of 10 KHz ultrasonic waves on the growth of the main tap root in Vicia faba seedlings, and they report that the observed reduction in the root growth rate cannot be ascribed to any small rise in temperature.

Lehmann et al.[103] report that the histological appearance of onion roots exposed to 1 MHz at 110 W/cm^2 was different from that produced by heat; but even then they do not rule out localized temperature increase as a possible factor.

(3) Direct Effects. It is possible that ultrasound directly affects some cell component and that the biological effect, including cell death, may be just an amplification of this primary damage.

It has been found that, depending on the intensity of ultrasound, microflows of varied strength appear in the cell, disturbing the spatial interaction of submicroscopic structures and leading to functional changes. High doses of ultrasound produce a disordered destructive shift in the cell ultrastructure leading to sharp disturbances in the physiological state of the cell, depressed growth, and even death.[16]

El'piner has shown that the mitochondria are ruptured immediately following irradiation. Such changes appear directly during sonication at a temperature (of the surrounding medium) which excludes the possibility of thermal coagulation of the protein structure of the cell and for ultrasonic intensities which exclude the possibility of cavitation.

Degradation of DNA occurs at intensities too low to produce cavitation or significant temperature increase. Such changes may be the result of direct interaction of ultrasonic energy with the polymer bond structure.

Burns[49,50] states that the mechanism for the inhibition of biosynthesis in sonicated yeast cells may be due to disruption of supramolecular organization in the cell. According to him, the simplest explanation for his results on the effects of sonication in yeast is that the cell membrane may become more permeable to certain small molecules during sonic irradiation.

Ravitz and Schnitzler[65] studied the effects of 85-KHz ultrasonic waves on the semitendinosus muscle of Rana pipiens. They observed a range of ultrastructural changes, the mitochondrial cristea and components of the sarcotubular system being the most sensitive. They postulate the generation of steady intracellular stress produced by ultrasonic waves to explain the results which were obtained under conditions that ruled out heating or cavitation as causative factors.

Lependin and Ustinova,[42] on the basis of a theoretical study on the mechanism of destruction of biological cells in an ultrasonic field, conclude that resonance vibrations of the cell in an ultrasonic field are quite probable. These resonance vibrations may lead to destruction of the cell membrane.

In conclusion, it may be stated that although transient cavitation and temperature increase may play a significant part in the biological effects of ultrasound at high intensities and low frequencies, many biological effects of ultrasound are nonthermal and noncavitational.

C. Explanation of Experimental Results

(1) Cell Death. Our results on the survival of mammalian cells are not due to transient cavitation since the doses we have used are about a factor of 100 below the cavitation threshold, nor are they

due to temperature as the temperature of the ambient medium did not exceed 37°C. However, we cannot exclude local high temperatures at interfaces which are not directly measureable by physical instruments.

We have concluded that the primary cause of cell death in cells exposed to 1.0 MHz frequency is damage to the structural or functional integrity of the cell membrane. This may be brought about by shearing action associated with bubble-induced eddying and related motion caused by stable cavitation, or the membrane damage may be the result of localized high temperature increases, which may occur due to the fact that maximum absorption of ultrasonic energy occurs at an interface--and the cell membrane is just such an interface.

The following observations support the hypothesis that membrane damage is the primary cause of cell death at 1.0 MHz frequency:

a) Eosin Y exclusion test. According to Goldstein and Okada,[99] eosin staining represents damage to the cell membrane. The results of the experiment on eosin uptake by sonicated cells indicate that cells began to die as early as 0 to 15 min after sonication and that most of the cells that are to be reproductively dead are already dead within two hours. It is unlikely that primary damage to some other cell substructure would become subsequently expressed as membrane damage so rapidly.

b) Phase-contrast microscopy shows characteristic vesicles around exposed cells within 2 min after exposure and comparison of scanning electron micrographs of control cells and of cells prepared for microscopy within 2 min after exposure shows that whereas the outer structure of control cells is smooth, that of exposed cells is

"bumpy", indicating that the membrane had collapsed on the internal cell organelles--possibly mitochondria.

c) When control cells are exposed to a hypotonic medium, they swell; but sonicated cells swell even in an isotonic medium, indicating that the membrane has become permeable to water. Also, certain molecules may leak out, as has been suggested by Burns. It is also possible that ultrasonication damages the viscoelastic properties of the membrane. The altered microscopic surface views attest to this.

d) Free radicals, if formed, are less than 10^{11} at doses as high as 10^{9} ergs/cm^{2}. Sonication of the medium itself, which could result in the formation of long-lived free radicals, was also ineffective.

e) The increased resistance of synchronized V79 cells in mitosis, as compared with the rest of the cell cycle, also leads us to believe that it is the membrane which is affected (see p. 68).

f) The insignificant difference in the survival of cells exposed in suspension or attached to the bottom of the petri dish may also be explained by assuming that cell death is due to membrane damage (see p. 44).

g) Lependin and Ustinova, on the basis of a theoretical study on the mechanism of cell death caused by ultrasonic waves, conclude that resonance vibrations of cells may lead to destruction of its membrane.

It is concluded that the integrity of the cell membrane is affected by ultrasonication. The damage may be to the membrane structures, such as pores, or to some components related to membrane

function, such as ATPase formation. No complete experiments were done to study the mechanism of cell death at frequencies other than 1.0 MHz; but, on the basis of the similarity of the microscopic appearance of cells exposed to 0.5, 1.0, and 2.0 MHz frequency, it is assumed that for all these frequencies the primary cause of cell death is membrane damage.

Also, again on the basis of the microscopic appearance of cells exposed to 0.1 and 3.3 MHz, it is assumed that coagulation of the protoplasm is the cause of cell death for these frequencies. We do not know what caused the coagulation of the protoplasm, but it does not seem to have been caused by temperature, because even for these frequencies the temperature of the medium did not exceed 37°C. It may have been caused by changes in the ionic composition of the protoplasm due to leakage of certain molecules, or due to cross-linking or denaturation of protein as a result of localized high temperatures.

(2) Dose-Rate Effect. The dose-rate effect is easily understood when one realizes that the term dose rate, as used in this study, refers to the intensity of ultrasound, which is related to the amplitude or maximum displacement of a particle from its position of rest; and the dose is the product of the displacement and the length of time this value of the displacement lasts.

If the amplitude of vibration is very small, it may have no effect--no matter how long it lasts. Above a threshold amplitude, the higher the amplitude the greater its effect; it probably would reach a plateau or peak and then become less effective. This has not been observed in our experiments, but Clarke et al.[66] have observed an anomalous dose-rate effect.

The shape of the survival curves can be explained by assuming that the cell population is heterogeneous in regard to its sensitivity to ultrasound. This is consistent with the fact that cells in different stages of the cell cycle have different sensitivities. However, no quantitative correlation was established between the survival curve and the distribution of cells in various stages and their sensitivities.

The survival curve shows a sharp initial decrease in survival with a much slower fall at higher doses. This is quite similar to the temperature increase caused by sonication. The initial rise in temperature is rapid, but subsequent increase is slow. One might therefore be tempted to say that temperature is responsible for cell death; however, the final temperature never exceeded 37°C. It is more likely that the survival curve and the time rate of temperature increase are both expressions of the nature of acoustic energy absorption.

Another factor influencing the effectiveness of ultrasound is the concentration of cells, as is well known in the case of microorganisms; but even at the highest doses, the cell concentration was not high enough to significantly affect the viscosity and thus the effectiveness of ultrasound.

We prefer to explain the shape of the curve by assuming that there are a fixed number of "sites" on a cell membrane which are vulnerable and that a certain number of these have to be inactivated before the cell will die. Initially all these sites are available, and the chance of their being damaged is good; but, with increasing dose, less are available to be damaged so that equal increments at higher doses are less effective.

These sites may be pores on the cell membrane. In an ultrasonic field these pores are stretched, which affects the membrane permeability; and if the amplitude is high enough, the pores may be irreparably stretched, thus creating holes in the membrane.

(3) Cell-Line Sensitivity. If the primary damage is to the membrane, then it is easy to say see why there should be no correlation between cell sensitivity and the origin of the cell line, its doubling time, the number of its chromosomes, or even the cell-size distribution. Cell sensitivity is probably related to some structural unit that has similar properties for the different cell lines. It cannot be DNA but may be mitochondria or microsmes or membrane units.

(4) Frequency Effect. That a certain frequency is more effective than another is probably due to a resonance phenomenon as discussed by Lependin and Ustinova. It is possible that the variation in the effectiveness of different frequencies may be an artifact of dosimetry; i.e., it may be due to variations in the energy absorbed by cells at different frequencies. However, that the greater effectiveness of 0.5 MHz frequency may be an artifact of dosimetry is ruled out by our experiments on the survival of *S. cerevisiae* exposed to the same set of frequencies. We have found 1.0 MHz to be the most effective of those frequencies for killing yeast cells.

(5) Dose Fractionation. Cells damaged by ultrasound are in a state of stress and are therefore probably more susceptible to a subsequent exposure. From various experiments we have concluded that ultrasound affects membrane permeability; and since cells are in a

continuous state of metabolic activity, the primary damage can be expected to get progressively worse with time, as has been shown by the eosin Y exclusion test. However, we do not know why the maximum sensitivity occurs about a half hour after the first exposure.

It would seem that even at the end of three hours, the cells do not recover from the initial damage, as has been demonstrated by the split dose technique to be the case with cells exposed to X-rays; but there is some 'recovery' from the 'sensitization' resulting from the initial damage.

(6) Synergism. There are three possible explanations for the synergistic effect of ultrasound and X-rays:

a) If the treatments are done simultaneously, the temperature increase produced by ultrasound leads to increased sensitivity to X-rays, as has been demonstrated by Bridges et al.[104] However, the temperature increase in our experiments, measured as less than 1°C, was not significant enough to explain the effect.

b) If ultrasound follows X-rays, the chromosome breaks induced by X-rays might be prevented from rejoining by the mechanical vibrations caused by ultrasound, as suggested by Conger.[105]

c) The interaction between the membrane damage caused by ultrasound and the nuclear damage caused by X-rays may be responsible for the synergistic effect, in the sense that one damage amplifies the other, possibly by interfering with repair mechanisms. Vibration causes appreciable changes in the cells, influencing their subsequent fate. It may be supposed that cells exposed to vibrations become

more reactive, and therefore any additional influence is capable of radically altering their biological properties. A cell with a damaged cell membrane may not be as able to withstand nuclear damage and a cell with its nucleus damaged may not be as capable of withstanding damage to its membrane, as a normal cell.

At present we do not understand why ultrasound followed by X-rays should be less effective than X-rays followed by ultrasound and why the synergistic effect should be greater if the treatments are separated by a half hour than if they follow immediately. Probably the differences are due to differences in the nature of the damage caused by the two agents, and in the nature and timing of the repair process for both cases.

(7) Cell Cycle. Cells in mitosis probably have a different membrane sensitivity than cells in S phase. This is based on the observations of Todd et al.[106] who have found that there is a significant difference in the microelectrode tip potentials on cell surfaces in cells in mitosis as compared with cells in the rest of the cycle. Cone[107] also reports that pronounced variation in cell volume and electrical potential accompany initiation of mitosis in vitro. Thus, if sonic effects are due to direct action on membranes, one would expect some variation of these effects with the cell-division cycle.

It is interesting to note that when synchronous cell populations are exposed to X-rays at different stages in their life cycle, cells in S phase are found to be most resistant. This too would seem to indicate that the mechanism of cell death due to sonication may be different from that of cell death due to X irradiation, which is due to nuclear damage. However, Clarke et al.[76] have reported that for

L5178Y mouse leukemia cells exposed to 1 MHz ultrasonic irradiation, cells in M phase are significantly more susceptible to disintegration than the average population.

(8) Growth Curve. Since the primary damage appears to be to the membrane, we believe that important nutrients may leak out during sonication; and the extension in the lag phase may be the period needed to resynthesize and accumulate those nutrients, and possibly to repair any sublethal damage. No effect on the doubling time is to be expected from membrane damage, which is what we have observed in our experiment.

VI. SUMMARY AND CONCLUSIONS

The colony-forming ability of cultured mammalian Cells (derived from Chinese hamster bone-marrow cell line M3-1, Chinese hamster lung cell line V79, human kidney cell line T-1, or Chang's human liver cell line) exposed to monochromatic ultrasonic vibrations of different frequencies (0.1, 0.5, 1.0, 2.0, 3.3 MHz) or to audiofrequencies has been studied to try to understand the nature of the biological action of ultrasonic energy at the cellular level. The combined effect of 150-kV X-rays and 1.0-MHz ultrasonic waves on M3-1 cells has also been studied.

A commercially available 1.0-MHz ultrasonic generator using a 10 cm^2 X-cut quartz crystal as a transducer was used for most of the experiments, after the oscillator had been redesigned to provide monitoring and independent control of the frequency, the dose rate (plate voltage), and the time of exposure. Later, another generator was specially designed to provide ultrasonic waves of 0.1, 0.5, 1.0, 2.0, and 3.3 MHz frequency using 10 cm^2 quartz crystals as transducers for 0.5, 1.0, 2.0, and 3.3 MHz and an 80 cm^2 barium titanate crystal for 0.1 MHz.

The experimental procedure involved plating an aliquot of a known number of cells into 35-mm plastic petri dishes. After four to six hours of incubation at 37°C, each dish was coupled to the 35-mm quartz crystal transducer (enclosed in a metal cup) using a thin layer of glycerine. The oscillator was tuned to the resonant frequency of

the crystal and the frequency monitored on a digital frequency meter. The samples were exposed to a given frequency and dose rate for a given time. The dose rate was controlled by adjusting the voltage in the plate circuit, and the plate current and voltage were recorded.

After incubation at 37°C for seven days (in the case of M3-1 and V79 cells) or 12 days (for T-1 and Chang's), the dishes were stained with 1% aqueous methylene blue solution for 30 min, rinsed with distilled water, dried overnight, and the number of visible colonies per dish counted.

To a limited extent, the effects on growth rate, dye uptake, size distribution, free-radical production, and microscopic alterations were also studied.

The following observations were made:

(1) The survival curves, in contrast with those for ionizing radiations, are nearly logarithmic; but the slope of the line decreases with increasing dose.

(2) The lethal effects are dose-rate dependent and have a threshold dose rate. For M3-1 cells at 1.0 MHz frequency, the threshold dose rate occurs around 0.125 W/cm^2.

(3) Different mammalian cell lines in vitro do not show appreciably different sensitivities.

(4) The shape of the survival curve for different frequencies, as well as for different cell lines, is similar.

(5) Using a synchronous V79 cell population, we found that M and early G-1 phases are more resistant and S phase more sensitive to ultrasound. About twice as many cells in M phase survive as do cells in S phase.

(6) Audiofrequencies at the dose rates used (of the order of 10^{-2} W/cm^2, which is the threshold for pain) have no lethal effects. The dose rates and doses used in the audiofrequency experiments were considerably below those shown to produce lethal effects for the ultrasonic frequencies.

(7) Dose-fractionation studies show that prior exposure sensitizes the cells to subsequent treatment, in contrast with what is observed with X-rays where cells show recovery. Maximum sensitivity occurs approximately 30 min after the first exposure, when cells are maintained at 37°C between exposures.

(8) Eosin Y exclusion test indicates sonicated cells begin to die within 15 min after exposure and that almost all the cells that will be counted seven days later as reproductive deaths are already dead within two hours after sonication.

(9) The lethal effects are observed only in the presence of a liquid medium during sonication. No lethal effects are observed if cells are sonicated in the 'frozen' state or in the 'damp' state, i.e., without medium.

(10) There is no difference in the sensitivity to ultrasonication whether the cells are in 'suspension' or attached to the dish, as long as they are also bathed in liquid medium.

(11) The temperature of the medium over a range of 0 to 25°C at the time of sonication does not affect the sensitivity of cells.

(12) Cells that survive sonication show a longer lag phase, but the doubling time is not affected.

(13) Microscopic examination shows that cells exposed to 0.5, 1.0, or 2.0 MHz appear the same. Microscopically, cells exposed to 0.1 MHz resemble those exposed to 3.3 MHz but appear different from cells exposed to 0.5, 1.0, or 2.0 MHz. Cells exposed to 0.5, 1.0, and 2.0 MHz frequency at intermediate doses detach from the petri dish and round off; many of the cells show characteristic vescicles around them. At very high doses, most of the cells disintegrate and appear as cell debris. Cells exposed to 0.1 and 3.3 MHz frequency remain attached to the dish even at high doses and retain their normal struc tural features, except that the cytoplasm appears coagulated.

Scanning electron microscopy shows characteristic bumpy outer structure for the sonicated cells as compared with the smooth outer structure of control cells.

(14) There is a small synergistic effect between ultrasound and X-rays. The degree of synergism depends on X-ray dose and the time interval between treatments, and is greater when ultrasound follows X-rays than when it precedes it.

(15) For M3-1 cells, 0.5 MHz is found to be the most effective of 0.5, 1.0, 2.0, and 3.3 MHz frequencies.

(16) Free-radical production if any, is less than 10^{11} free radicals at the doses used.

(17) Cell-size distribution studies show that cells exposed to ultrasound swell as though they had been exposed to a hypotonic medium.

From the above observations the following conclusions have been made:

(1) The lethal effects are not due to cavitation because the intensities used are much lower than those required to induce cavitation nor are they primarily due to temperature since the effects are observed even when the temperature does not exceed 37°C. We cannot completely exclude localized temperature increases at interfaces.

(2) There seem to be two mechanisms responsible for cell death:

a) For cells exposed to 0.5, 1.0, and 2.0 MHz frequencies, the primary cause of cell death seems to be damage to functional behaviour of membranous structures. This is indicated by the eosin Y test and cell-size distribution studies, as well as by microscopic observations.

b) For cells exposed to 0.1 and 3.3 MHz, cell death appears related to coagulation of protoplasm.

(3) Ultrasonic vibrations cause lethal damage as well as sub-lethal damage. There is a threshold dose rate for lethal effects.

(4) The effectiveness of ultrasound probably depends on both the frequency and the amplitude of the waves indicating a possible resonance phenomenon.

(5) Synergism between ultrasound and X-rays may be due to an interaction between the nuclear damage caused by X-rays and the damage to the cell membrane caused by ultrasound.

ACKNOWLEDGMENTS

During the course of this investigation I had the help, support, and encouragement of a number of people; I want to acknowledge specifically:

Dr. C. A. Tobias, for suggesting the problem for study and for general guidance.

Dr. M. R. Raju, for his guidance and personal encouragement, and for helpful discussions.

Dr. T. L. Hayes, the Chairman of the Thesis Committee, for his help during the course of the research and especially in scanning electron microscopy.

Dr. M. Alfert, for reading and criticizing the drafts of the manuscript.

Dr. P. W. Todd, for initiating me into the field of mammalian cellular radiobiology and cell culture.

Mr. A. Windsor, for the design, construction, and maintainance of the ultrasonic equipment.

Mrs. C. Lathrop and Mrs. W. M. Jackson, for their help in the laboratory in the earlier stages of the investigation.

Special thanks are due to Dr. Orr Reynolds who has suggested that the synergistic effect found between ultrasound and X-rays might be useful sometime in the future, for the treatment of cancer patients.

I am also thankful to Mrs. Yvonne Stone for editing the manuscript and to Mrs. Vicky Donelson for typing the thesis.

The research was supported jointly by the United States Atomic Energy Commission and by the National Aeronautics & Space Administration.

REFERENCES

1. A.E. Crawford, Ultrasonic Engineering Butterworths Scientific publications, Lond., 1960.
2. B. Carlin, Ultrasonics, McGraw-Hill Book Co., N.Y., 1960.
3. F.L. Thurstone, Three dimensional imaging by ultrasound holography (medical diagnosis), in Digest of 7th International Conference on Medical and Biological Engineering, Aug. 1967, Royal Academy of Engineering, Stockholm, Sweden.
4. A.F. Metherell, H.M.A. El-Sum and Lewis Larmore (Editors), Acoustical Holography, Vol. 1, Proceedings of the 1st International Symposium on Acoustical Holography, Dec. 1967, Plenum Press, New York 1969.
5. G. Daufin and M. Sainclivier, The use of ultrasonic vibrations for the removal of bacteria contained in milk film fixed on metal surfaces, Ann. Technol. Agr. (Paris) 16(3), 195-203, 1967. B.A. 15246, 1969.
6. J.L. Watkins, G.W. Sherrit and J.H. Ziegler, Predicting body tissue characteristics using ultrasonic techniques, J. Anim. Sci. 26(3), 470-473, 1967.
7. E.A. Campbell and Anne White, Estimation of total muscle in live cattle using ultrasonic measurements, Aust. Vet. J. 44(3), 97-100, 1968.
8. L.M. Anderson and R.C. Wahlstrom, Ultrasonic prediction of swine carcass composition, J. Anim. Sci. 28(5), 593-600, 1969.

9. J. Bta. Aparicio Macarro, Estimation of backfat thickness on live pigs by ultrasonics, Arch. Zootech. 16(62) 169-195, 1967. B. A. 14469, 1969.

10. A. Rittler, Ultrasonic measurements as part of the estimation of breeding value in swine, Z. Tierzucht Zuchtungsbiol. 84(2), 179-187, 1968. B. A. 93430, 1969.

11. E. Werhahn, On the suitability of different ultrasonic methods for the determination of meat and fat percentages in swine carcasses, Z. Tierzuecht Zuechtungsbiol. 85(2), 135-142, 1968, B. A. 85791, 1969.

12. E.W. Lauprecht, W. von Schutzbar and D. Flock, About the heritability of fat and muscle thickness measured ultrasonically in young boars, Z. Tierzuecht Zuechtsungsbiol. 84(1), 80-95, 1967. B. A. 28353, 1969.

13. A. Rittler, Ultrasonic measurements as an aid for the determination of breeding value in pigs: III. Estimation of meat and fat portions on the basis of ultrasonic measurements on living pigs, Z. Tierzuecht Zuechsungbiol., 85(1), 78-89, 1968. B. A. 121477, 1969.

14. R. Schuttert, K. Steenberg, N. Standal and H. Lien, Blood volume and ultrasonic measurements on live pigs destined for slaughter as a measure of meat and fat values of their carcasses, Acta. Agr. Scand. 18(1/2), 101-108, 1968.

15. B.P. Avakyan, Certain specific effects of ultraviolet rays and ultrasound on the basic microflora of wine. Dokl. Akad. Nauk. Arm. SSR, 47, 90-93, 1968. NSA 29939, 1969.

16. I.D. Rubtsova, Effect of ultrasound on the germination of seeds and on productivity of fodder beans, Biofizika 12(3), 489-492, 1967.

17. N.M. Suvorova, Effect of ultrasound on the menstrual function in women, Akush Ginekol 44(4), 44-47, 1968. B. A. 41031, 1969.

18. R. Gary Kirk, Interfacial reaggregation of ultrasonically disrupted erythrocyte membranes, Proc. N.Y. Acad. Sci. 60, 614-621, 1968.

19. J.J. Gordon and J.P. Ruthland, Solubilization of erythrocyte Acetylcholin-esterase by ultrasonic vibration, Nature 214, 850-851, 1967.

20. E.A. Davidson and T. Rosett, Some biological applications of ultrasound, in Ultrasonic Techniques in Biology and Medicine (Eds. B. Brown and D. Gordon), p. 206-219, Iliffe Books, Ltd., 1967.

21. J.A. Newell, Ultrasonics in Medicine, Phys. Med. Biol. 8(3), 241-264, 1963.

22. L. Balamuth, The application of ultrasonic energy in the dental field, in Ultrasonic Techniques in Biology and Medicine, (Eds. B. Brown and D. Gordon), p. 194-205, Iliffe Books, Ltd., 1967.

23. E. Kelly, Ultrasound in Biology and Medicine, Waverly Press, Inc., Baltimore, 1957.

24. E. Kelly, Ultrasonic Energy: Biological Investigations and Medical Applications, Univ. of Illinois Press, 1965.

25. W.J. Fry and F. Dunn, Ultrasound: Analysis and Experimental Methods in Biological Research, in Physical Techniques in Biological Research (Ed. W.L. Nastuk) Vol. 4, 261-394, Academic Press, N.Y. and Lond., 1962.

26. C.C. Grossman, J.H. Holmes, C. Joyer and E.W. Purnell (Editors), Diagnostic Ultrasound, Plenum Press, N.Y., 1966.

27. R.E. Goldberg and L.K. Sarin, Ultrasonics in Ophthalmology, W.B. Saunders Co., Philadelphia and Lond., 1967.

28. B. Brown and D. Gordon, Eds., Ultrasonic Techniques in Biology and Medicine, Iliffe Books Ltd., 1967.

29. C.R. Hill, The possibility of hazard in medical and industrial applications of ultrasound, Br. J. Radiol. 41, 561-569, 1968.

30. I.E. El'piner, Ultrasound: Physical, Chemical, and Biological Effects, Consultants Bureau, N.Y., 1964.

31. R.W. Wood and A.L. Loomis, The physical and biological effects of high-frequency sound waves of great intensity, Phil. Mag. 4, 417, 1927.

32. E.N. Harvey and A.L. Loomis, High-speed photomicrography on living cells subjected to supersonic vibrations, J. Gen. Physiol. 15, 147, 1932.

33. F. O. Schmitt, Protoplasma 7, 332-340, 1929.

34. W. Feindt, Uber die Ultraschallempfindlichkeit des Paramecium caudatum, Strahlentherapie 84, 611, 1951.

35. H.J. Dyer and W.L. Nyborg, IRE Trans. Med. Electron. 7, 163-165, 1960.

36. H.J. Dyer and W.L. Nyborg, Proc. Intern. Conf. Med. Electron., 3rd, 445-449, 1960.

37. D.E. Hughes and W.L. Nyborg, Cell Disruption by Ultrasound, Science 138, 108-114, 1962.

38. W.L. Wilson, F.J. Wiercinski, W.L. Nyborg, R.M. Schnitzler and F.J. Sichel, Deformation and motion produced in isolated living cells by localized ultrasonic vibration, J. Acoust. Soc. Am., 40(6), 1363-1370, 1966.

39. I.E. El'piner, G.A. Alimov and A.D. Shebaldina, Effect of ultrasonic waves on phase T2., Biofizika 12(5), 893-894, 1967.

40. Sara Z. Sudo and Martin Dworkin, Resistance of vegetative cells and microcysts of Myxococcus xanthus, J. Bacteriol. 98, 883-887, 1969.

41. B.I. Martins, B. Stackler, M.R. Raju and C.A. Tobias, Lethal and mutagenic effects of ultrasound on Saccharomyces cerevisiae, (to be published) 1971.

42. L.F. Lependin and V.A. Ustinova, Mechanism of destruction of biological cells in an ultrasonic field and the possibility of studying the physical properties of the cell membrane, Biofizika 12(2), 268-271, 1967.

43. A.G. Gordon, Mutations and ultrasound, in Ultrasonic Techniques in Biology and Medicine (Eds. B. Brown and D. Gordon), p. 220-246, Iliffe Books Ltd., 1967.

44. G.G. Selman and S.J. Counce, Abnormal embryonic development in Drosophila induced by ultrasonic treatment, Nature 172, 503-504, 1953.

45. E.B. Kirsten, H.H. Zinsser and J.M. Reid, Effect of 1 Mc ultrasound on the genetics of mice, IEEE Trans. on Ultrasonics Eng. 112-116, Dec. 1963.

46. O. Istomina and E. Ostrovskii, Effect of ultrasound on the development of plants, cited from Refs. 16 and 30.

47. D.J. Watmough, R. Oliver and Josephine Cavanagh, Effect of protracted ultrasonic irradiation on the growth of seedlings of Vicia faba, Nature 222, 773, 1969.

48. J. Jankowiak, J. Hasik, C. Majewski and R. Markowski, Influence of ultrasound on some histological and histochemical reactions in the liver of the rat, Am. J. Phys. Med. 37(3), 135, 1958.

49. V.W. Burns, Reversible sonic inhibition of protein, purine, and pyrimidine biosynthesis in the living cell, Science, 145, 1056-1058, 1964.

50. V.W. Burns, Effects of sonic irradiation on yeast, Radiation Research Supplement 7, 231-238, 1967.

51. V.M. Mityushin and I.E. El'piner, Effect of ultrasound on the cells of ehrlich ascites carcinoma, Biofizika 14(3), 502-505, 1969.

52. P. Slawinski, Effect of ultrasonics on iodine metabolism in guinea pigs, NSA. 49594, 1968.

53. P. Slawinski, Histologic studies on the thyroid gland in guinea pigs subjected to the action of ultrasound, Patol. Pol. 17(2), 147-154, 1966. B. A. 69457, 1969.

54. I. Hrazdira and M. Konecny, Functional and morphological changes in the thyroid gland after ultrasonic irradiation, Am. J. Phys. Med. 45, 238-243, 1966.

55. E.J. Valtonen, Influence of ultrasonic radiation in the medical therapeutic range on the fine structure of the liver parenchymal cell. An electron microscope study on mice, Arch.

Pathol. Anat. 343, 26-28, 1967. NSA. 50344, 1969.

56. E. J. Valtonen, A histological method for measuring the influence of ultrasonic energy on living tissue under experimental conditions, Acta Rheumatol Scand. 14(1), 35-42, 1968. B. A. 12120, 1969.

57. O.K. Basurmanova, A.A. Panov and I.E. El'piner, Effect of ultrasonic waves on nerve cells of the caterpillar brain, Dokl. Akad Nauk SSR 181(2), 476-477, 1968. B. A. 134728, 1969.

58. J. Hahn and R.H. Foote, Ultrasonic treatment of rabbit testes, J. Anim. Sci. 28(2), 230-232, 1969.

59. V.M. Andrianov, Effect of the ultrasonic vibration of small intensity on the sex gland structure in albino rats, B. A. 24311, 1969.

60. A.P. Covich and M. Tsukada, Electron microscopy of replicas from ultrasonicated Chara oosporangial walls, Bot. Mag (Tokyo) 82(970), 162-170, 1969. B. A. 128097, 1969.

61.(a) L.V. Kovalev, A. Study of the properties of Brucella subjected to ultrasound, B. A. 8888, 1969.

61.(b) L.V. Kovalev, Biological properties of individual cellular structures of Brucella obtained by ultrasonics, B. A. 8889, 1969.

62. D.A. Kleimenov, Antibody titers and interrelations between serum proteins in local and general ultrasonic treatment, B. A. 26155, 1969.

63. L.A. Chambers and E.N. Harvey, J. morph. physiol. 52, 155, 1931.

64. I.G. Shtrankfel'd, I.E. El'piner and A.G. Atanasov, Effect of high-intensity ultrasonic waves on G- and F- Actin, Biofizika 12(6), 1093-1094, 1967.

65. M.J. Ravitz and R.M. Schnitzler, Morphological changes induced in the frog semitendinosus muscle fiber by localized ultrasound, Exptl. Cell Res. 60, 78-85, 1970.

66. P.R. Clarke and C.R. Hill, Physical and chemical aspects of ultrasonic disruption of cells, J. Acoust. Soc. Amer. 47(2,part2), 649-653, 1970.

67. P.R. Clarke and C.R. Hill, Biological action of ultrasound in relation to the cell cycle, Expt. Cell Res. 58, 443-444, 1969.

68. K. Zapf, cited from Ref. 30, p. 265.

69. K.P. Garina, Mutagenic effect of ultraviolet light on Actinomycete spores after preliminary ultrasonic treatment, NSA 14147, 1969.

70. E. Spring, Increased radiosensitivity following simultaneous ultrasonic and gamma-ray irradiation, Radiology 93, 175-176, 1969.

71. K. Woeber, The effect of ultrasound in the treatment of cancer, in Ultrasonic Energy,(Ed. E. Kelly), Univ. of Illinois Press, Chicago 1965, p. 137-149.

72. B.I. Martins, Synergistic effect of X-rays and ultrasonic waves on mammalian cells cultured in vitro, Joint NASA-AEC Program in Space Radiation Biology, UCRL-17751, Sept. 1967, p. 37-40.

73. P.R. Clarke, C.R. Hill and Kay Adams, Synergism between ultrasound and X-ray in tumor therapy, Br. J. Radiol. 43, 97-99, 1970.

74. M.H. Repacholi, Electrophoretic mobility of tumor cells exposed to ultrasound and ionizing radiation, Nature, 227, 166-167, 1970.

75. T.T. Puck, P.I. Marcus and S.J. Ciecura, Clonal growth of mammalian cells in vitro: growth characteristics of HeLa cells with and without a 'feeder layer', J. Exptl. Med. 103, 273, 1956.

76. M.M. Elkind and G.F. Whitmore, the Radiology of Cultured Mammalian Cells, Gordon and Breach Science Publishers, Inc., N.Y. 1967.

77. L. Bergmann, Ultrasonics and their Scientific and Technical Applications, Trans. by H. S. Hatfield, John Wiley & Sons, Inc., N.Y., 1938.

78. R.W. Wood, Supersonics, The Science of Inaudible Sounds, Brown Univ. Press, R.I. 1939.

79. C. Kittel, Ultrasonics Research and the properties of matter, Physical Soc. Reports on Progress in Physics, Vol. XI, 205-247, 1948.

80. P. Vigoureux, Ultrasonics, Chapman and Hall Ltd., Lond., 1950.

81. E.G. Richardson and A.E. Brown, Ultrasonic Physics, Elsevier Publishing Co., Amsterdam and N.Y., 1962.

82. J. Blitz, Fundamentals of Ultrasonics, Butterworths, Lond., 1963.

83. V.A. Krasil'nikov, Sound and Ultrasound Waves, 3rd Ed., Trans. and published by the Israel Program for Scientific Translations, Jerusalem, 1963.

84. R.T. Beyer and S.V. Letcher, Physical Ultrasonics, Academic Press, N.Y. and Lond., 1969.

85. A.R. Peacocke and N.J. Prichard, Some biophysical aspects of ultrasound, in Progress in Biophysics and Molecular Biology, Vol. 18, (Eds. J.A.V. Butler and D. Noble), Pergamon Press, 1968, 185-209.

86. W.F. Fry and R.B. Fry, Determination of absolute sound levels and acoustic absorption coefficients by thermocouple probes--Theory, J. Acoust. Soc. Amer. 26(3), 294-310, 1954.

87. J.A. Newell, A radiation pressure balance for the absolute measurement of ultrasonic power, Phys. Med. Biol. 8(2), 215-221,1963.

88. C.R. Hill, Calibration of ultrasonic beams for bio-medical applications, Phys. Med. Biol. 15(2), 241-248, 1970.

89. Introduction to Vibration and its application p. 3 Textron Electronic, Inc. 1964.

90. R.C. Parker, Methods of tissue culture, 3rd Ed., Harper and Row, N.Y., 1961.

91. J. Paul, Cell and tissue culture, 3rd Ed., Williams and Wilkins, Baltimore, 1965.

92. E.N. Willmer, Ed., Cells and Tissues in Culture, Vol. I and II, Academic Press, N.Y. 1965.

93. P.W. Todd, Reversible and irreversible effects of ionizing radiations on the reproductive integrity of mammalian cells cultured in vitro Ph.D. thesis, Univ. of Calif. Berkeley, 1964.

94. E. Siegel, The effects of thyroid hormones on cultured mammalian cells, Ph.D. thesis, Univ. of Calif. Berkeley, 1966.

95. U. Madhvanath, Effects of densely ionizing radiations on human lymphocytes cultured in vitro, Ph.D. thesis, Univ. of Calif. Berkeley, 1971.

96. U.S. Demin, Effect of vibration and X-rays on cell nuclei of mammalian marrow, Radiobiology IV(4), 107-113, 1964.

97. P. Grognot, R. Loubier and A. Pfister, Un probleme original en biologie aerospetial: L'action des vibration mecaniques au niveau cellulaire, in Proc. 2nd Int. Symposium on Basic Environmental Problems of Man in Space, Paris, 1965. Springer-Verlag, Vienna, 1967, p. 229-233.

98. S.N. Romanov, Nature of Biological Effects of Vibration, Biofizika 12(1), 120-123, 1967.

99. R. Goldstein and S. Okada, Interphase death of cultured mammalian cells (L5178Y), Rad. Res. 39, 361-373, 1969.

100. C.R. Hill, P.R. Clarke, M.R. Crowe and J.W. Hammick, Biophysical effects of cavitation in a 1-MHz ultrasonic beam, in Ultrasonics for Industry, Conference Papers 1969, Illife House, 1969, p. 26-30.

101. E.H. Newcomer and R.H. Wallace, Chromosomal and nuclear aberrations induced by ultrasonic vibrations, Am. J. Bot. 36, 230, 1949.

102. E.H. Newcomer, cited from Ref. 43, p. 242.

103. J.F. Lehmann, E.J. Baldes and F.H. Krusen, Destructive effects of high-intensity ultrasound on plant tissues, in Ultrasound in Biology and Medicine, E. Kelly, Ed., Waverly Press, Inc., 1957, p. 177-190.

104. B.A. Bridges, M.J. Ashwood-Smith and R.J. Munson, Correlation of bacterial sensitivities to ionizing radiation and mild heating, J. Gen. Microbiol. 58, 115-124, 1969.

105. A.D. Conger, The cytogenic effect of sonic energy applied simultaneously with X-rays, Proc. Nat. Acad. Sci. U.S. 34, 470, 1948.

106. P. Todd, M.J. Steibel and T. Smyth, Jr., Microelectrode potentials on cell surfaces, Biophysical Society Abstracts, p. 145a, 1971.

107. C.D. Cone, Electroosmotic interactions accompanying mitosis initiation in sarcoma cells in vitro, Trans. N.Y. Acad. Sci. 31, 404-427, 1969.