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The Effects of Ultrasound on Human Lymphocytes: A Search for Dominant Mechanisms of Ultrasound Action.

by

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Abstract

Ultrasound is one of the most widely used tools in therapeutic and diagnostic medicine today. However, very little is known of its modes of action. Experiments were undertaken to determine whether ultrasound at a frequency and intensities close to those used in therapy produced measureable effects on human lymphocytes.

This work presents the results of investigations into the effects of ultrasound both within and on the surface of human peripheral blood lymphocytes exposed in vitro. Experiments were designed and performed with the aim of elucidating the predominant mechanisms of ultrasound on lymphocyte cultures.

Ultrastructural studies indicated that concanavalin A (Con A) - activated lymphocytes exposed to ultrasound (870 kHz, 4 W/cm^2, 30 min) did not suffer any gross damage. However ultrasound did appear to produce stresses within the Con A-treated cell that caused reversible disassembly of microtubules. Exposure of lymphocyte cultures to ultrasound at an intensity of 4 W/cm^2 produced an increase of 5°C above the ambient (37°C) temperature of the water bath. However heating was shown not to be one of the more important predominant mechanisms of action of ultrasound on the lymphocyte.

Evidence was produced to show that continuous wave, near field, 870 kHz ultrasound exposures at an intensity of 4 W/cm^2 for 30 min produce an immediate, significant ($P = 0.001$) reduction in the incorporation of ^{3}H-thymidine into lymphocytes activated with Con A for 2-3 days prior to sonication. This

effect was not observed in resting cells. At lower intensities a significant stimulatory effect on ^{3}H-thymidine incorporation occurs in activated lymphocytes some two to three days after exposure. These changes in incorporation of ^{3}H-thymidine did not appear to result from variations in the initial rates of transport of the radioactive precursor across the lymphocyte membrane. However, investigations of unscheduled DNA synthesis in activated lymphocytes suggested that ultrasound may be producing damage, either directly or indirectly to the DNA.

Simultaneous reductions in the incorporation of ^{3}H-thymidine, ^{3}H-leucine and ^{3}H-uridine were observed in activated lymphocytes following exposure to ultrasound. It is suggested that these reductions may be interrelated.

Ultrasound produced a significant reduction in the total binding of ^{3}H Con A to the lymphocyte cell surface. This result suggested that a study should be made of the effect of ultrasound on the mixed lymphocyte reaction (MLR). Exposure of responder cells at day 0 to ultrasound caused a reduction in the incorporation of ^{3}H-thymidine when this was measured at day 5. Exposure of stimulator cells to ultrasound using the same experimental conditions also produced a reduction in the incorporation of ^{3}H-thymidine at day 5. Exposure of both the responder and stimulator cells to ultrasound reduced the ^{3}H-thymidine incorporation at day 5 to a level substantially lower than if either the responder or stimulator cells alone were sonicated.

Cells were suspended in degassed media in an attempt to reduce ultrasound-induced cavitation. Both the experimental results and theoretical predictions suggest that mechanical stresses other than cavitation could be responsible for the effects produced in the lymphocytes by ultrasound.

Many of the results obtained from this work may have clinical significance. Further, a number of the experimental procedures and techniques have not previously been performed in the ultrasound field, making the results unique and difficult to compare to other works. However, the results are discussed and compared as far as possible to current theory on ultrasound actions in biological systems, and further experimental approaches are suggested.

Résumé

On présente ici les résultats de recherches portant sur les effets des ultrasons tant à la surface qu'à l'intérieur de lymphocytes humains de la circulation sanguine périphérique exposés in vitro. On sait que les ultrasons provoquent une élévation de la température du système exposé et sont responsables de contraintes mécaniques comme la cavitation et les courants acoustiques. Les expériences ont donc été conçues et effectuées dans le but de connaître les principaux mécanismes d'action des ultrasons dans les cultures de lymphocytes.

Des études de l'ultrastructure ont montré que des lymphocytes activés à la concanavaline A (Con A) et exposés aux ultrasons (870 kHz, 4 W/cm^2, 30 min) ne subissaient pas de dommages importants. Les ultrasons ont cependant été sources de contraintes, chez les cellules traitées à la Con A, qui se sont traduites par un démantèlement réversible des microtubules. L'exposition des cultures de lymphocytes aux ultrasons, à une intensité de 4 W/cm^2, a provoqué une élévation de la température de 5 ^oC par rapport à la température du bain d'eau (37,^oC). Ce réchauffement ne s'est cependant pas avéré être l'un des principaux mécanismes d'action des ultrasons sur les lymphocytes.

On a obtenu la preuve que des expositions en champ proche à des ultrasons, en onde entretenue, de 870 kHz et d'une intensité de 4 W/cm^2 pendant 30 minutes, se traduisaient

par une diminution immédiate et significative ($p = 0,001$) de l'incorporation de la thymidine-^{3}H dans les lymphocytes activés par de la Con A pendant 2 ou 3 jours avant le traitement aux ultrasons. Cet effet n'a pas été observé chez les cellules au repos. Il a été noté, aux plus faibles intensités, une stimulation significative de l'incorporation de la thymidine-^{3}H dans les lymphocytes activés environ deux ou trois jours après l'exposition. Ces modifications de l'incorporation de la thymidine-^{3}H ne résultaient pas de variations des vitesses de transport initiales du précurseur radioactif à travers la membrane lymphocytaire. Mais des études sur la synthèse de réparation de l'ADN chez les lymphocytes activés portent à penser que les ultrasons altéraient l'ADN qui était ultérieurement réparé.

Des réductions simultanées de l'incorporation de la thymidine-^{3}H, de la leucine-^{3}H et l'uridine-^{3}H ont été notées chez des lymphocytes activés après exposition à des ultrasons. On suggère la possibilité d'une interrelation entre ces réductions.

Les ultrasons ont provoqué une réduction significative de la fixation totale de la Con A-^{3}H à la surface cellulaire des lymphocytes. Il serait donc utile d'effectuer une étude portant sur les effets des ultrasons sur la réaction lymphocytaire mixte. L'exposition aux ultrasons des cellules réagissantes au jour 0 a provoqué une diminution de l'incorporation de la

thymidine-^{3}H quand celle-ci a été mesurée au jour 5. L'exposition aux ultrasons des cellules stimulatrices, dans les mêmes conditions expérimentales, a aussi provoqué une diminution de l'incorporation de la thymine-^{3}H au jour 5. L'exposition aux ultrasons des cellules réagissantes et stimulatrices a provoqué, au jour, 5, une réduction de l'incorporation de la thymine-^{3}H à un niveau substantiellement inférieur à celui noté après exposition d'un seul de ces deux types cellulaires.

Des cellules ont été mises en suspension dans un milieu dégazé dans le but de réduire la phénomène de cavitation par ultrasons. Les résultats expérimentaux et les prévisions théoriques portent à penser que des contraintes mécaniques autres que la cavitation pourraient être responsables des effets des ultrasons chez les lymphocytes.

Un bon nombre des résultats obtenus lors de ces travaux présentent une importance clinique. De plus, plusieurs de nos méthodes et techniques n'avaient jamais été utilisées dans le domaine de l'étude des ultrasons de sorte que les résultats obtenus sont uniques et difficiles à comparer avec ceux d'autres travaux. La discussion et la comparaison des résultats ont cependant été menées aussi à fond que possible, en demeurant dans le cadre de la théorie actuelle de l'action des ultrasons sur les systèmes biologiques, et d'autres approches expérimentales ont été proposées.

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Chapter 1. Introduction

In recent years applications of ultrasound in medicine have increased tremendously, especially in the diagnostic field. This has resulted partly from the commonly held contention that there is no risk to health from exposure of ultrasound.

Ultrasound was introduced to diagnostic medicine in mid-1950s and has been growing at such a rate that "with expanding services in ultrasound diagnosis, the frequency of human exposure is increasing with the potential that essentially the entire population may be exposed" (IRPA 1977). The Bureau of Radiological Health (U.S. Department of Health, Education and Welfare) estimates that by the early 1980s, every pregnant woman in the United States will undergo at least one ultrasound examination of the fetus (Stratmeyer and Stewart 1976).

Ultrasound therapy has been used for over 40 years in physiotherapy. It usually involves application of a hand-held ultrasound transducer to the injured area of a patient, and treatment with either a continuous or pulsed beam. The transducer head is generally moved over the area of injury to obtain as uniform a treatment distribution as possible.

The objective of ultrasound therapy is apparently to stimulate the blood flow within the injured region, which it appears to do effectively (Franklin et al 1977a, 1977b), and to provide deep heating to muscles and other tissues. Summer

and Patrick (1964) claim that the therapeutic effect of ultrasound is achieved by four specific effects:

(i) Thermal - a temperature rise within the tissue which is proportional to the input power and exposure time. Ultrasound seems to have the advantage that it is absorbed more in muscle than in fatty tissue.

(ii) Micromassage - this effect is caused by the mechanical reactions of the ultrasound in tissue, such as compression and dilation.

(iii) Volume reduction - as the ultrasound wave passes through the tissue it produces instantaneously small reductions in volume which are independent of frequency but proportional to the intensity.

(iv) Motion and amplitude - pressure waves set up stress patterns in tissue, producing reciprocal movement of cells.

Lehmann et al (1974, 1978) point out that the main therapeutic value of ultrasound is related to its selectivity of absorption. In soft tissue this absorption may be directly related to the protein content of the tissue (Piersol et al 1952). Lehmann et al (1974) claim that the benefit of ultrasound as a therapeutic agent is that it heats selectively the areas that one desires to be heated, including superficial bone, scar tissue within soft tissue, tendons and tendon sheaths, etc. They claim further, that ultrasound may accelerate diffusion process across biologic membranes, producing an increased rate of healing.

It is, however, very difficult to assess the benefit of ultrasound therapy, as Roman (1960) found. Of one hundred patients treated or sham-irradiated for lower back pain, bursitis of the shoulder and myalgia, 60% of those receiving ultrasound were categorized as good or normal, but 72.1% of the shams were in the same category.

1.1 The Problem and the Approach

1.1.1 The Problem

Therapeutic ultrasound is used at frequencies between 0.8 and 3 MHz (Lehmann et al 1974) and at intensities up to 4 W/cm^2. Pulsed and continuous wave modes are both in common use. Therapy devices have recently been the subject of extensive investigation. In Canada, Repacholi and Benwell (1979) found that most ultrasound therapy devices were not delivering the "dose" prescribed for the patient. The average treatment times varied between 1 and 20 minutes, typical spatial average intensities (cw mode) between 0.1 and 4 W/cm^2, timing errors went up to ±20% of setting and there were errors in the ratio of measured to indicated acoustic output over ±200%.

The problem arises from the facts that:

(i) millions of people are exposed to therapeutic ultrasound each year,

(ii) the intensities used in therapy are well within the range known to produce adverse biological effects,

(iii) the mechanisms of biological action of ultrasound in therapy have not been adequately investigated in a systematic manner, and

(iv) although the action of higher intensities (> 10 W/cm^2) of ultrasound have been investigated, very little is known of possible subtle effect at lower intensities.

1.1.2 The Approach

The major emphasis and purpose of this work has been to attempt to elucidate predominant mechanisms of action of ultrasound on an important biological model system, the human lymphocyte in culture. Experiments were designed to reproduce, to some extent, the situation existing for patients receiving ultrasound therapy. Human lymphocyte cultures exposed in vitro provide a reasonable analogy to peripheral blood lymphocytes exposed in vivo. Ultrasound exposure parameters used were at the upper limits of the therapeutic range. An apparatus was specially designed to allow:

(i) near field ultrasound exposures to be made;

(ii) cells to receive as even a distribution of acoustic energy as possible;

(iii) sterile conditions to be used throughout the experiment;

(iv) exposures to take place under free-field conditions.

(v) cell cultures to be contained without air bubbles. Thus the effect of ultrasound induced cavitation could be determined.

I attempted to demonstrate reproducible effects both on the cell surface and internally. Ultrasound is known to produce both thermal and non-thermal effects. End-points therefore had to be found that would allow one, not only to distinguish between these two effects, but to permit a more detailed investigation of an apparently dominant mechanism.

Chapter 2. Lymphocytes

2.1 General Description

The present studies were conducted on a population of small round cells called lymphocytes, a type of leucocyte, categorized as agranulocytes, because they have few if any prominent granules in the cytoplasm. Human peripheral blood lymphocytes have a diameter between 7 to 14 μm. In the resting state the nucleus is typically round or somewhat indented but may, on occasion, show a deep cleft. The chromatin of the nucleus is highly condensed and lies predominantly at the nuclear periphery. Nuclear pores are present and are generally free of dense chromatin (Mandel 1977). The nucleolus is usally visible only in suitably thin sections. The narrow rim of cytoplasm of small lymphocytes contains few organelles, generally several mitochrondia and a small Golgi zone that contains a pair of centrioles and sparely distributed free ribosomes. The rough endoplasmic reticulum is sparse and few microtubules may be seen under optimum conditions (Ling and Kay 1975). When lymphocytes are activated into cell division, their nuclei increase in size, the chromatin becomes more dispersed, and the nuclear outline more irregular; in activated cells, large nucleoli may be present. In activated cells the cytoplasm swells considerably, contains a greater concentration of organelles and large numbers of free ribosomes (Mandel 1977).

Although the lymphocytes are small they are very motile and are recognized to be an integral part of the human defence system against foreign cells and materials, being responsible for virtually all specific immune reactions (Roitt 1974). Cells having a similar morphology are found in the spleen, bone marrow, lymph nodes, tonsils, thymus and other areas, e.g. the Peyer's patches.

2.1.1. Origins

Bone marrow cells appear to contain the stem cells from which two major lymphocyte populations (B and T) are processed. The distinction between B and T cells is on the basis of presumed differences in ontogeny, cell surface markers and function (Mandel 1977). The point during ontogeny at which the stem cells become committed to the T or B cell pathway is still unclear.

Lymphocyte stem cells processed through the thymus are called thymus-derived or T-lymphocytes. These cells are concerned directly in cell-mediated immunity and indirectly with antibody formation by functioning as helper cells in the antibody response to many antigens.

In chickens, the bursa of Fabricius is responsible for the development of immunocompetence in cells destined to make humoral or circulation antibody, functioning predominantly as precursors of antibody secreting plasma cells. These cells are bursa-derived or B-lymphocytes. The equivalent of the bursa in man has not yet been identified, although the bone marrow has been implicated (Kincade and Moore 1977).

When an antigen enters the body the central importance of the lymphocyte is manifest through two different types of immunological reactions. Both B- and T-lymphocytes, after appropriate stimulation by an antigen, proliferate and undergo morphological changes. The B-lymphocytes develop into plasma cells which synthesize free antibody and release it into the

blood and other body fluids. Humoral or circulating antibodies act, for example, by direct combination with, and neutralization of, bacterial toxins, as well as by coating bacteria to enhance phagocytosis.

2.1.2. Cell Surface

The plasma membrane serves as the interface between the cell and its external environment. This membrane plays a vital role in recognition of molecular structures, direct contact with other cells, and in the initial phases of cell activation. Lymphocytes have plasma membranes which are predominantly a fluid mosaic of lipid and protein (Singer and Nicolson 1972) with some components anchored to micro-filements and microtubules (Loor 1979).

More than 25 different proteins (immunoglobulins and various glycoproteins) on the membrane serve as surface markers (Marchalonis 1977). These proteins are involved in conditioning the response of lymphocytes to external molecular stimuli. Distinct lymphocyte types are characterized by their surface markers and their differing capacities to respond to mitogens. B-lymphocytes bear readily detectable surface immunoglobulin molecules and have receptors for the third component of complement detected by the ability to form rosettes with sensitized sheep erythrocytes. T-lymphocytes lack these markers and form rosettes with unsensitized sheep erythrocytes. In humans a third type of lymphocyte - null cells (non-T, non-B cells) lack easily detectable surface immunoglobulins and fail to form rosettes with sheep erythrocytes (Boldt et al 1975).

Biochemical analyses of the plasma membrane indicates that it is composed of approximately equal quantities of lipid and protein, with carbohydrate constituting some 3 - 5%. A small amount of RNA exists in this membrane, but no DNA (Crumpton and Snary 1974).

Although it is known that for lymphocyte activation to occur, mitogens or antigens must bind to receptors exposed on the plasma membrane, the mechanism is still obscure. The plasma membrane appears to constitute the cellular organelle which regulates differentiation, growth and division in lymphocytes (Resch 1979).

Glycoproteins are responsible for the cell surface charge resulting from the ionic and highly polar groups on proteins protruding from the cell membrane. Non-polar groups are largely buried in the hydrophobic interior. It has been suggested (Santer 1977) that this layer has an important role in determining migratory behavior, antigenic reactivity, and in forming receptors for mitogens.

2.2 Activation of Lymphocytes

When human lymphocytes are cultured in vitro, they remain mitotically inactive. However, these resting lymphocytes can be activated by addition of mitogen, to undergo a series of biochemical and cytological events culminating in mitosis, as Nowell (1960) discovered 20 years ago. This is termed blastogenesis and is generally accepted to be a model of the in vitro proliferation process associated with the immune response.

While proceeding through blast transformation, the lymphocyte enlarges some 2-3 times its diameter in the resting state, to a diameter of 15 - 25 μ. During blastogenesis the densely packed heterochromatin in the nucleus decondenses, so that the ratio of euchromatin to heterochromatin changes from 0.56 to 10.8 (Cooper 1972).

While many systems are available for studying molecular events associated with the transition from resting to actively dividing cells, the lymphocyte is exceptional in having direct physiological significance to humans.

In the present studies, the mitogen used for activation was concanavalin A (Con A), a plant lectin derived from jack bean. It binds specifically to α-D-glucopyranosides, α-D-manno-pyrannosides, and to polysaccharides or glycoproteins containing such residues (Sharon and Lis 1972). Con A is a non-specific stimulant because blood or tissue T-lymphocytes of many different specificities are stimulated; such a response is said to be polyclonal and no requirement for prior sensitization to the substance concerned is necessary.

For activation of lymphocytes by a mitogen it is necessary that the mitogen bind to the cell surface. Although binding of the mitogen to the cell surface is a necessary first step in lymphocyte activation, it seems that the simple binding of the mitogen does not ensure that the lymphocyte will in fact be activated. Certain prerequisites must be met before activation can proceed (Stubo 1977).

1. A threshold number of mitogen molecules must bind to the surface of the lymphocyte to provide a sufficient stimulus.
2. There must be a linkage between events occurring at the cell surface and inner mechanisms for cell activation. For example, Stubo et al (1972) and Anderson et al (1972) have found that there are between 0.8 - 3 x 10^7 molecules of Con A bound per cell for saturation of murine thymocytes. However only 2.5 x 10^6 molecules of mitogen or approximately 20% are needed to initiate blast transformation and DNA synthesis.

Once the necessary binding has taken place, the cell must possess a mechanism by which information is relayed from the cell surface to the inner cell machinery to trigger blast transformation. Similarly, surface membrane movement and endocytosis occur shortly after mitogen binding, but their exact relationships to cell activation still remains obscure (Stubo 1977).

2.2.1 Early Effects of Mitogen

Activation of lymphocytes by Con A resembles in many ways specific antigen-induced lymphocyte stimulation (Wands et al 1976). Con A has been used to understand the mechanism by which lymphocytes are stimulated by a specific antigen. Since it has been shown that the mitogen needs to bind to the lymphocyte cell surface for blast transformation to occur, it is reasonable to expect that the initial effects of the mitogen on lymphocytes are observed as changes in the properties of the cell membrane.

Hulser and Peters (1971) showed that immediately after PHA administration, the normally high electrical resistance of the lymphocyte's membrane was reduced at sites of cell contact. In their studies to determine the mechanism by which PHA rapidly stimulates phospholipid metabolism of human lymphocytes, Fisher and Mueller (1971) found that a four-fold increase in the turnover of phosphatidyl inositol occurred within the first three minutes and was completely restricted to the lymphocyte plasma membrane. In their work on phospholipid metabolism in the plasma membranes of stimulated lymphocytes, Resch and Ferber (1972) and Resch et al (1973) found that stimulation of de novo synthesis of lecithin and neutral fats is also very rapid. Puntis (1977) reported a two-fold increase in phospholipid synthesis 12 h after addition of mitogen to human lymphocytes.

From studies of the incorporation of glucosamine into intermembrane glycoproteins of PHA stimulated lymphocytes, Hayden et al (1970) found that the membrane glycoprotein turnover is doubled in three hours. Some 5 to 30 minutes after the addition of mitogen increased sodium and potassium ion fluxes and increased oaubain sensitive ATPase activity occurs (Quastel 1970, Quastel and Kaplan 1970, Kaplan 1977, Averdunk 1972); as well as increased, fluxes of calcium ions (Whitney and Sutherland 1973, Kaplan 1977), glucose (Averdunk 1972), uridine (Peters and Hausen 1971), and amino acid (Van den Burg and Betel 1973).

The mechanisms by which Con A exerts its biological effect on lymphocytes may depend in part on the organization of its subunits, the number and type of binding sites, and valence. At physiological pH, Con A exists primarily as a tetrameric molecule comprising four identical subunits of approximately 25,000 molecular weight, each of which possesses a carbohydrate binding site (Edelman et al 1972). Studies by Gunther et al (1973) on a dimeric molecule of Con A and by Wands et al (1976) on a monovalent form of Con A indicated that both forms of the molecule bound to lymphocyte surface receptors as well as with the intact molecule, but displayed important biological differences. The dimeric form of Con A produced only weak agglutination of lymphocytes, and induced no cap formation by its glycoprotein receptors but was

mitogenic. (Immunoglobulin molecules are not rigidly fixed on the cell surface and can be drawn to one end of the cell to bind with an antigen. A "cap" at one end of the cell can be observed when a fluorescent labelled anti-immunoglobulin is added to B cells.) The same number of monovalent Con A molecules were bound to lymphocytes as the tetrameric form, but did not stimulate blast transformation or cytotoxicity, suggesting that Con A must bridge binding sites on the lymphocyte surface to induce lymphocyte activation.

There is evidence (Boldt et al 1975) that the number of receptor sites for any lectin on the surface of human lymphocytes is the same for each type of lymphocyte. Lymphocyte binding studies with ^{125}I-lectins indicate that human T, B, and Null cells bind equivalent amounts of each lectin. In particular, the number of Con A receptor sites on T, B and Null lymphocytes was determined to be 1.7×10^6/cell (Boldt et al 1975). This value is in reasonable agreement with the number of Con A binding sites found in other studies (Boldt et al 1972, Wands et al 1976, Krug et al 1973).

2.2.2 Metabolic Functions (DNA, RNA and Protein Synthesis)

2.2.2.1 Protein Synthesis

The rate of lymphocyte protein synthesis increases markedly after the addition of mitogen, a significant increase being observed as early as 2 - 3 h after stimulation (Kay et al 1971). The uptake of radioactive precursor was observed to rise about 2 fold after 4 h and 10 fold after 48 h (Wallace et al 1979), and appeared to be due to an increased rate of translation of pre-existing mRNA by pre-existing ribosomes. Studies on lymphocyte cytoplasm indicate that the low rate of protein synthesis in unstimulated cells is due to low activity of one or more protein initation factors, and the specific activity of these inhibitors is decreased after activation of lymphocytes by mitogen (Kay et al 1978, 1979).

Early de novo protein synthesis may be responsible, at least in part, for the observed increases in DNA polymerase and RNA polymerase activities. Along with a doubling of amino acid incorporation, the number of polysomes is increased one hour after stimulation (Hauser et al 1978).

2.2.2.2 RNA Synthesis

Synthesis and processing of RNA in lymphocytes has recently been reviewed by Kaplan et al (1979). Resting lymphocytes seem to maintain a basal level of RNA synthesis. When lymphocytes are activated by a mitogen, there is no increase in total RNA synthesis for some 4 - 6 h. Thereafter, it increases steadily for the next 40 h until a plateau is reached during S-phase.

Early reaction of lymphocytes to mitogen appear not to be on the initiation of new RNA chains, (Mitchell et al 1978), but rather on RNA processing including polyadenylation, splicing, methylation and capping, RNA export to the cytoplasm, and RNA binding to and translocation by polysomes (Schafer and Mitchell 1979).

2.2.2.3 DNA Synthesis

Resting lymphocytes are normally in G_0. When activated, the lymphocytes enter a growth phase G_1 in which there is no DNA synthesis. Subsequently the cells enter S-phase where DNA synthesis occurs, then G_2 in which DNA synthesis has been completed and the cells prepare for mitosis. The onset of DNA synthesis in human peripheral lymphocytes is delayed usually for at least 24 - 30 hours of incubation of the lymphocyte cultures with the mitogen. Bard et al (1978) have demonstrated that in human lymphocytes stimulated to proliferate by addition of mitogen, approximately 25% of the population enters blast transformation after 20 h and appreciable DNA synthesis did not occur until 36 h.

Soren (1973) found quite some variability in the time at which PHA sitmulated lymphocytes initiate DNA synthesis, reporting that most cells commenced synthesizing DNA between 48 and 72 hours of culture. However, some cells were in S-phase as early as 24 hours, while others did not enter their first S-phase until 120 hours of culture. This variability was confirmed in the findings of the present studies, where peak DNA synthesis occurred at varing times after cell stimulation. Generally the peak of incorporation of (^{3}H) thymidine occurred 2 - 4 days after stimulation and then trailed off.

There is evidence that DNA polymerase activity in PHA treated human lymphocytes increases immediately prior to replication of the DNA (Loeb and Agarwal 1971). This DNA polymerase activity increases 30 to 150 fold and probably results from de-novo synthesis of the enzyme.

Besides synthesis for normal DNA replication in lymphocytes activated in vitro with plant mitogens or specific antigens, there is evidence (Boldt et al 1977) for release of a large percentage of newly synthesized DNA into the culture medium. It is suggested that excreted DNA might serve a communicative role in cell-cell interactions among various lymphocyte subpopulations.

2.3 Mixed Lymphocyte Reaction

The mixed lymphocyte interaction was first reported by Bain et al (1964), who demonstrated that leucocytes from two unrelated individuals when mixed together resulted in the transformation of some of the cells into blasts typical of the proliferative response. To distinguish the response due to one population of cells (responders) stimulated by the other (stimulators), it is generally necessary to inhibit the DNA synthesis of the stimulators. The response observed will then be that of the uninhibited cell population. This inhibition can be achieved by mitomycin C (Bach and Voynow 1966) treatment of the stimulating cell population.

Stimulator lymphocytes must be viable, metabolically active cells at the time the MLR is set up, otherwise no activation occurs (Ling et al 1974). This would indicate that the stimulator is not merely a carrier cell on which the appropriate antigens are expressed.

T-cells are the responding cells in the autologous MLR, and may be stimulated by lymphocyte activating determinants (LADS) on allogeneic leucoyctes (Fainboim et al 1979). Beale et al (1979) reaffirmed the observation that T cells are the principle responder in autologous MLR, but found that macrophage are also necessary for mitogen stimulation.

A good review on the biological significance of the MLR has been completed by Nabholz and Miggiano (1977).

2.4 Unscheduled DNA Synthesis

Repair of damaged DNA in bacteria and mammalian cells has recently been reviewed by Hanawalt et al (1979). DNA in cells is continually subjected to alterations, either spontaneous or produced by some external agent. Damage to DNA is responsible for most mutagenesis and some lethality. Thus repair processes to DNA are essential for the cell's continuity (Hanawalt et al 1979).

Damage can be defined as any modification of DNA that alters its coding properties or its normal function in replication or transcription. Minor base changes may result in an altered DNA sequence while other types of damage may distort DNA structure and interfere with replication. To be repaired the damage must be recognized by a repair enzyme that can initiate a sequence of biochemical events leading to its elimination and the restoration of the intact DNA structure.

Mammalian cells are capable of incorporating nucleotides after single or double strand breaks in DNA chains, excising cyclobutane dimers or repairing other types of damage to DNA following exposure to chemicals or radiations. However, the nature of the DNA lesion determines the nature of the enzymic mechanisms responsible for the repair (Trosko and Chu 1975).

An example of one of the better known DNA repair processes is excision repair. In essence this process requires that: damage to the DNA be recognized by a specific enzyme; a phosphodiester strand scission made; the lesion excised; and the deleted section reconstituted using the intact complementary strand as a template. Possible DNA excision repair processes are shown in Figure 2.1 (Hanawalt et al 1979).

Certain physical and chemical agents can induce damage to the DNA in both strands at the same or neighbouring base pairs. In this case, neither strand can function as a template for error-free resynthesis. Nevertheless mammalian cells appear to repair this type of damage (Hanawalt et al 1979). An example of this is Mitomycin C, a chemical agent which produces covalent cross-linking of complementary strands of DNA (Iyer and Szybalski 1963) and is subsequently repaired (Sasaki 1978). Both Sasaki (1975) and Fujiwara et al (1977) have reported an unscheduled DNA synthesis in mammalian cells following treatment with Mitomycin C. Models that invoke both excision repair and strand exchange in the repair of covalent cross-linking of complementary strands of DNA have been proposed by Sasaki (1978) and Cole et al (1978).

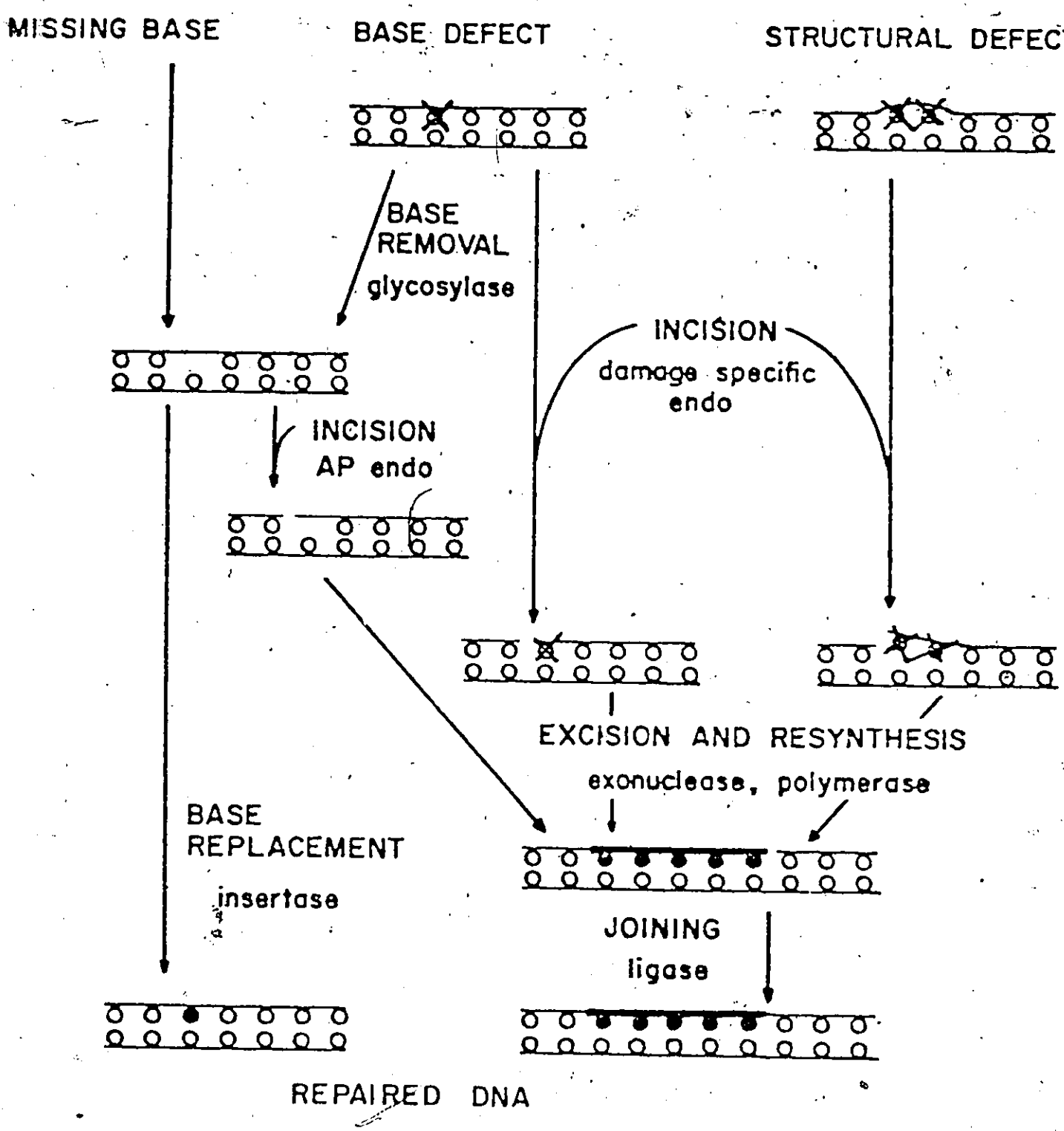


Figure 2.1 Possible DNA Excision Repair Process.

Resting lymphocytes have been found more susceptible to DNA damaging agents than activated cells. Only 5% of unstimulated lymphocytes survived for 6 days after treatment with 150 rads of X rays, while 30% of mitogen stimulated cells survived 1200 rads during the same period. Similarly, no resting lymphocytes survived treatment with 5 µg/ml nitrogen mustard, but 50% did so in mitogen stimulated cells (Ling and Kay 1975). The radiosensitivity of lymphocytes appears to be associated with its metabolically resting state and it was suggested that this was related to their incapacity to repair damage (Vaughan-Smith and Ling 1974, Timberlake et al 1976). DNA repair synthesis was demonstrated to occur in mammalian cell nuclei at G_1, G_2 and S-phase and in metaphase chromosomes (Stich and San 1970).

To date no single enzyme has been isolated from mammalian cells with the combined excision and polymerizing activities equivalent to E coli DNA polymerase 1 (Hanawalt et al 1979). However, Bertazzoni et al (1976) measured the levels of the DNA polymerases -α and -β in PHA stimulated lymphocytes during a 12 day period. Although both enzymes were present in detectable amounts in resting cells, the peak rate of DNA synthesis corresponded to a peak of DNA polymerase-α, increasing 20 fold over resting cell values. Evidence was produced to indicate that, although the levels of DNA polymerase-β increased with the DNA synthesis rate, it peaked some time after maximum DNA synthesis and seemed to correlate with UV induced DNA repair synthesis. Thus Bertazzoni et al (1976) suggested that the DNA polymerases had a tendency to specialize, the α-enzyme correlated with DNA replication and the β-enzyme assisting the cell to perform repair type synthesis.

DNA excision repair has been studied using a variety of methods. Monitoring the loss of radioactively labelled DNA or measuring the disappearance of lesions using chromatography provide direct measurement of excision repair (Hanawalt et al 1979). Sedimentation in alkaline sucrose gradients can be used to follow the initiation and completion of excision repair from the appearance and disappearance of strand breaks in DNA.

A number of methods have been developed to monitor DNA repair synthesis that do not depend upon a knowledge of the specific lesion being repaired. Combined density and radioactive labelling of newly made DNA, followed by separation on density gradients, allows repair synthesis to be determined by the radioactivity incorporated into unreplicated parental density DNA. The most useful but least quantitative method for determination of repair synthesis that circumvents the necessity for separation of DNA strands, is unscheduled DNA synthesis (Hanawalt et al 1979). This technique involves the incorporation of a radioactive precursor into DNA after damage has occurred and using an autoradiographic technique to allow observation of silver grains over the cell nucleus. However the interpretation of results with mammalian cells is complicated by the organization of DNA into chromatin (Trosko and Chu 1975). The substrate for damage and repair is a highly structured DNA-protein complex.

2.5 Nucleoside Transport

Transport is the unidirectional translocation of materials across the plasma membrane into the cell. In order to measure translocation mediated by membrane carriers one must measure influx of substrate (nucleoside) during the time interval before backflux of the nucleoside becomes significant. This interval may only last a matter of seconds. For experiments measuring only transport over a period of time, the quantities taken up will extrapolate back to zero (Berlin and Oliver 1975).

Rapid sampling techniques have enabled measurements of nucleoside transport to be taken in times as short as 4 seconds (Strauss et al 1976). Results from such methods applied to murine splenocytes and rat hepatoma cells have shown initial rates of transport, before backflux occurs, last as long as 60 s (Strauss et al 1977, Wohlueter et al 1978).

Resting human peripheral lymphocytes (Rudd - personal communication) and murine spleen cells (Strauss et al 1977) exhibit no apparent translocation of thymidine in a carrier-mediated fashion. These authors have also shown that Con A induces the appearance of two membrane transport systems for thymidine in lymphocytes "in vivo" as early as 24 h, corresponding to the onset of DNA synthesis.

2.6 Why Lymphocytes Used As Test System

Lymphocytes constitute some 30% of the white cell population in human blood, and are in continous circulation between the main blood stream and lymphoid tissue. About 1300 grams of lymphocytes exist outside the blood, 3 grams in the circulating blood, 70 grams in the bone marrow, and 100 grams in the lymphatic tissue (Ling and Kay, 1975). In any clinical exposure to ultrasound, therefore, it is inevitable that at least some of these cells will be subjected to sonication.

About 60% of the circulating lymphocytes have a lifespan measured in years (Buckton and Pike 1962) and may never divide under normal conditions; so that subtle or even delayed consequences of ultrasound action, such as short- or long-term impairment of lymphocyte functions, particularly their immune response, may well be significant. Their ubiquity and importance ensure that lymphocytes are a useful system for studying biological consequences of ultrasound action since their normal in vivo existence within (fluid) blood ensures that in vitro liquid culture irradiations may produce information relevant to the uncovering of dominant physical mechanisms of ultrasound action.

Perhaps one can gain an overall impression of the test system from the following ditty:

"THE LYMPHOCYTE

The LYMPHOCYTE is small and round
And innocent of face,
Which is extremely handy
For a cell that circulates.

Its size and shape allow our friend
The LYMPHOCYTE to squeeze
Through tissue spaces, nodes and spleen,
And small capillaries.

Into every nook and crevice
It wanders with its peers;
Its life is long: the LYMPHOCYTE
Can live for months, or years.

But when it meets a foreigner -
An antigen or graft -
The LYMPHOCYTE discards its cool
And goes completely daft.

It grows, expands, unfolds, 'turns on',
And even (so they say)
Prepares itself for fission
By making DNA.

The LYMPHOCYTE, that seemed so meek
Goes all the way to Hell,
And transforms to a flaming
PYRONINOPHILIC CELL!

- B. Bain - "

Chapter 3 Ultrasound

3.1 Characteristics

3.1.1 Nature

Ultrasound is a mechanical or acoustic radiation that cannot exist in vacuo; it requires a molecular medium in which to propagate.

Ultrasound propagates in waves at frequencies above 16 Hz. A wave is a disturbance whose position in space changes with time. One can describe the wave motion of the particles using the wave equation

$$\frac{d^2u}{dz^2} = \frac{1}{c^2}\frac{d^2u}{dt^2}$$

where u is a function of z describing the displacement of a particle in the medium at any given time t, as the wave moves in the z-direction with a velocity c. Wave motion causes a transfer of energy through the medium without a transfer of matter. This transfer is described by the wave equation. The mechanism of the energy transfer, information on interactions between the wave and the medium, and changes in the properties of the medium due to the wave, are all contained in the parameter c.

If we consider a wave moving along the z-axis, particles in the medium will be displaced u from this axis and can be described by a function of z and t where

$$u = f(ct-z)$$

Since an ultrasound transducer is usually driven with a sinusoidal wave of electrical impulses, u will be a simple harmonic displacement, so that an appropriate choice of function is

$$u = u_o \sin k(ct-z)$$

where $k = 2\pi/\lambda$ is called the wave number and λ is the wave length.

Since the energy transmission of ultrasound occurs via molecular stimulation of the medium, it can propagate in gaseous, liquid or solid media; it does so mostly via longitudinal or compressional waves, where the molecules vibrate to and fro in the direction of energy propagation. As energy passes through the molecular structure of the medium, the wave is formed by alternate areas of compression and rarefaction (relaxation) of molecules. Energy moves from molecule to molecule, rather than the molecules moving themselves. When the molecules undergo compression, there is an accumulation of energy, and conversely a reduction of energy in rarefaction.

For soft biological tissues such as fat, blood, muscle and organs, the velocity of ultrasound depends on the inhomogeneities of the medium, but generally lies between 1490-1610 m/s.

Absorption of ultrasound in a medium occurs when the vibrational energy is dissipated into internal molecular energy or as random molecular motions. There are many mechanisms by which ultrasound absorption occurs in a medium.

(i) Viscous Loss: ultrasound is strongly absorbed in viscous liquids because of friction between the moving particles or interfaces. The compressing action of the beam causes movement of some interfaces (particles) at 90° to the direction of the beam, resulting in the frictional loss of beam energy.

(ii) Hysteresis loss: a medium not behaving perfectly elastically causes extra energy to be required or dissipated to drive the particles of the medium through each wave cycle.

(iii) Relaxation: At given points in the cycle, ultrasound waves cause groups of particles to have an increased kinetic energy (particle velocity). The energy from these particles can be transferred to the next group of particles, which could be for example, in a state of higher internal molecular energy. At the beginning of the next part of the

wave cycle, the kinetic energy of the second group of particles due to the wave motion is reduced, causing energy to pass back to the first group of particles.

Energy transfer between the two groups of particles takes time, characterized by the relaxation time of the medium. The ultrasound intensity is reduced by this relaxation process.

(a) Thermal relaxation is the process by which energy is transferred to internal molecular energy and back.

(b) Conductive relaxation occurs when the transfer of energy takes the form of heat flowing from one region to another and back.

(c) Structural relaxation occurs when there is a passage of energy between two states of different physical structure.

Attenuation of the beam intensity and amplitude occurs exponentially according to the expression

$$I_x = I_o \, e^{-\mu x}$$

where I_x is the intensity of a progressive plane ultrasound wave, after travelling a distance x in the attenuating medium, I_o is its initial intensity (at x = o) and μ is the intensity attenuation coefficient of the medium. The larger the value of μ, the more rapid is the decrease in intensity of the beam in the medium.

The attenuation of the beam by a medium increases at higher ultrasound frequencies according to the general expression:

$$\mu_f = \mu_{f_o} (f/f_o)^n$$

where μ_f = intensity attenuation coefficient of the medium at frequency f (MHz), μ_{f_o} = intensity attenuation coefficient of the medium at reference frequency f_o, and n is a constant having value between 1 and 2 for most human tissue.

3.1.2 Generation of Ultrasound

Langevin produced the first dependable ultrasound source in 1918. He built a quartz transducer which utilized the piezoelectric effect to produce ultrasound with controlled frequency and intensity. When certain crystals are subjected to pressure or mechanical stress, an electrical potential appears on their surfaces -- the piezoelectric effect. Jacque and Pierre Curie first described this effect in 1880. In the following year, Lippmann found that there was a reverse piezoelectric effect: a mechanical distortion of the crystal occurred when an electrical potential was applied. Only crystals with an asymmetrical structure (possessing one or more polar axes), such as tourmaline, lithium sulphate, Rochelle salt, lead niobate and quartz, exhibit this effect.

Ultrasound can be generated by simple devices called transducers. The structure of a basic transducer used in most medical applications is shown in Figure 3.1. A cylindrical metal tube encloses a disc-shaped, piezoelectric crystal at one end. A thin metallic film is evaporated onto the front and back faces of this crystal to form the electrodes. Logically, the front electrode is earthed to the case to allow handling and application of the transducer to the patient skin without fear of electrical shock. The back electrode is connected to a wire carrying the electrical impulses. Backing material is used to damp crystal vibrations in the direction opposite to that of the useful beam. Similarly, acoustic insulating material is used to attenuate vibrations transmitted toward the casing of the transducer.

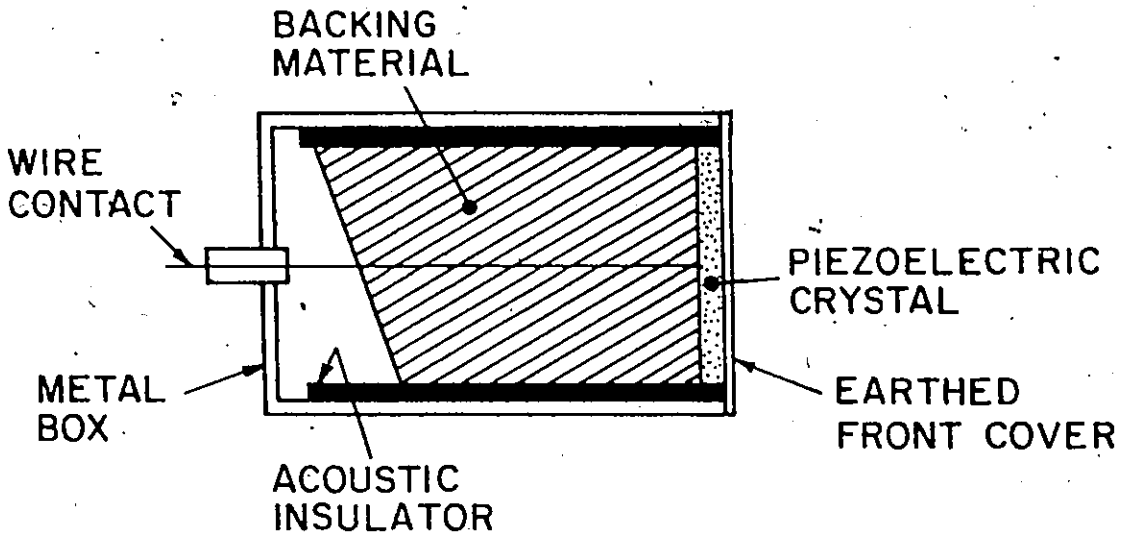


Figure 3.1

Structure of an Ultrasound Transducer.

3.1.3 Structure of Ultrasound Fields

The "ultrasonic field" of a transducer is the term used to describe the spatial distribution of its radiated energy. If a transducer is considered to be an ideal oscillating piston source, producing a continuous wave of ultrasound into water, the field produced will approximate the shape shown in Figure 3.2(a). The field is divided into two regions - the near field or Fresnel zone and the far field or Fraunhofer zone.

The near field is characterized by violent fluctuations in beam intensity along the central axis. A slow divergence of the beam occurs in the far field, characterized by a smooth falling-off of beam intensity, as is shown in Figure 3.2(b). Huygen's principle can be applied in the analysis of the field, where the surface of the transducer is considered to be an array of individual elements, each radiating spherical waves in the forward direction. Since each element moves synchronously with equal amplitudes, the crystal can be considered to be a piston, the surface of which vibrates in phase at constant amplitude. Using this analysis, the cross-sectional intensity distributions at selected positions along the central axis of the beam, and the theoretical distribution of the field can be determined (Figure 1.2(b)).

In the far field, the beam diverges at an angle Θ (called the Fraunhofer zone divergence angle) given by

$$\sin \Theta = 1.22 \lambda / D$$

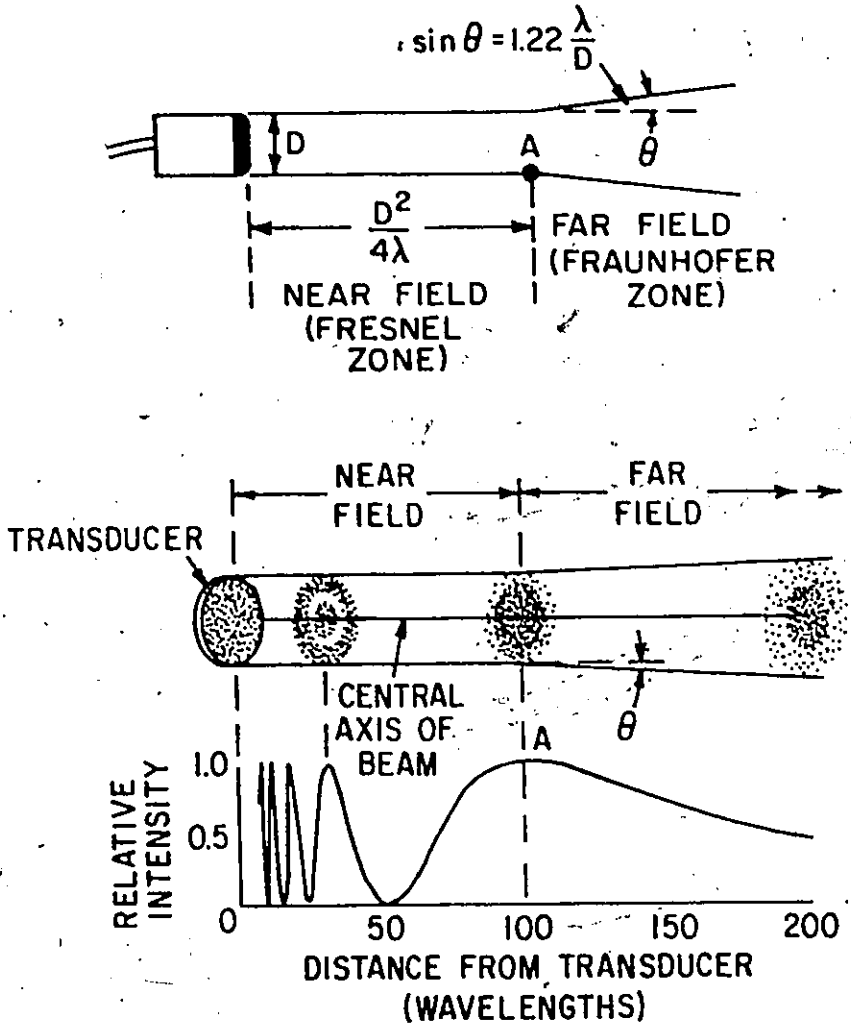


Figure 3.2

Structure of ultrasound fields: (a) The near and far field regions in relation to the transducer. (b) The field distribution of an ideal piston source generating a continuous wave. In this example the transducer has a diameter of 10 wavelengths. The ring diagrams represent the cross-sectional intensity distributions of selected positions along the central axis of the beam.

where λ = wavelength of the ultrasound beam and

D = diameter of the crystal.

The Fresnel zone extends from the crystal to a distance = $D^2/4\lambda$ when $D^2 >> \lambda^2$.

3.2. Mechanisms of Interaction

When any form of energy is absorbed by matter, it is partially converted into heat, the temperature elevation depending on the amount of energy absorbed. Thus one of the mechanisms of interaction of ultrasound with matter has to be thermal. Other mechanisms such as cavitation, stress, radiation force, radiation pressure, and radiation torque have also been observed in biological systems. Unfortunately the present state of knowledge on interaction mechanisms is very limited and so discussions of these cannot be given in the detail one would like to have. Excellent reviews of ultrasound mechanisms have been made by Nyborg (1978, 1979, 1980).

3.2.1 Thermal Mechanism

3.2.1.1 Volume Heating

When ultrasound interacts with matter, part of the energy of the beam will be absorbed and converted into heat. If there is no heat transfer, the rate at which heat is generated per unit volume within the matter is Q given by the equation $Q = \alpha I$; where α is the absorption coefficent and I is the intensity of a plane travelling wave. Without heat conduction away from the exposed medium the rate of temperature rise will be (Dunn 1965)

$$\frac{dT}{dt} = \frac{2\alpha I}{\rho C_m} \qquad 3.1$$

where dT/dt is the temperature rise per unit time, ρ is the density of the medium and C_m is the specific heat per unit mass.

Consider the example of an ultrasound beam of intensity 1 W/cm^2 exposing soft tissue. If ρ =1 g/ml, C_m = 4.2 J/g/°C and α is 0.1 Np/cm, the temperature rise dT/dt is then 0.048°C/s. The absorption coefficient of 0.1 Np/cm is approximately the value, for mammalian liver or brain at a frequency of 1 MHz.

Equation 3.1 is only valid for a stationary medium, so would not apply to a flowing liquid such as blood. Further, the equation applies only for an initial period, since with time, heat will be lost by conduction.

Consider the effect of heat conduction away from exposed matter. When the medium is initially sonicated, the temperature will rise linearally with time at a rate given by equation 3.1. As conduction occurs, heat flows away from the irradiated area affecting the rate of temperature rise. In time, the heat loss through conduction becomes significant, even at the point of direct irradiation. The temperature then rises more and more slowly and approaches a limiting value when equilibrium is achieved. Equilibrium occurs when the rate of temperature rise due to ultrasound equals the rate of temperature loss by heat conduction from

the irradiated medium. This phenomenon was observed in the present study and is shown in Chapter 4.

If the sonicated medium is a sphere, the equilibrium temperature T_c at the centre of the sphere is given by (Nyborg 1978)

$$T_c - T_o = \frac{\alpha I R^2}{K} \qquad 3.2$$

where T_o is the initial temperature, K is the thermal conductivity coefficient and R is the radius of the heated sphere.

The time required to reach the equilibrium temperature is

$$t = \rho C_m R^2/K; \qquad 3.3$$

where t is the time for the temperature at the centre of the sphere to rise to 80% of the equilibrium value. An estimate of time to reach the equilibrium temperature can be obtained for water where

$K = 0.006$ W/cm°C

$\rho = 1$ g/ml

$C_m = 4.2$ J/g/°C and

$\alpha = 0.1$ Np/cm.

If the intensity of the ultrasound beam is 0.1 W/cm^2 then from equations 3.2, 3.3

$T_c - T_o = 1.67R^2$ and

$t = 700\ R^2$

For a water sphere of radius R = 1 cm, the equilibrium temperature rise ($T_c - T_o$) at its centre is 1.67°C and the time (t) to reach this equilibrium is 700 seconds. The predictions of this simplified model have been shown to be comparable with experimental measurements (Lele 1975). This analysis assumes, however, that all the ultrasound energy is absorbed within the water sphere and that the exposures are conducted in the far field where acoustic intensity variations are gradual.

3.2.1.2 Surface Heating

Although the average temperature rise of a sonicated medium e.g. cell cultures as a whole may be small, ultrasound induced shearing and streaming forces may produce sufficient heat at the molecular level to cause denaturation. Let us consider this at the cell surface.

There exists the possibility of preferential membrane heating, since the primary effect of the ultrasonic beam is to impose an oscillatory motion on tissue, together with a sinusoidal pressure variation. A temperature increase is produced by the action of viscous forces between the cell membrane and the external medium. The oscillatory force is in dynamic equilibrium with the sum of the inertial forces (F) of the particle, according to Stoke's viscous drag formula, where

$$F = 6\pi N r V_r$$

N is the viscosity of the external fluid, r the radius of the cell (assumed spherical), and V_r the relative velocity between the cell and the external fluid.

The acoustic energy converted to heat per second (from Fry and Fry 1954) is

$$Q = \frac{1}{T_o} \int_0^{T_o} 6\pi N r (V_r)^2 dt$$

where T_o is the periodic time for the wave.

If the suspending liquid moves sinusoidally with respect to time, its motion can be represented by

$$\dot{x} = U \sin \omega t$$

where U is the particle velocity of the wave at frequency ω. Let ρ_f be the density of the fluid external to the cell, and ρ_c the average density of the cell, then

$$V_r = U \sin \omega t. \frac{(\rho_c - \rho_f)}{(\rho_c + \rho_f)}$$

For $\rho_c = 1.1$ and $\rho_f = 1.0$

$$V_r = \frac{U}{21} \sin \omega t$$

so the acoustic energy converted into heat per second is given by

$$Q = \frac{1}{T_o} \int_0^{T_o} 6\pi N r \frac{U^2}{(21)^2} \sin^2 \omega t \, dt$$

$$= 3\pi r N \frac{U^2}{(21)^2} \text{ erg/s} \qquad 3.4$$

Connolly and Pond (1967) quote the maximum cyclic velocity (in an aqueous medium) as $U = 11.5\ (I)^{\frac{1}{2}}$ cm/s where I is the intensity of the incident ultrasound beam in W/cm^2. The value of Q becomes

$$Q = 3\pi rN \frac{(11.5)^2}{(21)^2} \frac{I}{J} \times 10^{-7} \text{ cal/s}$$

The 10^{-7} factor converts ergs to joules.

For $I = 4$ W/cm^2, cell radius $r = 1.4 \times 10^{-3}$ cm (lymphocyte blasts) and N = 0.02 poise (from Ackerman 1962)

$$Q = 3.17 \times 10^{-13} \text{ cal/s}$$

This heating results from drag developed by a cell oscillating in a viscous fluid. Energy is dissipated in the boundary layer of medium surrounding the cell membrane. The boundary layer exists around an oscillating cell such that, out of this layer, no disturbance is detected from the cell oscillations in the ultrasonic field. Nyborg (1965) gives the boundary layer thickness d by the expression

$$d = \frac{(2N)^{\frac{1}{2}}}{(\rho_f \omega)^{\frac{1}{2}}} \qquad 3.5$$

For N = 0.02 poise, $\rho_f = 1.0$ and $\omega = 2\pi f = 2\pi \times (0.87 \times 10^6)$ Hz the value of d is approximately 0.2×10^{-3} cm.

Consider the case where all the heat goes into the boundary layer of a typical lymphocyte, the boundary layer having a thickness of 0.2×10^{-3} cm and the cell radius 1.4×10^{-3} cm. The mass (m) of the boundary layer ($\rho = 1$) assumed spherical is

$$m = \frac{4}{3}\pi[(r + d) - r]^3 \rho$$

$$= \frac{4}{3}\pi[(1.6 \times 10^{-3})^3 - (1.4 \times 10^{-3})^3] \times 1.1$$

$$= 5.66 \times 10^{-9} \text{ g}$$

The temperature rise (T) in the boundary layer, exposed to an intensity of 4 W/cm^2 is given by

$$Q = m \times C_m \times T \qquad 3.6$$

where C_m, the specific heat of the tissue, is approximately 1 cal/g/°C. Thus for $Q = 3.17 \times 10^{-13}$ cal/s and $m = 5.66 \times 10^{-9}$ g.

$$T = \frac{3.17 \times 10^{-13}}{5.66 \times 10^{-9}} \text{ °C/s}$$

$$= 5.6 \times 10^{-5} \text{ °C}$$

$$= 0.1\text{°C}$$

for a 30 min exposure if the ultrasound energy absorbed in the cell membrane occured without diffusion. This temperature rise indicates that at a frequency of 870 kHz an intensity of 4 W/cm^2 for 30 min causes heat production due to shear viscosity at the cell surface which could be considered as insignificant. Although preferential membrane heating can occur, the heat generated by the viscous forces is shared rapidly with the rest of the cell and its surroundings.

3.2.1.3 Summary of Thermal Mechanisms

One can summarize the thermal mechanism of action of ultrasound in the following way. Biological media subjected to an ultrasound field will experience a temperature rise, since heat is produced at a rate per unit volume proportional to both the ultrasound intensity at the point in question, and the absorption coefficient of the medium. Initially the temperature rises at a constant rate, but this rise slows as the temperature reaches an equilibrium value.

With the spherical model, it is found that the increase to the equilibrium temperature above the initial temperature is proportional to the square of the sphere's radius. The time required to reach this equilibrium temperature is also proportional to the square of the sphere's radius. A small body, uniformly exposed to ultrasound, will thus experience only a small temperature rise, but does so quickly. By contrast a body having a large radius uniformly exposed to the same ultrasound intensity reaches a higher final temperature but over a longer period of time.

Ultrasound energy converted to heat due to shear viscosity, generally results in a very small temperature rise at the cell surface, which is rapidly dissipated into the cell and its surroundings.

3:2.2 Cavitation

Under certain conditions, an ultrasound field can give rise to cavities or bubbles within the exposed medium. This phenomena is termed cavitation, and requires pre-existing nuclei of gas or vapor in the medium. Ultrasound induced cavitation is a complex activity which may include growth from pre-existing nuclei and eventual collapse of the bubble or cavity. When the bubble expands and contracts during the ultrasound pressure cycle, the surrounding medium flows inward and outward with velocities much greater than if the gas bubble was absent. The phenomenon of cavitation has recently been reviewed by Coakley and Nyborg (1978). There are two forms of cavitation:

Stable cavitation, or non-collapsing bubble formation, is important at relatively low intensities, and consists of simple breathing oscillations, or pulsations, in response to time varying pressure imposed on a nucleus of gas by the ambient ultrasound field. This time varying pressure is due to the alternating influence of the expansive and compressive phases of the ultrasound wave, and gives rise to streaming motions or acoustic microstreaming around the bubble. This can produce changes in the relative positions of intracellular organelles and breaks in cytoplasmic structure (Akopyan and Sarvazyan 1979).

Transient or collapse cavitation is more violent and occurs at higher ultrasound intensity levels. When the gas bubble or nucleus within the medium is acted on by an ultrasound field having a high pressure amplitude, it may collapse or implode. The gas nuclei expand and collapse from the alternate negative and positive pressure of the ultrasound wave. Kinetic energy given to a relatively large volume of liquid has, in the final stages of collapse, to be dissipated in an extremely small volume, and high temperatures and pressures result. When the gaseous material within the collapsing cavity is rapidly compressed; idealized thermodynamic calculations shows that for a compression in which no heat escapes the cavity, at the end of the cavity's existence, the final temperature is around 8000°K and pressures greater than 10,000 atmospheres. Sutherland and Verrall (1978) report that under actual conditions, all the heat does not remain trapped in the cavity during collapse, part is conducted away, producing temperatures of the order of 3500°K. Effects of biological systems can obviously be produced by the sudden changes in pressure or mechanical shocks during the bubble collapse.

Chemical changes are commonly produced by cavitation. The combination of high pressures and temperatures can generate aqueous free radicals and hydrated electrons within the sonicated medium and result in the disassociation of water vapor in the bubble during its contraction. Chemical interactions of biomacromolecules with these free-radicals often result, especially with hydrogen $H^{\cdot}$ and hydroxy $OH^{\cdot}$ and significantly altering their properties. This can be accompanied by the formation of such compounds as HNO_2, HNO_3 and H_2O_2 (Akopyan and Sarazyan 1979).

Equations for these reactions are given below.

$$H_2O \overset{\text{ultrasound}}{\rightleftharpoons} H^{\cdot} + OH^{\cdot} \qquad 3.7$$

$$RH \overset{\text{ultrasound}}{\rightleftharpoons} R^{\cdot} + H^{\cdot} \qquad 3.8$$

$$R^{\cdot} + H^{\cdot} \longrightarrow RH \qquad 3.9$$

$$R^{\cdot} + OH^{\cdot} \longrightarrow ROH \qquad 3.10$$

$$R^{\cdot} + R^{\cdot} \longrightarrow R\text{-}R \qquad 3.11$$

$$OH^{\cdot} + OH^{\cdot} \longrightarrow H_2O_2 \qquad 3.12$$

Equation 3.7 represents the ultrasound induced dissociation of water vapor into aqueous free radicals, while equation 3.8 represents the same type of dissociation of a typical macromolecule R from one of its hydrogen atoms. Both equations are reversible and if this reverse reaction occurs, you have repair (equation 3.9). However, if the $OH^{\cdot}$ radical combines with the macromolecular radical $R^{\cdot}$, or if the $R^{\cdot}$ combines with another $R^{\cdot}$, damage occurs (equation 3.10, 3.11) through

the formation of new macromolecules. At sufficiently high ultrasound intensities, where many of the water molecules are dissociated, the chances of forming toxic substances such as H_2O_2 (equation 3.12), a strong oxidizing agent causing further chemical changes in the biological medium, are increased.

In general, the lifetime of these radicals is very short (approximately 10^{-12} s) so in most cases dissociated molecules simply recombine. With high intensities of ultrasound, more highly reactive radicals are produced, increasing the chances of damage through various combinations of the free radicals.

Klibanov et al (1974) reported quite convincing evidence that the enzyme α-chymotrypain was inactivated irreversibly by reactive free radicals, produced by ultrasound induced cavitation. The authors determined the cavitation threshold to be approximately 1 W/cm^2 from the 880 kHz ultrasound field and inactivation of the enzyme occured at 2 W/cm^2. The addition of chloroform, an efficient donor of free radicals caused a sharp increase in the inactivation rate. In the presence of ethanol, which prevents free radical formation, ultrasound had practically no effect on enzymatic activity (Klibanov et al 1974).

The ultrasound intensities required to produce collapse cavitation exceeds a threshold value which varies considerably from one condition to another.

Hill (1972) defined cavitation as the condition in which three experimental criteria were simultaneously satisfied:

(i) DNA in aqueous solution was degraded by at least 50% of its initial molecular weight,

(ii) free iodine was released at a rate of at least 10^{-9} mol/s/cm from a solution of KI in the presence of CCl_4, and

(iii) the first order subharmonic of the driving frequencies was detectable by means of a focused hydrophone on the region of the exposed specimen.

With all criteria satisfied, Hill determined the cavitation threshold intensity for various frequencies (0.25 - 4 MHz) in air-equilibrated water for continuous wave ultrasound.

The threshold to produce collapse cavitation was found to vary from about 0.1 W/cm^2 at a frequency of 0.25 MHz to about 4 W/cm^2 at 4 MHz. The lower the frequency, the lower the intensity required to produce cavitation and vice versa.

Pulsing conditions were found to have a marked influence on cavitation (Hill and Joshi 1970). At shorter pulse durations the cavitation intensity threshold increased. Alternatively as the pulse duration decreased, the duty factor had to be increased to produce cavitation at a given intensity. At sufficiently short pulse durations, cavitation activity can be inhibited entirely. Higher ambient pressures cause higher threshold intensities for cavitation. For a 1 MHz continuous wave ultrasound beam, this threshold intensity varied from just under 1 W/cm^2 at an ambient pressure of 1 bar to much greater than 16 W/cm^2 at 1.75 bar. Thus increased ambient pressure provides a very effective means of inhibiting cavitation.

Cavitation can be produced more readily at higher temperatures. The threshold intensity for cavitation was found to decrease linearly on a log-log graph, with the volume of the irradiated liquid (Iernetti 1971).

The cavitational potential of a liquid is determined by the number of gas nuclei within the medium. Unfortunately this is not easily measured. Little is known whether animal tissue or cells contain these nuclei or gas bubbles. Thus one should not assume that cavitation induced biological effects observed from <u>in vitro</u> experiments on aqueous suspensions, would be expected in animals.

If biological tissue contains bubbles, it could absorb ultrasound energy readily, and thus effectively act as a heat source. This may account for the very high absorption coefficient of lung tissue being caused by gas bubbles in the lung (Dunn 1974). When ultrasound passes through a biological medium the rate of heat generation is proportional to the absorption coefficient. One would thus expect heating to occur preferentially in any tissue containing an appreciable number of bubbles resonant in the ultrasound field.

Consider the situation where ultrasound causes gas nuclei to pulsate in response to applied pressure oscillations. A gas nucleus can be approximated to a sphere of radius contracting to R - r and expanding to R + r, once during each ultrasound cycle, where r is the radial displacement amplitude and R is the mean bubble radius. The pulsating gas bubble may be analagous to a mass-spring model, the sphere acting similarly to a spring while a portion of liquid surrounding the sphere behaves like a mass (Nyborg 1978). If the pressure applied by the ultrasound is "in phase" with the bubble pressure, the bubble radius (and volume) is minimal when applied pressure is maximal and conversely the radius is maximal when the applied pressure is minimal. This condition occurs primarily at low ultrasonic frequencies, below a few hundred kilohertz. In the megahertz range, the phase difference between the applied ultrasound pressure and bubble pressure

differ in phase by 180°, i.e. the bubble radius increases when the applied pressure increases. Thus there must be a resonant frequency f_o where the phase difference is 90°. A general equation describing small bubbles exposed to ultrasound at the resonant frequency is given by (Coakley and Nyborg 1978)

$$\rho_o \omega^2_o R^2 = 3\gamma\,(p + 2\sigma/R) - 2\sigma/R; \qquad 3.13$$

where ρ_o is the density of water, ω_o ($= 2\pi f_o$) is the angular frequency at resonance, γ is the effective ratio of specific heats for the gas, p is the hydrostatic pressure and σ is the surface tension of the air-water interface. Equation 3.13 reduces to

$$f_o = 3260/R \qquad 3.14$$

if σ/R is much less than p, and γ is approximately 1.4. The latter assumption applies to air when the volume changes adiabatically (without change in temperature). Thus, equation 3.14 is approximately true for resonant air bubbles in water at frequencies up to a few hundred kilohertz. From this equation one derives a resonant ultrasound frequency of 65.2 kHz for a bubble having a mean radius of 50 μm. However, in the megahertz range, this adiabatic condition does not hold and the effective value of σ is not accurately known. For air σ lies between 1 and 1.4, the lower limit applying to very small bubbles oscillating isothermally.

Experimental measurement carried out by Miller (1977) indicates that a free air bubble of radius R = 3.5 μm has a resonant frequency f_o = 1 MHz. This value is consistent with the theory if one assumes a value of γ = 1.2.

Let us now return to the situation where a gas bubble in an ultrasound field with a mean radius of R is pulsating between R + r and R - r, where r is the radial displacement. Coakley and Nyborg (1978) have developed a theory describing the response of such a bubble assuming negligible surface tension effects. If at the site of a bubble, but in its absence, an ultrasound field exists producing pressure variations sinuoidally in time with frequency f and amplitude p_o, then, as was described above, the phase difference between the bubble radius and applied pressure is 180° if $f << f_o$ and 0° if $f >> f_o$. At resonance, the phase is 90° and $f = f_o$. The radial displacement r is given by:

$$r = r_o X,$$

where $$r_o = p_o R/3\gamma p,$$

$$X = [(1 - \Omega^2)^2 + \Omega^2\delta^2]^{-\frac{1}{2}}, \qquad 3.15$$

and $$\Omega = f/f_o$$

δ is the damping constant, which is a measure of the energy dissipation and is equal to the reciprocal of the oscillation quality factor. As mentioned above, at frequencies well below resonance $f << f_o$, Ω is much less than 1 and so the relative amplitude $X = r/r_o$ is unity. At resonance $f = f_o$, Ω = 1, then from equation 3.15 the relative amplitude X has the value of $1/\delta$.

As a bubble vibrates in a cavitating ultrasonic field, it extracts energy from the sound field applied to it. Some of this energy is converted irreversibly into heat and the rest is reradiated from the bubble as an outgoing spherical wave (Nyborg 1978). It has been shown that in water, a resonant bubble at 1 MHz in an ultrasound field of intensity 100 mW/cm^2 removes about 60 μW from the field. Of this, about 90% is converted to heat and the remaining 10% reradiated or scattered.

It has been observed by Nyborg et al (1975) that bubbles will attract each other if they are equal in size or if both are smaller than the resonant size. If one bubble is larger and the other smaller than resonant size, they repel if at large distances apart, but may attract at small separations, giving rise to the "pearl chain" effect. In a progressive ultrasound wave the radiation force propels the bubbles along the direction of propagation and the force is maximum if the bubble is resonant.

When a gas bubble pulsates, its motion is not usually spherical, either because of distortion by an adjoining solid boundary, or because of surface waves set up by the ultrasound field. Asymmetric or nonuniform oscillation of the air-liquid interface, at the surface of an air pocket or bubble, causes steady eddying motion to be generated in the immediately adjoining liquid. Since this motion is usually small, it is appropriately call microstreaming. Microstreaming can give rise to high velocity

gradients around air pockets at the surface of a solid boundary. If biopolymer molecules or small biological cells are suspended in the liquid, they may be swept into a region of high velocity gradient. The biological system will then be subjected to shearing action and damage may occur, such as fragmentation of macromolecules and membranes (Nyborg 1978).

Significant biological effects would be expected in suspensions near resonant bubbles, even at low intensity levels. This was recently reported by Miller et al (1978), who found that blood platelets tended to aggregate around artificial holes (forming gas bubbles) in a membrane. Previously, mechanical stresses produced by cavitation at low intensities of 20 kHz ultrasound were reported by Rooney (1970), who found that canine red cells were lysed by a single air bubble (at its resonant size). The intensity level was below that required for transient cavitation or free-radical production, but could be caused by shearing stresses set up by microstreaming near the bubble (Nyborg 1979).

3.2.3 Stress Mechanisms

Stress mechanisms or non-thermal, non-cavitational mechanisms have recently been reviewed by Dunn and Pond (1978). Ultrasound exposure produces various stresses within biological systems, the magnitude and significance depending on the detailed characteristics of the ultrasound field and the exposed biological systems.

Stresses or forces due to the ultrasound field acting on inhomogeneities in the medium can be considered in four categories (Dunn and Pond 1978):

1. buoyancy forces which are oscillatory, time-average to zero, and produce a radiation pressure on bodies having density differences from the surrounding medium,
2. displacement or radiation forces which have a non-zero time average and can cause an appreciable relative velocity between the inhomogeniety and the surrounding medium,
3. viscosity-variation forces or viscous stresses result in acousting streaming due to variations in viscosity over the cycle of the applied ultrasound, and

4. the Oseen force, another time-average force due to the dependence of drag on the second power of relative velocity. Stokes "viscous drag" formula for a sphere only accounts for the first power of the relative velocity.

The Oseen force can be considered as accounting for the fact that a moving particle leaves a wake whose energy per unit path length increases with the relative velocity. Viewed in another way, if one considers a pure sinusoidal ultrasonic wave travelling through a medium, it will suffer distortion. Thus the rate of change of momentum of a particle lying in the path of the ultrasound wave will be greater at the leading edge than at the trailing edge, and the particle will experience different forces (Oseen) at these edges (ter Haar and Wyard 1978). The Oseen force is given by (Westervelt 1951)

$$F_o = 6\pi Nr\ U\ (1 + k\ |U|)$$

where U is the fluid velocity, N the coefficient of viscosity, r the radius of the sphere and k a constant dependent on the degree of distortion. For cells suspended in water or blood plasma, the Oseen forces were reported to be negligible (ter Haar and Wyard 1978).

When ultrasound causes liquid to undergo oscillatory flow near a rigid boundary which imposes a non-slip condition on this flow, the viscous stress exerted on the boundary is the product of the velocity gradient and the shear viscosity coefficient N. This viscous stress S at the boundary varies sinusoidally in time with frequency ω, and has an amplitude given by Nyborg (1978)

$$S = \mu_o (\omega N \rho/2)^{\frac{1}{2}} \qquad 3.16$$

where μ_o is the free field velocity amplitude, ω is the angular frequency of the field (assumed continous and single-frequency), ρ is the density of the liquid and η its shear viscosity. For an ultrasound wave of frequency 1 MHz in water (ρ = 1, μ_o = 11.5 cm/s, N = 0.01 poise) and intensity 1 W/cm^2, then, using equation 3.16, the stress amplitude is 2040 dyn/cm^2. If boundary layers such as macromolecules or cell surfaces are in the liquid medium, viscous stresses of this size could act on them and be a source of damage. However, this stress is not constant since it reverses itself each half cycle of the wave. Oscillatory shear may give rise to steady fluid motion called acoustic streaming.

Non-thermal, non-cavitational stresses characteristically have a time independent component and arise from such phenomena as radiation pressure, radiation force, radiation torque and acoustic streaming. This has recently been reviewed by Nyborg (1979) and is summarized here.

3.2.3.1. Radiation Pressure

When ultrasound is generated in a medium, the pressure varies with time. At any given time, the pressure varies from point to point within the medium. For continuous wave ultrasound having one frequency, the pressure has a component which varies sinusoidally with time, and is called the first order contribution to the pressure. There is also a second order contribution consisting of two parts. One part, the second harmonic, varies sinusoidally with time and frequency of twice the fundamental frequency. The other part, called radiation pressure, is steady and independent of time (Nyborg 1979). The total pressure P produced by an ultrasound field can be considered as the sum of all the contributions;

$$P = P_0 + P_1 + P_2$$

Here P_0 is the zero-order contribution of the pressure, which exists in the absence of an ultrasound field, P_1 is the first order contribution and P_2 is the second order contribution. Third and subsequent order contributions are negligible. The time independent part of P, identified as the radiation pressure (P_{rad}), is like an increase in the static pressure brought about by the ultrasonic field, and so may be positive or negative. If the influence of viscosity can be neglected, P_{rad} can be written in terms of energy densities. The radiation pressure is equal to the time-averaged potential energy density minus the time-averaged kinetic energy per unit volume in the ultrasound field plus a constant.

$$P_{rad} = \langle PE \rangle - \langle KE \rangle + \text{constant}$$

For a plane travelling wave the time-averaged potential energy density and time-averaged kinetic energy per unit volume are equal, and equal to I/2C where I is the intensity and C is the velocity of the ultrasound. Hence the radiation pressure is a constant. For a plane standing wave the radiation pressure is a maximum in a series of planes one-half wavelength apart, and minimum in planes midway between successive maxima. The maxima of the radiation pressure occur when the pressure is a maximum in the standing wave, while the minima occur at velocity maxima. For a spherical travelling wave, the radiation pressure is constant, being typically zero, when the distance from the origin is large but decreases as this distance decreases. Near the origin or ultrasound source, the time-average kinetic energy per unit volume is large and dominates over the time-averaged potential energy density. Here the radiation pressure takes on relatively large values. Spatial variations in the radiation pressure can lead to forces on bodies in an ultrasound field and also a fluid flow through tubes or channels which connect one part of the ultrasound field to another.

There is evidence for the existence of radiation pressure being exerted by ultrasound pulses on brain tissue, giving rise to disturbances that can be sensed by the ear (Foster and Wiederhold 1978). Further, Gershoy and Nyborg (1973) have postulated that gradients of radiation pressure in sonicated plant tissue give rise to water flow in cytoplasmic channels.

3.2.3.2 Radiation Force

Material exposed to an ultrasound field is usually acted upon by a steady time independent force called the Radiation Force. When acting on a stationary body, this force is just the integrated result of the radiation pressure acting on all parts of its surface. However, if the material vibrates, there is an extra contribution to the radiation force. Consider the example of an ultrasound field exposing an inhomogeneous biological medium. In the medium, forces will be exerted on the inhomogeneities and on the containing wall, because of the ultrasound. In part, the force will be oscillatory in time, having the same frequency as the ultrasound, and a time average of this part is 0. However, there is another component of the force which is usually present and whose time average is not 0. This constant or time independent component is the radiation force. One uses this force to measure the acoustic output or intensity of an ultrasound device. In this application, the ultrasound beam interacts with a plane reflector, and the force can be measured. The intensity can then be computed using available theory (Nyborg 1980).

Gould and Coakley (1974) found that if a suspension is sufficiently dilute, so that the particles are widely spaced, the radiation force on each particle is exerted as if the other particles were absent. If the particles are closely spaced, they interact with each other by virtue of interparticle radiation force. Radiation force theory for closely packed particles has been reviewed by Nyborg (1978). The effect of radiation force on a close packed suspension of particles observed as a formation of particle chains in suspension - a pearl chain effect, was noted in section 3.3.2. Suspended particles form chains, since the ultrasound field in a fluid medium produces an excess positive or negative static pressure, which varies from point to point in the field. The excess static pressure at any given point is the radiation pressure. Gradients in the radiation pressure contribute to the radiation forces on the suspended particles. If small channels exist in the fluid, a difference in radiation pressure at the ends of the channels will cause fluid to flow within them.

Radiation force has been used to explain the flow produced by ultrasound in the cytoplasmic layer surrounding the large vacuole of a plant cell. Gershoy and Nyborg (1973) reported that a radiation pressure field set up in vacuoles of cells could cause intracellular flow of fluids between the vacuoles and the cytoplasmic layer.

If the influence of the viscosity of the medium is negligible on a small suspended particle (sphere), the radiation force F_{rad} can be expressed in terms of energy densities as follows (Nyborg 1979):

$$F_{rad} = vD\nabla\langle KE\rangle - v(1-\gamma)\nabla\langle PE\rangle \ , \qquad 3.17$$

where $\nabla\langle KE\rangle$ represents the gradient of the time averaged kinetic energy density of the ultrasound field, $\nabla\langle PE\rangle$ represents the gradient of the time averaged potential energy of the medium, γ is the ratio of the compressibility of the particle to that of the surrounding medium, F_{rad} is the radiation force on a sphere of volume v and density ρ, in a liquid of density ρ_o, and

$$D = 3(\rho - \rho_o)/(2\rho + \rho_o)$$

Equation 3.17 can be used to determine the radiation force on small particles in an ultrasound field. In a standing wave field exposing a suspension of particles of arbitrary density and compressibility, there is some distance (repeated along the path of the beam) where $F_{rad} = 0$, and where particles having the same characteristics collect. In principle cells having different density and compressibility could be separated in a standing wave field Apfel (1976).

Radiation force is an important mechanism by which ultrasound interacts with biological systems. An example of radiation force is the blood flow stasis phenomenon reported by Dyson et al (1974), where red cells in the blood vessels of chick embryo collected into parallel bands spaced at half wavelength intervals in an ultrasonic standing wave field.

3.2.3.3 Radiation Torque

Nyborg et al (1975) have observed spinning of intracellular bodies during exposure to ultrasound. When an ultrasound field is propagating within a liquid, a twisting action may be exerted on suspended objects, and on elements of the liquid itself. For asymmetrically shaped objects such as a rod or disc, this radiation torque varies with the orientation of the object relative to the oscillation direction of the surrounding liquid, so that the object tends to assume a favoured position. This favoured position being one in which the force on the object is least. Such an effect may be important when considering the effects of ultrasound on asymmetrically shaped cells, organelles or macromolecules.

According to theory (Nyborg 1979) this spinning is expected in fields existing at a boundary where a progressive ultrasound wave impinges obliquely and is reflected back. The velocity of spinning of the object is proportional to the ratio of the absorption coefficient for the material in this spherical body and the coefficient of shear viscosity for the surrounding fluid ($v \propto \frac{\sigma}{N}$).

Dyer (1972) observed that when the boundary of moss protonema was sonicated during cell division, the incipient cross-wall was set into steady rotation about the axis perpendicular to the protonemal axis. This rotation was probably due to radiation torque. Subsequence cell division proceeded in a number of instances, but some of the daughter cells and their progeny were abnormal.

Recently Martin et al (1978) have observed the effects of radiation torque in sonicated (2.1 MHz, 43 mW/cm^2) leaves of Elodea and root tip of Vicia faba. How radiation torque effects other macromolecular structures or organelles within or outside cells is at present unknown. However, it is certainly conceivable that such a mechanism of ultrasound could provide adverse biological effects at sufficiently high ultrasound intensities.

3.2.3.4 Acoustic Streaming

When an ultrasound field is propagating within a liquid, the particles of the liquid take part in an oscillatory flow. Near a boundary, the liquid moves to and fro in a direction parallel to the boundary. At the boundary itself the velocity of the liquid flow will be zero provided the boundary is a fixed rigid solid and "non-slip" conditions apply. A thin boundary layer may exist between the surface of the boundary and the liquid itself where the velocity gradient is large. For oscillatory flow in an ultrasonic field of frequency f, the characteristic thickness of this boundary layer is (Rooney, 1972)

$$d = \left[\frac{N}{\pi \rho_0 f}\right]^{\frac{1}{2}} \qquad 3.18$$

where N is the coefficient of shear viscosity and ρ_0 the density for the fluid. This is shown diagrammatically in Figure 3.3. The velocity gradient in the boundary layer can produce stresses of the order of 2000 dyn/cm^2. Viscous stresses of this magnitude would act on any biological molecules or cells which might exist in or near the boundary layer. Ultrasound fields which generate oscillatory shear in boundary layers give rise to small scale steady fluid motions called acoustic streaming. The particle velocity of

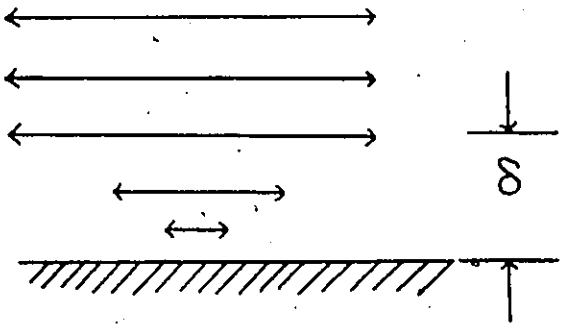


Figure 3.3

Boundary layer, of thickness δ, near a rigid boundry exposed to an ultrasound field.

components within a liquid medium exposed to a propagating ultrasound field can be expressed in a series of terms as shown below:

$$u = u_1 + u_2 + \ldots\ldots$$

The first order contribution u_1, varies sinusoidally in time with frequency f. The component u_2 has two parts; one a second harmonic which varies sinusoidally in time with the frequency 2f and the other is a steady time-independent component called the acoustic streaming. Such streaming has been observed as circulatory flow in the vacuoles of plant cells (Nyborg 1978). However, to set up this streaming there must be non-uniformity or asymmetry of some kind. For an ultrasound field propagating in a suspension of particles, relative motion occurs between the particles and the fluid, where boundary layers of streaming liquid are established around each particle and give rise to an acoustic streaming field; this consisting of an eddying pattern of small scale and is appropriately called microstreaming. This was shown as early as 1959 by Elder who demonstrated and analyzed four regimes of streaming as shown in Figure 3.4.

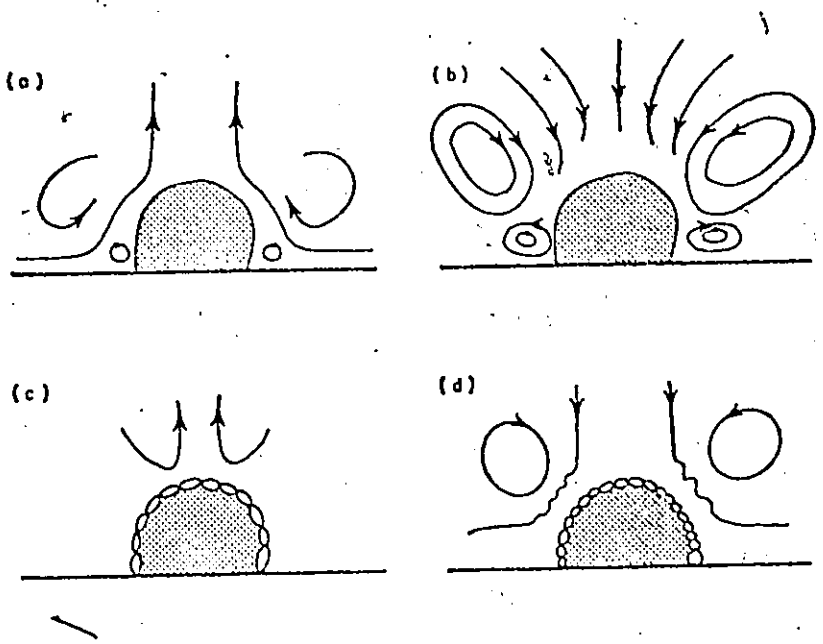


Figure 3.4

Four régimes of streaming. The bubble (which is shown stippled) is excited by the vibration of the surface to which it is attached. (a) A surface-contaminated bubble in a liquid of low viscosity, oscillating at low amplitude; (b) a pattern observed over a wide range of amplitudes and viscosities; (c) a pattern which occurs at low amplitudes in low viscosities, and at high amplitudes in high viscosities; its appearance coincides with the onset of the first surface mode and the dissolution of régime (b) at higher amplitude; (d) a pattern which occurs only at low viscosities, and which seems to represent a return to régime (b) as the amplitude becomes too large to permit the existence of a single stable surface mode. (From Elder, 1959).

For a solid rod or wire (cylinder) vibrating transversely in an ultrasound field, streaming occurs in symmetrical counter-rotating circulations as shown in Figure 3.5 Relatively high velocity gradients occur near the boundry. The viscous stress S associated with a velocity gradient G at the boundry of a cylinder of radius r_o, vibrating with angular frequency ω and displacement amplitude e_o is given by (Nyborg 1978).

$$S = \eta G = \eta \omega e_o^2 / r_o d \qquad 3.19$$

where d is the thickness of the boundry layer as defined by equation 3.18.

Early effects attributed to acoustic streaming were reported by Nyborg and Dyer (1960) who demonstrated migration of protoplasm towards a vibrating needle at 25 kHz in intact cells of Elodea. Selman and Jurand (1964) described the disorganization and subsequent recovery of the arrangement of the endoplasmic reticulum following irradiation for five minutes with 1 MHz ultrasound at intensities between 8 and 15 W/cm^2. More recently these stresses were suggested to have been responsible for such changes as

1) altered cell surface charge (Repacholi et al 1971)
2) altered cell membrane permeability (Chapman 1974)
3) reduction in cell half-life after reintroduction of cells into circulation (Rooney 1976)

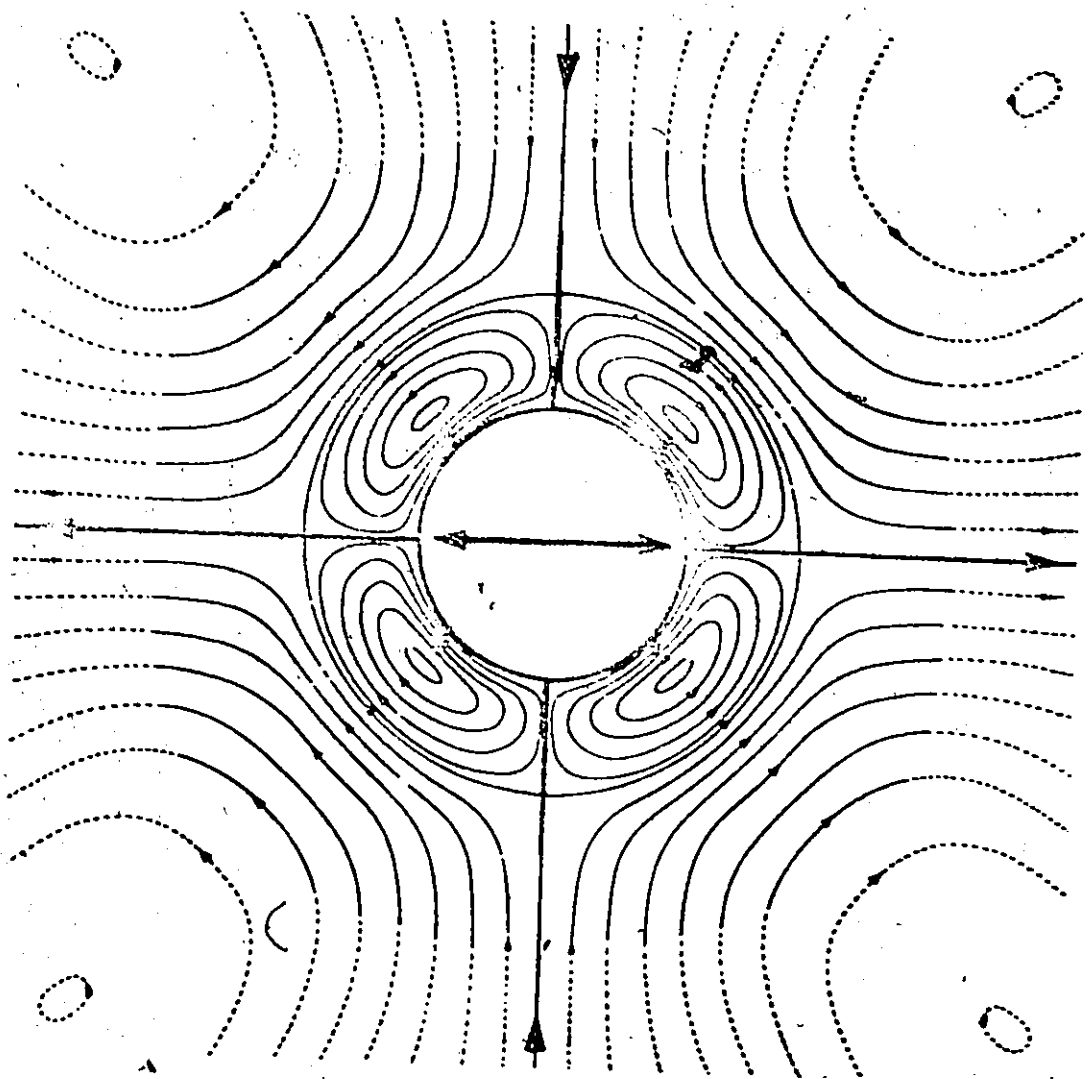


Figure 3.5

Acoustic streaming near a vibrating cylinder. Double headed arrow at center shows direction of cylinder oscillation. Other arrows show direction of steady fluid flow (Nyborg 1979).

4) separation from cells of small fragments (Apfel 1976)

5) rupture and fragmentation of cell membranes (Brown et al 1975, Williams 1972).

3.2.3.5 Surface Modes of Resonance

In 1960 Ackerman proposed that under certain conditions of ultrasound exposure, requiring transient cavitation, modes of resonance may occur at sites on the surface of biological cells. Presumably such sites have specific conditions which allow resonant absorption of energy from the ultrasound field to occur. Storm (1974) investigated the behavior of gas bubbles in gels exposed to ultrasound, and found oscillations occurring at the surface, superimposed on volume oscillations.

Watmough et al (1979) have reported circumstantial evidence for surface modes of resonance acting on the surface of HeLa cell nuclei. They re-examined electron micrographs of their previous work (Watmough et al 1977) on the effect of therapeutic ultrasound (750 kHz) on suspensions of HeLa cells. The sonicated cells appeared to have separated inner and outer membranes, notably on the nuclear membrane, at approximately regular intervals. Periodic changes in the chromatin were also observed close to the inner nuclear membrane. The ultrastructural studies also

revealed that some cells had burst nuclear membranes but intact cytoplasmic membranes, indicating direct ultrasound action with the nuclear membrane.

Although Watmough et al. (1979) have only produced circumstantial evidence for surface modes of resonance, it does appear to be a potential mechanism of ultrasound worthy of further investigation.

3.3 Ultrasound: Biological Effects

3.3.1 General

In reviewing the rather extensive literature on the biological actions of ultrasound, reported effects logically fall into distinct areas of biological organization. These could be arranged in order of increasing complexity, from macromolecules to complete multicellular organisms. For purposes of this thesis, this review will be restricted to ultrasound effects on macromolecules and cells only, will be made.

Only a few biological structures have been subjected to systematic examination for effects from ultrasound. Evidence presented in many cases should be considered as inconclusive since confirmation from independent laboratories has not been made. Estimates of ultrasound field intensities in living systems still suffer from a lack of accepted methods of measurements, and the inadequacy of many reports in stating the exact experimental conditions.

3.3.2 Macromolecules

The effects of ultrasound on biological molecules are either reversible or irreversible, depending generally on the beam intensity. High intensity levels can cause irreversible, disruptive events such as degradation or depolymerization of long chain biopolymers. This is usually recognized by a reduction in the molecular weight (Shea and Bradury 1973). Colson and Fredericq (1970) were able to show that at high intensities the frequency of the ultrasound is not the factor determining the efficiency of degradation of biomolecules.

Degradation may be related to the distinctiveness of the protein structure and its initial molecular weight (Zorina and El'piner 1972).

Peacocke and Pritchard (1968) claim that, in general, the higher the molecular weight of the biological molecule, the more susceptible it is to degradation in an ultrasonic field. Proteins and nucleic acids are not usually degraded to monomer units, but to lower molecular weight molecules dependent on the experimental conditions.

At lower intensity levels, the effects of ultrasound tend to be reversible. Events may be characterized by a dependence on the frequency of the ultrasound wave. Reversible effects usually involve some perturbation of a physico-chemical reaction.

One could classify the effects of ultrasound on biomolecules by mechanism of action: those due to the action of cavitation, resulting from the production of chemically active free radicals in the sonicated solution: and the mechanical vibration of large fragile molecules, giving rise to degradation. A good review of earlier work carried out in this area can be found in El'piner (1964).

The effect of ultrasound on some of the more important constituents of cells, proteins, nucleic acids and carbohydrates have been studied, and will be discussed here.

3.3.2.1 Proteins

Carstensen et al (1953) studied the effect of ultrasound in human blood, and found that the absorption of the acoustic beam was in direct proportion to the content of protein in the blood cells and plasma. Further, the acoustic properties of the blood were determined largely by the protein content. Later, Carstensen and Schwan (1959a) showed that, although most of ultrasound absorption occurs at the molecular level, there was a small contribution to the total absorption from the presence of intact cells. A relaxation process was suggested (Carstensen and Schwan (1959b) as being responsible for the acoustic absorption. Edmonds et al (1970) produced evidence to support this relaxation mechanism in the frequency range 33 kHz - 470 kHz.

Studies (Zana and Tondre 1972, White and Slutsky 1972, O'Brien and Dunn 1972) of ultrasonic absorption in various protein solutions at different pH levels have led to the conclusion that ultrasound absorption is sensitive to the overall conformation of the polypeptide molecule but probably not to the equilibrium between the helix and coil forms of proteins. There are still uncertainties as to the exact role of the protein conformation (White and Slutsky 1972, O'Brien and Dunn 1972) since evidence is still scant and subject to a number of interpretations.

When ultrasonic absorption measurements were performed (Zana and Tondre 1972) on solutions of poly-L-lysine, poly-DL-lysine and poly-L-ornithine at pH values in the range 5-13, and in the frequency range 1 - 115 MHz, absorption curves undergo a maximum at pH 11.4 - 11.5. The absorption maxima appear to be due to proton-transfer reactions on the side-chain amino group of the polypeptide. The effect of frequency on the high absorption of ultrasound in the polypeptide solutions enable determination of the rate constants and volume changes for the proton transfer reactions. The proton transfer or exchange may actually involve all the residues on protein side chains. An example of a proton transfer reaction on a carboxylic group is shown in the equation

$$-CO_2H \rightleftharpoons -CO_2^- + H^+$$

Later work (Yiv et al 1975) on ultrasonic absorption in adenosine 5'-di and triphosphate (5'ADP and 5'ATP) indicated that excess absorption in these nucleotide coenzymes is determined by proton exchange between different ionized forms of the nucleotides. These authors also claim that excess ultrasound absorption, via proton exchanges are likely to occur in cells, and involve nucleotides, nucleic acids and proteins at physiological pH levels

Sonication of various enzymes to different ultrasound exposure parameters have produced conflicting results. An analysis and comparison of exposure parameters indicate that, in general, where the irradiation conditions could produce cavitation, and hence highly reactive free radicals, enzyme inactivation was found.

3.3.2.2 Nucleic Acids

Much of the experimental results and conclusions about the absorption of ultrasound in protein solutions also applies to the nucleic acids.

Cavitating ultrasound fragments nucleic acids, and in the case of DNA, backbone cleavage occurs preferentially in the C-O and P-O bonds (Coakley and Dunn 1972). Results of ultrasound exposure of DNA in solutions indicated (Peacock and Pritchard 1968) that breaks in the strand structure occurred preferentially around the mid-point, lending support to the idea that, in ultrasound fields where cavitation was supposed to have been prevented, DNA degradation may result from viscous stresses established around the molecules in solution. These stresses probably result from the relative motion between two molecules of different density - the DNA and water molecules.

Further support for the ultrasonic induction of DNA degradation via the hydrodynamic shear mechanism comes from the work of Pritchard et al (1966). Their calculations indicate that DNA degradation by ultrasound under conditions of "stable cavitation" is mainly the result of shearing forces within the DNA solution.

Galperin-Lemaitre et al (1975) described the degradation of calf-thymus DNA by ultrasound at intensities as low as 200 mW/cm^2.

3.3.2.3 Carbohydrates

Ultrasound at sufficiently high exposure levels degrades starch into dextrins (short chain polysaccharides, generally 6 - 12 glucose units) (El'piner 1964). In general carbohydrates undergo depolymerization in an ultrasonic field at high intensities.

3.3.2.4 Summary

One can briefly summarize the observed ultrasound bioeffects at the macromolecular level as those due to

(i) degradation or depolymerization, most probably via the mechanism of cavitation, and

(ii) enzyme inactivation due to cavitation.

3.3.3 Cells

3.3.3.1 Cell Death

Ultrasound at sufficiently high intensity levels can completely destroy microorganisms, viruses, bacteria, animal and plant cells (Edmonds et al 1970, Young and Smithwick 1976, Peacocke and Pritchard 1968, Clarke and Hill 1970, Coakley et al 1971). Systematic investigations (Clark and Hill 1970, Coakley et al 1971, Hill 1972) of the physical and chemical aspects of cell disruption have shown that cavitation is the predominant mechanism.

Exposure of monolayers of Chinese hamster cells to continuous 990.5 kHz focused ultrasound at intensities above 400 W/cm^2 and long exposure times indicated that single cells could withstand up to 30 times greater intensities and up to 1000 times greater exposure times than any sonications producing lesions in mammalian tissues. Damage was attributed to the production of cavitation. Non-cavitational or non-thermal effects leading to cell death were not demonstrated in this case (Moore and Coakley 1977).

Studies on disruption and viability of HeLa and CHO cells exposed to 1 MHz ultrasound at intensities up to 30 W/cm^2 for 1 - 15 minutes reported (Kaufman et al 1977) indicated that the threshold for lysis was approximately 1 W/cm^2. For those cells surviving the treatment, there was a decrease in viability, possible decrease in proliferation rate and an increased incidence in giant cell formation. Lysis generally occurred within the first 5 minutes. An interesting feature of this work was the finding that an increased cell survival occurred after exposure to higher intensities, confirming the earlier work of Clarke and Hill (1970).

3.3.3.2 Cell Cycle

Only a few reports have been published on the variation of response of cultured mammalian cells to ultrasound, during different phases of the cell cycle. Clarke and Hill (1969) exposed mouse leukaemia cells to a 1 MHz beam having a peak intensity of 15 W/cm^2 and an average intensity (over the irradiated container) of 5 W/cm^2. Sublethal irradiation for 5 hours (pulsed 1 ms on, 10 ms off) had no significant effect on the progress of cells through the cell cycle. Cells exposed to 50% lethal ultrasonic irradiation for 10 seconds (continuous) were found to be more sensitive to disruption during mitosis. No clear evidence

of delayed death following lethal irradiation of cells has been reported (Hill 1974).

Nakamura et al (1977) have confirmed the increased sensitivity to cell death during mitosis. These authors also noted that the decoiling of chromosomes during mitosis increased significantly following ultrasound exposure.

3.3.3.3 The Cell Surface

Ultrasound has been shown (Repacholi 1970, Repacholi et al 1971, Taylor and Newman 1972, Joshi et al 1973, Chapman 1974) to act at the cell surface. Changes in the cell surface charge of mammalian cells were found to occur following exposure to ultrasound (Repacholi 1970, Repacholi et al 1971). This was later confirmed by Taylor and Newman 1972 and Joshi et al 1973. This effect was detected by measuring the electrophoretic mobility of Ehrlich ascities tumour cells. The reduction in mobility was found to be independent of the pulse length between 20 μs and 10 ms, and no further change in the electrophoretic mobility was found after 5 minutes total irradiation time. Repacholi (1970) suggested that the effect may be due to ultrasonic micro-streaming producing some alteration to the carbohydrate-rich layer of the cell surface. Evidence was presented that cavitation may not be the effective mechanism (Taylor and Newman 1972).

Joshi et al (1973) in subsequent work on cultured mouse lymphoma cells were also able to find a reduction of cell surface charge from electrophoretic mobility measurements, following exposure to ultrasound at 2 MHz and 10 W/cm^2 peak intensity (pulsed 1:0) for up to 5 minutes. These authors suggested that the changes in cell surface charge occurred as a result of cavitation.

Exposure of rat thymocytes kept at 39°C in vitro, to 1.8 MHz ultrasound at intensities above 1.0 W/cm^2, for 30 minutes produced an immediate decrease in the potassium content of the cells (Chapman 1974). Alteration of membrane permeability and presumably ultrasound induced changes to the cell membrane structure appear to have occurred. The author suggested that the effect was probably not attributable to heating, but may result from "microflow turbulence" (micro-streaming) at the membrane-liquid interface, influencing the passive diffusion rate of ions through the membrane, by causing transient changes in the ion concentration gradient either side of the membrane.

3.3.3.4 Chromosome Aberrations

In 1970 MacIntosh and Davey (1970) reported having found chromosome aberrations in human leucocyte cultures exposed to very low intensity levels of 2.25 MHz ultrasound. In a later paper (MacIntosh and Davey 1972), these authors claimed the threshold intensity of ultrasound was 8.2 mW/cm^2, below which no chromosome damage occurred. Their reports prompted a number of laboratories around the world to attempt confirmation of this effect, since diagnostic ultrasound was in widespread use and the implications of human health were potentially serious. Virtually all subsequent reports (Buckton and Baker 1972, Hill et al 1972, Oakley et al 1972, Watts et al 1972, Brock et al 1972, Braeman et al 1974) reached the same conclusion - in vitro exposure to cells to continuous or pulsed ultrasound at different frequencies, and even at higher intensities under cavitating conditions, does not increase the number of chromosome aberrations.

MacIntosh et al (1975) tried to reproduce, as closely as possible, their earlier work. They were unsuccessful, and suggested that the original aberrations were produced by an unidentified artifact, unrelated to the ultrasonic treatment.

A review of the literature on the possibility of ultrasound induced genetic hazards (Thacker 1973) concluded that, positive genetic effects seem possible only at very high ultrasound intensities where it is difficult or impossible to exclude heat shock. At ultrasound intensities presently used in medical applications, there is little likelihood that genetic effects will be found.

More recently, Liebeskind et al (1979a) have reported an increase in sister chromatid exchanges from exposure to human lymphocytes to 2 MHz pulsed ultrasound at a spatial average, temporal average intensity of 2.7 mW/cm^2 for 30 minutes. This result is in contrast with that of Morris et al (1978) who were unable to detect any increase in sister chromatid exchanges in human leucocytes exposed for 10 min to 1.05 MHz continuous wave ultrasound at intensities between 15.3 - 36 W/cm^2.

Although there do not appear to have been confirmed reports of chromosome aberrations in mammalian cells exposed to ultrasound, a number of studies on the broad bean Vicia faba suggest that ultrasound may induce non-breakage types of chromosome aberrations such as bridged, agglomerated and laggard mitotic figures (Cataldo et al 1973, Gregory et al 1974, Kokhar and Oliver 1975). Miller (1978) however failed to find any increase over control levels of chromosomal fragments (micronuclei) in Vicia faba root meristem cells exposed to ultrasound (1.1 MHz, 8 W/cm^2 peak, 1 min continuous exposure).

Liebeskind et al (1979b) have reported that exposure of synchronized HeLa S-3 cells to diagnostic levels of pulsed ultrasound (3 μs pulses at 200 Hz, 2.5 MHz frequency, an estimated spatial average, temporal average intensity of 16.9 mW/cm^2 and spatial peak, temporal peak intensity 35.4 W/cm^2, for times up to 40 minutes) produced unwinding of the DNA helix structure. Single strand breaks in the DNA were claimed to have occurred following the observation of unscheduled DNA synthesis using autoradiography. These authors also reported that breaks in the DNA were not observed on alkaline sucrose gradients. It is perhaps worth mentioning that single strand breaks to the DNA would not be detected using the autoradiographic technique. Unscheduled DNA synthesis is usually visualized using autoradiography following excision repair replication of DNA lesions.

3.3.3.5 · Cell Function

In recent years there have been a number of reports on functional changes in cells exposed to ultrasound. Of particular interest has been the action of ultrasound on DNA and protein synthesis. Prasad et al (1976) and Fung et al (1978) have reported changes in the DNA synthesis of cultured mammalian cells exposed to very low ultrasound intensities. Prasad et al (1976) reported that unexposed HeLa cells showed up to 14% more DNA synthesis than cells exposed to 1 MHz pulse-echo device for 10 minutes at an intensity of 4 mW/cm^2. Fung et al (1978), on the other hand, reported a dose-related biphasic effect of ultrasound. Exposure of lymphocytes to 2.1 MHz ultrasound at an intensity of 5 mW/cm^2 for 3 and 6 minutes stimulated DNA synthesis while longer exposure times (30 minutes) suppressed DNA synthesis. Both these results are difficult to interpret because the ultrasound induced effects on DNA synthesis are small compared to the errors encountered in the (^3H)-thymidine incorporation technique.

Enhancement of DNA synthesis in neonatal mouse tibial ephiphyses (chondrocytes) was observed after three 5-minute exposures, 24 hours apart, to 1 MHz ultrasound with an intensity of 1.8 W/cm^2 (Elmer and Fleischer 1974). It was not determined whether the stimulation in DNA synthesis was related to either DNA replication or repair. However, no statistically significant increase in radioactive proline incorporation into protein in the cartilaginous epiphyses was found. These authors suggested that ultrasound degraded the bone matrix, possibly by affecting the lysosomes, causing an early release of their hydrolytic enzymes. This explanation is possible since enzymes do play a role in the erosion of cartilage, and ultrasound has been observed to produce lysosomal swelling (Curtis 1972).

Belewa-Staikowa and Kraschkowa (1967), using an ultrasound therapy transducer for 5 minutes single exposures, found an increase in protein synthesis in hepatic, renal and myocardial tissue at acoustic intensities of 0.2 and 0.6 W/cm^2. However, they observed a depression of synthesis using an ultrasonic intensity of 1.0 W/cm^2.

Changes in protein content in the liver, small intestine and lung tissue, as well as slight changes in the blood serum proteins have been observed after partial body exposure of 75 adult guinea pigs to single doses of 800 kHz ultrasound for 10 minutes at intensities of 0.5 - 4 W/cm^2 (Bernat et al 1966). Changes in protein metabolism was found to be dose dependent and varied with the region of the animal treated with the ultrasound. Low ultrasonic intensities caused no change in protein composition while intensities of 2.5 - 4 W/cm^2 produced significant changes, especially in the liver and small intenstine.

Harvey et al (1975) observed in vitro stimulation of protein synthesis in human fibroblasts exposed to 1, 3 and 5 MHz ultrasound at intensities of 0.5 to 2.0 W/cm^2 for 5 minutes. These authors found that pulsed ultrasound (2 ms on, 8 ms off) produced greater stimulation then the continuous wave mode.

De la Maza et al (1976) studied the susceptibility of chromatin from normal and transformed cells to breakage by ultrasound, in an in vitro system in which DNA repair was reduced to a minimum. In both normal and transformed cells, the euchromatin fraction was found to be more susceptible to breakage. It was suggested that ultrasound (20 kHz - Branson sonifier Model S125, 8-11mA), having a low penetrating energy, would make the condensed heterochromatin less susceptible to breakage than the diffuse euchromatin.

3.3.3.6 Intracellular Compenents

The electron microsope (EM) has been used to great advantage to determine the effects of ultrasound on intracellular structures and organelles. The EM allows experimenters to use ultrasound intensities normally used in medical practice and possibly observe if subtle effects occur at the subcellular level.

Ultrastructural changes found in sonicated cells in vitro, by comparison to controls were: more free ribosomes, dilation of the rough endoplasmic reticulum, more cytoplasmic vacuolation, closer approximation of the membranes of adjacent cells, greater numbers of autophagic vacuoles, and damage to lysosomal and mitochondrial membranes (Harvey et al 1975). These effects were found in human fibroblasts exposed to ultrasound at 0.5 W/cm^2, 3 MHz pulsed 2/10 ms. HeLa cells exposed to therapeutic ultrasound of frequency 750 kHz, to power levels between 0.1 - 3 watts, for up to 5 minutes, showed damage in the plasma, nuclear and mitochondrial membranes as well as the endoplasmic reticulum (Watmough et al 1977).

Chater and Williams (1977) found *in vitro* evidence to suggest that therapeutic intensities of ultrasound may have the potential to trigger platelets to initiate or accelerate thrombogenesis. Exposing the cells to ultrasound frequencies of 0.75, 1.5 and 3 MHz and intensities between 0 and 5 W/cm^2 for up to 7 minutes, these authors found that the most effective exposure parameters were a frequency of 0.75 MHz and intensities above 2.5 W/cm^2. From 3 to 3.75 W/cm^2 there was a progressive increase in the amount of aggregation in the platelets. Damaged platelets were found in all irradiated samples, appearing swollen with lighter cytoplasm and a redistribution of organelles within the cells.

The subcellular organelle primarily affected by ultrasound appears to be the mitochondrion. Mitochondrial membrane disruption and swelling in cell systems exposed to ultrasound has been reported on a number of occassions (Hrazdira 1970, Hrazdira and Havelkova 1966, Stephens *et al* 1978). Typical intensities used in these studies were in the range of 1 - 1.5 W/cm^2 at frequencies of 0.8 - 2 MHz.

Tables 3.1 and 3.2 give a summary of ultrastructural effects produced by ultrasound exposure of biological systems in vitro and in vivo respectively.

Table 3.1. Ultrastructural Effects on Intracellular Components, Exposed to Ultrasound In Vitro .

Biological System	Exposure Parameters	Observation	Reference
Chicken muscle myofibrils	800 kHz, 2 W/cm^2, 2 - 5 min	Rupture of myofibrils	Samosudova and El'piner (1966)
Human fibroblasts	3 MHz, 0.5 W/cm^2, pulsed	Damage to lysosomes, mitochondria, rough ER, more autophagic and cytoplasmic vacuoles, more free ribosomes	Harvey et al (1975)
HeLa Cells	750 kHz, 0.1 - 3 W, up to 5 min	Damaged plasma and unclear membranes, increased cell debris	Watmough et al (1977)
Human blood	0.75 - 3 MHz, 0 - 5 W/cm^2, 5 min	From 3 W/cm^2 to 3.75 W/cm^2, increase platelet aggregation, the drug ASA increased the threshold for aggregation from 3 W/cm^2 to above 4 W/cm^2	Chater and Williams (1977)
		Mitochondrial swelling	Hrazdira (1970)

Table 3.2. Ultrastructural Effects of Ultrasound on Intracellular Components Exposed In Vivo

Biological System	Exposure Parameters	Observation	Reference
Human brain with tumours	1 mW/cm^2, 2 MHz, 1 h	nil	Garg and Taylor (1967)
Fetal cardiac and brain tissue	16 mW/cm^2, 1 MHz, 8 h	nil	Abramowski et al (1972)
Rat brain	5 mW/cm^2, 2 MHz, pulsed with ppr. 250 Hz, 2 h	nil	Oeckler et al (1975)
Rat testes	1 W/cm^2, 1.1 MHz, 10 min	Membrane changes, swollen mitochondria, large amounts of cell debris.	Dumontier et al (1977)
Rabbit larynx	Therapy freq., 3.5 - 5 W/cm^2, 3 min	Necrosis, intracytoplasmic vacuolation, destroyed mitochondria, pycnosis of nucleus,	Karduck and Wehmer (1974)
Rabbit arterial tissue	1 MHz, 6 - 12 min of 25 W/cm^2 to less than 1 s of 1500 W/cm^2	Vacuolation, necrosis, desquamation and mural thrombosis	Fallon et al (1973)
Mouse liver	1 MHz, 0.5 - 2 W/cm^2, 1 - 3 min	No effect of low doses. Necrosis, hemorrhage, loss of fine structure of cell organelles and loss of glycogen at high doses.	Valtonen (1967)
Rat liver	-	Fat degeneration, passive hyperaemia, loss of glycogen.	Jankowiak et al (1958)
Mouse liver	- , 12 W/cm^2, 15 s	Necrosis	Bell (1957, 1958)
Rat spinal cord	- , 25 - 50 W/cm^2 peak intensity, pulsed 1:10 for 1 - 15 min.	Endothelial damage, gross hemorrhage, disruption of lysosomes, cell destruction.	Taylor and Pond (1972a, 1972b)

Table 3.2. Cont'd

Biological System	Exposure Parameters	Observation	Reference
Rat bone marrow cells	-	Disruption of lysosomes, cell death.	Dvorak and Hrazdira (1966)
Chick embryo	1, 3, 5 Mkz, 0.1 - 12 W/cm^2 peak, 15 min	Damage to luminal aspect of plasma membrane, cell debris.	Dyson et al (1974)
Rabbit liver	1 MHz, 3 W/cm^2, 10 x 5 min	Increase in lysosomes	Jankowiak and Majewski (1966)
Rat liver	1 MHz, 3 W/cm^2, multiple 5 min	Increase in lysosomes	Majewski et al (1966)
Dog kidney	880 kHz, 1 W/cm^2, CW, 20 min	Swollen basal labyrinth, sicrovilli and mitochondria	Pinchuk et al (1971)
Mouse liver, pancreas, kidney and adrenal	2 Mkz; 1 W/cm^2, spat. average, 500 s C.W. or 4 W/cm^2 spat. peak, 25 s C.W., focused, or 4 W/cm^2 spat. peak, 125 s focused pulsed 1 in 5 with 1 ms bursts.	Changes to the mitochondria	Stephens et al (1978)
Germinating spores of Rhizopus nigricans	800 kHz, 1 W/cm^2, 10 min	Membrane changes, changes to mitochondria	Hrazdira and Havelkova (1966)

3.3.4 Tissue and Organs

When ultrasound is absorbed by any biological material, that material increases in temperature, depending on the exposure conditions. Biological material can absorb ultrasonic energy at very high rates. For example the absorption of 1 MHz ultrasound in liver is approximately 600 times greater than that in water; in striated muscle absorption is approximately 1000 times greater than water (IRPA 1977). The distribution of heating within tissues or organs depends generally on their size and shape, as well as the absorption coefficient of the material. The heat distribution also depends on the ultrasonic beam intensity distribution and frequency.

Ultrasound is absorbed in tissue primarily at the macromolecular level. Such absorption can, in turn, be modified by macromolecular interactions. However, the specific physical or chemical relaxation mechanisms responsible for the absorption of ultrasound are as yet unknown. The macroscopic inhomogeneities in tissue or organs affect ultrasound propagation and can lead to artifacts in certain methods of measurement of tissue absorption (Carstensen 1976). Localized heating by ultrasound at impedance discontinuities or tissue interfaces have been observed in many instances. The mechanism of mode conversion to shear waves at these discontinuities and

subsequent absorption of these waves in a very small distance was thought to provide an explanation for this localized heating. However, it has been found (Fizzell and Carstensen 1976) that negligible energy was converted to the shear wave mode at a discontinuity or interface.

When ultrasound passes through tissues it undergoes a complex series of interactions with the medium. These processes attenuate, scatter and refract the ultrasound beam from its initial path, generally at interfaces which are not distributed uniformly throughout the tissue. Further, the scattering interface may not intercept or alter the whole cross-section of the beam to the same extent. As a result, the scattered ultrasound beam within the tissue bears little resemblance to the initial beam at the entrance to the biological medium.

An in-depth review of the literature on biological effects of ultrasound has recently been drafted in a criteria document by Repacholi (1980). Other recent reviews have been completed by Dunn (1979, 1980).

Chapter 4 Methods and Materials

4.1 Lymphocyte Culture Technique

Peripheral venous blood from healthy human donors was withdrawn into 60 ml syringes containing 2-3 ml of sodium heparin (100 U/ml). The contents of each syringe was mixed with 40 ml of RPMI - 1640 culture medium (Flow Labs). This diluted blood mixture was then gently layered onto 15 ml of Ficoll-Paque (Pharmacia) in 50 ml plastic (Falcon) tubes. These tubes were then spun at 400 g for 30 min at 18°C. Following centrifugation each tube contained a yellowish upper layer of mostly plasma and platelets, a distinct band of lymphocytes, a clear area of Ficoll, and finally a pellet containing predominantly agglutinated red cells and granulocytes. The plasma layer was quickly removed to within 3-4 ml above the lymphocyte band. Lymphocytes were removed and transferred to clean, sterile 50 ml tubes.

Approximately 30 ml of RPMI 1640 culture medium was added to each 20 ml of lymphocyte rich solution, mixed well and centrifuged at 400 g for 20 min at 18°C. The supernatant was removed and the lymphocyte pellet resuspended with fresh culture medium into 15 ml sterile, plastic (Falcon) tubes. A second wash was carried out at room temperature, the lymphocytes resuspended in 5-10 ml of 0.83% NH_4Cl and allowed to stand for 5 min to lyse red blood cells. A few (2-3) ml

of fetal calf serum were layered on the bottom of the tube and the suspension pelleted again. The cells were resuspended in RPMI - 1640 culture medium containing 15% fetal calf serum and 1% penicillin (100 IU/ml) - streptomycin (100 µg/ml) (Difco Labs). Cells were cultured in plastic 75 cm^2 culture flasks (Corning) at a cell concentration of 1 x 10^6 lymphocytes/ml in 5% CO_2 in an incubator at 37°C. Sterile conditions were maintained throughout the separation and culturing procedure.

4.1.1 Cell Concentration and Viability Determinations

Cell concentrations were determined using a standard hemocytometer. Prior to counting, cells were diluted with 0.1% gentian violet in 0.2% acetic acid which stains the nucleus and lyses any remaining red cells. At least 200 cells were counted to determine cell concentrations for each sample. The leucocyte distribution was generally found to be 95-97% small lymphocytes, 2-4% neutrophils and about 1% monocytes, eosinophils or basophils. This distribution was found to be in agreement with that reported by Farnes and Barker (1977). For most experiments, the final cell concentration of the culture was 1 - 5 x 10^6 cells/ml.

Cell viabilities were routinely determined by diluting cell cultures with 0.5% trypan blue in 0.85% saline (Flow Labs). This dye is excluded by live cells.

Dead cells are observed to take up the dye and appear blue. Kaufman et al (1977) found that tests using trypan blue exclusion and colony-forming ability gave the same results for determining survival of mammalian cells exposed to ultrasound.

Since live cells passively exclude trypan blue, a few tests on viability were conducted using fluoroscene diacetate. Here 0.1 ml of fluoroscene diacetate (0.25 g in 10 ml acetone) was mixed with 10 ml of cells (in PBS). Live cells actively take up the dye and show as a bright fluoroscene green, while dead cells are colourless. In all tests conduced, viabilities using trypan blue and fluoroscene diacetate were the same.

4.1.2 Lymphocyte Activation

In experiments requiring activated lymphocytes, the cells were cultured in RPMI - 1640 culture medium supplemented with 15% fetal calf serum and 1% (100 IU/ml) penicillin - (100 µg/ml) streptomycin, at a concentration of 10^6 cells/ml, and incubated with 25 µg/ml concanavalin A (Con A) (Calbiochem) in a 5% CO_2 incubator at 37°C.

4.1.3 Preparation of Isotopes

Stock solutions (Amersham) of (^{3}H) thymidine, (^{3}H) uridine and (^{3}H) leucine were at a specific activity of approximately 50 Ci/mMol. For most experiments the stock solutions were diluted with RPMI 1640 culture medium or PBS to give final concentrations of 10 or 100 µCi/ml.

Stock solutions of (^{3}H) concanavalin A (New England Nuclear) had a specific activity of around 66 Ci/mMol and were diluted with 0.85% NaCl to 10 µCi/ml. 0.1 ml of this solution would then be added per 0.9 ml of culture to give a final activity of 1 µCi/ml.

4.1.4 Incorporation of Radioactive Precursors

To minitor DNA, RNA or protein synthesis in resting or stimulated lymphocytes, the cells were incubated with the appropriate radioactive precursor ((^{3}H) thymidine for DNA, (^{3}H) uridine for RNA and (^{3}H) leucine for protein) in 5% CO_2 at 37°C. The lowest concentrations needed to obtain good statistical results were used since high concentrations of radioisotope were previously suspected and later reported by Pollack et al (1979) to perturb the cell cycle progression of stimulated lymphocytes.

In most experiments, cells were incubated with 2 µCi/ml of (^{3}H) thymidine for 3 h, unless otherwise indicated. Incorporation of either (^{3}H) uridine or (^{3}H) leucine was carried out at a concentration of 20 µCi/ml for 3 h.

4.1.5 Determining Radioisotope Activity in Cells

4.1.5.1 Intracellular

Manifolds (Millipore) containing twelve wells each were used to layer cells onto glass microfibre filters (Whatman type GF/C), having a diameter of 2.4 cm. Prior to sampling, the manifold was cleaned thoroughly and each filter was moistened with phosphate buffered saline (PBS). A 1 ml sample of cells was washed onto the filters using about 30 ml of PBS. The supernatant and PBS washes were then vacuumed and washed through the filters leaving the labelled cells. Finally all cells were lysed with 5% trichoroacetic acid while layered on the filters so that the intracellular isotope which was not incorporated into the DNA, RNA or protein, was washed through the filter.

Filters were partially dried by vacuum, removed from the manifold and left to air dry. Dried filters were placed into glass vials, filled with 8 - 10 ml of scintillation solution (Scintilene, Fischer) and counted on the Beckman model LS233 liquid scintillation counter.

4.1.5.2 Bound (^{3}H) Concanavalin A

Manifolds (Millipore), containing twelve wells each, were used to layer samples of lymphocytes onto glass micro-fibre filters. Prior to sampling, the manifold was cleaned thoroughly and the filters were moistened with 5 ml of 0.5% bovine serum albumen (BSA) and vacuumed through the filter. This BSA treatment of the filters was repeated 3 times to reduce non-specific sticking of the isotope to the filters. When 1 ml sample of cells were placed in the wells on the filters, the empty tubes that contained the samples were filled three times with 10 ml of ice cold calcium and magnesium free phosphate buffered saline (CMF-PBS) and emptied into the wells. The supernatant and washes were then vacuumed and washed through the filters with 20-30 ml ice cold CMF-PBS, leaving the labelled cells to be counted.

The filters were dried, placed into vials, and 8-10 ml of "Scintilene" scintillation solution added for counting in a Beckman LS233 liquid scintillation counter.

To determine the percentage background counts from sticking or binding of the (^{3}H) concanavalin A (^{3}H Con A) to everything else except the cell surface, increasing numbers of the binding sites on the lymphocytes for Con A were bound using higher concentrations of methyl α-D-mannopyranoside (αmm) (Novogrodsky 1972).

When just culture medium and the ^{3}H Con A (no cells) were passed through the filters, the same percentage background was recorded. This meant that the background counts were due mainly to sticking of the ^{3}H Con A to the filters. Filters treated with medium and ^{3}H Con A were routinely used as blanks for all the binding experiments.

Using high temperatures to kill cells, it was determined that the ^{3}H Con A would bind equally well to both dead and live cells. Thus viability determinations were made for all experiments and corrections made where appropriate.

4.1.6 Procedure for Degassing Cell Suspension Medium

In experiments where ultrasonic cavitation was to be eliminated, the cells were suspended in degassed medium to ensure that no air bubbles (cavitation nuclei) were formed external to the cells.

PBS was degassed by autoclaving at 120°C for 20 min. Immediately following autoclaving the PBS was sealed from the air and cooled at 37°C for immediate use. The RPMI 1640 medium was degassed by leaving overnight in a Fischer vacuum oven (set at maximum vacuum). The degassing procedure did not significantly alter the pH of the medium (pH 7.40 to 7.45).

Both degassed PBS and 1640 medium were used for sonicating cells under conditions where cavitation should be significantly reduced externally to the cell. To ensure that the suspending medium did not contain gas bubbles, the cells were washed twice in degassed medium and resuspended in fresh degassed medium, prior to placing into the teflon disc. The disc was designed in such a way that it could contain the cells to be sonicated between plastic sheets, while not allowing air bubbles to enter.

To determine the degree of degassing that had occurred in the suspending media, measurements of gaseous oxygen content was made using a YSI Model 54ARC oxygen meter. This meter was temperature compensated and utilized and biochemical oxygen demand (BOD) probe. The instrument had been calibrated by the manufacturer and gave a read-out in parts per million (ppm) on a scale of 0 - 14.

Visual observation for gas bubbles near cells suspended in the degassed medium while in the teflon disc were made using a standard laboratory microscope.

4.2 Ultrasound Apparatus and Exposure Procedure

4.2.1 Ultrasound Generating Equipment

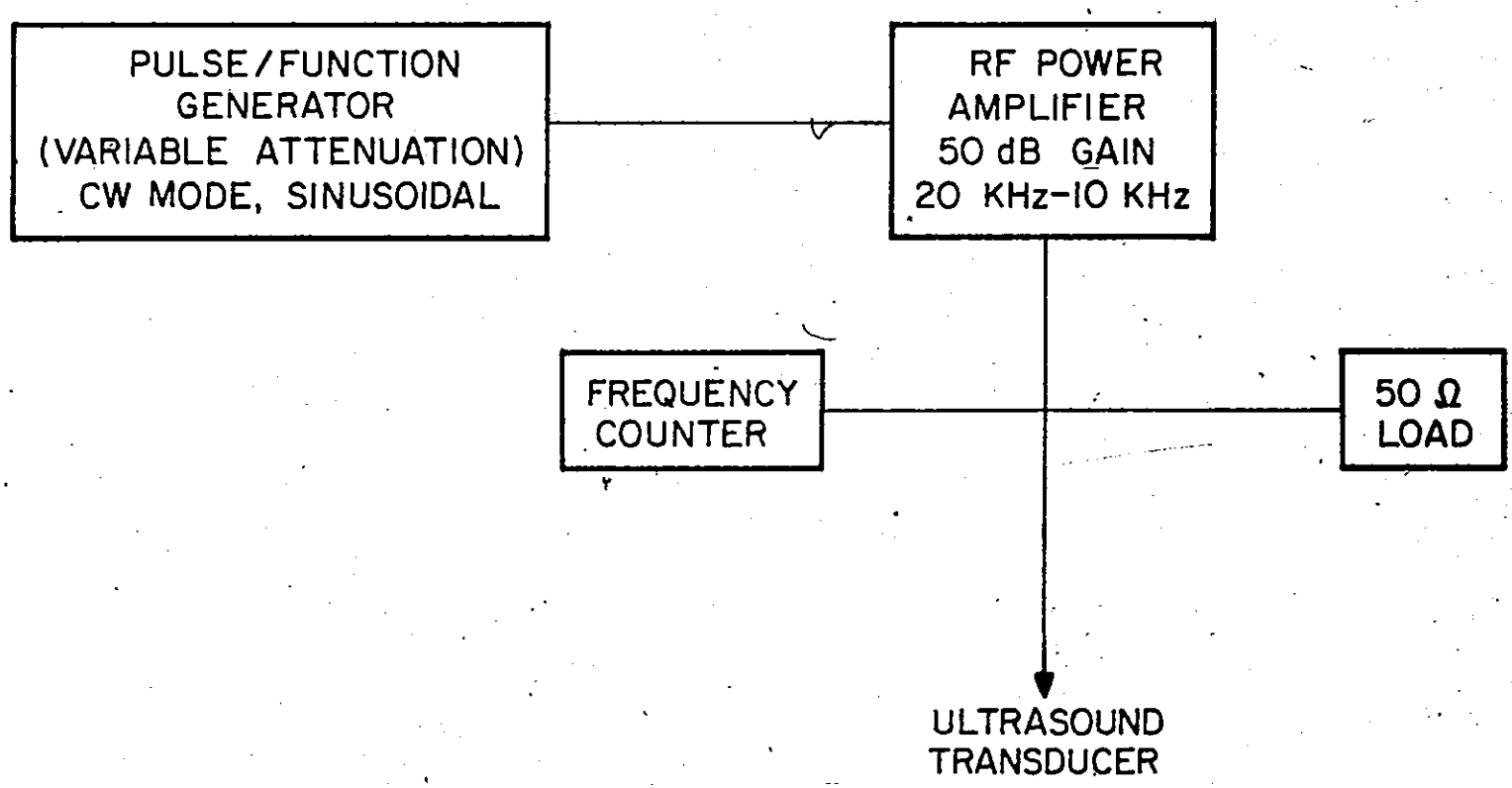
Ultrasound was generated with a Wavetec 20 MHz variable pulse/function generator model 145 providing sinusoidal, continuous waves into an RF power amplifier (EIN Inc.) model 204L. The power amplifier had a 50 dB gain and was loaded at the output with a 50 Ω load (Heath model HN-31). This resistive load allowed the power amplifier to operate near peak efficiency and drove a Siemens therapy transducer. The wave frequency was continuously monitored by a frequency counter (Hewlett Packard model 5382A).

A schematic diagram of the ultrasound generating equipment is shown in Figure 4.1. A photo of the apparatus connected for operation, with the water bath outside its incubator is shown in Figure 4.2.

4.2.2 Equipment Calibration

The ultrasonic power output of the transducer was calibrated with the kind assistance of the Bureau of Radiological Health, U.S. Department of Health, Education and Welfare, Rockville, Maryland. All calibrations under free-field conditions in a bath of degassed water were conducted at the resonant frequency of the transducer (870 kHz). The diameter of the radiating surface of the transducer was found to be 1.9 cm.

Figure 4.1



Block Diagram of Ultrasound Generating Equipment.

Figure 4.2

The exposure jig containing the disc of lymphocytes is placed in a water bath (at 37°C), in contact with the face of the therapeutic ultrasound transducer. Pimpled rubber and fibre-glass wool is used to absorb the transmitted ultrasound beam.

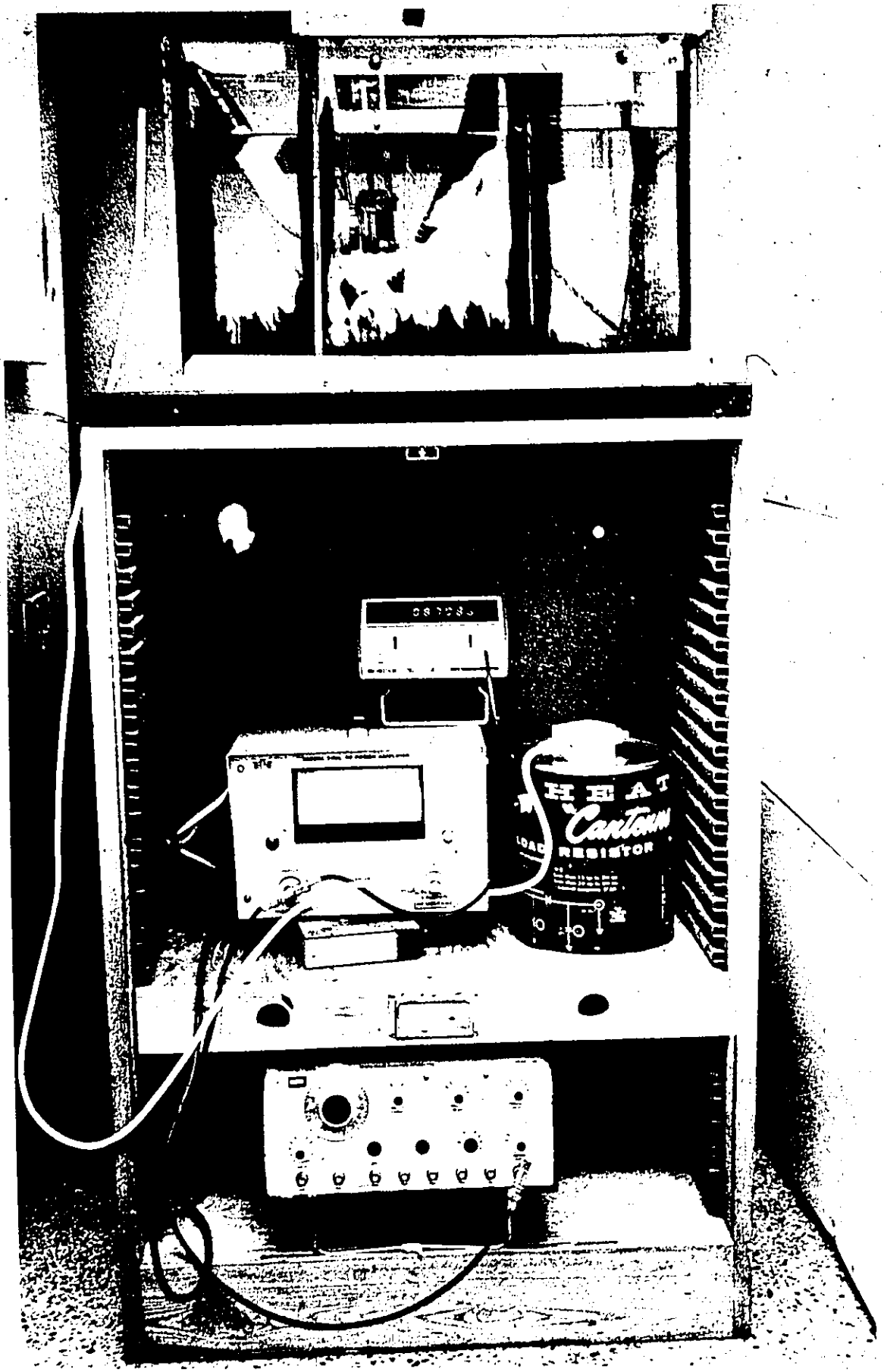

HEAT
Cantam
RESISTOR

Figure 4.3 shows the average output power of the ultrasound beam measured for each value of voltage applied to the piezoelectric crystal of the transducer. From this calibration curve, Figure 4.4 was derived giving the voltage applied to the transducer verses the output intensity of the beam. The average output intensity was calculated as the ratio of the output power to the active or effective radiating area of the transducer. This curve was then used as the standard for determining the acoustic output intensity. Periodic checks on the output power were made to ensure that there was no variation of output with time.

The fine structure of the ultrasound field, was determined using the Ultrasonovision (at the U.S. Bureau of Radiological Health). This uniquely accurate device utilizes the optical interferometry principle (Stewart 1980), and measures the acoustic intensity distribution. Figure 4.5 a gives the two dimensional distribution of the ultrasound intensity as it would appear at the centre of the teflon disc (cell culture container). The three dimensional ultrasound intensity distribution is shown in Figure 4.5 b. Measurements for Figure 4.5 were taken without the teflon disc in position.

From Figure 4.5, it is evident that the ultrasound field is quite non-uniform, primarily because exposures were made and measured in the near field, where the acoustic power fluctuates significantly. It was important therefore to ensure that the lymphocyte cultures were exposed at the same distance from the transducer. Further, cells

Figure 4.3

Transducer calibration curve: The voltage applied to the transducer versus the output power of the ultrasound beam measured at the resonant frequency of the transducer (870 kHz) under free-field conditions.

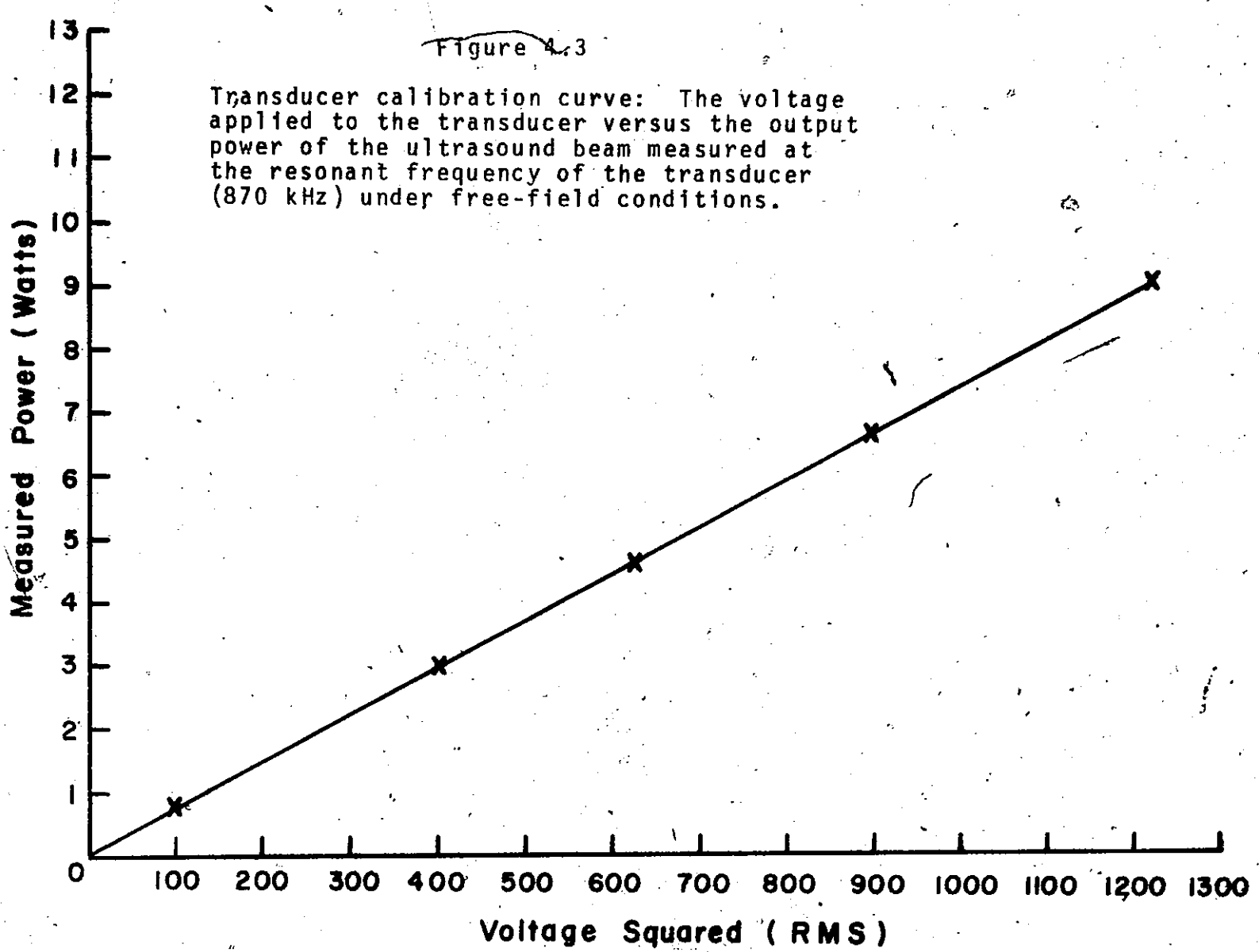


Figure 4.4

Curve of the applied transducer voltage versus the ultrasound output intensity, derived from Figure 4.3 by dividing the values of the output power by the effective radiating area of the transducer.

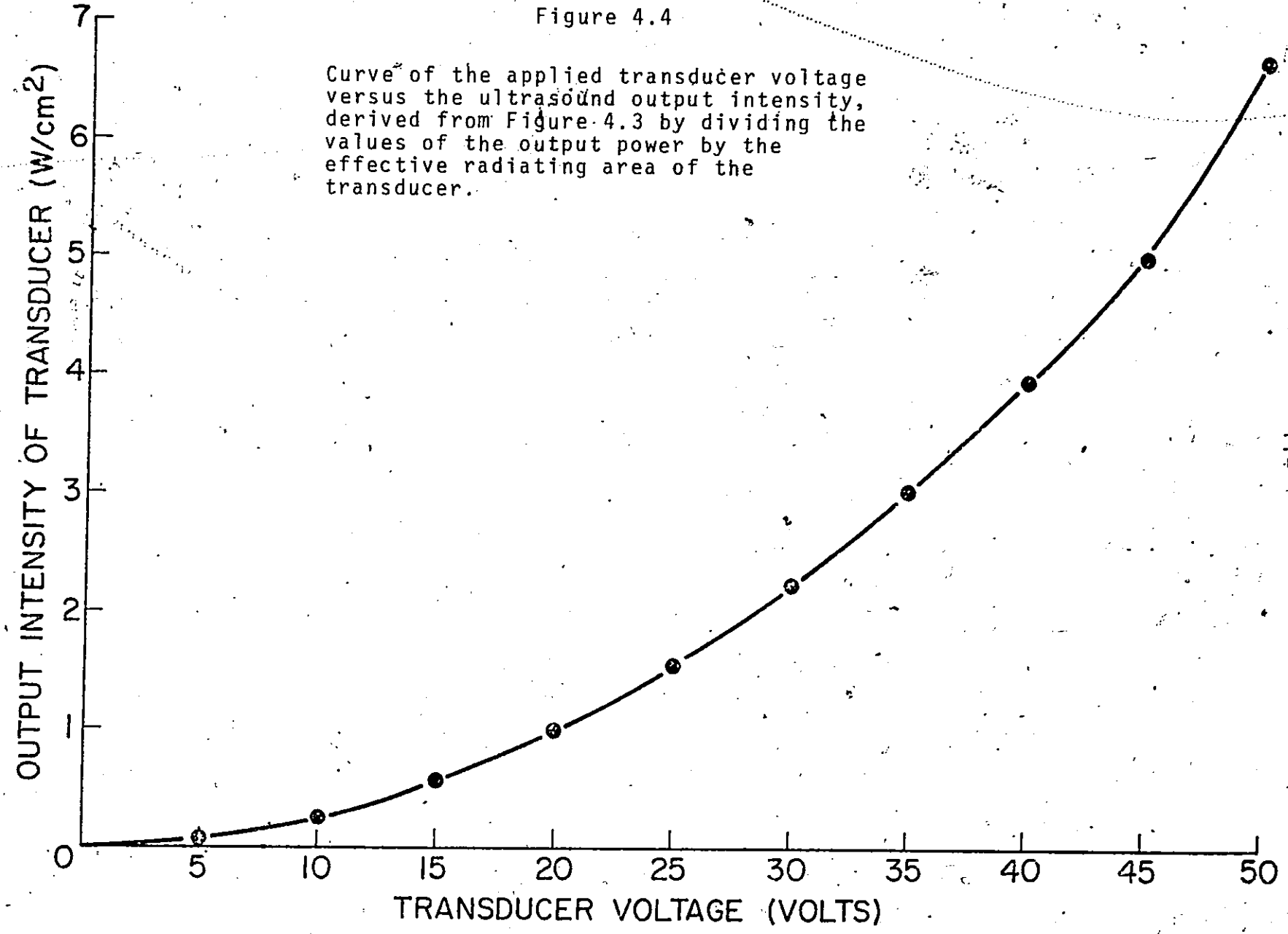
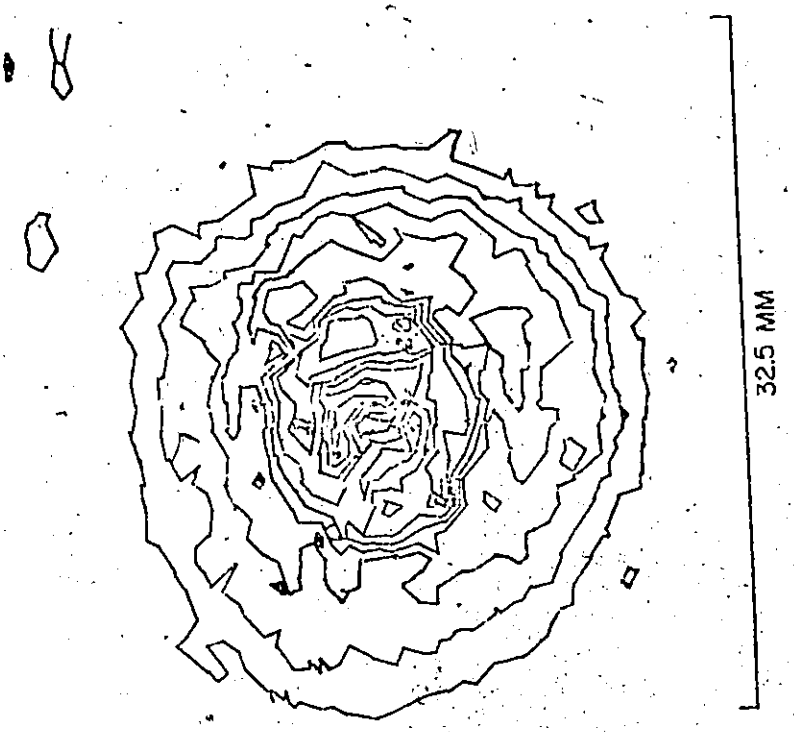


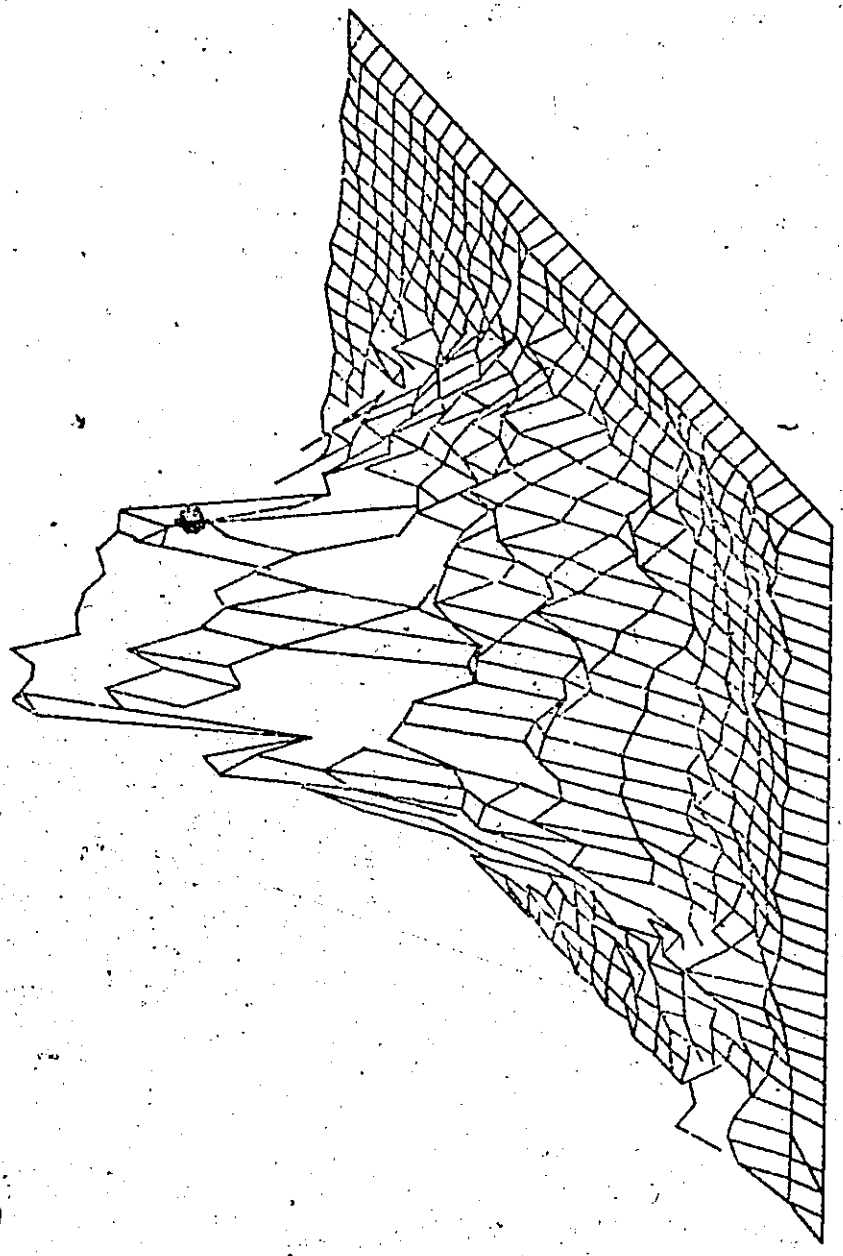
Figure 4.5

The field distribution of the ultrasound beam intensity as it would appear at the centre of the teflon disc.



a) Two Dimensional Distribution

Figure 4.5



b) Three Dimensional Distribution

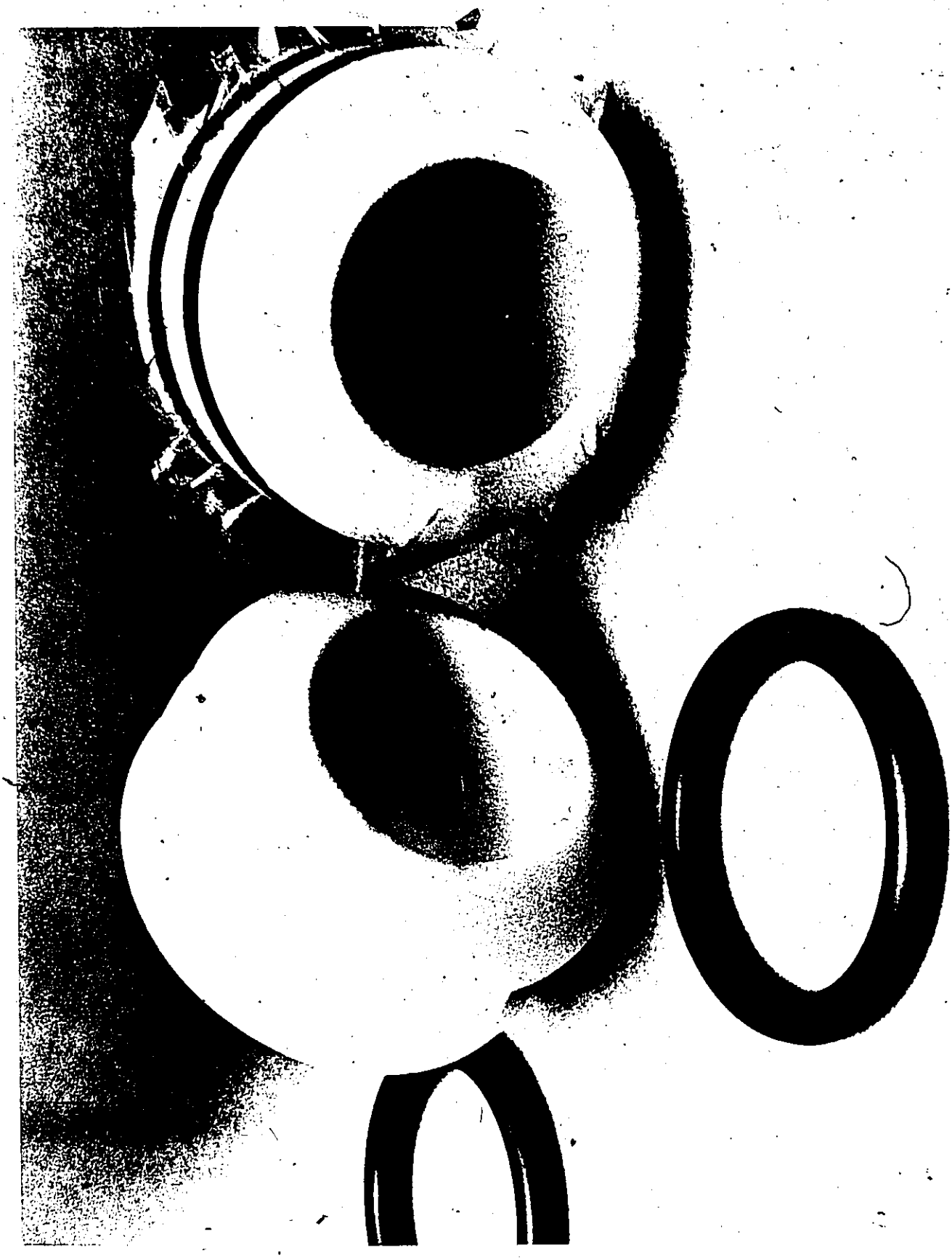
within the ultrasound field would receive a more even distribution of energy if they were moved through the field, and were prevented from sedimenting to the bottom of the container during exposure. This was achieved by designing an exposure jig that could be reproducibly positioned at the same distance from the transducer, and that rotated the cell cultures within the ultrasound field during exposure.

4.2.3 Cell Culture Container: Teflon Disc

Lymphocytes to be exposed to ultrasound were contained within a sterile, teflon disc (1.5 cm internal diameter X 2 cm long), closed at both ends by sterile plastic (NASCO, Whirl-Pak) held on by neoprene "O" rings. To contain the cells, a Whirl-Pak was cut and one of the sheets of plastic, with the sterile side inwards, was held to the disc with a neoprene "O" ring. The disc was then turned over and the cell culture poured in so that the fluid level made a meniscus above the top of the disc. A second sheet of plastic was then placed with its sterile side towards the cells, so that it formed a seal from the air with the top edge of the teflon disc. The second "O" ring was then pressed on in such a way that no air bubbles remain in or entered the container. Teflon discs with and without cells are shown in Figure 4.6. The plastic sheets on the ends of the teflon disc offer virtually zero attenuation to the ultrasound beam.

Figure 4.6

Teflon disc used to contain lymphocyte cultures under sterile conditions. The shape of the disc was designed in such a way that the lymphocyte cultures could be sealed from the air without air bubbles in the sample.



The teflon disc was specially shaped so as to minimize scattering of ultrasound into the container. At the front edge of the disc, the incident beam outside the centre of the disc would be scattered away from the cell cultures. Similarly for any beam scattered back towards the cells.

4.2.4 Cell Exposure Jig

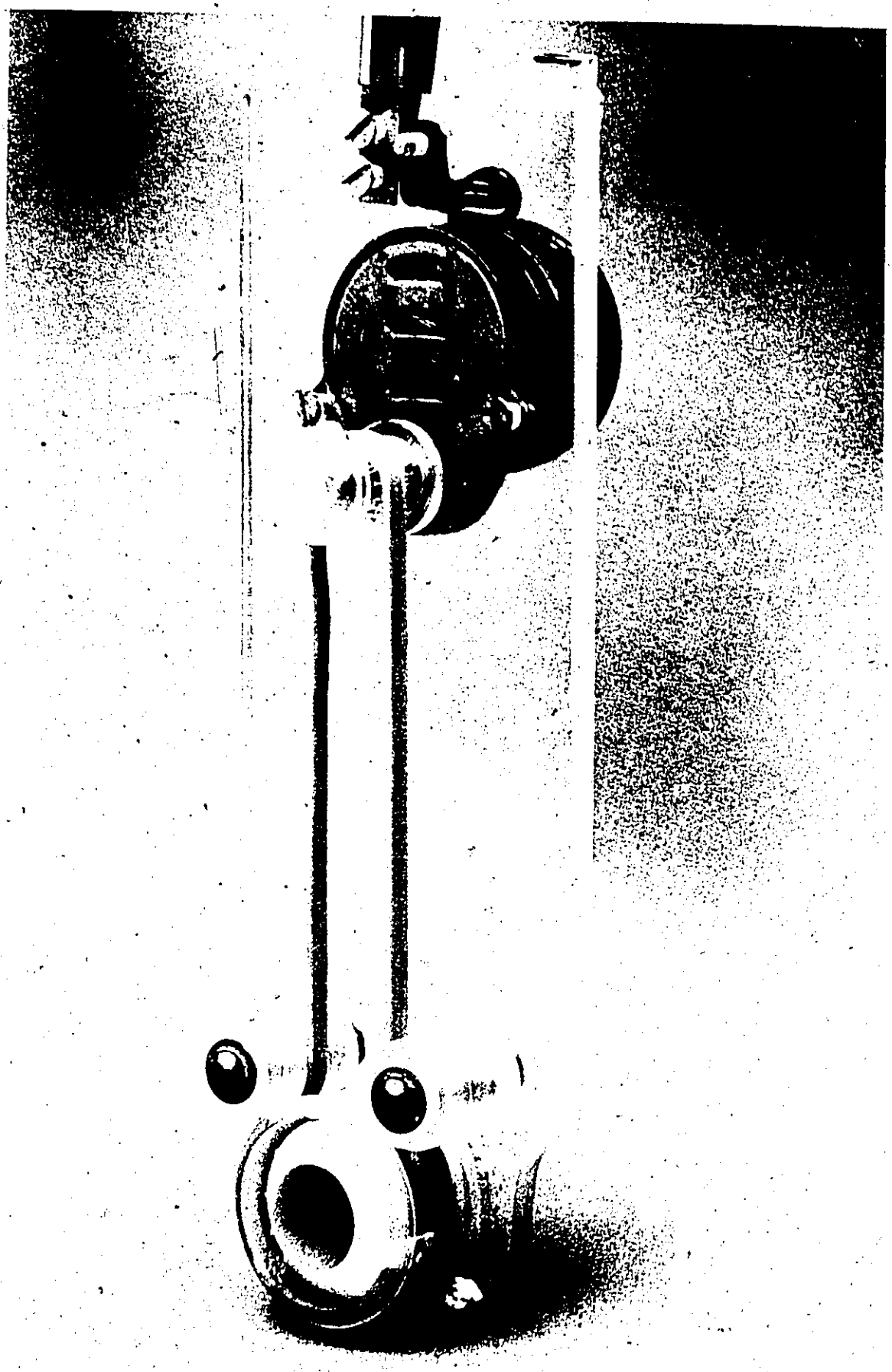
Prior to sonication, the teflon disc was placed, and held by friction, within the cell exposure jig, shown in Figure 4.7. The teflon disc was aligned to be parallel to the transducer face. The cell exposure jig was then positioned at a set distance from the transducer, 1.0 cm from the transducer face to the centre of the disc.

The cell exposure jig was constructed so that the teflon disc containing the cells could be easily inserted and removed. A hole on the side furtherest from the transducer allowed the ultrasound beam to pass through without scattering.

The exposure jig was constructed of plexiglas, with a small motor which rotated a plexiglas cylinder holding the teflon disc. The motor rotated the cell cultures at 1.6 revolutions per minute via a rubber belt drive. All metal screws were made of brass to prevent rusting.

Figure 4.7

Exposure Jig: a disc containing lymphocytes is placed inside a jig having a motor to rotate the cell suspension at 1.6 rpm during ultrasound exposure. The jig has a hole at the exit point of the ultrasound beam so that no part of the beam is reflected back into the culture.



4.2.5 Water Bath

Near-field ultrasound exposures were conducted in a bath containing degassed, distilled water at 37°C. To ensure that anechoic, free-field conditions were maintained (i.e. no scattering of the transmitted ultrasound beam back into the lymphocyte sample), fibreglass wool and pimpled rubber was arranged around the sides and rear of the bath as shown in Figure 4.8.

The bath was constructed with 2 walls forming 3 compartments: a wall holding the transducer which allowed the removal of the transducer without losing the degassed water, and a second wall which could be removed to allow far-field exposures to be conducted. Only the compartments with the transducer and the cell exposure jig were filled with degassed water.

The bath was normally inside an incubator kept at 37°C. A calibrated thermometer was always kept within the water bath to monitor temperature.

Distilled water was degassed in an autoclave for 20 min at 120°C. The water was then placed into a container, filled to the top, and sealed from the air. The container of water was then transported to the incubator and left overnight to reach a temperature of 37°C. This water was used to fill the bath and carry out ultrasound exposures the following morning. Sonications were always conducted with degassed water that had been exposed to the air for less than 4 h. It was noted by Taylor (1969) that water could be considered degassed for ultrasound exposures for periods up to 12 h in a large water bath.

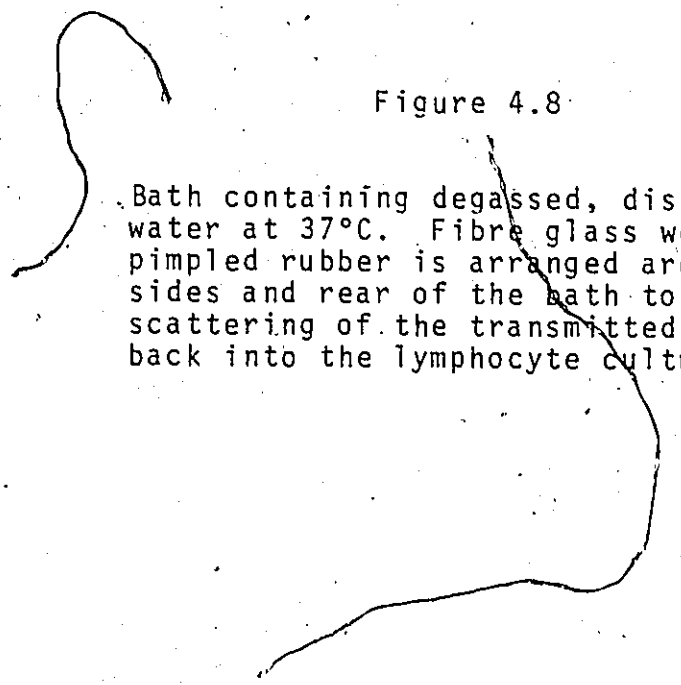
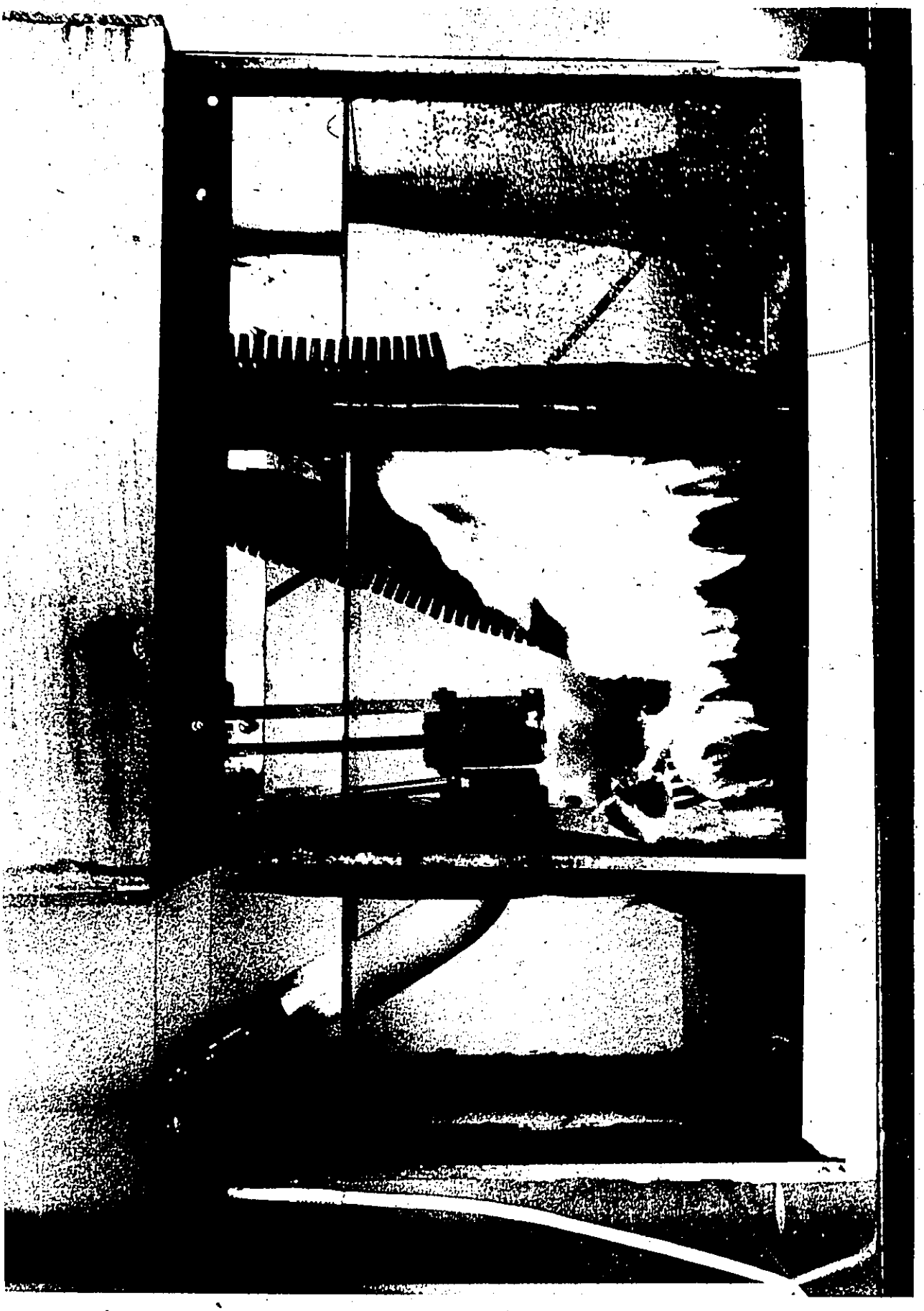


Figure 4.8

Bath containing degassed, distilled water at 37°C. Fibre glass wool and pimpled rubber is arranged around the sides and rear of the bath to mimimize scattering of the transmitted beam back into the lymphocyte cultures.



4.2.6 Ultrasound-Induced Temperature Rise Within Cell Cultures

Since one of the best known consequences of ultrasound action on biological media is heating, it was essential that the temperature rise be measured in cell cultures during sonication. To achieve this a calibrated fine wire thermocouple was connected to a Keithly electrometer voltmeter and a chart recorder, for continous readings of output. The thermocouple was placed through the plastic containing the cells and into the centre of the teflon disc, and positioned with its axis along the central axis, giving the peak intensity of the ultrasound beam. This not only allowed the cells to be rotated during sonication and temperature measurement in the water bath, but caused minimum perturbation to the ultrasound field. Prior to measurement, the temperature of the cells was allowed to become the same as the surroundings before sonication commenced.

4.3 Binding of (^{3}H) Concanavalin A to Lymphocytes

This experimental technique was developed primarily as a model for determining the mechanism of action of ultrasound at the cell surface. The technique is a modification of one developed by Jones (1973).

Prior to treatment, all lymphocytes were suspended at a cell concentration of 3-5 X 10^6/ml in calcium and magnesium free phosphate-buffered saline (CMF-PBS) at pH 7.5. Part of the culture was exposed to 870 kHz ultrasound at an intensity of 4 W/cm^2 for 30 min while in a water bath at either 30 or 37°C. Control samples underwent exactly the same procedure except that ultrasound was not generated. Samples of cells were also heated in a water bath at a temperature of 42.5°C for 30 min. Following each treatment, cell viability was determined by trypan blue dye exclusion and all cell concentration readjusted to 3-5 X 10^6 viable cells/ml. One ml samples were then pulsed with ^{3}H Con A (New England Nuclear) at 1 µCi/ml and incubated in a 5% CO_2 incubator at 37°C for predetermined periods up to 80 min.

After incubation, the cultures were harvested on a manifold (Millipore) according to the method of Jones (1973), and the levels of radioactivity determined in a liquid scintillation counter (Beckman model LS233).

4.3.1 Determination of the Number of Con A Binding Sites on Human Lymphocytes

To determine the number of binding sites for Con A on human lymphocytes, it was first necessary to calculate the counting efficiency of the liquid scintillation counter. This was achieved by adding 1 μCi of Con A to a filter paper within the counting vial, drying, and then adding the same volume of Scintilene as with experimental samples. The counting efficiency (E) was then determined using

$$E = \frac{\text{cpm (1 μCi counted in system)}}{\text{dpm (from 1 μCi)}}$$

The counting efficiency was found to be $(2.24 \pm 0.15) \times 10^{-2}$.

The number of Con A binding sites on the lymphocytes was determined knowing the activity of ^{3}H Con A bound to the cells, as measured by the liquid scintillation counter. This was converted to total disintegrations/min bound to the total number of cells/ml by dividing by the counting efficiency.

Converting the disintegrations/min to Ci and dividing by the specific activity of the ^{3}H Con A (Ci/mlMol) one obtains the number of m Mol of ^{3}H Con A in cells, or the number of m Mol/cell by dividing this by the total number of cells/ml. If the number of atoms of Con A bound per cell is equivalent to the number of binding sites/cell, then multiplying Avogadros number (atoms/mMol) by the number of mMol of Con A/cell gives the number of binding sites for Con A/cell.

4.4 Unscheduled DNA Synthesis - Autoradiography

To obtain an indication whether ultrasound produces damage to the DNA which could be observed as an unscheduled DNA synthesis, the following procedure was undertaken:

(i) lymphocytes were isolated and stimulated with Con A for 20 h, then

(ii) pulsed with (^{3}H) thymidine (10 µCi/ml) for 2 h, during which time the cells were exposed to 870 kHz ultrasound at 4 W/cm^2 for 30 min.

(iii) cells were washed twice in cold PBS, resuspended in 1 ml PBS + 1 ml 2% gluteraldehyde in 0.1 M PO_4 (pH 7.2) and left overnight

(iv) cells were pelleted, resuspended in cold PBS and left 30 min (on ice)

(v) step (iv) was repeated twice again

(vi) cells were pelleted, and resuspended in 4-5 drops of fetal calf serum

(vii) cells were smeared onto clean slides and air dried overnight

(viii) following 2 washes in PBS and 2 washes in distilled water, the cells were air dried

(ix) slides were dipped in diluted (1:1 water + 2% glycerol) nuclear emulsion (Kodak NTB-3), dried, wrapped light tight and kept in the refrigerator for 2-3 weeks

(x) Following development, fixing and washing, the cells on the slides were stained in a 2% solution of Giemsa for up to 15 min, then dried for silver grain counting.

Cells incubated with mitomycin C were used as positive controls for the experiment. Mitomycin C at concentrations of 15, 50 and 100 ng/ml were added to the cell cultures for 2 h. The mitomycin C treated cells were then washed twice in RPMI 1640 medium and pulsed with (^{3}H) thymidine (10 µCi/ml) for 2 h. All samples then underwent the autoradiography procedure commencing at step (iii).

4.5 Mixed Lymphocyte Reaction (MLR)

The MLR is widely used clinically as a preliminary test prior to tissue and organ grafting.

The MLR involves interactions at the cell surface and within the cell. Thus if ultrasound effects the MLR, it provides an opportunity to obtain further information on the mechanism of action of ultrasound by determining whether ultrasound acts predominantly at the cell surface or internally, to produce the effect.

The present procedure was modified to that described by O'Leary et al (1976). In brief, lymphocytes were isolated from two healthy donors - one made the responder (A) and the other the stimulator (B). Stimulator and part of the responder cells were incubated at 37°C with 25 µg/ml of mitomycin C for 30 min, then washed three times in RPMI 1640 medium supplemented with 10% fetal calf serum + 1% penicillin (100 IU/ml) - streptomycin (100 µg/ml). Incubation of cells with mitomycin C prevents normal DNA synthesis within the cells.

Samples of the stimulator (untreated with mitomycin C) and responder (treated and untreated with mitomycin C) cells were exposed to 870 kHz ultrasound at 4 W/cm^2 for 30 min, washed three times and resuspended in the supplemented medium.

Equal volumes of samples were combined and incubated at 37°C for 5 or 6 days. Where only one sample is referred to, it was combined with an equal volume of culture medium. Following this incubation period the samples were pulsed with (^{3}H) thymidine (2 µCi) for 4 h, washed, harvested and counted.

A stimulation index (SI) is determined to measure the degrees of stimulation between cells of the two tissues, and is given by

$$SI = \frac{AB_M - B_M}{AA_M - A_M} \quad \text{for the control cells}$$

$$SI = \frac{A_UB_M - B_M}{A_UA_M - A_M} \quad \text{for the sonicated cells.}$$

The subscript M refers to mitomycin treatment and U means exposed to ultrasound.

4.6 Preparation for Electron Microscopy

Human lympocytes were prepared for electron microscopy using standard fixation, dehydration and embedding procedures.

In brief the following procedures were conducted:

(a) Fixing immediately following sonication (within 10 sec).

(i) treated lymphocyte cultures were placed into an equal volume of 4% glutaraldehyde (Polysciences Inc.) in 0.1 M PO_4 (pH 7.0)

(ii) cells were pelleted and resuspended in 4% glutaraldehyde in 0.1 M PO_4 for 1.5 h at room temperature

(iii) cells were pelleted and resuspended in 0.05 M PO_4 (pH 7.0) for 10 min

(iv) step (iii) repeated three times

(v) cells were pelleted and resuspended in 1% OsO_4 in 0.05 M PO_4 (pH 7.0) for 1.5 h (on ice in fume hood)

(vi) three washes were carried out as in step (iii)

(vii) cells were pelleted, resuspended in 10% acetone and left for 10 min (on ice). This step was repeated using 30%, 50%, 70%, 90% acetone and finally 3 times in 100% acetone.

(viii) cells were pelleted, infiltrated with Spurrs and left overnight

(ix) next morning - cells were pelleted, Spurrs removed and infiltrated again with Spurrs

(x) same afternoon Beem capsules were loaded with cells, topped up with fresh Spurrs and heated overnight in a 55°C oven to cure

(xi) cells were then sectioned and placed onto copper grids and stained for EM. The staining procedure involved dipping the samples in 25% uranyl acetate (in methanol) for 10-15 min, washing in distilled water, and dipping in lead citrate (Reynolds) for approximately 10 min before washing and allowing the samples to dry before viewing.

(b) Fixing 30-60 min after sonication

- started at step (ii) and completed the entire procedure.

4.7 Thymidine Transport

Thymidine transport in human peripheral lymphocytes was measured by modification of the rapid sampling technique of Strauss et al (1976). Cells in RPMI-1640 culture medium (15% fetal calf serum + 1% pen-strep) were exposed to 870 kHz ultrasound at 4 W/cm^2 for 30 min, or were subjected to the same procedure but without ultrasound generation, then were washed twice and suspended in LMGB at a concentration of 10^7 cells/ml. LMGB consisted of 10 mM sodium phosphate buffer, pH.7.5, 0.9% NaCl, 0.1% bovine serum albumin (5 X crystallized (Sigma)) and 5 mM glucose (Fisher). Transport was measured by mixing equal volumes (0.25 X 0.25 ml) of cell suspension with thymidine at the desired concentration at 20 µCi/ml in LMGB. After the appropriate incubation time (15-100 s), 200 µl of reaction mixture was layered in a 400 µl microfuge tube (Canlab) containing 50 µl of 7% perchloric acid as the bottom layer and 150 µl of silicone oil (12 parts 550 fluid and 13 parts 510 fluid (Dow Corning). The tubes were spun at 10,000 rpm for 20 s using a Beckman microfuge. The microfuge tubes were frozen in ethanol/dry ice and the tips sliced into a scinitillation vial for counting. Cell digestion was accomplished overnight with 1 ml of Protosol (New England Nuclear) at 37°C. This was then neutralised with 100 µl glacial acetic acid and counted in 7 ml of scintillation fluid (Scintiverse, Fisher) with a Beckman Scintillation counter. Counting efficiencies, which ranged

between 24 - 35%, were determined by internal quench correction.

4.8. Fluorescence Analysis of DNA Unwinding (FADU)

This technique for analysis of breaks in the DNA was developed by Dr. H.C. Birnboim and Mr. J. Javcek at the Chalk River Nuclear Laboratories. These experiments were conducted with their assistance and cooperation.

Lymphocyte Isolation

1. Dilute 1 volume of blood (from healthy donor) with 3 volumes of 0.87% NH_4Cl in 10 mM Tris (BDH labs) at ph 7.2 for 20 min at 0°C.
2. Centrifuge at 400 g for 20 min at 0°C.
3. Wash in 0.87% NH_4Cl in 10 mM Tris at 0°C for 10 min.
4. Centrifuge at 400 g for 20 min at 0°C.
5. Suspend at desired cell concentration (1-5 x 10^6 cells/ml) in RPMI - 1640 (or modified Earle's salt solution) at 0°C.

Treatment

6. Expose cells to ultrasound in a water bath of degassed, distilled water at 0°C.
7. Wash cells twice in modified Earle's salt solution at 0°C.
8. Resuspend cells in inositol (25 ml of 0.5 M meso-inosital (BDH), 0.5 ml of 1 M phosphate buffer, 0.1 ml of 0.5 M $MgCl_2$, 24.4 ml H_2O and 2 to 3 drops of 1 N NaOH to raise pH to 7.2) for FADU assay, or in modified Earle's salt solution supplemented with glucose for repair analysis.

Analysis

To assay initial damage to DNA immediately after treatment, one continues straight on with the procedure below. For repair analysis, cells are incubated for varying periods of time after treatment before proceeding with the FADU assay.

9. Distribute 0.2 ml cells at 0°C into 12 tubes
   4 sets of 3 tubes marked: P,T and B.
   Note: P = sample of DNA partially unwound.
   T = sample of DNA kept totally in double stranded form (0% unwound).
   B = background DNA sample which is unwound 100%.
10. Add 0.2 ml of urea (9 M urea, 10 mM NaOH, 2.5 mM cyclodiamine tetra acetic acid, 0.1% sodium dodecyl sulphate to all samples, 0°C, for 10 min (this gently lyses cells to release DNA).
11. Add 0.4 ml of glucose (1 M glucose, 14 mM 2-mercaptoethanol) to all P samples (glucose prevents DNA denaturation) and vortex.
12. Add 0.1 ml of alkali (0.2 N NaOH in urea) to P samples for 30 min at 0°C, then transfer to 15°C water bath for 1 h (alkali unwinds nicked DNA).
13. Add 0.2 ml of alkali to T and B samples and vortex (note: samples are light sensitive - cover).
14. Shear samples T and B (using ultrasound cell disrupter (Heat Systems - Ultrasonics Inc.) for 2-3 sec), and leave at room temperature.
15. Add 0.4 ml glucose to samples P and B.
16. Shear samples P.

17. Add 1.5 ml of Ethydium Bromide (6.67 µg/ml EtBr in 13.33 mM NaOH) to all tubes and vortex.
18. Place all samples in 20°C water bath for 15 min.
19. Dry tubes, allow all samples to rise to room temperature and read in a Spectrofluorometer (Farrand Optical Co.).

4.9 Statistics

The modified t test (Blalock 1960) was used to determine the statistical significance between corresponding points in the data. This test takes into account any differences that may occur in the standard deviations (σ_1, σ_2). If the standard deviations are not compatable (a conservative assumption), then the degrees of freedom (df) for the t test determined using the formula

$$df = \frac{[\sigma_1^2/(N_1-1)+\sigma_2^2/(N_2-1)]^2}{[\sigma_1^2/(N_1-1)]^2/(N_1+1)+[\sigma_2^2/(N_2-1)]^2/(N_2+1)} \quad -2$$

where N_1 and N_2 were the number of samples used to determine the means $\bar{x}_1$ and $\bar{x}_2$, and standard deviations σ_1 and σ_2 respectively.

The value of t was determined using

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\sigma_1^2/(N_1-1) + \sigma_2^2/(N_2-1)}}$$

With the values of t and df known, the level of significance for the two-tail test was then determined by referring to tables listing the distribution of t for given degress of freedom.

The Kolmogorov-Smirnov two sample test (KS-test) (Siegel 1956) compares distributions as a whole by finding the maximum difference between the two empirical distributions and determining whether this difference is significant, given that the population distributions were equal.

Chapter 5 Results

Where appropriate the standard deviations are provided in the tables of results and as "error bars" in the graphs. In some cases error bars were drawn on one side of a point purely for clarity. In all cases the error bars should be assumed to have equal magnitude on each side of a point.

All statistical analyses, except the KS-test, compare corresponding points on the graphs under comparison.

5.1 Gross Effects

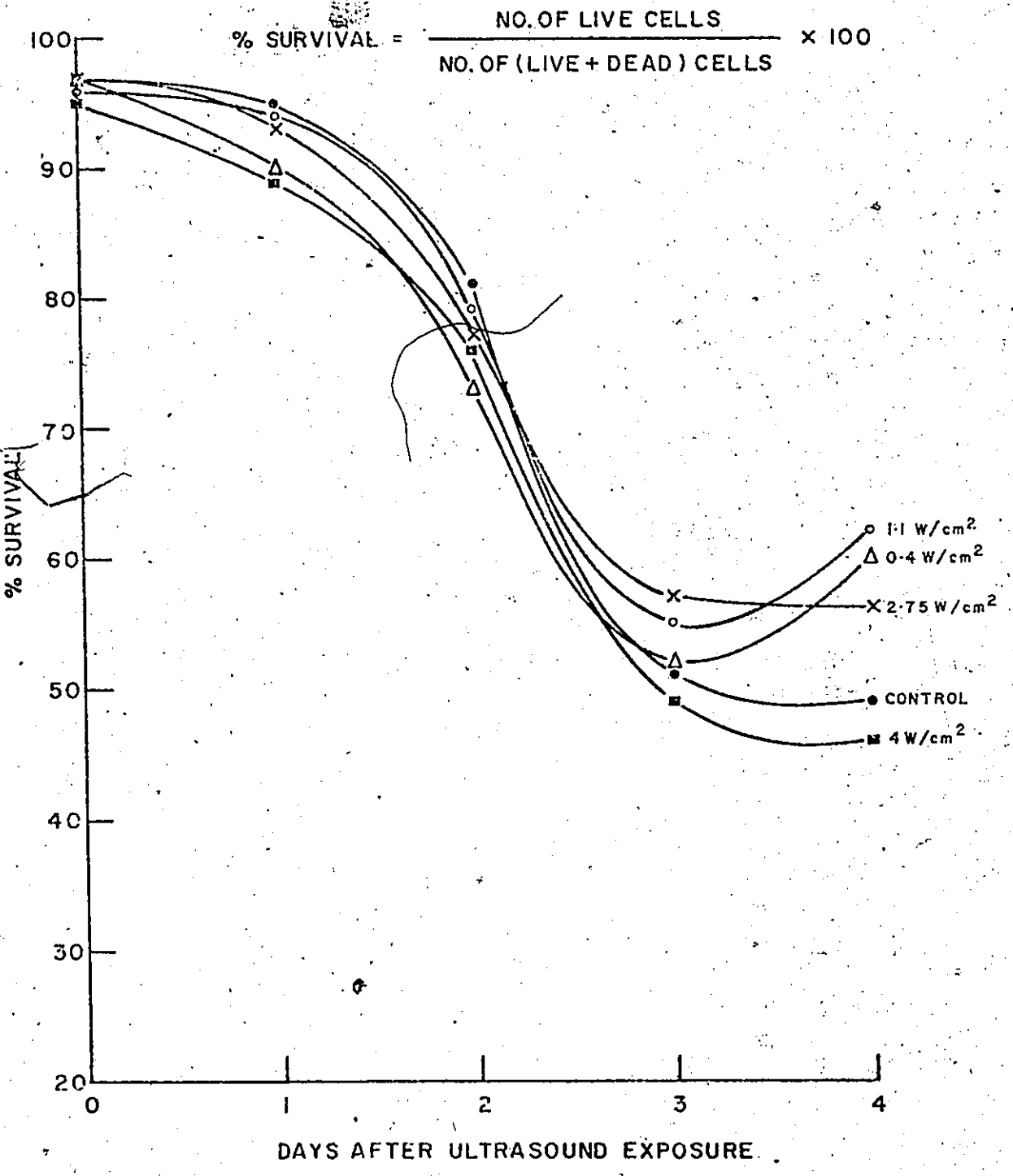
5.1.1 Cell Survival

Survival *in vitro* of human lymphocytes, stimulated for 2 days, then exposed to 870 kHz, near field, continuous wave ultrasound at varying intensities for 30 min was determined using trypan blue dye exclusion and is shown in Figure 5.1. It is evident that for ultrasound intensities up to 4 W/cm^2, the cell viability was not significantly affected by ultrasound. Statistical analysis of corresponding experimental points using the modified t test showed that survival up to 4 days after ultrasound at intensities up to 4 W/cm^2 was not significantly different ($P = 0.1$) than control values. Nevertheless, in all experiments cell number and viability were determined after treatments.

The survival of the cells in the present studies is in agreement with the findings of Harvey *et al* (1975) and Bleaney *et al* (1972).

Figure 5.1

CELL SURVIVAL CURVES FOR HUMAN LYMPHOCYTES STIMULATED WITH CONCANAVALIN A FOR 2 DAYS THEN EXPOSED TO 870 kHz CONTINUOUS WAVE NEAR FIELD ULTRASOUND OF VARYING INTENSITIES FOR 30 min



5.1.2 Temperature Rise

Exposure of living cells or biological fluids to ultrasound is known to produce an increase in temperature, the rise dependent on the acoustic intensity, duration and exposure conditions. The temperature rise of cultured human lymphocytes, exposed in a constant temperature water bath at 37°C to various intensities of 870 kHz, continuous wave, near field ultrasound, is shown in Figure 5.2. Temperature measurements were continued after termination of ultrasound generation.

Temperature determinations were made using cell concentrations varying from 10^6 to 10^7 cells/ml. Varying the cell concentration did not affect the temperature measurements.

One notes from Figure 5.2 that ultrasound at an intensity of 4 W/cm^2 produced an initial rapid rise in temperature for the first 3 min, then as thermal diffusion became significant, the rise became less rapid and an equilibrium temperature was reached within 6-7 min. Although Figure 5.2 indicates the temperature of the cells during the first 10 min, measurements were taken for the full 30 min, confirming the time at which thermal equilibrium was achieved.

The maximum temperature within the cell cultures was found to be 42°C when exposed to an intensity of 4 W/cm^2. This is 5°C above the ambient temperature of the water bath. Similarly intensities of 2.75 W/cm^2 and 1.10 W/cm^2 increased the temperature of the cells by 4.1°C and 2.2°C respectively.

TEMPERATURE RISE IN CELL CULTURES EXPOSED TO NEAR FIELD ULTRASOUND (870 kHz) AT VARIOUS INTENSITY LEVELS

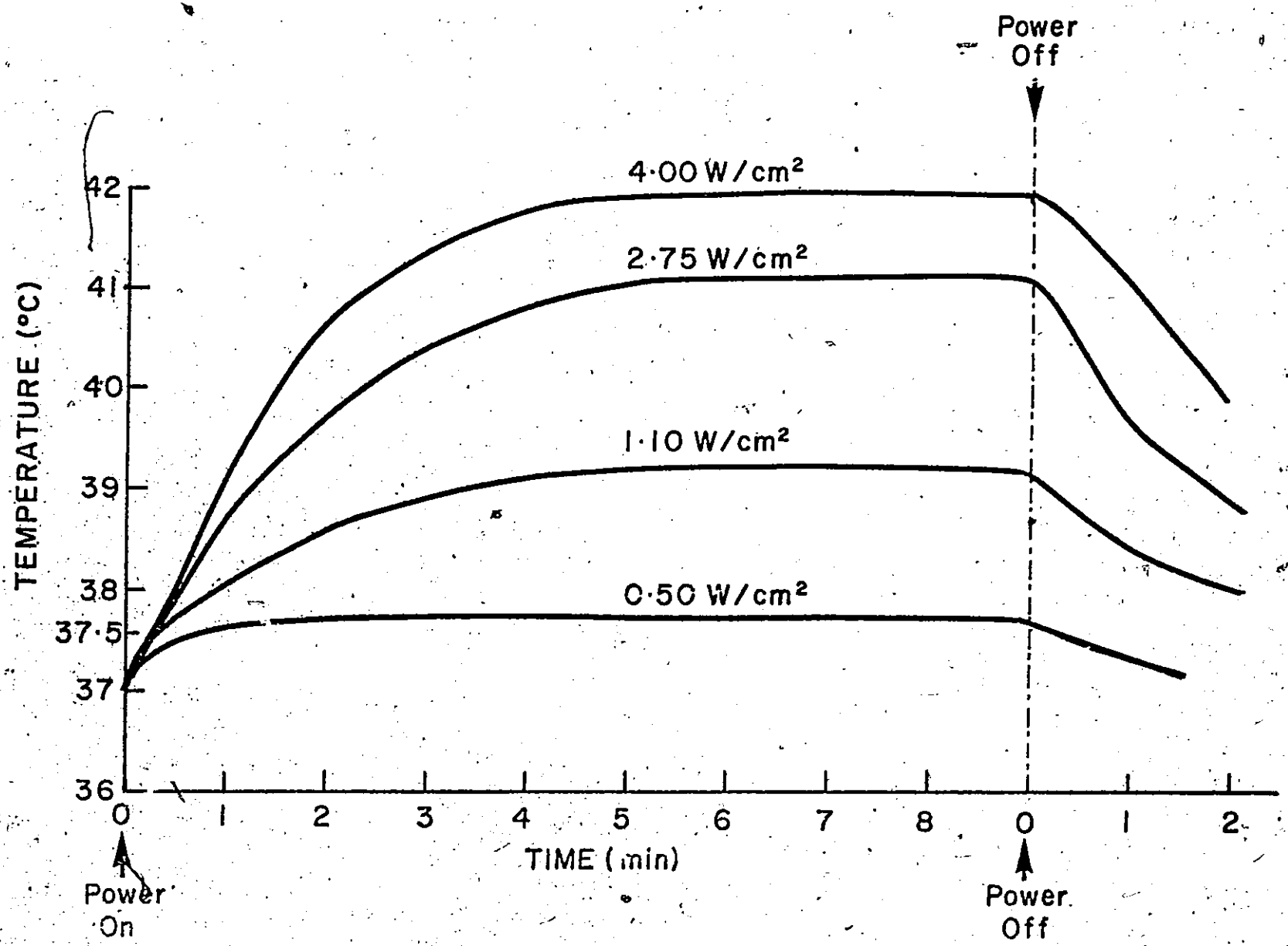


Figure 5.2

When the water bath temperature was lowered to 30°C, exposure of the cells to an ultrasound intensity of 4 W/cm^2 produced a temperature rise of 4.6°C. A lower final temperature would be expected when ultrasound exposure takes place in a lower temperature water bath because of thermal diffusion. However, one would expect that the temperature rise would still be 5°C. The discrepancy could be explained partially on the basis of experimental error, but more probably because of a change in the absorption coefficient of the water in the bath, due to an increased gas content at the lower temperature of 30°C.

When the ultrasound energy was removed the temperature of the cells initially dropped rapidly and then the fall tapered off until the water bath temperature was reached.

5.1.3 Ultrastructural Effects

An ultrastructural study was undertaken primarily to determine if ultrasound produced gross damage to activated lymphocytes. Fixing of the cells was carried out either immediately (within a few seconds) or within 30 - 60 min after treatment. Varying the time of fixation was done in an attempt to determine if immediate effects occurred that were later repaired.

Figure 5.3a shows the typical appearance of a normal activated lymphocyte which has not been treated. An extensive network of microtubules is noted radiating from the centriolar region as reported for mouse lymphocytes by Rogers and Brown (1979) and Rudd et al (1979). Examination of sonicated cells in Figures 5.3 b and c, reveals nuclei with the typically diffuse chromatin of blast cells, apparently undamaged nuclear or plasma membranes, endoplasmic reticulum, Golgi apparatus and mitochondria.

In cells where fixing was carried out immediately after sonication, there was no evidence of microtubules in the centriolar region near the nuclear notch (Figure 5.3b). When a comparison was made with cells that were fixed some 30 - 60 min after sonication, microtubules were again evident (Figure 5.3c). It appears that ultrasound caused dissassembly of microtubules by producing stresses that were strong enough to

break their weak hydrophobic bonds or by acting indirectly in some manner to produce the dissassembly. Within a few minutes the microtubules then began reassembly from the tubulin submits that were evidently not much affected by ultrasound.

No ultrastructural differences were detected between cells heated in a 42°C water bath for 30 min (Figure 5.3d) and the controls.

Figure 5.3(a)

Electron micrograph of untreated human lymphocytes stimulated for 3 days with Con A. Arrow indicates microtubules radiating from the centriolar region.

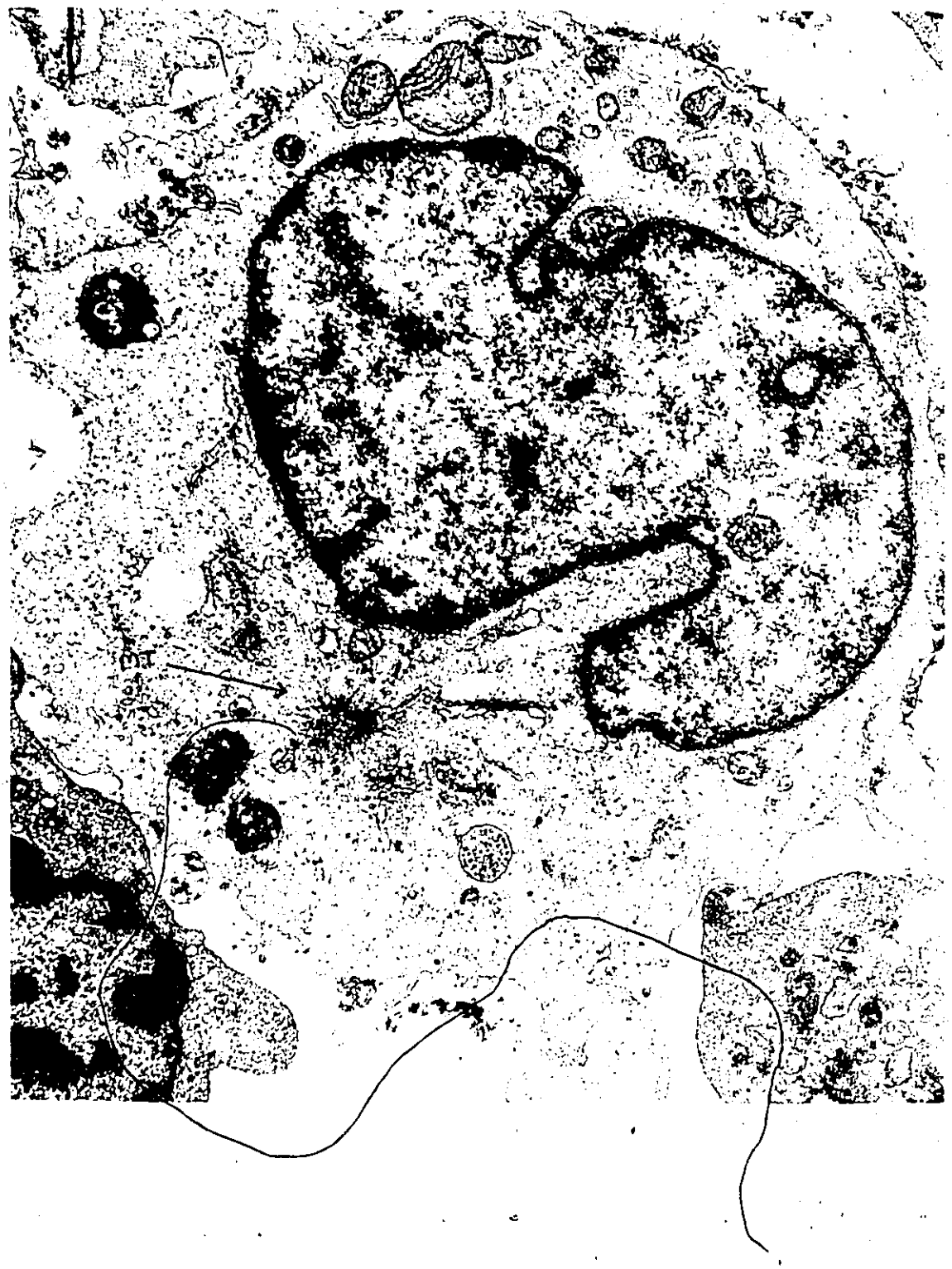


Figure 5.3(b)

Electron micrograph of human lymphocyte stimulated for 3 days then exposed to 870 kHz, near field, continuous wave ultrasound at 4 W/cm^2 for 30 min and fixed immediatey.

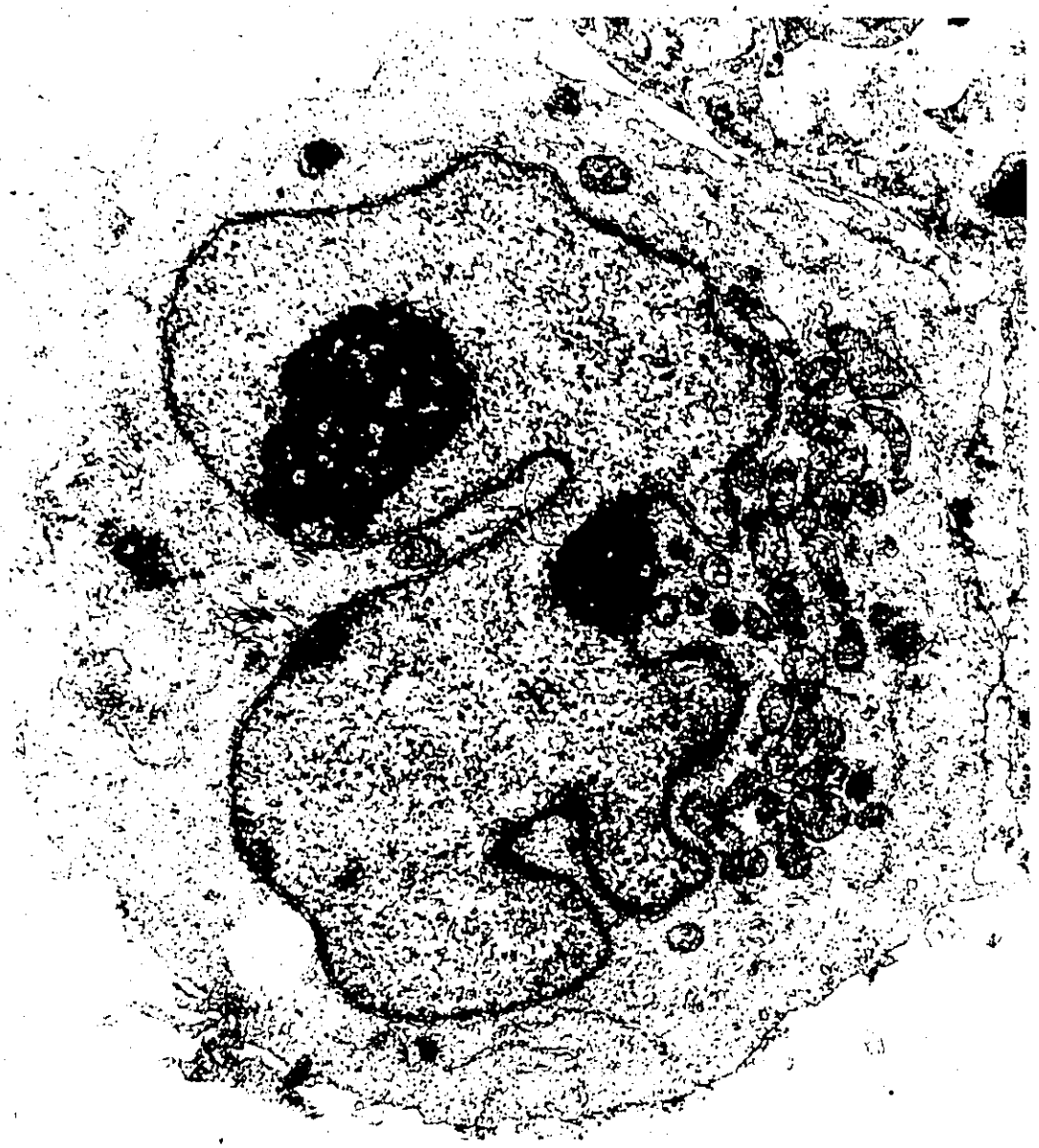


Figure 5.3(c)

Electron micrograph of human lymphocyte stimulated for 3 days then exposed to 870 kHz, near field, continuous wave ultrasound at 4 W/cm^2 for 30 min and fixed 30-60 min after.

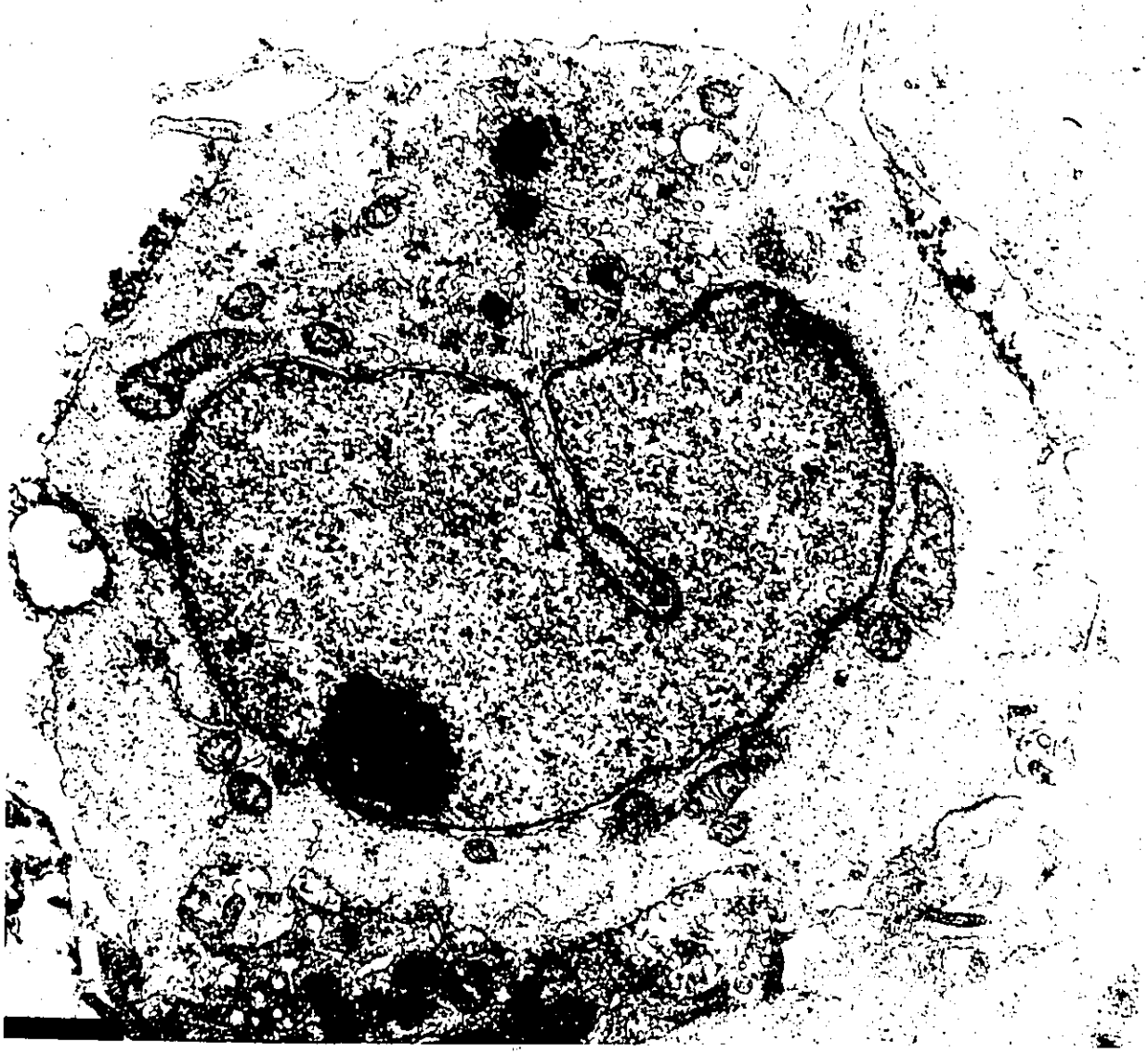
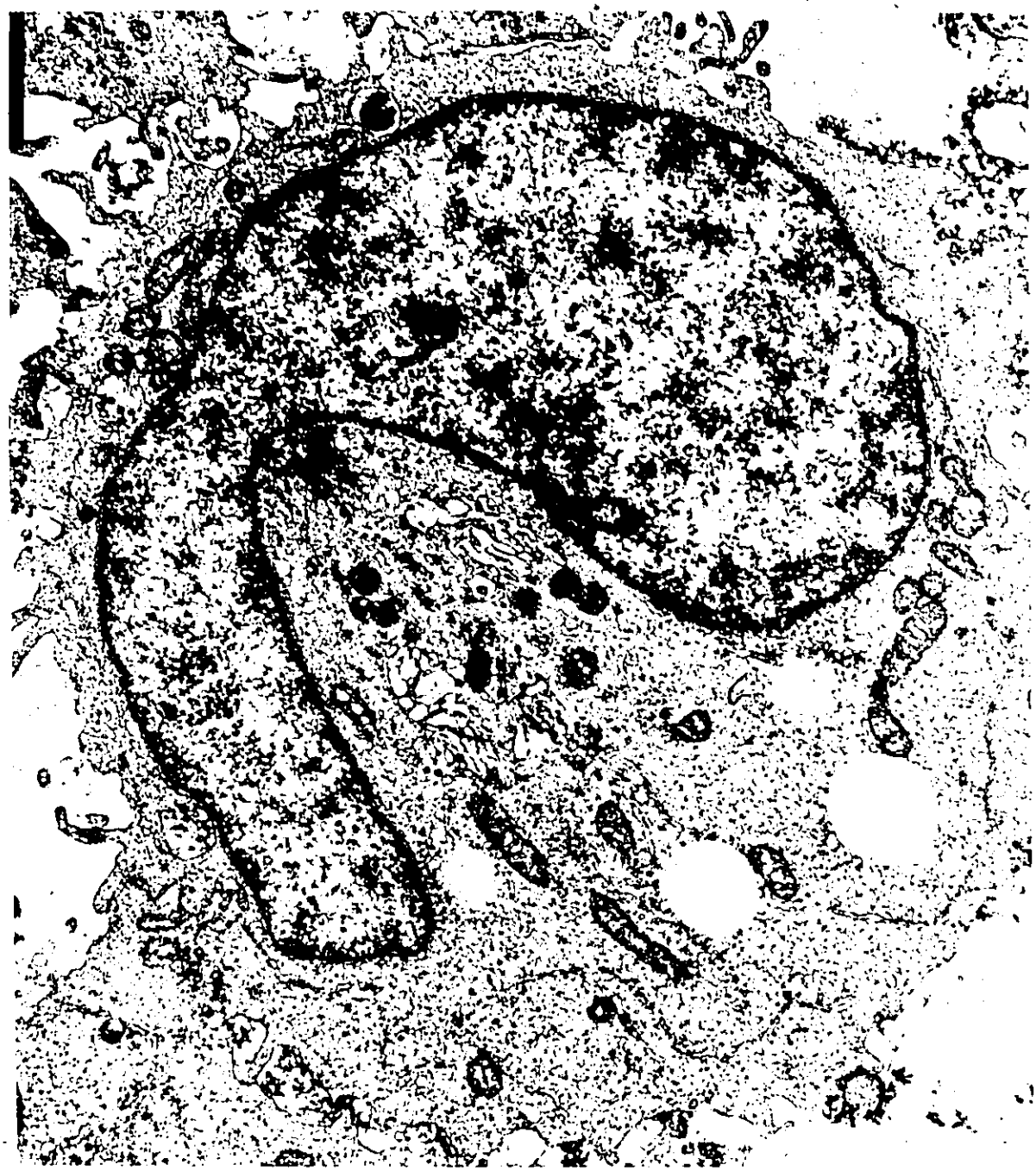


Figure 5.3(d)

Electron micrograph of human lymphocyte stimulated for 3 days then heated in a 42°C water bath for 30 min and fixed immediately after.



5.2 Intracellular Effects of Ultrasound

5.2.1 DNA Synthesis

It has been reported (Fung et al 1978, Prasad et al 1976) that ultrasound could affect DNA synthesis in cells both in vivo and in vitro. Thus it was decided to investigate this effect by measuring the incorporation of (^{3}H) thymidine into human lymphocytes in an attempt to determine a threshold level of exposure and obtain a reproducible end-point for the evaluation of the possible mechanisms of action of ultrasound.

5.2.1.1 Ultrasound Exposure at Varying Times After Activation

To assess the effect of ultrasound on (^{3}H) thymidine incorporation it was necessary to determine at what time after activation the cell population was most sensitive.

Incorporation of (^{3}H) thymidine into resting lymphocytes was unaffected by exposure to ultrasound up to an intensity of 4 W/cm^2. Both control and sonicated resting lymphocytes showed only basal levels of (^{3}H) thymidine incorporation when followed for up to 4 days after sonication. Further, if resting lymphocytes were exposed to 4 W/cm^2 ultrasound and then immediately activated with Con A, the same level of incorporation of (^{3}H) thymidine as the controls was found (Table 5.1).

Table 5.1

Incorporation of (^{3}H) thymidine (2 µCi/ml for 3 h) in lymphocytes exposed to 870 kHz ultrasound at 4 W/cm^2 for 30 min then activated with 25 µg/ml Concanavalin A.

Days After Sonication and Activation	*(Mean cpm ± SD) x 10^{-3}		
	0	1	2
Sonicated	5.4 ± 0.5	235 ± 16	383 ± 26
Control	5.0 ± 0.6	200 ± 20	340 ± 15

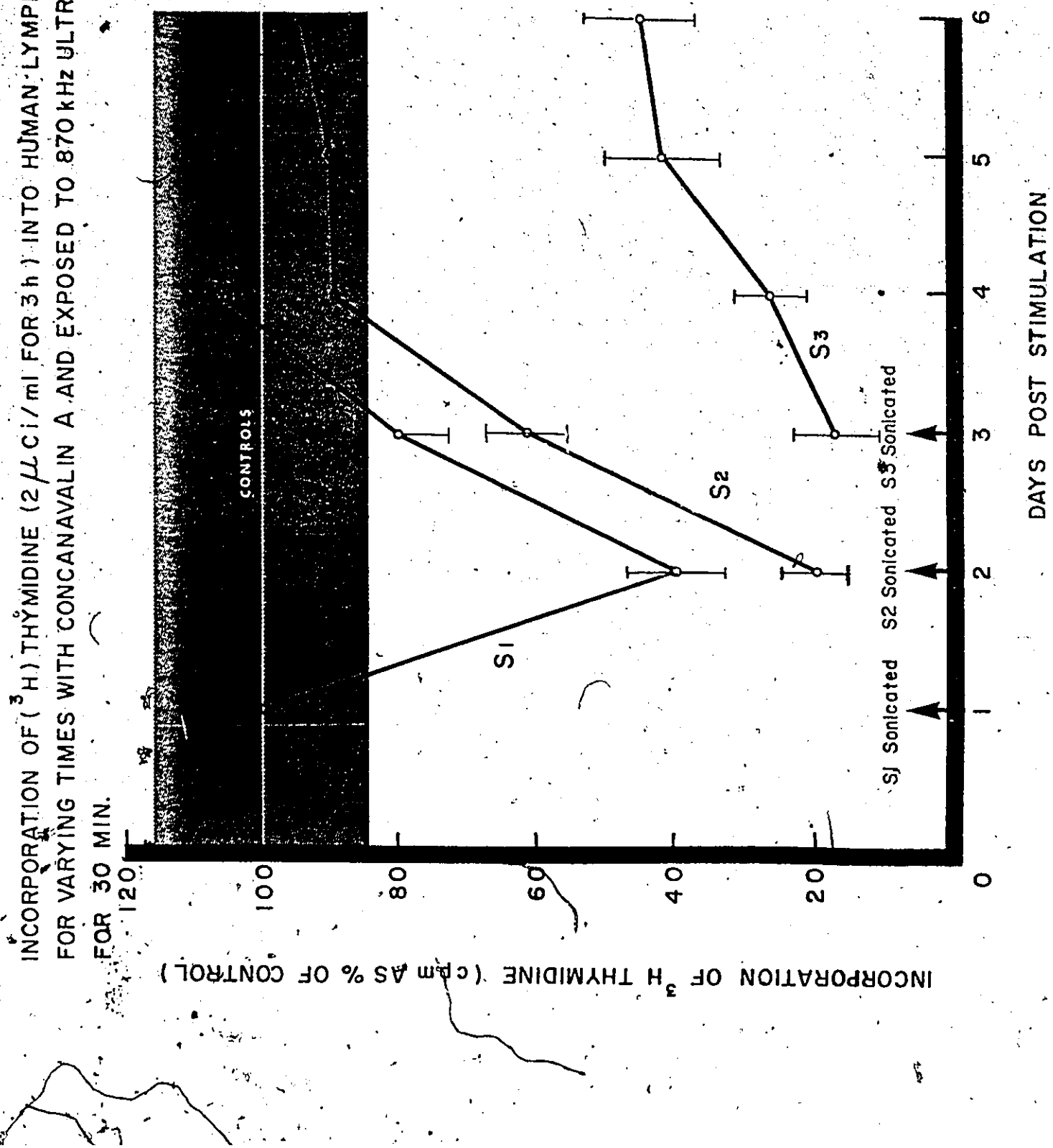
* mean of quadruplicate samples (counts per minute ± standard deviation).

When lymphocytes were exposed to ultrasound (4 W/cm^2 for 30 min) at varying times after activation, there was evidence that incorporation of ^{3}H-thymidine was inhibited (Figure 5.4). Bearing in mind the fact that activated lymphocytes do not undergo significant DNA synthesis until some 30 - 36 h after addition of the mitogen, one would not expect cells activated for only 24 h to show an ultrasound induced sensitivity to altered DNA synthesis. This is supported by the data on resting cells exposed to ultrasound, or in cells sonicated and then immediately activated (Table 5.1). Lymphocytes seem most susceptible to an ultrasound induced reduction in DNA synthesis 2-3 days after activation (Figure 5.4), corresponding to the period of peak DNA synthesis in the population (Repacholi et al 1979).

The initial reduction in the incorporation of (^{3}H) thymidine for both 2 and 3 day stimulated lymphocytes was found to be significantly different from the controls at the P = 0.001 level (modified t-test). However, cells sonicated after two days stimulation recovered to control values of (^{3}H) thymidine incorporation after a couple of days, while 3 day stimulated cells did not fully recover to control levels over the period examined.

An ultrasound intensity of 4 W/cm^2, the upper limit of the range used in therapy, was used in these experiments. It was thus of interest to determine the effect of lower intensities of the incorporation of (^{3}H) thymidine.

Figure 5.4


INCORPORATION OF (^{3}H) THYMIDINE (2 μCi/ml FOR 3 h) INTO HUMAN LYMPHOCYTES STIMULATED FOR VARYING TIMES WITH CONCANAVALIN A AND EXPOSED TO 870 kHz ULTRASOUND AT 4 W/cm^2 FOR 30 MIN.
CONTROLS
S1
S2
S3
S1 Sonicated
S2 Sonicated
S3 Sonicated
INCORPORATION OF ^{3}H THYMIDINE (cpm AS % OF CONTROL)
DAYS POST STIMULATION
120
100
80
60
40
20
0
1
2
3
4
5
6

5.2.1.2 Varying Intensities of Ultrasound

To determine the effect of ultrasound at varying intensities the incorporation of (^{3}H) thymidine into lymphocytes after sonication at intensities of 0 - 4 W/cm^2 for 30 min was obtained. The results are given in Figure 5.5. Experiments on 1 day activated cells were conducted to determine if stimulation of DNA synthesis occurred at lower intensities. It is evident that an exposure of 4 W/cm^2 is the only intensity to produce any substantial deviation from control values.

When lymphocytes were activated for 2 days before ultrasound exposure at intensities of 0 - 4 W/cm^2 for 30 min, significant deviations from control values of (^{3}H) thymidine incorporation occurred (Figure 5.6). As demonstrated previously, an intensity of 4 W/cm^2 produced an immediate significant ($P = 0.001$) depression of ^{3}H-thymidine incorporation. A similar immediate depression occurred on exposure to an intensity of 2.75 W/cm^2, although this population of cells rapidly returned to control levels of (^{3}H) thymidine incorporation.

It is interesting to note from Figure 5.6 that an immediate significant ($P = 0.001$) stimulation in incorporation of (^{3}H) thymidine occurred from exposure to an ultrasound intensity of 1.1 W/cm^2. A similar stimulation, significant at $P = 0.01$, occurred from exposure to an intensity of 0.5 W/cm^2.

Figure 5.5

INCORPORATION OF (^{3}H) THYMIDINE (2 μCi/ml FOR 3h) INTO HUMAN LYMPHOCYTES STIMULATED FOR 1 DAY WITH CONCANAVALIN A THEN EXPOSED TO 870 kHz ULTRASOUND (0-4 W/cm^2 FOR 30 min)

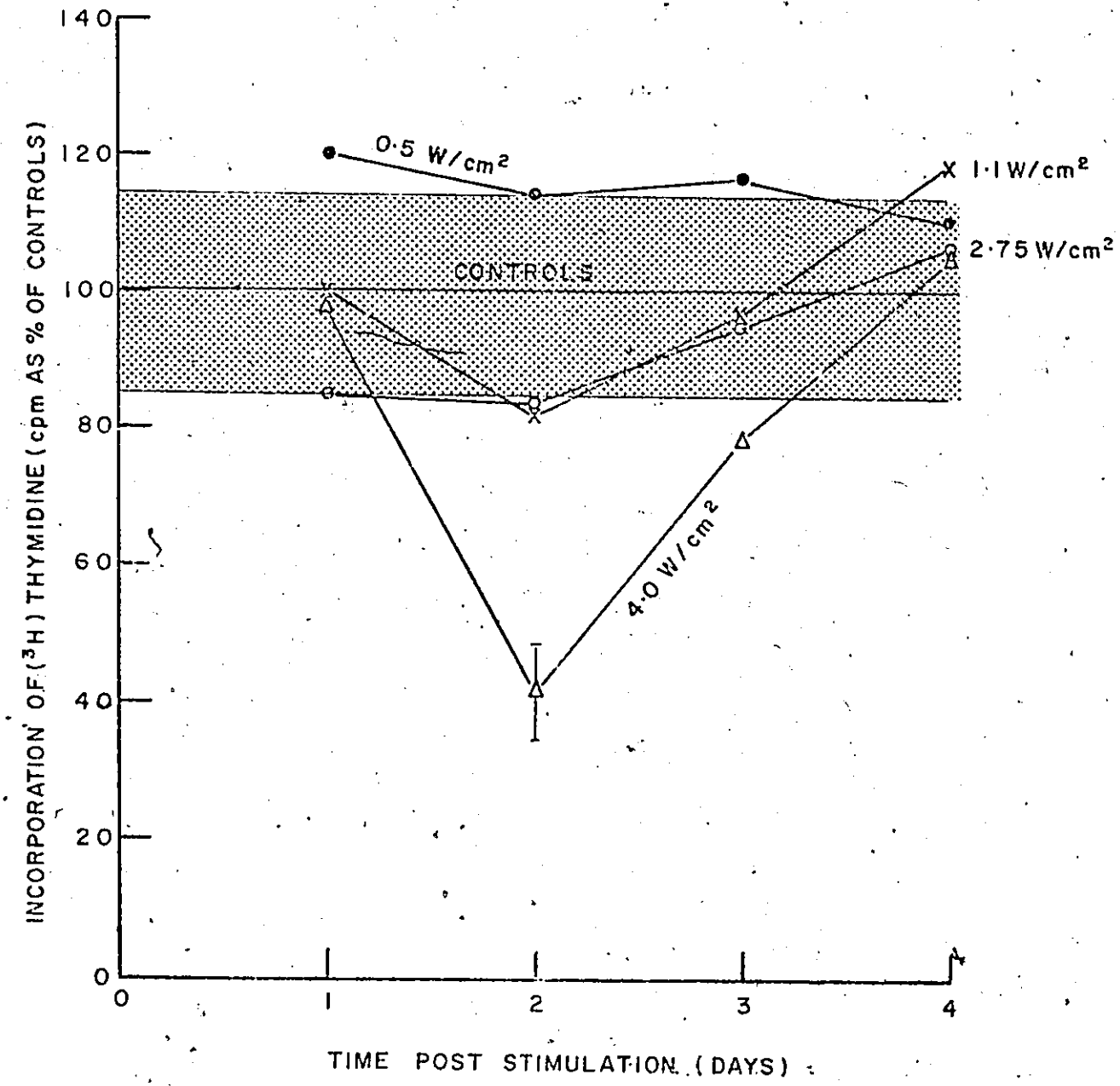
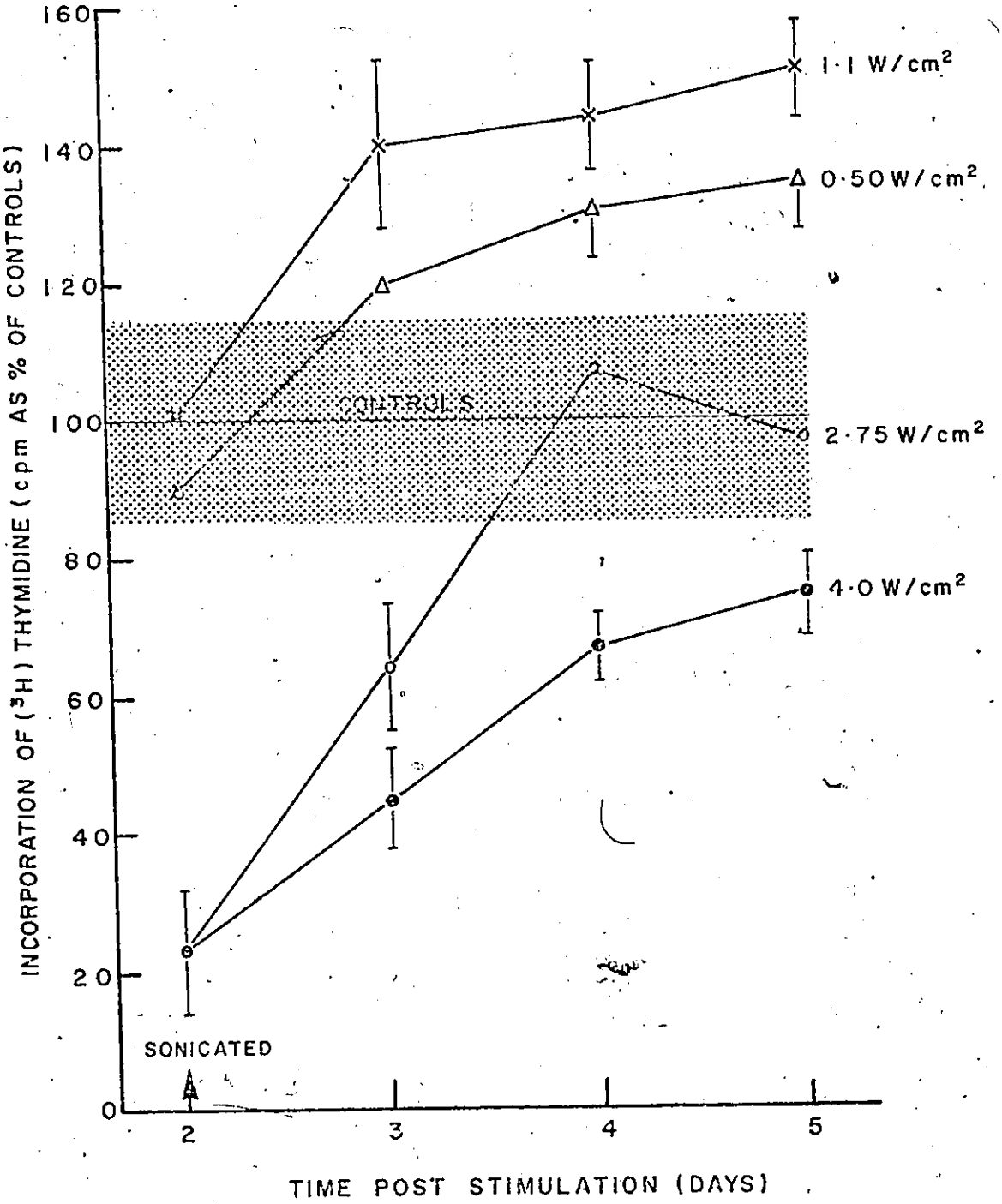


Figure 5.6

INCORPORATION OF (^{3}H) THYMIDINE (2 μCi/ml FOR 3h) INTO HUMAN LYMPHOCYTES STIMULATED FOR 2 DAYS WITH CONCANAVALIN A THEN EXPOSED TO 870 kHz ULTRASOUND (0-4 W/cm^2 FOR 30min)



When lymphocytes were activated for 2 days then the cells removed, the supernatant exposed to 4 W/cm^2 ultrasound and the cells resuspended in the sonicated medium, no deviation from control levels of (^3H) thymidine incorporation was found for up to 4 days after sonication.

Figure 5.6 can be interpreted to mean that the threshold ultrasound intensity to produce an immediate significant depression of (^3H) thymidine incorporation lies between 2.75 and 1.1 W/cm^2. It would also appear that if a reproducible significant initial depression of (^3H) thymidine was to be used as an end-point, an intensity of 4 W/cm^2 should be used with this biological system.

All ultrasound exposures were for 30 min, so it was of interest to determine if the same effects could be produced with shorter exposure times using an intensity of 4 W/cm^2.

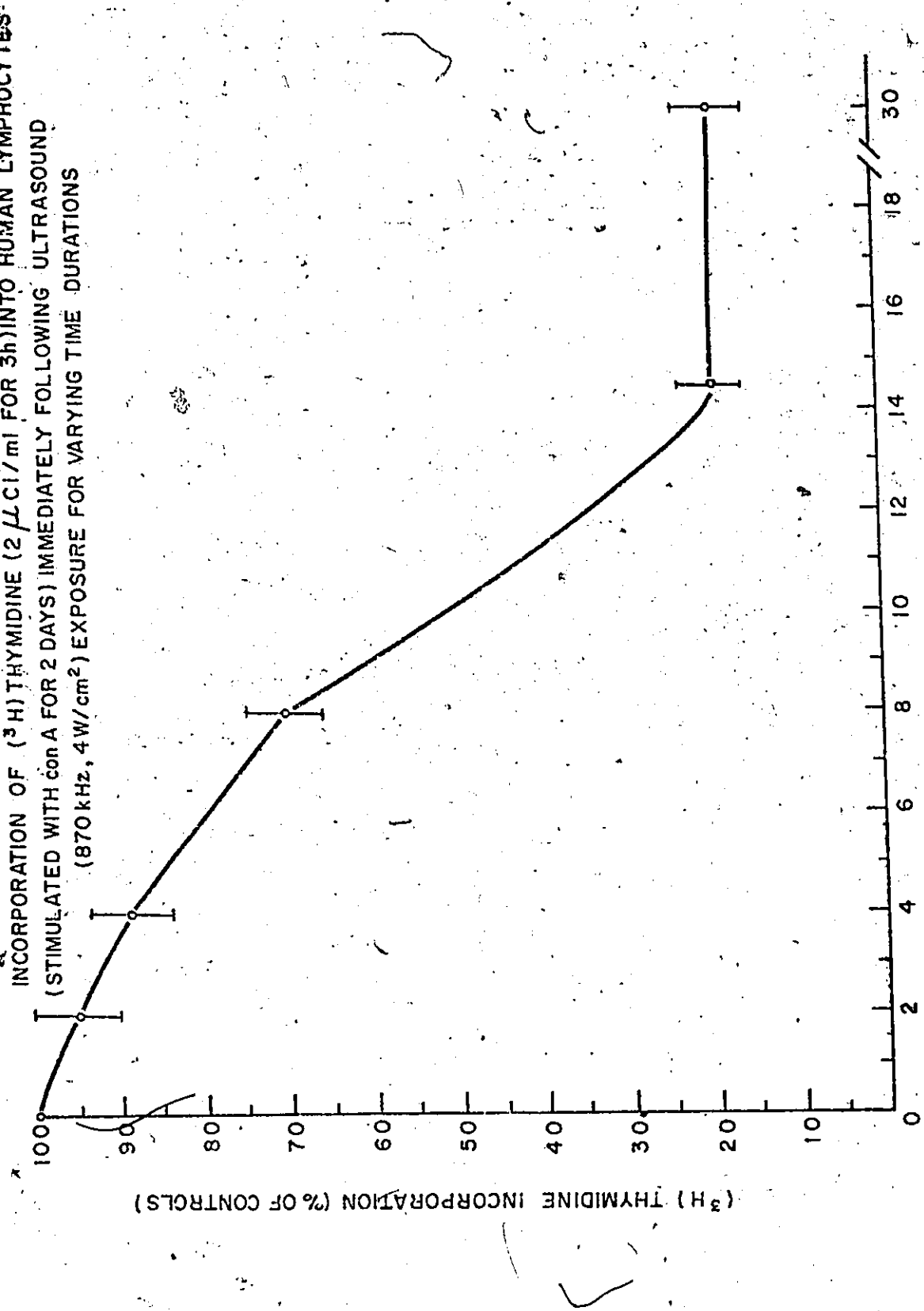
5.2.1.3 Varying Durations of Ultrasound Exposure

With the result from the previous experiments that a very significant inhibition of (^{3}H)-thymidine incorporation occurred in activated lymphocytes following exposure to 4 W/cm^2 for 30 min, it was of interest to determine if this inhibition would occur following shorter exposure durations. Such a result would allow the setting of fixed exposure parameters where a reproducible effect could be used to study the ultrasound mechanisms of action.

Incorporation of ^{3}H-thymidine into lymphocytes activated for 2 days then exposed to ultrasound (4 W/cm^2) for varying time durations is given in Figure 5.7. This time-threshold curve shows that more than 8 min of ultrasound exposure is required to produce the full depression in the incorporating of ^{3}H-thymidine observed after 30 min exposure.

A 15 min exposure to 4 W/cm^2 ultrasound was necessary to produce the maximum depression of ^{3}H-thymidine incorporation into activated cells.

Figure 5.7


INCORPORATION OF (^{3}H) THYMIDINE (2 μCi/ml FOR 3h) INTO HUMAN LYMPHOCYTES
(STIMULATED WITH con A FOR 2 DAYS) IMMEDIATELY FOLLOWING ULTRASOUND
(870 kHz, 4 W/cm^2) EXPOSURE FOR VARYING TIME DURATIONS
(^{3}H) THYMIDINE INCORPORATION (% OF CONTROLS)
100
90
80
70
60
50
40
30
20
10
0
2
4
6
8
10
12
14
16
18
30
DURATION OF ULTRASOUND EXPOSURE (min)

5.2.1.4 Intermission.

From experimental results obtained thus far on changes in the incorporation of (^{3}H) thymidine into activated lymphocytes after exposure to ultrasound the following conclusions can be drawn:

(i) To have any significant effect on (^{3}H) thymidine incorporation, lymphocytes must be stimulated for 2-3 days before exposure to ultrasound.

(ii) Varying the intensity of ultrasound exposure of activated lymphocytes produces quite unexpected results. The most reproducible end-point, depression of incorporation of (^{3}H) thymidine, occurred after an exposure to 4 W/cm^2. The threshold intensity for this end-point occurs between 1.1 and 2.75 W/cm^2, at least for the 30 min sonication time used in these experiments (Figure 5.6). A significant stimulation of (^{3}H) thymidine incorporation occurred in activated cells 3 days after being exposed to ultrasound at an intensity of 1.1 W/cm^2 (Figure 5.6).

(iii) The maximum depression of (^{3}H) thymidine incorporation into lymphocytes activated for 2 days occurred for ultrasound (4 W/cm^2) exposure times between 15 - 30 min.

(iv) A striking effect of the experiments was the immediate depression of incorporation of (^{3}H) thymidine into activated (2 day) lymphocytes exposed to 4 W/cm^2 ultrasound. There was no lag time for this depression to occur.

From the experimental data it can be concluded that a reproducible inhibition of (^{3}H) thymidine incorporation occurs in lymphocytes activated for 2 - 3 days and then exposed to 870 kHz ultrasound at 4 W/cm^2 for 30 min. These exposure conditions were then utilized to investigate how ultrasound was acting within the cell.

To determine what ultrasound was doing to inhibit (^{3}H) thymidine incorporation, a number of approaches had to be considered. It was possible that ultrasound could act at the cell surface to alter the transport of radioactive precursor across the cell membrane. If this were the case, the reduced incorporation of (^{3}H) thymidine could be due to less of the radioactive precursor being available for incorporation into the DNA.

It is well known that absorbed ultrasound enery is converted to heat. Since an intensity of 4 W/cm^2 was found to produce a temperature rise of 5°C (Figure 5.2), this thermal mechanism of ultrasound action needs further investigation (sections 5.2.1.6 and 5.2.1.7).

Ultrasound can also act via mechanisms of cavitation and mechanical stress. The mechanisms of biological action of ultrasound that occur inside the cell are difficult to distinguish. Problems arise through lack of information on the nature of cell contents. For example does the cell contain dissolved gases that could act as cavitation nuclei?

Is the arrangement of intracellular organelles so critical that acoustic streaming could have a significant effect? In an attempt to shed some light on these problems, two approaches were taken: (i) determine whether ultrasound produces the same end-point when the cells are suspended in a degassed medium, (ii) investigate ultrasound induced mechanical stress on the DNA molecules themselves by determining if direct damage occurs and is subsequently repaired.

Finally, could the reduced incorporation of (^{3}H) thymidine be due to an overall ultrasound induced depression of cell function. Such might be the case if ultrasound caused a "shake-up" of the cell and time was needed to either re-organize or repair damage. To investigate this, measurements were made of incorporation of (^{3}H) uridine and (^{3}H) leucine, precursors used for determining effects on RNA and protein synthesis respectively.

In all appropriate experiments, incorporation of (^{3}H) thymidine into lymphocytes activated for 2 - 3 days and then sonicated to 4 W/cm^2 for 30 min, was used as a positive control. If for some reason there was not an immediate significant inhibition of (^{3}H) thymidine incorporation demonstrated, the whole experiment was repeated.

5.2.1.5 Transport of (^{3}H) Thymidine

To determine if the ultrasound-induced inhibition of (^{3}H)-thymidine incorporation was due to a change in the rate of transport of (^{3}H)-thymidine across the membrane, experiments were undertaken to measure the amount of thymidine translocated into cells after 15 s exposure to the isotope. Minimal backflux from the cell occurs provided times of exposure to the isotope is less than 60 s (Strauss et al 1977, Rudd, M.Sc. Thesis, University of Ottawa 1980).

To determine if there was a difference in transport at varying concentrations of thymidine, cells were pulsed with different concentrations of thymidine. The double reciprocal plots (Lineweaver-Burk) presented in Figure 5.8 show that there was no significant difference between the control and sonicated cells in the transport of (^{3}H) thymidine at varying molar concentrations. The regression analysis completed for the data indicated that: for the control plot the slope ± standard error was 0.0059 ± 0.0015 and for the sonicated plot 0.0064 ± 0.0015. The correlation coefficient for fitting the control curve was r = 0.971 and for the sonicated curve r = 0.974. The maximum transport of substrate across the membrane (termed the V max) was found to be approximately 9 p mol/cell/s. The concentration of substrate at $\frac{1}{2}$V max (termed k_m) was found to be approximately 700 μM. Both these values are in good agreement with Strauss et al (1977) and Rudd (M.Sc. Thesis, University of Ottawa 1980).

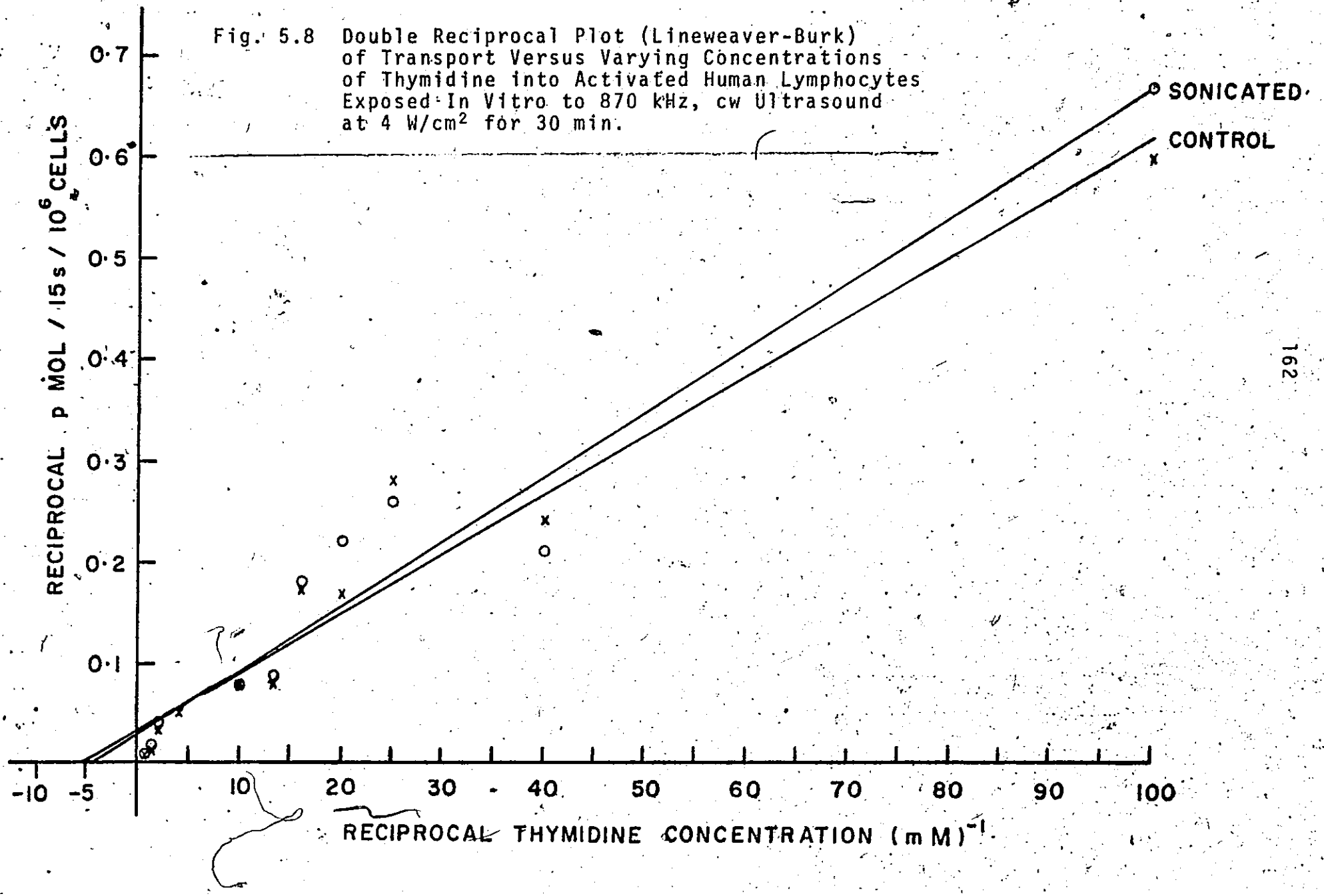


Fig. 5.8 Double Reciprocal Plot (Lineweaver-Burk) of Transport Versus Varying Concentrations of Thymidine into Activated Human Lymphocytes Exposed In Vitro to 870 kHz, cw Ultrasound at 4 W/cm^2 for 30 min.

5.2.1.6 Thermal Effects

Knowing that ultrasound exposure at 4 W/cm^2 for 30 min produced a temperature rise in the cells of 5°C, giving a final temperature of 42°C, one can determine if the ultrasound induced depression in the incorporation of (^{3}H) thymidine were due to heat alone by treating the cells in a 42°C water bath for 30 min.

Experiments were performed that compared the incorporation of (^{3}H) thymidine into lymphocytes stimulated for 2 days then exposed to 870 kHz ultrasound at 4 W/cm^2 for 30 min, with that into cells heated in a constant temperature water bath at 42°C for 30 min; typical results are given in Table 5.2. It is evident that treatment of cells with ultrasound produced a significant ($P = 0.001$) depression of (^{3}H) thymidine incorporation compared to the control levels; by contrast, heating in the 42°C water bath did not reduce the incorporation from the control level.

These data suggest that heating does not appear to be the predominant mechanism of action. However, the possibility that heating may be a contributing mechanism to effects caused by cavitation or acoustic streaming for example is not precluded by these results.

Table 5.2

Incorporation of (^{3}H) thymidine into human lymphocytes stimulated for 2 days then exposed to 870 kHz ultrasound at 4 W/cm^2 for 30 min, or heated in a 42°C water bath for 30 min.

Sample (medium)	*(cpm ± SD) x 10^{-3}
Control	204 ± 16
Sonicated	40 ± 3
Heated to 42°C	202 ± 15

* Counts per minute (cpm) ± standard deviation (SD) of 4 x 1 ml samples.

Experiments to determine if heating plays an auxiliary role in ultrasound action were conducted as follows:

(i) measurements of the temperature rise produced in lymphocytes during exposure to ultrasound were made at an ambient temperature of 30°C,

(ii) with the water bath at 30°C, the rise within the cells was measured to be approximately 4.6°C, giving a final temperature of 34.6°C, well below the physiological temperature of 37°C.

(iii) incorporation of (^{3}H) thymidine was measured in lymphocytes stimulated for 3 days and then exposed to 870 kHz ultrasound at 4 W/cm^2 for 30 min in a water bath at 30 or 37°C. Typical results of these experiments are given in Table 5.3. It is apparent from these data that ultrasound appears to be acting predominantly through some mechanism other than heating.

Table 5.3

Incorporation of ^{3}H (thymidine) into human lymphocytes stimulated 3 days then exposed to 870 kHz ultrasound at 4 W/cm^2 for 30 min in a water bath at 30 or 37°C.

Sample	*(cpm ± SD) x 10^{-3}
Control (37°C)	409 ± 30
Sonicated (37°C)	117 ± 6
Control (30°C)	416 ± 30
Sonicated (30°C)	114 ± 5

*- Counts per minute (cpm) ± standard deviation (SD) of quadruplicate samples.

5.2.1.7 RNA and Protein Synthesis

It was of interest to determine whether ultrasound could effect other and earlier events in blastogenesis than DNA synthesis, such as RNA and protein synthesis. Since DNA synthesis in the cell depends to some extent on previous synthesis of both RNA and protein, experiments conducted to determine the effect on RNA and protein synthesis could shed some further information on how DNA synthesis would be subsequently affected. Experiments were conducted on lymphocytes activated for 1 day since the rate of RNA and protein synthesis was much greater than that of DNA synthesis and so any effect obtained would not be confounded by this latter process.

Results of incorporation of appropriate radioactive precursors (uridine for RNA and leucine for protein) into lymphocytes activated for 1 day with Con A then exposed to 870 kHz ultrasound at 4 W/cm^2 for 30 min is shown in Figure 5.9. Following exposure to ultrasound there is an immediate, significant ($P = 0.01$) depression in the incorporation of (^{3}H) leucine, which remains for another two days after sonication. It is of interest to note that no immediate depression of (^{3}H) uridine incorporation occurs; up to a 24 h was necessary for this phenomenon to be observed. Incorporation of all precursors is suppressed significantly ($P = 0.01$) one day after exposure to ultrasound and only (^{3}H) thymidine incorporation recovers to control levels by the second day after sonication.

A statistically significant ($P = 0.01$) stimulation of DNA synthesis had been observed in activated lymphocytes 3 days after exposure to ultrasound at an intensity of 1.1 W/cm^2 (Figure 5.6). It was of interest therefore to determine if ultrasound at lower intensities produced a stimulatory effect in protein synthesis as had previously been reported by Harvey et al (1975).

The incorporation of (^{3}H) leucine into lymphocytes stimulated for 1 day then exposed to 870 kHz ultrasound (0 - 4 W/cm^2 for 30 min) is shown in Figure 5.10. A significant ($P = 0.01$) stimulation of (^{3}H) leucine incorporation occurred immediately following ultrasound exposure to 1.1 W/cm^2, but this incorporation returned to control levels within a few days. Although Harvey et al (1975) used fibroblasts and slightly different ultrasound exposure parameters, these data are in basic agreement with their results. Similar stimulations in protein synthesis have been reported by Webster et al (1978, 1980).

Figure 5.9

INCORPORATION OF RADIOACTIVE PRECURSORS INTO HUMAN LYMPHOCYTES STIMULATED FOR I DAY WITH CONCANAVALIN A PRIOR TO EXPOSURE TO 870 kHz ULTRASOUND AT 4 W/cm^2 FOR 30 min

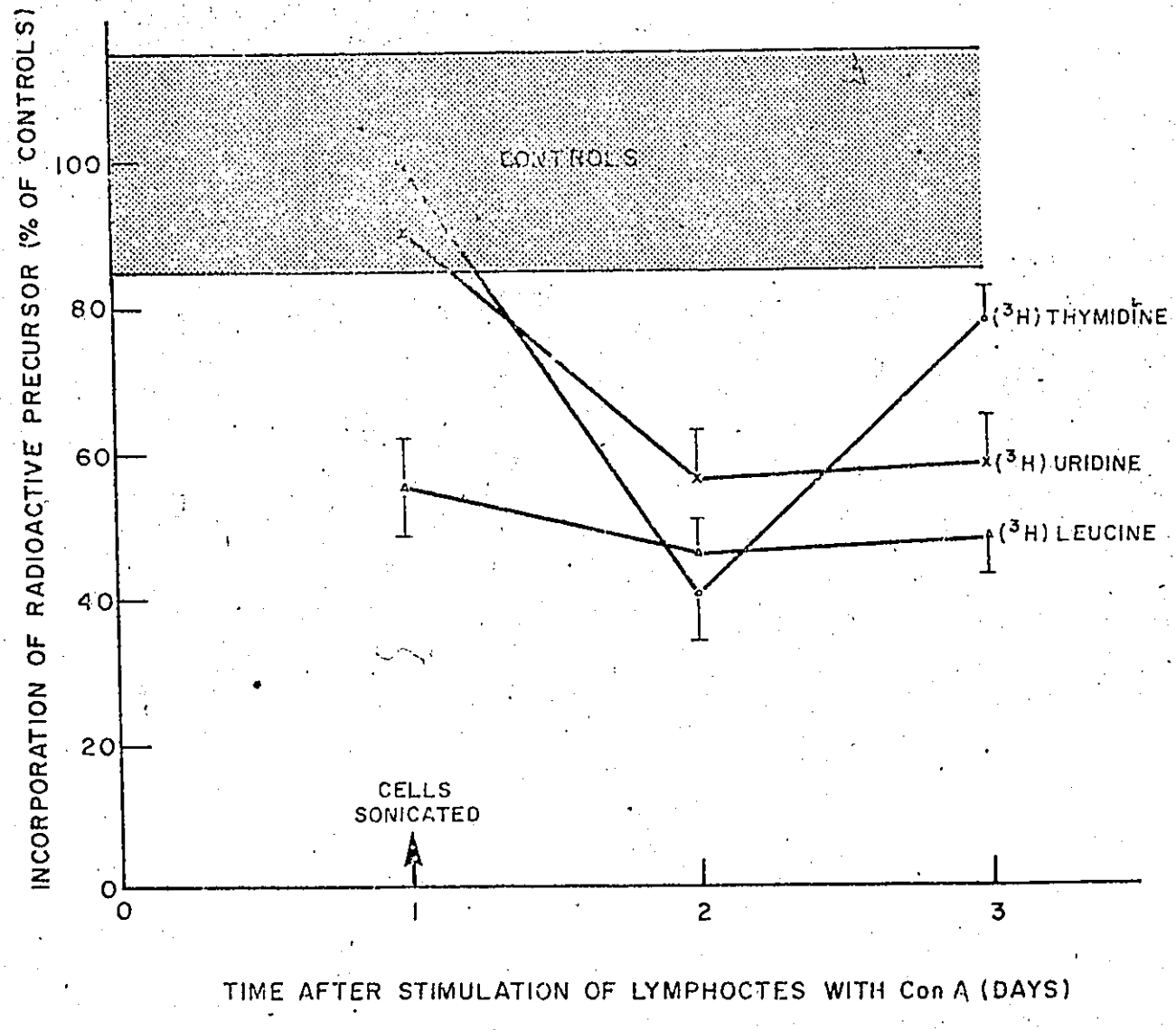
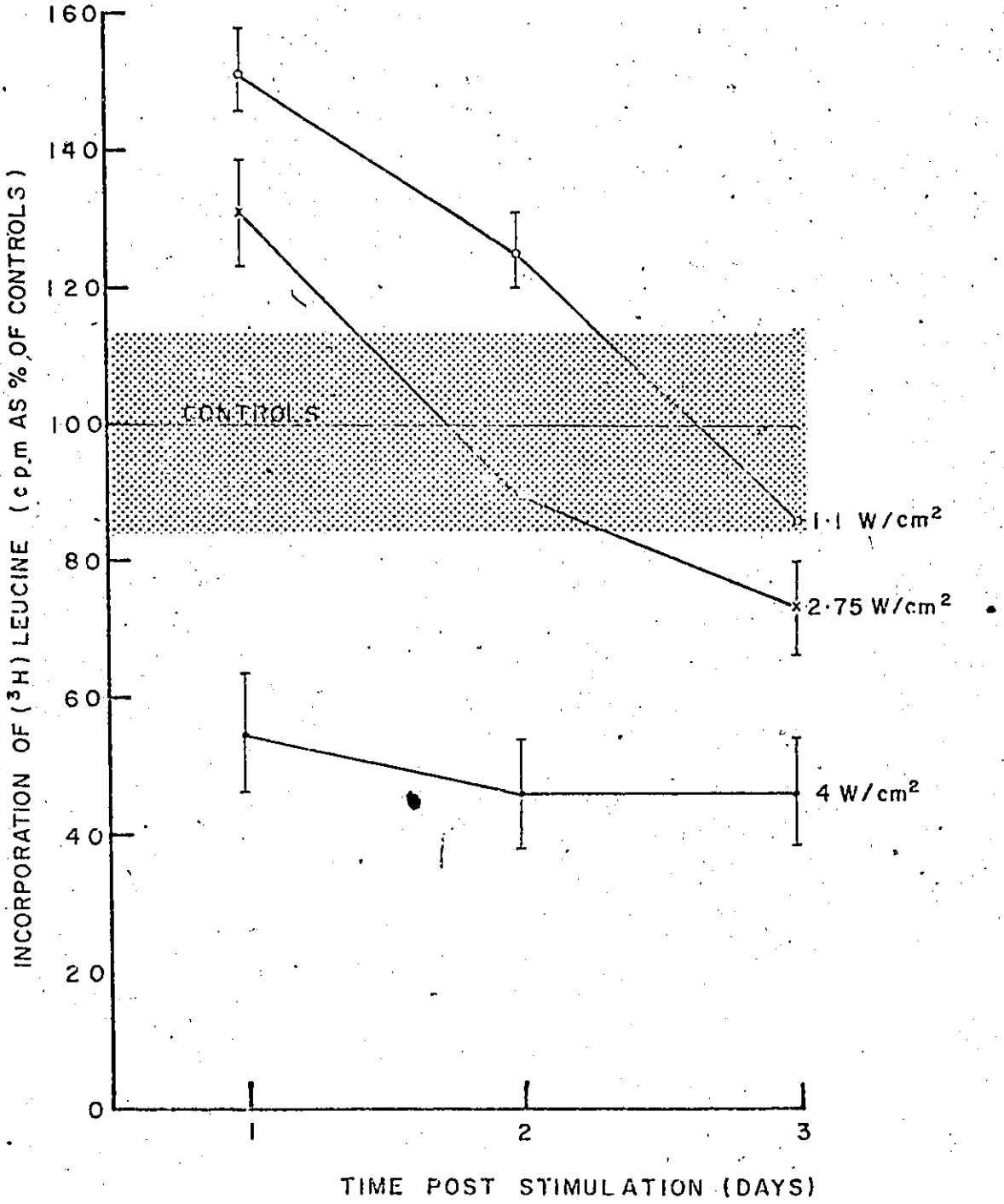


Figure 5.10

INCORPORATION OF (^{3}H) LEUCINE (2 μCi/ml FOR 3h) INTO HUMAN LYMPHOCYTES STIMULATED FOR 1 DAY WITH CONCANAVALIN A THEN EXPOSED TO 870 kHz ULTRASOUND (0-4 W/cm^2 FOR 30min)



5.2.1.8 Unscheduled DNA Synthesis

The immediate inhibition of (^{3}H) thymidine incorporation could be due to the ultrasound causing damage to the DNA, either directly or indirectly. To investigate whether mechanical stresses produced by ultrasound at 4 W/cm^2 for 30 min could cause reparable damage to the DNA of stimulated lymphocytes, an autoradiographic study was undertaken to determine whether unscheduled DNA synthesis occurred. Although this technique is a good indicator that damage to the DNA has occurred, it does not provide any information on the type of lesion that was repaired (Hanawalt et al 1979). Since there is evidence (see section 2.4) that very little repair synthesis takes place in resting cells, the lymphocytes were stimulated for 20 h into G_1 before exposure to ultrasound.

Using conventional autoradiographic techniques, a typical experiment showing the effect of ultrasound and mitomycin C on the DNA of lymphocytes is shown in Figure 5.11. Two other experiments were performed with similar results. The histograms show that in the control population, half the cells had no grains over the nucleus and 90% had 3 or less, with an exponentially shaped decline in the number of grains per cell. Mitomycin C is known to cause damage to the DNA, detectable as unscheduled DNA synthesis (Fujiwara et al 1977, Sasaki 1975) and was thus used as a positive control.* Unscheduled DNA synthesis is seen in the mitomycin C histogram as a second peak around 7 grains per cell.

* Mitomycin C induces low levels of unscheduled DNA synthesis (UDS). Higher levels of UDS may be more desirable for such a control.

Comparison of the histogram of the cells exposed to ultrasound with that for cells incubated with mitomycin C provides an indication that ultrasound could be producing damage to the DNA.

Statistical analysis of these distributions were done using the Kolmogorov-Smirnov two sample test (Siegel 1956). This test compares the histograms as a whole by finding the maximum difference between the two experical distributions and determines whether this difference is significant, given that the population distributions are equal. According to this test, the control and ultrasound histograms were found to be significantly different at the $P = 0.001$ level.

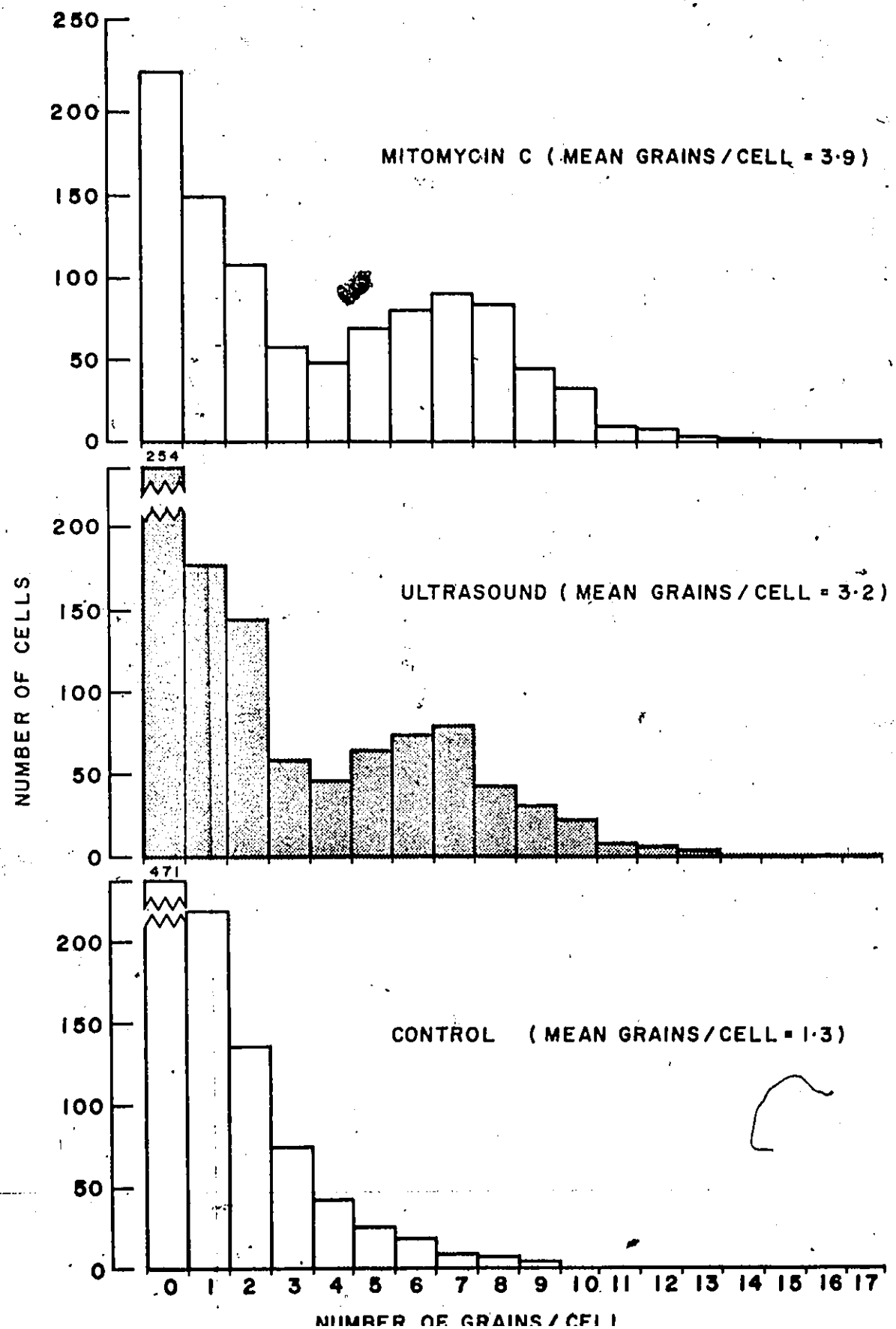
Using the binomial distribution, the standard deviation for the number of cells having 7 grains/cell in the ultrasound histogram is 8.5, giving a 95% confidence interval of 78 ± 17. For the control histogram the standard deviation at the same point is 3.0, giving a 95% confidence interval of 9 ± 6.

Fujiwara et al (1977) have compared the unscheduled DNA synthesis induced by UV and mitomycin C in normal human fibroblast lines. 10 J/m^2 of 254 nm UV exposure caused an average 33.8 grains/nucleus while 3 µg/ml of mitomycin C for 1 h caused 2.10 grains/nucleus. The latter finding is in reasonable agreement with the 2.6 grains/nucleus above control values, found using mitomycin C in the present study. It is not possible to make any direct comparisons of results

since the amount of unscheduled DNA synthesis will depend on a number of different variables such as the cell line, the stage in the cell cycle (Sotomayor et al 1979), time of exposure, type and concentration of the drug, and cell synchrony. Fujiwara et al (1977) used a synchronous population of fibroblasts while in the present a heterogeneous population of lymphocytes was used.

Figure 5.11

DNA REPAIR SYNTHESIS - HISTOGRAMS OF THE NUMBER OF GRAINS/CELL FROM THE UPTAKE OF ^{3}HTdR (10μCi/ml FOR 2h) INTO HUMAN LYMPHOCYTES EXPOSED TO ULTRASOUND (870 kHz, 4 W/cm^2, 30 min) OR MITOMYCIN C (100 ng/ml FOR 2h)



5.2.1.9 Fluorescence Analysis of DNA Unwinding (FADU)

Following the evidence from the autoradiography that ultrasound appeared to produce damage to the DNA, it was necessary to determine whether exposure of human lymphocytes to ultrasound at 4 W/cm^2 would produce damage in the DNA. For this the FADU technique developed by Birnboim and Javcek (personal communication) was used. The technique involves the post-treatment labelling of double stranded DNA by a fluorescing dye (ethydium bromide). The ethydium bromide dye intercalates only between the double strands of the DNA. Thus the measure of fluorscence from the sample relates directly to the degree of double strandedness of the DNA. Relative DNA damage is determined by an index (D) relating the degree of partially (P) unwound DNA with both the background (B = fully unwound) DNA and the completely double stranded (T) DNA samples. This index given by

$$D = \left(\frac{P - B}{T - B} \times 100\right) \%$$

is determined from the fluorescence given off by the P, B and T samples of each of the control and treated cells. The degree of damage to the DNA of the treated cells is given by ΔD, where

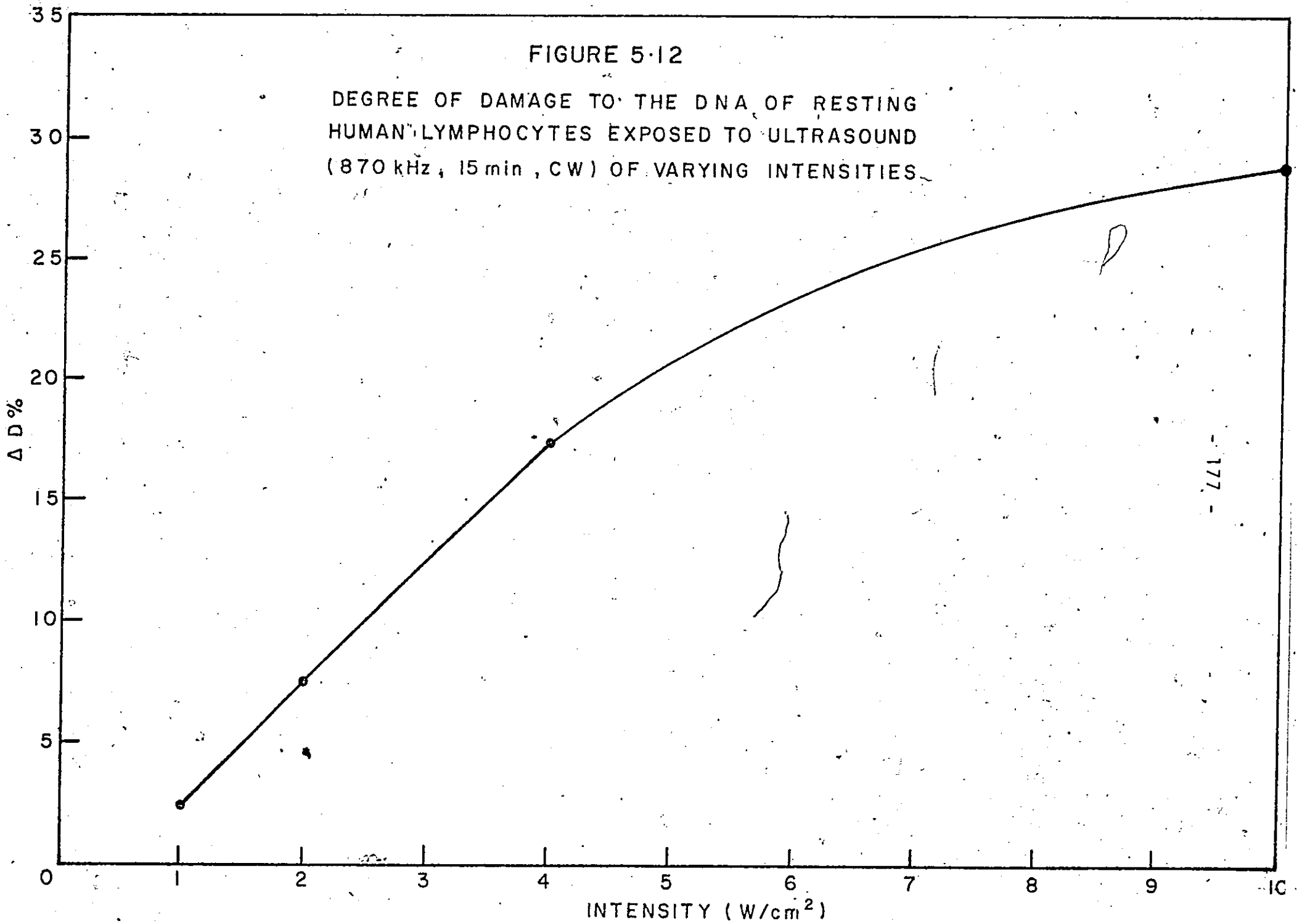
$$\Delta D = {}^{D}\text{control} - {}^{D}\text{treated}$$

Figure 5.12 gives the dose-response curve of ΔD for cells exposed to varying intensities of ultrasound for 15 min. The points on this graph are the result of three separate experiments.

Preliminary "repair" experiments indicated that cells exposed to 10 W/cm^2 for 15 min initially had a $\Delta D \doteq 28.44\%$, but after 30 min incubation at 37°C had a $\Delta D = 7.8\%$, implicating substantial repair to the DNA in this time period.

FIGURE 5·12

DEGREE OF DAMAGE TO THE DNA OF RESTING HUMAN LYMPHOCYTES EXPOSED TO ULTRASOUND (870 kHz, 15 min, CW) OF VARYING INTENSITIES



5.2.1.10 Suspension of Lymphocytes in Degassed Media

The primary objective of this work was to determine the predominant mechanisms of action of ultrasound on cells. From previous experiments, it appeared that heating was not the predominant mechanism of ultrasound action played some role, it would have been ideal if some facility was available to place the lymphocytes under a few atmospheres of pressure during sonication. This has been shown to be a very effective means of reducing cavitation (see Section 3.2). Another effective method of reducing cavitation would have been to utilize an ultrasound beam having pulses of short duration. However, the use of pulsed beams would not be compatible with the purpose of the experiment, which was to determine the mechanism of action of a continuous wave beam.

One can greatly reduce cavitation in the medium at the cell surface by performing sonications in degassed media. However, there is the possibility that gas bubbles or cavitation nuclei may exist or be formed within the cytoplasm. Although there is presently no evidence of this, the possibility cannot be excluded.

To ensure that the medium used to suspend cells was degassed, its O_2 content was determined. Using a YSI model 54ARC oxygen meter, the ppm value was determined on a weight basis. Thus a reading of 10 ppm of O_2 would indicate that 10 mg of O_2 was dissolved in 1000 g of water.

The oxygen content of RPMI 1640 medium placed in a vacuum oven overnight was found to be 4.7 ppm. By contrast, autoclaved PBS was found to have an oxygen content of approximately 0.6 ppm (the limit of accuracy of the meter).

The dissolved oxygen content of water at 37°C at equilibrium with the atmosphere is 6.8 ppm. Thus it is apparent that RPMI 1640 medium is only partially degassed. However the autoclaved PBS had an order of magnitude less oxygen. If cavitation played a role, one would expect that the differing degrees of dissolved oxygen in the medium would give rise to correspondingly different effects.

The results of experiments on the incorporation of (^{3}H) thymidine into lymphocytes stimulated for 2 days then suspended in either culture medium or degassed phosphate buffered saline and exposed to 870 kHz ultrasound at 4 W/cm^2 for 30 min is typified by those shown in Table 5.4. A statistically significant ($P = 0.001$) ultrasound-induced depression of (^{3}H)-thymidine incorporation in cells occurred even if the cells were suspended in degassed PBS. What is important from these results is the fact that the reduction in incorporation of (^{3}H)-thymidine caused by sonication of cells suspended in either degassed or aerated medium, is identical.

Similarly ultrasound-induced depression of (^{3}H)-thymidine incorporation into 3 day stimulated lymphocytes occurred to the same degree even if the culturing medium was partially degassed, as shown in Table 5.5. Thus the possibility that ultrasound-induced cavitation in the medium caused inhibitory effects at the cell surface has been reduced by these data.

Table 5.4

Incorporation of (^{3}H) thymidine into human lymphocytes stimulated for 2 days then exposed to 870 kHz ultrasound at 4 W/cm^2 for 30 min while suspended in culture medium or degassed PBS.

Sample (medium)	**(cpm ± SD) x 10^{-3}
Control (RPMI 1640 medium)	204 ± 16
Sonicated (RPMI 1640 medium)	40 ± 3
Control (degassed †PBS)	238 ± 26
Sonicated (degassed PBS)	29 ± 5

** Counts per minute (cpm) ± standard deviation (SD) of quadruplicate samples.

† Phosphate buffered saline (pH 7.2).

Table 5.5

Incorporation of (^{3}H) thymidine into human lymphocytes stimulated 3 days, then exposed to 870 kHz ultrasound at 4 W/cm^2 for 30 min while suspended in various media.

Sample (medium)	**(cpm ± SD) x 10^{-3}
Control (1640 RPMI)	176 ± 9
Sonicated (1640 RPMI)	47 ± 3*
Control (degassed 1640 RPMI)	169 ± 10
Sonicated (degassed 1640 RPMI)	42 ± 4*
Control (normal PBS)	183 ± 11
Sonicated (normal PBS)	52 ± 10*
Control (degassed PBS)	164 ± 7
Sonicated (degassed PBS)	44 ± 4*

* These values are not significantly different at the P = 0.1 level.

** Counts per minute (cpm) are the means ± standard deviation (SD) of 4 x 1 ml samples.

5.3 Effects At The Cell Surface

Experimental data presented so far have indicated that ultrasound at intensities up to 4 W/cm^2 acts primarily via some mechanism other than heat and may directly damage macromolecules. As described in Section 3.3, a number of researchers have reported effects of ultrasound at the cell surface. With the facility available to eliminate cavitation, suspension of cells in a degassed medium, an end-point showing the action of ultrasound at the cell surface could shed more light on predominant mechanisms.

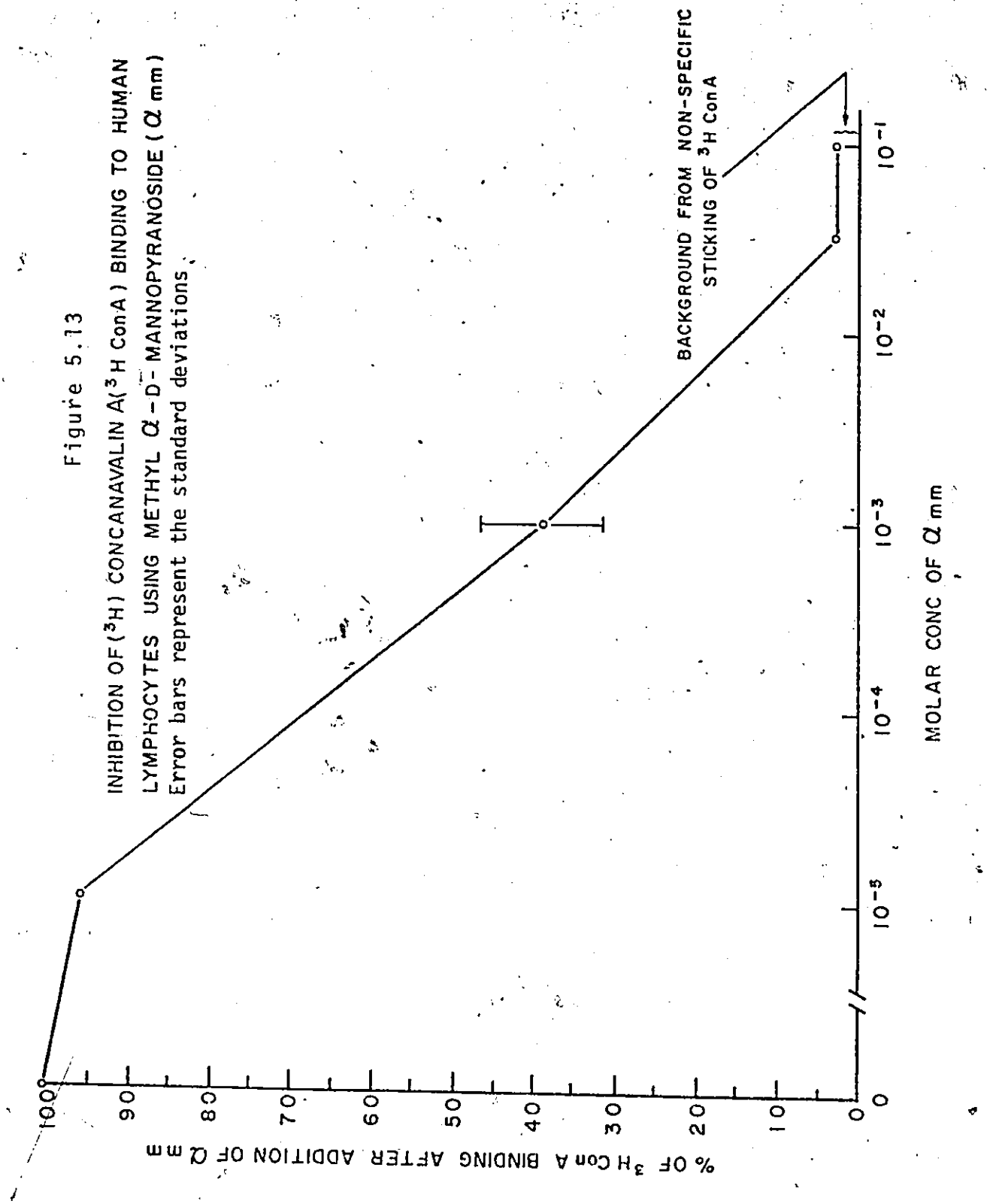
Some early experiments were conducted to determine if ultrasound altered the flux of $^{86}Rb^+$ (a co-gener of K^+) in activated lymphocytes. Although many experiments were performed, using the technique described by Hamilton and Kaplan (1977), and their results basically confirmed, ultrasound did not alter $^{86}Rb^+$ flux. Studies of immuno-fluorescent-labelling of lymphocytes after sonication, using a technique modified from that described by Sheldon et al (1973), gave inconclusive results and was considered to be a poorly reproducible end-point for a study on ultrasound mechanisms. Finally experiments were undertaken to determine if ultrasound would alter the binding of (^3H) Con A to the cell surface. Experimentation on a "surface" phenomenon would provide further evidence on whether cavitation was a predominant mechanism since cavitation most probably acted at the cell surface. Reduction of cavitation by suspending cells in a degassed medium would then give further information in its importance relative to acoustic streaming.

5.3.1 (^{3}H) Concanavalian A Binding

Control experiments were performed to determine the "background" counts from non-specific sticking of (^{3}H) Con A to dead cells, the glass fibre filters and the manifold wells. To quantitate this background methyl-α-D-mannopyranoside (αmm) was used to inhibit the normal binding of Con A to the cell surface. Figure 5.13 shows that the binding of Con A to the cell surface can be inhibited by 0.1 M mm. Further, the background counts from non-specific sticking of Con A is less than 5% of the total counts.

Figure 5.13

INHIBITION OF (^{3}H) CONCANAVALIN A(^{3}H ConA) BINDING TO HUMAN LYMPHOCYTES USING METHYL α-D-MANNOPYRANOSIDE (α mm)
Error bars represent the standard deviations.



5.3.1.1 Time Course of (^{3}H) Con A Binding

To investigate the kinetics of Con A binding to the lymphocyte cell surface, 1 μCi/ml of (^{3}H) Con A was incubated with lymphocytes for varying periods of time. The results are shown in Figure 5.14. From these data it appears that the binding of Con A to the lymphocyte surface proceeds normally for some 20 min, there not being a significant difference up to that time between the controls and the cells exposed to 870 kHz ultrasound at 4 W/cm^2 for 30 min. After 35 min incubation, the binding of (^{3}H) Con A was found to be significantly different ($P = 0.01$ using modified t-test between corresponding points) between these two populations.

This result was found to be a reproducible provided care was taken to ensure that the fibre glass filters were properly treated with 1% bovine serum albumen.

To determine whether the binding of Con A was compatible with other results in the literature, the number of binding sites on the cell surface was calculated as described in Section 4.3.1. As can be seen from Table 5.6, there is some "scatter" in the exact value, but the number of binding sites reported by others appears to be between 10^6 - 10^7/cell. In the present study 2×10^6 binding sites for Con A were found - in reasonable agreement with the most recent literature.

Figure 5.14

TIME COURSE OF ^{3}H con A BINDING TO HUMAN PERIPHERAL LYMPHOCYTES FOLLOW EXPOSURE TO 870 kHz ULTRASOUND AT 4W/cm^2 FOR 30 min. INCUBATION OF LYMPHOCYTES WITH ^{3}H con A WAS AT A TEMPERATURE OF 37°C

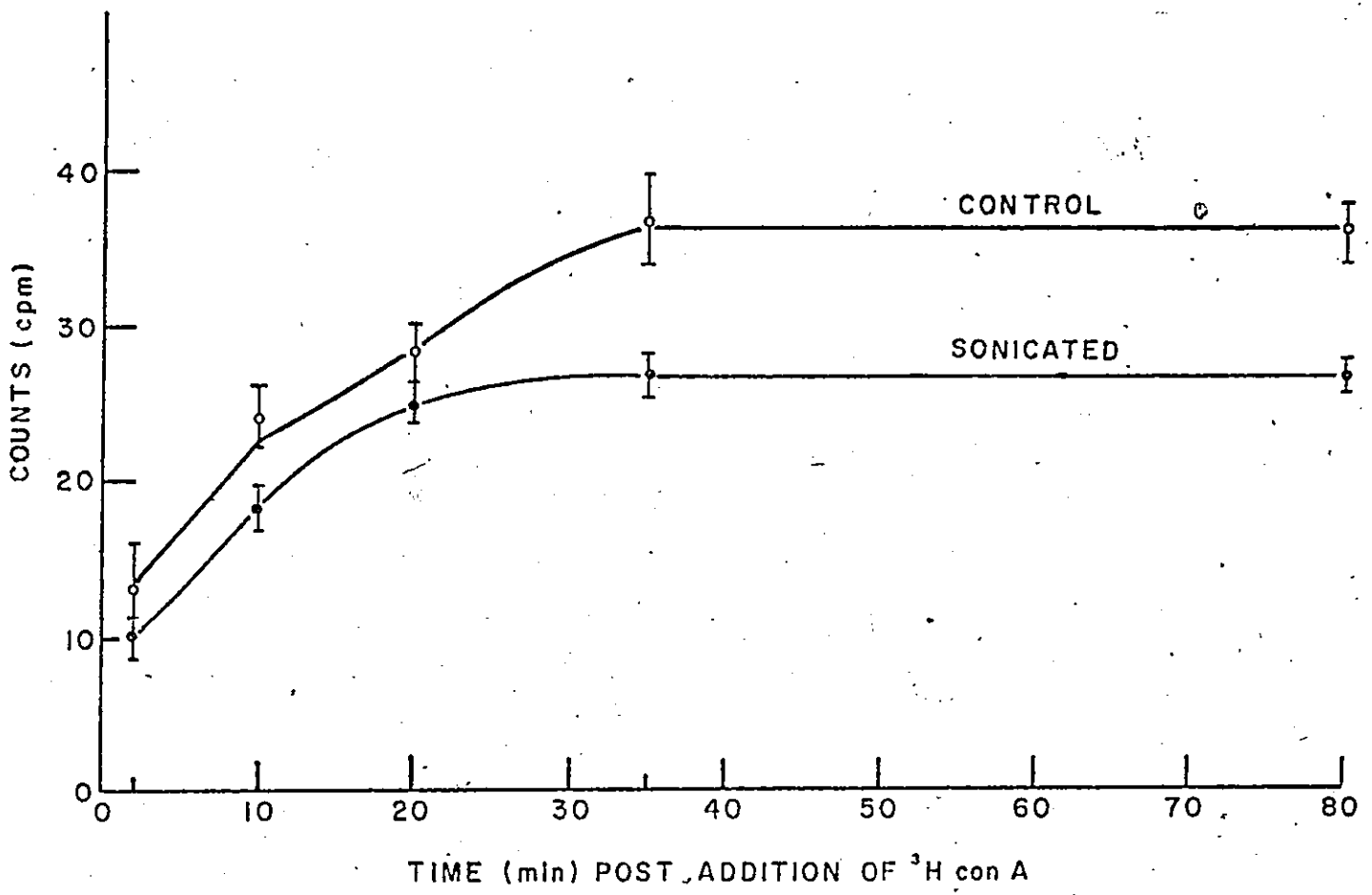


Table 5.6

Number of Con A binding sites on human lymphocytes - sample of values from the literature.

No. of Con A Binding Sites	Comments	Reference
$0.96 - 2.7 \times 10^6$	Assumed $MW_{Con\ A} = 55{,}000$ Studied two sub-populations of lymphocytes.	Boldt et al (1972)
1.02×10^6	Used both ^{131}I Con A and ^{3}H Con A in the determination.	Wands et al (1976)
1.7×10^6	Found that B and T lymphocytes have the same number of binding sites.	Boldt et al (1975)
$(1.8 \pm 0.2) \times 10^7$	Studies both transformed and untransformed lymphocytes.	Krug et al (1973)
$(2.0 \pm 0.4) \times 10^6$		Present study

5.3.1.2 Effect of Heating on (^{3}H) Con A Binding

To evaluate the possibility that ultrasound-induced heating was responsible for the reduction in (^{3}H) Con A binding, two approaches were taken. It was known that an ultrasound intensity of 4 W/cm^2 produced a temperature rise of 5°C in the cells, giving a final equilibrium temperature of 42°C. Thus experiments were performed where the cells were left in a 42.5°C water bath for 30 min. The results are given in Table 5.7. It is apparent that the increased temperature was not the predominant mechanism of ultrasound action to reduce (^{3}H) Con A binding.

A second approach was taken to determine if ultrasound-induced heating was a possible auxilliary mechanism. Exposing lymphocytes to ultrasound in a water bath at 30°C would result in a temperature rise within the cells of 4.6°C, short of their normal 37°C. Experimental results given in Table 5.7 indicate that ultrasound was just as effective at the lower temperature. Thus heating does not appear to be responsible for the ultrasound-induced reduction in the binding of (^{3}H) Con A.

Table 5.7

Concanavalin A binding to human lymphocytes exposed in water baths at 30 or 37°C to 870 kHz ultrasound (cw, 4 W/cm^2, 30 min), or heated in a water bath at 42.5°C, then incubated at 37°C for 45 min with (^{3}H) concanavalin A (1 μCi/ml).

Sample (water bath temp.)	*(cpm ± SD) x 10^{-3}
Control (37°C)	35 ± 2
Sonicated (37°C)	21 ± 1
Control (30°C)	33 ± 3
Sonicated (30°C)	20 ± 2
Heated (42.5°C)	35 ± 3

* counts per minute (cpm) are the means ± standard deviation (SD) of quadruplicate samples.

5.3.1.3 Effect of Cavitation

Ultrasound produces cavitation in a medium provided dissolved gases are present to form cavitation nuclei. This cavitation may be effectively reduced at the cell surface if the lymphoctyes are washed twice in degassed PBS and then resuspended in degassed PBS before exposure to ultrasound. The disc used to contain the lymphocytes had been specially designed to allow the cultures to be contained during sonication without air bubbles. Air bubbles were not visible when the disc of cells was carefully observed under a microscope.

The results of Con A binding to lymphocytes exposed to 870 kHz ultrasound (cw, 4 W/cm^2, 30 min) while suspended in normal or degassed PBS, then incubated at 37°C for 45 min with (^{3}H) concanavalin A (1 μCi/ml), are presented in Table 5.8. The same order of reduction in Con A binding, significant at the P = 0.01 level, is produced by ultrasound whether the cells are suspended in normal or degassed PBS. These data would indicate that cavitation may not be the dominant mechanism by which ultrasound is producing its effect at the cell surface.

Table 5.8

Concanavalin A binding to human lymphocytes exposed in a 37°C water bath, to 870 kHz ultrasound (cw, 4 W/cm^2, 30 min) while suspended in normal or degassed phosphate buffered saline (PBS) then incubated at 37°C for 45 min with (^{3}H) concanavalin A (1 μCi/ml).

Sample (medium)	**(cpm ± SD) x 10^{-3}
Control (normal PBS)	33 ± 3
Sonicated (normal PBS)	21 ± 2*
Control (degassed PBS)	32 ± 2
Sonicated (degassed PBS)	19 ± 1*

* These values are not significantly different at the P = 0.1 level.

** Counts per minute (cpm) are the means ± standard deviation (SD) of quadruplicate samples.

5.3.2 Mixed Lymphocyte Reaction (MLR)

The MLR is used clinically for the screening of compatible donors for transplantation. In essence there is a good correlation between graft rejection and blastogenic response in the MLR.

The MLR is a histocompatibility test that relies on interactions or recognition of sites between cells at the plasma membrane. Since evidence has already been produced that ultrasound affects Con A binding at the cell surface, it seemed that further information regarding the action of ultrasound might be obtained by investigating its effect on the MLR. Further, the MLR provides a unique test system that could provide information on the effect of ultrasound on the cell surface relative to that on the internal metabolism of the cell, since the MLR involves not only interactions at the surface but requires a living and metabolically active stimulating cell. In all the experiments, the same human donors were used for stimulator and responder cells.

The effect of exposing responder cells to ultrasound (870 kHz, cw, 4 W/cm^2, for 30 min) is given in Table 5.9. From this table it is evident that a high level of (^{3}H) thymidine incorporation was obtained from the control (AB_M). However, when the responder cells were exposed to ultrasound a significant ($P = 0.001$) reduction in this (^{3}H) thymidine incorporation occurs.

Clinically, a stimulation index is used to measure the degree of stimulation between the cell populations. Inserting the data obtained in these experiments, into the equation for the stimulation index (SI) given in Section 4.5, the SI values were found to be:

Table 5.9

Mixed lymphocyte reaction - effect of exposing the responder cells to 870 kHz ultrasound at 4 W/cm^2 for 30 min.

Sample*	†(cpm ± SD) X 10^{-2}
AB_M	201 ± 10
A_UB_M	18 ± 1
AA_M	28 ± 2
A_UA_M	5.2 ± 0.3
A_M	1.7 ± 0.2
B_M	1.4 ± 0.1

† counts per minute (cpm) ± standard deviation (SD) of quadruplicate samples

* A = responder cells

B = stimulator cells

M = treated with mitomycin C

U = exposed to ultrasound

SI = 7.6 for the control cells

SI = 4.9 for the sonicated cells

A donor would be considered for transplant if the SI was less than 5.

To investigate how ultrasound effected the MLR, experiments were performed where both the stimulator and responder cells were exposed to 870 kHz ultrasound at 4 W/cm^2 for 30 min. The results are presented in Table 5.10. As shown in the previous experiments (Table 5.9) a reduction in the incorporation of (^3H) thymidine occurred when the responder cells are exposed to ultrasound. When both the responder and stimulator cells are exposed to ultrasound, the incorporation of (^3H) thymidine was found to be lower than when just the responder cells were exposed to ultrasound.

Table 5.10

Mixed lymphocyte reaction - effect of exposing both the stimulator and responder cells to ultrasound (870 kHz, cw, 4 W/cm^2, 30 min).

Sample*	**(mean cpm ± SD) X 10^{-3}
AB_M	76 ± 5
AA_M	20 ± 2
AB_{MU}	28 ± 3
AA_{MU}	2.7 ± 0.4
A_UB_M	2.7 ± 0.2
A_UB_{MU}	1.5 ± 0.3

* A = responder cells
B = stimulator cells
M = treated with mitomycin C
U = exposed to ultrasound

** mean of quadruplicate samples ± standard deviation.

Chapter 6 Mechanisms of Action

This chapter presents a discussion of possible theoretical models of the experimental arrangement. These models are used to estimate the magnitude of the forces and stresses produced by ultrasound on the cell surface and within the cell. Experimental results are then compared with the stresses predicted by these models.

In summary, the theory predicts a temperature rise in the cell cultures that corresponds to the measured values. The experimental results suggest that a thermal mechanism alone could not account for the effects observed. Evidence from experiments indicates that cavitation may not be the primary mechanism of ultrasound action, pointing to a possible non-thermal non-cavitational mechanism. However, the present theory predicts that the forces and stresses produced by non-cavitating ultrasound may not be strong enough to directly break macromolecular bonds. Non-cavitating ultrasound may produce forces strong enough to cause, for example, conformational changes in the DNA, and to distort the shape of binding sites on the cell surface. However, since theoretical models that would more closely account for the complex structure and shape of DNA have not been developed, it is not possible to determine precisely the magnitude of ultrasound forces or state unequivocally that a combination of the various stresses due to non-cavitational streaming and twisting could not cause breaks in the DNA. Chapter 7 attempts to explain this further.

6.1 Thermal Mechanism

Experimental apparatus and procedures were designed to evaluate the mechanisms of action of ultrasound in producing the effects observed both within and on the cell surface. Heating produced in cells exposed to ultrasound was evaluated in two ways:

(i) heating activated cells for 30 minutes in a water bath at the maximum temperature (42°C) produced by ultrasound, and

(ii) exposing the cells to ultrasound in a water bath at a temperature of 30°C, so that with the heating produced by the exposure, the final temperature was still less than 37°C.

The experimental results suggest that ultrasound-induced heating did not directly cause the intracellular or cell surface effects observed. However, let us determine if the experimental results seem valid on theoretical grounds.

Figure 5.2 shows that the temperature rise in cell suspensions exposed to 4 W/cm^2 was 5°C and an equilibrium temperature of 42°C was attained in some 6 - 7 min. Equation 3.1 in Section 3.2.1.1 gives the expected rate of rise of temperature in a medium, provided no heat conduction occurs. Thus one can determine $\frac{dT}{dt}$ as the initial slope of the 4 W/cm^2 curve from Figure 5.2. The value was found to be 0.03°C/s. The absorption coefficient α for the medium containing the cells can be found using equation 3.1.

$$\frac{dT}{dt} = \frac{2\alpha I}{\rho C_M}$$

For $\frac{dT}{dt} = 0.03^{\circ}C/s$, $I = 4\ W/cm^2$, $\rho = 1\ g/ml$ and $C_M = 4.2\ J/g/^{\circ}C$ then $\alpha \simeq 0.015\ Np/cm$

Since heat conduction occurs, then considering the container of cells to be a sphere, the rise in temperature is given by equation 3.2.

$$T_c - T_o = \frac{\alpha I R^2}{K}$$

If $I = 4\ W/cm^2$, $\alpha = 0.015\ Np/cm$, $K = 0.006\ W/cm^{\circ}C$ and the radius of the exposure disc $R = 0.75$ cm, then

$$T_c - T_o \simeq 5.6\ C.$$

This value is in reasonable agreement with the measured temperature rise of $5^{\circ}C$. A lower temperature rise might be expected in the cells because ultrasound exposures were carried out in the near field where the intensity (I) fluctuates to a much greater extent than in the far field. Further the spherical model used to determine the temperature rise would not be exactly applicable to the cylindrical shape of the cell container.

The time required to reach the quilibrium temperature is given by equation 3.3

$$t = \rho\ C_M\ R^2/K\ .$$

Inserting the values given above, one obtains

$$t = 394\ s \text{ or } 6.5\ min\ .$$

This value is in good agreement with the measured result.

One can conclude from the above analysis that the measured values of equilibrium temperature and the time to reach this temperature are in reasonable agreement with theory.

In Section 3.2.1.2., it was calculated that only a very small temperature rise occurs at the cell surface and could not account for the results observed. Both the experimental results and theoretical predictions provide good evidence that a thermal mechanism is not predominant. This conclusion is in agreement with the heat sensitivity experiments conducted on human lymphocytes by Schrek (1966) and the ultrasound-induced heating effects found by Li et al (1977) and predicted by Lele (1975).

6.2 Stress at the Cell Surface

The exposure of cells to ultrasound while suspended in an aerated tissue culture medium at ambient pressure is conducive to cavitation, acoustic microstreaming and membrane vibration (Coakley and Dunn 1972). Shear and microstreaming forces produced by ultrasound are large near the boundaries of cavities (Nyborg 1965). In the present studies evidence was produced that ultrasound was indeed causing mechanical stresses (as described in Section 3.2) inside and at the surface of lymphocytes.

Evidence of ultrasound action at the cell surface was observed by the reduction in cell electrophoretic mobility as reported by Repacholi (1970) and Repacholi et al (1971). The temporal average, spatial average intensity of the ultrasound to produce this effect was 1 W/cm^2. Taylor and Newman (1972) confirmed this reduction in electrophoretic mobility and produced evidence that the mechanism of action was not cavitation. Joshi et al (1973) also confirmed the effect, but suggested that the reduced electrophoretic mobility was due to cavitation.

In the present studies, ultrasound at 4 W/cm^2 produced an apparent reduction in the number of binding sites for Con A on the cell surface. Experiments were performed using degassed media in an attempt to determine if ultrasound-induced cavitation played a predominant role in producing

these effects. The results suggested that cavitation may not be predominant. However, it was difficult to determine if cavitation was either suppressed or reduced, since at a frequency of 870 kHz, the size of bubble which would be resonant has a radius of approximately 4 μm (Nyborg 1977). Although every effort was made to ensure there were no gas bubbles in the degassed medium, only a very small amount of gas would be required to produce cavitation. Before discussing the role of cavitation further, let us consider if the ultrasound beam used in the present studies produced streaming forces strong enough to cause damage.

Cyclic stresses are set up in a cell suspension since the cells have a density greater than the medium and fail to oscillate freely in the ultrasound field. Under the ultrasonically-forced vibrations, there is a relative movement between the cells and the suspending medium, and the resultant force experienced by the cell is given by Stokes viscous drag formula,

$$F = 6\pi N r V_r$$

where N is the viscosity of the suspending medium, r is the radius of the cell (assumed spherical) and V_r the relative velocity between the cell and the suspending fluid. If ρ_f = density of the fluid, ρ_c = density of the cell, the relative velocity is given by

$$V_r = U \sin \omega t \left(\frac{\rho_c - \rho_f}{\rho_c + \rho_f} \right)$$

Connolly and Pond (1967) quote the velocity amplitude as $U = 11.5\ I^{\frac{1}{2}}$ cm/s, where I = intensity of beam (W/cm^2).

Thus $$V_r = 11.5\, I^{\frac{1}{2}} \sin \omega t \left(\frac{\rho_c - \rho_f}{\rho_c + \rho_f} \right)$$

For $\rho_f = 1.0$, $\rho_c = 1.1$ g/cm^3 and $I = 4$ W/cm^2.

$$V_r = 23 \sin \omega t \frac{1}{21}$$

The resultant viscous drag on the cell moving through the suspending fluid is

$$F = 6\pi N r \frac{23}{21} \sin \omega t \text{ dyn}$$

for each cycle of the wave.

Let N = 0.02 poise (for water N = 0.01) and the radius of the cell be 10^{-3} cm, then the maximum drag over the cycle is given by

$$F = 6\pi\ (2 \times 10^{-2})\ 10^{-3} \frac{23}{21} \text{ dyn}$$

$$= 4.1 \times 10^{-4} \text{ dyn} .$$

A constant force of between 10^{-3} and 10^{-4} dynes is required to break macromolecular bonds (El'piner 1964). It is not known whether cyclical forces of this magnitude produced at the cell surface could immediately produce breaks in molecules. However, such forces may have sufficient strength to distort molecular binding sites at the cell surface and produce the reduced binding observed. Cyclical forces act for a fraction of a second during each cycle, and so whether such forces could break molecules

immediately or act in some way to weaken molecular bonds over a longer time is questionable. It was shown in the experiments conducted to determine an exposure time - threshold that a gradual inhibition of (^{3}H) thymidine incorporation occurred (Figure 5.7). This suggests that if cyclic forces were responsible for producing damage to the biological molecules, they acted over a period of time, possibly by gradually weakening the bonds. The exact mechanism by which molecules could be weakened is unclear at present. However, there are reports in the literature (Kashkooli et al 1980) where the amount of inactivation of enzymes was found to depend on the duration of the ultrasound exposure. Kashkooli et al (1980) produced evidence to suggest that the enzyme inactivation was not the result of collapse cavitation or heating, but was probably related to the presence of acoustic microstreaming.

Let us now consider the forces and mechanical stresses produced at the cell surface by ultrasound in an attempt to determine if microstreaming could be involved. Basically the same theory is applicable to the determination of microstreaming stresses whether one considers the cell as a vibrating sphere or if a bubble is located near the cell surface and generates streaming forces as described in Section 3.2. For a cylinder or sphere of radius R vibrating with angular frequency ω and displacement amplitude r, the velocity gradient G is given by (Nyborg 1977).

$G = \omega\beta r^2/R$ where

$\beta = (2N/\omega\rho_o)^{-\frac{1}{2}}$ and

ρ_o and N are respectively the density and shear viscosity coefficient for the liquid.

For N = 0.01 poise, ρ_o = 1 g/cm^3, $\omega = 2\pi f$ where $f = 870 \times 10^3$ Hz in the present studies, then $\beta = 16.5 \times 10^3$. If the lymphoblast has a radius of 10^{-3} cm and the displacement amplitude is 0.5% of this radius, then the stress S is

$S = NG = 22.6$ dyn/cm^2

This stress would be produced by microstreaming at the cell surface when the cell vibrates in the medium under the action of the ultrasound field.

This is a constant stress produced by ultrasound microstreaming at the cell surface. Although the magnitude of the stress is relatively small, it acts in the present studies for some 30 min. One could compare this result with that of Brown et al (1975) who reported that hydrodynamic stresses of 150 - 250 dyn/cm^2 applied for 5 min caused the release of acid phosphatase and serotonin from human platelets causing aggregation. Krizan and Williams (1977) have shown that the same percentage haemolysis of red cells could be produced either by a high magnitude stress applied for a short duration or a lower magnitude of stress for a longer period of time.

The question arises whether a stress of 22.6 dyn/cm^2 applied for 30 min is likely to produce damage to the cell surface or cause distortions of molecular binding sites at the cell surface. It is possible that small stresses are needed to distort the shape of binding sites since Schmid-Schönbein and Wells (1969) have produced evidence that human erythrocytes are deformed by small shear stresses (less than 10 dyn/cm^2) to give prolate ellipsoids which are aligned parallel to the direction of fluid flow. A similar mechanism may be acting in the present experiments to account for the reduced Con A binding at the cell surface.

Using the same theory but considering a bubble of radius 4×10^{-4} cm at the cell surface that is undergoing stable cavitation in the ultrasound field, the stresses produced by the microstreaming around the bubble will be approximately

$$S = 900 \text{ dyn/cm}^2$$

for a displacement amplitude equivalent to 5% of the bubble radius. This is also a constant stress and should be of sufficient magnitude to produce effects at the cell surface (Brown et al 1975).

Consider the oscillatory flow set up by the ultrasound field at the boundary of the teflon disc. This disc can be considered as a rigid boundary which imposes a non-slip condition on the flow of the cell culture medium.

The viscous stress exerted at the surface of cells near this rigid boundary has an amplitude given by (Nyborg 1977):

$S = N\beta U_o$ where U_o is a constant

For $N = 0.01$ poise $\beta = 16.5 \times 10^3$ and $U_o = 11.5$ cm/s (for water) then

$S = 1901$ dyn/cm^2

This stress varies sinusoidally with time. This value of S is in agreement with Nyborg (1977) who states that for "... a plane travelling wave in water at frequency 1 MHz and intensity 1 W/cm^2, the stress amplitude is 2040 dyn/cm^2. Viscous stresses approaching this magnitude prevail throughout the boundary layer region and would act on any biomolecules or small cells which might exist there."

This is a cyclic stress which acts over the whole cell surface for approximately 0.5 μs in one direction and then in the opposite direction for the same period of time. What may be important is that although the stress acts for only 0.5 μs at a time, it does so 870,000 times per second in each direction. One cannot presently exclude that such stresses are causing the effects observed. It seems likely however, that higher levels of cyclic stress may be needed to produce the same effects as a constant stress.

The problem remains - are the observed effects at the cell surface caused by viscous stresses generated by the cell vibrating in the ultrasound field or are they due to stable cavitation-induced microstreaming around gas nuclei near the surface of the cell? In experiments designed to elucidate this problem, every precaution was taken to ensure that the cells were suspended in a degassed medium (having an oxygen gas content by weight of approximately 0.5 part per million). The results given in Table 5.7 suggest that if cavitation was truly eliminated, viscous stresses and not cavitation were responsible for the reduced binding of Con A to the cell surface.

Medium degassed to 0.5 ppm contains 0.5 mg of oxygen per 1000 l. Thus the 10 ml teflon disc of cells in degassed medium contained 3.5×10^{-7} ml of oxygen. For maximal cavitation to be produced by ultrasound in the medium, gas nuclei or bubbles would need to have a radius of approximately 4×10^{-4} cm. Thus the number of such bubbles that could exist in the 3.5×10^{-7} ml of oxygen is

$$\frac{3.5 \times 10^{-7}}{(3/4)\pi\ (4 \times 10^{-4})^3}$$

$$= 2.3 \times 10^{3} \text{ bubbles}$$

During experiments the teflon disc contained between $(1 - 5) \times 10^{7}$ cells. If one assumes that there were 10^{7} lymphoblasts exposed during any one experiment then if all bubbles had a 4μ radius (very unlikely) there would be approximately one cavitation

nucleus available for every 4000 cells. Allowing for a possible increased effectiveness caused by bubbles moving from cell to cell during rotation of the teflon disc, it seems unlikely that so few cavitation nuclei could cause the effects produced. This conclussion is in agreement with Chapman et al (1980) who used a degassed medium to expose suspensions of rat thymocytes to ultrasound. Their experiments also suggested that a non-thermal, non-cavitational mechanism was operative in producing changes in cell membrane permeability at an intensity of 2 W/cm^2.

6.3 Intracellular Stresses

In the near field of an ultrasound transducer rapid changes in pressure amplitude could produce significant stresses at particular times. Pressure amplitude variations could cause streaming, twisting of intracellular components (as observed by Dyer 1972), distortions and alterations of shape that could ultimately damage the DNA. Streaming produced by ultrasound has been observed experimentally near locally excited membranes (Jackson and Nyborg 1958), and within plant cells (Gershoy and Nyborg 1973). It seems reasonable to assume that similar effects occur within mammalian cells exposed in vitro to ultrasound. Streaming of cytoplasm has already been inferred in experiments where mammalian cells were observed to have damaged, disorganized and redistributed cell contents following sonication (Harvey et al 1975, Watmough et al 1977).

Levinthal and Davidson (1961) derived an equation for predicting the shearing stress S required to produce a break in a rod-like molecule:

$$S = \frac{16F \ln(L/r)}{3\pi L^2} \qquad \text{where}$$

F is the force required to break the molecule (generally equivalent to the bond strength between atoms in the molecule), "ln" means the natural logarithm, L is the length of the rod and r its radius.

If one assumes (Nyborg 1977) that the DNA molecule has a rod shape with length L = 2μ and radius r = 1 nm then stresses produced in the present experiments S = 2000 dyn/cm^2 will produce breaking forces $F = \frac{3\pi L^2 S}{16\ \ell n(L/r)}$

$= 4 \times 10^{-6}$ dyn

Constant forces of this magnitude would alone be unable to break the double helix structure of DNA molecules (Elpiner 1964, Levinthal and Davidson 1961). It is suggested that cyclical forces of this magnitude should have even less effect.

Consider the case where the DNA is clumped and for simplicity forms a dumbbell shape (ie 2 clumped balls joined by a rod). The theory for this model has been well described by Nyborg (1975). The tension exerted on this dumbbell, neglecting friction along the connecting rod, is given by

$F = f\ Gb \sin\Theta \cos\Theta$ for a dumbbell having a length of 2 b and two spheres of radius R. Θ is the angle at which the dumbbell is oriented to the direction of propagation of the ultrasound field, G is the oscillatory velocity gradient and f is given by $6\pi NR$. If similar stresses are generated by the ultrasound as determined in the previous section then

$G = 2 \times 10^5\ s^{-1}$

Assuming that one end of this dumbbell is anchored, then the maximum force will occur at $\Theta = 45^\circ$. With N = 0.01 poise, b = 10^{-3}cm, R = 5×10^{-4} cm.

$F \approx 10^{-4}$ dyn

This force is cyclical, is produced by a plane wave incident on the DNA dumbbells, and acts for a fraction of a second during each cycle. Again the question is raised whether such cyclical stresses are of sufficient magnitude to produce damage, even over a period of 30 min. It is questionable whether such forces could alone account for breaks in the DNA.

Although the structure of DNA is well known, the molecule does change configuration during various phases of the cell cycle. The two simplified models above represent some of the only available mathematical theory that has been developed to explain the forces on molecules in an ultrasound field. They indicate that DNA is probably more sensitive to breakage from ultrasound when the helical structure unwinds. Liebeskind et al (1979a) have produced evidence that ultrasound at a very low average intensity (6.6 mW/cm^2) may cause unwinding of the DNA helix. If this unwinding does take place then the DNA might become sensitive to damage by ultrasound from stresses similar in magnitude to that predicted by the dumbbell model. Evidence supporting the dumbbell model comes from de la Maza et al (1976) who, using density gradient centrifugation, found that the dispersed euchromatin of mammalian cells exposed in vitro was more sensitive to breakage by ultrasound than the highly condensed constitutive heterochromatin.

It is at present unknown if there exists gas nuclei within cells that could produce cavitation. However, cavitation outside the cell can produce effects within the cell since a vibrating bubble at the cell surface causes the cell membrane to vibrate, producing streaming of the intracellular contents (Nyborg 1978). Webster et al (1978, 1980) reported that ultrasound-induced stimulation of protein synthesis was due to cavitation. Their conclusions were based on the results of experiments where sonication was conducted under 2 atmospheres of pressure in an attempt to prevent cavitation. However their experiments did not appear to produce differences between sonicated and control cells that were sensitive enough to conclude which mechanism was acting.

Throughout this discussion, collapse cavitation has not been considered as a possible mechanism, because the instantaneous temperatures and pressures produced by this form of cavitation would almost certainly cause cell lysis. Since the survival of sonicated cells was not found to differ significantly from the controls, it seems reasonable to assume collapse cavitation was not a dominant mechanism of action in this study.

Ultrastructural studies suggested that ultrasound was responsible for the disassembly of microtubules. Microtubules are basically polymerized assemblies of tubulin subunits held together by hydropholic bonds (Pickett-Heaps and Tippit 1978). Ultrasound may have produced physical stresses

inside cells that were at able to overcome these bonds. Such a result may actually give some indication of the actual magnitude of these stresses. Pease (1941) reported that increased hydrostatic pressures of between 2000-3000 lb/sq in causes disassembly of microtubules. Reassembly occured after the release from pressures as high as 15,000 lb/sq in. To cause similar direct disassembly of microtubules, ultrasound would have to produce an equivalent static pressure. To metric terms, this stress or pressure approximates to 2×10^8 dyn/cm^2. There is no evidence that the ultrasound in the present study needs to produce pressures of such magnitude since part of the radiation pressure is cyclic in nature. Streaming motions set up within the cell could be responsible for disassembly of microtubules, however, indirect action of ultrasound cannot be excluded.

Ultrasound is known (Nyborg 1977, 1979, 1980) to cause twisting of intracellular organelles, probably due to radiation torque. This twisting action, together with stresses produced by acoustic streaming and radiation pressure all contribute to cause quite complex and widely varying tensions and stresses within the cell. The combination of all these ultrasound-induced stresses may be of sufficient magnitude to produce forces that could break the appropriate macromolecular bonds or cause other types of damage or alterations within the cell. Certainly this possibility could not be excluded from the theoretical analyses presented. However, ultrasound could conceivably be acting in an indirect manner to produce some of the effects observed. This will be discussed in the next chapter.

Chapter 7 Discussion and Conclusions

In the introductory chapters of this thesis it was indicated that although many experiments had been performed to determine the effects of ultrasound on various biological materials, very little work has been reported where a systematic approach has been adopted to delve below the "surface phenomenon". An outstanding example of this is the fact that only a few papers report on the effect of ultrasound on basic cell functions such as DNA synthesis. This thesis has attempted to narrow this gap in knowledge. With the initial finding that therapeutic ultrasound at 4 W/cm^2 could cause an immediate significant inhibition of (^3H) thymidine incorporation into human lymphocytes activated with Con A, work began with the primarily objective of elucidating the predominant ultrasound mechanisms involved in producing this inhibition.

From section 3.2 on mechanisms of action it was shown that ultrasound causes a number of phenomena to occur in biological materials, principally heating, cavitation and non-cavitational, non-thermal stresses. A number of experimental approaches were made to provide more information on these mechanisms and how they related to this highly repeatable inhibition of (^3H) thymidine incorporation.

7.1 Gross Effects

Exposure of human peripheral blood lymphocytes to ultrasound at an intensity of 4 W/cm^2 for 30 minutes did not appear to produce gross effects. The survival of sonicated cells over a period of 4 days was not significantly altered from that of the controls (Figure 5.1). This result is in agreement with the cell survival data of Harvey et al (1975) who exposed fibroblasts in vitro under similar conditions, and with Bleaney et al (1972) who found no reduction in survival of CHLF hamster cells exposed in vitro to ultrasound intensities up to 8.8 W/cm^2 provided the temperature remained below 45°C.

As mentioned in the materials and methods section, cell viabilities were routinely determined using the trypan blue dye exclusion technique. Kaufman et al (1977) had previously demonstrated that cell survival and reproductive capacity of mammalian cells exposed to therapeutic levels of ultrasound, using the trypan blue method or the colony forming ability, produced the same results. Further, tests on cell survival using fluoroscene diacetate also gave the same results as with trypan blue.

The cell survival data (Figure 5.1) was the first suggestion that the ultrasound in the present experiments was acting via a non-cavitational, non-thermal mechanisms since Moore and Coakley (1977) could not demonstrate cell death in vitro without ultrasound-induced cavitation or heating.

Gross ultrastructural damage was not observed, except for possible reversible disassembly of microtubules (Figure 5.3). Ultrasound effects on microtubules have not previously been reported, but would not be entirely unexpected considering the magnitude of the forces produced by ultrasound (see Chapter 6) and the fact that microtubules are composed of tubulin subunits held together by weak hydrophobic bonds (Dustin 1978). However, the possibility that ultrasound could be producing the disassembly of microtubules in an indirect manner cannot be discounted. Assembly of microtubules requires guanosine triphosphate (GTP) and divalent cations (Mg^{2+}, Ca^{2+}), Olmsted et al (1974). Ultrasound could, for example, alter the concentration of magnesium or calcium ions to the extent that disassembly of microtubules occurs since Chapman (1974) and Chapman et al (1980) have already reported that ultrasound at therapeutic levels could alter the concentration of potassium ions in mammalian cells. Further, changes in calcium permeability of muscle cells exposed to ultrasound have also been observed (Dunn and Oakley 1972).

7.2 Proposal of Action

Of the principle mechanisms of action of ultrasound; the predominant mechanism acting on activated human blood lymphocytes exposed to near field, 870 kHz, cw ultrasound at 4 W/cm^2 appears to be both non-cavitational and non-thermal, as explained in the previous chapter. Heating would appear to be eliminated as possible mechanism since using the FADU technique, alkali labile breaks (hereafter referred to as breaks) in the DNA were produced by ultrasound exposure at 0°C. These experiments indicated that the inhibition of (^{3}H) thymidine incorporation was probably due, at least in part, to physical stresses from ultrasound causing direct breaks in the DNA. The period of the lag time before normal incorporation of (^{3}H) thymidine occurs could partially be accounted for by subsequent repair to the DNA.

In preliminary FADU repair analyses it was found that although ultrasound produces damage to the DNA, it is rapidly repaired. Approximately 75% of the initial DNA damage was apparently repaired after 30 min of incubation of 37°C. One refers to an "apparent" repair of the DNA because the experimental technique merely indicates that less of the DNA lesions are seen and one assumes that repair has occurred (Douglas and Grant 1980). In other experiments using human blood lymphocytes treated with ionizing radiations, it was found (Birnboim, McLean - personal communications) that the repair process was biphasic. Following breaks in the DNA there is an initial rapid repair phase lasting less than 1 h followed by a much slower repair phase lasting many hours. This slower phase repair could account for the ultrasound-induced immediate inhibition of (^{3}H) thymidine incorporation and subsequent return to control levels.

There are many reports in the literature that ultrasound can cause breaks in the DNA. Hill et al (1969) reported the degradation of calf thymus and salmon sperm DNA after exposure to 1 MHz ultrasound at intensities greater than 0.4 W/cm^2. Irradiation of calf thymus DNA with 1 MHz ultrasound resulted in considerable damage, as observed in sonicated and control DNA samples examined by electron microscopy after exposure to as little as 200 mW/cm^2 (Galperin-Lemaitre et al 1975). The above experiments were performed on "naked" DNA solutions with the obvious difficulty of interpreting the results with respect to sonication of the normal DNA-protein complex found in eukaryotic cells. The intensities at which DNA degradation was observed were very much lower than 4 W/cm^2 value used in the present experiments. A number of studies on the broad bean Vicia faba suggest that ultrasound can induce non-breakage types of damage to DNA (Cataldo et al 1973, Gregory et al 1974, Kokhar and Oliver 1975). Thus it might not be unexpected that ultrasound at the intensities used in the present experiments could produce direct breaks or some other damage to the DNA.

Although unscheduled DNA synthesis was observed in Figure 5.11, and breaks in the DNA observed using the FADU technique (Figure 5.12), this does not necessarily mean that direct damage was done to the DNA by ultrasound. In Chapter 6 it was indicated that ultrasound may not be able to directly break bonds within the DNA, although such a possibility cannot be discounted. However, ultrasound does appear to

produce stresses that could break weak hydrophobic bonds and possibly change the conformation of the DNA molecule. Thus ultrasound may produce lesions or damage indirectly that could subsequently be observed as unscheduled DNA synthesis or breaks in the DNA. Two possible explanations for indirect induction of damage in the DNA by ultrasound are given.

Ultrasound has been reported (Harvey et al 1975, Jankowiak and Majewski 1966, Majewski et al 1966) to produce damage or alterations to lysomes at intensities up to 3 W/cm^2. It would appear likely that such damage might result in a partial release of hydrolytic enzymes. This could result in a small amount of damage to the DNA from hydrolytic enzymes that penetrate the nuclear membrane. Repair of this damage within the cell would take time and could explain the immediate inhibition and the subsequent gradual increase in the incorporation of (^3H) thymidine toward control levels observed in Figure 5.6. Repair of lesions in the DNA could be detected as unscheduled DNA synthesis.

Biologically-induced lesions in the DNA might also be mediated by ultrasound exposure as follows: streaming forces set up within the cell by ultrasound have already been shown to produce stresses able to break hydrophobic bonds (Chapter 6). DNA is losely combined or conjugated with nucleoproteins (histones and non-histones). Histones are relatively tightly bound to the DNA while the non-histones are bound with weak hydrophobic, ionic and hydrogen bonds, and van der Waals forces. Ultrasound-induced streaming could break these bonds causing a release of non-histones from the

DNA and change its conformation. Local changes in conformation of DNA might then be recognized and attached by specific repair endonucleases. Endonucleases could produce damage to the DNA that might show as unscheduled DNA synthesis or DNA breaks in sonicated cells. Evidence for such a proposal comes from Burkholder and Weaver (1975) who found that DNase digests extended chromatin at a faster rate than condensed chromatin. Endonucleases have been reported (Radman 1976; Gates and Linn 1977) that act upon DNA damaged by a variety of physical and chemical agents. Endonucleases that recognize these structural or chemical changes in DNA have been purified from mammalian cells (Hewitt and Meyn 1978).

Carstensen et al (1953) and Carstensen and Schwan (1959a) have already shown that the absorption of ultrasound in cells exposed in vitro, was in direct proportion to the protein content of the cells. It would not be unreasonable to expect that preferential absorption of ultrasound was occurring in the nucleoproteins compared to the nucleic acids and result in the preferential breaking of hydrophobic bonds that complex the nucleoproteins with the DNA and alter its conformation to the extent that it is recognized by specific endonucleases.

Evidence in the literature on this subject continues to be controversial because although Liebeskind et al (1979a) reported DNA repair synthesis, they were unable to observe breaks in the DNA on an alkaline sucrose gradient. This might now be explained by the fact that rapid repair of DNA breaks occurs after ultrasound exposure as indicated in the FADU experiments.

The results of exposure of 2 day activated lymphocytes to varying intensities of ultrasound (Figure 5.6) indicate that a threshold exists for this inhibition of (^{3}H) thymidine incorporation between 1.1 and 2.75 W/cm^2. One notes that the cells exposed to 2.75 or 4 W/cm^2 eventually recover to control levels of (^{3}H) thymidine incorporation. This suggests that whatever damage was caused by ultrasound was "repaired" by the cells. This is supported by the repair analyses in the FADU technique.

Damage could also take the form of disruption of normal metabolic pathways by, for example, displacement of ribosomes from endoplasmic reticulum or removal of DNA polymerases from their normal sites of activity. Enzymes of certain pathways may need to be confined to particular areas, such as membrane channels, to account for sustained rates of biosynthesis in the cell. Disruption of such channels by ultrasound streaming may contribute to the inhibition of biosynthesis. It could take the cell some time to recover fully to normal operation and this might show as a gradual recovery of (^{3}H) thymidine incorporation to control levels over a period of days.

Further evidence for disruption of enzyme pathways is given by Figure 5.9. It is known that a suppression of RNA synthesis will cause a corresponding decrease in protein synthesis (Ling and Kay 1975). Using lymphocytes to determine if ultrasound effects on RNA and protein synthesis will cause corresponding effects on DNA, is complicated by the fact that although a high proportion of the cells respond to mitogen by increased RNA and protein synthesis, only about half of these initiate DNA synthesis (Ling and Kay 1975). Nevertheless the induction of DNA synthesis is thought to be inhibited if ribosomal RNA synthesis is inhibited. Further, it appears that agents reported to inhibit or suppress DNA synthesis in stimulated lymphocytes probably act by preventing or depressing RNA, protein or nucleotide synthesis (Ling and Kay 1975).

Besides disruption of normal housekeeping processes within the cells, breaks to critical macromolecules, similar to the breaks produced in DNA, could contribute to the inhibition of (^{3}H) thymidine incorporation.

There is a lot of evidence for disruption of cell contents by ultrasound. Deformation, rotation, fragmentation of nuclei and nucleoli, and acoustic streaming of cytoplasm and nucleoplasm were observed when egg cells of marine invertebrates were subjected to 85 kHz ultrasound (Wilson et al 1966). Similar circular motions of cell contents were found by Hughes and Nyborg (1962) in the protozoan Tetrahymena pyriformis. Streaming, aggregation and rotation of intracellular particles and organelles of plant cells were observed under

the microscope during application of 80 - 90 kHz ultrasound Nyborg et al (1977).

Data from experiments measuring the incorporation of (^{3}H) uridine and (^{3}H) leucine into activated cells suggest that ultrasound caused an immediate depression of protein synthesis and a delayed inhibition of RNA synthesis (Figure 5.9). Such an effect on both protein and RNA synthesis could have a contributory effect on the inhibition of DNA synthesis. What is interesting in these results is that ultrasound produced an immediate depression of (^{3}H) leucine incorporation into lymphocytes activated for 1 day. Inhibition of (^{3}H) uridine incorporation did not occur until one day after sonication. One can gain a better perspective of the time-course of events by referring to Figure 7.1 (from Schafer and Mitchell 1979). Bearing in mind that resting lymphocytes maintain a basal level of RNA synthesis and turnover, one observes from Figure 7.1 that after addition of a mitogen, lymphocytes do not show an immediate increase in RNA synthesis but there is an initial lag period which may persist for at least 4 - 6 h. Thereafter RNA synthesis increases steadily for the next 40 h. On the other hand, protein synthesis begins immediately after addition of the mitogen (Schafer and Mitchell 1979). Significant DNA synthesis does not occur until some 30 h after addition of mitogen.

Suppression of protein synthesis and direct damage to protein molecules could cause a decrease in the amounts of DNA and RNA polymerases and result in a partial inhibition of both DNA and RNA synthesis some 24 h after sonication.

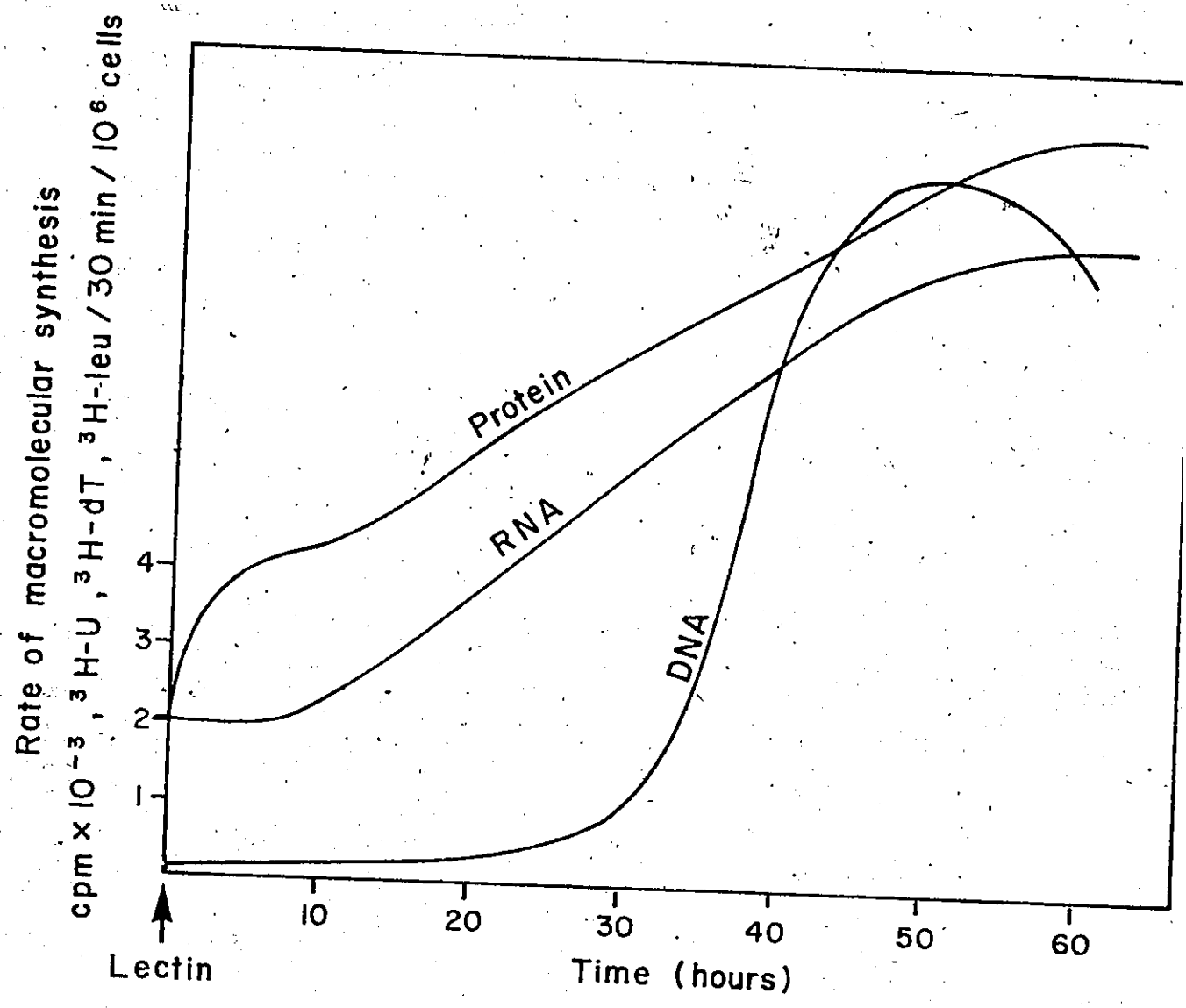


Figure 7.1. Schematic representation of the time course of macromolecular synthesis in lymphocytes. This diagram represents data from many experiments using lymphocytes from different species. (From Schäfer and Mitchell 1979.)

From Figure 5.6, one notes that a delayed stimulation of (^{3}H) thymidine incorporation occurred in cells exposed lower (0.5 and 1.1 W/cm^2) intensities of ultrasound. If the data in Figure 5.6 were plotted to give incorporation of (^{3}H) thymidine (as a percentage of controls) versus ultrasound intensity, the curves for various days after activation would have a shape similar to those reported on lymphocytes exposed to ultrasound by Crowell et al (1977). The present data also support the findings of Dyson et al (1970, 1976).

The delayed stimulation of (^{3}H) thymidine incorporation might be produced if ultrasound was responsible for an increase in DNA polymerase activity. This suggestion may seem more plausible when one notes that an immediate stimulation of (^{3}H) leucine incorporation occurs after exposure to lower intensity ultrasound (Figure 5.10). It is possible that an immediate stimulation in protein synthesis would have the effect of making more enzymes such as DNA polymerases available at later times.

Ultrasound exposure does not appear to alter the initial rates of transport of (^{3}H) thymidine across the cell membrane. The control rates of transport were found to agree with Rudd (M.Sc. Thesis, University of Ottawa 1980). A linear regression analysis of the Lineweaver-Burk plots for both sonicated and control transport rates indicated that there existed no significant difference between them. One cannot

conclude from these experiments that the amount of radioactive precursor available to both control and sonicated cells was the same since the experiments provided no information on the internal cell pools of the precursor. However, if the initial rates of thymidine transport were unaltered by ultrasound, it seems reasonable to suggest that both sonicated and control cells probably had the same amount of (^{3}H) thymidine available for incorporation.

The proposal that ultrasound acts directly through a non-cavitational non-thermal mechanism could explain the reduced Con A binding at the cell surface (Figure 5.13). An apparent reduction in the total number of Con A binding sites occurred in the sonicated samples since 30 - 40% less Con A was bound to the cell surface. A possible explanation is that ultrasound was directly damaging or altering the conformation of the binding sites on the lymphocyte surface so that they did not recognize the Con A.

Let us consider other possibilities that might explain this effect. The reduced binding could be due to an increase in the rate of internalization, breakdown and release of the tritiated structure of the Con A molecule. Do the lymphocytes quickly shed instead of internalizing the (^{3}H) Con A? Both these possibilities could explain the observed result.

It is known that Con A binding sites are randomly dispersed on the lymphocyte surface (de Petris 1975). The addition of Con A to lymphocytes initially induces clustering or patching of the Con A binding sites, then, depending on the experimental conditions, patching of Con A receptors in B or T lymphocytes is followed by capping (Schreiner and Unanue 1976). The caps usually take between 30 - 60 min to form, depending critically on the concentration and multivalent nature of Con A, and on crosslinking. Low concentrations of Con A, even levels incapable of saturating all binding sites, cap well (Yahara and Edelman 1973). Con A caps are observed on lymphocytes in active movement (Loor 1974, Unanue and Karnovsky 1974) and are situated on the uropod trailing the ameboid lymphocyte. Once formed, the lymphocyte's Con A cap is not rapidly cleared from the membrane; pinocytosis occurs, but is relatively slow (Loor 1974, de Petris 1975). For example de Petris (1975) reported that at Con A concentrations of 5 - 10 µg/ml, caps took approximately 15 min to form in splenocytes and persisted for at least 90 min at 37°C. This is a time frame longer than that of the experiment and so one would not expect the results to be explained by internalization and subsequent release of the ^{3}H label.

There is evidence for a role of the cytoskeletal system in the capping of Con A-binding sites. Capping of Con A is quite sensitive to the inhibitory influence of cytochalasin B (de Petris 1975, Unanue and Karnovsky 1974), suggesting that microfilament activity might be of central importance. Yahara and Edelman (1973) have implicated

microtubule-like proteins as exerting a controlling influence on the redistribution of Con A receptors. It has been suggested that Con A stimulates the physical anchoring of its receptors to microtubules, which prevents redistribution. (Edelman et al 1973). Since microtubules seem to be disassembled by ultrasound, one could not exclude the possibility that the reduced Con A binding to the cell surface may be in some way linked to this effect. Perhaps disassembly of the microtubules by ultrasound could cause a release of their anchoring of the Con A binding sites and allow some of the ^{3}H Con A to be released from the cell surface.

Shedding of the caps are reported only if the treatment is performed after the cap has been formed (Schrejner and Unanue 1976). In the present study the cells were sonicated prior to incubation with (^{3}H) Con A. Thus it is unlikely that shedding could explain the reduced (^{3}H) Con A binding. It seems more probable that this reduced (^{3}H) Con A binding occurs because ultrasound directly damages or distorts the molecular configuration of the receptor sites on the cell surface to the extent that many of the binding sites do not recognize the Con A molecule. Support for this proposal comes from the fact that ultrasound at a lower average intensity (1 W/cm^2) for 5 min has been shown to cause a reduced cell surface charge (Repacholi 1970, Repacholi et al 1971, Taylor and Newman 1972, Joshi et al 1973). This result suggests that ultrasound can cause direct damage to the glycoproteins (such as sialic acid residues) on the cell surface. The mechanisms by which ultrasound could produce this effect have already been discussed in Chapter 6.

A non-cavitational, non-thermal mechanism can also explain the effects of ultrasound on the mixed lymphocyte reaction. The capacity of lymphocytes to stimulate in the MLR is a highly labile metabolic process and not the mere display of the appropriate antigens at the cell surface (Christen et al 1975, Dornand and Kaplan 1976). The mixed lymphocyte reaction can be divided into three distinct parts:

(i) recognition of antigen (stimulator cells) by the responder cells and their binding to the cell surface,

(ii) triggering or transduction of a signal within the responder cell to indicate that binding to the antigen has taken place and to begin cell division, and

(iii) the metabolic response, measured by the incorporation of (^3H) thymidine.

Let us review the data collected thus far that would bear on the MLR response. It was shown that ultrasound did not effect basal DNA synthesis in resting cells or alter the rate of incorporation of (^3H) thymidine into cells exposed to ultrasound and then immediately activated with Con A (Table 5.1). It thus seems unlikely that ultrasound would act to inhibit the incorporation of (^3H) thymidine into responder cells (Table 5.12) by altering the metabolic response (part iii) of the MLR. The fact that sonication of the stimulator cells inhibited (^3H) thymidine incorporation suggests that ultrasound was probably not acting on the transduction of the signal within the responder cells

(part ii). Since ultrasound caused a lower incorporation of (^{3}H) thymidine in the MLR when both stimulator and responder cells were sonicated than if either the stimulator or responder cells alone were sonicated, could be explained on the basis of effects caused by ultrasound at the cell surface. Evidence has already been given for a non-cavitational, non-thermal mechanism acting at the cell surface (Con A binding experiments), and so a similar mechanism of ultrasound could also be effective in producing a similar result at the cell surface.

However, the MLR is not a straightforward reaction. Stimulator cells must be viable and metabolically active at the time of the MLR, otherwise no activation occurs (Ling et al 1974), and they are not merely a carrier cell on which the appropriate antigens are expressed (Dornand et al 1976, Dornand and Kaplan 1976, Christen et al 1975). T-cells are the principle responder in the autologous MLR but macrophage are also necessary for stimulation (Beale et al 1979). One could make a reasonable case for the macrophage being the cell that was damaged by ultrasound. Whatever function the macrophage has in the MLR could be altered by ultrasound to explain the results in Table 5.12. Although this possibility cannot be excluded, there is good evidence that ultrasound probably acted at the surface of both stimulator and responder cells to reduce the level of binding and hence stimulation of the MLR, observed as a reduced incorporation of (^{3}H) thymidine.

There were a few results obtained that were difficult to explain. In Table 5.1 it was shown that sonication of cells and subsequent activation with Con A, did not appear to alter the incorporation of (^{3}H) thymidine in the following days. In the MLR experiments sonication of the respondor cells prior to mixing with the stimulator population did produce a lower incorporation of (^{3}H) thymidine. A possible explanation for this apparent inconsistency is that the experiments were carried out over different time periods and repair of ultrasound-induced damage may have taken place in the first experiments (Table 5.1).

The apparent delayed stimulation of DNA synthesis (Figure 5.6) when cells are exposed to low intensities (0.5 and 1.1 W/cm^2) ultrasound is also difficult to explain. One might suggest that immediate stimulation of protein synthesis (Figure 5.10) could contribute to this effect.

Finally, there appears to be an inconsistency between the results of experiments indicating damage (Figure 5.12) and repair (Figure 5.11) to the DNA. It appears from Figure 5.11 that a large number of cells did not suffer any damage, but from Figure 5.12 that a typical dose-response relation exists. It should be pointed out however that the unscheduled DNA synthesis (UDS) experiments would not detect damage to single bases or "frank" breaks (physical breaks to the sugar-phosphate backbone of the DNA). The UDS technique will only detect damage if repair occurs to larger segments of DNA (incorporating many base pairs). This difference in the type of damage detected by the two methods could well explain the apparent inconsistency

in the results. However it is very difficult to determine the types of damage detected by either the UDS or FADU methods and so only limited interpretations can be made from the data.

7.3 Conclusion

Ultrasound appears to produce an immediate inhibition of (^{3}H) thymidine incorporation into activated lymphocytes predominantly by a non-thermal, non-cavitational mechanism. The most probable of these stress mechanisms being acoustic streaming since, as explained throughout this chapter, all data from the experiments could be accounted for by this mode of action. Although available theory does not convincingly predict that the forces produced by acoustic streaming are strong enough to directly break covalent bonds, evidence that these bonds are being broken comes from the FADU experiments (Figure 5.12). These forces appear to be able to break weaker macromolecular bonds as evidenced from the disassembly of microtubules.

Other evidence that suggests these streaming forces are capable of acting on macromolecules comes from experiments using radioactive labels. The results can be explained either on the basis of disruption of metabolic pathways or changes in the conformation of macromolecules both within and on the cell membrane. Although further experiments are needed to quantitate precisely the effect of acoustic streaming within the cell, increasing evidence is being produced that this mode of ultrasound action is important *in vitro* and should be investigated using *in vivo* systems.

Chapter 8 Directions

Experiments using the FADU technique should be continued to determine the threshold intensity production of the DNA breaks and evaluate its ultrasound frequency dependence. The same technique provides a rapid means of providing more information on the nature of the repair process. Some of the apparent contradictions in the literature may well be explained if this repair process is better quantitated. With the evidence that ultrasound produces breaks in the DNA, the FADU technique can be used as a rapid screening procedure to determine if such breaks occur to a greater extent in cells exposed to ultrasound in combination with other physical or chemical agents. The method might provide independent confirmation of reports that ultrasound and x-rays act in synergistically to produce enhanced DNA damage (Kunze-Muhl 1975).

The proposal that ultrasound shakes out non-histones from DNA to expose the double helix to endonucleases deserves further study. Ultrasound frequencies that result in a higher absorption of the acoustic power by proteins may result in greater DNA repair synthesis. The clinical significance of this result to ultrasound therapy suggests that further experimentation be conducted to determine the lower limit of intensity at which DNA repair replication occurs.

Since the ultrasound-induced changes to the MLR may possibly have clinical importance, it should be investigated to determine not only the threshold exposure parameters for this effect, but to obtain more information on the type of damage ultrasound is producing, to determine if other more clinically accessible agents could be found that would reproduce this suppressing effect.

The Con A binding experiments incorporate a sensitive model for distinguishing the various modes of action of ultrasound at the cell surface. Repeating the experiments using apparatus which allows sonications to be conducted at high pressures could give more evidence on the relative importance of cavitation. Capping of lymphocytes with fluorescent antibody and then exposing to ultrasound, could produce visual evidence of ultrasound effects at the cell surface. Such a technique could prove invaluable in the study of ultrasound mechanisms of action.

The present work was carried out with continuous wave ultrasound. Many recent studies have indicated that ultrasound may produce greater damage when pulsed. Clearly this should be investigated, especially since a significant number of procedures in both diagnostic and therapeutic ultrasound use pulsed beams having very high peak intensities. These investigations would provide badly needed information on the relative importance of the cw and pulsed exposure regimes as far as capacity to produce damage is concerned.

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