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Cellular inactivation by heat and shear

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Summary. Inactivation of Chinese hamster V79 cells in vitro by a temperature elevation to 43° C and with Couette shear flow was investigated. The shear stresses were chosen to mimic those produced by ultrasound of approximately 3 MHz and 3 W/cm^2 within the chambers employed by earlier investigators studying ultrasonic inactivation of cellular processes. The combined shear and thermal stresses produced survival curves exhibiting a summating effect among these two stresses and remarkably similar to the ultrasound/thermal survival curves.

Introduction

It is well established that ultrasound provides a most efficient and efficacious agent for producing hyperthermia in tissues (Lehmann 1982). Further, there is considerable interest in broader use of this technique for clinical applications wherein the maintenance of elevated temperatures has a therapeutic effect (Hahn 1978). In order that the full potentials of this modality be obtained, investigators have sought to study the basic mechanism of the physical interaction of ultrasound and tissue (Dunn 1982). In order to deal with simpler systems, investigators have chosen to study such mechanisms in cellular suspensions (Li et al. 1977; ter Haar et al 1980). However, because of the very low absorption coefficient of such suspensions, which would involve having to use unreasonably intense ultrasound fields to produce desired hyperthermia (Dunn and O'Brien 1976), investigators found it necessary to increase the temperature of the cellular suspension in a water bath while irradiating simultaneously the preparation with ultrasound. Thus, the temperature elevation is affected by a means other than by simple ultrasonic absorption, and mechanical effects are presumed to be present from the exposure to the high

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frequency sound. The general demeanor of these studies has been that the exposure to low megahertz ultrasound of the order of 3 W/cm^2 at the elevated temperatures does not give rise to cell lysis. Cells can be irradiated with the ultrasound for times exceeding 5 h at 37° C with no effect to cellular ability to produce colonies on incubation. However, at temperatures in the hyperthermia range, viz., 42° C–45° C, the increase in cell killing that results from the ultrasound radiation is greater than that which would be accredited to its heating effect alone (Li et al. 1977; ter Haar et al. 1980), e.g., heating to 43° C produced a surviving fraction of 10^{-1} in approximately 70 min while simultaneously heating to 43° C and "exposure to" 3 MHz ultrasound at 3 W/cm^2 produced the same surviving fraction in approximately 30 min (ter Haar et al. 1980). As the temperature elevation in these in vitro experiments is produced by other than ultrasonic means, in contradistinction to that which occurs in vivo, it may be questioned whether the same physical mechanisms are involved and how the results of the in vitro studies contribute to understandings of the in vivo events. The present study was undertaken with this in mind.

An observed feature of those in vitro ultrasound experiments is that the fluid medium is kept in motion by induced acoustic streaming, insuring that settling out of the cells, which are more dense than the suspending liquid, does not occur (ter Haar et al. 1980). Thus, while the convenience of the ultrasonic forces producing the mixing simplifies appreciably the irradiation procedure, viz., it is not necessary thereby for the investigators periodically to shake or otherwise stir the cellular suspension, it may be considered that the ultrasonically induced streaming can have had a significant influence on the results. It was the purpose of the present study to investigate this. Specifically, the flow pattern produced by the sound in typical ultrasound irradiation chambers was examined and the attending shear stresses estimated. Such shear stresses were then produced by a nonacoustic means, and samples so exposed while simultaneously heated in a water bath were studied.

Steady state acoustically induced streaming

It has long been known that mass transport is produced by steady state unidirectional sound fields in viscous compressible fluids. This follows from the fact that a unidirectional sound beam transports energy and momentum and the reduction in energy density, as occurs in an absorbing fluid, is compensated for by the induction of mass transport, i.e., streaming. The situation that existed in the experiments referred to previously (ter Haar et al. 1980) is one in which a relatively well-collimated traveling-wave acoustic beam propagates coaxially through the specimen chamber – a circular cylinder of radius greater than the sound beam. The flow generated in such a chamber was first studied by Eckart (1948) who considered a circular cylindrical tube with rigid walls and whose ends are closed by a material that permits the axial sound beam to enter and leave the tube without reflection. The tube is of such length that at its center all effects due to the ends can be neglected. The total flow through any cross section within the tube is zero, i.e., a counter flow is produced at the distal end of the chamber and

continues along the tube walls. The particle velocity of the sound beam is along the axial direction of the cylinder and the particle velocity amplitude is distributed smoothly, though it is not necessarily constant, over the beam cross section. For tractability, Eckart assumed that the attenuation of the beam was negligible, an assumption in full agreement with the studies of cell suspensions and, in actual fact, the reason for the necessity for utilizing the water bath as the heating agent. Further, it is assumed that for the prevailing conditions, i.e., in the low megahertz frequency region, the ultrasound beam has dimensions considerably greater than the wave length. It is further assumed that the volume of the vortex domain at the boundary of the sound beam is small in comparison with the volume occupied by the sound field and that any surface vortex disturbances introduce a negligible contribution to the streaming. Under these conditions of applicability, Eckart's theory has been found to be in fully satisfactory agreement with experimental results (Liebermann 1949).

Using the experimental conditions of ter Haar et al. (1980), measurements of the sound field distribution at the position in the field at the anterior surface of their specimen holder yielded a beam width at half-power of 0.7 cm, i.e., a ratio of peak to average intensity of 2.7. With this acoustic intensity distribution considered as the sound field input to the specimen chamber, calculations from Eckart's theory estimated that the streaming velocity gradient, in the specimen holder, produced a shearing stress of the order of 5 dynes/cm^2, for the 2 MHz sound field of 3 W/cm^2.

With these details in hand, it was decided to expose cell suspensions at hyperthermic temperatures in the range of these acoustically induced shear stresses.

The acoustically induced streaming is that of Poiseuille flow, i.e., the flow results from a gradient of pressure along the axis of flow and shear stresses result from a lateral distribution of the flow velocity. Further, because of the relative dimensions of the beam diameter and chamber diameter, the associated counter flow occurs over a larger volume, at the periphery of the chamber, than does the acoustically induced Poiseuille flow. The counter flow velocities, because these are distributed over a considerably larger volume, are much less than the primary flow velocities and not considered further in this discussion.

The production of Poiseuille flow by nonacoustic means would have involved the use of various kinds of pumps, which would have required extremely large volumes of cell suspensions for the actual amount of fluid in Poiseuille flow at any instant. Thus, the amount of time a cell could be considered in the shearing field would become a major concern.

For these various reasons, it was decided to make use of the much more simplified Couette flow for establishing the velocity gradients. Couette flow was established between two concentric cylinders in which the outer cylinder was held fixed and the inner cylinder rotated.

The Poiseuille flow induced by acoustic means was of such nature that only low Reynolds numbers developed, and turbulence was never established, i.e., only laminar flow existed. As with the Poiseuille flow, the Couette flow established was of such nature that only laminar flow conditions prevailed, i.e., the Taylor criteria that turbulence is not present was satisfied (Taylor 1923).

Materials and methods

Shear exposure

The shear device was comprised of an external precision cyclindrical glass tube, closed with a conical fitting at the bottom end, and of an internal rotor of chosen diameter to produce the appropriate shear rates. The precision glass cylinder was obtained commercially as 20 cm long sections of 30.0 mm inside diameter (obtained as Veridia precision bore tubing, Chance Bros. Ltd., Malvern Link, Worcestershire, England). The bottom closure assembly has been described in detail (Emery et al. 1978). Briefly, an O-ring is compressed against the inside wall of the glass tube by two stainless steel male-female shafts drawn tightly by a stainless steel screw. The precision glass cylinder, the three stainless steel members, and the rubber O-ring are easily assembled and disassembled and provide for rapid and thorough detergent washing, distilled water rinsing and alcohol sterilization. The rotor was fashioned with a Teflon pestle-head mounted on a stainless steel shaft. The base of the pestle-head fitted the 90-degree conical depression in the stainless steel bottom closure assembly. The length of the pestle was 12 cm. The diameter was accurately machined at 29.50 mm to provide a total gap of 0.49 mm with the inside diameter of the precision glass tubing, determined at 29.99 mm total. The variable speed motor employed to drive the rotor operated on two ranges, viz., 16–176 rpm on the low range and 180–1,980 rpm on the high range. The gap of 0.49 mm thereby provided for shear rates available between 50 s^{-1}–6,220 s^{-1}. Shearing rates from 69 to 760 s^{-1} were used in the experiments. The dimensions of the gap and the employed shear rates were such that, respectively, the surface-to-volume ratio and the developed shear stresses were below those for which cell-solid surface interactions have been considered significant for red blood cell damage (Leverett et al. 1972).

Cells

The cell line used in this work was the Chinese hamster lung fibroblast V79-379A, which were grown in suspension as described previously (Stratford and Adams 1977). Cells were maintained in log phase at concentrations between 10^5 and 10^6 cells/ml. The doubling time of 10–12 h required daily dilution.

The asynchronous cells were harvested from exponentially growing cultures and diluted to concentrations of 10^5 cells/ml in Dulbecco phosphate buffered saline A. The suspensions were introduced into the shear apparatus, all the components of which were stored in absolute alcohol and dried with forced air of the flow hood just prior to assembly and charging, by pipetting at the bottom with the rotor absent. The rotor was introduced with caution such that the suspension rose to its final position in the annular space between rotor and precision glass cylinder with a high degree of uniformity, i.e., bubbles, voids, etc., were not produced. All of this assembly procedure was carried out under a flow hood. The shear producing assembly was then placed in the water bath and

the rotor connected to the driving motor; the speed of rotation having been selected previously. Very great care was taken during this assembly and mounting procedure such that the trace of the menisci were not permitted to vary by more than a millimeter, over the perimeter of the rotor, on slow hand rotation. This was considered an essential portion of the procedure which had to be properly carried out, else the sample was discarded and another sample used to charge the shear device. This procedure was difficult to carry out during the early stages of the study, but experience allowed for only a few samples to have been discarded.

Several minutes were required for thermal equilibration of the sample, in the annular space of the shear device, upon placement of the charged unit in the water bath. This was determined originally by insertion of small thermocouples into the annular space and observing the thermal equilibration process. For example, it was determined empirically that the time course of equilibration from room temperature at 24° C to the bath temperature of 43° C followed the relation $\Delta T = 19 \exp(-0.74\,t)$ min. Thus, within 7 min the difference between the sample temperature and the bath temperature was essentially only 0.1° C. This equilibration time was allowed prior to beginning the shear treatment. The preliminary studies included estimating the temperature increase in the specimen gap due to the shear stress treatment. This was accomplished by the insertion of small thermocouples in the gap, with the specimen present, but with the rotor stopped. Herein, the ensemble was set to operate in the prescribed manner for approximately 1 h, the rotor was stopped for a period of approximately 15–30 s during which time the thermocouple was inserted and the difference between the temperature in the cylinder gap and the bath was observed. At the highest speed of rotation available, viz., 1,980 rpm, this temperature was of the order of 0.2° C.

After treatment by heat and shear, the rotor was gently removed from the shear assembly and the cells withdrawn by pipette. The cells were then resuspended, counted, serially diluted, plated in MEM + 15% fetal calf serum and incubated at 37° C in an atmosphere of 95% air + 5% CO_2 for 7 days and the colonies stained with methylene blue. Colonies so identified were scored as survivors and the surviving fraction was calculated as the number of colonies counted divided by the initial number of cells plated. The surviving fraction or plating efficiency of cells maintained at 37° C was routinely greater than 90%. A few treated samples were counted several times over a period of several hours after treatment to determine whether near-treatment-lysis occurred. No significant differences in counts, between those made immediately after treatment and those made several hours later, were found.

The viscosity of the cell suspensions was determined as a function of shearing rate and temperature using a Wells-Brookfield Microviscometer (cone and plate viscometer) model LVT, Brookfield Engineering Laboratories, Inc., Stoughton, MA. The nominal value of the viscosity of the cell suspensions in the temperature range of study was 50% greater than that of water. Variations with temperature and shearing rate was such that, over the values of these parameters considered in the study, deviations from this nominal value seemed not to be sufficient for estimating the shear stress. Thus, the shear stresses ranged from 0.7

to 8 dynes/cm^2; bracketing that estimated herein to have been produced by the reported ultrasound fields (ter Haar et al. 1980).

Results

Several sets of survival curves were obtained using the techniques described above. The plotted points of Figs. 1, 2, and 3 all represent the average of two experiments, each of which was comprised of three plating dishes whose number of colonies were averaged. The range of the individual surviving fraction values was, for each data point, less than 25% of the point-value shown plotted. Survival curves were determined for cell suspensions placed in stirred and unstirred flasks at 37° C and at 43° C and significant differences, based upon the stirring regime, were not obtained. Survival curves were also obtained for cell suspensions in 43° C stirred flasks and in suspensions in the charged shear device, but with the rotor not rotating. Again no significant differences were observed, i.e., the surviving fractions differed by less than a few percent over time periods of the order of 5 h. Next, survival curves were determined as a function of the bath temperature for cell suspensions in stirred flasks, over the temperature range from 37° C to 43° C. Figure 1 is a sample of these data from which it is seen that a thermal resistance region exists for bath temperatures below about 41.8° C, in that little inactivation occurs therein, in agreement with that of Dewey et al. (1977). Figure 2 shows survival at three shearing stresses and for cells in stirred flasks at 41.5° C, in the thermal resistance region; the shear stress is applied concurrent with the heat stress. Here it is seen that the shearing stress significantly affects cell survival for cells maintained in the thermal resistance region. Figure 3 shows the survival curves for five shearing stresses and for

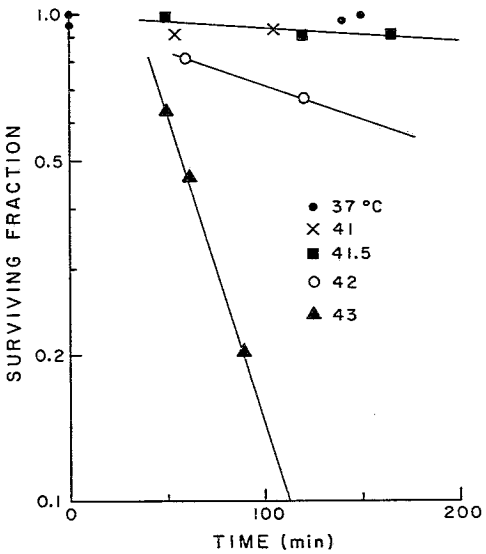

Fig. 1. Survival curves for Chinese hamster V79 cells. The parameter from curve to curve is temperature.

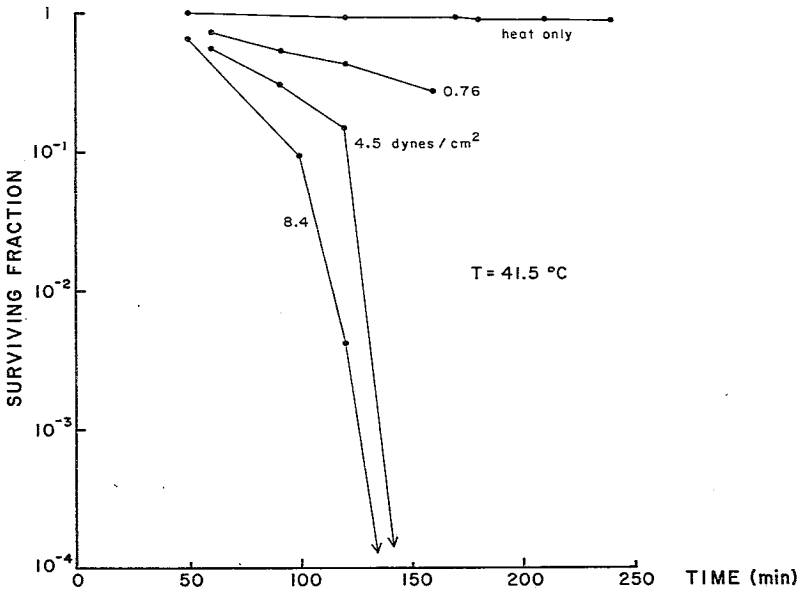

Fig. 2. Survival curves for Chinese hamster V79 cells at 41.5° C. The parameter from curve to curve is shear stress, applied concurrently with the heat stress. The arrows imply surviving fractions less than 10^{-4}.

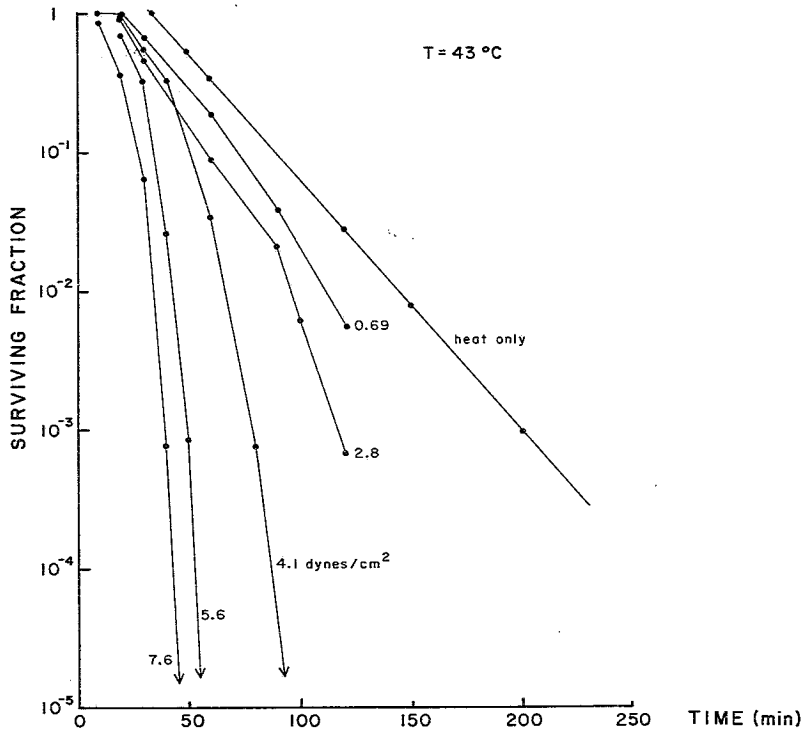

Fig. 3. Survival curves for Chinese hamster V79 cells at 43° C. The parameter from curve to curve is shear stress applied concurrently with the heat stress. The arrows imply surviving fractions less than 10^{-5}.

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