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BUBBLE FORMATION IN GUINEA PIGS AND AGAR GELS DURING ULTRASONIC IRRADIATION

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INTRODUCTION

The widespread use of ultrasound in medicine, both for diagnosis and therapy, requires that the interaction of ultrasound with tissue should be thoroughly understood. The mechanisms by which ultrasound produces biological effects are usually divided into thermal and non-thermal, with the principal source of non-thermal effects thought to be acoustic cavitation. Acoustic cavitation has been shown to produce biological changes in vitro [1,2,3]. Furthermore, the lethal and sublethal damage caused by ultrasonic irradiation in plant tissues and insects has been attributed to acoustic cavitation [4,5]. We have previously demonstrated that irradiation with 0.75MHz ultrasound at spatial average intensities below 1W.cm^{-2} causes the formation of stable bubbles in vivo [6,7,8]. It has also been shown that after ultrasonic irradiation localised regions of severe tissue damage appear [9]. In this paper we present evidence that the threshold intensity for the production of stable bubbles in vivo is not altered by increasing the ambient temperature at which irradiation is performed, from normal body temperature (37^{o}C) to that commonly experienced as a result of a typical hyperthermia treatment (43^{o}C). Increasing the ambient pressure, on the other hand, does increase the threshold intensity.

Observation of bubbles in vivo is far from straightforward, the number of bubbles formed is extremely variable and, in consequence, the number of animals required to give adequate statistics is large. We have, therefore, sought an alternative system in which bubble formation can be studied. There is considerable evidence to suggest that the formation of bubbles in gelatin following decompression is quantitatively similar to that observed in small animals [10,11,12]. We began by investigating the response to decompression of a number of different gels. We found the most reproducible results were obtained from agar gels and, accordingly, we have adopted these as our standard model system. We present evidence here to show that these gels do exhibit bubble formation during ultrasonic irradiation at intensities similar to those effective in vivo and may therefore provide a useful model for studying the phenomenon of acoustic cavitation under a variety of ultrasonic exposure conditions.

METHODS

a) In vivo experiments.

For all experiments male, Duncan Hartley derived, guinea pigs (University Park Farm) were used. They were selected from a stock pool as their weight reached 500 +/-10g. Before use they were fasted for 24hr. The guinea pigs were anaesthetised (Urethane, 1.5g/kg body weight, ip) for the duration of the experiment. A cross-section mid-way down the left hind limb was imaged using an 8MHz pulse-echo imaging system [13]. Before each experiment all hair was

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removed from the limb and a 25μm copper/manganin thermocouple was inserted into the limb such that it was central in the muscle and just above the section imaged by the diagnostic ultrasound. The guinea pig was placed in a perspex holder with the limb extended out and downwards from the body and secured in place. The diagnostic transducer (5mm diameter, focussed PZT-5A, 8MHz) was aligned 15mm behind the limb. Acoustic coupling was provided by a salt solution (NaCl 121mM, KCl 4.7mM, $MgSO_4$ 1.2mM). Acoustic cavitation was stimulated by irradiation with 0.75MHz continuous wave ultrasound using a 30mm diameter therapy transducer (Rank Sonacel Multiphon MkII). This transducer was positioned 25mm from the leg and perpendicular to the axis of the diagnostic transducer. The intensity of the ultrasound from the therapeutic transducer was increased in a stepwise fashion, with 5min given at each successive intensity. Ultrasound scanning with the diagnostic system began 5min before the start of irradiation with the 0.75MHz ultrasound and continued for the duration of the treatment. The ambient limb temperature was maintained by thermostating the acoustic coupling solution to the desired temperature of either 37 or 43°C. For the experiments at pressure, the entire experimental arrangement was placed inside a 35 litre pressure vessel and the pressure increased to 150kPa (absolute pressure) before the start of the experiment. The ultrasound images were recorded automatically on 35mm film at a rate of 1 every 2s. The films were subsequently coded, randomised and analysed 'blind'. Analysis (described in detail elsewhere [14]) comprised: construction of a control image from the images recorded prior to the irradiation with the 0.75MHz ultrasound, then identification of 'new' echoes (ie. those distinct from echoes from tissue interfaces recorded on the control images) by superposition of each subsequent frame onto this control. The location, time and duration of appearance of 'new' echoes was then recorded. An individual bubble was counted as a successive run of new echoes at a single site [14]. A minimum of 6 guinea pigs were used for each experimental series. The ultrasound intensity was measured using a tethered float radiometer [15] and the spatial average over the transducer face calculated. For the experiments at 37 and 43°C the spatial average intensities of the 0.75MHz ultrasound used were 60, 180, 300 and 680mW.cm^{-2}. For the experiments at 150kPa the intensities used were 180, 300, 680 and 1000mW.cm^{-2}. Acoustic pressure profiles were obtained using a calibrated PVDF (polyvinylidene difluoride) bilaminar membrane hydrophone (see fig 1).

b) In vitro experiments with Agar Gels.

The gels were prepared by adding 1.75g Agar Noble (Difco) to 100cm^3 cold tap water and gradually heated, in a water bath, to 100°C with continuous stirring. This produced a clear solution which was buffered to pH9, using Tri(hydroxymethyl)aminomethane (Sigma 7-9 Biochemical Buffer, Sigma). The gel solution was poured into cylindrical glass moulds 20mm in diameter and 20mm in height and the gels used within 6 to 24hrs. For the observation of bubbles the gels were supported on a glass stand with the plane face horizontal and the 0.75MHz therapy ultrasound transducer 40mm from the curved surface of the gel. Acoustic coupling was achieved by filling the surrounding tank with degassed water. Bubbles were observed either directly with a binocular dissecting microscope or with a television camera at a magnification of X10. The gels were individually irradiated for 5min at spatial average intensities of 60,

180, 300, 680 and 1000mW.cm^{-2}. Experiments were performed at ambient temperatures of 37 and 43°C. During irradiation the number of bubbles formed after each minute was noted. No significant difference was found between the number of bubbles observed using the microscope compared to the number observed using the television camera. Accordingly, for all subsequent experiments the television camera was used, both for reasons of convenience and because experiments could be recorded for further analysis.

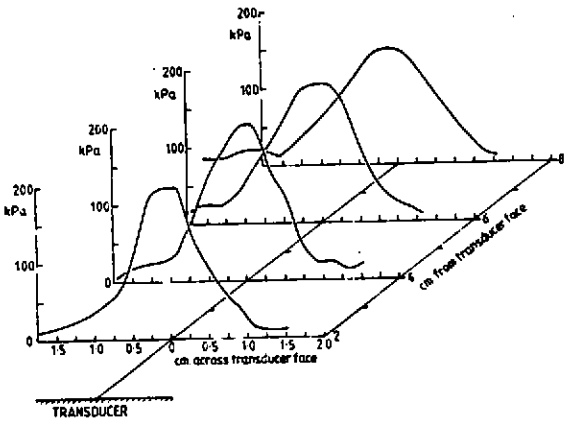

Fig 1. Transverse acoustic pressure profiles obtained using a calibrated PVDF membrane hydrophone at different positions in the field produced by the 0.75MHz transducer (Rank Sonacel Multiphon Mk II), using a spatial average intensity of 300mW.cm^{-2}.

An additional series of experiments was conducted using a clinical 2.25MHz diagnostic transducer (NE4328) driven by an Echo-encephalogram unit (Kretztechnik, Series 4100ME) to irradiate the gels. The pulse repetition frequency was set to 385Hz with the peak negative acoustic pressure 2.3MPa and the peak positive acoustic pressure 3.7MPa at the curved surface of the gel (40mm between transducer and gel). Details of the longitudinal variation of acoustic pressure from this transducer and an example of the waveform are shown in fig. 2. In these experiments the output power was fixed and the temperature of the gels was varied from 37 to 49°C. The total number of bubbles observed over a 20min irradiation period was counted.

RESULTS

a) In vivo experiments.

The great majority of the bubbles observed in the left hind limb of the guinea pig were less than 100μm in diameter (only bubbles >10μm diameter are recorded), with some (<15%) between 100 and 500μm in diameter. At the higher temperature (43°C) approximately twice as many sites of bubble formation were observed at each intensity than occurred at 37°C. For the experiments where the ambient pressure was increased to 150kPa the number of sites of bubble formation was similar to that observed at 37°C and atmospheric pressure (100kPa).

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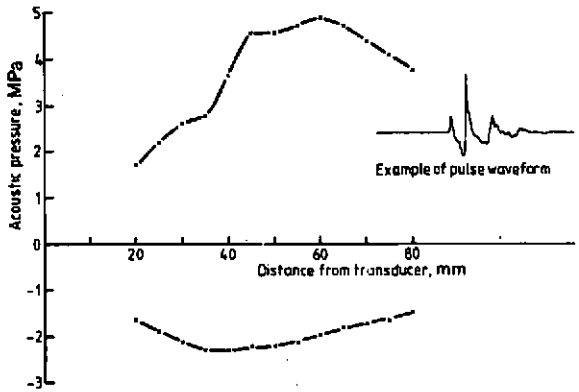

Fig 2. Longitudinal plots of acoustic pressure in the field produced by a 2.25MHz transducer (NE4328) driven by a Kretztechnik Echo-encephalograph Series 4100 ME measured using a PVDF bilaminar membrane hydrophone. Inset is an example of the wave form from this transducer.

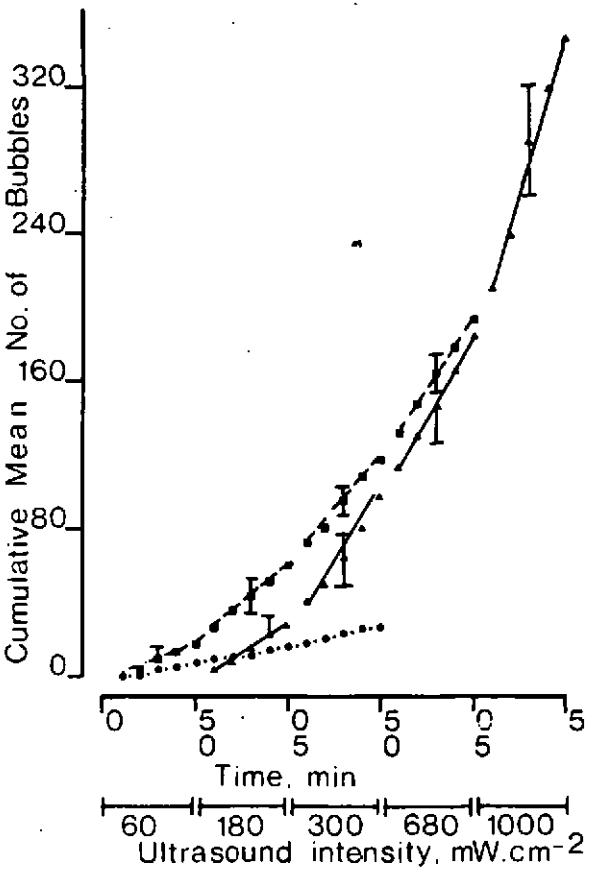

Fig 3. Cumulative mean number of bubbles observed in a cross-section of guinea pig hind limb: a) control experiments (circles), no irradiation with 0.75MHz ultrasound (mean values from 30 animals). b) temperature of leg 37°C and atmospheric pressure (100kPa) (squares) and irradiation with 0.75MHz ultrasound at spatial average intensities of 60, 180, 300 and 680mW.cm^{-2} with 5min at each intensity and a stepwise increase to the next intensity (mean values from 6 animals). c) temperature of leg 37°C and ambient pressure 150kPa (triangles) and irradiation with 0.75MHz ultrasound at spatial average intensities of 180, 300, 680 and 1000mW.cm^{-2} with 5min at each intensity and a stepwise increase to the next intensity (mean values from 7 animals). Bars show 1 standard deviation.

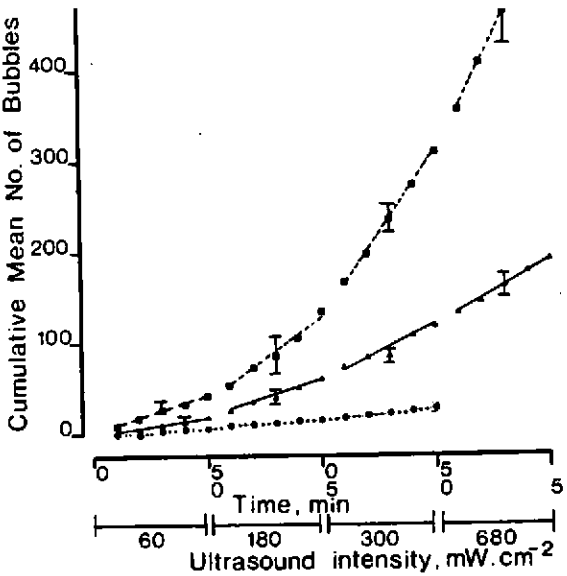

Fig 4. Cumulative mean number of bubbles observed in a cross-section of guinea pig hind limb: a) control experiments (circles), no irradiation with 0.75MHz ultrasound (mean values from 30 animals). b) temperature of leg 37°C and atmospheric pressure (100kPa) (triangles) and irradiation with 0.75MHz ultrasound at spatial average intensities of 60, 180, 300 and 680mW.cm^{-2} with 5min at each intensity and a stepwise increase to the next intensity (mean values from 6 animals). c) temperature of leg 43°C and atmospheric pressure (100kPa) (squares) and irradiation with 0.75MHz ultrasound at spatial average intensities of 60, 180, 300 and 680mW.cm^{-2} with 5min at each intensity and a stepwise increase to the next intensity (mean values from 6 animals). Bars show 1 standard deviation.

The cumulative bubble counts from the experiments at 37°C at atmospheric pressure (100kPa) and at 150kPa are shown in fig. 3. It should be noted that the experiments at 150kPa began with the 0.75MHz ultrasound intensity at 180 not at 60mW.cm^{-2}. The cumulative bubble counts from the experiments at 37 and 43°C are shown in fig. 4.

The major difference observed is that while the threshold for bubble formation appears to have increased with the increase in pressure the subsequent rate of accumulation of bubbles is similar to that at atmospheric pressure, whilst with increased temperature the threshold stays the same but the subsequent rate of accumulation is increased.

A total of 30 control experiments were performed in which the guinea pigs were prepared in the usual way and scanned for 15min with the 8MHz diagnostic ultrasound. These experiments provided baseline data which was compared to the data obtained during irradiation with the 0.75MHz ultrasound and were designed to reveal the extent of 'false positive' scoring of bubbles caused by animal movements during ultrasound scanning. This results in echoes from tissue

interfaces being intermittently recorded such that, on analysis, these echoes cannot be distinguished from those due to bubbles. The number of bubbles that occurred during the experimental procedures and the results of the control experiments are summarised in table 1.

Table 1. Mean number of Bubbles per min. in cross-section of Guinea Pig hind limb during irradiation with 0.75MHz continuous wave ultrasound: a) with limb temperature 37°C and ambient pressure atmospheric (100kPa), b) with limb temperature 37°C and ambient pressure 150kPa, c) with limb temperature 43°C and atmospheric pressure. (Standard deviation shown in brackets)

Ultrasound Intensity $mW.cm^{-2}$	Time (min)	Mean Number of Bubbles.min^{-1}			
		Control (No Ultra-sound) (SD) N=30	37°C		43°C
			100kPa (SD) N=6	150kPa (SD) N=7	(SD) N=6
60	1	0.5 (1.3)	0.8 (1.2)		7.8 (5.5)
	2	0.9 (1.9)	2.3 (2.2)		11.2 (8.9)
	3	1.4 (3.5)	6.0 (5.8)		7.3 (6.5)
	4	2.0 (4.0)	3.8 (4.1)		7.5 (3.0)
	5	1.9 (4.9)	4.2 (2.6)		8.3 (8.6)
180	1	2.3 (4.0)	8.7 (7.1)	3.3 (6.6)	13.3 (8.5)
	2	1.3 (2.6)	9.5 (8.3)	4.4 (6.6)	15.3 (11.0)
	3	1.9 (3.5)	7.7 (5.0)	6.7 (7.4)	14.0 (8.9)
	4	2.2 (3.9)	7.7 (5.5)	7.7 (11.3)	22.5 (13.4)
	5	1.9 (3.3)	8.7 (4.3)	5.4 (4.6)	28.2 (16.7)
300	1	2.3 (3.8)	12.3 (5.1)	12.7 (9.8)	30.3 (15.3)
	2	2.1 (3.2)	8.5 (2.2)	8.6 (5.9)	32.5 (15.5)
	3	2.3 (4.8)	14.5 (3.3)	14.6 (13.6)	38.7 (14.6)
	4	2.6 (4.4)	13.3 (4.8)	16.9 (15.4)	37.0 (11.1)
	5	1.9 (3.3)	9.0 (3.4)	16.3 (13.6)	37.5 (15.6)
680	1		14.5 (7.6)	16.7 (19.4)	43.8 (22.0)
	2		15.3 (7.1)	17.0 (18.6)	53.2 (24.0)
	3		17.0 (10.2)	15.9 (19.6)	53.3 (29.3)
	4		13.2 (5.6)	18.9 (17.5)	57.3 (19.5)
	5		14.7 (8.0)	18.9 (20.0)	46.8 (14.1)
1000	1			26.1 (26.6)	
	2			28.3 (31.7)	
	3			25.7 (18.7)	
	4			28.1 (21.8)	
	5			27.1 (24.5)	

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The temperatures in the guinea pig leg, were always 1-2°C lower than the temperature of the surrounding acoustic coupling medium (37 or 43°C) but in an individual animal were observed to vary by less than 0.3°C. The results presented are thus unlikely to be influenced by temperature fluctuations.

b) In vitro experiments.

The numbers of bubbles.cm^{-3} observed in the 1.75% agar gels at 37 and 43°C are shown in fig. 5. In the case of the experiments at 37°C only 4 gels per intensity were used. However, for the experiments at 43°C 30 gels were used at 60, 180 and 300mW.cm^{-2} and 6 gels at 680 and 1000mW.cm^{-2}. Despite some rather pronounced scatter in the results the trends, illustrated by the lines drawn, are clear. Increasing the intensity of ultrasonic irradiation causes an increase in the number of bubbles and, at a given intensity, the higher ambient temperature causes more bubbles to be formed. The gels exhibit the characteristic variability in the extent of bubble formation seen in vivo (see Table 1). A total of 24 gels were prepared as normal but before irradiation with 0.75MHz ultrasound at a spatial average intensity of 1000mW.cm^{-2} they were hydrostatically compresed to 2MPa. This pressure was maintained for 2min and they were then rapidly decompressed and irradiation begun 5min later. In none of the gels were any bubbles observed during the irradiation with the 0.75MHz ultrasound.

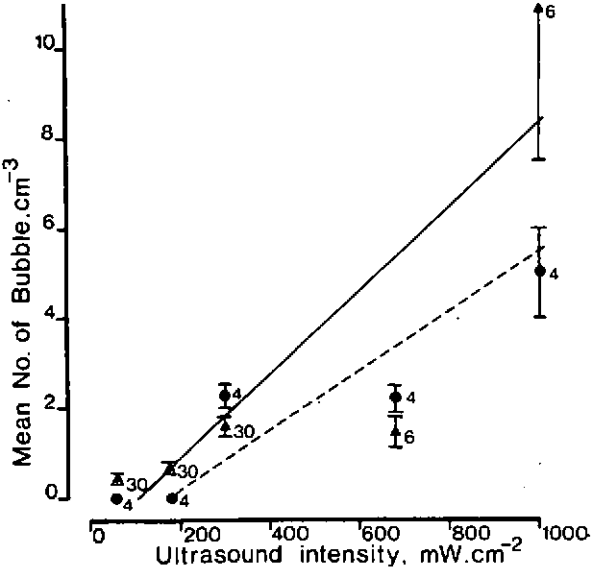

Fig 5. Mean number of bubbles over 5min in 1.75% agar gels (Agar Noble, Difco) irradiated with 0.75MHz ultrasound at spatial average intensities from 60 to 1000mW.cm^{-2} at temperatures of 37°C (circles) and 43°C (triangles). The number of gels at each intensity is shown at the appropriate mark. Bars show 1 standard error of the mean. The number of bubbles observed is expressed per cm^3 of gel. The gel volumes were typically 5cm^3.

The results of irradiating 1.75% agar gels with microsecond pulses of 2.25MHz ultrasound at temperatures from 37 to 49°C are shown in fig 6. Below 40°C no bubbles were observed, however above this temperature a progressive increase in the number of bubbles formed was seen with increasing temperature. It is possible that some of the bubbles observed at the highest temperature used (49°C) formed because of the reduced solubility of gas in the gel and experiments are in progress to test this hypothesis.

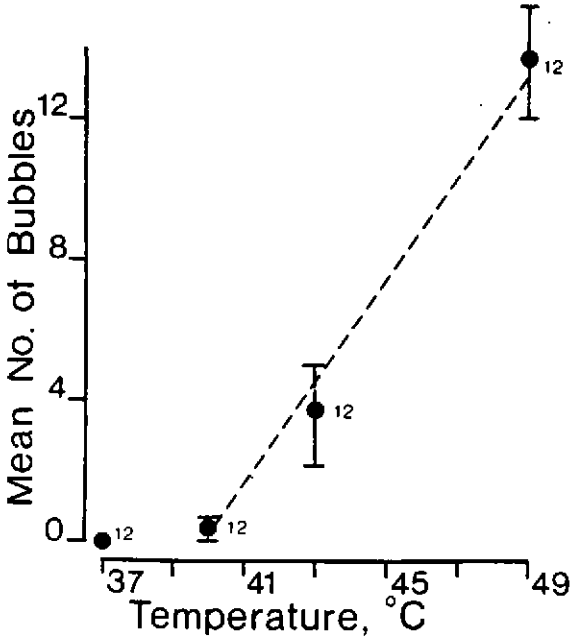

Fig 6. Mean number of bubbles over 20min in 1.75% agar gels (Agar Noble, Difco) irradiated with 2.25MHz pulsed ultrasound. Pulse repetition frequency was 385Hz, the peak negative acoustic pressure 2.3MPa and the peak positive acoustic pressure 3.7MPa at the face of the gel. 12 gels were used at each of the temperatures studied - 37, 40, 43 and 49°C. Bars show 1 standard error of the mean.

DISCUSSION

The number of bubbles observed in the guinea pig limb (Table 1) suggests that increasing the ambient pressure from atmospheric (100kPa) to 150kPa during irradiation with 0.75MHz ultrasound at 37°C increases the threshold intensity for bubble formation. In order to take account of the combined effects of time and intensity of ultrasonic irradiation the number of bubbles was compared to those in the controls using analysis of covariance [16]. This analysis revealed a significant difference (at the 95% confidence level) between the number of bubbles arising during ultrasonic irradiation at an intensity of 180mW.cm^{-2} when compared to controls. On the other hand when the ambient pressure was raised to 150kPa no significant difference is evident with ultrasonic irradiation at an intensity of 180mW.cm^{-2}. To achieve a

significant increase in the number of bubbles under these conditions required irradiation with 300mW.cm^{-2}. Thus, we estimate that the critical intensity threshold for bubble formation during ultrasonic irradiation at 37°C lies between 60 and 180mW.cm^{-2} and that raising the ambient pressure to 150kPa increases this threshold to 180 - 300mW.cm^{-2}. Using the established formula relating intensity (I) to acoustic pressure (P), assuming a plane wave approximation [17]:

$$I = P^2 / (2\rho c) \qquad (1)$$

equivalent threshold acoustic pressures can be calculated as 40 - 70kPa for bubble formation at atmospheric pressure and 70 - 100kPa for bubble formation at an ambient pressure of 150kPa.

Corresponding comparison of the number of bubbles formed during ultrasonic irradiation at 43°C gives an estimate for the threshold intensity as 60 - 180mW.cm^{-2} (equivalent acoustic pressure 40-70kPa). Thus the critical intensity thresholds for bubble formation are in the same range for both temperatures used (37 and 43°C). However, inspection of fig. 4 (and Table 1) shows that above this threshold more bubbles are formed, at a given intensity, when the higher temperature is used. Furthermore, at a given intensity, with the higher temperature the rate of accumulation of bubbles is greater.

The temperatures used span the range encountered in cancer treatment by hyperthermia, and in the course of ultrasound physio-therapy mild temperature elevations are expected. The specific biological implications of these findings remain to be determined.

In the 1.75% agar gels the critical intensity threshold for bubble formation, at both 37°C and 43°C, appears to lie between 60 and 180mW.cm^{-2} (equivalent acoustic pressures 40-70kPa) (see fig.5). In addition, at the higher temperature the trend is for more bubbles to be formed in the given time (5min), with this effect becoming more pronounced at higher irradiation intensities. There is, therefore, the same pattern of response to acoustic irradiation in the gels as is seen *in vivo*. Furthermore, the bubble formation threshold is in the same range. This, taken together with the observation that hydrostatic pressure pre-treatment of the gels prevents bubble formation in the same manner as is seen *in vivo* [18], suggests that the nuclei from which the bubbles originate are very similar. Quantitatively, it would appear that the number of bubbles (in equal volumes) is higher *in vivo* by a factor of 10 than *in vitro*. However, when the fact is taken into account that *in vivo* there is active transport of bubbles through the region studied this difference does not seem to be significant. We suggest, therefore, that agar gel is an ideal medium to study the way in which acoustic irradiation causes bubble formation without the limitations imposed by animal work.

There are indications from theoretical work [19] and more recently from experimental studies in water [20] that microsecond duration pulses, such as those associated with diagnostic ultrasound systems, may lead to acoustic cavitation. Indeed, we have found (fig. 6) that a clinical diagnostic system will generate bubbles at temperatures above 40°C. The negative acoustic

pressure excursion in the pulse at the face of the gel, with the system used, was 2.3MPa. Clearly, this finding indicates that further investigation of short duration pulsed systems should be undertaken, since there appears to be both a critical temperature as well as critical acoustic pressure for bubble formation and that these are inter-related.

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INTRACELLULAR DNA BREAKAGE: AN INVESTIGATION OF THRESHOLDS

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The effect of ultrasound on DNA structure and metabolism has been studied under a variety of conditions in different cell types. Repacholi (1981) has carried out detailed investigations into the effect of therapeutic ultrasound on DNA synthesis in stimulated human lymphocytes. He found that the incorporation of radioactive precursors into DNA was significantly inhibited but returned to normal over a period of 3 days. A large number of other investigations have shown that ultrasound at a variety of wavelenths and intensites does perturb DNA replication but unfortunately not in any reproducible fashion. Liebeskin (1979) and Repacholi & Kaplan (1980) have found that DNA repair synthesis is stimulated during and after pulsed ultrasound. These results and those obtained for the effect of ultrasound on DNA structure are variable and difficult to interpret.

It is a possibility that the various methodologies used are simply not sensitive enough to detect the relatively small amount of damage that ultrasound does to DNA. It was for this reason that we have used a DNA nucleoid analytical system to study the production (if any) of ultrasound induced single stranded breaks in DNA. Ultrasound may induce single standed breaks directly by physical interaction between the radiation and DNA. Alternatively the ultrasound might cause DNA damage where the various repair systems themselves produce single stranded breaks which may either be transient or long lived.

The DNA nucleoid method of Cook & Brazell (1976) was developed originally to study double stranded breaks in DNA. It has been successfully adapted to measure single stranded breaks on a neutral sucrose gradient by Weniger (1979). The cells whose DNA may have been damaged are gently lysed on the top of a neutral sucrose gradient (15-30% w/v). The cells release their DNA as a highly ordered structure which is stabilised by residual membranes. This DNA exhibits its normal supercoiled structure. Each and every single stranded break present in the DNA increases the possible free rotation of the DNA moleculre which therefore decreases the number of superhelical turns. This leads to a less compact structure which therefore has a decreased sedimentation constant. The nucleoid analysis is better than ten times more sensitive than the alkaline sucrose gradient analysis, i.e. it is possible to reproducibly detect 1 single stranded break per 10^{10} Dalton. Furthermore the centrifugation is carried out in the presence of ethidium bromide. This dye removes the negative supercoils but at the high concentration used in the gradient (30 g/ml) the ethidium bromide establishes positive supercoils so that the sedimentation behaviour of the DNA is similar to that at zero concentration of the dye. Thus no fractionation of the gradient is needed.

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Method

Insonation of the mouse L1210 cells was performed in cylindrical nylon chambers where the chamber was 4mm diameter and 10mm in length. When the cell suspension was in place within the chamber it was sealed in at each end with ultrathin polyethylene membranes and mounted with its axis vertical in a bath of degassed water at 8°C above a 3MZ transducer operated under CW. conditions. The transducer was 2.5CM in diameter and the beam foocussed to 5CM using a perspex lens with the chamber located at the focus. The total power was found to be 10.5W and, from hydrophone measurements of beam areas at lower powers, the spatial average intensity at the focus was estimated to be approximately 400W/CM^2. Significant temperature rises in the cells in suspension should not have occurred under these circumstances (Love & Kremkau,1980). Wave reflections from the water/air interface were reduced by the use of carpet. The cells were exposed to ultrasound for 30 minutes after which time they were lysed on a nucleoid gradient.

A linear sucrose (15-30% w/v) gradient was set up (13 mls) in Beckman SW27.1 ultracentrifuge tubes. The gradient contained 1.5ml NACl, 0.2M Tris. HCl pH 8.0. ImM EDTA Na_2 and 30 g/ml ethidium bromide. The sucrose was overlayered with 0.4mls lysis buffer (1.5M NaCl, 0.1M EDTAN_2 pH 8.0 and 0.7% Triton-X-100). Mouse L1210 cells (about 2 x 10^5 cells) in serum free medium were carefully layered on top of the lysis layer.

The ultracentrifugation was started after 30 minutes of lysis and centrifuged at 23,000 rpm for 90 minutes. Visualisation of the completed intact gradients was performed with an ultraviolet transilluminator at 350 nM.

Result and Conclusion

If the above ultrasound produced one or more single stranded breaks per 10^{10} Daltons the nucleoids so produced would not sediment as far down the gradient as nucleoids from undamaged cells. For each experiment, control cells (3 gradients) and isonated cells (3 gradients) were run simultaneously. A total of 37 experiments were carried out. In all these experiments the insonated nucleoids sedimented indistinguishably from the control cells. That is, there is no evidence that these doses of ultrasound cause DNA damage which results in single stranded breaks.

INTRACELLULAR DNA BREAKAGE: AN INVESTIGATION OF THRESHOLDS

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