Exp. Biol. (1968), **49**, 61–70
With 7 text-figures
Printed in Great Britain

THE EFFECT OF ULTRASONIC IRRADIATION ON THE SURVIVAL OF *DAPHNIA MAGNA*

BY P. N. T. WELLS

Department of Zoology, University of Bristol, and Department of Medical Physics, United Bristol Hospitals

(*Received* 26 *January* 1968)

INTRODUCTION

Recently, ultrasound has become quite widely used in medicine, both in surgery and diagnosis (see, for example, Newell (1963*b*)), but the mechanisms of the biological effects of ultrasonic irradiation are still the subject of speculation. It has been known for many years that ultrasonic irradiation can be lethal to small organisms. Wood & Loomis (1927) described how filaments of *Spirogyra* were torn to pieces by ultrasound at a frequency of 300 kHz., at an intensity of about 10 W./cm.$^{-2}$. Schmitt, Olson & Johnson (1928) irradiated Protozoa at a frequency of 750 kHz.: large specimens were killed, but small ones survived. Johnson (1929) noticed that the rate of destruction of Protozoa and red blood cells was accelerated when minute gas bubbles were formed in suspension by ultrasonic cavitation.

Harvey (1930) distinguished between two thermal effects. These were the heating of the medium due to the absorption of the ultrasound, which could be counterbalanced by cooling, and local heating at interfaces in rapid vibration, where thermal conduction was poor. He concluded that the mechanism of cell destruction by cavitation was due to the action of minute cavitated air bubbles in the medium. The following year, Harvey & Loomis (1931) observed the effects of ultrasonic irradiation on *Arbacia* eggs by high-speed photomicrography. The eggs were drawn out into spindle or tadpole shapes, which suggested that rapid tearing movements of the fluid might have been responsible for the destruction.

Ultrasound is absorbed as it passes through tissues. The rate of absorption increases with the frequency (see, for example, Dunn, (1965)). At medium and high frequencies, absorption can cause significant heating. For this reason, it is sometimes thought that the biological effects of ultrasound are due to heat alone. Thus, Herrick (1953) irradiated various biological structures with ultrasound at a frequency of 800 kHz., and at powers up to 10 W., from a 5 cm.^{2} radiator. She demonstrated the selective heating of nerve and bone, and described the blocking of nervous conduction. Differential study of various fibres in frog sciatic nerve indicated that an analgesic application was unlikely, and she concluded that heat was responsible for the effects which she observed. A similar conclusion was reached by Lehmann (1953), who also suggested that a decrease of the diffusion layer at an interface by mechanical stirring might be a mechanism for increased metabolite exchange. However, Fry (1953) analysed the various effects of temperatures produced during ultrasonic irradiation of nervous tissue, and gave evidence chosen to show that the biological effects could not

be satisfactorily explained by considering heat alone. On the basis of the results of experiments made using frequencies around 20 kHz., Hughes & Nyborg (1962) concluded that streaming and other activity around sonically induced bubbles is a cause of damage to living cells. Hawley, Macleod & Dunn (1963) also considered that streaming is important, but in their experiments on DNA, they were careful to eliminate cavitation. Curtis (1965) irradiated the livers of intact mice with ultrasound at a frequency of 1 MHz.: he thought that heat could have caused the histological damage which was found, in the intensity range 10 to 60 W. cm.$^{-2}$, but that some other effect became important at higher intensities.

In medical applications, the frequency of ultrasound is commonly in the range 1–5 MHz. At these frequencies, biological materials absorb ultrasound quite rapidly and it is important, from the point of view of possible hazard, to distinguish between the mechanical and thermal effects of irradiation. The investigations described in this paper were designed to differentiate between these two effects of ultrasonic irradiation, at a frequency of 3 MHz., on the survival of the water flea, *Daphnia magna*.

MATERIAL AND METHODS

Biological material

Colonies of about 500 specimens of the water flea *Daphnia magna* were collected as required from freshwater ponds, and stored in an aquarium of about 3000 ml. capacity. The aquarium was filled with pond water, which was changed every 2–5 days. A suspension of yeast was used to feed the colony, at the rate of about 1 mg. of yeast per animal per day. The temperature of the aquarium was held at about 18° C.

Animals of about 0·2 cm. length were visually selected for the experiments, and removed from the aquarium with the aid of a small pipette fitted with a rubber suction bulb.

Apparatus for irradiation

The apparatus shown in Fig. 1 was constructed to permit the irradiation of single specimens of *Daphnia*. The ultrasonic transducer was a disk of lead zirconate titanate of 0·95 cm. diameter and 0·07 cm. thickness. This thickness corresponds to a resonant frequency of about 3 MHz. The transducer mounting was surrounded by a jacket through which water was circulated for temperature control. The length of the water jacket in front of the transducer was 4·7 cm.: thus, all the Frésnel zone fell within the jacket. The jacket was terminated by a thin polythene window, which just dipped into the water-filled cavity in the Perspex block (*a*). The base of this cavity, which was cemented to block (*a*), is referred to as the upper polythene window in Fig. 1. The irradiation cell was placed in the water-filled irradiation chamber in block (*b*); this cavity was separated from block (*c*) and the circulating water in the absorbing system by the lower polythene window. An animal could be placed in the irradiation cell by removing the transducer assembly from its supporting bracket, and lifting block (*a*) from block (*b*). Careful reassembly prevented air bubbles from becoming trapped in the ultrasonic path.

Ultrasound leaving the irradiation chamber was arranged to travel into an absorbing system, to minimize the formation of standing waves. The absorber consisted of sheets of neoprene, arranged as a main line termination. The energy reflected from the

absorber was less than 0·1 % of the incident energy, measured by a pulse-echo method. The temperature of the irradiation chamber could be controlled by means of a pumped circulation through the absorbing system, the water jacket of the transducer, and a suitable heat exchanger.

The transducer was energized at resonance from a generator capable of delivering a maximum electrical power of about 50 W. The ultrasonic power was measured by a radiation pressure balance similar to that of Newell (1963*a*). The efficiency of the transducer was about 35%. The intensity distribution of the ultrasonic beam was

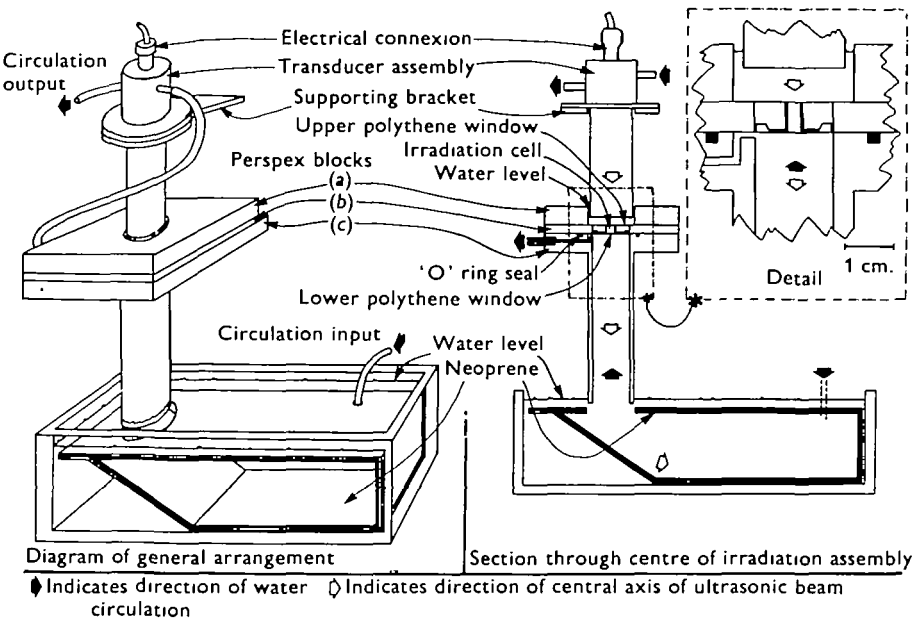

Fig. 1. Apparatus for irradiation of a single specimen of *Daphnia*.

measured by the thermocouple method of Fry & Fry (1954). Data calculated from the measurements are shown in Fig. 2. The solid black area on this graph represents the intensity variation within the irradiation chamber, with the relative intensity of unity corresponding to the intensity at the centre of the chamber. At any particular distance across the ultrasonic beam from its central axis, the greatest value of the relative intensity corresponds to the intensity at the upper polythene window (see Fig. 1), and the lowest value, to that at the lower polythene window. The intensity, expressed in W. cm.$^{-2}$, at the centre of the irradiation chamber was calculated to be 2·9 times the total ultrasonic power expressed in watts.

The irradiation cell, which restrained the animal in the centre of the ultrasonic beam within the irradiation chamber, is shown in Fig. 3. The cell was required to locate the organism accurately, but to have the minimum effect upon the ultrasonic beam. Experimental cells were constructed of polythene, P.V.C., Perspex, Tufnol and Nylon. Each cell was irradiated under identical conditions and the temperature rise in the centre was measured with a 50 μ diameter thermocouple, inserted through the two small holes in the walls of the central cylinder. A method similar to that of Hawley

Breyer & Dunn (1962) was used to construct the thermocouple. The Perspex cell was chosen for the subsequent experiments, because it caused the smallest temperature rise (8° C. in 2 min. with an ultrasonic power of 10 W.).

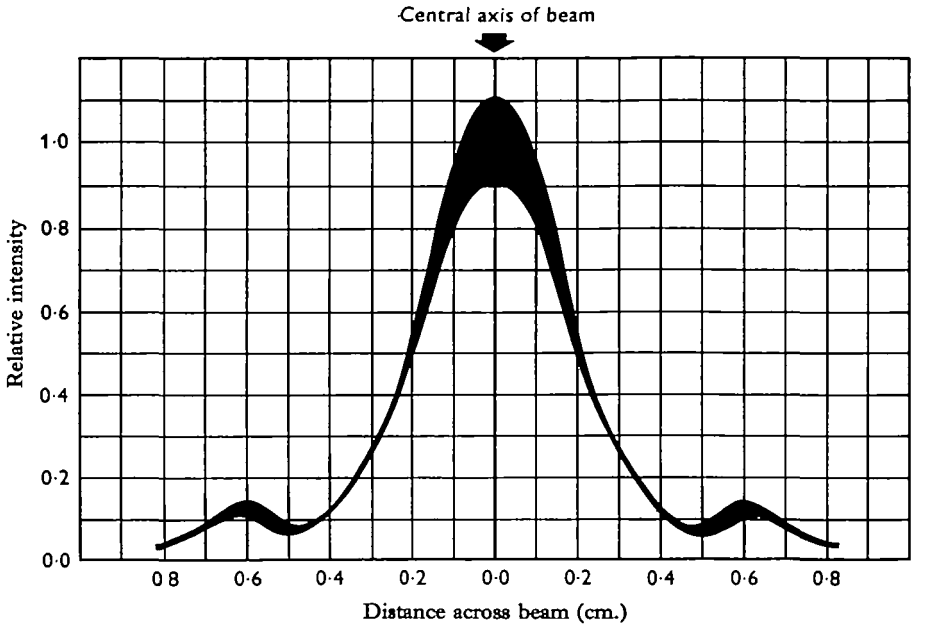

Fig. 2. Ultrasonic intensity distribution, within the volume of the irradiation chamber.

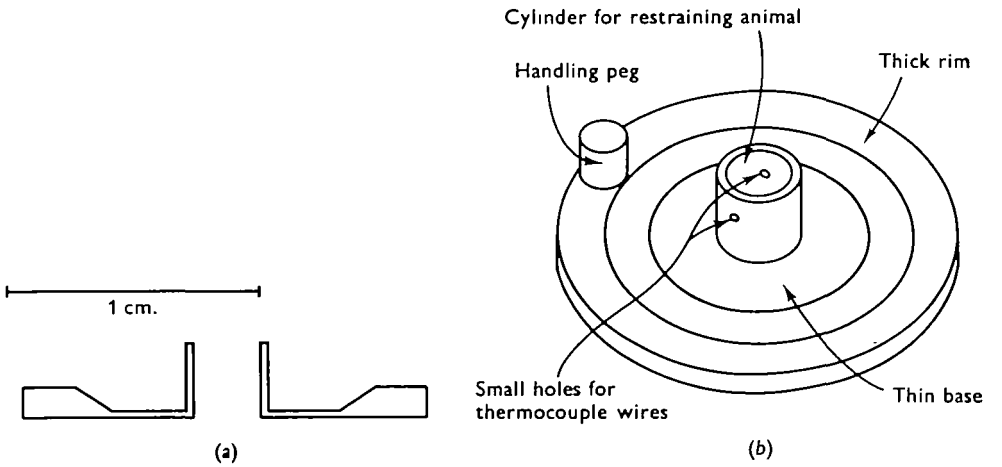

Fig. 3. The irradiation cell. (*a*) Section in plane of central axis of ultrasonic beam.

Temperature measurements

A technique was developed for the insertion of a 50 μ diameter thermocouple through the carapace of a specimen of *Daphnia*, so that the internal temperature might be measured. A binocular microscope and a micro-manipulator were used for this

procedure. Sometimes, an animal would survive the insertion, but, in any event, the temperature measurements were made within 1 hr. of the time of the insertion.

An animal thus impaled could be introduced within the irradiation cell, so that its internal temperature might be measured under various conditions of irradiation and cooling.

RESULTS

Two main groups of experiments were performed. The first was concerned with the immediate mortality which resulted from the exposure of *Daphnia* to a variety of ultrasonic and thermal conditions. The second was concerned with the survival time following exposure to heat and to ultrasound.

(i) *Short-term experiments*

Groups of animals were subjected to exposure for 2 min periods to elevated temperatures. The heat cycle was obtained by starting with the animals in a small volume of water, adding a larger volume of water at a temperature calculated to raise the mixture to the required level, and finally adding an even larger volume at a temperature calculated to return the mixture to the original temperature. The rate of change of temperature at the beginning and end of typical cycles was measured by means of a thermocouple imbedded within a single animal, as previously described; for example, a change of 20° C. was achieved within about 10 sec. Eleven groups of twenty animals were tested for mortality within the range 30–55° C.: the results are included in Table 1. The relevant data correspond to zero ultrasonic power. It can be seen that no animal died as a result of exposure for 2 min. to a temperature of 37·5° C.; but none survived exposure for the same time to a temperature of 40·0° C.

Table 1. *Survival of groups of* Daphnia *resulting from exposure for 2 min. periods to various thermal and ultrasonic conditions*

(The table shows the limits of ultrasonic power and temperature for 100% survival and 100% mortality.)

	100% survival			100% mortality		
Circulation temp. (°C.)	Ultrasonic power (W.)	Max. temp.* (°C.)	Max. temp.* range (°C.)	Ultrasonic power (W.)	Max. temp.* (°C.)	Max. temp.* range (°C.)
18·0	0	37·5	1·0	0	40·0	1·0
31·3	2	38·2	1·7	4	42·0	3·3
22·7	7	38·0	3·3	10	41·7	3·6
13·5	8	30·0	2·5	10	32·5	3·6

* Values measured and calculated from solution temperatures, and estimated from Fig. 5.

Recordings were made of the internal temperature, measured by means of thermocouples, of three separate animals, during 2 min. exposure within the irradiation cell, to various ultrasonic powers. A typical result, in which the several records are superimposed in time, is shown in Fig. 4. The maximum temperature rise which would occur within an animal subjected to irradiation at power levels of up to 15 W. was calculated from these data. The results are shown in Fig. 5.

The mortality which occurred as a result of irradiation for 2 min. at various powers in five groups of twenty animals was observed, with three different circulation temperatures. These data, together with the corresponding maximum temperatures reached

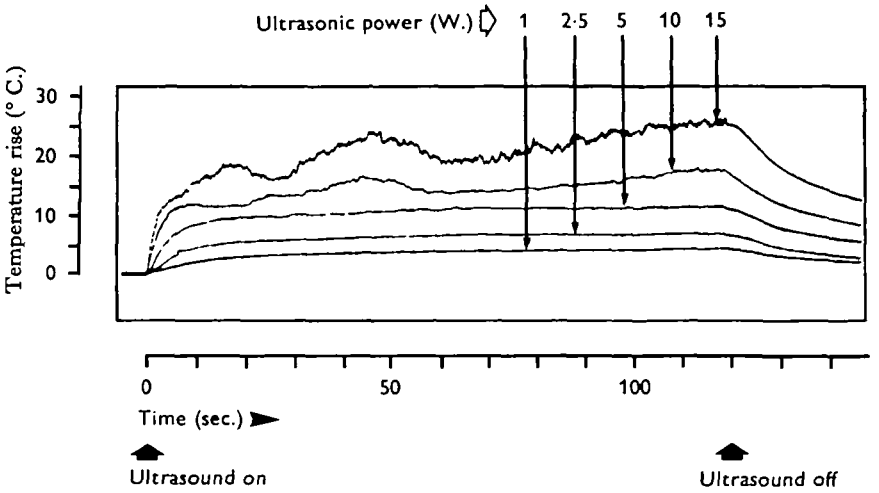

Fig. 4. Temperature changes induced in *Daphnia* by ultrasound: typical recordings.

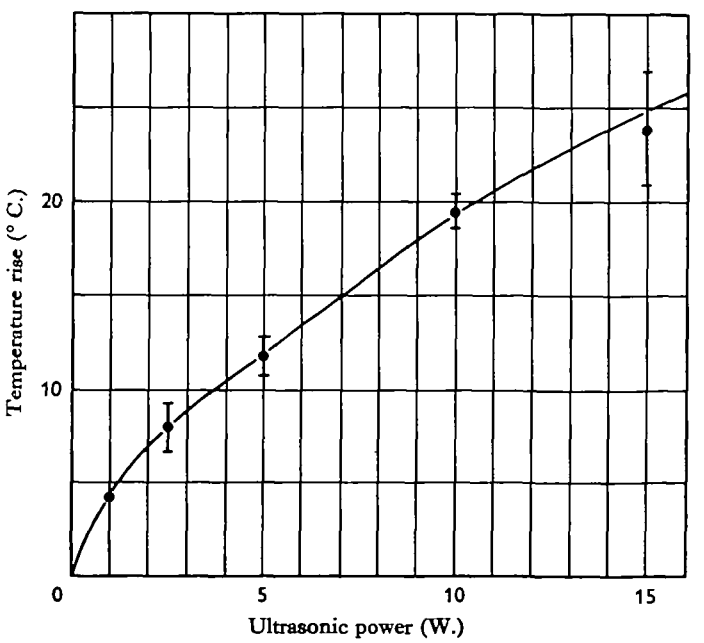

Fig. 5. Maximum temperature rise induced in *Daphnia* by ultrasound. 2 min. exposure periods.

by the animals during irradiation (estimated from Fig. 5), are included in Table 1. It can be seen that survival was heat-dependent at ultrasonic powers of up to 7 W.; at powers of 8 W. and more, survival was no longer heat-dependent alone, and, at

10 W., all the animals died even when the estimated maximum temperature during irradiation was below the level for 100% survival for heat exposure alone.

The results given in Table 1 are shown in graphical form in Fig. 6. This figure illustrates the limits for 100% survival and 100% mortality, for the combined effects of the various thermal and ultrasonic conditions investigated.

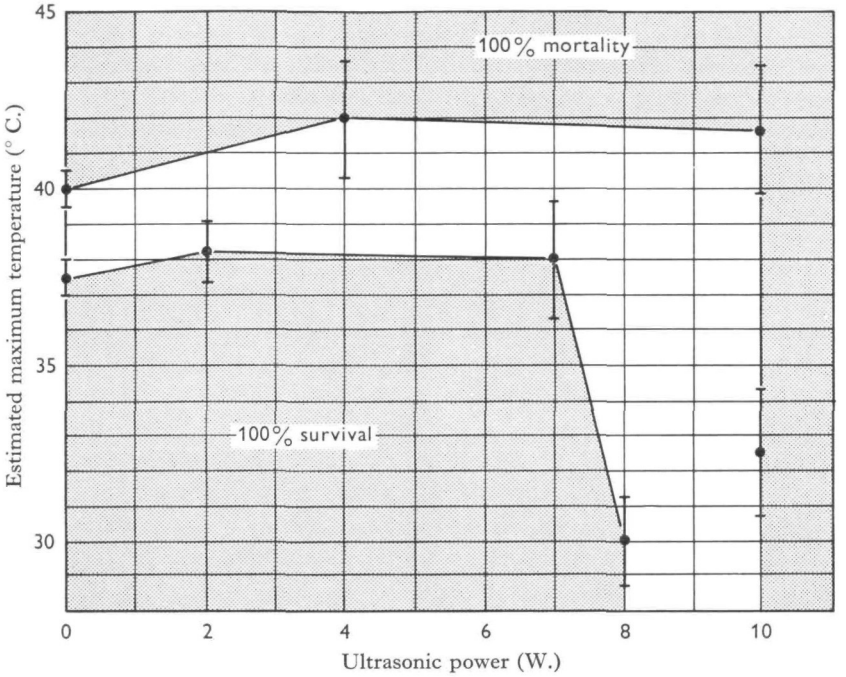

Fig. 6. Graph showing limits of 100% survival and 100% mortality of *Daphnia*, for various thermal and ultrasonic conditions. 2 min. exposure periods.

Table 2. *Mean survival time* (M.S.T.) *of groups of* Daphnia *following exposure for 2 min. periods to various thermal and ultrasonic conditions*

(Temperature maintained below the lethal level.)

Ultrasonic power (W.) or equivalent heat cycle	Heat alone		Ultrasound	
	M.S.T. (hr.)	S.D. (hr.)	M.S.T. (hr.)	S.D. (hr.)
1	126	30	170	26
3	154	32	148	32
5	134	36	132	30
7	—	—	14	4
10	106	46	2	—
13	—	—	0	—

(ii) *Long-term experiments*

Three groups of between thirty and sixty animals were selected for each ultrasonic power investigated. One group was set aside as a control. The animals in the second group were each irradiated for a period of 2 min. The circulation temperature of the

irradiation apparatus was controlled at about 18° C., except in the experiments where the ultrasonic power was greater than 5 W. For powers in excess of 5 W. the circulation temperature was chosen so that the estimated maximum temperature of the animal was well below the lethal level for heat alone. The third group of animals was subjected to a 2 min. heat cycle which raised the temperature to the maximum estimated (from Fig. 5) to have occurred in the irradiated group. A total of 2080 individual counts were made of 540 animals during these experiments.

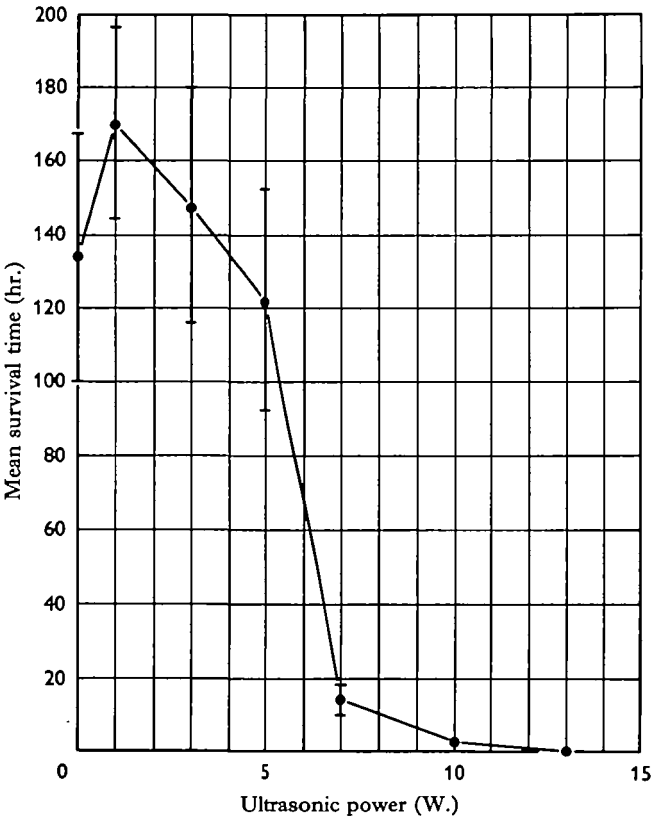

Fig. 7. Mean survival time of *Daphnia*, following ultrasonic irradiation. Temperature maintained below the lethal level. 2 min. exposure periods.

The number of animals which remained alive in each sample was counted at regular intervals, appropriate to the mortality rate. After each count the survivors were placed in fresh pond-water, and were fed at the rate of 1 mg. of yeast per animal per day.

The mean survival time of each group was calculated by probit analysis, and the results are set out in Table 2. It can be seen that there was no significant difference between the mean survival times of the control groups, the heated groups, and, for powers not greater than 5 W., the ultrasonically irradiated groups. At ultrasonic powers of 7 W. and more, the M.S.T. of the irradiated groups was significantly reduced. A graph of M.S.T. plotted against ultrasonic power is shown in Fig. 7: the M.S.T. corresponding to zero ultrasonic power is that of the control groups.

DISCUSSION

The temperature rise measured by a thermocouple inserted within the carapace of a specimen of *Daphnia* was probably an accurate indication of the maximum temperature rise which actually occurred anywhere in the body. It is difficult to imagine how the body surface temperature could have differed significantly from the internal temperature, for this would have required the existence of large temperature gradients.

Table 1 shows that, when subjected to heat alone, no animals died as a result of exposure to temperatures below 37·5° C., and none survived exposure to temperatures above 40·0° C. The rate of change of temperature in these experiments was rather more rapid than that experienced during the corresponding ultrasonic irradiations. Within the limits of experimental accuracy, survival following ultrasonic irradiation was temperature-dependent for circulation temperatures of 31·3 and 22·7° C. However, with a circulation temperature of 13·5° C., no animals survived irradiation at 10 W., although the estimated temperature (32·5° C.) was below the lethal level. Thus, there was a threshold level of power below which the immediate effect of irradiation on survival was purely thermal. It is interesting to note that streaming appeared to become significant at a power level of between 5 and 10 W. (see Fig. 4), and that the non-thermal cause of death might have been associated with this streaming effect. The threshold level for mechanical damage lay between 8 and 10 W., corresponding to an intensity at the centre of the irradiation cell of between about 23 and 29 W. cm^{-2}. The existence of this threshold is clearly demonstrated in Fig. 6, where 100% survival and 100% mortality are shown as stippled areas, with the conditions for partial survival lying between these limits.

The observations of mean survival time presented in Table 2 and Fig. 7 indicate that there was a threshold level of about 5 W. (under the conditions of the experiment), below which ultrasonic irradiation had no significant effect on survival. At powers greater than 5 W. the mean survival time was significantly reduced by a non-thermal effect.

It is important to realize that the particular ultrasonic power at which non-thermal effects were observed to become significant was probably very dependent upon the irradiation configuration. If the animals had been subjected to different forces (for example, if they had been irradiated in a medium of different viscosity or absorption, or with ultrasound of another frequency), then a different threshold level of power might have been found. Similarly, the size of the organism is important, for this determines the maximum shearing stress produced by a particular streaming system. Thus, the present results are quite compatible with the conclusions of Hughes & Nyborg (1962), that streaming around sonically induced bubbles is a cause of cell damage, and of Hawley, Macleod & Dunn (1963), that DNA in solution is degraded by the relative motion between the molecules and the medium induced by ultrasound. A fundamental difference between all these experiments is the difference in scale. In assessing the likelihood of non-thermal damage being produced by ultrasonic irradiation, the scale and character of the biological system are considerations just as important as the physical parameters of the ultrasonic field.

SUMMARY

1. Apparatus is described for the irradiation of *Daphnia magna* by 3 MHz. ultrasound, under controlled temperature conditions.

2. The lethal level for heat exposure was established for *Daphnia*.

3. It is shown that there was a threshold of irradiation level below which immediate death was due to heat.

4. Above the threshold level for non-thermal damage, streaming in the environment of the animal became considerable.

5. Long-term observations confirmed the existence of a threshold level for non-thermal damage during irradiation.

I should like to express my gratitude to Dr P. C. Caldwell for his guidance and encouragement, both during the course of the experiments described here, and in the preparation of this paper.

This work was financed in part by the Medical Research Council and by the Board of Governors of the United Bristol Hospitals.

REFERENCES

CURTIS, J. C. (1965). Action of intense ultrasound on the intact mouse liver. In *Ultrasonic Energy*, pp. 85–109. Ed. E. Kelly. Urbana: University of Illinois Press.

DUNN, F. (1965). Ultrasonic absorption by biological materials. In *Ultrasonic Energy*, pp. 51–62. Ed. E. Kelly. Urbana: University of Illinois Press.

FRY, W. J. (1953). Action of ultrasound on nerve tissue—a review. *J. acoust. Soc. Am.* **25**, 1–5.

FRY, W. J. & FRY, R. B. (1954). Determination of absolute sound levels and acoustic absorption coefficients by thermocouple probes. *J. acoust. Soc. Am.* **26**, 294–317.

HARVEY, E. N. (1930). Biological aspects of ultrasonic waves, a general survey. *Biol. Bull. mar. biol. Lab., Woods Hole* **59**, 306–25.

HARVEY, E. N. & LOOMIS, A. L. (1931). High speed photomicrography of living cells subjected to supersonic vibrations. *J. gen. Physiol.* **15**, 147–53.

HAWLEY, S. A., BREYER, J. E. & DUNN, F. (1962). Fabrication of miniature thermocouples for *uhf* acoustic detectors. *Rev. scient. Instrum.* **33**, 1118–19.

HAWLEY, S. A., MACLEOD, R. M. & DUNN, F. (1963). Degradation of DNA by intense, noncavitating ultrasound. *J. acoust. Soc. Am.* **35**, 1285–7.

HERRICK, J. F. (1953). Temperatures produced in tissues by ultrasound: experimental study using various technics. *J. acoust. Soc. Am.* **25**, 12–16.

HUGHES, D. E. & NYBORG, W. L. (1962). Cell disruption by ultrasound. *Science, N.Y.* **138**, 108–14.

JOHNSON, C. H. (1929). The lethal effects of ultrasonic radiation. *J. Physiol.* **67**, 356–9.

LEHMANN, J. F. (1953). The biophysical mode of action of biologic and therapeutic ultrasonic reactions. *J. acoust. Soc. Am.* **25**, 17–25.

NEWELL, J. A. (1963*a*). A radiation pressure balance for the absolute measurement of ultrasonic power. *Physics Med. Biol.* **8**, 215–21.

NEWELL, J. A. (1963*b*). Ultrasonics in medicine. *Physics Med. Biol.* **8**, 241–64.

SCHMITT, F. O., OLSON, A. R. & JOHNSON, C .H. (1928). Effects of high-frequency sound waves on protoplasm. *Proc. Soc. exp. Biol. Med.* **25**, 718–20.

WOOD, R. W. & LOOMIS, A. L. (1927). The physical and biological effects of high-frequency sound waves of great intensity. *Phil. Mag.* 7th series, **4**, 417–36.