An Ultrasonic Dosage Study: Functional Endpoint

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This paper constitutes an initial report on an elaborate ultrasonic dosage study which has been undertaken at the Bioacoustics Laboratory of the University of Illinois. The completed study will include dosage relationships as a function of base temperature of the tissue, hydrostatic pressure and frequency.

The equipment, both mechanical and electronic, has been developed over the past several years. Instrumentation for such a study must be of a precision nature if quantitative data are to result for elucidation of mechanisms. It is possible to realize accurately reproducible results on a suitably prepared and precisely irradiated biological preparation.

It is the purpose of this paper to describe the experimental arrangement, technique of preparation and accuracy of results obtainable with such a suitably designed system.

The type of results obtainable from such a study will be illustrated with data taken at a base temperature of 10°C, a hydrostatic pressure of one atmosphere and a frequency of 982 kc/s.

The subject used for this study is an intact mouse, approximately 24 hours after birth. The mice used are of the LaFl strain, which is an impure strain, so that brown, white and gray mice are utilized indiscriminately. The mice are cooled to render them inactive so that they can be accurately positioned in the mouse-holder. They are placed in the sound tank which utilizes degassed mammalian saline, as the acoustic transmitting medium. The temperature of the saline is accurately controlled to a few tenths of a degree centigrade.

The mice are positioned in the sound field such that the axis of the beam is centered on the third lumbar vertebra in order to produce motor paralysis in the hind legs. The animals are allowed several minutes, which is sufficient time, to reach their equilibrium temperature before exposure to the sound. The exposure is transcutaneous.

The sound field is calibrated each day, just before the animals are exposed, by the method described by Dr. Fry (Fry and Fry, 1954a; Ibid., 1954b). This makes it possible to precisely determine the acoustic intensity
Fig. 1. Scheme for presenting the data. A "dot" represents an unparalyzed animal and an "X" represents a paralyzed animal. Animals which appear in the higher dosage region "A" are always paralyzed. Those which fall in the lesser dosage region "B" are never paralyzed and those which appear in the threshold region "C" are distributed between paralyzed and unparalyzed.

at which the mouse is exposed. In this paper, ultrasonic dosage is specified by the acoustic intensity and the time duration of the exposure.

The functional endpoint observed on these animals after exposure to acoustic energy is paralysis of the hind legs; that is, lack of motor response. Anesthesia is also observed; however, correlation between lack of sensory response and ultrasonic dosage is not the subject of this study.

Fig. A-1. Sigmoidal distribution of percentage of mice paralyzed as a function of the reciprocal of the duration of exposure at constant acoustic intensity.
Fig. 2. The ultrasonic dosage threshold curve for base temperature of 10° C. The midline of the threshold region is extended to show the variations at the extremities of the linear portion. The indicated temperature rises were measured by imbedded thermocouples in the spinal cords of irradiated mice.

The results can be plotted on a coordinate system which has the reciprocal of time as the ordinate and the square root of the acoustic intensity as the abscissa (dosage graph), for example, by placing a “dot” at the proper point to represent an unparalyzed animal and an “X” for a paralyzed animal. Fig. 1 illustrates the schemes for presenting these data. When this is done it is possible to place two straight lines, having a common value for acoustic intensity at infinite time, as boundaries for a threshold region. Thus essentially all animals which fall in the higher dosage region “A” are paralyzed and essentially all those which fall in the lesser dosage region “B” are not paralyzed. The animals which appear in the threshold region “C” are distributed between paralyzed and unparalyzed.¹

¹ This description is readily expressed in more precise terms as follows: If the raw data at one intensity are treated by probit analysis (Finney, 1952), the type of curve illustrated by Fig. A-1 results. The mice considered in this analysis were irradiated at a constant acoustic intensity for various exposure times. The distribution of the number of animals paralyzed is plotted as a function of the reciprocal of the exposure time. Each point represents approximately 20 animals. The standard deviation is of the order of a few percent. From curves such as these, it is possible to define the threshold region in a more precise fashion than stated above. Thus, from a series of curves (at different acoustic intensities) such as Fig. A-1, one can obtain the reciprocal of the exposure time for 10% and 90% of the animals paralyzed. The collection of these two sets of values define two curves on the dosage graph and the region between these two curves is defined arbitrarily as the threshold region.

Fig. A-2 shows the threshold region determined in the above fashion plotted on the dosage graph.
At the present time we have rather extensive data at a hydrostatic pressure of one atmosphere, a frequency of 982 kc./s., and a base temperature of 10° C. These data show that the slopes of these two straight lines differ by approximately eighteen percent. See Fig. 2. In the region of dosages of intensities less than 50 watts/cm.², corresponding to time durations greater than 20 seconds, the slope of the borders of the threshold region appears to increase gradually, probably indicating that excessive temperatures developed at these long exposure times are the primary cause of the effect on the tissue.

The region between 50 watts/cm.², corresponding to 20 seconds time duration, and 120 watts/cm.², corresponding to one second time duration, appears to exhibit a linear relationship between the reciprocal of time duration and the square root of intensity, indicating that in this region a second process has become important. For dosages at intensities greater than 130 watts/cm.², (time durations less than one second) the relationship between

![Graph](image)

**Fig. A-2.** Threshold region for paralysis of the hind legs of mice under ultrasonic irradiation. The indicated temperature rises were measured by imbedded thermocouples in the spinal cords of irradiated mice. Time and intensity are also indicated on the coordinate axes.
the reciprocal of time and the square root of intensity deviates from linearity in that the slope of the threshold region appears to increase further. Thus, it appears that different processes may be involved in the different ultrasonic dosage regions lying in the range from, say 30 watts/cm.$^2$ (1,000 seconds) to 250 watts/cm.$^2$ (0.10 second).

Measurements have been made of the temperature rise in the spinal cord of the mice as a function of ultrasonic dosage. This was accomplished by imbedding small thermocouples in the cord. The results of these temperature measurements are indicated at the corresponding dosage coordinates in Fig. 2. Considering these temperature increases in the cord in conjunction with the value for the base temperature of the animal, 10$^\circ$ C., it can be concluded that temperature rise is not the primary factor for the observed functional changes in the mice in the linear portion of the threshold region.

An outline of the preparation and technique and a discussion of the instrumentation will now be presented.

The mice are selected for this study 24 hours after birth, plus or minus five hours. They range in weight from 1.2 to 1.4 grams. It is not possible to determine the subsequent color of the mice at this stage so that white, gray and brown mice are irradiated indiscriminately. Males and females are also irradiated indiscriminately. Young mice were chosen for this study for the following reasons: (1) They are essentially poikilothermic, that is, they possess virtually no temperature control mechanism, so that they can
be carried through reversible temperature cycles, with the temperature being reduced to nearly zero degrees Centigrade, without producing either morphological or permanent physiological changes. (2) Ossification is not complete. As determined by standard staining techniques, the tissue overlying the dorsal side of the cord is soft tissue, while that over the lateral and ventral sides shows a slight degree of ossification. Therefore, acoustic absorption in the structures surrounding the spinal cord does not result in the large temperature increases characteristic of irradiated adult mice. (3) These animals are small in size so that it is possible to irradiate the desired region with a nearly uniform acoustic intensity with a single controlled ultrasonic pulse.

The equipment comprises a sound tank which is immersed in a deep-freeze unit for maintaining the desired temperature within a few tenths of a degree Centigrade. The overall arrangement of the equipment is shown in Fig. 3. Hydrostatic pressures from one to twenty atmospheres can be developed in the tank. A coordinate system is provided in the tank to accurately position either the calibrator or the mouse. An accurately machined tongue is used to attach either the calibrator or the mouse-holder to the coordinate system. Fig. 4 is a view looking into the tank. The calibrator is shown attached to the coordinate system. A cross-hair attachment can also be fastened to the tongue. The intersection of the cross-hairs has been set so that it coincides with the position previously occupied by the thermo-

Fig. 4. A view looking into the sound tank. The calibrator is shown mounted on the coordinate system.
couple junction of the calibrator. With adjustments provided on the mouse-holder, together with the tongue and the cross-hair attachment, it is possible to place almost any part of the mouse in the position occupied by the calibrator thermocouple junction in the sound field. Fig. 5 shows the mouse-holder and tongue assembled. Fig. 6 is a close-up view showing the cross-hair attachment in position.

The method of probing and calibrating the sound field is the standard procedure employed at this laboratory (Fry and Fry, 1954a; ibid., 1954b). A calibration of the sound field is performed daily.

The mice are irradiated at the third lumbar vertebra. Since it is desirable to use as a functional endpoint motor paralysis of the hind legs, the region of the spinal cord which must be altered is that containing the neurons associated with the femoral, sciatic and obturator nerves. This region, at which the axis of the beam is centered, has been determined by acoustic means.

For a study of this type it is highly desirable to have an acoustic field of almost uniform intensity over the region to be affected. An unfocussed quartz crystal is used to develop the traveling wave field. Fig. 7 shows the transverse and vertical patterns of the beam. At 5 percent below the peak intensity, the vertical beam width (along the length of the cord) is 2.6 mm. At 10 percent down, the beam width is 3.2 mm., and at half-power the beam width is 7.1 mm.
Fig. 6. A close-up view showing the cross-hair attachment in position. The two knurled knobs on the right permit almost any part of the animal to be positioned under the intersection of the cross-hairs.

In the lumbar region, the vertebral segments are 0.67 mm., measured from corresponding edges. Thus, nearly four vertebral segments of the cord are irradiated with an intensity variation of no more than 5 percent.

In preparing the animal for irradiation, the mouse is first cooled down to render it dormant so that it can be properly positioned in the mouseholder and remain in that position until the termination of exposure. When the mouse is sufficiently cooled, it is placed in the holder which firmly

Fig. 7. The transverse and vertical field patterns developed by the unfocused quartz crystal. The cord lies along the vertical direction.
holds the head, the hind legs, and the tail. The mouse is then fully extended to reduce possible lateral movement. Fig. 8 is a close-up view showing a mouse mounted in the mouse-holder.

The mouse-holder, the tongue and the cross-hair attachment are then assembled (see Fig. 6) and placed over an intense, cool light source. With such an arrangement it is possible to see clearly the vertebrae from the second lumbar through the sacral and caudal regions. The mouse-holder is then adjusted to place the center of the third lumbar vertebra under the intersection of the cross-hairs. The cross-hair attachment is then removed and the mouse-holder supporting the mouse is placed in the sound tank.

Concerning the accuracy of placement of the third lumbar vertebra with respect to the axis of the sound beam, the following statements can be made: (1) The accuracy of the machined parts is ± 0.002 in. or ± 0.05 mm. (2) The axis of the beam can be determined to ± 0.1 mm. (3) The maximum uncertainty in locating the center of the third lumbar vertebra, which is approximately 0.6 mm. in length, is ± 0.1 mm. Thus the overall uncertainty in the position of the center of the third lumbar vertebra with respect to the axis of the sound field is ± 0.25 mm. Since the beam width at 95 percent of the peak intensity is 2.6 mm., it appears that the overall accuracy of positioning the animal in the sound field is adequate.

Since these animals are essentially poikilothermic they will come to temperature equilibrium above the temperature of the environment, the exact amount being a function of their age and the temperature of the surround-
ings. For these animals and the temperature considered here—one day old mice at 10° C.—the equilibrium temperature is approximately 10.2° C. A few minutes times is sufficient for the animals to reach their equilibrium temperature. A single acoustic pulse of rectangular envelope, having a rise time of several microseconds and predetermined acoustic intensity and time duration is then initiated.

After the cessation of the sound, the animal-holder is removed from the tank. The mouse is removed from the holder and rapidly warmed to room temperature. The animals are examined for paralysis or overt movements approximately 15 minutes after exposure and again after 6 hours.

In conclusion, it thus appears that the precision ultrasonic dosage study planned by this laboratory is possible. The instrumentation and technique for obtaining accurately reproducible results with a suitably prepared biological specimen have been developed and demonstrated.

The preliminary results obtained at a base temperature of 10° C., hydrostatic pressure of one atmosphere and frequency of 982 kc./sec., indicates that several processes may be involved in producing changes in the central nervous system in the ultrasonic dosage range from 25 watts/cm.² and 1,000 seconds to 250 watts/cm.² and 0.1 second.

References


Dr. Hueter: In our dosage study which utilizes adult mice, we have occasionally been troubled with the following problem. We increased the dosage, e.g., the sound amplitude, in order to obtain an additional point on the sigmoid curve. We found that at the higher dosage the experimentally determined point was considerably lower than was anticipated and at a still higher dosage the point appeared in the expected region.

We found that at a certain dosage level cavitation developed in the transmitting medium between the animal and the transducer. We had to increase the dosage in order to get through the cavitation screen with sufficient energy to carry out the experiment.

Dr. Dunn: The experiments which we described were conducted in the absence of cavitation. The degree of reproducibility obtained appears to
bear this out. However, we might indicate how we determined that we were not being troubled by cavitation.

In the course of this experimentation, we made a rather extensive set of temperature measurements by embedding small thermocouples in the mouse cord. The location of the thermocouple was determined after the animal was sacrificed. The soft tissue was cleared and the bone stained with alizarin red. The specimen was then viewed under a microscope and the thermocouple junction was located quite accurately with respect to the vertebral structure.

We irradiated the animals, which contained imbedded thermocouples, at varying dosages. The output of the thermocouple was fed to a recording galvanometer which provided us with a permanent record of the temperature rise versus time function. Now, on some occasions, we did have cavitation present. Figure D-1 is a photograph of such a galvanometer record which shows the type of response obtained both with and without cavitation present.

![Graph showing temperature rise versus time](image)

Fig. D-1. Photograph of a galvanometer recording of the response of a small thermocouple imbedded in a mouse cord during irradiation with sound. The record shows the response obtained both with and without cavitation present.

Note that in the absence of cavitation the temperature rise versus time curve has the characteristic shape which Dr. Fry has described, i.e., there is an initial rapid rise due to viscous forces and a subsequent linear rise due to acoustic absorption in the vicinity of the thermocouple. When cavitation sets in, the galvanometer response becomes very erratic with the curve rising to rather high values and presumably high temperatures are developed. Referring to Fig. D-1, after 8.27 seconds of acoustic irradiation at 71 watts/cm² the temperature rise was 22.2° C. The maximum rate of rise during this time was 10.0° C./sec, which occurred at the start of the irradiation period and which was caused by the viscosity effect. During the initial cavitation blast, the temperature rose 13.4° C. in 0.17 second, for a total rate of 78.8° C./sec.

In our experimental procedure, the vacuum tube voltmeter, which meters a calibrated portion of the voltage impressed across the quartz crystal transducer, is always observed. Now, when cavitation occurs, there is a reaction back on the crystal which is reflected into the electronic circuit.
This is readily observed on the vacuum tube voltmeter. The results which we just presented were carried out in the absence of any phenomena suggestive of the presence of cavitation.

Dr. Huetter: In your 4-beam irradiation system, you have an intensity level of 16 watts/cm.² at the transducer. These levels increase toward the dura and it would appear that you should at least develop occasional cavitation or gas bubbles.

Dr. Fry: Such gas bubbles are observed when the water in the transmission path becomes gassy which means that it is being forced to serve its purpose too long. Our experiments are performed in the absence of such undesirable conditions. We are careful in our preparation and handling of the degassed water.

Dr. Baldes: What temperatures did you actually get in your thermocouple recording at 10° C.?

Dr. Dunn: The temperatures measured, of course, are highly dependent upon the dosage, i.e., the time duration of irradiation and the acoustic intensity. For example, at 54 watts/cm.² and 7.70 seconds irradiation time, the measured temperature rise was 16.5° C. At 112 watts/cm.² and 1.25 seconds irradiation time, the temperature rise was 6.1° C. Both of these values are in the threshold region. High temperature rises can certainly be obtained. For example, at 54 watts/cm.² and 20.00 seconds time duration of irradiation the temperature rise was 29.0° C. This is well beyond the threshold region where all animals are paralyzed. Conversely, in the lesser dosage region where animals are never paralyzed, at 54 watts/cm.² and 1.00 seconds irradiation time, the measured temperature rise was 2.43° C. These values are observed at the positions in the cord of highest temperature rise, i.e., the ventral side of the cord.

It is interesting to compare these temperature rises in the mouse cord with those in onion roots previously described by Dr. Lehmann. Dr. Lehmann observed temperature increases in onion roots of the order of 150° C. when irradiated with 1 mc./s. ultrasound at 110 watts/cm.² for five minutes. His experiments were carried out under a high hydrostatic pressure.

Dr. Carsten: I wonder if someone would comment on the reason for the time lag between the onset of this cavitation effect and the application of the sound field which was just described. Is it presumably that there is a minimum time requirement for the bubbles, or whatever we have here, to grow to the proper size to produce an observable effect or is there perhaps a time rate of production of bubbles which requires a particular time interval to reach a sufficient concentration to produce an observable effect?

Dr. Nyborg: I think Dr. Leonard's experiments are illuminating. He used focussed sound and studied the cavitation at the focus. He found that
considerably greater sound pressures were necessary to produce cavitation under these conditions than were necessary under other experimental conditions. He explained this by the fact that in the focal region, the fluid in the region of high sound pressure moves at very high velocities. This is a kind of acoustic streaming which means that part of the fluid does not remain in the focal region very long and consequently the bubbles do not have sufficient time to grow to the proper size to produce an effect.

Dr. Rosenberg: I personally feel that the time required for the bubbles to grow to a particular size to be able to produce the effect is most important. In the plane wave case it is definitely the growth time that is important. This is also true of acoustic streaming. Concerning the population, I feel that unless one employs a focussed beam with an extremely small focal region, the population in all such cases, is large. In the case of a focussed beam of exceedingly small focal region, one may have to allow adequate time for both the growth of the bubbles and the build-up of the population before the effect can be observed.

Dr. Huetter: With respect to your mouse data, you indicated that the threshold region displays three different shapes, a linear region sandwiched between two non-linear regions of increasing slope. I wonder if one could not argue that the entire threshold region has a parabolic shape.

Dr. Dunn: One may, of course, argue this, however, the experimental evidence appears to be otherwise. We should like to point out, in comparing your mouse data with ours, that with respect to the duration of time of irradiation, there is a reasonable correspondence. Our data, for single shots of sound, shows a deviation from the linear portion beyond approximately 10 seconds of exposure to the sound. Our data covers irradiation times from one second to beyond 1,000 seconds. For your data, let us consider the length of time for which the sound is actually on, e.g., for 10 pulses, each of 0.4 second duration, pulsed at the rate of one per second. Now, your data covers total irradiation only from 4 seconds to 24 seconds, i.e., 10, 30 and 60 pulses. While your data seems to follow a parabolic relationship, the greatest curvature appears to occur beyond approximately 8 or 10 seconds of time during which the animal is actually exposed to the sound. Perhaps if your data extended over a wider range especially to shorter total exposure times you might have found a linear range as we did.