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Morphology of Ultrasonically Irradiated Skeletal Muscle

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Introduction by Dr. Herrick

The history of the application of physical agents to medicine has not been a happy one partly because such agents have been applied in some instances before their basic action on the biological system was thoroughly understood. A most encouraging aspect of this meeting is the emphasis on understanding the basic mechanisms involved in the observed effects of ultrasound. The next paper concerns the application of electron microscope techniques in order to determine the effects of ultrasound on muscle. It will be presented by Dr. Eggleton.

Studies involving the use of ultrasound as a research tool to produce differential and controlled changes in biological tissue are of particular interest to this laboratory. The research program has included an investigation of the use of high-intensity ultrasound to produce functional changes in striated skeletal muscle (1, 2). In the present study, the morphological ultrastructure of ultrasonically irradiated skeletal muscle is being investigated with the aid of the electron microscope. It is hoped eventually to relate structural changes with corresponding functional changes and possibly with basic muscle mechanisms.

Although the ultrasound equipment used in these experiments was designed primarily for the production of focal lesions in the CNS, it was readily adapted to the requirements of this study. This equipment consists of a single-beam focusing transducer driven at 4 Mc by a feedback-controlled amplifier. More detailed descriptions of the equipment will be given later in this symposium by F. J. Fry and G. H. Leichner of this laboratory. Sound pressure amplitudes in the range of 50 atm were applied to the muscle for periods of the order of 0.8 sec. A beam-width characteristic of the transducer was 0.6 mm to the half-power point. The specimen thickness was approximately 1 mm.

Because there are many systems of muscle nomenclature, perhaps it is appropriate first to describe the terms used here. Figure 1 shows a portion of three myofibrils. Note the sarcomere between two "Z" bands. The "A" band is the dark area, and the "I" band is the light area. The "H" zone is the lighter portion of the "A" band, and the "M" line is in the center of the "H" zone. It is generally thought that the protein, myosin, occupies the "A" band and is composed of filaments 100 A in diameter and that the protein, actin, is the principal ingredient of the "I" band which is composed of 50-A-diameter filaments. The actin presumably extends into the "A" band, giving rise to a zone of overlap. Note further that the sarcomere is of the order of 2 μ long and 0.5 μ in diameter.

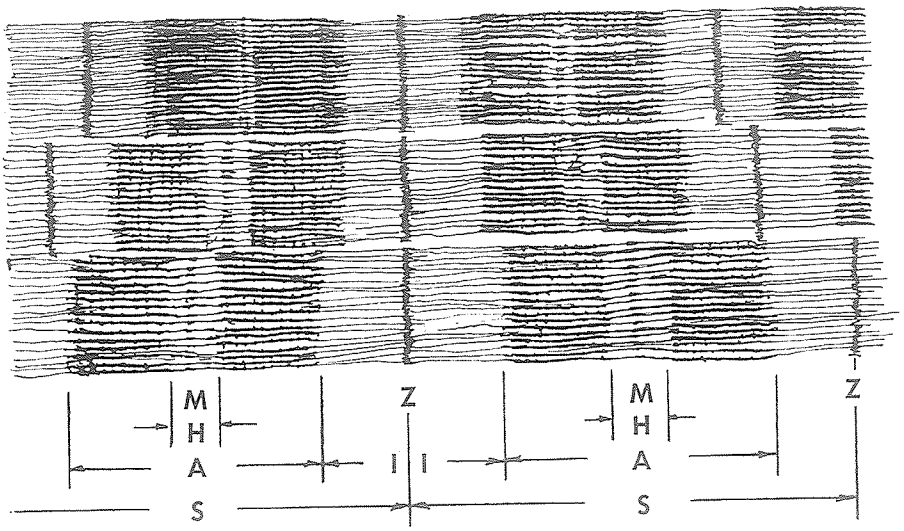

FIGURE 1. Diagram of ultrastructure of frog sartorius muscle showing nomenclature used in this paper.

It has been found that ultrasound lesions in muscle are, as might be predicted, relatively complex. One of the more interesting phenomena observed is the loss of band structure within the lesion. It is very common to find along the edge of the lesion, as shown in Figure 2, a normal-appearing fiber next to a fiber in which the band structure is destroyed. Longitudinal filaments about 100 A in diameter can be seen in this area. The filaments appear to be continuous rather than discontinuous as might be predicted from the Huxley-Hanson model (3). It is further noted that this change in band structure usually occurs at the fiber boundary. This observed condition cannot be explained in terms of sound or heat temperature gradients across the small (0.1 μ) gap between fibers.

To understand the changes caused by high-intensity ultrasound, it is first necessary to have more information on the effect of heat on muscle since ultrasound causes heating of the tissue. Accordingly, several series of excised frog

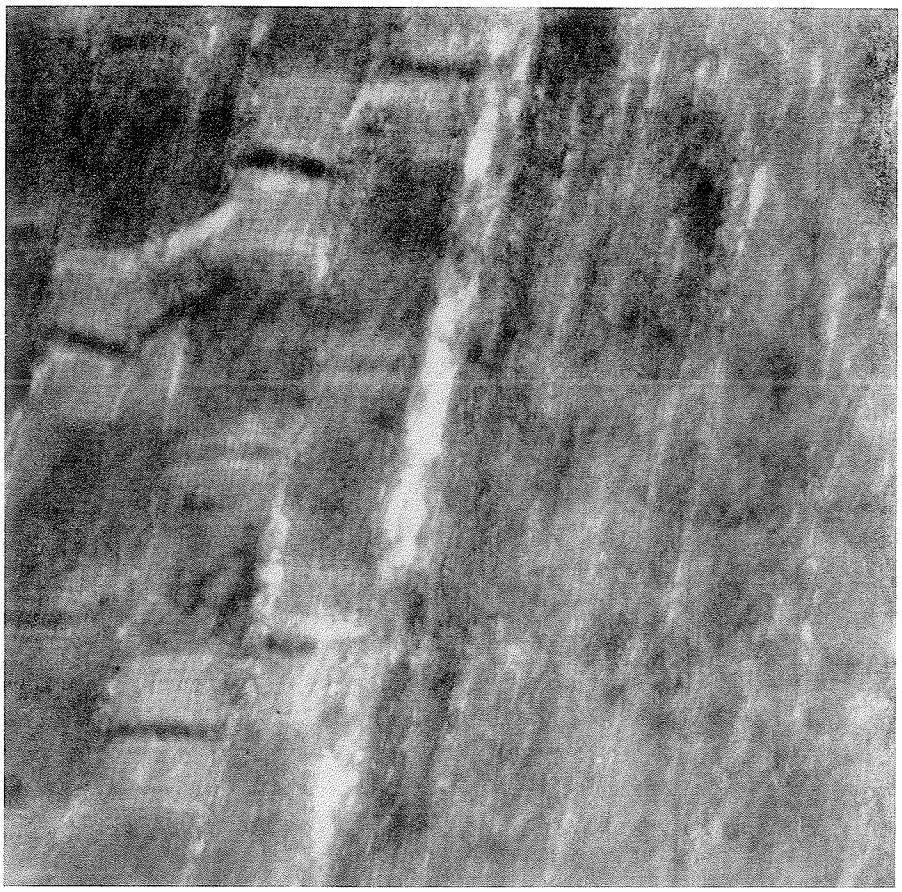

FIGURE 2. An ultrasonically irradiated muscle showing a normal-appearing fiber adjacent to a fiber in which the band structure is destroyed.

sartorius muscles were immersed in heated Ringer's solution that ranged in temperature from 35 to 55°C. In one series, the muscles were immersed in the heated Ringer's solution for a period of 2 min. In other experiments, a heat exposure of 15 sec was used. As a means of following dimensional changes in the muscle during the various processing procedures, three fine silk threads were tied in the edge of the muscle in such a configuration that both lateral and longitudinal changes could be determined (Figs. 3, 4). The muscles were held at about 20% stretch during the heat exposure and subsequent fixation and dehydration procedures.

All muscles were fixed in veronal-buffered osmium tetroxide solution, dehydrated in graded alcohols, and imbedded in methacrylate. Some of the imbedded specimens were sectioned into 25 slices and mounted on microscope slides with a water base mounting medium (Fig. 5). The mounted sections were scanned under the light microscope and selected areas were dissected out of the specimen slice and secured to a mounting tip using dental wax

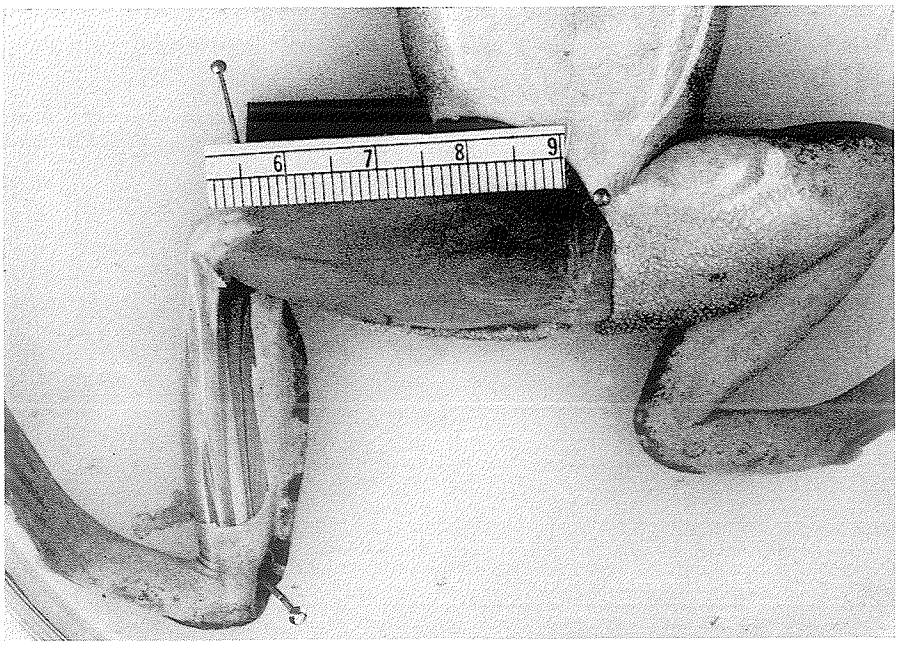

FIGURE 3. Markers were placed in the edge of the sartorius muscle so that dimensional changes could be followed through the processing of the muscle. Dimensions were recorded for various postural configurations.

(Figs. 6, 7). The selected specimen area was sectioned in a Porter-Blum microtome for examination in the electron microscope.

It was immediately evident upon a cursory examination of the sections of heat-treated muscles that the band structure appeared normal in specimens subjected to temperatures up to about 40°C. Muscles exposed to temperatures of about 45°C or higher contained no normal bands. Between these two temperatures there was a gradual transition in the percentage of the muscle fibers with normal bands. The structure shown in Figure 8 is typical of a muscle subjected to 43.25°C. Note that the bands have disappeared, but the longitudinal filaments are still intact. A small percentage (5 to 10%) of the volume of this specimen contained rather normal-appearing bands as shown in Figure 9. Figure 10 is an example of the most intact part of 43.75°C muscle as found in this particular specimen. An occasional isolated sarcomere can be seen. The longitudinal filaments are generally present, but the myofibrils are not always distinct. It should be noted that in any given heat-treated muscle there is a spectrum of effects. In the series of micrographs shown in Figure 11 (a 43.75°C muscle), myofibrillar structure is clear in #1 but is not present in #4. Mitochondria are present in all cases and appear normal morphologically although no measurements were made to determine their condition. It was also noted that the change in ultrastructure was accompanied by a change in the gross appearance of the muscle from a translucent to an opaque white.

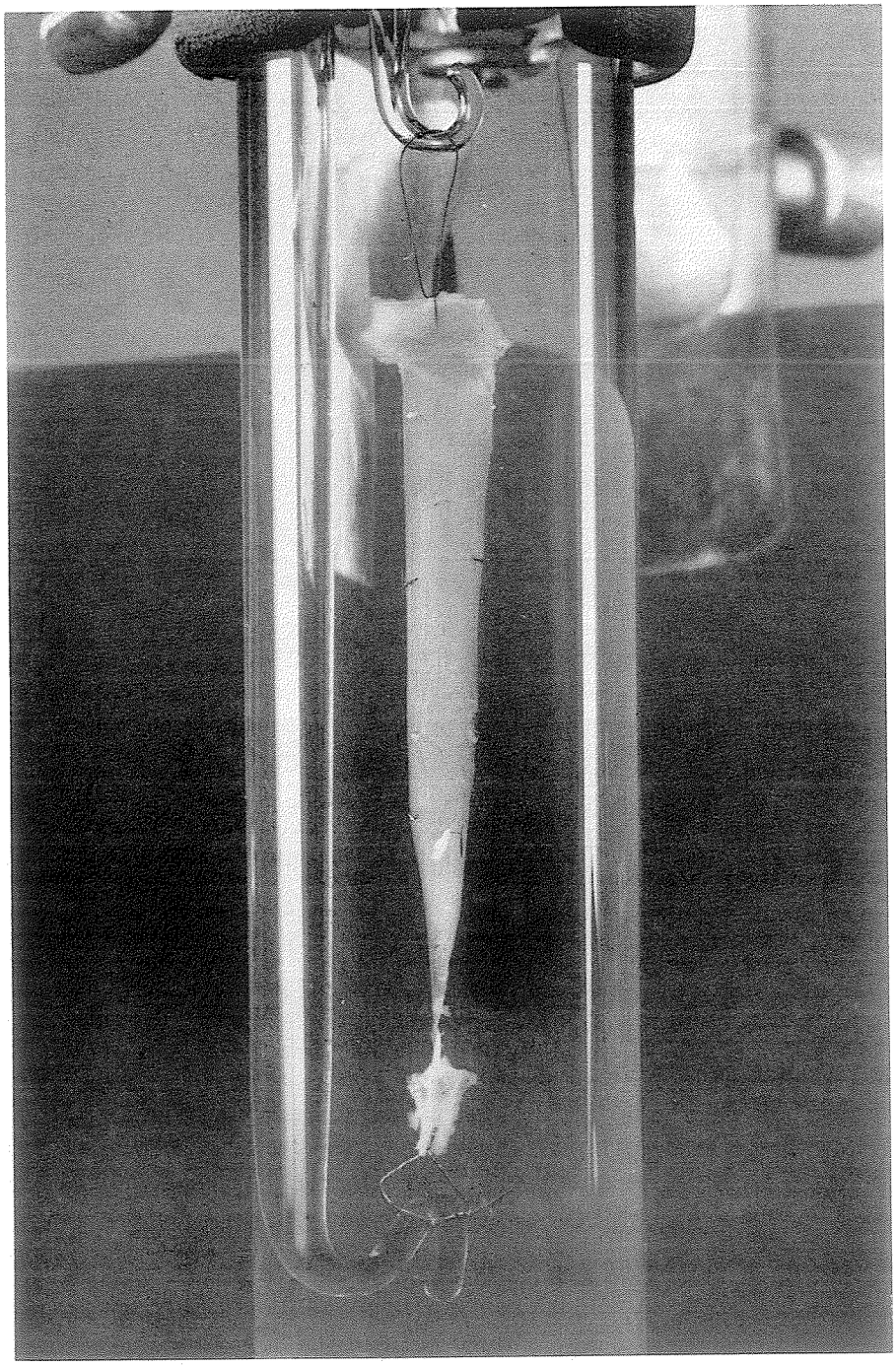

FIGURE 4. Muscles were held at various degrees of stretch during the heat exposure and subsequent fixation and dehydration procedures.

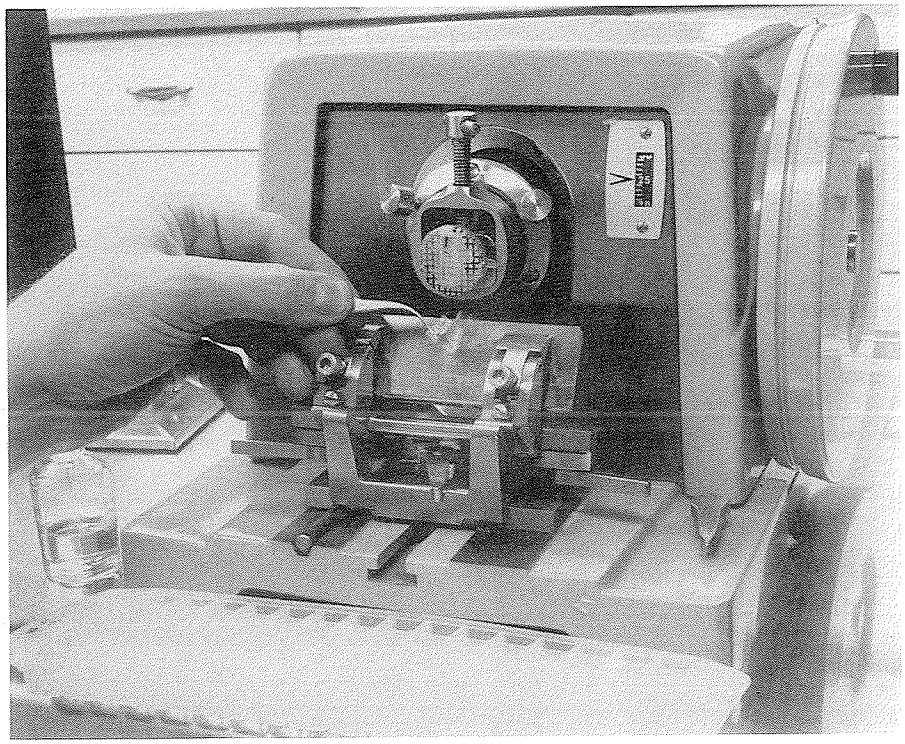

FIGURE 5. The muscles were imbedded in methacrylate and sectioned into 25-μ slices. The mounted slices could be scanned under the light microscope for areas of interest.

A corresponding series of pictures of muscle cross-sections is shown in Figure 12. Note that in #1, the myofibril is still clearly defined by the sarcoplasmic reticulum, the filaments are in a systematic array, and there are no large interstitial spaces in this part of the muscle. Succeeding pictures show increasing amounts of disorder with large interspaces present. In #4 there is no myofibrillar structure and many filaments are indistinguishable against a general background of debris. At higher temperatures the conditions shown in #4 become more prevalent. Also, at the higher temperature there is abnormal band structure such as that shown in Figure 13. This muscle was immersed for 2 min in a Ringer's bath which had been heated to 50°C. The fact that the muscle was unrestrained may explain the short sarcomere length, although not all unrestrained heat-treated muscles have contracted sarcomeres. The important features to be noted are the presence of the transmyofibrillar bridges and the general form of the myofibril. This is typical of perhaps 10 to 25% of this muscle. No longitudinal filaments are discernible in this muscle.

More than one mechanism is involved in the effect of heat on muscle. The following observations are noted: (1) Loss of band structure is evidence of damage to muscle and may result from various nutritional deficiencies (4)

FIGURE 7. The specimen is transferred to the mounting tip and secured in place with dental wax. The specimen may then be further sectioned in the ultramicrotome.

FIGURE 6. The technique for cutting specimens from the 25-μ-thick sections is illustrated. A razor blade fragment is used for this purpose.

as well as from heat and ultrasound. This loss of structure apparently is a property of the fiber, that is, all myofibrils within a fiber lose their bands while the fibers on either side may be quite normal in appearance (4). (2) Fibers toward the outside of the heat-treated muscle are more likely to retain their band structure than are central fibers. (3) The time constant for the

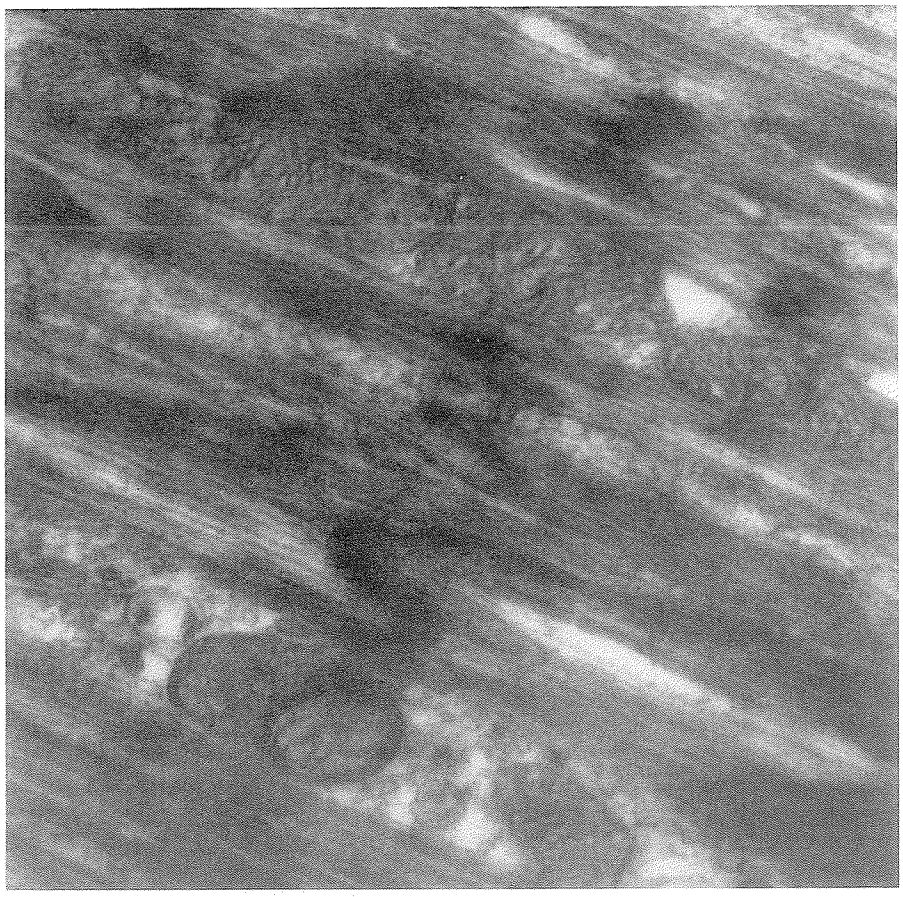

FIGURE 8. Structure typical of muscle subjected to 43.25°C. Bands have disappeared, but the longitudinal filaments are still intact.

primary action of heat on muscle would seem to be short – possibly less than 15 sec. (4) The lengths of the sarcomere "A" band and "I" band do not change significantly for muscles (held at 20% stretch) exposed to temperatures up to approximately 43°C. (5) The center-to-center distance of the filaments measured in the "H" zone are about the same for muscles exposed to various temperatures up to approximately 46°C. Filaments have not been found in muscles heated above 46°C. (6) A type of damage to the muscle is observed in which band structure is destroyed but longitudinal filaments remain intact.

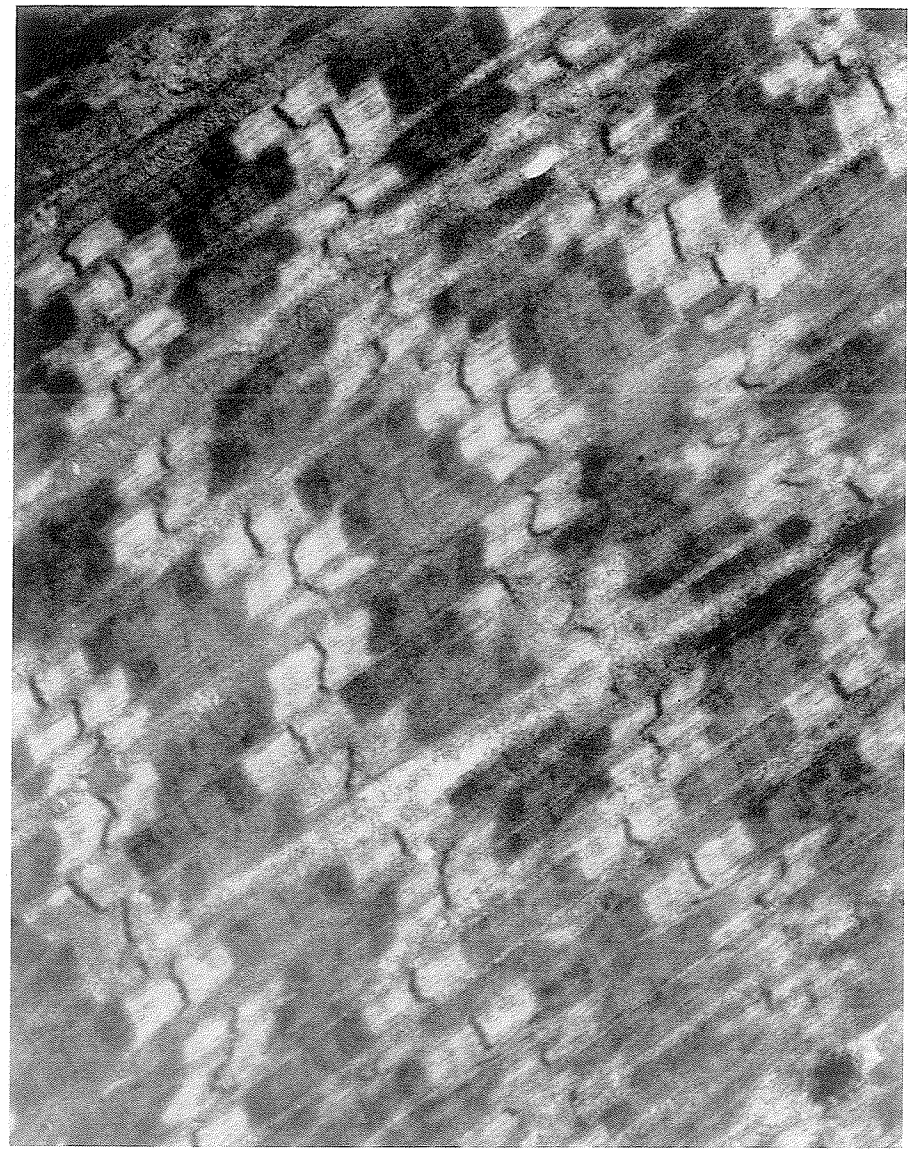

FIGURE 9. A small percentage of the muscle heated to 43.25°C shows normal appearing band structure.

One hypothesis which is being considered to explain these observed changes is that some agent already present within the muscle fiber is responsible for the loss of band structure. This agent, after the release by any one of several "stimuli," might diffuse along the fiber affecting all the myofibrils within it but does not readily diffuse across the sarcolemma into adjacent fibers. Perhaps the myosin of the "A" band is mobilized by this agent to provide a means for the myosin to diffuse into the "I" band where it combines

FIGURE 10. Muscle heated to 43.75°C is illustrated showing the most intact structure in this particular specimen.

with the actin to form an actomyosin complex, thus resulting in the general appearance shown in Figure 14.

It was reported by Jensen (5) in 1914 that frog sartorius muscle exhibits reversible and irreversible heat contraction. The reversible heat contraction takes place between 37 and 39°C and the irreversible thermal rigor takes place between 39 to 45°C. Later, Mirsky (6), in working with myosin isolated from frog muscle, found myosin denatures in two distinct steps. He found a pronounced change in solubility occurring at 37°C. As the myosin is heated to temperatures over 39°C, a second change takes place; beginning at about 41°C, the gel draws together into a number of firm opaque clumps. As previously noted, the authors of this paper found what appears to be a two-step

FIGURE 11. This series of micrographs shows the spectrum of effects found in a muscle heated to 43.75°C. The micrographs are arranged in order of increasing amounts of destruction of muscle ultrastructure: 1, least evidence of destruction; 2, 3, intermediate level of destruction; 4, complete destruction.

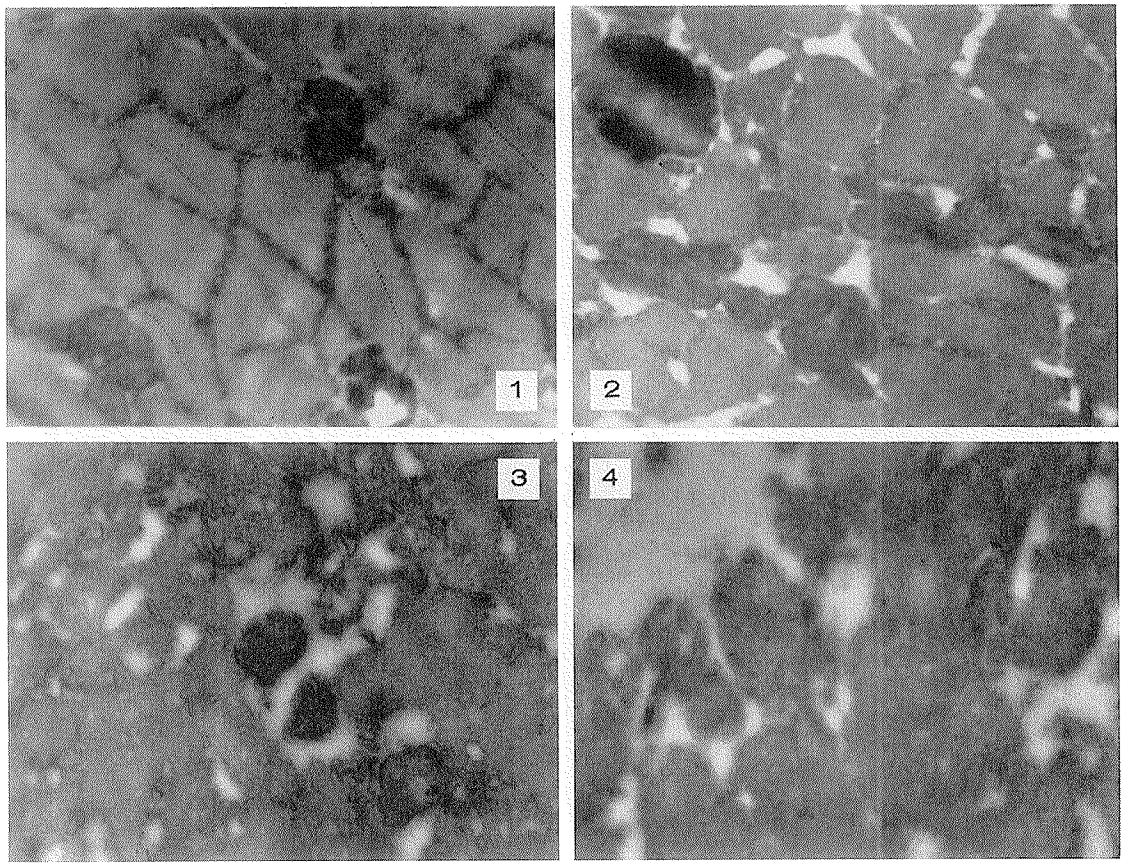

FIGURE 12. This series of cross-sectional micrographs illustrating a spectrum of effects corresponds to the similar series shown in Figure 11.

change in ultrastructure of the frog muscle exposed to high temperatures. In the temperature range of 40 to 44°C it was found that the band structure disappears leaving 100-A-diameter longitudinal filaments clearly visible while in the temperature range from 44 to 46°C, the filaments are found to disappear.

Another type of damage to muscle which has been observed and reported on by previous authors (7) is noted again here because it plays a part in the general configuration of the ultrasound lesion. Figure 15 shows a single fiber damaged by mechanical stretching of the fiber, and Figure 16 shows the same type of damage produced by ultrasound. It appears that the myofibrillar bun-

FIGURE 12. This series of cross-sectional micrographs illustrating a spectrum of effects corresponds to the similar series shown in Figure 11.

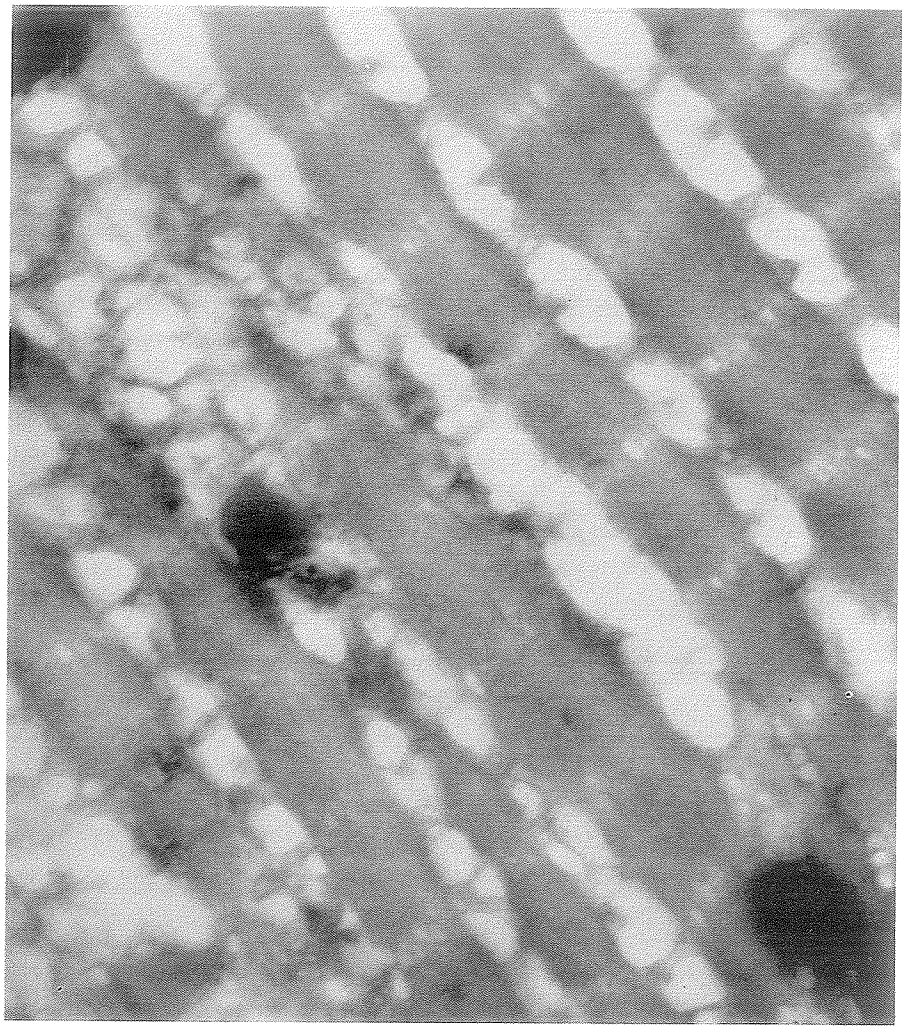

FIGURE 13. Muscle immersed for 2 min in Ringer's bath heated to 50°C is illustrated. Transmyofibrillar bridges and remnants of band structure are in evidence.

dle is under stress and is in equilibrium with the constraining force of the sarcolemma. The breaking strain of the sarcolemma is considerably greater than that of the myofibrillar bundles, thus when the sarcolemma is stretched, the adhering bundles stretch with it until the breaking stress of the bundles is exceeded. When this occurs, the structural part of the bundles (the filaments) apparently contracts and forms a series of compact masses at distributed sites within the sarcolemma, and the medium which surrounds the filaments in the intact fiber now fills the spaces between the compact masses. The area of these compact masses can be measured and the volume estimated. The values obtained may be compared with the estimated volume of intact fiber based on measurements outside of the damaged area and before the fiber was damaged. Table I shows some examples of these volume changes which result from this process.

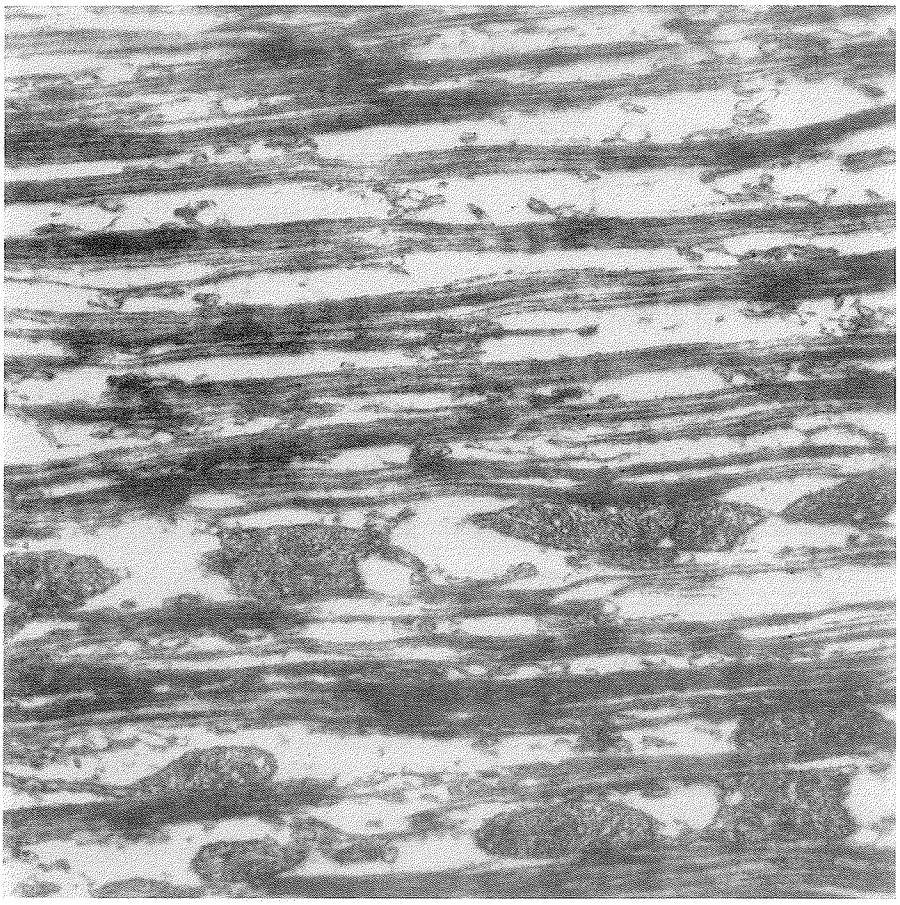

FIGURE 14. Electron micrograph of frog sartorius muscle exposed to 42°C bath for 15 sec. It should be noted that the filaments are continuous for a distance in excess of the sarcomere length.

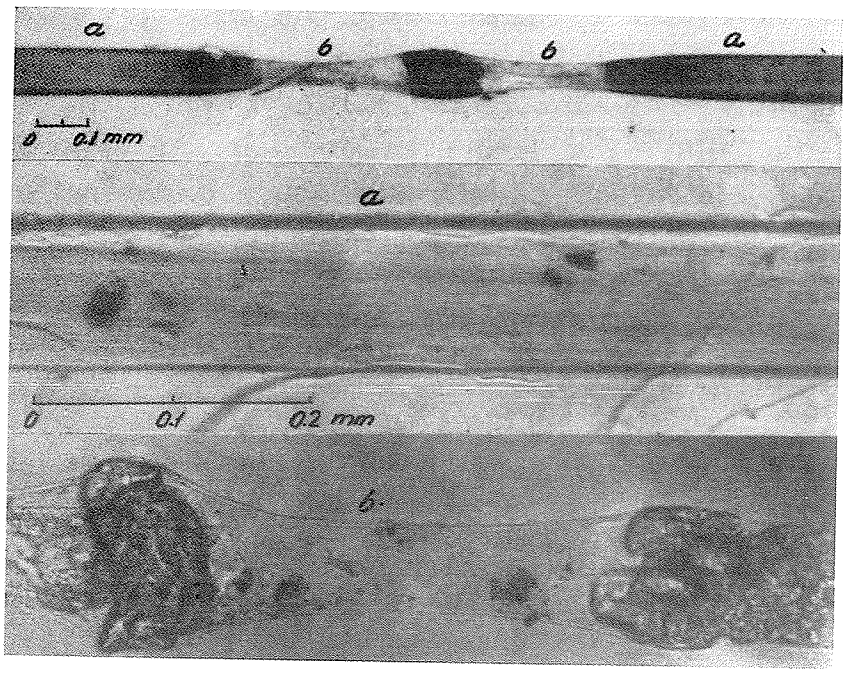

FIGURE 15. Photomicrographs of fibers damaged by mechanical stretching of the fiber (after Buchthal, 1951). a, undamaged fiber; b, empty sarcolemma tube.

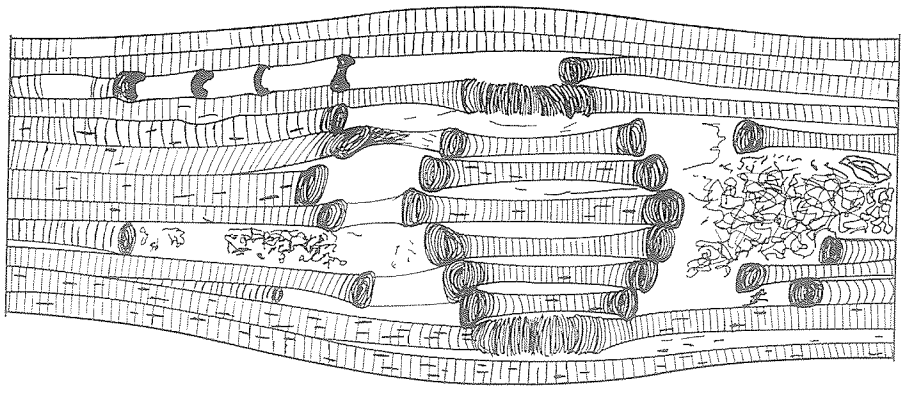

FIGURE 16. Damage produced by ultrasound similar to the damage illustrated in Figure 15. Sketch based on microscopic observations.

Another process which may be contributing to the structure of the ultrasonic lesion in muscle is "heat fixation." Biologists have long used heat as a means of fixing tissue. Focused ultrasound causes a rapid increase in temperature within the central part of the muscle and is calculated to be about a 46°C rise above 19°C ambient temperature for the sound levels used here. The gross configuration of the high-level focused ultrasound lesion in muscle is

TABLE I. VOLUME CHANGES IN MUSCLE FIBERS RESULTING FROM BREAKS IN THE MYOFIBRILLAR BUNDLES.

Muscle no.	*Estimated volume undamaged*	*Measured volume damaged*	*Ratio* V_o/V_i
1.	$3.35 \times 10^6 u^3$	$2.25 \times 10^6 u^3$	1.5
2.	$4.26 \times 10^6 u^3$	$2.06 \times 10^6 u^3$	2.0
3.	$5.15 \times 10^6 u^3$	$2.72 \times 10^6 u^3$	1.9
4.	$8.18 \times 10^6 u^3$	$1.96 \times 10^6 u^3$	4.1

characterized by an island of relatively intact tissue surrounded by a moat within which there is extensive tissue damage. It seems reasonable to suppose that the structure within the central island has been preserved due to heat fixation. This conclusion is supported by the observation of an *immediate* opacity change in the tissue. In the island, the sarcomere length is consider-

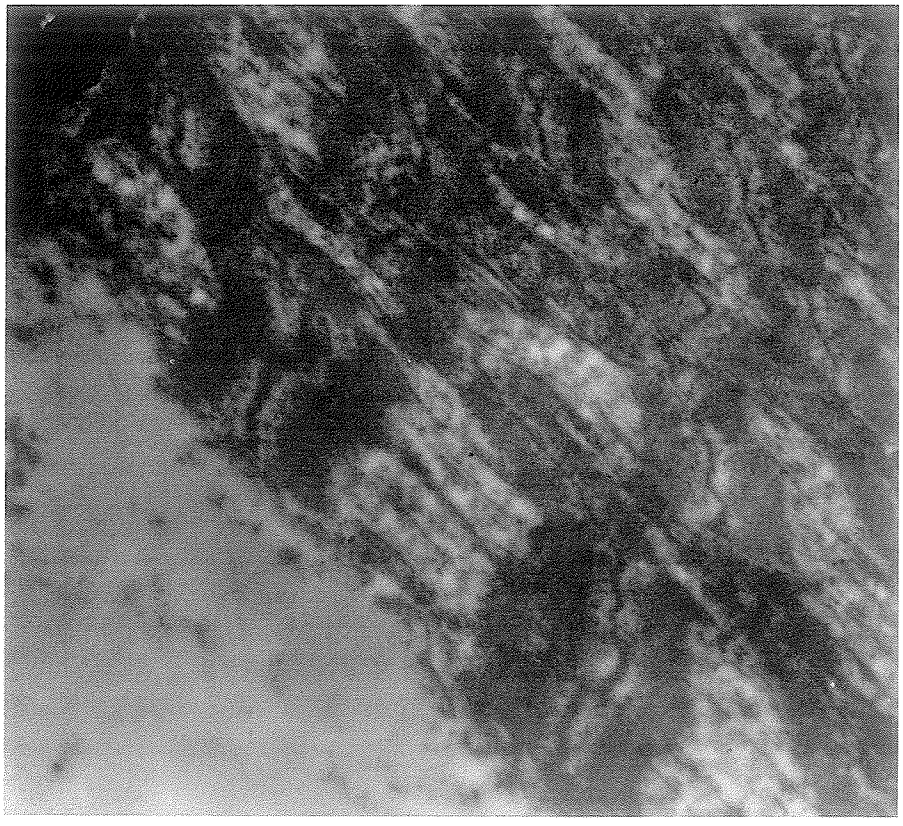

FIGURE 17. This micrograph illustrates the type of damage found in the center of an ultrasound lesion. The bands appear to be broken in the region of the H-zone.

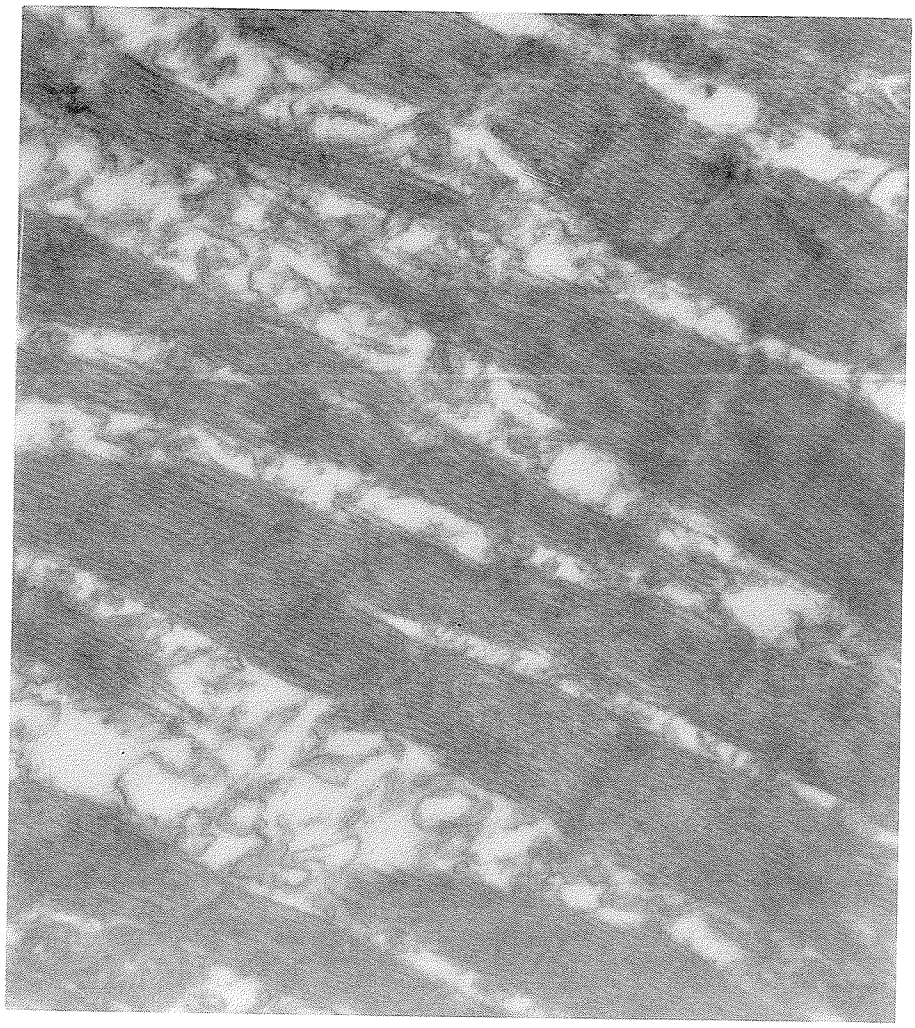

FIGURE 18. A micrograph illustrating an area from the center of an ultrasound lesion in which longitudinal filaments are present but band structure has disappeared.

ably increased, and this is explained if one assumes that the *tensile* strength of the *heated* portion of the fibers is much less than that characteristic of the unheated portion of the fibers. The muscle is held under slight tension, and as a result of this tension, the unheated portion stretches the heated portion to the point where the fibers rupture and contract away from the lesion, forming a moat. Figure 17 shows a modified band structure taken from the center of an island. If the "Z" membrane is the dark line, then each myofibril has been broken at the "M" line. Figure 18 shows an area in which the bands are not present. One of the areas of greatest damage is shown in Figure 19, but even at this level of destruction one can find evidence of mitochondria being present.

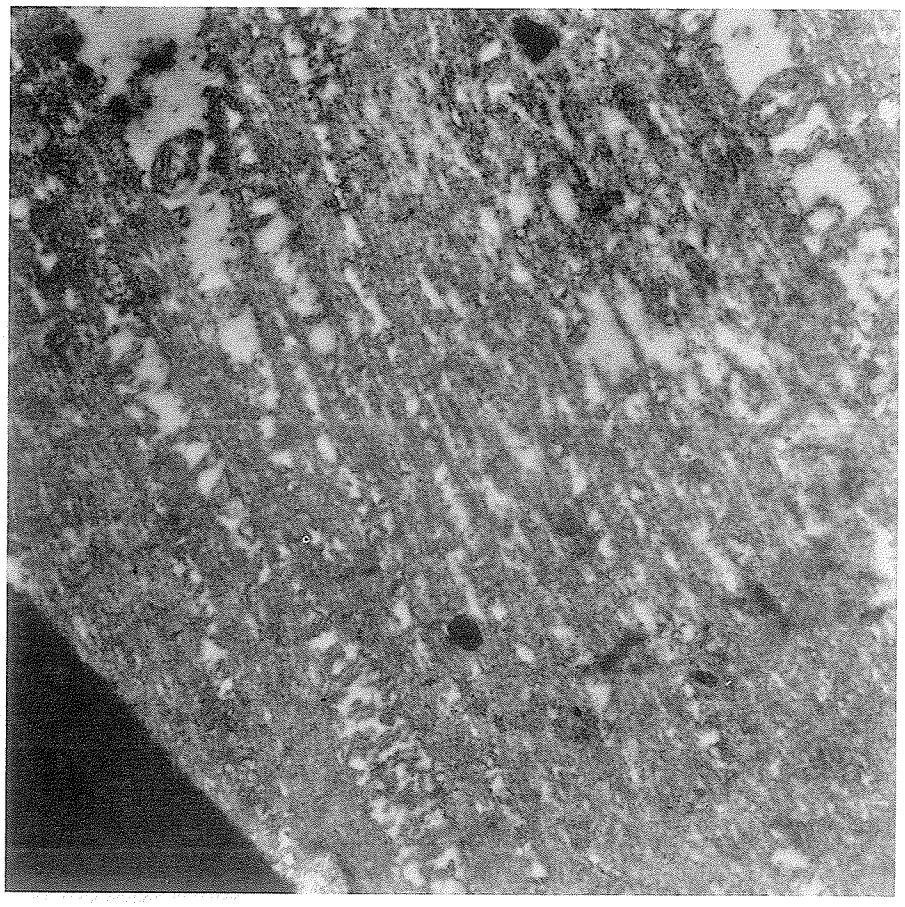

FIGURE 19. This electron micrograph illustrates an area of great damage within an ultrasound lesion. Note that mitochondria are present.

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DISCUSSION

DR. CURTIS: I wonder if you would make a general statement about the range of doses that you used in terms of intensities or exposure. I wasn't clear on this point.

DR. EGGLETON: The intensities that we have used are in the range of 40 to 60 atm of sound pressure amplitude. The length of time of exposure was of the order of 1 sec.

DR. CURTIS: All single pulses?

DR. EGGLETON: Yes. In order to get more lesions per muscle, we spaced the lesions 1 mm apart. The lesion diameters were small compared to 1 mm.

DR. CURTIS: I have a question concerning the fixation. Were these 25-μ sections that you cut fixed in osmium?

DR. EGGLETON: The whole muscle was fixed in osmium, imbedded in methacrylate, sectioned on a conventional microtome, and then mounted on a microscope slide with a water base medium. We store most of our specimens in this manner. In this way we are able to scan large volumes of tissue and select portions of interest without spending a great deal of time searching with the electron microscope.

DR. CURTIS: I realize that this is a basic problem in using the electron microscope, but you are also confronted with the problem of fixation. How do you fix large pieces of muscle in osmium?

DR. EGGLETON: Fortunately, frog sartorius muscle is quite thin (not more than 1 mm in thickness); therefore it is easily possible to study fixation as a function of depth in the muscle. For example, the first section shows the fixation at a depth of 25 μ, the second at 50 μ, etc. We find that the muscle is quite well fixed throughout.

DR. FORREST: I would like to ask what, if anything, ultrasound has shown in relation to the Huxley model, and if you would like to make any comment about other models and possible future applications.

DR. EGGLETON: One of the more interesting phenomena present in some of these micrographs of muscle in which there is a loss of band structure is that the longitudinal filaments which remain after the disappearance of band structure are continuous for more than the sarcomere length. The Huxley model indicates that these fibers would be discontinuous. The model proposes that the 100-A myosin filaments extend the length of the "A" band and the 50-A actin filaments extend the length of the "I" band into the "A" band, producing a zone of overlap. These filaments are not connected and cannot account for the continuous filaments observed. In seeking an explanation for this, one can speculate that either the filaments are formed de nova or that they are continuous in normal muscle. The center-to-center distance between adjacent "A" and "I" filaments in normal muscle is the same as the spacing between filaments in muscle in which bands have been destroyed. However, it is difficult to understand how a contractual mechanism can be constructed from this data.

DR. FORREST: What prompted you to use 4 Mc instead of some other frequency?

DR. EGGLETON: Principally because the 4-Mc equipment was available to us and ready to use. It has the advantage of producing a small lesion appropriate to the size of the specimen we were using.

DR. HUGHES: May I make a suggestion? Since you have the material, it might be worthwhile to make frozen sections and estimate the water and dry weight in these by means of its appearance. Then you could relate this to changes certain of us have found in working with heated proteins. You might do this quite easily and see what, in fact, is happening to the protein in the muscle.

DR. EGGLETON: That's a good idea. Thank you for the suggestion.

DR. NYBORG: I get the impression from looking at some of the micrographs that some of the strands might be twisted.

DR. EGGLETON: Twisted around each other?

DR. NYBORG: Just out of shape.

DR. EGGLETON: Yes, as the muscle is heated to higher temperatures, there is an increasing amount of disorder in the specimen.

DR. NYBORG: I was thinking primarily of the ultrasonically treated muscles. They appear different to me than the heat-treated ones.

DR. EGGLETON: Regions can be found in the ultrasonically treated muscles that are quite similar to the heat-treated ones.

DR. GERSTEN: Did I understand you to suggest the possibility that some substance might be diffused and that this would account for this process? What evidence do you have for this?

DR. EGGLETON: We have no evidence for it, but suggested this as a possible hypothesis.

DR. WEISSLER: Did you do any experiments on cooled muscles? If the muscles were at a temperature of 2 or 5°C, presumably 0.8-sec exposure might not be enough to raise the temperature to 46°C. It would be interesting to see then whether any damage resulted. This might be one way of distinguishing thermal from other kinds of damage.

DR. EGGLETON: The temperature rise for the sound intensities we used was calculated to be about 46°C. Even an ambient temperature of 0°C would be sufficient to take it into the region of destruction.

DR. CURTIS: It would be interesting to know what changes take place at the structural level when one irradiates along the axis of the muscle fiber. It would be interesting to correlate. Have you done this?

DR. EGGLETON: No, we haven't. I agree that it would be an interesting study.