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Argon Laser Micro-irradiation of Mitochondria in Rat Myocardial Cells in Tissue Culture
IV. Ultrastructural and Cytochemical Analysis of Minimal Lesions*

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K. P. ADKISSON, D. BAIC, S. L. BURGOTT, W. K. CHENG AND M. W. BERNS. Argon Laser Micro-irradiation of Mitochondria in Rat Myocardial Cells in Tissue Culture. IV. Ultra-structural and Cytochemical Analysis of Minimal Lesions. *Journal of Molecular and Cellular Cardiology* (1973) **5**, 559–564. Laser irradiated mitochondria in rat myocardial cells were analysed cytochemically for succinic dehydrogenase and with the electron microscope for ultrastructure. The least severe lesion type did not alter the SDH activity of the mito-chondria. The major ultrastructural alteration observed was an electron-dense region corresponding to a phase-dark spot in the light micrographs. This lesion appeared to be restricted in depth and width within the irradiated structure. The damage was primarily localized to the intercristae matrix. The cristae appeared normal.

KEY WORDS: Laser; Mitochondria; Myocardial; Electron microscope.

1. Introduction

In previous reports in this series we described various alterations in morphology and contractility of cultured myocardial cells following selective argon laser micro-irradiation (0.5–1 μm spot) of single mitochondria [*1, 3, 4*]. It was demonstrated that three different types of light-microscope lesions could be produced in the mitochondria, and that these lesions could be correlated with the amount of laser energy and/or the optical density of the target mitochondrion [*3*]. The "least severe" lesions appeared as a general phase "paling" or blanching of the entire irradiated structure, often with a small phase-dark spot at the center. The "moder-ate" lesion-type resembled a hole with a dark border, and the "severe" lesion appeared to be destruction of the entire organelle with only a vacuole remaining [*1*]. Specific changes in the contractile state of the cell were associated with the moderate and severe lesions. These responses varied from a slight increase in beat rate to the precipitation of a violent, uncoordinated contractile state termed

"fibrillation." A large percentage of the cells recovered from their altered contractile state and returned to a rhythmic beat rate often identical to the pre-irradiation rate. Salet [*10*] has described a similar acceleration of beat rate followed by a return to the pre-irradiation rate in heart cells microirradiated with a green (5300 Å) neodymium laser beam. No contractile changes were observed with the least-severe lesions, even when as many as 30 mitochondria were irradiated. This last result was particularly surprising since it was obvious from the phase micrographs that the alteration to the mitochondria was considerable. To achieve a better understanding of the structural and functional changes produced by laser micro-irradiation, further experiments were undertaken employing techniques of electron microscopy and cytochemistry.

We will devote this report only to an analysis of the least-severe lesion type. Admittedly it would be desirable to present data on all three lesion types in one report. However, the technique of single cell recovery (and, indeed, single organelle recovery) with the electron microscope is a tedious and time-consuming procedure. Consequently, we felt that as thorough an analysis as possible of each lesion-type should be undertaken. Indeed, if an adequate understanding of the light microscope changes and the physiological responses of the cell is to be achieved, a precise knowledge of the ultrastructural changes is necessary. This information is also essential to an adequate interpretation of the nature of the interaction of the laser radiation with the target structure.

2. Materials and Methods

Cell culture was performed according to the procedures of Mark and Strasser [*9*]. The ventricles of 2 to 4-day old rat hearts were subjected to multiple trypsinization and the pooled cell suspension was injected in Rose culture chambers. Culture medium was minimal essential Eagle's medium supplemented with non-essential amino acids, 10% fetal calf serum, streptomycin, and penicillin. pH was maintained between 7.2 and 7.4 using a standard bicarbonate buffer system. After incubation at 37°C for 2 days, numerous contracting myocardial cells were attached to the bottom coverglass of the Rose chamber. Cells for electron microscopy were grown in Rose chambers on coverslips that had been coated with silicone (Siliclad). These coverslips were dipped into a 1% solution of silicone, sterilized, and then used as the bottomplate of the Rose chamber.

Micro-irradiation was conducted with an argon laser microbeam system [*2*]. The beam consisted of the mixed wavelengths (488 nm and 514 nm) of the argon emission spectrum. Beam configuration was in single mode, thus assuring a gaussian energy distribution across the focused spot. An oil immersion Zeiss Neo-fluar objective (100 ×, N. A. 1.3) was used to focus the beam down to a 0.5 to 1 μm spot. Energy measurements with a calibrated vacuum photodiode indicated a power density of 1.5 to 3 W in the focused microspot. Pulse duration was 50 μs.

PLATE 1. Phase micrograph of myocardial cell pre-irradiation. Target mitochondria indicated by arrows; small portions of the cytoplasm of adjacent cells are also visible. × 1000.

PLATE 2. Same cell 30 s post-irradiation; arrows indicate five irradiated mitochondria that have small phase-dark "spots" at the site of irradiation, and a general paling of the entire organelle. The mitochondrion closest to the nucleus in Plate 1 paled upon irradiation but did not have a dark "spot". × 1000.

PLATE 3. Same cell following succinic dehydrogenase cytochemistry; note high levels of SDH in all of the irradiated mitochondria (see text for further discussion). × 1000.

PLATE 4. Typical irradiated mitochondrion. Note the orderly cristae pattern extending through the electron dense region and attached to the mitochondrial membrane. Also note the small light areas in the intercristae spaces that may represent regions devoid in material, or region incapable of binding the stain. × 25 000.

PLATE 5. Phase micrograph of unirradiated cell.

PLATE 6. Phase micrograph of irradiated cell. Target mitochondria represented by arrows 1, 2, 3. × 1200.

PLATE 7. Irradiated mitochondrion 1. Note the electron-dense circular lesion 0.5 μm in diameter (arrow No. 1) also note that there may be some damage (secondary) to the outer mitochondrial membrane. × 25 000.
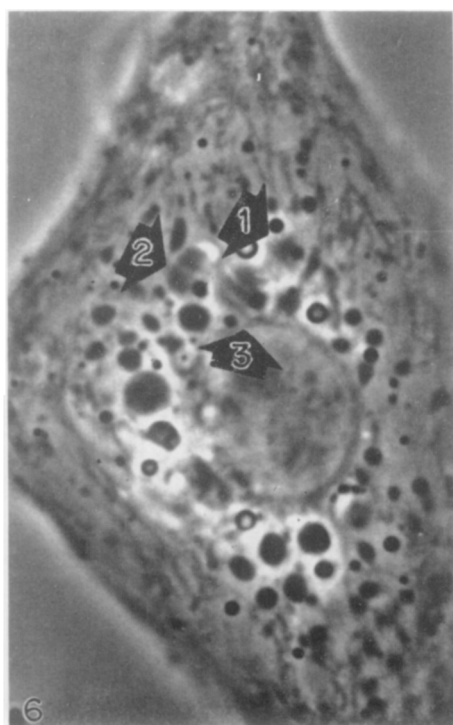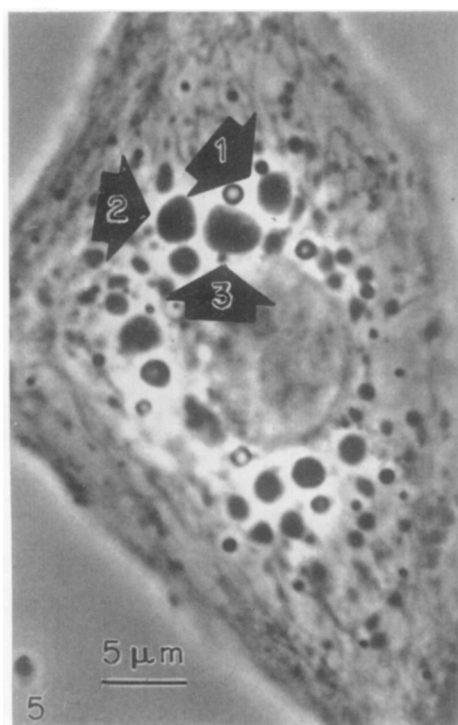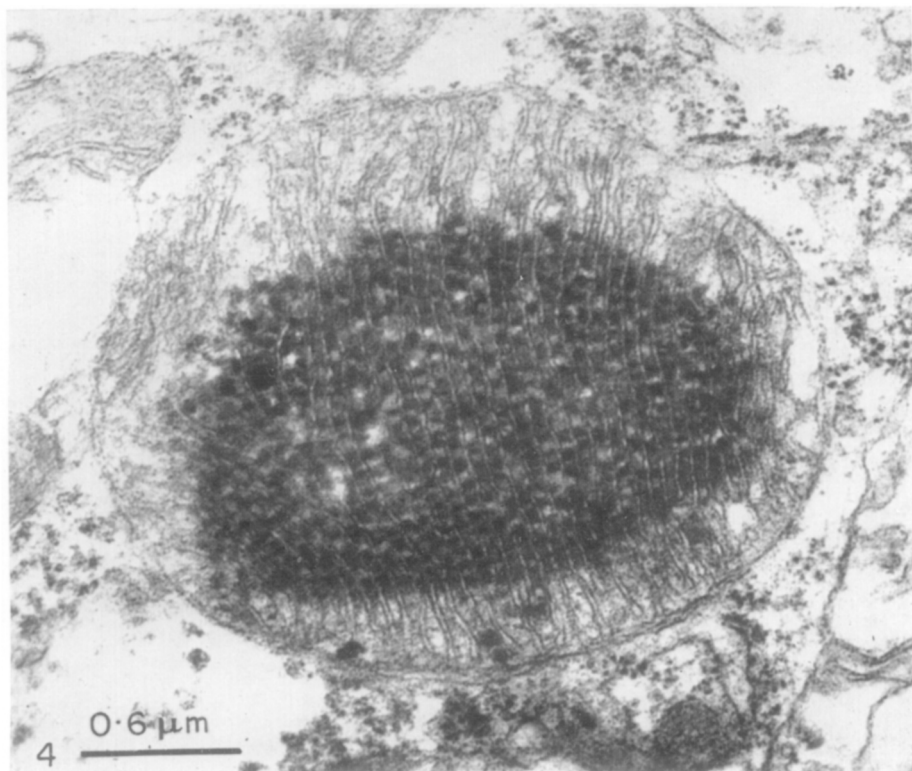
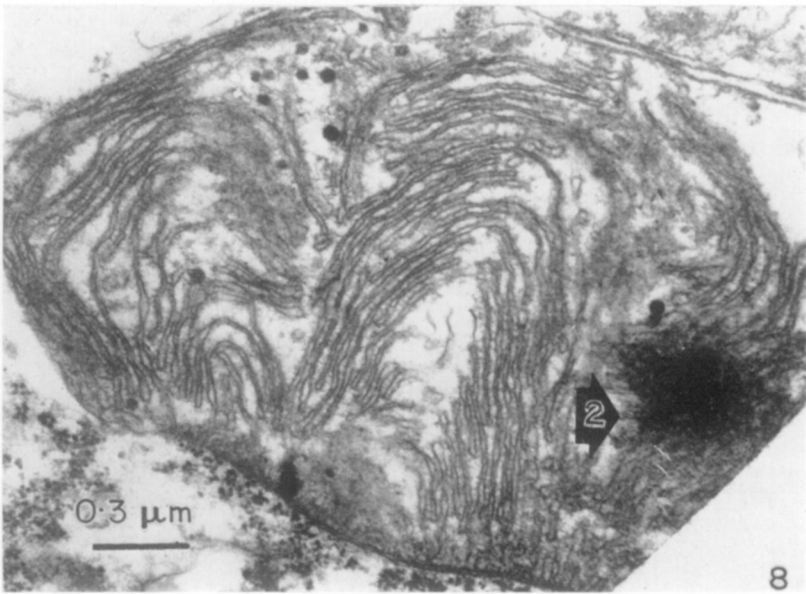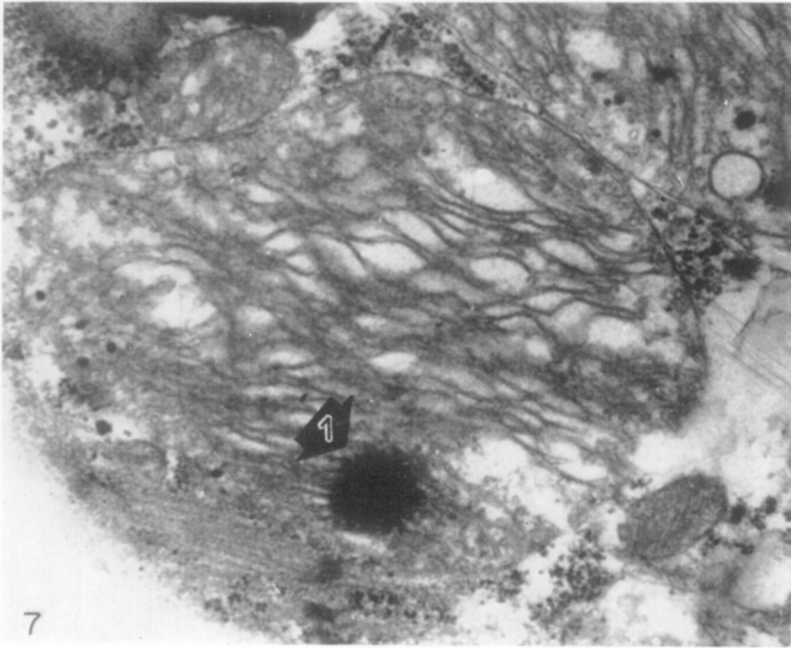PLATE 8. Irradiated mitochondrion 2. Note small dark lesion (Arrow No. 2) similar to mitochondrion 1. × 25 000.

PLATE 9. Irradiated mitochondrion 2. Section through different plane reveals absence of lesion. × 10 500.

PLATE 10. Irradiated mitochondrion 3. Note absence of small dark lesion. × 25 000.

PLATES 1–3

PLATES 4–6

0·3 μm
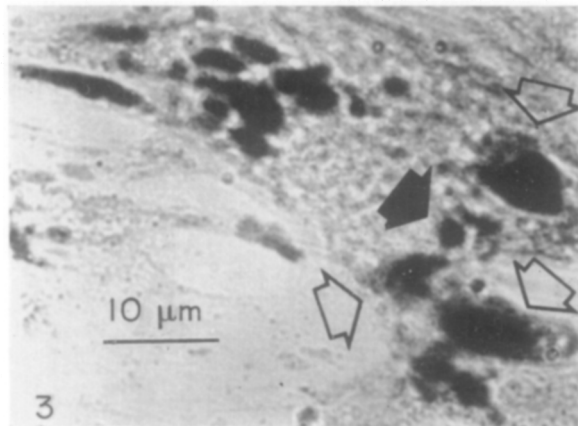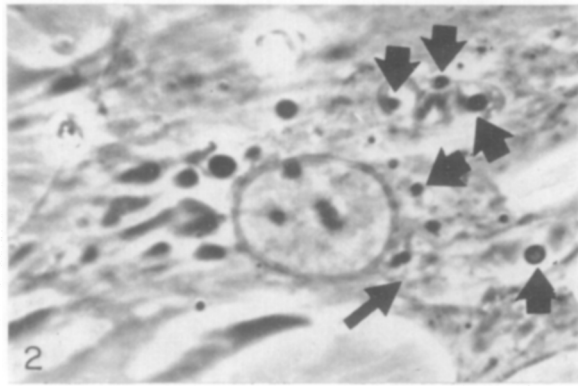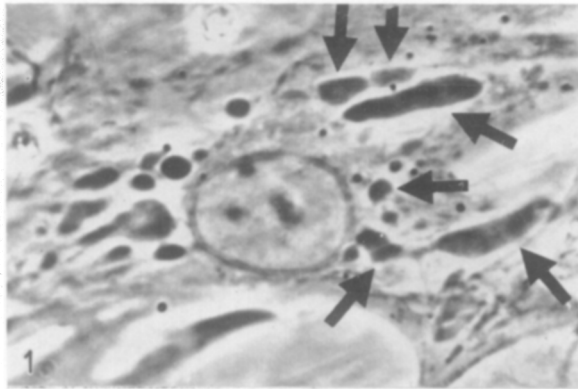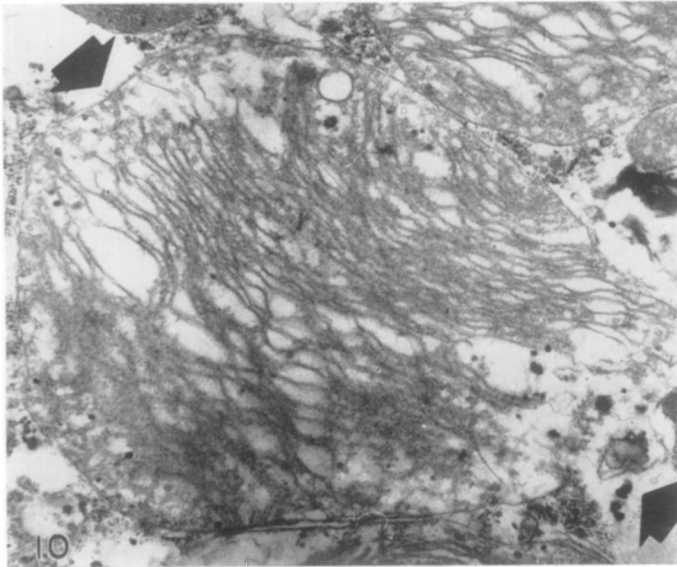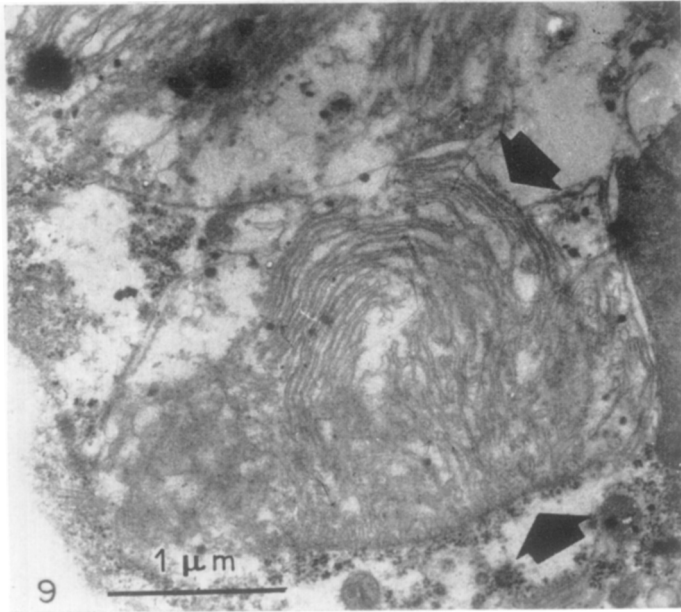
PLATES 7 and 8

PLATES 9 and 10

Irradiation was carried out by locating the image of the target cell on a television monitor that projected the microscope field of vision. By moving the microscope mechanical stage the target mitochondrion was moved directly under a cross hair on the screen that indicated the focal spot of the laser beam. Since the closed circuit TV was combined with a time-lapse video tape system, it was possible to tape each irradiation experiment for subsequent review and analysis. In addition, 35-mm still photos were taken before and after each mitochondrial irradiation.

Cytochemical analysis for succinic dehydrogenase activity was performed immediately following and up to 6 hours post-irradiation. The Rose chamber was disassembled, and the cover-slip with the irradiated cell was placed for 1 h at 37°C in an incubating solution of dinitro blue tetrazolium and sodium succinate prepared according to the method of Humason [8]. The cells were rinsed in cold Hank's balanced salt solution, fixed in 10% formalin for 10 min, rinsed in water, placed 2 min in 10% ethanol, 2 min 40% ethanol, and rinsed again in water before mounting in glycerine. Cells were relocated and examined with a phase and bright field microscope, and SDH activity assayed qualitatively by the degree of blue precipitate. Controls for each set of irradiated mitochondria were the unirradiated mitochondria within the same cell, and adjacent unirradiated cells. Previous experiments have demonstrated that irradiation with the same energy levels employed in this study does not affect the heart cell if the site of irradiation is outside the mitochondrion [1, 3, 4].

Prior to fixation for electron microscopy the irradiated cell was carefully marked to ensure subsequent relocation. A small circle of both india ink and ink from a "Sharpie" marking pen was drawn on the outer surface of the Rose chamber around the irradiated cell. A larger circle (about 1 to 2 cm in diameter) was scribed around the cell with a diamond pen. The coverglass was then fractured. The circular glass disc with the marked irradiated cell was rinsed with 0.2 M-cacodylate–HCl buffer by pipetting fluid over the surface of the cells several times. The glass disc was next placed cell side down on a fixative solution of glutaraldehyde (2%)–acrolein (4%) mixture buffered with a 0.2 M-sodium cacodylate buffer (pH 7.2) for 1 h at room temperature. Fixation was usually initiated within five minutes of irradiation. Following fixation the disc (with attached cells) was stored in buffer. The disc containing the irradiated cell was washed 10 min in water and post-fixed for 1 h in Caulfield's buffered osmium tetroxide with sucrose at 4°C [7]. After osmium fixation the cells were washed in three changes of H_2O, dehydrated in a graded series of ethanol (40 to 100%) treated with a 50:50 ethanol (100%): propylene oxide mixture, and given two changes of 100% propylene oxide (15 min each). The disc was removed from the propylene oxide and immediately a drop of EPON/ propylene oxide (50:50) was pipetted onto the cells. Next a 5 mm high piece of sliced polyallomer tube (4.5 ml Spinco Beckman tube) was placed around the cell and carefully glued (using Duco cement) to the disc. The cement was allowed to dry and the ring was filled about half way with more propylene oxide/EPON

mixture and allowed to set overnight. The next day fresh EPON (53 cm³ EPON, 25 cm³ DDSA, 33 cm³ NMA, 44 drops DMP-30) was poured into the ring and placed at 60°C to allow partial polymerization for 10 to 18 h [6]. A small circle was then scratched on the polymerized EPON above the irradiated cell (the cell surface was still closest to the glass disc). The preparation was next placed on a block of dry ice causing the EPON containing the cells to separate from the gloss in a few seconds. Final polymerization of the specimen was done at 60°C for 2 days. After polymerization was complete the EPON block was examined under low power phase and the irradiated cell relocated. A final mark indicating the position of the cell was placed on the cell side surface of the EPON disc. The small EPON disc was next glued to a larger EPON block with Epoxy glue, trimmed and then thin-sectioned parallel to the horizontal surface of the cell using a MT-2 ultramicrotome. Sections were picked up on 200 mesh grids with a carbon coated formvarfilm and stained for 40 min at 40°C with 4% uranyl acetate in 40% ethanol. Sections were examined and photographed using either a Zeiss EM 9 S-2, or an RCA EMU 4 electron microscope.

3. Results

Succinic dehydrogenase activity

The results of the SDH histochemical analysis were the same for cells assayed immediately following and up to six hours post-irradiation. A typical cell is illustrated in Plates 1 to 3. In the pre-irradiation state the large mitochondria appear as phase-dark bodies (Plate 1). Six of these mitochondria on the same side of the cell were irradiated (Plate 2). All of the lesions were of the least-severe type—resulting in the phase-lightening of the organelle. A single small phase-dark spot can be detected in five of the irradiated mitochondria (arrows, Plate 2). The irradiated mitochondrion closest to nucleus (Plate 1) does not have a dark spot. SDH analysis of the cell demonstrates that even though the irradiation resulted in a substantial light microscope morphological effect to the mitochondria, the enzyme activity was not affected (Plate 3). All six of the irradiated organelles maintained high levels of SDH activity as indicated by the dark staining reaction. The three mitochondria in the upper portion of the cell appear as one dark-staining mass.

Electron microscopy

Fifteen irradiated mitochondria in five cells were relocated. The predominant feature that delineates these organelles from unirradiated control mitochondria is the presence of an electron dense area localized within a discrete region of the mitochondrion (Plate 4). This electron dense area corresponds precisely in location with the "phase dark" spot observed immediately following irradiation. Two additional good examples of the phase microscope lesions and the corresponding ultrastructural regions are presented in Plates 5, 6, 7 and 8, arrows 1 and 2.

A major feature of the lesion appears to be an alteration of the intercristae matrix. This is the material that presents the highest electron density. The cristae can be seen passing through this region and attaching to the mitochondria membranes (Plate 4). It is possible that the general organization of the cristae (see Plates 7 and 8) are more disorganized (less lamellae-like) in the irradiated structures. However, similar patterns of disorganization and irregular spacing were observed in unirradiated mitochondria, whereas the electron dense matrix lesion was never observed in hundreds of control mitochondria.

Serial sections through the irradiated mitochondrion demonstrated that the localization of the lesion was quite restricted in depth as well as width. For example, lesion 2 (arrow 2, Plate 8) is not detectable in the same mitochondrion in a section through a slightly different level (Plate 9). The cristae appear to be normal Similarly, irradiated mitochondrion No. 3, Plate 6, does not present the typical electron dense lesion in the corresponding electron micrograph (Plate 10). Obviously this section was not through the plane containing the lesion.

4. Discussion

The most frequently observed ultrastructural change was the production of an electron dense area in a localized region of the irradiated organelle. The electron dense area appears to correspond to the phase-dark spot visible in the living state. In fact, the diameter of these small lesions (see lesion No. 1, Plate 7) may be as small as 0.5 μm. This size compares favorably with a measured light-microscope diameter of 0.5 to 1 μm for threshold lesions in blood cells [5]. Close examination of the micrographs reveal that the cristae continue through this region in a rather orderly, lamellae-like pattern. It would seem that the electron dense material is between the cristae membranes. Whether this electron dense material is produced by denaturation, and/or coagulation of the intercristae matrix, or by increasing the affinity of this material for the stain by some other mechanism is not clear. It has been suggested that the production of electron dense material following laser micro-irradiation is characteristic of thermal denaturation [11, 12]. Furthermore, it has been suggested that in our earlier experiments a temperature rise of 100°C at the focal point was likely [10]. Certainly a temperature rise of this magnitude would create considerable thermal damage not only to the entire irradiated structure (cristae as well as intercristae matrix), but to adjacent organelles and cytoplasm. The lesions described in this manuscript are not only localized to one portion of the mitochondrion within the plane of the micrographs, but also we have demonstrated that the lesions may be localized to only a limited area in depth as well (see lesion No. 2, Plates 8 and 9). These results would suggest that if, indeed, the lesions are produced by a thermal effect, the heating is not of such great magnitude as has been predicted [10]. Further evidence suggesting that the integrity of the cristae is

at least partially maintained, is the succinic dehydrogenase assay which demonstrates high levels of SDH activity in the irradiated organelle. Succinic dehydrogenase is generally believed to be located in the cristae membranes. Why and how the matrix between the cristae is damaged without affecting the cristae is, indeed, an intriguing (but yet unanswered) question.

In view of the results presented in this manuscript, the results of earlier functional studies are not surprising. Since the alterations are confined to only small regions within the irradiated organelles, the lack of an observed contractility change is reasonable. Other organelles should be able to function normally, and it even seems possible that the unirradiated regions of the irradiated mitochondrion could function normally. Further ultrastructural analysis of cells with moderate and severe lesions is currently in progress.

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