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Authors

Rattner, JB
Lifsics, Miriam
Meredith, Stephanie
[et al.](#)

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**Argon Laser Microirradiation of Mitochondria
in Rat Myocardial Cells
VI. Correlation of Contractility and Ultrastructure**

J. B. RATTNER, MIRIAM LIFSICS,
STEPHANIE MEREDITH AND MICHAEL W. BERNS

Developmental and Cell Biology, University of California, Irvine, California 92664, U.S.A.

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J. B. RATTNER, M. LIFSICS, S. MEREDITH AND M. W. BERNS. Argon Laser Microirradiation of Mitochondria in Rat Myocardial Cells. VI. Correlation of Contractility and Ultrastructure. *Journal of Molecular and Cellular Cardiology* (1976) **8**, 239–248. A correlation is made between individual laser microbeam lesions (0.25–1 μm in diameter) in single mitochondria of myocardial cells and alterations in cellular contractility. A moderate lesion is described ultrastructurally that has three concentric zones of damage. In addition, the outer mitochondrial membrane is broken, and the intercrisetae matrix outside the lesion may be affected. Two kinds of weak lesions are described. No concentric zones of damage are observed, and the cristae and mitochondrial membranes outside the lesion area are not grossly affected. All cells that exhibited an alteration in contractile patterns had moderate lesions. A correlation between laser energy density and type of lesion was observed. The nature of the lesions and explanations of the contractile responses are discussed.

KEY WORDS: Laser; Mitochondria; Contractility; Ultrastructure; Microirradiation; Cell cultures.

1. Introduction

Intense laser light can be focused to an effective damage-producing spot of 0.25 μm within living cells [3, 4]. When such a beam is focused into a single large mitochondrion of a myocardial cell, various changes in mitochondrial morphology and cellular contractility are observed [1, 5–8]. Light microscope observations suggest at least four different kinds of mitochondrial damage. These are divided into three different general categories: “severe,” “moderate” and “weak.” Two types of lesions are classified as “weak”: (1) a phase dark spot within a generally “paled”* mitochondrion, (2) a completely paled mitochondrion. The moderate lesion appears as a phase dark spot with a phase-light central region. The severe lesion appears to be destruction of the entire irradiated mitochondrion and generally results in rapid cell death.

In addition to the apparent morphological alterations to the irradiated mitochondrion, contractile changes have been observed [5, 7]. These changes follow

* The term “paled” is used to indicate that the mitochondrion, as viewed with the phase microscope, has undergone a considerable decrease in optical phase density.

definite sequential patterns (see summary in Figure 1). In order to more fully understand and interpret these responses, it is necessary to examine single irradiated cells with the electron microscope.

In this report, we will describe the ultrastructural changes in irradiated cells and correlate these changes with the observed contractile responses.

2. Materials and Methods

An argon laser microbeam was employed in these studies [2]. Primary wavelengths of 488 nm and 514 nm were focused by a $\times 100$ oil immersion Neofluar objective to spot diameters of 0.25–1.0 μm . Total laser output was 19.7 W per 50 μs pulse. Calibrated neutral density filters were used to attenuate the laser output to energy densities of 6.7 μJ , 10 μJ , and 12.6 μJ in the focused spot. In all experiments, the mitochondrion was irradiated for one 50 μs pulse. A time-lapse videotape system combined with the laser microbeam provided continual recording before, during and after irradiation. Data on the contractile responses were abstracted from the videotape.

The heart cell cultures were established in Rose multipurpose culture chambers according to the procedures of Mark and Strasser [10]. Ventricles from 2–4 day old rats were cut into small pieces (1–2 mm^3) and subjected to stepwise enzymatic digestion in 0.1% Viokase. Following resuspension in supplemental minimal essential medium and adjustment of pH to 7.2–7.4, the cells were injected into the culture chambers. After incubation at 37°C for 2 days, numerous contracting myocardial cells were observed attached to the bottom glass plate of the chamber. Medium was changed every 2–4 days.

Irradiation of cells was accomplished by placing one of the chambers on the laser microbeam stage. A constant air curtain incubator was used to maintain the chamber at 37°C. The target mitochondrion was moved under a crosshair on the TV monitor by gently moving the mechanical stage of the microscope. Pre-alignment of the system established that the laser beam was focussed directly under the crosshair. The cells either were recorded on videotape for several minutes pre- and post-irradiation in order to document the contractile responses, or they were fixed within 1 min after irradiation for electron microscopy.

The monolayer cultures grown on 1% siliclad coverslips were fixed *in situ* after irradiation in 3% glutaraldehyde buffered in Millonig's phosphate buffer, pH 7.4, and post-fixed in 1% OsO₄ buffered in a similar manner. Cells were embedded according to the method of Brinkley *et al.* [9, 11]. The irradiated cell was relocated, scored with a diamond objective marker, cut and cemented to an Epon block. Serial sections in the silver range were cut on an LKB Ultratome III, collected on copper slotted grids coated with formvar and carbon, and examined on a Siemens Elmiskop 1A operated at 60 KV.

3. Results

Light microscope morphology and contractility

Two types of mitochondrial lesions were produced: a dark spot within an otherwise "paled" mitochondrion and a dark spot with a phase light central zone. The former lesion would be classified as "weak" and the latter as "moderate" by our previous classification scheme [6]. A correlation among the lesion type, laser energy density, and contraction change was observed (Table 1). There was a predominance of weak

TABLE 1. Correlation of lesion type, laser energy density and contractility

Lesion type	Laser energy†			Change in contraction	Remains rhythmic
	6.7 μ J	10.0 μ J	12.6 μ J		
Weak	12*	5	1	0	18
Moderate	0	2	15	14	3

* Number of cells.

† Energy density in 0.5 μ focused spot.

lesions (12/18) with the lowest energy level (6.7 μ J) and of moderate lesions (15/17) with the highest energy (12.6 μ J). Most of the contractility‡ changes (14/17) occurred in cells with moderate lesions, and all of the cells with weak lesions continued to contract rhythmically. Cells irradiated in non-mitochondrial regions of the cytoplasm with all three energy densities never exhibited a contractility change.

Cells undergoing sequential contraction changes exhibited six different patterns (Table 2). Of the nine cells where detailed timing data was extracted from the videotapes, eight cells exhibited the "fibrillation"§ response. In addition, eight cells returned to a rhythmic rate of contraction. However, the post-irradiation rhythmic contraction rates were often considerably different than the pre-irradiation rates. The mean change in beats/minute for this group was 42. This contrasts considerably with the cells that had weak lesions and exhibited no sequential change in contractility (Table 3). In this group of cells, the mean change in beats/minute was 10.7. It is significant to note that seven (33%) of these cells had a post-irradiation beat rate identical to the pre-irradiation rate. This group appeared to be very similar to unirradiated control cells where the mean change in beats per minute was 9. In establishing the mean change in beat rate, an absolute value in change in frequency has been used regardless of whether the change was (+) or (-). There appeared to be no consistent pattern with respect to either an increase or decrease in beat frequency.

‡ A contractility change is defined as an observed change in the beat frequency. This either may be an increase, decrease, or induction of an irregular beat pattern.

§ Fibrillation of a single cell is defined as an uncoordinated, rapid, irregular contraction pattern of a single cell.

TABLE 2. Sequential changes following laser microirradiation of one mitochondrion*. (All lesions were moderate)

Cell #	Pre-irradiation beat frequency (beats/minute)	Sequential changes†	Change‡
1	{	42 → Stp (3s) → Fib (38s) → Ir (19s) → Rhy 36 (beats/min)	(-) 6
2		40 → Stp (67s) → Fib (10m) → Ir (40s) → Rhy 36	(-) 4
3	{	40 → Fib (19s) → Ir (11s) → Rhy 60	(+) 20
4		34 → Fib (9s) → Ir (22s) → Rhy 53	(+) 19
5	{	100 → Fib (6s) → Stp (10s) → Rhy 35	(-) 65
6		7 → Fib (30s) → Rhy 90	(+) 83
7	{	10 → Fib (28s) → Rhy 90	(+) 80
8		60 → Fib (3s) → Stp (indefinite)	(-) 60
9	28 → Stp (33s) → Ir (29s) → Rhy 72	(+) 42	
		$\bar{X} = 42$	

* Sequences on only 9 of the 14 cells are presented because on 5 cells, accurate timing data could not be extracted from the videotape because of mechanical malfunction.

† s = seconds (time in parentheses is the time duration of that particular response), m = minutes.

‡ Change = change in beats/minute; \bar{X} is calculated using absolute value regardless of whether (+) or (-).

Stp = Stop.

Fib = Fibrillate.

Ir = Irregular contractions.

Rhy = Rhythmic contractions (beats/minute).

TABLE 3. Rhythmic contractility following laser microirradiation of one mitochondrion

Pre-irradiation rate (Beats/min)	Post-irradiation rate	Change*
45	54	(+) 9
60	48	(-) 12
40	34	(-) 6
48	74	(+) 26
90	80	(-) 10
24	51	(+) 27
53	54	(+) 1
84	84	0
66	78	(+) 12
72	60	(-) 12
90	90	0
72	72	0
60	60	0
60	60	0
120	120	0
132	138	(+) 6
150	150	0
198	168	(-) 20
132	126	(-) 6
48	60	(+) 18
150	210	(+) 60
		$\bar{x} = 10.7$

* \bar{x} is calculated using absolute value regardless of whether (+) or (-).

Moderate lesion ultrastructure

Pre- and post-irradiation light micrographs of mitochondria exhibiting typical moderate lesions are presented in Plates 1 and 2. The two mitochondria labeled moderate (M_1 and M_2 , Plate 2) have the characteristic dark spot with a light central zone. Both mitochondria are markedly pale in their non-lesion areas when compared to the pre-irradiation pictures of the same regions. This is particularly clear in mitochondrion M_2 where the mitochondrial material along one side of the lesion (small arrow, Plate 2) is much paler than pre-irradiation.

The ultrastructure of the two mitochondria with moderate lesions is depicted in Plates 3 and 4. Both lesions have a light central zone which appears to contain remnants of cristae membranes. A second region of considerable electron density surrounds the central light zone. In some portions of this zone, there appear to be electron dense, lamellar-like structures which may be cristae or intracristae material. They are more clearly discernible in a thin section through a slightly different plane of M_1 (see inset in Plate 3).

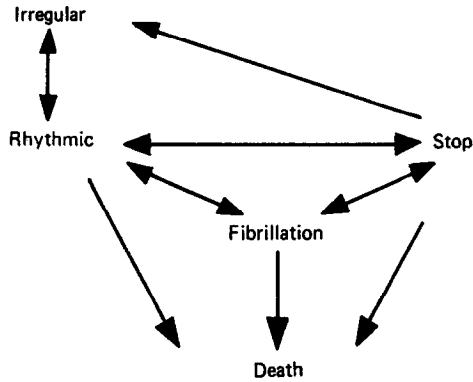


FIGURE 1. Contractile sequences observed in single myocardial cells after laser microirradiation. All cells initially started at rhythmic. The arrows indicate the observed sequential changes.

TABLE 4. Contraction changes of control cells

Time 0 (beats/min)	30 min	Change*
60	60	0
60	64	(+) 4
24	20	(-) 4
60	60	0
32	40	(+) 8
96	120	(+) 24
62	88	(+) 26
76	60	(-) 16
80	64	(-) 16
112	104	(-) 8
56	48	(-) 8
68	72	(+) 4
112	116	(+) 4
136	124	(-) 12
80	68	(-) 12
120	128	(+) 8
64	64	0
		$\bar{x} = 9$

* \bar{x} is calculated using absolute value regardless of whether (+) or (-).

A final lesion zone of moderate electron density surrounds the innermost electron dense region (see Plates 3 and 4). Much of the increased electron density of this lesion zone appears to be in the matrix between the cristae. Cristae from the unirradiated region of the mitochondrion can be seen penetrating into this lesion

PLATE 1. Phase micrograph of pre-irradiation cell. Arrows indicate target mitochondria. ($\times 4800$).

PLATE 2. Post-irradiation cell. M_1 and M_2 are mitochondria with moderate lesions. W_1 is a mitochondrion with a weak lesion. ($\times 4800$).

PLATE 3. Electron micrograph of mitochondrion M_1 with moderate lesion. Inset is from an alternate serial section. ($\times 55\ 000$).

PLATE 4. Electron micrograph of mitochondrion M_2 with a moderate lesion and of mitochondrion W_1 with a weak lesion. ($\times 42\ 500$).

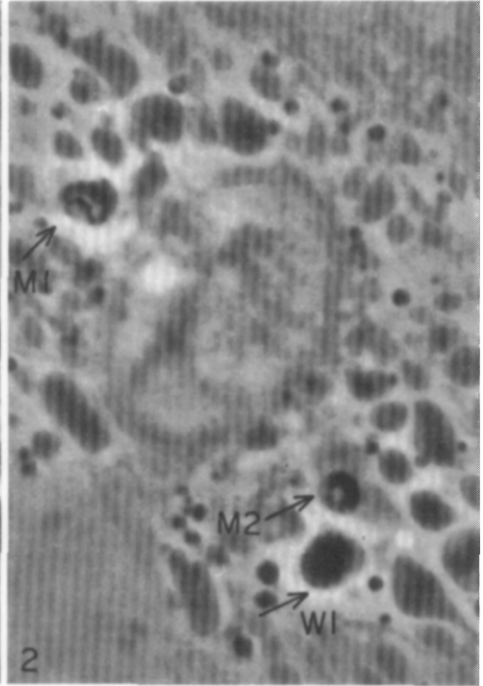
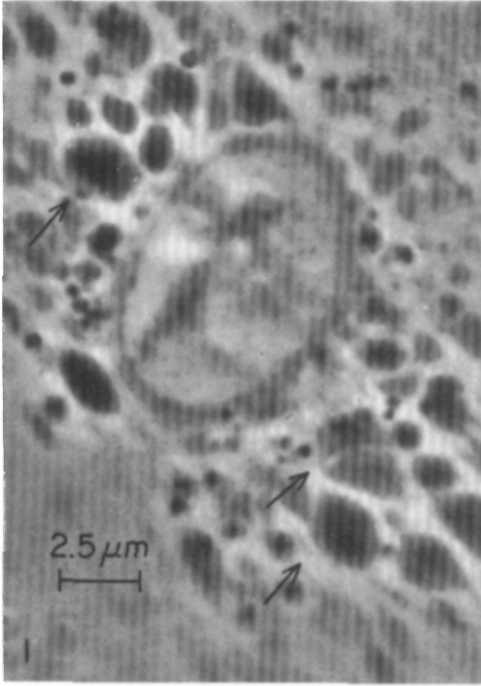
PLATE 5. Light micrograph of pre-irradiation cell. Arrows denote target mitochondria. ($\times 4000$).

PLATE 6. Post-irradiation micrograph indicating (arrows) three mitochondria with weak lesions. ($\times 4000$).

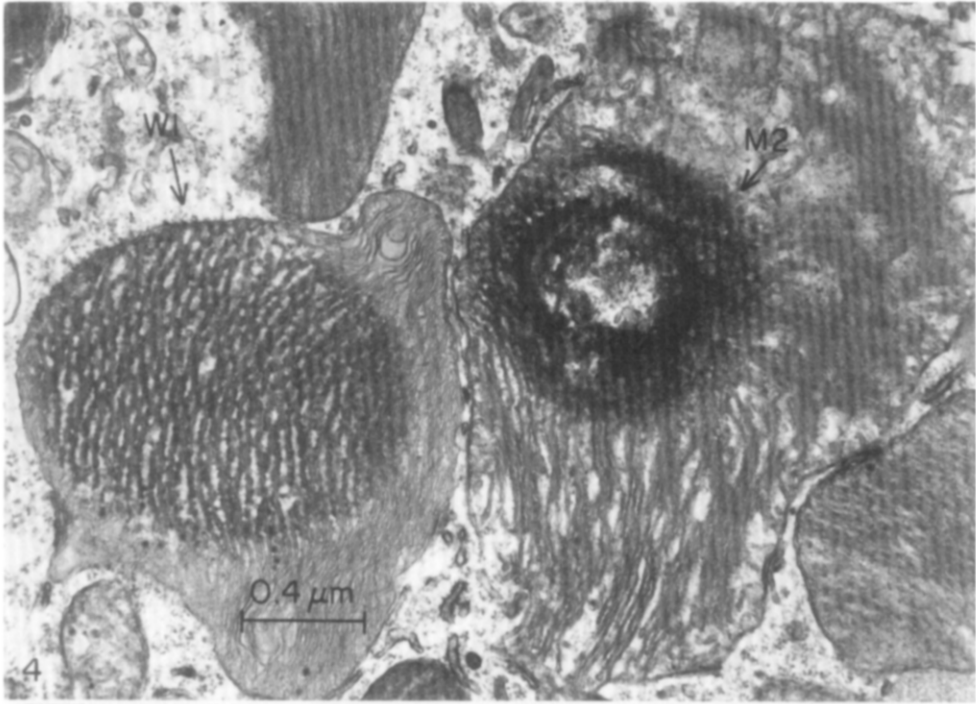
PLATE 7. Electron micrograph of mitochondria with weak lesions W_3 and W_4 . Small arrow indicates a mitochondrion that was exposed to radiation focused into mitochondrion W_4 . Note the small lesion in this organelle. ($\times 55\ 000$).

PLATE 8. Mitochondrion W_2 with a small weak lesion, arrow. ($\times 27\ 000$).

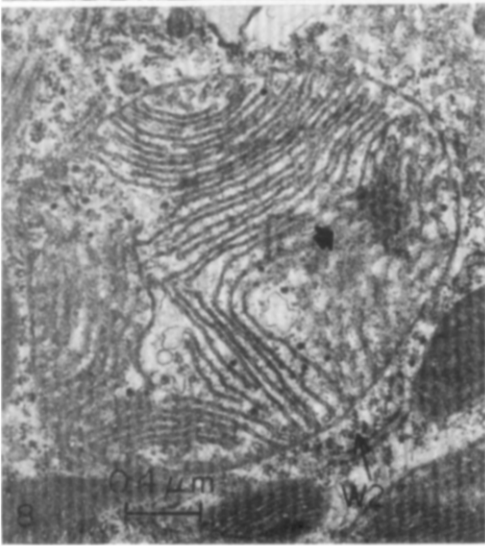
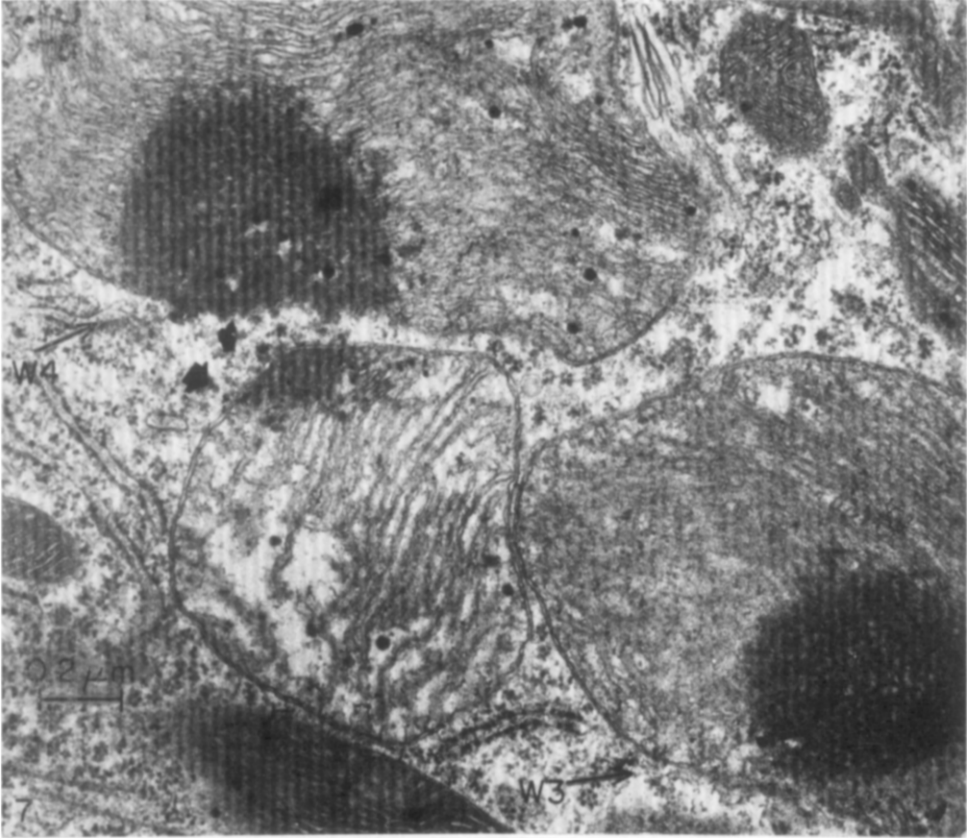
PLATE 9. Alternate serial section of mitochondrion W_2 . Note the absence of the small electron dense lesion present in the preceding section. ($\times 27\ 000$).



PLATES 1-3



PLATES 4-6



PLATES 7-9

zone, and in some regions (see inset, Plate 3), they appear to penetrate all the way through it. In addition to the three concentric zones of the moderate lesion, the outer mitochondrial membranes appear to be broken in numerous places. Furthermore, in several areas outside the lesion zone but within the irradiated mitochondrion, there appears to be a reduced amount of electron dense material in the intercrisae spaces. This may explain the paling phenomenon that is often observed in the unirradiated portions of the mitochondrion.

Weak lesion ultrastructure

One weak lesion was produced in the previous cell (labeled W_1 in Plates 2 and 4), and three weak lesions (W_2 - W_4) were produced in the cell depicted in Plates 5-9. When viewed with the phase contrast microscope, all four of the weak lesions appeared as a dark spot within an otherwise paled mitochondrion. The paling of the rest of the mitochondrion is particularly clear in lesion W_2 and W_4 (Plate 6). However, when viewed with the electron microscope, lesion W_1 appeared different than W_2 , W_3 , and W_4 . Lesions W_2 - W_4 appeared to contain alternating areas of electron dense and electron light material. The cristae appeared broken down in these regions as no lamellar membrane pattern was evident. However in W_1 , cristae could be seen traversing completely through the lesion area. These cristae membranes appeared to have greater than normal electron density and considerably more spacing between.

The general integrity of the mitochondria with weak lesions was maintained. The outer mitochondrial membranes appeared to be mostly intact, and the cristae outside the lesion area were organized in a lamellar-like pattern. The lack of alteration outside of the lesion area was particularly clear in mitochondria W_1 (Plate 4), W_3 and W_4 (Plate 7). In mitochondrion W_2 (Plates 8 and 9), there appeared to be some degree of cristae disorganization. But even within this organelle, several areas with lamellar cristae patterns were evident. Serial sections through this mitochondrion revealed that the electron dense lesion material (small arrow, Plate 8) was limited in depth (Plates 8 and 9) within a single mitochondrion. A final observation on the weak lesion is that a fifth weak lesion (small arrow, Plate 7) was produced. This lesion was produced in a mitochondrion adjacent to W_4 . Apparently the focal point of the laser beam extended over this mitochondrion which was not visible with the light microscope. The general ultrastructure of this mitochondrion and lesion was similar to W_2 .

4. Discussion

There is a definite correlation between lesion type and change in contractility. All of the cells that exhibited a sequential change in contractility had moderate lesions placed in one mitochondrion. No cells with weak lesions went through one of the

sequential contraction patterns. However, a moderate lesion does not automatically produce a contraction change. Three cells with moderate lesions did not exhibit altered contractility.

As in earlier studies, the type of contractility responses were quite variable. In the studies reported here, six different sequences were observed. Of the nine cells carefully followed, eight exhibited the uncoordinated response termed "fibrillation", and seven of these eight returned to a rhythmic rate of contraction within several minutes of irradiation. Though some of these cells returned to rates very close to the pre-irradiation rate, others varied considerably. This contrasts greatly with the control cells and the irradiated cells that did not exhibit contractile changes.

In addition to a correlation between lesion type and contractility change, there also appears to be a correlation between energy density and lesion type. There was a preponderance of moderate lesions with 12.6 μJ and weak lesions with 6.7 μJ .

The ultrastructural studies reveal that the moderate lesion has three concentric zones: (1) an inner light central region that contains some disrupted cristae, (2) a middle dark electron dense zone, and (3) an outer region containing both electron dense and electron light material and also some visibly altered cristae. Mitochondria with moderate lesions have varying degrees of cristae disorganization outside the lesion itself, and the outer mitochondrial membranes appear to be broken in numerous places.

Lesions classified as "weak" with the light microscope have varied ultrastructure. One kind of weak lesion (W_2 - W_4) appears as an electron dense region without any evidence of cristae and with scattered light areas throughout. The other weak lesion (W_1) appears to have an altered, but definite, cristae pattern. It is possible that the two types of weak lesions merely reflect a gradation of damage with the least amount of damage (non-destruction of cristae) occurring in lesion W_1 . This possibility is further suggested by the fact that the outer electron dense zone of the moderate lesions (Zone 3 above) is almost identical to the more severe weak lesion. In addition, careful examination of this outermost zone (see M_1 , Plate 3) reveals areas that appear to have cristae patterns similar to those found in the weakest lesion (W_1). The observations on the weak and moderate lesions indicate a pattern of decreasing damage emanating from the center of the moderate lesion. The observation that the outer region of the moderate lesions look similar to the weak lesions supports this idea.

The ultrastructural studies do not provide a definitive explanation for the observed contractile changes. However, the fact that the weak lesions do not result in much disruption to the irradiated mitochondrion other than in the actual lesion area itself does agree with a lack of observed contractile response. The more severe alterations produced by the moderate lesions, such as, general cristae disruption and mitochondrial membrane breakage, does allow one to more easily reconcile the contractility changes. The morphology of the moderate lesion suggests a thermal effect. If a temperature rise were occurring at the focal point, a thermal gradient

would be established. The three concentric levels of damage, with the greatest alteration in the center, are characteristic of a thermal gradient. The actual precipitation of contractile change could be due to heat transfer to the above (or below) electrically active cell membrane. A transient effect on the cell membrane could result in the abnormal contractile sequences followed by a return to rhythmicity. On the other hand, it is also possible that the heat dissipating from the focal point produces an effect only on the irradiated mitochondrion. This view is supported by lack of damage to organelles and other cytoplasmic inclusions near the damaged mitochondrion. Under these conditions, it is possible that the contractility changes are produced by a release of calcium from the irradiated mitochondrion. Such an efflux of calcium into the general cell cytoplasm could conceivably result in a temporary alteration in cellular contractility. Another possibility suggested by Salet [12] is that the laser energy is converted into usable chemical energy in the form of ATP which is subsequently used to drive the cell into an altered state of contractility. Electrophysiological studies are currently being undertaken in order to help elucidate this question.

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