Some Effects of Extreme Shortening on Frog Skeletal Muscle

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In 1940 Ramsey and Street found that if isolated fibers of frog muscle were stimulated to shorten to more than one-third of the length at which maximum isometric tension was developed, some of their reactions were permanently changed. Probably the most important of these changes was an alteration in the relation of fiber length and isometric tension. Active tension developed at shorter lengths than before, and maximum isometric tensions were about 50% of normal. At that time, we believed that it was the contractile proteins that were affected because we could find no significant change in passive tension or in excitation, but this was not sufficient evidence to prove the point. Now that electron microscopy has so greatly increased the information on the fine structure of muscle, it seemed worthwhile to undertake further studies on muscle that has shortened below its normal limits. In 1940, we applied the term "delta state" to all such muscle, but in this paper we apply it only to muscle which has shortened to 35% or less of optimum tension length. This article will describe the results obtained to date in an electron microscope study of shortened muscle and will also include measurements of the resting oxygen consumption of delta state frog sartorius muscles.

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MATERIALS AND METHODS

Electron Microscopy

Semitendinosus muscles (*Rana pipiens*) were isolated and equilibrated overnight in frog Ringer's solution. The next day small bundles consisting of three or more muscle fibers were dissected from them. These were stimulated to shorten partly or fully as described in the figure legends.

The bundles were fixed for two hours by adding sufficient glutaraldehyde to the frog Ringer bath to bring the concentration to 2.5% and post fixed in 2% OsO_4 with phosphate or veronal acetate buffer for three to four hours. They were then washed in distilled water, dehydrated in a graded series of acetone solutions and embedded in Araldite. Sections of 600 to 800 Å in thickness were cut with a Porter-Blum microtome (MT-2) using glass knives. The sections were stained with $KMNO_4$ (Lawn, 1960) or lead citrate (Reynolds, 1963) and viewed with an RCA EMU 3 E or 3 G electron microscope.

Oxygen Consumption

Sartorius muscles of medium sized frogs (*R. pipiens*) were measured in situ. Both muscles were dissected out, but were left attached to the pelvis and placed in frog Ringer's solution to equilibrate overnight at 4 C.

The next day tetanic isometric tension was recorded with the muscles held at the length measured as maximum in the body: for sartorius this is approximately optimum tension length. (Grass stimu-

lator: stimulus duration, 1 msec; frequency 80/sec for 0.2 seconds, temperature 18 C). Tension was recorded by a suitable strain gauge. Then both muscles were given three two-second tetani at 10-minute intervals; one muscle was allowed to shorten freely, the other was held at maximum body length.

Resting oxygen consumption was measured by standard methods. Right and left muscles from each frog were paired because resting oxygen consumption of muscles from different frogs normally varies by at least a factor of two. After the tetani were administered, the muscles were cut off the pelvis, weighed, and placed in Warburg flasks. Oxygen consumption was measured at 18 C for 4 to 5½ hours.

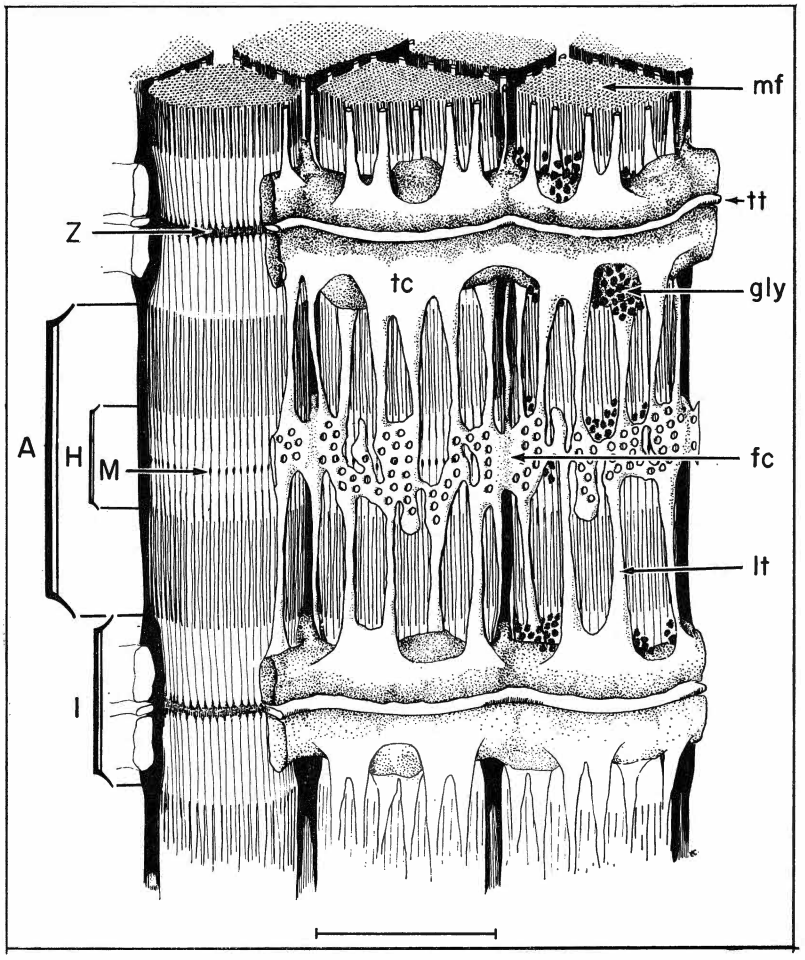

Fig. 1—Three-dimensional reconstruction of the sarcoplasmic reticulum (SR) associated with several myofibrils of frog sartorius muscle. Reproduced by permission of the Rockefeller University Press (Peachey, 1965) with our lettering added. The myofibrils (mf) show a transverse banding pattern with the Z lines at the centers of the light I bands (I). The A band (A) has a central light H zone (H) and denser outer regions where the thick and thin myofilaments overlap, and a dark M line (M) at its center. The fenestrated collar (fc), or H band cisterna, of the SR connects to the terminal cisternae (tc) by the longitudinal tubules (lt). Transverse tubule (tt). Glycogen granules (gly). Dimensions: sarcomere, Z line to Z line-2.65 μ; A band (thick myofibrils) $\sim$1.6 μ; thin myofibrils $\sim$1 μ. Bar $\sim$1 μ.

Three series were done: 1) Normal paired with delta long (the shortened muscle was slowly extended to its original length and active tension during a brief, 0.2-second tetanus was recorded at that length). 2) Normal paired with delta short (the shortened muscle was not re-extended and the last two-second tetanus was given in the Warburg flask). 3) Delta short paired with delta long.

RESULTS

Electron Microscopy

We have found evidence that it is the contractile proteins (the interdigitating filaments) which are most affected by extreme shortening and have also demonstrated that the sarcolemma is tied in to each sarcomere at the level of the M line, as well as at the Z line. Before describing these results we shall

Fig. 2—Normal, longitudinal section, a relatively smooth sarcolemma (S) is present. The usual cross banding is evident (A,I,Z, and M). Mitochondria (Mi), lipid (L) and glycogen (electron dense granulation) occupy the intefibrillar space. Triads (T) are formed by the transverse tubule centrally and terminal cisternae of the sarcoplasmic reticulum laterally. Pb stained. [Figs. 2 through 7, labeling as in fig. 1. Frog semitendinosus muscle.]

outline the presently accepted concepts of the fine structure of a striated, frog muscle fiber. (The structure of mammalian muscle fibers is essentially the same, the main differences being in the location of the transverse tubule system).

Each muscle fiber is enclosed by its sarcolemma, a four layered elastic sheath which terminates in micro-tendons; its innermost layer is the plasma membrane of the cell (Mauro and Adams, 1961). The myofibrils largely fill the interior and are believed to extend from one end of the fiber to the other. Figure 1 is a three dimensional reconstruction (Peachey, 1965). The myofibril on the left shows the interdigitating thick and thin myofilaments (H. Huxley and Hanson, 1954; A. Huxley and Niedergerke, 1954) and their relation to the Z and M lines; the others show the related sarcoplasmic reticulum (SR) and transverse tubule system. The transverse tubules form a network which transverses the fiber at the Z lines and has openings to the fiber surface. (Francini-Armstrong and Porter, 1964; H. Huxley, 1964). The SR is homologous to the endoplasmic reticulum of other cells; it sheaths the myofibrils and its network extends across the width of the fiber at each sarcomere level. In longitudinal section, the transverse tubule with the associated terminal cisternae of the SR form a "triad."

When sarcomere length is 2.0 to 2.2 μ, maximum isometric tension develops if the muscle is appropriately stimulated. Within the normal range of motion of a muscle, changes in fiber length, whether active or passive, correlate with changes in sarcomere length and with the amount of overlap of the thick and thin filaments, without change in length of the filaments. When a sarcomere has shortened to 1.6 μ, the ends of the thick filaments are touching the Z line; further shortening involves crumpling or coiling of the filaments.

Figure 2 is a longitudinal section of normal semitendinosus, showing typical cell structures, including the sarcolemma, while figure 3 shows a bit of the edge of a fiber from a small bundle that was stimulated to shorten to a sarcomere length of about 1.3 μ. Sufficient Glutaraldehyde was added to the Ringer's solution to bring the concentration to 2.5% while stimulation continued. The Z line, presumably, is obscured by material of the filaments piling up near it and the M line by the presence of a double array of thin filaments which have slid into it. The most striking feature is the festooning of the sarcolemma. Festooning at the Z line was first described about a hundred years ago (Tiegs, 1955). In so far as we know, festooning at the M line has not been observed before. Its occurrence strongly suggests that the H-band cisterna, or fenestrated collar of the SR, adheres to the sarcolemma.

Figures 4, 5, 6, and 7 are all of the same specimen. This was a bundle of three fibers which were stimulated to shorten from 14 mm to about 4 mm by three two-second tetani. Such long stimulation is not necessary for complete shortening of a small bundle of fibers; it was done so that the specimen would be comparable with the delta state sartorius muscles. The bundle was stretched slightly after shortening and fixed at a fiber length of 6.5 mm. The structures shown are indicated in the figure legends. Festooning of the sarcolemma at the Z and M lines is definite, indicating that the transverse systems have not been disrupted. The mitochondria with their cristae are intact and transverse tubules and sarcoplasmic reticulum and the triads they form are easily recognized and appear normal. However, the thick myofilaments are less than 1 μ long and somewhat disarrayed.

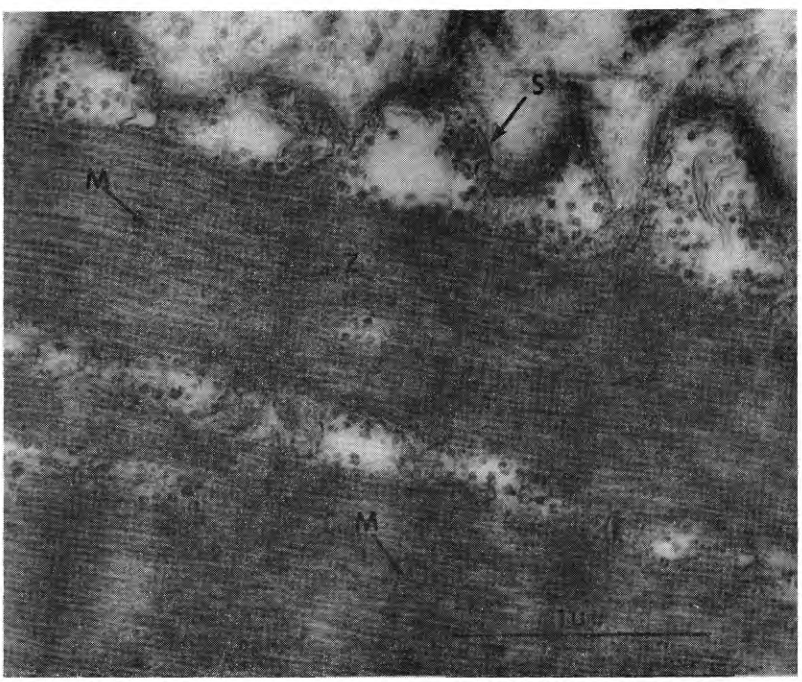

Fig. 3—Longitudinal section of the edge of a muscle fiber fixed at a sarcomere length of 1.3 μ while being tetanized. Shortening has obscured the cross-banding; Z lines are not distinct due to thick filaments of adjacent sarcomeres abutting at that point. M band is present. Note the pronounced festooning of the sarcolemma produced by shortening. This preparation indicates an attachment of the sarcolemma to intracellular structures at the level of the M line as well as at the Z line. $KMnO_4$ stained.

Resting Oxygen Consumption

In these experiments, we considered that a muscle was in the delta state if maximum isometric tension decreased at least 30% after the shortening procedure; usually it decreased 50%.

When using whole muscle it is necessary to remember that one is dealing with a population of fibers. We think the loss of tension is not due to failure of half the fibers to react for several reasons. The three main ones are:

a) Visual observation with low magnification confirms that most fibers shorten.

b) It is well known that a quick stretch of 2 or 3 mm applied to a normal muscle during a tetanus at optimum length results in a considerable increase of tension, as much as one-fourth to one-third of the maximum. Quick stretch of delta state single fibers or whole muscle results in only a slight increase of tension and never restores the maximum.

c) After shortening, the relation between length and tension is the same for sartorius muscle as for delta state single fibers of muscle (Ramsey and Street, 1940). Figure 8 shows a graph of such an experiment. Its details are explained in the legend.

The data for the resting oxygen consumption are given in table 1. There is considerable variation, but since the oxygen consumption of delta state muscle always exceeded that of its normal pair, and delta short always exceeded delta long the differences are definitely significant. The probability that these observations could occur by chance is less than 0.005 for the first and third sets and 0.05 for the second.

DISCUSSION

The finding that the sarcolemma is quite firmly tied in to the sarcomeres at the M line as well as at the Z line is an interesting one. According to Tiegs (1955), the attachment at the Z line, indicated by festooning, was observed as far back as 1859. It seems likely that the network of transverse tubules is part of this tie. He describes the M line as "exceedingly delicate," and pictures it as sometimes extending to the sarcolemma. Dorn (1965) says he has often observed invaginations at the M line as well as at the Z line in guinea pig muscle, but does not illustrate it.

While the sliding filament model of muscle structure has been accepted for years as the best one available, some aspects of mechanical coupling have never been clarified. For example, how can the unattached array of thick myofilaments stay neatly in place in the middle of the sarcomere while generating forces of 2 to 4 kg/cm^2 of *fiber* cross sectional area? A transverse network at the M line could play a role in positioning these filaments and there is considerable evidence for the existence of a complex network there. Franzini-Armstrong and Porter (1964) have pictured cross connections between the thick filaments at the M band. The cisternae of the SR, located at the middle of the sarcomere have continuity across the transverse width of the muscle fiber (Bennett, 1960; Peachey, 1965), and Bennett (1955) and Foulks (1965) have shown that they often connect to the M band. We also have seen this in our preparations (fig. 5). It seems likely that these cisternae adhere to the sarcolemma at the circumference of the muscle fiber but serial sections will be necessary to prove the point.

In some circumstances, active tension, even maximum isometric tension, is transmitted laterally from myofibril to sarcolemma and tendon (Street and Ramsey, 1965). It is possible that the transverse networks are involved in this mechanical coupling, in spite of their delicacy.

What happens to the interdigitating filaments when a sarcomere shortens below 1.6 μ is a problem we are still working on. Micro-

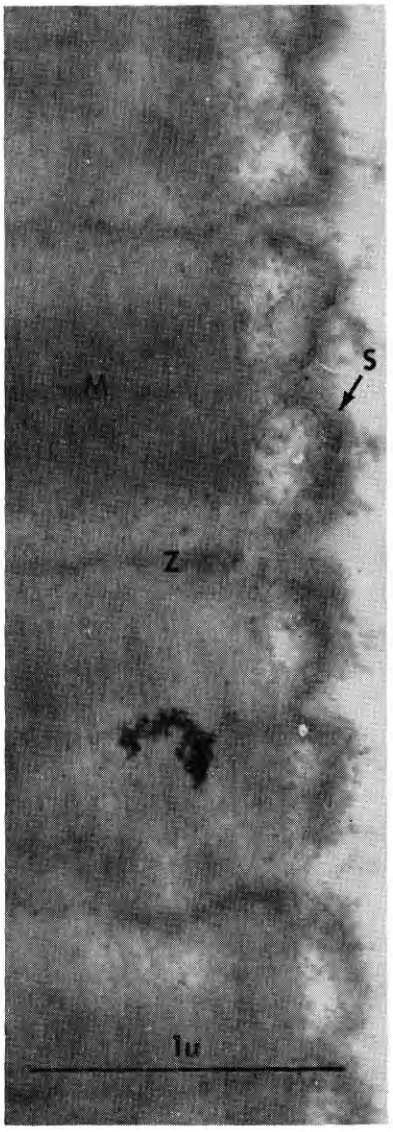

Fig. 4—The sarcolemma still shows festooning, indicating that the attachments in the regions of the Z and M lines are not broken. $KMnO_4$ stained. (Figs. 4, 5, 6, and 7 are all from the same preparation. Three muscle fibers were stimulated to shorten fully, as described in the text, slightly re-extended, and fixed at a sarcomere length of about 1 μ. Delta state muscle.)

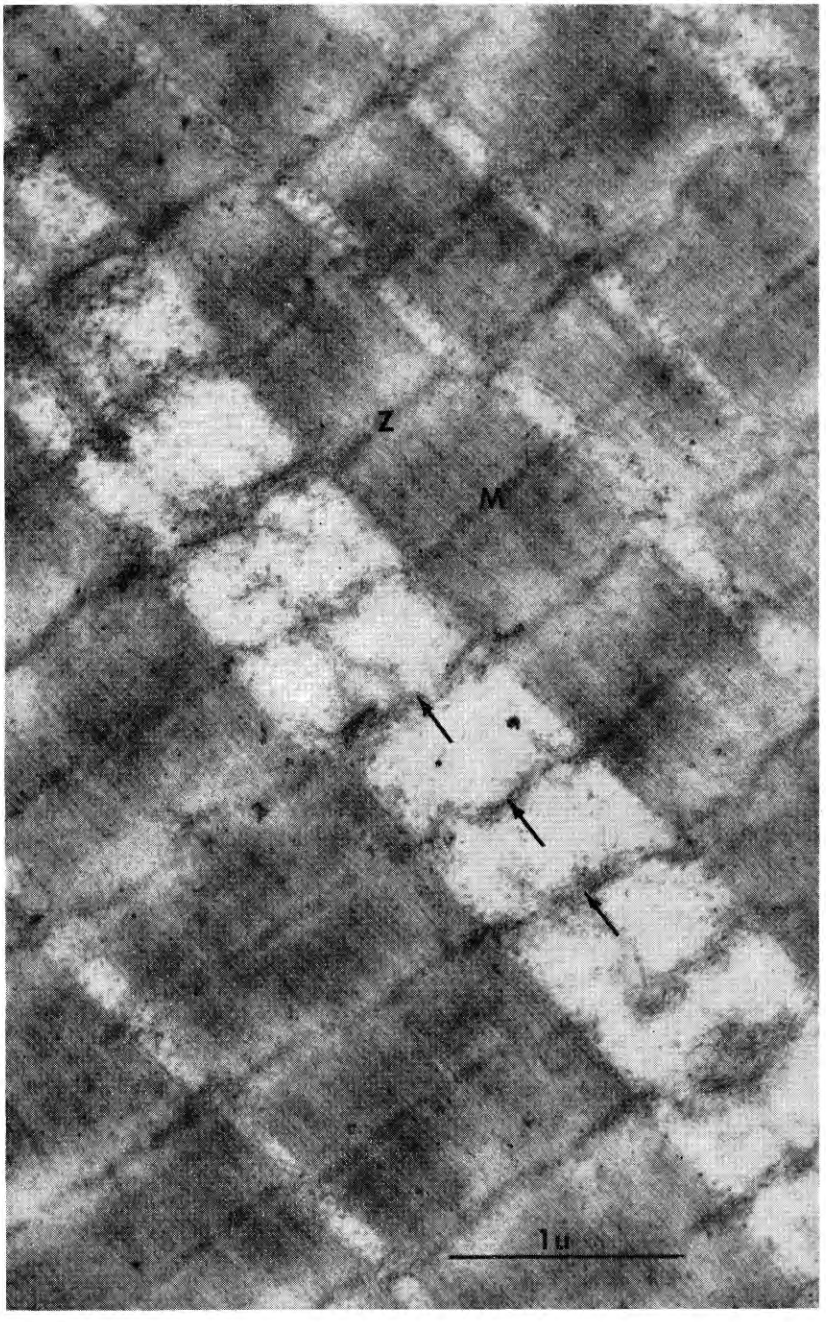

Fig. 5—This micrograph demonstrates myofibril connections at M and Z lines by strands of sacoplasmic reticulum transversing the interfibrillar space (arrows). $KMnO_4$ stained.

graphs of re-extended delta state muscle show the M line still clear and straight, which suggests the cross connections between the thick filaments are not broken. Galey (1964) has published pictures of contractures in frog semitendinosus which show thick filaments varying in length from 0.4 μ to 1.6 μ. Perhaps when the thick filaments shorten they stay short even when the muscle is re-extended.

The increase in resting oxygen consumption of delta state muscle is interesting, but so far unexplained. Fischer (1931) clearly showed that when normal muscle, contracting isotonically, shortened by 10% of its length, the activity oxygen consumption was markedly reduced. In fact, it was this early work of Dr. Fischer's that stimulated our interest in the oxygen consumption of delta state muscle.

SUMMARY

We have established that the sarcolemma of frog skeletal muscle is so firmly tied in at each sarcomere level near the M line, as well as near the Z line, that it is thrown into folds or festoons when the fibers shorten. The attachment is not broken even when the fibers shorten to 25% of optimum tension length. Such extreme shortening affects both the morphology and physiology of the muscle; the morphological change seems to be limited to the myofilaments. The physiological effects in frog sartorius muscle include an increase in resting oxygen consumption and changes in the relation between fiber length and isometric tension similar to those found in isolated muscle fibers.

Acknowledgments

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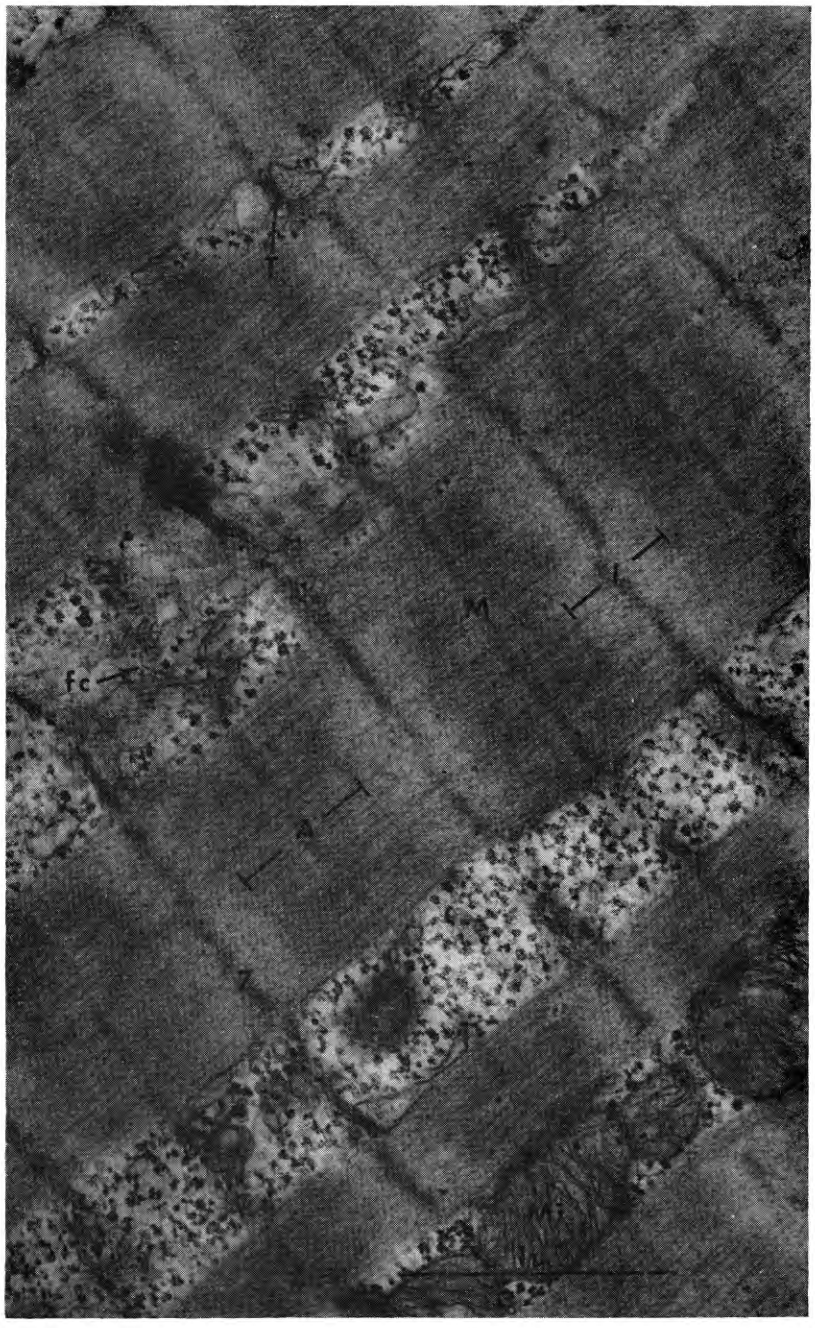

Fig. 6—Mitochondria, triads and fenestrated collar appear normal. The A band is reduced to a length of 0.6 μ in places and its outline is irregular. Pb stained.

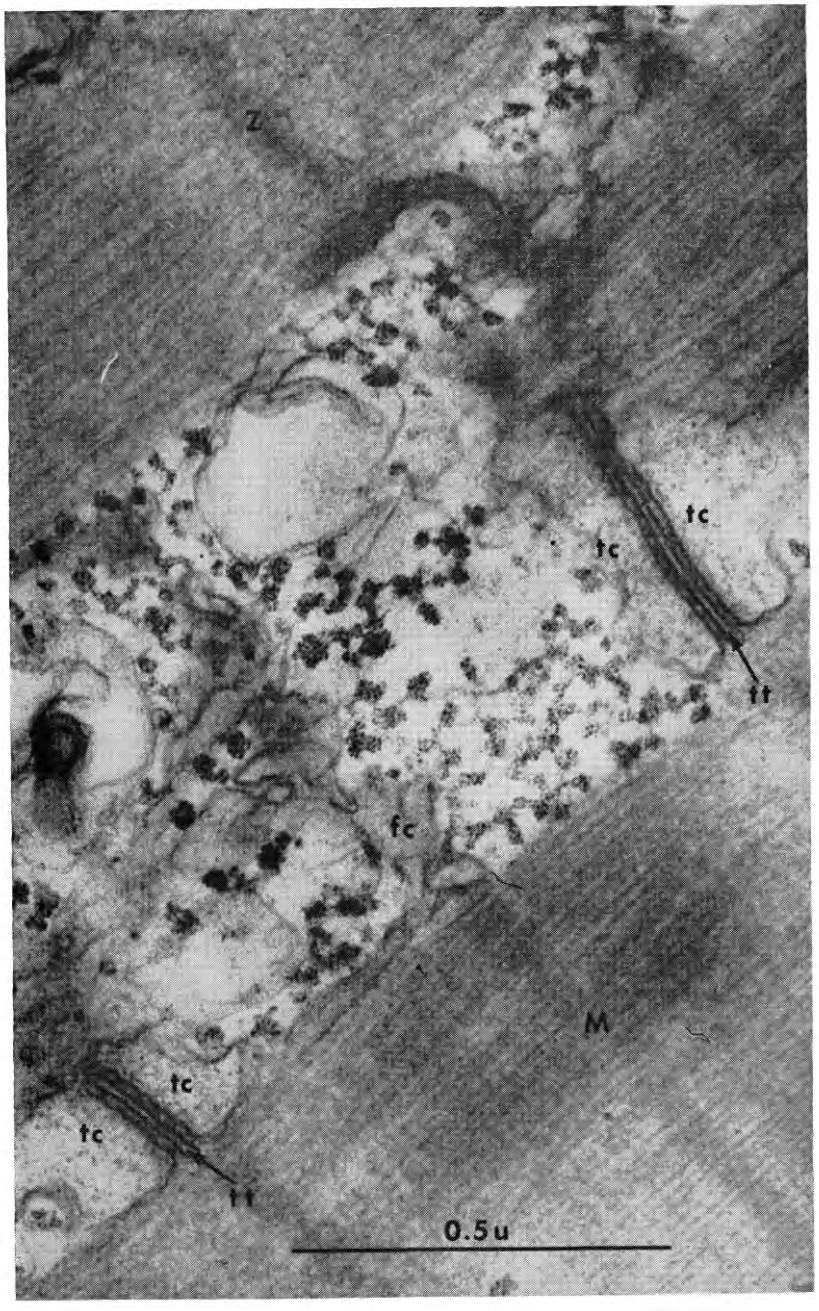

Fig. 7—This micrograph illustrates in more detail the quite normal appearance of the transverse tubules, terminal cisternae, and fenestrated collar region in delta state muscle. Pb stained.

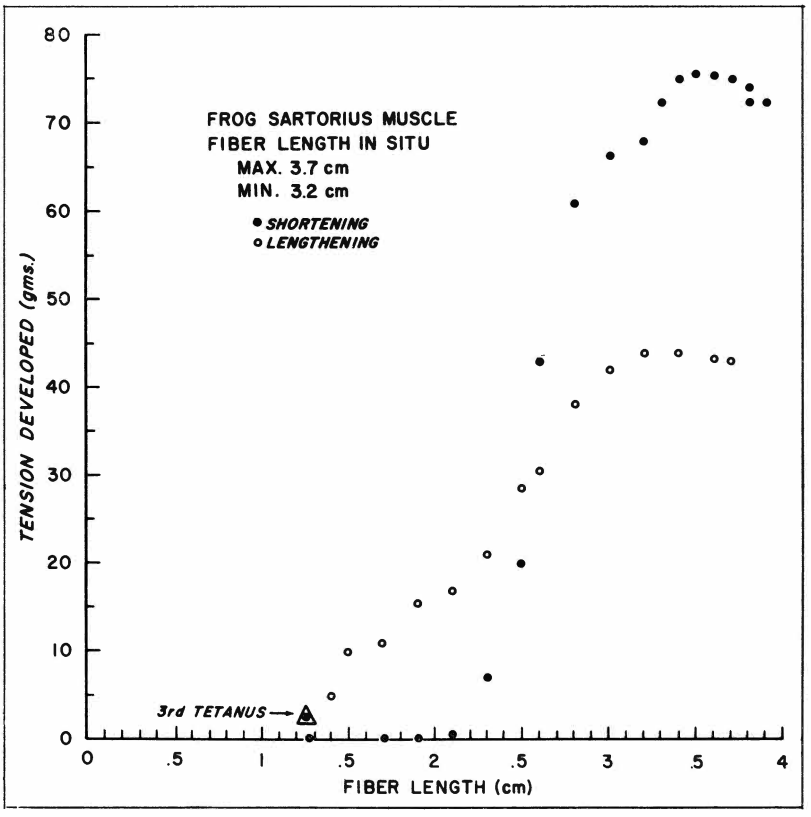

Fig. 8—Graph showing the relation between fiber length and isometric tension. All tetani were 0.2-sec except the three at the shortest length which were 2 sec each. Average sarcomere length (SL) was 2.4 μ at fiber length 3.7 cm. Calculated SL at fiber length 1.3 cm. is 0.8 μ. Shortening tensions (solid circles) and lengthening tensions (open circles) were measured in successive steps. The shape of the curve for re-extension after extreme shortening is the same as that seen in delta state single fibers of muscle. T 18 C.

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TABLE 1
Resting Oxygen Consumption of Frog Sartorius Muscle (μ l/g/hr)

Exp. No.	Normal	Delta Long	Percent
5	54.2	74.1	137
18	44.4	100.9	227
22	62.9	69.3	110
28	51.9	81.4	157
29	55.0	71.1	129
29	44.4	57.7	130
30	38.2	72.1	189
30	41.1	60.4	147
31	44.2	64.8	146
32	50.6	66.3	131
33	36.2	63.1	174
34	41.8	63.8	152
35	33.1	59.8	181
35	32.3	71.5	221
34	54.3	62.0	114

Average % $\frac{\text{Delta Long}}{\text{Normal}}$ = 156

Exp. No.	Normal	Delta Short	Percent
9	48.6	122.5	252
9	42.2	69.7	165
10	59.8	92.0	154
16	64.1	147.4	230
31	38.9	83.9	216
32	38.8	84.9	219

Average % $\frac{\text{Delta Short}}{\text{Normal}}$ = 206

Exp. No.	Delta Long	Delta Short	Percent
6	68.1	108.1	159
6	67.8	87.8	128
11	65.7	86.3	131
15	99.2	102.9	104
15	90.2	114.0	126
17	91.2	101.1	111
17	108.7	124.1	114
19	84.5	91.3	108
19	106.3	133.6	126
23	106.8	122.9	115
23	124.1	134.7	108
24	77.6	116.3	150
24	82.6	118.8	144
25	122.4	163.8	134
26	121.8	183.5	151

Average % $\frac{\text{Delta Short}}{\text{Delta Long}}$ = 127

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