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A LESION OF THE TRANSVERSE TUBULES OF SKELETAL MUSCLE

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SUMMARY

1. A method is described by which a selective lesion can be made *in vitro* in the transverse tubules of frog skeletal muscle.

2. The method consists of exposing the muscle for 1 hr or more to a buffered salt solution made hypertonic by the inclusion of 400 mM glycerol and then returning the muscle to an isotonic salt solution. The lesion is induced during the washout of the glycerol.

3. Electron micrographs reveal that the lesion consists of a rearrangement of the T-system membranes in such a way that the continuity of the tubules is lost. The membranes appear to coalesce into large vesicles scattered irregularly throughout the sarcoplasm.

4. The glycerol treatment results in a depression of the resting potential of up to 30 mV. The treated fibres are depolarized by high concentrations of K as are normal muscle fibres.

5. T-tubule lesioned fibres are unable to respond mechanically either to electrical stimulation or to elevated K but they do contract in the presence of caffeine and relax when the caffeine is removed.

6. Problems concerning the variability of the procedure are presented and certain considerations concerning the mechanism of the effect are discussed.

INTRODUCTION

The depressant effect of hypertonic solutions on the excitability of skeletal muscle was recognized long ago (Overton, 1902; Steggarda, 1927) but the fact that the effects are easily reversible became apparent much more recently (Howarth, 1957; Hodgkin & Horowicz, 1957). Hodgkin & Horowicz reported that the reversible blockade of the twitch in hypertonic solutions is not accompanied by any alteration of the electrical activity of the muscle cells. Since then hypertonic conditions have been used in

micro-electrode studies of muscle action potentials in order to eliminate the nuisance of rapid cell movement.

Fujino, Yamaguchi and co-workers (Fujino, Yamaguchi & Suzuki, 1961; Yamaguchi, Matsushima, Fujino & Nagai, 1962) observed that solutions made hypertonic with glycerol or urea do not affect muscles in the same way as do solutions made hypertonic with sucrose or NaCl. The blockade of the twitch induced by hypertonic glycerol solutions was found to be transient; the ability to twitch recovers while the muscle continues to be bathed in the hypertonic medium. Under these circumstances the electrical activity is also unaltered. This phenomenon was called the glycerol effect and was suggested to involve hypertonic interruption of the excitation–contraction coupling link which could be restored by glycerol and, to a lesser extent, by urea. These authors also noticed another phenomenon, namely that the return of a muscle to an isotonic Ringer solution following hypertonic glycerol treatment induced an irreversible loss of the twitch which, like the glycerol effect, was unaccompanied by any affect on resting or action potentials. Electron microscopic studies we have undertaken have provided an explanation for this irreversible loss of the twitch, which we will refer to as the glycerol removal effect. A preliminary account of this work has already been reported (Howell & Jenden, 1967).

METHODS

The methods employed involved the use of conventional techniques both in the areas of physiology and electron microscopy. Two different systems for tension measurement were used. The first was an RCA 5734 transducer tube mounted either vertically over a glass tissue bath or horizontally in a lucite chamber, the latter assembly permitting the insertion of micro-electrodes into the muscles. Twitches were recorded photographically from an oscilloscope tracing and slower contractions were followed on a strip chart recorder. In some experiments twitches and contractures were followed on a Grass Instrument Company polygraph. The second system was an E & M myographic transducer used in conjunction with an E & M Physiograph IV. In a few experiments isotonic measurements were made using a G. L. Collins (Long Beach, California) displacement transducer. In the experiments using the RCA transducer, muscles were stimulated supramaximally with pulses of 1–2 msec duration through platinum electrodes located 1 cm apart. The electrodes were capacitatively coupled to the stimulator. In the experiments using the Physiograph, the muscles were stimulated in air with platinum wire electrodes connected to a Harvard induction coil stimulator. Responses to both the make and the break shocks are visible on the records. Resting potentials were obtained from sartorius muscles with glass micropipettes filled with 3 M-KCl and having resistances of at least 10 MΩ. Potentials were read on a Keithley electrometer (input impedance, 10^{14} Ω).

Most experiments were performed on toe muscles (extensor longus digitorum IV) of *Rana pipiens* throughout the year. Experiments were performed with the sartorius of *R. pipiens* in order to obtain resting potentials and these experiments were done in the summer. The experiments using the Physiograph were performed on toe muscles (extensor longus digitorum IV) of *R. catesbiena* and were performed in the spring.

Muscles were excised and used immediately or permitted to stand overnight in frog Ringer at 2–5° C. The frog Ringer employed contained NaCl, 125 mM; KCl, 2·5 mM; and $CaCl_2$, 1·8 mM. It was modified by the addition of diaminoethanetetra-acetic acid (EDTA), 0·01 mM, and D-tubocurarine chloride, 2×10^{-5} g/ml. The EDTA was included in an attempt to protect the muscles from traces of heavy metal ions, but experiments performed without EDTA indicated that its presence was not required to observe the glycerol removal effect. The Ringer solution was buffered with either 3 mM phosphate or 3 mM-N-Tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid (TES) at pH 6·8–7·0. Hypertonic glycerol solutions contained 400 or 420 mM glycerol in addition to the constituents of the normal Ringer. Likewise, elevated K solutions and caffeine solutions were prepared by adding the additional KCl or the caffeine to the normal Ringer solution. All experiments were performed at room temperature which was 20–22° C.

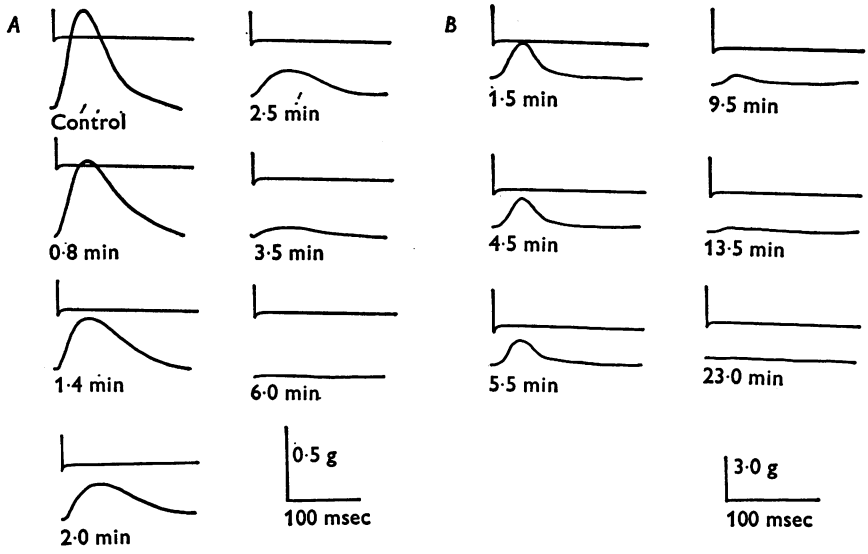
For electron microscopy all muscles, both experimentals and controls, were fixed by replacing the Ringer solution in the chamber with a Ringer solution to which 5 % glutaraldehyde had been added. After 1 hr of fixation the muscles were returned to Ringer and kept refrigerated until muscles from several experiments had accumulated. The muscles were post-fixed in 2 % OsO_4 for 1 hr and dehydrated through a series of alcohols in the conventional manner. They were embedded in Epon and sectioned on a Sorvall MT-2 microtome. Sections were examined on an Hitachi HU-11A electron microscope.

RESULTS

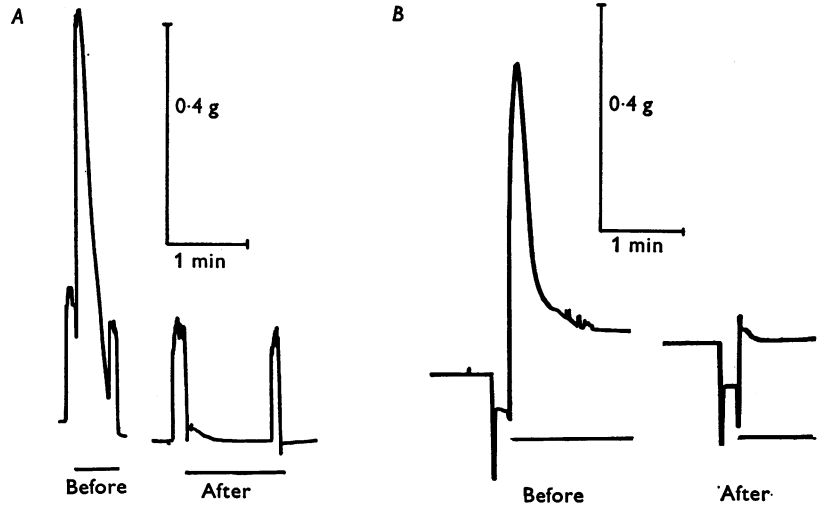
Physiological observations. The disappearance of the twitch in Ringer solution following a 1 hr exposure to glycerol Ringer is illustrated in Text-fig. 1 for both the toe muscle and the sartorius. The phenomenon takes place more rapidly in toe muscles than in the larger sartorius muscles; in the toe muscle twitch responses disappear within 6 min after being reimmersed in normal Ringer solution. The response to elevated K also disappears and this is illustrated in Text-fig. 2. The concentration of KCl used was always between 60 and 75 mM. The solution change artifacts appear different in the two muscles because of the different recording devices used; the toe muscle recording was obtained with the muscle mounted vertically while the sartorius was held horizontally during the experiment.

Despite the loss of the twitch and the potassium contracture, the muscle response to caffeine was undiminished after glycerol removal. Text-figure 3 shows caffeine contractures in the toe and sartorius muscles after glycerol removal, and in Text-fig. 4 contractures both before and after treatment with glycerol can be seen in *Rana catesbiena* toe muscles; also illustrated is the diminution of the twitch, the tetanus and the KCl contracture. The tetanic responses were elicited with stimuli given at 50 Hz. After glycerol removal a residual tetanic response was observed (less than 5 % of the control tetanus) but the twitch is just barely detectable on the records.

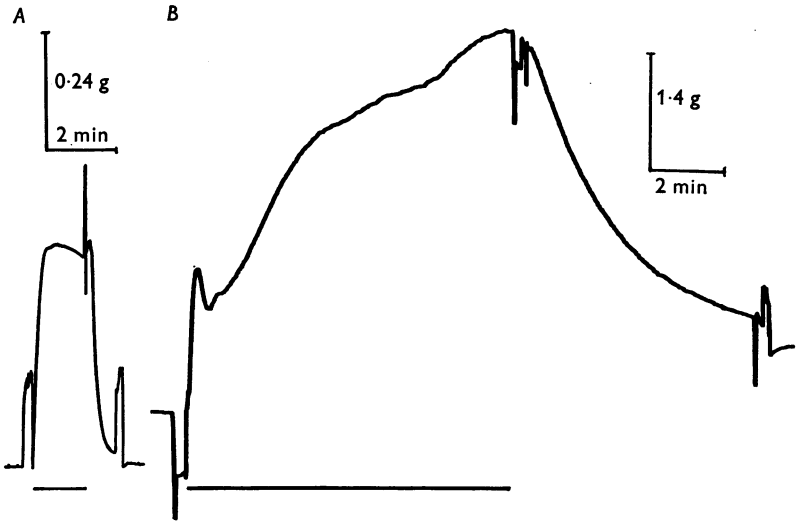
The minimum durations of exposure to glycerol which were used were 45 min for toe muscles and 1 hr for sartorius. Some muscles were left as



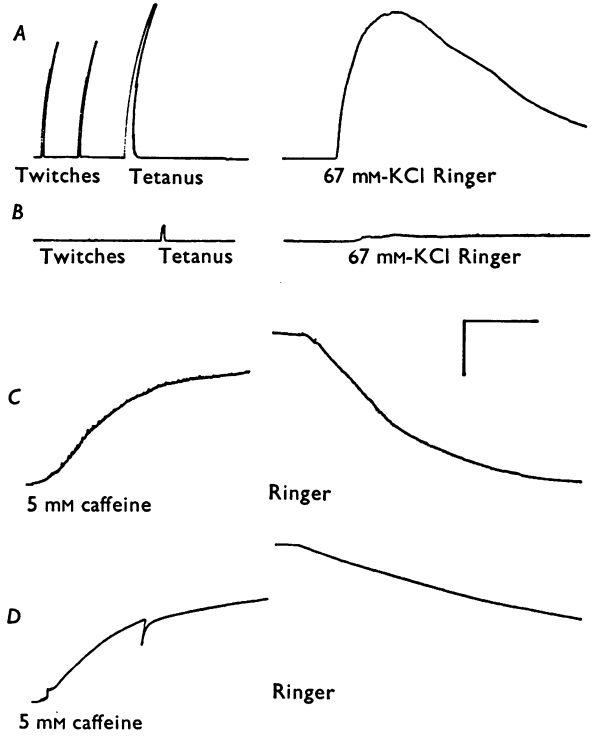
Text-fig. 1. Disappearance of the twitch after transferring a toe muscle (*A*) and a sartorius muscle (*B*) from 400 mM glycerol Ringer (control) into normal Ringer. Times represent duration of exposure to Ringer after the transfer.



Text-fig. 2. Disappearance of the K contracture as a result of glycerol removal. Toe muscle in *A*; sartorius in *B*. Bars indicate period of exposure to 60 mM-KCl Ringer in *A* and to 75 mM-KCl Ringer in *B*. Solution change artifacts are different in the two experiments because the recording apparatus used for the two muscles was not the same. In *B* the solution change artifacts for replacing the contracture solution with Ringer do not appear because the contracture solutions were kept in the bath long enough to make membrane potential measurements.

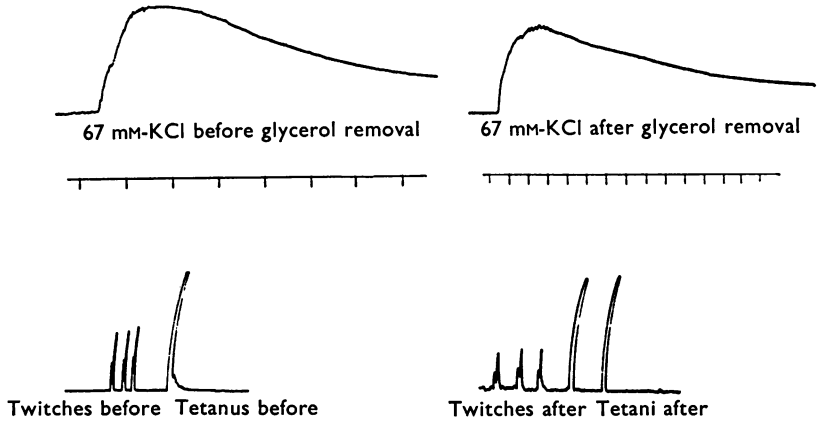


Text-fig. 3. Caffeine contractures in toe muscle (*A*) and sartorius (*B*) after glycerol removal. Bars indicate duration of exposure to 1·5 mg/ml. (7·75 mM) in *A* and to 1·0 mg/ml. (5·15 mM) in *B*.



Text-fig. 4. Twitch, tetanus, K contracture and caffeine contracture in bullfrog toe muscle before and after glycerol removal. Records *A* and *C* before, records *B* and *D* after glycerol removal.

long as 72 hr in cold glycerol Ringer in which they exhibited vigorous twitches and these, too, became unable to twitch when they were returned to Ringer. The glycerol removal effect appears to be irreversible. In several experiments glycerol treated muscles were allowed to stand for several hours in Ringer. Restoration of the twitch was never observed, even when muscles were left overnight. Re-exposure to glycerol was also ineffective in restoring the twitch.

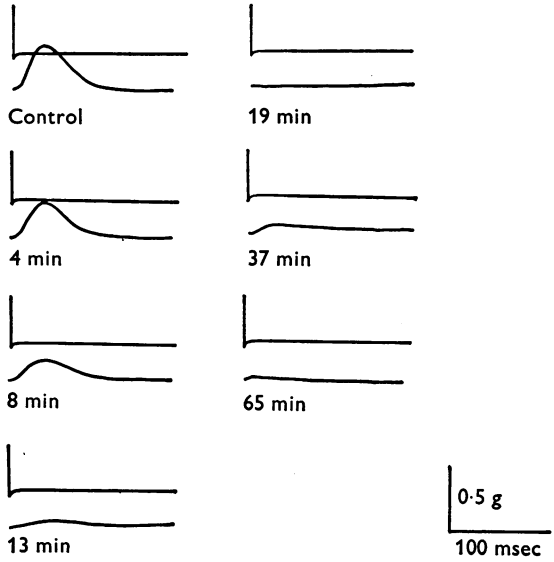


Text-fig. 5. Persistence of the twitch and K contracture after slow glycerol removal. The following series of glycerol Ringer was used (mM): 420, 370, 320, 270, 220, 170, 120, 70, 0. Removal was complete in 120 min. Time marks at 5 sec intervals.

With the toe muscle removal of 800 mM glycerol as well as 400 mM glycerol causes the disappearance of the twitch but removal of 220 mM glycerol produces only a partial (approx. 65 %) inhibition of the twitch. Removal of 400 mM urea is also effective in abolishing the twitch although the disappearance of the twitch is much slower when urea is used in place of glycerol. Removal of 400 mM ethylene glycol produces only a 50 % inhibition of the twitch. Reducing the temperature during glycerol removal to 0–2° C does not prevent the loss of the twitch although it does slow it down. Alterations in the buffer used or in Ca concentrations between 0·18 and 10 mM do not influence the course of the glycerol removal effect. The glycerol removal effect could be obtained in either TES or imidazole buffer as well as phosphate.

The ability to twitch can survive glycerol removal if it is done gradually. Text-figure 5 illustrates an experiment in which muscles, instead of being transferred from 420 mM glycerol Ringer directly into normal Ringer, were transferred from the 420 mM glycerol to normal Ringer gradually through a graded series of glycerol solutions. Only a small decrement in

tension development can be seen and this may reflect general deterioration of the muscle during the 6 hr experiment rather than an effect of glycerol removal. Some experiments were performed with altered osmotic environments for the muscles during glycerol removal. In these experiments, instead of transferring the muscle from the hypertonic glycerol Ringer directly into normal Ringer, the muscles were transferred into Ringer solutions devoid of glycerol but made hypertonic by the addition of sucrose or other non-penetrating agents. The twitch responses in one



Text-fig. 6. Glycerol removal in the presence of 400 mM sucrose. Between 19 and 37 min after the glycerol Ringer was exchanged for the sucrose Ringer, the muscle was returned to Ringer. A weak and transient restoration of the twitch can be observed.

such experiment are illustrated in Text-fig. 6. Replacement of the glycerol solution with 400 mM sucrose Ringer abolishes the twitch. Twenty-three minutes after the glycerol solution was exchanged for the sucrose solution, the sucrose solution was exchanged for normal Ringer. At that point a partial restoration of the twitch occurs, but shortly after that the twitch disappears once more, leaving only a very small residual response comparable to that seen in the other glycerol removal experiments. Different concentrations of sucrose from 120 to 400 mM were tried and at no concentration was the twitch loss prevented. Similar experiments were performed using mannitol and sodium sulphate instead of sucrose. Even when the muscles were kept in the hypertonic solution for 1–1½ hr, during which glycerol efflux must be very nearly complete, the twitch

could not be restored in normal Ringer. In one experiment 400 mM mannitol was added before the glycerol was removed and this procedure also failed to prevent the irreversible loss of the twitch.

Measurements of resting potentials in sartorius muscles before and after glycerol removal show a decrement following glycerol removal. In the first two rows of Table 1 are membrane potentials taken from glycerol treated muscles and from contralateral control muscles which were soaked in normal Ringer during the time the experimental muscles

TABLE 1. Effect of glycerol removal on resting potentials in sartorius

Controls	Resting potential in glycerol	Resting potential after glycerol removal	Decrement in resting potential from glycerol removal	Resting potential in glycerol treated muscles in 75 mM-KCl
89·27* (15) ±0·83	—	64·26 (14) ±2·04	24·89	—
86·31* (13) ±0·93	—	72·08 (12) ±1·38	14·23	25·00 (8) ±0·68
86·17 (6) ±1·62	—	58·75 (12) ±1·26	27·42	26·50 (8) ±2·20
92·25 (4) ±0·63	88·63 (8) ±2·47	53·62 (26) ±3·10	35·01	30·13 (8) ±2·81
90·00 (4) ±1·22	92·67 (6) ±1·20	71·46 (13) ±2·77	21·21	30·17 (18) ±1·49

* Indicates contralateral control muscles; other controls are values from the experimental muscle before it was exposed to glycerol.

Results are given as the mean ±S.E. of mean; the number of fibre penetrations is in parentheses.

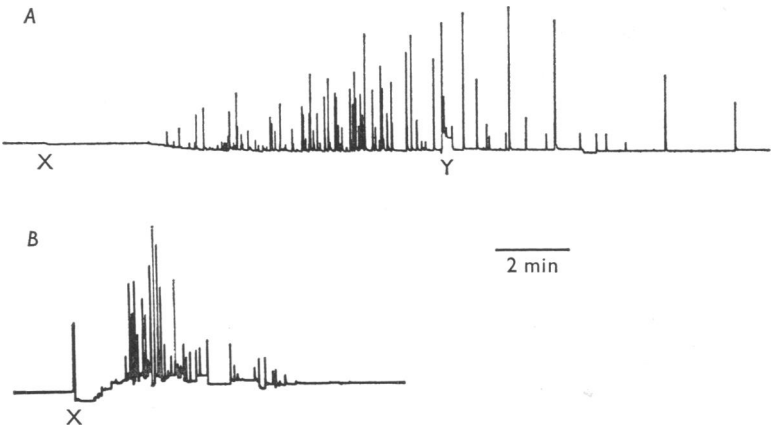
were being exposed to hypertonic glycerol. In these two experiments resting potentials in the treated fibres were lower than in the contralateral controls by 24·89 and 14·23 mV. Membrane potentials recorded from fibres before and after glycerol treatment are shown in the bottom three rows of Table 1 and these tend to show a larger decrement after glycerol removal.

In these muscles the loss of responsiveness, both to electrical stimulation and to high K, can be in part attributed to the low resting potentials. Many of the fibres, particularly in the muscles represented in rows 3 and 4 of Table 1, were depolarized by glycerol removal past the −55 mV mechanical threshold (Hodgkin & Horowicz, 1959). However, this cannot be the explanation for the decrease either of the twitch or of the K contracture which occurred in the sartorius muscles whose resting potentials are given in rows 1 and 2 of the Table. In these muscles, whose mean resting potentials were 64·26 and 72·08 mV, only one of fourteen and two of thirteen fibres sampled were below −55 mV. In the same muscles the maximum amplitude of the twitch was reduced by 95% and that of the K contractures by 90%. Furthermore, action potentials in glycerol treated muscles have been demonstrated both by Fujino *et al.* (1961) and by Gage & Eisenberg (1967) in the absence of any mechanical response.

Resting potential measurements of the muscles while they were in the hypertonic glycerol Ringer indicated that the presence of glycerol had

no deleterious effect at all on the resting potentials. The decrement in potential did not occur until glycerol was removed. The right-hand column in Table 1 indicates the fact that the glycerol treated muscles were still capable of being depolarized by elevated K concentrations although the depolarization was not accompanied by any mechanical response.

A phenomenon which is probably related to the decrement in resting potentials is illustrated in Text-fig. 7. These are contractions recorded



Text-fig. 7. Isotonic displacement records of sartorius in *A* and toe muscle in *B* during glycerol removal. At X 400 mM glycerol Ringer was exchanged for a normal Ringer, fresh Ringer at Y. 200 mg loads.

with a displacement transducer just after the muscle has been transferred from hypertonic glycerol to normal Ringer. They are spontaneous in that they occur in the absence of any applied electrical stimulus. They last longer in the sartorius than in the toe muscle and, under the loads used (approx. 100 mg), do not exceed 50 % of the shortening achieved in a 75 mM-K contracture.

Morphological observations. Electron microscopic examination of muscles subjected to the glycerol removal revealed a dramatic alteration in the appearance of the transverse tubular system (T-system) with little or no change apparent in other organelles. Most triads appeared to be lacking central elements altogether (Pl. 1, fig. 2; Pl. 1, fig. 3; Pl. 2, fig. 2; compare with control in Pl. 1, fig. 1), while at other triadic sites the central elements appeared as enormous, swollen vesicles (Pl. 1, fig. 3; Pl. 2, fig. 1; Pl. 2, fig. 2; Pl. 3, fig. 1). The swollen vesicles could be identified as T-tubules by virtue of the normally appearing contacts they made with the terminal cisterns of the sarcoplasmic reticulum (SR). They also appeared completely empty in contrast to the SR whose terminal cisterns contain a granular substance of unknown composition and function. In some cases the

membranes of the vesicles appeared to be ruptured in one or more places (Pl. 3, fig. 1). An extreme case of this appears in Pl. 3, fig. 2, where the membrane fragments formed by the rupture of the swollen vesicle have resealed into smaller vesicles whose distribution retains the outline of the original vesicle. The largest vesicles observed were about 3 μ long and these occurred near the periphery of fibres.

The morphology of other subcellular elements in the treated muscles appeared normal. The myofibrils appeared unchanged and the mitochondria were not markedly swollen. The terminal cisterns of the SR exhibited distortion which was probably induced by movement of the T-tubules during the glycerol removal. The terminal cisterns were identifiable because of their granular content even when they took very unusual outlines (arrows in Pl. 1, fig. 2; Pl. 3, fig. 2). In many triads their distortion was slight with the only conspicuous change being the absence of the T-tubules. In these triads some pairs of terminal cisterns tended to move apart while other pairs moved toward one another almost to the point of coalescence of their membranes. In the latter case the crisp outlines of the membranes were usually lost, perhaps as a consequence of the mechanical rupture of the triadic junctions between the T-tubules and the SR. Except for the distortions of the terminal cisterns, the SR remained unaffected by the glycerol removal. Profiles characteristic of normal fenestrated collars were always observed in these muscles (Pl. 3, fig. 3). For a thorough report on the electron microscopic appearance of normal frog muscle see Peachey (1965).

The morphological picture which emerges is that of a rather selective lesion of the T-system in which the inward continuity of the tubules of the T-system is lost. Current knowledge of the T-system suggests that the T-tubules provide an inward conduction pathway for electrical activity which permits the simultaneous contraction of the myofibrils throughout the entire cross-section of the cell in response to an electrical event at the surface. The disappearance of the twitch after glycerol removal can therefore be accounted for by disruption of the continuity of the T-tubules.

The variability of the glycerol removal effect. A degree of variability is encountered in the glycerol removal effect which may limit the applicability of the procedure. Variability is encountered both from muscle to muscle and from fibre to fibre within a single muscle. The experiments with stepwise removal of glycerol suggest that some minimum concentration gradient of glycerol is necessary to produce the rearrangement of the T-system. It is clear that when glycerol Ringer is exchanged for normal Ringer the glycerol concentration will fall more slowly in the extracellular spaces which are deep in the muscle than in those around superficial fibres. It is not surprising, then, that small residual contractile responses were

occasionally seen, responses which presumably reflect the survival of excitation–contraction coupling in deep fibres. Fibre to fibre variation was observed both in the resting potential measurements and under the electron microscope. Resting potentials tended to show greater variation after treatment, although, of the experiments where other variables were eliminated, the difference in variation was significant in only one. Morphologically, in the sartorius muscles examined it was always possible to find fibres which exhibited primarily normal triads.

In the toe muscle, where diffusion distances are considerably less, this difficulty might be expected not to occur. However, residual contractile responses were seen in toe muscles, e.g. the tetanic responses in Text-fig. 4. The residual responses to single stimuli had slower time courses than twitches before glycerol treatment. In view of the report by Stefani & Steinbach (1968) that slow muscle fibres were unaffected by glycerol treatment, it is likely that the residual responses reflect activity of the slow fibres. Whether or not the lesion is absolutely complete in the twitch fibres of the toe muscle cannot be established on structural grounds alone. A marker technique similar to that employed by Eisenberg & Eisenberg (1968) will be required to demonstrate the extent of the T-tubular disruption.

Considering the variability which is encountered among fibres in the sartorius and possibly also in the toe muscle, caution must be exercised in the use of glycerol treated muscles in studying whole muscle parameters such as has been attempted by Van der Kloot (1968). In studies of the properties of single fibres the fibres can be appropriately selected. Gage & Eisenberg (1967) and Eisenberg & Gage (1967) used only surface fibres of the glycerol treated sartorius in order to establish that the electrical properties, particularly the capacitance, of the treated muscles are consistent with the absence of T-tubules. One final type of variability which was encountered is that once during the course of our experiments, glycerol removal simply failed to have any effect. This occurred in several successive experiments before the glycerol removal effect was one again observed, and the failure was never again encountered. The cause for the failure is totally obscure, but it has been reported to the author to have occurred in the hands of other investigators.

DISCUSSION

The mechanism of the glycerol removal effect. The mechanism of the T-system rearrangement is not at all clear. The rearrangement from a system of narrow tubules into a number of isolated vesicles which occurs during the washout of glycerol tends to maximize the volume of

the T-system. This suggests the involvement of an osmotically induced bulk flow of water into the T-system from the sarcoplasm. Such a bulk flow will occur, if during glycerol removal, a glycerol concentration gradient develops across the T-tubular membranes such that the glycerol concentration within the T-system exceeds the concentration in the sarcoplasm. The concentration gradient would only need to be transient in order to produce the observed effects.

During the exposure of the muscle to glycerol, glycerol penetrates into cells. This has been shown by osmotic methods (Overton, 1902; Krolenko & Adamjan, 1967) and by isotopic methods (Bozler, 1961). No kinetic data are available concerning glycerol penetration of the toe muscle. However, the half-time for glycerol penetration into sartorius muscles is about 20 min; about 2 hr are required for equilibration (Bozler, 1961). These data suggest that within 1–1½ hr of exposure to glycerol, equilibration of the glycerol throughout the cytoplasm of both the toe muscle and the sartorius is nearly complete. When the muscle is returned to normal Ringer, water enters the muscle and glycerol begins to diffuse out. If either of these movements occurs more rapidly between the sarcoplasm and the exterior than they do between the T-system and the exterior, an osmotic gradient will develop across the T-system membranes in such a way as to cause swelling of the T-tubules.

It does not seem likely that glycerol diffusion from the cytoplasm could be faster than diffusion from the T-tubules. Movement of glycerol into and out of cells is relatively slow and this has been interpreted to mean that cell membranes are only poorly permeable to glycerol. On the other hand the T-tubules are quite accessible to substances in the extracellular fluid. Endo (1966) has shown by fluorescence microscopy that lissamine rhodamine B 200, mol. wt. 558, diffuses out of the T-tubules of single cells in two phases each with half-times of less than 30 sec even in fibres 93 μ in diameter. Estimates of ion movements from the T-tubules give considerably shorter half-times (Hodgkin & Horowicz, 1960; Adrian & Freygang, 1962; Freygang, Goldstein & Hellam, 1964) indicating that efflux from the T-system can be very rapid.

A more probable origin of the required osmotic gradient may be found in the water movement. When muscle cells are placed in hypertonic glycerol they shrink and on return to Ringer they swell. These volume changes represent the efflux and influx of water and they occur much more rapidly than the fluxes of glycerol. The influx of water which occurs when the muscle is returned to Ringer dilutes the cytoplasm very rapidly, perhaps more rapidly than the diffusion of glycerol from the T-tubules. By this means a transient concentration gradient across the T-system membranes might arise, causing water to move from the cytoplasm into

the T-tubules and producing the rearrangement of the T-system observed after glycerol removal. However, in the experiments in which water movement was prevented during glycerol removal by the presence of non-penetrating non-electrolytes, the twitch loss was not prevented, rendering any such explanation unlikely. In these experiments the muscles were not examined morphologically or with micro-electrodes and the possibility remains that the combined presence of glycerol within the cells and the non-penetrating agent outside the cells damages the fibres in some way other than T-tubular rearrangement.

Despite the difficulties in proposing the precise mechanism by which the T-system is disrupted, it does seem that some sort of osmotic phenomenon is involved. The following observations point in that direction.

1. It is not specific to glycerol. It also occurs with urea, which like glycerol moves into and out of cells slowly.

2. It does not occur with agents that do not penetrate cells, such as sucrose. It occurs only incompletely with agents that pass rapidly into and out of cells, such as ethylene glycol.

3. It does not result from the presence of glycerol, but rather from its removal.

4. It does not occur if glycerol is withdrawn stepwise, so that the osmotic gradients are reduced in magnitude.

In considering osmotic events which might occur at the T-system membranes, several uncertainties are encountered. The most obvious uncertainty arises from the morphological relationship between the membranes of the T-system and the membranes of the SR. The greater portion of the surface area of the T-system membranes form triadic junctions with SR membranes (Peachey, 1965) leaving only 20 % of the T-system membranes for direct osmotic interaction with the cytoplasm. It may be safe to assume that the latter 20 % has permeability properties similar to the sarcolemma but the permeability properties of the remainder of the T-tubular surface may be substantially altered by the junctions formed with SR membranes. For instance, Luft (1966) has shown that the cationic dye, ruthenium red, which does not penetrate cell membranes, does appear to cross the T-tubular membranes to gain access to the inside of the terminal cisterns of the SR. He has also shown that the dye, which selectively stains mucopolysaccharides, stains the interior of the T-system heavily, suggesting the presence of this material within the T-tubules. What effect mucopolysaccharides might have on the diffusion characteristics of various substances within the T-system is not known. These studies make it obvious that considerably more information is needed before the functioning of the T-tubules can be properly understood.

Eisenberg & Eisenberg (1968) have demonstrated that the T-system

becomes inaccessible to the marker, horseradish peroxidase, following glycerol removal. If the closing off of the T-tubules occurs during the initial stages of glycerol efflux, equilibration of the T-tubular contents directly with the extracellular fluid would be prohibited. By this means it is at least conceivable that some glycerol might be trapped within the T-system to produce the water movements responsible for vesiculation.

Breakage of the T-tubules at the cell surface, followed by a resealing of the sarcolemma, may be responsible for the decline in resting potentials observed in glycerol treated fibres, since transient breaks should permit some leakage of ions down their electrochemical gradients into or out of the cells. The extent of leakage undoubtedly varies from fibre to fibre, thus accounting for the tendency toward greater variability of resting potentials after glycerol removal. The observation of spontaneous twitching in muscles under light loads during glycerol removal (Text-fig. 7) is also consistent with the idea that ionic leakage occurs. The irregular burst of twitches probably results from action potentials which are induced by ionic leakage during the separation of the T-tubules from the cell surface. Action potentials will be capable of eliciting contractile responses as long as some T-tubules retain their inward continuity.

A light microscopic study of muscle fibres following washout of non-electrolytes has been reported by Krolenko, Adamjan & Shwinka (1967). They describe vacuolization in the sarcoplasm after washing out a variety of penetrating non-electrolytes. The vacuoles they observed were 1–3 μ in diameter which corresponds to the largest of the swollen T-tubules which have been observed in this study. Their distribution corresponds to the distribution of the severely swollen T-tubules observed in this study in that the largest vacuoles generally occurred in the periphery of the fibres. During glycerol removal with single twitch fibres from the iliofibularis, structural changes began in 1–2 min and vacuolization was completely developed in 10–20 min. Since loss of the twitch in whole toe muscles is near completion in 6 min, the twitch loss probably occurs in the early phases of vacuolization. Krolenko *et al.* (1967) tested the irritability of the treated fibres by stimulating them mechanically. In some they observed that the whole fibre shortened normally while in others only local contractions could be obtained. In their work all the non-electrolytes studied were used at a concentration of 220 mM. With toe muscles 220 mM is not a sufficient concentration to produce a complete inhibition of the twitch. Had they used 400 mM glycerol they would probably have only rarely observed complete contractile responses. They observed a decline in resting potential of 20–30 mV in only 30 % of the fibres but this too may be a function of the concentration of glycerol which they used. Kutscha,

Pauschinger & Brecht (1963) observed a more severe decline in resting potentials after washing out 1 M urea from sartorius muscles.

In experiments similar to the ones reported here, Krolenko *et al.* (1967) attempted to prevent vacuolization by washing out the non-electrolytes under conditions where swelling of the fibres did not occur. They too found that washing out the glycerol in the presence of Ringer made hypertonic with NaCl did not prevent vacuolization. Fibres loaded with glycerol in solutions isosmotic to Ringer in which glycerol replaced most of the NaCl still showed vacuolization when the glycerol was washed out in normal Ringer despite the fact that no swelling occurred during the glycerol removal. Krolenko *et al.* also pointed out the fact that water movement induced with non-penetrating agents does not produce vacuolization and concluded that water movements associated with swelling are not responsible for vacuolization. One final comment needs to be made about the paper by Krolenko *et al.* and that concerns the fact that the vacuolization as they observed it under the phase contrast microscope could be reversed by reimmersion of the fibre in hypertonic glycerol. The experiments reported here indicate that the inhibition of the twitch and K contracture produced by the removal of 400 mM glycerol cannot be reversed. Even if the vesicles which are formed from T-system membranes during glycerol removal can be reduced in size so as to eliminate the vacuoles visible under the light microscope, the inward continuity of the T-system apparently cannot be reestablished in isolated muscles.

The role of the T-system in excitation–contraction (E–C) coupling. The evidence that the T-system provides the E–C coupling link has been indirect. From a morphological point of view the T-tubules are ideally situated to provide the structural basis for a physical mechanism of E–C coupling which Hill (1949) predicted would be necessary to account for the speed of development of the active state. Furthermore, membrane sites sensitive to low level stimulation coincide with the places where the T-tubules open to the exterior (Huxley & Taylor, 1958). The results reported here provide more direct confirmation of the role of the T-system in E–C coupling. In muscles whose T-system can be seen under the electron microscope to be disrupted in otherwise intact cells, E–C coupling is lost. Depolarizations of the membranes, whether induced chemically or electrically, fail to activate the contractile machinery, whereas caffeine, which bypasses the normal coupling link by acting directly on the SR to cause release of Ca, is still able to elicit contractions. These observations leave no doubt as to the essentiality of the T-system in E–C coupling.

The potassium contracture. The mechanisms involved in K contractures are generally taken to be very closely related to, if not identical with, the mechanism of the twitch. However, conditions have been reported under

which either the twitch or the K contracture, but not both, is inhibited (Csapo & Suzuki, 1958; Sten-Knudsen, 1960; Sandow, 1955; Fujino & Fujino, 1964). Lorkovic (1962) concluded that K contractures, unlike twitches, were dependent upon influx of extracellular Ca. His conclusion was based on his observation that both the Ca influx, over and above the resting value, which occurs during K contractures and the magnitude of K contractures are dependent upon the extracellular Ca contractures.

The results reported here do not tell us whether the mechanisms of coupling in twitches and in contractures are identical, but they do indicate that both are dependent upon the presence of an intact T-system. Potassium contractures, like twitches, are almost completely inhibited by glycerol removal, although depolarization is not prevented. This inhibition clearly indicates that K contractures cannot be dependent upon calcium influx across the cell membrane, although it is possible that Ca within the T-tubules, a space continuous with the extracellular fluid, does play a role in K contractures. Brecht, Barbey, Kutscha & Pauschinger (1961) have shown that the initial phase of K contractures is actually an asynchronous tetanus and only the second phase is augmented by high extracellular Ca. These authors point out that the second phase is variable and sometimes non-existent. Occasionally weak and slow responses to KCl were seen after glycerol removal in the experiments reported here and this may represent the persistence of the second phase. In any case, it is clear that the contribution of Ca influx across the sarcolemma to the K contracture must be small. The primary response to elevated K evidently involves inward conduction along the T-tubules.

The caffeine contracture. The observation that caffeine contractures can be elicited in glycerol treated muscles is consistent with the idea that the site of action of caffeine is within the cell rather than at the surface (Caldwell & Walster, 1963; Gruener, 1967). Caffeine is known to mobilize the intracellular stores of Ca (Bianchi & Shanes, 1959) and Herz & Weber (1965) and Carvalho & Leo (1967) have shown that caffeine can cause Ca release from isolated reticular fractions of skeletal muscle. It is still not clear, however, whether caffeine induces Ca release from the SR directly or indirectly, via an effect on the T-tubules.

Lüttgau & Oetliker (1968) have argued that the site of action of caffeine is the T-tubules. However, the fact that caffeine contractures are undiminished by glycerol removal suggests that the SR rather than the T-tubules is the site of action of caffeine. Most of the triadic junctions are disrupted in the treated fibres, greatly reducing the area of contact between the T-system and the SR. If the caffeine effect depended upon intact triadic junctions, the intensity of the response to

caffeine should decline after glycerol removal. Since this does not occur, it seems likely that caffeine acts directly upon the SR membranes to induce the leakage of Ca from the SR.

Note added in proof

Since the submission of this paper for publication several relevant articles have appeared or have been submitted. Full accounts of the work of Eisenberg, B. & Eisenberg, R. S. (1968) (*J. cell Biol.* **39**, 451–467) and Eisenberg, R. S. & Gage, P. W. (1969) (*J. gen. Physiol.* In the Press) are or will shortly be available and a thorough electron microscopic study by Krolenko, S. A. (1968) (*Cytology* (*USSR*) **10**, 803–811) using ferritin as a marker has provided results similar to those reported here as well as some additional observations.

The work presented here represents portions of a dissertation submitted in partial fulfilment of the degree Doctor of Philosophy in Pharmacology at UCLA.

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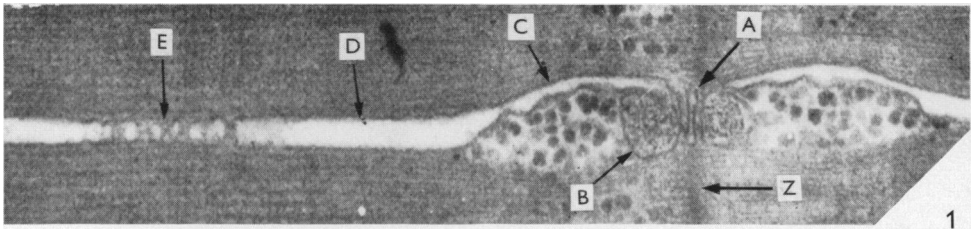
EXPLANATION OF PLATES

PLATE 1

Fig. 1. Normal muscle. Parts of the SR are labelled according to Peachey's (1965) terminology. A, T-tubule; B, terminal cistern; C, intermediate cistern; D, longitudinal tubule; E, fenestrated collar; Z, Z-disc.

Fig. 2. Glycerol treated muscle. 'Triads' without intermediate elements are indicated by open arrows.

Fig. 3. Glycerol treated muscle. Arrows indicate triadic junctions. In the centre appears a fenestrated collar. T-tubules are not seen at most 'triadic' sites.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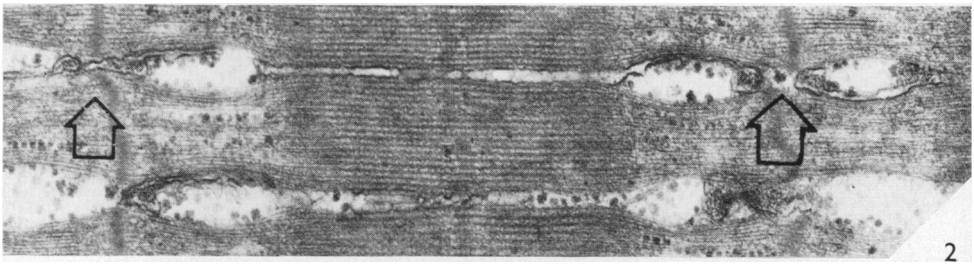
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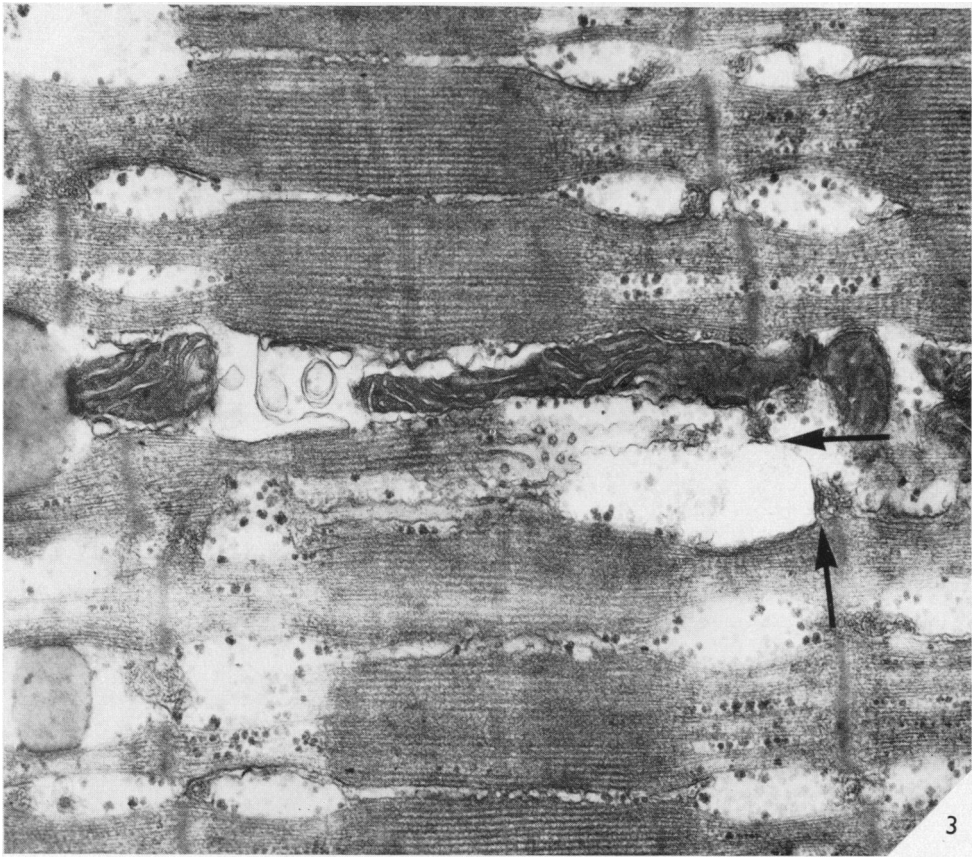
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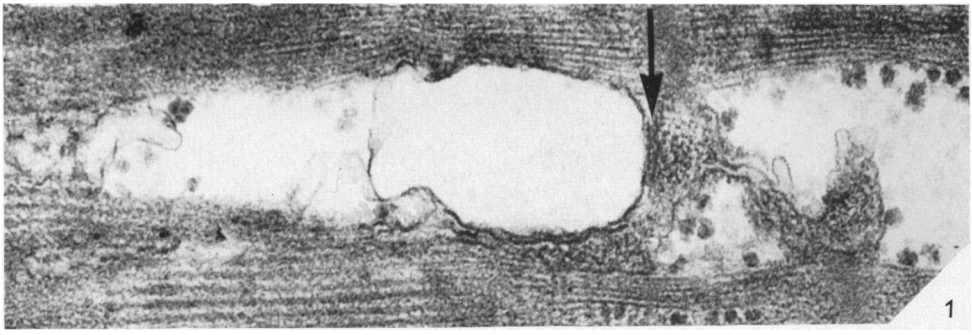
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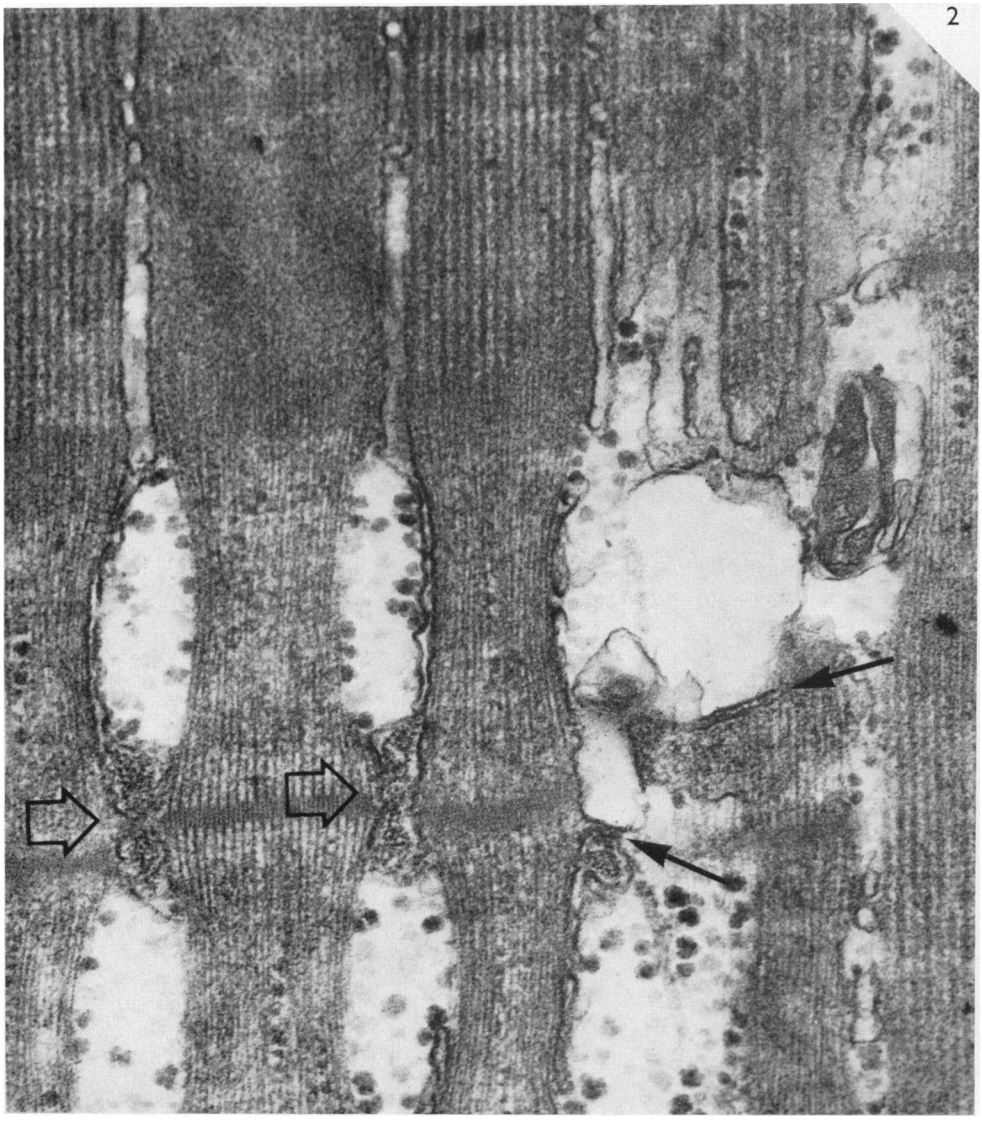
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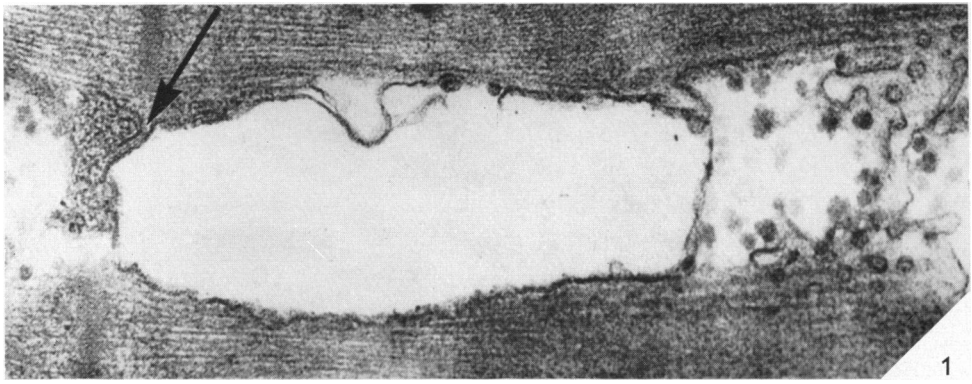
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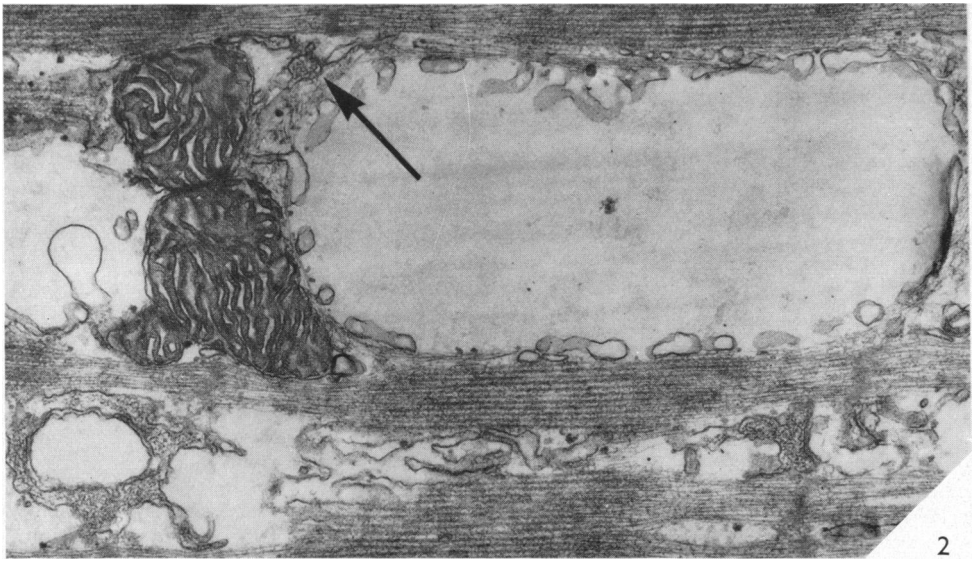
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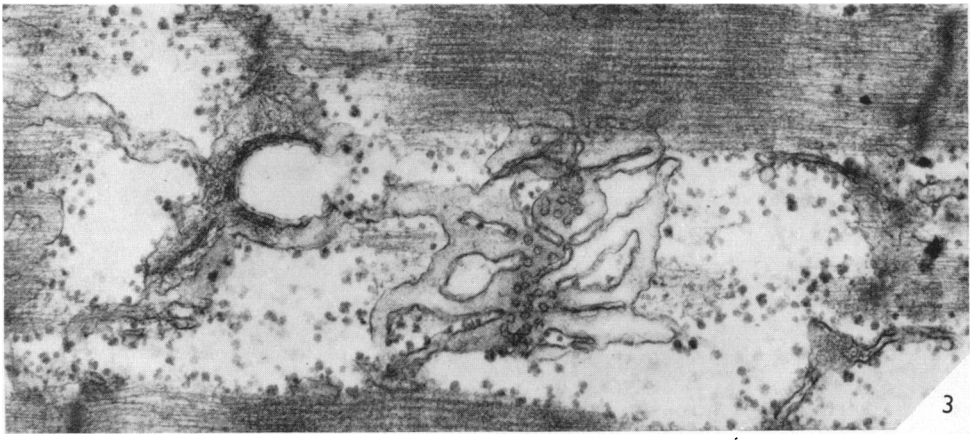
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PLATE 2

Fig. 1. Glycerol treated muscle. Junction between terminal cistern and swollen T-tubule indicated by arrow.

Fig. 2. Glycerol treated muscle. Irregularly swollen T-tubule with triadic junctions indicated by small arrows. Triads without T-tubules are indicated by open arrows.

PLATE 3

Fig. 1. Glycerol treated muscle. Junction between a terminal cistern and a swollen T-tubule is indicated at the arrow.

Fig. 2. Glycerol treated muscle. Terminal cistern at arrow. A bizarre configuration of a terminal cistern can be seen in the lower left.

Fig. 3. Glycerol treated muscle. Fenestrated collar of SR appears normal despite considerable distortion of adjacent terminal cistern.