

SELECTIVE DISRUPTION OF THE SARCOTUBULAR SYSTEM IN FROG SARTORIUS MUSCLE

A Quantitative Study with Exogenous Peroxidase as a Marker

BRENDA EISENBERG and ROBERT S. EISENBERG

From the Department of Physiology, Duke University Medical Center, Durham, North Carolina 27706. The authors' present address is the Department of Physiology, University of California at Los Angeles, Los Angeles, California 90024

ABSTRACT

Skeletal muscles which have been soaked for 1 hr in a glycerol-Ringer solution and then returned to normal Ringer solution have a disrupted sarcotubular system. The effect is associated with the return to Ringer's since muscles have normal fine structure while still in glycerol-Ringer's. Karnovsky's peroxidase method was found to be a very reliable marker of extracellular space, filling 98.5% of the tubules in normal muscle. It was interesting to note that only 84.1% of the sarcomeres in normal muscle have transverse tubules. The sarcotubular system was essentially absent from glycerol-treated muscle fibers, only 2% of the tubular system remaining connected to the extracellular space; the intact remnants were stumps extending only a few micra into the fiber. Thus, glycerol-treated muscle fibers provide a preparation of skeletal muscle with little sarcotubular system. Since the sarcoplasmic reticulum is not destroyed and the sarcolemma and myofilaments are intact in this preparation, of the properties of the sarcolemma may thus be separated from those of the tubular system.

INTRODUCTION

It has been clear for many years that skeletal muscle fibers must contain a specialized system for linking excitation of the surface membrane with contraction in the depths of the fiber (14). Indeed, in frog skeletal muscle Huxley and Taylor (18) postulated a specialized conduction system located at the Z line before the fine structure of muscle had been examined in the electron microscope. The structure of this conducting system is now known (2, 23). In frog sartorius muscle a system of tubules arises as invaginations of the

sarcolemma and invades the fiber in the plane of the Z disc, branching so as to surround the myofibrils. This system is called the T system or the sarcotubular system. The evidence that the tubules are, in fact, invaginations of the sarcolemma is indirect, the aperture of the tubule not having been convincingly observed in this tissue. The evidence that the tubular lumen is open and thus that the tubular membrane is continuous with the sarcolemma is that various extracellular markers fill the lumen of the transverse tubules (5, 15, 19, 22).

A possible explanation of the difficulty of observing the opening of the tubule in material prepared for observation in the electron microscope is found in the work of Huxley (19), who used ferritin as an extracellular marker. If a muscle is fixed in glutaraldehyde for 20 min to 2 hr and then exposed to ferritin, no ferritin is found in the tubules. It thus seems likely that glutaraldehyde fixation seals off the opening of the tubules.

Many of the electrical properties of muscle have been explained in terms of the properties of the tubular system. In particular, the large capacitance of muscle fibers (3, 6, 8), the after potential which follows a single action potential (12), the after potential that follows a train of action potentials (9), and the slow potential change produced by a prolonged pulse of hyperpolarizing current (1) have been attributed to the tubular system. In addition, the tubule membranes may play a role in some of the permeability properties of muscle fibers, particularly the peculiar potassium system (16), called anomalous rectification.

In the absence of a method of selectively removing the sarcotubular system it has been difficult to test any of these ideas directly. Howell and Jenden (17), following an incidental observation of Fujino et al. (11), discovered a treatment which disrupted the transverse tubules. They reported that the sarcotubular system is absent in muscle fibers which have been soaked for 1 hr in a Ringer solution to which 400 mM glycerol had been added, and then returned to normal Ringer solution. The electrical properties of these treated fibers have been examined (4, 12) with a view to localizing the many unusual electrical properties of skeletal muscle. Interpretation of these electrical results and, indeed, the usefulness of this preparation depends on the extent of destruction of the sarcotubular system in such treated fibers. It is not sufficient to note the presence or absence of a tubular profile since this membrane is not necessarily connected to the sarcolemma. The amount of damage is measured here by determining the amount of the sarcotubular system which is still connected to the extracellular fluid after glycerol treatment. Horseradish peroxidase, rendered visible in the electron microscope by the method of Graham and Karnovsky (13), was used as an extracellular marker in these experiments. Our results show that very little of the sarcotubular system remains connected to the extracellular space in glycerol-treated muscle fibers. Thus, the

properties of treated muscle fibers essentially represent the properties of a smooth cylinder of the surface membrane.

METHODS

Glycerol Treatment

Sartorius muscles, tied to a bar, were placed in glycerol-Ringer's (115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂, 3.0 mM sodium phosphate buffer pH 7.0 and 400 mM glycerol) for 1 hr at room temperature (17–20°C). After this treatment, the muscle was rapidly transferred to normal Ringer's.

Tetrodotoxin (TTX) (10⁻⁷ g/ml) was added to both glycerol-Ringer's and normal Ringer's to block spontaneous contractions which otherwise often occur when the muscle is first returned to normal Ringer solution (Gage and Eisenberg, unpublished observation). It has been reported (4, 17) that destruction of the transverse tubular system occurs not in the glycerol-Ringer solution, but only after the muscle has been returned to normal Ringer's. Some muscles were transferred directly from glycerol-Ringer's to fixative to confirm this finding (see Results); in order to have good preservation, it was found necessary to add 400 mM glycerol to the fixative. Muscles fixed this way are referred to as "in glycerol" muscles; muscles fixed (see Standard Fixation) 1 hr after return to normal Ringer's are called "glycerol-treated" muscles.

Sampling of Tissue

In these experiments muscles were treated in a variety of ways before, during, and after fixation. The important features common to all techniques were concerned with sampling problems. One of the essential difficulties in relating morphological findings to physiological findings is that morphological results are normally based on samples from a very small part of a few fibers from an undetermined location in a muscle, whereas the physiological results are based on measurements from many fibers on one surface of the muscle. In these experiments care was taken to study the morphology of those fibers normally used in physiological experiments. Only the central third of the muscle was embedded and only those fibers on the deep surface of the muscle were examined. The other surface of the muscle, which lies just under the skin in the frog, is not used in studies using micro-

electrodes because of its dense covering of connective tissue. It is interesting to note that this covering was also found to act as a barrier to penetration of glycerol, peroxidase, and fixative in our experiments.

It is clear that the surface fibers could be easily identified in a transverse section cut across the entire muscle. Transverse sections are, however, unsuitable for studies concerned primarily with the transverse tubular system since most fibers will not be sectioned through a Z disc. Longitudinal sections of random orientation are also unsuitable because they give little information about the location of a fiber. Oriented longitudinal sections, cut so that the section extends from the skin surface to the depth of the muscle (see Fig. 1), permit the identification of the location of a fiber. All results presented here are from such sections.

In order to cut the required sections, precaution had to be taken during the processing of the tissue preceding sectioning. The muscle was cut into four strips parallel to the fibers. The strips were separated and cut into 1–2 mm lengths. These pieces were small enough for penetration of the fixatives and reagents, yet each piece could be embedded in a flat mold so that sectioning of the Araldite block in a particular oriented plane gave the

desired profile of layers (Fig. 1). In every experiment a surface fiber, and often a few fibers from the next two layers, were examined from each of the four strips.

Dissection and Standard Fixation

Sartorius muscles from small frogs were dissected, particular care being taken not to bruise or otherwise injure the surface fibers. Muscles were tied to a glass bar at "resting" length, i.e. that length at which the muscle was just taut. Fixation required 1.5–2.0 hr in a 5% glutaraldehyde solution to which 0.1 M sodium cacodylate (pH 7.2) and 2 mM CaCl_2 had been added (23). The muscles were left overnight at 4°C in buffer wash (0.1 M sodium cacodylate, pH 7.2, 2 mM CaCl_2 and 10% sucrose) and were then cut into small pieces (see Sampling of tissue) and postfixed in 10% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.2. In some experiments these small pieces of fixed tissue were stained with uranyl acetate, called staining en bloc (7). The pieces were oriented as described above, and then embedded in Araldite. Sections were cut with glass and diamond knives on a Porter-Blum microtome (MT-1) (Ivan Sorvall, Inc., Norwalk, Conn.). Thick sections (1 μ) were stained with toluidine blue and ob-

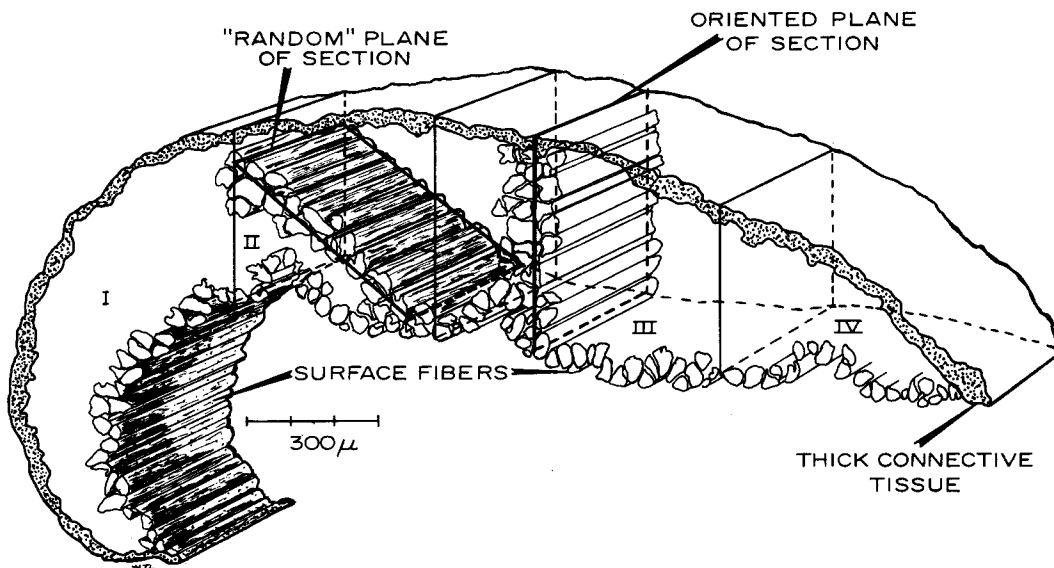


FIGURE 1 Three-dimensional reconstruction of frog sartorius muscle showing how sections were obtained. Four strips (I, II, III and IV) were cut parallel to the longitudinal axis. All micrographs in this paper are taken from oriented sections like that shown in strip III. Longitudinal sections in a "random" plane, as in strip II, were rejected. The cross section of the muscle, used in the front face of this figure, was traced from a light micrograph.

served with the light microscope to confirm that the orientation procedures had been correctly performed. Thin sections for electron microscopy were either unstained, stained with uranyl acetate, or stained with uranyl acetate and then lead citrate (27). Sections were mounted on carbon-coated grids and observed in a Philips EM-200 (Philips Electronics & Pharmaceutical Industries Corp., New York) with a single condenser, 20 μ aperture, and accelerating voltage of 60 kv.

Peroxidase Method of Marking Extracellular Space

The peroxidase method of Graham and Karnovsky (13) was found to be a cheap, convenient, and reliable method of marking the sarcotubular system in skeletal muscle (see also 21, 26). Normal untreated sartorius muscles were exposed for 30 min to a Ringer solution to which 0.05% horseradish peroxidase (Sigma Chemical Company, St. Louis, Type II) had been added. This solution often also contained TTX. After glutaraldehyde fixation and buffer wash, small pieces of fixed muscle were placed in a solution containing 0.05% 3,3'-diaminobenzidine tetrahydrochloride (Sigma), 0.01% H_2O_2 , and 0.05 M Tris-maleate buffer (pH 7.6). Three brief washes in distilled water preceded the 2-hr postfixation at 0°C with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.2). Peroxidase itself is not visible in the electron microscope, and neither, presumably, is the reaction product resulting from incubating diaminobenzidine and hydrogen peroxide with peroxidase. The electron-opaque material observed with the electron microscope is probably osmium which has reacted with this reaction product (25).

The peroxidase method was used to determine the number of tubules still connected with the extracellular space in glycerol-treated muscle fibers. In these experiments muscles were soaked for 1 hr in TTX glycerol-Ringer's, then for 30 min in TTX-Ringer's and finally for 30 min in TTX-Ringer's to which 0.05% peroxidase had been added. Several muscles were exposed to peroxidase while they still remained in glycerol-Ringer's, to determine whether the sarcotubular system is still connected to the extracellular space in this solution. These muscles were soaked for 30 min in glycerol-Ringer's, then for 30 min in glycerol-Ringer's to which peroxidase had been added, and then transferred to glutaraldehyde fixative. The appear-

ance of such in glycerol fibers depended on whether standard fixative or fixative modified by adding 400 mM glycerol had been used. The latter fixative was found to give better but not perfect fixation.

Methods of Counting

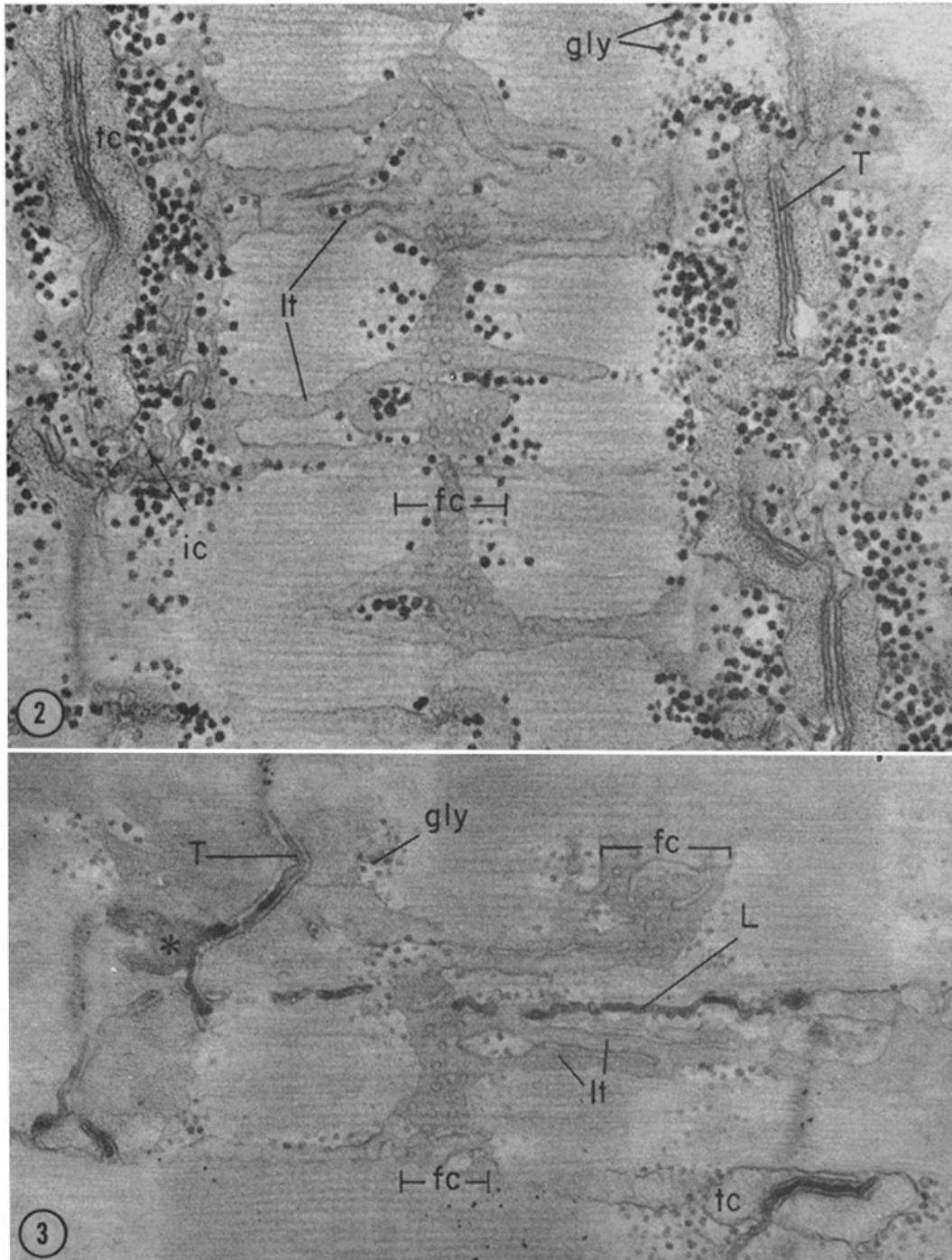
Unstained or lightly stained thin sections (silver) were used to count the peroxidase-filled tubules, since the contrast between the dense peroxidase reaction product and the lightly stained muscle made counting much easier. In each block of tissue the surface fiber was located, as described above, and micrographs of the fiber were taken. Second- and third-layer fibers were identical in appearance to the surface fibers and were used in counting if the sarcolemma was surrounded by a dense ring of peroxidase. A montage of each fiber was made at a final magnification of about 20,000. The apparent diameter of the fiber was estimated and three sets of counts were made on each normal muscle fiber: one for all the filled tubules (called x), one for all the empty tubules (called e), and one for those sites which had no tubules (called n).

Occasionally a tubule was not cut transversely, but longitudinally, so that it was seen for the width of one or more myofibrils (Fig. 4). These tubules were counted according to the number of interfibrillar spaces they crossed: that is, a tubule extending over one entire fibril and crossing two interfibrillar spaces was counted as two sites.

The counting was quite reproducible, the precision being better than 1%. In glycerol-treated muscle fibers the presence of disorganized tubules (see Results) made it impossible to distinguish between e and n sites. Thus, these sites were counted together as ($e + n$) sites. Each time a filled tubule was found, its depth from the sarcolemma was noted.

RESULTS

The fine structure of normal frog sartorius muscle has been well described (23). Occasionally most of this structure can be seen in a single longitudinal section (Fig. 2). Such a fortuitous section passes just above a myofibril, cutting through a substantial amount of the sarcotubular system and sarcoplasmic reticulum. The transverse tubule is situated at the Z line and is continuous from one myofibril to the next. The tubule branches in two planes; it branches in the transverse plane and forms a ring which surrounds most of the Z disc;



FIGURES 2 and 3 Normal muscle longitudinal section with long axis of the fiber oriented horizontally. Fig. 3 has peroxidase reaction product filling the sarcotubular system. (*T*) transverse sarcotubule. (*L*) longitudinal sarcotubule. (*t.c.*) terminal cisternae. Zone of intermediate cisternae (*i.c.*) indicated by arrow. (*l.t.*) longitudinal tubule of reticulum. (*f.c.*) fenestrated collar. (*) possible frontal section of flattened sarcotubular cisterna filled with peroxidase. (*gly*) glycogen granules. Fig. 2, $\times 50,000$; Fig. 3, $\times 45,000$.

and its branches in the longitudinal planes, (Figs. 3 and 12) perhaps so that it can pass near the Z line even when the Z lines of adjacent myofibrils are not in register. The longitudinal membrane structures which are not continuous with the transverse tubules are called the sarcoplasmic reticulum. The component of the sarcoplasmic reticulum which is immediately adjacent to the transverse tubule is called the terminal cisterna or lateral sac. The characteristic structure formed by two terminal cisternae in close apposition to a transverse tubule is called a triad. The terminal cisterna is continuous with the intermediate cisterna, and the latter branches into longitudinal tubules in the outer part of the A band. (This tubular component of the sarcoplasmic reticulum should not be confused with the occasional longitudinal elements of the so called "transverse" sarcotubular system, Fig. 3). The longitudinal tubules of reticulum then fuse to form the fenestrated collar in the central region of the A band.

Since our analysis depends on a quantitative estimate of the number of tubules accessible to the external medium, it was important to use a reliable marker of extracellular space. Ferritin was not used because it was thought to be rather capricious (24). The sodium localization method used on muscle by Zadunaisky (28), which depends on the observation of sodium pyroantimonate precipitate, was found to be most unreliable in our hands; in only 1 out of 10 experiments (normal muscle) was any precipitate found in the sarcotubules, and then only 20% of the tubules were filled. However, Figs. 2 and 12, which show the structure of normal muscle, were taken from these negative experiments with pyroantimonate.

On the other hand, the peroxidase method for marking extracellular space was most successful. The peroxidase marker formed a dense ring around all the surface fibers in the muscle. The reaction product was not found below the fourth- or fifth-layer fibers nor below the sheet of connective tissue which covers the surface of the sartorius and lies just under the skin of the frog. We think that this result is due to the failure of diaminobenzidine to penetrate. Only those fibers which were surrounded with a dense ring of peroxidase were used in this study, but all surface fibers fulfilled this criterion. The sarcotubular system was filled with electron-opaque material (Fig. 3, 4, and 5) and was thus continuous with the extracellular medium, as has been described many times previously. Longi-

tudinal branches of the tubular system were seen in about 1% of the sarcomeres, although this number obviously will depend greatly on the amount of stagger in the Z lines of adjacent myofibrils. Peroxidase was absent from the sarcoplasmic reticulum, which further confirms a separation of this compartment from the sarcotubular system. In some places a transverse tubule is not seen in the space between myofibrils near the Z line. Such an absence of a profile of the transverse tubule implies that not every myofibril is surrounded by a complete ring of transverse tubule. Table I shows the results of counting the various classes of tubules (see Methods) in many muscle fibers. The depth of the single muscle fiber in the whole muscle is indicated. Some 84% of the sites where profiles of the transverse tubule are expected to be seen do, in fact, contain tubules *filled* with peroxidase. This latter number is important since it forms the basis for the interpretation of the number of filled tubules in glycerol-treated muscle fibers. The peroxidase method is clearly very reliable, the peroxidase filling some 99% of all the tubules observed, with little variation from fiber to fiber (standard error of the mean for nine fibers, 0.6%; maximum deviation observed, 3.8%). Thus, the peroxidase method is suitable for determining what fraction of the tubular system is connected to the outside in glycerol-treated muscle fibers.

The appearance of muscle fibers fixed while they were still in glycerol-Ringer solution, before the sarcotubular system had been disrupted, depended on the fixative used. Muscle fibers which were fixed in glutaraldehyde to which 400 mM glycerol had been added were normal in appearance (Fig. 5), both the sarcotubular system and the sarcoplasmic reticulum being essentially indistinguishable from normal. It was not possible to determine, with any degree of confidence, whether these fibers were shrunken since considerable variation in fiber size was noted from one normal frog to another. Fixation in standard glutaraldehyde fixative (to which 400 mM glycerol had *not* been added) gave fibers which were grossly swollen in appearance, but fibers deeper in the muscle were much more normal. This finding may be explained by the osmotic shock involved in transferring a muscle from hypertonic glycerol-Ringer's to a fixative designed for muscles in isotonic solutions. Irrespective of the degree of swelling of the fiber, the sarcotubular system was intact

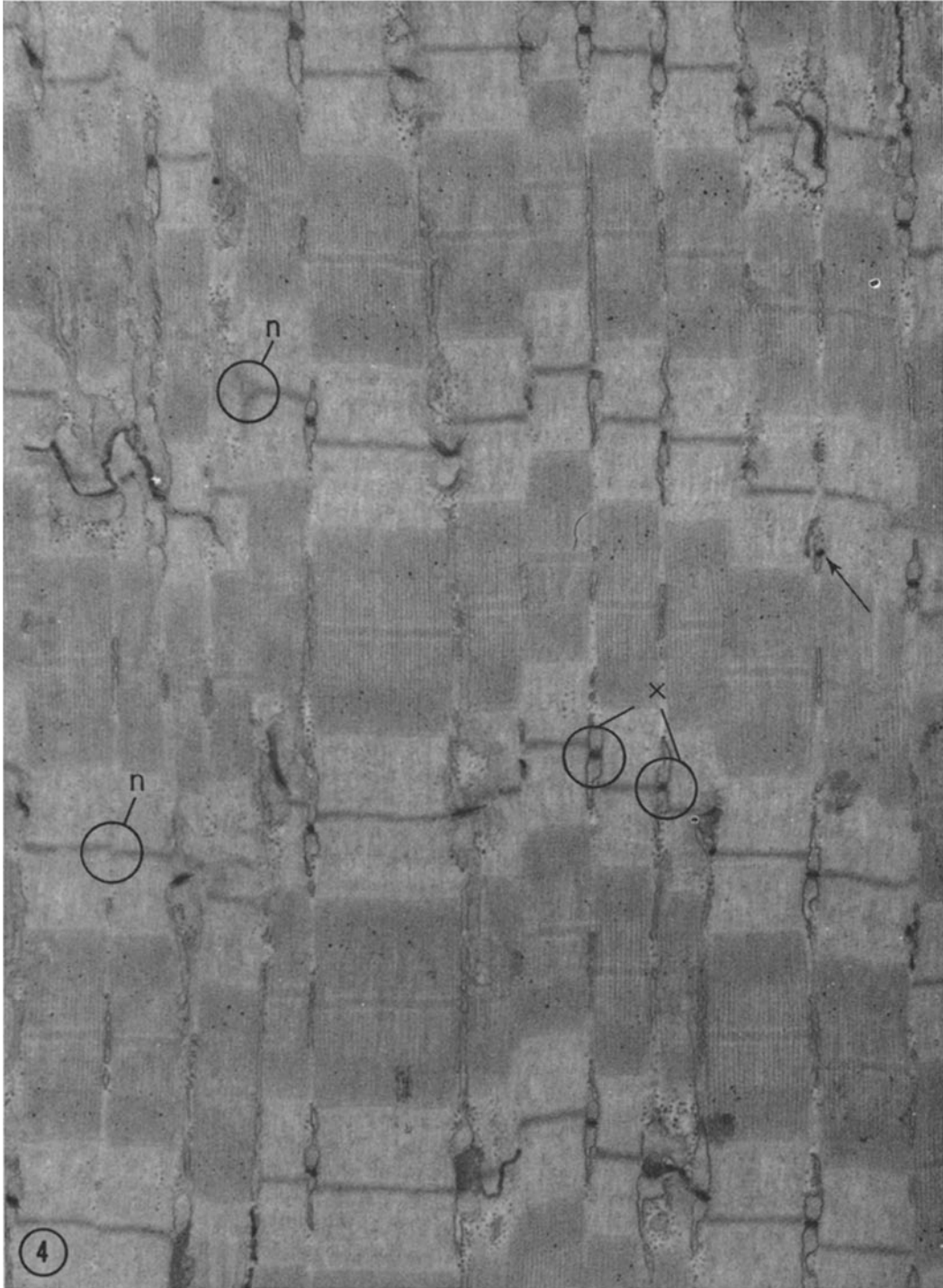


FIGURE 4 Normal muscle longitudinal section with long axis of the fiber oriented vertically. Peroxidase reaction product fills the sarcotubular system. (x) examples of triads in which the central element was filled with peroxidase (x-sites). (n) examples of "no tubule sites." Arrow marks triad which happens to be located at the A-I junction. Long lengths of tubule were counted as described in the text. $\times 21,000$.

TABLE I
Normal Muscle

Muscle	Depth of fiber from surface	No. of filled tubules (x)	No. of empty tubules (e)	No. of sites without tubules (n)	Peroxidase reliability	Fraction of sites at which tubules are found,	Fraction of sites at which filled tubules are found,
					$\frac{x}{x+e}$	$\frac{x+e}{x+e+n}$	$\frac{x}{x+e+n}$
					%	%	%
A	1	71	4	28	94.7	72.8	68.9
	3	87	1	17	98.9	83.8	82.9
	4	104	0	26	100.0	80.0	80.0
B	1	80	0	11	100.0	87.9	87.9
	3	32	1	7	97.0	82.5	80.0
C	1	191	3	40	98.5	82.9	81.6
	2	349	6	49	98.3	87.9	86.4
	3	162	2	29	98.8	85.0	89.9
	4	222	0	24	100.0	90.2	90.2
Mean, %					98.5	83.7	82.4
Standard Error of Mean, %					0.6	1.7	2.1

Means were computed on a unweighted basis, i.e. each fiber was given equal weight. If the data from each fiber are weighted according to the number of observations made, the means are not changed significantly.

and filled with peroxidase. Thus, Table II includes data from both sets of experiments.

Peroxidase was added to the glycerol-Ringer solution before fixation (see Methods). The results of counting the number of filled tubules, empty tubules, and sites without tubules are shown in Table II. The data seem to be indistinguishable from those taken from normal muscles. For purposes of comparison with glycerol treated muscle fibers, some of the data in the two tables were lumped together. In particular, the combined figure for the "fraction of sites at which there were filled tubules" is 81%.

Soon after a glycerol-soaked muscle is returned to Ringer's it becomes opaque and often gives spontaneous twitches (unless TTX is used to block muscle action potentials). This glycerol-treated muscle bears no resemblance to the classical glycerinated preparation of myofibrils, since glycerol-treated fibers have relatively normal resting potentials and action potentials (4) and therefore have an intact sarcolemma. Furthermore, these fibers give caffeine contractures¹ (17). Light microscopy reveals a considerable variation in structure from fiber to fiber: a few fibers appear normal while

many have "vacuoles" or swollen spaces between the myofibrils. The fine structure observed with the electron microscope shows a similar range of fibers: some fibers are normal in appearance except for the lack of sarcotubular system, and others show evidence of general disruption. It is natural to suppose that the latter case corresponds with those fibers found to have low membrane potentials, but direct proof of this identification has not been attempted. The fine structure is often that shown in Fig. 6. In this low magnification survey picture it is evident that the muscle striations are normal and that there is only a little swelling between myofibrils; the mitochondria are broken, however. At higher magnification (Figs. 7, 8, and 9), the filaments appear normal¹ and the disruption of the

¹A. Sandow and E. S. Geffner (personal communication) have recently measured the tension produced by application of 5 mM caffeine to glycerol-treated toe muscle fibers. They find that there are large caffeine contractures in these fibers, the average peak tension being 62% of normal. It thus seems likely that those parts of the sarcoplasmic reticulum involved in the release of Ca^{++} and the filaments are reasonably normal.

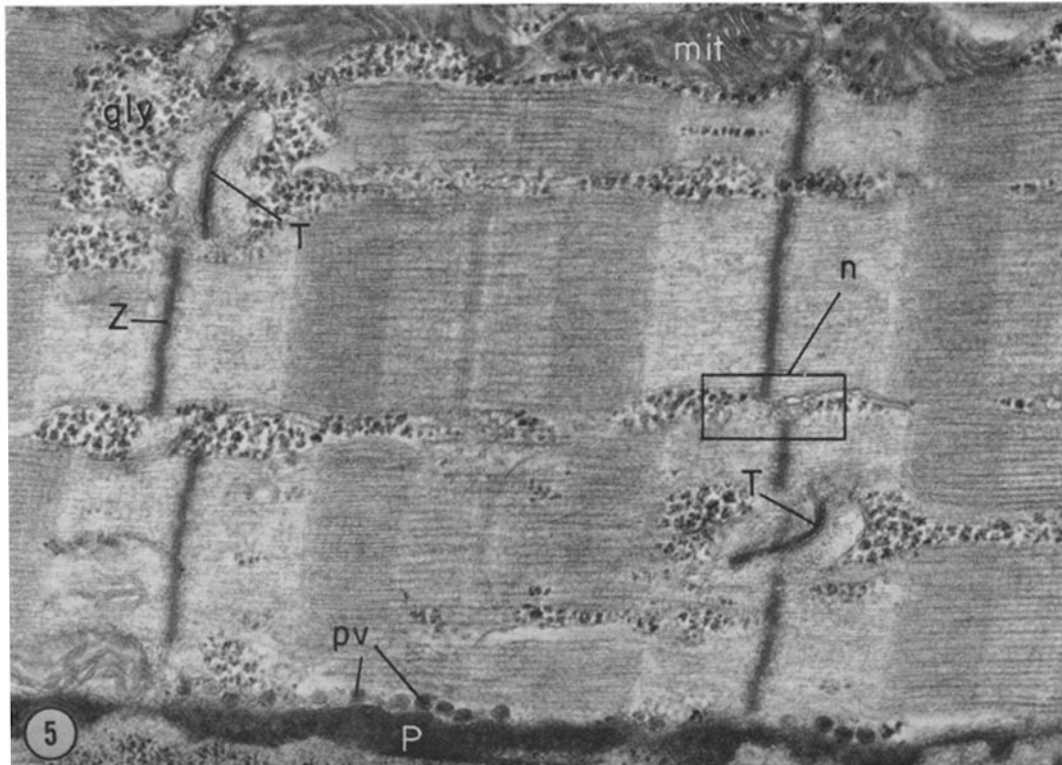


FIGURE 5 "In glycerol" muscle: treated with peroxidase and fixed with glutaraldehyde + 400 mM glycerol. Longitudinal section with long axis horizontally oriented. Peroxidase reaction product (*P*) seen in the extracellular space outside the sarcolemma, and in the sarcotubular system (*T*). Muscle is normal in appearance. Note mitochondrion (*mit*) and pinocytotic vesicles (*p.v.*), glycogen granules (*gly*), 'no-tubule' site (*n*). This section was stained with lead citrate and then uranyl acetate, thus producing particularly dark Z lines ($\times 32,000$).

transverse tubular system can be clearly seen: a typical triad no longer contains three elements since the transverse tubule is usually missing: however, many triads have small remnants of tubules. Some intermediate cisternae remain intact, but most are broken. The fenestrated collar seems the least damaged structure of the reticulum, usually being indistinguishable from normal. The fibers shown in Figs. 8 and 9 are more severely altered: the terminal cisternae are damaged and the space between myofibrils is swollen, especially at the AI junction.

The glycerol-treated muscle fiber in Figs. 8 and 9 had been exposed to peroxidase to mark the extracellular space. The peroxidase marker is found in a dense ring around the sarcolemma. The sarcolemma had normal substructure (i.e. it consisted of two dense lines) and was seldom broken, even

in the most severely damaged fibers. On 15 fibers, counts were made of the number of tubules filled with peroxidase (see Table III). The average number of filled tubules after glycerol treatment was $2.6 \pm 0.7\%$ (mean \pm standard error of the mean), compared with 81% in muscles with normal structure (combined data from Tables I and II). That is to say only 2.6% of the sites where tubules might be expected to be found did, in fact, contain filled tubules. Thus, the apparent fraction of the tubular system remaining in the glycerol-treated fibers is $2.6 \div 0.81 = 3.2\%$. Presumably, 97% of the tubular system no longer contributes to the electrical properties of glycerol-treated muscle fibers, nor is involved in excitation-contraction coupling

3.2% is, in fact, an overestimate of the amount of the tubular system still continuous with the

TABLE II
Muscles in Glycerol (Tubules Intact)

Muscle	Depth of fiber from surface	No. of filled tubules (x)	No. of empty tubules (e)	No. of sites without tubules (n)	Peroxidase reliability.	Fraction of sites at which tubules are found,	Fraction of sites at which filled tubules are found,
					$\frac{x}{x+e}$	$\frac{x+e}{x+e+n}$	$\frac{x}{x+e+n}$
					%	%	%
A	1	36	4	5	90.0	88.9	80.0
B	1	251	6	65	97.7	79.8	78.0
	2	152	0	23	100.0	86.9	86.9
	3	135	1	29	99.3	82.4	81.8
	4	199	2	26	99.0	88.5	87.7
C	1	140	4	19	97.2	88.3	85.9
	1	264	6	63	97.8	81.0	79.3
	2	146	28	34	83.9	83.7	70.2
	2	467	31	92	93.8	84.4	79.1
	2	314	41	85	88.5	80.7	71.4
Mean, %					94.7	84.5	80.0
Standard Error of Mean, %					1.7	1.0	1.9

Means were computed on an unweighted basis, i.e. each fiber was given equal weight. If the data from each fiber are weighted according to the number of observations made, the means are not changed significantly.

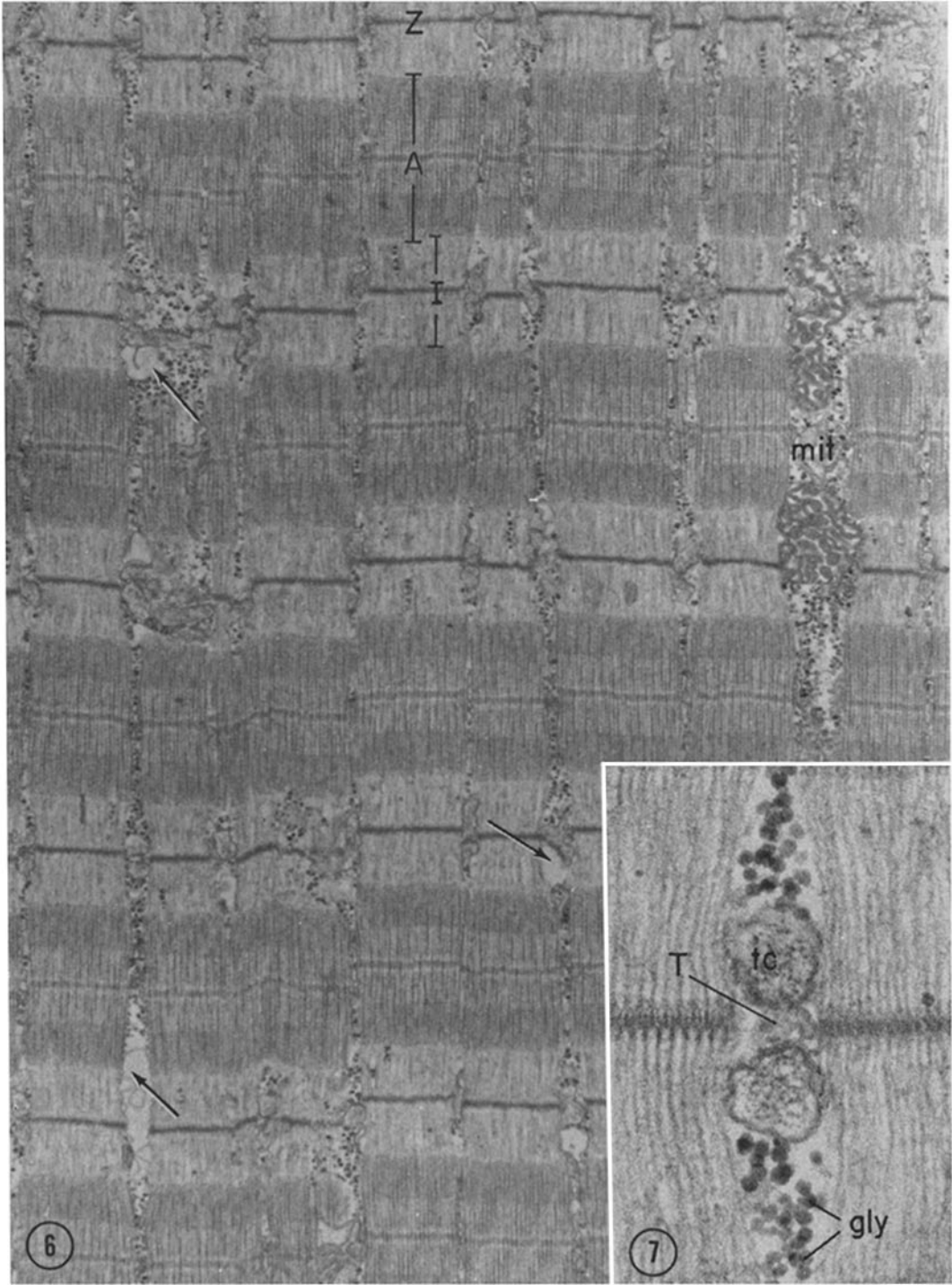
extracellular medium because of sampling errors associated with the distribution of the few tubules remaining in treated muscle fibers. Fig. 10 is a histogram showing the location of the few filled tubules. It is clear that almost all of the tubules remaining are stumps, not extending farther than a few micra into the interior of the muscle fiber. The interpretation of this histogram would be complicated if isolated filled tubules were common, particularly if they were found in the depths of the fiber. Fortunately, this is not the case; indeed, isolated filled tubules were almost never found. Thus, the distribution of remaining tubules can be represented in a crude approximation as an annulus (Fig. 11), the thickness of which in relation to the fiber radius represents the fraction of tubules remaining. Such an approximation incorrectly assumes that every site in the outer part of the fiber has a filled tubule, but will give correct results if the probability of finding a filled tubule is independent of position in the annulus and is constant from fiber to fiber. There are not enough data available to justify more precise models. In such a simple model random longitudinal sections will tend to overestimate the relative size of the annu-

lus, for two reasons: first, since most sections do not pass through the center of the fiber, the apparent diameter of the fiber (actually the chord of the circle) will be less than the actual diameter; second, since the section will not, in general, cut through the annulus at right angles, the apparent absolute size of the annulus will be an overestimate. This effect is quantitatively analyzed for idealized geometry in Appendix I and is shown not to be trivial. It is likely that the effect is of the order of 50% in our case; thus, the true figure for the fraction of tubular area remaining connected to the extracellular space in glycerol-treated muscle fibers is likely to be about $3.2\% \times 0.5 = 1.7\%$.

It should be mentioned that several variables which might conceivably affect the degree of tubular destruction have not been studied in these experiments. In particular, the effects of temperature, stretch, and speed of solution change were not examined.

DISCUSSION

In the course of these experiments an interesting observation was made concerning the structure of normal muscle. Fig. 12 shows a transverse tubule



FIGURES 6 and 7 Glycerol-treated muscle. Muscle soaked in Ringer's + 400 mM glycerol for one hour, then returned to normal Ringer for 30 min, and then fixed. The A band, I band and Z lines are normal. The mitochondrion is disrupted (*mit*). Arrows point to swellings in intermyofibrillar spaces. Fig. 7 (inset) shows the remnant of a sarcotubule (*T*); terminal cisternae (*tc*) and glycogen (*gly*) are present. Fig. 6: $\times 17,000$; Fig. 7: $\times 93,000$.



FIGURES 8 and 9 Glycerol-treated muscle with disrupted sarcotubular system. Peroxidase reaction product (*P*) is seen outside the sarcolemma, but the disruption of the sarcotubular system (arrows) presumably prevents peroxidase from filling the remaining "intact" transverse sarcotubules (*T*) and swollen sarcotubules (*S.T.*). In Fig. 9 the swollen tubule in the lower left corner is identifiable by the characteristic scalloped edge where the tubule is attached to the terminal cisterna (*t.c.*). Intermediate cisternae (*i.c.*) are swollen or broken; the fenestrated collar (*f.c.*) is intact. Fig. 8: longitudinal section vertically oriented, $\times 41,000$. Fig. 9: longitudinal section diagonally oriented, $\times 41,000$.

TABLE III
Treated Muscle Fibers (Disrupted Transverse Tubules)

Muscle	Depth of fiber from surface	Apparent diameter, μ	No. of filled tubules (x')	No. of unfilled sites ($e' + n'$)	Fraction of sites at which filled tubules are found	Fraction of tubules* connected to surface
					$\frac{x'}{x' + e' + n'}$	$\frac{x'/(x' + e' + n')}{x/(x + e + n)}$
					%	%
A	1	40	7	263	2.6	3.1
	1	25	18	536	3.3	3.9
	2	15	17	200	7.8	9.5
	2	>25‡	6	222	2.6	3.2
	2	38	0	128	0	0
	2	20	5	253	1.9	2.3
	3	>30‡	3	281	1.1	1.3
B	1	47	3	354	0.8	1.0
	1	55	0	392	0	0
	1	>20‡	7	119	5.6	6.7
	2	50	0	56	0	0
	2	40	23	253	8.3	10.5
	3	25	4	89	4.3	5.2
C	1	>25‡	0	226	0	0
	2	40	3	311	1.0	1.2
Mean, %					2.6	3.2
Standard Error of Mean, %					0.7	0.9

Means were computed on an unweighted basis, i.e. each fiber was given equal weight. If the data from each fiber are weighted according to the number of observations made, the means are not changed significantly.

* $x/(x + e + n)$ is taken as 81.1% from combined data in Tables I and II.

‡ The apparent diameter is greater than this value, since the edge of the fiber was obscured by a grid bar.

which evidently throws off two branches on either side of the Z line; these branches then form flattened sarcotubular cisternae. Peachey (23) and Peachey and Schild (24) have reported other less complex branching structures of the sarcotubular system. If structures like these occur fairly often, they might explain, at least in part, the finding of Endo (5) and D. K. Hill (15) that most of the I band seems to be accessible to some extracellular markers. Fig. 3 shows peroxidase filling this sarcotubular cisterna.

The fine structure of in glycerol muscle fibers is not very consistent; sometimes enlarged intermyofibril spaces are shown, sometimes not. We cannot decide whether these swellings are caused by "bad fixation" or whether they represent a true phenomenon. In all fibers examined the tubular system was intact and the results of the peroxidase

experiments show that the tubular lumen was continuous with the extracellular space. If glycerol remains outside the fiber in a 1 hr soak, the muscle would shrink and the sarcotubule would be swollen as in other hypertonic solutions (10, 20). On the basis of weight measurements, Fujino et al. (11) have suggested that glycerol does not penetrate, and Eisenberg and Gage (4) have interpreted their internal resistivity measurements on this hypothesis. In some fibers, however, the tubules are not swollen. If glycerol crosses the sarcolemma quickly enough so that it is essentially in equilibrium after 1 hr, the muscle fiber would not be shrunken and thus swollen tubules would not be expected.

The structure of muscle fibers which have been returned to Ringer solution from glycerol-Ringer's is strikingly different from that of normal muscle. The tubular system is destroyed; its morphology is

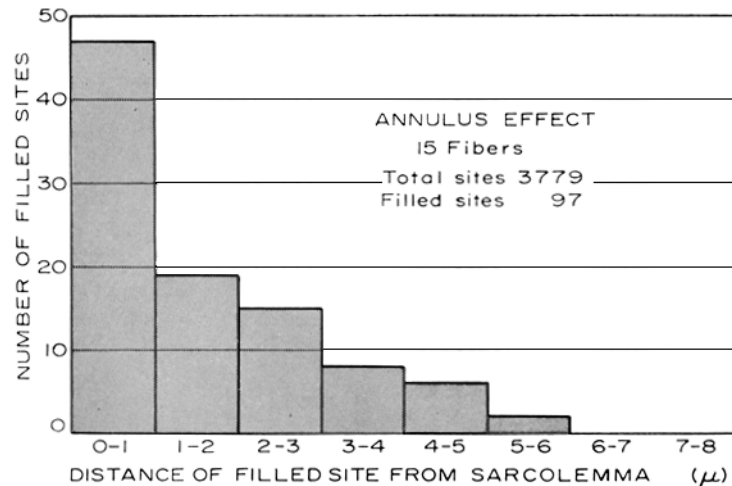


FIGURE 10 Histogram to show the location of the few filled tubules remaining after glycerol treatment (see text for details).

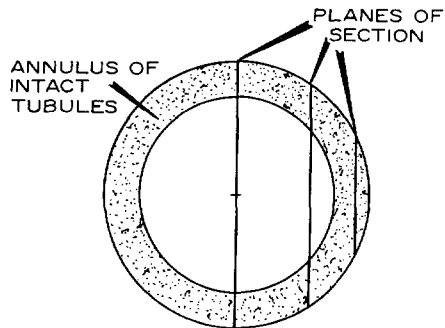


FIGURE 11 A diagram showing location of longitudinal section. Note that most longitudinal sections will not pass through the center of the fiber and thus will overestimate the size of the annulus (stippled).

drastically altered and only a few tubules are left continuous with the extracellular space. It is important to note that the sarcoplasmic reticulum is not untouched by this treatment: mild damage occurs in the terminal cisternae and fenestrated collar, and severe damage occurs in the intermediate cisternae. Thus, those properties of the muscle which require an intact sarcoplasmic reticulum (e.g. caffeine contractures) would be expected to be somewhat impaired in this preparation.

It is of particular importance to have an estimate of the area of the sarcotubular membrane still left connected to the extracellular medium and thus possibly functional. In the Results section, it is

concluded that about 2% of the tubules are still connected after treatment (the best estimate after making a correction for the "annulus effect"). In order to use this figure in interpreting the properties of glycerol-treated muscle fibers, it is necessary to calculate what fraction of the outer membrane area this 2% represents. Peachey (23) estimates that for a 50- μ fiber (our average diameter) the area of the membranes of the transverse tubular system is 3.5 times that of the sarcolemma, and this number has recently been modified to 4.5 in order to account for branching of the tubules (24). However, we find that 16% of the triadic sites where tubules had been assumed to be present do not, in fact, contain tubules (our *n*-sites). Therefore, we use a somewhat different figure for the ratio of tubular to surface area in a 50 μ fiber, namely $4.5 \times 0.84 = 3.8$. Hence, the fraction of the tubular system remaining in glycerol-treated fibers (2%) represents $3.8 \times 2\% = 7.6\%$ of the outer membrane area. The presence of this much area has some effect on the quantitative interpretation of the electrical properties of treated muscle fibers. The contribution of tubule area in glycerol-treated fibers is not sufficient to influence the qualitative findings of Gage and Eisenberg (12) concerning excitation-contraction coupling or their conclusion concerning slow electrical phenomena.

It must be remembered that the numerical estimates are based on random sampling of surface fibers, whereas the physiological data are based on fibers selected for membrane potentials above 70

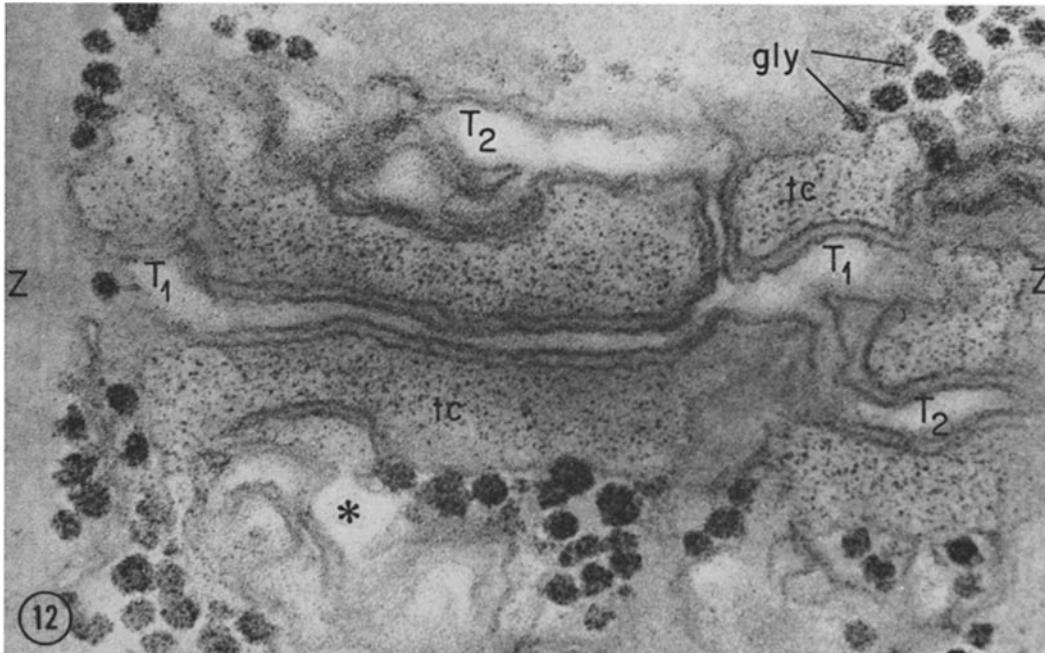


FIGURE 12 Normal muscle showing the complexity in the triadic region. Longitudinal section, fiber axis oriented vertically. The Z line runs horizontally in this figure. The transversely oriented sarcotubule (T_1) branches in the longitudinal direction, then bends to form a sarcotubular cisterna (T_2) and probably another cisterna (*). The terminal cisternae (*tc*) have a particularly speckled appearance, possibly a calcium pyroantimonate precipitate. Glycogen granules (*gly*) are prominent. $\times 120,000$.

mv. If there is some correlation between membrane potential and the number of tubules remaining, quantitative application of our data to physiological results could be in error. We note that the highest per cent of tubule area remaining is 10.5% (correcting for the annulus effect gives 5.3%), or about 15% tubular area referred to the sarcolemma area. However, 27% of the fibers had no intact tubules connected to the outside.

Finally, it is necessary to discuss the limitations of this preparation. First, in the absence of information about the mechanisms of the glycerol treatment it is unwise to apply this technique to other tissues or to modify the technique without due caution; the area measurements described here apply only to frog sartorius muscle treated as we have described. Secondly, only surface fibers have been studied here; deep fibers appear to have more tubules remaining. This point, taken in conjunction with the finding of a rather high scatter in resting potentials (4), suggests that experiments

which measure the properties of a whole glycerol-treated muscle cannot easily be interpreted.

Appendix: The Annulus effect

Since the few tubules remaining in the treated muscle fibers all lie very close to the outer membrane and since most longitudinal sections do not pass through the center of the fiber, the raw data given in Table III will tend to overestimate the number of tubules left (Fig. 11). This appendix is devoted to a quantitative analysis of this effect.

Fig. 13 shows the geometry of the system of interest and represents a magnified section of part of the fiber drawn in Fig. 11. The length of the line AB (the radius of the fiber) is called a , and the length of the line AC (the inner radius of the annulus) is called $a - d$. The thickness of the annulus is called d , the apparent size of the annular region as seen in a random longitudinal section is x (i.e., line BC), and the apparent diameter is given by $2z$ (i.e., twice the length BD). Finally, the displacement of the section from the center of the circle (i.e. line AD) is called w .

Considering triangles ABD and ACD respectively

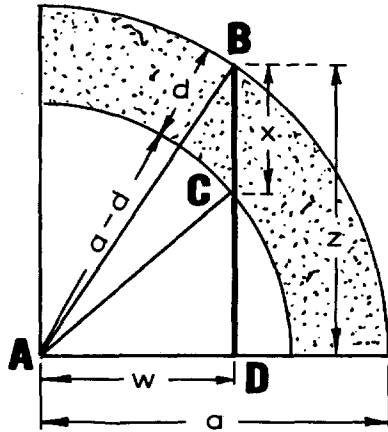


FIGURE 13 The geometry of a quadrant of a muscle fiber in cross section showing location of section. See text for definitions used in the analysis of the annulus effect.

it can be seen that:

$$w^2 = a^2 - z^2$$

$$w^2 + (z - x)^2 = (a - d)^2$$

These equations can be solved to give the true fractional size of the annulus (i.e., d/a) in terms of the apparent fractional size (x/z) and the ratio of the apparent diameter of the section to the true diameter (z/a).

$$\frac{d}{a} = 1 - \left[1 + \left(\frac{z}{a} \right)^2 \left(\frac{x}{z} \right)^2 - 2 \left(\frac{x}{z} \right) \left(\frac{z}{a} \right)^2 \right]^{1/2} \quad (1)$$

or approximately (if x/z is sufficiently small)

$$\frac{d}{a} = \frac{x}{z} \left(\frac{z}{a} \right)^2 \quad (2)$$

Some idea of the true value of d/a in our case can

REFERENCES

- ADRIAN, R. H., and W. H. FREYGANG. 1962. The potassium and chloride conductance of frog muscle membrane. *J. Physiol., (London)*. 163:61.
- ANDERSSON-CEDERGREN, E. 1959. Ultrastructure of motor end plate and sarcoplasmic components of mouse skeletal muscle fiber as revealed by three-dimensional reconstructions from serial sections. *J. Ultrastruct. Res.* (Suppl. 1) 5.
- EISENBERG, R. S. 1967. The equivalent circuit of single crab muscle fibers as determined by impedance measurements with intracellular electrodes. *J. Gen. Physiol.* 50:1785.
- EISENBERG, R. S., and P. W. GAGE. 1967. Frog skeletal muscle fibers: changes in electrical properties after disruption of transverse tubular system. *Science*. 158:1700.
- ENDO, M. 1966. Entry of fluorescent dyes into the

be found by substituting average values in equation (2). If the average diameter of the muscle fibers used is about 50μ , as transverse sections indicate, and the average apparent diameter is 36μ , the number (3.2%) given in Table III as the fraction of the transverse tubular system remaining connected to the outside is an overestimate, the true number being $d/a = 0.032 \times (36/50)^2 = 1.7\%$.

Thus, the best estimate for the amount of tubular area remaining in treated fibers is about 1.7% of normal.

Our correction is only a coarse approximation since the figure for the true diameter is likely to be in serious error because of the severe variation in diameter of the fibers from muscle to muscle, and also since our whole analysis requires muscle fibers to be circular in cross section, which requirement few fibers even approximately fulfill.

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Note:

We have recently been informed of a paper in the Russian literature by S. A. Krolenko, S. YA. Adamyan, and N. E. Shvinka, 1967, entitled Vacuolization of skeletal muscle fibers. 1. Vacuolization after efflux of low molecular non-electrolytes (*sic*). *Tsitologiya* 9:1346. This paper describes the morphology of glycerol-treated muscle fibers observed with the light microscope. A further paper, from the same laboratory, on the fine structure as seen in the electron microscope is to appear, we are told, in the July 1968 issue of *Tsitologiya*.

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- sarcotubular system of the frog muscle. *J. Physiol., (London)*. 185:224.
6. FALK, G., and P. FATT. 1964. Linear electrical properties of striated muscle fibers observed with intracellular electrodes. *Proc. Roy. Soc. (London), Ser. B*. 160:69.
 7. FARQUHAR, M. G., and G. E. PALADE. 1965. Cell junctions in amphibian skin. *J. Cell Biol.* 26:263.
 8. FATT, P. 1964. An analysis of the transverse electrical impedance of striated muscle. *Proc. Roy. Soc. (London) Ser. B*. 159:606.
 9. FREYGANG, W. H., JR., D. A. GOLDSTEIN, and D. C. HELLAM. 1964. The afterpotential that follows trains of impulses in frog muscle fibers. *J. Gen. Physiol.* 47:929.
 10. FREYGANG, W. H., D. A. GOLDSTEIN, D. C. HELLAM, and L. D. PEACHEY. 1964. The relation between the late after-potential and the size of the transverse tubular system of frog muscle. *J. Gen. Physiol.* 48:235.
 11. FUJINO, M., T. YAMAGUCHI, and K. SUZUKI. 1961. "Glycerol effect" and the mechanism linking excitation of the plasma membrane with contraction. *Nature*. 192:1159.
 12. GAGE, P. W., and R. S. EISENBERG. 1967. Action potentials without contraction in frog skeletal muscle fibers with disrupted transverse tubules. *Science*. 158:1702.
 13. GRAHAM, R. C., and M. J. KARNOVSKY. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* 14:291.
 14. HILL, A. V. 1948. On the time required for diffusion and its relation to processes in muscle. *Proc. Roy. Soc. (London) Ser. B*. 135:446.
 15. HILL, D. K. 1964. The space accessible to albumin within the striated muscle fiber of the toad. *J. Physiol., (London)*. 175:275.
 16. HODGKIN, A. L., and P. HOROWICZ. 1960. The effect of sudden changes in ionic concentrations on the membrane potential of single muscle fibers. *J. Physiol., (London)*. 153:370.
 17. HOWELL, J. N., and D. J. JENDEN. 1967. T-tubules of skeletal muscle: morphological alterations which interrupt excitation contraction coupling. *Federation Proc.* 26:553.
 18. HUXLEY, A. F., and R. E. TAYLOR. 1958. Local activation of striated muscle fibers. *J. Physiol. (London)*. 144:426.
 19. HUXLEY, H. E. 1964. Evidence for continuity between the central elements of the triads and extracellular space in frog sartorius muscle. *Nature*. 202:1067.
 20. HUXLEY, H. E., S. PAGE, and D. R. WILKIE. 1963. An electronmicroscopic study of muscle in hypertonic solutions. *J. Physiol., (London)*. 169:312. Appendix of Dydyńska, M. and Wilkie, D. R.
 21. KARNOVSKY, M. J. 1965. Vesicular transport of exogenous peroxidase across capillary endothelium into the T-system of muscle. *J. Cell Biol.* 27:49A.
 22. PAGE, S. 1964. The organization of the sarcoplasmic reticulum in frog muscle. *J. Physiol., (London)*. 175:10P.
 23. PEACHEY, L. D. 1965. The sarcoplasmic reticulum and transverse tubules of the frog's sartorius. *J. Cell Biol.* 25 (Pt. 2):209.
 24. PEACHEY, L. D., and R. F. SCHILD. 1968. The distribution of the T-system along sarcomeres of frog and toad sartorius. *J. Physiol., (London)*. 194:249.
 25. REESE, T. S., and M. J. KARNOVSKY. 1967. Fine structural localization of a blood-brain barrier to exogenous peroxidase. *J. Cell Biol.* 34:207.
 26. SOMMER, J. R., and E. A. JOHNSON. 1968. Purkinje fibers of the heart examined with peroxidase. *J. Cell Biol.* 37:507.
 27. VENABLE, J. H., and R. COGGESHALL. 1965. A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* 25 (Pt. 1): 407.
 28. ZADUNAISKY, J. A. 1966. The location of sodium in the transverse tubules of skeletal muscle. *J. Cell Biol.* 31:C11.