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Morphology and Accessibility of the 'Transverse' Tubular System in Frog Sartorius Muscle after Glycerol Treatment

C. Franzini-Armstrong, R. A. Venosa, and P. Horowicz

Department of Physiology, University of Rochester,
School of Medicine and Dentistry, 260 Crittenden Boulevard,
Rochester, New York 14642

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Summary. Sartorius muscles were exposed to a hypertonic Ringer's fluid containing 400 mM glycerol and subsequently returned to normal Ringer's fluid. Employing lanthanum as an extracellular marker and after suitable preparation, sections of muscle fibers were examined with an electron-microscope. Extensive alteration in 'transverse' tubular morphology occurs after glycerol treatment. The number of sites at the Z line level usually containing complete triads decreases by about two-thirds. In about half of the sites with altered morphology no 'transverse' tubules were present, while in the other half the sites were completely empty. Fibers at all depths in glycerol-treated muscles were equally affected. The remnants of the 'transverse' tubules which remain were not very accessible to extracellular lanthanum. Estimates based on measurements of the presence or absence of lanthanum indicate that glycerol treatment disconnects 90% of the 'transverse' tubules from the external solution. The remaining tubules connected to the external solution are largely but not entirely located in a surface layer of about one-tenth the fiber radius in depth.

The aim of this study was to provide quantitative information on the extent of damage suffered by the 'transverse' tubular system in whole sartorius muscles exposed to a hypertonic Ringer's fluid containing 400 mM glycerol and subsequently returned to normal Ringer's fluid. This treatment is known to produce both uncoupling of contraction from excitation (Fujino, Yamaguchi & Suzuki, 1961; Howell & Jenden, 1967; Howell, 1969) and a reduction in the low frequency membrane capacity (Eisenberg & Gage, 1967; Gage & Eisenberg, 1969; Hodgkin & Nakajima, 1972).

The morphology of glycerol-treated fibers has been studied in detail by several investigators, often with the aid of extracellular tracers (Krolenko, Adamjan & Shwinka, 1967; Eisenberg & Eisenberg, 1968; Howell, 1969; Krolenko, 1969; Nakajima, Nakajima & Peachey, 1969, 1973; Niemeyer &

Forssmann, 1971; Dulhunty & Gage, 1973*a*). From these studies it is generally accepted that a prolonged exposure to glycerol-containing solutions followed by return to normal Ringer's fluid produces an interruption in the continuity of the 'transverse' tubular system at or very close to the surface of the fibers. Interestingly, shorter exposure times and/or the use of lower concentrations of glycerol produce incomplete damage to the 'transverse' tubular system (Dulhunty & Gage, 1973*b*) and allow recovery of excitation-contraction coupling (Krolenko & Fedorov, 1972; Zachar, Zacharova & Adrian, 1972). In one case, preservation of a fairly intact 'transverse' tubular system following prolonged exposure to 400 mM and return to normal Ringer's fluid has been reported (Nakajima *et al.*, 1973).

The studies described in this report were initiated to determine how much of the 'transverse' tubular system is accessible to the extracellular space in the glycerol-treated muscles used for sodium flux measurements reported in a previous paper (Venosa & Horowicz, 1973). Two questions, for which the literature did not provide unequivocal answers, were considered. The first dealt with the reliability of the method when applied to whole muscle. In using such preparations it is important to be certain that fibers at the center of a muscle have as much of their 'transverse' tubular system disrupted as have fibers at the surface. The adequacy of the method is very likely to depend on such factors as the specific muscle used, the size of the muscle, and the length of exposure to the different solutions. Our data apply only to small sartorius muscles after being exposed at least for 1 hr to hypertonic Ringer's fluid containing 400 mM glycerol and then returned to normal Ringer's fluid for at least 1.5 hr. The muscles weighed about 60 mg, had an average length of about 29 mm, contained a total of about 600 to 700 fibers, and had no more than 15 layers of fibers in the thickest part. Within these restrictions we found that fibers at all depths were uniformly "detubulated".

The second question considered dealt with the extent of damage of the 'transverse' tubular system and the effect of glycerol treatment on the sarcoplasmic reticulum. Only one-third of the original 'transverse' tubular membrane was located at the original site in the center of the triad, regardless of fiber location. Such tubular membrane as remained was not entirely intact. Only 10% of the original 'transverse' tubules remained connected to the surface, the other 90% was effectively disconnected from the external solution. On the other hand, the morphology of the sarcoplasmic reticulum was not much affected by glycerol treatment. For the most part, our data agree with those obtained by Eisenberg and Eisenberg (1968) for superficial fibers of frog sartorii.

Materials and Methods

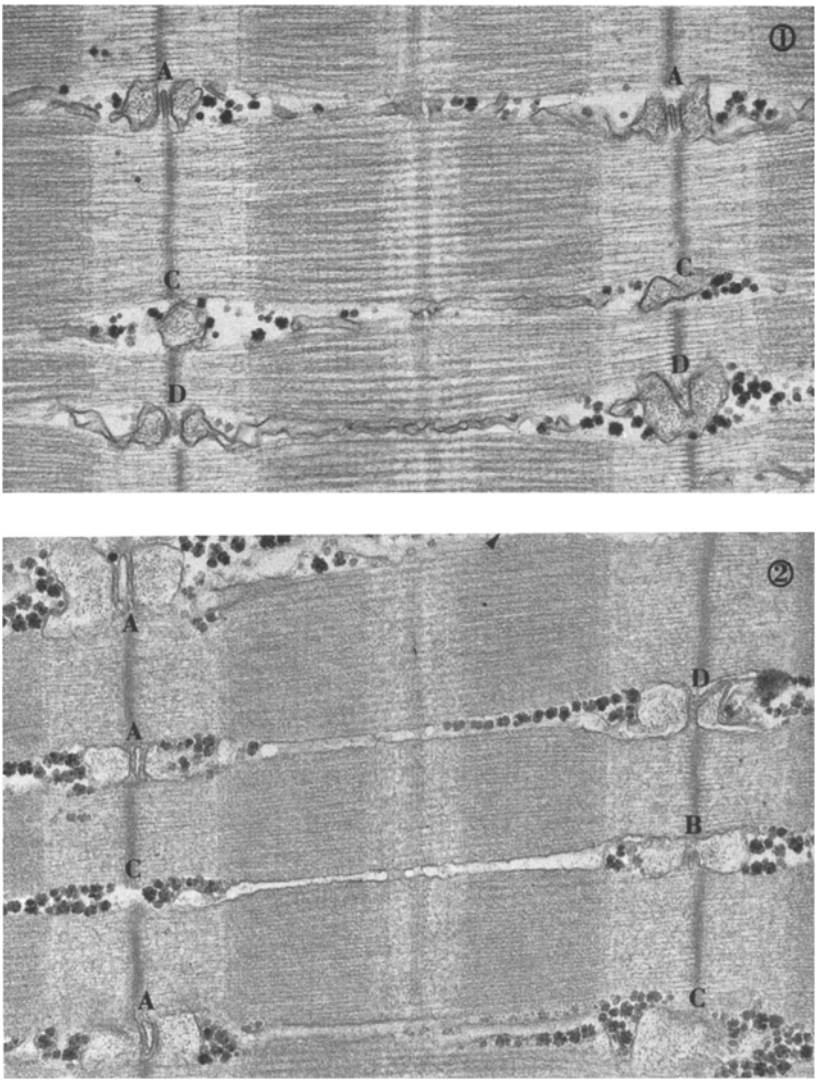
Sartorius muscles were immersed either in Ringer's fluid (controls) or in Ringer's fluid containing 400 mM glycerol for 70 to 90 min and then transferred to Ringer's fluid for 75 min or longer (*see* Venosa & Horowicz, 1973 for details). Included in Table 2 are two muscles exposed to a high calcium, high magnesium solution (Eisenberg, Howell & Vaughan, 1971). The muscles were fixed for 2 hr in peroxide-4% glutaraldehyde (Peracchia & Mittler, 1972), containing 0.1 M phosphate buffer, at pH 7.2, followed by 2% osmium tetroxide, dehydration in ethyl alcohol, and embedding in Epon. Four muscles, two controls and two glycerol-treated, were exposed for 15 min to 1% lanthanum nitrate in 125 mM NaCl (Mirolli & Crayton, 1968). The pH of the solution was then raised by addition of NaOH until it became translucent, and the muscles were then fixed by glutaraldehyde and osmium solutions, both containing colloidal lanthanum essentially according to the method of Revel and Karnovsky (1967). At the moment of embedding, the muscles were divided length-wise by a cut perpendicular to the inner and outer surfaces, through the thickest portion of the muscle. Blocks were obtained by dividing the two muscle halves into 2- to 3-mm segments, which were embedded so that the exposed center of the muscle became the cutting face. Longitudinal sections included the whole thickness of the muscle (12 to 15 fibers). Fibers on either edge of the section were originally located on the inner and outer surfaces of the muscle, whereas fibers in the center of the section were situated close to the center of the muscle. Fibers in positions 1 to 4 on either edge were classified as superficial, all others as deep (Tables 1 and 2). Where orientation of the section was not determined accurately, fibers were considered located at random.

Sections were stained in lead solution (Sato, 1968) and three to five pictures at a 10,000 × magnification were taken for each fiber with an AEI 801 electron-microscope. Areas containing large numbers of mitochondria, expanses of tangentially cut sarcoplasmic reticulum and (in glycerol-treated fibers) large vacuoles were avoided. Otherwise pictures were taken at random, except when information on the surface areas of individual fibers was sought (Tables 3 and 5).

Results

In frog striated muscle, triads are located at the Z line level and most of the 'transverse' tubular profiles occur within this structure (Eisenberg, 1972). A fairly precise quantitative estimate of 'transverse' tubules distribution can thus be obtained by examining only structures located at the Z line level.

For quantitative purposes, the interfibrillar spaces at the Z line level have been divided into four groups. The micrographs used for illustrations were taken from glycerol-treated muscles in which all four categories were represented. First, there were the complete triads (*A*). These were profiles in which the lateral sacs of the triad, belonging to the sarcoplasmic reticulum were regularly arranged on either side of a clearly distinguishable 'transverse' tubule (Figs. 1 and 2). To fall into this category, the tubule had to have a patent opening, it must not have been greatly enlarged, and it had to have a sharply defined limiting membrane. Clear 'transverse'



Figs. 1 and 2. Longitudinal sections of two glycerol-treated fibers. Profiles at the Z line level are marked with letters corresponding to their classification in the text. Notice *D*-type triads, where the 'transverse' tubule is completely absent. Some material, probably remnant of the junctional feet, is visible in the gap. × 40,000

tubular profiles accompanied by one, rather than two, lateral sacs (dyad) were also included in this category.

Second, there were the possibly complete triads (*B*). Triads and dyads in this group are similar to group *A*, but the 'transverse' tubule was either very small or it had walls that were not clearly visible (Fig. 2). Inclusion of a

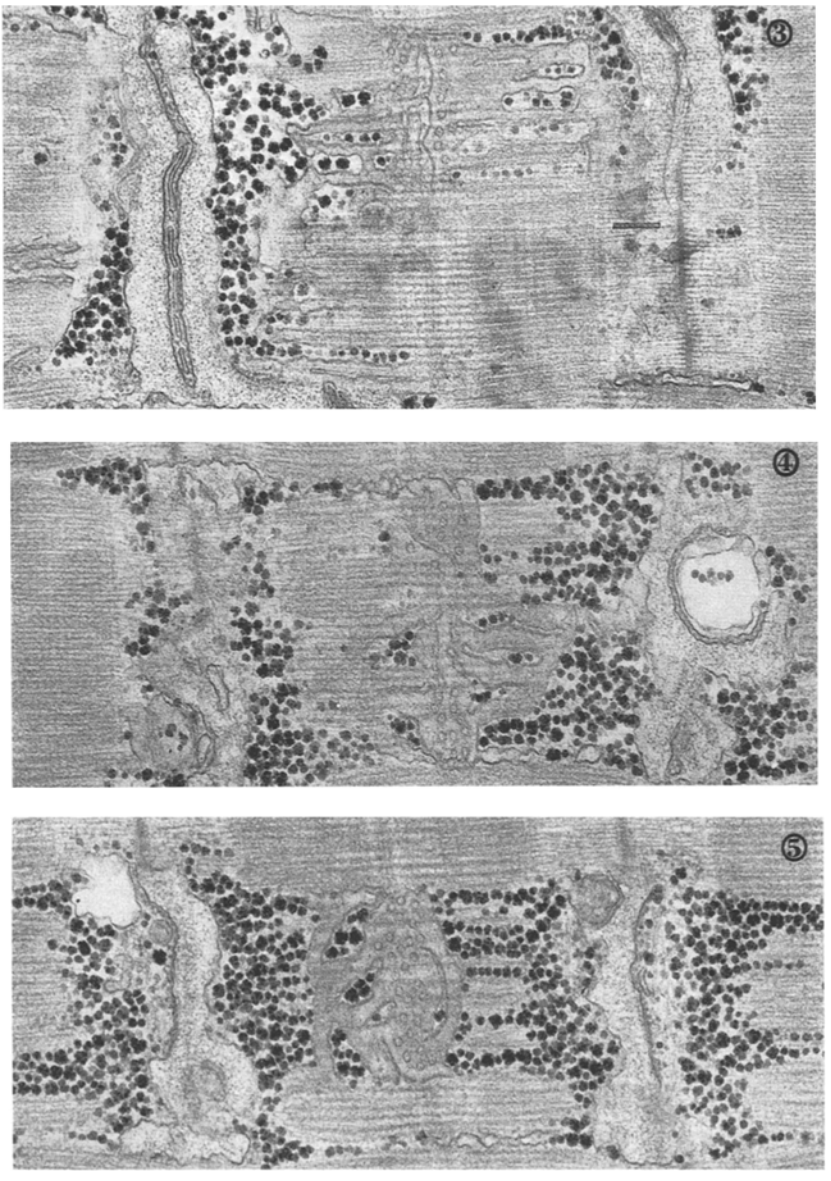
small number of profiles in this category in control muscles was probably due to the fact that the sections were not very thin and obliquely or tangentially cut tubular profiles were not easily distinguishable. In glycerol-treated fibers many profiles in this category had either an interrupted tubule or one with a considerably reduced lumen.

Third, there were no triads (*C*). In control, as well as in treated, muscles the interfibrillar spaces at the *Z* line level were often occupied by glycogen granules, undifferentiated sarcoplasm, and sarcoplasmic reticulum elements which ran longitudinally without forming a triad (Figs. 1 and 2). All these spaces were classified in this group. Interfibrillar spaces occupied by mitochondria and those in areas where the cross-striation went out of register were not included in the counting.

Fourth, there were triads lacking 'transverse' tubules (*D*). Profiles of this sort were almost exclusively found in glycerol-treated fibers (Figs. 1*A* and 5). They consisted of either two or one regularly oriented lateral sacs of the triad accompanied by an empty space in the area usually occupied by a 'transverse' tubule. The junctional feet of the sarcoplasmic reticulum were often still clearly visible in these triads. Triads and dyads with a largely swollen tubule were also counted in this category (Fig. 4), but these were few in the pictures examined, because as mentioned above, areas filled with them were avoided.

In Tables 1 to 4 all numbers are expressed as percentages of the total number of sites counted.

Control muscles served the purpose of checking on the quality of preservation achieved, particularly with regard to the fibers deep in the muscle, and for obtaining an estimate of the number of sites at which a 'transverse' tubule was present in untreated muscles (Table 1). Two such control muscles (# 1 and # 2 in Table 1) were fixed and embedded concomitantly with the glycerol-treated muscles. The morphology of deep and superficial fibers in these muscles is compared in Table 1 with that of randomly sectioned fibers from another normal muscle fixed by the same method at an earlier date (muscle # 3). No significant differences exist between the groups of fibers, indicating that fixation was reliable. The majority of profiles fall into the complete triad category. However, approximately 20% of the sites do not have 'transverse' tubules and triads in them indicating that the 'transverse' tubular network is far from complete at each *Z* line level. The total percentage of profiles in categories *A* and *B* (about 81%) is very close to the "fraction of sites at which tubules" were found in a similar study by Eisenberg and Eisenberg (1968).



Figs. 3–5. Examples of variable degrees of 'transverse' tubule damage. In Fig. 3, at left, 'transverse' tubular system is present, but interrupted. In Fig. 4 at left, the triad is disarranged and two 'transverse' tubule segments are visible. At right the tubule is vacuolated. In Fig. 5 the tubules are totally absent. × 40,000

When glycerol-treated muscles are considered (Table 2), the most obvious differences are in categories *A*, *C* and *D*. The percentages of complete triads decreased by about two-thirds. Approximately half of these triads can be

Table 1. Triad statistics of control muscles[a]

Muscle ref.	No. superficial fibers	No. deep fibers	No. random fibers	Complete triads (%) (*A*)	Possible complete triads (%) (*B*)	No triads (%) (*C*)	Triads lacking T System (%) (*D*)	Total sites counted
1 (417)[b]	3			71	7	21	1	276
		3		76	6	19	0	302
2 (431)[b]	3			69	3	27	0	209
		2		81	1	18	0	131
3 (620)			4	79	3	17	0	365
Mean ± SEM (%)				77.2 ± 8.3	3.6 ± 3.5	19.8 ± 6.6	0.3 ± 1.0	

[a] The meaning of the categories *A* to *D* is explained in the text.
[b] Muscles 1 and 2 were fixed concomitantly with muscles 1 and 2 of Table 2.

Table 2. Triad statistics of glycerol-treated muscle

Muscle ref.	No. superficial fibers	No. deep fibers	Complete triads (%) (*A*)	Possible complete triads (%) (*B*)	No triads (%) (*C*)	Triads lacking T System (%) (*D*)	Total sites counted	Time in glycerol (min)	Time in Ringer's (min)
1 (418)	4		26	9	42	23	438	75	75[b]
		4	19	8	50	23	430		
2 (430)	4		23	4	48	25	272	70	150
		4	22	4	44	29	249		
3 (415)[a]	7		34	11	13	28	705	90	390
		6	36	9	32	23	768		
4 (416)	8		24	8	35	32	892	90	390[b]
		7	19	9	35	37	999		
Mean ± SEM (%)	23		27.3 ± 10.2	8.2 ± 4.0	35.7 ± 10.0	26.0 ± 10.6			
		21	24.8 ± 11.5	7.9 ± 4.0	39.7 ± 11.0	27.4 ± 11.3			

[a] One fiber in this muscle had 75% profiles in category *C*. We considered the glycerol treatment to have failed in this fiber and thus did not include it in the statistics.
[b] 75 to 90 min were in high calcium, high magnesium Ringer's.

traced to category *D*; that is, in many instances, the 'transverse' tubules were no longer present, but the lateral sacs of the triad preserved their original structural relationship to the fibrils and to each other (Fig. 5).

Interestingly, even when muscles were stored for a long period following glycerol treatment (e.g., muscles 3 and 4 in Table 2) the number of profiles in category *D* was unchanged. A small number of triads moved from category *A* to *B*. These are places at which the tubular membrane may be partially destroyed, but is sufficiently visible to form a weak 'transverse' tubular profile (Fig. 3, right). Slightly less than one-third of the original triads are found in category *C* after glycerol treatment. For these it must be assumed that once the tubule was absent from the center of the triad, the sarcoplasmic reticulum changed its location relative to the fibrils. It is not clear why only half of the damaged triads behaved in this way.

About one-third of the original triads still had a clear 'transverse' tubular profile following glycerol treatment. These profiles, however, differ in significant details from those in the control muscles. In sections tangent to the triads, the tubules are fractionated into short segments (Figs. 3 and 4). In sections which cut across the triad, the cross-sectional shape and area of the tubules vary considerably; from small round tubules, such as those that osmium tetroxide fixation would produce in normal muscle, to enlarged profiles which in normal fibers were found only at the surface. These enlarged profiles were probably intermediate between a normal 'transverse' tubular system and the very large vacuoles which were scattered throughout glycerol-treated fibers. Hence, although one-third of the original tubular profiles preserved their original disposition in the triad, the continuity of the tubular network was probably largely interrupted.

The fate of the missing tubular membrane must remain a mystery, since identification of tubular profiles away from the triad is almost impossible without the use of tracers and extracellular tracers did not penetrate into the majority of the remaining 'transverse' tubular profiles in glycerol-treated fibers (vide infra). Part at least of the tubular membrane lined the large vacuoles, which in this as in previous studies (Eisenberg & Eisenberg, 1968; Howell, 1969; Krolenko, 1969) can be identified as 'transverse' tubular elements owing to the preservation of their specific junctions with the sarcoplasmic reticulum membranes. The number of vacuoles in individual fibers was highly variable. Some of them seemed to be in the process of forming labyrinthine structures, not unlike those formed by the 'transverse' tubular system during muscle differentiation (Fig. 6; *see* Krolenko, 1969).

Below it will be seen that 'transverse' tubules in the surface layers of glycerol-treated fibers are more penetrated by lanthanum than those in deeper layers. Therefore, the morphology of the interfibrillar sites near the surface was specifically examined. Table 3 presents the data and it is clear

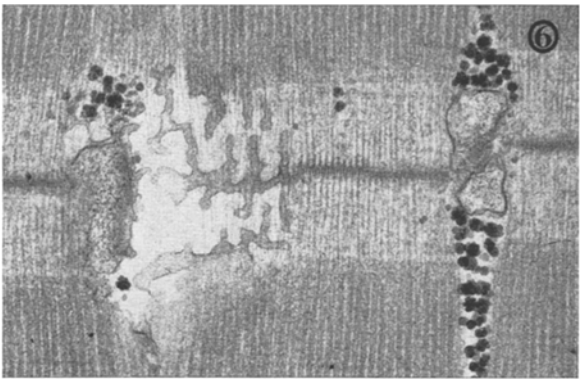


Fig. 6. This figure illustrates the convoluted surface of one of the vacuoles formed by swelling of a 'transverse' tubule. Images like this are fairly frequent. × 40,000

Table 3. Triad statistics near the surface in glycerol-treated muscles

Classification	Depth of sites in terms of the number of myofibrils below the plasma membrane						Mean ± SEM
	Surface	1	2	3	4	5	
A, Complete triad	7	38	36	36	36	25	
	30%	33%	31%	32%	30%	24%	30.0 ± 1.3
B, Possible complete triad	3	9	9	14	15	5	
	13%	8%	8%	12%	12%	5%	9.7 ± 1.3
C, No triad	5	30	47	38	48	45	
	22%	26%	40%	33%	40%	42%	33.8 ± 3.4
D, Triads with no T-tubules	8	39	25	26	22	31	
	35%	34%	21%	23%	18%	29%	26.7 ± 2.9
Total no. of sites counted	23	116	117	114	121	106	

that the distribution between the categories was the same as that found in the more centrally located sites (*see* Table 2).

Deep and superficial fibers did not differ in their general appearance and in the number of interfibrillar spaces which belong to the different categories, nor did the four muscles examined differ significantly from one another. Since two of these had been in normal Ringer's fluid for an extended period of time prior to fixation, it must be concluded that for the experimental conditions described there was no recovery of tubular continuity following the initial damage.

By way of contrast, the sarcoplasmic reticulum did not suffer any appreciable damage from glycerol treatment. Continuity of the lateral sacs

Table 4. 'Transverse' tubular profiles containing lanthanum

Muscle ref.	No. superficial fibers	No. deep fibers	No. random fibers	Filled T profiles inside fibers (%)	Total T profiles counted
1	2			95	108
(Control)		2		97	85
2 (Control)			9	96	428
1 [a] (Glycerol-treated)			7	9	67
2 (Glycerol-treated)			8	9	93

[a] One fiber in this muscle had the majority of T profiles filled with lanthanum. Presumably detubulation was not complete. This fiber was not included in the Table.

Table 5. 'Transverse' tubular profiles containing lanthanum near surface

	Depth of site in terms of number of myofibrils below surface membrane									
	Surface	1	2	3	4	5	6	7	8	9–10
A. Glycerol-treated muscles										
No. filled profiles	7	27	35	27	17	14	11	6	2	3
No. empty profiles	4	26	31	39	44	29	24	18	16	27
Fraction of filled profiles	0.64	0.51	0.53	0.41	0.28	0.33	0.31	0.25	0.11	0.11
B. Control muscles										
No. filled profiles	13	47	43	37	34	26	21	16	6	16
No. empty profiles	0	5	1	2	4	5	2	4	3	2
Fraction of filled profiles	1.00	0.90	0.98	0.95	0.89	0.84	0.91	0.80	0.67	0.89

of the triad with the longitudinal tubules and of these with the fenestrated collar was as complete as in control fibers (Figs. 3 to 5).

In many experiments involving glycerol-treated fibers the crucial question is not how much 'transverse' tubular membrane is still, at least apparently, intact, but to what extent are the remaining tubules connected to the external

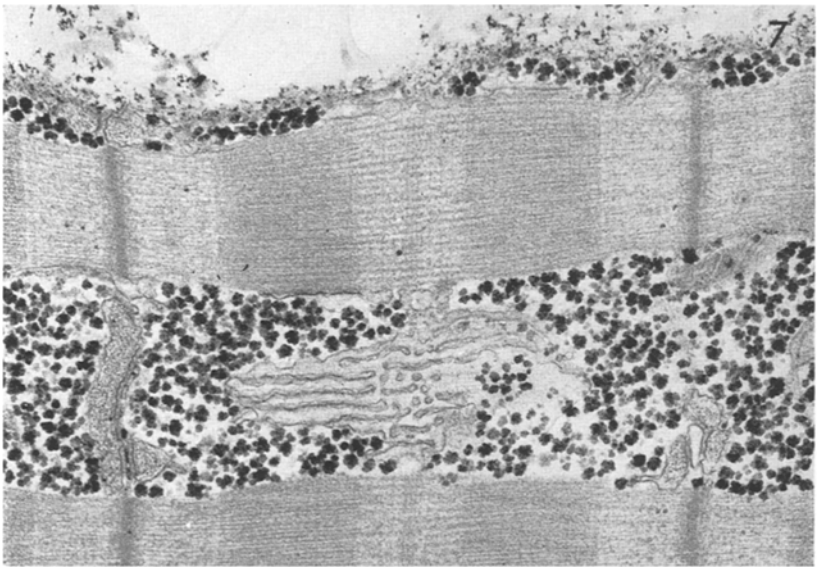


Fig. 7. Longitudinal section near the surface of a glycerol-treated, lanthanum infiltrated fiber. Notice one filled and one empty 'transverse' tubule profile. At top, left there is a triad remnant without tubule. × 40,000

solution. We were able to overcome the difficulty intrinsic to most currently used extracellular tracers (i.e., the fact that they are effective only in the superficial layers of the tissue) by employing a slight modification of the lanthanum infiltration technique, as suggested by Mirolli and Clayton (1968). In the control muscles, all fibers at every depth were surrounded by a visible deposit of lanthanum and a high percentage of the visible 'transverse' tubular profiles contained the tracer (Table 4, top).

In glycerol-treated fibers, the percentage of tracer-filled tubules depends on their location in the fiber. Tubules near the surface (for a depth of about 7 μ) were more likely to be filled (Fig. 7) than those close to the center of the fiber. This is shown in Tables 4 and 5.

Discussion

One aim of this study was to determine whether all of the fibers in a muscle like the sartorius were equally affected by glycerol treatment. The data in Table 2 indicates that for the size of muscles used and for the protocol chosen there was no difference in the altered morphology produced by glycerol treatment between superficial and deep fibers.

The other major aim was to quantitate the number of 'transverse' tubules remaining undamaged and accessible to the extracellular fluid after

glycerol treatment. It is clear from the results that there was significant alteration in the morphology of the 'transverse' tubular system after glycerol treatment. When extracellular lanthanum is present, glycerol-treated fibers exhibit two regions of different accessibilities. From the data in Tables 4 and 5 it is apparent that in random sections the 'transverse' tubules within a depth of seven myofibrils are more accessible to lanthanum than are deeper tubules. The overall fraction of remaining tubules which are filled within a depth of seven myofibrils is 0.40 $\left[\frac{144}{359}\right.$, *see* Table 5$\left.\right]$. For deeper layers the fraction of remaining 'transverse' tubules which are filled falls to 0.09 (*see* Table 4). The mean breadth (not diameter) of the fibrils was estimated by measuring their apparent thickness in random sections. The result was 1.08 ± 0.02 (mean $\pm$ SEM; $n = 512$). Hence a depth of seven myofibrils in a section is equivalent to a distance of 7.56 μ. The fiber diameters in six sartorii were measured and the mean diameter was 55 ± 2 μ (mean $\pm$ SEM).

To determine the depth of the surface shell which is more accessible to lanthanum it is important to note that a random section will only occasionally pass through the center of a fiber and hence the average random section will be displaced from the center. Fig. 8 illustrates a method of determining the radius of the inner core of less accessible tubules, and hence the depth of the outer shell, on the assumption that fibers have a circular cross-section. The radius of the inner core is given by

$$R_i^2 = d^2 \sin^2\theta + (R_0 - d\cos\theta)^2$$

or

$$R_i^2 = R_0^2 + d^2 - 2R_0 d\cos\theta \tag{1a}$$

where R_i is the radius of the inner core, R_0 is the radius of the fiber, d is the depth of the outer shell in the average random section, and θ is the angle between the radius and the chord generated by the average random section. From Fig. 8 it can be seen that $\cos\theta = \langle C\rangle / 2R_0$ where $\langle C\rangle$ is the chord length generated by the average random section. Since $\langle C\rangle = \pi R_0/2$ (*see* Appendix), then $\cos\theta = (\pi/4)$ and substituting in Eq. (1a) it follows that

$$R_i^2 = R_0^2 + d^2 - (0.5)\pi d R_0. \tag{1b}$$

From Eq. (1b) the calculated R_i is 22.1 μ when $d = 7.56$ μ and $R_0 = 27.5$ μ.

To determine the fraction of the total potential 'transverse' tubular volume accessible to lanthanum in either normal F_{av}^n or glycerol-treated F_{av}^g muscles, the inner core and outer shell are considered independently. These

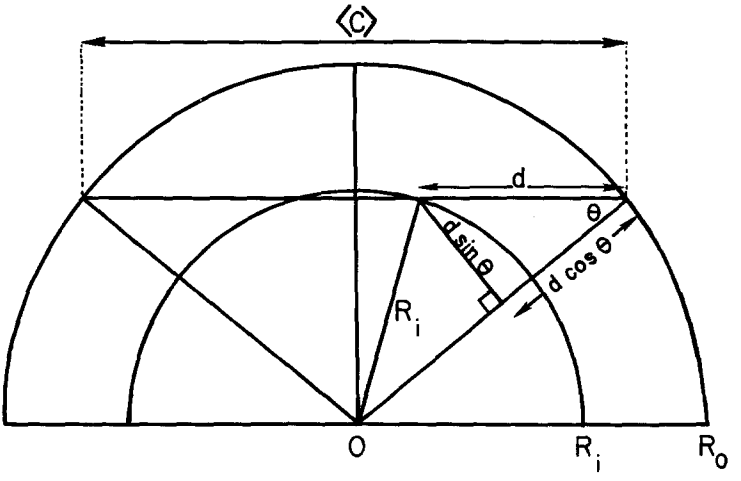


Fig. 8. Assumed geometry for the calculation of the inner core radius (R_i) from the measured depth (d) of the outer shell for the average random longitudinal section. $\langle C \rangle$ is the chord determined by the average random section. The perpendicular distance to center of average random chord is $X_{\langle C \rangle} = R_0 \left(1 - \frac{\pi^2}{16}\right)^{\frac{1}{2}}$

fractional volumes are given by

$$F_{av}^{n} = V_i \cdot S_i^n \cdot A_i^n + V_0 \cdot S_0^n \cdot A_0^n \tag{2a}$$

and

$$F_{av}^{g} = V_i \cdot S_i^g \cdot A_i^g + V_0 \cdot S_0^g \cdot A_0^g \tag{2b}$$

where V_i is the fraction of volume which is inner core

$$\left[= \left(\frac{R_i}{R_0}\right)^2 = \left(\frac{22.1}{27.5}\right)^2 = 0.65 \right];$$

V_0 is the fraction of volume which is outer shell

$$\left[= 1 - \left(\frac{R_i}{R_0}\right)^2 = 0.35 \right];$$

S_i^n is the fraction of inner core sites containing 'transverse' tubules in normal muscle [$A+B$, Table 1 = 0.81]; S_0^n is the fraction of outer shell sites containing 'transverse' tubules in normal muscle [$A+B$, Table 1 = 0.81]; S_i^g is as S_i^n except in glycerol-treated muscles [$A+B$, Table 2 = 0.36]; S_0^g is as S_0^n except in glycerol-treated muscles [$A+B$, Table 3 = 0.40]; A_i^n is the fraction of tubules present and accessible to lanthanum in the inner core in normal muscle [Table 4 = 0.96]; A_0^n is as A_i^n except in outer shell [Table 5 = 0.91]; A_i^g is as A_i^n except in glycerol-treated muscles [Table 4 = 0.09]; and A_0^g is as A_0^n except in glycerol-treated muscles [Table 5 = 0.40]. Substituting these values into Eq. (2a) and (2b), one gets $F_{av}^n = 0.76$ and $F_{av}^g = 0.08$. This indicates that, using lanthanum as the extracellular tracer, 10% of the

total original 'transverse' tubular system remained after glycerol treatment. In the outer shell 23% of the 'transverse' tubules remained accessible while in the inner core only 4% remained accessible after glycerol treatment.

Eisenberg and Eisenberg (1968) using peroxidase as the extracellular marker found that only 2% of the 'transverse' tubules remained accessible after glycerol treatment. When compared with the 10% found in this study using lanthanum, it seems possible that the openings of the remaining tubules are sufficiently 'restricted' to prevent entry of peroxidase. Since the distribution of peroxidase within the 'transverse' tubules of normal muscles found by Eisenberg and Eisenberg (1968) is the same as the distribution of lanthanum in the normal muscles of this study, this suggests that the 'transverse' tubules which remain after glycerol treatment have a greater than normal 'access' resistance (Peachey & Adrian, 1973). Recent experiments from two other laboratories also suggest that detectable amounts of 'transverse' tubules remain after glycerol treatment. In the experiments of Nakajima *et al.* (1973) repolarization is slow upon reduction of $[K]_0$ in glycerol-treated fibers after long-term equilibration in high $[K]_0$. This was taken to indicate that 'transverse' tubules are present after glycerol treatment and they have a high 'access' resistance. In impedance measurements on glycerol-treated fibers Valdiosera, Clausen, and Eisenberg[1] find that 10% of the 'transverse' tubules remain connected to the external solution through a much higher than normal resistance.

In summary, the morphometric data using lanthanum as an extracellular tracer indicates that glycerol treatment disconnects about 90% of the total 'transverse' tubules from the external fluid space. The remaining 10% of the 'transverse' tubules connected to the external fluid space are largely found in a surface layer of about one-fifth the fiber radius in depth. In this outer shell about one-fourth of the 'transverse' tubules remain connected to the external fluid. Within the deeper core, about one twenty-fifth of the tubules remain intact and connected to the external fluid space. The disconnected tubules in the interior of the fiber show considerable fractionation and some swelling. There was little alteration of the sarcoplasmic reticulum. The effect was the same in all fibers examined except for two out of a total of 79 in which tubules seemed to be largely intact (*see* Tables 2 and 4).

Appendix

The aim of this section is to evaluate the average length of randomly generated chords in muscle fibers or fibrils whose cross-section are taken

1 R. Valdiosera, C. Clausen, and R. S. Eisenberg, 1973. The impedence of frog skeletal muscle fibers in various solutions. (*In preparation.*)

to be circular. This assumption is not strictly true but is taken to provide a first order approximation. The other major assumption used is that the perpendicular distances from the center of the circle x of the random chords have a uniform distribution of probability in the interval 0 to R_0, where R_0 is the radius of the circular cross-section. The perpendicular distance of any chord must lie within this latter interval, which is thus the fundamental interval. The assumption of a uniform distribution of perpendicular distances signifies that the probabilities of finding chord distances within two mutually exclusive subintervals of the fundamental interval are equal if the lengths of the subintervals are equal. On this assumption, the probability distribution function for the perpendicular distances of the chords $P(x)$, i.e. the probability that a given distance will not exceed or will be less than x, is given by $P(x)=x/R_0$. Hence, the probability density function $p(x)$ is given by

$$p(x)=\frac{d}{dx}[P(x)]=\frac{1}{R_0}. \tag{A.1}$$

The mean value of the chord lengths is given by

$$\langle C\rangle=\int_0^{R_0} C(x)\cdot p(x)\cdot dx, \tag{A.2}$$

where $C(x)$ is the chord length at a perpendicular distance x from the center (*see* Cramér, 1946). From simple geometry it follows that

$$C(x)=2\sqrt{R_0^2-x^2}. \tag{A.3}$$

Substituting equations (A.1) and (A.3) into equation (A.2) one obtains

$$\langle C\rangle=\frac{2}{R_0}\int_0^{R_0}\sqrt{R_0^2-x^2}\cdot dx. \tag{A.4}$$

Since $\int_0^{R_0}\sqrt{R_0^2-x^2}\,dx=(\pi R_0^2)/4$, the desired solution is

$$\langle C\rangle=(\pi R_0)/2. \tag{A.5}$$

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