SURFACE FEATURES OF STRIATED MUSCLE
II. GUINEA-PIG SKELETAL MUSCLE

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SUMMARY

A study of the structure of the terminal part of the T-tubule system and the distribution of subsarcolemmal caveolae was undertaken in guinea-pig psoas muscle. The results were correlated with the array of cell surface features as revealed by frozen-etched, shadowed replicas of the cell membrane.

Freeze-etching revealed numerous small rounded objects overlying the Z- and I-bands and the A—I junction regions of the sarcomeres. These objects appeared as pits or excrescences, depending on whether the cell membrane was viewed from outside or inside the cell, and were interpreted as apertures in the membrane.

Conventional thin sections demonstrated the presence of numerous subsarcolemmal caveolae with a similar distribution to the rounded features seen in the replicas. Such sections also showed that the T-tubules, lying at the A—I junctions, seem to change direction when approaching the cell surface and may occasionally appear to branch in the subsarcolemmal region. The T-tubules often terminated in caveolae. Caveolae were sometimes seen in direct communication with the extracellular space. No simple direct communications of T-tubules with the cell surface were observed.

After treatment of the muscle with lanthanum during fixation, thin sections revealed apparently continuous dense deposits from the cell surface, through the caveolae to the T-tubule proper. It thus appears that each T-tubule communicates indirectly with the extracellular space via one or more subsarcolemmal caveolae.

INTRODUCTION

In mammalian skeletal muscle cells, communication between the transverse (T) tubules and the extracellular space has proved difficult to demonstrate directly by electron microscopy in spite of improved preservation of membranes with aldehyde fixatives, and the number of published examples of such communications is very small indeed. Walker & Schrodt (1965, 1966) have shown two examples from rat skeletal muscle; continuity of plasmalemma and T-tubule membrane is present in only one of these and the appearances in both cases are complicated by the 'convolutions' formed by the T-tubules in the sarcolemmal region. In the example from rat skeletal muscle shown by Côte, Sandborn & Bois (1966) a presumptive T-tubule opens to the exterior at about the M-band level, an unusual place for a T-tubule in this type of muscle where the T-tubules lie at the A—I junctions (Porter & Palade, 1957; Andersson-Cedergren, 1959). Shafiq, Gorycki, Goldstone & Milhorat (1966) were able to demonstrate continuity of T-tubule and plasma membrane in human...
skeletal muscle cells, but such instances were comparatively rare. By analogy with other types of skeletal muscle (see review by Smith, 1966) such as that of fish (Franzini-Armstrong & Porter, 1964) it can be inferred that also in mammalian skeletal muscle the T-tubules communicate with the extracellular space. Luft (1966), in fact, has indirectly demonstrated this, using ruthenium red as a marker in the same way as Huxley (1964) and Page (1964) had done previously with ferritin in amphibian and fish skeletal muscle.

Thus, communication between T-tubules and extracellular space in mammalian skeletal muscle is no longer in doubt, but the manner of termination of the T-tubules at the surface of the cell remains to be clarified. In the present study in addition to conventional electron-microscope techniques, two special techniques have been used to investigate this problem—staining with lanthanum nitrate (Doggenweiler & Frenk, 1965; Revel & Karnovsky, 1967) and freeze-etching (Moor & Mühlethaler, 1963).

MATERIALS AND METHODS

Small portions of psoas muscle were obtained from guinea-pigs anaesthetized with pentobarbitone (40 mg/kg) intraperitoneally.

The freeze-etch technique was the same as that used in the earlier study on cardiac muscle (Rayns, Simpson & Bertaud, 1968).

For conventional electron microscopy, Millonig’s buffer (Millonig, 1961) containing 2% glutaraldehyde and 2% formaldehyde (1460 m-osmoles, pH 7.4) was dripped on the muscle in situ for 3 min and small portions of tissue were then excised, fixed for 1 h in the same fixative, washed in buffer (342 m-osmoles), post-fixed with 2% osmium tetroxide in Millonig buffer (188 m-osmoles, pH 7.4) and embedded in Epon (Luft, 1961). Thin sections were counterstained with lead citrate (Reynolds, 1963).

The lanthanum staining was essentially as described by Revel & Karnovsky (1967), but the initial fixation was with 2% glutaraldehyde and 2% formaldehyde in cacodylate buffer (955 m-osmoles), dripped on the muscle in situ for 3 min. The tissue was then excised and fixed for 2 h in the same fixative, and washed in buffer (179 m-osmoles) for 20 min. Post-fixation was with 1% osmium tetroxide in colidine buffer containing also 1% lanthanum nitrate (203 m-osmoles) for 2 h. The colidine buffer was purified by the method of Bennet & Luft (1939). Osmolalities were measured on a Fiske osmometer.

The replicas were viewed in a Philips EM 200 electron microscope and the sections in a Hitachi HU 11 A.

RESULTS

Freeze-etch studies. These were successful in demonstrating the cell membranes from both external (Figs. 1, 2) and internal (Figs. 3–5) aspects, as in the previous study on guinea-pig myocardial cells (Rayns, Simpson & Bertaud, 1967, 1968). To identify the aspect from which a membrane is being viewed, the direction of shadowing must first be determined. This is deduced from the negative shadows cast by small raised objects such as collagen fibres or myosin filaments.
When the direction of shadowing has been determined, it is possible to interpret the general topography of membrane surfaces. For example, the membranes seen in Figs. 1 and 2 are convex towards the viewer, while those seen in Figs. 3–5 are concave. As mammalian skeletal muscles cells are approximately cylindrical in shape, it follows that replicas showing cell membranes with a convex form are most probably exhibiting the membranes from the external aspect. Conversely cell membranes with a concave form are membranes seen almost certainly from the internal aspect.

It is also possible, when the direction of shadowing is known, to elucidate the numerous features associated with the cell membranes. Depressions or pits in a membrane surface are seen as rounded objects with a pale shadow on the side nearest to the source of shadow-casting and a dark shadow on the side farthest from the source (Figs. 1, 2). The heavy shadowing metal, in fact, has failed to fall on the nearer side of the pit and has piled up against the farther side. Conversely, excrescences on a membrane are seen as rounded objects with a dark shadow on the side nearest to the source of shadow-casting and a pale shadow on the side farthest from the source (Figs. 3–5). In other words, the heavy metal has piled up against the nearer side of the small excrescence and has not fallen on the farther side.

The membrane replicas fell into two classes: those with a predominance of pits (Figs. 1, 2) and those with a predominance of small excrescences (Figs. 3–5). It was found that the pits were associated with the convex or outer surface of the cell membranes and that the excrescences were associated with the concave or inner surface of the cell membranes. The concave surfaces also carried some small crater-like structures. No structures were seen which could be readily identified as T-tubule apertures.

The distribution of the pits and excrescences varied considerably in different areas of membrane. An impression of the myofibrillar cross-banding, i.e. the A- and I-bands, could often be seen on the membrane (e.g. Fig. 5, also Figs. 1, 3, 4) and this permitted an orientation of the membrane characteristics in relation to the myofibrils. In some instances (e.g. Figs. 1, 3, parts of 4, 5), the pits in the membrane were clearly distributed in relation to the sarcomere pattern, with very few pits in the membrane overlying the A regions, with many pits in the region of the A-I junctions, and with a moderate number of pits in the I and Z regions. However, in other instances (e.g. Fig. 2, parts of Figs. 4, 5) the pits appeared to be randomly distributed; this seemed to be the case especially in places where the impression of the sarcomere pattern on the membrane was absent. It is possible that these parts of the membrane had been apposed not to myofibrils but to other cellular contents such as the nucleus or mitochondrial masses.

In summary, therefore, the freeze-etch studies have shown numerous apertures in the sarcolemma, mainly over the regions of the A–I junctions, the I-bands and the Z-bands. No orderly array of T-tubule apertures was seen.

**Sectioned material, conventional staining.** Attention was focused particularly on the surface of the muscle cells. The sarcolemma had the usual appearance of a plasma membrane and basement membrane. In some places it lay directly against the outermost myofibril but it was more usual to find caveolae, glycogen granules and elements
of the sarcotubular system between the sarcolemma and the outermost myofibril (Fig. 8). The caveolae varied in diameter but were usually from about 300 Å to 1500 Å, and were usually electron-translucent, like the T-tubules. Examples were seen of communication between the caveolae and the T-tubules (Figs. 8, 10, 12); other caveolae appeared to communicate with the extracellular space (Figs. 8, 10, 11). The structures interpreted as belonging to the sarcotubular system (Figs. 9, 11) were less electron-translucent, presumably because of their narrower lumina. Localized masses of mitochondria were also quite commonly seen under the sarcolemma (Figs. 7, 13), the mass being usually 0.5-1 μ thick and several sarcomeres long. Subsarcolemmal caveolae were present also over such mitochondrial masses, distributed in a random fashion, and T-tubules could sometimes be seen lying among the mitochondria.

In some places the caveolae appeared not to be distributed in any relation to the sarcomere pattern (Fig. 8). More usually, there was a tendency for the caveolae to be more numerous in the region of the Z-bands and I-bands than in the region of the A-bands (Figs. 9-12).

The T-tubules within the cell were seen, as expected, at the A-I junctions. At the edge of the cell, however, it was noted quite frequently that the T-tubules changed direction (Figs. 9, 11) to run parallel to the sarcolemma for a short distance; in effect, they joined up with caveolae. In other instances, instead of approaching the sarcolemma at right angles, the T-tubule lay obliquely, approaching the sarcolemma more at the Z level. No instances of T-tubules communicating directly in a simple fashion with the extracellular space were noted. The T-tubules appeared to be somewhat distended near the surface of the cell (Figs. 10, 12), and the subsarcolemmal caveolae were rounded and possibly dilated—at least in comparison with their appearances after lanthanum staining (see below).

In summary, these conventional electron micrographs have shown T-tubules lying at the A-I junctions but approaching the sarcolemma in an irregular fashion. The T-tubules appear usually to terminate in subsarcolemmal caveolae which in turn communicate with the extracellular space. The caveolae tend to be most common over the I-bands and A-I junction regions; the presence of mitochondria in the subsarcolemmal region modifies this arrangement.

**Sectioned material, lanthanum staining.** The lanthanum was evident as a dense deposit on the sarcolemma, in the subsarcolemmal caveolae and in the T-tubules (Figs. 14-20).

On the sarcolemma the layer of electron-dense material was of variable thickness, up to about 1000 Å, with an irregular ‘shaggy’ surface sometimes incorporating collagen fibres. The surfaces of collagen fibres were densely stained (Figs. 16, 20).

The subsarcolemmal caveolae were well demonstrated by the technique. At places where myofibrils lay directly under the sarcolemma, the caveolae tended to be much more numerous at the I levels than at the A levels (Figs. 14, 17) but this was not invariable. They were of irregular shape, 250-1400 Å in diameter, often partly flattened and lying with their long axes parallel to the sarcolemma. Neighbouring caveolae were sometimes interconnected (Figs. 14, 17, 18) and connexions between
Skeletal-muscle surface features

caveolae and the lanthanum-stained material outside the cell were sometimes seen (Fig. 14). Some vesicular and tubular structures lying under the sarcolemma were not stained with lanthanum and were tentatively classed as belonging to the sarcotubular system (Figs. 14, 17, 18).

The T-tubules were usually well outlined by the lanthanum near the surface of the cell (Figs. 14–19) but deeper penetration of the marker into the T-tubule system was patchy. Occasional T-tubules at the surface had not taken up the lanthanum (Figs. 14, 16). No direct communication between a T-tubule and the extracellular space was seen with this technique and at the cell surface the T-tubules usually seemed to terminate in subsarcolemmal caveolae (Figs. 15–18).

In Fig. 15 a T-tubule appears to bifurcate in the subsarcolemmal region. Further evidence of terminal branching of the T-tubule may be present in Fig. 20, which shows a partly tangential section of a cell, thus demonstrating en face the structures lying just under the sarcolemma. There are clearly a number of caveolar structures which have been penetrated by the lanthanum; two of them have a distinct 'cloverleaf' shape and could represent the ends of T-tubules each branching into three parts.

The lanthanum deposit did not always fill the T-tubule lumen completely (Figs. 17, 19), the centre of the lumen being more or less free of deposit. The adjacent scalloped membrane of the sarcotubular system lying up against the T-tubule could be clearly seen only after lead staining and the lanthanum did not appear to have penetrated beyond the confines of the T-system.

In summary, the lanthanum staining has outlined the T-tubules and the subsarcolemmal caveolae and has confirmed that the T-tubules usually terminate in caveolae which in turn communicate with the extracellular space. There is a possibility that the T-tubules branch in the subsarcolemmal region and thus each communicates with more than one caveola.

DISCUSSION

The combined use of various electron-microscopical techniques in the present study has helped to clarify the relationship of the T-tubules to the cell surface in one type of mammalian skeletal muscle.

The results obtained with conventional staining techniques have re-emphasized the difficulty of finding T-tubules opening directly to the exterior of the cell. They have demonstrated how the T-tubules often change direction a little at the periphery of the cell and do not necessarily approach the sarcolemma perpendicularly at the A–I junction. The many caveolae present under the sarcolemma, particularly at the regions of the I-bands and A–I junctions, have also been demonstrated.

The lanthanum staining has indicated the structures which are in communication with the extracellular space. It has confirmed that the T-tubules seldom if ever open directly to the exterior at the A–I junction but either terminate in a subsarcolemmal caveola or—and this may be essentially the same thing—run parallel to the sarcolemma for a short distance before opening to the exterior. The entry of lanthanum into the subsarcolemmal caveolae indicates that they communicate with the extra-
cellular space and serves to differentiate them from other tubular or vesicular structures under the sarcolemma, which probably belong to the sarcotubular system. Failure of the lanthanum to penetrate into a vesicular structure does not, however, indicate with complete certainty that there is no communication between the structure and the extracellular space.

The freeze-etch replicas of the cell membrane have shown that there is no regular array of T-tubule apertures in this mammalian skeletal muscle. The replicas have demonstrated instead a profusion of pits on the outer surface of the membrane and a profusion of excrescences on the inner surface of the membrane. These membrane features, which are much smaller than the pits and stumps representing T-tubule apertures in cardiac muscle (Rayns et al. 1967, 1968), are distributed mainly at the levels of the I-bands and A-I junctions. They are not readily classifiable into T-tubule and other apertures on the basis of position, shape or size; yet it is clear that a proportion of them at least must be apertures leading into the T-system. It seems reasonable to assume that in fact all the pits in the external surface of the cell membranes are apertures of one sort or another. In view of the frequent occurrence of caveolae in the subsarcolemmal region (as seen in sectioned material), and in view of the fact that the caveolae are in communication with the extracellular space, it seems logical to conclude that those pits which are not apertures of T-tubules must represent apertures of caveolae.

The crater-like structures seen on the inner surface of the cell membrane can be interpreted as the remains of caveolae fractured very close to the membrane. The excrescences are interpreted as the remains of caveolae fractured less close to the membrane.

The number of apertures seen in the cell membrane in frozen-etched material is clearly much larger than the number of T-tubules approaching the surface of the cell. The number of subsarcolemmal caveolae seen in the sectioned material is also much larger than the number of T-tubules, but this could possibly be explained by the caveolae being tortuous channels appearing more than once in a given section or being large enough to appear in many sections. The questions seem to be, therefore, whether a caveola can have more than one opening to the exterior, whether a T-tubule communicates with more than one caveola, and whether caveolae exist which do not communicate with a T-tubule. Possible evidence that a T-tubule may branch near the sarcolemma and thus communicate with two or more caveolae is shown in Fig. 15, and in Fig. 20. In Fig. 15 a terminal bifurcation of the T-tubule is fairly definite; Fig. 20 is more difficult to interpret but it seems to give an en face view of the subsarcolemmal structures and it is tempting to interpret the two ‘clover-leaf’ structures as the ends of transverse tubules dividing into three branches. Each of these branches might form, or open into, a caveola. A comparable tangential section of a developing chick skeletal muscle cell (but without lanthanum) has recently been published (Ezerman & Ishikawa, 1967); this shows both simple, round T-tubule apertures and also a rosette-like T-tubule aperture which is a little reminiscent of the ‘clover-leaf’ structures described above.

If in fact it is true that each T-tubule splits to form 2-4 caveolae, then the number
of apertures seen in freeze-etched material should be approximately 3 times the expected number of T-tubule apertures. There appear in fact to be many more apertures than that. A possible explanation for this could be that some of the apertures belong to pinocytotic vesicles, as in mammalian myocardial cells (Rayns et al. 1968). However, while pinocytotic vesicles are readily found in sections of mammalian myocardial cells (Simpson, 1965) and amphibian skeletal muscle cells (Page, 1965), no structures resembling pinocytotic vesicles were seen in the sectioned material in the present study. The caveolae in this material are much more variable in size and far less regular in shape than pinocytotic vesicles.

It appears, therefore, that the past difficulties in demonstrating continuity between T-tubule membranes and plasmalemma in mammalian skeletal muscle cells have not been due solely to problems in fixation of membranes but have in large part been due to the complicated structural arrangements in the subsarcolemmal region. A rather similar arrangement of subsarcolemmal caveolae and convoluted terminations of T-tubules is present also in amphibian skeletal muscle cells (Franzini-Armstrong, 1964; Peachey, 1965); the number of caveolae and the tortuosity of the T-tubules seem in fact to be even greater in amphibia.

The caveolae have not received a great deal of attention in the past but it is evident that they represent a very considerable area of membrane and that they should also be taken into account when calculations are made of the space within cells which is accessible to the extracellular fluid. The volume of the caveolae in fixed material, however, may depend in some way on the fixation, as they appear much less distended in the lanthanum-stained preparations (Figs. 14-18) than in the conventionally stained ones (Figs. 8-13). The reason for this difference is not clear but it may lie in the types of buffer used or in the differing osmolalities of the fixatives.

The functional significance of the caveolae and of the rather complicated mode of communication between the T-tubules and the extracellular space must at present be a matter for speculation. The most immediately obvious point is that the tortuous and indirect nature of the communication with the external environment of the cell must reduce the rate of diffusion of solutes into and out of the T-tubules. In fact, the failure of lanthanum to penetrate occasional T-tubules (e.g. Figs. 14, 16) could perhaps be taken as evidence of this. However, it seems more likely that the structural arrangements have some electrophysiological significance related to the spread of activation into the T-tubule system.

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(Facing p. 482)
**PLATES**

Figs. 1–5 show replicas of frozen-etched skeletal muscle cells. The double-headed arrows indicate the direction of shadowing.

Figs. 6–11 are electron micrographs of aldehyde-fixed tissue, post-fixed with osmium, Epon embedded, sectioned and counterstained with lead citrate.

Figs. 14–20 are electron micrographs of aldehyde-fixed tissue, post-fixed with osmium and lanthanum, Epon-embedded, sectioned and counterstained with lead citrate.

**ABBREVIATIONS ON PLATES**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tr>
<td>a</td>
<td>aperture</td>
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<tr>
<td>c</td>
<td>caveola</td>
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<tr>
<td>cl</td>
<td>'clover-leaf' structure</td>
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<td>e</td>
<td>extracellular space</td>
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<td>f</td>
<td>myosin filaments</td>
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<td>in</td>
<td>invagination</td>
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<td>n</td>
<td>nucleus</td>
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<td>pi</td>
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<td>po</td>
<td>outer surface of plasmalemma</td>
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<td>sr</td>
<td>sarcotubular system</td>
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<td>t</td>
<td>T-tubule</td>
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Fig. 1. Outer surface of a skeletal muscle cell (po). The surface is convex towards the viewer. The long axis of the cell and the sarcomere pattern are shown by the line, the crossbars of which indicate the approximate positions of the Z-lines. The small irregularities (a) in the surface can be identified as pits (see text) and these represent the apertures of caveolae or T-tubules. They are distributed mainly in the I-bands and over the A–I junctions. At the lower left corner, part of the obliquely fractured interior of another cell is seen. \( \times 11750 \).

Fig. 2. Outer surface of another skeletal muscle cell (po), showing a much less regular distribution of apertures (a). There seems to be no certain way of differentiating types of aperture. \( \times 14800 \).

Fig. 3. Inner surface of plasmalemma (pi) of a skeletal muscle cell. The surface is concave towards the viewer. The long axis of the cell and the sarcomere pattern are shown by the line, the crossbars of which indicate the approximate positions of the Z-lines. The small irregularities in the surface (in) can be identified as excrescences rising up from the plasmalemma, i.e. towards the interior of the cell; they represent the small invaginations of the plasmalemma which form the apertures of caveolae. In this example, these invaginations are confined fairly strictly to the I-band and A–I junction regions. Part of the interior of another cell, fractured semi-longitudinally, is seen at the upper edge of the figure. \( \times 6800 \).

Fig. 4. Inner surface of plasmalemma of another cell. The sarcomere pattern is well seen at the lower part of the figure, and here the distribution of invaginations is again mainly in the I-band regions. At the upper part of the figure the sarcomere pattern is not well seen, and the distribution of invaginations is a random one. \( \times 11750 \).
Fig. 5. Low-power view of inner surface of plasmalemma (pi) again showing in one area (mid and left) the sarcomere pattern and the sarcomere-related distribution of invaginations. In another area (top right) the sarcomere pattern cannot be discerned and the distribution of invaginations is random. At lower right, part of the membrane has been removed in the fracturing process, and the outer surface (po) of another cell appears to have come into view. x 4800.

Fig. 6. Subsarcolemmal region of a contracted skeletal muscle cell, showing a myofibril lying immediately under the cell membrane. Subsarcolemmal caveolae (c) are present, mainly in the region of the I-band and A-I junctions. x 12,500.

Fig. 7. Subsarcolemmal region of another contracted cell, showing mitochondria (m) and a nucleus (n) lying in the subsarcolemmal region. The subsarcolemmal caveolae (c) here appear to be randomly distributed, as in upper right part of Fig. 5. x 12,500.
Figs. 8–13. Subsarcolemmal regions from longitudinal sections. × 40,500.

Fig. 8. subsarcolemmal caveolae (c), rather haphazardly distributed. Two caveolae communicate with the extracellular space (arrows); other examples of this are seen in Figs. 10, 11 and 13.

Fig. 9. A T-tubule appears to communicate with a subsarcolemmal caveola (c). Sarcotubular elements (sr) are seen here and in Fig. 11.

Fig. 10. Two T-tubules become widened and irregular as they approach the cell surface. One of them may be communicating directly with the exterior of the cell (arrow).

Fig. 11. The lower T-tubule (t₁) either changes direction to run parallel with the cell membrane for a short distance or else communicates with a caveola. The next T-tubule (t₂) shows evidence of a structure within its lumen.

Fig. 12. Caveolae distributed over A–I junctions and I-bands, as also in Figs. 9 and 11. The T-tubule at the centre may be communicating with the extracellular space but is also in communication with a caveola (c).

Fig. 13. Mitochondria (m) in subsarcolemmal region, thus complicating the distribution of caveolae and T-tubule terminations.
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Figs. 14–18. Subsarcolemmal regions from longitudinal sections. Dense deposits of lanthanum on cell surfaces, subsarcolemmal caveolae (c), and T-tubules (t).

Fig. 14. T-tubules running an irregular course near the cell surface and approaching the plasmalemma without making actual contact. One T-tubule (t₁) has not been penetrated by the lanthanum. At least one caveola (c₁) seems to communicate with the extracellular space. × 45000.

Fig. 15. A T-tubule (t) branches as it nears the plasmalemma. × 30000.

Fig. 16. One T-tubule (t) terminates in a caveola. Another (t₂) has not been penetrated by the lanthanum. Negatively stained collagen fibres can be seen outside the cell. × 30000.

Figs. 17, 18. Examples of T-tubules (t) changing direction as they near the cell surface. × 30000.
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Fig. 19. Longitudinal section demonstrating deeper penetration by lanthanum into T-tubules. The centre of the lumen of some T-tubules is free of lanthanum deposit. $\times 30000$.

Fig. 20. Semilongitudinal section which becomes tangential for a short distance, thus showing subsarcolemmal structures en face. The two 'clover-leaf' structures (cf) could be branching T-tubules. Collagen fibres can be seen outside. $\times 43500$. 