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T-TUBULE SWELLING IN HYPERTONIC SOLUTIONS: A FREEZE SUBSTITUTION STUDY

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SUMMARY

Striated muscles from *Rana pipiens* have been exposed for variable periods of time to Ringer solutions made hypertonic by addition of either sucrose or sodium chloride. The muscles have been rapid-frozen and then prepared for electron microscopy by either freeze-substitution, freeze-fracture or cryoultramicrotomy.

The only compartment greatly affected by hypertonicity is the transverse tubular system, which is visibly swollen. None of the elements of the sarcoplasmic reticulum increase in size.

INTRODUCTION

Striated muscle fibres contain two major membrane systems: the T-tubules that are invaginations of the surface membrane, and the sarcoplasmic reticulum (SR) that is a structure equivalent to the endoplasmic reticulum (ER) of other cell systems (Porter & Palade, 1957). The two membrane systems are in structural apposition at the site of the triadic junction (Franzini-Armstrong, 1970), where the T-tubules are separated from the terminal cisternae of the SR by a 10–12 nm gap that is traversed by electron dense 'feet'. The triadic junction is the site where the electrical signal of the T-tubule action potential (Costantin, 1970; Gonzalez-Serratos, 1971; Bastian & Nakajima, 1974) is thought to trigger the release of calcium from the SR, thus activating contraction. Therefore, the nature of the communication between the T-tubule and SR membrane system is of fundamental physiological interest.

Three major types of experimental evidence suggest that the SR is indeed not continuous with the extracellular fluid in the T-tubules: (1) measurements of surface capacitance are too small to be consistent with an electrical continuity of the SR membrane (Falk & Fatt, 1964; Costantin, 1975); (2) extracellular markers, such as ferritin, enter the T-tubules but not the SR (Page, 1964; Huxley, 1964); (3) electron probe analysis of cryosections of resting muscle shows that the sodium and chloride concentrations of the SR are not comparable to those in the extracellular fluid (Somlyo, Shuman & Somlyo, 1977*a*, *b*). Furthermore, while the openings of the

T-tubules have been visualized in thin sections (Smith, 1961; Franzini-Armstrong & Porter, 1964), communications of the SR with the extracellular space, either directly or indirectly through T-tubules or caveolae, have never been observed.

In contrast to the above, however, there are also three lines of evidence suggestive of direct communication of the SR with the extracellular space contained in the T-tubule. These are (1) the presence of kinetically defined compartments of sodium and chloride that show efflux rates compatible with a compartment communicating with the extracellular space and having the approximate volume of the SR (Conway, 1957; Harris, 1963; Keynes & Steinhardt, 1968; Rogus & Zierler, 1973; Makino & Page, 1975); (2) the osmotic behaviour of striated muscle in hypertonic solutions (Dydynska & Wilkie, 1963; Blinks, 1965); (3) the swelling of the SR in muscles chemically fixed with acrolein, following incubation in hypertonic solutions (Birks & Davey, 1969). This evidence, however, is subject to the uncertainties of the structural identification of the kinetically and osmotically identified compartment and to the objection that swelling of the SR, or of any other structure, in chemically fixed muscles may represent the effect of fixation and not be representative of the living state (Birks & Davey, 1972).

Chemical fixation of tissues can be avoided by the use of rapid freezing techniques (Eranko, 1954; Van Harreveld & Crowell, 1964; Christensen, 1971; Appleton, 1974; Heuser, Reese, & Landis, 1976; Somlyo *et al.* 1977*a*). In a previous study in one of our laboratories (Somlyo *et al.* 1977*a*), it was found that in freeze-dried cryosections of frog muscle incubated in hypertonic solutions frequent vacuoles were present, usually within the I bands, and containing high concentrations of extracellular solutes (sodium, chloride, sucrose or isethionate). However, the image detail in unstained or osmium vapour stained freeze-dried cryosections was insufficient to determine with certainty whether the T-tubule or the SR membrane system was swollen by the hypertonic solutions. Therefore, we decided to explore this question with the use of rapid-freezing followed by freeze-substitution (Feder & Sidman, 1958; VanHarreveld & Crowell, 1964; Van Harreveld *et al.* 1965; Heuser & Reese, 1976) in the hope that this technique would provide better image detail in stained sections and also preserve the living state more faithfully than would conventional chemical fixation. Furthermore, the latter assumption could be verified by comparing freeze-substituted sections with freeze-dried cryosections and with replicas of freeze-fracture material that was not subjected to any chemical fixation prior to freezing. These expectations have been fulfilled and we have identified regions of the T-tubule system as the predominant site of swelling in muscles bathed in hypertonic solutions.

METHODS

Frog (*Rana pipiens*) striated muscles (sartorius, pectoralis and extensor digitorum longus IV) were studied after incubation either in normal Ringer solution or in Ringer solution made hypertonic with sucrose (up to 2·5 × hypertonic) or with sodium chloride (up to 2·2 × hypertonic, for a total of 270 mM sodium chloride) for 15–30 min. The composition of the frog Ringer solution was sodium chloride 116 mM; potassium chloride 3 mM; calcium chloride 1·2 mM; sodium bicarbonate 2 mM, and pH 7·2 or sodium chloride 111 mM; potassium chloride 2 mM; calcium chloride 1·8 mM; Hepes 5 mM; Dextran 3 mM.

Tissues were rapidly frozen by one of two methods: with the first method (I), the muscles mounted on thin aluminium disks were applied to the surface of a highly polished copper block

cooled with liquid helium (Van Harreveld & Crowell, 1964; Heuser, *et al.* 1976); with the second method (II) muscles were mounted on a stainless-steel mesh holder and shot at 60 cm/sec into Freon 22 supercooled to -164 ± 2 °C (Somlyo *et al.* 1977*a*). Frozen muscles were treated in three ways: (1) *freeze-substitution*, in which the water in the frozen material was substituted with acetone containing 5–10 % osmium tetroxide at -80 °C for 3 days and then gradually warmed up; the muscles were embedded in either Epon, Spurr or Araldite; (2) *freeze-fracture*, in which the muscles were fractured, shadowed and replicated in a Balzer's unit; control muscles only were examined by this technique; (3) *cryoultramicrotomy*, which has been described in detail (Somlyo *et al.* 1977*a*). It should be emphasized that neither cryoprotectants nor fixatives were used to preserve the muscles.

RESULTS

The best rapidly frozen muscles yielded sections free of ice crystals to a depth of approximately 5–10 μm. At a greater depth, at the boundary of visible ice crystal formation, the first region of distortion was at the Z-lines and at the A-I junctions. Regions free of disruptive ice crystals were observed in freeze-fracture, in frozen-dried thin sections as well as in freeze-substituted material, indicating that the small size of ice crystals was not due to their being merely obliterated during the freeze-substitution process.

In control muscles the triads (Pl. 1, figs. 1 and 2) consisting of the central T-tubule and lateral sacs continuous with longitudinal tubules were readily identifiable and in a position identical to that following conventional fixation. When sectioned at right angle to its long axis, the profile of the T-tubule in muscles frozen by method II was oval, thus resembling its appearance in glutaraldehyde and acrolein fixed preparations; in muscles frozen by method I the T-tubules had a round cross-section. In muscles too vigorously blotted before freezing, occasionally dilated T-tubule areas were observed; these changes resembled, to a lesser degree, the changes observed in muscles incubated in hypertonic solutions. In muscles which were freeze-fractured, the shape of T-tubules was the same as in those freeze-substituted (compare figs. 1 and 2). The appearance of the SR differed slightly from that of conventionally fixed material; the SR occupied a smaller volume in freeze-substituted material and the lumen of the longitudinal sacs appeared often totally obliterated (Howell, 1974; Wallace & Sommer, 1975; J. Heuser, unpublished results). The small volume of the SR, compared to conventionally fixed materials, was also observed in freeze-fractures of frog muscle (Pl. 1, fig. 2) and in unfixed freeze-dried cryosections of toadfish striated muscle (Somlyo *et al.* 1977*b*).

The T-tubules in the hypertonically treated muscles were significantly different from controls. There was marked swelling and vacuolation of the T-tubule system but not of the SR in all muscles incubated in hypertonic solutions (Pls. 2, 3 and 4). The extent of swelling was very variable within the same field of view, and normal appearing T-tubule profiles frequently communicated with large vacuoles (Pls. 2 and 3). The vacuolated T-tubules often appeared as paired structures at the Z-line (Pl. 2, fig. 2, Pl. 4), thus having an identical appearance to that observed in cryosections (Pl. 3, fig. 1 and Somlyo, Shuman & Somlyo, 1977*a*). At higher magnifications, the continuity of the lumen of such paired vacuoles with the T-tubules was evident (Pl. 3, fig. 2 and Pl. 4). The lateral sacs of the terminal cisternae were frequently seen as narrow structures compressed by the grossly distended portions

of the T-tubule system (Pl. 2). The identification of the compressed lateral sacs was further verified by the presence of foot processes connecting them with the swollen T-tubules (Pl. 4) and through their continuity with the longitudinal SR. In the early stages of T-tubule swelling, the appearance of paired vacuolation at the T-tubules could be shown to be due to 'herniation' of the T-tubules to either side of the Z-line around lateral sacs of the SR (Pl. 3, fig. 2 and Pl. 4). Some vacuoles were also present in regions other than the Z-line and were not associated with elements of the SR; these are thought to represent swellings of the longitudinal oriented elements of the T-tubular system (Peachey, 1976). The possibility that the swelling of T-tubules is an artifact due to freeze-substitution can be excluded by the similar distribution and extent of the vacuoles in cryosections of similarly treated muscles (see Pl. 3, fig. 1; Somlyo *et al.* 1977*a*) and by the normal appearance of control muscles in freeze-fracture replicas (Pl. 1, fig. 2) as well as in freeze-substituted material. The possibility that the T-tubule swelling is due to the transfer of water from the fibre to the T-tubule lumen during freezing can also be excluded, since it does not occur in the more highly hydrated control muscles (Pl. 1).

DISCUSSION

Our findings show that the T-tubule system swells in muscles incubated in solutions made hypertonic by either sucrose or sodium chloride, but the SR does not. Interestingly, three independent groups of investigators (Huxley *et al.* 1963; Freygang, Rapoport & Peachey, 1967; Birks & Davey, 1972) succeeded in preserving the swollen shape of T-tubules following sucrose-hypertonic Ringer by using osmium tetroxide as a primary fixative. In this particular instance osmium seems to preserve structures more faithfully than other fixatives that produced artifactual swelling of the SR, yet were interpreted as showing faithful representation of the *in vivo* structure (Birks & Davey, 1969). It is not clear why the T-tubules do not appear swollen when the Ringer solution is made hypertonic by the addition of sodium chloride, and the primary fixative is osmium (Freygang *et al.* 1967). Our data indicate, on the contrary, that T-tubule swelling due to sodium chloride is quite prominent. In cardiac muscle, swelling of the T-tubules, as well as of the terminal cisternae, was present after hypertonic treatment and osmium (Page & Upshaw-Earley, 1977), while only T-tubule swelling was present after hypertonic aldehyde fixation (Sperelakis & Rubio, 1971).

Volume changes in whole muscle and in single fibres exposed to solutions of variable osmolarity have been interpreted to show that muscle fibres behave like perfect osmometers with an apparent solvent volume of 67 % of the volume in normal Ringer (Dydynska & Wilkie, 1963; Blinks, 1965). Of the remaining 33 %, 20 % was calculated to be occupied by solid. The possibility was considered that at least part of the remaining 13 % represented sucrose space within the outlines of the fibre (Blinks, 1965). The 13 % could be fully accounted for *in hypertonic solutions* if the T-system volume increased from less than 1 to 13 % in going from an isotonic solution to a solution of infinite tonicity. However, our findings do not account for the extrapolated 13 % volume in isotonic or hypotonic Ringer solution (Dydynska & Wilkie, 1963; Blinks, 1965). Therefore, to account for 13 % of fibre volume in normal solutions alternative explanations, such as the existence of a significant fraction of

bound water, or the effects of mechanical rigidity opposing osmotic forces (Blinks, 1965), appear more plausible. The fact that the filament lattice volume behaves as an osmometer in a manner similar to the whole fibre and has an osmotically inactive volume of 38 % (Rome, 1968) also supports the latter interpretation.

The swelling of the T-tubules in hypertonic solutions implies that the added solutes are entering the T-system and that the osmotic flow of water into the T-tubules is more rapid than its exit from the T-tubule mouths. The resistance to the efflux of water may also be related to the tortuosity of the T-system (Peachey, 1976). The T-tubule surface area per fibre cross-sectional area increases as the fibre volume decreases and this 'extra' membrane could be partially taken up by the swelling and is also free to bulge into regions of the intermediate reticulum.

Our results are confirmed by other recent evidence, indicating that previous apparent proofs of continuity between SR and T-tubules were fallacious. The compartment of extracellular composition and occupying approximately 13 % of muscle volume based on efflux rates (Conway, 1957; Harris, 1963; Keynes & Steinhardt, 1968; Rogus & Zierler, 1973; Makino & Page, 1975) can no longer be assigned to the SR. Recent studies of the Cl content of amphibian muscle are incompatible with sequestration of Cl in the SR (Macchia, Page & Polimena, 1978). Preliminary reports based on the analysis of efflux components, using either single fibres (Neville, 1978), or a tracer (horseradish peroxidase) whose location can be identified in the electron microscope (Rubin & Neville, 1978), also indicate that none of the compartments accessible to the extracellular space are identifiable as the SR. Two extracellular space tracers had been described to penetrate into the SR (presumably from the T-tubules) on the basis of electron microscopic evidence (Luft, 1971, for ruthenium red, RR; Rubio & Sperelakis, 1972, for horseradish peroxidase, HRP). In the case of RR, it was shown that this molecule slowly penetrates across the surface membrane (Luft, 1971). Once inside, the tracer is specifically taken up by the sarcoplasmic reticulum, as indicated by the fact that 'staining' of the SR occurs when RR is applied to a skinned fibre (C. Franzini-Armstrong, unpublished observations) and that very intense SR 'staining' results from prolonged exposure to RR (Howell, 1974). In the case of HRP it was found that SR gives a positive peroxidase reaction even in the absence of extrinsic HRP (Peachey *et al.* 1974; Forbes *et al.* 1977). Thus, no evidence, either direct or indirect, indicating continuity between the lumina of SR and T-tubules remains unchallenged to date.

We conclude that a long standing controversy has been resolved by the use of rapid freezing. Our findings that T-tubules and not the SR swell in hypertonic solution is consistent with the assumption that the T-tubules communicate with the extracellular space; they also provide further evidence that the SR system is isolated from the extracellular space in muscles in hypertonic solutions, as it is in normal resting muscle, where the elemental composition of the SR has been directly measured (Somlyo *et al.* 1977*a*, *b*).

Note added in proof.

Since the acceptance of this manuscript, the preservation of the swollen T-system and normal SR, in muscles incubated in hypertonic solutions, was achieved by fixation in similarly hypertonic solutions (O'Brien & Davey, 1978).

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EXPLANATION OF PLATES

The muscles illustrated in Pls. 1 and 2, fig. 1 were frozen by method I and the remaining figures by method II.

PLATE 1

Fig. 1. Longitudinal section of a control muscle, freeze-substituted. Sarcoplasmic reticulum (SR) and transverse tubules (T) are very similar to those seen following chemical fixation. There is no visible ice crystal damage in this image. × 25,000.

Fig. 2. Freeze-fracture of a rapid-frozen control muscle. Compare shape of SR and T-tubules with those in fig. 1. There is some minor ice crystal formation in the filament space. Particle distribution on protoplasmic and luminal leaflets of SR and T-tubules is the same as seen following chemical fixation. E, luminal face; P, protoplasmic face. × 41,000.

PLATE 2

Fig. 1. Swollen T-tubules in muscles incubated in hypertonic Ringer + 200 mM-sucrose (for 9 min) before freezing. Dilated vacuoles are at the I-band level. One vacuole (arrow) is continuous with the T-tubule; other T-tubules show various degrees of swelling. Freeze-substituted preparation. × 20,000.

Fig. 2. Vacuolation in muscle incubated in hypertonic solution (2·2 × normal sodium chloride Ringer for 30 min) before freezing. The vacuoles, some of them paired, are located predominantly in the I-band region. Freeze-substituted preparation. × 26,000.

The vacuoles, which arise from the T-tubule system, frequently compress the terminal cisternae and intermediate SR by ballooning into the adjoining glycogen regions (see Pl. 3, Fig. 2).

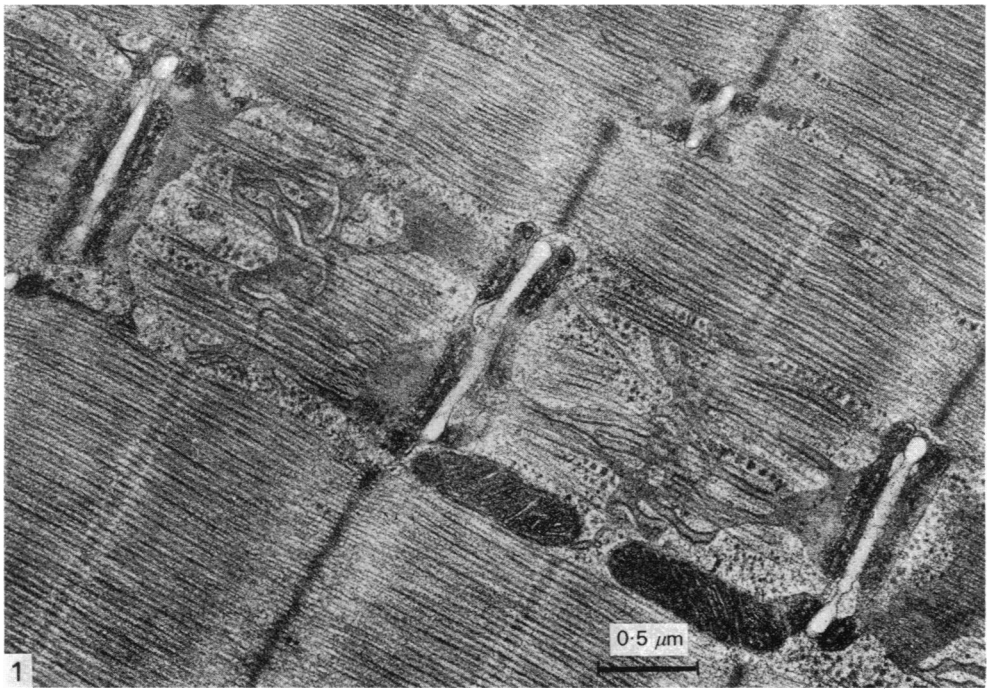
PLATE 3

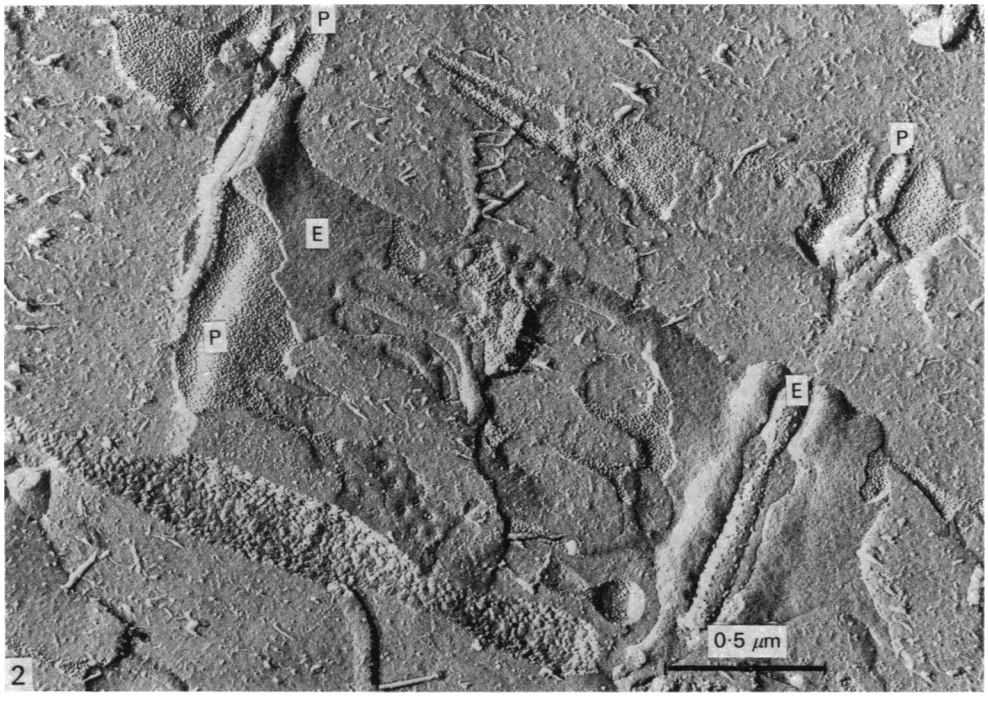
Fig. 1. Freeze-dried cryo section of muscle incubated in hypertonic solution (2·2 × normal sodium chloride for 30 min) before freezing. The distribution of vacuoles at the I-band, some of them paired, is similar to that observed in the freeze-substituted (Pl. 2, fig. 2 and Pl. 4) preparation. × 8,300.

Fig. 2. Oblique section of muscle incubated in hypertonic (1·8 × normal sodium chloride for 15 min) solution before freezing. A dilated T-tubule flanked by two terminal cisternae (arrows) is continuous with the vacuole. Freeze-substituted preparation. × 40,000.

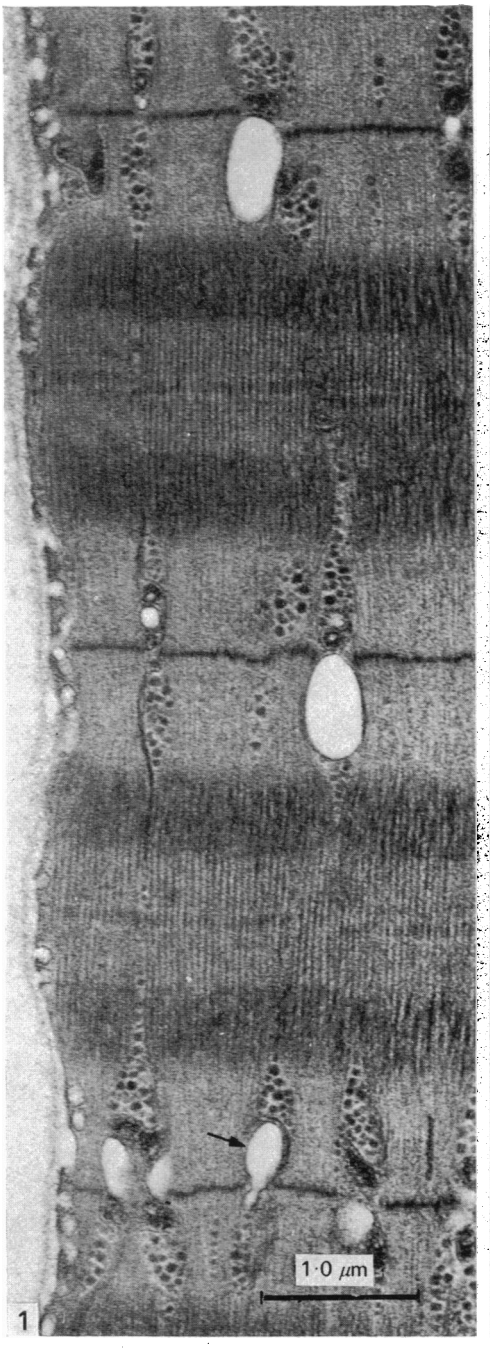
PLATE 4

Fig. 1. High magnification view of a longitudinal section of muscle incubated in hypertonic (2·2 × normal sodium chloride for 30 min) solution before freezing. Note the continuity of the T-tubule (arrow) with the dumbbell shaped membrane-bound vacuole adjacent to the terminal cisternae. There is some evidence of foot processes between the terminal cisterna (tc) and the dumbbell shaped vacuole. Freeze-substituted preparation. × 43,000.





C. FRANZINI-ARMSTRONG AND OTHERS (*Facing p.* 140)



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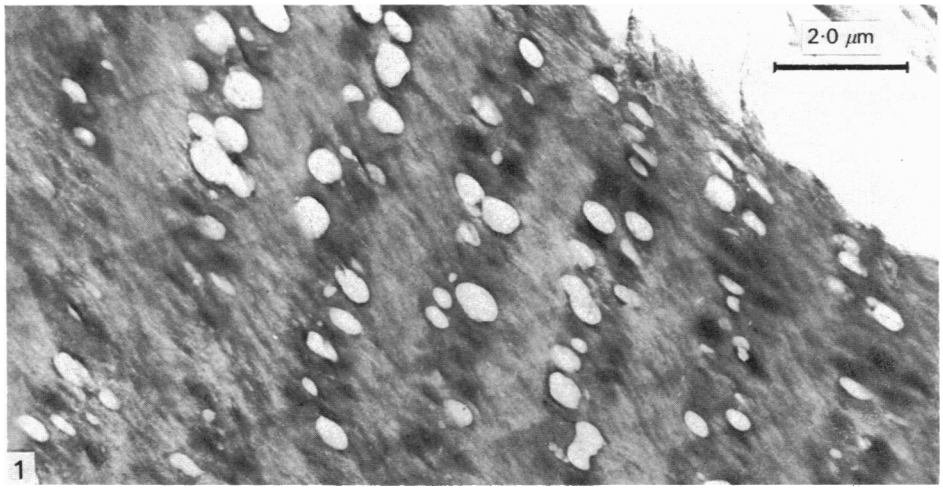

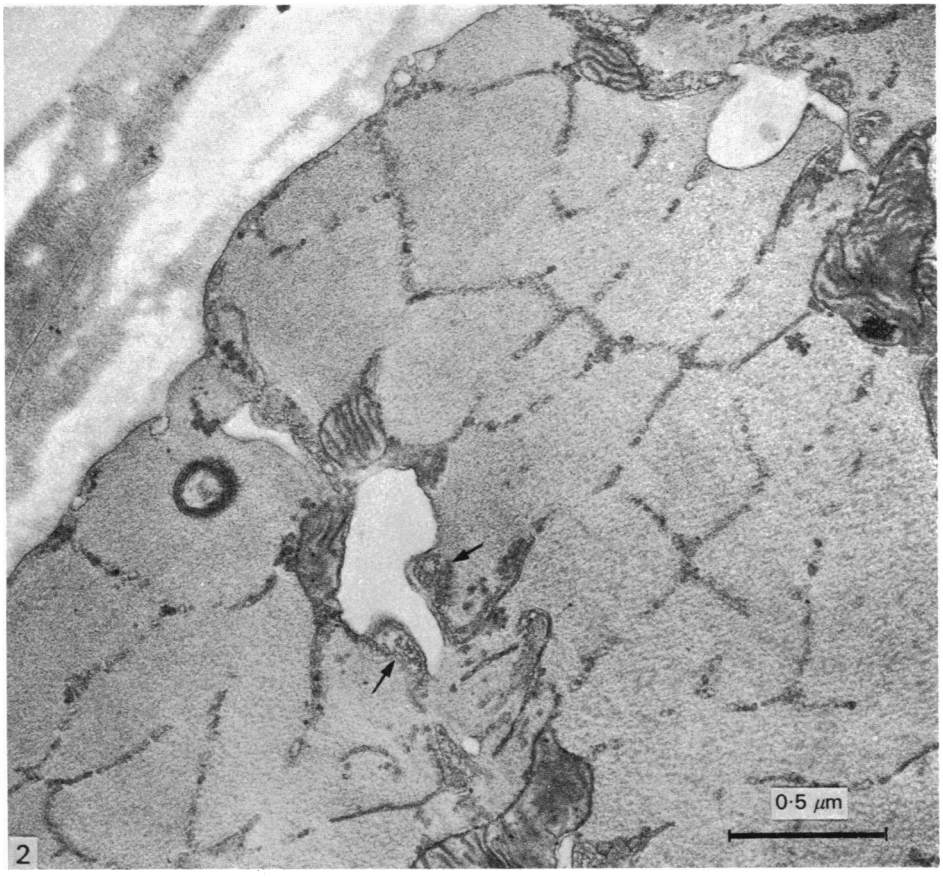

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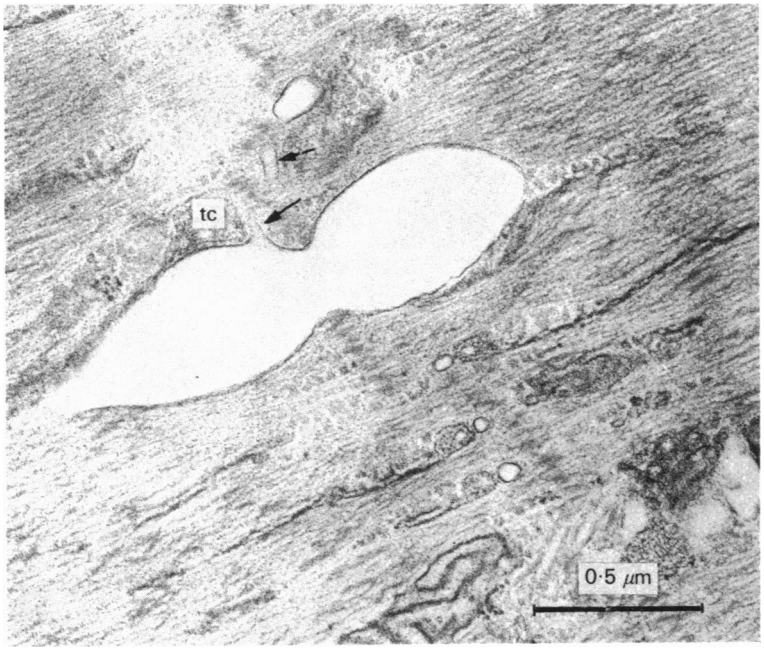
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