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CONTRACTURES AND SWELLING OF THE TRANSVERSE TUBULES DURING CHLORIDE WITHDRAWAL IN FROG SKELETAL MUSCLE

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Recent electron microscopic studies have shown that the transverse tubules (central elements of the triads) in striated muscle are invaginations of the sarcolemma (Franzini-Armstrong & Porter, 1964) and that their lumen is continuous with the extracellular fluid (Endo, 1964; H. E. Huxley, 1964). A. F. Huxley and his colleagues (Huxley & Taylor, 1955, 1958; Huxley & Straub, 1958) found that localized depolarization of very small areas of the surface membrane of striated muscle produced contractions within the subjacent sarcomere only when the micro-electrode was applied to points on the fibre surface corresponding to the position occupied by the triads of the sarcoplasmic reticulum. Together, these observations provide strong support for the hypothesis, originally advanced on morphological grounds, that the triads (Bennett, 1955; Edwards, Ruska, Souza-Santos & Vallejo-Freire, 1956; Porter, 1956; Porter & Palade, 1957), and more recently that their central elements, the transverse tubules (Andersson-Cedergren, 1959; Fawcett & Revel, 1961; Revel, 1962; Falk & Fatt, 1964), furnish a channel for the inward spread of current during surface depolarization.

The possible similarities and differences between the ionic permeabilities of the surface membrane and the membrane lining the transverse tubules has attracted considerable comment (Hodgkin & Horowicz, 1960*a*; Adrian & Freygang, 1962; Girardier, Reuben, Brandt & Grundfest, 1963; Freygang, Goldstein & Hellam, 1964; Falk & Fatt, 1964). Frog skeletal muscle is known to be relatively permeable to chloride, this ion carrying about two-thirds of the transmembrane current when the membrane potential is near its resting level (Hodgkin & Horowicz, 1959; Hutter & Noble, 1960). Hodgkin & Horowicz (1960*a*) suggested that chloride-permeable sites in frog skeletal muscle might be limited to the surface membrane. Grundfest and his colleagues (Girardier *et al.* 1963) showed that the transverse tubular system of crayfish muscle swells under circum-

stances which involve an efflux of chloride ion, and concluded that the walls of this system are selectively permeable to chloride.

During an examination of the contractures produced in frog skeletal muscle following withdrawal of chloride from the external medium, we have obtained results which suggest that chloride efflux in this tissue might occur at subsurface sites as well as across the fibre surface. We also have found that chloride withdrawal causes a marked enlargement of the transverse tubules of the triads in this tissue. Some of these results have already been briefly reported (Foulks & Perry, 1965).

METHODS

All experiments were done on frog (*R. pipiens*) striated muscle at room temperature *in vitro*. Frogs were obtained from General Biological Supply House, Chicago, during all seasons of the year. Most experiments were performed on the long extensor of the fourth toe, a slender muscle consisting predominantly of fast (phasic) fibres (Gray, 1958). Relaxation from maximum potassium contractures was prompt and virtually complete in the vast majority of specimens which we have studied, indicating that most toe muscles contain very few slow (tonic) fibres (Kuffler & Vaughan Williams, 1953). Parallel observations with muscles containing a greater proportion of slow fibres were carried out on tonus bundles dissected from frog iliofibularis muscles as described by Sommerkamp (1928).

Contractile tension was recorded isometrically, using Sanborn mechanotransducers, amplifiers, and direct-writing oscillographs. The various media used consisted of isotonic variants of a conventional frog Ringer's fluid containing 0·1 mg/ml. tubocurarine chloride. The pH of all solutions was adjusted to 7·1 with a Beckman Zeromatic pH meter immediately before use. 1·08 mM calcium was used as the normal extracellular concentration of this ion (Boyle & Conway, 1941; Frank, 1960), rather than 1·8 mM (a concentration which has been employed by numerous investigators). Otherwise the composition of this medium was (mM): Na, 114·3; K, 2·47; Mg, 1·2; HCO_3, 2·38; SO_4, 1·2; PO_4, 0·087; glucose 11·1. Choline chloride or potassium chloride Ringer's solutions were made by replacing all the sodium with the specified cations. Sodium acetate or potassium acetate Ringer's solutions were made by replacing all the chloride with acetate. In sucrose-Ringer's fluid all sodium and chloride were replaced by sucrose, potassium was introduced as bicarbonate and phosphate, and the sulphate salts of calcium and magnesium were employed so as to keep the solution chloride-free. Contracture procedures were preceded by a brief (1–2 min) immersion in choline chloride Ringer's solution in order to eliminate sodium influx. Chloride-withdrawal contractures were produced by transfer from choline chloride Ringer's fluid to sucrose-Ringer's fluid. A 15 min interval of rest was allowed between successive contractures. Calcium depletion was carried out using solutions to which no calcium salts had been added, but no effort was made to exclude all traces of calcium from these media.

For electron microscopic observations, muscles were attached to plastic holders and maintained at constant length during exposure to experimental and preparative solutions. The muscles were fixed in 1 % osmic acid buffered with 0·14 M veronal acetate, embedded in an epoxy mixture containing Maraglas 655 and Cardolite NC-513 (Spurlock, Kattine & Freeman, 1963), sectioned with a Porter-Blum ultra-microtome at 500–900 Å, stained with lead hydroxide (Karnovsky, 1961), examined and photographed under a Siemens-Elmiskop-I microscope at 60 kV and an objective aperture of 50 μ. When ferritin was used as a marker, 30 % solutions of the protein (Nutritional Biochemicals) were prepared according to the procedure described by H. E. Huxley (1964). Muscles were exposed to 30 % ferritin in normal Ringer's fluid for 2 hr before transfer to other experimental solutions which also contained 30 % ferritin.

RESULTS

Chloride-withdrawal contractures. The contractures which develop in frog skeletal muscle following the abrupt removal of chloride from the medium have been noted by other investigators (Hodgkin & Horowicz, 1959; Falk & Landa, 1960), but systematic observations of this contractile response have not been reported.

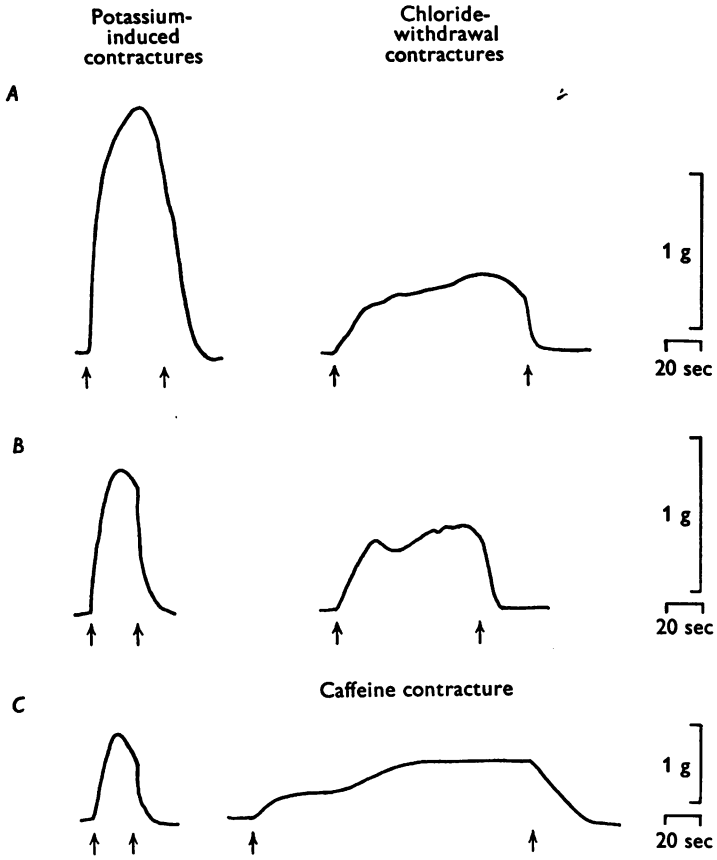
We have observed chloride-withdrawal contractures in more than 100 frog toe muscles. Typical responses are illustrated in Text-figs. 1–6. The peak tension developed by frog-toe muscle during these contractures varied considerably from one specimen to another. The average tension developed following chloride removal was 48% of that observed during maximum potassium-induced contracture (47 muscles, S.D. ± 23%). The magnitude of these contractures generally was stable (reproducible) in a particular muscle, although in a few preparations repetition resulted in successively smaller contractures.

Chloride-withdrawal contractures were uniformly enhanced (seven experiments) by brief prior exposure to an increased external concentration of potassium when there was no accompanying change in the chloride concentration of the medium (Text-fig. 2). Maximum potentiation was produced by pre-contracture exposure to 10–15 mM potassium for at least 2–3 min and this effect persisted undiminished even after the muscles were returned to a medium containing a normal external potassium concentration for 1–2 min before chloride removal.

Chloride-withdrawal contractures in frog-toe muscle also were potentiated by exposure to a number of drugs, including local anaesthetics such as cocaine (0·3 mM) (seven experiments), and general anaesthetics such as pentobarbitone (0·2–1·2 mM) (thirteen experiments). These agents invariably produced a substantial increase in the magnitude of chloride-withdrawal contractures, even in preparations which showed unusually small control contractures. The peak tension level in potentiated chloride-withdrawal contractures often approached that developed during maximum potassium-induced contractures (Text-fig. 2).

Chloride-withdrawal contractures were reduced or prevented, and when already under way were interrupted and suppressed, when the external calcium concentration was elevated to 16 mM (eleven experiments) (Text-fig. 3*A*). On the other hand, reduction of external calcium concentration or transfer to a calcium-free medium often (five of fifteen experiments) resulted in a temporary augmentation of chloride-withdrawal contractures. On several occasions, this effect was prominent and persisted for a considerable period of time (> 30 min) (Text-fig. 4*B*, Text-fig. 5*C–D*). Chloride-withdrawal contractures could be alternately suppressed

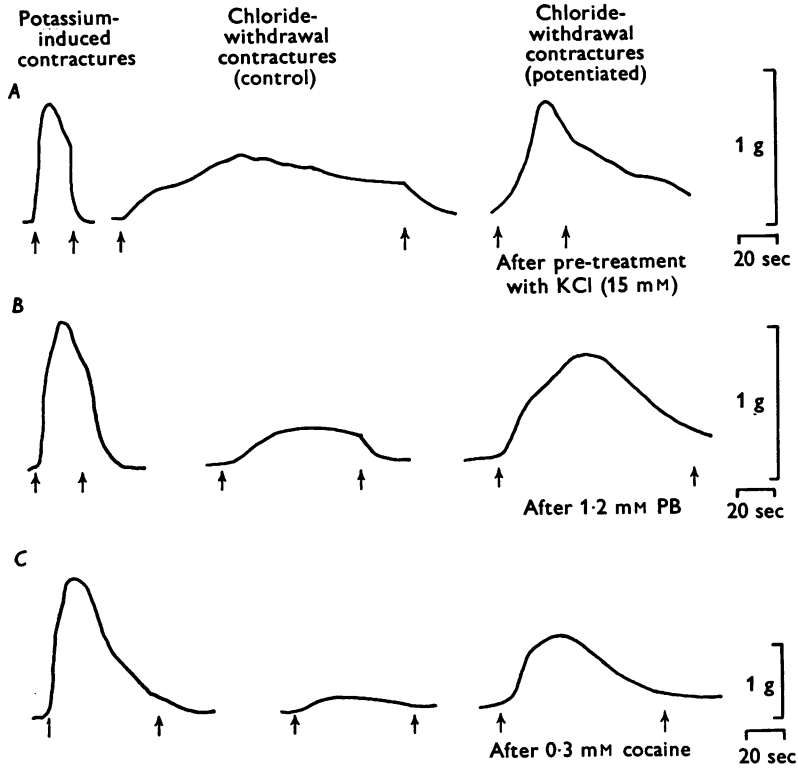
and renewed for several cycles by successive elevation and lowering of the external calcium concentration (Text-fig. 3*B*). Submaximum potassium-induced contractures behaved in the same way when exposed to similar variations in the external calcium concentration.



Text-fig. 1. Typical patterns of tension development in frog-toe muscle during contractures produced (*A* and *B*) by chloride withdrawal (transfer from choline chloride Ringer to sucrose-Ringer) and (*C*) by exposure to 3·0 mM caffeine in choline chloride Ringer. Contractures induced in each muscle by potassium chloride (KCl 60 mM) are shown on the left. (In this and subsequent figures direct tracings have been made from original oscillograph recordings. These have been retouched to eliminate the artifacts which accompany emptying and refilling of the muscle bath. In each instance the small arrows beneath the records indicate the introduction and subsequent removal of the contracture-inducing solution.)

In spite of the contracture-potentiating effect of a reduced concentration of external calcium, more prolonged calcium depletion always resulted in progressive loss of contracture capacity, and some decline in the magnitude

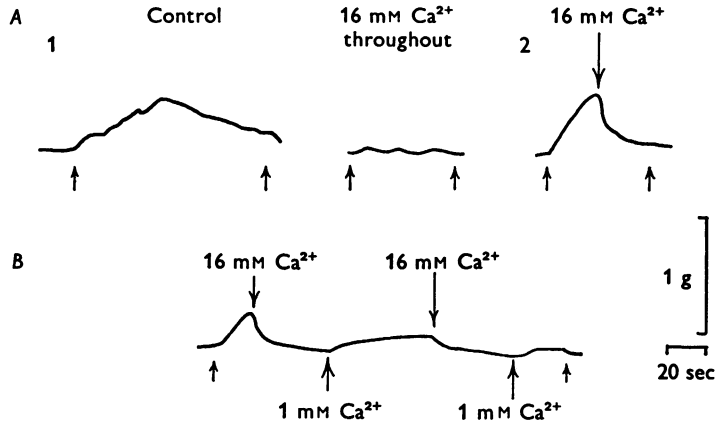
of chloride-withdrawal contractures commonly was observed fairly soon after transfer to a calcium-free medium (Text-fig. 5*A–B*). Preliminary observations (Foulks & Perry, 1965) showed that the rate of loss of contracture capacity during the early stages of calcium depletion was significantly slower for contractures following chloride removal than for maximum potassium-induced contractures. The considerable difference in the



Text-fig. 2. Potentiation of chloride-withdrawal contractures (transfer from choline chloride Ringer to sucrose-Ringer) in frog-toe muscle following (*A*) prior exposure to 15 mM potassium chloride (KCl) in choline chloride Ringer for 4 min followed by return to 2·5 mM potassium 1 min before withdrawing chloride; (*B*) exposure to 1·2 mM pentobarbitone (PB) for 2 min before and during chloride withdrawal; (*C*) exposure to 0·3 mM cocaine for 2 min before and during chloride withdrawal. Control potassium-induced and chloride-withdrawal contractures are shown on the left.

magnitude of these two types of contracture before calcium depletion gives rise to uncertainty in interpreting this result, since submaximum potassium-induced contractures also may be potentiated during initial exposure to calcium-free media (Text-figs. 4*A*, 2; 5*D*). In a subsequent comparison between chloride-withdrawal contractures and submaximum potassium-

induced contractures of comparable initial magnitude, an appreciable lag in the rate of decline of chloride-withdrawal contractures during calcium depletion was again noted (Text-fig. 5).



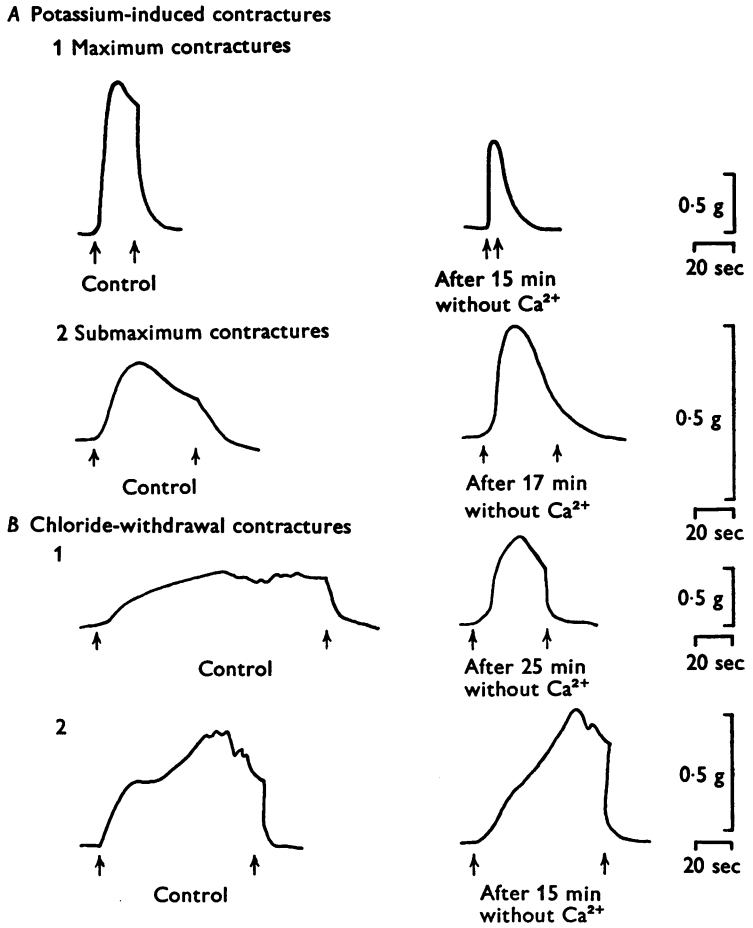
Text-fig. 3. The effect of altered external calcium (Ca^{2+}) concentration on chloride-withdrawal contractures (transfer from choline chloride Ringer to sucrose-Ringer). *A* 1, prevention of contracture by exposure to elevated calcium for 1 min before and during chloride withdrawal; *A* 2, abrupt interruption of chloride-withdrawal contractures upon elevation of the calcium concentration at the point indicated. *B*, alternate suppression and resumption of contracture as external calcium concentration is elevated or returned to normal. The large arrows above and below the records indicate points at which the calcium concentration was changed without otherwise altering the composition of the medium.

When chloride-withdrawal contractures had been depressed by calcium depletion, contracture capacity was rapidly regained upon calcium repletion, a substantial degree of restoration taking place within 30–60 sec. This rate of recovery is comparable to that seen with potassium-induced contractures under similar circumstances (Frank, 1960).

The time course as well as the magnitude of chloride-withdrawal contractures varied considerably from one muscle to another. Peak tension frequently was not reached until a minute or longer after transfer to a chloride-free medium. The time course of chloride-withdrawal contractures is similar to that of submaximum potassium-induced contractures of comparable magnitude and presumably reflects incomplete membrane depolarization (Hodgkin & Horowicz, 1960*b*). When chloride-withdrawal contractures were potentiated by drugs or by prior exposure to elevated external potassium concentrations, the rate of tension development was increased and the duration of the contracture was reduced (Text-fig. 2).

In some preparations, rather irregular fluctuations in tension were observed during the peak phase of chloride-withdrawal contractures (Text-

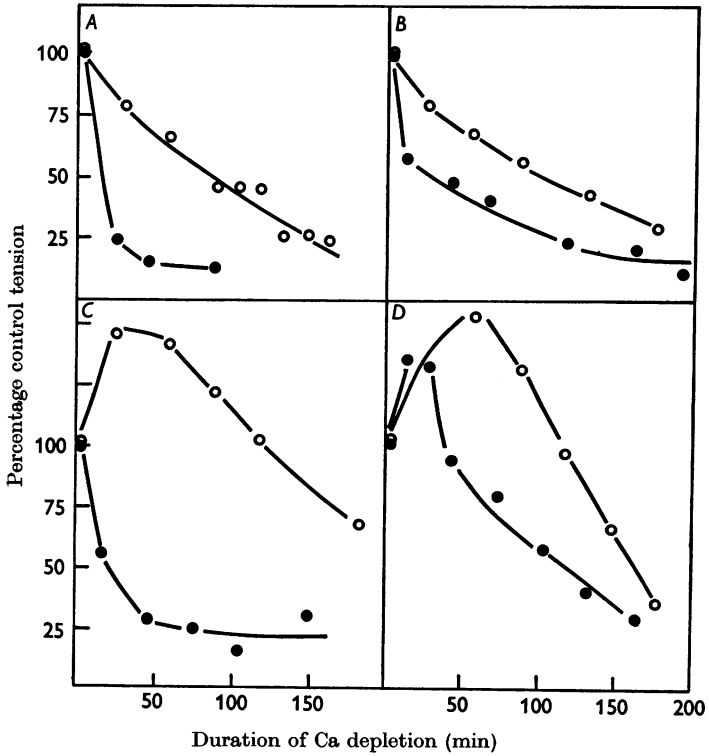
fig. 1). However, tension development often followed a smooth course and frequently showed two distinct phases; an initial fairly rapid increase in tension, a brief plateau or an interval of greatly slowed tension develop-



Text-fig. 4. Comparison of the effect of calcium (Ca^{2+}) depletion on potassium-induced contractures and those resulting from chloride-withdrawal (transfer from choline chloride Ringer to sucrose-Ringer). *A* 1, maximum potassium-induced contractures (KCl 60 mM) before and after calcium depletion; *A* 2, submaximum potassium-induced contractures (30 mM-KCl) in another muscle before and after calcium depletion. *B*, two experiments which illustrate the potentiating effect on chloride-withdrawal contractures which sometimes is observed during exposure to calcium-free media. Experiments *A* 1 and *B* 1 were performed on the same muscle.

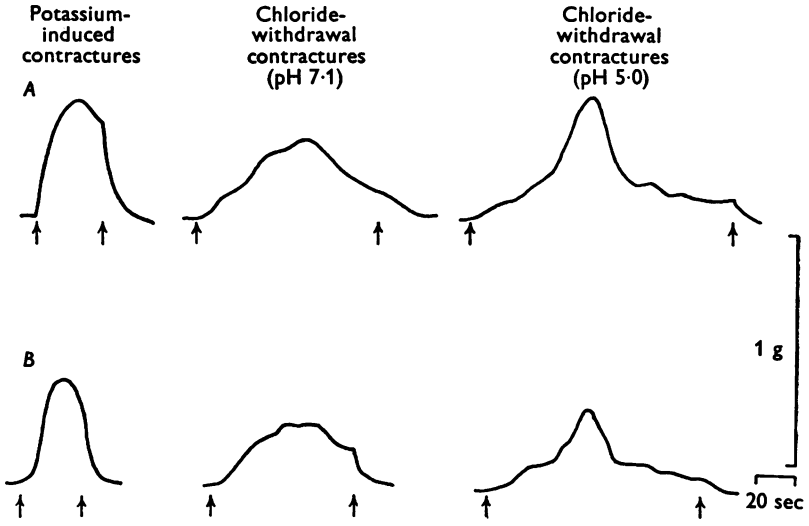
ment, followed by a phase of renewed rapid contraction (Text-figs. 1, 2, 4). A similar pattern of tension development was frequently observed during caffeine-induced contractures (Text-fig. 1*C*).

Brooks & Hutter (1964) reported that chloride efflux from frog-toe and sartorius muscles was greatly slowed in an acid medium. We found no consistent change in the magnitude or pattern of tension development of chloride-withdrawal contractures in toe muscles subjected to altered extracellular pH. However, in two of six muscles which were exposed to pH 5·0 for 15 min before chloride removal, the separation of tension development into two distinct phases was accentuated, the initial phase being retarded and the second phase accelerated (Text-fig. 6).



Text-fig. 5. Four experiments illustrating the slower rate of decline of contracture magnitude during prolonged calcium depletion for contractures produced by chloride withdrawal (○) as compared with those produced by elevated external potassium (●). The potassium concentration used in these experiments was selected in each case so that the magnitude of control potassium-induced contractures before calcium depletion was the same as that of control chloride-withdrawal contractures. The potassium concentrations which met these requirements were: *A*, 40 mM; *B*, 40 mM; *C*, 33 mM; and *D*, 25 mM. The magnitudes of the initial contractures (before calcium depletion) in each of these experiments, expressed as per cent of the peak tension developed during maximum potassium-induced contractures, were: *A*, 62 %; *B*, 84 %; *C*, 43 %; and *D*, 38 %. The muscles remained in calcium-free choline chloride Ringer's solution during the intervals between contractures in order to maintain adequate resting membrane potentials.

The response of the tonus bundle of the frog iliofibularis muscle to chloride withdrawal also was examined. Peachey & Huxley (1962) reported that these bundles contain a mixture of varying proportions of slow and fast fibres. In general, these bundles produced very small contractures on chloride removal. In ten of eleven specimens, the magnitude of chloride-withdrawal contractures was less than 6% of the size of the maximum potassium-induced contracture. Potentiating procedures also were less

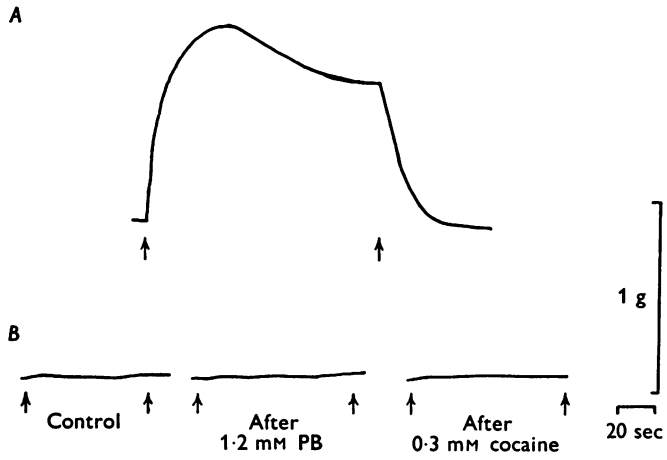


Text-fig. 6. Two experiments in which the separation of contracture tension into two distinct phases was accentuated when chloride removal was preceded by 15 min exposure to an acid pH (5·0).

effective than with toe muscle. In three of eight specimens in which little or no increase in tension was discernible following chloride removal, pentobarbitone and cocaine were ineffective in augmenting chloride-withdrawal contractures (Text-fig. 7*B*). These particular bundles appeared to contain a relatively small proportion of fast fibres (as indicated by their very small twitch response to electrical stimulation, and their persistent maintenance of potassium-induced contracture tension at near maximum height (Text-fig. 7*A*) (see Kuffler & Vaughan Williams, 1953)).

Electron microscopic examination of the transverse tubular system. The central element of the reticular triads in normal frog striated muscle typically is about 300–400 Å in diameter, although in ferritin-treated muscles we observed a few transverse tubules with diameters as large as 600–700 Å. These tubules expanded rapidly after transfer to isotonic sucrose-Ringer's fluid. Although the extent of the enlargement of the transverse tubules varied from one site to another, some degree of swelling

occurred throughout this system. Tubule diameters two to four times greater than normal were the rule, and typical triads in which the central element approximated the size of the lateral cisternae on either side were not uncommon (Pls. 1–4). Intracellular ferritin appears to be confined to the transverse tubules (H. E. Huxley, 1964). Its presence permits a positive identification of the central elements of the triads, when swelling has rendered them indistinguishable from the lateral and longitudinal components on purely morphological grounds. In some instances the shortest diameter of ferritin-containing vesicles was as large as 5000 Å



Text-fig. 7. Potassium and chloride-withdrawal contractures in a tonus bundle of the frog iliofibularis which appeared to have few phasic fibres. *A*, prolonged contracture in potassium chloride (KCl 60 mM). *B*, lack of response to simple chloride withdrawal and failure to produce appreciable chloride-withdrawal contractures even after exposure to concentrations of pentobarbitone (PB) and cocaine, which invariably produce a marked potentiation of chloride-withdrawal contractures in toe muscles. (Compare with Text-fig. 2.)

(Pls. 3, 4). Substantial degrees of tubular enlargement were evident after as little as 30 sec exposure to the chloride-free medium, and the degree of effect observed after 1–2 min exposure to sucrose-Ringer's fluid was not increased by longer intervals (up to 20 min) of chloride withdrawal (Pls. 1, 3). Similar effects were observed following transfer from normal Ringer's fluid to Ringer's fluid in which all of the chloride was replaced with the impermeant acetate anion (Pl. 4, fig. 1). Very large ferritin-containing vesicles (4000–5000 Å) also were observed in muscles transferred from isotonic potassium chloride-Ringer's fluid to isotonic potassium acetate-Ringer's fluid (Pl. 4, figs. 3, 4).

No change in the dimensions of the lateral cisternae of the triads or the longitudinal components of the sarcoplasmic reticulum was observed

following chloride removal. In a few instances, large electron-dense particles resembling ferritin appeared to lie within the lumen of lateral elements of the triads, but the concentration of these electron-dense particles was much less than in the adjacent central element. Scattered electron-dense particles sometimes were encountered in the sarcoplasmic reticulum of lead-stained muscles not treated with ferritin (see also Porter, 1954). They probably were glycogen granules (Fawcett, 1958; Revel, Napolitano & Fawcett, 1960; Fawcett & Revel, 1961; Revel, 1962). The background of amorphous material in the lateral cisternae made uncertain the distinction between glycogen and ferritin particles. If channels of continuity do exist between the lumina of the central and lateral elements of the triads, chloride withdrawal did not enlarge them sufficiently to allow the passage of appreciable numbers of clearly identifiable ferritin particles.

The appearance of the longitudinal components of the sarcoplasmic reticulum was not changed by transfer to sucrose or sodium acetate Ringer's fluids, although these structures were considerably disarranged in muscles which had been exposed to isotonic potassium chloride-Ringer's solution for an interval of 1 hr (Pl. 4, figs. 3–5). Under these circumstances a substantial swelling of the entire fibre takes place as the result of salt and water uptake (Boyle & Conway, 1941), and the myofibrils become rather widely separated by fluid-filled spaces (Harris, 1961). Bands of sarcoplasmic reticulum lying transversely across the space between adjacent myofibrils at the level of the H-band were prominent in such muscles (Pl. 4, figs. 3–5), and the continuity of this sarcoplasmic structure across several adjacent myofibrils often was evident (Pl. 4, fig. 5). The profile of these reticular strands was continuous with that of the M-band of the myofibrils. At a few sites, this reticular element appeared to have been displaced, and the M-band was no longer clearly discernible (Pl. 4, fig. 5).

DISCUSSION

Contracture potentiation as the result of altered chloride and potassium efflux. It seems reasonable to assume that the magnitude of chloride-withdrawal contractures reflects the relative rates of chloride and potassium efflux, which will determine the extent of the depolarization accompanying this procedure. The chloride permeability of frog skeletal muscle is believed to be rather stable (Hodgkin & Horowicz, 1959, 1960*a*; Adrian & Freygang, 1962), although Adrian (1962) has called attention to the considerable variation in chloride permeability from one muscle to another. Varying degrees of chloride permeability could readily explain the rather marked variation between muscles in the magnitude of chloride-with-

drawal contractures, although variation between muscles in their permeability to potassium and other ions is also possible.

The reciprocal role of potassium and chloride efflux in the production of chloride-withdrawal contractures is illustrated by drug-induced potentiation of chloride-withdrawal contractures. Evidence that local anaesthetics (as well as some other drugs) interfere with potassium influx in skeletal muscle has been reported by several investigators. The increase in volume (weight) of frog sartorius when immersed in isotonic potassium chloride-Ringer is retarded by exposure to a number of drugs, including local anaesthetics (Shanes, 1950) and pentobarbitone (Foulks & Perry, unpublished results). In media containing no chloride (choline-sulphate Ringer's fluid) exposure to 0·1 % (*ca.* 3 mM) cocaine resulted in a prompt reduction in the membrane potential of frog sartorius fibres from −90 to −51 mV (Adrian & Freygang, 1962), indicating a reduction in potassium permeability. Thus, the augmentation of chloride-withdrawal contractures which is produced by these drugs can be explained by their ability to retard potassium outflow from frog-muscle fibres and thereby to enhance the depolarizing effect of chloride efflux.

The potentiation of chloride-withdrawal contractures by previous immersion in media containing 10–15 mM potassium also may be explained on the basis of a change in the relative rates of chloride and potassium efflux. Repolarization should be virtually complete following brief prior exposure to such a modest increase in the external potassium concentration (Hodgkin & Horowicz, 1960*a*). The membrane which encloses frog striated muscle fibres behaves in accordance with the Donnan equilibrium (Boyle & Conway, 1941; Adrian, 1960), an increase in the concentration product of external potassium and chloride leading to an uptake of these ions by the muscle fibre. Under these circumstances, the normally low cellular content of chloride can be significantly increased. Subsequent removal of external chloride would then be associated with an increased transmembrane concentration gradient for chloride, and this should lead to an increased chloride efflux. Any increase in the potassium concentration gradient would be relatively small, and should reduce rather than promote potassium efflux (Adrian, 1962), an effect which also would enhance depolarization.

Contracture tension in relation to external calcium concentration. Sufficiently prolonged calcium depletion results in a progressive decline in the contracture capacity of frog skeletal muscle (Frank, 1960). However, even brief exposures to alterations in the calcium concentration of the medium give rise to characteristic changes in the time course and magnitude of contractures. High external calcium causes an increase in the threshold concentration for potassium-induced contractures (Fleckenstein & Hertel,

1948; Adrian & Freygang, 1962) and also increases the potassium concentration required to produce more complete degrees of contracture (Lüttgau, 1963). On the other hand, brief exposures to reduced external calcium concentration may enhance submaximum potassium-induced contractures (Frank, 1960; Lüttgau, 1963), although maximum potassium-induced contractures may be depressed. The conclusion that the potassium concentration required to produce varying degrees of contracture is proportional to the external calcium concentration has been amply confirmed by unpublished observations in our laboratory. (Our solutions all contained magnesium (1·2 mM), whereas the solutions used by Adrian & Freygang (1962), and by Lüttgau (1963), apparently did not.)

Chloride-withdrawal contractures resemble submaximum potassium-induced contractures in their response to abrupt changes in the external concentration of calcium ion, both being depressed by a high external calcium concentration, and enhanced by a moderate or brief reduction in the external calcium concentration. It seems unlikely that the contracture-modifying effects of brief alterations in the external calcium concentration are the result of changes in fibre permeability to potassium or chloride. The chloride conductance of frog sartorius fibres is not altered by changes in the external calcium concentration (Adrian, 1962, p. 1226). High external calcium was found not to reduce the potassium conductance of frog sartorius muscle membrane either in sodium chloride Ringer's fluid (Jenerick, 1957), or in choline-sulphate Ringer's fluid (Adrian & Freygang, 1962). It seems clear that the external calcium concentration exerts an influence on the level to which the membrane potential of frog muscle must be reduced in order to release the contractile process, and that this effect is independent of the nature of the ionic flux which is responsible for depolarization.

Superficial and internal sites of excitation-contraction coupling during chloride-withdrawal contractures. The events which intervene between depolarization and tension development have been referred to as excitation-contraction coupling. A substantial body of evidence indicates that the release of calcium ion into the sarcoplasm is an essential step in this process (Shanes, 1958; A. F. Huxley, 1964). The observations presented here suggest that chloride-withdrawal contractures are dependent, at least in part, upon coupling events occurring within the fibre interior. The fact that agents such as caffeine can produce undiminished contractures after degrees of calcium depletion which substantially depress potassium-induced contractures (Frank, 1960) has been explained by concluding that caffeine-induced contractures involve the discharge of calcium from sites which are depleted more slowly, presumably from some internal source. A similar conclusion seems warranted with respect to chloride-

withdrawal contractures, which also are somewhat more resistant to the depressant effect of prolonged calcium depletion than are potassium-induced contractures. Chloride-withdrawal contractures also resemble those produced by caffeine in their tendency to display a biphasic pattern of contraction. It is tempting to relate these two successive phases of tension development to superficial and internal (tubular) sites of calcium release.

Our results with slow-muscle bundles point to an apparent parallel between the degree of development of the transverse tubular system and the capacity for chloride-withdrawal contractures in frog skeletal muscle. Peachey & Huxley (1962) showed that the sarcoplasmic reticulum of frog slow (tonic) muscle fibres is much more sparsely developed than that of fast (phasic) muscle fibres, and that typical triads occur infrequently in slow fibres. The ability of slow fibres to develop chloride-withdrawal contractures appears to be much less than that of fast fibres, even in the presence of potentiating agents. This suggests that frog slow-muscle fibres may have a relatively low chloride permeability. However, fast and slow-muscle fibres differ in other respects. Slow fibres have a relatively low membrane potential (Kuffler & Vaughan Williams, 1953), apparently as a result of a high sodium permeability (Kiesling, 1960). It is therefore premature to draw conclusions as to the relative chloride and potassium permeabilities of these two types of frog muscle.

These observations provide circumstantial evidence that the transverse tubules may provide an important site of chloride efflux and of excitation-contraction coupling when chloride-withdrawal contractures are produced in frog toe muscle. The biphasic character of chloride-withdrawal contractures in frog toe muscle suggests that two sites of chloride efflux, one superficial and another more internally placed, may be involved. The rapid restoration of considerable capacity for chloride-withdrawal contractures following return of external calcium to normal levels after a preceding interval of calcium depletion also suggests some contribution to excitation-contraction coupling from relatively superficial sites. The effect of exposure to an acid medium on the two phases of tension development during chloride-withdrawal contractures suggests that any acid-induced interference with chloride efflux may be confined to the surface membrane.

Transverse tubular swelling as the result of chloride withdrawal. The transverse tubules of frog skeletal muscle resemble those of crayfish striated muscle (Girardier *et al.* 1963) in the enlargement which they undergo upon transfer to a chloride-free solution. In an isosmotic medium, swelling of the transverse tubules presumably requires the rapid transfer of ions (or other solute) from the sarcoplasm into the tubular lumina. The removal of chloride from the external medium leads to an outward move-

ment of both chloride and potassium ions, potassium outflow taking place as a consequence of the depolarization which accompanies chloride efflux. The swelling of the transverse tubules which is produced by chloride withdrawal demonstrates that the walls of these tubules are a site of rapid ionic efflux under these circumstances. However, when both potassium and chloride ions leave the fibre, the possibility that either ionic efflux might be confined to the fibre surface cannot be excluded, since, as Girardier *et al.* (1963) pointed out, current flow between surface and tubular sites of ion efflux could be maintained by means of an equal and opposite movement of anions and cations through the extracellular fluid and the tubular orifices.

In studies on single twitch fibres of frog muscle, Hodgkin & Horowicz (1960*a*) observed that changes in membrane potential in response to alterations in the external chloride concentration were more rapid than those associated with modification in the external potassium concentration, and concluded that sites which are sensitive to potassium ions are less accessible than those which are sensitive to chloride. However, when the external concentration of either ion was changed, an initially rapid alteration in membrane potential was followed by a slower drift to its final level, and the lag in potential change in response to altered potassium concentration was more prominent than that seen with changes in external chloride concentration only when the external potassium concentration was reduced. This result, as Hodgkin & Horowicz (1960*a*) pointed out, may be associated with the asymmetrical properties of the twitch muscle membrane with respect to potassium transfer, so-called anomalous rectification (Katz, 1949). Adrian & Freygang (1962) suggested that retarded potassium efflux might be largely a property of the walls of the sarcoplasmic reticulum. These workers suggested a model in which an 'intermediate compartment' is lined by a membrane possessing little permeability to chloride. The possibility that this compartment might correspond to the transverse tubule was raised (Adrian & Freygang, 1962; Freygang *et al.* 1964). However, chloride impermeability is not an essential feature of the proposed model, having been introduced in part to minimize the volume changes which otherwise would have been expected in this compartment. The electron microscopic studies reported here show that substantial changes in the volume of the transverse tubular system in frog muscle may in fact occur.

Girardier *et al.* (1963) showed that the transverse tubules of crayfish muscle are specifically permeable to chloride, since tubular swelling in this tissue was produced when a hyperpolarizing current was passed across the fibre membrane by means of a micro-electrode, i.e. under circumstances in which ionic efflux is confined to anions. Until similar experiments are

performed on frog muscle, the suggestion that the transverse tubules in this species also are permeable to chloride must remain unsettled.

The sarcoplasmic reticulum and the M-band. The portion of the sarcoplasmic reticulum which encircles the sarcomere at the level of the H-band has been termed the central (Porter & Palade, 1957) or H-band (Franzini-Armstrong, 1963) cisterna, and this structure seems to have continuity across the entire transverse width of the muscle fibre (Edwards *et al.*1956; Porter, 1956; Porter & Palade, 1957). Our observations confirm this view and indicate that the location of the H-band cisterna corresponds to the position of the M-band. Bennett (1955) noted that 'it is not unusual to see the sarcoplasmic reticulum connecting with the M-band of the myofibril'. The connexion between this transverse band and the longitudinal elements of the sarcoplasmic reticulum sometimes was clearly seen in preparations in which the normal structural relations remained intact (Pl. 4, fig. 1).

The M-band is characterized by thickenings of the thick filaments (Stenger & Spiro, 1961), and by cross-bridges directly linking these filaments (Franzini-Armstrong & Porter, 1964). Nevertheless, it is apparent that the H-band cisterna also makes an important contribution to the density of the M-band region in sections as thick as these (500–900 Å). In both swollen and intact fibres, the profiles of the H-band cisterna and the M-band appeared to coincide. The intensity of the M-band is substantially reduced when the transverse strand of the sarcoplasmic reticulum is not present (Pl. 4, fig. 5). Slow fibres, whose sarcoplasmic reticulum is poorly developed, do not possess M-bands (Peachey & Huxley, 1962).

SUMMARY

1. The purpose of this investigation was to assess the chloride permeability of the transverse tubules (central elements of the reticular triads) in frog skeletal muscle. Functional and structural responses to the replacement of chloride with impermeant substitutes furnished the experimental basis for this study.

2. The magnitude of chloride-withdrawal contractures in frog-toe muscle was potentiated by procedures which amplify the transmembrane concentration gradient for chloride (prior loading in high potassium media), or which retard potassium efflux (exposure to drugs such as cocaine and pentobarbitone).

3. Chloride-withdrawal contractures were suppressed by high external calcium, and temporarily facilitated by low external calcium. This is attributed to the influence of external calcium concentration on the relation between contracture tension and membrane potential.

4. Contractures produced by chloride withdrawal showed a slow time

course in which tension frequently develops in two successive phases. The diminution of contracture tension as a result of calcium depletion occurred more gradually with chloride-withdrawal contractures than with potassium-induced contractures. These characteristics are similar to the corresponding features of caffeine-induced contractures and are attributed to excitation-contraction coupling at sites located within the interior of the muscle fibre, presumably the transverse tubule.

5. Slow muscle fibres, which have poorly developed triads and a sparse sarcoplasmic reticulum, also display poor contracture responses to chloride withdrawal.

6. These observations suggest that the transverse tubules in frog skeletal muscle may have an appreciable chloride permeability.

7. Electron microscopic observations of frog-toe muscle fibres, including muscles exposed to media containing ferritin, show that chloride withdrawal leads to a prompt and marked enlargement of the transverse tubular system. This finding is compatible with (but does not prove) the hypothesis that these tubules are permeable to chloride.

8. The H-band cisterna seems to make an important contribution to the density of the M-band in thick sections.

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EXPLANATION OF PLATES

Electron micrographs of frog-toe muscle following chloride withdrawal

When chloride was removed by exposure to sucrose-Ringer's solution, this transfer was always preceded by immersion in choline chloride-Ringer for 2 min. When ferritin was used, muscles were exposed to 30 % ferritin in frog Ringer for 2 hr before transfer to experimental media, all of which contained 30 % ferritin. When chloride was withdrawn in high potassium solutions, the muscle was immersed in isotonic potassium chloride for 1 hr before the substitution of acetate for chloride.

The following symbols have been used: T = transverse tubule. C = lateral cisterna. L = longitudinal components of sarcoplasmic reticulum. Z = Z-line. M = M-band.

PLATE 1

Fig. 1. Control: Choline chloride Ringer's fluid (7 min).

Fig. 2. Sucrose-Ringer's fluid (5 min).

Fig. 3. Sucrose-Ringer's fluid (2 min).

Fig. 4. Sucrose-Ringer's fluid (1 min).

Magnification: × 22,500.

PLATE 2

Fig. 1. Control: Choline chloride Ringer's fluid (5 min).

Fig. 2. Sucrose-Ringer's fluid (2 min).

Magnification: × 31,500.

PLATE 3

Ferritin treated muscle

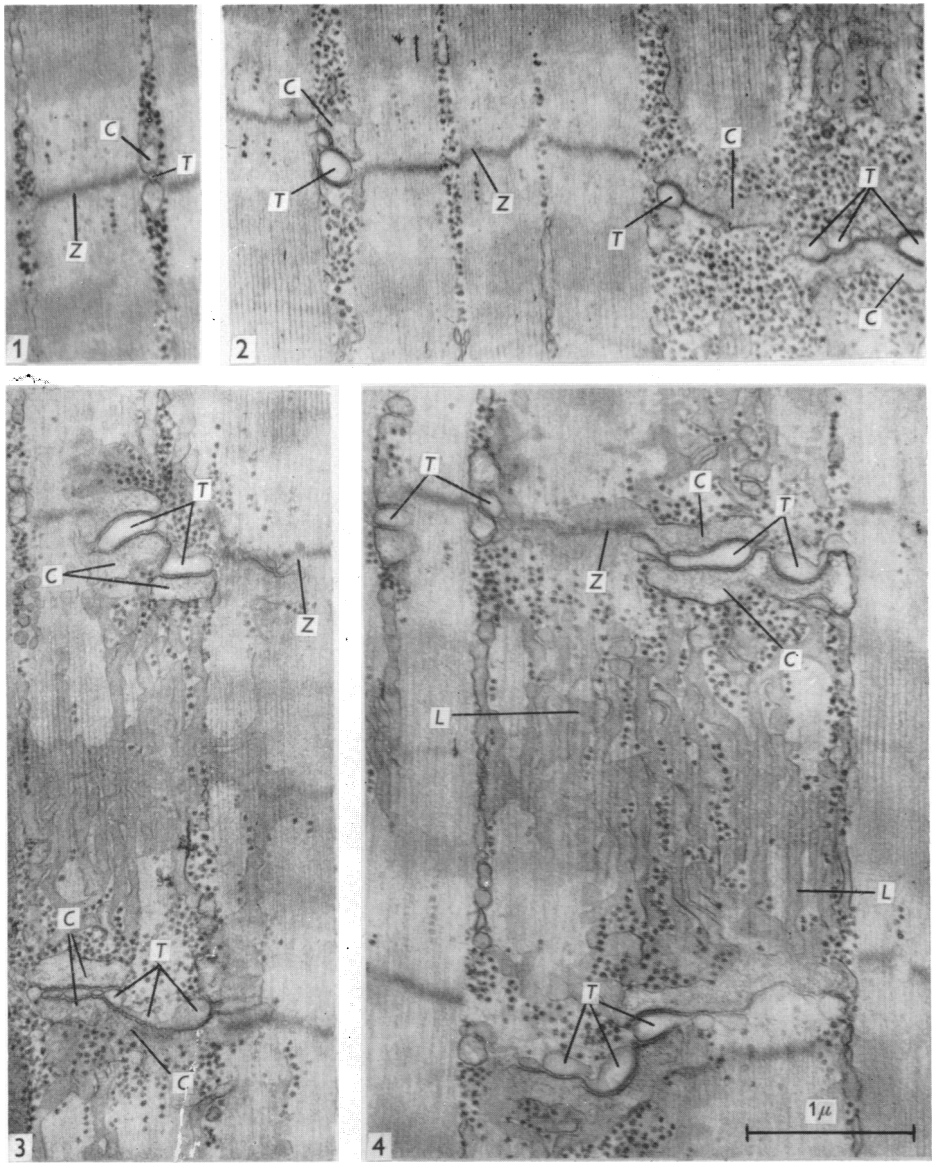
Fig. 1. Control: Normal Ringer's fluid (20 min).

Fig. 2. Sucrose-Ringer's fluid (20 min).

Fig. 3. Sucrose-Ringer's fluid (20 min).

Fig. 4. Sucrose-Ringer's fluid (20 min).

Magnification: × 36,000.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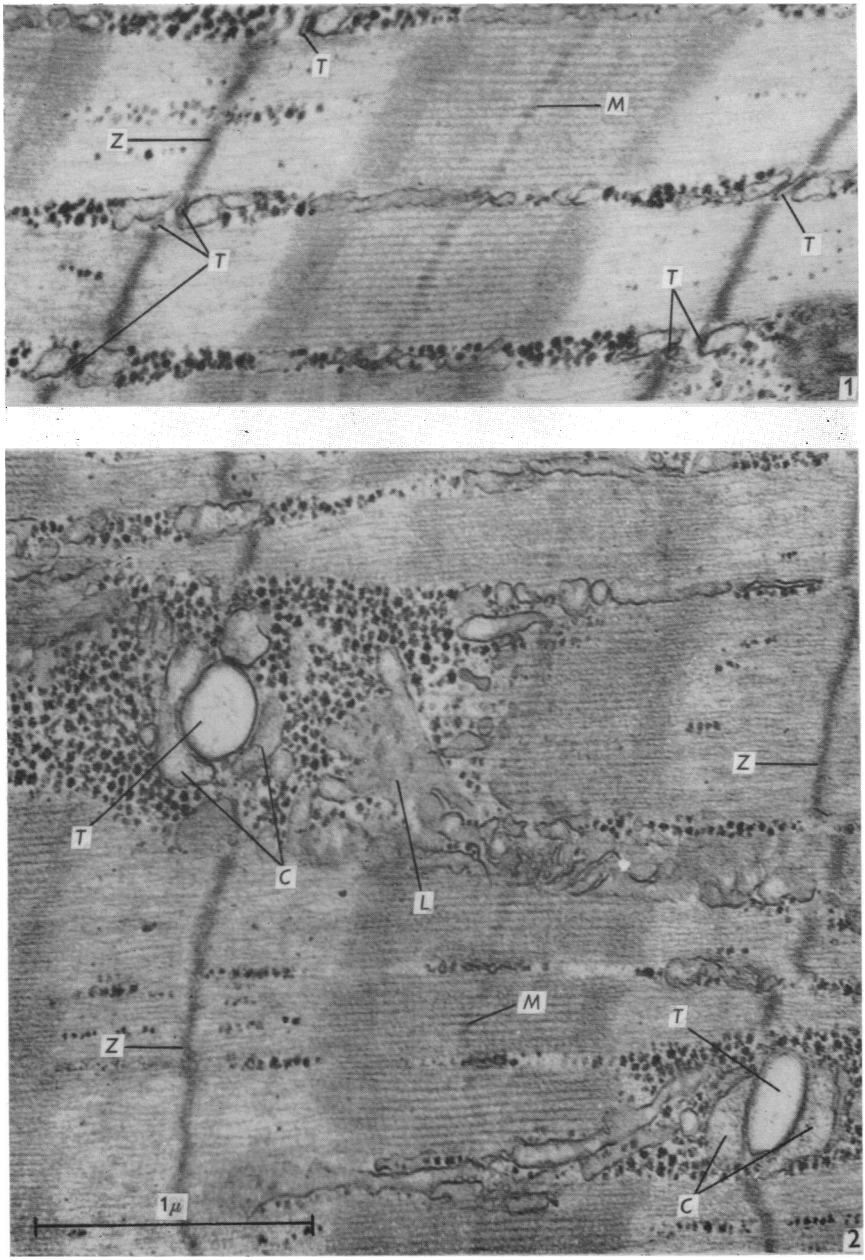
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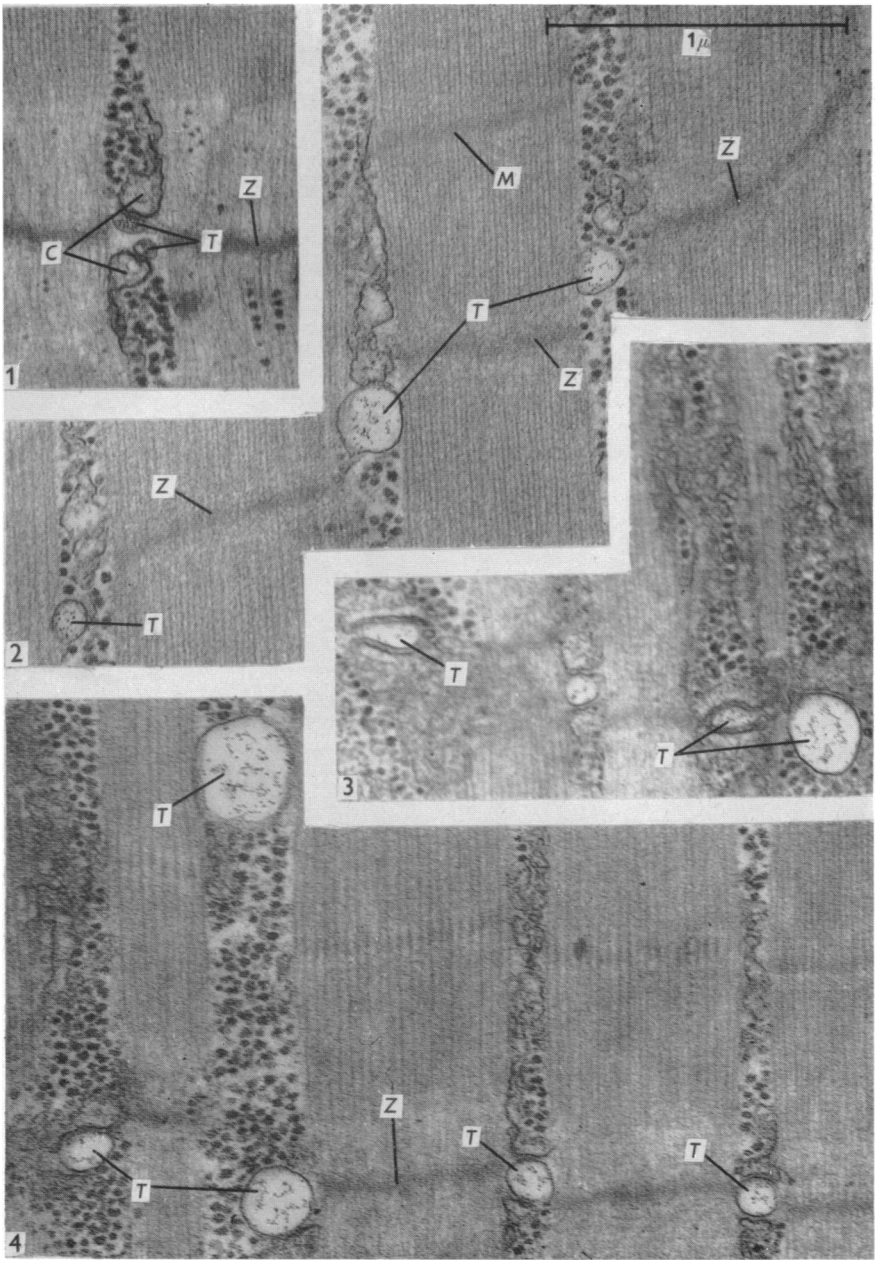

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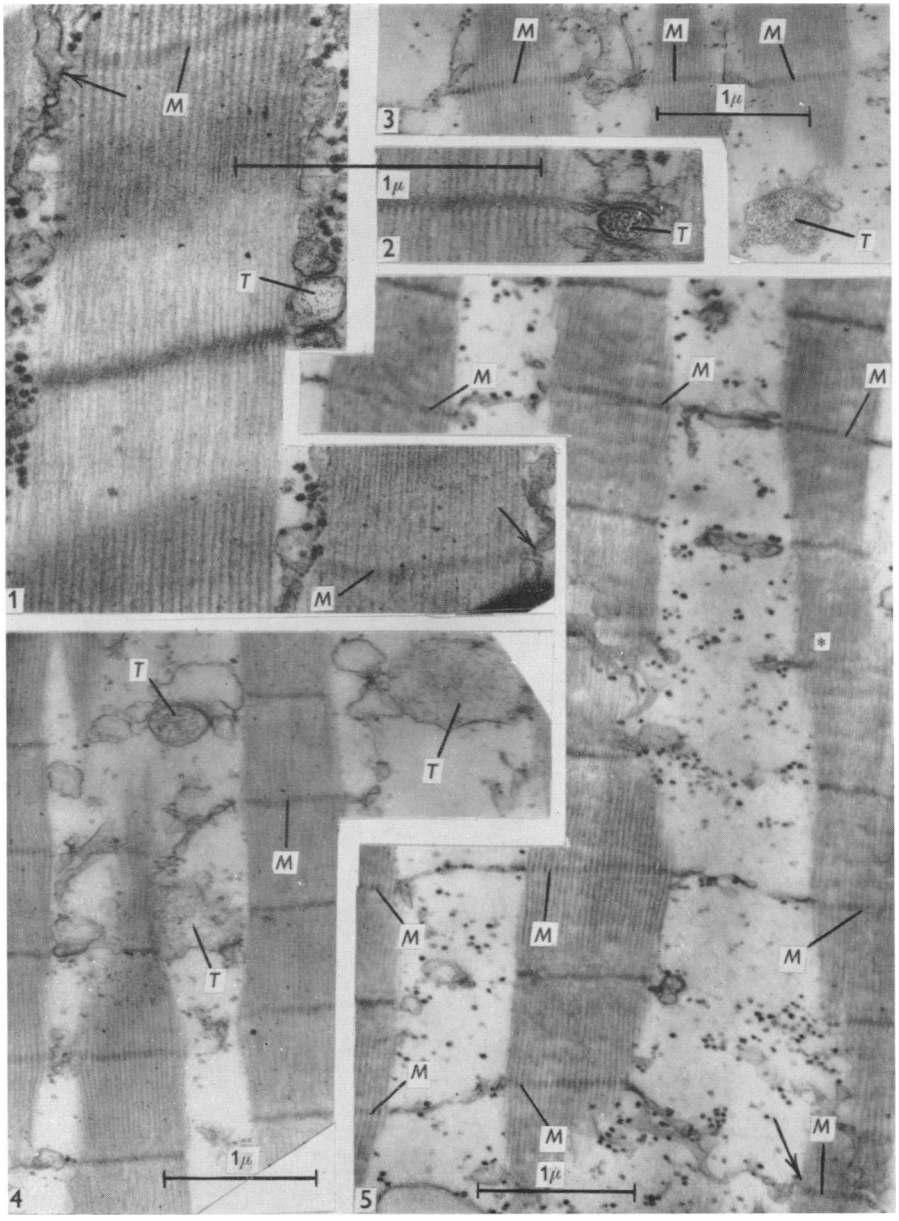

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PLATE 4

Ferritin treated muscle. The arrows indicate points of junction between strands of sarcoplasmic reticulum and M-bands.

Fig. 1. Sodium acetate Ringer's fluid (20 min).

Fig. 2. Potassium acetate Ringer's fluid (2 min).

Fig. 3. Potassium acetate Ringer's fluid (20 min).

Fig. 4. Potassium acetate Ringer's fluid (20 min).

Fig. 5. Potassium acetate Ringer's fluid (20 min). The asterisk indicates an H-band whose transverse strand has been disrupted.

Magnification: Figs. 1–2 × 33,000, Figs. 3–5 × 16,500.