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Composition of vacuoles and sarcoplasmic reticulum in fatigued muscle: Electron probe analysis

(fatigue/cryo-ultramicrotomy/mitochondrial calcium/striated muscle/muscle calcium)

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ABSTRACT **Electron probe analysis, cryo-ultramicrotomy, and freeze-substitution were used to determine the nature of vacuolation and the subcellular composition in fatigued frog skeletal muscle fibers. The vacuoles caused by fatigue were part of the T-tubule system and contained high concentrations of NaCl. The calcium concentration in the terminal cisternae was higher than previously measured normal resting values. Mitochondrial calcium content was relatively low (mean ± SEM, 2 ± 2 mmol/kg dry weight). Fiber NaCl was increased. It is concluded that fatigue is not due to the depletion of calcium stores from the terminal cisternae or to uncoupling of mitochondria due to calcium loading but may be caused by multiple mechanisms including failure of the T-tubule action potential.**

Prolonged tetanic stimulation of vertebrate striated muscles induced a state (hitherto referred to as fatigue) during which the contractile force first declines and subsequently the muscle becomes mechanically refractory to further stimulation. Fatigue is not due to failure of the action potential mechanism (1–3) or to the depletion of high-energy phosphates (4, 5), and it is associated with vacuolation detectable by light microscopy (6, 7). The purpose of the present studies was to establish the ultrastructure of these vacuoles and their contents and to determine whether fatigue was due to the depletion of Ca^{2+} from the terminal cisternae of the sarcoplasmic reticulum or to abnormal accumulation of Ca^{2+} by the mitochondria, or to both.

The preliminary account of some of these findings has been presented (8).

METHODS

Single fibers were isolated from the semitendinosus muscle of *Rana temporaria* or *R. pipiens* for the physiological experiments, and bundles of 8–12 fibers of *R. pipiens* were prepared for cryo-ultramicrotomy and electron probe analysis. The muscles were suspended in frog Ringer's solution in a chamber (9) and stretched to 2.8-μm sarcomere length. Force was recorded with an RCA 5734 transducer. The stimulation consisted of periodic tetanization at 40–50 supramaximal shocks per sec during 0.3 sec of every 1 sec, until the tension was nearly 0.

Intracellular pH measurements were made on both control and fatigued fibers (8–20 min after fatigue) by measuring the potential between a commercial pH microelectrode (Microtode, David Kopf, Ints. Tejunga, CA) and a glass micropipette filled with 3 M KCl and inserted in the same muscle fiber. Each microelectrode was connected to a cathode follower (W-P Instruments Co.) and the outputs were recorded differentially. Before and after each experiment, the pH microelectrode was calibrated against the glass micropipette with four different standard pH solutions.

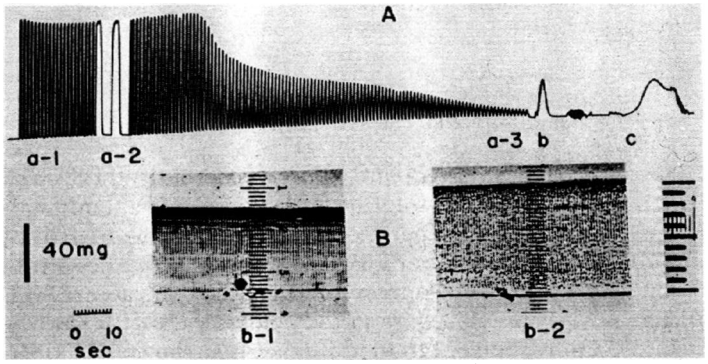

FIG. 1. Development of fatigue. (*A*) a-1, control tetanic stimulation; a-1 to a-3, periodic tetanic stimulation (50 shocks per sec during 0.3 sec of every 1 sec); b, K contracture obtained by equimolar replacement of NaCl by 80 mM KCl; c, caffeine contracture stopped by washing with Ringer's solution. a-2 was recorded 5 times faster. Fiber diameter: before the sequence was started, 77 μm; after the sequence ended, 108 μm. Temperature, 20°. (*B*) Light micrograph of a single fiber before (b-1) and after (b-2) the development of fatigue. Note the increase in fiber diameter and the appearance of vacuoles in b-2. (Stage micrometer calibration, 100 μm between extreme bars.)

Fiber bundles used for electron probe analysis were mounted with one tendon attached to an end of the stainless steel mesh holder used for freezing and the other to the mechanotransducer. When fatigue was established, the tendon attached to the transducer was released and hooked to the other end of the mesh holder; the holder was rapidly transferred to the air gun, frozen within 60–150 sec in Freon 22 supercooled to −164° ± 2°, and cryosectioned for electron probe analysis (10, 11). The details of the method for quantitative electron probe analysis have been published (12, 13). Analyses were done on a liquid N_2-cooled stage at approximately −160°.

Freeze-substitution in acetone containing 5–10% osmium tetroxide was performed over 3 days at −80°, followed by warming to room temperature and infiltration with Spurr's resin (14).

RESULTS

Physiological Experiments and Light Microscopy. Fatigue of a frog semitendinosus fiber is illustrated in Fig. 1*A*. When

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Table 1. Elemental content of muscles

Conditions*	No. of analyses	Stroph.	Content, mmol/kg dry weight Na	Mg	P	S	Cl	K	Ca
Cytoplasm, control (small probes)	30	–	29 ± 13	.39 ± 5	317 ± 12	216 ± 9	42 ± 4	488 ± 16	1 ± 2
Cytoplasm, fatigue	11	–	101 ± 27	57 ± 12	299 ± 12	267 ± 10	142 ± 7	368 ± 11	0 ± 4
(small probes)	7	+	120 ± 36	42 ± 15	216 ± 13	270 ± 12	118 ± 8	285 ± 11	2 ± 5
Cytoplasm, fatigue	26	–	140 ± 8	47 ± 3	290 ± 3	224 ± 2	133 ± 2	332 ± 3	8 ± 1
(large probes)	10	+	101 ± 13	33 ± 5	236 ± 5	200 ± 4	110 ± 3	251 ± 4	5 ± 2
Terminal cisternae, control (small probes)	30	–	44 ± 14	40 ± 6	449 ± 16	208 ± 0	54 ± 5	587 ± 19	66 ± 5
Terminal cisternae, fatigue	15	–	169 ± 20	64 ± 9	483 ± 11	256 ± 7	120 ± 5	523 ± 10	102 ± 4
(small probes)	13	+	164 ± 13	59 ± 6	379 ± 6	235 ± 4	106 ± 3	396 ± 5	74 ± 2

Ten muscles were fatigued without and three with strophanthidin or ouabain (0.3 mM for 30–60 min). Control values are from Somlyo *et al.* (10). Data are shown as means ± SEM.
* Small probes, 50–100 nm; large probes, 0.5–10 μm.

tension was nearly 0, a K contracture was produced followed by a wash with Ringer's solution and exposure to 2.6 mM caffeine. In seven similar experiments, the average mean (±SEM) K contracture tension after fatigue was 32 ± 7.5% of controls; the rate of tension development in the same contractures was 16.1 ± 3.9% of previously reported control values (9, 15).

Light microscopy of fatigued single fibers showed vacuolation and an increase in total fiber volume by approximately 80% (Fig. 1*B*). The vacuolation was not blocked by pretreatment (30–60 min before stimulation) with 0.3 mM ouabain or strophanthidin. The changes in tetanic tension, fiber diameter, and vacuolation due to fatigue were reversible; within 15 min after the end of fatigue, 30% of the control tetanic tension could be obtained. The vacuoles were difficult to find 30 min after fatigue.

Electron Microscopy of Frozen-Dried Sections and Freeze-Substituted Material. Frozen-dried sections of fatigued muscle (Fig. 2) clearly showed the sarcomeres, mitochondria,

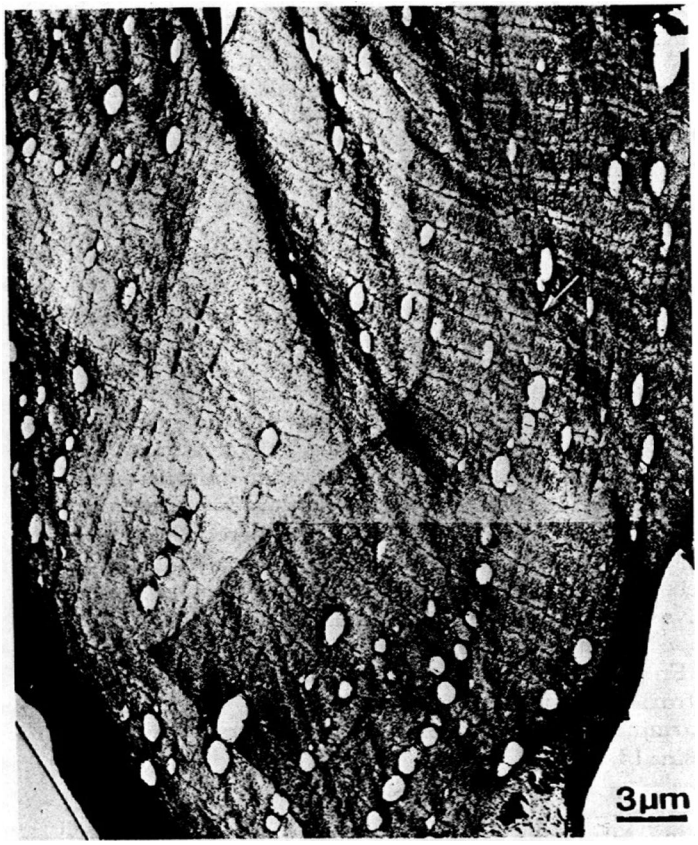

FIG. 2. Montage of unstained frozen-dried section of a fatigued muscle fiber. The vacuoles frequently occurred in longitudinal rows, occasionally at sites of vernier shifts (arrow) which are regions associated with mitochondria and longitudinal T system (32). Vacuoles also occurred in the region of the triads.

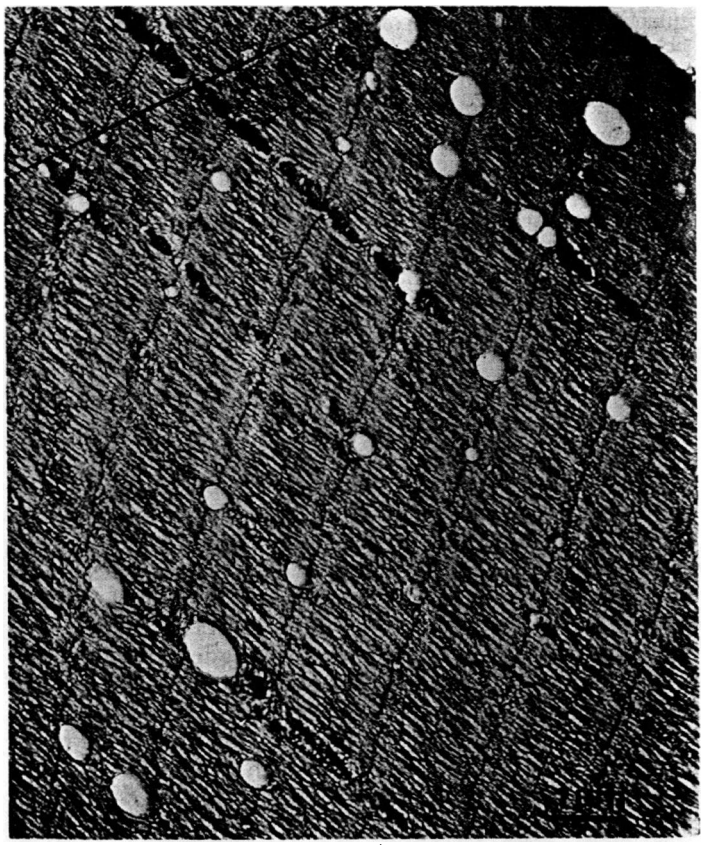

FIG. 3. Unstained frozen-dried longitudinal section of a fiber fatigued after 60-min exposure to 0.3 mM strophanthidin. Vacuoles are present. Arrows, mitochondria.

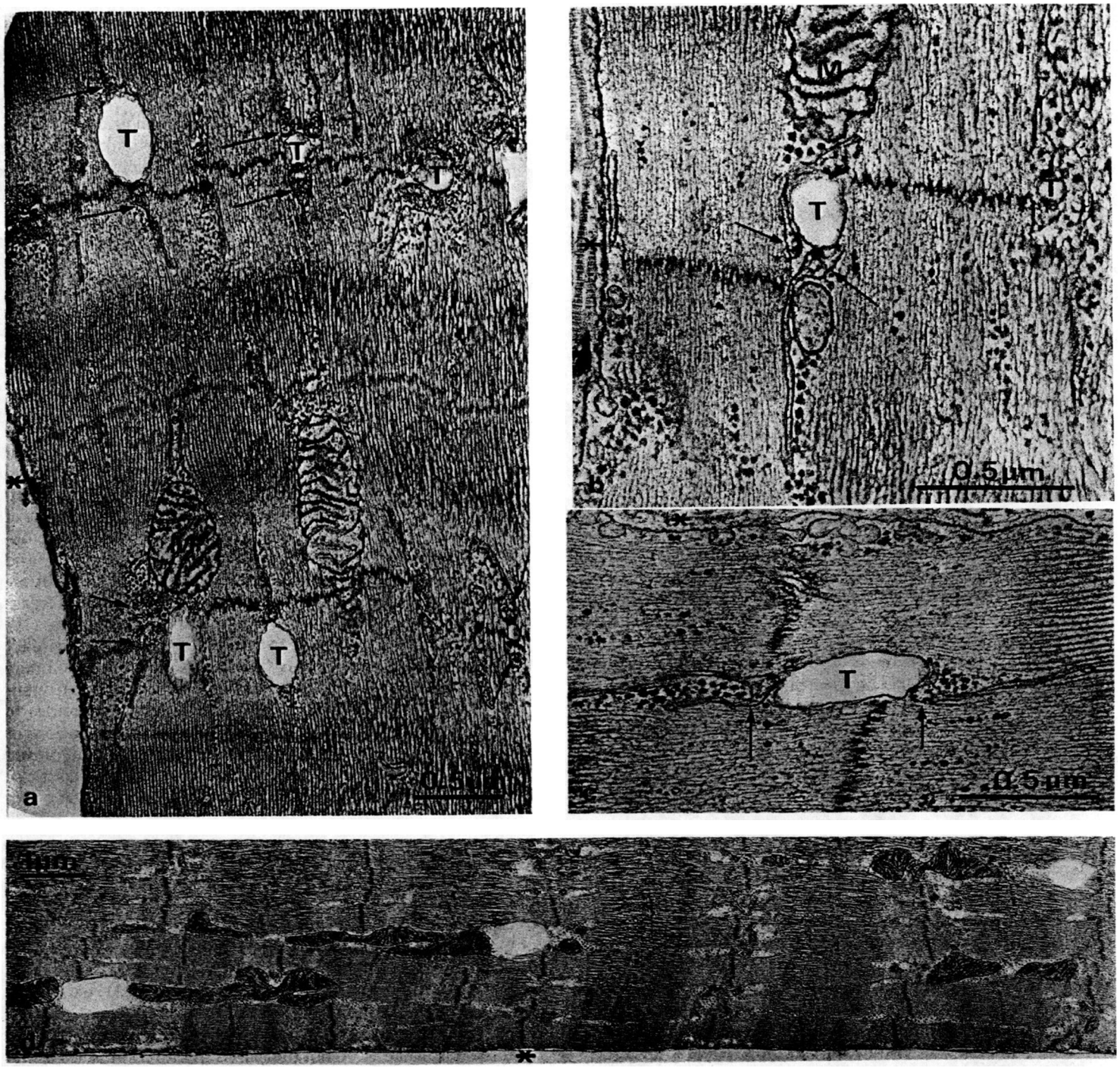

FIG. 4. Freeze-substituted fatigued fibers, showing swollen and vacuolated T-tubules (T) and normal sarcoplasmic reticulum and mitochondria (M). Arrows, terminal cisterna; *, best frozen (ice crystal-free) outer aspect of muscle.

and terminal cisternae of the sarcoplasmic reticulum described in unfixed sections of normal muscle (10, 11), although small ice crystals were more common in fatigued than in normal muscle, presumably due to the swelling of the fatigued fibers. The most striking finding in fatigued fibers was the presence of vacuoles, most commonly located in longitudinal rows between mitochondria. Single and occasional paired vacuoles at the Z lines were also seen. Ouabain or strophanthidin did not block the development of vacuoles (Fig. 3). The presence of normal terminal cisternae suggested that the swelling did not originate in the sarcoplasmic reticulum.

The same extent and distribution of the vacuoles were observed in freeze-substituted sections of the same muscles (Fig. 4). At higher magnifications in freeze-substituted sections, the vacuoles were seen to be membrane-bound and continuous with a swollen T-tubule system and sometimes adjacent to (but not continuous with) displaced elements of the sarcoplasmic reticulum (Fig. 4*b*).

Electron Probe Analysis. The cytoplasmic elemental concentrations in fatigued muscles are shown in Table 1. Compared with normal values, fatigued muscles gained Na and Cl and lost K. Muscles treated with ouabain or strophanthidin did not gain more Na. The cytoplasmic Ca concentrations measured with small probes are subject to the error due to occasional inclusion of longitudinal sarcoplasmic reticulum in the cytoplasmic regions analyzed, because the fibrils are narrow at long sarcomere

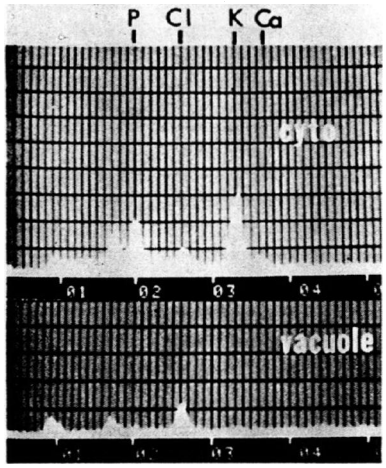

FIG. 5. Spectra of a region of cytoplasm (*Upper*) and of a neighboring vacuole (*Lower*) in a fatigued semitendinosus fiber. The number of x-ray counts on the ordinate are shown for the energies (keV) on the abscissa. Note the high Cl in the vacuole and high K in the cytoplasm.

lengths. Further experiments will be required to obtain an accurate estimate of cytosolic Ca in fatigued fibers.

The vacuoles had high Na and Cl contents but low organic (as judged by x-ray continuum count) content. Typical spectra obtained from a vacuole and from the adjacent cytoplasm are shown in Fig. 5. However, the contents of large aqueous domains are not homogeneously preserved in frozen-dried sections; they either form threads across the vacuole or are precipitated on its walls (10). The spectra obtained from threads were frequently of NaCl, but some of the analyses of edges of vacuoles gave rise to "mixed signals" to which both the vacuole contents and portions of cytoplasm contributed. Nevertheless, whether comparisons with adjacent cytoplasm were made on the basis of concentration or in terms of the absolute elemental mass (proportional to the total characteristic peak counts generated by identical probe parameters), the vacuoles had a characteristically higher NaCl content than did the cytoplasm.

The elemental composition of the terminal cisternae of fatigued muscle (with or without strophanthidin treatment) and of paired adjacent cytoplasmic regions (within 100–150 nm of terminal cisternae) analyzed with 50- to 100-nm probes also is shown in Table 1. The terminal cisternae had higher Ca and P contents than previously found in resting muscles (10, 11). The differences and similarities between sarcoplasmic reticulum and fiber composition in fatigued muscle were the same as those found previously (10, 11) in normal resting muscle: Ca, K, and P concentrations were significantly higher in the terminal cisternae, and the Na and Cl concentrations were comparable to the cytoplasmic values.

The mitochondria were not Ca-loaded in fatigued muscles but contained only 2.2 ± 1.7 mmol/kg dry weight (mean ± SEM, $n = 14$).

pH. The mean intracellular pH in control sartorii (15 measurements in two muscles) was 7.31. In fatigued muscles, the mean pH was 6.27 (45 measurements in three muscles from three frogs).

DISCUSSION

We have demonstrated that vacuolation involves the T-membrane system and that the Ca concentration in the terminal cisternae is not decreased in fatigued muscles. Through the use of rapid freezing techniques we have avoided objectionable aspects of chemical fixation that may alter the distribution of swollen cellular structures (ref. 16; C. Franzini-Armstrong, J. E. Heuser, T. S. Reese, A. P. Somlyo, and A. V. Somlyo, unpublished data.). The communication of the vacuoles with unswollen elements of the transverse T system and their occasional relationship to adjacent sarcoplasmic reticulum together with the results of electron probe analysis of their contents (see below) are all compatible with their identification as part of the T-tubule system. The preferential location of the vacuoles in the longitudinal T system (17), rather than at the T tubules of the triads, is reminiscent of the vacuolation observed during the efflux of permeant solutes from muscle (18, 19).

The vacuoles were high in Na and Cl content. Because the vacuolation itself was not blocked by ouabain or strophanthidin, it is unlikely that it is mediated by the Na,K pump. It may be due to the movement of permeant metabolites into the T-tubule system, followed by the entry of extracellular water and, through solvent drag, extracellular electrolytes. Exit flow may be impeded by compression of the transverse elements and mouths of the T tubules due to swelling of the fibers. Our findings suggest that the slow inulin efflux with complicated kinetics observed by Gillis (20) in fatigued muscle represents the escape of the tracer from T-tubule vacuoles. The results of electron probe analysis are also in agreement with those obtained with atomic absorption spectrophotometry (21) and showing that fatigued muscles gain Na and lose K; in addition, our results show a gain of Cl by fatigued muscle. The increase in Cl_i may have been due to the operation of a Cl pump (10) becoming more effective due to the decrease in Cl permeability caused by the decrease in pH (22, 23). Alternatively, Cl may have entered in association with Na to maintain electroneutrality. Fatigued muscles treated with cardiac glycosides to inhibit the Na pump did not contain higher concentrations of Na than did untreated fatigued muscles, suggesting that, during fatigue, the Na pump is nearly completely inhibited. This is also in agreement with Gillis' observation (21) that extrusion of Na from fatigued frog muscle was incomplete even after recovery for 7½ hr, also ascribed to suboptimal function of the Na pump.

The Ca content of the terminal cisternae was significantly higher in fatigued than in unstimulated muscles, possibly due to the decreased pH (to 6.27) in fatigued muscle. The amount of Ca bound by fragmented sarcoplasmic reticulum is increased 50% with a similar decrease in pH (24). Therefore, some or all of the increased Ca content of fatigued muscle (21) can be accounted for by the increased stores in the sarcoplasmic reticulum. However, because control values of the Ca content of the terminal cisternae were not measured in paired muscles of the fatigued preparations, the possibility of frog-to-frog variations contributing to the higher Ca in the sarcoplasmic reticulum of fatigued muscles has not been excluded.

Mitochondrial Ca content in fatigued muscles was relatively low, indicating that uncoupling due to mitochondrial Ca loading is not a mechanism of fatigue.

The mechanism of fatigue remains to be established, although the present study clearly shows that it is not due to depletion of Ca from the terminal cisterna. Alternate mechanisms that may contribute to fatigue are (*i*) failure of the action potential in the T tubules (25, 26) secondary to their swelling and (*ii*) decreased affinity of a binding site (i.e., troponin) for Ca due to the decreased pH (27, 28) caused by the increase in muscle lactic acid (29, 30). The persistent response of fatigued muscles to high K or caffeine (refs. 5 and 31 and present study) and the high Ca content of terminal cisternae are both com-

patible with failure of the T-tubule action potential as a mechanism of fatigue.

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