SARCOPLASMIC RETICULUM OF AN UNUSUALLY FAST-ACTING CRUSTACEAN MUSCLE

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ABSTRACT

The fast-acting, synchronous "remotor" muscle of the lobster second antenna was examined by light and electron microscopy and was found to have a more profuse sarcoplasmic reticulum (SR) than any other muscle known. Myofibrils are widely separated from one another and occupy only about one-fourth of the volume of the muscle; most of the remaining volume is taken up by the SR, which resembles the smooth-surfaced reticulum of steroid-secreting cells. Dense granules (0.03–0.1 μ in diameter) are scattered through the reticulum. T-tubules penetrate into the fibers and form dyads along the A bands of myofibrils; however, ferritin-labeling experiments show that the volume of the T-system is very small compared with that of the SR. Myofibrils are $\sim$0.5 μ $\times$ 1.0 μ in cross section and consist of thick filaments, which appear tubular except at the M region, and thin filaments, which are situated midway between neighboring thick filaments. The ratio of thin to thick filaments is 3:1. The extreme development of the SR in this muscle is discussed in relation to the exceedingly short duration of the contraction-relaxation cycle.

INTRODUCTION

Comparisons of fast and slow vertebrate striated muscles have shown that fast-acting fibers are characterized by a prominent system of "T-tubules" and by the regular occurrence of "triads" and "dyads" in relation to the band pattern of the myofibrils (8, 11, 17, 24). The relative volume of the sarcoplasmic reticulum (SR) is also somewhat larger than in slow muscles, but this difference is not very striking; even in the fastest vertebrate muscle fibers, the percentage of cell volume occupied by SR is relatively small, amounting to no more than 10% of the total volume (24). These generalizations apply also to invertebrate muscles (7, 22, 30), except for the asynchronous flight muscles of insects, which lack SR almost altogether (29).

The present report describes the structure of a lobster muscle which is responsible for sound production at fundamental frequencies of 100–130 cycles per second (9). The muscle contracts synchronously at these rates and has a contraction-relaxation cycle comparable in duration to that of the fastest vertebrate muscles (14, 15). As expected, T-tubules and dyads are prominent, and the myofibrils are typical of those in other fast-acting invertebrate muscles (3, 6, 22, 29). However, the most outstanding ultrastructural feature of the lobster muscle, which was quite unexpected, is an extraordinarily profuse sarcoplasmic reticulum, the volume of which greatly exceeds that occupied by the myofibrils. No other example of a muscle with such a voluminous SR is known among vertebrates or invertebrates. The significance of this structural specialization is discussed in relation to the physiological characteristics of this muscle which are reported separately in the accompanying paper (15).

METHODS

The dorsal carapace surrounding the second antenna of the lobster (*Homarus americanus*) was removed with the antenna, and the fleshy "remotor" muscle (27) was cleaned and either flooded with fixative in situ or dissected free of its attachments and immersed in fixative. Three fixation procedures were used. In one, the tissue was exposed first to a solution of approximately 4% glutaraldehyde (biological grade) in 0.1 M phosphate buffer (pH 7.5). After 1–3 hr, the specimens were rinsed in either sea water or 0.9% NaCl. The large fast portion of the muscle was separated from the smaller, anatomically separate, slow component and cut into 1 mm strips which were then postfixed in phosphate-buffered or Veronal-acetate-buffered 1–2% OsO_4 (pH 7.5) for an additional 1–3 hr. In the second procedure, the buffered OsO_4 solution was used as a primary fixative for 1–3 hr, and in the third, the fixative consisted of 3% $KMnO_4$ for 4 hr. The fixed strips of muscle were rinsed in sea water or 0.9% NaCl, dehydrated in a graded series of methanol solutions followed by absolute propylene oxide, and embedded flat in Araldite 502 (Ciba). 1-μ sections were stained for light microscopy with toluidine blue for survey purposes, or for glycogen with a solution of 4% iodine in 8% aqueous potassium iodide, or by the periodic acid-Schiff (PAS) method. In order to estimate the percentage of the muscle occupied by myofibrils, photomicrographs of toluidine blue-stained transverse sections were enlarged to a magnification of 2400–8000 × and overlaid with transparent graph paper (20 subdivisions per inch). The area overlying myofibrils was pencilled in and calculated as percentage of total area. Thin sections were stained with uranyl acetate and lead hydroxide and examined in a Zeiss EM 9, a Siemens Elmiskop, or a Philips EM 300 electron microscope at 40, 60, or 80 kv.

In one set of experiments, muscle specimens were soaked in ferritin solutions prior to fixation. Ferritin was obtained from Nutritional Biochemicals Corp. (Cleveland, Ohio) (2 × crystallized, cadmium free) in aqueous solution (approximately 0.1 g/ml). One part of this solution was mixed with one part natural sea water boiled down to half its original volume. The muscles were removed from the animals intact and still attached to the carapace and base of antenna and were submerged in the ferritin solution for periods of 15–150 min, and then were fixed in glutaraldehyde and osmium tetroxide as described above. In order to avoid spread of ferritin along artifactually produced pathways, the muscles were not dissected until after removal from the osmium tetroxide. The ferritin-containing specimens were stained with uranyl acetate only, in order to avoid possible confusion between ferritin molecules and fine lead-stain deposits.

OBSERVATIONS

Grossly the fast muscle has a translucent, white color and is composed of coarse fascicles which run approximately parallel to the muscle axis and impart a corrugated appearance to its surface. In transverse sections (Fig. 1) muscle fascicles are seen to be surrounded by a thin perimysium from which connective tissue septa extend inward and branch repeatedly. The smallest subdivisions created by the septa are ~50–100 μ in diameter. However, the septa are discontinuous, and consequently, the subdivisions remain interconnected and constitute a syncytium at least several hundred microns in diameter. The limits of the syncytium are not clearly defined. Multiple nuclei occur both along the surface of the fascicle and in its depths.

Histologically the most distinctive aspect of the crosss-sectioned muscle is its mottled texture (Fig. 2). Innumerable dense, polygonal profiles, which represent sections of myofibrils, are distributed randomly throughout a pale, homogeneous, nonbasophilic matrix which is PAS negative and has no affinity for iodine. The myofibrils occupy ~23% of the total cross-sectional area of the muscle. Individually their profiles measure approximately 0.5 μ × 1.0 μ and they display some tendency to form short rows. Although the rows themselves are separated by ~2 μ or more, the members of each row are much closer together (approaching 0.1 μ). For this reason, longitudinally-sectioned myofibrils may appear closely associated in one part of a section and widely spaced in another.

Sarcoplasmic Reticulum

Even in low-power electron micrographs (Fig. 3), it is immediately obvious that the homogeneous matrix surrounding the myofibrils is composed primarily of membranous profiles rather than undifferentiated sarcoplasm, mitochondria, or particulate accumulations. If myofibrils were not present, this membranous system would closely resemble that which occurs in steroid-secreting cells (5). It consists of innumerable sacs in close apposition to one another, the profiles of which are either elongated or circular (Fig. 4). Their pattern is consistent with the appearance of a section through either an interconnected reticulum of cisternae or multiple elongated vesicles. By and large the membranes do not have a significant particulate component associated with them but in some locations (Fig. 5) a moderate number of

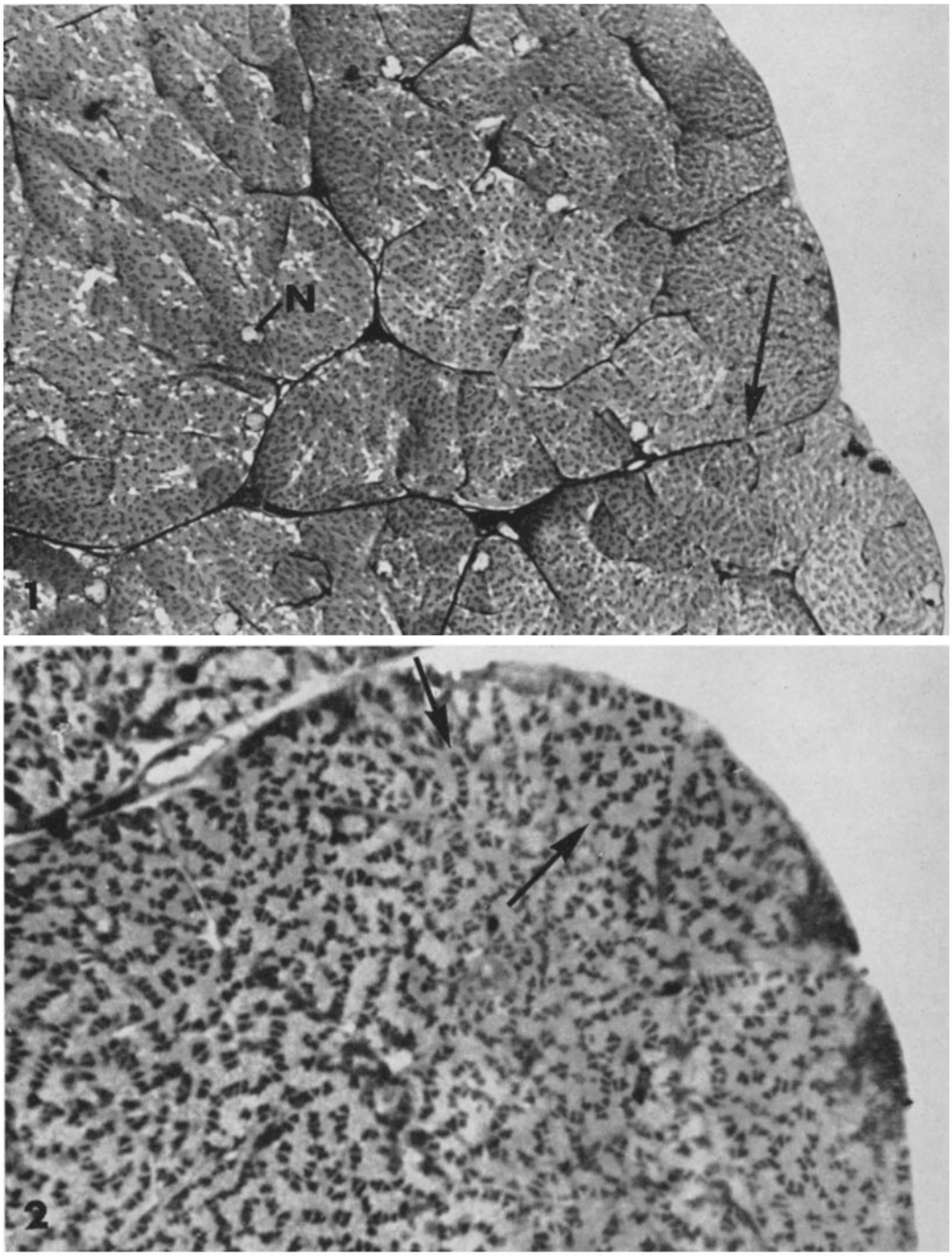


FIGURE 1 Survey photomicrograph of transverse section. The muscle is subdivided by dark connective tissue septa into irregular polygons ~50–100 μ in diameter. The septa appear to be discontinuous and the polygonal areas confluent. Even the one long septum which seems to separate two fibers from each other has a discontinuity in it (arrow). The mottled aspect of the muscle is obvious. *N*, nucleus. × 275.

FIGURE 2 High power photomicrograph of transverse section. The sections of myofibrils appear as dense angular profiles against a pale background. In some places the myofibrils are aligned in rows (arrows). × 1600.

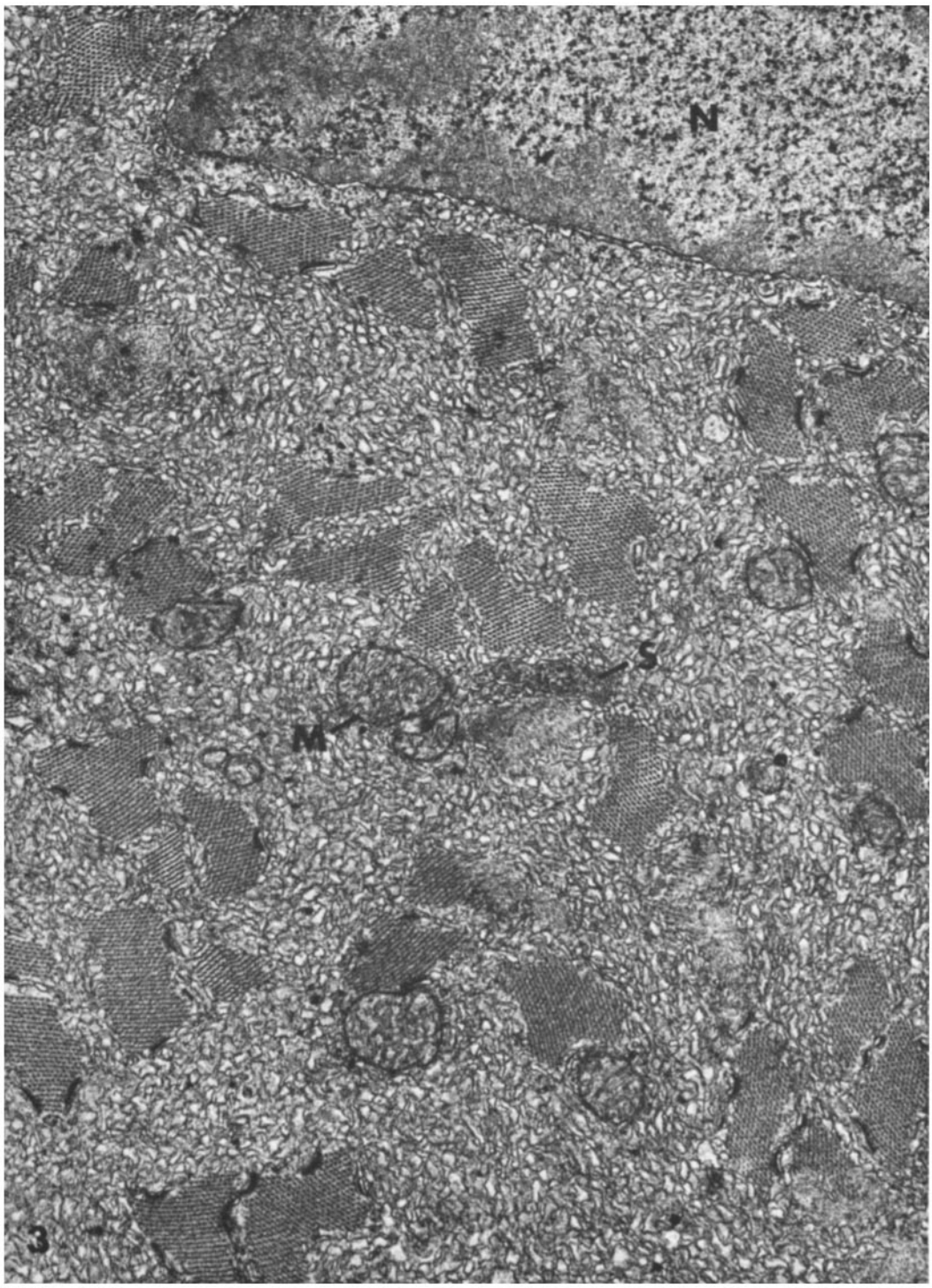


FIGURE 3 Survey electron micrograph of transverse section. Part of a nucleus (*N*) is at the top. The sarcoplasm surrounding the myofibrils is filled up with innumerable cisternae. Mitochondria (*M*) and part of a connective tissue septum (*S*) are also visible. The linear densities along the edges of the myofibrils are dyads. × 15,000.

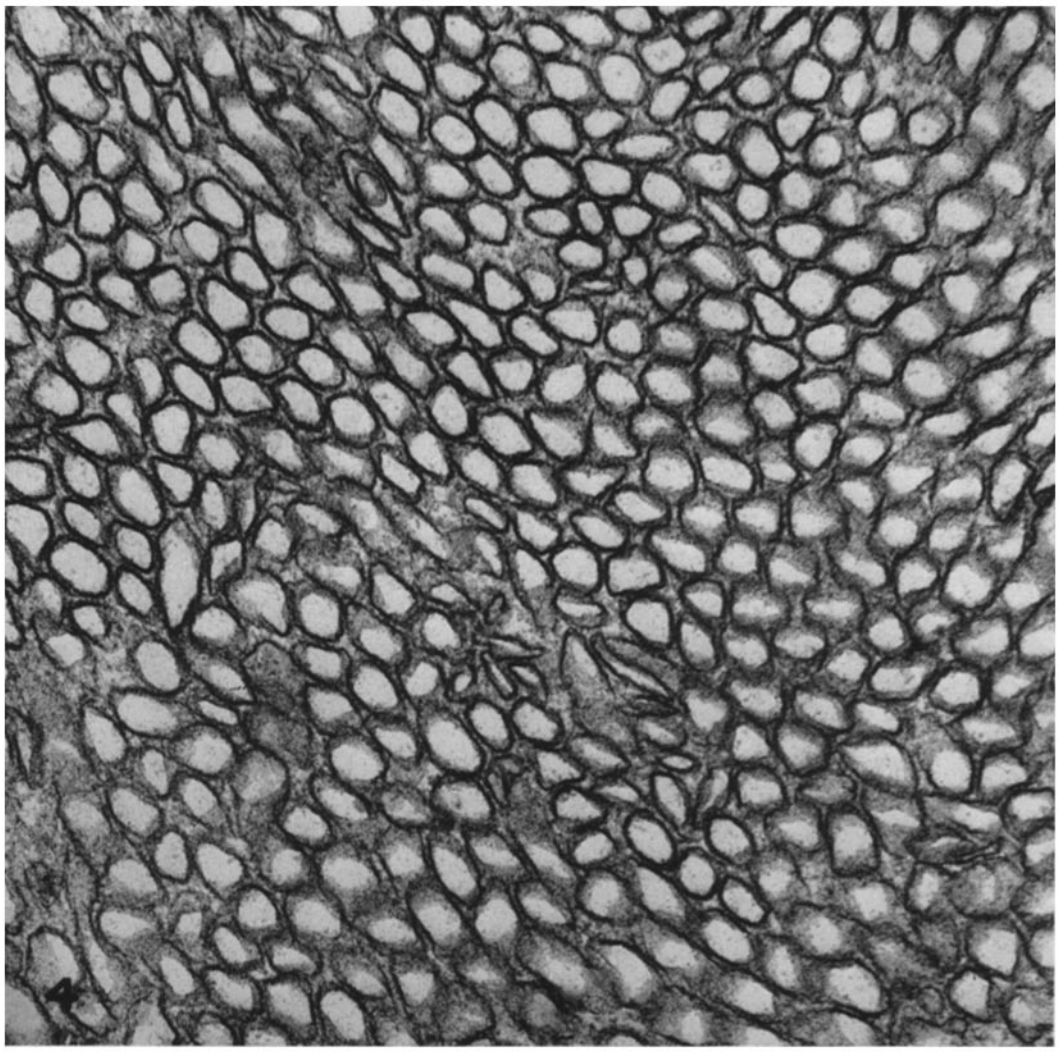
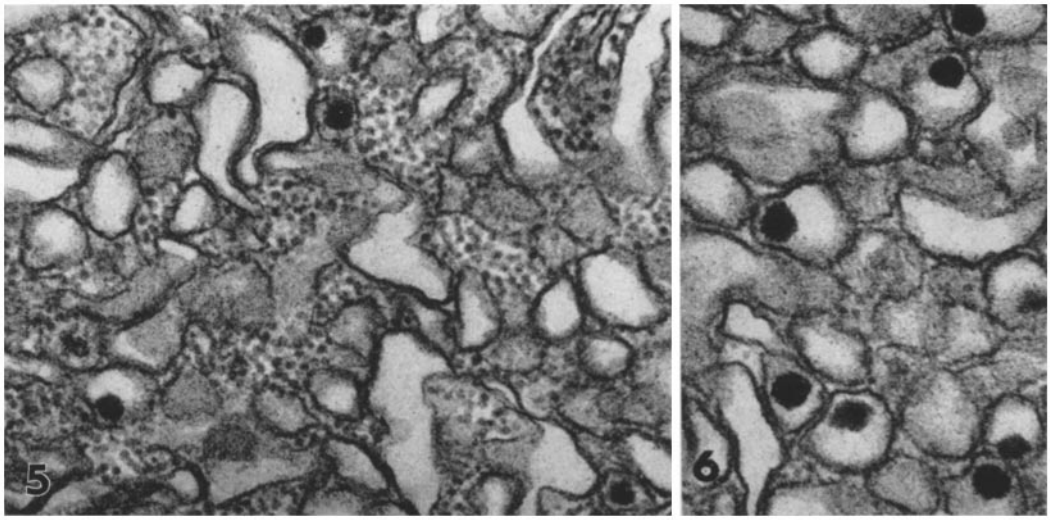


FIGURE 4 Sarcoplasmic reticulum. The vesicles and cisternae here are smooth surfaced, close together, limited by a single membrane, and apparently empty. × 66,000.

particles ~150–200 A in diameter lie in the interstices between the sacs apparently not attached to the outer surfaces of the membranes. The particles are stained by uranyl acetate but have no special affinity for lead stains. They do not form chains and rosettes. (The sarcoplasmic reticulum of vertebrate muscles also has comparable particles associated with it in approximately the same proportion [16].) In permanganate-fixed muscles, some particles are still visible immediately around the myofibrils but very few are preserved elsewhere.

The membranous sacs appear empty for the most part but scattered through them are dense intracisternal granules which are approximately 0.03–0.1 μ in diameter (Figs. 3 and 6). In one instance, a granule was seen in the SR half of a dyad. These granules are denser and smaller than the "specific" granules that occur in muscles of the vertebrate cardiac atrium (13), but are similar to the divalent cation granules that accumulate in mitochondria (18) (Fig. 7). They are not noticeably concentrated near the cell surface, myofibrils, or mitochondria, and no evidence for their



FIGURES 5 and 6 Sarcoplasmic reticulum.

FIGURE 5 The cisternae here are more widely spaced and are surrounded by ~150–200 A particles which appear not be attached to the membranes and which do not form rosettes. Four intracisternal granules are visible. × 69,000.

FIGURE 6 Only a few extracisternal particles are visible but seven very dense intracisternal granules are present. The latter are loosely bounded by the cisternal membrane and measure approximately 0.06 μ in diameter. × 77,000.

extrusion from the cells has been seen. Granules of this size have not been reported in the SR of vertebrate muscles. However, in some vertebrate preparations, "terminal cisternae" contain deposits of an unknown finely granular material (12).

T-System and Dyads

The connective tissue septa which penetrate into the muscle taper from several microns in width down to less than a half micron and contain multiple strands of a dense amorphous material (Fig. 7). If the muscle cell plasma membrane lining a septum is followed it can sometimes be seen to fold inwards, producing a narrow T-tubule (1), ~100 A wide, which is continuous with the connective tissue space. In occasional fortunate sections one can follow such a tubule from its origin at a connective tissue septum to the surface of a myofibril where it forms a typical dyad with a cisterna of the SR (Fig. 7). Occasionally, triads (20) are formed. The apposed membranes that form a dyad are strictly parallel to each other, and the interspace between them usually contains an intermediate density. The over-all width of the membrane pair is, however, ~250–300 A, indicating that these membranes do not form tight junctions with one another. The luminal surface of the SR membrane at the dyad sometimes has a radially oriented fuzzy coating of unknown significance. Such dyads form an interrupted collar around the A bands of the fibrils, usually with the SR component interposed between the T-tubule and the surface of the fibril. Dyads have been identified only in immediate proximity to the myofibrils and not in the interfibrillar regions. Relatively few mitochondria occur scattered through the muscle, and they seem to be preferentially associated with the plasma membrane but frequently adjoin T-tubules at dyads (Figs. 3 and 8).

An obvious question that arises from these observations is: What proportion of the membranous system in this muscle belongs to the sarcoplasmic reticulum and what part is continuous with the T-system? In certain fortunate instances, as described above, one can trace a T-tubule inward from the cell surface for some distance but not very far, and on the basis of morphology alone

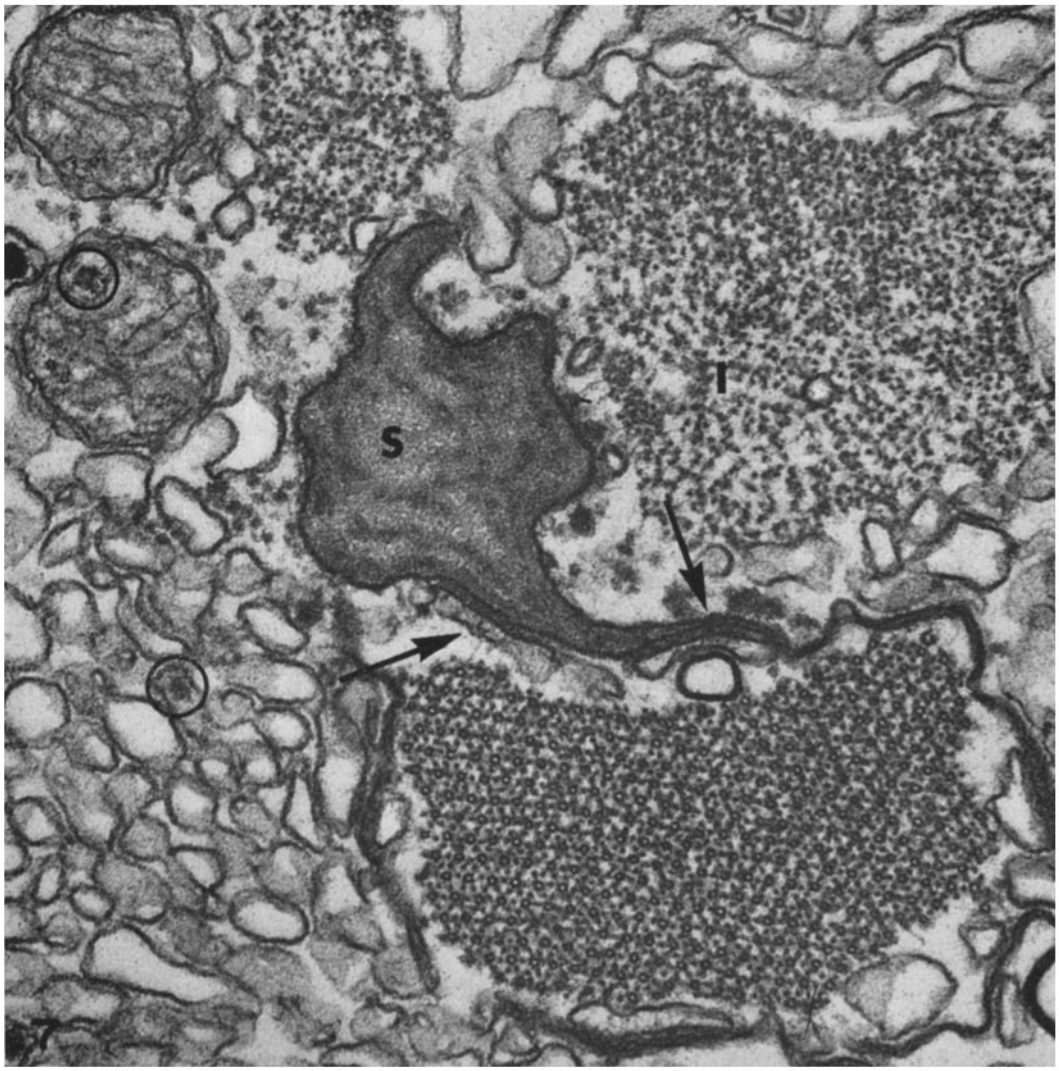


FIGURE 7 Connective tissue septum (*S*) funneling into a T-tubule. The limiting membrane of the septum forms two dyads (arrows) adjacent to a myofibril. Several intracisternal granules are visible in the SR, one of which (lower circle) closely resembles an intramitochondrial granule (upper circle). *I*, cross section through I band. × 75,000.

there is no way of distinguishing its further ramifications from cisternae of sarcoplasmic reticulum. Neither the limiting membrane nor the lumen of the T-tubule is distinctive. In order to determine the relative volume of the T-system, specimens of muscle were soaked in ferritin solutions on the assumption that the ferritin molecules would diffuse through the lumen of the T-system as they do through the T-tubules of other muscles (12) and through other narrow aqueous channels (26). Examination of thin sections of these soaked specimens (Fig. 8) showed the great majority of the membranous sacs within the muscle cells to be devoid of the tracer; only a small proportion of the profiles were found filled with ferritin molecules, usually immediately around myofibrils and often at dyads (Fig. 8, inset). Ferritin-containing profiles could be identified both near the cell surface and in

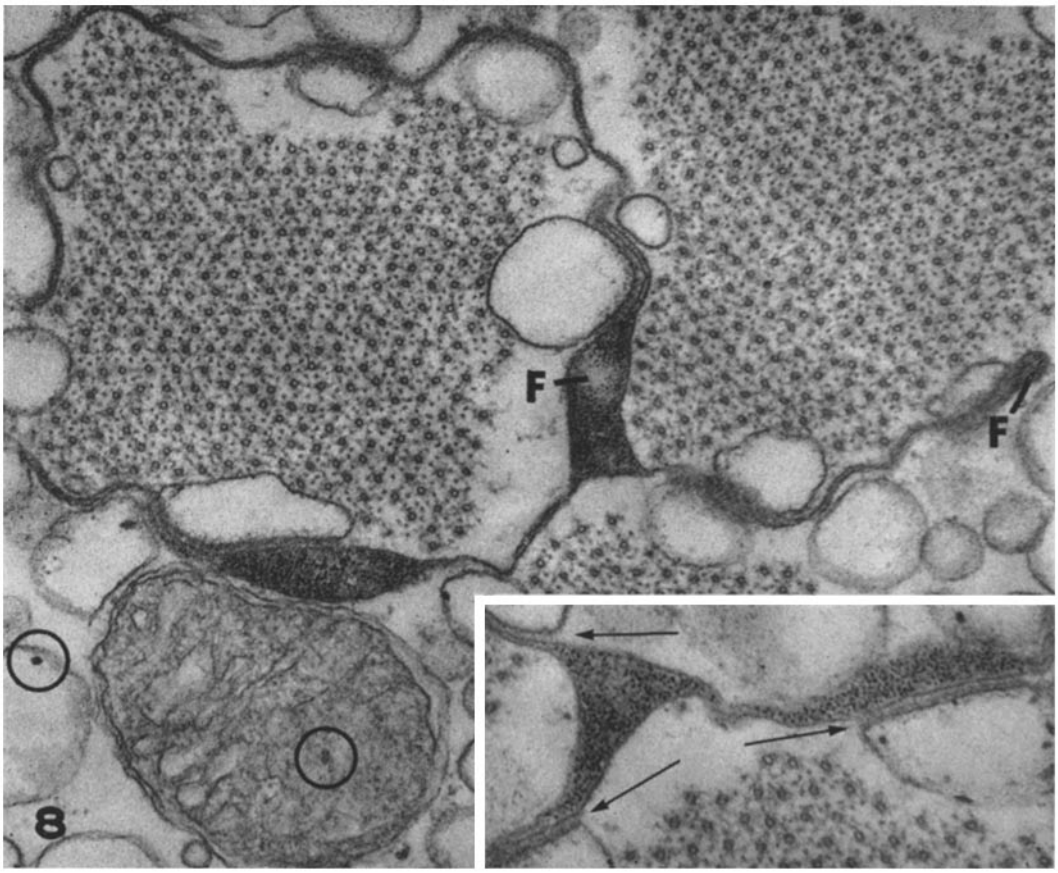


FIGURE 8 T-system containing ferritin. The caliber of the T-tubules is variable in this picture. Large numbers of dense ferritin molecules (*F*) have accumulated in the expanded portions. The surrounding SR cisternae are dilated and contain some dense inclusions (left circle) larger than ferritin molecules, but similar to intramitochondrial granules (right circle). × 63,000. Inset: T-tubule branching into three limbs each of which forms a dyad (arrow) with a cisterna of SR. × 74,000.

its depths. The findings confirm the supposition that a T-system penetrates into the depths of the muscle but indicate that the great bulk of the membranous system in this muscle corresponds to sarcoplasmic reticulum and is not continuous with the T-system. In the ferritin-treated specimens (Fig. 8) it was noted incidentally that intracisternal granules were encountered more frequently but were considerably smaller than in untreated muscle.

Myofibrils

In longtiduinal sections (Fig. 9) the myofibrils of the lobster fast antennal muscle resemble those of other fast invertebrate muscles (3, 6, 22, 29), except that the M band is very prominent and the A band is relatively long (3.2 μ). This is twice the length of the A band of vertebrate striated muscles (24). Oblique, nearly transverse, projections or ridges extend from the thick filaments with a repeat period of $\sim$ 400–450 A (Fig. 14).

In cross sections the thick filaments resemble microtubules; i.e., they look hollow (Fig. 13). However, they are only $\sim$165 A in diameter, except at the M band where they appear solid and are thicker (255 A) (Fig. 10). The thick filaments form a very regular hexagonal lattice with a center-to-center distance of $\sim$400 A. Thin filaments, which are approximately 80 A in diameter, are also in regular hexagonal array in the A band,

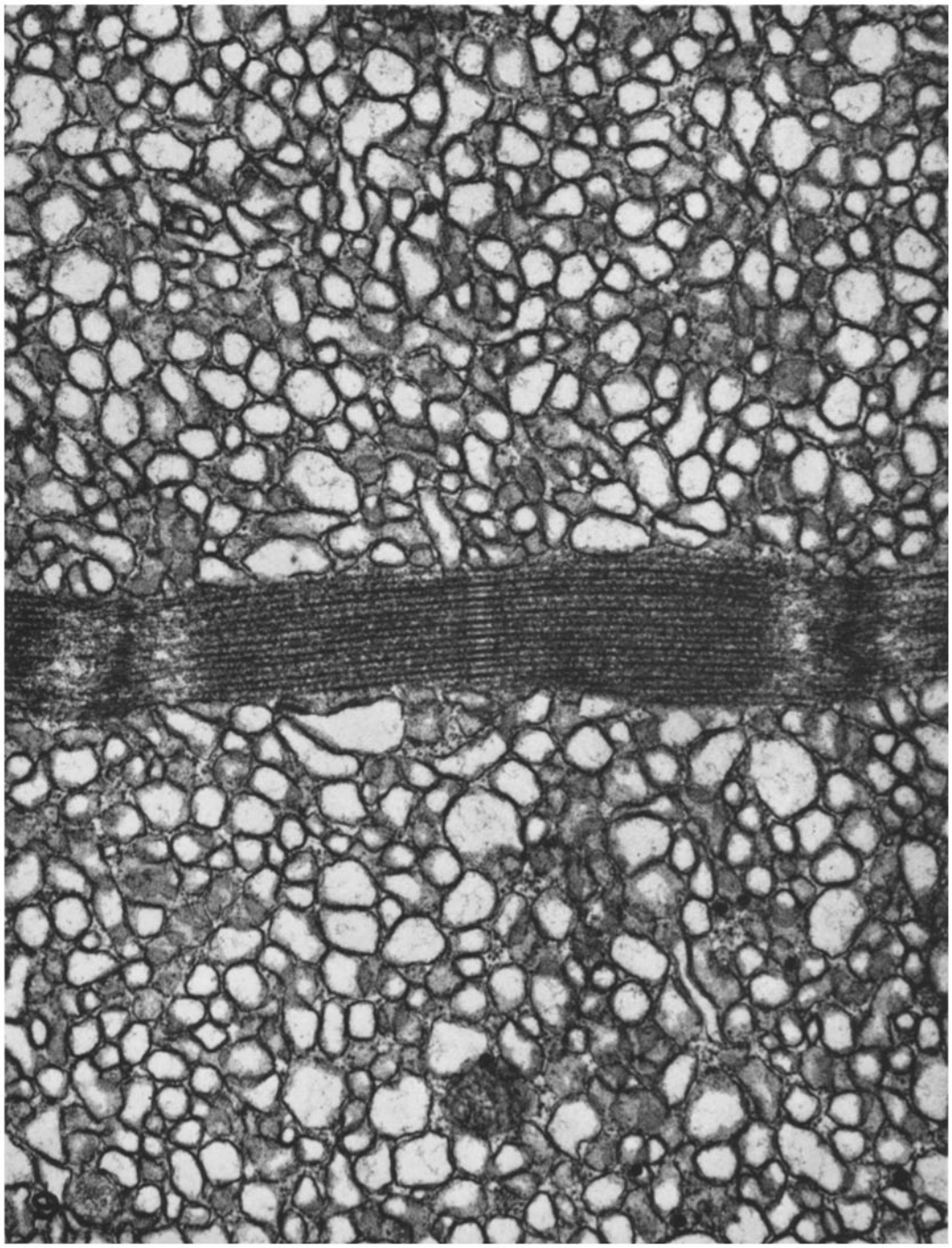
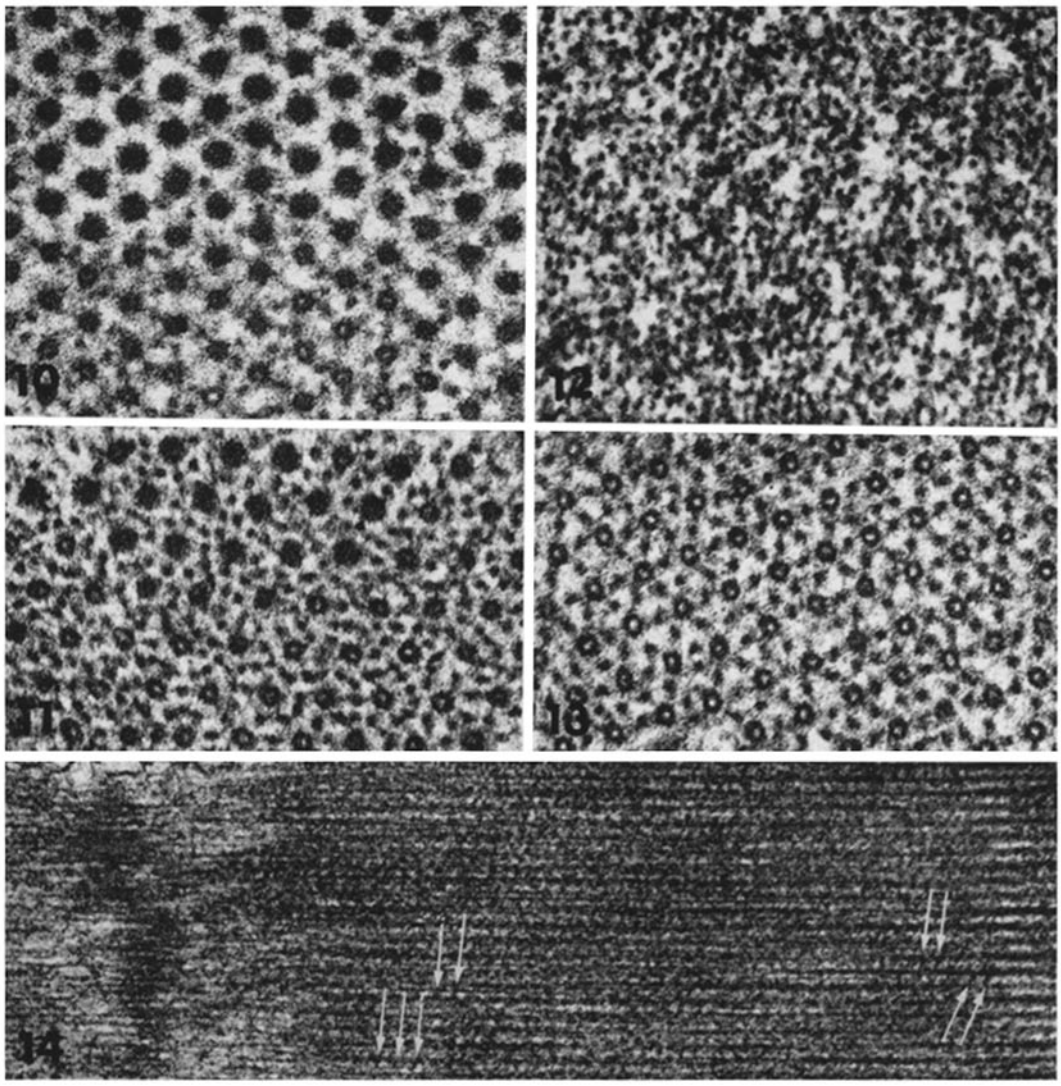


FIGURE 9 Longitudinal section of myofibril. One sarcomere is shown which has a very prominent M band in the center and rather broad Z bands at the sides. Here, as in transverse sections, the SR consists of short disconnected profiles rather than elongated cisternae. × 35,000.



FIGURES 10–14 Details of myofibrils.

FIGURE 10 Transverse section through M band. Thick filaments which are in hexagonal array appear solid and measure ~255 A in diameter. × 167,000.

FIGURE 11 Transverse section through M band. In this case, the thick filaments are surrounded by a double complement of thin filaments. × 167,000.

FIGURE 12 Transverse section through I band. Some of the thin filament profiles appear to be matted together by an amorphous material of medium density. The ends of several thick filament profiles are present. × 105,000.

FIGURE 13 Transverse section through A band. Thick filaments are in hexagonal array and appear microtubular. Thin filaments are located midway between the members of each pair of thick filaments. × 167,000.

FIGURE 14 Longitudinal section of myofibril. Portions of Z, I, A, and M bands are shown (from left to extreme right). Periodic projections from thick filaments are shown by arrows. × 56,000.

but are located midway between adjacent thick filaments rather than at the trigonal points (Fig. 13). The ratio of thin to thick filaments in this muscle is therefore 3:1. In cross sections through I bands, the thin filaments appear disordered (Fig. 12). At the M band, thin filaments are ordinarily absent but in some sections a double complement of thin filaments occurs at the mid-sarcomere level (Fig. 11), indicating that the sarcomeres are capable of shortening beyond the point at which thin filaments from opposite sides of the same sarcomere begin to overlap each other.

The slow component of the antennal muscle will not be described in detail here because it is typical of other crustacean muscles in all respects and these have been reported on extensively elsewhere (e.g. 7, 22). In brief, it contains T-tubules and SR but these structures are relatively sparse and occupy only a very small proportion of the cross-sectional area of the muscle. Thick filaments appear solid all along their length and are longer and thicker than those in the fast muscle. The ratio of thin to thick filaments is closer to 5:1 than 3:1, and the filament lattice is not nearly so regular as that of the fast muscle, which is virtually in crystalline array.

DISCUSSION

This report describes the structure of a fast-acting, synchronous muscle which is unique in that the major part of its cell volume is occupied not by myofibrils but by an extensive system of smooth-surfaced membranes. It is shown, moreover, that these membranes can be resolved into two components: a T-system connected to the extracellular space, and an enormous sarcoplasmic reticulum. Neither the myofibrils nor the T-tubules are remarkable in comparison with the equivalent structures in other fast muscles, but the sarcoplasmic reticulum is extraordinary in that its surface and volume are many times greater, relative to the myofibrils, than in any other muscle known. If hypertrophy of structure indicates hypertrophy of function, then the obvious question raised by the observations is whether this unprecedented sarcoplasmic reticulum represents an adaptation for high speed, or whether it has an entirely separate function in this muscle.

Calcium Flux

The available evidence indicates that contraction in synchronous striated muscles is initiated by the movement of calcium into the sarcoplasm surrounding myofibrils, and relaxation by the removal of that calcium by the sarcoplasmic reticulum (10, 19). The rate of calcium uptake presumably depends on the specific calcium binding or transporting activity of the SR membranes lining the sarcoplasm and on the surface area of the SR membranes relative to sarcoplasmic volume. Thus, in a muscle with a very short relaxation time, it is not surprising to find an SR with a very large surface area. A question arises, however, as to whether SR cisternae that are removed from the immediate vicinity of the myofibrils are close enough to be of any use in regulating calcium ion concentration within and around them.

This question has been answered theoretically by Van der Kloot (32), who calculated changes in calcium ion concentration at the center of a hypothetical 1 μ myofibril as a function of time and of the amount of surrounding sarcoplasmic reticulum. Assumptions about the rate of calcium uptake were based on previous experiments on lobster SR (31). In the case where the layer of SR surrounding the myofibril was assumed to be 0.2 μ thick, a 20% change in calcium concentration took approximately 2½ times as long as it did when the SR was assumed to be 0.4 μ thick and almost four times as long as when the layer of SR was taken to be 1 μ thick. These calculations suggest that the potential usefulness of the SR with respect to calcium exchanges has not been exceeded in this muscle.

A more troublesome question is why the SR of this particular muscle is so much more abundant than that of other fast-acting synchronous muscles a number of which, both vertebrate (4, 24, 28) and invertebrate (7, 22, 23, 30), can operate at rates approaching that of the muscle described here. Morphologically, such muscles are characterized by closely spaced and regularly occurring dyads and triads, and in Lepidoptera by elements of the SR which extend in among the myofilaments at the level of the M band (2, 23). However, in none of them is the SR particularly noluminous relative to the myofibrils (cf. Fig. 1 in reference 24).

Two possible bases for the enormous development of the SR in the lobster muscle are worth mentioning: (*a*) Membrane depolarization in this muscle is accompanied by an influx of calcium ions from the extracellular fluid (15). The SR may therefore serve the dual function of taking up the calcium that enters from outside as well as the

calcium that is released by the SR itself. (*b*) The temperature of the water in which lobsters live may vary from less than 5°C to ~25°C. If sound production constitutes defensive behavior, as Fish suggests (9), or is used for echolocation or some other vital function, then the sound-producing muscle may be equipped with an excess of SR in order to insure function at high rates even at extremes of temperature or under other conditions which might tend to reduce the maximum specific rate at which calcium is exchanged by the SR membranes.

Another question raised by the morphology of this muscle concerns the signal for calcium exchanges by the SR. If the entire SR participated in alternately taking up and releasing calcium at a frequency of 100 cycles per second, then a signal would have to reach all parts of the SR synchronously at this rate. Dyads may serve to transmit either AC or DC signals or a combination of the two from sarcolemma to SR; however, it is not clear whether the SR consists of intercommunicating cisternae or isolated vesicles. In the latter case, it is difficult to see how an electrical signal could spread throughout the SR.

This problem would be circumvented if contraction were triggered directly by the calcium ions entering the fiber from the extracellular space across the sarcolemma and T-tubule membrane and if the SR were concerned only with relaxation, i.e., with the removal of calcium from the sarcoplasm and its eventual extrusion from the cell. No signal would then need to reach the SR from the cell surface. Alternatively, the particular cisternae that form dyads might be coupled to the T-tubules but the remainder of the SR not coupled. In this case the terminal cisternae, immediately around the A band, would be concerned with alternating calcium release and uptake, and the rest of the SR only with the uptake and extrusion of calcium. Presumably all parts of the SR are connected, at least intermittently, so that inequalities in the distribution of the calcium that might develop within the SR would be only temporary.

Other Functions

Aside from whatever role it may play in calcium exchanges, all or part of the sarcoplasmic reticulum in these muscle fibers may have other functions as well. Mitochondria are relatively sparse and usually are not closely associated with the myofibrils. The possibility exists, therefore, that energy metabolism in this muscle entails microsomal enzymes. On the other hand, the paucity of mitochondria may indicate merely that the muscle does relatively little work and not that it depends on special substrates or enzymes associated with the SR.

The presence of granules within the SR cisternae and of some ribosomes outside suggests the additional possibility that these muscle cells may be engaged in the manufacture of a secretory product. In principle, the coupling of two seemingly unrelated functions (contraction and secretion) in the same cell could serve a useful purpose if it were advantageous for these functions to coincide. However, dense inclusions have also been reported in mitochondria (18) and in isolated SR vesicles which have accumulated calcium (10), and the densities described here may therefore represent nothing more than deposits of calcium or a calcium-binding material. Whatever free ribosomes are present among the sacs of SR may thus be concerned not with the synthesis of a secretory product but with the replenishment of the SR membrane itself or of an ATPase or calcium-binding protein associated with the SR, or with the production of actin and myosin. The possibility of the synthesis and secretion of a steroid, as suggested by the similarity of the SR to the ER of steroid-secreting cells (5), cannot be excluded.

Although the bulk of the extraordinary SR in this muscle may ultimately prove to have some function unrelated to the control of the contraction-relaxation cycle, the simplest assumptions for the present are that the SR here as in other muscles controls myofibrillar activity by regulating calcium concentration in the sarcoplasm, that this muscle is one of the fastest if not the fastest synchronous muscle known, and that the enormous surface area of the SR serves to produce correspondingly rapid changes in the calcium concentration of the sarcoplasm. Contraction rates of 100–130 c.p.s. are probably not even the maximum possible in this muscle. As Mendelson suggests (15), relaxation begins so quickly that were it not for a transmission block at the myoneural junction, synchronous responses could probably still be elicited at several times this rate. Why this muscle has evolved as a synchronous muscle rather than as an asychronous muscle is not clear. Apart from the fact that asynchronous muscles exist only among insects and have not been reported among crustaceans, it may also be significant that the contraction rate of

synchronous muscles is determined directly by the nervous system and is not dependent upon the mechanical properties of a resonant system (21). Thus the animal has greater control over sound production, in that it can readily regulate not only the pattern of the sound but also the pitch.

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