ULTRASTRUCTURE OF BARNACLE GIANT MUSCLE FIBERS

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ABSTRACT

Increasing use of barnacle giant muscle fibers for physiological research has prompted this investigation of their fine structure. The fibers are invaginated by a multibranched system of clefts connecting to the exterior and filled with material similar to that of the basement material of the sarcolemmal complex. Tubules originate from the surface plasma membrane at irregular sites, and also from the clefts They run transversely, spirally, and longitudinally, making many diadic and some triadic contacts with cisternal sacs of the longitudinal sarcoplasmic reticulum. The contacts are not confined to any particular region of the sarcomere. The tubules are wider and their walls are thicker at points of contact with Z material. Some linking of the Z regions occurs across spaces within the fiber which contain large numbers of glycogen particles. A-band lengths are extremely variable, in the range 2.2 μm–20.3 μm (average 5.2 μm) Individual thick filaments have thin (110 Å) hollow regions alternating with thick (340 Å) solid ones. Bridges between thick filaments occur at random points and are not concentrated into an M band The thin:thick filament ratio is variable in different parts of a fiber, from 3:1 to 6:1. Z bands are basically perforated, but the number of perforations may increase during contraction.

INTRODUCTION

It was discovered in 1962 (Hoyle and Smyth, 1963) that large, mature specimens of the giant barnacle *Balanus nubilus* each contain several giant cross-striated muscle fibers of from 0.5 mm to a maximum of more than 3.0 mm in diameter, each with an individual apodeme, which are especially suitable for certain kinds of physiological studies. It has since been shown that individual fibers may be cannulated and that mechanical as well as electrical responses may be studied simultaneously (Hagiwara and Naka, 1964; Edwards et al, 1964, Ashley, 1967; Ashley and Ridgway, 1970). The fibers are proving especially useful for quantitative studies of the excitation-contraction coupling mechanism (Hagiwara et al, 1968, Ashley and Ridgway, 1970), of the binding of ions by membrane (Hagiwara and Takahashi, 1967), and of a variety of cellular contents (Hinke and McLaughlin, 1967; Gayton and Hinke, 1968).

To date, electron microscope studies of the fibers have been published only in relation to answering specific questions, such as the basis of reversible supercontraction of which the fibers are capable (Hoyle, et al, 1965). They have most recently been used to illustrate the relatively slight damage done to the sarcoplasmic reticulum and cleft system by the insertion and utilization of an axial microsyringe (Bittar et al, 1972). Hence we decided to prepare a general survey of

their ultrastructure to facilitate interpretation of the physiological results.

MATERIALS AND METHODS

Giant barnacles are now readily and reliably available commercially (Pacific Biomarine Supply, P.O. Box 536, Venice, Calif. 90291; or Cleve Vandersluys, Friday Harbor, Wash. 98250) and can be shipped by air freight to cities in many parts of the world.

For electron microscopy, freshly dissected scutal depressor muscle fibers were fixed in a solution of 2.5% glutaraldehyde in either seawater or 0.2 M Millonig phosphate buffer containing 0.14 M NaCl After a 3-h wash in seawater or phosphate buffer (containing 0.3 M NaCl) the fibers were postfixed in a 1% solution of osmium tetroxide in the same solvent The fibers were dehydrated either in alcohol, followed by three changes of propylene oxide, or in acetone, and finally embedded in Epon 812. Silver-gray sections were cut with a diamond knife on a Sorvall MT-2 ultramicrotome, mounted on bare copper grids, stained with uranyl acetate and lead citrate, and examined in a Siemens Elmiskop 1A electron microscope.

For light microscopy, thick sections of the same Epon blocks were examined with a phase contrast microscope.

RESULTS

General Appearance

The depressor scutorum rostralis and also the depressor tergorum comprise pairs of muscles containing only rather homogeneous, translucent white fibers which are the subject of this report. Similar fibers comprise the bulk of the depressor scutorum lateralis fibers, but these two muscles also have a thin band of much thinner fibers on their outer margins. These smaller fibers have a distinctly pink color, attributable to a hemoglobin-like molecule (Southward, 1963). They serve to rotate the body and probably function during feeding. We shall not describe these specialized fibers in the present paper.

At low magnifications the large white fibers have a punctate appearance superficially resembling that of vertebrate "Fibrillenstruktur" (Kruger, 1952) fibers (Figs. 1 and 2). Closer inspection reveals that the divisions are much coarser than those occurring in vertebrate fibers and that further divisions occur within the coarser myofilament clusters. The richly anastomosing network dividing up the fiber represents an extensive system of invaginated clefts. The whole, which we shall refer to collectively as the cleft system, is effectively open to the exterior. Nevertheless, when cannulated and injected with fluid the fiber does not "leak," but rather expands, even to several times its basic size, before bursting. The cleft system and its larger branches are filled with the same kind of material as that which forms the sarcolemma

In many places the appearance in transverse section shows clusters of myofibrils to be completely surrounded by cleft space (Fig. 1) However, after passing through only a few sarcomere lengths longitudinally, the appearance is dramatically changed and the same bundle cannot be recognized. The reason is that each cleft runs longitudinally only for a limited distance before dividing or diverting and perhaps also narrowing and terminating. Within a cluster of myofilaments surrounded by cleft channels a partial further division may be seen. This is associated with invasion of the myofilament clusters by sarcoplasmic reticulum (SR) and denotes elements comparable to the "fibrils" of vertebrate fast muscle fibers. However, the barnacle SR is fragmentary and does not divide the contractile elements into segregated clusters.

The Cleft System

We are satisfied that there is no ordering of the arrangement of the cleft system within a fiber It is remarkable how different in detail are the forms of cleft systems in different fibers, yet each fiber has about an equal amount of total cleft space. We estimated the volume of the cleft space graphically, by tracing sections onto squared paper and measuring the areas The average result was that cleft space represents 8% of the total volume of the fiber. The method is extremely tedious and our sample size was too small to permit a statistical treatment Earlier estimates (also on small samples) made by Selverston (1967 b and unpublished) and by Ashley (unpublished) by weighing electron micrograph prints before and after cutting out the cleft areas yielded higher values than the present ones, but it should be borne in mind that not only are there large differences between fibers, but also different overall and local changes during fixation. As a result it will be extremely difficult to give really reliable estimates. Cleft space is present at any level down to the central core of the fiber, its width varies continually and it is not always

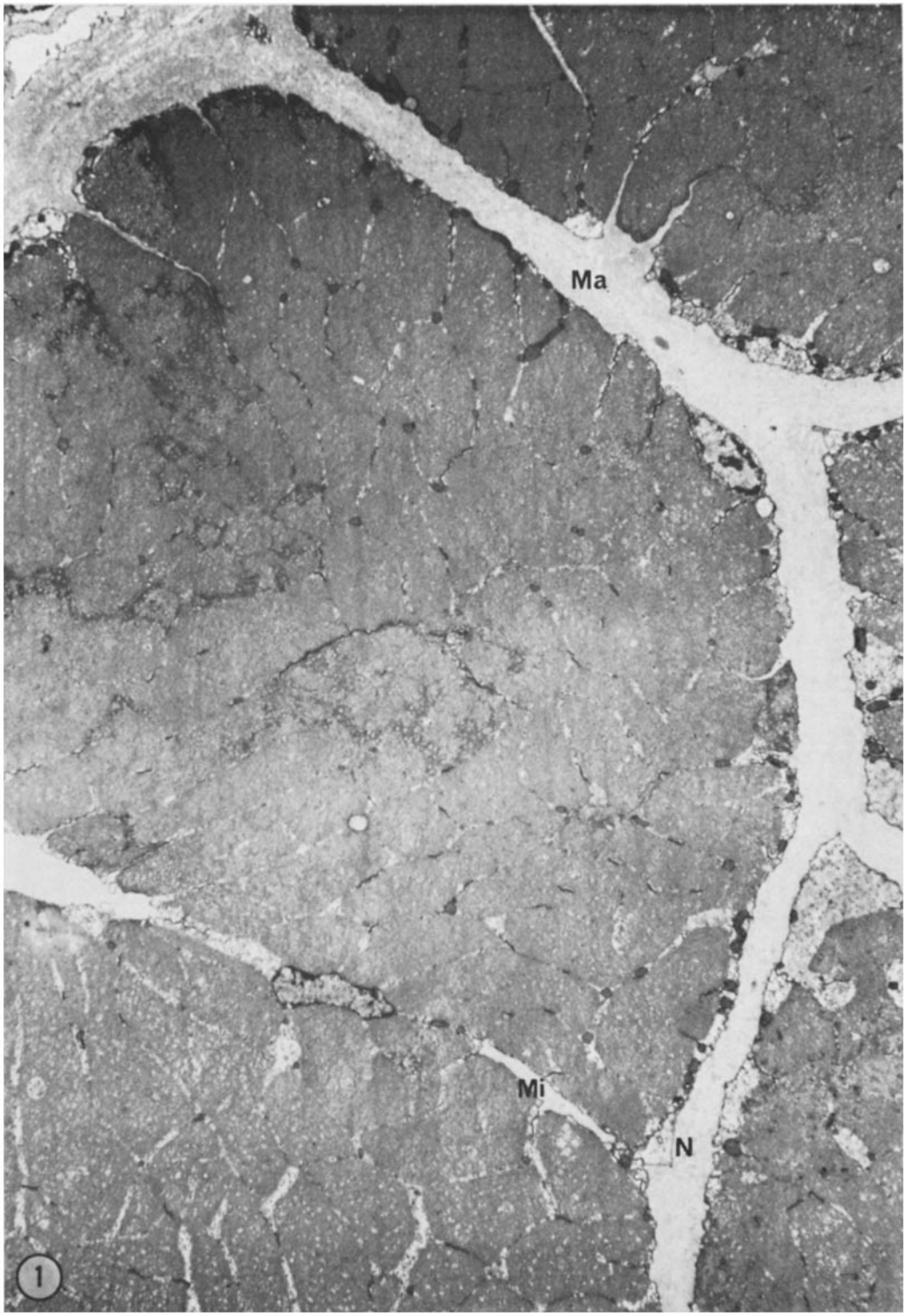

FIGURE 1 Transverse section. Giant barnacle retractor muscle fiber. A small portion of the outer surface of the fiber is seen at top left. The large, branching channel (*Ma*) which is a major cleft invaginates from the surface Finer branches, termed minor clefts (*Mi*) emerge from the major clefts. The denser regions, with perforations, seen to the left of the micrograph, are Z regions. A motor nerve axon (*N*) within the cleft is seen at the bottom of the micrograph. × 7200.

greatest near the periphery. A cleft space in the center of a fiber may be as wide as 3 μm An approximate classification of clefts seems possible since one kind are present as deep furrows with a direct longitudinal opening to the exterior while the other are branches of the former which are cut off from direct contact with the exterior membrane (Fig. 1) We shall refer to the largest clefts, which are directly invaginated, as major clefts, smaller ones, or subsidiary branches, will be termed minor clefts (see also Hoyle and McNeill, 1968 a)

All clefts are filled with a mucopolysaccharide-like substance, as judged by staining reactions, which is identical to the material which forms the thick basement layer of the sarcolemma This is confirmed by its appearance in electron micrographs.

The clefts are especially conspicuous at the level of Z lines, and Z material adheres closely to the cleft membrane (Figs. 2 and 3 b). At least one small portion of mucopolysaccharide-filled cleft space is attached to a Z line, the extent of the contact varying from a small fraction (3–6%) of the Z perimeter, to as much as 30%. Branches of the cleft system make special contacts with the Z regions of each sarcomere (Fig. 3 b). There is thus a continuous network internally of anastomosing cleft material of the same nature as the sarcolemma, to which the contractile material is attached by way of slight contacts at the Z lines

The Excitatory Tubular (T or E) System

This is the system of flattened tubules which in vertebrate systems has come to be known as the T system. The T might stand for "tubular," though it was intended to mean "transverse" (Andersson-Cedergren, 1959), since in many vertebrate striated muscle fibers they run radially. It is now recognized that in both vertebrates and invertebrates some of the tubules run only or mainly longitudinally, or even spirally within the fiber, and we have suggested (Hoyle et al, 1966) that the letter E for "excitatory" or "externally-connected" would be more appropriate since their function is to transmit excitation inwards and their lumens are open to the outside. The "tubules" are actually highly flattened. When they run transversely the long axis of the tube section is longitudinal, but when they run longitudinally the sectional long axis is radial. All are formed from plasma membrane which is continuous with that of the surface or of the cleft system. The function of the tubular system is to conduct electric excitation inwards (Gage and Eisenberg, 1967) and to make functional contact with the SR. The latter sequesters calcium ions but releases calcium after excitation via the tubular system thereby mediating excitation-contraction coupling (Sandow, 1965).

For some amphibian fibers, which do not have invaginating clefts, it has been shown that the T system opens to the exterior at regularly-arranged points around the circumference and only at a longitudinal level corresponding to an underlying Z line (Peachey, 1965). In the barnacle giant fibers there is no regularity in the positions of the openings either around the circumference or longitudinally. they are distributed randomly over the entire surface.

Z Tubules

A system of tubules making contact with Z bands was first described in large crab (*Carcinus*) muscle fibers by Peachey and Huxley (1964) and referred to by them as Z tubules (see also Peachey, 1967). It is doubtful if they deserve to be distinguished from other transverse tubules, however, since they are undoubtedly connected to the latter, and also to longitudinally-running tubules emanating from the cleft system (Fig 4) The tubules contacting Z regions always widen at the region of contact with Z material, and are filled with a dense, fine, granular substance similar in appearance to sarcolemmal basement material (Fig 3 a). The membranes are also thicker and more densely-staining in the contact region, forming a desmosome-like band. T(E) system tubules open into clefts along their entire length and clefts terminate in one or more tubules Thus it is sometimes difficult to determine exactly which is cleft and which is T(E) system. The system can be distinguished both by its narrowness and by the absence of the sarcolemma-like material inside it When the tubule system contacts Z material, however, it is wider and filled with sarcolemma-type mucopolysaccharide In some cases these Z region contacts are undoubtedly made by parts of the cleft system, with which the tubular system makes contact But in other instances there is no nearby connection to a cleft and we can consider the Z contact a special modification of a tubule.

Tubular System—Cisternal Element Contacts

Contacts between excitable membrane and SR begin with the surface membrane and the cleft

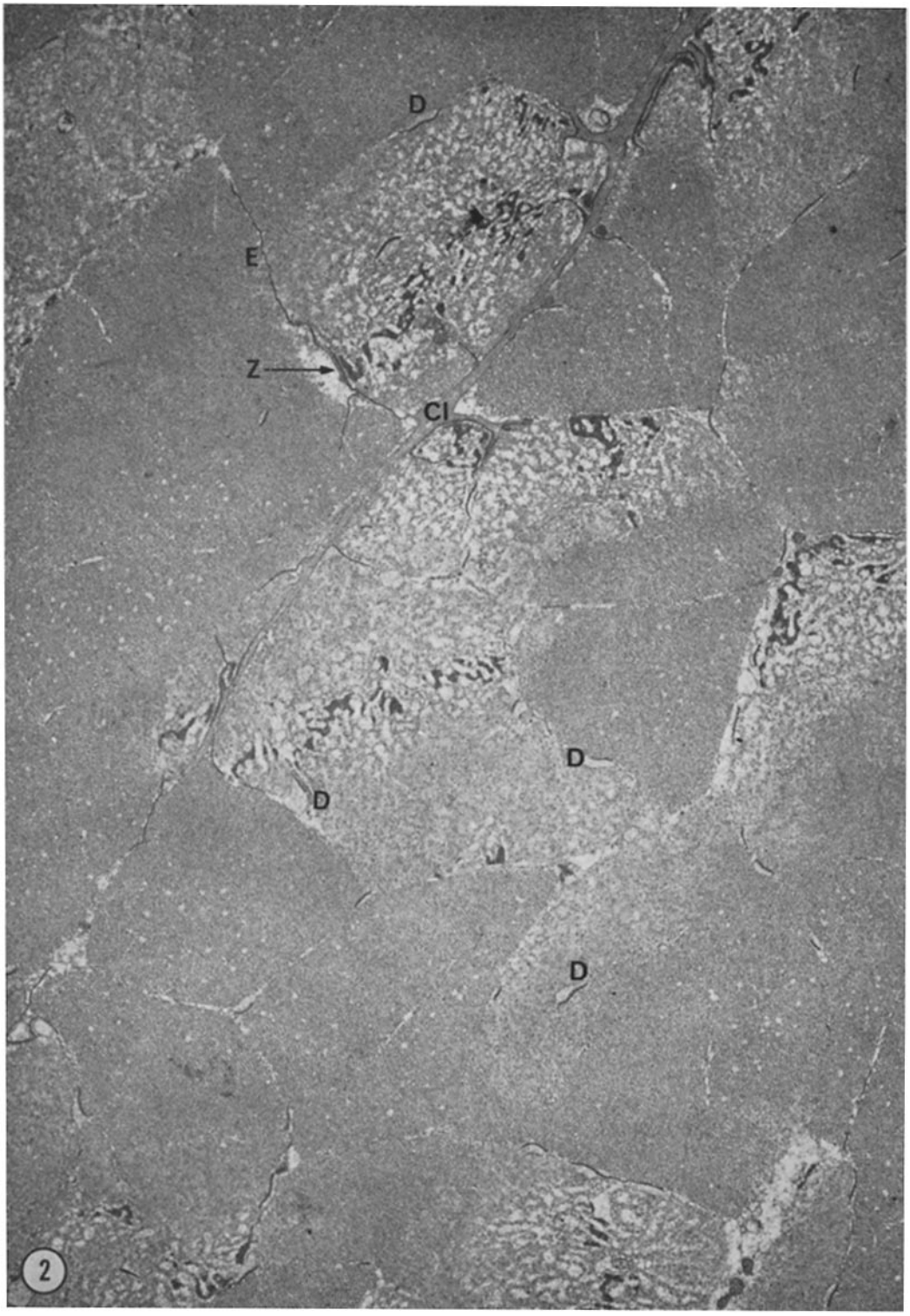

FIGURE 2 Transverse section near center of fiber shows a minor cleft (*Cl*) with excitatory tubular system branches (*E*), one of which widens as it contacts a Z region (Z arrow). Several diadic contacts (*D*) between the longitudinal elements of the SR and longitudinal branches of the excitatory tubular system are shown. × 8400.

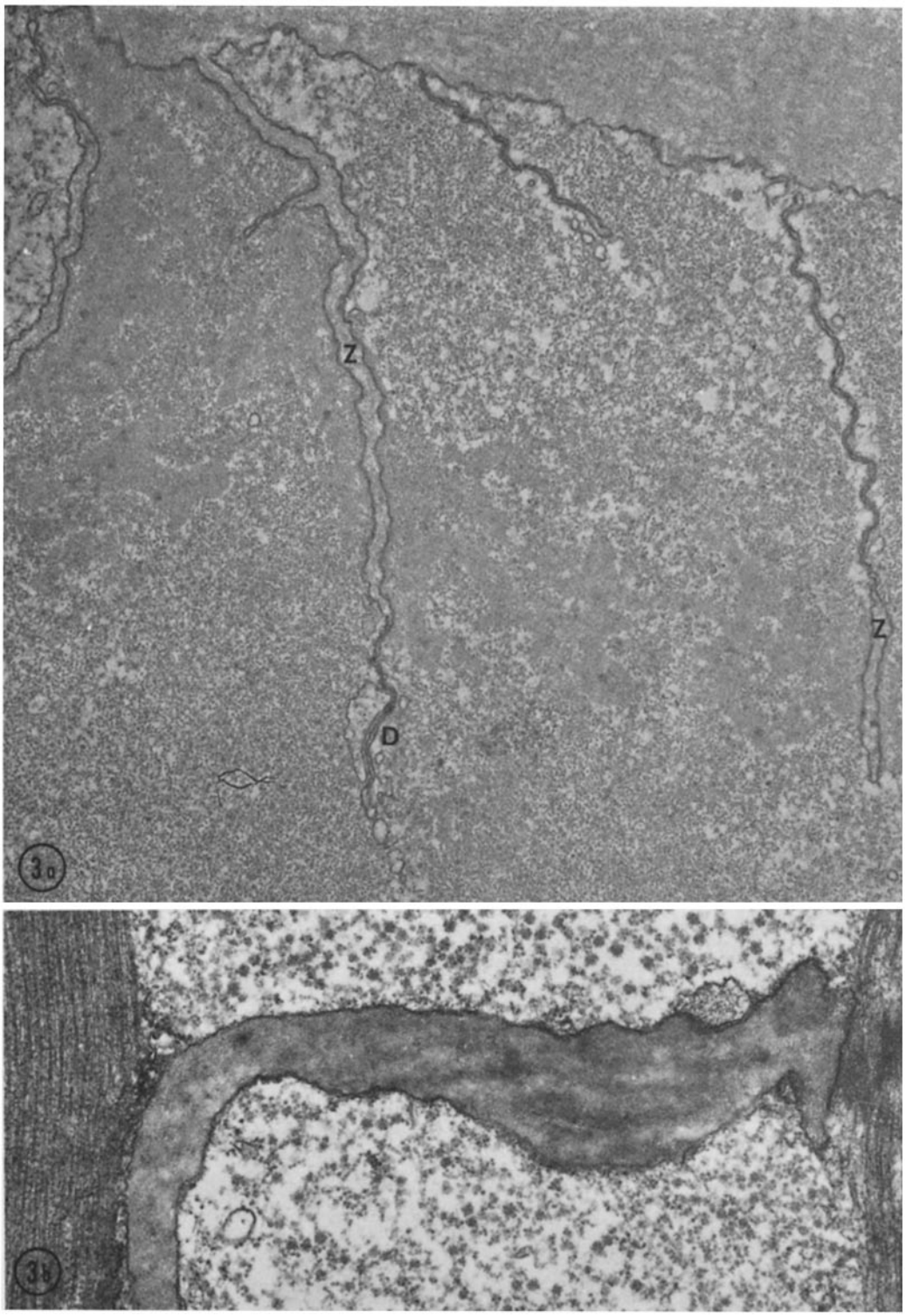

FIGURE 3 Z tubules Fig. 3 a, Section showing branches of the excitatory tubular system entering the fiber from the surface (at top right) at the level of a Z band Wherever the tubule abuts a Z region it is wide. The same tubule is narrow elsewhere, and makes diads (*D*) with the reticulum. × 33,900. Fig. 3 b, Part of the excitatory tubular system seen in longitudinal section at the level of a Z band The tubule is wide and filled with dense material similar to that forming the sarcolemmal basement membrane. It straddles a large space containing glycogen particles as it links two Z regions. × 33,250.

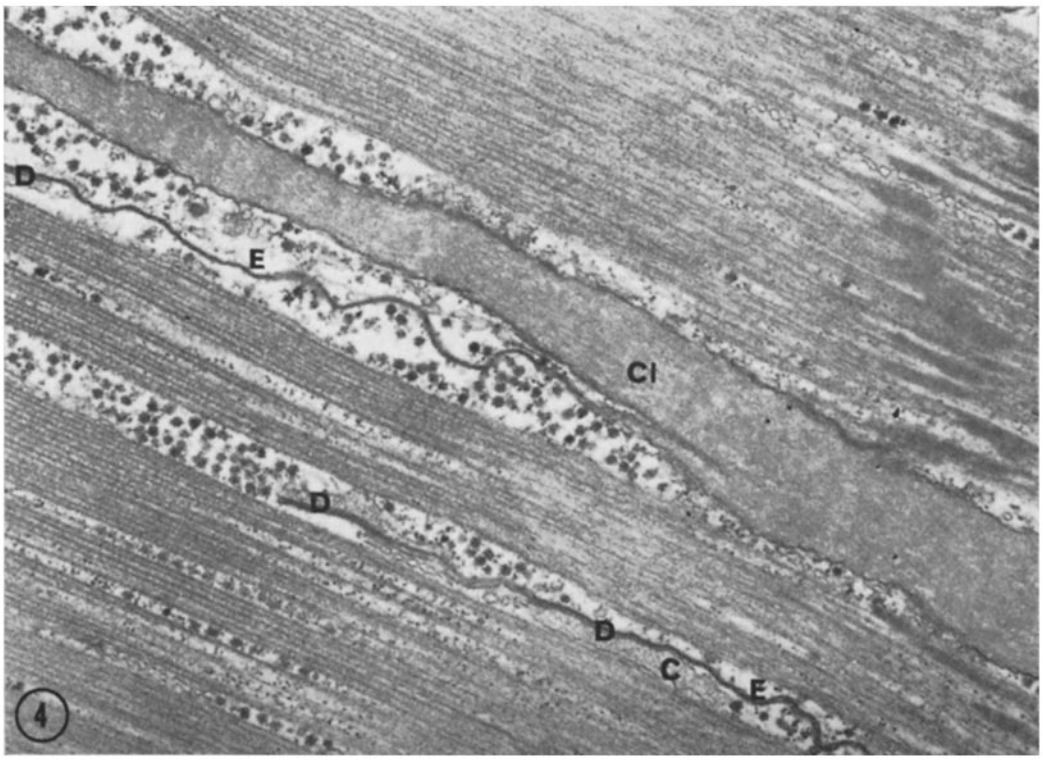

FIGURE 4 Longitudinal section showing origin of longitudinal elements of excitatory tubular system (*E*) from cleft system (*Cl*). They make diadic contacts (*D*) with cisternal sacs (*C*) of longitudinal SR Note numerous glycogen granules between myofilament clusters × 22,600.

system. These take the form of simple diads, and they occur sporadically anywhere along the circumference of the fiber or along a cleft

The major points of contact are, however, with the tubular system. These can occur either when the latter is running transversely, obliquely, or longitudinally, in the form of diads (Figs 4 and 5). The locations of these diads do not appear to bear any fixed relationship to the general morphology of the sarcomere. They are most commonly located toward the outer part of an A band, but can be in the center of the A band or in an I band close to a Z band. More than two may occur in the same section of a single myofilament cluster, at different A-band positions. This is especially the case when a portion of the tubular system runs longitudinally for some distance (Fig. 4). The tubule may run along one or even two complete sarcomeres, making diadic contacts serially.

At the few places where the SR is bilayered, the tubule runs between the layers giving diads on either side, or even simultaneously In the latter case the resulting contacts form a triad (Figs. 5 b, 5 c). Both the central tubular system element and the cisternal elements must take the form of long, flattened sacs since a similar appearance is obtained in both transverse sections and some of the longitudinal sections. The latter are ones which lie along a radius perpendicular to the tangential plane which would pass through the reticulum.

The sizes and shapes of cisternal sacs vary greatly, even when it is evident that they are not swollen. They may have a slit-like cross-section not more than 300 Å across, or an oval or even pear-shaped profile more than 1000 Å across. They contain numerous small granules, from 60 to 70 Å in diameter. Cisternal sacs swell very readily if the fixation is not perfect, for reasons which are not clear, but may relate to selective permeability properties of the membrane which permit certain ions, possibly chloride, to enter the sac. This movement is followed by massive water entry until the

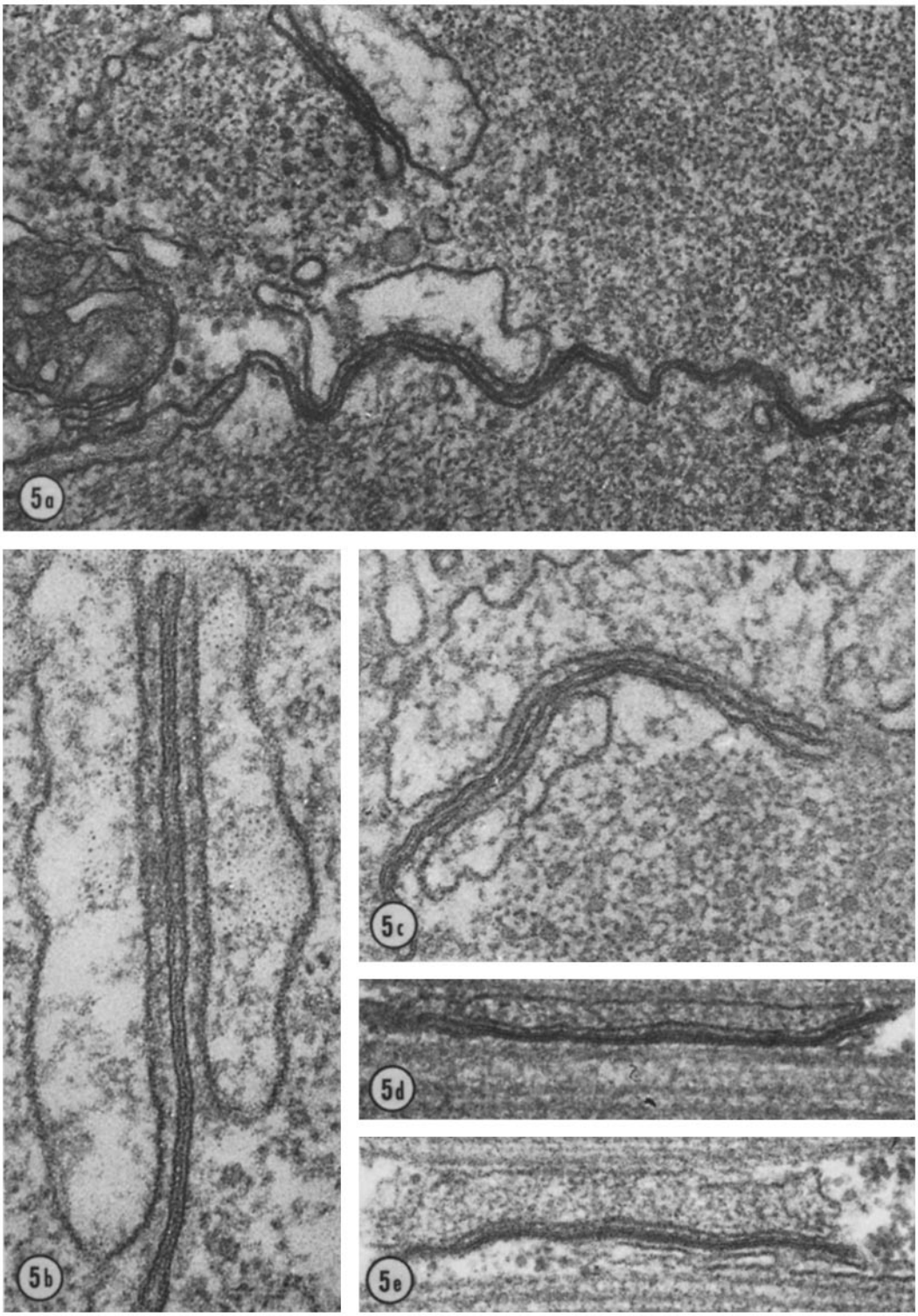

FIGURE 5 Details of excitatory tubular system/SR contacts. Figs 5 a, 5 d, and 5 e are diads; 5 b and 5 c are triads. The flattened, wide nature of these contacts is emphasized by the similarity in their appearance when seen in transverse (Figs. 5 a, 5 b, 5 c) compared to longitudinal (Figs. 5 d, 5 e) sections 5 a, × 75,250; 5 b, × 145,250; 5 c, × 107,500; 5 d, × 75,250; 5 e, × 75,250.

rounded sacs balloon into spaces scores of times greater in volume than their original ones (Selverston, 1967 a).

The lumen of the tubule is often collapsed in the micrographs, giving a similar appearance to a "tight junction" (Figs. 5 b, 5 c, and 5 e). The staining density of the plasma membrane of the tubule is always intense and in marked contrast to the membrane of the reticulum It appears to be appreciably thicker than the latter· 100 Å compared with only 60–70 Å.

The gap between the tubule and the cisternal element is about 250 Å wide. Running in the center of the gap is a fine line of dense material; projections or "feet" (Franzini-Armstrong, 1970 a) appear to occur at intervals (Fig. 5 b), bridging the gap.

The Sarcoplasmic Reticulum (SR)

Since we have good information regarding the total calcium content of barnacle giant fibers (Ashley, 1967) as well as aspects of the kinetics of release (Ashley and Ridgway, 1970) it is of particular importance to get a good overall picture of the SR. The reticulum is surprisingly sparse, almost vestigial compared with some other crustacean muscles (e g. Hoyle and McNeill, 1968 a; Atwood, 1967) but is similar in appearance and opens into characteristic, large terminal cisternae (Fig 6). Not only are the myofilament clusters quite large, frequently of the order of 4–5 μm across, they are very poorly defined at all levels of the sarcomere There is commonly only a single layer of SR, with numerous, large perforations, and nothing like a complete envelope around any given myofilament cluster. A double layer occurs only in rare patches, which are always close to a T tubule.

The total fiber calcium is equivalent to 1 mM/kg wet weight The SR constitutes less than 0 5% of total fiber volume as determined from the micrographs. If all of the calcium were sequestered evenly within it the concentration would be more than 0.2 M, or if held mainly in the cisternal elements a few times molar strength. It would seem probable then that much of the calcium is bound to the cleft membrane.

The Myofilaments

In the light microscope the fibers are obviously striated, but the Z lines are not in register across the fiber and it is difficult to assign an accurate value to either average A-band or average sarcomere lengths. An early value for average

FIGURE 6 Surface view of SR and contact with the tubular system. Note the oblique angle made by the tubules. The large terminal cisternae (*C*) are filled with very fine granular material. × 39,950.

sarcomere length based on light micrographs was 8 μm (Hoyle and Smyth, 1963) with 4–6 μm A bands. After fixation which is good for electron microscopy some sarcomeres are markedly longer than others while a few appear shortened. This appearance could be due to various extents of overlap of filaments However, electron microscopy of thin sections leaves no doubt that the A bands are themselves of markedly unequal lengths (Fig. 7) The mean value, which we have determined only approximately, is 5 2 μm, in agreement with earlier light microscopy

The I filament lengths are even more variable than the A filament lengths and impossible to determine from sections of normal sarcomeres because at no sarcomere length can we discern a clear H zone However, when the fibers are stretched to 200% rest length the I filaments are found to have been largely removed from overlap with myosin and I band maximum lengths can then be determined. In the 8 μm sarcomeres the I filaments range from 3.5 μm to 5 μm A few of the I filaments, which are clustered together, may be of even greater length, for some longitudinal elements overlap the thick filaments even in sarcomeres stretched to 200% rest length. These extra-long I filaments have a length from their Z attachment equal to or slightly longer than the A filament length and consequently should be in extensive double overlap even in the resting sarcomere. We were surprised to find, however, that sarcomeres of widely different lengths tend to be grouped together rather than randomly mixed There is a gradual, rather than an abrupt, transition from the very long or the very short to the average, both longitudinally and laterally

The longest sarcomere length determined in a myofilament cluster which was not unduly stretched, was 20.3 μm with an A band of 14.3 μm The shortest was 3 9 μm with an A band length of 2 2 μm The lengths of components of individual sarcomeres were often unequal, there being a progressive decrease in A filament length from one margin of the fibril to the other (Fig. 7 a).

Individual A filaments were found to differ greatly in length even when the overall sarcomere length was constant This makes the margin of the A band appear ragged and ill-defined (Fig 7 a). Myofilament clusters show a lot of variation in this regard. Some contain thick filaments with a rather uniform diameter of about 200 Å, while others show a wide range, from 110 Å to 340 Å in a single cluster In a series of sections the distribution of the filaments of different thicknesses changes.

The thick filaments, measured in a typical transverse section across an overlap zone, varied from 110 Å to 340 Å in diameter (Fig 8 b) The filaments undoubtedly taper towards their outer ends, and are relatively thick in the central regions. They are also not well-aligned with each other so that the sarcomeres have a ragged appearance in longitudinal sections Nevertheless, it is not possible to interpret the variation in thickness on the basis of their being of simple cigar-shape. Some of the thick filaments, both the thicker and thinner ones as seen in transverse sections, were dense-cored, while others, including almost all the thinner ones, had a hollow core (Fig 8 b)

In very thin longitudinal sections alternating thick, thin, solid, and hollow-core regions can be discerned along the entire length of the same thick filament (Fig. 8 a). From these observations we conclude that individual A filaments are comprised of varying regions along their length, ranging from thin and hollow (110 Å) to thick and solid (340 Å).

Not only are there large variations in thick filament diameter, there are also wide variations in the numbers of thin filaments in orbit around them While as many as 15 thin filaments orbit a thick one in some regions (a 6:1 ratio), in other regions there may be only one or two, and all possible intermediates occur Some myofilament clusters have greater numbers of thin filaments than others, so that thin:thick filament ratios occur in the range from 3:1 to 6:1

Nature of Z Bands

It was demonstrated by Hoyle et al , (1965) that the remarkable phenomenon of reversible supercontraction (down to 16% rest length) which is shown by barnacle giant muscle fibers is possible because the thick filaments can penetrate the Z bands Thick filaments from adjacent sarcomeres overlap across a Z region in the supercontracted condition The regions expand laterally during supercontraction This might occur simply by the Z region being pushed apart as the filaments force their way through existing holes in the Z region But it was shown by Selverston (1964) that Z regions of isolated myofibrils can expand in advance of shortening during treatment with adenosine triphosphate (ATP), suggesting that an active

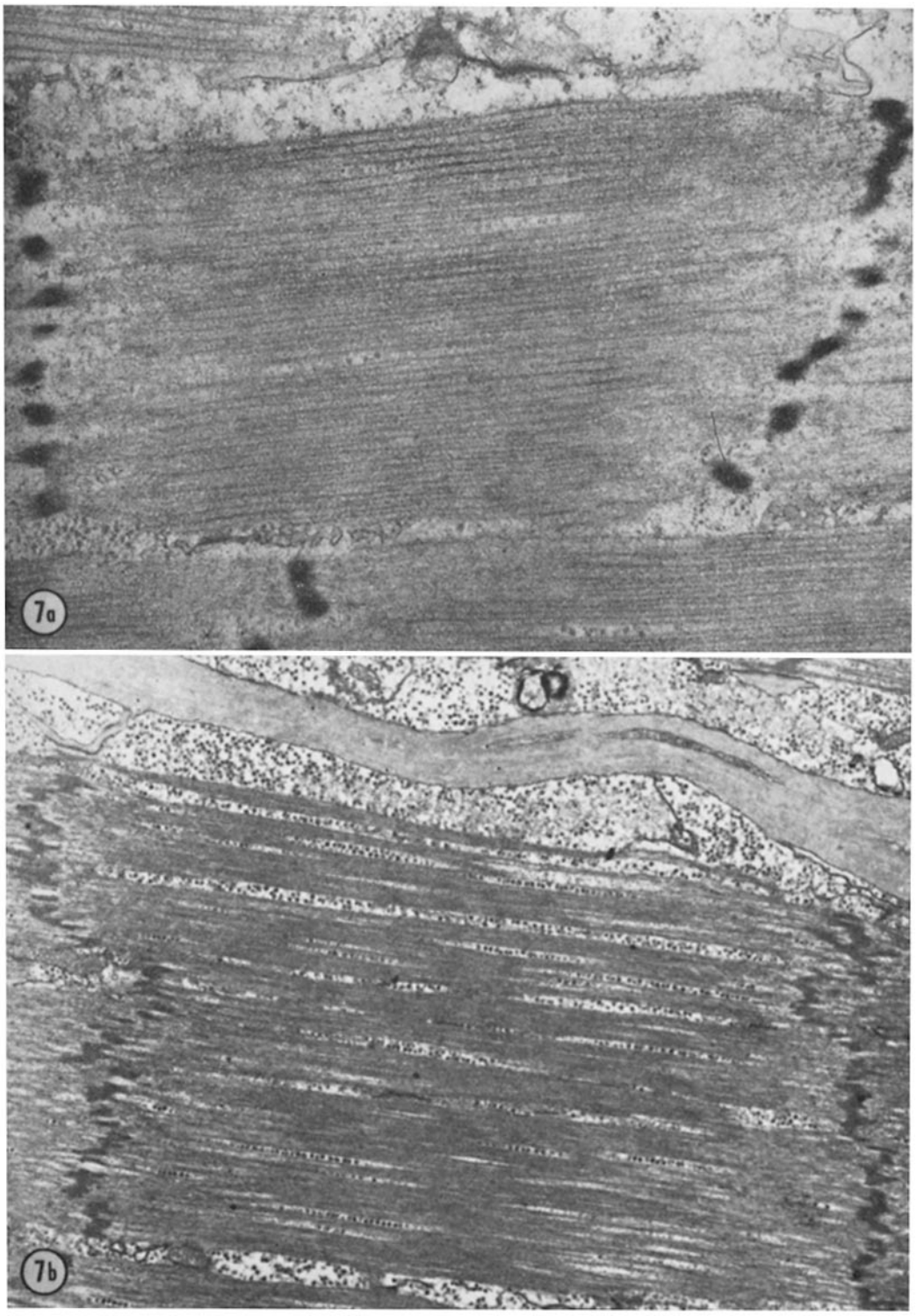

FIGURE 7 Longitudinal section. Two sarcomeres at different magnifications to show extreme difference in sarcomere length (SL). 7 a, × 22,500, SL = 5 μm; 7 b, × 9300, SL = 12 μm.

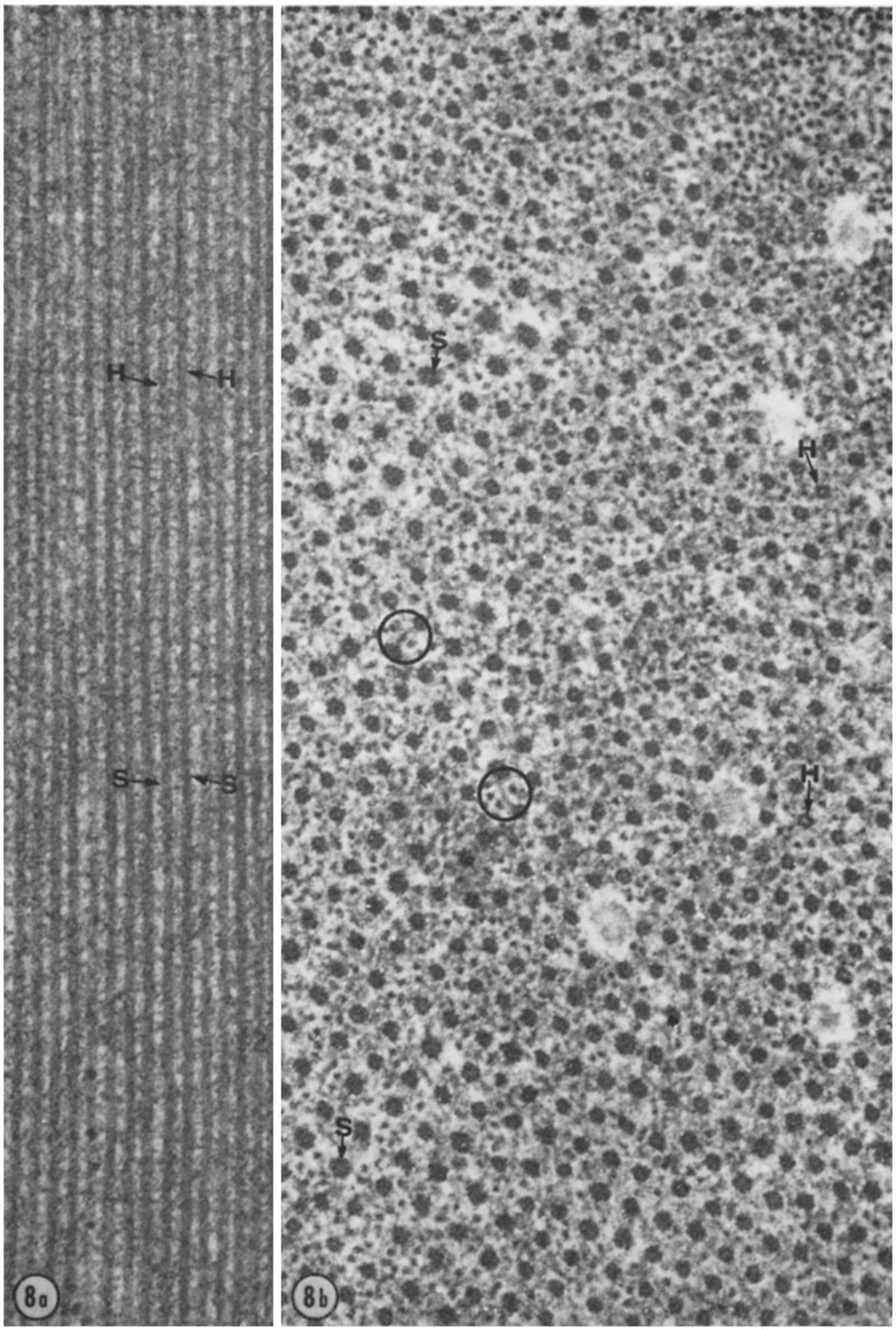

FIGURE 8 Fig. 8 a, Longitudinal section to show alternation of thin, hollow segments (*H*) and thick, solid segments (*S*) on the same thick filament. Fig. 8 b, Transverse section at high magnification to show wide range of profiles of thick filaments, from thin, hollow (*H*) to thick, solid (*S*). Barnacle fibers lack an M band, but the thick filaments are linked together by cross-bridges which occur apparently at random, along the entire length of the A band. Examples are encircled. 8 a, × 75,250; 8 b, × 110,150.

change may be associated with the expansion. An important question relates to the perforations in the Z region. While it is evident that perforations, which we shall call residual, occur naturally in the Z regions, these are few in number and relatively far apart. It is not possible to determine their extent from a single transverse section since the Z regions are extremely wavy, as seen edge on. Some areas of Z region have but a single perforation per 1 μm^2 area. When we examined them in the supercontracted state we found as many as 60 perforations, of various sizes, per 1 μm^2 of disc (see Fig. 8 in Hoyle et al., 1965). Since the disc in supercontracted fibers is more than twice its resting diameter, this corresponds to 240 perforations in 1 μm^2 of a resting disc. We have never seen more than 20 perforations in 1 μm^2 of disc fixed at extended length and the number is usually much less. We therefore conclude that the number of perforations present in the Z regions increases during the expansion accompanying extensive contraction.

The question arises as to how the pattern of residual gaps or holes is established. No two Z regions are exactly alike. The I filaments emerge from overlap with the thick filaments in an irregular array. As they approach close to the Z region, small clusters of them tend to collect in a square array, with about 150 Å separation in the lattice planes (Fig. 9). In the Z region proper there is a lot of dense material which obscures the details. However, two observations have been made; first, that there are very fine diagonal filaments linking I filaments; second, that the I filaments do not continue into the Z region but give way to a larger number of much finer ones. It is around these finer filaments that the dense material is accumulated.

Mitochondria and Nuclei

The principal location of the mitochondria is in the region immediately underlying the circumferential sarcolemma, where they form a layer two to three mitochondria deep (Fig. 10 a) They also

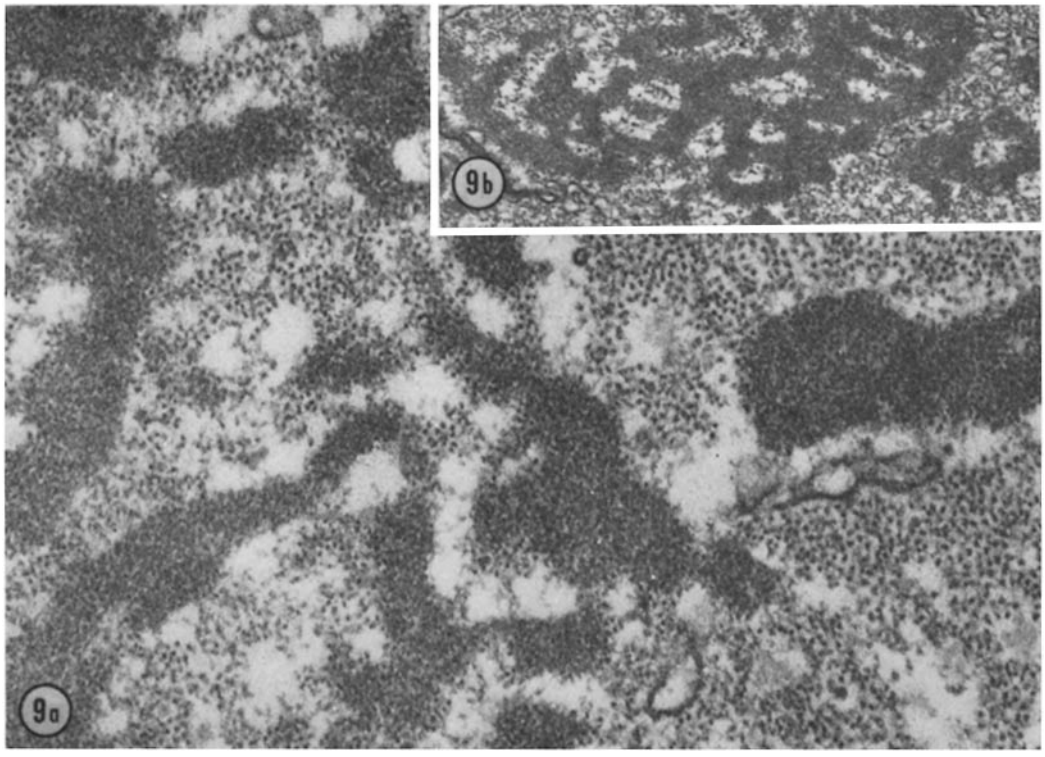

FIGURE 9 Transverse section through a typical Z region. The insert at the top shows a more compact form, with few perforations, at lower magnification. At the higher magnification a woven texture, with fine diagonal filaments, can be discerned in the dense Z material 9 a, × 75,250; 9 b, × 39,550.

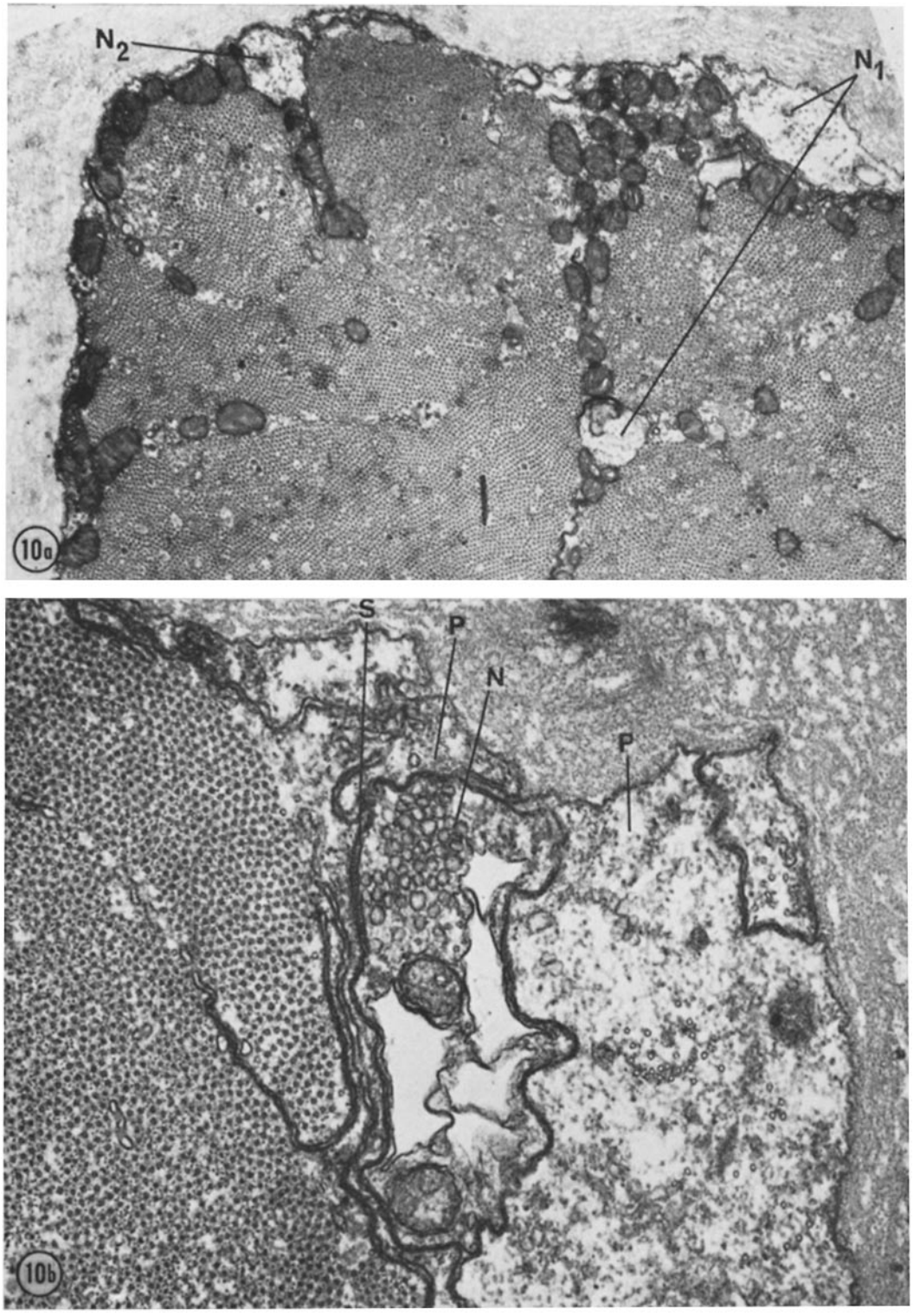

FIGURE 10 Innervation and neuromuscular junctions. Fig. 10 a, Two motor axons of different size N_1 and N_2, innervate the fiber after losing their sheath cell covering and passing through the basement material of the sarcolemmal complex. A small branch derived from N_1 is seen within the muscle fiber after entering along a minor cleft. Fig. 10 b, Details of a synaptic contact (S). Projections (P) from the muscle fiber contact with the nerve terminal (N). 10 a, × 18,550, 10 b, × 39,550.

line many of the major clefts. A few others occur between myofilament clusters, but they are not regularly placed.

Nuclei are few in numbers, large, and occur both at the surface of the fiber and adjacent to the larger clefts (Fig. 1).

Glycogen

The glycogen content of fresh barnacle fibers is extremely high, and glycogen particles are a conspicuous feature of all sections, occurring singly, in clusters, and in characteristic rosettes (Fig 4). They occur at any level in the sarcomere, though in stretched fibers they become concentrated in the region of the I bands closest to the A band. They seem to be completely free to move within the fiber and are simply squeezed out physically from both A band and I band as the myofilaments are brought closer together. In order to accommodate the particles, longitudinal spaces occur between myofilaments, which curve around the rows of particles These spaces are cigar-shaped, containing several rosettes side-by-side at their centers, but a single row at their outer ends. They penetrate the Z region at the residual perforations. There are places in some I bands where the thin filaments are crushed close together, since the space occupied by the glycogen is so great.

Neuromuscular Junctions

Each giant mucle fiber is known to receive two axons, both excitatory, one producing a larger excitatory postsynaptic potential (epsp) than the other, but otherwise not having greatly dissimilar properties (Hoyle and Smyth, 1963). We have been surprised, in these investigations, at how seldom we have found nerve fibers and neuromuscular junctions in our electron micrographs of giant fibers and conclude that there are relatively few nerve terminals per fiber.

The two axons dip through the basement membrane material to contact the sarcolemma and are apparently naked (lacking in Schwann cell covering) as they run along the surface of the fiber very close together and branching (Fig. 10 a). The smaller branches then separate and each axon runs in a deep groove, making synaptic contacts. Such terminals are about 1 μm across and are filled with synaptic vesicles (Fig 10 b). Both the major axons and the preterminal fine branches contain only a few vesicles, but they also contain a large number of microtubules.

Branches of nerve, some of them very fine (less than 0 5 μm in diameter), are found in small clefts down into the heart of the fiber. However, whether or not these fine branches are anchoring only or are also functional synaptically we cannot ascertain They do not contain the typical clusters of vesicles normally associated with sites of synaptic transmission.

The vesicles present in some terminals were of a wide size range, from about 250 Å to as large as 2000 Å. Some had a round shape, but others were of mixed shapes, including many oval ones. Oval vesicles have been suggested by Uchizono (1968) to be characteristic of inhibitory synapses, but there is no evidence for inhibitory junctions in the barnacles. In places where the nerve terminal lies near the surface and has not become deeply embedded, a lateral projection from the muscle fiber may arise and come into close contact with the terminal. These projections contain not only granules, each about 120 Å in diameter, but also longitudinally-running microtubules. The projections resemble those described for certain insect neuromuscular systems by Hamori (1963). They are, however, much less regularly arranged.

DISCUSSION

The general features of the fine structure of barnacle giant muscle fibers are similar to those which have previously been found for other large diameter crustacean muscle fibers These include the extensive invaginated cleft system which is also found in crab muscle (c f. Peachey, 1967; Atwood, 1967, Selverston, 1967 a, 1967b), variable sarcomere lengths (Franzini-Armstrong, 1970 b, Hoyle and McNeill, 1968 a), and intra-muscle-fiber nerve terminals (Hoyle and McNeill, 1968 b). Perforated Z lines permitting extensive reversible supercontraction were first described by us in the earlier report on barnacle ultrastructure (Hoyle et al , 1965) but have since been described also for insect visceral muscles (Osborne, 1967, Rice, 1970) and for moth alary muscle (Sanger and McCann, 1968).

A feature of the barnacle fiber ultrastructure which is so far unique, however, is the nature of the thick filaments In no other material have thick filaments been described which have several thick regions as well as hollow parts along the length of one myofilament. Muscle fibers of several different crustaceans have thick central regions and hollow, tapering, outer regions. They include copepod muscle (Bouligand, 1962), *Podophthalmus*

eye raiser (Hoyle and McNeill, 1968 a), and *Hemisquilla* raptorial leg flexor (McNeill et al., 1972) In contrast to the barnacle, these fibers are uniform and extremely regularly arranged. They are cross-connected by two sets of bridges, forming a pair of M lines, on either side of a clear zone which is at the exact center of the fiber

In barnacle fibers the thick regions probably correspond to those of the regular double M band fiber types Pepe (1971) has suggested that these are formed by overlapping of the light meromyosin (tail) fractions of the myosin molecules instead of abutting, as is the case in other kinds of muscle This can at once explain the double M band appearance as well as the double thickness. No M bands can be discerned in barnacle giant fibers, but bridges between thick filaments are seen in transverse sections, especially from nonoverlapping regions of A bands in heavily stretched fibers (Fig. 8 b). These bridges do not occur at hollow-hollow region abutments but between solid and hollow regions and solid and solid regions If M bridges occur only where myosin molecules of opposed polarity abut (Pepe, 1971) this means that barnacle thick filaments are composed of myosin molecules which do not all point outwards from their centers Instead, it would be necessary to suppose that there are alternating regions with opposite orientations

If substantiated, this type of molecular architecture would present severe problems when viewed in terms of the cross bridge movement hypothesis of contraction (Huxley, 1957), because the regions would produce opposite directions of movement in the same filament. As a counter to this, however, there is the anomaly that at rest length and below it there is present in the normal I/A overlap zone a number of I filament ends from the other half of the sarcomere due to the extreme length of some of them. If these ends link only with the oppositely-directed myosin molecules of the other half of the same sarcomere then the forces generated will be in the right direction

Of even greater difficulty for reconciliation with the assumption that cross-bridges generate force is the finding that the sarcomere lengths can be so different This is common to other crustacean muscles, though to a lesser extent than in the barnacle fibers However, the problem is common to all fibers in which growth is actively occurring As muscle fibers increase in length, they do so by adding new sarcomeres to their ends. The problem as to how mechanical continuity is achieved has not yet been resolved There are additional problems caused by the expectation that on the cross-bridge movement hypothesis the force developed by a sarcomere must be linearly proportional to its length. By contrast, its speed of movement will be inveresely proportional to length. Also, the time taken for longitudinal diffusion of activator calcium ions from release sites to the bridges lying in the centers of the sarcomere will be directly proportional to fiber length Both contraction and relaxation times for short sarcomere fibers are briefer than for longer ones. Studies on single fibers of another crab indicated a logarithmic relationship between total twitch time and sarcomere length (Hoyle, 1967).

In the early stages of contraction the short sarcomere elements should be fully activated and contracted, pulling out their partly-activated, long-sarcomere neighbors But they themselves will soon be extended by the longer ones in series with them. The invaginated cleft system, since it provides an elastic internal skeletal element attached to the Z regions may, in part, counteract the stresses so developed Alternatively, it would be necessary to postulate elastic filaments running longitudinally within the sarcomeres

Direct physical and physiological tests have been made on the fibers of *B nubilus* in an attempt to determine whether or not the anticipated internal distortion occurs and results in extension of internal elastic elements. These results (Hoyle and Abbott, 1967, Hoyle, unpublished) gave a clear indication of a large effective longitudinal elasticity associated with something other than the sarcolemma and probably lying within the contractile apparatus

When isolated fibrils were made to shorten, by adding ATP, they did so unevenly (Selverston, 1964). Some sarcomeres totally lacking in surrounding sarcolemma or even SR were stretched out by their more strongly-contracting neighbors to lengths 2.5 times their rest length Electron micrographs will be presented in a subsequent paper to show that there is still overlap between thick filaments and the few extra-long thin filaments in this material, but that there are very few cross-bridges to bear the tension It has been suggested that an additional, elastic, ultrathin filament in parallel with the I and A filaments carries the major part of the elastic load (Hoyle, 1967, McNeill and Hoyle, 1967). The inequalities in both A and I filament lengths as well as the variations of thick filaments along their lengths

and the absence of an M band, can perhaps all be explained on the basis of a weak and irregular regulation of the polymerization of the component molecules during development

It is interesting to speculate whether the various aberrations are the result solely of mishap or whether they have, in fact, some functional value. The biological role of the giant fibers is as yet unknown. Strong withdrawal into the shell may be protective, but a more dramatic role has suggested itself. When disturbed, a giant barnacle sometimes ejects a powerful jet of seawater with such force that it lands several feet away This would possibly serve in nature to scare away a potential predator. The jet is achieved by a strong contraction in the white depressor muscles which leads to compression of the fluid contents of the body cavity. A part of the membrane containing the fluid ruptures under the pressure and water is released. The muscles cannot contract sufficiently rapidly to be able to play a significant role in accelerating the water mass.

We would like to suggest that the force is supplied by sudden shortening of elastic elements in the muscles, which have previously been fully stretched out during the slow contraction to full tetanus of the retractors. These have a stretched length under tetanus equal to about 14% of the fiber length and displace a volume of as much as 10 ml in a large barnacle. The total maximum force produced by the six muscles in a large barnacle is more than 600 kg.

On this hypothesis, unequal contractions of individual sarcomeres provide the basis for extensive internal stretching which is needed to realize the elastic potential The nature of the elastic material is unknown The existence of an elastic, very thin filament in muscle was first suggested by Hanson and Huxley (1955) They postulated that it links I filaments of opposite half sarcomeres across the center of the sarcomere Ernst and Benedeczky (1962) and Guba et al (1968) suggested that a continuous elastic strand forms the core of the thick filaments, while Hoyle (1967) and McNeill and Hoyle (1967) have proposed that it may run from Z line to Z line in parallel with the I and A filaments

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