STRUCTURE AND FUNCTION OF THE TUBULAR SYSTEM IN CRUSTACEAN MUSCLE FIBERS

by

ALLEN I. SELVERSTON

A THESIS

Presented to the Department of Biology
and the Graduate School of the University of Oregon
in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

June 1967

ACKNOWLEDGMENTS

I wish to thank Dr. G. Hoyle for help and encouragement throughout the work. I am also indebted to Dr. H. Grundfest, John Reuben, Philip Brandt and Harold Atwood for their invaluable advice. I am most grateful to Professor Andrew Huxley for help in deriving the formula for determining membrane capacitance from the action potential foot as well as many helpful suggestions. This research was supported by a Physiology Training Grant to the University of Oregon and in part by a Grass Fellowship in Neurophysiology.

TABLE OF CONTENTS

CHAPTER I

INTRODUCTION

Probably the two most pressing unsolved problems in muscle physiology are the mechanism of interaction between actin, myosin and other muscle proteins, and the processes by which surface membrane depolarization is coupled to the activation of this mechanism. This thesis will present a correlated morphological and physiological investigation of the latter question in striated muscle fibers of the Class Crustacea. Particular emphasis will be placed on representative tonic and phasic fibers, because of the large amount of background information available for them, as well as my own interests in the unique problems these large fibers pose.

It is now generally accepted that the transverse tubular system (TTS) provides the pathway for the inward passage of excitation. This conclusion is derived mainly from the ultrastructural evidence of the last ten years. At the present time the morphology of the TTS is well in advance of its physiology, although many suggestions pertaining to its function have been advanced and will be discussed fully.

A review of the historical developments which led to the concept of the TTS as a discrete entity begins in the early 1800's with the work

of Bowman (1840), Kolliker (1846) and other light microscopists. Their ideas about the presence of contractile fibrils embedded in a fluid matrix were challenged by Retzius (1881), Carnoy (1884) and Cajal (1888), who felt the fibrils were in reality coagulation artifacts caused by fixation. Although their interpretations varied, they were unanimous on one point, that the fibrils were really a semi-fluid matrix which was surrounded by different kinds of solid reticulum. Retzius actually implicated this reticulum in impulse conduction, although it should be pointed out that he felt the reticulum was a network within the fiber which was an extension of the excitatory nerve supply. Carnoy indicated he felt the reticulum was the contractile component, an idea which found support in many early accounts, particularly in the works of VanGehuchten (1886). Butschli and Schewiakoff (1891) were among the first to examine insect and crustacean fibers. The reticular meshworks which they describe were thought to be formed by the fluid protoplasmic matrix during fixation.

Differential staining of fibers by Rollett (1888), provided strong evidence for the existence of fibrils within the sarcoplasm, and that the sarcoplasm might contain a separate reticular system. Rollett's views gained little acceptance among the "fluid matrix" school, but were seized upon by Veratti (1902) and elaborated upon in his now famous memoir. In addition to many illustrations of reticula from a wide

variety of muscle fibers, Veratti supported Rollett in objecting to Van Gehucthen and his colleagues by emphasizing that the reticula which they described was not a special differentiation of the sarcoplasm, but represented structures seen when the sarcoplasm was stained. Veratti's illustrations show clearly that the reticular structures were not responsible for the striations as was previously thought, but were in fact separate and only spatially related to them. Three main conclusions can be drawn from Veratti's work. First, the reticulum is a separate entity within the sarcoplasm, shown clearly by the fine reticular filaments obtained with Golgi silver impregnation techniques. Second, the reticulum is arranged in phase with the striations of the fibrils. Third, the reticular elements remain intact during all phases of contraction.

Although the period between the turn of the century and the 1930's saw notable advances in histologic methodology, little progress was made in elucidating the structure of the sarcoplasmic reticulum beyond the level which Veratti had achieved. The Golgi apparatus was found to be a component of almost all cells examined and most cytologists felt the reticulum of muscle cells to be homologous with it. Until the advent of the thin sectioning techniques employed in electron microscopy the very existence of a sarcoplasmic reticulum remained in doubt.

Rediscovery of the sarcoplasmic reticulum by Bennett and Porter

(1953), led to a revitalization of interest in its structure and function. While the electron micrographs in this early paper were crude by present standards, they clearly indicated a system of tubules and vesicles interposed between the myofibrils. Preliminary investigations by Ruska (1954) on insect leg muscles, Bennett (1955) and Porter (1956), all pointed to the existence of a sleeve of Sarcoplasmic reticulum (SR), surrounding each fibril. The first definitive reports were published by Porter and Palade (1957), Anderson (1957) and Moore and Ruska (1957). The correspondence between the reticula observed by Veratti in the light microscope and the electron micrographs of the SR now available, was established. The SR was described as a collar surrounding the fibrils and having a dilated "cisternal" portion either at the Z-line or the A-I junction, depending on the kind of muscle examined. The concept of the triad was introduced by Porter and Palade (1957) to describe the longitudinal sections which had an intermediate vesicle flanked by two cisternal elements. The SR was thought to be homologous with the endoplasmic reticulum of other cells since its membranes were found to be continuous with the nuculear envelope (Moore and Ruska, 1957). The intermediate vesicle of the triad was investigated in serial sections by Anderson-Cedergren (1959), and found to represent a transversely sectioned radial convoluted tubule which was part of a system of radially oriented tubules to which she

gave the name transverse tubular system (TTS). Although she proved the T-system tubule was discontinuous with the cisternae, she found no evidence that it was connected to the plasma membrane of the fiber. The suggestion that the SR could serve as a pathway for the inward spread of excitation had already been made (Edwards, et al., 1956; Porter and Palade, 1957). With the discovery of the TTS came the concomitant experiments of A. F. Huxley's group which utilized small current-passing micropipettes to depolarize small areas over the sarcomere (Huxley and Taylor, 1955, 1958; Huxley and Straub, 1958). When the areas over which triads had been localized were depolarized, graded contractions of the sarcomeres were observed to a depth of about 10 μ. The fibers which had triads at the Z-line (e.g., frog), could only be made to contract when the micropipette was directly over the Z-line. Contractions of half the sarcomere could be produced when the micropipette was over the triads which were located at the A-I junction (e.g., lizard). These experiments were the strongest evidence ever gathered to tie the TTS with E-C coupling, and remain today as the most direct evidence.

Peachey and Porter (1959) had suggested that two mechanisms were utilized in the transmission of excitation in vertebrate smooth and striated muscle. Since the former had a slow contraction speed, ample time was available for a diffusion process. But for rapidly con-

tracting striated fibers, Hill (1948, 1949) had shown diffusion to be too slow to account for the initiation of activity. When extremely fast-acting muscles were examined, it became apparent that there was a positive correlation between the development of the SR and TTS and speed of contraction (Revel, 1962; Fawcett and Revel, 1961). In the longitudinal body muscles of Amphioxus however, the flat form of the muscle allowed the sarcolemma to be in close proximity to the fibrils and a tubular system was not found (Peachey, 1961).

Reports of studies on the ultrastructure of fast and slow frog fibers, the physiology of which had been elucidated by Kuffler and Vaughn Williams (1953), revealed structural differences which correlated with the functional ones. Peachey and Huxley (1962) and more recently Page (1965) have shown that in the phasic fibers there is a well developed TTS and SR, while the tonic fibers have a poorly developed SR and virtually no tubular system.

Another pertinent morphological feature of the T-system in vertebrate muscle which has become well established within the past few years is the connection between the tubular lumen and the exterior of the fiber.

Clear evidence for this has been provided by Franzini-Armstrong for fish skeletal muscle (1964), although it had been suggested previously for myocardial muscle by Simson and Oertelis (1961), and for insect

muscle by Smith (1961). The rapidity of exchange between the interior of the tubules and the external solution has been shown by Endo (1964), who observed the rate at which fluorescent dye fills the tubular system. In a similar type of experiment on frog fibers, H. E. Huxley (1964) was able to demonstrate directly the continuity between the tubular system and the extracellular space by soaking the muscle in ferritin containing Ringer solution, and showing that the electron-dense ferritin was found only in the TTS but not elsewhere.

The ultrastructure of crab muscle fibers has been investigated by A. F. Huxley and Peachey (1964). They pointed out the fact that the tubular system did not originate only from the surface of the fiber but also from sarcolemmal invaginations. They described two kinds of tubules, one which went to the Z-line and another which formed diads with dilated portions of the SR. The diads, which had been described previously by Edwards, et al. (1958) in Cicada tymbal muscle fibers, synchronous (Smith, 1961), and asynchronous (Smith, 1961) insect flight muscles, copepod (Fahrenbach, 1963) and ostracod (Fahrenbach, 1964) and crayfish (Peterson and Pepe, 1961; Brandt, et al., 1965), were homologous to the triads previously described by Porter (1956). In crab, the diads were found to locate at the A-I junctions and localized depolarization by A. F. Huxley produced results found earlier in lizard (Huxley and Straub, 1958). In addition, placement of the

electrode down into the clefts allowed spread of contraction in a longitudinal direction, a result interpreted as being due to a more diffuse spread of current.

With this review of the developments which led to the suggestion of the T-tubules as a possible pathway for the spread of inward excitation, I want to consider now the theories which have been developed within the past decade to explain this role from a more physiological standpoint. Because the small size of the T-system does not allow direct experimentation, all of the proposed mechanisms have resulted from the interpretation of indirect evidence.

The most compelling evidence for the transmission of inward excitation by the TTS are the local-activation experimens of Huxley and Taylor (1958) and Huxley and Straub (1958) which I have already mentioned. The pertinent observations were that the locally excitable surface spots coincided with the positions of the triads in frog and lizard. Small outward currents at these spots caused contractions of the underlying sarcomeres which did not spread to adjacent sarcomeres. Furthermore, the inward spread was graded, i.e., the depth to which contractions occurred depended upon the strength of depolarizing current used. The idea that the walls of the tubules behaved electrotonically suggested that its membrane had characteristics which were different from the "all-or-none" membrane of the sarcolemma.

The local activation experiments provided a strong impetus to the investigation of T-system physiology. It was easy to recognize how, in response to a depolarization of the outer membrane, current might flow from the sarcoplasm across or down the tubular membrane. It was of interest therefore, to identify the ions participating in this current and to find out at which points in the membrane tubule cisternal pathway they were acting.

Hodgkin and Horowicz (1960) attempted to resolve this problem by studying the effects of sudden alternations of the external medium on the resting membrane potential. They reasoned that sudden changes in the concentrations of ions which permeated the outer membrane should cause very rapid changes in the membrane potential, while those ions which permeated the membranes of the tubular system should cause a slower change in the T-system concentrations because of diffusion times, and therefore a slower change in the membrane potential. They found that changes in Cl concentrations in either direction caused immediate changes in the membrane potential (half-time, 0.3 sec.). Changes to lowered K concentrations, however, had a half-time of 5 seconds, and while potential changes in elevated K concentrations were less rapid, they were still greater than those in changes in either direction of Cl concentration. Their results suggested that the Cl permeability was primarily along the outside of the fiber while the K permeability was

located in some special region which required time for diffusion before K^+ would affect the potential.

Adrian and his colleagues have assumed that any current flowing in the TTS membrane would be in parallel with the current flowing in the surface membrane (Adrian, 1964). Their experiments have concentrated on looking for the electrical properties which could be assigned to this parallel pathway in muscle fibers. Several early experiments served as a starting point for their investigations. Katz (1948) had shown that when a frog fiber was placed in isotonic potassium sulfate, the membrane allowed the passage of large inward currents but only small outward currents (Anomalous rectification). Since in this solution K^+ is the only ion that can carry current across the membrane, the membrane behaves as a rectifier for K^+. As has already been mentioned, Hodgkin and Horowicz concluded that K^+ permeability was small for outward K^+ movements but large for inward K^+ movements. Jenerick (1959) demonstrated a delayed rectification for depolarizing currents and Nakajima, et al. (1962), showed that in 40mM K_2SO_4, delayed rectification was inactivated and followed by anomalous rectification.

One explanation for these results was a time varient change in K^+ conductance during maintained depolarization. An alternative possibility suggested by Adrian was the existence of two parallel membrane systems, one whose conductance increases on depolarization and is inactivated as

the depolarizing current continues, and another which could pass large inward K currents and small outward K currents. Adrian and Freygang (1962) have suggested that these two membranes exist independently because they could detect anomalous rectification both before delayed rectification occurred and after it had been inactivated.

Another critical experiment performed by Adrian (1963) was the replacement of the internal K with Rb. The voltage-current curves obtained with isotonic $RbSO_4$ bathing the fiber indicated no rectification but instead a high resistance to Rb currents in both directions. However, when the fiber was placed in normal Ringer's solution, or Ringer's solution in which the KCl had been replaced with RbCl, normal action potentials were obtained. Adrian interpreted these results to mean that the repolarizing phase of the action potential could utilize Rb, but the pathway which allows large inward currents could not allow inward Rb movements. The different selectivities suggested that two separate mechanisms are involved. The intermediary space which Freygang postulated accumulates K during long trains of spikes and is responsible for the late after-potential which followed the train (Freygang, et al., 1964). When the fibers were loaded with Rb however, the late after-potential was abolished. The late after-potential could be prolonged by soaking the fiber in hypertonic or low-chloride Ringer, a change accompanied by swelling of the TTS.

Adrian's results are consistent with the hypothesis of a T-system connected to the external solution having a wall which allows large inward K movements but through which outward K movements are restricted.

Hodgkin and Horowicz (1960) suggested that the shape of K contracture curves for single frog fibers could be explained on the basis of the release of an activator from a limited store of precursor and inactivation by removal of this activator. They considered the possibility of this precursor being a Ca-complex with a net negative charge and held in the T-system lumen by the potential across its walls. An action potential on the surface would short circuit this potential and the Ca-complex would flow into the sarcoplasm. The decrease in K permeability during activation would simply act to increase the flow of the Ca inward. Adrian was able to explain almost all of his experimental results on the basis of this hypothesis.

Another suggestion of a mechanism which would provide rapid inward conduction has been put forward by Girardier, Reuben, Brandt and Grundfest (1963). They have been able to demonstrate that the TTS membrane in gradedly-responding crayfish fibers is selectively permeable to Cl. By causing Cl to leave the sarcoplasm, the TTS was found to swell and vesiculate. The Cl withdrawal was achieved by either first loading the fibers with KCl and then transferring them to a Cl-free

medium or by passage of long duration hyperpolarizing currents through a microelectrode. In the latter case K was made to enter the cell at the surface, while CL passed from the sarcoplasm into the tubules, where water, moving through an electrosomotic gradient, caused them to swell. Subthreshold depolarizing currents or outward currents passed through a K-propionate electrode produced no swelling of the TTS. Foulks, et al. (1965), showed that sudden Cl withdrawals produced contractures in frog phasic fibers, but not in the tonic ones. The contractures were also associated with swelling of the TTS. Grundfest's group was able to show that procaine-induced spikes were of low conductance in a Cl-free medium and tension production was small. There was no change in the amplitude or duration of the spike when fibers were placed in a Cl-Ringer, but the conductance and tension development became high (Garcia, et al., 1966).

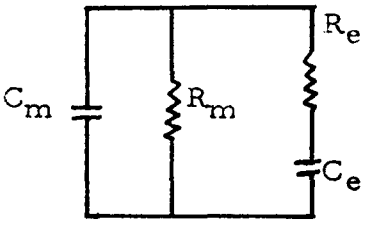
The presence of a spatially separated membrane with a specific permeability to anions suggested a different mechanism for the spread of inward conduction. Since the ionic concentrations in the tubules would be identical with the external solution, a Cl-diffusion potential would be set up across the tubular wall in the same way that a K-potential is established across the wall of the plasma membrane. Both would be polarized negatively on the sarcoplasmic side. In the resting state the K and Cl batteries would be opposed and no current

would flow. When the surface membrane was depolarized, the batteries would be in series since the tubular membrane was electrically inexcitable, and a current loop would be established. The current flow would be in such a direction as to depolarize the tubular membrane and could thus act as a stimulus for contraction.

Falk and Fatt have suggested another hypothesis for the role of the TTS based on their analysis of the electrical properties of frog and crayfish striated fibers. It had been known for some time that the capacitance of muscle fiber membranes was unusually high for a dielectric membrane. For example, Katz (1948) found the value to be 5 $\mu F \cdot cm^2$ in frog fibers. In crayfish (Fatt and Ginsborg, 1958) the value was 20 $\mu F/cm^2$ and in some crabs it reached a value of 40 $\mu F/cm^2$ (Fatt and Katz, 1953). The large values expressed per unit area of surface membrane could have been the result of the tubular membrane effectively increasing the surface area. Falk and Fatt (1964) tried to separate the two capacitances by setting up a model which had a series resistance and capacitance representing the TTS in parallel with a parallel resistance and capacitance which represented the plasma membrane. The presence of a resistance in series with one of the capacitances, but not the other would allow the two capacitances to be separated by plotting their impedances at different frequencies on a complex plane plot. The impedance-locus plots which they obtained

did, in fact, show two dispersions from which they were able to calculate the values of the elements of their model.

	C_m $\mu F/cm^2$	Ce	R_m cm^2	Re
Frog	2.6	4.1	3100	330
Crayfish	3.9	17	680	35
Carcinus	9	47	173	21



m = membrane plasma
e = TTS membrane

The frog and crayfish values are from Falk and Fatt (1964). The Carcinus values were obtained by similar analysis by Eisenberg (1965). The model on the right represents the inside-outside admittance of the muscle fiber.

It should be noted that their figures were based on complicated equations which were required to be corrected for the stray and distributed capacitance of their experimental arrangement. Their raw data actually measured quite different electrical components of the fiber and the figures they arrive at are, at best, approximations based on indirect evidence. Their hypothesis assumes that two membrane systems can be represented by an analog in which the potential across the capacitance of the tubular wall would be discharged by a depolarization of the

surface membrane. Positive current would originate at the inside of the depolarized plasma membrane and the flow through the myoplasm, the resistance and capacitance of the TTS would pass through the lumen of the tubule to the outside. The capacitance of the TTS membrane (C_e) would have a depolarizing potential impressed on it with a lag determined by its RC product. The potential across C_e would have a time course similar to the action potential, but distorted somewhat by rising and falling more slowly and having a small peak amplitude. The mechanical threshold would still be reached within the time necessary to be consistent with the high speed of activation of contraction. Their model also suggests that if control of contraction is effected by the potential across C_e, junctional potentials with different time courses, such as are known to exist in crustacean fibers, would produce different contraction rates. According to their model, J. P.'s with fast time courses would be less effective in initiating contraction than slower ones of the same amplitude (Falk and Fatt, 1965).

The basis for the relatively high membrane capacitance of cardiac Purkinje fibers has been investigated by Fozzard (1966). He compared the values obtained from analysis of the foot of the propagated action potential, a method originally suggested by A. F. Huxley (Hodgkin and Huxley, 1952) and developed by Tasaki and Hagiwara (1957), to determine the membrane capacitance of toad muscle fibers, to the values

obtained from cable analysis equations developed by Hodgkin and Rushton (1946) and refined for use in the Purkinje fiber by Weidmann (1952). The square wave value was 12.8 $\mu F/cm^2$ and that from the foot of the action potential was 2.4 $\mu F/cm^2$. The latter value was assumed to represent the capacitative filling of the surface membrane and corresponds to a frequency of about 300-600 c/s, while the former represents the total capacitance at a frequency of about 8 c/s. His results were consistent with the Falk and Fatt model of one capacitance in parallel with the membrane resistance, and the other in series with a small resistance thought to represent the content of the tubules.

When the capacitance of fast and slow frog fibers was investigated by Adrian and Peachey (1965), they found that the slow fibers did not show the large capacitance of the twitch fibers, the value being about three times as large in the twitch fibers.

In frog twitch fibers Peachey (1965) has calculated the ratio of the TTS area to outer surface area to have a value of 7, while in slow fibers the ratio is only one. Similarly, the high value of crab fiber capacitance can be related to the extensive system of infoldings in addition to the TTS area. The comparisons cited by Peachey support the idea that the low frequency capacitance can be correlated with the ratio of TTS area to surface area, and that the capacitance values for the TTS found by Falk and Fatt in frog and crayfish and subsequently

by Eisenberg (1965) in Carcinus, can be substantiated by the direct approximation of TTS areas from electron micrographs.

During the course of this introduction I have alluded to several investigations which were concerned with the structural and functional differences in tonic and phasic frog muscle fibers. Largely through the investigations of our laboratory, it has become apparent that crustacean fibers display an even greater heterogeneity in both structure and function. The large size of single crustacean muscle fibers makes it possible to utilize techniques which are impossible in the smaller vertebrate fibers. Comprehensive studies of the electrical and mechanical responses of single innervated fibers from the closer and extensor muscles of the crab Cancer magister have been published (Atwood, et al., 1965; Atwood, 1965). A similar study on the accessory flexory muscle has been made by Atwood and Dorai Raj (1964). It was possible to categorize fibers, on the basis of their tension development, into tonic, phasic and intermediate. Tonic fibers had long time and length constants, low resting potentials, did not spike on direct or indirect stimulation and could maintain a potassium contracture for many minutes. The fast fibers could develop tension and relax at much faster rates, would give spikes on indirect stimulation, and spike or show graded responses with direct depolarization. In general, the membrane characteristics were just the opposite of those associated

with the tonic fibers. Cohen (1963) first observed the histological differences between these fibers, the tonic fibers having long sarcomeres and the phasic fibers having shorter ones. In a detailed histological study of the different fibers in the accessory flexor muscle of Cancer magister Dorai Raj (1964) was able to show that tonic fibers have their myofibrils clumped into a "felderstruktur" arrangement (Kruger, 1949), while phasic fibers were typically "fibrillenstruktur" in appearance.

In the broad view, mechanisms underlying E-C coupling may not be the same for all types of striated muscle fibers. In fibers with Ca-K action potentials, Ca entering the membrane may be sufficient to activate the fibrils, or it might serve as a primer for SR calcium release. Tonic fibers could spread activation inwards by an entirely different mechanism than phasic fibers.

To determine if there are general phenomena common to tonic and phasic crustacean fibers, combined ultrastructural and physiological studies have been carried out. At the present time, the ultrastructure of the various types of crustacean fibers has not been examined in detail. The initial portions of this thesis therefore, deal with the ultrastructure of Balanus and Carcinus fibers, with particular emphasis on the structure of the T-system and sarcoplasmic reticulum. Unlike the striking differences displayed in vertebrate fast and slow

fibers, both the tonic Balanus and the Carcinus twitch fiber have well developed tubular and SR systems. Both also have an extensive array of sarcolemmal invaginations permeating the fiber interior. The channels between the external bathing solution and the sarcoplasmic reticulum were identical in both types of fiber, although somewhat better developed in Carcinus.

Because of the diversity of fiber types within single crustacean muscles, the neuromuscular responses of the five types of Balanus fiber were examined in some detail. Differences between white and pink fibers were especially noted. In the final sections, two physiological approaches were used in an attempt to elucidate the mechanisms by which activation spreads down the tubular system to the diad. An accurate determination of membrane capacitance was made in order to define its role in the coupling process. The large values of crustacean fiber membrane capacitance were found to be due to underestimating the amount of membrane contained in single fibers. Permeability of the tubular system membranes was examined also to see if it was different from that of the surface membrane. The method used was that of Girardier, et al. (1963), which consisted of swelling the tubules by establishing a Cl gradient between the sarcoplasm and the tubule lumen. The finding of a permselectivity to Cl ions suggested a means by which a current loop could transmit excitation inwards.

CHAPTER II

MATERIALS AND METHODS

Balanus Morphology

Specimens of Balanus nubilus were obtained by dredging from the waters near Friday Harbor, Washington. They were stored in large tanks of running sea water at the Friday Harbor laboratories, or kept in a cold room in small aquaria containing well aerated sea water.

For examination by light microscopy, fibers were held at rest length, or slightly stretched and fixed in Bouin's solution for twelve hours or longer. Picric acid was removed by soaking the fixed fibers in a solution of lithium carbonate in 70% ethanol prior to dehydration and embedding. Paraffin sections were cut at 6-8 μ and stained with Masson's trichrome.

Fixation for electron microscopy was with 3% gluteraldehyde in sea water or in Palade's buffer with 20% sucrose added. After a three hour wash in sea water or buffer, the fibers were post-fixed in 1% OsO_4 in either of the same vehicles used for the gluteraldehyde. Dehydration in alcohol was followed by three changes of propylene oxide. Fibers were embedded in epon and silver-gray sections cut

with a diamond knife on a Sorvall MT-2 ultramicrotome. Grids were stained with uranyl acetate and lead citrate and examined in a Siemens Elmiskop 1A electron microscope.

Thick sections of epon-embedded material were also examined in the phase-contrast microscope, especially when mapping of the cleft systems was to be performed.

Carcinus Morphology

Vigorous male specimens of Carcinus maenas were obtained from the waters in the vicinity of Woods Hole and kept in tanks of fresh running sea water at a temperature of about 18°C. Single fiber preparations were made from the closer carpopodite muscle. Every fiber was first tested to see if it would support a propagated action potential. In some preparations twitch tension was also monitored by means of a RCA 5734 transducer tube (see Figure 2). The fibers were bathed in a physiologic saline solution with the following ionic composition: NaCl 520, KCl 10, $CaCl_2$ 15, $MgCl_2$ 8, $NaHCO_3$ 3, (all mM/L). The pH was adjusted to about 7.4.

Two methods were employed to produce swelling of the TTS. Both depend on establishing an electrochemical gradient across the membranes of the TTS for Cl, so that there would be an outflow of Cl from the sarcoplasm into the lumen of the tubules. In the first method,

the internal concentration of Cl was increased by soaking the fiber in a high (120 mM) potassium Ringer solution for about forty-five minutes. The solution was then changed to one which contains the normal concentration of KCl or one which has had the Cl replaced by propionate, the darkening of the fiber which occurs when the tubular system swells could be observed visually with the aid of transmitted light, and the fixative could be poured on at the appropriate stage.

In the second method, long pulses of inward current were passed through a microelectrode. The membrane potential was monitored with another electrode and the current was measured as a voltage drop across a resistor. Swelling was produced when the microelectrode was filled with 3M KCl, but not when filled with 3M K propionate.

Primary fixation was with glutæraldehyde, either in a .02% solution in Palade's buffer for forty-five minutes followed by post-fixation in 1% OsO_4, or in a 3% solution in Palade's buffer with 20% sucrose added. In the latter method, the fibers were washed in a 20% sucrose-buffer solution and postfixed in 1% OsO_4 + 20% sucrose.

After dehydration in ethanol and propylene oxide the fibers were embedded in epon and sectioned. Thin sections were stained with lead citrate and uranyl acetate and examined with a Phillips 200 electron microscope at the Marine Biological Laboratories, Woods Hole.

Physiological Recording

Balanus fibers were prepared according to the dissection procedure described by Hoyle and Smyth (1963). Nerve trunks to whole muscles were separated from their surrounding connective tissue and pulled into suction electrodes for stimulation. Single adductor fibers were isolated by removal of all adjacent fibers, leaving only the scutal attachments intact. Tension was monitored in these fibers by placing a forceps attachment to an RCA 5734 transducer tube in contact with one of the scutes, while firmly anchoring the other one. Similarly, depressor fibers were isolated by separation from the other fibers in the bundle and clamping the transducer forceps on the tendon.

Glass capillary microelectrodes filled with 3M KCl were used to monitor membrane potential changes and apply current directly. Recording electrodes were of the order of 7-15 MΩ resistance and those with tip potentials greater than 5 mV were discarded. Stimulating electrodes had somewhat smaller resistances and were checked first to see if they would pass inward and outward currents equally well and without distortion.

Membrane "cable" properties were calculated following the method of Fatt and Katz (1953) (Figure 1). The stimulating electrodes were placed as close as possible to the recording electrodes and

monitored hyperpolarizing currents were passed into the fiber while the recording electrode was moved down the fiber known distances. Because the electrotonic potential decays exponentially, the following methods of calculation were employed.

Symbols Used

τ_m = Time constant in msec.

I = applied current in μA.

V_o = recorded membrane voltage in mV with no electrode separation

C_m = membrane unit capacitance in $\mu F / cm^2$

d = mean fiber diameter in mm.

V = membrane voltage in mV

λ = length constant in mm

R_m = membrane unit resistance in $\Omega\ cm^2$.

R_i = specific resistance of sarcoplasm in Ω cm.

x = interelectrode distance in mm.

(1) Length constant -

$$\lambda = \frac{x_1}{\mathrm{Ln} V_o / V_{x1}}$$

Where V_{x1} is the recorded membrane voltage at an electrode separation of x_1 mm.

(2) Membrane resistance - R_m

$$R_m = \frac{\pi d \ \lambda \ V_o}{I_o}$$

(3) Internal resistance - R_i

$$R_i = \frac{R_m \, d}{4 \lambda^2}$$

(4) Time constant - τ_m

Calculated by plotting the time of 1/2 amplitude of recorded potential against inter-electrode distance (Hodgkin and Rushton, 1946). Doing this for several distances gives the mean velocity of propagation at 1/2 amplitude (V 1/2), then

$$\tau_m = \frac{2 \lambda}{V \, 1/2}$$

(5) Membrane capacitance - C_m

$$C_m = \frac{\tau_m}{R_m}$$

The circuit employed for making these measurements is shown in Figure 1A.

Twitch fibers from Carcinus were isolated by first cutting away the lateral portions of the meropodite cuticle and completely removing the opener muscle. Fibers from the closer carpopodite muscle were then dissected away one by one until a single fiber was left, attached to the tendon, which was cut distally. The transducer forceps were

attached to the cut tendon and a small amount of stretch applied to the fiber. The fiber was stimulated directly with outward (depolarizing) currents and membrane spiking and twitch tension monitored. Microelectrodes were floated or fitted with rubber collars in order to prevent them from being dislodged by the movement of the fiber. If the fiber was judged to be in good condition, cable properties were measured in the same way as has been described for Balanus.

Membrane capacitance was also determined from the foot of the propagated action potential as described by Fozzard (1966). Conduction velocity was measured by two electrodes at least 0.5 cm. apart (see Figure 1B). The time constant of the foot of the action potential was measured by recording the conducted action potentials at high sweep speeds (Figure 3) and plotting the early voltage values on semi-logarithmic paper or by measuring the time required for the exponentially rising phase of the action potential foot to come to 1/e of its final value. From these values, the capacitance apparently being filled by the foot of the action potential could be calculated according to the following formula:

$$(1) \qquad c_m = \frac{1}{U^2 r_2 t}$$

where U = velocity of conduction
r_2 = internal resistance
t = A.P. foot time constant

The derivation of the formula is as follows:

$$i_2 = -\frac{1}{r_2}\frac{dV}{dX}$$

, where i_2 represents the current flowing through the interior of the fiber and r_2 represents the internal resistance.

$$i_m = -\frac{di_2}{dx}$$

, where i_m is the current flowing longitudinally and transversely across the membrane per unit length.

Since $$\frac{di_2}{dx} = \frac{1}{r_2}\frac{d^2V}{dx^2}$$

Then $$i_m = \frac{1}{r_2}\frac{d^2V}{dx^2}$$

The equation which describes the foot of the action potential is:

$$V = Ae^{-Kx + K\,Ut}$$ (Hodgkin and Huxley, 1952)

where $K = \frac{1}{T}$ (inverse of A. P. foot time constant)

and U = velocity.

At condition x, $V = Ae^{-Kx}\ e^{K\,Ut}$

if $T = \frac{1}{K\,U}$, $V = A'e^{K\,Ut} = A'e^{\,t/T}$

$$\frac{dV}{dt} = K\,UA'e^{K\,Ut} = K\,UV$$

$$V = Ae^{K\,Ut}e^{-Kx} = A''e^{-Kx}, \quad \frac{dV}{dX} = -A''Ke^{-Kx},$$

$$\frac{d^2V}{dx^2} = A''K^2e^{-Kx} = K^2V$$

$$i_m = \frac{1}{r_2}\frac{d^2V}{dx^2} = \frac{K^2}{r_2}V = \frac{V}{r_m} + c_m\frac{dV}{dt} = V\left(\frac{1}{r_m} + K_U c_m\right)$$

therefore, $$\frac{K^2}{r_2}V = V\left(\frac{1}{r_m} + K_U c_m\right)$$

$$\frac{K^2}{r_2} = \frac{1}{r_m} + K_U c_m$$

and, $$c_m = \frac{1}{K\,U}\left(\frac{K^2}{r_2} - \frac{1}{r_m}\right) = \frac{K}{Ur_2} - \frac{1}{K_u r_m} = \frac{1}{U^2 r_2 t} - \frac{t}{r_m}$$

$\frac{t}{r_m}$ will be negligible so that $c_m = \frac{1}{U^2 r_2 t}$ (equation 1)

where c_m and r_2 are expressed per cm. length.

It was found more useful to express c_m per unit membrane area. This can be accomplished by letting $c_m = C_m \times 2\pi a$, where a = radius, and since $r_2 = \frac{R_i}{\pi a^2}$

so that $2\pi a C_m = \frac{K \pi a^2}{U^2 R_i}$ where $K = \frac{1}{t}$

and $C_m = \frac{K_a}{2R_i U^2}$.

If we wish to include the increased area contributed by the clefts,

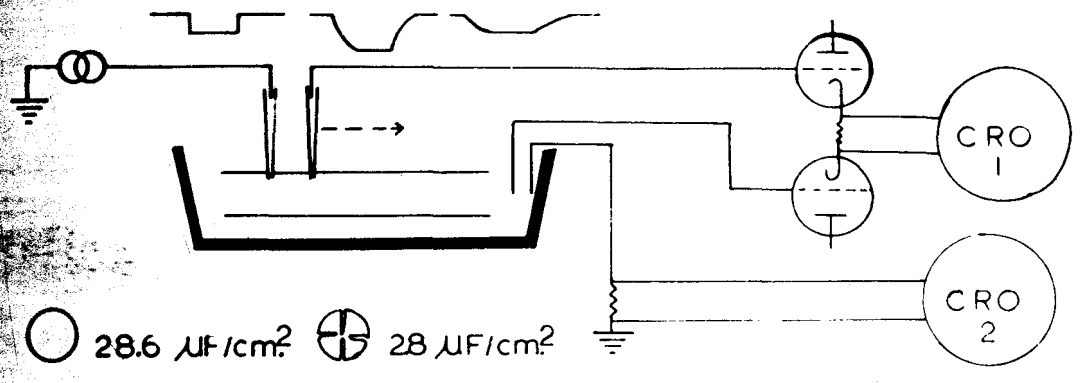
let A = cross-sectional area

P = circumference of cross section including clefts

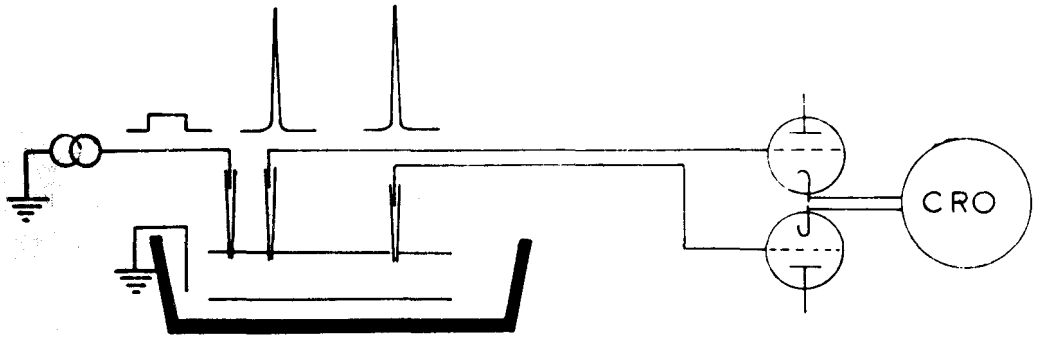
then $r_2 = \frac{R_i}{A}$ and $C_m = c_m P = \frac{A}{U^2 R_i t}$

$C_m = \frac{A}{P U^2 R_i t}$ where C_m is expressed in $\mu F/cm^2$.

Figure 1. Circuit diagrams for measuring membrane cable properties (upper) and capacitance from foot of the action potential (lower). In the upper diagram, current is delivered to the fiber through one microelectrode and the membrane potential recorded at various distances through another via a cathode follower headstage. Current is monitored on the second beam of the oscilloscope across a 10 K Ω resistor. In the lower diagram, depolarizing pulses are used to generate propagated action potentials which are then recorded at two different positions on the fiber. The values shown are for Carcinus membrane capacitance before and after correction for cleft area.



○ 28.6 μF/cm² ⊕ 28 μF/cm²



○ 6.1 μF/cm² ⊕ 1,5 μF/cm²

Figure 2. Response of a Carcinus twitch fiber to direct intracellular stimulation. A) shows overshooting spike response. Upper trace is 0 potential, middle is membrane potential and lower is current. In B, the upper trace shows the membrane potential change while the lower trace indicates tension. Note there is no graded tension development in response to sub-threshold depolarizing currents. The step voltage at the beginning of the membrane potential traces is a 10 mV calibrating pulse.

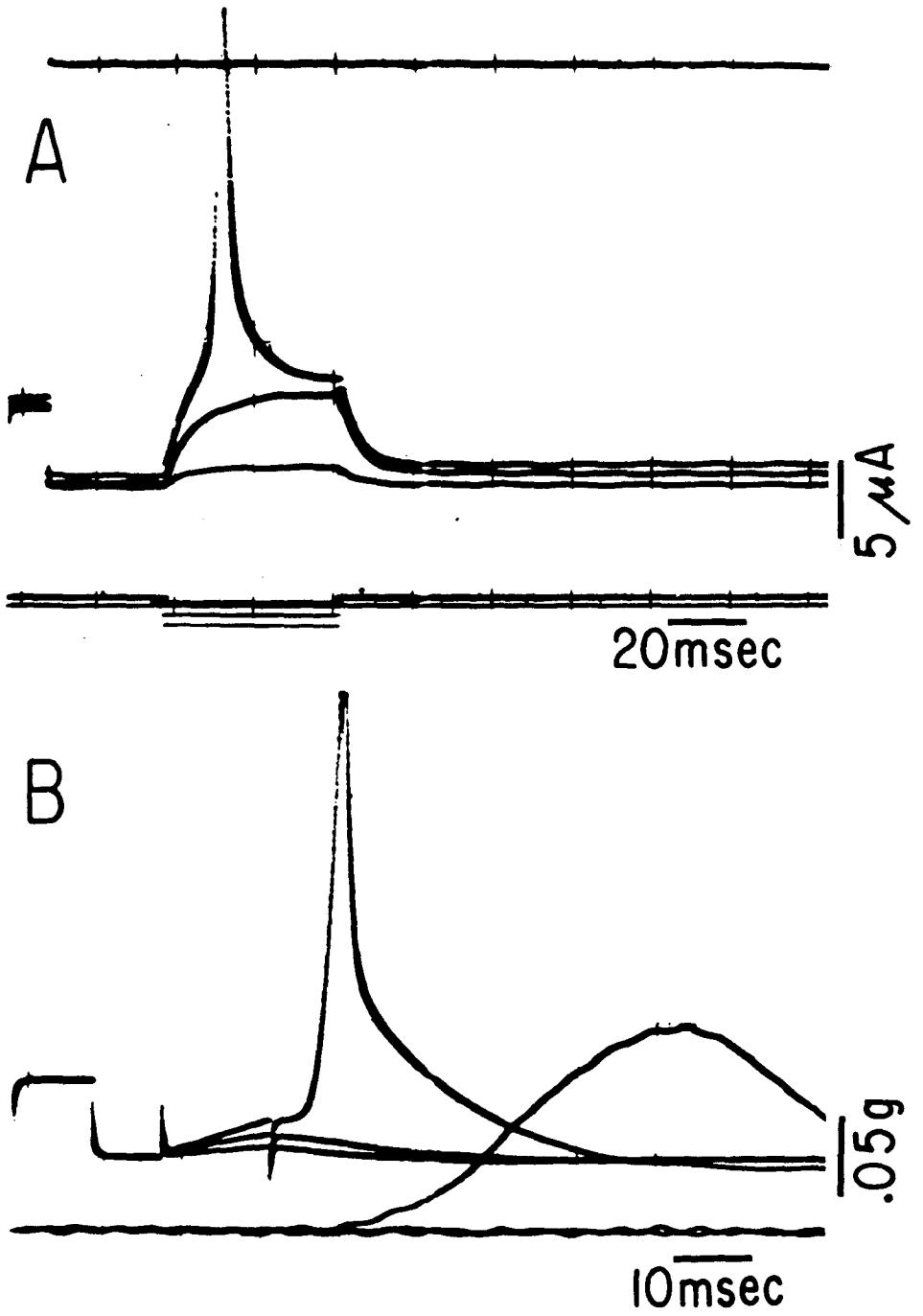
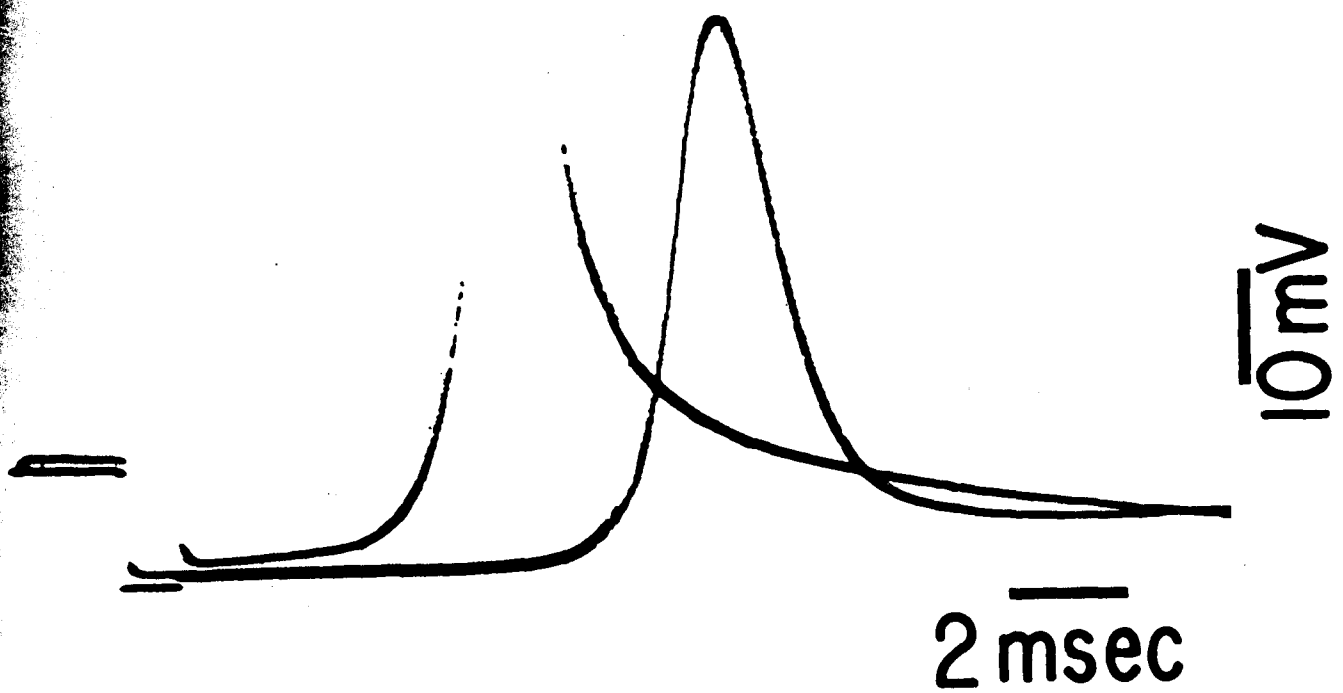

A
5 μA
20 msec
B
.05g
10 msec

Figure 3. Propagated action potentials along the surface of a Carcinus twitch fiber recorded from two different locations on the membrane. This type of record was used to determine the conduction velocity and time constant of the action potential foot for capacitance measurements.


10 mV
2 msec

CHAPTER III

RESULTS AND DISCUSSION

Results

A thorough analysis of the mechanisms underlying excitation-contraction coupling requires a correlated morphological and physiological approach. A complete anatomical study of two types of crustacean fibers, the large gradedly-contracting fibers from the barnacle Balanus nubilus and small twitch fibers from the shore crab Carcinus maenas, is described first. Aspects of the morphology such as the tubular system and sarcoplasmic reticulum are especially relevant to the inward spread of excitation and are assessed and evaluated in separate discussions following each descriptive section.

While the electro-mechanical properties of Carcinus twitch fibers have been described previously (Atwood, Hoyle and Smyth, 1965), only a preliminary account of Balanus muscle fiber physiology is available (Hoyle and Smyth, 1963). Inasmuch as the giant fibers of Balanus have become a widely used preparation for the investigation of muscle membrane properties (Hagiwara, et al., 1964) and excitation-contraction coupling (Edwards, et al., 1964), it was of interest to examine more

completely the electro-mechanical responses of not only the depressor fibers, but also the adductor fibers and the various pink fibers. This investigation makes up the third section and is also discussed separately.

As emphasized in the introduction, it is possible to study the function of the tubular system by many approaches, all of them unfortunately indirect. Three questions which might be asked are how the large capacitance of crustacean fibers is distributed and might function in E-C coupling and what are the tubular permeability characteristics of crustacean fibers and how might tubular ionic fluxes be responsible for transmission of excitation inwards? In the final section, a series of experiments designed to accurately measure fiber capacitance and tubular permeability are described. The significance of these findings to a proposed coupling hypothesis is treated in the discussion.

General Description of Balanus Fibers

A preliminary account of the gross anatomy for Balanus has been given by Hoyle and Smyth (1963). I wish to consider in some detail the ultrastructure of the five groups of fibers which can be distinguished. The most conspicious of these are the white depressors, the so-called "giant" fibers. There are three pair of depressor muscles, the depressor scutorum rostralis, the depressor scutorum lateralis and the depressor tergorum. On the medial aspect of the lateral depressors

one finds a flag of thin pink fibers having the same origin and insertion as the white fibers. The adductor scutorum muscle serves to close the scutal plates, and while the fibers are only about one third the length of the depressors, they have the same diameter. The adductor muscle is enveloped by a double-walled membrane (Figure 4). Attached to this membrane, above and beneath the adductor, are found four bands of small pink fibers which I shall call the pink adductors. There is one additional pair of pink muscles which serves to rotate the body relative to the scutum and which probably functions during feeding.

White Fibers

In very large specimens, the diameter of the white depressors may exceed 3 mm.; the adductor fibers are slightly smaller. The resting lengths are 3 to 4 cm. for the depressors and 2 cm. for the adductors. With the aid of transmitted light, it is possible to see longitudinal invaginations which may extend 4-5mm. along the fiber. Light microscopy reveals that the myofibrils are dispersed uniformly throughout the fibers of all the white depressors (Figure 5). This punctate appearance is similar to the "fibrillenstruktur" arrangement of Kruger (1949). Although this myofibrillar configuration has been associated with phasic fibers in other crustaceans (Cohen, 1963), it is not limited to only fast fibers.

Figure 4. Diagrammatic representation of interior surface of scutal-tergal plates with body still attached. The white adductor muscle is covered with a bi-layered heavy membrane which has been removed from the right half in the drawing. Two flags of thin pink fibers are attached to the membrane above and below the adductor muscle. In addition, two bundles of pink fibers are found connecting the shell to the body. The motor nerve supply to all the fibers is readily accessible.

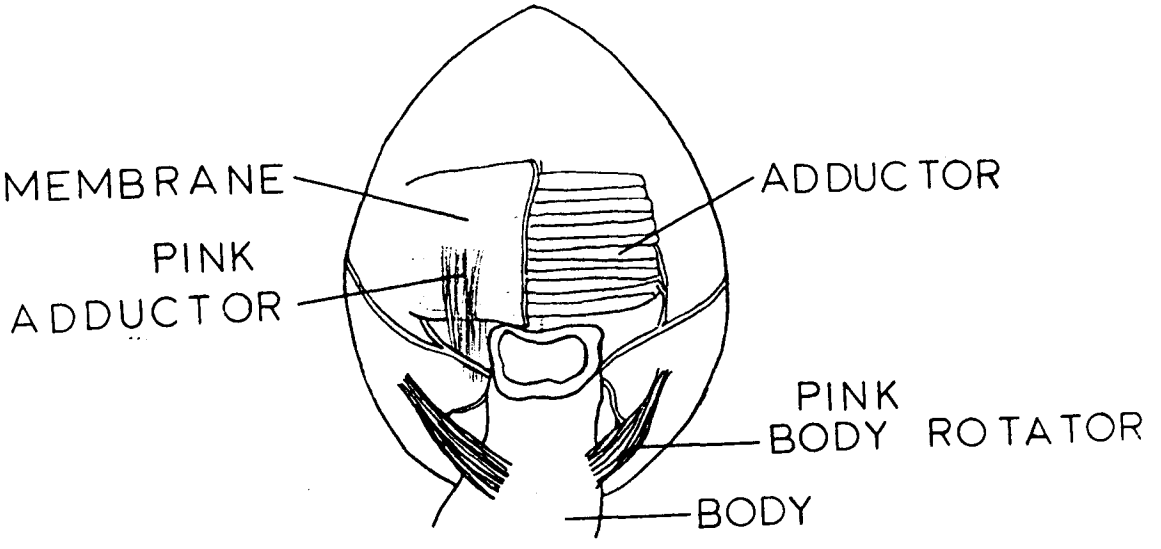
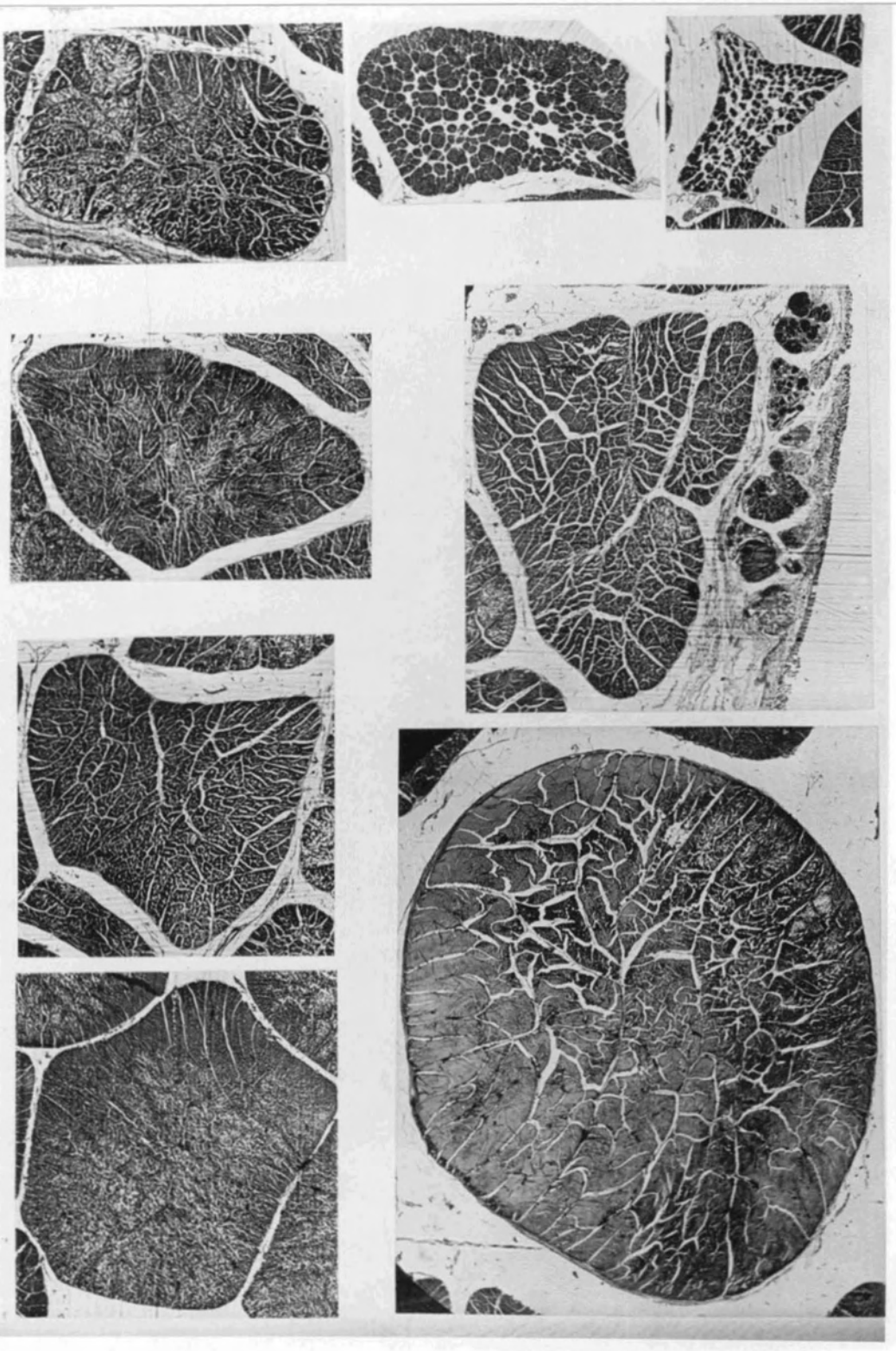

MEMBRANE
PINK
ADDUCTOR
ADDUCTOR
PINK
BODY ROTATOR
BODY

Figure 5. Light photomicrographs of the various types of white Balanus fibers. The two in the lower left hand corner are typical depressor fibers, while the remainder are types found in the adductor muscle. The two in the upper right hand corner are relatively rare. Note the large increase in surface area due to the sarcolemmal invaginations.



There is considerable variation of sarcomere length in the depressor fibers, the mean being 10 μ. This value compares favorably with the 10 μ sarcomeres of tonic fibers found in the crab accessory flexor muscle by Dorai Raj (1964), and the 10-14 μ sarcomeres found in the slow fibers of the crab closer muscle (Atwood, Hoyle and Smyth, 1965). The white adductor fibers have approximately the same sarcomere lengths as the depressors, but their cross-sectional appearance is extremely variable (Figure 5). About 20% of the adductor fibers have the same myofibrillar arrangement as the depressor fibers. The remainder have their myofibrils clumped in the "felderstructur" arrangement, or disposed in configurations (Figure 5), which are intermediate between punctate and clumped.

Pink Fibers

All of the pink fibers are less than 1 mm. in diameter, and occur in bundles which are one or two layers thick. They are surrounded by more connective tissue than the white fibers, so that dissection of single fibers is more difficult. Lateral pink fibers are the same length as the white depressors, but those attached to the adductor muscle membrane are only about 1 cm. in length (see Figure 4).

Pink fiber sarcomeres are a bit longer than the white fiber sarco-

meres, averaging 11.5 μ, and myofibrils are arranged in small clumps (Figure 6), with large areas of sarcoplasmic matrix containing large numbers of mitochondria interspersed between them. Their pink color is due to the cytochromes of these mitochondria.

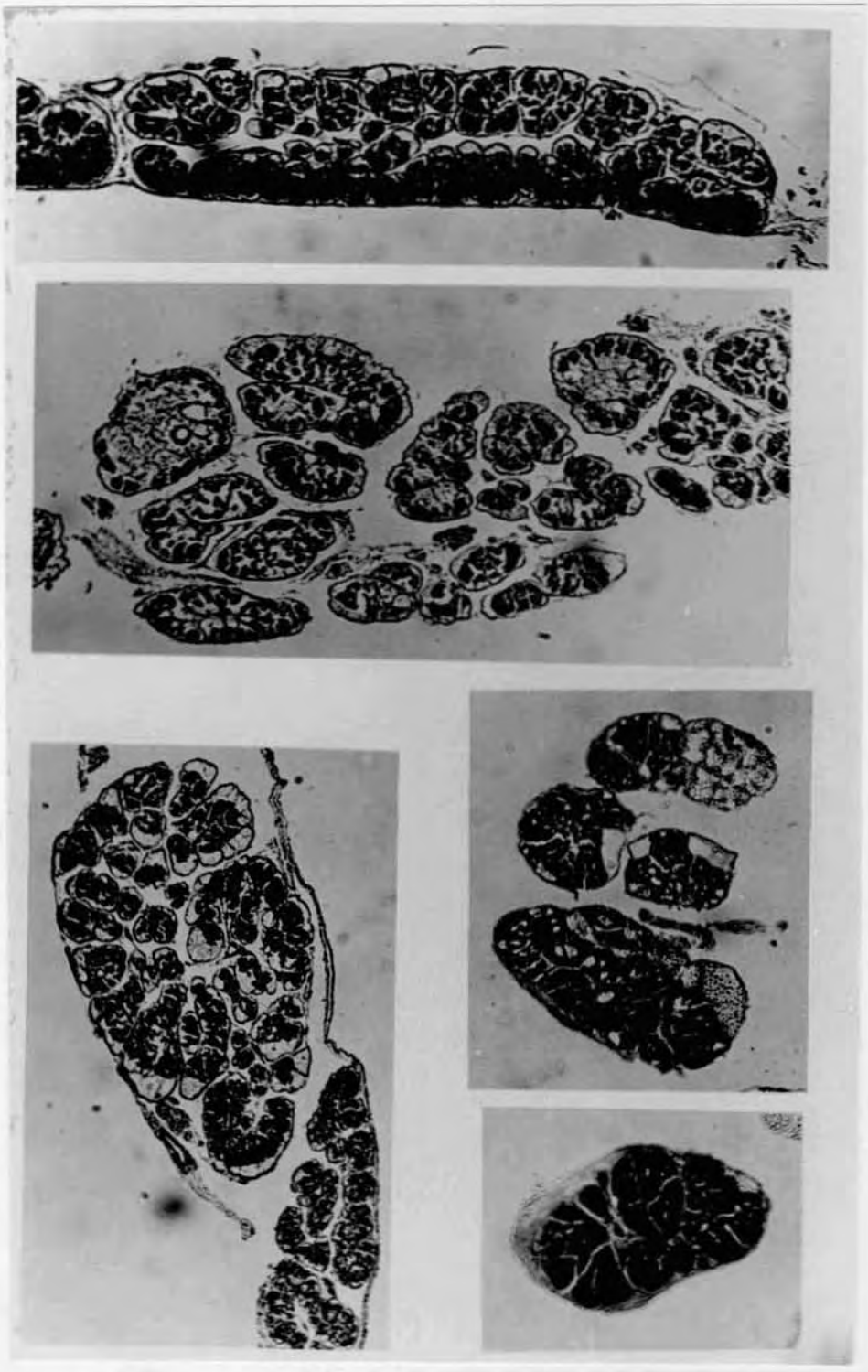
Sarcolemma

The plasma membrane has the dimensions and appearance of a typical unit membrane, 75-90 A thick, depending upon the type of fixation employed. Lying above the unit membrane is a wider band of basement material, about 0.5 μ in thickness. Both the unit plasma membrane and the basement material are invaginated longitudinally to form deep clefts which ramify and become confluent within the fiber (Figures 5, 12). Enlarged cross sections allow quantification of the increased surface area, and these results will be discussed in a later section.

One often sees axon branches lying deep within the clefts (Figure 16), but structures having the characteristics of synaptic contacts have thus far not been encountered.

Mitochondria are found between the fibrils and the sarcolemma in both the white and pink fibers, but the largest numbers of them are at the periphery of the pink ones. Where there are indentations of the

Figure 6. Cross sections of Balanus pink fibers. Upper two are from the lateral depressor, lower left is a body rotator and the two in the lower right hand corner are pink adductors. There is considerable clumping of the myofibrils and more undifferentiated sarcoplasm than in the white fibers.



sarcolemma, the mitochondria are found in clusters along the membrane (Figure 19).

Sarcomere Ultrastructure

Z Line

The Z lines of the barnacle fibers are unique, and their structure has already been considered in a previous publication (Hoyle, McAlear and Selverston, 1965). Instead of a regular network composed of unwound actin filaments (Knappeis and Carlson, 1962), the barnacle Z line is composed of dense amorphous particles (Figure 16), very similar to the dense bodies reported by Hanson and Lowey (1961), for an oyster adductor muscle. They vary in thickness from .1 to .3 μ when viewed in longitudinal sections, and present a patchwork appearance in cross sections (Figure 7). Previous investigations have shown that when the fibers are "super-contracted" via neural stimulation, thick filaments can pass through them (Hoyle and McAlear, 1963). Potassium contractures and ATP-induced contractures of glycinerated fibers did not permit passing through, however, and thick filaments were instead bent back at the Z line (Selverston, 1964).

Thick Filaments

The A-band is formed by thick filaments 140 A in diameter, tapering to a point at their ends. Neither an M-band or H-zone is present, and the lateral borders of the A-band are not sharply delineated (Figure 16).

Cross sections of thick filaments often appeared hollow, as has been reported by many others (e.g., Auber and Couteaux, 1963). In Carcinus twitch fibers, individual light meromyosin monomers could be seen around the hollow core, but this was never observed in Balanus.

While there is no question of thick-thin filament interdigitation, periodic actomyosin cross-bridges are not conspicious (Figure 16). A poor crystalline array of contractile proteins is common to crustacean fibers, so that the absence of discrete bridges is not surprising.

Thin Filaments

Thin filaments about 70 A in diameter originate from the dense Z-particles, but do not appear to contribute to the Z-lines, as is the case for vertebrates (Figure 16). Thin filaments interdigitate with the thick filaments, extending about one third of the way into the A-band. Despite the absence of thin filaments in the central portion of the A-band, there is no H-zone. Figure 25 illustrates several cross

Figure 7. White depressor fiber, cross section. The sarcolemmal invagination, SI, is always identifiable by the presence of basement material. Various portions of the sarcomere are transected, because of the longitudinal displacement of sarcomeres produced by the fixation process. Note the diads formed by the SI, as well as by tubules. X 35,000.

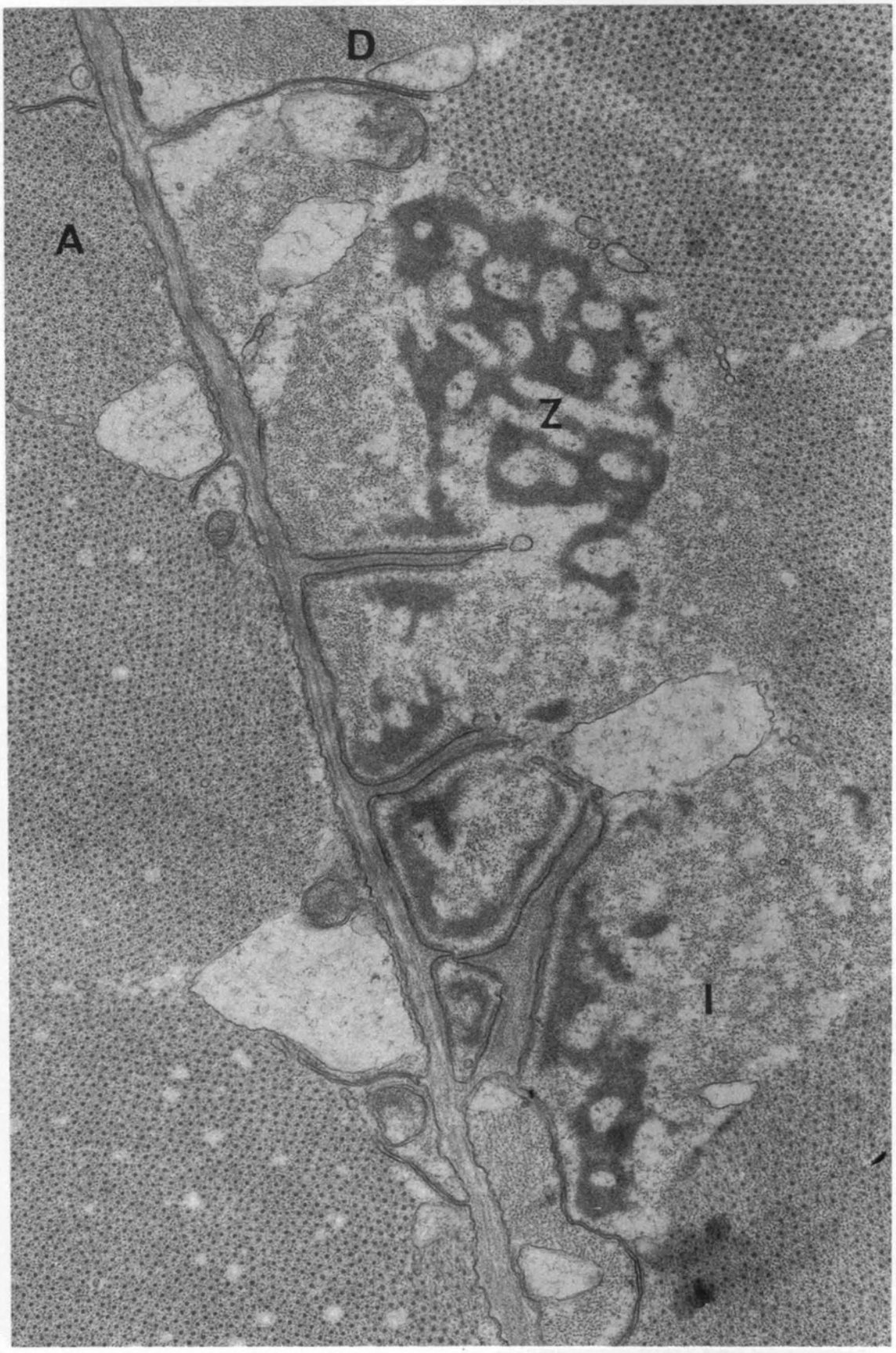
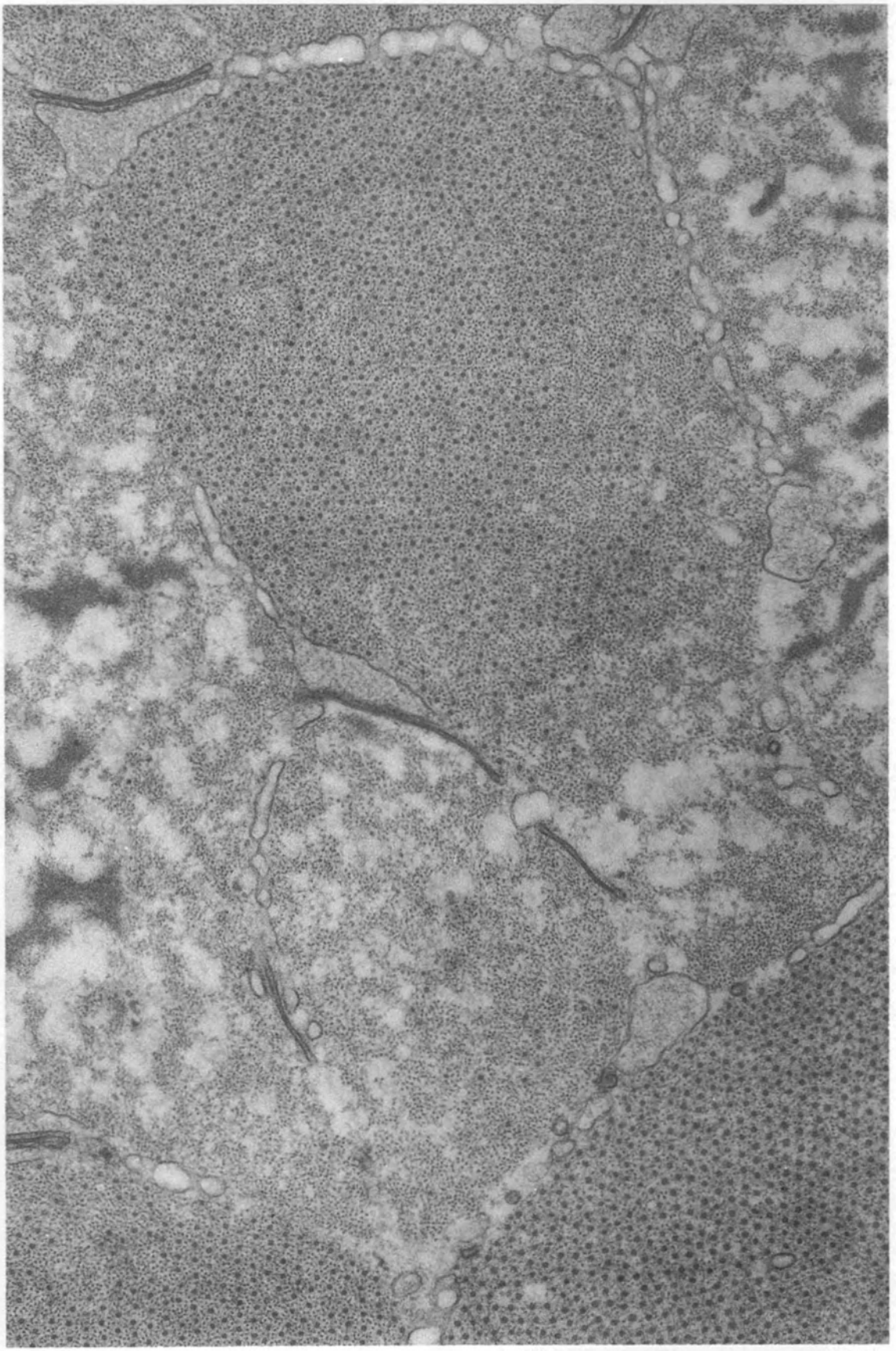
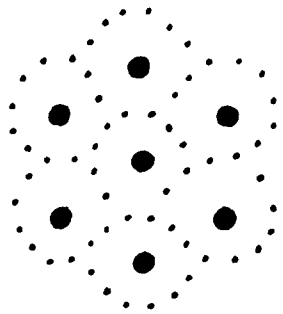

D
A
Z
I

Figure 8. White depressor fiber, cross section. The central fibril is transected at the A-I junction and illustrates the number of diadic contacts surrounding it. Note how the fibrils tend to merge with one another. Large numbers of thin filaments can be seen in orbits around thick filaments. X 52,500.



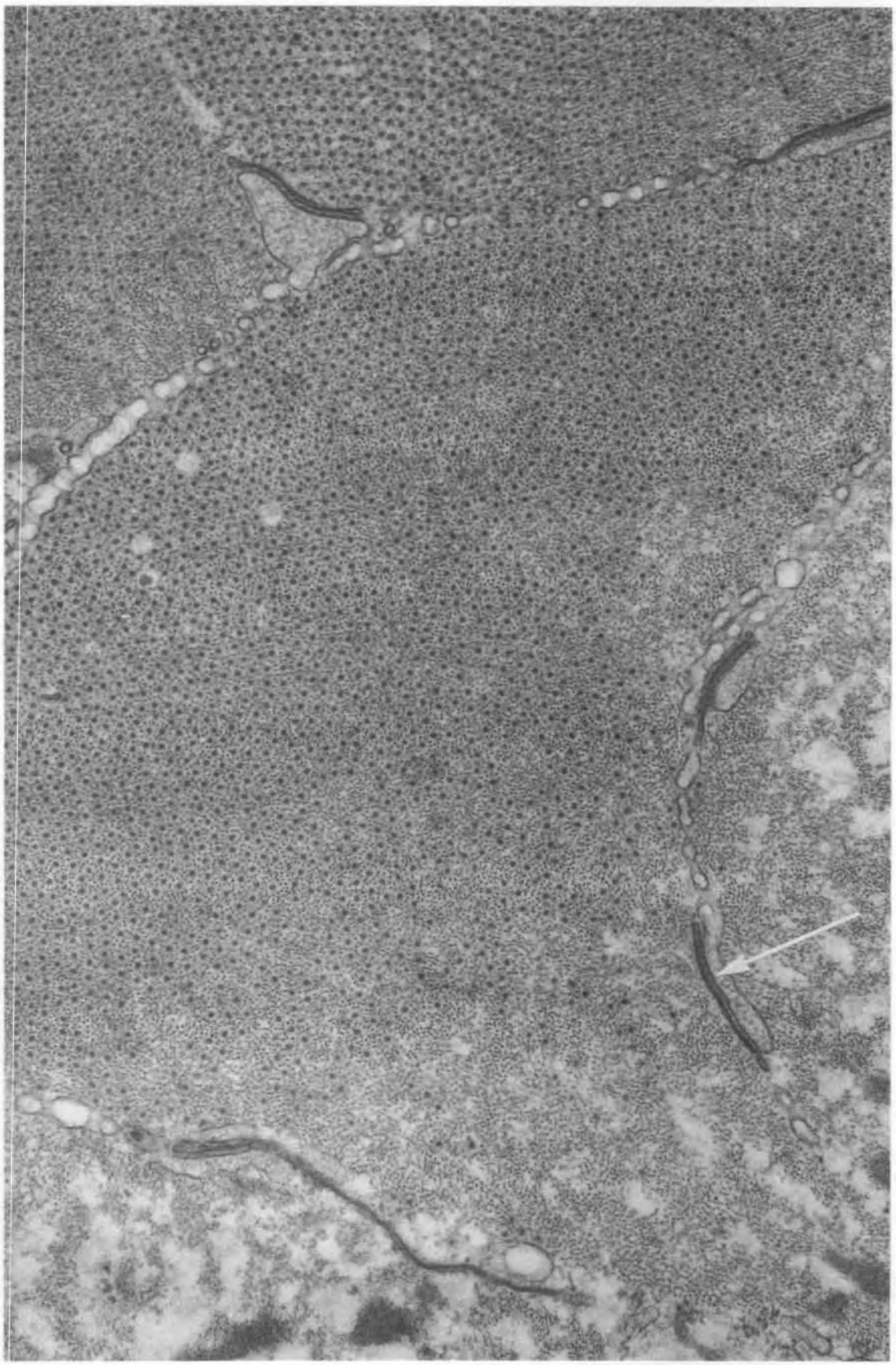
sections through a Balanus white depressor fiber and a twitch fiber from Carcinus at the level of the thick-thin filament overlap zone. In both cases one can see large numbers of thin filaments in orbits around the thick filaments. By direct count, the ratio of thin to thick filaments in Balanus is 5:1, with orbits arranged thus:



A similar pattern has been described by Smith, et al. (1966) for insect visceral muscles although the original observation was made by Tosselli (1965).

While the largest number of thin filaments reported for arthropods has been 12 (Auber, 1966), one frequently sees up to 14 in Balanus. Auber (1966) has tried to correlate the frequency of contraction with the number of thin filaments, the very fast fibrillar muscles of insects having smaller ratios, but at this stage I feel any such correlation is premature.

Figure 9. White depressor fiber, cross section. General features of fibril relationship to tubule and SR. There is a well developed collar delineating the fibril, interrupted at frequent intervals by diads. Note the dense line between the tubule and cisternae (arrow), and the large number of thin filaments. X 52,500.



Sarcoplasmic Reticulum

The sarcoplasmic reticulum (SR), consists of a fenestrated envelope surrounding each myofibril (Figures 8, 9). At the level of the A-I junction, the SR becomes slightly dilated and the fenestrations become more elongated and less frequent. The collar itself is 500 A thick and the fenestrations are variable in size from 350 A to 1500 A. Because the myofibrils often have some flat sides, longitudinal sections frequently show portions of the SR surface (Figures 13, 14). There is no indication that the pores connect the interior of the SR with the sarcoplasm as has been suggested by Franzini-Armstrong (1963).

The interior of the cisternal vesicle appears more granular than the interior of the adjacent SR (Figures 8, 9). This granularity has also been noted in other fibers (Birks, 1965, Peachey, 1965).

The SR of the pink fibers is more poorly developed than in the white fibers, as can be seen in both the longitudinal and cross sections (Figures 18, 20), however, the number of diads is not diminished. The cisternal portions of the SR form diads with the tubular system at the A-I junctions. In addition to these, diads are formed by the cisternae with the invaginated plasma membrane, or with the plasma membrane at the surface of the fiber.

Tubular System

In Balanus, the tubular system in both white and pink fibers is well developed when compared with other gradedly-contracting fibers (e.g., Page, 1965, Peachey and Huxley, 1962, Hess, 1961). Tubules are generally oriented inwards from the periphery, but there is no distinct transverse plane which they follow. Some may be purely transverse, while some may be longitudinally oriented, and still others display some intermediate variation of orientation.

The tubules are a morphological continuation of the surface plasma membrane to the diad. Implicit in this suggestion is that they are open to the exterior solution, however, as I shall demonstrate later, it does not imply that the tubular membrane has the same permeability characteristics as the sarcolemmal membrane.

Figure 7 illustrates typical sarcolemmal invagination of a white adductor fiber. The same features apply to the white depressors and the pink fibers also. Several characteristics can always be used to identify the invaginations. Basement material accompanies the plasma membrane inwards, but terminates at the origin of the tubules (Figures 1, 10, 11). Once they leave the clefts, the tubular diameter remains more or less constant at Ca. 300 Å. When they pass through Z-lines, the tubules show an increased electron density along their

Figure 10. White depressor fiber, cross section. Relation of sarcoplasmic invagination, tubules and sarcoplasmic reticulum. X 35,000.

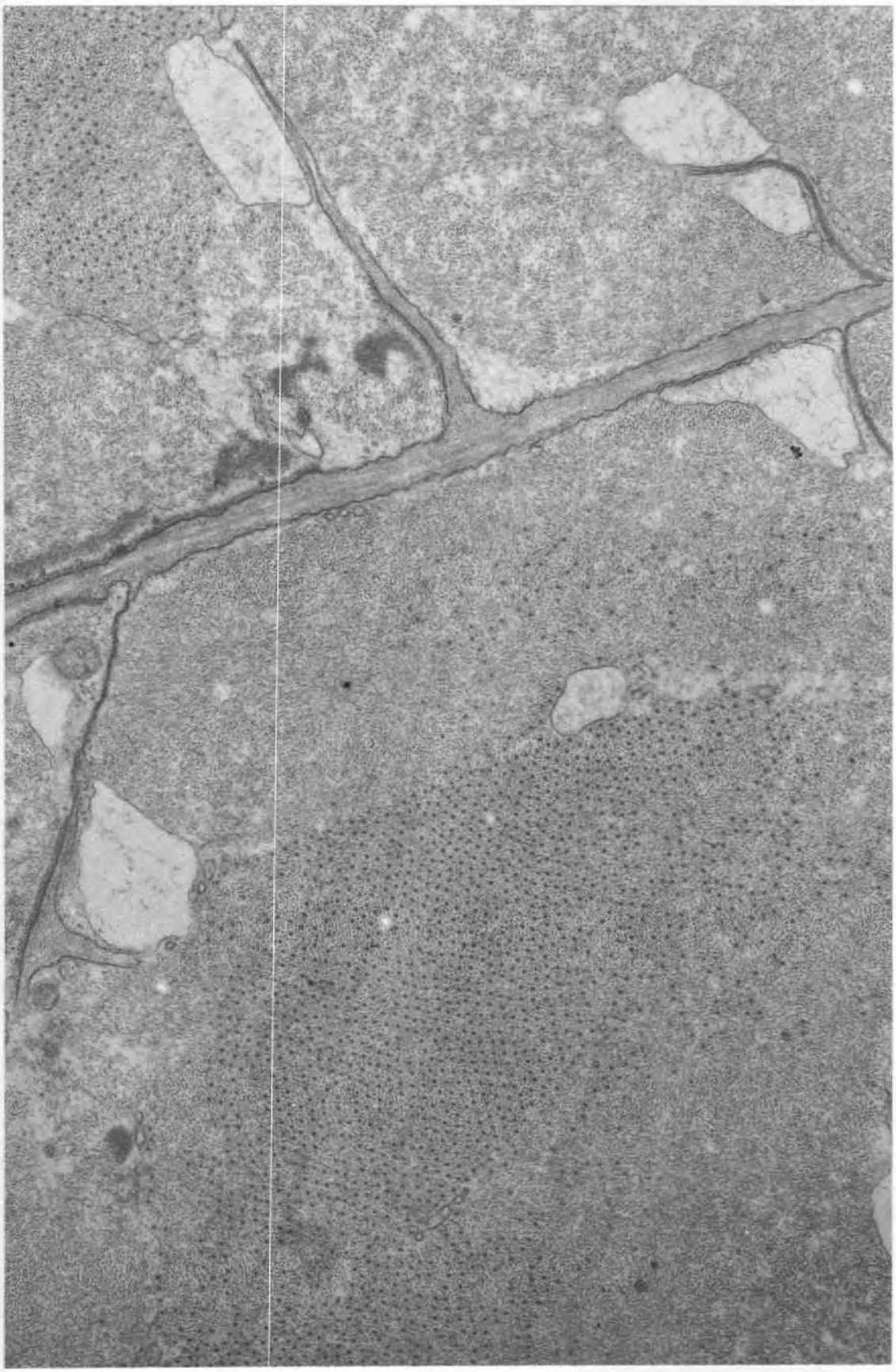
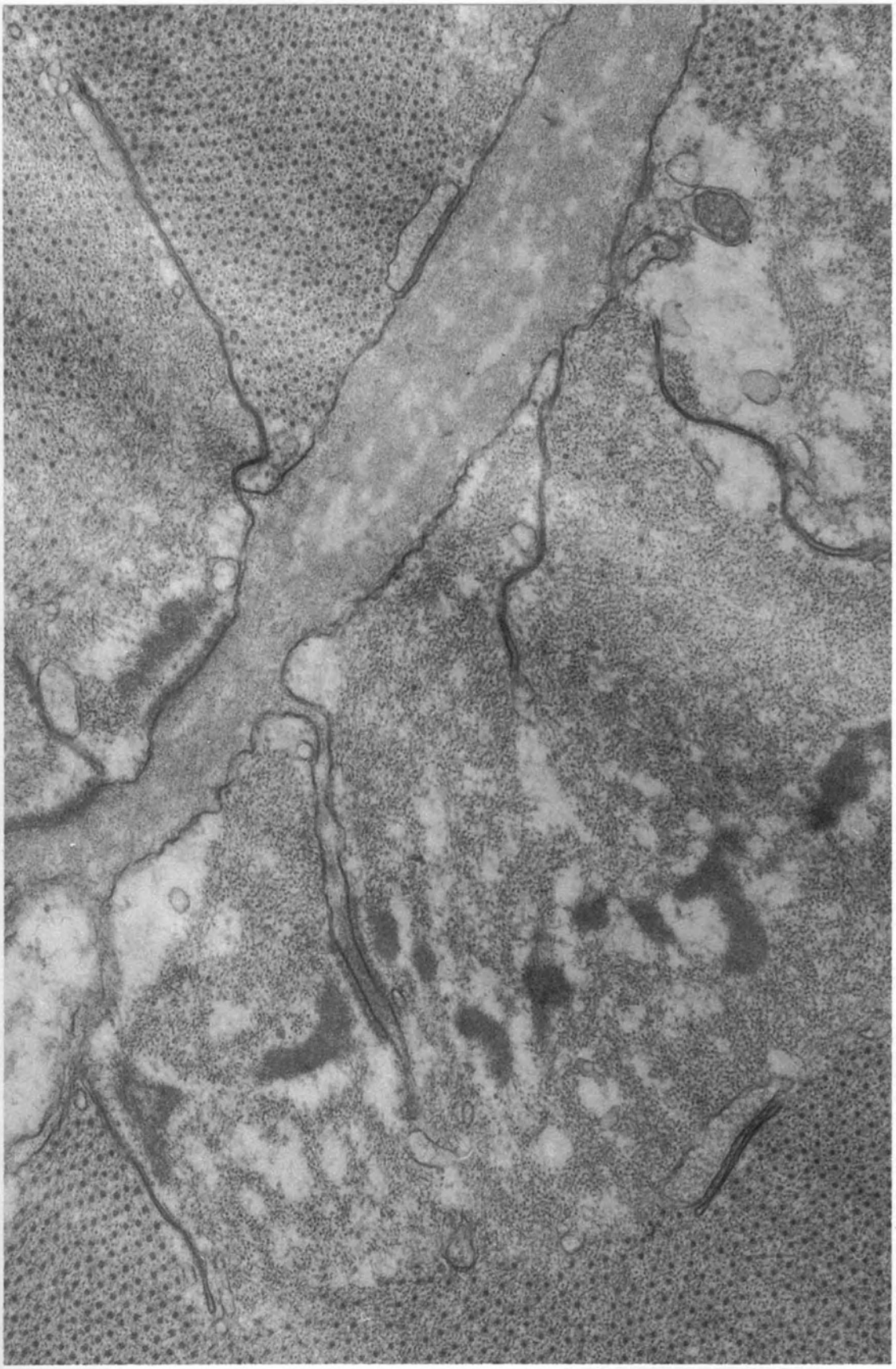


Figure 11. White depressor fiber, cross section. Relation of sarcoplasmic invagination, tubules and sarcoplasmic reticulum. X 52,500.



outer walls. This darkening is especially noticeable along the walls of the clefts (Figure 7) when they pass through Z lines. Figures 8 and 9 illustrate several transverse tubules forming diads. One can see that they soon go out of the plane of section and may run diagonally or longitudinally (Figures 16, 21, 23) for several hundred μ before forming diads. One tubule may form several diads (e.g., Figure 23) before finally terminating blindly.

Longitudinal and transverse tubule-cisternal contacts are shown in Figures 21, 23. The regular arrangement of the tubules with respect to the sarcomeres can be seen in Figure 14, where they appear at intervals of 2.1 μ.

Diads

Typical diads are shown in Figures 7, 8, 9, 14, and 23. The gap between the tubule and the cisterna is constant at 200 Å. The material filling the gap is rather electron dense, however, a faint line which has been described by Hoyle (1965), may be distinguished midway between tubular and cisternal membranes (Figure 9).

The interior of the cisterna contains a granular material (Figures 8, 9) as has been mentioned previously. The appearance of the cisternal contents relative to the interior of the remaining SR might indicate that this element subserves a different function, most tantalizing

of which could be the storage and release of calcium.

The position of the diad is usually at or near the A-I junction, but enough variation exists not to make this a hard rule. Most often these other locations are somewhere along the A-band (Figure 23). The cisternae may make contact with the tubules in several places around the myofibril (Figure 9). The average number of diads per A-I unit is four, however, up to seven have been counted, making in some cases, a total of fourteen contacts per sarcomere.

Although no ferritin or dye experiments were attempted to prove the patency of the tubules, their morphology indicates a continual open pathway between the external solution and the diad.

Discussion

The ultrastructure of Balanus fibers is in general typical of crustacean muscle fiber structure. The fundamental constituents of all striated muscles are present, although often modified in form. Myofibrils consisting of interdigitating thick and thin filaments divided into sarcomeres by Z-bands, and surrounded by an envelope of sarcoplasmic reticulum form the basic structural pattern. The absence of an H-zone and a M-line is also common to the vertebrate slow fiber (Peachey and Huxley, 1965). The tubular system forming a separate compartment within the fiber, participates in the formation of diads

Figure 12. White adductor fiber, cross section. Several invaginations can be seen entering the fiber from the surface, one almost completely enveloping a fibril at the level of the Z-band. Cisternae appear slightly swellen due to the low tonicity of the fixative, 5% gluteraldehyde in barnacle Ringer. X 21,000.

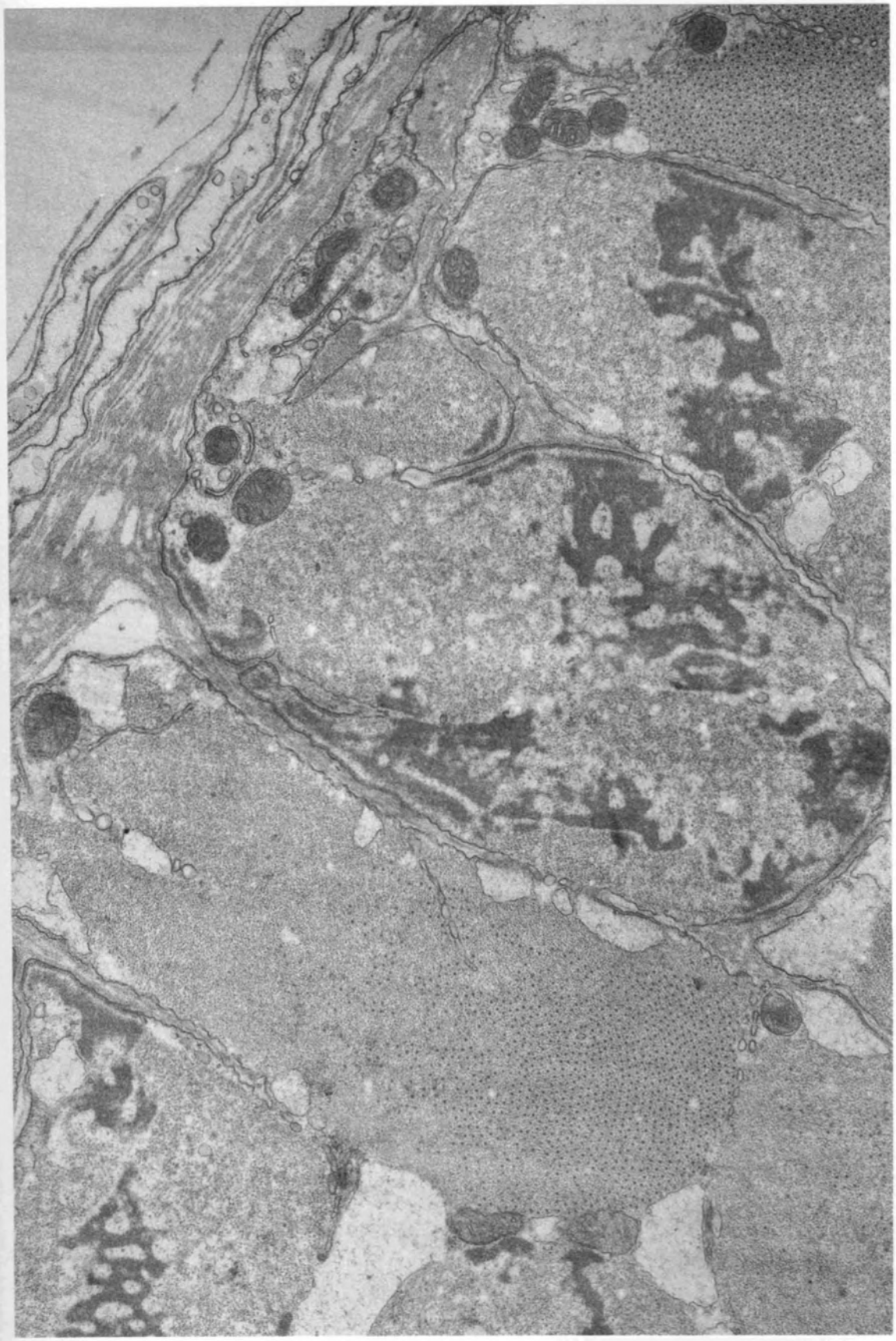


Figure 13. White depressor fiber, longitudinal section. General features of sarcoplasmic reticulum collar. The cisternal portion is indicated (C). Segments of tubules are identified in the upper right-hand area (T). X 50,000.


C
SR
T

Figure 14. White depressor fiber, longitudinal section. The two components of the SR can be seen in this section. The fenestrated collar is indicated (SR), and the connecting, non-fenestrated cisterna (C), is observed to be in close proximity to the excitatory tubules (arrows). X 50,000.

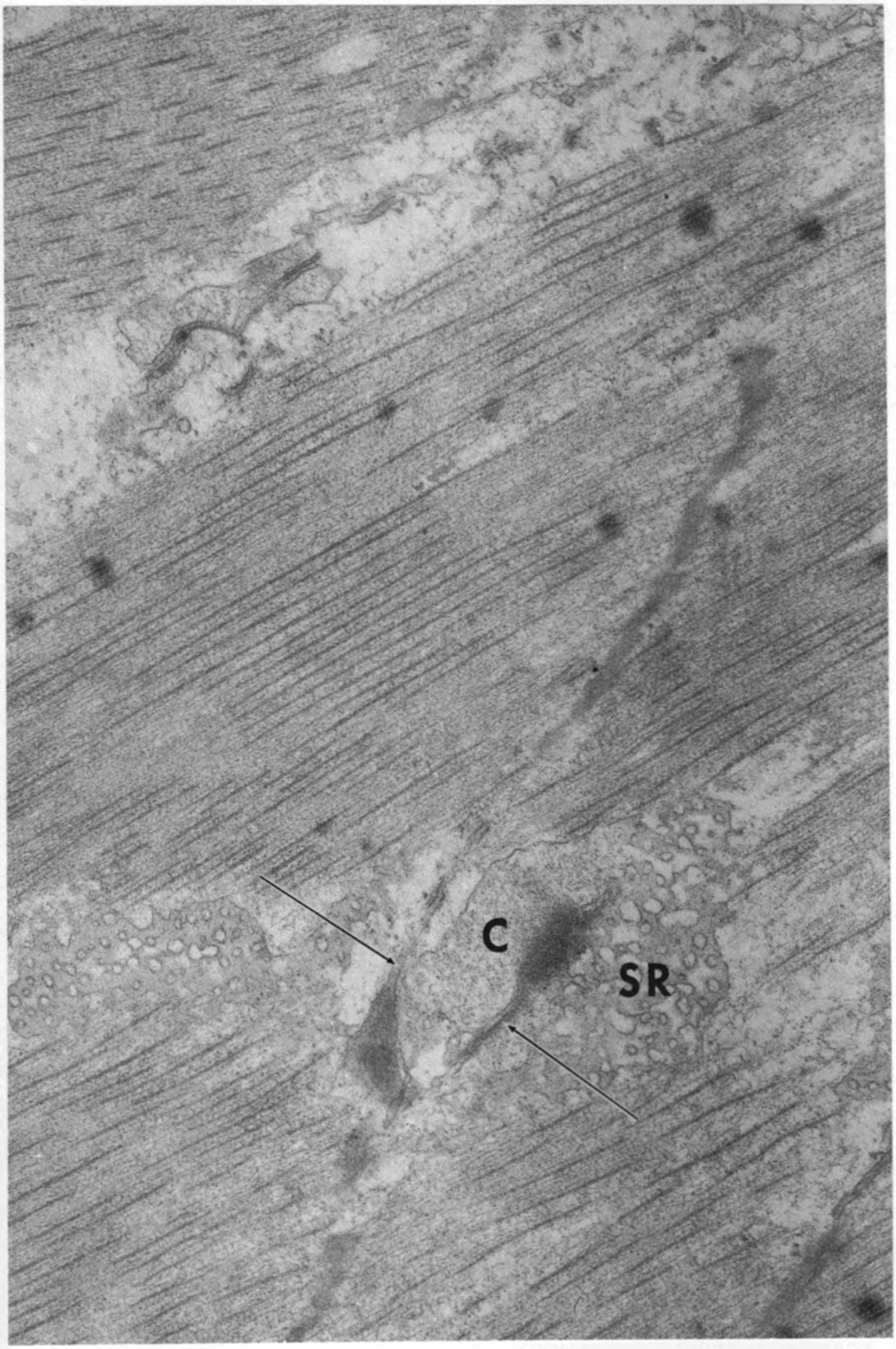

C
SR

Figure 15. White depressor fiber, longitudinal section. A tubular element is shown running just to the outside of the cisternal portion of the SR. The granular content of the interior of the cisternae is not visible, except for a small portion indicated by the arrow. X 90,000.

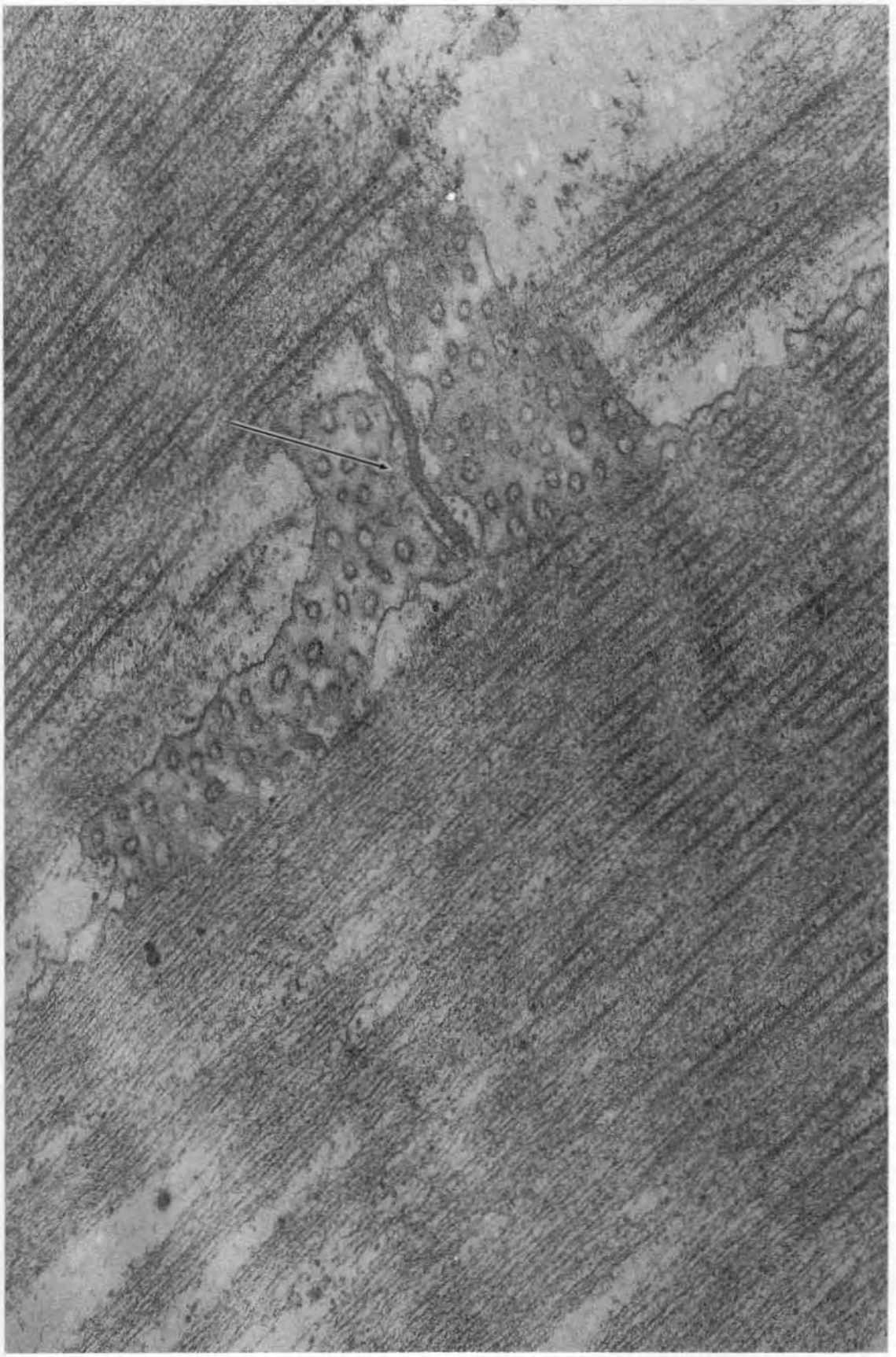


Figure 16. White depressor fiber, longitudinal section. A large sarcolemmal invagination can be seen containing neural elements (N). Note the attachments of the thin filaments to the dense bodies constituting the Z-line. X 45,000.

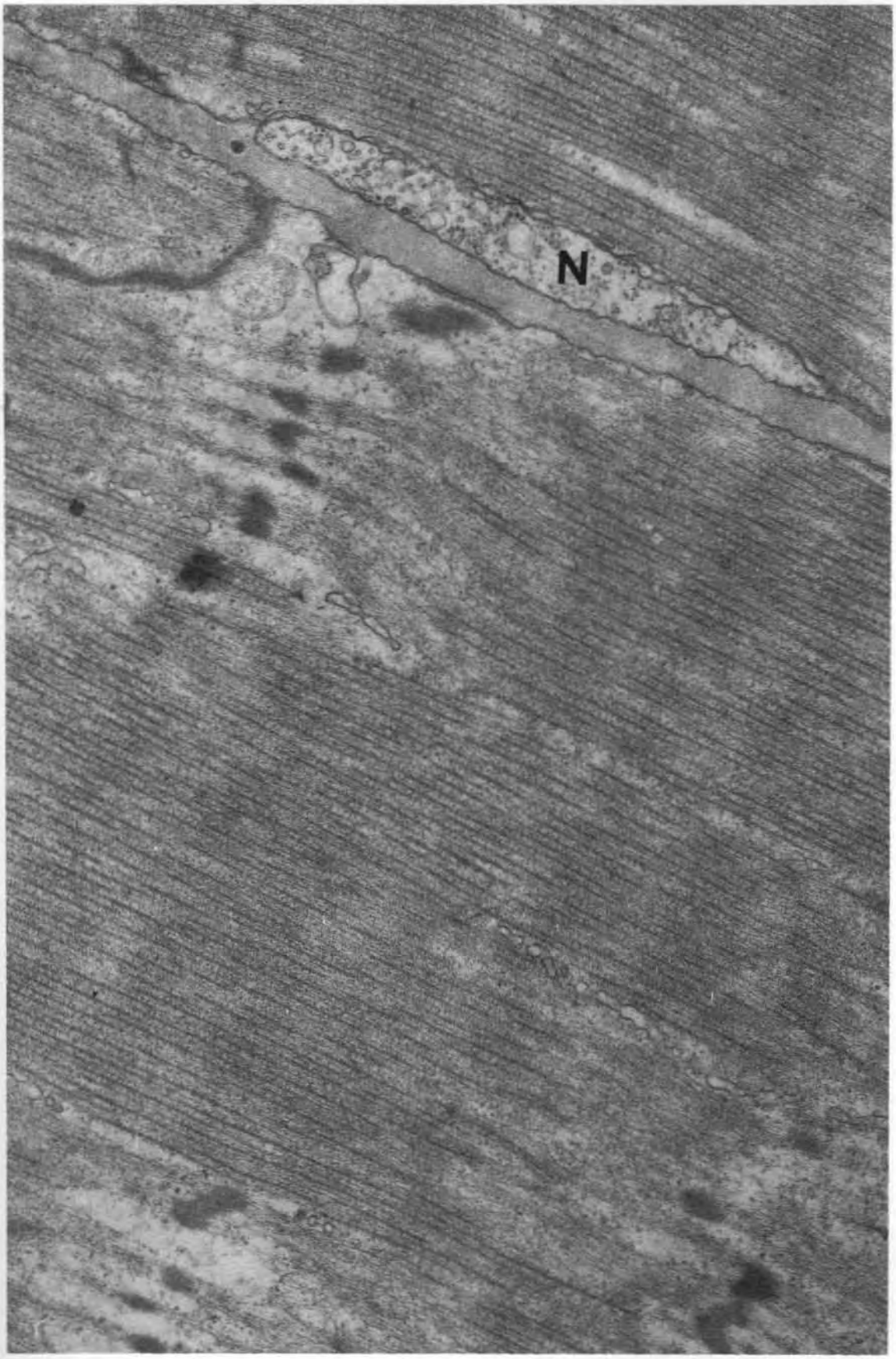

N

Figure 17. Pink adductor fiber, cross section. A large, vesicle-containing inclusion, in a sarcoplasmic invagination. Diads are indicated (D). X 35,000.

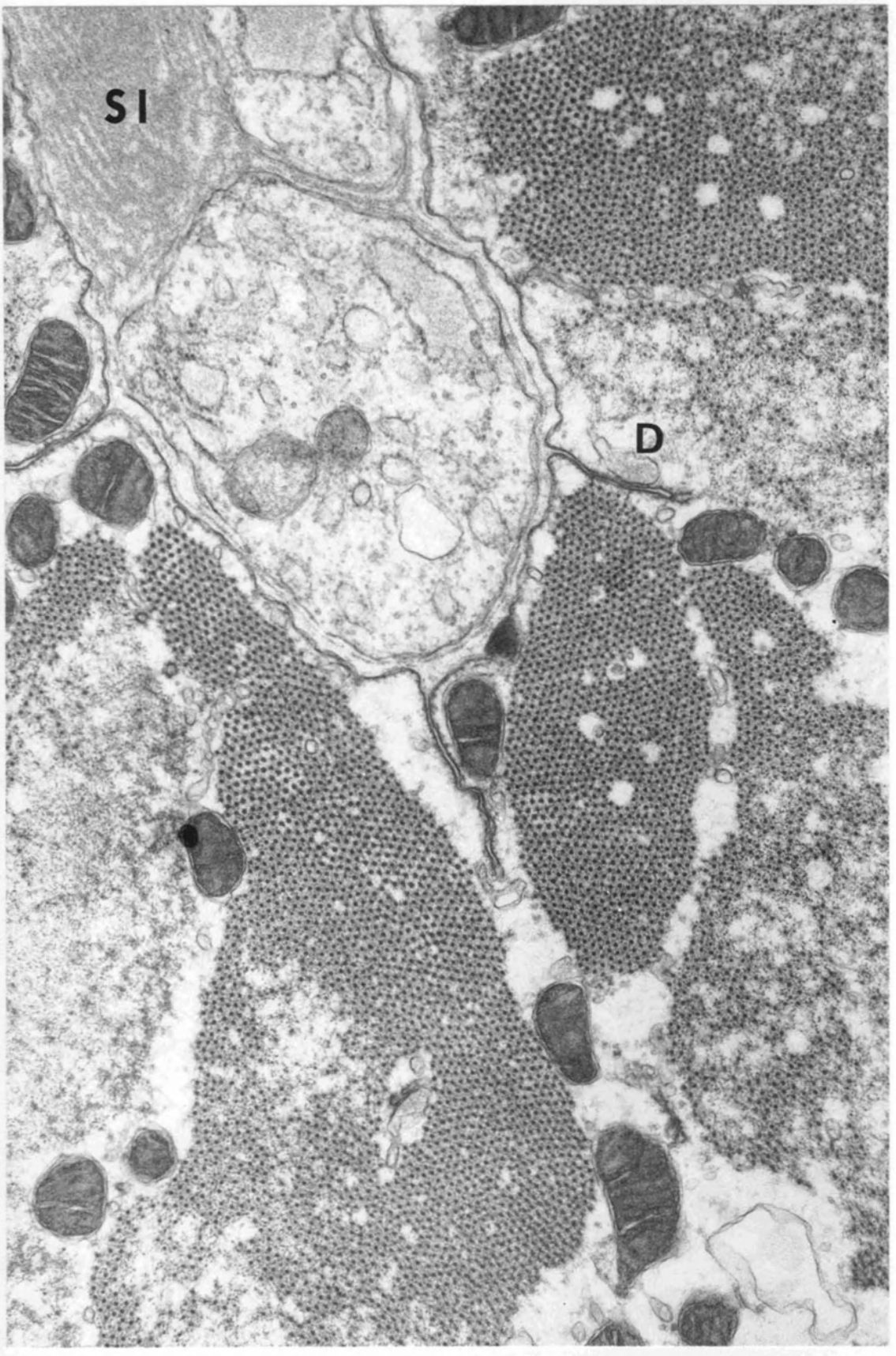
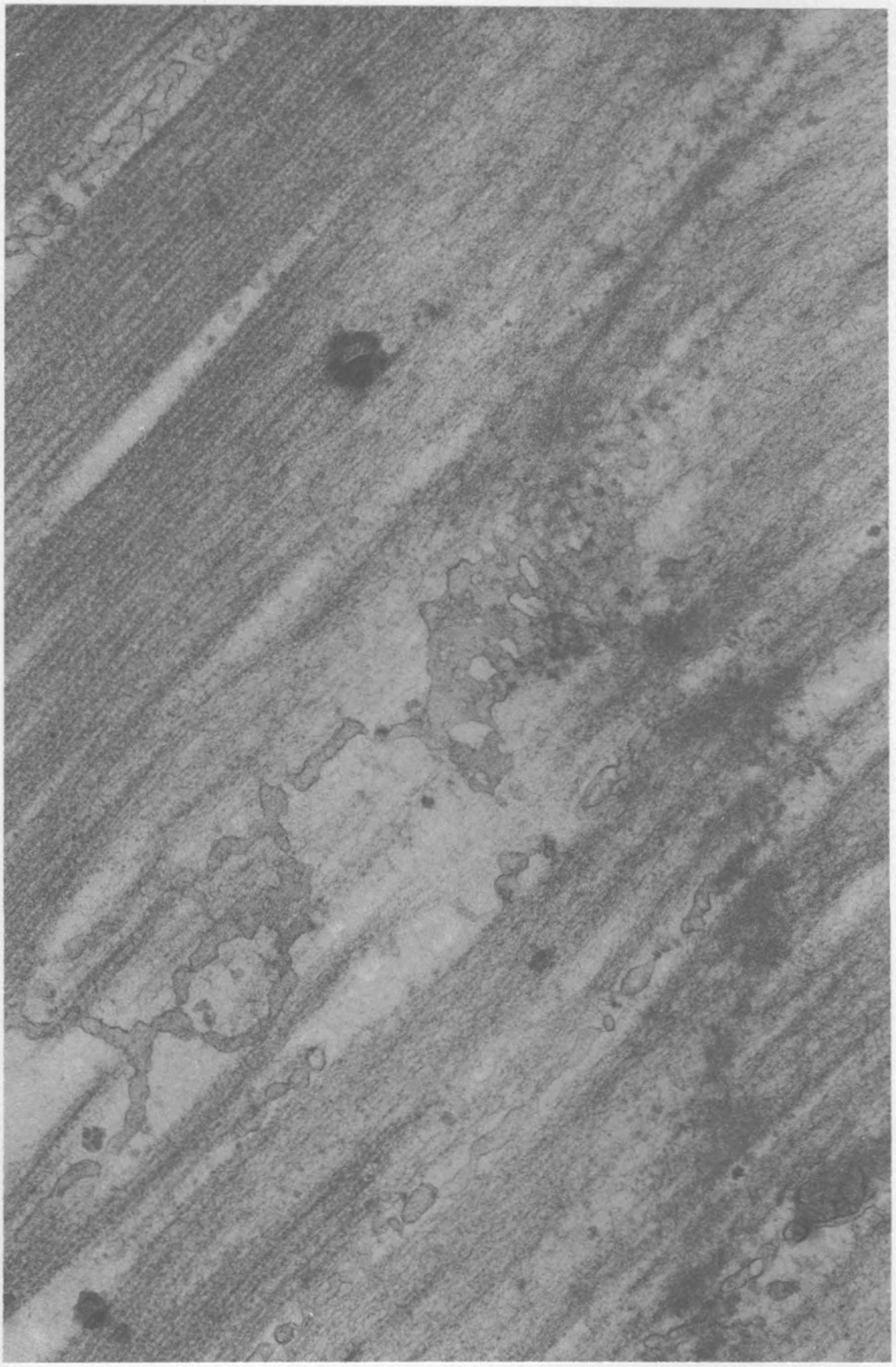

SI
D

Figure 18. Lateral pink fiber, longitudinal section. The sarcoplasmic reticulum is less well organized than in the white fibers, but is present nevertheless in abundant quantities. Note the considerable variation in pore size. X 56,000.



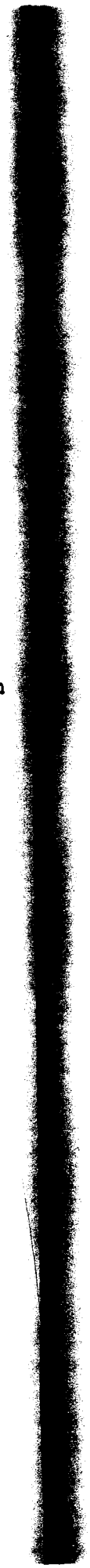


Figure 19. Pink adductor fiber, cross section. Sarcolemmal invagination at edge of fiber. Note large numbers of mitochondria and the diad (arrow). X 35,000.

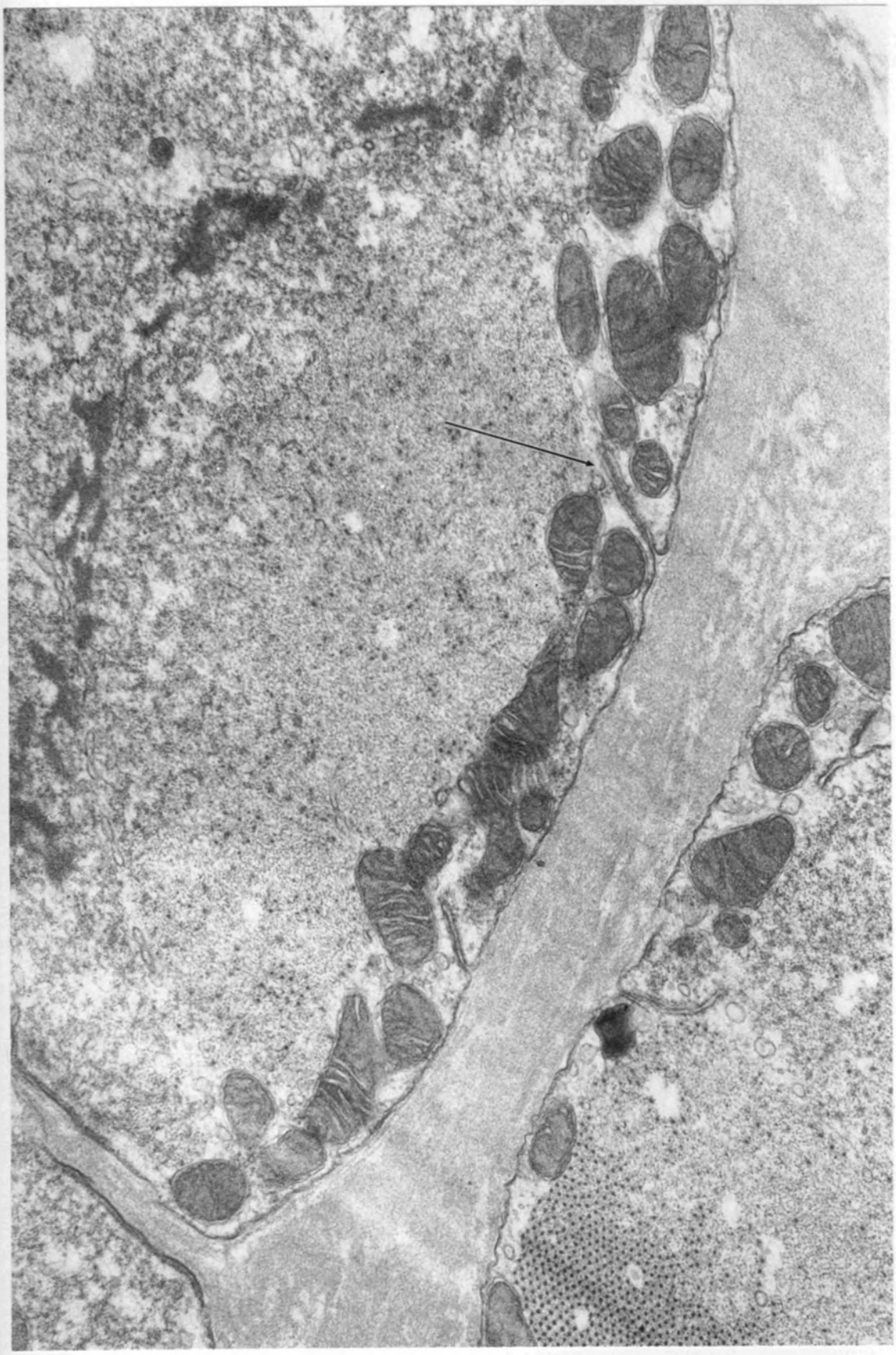


Figure 20. Pink lateral fiber, cross section. The poorly developed SR is evident in this section. Diads (D) are numerous, however, and similar in morphology to the ones found in white fibers. X 45,000.

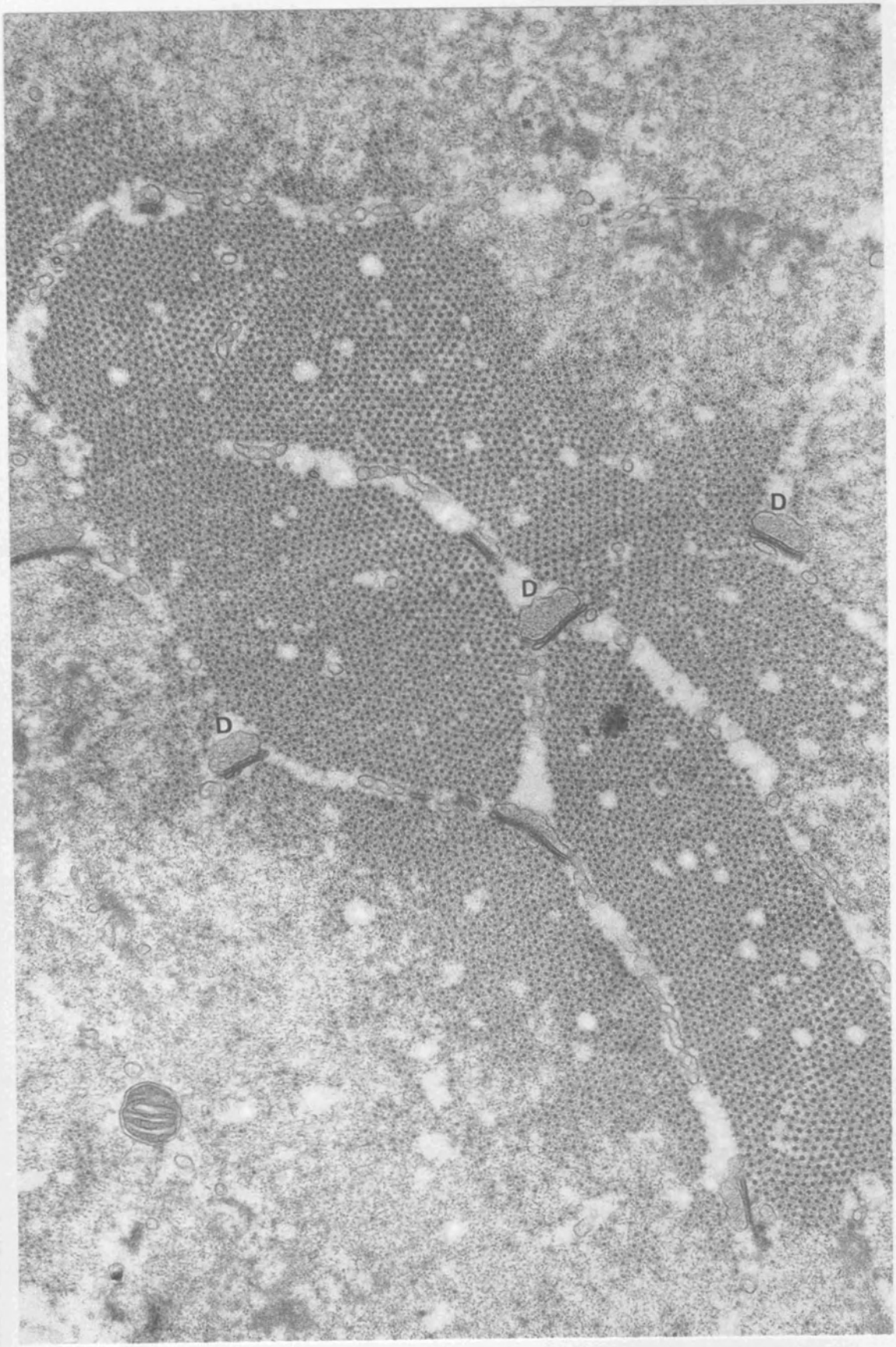

D
D
D

Figure 21. Pink depressor fiber, longitudinal section. Longitudinally running tubule which has left the SI and has formed a triad (TR) with the SR. X 28,000.

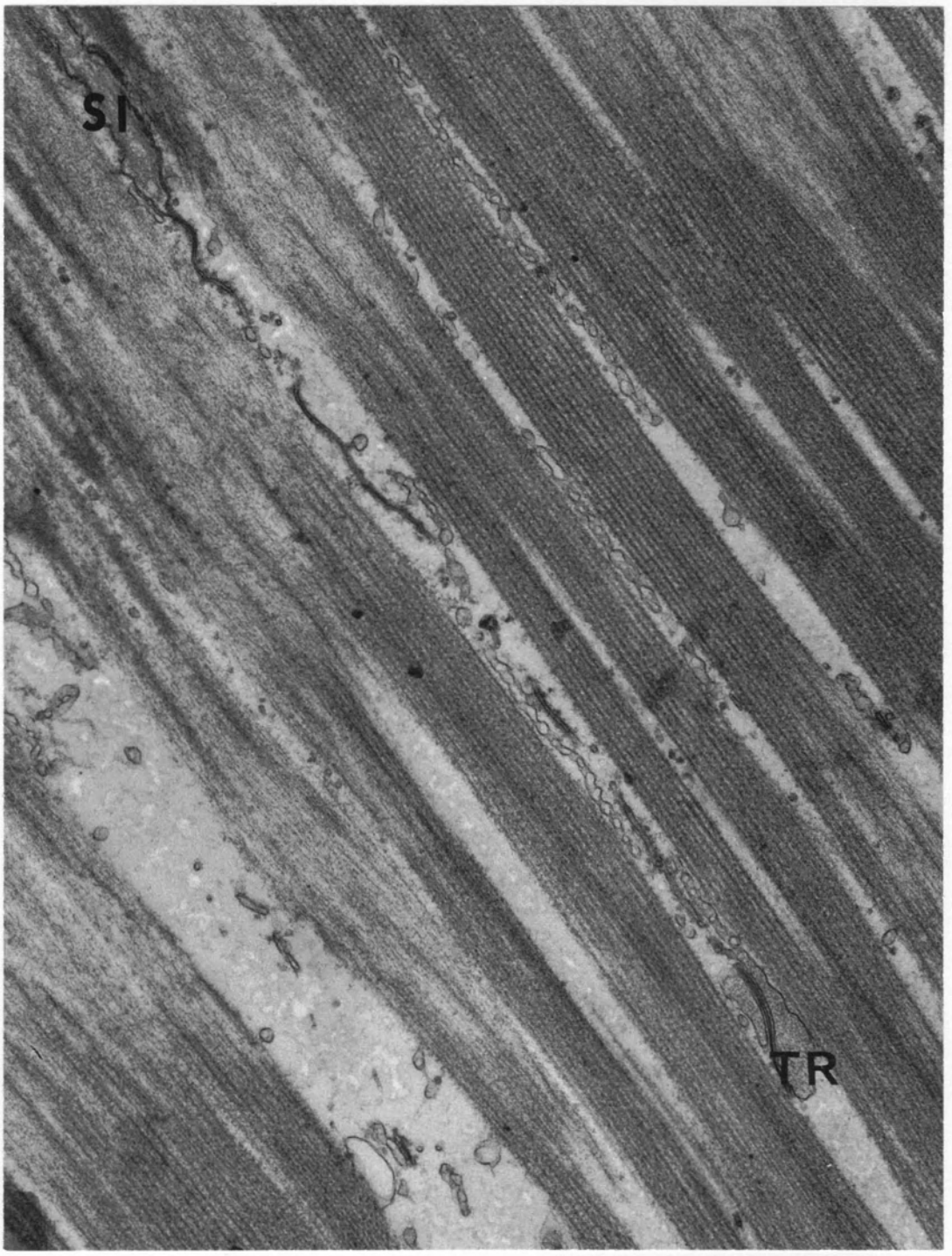

SI
TR

Figure 22. Pink lateral fiber, cross section. The sarcolemmal invagination is observed to be funnel shaped at the surface and is characteristically lined with mitochondria. Two axonal processes near the mouth of the invagination are marked (AX). X 45, 000.

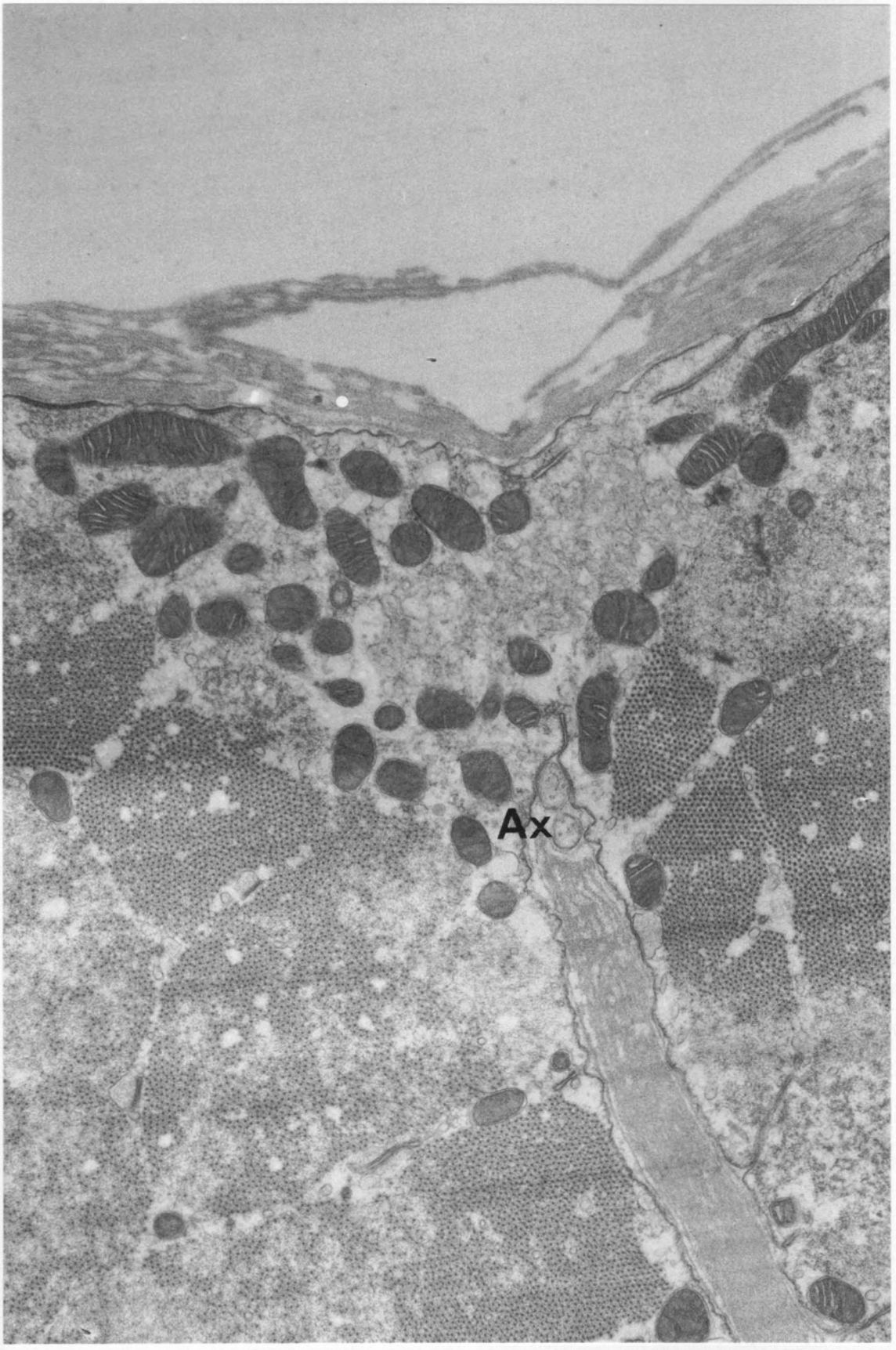

Ax

Figure 23. Lateral pink fiber, longitudinal section. Tubules, which originate from the sarcollemal invaginations can be seen running in and out of the plane of section between the myofibrils. One tubule forms many contacts with the cisternal portion of the sarcoplasmic reticulum, most often at the A-I junction, but sometimes at positions anywhere along the A-band. Note the granular, amorphous matrix within the cisternae, and how it grades into the fenestrated portion of the SR (arrow), X 42,000.

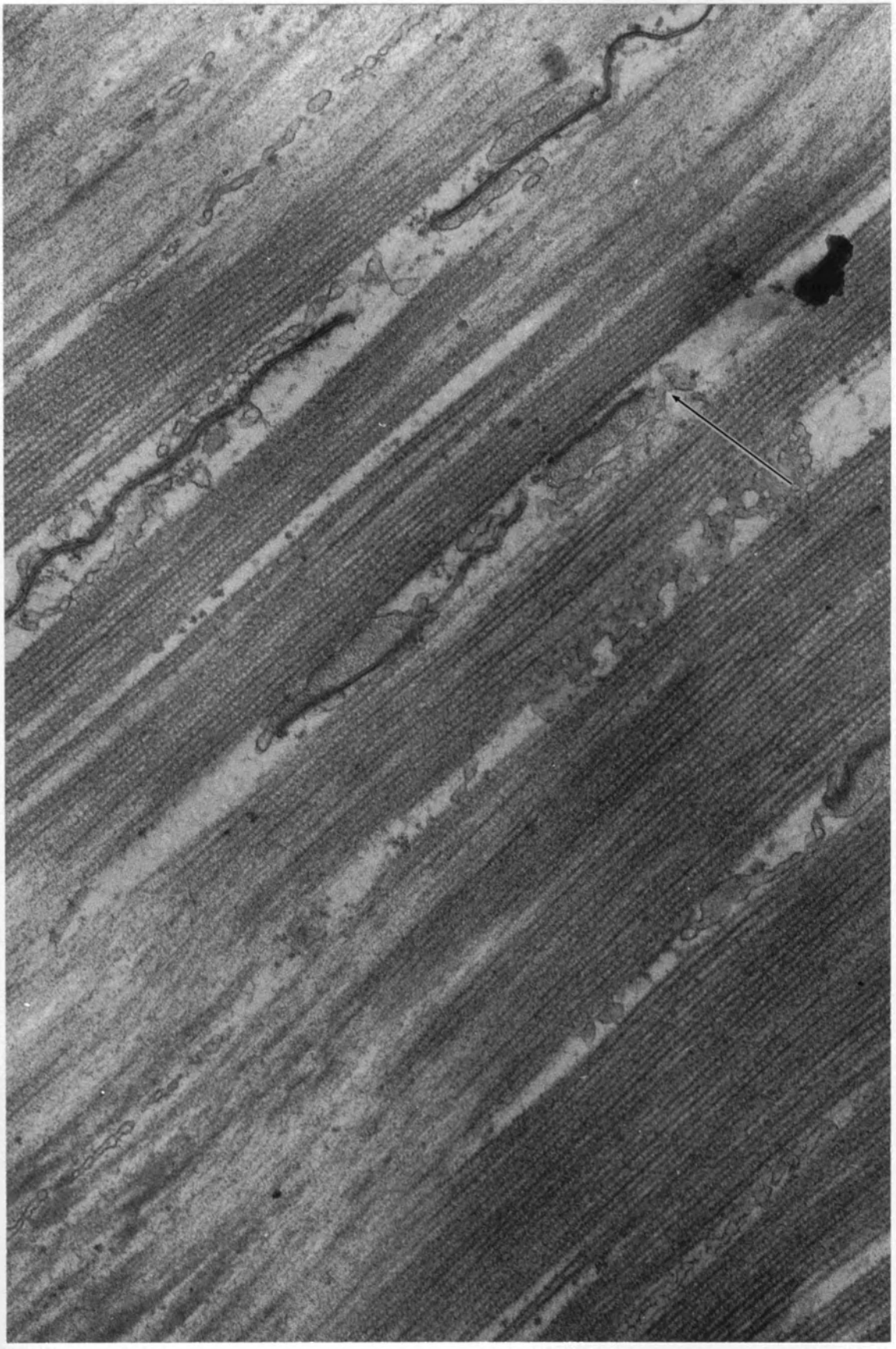


Figure 24. Lateral pink fiber, longitudinal section. Two sarcolemmal invaginations can be seen, running diagonally. Their membranes show an increased density at points opposite Z-lines, and occasionally one observes inclusions in them which are probably axonal projections. Large numbers of mitochondria often follow the invaginations. Several diads (arrows), and a tangential portion of the sarcoplasmic reticulum can be seen. X 21,000.


SR

with the dilated cisternal portions of the sarcoplasmic reticulum at the A-I junctions. As in all other striated fibers, the tubules are open to the outside of the fiber so that their internal ionic composition is probably equivalent to that of the external bathing solution.

Whether or not the myofibril is a discrete entity has been challenged by some investigators on the ground that in many fibers there is not a distinct separation between groups of myofilaments. This merging of sarcomeres is especially noticeable in crustacean fibers (Figures 7, 26), where separation of fibrils is accomplished by the sarcoplasmic reticular collars. There are several lines of evidence which contradict this view. In insect fibrillar flight muscles, for example, the fibrils are extremely distinct, separated by mitochondria and glycogen. In crayfish slow fibers the separation between fibrils is less distinct, but the majority are completely surrounded by SR. In cross sections of Balanus fibers one often sees merging of adjacent sarcomeres, however after glycerination, there is complete separation of individual myofibrils (Hoyle, McAlear and Selverston, 1965). Examination of glycerinated fibrils which have been separated by a Waring blendor and viewed with the aid of a phase-contrast microscope, reveal thousands of fibrils, all of about equal diameter and of various lengths, the length depending on the time in the blendor. The only way to answer this question conclusively

would be to demonstrate one continuous fibril from one end of the fiber to another, a feat which no one has as yet had the patience to perform.

Because the SR collar is not perfectly round, longitudinal sections are often cut through extended sheets of this material so that its structure and relationship to the fibrils was easily determined. The SR of the white fibers was better developed than that of the pink fibers. The SR from the Balanus pink fibers was markedly reduced from that of the white fibers. There were large numbers of diads however and a well developed tubular system. The SR collars, extending from sarcomere to sarcomere without interruption, contained pores of varying size and shape. The number of pores was decreased at the cisternae and an abrupt change in the inner composition of the cisternae, compared with the remainder of the SR, was evident.

The assumption which has been made by many electron microscopists is that there is a positive correlation between the extent of SR organization and speed of contraction and relaxation (Revel, 1957; Franzini-Armstrong, 1964). That this assumption may be an overgeneralization is demonstrated clearly in Balanus which has a relatively long relaxation time of 200-300 msec and a well developed SR. Another case where this assumption has not held up is in the extraocular muscles of the cat. Pilar and Hess (1966) demonstrated that

the fast fibers contained abundant quantities of SR separating the fibrils and a well developed tubular system forming triads at the A-I junction. In the slow fibers, however, the fibrils were not separated by SR, which was markedly reduced, while triads and tubules were absent entirely. Bach-y-Rita and Ito (1966) have shown that the small, multiply innervated extraocular fibers were capable of propagating all-or-none impulses. When fibers from extraocular muscles were reexamined by Peachey (1966), he claimed that there was no substantial difference in the ultrastructure of fast and slow fibers, although he published no electron micrographs to substantiate this conclusion.

The actual biochemical differences in SR from different invertebrate fibers, especially with regard to their calcium binding rates, has scarcely been looked at. Hasselbach (1966) has compared SR fraction activities of rabbit and Balanus SR and found the Ca uptake was lower in Balanus than in rabbit. His experiments were all done at room temperature however, so that optimal enzymatic activity of the Balanus SR might not have been achieved.

The fenestrations of the SR collar are variable in size (Figure 14) from 350 A to 1500 A in diameter. They do not open to the sarcoplasm but serve instead to increase its surface area. Since these cisternae contain a granular material within them, clearly differentiating them from the remainder of the SR, one might be tempted to speculate that

this material is somehow related to calcium storage, and that it would serve to release calcium during activation by the tubules. This suggestion is strongly supported by the autoradiographic studies of Winegrad (1965) which show that during contraction calcium moves from the diads to the A-band. In addition, Hasselbach (1964) and Constantin (1964) have shown that Ca ions sequestered by the SR during relaxation are concentrated in the terminal cisternae.

One of the most striking features of Balanus fibers is the extent of sarcolemmal invagination. These invaginations have probably developed from the fusion of many small fibers, the roots of which are often distinguishable at the base. Running longitudinally, the invaginations branch into smaller and smaller subdivisions, much like the root system of a tree, except that many of them connected with others within the fiber. The invaginations are easily distinguished from the ubiquitous tubules by the presence of basement material, a continuation of the surface basement material. Tubules leave the invaginations from their most peripheral to their deepest portions, the basement material always terminating at the beginning of the tubule.

While the sarcoplasmic reticulum is a specialization of the endoplasmic reticulum common to all cells (Porter and Palade, 1957), the tubular system appears to be derived from, and in continuity with, the plasma membrane of the sarcolemma.

Tubules are of uniform size, in contrast to the variable size of the sarcolemmal invaginations. While, in vertebrate material, the tubules are transversely oriented, in Balanus they are found to run both transversely and longitudinally. One tubule may form many diads, and diads are also observed along the walls of the clefts and under the plasma membrane at the surface of the fiber.

The most conspicious feature of the Balanus pink fibers are large numbers of mitochondria. They are located principally under the sarcolemma and along the walls of the clefts. Few are found dispersed among the myofibrils. Hoyle (1966) has described pink fibers in the swimming muscle of the Pacific crab Portunus which also contain large numbers of mitochondria, and are much slower than the white fibers. However, mitochondria are often found in very fast asynchronous insect fibrillar fibers (Smith, 1966) so that there appears to be no obvious relation between mitochondrial content and speed of contraction. I believe a more fruitful approach would be a comparative study of the fiber components directly involved in the contraction-relaxation cycle. For example Varga (1962) has described myosins from tonic fibers which have lower ATPase activities and higher choline esterase activity than myosin from twitch fibers.

Sarcomere lengths for the white fibers averaged 10 μ while those of the pink fibers were 11.5 μ, both within the range described by

Atwood, Hoyle and Smyth (1965) for slow fibers. Classification into a "fibrillenstruktur" and "felderstruktur" arrangement (after Kruger, 1949) is difficult since there are many configurations the myofilaments can assume (see Figure 5). The white depressor fibers are generally punctate in appearance, an organization usually attributed to twitch fibers. Many of the white adductor fibers are similar, but in addition a large number of felderstruktur types were evident. Pink fibers were always of felderstruktur arrangement.

The tubular-cisternal (TC) junction consists of the apposed membranes separated by a gap of ca. 150-200 A. An electron-dense line, similar to that described by Hoyle (1966) is found midway between the two membranes, but otherwise the junction is free of structural elements. No indication of any continuity between the membranes was found suggesting that electrotonic flow of current between the tubular system and the SR would be impeded. Many electron microcopists have attempted to demonstrate tight junctions or bridges joining the tubule-cisternal membranes. For example, Smith (1966) described "blocks" within the T-C gap of insect muscle fibers. Fahrenbach (1966) as well as Walker and Schrodt (1966) have published micrographs suggesting tight junctions (zona occludens) provide continuity. While some differences may be attributed to variation in fixation methods, the size of the gap in Balanus remains constant for both glutaraldehyde and osmium

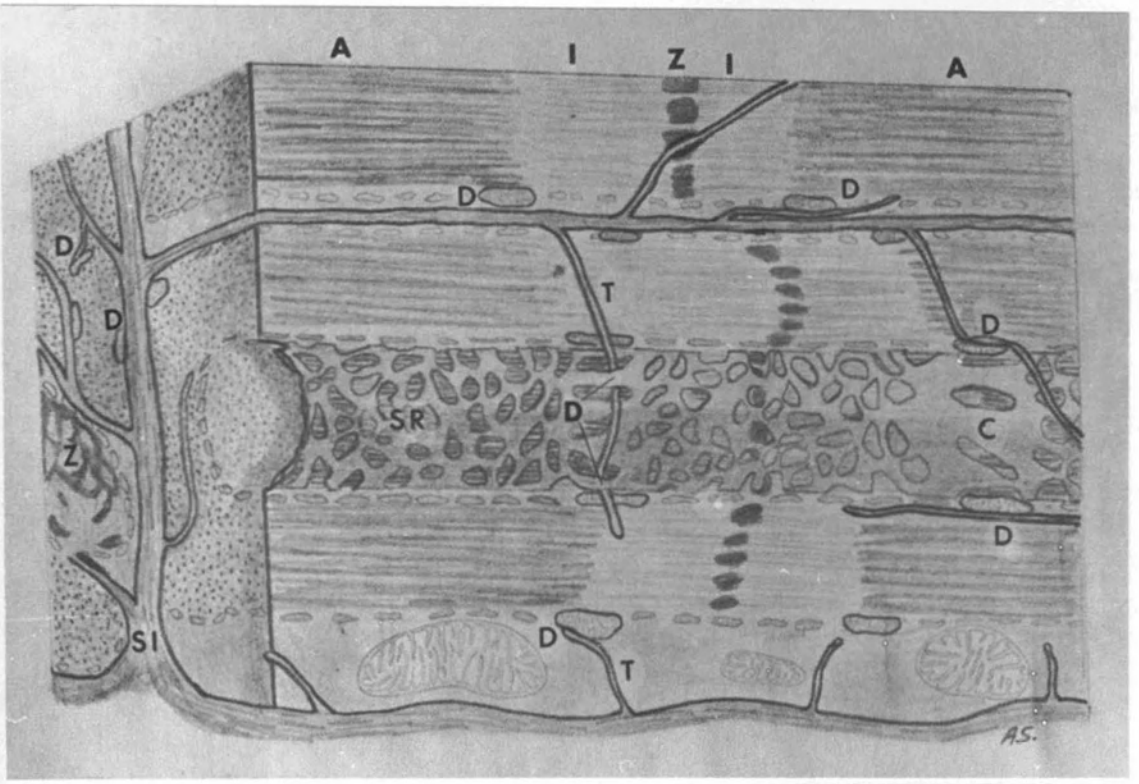
fixation at various tonicities. The question of how excitation bridges the T-C gap in Balanus as well as in many other fibers must remain unanswered.

Synaptic contacts were extremely difficult to find, but occasionally were seen both at the surface and deep within the clefts. Their structures were atypical and only the presence of vesicles suggests that they are synapses. It might be possible to locate the synapses by searching the fiber with a low-resistance external microelectrode while stimulating indirectly and fixing those precise areas in which synaptic currents were found.

General Description of Carcinus Twitch Fibers

The average diameter of a single Carcinus fiber in Ringer solution is about 0.45 mm, as measured through an eyepiece micrometer. This method, although widely employed, has obvious shortcomings because the fibers are not perfectly cylindrical, but vary from highly elliptical to irregularly round. Their length is approximately 7 mm when the carpopodite is in its resting position. Longitudinally oriented invaginations of the sarcolemma are observed when the fiber is viewed with the aid of transmitted light. The infoldings, as well as a "fibrillar-structure" arrangement of the myofibrils are evident in thick epon cross

Figure 24B. Composite representation of the fine structure of the barnacle white fiber. The fenestrated collar of sarcoplasmic reticulum (SR) is shown surrounding one myofibril. The cisternal portion of the SR is indicated at C, and forms diads (D) with either the excitatory tubules (T), or directly with the membrane of the sarcolemma. The sarcolemmal invagination (SI) is distinguished by the presence of a basement membrane. A bands (A), I bands (I) and Z lines (Z) are indicated. (Modified from Brandt, et al., 1963).


A
I
Z
I
A
D
D
D
D
T
D
SR
D
C
Z
D
SI
D
T
A.S.

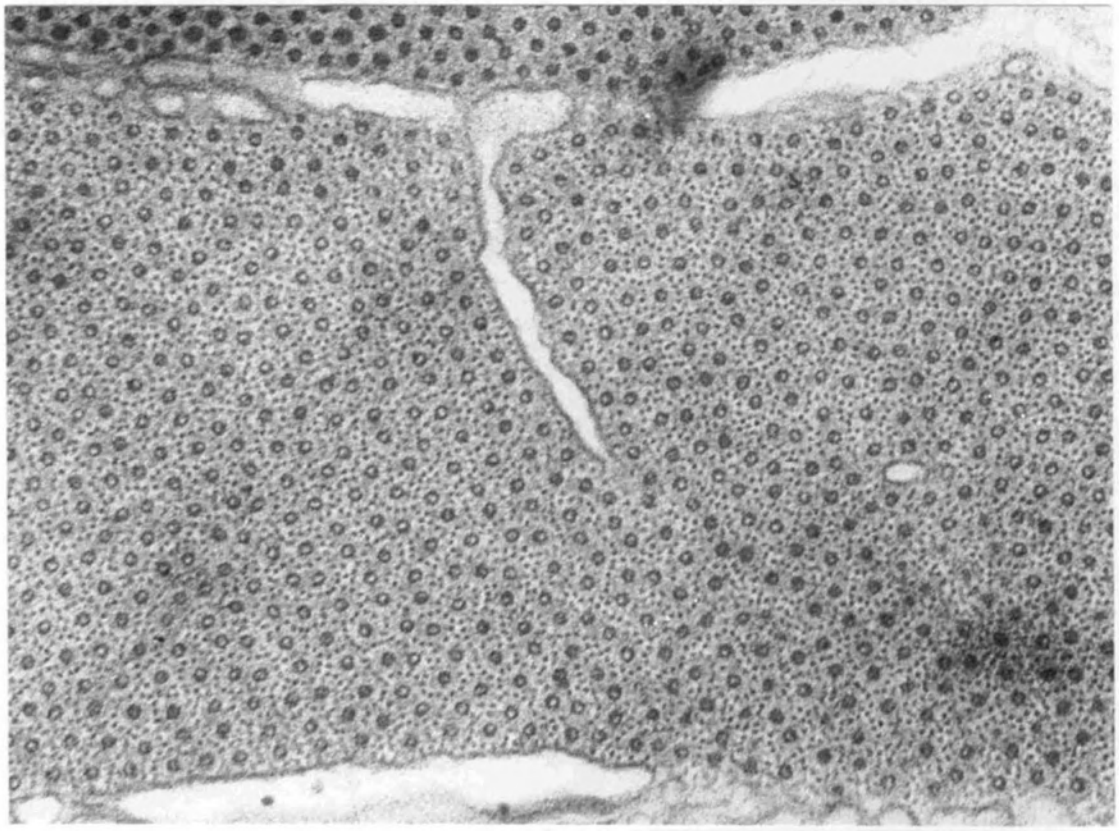
sections. Sarcomeres are about 5 μ long in phasic fibers fixed at rest length. In gradedly contracting fibers from the same muscle the sarcomere length was approximately 9.2 μ.

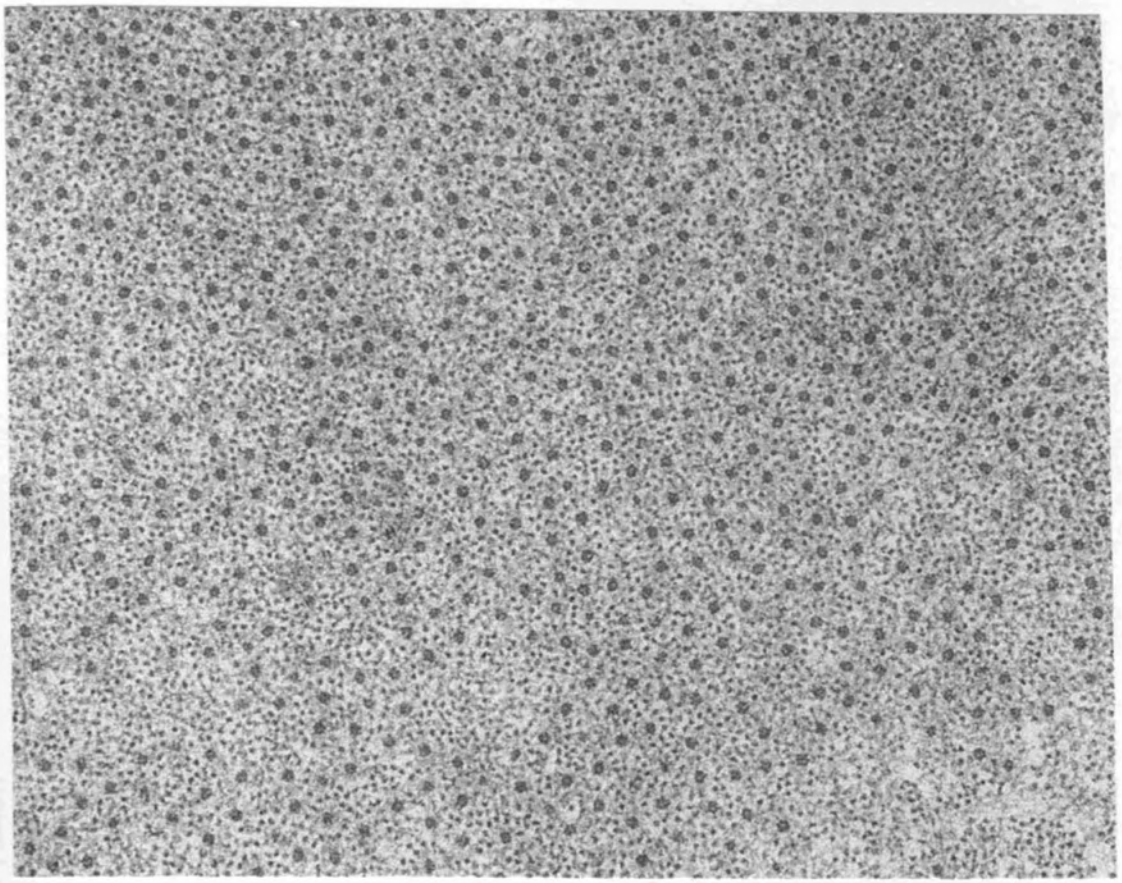
Two separate membrane systems are present. The first is a tubular system (TS) arising from the sarcolemma, both at the surface and from the sarcolemmal invaginations. The second is a longitudinally oriented fenestrated collar, the sarcoplasmic reticulum (SR), surrounding each fibril.

Sarcolemma

The sarcolemma in Carcinus fibers consists of two parts, a "unit" plasma membrane 80 Å thick and an outer layer of basement material about .14 μ in thickness. The sarcolemma is invaginated longitudinally (see Figures 26 and 30). Many of the invaginations become confluent within, so that a ramifying system completely permeates the interior of the fiber. Since the invaginations can be seen to be patent to the exterior, no portion of the myofibrillar apparatus is very far removed from the external environment. The extent of increase in surface area due to sarcolemmal infolding has been estimated by enlarging photomicrographs of cross-sections and utilizing a map measurer to trace the actual distances involved. On the average, the increased sur-

Figure 25. Comparison of overlap zones in Carcinus (upper) and Balanus (lower) fibers. Both have 12-14 thin filaments in orbit around each thick filament. The thin filament orbits are much more regular and uniform in appearance in Carcinus than in Balanus. Magnification, upper X 90,708, lower X 90,000.





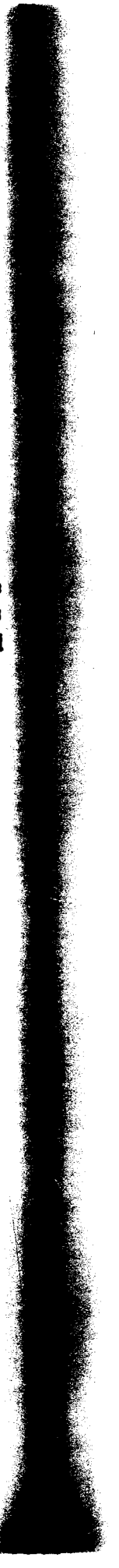
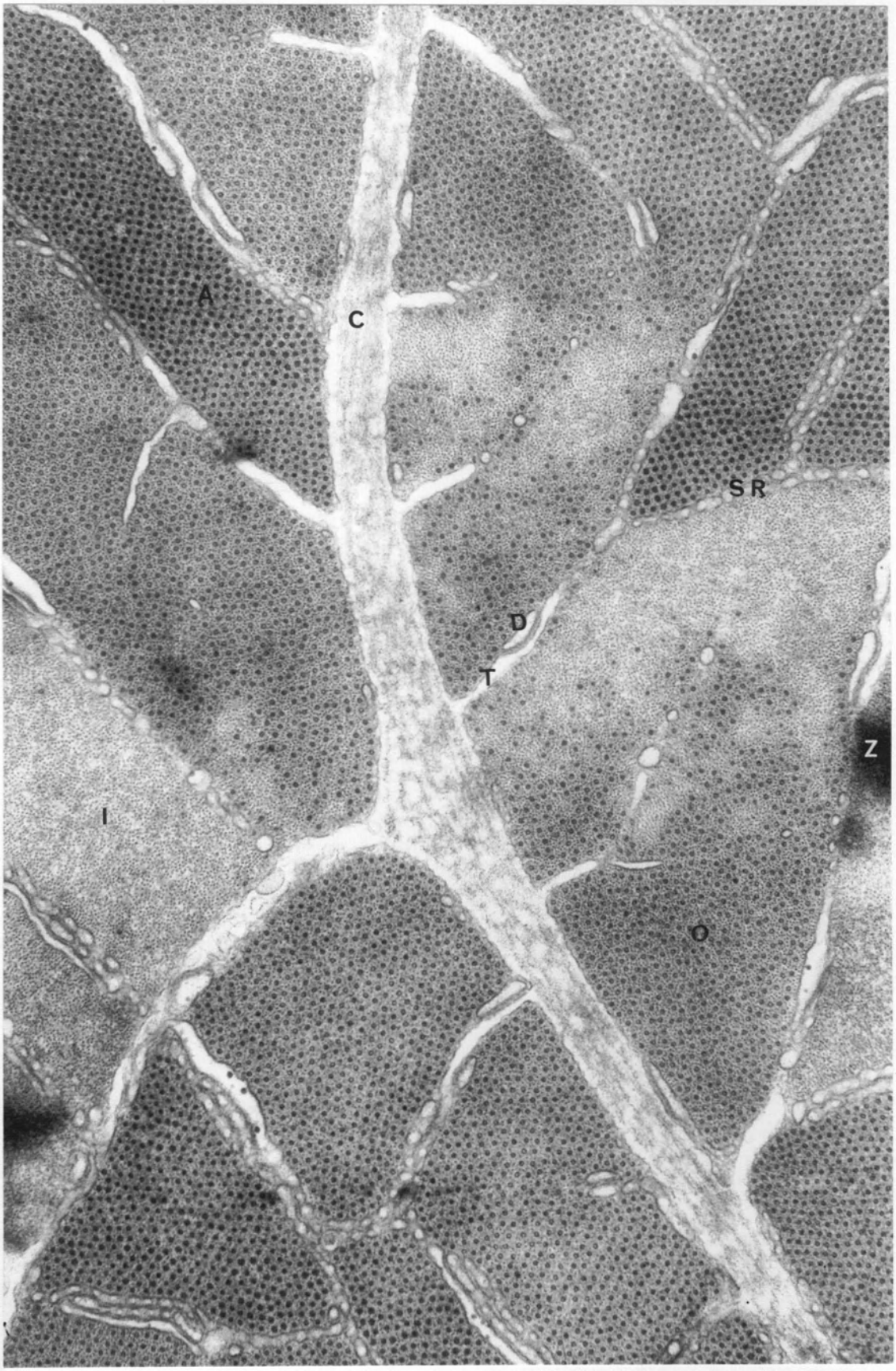


Figure 26. Carcinus twitch fiber cross section. General survey electron micrograph showing cross sections of various regions of the sarcomere; Z-disc (Z), I-band (I), A-band (A) and overlap area (O). The large medial cleft (C) also called a sarcolemmal invagination, gives rise to smaller tubules (T) which form many diads (D) with cisternal portions of the sarcoplasmic reticulum (SR). Clefts can be distinguished from the tubules by the presence of a basement membrane. Note the large numbers of thin filaments surrounding the thick filaments and the hollow appearance of some of the larger filaments. Magnification X 37,820.


A
C
SR
D
T
Z
I
O

face area is ten times that of the circumference determined by measuring the fiber through an eyepiece micrometer. This increased surface area does not include the surface area contributed by the TS, which is continuous with the sarcolemma and would therefore cause the value to be somewhat higher.

Beneath the sarcolemma one often finds areas which are devoid of myofibrils and which contain clusters of mitochondria. These areas appear to be filled with a granular material of low electron density.

Sarcomere Ultrastructure

Z-line

The Z-lines of Carcinus fibers are about 1 μ in width and do not have the "zig-zag" appearance associated with the Z-lines of vertebrate phasic fibers (Franzini-Armstrong and Porter, 1964; Knappis and Carlson, 1962). In vertebrates the "zig-zag" appearance is due to an unraveling of actin monomers at the Z-line. In Carcinus Z-lines appear to be made up of parallel overlapping thin filaments, embedded in an amorphous matrix which gives the Z-line an increased electron density. In longitudinal sections they present a somewhat wavy appearance, possibly due to unequal contractures resulting from penetration of the fixative. They closely resemble the structure of the

Figure 27. Carcinus twitch fiber, longitudinal section. The general features of the band pattern are shown in this survey electron micrograph. A-bands (A), I-bands (I), and Z-discs (Z), are similar to those found in vertebrate striated fibers. Conspiciously absent are H-bands and M-lines however. The sarcoplasmic reticulum (SR) can be seen between fibrils, and some diads are observed at the A-I junctions. The longitudinal displacement of myofibrils relative to one-another is probably due to unequal activation during fixation. Magnification X 21,000.


D
I
A
Z
SR

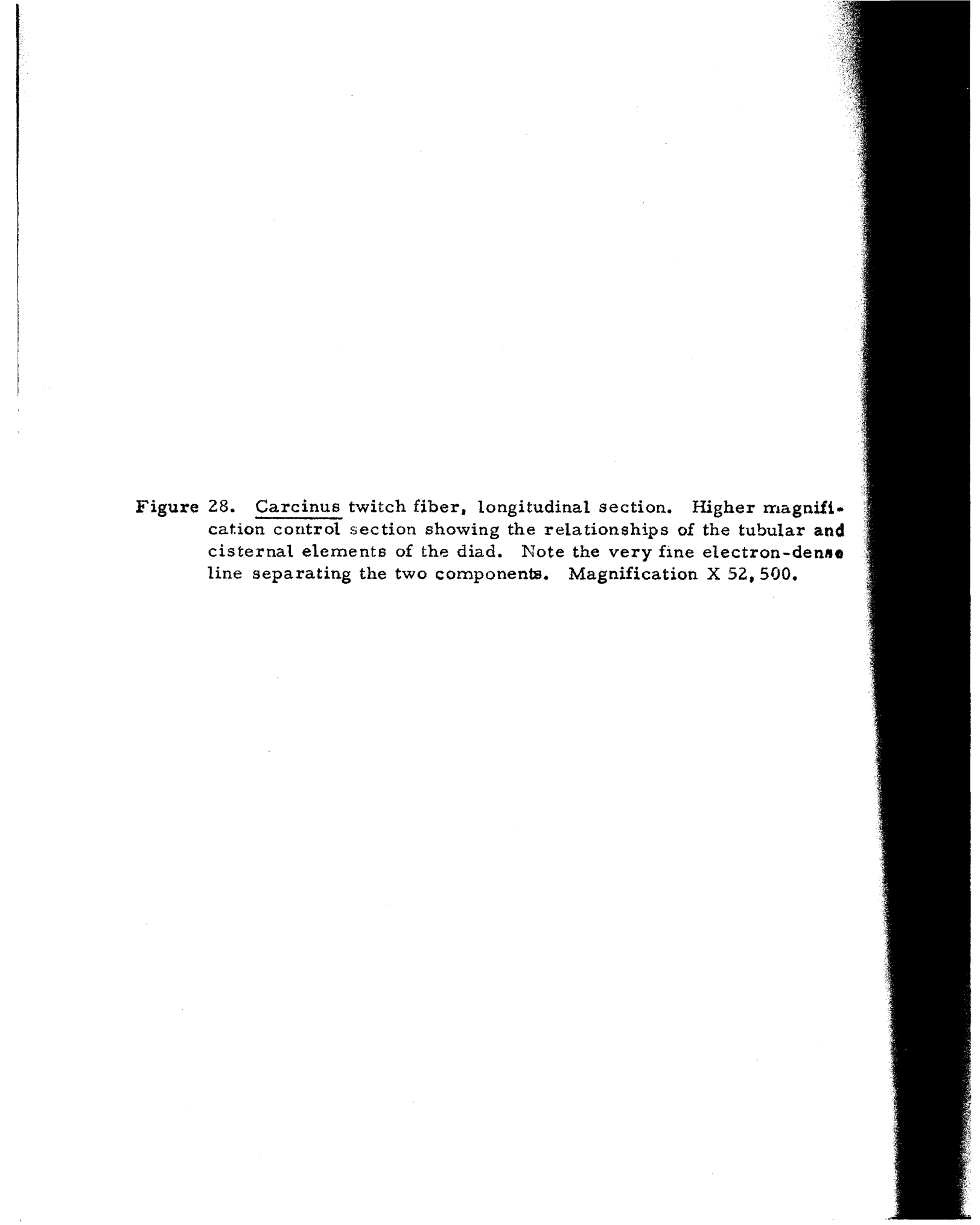


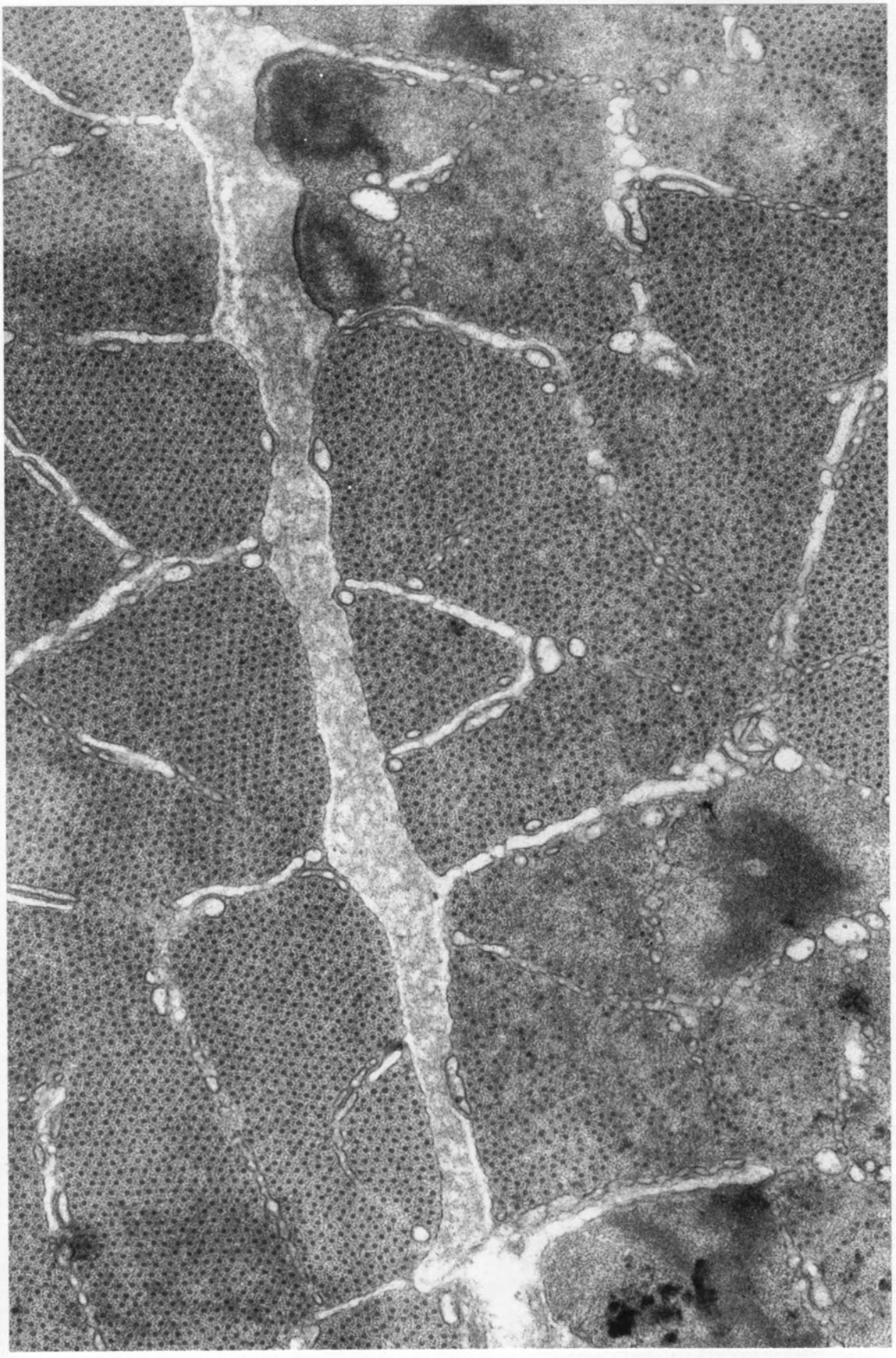
Figure 28. Carcinus twitch fiber, longitudinal section. Higher magnification control section showing the relationships of the tubular and cisternal elements of the diad. Note the very fine electron-dense line separating the two components. Magnification X 52,500.

Z-lines of frog slow fibers which were described by Page (1965) as having neither filaments or internal structure, but consisting of only a dense amorphous region. Figure 28 shows the penetration of I-band filaments into the Z-line. In this respect they appear very similar to glutaraldehyde fixed cockroach femoral muscles, in which traces of thin filaments can also be seen (Hagopian, 1966).

Thick Filaments

The A-band is formed by tapered thick filaments having a diameter of 150 Angstroms at their central portion tapering to a value of about 130 Angstroms at their ends. There is a slight asymmetry of the filaments toward either Z-line which may be associated with the absence of an M-band. In cross section, when just the right combination of orientation and staining is achieved, the thick filaments appear hollow, as has been described by Hodge (1955), Smith (1961), Huxley and Hanson (1957), Auber and Couteaux (1963), Bouligand (1963), and Fahrenbach (1963). At higher magnifications individual particles of about 40 Angstroms can be resolved around the periphery of the filament (Figure 26). These are probably the light meromyosin molecules suggested by Huxley (1963).

Figure 29. Carcinus fiber, cross section of control. Large numbers of diads are seen along the tubules and between the SI wall and the myofibrils. The cause of the SI membrane darkening near the Z-disc is not known. Magnification X 37,607.



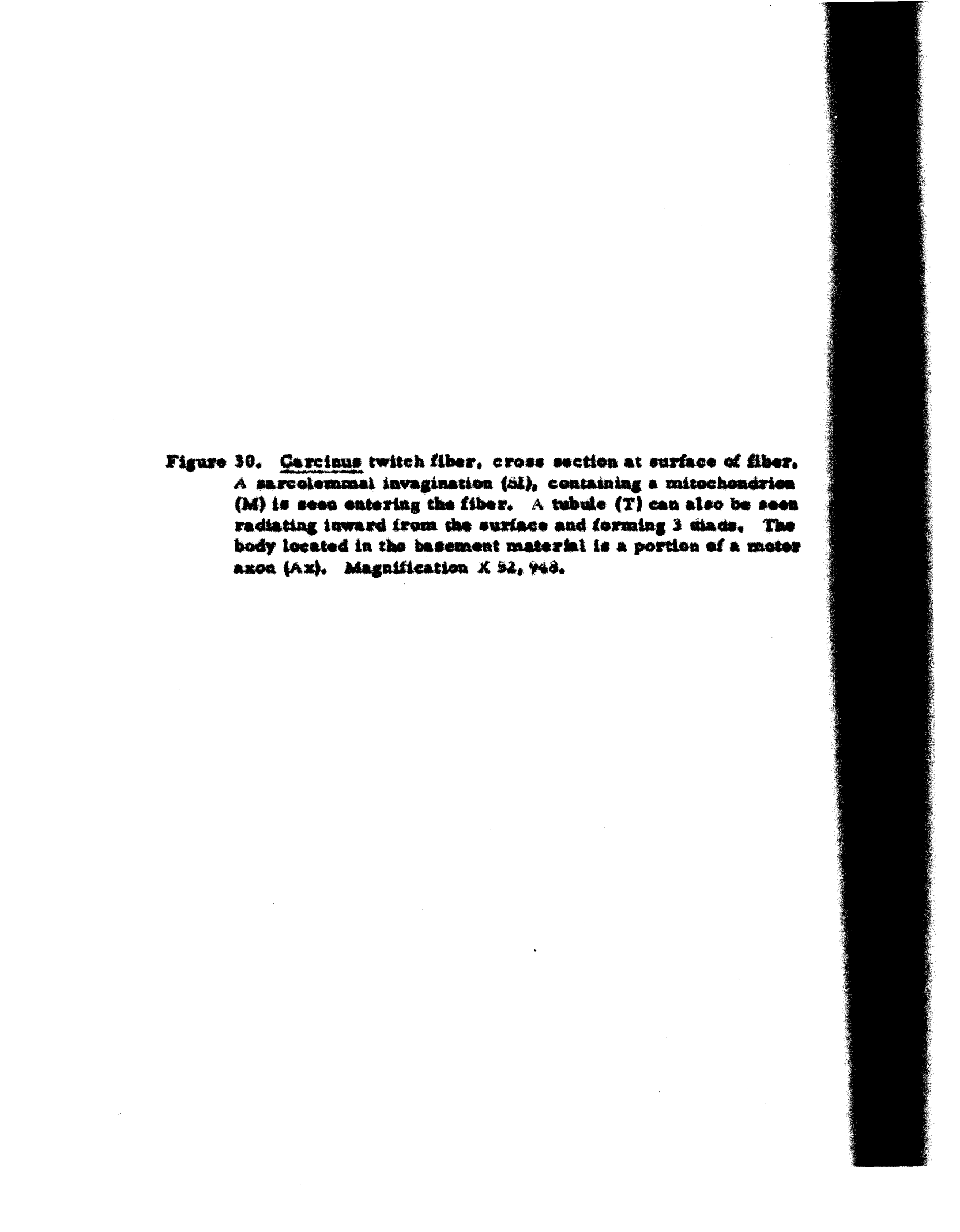
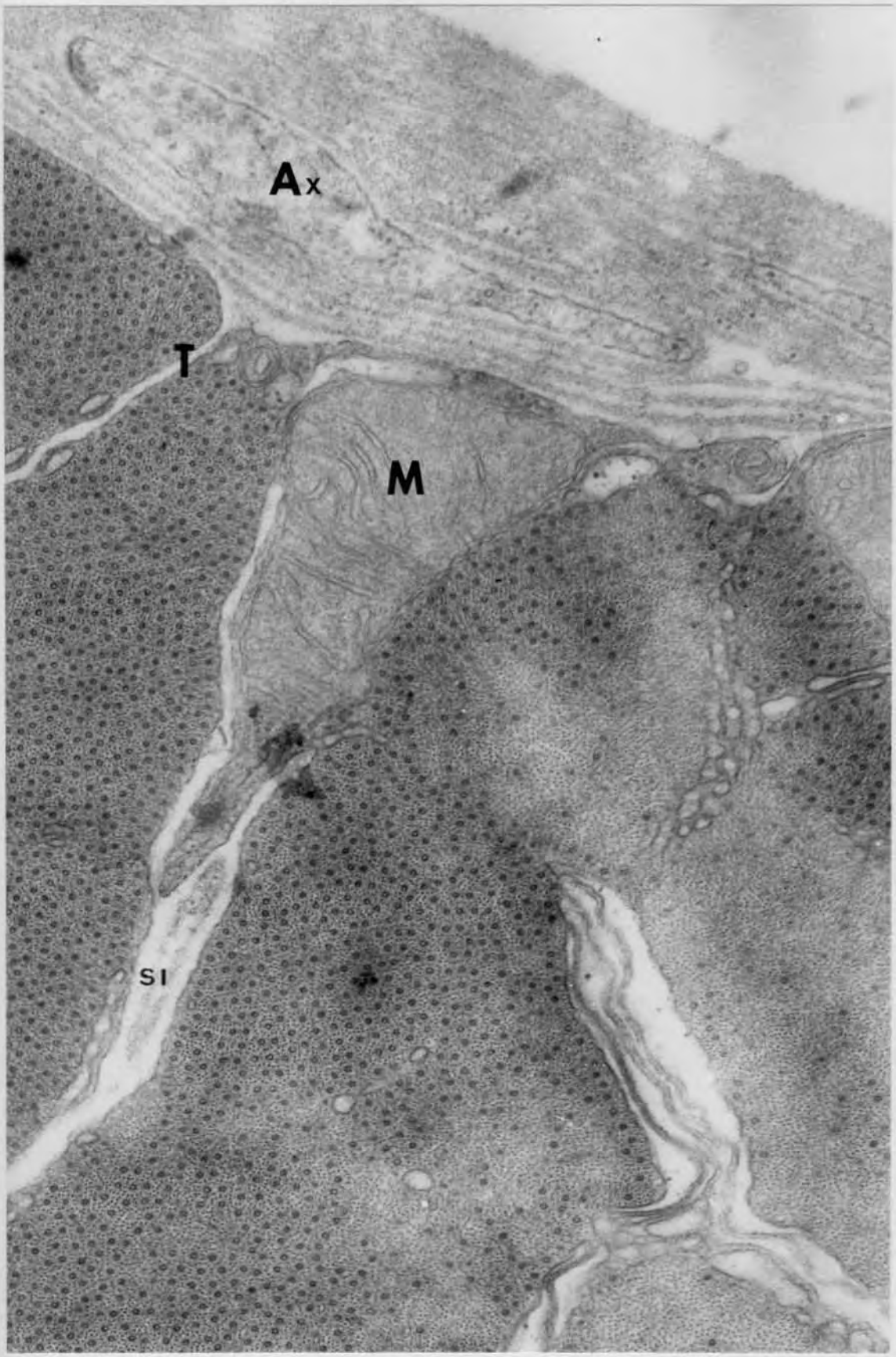


Figure 30. Carcinus twitch fiber, cross section at surface of fiber. A sarcolemmal invagination (SI), containing a mitochondrion (M) is seen entering the fiber. A tubule (T) can also be seen radiating inward from the surface and forming 3 diads. The body located in the basement material is a portion of a motor axon (Ax). Magnification X 52, 948.


Ax
T
M
SI

Thin Filaments

Thin filaments with a diameter of 80 A extend from the Z line to a point approximately one third of the way into the A-band when the fiber is fixed at rest length. Despite the termination of thin filaments at this point, absence of a discrete H-zone is similar to that described in other arthropods without H-zones (Hagopian, 1966; Smith, 1966; Brandt, et al., 1965) and in slow fibers of the frog (Page, 1965; Peachey and Huxley, 1962). In cross sections of fibers which were fixed without shortening, four separate zones can be distinguished (Figure 26). Z-lines which are characteristically dense and usually not entirely within the plane of section, (Figure 26); I-bands, lying next to Z-lines and composed entirely of thin filaments; A-bands, located in the middle portion of the sarcomere and composed of only thick filaments in their central region; and thick and thin filaments in the overlap region. The ratio of thin to thick filaments is 5:1, greater than the vertebrate ratio of 2:1. A similar ratio has been reported by Smith, et al. (1966) for insect visceral muscles, and by Hagopian (1966) in roach femoral muscles. Each thick filament is surrounded by orbits of from 10 to 14 thin filaments (Figure 25). The physiological significance of the large ratio of thin to thick filaments in some arthropod muscles remains unknown.

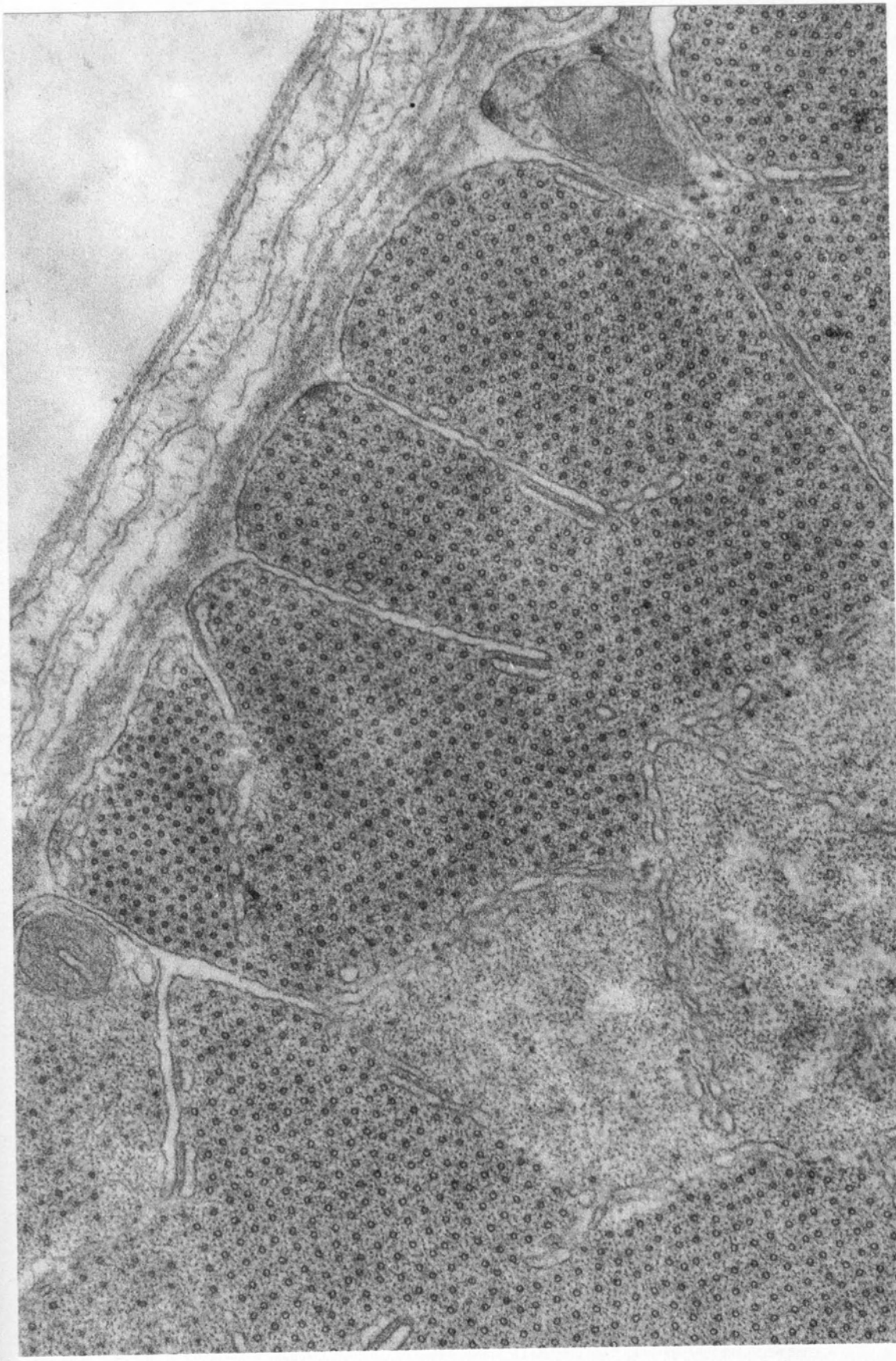
Sarcoplasmic Reticulum

The sarcoplasmic reticulum consists of a fenestrated collar surrounding each fibril, very similar in form to that described for crayfish by Brandt, et al. (1965), except that it extends the entire length of the sarcomere, being continuous from one sarcomere to another. The reticular collar becomes dilated into cisternae around the myofibril at the A-I junction (Figure 28). The cisternae form diads with the tubular system, the detailed morphology of which will be described in another section. The fenestrations are all about 250 A in diameter with only slight variation in size. They connect inner and outer membranes of the collar without opening to the outside, so that the reticulum may be considered a closed compartment. As in Balanus, no evidence for connections to the sarcoplasm through the pores, as described by Franzini-Armstrong (1963) for fish muscle, was found. The fenestrations probably serve to increase the surface area of the reticulum and thus expedite ionic exchange.

Tubular System

This system consists of tubules which originate from the sarcolemma and pass through the myofibril to terminate in diadic contacts with the cisternae of the SR. The tubules which come from the fiber

Figure 31. Carcinus twitch fiber, control cross section at surface. The tubules which form diads are obviously patent to the exterior although they lie under the thickened basement material. Magnification X 60,512.



surface (Figure 31) have a diameter of .01 μ. Those which come from the anastomosing network of sarcolemmal invaginations (Figures 26 and 29) are about .04 μ in diameter. The majority of tubules leave from the invaginations. They are easily distinguished from the invaginations by the absence of basement material, although the plasma membrane is continuous. While the fibers which were examined came from the antagonistic muscle, no evidence was found for a separate system of Z-tubules such has been described by Peachey and Huxley (1964) for Carcinus. Many of the tubules which left the invaginations did pass through Z-discs (Figure 29), and continued on to form the usual diadic contacts at the A-I junctions. Wherever tubules or invaginations did contact the Z disc, there was an increased electron density of the adjoined membranes. The openings of the tubules into the sarcolemmal invaginations in effect connect the entire TS to the external solution. The TS membrane of Carcinus conforms to the suggestions of Falk and Fatt (1964), Peachey and Huxley (1964), that, being continuous with the sarcolemmal membrane, it effectively increases its surface area. Morphologically, the TS is ideally suited for the performance of a coupling function between the sarcolemma and the contractile apparatus.

Diads

Whether the tubules which were just described come from the surface or from the invaginations, they form identical diadic contacts with the dilated portion of the SR. In this respect, they are entirely similar to the crayfish diad described by Brandt, et al. At each A-I junction there may be several diads surrounding the fibril (Figure 29). The unit membrane of the tubule is about 80 A while that of the cisternum if 95 A. The gap between the two membranes if 150 A (Figure 28). Clearly visible midway within the gap is a weakly osmiophilic thin band previously described by Hoyle for a variety of arthropod diads (1965).

While the great majority of diads were formed by the tubules, as in Balanus many were found between the cisternae and the sarcolemma, both at the surface and in the invaginations (see Figure 29). This was found also to be the case in crayfish by Brandt, et al. (1965). Since the morphology is exactly the same in either case, it is possible that these diads are responsive to propagated action potentials (Figure 2) as well as to the graded electrotonic currents which have been suggested by Huxley (1964) to be found in the TS.

A further distinguishing feature of the diads is the presence of a granular amorphous material located within the cisternae. This sub-

stance is not found within the remainder of the SR. A similar meshwork has been described by Birks (1965) in the cisternae of frog twitch fibers.

Discussion -- Carcinus Twitch Fiber Morphology

The morphological features of Carcinus twitch fibers resemble closely those described by Brandt, et al. (1965) for crayfish graded fibers. The sarcolemma of both types is extensively infolded and both have a well developed transverse tubular system. While the tubules which originate at the fiber surface were smaller than those continuous with the sarcolemmal invaginations (.01 μ and .04 μ respectively), their permeabilities and relationships to the cisternal elements were identical. Since they are both transversely oriented, a further subdivision into radial and transverse tubules was not felt necessary. The connections between the tubules originating at the surface and the plasma membrane were not as wide as those described by Peterson and Pepe (1961) for crayfish but were similar to those described by Brandt, et al. (1965). The tubules which originated from the invaginations were all about the same diameter and all formed diadic contacts. The fenestrated collar of SR in Carcinus is well developed, extending the entire length of the fibril as has been shown by Peachey (1965). Large numbers of diads are found at the A-I junctions, being

formed by dilated portions of the SR and the terminations of the TS, the sarcolemma, and the sarcolemmal invaginations. This raises the question whether or not the diads, which are in "contact" with membranes which are known to support propagated action potentials operate in a way similar to those in contact with the tubular system.

The structural differences in frog tonic and phasic fibers has been investigated by Adrian and Peachey (1966) and by Page (1965). A similar study has been reported for garter snakes by Hess (1965). In all cases, fast fibers were characterized by extensive TTS and a well developed SR. Slow fibers however, were found to have a poorly developed SR and a paucity of diadic connections, in some cases, none at all. The slow fibers could be further distinguished by the absence of an H-zone and M-line.

The Carcinus twitch fibers share features which are common to both tonic and phasic vertebrate fibers. The TS and the SR are well developed but the A-bands are more similar in appearance to tonic fibers. The sarcomere lengths, while long (5 μ) compared to vertebrate twitch fibers, fall within the range described by Atwood, Hoyle and Smyth (1965), Atwood (1963), and Dorai Raj (1964) for crustacean twitch fibers. In addition, their cross-sectional appearance at low magnification is similar to the punctate appearance of the myofibrils

often called fibrillenstructur, after Kruger (1949), and described by Cohen for phasic fibers in Cancer (1963). The salient features of both Balanus and Carcinus ultrastructure with respect to E-C coupling is that a continuous pathway exists from the surface plasma membrane to the calcium storage sites around the myofibrils. Because of the invaginated surface membrane, the high resistance tubular pathways are kept short. No bottlenecks occur at the openings of the tubules, and plasma membrane continuity is maintained until the diad is reached.

Neuromuscular Physiology of Balanus

A preliminary report of the anatomy and physiology of the depressor and adductor fibers (Hoyle and Smyth, 1963) has shown several unique features of the neuromuscular system. The adductor muscle received excitatory innervation from both ends which did not overlap in the center of the fibers. P.s.p.'s at constant stimulus intensity were largest close to the shell and became smaller towards the center of the fiber. All of the fibers which were examined, responded to indirect (neural) stimulation with postsynaptic potentials (p.s.p.'s) of different sizes which summated but which did not show the facilitation common to other crustacean fibers. Occasionally, large p.s.p.'s gave rise to secondary graded electrically excited spikes. The

mechanical responses consisted of small twitches to single p.s.p.'s which summated at increasing frequencies of stimulation. The adductor muscle was innervated by two to four axons and the depressor muscle by two. No inhibitory axons were found.

In addition to the large white fibers which were studied by Hoyle and Smyth, there are present in Balanus 8 flags of thin pink fibers, which in some cases function synergistically with the white fibers and in other cases, alone. Pink fibers also have been seen in the large swimming muscle of the Hawaiian crab, Portunus sanguinolentis (Hoyle, 1966), in the leg muscles of the common roach, Periplaneta americana (Jacklett, 1966), and the water bug, Lethocerus collosicus (Walcott, 1966). The color of these fibers can be correlated with large numbers of mitochondria (see section on electron microscopy of Balanus) and their associated enzymes, particularly the cytochromes. Spectographic analysis of lysed fibers produce curves with the same absorption peaks as human hemoglobin (Willows, 1965), but the biochemistry of these fibers has not been examined sufficiently enough to make any firm statement about the pigment content.

When the electrical and mechanical responses of the pink fibers are compared with those of the white ones, several differences become apparent. How the barnacle exploits these differences will be considered in the discussion. To begin, I want to first review the general

features of the largest and most numerous white fibers and then go on to the various pink muscles.

White Depressors

Six muscle bundles serve to depress and rotate the scutal-tergal plates. Their function is unlike that of any other crustacean muscle since the scutes are held up by a column of fluid under pressures as high as 430 mm of Hg (Tait and Emmons, 1925). All of the depressors contracting synchronously can retract the scutal-tergal plates, or rotate and tip them during feeding. Electromyograms were made of feeding barnacles during the present work and indicate that the muscles receive a constant barrage of impulses (Figure 32) similar to those found by Kennedy in the tonic flexor system of crayfish (Kennedy, 1965). The depressors all have small p.s.p.'s, usually less than 5mV (Figure 33). Tension could not be elicited in these fibers until summed p.s.p.'s depolarized the fibers to about 20mV. The rate of rise of tension was linearly related to the frequency of stimulation, and fell off rapidly at the termination of depolarization. With plate stimulation, current pulses of 10msec. produced twitches of about 400msec. duration which fused at 6/sec. (Figure 34). Maximum tetanic tension could be obtained at frequencies above 12/sec., the force produced being about 4.5Kg/cm^2. The rate of force develop-

ment in response to direct intracellular stimulation with outward current again depended upon the level of depolarization, the threshold being about 20mV (Figure 35).

The lateral and rostral depressors were identical in both electrical and mechanical responses to all forms of stimulation, but the tergal depressors produced larger p.s.p.'s of from 20-25mV (Figure 36). The resting potentials were also slightly higher, averaging 68mV compared to the other depressors averaging 60mV. The threshold for contraction (Ec) was higher, requiring about 22mV of depolarization to initiate contraction.

While only one, of approximately 50 depressor fibers which were examined, produced all-or-nothing spikes, the membrane potential for spike production could be demonstrated by lowering the internal Ca activity by Hagiwara's method. (Hagiwara, et al., 1964). Figure 37 illustrates the spike potentials produced in response to direct stimulation after the fiber had been filled with a citrate buffer solution.

White Adductors

These graded fibers were described fully by Hoyle and Smyth, (1963) and my results essentially confirm theirs. Figure 38 illustrates the electrical and mechanical responses of isolated adductor fibers.

Figure 32. A-E, electromyograms of different Balanus muscles obtained from unrestrained animals while feeding. A & B are white lateral depressors, the large deflections in A being movement artefacts. C is from the rostral depressor and D from the depressor tergorum and E from the adductor muscle. F shows PSPs from a single adductor fiber in a dissected preparation with the nerve supply still connected to the ganglion. Tension of the whole muscle is monitored on the lower trace. There is a continual barrage of PSPs, with occasional bursts associated with tension development. The intracellular records show a continuous alteration of the membrane potential by PSPs with occasional summating bursts giving rise to slow graded twitches. Time marks in A, B & E are 30 sec. and 10 sec. in F, C & D time marker (lower trace) is 1/sec. F reads from right to left.

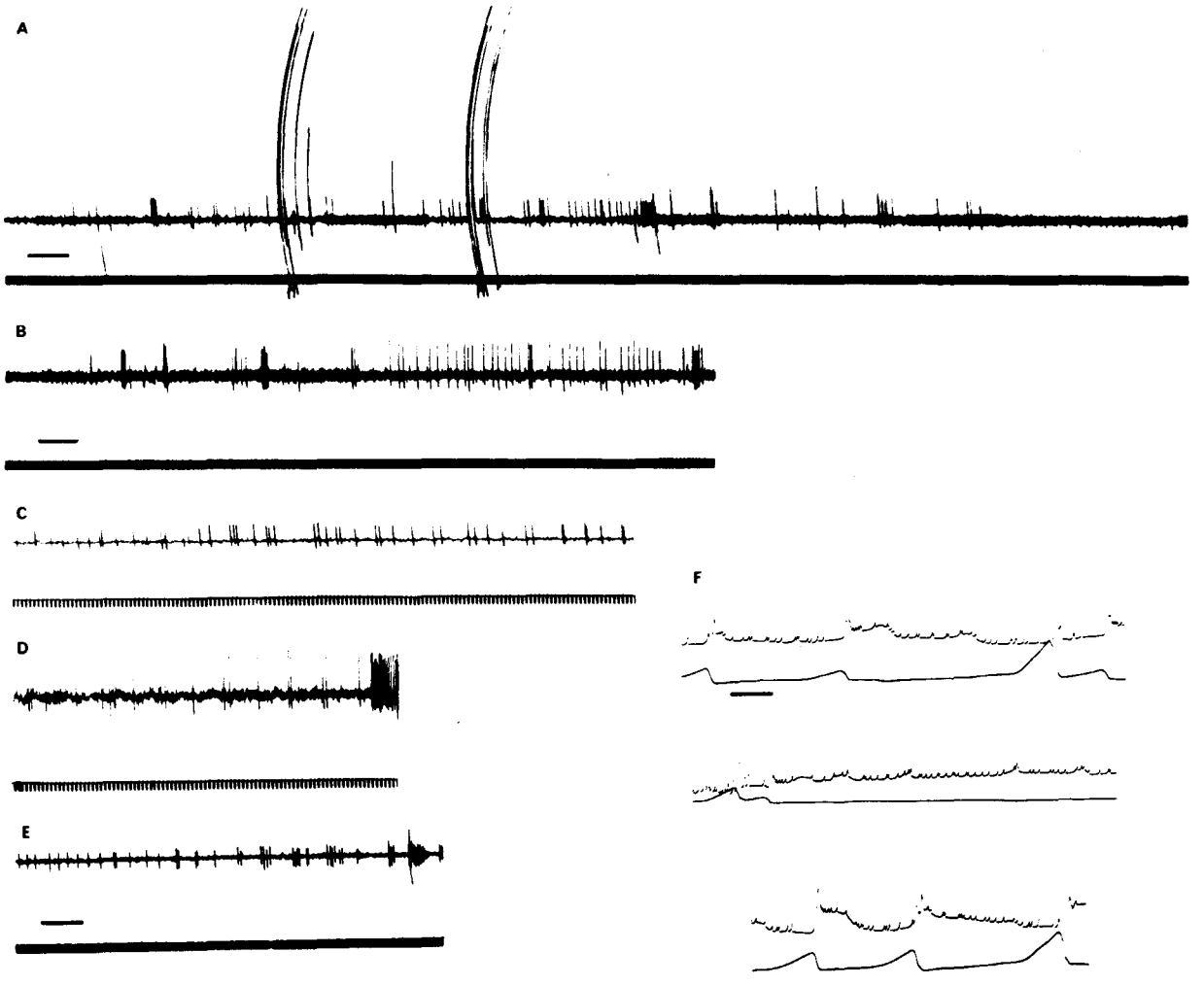

A
B
C
D
E
F

Figure 33. Electrical and mechanical responses of lateral depressor white fibers to indirect stimulation via the motor nerve. Frequencies in A; 25/sec., 40/sec., 50/sec., 77/sec.; B; 1 & 2 20/sec., 31/sec., 40/sec., 50/sec., 62/sec., 77/sec. Calibration A: 20mV - .75 gm-200msec., B: 5mV-.75gm-100m

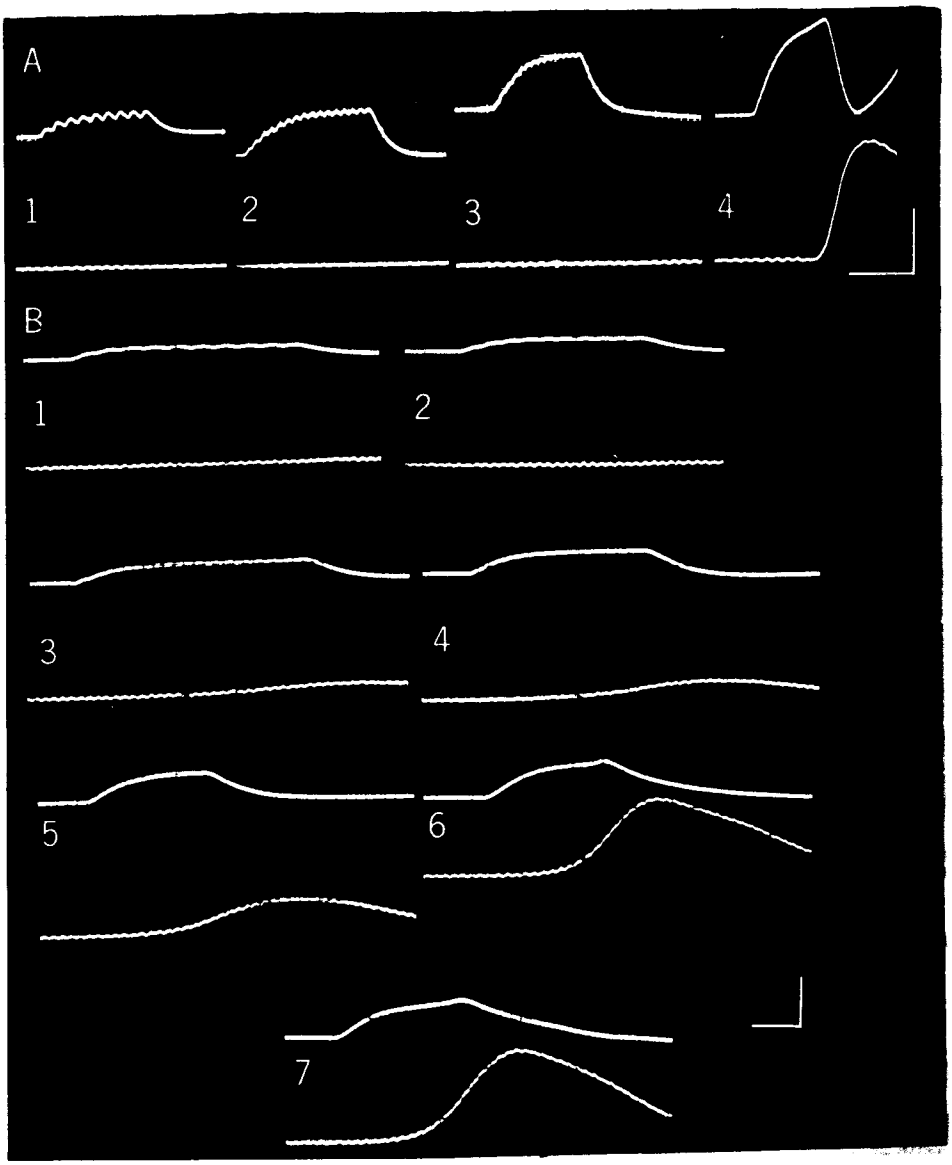

A
1
2
3
4
B
1
2
3
4
5
6
7

Figure 34. Genesis of tension in white lateral fibers. Stimulation by silver-silver chloride plate electrodes parallel to fiber. Frequencies of stimulation are 2/sec., 4/sec., 6/sec., 8/sec., 10/sec., 12.5/sec. Calibration 1 sec. & 1 gm.

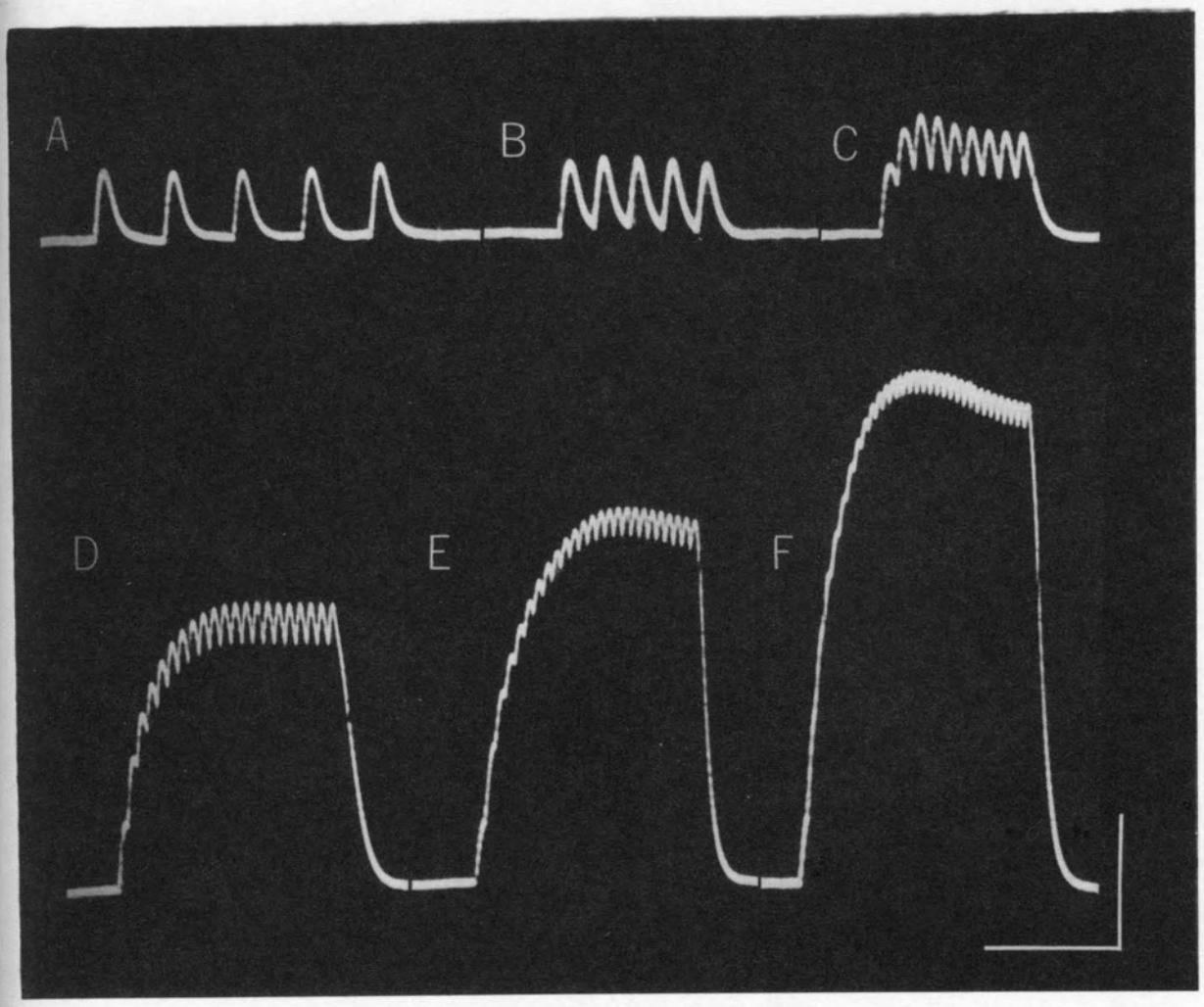

A
B
C
D
E
F

Figure 35. Tension development in white lateral depressor fiber in response to direct stimulation. E_c at approximately 20mV depolarization. Some distortion of the membrane potential is caused by movement of the fiber. Calibration 20mV-1gm-100msec.

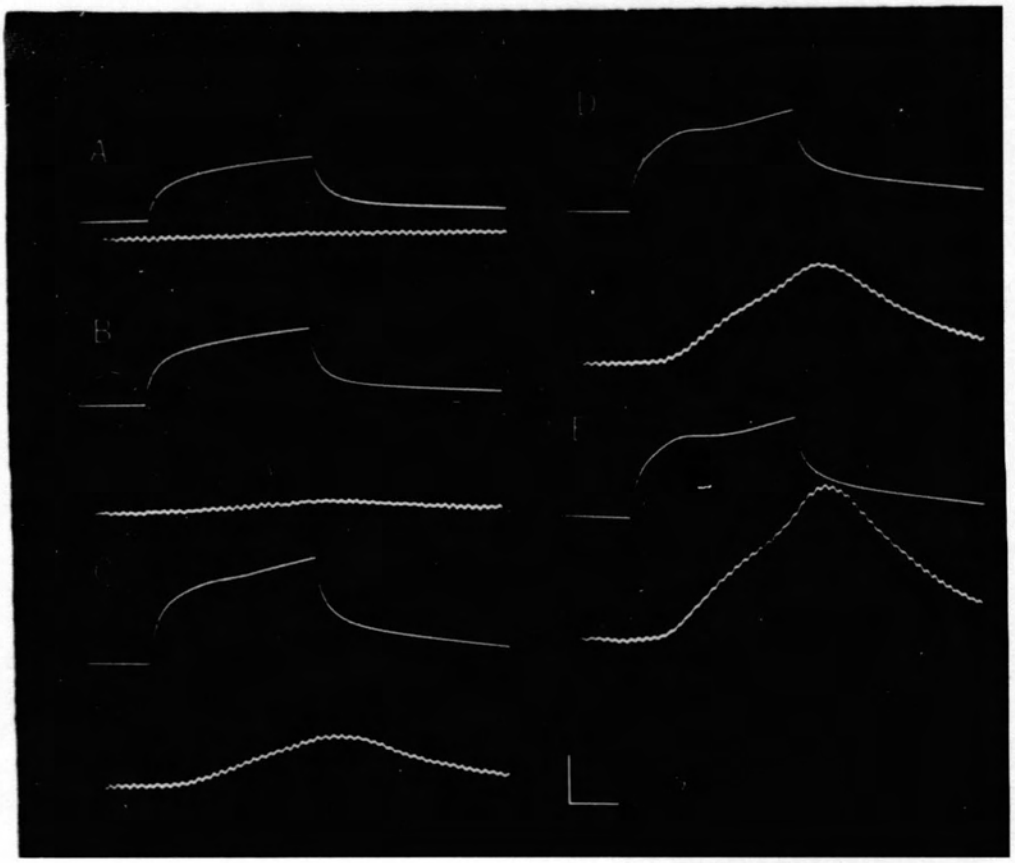

A
B
D
L

Figure 36. Development of tension in single fibers of depressor tergorum muscle. Single P. S. P. s never elicited tension. At frequencies above 12/sec. in A, summation without facilitation will depolarize the fiber to the E_c, approximately 22mV. Frequency of stimulation in A: 10/sec., 12.5/sec., 20/sec., 20/sec. B: similar to A, 1 shows no tension developed in response to a single large P. S. P. 3 & 4 both at 10/sec., 4 having a longer duration burst. Note the different thresholds for tension development. Calibration: A, 5mV-1gm-200msec.; B: 100msec.-20mV-1gm.

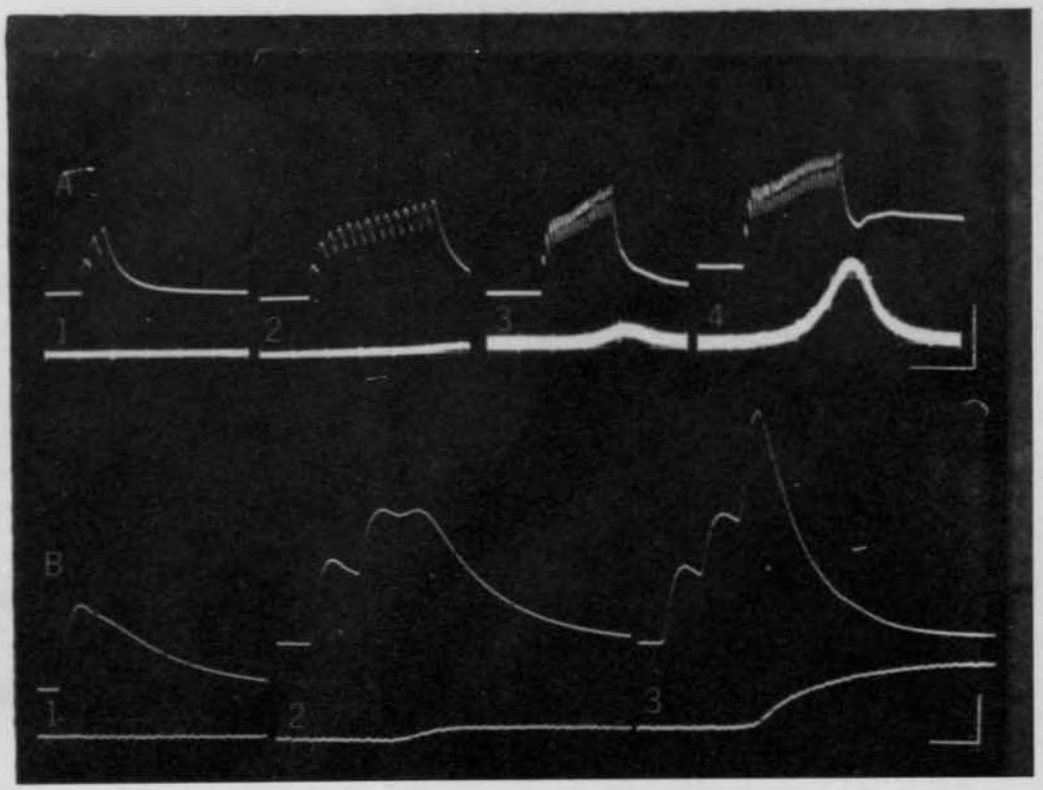
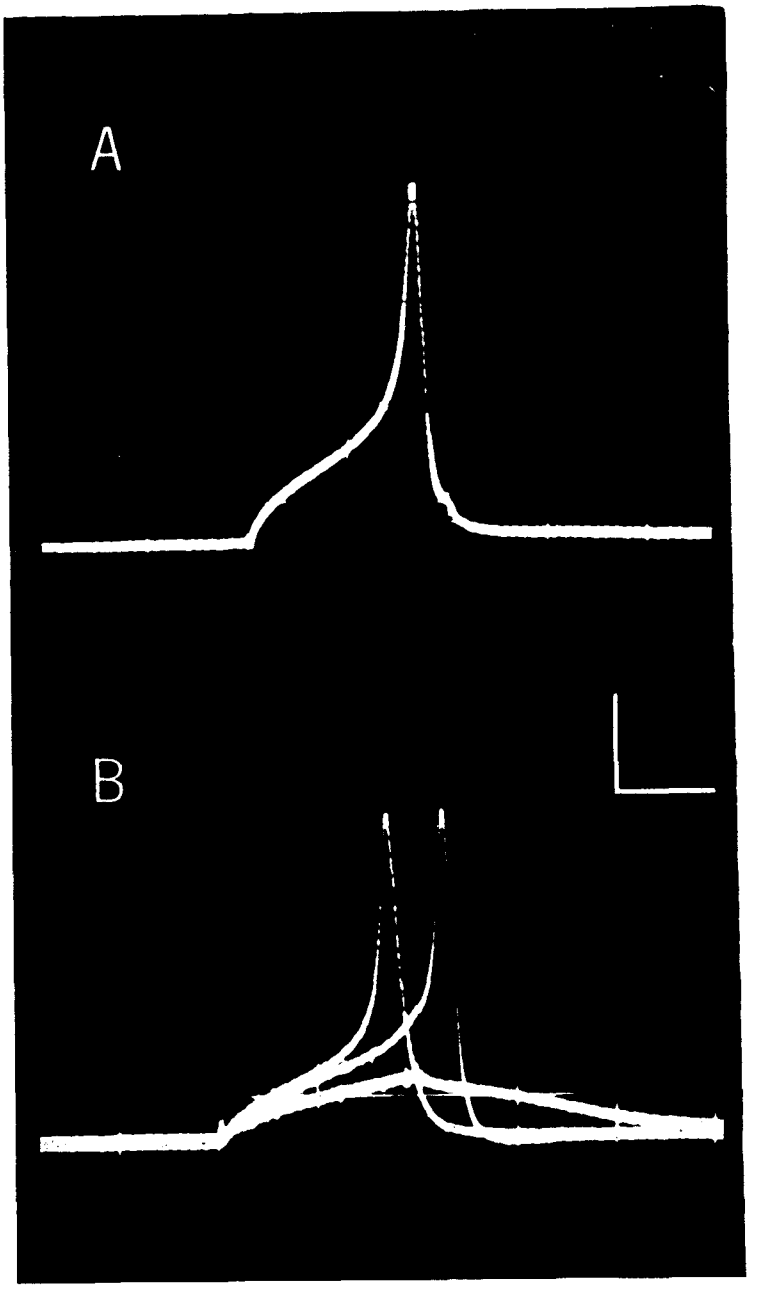

A
1
2
3
4
B
1
2
3

Figure 37. Development of non-overshooting spike potentials by reduction of internal Ca. Fibers were cannulated and injected with 137 mM potassium citrate prior to direct depolarization. Calibration: 10mV-50msec.


A
B
L

Four sizes of p. s. p. 's with different decay times could be obtained, but over 85% responded with three only. These could get quite large, up to 35mV, which exceeded the Ec, and produced twitches of about 120msec. duration. Often, active responses (Figure 38, E-c) were generated by large p. s. p. 's but paired p. s. p. 's at decreasing shock intervals seldom were converted to active responses. Twitch-tetanus ratios were identical to those described by Hoyle and Smyth.

Lateral Pink Depressors

A single-layered flag of pink fibers is found on the interior aspect of the lateral depressor muscles. Their origin and insertion are the same as the white lateral depressors. P. s. p. 's are never greater than 5mV, and summation without facilitation occurs at increasing frequencies of stimulation (Figure 39). As in the white fibers, tension was never produced by single p. s. p. 's, but required summation to about 18mV of depolarization. Fusion of tension responses occurred above 4/sec. with a maximum tension of 15gms. at frequencies above 16/sec. Decay of tension after indirect stimulation lasted about 250msec.

Pink Adductors

These fibers are located above and beneath the adductor muscles,

Figure 38. Electrical and mechanical responses of single white adductor fiber preparations. A, B & C show P. S. P.'s in response to various intensities of neural stimulation. The majority of fibers were innervated by three motor axons. A few were supplied by only two (D) and occasionally a fiber could be seen with four. Tension could always be registered in response to a single P. S. P. as shown in E, a-c. The time course of contraction varied from fiber to fiber, however. F, shows the summation of two paired P. S. P.'s with decreasing intervals. No facilitation of response was observed. Calibrations: upper, 40msec. -20mV, middle, 20msec-10mV-1 G., lower, 20msec-10mV.

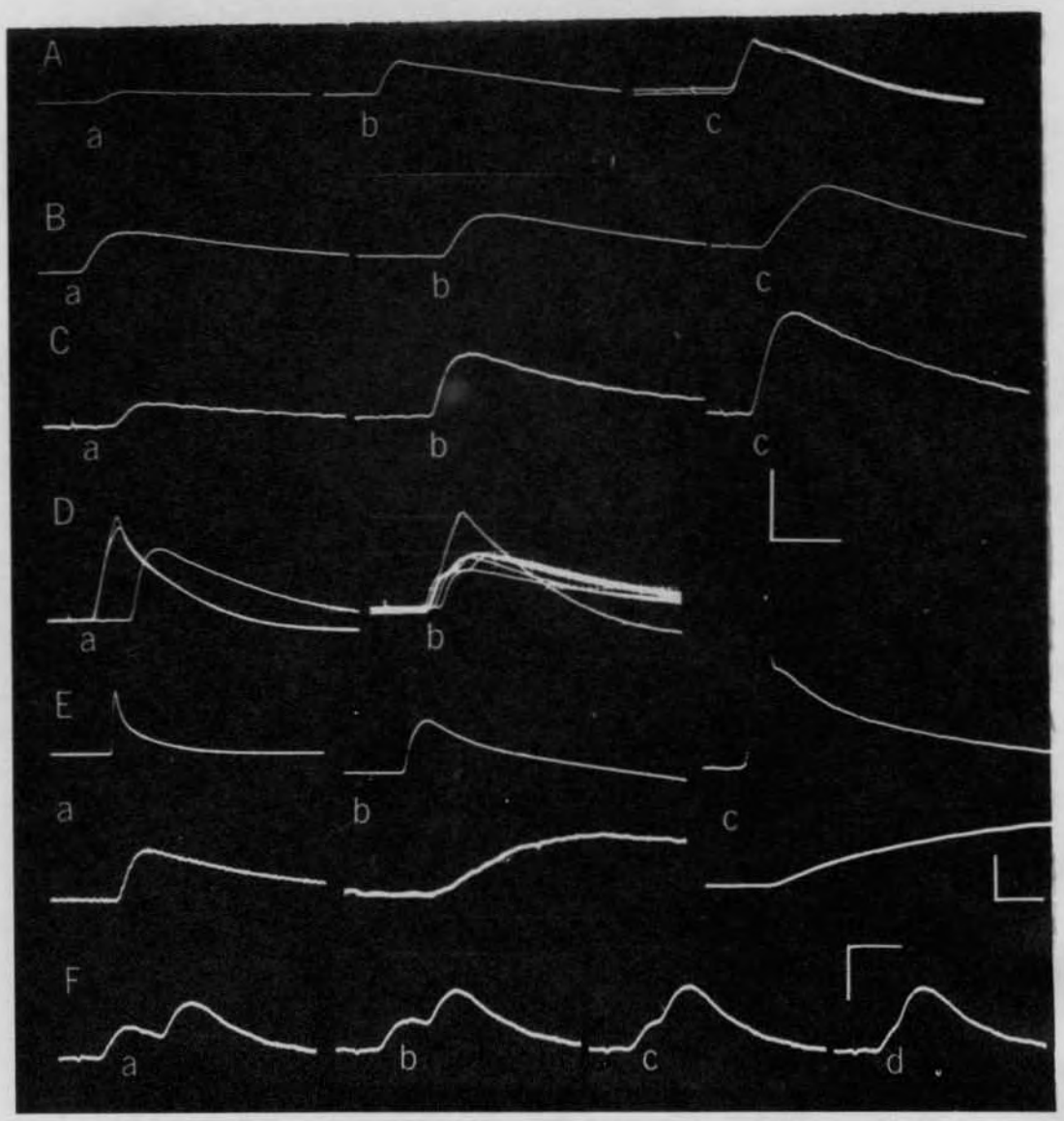
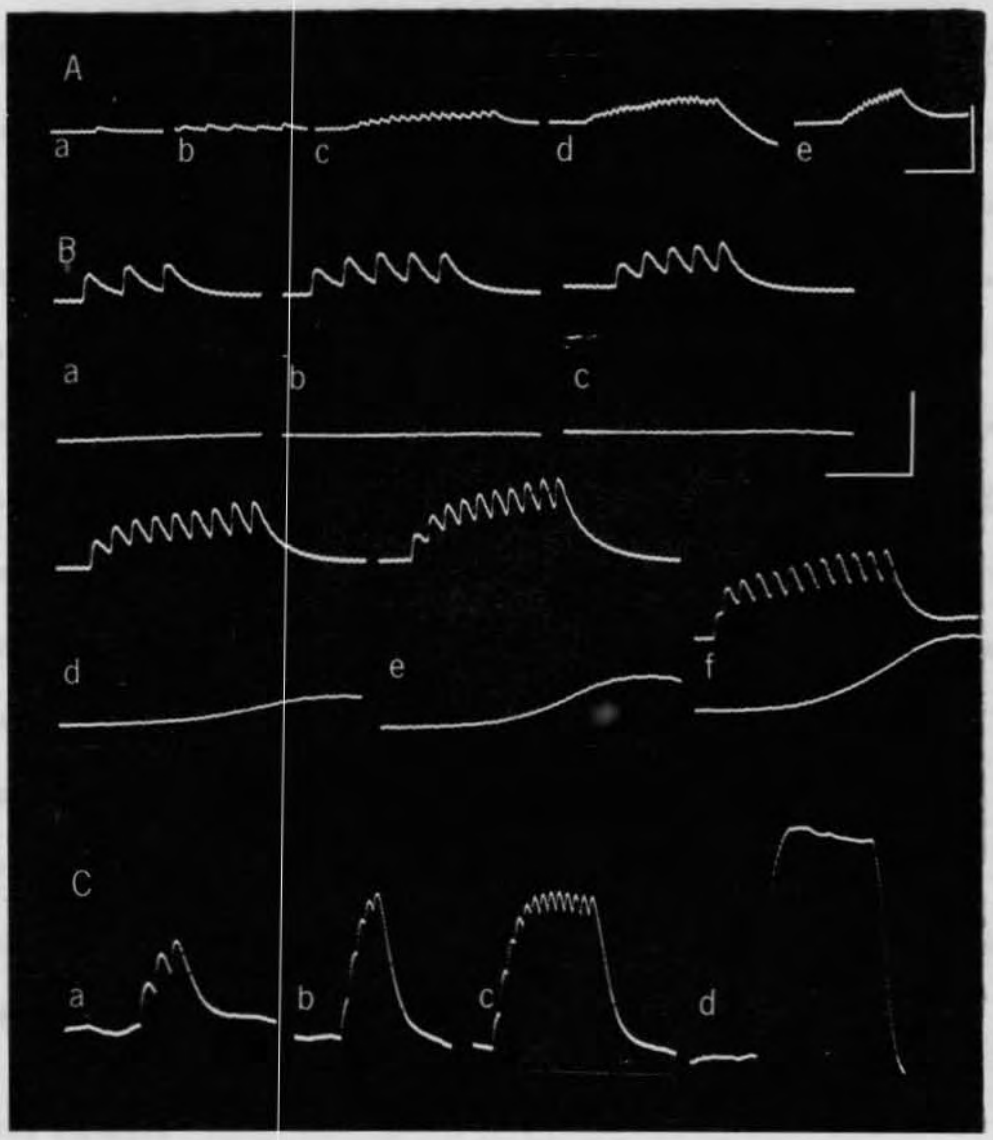

A
a
b
c
B
a
b
c
C
a
b
c
D
a
b
E
a
b
c
F
a
b
c
d

Figure 39. Mechanical and electrical responses of pink lateral depressor fibers. A shows small P.S.P.'s summating at increasing frequencies. B indicates the development of tension at 18mV depolarization. C represents the genesis of tetanus with plate stimulation. Fusion occurs at 4/sec. (C) and maximum tension is at 16/sec. (D). a is 2/sec. and b is 4/sec. Calibration: A & B, 200msec-10mV-(1gm). C is 1 sec. and 5 gm.


A
a
b
c
d
e
B
a
b
c
d
e
f
C
a
b
c
d

originating on the scutal plate and inserting on the transparent sheath enveloping the adductor muscle (See Figure 4). When stimulated, they pull the sheath down tight over the adductor muscle, but I was not able to determine for what purpose. The fibers respond to neural stimulation with very large p. s. p.'s of up to 40mV. (Figure 40). Single shocks cause twitches of up to 600msec. duration. Active responses were seen often and could be elicited regularly by decreasing the paired shock intervals (Figure 40, C-1-2). The pink adductor fibers, as well as all the other Balanus fibers, required Ca in the bathing saline. Removal of Ca (Figure 41) caused a reversible diminution and eventual abolition of both electrical and mechanical responses. Tension responses to plate stimulation are shown in Figure 42. These fibers were relatively weak, but a tension of several grams per fiber could be developed.

Pink Body Rotator

This third group of pink fibers are attached to the tergal shell and insert on the sides of the body (Figure 4). From their anatomical location, they appear to rotate the body and feeding apparatus. Stimulation of the large nerve trunk leading to them from the subesophageal ganglion, produces medium to large p. s. p.'s usually greater than Ec. As in the pink adductors, twitch durations are very long, up to

Figure 40. P. S. P.'s at different stimulus intensities of neural stimulation in upper pink adductor fiber. B & C are paired shocks to nerves delivered at decreasing intervals. In B, there is no change in the shape of the second response, while in C (different fiber), there is a conversion of the second P. S. P. to an active response. Calibration: A, 20mV-40msec., B, 5mV-20msec., C, 10mV-20msec.

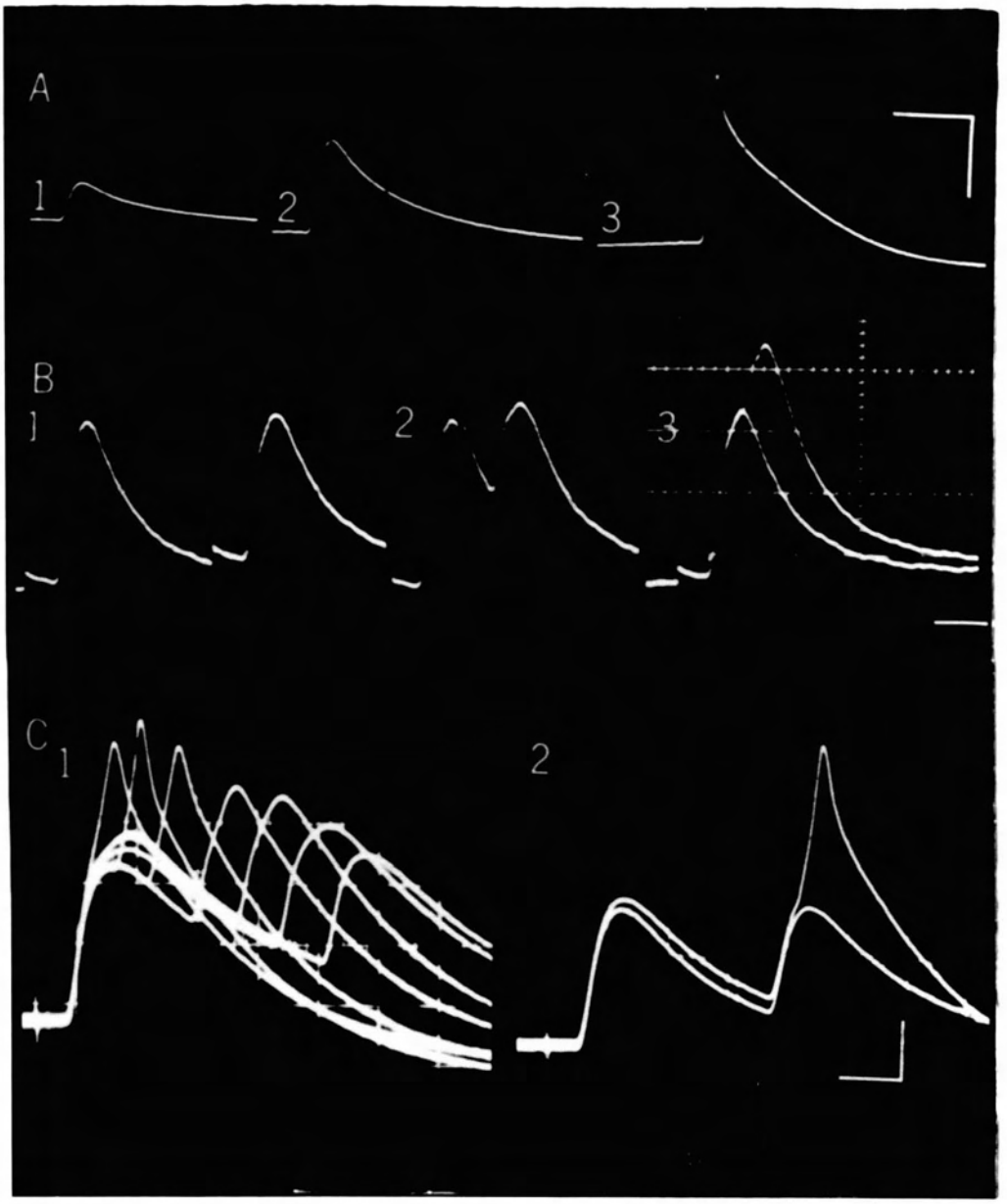

A
1
2
3
B
1
2
3
C
1
2

Figure 41. Dependence of P. S. P. and tension on external Ca activity is shown in this series of electrical and mechanical records of a single pink adductor fiber. A is the normal response to a single shock. B shows the decrease in P. S. P. amplitude and tension after 30 seconds in O Ca saline. C is 1 minute after transfer and in D, 5 minutes after transfer, both tension and membrane response have been abolished. E shows the normal response after return to Ca saline. Calibration: 10mV-.5gm-200msec.

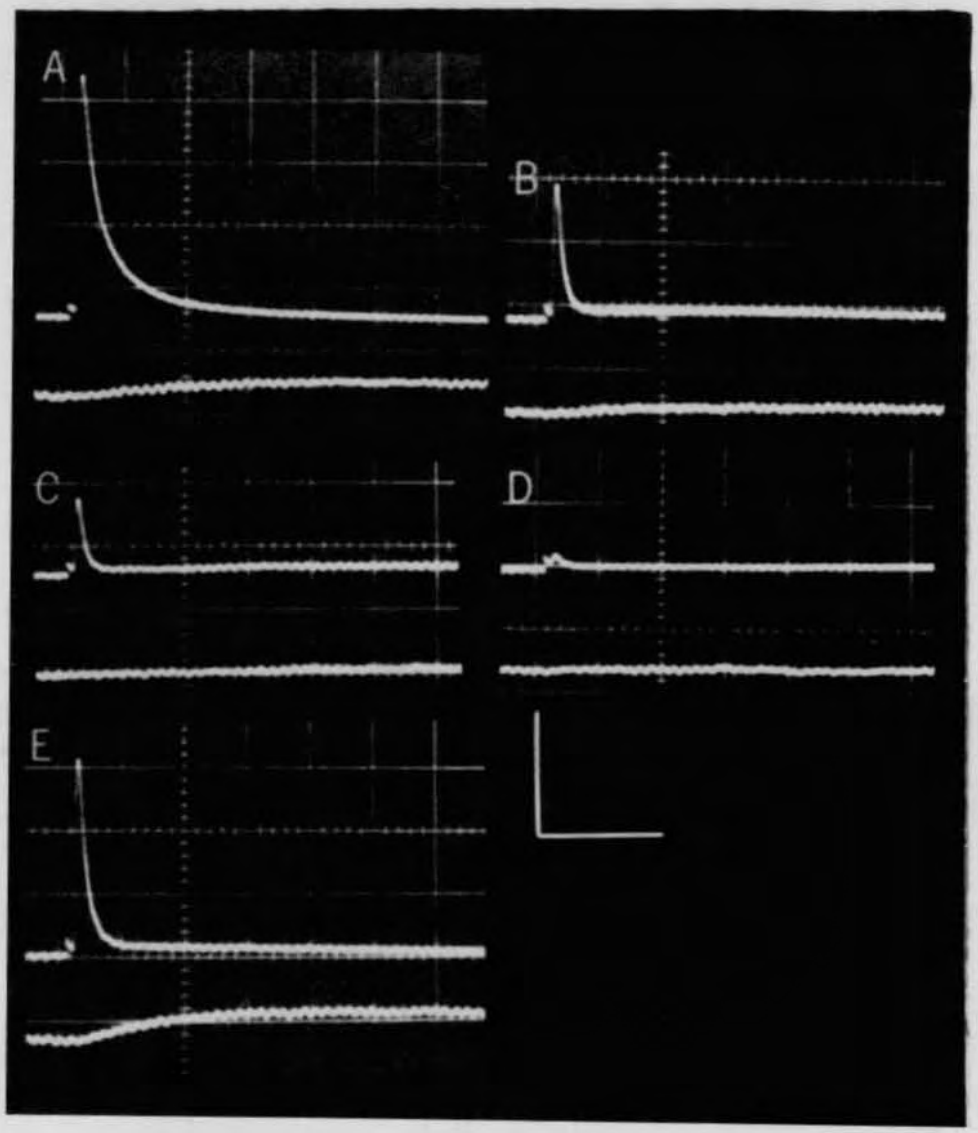
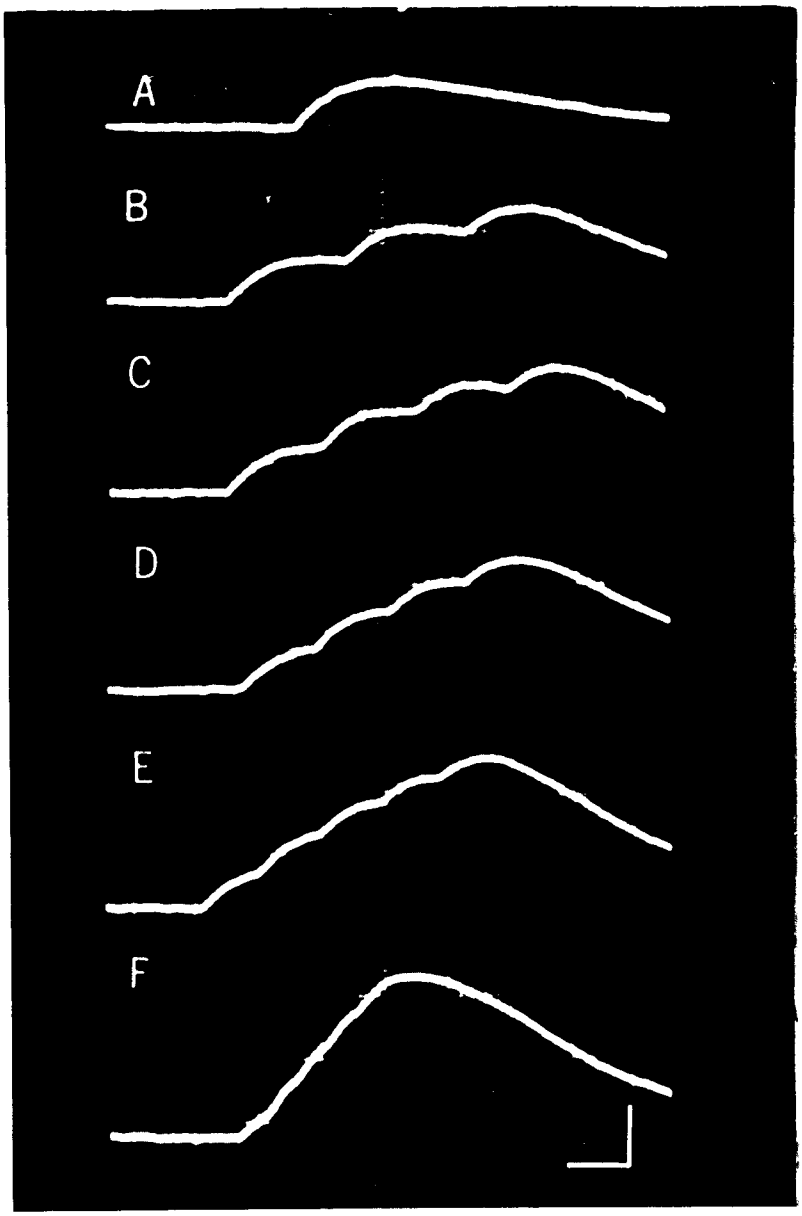

A
B
C
D
E

Figure 42. Tension responses of pink adductor fiber to stimulation via plate electrodes. Frequencies: 2.5/sec., 3.1/sec., 4/sec., 5/sec., 10/sec. Calibration: .5gm-100msec.


A
B
C
D
E
F

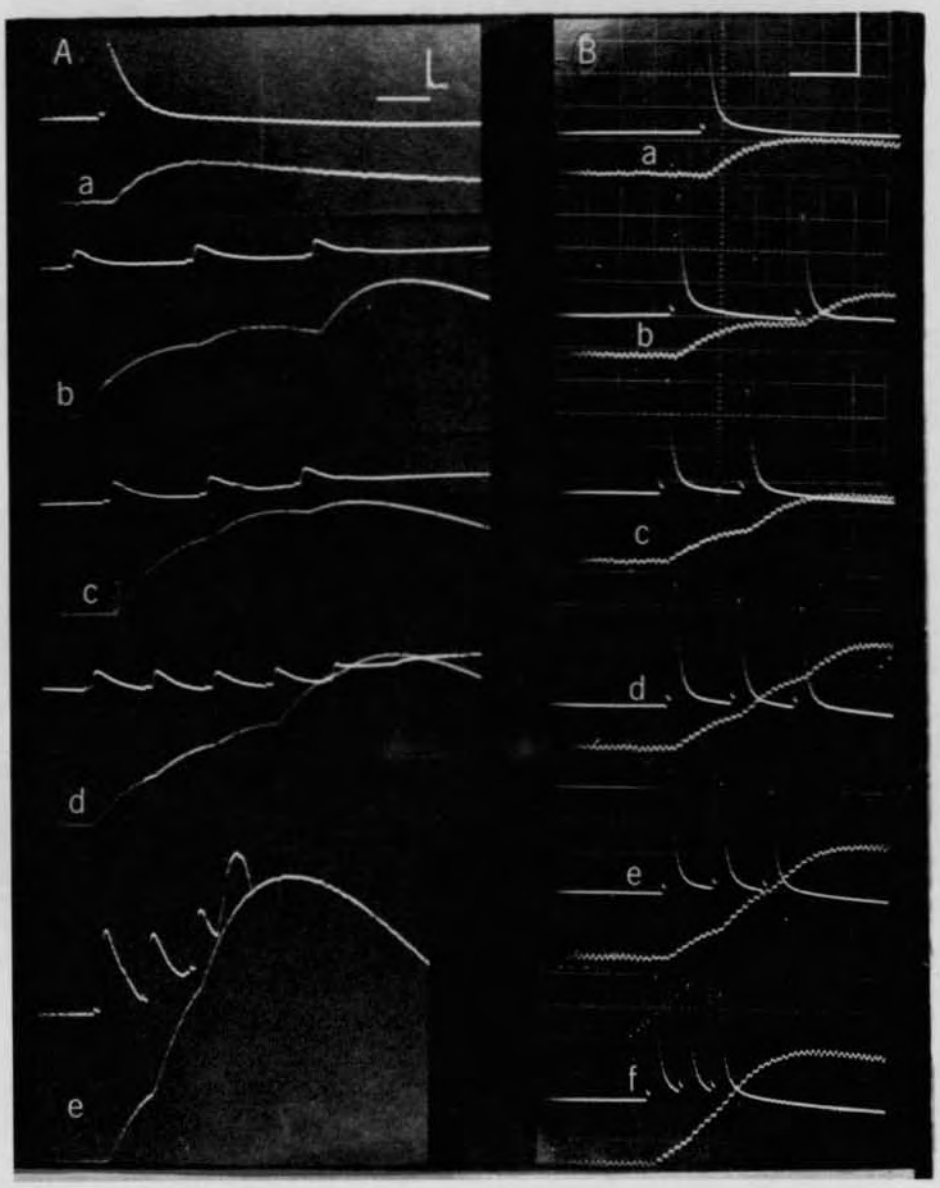
600msec. (Figure 43). These long durations allow summation of tension responses at frequencies as low as 4/sec., i.e., there is considerable tension summation without p. s. p. summation. While most of the fibers twitched in response to single shocks, a few required depolarization to above 25mV. Paired shocks at close intervals produced large spikes which always led to long duration twitches (Figure 44). All of the electro-mechanical studies are summarized in Table 1.

Discussion -- Balanus Neuromuscular Physiology

The crustacean motor control systems which have been described so far seem to fall into two broad categories. Limb muscles appear to be made up of mixed populations of fibers innervated by slow, fast or inhibitor axons. (Atwood, Hoyle and Smyth, 1965; Raj, 1964). Phasic fibers usually receive predominantly fast axon innervation while tonic fibers are predominantly innervated by slow axons. Abdominal musculature control, however, is achieved by separate exclusively tonic and phasic muscles, with homogeneous fiber types, each systems with its own separate innervation (Abbott and Parnas, 1965; Parnas and Atwood, 1966; Kennedy and Takeda, 1965).

Balanus depends entirely upon a slow system to manipulate its scutal-tergal plates through a narrow range of movements, all of which

Figure 43. Aa-Ae are mechanical and electrical responses of a single pink body rotator fiber to nerve stimulation. A slow contraction is produced following a single p. s. p. of about 7mV. Tension responses are summated even at low frequencies. Frequencies shown are: 4.3/sec., 5.4/sec., 8.7/sec., 10/sec. Calibrations in a & e are 5mV-.5gm-100msec., in g-d, 10mV-.5gm-100msec. Ba-Bf are similar responses in an upper pink adductor fiber. Frequencies are 2.5/sec., 4/sec., 5/sec., 6.2/sec., 10/sec. Calibration: 200msec. -10mV-.5gm.


A
L
a
b
c
d
e
B
a
b
c
d
e
f

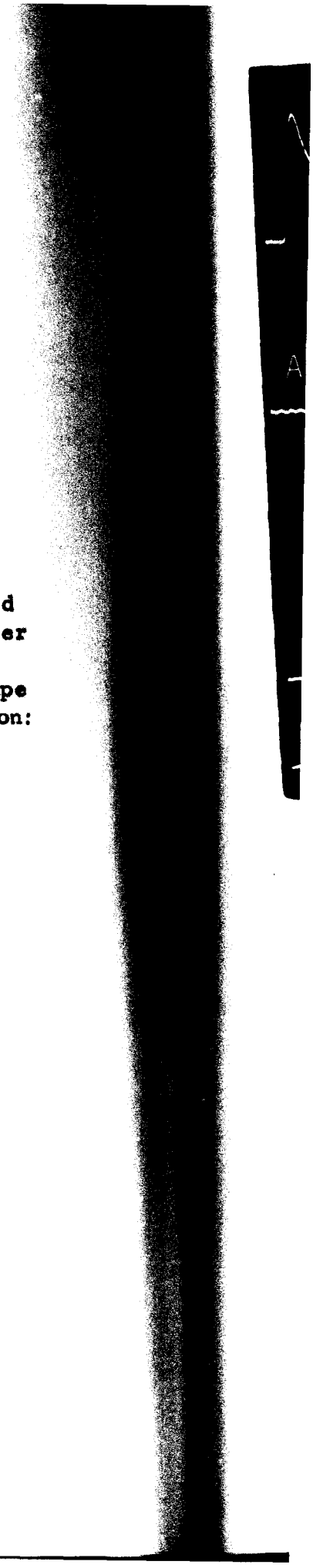


Figure 44. Pink body rotator fiber stimulated indirectly with paired stimuli at decreasing intervals. Tension, registered on lower trace begins at 26mV depolarization. The rate of tension development increases with decreasing intervals and the shape of the superimposed P. S. P. is markedly altered. Calibration: 10mV-. 5gm-20msec.

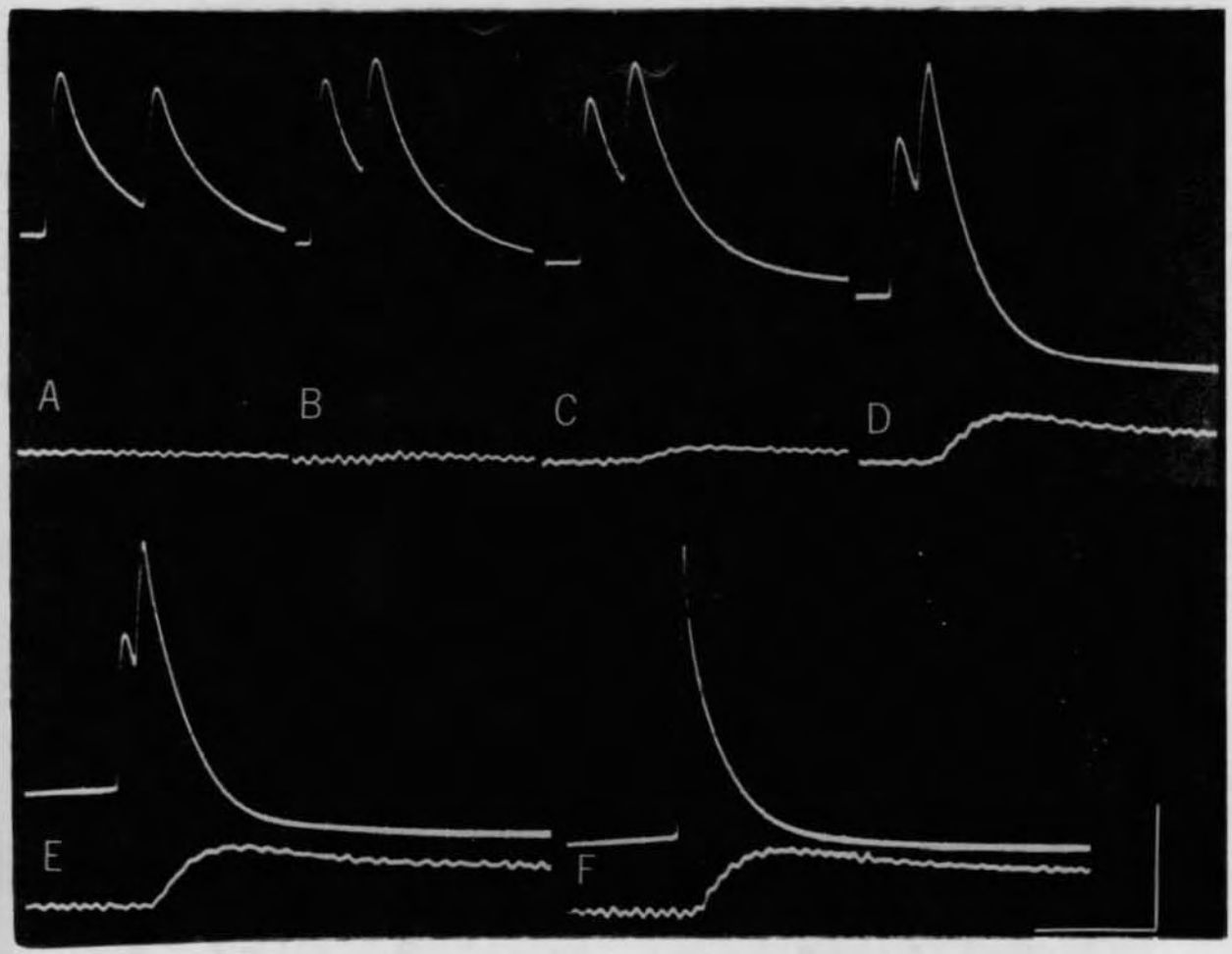

A
B
C
D
E
F

TABLE 1

ELECTRO-MECHANICAL RESPONSES OF BALANUS FIBERS

	W. Depressor	W. Adductor	P. Depressor	P. Adductor	P. Body Rotator
R. P.	60mV	58mV	40mV	66mV	66mV
Twitch Time	---	120-200msec	---	to 600 msec	to 600 msec
Fusion Frequency	6/sec	*	4/sec	10/sec	4/sec
Response to single p. s. p.	--	-	-	-	-
E_c	5-20mV	5mV	18mV	*	5-26mV
Active Response	-	-	-	-	-
Risetime of psp	slow	fast to slow	fast	fast to slow	fast
Size of psp	2-5mV	5-35mV	2-5mV	40mV	6-35mV

are devoted to feeding and respiration. Crisp and Southward (1961) have investigated the feeding movements using high speed cinematography and describe four phases of activity, two of which involve the use of the adductor and depressor muscles. The adductor muscle is used to bring the scutes together after they have been opened by a combination of depressor activity and internal hydrostatic pressure. In addition to feeding movements, the depressors hold the scutes open slightly when the barnacle is out of water in order to allow oxygen to permeate the bubble formed at the opening. The scutes may be held slightly open in this way for many hours without apparent fatigue.

In place of the flexor-extensor antagonistic systems found in other crustaceans, the cirripedes rely on fluid pressure to extend the scutal plates. Pressures recorded manometrically by Tait and Emmons (1925) on Balanus nubilis were routinely 200-350mm/Hg, with their highest value being 430mm. The lack of antagonistic muscles may be the reason no inhibitory axons were found (Hoyle and Smyth, 1963).

A large variety of post-junctional potentials are found, both large and small, but are unlike other slow systems in that facilation at increasing frequencies of stimulation does not occur. Tension is produced in response to single p. s. p.'s in the white and pink adductor fibers, but in no others. The usual case is for the summed p. s. p.'s

to reach a critical level of depolarization, Ec, which seems to be variable from fiber to fiber, but in the range of between 5 to 25mV.

Recording from single fibers with intact CNS innervation, or EMGs from whole muscles of intact animals, revealed a continual background of spontaneous activity, characteristic of systems contracting in graded fashion (Kennedy and Takeda, 1965). The electrical activity of intact preparations showed the membrane potential to be in a state of continual variation due to summating p.s.p.'s at irregular intervals. Occasionally these were interrupted by large depolarisations due to summed p.s.p.'s which were correlated with large increases in tension. But, as described by Orkland (1962) and Atwood (1963), tension was a continuous function of the membrane potential, even at very low levels of activity.

Pink fibers, their color being due to the cytochromes contained in large numbers of mitochondria, were found on the medial surface of the lateral depressors, attached to the sheath surrounding the adductor muscle and alongside the body.

Pink fibers were generally slower than the white fibers, maintaining K contractures for longer periods and having much longer twitch durations. It is not possible to correlate this physiological difference with the large numbers of mitochondria at the present time.

Calcium was found to be a necessary constituent of the bathing saline for the production of p. s. p. 's and tension development. If fibers were soaked in an O- Ca saline, p. s. p. 's and mechanical responses were abolished. Both would return when Ca was replaced, provided the fiber had not remained in the O - Ca saline too long.

On the basis of contraction-relaxation time, the white adductor fibers could be classified as fast-followers (after Atwood, et al., 1965). No all-or-nothing spiking fibers were found, but only gradedly responding fibers which, as in the case of the adductors were fast, or as in the remainder of the fibers, were slow.

Permeability of the Tubule Membrane

Swelling Induced by Chloride Removal

When the Ringer solution bathing a Carcinus fiber was removed and a Cl free Ringer introduced, the membrane underwent a transient depolarization of 20mV, returning to the resting potential level with a time constant of about 20 minutes (Figure 45). This amount of depolarization was within the range reported by Reuben, et al. (1964), for crayfish fibers, but smaller in magnitude than that shown for frog fibers (Hodgkin and Horowicz, 1960). The rate of initial depolarization is not instantaneous due to a longer diffusion path brought about by the exten-

Figure 45. Chloride transient response in Carcinus fiber when propionate saline is substituted for Cl saline at arrow. The approximately 20 mV depolarization is followed by a gradual return to the resting potential as the ions are redistributed.

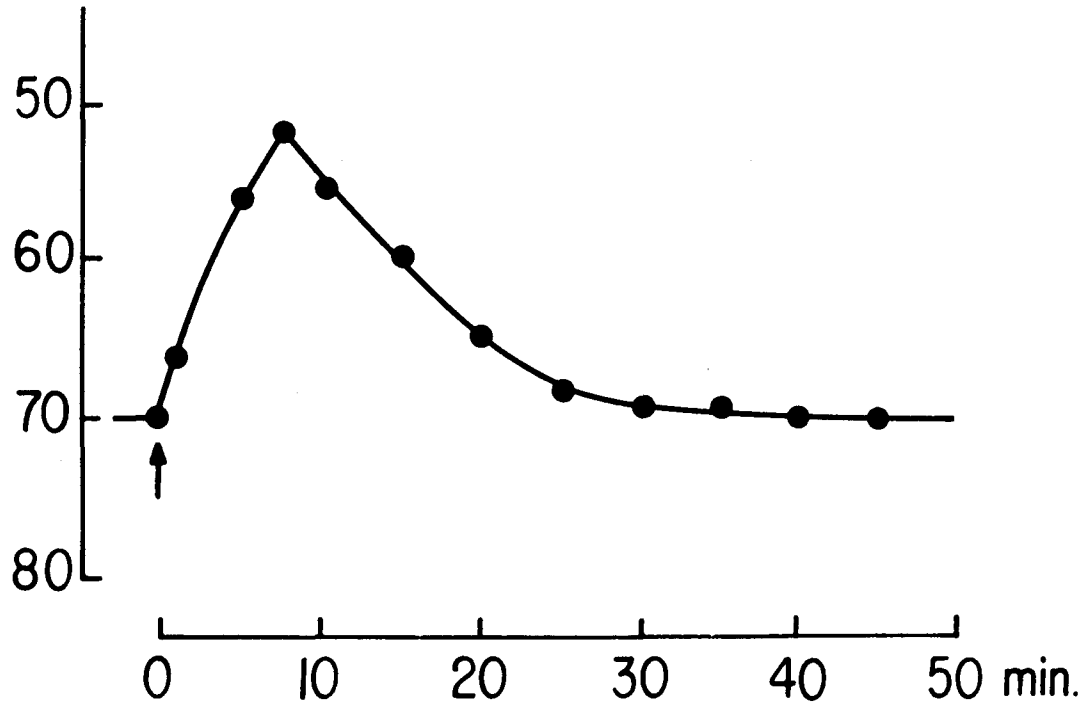
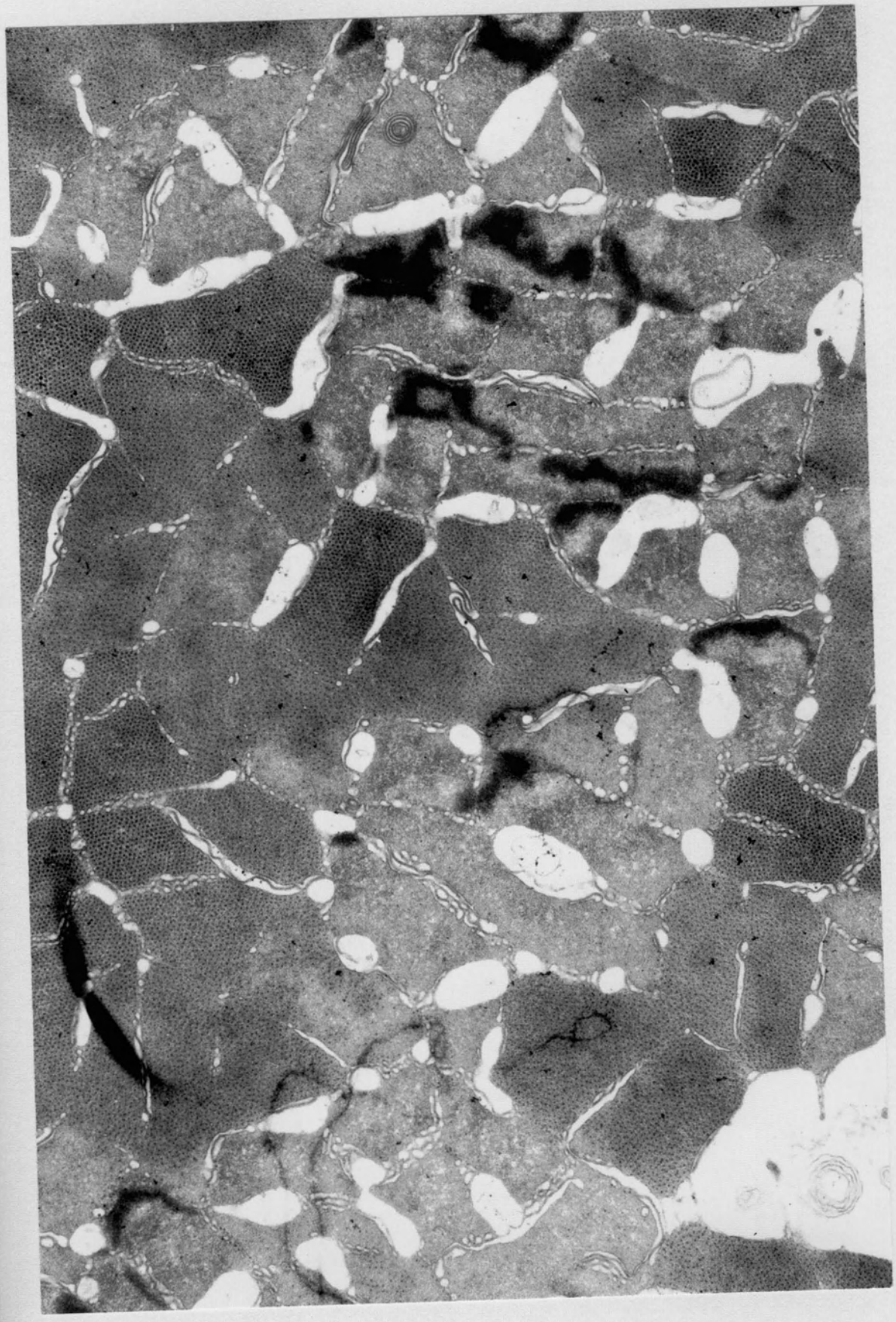

50
60
70
80
0
10
20
30
40
50 min.

Figure 46. Carcinus twitch fiber, cross section. Low power survey electron micrograph of a fiber which had been reversed from a 120 KCl Ringer to a K Propionate Ringer. The swollen organelles appear evenly dispersed throughout the fiber and are the cause of the darkening observed through the dissecting microscope. The myofibrils and the sarcoplasmic reticulum are unaffected by the reversal process, the vesicles being due solely to swollen tubules. Magnification X 18,476.



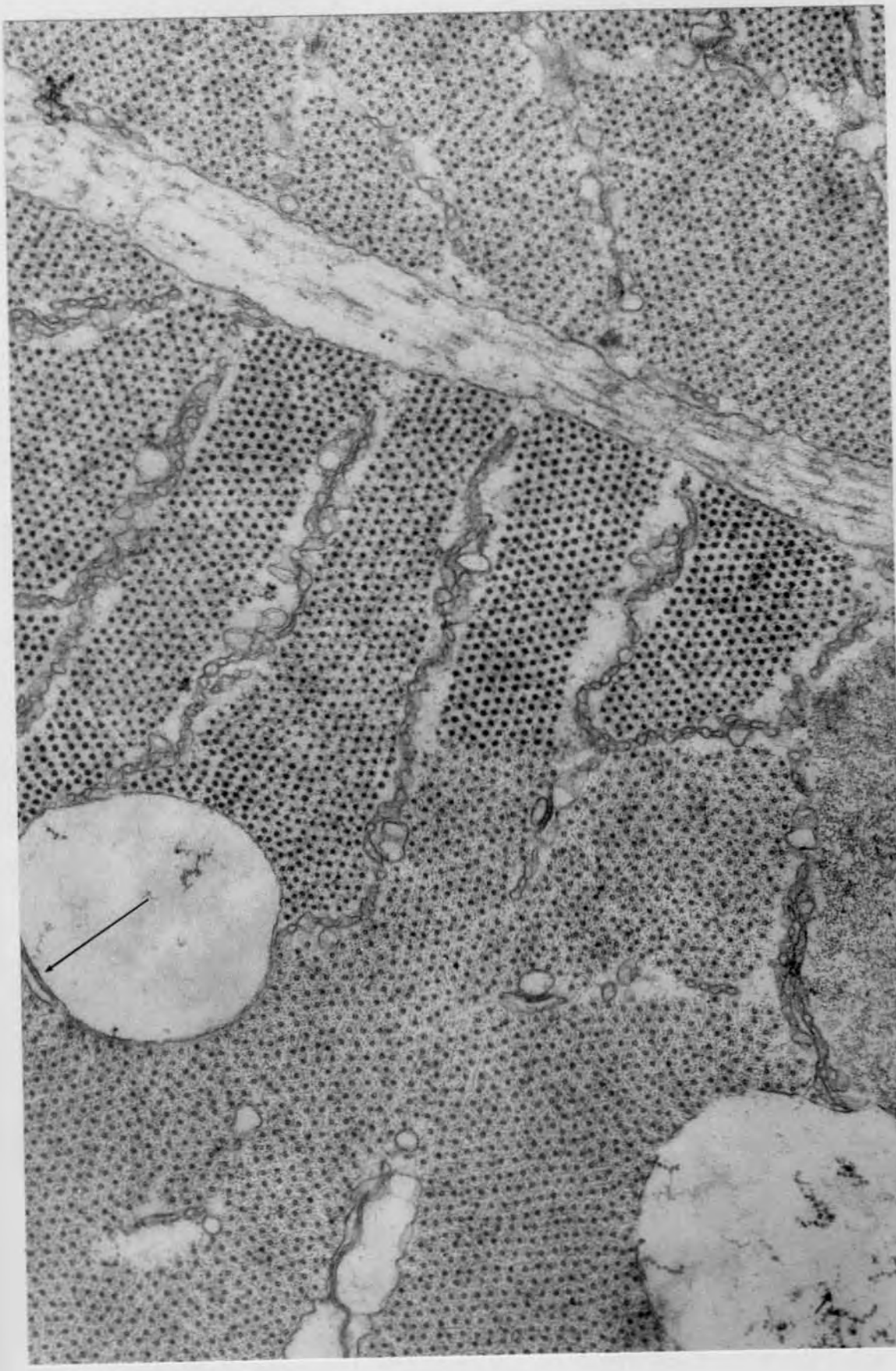
a
elles
e of
he
he
ules.

Figure 47. Carcinus twitch fiber, cross section with same treatment as in Figure 46, but at higher magnification to illustrate the vesiculated tubules more clearly. Magnification X 24,635.

sive system of sarcolemmal invaginations. The membrane potential changes were accompanied by a slight shrinkage of the fiber as Chloride was redistributed, and a transient swelling when fibers were replaced in a normal Chloride Ringer.

The internal Chloride concentration was increased by soaking the fibers in a 120 mm KCl Ringer until their rate of swelling had stabilized. They were then transferred to a normal Ringer or to a low Chloride Ringer. Changes in diameter and optical density could be observed through a microscope and photographed at any stage. The gradual darkening of the fiber occurred at about the same rate as described by Reuben, et al., for crayfish. Fibers were fixed for electron microscopy at various stages of darkening. When sections were examined in the light microscope, the fiber appeared full of small vesicles. The higher resolution of the electron microscope revealed the fact that only the tubular system had undergone swelling, particularly the terminal portions of this system (Figures 46, 47). The extent of tubular swelling could be correlated with the stage of darkening the fiber was in at the time it was fixed. Those which appeared very dark and grainy in the dissection microscope showed almost complete vesiculation of the terminal tubules in the electron microscope (Figures 47, 48). Those which were fixed in earlier stages of darkening still had intact tubules, but ones which were obviously swollen. In all

Figure 48. Carcinus twitch fiber, cross section. Vesiculation due to 120 KCl reversal process. The tubular openings to the sarcolemmal invagination appear to have sealed off, and vesicles have isolated themselves. A non-swollen cisternae (arrow) is shown still forming a diadic contact. Magnification X 45,384.



cases only the tubular components of the diads were swollen, while the appositional membrane of the cisternae and the cleft between them were identical with control fibers.

Swelling Induced by Inward Currents

Passage of inward current through a KCl filled microelectrode (0.5 to 1 μA for from 10 to 30 minutes), produced a more localized tubular swelling. Darkening of the fiber directly beneath the microelectrode could be observed while the current was being passed. Fibers were again fixed at various stages of darkening for examination of their ultrastructure. The amount of swelling and vesiculation was found to be greatest when sections were made at a point near where the microelectrode had been inserted and became less as sections were made further away from this point (Figures 51, 52). Outward (depolarizing) currents which were below threshold had no effect on the TS morphology no matter how long they were applied. The swellings were identical if the inward currents were applied in one long DC pulse, or in shorter pulses of about 500 msec. duration. Microelectrodes filled with 3M K propionate did not cause swelling of the tubules, nor did longitudinal currents passed through two intracellular electrodes.

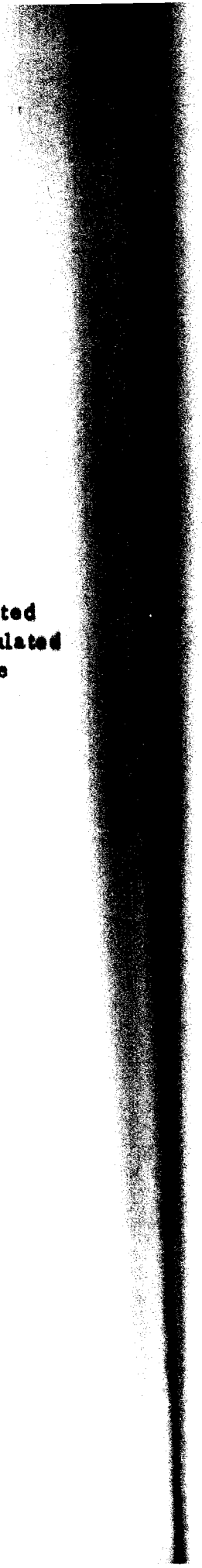


Figure 49. Carcinus twitch fiber, cross section at surface of vesiculated fiber. (120 K reversal). Note not all of the tubules have vesiculated while others have swollen to such an extent that their walls have collapsed. Magnification X 42,358.

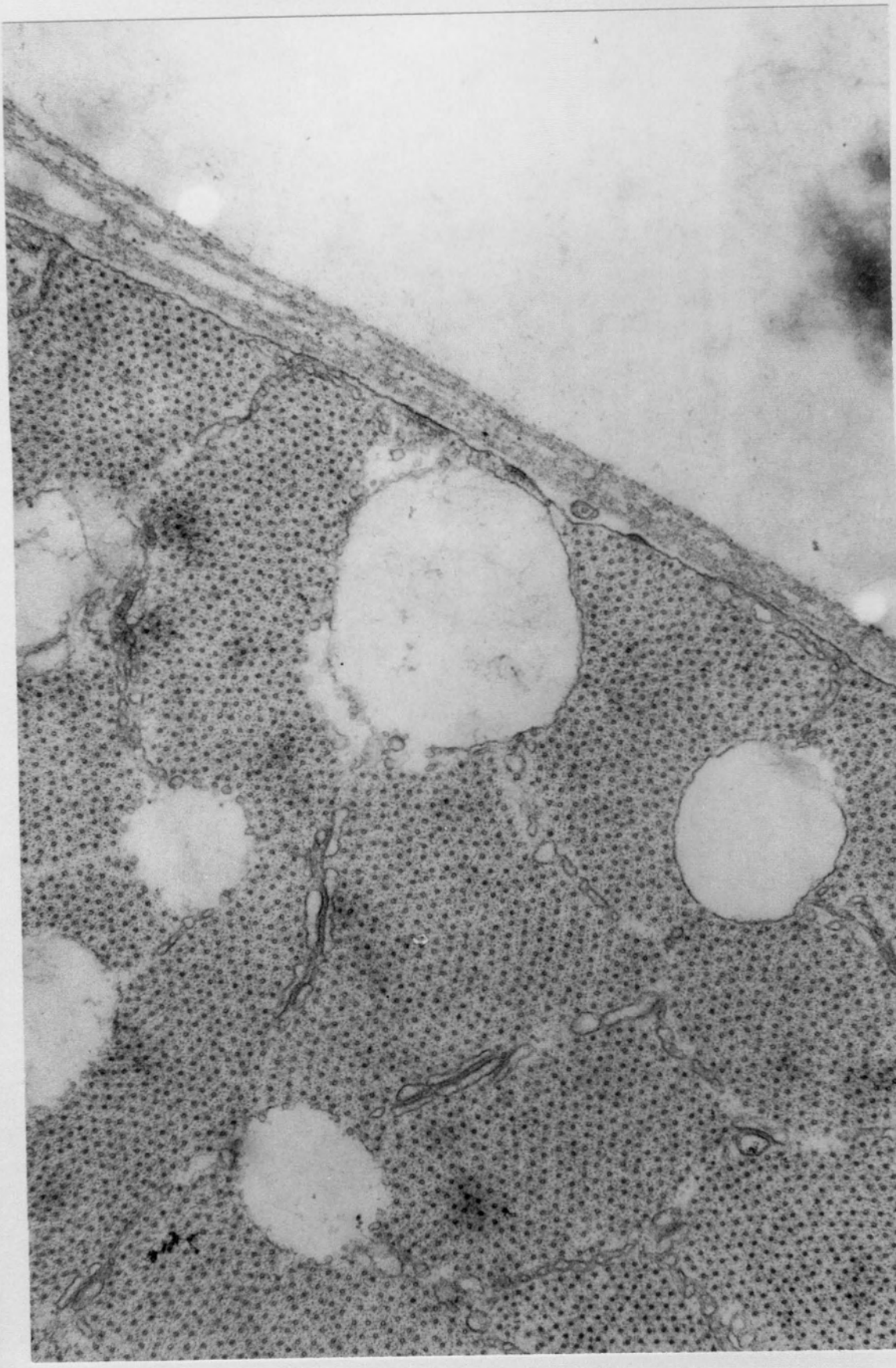


Figure 50. Carcinus twitch fiber, high magnification cross section through a vesicle produced by the 120 KCl reversal process. Note the unswollen sarcoplasmic reticular collar adjoining it. Magnification X 114, 976.

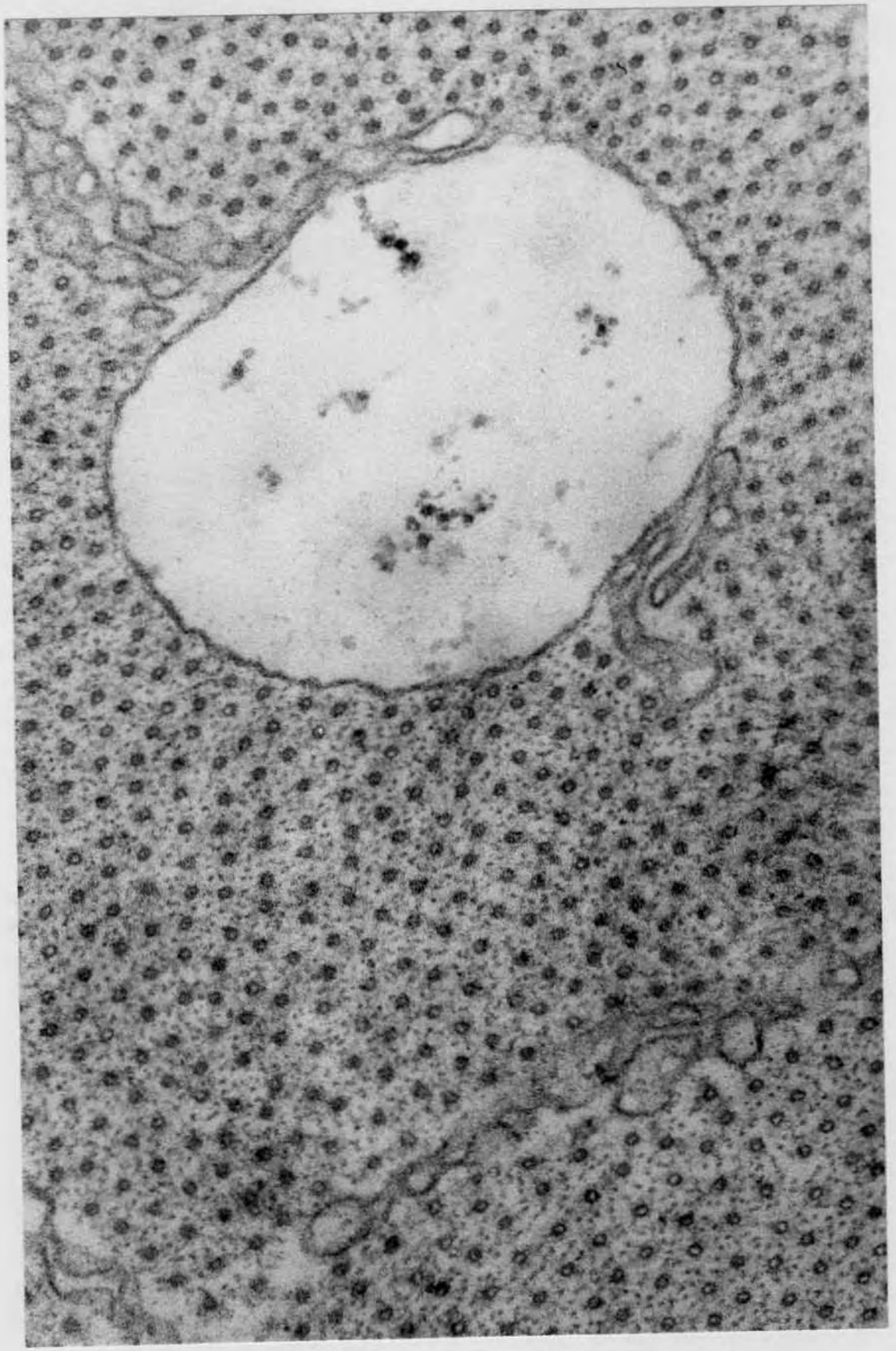
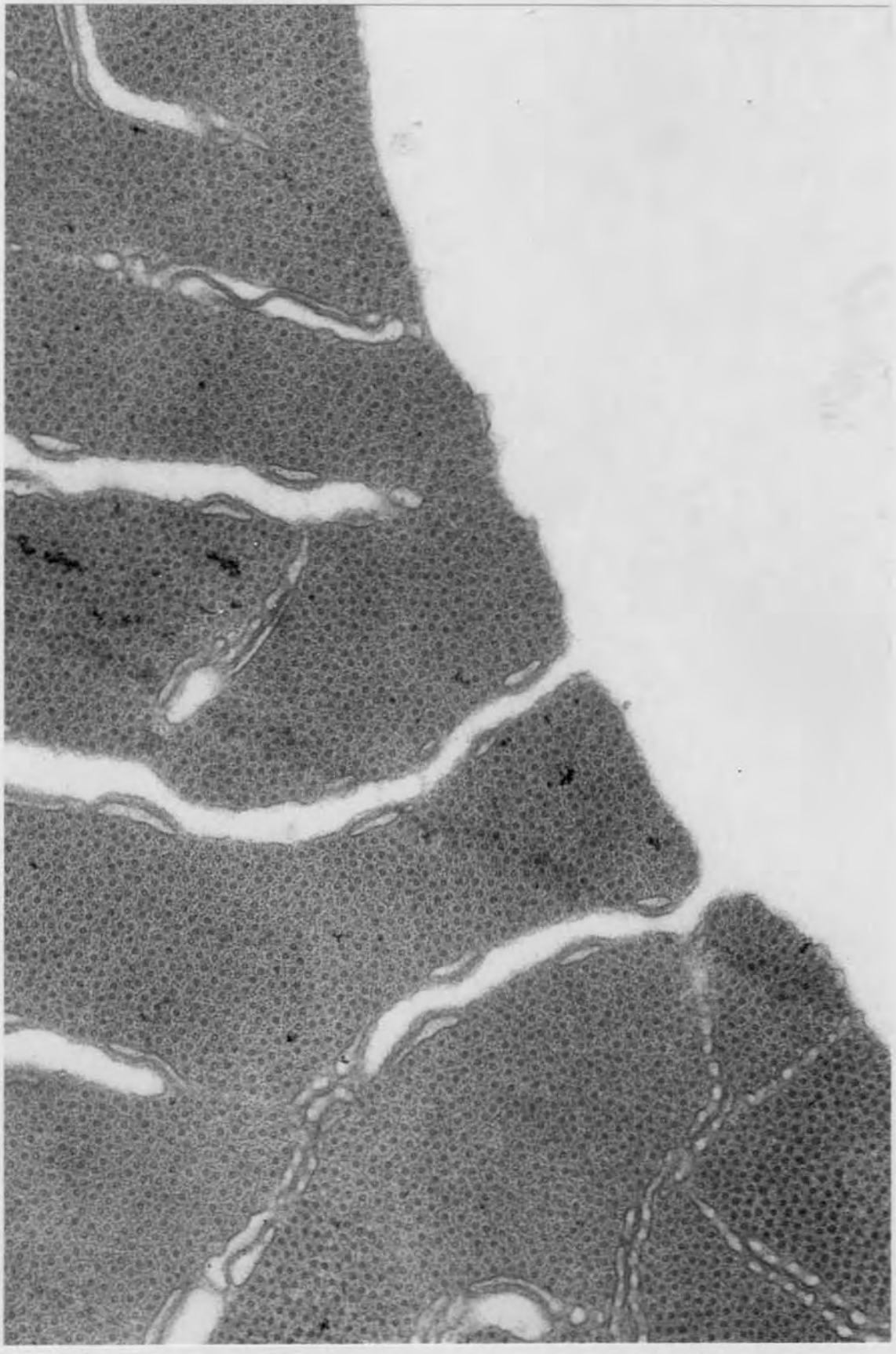


Figure 51. *Carcinus* twitch fiber, cross section. Swollen tubules produced by passage of hyperpolarizing current through a microelectrode approximately 1 mm. from point of section. Large clear area is a sarcolemmal invagination which has swollen itself in this case. Note normal appearance of SR and diads. Compare with Figure 29. Magnification X 76,000.



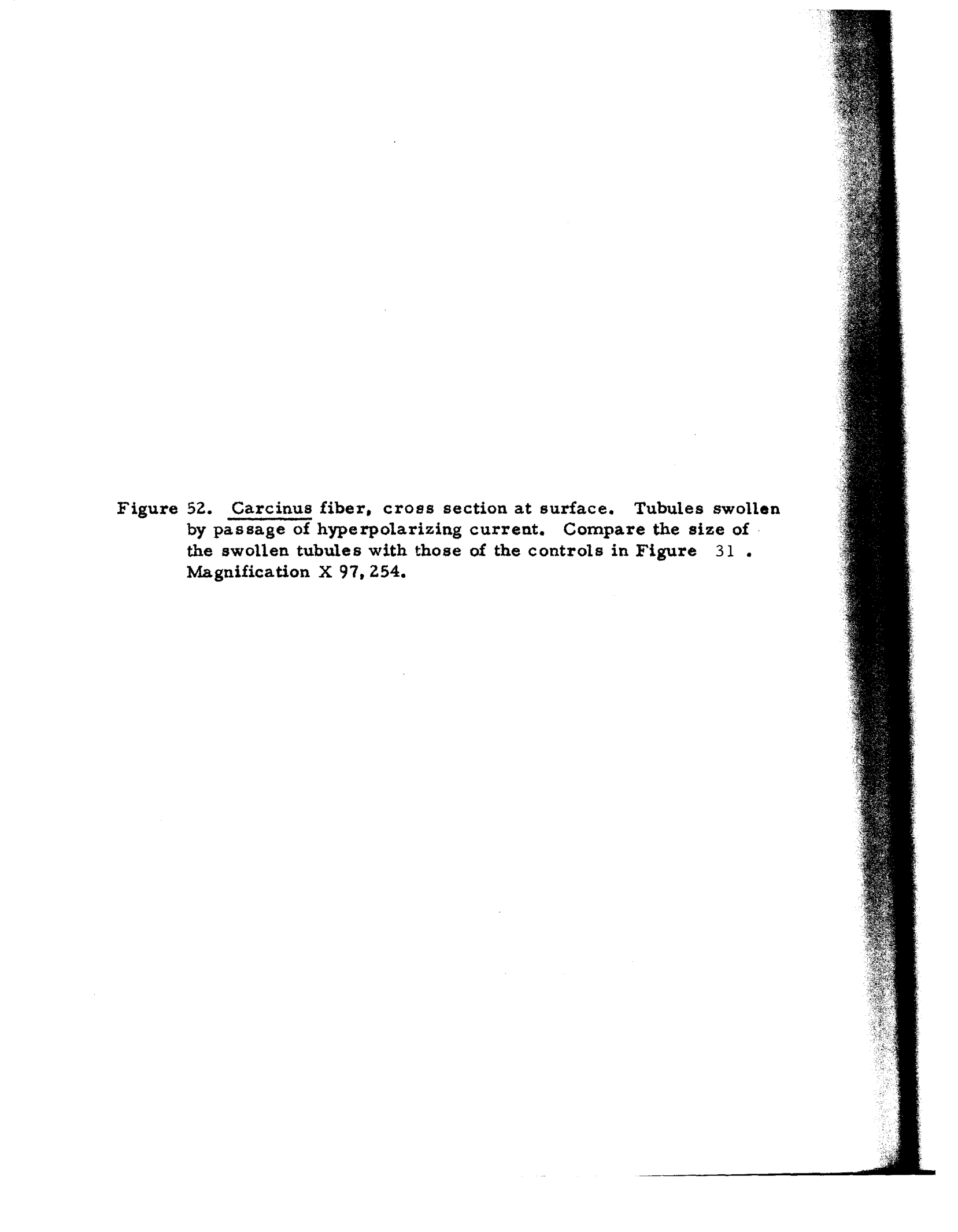


Figure 52. Carcinus fiber, cross section at surface. Tubules swollen by passage of hyperpolarizing current. Compare the size of the swollen tubules with those of the controls in Figure 31. Magnification X 97,254.

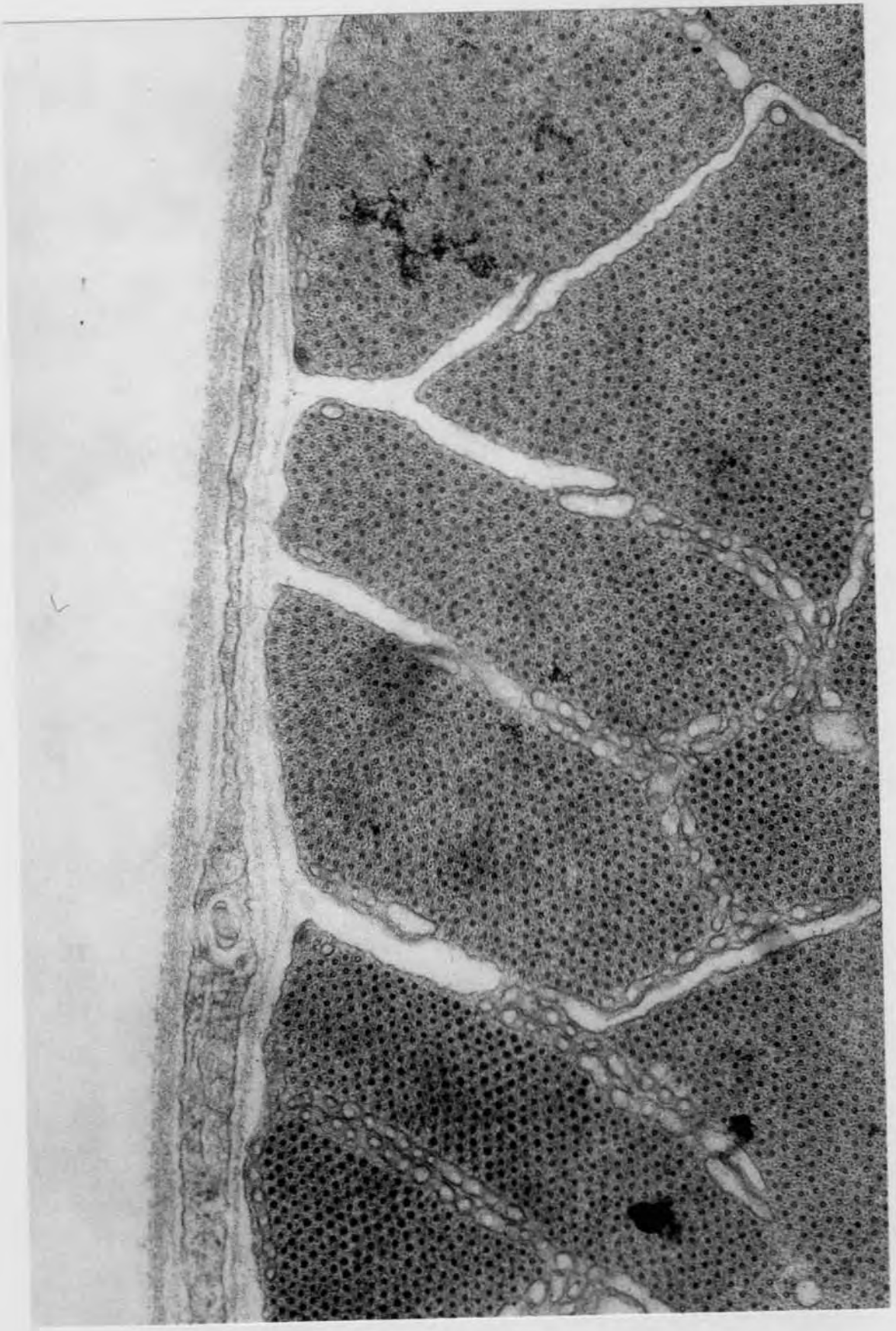
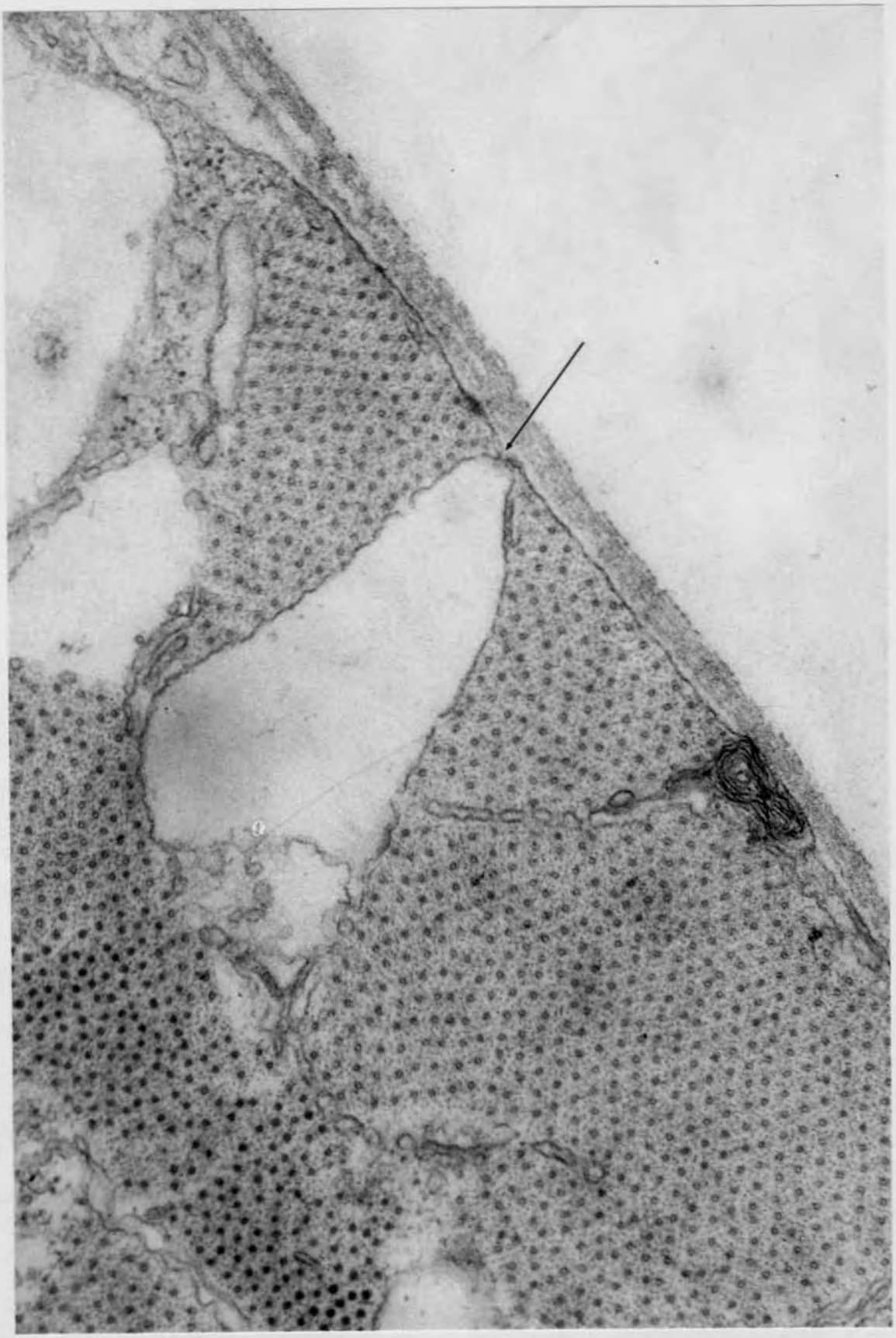


Figure 53. Carcinus fiber, cross section at surface. Vesiculated tubule can still be observed to be connected to the outside (arrow). Magnification X 60,512.



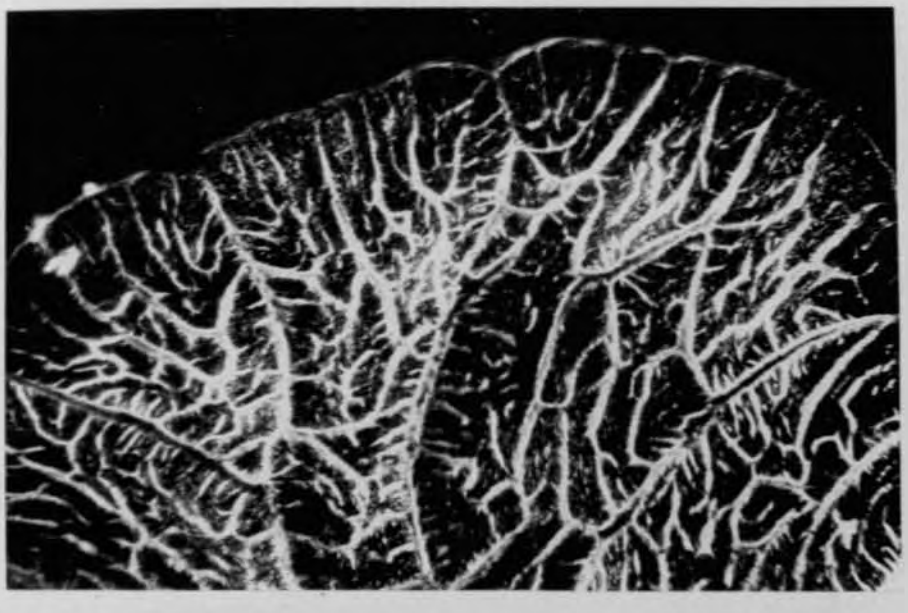
Membrane Capacitance

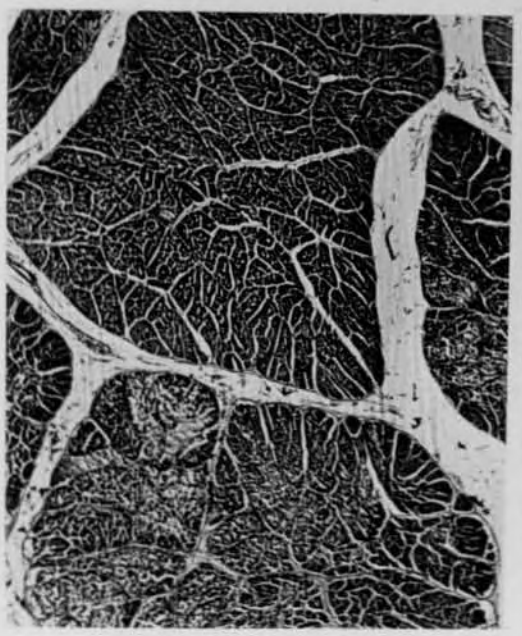
Capacitative values obtained from rectangular pulse analysis (Table 2) are higher than those reported for Balanus by Hagiwara, et al. (1964) by a factor of two and are much more similar to the values reported by Atwood (1963) for slow closer fibers of Carcinus (41.4 $\mu F/cm^2$). The high membrane capacitance of crustacean fibers could result from a folding of the surface membrane inwards, as originally suggested by Fatt and Katz (1963), so that the measured diameter would underestimate the true surface area of the fiber. There are two other possible explanations for the high capacitance of these fibers. It is possible that the unit membrane is considerably thinner than that found in other excitable membranes, or that the lipid composition of the membrane is different enough to increase the dielectric constant. Electron microscopy of crustacean muscle fibers does not support the first idea since the dimensions of the sarcolemmal unit membrane are about the same as those found in squid axon membranes. There have been some reports of high Lethicin concentrations in the sarcolemmal fraction of rabbit muscle (Waku, 1964), but there is no definitive biochemical evidence at present which would support the second suggestion. It is unlikely that even if there were a different lipid composition in the crustacean

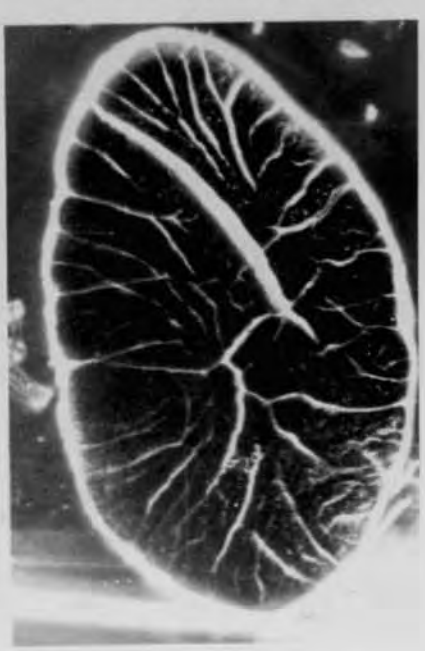
fiber membrane, it would be sufficient to account for such a large increase in apacitance. It is much more probable that this value results from an underestimation of the surface area.

The most common method of determining the diameter of these fibers is to use a calibrated eyepiece micrometer in the dissecting microscope. The crustacean fiber, however, varies from oval to polyhedral or even triangular in shape, so that this estimate at best is a rough approximation. What has never been estimated is the large increase in surface area brought about by infolding of the sarcolemma. These infoldings are clearly visible in the dissecting microscope when the fibers are viewed with the aid of transmitted light. Since they are connected directly to the exterior and are also connected to a large number of tubules, the combination serves to increase the surface area of the fiber many times. In order to correlate the relationship between the sarcolemmal invaginations and the membrane capacitance quantitatively, large cross sections of photomicrographs were utilized (Figure 54). The increase in effective diameter was determined by tracing out the cleft systems of a large number of Balanus depressor fibers with a map measurer. The mean values obtained indicated an increase of 14 times the circumference, or 4.5 times the fiber diameter. The results of employing this factor in the equation for R_m, increases this value and reduces the value of the membrane capacitance to 8 $\mu F/cm^2$, (Table 3).

Figure 54. The increased surface areas due to the sarcolemmal invagination in different crustacean fibers are shown clearly in these light micrographs. The upper fiber is from the Alaskan king crab, Paralithoides, the lower left is from Balanus and the lower right from Carcinus. Because of the shape of the fibers and their large surface areas, the use of fiber diameter in determining electrical constants introduces a large error.







If the increased area of the tubules were also considered in the calculation, the capacitance value would be still lower. Peachey (1965) has estimated this area for the transverse tubular system of the frog fast fiber. The arrangement of tubules in these fibers are of sufficient regularity to permit this approach. The tubular arrangement in both Balanus and Carcinus, however, makes this method of estimation extremely difficult. As can be seen in the electronmicrographs of both fibers, the size of the myofibrils (Figures 12, 26) as well as the arrangement of the tubular system (Figure 24B) is extremely variable.

The membrane capacitance determined by the square wave method corresponds to a frequency of 8-10 cps. It has been known for some time that the capacitance can be measured at a higher frequency by utilizing the foot of the propagated action potential (C_{AP}) (Tasaki and Hagiwara, 1957). Since this was not possible for Balanus, whose fibers never show propagated activity, the capacitance, measured by both the rectangular pulse method and the action potential foot method was determined for twitch fibers of Carcinus maenas. As can be seen in Figure 54, these fibers also have an extensively developed cleft system, which increases its effective diameter about 3 times. The values which were obtained are shown in Tables 4 and 5 before correction for the clefts and in Table 6 and 7 after the correction had been made. The capacitance calculated from square wave analysis (corre-

TABLE 2

MEMBRANE PROPERTIES OF BALANUS DEPRESSOR FIBERS (SIMPLE DIAMETER USED IN CALCULATIONS)

Fiber No.	Diameter (mm.)	λ (mm.)	R_m (Ωcm^2)	R_i (Ωcm)	τ_m (msec.)	C_m ($\mu F/cm^2$)
4136 A	1.6	2.4	2410	168	95.8	40
B	1.3	4.5	2820	450	23.6	8.3
C	.8	4.8	2410	210	40	16.5
D	1.2	3.4	2660	690	106	40
E	1.2	3.0	2260	750	85.7	38
F	1.2	3.5	2640	650	100	38
G	1.0	3.3	1550	356	88	57.5
H	.8	4.2	1900	116	126	65
I	.8	2.7	3400	96	76	22.4
Mean	-	3.5	2198	387	82	36.2

TABLE 3

MEMBRANE PROPERTIES OF BALANUS DEPRESSOR FIBERS (INCREASED AREA OF CLEFTS USED IN CALCULATIONS)

Fiber No.	Diameter (mm.)	λ (mm.)	R_m (Ωcm^2)	R_i (Ωcm)	τ_m (msec.)	C_m ($\mu F/cm^2$)
4136 A	1.6	2.4	10,845	766	95.8	8.9
B	1.3	4.5	12.690	2025	23.6	1.8
C	.8	4.8	10,845	945	40	3.6
D	1.2	3.4	11,970	3105	106	8.9
E	1.2	3.0	10,170	3375	85.7	8.4
F	1.2	3.5	11,880	2925	100	8.4
G	1.0	3.3	6,975	1602	88	12.7
H	0.8	4.2	8,550	5220	126	14.4
I	0.8	2.7	15,300	4320	76	4.9
Mean	-	-	11,025	2698	82	8.0

TABLE 4

MEMBRANE PROPERTIES OF CARCINUS TWITCH FIBERS (SIMPLE DIAMETER USED IN CALCULATIONS)

Fiber No.	Diameter (mm.)	λ (mm.)	R_m (Ωcm^2)	R_i (Ω cm)	τ_m (msec.)	C_m ($\mu F/cm^2$)
836A	.53	.59	4400	167	1.3	29.7
836B	.38	.82	2920	414	1.3	43.8
846A	.41	.55	4835	163	2.2	45.0
846B	.30	.48	4220	137	3.5	32.0
846C	.38	.61	4655	119	2.7	58.0
846D	.41	.43	4645	369	2.2	47.0
Mean	.40	.58	4279	228	2.2	50.0

TABLE 5

MEMBRANE CAPACITANCE OF CARCINUS TWITCH FIBERS DETERMINED FROM FOOT OF ACTION POTENTIAL (SIMPLE DIAMETER USED IN CALCULATIONS)

Fiber No.	Diameter (mm.)	θ (M/sec)	τ_{AP} (msec.)	C_{AP} ($\mu F/cm^2$)
836A	.53	22.7	6.2	10.8
836B	.38	19.7	5.5	5.3
846A	.41	27.6	4.7	6.8
846B	.30	16.9	5.0	7.2
846C	.38	18.5	5.3	2.1
846D	.41	33.0	5.3	2.3
Mean	.40	23.0	5.3	5.7

TABLE 6

MEMBRANE PROPERTIES OF CARCINUS TWITCH FIBERS (INCREASED AREA OF CLEFTS USED IN CALCULATIONS)

Fiber No.	Diameter (mm.)	λ (mm.)	R_m (Ωcm^2)	R_i (Ω cm)	τ_m (msec.)	C_m ($\mu F/cm^2$)
836A	.53	.59	13,200	501	1.3	9.9
836B	.38	.82	8,760	1242	1.3	14.6
846A	.41	.55	14,505	489	2.2	15.0
846B	.30	.48	12,660	411	3.5	27.3
846C	.38	.61	13,965	357	2.7	19.3
846D	.41	.43	13,935	1107	2.2	15.6
Mean	.42	.58	12,837	684	2.3	14.7

TABLE 7

MEMBRANE CAPACITANCE OF CARCINUS TWITCH FIBER DETERMINED FROM FOOT OF ACTION POTENTIAL (INCREASED AREA OF CLEFTS USED IN CALCULATIONS)

Fiber No.	Radius (mm)	Area (mm^2)	τ_{AP} (msec.)	C_{AP} ($\mu F/cm^2$)
7206	.26	.021	5.2	1.1
7316	.23	.016	5.5	1.8
836A	.26	.021	6.2	1.2
836B	.19	.011	5.5	1.7
846A	.20	.012	4.7	.8
846B	.15	.007	5.0	2.8
846C	.19	.011	5.3	1.7
846D	.20	.012	5.3	1.2
Mean	.21	.013	5.3	1.5

sponding to a frequency of 8 cps) was 50.0 $\mu F/cm^2$ when the fiber was treated as a cylindrical core conductor cable, and 14.7 $\mu F/cm^2$ when the added surface area of the clefts was considered in the calculations. The capacitance calculated from the foot of the action potential (300-600 cps) and representing the capacitative filling of only that membrane which supports the action potential was 5.7 $\mu F/cm^2$ for a perfectly cylindrical fiber and 1.5 $\mu F/cm^2$ when the cleft area was considered. Once again the values do not include the area of the tubular walls. The corrected AP foot value of 1.5 $\mu F/cm^2$ can be compared with the 9 $\mu F/cm^2$ found by Eisenberg (1966) for Carcinus fibers determined from AC impedance measurements. His calculations assumed the fibers to be perfect cylinders however. If we assume that the action potential foot fills the capacitance of the walls of the surface membrane and clefts only, and I shall present evidence for this later, then the CAP, after correction for the increased cleft area would approach very closely the true membrane capacitance of the fiber.

Effect of Tubular Swelling on Capacitance

Fibers in which the tubular system had been swollen by either of the methods previously described were still able to support propagated action potentials. Membrane capacitance values determined from the

foot of the action potential were identical with control fibers, 1.5 $\mu F/cm^2$. Values for capacitance determined by cable analysis increased from 14.7 $\mu F/cm^2$ to 70 $\mu F/cm^2$, an approximate five fold increase. The amount of swelling which was induced could be correlated with increases in capacitance, small amounts of swelling produced smaller increases in capacitance. The values which were obtained can be compared to the increase in tubular capacitance C_e, described by Freygang (1966). Utilizing AC impedance measurements which did not allow potential differences in the microelectrodes and therefore minimized tip capacitance errors, he found the capacitance of swollen tubules increased approximately 6.2 times while the membrane capacitance showed no significant difference from the controls.

Discussion -- Permeability of Tubular System Membrane

The findings of Girardier, et al., of an anion-permselective membrane in the tubular system of crayfish graded fibers can now be extended to the phasic muscle fibers of the crab. Since Cl is the principal intracellular permeable anion, it is probably also the ion selectively permeating the TS membrane. The electron micrographs leave no doubt as to the precise location of the swellings and vesiculations. This is best demonstrated in fibers which have been fixed after passage of a local hyperpolarizing current. Since the current flow decreases ex-

ponentially away from the microelectrode, sections directly beneath the microelectrode show the greatest amount of vesiculation, while those taken more distally show the least amount. In these peripheral areas, the tubular system is only slightly swollen. This can be seen in tubules which open to the sarcolemmal clefts as well as those which are open directly to the external surface of the fiber. Sections taken closer to the microelectrode indicate a much greater increase in the extent of swelling and also some vesiculation of the tubules. These vesicles are much larger and more prominent directly beneath the microelectrode. It appears as if the greatest amount of vesiculation occurs at the segment of the tubule closest to the diad, but they can also be observed close to the clefts and directly beneath the sarcolemma.

As in the case of crayfish fibers, the electrophysiological evidence provides clear proof that the TS membrane is permeable to Cl. Only outward currents cause swelling, and the lumens of the tubular system have been shown to be connected directly to the external solution. When propionate was substituted for Cl in the current-passing microelectrode, no swelling occurred. The structural arrangement would allow current to flow between the sarcoplasm and the exterior of the fiber through the walls of the TS. The efflux of Cl under conditions in which there is no change in osmolarity, requires that there

be a potential across the tubular membrane. Since K and Cl activities are determined by a Donnan equilibrium, the potential should be E_{Cl} and very close to the resting potential. As in crayfish, the inhibitory post-synaptic potentials which reflect a Cl activation are very small (Florey and Hoyle, 1963).

The equivalent circuit postulated by Falk and Fatt (1964) to account for their impedance-locus data requires the tubular resistance to be in series with the tubular capacitance. In crab, however, the response of the TS to direct current and electrochemical driving forces would make such a barrier to steady ionic efflux highly unlikely.

The circuit for Carcinus twitch fibers is represented in Figure 55. The plasma membrane is composed of the conventional resistance and capacitance in parallel. In parallel with this component is the parallel resistance and capacitance of the TTS which are in series with the resistance of the tubular lumen.

Mechanism of Tubular System Swelling

The mechanism which causes swelling is essentially the same as has been described by Girardier, et al. (1963) for crayfish fibers. In the resting condition, the potential across the tubular membrane would be close to the equilibrium potential for Cl, while the potential

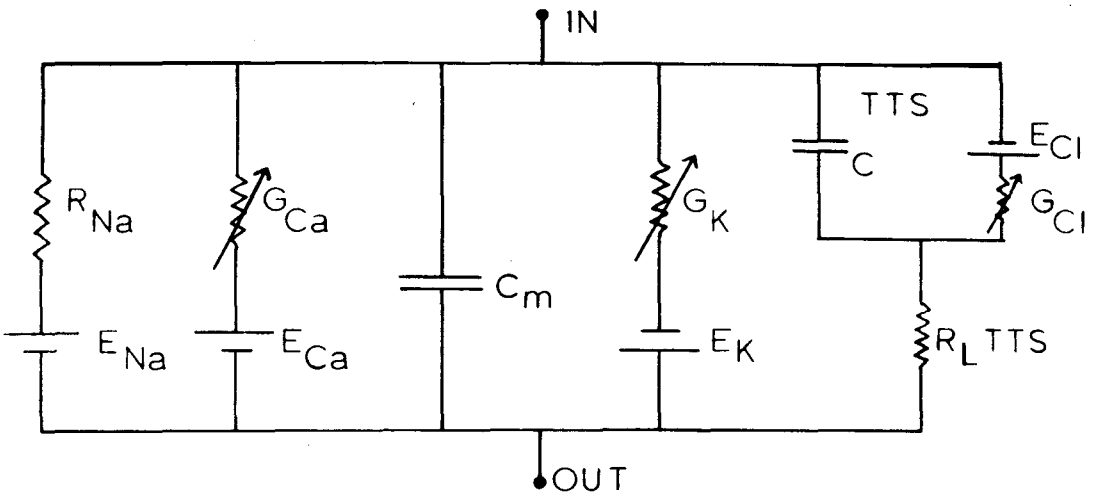


Figure 55. Equivalent circuit for crab twitch muscle fiber. E_{Ca}, E_K, and E_{Cl} represent ionic batteries for the sarcolemma and TS membranes. G_{Ca} and G_K represent the variable resistances of the plasma membrane. R_mTS and R_LTTS are the resistances of the tubular membrane and lumen respectively. C_m is the capacitance of the plasma membrane and C_{TTS} is the capacitance across the tubular walls.

across the plasma membrane would be close to the K equilibrium potential. If fibers which have had their internal KCl concentrations increased are subjected to a sudden decrease in the external Cl concentration, the tubular membrane, but not the plasma membrane, will be depolarized. Because the membranes are separated, current flows between them are carried mainly by efflux of Cl across the tubular wall. The accumulation of Cl within the tubular lumen causes water to enter

osmotically and swelling of the tubules occurs. Similarly, increasing the internal Cl concentration by passage of inward current produces an efflux of Cl from the sarcoplasm which accumulates salt within the tubules. The necessary and sufficient condition to induce swelling, whether by changing the ionic concentrations or by applied current, is the movement of Cl from the sarcoplasm into the tubules. In crayfish, however, it seemed that the TTS membrane was distinct from the membrane of the radial tubules, i.e., those originating at the surface, in terms of Cl permeability. This distinction does not seem to be a valid one for crab, where all tubules show some degree of swelling. One possibility is that water entering the tubules through osmotic forces may not reflect a Cl permeability for all parts of the tubule membrane. However, the uniformity of swelling indicates that the entire tubular system is Cl permselective. Another possibility is that there is an electroosmotic water movement because of the presence of fixed charges (Hober, 1945). This mechanism holds that the water will also follow the movement of Cl into the tubules.

Role of the TTS in E-C Coupling

On the basis of a Cl-permselective tubular membrane, Girardier (1963) has suggested a mechanism by which excitation could be trans-

ferred from the cell surface to the contractile proteins. Figure 56 shows diagramatically the electrical basis for this hypothesis.

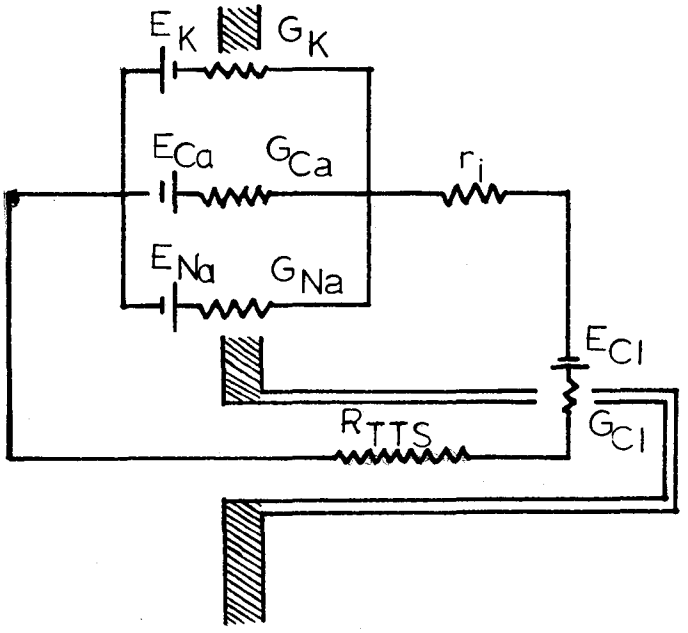


Figure 56. Diagram of plasma membrane and tubular system explaining Girardier's hypothesis of E-C coupling. Tubular walls are open and plasma membrane walls are hatched. The electrical equivalent of the two membrane complex is shown. The explanation is in text.

The surface membrane consists of a sodium, potassium and calcium battery (see Figure 55), associated with series variable resistances to account for conductance changes during excitation. At rest, the e. m. f. across the plasma membrane is due to the K battery and the potential is approximately equal and opposite to the Cl battery e. m. f. of the tubule membrane. The membrane of the tubule contributes a small resistance component and the lumen of the tubule, because of its small diameter also can be represented by a resistance. The circuit

is completed by another resistance r_i, the value of which is a function of the sarcoplasmic resistance.

During activity, depolarization of the fiber caused by increased Ca conductance makes the plasma membrane inside positive, but because the tubule membrane is Cl-selective, its e.m.f. does not change. The imbalance in potentials would produce a current between the plasma membrane and the tubule. The current across the tubule membrane would be carried primarily by influx of Cl ions and would account for a shunting of most of the action current inward. By this mechanism, calcium might be accumulated along the tubule walls, or the current might be responsible for release of calcium from the cisterna.

Girardier developed this mechanism on the assumption that the tubules were open to the outside without direct morphological evidence that this was the case. In addition, he postulated only the terminal portions of the tubule system were permselective to chloride since it appeared that only this segment of the tubule underwent swelling in response to efflux of chloride from the fiber.

The ultrastructure of Carcinus fibers indicates that both the tubules originating at the surface of the fiber as well as those arising from the sarcolemmal invaginations are open to the outside and therefore are able to serve as a current pathway. There is no separation of radial and transverse tubules, but instead all tubules appear equal in terms of Cl permeability.

It has been noted by A. F. Huxley and his colleagues that the inward spread of activation involves the transition of an all-or-none action current to one which is graded in nature (Huxley, 1964). The large number of cisternal elements which form diad-like contacts with the plasma membrane, raises the possibility of a direct influence of the action-potential on the release of calcium from the SR. On the basis of impedance-locus measurements of Carcinus fibers, it would seem that the largest portion of the current is carried by the tubular system (Eisenberg, 1965), so that the influence of the spike may be minimal.

Swelling of the TTS in frog muscle has also been described by Foulks, et al. (1965) in response to Cl efflux from the sarcoplasm into the tubules. The contracture tension developed in these fibers due to Cl withdrawal was very similar to caffeine induced contractures in which the site of drug action is presumed to be intracellular, possibly at the tubule.

The presence of a tubular system permeable to Cl and a high inward K conductance across the plasma membrane during excitation allows a current flow to be established for the efficient inward spread of excitation. A current of this type is suggested by studies of procaine induced spikes in crayfish (Garcia, et al., 1966). When the spikes are produced in the absence of Cl, tension production is very low, as is the membrane conductance. With Cl present, however, there is little change in the shape of the spike, but tension and conductance both increase.

Membrane Capacitance

The apparent separation of tubular and membrane capacitance by Falk and Fatt has provided considerable impetus to the investigation of muscle fiber capacitance. If indeed the greatest amount of the capacitance is located in the tubular system, it is easy to explain the high values which have been obtained in crustacean fibers. In addition it becomes possible to account for a large number of membrane responses such as different p. s. p. decay times and the effects of fast and slow rise times of p. s. p. 's on the rates of tension development. Falk and Fatt have postulated a mechanism of excitation-contraction coupling based on the discharge of the tubular capacitance by a short-circuiting of the tubular lumen by the action potential.

It is quite clear from the values obtained for Balanus fiber capacitance by square pulse analysis (Table 2), that the high values are obtained by underestimating the true surface area of the fiber. Furthermore, separation of the tubular and membrane capacitance, which have time constants of comparable magnitude, would be impossible to observe by transient response methods which are obscured by capacitative artifacts. It is also obvious that the measured value of capacitance is dependent on the frequency of the signal used to measure it. By comparing cable analysis values with those obtained from analysis of the foot of the

propagated action potential, one can achieve a rough separation of tubular and membrane capacitance values.

Since there is no change in surface membrane capacitance when the tubules are swollen, it is reasonable to assume that the action potential fills only the capacitance of the surface and cleft membranes. Because the cleft area can be estimated with some degree of accuracy by direct mapping, the corrected values of capacitance determined by AP foot measurements are the most accurate.

It was originally hoped that the capacitance of slow and fast crustacean fibers could be correlated with the relative development of the tubular system. This has been investigated in frog tonic and phasic fibers by Adrian and Peachey (1965). Although not rigorously quantifiable, they found the capacity was about three times as large in the fast fibers as in the slow ones, and they described the fast fiber tubular system as being much better developed than in the slow fiber.

It appears, in comparing Balanus and Carcinus fibers, that the extent of increase in surface area due to infolding of the surface membrane contributed more to the values of capacitance than did the development of the tubular system. The tubular system in both fibers was extensive, but difficult to measure by mapping because the tubules have variable lengths and irregular courses. If one uses Peachey's estimate for frog fibers in that they increase the surface area about seven times (Peachey,

1965), then values of about 2 $\mu F/cm^2$ are obtained for both Balanus and Carcinus by square wave analysis.

The membrane model suggested by the tubular permeability data (Figure 55) could very likely produce the dispersions described by Falk and Fatt (1964) for crayfish fibers, and still allow for passage of currents during activation.

Conclusions

The ultrastructural analysis of both Balanus and Carcinus fibers suggests that the pathway for the inward spread of activation occurs via the cleft and tubular systems. The neuromuscular properties of Balanus and Carcinus fibers are those of tonic and phasic fibers respectively. While the fibers are conveniently separated physiologically, their ultrastructure indicates features common to both. The correlation between the development of the sarcoplasmic reticulum and the tubular system with the speed of contraction and relaxation does not appear nearly as definitive as in vertebrate muscle. The location of spacially separated membranes with different permeabilities, allows the establishment of a current loop between the inside and outside of the fiber which can carry current inward, linking surface depolarization to release of Ca at the diad.

As long as much of the fiber capacitance is located within the

tubular membranes, one cannot rule out the possibility of its discharge contributing to the inward spread of excitation. The final process in the coupling link is the increase in calcium ion concentration within the sarcoplasm. Whether the calcium influx during the graded p.s.p. or propagated spike is sufficient to raise the calcium concentration to 7×10^{-7}M, which Hagiwara (1966) calculates is necessary to cause tension development, remains unexplored. It may be that a combination of "membrane" calcium and SR calcium are required to trigger contraction. The recent techniques of Jobsis and O'Connor (1966), which permit the analysis of calcium kinetics with respect to tension development, may provide an answer for crustacean fibers.

Finally, when a careful examination is made of all the factors involved in differentiating tonic and phasic fibers, and how any of these may influence E-C coupling, one may realise that an almost infinite number of combinations and gradations exist. The blending of membrane conductance properties, structural and chemical differences and passive electrical properties which determine the final "classification" of a muscle fiber will occupy comparative physiologists for many years.

BIBLIOGRAPHY

Abbott, B. C. and Parnas, I., 1965. Electrical and mechanical responses in deep abdominal extensor muscles of crayfish and lobster. J. Gen. Physiol., 48:919.

Adrian, R. H. and Freygang, W. H., 1962, Potassium conductance of frog muscle membrane under controlled voltage, J. Physiol., 163:104.

Adrian, R. H., 1963, The replacement of potassium by rubidium in striated muscle fibers, J. Physiol., 169:16P.

Adrian, R. H., 1964, Membrane properties of striated muscle and the initiation of contraction, In: The Cellular Functions of Membrane Transport, J. F. Hoffman, Ed., Prentice-Hall, N. J.

Adrian, R. H. and Peachey, L. D., 1965, The membrane capacity of frog twitch and slow muscle fibers, J. Physiol., 181:324.

Anderson, E., 1957, The tubular system in the striated muscle, In: Proceedings of the Stockholm Conference on Electron Microscopy, F. S. Sjostrand and J. Rhodin, Eds., Acad. Press, Inc., N. Y.

Anderson-Cedergren, E., 1959, Ultrastructure of motor plate and sarcoplasmic reticulum components of mouse skeletal muscle fiber as revealed by three-dimensional reconstruction from serial sections, J. Ultrastr. Res., Suppl. 1; 1.

Atwood, H. L. and Dorai Raj, B. S., 1964, Tension development and membrane responses in phasic and tonic muscle fibers of a crab, J. Cell. Comp. Physiol., 64:55.

Atwood, H. L., 1965, Characteristics of fibers in the extensor muscle of a crab, Comp. Biochem. Physiol., 14:205.

Atwood, H. L., Hoyle, G. and Smyth, T., 1965, Mechanical and electrical responses of single innervated crab muscle fibers, J. Physiol., 180:449.

Auber, J. and Couteaux, R., 1963, Ultrastructure de la strie Z dans des muscles de dipteres, J. De Micr., 2:309.

Auber, J., 1966, Myofibrils of insects, Symposium on the Comparative Aspects of Muscle, A.A.A.S. Meeting, Washington, D.C.

Bach-y-Rita, P. and Ito, F., 1966, In vivo studies on fast and slow muscle fibers in the cat extraoccular muscles, J. Gen. Physiol., 49:1177.

Bennett, H. S., 1953, The structure of striated muscle as seen in the electron microscope, In: Structure and Function of Muscle, G. Bourne, Ed., Acad. Press, Inc., N. Y.

Bennett, H. S. and Porter, K. R., 1953, An electron microscopic study of sectioned breast muscle of the domestic fowl, Am. J. Anat., 93:61.

Bennett, H. S., 1955, Modern concepts of structure of striated muscle, Am. J. Phys. Med., 34:46.

Bouligand, Y., 1963, Les ultrastructures musculaires des copepodes. II Membrane sarcoplasmique, reticulum sarcoplasmique et jonction neuromusculaire chez les cyclops, J. Micr., 2:197.

Bowman, W., 1840, On the minute structure and movements of voluntary muscle, Phil. Tr. Roy. Soc., London, 130:457.

Brandt, P. W., Girardier, L., Reuben, J. P., and Grundfest, H., 1965, Correlated morphological and physiological studies on isolated single muscle fibers, J. Cell. Biol., 25:233.

Butschli, O. and Schewiakoff, W., 1891, Uber den feineren bau der quergestreiften muskeln von arthropoden, Biol. Centr., 11, 33.

Carnoy, J. B., 1884, La biologie cellulaire, etude comparee de la cellule dans les deux regnes, Lierre, Van In, pp. 189.

Cajal, S. R., 1888, Observations sur la texture des fibres musculaires des pattes et des ailes des insects, Internat. Monatschr., Anat. u. Physiol., 5:205.

Cohen, M. J., 1963, Muscle fibers and efferent nerves in a crustacean receptor muscle, Quart. J. Micr. Sci., 104:551.

Constantin, L. L., 1965, Calcium localization and the activation of striated muscle fibers, Fed. Proc., 24:1141.

Crisp, D. J. and Southward, A. J., 1961, Different types of cirral activity of barnacles, Phil. Trans. Roy. Soc., London, Ser. B., 243:271.

Dorai Raj, B. S., 1964, Diversity of crab muscle fibers innervated by a single motor axon, J. Cell. Comp. Physiol., 64:41.

Edwards, C., Chichibu, S., and S. Hagiwara, 1964, Relation between membrane potential changes and tension in barnacle muscle fibers, J. Gen. Physiol., 48:225.

Edwards, G. A., Ruska, H., Souza-Santos, P. and Vallejo-Freire, A., 1956, Comparative cytophysiology of striated muscle with special reference to the role of the endoplasmic reticulum, J. Biophys. Cytol., 10:89.

Edwards, G. A., Ruska, H. and E. De Harven, 1958, Neuromuscular junctions in flight and tymbal muscles of the cicada, J. Biophys., Biochem. Cytol., 4:251.

Eisenberg, R., 1965, A. C. Impedance of single muscle fibers, Ph. D. Thesis, University College, London.

Endo, M., 1964, Entry of a dye into the sarcotubular system of muscle, Nature, 202:1115.

Fahrenbach, W. H., 1963, The sarcoplasmic reticulum of a striated muscle of a cyclopoid copepod, J. Cell Biol., 17:629.

Fahrenbach, W. H., 1964, A new configuration of the sarcoplasmic reticulum, J. Cell Biol., 22:477.

Fahrenbach, W. H., 1965, Sarcoplasmic reticulum: ultrastructure of the triadic junction, Science, 147:1308.

Falk, G. and Fatt, P., 1964, Linear electrical properties of striated muscle fibers observed with intracellular electrodes, Proc. Roy, Soc. B., 160:69.

Falk, G. and Fatt, P., 1965, Electrical impedance of striated muscle and its relation to contraction, In: Studies of Physiology, Springer-Verlag, 64-70.

Fatt, P. and Katz, B., 1953, The electrical properties of crustacean muscle fibers, J. Physiol., 120:171.

Fatt, P. and Ginsborg, B. L., 1958, The ionic requirements for the production of action potentials in crustacean muscle fibers, J. Physiol., 142:516.

Fawcett, D. W. and Revel, J. P., 1961, The sarcoplasmic reticulum of a fast acting fish muschel, J. Biophys. Biochem. Cytol., 10:89.

Florey, E. and Hoyle, G., 1961, Neuromuscular synaptic activity in the crab, Cancer magister, In: Nervous Inhibition, E. Florey, Ed., Pergammon Press, N. Y.

Foulks, J. G., Pacey, J. A. and F. A. Perry, 1965, Contractures and swelling of the transverse tubules during chloride withdrawal in frog skeletal muscle, J. Physiol., 180:96.

Fozzard, H. A., 1966, Membrane capacity of the cardiac Purkinje fiber, J. Physiol., 182:255.

Franzini-Armstrong, C., 1963, Pores in the sarcoplasmic reticulum, J. Cell Biol., 19:637.

Franzini-Armstrong, C., and Porter, K. R., 1964a, Sarcolemmal invaginations and the T-system in fish skeletal muscle, Nature, 202:355.

Franzini-Armstrong, C., and Porter, K. R., 1964b, The Z-disc of skeletal muscle fibrils, Z. fur Zellforsch., 61:661.

Freygang, W. J., Jr., Goldstein, D. A., Hellam, D. C., and L. D. Peachey, 1964, The relation between the late after-potential and the size of the transverse tubular system of frog muscle, J. Gen. Physiol., 48:235.

Freygang, W., 1966, Effect of swelling on the TTS on the impedance of frog sartorius muscle, Fed. Proc., 25:332.

Garcia, H., Reuben, J., and P. Brandt, 1966, Spikes and E-C Coupling in crayfish muscle fibers, Fed. Proc., 25:332.

Girardier, L., Reuben, J. P., Brandt, P. W., and Grundfest, H., 1963, Evidence for anion-permselective membrane in crayfish muscle fibers and its possible role in excitation-contraction coupling, J. Gen. Physiol., 47:189.

Hagiwara, S. and Naka, K., 1964, The initiation of spike potential in barnacle muscle fibers under low intracellular Ca., J. Gen. Physiol., 48:141.

Hagiwara, S., and Nakajima, S., 1966, Effects of the intracellular Ca ion concentration upon the excitability of the muscle fiber membrane of a barnacle, J. Gen. Physiol., 49:807.

Hagopian, M., 1966, The myofilament arrangement in the femoral muscle of the cockroach, Leucophaea maderae, Fabricus, J. Cell Biol., 28:545.

Hanson, J., and Lowey, J., 1961, The structure of the muscle fibers in the translucent part of the adductor of the oyster Crassostrea angulata, Proc. Roy. Soc. B., 137:273.

Hasselbach, W., 1964, Relaxation and the sarcotubular calcium pump, Fed. Proc., 23:909.

Hasselbach, W., 1966, Structural and enzymatic properties of the calcium transporting membrane of the sarcoplasmic reticulum, Ann. N. Y. Acad. Sci., 137:1041.

Hess, A., 1965, The sarcoplasmic reticulum, the T-system and the motor terminals of slow and twitch muscle fibers in the garter snake, J. Cell Biol., 26:467.

Hill, A. V., 1948, On the time required for diffusion and its relation to processes in muscle, Proc. Roy. Soc. B., 135:446.

Hill, A. V., 1949, The abrupt transition from rest to activity in muscle, Proc. Roy. Soc. B., 136:399.

Hober, R., 1945, The physical chemistry of cells and tissues, Blakiston Co., Phil., Penn.

Hodge, A., 1955, Studies on the structure of muscle, III Phase contrast and electron microscopy of dipteran flight muscle, J. Biophys. Biochem. Cytol., 1:361.

Hodgkin, A. L. and Rushton, W. A. H., 1946, The electrical constants of a crustacean nerve fiber, Proc. Roy. Soc. B, 133:444.

Hodgkin, A. L. and Huxley, A. F., 1952, A quantitative description of membrane current and its application to conduction and excitation in nerve, J. Physiol., 117:500.

Hodgkin, A. L. and Horowicz, P., 1960a, The effect of sudden changes in ionic concentrations on the membrane potential of single muscle fibers, J. Physiol., 153:386.

Hodgkin, A. L. and Horowicz, P., 1960b, Potassium contractures in single muscle fibers, J. Physiol., 153:385.

Hoyle, G. and Smyth, T., 1963, Neuromuscular physiology of giant muscle fibers of a barnacle, Balanus nubilus, Darwin, Comp. Biochem. Physiol., 10:291.

Hoyle, G. and McAlear, J., 1963, Mechanism of supercontraction in a striated muscle fiber, Science, 141:712.

Hoyle, G., McAlear, J. and A. Selverston, 1965, Mechanism of supercontraction in a striated muscle, J. Cell Biol., 26:621.

Hoyle, G., 1965, Nature of the excitatory sarcoplasmic reticular junction, Science, 149:70.

Hoyle, G., 1966, Unpublished data.

Huxley, A. F. and Taylor, R. E., 1955, Function of Krause's membrane, Nature, 176:1068.

Huxley, A. F. and Straub, R. W., 1958, Local activation and interfibrillar structures in striated muscle, J. Physiol., 143:40.

Huxley, A. F. and Taylor, R. E., 1958, Local activation of striated muscle fibers, J. Physiol., 144:426.

Huxley, A. F. and Peachey, L. D., 1964, Local activation of crab muscle, J. Cell Biol., 23:107A.

Huxley, A. F., 1964, The links between excitation and contraction, Proc. Roy. Soc. B., 160:486.

Huxley, H. E. and Hanson, J., 1957, Preliminary observations on the structure of insect flight muscle, In: Proceedings of the Stockholm Conference on Electron Microscopy, F. S. Sjostrand, Ed., Academic Press, Inc., N. Y.

Huxley, H. E., 1963, Electron microscopic studies on the structure of natural and synthetic protein filaments from striated muscle, J. Mol. Biol., 7:281.

Huxley, H. E., 1964, Evidence for continuity between the central elements of the triads and extracellular space in frog sartorius muscle, Nature, 202:1067.

Jacklett, J., 1966, Unpublished data.

Jenerick, H. P., 1959, Control of membrane ionic currents, J. Gen. Physiol., 42:923.

Jobsis, F. F. and O'Connor, M. J., 1966, Calcium release and reabsorption in the sartorius muscle of the toad, Biochem. Biophys. Res. Comm., 25:246.

Katz, B., 1948, The electrical properties of the muscle fiber membrane, Proc. Roy. Soc. B., 135:506.

Kennedy, D. and Takeda, K., 1965, Reflex control of abdominal flexor muscles in the crayfish. II The tonic system, J. Exp. Biol., 43:229.

Knappeis, G. G. and Carlsen, F., 1962, The ultrastructure of the Z disc in skeletal muscle, J. Cell Biol., 13:323.

Kolliker, A., 1866, Ueber die Cohneim'schen felder der muskelquerschnitte, Z. Wissensch. Zool., 16:374.

Kruger, P., 1949, Die innervation der telanischen und tonischen fasern der quergestreiften skeletmusculatur des wirbeltiere, Anat. Anz., 97, 169.

Kuffler, S. W. and Vaughan Williams, E. M., 1953, Properties of the slow skeletal muscle fibers of the frog, J. Physiol., 121:318.

Moore, D. H. and Ruska, H., 1957, Electron microscope study of mammalian cardiac muscle cells, J. Biophys. Biochem. Cytol., 3:261.

Nakajima, S., Iwasaki, S. and K. Obata, 1962, Delayed rectification and anomalous rectification in frog skeletal muscle membrane, J. Gen. Physiol., 46:97.

Orkand, R. K., 1962, The relation between membrane potential and contraction in single crayfish muscle fibers, J. Physiol., 161:143.

Page, S., 1965, A comparison of the fine structure of frog slow and twitch muscle fibers, J. Cell Biol., 26:477.

Parnas, I. and Atwood, H. L., 1966, Phasic and tonic neuromuscular systems in the abdominal extensor muscles of the crayfish and rock lobster, Comp. Biochem Physiol., 18:701.

Peachey, L. D. and Porter, K. R., 1959, Intracellular impulse conduction in muscle cells, Science, 129:721.

Peachey, L. D., 1961, Structure of the longitudinal body muscles of Amphioxus, J. Biophys. Biochem. Cytol., 10 (Suppl.), 159.

Peachey, L. D. and Huxley, A. F., 1962, Structural identification of twitch and slow striated muscle fibers of the frog, J. Cell Biol., 13:177.

Peachey, L. D. and Huxley, A. F., 1964, Transverse tubules in crab muscles, J. Cell Biol., 23:70A.

Peachey, L. D., 1965, The sarcoplasmic reticulum and transverse tubules of the frog sartorius, J. Cell Biol., 25:209.

Peachey, L. D., 1966, Fine structure of two fiber types in cat extraocular muscles, J. Cell Biol., 31:84A.

Peterson, R. P. and Pepe, F. A., 1961, The relationship of the sarcoplasmic reticulum to sarcolemma in crayfish stretch receptor muscle, Am. J. Anat., 109:277.

Pilar, G. and Hess, A., 1966, Differences in internal structure and nerve terminals of the slow and twitch muscle fibers in the cat superior oblique, Anat. Rec., 154:243.

Porter, K. R., 1956, The sarcoplasmic reticulum of muscle cells of amblystoma larva, J. Biophys. Biochem. Cytol., 2(Suppl):163.

Porter, K. R. and Palade, G. E., 1957, Studies on the endoplasmic reticulum. III Its form and distribution in striated muscle cells, J. Biophys. Biochem. Cytol., 3:269.

Retzius, G., 1881, Zur kenntniss der quergestreiften muskelfaser, Biol. Untersuch., 1:1.

Reuben, J. P., Girardier, L. and H. Grundfest, 1964, Water transfer and cell structure in isolated crayfish muscle fibers, J. Gen. Physiol., 47:1141.

Revel, J. P., 1962, The sarcoplasmic reticulum of the bat cricothyroid muscle, J. Cell Biol., 12:571.

Rollett, A., 1888, Ueber die flossenmüskeln des seepferdchens und uber muskel struktur in allgemeinen, Arch. Mikr. Anat., 32:233.

Ruska, H., 1954, Electronenmikroskopischer beitrag zur histologie des skelettmuskels kleiner sangetiere, Z. Naturforsch., 95:358.

Selverston, A., 1964, Mechanism of contraction in barnacle muscle, Master's thesis, University of Oregon.

Simson, F. O. and Oertelis, S. J., 1962, The fine structure of sheep myocardial cells; sarcolemmal invaginations and the transverse tubular system, J. Cell Biol., 12:91.

Smith, D. S., 1961a, The structure of insect flight muscle. A study made with special reference to the membrane system of the fiber, J. Biophys. Biochem. Cytol., 10 (Suppl):123.

Smith, D. S., 1961b, The organization of the flight muscle in a dragonfly Aeshna sp. (Odonata), J. Biophys. Biochem. Cytol., 11:119.

Smith, D. S., 1965, The organization of flight muscle in an aphid Megoura viciae, J. Cell Biol., 27:379.

Smith, D. S., Gupta, B. L. and Smith, U., 1966, The organization and myofilament array of insect visceral muscle, J. Cell Sci., 1:49.

Smith, D. S., 1966a, The organization of flight muscle fibers in the odonata, J. Cell Biol., 28:109.

Smith, D. S., 1966b, The organization and function of the sarcoplasmic reticulum and T-system of muscle cells, Prog. in Biophys. and Mol. Biol., 16:109.

Tait, J. and Emmons, W. F., 1925, Experiments and observations on crustacea. Part VI The mechanism of massive movement of the operculum of Balanus nubilus, Proc. Roy. Soc. Edinb., 45:42.

Tasaki, I. and Hagiwara, S., 1957, Capacity of muscle fiber membrane, Am. J. Physiol., 188:423.

Toselli, P. A., 1965, The fine structure of the fully developed intersegmental abdominal muscles of Rhodnius procixus, Anat. Rec., 151:427.

Van Gehuchten, A., 1886, Etude sur la structure intime de la cellule musculaire striee, Cellule, 2:293.

Varga, E., Kover, A., Kovacs, T., Jokay, I. and T. Szilagy, 1962, Differentiation of myosins extracted from tonic and tetanic muscles on the basis of their antigenic groups, Acta Physiol. Acad. Sci. Hung., 22:21.

Veratti, E., 1902, Investigations on the fine structure of the striated muscle fiber, (Translation of paper of 1902 by Bruni, C., Bennett, H. S. and deKoven, D.), J. Biophys. Biochem. Cytol., 10(Suppl):3.

Waku, K. and Nakazawa, Y., 1964, On the lipids of rabbit sarcoplasmic reticulum, J. Biochem., 56:95.

Walcott, B., 1966, Unpublished data.

Walker, S. M. and Schrodt, C. R., 1966, T System connections with the sarcolemma and sarcoplasmic reticulum, Nature, 211:935.

Weidmann, S., 1952, The electrical constants of Purkinje fibers, J. Physiol., 118:348.

Willows, A. O. D., 1965, Unpublished data.

Winegard, S., 1965, The location of muscle calcium with respect to the myofibrils, J. Gen. Physiol., 48:997.

Typed by: Nancy McLain