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ABSTRACT

Electron microscopy of muscles soaked in Ringer solution containing horseradish peroxidase shows a considerable penetration of peroxidase into the T-tubes, terminal cisternae and the longitudinal tubules of sarcoplasmic reticulum. Taken together with observations on the effect of hypertonic solution on the dimensions of the T-tubes and the terminal cisternae it appears that there is a continuity between the sarcoplasmic reticulum and the external solution, probably through connections with the T-tubes at the triad junction. These findings conflict with other reports that the probe molecules do not penetrate into the longitudinal elements of SR. The reason for these discrepancies is not yet clear. One factor which may limit the penetration of the probe molecules in the sarcoplasmic reticulum is the fluid dynamics occurring within that system.

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CHAPTER I

REVIEW OF THE LITERATURE

A. The Structure of Sarcoplasmic Reticulum

The study of structure and function of muscle had its origins in the 1800's. Golgi, Bowman, Gerlach, Retzius, von Gehuchten and Cajal are some of the scientists involved in this field at that early time.

The first report of a reticular structure between the fibrils was made by Bowman(1840). Gerlach (1877) also observed this reticular structure within the muscle cell and proposed that it is the continuation of the end plate nerve supply. Retzius (1881) and Bremer (1883) similarly implicated the system in impulse conduction. Carnoy (1884) suggested that the properties of irritability and contractility reside in the reticular system. In 1890, Haswell described more closely the reticular system, suggesting an existence of a longitudinal component as well as a transverse membrane occurring in the region which is presently called the Z band. Fusari (1895) also described a series of longitudinal and transverse reticula in striated muscle. In 1902, Veratti made an assessment of previous work dealing with the reticular structure in the muscle of various species. He concluded that the reticulum is found within the sarcoplasm, is distributed between and around the fibrils and is oriented in phase with the striations.

Between 1911 (Luna) and 1937 (Eastlich), there was a question as to whether the Golgi apparatus seen near the nuclear region of some preparations was of the same nature as the reticular structure described by the earlier workers. It was only in 1940 (Sosa and Menagazzi) that the independent existence of Golgi complex and the sarcoplasmic reticulum was accepted. From this time until the development of the electron microscope very little attention was paid to the reticular system.

With the introduction of the electron microscope, the reticular structure of muscle was re-discovered. The first work on this field using an electron microscope was performed by Bennett and Parker (1953) on pigeon breast muscle. The reticulum was discovered to be vesicular in nature and clustered in the I band region of the muscle cell. A new term was brought in to describe this vesicular structure; it was called "the sarcoplasmic reticulum". It was proposed that the sarcoplasmic reticulum functioned in the movement of substances into and out of the myofibrils from the sarcoplasm during the process of contraction. Edwards et al. (1956) described the sarcoplasmic reticulum in the muscles of insects, birds, reptiles and mouse. The sarcoplasmic reticulum was described as tubular. Tubules were found in the region of the I band and in some cases were intimately associated with the Z line. It was suggested that the function of the sarcoplasmic reticulum may be that

of a transport system, as well as an impulse conducting system to the fibrils.

Porter and Palade (1957), studying the sarcoplasmic reticulum in a striated muscle cell, added several new concepts to muscle morphology. The term "Triad" was introduced to describe a unit of sarcoplasmic reticulum having a central element perpendicular to the long axis of the fiber, and on either side a longitudinal element extending parallel to the long axis of the fiber. Another term "terminal cisterna" referred to an enlarged end of longitudinal tubule in close apposition to the central element of the triad. "Intermediate vesicle" was the central element of the "triad".

Andersson-Cedergren (1959), using mouse skeletal muscle, performed a detailed study of the sarcoplasmic reticulum from a reconstruction of serial sections. Another term was introduced, "the T tube or transverse tube", equivalent to the intermediate vesicle of Porter and Palade (1957). This detailed study resulted in the following conclusions: the T (transverse tube) system forms a continuous network throughout the fiber and is located at the A-I band junction. The width of the T tube is approximately 300 Å and it is found to have no connection with the rest of the sarcotubular system. The longitudinal tubules of the A and I bands with the T tube between them form the so called "triad" of the A-I band junction. Subsequent studies by Fawcett and

Revel (1961) and Smith (1961), indicated that the position of the triad depends very much upon the functional nature of the muscle. Thus, a muscle capable of a high frequency contractions (i.e. phasic muscle) has the triad located at the A-I band junction and also has two triads per sarcomere. Slower (tonic) muscles, as well as the cardiac muscle, have one triad per sarcomere and this is located at the Z line only.

Because of its high density, regularity of structure and clarity, Peachey (1965) chose to describe the sarcoplasmic reticulum in frog skeletal muscle. He presented the following picture of the system:

1) The triads are located in the region of the Z line. The central element of the triad is the T tube, which runs perpendicular to the long axis of the fiber. The outer elements of the triad are called the terminal cisternae, and they have a greater diameter in the longitudinal section than the T tube. The junction of the T tube and the terminal cisternae is characterised by dense connections or bridges, 200 A apart forming a vesicular appearance. The terminal cisternae are usually filled with amorphous matter.
2) The intermediate cisternae are parts of the longitudinal sarcoplasmic reticulum located exclusively in the I band region and connect the terminal

cisternae with the longitudinal tubules in the A band.

3) Longitudinal tubules are found in A band region. A fusion of longitudinal tubules near the center of the A band forms a collar around each individual myofibril. This collar is usually pierced by fenestrations of 300-400 A in diameter. It is presumed that there is a continuity of sarcoplasm of adjacent fibrils between these pores.

B. Early Theories of the Function of Sarcoplasmic Reticulum

With the discovery of sarcoplasmic reticulum by Bowman(1840) arose the question of its function. Gerlach (1866) proposed that the reticular system is a continuation of the motor end plate nerve supply. Retzius (1881) and Bremer (1883) both subscribed to this theory, and in their respective works related the function of the reticulum to the conduction of impulses. Carnoy (1884) went even further and suggested that the property of contractility resides in this system as well. Cajal (1890), studying the fibrillar flight muscle of an insect, proposed that the reticulum is a very fine extension of the tracheal tubes into the substance of the muscle. Being filled with air, it would play a role in the respiration of the muscle. Veratti (1902) in his summary of the work done on the reticulum up to that time discounted the theories of impulse conduction by the reticulum due to the lack of sufficient evidence.

A major hypothesis on the nature of the function of the reticulum was put forward by Holmgren (1908). This hypothesis regarded the reticulum as a series of pseudopodia sent into the substance of the fiber by a trophocyte external to the cell. The purpose of this pseudopodial network was a nutritive one in function. The network would change from solid to liquid depending upon the nutritional need of the cell. This was the "Trophospongium" concept of reticular function. Because of lack of evidence in other cell types having similar tubular network, Holmsgren's trophospongium theory was rejected.

The discovery of the electron microscope permitted scientists to have a closer look at the structure of the sarcoplasmic reticulum. It also permitted them to formulate new theories regarding its function. Following the preliminary work of Porter (1956) and Porter and Palade (1957) on the structure of the sarcoplasmic reticulum, Huxley and Taylor (1958) proposed another major theory regarding the function of the reticular system. Using a micropipette as an electrode, they stimulated selected regions of the muscle fiber. It was discovered that the stimulation of the fiber in the region of the triad, produced a contraction of one sarcomere in width with an inward spread of 10μ. Stimulation of the longitudinal tubule region produced no contraction. At first, it was thought that the A line was responsible for the impulse conduction. However,

muscles having the triad located at the A-I band junction (e.g. a crab muscle) produced a contraction only if stimulated at the A-I band junction. Stimulation at the Z line produced no results. The resulting evidence pointed to T tube as the structure most probably responsible for the inward conduction of electrical impulses. Such a theory offered a convenient solution to the problem encountered by A.V. Hill (1948). Studying the diffusion rates of substances into and out of the muscle fiber during its activation, he suggested that the activation of the muscle fiber could not result from the diffusion of activating substance across the surface membrane. The time for diffusion of this substance into the core of the fiber would be too long to account for the very short time interval between the stimulus and the contraction. A T tube would provide a quick route for the spread of excitation into the core of the fiber.

C. Present Status of Sarcoplasmic Reticulum

a) T tube system

Much of the speculation as to the function of the T tube system was supplied by the analysis of ionic currents in and out of the muscle fiber during changes in membrane potential.

Since the implication of the T tube system in an inward spread of electrical impulse in a muscle (Huxley and Taylor 1958) it was proposed that this system is

responsible for carrying a part of the current, previously attributed only to the surface sarcolemma. The evidence presented by Hodgkin and Horowicz (1960a) and Adrian and Freygang (1962a) seemed to indicate that the T tubes carry K^+ current. Other electrical studies, supplemented by observation of morphological changes during the electrical activity of the muscle (Girardier et al 1963; Freygang et al 1964a, 1964b; Foulks and Pacey 1965; Sperelakis and Shneider 1968; Gage and Eisenberg 1969) clearly implicate the T tube system in ionic movements, but there still exists some doubt as to whether it is the Cl^- current or only K^+ current that is carried by the T tubes. The bulk of the evidence favors the K^+ current, but species differences may occur.

The relationship between the T tube system and the extracellular space has been investigated by several workers. Huxley (1964) using horse spleen ferritin of M.W. 750,000 and 110 Å diameter was the first one to demonstrate a connection between the extracellular space and the T tubes. Muscles soaked in ferritin for various lengths of time clearly exhibited the penetration of ferritin into the T tube system. Subsequently Hill (1964) using radioactive albumen; Endo (1964, 1966) using luminescent dye Lissamine Rhodamine B200, Page (1964) using ferritin and Eisenberg and Eisenberg (1968) using horseradish peroxidase confirmed this initial finding. These results

indicate indirectly that the T tubes open as pores at the surface of the sarcolemma and that the walls of the T tubes are in direct continuity with the sarcolemma. Such a continuity has been demonstrated directly in muscles of other species. (Fawcett and Revel 1961; Franzini-Armstrong and Porter 1964; Walker and Shrodt 1965).

The diffusion characteristics of substances into the T tubes also have been investigated. Huxley (1964) observed that the resistance to the diffusion of ferritin seemed to be located at the mouths of the T tubes. Endo (1966) after the analysis of distribution of luminescent dye across the fiber diameter concluded that the resistance to the diffusion of the dye was located along the T tubes as well as at their mouths, since the dye concentration increased or decreased more rapidly in the outer parts of the fiber than in the centre.

b) <u>The longitudinal component of the sarcoplasmic reticulum</u>

In contrast to T tube system, less is known about the longitudinal component of the SR i.e. the terminal cisternae, longitudinal tubules and the fenestrated collar.

Since the demonstration of local contraction of a muscle by intracellularly applied calcium (Neidergerke 1955), evidence has been accumulating pointing to the longitudinal elements of the SR as the storage and release sites of muscle Ca^{++}. Ebashi and Lipman (1962) isolated

SR fragments from a skeletal muscle and discovered that the fragments concentrated Ca^{++} from the surrounding medium in the presence of ATP. This suggested that the SR has a similar function in vivo. Costantin et al (1965) demonstrated in a skinned muscle the deposit of calcium oxalate in the terminal cisternae of the SR. These electron-dense deposits did not appear in any other part of the muscle. Perfusion of the muscle with Ca^{++} increased the frequency of the electron-dense deposits in the terminal cisternae.

Winegrad (1965a,b) demonstrated by autoradiography the distribution of muscle Ca^{++}. The results indicated that 38% of muscle Ca^{++} was localised in the centre of the I band, a region of the location of the triad in frog muscle. When stimulated, less active muscles tended to have a higher concentration of Ca^{++} in the I band region than their more active counterparts. A small quantity of Ca^{++} was also located in the A band region. It was suggested that the Ca^{++} in the I band region is found outside of the myofibrils i.e. the SR, whereas Ca^{++} in the A band region was within the myofibrils and bound to the contractile proteins. In later work, Winegrad (1968) confirmed these initial suggestions. He also demonstrated that Ca^{++} in the SR is bound in two regions: a) in the intermediate cisternae and the I band region and b) in the intermediate cisternae and the longitudinal tubules in the A band region. The Ca^{++} stores in the latter sites are easily released and

replenished during contraction and relaxation phases of the muscle. The stores of Ca^{++} in the terminal cisternae appear to be bound more firmly and equilibrate slowly with the total Ca^{++} content of the SR i.e. the T tubes, the intermediate cisternae and the longitudinal tubules.

The relationship between the Ca^{++} movement in the SR and the electrical activity has been demonstrated by Lee et al (1966). The SR fraction of skeletal and cardiac muscle showed uptake of Ca^{++} similar to that described by Ebashi and Lipman (1962). When a current was passed through that fraction, the uptake of Ca^{++} decreased, and when the current was stopped, Ca^{++} uptake increased. This phenomenon was dependent directly upon the frequency of stimulation and the voltage of the stimuli. Present theory suggests that Ca^{++} is accumulated and released from the walls of T tubes and terminal cisternae of the SR. Though these results must be regarded with some caution they nevertheless demonstrate a current-dependent Ca^{++} movement in SR preparations. It is possible that this is related to the chain of events starting with current flow in the T tubules and release of stored calcium from the SR.

c) <u>The junction between the T tube system and the terminal cisternae of the SR</u>

In their comprehensive study of the sarcoplasmic reticulum, Porter and Palade (1957) and later Andersson-

Cedregren (1959) described the triad region as two opposing membranes of the terminal cisternae 500 Å apart with an interposed tubular structure (T tube) approximately 300 Å wide in longitudinal axis. This, therefore, indicated that the distance between the walls of the T tube and the terminal cisternae is approximately 100 Å wide on either side.

In a later study, Fahrenbach (1965) described the junction of the two membranes in human rectus abdominis muscle. He suggested that the triad junction is composed of two membranes (one of T tube and one of the terminal cisternae) 45-60 Å thick separated by a space of 110 Å in which a 3rd structure 40 Å thick was located. This arrangement of membranes was compared to a tight junction (macula adherens) illustrated in other cell types. It was also compared to zonula occludens. However, since the space between the two membranes (110 Å) was larger than in a typical tight junction, and since the intermembranous space was filled with what appeared to be a cementing susbtance, it was more likened to zonula occludens. This could mean that such a junction had low resistance and would allow an impulse conduction from the T tube into the terminal cisterna. Hoyle (1965) studying the triadic junction in insects described it as being compoased of: 75 Å wide double

layered membrane of the T tube, 56 Å wide double layered membrane of the terminal cisterna with 100 Å space separating them. He concluded that a simple transmission of electrical impulses is improbable in this region. Walker and Shrodt (1966) in their analysis of the triadic junction in rat skeletal muscle described it as scalloped in appearance, a description previously used by Revel (1962) and Fahrenbach (1965). The two opposing membranes are double-layered without a third structure in the inter-membrane space as described by Fahrenbach. The scalloped appearance is due to dense bridges between the two membrane systems. The periodicity of the dense bridges is approximately 200 Å. The less dense areas have convex membranes at their extremes and are totally enclosed by membranes, i.e. do not have continuity with the sarcoplasm. Because of these convexities at the end of the less dense regions the distance between the two membrane system varies from 80-100 Å in dense areas to a slightly larger distance in the less dense areas. Kelly (1969) essentially confirmed these findings studying the triad junction structure in young newts and adult frogs. He also suggested that the third structure seen in the intermembranous space by Fahrenbach (1965) and Walker and Shrodt (1966) results from the condensation of an amorphous substance found in the intermembranous space. This arrangement suggests that a physiological tight

junction exists and that a transmission of current is possible between the T tube membrane and the membrane of the terminal cisterna.

d) The permeability of the triadic junction

If the triadic junction does indeed exhibit the continuity of membranes between the T tube system and the terminal cisternae as demonstrated by Walker and Shrodt (1966) it is reasonable to question whether there is a possible communication between the two structures.

An indication of whether such a connection does exist was made possible by experiments in which various molecules were used as the extracellular space markers, as well as experiments in which volume changes of the SR were observed in response to solutions of various tonicities.

Tasker et al (1959) using inulin, sucrose and albumen as the extracellular space markers calculated the volume of the extracellular space to be 24.8%, 26.5% and 21.9% of total fiber volume respectively, the difference between the values not being significant. Harris (1963) in his analysis of chloride ion distribution in a muscle estimated the volume of the SR to be 12% of total fiber volume. The sucrose space of the toad fiber as measured by Didynska and Wilkie (1963) was calculated to represent 34.0% of total fiber volume. The authors suggested, however, that the difference in values of the sucrose space as compared to that to Tasker et al (1959) could have

been due to differences in the technique of handling the muscles. It was also observed by the authors that the volume of space accessible to sucrose increased in hypertonic solution. Blinks (1965) using an isolated fiber and optical measurements estimated the volume of sucrose space to be only 13%. The difference in the volume estimates are most probably due to the fact that a whole muscle was used in experiments of Didyniska and Willie (1963) and only a single muscle fiber by Blinks (1965) making his estimates more valid. The electron microscopy of these hypertonic muscles as performed by Huxley et al (1963) demonstrated a considerable swelling of the T tubes and the terminal cisternae. It was proposed that sucrose enters these spaces and draws water from the sarcoplasm to offset the osmotic imbalance caused by movement of molecules into these spaces. The distribution of swellings was not uniform throughout the width of the fiber. Huxley (1964) demonstrated a connection between the T tube system and extracellular space using horse spleen ferritin. Although these large molecules (110 $\mathring{A}$ diameter) penetrated into the T tubes, none were found in the terminal cisternae, suggesting that they are not part of the extracellular space. Similar results were observed by Page (1964). D.K. Hill (1964) used radioactive albumen to map out the space accessible to it in a toad muscle. The results indicated that the somewhat smaller molecule

of albumen (81 Å diameter) had two peaks in their distribution along the length of a sarcomere. The first peak was found in the Z line region and the second at the A-I band junction. The total volume accessible to albumen was calculated to the 0.12-0.28% of total fiber volume, a value quite small as compared to the volume of extracellular space estimated previously. This value corresponded closely to an estimated volume of T tubes (0.3% of fiber volume). However, due to the fact some tracer was found well outside of the Z-line region where the T tubes are found, it was suggested that the space at the A-I junction may be a part of the longitudinal tubule system filled by connections with the T tubes. The possibility that the A-I junction compartment was caused by radiation damage was not excluded.

The observation on the nature of distribution of fluorescent dye in frog muscle SR provided more information on its nature as well as the characteristics of fluid movement within it. Endo (1966) using luminescent dye Lissamine Rhodamine B200 observed the entry of the dye into the I band region of a muscle. The width of the luminescent band within an I band was estimated to be 0.1 μ after allowing for large error due to the limitations of light microscope. It was suggested that some part of the triad system located in the I band was filled with the dye. The volume of space accessible to it was calculated to be

1-2% of the fiber volume, which is larger than the volume of T tubes (0.3% of fiber volume) and smaller than the combined volume of the triad i.e. T tube plus two terminal cisternae (5% of fiber volume) as estimated by electron microscopy (Page 1964, Peachey 1965). One possible explanation mentioned was that the dye enters a portion of lateral elements of the triads. In hypertonic solution, in agreement with observations of Huxley et al (1963), the dye space increased in size. Furthermore, the analysis of data from wash-out experiments indicate that the wash-out curve has two components - a fast initial one, followed by a slower one with the total wash-out time of 1-2 min. It was decided that the shape of the wash-out curve was consistent with the assumption that the resistance to escape of the dye is located in three areas: a) at the mouths of the T tubes (as previously suggested by Huxley in 1964); b) along the tubules e.g. tortuosity of the T tubes and c) resistance at the junction between the T tubes and "extra spaces". (Endo 1966).

Recently Eisenberg and Eisenberg (1968) used horseradish peroxidase (HRPO) to test for the completeness of disruption of the T tube system by glycerol treatment. The use of HRPO as an extracellular space marker was used by Strauss (1964c,d) in light microscopy with very good results. An adaptation of this technique for electron microscopy by Graham and Karnovsky (1966) proved to be very

successful. The small molecular weight of 40,000 (Keilin and Hartree, 1951) makes it a good penetrating molecule. Its reaction product with benzoate, catalysed by H_2O_2 becomes electron dense and can be visualised directly. Eisenberg and Eisenberg (1968) reported consistent filling of T tubes with HRPO - up to 99% of observed sites. They did not observe, however, any penetration of HRPO into the lateral elements of the SR. Peachey and Shild (1968) in an attempt to identify the albumen space at the A-I band junction described by Hill (1964) performed a comparative study of frog and toad muscle SR to assess whether species differences could account for Hill's observations. Using ferritin soaked muscles the distribution of ferritin particles along the length of a sarcomere failed to uncover such a space, since most of the particles were located in the Z line region. The work of Birks and Davey (1969) on the effects of solutions of various tonicities on the frog muscle again showed that the size of the SR increases in hypertonic solutions, a fact earlier demonstrated by Huxley (1963). They attribute the increase in size of the SR directly to the osmotic imbalance caused by particles moving into the various compartments of the SR, suggesting that the particles do so through a connection between the T tubes and the terminal cisternae, and that the dense connecting bridges between these two compartments of SR as described previously by Fahrenbach (1965) are in

effect selectively permeable pores.

One can gather from all this information that the question as to whether or not there is a connection between the ECF and more specifically between the T tube system and the terminal cisternae is far from being solved. The experiments of Tasker et al (1959), Huxley (1963), D.K. Hill (1964), Endo (1966) and Birks and Davey (1969) indicate the possible existance of such a connection. On the other hand, experiments in which large tracer molecules have been used e.g. Huxley (1964), Page (1964) and Eisenberg and Eisenberg (1968) seem to indicate that such a connection is not present, or at least is not detectable with molecules of large size.

D. Objectives of the present work

It has been shown that there is a variety of conflicting evidence relating to the structure and function of SR.

There are also wide discrepancies in the estimates of the apparent extracellular space and morphometrically determined volumes of different parts of the tubular system within muscle fibres.

Electron-microscopic studies of fixed sections of frog muscle have repeatedly failed to demonstrate regular, clearly defined channels both between different parts of the tubular systems and between the tubular system and the external fluid. The use of probe molecules thus

assumes a critical importance in defining the continuity of spaces within the living muscle. The location of probe molecules still depends upon electron-microscopy and associated proceedures such as fixation which may result in artefacts. The distribution of probe molecules after fixation should however give a clear idea of continuity of spaces in the living muscle.

Direct electron-microscopic demonstration of probes within cellular compartments is only possible in the case of large electron-dense molecules such as ferritin. The penetration of smaller, less dense molecules can be shown either by their reaction products after fixation or by indirect effects such as the osmotic swelling of the compartments penetrated.

In spite of the critical importance of defining the continuity of tubular compartments, the experimental evidence remains confined to a few, frequently quoted papers (Eisenberg and Eisenberg (1968), Huxley (1964), Birks and Davey (1969)).

This work was undertaken to reinvestigate the penetration of probe molecules into the tubular system of intact muscle fibers. Two types of probe molecules were chosen, sucrose and horseradish peroxidase, (HRPO), Sucrose because it is near the lower limit in dimensions of uncharged polar molecules which do not readily penetrate cell membranes, HRPO, because

it is close to the dimensions of albumin which appears to penetrate deeply in the I band region (Hill 1964) and yet which Eisenberg and Eisenberg (1968) have found under the conditions of these experiments to be located only in the T tubular system.

The use of these two very different probes should help to define the regions of continuity within the muscle fibre and at least set limits on the size of the channels which mayy connect them.

CHAPTER II

METHODS

A. Muscles

The sartorius muscles of frogs (Rana pipiens) were isolated together with their attachment at the pelvic bone, which was subsequently split in the middle to produce a pair of muscles. The pelvic bone chip was pierced by size 12 hypodermic needle to provide the means of attachment onto a glass frame. The opposite tendon was tied with a length of thread to form a loop for the attachment of the lower end of the muscle. Each muscle was mounted onto a glass frame at 1 to 1.5 times its normal resting length. The mounted muscle was placed in a container made out of a 5 cc syringe equipped with a stopcock to permit easy evacuation of solutions (Fig. 1).

B. Subjecting the muscles to hypertonic solutions

After the mounted muscles were placed in their containers, they were allowed to equilibrate for one hour in normal Ringer solution. This was done to allow the ionic composition of the undamaged fibers to return to normal. During the isolation procedures some fibers are inevitably damaged causing areas of high $[K^+]$ in the extracellular space, thus affecting the neighboring undamaged fibers. The equilibration is therefore an essential step in obtaining a good preparation.

Following the period of equilibration the

Fig. 1

Mounting of muscle for experimental procedures

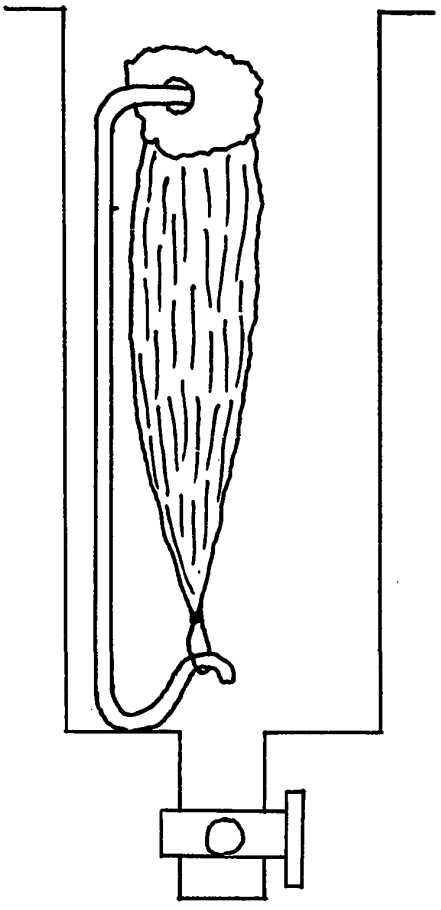


TABLE 1

COMPOSITION OF RINGER SOLUTIONS

(mM per liter)

	Ca	K	Na	Cl	HPO_4	H_2PO_4	Sucrose	Relative Tonicity
Normal Ringer	1.8	2.5	120	120	2.15	0.85	-	1
Hypertonic Ringer	1.8	2.5	120	120	2.15	0.85	232	2

experimental muscles were placed in the hypertonic Ringer solution for 1/2 hour while the control muscles remained in normal Ringer solution for the same period of time. The composition of both the normal and the hypertonic Ringer solutions is given in Table I. At the end of the 1/2 hour both the experimental and the control muscles were processed for electron microscopy.

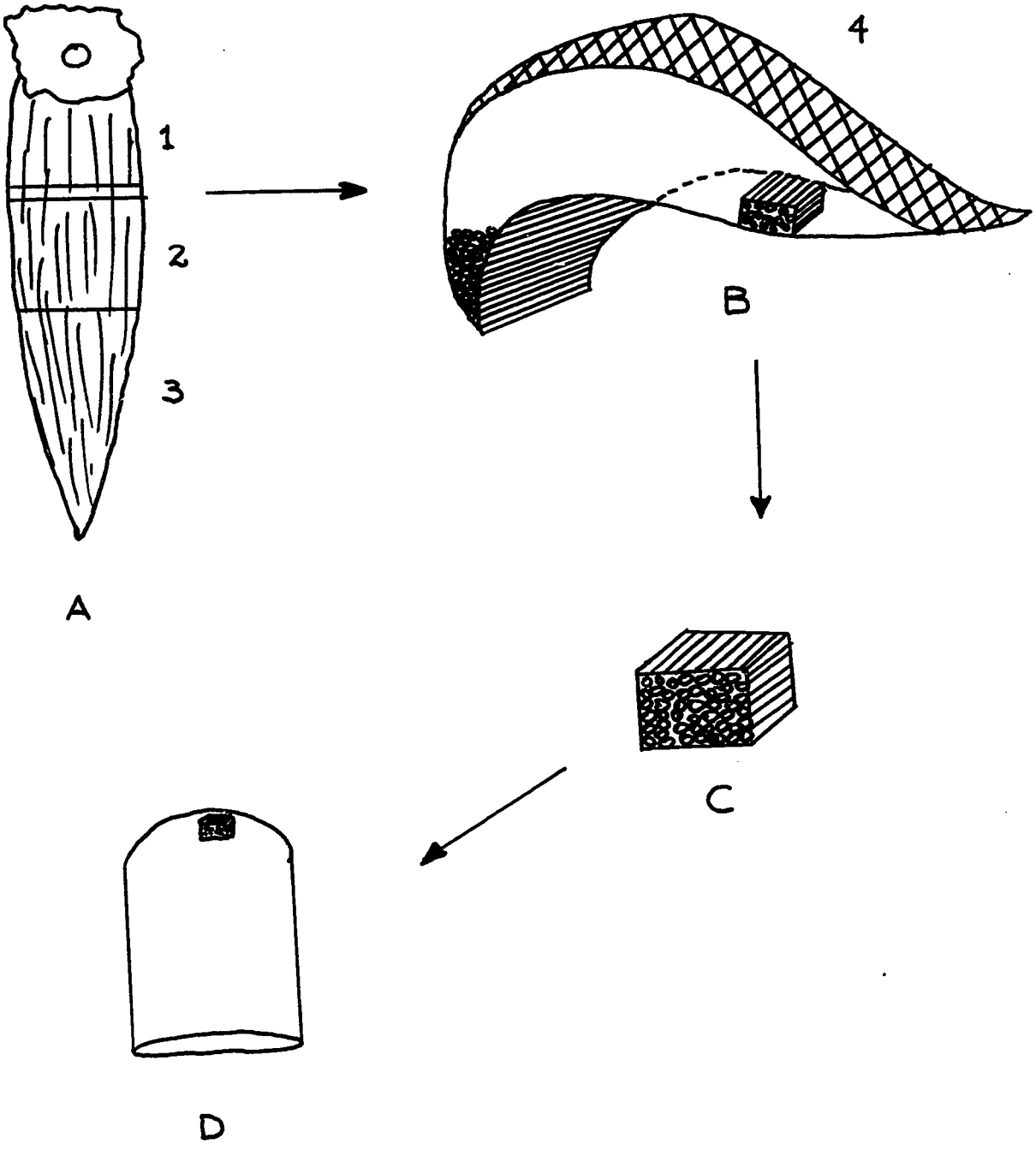
C. Horseradish peroxidase (HRPO) as an extracellular space marker

Horseradish peroxidase (HRPO) type II (Sigma Chemicals Co.) was used as a probe molecule (Eisenberg and Eisenberg, 1968). Normally, the isolated sartorius is covered byaconnective tissue sheath on both sides with the anterior surface having more dense covering than the posterior surface. This usually presents a diffusion barrier to the HRPO molecule. In order to facilitate the diffusion of HRPO into the fibers, the sartorius muscle was split longitudinally along one side (Fig. 2 (a)(b)) exposing a layer of fibers free of connective tissue. All visibly damaged fibers were removed at that time and then the muscle was allowed to equilibrate for 1 hour. The control muscle was prepared similarly.

Following the equilibration, the experimental muscle was immersed in Ringer solution containing 0.05% HRPO for periods of time between 1/2 - 1 hour, while the control muscle remained in normal Ringer for the same

Fig. 2

Processing of muscle for electron microscopy.
(Hypertonic Ringer solution experiments).


1
2
3
4
A
B
C
D

period of time. Subsequently both muscles were immersed in 4% glutaraldehyde fixative for 1 hour. Following this, both muscles were washed briefly (20 sec) in 0.1 M sodium cacodylate buffer of pH 7.3 and osmotic pressure of 460 mOsm/Kg H_2O as determined by freezing point depression. At this time all the remaining damaged fibers which have been made visible by the action of the fixative were removed from the cut surface. Also at this time several small samples of 4 fiber layers thick, 6-10 fiber layers high and 1-2 mm long were removed with a sharp razor blade (Fig. 2 (c)(d)). The samples of experimental muscle were then placed in 3,3' diaminobenzidine (Sigma Chemical Co.) in 0.05 M-Tris buffer (pH 7.6) together with 0.01% H_2O_2 for 1/2 hour at the end of which they were rinsed in distilled H_2O. Both the samples of the control and the experimental muscles were subsequently post-fixed in 1% OsO_4 at 4°C for 1 hour and processed for electron microscopy (Fig. 3(e)).

The shape of the samples as described above ensured that only the fibers closest to the cut surface would be analysed. In order to be certain that it is the reaction product of HRPO and 3,3' DAB that is actually visualised in microscopy, two HRPO control muscles were prepared. All the steps were identical with those of the experimental muscle except HRPO was omitted from the Ringer solution.

D. Treatment with ouabain

In ouabain-treated muscles, all the procedures used were identical to those described above for the experimental muscles, except ouabain was added to HRPO containing Ringer solution in the concentration of 10^{-5}M.

E. Wash-out experiments

The initial preparation of the muscles in this category was identical to that of the experimental muscles up to the point ending with the 1/2 hour soaking in the HRPO containing Ringer. Following this the muscles were treated according to the two categories:

a) Normal wash-out. At the end of the 1/2 hour soak in HRPO containing Ringer the muscle was re-immersed in HRPO-free Ringer for the period of 1/2 hour.

b) Hypertonic wash-out. At the end of the 1/2 hour soak in HRPO containing Ringer the muscle was re-immersed in hypertonic HRPO-free Ringer, the composition of this is described in Table I.

F. Electron microscopy

At the termination of all the experimental procedures in various categories as described above, whole muscles were fixed for 1 hour in 4% glutaraldehyde fixative in phosphate buffer of pH 7.2 and osmolarity of 980 mOsm/Kg H_2O determined by freezing point depression (Sabatini et al 1963). Following this initial fixation, small samples were excised from the muscles, as described avobe in the section

on HRPO, as an extracellular space marker. In the hypertonic experiments, the whole muscle was divided into 3 parts (Fig. 3 (a)) and a sample 1-2 mm^3 was removed from the posterior surface of the muscle after the removal of all visibly damaged fibers in that area (Fig. 3 (b)(c)). The samples from the HRPO experiment were briefly (30 sec) washed in 0.1 M sodium cacodylate buffer as described previously, whereas the samples from the hypertonic experiment group received similar treatment in phosphate buffer (pH 7.2) and all samples were subsequently post-fixed in 1% OsO_4 fixative (Millonig 1963) at 4°C for 1 hour.

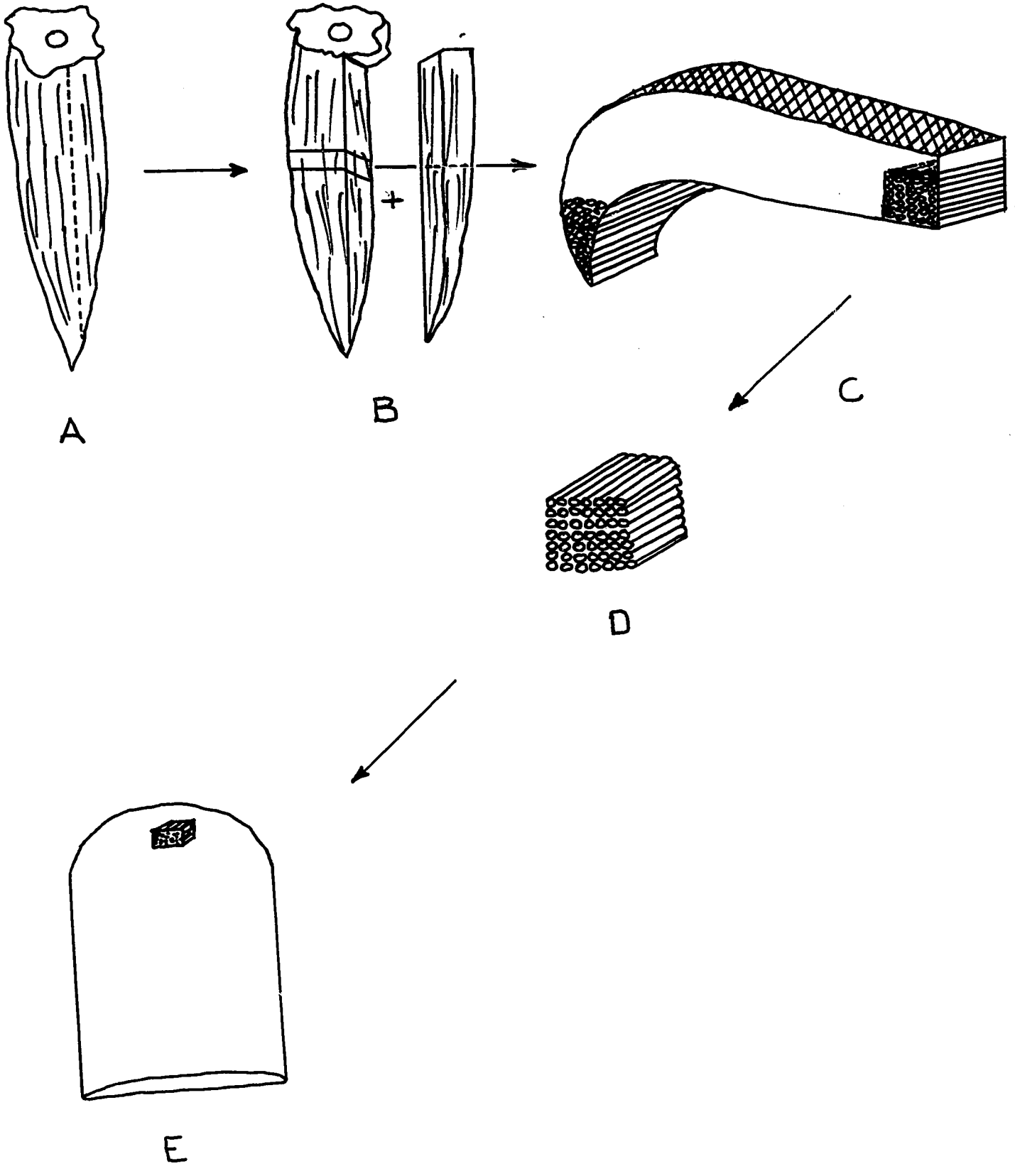
Following the fixation, the majority of the samples were dehydrated in ethanol of graded concentration from 50-100% followed by propylene oxide and then embedded in Araldite 502 (Ladd Laboratories) as described by Coulter (1967) in gelatine capsules (Fig. 2 (e), 3 (d)). In some cases, Vestopal (Jaegerss, Switzerland) was also used as an embedding medium. The embedded samples were then sectioned with a glass knife on a Reichard OmU_2 ultramicrotome. The sections were mounted on grids with carbon coated Formvarr film. The grids were of two types:

a) 200 mesh copper grid (Ladd Laboratories) for majority of observations.

b) Longitudinal bar grids without cross bars (Veco Ziefplaten Fabriek, Holland) to permit taking micrographs of whole cross-sectional areas of a single fiber.

Fig. 3

Processing of muscle for electron microscopy (HRPO tracer experiments).


A
B
C
D
E

The sections were stained with a saturated solution of uranyl acetate in 50% ethanol (pH 4.4) for 30 min. at room temperature and counterstained with Reynold's lead citrate (pH 12.0) for 20 min. at room temperature, and examined in Siemens IA Elmiscop at the accelerating voltage of 80 V, objective aperture of 25μ.

G. Measurements

(i) Hypertonic experiments

A number of micrographs of random areas were taken from each of the 3 parts of the muscle. The size of the T tubes was measured longitudinal to the long axis of the fiber, and always the largest diameter was measured. The cisternae were measured at right angles to the longitudinal axis of the fiber (Fig. 4). The measurements were taken from an 8 x 10^{11} photograph using (Bausch and Lomb) 8 x magnifier with a microgrid eyepiece. The results obtained from the 3 parts of each muscle were combined to give a representative picture of the size of T tubes and terminal cisternae in the whole muscle.

(ii) HRPO tracer experiments

Micrographs of random areas of fibers containing HRPO were taken for observation and comparison with the control fibers.

For the measurements of the number of sites containing HRPO, whole cross-sections of fibers were

assembled from serial micrographs. An area equal in width to one sarcomere length (from Z line to Z line) and running across the fiber diameter was chosen as a standard area for counting the sites containing HRPO. Theoretically, therefore, this gives 2 T tubes, 2 terminal cisternae, and 2 sarcoplasmic reticulum (SR) tubules per sarcomere as a number of potential sites (Fig. 5). The number of peroxidase containing sites per 100 available sites was calculated.

Since the isolated sartorii were small, it was taken that the average diameter of a muscle fiber would be 50μ and only the fibers of size close to the average diameter were examined.

Fig. 4

Triad system. Measurements of T-tubes and terminal cisternae in hypertonic Ringer solution experiments.

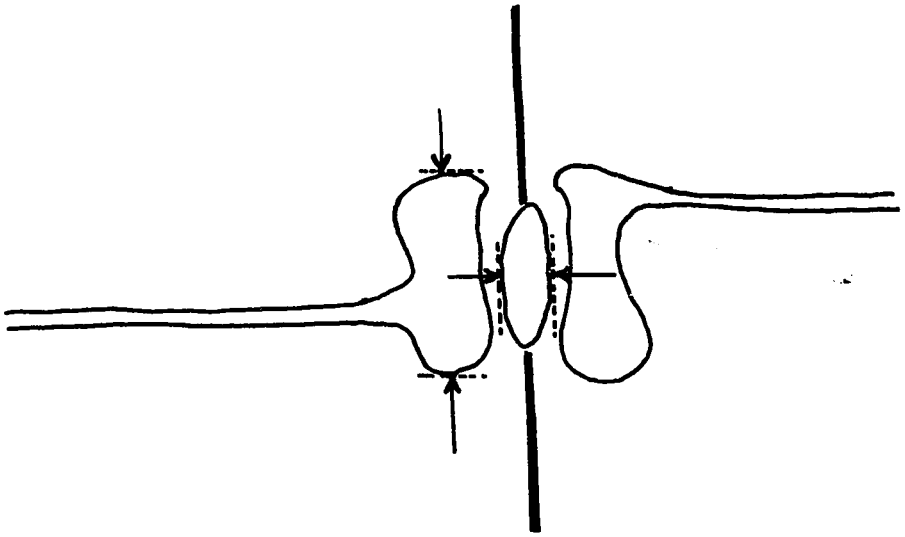
Fig. 5

Sarcomere (schematic). Calculation of HRPO distribution in muscle fiber. Numbers represent the sites where HRPO location was counted.

1 - T-tube

2 - terminal cisterna

3 - longitudinal tubules



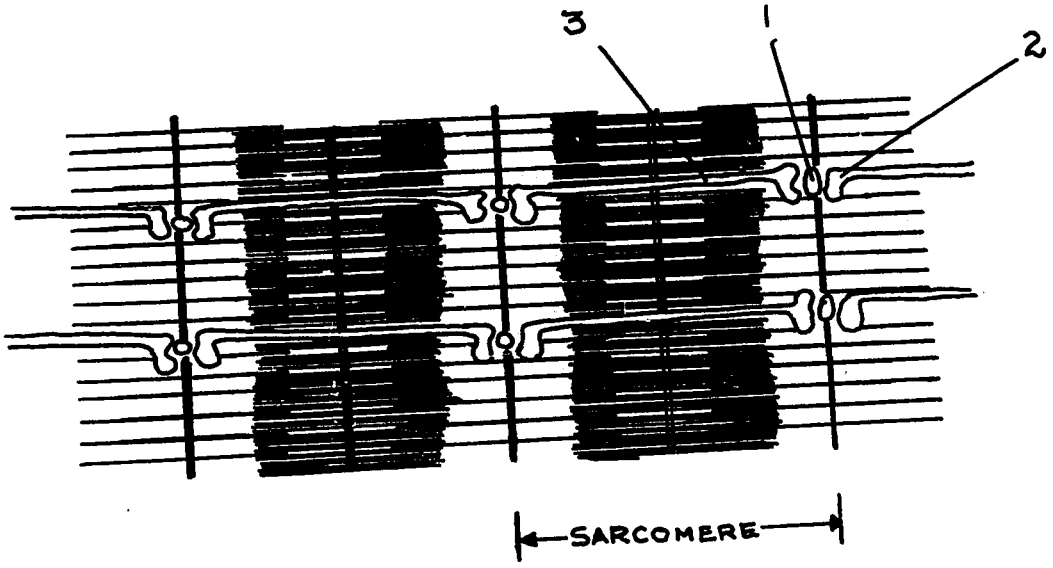

3
1
2
SARCOMERE

TABLE 2

DIMENSIONS OF T-TUBES AND TERMINAL CISTERNAE

AND THE EFFECT OF HYPERTONIC SOLUTION

	Experiment No.	Control (C) mean ± S.D. N	Control (C) mean ± S.D. (A°)	Hypertonic (H) mean ± S.D. N	Hypertonic (H) mean ± S.D. (A°)	H/C	P
A. T-tubes	6	40	429 ± 114	61	526 ± 230	1.22	0.05-0.02
	8	286	298 ± 171	284	778 ± 546	2.61	< 0.01
	9	228	408 ± 188	257	588 ± 341	1.44	< 0.01
	10	369	343 ± 123	227	593 ± 359	1.73	< 0.01
	11	697	296 ± 125	422	683 ± 423	2.30	< 0.01
B. Lateral cysternae	6	27	805 ± 476	48	1276 ± 939	1.52	< 0.01
	8	337	1545 ± 440	341	2028 ± 902	1.33	< 0.01
	9	227	1780 ± 604	287	2501 ± 1103	1.40	< 0.01
	10	421	1567 ± 701	296	1902 ± 725	1.23	< 0.01
	11	898	1833 ± 705	710	1953 ± 856	1.07	< 0.01

CHAPTER III

RESULTS

A. The effect of hypertonic solution

Fig. 6 shows a typical appearance of sartorius muscle following 1/2 hour immersion in normal Ringer solution, as compared with a muscle soaked for the same period of time in hypertonic Ringer solution (Fig. 7). The increase in size of the diameter of T-tubes (T) and terminal cisternae (TC) is readily observed.

In a series of five experiments using paired muscles there was a mean increase of 88% in the diameter of the T-tubes and 31% increase in the diameter of the terminal cisternae (Table 2). Due to the fact that the size of these compartments is not uniform in any one section, as well as the plain of the cut may vary a certain amount from true longitudinal orientation, it was necessary to perform a large number of measurements (from 27 to 898) to obtain statistically valid values.

B. The penetration of peroxidase into the tubular system

In a series of 13 experiments, one experiment failed to show any penetration of HRPO into the muscle. Of the remaining 12, one showed HRPO confined exclusively to the T-tube system, whereas in 11 experiments the HRPO was found in the T-tubes, terminal cisternae and the longitudinal tubules of the SR.

Fig. 8 shows the tracer in the T-tube, whereas Fig. 9 shows it in the terminal cisternae and the

Fig. 6

Longitudinal section of normal muscle fiber x 75000

T - T tube

TC - terminal cisterna

SR - sarcoplasmic reticulum

gly- glycogen granules

Z - Z line


TC
Z
gly
SR
T

Fig. 7

Longitudinal section of muscle fiber exposed to hypertonic Ringer solution x 75000

T - T tube

TC - terminal cisterna

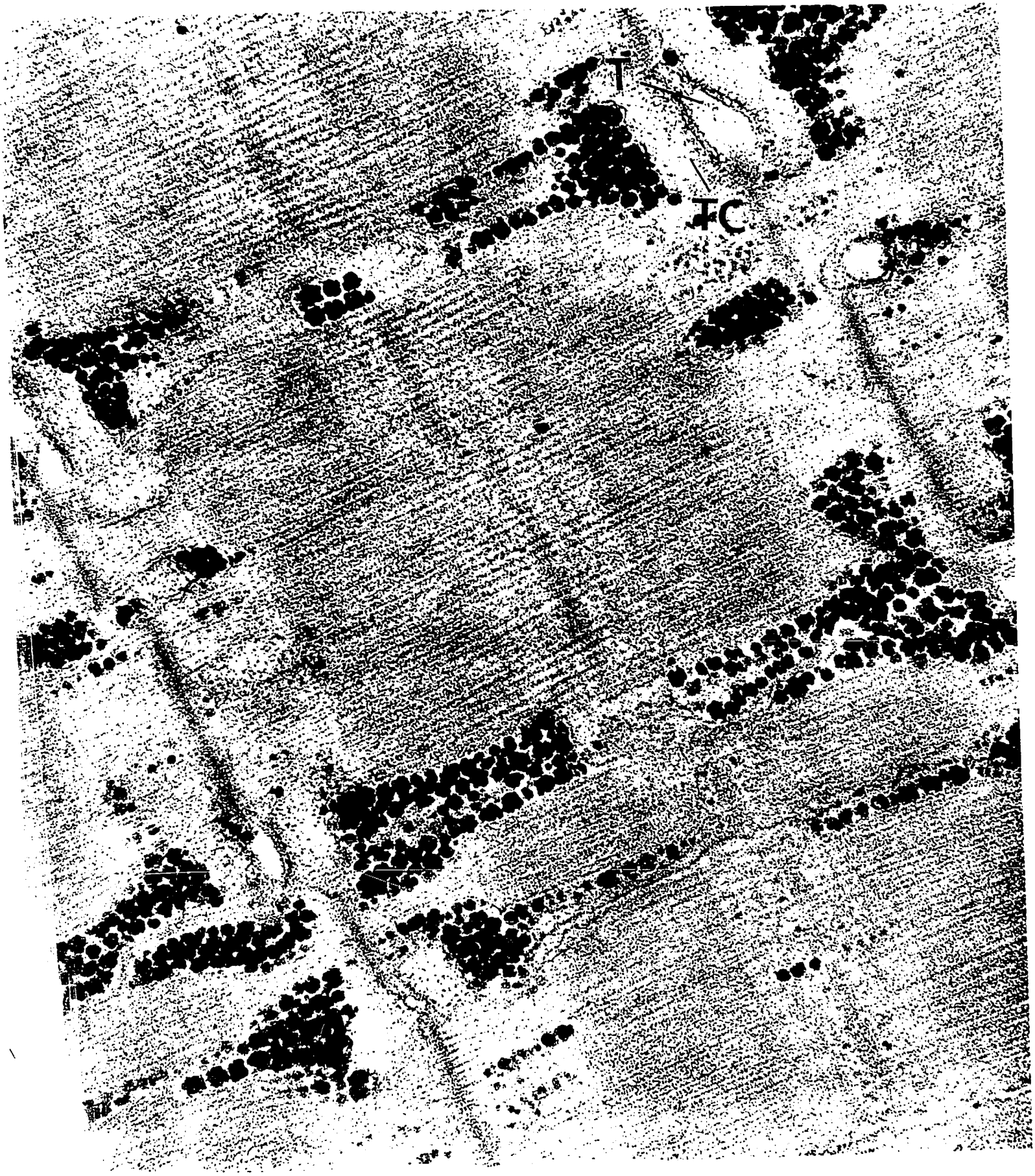

T
FC

Fig. 8

Triad system. HRPO located in T-tube x 210000

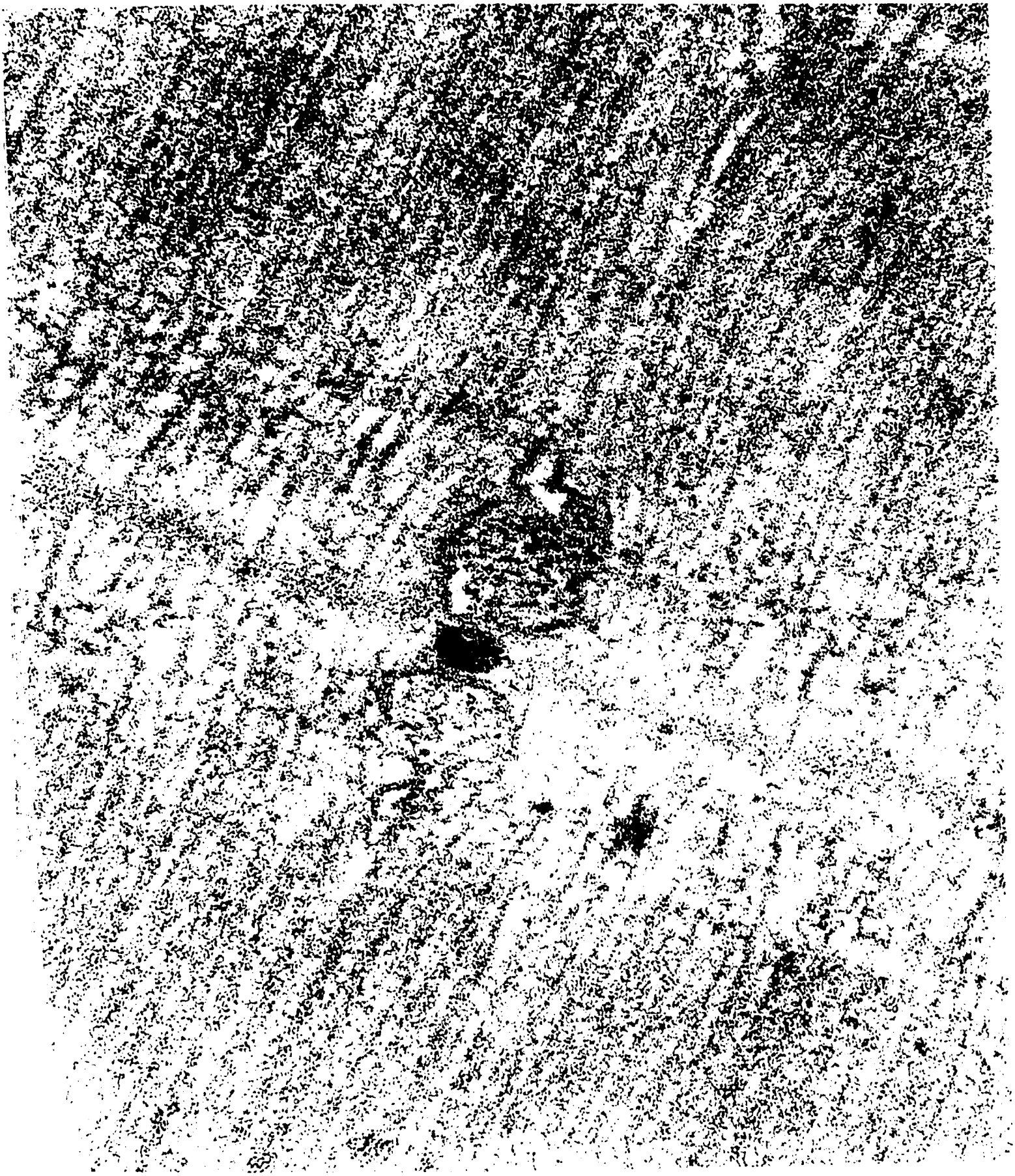
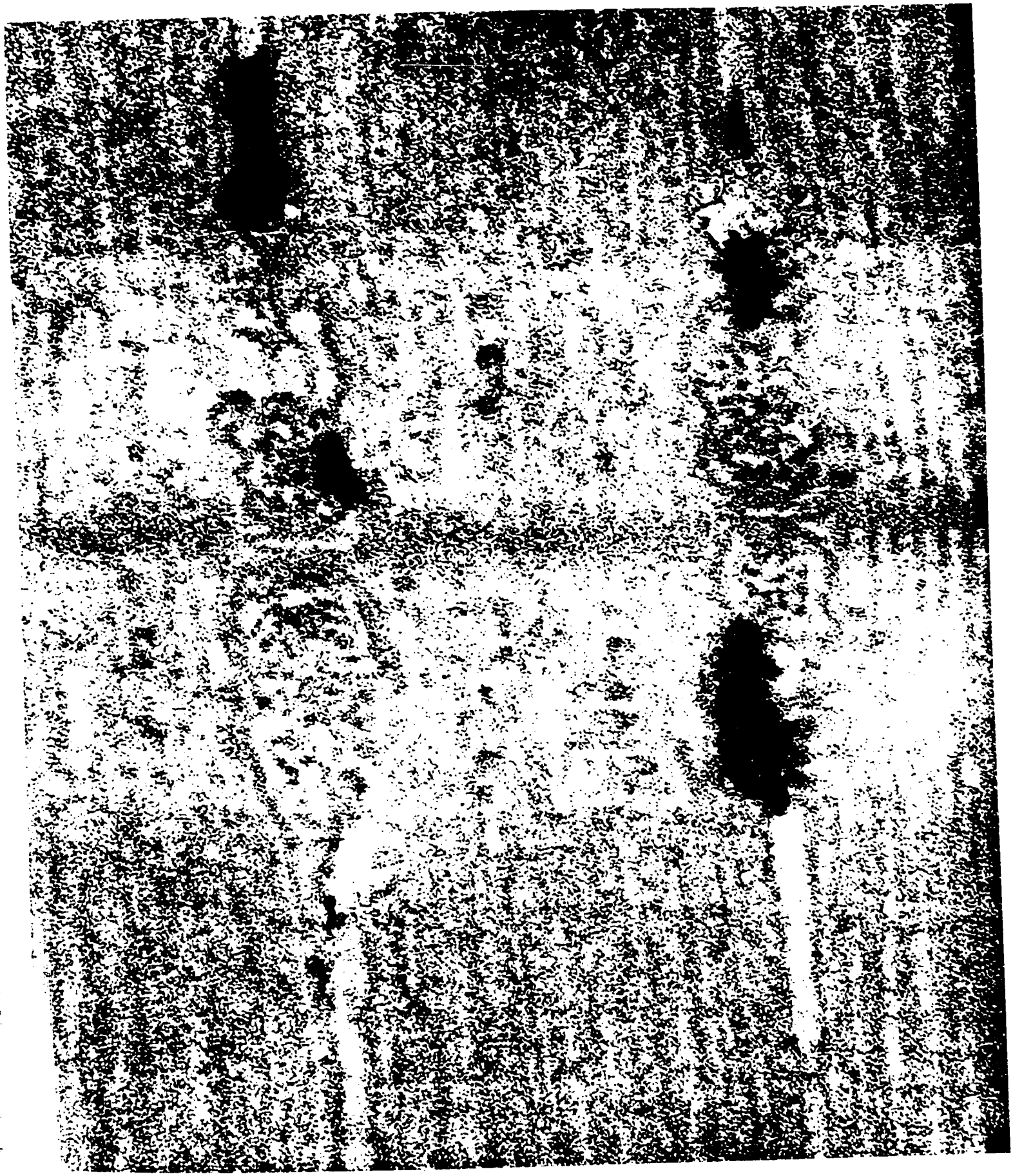


Fig. 9

The location of HRPO in lateral elements of SR x 210.000



longitudinal tubules of the SR. In order to demonstrate the HRPO tracer more clearly in the various compartments and to avoid confusing it with dark showing glycogen granules in fully stained sections, micrographs of unstained sections were obtained. They indicate very clearly the electron-dense deposits of HRPO products in various compartments (Fig. 10).

The number of peroxidase containing sites varied from muscle to muscle, from fiber to fiber and even within one fiber. This is probably due, in part at least, to different diffusion distances and to varying loss of peroxidase during fixation procedure. To obtain a more objective assessment of the extent of peroxidase penetration, counts were made right across the reconstructed cross sections of fibers by assembling serial micrographs. Profiles of peroxidase filling frequency are shown in Fig. 11 A-D, representing four fibers from four different experiments. A-C represent normal fibers and D represents a normal HRPO soaked fiber which has been further washed in normal Ringer 1/2 hour.

C. Peroxidase wash-out

Immersion of muscle for a standard period of time in Ringer solution containing peroxidase and a subsequent reimmersion in peroxidase free Ringer solution showed that the peroxidase disappeared from the fibers quite rapidly, and in four experiments the fibers examined were

Fig. 10

Unstained longitudinal section of muscle showing the location of HRPO x 100.000

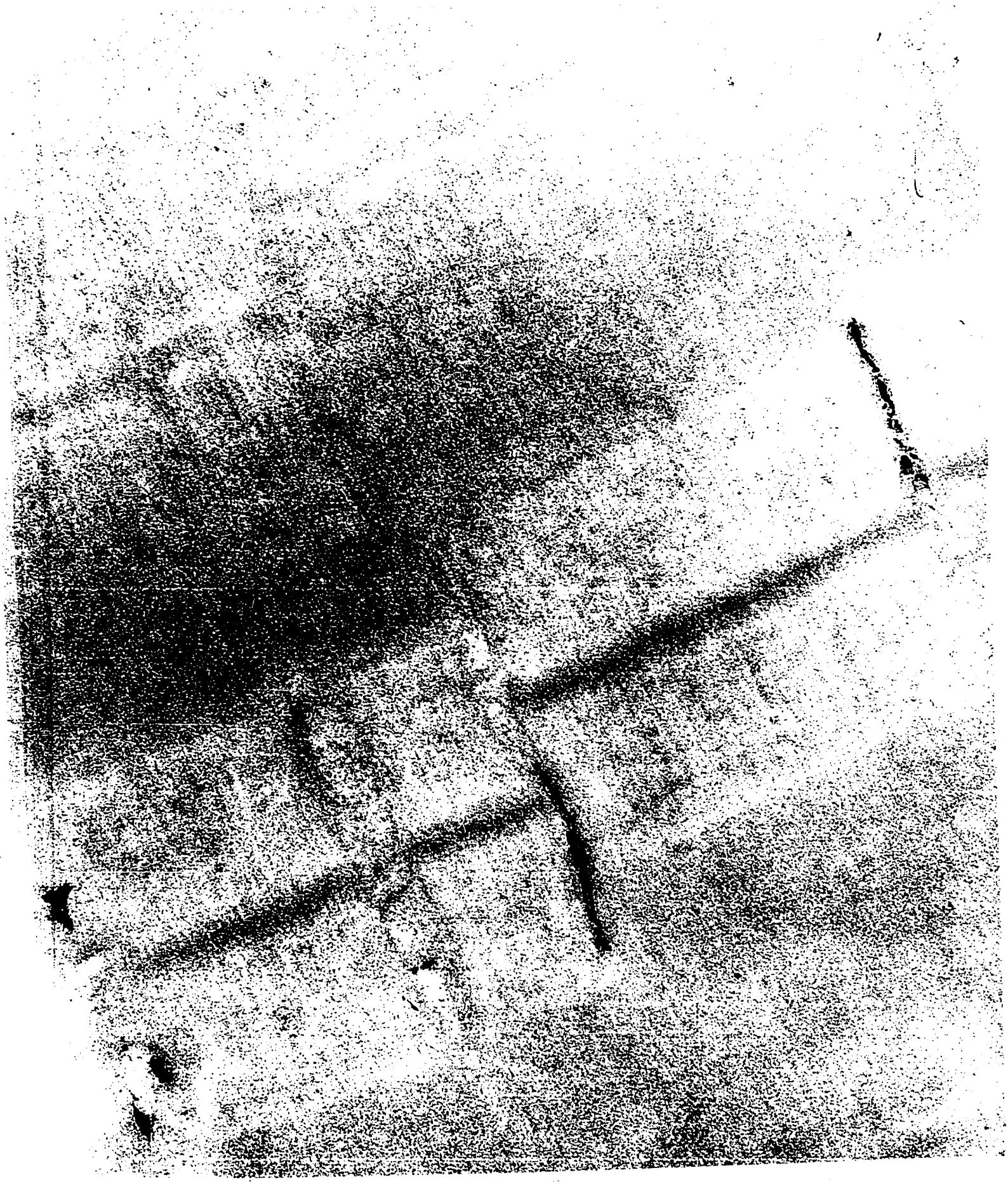


Fig. 11 A-D

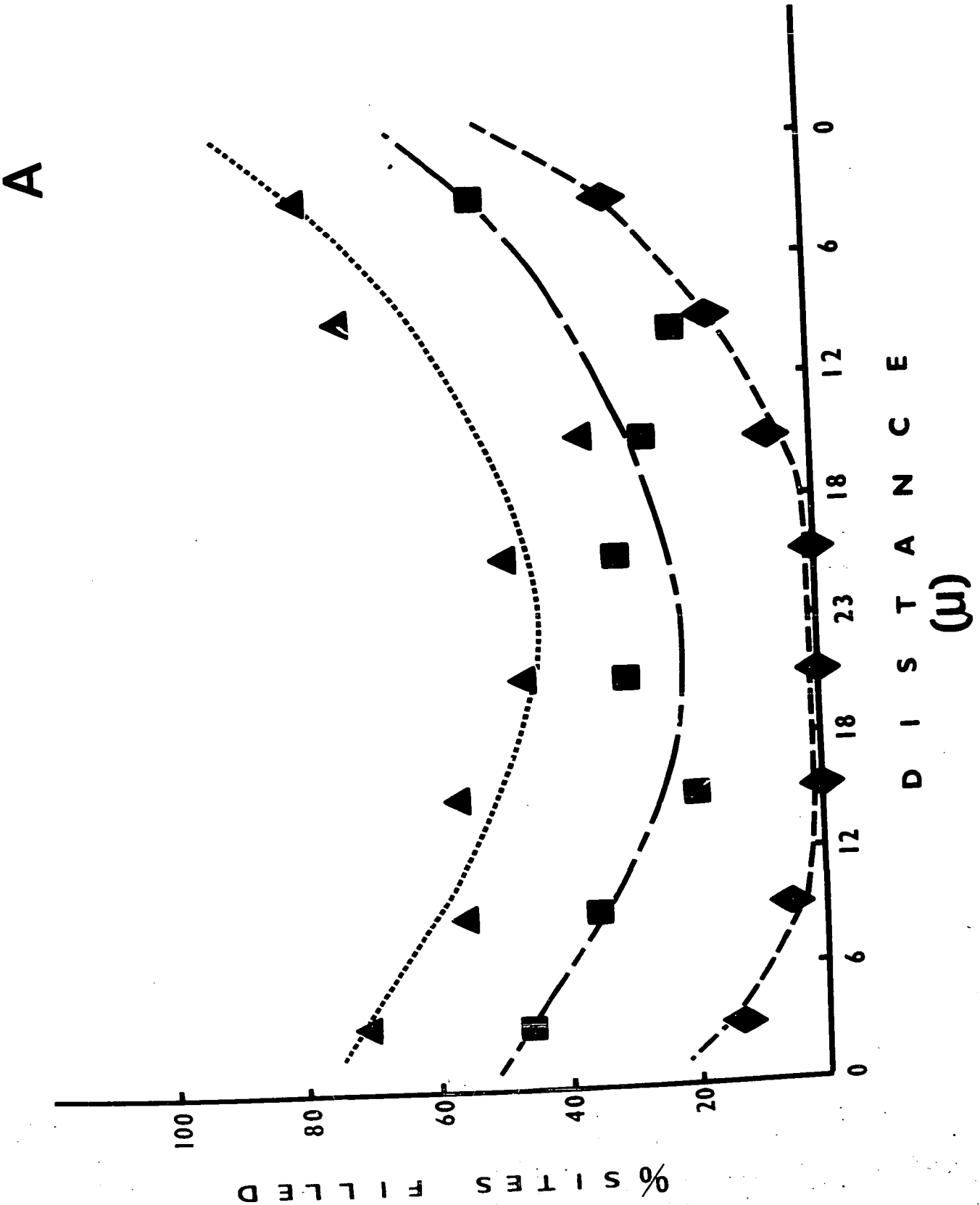
Distribution of peroxidase-filled structures in frog sartorius muscle.

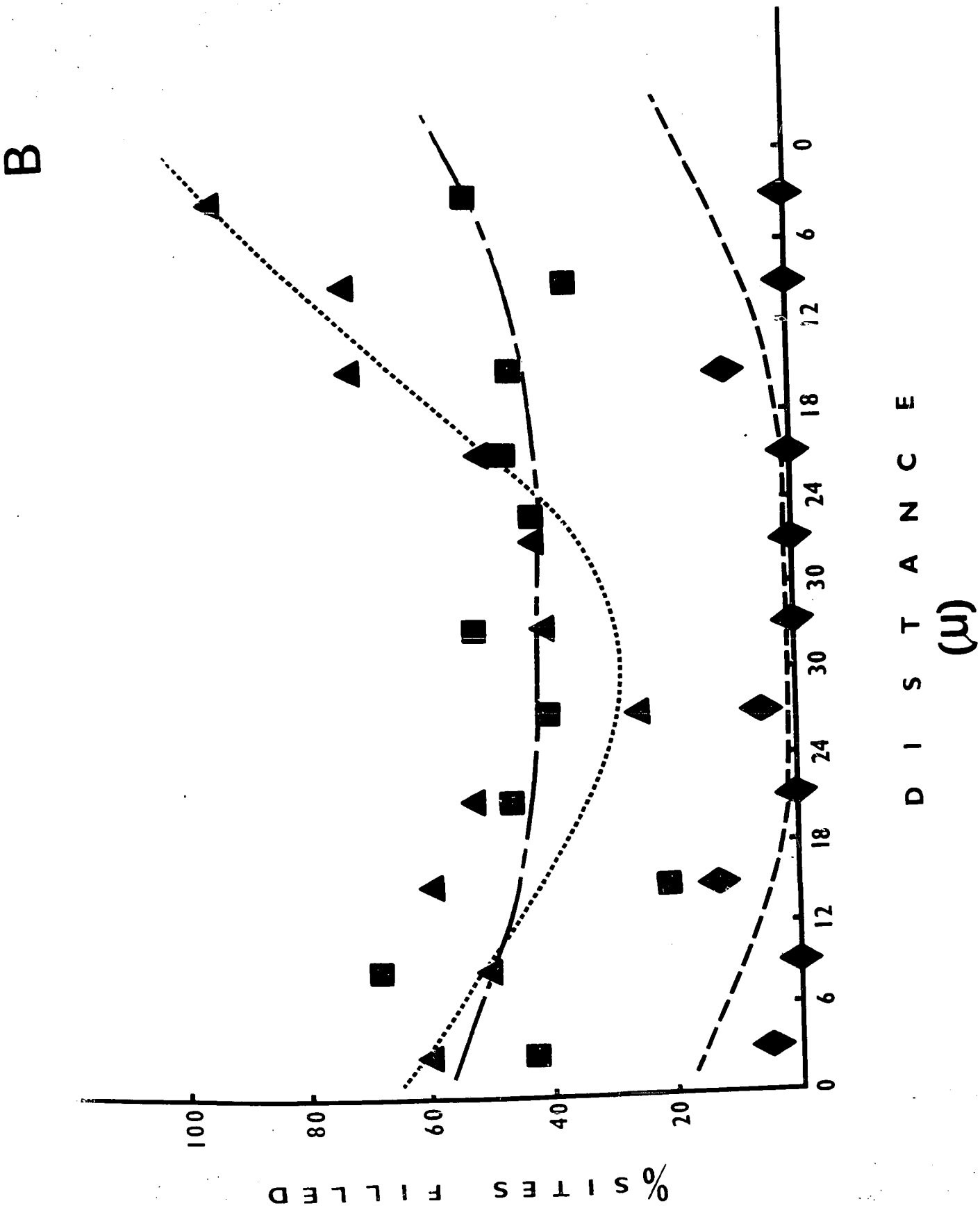
A-C normal fibers

D following 20 min. wash

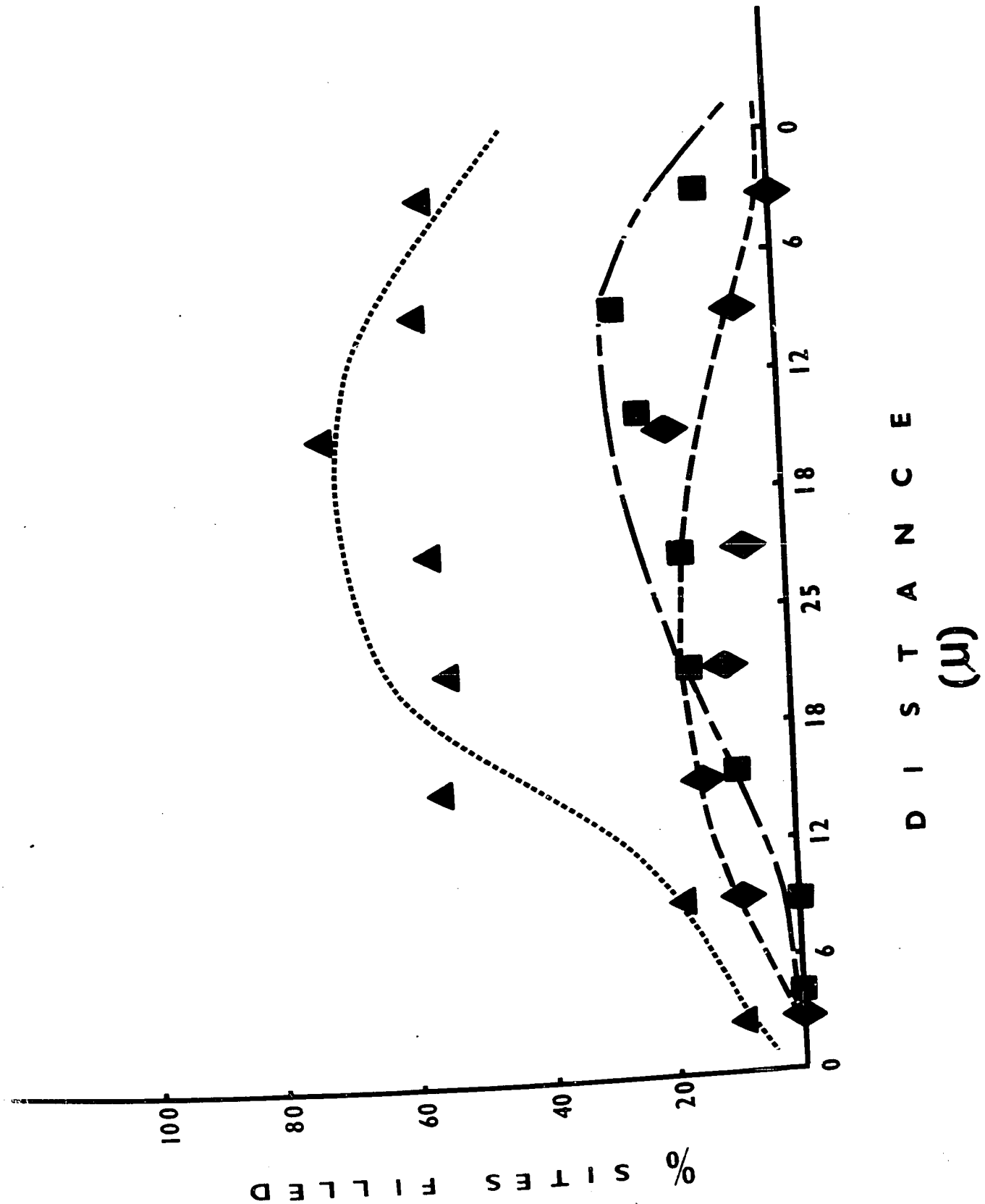
Symbols:

diamonds	-	T tubes
triangles	-	cysternae
squares	-	sarcoplasmic reticulum
solid	-	normal fibers
open	-	washed fiber
abscissa	-	distance from outer edges

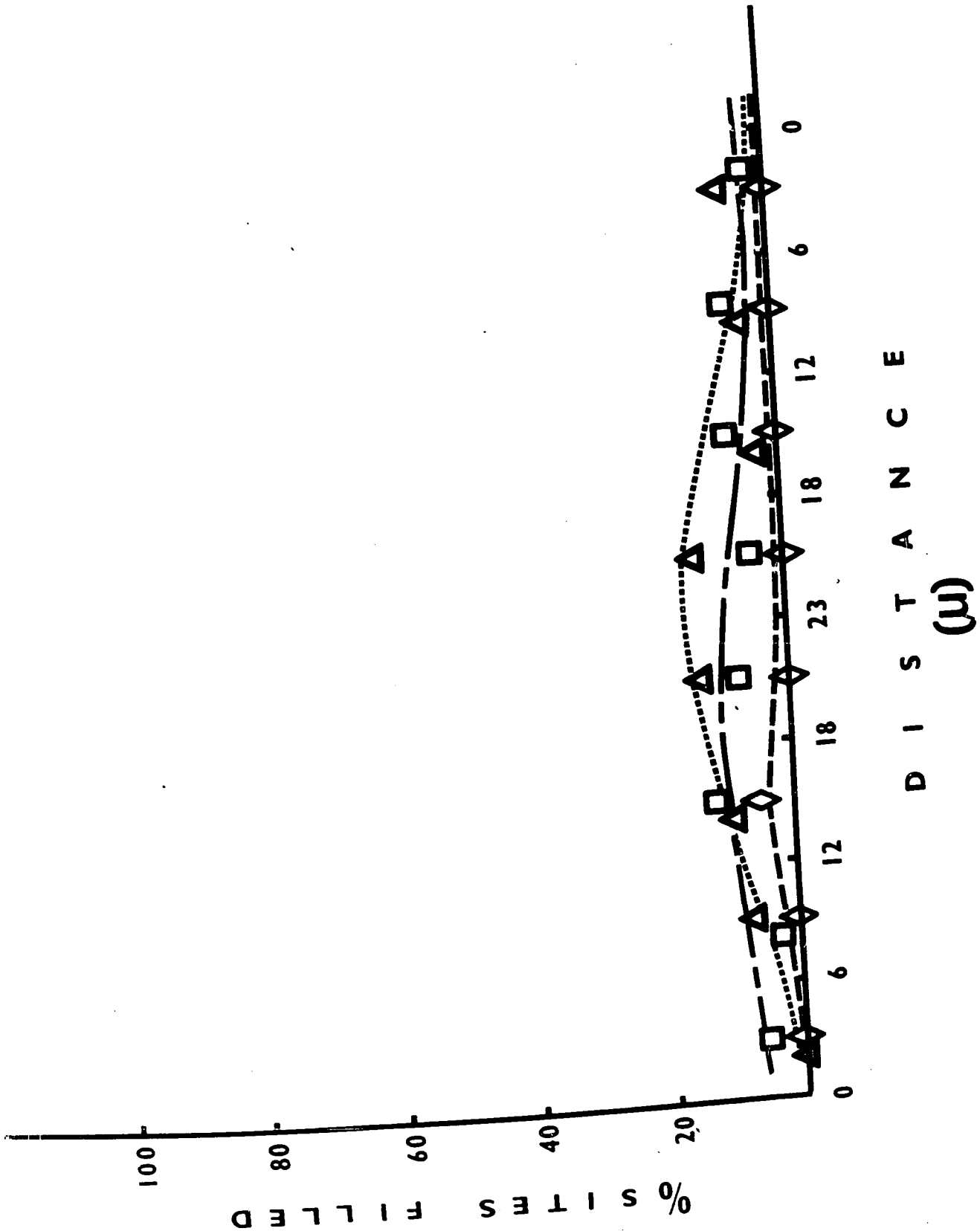

A
% SITES FILLED
100
80
60
40
20
0
6
12
18
23
18
12
6
0
DISTANCE
(μ)


B
% SITES FILLED
100
80
60
40
20
0
6
12
18
24
30
30
24
18
12
6
0
DISTANCE
(μ)

C



D



almost free of it after a wash period of 20 min. duration. Fig. 11 D indicates the typical effect of wash-out on the peroxidase distribution through a cross-section of a single fiber in one experiment. Fig. 12 shows the location of remaining peroxidase in a wash-out experiment.

D. The effect of ouabain on peroxidase penetration

Since it has been demonstrated in previous experiments that the longitudinal elements of the SR are penetrated by the peroxidase molecules, a question arose as to the mechanism of penetration of the molecules into that portion of the SR. Since ouabain is known to inhibit Na^+ pump and therefore perhaps an associated water flow, it was decided to see whether ouabain would have any effect on the penetration of HRPO molecules in the muscle fiber. In the presence of ouabain, peroxidase is still accumulated in SR (two experiments) and the penetration was generally further along the longitudinal tubules and was often found in the A band region which was quite uncommon in the absence of ouabain (Fig. 13,14).

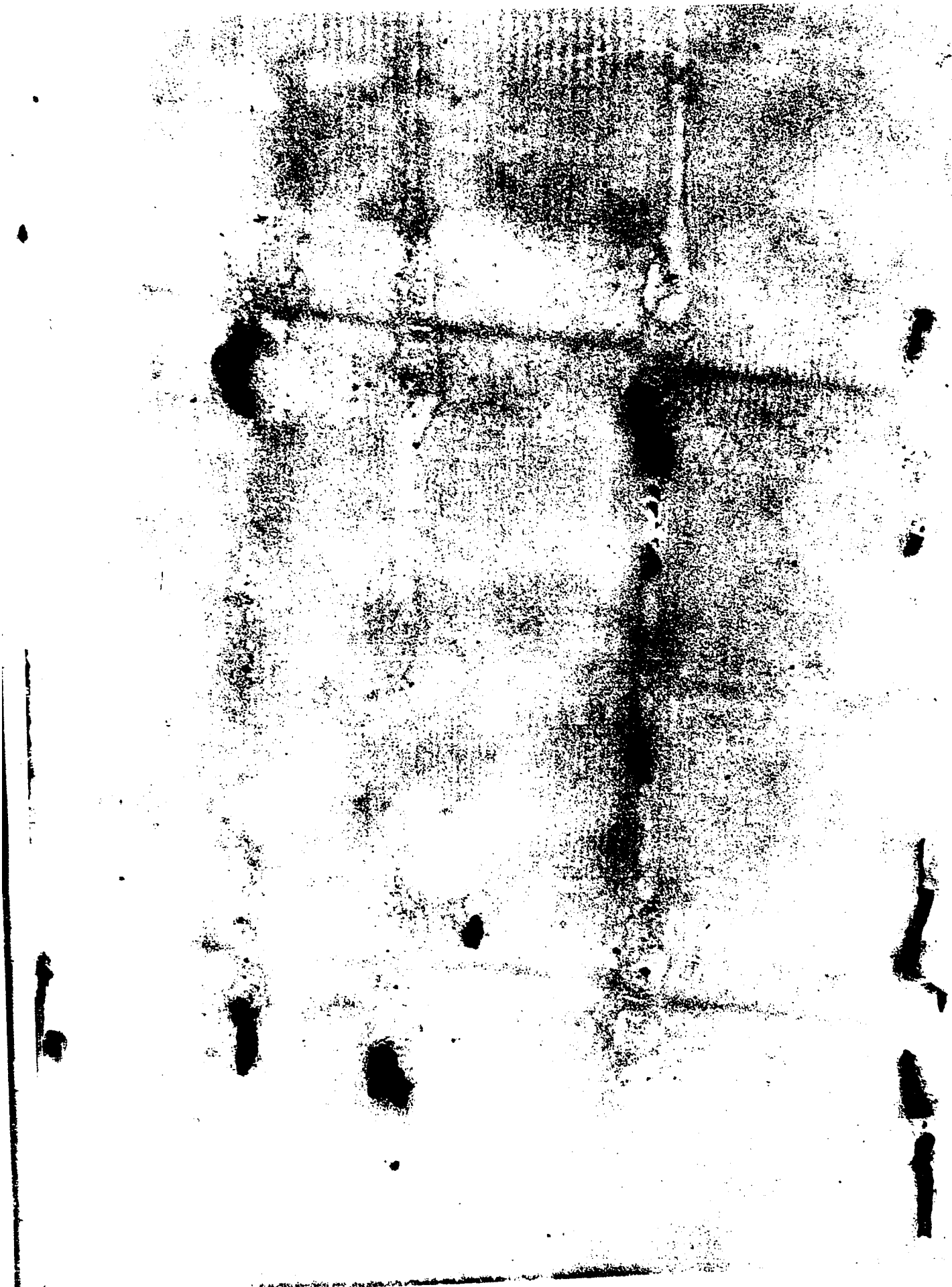
Fig. 12

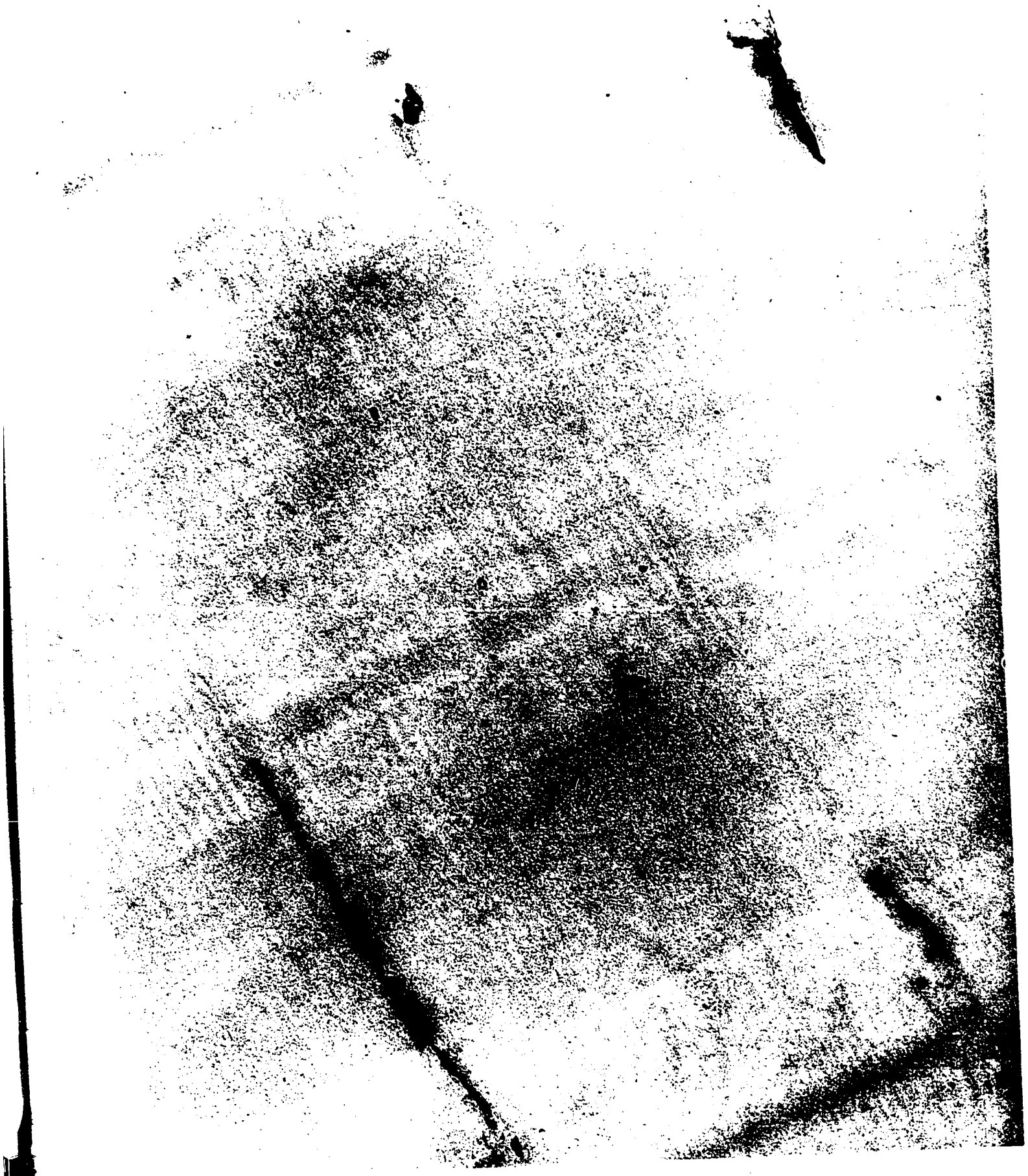
Longitudinal section of a muscle washed for 20 min. in Ringer solution following the exposure to HRPO x 75000

The location of remaining HRPO indicated by arrows.

Fig. 13 and 14

Unstained longitudinal sections of muscle soaked in HRPO containing Ringer solution together with 10^{-5}M ouabain x 100.000





DISCUSSION

It is now generally accepted that the T-tube system of muscles in a variety of species is in direct continuity with the surface sarcolemma and therefore forms a part of the extracellular space. The status of the terminal cisternae and the longitudinal tubules of the SR in relation to the extracellular space is not clear. The morphological studies of Porter and Palade (1957), Andersson-Cedergren (1959), Peachey (1965), Fahrenbach (1965) and others failed to show any connections of the terminal cisternae and the longitudinal elements of the SR with the extracellular space. Furthermore, when tracer molecules which could be visualised by EM (such as ferritin used by Huxley 1964 and Page 1964 and HRPO used by Eisenberg and Eisenberg 1968) were utilised to map out the spaces accessible to them in a muscle, these consistently remained confined to the T-tube system, none having penetrated into the terminal cisternae or the longitudinal elements of the SR. Hill (1964) and Endo (1966) using radioactive albumen and luminescent dye Lissamine Rhodamine B200 respectively indicated that these molecules penetrated some other compartment besides the T-tubes. They found a considerable amount of tracer molecules in the I band region. However, due to the fact that only light microscopy was used in these observations, the

nature of the compartment or compartments penetrated by these molecules could not be established clearly. A suggestion that the terminal cisternae of the SR were involved was made at that time. The supporting evidence for such a suggestion is found in the experiments dealing with volume changes of the SR of muscle under the condition of varied tonicities of the extracellular fluid (ECF). Huxley (1963), Didynska and Wilkie (1963), Sperelakis and Shneider (1968), Birks and Davey (1969) all showed that the volume of both the T-tubes and the terminal cisternae of the SR increase in size when the muscle is exposed to hypertonic sucrose Ringer solution. Birks and Davey (1972) recently pointed out that the swelling of T-tubes occurs artefactually in situations where sucrose is present in Ringer solution under either isotonic or non-isotonic conditions and subsequently fixed in OsO_4 only. They also point out that fixation in aldehyde fixatives prior to fixation with OsO_4 eliminates this artefact. Thus the increase in size of SR observed in this work is most probably valid, since extensive glutaraldehyde fixation prior to OsO_4 was used (see Methods). Birks and Davey (1969) suggested that the most probable mechanism of swelling of the terminal cisternae of the SR is due to penetration of the sucrose molecules into the terminal cisternae of the SR, most probably through the membrane junction between the T-tubes and the terminal

cisternae. This results in an osmotic gradient which draws water towards that compartment causing it to enlarge. However, if sucrose penetrates into these spaces, it is reasonable to question why the route of access for sucrose molecules into these spaces does not provide the means for equilibration of pressure caused by intracellular water entering these spaces. The mechanism of this apparent anomaly is still not known.

If one considers all these observations in the light of molecular weights (M.W.) of various molecules used, the results are not necessarily contradictory as it appears at first. The ferritin molecule used by Huxley (1964) and Page (1964) has a M.W. of 750,000 and is approximately 110 Å in diameter. The albumen molecule used by D.K. Hill (1964) has a M.W. of 73,000 and has an estimated diameter of 81 Å. This molecule was found to be present in the regions of the I band, the Z line and outside of the I band. Here a possibility arises that a molecule of this size may penetrate compartments other than T-tubes which are found only in the Z-line region in frog muscle. Similarly, the luminescent dye Lissamine Rhodamine B200 and M.W. of 558 used by Endo (1966) appeared to penetrate into spaces of estimated volume of 1-2% of total fiber volume. This is more than the estimated volume of the T-tube system which was calculated to be 0.2 to 0.3% of total fiber volume. (Page, 1964; Peachey, 1965). Here

again a suggestion was made that the dye molecules penetrate some compartment other than T-tubes. Tasker et al (1959) also reported that the space available to low M.W. molecules such as sucrose inulin and albumen was greater than the estimated volume of the T-tube system (see Introduction).

On the basis of these observations, the following assumptions can be made:

(1) If pores do exist between the T-tubes and the terminal cisternae, their size is at least 81 Å (the size of albumen molecule) but less than 110 Å (the size of ferritin molecule).

(2) Molecules larger than 110 Å do not penetrate these pores.

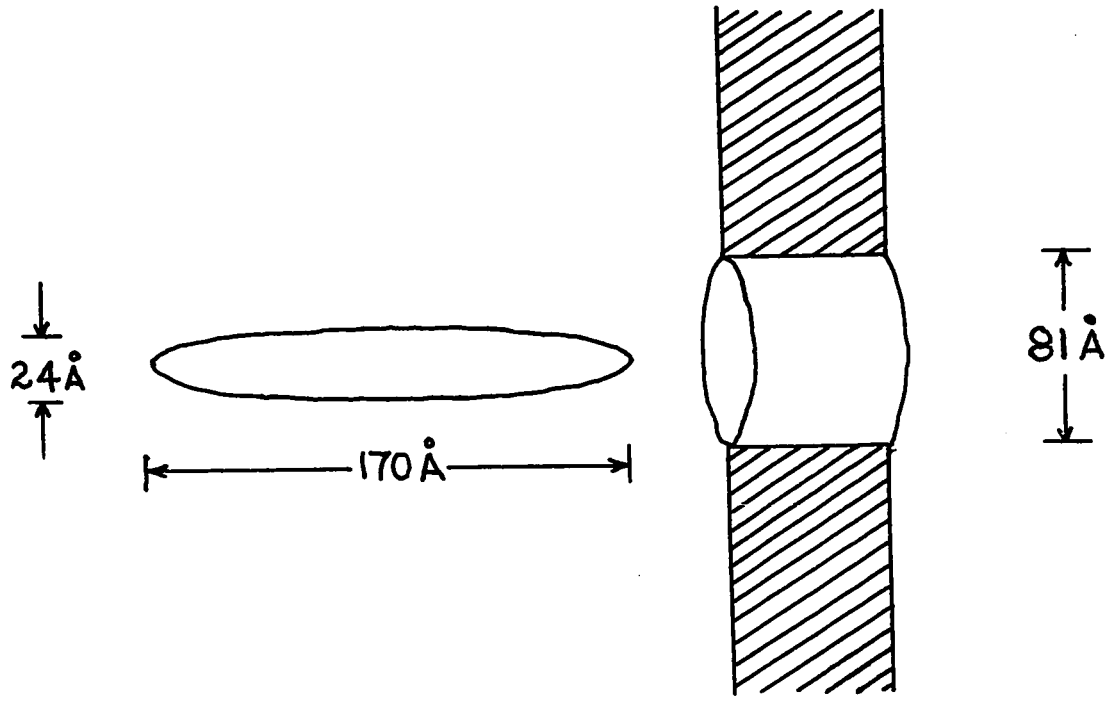
(3) Small molecules, such as sucrose, inulin and luminescent dye Lissamine Rhodamine B200 penetrate these pores readily.

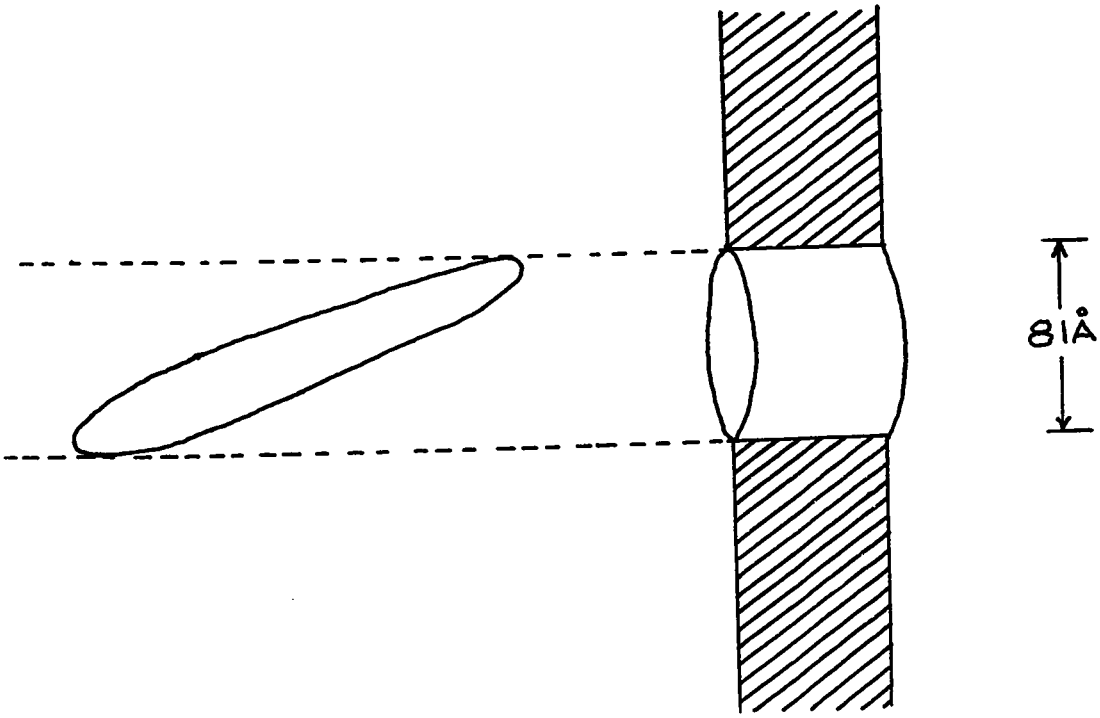
(4) Molecules of HRPO (M.W. 44,000) which have physical characteristics of prolate ellipsoid with an axial ration of 7 (Lemberg and Legge, 1949) have approximate dimensions of 24 x 170 Å. These molecules should be able to penetrate these pores if they approach a pore "head-on" or present a frontal profile of no more than the actual size of the pore (Fig. 15).

The first part of this work indicates that in muscles

Fig. 15

Theoretical model showing penetration of a pore by HRPO molecule.


24Å
170Å
81Å


81Å

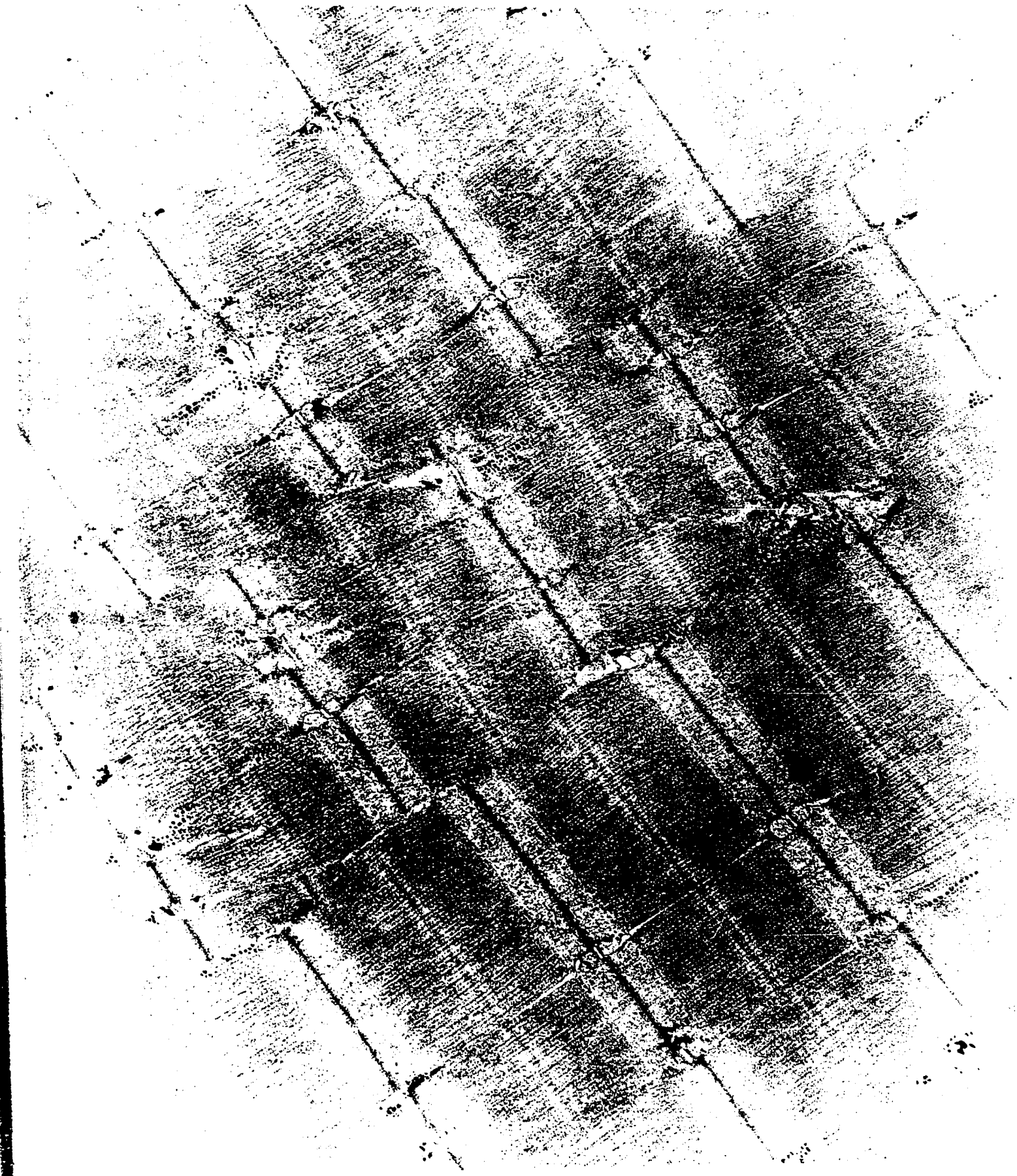
soaked in Ringer solution made hypertonic with sucrose, there is a mean increase in size of T-tubes by 86%, and an increase in size of terminal cisternae by 30% (Table I). Although the increase in size of these compartments does not in itself prove the penetration of sucrose molecules into these compartments, it certainly strongly points in that direction. One may question whether such a swelling is not due to water movement out of the fiber under hypertonic conditions, resulting in excess accumulation of water in these regions. This seems unlikely since the swelling persists after the equilibration of the muscles in hypertonic solution i.e., when there is expected to be no net transfer of water in or out of the fiber. These results, therefore, suggest that sucrose molecules penetrate both the T-tubes and the longitudinal elements of the SR, and the resulting osmotic gradient draws water out of the fiber into these compartments.

This suggestion is very strongly supported by the second part of this work. The results clearly demonstrate the presence of electron dense deposits of HRPO reaction products in the T-tubes, terminal cisternae and the longitudinal tubules of SR of apparently normal, undamaged fibers (Fig. 16).

To explain this consistent finding of HRPO in all parts of the SR the following possibilities may be considered:

Fig. 16

Low magnification of longitudinal section of a fiber soaked for 1 1/2 hrs. in 0.10% HRPO Ringer solution x 20000



(1) The fibers were damaged in some way during the processing of the muscle causing an internal damage which permitted the entry of HRPO molecules into spaces otherwise not accessible to it.

(2) More specifically, the membranes between the T-tube system and the longitudinal components of the SR were disrupted by some procedure.

(3) HRPO molecules penetrate not only the T-tubes, but the surface sarcolemma and diffuse uniformly throughout the muscle fiber. HRPO would then appear only in regions which contain some other substance (ex. Ca^{+2})which may act as a catalyst in making HRPO the peroxidase reaction products electron dense. Relative concentrations of this catalytic substance would then make HRPO visible in varying degress.

(4) Actual pores exist between the T-tube system and the longitudinal components of the SR.

The first possibility is unlikely since the fibers clearly show all the usual structural features in a well preserved state without apparent swelling or vesiculations. Whole intact cross-sections of fibers have been reconstructed from serial micrographs. Peroxidase appears in the central region of these fibers which were clearly not adjacent to any damaged areas.

The second possibility is more difficult to analyse. If there is a disruption of the membranes of the T-tube system one would expect to find some leakage or penetration of HRPO molecules into the sarcoplasm surrounding the T-tube system which is not the case. On the other hand, although the high power micrographs show good preservation of the T-tube membranes and the membranes of the longitudinal elements of the SR, their integrity is by no means always clear. The following factors may contribute to the lack of clear membrane definition in some sections.

(a) the buffer wash following glutaraldehyde fixation was too brief (30 sec) to displace the fixative from the tissue. Subsequent post-fixation in OsO_4 could have failed to produce full penetration, thus producing certain loss of detail (Pease, 1964).

(b) H_2O_2 used for the development of the HRPO after glutaraldehyde fixation but before the OsO_4 could have contributed to the loss of integrity of membranes.

(c) $CaCl_2$ was not used in OsO_4 fixative. It was pointed out that Ca^{+2} could play a role in better preservation of the membranes (Peachey 1964).

(d) Araldite 502 used in embedding of the tissues has relatively high density. One of its drawbacks is the difficulty in staining of the sections embedded in it. Prolonged staining time was

used but it is possible that the contrast achieved was not high enough to outline the membranes entirely.

The third possibility is difficult to discount. However, the literature review ex. Graham and Karnowsky (1966), Strauss (1964 c,d) and Forssmann (1969) failed to mention any role that other substances ex. Ca^{++} may have on the visualisation of HRPO reaction products.

The fourth possibility remains. Fig. 17 indicates that such pores may indeed exist. Very strong support for this statement is offered by recent work of Rubio and Sperelakis (1972). Some time after the original observation of penetration of HRPO into compartments of SR other than T-tubes was made in this work, these authors published results which also indicate that the terminal cisternae and the longitudinal tubules were penetrated by HRPO molecules.

If it is assumed that the T-tube system and the longitudinal elements of the SR are interconnected by a system of pores, this would necessarily mean that the membrane function at this point would be in effect a low resistance pathway for stimuli propagated along the walls of the T-tube system. This may appear to contradict the measurements of conductivity of internal membrane system ex. Falk and Fatt (1964) who calculated that the walls of T-tube system have a high resistance. It is still possible to have a membrane system with high current

Fig. 17

Triad system with a possible pore connecting the T tube with TC x 210.000

resistance and yet allow large molecules to diffuse through it. Such a condition would be possible:

(a) if only a small number of pores exist in that region

(b) if the pores are long enough to have resistance of their own

(c) if specific conductance of fluid in the pores is low

Furthermore when Adrian et al (1969) calculated the length constant of the T-tubes from inward spread of contraction in the muscle, it appeared that the length constant was much shorter than predicted from the measurements of T-tube diameter and wall conductance. The length constant was also suprisingly unaltered by large changes in conductances of the external solutions. These facts made it clear that the present model of the T-tube system and its relation to transmembrane current flow is still incomplete and that the findings of Falk and Fatt (1964) may not have such a simple interpretation. These conflicting findings did in fact lead Adrian et al (1969) to suggest that the outer elements of triads may be capable of transmitting the effect of surface depolarization inwards. This suggests some sort of electrical continuity between the external fluid and the terminal cisternae quite apart from the T-tubes.

The question now arises as to the differences in observations between this work and that of Eisenberg and Eisenberg (1968), since the authors found HRPO

in T-tubes only, none having penetrated into the terminal cisternae or longitudinal tubules of the SR. One can assume that this difference in observations is most probably due to the differences in methods:

(1) the muscles were exposed to HRPO containing Ringer solutions for lengths of time varying from 1/2 hr to 1 hr in contrast to only 1/2 hr soaking time by Eisenberg and Eisenberg. It appears therefore that longer periods of exposure to HRPO are necessary to achieve the penetration of HRPO into the longitudinal elements of the SR.

(2) an attempt was made to expose large areas of the fibers unhindered by fibrous covering enveloping the whole muscle, especially on the superior aspect. This was achieved by cutting the whole muscle longitudinally and removing all the damaged fibers (see Methods). Eisenberg and Eisenberg used the inferior portion of the muscle which had a lesser amount of the fibrous cover than the superior portion of the fibers. The small amount of the fibrous covering of the muscle in that region probably still offered some resistance to diffusion of HRPO molecules.

It is interesting to consider the distribution of the HRPO in the SR. If the SR of the muscle consists of a series of relatively straight tubes with a system of

pores connecting the transverse system (T-tubes) with the longitudinal system (terminal cisternae and the longitudinal tubules) and assuming that there is no active transport involved in this system of membranes as well as no fluid shifts within this system under normal conditions, then one may expect that the distribution of the tracer molecules small enough to penetrate these pores would be quite uniform. Fig. 11 A-C indicates a typical distribution of HRPO in T-tubes, terminal cisternae and the longitudinal tubules. One can readily observe that the distribution is not uniform. The phenomenon is probably due to several factors, some due to the inherent structure of the muscle fiber and some due to artefacts arising from the procedures that the muscle fiber was subjected to so that the tracer could be visualised.

In the first category the following may be considered:

(1) the size distribution of pores between the T-tubes and the terminal cisternae. If one assumes that the mean pore diameter between the two compartments is approximately 81 $\mathring{A}$ (size of albumen molecule), there are probably pores larger than and smaller than 81 $\mathring{A}$. It is reasonable to assume that this would result in smaller accumulation of the tracer in some regions than others, perhaps so small as not to be detected by the present methods.

(2) not all pores between the two compartments may be opened under normal conditions. This would result in accumulation of the tracer molecules in some sites and not others, thus giving rise to non-uniform distribution. Indeed such a suggestion finds support in the experiments by Endo (1966) and very recently by Rubio and Sperelakis (1972). Endo observed an increase in luminescence of a fiber exposed to luminescent dye in hypertonic solution. It was unresolved at that time whether this increased luminescence was due to the enlargement of the T-tube system in hypertonic solution or some other mechanism. Rubio and Sperelakis (1972) reported a qualitative increase in the number of terminal cisternae filled with HRPO under hypertonic conditions as compared with the number of terminal cisternae filled with the tracer under normal conditions. It appears, therefore, that some conditions, such as hypertonicity, seem to open up pores between the T-tube and the terminal cisternae which are closed under normal conditions. It is logical to assume therefore, that the increased amount of luminescence within the muscle under hypertonic conditions observed by Endo was due to opening up of the pores between the two compartments which were previously closed.

(3) the distribution of tracer molecules is not entirely determined by pores, but by flow dynamics. It is clear from the experiments with hypertonic solutions that considerable volumes of fluid can be transferred across the tubular membranes which must lead to pressure gradients along the tubules. If the openings of T-tubes to ECF exist as was demonstrated by Huxley (1964), Endo (1966) Eisenberg and Eisenberg (1968), Rubio and Sperelakis (1972) and others, this must result in a bulk flow of fluids within the T-tube system. Endo (1966) proposed several sites at which there may be a resistance to diffusion of molecules into and out of fibers. They were located

a) at the mouths of T-tubes
b) along the length of T-tubes and
c) at the points of access from the tubules to additional spaces accessible to tracer molecules. The bulk flow of fluids and diffusion of molecules in such a system certainly would not result in a uniform distribution.

The experiments with ouabain seem to support the suggestion that bulk flow of fluid occurs within the T-tube system, as well as between the longitudinal elements of the SR and the T-tube system. Ouabain is

known to inhibit transport of Na^+ across membranes, Caldwell and Keynes (1959). In two experiments where ouabain was used it was observed that HRPO penetrated further along the longitudinal tubules of the SR, even as far as the M line (Fig. 14) than in experiments where HRPO was used without ouabain. This observation could be consistent with the presence of ouabain-sensitive active transport of Na^+ and perhaps H_2O with the general direction of flow of fluids from the longitudinal components of the SR into the T-tube system via the triad junction and then out to the external medium. Suppression of active transport by ouabain will then tend to promote a process of diffusion into the terminal cisternae and the longitudinal tubules of the SR through a system of pores. The geometry of these openings and the extent of local active transport would lead to differences in the peroxidase entry at the triad junction resulting in non-uniform distribution of the tracer molecules.

In the second category i.e. the non-uniform distribution of tracer due to possible artefactual causes the following may be considered:

(1) Insufficient exposure of the muscle to HRPO. Although the length of time that a muscle was exposed to HRPO lasted up to 1 hr, this possibly was not long enough for all the available sites to be filled. Indeed, it was pointed out by

Rubio and Sperelakis (1972) that long exposure times are required to achieve good penetration. However, since the authors did not have any data regarding the distribution of the tracer molecules within the compartments available to it, it is difficult to assess whether longer exposure times have an effect on the distribution of the tracer.

(2) these same authors point out that

a) glutaraldehyde diminishes the enzymatic activity of HRPO and both the duration of exposure and the concentration of glutaraldehyde are important. Since 1 hr of 4% glutaraldehyde fixation was used (as compared to 20 minutes of 1.5% glutaraldehyde fixation used by the authors) it is conceivable that HRPO in certain sites was inactivated to the extent that it did not appear in the final micrographs, again giving rise to a non-uniform distribution of the tracer.

and b) uranyl acetate removed some electron-dense deposits of HRPO reaction products especially in the regions where the deposits were in low concentration.

Since the calculations of the distribution of tracer in this work was performed on unstained cross-sections of whole fibers, this last factor probably did not have a bearing on these calculations.

One consistent observation made in this work is the virtual absence of HRPO in T-tubes, with the tracer still present in the longitudinal elements of the SR. This is most probably due to wash-out of the tracer during fixation procedure. Huxley (1964) reported that T-tubes remain open in the early stages of fixation and thus may lead to loss of tracer from the T-tubes, especially if there is an associated water movement within the T-tubes at that time. Tasker et al (1959) described that the sucrose space in frog sartorius increases slowly with increased exposure to sucrose. If we are to assume that this increased sucrose space is due to diffusion of sucrose molecules into the longitudinal elements of SR, then the diffusion rate into these spaces is quite low. It would be reasonable to also assume that the outward diffusion of molecules from these spaces would also be quite slow. HRPO molecules, being much larger than sucrose molecules, having diffused into these spaces would also diffuse out very slowly and perhaps this is the reason why it still appeared in the longitudinal elements of the SR after most of it has been washed out from the T-tubes.

If one accepts the assumption that the longitudinal elements of the SR are connected by a system of pores with the T-tubes as the results of this work indicate, then it is logical to assume also that the contents of the longitudinal elements of the SR are identical to those of the ECF. In such a case an electrical potential is expected to exist across the membranes of these compartments and the sarcolemma. Such a state of affairs certainly would have to be taken account of in interpreting the mechanism of excitation contraction coupling.

SUMMARY

1. In muscles exposed to hypertonic sucrose Ringer solutions both the T-tubes and the terminal cisternae increase in size.
2. Together with the consistent presence of HRPO in the T-tube, terminal cisternae and longitudinal tubules of the SR this seems to indicate that a functional connection exists between these compartments.
3. The distribution of HRPO is non-uniform throughout the fiber.
4. Several factors, both artefactual and functional can explain this non-uniform distribution of the tracer molecules.

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