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BINDING SITES ASSOCIATED WITH MEMBRANE
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THE CYTOCHEMICAL LOCALIZATION AND CHARACTERIZATION
OF POTENTIAL CALCIUM BINDING SITES ASSOCIATED
WITH MEMBRANE SYSTEMS OF MUSCLE

By

Margaret Ann Goldstein

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[Signature]

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THE CYTOCHEMICAL LOCALIZATION AND CHARACTERIZATION OF POTENTIAL CALCIUM BINDING SITES ASSOCIATED WITH MEMBRANE SYSTEMS OF MUSCLE

INTRODUCTION

The muscle cell is a popular system for studying relations of structure and function at the subcellular level and for good reasons. Two distinct morphological systems, the contractile elements and the membrane components, are easily recognized in the electron microscope. These same two systems have been isolated and to some extent characterized by biochemical and physiological studies. Data obtained from each of these approaches can be correlated at the level of one common subunit, the sarcomere, making feasible studies in depth on the molecular basis of contractile activity.

MORPHOLOGY OF CONTRACTILE SYSTEM

A comparison of striated muscles from animals of many different phyla, classes and species reveals a common pattern of organization (Peachey, 1968). There is always an ordered array of thick filaments surrounded by thin filaments. While the arrangement of thick filaments is precisely hexagonal in all striated muscle, the number and arrangement of thin filaments around the central thick filament is somewhat variable. In vertebrate muscle and insect flight muscle a hexagonal array of six thin filaments is seen, but in certain invertebrate muscle, orbital arrays of nine to twelve thin filaments appear in cross section. The overlap of thick and thin filaments, together with the placement of myofibrils in lateral registry, gives rise to a typical banding pattern. Visible at the light microscope (LM) and electron microscope (EM) levels these bands, A, I, Z, H and M, are used as landmarks to identify various regions of the sarcomere.

The thick filaments are composed mainly of myosin molecules and
have extensions (the cross bridges) containing most or all of the heavy meromyosin with its associated ATPase activity (Pepe, 1966 and 1967). The thin filaments contain primarily actin molecules, some tropomyosin and three recently isolated muscle proteins -- troponin, \(\alpha\)-actinin and \(\beta\)-actinin (Peachey, 1968).

**MORPHOLOGY OF MEMBRANE SYSTEMS**

**Sarcolemma and T-system**

A system of membranous tubules (T-system) primarily oriented in the transverse direction with respect to the long axis of the fiber has been observed consistently in vertebrate muscle. Continuity of these tubules with the muscle cell membrane or sarcolemma has been demonstrated repeatedly among different muscle types and is thought to represent a general finding. Morphological continuity between the sarcolemma and T-system was first seen in the electron microscope in fish muscle by Franzini-Armstrong and Porter (1964a,b). Subsequent reports of continuity in fish muscle have appeared (Jasper, 1967, and Kilarski, 1967). Convincing images of sarcolemmal invaginations in amphibian and mammalian muscles have been more difficult to obtain. However, continuity of the T-system with the surface membrane of frog muscle is indicated from experiments using the electron opaque ferritin molecule (Huxley, H. E., 1964; Page, 1964; Peachey and Schild, 1968) and a fluorescent dye (Endo, 1964). A recent study on surface membranes of guinea pig muscle using the freeze-etch method, has revealed apertures along the sarcolemma corresponding to openings of the T-system so convincingly demonstrated with a lanthanum tracer technique (Rayns, et al., 1968). Additional evidence that the T-system is derived from the sarcolemma comes from studies on the cytodifferentiation of muscle (Ezerman and Ishikawa, 1967; Shimada, et al., 1967; Ishikawa, 1968).
The invagination of the sarcolemma at regular intervals forming the T-system means a substantial increase in total surface area of the muscle cell. In the case of frog sartorius, Peachey (1965b) calculated that for a fiber 100 μ in diameter, the T-system provides a surface area seven times greater than that of the outer surface or sarcolemma proper. Hence, the T-system augments the total surface area to about eight times the value calculated for a simple cylinder.

Sarcoplasmic Reticulum

The sarcoplasmic reticulum (SR) is a network of anastomosing tubules and cisternae that is wholly intracellular. This membrane system corresponds to the smooth endoplasmic reticulum of other cell types (Porter and Palade, 1957; Porter and Franzini-Armstrong, 1965). The SR functions in glycogen metabolism (Fahimi and Karnovsky, 1966); it appears to be a derivative of granular endoplasmic reticulum (Ezerman and Ishikawa, 1967; Shimada, et al., 1967); and it is never continuous with the cell surface (Peachey, 1968).

The SR is composed of repeating membrane systems which parallel the ordered contractile elements. The subunit that is common to both the SR and the contractile apparatus is the sarcomere, which is the portion of the myofibril that extends from one Z line to the next. In vertebrate muscle each subunit of SR has three morphologically distinct areas. The first of these, the terminal cisternae, are located either in the I band on either side of the central T tubule at the Z line or in the A band on either side of the T tubule at the A-I junction. The longitudinal tubules occur in the region of the A band and connect the terminal sacs with the third portion of the SR, the central cisternae or tubular elements which are located at the M band.

Specialized regions of contact between the terminal sacs and the T-tubules occur at regular intervals in adult muscle. They appear early
in development (Ezerman and Ishikawa, 1967) and take the form of dyads, triads, or pentads (see Diagram III) (Peachey, 1968). The most common arrangement is the triad which consists of two terminal sacs of the SR with an intermediate T tubule. Periodic dense structures between the membranes forming the triad have been observed (Revel, 1962; Fahrenbach, 1965; Kelly, 1967; Armstrong, 1968). The morphological similarity of these regions to septate junctions between epithelial cells (Wiener, et al., 1964) has prompted Fahrenbach (1965) to suggest that ionic coupling occurs at the triad as it does in septate junctions (Loewenstein, et al., 1965).

PHYSIOLOGY OF MEMBRANE SYSTEMS

The importance of calcium ions in the contraction mechanism of striated muscle has been established (Heilbrunn and Wiercinski, 1947; Niedergerke, 1955; Ebashi, 1961; Hasselbach and Makinose, 1961; Podolsky and Costantin, 1964; Sandow, 1965; Ebashi and Endo, 1968). It is generally accepted that the contractile activity of muscle is regulated by the level of ionized calcium in the sarcoplasm surrounding the myofibrils (Hasselbach, 1964b; Weber, 1966; Weber and Herz, 1968). The intracellular translocation of calcium ion is determined by the membrane systems outlined previously, i.e. sarcolemma, T-system and SR. Excitation-contraction coupling is associated with the sarcolemma and the T-system. Calcium release and calcium accumulation are associated with the sarcoplasmic reticulum.

**Excitation-Contraction Coupling**

The sequence of events which take place between the depolarization of the sarcolemma and the subsequent release of calcium from the sarcoplasmic reticulum is called excitation-contraction coupling (e-c coupling). After membrane depolarization the stimulus spreads to the
interior of the fiber via the T-system and calcium is subsequently released from the terminal sacs of the SR (Huxley and Taylor, 1955; Huxley, 1957; Huxley and Straub, 1958; Huxley and Taylor, 1958; Podolsky and Costantin, 1964). Since there are specialized regions of contact between the T-system and the terminal sacs of the SR throughout the interior of the fiber, i.e. triads, excitation contraction coupling is thought to occur wherever a triad is located. Moreover, the presence of pentads in muscles having both extremely fast contraction and extremely fast relaxation times (Revel, 1962) suggests that e-c coupling is facilitated by the increased area of contact obtained when two T-tubules are placed between three terminal sacs of the SR to form the pentad (see Diagram III). Ionic currents in the T-system are thought to be responsible for the release of calcium in the following way (Peachey, 1966). During depolarization of the surface membrane, positive current may flow from the sarcoplasm into the sarcoplasmic reticulum, then into the terminal sacs and from here into the T-system and outward to the sarcolemma. It has been suggested that the major part of the current flowing into the T-tubules comes from the terminal sacs, since in the frog sartorius eight-tenths of the surface area of the transverse tubules are in contact with the terminal cisternae (Peachey, 1965). The morphological similarity of the contact region to septate junctions between epithelial cells (Wiener, et al., 1964) has led to the speculation that ionic coupling via a high conductivity pathway takes place at the triads in muscle (Fahrenbach, 1965) as it does through the septate junction of epithelial cells (Loewenstein, et al., 1965.) The high ionic permeability of triad regions suggested by Fahrenbach (1965) would facilitate even more the passage of ionic currents from the terminal sacs to the T-system. The exact coupling mechanism between the proposed current through the SR and the release of calcium ions is
not known. However, there is evidence from experiments using muscle
potentiators that depolarization of the surface membrane may determine
quantitatively the amount of calcium released by the SR (Sandow, et al.,
1965; Sandow and Isaacson, 1966; Bianchi, 1968).

**Accumulation and Release of Calcium by the Sarcoplasmic Reticulum**

Numerous studies on isolated sarcoplasmic reticulum have shown that
these membranes actively transport calcium (Ebashl and Lipmann, 1962;
Weber, et al., 1963; Hasselbach, 1964a; Martonosi and Feretos, 1964a, b;
The transport process is inhibited by agents, such as phospholipase c,
that disrupt the membrane and by compounds that affect the ATP level
(Martonosi, 1964). In vitro studies on isolated actomyosin as well as
studies on living fibers show that the contractile proteins are in a
relaxed state if the free Ca\(^{++}\) concentration is below 10\(^{-7}\) M (Weber and
Herz, 1963; Portzehl, et al., 1964). Moreover, the vesicles of the SR
can accumulate Ca\(^{++}\) from an initial external concentration in the order
of 10\(^{-5}\) M until levels well below 10\(^{-7}\) M are reached (Hasselbach,
1966). Most workers, therefore, have concluded that the active re-
moval of Ca\(^{++}\) by the SR causes relaxation (Peachey, 1968). Additional
support for this idea comes from a spectrophotometric study by Jobsis
and O'Conner (1966). They showed ionized calcium appears in the sarco-
plasm 1 to 5 msec. after the stimulus, peaks at 55-75 msec. and is half
gone from the sarcoplasm slightly before peak tension development.
Therefore, ionic calcium is present during most of the latency period
and the disappearance of ionic calcium parallels the disappearance of
the active state.

The presence of Ca\(^{++}\) in the SR has been established by two dif-
ferent techniques. Calcium in the terminal sacs of the SR was
demonstrated cytochemically at the level of the electron microscope in
the form of its insoluble oxalate salt (Costantin, et al., 1965). Using autoradiography and a very rapid freeze-dry method, Winegrad (1965a, b) localized Ca^{45} over regions of the terminal sacs during relaxation and near the active sites of the myofilaments during contraction. In a more recent study Winegrad (1968) has shown that in the early phases of relaxation most of the Ca^{45} is located in the A band between the myofibrils while in the later phases of relaxation the Ca^{45} has accumulated over the I bands. This suggests that Ca^{++} is actively taken up by the longitudinal tubules and transferred to the terminal sacs for storage and subsequent release (see Diagram III). It is of particular interest that the large accumulations of calcium at the end of the relaxation cycle are located in the terminal cisternae. These are the very regions most probably involved in the final step of excitation-contraction coupling.

STATEMENT OF THE PROBLEM

Membrane depolarization and ionic currents in the T-system lead to the subsequent release of calcium ions from the SR. The calcium ions interact with the actomyosin system and contraction occurs. Relaxation takes place when calcium reaccumulates in the SR.

The molecular basis for these events is completely unknown. It is hoped that cytochemical studies of muscle will reveal cellular constituents which play a role in the contraction-relaxation cycle as outlined here.

In the present work, three cytochemical techniques for the ultrastructural localization of tissue polyanions were applied to skeletal muscle cells representing different vertebrate classes and different functional categories. The polyanionic material was associated with the sarcolemma, the T-system and the sarcoplasmic reticulum of each and every muscle fiber studied. Both the distribution and the amount
of polyanionic substance associated with these physiologically important membrane systems support the idea that the reactive material serves as an anionic substrate for binding cationic calcium.
MATERIAL AND METHODS

PREPARATION OF TISSUE

Adult vertebrates from three different classes and a cephalochordate were studied. The particular muscles used were chosen because they exhibited varying amounts of sarcoplasmic reticulum, and published morphological and physiological observations were available. Branchial muscle and extraocular muscle from Fundulus grandis, the sartorius muscle from Rana pipiens, diaphragm muscle from the albino laboratory rat and longitudinal body wall muscles from Branchiostoma caribaeum were examined. The animals were pithed or killed and the muscles were exposed. The excised muscle was quickly immersed in several drops of fixative and cut into one millimeter cubes.

All of the muscles except the longitudinal body wall muscles of Amphioxus were fixed in either 1% osmium tetroxide in cold phosphate buffer (Millonig, 1961) containing 2.5 mM CaCl₂ at pH 7.2-7.4 for 90 minutes or a mixture of 1% osmium tetroxide and 2.5% glutaraldehyde (Trump and Bulger, 1966) in cold phosphate buffer (Millonig, 1961) containing 2.5 mM CaCl₂ at pH 7.2 for 60-90 minutes. Good preservation of the acid mucosubstances was obtained with osmium fixation. However, vesiculation of known tubular membrane systems was sometimes observed. The fixative containing both osmium and glutaraldehyde gave satisfactory preservation of polyanionic substances, and in addition gave optimal preservation of the muscle cell membranes. Hence, this technique was useful for routine morphological studies as well as cytochemical procedures. Amphioxus muscles were fixed in either 3% acrolein (Sabatini, et al., 1963) or 3% glutaraldehyde (Sabatini, et al., 1963) in phosphate buffer (Millonig, 1961) at pH 7.4 for 90 minutes at room temperature. After a series of buffer rinses, the
muscles were post fixed in 1% osmium tetroxide prepared in the same buffer for 90 minutes at 4°C.

After fixation, all tissues were rapidly dehydrated through increasing concentrations of ethanol. Some tissue was embedded in a mixture, composed either of three parts butyl- and one part methyl-methacrylate or straight butyl methacrylate partially prepolymerized with heat. Final polymerization was achieved by exposure to 45°C heat or UV light. All the other tissue was embedded in Epon 812 (Luft, 1961).

For light microscopy, thick sections (0.5 -1μ) were cut with glass knives on a Model MT-2 Porter Blum microtome. These sections were stained with a 1:1 mixture of methylene blue and Azure II (Richardson, et al., 1960) and examined with a Zeiss Ultraflot microscope with either bright field or phase optical systems.

For electron microscopy thin sections (500-800 Å) were cut with a diamond knife on a Model MT-2 Porter Blum microtome. These were conventionally stained with 2% uranyl acetate adjusted to pH 5 with 1 N NaOH for five minutes and post stained with lead citrate (Reynolds, 1963) for 2-5 minutes. The sections were examined at 60 kv with a Phillips 200 electron microscope or at 50 kv with an RCA EMU-3F.

CYTOCHEMICAL PROCEDURES

Thin sections of methacrylate-embedded material were stained with colloidal thorium dioxide (Thorotrast) (Revel, 1964). According to this technique thin sections were transferred by loop from the knife trough through each of the following solutions: 30% acetic acid (pH ~2) for at least 5 minutes; 1% Thorotrast in 30% acetic acid for 5 minutes, and rinses of 30% acetic acid for 5 minutes each and distilled water for 5 minutes. The sections were retrieved on a copper grid, dried, and stabilized with a thin film of evaporated carbon. Unstained control sections were transferred directly from the knife
tough to the grid. Throughout this procedure gentle handling was absolutely essential. Repeated failure to pick up sections from any one of these solutions resulted in the displacement of thorium from reactive sites and its nonspecific adsorption elsewhere on the surface of the section. The introduction of fine mesh gold grids which do not react with the acetic acid as well the copper ones, made it possible to stain the sections on grids, thus eliminating the tedious transfer of sections by loop. This newer modification proved useful for Epon embedded material since much longer staining times (1-2½ hours) were then convenient. It had been noted before (Revel, 1964) that methacrylates were much more permeable to surface stains than the epoxy embedding media. However, since some authors (Curran and Clark, 1963) had reported significant staining of Epon embedded sections using colloidal iron, it seemed likely that some thorium would be observed after prolonged staining times. Although the amount of staining in Epon sections was somewhat reduced, it is noteworthy that the quality of morphological preservation was greatly improved.

Another technique using a heavy metal colloid was employed (Wetzel, et al., 1966). Blocks of tissue that had been fixed in 1% osmium tetroxide plus 2.5% glutaraldehyde were rinsed in 7.5% sucrose, frozen onto chucks with dry ice and sectioned in a cryostat set at \(-15^\circ\text{C}\). Sections (80-160\(\mu\) thick) were sandwiched between two pieces of fibrous lens paper tied over the end of a segment of 10 mm diameter glass tubing (Wetzel, et al., 1966) to facilitate transfer from one solution to the next. The sections were then immersed in a staining solution of 1 volume of glacial acetic acid to 3 volumes of dialyzed iron stock solution (Rinehard and Abul-Haj, 1951) at pH 1.8-2.0 for 16-20 hours. Control sections were immersed in 0.9% NaCl acidified to pH 1.8 with glacial acetic acid for similar time periods. After
five or six brief rinses in 30% acetic acid, the sections were dehydrated and embedded in Epon 812 (Luft, 1961).

Both the colloidal thorium and the colloidal iron techniques are essentially similar to the classical Hale reaction (Hale, 1946). The positively charged colloidal micelles bind with acidic groups in the tissue. Since the staining is done at a pH of about 2, only the strong acidic groups remain dissociated and hence, free to interact with the colloidal particles.

Ruthenium red, long known to botanists for its reactivity with acid polysaccharides such as plant pectins, was recently introduced as a cytochemical stain for polyanionic materials at the electron microscope level (Luft, 1965a). According to this procedure, purified ruthenium red is added to the glutaraldehyde fixative and to the osmium solution used for post fixation (Luft, 1965a). The tissue is then dehydrated as usual and embedded in Epon.

Ruthenium red is a small polyvalent cation with a molecular weight of about 858. It is a crystalline complex of ruthenium, oxygen and ammonia that forms a true solution, not a colloid. The exact details of the staining mechanism are not known, but ruthenium red forms a complex with osmium which imparts sufficient electron opacity to make the stain useful at the electron microscope level. Luft has proposed that 1) ruthenium red is bound to acidic polysaccharides; 2) the bound ruthenium is oxidized by osmium tetroxide to ruthenium brown which in turn oxidizes the polysaccharide; 3) ruthenium brown is reduced back to ruthenium red when it reacts with the polysaccharides. Hence, the overall reaction is an oxidation of the polysaccharide with an equivalent reduction of osmium to lower, insoluble products.

Differences in the staining procedure used for each of the three cytochemical localizations should be emphasized at this point. Fixed
and embedded tissue is stained in the case of the thorium dioxide procedure, whereas fixed and frozen material is stained in block before embedding in the colloidal iron technique. In contrast, the ruthenium red stain is included in the fixative solutions and penetrates the tissue presumably before fixation is completed. Thus, in each case the stain is introduced at a different stage during the tissue processing. There is a second important difference between the two Hale type reactions. The colloidal thorium dioxide micelles have equal opportunity to react with all tissue components within the section, whereas the colloidal iron particles must penetrate a membrane compartment inside the cell (the SR) either by way of the T-system and terminal sacs of the SR or through the sarcolemma and sarcoplasm to the SR. Problems of penetration may occur with the ruthenium red technique as well as with the colloidal iron procedure. Ruthenium red is known to enter the T-system and penetrate the SR terminal cisternae in the region of the triad (Luft, 1966a). It has not been seen outside the SR except where the sarcolemma obviously has been disrupted.

TRACER TECHNIQUES

Each half of a transected amphioxus (Branchiostoma caribaeum) was injected subcutaneously with 0.2-0.3 ml of horseradish peroxidase (Sigma Type II) (Karnovsky, 1967) 5 mg/0.5 ml Ringer solution. After one hour both halves were fixed in 6% glutaraldehyde in 0.2 M cacodylate buffer at pH 7.2 with 10 mM Ca at room temperature for 4 hours. The tissue was rinsed in 0.2 M cacodylate buffer overnight, then cut into 1-2 mm slabs and incubated in a mixture of 5 mg DAB (3,3′ diaminobenzidine hydrochloride, Sigma) in 10 ml of 0.05 M Tris-HCl at pH 7.6 and 0.1 ml of 1% H₂O₂ (freshly prepared from a 30% stock solution of superoxol H₂O₂) for 15 minutes at room temperature. For the control tissue the H₂O₂ was omitted at this stage. Following post fixation in 1% osmium
tetroxide in 0.2 M cacodylate buffer at pH 7.2 with 10 mM Ca\textsuperscript{++} for 90 minutes at room temperature, the tissue was dehydrated in ethanol and embedded in Epon as usual (Luft, 1961).

A whole amphioxus (B. caribaeum) was placed in 3 % glutaraldehyde in 0.2 M cacodylate buffer at pH 7.6 mixed 1:1 with 4 % lanthanum nitrate in distilled H\textsubscript{2}O neutralized with 0.1 N NaOH (Revel and Karnovsky, 1967). After two hours the animal was cut into 2 mm slabs and the tissue was returned to the fixative and left overnight. After several rinses during one hour in lanthanum and cacodylate buffer with 5 mM Ca\textsuperscript{++}, and post fixation in 1 % osmium tetroxide in 0.2 M cacodylate buffer at pH 7.2 at room temperature, the tissue was quickly rinsed in 50 % ethanol and dehydrated rapidly and gradually to 100 % ethanol. The tissue was embedded in Epon (Luft, 1961).

AUTORADIOGRAPHY

The minnow Fundulus grandis was narcotized with MS 222, the heart was exposed and 0.1-0.2 ml of Na\textsubscript{2}S\textsuperscript{35}O\textsubscript{4} 100\mu c/0.1 ml Ringer's solution was injected into the conus arteriosus. After 5, 15 and 30 minutes, the inferior oblique eye muscles were excised and fixed in 1 % osmium tetroxide in Millonig's phosphate buffer (Millonig, 1961) at pH 7.4. The tissue was then dehydrated in ethanol and embedded in Epon (Luft, 1961). Thick sections (0.5-1\mu) were placed on light microscope slides. Then the slides were dipped in Ilford L-4 emulsion diluted 5 parts water to 1 part emulsion and heated to 45\degree C. After the slides were dry, they were placed in a black plastic slide box containing dessicant sealed with black plastic tape and stored for 6 weeks. The emulsion coat was developed in a mixture of 2 parts distilled H\textsubscript{2}O to 1 part filtered Dekto! for 1 minute.
NEURAMINIDASE DIGESTION

Frozen sections of formaldehyde-glutaraldehyde-fixed extraocular muscle (Karnovsky, 1965) were treated with *Vibrio cholerae* neuraminidase (Pierce Chemical Company, Rockford, Illinois) 500 units/ml, pH 5.3, for 16 hours at 40°C (Spicer, et al., 1967) just prior to colloidal iron staining. Control muscle sections were incubated for 16 hours at 40°C in enzyme that had been boiled for 10 minutes.
RESULTS

PRINCIPAL CYTOLOGICAL CHARACTERISTICS OF THE MUSCLES

The skeletal muscle cells used in this study contain 1) contractile fibrils made up of a geometric array of thick and thin filaments which form in lateral register a characteristic pattern of A, I, H, Z and M bands (Diagram I and II, Fig. 1, 2), 2) a network of anastomosing tubules and cisternae which surrounds the individual myofibrils and is called the sarcoplasmic reticulum (SR) since it corresponds to the smooth endoplasmic reticulum of other cells (Diagram I, II, and III), 3) a series of invaginations of the sarcolemma primarily in the transverse direction which is called the transverse tubular system (T-system), 4) mitochondria and nuclei, and 5) ground substance containing B-glycogen, ribosomes, etc.

The myoneural junction, where the nerve makes contact with a portion of the sarcolemma, has also been observed in fish branchial and extraocular muscles and frog sartorius muscle.

**Branchial Muscle of Fundulus grandis**

Branchial muscle of *Fundulus grandis* resembles other muscles responsible for undulatory movements such as the truncal muscles of fish in general. The flat ribbon-like myofibrils, frequently branch, and are radially arranged (Fig. 3, 4, 5). Tubules of the T-system are seen at regular intervals at the level of the Z line (Diagram I). The SR is well developed and each sarcomere contains two terminal sacs connected to the central cisternae (or fenestrated collar) by a network of longitudinal tubules. The terminal sacs of adjacent sarcomeres together with a central transverse tubule of the T-system form a triad at each Z line. Great numbers of mitochondria are found grouped together just beneath the sarcolemma, while a few occur randomly.
between myofibrils in the interior of the fiber. The nuclei are peripherally placed and have an elongated shape (Fig. 6). The branchial muscle thus resembles truncal muscle of the tench (Kilarski, 1967) and tail muscle of the Mollie (Franzini-Armstrong and Porter, 1964a and 1964b). The myoneural junction is of the simple type with a synaptic cleft of about 200-300Å. The subjunctional sarcolemma lacks the infoldings so characteristic of amphibian (Birks, et al., 1960) reptilian (Hess, 1965) and mammalian (Anderson-Cedergren, 1959) skeletal muscle. Also, the sarcoplasm in this region is not differentiated into a "sole plate", and the sarcolemma is quite close to neighboring myofibrils in this region (Fig. 7). In the longitudinal section seen in Fig. 7, a finger-like projection of the axon into the muscle fiber has been cross cut so that the sarcolemma appears to surround the axon.

**Extraocular Muscle of Fundulus grandis**

Two different types of extrinsic eye muscle fibers of fish have been reported. Reger described fibers from Fundulus heteroclitus (a species closely related to the one used in this study, *Fundulus grandis*), which contain a highly developed sarcoplasmic reticulum with triads at the A-I junction (Reger, 1961). Kilarski examined extraocular muscles from the tench (*Tinca tinca* L.) and found two fiber types. Cross sections revealed large white fibers containing ribbon-like myofibrils radially arranged and small red fibers containing irregularly packed fibrils which had a polygonal shape. The mitochondria of the white fibers were grouped together just beneath the sarcolemma and had poorly developed cristae; while the mitochondria of the red fibers (so named for the appearance of red pigment attributed to cytochromes and myoglobin in fresh muscle) were more numerous, were located among the myofibrils as well as beneath the sarcolemma and had
a great number of well-developed cristae. Longitudinal sections of
the white fibers revealed a sarcoplasmic reticulum with triads occurring
at every Z line, whereas in the red muscle the triads were located at
the A-I junction. Significant from the standpoint of this study was
Kilarski's observation that the terminal sacs of the SR contained large
accumulations of granular or fibrous material while the longitudinal
tubules and central cisternae seemed to be devoid of this material. He
also noted that material contained in the T-tubules was less dense than
the material in the SR and that it seemed identical to the surface coat
on the sarcolemma.

The most predominant morphological fiber type seen in the extra-
ocular muscles of Fundulus grandis is a twitch fiber with abundant
glycogen, a well-developed SR and triads at the A-I junction (Fig. 8,
Diagram II). Glycogen granules are seen beneath the sarcolemma, between
the myofibrils and in the I band between the myofilaments (Fig. 9).
Continuity of the T-system with the sarcolemma has been observed in this
fiber (Fig. 10). At the present time this image represents the first
and only clear example of an invagination of the sarcolemma which forms
a triad with the terminal sacs of the SR at the edge of the A band.

Fibers of this type vary in diameter and in mitochondrial content
(Fig. 11-15). Some fibers contain a few mitochondria peripherally
placed (Fig. 16), while others contain numerous well-developed mito-
chondria placed between the myofibrils as well as beneath the sarcolemma
(Fig. 17). In general, this fiber type corresponds to the red muscle
described by Kilarski.

Another twitch fiber type observed was one with a few mitochondria,
with ribbon-like myofibrils arranged radially, and with a well-developed
SR and triads at every Z line (Fig. 18, 19). This fiber type was
similar to the large white fibers described by Kilarski (1967).
Continuity of the T-system with the sarcolemma in this fiber type from tench extraocular muscle was observed by Kilarski (1967) also. It should be emphasized that both fiber types have the granular material in the terminal sacs of the SR (Fig. 10, 19) similar to that found in tench extrinsic eye muscle (Kilarski, 1967). The myoneural junctions (Fig. 20) in the extraocular muscle are quite similar to those seen in branchial muscle (Fig. 7) and extraocular muscle of a closely related species Fundulus heteroclitus (Reger, 1961).

Although slow fibers have been found in mammalian extraocular muscle (Hess, 1961 and Hess and Pilar, 1963) such slow fibers in fish extraocular muscle have not been reported and were not seen in the course of this study. However, their presence has been predicted on the basis of slow steady state contractions similar to tonus contractions, known to occur (Reger, 1961).

**Segmental Trunk Muscles of Branchiostoma caribaeum**

The segmental trunk muscles of Amphioxus (*Branchiostoma caribaeum*) have the least amount of sarcoplasmic reticulum of any muscle examined during the course of this study. The very presence of the SR has been disputed. Peachey (1961) reported muscle lamellae made up of individual fibers of a single type, each fiber containing a single myofibril. He described a series of vesicles beneath the sarcolemma which he thought were for the most part sarcolemma. He suggested that the absence of SR could be explained by the fact that the maximum distance required for diffusion of substances such as Ca\(^{++}\) from the sarcolemma was only 0.5\(\mu\) in this muscle. He concluded that since diffusion was an adequate explanation for movement of Ca\(^{++}\) to active sites in the actomyosin system there was no necessity for a tubular system to be involved in excitation-contraction coupling. However, at the time of Peachey's
publication, there was no conclusive evidence that the T-system and not the SR, was continuous with the sarcolemma. This same author (Peachey, 1968) and others (Franzini-Armstrong and Porter, 1964b and Huxley, 1964) now regard the T-system as an entity separate and distinct from the sarcoplasmic reticulum and believe that the T-tubular system plays the primary role in excitation-contraction coupling. The absence of the T-system in Amphioxus is also suggested by the lower $K^+$ permeability of this muscle, since the T-system has been associated with a high resting $K^+$ permeability in frog twitch fibers (Geduldig, 1965).

The question of whether or not vesicles beneath the sarcolemma are in fact elements of the sarcoplasmic reticulum remains. Flood (1968) in the most recent morphological study on Amphioxus describes not just one fiber type but two distinct types and a third intermediate type. He points out that vesicles ranging both in size and distribution were found in all types of muscle fibers but the arrangement of these vesicles never resembled the sarcoplasmic reticulum of other striated muscle.

Using the criterion that vesicles of the T-system would be continuous with the sarcolemma, whereas the vesicles of the SR would not be (Peachey, 1968), two different tracer techniques (Karnovsky, 1967 and Revel and Karnovsky, 1967) were used during this study to demonstrate which of the vesicles were derived from the sarcolemma. The presence of lanthanum nitrate and horseradish peroxidase in vesicles next to the sarcolemma and the absence of these same tracers in vesicles in the immediate vicinity (Fig. 22, 23, 24), suggests that there are two types of vesicles. Although they are both equally near the sarcolemma, one type of vesicle is separate from this membrane and does not have access to the tracer. The other type of vesicle is or has been continuous with the sarcolemma. It is not possible to say
on the basis of morphological evidence alone whether the vesicles continuous with the sarcolemma represent the T-system, because no distinct apposition in the form of diads or triads between these vesicles and the presumed SR have been regularly observed. However, occasional regions showing even spacing between the sarcolemma and the vesicle may be significant (Fig. 25). The association of glycogen with many of these vesicles (Fig. 25) (Flood, 1968 and Peachey, 1961) is consistent with the idea of a sparse sarcoplasmic reticulum in this muscle since it is well known that the SR plays a role in glycogen metabolism (Fahimi and Karnovsky, 1966). Whether or not the postulated SR of Amphioxus functions in calcium release, uptake or storage is not known, since biochemical studies of muscle relaxation have not yet been performed.

CYTOCHEMICAL STUDIES

It should be emphasized that in the following description of results the word stain is used to indicate a specific cytochemical reaction. The uranyl and lead salt solutions used to enhance overall contrast of morphological features in electron micrographs will be referred to here as conventional stain when used alone, or as counterstains when used after a cytochemical stain. It should also be noted that in general there is less contrast in micrographs of muscle treated only with cytochemical stains than there is in micrographs of conventionally stained muscle.

Thorium dioxide

Branchial Muscle of Fundulus grandis

Low pH thorium dioxide staining of thin methacrylate sections of fish branchial muscle shows localized thorium micelles over regions occupied by the sarcoplasmic reticulum (Goldstein and Philpott, 1966,
Philpott and Goldstein, 1967). The overall morphology of sections of branchial muscle embedded in methacrylate is not as good as that seen in Epon sections, but the preservation is adequate for relating sites of thorium localization to the muscle fine structure (Fig. 26). Particles are also found over smooth-surfaced membranes that occupy the peripheral sarcoplasm and over the surface coat of the sarcolemma. The colloidal particles have a somewhat angular contour with a diameter ranging from 50-80 Å.

The thorium dioxide shows no affinity for myofilaments and there is no apparent staining of mitochondria nor T-tubules. The absence of stain in these areas attests to the specificity of the stain for anionic binding sites in the SR, since all areas of the tissue section are equally exposed to the stain. The presence of thorium micelles closely associated with membranes of the sarcoplasmic reticulum indicates that these membranes possess strong anionic sites which react with the positively charged thorium particles at low pH. The overall quality of the fine structure of the branchial muscle in sections treated with thorium dioxide and in untreated control sections (Fig. 27) is identical.

**Extraocular Muscles of Fundulus grandis**

Although the branchial muscle from Fundulus grandis has a well-developed SR, the twitch fibers of the extraocular muscle have a much more highly developed SR. Cross sections of this muscle reveal numerous profiles of the sarcoplasmic reticulum. Treatment of cross sections of methacrylate embedded material with thorium dioxide at low pH reveals intense staining of the SR (Fig. 28). The colloidal particles are often arranged in linear patterns which outline the cross cut tubules of the SR (Fig. 29). These linear arrays are too
extensive to result from random packing of the stain particles (Revel, 1964) and are interpreted as membrane associated binding sites. Occasional isolated micelles are scattered over other parts of the cell, and although these most likely represent spurious deposits, it nevertheless remains as a possibility that they denote the presence of small amounts of anionic materials. It is obvious from the micrographs that thorium dioxide does not react significantly with the myofilaments (Fig. 30, 31). In addition to the reaction with the membranes of the SR, the sarcolemma and its associated surface coat also bind thorium micelles. Profiles of the transverse tubular system are more numerous in this muscle than in the branchial muscle because the invaginations of the sarcolemma which form the tubules occur at each A-I junction. Thus, the T-system can be seen more easily in oblique sections. Thorium dioxide micelles are also localized over the membranes of this tubular system to about the same degree as seen over the SR.

Due to the spindle shape of the small extraocular muscle fibers, longitudinal sections which graze the edge of the myofibril are obtained more often in this muscle than in branchial muscle. Extensive regions of the T-system can be seen in these longitudinal sections. Sections treated with thorium dioxide once again show staining of the T-system tubules (Fig. 32). These same longitudinal sections of extrinsic eye muscle reveal that the granular material in the terminal sacs of the SR which stains readily with lead salts also binds thorium dioxide. This material corresponds to the amorphous material preserved in the terminal sacs of other muscles such as frog sartorius (Huxley, 1964 and Peachey, 1965).

Epon sections of extraocular muscle were also treated with thorium dioxide and although depositions were not as intense as with methacrylate embedded material, the results were consistent in every respect.
with the conclusion that the membranes of both tubular systems are stained. (See Fig. 33 stained and Fig. 34 unstained control)

Sections of Epon-embedded Amphioxus muscle stained with thorium dioxide at low pH revealed membrane staining of the sarcolemma and the vesicles underneath the sarcolemma (Fig. 35). The presence of concentrations of polyanions was again indicated by the low pH thorium stain.

**Colloidal Iron**

Another Hale-type reaction (Hale, 1946) was employed. The dialyzed iron technique (Wetzel, et al., 1966) has the advantage that the colloidal micelles are smaller and the fine precipitate can be localized more precisely with respect to muscle cell membranes. However, it has the disadvantage of inducing artifacts due to freezing and prolonged acid treatment directly on the tissue.

Muscles of the same functional categories used for the thorium dioxide technique were treated with the colloidal iron procedure. Muscle fibers with triads located at the Z (frog sartorius and fish extraocular) and fibers with the triads at the A-I junction (fish extraocular) were compared after colloidal iron treatment.

Despite the harsh treatment of freezing and prolonged acid treatment in block, the essential elements of muscle fine structure of frog sartorius muscle and fish extraocular muscles have been preserved. The membranes of the SR are intact and show little or no swelling. The very light areas near the SR are probably "blank" spaces representing glycogen loss from the muscle during the prolonged acid treatment (Orrell and Bueding, 1964). However, since this same region near the SR contains proportionally more water than the areas occupied by the filaments, some ice crystal damage may be present also. The mitochondria are seemingly well preserved. Although the myofilaments appear somewhat
disorganized in cross section, the typical banding pattern of thick and thin filaments can be seen in oblique and longitudinal sections.

Thin sections of frog sartorius muscle treated with colloidal iron reveal a characteristic staining pattern. Images at low magnification of oblique sections (Fig. 36, 37) that have not been counterstained show very heavy deposits of colloidal iron located on the surface coat of the muscle cells and on the sarcolemma. Iron particles are also seen along the plasmalemma and the Golgi membranes of a fibroblast (Fig. 37) which is known to secrete acid mucopolysaccharide as well as monomeric collagen (Grossfield, et al., 1957, Yardley and Brown, 1965). In the muscle cell the iron stain is localized over regions occupied by the SR. However, the details of the membrane fine structure are masked by the stain. Dense accumulations of the stain are seen in the I band on either side of the Z line as well (Fig. 37). At higher magnification, the actual membrane staining of the SR can be seen in cross sections, especially where the leaflets of the membrane have been lightly counterstained. Figure 38 lightly counterstained with uranyl acetate is compared with Figure 39 counterstained with both uranyl acetate and lead citrate.

The same characteristic pattern can be seen in cross sections of fish extraocular muscle treated with the colloidal iron procedure. The fine colloidal particles are evenly deposited over the membranes of the SR and on the sarcolemma (Fig. 40, 41). Two cross sections of the control extraocular muscle run in parallel with the colloidal iron procedure are shown in Figures 42 and 43. Figure 42 is unstained and Figure 43 has been stained conventionally with uranyl and lead salts. Longitudinal sections reveal that the I band stains in a similar manner to that seen previously with frog sartorius muscle. In addition, sections which show triad regions (Fig. 44) reveal colloidal iron
deposits on the membranes of the T-system as well as on the membranes of the SR. The clumps of material preserved in the terminal sacs of the SR also react with the iron micelles as they did with the colloidal thorium dioxide. The results obtained with colloidal iron treatment not only confirm the reactions seen with colloidal thorium but reveal an additional reactive site as well.

**Ruthenium Red**

Application of the ruthenium red technique (Luft, 1965a) to extraocular muscle of *Fundulus grandis* gives results similar to those obtained with the Hale-type reactions. Dense accumulations of ruthenium red positive material are seen along the sarcolemma, in the synaptic cleft of the myoneural junction and in the T-tubules (Fig. 45). Increased density of the sarcolemma and SR membranes of the terminal sacs in these regions is interpreted as ruthenium staining also (Fig. 47). The granular material in the terminal sacs of the SR, which is consistently preserved in glutaraldehyde-osmium fixation, appears ruthenium red positive (Fig. 45-49). Comparison of ruthenium treated material with unstained Epon sections of similarly fixed material (Fig. 34) gives further evidence of this staining in the terminal sacs. In addition, spherical deposits of varying size and density are regularly found in the tubules of the SR. They are thought to be related somehow to the staining process because they are only seen over the stained granular material in the terminal sacs and are elsewhere excluded. These results are consistent with Luft's (1966a) observation that the membranes of the T-system and terminal sacs offer no barrier to the penetration of ruthenium red in the region of the triad.

The density pattern of the ruthenium deposits along the sarcolemma, within the T-system and terminal sacs of mouse diaphragm muscle
(Luft, 1966a) are similar to those seen in this study. However, the very dense homogeneous deposits seen here in the SR longitudinal tubes of fish extraocular muscle were not observed by Luft in diaphragm muscle. In a more recent paper Luft said, "under certain circumstances, ruthenium red appears intracellularly producing very interesting density distributions which are, at present, uninterpretable".1

In summary, two comparisons can be drawn from the results. The application of two closely related techniques to several different muscles gives a consistent staining pattern. For example, thorium dioxide is localized over the sarcolemma and the membranes of the T-system and SR in fish branchial and extraocular muscle and amphioxus trunk muscle. The most intense staining is seen in the muscle that has the most highly developed membrane systems, i.e., the fish extraocular muscle. Likewise, colloidal iron treatment of frog sartorius and fish extraocular muscle reveals a similar reaction pattern.

A comparison of three different cytochemical techniques applied to the same muscle (fish extraocular) shows that in all cases the sarcolemma, the T-system and its contents, and the SR and its contents are stained. The colloidal iron technique reveals an additional significant reactive component in the I band of muscle.

The application of cytochemical techniques to muscle cells for demonstrating tissue polyanions at the EM level reveals a pattern of localization that is consistent and reproducible.

DISCUSSION

COMMENTS ON THE NATURE OF THE REACTIVE SITES

All three of the cytochemical techniques mentioned here have been used on tissue known to contain certain classes of substances and specific macromolecules. For example, Revel (1964) showed that the matrix of cartilage which consists of acid mucopolysaccharides such as hyaluronic acid and chondroitin sulfate is stained readily by colloidal thorium dioxide. Moreover, he verified that the stained material observed in the EM was hyaluronic acid by showing that digestion with hyaluronidase resulted in loss of stainability with the thorium dioxide. Methylation of the cartilage tissue also completely abolished the stainable material. Since methylation removes sulfate groups (Kantor and Schubert, 1957) and esterifies carboxyl groups (Frankel-Conrat and Olcott, 1945), he concluded that the positively charged colloidal micelles reacted with the strong acidic groups in the acid mucopolysaccharides, such as uronic carboxyl and/or sulfate. Revel showed that another class of tissue substances, the acidic glycoproteins, were also stained by the thorium dioxide technique. Deposits of thorium micelles were associated with the surface coating of the microvilli of intestinal absorptive cells. Still another class of tissue polyanions, the epithelial mucins, reacted with the thorium as seen by the presence of stain over mucous droplets of the goblet cells of the intestine (Revel, 1964).

Likewise, the colloidal iron technique has revealed the same tissue components. Matukas, Panner and Orbison (1967) for example, demonstrated that cartilage matrix stained with colloidal iron, and further, that after hyaluronidase treatment, the staining was abolished. Gasic and Berwick (1963) have shown that the surface coat of ascites
tumor cells which contains acidic sialomucins reacted with colloidal iron, and furthermore, that this staining was abolished with neuraminidase. The surface coat of fibroblasts also stained with colloidal iron (Yardley and Brown, 1965). Various epithelial mucins, particularly those located intracellularly, have been studied with the colloidal iron procedure (Wetzel, et al., 1966 and Spicer, et al., 1967) and nonsulfated as well as sulfated mucins react with the colloidal iron.

The third cytochemical stain employed in this study, ruthenium red, also reacts with these groups of tissue polyanions. Luft has shown that acid mucopolysaccharides (Luft, 1965b) and acidic glycoproteins (Luft, 1966a,b) are ruthenium red positive. He states that polymers which contain large numbers of acidic groups react with the polyvalent cation, ruthenium red.

From the foregoing observations it can be seen that in all three cytochemical techniques the "stains" react with strong acidic groups in the different tissues. These strong acidic groups are most probably 1) sulfates -- found in chondroitin sulfate of cartilage (Grossfield, et al., 1957) or sulfated epithelial mucins (Wetzel, et al., 1966) and 2) carboxyls -- found in hyaluronic acid of cartilage or sialic acid of glycoprotein surface coats and sialomucins. The present studies do not eliminate the possibility of phospholipids, some of which have phosphate groups possessing extremely low pKa's (Oncley, 1959). The specificity of the Hale-type reaction (colloidal thorium and colloidal iron) for these acid groups (sulfate and/or carboxyl) depends on adjusting the pH of the staining solution to a value (pH 2) corresponding to their pKa values (Szirmai, 1963). It is thought that the heavy metal colloidal micelles act as cations and bind electrostatically to the anions in the tissue at this low pH. On the other hand, ruthenium red is used at neutral pH's. Its specificity is attributed to the greater interaction
between the ruthenium red polycation and tissue polymers with a high charge density like chondroitin sulfate (Luft, 1965a).

A survey of the results obtained on other cell types indicates that in the case of extracellular polyanions, acid mucopolysaccharides, such as hyaluronic acid and chondroitin sulfate, are usually found in connective tissue while glycoproteins, such as neuraminic acid, most often occur as secretory products of or as surface coats of epithelial tissues. Polyanions of both types have been observed intracellularly. Thus, it is likely that either acid mucopolysaccharides or glycoproteins or both classes of polyanionic materials are associated with the membranes and within the T tubules and SR cisternae of the muscle cells studied.

The task of distinguishing between acidic polysaccharides and glycoproteins, between sulfate and carboxyl groups, at the EM level is a difficult one. Some preliminary attempts to characterize the stainable groups have been made. It was hoped that the use of autoradiographic techniques with $S^{35}O_4$ on extraocular muscle would show the distribution of label incorporated into the polyanions. Significant incorporation was not observed during the incubation times used, and the results were inclusive. However, studies on labelling of epithelial sulfomucins (Spicer and Lev, 1964) indicate that much longer incubation times (6-20 hrs.) may be required, even with large doses of isotope.

The use of enzyme digestion techniques seems even more promising. In a pilot experiment, treatment on frozen sections of formaldehyde-glutaraldehyde fixed material with neuraminidase from *Vibrio cholerae* and subsequently stained at low pH with colloidal iron did not prevent iron staining. Staining associated with the membranes of the T system and sarcolemma appeared somewhat reduced. No effect on I band staining was observed.
Dougherty (1967) reported that rabbit psoas muscle, chicken breast muscle and frog sartorius muscle all showed 1 band staining with colloidal iron and furthermore, that after methylation for 18 hrs. at room temperature followed by 2 hrs. at 60°C, the staining was significantly reduced. Hyaluronidase treatment had no apparent effect on 1 band staining. Dougherty (1967) suggested that sialomucins occur within the 1 bands. In a more recent paper, however, Dougherty (1969) concluded from a more thorough study at the light microscope level that sialomucins are not present in the myofibrils of the organisms studied. He suggested that a hyaluronic acid-like molecule occurs in the 1 band of fish, chicken, and frog skeletal muscles and that sulfated mucopolysaccharides occur as an additional component in the 1 bands of fish, chicken, and frog, but not in rabbit muscle 1 bands. In support of the presence of these two types of acid mucosubstances, he reported that treatment of muscle sections with Vibrio cholerae neuraminidase and testicular hyaluronidase did not prevent Alcian blue staining; however, saponification prior to hyaluronidase treatment did reduce 1 band staining in all of the muscles studied.

Also, there have been attempts to characterize the staining groups of surface coat both at the light microscope level and the electron microscope level. Goldstein (1959) found diastase-resistant PAS-positive, Alcian blue-positive, material outlining striated muscle fibers and Z bands in several animal species. In addition to these stained areas, Zacks (1961) found Alcian blue, PAS-positive material in the synaptic cleft of the neuromuscular junction. Quite recently Zacks (Zacks and Sheff, 1968) reported that the Alcian blue staining is blocked by methylation. On the basis of results obtained with the Alcian blue-aldehyde fuchsin staining sequence and with induced sulfation, he concluded that the noninnervated sarcolemma contains
primarily neutral polysaccharides. He also found that incubation with collagenase and hyaluronidase removed the Alcian blue staining on the sarcolemma whereas incubation with sialidase and sulfatase did not. Studies at the EM level showed loss of the "basement membrane" after hyaluronidase or collagenase treatment (Zacks and Sheff, 1968).

These studies indicate the presence of strong carboxyl and/or sulfate groups in the portion of the surface coat called the basement membrane. However, the resolution of the enzyme techniques at the light microscope level is not sufficient to determine what groups may be present in the glycoproteins which are presumably much more intimately associated with the sarcolemma. It is hoped that continued improvement of enzyme techniques at the EM level will make the determination of these acid groups more feasible.

POSSIBLE ROLE OF THE REACTIVE SUBSTANCES IN MUSCLE CELL FUNCTION

Stainable Material Associated with the Sarcolemma and T System

The three cytochemical techniques used in this study reveal the presence of polyanionic material in the surface coat of muscle cells (Fig. 30, 37, 46). The association of polyanions such as acid glyco- or mucopolysaccharides with the cell surface has been observed in many cell types (Bennett, 1963 and Fawcett, 1962). Current theory suggests that these substances play a significant role in cellular ion regulation and transport phenomena (Philpott, 1968). By mechanisms such as filtration, selective binding and ion exchange, they influence the composition of the environment in the immediate vicinity of the plasmalemma. Also, these polyanions may play a role in transport phenomena through mechanisms which include hydration of surfaces, osmotic buffering, regulation of diffusion by viscosity and surface charge, and reversible mechanochemical conversion (Katchalsky, 1964; Reichenberg, 1963; and Schubert, 1964).
In addition to the polyanionic material of the surface coat, the results of this study indicate the presence of strong acid groups more closely associated with the sarcolemma (Fig. 36, 37, 46). Polyanions closely associated with the cell membrane have been demonstrated in other studies besides this one. Luft (1966b) demonstrated that the outer leaflet of the plasmalemma of capillary endothelial cells stained with ruthenium red. Revel and Ito (1967) observed thorium staining directly on the plasma membrane of the erythrocyte and interpreted this as further evidence of the sialic acid groups known to be present in these membranes (Wallach, 1967). Benedetti and Emmelot (1967) demonstrated staining of sialic acid groups in the outer leaflet of isolated liver plasmalemma with the colloidal iron technique. Karnaky (unpublished observations) has observed staining of the plasmalemma and tubular system of the teleost chloride cell with the colloidal iron technique.

Cook (1968) considers the sialic acid-containing glyco- or mucoproteins in the plasmalemma as an integral subunit of the membrane. Of particular interest here is the role that fixed membrane negative charges, such as those present in sialic acid residues, play in altering membrane permeability ratios to the Na⁺ and K⁺ (Diamond and Wright, 1968; Wright and Diamond, 1968). These investigators proposed an attractive theory which when extended to the studies reported here may account for 1) properties of the sarcolemma associated with Na-K transport activities in conduction of the action potential, and 2) properties of the T-system responsible for excitation-contraction coupling.

Diamond and Wright developed a model system based on the fact that alkali cation permeability sequences in biological membranes closely resemble those for artificial membranes, whose selectivity
patterns depend upon the field strength of membrane negative charges. From the model system, they predicted that if at pH 7 the membrane was negatively charged, then as the pH was lowered to 2 and negative charges were successively blocked there would be increased chloride permeability and a decrease in the permeability ratio, $\text{PK/PNa}$. Observed changes in membrane charge of rabbit gall bladder epithelium substantiated their predictions. At neutral pH the membranes were negatively charged and were selectively permeable to cations but at low pH the membranes were positively charged and selectively permeable to anions. Furthermore, they found that increased Ca$^{++}$ concentration had the same effect as lowering the pH. They concluded that H$^+$ and Ca$^{++}$ may block the same sites, particularly in nerve where the effects of lowered pH and increased Ca$^{++}$ concentration on permeability and conductance are qualitatively the same.

The Diamond and Wright scheme is compatible with observed phenomena in certain muscle cell membranes. Hagiwara and coworkers (1968) recently analyzed the membrane potential and conductance of the giant muscle fiber of a barnacle (Balanus nubilus Darwin) in relation to changes in external and internal pH changes. They found a sharp increase in membrane conductance associated with a large increase in conductance to Cl ions when the external pH was lowered to values below 5.0. Also they reported that in a Cl-depleted muscle fiber the K conductance decreased with decreasing pH for the whole range of pH examined. These results supported the idea that when the membrane has fixed positive charges, the passage to anions is facilitated but the passage of cations is retarded. Hagiwara, et al. suggested that the variety of Cl permeabilities, relative to K permeabilities, seen in different excitable membranes may reflect a diversity of membrane fixed charges and furthermore, that the different Cl and K permeabilities
would be found if the fixed charges of the membrane were changed. Therefore, the localization of reactive material closely associated with the sarcolemma in this study indicates negative charges in or on the membrane, and furthermore, these negative charges may play a role in altering membrane permeability as suggested by Diamond and Wright.

The polyanionic material associated with the membranes of the T-system demonstrated in this study may also play a role in altering membrane permeability of the T-system membranes. There are two studies which support the idea that the T-system like the sarcolemma is indeed anion permselective (Brandt, et al., 1968, and Freygang, et al., 1964). From these experiments on Cl⁻ flux it is apparent that the T-system tubules swell when the muscle loses Cl⁻ and shrinks when the fiber is gaining Cl⁻. It is puzzling then that the measured Cl⁻ conductance of the T-system is small. Studies on the membrane capacitance, conductance and ion permeability of frog sartorius muscle suggest striking differences between the properties of the sarcolemma and those of the T-system tubular membranes. Hodgkin and Horowicz (1959) have shown that the resting frog muscle membrane is about twice as permeable to Cl as to K ions. Furthermore, they suggested that the chloride conductance, which makes up two thirds of the total membrane conductance, is located principally in the exposed surface membrane (Hodgkin and Horowicz, 1960). Eisenberg and Gage (1968) recently measured frog sartorius membrane capacitance and conductance before and after selectively destroying the T-system with glycerol treatment. They found that indeed the chloride conductance of the transverse tubular system is quite small; yet the potassium conductance and the capacitance of the T-system membranes is about half that of the surface membrane (Eisenberg and Gage, 1968). They
suggested that if there are fixed negative charges in the material which fills the T-tubules, the measured chloride conductance might be smaller than the true membrane chloride conductance. They also suggested that if the T-system had special properties in selective regions such as the triad, the reduced potassium conductance and capacitance of the tubular system might then be explained. Different permeabilities in the T-tubular membranes could be explained if the fixed charges in the membrane were changed. According to the Daimond and Wright hypothesis concerning membrane fixed charges, one could speculate that the negatively charged groups associated with the T tubular membranes demonstrated in this study may be blocked by Ca\textsuperscript{++} especially in selective regions such as the triad. The T tubular membrane would then be permselective to anions such as Cl\textsuperscript{−}. It is of interest that in crab muscle fibers the swelling of T-tubules associated with Cl\textsuperscript{−} efflux initiates and becomes largest at regions of close association with the SR (Brandt, et al., 1968). Therefore, it may be significant that the anion perselectivity of the T-system is greatest in the region of contact with the SR terminal sacs which are also the region of highest calcium concentration in fully relaxed muscle (Winegrad, 1968).

Reactive Material on the Membranes of the Sarcoplasmic Reticulum

The demonstration of strong acid groups closely associated with the SR membranes in these studies indicates the presence of potential Ca\textsuperscript{++} binding sites. Microsomal fractions containing SR membranes (Sampson and Karler, 1963) bind calcium ions. Moreover, the binding properties of these microsomal fractions are similar to those of a cation-exchange resin because 1) there are a limited number of adsorption sites, 2) there is competition between different cations
for the binding sites and 3) there is greater selectivity of the microsomes for a divalent cation. Since acid mucopolysaccharides and glycoproteins have binding properties similar to cation-exchange resins (Schubert, 1964), it is of particular interest that hexosamine, a constituent of acid mucopolysaccharides and glycoproteins, has been demonstrated in the large (15,000-41,000 g.) microsomal fraction identified in the EM as "sarcotubular material", containing SR membranes. Furthermore, these same isolated vesicles displayed calcium pumping activity (Seraydarian and Mommaerts, 1965). On the basis of studies with microsomal fractions, Ebashi and coworkers (1968) have suggested for sometime that Ca\(^{++}\) is bound to the membranes of the SR and that this step precedes active transport.

Winegrad's data on whole muscle cells also indicates that calcium is bound to the SR and that this step may precede active transport. In an autoradiographic study using a freeze-dry, vapor-fixation technique, Winegrad (1968) has shown that the Ca\(^{45}\) uptake in muscle recovering from a tetanus indicates that there are a sufficient number of binding sites in the sarcoplasmic reticulum to produce relaxation without ion transport. Winegrad's data indicates that the calcium-sequestering system which operates during relaxation is located in the longitudinal tubules and intermediate cisterna of the SR. Furthermore, Winegrad suggests that there are at least two different kinds of binding sites in the SR. Since the amount of Ca\(^{45}\) in the SR seems to depend on the specific activity of Ca\(^{45}\) in the sarcoplasm, Winegrad has concluded that the binding sites for calcium in the longitudinal tubules and the intermediate cisterna are in equilibrium with the sarcoplasmic calcium and that these binding sites are different from those in the terminal sacs of the SR. The polyanionic material associated with the membranes of the SR may indeed be calcium binding sites in equilibrium with
sarcoplasmic calcium and may differ from those binding sites in the granular material in the terminal sacs.

Reactive Material in the Terminal Sacs of the Sarcoplasmic Reticulum

The stained polyanionic material in the terminal sacs of the SR may also play a role in Ca$^{++}$ binding. In vitro experiments with microsomal fractions indicate that part of the calcium inside the SR is bound (Carvalho, 1966; Weber, et al., 1966). It is not clear, however, whether the granular material in the terminal sacs of the SR of whole muscle (see Fig. 10, 32, 47, 48) would survive the isolation procedure for SR vesicles. Winegrad's autoradiographic data on whole muscle cells (1968), however, indicates that in the early phases of relaxation the calcium is actively taken up into the longitudinal tubules of the SR and subsequently transferred to the terminal sacs. After depolarization the calcium is released from the terminal sacs. Winegrad has suggested that the calcium binding sites in the terminal sacs of the SR are in equilibrium with the calcium inside the SR.

The calcium transport activity of the SR depends not only on the Ca$^{++}$ concentration external to the SR vesicles, i.e. in the sarcoplasm, but on the internal level of free calcium ion as well (Weber, et al., 1966). If the polyanionic materials in the terminal sacs of the SR serve as calcium binding sites, then the movement of calcium ions from the sarcoplasm to the inside of the longitudinal tubules of the SR and from the longitudinal tubules to the terminal sacs would be facilitated. Some means of regulating the Ca$^{++}$ concentration inside the SR must be present and furthermore, this must be related to the electrical events that couple the depolarization of the sarcolemma to the release of Ca$^{++}$ into the sarcoplasm.
There is direct evidence that electrical stimulation of SR membranes of microsomal fractions releases stored Ca\(^{++}\) and inhibits the uptake of Ca\(^{++}\) (Lee, 1965). Furthermore, the degree of Ca\(^{++}\) release and the degree of inhibition of Ca\(^{++}\) uptake is dependent on the voltage and the frequency of the stimulation (Lee, 1966). It is possible that the current alters the binding properties of the reactive sites in the terminal sacs and brings about the release of calcium from these sites. The concentration of free calcium ion inside the SR then transiently rises so that calcium may diffuse outward into the sarcoplasm. At the same time the increased concentration of Ca\(^{++}\) inside the SR would slow down the rate of calcium accumulation by the longitudinal tubules. The active uptake of Ca\(^{++}\) by the SR membranes which then follows, restores the Ca\(^{++}\) to the terminal sac compartment and it is bound once more to the reactive sites. If the stained material in the terminal sacs is a protein-polysaccharide complex, then the mechanism for the interaction between current and calcium binding sites may involve the protein of this presumed protein-polysaccharide complex. Such a suggestion comes from studies on the calcium binding properties of protein polysaccharide complexes of cartilage. Woodward and Davidson (1968) have found that the remarkable Ca\(^{++}\) binding properties of the SO\(_4^{--}\) groups of the polysaccharide are present only when the polysaccharide is covalently linked to protein. If the protein acts by restricting the movement of the polysaccharide chains for more effective chelation, as these authors conclude, a conformational change in the protein would alter the calcium binding of the reactive sites on the polysaccharide.

From the previous discussion it can be seen that the membrane-associated polyanions demonstrated in this study may very well indeed play a role in the intracellular translocation of Ca\(^{++}\) necessary for
the contraction-relaxation cycle. It is premature to speculate in
greater detail on the physiologic role of the tissue polyanions
revealed in this study.
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KEY TO ABBREVIATIONS USED IN FIGURES

a - arteriole
A - A band
g - glycogen
H - H band
I - I band
LT - longitudinal tubule of the SR
m - mitochondrion
M - mid line of sarcomere
n - nucleus
ne - nerve ending
SR - sarcoplasmic reticulum
TS - terminal sac of SR
TT - transverse tubule
Z - Z band
Diagram I

A three dimensional reconstruction of a twitch fiber of extraocular muscle from *Fundulus grandis* showing the arrangement of the two membrane systems - the transverse tubular system (TT) and the sarcoplasmic reticulum (SR) - with respect to the banding pattern of the contractile elements. The upper cross section at the level of the A band shows a row of vesicles, representing cross cut tubules of the SR, which surrounds the myofibril. The lower cross section at the dense Z line reveals the invaginations of the sarcolemma forming the T-system. The surface view of the myofibril shows the triad, consisting of a central T tubule at the Z line between two terminal sacs (TS) of the SR. Two other regions of the SR, the longitudinal tubules and the central cisternae are also seen in this view. The granular material often preserved in the terminal sacs is seen inside the SR at the I band level. A longitudinal section through several myofibrils shows profiles of the SR between the myofibrils and cross cut transverse tubules at the Z line.

Diagram II

A three dimensional reconstruction of a twitch fiber of extraocular muscle from *Fundulus grandis* showing the arrangement of the two membrane systems in this second fiber type. The triad is located at the edge of the A band near the A-I junction. Compare with Diagram I.
Diagram III

A diagram showing the arrangement of triads and pentads seen in the muscle fibers used in this study. The upper triad, located at the Z line, represents the type seen in fish branchial muscle, fish extraocular muscle and frog sartorius muscle. The lower triad, located at the edge of the A band, represents the type seen in fish extraocular muscle and rat diaphragm muscle. Three terminal sacs and two T tubules form a pentad at the lower right. Between two terminal sacs of the SR a branching T tubule in turn surrounds a third terminal sac. The pentad has been seen in rat diaphragm muscle, fish extraocular muscle and occasionally in frog sartorius muscle.
Figure 1

Longitudinal section of diaphragm muscle fiber from *Rattus norvegicus* stained with uranyl acetate and lead citrate to show typical banding pattern. The A band contains overlapping thick and thin filaments, whereas the I band contains only thin filaments. The unit of contraction, the sarcomere, extends from one Z line to the next Z line and includes the surrounding membrane systems. Magnification approximately 23,500.

Figure 2

Two complete sarcomeres of an extraocular muscle fiber from *Fundulus grandis* are seen in longitudinal section. The H zone contains only thick filaments, and cross attachments between the thick filaments in the middle of the sarcomere make up the M band. The section has been conventionally stained with uranyl acetate and lead citrate. Magnification approximately 32,000.
The myofibrils of a branchial muscle fiber from *Fundulus grandis* in cross section. Each myofibril is surrounded by membranes of the sarcoplasmic reticulum (SR). In the region of the A band cross cut longitudinal tubules of the SR are seen. In the region of the I band portions of the terminal sacs of the SR are apparent. The section has been conventionally stained with uranyl acetate and lead citrate. Magnification approximately 25,500.
Figure 4

A cross section of a branchial muscle fiber from *Fundulus grandis* similar to Figure 3. Thick and thin filaments are seen in the A band, only thin filaments are seen in the I band on either side of the Z. The section has been stained with uranyl acetate and lead citrate. Magnification approximately 33,000.

Figure 5

Another conventionally stained cross section similar to Figures 3 and 4 but at a still higher magnification. The hexagonal array of thick and thin filaments around a central thick filament is at the arrow. Another arrow at the left points to a series of attachments between thick filaments in the region of the M band. Magnification approximately 42,000.
Figure 6

A tangential section of a branchial muscle fiber from *Fundulus grandis* stained with uranyl acetate and lead citrate. The nucleus is seen at the upper left just beneath the sarcolemma. Magnification approximately 28,500.
Figure 7

Longitudinal section of a branchial muscle fiber from *Fundulus grandis* showing a myoneural junction with a synaptic cleft of 200-300 Å. The subjunctional sarcolemma lacks the infoldings so characteristic of other vertebrate skeletal muscle. A finger like projection of the nerve ending into the muscle fiber has been cross cut so that the sarcolemma appears to surround the nerve ending. The section has been stained with uranyl acetate and lead citrate. Magnification approximately 28,500.
Figure 8

Longitudinal section of a fast extraocular fiber from *Fundulus grandis* stained with uranyl acetate and lead citrate, showing the highly developed sarcoplasmic reticulum and abundant glycogen. Each triad, consisting of two terminal sacs of the SR and an intermediate T tubule, is located at the edge of the A band. At the top of the picture a large portion of the triad can be seen on top of the myofibril. The arrow points to a very thin section of SR membrane superimposed on the thick and thin filaments. Profiles of several triads between the myofibrils are starred. Magnification approximately 28,500.
Figure 9

Longitudinal section of an extraocular muscle fiber from *Fundulus grandis* stained with uranyl acetate and lead citrate. The glycogen granules are beneath the sarcolemma, between the myofibrils and in the I band between the myofilaments. Magnification approximately 25,500.
Figure 10

A longitudinal section of an extraocular fiber from Fundulus grandis which grazes the edge of a myofibril. A large portion of several triads can be seen. The arrow points to an invagination of the sarcolemma which forms the transverse tubule seen between two terminal sacs of the SR. The section has been stained with uranyl acetate and lead citrate. Magnification approximately 36,500.
Figure 11

Light micrograph of a transverse section of extraocular muscle from *Fundulus grandis* stained with methylene blue and Azure II. The myofibrils within each of the muscle fibers is stained, while the light areas between the myofibrils are the regions occupied by the SR. The nucleus of the large fiber at the top is just beneath the sarcolemma. An arteriole is at the upper left. Magnification approximately 1,600.

Figure 12

Light micrograph of a transverse section of extraocular muscle from *Fundulus grandis* treated with the Periodic Acid-Schiff reaction to show neutral and acidic polysaccharides with vicinal hydroxy groups. The myofibrils are unstained, while the regions between the myofibrils are stained. Compare with Figure 11. Magnification approximately 1,600.
Figure 13

Light micrograph of a longitudinal section of extraocular muscle from *Fundulus grandis* stained with methylene blue and Azure II. A capillary wrapped in serpentine fashion around a muscle fiber is seen at the top. Magnification approximately 550.

Figure 14

Light micrograph of a longitudinal section of extraocular muscle from *Fundulus grandis*, stained with methylene blue and Azure II. The muscle fibers vary in diameter and mitochondrial content. Magnification approximately 700.
Figure 15

Light micrograph of a longitudinal section of extraocular muscle of *Fundulus grandis* stained with methylene blue and Azure II. In the large fiber at the top, the A, I, and Z bands as well as rows of mitochondria between the myofibrils, are visible. Red blood cells can be seen in the capillary in the middle. Magnification approximately 1,400.
Figure 16

Longitudinal section of extraocular muscle from *Fundulus grandis* stained with uranyl acetate and lead citrate, showing fibers which contain few mitochondria. These mitochondria are usually found just beneath the sarcolemma. Magnification approximately 37,500.
Figure 17

This longitudinal section of an extraocular muscle fiber from *Fundulus grandis* stained with uranyl acetate and lead citrate shows rows of mitochondria between the myofibrils as seen in Figure 15. The mitochondria are well developed with numerous cristae. Magnification approximately 37,500.
Figure 18

Longitudinal section of an extraocular muscle fiber from *Fundulus grandis* stained with uranyl acetate and lead citrate showing triads located at the Z line. Magnification approximately 32,000.
Longitudinal section of an extraocular muscle fiber from *Fundulus grandis* similar to Figure 18. The longitudinal tubules and the terminal sacs of the SR are labelled. The granular material often preserved in the terminal sacs can be seen clearly in this lead stained section. Magnification approximately 64,000.
Figure 20

Longitudinal section of an extraocular muscle fiber from *Fundulus grandis* stained with uranyl acetate and lead citrate. The myoneural junction at the top of the figure is quite similar to the simple type seen in Figure 7 in branchial muscle. Magnification approximately 13,000.
Figure 21

A portion of a longitudinal body wall muscle of *Branchiostoma caribaeum* seen in longitudinal section. Each fiber contains a single myofibril as seen here. Some of the vesicles seen just beneath the sarcolemma are presumed to be SR. The banding pattern is quite similar to that seen in vertebrate muscle (Figures 1, 2, 27). The section has been stained with uranyl acetate and lead citrate. Magnification approximately 24,000.
Figure 22

Longitudinal section of body wall muscle from *Branchiostoma caribaeum*. Electron-opaque tracer horseradish peroxidase is seen in the extracellular space between two fibers and in some of the vesicles just beneath the sarcolemma (arrows). These vesicles were derived presumably from the sarcolemma. Magnification approximately 14,000.
Figure 23

Longitudinal section of body wall muscle from *Branchiostoma caribaeum* similar to Figure 22. The tracer horseradish peroxidase is found in the extracellular space between two fibers but is not found in the vesicles just beneath the sarcolemma. These vesicles presumably represent SR. Magnification approximately 26,500.

Figure 24

Longitudinal section of body wall muscle from *Branchiostoma caribaeum* similar to Figures 22 and 23. The tracer horseradish peroxidase is located in a few of the vesicles near the sarcolemma. Magnification approximately 12,000.
Figure 25

A portion of two sarcomeres of a body wall muscle fiber of *Branchiostoma caribaeum* stained with uranyl acetate and lead citrate. Glycogen granules are associated with the vesicles just beneath the sarcolemma. Even spacing between a vesicle believed to be SR and the sarcolemma is seen in two fibers near the Z line (arrows). Magnification approximately 52,500.
Figure 26

Longitudinal section of a branchial muscle fiber from *Fundulus grandis* embedded in methacrylate and treated with thorium dioxide at pH 2. The section has not been counterstained. Thorium micelles are localized in regions occupied by the sarcoplasmic reticulum. Thorium particles are not seen over the myofibrils or the mitochondria. Compare with Figure 27, the unstained control section. Magnification approximately 19,500.

Figure 27

Longitudinal section of a branchial muscle fiber from *Fundulus grandis* embedded in methacrylate and unstained. The essential features of the muscle cell fine structure have been preserved. Magnification approximately 23,000.
Figure 28

A cross section of an extraocular muscle fiber from *Fundulus grandis* embedded in methacrylate and treated with thorium dioxide at pH 2. Numerous profiles of the highly developed SR are seen. Thorium particles are associated with the membranes of the SR as well as within the tubules. Magnification approximately 28,500.
Figure 29

A cross section of an extraocular muscle fiber from *Fundulus grandis* embedded in methacrylate and treated with thorium dioxide at pH 2. Thorium micelles are closely associated with the membranes of the SR. Magnification approximately 28,000.
Figure 30

Another cross section of an extraocular muscle fiber from Fundulus grandis embedded in methacrylate and treated with thorium dioxide at pH 2. The thorium micelles are again associated with the membranes of the SR. Compare with Figure 31, the unstained control. Magnification approximately 27,500.

Figure 31

A cross section of an extraocular muscle fiber from Fundulus grandis embedded in methacrylate. The section has not been stained. The essential features of muscle cell fine structure have been preserved. Magnification approximately 27,500.
Figure 32

Longitudinal section of an extraocular muscle fiber from *Fundulus grandis* embedded in methacrylate and treated with thorium dioxide at pH 2. Numerous thorium deposits are seen over the membranes of the SR. In addition thorium particles are seen on the membranes of the T-system and associated with the granular material noted previously in the terminal sacs of the SR. Magnification approximately 28,500.
Figure 33

Longitudinal section of an extraocular muscle fiber from *Fundulus grandis* embedded in Epon and treated with thorium dioxide at pH 2. Thorium particles are associated with the membranes of the SR and the T-system. Compare with Figure 34, unstained control. Magnification approximately 43,000.

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Figure 34

Longitudinal section of an extraocular muscle fiber from *Fundulus grandis* embedded in Epon. The unstained section shows that the essential features of muscle fine structure have been preserved. Magnification approximately 43,000.
Figure 35

A longitudinal section of body wall muscle from *Branchiostoma caribaeum* embedded in Epon and treated with thorium dioxide at pH 2. Numerous thorium particles are associated with the sarcolemma and the vesicles beneath the sarcolemma. Magnification approximately 25,500.
Figure 36

A tangential section (similar to Figure 6) showing two fibers of sartorius muscle from *Rana pipiens* treated with colloidal iron at pH 2. Deposits of the colloidal iron are seen along the sarcolemma, associated with the membranes of the SR and in the region of the I band. The unstained A band appears lighter than the I band in this image. Compare with Figure 6 which shows the A band darker than the I band as it usually appears in conventionally stained sections. Magnification approximately 9,000.
Another tangential section of sartorius muscle from Rana pipiens treated with colloidal iron at pH 2. Colloidal iron deposits are seen along the sarcolemma, associated with the membranes of the SR and in the I bands. Also colloidal iron deposits are seen, as expected, in the fibroblast between the two muscle cells. Magnification approximately 25,500.
Figure 38

This cross section of sartorius muscle from *Rana pipiens* treated with colloidal iron at pH 2 shows iron particles associated with the membranes of the SR. The section has been lightly counterstained with uranyl acetate. Magnification approximately 32,000.

Figure 39

Another cross section of sartorius muscle from *Rana pipiens* treated with colloidal iron at pH 2 shows iron particles associated with the membranes of the SR. This section has been counterstained with both uranyl acetate and lead citrate to reveal membrane outlines. Compare with Figure 38. Magnification approximately 32,000.
Figure 40

A cross section of extraocular muscle from *Fundulus grandis* treated with colloidal iron at pH 2. Iron particles are associated with the sarcolemma and the membranes of the SR. Magnification approximately 38,000.
Figure 41

Another cross section of extraocular muscle from *Fundulus grandis* treated with colloidal iron at pH 2 showing iron particles on the membranes of the SR. Magnification approximately 42,500.
Figure 42

A cross section of control extraocular muscle from *Fundulus grandis* treated with 0.9% NaCl acidified to pH 2. The essential features of fine structure have been preserved. Magnification approximately 38,000.

Figure 43

Another cross section of control extraocular muscle from *Fundulus grandis* treated with 0.9% NaCl acidified to pH 2. The characteristic features of muscle are seen particularly well in this section which has been counterstained with uranyl acetate and lead citrate. Magnification 38,000.
Figure 44

A longitudinal section of extraocular muscle from *Fundulus grandis* treated with colloidal iron at pH 2. Iron particles are associated with the membranes of the T tubules, the membranes of the SR and the preserved material in the terminal sacs of the SR. Magnification approximately 40,500.
Figure 45

Longitudinal section of an extraocular muscle fiber from *Fundulus grandis* treated with ruthenium red. The ruthenium red is found along the sarcolemma, in the synaptic cleft, in the T-system, and associated with the granular material in the terminal sacs of the SR. Dense round deposits of ruthenium red material are seen within the sacs and tubules of the SR. Magnification approximately 37,500.
Figure 46

Another longitudinal section of an extraocular muscle fiber from *Fundulus grandis* treated with ruthenium red. As seen before, the ruthenium red is in the T tubules and associated with the granular material in the terminal sacs of the SR. The dense round deposits are seen not only in the longitudinal tubules of the SR but associated with the granular material in the terminal sacs as well (arrows). Magnification approximately 37,500.
Figure 47

Another longitudinal section of an extraocular muscle fiber from *Fundulus grandis* treated with ruthenium red. The reactive material is seen within the T tubules, the terminal sacs of the SR and the longitudinal tubules of the SR. The membranes of the terminal sac in the region of the triad at the top of the micrograph appear more dense than the membranes of the T tubule, especially when compared to an unstained section like Figure 34. Magnification approximately 37,500.
Figure 48

Another longitudinal section of an extraocular muscle fiber from *Fundulus grandis* treated with ruthenium red. The reactive material is seen within the T tubules, the terminal sacs of the SR and the longitudinal tubules of the SR. Magnification approximately 37,500.
Another longitudinal section of an extraocular muscle fiber from *Fundulus grandis* treated with ruthenium red. The reactive material in the terminal sacs of the SR appears quite dense and should be compared to an unstained section such as Figure 34. Magnification approximately 37,500.