evidence to eliminate the possibility that the $Hg(SCN)_4^{--}$ anion is acting as an Hg^{++} buffer and that the precipitating species is the Hg^{++} cation.

MAYO E. BROWN
Department of Pathology,
Harvard Medical School,
Boston, Massachusetts

FRED ROTHSTEIN
Department of Physiology,
Tufts Medical School,
Boston, Massachusetts

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Sarcoplasmic Reticulum of Striated Muscle: Localization of Potential Calcium Binding Sites

Abstract. *Fish branchial muscle stained at a low pH with thorium dioxide shows localization of the stain over the sacroplasmic reticulum. Binding of the positively charged thorium micelles with dissociated acid groups of polyanions in this region suggests a possible mechanism for the storage and release of divalent cations such as calcium.*

The importance of calcium ions in the contraction mechanism of striated muscle has been established (*1*). Membrane depolarization spreads to the interior of the fiber via the transverse tubular system (T-system) and causes the subsequent release of calcium ions from the sarcoplasmic reticulum (SR) into the sarcoplasm (*2, 3*). The calcium ions then activate the adenosine triphosphatase of the actomyosin system, and contraction occurs. Relaxation takes place when calcium reaccumulates in the SR (*4*). The exact mechanisms of these steps are not known. Depolarization may extend to the membranes of the SR via bridges between the transverse tubules and the terminal sacs of the SR located at the triads (*5, 6*). The subsequent release of calcium ions by the SR may or may not be immediately dependent on such a depolarization (see *7*). Also the steps involved in the uptake of calcium by the SR have not been clearly defined (*8*).

The data of Hasselbach (*9*) show that calcium ions are accumulated inside the vesicles of the SR, and recent studies by Weber *et al.* (*10*) suggest that part of this calcium exists in a bound form. The staining technique with thorium dioxide discussed in this paper demonstrates potential binding sites for calcium localized in the regions occupied by the SR.

Branchial muscle from *Fundulus grandis* was fixed for 90 minutes in cold, phosphate-buffered (*11*) 1 percent osmium tetroxide at a *p*H of 7.4. After being rapidly dehydrated through increasing concentrations of ethanol, tissue was embedded in a prepolymerized methacrylate mixture composed of three parts butyl- and one part methylmethacrylate. Thin sections were cut on a Model MT-2 Porter Blum microtome and stained with colloidal thorium dioxide (Thorotrast) (*12*). This technique is essentially similar to the classical Hale reaction (*13*) in that positively charged colloidal particles bind with acidic groups in the tissue. Since the staining is done at a *p*H of about 2, only strong acidic groups remain dissociated and, hence, free to interact with the positively charged thorium dioxide micelles.

Unstained control sections were transferred directly from the distilled water of the sectioning trough to copper grids. All sections were stabilized by a thin film of evaporated carbon before being examined at 60 kv with a Philips 200 electron microscope.

The overall morphology of the branchial muscle is not of the same high quality as that obtained with conventional preparations of striated muscle embedded in epoxy. In general, the epoxies are not as permeable to surface stains as the methacrylates, making them unsuitable for use here. Nevertheless, familiar features of striated-muscle fine structure are easily recognized in the methacrylate sections (Figs. 1 and 2), and the preservation is certainly adequate for relating sites of thorium localization to the muscle fine structure.

The longitudinal section of *Fundulus* branchial muscle (Fig. 1) shows that this moderately fast acting muscle has a well-developed SR with the triad located at the level of the Z-line. Although some of the mitochondria are quite long, they are not particularly numerous. These observations generally support Porter and Franzini-Armstrong's (*5*) correlation between ultrastructure and metabolic needs for skeletal muscles that contract rapidly but in short bursts.

Figure 2 shows localized thorium micelles over regions occupied by the sarcoplasmic reticulum. At this magnification it is difficult to ascertain whether these particles are directly associated with the membranes or whether they are dispersed over the spaces of the cisternae. A survey of images at higher magnifications, however, indicates a greater tendency for the particles to be directly associated with the membranes. That the micelles are seldom concentrated over the dilated terminal sacs of the SR further supports this interpretation. Also attesting to the high specificity of the stain for binding sites of the SR is the finding that neither the membranes of the T-system nor the mitochondrial membranes react with the colloidal thorium. Particles are, however, found over smooth-surfaced membranes that occu-

py the peripheral sarcoplasm. It is apparent from the rather large field seen in Fig. 2 that the stain does not react with the myofilaments. The fine structure of the branchial muscle in sections treated with thorium dioxide and in untreated control sections is identical. Thorium micelles are, of course, absent in the control sections.

From the foregoing observations it is clear that the membranes of the SR possess strong anionic sites that react with the positively charged thorium dioxide micelles. There are several reasons for believing that the anionic sites represent an acid mucopolysaccharide or a glycoprotein. In Revel's (*12*) initial application of the thorium dioxide staining procedure to cartilage, there could be little doubt that the stain was specific for acid mucopolysaccharides. Treatment with hyaluronidase and methylation served as controls, and Revel concluded that the colloidal micelles react with strong acidic groups such as uronic carboxyl, or sulfate of acid mucopolysaccharides, or both.

Further evidence comes from sections of muscle embedded in epoxy and stained with methylene blue-azure II (*14*) which show metachromatic zones where the SR is found due to the presence of polyanions in this region. Another cytochemical technique which demonstrates anionic binding sites in striated muscle was described by Luft (*15*). Ruthenium red, a crystalline ionic complex with a net positive charge, is strongly bound by acid mucopolysaccharides and surface-associated glycoproteins. Luft found ruthenium red in the terminal sacs of the SR as well as in the T-system. It is likely that Luft's ruthenium-positive material corresponds to an amorphous substance frequently preserved in the terminal sacs (*3, 16*). The biochemical studies of Seraydarian and Mommaerts (*17*) on rabbit striated muscle are also relevant. Hexosamine, a constituent of mucopolysaccharides and glycoproteins, was demonstrated in the large (15,000 to 41,000*g*) microsomal fraction identified in the electronmicroscope as sarcotubular material. Moreover, it is significant that the isolated vesicles and membranes of this fraction displayed "calcium pumping" and "relaxing factor" activity.

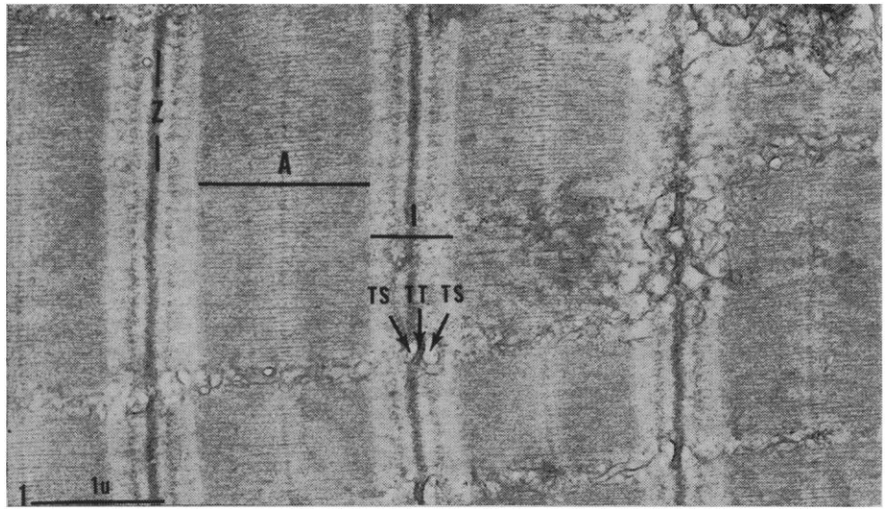

Fig. 1. Unstained control section of branchial muscle from *Fundulus grandis* showing A- and I-bands. Longitudinal elements of the sarcoplasmic reticulum (SR) are seen between the myofilaments. The terminal sacs (TS) of the SR which form the lateral elements of the triad are seen in the I-band. The central element of the triad, the transverse tubule (TT), is located at the level of the Z-line. The triad is indicated by three arrows. Fixed in phosphate-buffered osmium.

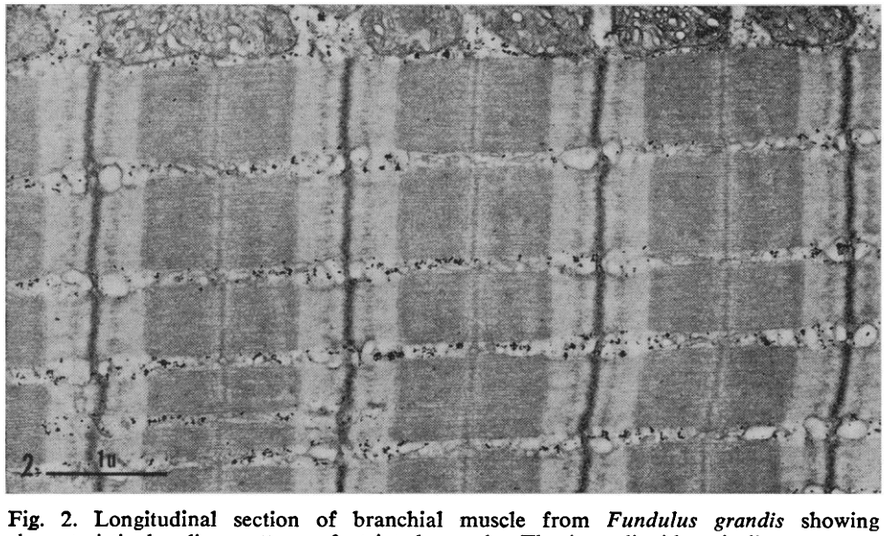

Fig. 2. Longitudinal section of branchial muscle from *Fundulus grandis* showing characteristic banding pattern of striated muscle. Thorium dioxide micelles are seen over regions occupied by the sarcoplasmic reticulum, but not over the myofilaments or mitochondria. Fixed in phosphate buffered osmium and stained only with thorium dioxide.

The presence of Ca^{++} in the SR has been established by two different techniques. Calcium accumulations were cytochemically demonstrated within the terminal sacs of the SR by Constantin *et al.* (*18*). Furthermore, in an autoradiographic study by Winegrad (*19*), Ca^{45} was localized over regions occupied by the terminal sacs during relaxation and near the active sites of the myofilaments during contraction. While our results suggest that potential calcium binding sites are located on the membranes of the SR, they do not exclude the possibility that calcium and perhaps also a mucopolysaccharide-like substance are present within the cisternae proper. Whether the relatively even distribution over longitudinal tubules and terminal sacs of the SR demonstrated by this technique is due to differences in methodology or whether it reflects the physiological distribution of potential binding sites in this muscle is not known at the present time.

Our demonstration of potential binding sites for calcium associated with the SR, and the localization of calcium (*18*) and a mucopolysaccharide-like substance (*16*) in the vesicles of the SR are compatible with the role of the SR in calcium accumulation and storage.

CHARLES W. PHILPOTT
MARGARET A. GOLDSTEIN
Biology Department,
Rice University, Houston, Texas 77001

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Deuterium Oxide: Direct Action on Sympathetic Ganglia Isolated in Culture

Abstract. *Immature ganglia from chicks and rodents were maintained as organized, developing cultures for 2 months or more, during which time they were continuously exposed to deuterium oxide in their medium. Observations of the living cell communities with the light microscope indicated that deuteration within viable limits (up to 25 percent) accelerates and increases the growth of sympathetic neurons and favors their repeated subdivision as a very large size is attained, thus inducing them to recapitulate cyclically the early stages of neurogenesis. Living deuterated cells appear more opaque and heteromerous than control neurons; furthermore, electron micrographs reveal an unusual abundance of granular and fibrillar elements in the nuclei of both neurons and supporting cells. Sheaves of complexly organized fibrillar components appear in the neuronal perikaryon; and ribosomes, Golgi elements, and microtubules are conspicuously numerous. Both fine structure and function of these ganglia therefore appear to have been modified directly by action of the deuterium isotope.*

Soon after the deuterium isotope of hydrogen (mass 2) was discovered, and its biological significance began to be explored, Barbour (*1*) reported a sympathicomimetic effect in mice whose normal body water had been progressively replaced by D_2O to the sublethal extent of 20 percent as they received the compound with their drinking water. In this and subsequent studies summarized by Thomson (*2*), it was observed that with 15 to 20 percent replacement by D_2O the animals exhibited piloerection and exophthalmos and became hyperexcitable and hard to handle. Above this range, combativeness was very pronounced, and convulsions frequently followed stimulation. At 30 percent replacement the mice became comatose and refused to eat; death ensued as the ratio of heavy to normal water increased further. Anatomical and histological studies of lethally exposed animals have shown a variety of organs and systems to be modified, including the cortex but not the medulla of the adrenal (*3*). Bachner *et al.* (*4*) confirmed in mice the earlier reports of sympathetic erethism which was followed by lethargy, convulsions, and death as replacement of body water by D_2O advanced to one-third or more; the nervous system, which appeared grossly normal in these mice at autopsy, was not examined microscopically, nor were the parotid glands. The submandibular salivary gland, however, in histological section showed destructive changes, especially in its ductular epithelium.

Notwithstanding these highly suggestive incidental findings, very little study has been directed in vivo toward the deuterated nervous system per se, and none previously, as far as we know, toward deuterated nervous tissue isolated in culture (*5*). We therefore undertook to expose long-term organized cultures of sympathetic chain and ganglia to deuterium oxide, administered directly by incorporation into the feeding medium, for periods of 2 months or longer. The normal development of sympathetic ganglia in vitro has been under study for several years in this laboratory (*6*), as has their response (*7*) to continued administration of the nerve growth factor (NGF) isolated by Cohen and Levi-Montalcini from the ductal portion of the mouse submandibular salivary gland (*8*, *9*).

Superior cervical, stellate, and chain ganglia were explanted from embryonic chicks (14 to 16 days) and newborn rats and mice onto collagen-coated coverslips and were carried in Maximow double-coverslip depression-slide assemblies up to 83 days without passage or transfer. In this series 440 cultures were used. The biological feeding medium (equal parts of human placental serum, bovine serum ultrafiltrate, and saline extract of chick embryos of 9 days) was supplemented with glucose to a total concentration of 600 mg per 100 ml of medium. The D_2O was incorporated in a fourth fraction so as to compose 5, 12½, 25, or 33 percent of the complete medium. In three types of controls this fraction was replaced by normal distilled water, isotonic balanced saline solution (BSS), or NGF (Abbott), 2 unit/ml. Cultures were incubated at 35.5°C; twice weekly they were washed briefly in BSS and supplied with fresh medium. They were observed daily and photographed repeatedly in the living state. These observations on specific cultures with the light microscope also provided background data for electron micrographs of the same cultures.

The developmental sequence for autonomic ganglia cultured in normal media (BSS and water controls), as observed serially with the light microscope in living material, is outlined as follows:

First, sporadic bursts of fine neurites form a smooth outgrowing network after 48 hours; their interneuritic spaces are then slowly invaded by cells of supporting-tissue type until by 9 to 10 days the explant has thinned sufficiently for the relatively immobile neurons to be discernible. These consist of groups of very small cells so closely packed inside capsules as to appear epithelial; at this time the round nucleus is surrounded by a thin rim of cytoplasm, and the whole cell may be 12 μ in