

THE FINE STRUCTURE OF NEUROMUSCULAR JUNCTIONS AND THE SARCOPLASMIC RETICULUM OF EXTRINSIC EYE MUSCLES OF *FUNDULUS HETEROCLITUS*

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ABSTRACT

The extrinsic eye muscles of the killifish (*F. heteroclitus*) were fixed in OSO_4 (pH 7.6) and subsequently dehydrated, embedded, and sectioned for electron microscopy. The fine structures of neuromuscular junctions and of sarcoplasmic reticulum were then observed.

The neuromuscular junction consists of the apposition of axolemma (60 to 70 Å) and sarcolemma (90 to 100 Å), with an intervening cleft space of 200 to 300 Å, forming a synaptolemma 400 to 500 Å thick. The terminal axons contain synaptic vesicles, mitochondria, and agranular reticulum. The subsynaptic sarcolemma lacks the infolding arrangement characteristic of neuromuscular junctions from other vertebrate skeletal muscle, making them more nearly like that of insect neuromuscular junctions. A comparison between the folded and non-folded subsynaptic membrane types is made and discussed in terms of comparative rates of acetylcholine diffusion from the synaptic cleft and resistances of the clefts and subsynaptic membranes. The sarcoplasmic reticulum consists of segmentally arranged, membrane-limited vesicles and tubular and cisternal elements which surround individual myofibrils in a sleeve-like arrangement. Triadic differentiation occurs at or near the A-I junction. Unit sleeves span the A and I bands alternately and consist of closed terminal cisternae interconnected across the A and I bands by tubular cisternae. The thickness of the sarcoplasmic membranes increases from 30 to 40 Å in intertriadic regions to 50 to 70 Å at the triads. The location of the triads is compared with previously described striated muscle from *Ambystoma* larval myotomes, cardiac and sartorius muscles of the albino rat, mouse limb muscle, chameleon lizard muscle, and insect muscle, with reference to their possible role in intracellular impulse conduction.

INTRODUCTION

Studies on the fine structure of skeletal muscle from widely different vertebrate and invertebrate sources have revealed a basic similarity in organization, particularly in the morphology of the contractile elements (Hodge, 1). Studies on different functional and phyletic types of muscle, however, have revealed differences in the numbers, size, and structure of mitochondria in muscles with different contractile rates (Edwards *et al.*, 2), the morphology of the sarcoplasmic reticulum in muscle from different species (Porter and Palade,

3), the morphology of the contractile elements from muscle with different conduction properties (Ruska, 4), and the structure of neuromuscular junctions in various vertebrate and invertebrate species (Edwards and coworkers, 5-7; Reger, 8, 9; Robertson, 10; and Andersson-Cedergren, 11). The significances of these differences are not understood. Extension of the studies on the fine structure of muscle to a wide variety of functional and phyletic types should assist in the elucidation of these differences.

The specialized properties of extrinsic eye mus-

cles, compared to other types of vertebrate skeletal muscle, are known (Hinsey, 12, and Tiegs, 13) and include properties which are directly related to their function in high precision movement and steady prolonged postural adjustments of the eye-ball. Thus, they exhibit a slow steady state of contraction (tonus or acetylcholine contraction reaction) (Duke-Elder and Duke-Elder, 14) similar to tonus muscle systems of frog skeletal muscles (Kuffler and Williams, 15).

It is the purpose of this paper to present information on the fine structure of both the neuromuscular junction and the sarcoplasmic reticulum of *Fundulus* extrinsic eye muscle, since the physiological properties which distinguish vertebrate extrinsic eye muscles are properties involving both junctional potentials and muscle fiber conduction.

MATERIALS AND METHODS

Orbits of freshly decapitated (5 to 10 seconds) *Fundulus heteroclitus* were flooded by injection with fixative (2 per cent OsO_4 in veronal-acetate buffer, pH 7.6, to which 0.41 gm. of sucrose and 1.5 mg. of CaCl_2 were added per 5 cc. of fixative). The extrinsic eye muscles were immediately (1 to 2 minutes) exposed, excised, and fixed in more of the same fixative at 0–4° C. for 2 hours. After fixation the tissue was rinsed 5 minutes in buffer, dehydrated in 5 minute changes of successive 10 per cent grades of methanol (beginning with 10 per cent), and embedded in a prepolymerized, (2 per cent lucidol-benzoyl peroxide), 3:7 mixture of methyl and *n*-butyl methacrylates with completion of polymerization at 60–65° C.

Sections exhibiting gold, silver, or gray interference colors were cut with glass knives on a Servall, Porter-Blum microtome onto 50 per cent acetone, mounted on collodion-coated slit grids, sandwiched

with another layer of collodion, and examined at 80 or 100 KV in a Philips EM100A electron microscope fitted with a 30 micron objective aperture. Micrographs were made on Kodak Contrast Lantern Slide Plates at initial magnifications of 2,000–30,000 at exposures of 1 to 4 seconds and were photographically enlarged up to 5 times.

OBSERVATIONS

Neuromuscular Junction: The neuromuscular junction of *Fundulus* extrinsic eye muscle consists of the apposition of axolemma (*AL*) and sarcolemma (*S*) (Fig. 1), forming a synaptolemma 400 to 500 Å thick. The dense presynaptic axolemma is 60 to 70 Å thick, and the dense subjunctional sarcolemma is 90 to 100 Å thick, with an intervening cleft space of 200 to 300 Å. The subjunctional sarcolemma lacks the infolding so characteristic of other vertebrate skeletal muscle studied in amphibian, reptilian, and mammalian skeletal muscle. The subjunctional sarcoplasm is more sparse and not differentiated into a sole-plasm region as in other vertebrate neuromuscular junctions examined; thus the subjunctional sarcolemma directly abuts the neighboring myofibrillae with their investing sleeves of sarcoplasmic reticulum (*R*, Fig. 1, and *R*₁, *R*₂, Fig. 2).

Neurilemmal cells (*N*, extreme left, Fig. 1) cover the non-synaptic surface of the terminal axons. Neurilemmal cells covering the terminals are branched and sections through such branched processes may be seen at *X* in Figs. 1 and 2. The branched processes are sometimes situated between axolemma and sarcolemma (*X*₁, Fig. 1).

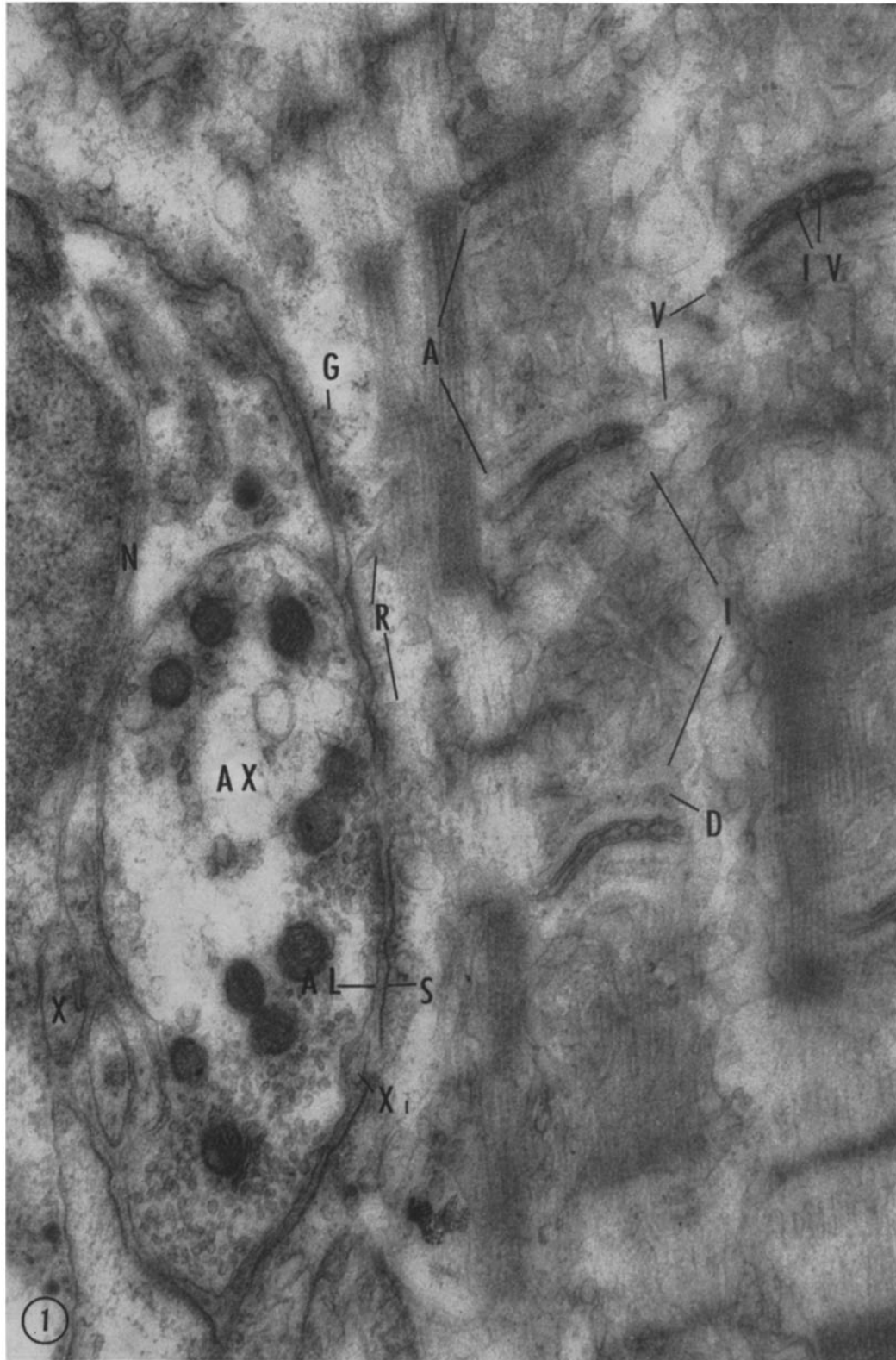
The terminal axons contain mitochondria (*M*₁), synaptic vesicles (*V*), and agranular reticu-

Explanation of Figures

All figures are longitudinal sections through *Fundulus* extrinsic eye muscle fibers.

FIGURE 1

This figure shows a portion of a muscle fiber at the level of the neuromuscular junction. A neurilemmal cell (*N*) with branched processes (*X*), one of which (*X*₁) is interposed between axolemma (*AL*) and sarcolemma (*S*), overlies the innervating axonal terminal (*AX*). Portions of the sarcoplasmic reticulum (*R*) and small granules (*G*) are found in the subjunctional sarcoplasm. The elements of sarcoplasmic reticulum which alternately span the A band (*A*) and the I band (*I*) consist of tubular and cisternal elements (see also Fig. 6). The intermediary vesicular element (*IV*) of the sarcoplasmic reticulum is seen to be continuous between triads of adjacent myofibrils (*V*). The dense regions (*D*) characteristic of the membranes of the triadic terminal cisternae are visible in all triads. × 40,500.



lum (*AR*) (Fig. 2). The subjunctional sarcoplasm contains granules (*G*, Fig. 1) and portions of sarcoplasmic reticulum (*R*, Fig. 1, and *R*₁, *R*₂, Fig. 2). Mitochondria in the axon and muscle fiber (*M*₁ and *M*₂, Fig. 2) differ in size and morphology, those in the muscle fiber being larger and having fewer cristae per unit cross-sectional area.

Sarcoplasmic Reticulum: The morphology of the sarcoplasmic reticulum is similar to but differs in some details from that described for muscle from the caudal myotomes of *Ambystoma* larvae and cardiac and sartorius muscles of the adult albino rat (Porter and Palade, 3). The reticulum consists of a segmented repeating pattern of membrane-limited vesicles and anastomotic tubular and cisternal elements which surround individual myofibrils in a sleeve-like arrangement (see Fig. 3). The sleeve-like investment is interrupted transversely at successive A-I junctions which are spaced at approximately equal intervals along the length of the myofibrils. The terminology formulated by Porter and Palade (3) will be adhered to in the following description.

The pattern of the system is clearly evident in Figs. 1, 4, and 6. The network of sarcoplasmic reticulum which extends longitudinally from one triad to the next alternately spans the A and I bands (*A*, *I*, Figs. 1, 4, and 6). The "unit sleeves" which span the A band (*A*, Fig. 6) consist of closed terminal cisternal elements (*TC*, Fig. 6) interconnected across the A band by tubular elements (*TE*, Fig. 6) which join in a common central cisterna (*CC*, Fig. 6) at the middle of the A band. The cisternal and tubular elements which span the I band (*I*, Figs. 1, 4, and 6) are similar to those of the A (compare A and I regions, Figs. 1, 4, and 6; see Fig. 3) but the tubular elements of the I are less uniform in diameter and regular in disposition than those of the A bands.

At, or near, the A-I junctions are situated highly differentiated regions termed triads by Porter and Palade (3). Portions of eight triads, for exam-

ple, occur in the section shown in Fig. 4. A triad consists of the apposition of enlarged paired cisternal elements called terminal cisternae (*TC*, Figs. 4 and 6), between which are situated intermediary elements termed the intermediary vesicles (*IV*, Figs. 4 and 5). The terminal cisternae face each other at distances ranging between 450 to 750 Å (arrows, Fig. 5). The intermediary vesicles (*IV*, Figs. 4 and 5) vary from 200 to 300 Å in width and sometimes appear tubular rather than vesicular in shape (*IV*, Figs. 5 and 6). The membranes of the terminal cisternae and intermediary vesicles appear denser and thicker (50 to 70 Å) at their level of contact than the sarcoplasmic reticular membranes elsewhere (30 to 40 Å). The intermediary vesicles extend into the intermyofibrillar spaces (*V*, Figs. 1 and 4). The intermediary element appears to extend transversely across the muscle fiber (*V*, Fig. 1), although its continuity is difficult to follow in the micrographs.

DISCUSSION

Neuromuscular Junction: The most obvious difference in the neuromuscular junctional fine structure of *Fundulus* extrinsic eye muscle as compared with the neuromuscular junctions of skeletal muscle of other vertebrates is the absence of subjunctional infoldings of the type described in mouse intercostal (Reger, 9, and Andersson-Cedergren, 11), gastrocnemius (Reger, 8) and abdominal (Andersson-Cedergren, 11) muscle, lizard limb (Robertson, 10) muscle, and frog gastrocnemius (Reger, 8) muscle. In this respect the neuromuscular junction of *Fundulus* extrinsic eye muscle more nearly resembles the neuromuscular junctions of invertebrates studied in the leg of the wasp (*V. carolina*) (Edwards *et al.*, 5), the flight and tymbal muscles of the cicada (*T. linnei*) (Edwards *et al.*, 6), and the abdominal muscles of the cockroach (*B. germanica*) (Edwards, 7). Thus, at least two types of skeletal neuromuscular junc-

FIGURE 2

This figure shows another muscle fiber at the level of the neuromuscular junction. Synaptic vesicles (*V*), mitochondria (*M*₁), and agranular reticulum (*AR*) are found in the axonal terminal. Portions of a neurilemmal cell are seen at *X*. Portions of vesicular (*R*₁) and tubular (*R*₂) sarcoplasmic reticulum are found in the region of the subjunctional sarcoplasm. Mitochondria (*M*₂) of the muscle fiber are larger and have fewer cristae per unit cross-sectional area than the mitochondria (*M*₁) of the axonal terminal. × 40,500.



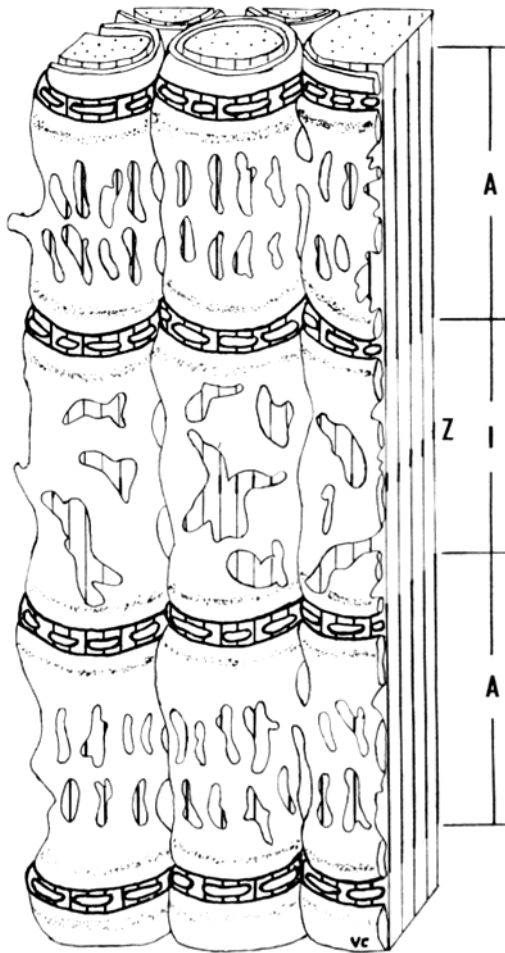


FIGURE 3
This figure is a three dimensional schematic diagram to summarize the findings on the morphology of the sarcoplasmic reticulum of *Fundulus* extrinsic eye muscle and its relationship to the myofibrillae. A band (A), I band (I), Z line (Z). Approximately $\times 60,000$.

tions appear to exist: (1) that with infolded subjunctional sarcolemma, and (2) that with non-folded subjunctional sarcolemma. Whether any functional differences exist between these two types of neuromuscular junctions or whether the difference in subjunctional morphology is a functionally insignificant species variation is an open question. The mathematical treatments given by Eccles and Jaeger (16) on acetylcholine diffusion rates and subsynaptic resistances in terms of synaptic and junctional fine structural morphology, however, seem to help explain the role subjunctional sarcolemmic infoldings have in neuromuscular transmission. It is, therefore, of interest to compare the two types of neuromuscular junctions in terms of acetylcholine diffusion rate from the junctional cleft and resistance of cleft and subjunctional sarcolemma calculable by the methods of Eccles and Jaeger (16).

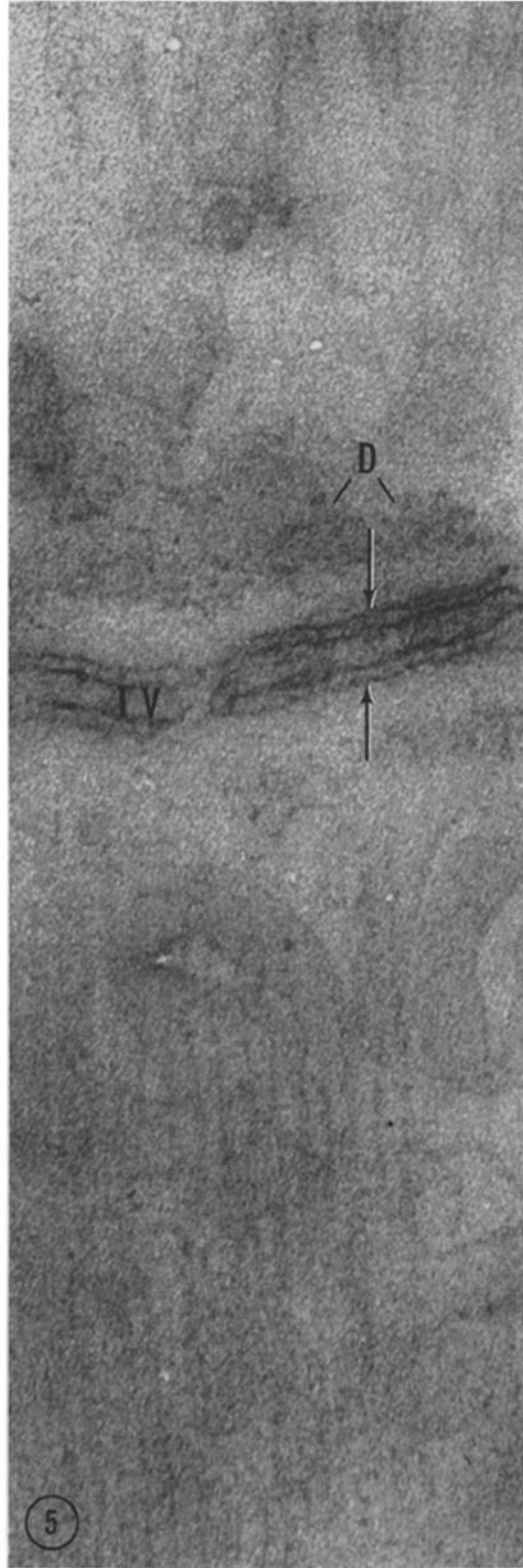
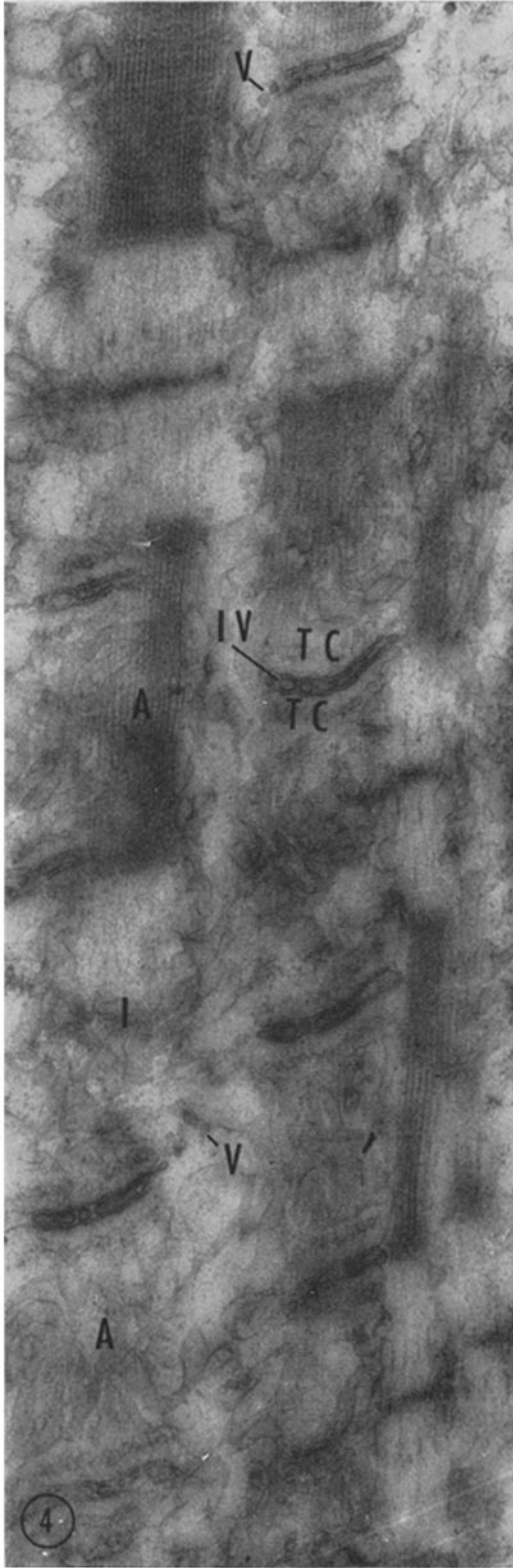
Eccles and Jaeger (16) have formulated equations which define, to a first approximation, acetylcholine diffusion rates and conductance capacities of various central nervous system and peripheral synapses in terms of their dimensions and geometrical configurations based on their fine structure. In the case of the neuromuscular junction it was shown that the subjunctional infoldings, by increasing synaptic interspace mean width and subjunctional surface area approximately fourfold, should result in a lower rate of acetylcholine diffusion from the cleft and lowered resistance of the cleft and total subjunctional membrane to the passage of electric current, as compared to an identical strip length of non-folded junctional contact of axolemma and sarcolemma. Although these differences cannot be precisely quantitated until serial sections of several entire neuromuscular junctions of both the folded and non-folded subjunctional membrane types are

FIGURE 4

This figure shows portions of two myofibrillae with associated sleeves of sarcoplasmic reticulum alternately spanning the A (A) and I (I) bands. The triad consists of the apposition of two terminal cisternal elements (TC) from each segment interposed between which there is an intermediary vesicular element (IV). The intermediary vesicular element is seen to extend into the intermyofibrillar space (V). $\times 27,500$.

FIGURE 5

This figure shows a triad at higher magnification. The dense element (D) at the level of the terminal cisternae appears to be granular or vesicular. The arrows point to the membranes of two facing terminal cisternal elements between which the intermediary element (IV) is situated, here more tubular than vesicular. The triadic membranes are 50 to 70 Å thick and increased in density. $\times 144,000$.



made, general suggestions of functional interest seem justifiable.

According to Eccles and Jaeger's (16) formulae and calculations the type of non-folded neuromuscular junction described here and in insect muscle should have: (1) a higher rate of acetylcholine diffusion from the junctional cleft, and (2) a higher resistance of cleft and subjunctional sarcolemma. These two factors should provide for faster synaptic decay rates and smaller end-plate potentials. Whether the neuromuscular junction of *Fundulus* extrinsic eye muscle does, in fact, exhibit faster synaptic decay rates and smaller end-plate potentials is unknown, since no physiological data exists for *Fundulus* extrinsic eye muscle. Insect muscles with no subjunctional infoldings as described by Edwards and coworkers (5-7) do, however, exhibit functional properties different from most vertebrate skeletal muscle, including small end-plate potentials and non-propagated localized muscle response (Hoyle, 17). Skeletal muscles of many invertebrates, in fact, as well as some frog skeletal muscle (Kuffler and Williams, 15), exhibit functional properties which include small end-plate potentials (the so called slow or tonus system; see Kuffler and Williams, 15). A study of the fine structure of the neuromuscular junctions of some of these slow (tonus) systems might prove helpful in verifying some of the calculations made by Eccles and Jaeger (16) with respect to the neuromuscular junction. The difficulty in such a study, however, is the fact that those muscles which exhibit the slow (tonus) response also exhibit the fast (twitch) response; therefore it is difficult to know which fiber one is observing in such a fine structural study. Preliminary studies by Reger (18) on neuromuscular junctions from the tonus bundle of *Rana pipiens* iliofibularis and albino mouse extrinsic eye muscles, both of which exhibit the slow or tonus response (Tiegs, 13), have shown subjunctional sarcolemmic infolding,

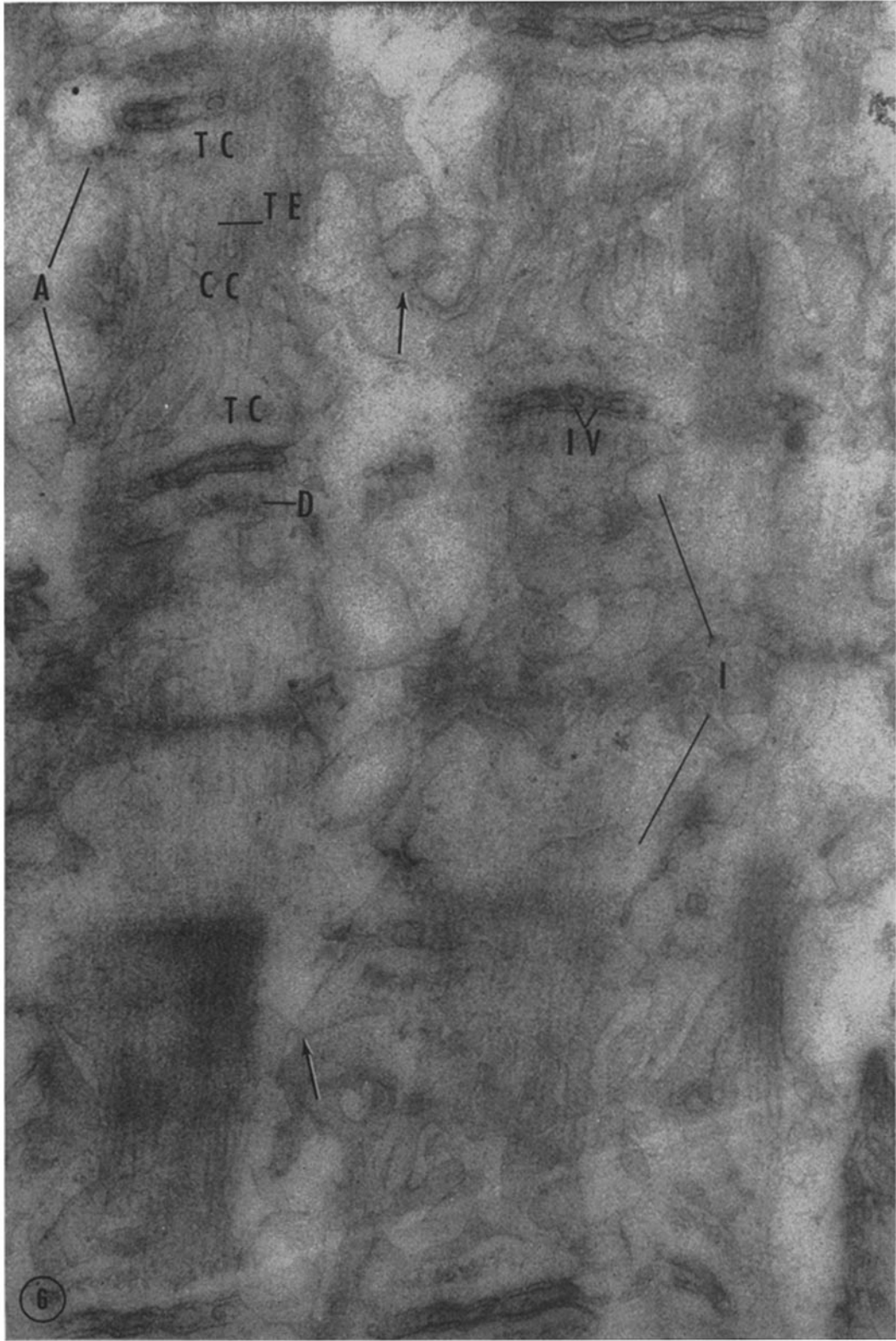
albeit considerably reduced, in contrast to the situation in *Fundulus* extrinsic eye muscle. The subjunctional surface area increase in frog tonus bundle and mouse extrinsic eye muscle is one- to twofold compared to the fourfold increase in mouse gastrocnemius and the threefold increase in frog gastrocnemius.

If differences in acetylcholine diffusion rates are responsible for differences in synaptic decay rates and end-plate potentials they may be so, because of differences in time-concentration products in the action of depolarizing the subjunctional sarcolemma. Since, however, the neurilemmal cells, which are found so intimately associated with the axonal terminals, may act as barriers to the diffusion of acetylcholine from the junctional cleft, to prolong its action on subjunctional membranes of either type, the diffusion effect in the sense of Eccles and Jaeger (16) may not be significant. The function of acetylcholinesterases in synaptic decay, furthermore, is well known (Eccles, Katz and Kuffler, 19; Eccles and MacFarlane, 20; and Fatt and Katz, 21). It cannot be unequivocally stated, therefore, that the acetylcholine diffusion rate alone is responsible for given synaptic decay rates or end-plate potentials, as Eccles and Jaeger (16) themselves pointed out.

Sarcoplasmic Reticulum: The fine structure of the sarcoplasmic reticulum of *Fundulus* extrinsic eye muscle is similar, but not identical, to that reported by Porter and Palade (3), in *Ambystoma* larval muscle and rat sartorius and cardiac muscle, and by Andersson-Cedergren (11), in mouse skeletal muscle. Triads are located at the level of the the A-I junction and two unit sleeves span alternately the A and I bands. Since the possible importance of the sarcoplasmic reticulum in intracellular conductance has been repeatedly stressed (Retzius, 22; Barer, 23; Bennett, 24; and Porter, 25) and since the level of the triad and its associated transversely oriented intermediary element

FIGURE 6

In this figure the plane of section passes tangentially through two parallel sarcoplasmic sleeves. As in previous figures the intermediary element (*IV*), the dense regions (*D*) of the terminal cisternae (*TC*), and the spanning sleeves of the A (*A*) and I (*I*) bands may be seen. The sleeve spanning the A band (*A*) may be seen to consist of terminal cisternal elements (*TC*) continuous across the A band via tubular elements (*TE*) which join in the middle of the A band span to form a common central cisternal element (*CC*). A similar organization spans the I bands (*I*) but the interconnecting elements are much more randomly arranged. Interfibrillar anastomoses between both A band spanning sleeves are clearly seen (arrows). $\times 67,500$.



is being increasingly implicated as significant in terms of intracellular conduction (Porter and Palade, 3; Huxley and Taylor, 26; Ruska *et al.*, 27; Peachey and Porter, 28; and Andersson-Cedergren, 11) a knowledge of the location of the triads in different muscle types may be useful in predicting the physiological properties of muscles not yet physiologically investigated. For example, triads are located at the Z line in *Ambystoma* larval muscle (Porter and Palade, 3), rat cardiac muscle (Porter and Palade, 3), and frog limb muscle (Andersson, 29), whereas triads are located at or near the A-I junction in chameleon lizard muscle (Robertson, 30), rat sartorius muscle (Porter and Palade, 3), mouse limb muscle (Andersson-Cedergren, 11), and insect muscle (Edwards *et al.*, 6). To what degree the location of the triads determines the special properties of intracellular conduction must await further correlated fine structural and electrophysiological investigations.

Using micropipette stimulation techniques Huxley and Taylor (26) in frog muscle, and Huxley and Straub (31) in lizard muscle, have shown that the only area in which localized excitation could be elicited corresponded to the level of the triad, *i.e.* at the Z line in frog muscle and the A-I junction in the lizard. Thus, they were led to conclude that some component of the triad was responsible for the conduction of excitation to the myofibrils. This component may be the

intermediary element which is transversely oriented across the muscle fiber, is continuous across the muscle fiber (Andersson-Cedergren, 11), and approaches the sarcolemma (Porter and Palade, 3). If the transverse intermediary element is, in fact, an intracellular conductor, the suggestion made by Andersson-Cedergren (11) that the contact of intermediary vesicles (T system) with the terminal cisternae at the level of the triad is "synaptic" in nature warrants further investigation. On a purely morphological basis the thickenings of the triadic membranes reported here and by Porter and Palade (3) in *Ambystoma* bear some similarity to the thickenings of apposed synaptic membranes of synapses and neuromuscular junctions.

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