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Study on the Efferent Innervation of the Body Wall Musculature of Lumbricus Terrestris (L)

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STUDY ON THE EFFERENT INNERVATION
OF THE BODY WALL MUSCULATURE OF
LUMBRICUS TERRESTRIS (L.)

by

Carol Aslam

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BIOGRAPHY

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INTRODUCTION

A. A General Description

The earthworm *Lumbricus terrestris* (L.) on which this study was done is an oligochaete of the phylum Annelida. It is characterized by 100-180 externally visible segments and has a length of approximately 150 millimeters. The shape of this worm is cylindrical and flattened ventrally. The dorsal surface is a shade of deep brown bisected by a median longitudinal dark line running the entire length of the animal that is the mid-dorsal blood vessel. The anterior end exhibits a small knob which is called the prostomium, or lip. The body wall is composed of several layers of tissue including a thin transparent cuticle which covers the whole external surface. Beneath the epidermis are the two muscle layers which are the main constituents of the body wall. The outer circular layer is 500u thick with fibers oriented circumferentially, while the inner longitudinal muscle layer is thicker (1000u) and composed of pinnately arranged bundles. The muscle cells in both layers are morphologically identical (Mill and Knapp, 1970). Both muscle layers are interrupted at the border of each somite by a connective tissue septal wall.
that partially divides the body celom into individual compartments. The celomic cavities are lined by peritoneum and are filled with fluid which acts as a hydrostatic skeleton, rendering the body semi-rigid.

The organization of the nervous system on which this study has been done is intermediate between the nerve net of the radially symmetrical coelenterate and the well organized central nervous system of the arthropods (Dorsett, 1964). The central nervous system consists of: (1) a bilobed suprpharyngeal ganglion (the "brain") situated dorsally at the level where the oral cavity joins the pharynx (somite three); (2) a pair of circumpharyngeal connectives that extend from this dorsally situated "brain" to the ventral region where they fuse to form the paired subpharyngeal ganglion (somite four) and (3) a set of nerve cords originating from this paired structure, fused at somite four that continued along the entire length of the body on the ventral floor of the celom, as the ventral nerve cord. One segmental ganglion in each somite can be seen with a dissecting microscope (Adey, 1951). In each somite three pairs of nerves course in a ventro-lateral direction toward the body wall musculature (Dawson, 1920; Drewes and Pax, 1973).

Collectively, the lateral nerves are called the segmental nerves and typically they are present in each segment. These lateral nerves contribute to the innerva-
tion of the body wall musculature and the mucous gland cells. This is accomplished by means of: (a) a nerve ring located between the circular and longitudinal muscle layers, (b) through a subepidermal plexus located between the epidermis and the circular muscle layer and (c) an internal nerve net located in the body wall musculature. The internal nerve net is a plexus of nerve fibers which surround muscles in the circular and longitudinal layers. The subepidermal plexus and internal nerve net are in communication with each other throughout the length of the earthworm (Hess, 1925).

B. The Problem

While earlier research on the neuromuscular system of the earthworm was concerned with the general features of neuromuscular junctions, (Retzius, 1892; Smallwood, 1926) contemporary studies have concentrated on its fine structure (Mill and Knapp, 1970, Rosenbluth, 1972). The most recent investigation has revealed the presence of neuromuscular junctions in the earthworm consistent with features demonstrated in a variety of other species (Rosenbluth, 1972). Rosenbluth (1972) described the characteristics of neuromuscular junctions by: (i) morphology of axonal vesicles, (ii) gap width and (iii) postjunctional membrane specialization. Nevertheless, these morphological features proved inadequate to characterize specific
functions (e.g., fast versus slow muscular contraction; excitatory versus inhibitory responses, etc.). Physiological studies indicated that responses observed in the body wall muscle were a result of dual innervation (Dorsett, 1964; Hidaka, Ito, Kuriyama and Tashiro, 1969; Drewes and Pax, 1974).

The purpose of this study is to substantiate earlier descriptions of neuromuscular junctions, using light and electron microscopy. Earlier studies have failed to relate neuromuscular junctions with either a plexi system (a vestige of the nerve net) or the centrally organized segmental nervous system which may dually innervate the body wall musculature of Lumbricus terrestris (L.). This study will attempt to show that earthworm body wall muscle is dually innervated by plexi (whose possible origin is being described for the first time) and the segmental system. Further morphological assessment can then be made on the bases of end plate morphology, gap width and post-junctional membrane specialization. These two systems may well represent the morphological basis for the differing physiological properties which have not been previously identified morphologically, nor adequately explained.
REVIEW OF THE LITERATURE

Since the first histological studies on the earthworm by Retzius (1892) there have been numerous histological, biochemical and physiological studies on the nervous system of annelids (Hess, 1925, 1925a; Smallwood, 1926, 1928; Stough, 1926; Bullock, 1945; Adey, 1951; Wilson, 1960; Hidaka, Ito, Kuriyama, Tashiro, 1969; Mill and Knapp, 1970, Ehinger and Myhrberg, 1971; Myhrberg, 1972; Rosenbluth, 1972; Drewes and Pax, 1974). This review will also include those studies on the neuromuscular junction in Lumbricus terrestris (L.).

A. The Ventral Nerve Cord

The nervous system of the earthworm has been the subject of numerous studies and reviews (Retzius, 1892; Langdon, 1895; Hess, 1925; Smallwood, 1926; Stough, 1926; Bullock, 1945; Adey, 1951; Horridge, 1959; Wilson, 1960). The consensus of these investigators was that the ventral portion of the central nervous system was composed of a giant fiber system and a ventral nerve cord that arose at the level of the fourth segment and continued to the caudal end of the body. The giant fiber system and the ventral nerve cord have been functionally coupled to the startle response as well as to
reflexes concerned with slower ambulatory and burrowing movements respectively (Bullock and Horridge, 1965). There were three giant fibers in the ventral nerve cord; one median and two lateral ones. The size of the central fiber as a rule was consistent throughout the cord while the lateral fibers are subject to fluctuations (Stough, 1926). The two lateral fibers were freely interconnected throughout the cord (Stough, 1926; Bullock, 1945).

Bullock (1945) has shown that in Lumbricus, a tactile stimulation of the anterior 35-40 segments of the body excited the median fiber, while stimulation of the body caudal to segment 40 excited the lateral fibers. The lateral giant fiber system was probably intended primarily to subserve anterior conduction of stimuli from the posterior segments while the median fiber conducted impulses from the anterior region to the posterior part (Bullock, 1945).

On transverse section, the ventral nerve cord was found to contain three types of cell bodies: unipolar, bipolar and multipolar (Adey, 1951; Bullock and Horridge, 1965). In transverse section, centrally located nerve cells were within an encircling neuropile mass that in turn was enclosed within a peripheral circle of nerve cell bodies comprising the rind. These cells, the central as well as the peripheral ones were either motor or inter-
neuron cell bodies. On the strength of their location they could not be segregated into functional groups.

(i) Interneurons

Sensory information is relayed from the periphery (epidermis) to the ventral nerve cord in the following way; upon receiving a stimulus, receptor cells in the epidermis propagate an impulse through their axons which synapse on interneurons in the segmental ganglia. (These axons are the afferent portion of the lateral nerve in every segment). The interneurons relay information to the giant fiber system. The giant fiber system is the sensory and motor integrator of the central nervous system (Bullock and Horridge, 1965).

(ii) Motor Neurons

Motor neurons are characterized by a cell body with a diameter of 20u-60u, which possesses an axon that passes toward the adjacent segmental nerve. The axon cleaves into two branches; one courses in the region of the giant fiber system while the other enters the segmental nerve. Adey, (1951) assumed that these cells were at least part of the efferent pathway to the musculature of the body wall.

B. The Peripheral Nervous System

The peripheral nervous system is composed of: (1) the segmental innervation and (2) the peripheral plexus.
(1) Segmental Innervation

Each segment of the earthworm is supplied by three pairs of lateral nerves whose axons originate in the cell bodies of the ventral nerve cord. Hess (1925) found that these lateral nerves send branches to one segment only.

Bullock and Horridge's studies (1965) indicate that all three pairs of lateral nerves contain afferent and efferent fibers. Cell bodies of the afferent fibers are located in the periphery, in or beneath the epidermis, in the muscles, or other tissues along the course of the nerves, but never in the central nervous system (Bullock and Horridge, 1965). The afferents are most heavily pronounced in the second lateral nerve while the efferents are most heavily pronounced in the third. In each segment the first lateral nerve arises more rostrally than the second and the third. The first lateral nerve is responsible for innervation of the segment it arises from and the caudal-most portions of the adjacent cranial segment. The second lateral nerve innervates only the segment from which it arises, while the third lateral nerve innervates its own segment along with the anterior portions of the next most caudal segment (Drewes and Pax, 1973). Upon entering the body wall musculature, branches are given off to the longitudinal muscle layer at its celomic border (Smallwood, 1926). The bulk of the segmental branches course between
the circular and longitudinal muscle layers of the body wall, forming a nerve ring. The nerve ring has been shown to send branches into the muscle layers as well as branches to the subepidermal plexus and to the sensory receptors in the epidermis (Hess, 1925; Smallwood, 1928).

The subepidermal plexus is an accumulation of anastomosing nerve fibers that show no signs of being segmental in arrangement (Hess, 1925). These fibers communicate with an internal nerve net (located within the circular and longitudinal muscle layers) and with the ventral nerve cord where they form synaptic connections with other neurons. It appears that part of the subepidermal network can be classified as efferent in character as it sends fibers into the epidermis and innervates mucous gland cells (Dawson, 1920; Smallwood, 1926).

Therefore, the segmentally arranged portion of the peripheral nervous system not only innervates (i) the body wall musculature in a segmental manner but also contributes to the formation of (ii) the nerve ring, (iii) subepidermal plexus and (iv) internal nerve net, the latter two of which are not segmental in distribution.

(2) The Peripheral Plexus
The peripheral plexus includes: (a) axons originating from sensory receptor cell bodies of the epidermis, (b) the internal nerve net of the body wall musculature, (c) the
afferent portion of the subepidermal plexus and (d) inter-
neurons.

Sensory receptor cells (a) or "sense cells" of the
epidermis send impulses directly to the internal nerve
plexus within the body wall muscle layers as well as to the
central nervous system. The impulses may pass directly
from the epidermal cells to muscles without synaptic rela-
tions in the central nervous system (Hess, 1925). There­
fore, Hess (1925) concluded that the so-called "sense
cells" of the epidermis appear to be the cell bodies of
sensory neurons. Experiments which involve removal of the
suprapharyngeal ganglion, subpharyngeal ganglion and vent­
ral nerve cord showed that the (b) internal nerve net, to­
gether with the (c) subepidermal nerve plexus was capable
of transmitting tactile impulses over as many as six som­
ites. With heat as a stimulus, impulses were transmitted
over as many as 15 somites (Hess, 1925).

Interneurons (d) have been reported as multipolar
and bipolar (Bullock and Horridge, 1965). They are found
in or between the two peripheral muscle (circular and long-
itudinal) layers or along peripheral nerves and give origin
to relatively long processes that originate from each pole.
Morphological characteristics and distribution of these
interneurons indicate that more than one physiological
function may be attributed to this system (Bullock and
Eorridge, 1965).

On the strength of a variety of staining techniques, Dawson (1920) observed a vast array of intermuscular nerve cells (interneurons) in the earthworms Helodrilus caliginosus and Allolobophora (Eisenia) foetida. He (Dawson, 1920) hypothesized that these nerve cells represent vestiges of a primitive nerve net characteristic of lower invertebrates and outlying cells which phylogenetically have not been incorporated in the ventral nerve cord.

On the strength of previous investigations and his own work, Heas (1925) concluded that the earthworm represents a transitional stage between those animals that have a nerve plexus and those with a well-defined central nervous system, i.e., from the plexus system to the synaptic system.

C. Muscle-Nerve Relationships

(1) Preterminal Nerve Fiber Characteristics

Retzius, (1892) using the Golgi method demonstrated the first type of finely branched nerves with varicosities that end in minute knobs. His camera lucida drawings indicate that he may have observed both multiterminal and polyneuronal innervation. Smallwood (1926) described a second type of nerve termination characterized by a cluster of branches. These nerves also end with a knob-like terminal and were assumed to be true motor endings. Using levadite
and gold chloride stains, Smallwood (1926) found these very minute branches as passing from the nerve ring to the longitudinal muscle layer. This type of ending has since been described as an "en grappe" ending (Gray, 1957; Dorsett, 1964).

(2) Terminal Nerve Fiber Characteristics

Both types of endings, cluster ending and fine branching, (Smallwood, 1926; Retzius, 1892) were demonstrated to be multiterminal with a variety of staining methods. Thus, morphological evidence indicates that in oligochaetes a relatively small number of motor neurons innervate a large number of muscle fibers. [There could be as few as 75 nerve cells in a single segmental ganglion sending fibers to the 1500 effector cells in each somite (Smallwood, 1926)]. This situation is similar in the polychaeta Nereis and Harmothol, where only a small number of motor axons leave the segmental ganglia (Horridge, 1959).

Using the electron microscope, Mill and Knapp, (1970) have described "muscle tails" in Lumbricus terrestris (L.) which are devoid of contractile elements and structurally differ from those fibers which connect the muscle to connective tissue. These "muscle tails" were characterized by the absence of fibrillar bundles and specialized sarcoplasm. Many of these "muscle tails" have been seen associated with a single axonal ending.
Considering the light and electron microscopic evidence, it is possible for a nerve to contact more than one muscle and for one muscle to form junctions with more than a single nerve (Retzius, 1892; Smallwood, 1926; Mill and Knapp, 1970).

(3) Physiological Implications
Polyneuronal innervation in the earthworm is indicated by the observations of minute excitatory and inhibitory potential changes in the body wall muscles, which on rare occasions could be recorded from the same cell (Hidaka, Ito, Kuriyama, Tashiro, 1969). A further indication of dual innervation is the capacity of certain muscles for fast and slow contraction (Horridge, 1959; Wilson, 1960). This is caused presumably by having a dual innervation similar to the one reported in the crustacea (Uchizono, 1967).

Dorsett (1963), and subsequently Mill and Knapp, (1970) have suggested that if the nerve endings found in Lumbricus terrestris (L.) by Retzius (1892) and Smallwood, (1926) do represent two different types of motor endings, these may provide an anatomical basis for the slow and fast systems of muscular contractions.

(4) Neuromuscular Junctions
While studying the ultrastructure of the earthworm body wall musculature Mill and Knapp (1970) found only a single type of myoneural junction. Rosenbluth (1972) described a second type of ending. Rosenbluth (1972) proposed that
Type I junctions are the sites of cholinergic transmission, characterized by clear neurosecretory vesicles (measuring 50nm in diameter), and a gap measuring 100nm in width whereas the Type II junctions were characterized by (among others) dense core vesicles (measuring 100nm in diameter) and a gap width of 15nm or thereabouts. These characteristics indicated that Type II junctions were probably catecholaminergic.

D. An Account of Secretory Vesicles Within the Earthworm Nervous System

(1) Central Nervous System

Perikarya and their associated neuropile mass were of two varieties. The first contained large dense vesicles measuring 120-350nm in diameter and was neurosecretory in function (Myhrberg and Ehinger, 1971-1972). The second contained dense cored vesicles measuring 70-120nm in diameter and contained the transmitters norepinephrine and dopamine, as well as smaller granular vesicles measuring 60-90nm in diameter and containing the transmitters norepinephrine, dopamine and 5-hydroxytryptamine (Myhrberg and Ehinger, 1971-1972). The vesicles containing catecholamine transmitter substances have been associated with sensory reflexes (Clark, 1967).

(2) Peripheral Nervous System

Ehinger and Myhrberg (1971) also reported the ultrastruc-
tural localization of dopamine within the receptor cells of
the epidermis and the subepidermal nerve plexus of the
peripheral nervous system.

Axon terminals associated with the innervation of
the body wall musculature contained an abundance of clear
spherical vesicles (measuring 50nm in diameter) were be-
lieved to contain the transmitter acetylcholine (Rosen-
bluth, 1972). Acetylcholine has been shown to be the ex-
citatory transmitter in the body wall musculature of
*Lumbricus terrestris* (L.) (Hidaka, Ito, Kuriyama and
Tashiro, 1969).
MATERIAL AND METHODS

A. Description of the Animal

Live, sexually mature specimens of *Lumbricus terrestris* (L.) were obtained from local bait shops. It has been noted that peripheral axons associated with neuromuscular junctions give off many branches to the muscles, and their endings are dendritic in fully-grown worms but bud-like in young worms (Mill and Knapp, 1970). The presence of well developed clitellum in segments 31-37 is the external sign of sexual maturity (Elliott, 1968). Therefore, only animals with a well-developed clitellum have been used.

B. Care

Animals were kept in large plastic containers (8x11x6 inches) filled with three inches of moist potting soil, and fed oak leaves. The leaves were collected dry in the fall and first soaked in tap water prior to placing in a two-inch layer over the potting soil. Old leaves and apparent sick animals were routinely discarded. Containers were covered with tight fitting aluminum foil which had regularly spaced air holes. In this manner specimens were satisfactorily maintained in a refrigerator at 13°-15° C for six months.
C. Light Microscopy

Survey studies on transversely sectioned earthworms were undertaken for general orientation. Staining techniques for these sections included: Masson's trichrome, toluidine blue and Kluver Barrera's method (Luna, 1968). Earthworms selected for light microscopy were anesthetized by placing the animals in an aqueous 5% urethane solution for 20 minutes prior to dissection. Tissues were fixed in 10% Formalin 24 hours/cubic centimeter, washed in tap water, dehydrated in baths of ethyl alcohol of increasing strength, embedded in paraffin wax and serially sectioned at 8u with a rotary microtome. To demonstrate the nervous elements in pilot studies the silver staining method of Bodian (1936) was used. Subsequently, however, because of the capriciousness of the method, it was used only for survey work. Gregory (1974) attributed inconsistencies in staining to impurities and aging of the protargol (silver protein).

Fitzgerald (1964) reported that following Formalin fixation the silver impregnation of the cyton is poor and the staining of the nervous elements in the central nervous system is rather inconsistent. This inconsistency was eliminated by the method devised by Fitzgerald (1964). Fitzgerald's (1964) double impregnation technique was successful in demonstrating differentiation in the central nervous system and apparent complete staining of somatic
motor and sensory fibers. Since this is a rarely used technique, a description of the method follows:

**Fixative Solution**

- Picric acid, saturated in 90% alcohol 70%
- Formaldehyde 25%
- Glacial acetic acid 5%

Following fixation for one to three days, tissue blocks were transferred directly to absolute ethanol for 24 hours (four changes), cleared in benzene and embedded in 58°F paraffin wax. Sections were cut at 15u, mounted on albuminized slides, and stored overnight at 37°C. Slides were allowed to stand at room temperature for 20 minutes, then treated in the following manner:

1. Hydration in solutions of decreasing concentrations of ethanol was accomplished within a thirty minute interval after initial clearing in xylene;

2. Slides were rinsed in distilled water and placed in a 10% solution of silver nitrate for 1.5-4 hours at 56°C;

3. Subsequently, slides were allowed to stand at room temperature for 20 minutes followed by decantation of the silver nitrate solution and washed in three changes of distilled water for 1.5 minutes;

4. Slides were then stored at 37°C for eighteen hours in a protargol solution which was made up within
thirty minutes of use by dusting 200 mg. of "protargol-S Winthrop" onto 100 ml distilled water;

5. Prior to development, slides were allowed to stand at room temperature for 20 minutes, then rinsed in distilled water for five to ten seconds;

6. The first step of development was accomplished by placing the slides in a mixture of 10% sodium sulphite (anhydrous) containing 1% hydroquinone for a period of three to five minutes;

7. Following the initial development, slides were washed in running tap water for 10-15 minutes and then rinsed briefly in distilled water;

8. Toning of the sections was accomplished during a 10-minute interval in a 0.5% aqueous gold chloride solution containing one drop of glacial acetic acid per 100 ml;

9. Following a two to three minute wash in distilled water, the slides were further developed in either 50% alcohol containing three drops of aniline oil per 100 ml for 20-30 seconds or in a 2% aqueous oxalic acid solution for four to five minutes;

10. Slides were washed again in distilled water for one to two minutes, then fixed in 5% sodium thiosulphate for five to ten minutes;

11. Slides were then dehydrated, cleared, and mounted in the usual manner.
Serial sections of 15μ were obtained through the first five to seven segments in either transverse, longitudinal or parasagittal planes. Micrographs were obtained on a Zeiss photomicroscope with initial magnifications ranging from 10 to 400 times, with subsequent photographic enlargements to 1200 times.

D. Electron Microscopy

Appropriate tissue samples were obtained from animals anesthetized in 5% urethane. The procedure was as follows:

1. The tissue was fixed in 2.5% gluteraldehyde in Sorensen's buffer (1909) at pH 7.4 for one hour and post fixed in 1% osmium tetroxide in the same buffer for an additional one to two hours;

2. The tissue was dehydrated in increasing grades of acetone or ethanol at 4°C until absolute concentration was reached, then was kept at room temperature;

3. The tissues were cleared in propylene oxide;

4. The tissue was infiltrated and embedded in Epon 812 (Luft, 1961). Care was taken to orient the tissues carefully so that they could be cut in planes that one could relate to the axis of the animal;

5. Specimen blocks were incubated at 60°C for three days. Variations in this procedure included addition of 0.045 gm. of sucrose per one ml of fixative (Caulfield, 1957).
Additional preparation of the tissue was achieved in the following ways:

1. Anesthetized earthworms were flooded with the above described gluteraldehyde fixative at room temperature during dissection and appropriate samples no larger than one cubic millimeter were obtained from the body wall musculature.

2. Whole earthworms were perfused by means of a tuberculin syringe with 2.5% gluteraldehyde buffered with Sorensen's buffer at pH 7.4 that contained 4.5% sucrose, and subsequently chilled to 4°C. After 45 minutes, earthworms were removed from the fixative, portions of the ventral nerve cord with segmental nerves and body wall musculature attached were dissected out. These tissues were returned to the gluteraldehyde solution for an additional one-half hour and post fixed in osmium tetroxide with the same buffer and sucrose concentrations.

Sections were obtained on either a Sorvall Porter-Blum (MT1) or on a Reichert (OMU2) ultramicrotome. One micron sections stained with toluidine blue were used for survey purposes. Thin sections ranging from 80nm to 100nm were mounted on 200 or 300 mesh copper grids. Contrast was enhanced with uranyl acetate (Swift and Rash, 1948), or a combination of uranyl acetate and lead citrate (Reynolds, 1963); and examined with an RCA EMU 3F-2 electron microscope.
at an accelerating voltage of 50KV. Electron micrographs were obtained at initial magnifications ranging from 2000 X to 22,000 X with subsequent photographic enlargements to 88,000 X original size.
RESULTS AND OBSERVATIONS

A. General Organization of the Peripheral Nervous System, Light Microscopy

Silver impregnation studies of 20 animals (segments one through seven) were obtained in either transverse or longitudinal sections. In transverse sections segmental nerves were observed extending from the ventral nerve cord to the periphery (Fig. 1). In longitudinal sections it was possible to detect the single first and "double" second and third nerves. The second and third nerves invariably separated before entering the body wall musculature (Fig. 2). Tracing these nerves from the cord to the nerve ring, the two types of fibers, thick and thin, were observed in all cases (Fig. 3).

Fibers, heavily impregnated with silver dye, were observed coursing from the nerve ring into the septum of the pinnately arranged bundles of the longitudinal muscle layer (Fig. 4). From the septum the nerve fiber traveled a short distance between the muscle fibers and abruptly terminated by a spray which ended in knobs on the sarcolemma (Fig. 4).

The internal nerve net was observed in transverse as well as in longitudinal sections of the body wall musculature (Figs. 5, 6). This network was characterized
by apparently anastomosing fibers and varicosities. It was also noted that almost all nerve fibers were wavy in appearance and occasionally spirally arranged (Figs. 3, 4, 5 and 6). Higher magnification frequently resolved an apparent knobbed process to be a "kink" in the fiber (Fig. 6).

Cell bodies were seen along the length of the segmental nerve (Fig. 3) and apparently lying on the surface of the muscles in the longitudinal muscle layer (Fig. 7). These, presumably intermuscular nerve cells, were irregularly shaped and on occasion appeared to issue processes which seemed to contribute to the formation of the internal nerve net of the body wall musculature (Fig. 7).

B. Fine Structure of the Peripheral Nervous System, Electron Microscopy

The most striking and also the most consistent feature throughout the length of one lateral nerve and portions of six other lateral nerves, when studied in transverse sections, was the existence of two populations of axons.

The first type was an irregularly shaped axon of almost uniform size whose appearance could be compared to a pavement stone (Fig. 8). The average width of the axons at their narrowest and widest margins measured 350nm and 800nm respectively. Approximately 50 axons were measured across the narrowest and widest margins. The narrowest margin
ranged from 69nm to 460nm with an average width of 350nm. The wider margin ranged from 460nm to 1170nm with an average width of 800nm. Although the lateral nerve was sectioned transversely, individual axons (observed in light microscopic studies) did not course in a straight plane, but were assumed to be wavy in form. Due to this light microscopic observation, it was assumed that the axons, in many cases, were cut obliquely and possibly sectioned more than once. The axoplasm showed little density and contained several identifiable inclusions: neurotubules, mitochondria, agranular endoplasmic reticulum and smooth walled vesicles. In any one plane of section, this pavement stone shaped axon was always observed to contain neurotubules but not always mitochondria or vesicles. The population of mitochondria in any axon ranged from zero to five with the average being one to two mitochondria/axon, measuring approximately 100nm in diameter.

The second variety of axon was smaller, tubular shaped and demarcated by a smooth border (Fig. 9). Its average width at its narrowest and widest margins measured 110nm and 370nm respectively. The measurements were obtained in the same manner as the larger axon. The narrowest margin ranged from 46nm to 276nm with the average width being 110nm. The wider margin ranged from 138nm to 805nm with the average width being 370nm. The axoplasm appeared more dense, with a greater abundance of neurotubules than
the larger pavement stone axon mentioned above. Mitochondria and agranular endoplasmic reticulum were not as frequent as in the larger axon. More than one mitochondrion was rarely observed in any one plane of section in these smaller axons.

In both varieties of axons the axolemmae were 20nm apart without any intervening cellular process (Figs. 8, 9).

The two types of axons were generally seen arranged in bundles of their respective types (Figs. 8, 9). At times mixed bundles of axons could be observed originating from the main lateral nerve (Fig. 10). The gliosome supporting cells form a connective tissue lamella (Fig. 10). Frequently, contact zones characterized by a thickening of opposing cell membranes were observed in this supportive lamella (Fig. 10).

Approximately twenty animals were observed in the electron microscopic study of the body wall muscle. In the longitudinal muscle bundles the septal nerve contained:

(a) processes which were almost devoid of secretory vesicles, but contained mitochondria, microtubules and profiles of agranular endoplasmic reticulum (Fig. 11);

(b) axons with a smooth border which contained either clear spherical secretory vesicles measuring 50nm in diameter or dense vesicles measuring 100nm in diameter (Fig. 12);
(c) axons with an irregular border which contained predominantly clear spherical vesicles measuring 50nm in diameter (Fig. 13).

The septal nerve was observed to course in the septum of the pinnately arranged muscle fibers of the longitudinal muscle layer along with various blood vessels.

The internal nerve net was observed in transverse and longitudinal sections (Figs. 14, 15) to contain:

(a) cell processes characterized by profiles of agranular endoplasmic reticulum, neurofilaments and occasionally clear spherical vesicles (measuring 50nm) as well as dense cored vesicles (measuring 100nm) (Fig. 14);

(b) small tubular shaped axons contained within a smooth border (Fig. 14).

A gliosome supportive cell layer was observed to form a thin connective tissue lamella. The entire bundle was enveloped by a sheath of collagen fibers.

Neuromuscular junctions resembling motor end plates in skeletal muscle were observed along the length of septal nerve bundles. In any one pinnate muscle bundle, the septal nerve could be observed to form neuromuscular junctions with as many as twenty muscle fibers and tails. In some instances the septal nerve was observed without formation of neuromuscular junctions. The septal nerve was composed exclusively of non-myelinated axons (Fig. 13).
The similarity between Type I junction and motor end plates in vertebrate skeletal muscle is due to the overwhelming presence of clear spherical vesicles (measuring 50 nm in diameter). Within the nerve endings, however, unlike vertebrate motor end plates, microtubules and neurofilaments were absent from the junctional axoplasm. The scalloped postjunctional membrane displayed surface projections measuring 20nm in length (Fig. 16). The space (junctional gap) between the two cell membranes contained an amorphous ground substance which appeared to "layer" parallel with the postjunctional membrane. The junctional gap proper measured approximately 85-120nm in width. (Fig. 13).

This type of junction (Type I), occurred along the length of the septal nerve, was observed to contact muscle tails as well as individual muscle fibers (Fig. 13).

Occasionally the axons contained dense cored vesicles. On the micrograph which is included in this thesis, a small nerve bundle incompletely ensheathed by supportive cells was observed to form a neuromuscular junction between the exposed axons and the adjacent muscle cell (Fig. 17). The interesting feature of this junction was the presence of five axons apparently all of them in contact with the muscle. One of the axons displays predominantly dense cored vesicles (approximately 100nm in diameter), a second was mixed, while the other three contain
predominantly clear spherical vesicles (measuring 50nm in diameter). Microtubules and neurofilaments were absent from the junctional axoplasm. The scalloped postjunctional membrane displayed specializations which include cytoplasmic filaments (Fig. 18) and the previously described peripheral projections measuring 20nm in length (Fig. 17). The distal ends of the projections were frequently interconnected by a thin linear density (Fig. 16). The presynaptic element averages approximately one micron across (per axon) at the point of contact with the muscle.

Neuromuscular junctions characterized by Rosenbluth (1972) to contain dark secretory vesicles and to exhibit a narrow junctional gap were not observed in the earthworms studied. However, a different nerve-muscle relationship was observed in the series of earthworms studied. This nerve-muscle relationship was characterized by single unsheathed axons in close apposition to a muscle cell. The axon was filled with clear spherical vesicles of 50nm in diameter. The nerve and muscle cell membranes did not appear to exhibit any specialization (Fig. 19).
DISCUSSION

The goal of this study was to identify neuromuscular junctions with light and electron microscopy and to unravel the relationship between the dual innervation (plexi and segmental system) of the earthworm body musculature. A second aim was to observe the structure of the axonal end-plate and attempt to assess the functional differences, if any, that may exist between the neuromuscular junctions most frequently observed, on the strength of their morphology. The two morphological techniques used in the study were light and electron microscopy.

A. Light Microscopic Methods

Since the start of this work, the most efficient method (technique) to illustrate the structure of nerve terminals was sought. The silver impregnation method of Fitzgerald (1964) was selected as the most effective because with it, metallic silver is absorbed not only by axons, but also by the end terminals themselves. This was borne out in this study. Following silver staining, absorption differences between the fibers in the central nervous system and the fibers in the peripheral nervous system were noted, even with low-power observation. The peripheral fibers appeared wider (thicker) than fibers in the ventral nerve cord.
This was due to the fact that peripheral axons absorb silver more rapidly than does the central nervous system. A similar phenomenon was observed by Adey (1951). He reported that with methylene blue staining preparations, the peripheral nerves in *Lumbricus megacolex* stained more rapidly than the fibers associated with the central nervous system. It was also apparent that when using the standard Bodian's technique the septal nerve and accompanying blood vessel were equally impregnated, making differentiation between the two difficult. The advantage of using the Fitzgerald modification of the Bodian stain was that peripheral nerve fibers appeared to be more consistent in size. Its disadvantage (in the peripheral nervous system) was the difficulty in determining if an apparent fiber represented a single axon or several fibers travelling together, owing to heavy impregnation. Therefore, employment of this stain had the obvious advantage of resolving terminals, but did not resolve the question of whether the elements in the nerve net were single or multiple fibers. Possibly because of fixation artifact, a single fiber could often be observed breaking away from the main trunk and returning immediately, indicating that the "single" nerves observed were actually several fibers. For this reason, measurements of nerve bundles or endings could not be trusted and are not included in this study. The Fitzgerald method stained the elements of the body wall in the following manner: Blood
absorbed silver at such a rate that the enclosed individual axons were not able to be observed.

B. Electron Microscopic Methods
Although the fixative fluid was buffered to the physiological level of the earthworm (pH 7.4), fixation artifacts (e.g., membrane separation, leaching, etc.) were repeatedly observed. The tonicity of the fixative solution appeared to have a direct effect on the appearance of the fixed tissue. In this regard, the use of 4.5% sucrose improved the membrane separation and leaching of connective tissue (Caulfield, 1957).

C. Histological and Ultrastructural Findings
Lateral nerves containing thick and thin fibers have been observed with both techniques. Electron microscopic studies have shown that in the lateral nerve (a component of the segmentals), both large and small axons contain light and dark vesicles. A predominance of dark vesicles (100nm in diameter) were frequently associated with the tubular shaped smaller axons, although clear vesicles were also present but in a much lower number. Available evidence indicates that dark microvesicles, (containing catecholaminergic transmitter substances) are associated with sensory reflexes (Clark, 1967). Rosenbluth, (1972) has proposed that axons containing dense vesicles may form inhibitory catecholaminergic neuromuscular junctions.
It is assumed that dark vesicle filled axon terminals represent a "direct line" between sensory receptor cells and motor terminals, involving at most one relay. This could represent a peripheral inhibitory reflex arc.

The larger pavement-stone type of axons described for the first time in this study most frequently contained a predominance of clear spherical vesicles (measuring 50nm in diameter). The available evidence indicates that these vesicles contained acetycholine transmitter which is the excitatory transmitter of the body wall musculature (Hidaka, Ito, Kuriyama, Tashiro, 1969; Rosenbluth, 1972).

Cell processes were observed in the periphery of lateral nerves. These structures could be the axons or dendritic processes of interneurons, although the perikaryon of the interneurons was never identified as such in electron microscopic studies of the lateral nerve. Therefore, confirmation of the presence of interneurons along the lateral nerves cannot be made in this study.

The nerve ring that is present between the circular and longitudinal muscle layers was observed to be composed of the same elements as the lateral nerve, i.e., bundles of small tubular shaped axons, bundles of larger pavement-stone shaped axons and cell processes, all of them invested in a supportive lamella. In addition, cell bodies of varying sizes and shapes were observed in the nerve ring.
These, presumed interneurons, usually displayed at least two poles. On occasion they could be observed sending one of their processes to the periphery of the pinnately arranged muscle bundles of the longitudinal muscle layer. These processes (dendrites or axons) were observed to be incorporated into the internal nerve net, results heretofore not mentioned.

The septal nerve in light microscopic studies appeared to be a single thick fiber which continued along the length of the septum within the longitudinal muscle layer. Electron microscopic studies have shown the septal nerve to be composed of many fibers, almost exclusively axons, and was the origin of Type I neuromuscular junctions along its entire length. These Type I terminations could end either on muscle fibers or on muscle tails. In some instances small tubular shaped axons which were not associated with neuromuscular junctions were also observed. Large cell processes, usually devoid of secretory vesicles were occasionally present in the septal nerve.

Under the light microscope the internal nerve net was observed to be composed of multiple anastomosing fibers, each showing some varicosities. This nerve net appeared to "lay" on the surface of the body muscle. Electron microscopic studies, primarily in the longitudinal muscle layer, have shown the internal nerve net (surrounding each pinnate muscle bundle) is composed of many axons.
and processes (dendrites or axons). Some of the elements within the internal nerve net were cytoplasmic processes; the others bundles of tubular shaped axons; all ensheathed by supportive cells. No cell bodies that could be described as interneurons were observed to be associated with the cytoplasmic processes. In some areas supportive cells resembling large fibroblasts were observed. These could be the "interneurons" light microscopists observed. These cells could also represent the varicosities observed in light microscopic studies. No pavement-stone shaped axons could be identified in the electron microscopic studies of the internal nerve net.

The term "internal nerve net," used by previous authors, morphologically denoted anastomosing nerve cells and axons and functionally denoted a diffusely conducting system. This study has shown the internal nerve net was actually composed of many axons which do not share the same axoplasm. Hess (1925) has observed that the plexus system was not diffusely conducting (from one end to the other end of the animal) but rather had a local effect restricted to not more than 15 somites. Intermuscular nerve cells, a component of a nerve net, could not be identified. "Internal nerve net" is therefore considered a misnomer and is more properly named internal muscle plexus.

Occasionally a branch of the internal nerve net
could be observed extending into the septum of the pinnately arranged longitudinal muscle bundles. This branch, composed of many axons, coursed between individual muscle fibers until it reached the septum and once within it, it would accompany the septal nerve. The neuromuscular junctions associated with the internal nerve net occurred in the periphery of the pinnate muscle bundles. They were exclusively Type I junctions characterized by axon terminals filled with clear spherical vesicles, a wide junctional gap, and a postjunctional membrane specialization.

The internal nerve net observed in this study confirms the nerve terminals described by Retzius (1892) and Smallwood (1926). These finely branching nerve terminals described by the previous authors are the structures which end in knobbed processes were also observed in this light microscopic study. This substantiates the findings of not only Smallwood (1926) in Lumbricus, but also Retzius (1895) in polychaetes, Dorsett (1963, 1964) in *Nereis Vierens* and *Nereis Diversicolor*, Gray (1957) in the tonic muscle of *Rana temporaria* and Cole (1957) in *Natrix oreythrogaster* and *Felis domesticus*.

With regard to nerve termination, electron microscope study revealed one predominating type of neuromuscular junction (Type I). These junctions were observed in the septal region as well as in the periphery of the pinnately
arranged muscle bundles of the longitudinal muscle layer. The axons located in the septum were observed to contact muscle fibers and muscle tails along the entire length of the septum. The limiting membrane of the axons forming Type I junctions was always the irregular variety paralleling observations of the pavement-stone type axon localized in the lateral nerves. The junctions were characterized by a wide junctional gap, specialized postjunctional membrane and axon terminals, predominantly containing clear spherical vesicles. This latter observation is consistent with neuromuscular junctions reported by Mill and Knapp (1970) and Rosenbluth (1972) in Lumbricus; Farnesi and Vagnetti (1975) in Branchiobdella pentodonta; and Rosenbluth (1973) in leech.

Infrequently a Type I junction observed in the septal region was composed of more than one axon terminal. This contained two types of axons which contained either clear or dense vesicles (Fig. 18). According to previous authors, these axons may originate from (at least) two different perikarya, indicating polyneuronal origin and innervation (Clark, 1967; Myhrberg, 1972). Parallel with this information it has been reported that more than one transmitter substance may be liberated at a synapse (Pellegrini de Iraldi and De Robertis, 1962). On the strength of this study it is suggested that this nerve
ending may have a dual function. It is assumed that this junction not only represents polyneuronal innervation but may also be an example of a mixed excitatory and inhibitory termination which, excepting this study, has only been substantiated physiologically (Hidaka, Ito, Kuriyama and Tashiro, 1969).

Type II neuromuscular junction (Rosenbluth, 1972) could not be confirmed in this study although occasionally single non-myelinated axons containing predominantly clear spherical vesicles or dense vesicles could be observed coursing near the border of muscle fibers. The vesicles appeared to be evenly spaced along the axon. The axon formed an "apparent" neuromuscular junction with the muscle fiber. The junctional cleft was narrow. No postjunctional membrane specialization could be confirmed in this study. This "junction" may represent axons which merely course near the border of muscles. Due to the rarity and inconsistency of these "junctions" this finding was not interpreted as a second type of neuromuscular junction, although the possibility cannot be excluded.

Interneurons have been reported to occur along the length of the lateral nerve, nerve ring, subepidermal plexus and in, or between body wall muscles (Dawson, 1920; Bullock and Horridge, 1965). Certain cells were observed in this light microscopic study which appear to confirm this.
However, examination with the electron microscope did not confirm it. There was an abundance of large, irregularly shaped supportive cells in these areas, but there were no nerve cells and consequently could not be interneurons.

The pavement-stone shaped axons of the lateral nerve that were observed in the nerve ring and septal nerve contained predominantly clear spherical vesicles. It is assumed that their axon terminals containing the clear spherical vesicles form the neuromuscular junctions responsible for excitatory efferent innervation of the earthworm body wall musculature.

The tubular shaped smaller axon in the lateral nerve was not observed forming neuromuscular junctions. Axons of similar morphological characteristics were observed in the nerve ring and formed almost entirely the subepidermal plexus. These axons invariably contained dense vesicles. It is the assumption of this study that these smaller tubular shaped axons are the afferent pathway from the epidermal sense cells to either a central connection (via the lateral nerves) or to a peripheral interneuron (located in the nerve ring) which in turn may have local effects on the body wall musculature. This pathway could well represent the integration of sensory and motor functions in the peripheral plexus system.
SUMMARY AND CONCLUSIONS

The two nervous systems (segmental and plexi) contributing to the innervation of the body wall musculature of Lumbricus terrestris (L.) were studied by light and electron microscopy. The results of this investigation substantiate earlier histological features of neuromuscular junctions. Confirmation of an "en grappe" ending is consistent with findings reported by Smallwood (1926) in Lumbricus terrestris (L.) and Gray (1957) in Rana temporaria. The observation of a finely branching network system, the internal nerve net, confirms the results by Retzius (1892) in Lumbricus.

Light and electron microscopic studies on the peripheral nervous system revealed two populations of axons comprising the lateral nerves in each segment: the larger "pavement stone" shaped axons and the smaller "tubular" shaped axons and cytoplasmic processes. The nerve ring was also composed of the two varieties of axons and the cytoplasmic (dendritic or axon) processes. The latter processes were frequently incorporated into the internal nerve net (plexi system).

The nerve ring has been observed to give rise to the septal branches which penetrate the connective tissue.
septum of longitudinal muscle bundles. Examination of septal nerve bundles, as in the nerve ring and lateral nerves again showed the existence of large and small axons. The more abundant larger axons were observed to form neuromuscular junctions (Type I) along their length. These junctions resembled vertebrate skeletal muscle innervation due to the presence of axon terminals filled with clear spherical vesicles and a wide junctional gap and a specialized postjunctional membrane. The clear spherical vesicles are believed to contain acetylcholine, the excitatory transmitter of earthworm body wall musculature (Rosenbluth, 1972; Hidaka, Ito, Kuriyama and Tashiro, 1969). This observation substantiated earlier studies on earthworm neuromuscular junctions (Mill and Knapp, 1970; Rosenbluth, 1972).

Smaller tubular shaped axons in the septum of longitudinal muscle bundles were not observed to form neuromuscular junctions.

Examination of the internal nerve net revealed a supportive network containing many non-myelinated nerve fibers (tubular shaped axons and cell processes). The term "internal nerve net" presupposes fibers and nerve cell bodies that may be anatomically anastomosing and functionally, diffusely conducting. Hess's work (1925) has shown the internal nerve net to have local effects only, and this study has shown that the axons and cytoplasmic processes do
not anastomose. Intermuscular nerve cells were not identified. Therefore, the internal nerve net is more properly named the internal muscle plexus. Occasionally branches of this internal muscle plexus were observed entering the connective tissue between the pinnate muscle bundles. In the periphery as well as in the septum, neuromuscular junctions resembling vertebrate skeletal muscle innervation were observed. Light and electron microscopic studies confirm that the neuromuscular junctions of the body wall muscle are multiterminal (Smallwood, 1926; Dorsett, 1963; Mill and Knapp, 1970; and Rosenbluth, 1972).

Type II neuromuscular junctions reported by Rosenbluth (1972) could not be confirmed by this study.

Infrequently, a neuromuscular junction with the previously described characteristics contained, in addition, axon terminals filled with dense vesicles. As the variety of vesicle population is constant from soma to axon terminal (Myhrberg, 1971), this junction represents polyneuronal innervation and possibly an example of a mixed excitatory and inhibitory termination. This observation contributes to the physiological evidence already on hand that earthworm muscle cells are capable of inhibitory and excitatory junction potentials (Hidaka, Ito, Furiyama and Tashiro, 1969).

Large "pavement stone" shaped axons localized in the lateral nerves, nerve ring, and septal bundle represent
the segmentally arranged portion of the peripheral nervous system. The neuromuscular junctions formed by this variety of axons filled with clear spherical vesicles (containing acetylcholine) are responsible for the excitatory motor responses in earthworm body wall muscle. The centrally organized, segmentally arranged portion of the nervous system is believed to be the dominant system in the innervation of the body wall muscle.

The smaller "tubular" shaped axons were most abundantly observed in the subepidermal plexus. On the strength of this study, this variety of axons most likely represents the afferent pathway from epidermal sensory receptor cell bodies. These axons follow two courses; some are directed to interneurons in the nerve ring, while others are directed centrally through the lateral nerves. Interneurons and tubular shaped axons (comprising a portion of the peripheral plexi system) are assumed to represent the nervous mechanism responsible for the local control of the earthworm body wall muscle.
BIBLIOGRAPHY


PLATES AND FIGURES
Figure 1. Schematic representation of peripheral pathways in the body wall of *Lumbricus terrestris* (L.), after Hess (1925)

GF - Giant Fiber System
VNC - Ventral Nerve Cord
IN - Interneuron
LN - Lateral Nerve
MN - Motor Neuron
EG - En Grappe Ending
INN - Internal Nerve Net
LM - Longitudinal Muscle Layer
SB - Septal Branch
NR - Nerve Ring
CM - Circular Muscle Layer
SEP - Subepidermal Plexus
SC - Sense Cell (Receptor)
MC - Mucous Gland Cell
PLATE II

EXPLANATION OF FIGURES

Figure 2. Photomicrograph of the ventral nerve cord and lateral nerves (longitudinal section). The single first nerve (arrow) and the "double" second and third nerves (double arrow) comprising the segmental nerve in each somite are visible. X52

Figure 3. Longitudinal section of a second and third lateral nerves coursing together; demonstrating coarse and fine fibers. At this stage the origin of fibers cannot be determined any longer. Unidentified cells are visible along the pathway of these fibers. X1200
PLATE III

EXPLANATION OF FIGURES

Figure 4. Photomicrograph of the earthworm body wall (transverse section). An "en grappe" ending with motor end plates (arrow) is visible near the base of the longitudinal muscle layer. X800

Figure 5. Longitudinal section demonstrating the internal nerve net associated with the body wall musculature. X1200
PLATE IV

EXPLANATION OF FIGURES

Figure 6. Photomicrograph of the earthworm longitudinal muscle layer (longitudinal section), demonstrating apparent varicosities associated with the internal nerve net. Observe also "kink" (arrow) referred to in the text. X800

Figure 7. Photomicrograph of the earthworm longitudinal muscle layer (transverse section), demonstrating an apparent "interneuron" (C) X800
EXPLANATION OF FIGURE

Figure 8. Electron micrograph demonstrating a bundle of "pavement stone" shaped axons within the lateral nerve (transverse section). The axons show an irregular border and contain microtubules, neurofilaments and flattened membranous sacs. X44,000
Figure 9. Electron micrograph demonstrating a bundle of "tubular" shaped axons within the lateral nerve (transverse section). The axons exhibit a smooth border and contain numerous neurofilaments. The clefts between the axons are free of formed elements. X44,000
PLATE VII

EXPLANATION OF FIGURE

Figure 10. Lateral nerve (transverse section). Axon bundles are ensheathed in a supportive lamella which contains prominent gliosomes (G). The entire bundle is enveloped by a sheath of collagen fibers. X8400

P - boundary of nerve bundle
B - small axon bundle
Arrow - dense bodies
PLATE VIII

EXPLANATION OF FIGURE

Figure 11. Connective tissue septum of the longitudinal muscle bundle which is penetrated by a septal nerve branch. Two kinds of nerve fibers are visible, one containing predominantly dense cored vesicles (D), and a second containing microtubules, and profiles of agranular endoplasmic reticulum. X32,000
Figure 12. Connective tissue septum of the longitudinal muscle which contains a bundle of smooth "tubular" shaped axons. Prominent gliosomes are visible, partially ensheathing the nerve bundle. X22,400

Figure 13. Neuromuscular junction associated with a radial nerve bundle forming contact with muscle fibers and tails along its pathway. Arrow indicates instances in which a linear density is seen in the junctional cleft parallel to the postjunctional membrane. X32,000
Figure 14. Electron micrograph of the internal nerve net (transverse section). Small "tubular" shaped axons (arrow) and cell processes (double arrow) which are characterized by profiles of agranular endoplasmic reticulum (ag), and clear spherical vesicles (v) which occasionally are visible. The entire bundle is enveloped by a sheath of collagen fibers. X22,400
Figure 15. Internal nerve net (longitudinal section) demonstrating a gliosomal (G) supportive lamella and axon bundles containing dense vesicles (V). X32,000
Figure 16. The scalloped postjunctional membrane exhibits rows of projections approximately 20 nm in length (arrow). The distal ends of the projections are interconnected by a thin linear density. X88,000
Figure 17. Neuromuscular junction occurring between a muscle fiber and exposed axons of a small nerve bundle incompletely ensheathed by supportive cells. Axons are packed with vesicles at the point of contact, with either clear spherical vesicles (measuring 50nm in diameter) or dense vesicles (measuring approximately 100nm in diameter). The junctional membrane is scalloped and exhibits a cytoplasmic coating. X22,400
Figure 18. Membrane specialization associated with a neuromuscular junction. The axonal membrane appears to be rippled. The cytoplasmic surface of the postjunctional membrane appears to be the site of insertion of fine obliquely oriented cytoplasmic filaments (arrow). X64,000

Figure 19. Electron micrograph demonstrating close apposition between a muscle fiber and an axon containing clear spherical vesicles. The nerve and muscle cell membranes are separated by a narrow gap which contains no basement membrane. X44,000
The thesis submitted by Carol A. Aslam has been read and approved by the following Committee:

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The final copies have been examined by the chairman of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science in Anatomy.

01/07/76
Date

Chairman's Signature