INNERVATION OF THE FIBRILLAR FLIGHT MUSCLE OF AN INSECT: *TENEBRIO MOLITOR* (COLEOPTERA)

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ABSTRACT

The structure of peripheral nerves, and the organization of the myoneural junctions in flight muscle fibers of a beetle is described. The uniaxonal presynaptic nerve branches display the "tunicated" structure reported in the case of other insect nerves and the relationship between the axon and the lemnoblast folds is discussed. The synapsing nerve terminal shows many similarities with that of central and peripheral junctions of other insects and of vertebrates (*e.g.*, the intra-axonal synaptic vesicles) but certain important differences have been noted between this region in *Tenebrio* flight muscle and in other insect muscles. Firstly, the axon discards the lemnoblast before the junction is established and the axon effects a circumferential synapse with the plasma membrane of the fiber, which alone shows the increased thickness often observed in both pre- and postsynaptic elements. Secondly, in addition to the synaptic vesicles within the axon are present, in the immediately adjacent sarcoplasm, great numbers of larger postsynaptic vesicles which, it is tentatively suggested, may represent the sites of storage of the enzymatic destroyer of the activating substance similarly quantized within the intra-axonal vesicles. The spatial relationship between the peripherally located junctions and the portion of the fiber plasma membrane internalized as circumtracheolar sheaths is considered, and the possible significance of this with respect to impulse conduction is discussed briefly.

INTRODUCTION

Despite structural differences which exist between various insect muscles, all have a common feature that distinguishes them from vertebrate striated twitch fibers, namely that the myoneural junctions are established at several points on each fiber, rather than in a single motor end-plate. The fine motor nerve branches reaching the insect muscle may each, as is often the case in skeletal muscle, end in a complex conical structure (Doyère's cone), or may ramify over the fiber as slender arborizations, the diameter of which approaches the limit of resolution of the light microscope (Morison, 1927, 1928). The latter situation is characteristic of the orders Coleoptera, Hymenoptera, and Diptera; those orders in which fibrillar flight muscle is highly developed.

In tubular or non-fibrillar flight muscle,[1] elec-

[1] The term "fibrillar" is frequently used to descibe the indirect flight muscles of members of certain insect groups, notably Coleoptera, Diptera, and Hymenoptera; muscles which move the wings not by acting directly on the wing bases, but by the mechanical deformation of the exoskeleton of the wing-bearing segment. The term originated through von

Received for publication, May 16, 1960.

tron microscopic studies of the myoneural junction have been made on the wasp leg (Edwards, Ruska, and de Harven, 1958*a*) and on the intersegmental muscle of the cockroach (Edwards, 1959). Pringle (1954) showed that the tymbal muscles of cicadas exhibited certain physiological features associated with fibrillar flight muscle of higher insects. Edwards, Ruska, and de Harven (1958*b*) examined the myoneural junctions in both tymbal and indirect flight muscle of the cicada *Tibicen*, and found them to be similar to each other, though differing somewhat from the junctions in typical tubular muscle. On a basis of the general features of these muscles, for example the large sarcosomes and "internalized" tracheolar supply, Edwards and his coworkers agreed that these should be termed "fibrillar," by analogy with the flight muscles of higher insects. On the other hand, they described a sarcoplasmic reticular system, extensive in the case of tymbal muscle and more restricted in indirect flight muscle fibers, which differs from that occurring in *Tenebrio* (Smith, 1960). Furthermore it is evident that the myoneural junctions in *Tenebrio*, described here, also differ from those of the cicada, and it is important that such differences be considered and their significance assessed, before all these muscles are grouped together in the same category. While the present work contributes to the structural aspects of the problem, correlation with functional considerations awaits further study.

Siebold's observation, in the middle of the last century, that these flight muscles were easily fragmented into their component fibrils, unlike the wing and body muscles of other insects. Amongst the other characteristics of fibrillar muscle may be mentioned the well developed internalized tracheolar system of the fiber, the randomly scattered nuclei, and the large diameter of the fibrils and of the fibers.

The muscles of the legs and of the rest of the body (*e.g.* intrasegmental muscles) have been styled "tubular," since their nuclei usually occupy only the core of the fiber. In this case, the interfibrillar sarcoplasm is greatly reduced, the fiber is typically narrow, and the tracheal supply is superficial. A further term, "close packed," has been applied to flight muscles of Orthoptera and Leptidoptera, where the radial arrangement of fibrils found in tubular muscle has been lost, and where the nuclei are situated peripherally. The peculiarities of fibrillar muscle have been correlated with the high metabolic demands made by the flight mechanism of the higher insects.

It should be stressed that these terms are approximate rather than absolute; that structural differences in detail occur between muscles generally referred to in a single category. For an excellent review of the structure and physiology of insect muscle see the monograph by Pringle (1957).

MATERIALS AND METHODS

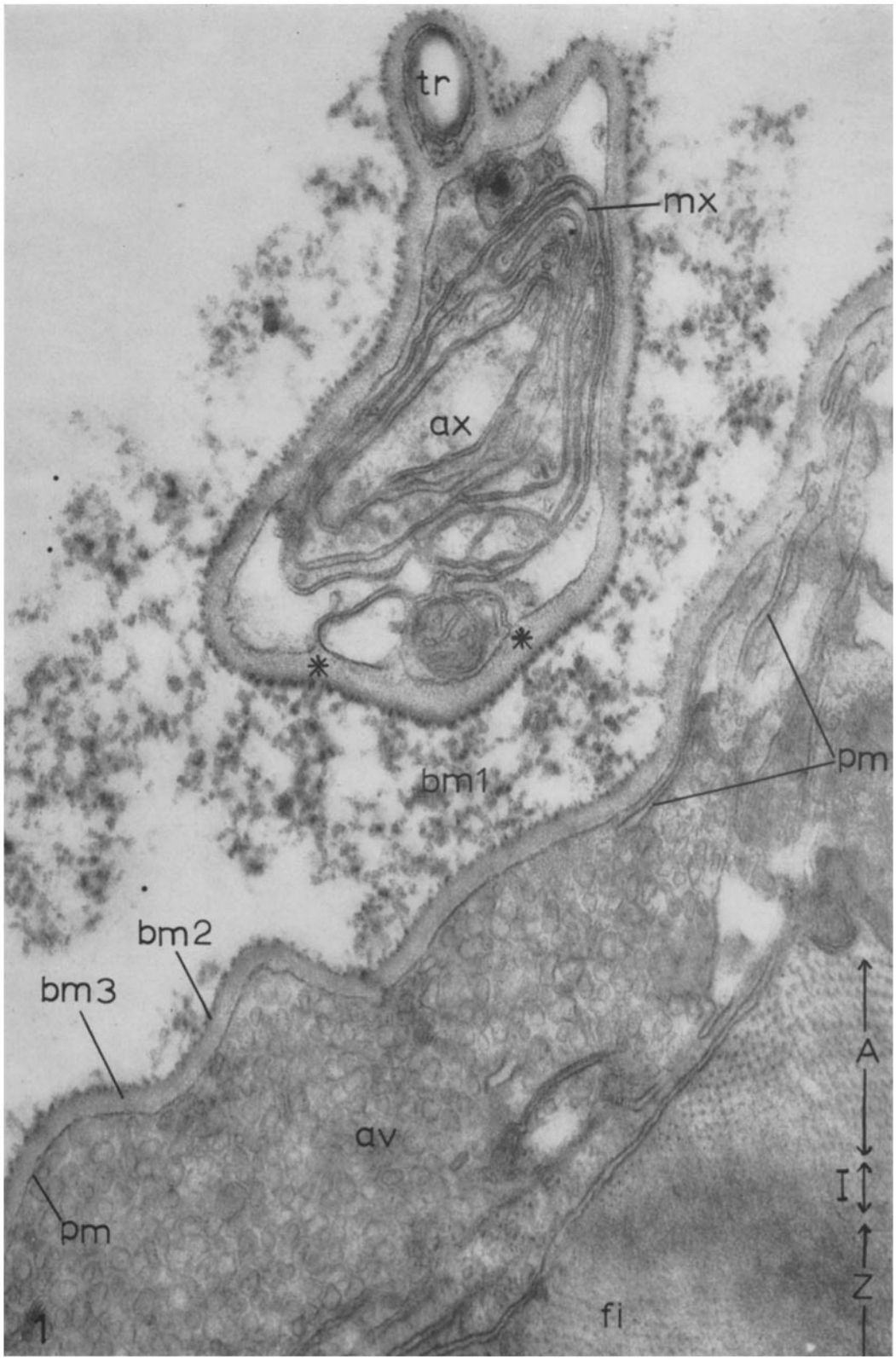
The tergosternal and basalar pleural muscles of fully hardened adults of the mealworm beetle, *Tenebrio molitor*, were employed. The freshly separated thorax was bisected medially in sucrose-con-

FIGURE 1

Electron micrograph of a peripheral motor nerve branch, approaching the surface of a fiber of fibrillar flight muscle of *Tenebrio molitor*. The mesaxons (*mx*) envelop the axon (*ax*) in an irregular series of folds; the so called "tunicated" arrangement (Edwards *et al.*, 1958*a*). Two mesaxon origins are present, and are indicated by asterisks. A tracheole (*tr*) accompanies the axon, and the tracheoblast and lemnoblast basement membranes are confluent at this point. Note also the similarity between the basement membrane of the lemnoblast and of the sarcolemma. In the case of the latter, the diffuse unorganized component *bm1*, sometimes observed in this muscle, is unusually well developed here. Fibrillar organization is not apparent in either of the compact regions of the basement membrane *bm2* and *bm3*.

The plasma membrane of the muscle cell (*pm*) lies immediately beneath the basement membrane of the sarcolemma, and is deployed in a series of complex folds (*pm*). The densely packed vesicles (*av*) are thought to represent sarcoplasmic aposynaptic vesicles, surrounding an axon which itself lies out of the plane of section, (*cf.* Fig. 13).

The myofibril at the lower right (*fi*) is sectioned through a Z band, and the A band and very short I band are also seen. × 53,000.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taining 1 per cent OsO_4 buffered with veronal-acetate at pH 7.7. The material was fixed at 0°C. for 90 minutes and then transferred directly to 70 per cent ethanol, at which point individual fibers were isolated, and their dehydration completed. Small segments of single fibers were embedded in 25:75 methyl:butyl methacrylate containing 1 per cent luperco and 0.01 per cent uranyl nitrate. Sections were cut on a Porter-Blum microtome and examined in an RCA-EMU-2 and in a Siemens Elmiskop 1. Contrast in the specimens was enhanced by "staining" with lead hydroxide for from 2 to 5 minutes according to the method of Watson (1958) and Peachey (1959).

RESULTS

The Fibrillar Flight Muscles

A brief description of the organization of *Tenebrio* flight muscle will suffice here, as a detailed account appears elsewhere (Smith, 1961). The mean fiber diameter is about 200 μ in the mature adult, and as is typical of fibrillar muscle, the fibrils are large (about 2 μ in diameter) and the sarcosome content is very high; the latter accounting for almost one-half of the total cross-sectional area of the fiber. Tracheolar penetration is extensive at all depths within the fiber. Synapses between the plasma membrane of the fiber and the motor nerve branches are established exclusively at the periphery of the fiber.

Peripheral Nerves and Presynaptic Areas

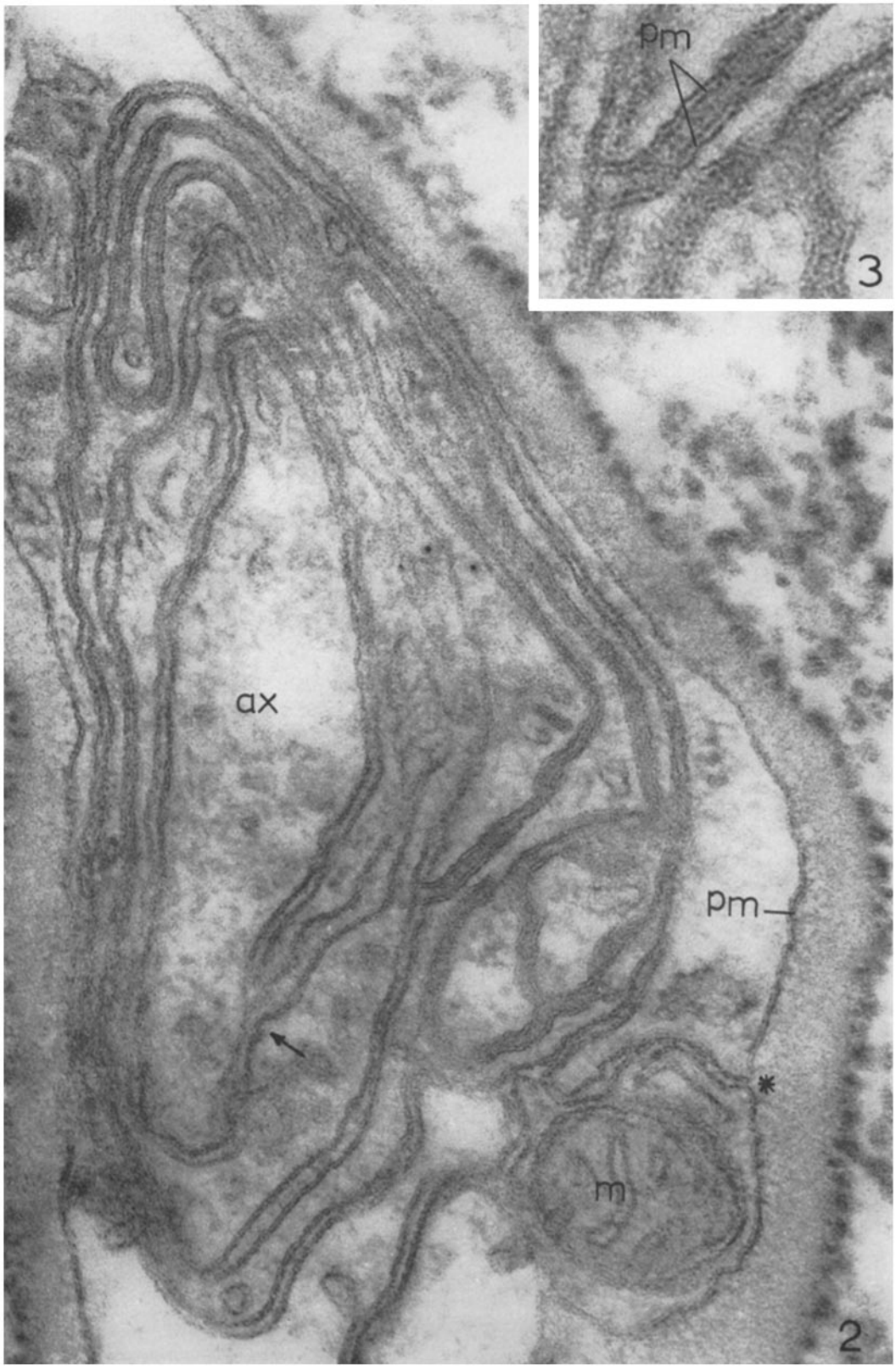
The main motor nerve trunks divide in their passage to the muscle into fine branches, several of which associate with each fiber. The number of myoneural junctions occurring at the surface of a single fiber has not been ascertained, but in any field including an entire profile of a transversely sectioned fiber, four or five synapsing or presynaptic axons are commonly seen.

The nerve branch, before it reaches the surface of the fiber, shows the "tunicated" organization described in other insects by Edwards, Ruska and de Harven (1958*a*, *b*) and Edwards (1959). The finest of these branches contain but a single axon, as little as 0.5 μ in diameter, bounded by a loosely folded mesaxon system derived from the invaginated plasma membrane of the lemnoblast. Transverse sections (Figs. 1, 2, and 7) show frequent bifurcations of the mesaxon, sometimes involving the separation of apposed plasma membrane surfaces and furthermore, longitudinal sections of nerve branches near the surface of the fiber (Figs. 5 and 6) frequently show that the number of mesaxon profiles on each side of the axon is unequal. Usually, more than one mesaxon is seen in a branch containing only a single axon (Fig. 1). The relationship between adjacent units of the invaginated membrane is essentially as has been described by Edwards and his coworkers in *Vespa*. Each unit membrane shows the characteristic triple-layered (25-25-25 A) organization described by Robertson (1959), and each pair of membranes is separated by a space of 75 to 150 A containing material of higher density than the surrounding cytoplasm of the lemnoblast (Fig. 3). This relationship is, however, periodically disrupted by the intercalation of lacunae formed through separation of adjacent plasma membranes of a mesaxon; and these lacunae may be larger than the axon itself (Fig. 7).

The organization of the membranes of the lemnoblast deserves further comment and, regarding this, there are two possibilities concerning the complications found in insect tunicated nerve. The irregular mesaxon profiles described may

FIGURES 2 and 3

Higher power electron micrographs of the peripheral nerve shown in Fig. 1. Note the triple-layered organization (25-25-25 A) of the lemnoblast plasma membrane (*pm*), and the origin of a mesaxon (*). The arrow denotes the end of the mesaxon fold, the membranes of which diverge to envelop the axon; a space of about 75 A separating the axonal and lemnoblast plasma membranes. The 75 to 100 A space between adjacent regions of invaginated membrane in the mesaxon folds (Fig. 3) is filled with amorphous material of greater density than the surrounding cytoplasm. The axon (*ax*) contains an ill defined granular material, and only one or two vesicles. A lemnoblast mitochondrion is seen at *m*. Note the organization of the basement membrane, described more fully in the caption to Fig. 1. Fig. 2 × 140,000. Fig. 3 × 280,000.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either represent the membranes of a single rolling lemnoblast fold as in myelinated nerve, but with many indentations and perhaps fenestrations, or of a number of folds lapped loosely around the single axon. The former suggestion appears (in the present case) to be the more probable as, if multiple rather than single irregular lemnoblast folds are involved, then, in contrast to what has been found, not all of the multiple mesaxons or of the branches of bifurcating mesaxons of each lemnoblast would ultimately rejoin each other.

The outer unit membrane of the lemnoblast, from which the mesaxons invaginate, lies immediately beneath the basement membrane, which is indistinguishable from the basement membrane component of the muscle fiber sarcolemma. In each instance, the inner layer, 500 to 1000 A in depth, is of relatively low density (in material "stained" with lead hydroxide), and in which fibrillar organization has not been observed. Outside this lies a second component usually between 250 and 300 A in depth which is denser and more irregular. This layer is, however, indistinct in "unstained" preparations, and is occasionally very restricted even after treatment with lead hydroxide (Fig. 7). A randomly distributed coarsely granular material is sometimes observed outside this layer, and may be regarded as a third component of the basement membrane (Fig. 1). The basement membrane of the lemnoblast frequently though not invariably surrounds a small tracheolar branch (Fig. 1), around which lie the outer tracheoblast plasma membrane, and the inner membrane (probably a "mestracheon"; Edwards, Ruska, and de Harven, 1958*c*), within which the cuticular lining of the intima is laid down.

The lemnoblast basement membrane surrounding the presynaptic axon fuses with the basement membrane of the sarcolemma (Figs. 4 and 7), but this does not affect the complex mesaxon or the cytoplasmic components of the nerve fiber. A few small mitochondria occur in the lemnoblast cytoplasm, and are very occasionally seen in the axon itself, and the lemnoblast matrix also contains particles, presumably of ribonucleoprotein, which are more usually free than membrane-bound. The appearance of the axonal cytoplasm, which contains granules and, rarely, occasional vesicles, is similar both before and after fusion has taken place between the peripheral nerve, and the sarcolemma.

Up to this point then, the peripheral nerve and presynaptic axon in *Tenebrio* flight muscle does not differ markedly from the situation described in the case of other insects. The distinction only becomes apparent when examination is made of the axon as

FIGURE 4

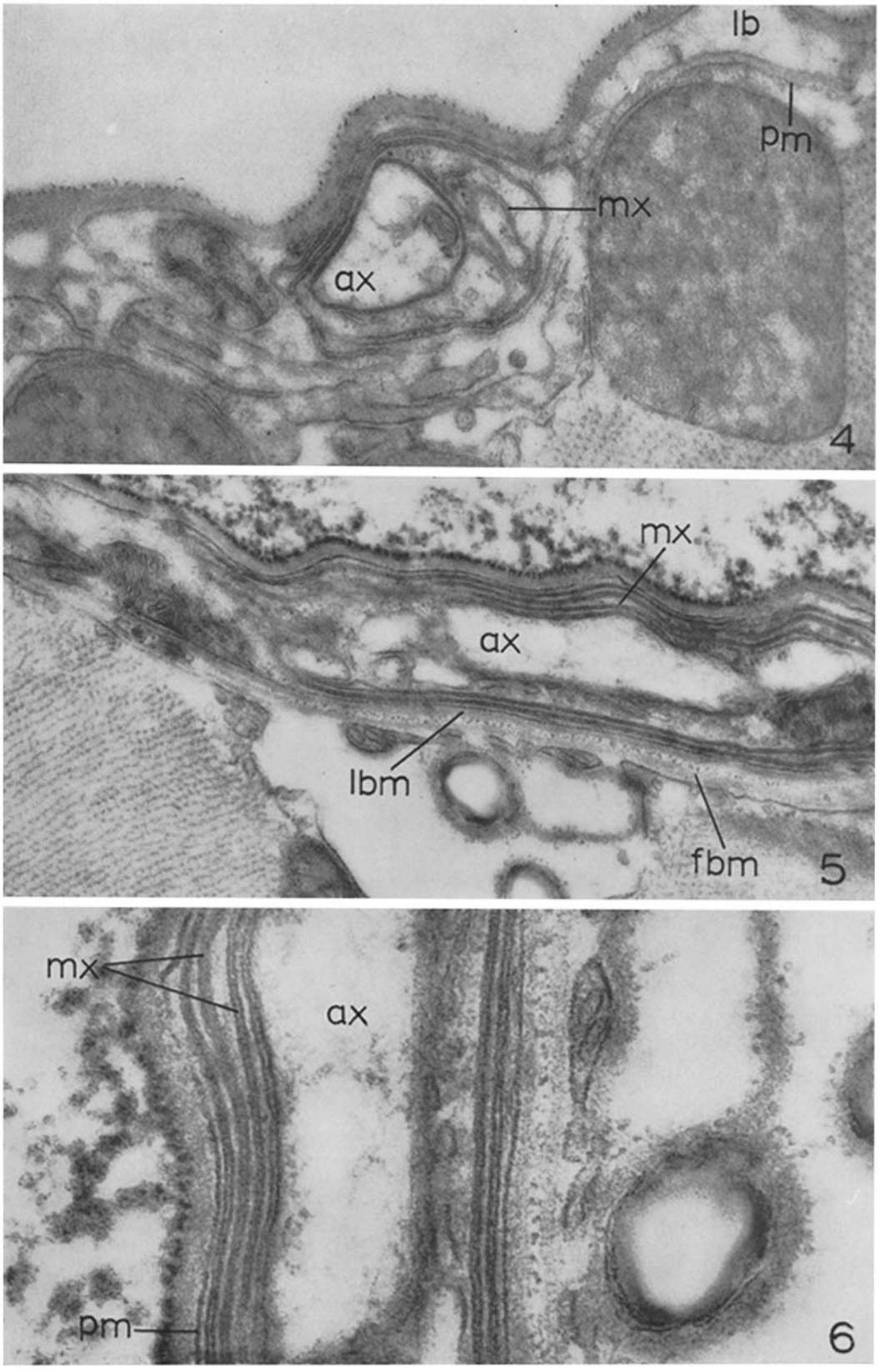
Showing a presynaptic axon in *Tenebrio* flight muscle, which is still surrounded by the mesaxon folds (*mx*) of the lemnoblast (*lb*) which extends laterally. The axon lies beneath the basement membrane of the sarcolemma; a consequence of the fusion of this with the lemnoblast basement membrane. In such presynaptic axons, vesicles are virtually absent, and mitochondria, such as that seen here, are of rare occurrence. The plasma membrane of the fiber (*pm*) lies just beneath the lower surface of the lemnoblast. × 45,000.

FIGURE 5

A longitudinal section of a peripheral nerve branch close to, though not yet fused with, the surface of a flight muscle fiber of *Tenebrio* flight muscle. The axon (*ax*) is devoid of vesicles, and is surrounded by profiles of mesaxon folds (*mx*) and the basement membrane of the lemnoblast (*lbm*), which is distinct from that of the sarcolemma of the fiber (*fbm*). × 40,000.

FIGURE 6

An enlargement of a portion of Fig. 5. Note the inequality in the number of mesaxon profiles on each side of the axon (*ax*), attesting to the irregularity with which these folds envelop the axon. Note also the single unit membrane lying beneath the basement membrane of the lemnoblast (*pm*) and the paired membranes of the invaginated mesaxons (*mx*); each pair being separated from its neighbors by a narrow zone of cytoplasm. × 93,000.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it approaches and effects synapse with the muscle fiber.

The Synaptic Region

The appearance of vesicles within the synapsing axon is characteristic both of vertebrate and invertebrate nerves, and while it is frequently assumed that these vesicles represent the site of storage of acetylcholine (in cholinergic nerves) this has yet to be rigorously demonstrated. As in all presynaptic axons, such vesicles are virtually absent in *Tenebrio* both in the free peripheral nerve branches, and in the axons lying beneath the basement membrane of the fiber but still isolated from the plasma membrane of the muscle, by the presence of the lemnoblast. Fig. 4 shows a small axon, lying beneath the dense layers of the sarcolemma, surrounded by folds of its complex mesaxon and in which both pre- and postsynaptic vesicles are almost entirely lacking.

The unique feature of the myoneural junction in the muscle of this insect is apparent when the synapse is neared and completed, and resides not in the nature of the structure of the synapsing axon, but rather in that of the muscle cell in the synaptic and postsynaptic area. In all other insect muscles studied (Edwards *et al.*, 1958*a*, *b*; Edwards, 1959), the synapsing axon was found to be capped dorsally by the persisting lemnoblast, which leaves only the ventral aspect of the axon in apposition with the plasma membrane of the fiber, with which a longitudinal synapse is effected. In *Tenebrio*, on the other hand, the lemnoblast is shed before the synaptic region is reached, and the naked axon is thereafter allowed an uninterrupted circumferential synapse with the muscle.

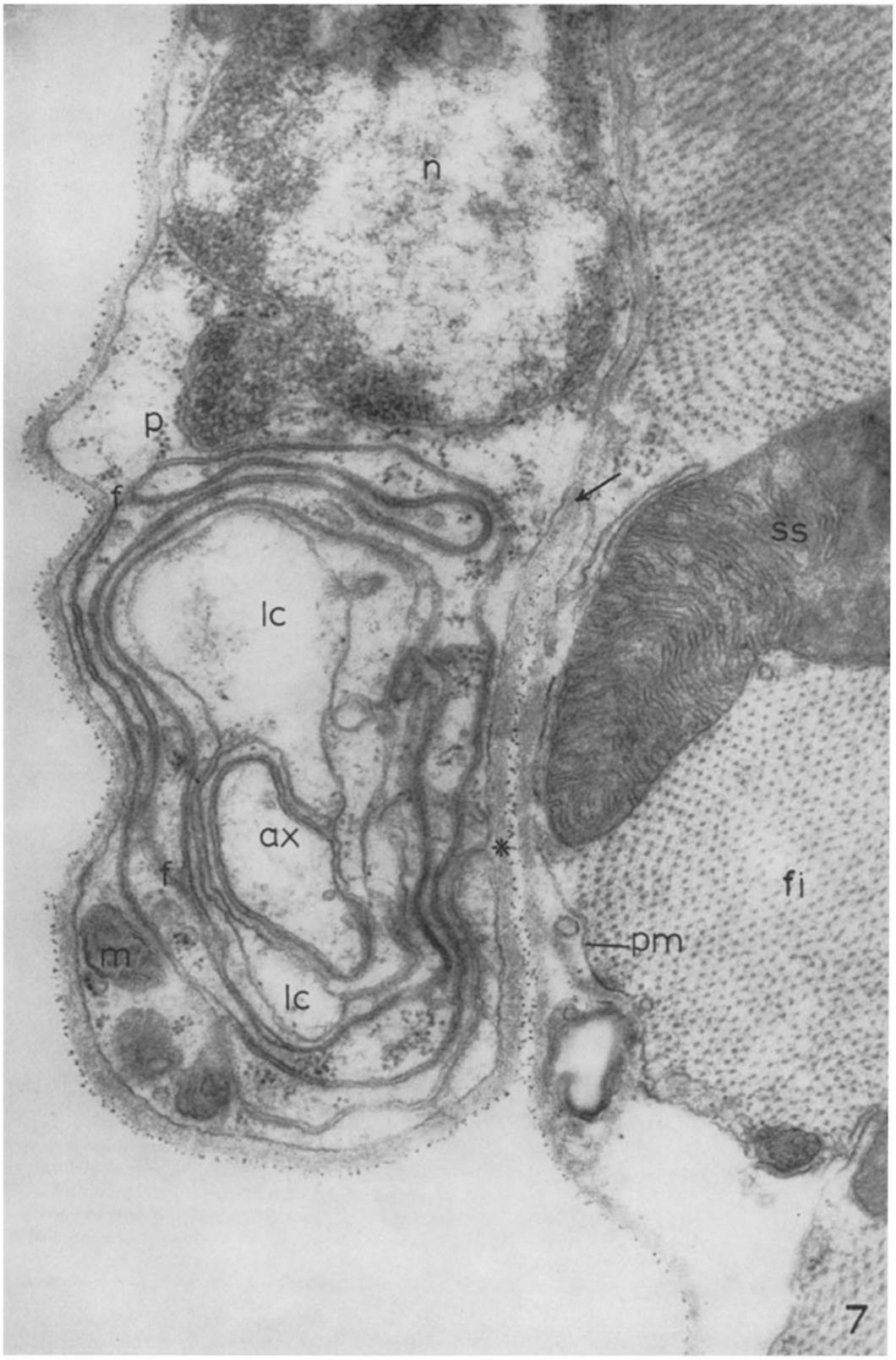
Figs. 8 and 9 illustrate axons close to the commencement of synapse. In the latter, the lemnoblast folds have been almost discarded, and only a short mesaxon is present. In the former, the lemnoblast has been entirely shed by the axon though the basement membrane of the lemnoblast terminations are still to be seen, fusing laterally with the basement membrane of the sarcolemma. In each figure, the axon contains a mitochondrion and numbers of synaptic vesicles. Beneath the axon lies a series of membrane folds enclosing cytoplasm containing many vesicles larger than those within the axon. The complexity of these irregular folds may be seen by comparing the three serial sections (about 600 to 1000 A apart) shown in Figs. 15 through 17. Examination of many such fields has established that these periaxonal profiles represent complex convolutions *of the plasma membrane of the muscle fiber*, and that synapse may thus occur all around the axon.

In Fig. 11 is seen a relatively large axon, surrounded by profiles of numerous pseudopodia through which is clearly established a direct and uninterrupted path to the body of the fiber. The axon is packed with synaptic vesicles (about $7500/\mu^3$) together with mitochondria, and is bounded by a typical 75 A plasma membrane. Around this, at a distance of no more than 75 to 100 A, lies the plasma membrane component of the muscle which contributes to the synapse and which is thickened in this region. Whereas in cicada indirect flight muscle, Edwards and his coworkers (1958*b*) reported an apparent thickening in *both* apposed membranes of the synapse, there is no doubt that here it is that of the muscle which alone shows this modification. The morpho-

FIGURE 7

In this micrograph, the axon (*ax*) is still surrounded by folds of lemnoblast cytoplasm just as in a peripheral nerve branch, but the basement membranes of lemnoblast and muscle fiber have become confluent (arrow). A mesaxon origin is indicated by the asterisk: the mesaxon show numerous bifurcations, as at *f*, and also lacunae (*lc*) where the component membranes of the mesaxon separate for a short distance. Small mitochondria (*m*) are present within the lemnoblast cytoplasm, and the groups of unattached particles (*p*) are presumed to be of ribonucleoprotein. *n* represents a lemnoblast nucleus. Note that the axon contains only two or three vesicles; a sparseness characteristic of the presynaptic regions.

The plasma membrane of the fiber (*pm*) bounds the peripheral layer of sarcosomes (*ss*) and fibrils (*fi*). × 48,000.


n
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7

logical significance of this feature is not clear, though it appears (see Fig. 12) that the thickening is achieved by intercalation of a layer of dense material about 75 A in thickness, on the inner surface (*i.e.* the surface facing the axon plasma membrane) of the highly osmiophilic fiber membrane.

The intra-axonal vesicles are quite distinct in appearance from the postsynaptic vesicles occurring in the surrounding sarcoplasm. The former are smaller, 250 to 450 A in diameter and contain material of relatively high density. The latter, on the other hand, are not only larger but have a much wider size-spectrum (from 450 to 1200 A diameter), and the density of their contents also varies considerably, many appearing to be devoid of any dense included material (Figs. 1, 10, 13, and 14, etc.). The most striking feature of these synapses, however, lies in the enormous number of postsynaptic vesicles in the cytoplasm of the muscle fibers. These are by no means restricted to the immediate vicinity of the axon, as is clearly seen in Fig. 10 where a naked synapsing axon is placed at the edge of a vesicle-filled region 1 μ in depth extending for more than 6 μ laterally from the axon, while Fig. 13 illustrates to good advantage the size and spatial relationships of the synapsing axon, the postsynaptic area, and the underlying myofibrils and sarcosomes. While small numbers of "aposynaptic granules" 150 to 200 A in diameter similar to those reported in other insect myoneural junctions, occur in the postsynaptic zone in *Tenebrio*, (Figs. 11, 14 to 17), the additional extreme elaboration of vesicles situated round a naked axon constitutes an important and novel structural feature, demarcating these junctions from all others hitherto described. In Fig. 13 the lemnoblast is still present, though no longer associated with the axon, and a tightly packed area of sarcoplasmic postsynaptic vesicles is interposed between the axon and the peripheral layer of fibrils and sarcosomes.

The extensiveness of the postsynaptic vesicular zone around the axon produces such images as in Fig. 1. High concentrations of closely packed vesicles (about 2500/μ^3) are often observed, lying just below the sarcolemma and apparently not immediately associated with an axon. But where

FIGURE 8

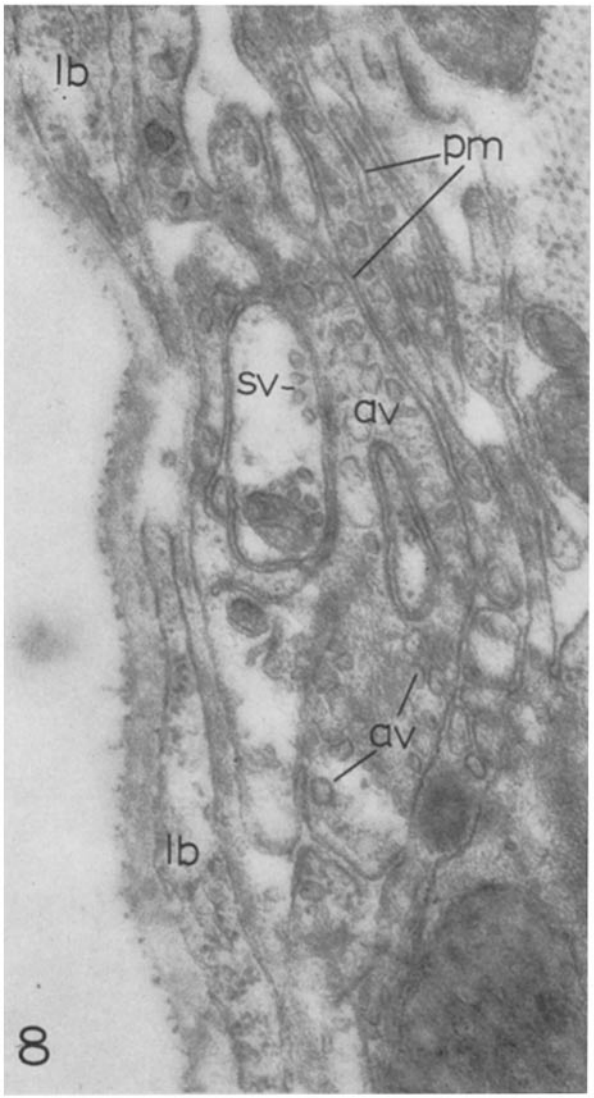
An electron micrograph of a transversely sectioned flight muscle fiber of *Tenebrio* showing an axon which has apparently just commenced synapse. The lateral terminations of the lemnoblast, which no longer surrounds the axon, are seen at *lb*. The axon contains a mitochondrion and a few synaptic vesicles (*sv*), and in the sarcoplasm surrounding it are present a number of large aposynaptic vesicles (*av*). As is characteristic of myoneural junctions in this muscle, the fiber plasma membrane (*pm*) in the area of synapse is folded in a complex manner. × 40,000.

FIGURE 9

An axon in *Tenebrio* flight muscle close to the start of synapse. A short mesaxon (*mx*) persists, and the axon is thus still surrounded by lemnoblast cytoplasm, which is subsequently discarded, allowing the close apposition of muscle and nerve plasma membranes. Even at this stage, a number of vesicles appear within the axon. × 24,000.

FIGURE 10

A synapsing axon, within which are seen mitochondria and large numbers of synaptic vesicles (*sv*). Note that the pseudopodia surrounding the axon are continuous (at arrow) with the typical sarcoplasm. Extending for a distance of several microns from the axon itself lie many aposynaptic vesicles (*av*) interspersed with isolated profiles of the intensely folded fiber plasma membrane (*pm*). This electron micrograph may be compared with that shown in Fig. 1, where a similar concentration of vesicles occurs; presumably the aposynaptic vesicles associated with an axon lying out of the plane of section. The whorled membranous structure seen in this figure is not a typical component of the junction, and its significance is unknown. × 40,000.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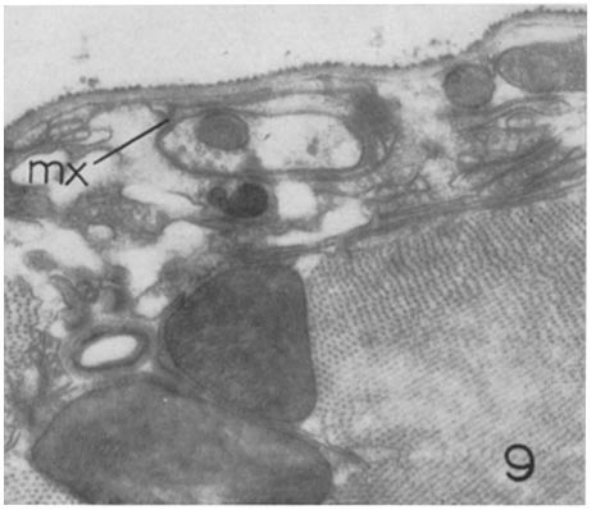
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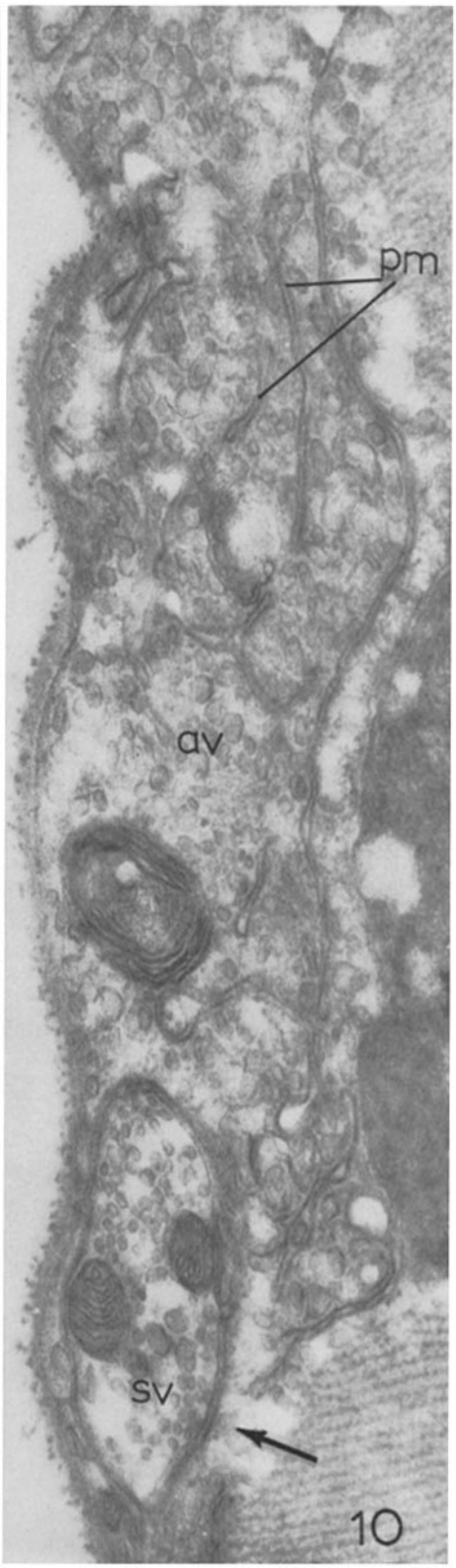
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such regions occur, a peripheral nerve is usually present in the vicinity, as in this figure, and it is supposed that these vesicle-filled areas represent postsynaptic regions associated with axons persuing a course which lies out of the plane of section.

DISCUSSION

While a considerable amount of information is now available concerning the fine structure of both central and peripheral components of vertebrate and invertebrate nervous systems, it is evident that relatively little progress has been made in effecting a synthesis between this and corresponding physiological data. This difficulty is, unfortunately, met with in the case of the myoneural junction.

All myoneural junctions achieve the more or less close apposition of axon and fiber plasma membrane, presumably to facilitate membrane depolarization by the chemical activator (*e.g.* acetylcholine) released by the synapsing axon. The lemnoblast elements stop short of the ending in all myoneural junctions so far studied and, furthermore, the synaptic contact is more intimate in insects, because the interposition of the basement membrane found in vertebrates (Palade, 1954; Robertson, 1956; Reger, 1957, 1958, 1959; and Andersson-Cedergren, 1959) is lacking. In all insect muscles so far examined including the flight muscles of *Tenebrio* described here, a more intimate apposition of plasma membranes is allowed by the absence, from the junction, of basement membrane material; a situation apparently paralleled in mammalian smooth muscle (Caesar, Edwards, and Ruska, 1957). Further variation occurs in the architecture and distribution of the synaptic area. All investigations of vertebrate "twitch" fibers have shown that in the end-plate region, the muscle surface is infolded in a series of convolutions or synaptic grooves within which are situated the axon branches. This modification is not found in insect myoneural junctions.

All insect muscles are alike in that each fiber receives several fine branches of the motor nerve or nerves, each of these branches forming a separate myoneural junction at the fiber surface. While a

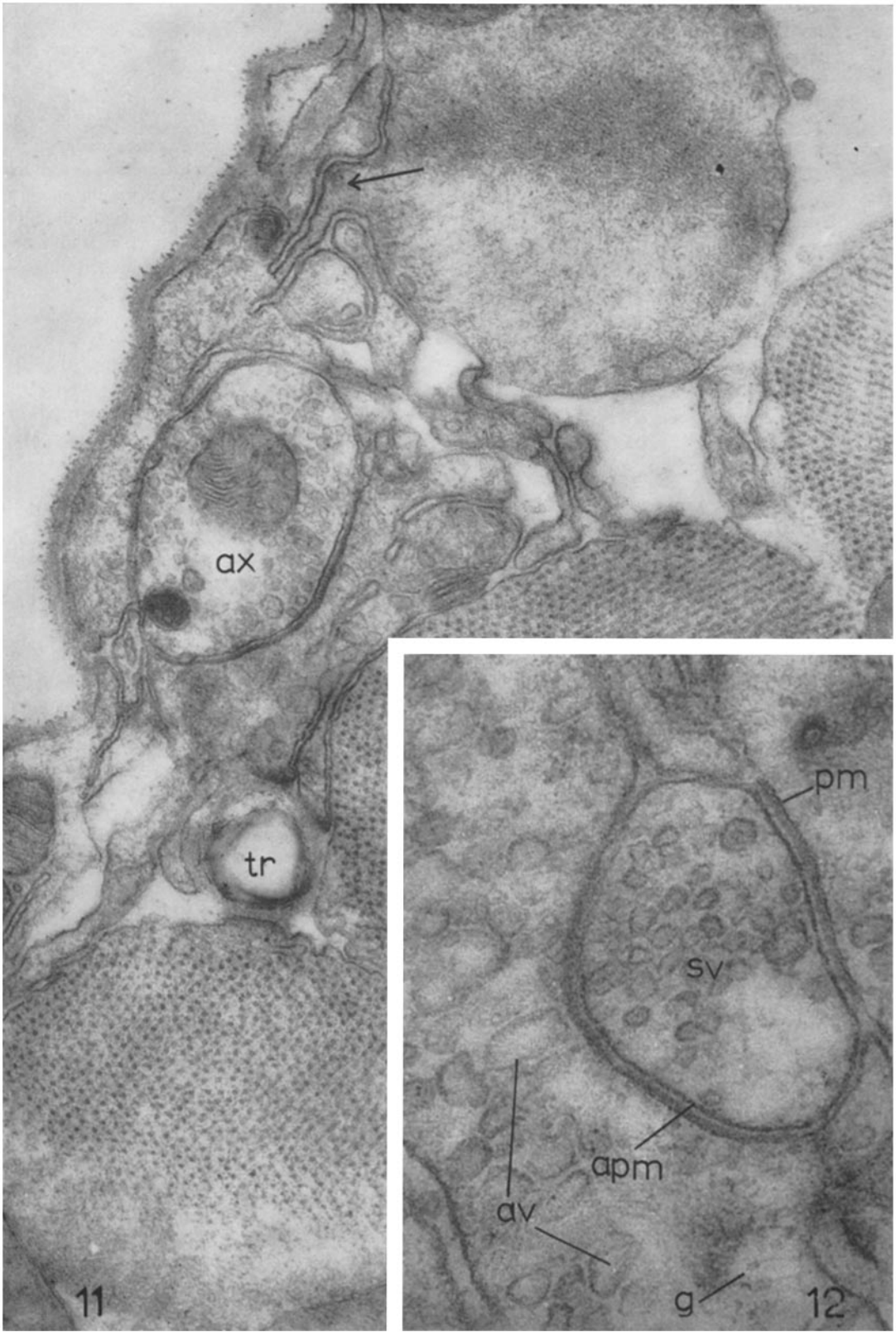
FIGURE 11

Another example of a neuromuscular junction in fibrillar flight muscle of *Tenebrio*. In this case fewer aposynaptic vesicles are present in the section than in other instances, but the abundance of synaptic vesicles within the axon (*ax*) should be noted. Continuity between the area immediately surrounding the axon and the bulk of the sarcoplasm is clearly demonstrated by the narrow isthmus indicated by an arrow. The lemnoblast has been completely discarded, and the axon, surrounded now by sarcoplasm, effects a circumferential synapse running parallel with the long axis of the fiber. *tr* represents a tracheole in transverse section. × 40,000.

FIGURE 12

A higher magnification electron micrograph, illustrating the apparent localized thickening of the postsynaptic (fiber) membrane at the point or region of synapse. This seems to be effected through the apposition of a 75 A layer of dense material upon the fiber plasma membrane (*pm*), while that of the axon (*apm*) is unmodified. In other insect myoneural junctions (Edwards *et al.*, 1958*a*, *b*; Edwards, 1959) both synapsing membranes are thickened, but the presence of a persistent lemnoblast cap restricts the region of junction to the inner surface of the axon.

Note the synaptic (*sv*) and aposynaptic vesicles (*av*). The latter are larger and have a greater size range, and also show considerable variation in the density of their contents. The view that the synaptic vesicles may be the sites of liberation of the activator is adopted here, and it is suggested that the enzymatic destroyer of this, acetylcholine esterase or a functional analogue, may be similarly quantized in the aposynaptic vesicles. A few granules (*g*) present in the cytoplasm surrounding the axon may be identical with the "aposynaptic granules" described in other neuromuscular junctions. × 100,000.


ax
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similar situation is met with in crustacean and other invertebrate muscles and in vertebrate tonic fibers, in twitch fibers only a single end-plate is present, at which a propagated change in membrane potential is initiated. No such propagated spread occurs in insect muscles; local areas of depolarization are initiated at each myoneural junction. In such muscles, the nerve endings may occur at specialized regions (Doyère's cones) [prominencies at the surface of the fiber apparently covered by the fused basement membranes of lemnoblast and muscle cell] or may simply be represented by extremely fine branches, ramifying across the surface of the fiber and ultimately penetrating beneath the basement membrane. The latter condition is characteristic of fibrillar flight muscle, though not exclusively confined to it, while most skeletal muscles appear to be supplied at Doyère's cone endings. Graded response in skeletal muscles of arthropods and some other invertebrates is effected by multiple innervation of each fiber by "fast," "slow," and, in crustacea, "inhibitory" axons. The double innervation of insect muscle was first recognized by Mangold (1905), and received physiological confirmation through the work of Pringle (1939) and others. A review of this subject is given by Hoyle (1957).

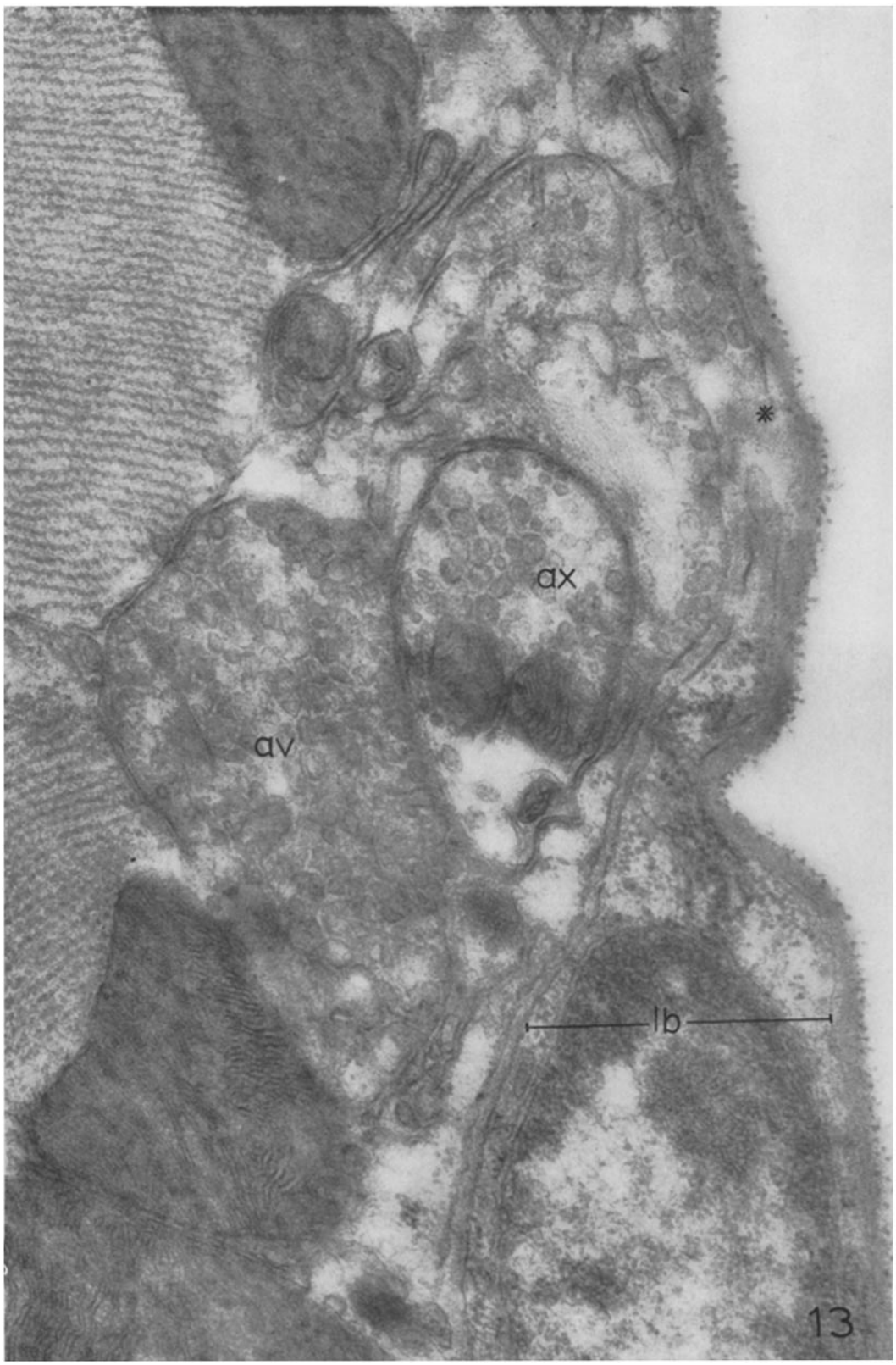
All electron microscopic investigations of insect muscles have confirmed the multiterminal nature of the innervation. However this should not be confused with polyneuronal supply. The peripheral nerve branches to cockroach intersegmental fibers (Edwards, 1959) were found to contain one large central axon and up to seven additional smaller axons, enclosed within a common lemnoblast. Furthermore, Edwards showed that up to four complexes of from one to three lemnoblasts, each containing a number of axons, contacted each fiber. Edwards' work affords the only published micrographs of what appear to be profiles of the complex lateral expansions of Doyère's cone endings. While it is entirely probable that each such ending contains both "slow" and "fast" neuron branches, physiological evidence has not so far suggested that "slow" and "fast" axons occur within the same nerve branch, enclosed within a common lemnoblast, though these elements may be closely associated spatially at the myoneural junction. On the other hand in cicada flight and tymbal muscle (Edwards *et al.*, 1958*a* and *b*) and in *Tenebrio* flight muscle, multiple axons within a common lemnoblast are not found, and uniaxonal junctions are made with a fiber at frequent intervals. The difference between this situation and that described for the cockroach is striking.

Darwin and Pringle (1959) point out that no physiological evidence has been obtained for the existence of "fast" and "slow" double innervation of fibrillar flight muscle. They found, however, that the single nerve supply of the basalar muscle of the beetle *Oryctes* is of the "slow" facilitating type, whereas flight muscle of the flies *Sarcophaga* (Boettiger and McCann, 1953; Boettiger, 1957), *Calliphora* (Pringle, 1949), *Calliphora*, *Lucilia*, *and Eristalis*, and of the wasp *Vespa* (Roeder, 1951), and also the tymbal muscle of the cicada *Platypleura* (Pringle, 1954) all prove to be "fast." At present, cytological characterization of these two types of myoneural junction cannot be made, but it is evident that examination of selected examples, the physiology of which is known, may well clarify the structural basis of this functional division.

Whether the nerve branch ends in a Doyère's cone or whether it takes part in a longitudinal junction with the fiber, two common features are evident. Firstly, light and electron microscopy support the view that basement membranes of lemnoblast and fiber fuse, resulting in the direct apposition of synapsing plasma membranes. Secondly, no indisputable evidence exists for the penetration of the nerve into the body of the fiber. Tiegs (1955) found that the fibrillar flight muscle

FIGURE 13

An electron micrograph of a synapsing axon (*ax*) associated with which is a particularly compact region filled with aposynaptic vesicles (*av*) and which may be compared with Fig. 1. A lemnoblast extension is present (*lb*) but is no longer accompanying the axon. The asterisk denotes a region of fusion between the basement membrane of the lemnoblast and of the muscle fiber. Note the extensive folding of the fiber plasma membrane around the synaptic zone. × 55,000.


ax
av
lb
13

fibers of certain flies appeared to be of very great size reaching 1800 μ in *Rutilia potina*, one of the species in which innervation was examined. It is possible, however, that these giant fibers are in reality compound structures enclosed within a common basement membrane, as the topography of the plasma membrane cannot be observed in the light microscope, and Tiegs mentions the suggestive fact that the fibers are broken up into smaller units by large tracheal trunks. If these smaller units are the true fibers, the nerve endings described by Tiegs as occurring deep within the fiber may actually be situated at the true fiber surface. This point is currently being investigated with the electron microscope in the fly *Phormia*.

The work of Marcu (1929) is frequently quoted as a demonstration of nerve penetration. He examined muscles of Diptera, Coleoptera, and Orthoptera in sections stained with silver according to the methods of Golgi and Cajal, and described thick "trunks" between the fibers, giving rise to brush-like endings within the fibers; up to 20 per mm. in the case of fly muscle. Although he states that skeletal and flight muscles are alike in this respect, it is not clear precisely which muscles were examined and figured. Silver impregnation methods for nerves also stain the tracheal system of insect muscles, but no mention of tracheae or tracheoles appears throughout the work. Penetration of the fiber by tracheoles occurs in flight muscles of members of the three orders studied and the "brushes" of silver-stained filaments figured correspond precisely with the tracheolar topography within these muscles.

In the few instances so far examined with the electron microscope, the myoneural junctions have proved to be restricted to the periphery of the fiber and, while it is not proper to generalize from so small a sample, additional doubts have thus been cast upon the existence of more deeply situated endings.

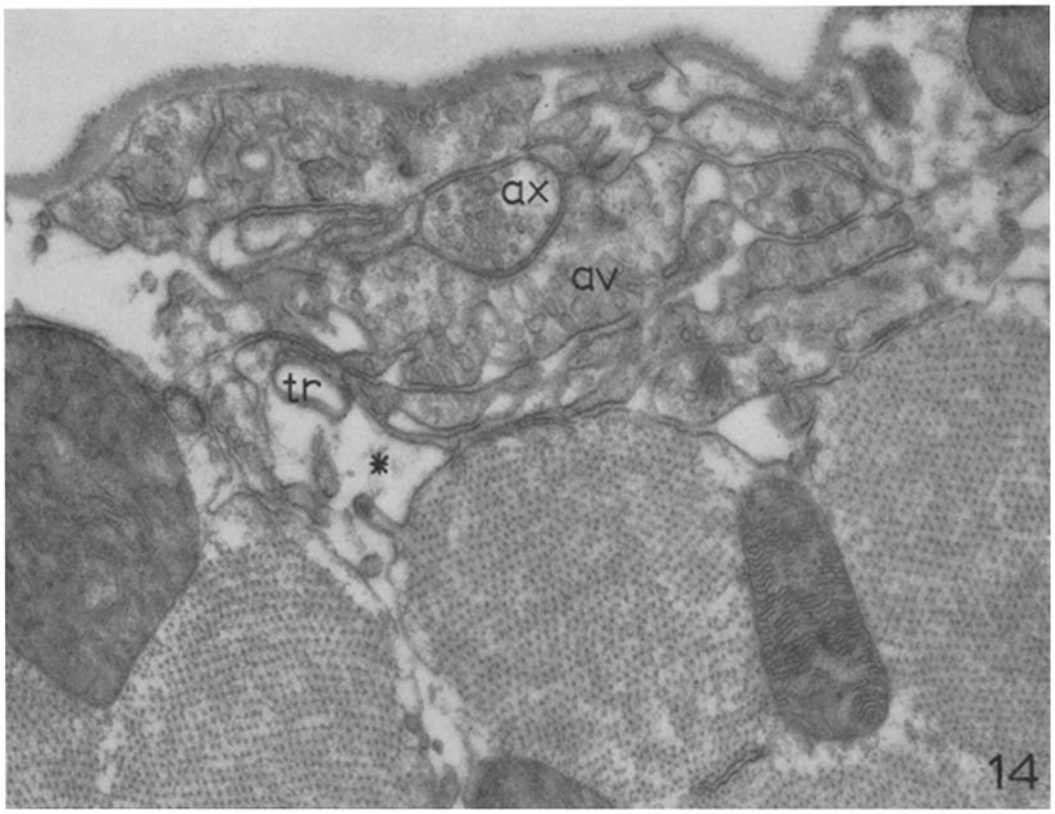
While it is clear that all myoneural junctions involve close juxtaposition of plasma membranes of fiber and axon, neither the morphological pathway taken by the membrane depolarization initiated at the junction, nor the precise nature of the coupling between depolarization and contraction, have been elucidated. As Palay (1956) points out, certain features of synaptic junctions appear to be common to all, whether axo-somatic or axo-dendritic, vertebrate or invertebrate. The apposition of plasma membranes of the pre- and post-synaptic cells has already been mentioned. In vertebrate neuromuscular junctions, the distance between these membranes is enlarged to about 600 A by the intercalation of basement membrane material. When this layer is absent, the apposition is more intimate: 200 A in central synapses of the rat brain (Palay, 1956), apparently about 200 to 300 A in the central nervous system of the cockroach (Hess, 1958), 200 A in mouse smooth muscle (Caesar *et al.*, 1957), 150 A in intersegmental muscle of the cockroach (Edwards, 1959), 100 A in similar regions in the wasp leg (Edwards *et al.*, 1958*a*), and as little as 50 to 100 A in fibrillar muscle of *Tenebrio*. It is possible that the reduction

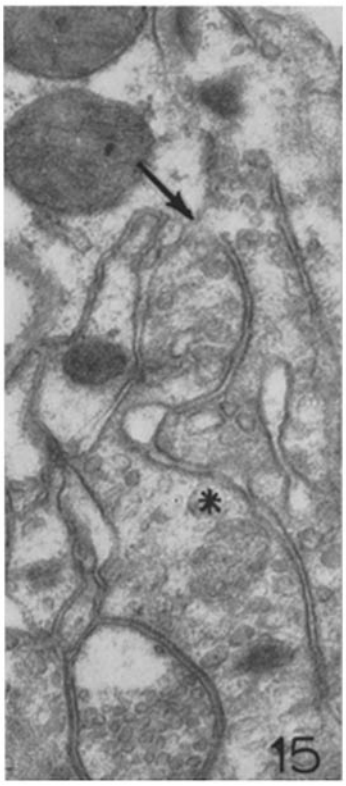
FIGURE 14

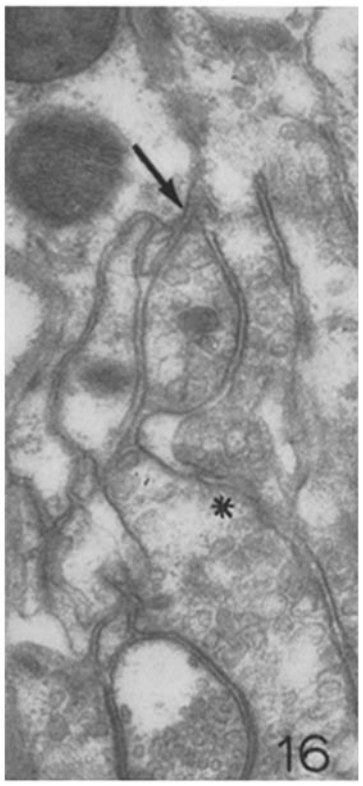
A low power electron micrograph illustrating practically the entire extent of a neuromuscular junction in *Tenebrio* fibrillar flight muscle. The small axon (*ax*) is surrounded by sarcoplasm containing large numbers of aposynaptic vesicles (*av*) and plasma membrane profiles. A tracheole is present (*tr*), lying within a plasma membrane-defined cavity (*). It is suggested that the membrane of the fiber is drawn inwards to all depths in the form of sheaths surrounding the invading tracheoles, and that this disposition affords the morphological pathway for impulse conduction inside these very wide fibrillar flight muscles. × 30,000.

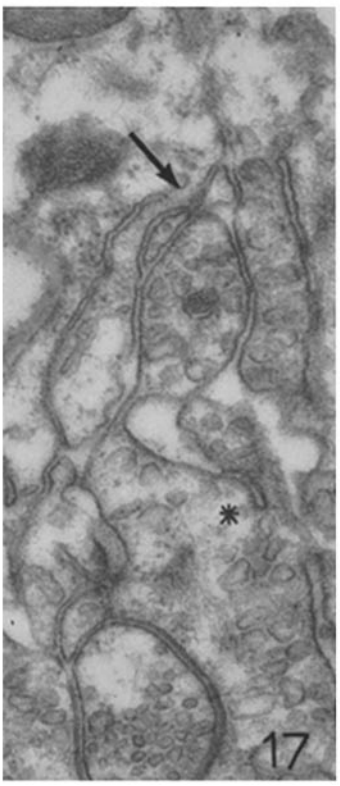
FIGURES 15 to 17

A series of three serial sections (about 600 to 1000 A apart) of the axon and associated sarcoplasm shown in Fig. 14. These fields give an indication of the complexity of plasma membrane folding in the aposynaptic region; compare, for example, the membrane profiles at the points indicated by arrows and asterisks. × 42,000.


ax
av
tr
*
14


*
15


*
16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17

of this interspace, which has been found in all insect myoneural junctions so far examined, may be reflected in differences in the details of synaptic transmission.

A second common feature mentioned by Palay is the increased concentration of mitochondria in the synapsing axon, found in insects and vertebrates alike, and thirdly, the universal appearance of vesicles within the axon, whether the synapse is central or peripheral. It is generally supposed that membrane depolarization is initiated by the release of a chemical mediator (acetylcholine or an analogue) from the axon at synapse. Many authors have lighted upon these "synaptic vesicles" as bearer of this substance, an inference supported by the arguments of Fatt (1954) who showed that on physiological grounds, the apparatus for mediator-release must be subdivided into many units at each ending. Considerable conformity in the size and appearance of these vesicles exists between various nerve-muscle junctions. In mammalian and amphibian muscle, Reger (1957) found their diameter to be 200 to 600 A; in reptile muscle the corresponding figure is 300 to 500 A (Robertson, 1956); in skeletal muscle of the mouse 450 A (Andersson-Cedergren, 1959); 250 A in the wasp leg (Edwards *et al.*, 1958*a*), and 250 to 450 A in *Tenebrio* flight muscle junctions. The only available figures for their concentration are $4000/\mu^3$ in the wasp leg, and about $7500/\mu^3$ in *Tenebrio*; both figures amply meeting the requirements of Fatt's concept of mediator release. Before too ready a comparison is drawn between vertebrate and insect myoneural junctions however, it should be stressed that while the importance of acetylcholine and cholinesterase is evident in vertebrate junctions, the role of these substances in central and peripheral synapses in insects requires clarification. Mikalonis and Brown (1941) and Tobias *et al.* (1946) found far greater concentrations of acetylcholine in the cockroach central nerve cord than in the mammalian central nervous system, and cockroach ganglia and honey-bee brain were found to contain more cholinesterase than mammalian autonomic ganglia (Richards and Cutcomp, 1945). However, Wigglesworth (1958) could not detect esterase activity, histochemically, in motor endings of *Rhodnius* intersegmental muscle. This controversial subject is discussed fully by Roeder (1953). A solution to the problem might well be provided by application of techniques for visualizing sites of cholinesterase activity in the electron microscope (Barrnett and Palade, 1959; Lehrer and Ornstein, 1959), in a study of these myoneural junctions.

While the synaptic vesicles within the axon are a constant feature, more variation is met with in the organization of the immediately postsynaptic area of the junction; the presumed site of enzymatic destruction of the mediator. Palay (1956) found, in central synapses in the rat brain, "no characteristic orientation or aggregation of cytoplasmic elements" in this region. Densely osmiophilic postsynaptic granules, 50 to 150 A in diameter, occur in the postjunctional region in the wasp leg muscle (Edwards *et al.*, 1958*a*), in flight and tymbal muscles of the cicada (Edwards *et al.*, 1958*b*), and in cockroach intersegmental muscle fibers (Edwards, 1959). A vesicular component of this area, the "sole plasm," was found by Reger (1957) in mammalian and amphibian muscle and in the mouse, Andersson-Cedergren (1959) describes postsynaptic vesicles which are somewhat *smaller* than the vesicles inside the axon, and rather sparsely distributed in the sarcoplasm adjacent to the junctional folds in the end-plate. None of these instances parallels the great concentration of vesicles (about $2500/\mu^3$) occurring around the synapsing axons in *Tenebrio* flight muscle. It is tempting to hypothesize that these vesicles are the postsynaptic counterpart of those of the axon, containing cholinesterase or an analogous substance, in this case quantized as in the case of the mediator. But at the present time, no obvious correlation exists between this unusual morphological feature and what is known of the physiology of the muscle. Although fibrillar muscle may have a very high contraction frequency (from 50 to 150 cycles per second in beetles to over 1000 c./s. in the fly, *Forcipomyia*; Sotavalta, 1947), it is well known (Pringle, 1949; Boettiger, 1951; Roeder, 1951) that this rate is the result of the architecture of the thoracic exoskeleton and wing articulations, and that the rate of motor impulses is far below the contraction frequency. Also, these muscles are not especially "fast" in the sense of the rate of development of tension. Thus their contraction-physiology, as far as is now known, involves no special features which may be correlated with their specialized myoneural junctions.

Perhaps the greatest challenge to cytological analysis is the pathway of impulse conduction within the fiber. A motor impulse in a twitch fiber can initiate a propagated change in membrane potential, and in an insect muscle fiber, an area of membrane depolarization at each myoneural

junction; depolarizations in each instance being rapidly followed by contraction. If Hill's (1948, 1949) calculations are accepted, then in vertebrate fibers and even more in the very large fibers of fibrillar flight muscle, membrane excitation-contraction coupling cannot, because of the time-factors involved, be the direct result of diffusion of a substance liberated inside the peripherally activated cylindrical plasma membrane. It is thus natural to look for a sarcoplasmic component through which excitation may be channeled; drastically reducing the maximum membrane-to-fibril distance. It is possible (Smith, 1961) that, in the flight muscle of *Tenebrio*, such a pathway may be provided by an internalized plasma membrane system drawn into the fiber with the tracheoles, so richly permeating the fiber that the mean distance between any point in the fiber and a plasma membrane surface is less than the diameter of the individual fibrils. The suggested steps in impulse conduction and excitation are as follows.

Motor impulses pass from the thoracic ganglion centers along the main nerve trunks, which divide to form smaller branches, until ultimately the impulse is distributed to many points on the fiber by uniaxonal myoneural junctions. The activator (acetylcholine or a functional analogue) is released from the synaptic vesicles within the axon, and local areas of depolarization are set up. The enormous concentration of vesicles in the post-synaptic sarcoplasm cannot definitely be accounted for at present, but it is tentatively suggested that they may represent "quanta" of cholinesterase, or other enzymatic destroyer of the mediator. While a propagated membrane potential does not occur along the entire surface of the fiber, as in vertebrate twitch fibers, it is supposed that local depolarization of the fiber membrane, effected across the 50 to 100 A "synaptic gap," travels into the fiber along the plasma membrane sheaths drawn in with the internalized tracheoles. If the concept of activation of the fibrils *via* one or more intermediary substances liberated inside the activated membrane is accepted, then this sequence of events would result in the production of such substances throughout the fiber after a negligible time interval following the arrival of the impulse at the distributed myoneural junctions.

It is possible that in this mechanism lies the explanation of the great variation in the fiber diameter of fibrillar muscle, and for their generally large size. Within the Coleoptera fiber diameters of 2 to 300 μ are frequent while in other insects this value may be exceeded. If the system suggested here indeed operates in these muscles, then the size of the fibers ceases to be a limiting factor in excitation-contraction coupling considerations.

The author gratefully acknowledges the technical assistance of Miss Stephanie Walser.

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