

CORRELATION OF FINE STRUCTURE AND PHYSIOLOGY OF THE INNERVATION OF SMOOTH MUSCLE IN THE GUINEA PIG VAS DEFERENS

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ABSTRACT

An electron microscope study of the innervation of smooth muscle of the guinea pig vas deferens was undertaken in order to find a structural basis for recent electrophysiological observations. The external longitudinal muscle coat was examined in transverse section. Large areas of the surfaces of adjacent muscle cells were 500 to 800 Å apart. Closer contacts were rare. A special type of close contact suggested cytoplasmic transfer between neighbouring cells. Groups of non-myelinated axons from ganglia at the distal end of the hypogastric nerve ramified throughout the muscle. Some small axon bundles and single axons lay in narrow fissures within closely packed muscle masses. Many axons contained "synaptic vesicles." About 25 per cent of the muscle fibres in the plane of section were within 0.25 μ of a partly naked axon; of these 15 per cent were within 500 Å of the axon, and about 1 per cent made close contact (200 Å) with a naked axon. It is unlikely that every muscle fibre is in close contact with an axon, and it is not possible for every fibre to have many such contacts. Muscle fibres are probably activated by both diffusion of transmitter from naked portions of axons a fraction of a micron distant, and electrotonic spread of activity from neighbouring cells.

INTRODUCTION

Recent electrophysiological recordings from single smooth muscle cells in the guinea pig vas deferens, during stimulation of its sympathetic motor supply in the hypogastric nerve (Burnstock and Holman, 1960, 1961 *a*, and *b*, 1962 *a* and *b*), have shown that the mechanism of transmission of excitation from nerve to smooth muscle has many features in common with those established for the skeletal neuromuscular junction (Katz, 1962). There are some differences, however. Each smooth muscle cell in the vas deferens is influenced by many

hypogastric nerve fibres, and the transmitter at sympathetic nerve endings is noradrenaline. This paper describes the electron microscopic appearance of this smooth muscle and its innervation in the normal guinea pig, in an attempt to decide whether each muscle cell is innervated by many discrete nerve endings, or whether there is some other structural basis for the release of transmitter. Comparison is made with reports on the structure and innervation of smooth muscle in other organs, and with Richardson's (1962) findings on the vas

deferens of the rat. A preliminary account of this work has been published in abstract (Burnstock, Holman, and Merrillees, 1962).

A further report will deal with changes in fine structure associated with various physiological and pharmacological treatments of this tissue.

MATERIALS AND METHODS

Young, but mature, male guinea pigs aged about 6 months were used. They were taken from a common enclosure containing males only. They were either stunned and bled, or anaesthetized with ether, chloral hydrate, or pentobarbitone. Each vas deferens was exposed, split longitudinally on a director to improve penetration of the fixative, and fixed in a gently stretched condition. The start of fixation of two specimens was deliberately delayed for 4 minutes after removal, to see whether a rapid start is important for the preservation of vesicles in axons. One of these specimens was exposed to the air; the other was covered by folds of exposed small intestine. The two specimens were then treated in the same way as the others, which were in fixative 30 or 40 seconds after the first general or local interference with blood flow.

Fixation was commenced in ice cold 2.5 per cent osmium tetroxide with *s*-collidine buffer (Bennett and Luft, 1959), with Richardson's (1962) modification of Dalton's (1955) buffer, or with Millonig's (1962) phosphate buffer. After 15 minutes, several transverse sections about 1 mm thick were cut from the partly fixed tissue and returned to cold fixative for about 1 hour. Strips of external longitudinal muscle coat, easily recognizable in fixed material, were cut from these segments while still in the fixative. Small portions of the middle circular muscle coat sometimes remained adhering to the longitudinal muscle. This final division into blocks was delayed until the tissue was in 50 per cent acetone in a number of animals when collidine was used. The remaining collidine-buffered specimens and most of those treated with chromate and phosphate buffers were passed through ascending grades of acetone very rapidly at room temperature so that dehydration was completed in 10 minutes or less. A similarly rapid dehydration was done with methanol on two specimens. Those tissues that were divided while under 50 per cent acetone were dehydrated for 1 hour. Two lots of chromate-buffered material were also dehydrated for 1 hour. The strips were embedded in Araldite according to the method of Luft (1961) with the following modification: acetone was used as the diluent, because propylene oxide, when used as a reactive diluent for impregnations lasting 12 to 24 hours, was found to interfere seriously with the staining of Araldite sections by heavy metals (Merrillees and Ham, unpublished). Because acetone

very effectively lowers the viscosity of the plastic mix, very little is required. For long impregnations, one part of acetone was used with four parts of plastic for 1 hour, and was followed by one part of acetone to eight or ten parts of plastic rotating in a roller tube at 1 revolution per minute at room temperature overnight. For short impregnations lasting 4 hours, one part of acetone was used with roughly ten parts of plastic for about 20 minutes and the mixture was then sucked away and replaced by plastic only. The tubes were rotated for the remainder of the 4 hours at room temperature. The Araldite remained quite liquid. When methanol was used for dehydration it was also used as the diluent. Those tissues that were dehydrated for 1 hour were impregnated overnight; those that were rapidly dehydrated were impregnated for 4 hours. For orientation for transverse sectioning, the strips of muscle, having sunk to the bottom of filled capsules, were guided into axial holes drilled into Araldite platforms previously prepared in the bottoms of the capsules.

The sections, producing interference colours of very pale gold, were cut with glass knives on a A. F. Huxley-pattern Cambridge microtome. The speed of cutting of Araldite is extremely important. To avoid chatter, a cutting speed of less than 1 cm per minute is desirable. These blocks, 400 μ in the vertical axis of the block face, passed the knife edge in about 4 seconds. They were stained with lead salts (Karnovsky, 1961, method 1) and examined in a Siemens Elmiskop 1 electron microscope.

OBSERVATIONS

The axons and muscle fibres in the outer longitudinal muscle coat of the guinea pig vas deferens run for long distances closely packed in parallel. It would seem, therefore, that the best way to study this tissue would be in longitudinal section. We attempted to do this, but were unable to estimate the relative numbers of muscle fibres and axons because small undulations of the axons take them in and out of the plane of section: fragments of axon are rarely longer than 1 or 2 μ , and one cannot be certain that a series of profiles close to a muscle fibre are, in fact, all parts of one axon. This becomes very important in relation to multiple innervation. Furthermore, in longitudinal sections, the plasma membranes are cut obliquely whenever a section through a fibre is approaching a tangential plane. This causes considerable confusion, especially if the membranes are wrinkled by muscular contraction. Again, the characteristic vesicles of the axons are not uniformly distributed, and hence a nearly tangential slice is often hard to interpret if there are no vesicles in that plane.

The muscle cells in the longitudinal coat are arranged in a long spiral at a small angle to the axis of the vas deferens, and although, in general, neighbouring fibres are close to parallel, the very close packing and mutual pressure forces each individual fibre into irregular crevices between its neighbours. This type of deformity, which changes from place to place along a fibre, together with small undulations in groups of fibres, makes it very difficult to produce large areas of perfectly longitudinal sections.

Profiles become easier to interpret with increasing obliquity of section. We, therefore, decided to use transverse sections in which both muscle fibres and axons can be counted with certainty. One can be satisfied that there are neither more nor less than a certain number in a given plane. This is not possible with longitudinal sections, particularly in respect to axons. Admittedly, transverse sections have limitations. Branching of axons cannot be recognized, and the length of contact between axon and muscle cannot be measured. However, random longitudinal sections are little better than transverse sections because of axonal undulation. Although changes in shape or diameter can be recognized only in longitudinal sections, many apparent examples of these changes are again the effect of slight undulation through the plane of section.

It seems reasonable to suppose that the appearance of the random profiles, through a large number of overlapping muscle fibres in a transverse section, would be similar to the appearance of the profiles in an equally large number of random sections through a single muscle fibre if those profiles could be displayed in one plane. This supposes that all the fibres are similar and that all the observed events occur on each fibre. This is equivalent to random sections through an idealized fibre and its environment, and it is a useful basis for conjecture. If the pattern is always the same, the observations become more significant. In the last 2 years we have examined many hundreds of grid squares from sections derived from several blocks taken from each of thirteen guinea pigs. Within narrow limits, in normal animals, the pattern has always been the same.

The Muscle Cells

These electron microscopic observations refer to the outer longitudinal muscle coat of the vas deferens. In general, the structure and arrange-

ment of the smooth muscle fibres are similar to those described by Caesar, Edwards, and Ruska (1957) in the gall bladder, uterus, and urinary bladder of the mouse.

The smooth muscle of the vas deferens of the guinea pig is arranged in large bundles subdivided by narrow clefts that contain a few collagen filaments, occasional delicate processes of fibroblasts, and branches of the nervous and vascular supply. Within the small bundles the muscle fibres are discrete. They are closely packed in an overlapping array with their long axes parallel, and in transverse section they are polyhedral. For considerable distances around the periphery of the fibres their surfaces are relatively smooth, and the separation between neighbouring muscle cells is generally between 500 and 800 Å (Figs. 1 and 2). Basement membrane material and scattered collagen filaments fill this narrow intercellular space. In addition, numerous wider spaces occur, and, within these, the basement membrane is confined to the cell surface. Most of the very small nerve bundles, containing one to three axons, are found in the small irregular tissue spaces between three or four neighbouring muscle cells (Figs. 1 and 2).

Like Caesar *et al.* (1957), Bergman (1958), Prosser, Burnstock, and Kahn (1960), and Rhodin (1962), we have not found intercellular bridges with cytoplasmic continuity of the type described by Mark (1956) in rat uterus, and by Thaemert (1959) in rat gut-muscle. In this study, a few examples of close contact between neighbouring cells have been found, where, for a distance of 0.5 μ or less, there was a separation of less than 200 Å between the plasma membranes. This type of close contact sometimes occurred on the summit of a projection of the surface of one of the cells, and would appear to be similar to the "intercellular bridge" that Bergman described in the rat. The special case of intrusions of one cell into another is described below. Whereas dense attachment areas were well preserved between the endothelial cells of all the blood vessels encountered, none have been found between the muscle cells of the vas deferens. Nothing resembling the "nexus" in circular muscle of cat intestine, described by Dewey and Barr (1962), has been found: the neighbouring plasma membranes are never fused, and are always separated by a gap greater than 100 Å.

The muscle cells appear to be attached to each

other by means of the basement membrane-filled zone, 500 to 800 Å wide, between the cells. There is evidently considerable bonding between the cells through this zone. If the edge of the specimen block is examined where the tissue was cut with razor blades before embedding, one can find many examples of a considerable length of plasma membrane, with *caveolae intracellulares* (micropinocytotic vesicles) still attached, quite undisturbed in its relationship to the neighbouring plasma membrane, although the rest of the cell has been torn away (Fig. 3).

The tapering ends of the fibres rarely interdigitate, and do not make any closer contact than is permitted by the basement membrane zone, although the end of one cell may be accommodated in a groove in the belly of another. In the tapered end of a cell the filaments are closely packed, thus increasing the density of the cytoplasm and tending to exclude all other organelles (Fig. 4). The dense bodies (see below), which are scattered amongst the myofilaments through the main portion of the cell, are not present in the extremities, but the similar dense material that is found in small patches against the inside of the plasma membrane, in the main part of the muscle cell, becomes an almost continuous lining to the plasma membrane. Many of the tapered ends of the cells contain gutters and tunnels parallel to the long axis of the cell. These contain basement membrane, but, although the contours of the muscle cell bear a remarkable resemblance to those of the skeletal muscle fibre at the myotendinous junction, the invaginations contain

little or no collagen. It should be noted that, in a longitudinal section involving the gutters and tunnels, the end of the fibre will appear to break up into thin processes or digitations. In fact, few fibres branch at their tips.

The contractile filaments of the cell are arranged in ill defined groups, roughly parallel to the long axis of the fibre. The groups tend to merge and interweave, and individual filaments quickly leave the plane of section. At times, the interweaving produces the appearance of a feltwork. The concentration of filaments varies markedly from cell to cell and even in different areas within a cell. The sections used in this investigation were too thick for a useful study of the filaments.

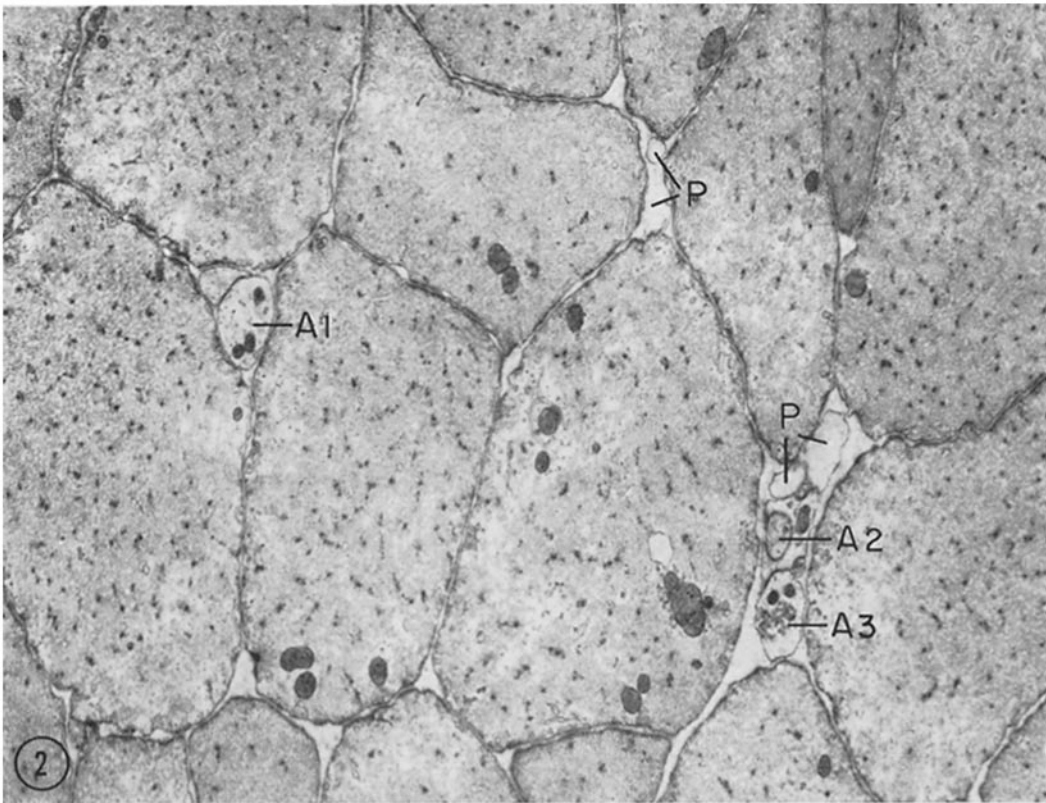
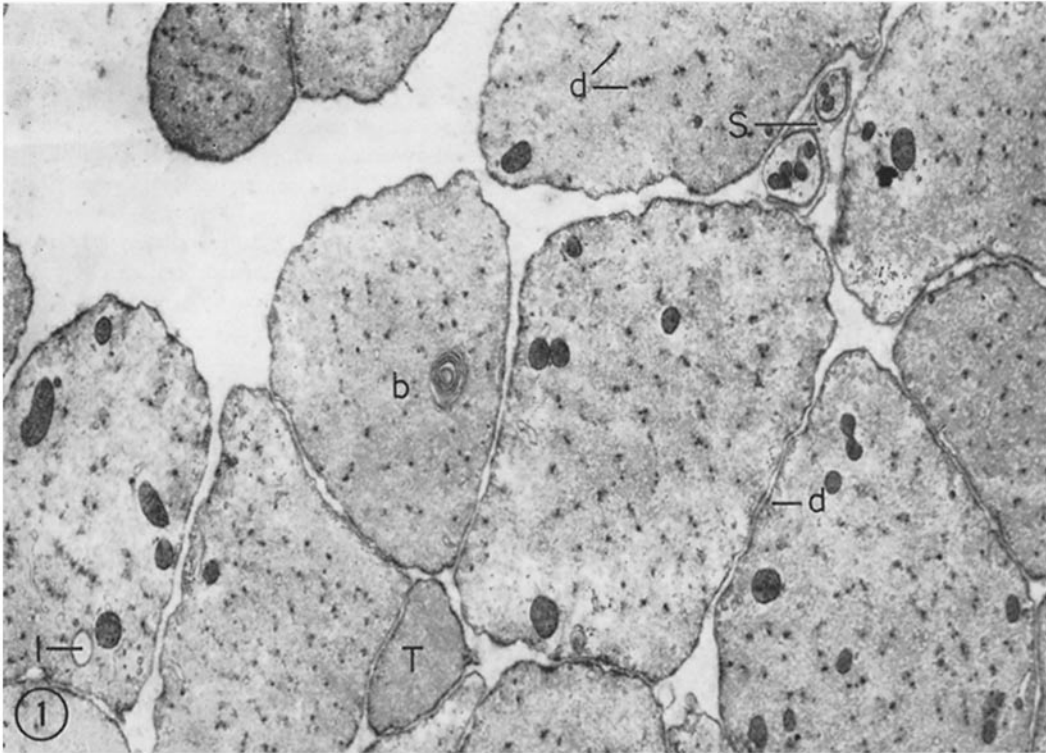
Small, irregular dense bodies, of unresolved structure, elongated in the direction of the long axis of the cell, are scattered at frequent intervals through the sarcoplasm, some of them lying against the plasma membrane (Figs. 1 and 2). Mark (1956), Caesar *et al.* (1957), Pease and Molinari (1960), Prosser, Burnstock, and Kahn (1960), Rhodin (1962), and others have described them. Pease and Molinari demonstrated myofilaments passing into well developed densities attached to the plasma membrane in vascular muscle, and Caesar *et al.* found that the densities along the plasma membrane in mouse uterus and urinary bladder were, although rudimentary, often lying opposite corresponding densities against the plasma membrane of the neighbouring cell. In the vas deferens, numerous dense bodies are conspicuous throughout the sarcoplasm, and some of these lie against the plasma membrane;

FIGURE 1 This and the following figure illustrate the general appearance of transverse sections through the outer longitudinal muscle coat of the guinea pig vas deferens. This figure is of an area of rather looser packing than that in the following figure. In the original section the fields of Figs. 1 and 2 were separated by a distance equal to twice the width of the plate. The intervening area was devoid of axons.

The outlines of the muscle fibres are relatively smooth, and there is no cytoplasmic continuity between fibres. Small irregular densities (*d*) are scattered through the cytoplasm except in the tapering end of the fibre *T*. Similar dense patches occur against the inside of the plasma membrane. Plasma membranes are separated by from 500 to 800 Å of basement membrane. *S* is a portion of Schwann cell with two axons, *b* an unnamed membranous body, and *I* is probably an intrusion of one muscle cell into another. $\times 10,000$.

FIGURE 2 A typical field of close packed fibres. The extracellular space is very small, and much of it is completely occupied by basement membrane material.

A1 to *3* are axons associated with Schwann cell cytoplasm. *A1*, which is almost naked, is in a typical position, lying between three cells. *A3* contains many "synaptic" vesicles. Empty profiles, *P*, are probably processes of muscle cells: this type of body was frequently ruptured. $\times 10,000$.



but in the latter position they are small, they do not appear to give attachment to many myofibrils, and, often, they are not matched by others in the neighbouring cell.

The common organelles, including Golgi bodies, centrioles etc., described by Caesar *et al.* (1957), are present in the muscle cells of the vas deferens, and they have the usual form. Most of the mitochondria, and vesicles of the endoplasmic reticulum, are concentrated about the nucleus or close to the cell membrane, but some are scattered through the cell. Their form and distribution are little affected by the proximity of nerve fibres (see below). Ribosomes are common, either attached to the surface of endoplasmic reticulum, thus forming rough-surfaced vesicles or ergastoplasm, or unattached in scattered groups through the cytoplasm. Ergastoplasm is most common in the central area surrounding the Golgi zone, and some of the examples are long and complex. *Caveolae intracellulares* are randomly distributed along the plasma membranes (Fig. 16); they may be crowded or relatively few.

Rounded membranous structures of unknown significance were found in a number of animals, in considerably less than 1 per cent of the cells in a transverse section (Figs. 1 and 5). The fine detail was not well resolved, but they appear to be concentric flattened *cisternae*, each *cisterna* being separated from the next by a narrow zone containing a row of granules, which, although they appear to be rather smaller than ribosomes, suggest that these bodies are complex formations of ergastoplasm. Long "strips" of typical ergastoplasm, several microns long, are common in the muscle cells.

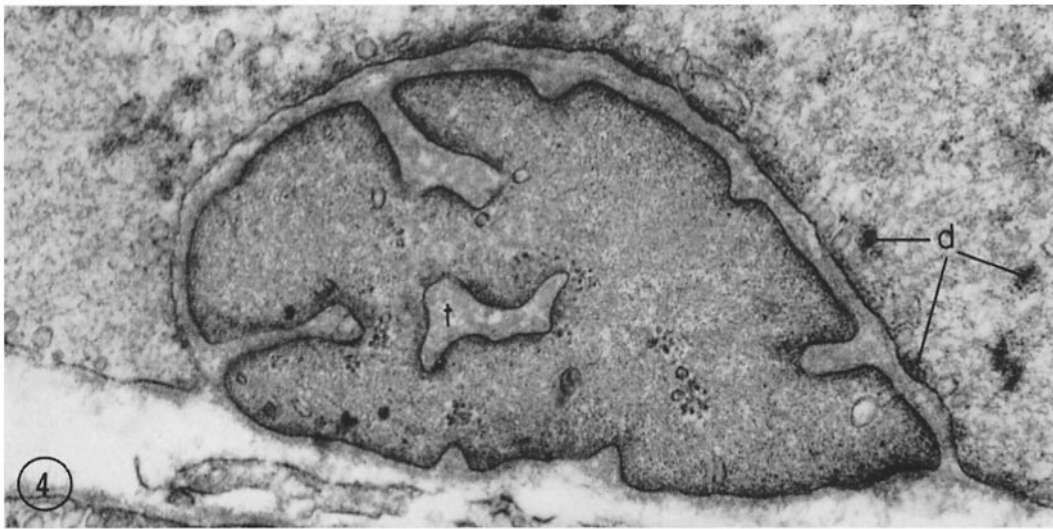
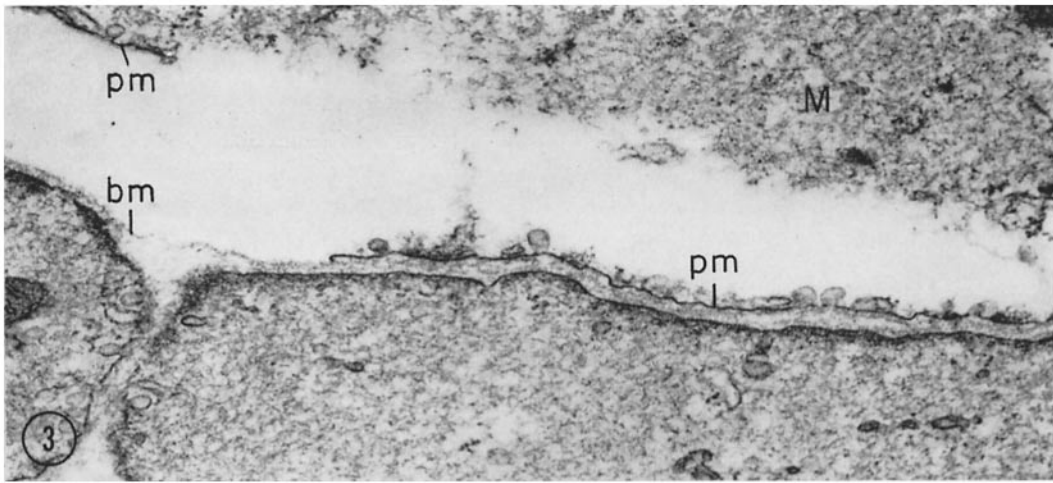
A number of rounded profiles, ranging in size

from $\frac{1}{2}$ to 2 μ , have been found within the muscle cells in this study. A few cells contained two or three examples. Some, if not all, arise from intrusions of large cytoplasmic processes from one muscle cell into another (Fig. 6). The very closely opposed plasma membranes of the two cells limit the intrusions. The distance between the mid-lines of the two membranes, at their closest approach, is about 120 A, which suggests a separation of about 50 A between the membranes. The membranes in this situation are very dense. The interior of the intrusions is always of very low density, and appears to be an amorphous precipitate in the fixed material, but sometimes there are a few small vesicles, fragments of membranes, and, occasionally, what seems to be a disintegrating mitochondrion. The contents of the invaded cell, immediately surrounding the intrusion, never show any signs of disturbance such as compression or cleavage, or orientation suggesting flow. The intrusions are partly or wholly enclosed by the invaded cell (Figs. 7 and 8), usually with no visible attachment to the cell of origin, but these appearances can be explained by the plane of section missing the typically narrow neck of attachment. The majority of the attachments are thought to be narrow necks, because, although hundreds of intrusions have been observed, the connection with the cell of origin is only very rarely seen, and in transverse sections the connection is always relatively narrow unless the intrusion is itself small. Most of the intrusions are thought to be rounded because they have a similar appearance in both transverse and longitudinal sections. Occasionally, however, in a longitudinal section an elongated elevation bears an intrusion-like relationship to a depression in a neighbouring

FIGURE 3 A cell (*M*) has been torn from its neighbours before the tissue was embedded. Towards the right, its plasma membrane (*pm*) and basement membrane are still attached to the neighbouring cell in a normal fashion. Towards the left, its plasma membrane has parted from the basement membrane (*bm*) and remains attached to the cell. $\times 35,000$.

FIGURE 4 A transverse section through the tapering end of a smooth muscle fibre lying in a hollow in another fibre. The filamentous material is becoming uniformly compacted at the expense of other organelles, including the scattered densities (*d* in the neighbouring fibre). A similar dense material, normally found in patches against the inside surface of the plasma membrane, now forms an almost continuous peripheral zone. Deep grooves and tunnels (*t*), filled with basement membrane material, frequently penetrate the tips of the fibres, parallel to the long axis. $\times 35,000$.

FIGURE 5 A type of membranous body of unknown significance. It is probably ergastoplasm. Some examples were two or three times this size. $\times 45,000$.



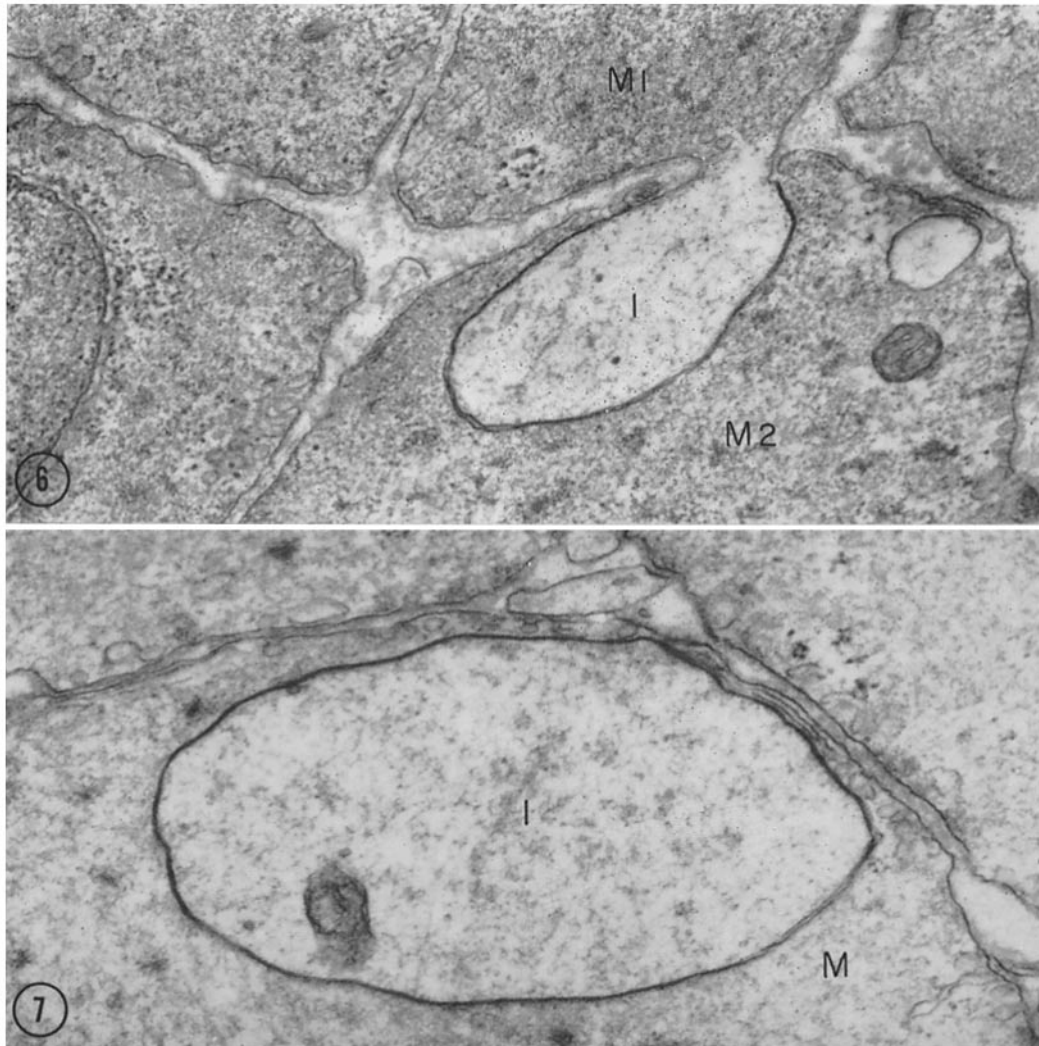


FIGURE 6 An intrusion (*I*) of a process of a muscle cell (*M1*) into a neighbour (*M2*). It contains no recognizably organized myofilaments. The plasma membranes of both cells still confine the intrusion, and are dense and closely applied. $\times 35,000$.

FIGURE 7 In this plane of section the intrusion (*I*) is wholly buried in the muscle cell (*M*). No *caveolae intracellulares* are found in the plasma membranes associated with these intrusions. The distance between the mid-lines of each membrane at their closest approach is about 120 A, and the separation is barely resolved on the original print at this magnification. The intrusion contains some amorphous material of low density, several small vesicles, and, possibly, a mitochondrial remnant. No disturbance suggesting local compression has ever been seen in the cytoplasm of the invaded cell. $\times 35,000$.

cell. Some intrusions are found isolated, deep within a cell; and some structures of similar form and density, but with modified membranes, are likewise deeply buried. The membranes in the latter are of normal density, they are not closely

applied, and, particularly in the case of the inner one, they are more or less convoluted and broken (Figs. 8 and 9). An intermediate condition, with part of the peripheral membranes condensed and the remainder separated, also occurs. The ex-

amples with the most convoluted membranes are obviously in the central area of the cell, surrounded by stacks of Golgi vesicles, free ribosomes, ergastoplasm, and mitochondria.

Numerous small "empty profiles," from 0.1 to 1 μ in diameter, are found in the small spaces between the muscle fibres (Figs. 2 and 15). They are evidently fragile, because most of them had broken membranes in this material. They have little or no basement membrane, and their sub-

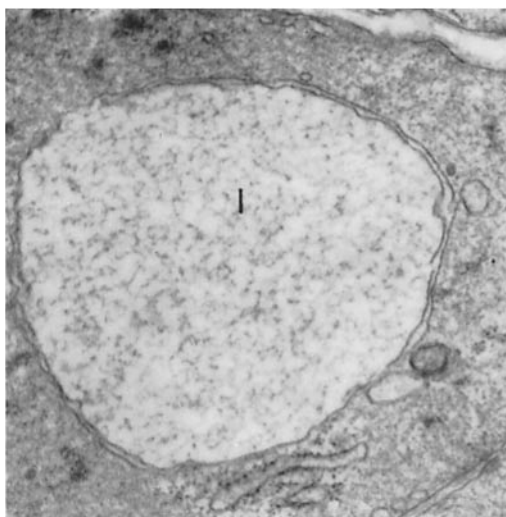


FIGURE 8 This type of body (I), lying deeper in a muscle cell, is probably the next stage in the history of an intrusion, and bodies intermediate in appearance between this one and that in Fig. 7 have been found. The dense closely applied membranes have become separated and less dense, and are now convoluted. $\times 24,000$.

stance is of very low density and almost featureless. Some, if not all of these, are processes of muscle plasma membrane, attached to the muscle by very narrow necks that are usually outside the plane of section.

Muscle Cells Following Delayed Fixation

With few exceptions, the muscle cells in the two specimens that were not placed in fixative until 4 minutes after removal were normal. A few cells showed mechanical damage in the form of broken cell membranes. The vas deferens from the other side of one of these animals was placed in fixative within 42 seconds after removal, and showed many intrusions of one muscle cell into another, whereas

in the other vas deferens, following delayed fixation, there were few intrusions.

The Nerve Fibres

The motor nerve supply reaches the vas deferens through a number of fine branches arising from a group of small ganglia situated along the bundles of the hypogastric nerve or plexus (personal observation). Most of the ganglia form a compact mass where the nerve crosses the superior vesical vessels in the angle between the seminal vesicle and the vas deferens, a few millimeters from the converging proximal ends of these organs. At this point, the vessels break up into many branches, which fan out through the tangle of nerve bundles in the ganglionic zone to supply the vas deferens, the seminal vesicle, the urinary bladder, and other associated organs. Small nerves, derived from the ganglia, follow the general course of the vessels in the serous coats of the organs. The ganglia are crowded with sympathetic nerve cells.

Electron microscopic observation reveals typical autonomic axon bundles, derived from the small nerve trunks in the serous coat, similar to those described by Gasser (1952, 1958), Hess (1956), and Elfvin (1958, 1961) in other sites, penetrating spaces between the large muscle bundles of the vas deferens.

Almost all the axons in the small trunks are unmyelinated, and all are invaginated into the surface of Schwann cells (Figs. 10 and 11). A very few small myelinated fibres, between 2 and 3 μ in diameter, were found in this material. The Schwann cells are enclosed in a sheath of longitudinally arranged collagen filaments completely surrounded by a very delicate sheath of the capsular-sheet cells of the perineurium. Each Schwann cell contains a number of unmyelinated axons, most of which are almost surrounded by Schwann cell cytoplasm. No basement membrane is included in the 200 A space between the plasma membranes of Schwann cell and axon. Some axons, however, are not entirely submerged (Fig. 10), and a portion of the plasma membrane of each of these axons is separated from the tissue space by only the basement membrane, which passes from the Schwann cell onto the axon. Many of the axons are not separated from each other by Schwann cell, and their plasma membranes, therefore, lie about 200 A apart.

The unmyelinated axons vary in diameter from about 1.75 to 0.2 μ . They contain large and small

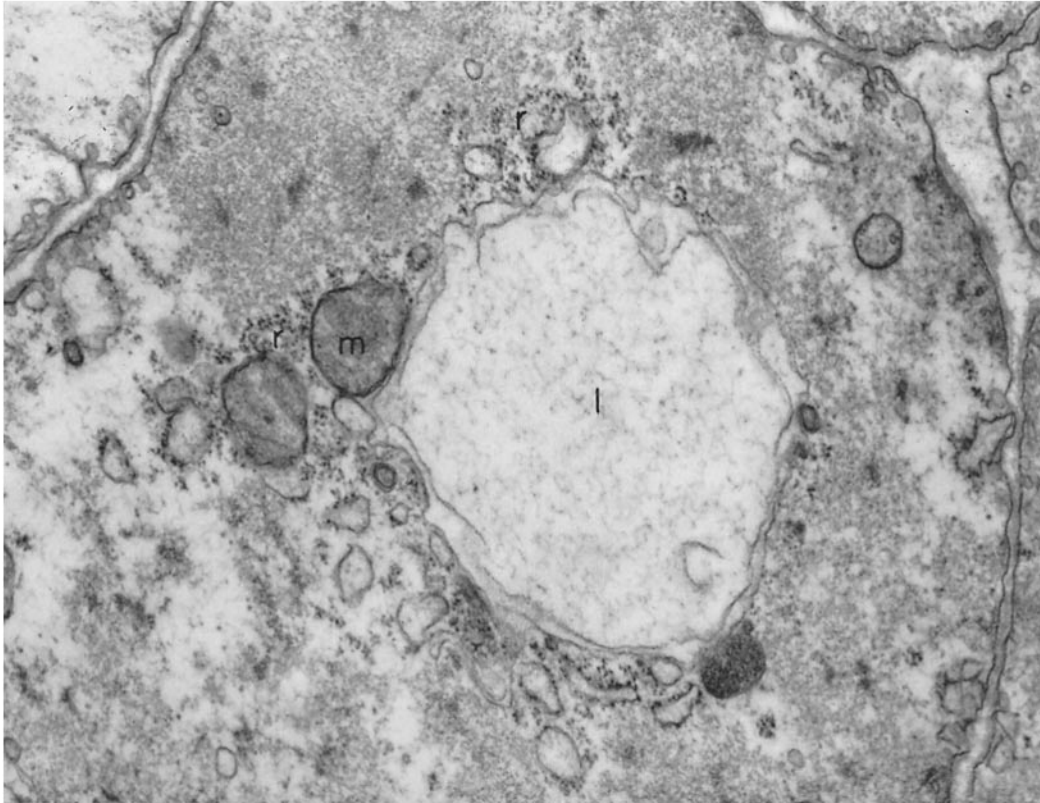


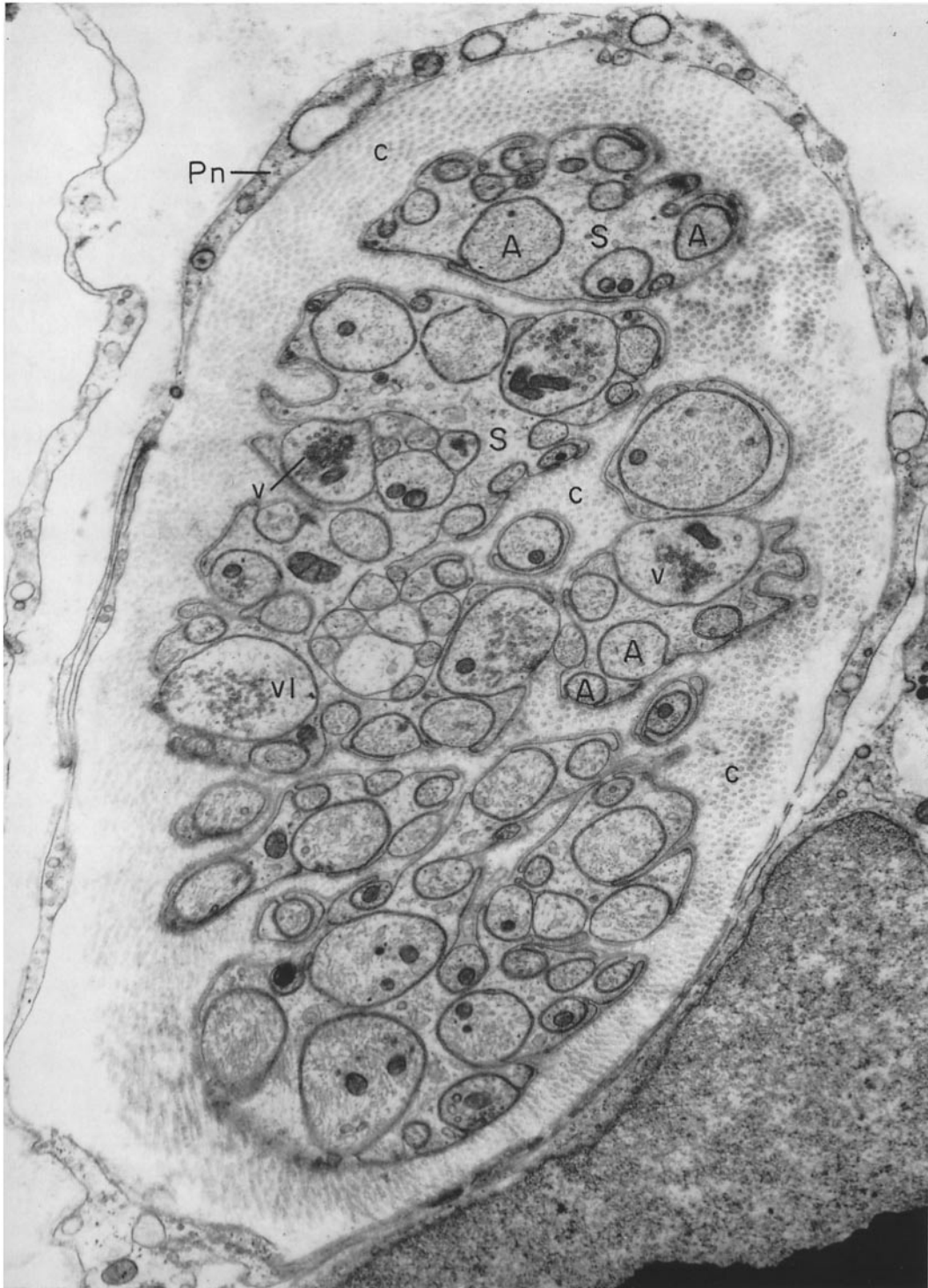
FIGURE 9 This is, perhaps, the fate of an intrusion. The large vesicle (*l*), bounded by obliquely cut, convoluted membranes that seem to be breaking up, is in the central region of the cell, surrounded by mitochondria (*m*), vesicles, and dense ribosome and glycogen particles (*r*). $\times 26,000$.

neurofilaments and a few mitochondria. Some of the medium-sized and small axons contain many round or oval vesicles between 300 and 600 Å in diameter (Figs. 10 and 11), similar to the pre-synaptic vesicles in the central nervous system (De Robertis and Bennett, 1955; Palay, 1956) and in motor endings in skeletal muscle (Robertson, 1956), and previously reported in fibres of the

autonomic system in smooth muscle (Caesar *et al.*, 1957; Richardson, 1958, 1962; Gansler, 1961) and in other tissues. The axons containing vesicles have fewer neurofilaments. This sometimes causes a conspicuously low density of the axoplasm (Fig. 10).

Although sudden variations in the concentration of vesicles within an axon are common, no con-

FIGURE 10 A small nerve trunk from the serous coat of the vas deferens. Schwann cells (*S*), containing numerous unmyelinated axons (*A*), lie in a reinforcing sheath of collagen filaments (*c*) within a complete investment of perineurium (*Pn*), which, in this case, is only a single layer of attenuated, squamous, capsular-sheet cells. There are no endoneurial fibroblasts in small trunks like this. Some of the axons are in contact with each other. A few of the axons contain clumps of "synaptic" vesicles (*v* and *v1*); others contain thick and thin neurofilaments. Those that have vesicles tend to have fewer filaments. The vesicles in some axons (*v*) are larger than those in others (*v1*). The vesicles *v* are clumped: clumping is common and causes an uneven distribution down the length of an axon, and different concentrations in adjacent sections. Fixation delayed 4 minutes. $\times 13,500$.



sistent relationships can be seen, in the present study, between the presence of "synaptic" vesicles and whether or not the axon is partly uncovered at the surface of the Schwann cell. Furthermore, in this type of preparation the varicosities commonly seen in small autonomic fibres with the light microscope would not be recognisable without a study of many serial sections, and, therefore, the presence of vesicles in an axon could not be related to sites of varicosities.

Small nerve bundles, containing two or three Schwann cells, still accompanied by a collagen sheath but with an incomplete cover of perineurial-cell cytoplasm, pass between the large and small muscle masses in narrow connective tissue planes that contain a few collagen filaments and an occasional sheet of fibroblast cytoplasm. In some of these small bundles, many of the axons contain "synaptic" vesicles, whereas other bundles have as few vesicle-containing axons as the larger trunks (Figs. 12 and 13). In some axons there are a few larger vesicles, up to 1000 Å in diameter, among the smaller ones. The larger vesicles have a higher internal density in the form of indistinct granular material or one or two small vesicles (Figs. 12 and 18). They are similar to the "neurosekretorische Elementargranula" in the axons of the myenteric plexus of the guinea pig (Hager and Tafuri, 1959), and, although of lower density in our material, and sometimes containing membranous structures, they may correspond to the Type I granules in the classification of Grillo and Palay (1962). Some of the vesicles between 400 and 600 Å contain a

faint ill defined particle, usually less than 100 Å in diameter; they can be seen in several of the original prints but the contrast may be insufficient for reproduction. This type is best seen in Fig. 19. Some of these very small granules appear to be attached to the walls of the vesicles. If this is so, the orientation of the vesicle within the section would determine the apparent position of the granule within it. These granulated vesicles resemble those of Type III described by Grillo and Palay (1962) (Fig. 19, 3). A small dense thickening was found in the walls of some of the 400 to 600 Å vesicles. The thickenings were inconstant and may have been cutting-artefacts. If, however, they are true structures, any that lies in the "roof" or "floor" of a vesicle might resemble a granule within the vesicle.

The majority of the vesicles contain no granules.

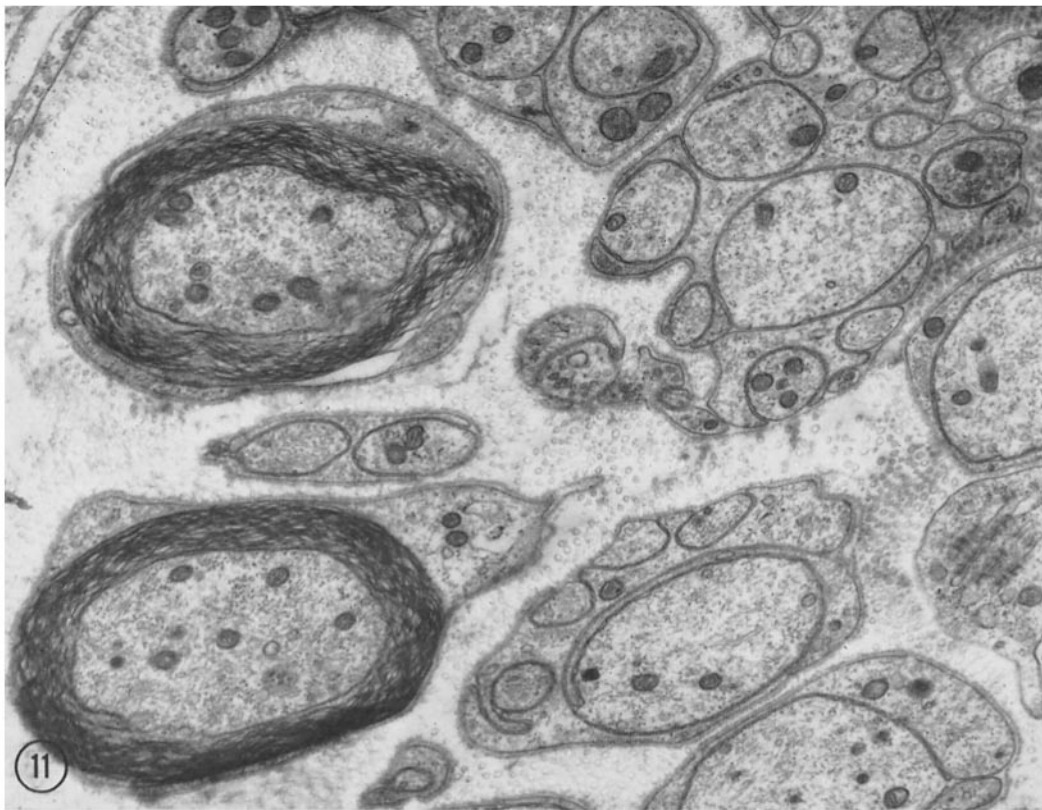
The granulated vesicles typical of the guinea pig vas deferens differ from those in the rat since many of the latter show conspicuous granules (Richardson, 1962), probably Type II of Grillo and Palay (1962). To assess the effectiveness of our techniques for the demonstration of granules, we used Richardson's (1962) chromate-buffered osmium tetroxide, with both long and short dehydrations and impregnations, on segments of vas deferens from each of 3 white rats. Many Type II vesicles containing conspicuous granules were found in all cases (Fig. 14).

Small bundles of a few axons, contained in a single thin sheath of Schwann cell cytoplasm that frequently fails to cover the whole surface of each

FIGURE 11 Portion of a nerve trunk in the serous coat, close to the one in Fig. 10. It includes two of the very few myelinated axons that were found in the vas deferens. This trunk was twice the diameter of that in Fig. 10, and few of the axons contained vesicles. With the possible exception of the separation of myelin lamellae in this figure, and a few breaks in the plasma membranes in Fig. 10, no damage has resulted from delaying fixation 4 minutes. $\times 14,500$.

FIGURE 12 A medium-sized intramuscular bundle of axons within a single Schwann cell (S). There is no perineurial sheath. The lower axons contain "synaptic" vesicles. There is a Type I vesicle at *i*. The axons *A1* and *A2* have the appearance of an axon-axon synapse because of the large number of vesicles in *A1*, but the significance of the crowded vesicles may be related more to the proximity (800 Å) of this uncovered axon (*A1*) to the muscle cell (*M*). Most of the axons in bundles of this size have few vesicles in the plane of section, but they resemble the vesicle-containing axons of the larger trunks in that they have very few large neurofilaments. The small profiles (*N*), less than 0.25μ in diameter, are probably small axons.

Apart from some fractures in the plasma membranes, this material appears to be within normal limits. Fixation delayed 4 minutes. $\times 20,000$.



axon, penetrate the small muscle bundles (Figs. 1 and 2). The exposed axon surface is covered by basement membrane. These groups of two or three axons are most commonly found in the small irregular spaces bounded by three or four muscle cells, and here the majority of the axons are crowded with vesicles. In this situation, the axons are within less than 0.25μ of the muscle cells, and the Schwann cell covering, often very incomplete, is only a small fraction of a micron thick.

them from the muscle (Fig. 16). A few naked axons crowded with vesicles, some of which are granular and similar to those described above, are found in close contact with muscle cells: a space of about 200 \AA , devoid of basement membrane, separates the plasma membranes, and the axon is often contained in a gutter in the muscle cell surface (Figs. 17 to 19). When this type of contact exists, there is, sometimes, a flattened *cisterna* of the sarcoplasmic reticulum in more or less intimate relationship to the plasma membrane lining the

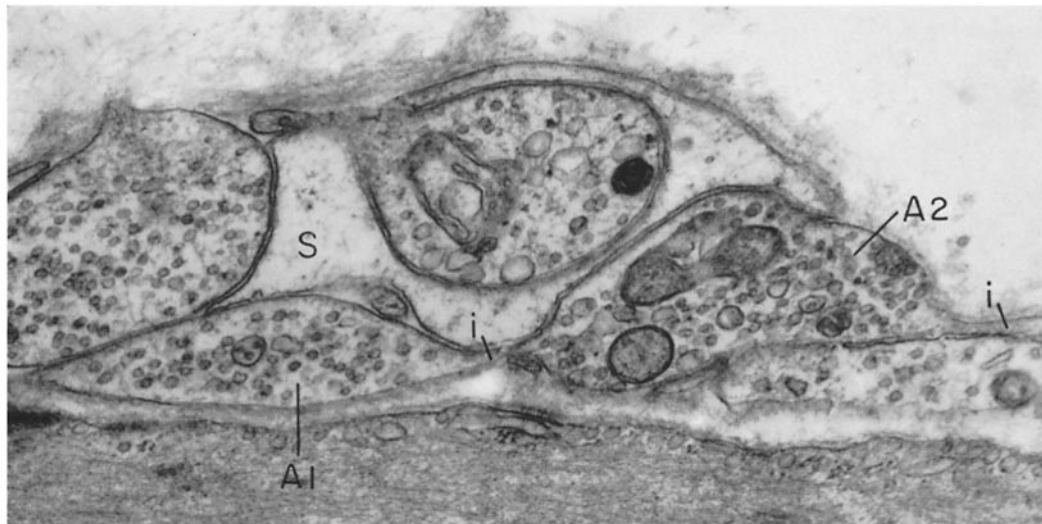


FIGURE 13 A medium-sized intramuscular bundle of axons, also contained in a single Schwann cell (S) (compare Fig. 12). In this example, all the axons are crowded with vesicles of various sizes and densities. This is from an obliquely longitudinal section, and illustrates some difficulties of interpretation of longitudinal sections. Small irregularities in the contours of the axons, caught in the plane of section as the axons meander through it, give the appearance of regions of small diameter. Thus, A1, A2, and a third expanded portion of the profile outside the figure to the right are joined together by "isthmuses" (i). The axon, however, was probably fairly uniform in diameter. It is not possible to be sure of the individuality of axons in a section like this. $\times 27,000$.

Partly or completely naked axons pass into the narrow spaces between neighbouring muscle cells. Single axons, containing crowded vesicles, both granular and non-granular, and an occasional mitochondrion, approach the surface of the muscle cells very closely. Some are separated from the muscle cell by remnants of Schwann cell cytoplasm a few hundred angstrom units thick, and by the basement membranes of Schwann and muscle cells (Fig. 15). Others have lost the Schwann cell on the side closest to the nearest muscle cell, and only basement membranes and an almost obliterated intercellular space separate

gutter. In both our rat and guinea pig material, the subsynaptic *cisternae* were rare when the muscle fibres had smooth outlines suggesting relaxation. After chromate and phosphate buffers, there was more contraction with folding and convolution of the plasma membranes, and in these specimens there were more subsynaptic *cisternae*. Richardson (1962) found them commonly in the rat. They were considerably less common in our guinea pig material.

When there is a 200 \AA space between nerve and muscle there are usually no *caveolae intracellulares* in the muscle plasma membrane under the axon;

but apart from this, and the inconstant presence of subsynaptic *cisternae*, the muscle cell shows no morphological signs of reaction to the proximity of the axon.

In order to assess relationships, montages of the whole area within grid squares were assembled, using a magnification of 15,000. Figs. 1 and 2 are two frames from one of the assemblies. That grid square measured $75 \times 75 \mu$. It contained one small arteriole at the outer edge of the muscle mass, and one capillary. There were 363 muscle

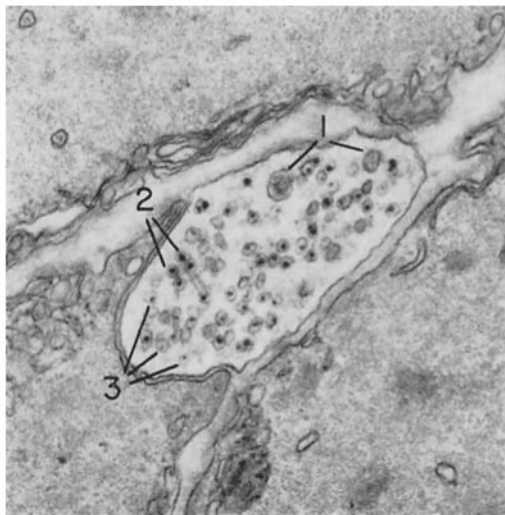


FIGURE 14 An axon from the vas deferens of a rat. The large dense granules of the Type II (2) vesicles are conspicuous. Types I (1) and III (3) are also present. The axon is 200 A from one muscle cell and 500 A from another. The convoluted plasma membranes are characteristic of our chromate-buffered material. $\times 35,000$.

fibres, 11 of which were cut through the nucleus. There were 103 axons associated with the muscle, and, of these, 25 were single. Of these 25 axons, 11 were naked, *i.e.* devoid of Schwann cell, whereas the other 14 were partly covered, *e.g.* the single axon in Fig. 2. Three of the single axons, all naked, were within 200 A of the muscle cells. Thus, in the plane of the transverse section, 3 muscle cells, less than 1 per cent of the total, were within 200 A of an axon; 46 muscle cells, however, were separated by only 500 A of basement membrane from the bare surface of naked or partly naked axons (27 axons were involved), and this was included in a total of about 90 muscle cells that

were within 0.25 μ of a portion of bare axon surface.

The number of axons in the small bundles in the same grid square is given in Table I. In counting the axons, care was taken to exclude the "empty profiles" presumed to be muscle processes; and a few of the very small profiles resembling axons, 0.1 μ in diameter (Fig. 12), were excluded because of uncertain identification. None of the latter were included in the total of single axons, and, in fact, they were rarely seen alone.

There were 5 axons associated with the two blood vessels in the grid square (one bundle with 2 axons and one with 3). They have not been included in the count, although they lay within a fraction of a micron of the nearest muscle cells, and were identical in appearance with the other axons in the field.

If Figs. 1 and 2 are taken together as though they were one, they give an accurate impression of the general distribution of axons and muscle cells in the longitudinal muscle coat, although, of course, some areas are a little more or a little less richly supplied with nerves than this grid square which is considered typical. Column B in Table II is an analysis of the area described above. Columns A and C are from montages showing a poorer and "richer" innervation. The area summarized in column C was unusually rich in 200-A axon-muscle relationships, but in other respects it had a slightly poorer innervation than the specimen in column B. The difference in the number of muscle fibres in these three areas was largely due to fixation in different states of contraction. The more relaxed muscle fibres in column A were longer and thinner and, therefore, more fibres appeared in cross-section in a standard area.

No attempt was made to count the vesicles in axon profiles. The number of vesicles is greatly affected by the local thickness of the section, because in most sections the diameter of the vesicles is relatively small. This was certainly the case in this study of very pale gold sections, which are about 1,000 A thick (Peachey, 1958).

Applying a name to all the very small axon-like profiles, down to less than 0.1 μ in diameter, poses a problem. They are found indenting the surface of Schwann cells containing obvious axons (Fig. 12) and, sometimes, they are tucked in between a single axon and the edge of its little cover of Schwann cell cytoplasm (Fig. 16). They rarely occur alone. The majority of them are about

0.2 μ in diameter, but there does seem to be a continuous series of diameters between these objects and obvious axons 0.5 μ and more in diameter. The small profiles usually have a higher density than the recognizable axons. They may contain one or two small vesicles of the synaptic type, or a number of irregular dense bodies about 200 A in diameter, which are probably transversely cut coarse neurofilaments (some of these are tubular).

Axon structure was as little affected by delayed fixation as was the structure of the muscle cell. The vesicles appeared normal. Some plasma membranes had small breaks, and the myelin lamellae in a pair of myelinated nerve fibres were separated (Fig. 11).

DISCUSSION

The relations between fine autonomic nerves and the smooth muscle of the guinea pig vas deferens described above are essentially similar to those seen in the gall bladder, urinary bladder, and uterus of the mouse (Caesar *et al.*, 1957), rabbit intestine (Richardson, 1958), human vermiform

appendix (Yamamoto, 1960), guinea pig colon, frog stomach, and rat uterus (Gansler, 1961), and rat vas deferens (Richardson, 1962). Axons, many of them packed with vesicles, run between the muscle cells, singly or in bundles, and more or less enclosed in Schwann cell cytoplasm. A few of the single axons lie in close apposition (200 A) to the muscle membrane. These are naked and always contain vesicles.

Richardson (1962) has described two types of vesicles in axons of the rat vas deferens. The "agranular vesicles" are relatively uniform in size (450 to 600 A) and resemble those of the skeletal neuromuscular junction. Richardson states, however, that "it would be altogether premature to suggest that endings containing this type of vesicle exclusively are cholinergic in function." The second type, "granular vesicles," vary in diameter from 300 to 900 A. Richardson has discussed the possible function of vesicles in relation to recent work on the identification of catecholamines in "granule-fractions" derived from differential centrifugation of homogenized adrenal medullary cells and sympathetic nerves.

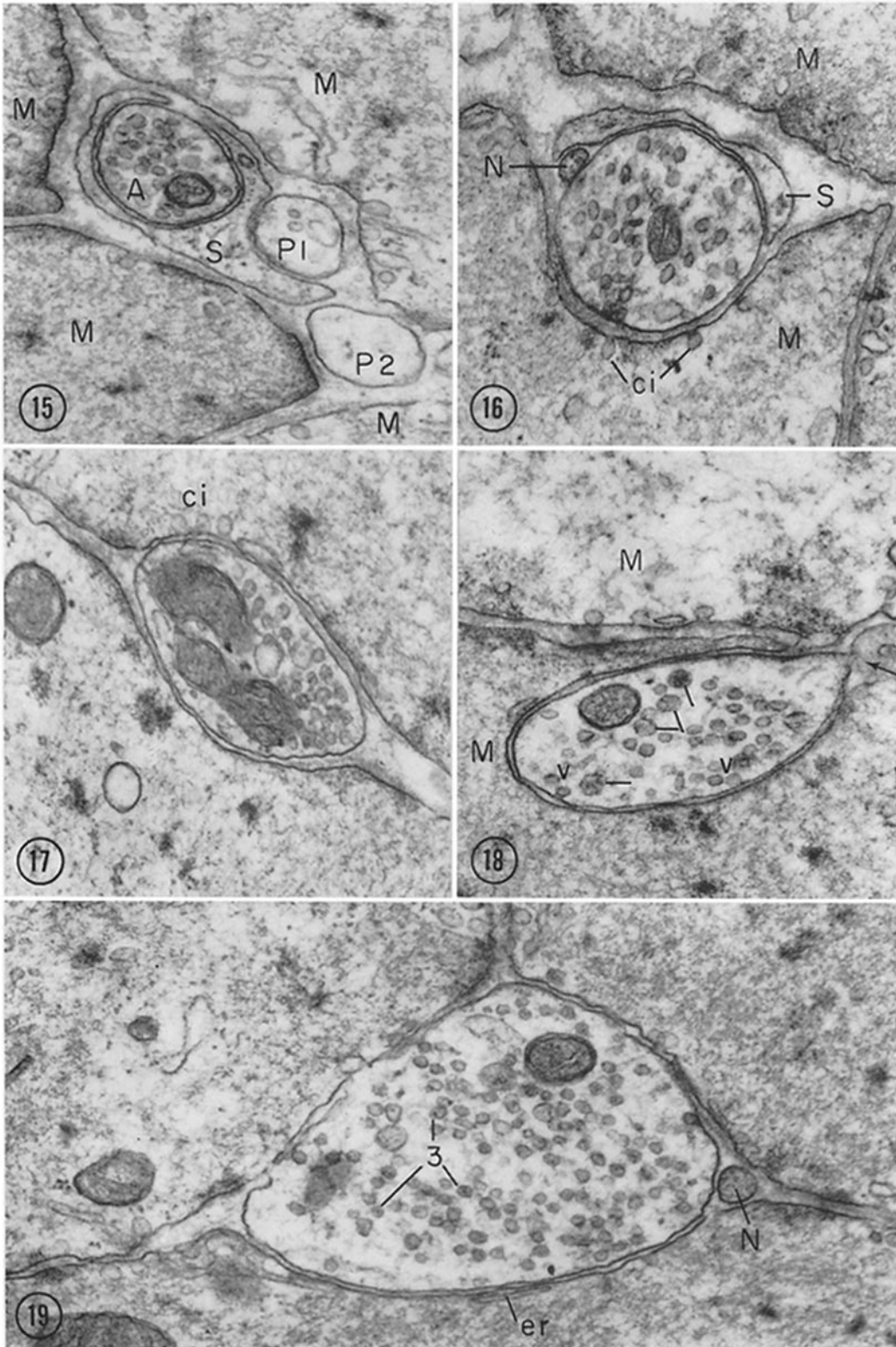
FIGURE 15 A single axon (*A*) and two "empty profiles" (*P1* and *P2*) lie between four muscle cells. The axon is almost surrounded by Schwann cell cytoplasm (*S*) and contains "synaptic" vesicles between 300 and 600 A in diameter, and a mitochondrion. The identification of *P1* is uncertain: it may be an axon. Fixation delayed 4 minutes. $\times 35,000$.

FIGURE 16 A section through an axon 0.8 μ in diameter, containing vesicles, and partly covered by a thin sheet of Schwann cell cytoplasm (*S*). The plasma membranes of nerve and muscle (*M*) are separated by basement membrane between 350 and 650 A thick. A fortuitous resemblance to a simple motor end-plate is enhanced by the contour of the plasma membrane of the muscle, which is complicated by *caveolae intracellulares* (*ci*). The small profile (*N*) is about 0.1 μ in diameter, but it may be a minute axon. $\times 35,000$.

FIGURE 17 An axon containing vesicles and mitochondria, but devoid of Schwann cell, is coming into close apposition with two muscle cells. On both sides the basement membrane is being displaced. When the nerve and muscle plasma membranes approach to within 200 A, *caveolae intracellulares* (*ci*) are usually absent; but here, and also in Fig. 19, there are several. Fixation delayed 4 minutes. $\times 35,000$.

FIGURE 18 An axon almost buried in the surface of a muscle fibre (an unusual condition). The axon contains vesicles and a mitochondrion. Four large vesicles (*lines*), although of low internal density, are probably Type I. At the arrow the cell boundaries are confused because the membranes are cut obliquely. The two vesicles (*v*) are lying against the plasma membrane of the axon, but are not opening through it. $\times 35,000$.

FIGURE 19 Another unusual case in which a large naked axon is in close apposition (*ca.* 200 A) with three muscle fibres at once. The large size of this axon may be due to a varicosity. The small profile (*N*) is similar to those in Figs. 12 and 16. There is a small portion of a subsurface *cisterna* of the endoplasmic reticulum at *er*. Fixation delayed 4 minutes. $\times 35,000$.



Support for Richardson's suggestion that "granular vesicles" contain noradrenaline has come from studies on the localization of tritiated noradrenaline by electron microscopic autoradiography of the rat pineal (Wolfe *et al.*, 1962).

A broad comparative study is needed to distinguish the types of nerve-muscle relationships which may be characteristic of sympathetic or parasympathetic, and excitatory or inhibitory innervation. In fact, up to the present time no attempts have been made to distinguish the fine structure of afferent from that of efferent nerves in smooth muscle.

Apart from their probable function in relation to the storage of transmitter, the role of the

vesicles are wide-spread, but are far from uniform in distribution. The distribution and variations in density of the vesicles are consistent with the micrographs that Falck (1962) obtained by fluorescent methods, showing the distribution of catecholamines in the vas deferens of the guinea pig.

The occurrence of spontaneous miniature junction potentials and the presence of vesicles in the axons of the ground plexus suggest that the transmission processes in the vas deferens and the skeletal neuromuscular junction are essentially similar. In this smooth muscle, however, junction potentials and miniature junction potentials can be recorded in every cell throughout the tissue.

TABLE I
*The Number and Content of Axon Bundles
in a 75 μ Grid Square*

No. of axons per bundle	No. of bundles	Axon total
13	1	13
10	2	20
9	1	9
6	2	12
4	3	12
3	2	6
2	3	6
1	25	25
		103

vesicles in the actual process of transmission has been much discussed recently (Gray and Whittaker, 1962; De Robertis *et al.*, 1962; Eccles, 1961; Richardson, 1962). Katz and his co-workers (Katz, 1958 *a*) have shown that acetylcholine is released from the nerve terminals at the skeletal neuromuscular junction in a quantal fashion, *i.e.* in "packets" of uniform size, each packet giving rise to a miniature junction potential. Since those nerve endings are loaded with vesicles of a fairly uniform size, it has been suggested that each vesicle corresponds with a packet of acetylcholine (Palay, 1956). A spontaneous discharge of miniature junction potentials has been recorded from smooth muscle cells of the vas deferens, and it has been suggested that this is due to the release of packets of noradrenaline from the axons of the ground plexus (Burnstock and Holman, 1961 *a*, 1962 *a*). In the axons in the vas deferens the

TABLE II
An Analysis of Three Grid Squares

Specimen	A	B	C
Capillaries	—	1	—
Muscle fibres	446	363	322
Muscle nuclei	11	11	18
Total number of axons	40	103	87
Axons in bundles of 3 or less	34	37	35
Single axons	22	25	21
Muscle fibres per single axon	20	14	15
Axons 200 A from muscle fibre	2	3	6

This implies that each cell must have its own nerve ending or be coupled electrically to a neighbouring innervated cell. Electrophysiological studies indicate that each axon in the hypogastric trunk can influence the activity of many different smooth muscle cells, because it is found that a change in the number of stimulated fibres causes a similar change in the amplitude of the junction potential in all cells. This convergence of each axon on each single or small group of smooth muscle cells could be brought about in a number of different ways. Each axon may branch extensively and send processes to each group of cells, so that every cell has a multiple innervation. Lundberg (1958) believed this as a result of his microelectrode studies on salivary gland cells. Or the convergence mechanism may be partly or wholly centred in the ganglion, in the distal end of the hypogastric trunk, from which we have traced nerve bundles passing to the vas deferens. There is both pharmacological and physiological evidence for the

presence of a ganglionic relay. Sjöstrand (1962 *a*) found that the response of the vas deferens to hypogastric nerve stimulation was inhibited by ganglionic blocking agents. He also found (1962 *b*) that the catecholamine content of the vas deferens was not significantly altered by dividing the hypogastric nerve. Burnstock and Holman (1962 *b*) obtained a normal discharge of miniature junction potentials in many smooth muscle cells after hypogastric division. Finally, it is conceivable that some interaction might occur between neighbouring axons in the nerve trunks, where axon to axon contacts are quite common. Nevertheless, it is hard to understand how uniformly graded junction potentials can arise in all the cells unless there is a dense distribution of functional nerve endings, *i.e.* of axonal regions from which transmitter is released.

Table II demonstrates that it is difficult to define richness of innervation. The total number of axons is probably misleading because, as was the case in the middle column, some areas have large bundles containing 12 or 15 axons that are probably passing to distant sites. If the larger bundles are excluded, the count becomes more uniform from specimen to specimen. There is probably no significant difference between the three specimens if only the very small bundles or even single axons are considered, *e.g.* from 14 to 20 muscle fibres per single axon. In our material, the commonest proportion was between these two figures.

We have observed close apposition (200 Å) between an axon and a muscle cell in about 1 per cent of muscle profiles. The number of close contacts cut in a transverse section will depend upon both the number of muscle cells that have close contact, and the length of each muscle fibre involved in the contact. In 363 muscle profiles, in the montage considered most typical, there were 11 nuclei. The length of the nucleus in the relaxed muscle of the vas deferens is about 15 μ . Therefore, if each muscle fibre had a close contact about 15 μ in length with an axon, one would expect to find equal numbers of close contacts and nuclei in a transverse section. This is never so. There are three or four times as many nuclei as there are close contacts. This could happen if each muscle cell had only 1 contact 3 to 4 μ long. Against this, however, one might expect more than the observed number of 25 single axons in the field, *i.e.* more than 1 axon profile to 14 muscle fibre profiles, if a nerve ending passes to each muscle fibre, or even

if an axon, following a winding pathway, connects with each muscle fibre in the group. We have found no evidence of single axons winding across the direction of the long axes of the muscle fibres: the axons run roughly parallel to the muscle fibres over long distances after an initial short oblique course as they leave their parent bundles.

It seems improbable, therefore, that an axon makes passing contact with, or supplies a lateral branch or ending to, every muscle fibre in its vicinity. Thus, the mechanism by which each muscle fibre is influenced by *many* nerves is certainly not close contact between each muscle fibre and many nerve endings or axons.

Without serial sections it is not yet possible to recognise a nerve terminal. Richardson (1962) called the axon-like profiles within 200 Å of the muscle fibre nerve endings. He observed, in longitudinal section, swollen segments or terminals of thin axons lying in grooves in the muscle fibres. He thought it likely that some neurites may emerge from their grooves to form further synaptic contacts (endings *en passage*) with adjacent muscle fibres. In the muscle fibre immediately below many of these endings he found flattened *cisternae* of the sarcoplasmic reticulum, in close apposition to the sarcolemma. In the guinea pig vas deferens, however, this precise arrangement of reticulum is very uncommon, although elongated *cisternae* are often seen elsewhere near the plasma membrane of the muscle cells (Fig. 12). Because we cut our material transversely, we are unable to confirm or deny Richardson's observation that the endings are swollen segments or terminals of thinner fibres. Apart from the very small profiles of 0.1 to 0.2 μ , which may be thin fibres destined to swell into endings, we have found no obvious difference in size between axons in close apposition to muscle cells and those more remote. The very small profiles are far more commonly seen associated with another axon, rather than in a position suggesting that they have left the nerve bundle and are about to swell into solitary endings.

The naked axons 200 Å from the muscle may be terminals; but, it is quite possible that these close contacts are only episodes along axons that, for most of their length, have a patchy Schwann cell cover. The naked and partly naked axons appear to be identical in structure. All the naked and most of the partly naked axons contain many agranular and a few granular vesicles. There seems no reason why the release of transmitter should be limited to those regions of bare axon which make

intimate contact with the smooth muscle membrane. It may well be that the transmitter can pass through the basement membrane surrounding those parts of the axon which are free of Schwann cell, but situated more than 200 Å from the muscle membrane.

Although each axon may be able to influence many different smooth muscle cells in this way, the paucity of naked or partly naked axon profiles in the guinea pig vas deferens suggests that some cells may be excited indirectly, *i.e.* by passive spread of current from other cells which are more favourably placed in relation to a naked axon. There are neither cytoplasmic bridges nor fusions of plasma membranes in this tissue. Distances less than 500 Å between neighbouring muscle plasma membranes are so rare that they can probably be neglected as major pathways for the spread of excitation. We do not think that the "intrusions" should be classed as important pathways for excitation in the vas deferens: they are seen in less than 1 per cent of the cell outlines. Moreover, it may be that a few cells have many intrusions, rather than that intrusions occupy 1 per cent of each muscle cell, since several intrusions are sometimes seen in one cell. Their necks of attachment are probably very narrow and would have a very high electrical resistance similar to that which has been calculated for the equally small areas of contact ("bridges") between muscle cells of the gastro-intestinal tract (Prosser, Burnstock, and Kahn, 1960). We suggest that the intrusions may be a mechanism for the transfer of material between cells (as a consequence, perhaps, of the long distances between capillaries), but this depends on the interpretation of Figs. 6 to 9. We think that the bodies identified by Yamamoto (1960) as axons within muscle cells of the vermiform appendix were intrusions from neighbouring muscle cells. Richardson (1962) also believed them to be muscular.

In the absence, therefore, of cytoplasmic bridges, and in the virtual absence of intermembranous contacts between muscle cells, transfer of excitation from cell to cell must be by electrotonic spread of current in a "functional" electrical syncytium, through the large areas of 500 to 800 Å intercellular space filled with basement membrane. Smooth muscles of other viscera also behave as "functional" syncytia (Bozler, 1948), although intermuscular contacts may be different (Dewey and Barr, 1962; Rhodin, 1962).

In the vas deferens the delay between stimulation of the hypogastric nerve and the appearance of a junction potential is long and variable (20 to 100 msec: Burnstock and Holman, 1961 *a*); and the junction potentials may have considerably longer time-courses than the spontaneously arising miniature junction potentials. Both these observations may be accounted for by the different lengths of the pathways along which nerve impulses might pass through various axons of the ground plexus, and by the different distances and directions of sources of electrotonic current from neighbouring fibres.

We, therefore, believe that transmitter-substance is released from many areas of bare axon-surface in the ground plexus. Many of these areas are within a fraction of a micron of muscle membrane. This should produce high concentrations of transmitter over fairly discrete regions of the smooth muscle cell surface, as well as a general increase in the concentration of transmitter in the extracellular space. This concept has some similarity with that developed by Rosenblueth (1950). The consequences of Rosenblueth's theory have been discussed recently by Hillarp (1960).

At the skeletal neuromuscular junction, whereas part of the local muscle membrane is only 500 Å from the axon, much of it is up to 1 μ away because of its complex infolding. Yet each quantum of acetylcholine exerts its action in a fraction of a millisecond (Katz, 1958 *b*). Our own calculations (Pugsley *et al.*, unpublished) indicate that the time taken for the diffusion of a similar packet of noradrenaline over a distance of less than 1 μ is probably much shorter than the time required for the spontaneous miniature junction potentials in the vas deferens to reach maximum amplitude.

Many of the naked axons in our preparations were found in extremely narrow crevices filled with basement membrane, lying deeply within the bundles of smooth muscle cells. These small spaces communicate with the larger extracellular spaces between the bundles by narrow tortuous channels, also filled with basement membrane, which may provide barriers to the diffusion of drugs when these are added to a solution bathing the tissue. On the other hand, naked axons also occur in some of the larger extracellular spaces where drugs would have easy access to both naked axons and smooth muscle membranes in their vicinity. These factors may be important in

determining the site and mode of action of drugs at autonomic nerve endings.

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