ULTRASTRUCTURAL INTERRELATIONSHIPS OF NERVE PROCESSES AND SMOOTH MUSCLE CELLS IN THREE DIMENSIONS

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ABSTRACT

The muscularis externa of the intestinal wall of frogs was fixed in osmium tetroxide, embedded in Vestopal-W, serially sectioned for electron microscopy, and stained with uranyl acetate. A method to obtain individually mounted and properly positioned serial sections is described. The three-dimensional techniques used during the course of this investigation demonstrate that it is possible to examine carefully relatively large areas of tissue on individual serial sections with the electron microscope and subsequently to construct montages of electron micrographs of pertinent areas from each section. Several carefully rendered interrelationships of nerve processes and smooth muscle cells in three dimensions are exhibited and described. Recent studies of other neuro-effector relationships are discussed in relation to the present status of the nature and organization of the autonomic nervous system in visceral organs.

INTRODUCTION

Information concerning the ultrastructural interrelationships of nerve and muscle in a variety of vertebrates has been revealed in recent years with the use of the electron microscope. Such information was first reported in skeletal muscle tissue (5, 48, 49, 57) in which vesiculated nerve processes were found lodged within depressions of the surface of skeletal muscle fibers. Junctional folds were seen extending into the sarcoplasm of the fiber from the bottom of these depressions. Subsequent reports (1, 3, 12, 14, 50, 52, 80) have described these features in greater detail and with more clarity. Several reports (39, 44, 51) described the fine structure of neuromuscular relationships in so called tonus skeletal muscle. These relationships resembled those just mentioned, except that junctional folds were absent and the depressions were not so extensive. Several electron microscopic studies (4, 13, 23, 26, 71) have demonstrated a close relationship between nerve processes and cardiac muscle cells, but others (19, 29, 40, 46, 70, 73) have indicated a more distant association. Recently, however, a preliminary report (69) has demonstrated intimate relationships of two types of vesiculated nerve processes with the surface of cardiac muscle cells.

Information concerning the fine structure of neuromuscular relationships in smooth muscle tissue was not forthcoming until 1957 when Caesar, Edwards, and Ruska (11) indicated the presence of such relationships in the muscular wall of the mouse intestine. Since that time numerous reports (10, 17, 20, 22, 27, 32, 33, 35, 37, 41, 53–56, 64, 65, 67, 68, 77, 78) have described various features of neuromuscular relationships in smooth muscle tissue of different organs and animals, the most significant of which are the reports by Richardson (55, 56) on the ductus deferens of the

rat and the iridial muscle of the rabbit, by Merrillees et al. (35) on the ductus deferens of the guinea pig, by Taxi (65) on the ductus deferens of the rat, nictitating membrane and ciliary muscle of the cat, and the intestinal wall of the mouse, and by Thaemert (67) on the muscularis externa of the wall of the gastrointestinal tract and the urinary bladder of rats. These papers have described the existence of intimate relationships between vesiculated nerve processes and smooth muscle cells.

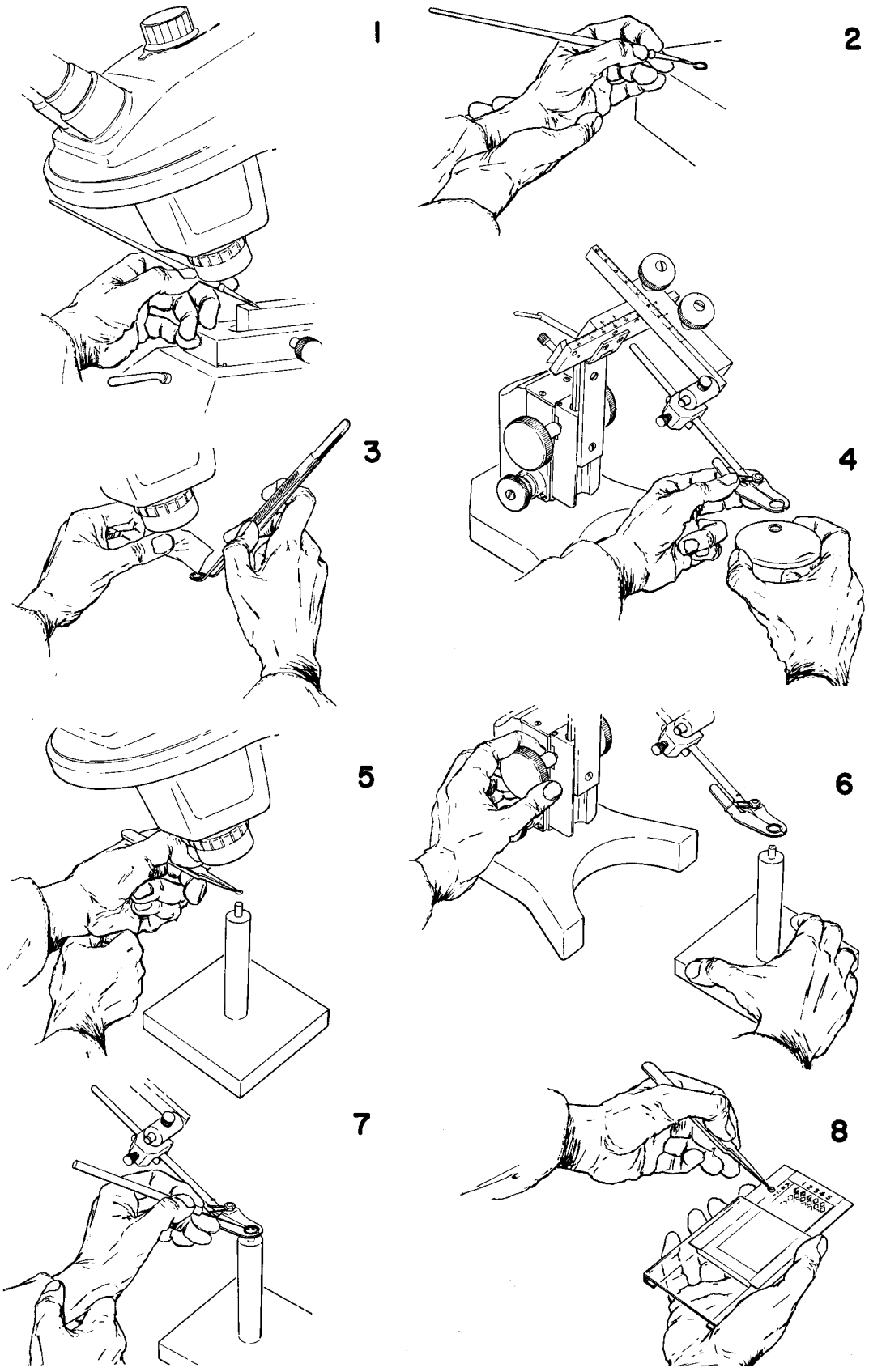
The above-mentioned smooth muscle studies clearly indicate that a great deal of additional information is needed regarding the ultrastructure and distribution of the end formation of the autonomic nervous system. This is especially true for the gastrointestinal tract, for which this kind of information must be forthcoming in order to aid in the explanation of evidence obtained from electrophysiological and pharmacological experiments (2, 7–9, 21, 30, 38, 47, 58, 62, 66, 72). The present report will describe a method whereby the relationships of nerve processes to smooth muscle cells can be determined through the study of serial sections of tissue with the electron microscope and through subsequent three-dimensional reconstruction. This method is a modification of techniques previously used (1, 16, 34, 60, 61, 74). This report will also reveal some preliminary results obtained from a continuing three-dimensional study and demonstrate additional two-dimensional relationships of vesiculated nerve processes with smooth muscle cells. A portion of this study has been previously published in abstract (68).

MATERIALS AND METHODS

All smooth muscle tissue used for this study was obtained from the muscularis externa of the wall of the gastrointestinal tract of frogs (*Rana pipiens*). 1% osmium tetroxide solution (pH 7.5), buffered with veronal acetate, was injected into the pleuroperitoneal cavity immediately after severance of the spinal cord at the base of the brain. Shortly thereafter, long segments of the gut (now stained with osmium) were removed from the opened pleuroperitoneal cavity and placed in cold fixative. While there, these long segments were subdivided into pieces 5 mm in length and fixed for 90 min. After fixation, the tissues were dehydrated in ethyl alcohol and embedded in Vestopal-W.

In preparation for a three-dimensional study, serial sections with dimensions of 1 mm^2 and larger are cut from blocks of embedded tissue with glass knives. Most of the sections display interference colors of silver or gold. Each section is separated from the adjoining one, picked up individually from the surface of the water with a sable-hair brush (Fig. 1), and transferred to a small drop of water which is supported by a carbon-coated parlodion membrane stretched across a ring[1] with a diameter of 5 mm (Fig. 2). The water is then slowly removed with tufts of filter paper (Fig. 3) while keeping the tissue section in the center of the membrane. After the water is removed, the ring with the membrane-supported section is picked up by a specially designed forceps which is fastened to the arm of the micromanipulator (Fig. 4). A clean copper disc (specimen mount) with a diameter of 3 mm and a 0.5-mm hole in its center is placed atop a lucite stand which is just 3 mm in diameter at its top (Fig. 5). This stand with the copper disc is then moved beneath the 5-mm ring which is held at the end of the arm of the micromanipulator (Fig. 6). Adjustments of the position of the lucite stand in relation to the 5-mm ring with its tissue section are made so that the tissue section is directly above the 0.5-mm hole in the copper disc. A specific area of the muscularis externa is then placed over the hole in the disc with the use of the controls of the micromanipulator while viewing this procedure through a dissecting microscope. After the section is in contact with the disc, the supporting membrane is then carefully broken around the circumference of the disc (Fig. 7). The disc with the affixed section, after staining with uranyl acetate, is then filed away for subsequent use (Fig. 8). The foregoing procedure is carried out for each succeeding tissue section in the series. The sections are then viewed in a Philips EM100-B electron microscope with a single condenser. A selected area of the muscularis externa is chosen. Numerous electron micrographs of this area are made from each succeeding section. Montages of these electron micrographs are constructed and all neuromuscular relationships within this area are followed from one section to the next. In this way, it is possible to visualize these interrelationships in three dimensions. However, in order to determine the detailed three-dimensional status of these relationships it is necessary to reconstruct them carefully from each succeeding section.

[1] The parlodion membrane is applied to the rings by immersing a group of thirty rings, supported on a piece of window screen which is resting on a microscope slide, beneath the surface of water in a bowl. Two drops of a 3% solution of parlodion in amyl acetate are dropped on the surface of the water. After formation of the membrane, the water level in the bowl is lowered by siphoning the water with the use of a rubber hose until the membrane covers all the rings. After the rings with their membranes are dry, vaporized carbon is applied in a high vacuum evaporator in order to stabilize the membranes.



FIGURES 1 to 8 Illustrations depicting the procedure used for obtaining properly mounted serial sections for subsequent three-dimensional studies.

This is done by tracing the pertinent structures from electron micrographs on vellum paper. These tracings are overlaid with a grid-plane and subsequently redrawn on a perspective grid-plane. These tracings are then connected to one another in the most logical manner to form an illustration in perspective. This technique has been previously described (37).

RESULTS

An area, 55 by 100 μ, of the inner layer of the muscularis externa of the duodenum of a frog, which included a portion of the adjacent myenteric plexus, was followed sequentially from section to section on electron micrographs. These micrographs were fitted together to construct montages of this area. The total depth of the tissue cut, represented by all sections and areas of missing sections, amounted to a total of 44 μ. Some of the sections were lost during the transfer from the water trough of the microtome to the copper discs, and others were lost by breakage of the tissue sections and their supporting membranes while in the electron microscope. The largest gap encountered, because of these losses, was 5 μ. Despite these losses, it was possible to follow several neuromuscular contiguities for varying distances.

In Fig. 9, a nerve process, partially enveloped by a portion of a Schwann cell, is closely applied to the surface of a protrusion of a smooth muscle cell. This relationship is more evident in Fig. 10, in which the illustration is divided into three unequal slices to show the three horizontal surfaces representing the corresponding electron micrographs in Figs. 11 to 13. The illustrations and the micrographs clearly show that the nerve process extends from a position 400 mμ away from the innervated muscle cell to a position of intimate contact with a protrusion of the innervated muscle cell. The area of contact is 1.25 μ in length. Since there is a space of 1 μ of missing sections 300 mμ below the lower horizontal surface shown in Fig. 10 and Fig. 13, it is not known whether this nerve process is ending. However, beyond this space no trace of the nerve process or its Schwann cell investment can be detected. The illustration is continued for 1.95 μ beyond the space of missing sections. The total thickness of all sections and areas of missing sections used in the construction of this illustration is 6.1 μ, which represents approximately 60 sections.

The Schwann cell process, as seen in Figs. 9 to 13, is slender and closely applied to the nerve process in the upper portion of the illustrations; then, as the nerve process makes contact with the protrusion of the smooth muscle cell, the Schwann cell process expands and almost completely surrounds the nerve process except for the area of contact with the protrusion. The origin of the nerve process could not be determined. The expanded portion of the Schwann cell process also prevents the nerve process from making contact with the expanded portion of a dendrite which is situated between the Schwann cell–invested nerve process and the smooth muscle cell in the left portion of the illustrations and micrographs. The dendrite is continuous with the perikaryon of a neuron which lies in the periphery of the myenteric plexus approximately 10 μ away. The dendrite is identified as such because it contains characteristic cisternae of

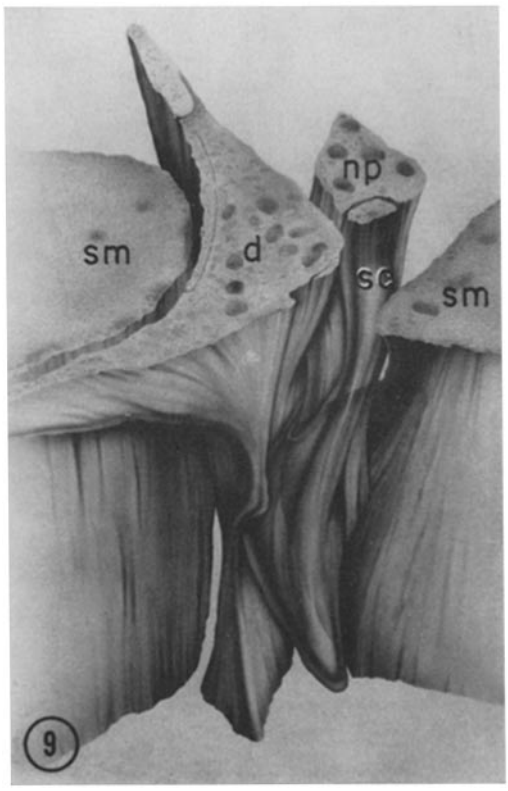


FIGURE 9 This three-dimensional illustration was drawn in perspective from serial sections of the inner layer of muscularis externa of the duodenum of a frog. A vesiculated nerve process (*np*) with a Schwann cell investment (*sc*) and a dendrite (*d*) are situated between two smooth muscle cells (*sm*). The sections and the areas of missing sections responsible for this illustration amount to a combined thickness of 6.1 μ.

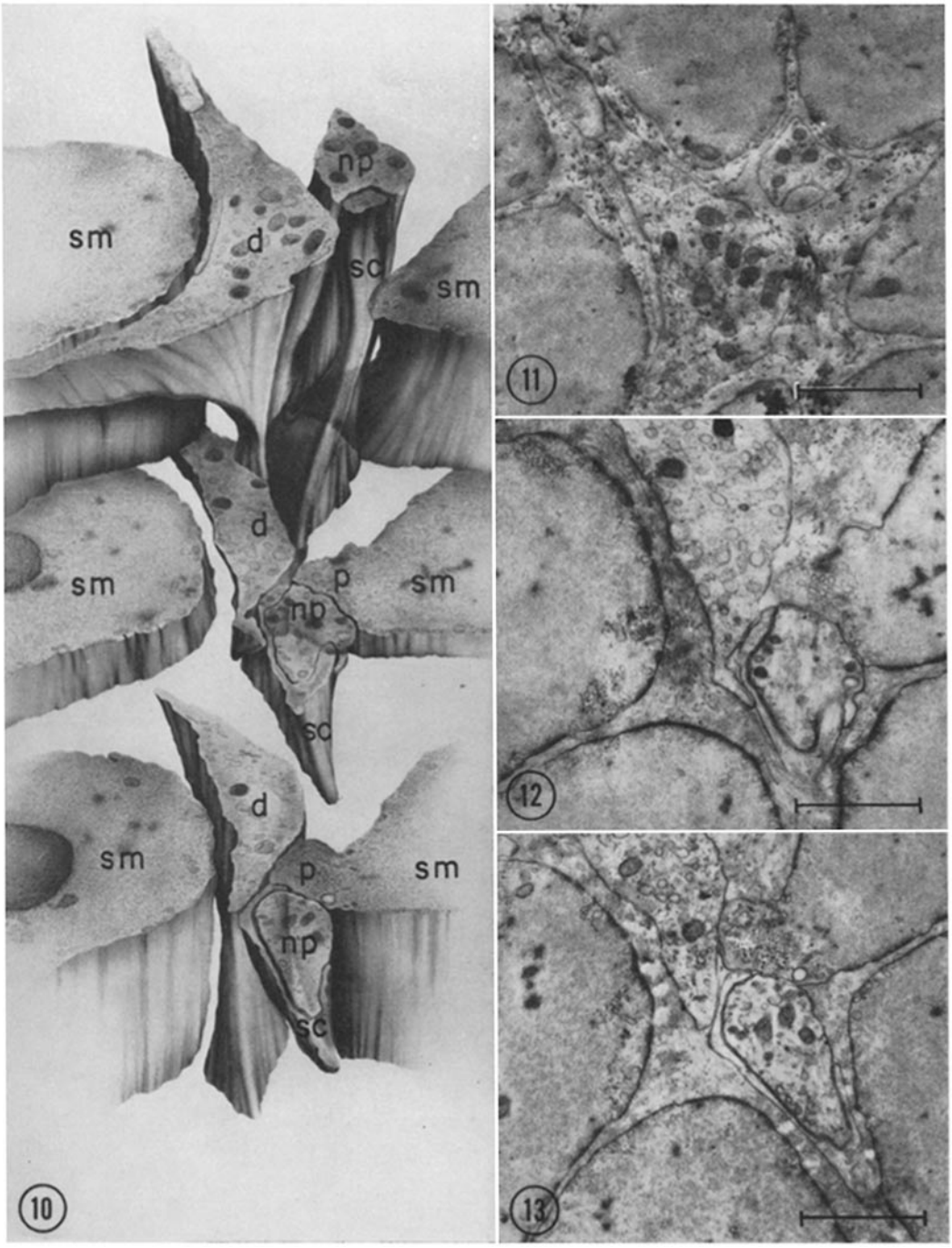


FIGURE 10 This three-dimensional illustration depicts the result of horizontally dividing the illustration of Fig. 9 into three slices. These three slices were then moved out of register with respect to each other and separated so that each slice is situated above the other. This permits the demonstration of three surfaces. *sm*, smooth muscle cell; *np*, vesiculated nerve process; *sc*, Schwann cell investment; *d*, dendrite; *p*, protrusion of smooth muscle cell.

FIGURES 11 to 13 Electron micrographs which represent the three horizontal surfaces of Fig. 10. The scale in the lower right corner of the micrographs indicates 1 μ.

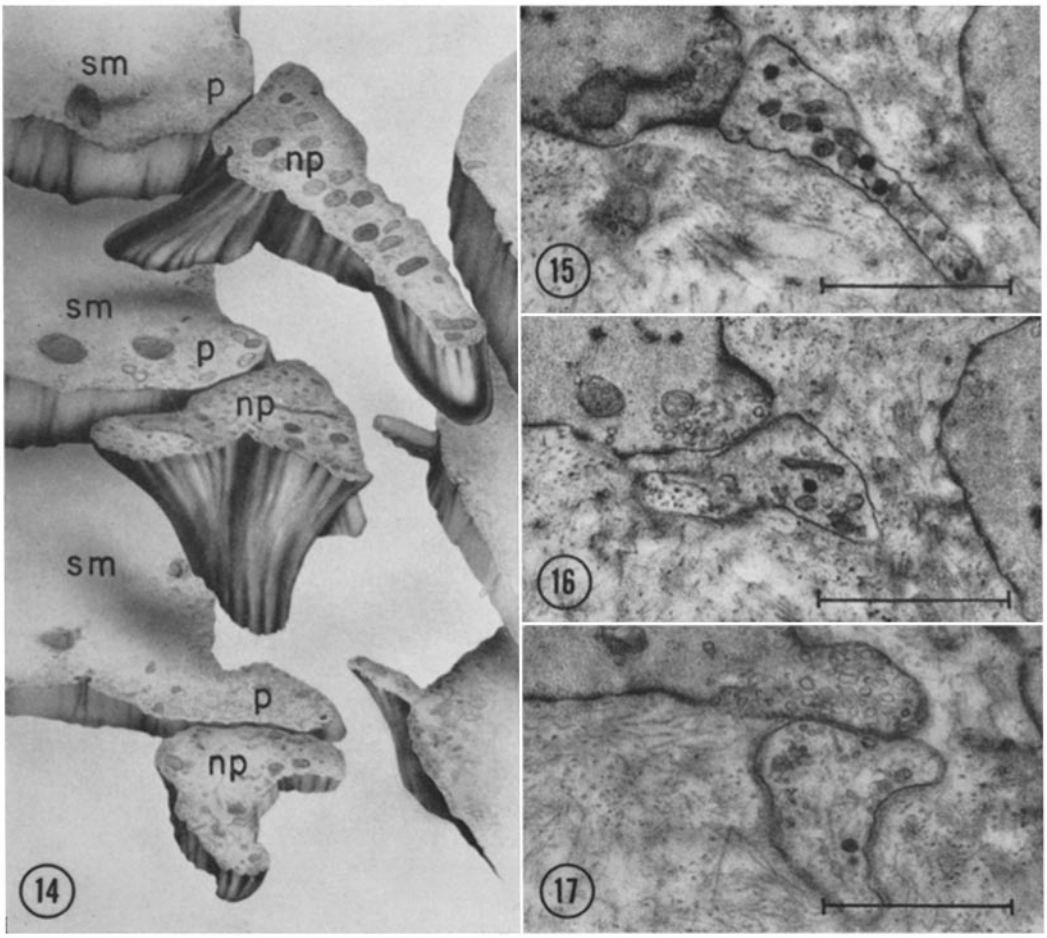


FIGURE 14 This three-dimensional illustration resulted from the same procedure as used in the construction of Fig. 10. If one imagines the slices of tissue one on top of the other a composite illustration would be mentally contrived, similar to the illustration of Fig. 9. A vesiculated nerve process (*np*) without a Schwann cell investment is closely applied to the surface of a protrusion (*p*) of a smooth muscle cell (*sm*). This illustration was drawn in perspective from serial sections of the inner layer of the muscularis externa of the duodenum of a frog. The sections and areas of missing sections responsible for this illustration amount to a combined thickness of 1.9 μ.

FIGURES 15 to 17 Electron micrographs which represent the horizontal surfaces of Fig. 14. The scale in the lower right corner of the micrographs indicates 1 μ.

slightly granular endoplasmic reticulum and free ribonucleoprotein granules which are prominent in many other short and long processes of neurons within the myenteric plexus and between the peripherally located smooth muscle cells of the inner layer of the muscularis externa. Even though this dendrite and many others are contiguous with the surface of smooth muscle cells, it is not known whether such dendrites act as receptors for afferent stimuli. The apparent semitransparency of the nerve and Schwann cell processes in Figs. 9 and 10 permits the visualization of the edges of both the smooth muscle cell and the Schwann cell process.

The illustration in Fig. 14 and the supporting electron micrographs in Figs. 15 to 17 demonstrate the intimate contact of a nerve process (without a Schwann cell investment) with a protrusion of a

smooth muscle cell. Despite the irregularity of the surface of the nerve process, it remains intimately associated with the smooth muscle cell. The total thickness of all tissue sections and areas of missing sections represented in the illustration of Fig. 14 is 1.9 μ, or approximately 20 sections, and, therefore, the nerve process is in contact with the surface of the smooth muscle cell for at least that extent. The uppermost surface in the illustration of Fig. 14 represents the first section cut from a block of tissue. A 1-μ gap of missing sections exists beneath the lowest border of the illustration; therefore, the total extent of the nerve-muscle contact is not known.

The total thickness of all sections and the area of missing sections in Fig. 18 is 1.05 μ, or approximately 10 sections. This illustration shows two vesiculated nerve processes intertwined one with the other, one of which lies near the surface of a smooth muscle cell. In the electron micrograph of Fig. 19, basement membrane material is apparent on the surface of the nerve process which is near the smooth muscle cell on the right.

Three slender nerve processes, shown in the illustration of Fig. 20 and in the supporting electron micrographs of Figs. 21 to 23, make intimate contact with a protrusion of a smooth muscle cell. The electron micrograph in Fig. 21 was taken from a section which was slightly compressed. The uppermost surface of the corresponding illustration in Fig. 20, however, was drawn in such a way as to compensate for this compression so that it would fit the succeeding sections. The lower horizontal surface in the illustration of Fig. 20 and the electron micrograph of Fig. 23 show that the slender nerve process on the left is emerging from or retreating into a groove provided by the Schwann cell investment. The large nerve process, which is not in contact with the smooth muscle cell and which is completely surrounded by the Schwann cell investment, is shown to divide into two smaller processes. The apparent semitransparency of the Schwann cell investment and the nerve processes permits the visualization of the position of the nerve processes and the relationship of one to the other. The total thickness of all tissue sections and areas of missing sections in Fig. 20 is 1.9 μ or approximately 20 sections. This corresponds to the total thickness of the illustrations in Fig. 14, since they were all derived from the same sections.

The electron micrographs of Figs. 24 to 29 show various types of intimate relationships of vesiculated nerve processes and smooth muscle cells. They represent several types of nerve processes, in addition to those previously described in three di-

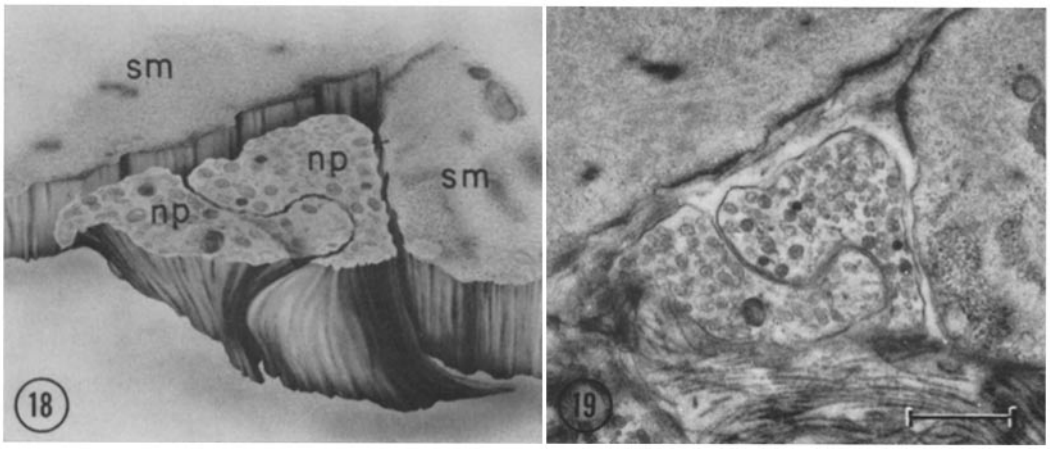


FIGURE 18 This three-dimensional illustration was drawn in perspective from serial sections of the inner layer of the muscularis externa of the duodenum of a frog. The sections and areas of missing sections responsible for this illustration amount to a combined thickness of 1.05 μ. *np*, vesiculated nerve process; *sm*, smooth muscle cell.

FIGURE 19 An electron micrograph which represents the top surface of the illustration of Fig. 18. Notice the abundance of vesicles within the two intertwined nerve processes. Some of the vesicles contain granules. The scale in the lower right corner of the micrograph indicates 1 μ.

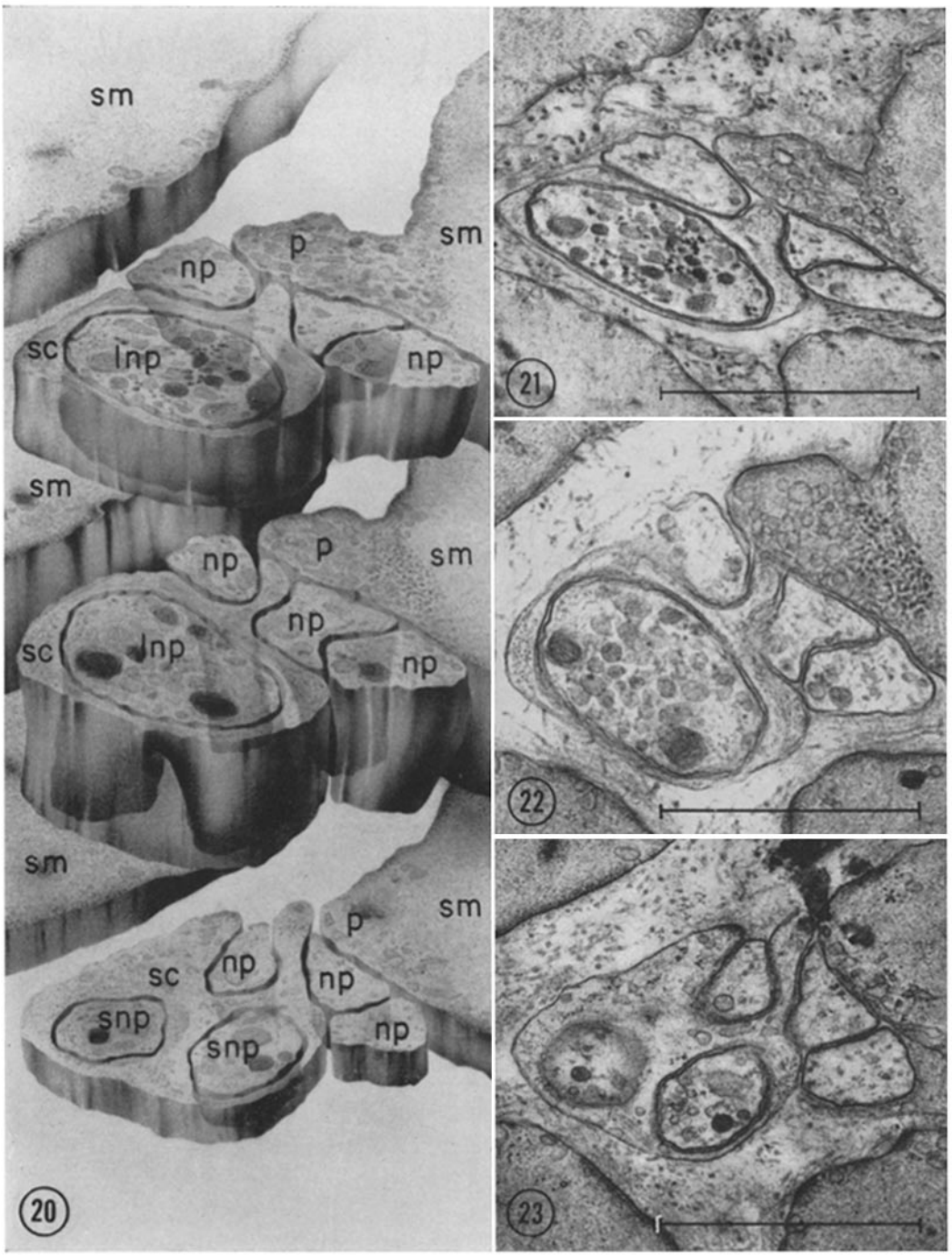
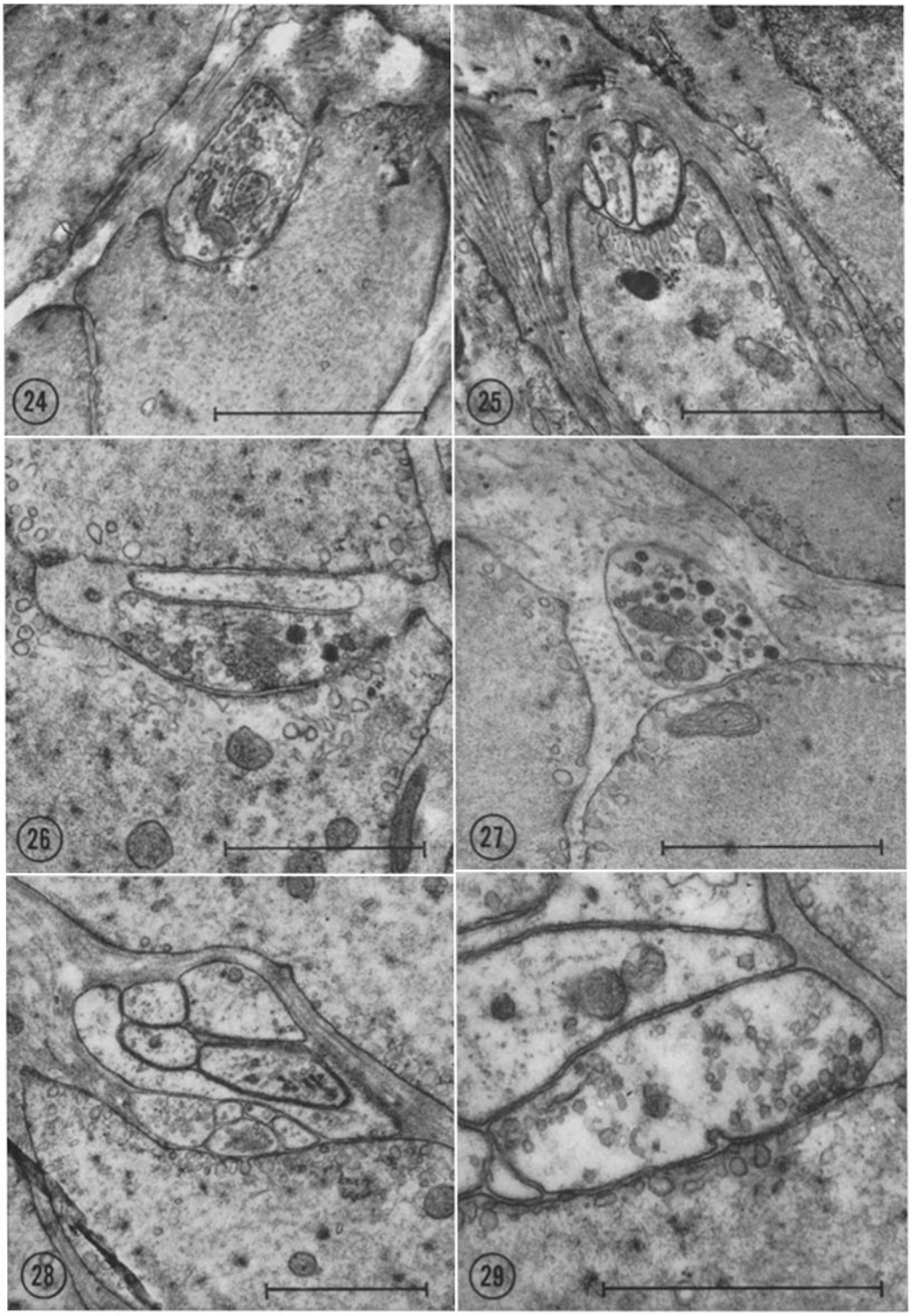


FIGURE 20 This three-dimensional illustration resulted from the same procedure used in the construction of Figs. 10 and 14. If one imagines the slices of tissue one on top of the other a composite illustration would be mentally contrived, similar to the illustration of Fig. 9. Three slender nerve processes (*np*) make intimate contact with a protrusion (*p*) of a smooth muscle cell (*sm*). *lnp*, large nerve process; *snp*, small nerve process; *sc*, Schwann cell investment. This illustration was drawn in perspective from serial sections of the inner layer of the muscularis externa of the duodenum of a frog. The sections and areas of missing sections responsible for this illustration amount to a combined thickness of 1.9 μ.

FIGURES 21 to 23 Electron micrographs which represent the horizontal surfaces of Fig. 20. The scale in the lower right corner of the micrographs indicate 1 μ.



FIGURES 24 to 29 These electron micrographs demonstrate the various types of neuromuscular relationships observed in the inner layer of the muscularis externa of the duodenum of a frog. The scale in the lower right corner of the micrographs indicate 1 μ.

mensions, which occur in the smooth muscle tissue of the wall of the gastrointestinal tract of frogs. A single vesiculated nerve process in Fig. 24 is situated within a deep depression. Fig. 25 shows three vesiculated nerve processes, which are contiguous with one another, lying within a relatively deep depression of the surface of the muscle cell. Elongated "caveolae intracellulares" can be seen streaming from the contact area within the periphery of the muscle cell. The vesiculated nerve process in Fig. 26 is situated within a shallow depression. In Fig. 27, the vesiculated nerve process is merely apposed to the surface of the muscle cell. Several vesiculated nerve processes in Fig. 28, which are in the periphery of a small nerve bundle, are positioned within a shallow depression. A small invagination of the plasma membrane of the muscle cell occurs opposite a nerve process.

The vesiculated nerve processes just described vary in size and in the number and kinds of vesicles which they contain. In Figs. 11 to 13, 15 to 17, 21 to 23, 25, and 29 the nerve processes in contact with muscle cells contain relatively fewer vesicles than the nerve processes in the electron micrographs of the remaining figures. A number of granular vesicles are present within the nerve processes of Figs. 19 and 27, whereas in the other figures the vesicles are all agranular with just a few exceptions. Schwann cell association is lacking in Figs. 15 to 17, 19, 24, 25, and 27.

DISCUSSION

The three-dimensional techniques used during the course of this investigation demonstrate that it is possible to carefully examine relatively large areas of tissue on individual serial sections with the electron microscope and subsequently to construct montages of electron micrographs of pertinent areas from each section. Together with the additional procedure of carefully rendering the interrelationships of nerve processes and smooth muscle cells in three dimensions (37), these techniques will help to determine the complex nature of the terminal ramifications of the autonomic nervous system in the wall of the gastrointestinal tract, the complete knowledge of which has eluded the efforts of scores of investigators for many years (28). These procedures are not entirely new. They have recently been used, in a modified form, by several investigators (1, 16, 60, 61, 74). Their methods, however, differed from the present techniques in that they depended upon the use of serial sections of small dimensions so that many sections could be placed on one and the same specimen mount (grid). Such methods have the advantage of permitting the investigator to view and study many sections without changing specimen mounts in the electron microscope and, therefore, will save valuable time and effort. On the other hand, those methods are limited because only a very small and restricted area of tissue can be followed sequentially from one section to another. Therefore, in the present study, in which nerve processes must first be located and then traced for lengths of several microns, the multiple-section technique is not feasible.

Even though the nerve processes in the smooth muscle tissue of the muscularis externa of the gastrointestinal tract do not seem to be so numerous as those found in the wall of the ductus deferens (10, 35, 55, 65), in the iris (17, 41, 56) and ciliary body (27, 65), several nerve-muscle contact regions in the inner circular layer of the muscularis externa were traced for varying distances. In each of the three-dimensional illustrations and their accompanying electron micrographs of these nerve-muscle contacts, it is shown that these contiguities extend for several microns. The paucity of nerve processes in the intestinal wall is very evident when studying many tissue sections, but it is premature to state that each smooth muscle cell is not innervated or that intimate contact of nerve and muscle is not necessary in the propagation of the nerve impulse. Perhaps the greatest dispute in recent literature, regarding the nature and organization of the neuro-effector relationships in visceral organs, is concerned with these unknowns. Merrillees et al. (35), on the basis of their ultrastructural studies of the guinea pig vas deferens, contend that close apposition between nerve processes and smooth muscle cells occurs in only 1% of the muscle profiles. They concluded, therefore, that "it seems improbable that an axon makes passing contact with, or supplies a lateral branch or ending to, every muscle fiber in its vicinity." Richardson (55), in a report of his work on the wall of the rat vas deferens, infers that each and every smooth muscle cell possesses an ending from a nerve process, since he encountered many neuromuscular contacts. In a comparative study of the intestine, the vas deferens, the ciliary muscle of the eye and the retractor muscle of the nictitating membrane, Taxi (65) contends that the innerva-

tion of the intestine is not mediated by definite synapses of nerve and muscle, whereas the vas deferens and the ciliary muscle are innervated by definite neuromuscular junctions and the retractor muscle of the nictitating membrane perhaps represents an intermediary type. Merrillees et al. (35) also stated that "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. This implies that each cell must have its own nerve ending or be coupled electrically to a neighboring innervated cell." Since they observed no cytoplasmic bridges and virtually no intermembranous contact or nexuses (2) between smooth muscle cells of their preparations, they concluded that "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-A intercellular space filled with basement membrane." Taxi (65) assumes that only certain smooth muscle cells exhibit a close relationship (less than 500 A) with a nerve fiber within the wall of the intestine and that many smooth muscle cells are stimulated either by a substance diffusing from nerve bundles or by a direct transmission from one smooth muscle cell to another. Electrophysiological studies have indicated that propagation of the wave of excitation or action potential occurs between smooth muscle cells (2, 9, 21, 38, 47, 72 and others). Recently, however, Sperelakis and Tarr (62), as the result of an important investigation using double microelectrodes, reported a weak electrotonic interaction between neighboring visceral smooth muscle cells in cat intestine and therefore they concluded that "current flow through one cell does not have substantial effect on the transmembrane potential of adjacent cells." This electrophysiological evidence supports the view that each intestinal smooth muscle cell must be influenced directly by nerve processes or the transmitter substances from these processes.

Nerve processes of the autonomic nervous system may contain "synaptic" vesicles ranging in number from a few to many. Such vesicles, ranging in size from 30 to 100 mμ in diameter, are described as agranular or granular, depending upon whether or not they contain electron-opaque cores. These cores may vary in diameter from 20 to 80 mμ. Granular vesicles are prevalent in the nerve processes within the smooth muscle tissue of the vas deferens (10, 35, 55, 65), arterioles (32, 33) and dilator muscle of the iris (56), and within the adrenal medulla (79), pineal gland (15, 75) and cardiac muscle (4, 12, 26, 40, 69). Each of these tissues, however, also contains nerve processes with agranular vesicles. Within glands (6, 59, 76), peripheral ganglia (22, 24, 63, 67), the smooth muscle tissue of the gastrointestinal tract (22, 53, 54, 64, 65, 78), and the sphincter pupillae (17, 56), the nerve processes contain vesicles which are predominantly agranular. Currently, only agranular vesicles have been reported to be present in the nerve processes of the smooth muscle tissue of the urinary bladder (67), ciliary muscle (27, 65), and lung (10), but with further study it is possible that some granular vesicles may be detected.

With the use of fluorescence methods (18, 25, 31, 42, 43) for the histochemical demonstration of catecholamines, it has been determined that neurons and nerve processes which contain catecholamines are present in various visceral organs. These methods have shown that the more intense the fluorescence the greater the amount of catecholamines present. This information, when compared with electron microscopic studies, implies that those organs whose nerve processes exhibit intense fluorescence are predominantly endowed with granular vesicles. Nerve processes containing granular vesicles are infrequently seen in the muscularis externa of the gastrointestinal tract. This finding correlates well with the fluorescence study of Norberg (42) in which it was found that fluorescent fibers were scarce in the muscle layers of the intestine. However, he did detect intense fluorescence within the myenteric plexus in which nerve processes with granular vesicles are more frequently found. In addition to the fluorescence methods for the identification of adrenergic fibers, the thiocholine method for the detection of cholinergic fibers has been used with some success in the anterior segments of the rabbit eye (31). Heavy staining for nerve processes containing acetylcholinesterase was seen in the sphincter pupillae and ciliary muscle. According to electron microscopic studies, these structures contain nerve processes which are predominantly endowed with agranular vesicles (17, 27, 56, 65).

In a study by Potter and Axelrod (45), ho-

mogenates of heart, vas deferens, submaxillary salivary gland, and pineal body were made of these organs from rats injected with tritiated norepinephrine. It was subsequently determined that the endogenous and the tritiated norepinephrine in these tissues were primarily localized in particles associated with the microsomal fraction. In an electron microscopic study of the microsomal fraction of rat hearts by Michaelson et al. (36), it was determined that vesicles with diameters of 50 mμ were present within the fraction. A few of the vesicles contained electron-opaque cores. This evidence seems to implicate vesicles in the storage of catecholamines within the tissues of the heart. Wolfe et al. (75), with the use of autoradiography and electron microscopy, have shown that, after the injection of tritiated norepinephrine into rats, sections of their pineal glands revealed that only nerve processes containing granular vesicles accumulated the injected norepinephrine. This observation prompted these investigators to conclude that norepinephrine resides in the electron-opaque core of the granular vesicle and that the presence of granular vesicles can be used as one criterion for the identification of adrenergic sympathetic axons in electron micrographs.

It appears from the foregoing discussion that it may be possible to distinguish between sympathetic and parasympathetic nerve processes on the basis of their vesicular content. However, since the essential details of the nature and organization of the autonomic nervous system and its relationship to effector cells have not as yet been determined by the studies discussed here and by the present investigation, it is obvious that much more diligent work must be forthcoming. The serial sectioning of visceral organs with subsequent three-dimensional reconstruction appears to be the best procedure to accomplish this task.

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