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C. ELECTRON MICROSCOPY OF SYMPATHETIC TISSUES

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The fortuitous circumstances which first led me to use the word *tissue* when asked to submit a title for this review have already been forgotten, but a more thoughtful, a more deliberate choice of words would not have yielded one more appropriate. For a tissue is characterized not only by the nature of its component cells but also by the way in which these cells are specifically related to each other and to other structures in their immediate environment. This in essence has been the concern of electron microscopical investigations of the sympathetic nervous system. One of the primary aims of this account will be to abstract the available morphologic data for a tentative picture of the sympathetic neuron in all of its different parts. Since a distinctive feature of any neuron is its contacts with other cells, considerable attention is given to the junctions formed by other neurons with the sympathetic ganglion cells and to the relationships of the sympathetic fibers to the effector cells of the peripheral organs. Implicit in this statement is the notion that morphologic criteria exist whereby the sympathetic fibers can be distinguished from other autonomic fibers at the periphery. The evidence supporting this idea will be reviewed critically.

The well known morphologic criteria for identifying synapses in the central nervous system apply also to the synapses located in the sympathetic ganglia of fish (95), amphibia (21, 75, 91, 94, 95, 99, 109), reptiles (87, 94, 95), birds (94, 95) and mammals (3, 12, 29, 42, 44, 76, 89, 105). Briefly stated these are: a direct apposition of the pre- and postsynaptic processes separated by an interspace of 80 to 250 Å; localized thickenings of the apposed membranes, due apparently to an aggregation of opaque material in the subjacent cytoplasm; a congregation of small vesicles 200 to 600 Å in diameter found in the presynaptic process only and clustered preferentially at the synaptolemma in association with the membrane thickenings. The location of the synapses on the surface of the ganglion cell differs not only from species to species, but also from one ganglion to another within the same species. Thus, in mammals where the neurons give rise to multiple, branching protoplasmic processes of considerable length, the synaptic contacts are primarily axodendritic in type, while on the unipolar ganglion cells of amphibia axosomatic synapses prevail. The thin sections of electron micrography and the complex geometry of the multipolar neurons have militated against providing more than fragmentary information on the number and spatial arrangement of the synaptic bulbs on the surface of the neuron. Multiple bulbs with their plasma membranes separated by a thin glial process or merely by the 100 to 150 Å interspace usually found between contiguous epithelial cells have been observed on the soma of amphibian neurons (91,109) and on the dendrites of reptilian gan-

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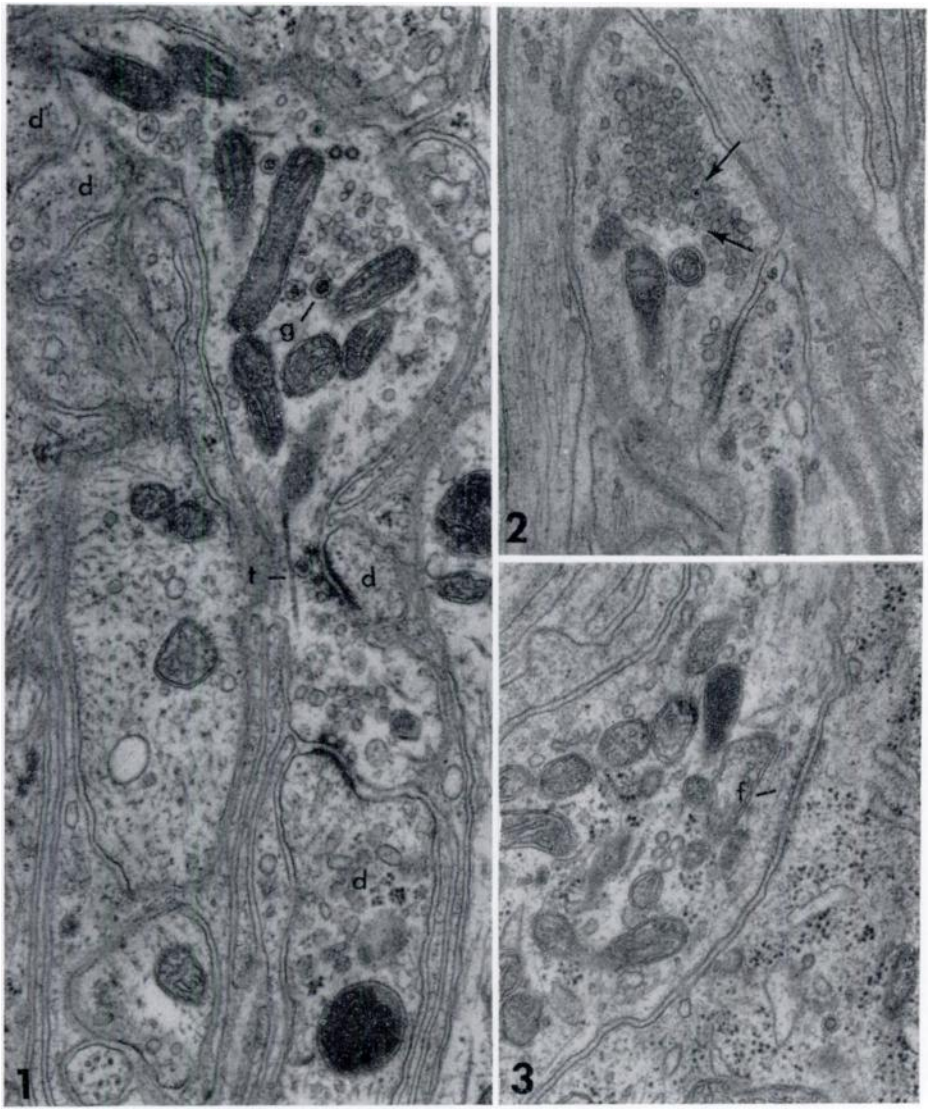
glion cells (87). In mammals a single preganglionic fiber may run for some distance a parallel or winding course around the dendrite, establishing *en route* multiple synaptic contacts at irregular intervals (29, 44). These endings *en passage* are established through local dilations or beaded enlargements containing swarms of vesicles, which may or may not involve an interruption of the longitudinally oriented neurotubules which are such a prominent component of the axoplasm of the terminal neurites (fig. 1). Although several fibers appear to synapse on a given dendrite, their origin from one or more parent axons has not been ascertained. The best data on the maximum length of the synaptic membranes have been obtained in the cat from serial sections through 10 complete synapses (29); they range from 0.6 to 2.1 μ (mean of 1.2 μ) with the specialized membrane thickenings occupying 25 to 50 % of the total surface.

In addition to the agranular vesicles mentioned previously, a variety of other components have been identified in the presynaptic processes, including mitochondria, dense particles about 300 Å in diameter tentatively identified as glycogen on the basis of their staining properties (29, 75, 91, 95, 109), complex or "coated" vesicles which, by analogy with similar structures elsewhere (83), are thought to arise by a pinching off of specialized regions of the surface membrane in a process of micropinocytosis (44), highly opaque granules about 0.2 to 0.4 μ large (29), neurotubules (29) and granule-containing vesicles (29, 42, 75, 87, 91, 95, 99, 109). These sundry cytoplasmic inclusions are usually at some distance from the synaptolemma. Of particular interest are the granulated vesicles about 900 Å large which occur in the ganglionic synapses of most vertebrates. Their concentration per bulb varies, but most processes contain 10 or less in single sections. In the superior cervical ganglion of the rat granulated vesicles were seen in 82 % of a total of 185 synapses, and the complete disappearance of synaptic boutons of this kind 130 hr after transection of the cervical sympathetic trunk supports the interpretation arrived at from the examination of normal material that these are preganglionic fibers (42, 44). The few synapses which resist degeneration display the morphologic characteristics of the residual 18 % of normal synapses, namely, small agranular vesicles in which a minute particle about 150 to 200 Å in diameter is visible occasionally (fig. 2). These resistant fibers, which have been seen on both the soma and small dendrites, are thought to arise from neurons within the ganglion, perhaps recurrent collaterals which function in integrative mechanisms.

Other variations in synaptic morphology involve the membrane thickenings of the pre- and postsynaptic processes. As in the brain (101), these may be absent (29), symmetrical (109), or distinctly wider and denser on the postsynaptic side (29, 44, 95). The last type is by far the most common. A special disposition of these various forms on the surface of the neuron has not yet been detected. Of possible significance in the uptake of macromolecules from the synaptic cleft are inpocketings of the plasma membrane adjacent to the postsynaptic thickening (29, 44, 91). In a minority of amphibian synapses the postsynaptic thickening is associated with one or two dense strata about 300 Å thick which lie parallel to each other and to the plasma membrane at distances of a few hundred Ångstrom

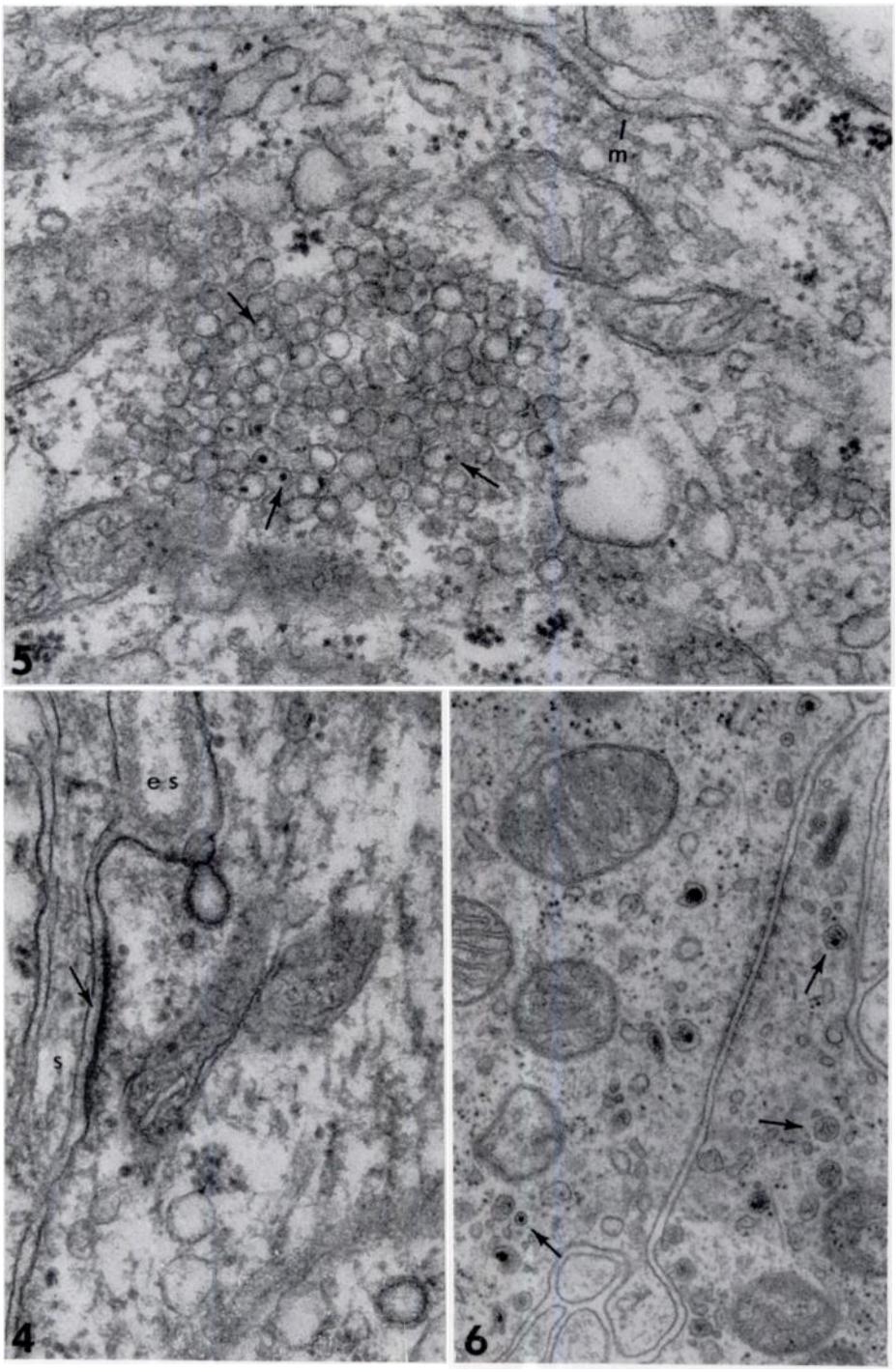


All micrographs are taken from the superior cervical ganglion of the rat.

FIG. 1. A preganglionic fiber in synaptic contact with four dendritic processes (d). The membrane thickenings at the lower two synapses are asymmetrical and distinctly thicker and denser on the postsynaptic side. Numerous mitochondria and agranular and granular (g) vesicles are visible in the axoplasm. In the slender stalk connecting the axon swellings a neurotubule (t) can be seen. (× 34,000)

FIG. 2. An axodendritic synapse which has survived degeneration at 130 hr after preganglionectomy. Dense inclusions (arrows) can be seen in a few synaptic vesicles. (× 34,000)

FIG. 3. Dendro-somatic contact. The membrane thickenings are symmetrical and not immediately associated with any vesicles, though fine filaments (f) are visible in the subjacent cytoplasm on the dendritic side. Note the accumulation of mitochondria in the dendrite. (× 34,000)



FIGS. 4–6

units (91, 94, 95). This subsynaptic band is of particular interest not only because it is one of few known examples of a special structure uniquely associated with the postsynaptic membrane, but also because its morphologic integrity is dependent upon an intact synapse (94, 95). Further studies are needed to determine whether the postsynaptic thickenings which persist in mammals 130 hr after preganglionectomy (fig. 4) will also fragment and disappear later in degeneration.

It has become increasingly apparent in recent years that close contacts between neurons of the central nervous system occur at sites other than the morphologically differentiated regions commonly recognized as synaptic junctions (15, 101). With the exceptions of Elfvin's study on the superior cervical ganglion of the cat (28) and the brief mention by Richardson of an unconventional dendro-somatic contact in the myenteric ganglia of the rabbit (79), the occurrence of such contacts in the autonomic nervous system has not been fully recognized or adequately emphasized. The cell bodies and processes of sympathetic neurons come into direct and mutual contact with each other, sometimes over extensive areas, to establish soma-somatic, dendro-somatic, and dendro-dendritic contacts. Although contacts between the perikarya of adjacent neurons are somewhat rare, the other contacts vie in frequency with the synaptic junctions (28, 44). The contiguous plasma membranes usually show one or more specialized areas of membrane thickenings which are of a distinctly different form from those at the synapse: they are never directly related to aggregates of vesicles and are without exception symmetrical, appearing either as simple bands of opaque material or as a series of dense masses apposed to the surface membranes in an organized pattern (fig. 3). Morphologically identical contacts have been observed in the sphenopalatine ganglion, while the specialized membranes seem to be absent at similar contacts in the ganglia of the adrenal medulla and of Auerbach's and Meissner's plexuses (44). In the enteric ganglia, however, where the glial and neural elements are intermeshed and tightly packed in a pattern (45, 79, 90) which resembles somewhat that seen in the brain and in the developing sympathetic ganglia (77), the perikaryal surfaces are often apposed to each other in broad membrane-to-membrane contacts. Whether the contacting processes arise from a single neuron as well as from separate neurons is unknown. It is interesting to speculate that such contacts, which almost certainly are sites of adhesion and possibly of cell interaction, occur between neurons intimately related to each other topographically and perhaps genetically during development.

FIG. 4. Dendrite with postsynaptic thickening at 130 hr after preganglionectomy. The presynaptic process has disappeared completely and is now replaced by Schwann cell cytoplasm (s), which no longer insulates the synaptic area from the extracellular space (es). Still attached to the postsynaptic thickening are remnants of the "gap substance" (arrow) often visible in the normal synapse. (× 69,000)

FIG. 5. A cluster of small vesicles located in the peripheral neuroplasm of a ganglion cell at some distance from the surface membrane (m). Dense inclusions are visible in some vesicles (arrows). (× 69,000)

FIG. 6. Dendro-somatic contact with symmetrical membrane thickenings. Compare the large mitochondria in these unusual neurons with those in figure 3. Cytoplasmic granules vary in appearances (arrows) and are surrounded by a single membrane. (× 34,000)

Before dealing with the problem of the identification of sympathetic fibers, I should like to summarize briefly the structure and relationships of autonomic nerves in general to the effector cells of the peripheral organs. In the very fine nerve bundles which ramify within the glandular and muscular tissues, a series of varicosities or beaded enlargements, containing clusters of vesicles and mitochondria, appear at irregular intervals along the terminal length of the individual axons, which are enveloped only partially by Schwann cell cytoplasm, and which in their most distal portions may lack a Schwann sheath, even a basement membrane (37, 58, 59, 80, 82, 97). The most striking difference between these terminal axons at the periphery and those in the sympathetic ganglia is their Schwann cell covering; naked axons are totally absent in the ganglia, and the preganglionic fibers, *especially* at their varicosities, are insulated from the surrounding connective tissue by a layer of glial cytoplasm. It is primarily through the beaded enlargements that the axons approximate the surfaces of the effector cells. Reports on the degree of this approximation differ with the organ and the species.

Direct contacts involving a membrane-to-membrane apposition with a gap of only 180 to 250 Å have been observed in smooth [intestine (7, 42, 78, 79, 97, 107, 108), vas deferens (55, 59, 80), intrinsic eye muscles (35, 49, 54, 82), urinary bladder (10)] and cardiac [conducting system (11, 98), striated musculature of the pulmonary vein (50)] muscle and in various exocrine [salivary and lacrimal (84), pancreatic acinar (86)] and endocrine [pancreatic islets (4, 86), adrenal medulla (16, 17, 22, 30)] glands. The areas of contact commonly involve the formation of a groove or depression on the surface of the effector cell. The contacting membranes lack specializations, so that the junctional folds characteristic of the motor endplate of skeletal muscle as well as the membrane thickenings of interneuronal synapses are missing. A noteworthy exception is the junction formed on the adrenal medullary cells, which, by the presence of thickenings at the contacting membranes as well as by the envelopment of the nerve endings by glial cytoplasm, is more closely related to the ganglionic synapse than to the neuroeffector junction; this difference may have its explanation in the fact that the sympathetic neurons and the medullary cells are closely related developmentally, both being derived from the neural crest (16). Through one or more enlargements a single fiber may contact several effector cells (18, 50, 55, 80, 82, 84, 98). On less secure ground is the question of the polyaxonal innervation of a single cell (7, 18, 80, 84, 86).

Indirect contacts only, in which a space of 400 to several thousand Ångstrom units occupied by elements of the connective tissue is interposed between the beaded enlargement and the surface of the effector cell, have been observed in vascular smooth muscle (1, 9, 38, 57, 58, 71, 86, 103, 110) and in various other organs [nictitating membrane (35), frog (40), mouse (55, 56, 64, 92) and guinea pig (45, 88) digestive tract, myocardium (37, 51, 52, 63, 100), uterus (40), rat dilator pupillae (65), and the submandibular (39, 84), sweat (62, 106) and thyroid (8) glands]. The indirect contacts are decidedly more common in all organs studied up to this time. Although few quantitative estimates of the

relative numbers of axons and effector cells have been made (59), it seems unlikely in some tissues that every effector cell can have a close relationship with an axon. The possibility that specialized areas at the membranes of effector cells in contiguity may serve to conduct excitation from one effector cell to another has been suggested by some (25, 26, 35, 68, 80, 82, 92, 96) and challenged by others (59).

The alternative hypotheses of autonomic innervation which have emerged from these studies have been discussed by Richardson (82). The questions raised in the various hypotheses are: Would a more exhaustive search or serial sections reveal direct contacts with the effector cells by each postganglionic fiber? What is the functional interpretation of the partial or complete lack of sheaths around the terminal axons? Is the transmitter substance released at the contact junctions only, or does this release occur from all beaded enlargements without special reference to the anatomical approximation of the effector cell? Does the site of release vary with the chemical transmitter? What is the cytologic localization of acetylcholinesterase?

The recognition of granular as well as agranular vesicles in the peripheral autonomic fibers suggested three interesting possibilities: 1) that the presence of granulated vesicles might be an identifying characteristic of autonomic fibers of different kinds, 2) that the granulated vesicles might be the storage site of a transmitter substance, 3) that the dense inclusion might provide a valuable natural marker for determining with greater certainty than has been possible up to this time the mode of formation of synaptic vesicles.

Unlike the agranular vesicles, which are fairly uniform in appearance, the granular vesicles constitute a heterogenous group in which differences in size, density, and other structural and distributional characteristics are the rule rather than the exception. The most conspicuous differences, though certainly not the only differences, between the two major varieties of granulated vesicle acknowledged by most investigators are their size and their density (6, 42, 59, 82, 88). The larger of the two measures on the average 900 Å and encloses centrally a finely granulated droplet of variable density, which, however, almost never attains the intense osmiophilia of the second kind of granule, which is only half as large. The larger vesicles have been seen in the autonomic nervous system of almost every organism studied to date (18, 29, 42, 46, 47, 50, 59, 75, 82, 87, 88, 95, 97, 99, 107, 109), while the smaller ones have been found only in mammals (6, 13, 23, 24, 35, 42, 55, 57–59, 61, 63, 80, 82, 88, 102, 103, 110). The granulated vesicles of many fibers appear to consist of one kind or the other, and they occur above all in the terminal parts of the axons in association with a variable number of agranular vesicles, 200 to 600 Å in size. In the axoplasm of the larger nerves located both within (42, 80, 82, 88) and outside the peripheral organs (27, 42, 74), they are scattered either singly or in small clusters or rows among the filaments and mitochondria. Fibers with dense-cored vesicles may also contain a few larger vesicles with a dense inclusion identical to that described above or somewhat different in its detailed morphology (42, 58, 59, 80, 82).

Small nerves in the peripheral organs are composed of a spectrum of fibers:

sympathetic and parasympathetic, excitatory and inhibitory, afferent and efferent. The lack of precise information on the distribution of these different fibers within the organ has made their identification at the level of the electron microscope little more than an educated guess. This topographical problem was circumvented to some extent by Richardson (82), who studied the innervation of the dilator and sphincter pupillae of the rabbit iris, organs which have the physiologic and the pharmacologic parameters of a sympathetic and parasympathetic innervation, respectively. The finding of distinct morphologic differences between the two sets of fibers led him to conclude that fibers containing dense-cored vesicles typify an adrenergic innervation while those with agranular vesicles only and perhaps with agranular vesicles mixed with a few of the large granulated vesicles are characteristic of a cholinergic innervation.

This identification of adrenergic fibers receives support from a number of observations and experiments. Fibers with dense-cored vesicles occur frequently in tissues with a rich sympathetic innervation (35, 57, 61, 80), and in some instances their degeneration has been observed after sympathetic denervation (44, 73). Reserpine and guanethidine caused a marked reduction in the number of dense-cored vesicles in the preterminal and terminal axons of the rat pineal (13, 72, 73) and the vas deferens (13, 81). This depletion was largely prevented by the monoamine oxidase (MAO) inhibitor iproniazid (13, 73), which by itself resembled pyrogallol (73) in having a slight or no effect on the morphology of the granules. The number of granulated vesicles and the levels of norepinephrine (NE) measured chemically in the rat vas deferens were in good agreement up to 2 weeks after a single dose (5 mg/kg) of reserpine (81). The administration of dopa or dopamine, but not of 5-hydroxytryptophan, increased the number of granulated vesicles in the pineal fibers (73). In an ingenious application of electron microscopical autoradiography Wolfe *et al.* (102, 103) demonstrated a specific localization of H^3-NE in the region of granulated vesicles contained within axons of the rat pineal and atrial myocardium. Subsequent studies with flourescence microscopy in combination with fluorimetric determinations of 5-hydroxytryptamine (5-HT) levels under normal and experimental conditions have shown that the terminal, but not the proximal regions of the sympathetic nerves of the rat's pineal body are unique in containing not only NE but also 5-HT, which they apparently take up from the adjacent parenchymal cells (5, 69). No morphologic differences have been reported between the pineal fibers (13, 24, 61, 102) and the fibers tentatively identified as adrenergic elsewhere.

While this evidence is consistent with the proposed identification of adrenergic fibers, other studies on the guinea pig vas deferens (59) and on the dilator muscle of the rat iris (47, 65), which are known to have a rich sympathetic innervation (36), have failed to show impressive numbers of granule-containing axons. Although the explanation could lie in the techniques of tissue preparation, which may differ in their requirements with the species as well as with the organ, clearly it would be premature to assume that axons with only agranular vesicles are necessarily cholinergic. The only site where dense-cored vesicles have been found (44) and where fluorescence microscopy has failed to demonstrate adrenergic

fibers (66) is in occasional axosomatic synapses in the ganglia of Meissner's plexus.

The terminal axons in the sphincter pupillae (82), in the adrenal medulla (16, 18, 42), and in several sympathetic (29, 42, 75, 87, 95, 99) and parasympathetic (42, 87) ganglia are qualitatively alike with regard to their content of synaptic vesicles. However, the relative number of granule-containing axons in these tissues differs widely. As mentioned previously, the minimal estimate in the rat's superior cervical ganglion is 82%, while most bulbs in the sphincter pupillae (35, 47, 82) contain only the usual synaptic vesicles and resemble therein the nerve endings at the motor endplate of skeletal muscle. There is at present no explanation for the anatomical differences in these fibers, which are commonly presumed to be cholinergic. The application of recently developed techniques for the localization of cholinesterase activity with the electron microscope (2, 53) could help in the further identification of autonomic fibers, provided that the cytologic preservation, particularly of all vesicular components, is acceptable.

With regard to the chemical nature of the different kinds of synaptic vesicle found in autonomic nerves, only the most common of which have been dealt with here (27, 42, 107), the cytophysiologic and autoradiographic evidence cited previously in relation to the identification of adrenergic fibers suggests that the small dense-cored vesicles themselves contained catecholamine, probably NE. There is no such evidence, however, for the larger granule-containing vesicles as found typically in the sympathetic and parasympathetic ganglia and in the adrenal medulla. Since the nerve granules in the adrenal medulla stain differently from the presumed NE-containing granules of the chromaffin cells and since preganglionectomy has no visible effect on the NE content of the rat's superior cervical ganglion as determined by fluorescence microscopy (32, 67), these granules probably do not contain a primary monoamine; the presence of epinephrine, however, cannot be excluded (18). Catecholamine storage particles isolated from homogenates of bovine splenic nerve (33) and rat heart tissue (34, 41, 60) not only contain a mixture of agranular and granular vesicles, but the granular vesicles themselves are also mixed in most preparations. This topic will be discussed elsewhere in this symposium and will not be considered here.

Of the various organelles and numerous inclusions found in sympathetic neurons (20, 28, 43, 48, 70, 75, 76, 85, 93, 109), of particular interest to the present discussion are those structures related, or possibly related, to the question of a perikaryal origin of synaptic vesicles and to the identification of cholinergic and adrenergic neurons. Most neurons lack granules or vesicles which can be related on morphologic grounds alone to those seen in the synaptic boutons. In the perikarya (44) and processes (28, 44) of some cells, however, are found multiple aggregates of clear vesicles about 500 Å in diameter located preferentially in the peripheral neuroplasm, usually at some distance from the plasma membrane. A clear relationship between these vesicles and other membranous structures of the cell has not been observed. A small fraction of the vesicles forming a cluster may contain a small dense particle 100 to 200 Å in size (fig. 5). Sparsely dispersed in the cytoplasm of other processes (28) and cells (19, 42, 95), partic-

ularly in the region of the Golgi membranes, are moderately dense granules enclosed in vesicles 60 to 150 mμ large. The similarities between these perikaryal vesicles and those in the synaptic boutons is obvious. Less obvious are the differences: the dense inclusions found centrally often differ slightly in size or density from those encountered in many synapses. However, the variation in morphology at the periphery [which may be related to a physiologic cycle of activity (6, 46, 88)] is sufficiently great to include forms identical to those found in the cell bodies. The hypothesis that the granular vesicles are synthesized in the cell body and transported along the axon to the terminal swellings is supported by these observations, by the peculiarities of their distribution in other parts of the neuron (27, 42, 74) and by their piling up at the cut ends of sympathetic nerves (44).

While the cells mentioned above are unquestionably neurons, other cells in the superior cervical ganglion of the rat have the morphologic characteristics of both neurons and chromaffin cells (fig. 6). Although they contain numerous polymorphic membrane-limited granules which resemble closely, except in size, the adrenal medullary granules of the same species (14, 17, 30, 31, 104), their numerous long processes, which are encapsulated by Schwann cell cytoplasm, and the nature of the contacts they establish with cells of their own kind and with "typical" neurons (which resemble in all respects the symmetrical contacts found between unequivocal sympathetic nerve cells) indicate that they are true neurons. Typical synapses formed by preganglionic fibers have been seen on their cell bodies. An identity between these cells and the intensely fluorescent neurons described by Norberg and Hamberger (67) seems probable on the basis of their rarity, their occurrence in groups, and their size, which is somewhat less than that of the average ganglion cell. Conclusive morphologic evidence for their neuronal nature must await the clear demonstration of their granules in synaptic boutons, which, of course, could be expected to terminate on other neurons, on effector cells, or on capillaries. Should the last possibility obtain, then the already vague boundaries between the neural and glandular nature of sympathetic tissues would become imperceptible.

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