THE FINE STRUCTURE OF INHIBITORY SYNAPSES IN THE CRAYFISH

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ABSTRACT

Physiological investigations have shown that the synaptic input to the sensory neuron of the stretch receptor in the abdominal muscles of the crayfish is purely inhibitory. This neuron was chosen, therefore, as a site in which to study the fine structure of inhibitory synaptic endings. It was hoped that this fine structure might (*a*) provide a morphological prototype for the study of more complex synaptic systems and (*b*) reflect the inhibitory mechanisms. Stretch receptors were fixed *in situ* in buffered OsO_4, dehydrated, and embedded in Araldite. Both cross and longitudinal sections were examined after staining with phosphotungstic acid. The inhibitory endings were easily identified by their great similarity to previously described excitatory endings. Small circular profiles (synaptic vesicles) about 460 A in diameter and an accumulation of mitochondria were consistently observed within the presynaptic endings. An increased osmiophilia of pre- and postsynaptic membranes, where they were in apposition, was also seen. The only observed difference between these inhibitory endings and excitatory endings, described by other authors, was the variable presence of a latticework of 230 A tubules in the connective tissue immediately adjacent to the inhibitory endings. Inhibitory endings were observed on all parts of the sensory neuron except the axon.

INTRODUCTION

In recent years many synapses have been examined and characterized. These include central nervous system (17), peripheral (2, 4, 18), and ganglionic (7–9) synapses. In all cases known to the author three features have come to characterize the presynaptic terminal. These are (*a*) small circular profiles (300 to 600 A in diameter) or synaptic vesicles, (*b*) an increased osmiophilia of the pre- and postsynaptic membranes, and (*c*) an accumulation of mitochondria.

In all the previous electron microscope studies of synaptic morphology, little consideration of the physiological effect of the ending has been taken into account, *i.e.*, whether its function is to inhibit or excite the postsynaptic cell. In some cases, such as the vertebrate motor end-plate, it is known that there is no direct inhibition and that the synaptic action is excitatory. On the other hand, the central nervous system exhibits both facilitation and inhibition, and, therefore, the action of a particular synapse would be unknown.

The purpose of this investigation was, therefore, to explore the morphology of a known inhibitory synaptic ending. The stretch receptor in the abdominal musles of the crayfish was chosen as material. An investigation of this kind might be expected to provide a prototype of the fine structure of the inhibitory synapse for the investigation of more complicated synapses such as those in the central nervous system. Also it was hoped that this approach might yield some information regarding inhibitory synaptic mechanisms. The

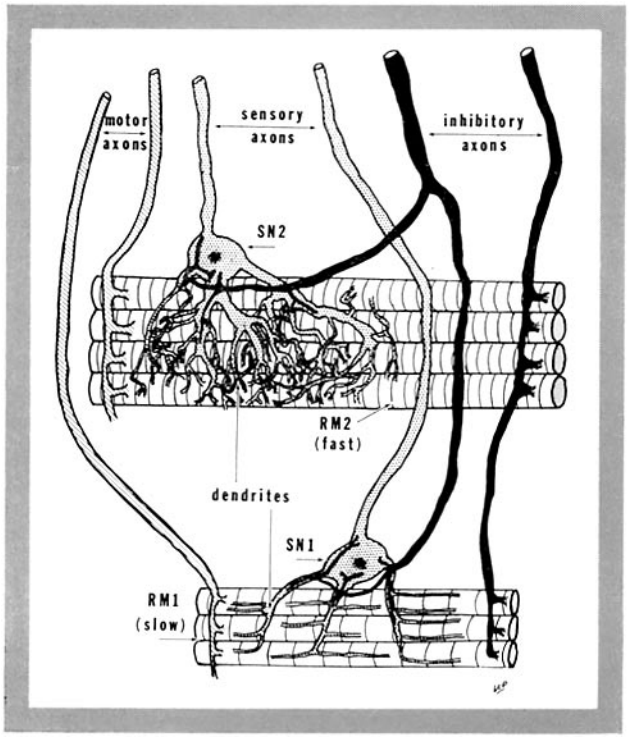


FIGURE 1
A diagram of the paired crayfish stretch receptors. See text for description.

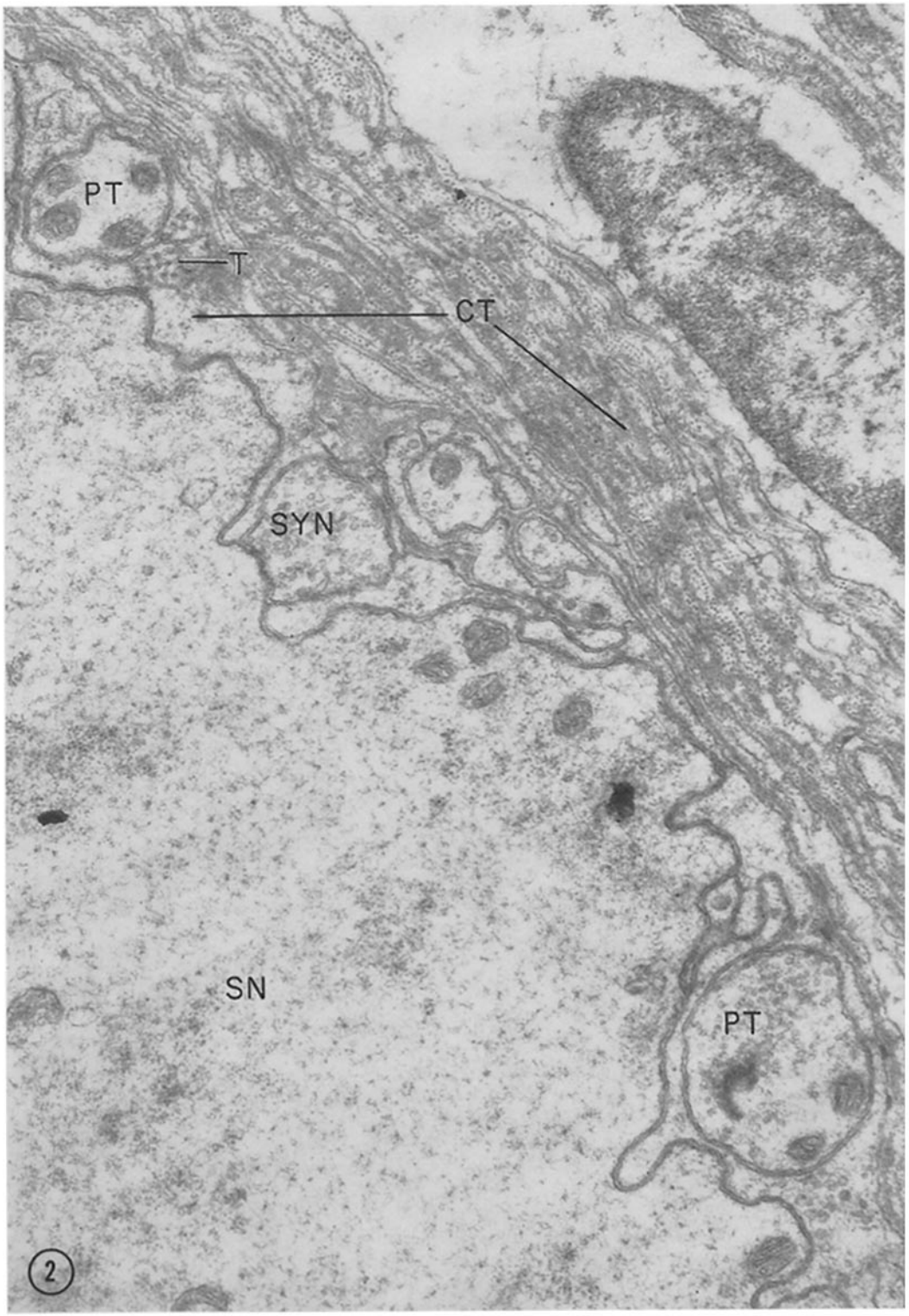
latter aim was based on the recently hypothesized relationship of synaptic vesicles to acetylcholine transmission (5, 6, 9, 19).

METHODS

The crayfish used in this study, *Orconectes virilis*, were obtained from Wisconsin in the summer and fall. Animals that had been left in the refrigerator overnight to cool, were dissected and selected tissues were fixed as follows. The abdomen was detached and the tergum removed by cutting it away at the dorsolateral corner on both sides. Usually the ventral musculature and gut were stripped off as the tergum was removed. This left the dorsal musculature (including the stretch receptors) exposed and *in situ* on the tergum. The tergum was then clamped into a special dissecting chamber (chamber, instruments, and fluids were all ice cold) and flooded with Van Harreveld's solution. The stretch receptors were dissected free of connective tissue, and the Van Harreveld's solution replaced with fixative. The chamber was placed on ice for the remainder of the fixation period (1 to 1½ hours), the fixing fluid being replaced with fresh fixative every 15 to 20 minutes. At the end of the fixing period, the receptors were stiff enough to dissect out without loss of shape. They were then dehydrated through increasing concentrations of ethanol and embedded. The fixative used was 1.5 per cent osmium tetroxide buffered to pH 7.4 with s-collidine (3). This gave as good, if not better, preservation when prepared in distilled water, than when various concentrations of sucrose were added. The embedding material was Araldite epoxy resin. The embedding was usually done in polyethylene molds. This made it easier to orient the receptors for sectioning than when they were embedded in gelatin capsules.

FIGURE 2

A micrograph of an area near the border of a sensory neuron (*SN*). The inhibitory endings (*SYN*) are seen in the first layer of connective tissue (*CT*). Preterminal (*PT*) endings are also present. In the upper left portion of the micrograph are seen parts of the 230 A tubular lattice system (*T*). At the upper right is a connective tissue cell nucleus. × 22,500.


PT
T
CT
SYN
SN
PT
2

Gold, silver, and gray sections were cut on a Porter-Blum microtome, using glass knives, and were examined with a Hitachi HS-6 electron microscope. Routinely sections were stained in a 1 per cent solution of phosphotungstic acid (PTA) in absolute ethanol. After immersing the grid for 1 to 2 minutes, it was washed for several minutes in two changes of absolute ethanol. In some cases a thin layer of carbon was then evaporated on top of the grid. No substrate, formvar or collodion, was used on the grids.

RESULTS

The Stretch Receptor

With the light microscope, the stretch receptor has been studied in lobsters by Alexandrowicz (1) and in the crayfish by Florey and Florey (13). In each abdominal segment of these animals there are four receptors, two on each side of the body. For purposes of orientation a brief description of the stretch receptors is included here. Fig. 1 illustrates the main structures of the paired receptors. Each receptor consists of several muscle fibers (*RM*) and a large sensory neuron (*SN*). The dendrites of the sensory neuron come off the cell body in three to five main trunks which then divide into branches and finally end in numerous, very small twig-like processes (not shown). These dendritic processes are heavily covered with connective tissue which in turn is embedded between the muscle fibers. The axons innervating the receptors provide inhibitory innervation to the sensory neuron cell body and dendrites, inhibitory innervation to the muscle fibers, and motor innervation to the muscle fibers. Also illustrated are the two sensory axons which run centrally from the sensory neurons.

The paired receptors differ from each other both physiologically and morphologically. As indicated in Fig. 1, one receptor organ has fewer muscle fibers which are smaller and of greater sarcomere length, and are physiologically "slow" (*RM* 1). The sensory neuron (*SN* 1) associated with *RM* 1 is slow-adapting and its dendritic branches show a definite orientation parallel to the muscle fibers. The other receptor organ has more muscle fibers (*RM* 2) which are larger in diameter and more finely striated. These muscle fibers give a fast or more "twitch-like" response to stimulation. The dendritic processes of the sensory neuron (*SN* 2) associated with *RM* 2 do not show the well defined orientation parallel to the muscle fibers. *SN* 2 adapts rapidly to stimulation.

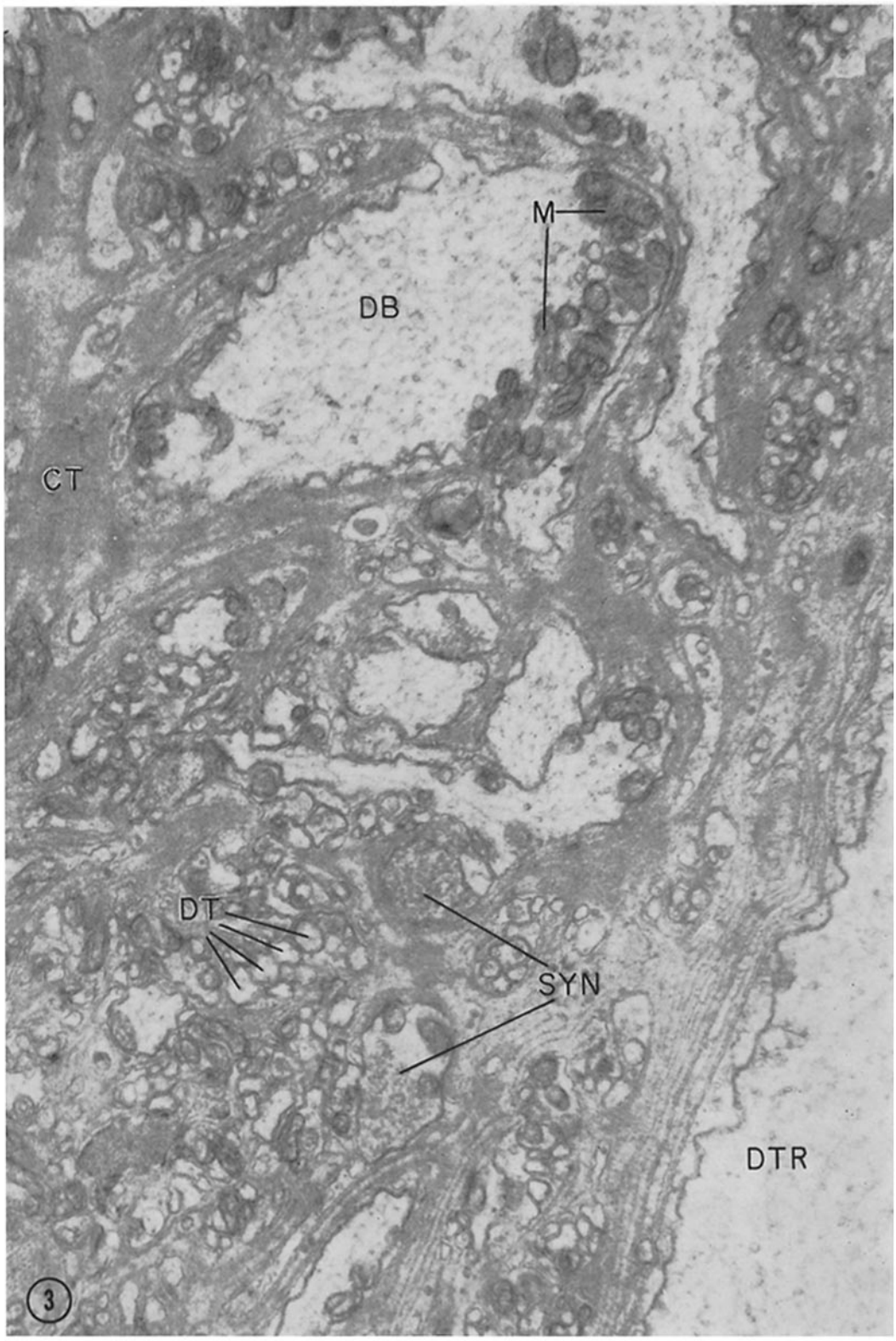
Muscle stretch or contraction produces a mechanical distortion of the dendritic branches which in turn sets up a generator potential. This is conducted to a point several hundred microns along the axon, where a propagated impulse is initiated (12). Repeated physiological investigations (11, 14, 15) have demonstrated that the synaptic input to the sensory neuron is purely inhibitory, and it is for this reason that this cell was chosen as a site in which to study inhibitory synaptic morphology.

Location and Distribution of Endings

The inhibitory endings are located in the first of several layers of connective tissue surrounding the sensory neurons and their dendrites. The endings appear as simple oval-shaped membrane-bounded areas (Figs. 2 to 5). They are invaginated into the connective tissue cell much as so-called unmyelinated fibers are invaginated into Schwann cells in other animals. What might correspond to a "mesaxon" is often apparent, usually forming complicated double-membraned figures. Often processes can be seen (embedded in the connective tissue cells) which may or may not contain mitochondria and vesicles and which are not in apposition to the sensory neuron. From their appearance it may be assumed that these processes are terminal fibers just short of the synaptic ending; they will be referred to as preterminal fibers (Fig. 2, *PT*). From the presence of the preterminal fibers and from the fact that usually the endings occur in groups of two to four along the sensory neuron, it is concluded

FIGURE 3

A micrograph of an area near one of the large dendritic trunks (*DTR*). A dendritic branch (*DB*) and dendritic twigs (*DT*) are indicated. The inhibitory endings shown (*SYN*) are innervating the dendritic twigs. Note the connective tissue (*CT*) in which the whole system is embedded. *M*, mitochondria. × 18,000.


M
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DTR
3

that the inhibitory branch probably divides just before terminating into several smaller twigs which form the synaptic endings. The short dimension of these ovoid endings rarely exceeds 2 microns, the long dimension usually ranging from 2 to 4 microns. If the ending consisted of a fiber in contact with the neuron over a long distance, its long dimension of 4 microns should occasionally be exceeded when the plane of section is parallel to the direction of the fiber. Since this never occurs, it is assumed that the ending is of a simple end-foot type. Serial sections support this conclusion. In fact the actual distance over which the pre- and postsynaptic membranes are in apposition is rarely more than 1 micron. The distance across the synaptic cleft varies from 200 to 250 A.

An idea of the distribution of the inhibitory endings on the neuron was gained from cross and longitudinal sections through the whole stretch receptor. The sensory neuron may be divided into several synaptic fields for ease of description: (*a*) the cell body, (*b*) the axon hillock and initial parts of the sensory axon, (*c*) the large dendritic trunks, (*d*) the dendritic branches, and (*e*) the dendritic twigs of very small diameter (Figs. 3 and 5). The last three categories are rather clearly defined in observed sections. The large dendritic trunks apparently divide into several large branches, each of which gives rise to many very slender (0.05 to 0.3 micron in diameter), long dendritic processes. That the small twigs arise from dendrites was confirmed when transition zones were observed between dendritic branches and twigs. The small twigs usually parallel one another, and the muscle fibers, in groups of processes. These are embedded in connective tissue between the muscle fibers.

The inhibitory endings are about equally distributed in all five of these synaptic areas. However, there is probably a greater number of endings, per unit area of postsynaptic membrane, on the cell body than on the dendritic twigs, since several twigs may synapse with one ending. There was no notable difference between inhibitory endings on the "slow" (*SN* 1) and "fast" (*SN* 2) cell bodies.

Morphology of Inhibitory Endings

The identification of the above mentioned membrane-bounded areas as synaptic endings rests on several features: (*a*) They are the only irregularities seen in the connective tissue layer around both the cell body and the dendrites; (*b*) they have an accumulation of the characteristic circular profiles or vesicles; (*c*) they show an accumulation of mitochondria; and (*d*) they reveal an increased osmiophilia which is variably present at the opposing membranes. The supposition that they are inhibitory endings is based on physiological data which indicate that the only endings on this neuron are inhibitory.

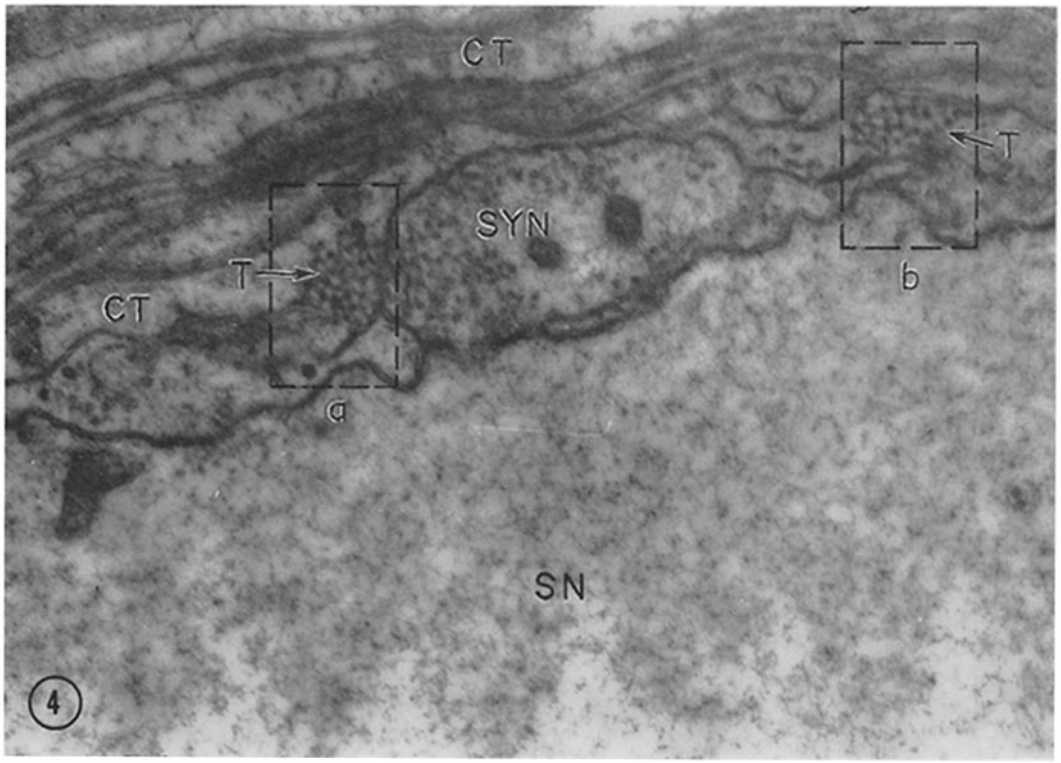
The circular profiles in the synaptic endings are 420 to 500 A in diameter. They appear to be within the same size range as the profiles reported by other authors for other endings, but a slight variation is probably to be expected both from changes during preparation and from differences in the physiological history of the endings (9). Whether these profiles represent tubules or vesicles is probably a moot point, but none of the profiles seen in the material reported here have

FIGURE 4

A single inhibitory ending (*SYN*) on a sensory neurone (*SN*). *CT*, connective tissue. The lattice-like arrangement of the 230 A tubules (*T*) may be seen here, and at higher magnification in Figs. 4 *a* and 4 *b*. Synaptic vesicles and mitochondria are present in the ending. × 26,000.

FIGURES 4 *a* AND 4 *b*

Higher magnification (× 95,000) micrographs of the tubular elements in areas *a* and *b* in Fig. 4. That these are tubules, as distinct from vesicles, may be seen in both figures. This fact and the difference in diameter preclude any direct relationship of the tubular lattice to synaptic vesicles. The lattice-like arrangement of the tubules is seen more clearly in Fig. 4 *b*, where the plane of section roughly parallels the plane of the lattice, whereas in Fig. 4 *a* it is oblique to the plane of the lattice.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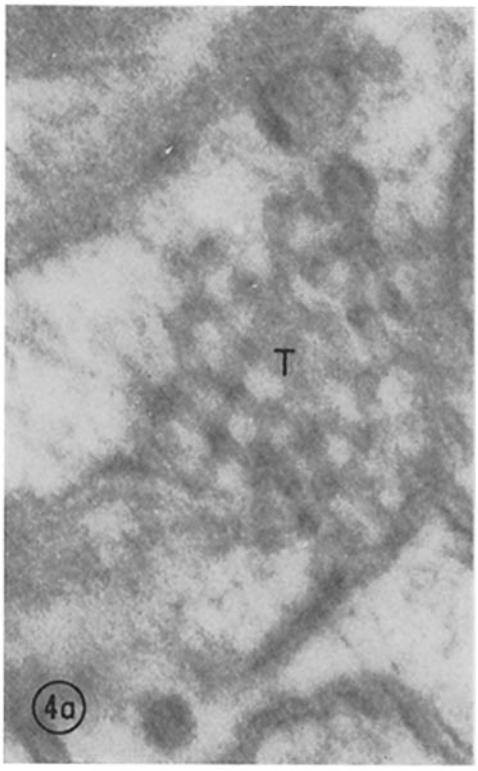
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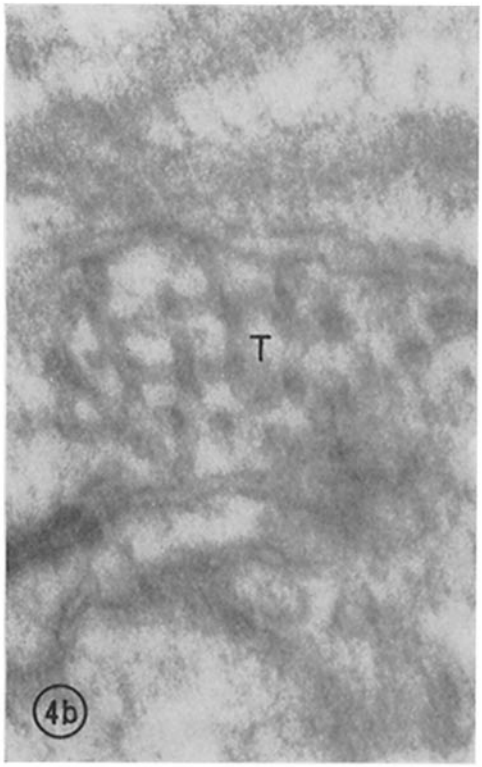
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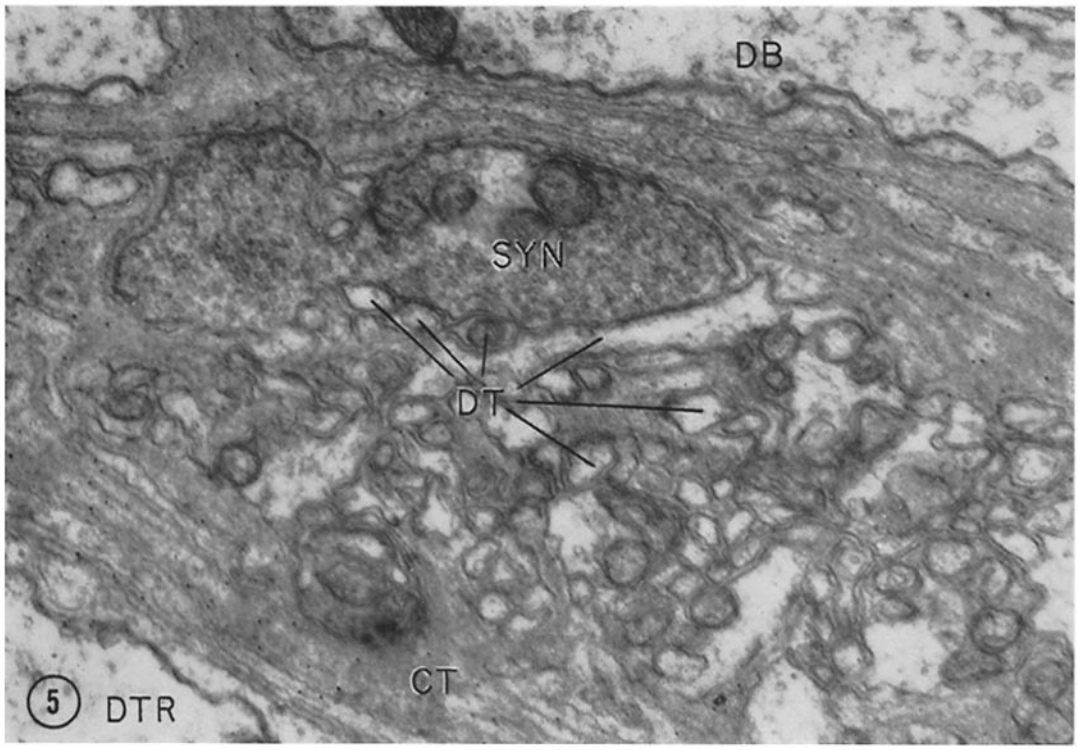


FIGURE 5

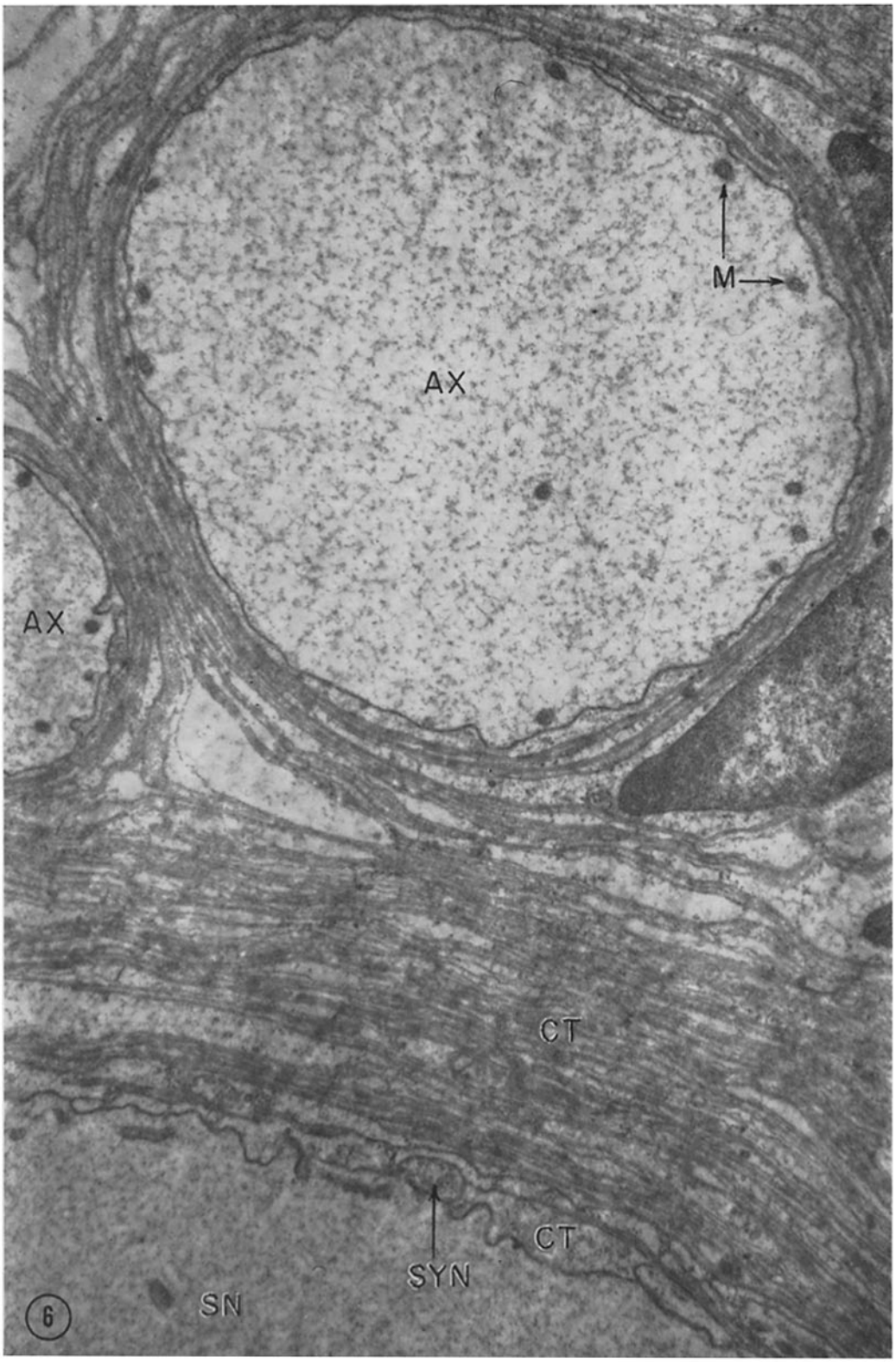
An electron micrograph of an area bordering one of the large dendritic trunks (*DTR*). Also indicated is a dendritic branch (*DB*) and numerous small dendritic twigs (*DT*). An inhibitory ending innervating several of these twigs is indicated (*SYN*). × 30,500.

had a tubular appearance, even in relatively thick sections. The distribution of vesicles is variable from ending to ending and also within the same ending. The numerical density is always higher in an ending which is clearly in apposition to the postsynaptic membrane. As has been described in other endings, there is usually an increased number of vesicles in that part of the ending where the increased osmiophilia is also present. The number of mitochondria in the synaptic ending varies from none to four.

These inhibitory endings differ from other endings, described by other investigators, in having in their proximity what might be described as a tubular lattice (*T* in Figs. 2 and 4). The lattice consists of an accumulation of tubular elements of quite constant diameter of about 230 A, located in the cytoplasm of the cells in the first layer of connective tissue in which the endings are embedded, not within the synaptic endings. They have been observed only in the immediate vicinity of the endings or preterminal processes, never elsewhere in the first connective tissue layer. It is because of this characteristic location that they are thought to be in some way associated with the synaptic ending. The tubules are often

FIGURE 6

A micrograph showing two axons (*AX*) near the border of a sensory neuron (*SN*). In the large axon may be seen neurofibrils and mitochondria (*M*). Outside the axolemma may be seen several layers of Schwann cell with the Schwann cell nucleus at the far right. Around the sensory neuron the alternating layers of connective tissue cytoplasm and connective tissue filaments (*CT*) may be seen. An inhibitory ending is indicated (*SYN*). × 14,400.


M
AX
AX
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SYN
SN
6

very close to the presynaptic terminal membrane and in some cases actually appear to be in contact with it. They have been observed on all sides of the ending except between the ending and the postsynaptic neuron.

When seen in relatively thick sections, these tubules appear to be woven together into a three-dimensional lattice or meshwork (Fig. 4 *b*). In some cases this organization is well developed, whereas in others it is not clear. The definite tubular, as opposed to vesicular, form of these elements, plus the difference in diameter, excludes any direct relationship between the tubular lattice and the synaptic vesicles.

The tubules do not form a complete sleeve around the presynaptic terminal, but seem to occur in somewhat isolated groups. Perhaps in three dimensions they are completely connected around the endings, but this has not been observed. The tubules are not always present. They are seen near most endings on the cell body, but less frequently near endings on the dendritic branches. They have been observed in some instances to continue through serial sections for several microns.

Morphology of Axons

No consistent difference was noted between inhibitory axons and excitatory axons. Only one type of axon, among all axons seen, including those immediately adjacent to the sensory neuron cell body, was evident in this material (Fig. 6). All axons, both near to and far away from the cell soma, consist of an axolemma surrounding axoplasm which contains filaments and usually a ring of mitochondria just beneath the axolemma. The axon is surrounded by a Schwann cell and several layers of double membranes. These membranes probably represent a mesaxon, indicative of a wrapping of the Schwann cell around the axon, but in most cases this was not clear enough to follow from the axon to the outside.

Morphology of Muscle Innervation

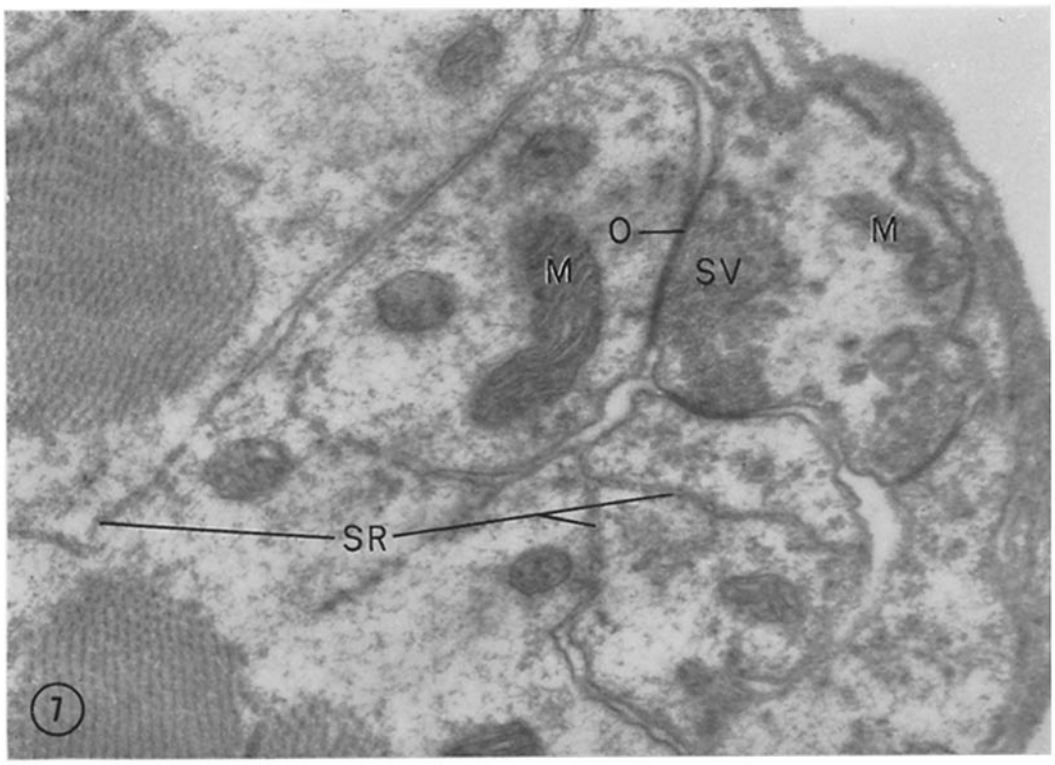
In the Introduction it was mentioned that the muscle portions of the stretch receptor have both excitatory and inhibitory innervation. It was hoped that knowledge of the morphology of inhibitory endings on the sensory neuron might provide some identifying feature which could then be used to differentiate between excitatory and inhibitory endings on the muscle. Since, as described above, inhibitory endings in this system seem to have no identifiable characteristics (except perhaps the tubular lattice in the neighboring connective tissue cell), there was no way of distinguishing between these two types of endings on the muscle. In agreement with this is the fact that only one morphological type of neuromuscular junction was observed.

According to the description of Florey and Florey (13), the muscle innervation is derived from one large axon and several smaller axons. The one large (7 to 10 microns) axon runs toward the cell body of *SN* 2, where it bifurcates, sending its branches in opposite directions along *RM* 2 (Fig. 1). This axon probably provides the sole motor innervation of *RM* 2. *RM* 1 is innervated by several of the smaller (1 to 3 microns) axons all along its surface. The smaller branches of these innervating fibers have also been shown clearly by Maynard and Maynard (16), using a histochemical method for detecting cholinesterase. A picture somewhat in contrast to this innervation pattern is provided by the electron microscope. *RM* 2 (the muscle innervated by a single large axon) shows many more end-plates than *RM* 1 (which is innervated by several smaller fibers). Estimates indicate that there are considerably more neuromuscular junctions on *RM* 2 than on *RM* 1. The endings on *RM* 2 are essentially the same as those on *RM* 1.

The presynaptic nerve terminals (Figs. 7 to 9) may be either superficially applied to the muscle fiber or located in a deep trough in the muscle.

FIGURES 7, 8 AND 9

Electron micrographs of three different neuromuscular junctions. The characteristic synaptic vesicles (*SV*), mitochondria (*M*), and increased osmiophilia (*O*) are indicated at the nerve terminal. These micrographs show that subsynaptic folds are absent. However, deep infoldings of the sarcolemma are present which, it is suggested, are continuous with the sarcoplasmic reticulum (*SR*). Figs. 7 and 8, × 28,000. Figs. 9, × 22,500.


O
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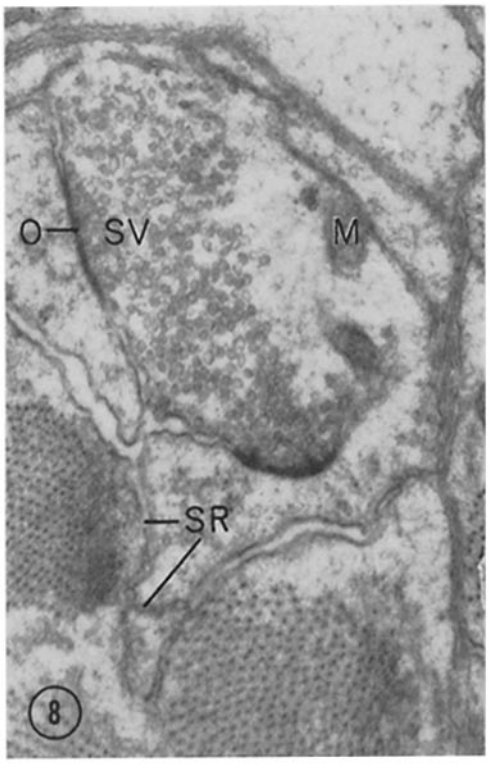
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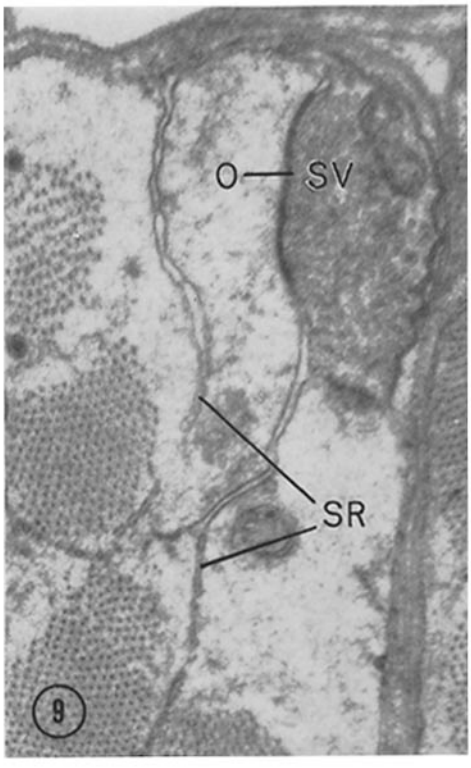
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In some cases they appear to be almost embedded in or incompletely enveloped by the muscle fiber. However, they are not hypolemmal. Sometimes two or three endings may be seen to be near one another on the same fiber in a section. This appearance suggests that probably the final preterminal branch ends in several separate endings. The connective tissue which covers the nerve ending is seen to be continuous with that which covers the rest of the muscle fiber, *i.e.*, there is no special Schwann cell continuation from the axon which covers this part of the muscle fiber.

The endings themselves contain the usual mitochondria, circular profiles, and often an increased osmiophilia or darkening of the synaptic membranes. The vesicles at the neuromuscular junctions seem to be somewhat larger than those observed in the endings on the sensory neurons. At the nerve-muscle junctions the vesicles average about 500 A in diameter. Also there usually appear to be more of them, per unit area, at the neuromuscular junctions than at the inhibitory synapses on the sensory neuron. Again the vesicles are usually most heavily congregated in areas near the increased osmiophilia of the synaptic membranes. The sarcolemma opposite the nerve ending usually shows deep sheet-like infoldings which are continuous with the sarcoplasmic reticulum of the muscle fiber. In addition to other differences, these infoldings are neither so numerous nor so regular as the subsynaptic folds which have been described at other motor endplates. However, in the crayfish they are considerably deeper. This difference in appearance does not exclude the possibility that they are in some way analogous structures.

The 230 A tubular latticework, mentioned above in connection with the inhibitory endings on the sensory neuron, was not observed at the neuromuscular junction.

DISCUSSION

The inhibitory synaptic endings described in this paper are morphologically essentially the same as excitatory endings previously described by other authors. The two types of endings are similar with respect to (*a*) the size and configuration of the terminal ending, (*b*) the presence of synaptic vesicles within the presynaptic terminal, (*c*) the accumulation of mitochondria, and (*d*) the increased osmiophilia of the synaptic membranes. The only differences noted are the specializations of the connective tissue (or Schwann cell) layer surrounding the sensory neuron: (*a*) the manner in which the presynaptic terminal is embedded in the connective tissue layer, and (*b*) the variable existence of what might be called a tubular lattice.

The synaptic vesicles seen previously at excitatory synapses have recently come to be correlated with chemical transmission (5, 6, 9). The hypothesis, briefly, is that the synaptic vesicles contain acetylcholine or some chemical transmitter, and that when an impulse arrives at the presynaptic terminal it triggers the movement of a group of these vesicles toward the presynaptic membrane and the release of their contents either inside or outside this membrane. All that may be derived from the present work is that it is an interesting coincidence that vesicles supposedly containing acetylcholine have the same morphological characteristics as those at a known non-cholinergic inhibitory junction. At present the substance which most effectively mimics the action of inhibitory stimulation to the stretch receptor, and which is found normally occurring in this organ, is gamma-aminobutyric acid (10).

The fact that most of the inhibitory endings appear on the cell body and dendritic trunks of the sensory neuron at first seems surprising, in view of the light microscopic description of the accessory fiber branching at the base of the dendrites (13). This may be explained by the fact that inhibitory branches travel back around the cell body between the connective tissue layers. Because of the extensive distribution of synaptic endings on the sensory neuron, there is no need to assume that inhibitory electrical events, as recorded in the cell body, are particularly diminished or attenuated (15). The inhibitory potential changes recorded in the cell body are probably very close to the true potential changes. In this regard, it was thought that it might be of interest to estimate the total percentage of postsynaptic membrane in contact with the inhibitory endings. Because of the wide variability in the number of endings in various sections, this proved to be quite difficult. All that may be said is that certainly much less than 1 per cent of the total membrane of the sensory neuron (exclusive of the axon) is in apposition to the presynaptic inhibitory terminals. By contrast, for the Mauthner cell of *Amblystoma* larvae, a large association neuron in the medulla of these animals, the compa-

rable figure would be in excess of 95 per cent, both excitatory and inhibitory endings being involved (Peterson, unpublished observations).

This investigation was supported by United States Public Health Service grant 2G-281.

Received for publication, December 29, 1960.

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