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Richard A. Everett for the Master of Science

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Title: PERISYMPATHETIC NEUROSECRETION IN THE AMERICAN COCKROACH: AN ELECTRON MICROSCOPIC STUDY

Abstract approved: Katherine N. Swallow

An electron microscope study of the ultrastructural changes due to dehydration and starvation in the cockroach abdominal perisympathetic organs was conducted. The main purpose of the study was to determine the mechanisms of release of the neurosecretory product stored in the perisympathetic organs.

The perisympathetic organ's general profile reveals three types of axon endings containing three mutually exclusive types of neurosecretion. Type I contains large classical electron-dense granules. Type II contains small electron-dense granules and electron-translucent vesicles. Type III contains only intermediately sized electron-translucent vesicles.

Stressing the cockroach by dehydration and starvation causes ultrastructural changes in Type II terminals. During normal conditions and early stages of stress, the mechanism of neurosecretory release is exocytosis. As stress continues, the preferred mechanism of neurosecretory release shifts to molecular dispersion. These conclusions are based on changes

in the granule to vesicle ratio, changes in the frequency of release sites, paling of granules, and variations in the distribution of microvesicles. When nutrient reserves are apparently dissipated, autolytic activity begins and is denoted by the presence of multivesicular and multilamellar bodies.

A secondary purpose of this study was to describe previously undescribed phenomena in the fine structure supportive of the neurohemal function of the perisympathetic organs. The perineurium, a barrier to large diffusing molecules, is missing. The glial structural integrity is maintained by desmosomes and septate desmosomes which allow neurohormone to pass freely through mesaxons and glial extracellular spaces. No tight junctions, which restrict the passage of large diffusable molecules, are found. The neurosecretory release structures referred to as "synaptic sites" and omega-shaped profiles are present. The tracheal supply is sparse which would be expected in tissue where the main function is storage and release of neurohormone.

PERISYMPATHETIC NEUROSECRETION IN THE AMERICAN COCKROACH: AN ELECTRON MICROSCOPIC STUDY

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In Partial Fulfillment
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by
Richard A. Everett
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Katherine N. Snalley
Approved for Major Department

Harold E. Wurst
Approved for Graduate Council

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INTRODUCTION

Neurosecretion refers to the synthesis, storage, and release of hormones by cells which possess the definable characteristics of neurons (Gosbee et. al., 1968). The neurosecretory material is synthesized by rough endoplasmic reticulum and packaged by the Golgi apparatus in the perikaryon of the neurosecretory cell (Normann, 1965). The neurosecretory product is packaged in spherical membrane-bound electron-dense granules ranging from about 1000 to 3000 Å in diameter (Scharrer, 1962). Occasionally the neurosecretory product appears as clear vesicles rather than granules. The granules (or vesicles) are channelled into the axons and transported along them to be stored in the bulbous endings of the axons. A group of neurosecretory cells and their axons and endings comprise a neurosecretory system.

The neurosecretory axons branch profusely near their endings so that there are many more axon terminals than axons giving rise to them (Brady and Maddrell, 1967). If the axon terminals form a discrete anatomical structure, it is known as a neurohemal organ (Knowles and Carlisle, 1956) and is the site of both the storage and the release of the neurosecretory product. However, a discrete neurohemal organ is not required for release, as the axon terminals may be diffusely distributed (Finlayson and Osborne, 1968; Hinks, 1975) or may form junctions with visceral muscles or other effector organs (Osborne, 1970; Miller and Rees, 1973).

The neurosecretory neuron plays an important role in neuroendocrine correlation; whenever the central nervous system communicates to an endocrine organ, its immediate channel is almost invariably a neurosecretory cell (Hagadorn, 1967). A deeper knowledge of the structure and function of neurosecretory cells should contribute to the understanding of the interrelationships between the two major mechanisms of coordination, the nervous and endocrine systems. Invertebrate preparations are of great utility in the study of many aspects of neurosecretion. In the present study, ultrastructural changes in an insect neurosecretory system have been observed during a period of neurohormone release.

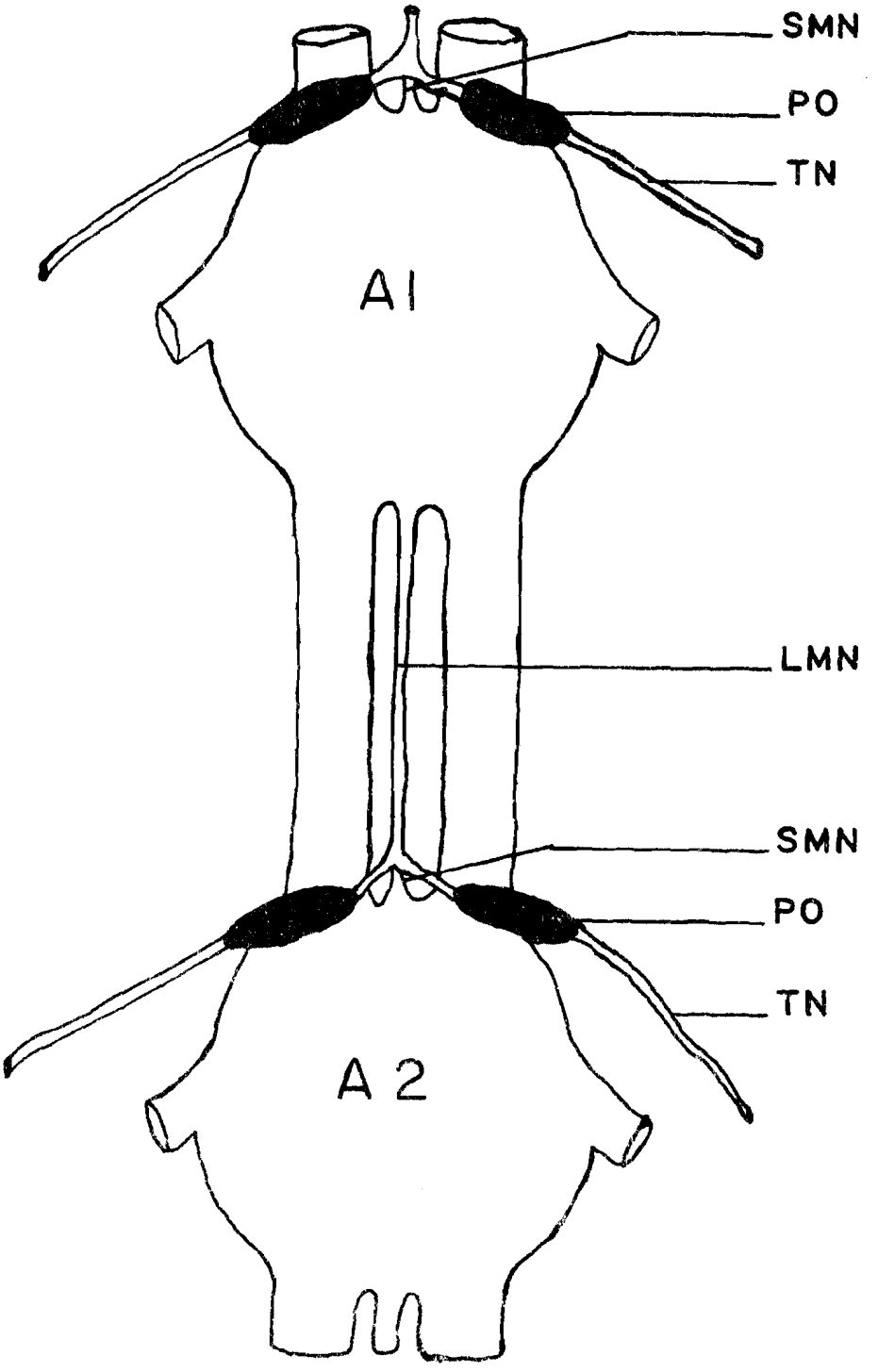
Slight thickenings or small spindle-shaped swellings are associated with the median nervous system in Periplaneta americana and other insects. The existence of these segmentally arranged swellings has been known for a very long time (Lyonet, 1762) and their function was speculated as being "instrumental in the liberation of certain substances into the blood" (Alexandrowicz, 1952). This function was corroborated in the median nerve swellings of two stick insects (Raabe, 1965, 1966) and the locust (Chalaye, 1966) where histological and histochemical evidence suggested that the swellings were indeed neurohemal. In addition, the median nerve swellings in phasmids (Raabe and Ramade, 1967), the stick insect Carausius morosus, the locust Schistocerca gregaria, and the cockroach Periplaneta americana (Brady and

Maddrell, 1967) all have the fine structure of neurohemal organs.

These neurohemal organs, which have been named the perisympathetic organs (Raabe and Ramade, 1967), are the focus of this research. Figure 1 shows the perisympathetic organs associated with the first and second abdominal ganglia in Periplaneta americana. A long median nerve extends posteriorly from each abdominal ganglion to just in front of the following ganglion where it branches to form two tranverse nerves which pass through the swellings formed by the perisympathetic organs. The axons of spiracular motor neurons pass down this long median nerve, through the swellings, and out the transverse nerve to the spiracle (Case, 1957). A short median nerve passes anteriorly from each ganglion and branches to enter the juxtaposed swellings. This short median nerve contains the neurosecretory axons which end in the perisympathetic organs (Smalley, 1970).

The fine structure of the perisympathetic organs in Periplaneta has been studied previously by Brady and Maddrell (1967) and by Fitch (1972). The study by Brady and Maddrell was primarily concerned with demonstrations of the neurohemal nature of the organs in several insect species and did not describe the detailed structure of the organs in Periplaneta. One purpose of this research was to provide a detailed study of the fine structure of these organs, especially as it relates to their function as neurohemal organs.

Figure 1. A schematic representation of a portion of the ventral nerve cord in Periplaneta americana. Key: first abdominal ganglion (A1), second abdominal ganglion (A2), long median nerve (LMN), short median nerve (SMN), transverse nerve (TN), and perisympathetic organ (PO).


SMN
PO
TN
A1
LMN
SMN
PO
TN
A 2

According to the previous investigators, the principal type of neurosecretory ending in the perisympathetic organs of Periplaneta contains both electron-dense granules and electron-translucent vesicles. Fitch (1972) discovered that the proportion of granules to vesicles seemed to vary under different experimental conditions. This suggests that the granules might be transformed into vesicles during the release process. Since evidence for such transformation had not been reported previously, the present study was designed to thoroughly investigate the mechanisms of release of neurosecretory products in the perisympathetic organs of Periplaneta.

Since Fitch (1972) found that the neurosecretory contents of the abdominal perisympathetic organs in Periplaneta were depleted by dehydration and starvation, these stresses were used to stimulate the release of neurosecretory materials. Perisympathetic organs were removed from animals which had been dehydrated and starved for various periods of time and were examined for evidence of neurohormone release. Changes in the proportion of granules to vesicles were substantiated by statistical methods.

METHODS AND MATERIALS

ANIMALS

Periplaneta americana Linnaeus, the American cockroach, was used exclusively in this study. Although there were apparently no sex-associated variations in earlier observations of the perisympathetic organs (Fitch, 1972), only adult males were used as experimental animals. All animals were kept on a day-night cycle of fifteen hours light and nine hours dark. Control animals were taken directly from stock cultures which were fed a diet of Gaines dog food, oatmeal, and apples. Since Fitch (1972) discovered that irregular feeding caused changes in the ultrastructure of the perisympathetic organ, an effort was made to provide the stock cultures with food and water at all times.

Experimental animals were isolated from food and water in white, small laboratory animal cages with ventilated aluminum foil secured over the open top. No attempt was made to control temperature or humidity, but the experimental animals were kept in a laboratory office at room temperature where those variables changed little. Animals were dehydrated and starved for four, seven, ten, and thirteen days before being sacrificed and prepared for ultrastructural studies.

ELECTRON MICROSCOPY

Processing perisympathetic organs for EM study. The animals were sacrificed by an intra-abdominal injection

(0.5 cc) of ice-cold 4% glutaraldehyde fixative buffered with 0.1 M cacodylate (pH 7.4). After all movement ceased, the animal was decapitated, trimmed of all appendages, and pinned to a wax-filled petri dish for dissection. The dorsal abdominal body wall was removed and the abdominal cavity flushed with more ice-cold 4% cacodylate buffered glutaraldehyde. The perisympathetic organs associated with the first and second abdominal ganglia were rapidly removed and placed in ice-cold fixative for one-half hour. The tissue was then washed in ice-cold 0.1 M cacodylate buffer (pH 7.4) and post-fixed in ice-cold 1% osmium tetroxide buffered with 0.2 M cacodylate (pH 7.4) for one-half hour. After dehydrating in an ethanol series and rinsing in propylene oxide, the tissue was embedded in an Epon-Araldite mixture. The Epon-Araldite mixture consisted of 3.75 parts Epon 812 and 6.25 parts Araldite 6005 and 1 part dibutyl pthalate. To 7 ml. of Epon-Araldite mixture, 3 ml. of DDSA were added as a hardener and 0.3 ml. of DMP-30 was added as an accelerator.

After curing a minimum of twelve hours at 50 to 55°C, the Epon-Araldite blocks were allowed to continue curing at room temperature for 7 to 10 days before silver sections were cut with glass knives on an LKB Ultramicrotome I. The thin sections were stained one-half hour with 2% uranyl acetate and 5 minutes with lead citrate (Reynolds, 1963). Photographs were taken with a Hitachi HS-8 electron microscope and routinely enlarged three times. Photographs were taken of

many sections through different parts of the tissue to generate statistically significant data from a large number of endings.

Problems unique to the material. The size of the perisympathetic organs ($\leq$ 100 um) presented many technical problems. Greatest among them was the tendency to lose the tissue during the many changes of fluid during processing. Tissue has been commonly stored and processed in small glass vials and the fluid pipetted out, leaving the tissue in the vial. When this technique was used on perisympathetic organs, the tissue was often drawn into the pipette and lost. During this investigation, it was found that the use of a shallow, wide-mouthed vial produced more satisfactory results. It could be tipped to one side or the other making it possible to pipette out the fluid while at the same time observing the procedure under a binocular dissecting scope.

Interpretation of the EM data. The collection of EM data in this research was three-fold. First, the cytological phenomena which cooroborate the neurohemal function of the perisympathetic organs were described, such as, the sparse distribution of tracheoles and the presence of large molecule penetratable desmosomes and septate desmosomes.

Secondly, hundreds of photographs were surveyed to locate any of the known cytological structures of neurosecretory release such as "synaptic sites" (Johnson, 1966a) or omega-shaped profiles in the axolemma (Normann, 1965).

Furthermore, attention was given to find any other morphological evidence which might be interpreted as neurosecretory release.

Thirdly, since Fitch (1972) suggested that the electron-dense granules may empty themselves to become electron-translucent vesicles, large samples were collected to produce statistically significant counts of granules and vesicles. Five animals from each of the dehydrated and starved and control groups were used, and the granules and vesicles of 40 to 45 axon terminal profiles were counted from each animal. The area of each axon profile counted was measured directly from the photographs with a compensating polar planimeter and converted to square microns (u^2). The counted granules (G) and vesicles (V) were expressed as percentages (%G and %V), as a ratio (G/V), and in terms of area ($G+V/u^2$). The data from the five animals within each group were analyzed statistically to determine the mean, standard deviation, and the levels of significance from Student t-test scores.

RESULTS

ELECTRON MICROSCOPY

Ultrastructure of the normal perisympathetic organ. The perisympathetic organ is enveloped by a connective tissue sheath called the neural lamella, which appears as an amorphous ground substance containing collagen-like fibrils. The neural lamellar sheath makes many infoldings or ramifications into the organ forming an extensive stroma (Figure 3). Neurosecretory terminals containing classical electron-dense granules, 1250 to 2075 Å in diameter, are almost always associated with the stroma of the neural lamella. These endings, which will be referred to as Type I endings, are either completely enveloped by the stroma or lie closely positioned beneath it (Figure 2). Also associated with the outer sheath are the sparsely distributed tracheoles (Figures 2, 3, 4, and 9) which supply the cells of the organ with oxygen and remove carbon dioxide. In other parts of the nervous system a specialized layer of glial cells called the perineurium is found lying just beneath the neural lamella. However, the perineurium, as well as the outer fat body sheath associated with the ganglia and their connectives, is absent from the perisympathetic organs.

The major portion of the organ consists of profiles of a second type of neurosecretory ending (Type II; Figures 2, 3, and 4). These Type II endings contain small electron-dense granules, 625 to 1650 Å in diameter. The Type II endings are

Figure 2. Electron micrograph of a perisympathetic organ showing a view at the surface. Key: Type I axon (I), Type II axon (II), neural lamella (NL), hemocoele (HC), mitochondria (M), rough endoplasmic reticulum (rER), tracheole (Tr) showing tubercles (short arrows), branching of an axon (double-headed arrow), and nucleus of a glial cell (gN). 24,000 X

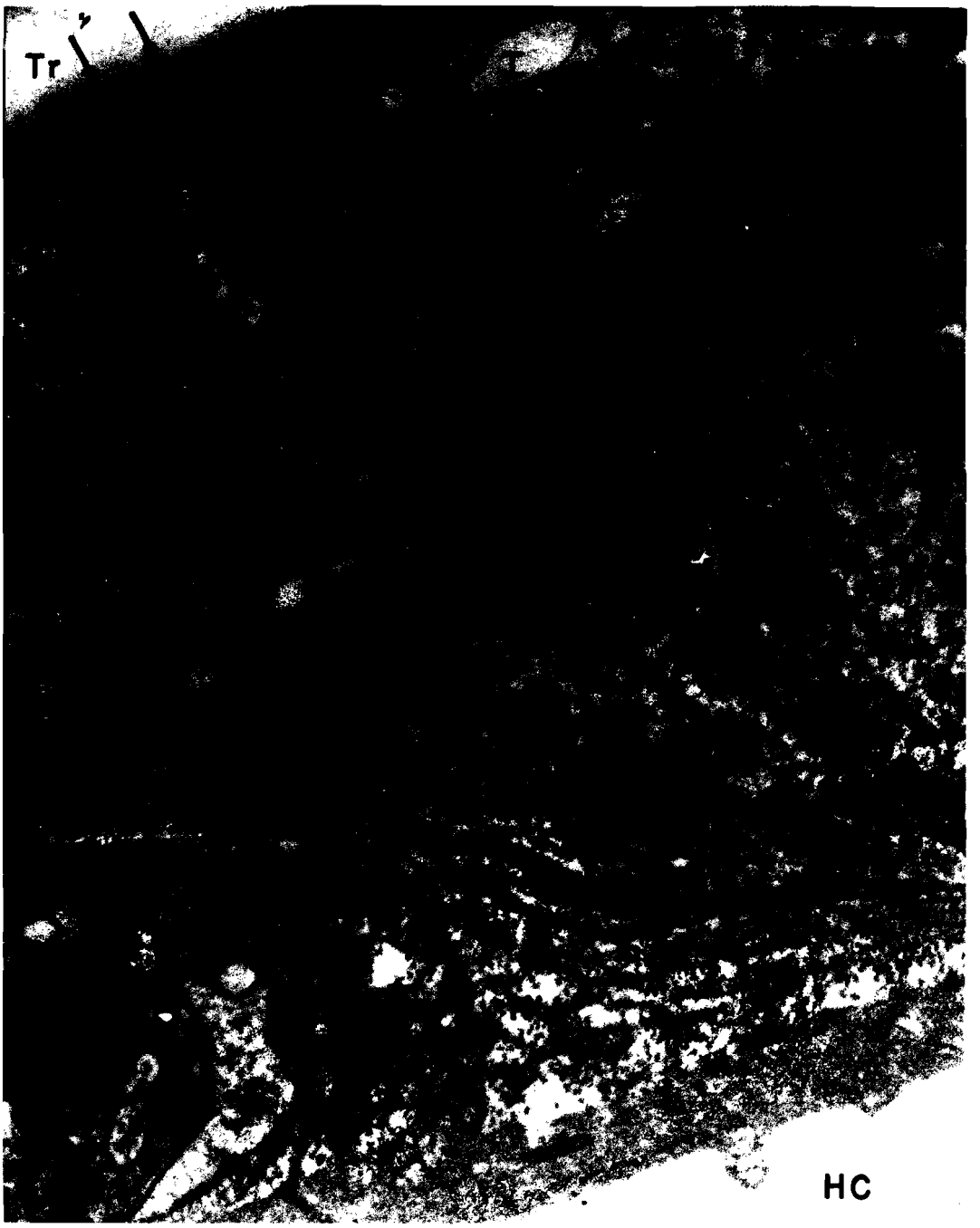

Tr
HC

Figure 3. Electron micrograph of a perisympathetic organ showing ramifications of the neural lamella (stroma). Key: Type I axon (I), Type II axon (II), stroma (S), mesaxon (Ma), desmosome (D), tracheole showing oblique section of a helically arranged taenidial fold (Tr), and a tracheolar end cell nucleus (tN). 20,000 X

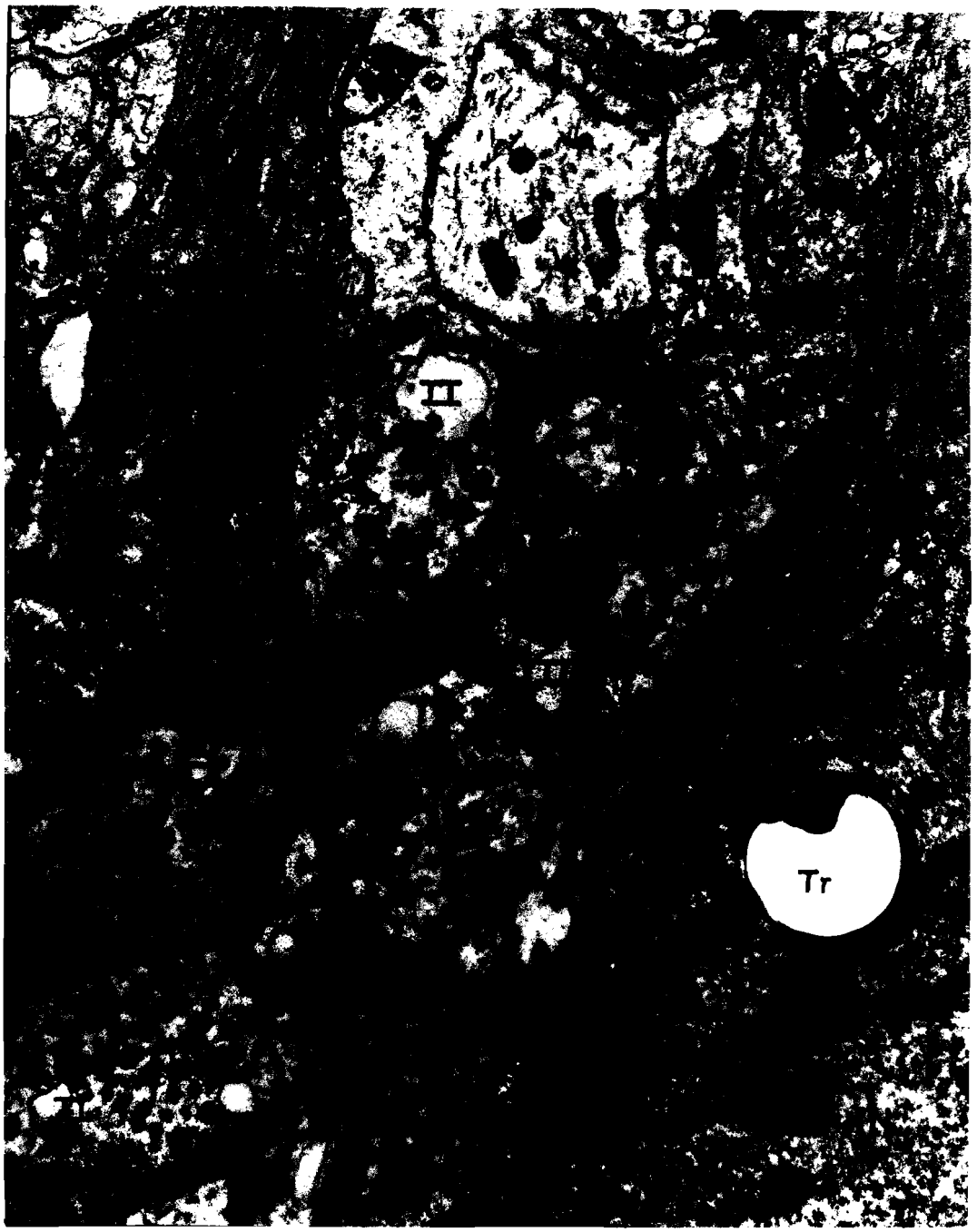
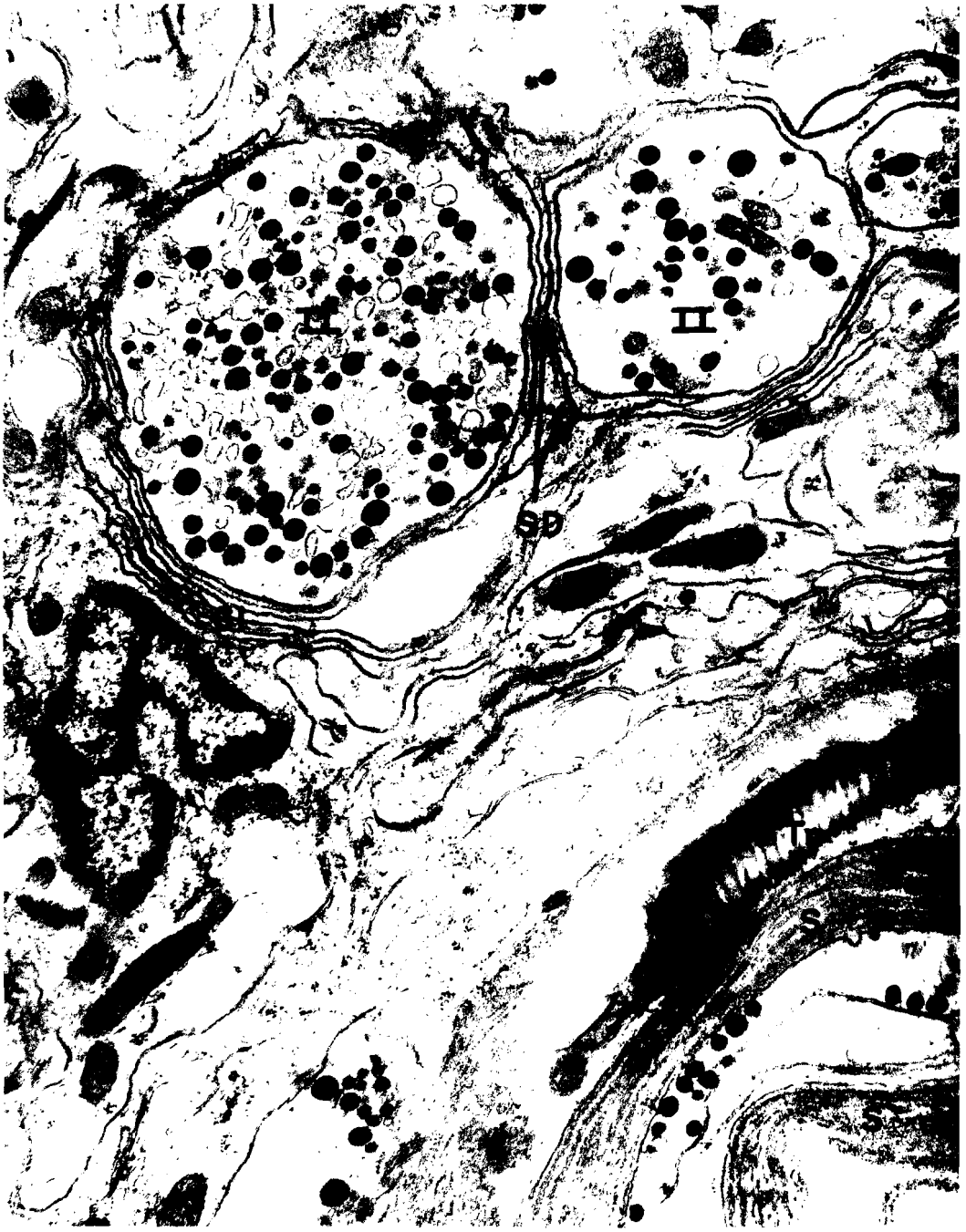

II
Tr

Figure 4. Electron micrograph of a perisympathetic organ showing tracheole and septate desmosomes. Key: Type II axon (II), septate desmosome (SD), tracheole showing annular taenidial rings (Tr), and attenuated folds of a glial cell (Gl). 24,000 X


II
II
SD

highly branched (Figures 2, 7, 11, and 12) and ensheathed with glial cells. The attenuated glial cell processes spiral around the cylindrically-shaped axons and bulbous endings forming an invagination in which the axon or ending lies. This invagination is linked to the surface of the glial cell by an extracellular channel, called the mesaxon (Figure 3). Septate desmosomes (Figures 4 and 12) and desmosomes of the zonula adherens type (Figure 3) are frequently seen. These structures apparently maintain the integrity of the glial structure. Tight junctions are not found. The smaller endings, and, particularly those close to the neural lamella, either do not have any glial sheath or are partially covered or share a glial cell with other axons. Toward the center of the organ, the endings have an unshared glial cell with many spiral folds. Large spiracular motor axons toward the center of the organ have many compacted glial cell folds (Figure 5).

The Type II neurosecretory axons contain neurotubules which are aligned parallel to the longitudinal axis. They are closely associated with the granules in transit (Figure 6) from the perikaryon to the axon terminal (Figure 7).

A possible new type of neurosecretory material was discovered in this study (Type III, Figure 8). The Type III endings are associated with the neural lamella, as are the Type I endings, but are packed only with electron-translucent vesicles ranging from 800 to 1450 $\mathring{A}$ in diameter. They are rarely found and have not previously been described in the

Figure 5. Electron micrograph of a perisympathetic organ showing Type II axons and spiracular motor axons. Key: Type II axon (II), spiracular motor axon (SMA), compacted glial cell folds (Gl), mitochondria (M), and neurotubules (Nt). 24,000 X

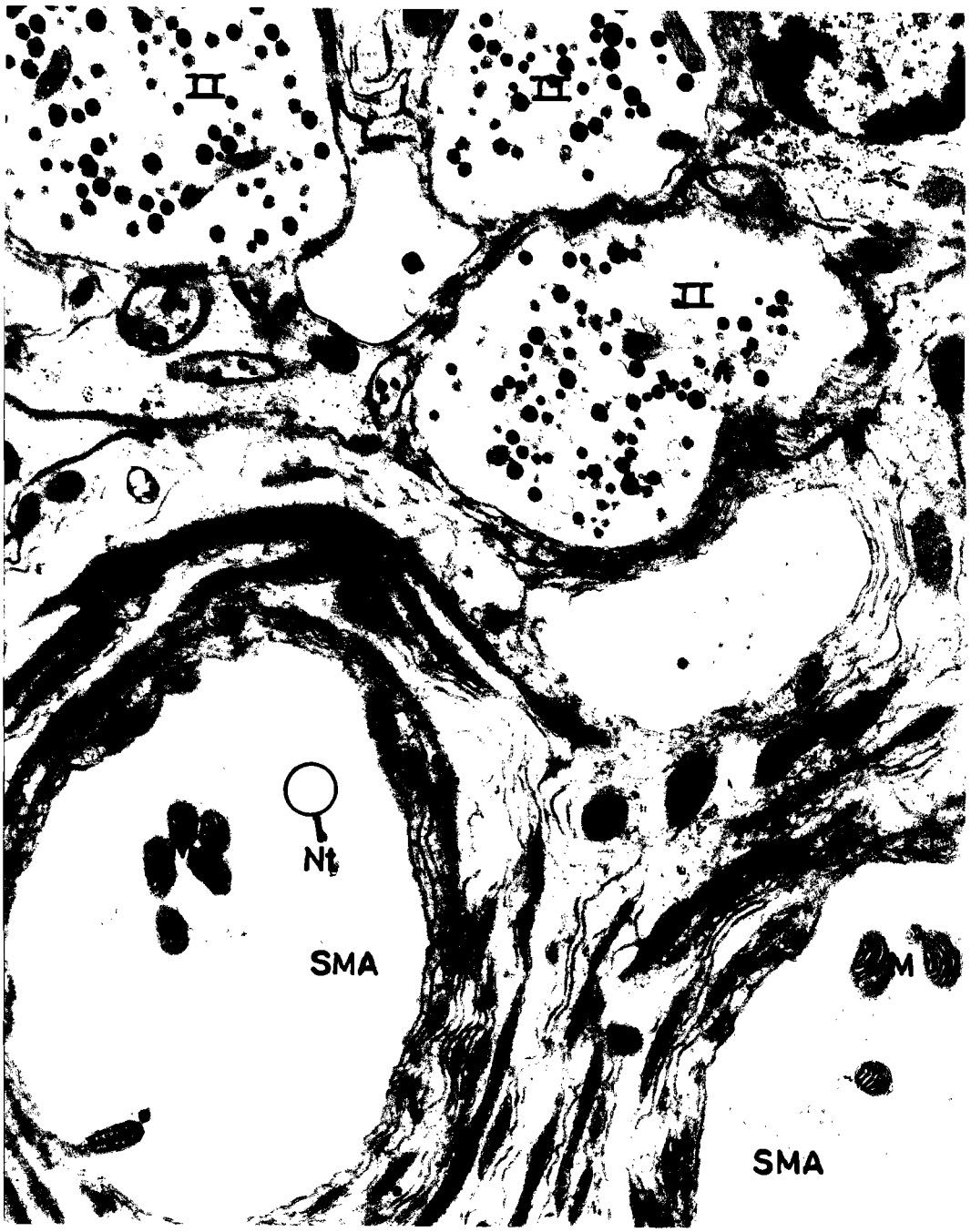

II
II
II
Nt
SMA
M
SMA

Figure 6. Electron micrograph of a perisympathetic organ showing longitudinally arranged neurotubules. Key: neurotubules (Nt), electron-dense granules (G), and glial cell folds (Gl). 24,000 X


Nt

Figure 7. Electron micrograph of a perisympathetic organ showing neurotubules arriving at an axon ending. Key: Type II axon (II), neurotubules (Nt), branching of the axon (double-headed arrows). 24,000 X


II
II

Figure 8. Electron micrograph of a perisympathetic organ showing a previously undescribed Type III axon. Key: Type I axon (I), Type III axon (III), stroma (S), and a tracheole (Tr) showing tubercles (short arrows). 24,000 X


I

perisympathetic organs of Periplaneta (Brady and Maddrell, 1967; Smalley, 1970; and Fitch, 1972).

Ultrastructure of the perisympathetic organ during neurohormone release. The Type I endings are not affected by the experimental conditions or this research (Figure 9). Ultrastructural changes, suggestive of neurohormone release, are found in Type II neurosecretory endings of dehydrated and starved animals. "Synaptic sites" denoted by a thickening of the axolemma and juxtaposed clusters of "synaptic vesicles", 250 to 350 Å in diameter, are found in the earlier stages of release, such as in the four day dehydrated and starved animal shown in Figure 11. When found in the seven day (Figure 12), the ten day (Figure 13), and the thirteen day animals (Figure 14, the "synaptic vesicles" tend to be dispersed throughout the axon profile. Compare these profiles with those of a normal animal in Figure 10. These small vesicles have been previously described by Johnson (1966a) as being involved in neurohormone release. In addition, omega-shaped profiles in the axolemma have also been found in actively releasing tissue (Figures 12 and 14). Normann (1965) described these structures as being indicative of exocytosis where the limiting membrane of the granule fuses with the axolemma, expelling the contents of the granule into the extracellular space. Although both the "synaptic sites" and omega-shaped profiles are rare, they are more common in the earlier stages of dehydration and starvation.

Figure 9. Electron micrograph of a perisympathetic organ showing Type I axons in a thirteen day dehydrated and starved animal. Key: Type I axon (I), Type II axon (II), neural lamella (NL), hemocoele (HC), and a tracheole (Tr). 12,000 X

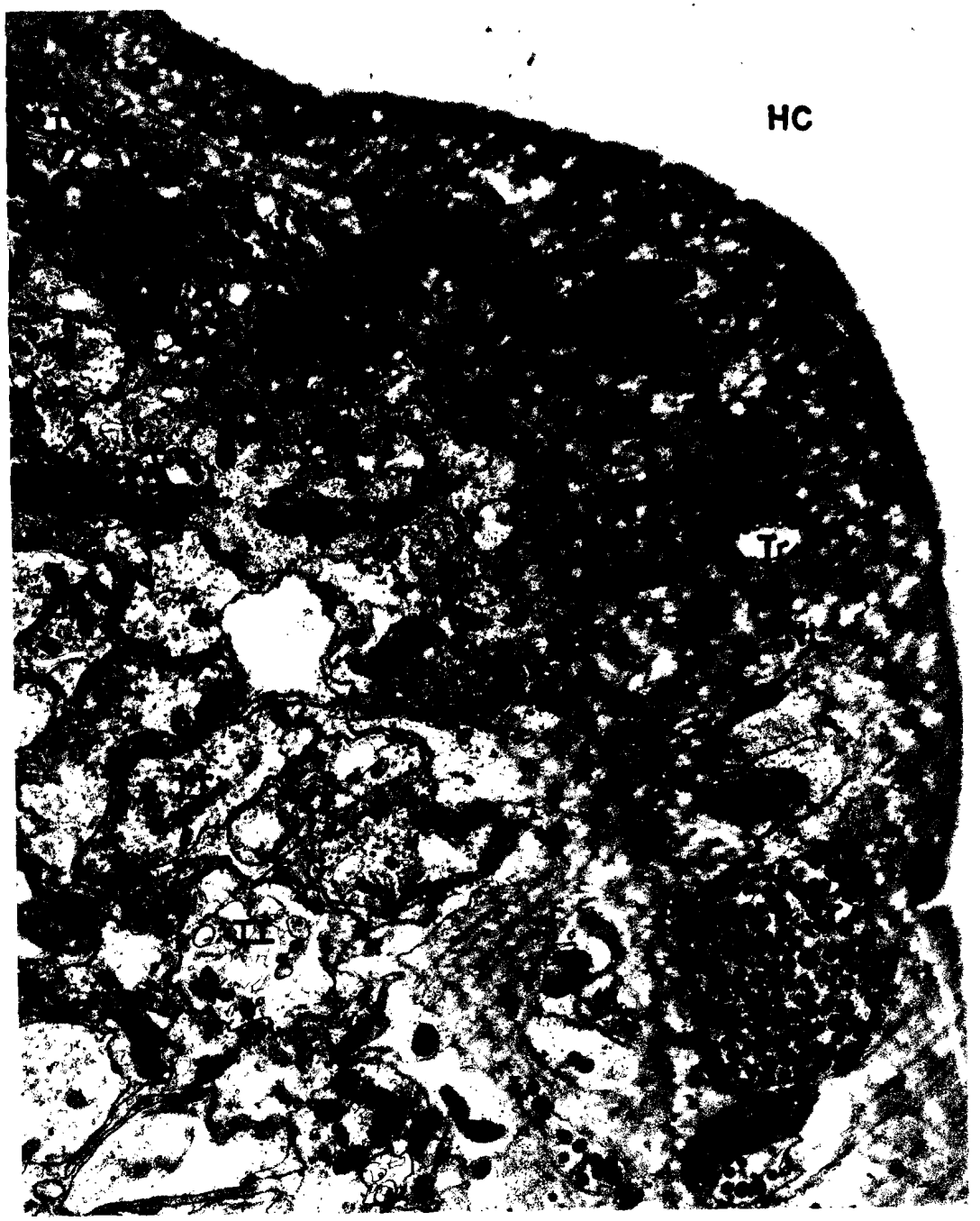

HC

Figure 10. Electron micrograph of a perisympathetic organ of a typical normal animal. Key: granules (G), vesicles (V). All are Type II axons. 24,000 X


G
V

Figure 11. Electron micrograph of a perisympathetic organ of a typical four day dehydrated and starved animal showing "synaptic sites". Key: granules (G), vesicles (V), "synaptic sites" or microvesicle clusters (MVC), and branching of the axon (double-headed arrows). All are Type II axons. 24,000 X

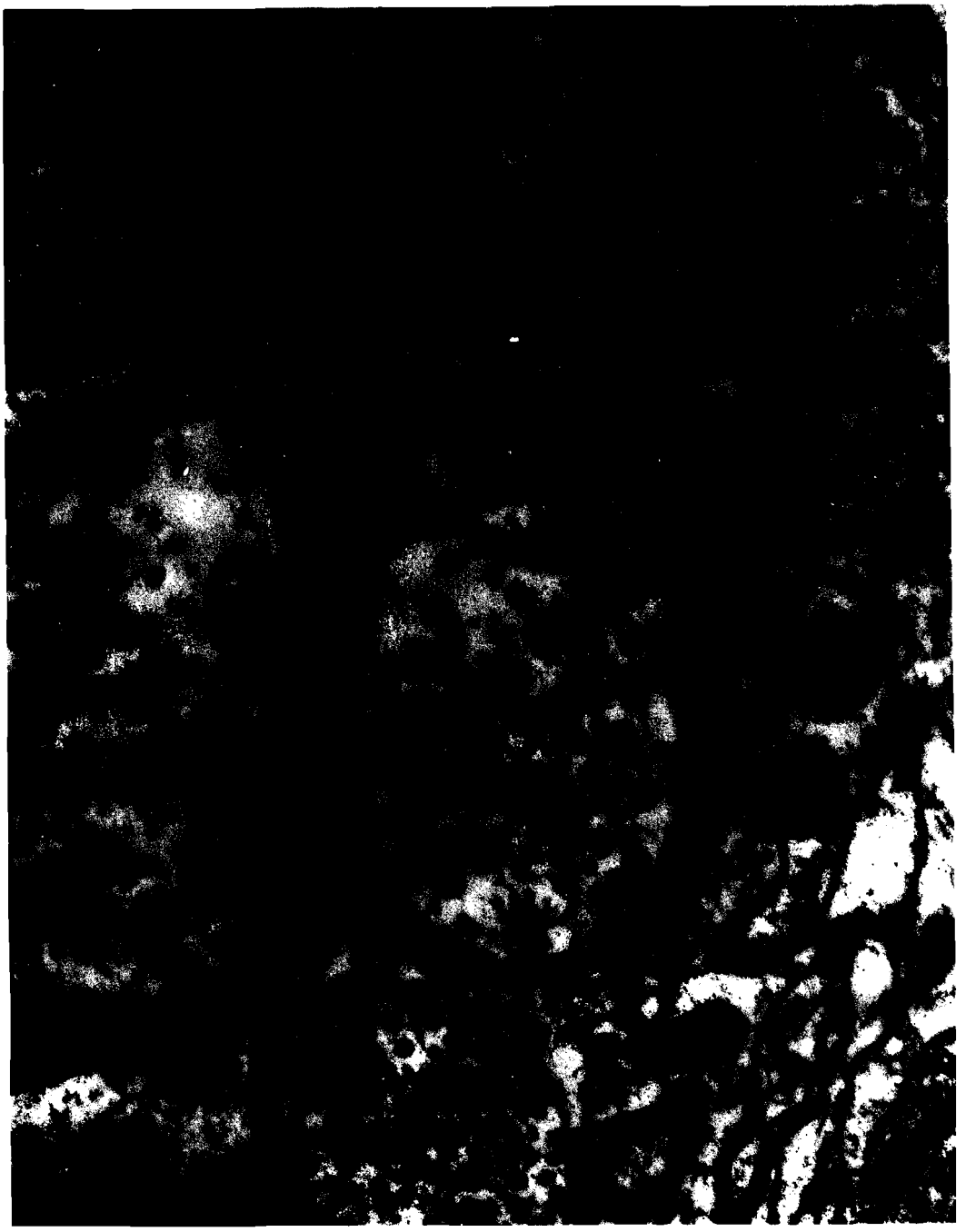


Figure 12. Electron micrograph of a perisympathetic organ of a typical seven day dehydrated and starved animal. Key: septate desmosomes (SD), branching of the axon (double-headed arrows), microvesicles (MV), and omega-shaped profiles (OP). All are Type II axons. 24,000 X

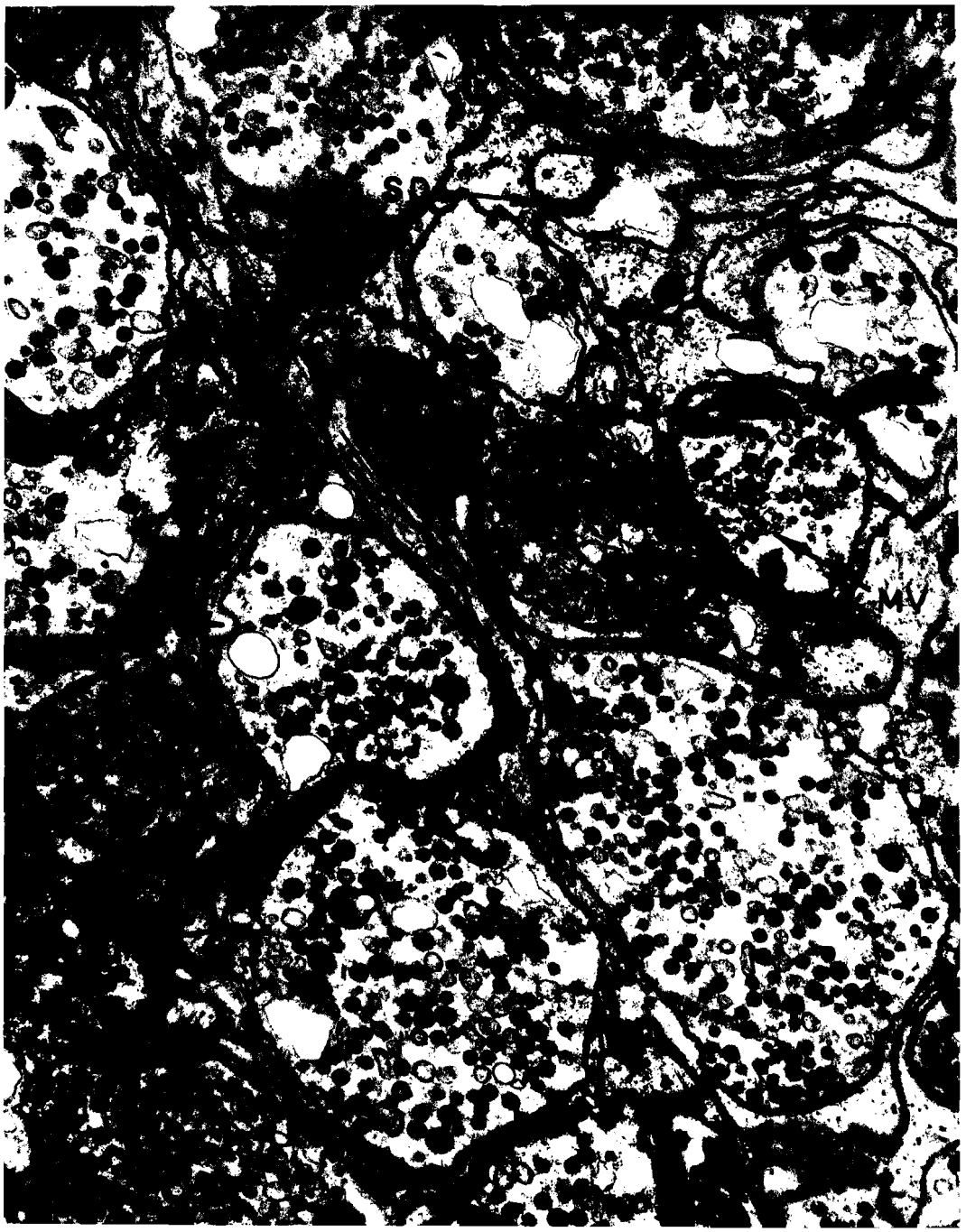

MV

Figure 13. Electron micrograph of a perisympathetic organ of a typical ten day dehydrated and starved animal. Key: microvesicles (MV). All are Type II axons. 24,000 X

Figure 14. Electron micrograph of a perisympathetic organ of a typical thirteen day dehydrated and starved animal. Key: microvesicles (MV), and omega-shaped profile (OP). All are Type II axons. 24,000 X


MV

As the stress of dehydration and starvation progresses, he electron-dense granules, which appear uniformly dense and paque in the normal animal (Figure 10), begin to vary in pacity and density. This is illustrated in Figure 15. Some ranules are extremely dark-staining (Figure 15, A); some how a slightly mottled appearance (B); some develop a clear uter ring just inside the limiting membrane (C); some ppear to be slightly stippled, but mostly filled (D); and ome are only partially filled with stippled appearing mater- al (E).

During the final days that the cockroach is able to ndure dehydration and starvation (10 to 13 days), multi- esicular structures appear (Figures 16A and B) as well as ultilamellar structures consisting of concentric rings of embraneous material (Figure 18). High magnification of the ultivesicular structures shows that both granules and esicles are entrapped within doubled-layered membranes Figure 17). As the development of these structures pro- gresses, many rings of membranes are laid down, mitochondria are entrapped, and vesicles and membraneous cisternae come nto contact with the outer lamellar rings to perhaps, become part of the developing multilamellar structure (Figure 18). Death usually occurs at 13 to 15 days. The main activity within the neurosecretory axons during this time appears to be the collection of membraneous material by the multilamellar bodies.

Figure 15. Electron micrograph of a perisympathetic organ showing varying degrees of electron density and opacity. Key: granules (A, B, C, D, E; see text), vesicles (V), microvesicles (MV), mitochondria (M), and short microtubules (Nt). From a Type II ending of a ten day dehydrated and starved animal. 57,000 X

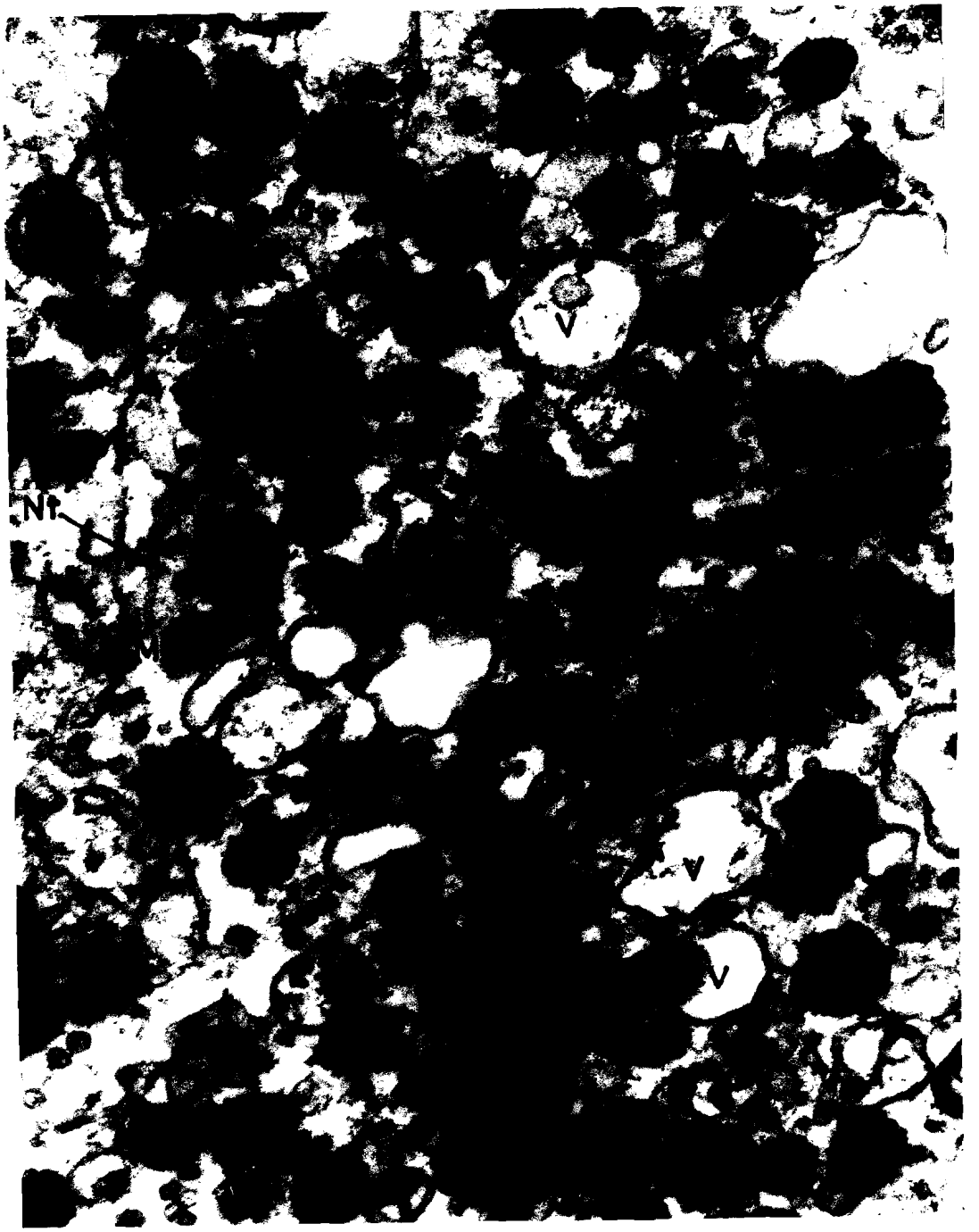

V
Nt
V
V

Figure 16A. Electron micrograph of a perisympathetic organ showing multivesicular bodies in a ten day dehydrated and starved animal. Key: Type I axon (I), Type II axon (II), multivesicular body (MVP), and stroma (S). 24,000 X

Figure 16B. Electron micrograph of a perisympathetic organ showing a multivesicular body being invested by multilamellar membrances. Key: multivesicular body (MVB). All are Type II axons. Ten day dehydrated and starved animal. 24,000 X

A.

B.

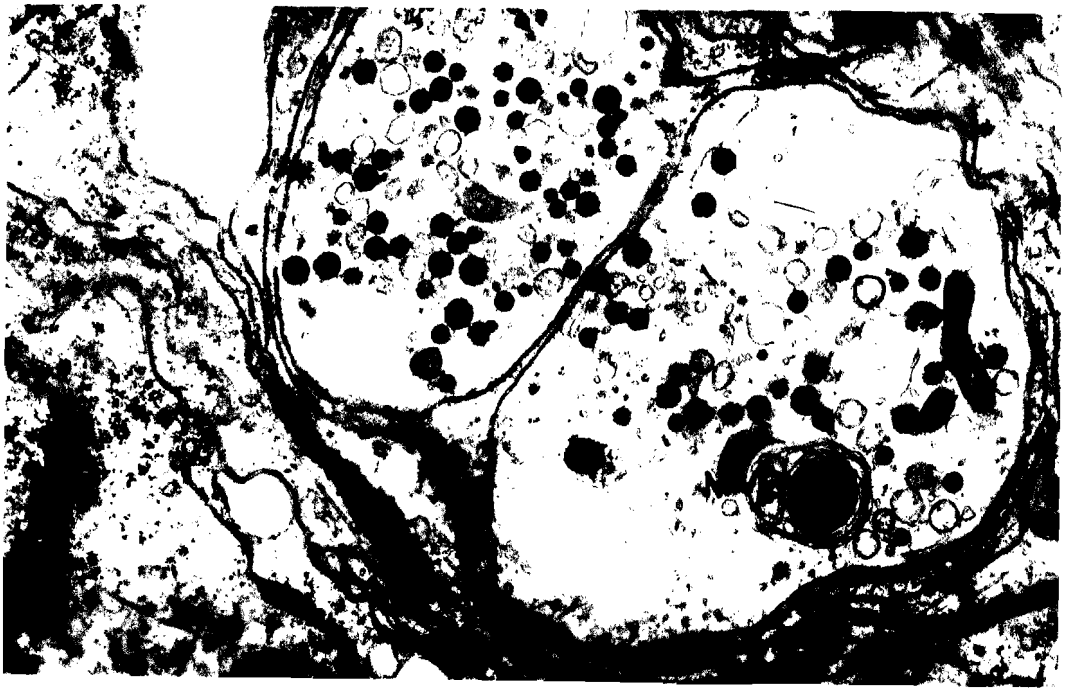
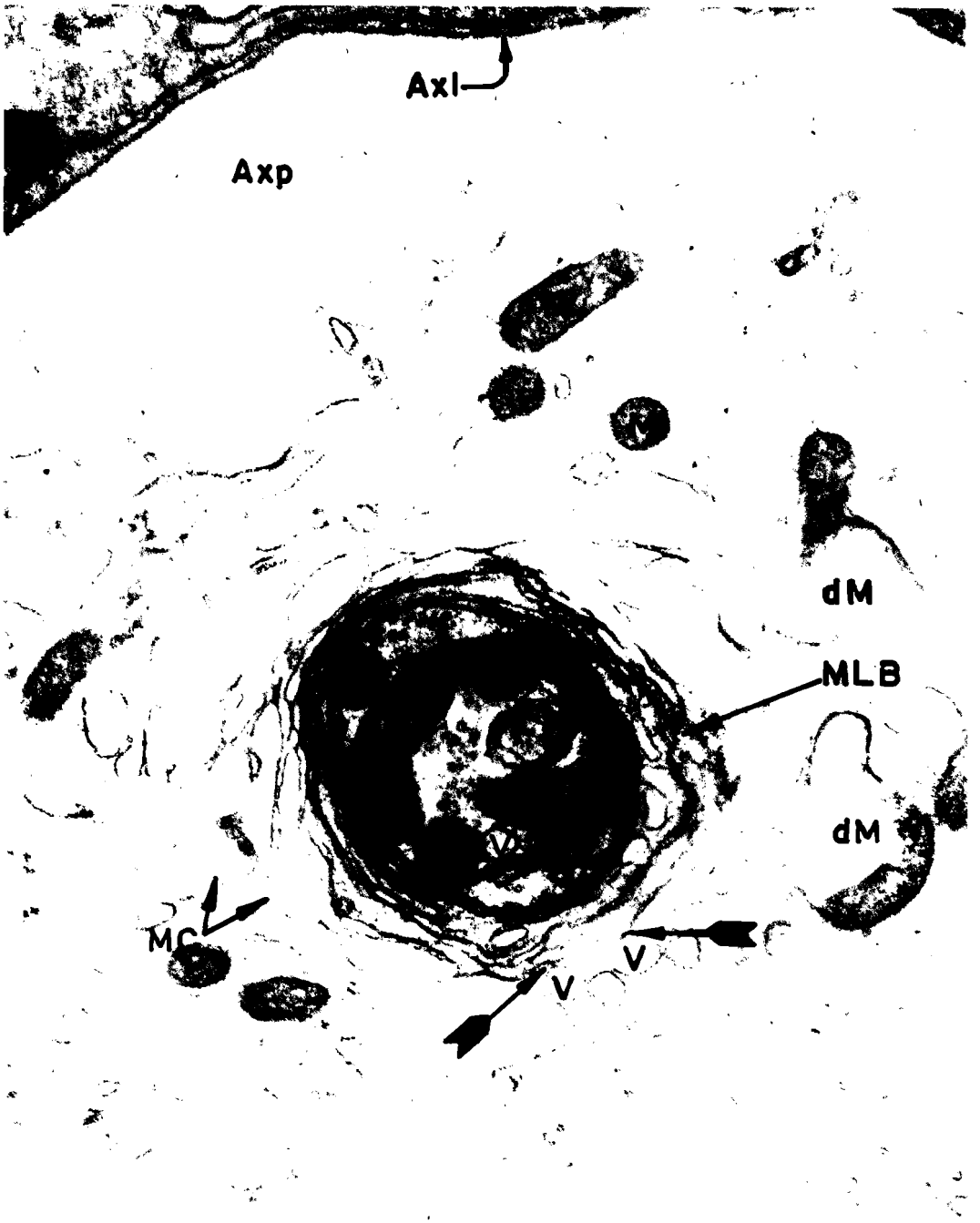


Figure 17. Electron micrograph of a perisympathetic organ showing double-layered membrane of multivesicular body containing granules and vesicles. Key: multivesicular body (MVB), granules (G), vesicles (V), and mitochondria (M). Type II axon from a thirteen day dehydrated and starved animal. 57,000 X

Figure 18. Electron micrograph of a perisympathetic organ showing a multilamellar body containing mitochondria in a thirteen day dehydrated and starved animal. Key: multilamellar body (MLB), vesicles (V), mitochondria (M), membraneous cisterne (MC), axoplasm (AxP), axolemma (AxL), degenerating mitochondria (dM), contact points between vesicles and lamellar rings (arrows). Type II axon. 48,000 X


Axl
Axp
dM
MLB
dM
MC
V
V
V

Granule-vesicle relationships in Type II endings of the perisympathetic organs during neurohormone release. The numbers and proportions of granules and vesicles in Type II endings change significantly during dehydration and starvation. In the normal animal there are an average of 30.9 granules and vesicles per sq. u with more than 75% of these being granules (Table I). As dehydration and starvation progresses there is a decrease in the %G and a corresponding increase of the %V until after 13 days there are only 10.5 $G+V/u^2$ with only about 35% of those being granules (Table I, Figure 19). The progression of the G/V ratio (Figure 20) shows that there is a rapid change in the relationship of granules to vesicles for the first seven days with the most rapid change occurring in the first four days.

A graphic representation of the numbers of granules, vesicles, and total granules plus vesicles per given area (G/u^2, V/u^2, and $G+V/u^2$ respectively) demonstrates the most enlightening discovery of this research (Figure 21). Between 0 and 4 days the total number of granules and vesicles declines rapidly. The difference between the control value (day 0) and the 4 day value is significant at the $p \leq 0.05$ level (Table IV). This is due primarily to a substantial decrease in the number of granules. During this same interval, the number of vesicles does not change appreciably. Between 4 and 10 days the total number of granules and vesicles remains relatively constant. However, during this time period there is a moderate decrease in the number of granules

TABLE I. Comparison of granule—vesicle relationships in control and experimental animals.*

	% G	% V	G / V	G/μ^2	V/μ^2	$\frac{G+V}{\mu^2}$
CONTROL	76.8 ± 5.0	23.2	4.00 ± 1.07	23.2	7.7	30.9 ± 8.6
4 DAY	62.0 ± 11.8	38.0	1.96 ± 0.81	13.1	8.1	21.2 ± 3.1
7 DAY	49.2 ± 18.1	50.8	1.28 ± 0.92	11.0	11.4	22.4 ± 3.5
10 DAY	46.0 ± 12.4	54.0	1.06 ± 0.47	10.5	12.4	22.9 ± 8.1
13 DAY	35.8 ± 12.3	64.2	0.71 ± 0.40	3.8	6.7	10.5 ± 8.1

The values are either means or means plus or minus the standard deviation.
*The experimental animals were dehydrated and starved N number of days.

Figure 19. Graph of %G and %V versus the number of days animals were dehydrated and starved. Vertical bars represent standard deviation.

Figure 20. Graph of the granule-vesicle ratio versus the number of days the animals were dehydrated and starved. Vertical bars represent standard deviation.

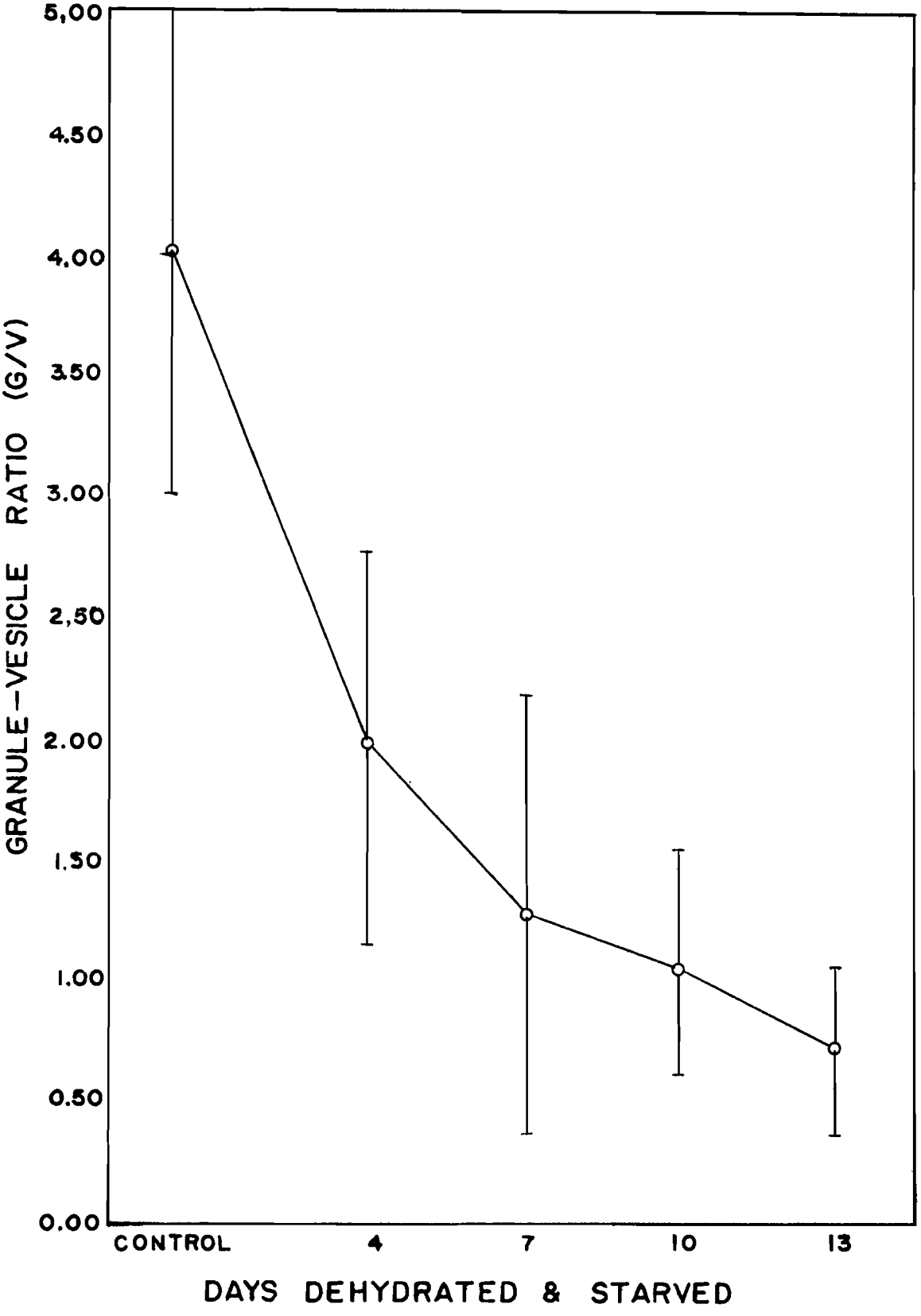

5.00
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
GRANULE-VESICLE RATIO (G/V)
CONTROL
4
7
10
13
DAYS DEHYDRATED & STARVED

Figure 21. Graph of granules and/or vesicles per square micron versus the number of days the animals were dehydrated and starved. Vertical bars represent standard deviation.

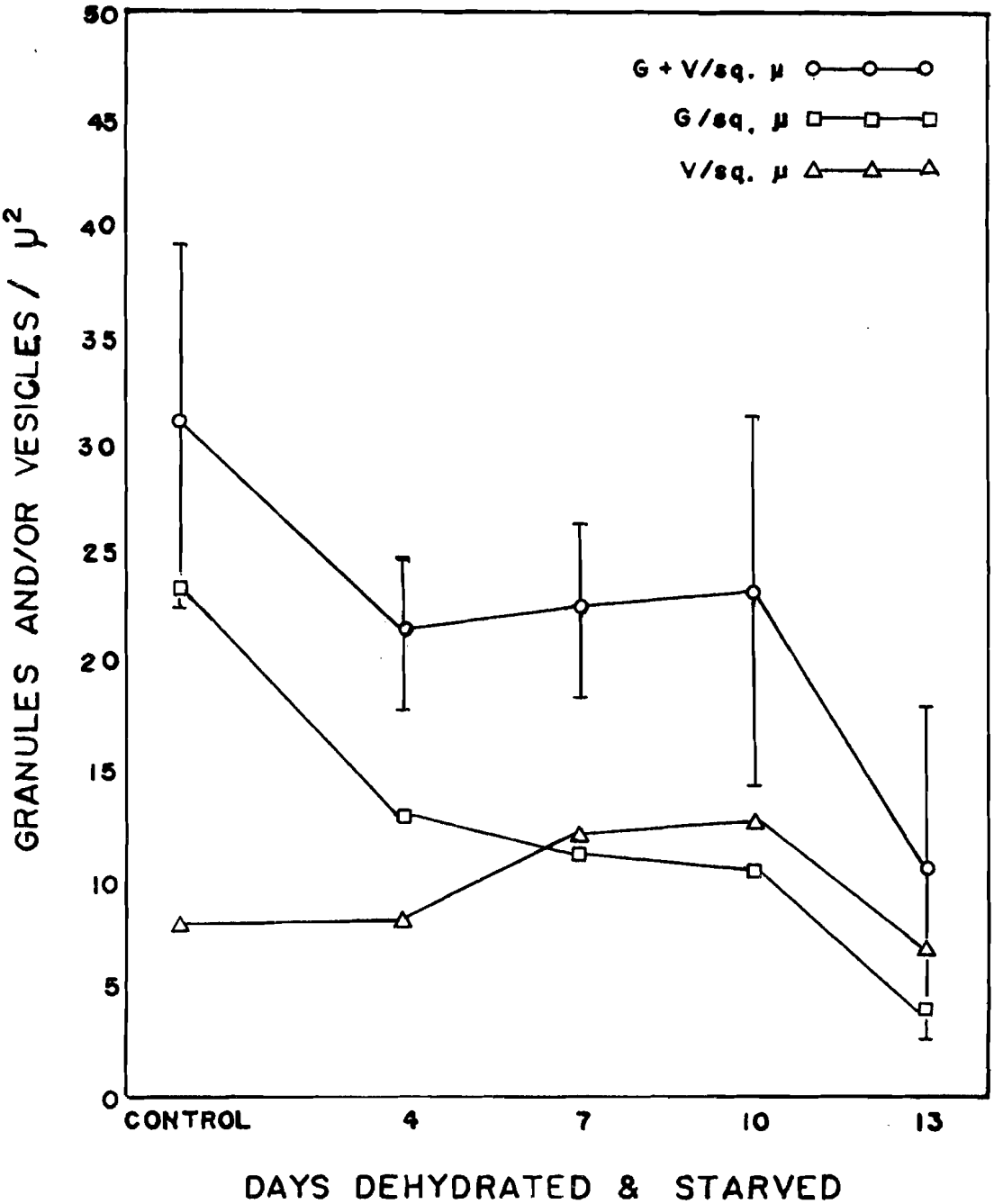

G + V/sq. μ
G/sq. μ
V/sq. μ
GRANULES AND/OR VESICLES / μ²
0
5
10
15
20
25
30
35
40
45
50
CONTROL
4
7
10
13
DAYS DEHYDRATED & STARVED

TABLE II. Comparison of the p values for the category % G.

	4 DAY	7 DAY	10 DAY	13 DAY
CONTROL	0.037	0.013	<0.001	<0.001
4 DAY		>0.500	0.074	0.010
7 DAY			>0.500	0.209
10 DAY				0.231

TABLE III. Comparison of the p values for the category G/V.

	4 DAY	7 DAY	10 DAY	13 DAY
CONTROL	0.010	0.005	<0.001	<0.001
4 DAY		0.249	0.067	0.015
7 DAY			>0.500	0.242
10 DAY				0.242

TABLE IV. Comparison of the p values for the category $\frac{G+V}{\mu^2}$.

	4 DAY	7 DAY	10 DAY	13 DAY
CONTROL	0.046	0.080	0.174	0.007
4 DAY		>0.500	>0.500	0.027
7 DAY			>0.500	0.017
10 DAY				0.044

and a corresponding increase in the number of vesicles. Between 10 and 13 days there is a significant decline ($p \leq 0.05$) in the total number of granules and vesicles. In this case a decrease of both granules and vesicles accounts for the decline.

The standard deviations from Table I are included in the graphic representations in Figures 19 to 21. They tend to be somewhat large. The standard deviation was not determined in every case; for example, only the standard deviation for $G+V/u^2$ was calculated. The variance from the mean values of G/u^2 and, for that matter, V/u^2 are reflected in that of %G in Figure 19 and Table I.

Tables II, III, and IV compare the p-values of the categories %G, G/V, and $G+V/u^2$ respectively. To summarize the data, the control group is significantly different ($p \leq 0.05$) from each of the four experimental groups in both categories of %G and G/V. The only other significant difference in these categories are between the 4 and the 13 day animals. In the $G+V/u^2$ category, the thirteen day animals are significantly different ($p \leq 0.05$) from each of the other experimental groups and the control. The difference between 0 and 4 days is likewise significant.

DISCUSSION

ULTRASTRUCTURE OF THE PERISYMPATHETIC ORGAN RELATING TO ITS NEUROHEMAL FUNCTION

The abdominal perisympathetic organs of Periplaneta are neurohemal in function. This fact is well established by the work of Brady and Maddrell (1967). This research substantiates their results and conclusion. In addition, this present study describes other cytological phenomena condusive to the neurohemal function of the organ. First, the perineurium is absent in the perisympathetic organs. The presence of the perineurium in the central nervous system is thought to establish an effective "blood-brain" type barrier (Lane, 1974). Lane and Treherne (1972) have shown that the tight junctions of the perineurium are not penetrated by the electron-opaque tracer molecules microperoxidase and lanthanum. Since the neural lamellar sheath is known to be permeable (Treherne, 1962), the absence of a perineurium provides easy access to the hemolymph for the released neurohormone.

Secondly, desmosomes and septate desmosomes do not hinder the diffusion of neurosecretory material. The desmosomes and septate desmosomes are located either along the mesaxon (Figure 3) or between adjacent glial cells (Figures 4 and 12) to maintain the glial cells structural integrity. Lane and Treherne (1972) discovered that the electron-opaque tracer molecule lanthanum penetrates the intercellular clefts between glial cells and axons of a peripheral abdominal nerve

in Periplaneta. Therefore, since only desmosomes and septate desmosomes and no tight junctions are found in the perisympathetic organ, the released neurohormone apparently can pass freely along the mesaxons to the stroma. Neurosecretory release sites denoted by microvesicle clusters adjacent to the axolemma are indeed found opposed to glial cells (Figure 11). Brady and Maddrell (1967) point out the fact that very few Type II axon terminals are found facing the stroma. The neurohormone does not then necessarily need to be released from axons in direct contact with the stroma, and may be a readily diffusable molecule.

Thirdly, omega-shaped profiles and "synaptic vesicles" (microvesicles), which have both been associated with neurohormone release (Normann, 1965, and Johnson, 1966a), are found in the perisympathetic organ of Periplaneta. While Brady and Maddrell (1967) described these structures in Carausius, they did not describe them in Periplaneta.

Finally, the tracheal supply to the perisympathetic organ is not abundant. Smith (1968) has suggested that the amount of trachea supplied a tissue is commensurate with its relative need for oxygen. The sparse tracheal supply of the perisympathetic organ suggests minimal oxidative metabolism. This is consistent with the conclusion that the organ is neurohemal, that is that it stores and releases neurohormone into the hemolymph.

The predominant characteristic of the neurosecretory axons of the perisympathetic organ is the presence of electron-dense granules. Their contents are sequestered within a limiting membrane which may provide protection for the hormone against destruction by cytoplasmic factors. Alternately, sequestration may lead to more efficient transport of small molecules from the cell body to the terminals, or it may prevent the hormones from interfering with normal cell function (Normann, 1974). The synthesis and transport of the granules seem to be independent of one another (Berlind, 1977), and the transport of the granules along the axon is apparently under the influence of the neurotubules (Loh et. al., 1975) aligned parallel to the longitudinal axis of the axon (Figure 6).

Three types of neurosecretory products are elaborated in three mutually exclusive types of endings. Type I (Figure 2) are the classical electron-dense granules 1250 to 2075 Å in diameter. These granules seem to be completely undisturbed by dehydration and starvation (Figure 9). These large classical granules are associated with A-type fibers and are probably peptidergic (Bunt and Ashby, 1967). Although A-type cells have been found in the ventral ganglia of Periplaneta (Füller, 1960) and peripherally along the transverse nerve (Finlayson and Osborne, 1968), no positive connection has been made as to where the peptidergic cell bodies giving rise to these Type I axon endings are located.

Type II endings (Figure 2) contain both electron-dense granules 625 to 1650 Å in diameter and electron-translucent vesicles 825 to 1875 Å in diameter. Although Brady and Maddrell (1967) assume both granules and vesicles to contain neurohormone, this study provides evidence suggesting that the vesicles are merely empty granules. This idea is developed later in the discussion. Small electron-dense granules, such as Type II, are associated with B-fibers and considered aminergic (Bunt and Ashby, 1967). Smalley (1970) and Fitch (1972) describe uptake mechanisms for dopamine in the perisympathetic organs of Periplaneta, but Fitch (1972) could not show by flourescence microscopy that a catecholamine was present in the normal organ. Therefore, a biogenic amine other than a catecholamine is probably involved. Miller (1975) developed a broad generality stating that those cells forming neurohemal organs in insects are most often associated with the peptidergic A-fibers while those innervating the visceral muscles and organs are associated with aminergic B-fibers. American cockroach perisympathetic organs seem to be exceptions to Miller's generality.

Type III endings (Figure 8), heretofore undescribed in Periplaneta, are packed only with electron-translucent vesicles 825 to 1475 Å in diameter. This type of vesicle has been associated with positive azocarmine staining and C-type neurosecretory cells (Brady and Maddrell, 1967). Since C-type cells have been described in the ventral ganglia

(de Bessé, 1967; Brady, 1967) and small amounts of azo-carmine positive staining have been found in the long median nerves (Brady and Maddrell, 1967), perhaps a Type III system exists where the cell bodies lie in the ganglion anterior to the perisympathetic organ containing the endings.

For a description of A-, B-, and C- type neurosecretary cells and fibers, see the review by Delphin (1965).

INTER-RELATIONSHIPS OF THE CYTOLOGICAL AND NUMERICAL DATA

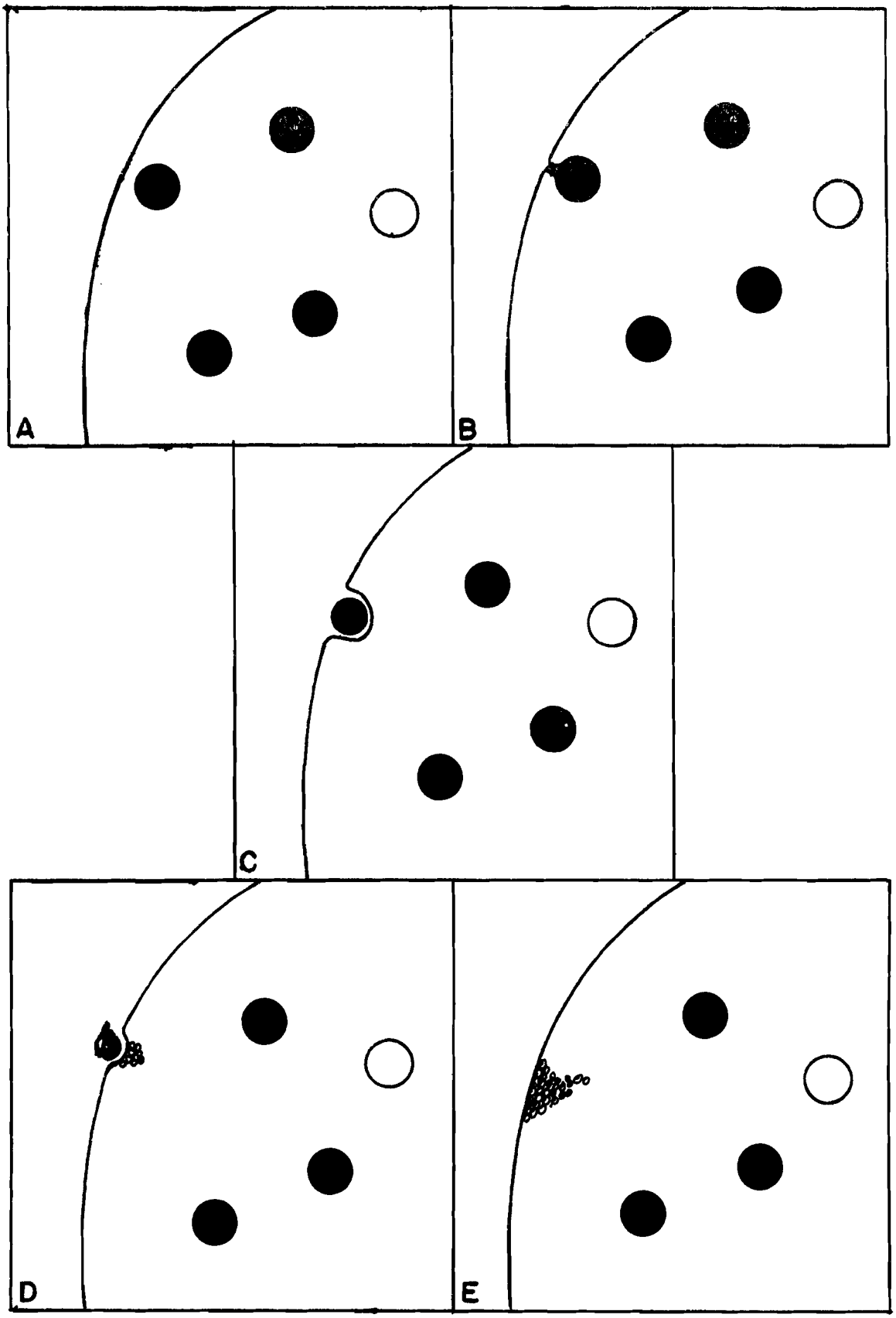
Comparison of the cytological data reviewed earlier and the numerical data summarized in Figure 21 lends itself to an interesting interpretation. The changes which take place during the 0 to 4 day interval are accompanied by the presence of the exocytotic stages of omega-shaped profiles (Figure 12) and microvesicle clusters (Figure 11). It should be pointed out that both structures are seldom found, perhaps resulting from their rapid occurence, but are found most often in the first two intervals. The significant difference in the total number of granules and vesicles over the first interval, caused almost entirely by the decrease in the number of granules and accompanied by omega-shaped profiles and microvesicle clusters, strongly suggests that exocytosis is the preferred mechanism of release during the 0 to 4 day interval.

In exocytosis, the limiting membrane of the granule fuses with the axolemma and the contents of the granule are extruded into the extracellular spaces. The membrane then

collapses and ruptures, and the remnants round up in microvesicles. Normann (1970) produced persuasive micrographs of the stages of exocytosis which are represented schematically in Figure 22. The function of the microvesicles as shown by Normann (1970) is the retrevial of the granule membrane that had been fused to the axolemma. This is a much different concept than those proposed and endorsed by other researchers (Holmes and Knowles, 1960; Koelle, 1961; Scharrer and Kater, 1969). Bunt and Ashby (1967) proposed that the microvesicles originate as inpocketings of the axolemma, and the retrievable mechanism is convincingly demonstrated by the uptake of ferritin, an electron-opaque tracer molecule, by the microvesicles (Smith, 1970, 1971). Ferritin was found only in the extracellular space and microvesicles; never in the axoplasm.

Evidence for exocytosis has been known for some time for animals other than insects, for example in the release of zymogen granules from the apical surfaces of exocrine pancreatic cells (Palade, 1959); in the release of adrenalin from the adrenal glands of vertebrates, both morphological evidence (de Robertis and Vas Ferreira, 1957) and compelling physiological evidence (Douglas and Poisner, 1966); and in release of pituitary secretion in the platyfish (Weiss, 1965). In addition to this present study, there is direct evidence for this type of release in insects, in the corpus cardiacum of Calliphora (Normann, 1965) and of Carausius (Smith and Smith, 1966), in the release of the diuretic hormone of

Figure 22. Schematic representation of the stages of exocytosis as described by Normann (1970). Key: A. newly established connection; a very delicate membrance. B. membrane fuses; axolemma is drawn inward. C. omega-shaped profile. D. membrane collapses and ruptures with remnants rounding up in microvesicles. E. microvesicle cluster; membrance retrieval.


A
B
C
D
E

Rhodnius (Maddrell, 1966), and in the release of neurosecretory material in the abdominal neurohemal organs of Carausius (Brady and Maddrell, 1967).

The release of neurohormone by exocytosis is thought to be induced by nerve impulses. In Blaberus (Wigglesworth, 1964) and in Periplaneta (Gosbee et. al., 1968), as well as other animals (Cooke, 1967; Finlayson and Osborne, 1975), electrical stimulation leads to the discharge of neurohormone from the neurosecretory cells and, in addition, high concentrations of potassium, which might be expected to stimulate the depolarization by nerve impulses, likewise lead to depletion. When a nerve impulse depolarizes the axon membrane, that makes it possible for the granule membrane to fuse with the cell membrane (Normann, 1965). This is supported by the fact that the frequency of "synaptic sites" (microvesicle clusters) and omega-shaped profiles increase when neurosecretory cells are experimentally induced to release neurohormone (Normann, 1969). In addition, the action potentials of some neurosecretory cells are known to have a slower rate of firing and a longer duration (Finlayson and Osborne, 1975), correlating possibly with the mechanics of exocytosis. In neurosecretory cells the granules are large and located some distance from the axolemma making a long, slow impulse most effective in attracting the granule to the axolemma. In a motor, sensory, or integrating neuron the snyaptic vesicles are very small in comparison to neurosecretory granules and are located close to the axolemma

making a short, fast impulse sufficient to induce exocytosis of synaptic vesicles.

A frequently observed feature of exocytosis is the presence of intact extracellular neurosecretory droplets (Smith and Smith, 1966; Smith, 1970; Normann, 1970; Finlayson and Osborne, 1975). These droplets were not seen in the present study. A possible explanation for this difference may have to do with the fluidity of the neurosecretory material or how readily the complex may be dissociated. The more fluid or readily dissociable a neurosecretory material is the less likely it would be seen as an intact extracellular droplet.

The objection to this mechanism is that in some areas of presumed release no instances of the characteristic omega-shaped profiles of exocytosis could be found (Scharrer, 1963, 1967a, 1967b, 1968; Barer and Lederis, 1966; Johnson, 1966a, 1966b; and Bowers and Johnson, 1966). However, if the membrane coalescence involved in the release were over quickly, then examples might only rarely be found as in this study. This might explain why no omega-shaped profiles have been found at the apparent synapses of neurosecretory axons.

Although exocytosis may indeed continue through all or parts of the 4 day to 10 day interval, the cytological and quantitative evidence suggests that another mechanism of release is preferred. The following facts indicate that release during the 4 to 10 day interval is diffusion or

molecular dispersion. The total number of granules plus vesicles is relatively stable (21.2 G+V/u^2 to 22.9 G+V/u^2 over the interval), while the number of granules alone and the number of vesicles alone interchange (13.1 G/u^2 to 10.5 G/u^2, and 8.1 V/u^2 to 12.4 V/u^2 over the interval). Structures of exocytosis are now rarely found, and when microvesicles are found, they tend to be dispersed throughout the axoplasm (Figures 12, 13, and 14). Granules exhibit diversity in opacity and density accompanied by darkening of the cytoplasmic background (Figure 15).

It was noticed a number of years ago (Gerschenfeld et. al., 1960) that under conditions of experimentally induced hormone release from the posterior pituitary, the electron-dense contents of the neurosecretory granules were leached, leaving behind empty vesicles. Since then, a similar loss of electron opacity of granules following stimulation of neurosecretory cells has been reported many times (Bunt and Ashby, 1968; Douglas et. al., 1971). It has been stated that the different degrees of electron opacity of the granules may be correlated with the hormonal substance they carry or represent (Lane and Treherne, 1972). Some researchers (de Robertis, 1964; Barer and Lederis, 1966) have suggested that the differences in electron opacity may reflect stages of neurohormone release in which the neurohormones are dispersed in a diffusable form into the cytoplasm from whence they are discharged to the exterior through the cell membrane.

An increased electron density of the cytoplasmic background has been reported in conjunction with the paling of granules (Weitzman, 1969; Scharrer and Kater, 1969). This should not be confused with the paling of granules in intrinsic corpus cardiacum cells as they move from the perikaryon to the short stubby cell processes (Scharrer, 1963). In this case, the paling of granules do not result in the release of neurohormone, but suggest further chemical changes take place while the granule is in transit.

It is possible that the paling of neurosecretory granules under conditions of probable release might be a result of local ionic changes caused by nervous activity. The passage of many nervous impulses along a small axon would be expected dramatically to alter the internal sodium and potassium concentrations (Katz, 1966). At the ending of such an axon, the surface area/volume ratio is even higher, so nervous activity there would have a greater effect on the internal ionic concentrations. This in turn might well alter the configuration and so the staining properties of the contents of the neurosecretory granules (Barer and Lederis, 1966), without necessarily involving the loss of their contents. Small osmophilic components may leach into the cytoplasm resulting in paling (Andrews et. al., 1971). However, these ionic and osmophilic mechanisms may be the means by which a molecular dispersion mechanism operates.

Maddrell (1970) offers three objections to diffusion as a mechanism of neurohormone release. First, the neurosecretory product must traverse the granule membrane, cross the cytoplasm and penetrate the relatively impermeable plasma membrane. Second, the neurohormones have potent actions on biological systems and it seems paradoxical to invoke the release of such compounds inside cells which may themselves be affected. Third, the inevitable dilution of the neurosecretory material by the cytoplasm would reduce the rate at which effective concentrations outside the cell could be achieved. While these are serious objections, it is the view of this author that each can be overcome.

While it is true that the axon membrane is normally quite impermeable, nerve impulses may alter the permeability of the membrane or activate a gating mechanism to allow passage of the neurohormone from the terminal. Alternately the neurohormone may be stored as a complex of smaller fractions which may be induced to cleave and then be capable of passing through the membrane. It is not certain that the neurohormone would have any deleterious effects on the axoplasm if released internally. However, possible deleterious effects could be minimized by complex formation. Calcium is known to be an essential factor in excitation-secretion coupling (Thorn, 1970), and it could form a complex with the neurosecretory material in the axoplasm to temporarily inactivate it. Finally, if diffusion were to take place one

granule at a time, Maddrell's third objection mentioned above would make sense. However, the results of this study show that the release of neurosecretory material from the granules takes place in mass. The large amount of neurohormone released into the axoplasm would probably overcome dilution and set up a diffusion gradient with the glial environment.

Berlind (1977) cautions that conclusions based on the changes in density of granular contents or gross morphology of granules in the terminals should be made conservatively. Such changes may result from a variety of nonphysiological factors including the duration of exposure to various chemicals, the pH of the fixatives and buffers, the choice of buffers (Fitch, 1972), and the temperature of the fixatives (Douglas et. al., 1971). While a reasonable doubt may exist concerning causes for granule paling, the combined evidence of this study favors molecular dispersion or diffusion during the 4 day to 10 day interval. Incidentally, the idea of more than one concommitant mechanism of release is not new. It has been previously suggested that different mechanisms exist not only in different cells of a single organism (Cassier and Fain-Maurel, 1970; Juberthie and Juberthie-Jupeau, 1974), but also within the same cell of a single organism (Weitzman, 1969).

The 10 to 13 day interval is apparently representative of a dying animal. Notice that the slope of the change in granules plus vesicles over the interval is very steep, and

the slopes of granules alone and vesicles alone are similar (Figure 21). This is probably reflective of the indiscriminate engulfing of granules and vesicles in multivesicular and multilamellar bodies (Figures 16A and B, and 17). Although the neurosecretory terminals of vertebrates have a much more ordered microanatomy (Polenov and Garlov, 1971), they contain multivesicular bodies and multilamellar bodies in their "life cycle" similar to those seen in this study (Figures 16A and B, 17, and 18). They are considered to be functioning in a process of autolytic reorganization (Dellmann, 1973; Dellmann et. al., 1974). It has been assumed that there is a connection between these bodies and lysosomes (Kurosumi et. al., 1964; Pilgrim, 1970). It has also been suggested that these bodies originate from mitochondria in the process of degeneration (Lederis, 1964; Belenky, 1969; Figure 18). The double-layered membrane of the multivesicular body seen in Figure 17 is supportative of this idea. Beattie (1976) has described multilaminate structures in cells of the neurohemal organ in the wall of the ampulla of the antennal pulsatile organ in Periplaneta which are undergoing autolysis.

This 10 to 13 day interval probably represents a critical phase for the survival of the cockroach. Reorganization of the terminal is attempted, but because of depleted sources of nutrient the energy-requiring restoration is interrupted and is overtaken by the ensuing death of the animal.

As previously noted, the values for standard deviation are large. This is probably due either to differences in individual organisms or factors such as temperature and humidity which were not finely controlled.

PHYSIOLOGICAL ROLE OF THE PERISYMPATHETIC ORGAN

In Periplaneta, neurosecretions by the corpus cardiacum and the terminal abdominal ganglion are involved in the control of diuresis (Mills, 1967), antidiuresis (Wall and Ralph, 1965; Wall, 1967), fat body metabolism (Steele, 1961; Wiens and Gilbert, 1965), tanning (Mills et. al., 1965), and integumentary transpiration (Treherne and Willmer, 1975). Previous studies on the perisympathetic organs in Periplaneta have revealed antidiuretic properties (de Bessé and Cazal, 1968) while in other insects these organs are associated with tanning (Delachambre et. al., 1972; Grillot et. al., 1976), blood protein levels during imaginal life (Raabe and Demarti-Lachaise, 1973), heart rhythm, diuresis, and are conspicuously located on the nerve innervating the moulting gland (Raabe, et. al., 1973).

The physiological role of the abdominal perisympathetic organs in Periplaneta is not unequivocally determined. One possibility is that it controls trehalose levels in the hemolymph. DDT-treated Periplaneta produced increased levels of trehalose (Granett and Leeling, 1972), and show a dramatic depletion of the small electron-dense granules (Type II) in the perisympathetic organs (Fitch, 1972). Trehalose

concentration is also elevated when electrically stimulated fluid secreted from the corpus cardiacum is injected into whole animals (Normann and Duve, 1969). The hyperglycemic factor is secreted from the intrinsic cells of the corpus cardiacum containing small electron-dense (aminergic) granules (Highnam and Goldsworthy, 1972). This suggests that the neurohormone which stimulates elevated levels of trehalose concentrations is secreted from small electron-dense granules.

Another recent study has shown that glycogen metabolism of cockroach nerve cord appears to be under control of hormones secreted from the corpus cardiacum (Hart and Steele, 1973). Similarly, the injection of octopamine into intact cockroaches in amounts as low as 25 nM caused severe depletion of nerve cord glycogen (Robertson and Steele, 1972, 1973a). Metabolism of glycogen is controlled by the rate-limiting enzyme phosphorylase. Nerve cords which have been treated in vitro with corpus cardiacum extract or octopamine show a significantly higher level of phosphorylase activity than do control tissues (Hart and Steele, 1973; Robertson and Steele, 1973a). Since it is known that several peptide hormones as well as some catecholamines and indoleamines stimulate the production of cyclic-AMP from ATP in the presence of an adenyl cyclase (Harrow and Mazur, 1966), the available evidence suggests that regulation of phosphorylase activity, and ultimately glycogen metabolism, could be achieved by alterations in the intracellular level of cyclic-AMP. Treatment

of nerve cords with cyclic-AMP or caffeine, which is known to cause its accumulation, results in augmented levels of phosphorylase activity (Hart and Steele, 1972).

Since the perisympathetic organs of Periplaneta have an uptake mechanism for dopamine (Smalley, 1970), but do not normally show the fluorescence associated with this catecholamine (Fitch, 1972), it is possible that they contain a monophenolic amine such as octopamine. Relatively high concentrations of octopamine have been found in the nerve cord of Periplaneta (Robertson and Steele, 1973b). Since the cockroach nerve cord contains an adenyl cyclase specific for octopamine (Nathanson and Greengard, 1973), it is possible that octopamine might be involved in glycogen metabolism by stimulating the production of cyclic-AMP.

In summary, elevation of the concentration of trehalose, a product of glycogen metabolism in insects, is stimulated by neurohormone released by small electron-dense granules. The biogenic amine octopamine stimulates glycogenolysis, and is present in relatively high concentrations in Periplaneta nerve cord. The following hypothesis may then be suggested as a possible physiological role of abdominal perisympathetic organs in Periplaneta. The biogenic amine octopamine is secreted from the small electron-dense granules of Type II axon terminals of the perisympathetic organs. In the presence of its specific adenyl cyclase, octopamine stimulates the production of cyclic-AMP which in turn mediates the phosphorylase activity that produces trehalose from stored glycogen.

In view of the findings of de Bessé and Cazal (1968) which associate perisympathetic organs of Periplaneta with antidiuresis, suggesting that the perisympathetic organs are involved in glycogenolysis seems contradictory, especially since the findings of this research were a result of both dehydration and starvation. It is for just that reason this suggestion was made. Certainly, further tests along the lines of this research should be conducted using dehydration only and starvation only; but, since a single hormone may have more than one function depending on its level of concentration in the blood (Kramer, personal communication), the neurohormone represented by the Type II granules of the abdominal perisympathetic organs in Periplaneta may indeed have a dual role in antidiuresis and glycogenolysis.

SUMMARY

The abdominal perisympathetic organ of Periplaneta americana are neurohemal organs. This study describes several cytological phenomena which are consistent with their neurohemal function. The perineurium, a barrier to large diffusing molecules, is missing. The glial structural integrity is maintained by desmosomes and septate desmosomes which allow neurohormone to pass freely through mesaxons and glial extracellular spaces. No tight junctions, which restrict the passage of large diffusable molecules, are found. The neurosecretory release structures referred to as "synaptic sites" and omega-shaped profiles are present. The tracheal supply is sparse which would be expected in tissue where the main function is storage and release of neurohormone.

The perisympathetic organ's general profile reveals three types of axon endings containing three mutually exclusive types of neurosecretion. Type I contains large classical electron-dense granules. Type II contains small electron-dense granules and electron-translucent vesicles. Type III contains only intermediately sized electron-translucent vesicles.

Stressing the cockroach by dehydration and starvation causes ultrastructural changes in Type II terminals. During normal conditions and early stages of stress, the mechanism of neurosecretory release is exocytosis. As stress continues, the preferred mechanism of neurosecretory release shifts to

molecular dispersion. These conclusions are based on changes in the granule to vesicle ratio, changes in the frequency of release sites, paling of granules, and variations in the distribution of microvisicles. When nutrient reserves are apparently dissipated, autolytic activity begins and is denoted by the presence of multivesicular and multilamellar bodies.

The physiological role of the neurohormone contained in Type II electron-dense granules may be antidiuretic or glycogenolytic or both. Studies using dehydration only and starvation only need to be carried out to test this hypothesis.

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