# AXONAL AGRANULAR RETICULUM AND

# SYNAPTIC VESICLES IN CULTURED

# EMBRYONIC CHICK SYMPATHETIC NEURONS

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## ABSTRACT

Cultured chick embryonic sympathetic neurons contain an extensive axonal network of sacs
and tubules of agranular reticulum. The reticulum is also seen branching into networks in
axon terminals and varicosities. The axonal reticulum and perikaryal endoplasmic reticulum
resemble one another in their content of cytochemically demonstrable enzyme activities
(G6Pase and IDPase) and in their characteristic membrane thicknesses (narrower than
plasma membrane or some Golgi membranes). From the reticulum, both along the axon and
at terminals, there appear to form dense-cored vesicles ranging in size from 400 to 1,000 Å in
diameter. These vesicles behave pharmacologically and cytochemically like the classes of
large and small catecholamine storage vesicles found in several adrenergic systems; for
example, they can accumulate exogenous 5-hydroxydopamine. In addition, dense-cored
vesicles at the larger (1,000 Å) end of the size spectrum appear to arise within perikaryal
membrane systems associated with the Golgi apparatus; this is true also of very large (800–
3,500 Å) dense-cored vesicles found in some perikarya.

## INTRODUCTION

Despite its widespread occurrence (cf. 88) little has been established about the functions of the agranular membrane-delimited tubules and sacs found in axons and often referred to as axonal smooth endoplasmic reticulum (ER) or agranular reticulum. For example, the relationships between this system and perikaryal membrane systems have not been established. On general grounds, Palay (82) early suggested that the axonal reticulum might serve as a source of synaptic vesicles. Cytochemical studies (44, 48, 96, 110) indicated that the axonal reticulum is a likely transport route for acid hydrolases, and several workers (55, 99) have demonstrated acetylcholinesterase in the sacs and tubules. It has been pointed out (43, 44, 55) that segregation of materials in the reticulum might ex-plain some of the heterogeneity of transport rates in axons (59, 80).

Our interest in the axonal reticulum of chick sympathetic axons derives from our initial observations of frequent continuities between large dense-cored vesicles and elements of the reticulum (44, 98). There is some evidence suggesting the origin of some "synaptic vesicles" in perikarya (17, 35, 36, 37, 104) and their transport down the axon, perhaps along microtubules (3, 42, 95). But this in no sense rules out other sites of origin and modes of transport. For example we have demonstrated that some of the vesicles in synaptic regions are participants in endocytosis-like events that seem to be linked to transmission (47, 51) and

others have recently published confirmatory findings (14, 39, 40).

The present report concerns a morphological and cytochemical study of cultured embryonic chick sympathetic neurons. In these cells, the axonal reticulum is a network of tubules and sacs found all along the axon, including axon terminals and varicosities. Our findings indicate that perikaryal and axonal ER resemble one another in several respects, including cytochemically demonstrable enzyme activities. From the axonal reticulum there appear to form both the larger and smaller categories of dense-cored vesicles found at the axon terminals. These vesicles have cytochemical characteristics usually associated with catecholamines (cf. 7, 8). There also are indications that some catecholamine-containing vesicles are "packaged" near the Golgi apparatus of perikarya.

## MATERIALS AND METHODS

### Preparation of Cultures

Lower thoracic and lumbar sympathetic chain ganglia dissected from 10-day old White Leghorn chick embryos (Shamrock Farms, North Brunswick, N. J.) in Hanks's Basic Salt Solution (International Biological Laboratories, Bethesda, Md. [IBL]) were collected in a modification of the culture medium of England (22) containing 70% Medium 199 (IBL), 30% fetal bovine serum (IBL), 50 units/ml of Nerve Growth Factor (63) (Wellcome Research Laboratories, Beckenham, England), and 1% antibiotic-antimycotic (Grand Island Biological Co., Grand Island, N. Y.). Ganglia from two to three embryos were used for each tissue culture dish to be plated; the ganglia were minced into 1 $mm^2$ fragments and incubated at 37°C in a modified trypsin-collagenase dissociation medium of Coon (15) for 60–90 min. Tissue was dissociated in fresh culture medium by vigorous pipetting and washed in fresh medium. This procedure yields a suspension of single, rounded cells and a few groups of small aggregates of rounded cells. 0.2 ml of this suspension was placed on UV-sterilized, collagen-coated (10), 30-mm Falcon Petri dishes (Falcon Plastics, Oxnard, Calif.) containing 1.8 ml of culture medium and maintained for up to 8 days in a National incubator (National Appliance, Portland, Oreg.) at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air.

### Drug Treatments

5 × $10^{-5}$ M 5-hydroxydopamine (Hoffman-La Roche, Inc., Nutley, N. J.) was added to the medium of some cultures 6–16 h before fixation (102). In some cases a monoamine oxidase inhibitor, 5 × $10^{-5}$ M iproniazid or nialamide (Sigma Chemical Co., St. Louis, Mo.), was simultaneously present (53). In the longer term treatments, medium and drugs were replaced after the first 6–8 h to prevent a buildup of oxidized amine by-products that could harm the cultures (89).

A few 4- and 7-day old cultures were exposed to 25–250 μg/ml of reserpine (53) (Serpasil; Ciba Pharmaceutical Co., Summit, N. J.), added to the culture medium 3–4 h before fixation. Longer treatments proved harmful to the cells.

Other 4- and 7-day old cultures were treated with 5 × $10^{-5}$ M noradrenaline bitartrate (53) (Sigma Chemical Co.), added to the culture medium 16 h before fixation. In some of these cultures 5 × $10^{-5}$ M nialamide was added simultaneously.

### Routine Fixation and Electron Microscope Preparation

For routine examination, cultures were fixed in 0.1 M phosphate-buffered (pH 7.2) 2.5% glutaraldehyde (92) at room temperature for 30 min and then on ice for an additional 30 min. Cultures were then briefly rinsed in cold 0.2 M phosphate buffer, postfixed on ice in 1% osmium tetroxide in 0.1 M phosphate buffer at pH 7.2, rinsed in cold 7.5% sucrose, stained with uranyl acetate en bloc (27) at room temperature, rinsed in cold 7.5% sucrose, and dehydrated in a graded series of ethanols. After 100% ethanol, Epon (66) was added directly to the plastic culture dish according to the method of P. Claude (personal communication), left overnight at room temperature, and then polymerized at 60°C for 24 h. The plastic dish was then pried away. This embedding procedure provides flat orientation of the small axon bundles and groups of perikarya. Propylene oxide steps, which dissolve plastic, are not necessary for this thin tissue.

### Cytochemical Preparations

PHOSPHATASES: For most phosphatase incubations, tissue was fixed for 4–10 min on ice in 0.1 M cadodylate-buffered (pH 7.2) 2% glutaraldehyde then rinsed overnight in cold (pH 7.2) 0.1 M cacodylate buffer. Both fixes and rinses often contained the substrate to be used in the cytochemical incubation at a concentration of 6 mM. Before incubation the tissue was rinsed briefly in 7.5% sucrose, frozen for 30 s on the head of a freezing microtome, and rinsed in 0.1 M cacodylate buffer (pH 7.2) with 7% sucrose.

The short glutaraldehyde fixation with substrate present was suggested by the work of Leskes et al. (61) on glucose-6-phosphatase (G6Pase). This pro-

cedure provides reasonably good morphological preservation for electron microscopy and extensive cytochemically demonstrable enzyme activity. Leskes et al. have shown that in hepatocytes almost 80% of the biochemically assayable G6Pase activity is retained after similar procedures.

For some acid phosphatase preparations, plates were fixed for 1 h in 0.1 M cacodylate-buffered 2% glutaraldehyde with 1% sucrose at pH 7.2 (72), rinsed overnight in 0.1 M cacodylate at pH 7.2 with 7% sucrose, and then briefly frozen on the head of a freezing microtome. The longer fixation provided superior morphological preservation for the acid phosphatase studies.

Cultures were incubated in the following media to demonstrate phosphatase activities (usual incubation time is indicated in parentheses): G6Pase in a modified Wachstein-Meisel (106) medium containing 2 mM glucose-6-phosphate (G6P) sodium salt (Sigma Chemical Co.), 2 mM lead nitrate, 0.05 M Tris-maleate buffer adjusted to pH 6.75 (60–90 min), or in the Leskes medium (61) (75 min); inosine diphosphatase (IDPase) in the Novikoff-Goldfischer medium (32, 77) (30–120 min); acid phosphatase with 5'-cytidine monophosphate (CMP) (P-L Biochemicals, Milwaukee, Wisc.) (74) or β-glycerophosphate (βGP) (Sigma Chemical Co.) as substrate with

the Gomori medium (34) using acetate or Tris buffer (4) adjusted to pH 5.0 (60–90 min).

All enzyme reactions were carried out at 37°C with 5% sucrose added to the medium except for the Leskes G6Pase incubation, which was done at 27°C without any sucrose.

For phosphatase controls, media were used complete except for substrate. In addition, control acid phosphatase incubations were performed using the full medium to which 0.01 M NaF was added. Also, in some material prepared for G6Pase study (including fixation and rinses with G6P), 5'-CMP was substituted for G6P in the final incubation medium. Thiamine pyrophosphate (TPP) (Sigma Chemical Co.) was similarly substituted for inosine diphosphate (IDP) (Sigma Chemical Co.) as substrate in an otherwise complete IDPase medium (32, 77).

Light microscope samples of phosphatase-incubated cultures were visualized with dilute ammonium sulfide after incubation (or in a few cases by treating 1 μm thick Epon sections with concentrated ammonium sulfide for 1 min [87]). In addition, heavy deposits of reaction product can often also be detected by phase microscopy of Epon-thick sections not treated with sulfide.

Incubations for electron microscopy were stopped by transferring the cultures to 0.1 M cacodylate-

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Figs. 20–25 are light micrographs. Bar length is 5 μm. The remainder of the figures are electron micrographs. Bar length is 0.5 μm.

FIGURE 1    Electron micrograph of a region near the terminal of a week old culture that was not specially treated. A multivesicular body (MV) is seen near a cluster of small, clear vesicles (V), some of which (arrows) contain a tiny core. Also present are large dense-cored vesicles (D), narrow tubular elements of agranular reticulum (R), as well as an elongate broad tubule (T). Stained with uranyl and lead. × 60,000.

FIGURE 2    Terminal region from a week old culture not specially treated. Small, clear vesicles (V) are seen; a few contain a tiny core (arrow). Large dense-cored vesicles are seen at D. A network of branching tubular agranular reticulum (R) is present. Note subsurface cistern (SC) in an adjacent cell and density (DE) in the region of contact between the terminal and an adjacent cell. Stained with uranyl and lead. × 54,000.

FIGURE 3    Electron micrograph of a portion of an axon from a preparation exposed to reserpine for 3 h. A cluster of the larger class (750 Å diameter) of dense-cored vesicles is seen in which the cores appear as if depleted of their dense material (arrow). A pale corelike structure is retained. M indicates part of a mitochondrion and R, part of a cisterna of agranular reticulum. Stained with uranyl and lead. × 84,000.

FIGURE 4    Terminal region from a culture treated with 5-hydroxydopamine and iproniazid for 6 h. Small, clear vesicles (V), large dense-cored vesicles (D), and smaller dense-cored vesicles (arrow) are seen. Some of the small dense-cored vesicles are virtually entirely filled with electron-opaque material and some are only partially filled. Density (DE) is seen in the region of contact between the axon and an adjacent cell. Stained with uranyl and lead. × 66,000.

FIGURE 5    Electron micrograph of material from a culture treated with noradrenalin and nialamide and incubated with the Woods chromaffin reagents (120 min). The preparation was not exposed to osmium or uranyl subsequent to the chromaffin reaction (hence the absence of membrane electron density). A cluster of chromaffin-reactive cores is seen (arrow). The cores are approximately 250 Å in diameter and belong to the smaller class of dense-cored vesicles. Lead stained. × 40,000.
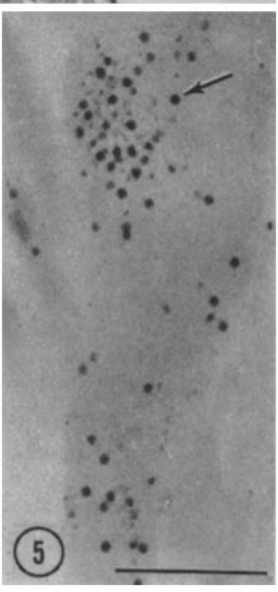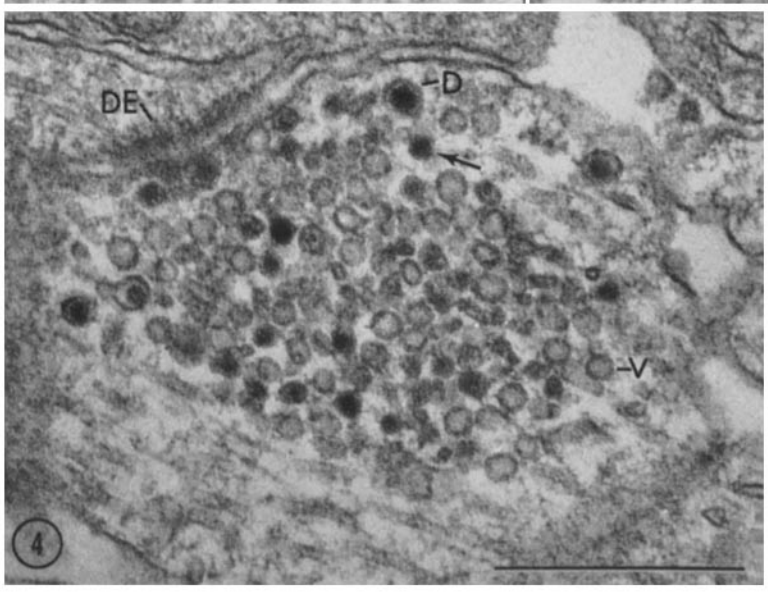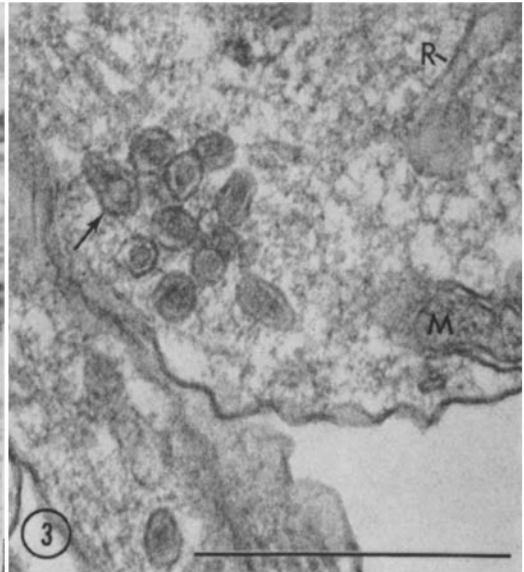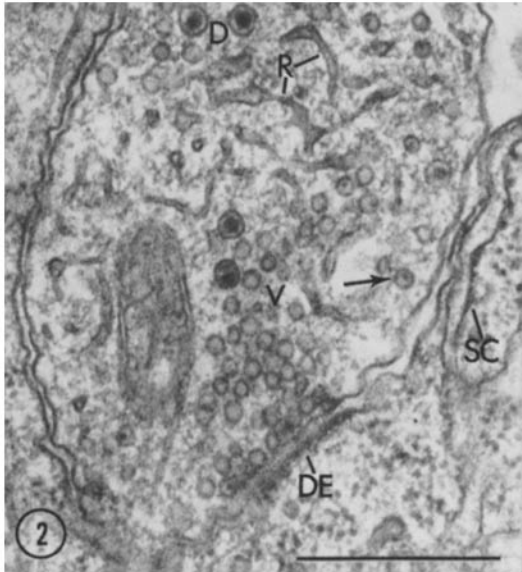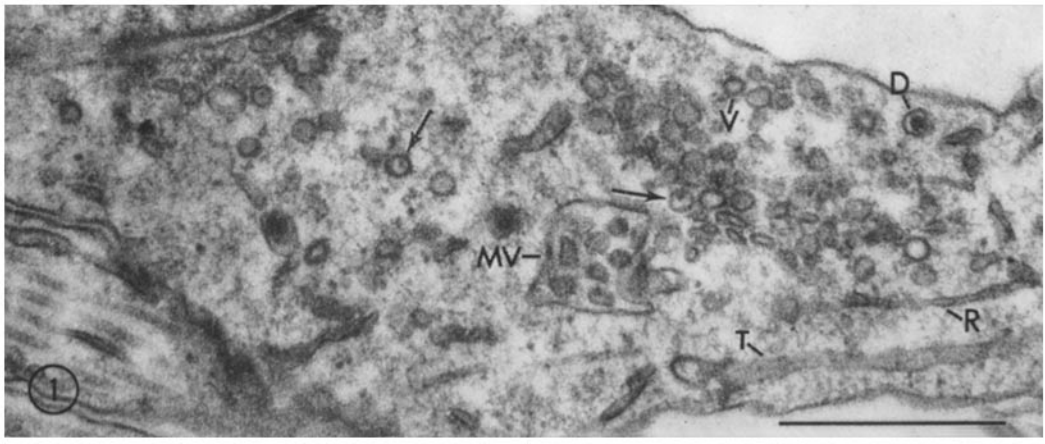
buffered 1% osmium tetroxide at pH 7.2 on ice. Material was then prepared for electron microscopy as described above.

CHROMAFFIN REACTION: Controls and cultures exposed to $5 \times 10^{-5}$ M noradrenalin bitartrate (Sigma Chemical Co.) or noradrenalin and $5 \times 10^{-5}$ M nialamide were incubated for the chromaffin reaction of Woods (112) using potassium dichromate at pH 4.0. Postosmicated and non-osmicated materials were examined. No uranyl soak was used. Otherwise, cultures were prepared for electron microscopy as above.

## Comparisons of Membrane Thickness

These were done on electron microscope sections of uranyl-soaked material stained with uranyl and lead. We selected membrane regions which showed sharp unit membrane structure and seemed to be cut transversely at points where the overall structure showed little curvature. Since our primary concern was with relative dimensions, we determined the ratios of diameters of Golgi apparatus (the thicker membranes), ER, and plasma membranes within the same perikarya and compared these with similar data for axonal reticulum and plasma membranes obtained from the same sections as were used for the perikarya. Measurements of the photographs were made with a Bausch and Lomb 7x ocular micrometer (Baush and Lomb Incorporated, Rochester, N. Y.) ruled with 0.1 mm divisions. The differences measured are readily reproducible and can be detected by careful visual inspection. For rough quantitation, we used sections with silver interference color and used micrographs at magnifications of ×160,000 and measured roughly 50 regions of each type of membrane.

## Electron Microscopy

Regions of embedded plates were selected under a dissecting microscope, cut out, and glued in proper orientation to blank holder blocks for sectioning. Sections were cut parallel to the plane in which the axon bundles and perikarya lie, on a Porter-Blum MT2 ultramicrotome (Ivan Sorvall Inc., Norwalk, Conn.), and collected on naked 300-mesh copper grids or on Formvar- and carbon-coated slot grids. Sections were unstained or stained with lead citrate (105) or with uranyl acetate in 50% ethanol (107) and lead citrate and were examined with either a Siemens 101 or an RCA EMU 3F electron microscope. Electron micrographs were taken at initial magnifications of ×4,000–40,000.

## RESULTS

Most of our attention was focused on cells 4–7 days after explantation. Unless otherwise noted, the descriptions refer to such cells.

## Light Microscopy

The overall behavior of embryonic sympathetic neurons in culture has been described elsewhere (23, 63). Briefly, the dissociated cells clump into small aggregates, attach to the culture dishes within 12 h, and grow axons for the next few days. The axons form bundles, free of recognizable Schwann cells and often appear as if running between clusters of perikarya. Fibroblastic cells also appear in the cultures (22).

The results to be described below indicate that the neurons can accumulate catecholamines. Additional autoradiographic and fluorescence microscopic information is outlined elsewhere (51); in

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FIGURES 6 a and b Two consecutive sections of an axon bundle from a week old culture that was not specially treated. Reference points are indicated by A, B, C, and microtubules by T. The short sacs of agranular reticulum seen in 6 a (arrows) are actually part of a longer portion of agranular reticulum as is evident by comparison with 6 b. Stained with uranyl and lead. × 30,000.

FIGURES 7 a and b Two consecutive sections through an axonal varicosity filled with small and large dense-cored vesicles (from material treated with 5-hydroxydopamine). Reference points are indicated by A, B, and C. Microtubules are indicated by T. Comparison of 7 a and 7 b indicates that the tubular elements of agranular reticulum in the varicosity (arrows) are branches from a small cisterna of reticulum (R). The reticulum appears to enter the varicosity (7 b) from the axon at N. Note the attachment of a large dense-cored vesicle to a tubule of reticulum (D in 7 a). A configuration that probably is similar is seen at F in 7 b. × 56,000.

FIGURE 8 Cluster of vesicles in an axon from a 4-day old culture treated with 5-hydroxydopamine for 6 h. A dense-cored vesicle of the smaller class is seen attached to agranular reticulum (R). Microtubules are indicated by T and the axon plasma membrane by P. × 88,000.
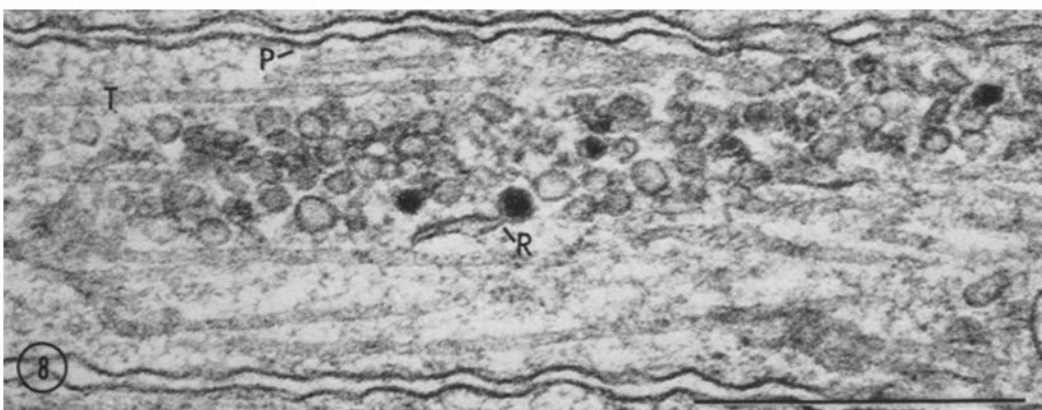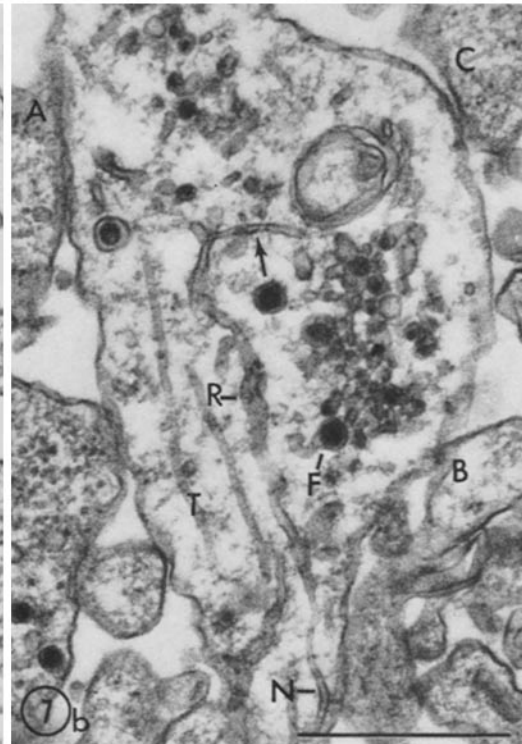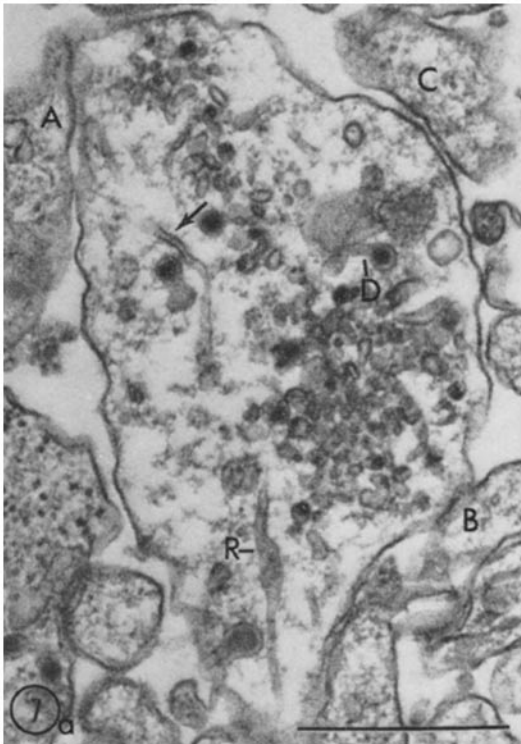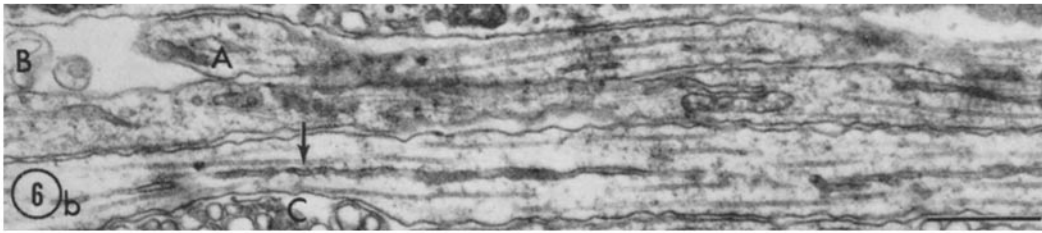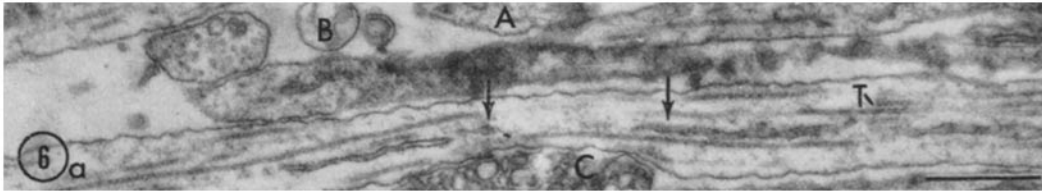
accumulating catecholamines the neurons behave as expected from studies in other laboratories (13, 22, 28).

As the cultures mature, perikaryal organelles undergo distributional shifts similar to those usually associated with recovery from chromatolysis (49, 64); for example, during the first week in culture, nuclei generally move from an initial peripheral location (Figs. 20, 22) to the center of perikarya (Figs. 21, 25) and lysosomes seen in acid phosphatase preparations (Figs. 20, 21) as well as Golgi apparatus demonstrable in thiamine pyrophosphatase (TPPase) studies (Figs. 22, 25) shift from a central to a perinuclear distribution.

## Electron Microscopy

### PERIKARYA

Many of the general ultrastructural features of the perikarya of chick sympathetic neurons as well as the lack of recognizable Schwann cells at the stages examined have been noted elsewhere (62). Perikarya contain the usual organelles (mitochondria, Golgi apparatus, ER, lysosomes, etc.) as well as dense-cored vesicles, usually with diameters approaching 1,000 Å. The shifts in organelles seen by light microsopy as the cultures mature are readily seen in the electron microscope. Additionally, within 1 wk in culture, the organiza-

tion of rough ER is seen to change from scattered patches, with few attached ribosomes, to a more elaborate ribosome-studded network. As in other neuronal systems, ER is closely associated with the inner and outer faces of the Golgi apparatus (cf. 43, 44, 49, 76, 79, 83) and occasionally continuities between ribosome-studded membrane systems and smooth sacs in the middle of the Golgi stacks are encountered as well (Fig. 9). ER cisternae ("subsurface cisternae;" reference 91) are also frequently seen adjacent to plasma membrane (Fig. 2); the face closest to the plasma membrane is ribosome free, and two such cisternae often appear facing one another across the intercellular space between two adjacent perikarya.

Occasional cells with very large (800–3,500 Å diameter), dense-cored vesicles are seen among other perikarya (Fig. 11); similar cells have been noted in other preparations (62, 68, 93). Observations on this cell type focused on the perikarya; we have not extensively studied the processes.

### AXONS

Axons can be distinguished from dendrites by several features including their presence in distinctive bundles and the absence of rough ER. As detailed below, the axons contain mitochondria, microtubules, filaments, multivesicular bodies,

FIGURE 9   Golgi apparatus in the perikaryon of a week old culture that was not specially treated. Ribosomes (arrow) are seen attached to a sac that forms part of the stacked saccules of a region of the Golgi apparatus. $M$ indicates a mitochondrion and $T$ a portion of a microtubule. $\times$ 60,000.

FIGURE 10   Portion of a perikaryon of a 4-day old culture. A large dense-cored vesicle ($D$) (approximately 1,000 Å in diameter) appears as if forming in a saccule associated with the Golgi apparatus ($G$). Part of a mitochondrion is seen at $M$. $\times$ 58,000.

FIGURE 11   Electron micrograph of a portion of one of the cells which contain very large (800–3,500 Å diameter), chromaffin-reactive, dense-cored vesicles. The image suggests that these vesicles arise in Golgi-associated membrane systems. Dense material, similar to the vesicle contents, is seen accumulating in cisternae or saccules near the inner aspect of the Golgi apparatus (arrows). A very large, dense-cored vesicle ($L$) which may be newly formed is also present in this region. At $S$, a configuration is seen that may reflect splitting of the very large vesicles. $M$ indicates a mitochondrion, $N$, nuclear envelope. $\times$ 41,000.

FIGURE 12   Electron micrograph of week old material exposed to 5-hydroxydopamine. Larger (750 Å diameter) dense-cored vesicles are seen in an axonal varicosity. Three of these (arrows) are seen attached to elements of agranular reticulum. Stained with uranyl and lead. $\times$ 52,000.

FIGURE 13   Electron micrograph of an axon from the sympathetic chain ganglia of a 10-day old chick embryo (not cultured). Large dense-cored vesicles are seen ($D$) in an axon. One of the vesicles is attached to a tubule (arrow) of agranular reticulum (cf. Fig. 12). Stained with uranyl and lead. $\times$ 73,000.
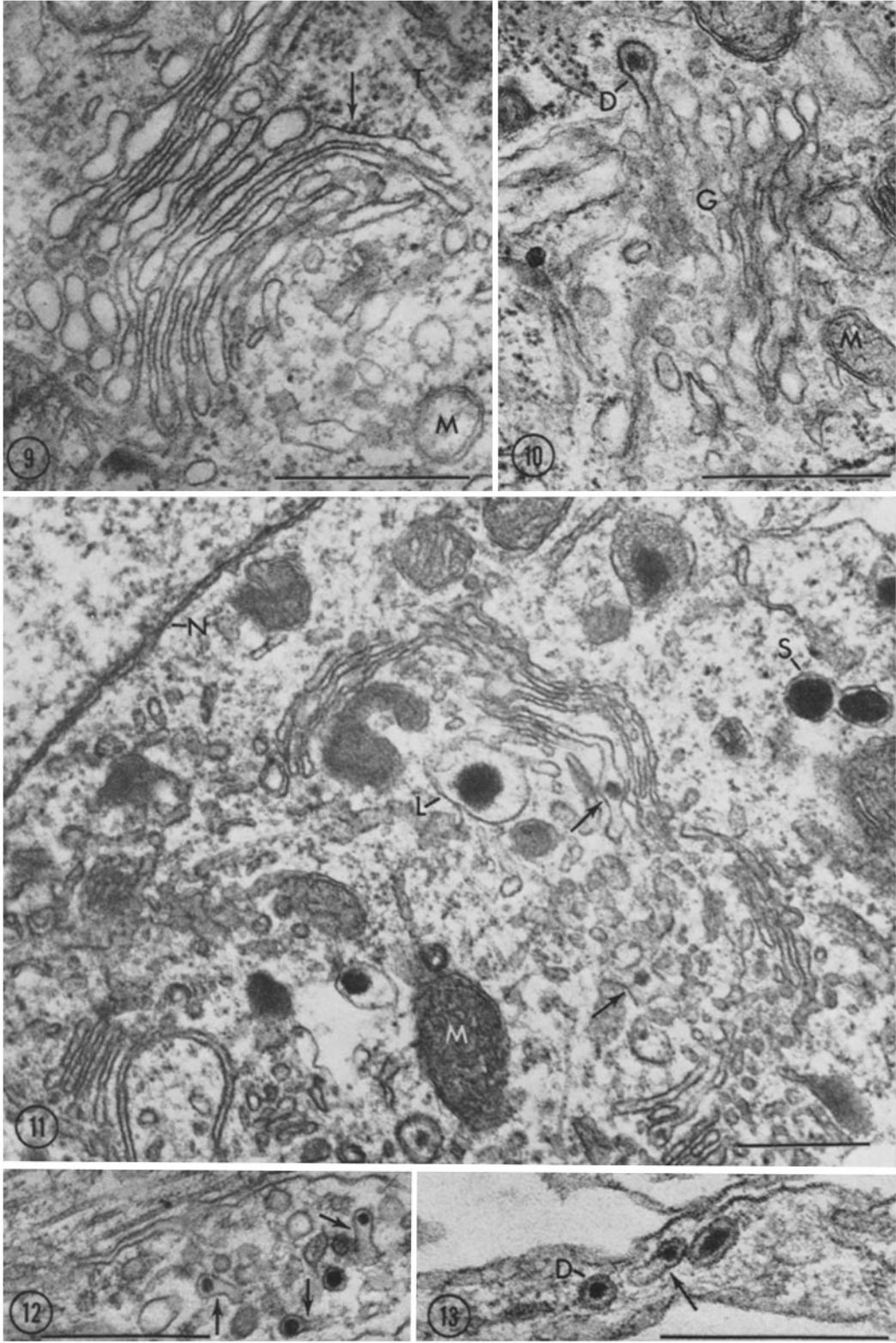
dense-cored vesicles, and sacs and tubules of agranular reticulum.

## TERMINALS AND VARICOSITIES

After 4 days in culture, structures morphologically resembling vesicle-filled terminals and varicosities appear. In what follows, "terminal" refers to an enlarged axon region full of vesicles. These axon regions contain mitochondria, microtubules, multivesicular bodies (Fig. 1), occasional autophagic vacuoles, sacs and tubules of agranular reticulum (Fig. 2), along with numerous vesicles ranging in diameter from 400 to 1,000 Å. Sometimes, electron-opaque material is seen along the cell borders where these axon regions abut on other cells (Figs. 2, 4). This density is not typical of adrenergic endings near smooth muscle (cf. 25) nor is the physiological significance of the contacts known. The density may reflect embryonic contacts similar to those seen in developing cultured central nervous systems (CNS) (12, 70).

In ordinary preparations, dense-cored vesicles at the larger (700–1,000 Å) end of the size spectrum are found, along with many small, clear vesicles (Figs. 1, 2). Occasionally, a few of the small vesicles have a tiny core that appears as a dot (Figs. 1, 2); apparently, the routine methods we use do not adequately preserve the cores of these vesicles. In 5-hydroxydopamine-treated material the dense-cored vesicles of the larger variety are seen as well as some small, clear vesicles, but many of the smaller vesicles (400–600 Å in diameter) now show distinct dense cores (Figs. 4, 14). The smaller vesicles filled with electron-opaque material appear in the 5-hydroxydopamine-treated preparations whether or not a monoamine oxidase inhibitor was also present in the medium. In other respects, the appearance of the 5-hydroxydopamine-treated cultures was "normal" as compared with controls.

Numerous clusters of smaller and large dense-cored vesicles are seen in material exposed to noradrenalin and nialamide and then incubated with the chromaffin reaction of Woods (112). As also noted in other adrenergic systems (cf. 113), chromaffin-reactive cores can be seen in material not exposed to osmium tetroxide (Fig. 5), as well as in material that was exposed to osmium. In

FIGURE 14 Cluster of vesicles in a terminal region of a week old culture treated with 5-hydroxydopamine and nialamide for 6 h. The axon plasma membrane is seen at P. Clear vesicles (V) as well as large (L) and small (S) dense-cored vesicles are present. The configuration at the arrow appears to be a small dense-cored vesicle at a stage before separation from the agranular reticulum (see Fig. 8). Stained with uranyl and lead. × 82,000.

FIGURE 15 Portion of an axonal varicosity from material treated as in Fig. 14. At arrow, a small dense-cored vesicle is seen as if about to bud from a tubule (T) of the agranular reticulum. Stained with uranyl and lead. × 90,000.

FIGURE 16 Portion of an axonal varicosity from material treated as in Fig. 14. A dense-cored vesicle is seen attached to a tubule of agranular reticulum (arrow). Note also the other elements of the reticulum; a flattened saccule (F) and portions of a branching tubular network (R). Stained with uranyl and lead. × 57,000.

FIGURE 17 Portion of an axon from a day old culture treated with 5-hydroxydopamine. Electron-opaque material is seen accumulating in a tubule of agranular reticulum (R) associated with what seems to be a forming larger dense-cored vesicle. Microtubules are seen at T. Stained with uranyl and lead. × 58,000.

FIGURE 18 Portion of a perikaryon from a week old culture incubated for acid phosphatase activity (90 min with βGP as substrate). Reaction product is seen in Golgi-associated membrane systems including GERL (arrows) and some other saccules of the Golgi apparatus (G). Face views of some of the sacs are seen at GF (cf. 79). M indicates a mitochondrion. Lead stained. × 39,000.

FIGURE 19 Portion of a perikaryon from a week old culture incubated for TPPase activity (90 min). Reaction product is seen in portions of the Golgi apparatus (G). Similar saccules show IDPase activity. No reaction product is seen in a dense body (D) or in the nuclear envelope (N). A mitochondrion is seen at M. Lead stained. × 34,000.
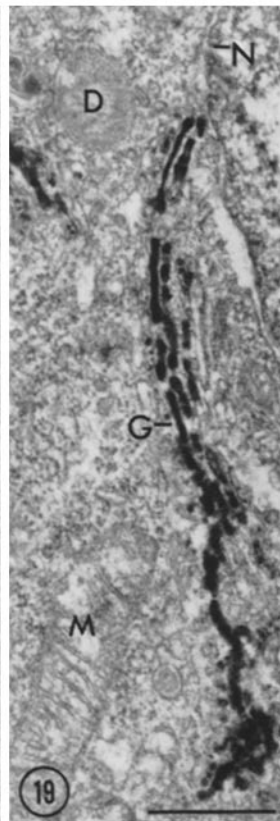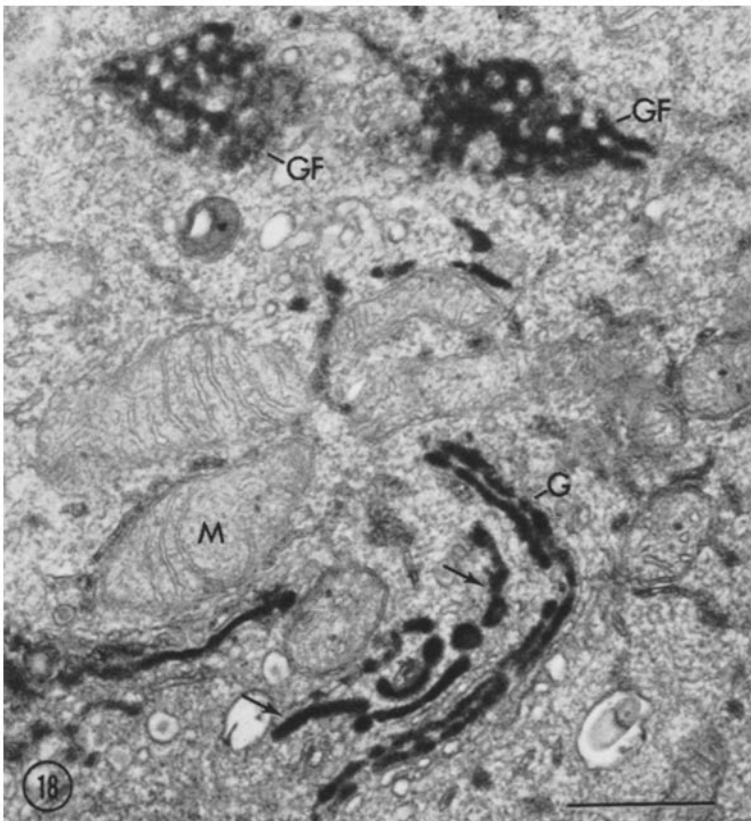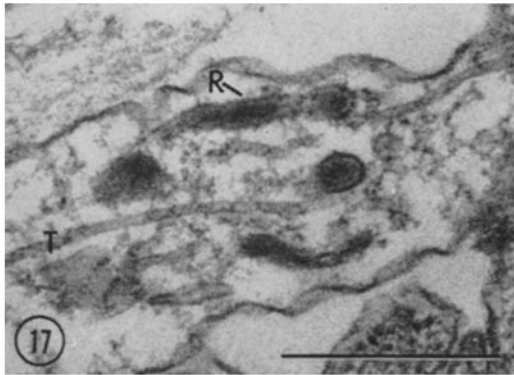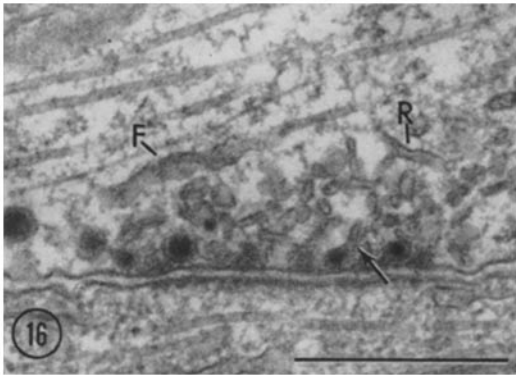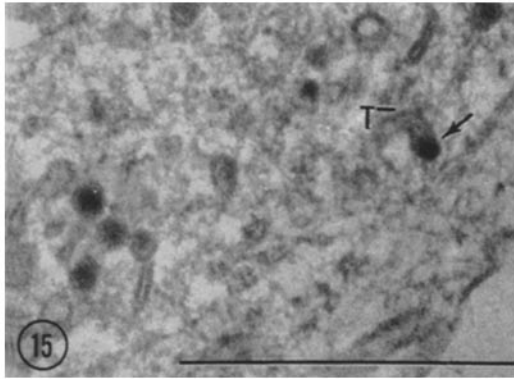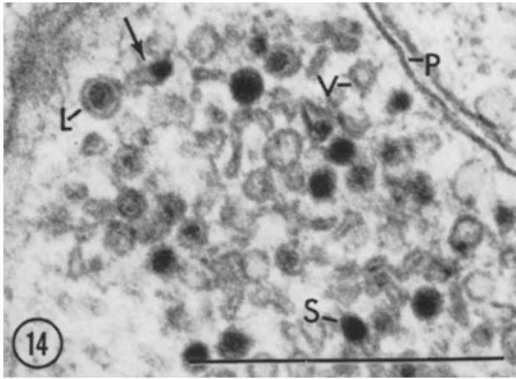
preparations not treated with noradrenalin and nialamide, only the very large, 800–3,500 Å diameter vesicles seen in occasional cells react positively; in these preparations when dichromate was omitted from the Woods medium no chromaffin-reactive cores were noted (69).

Cores of the large axonal, dense-cored vesicles show diminished electron density after a 2–4 h treatment with reserpine (Fig. 3); a pale shell-like "ghost" core appears to be retained. Reserpine treatment also produces dilations of perikaryal ER and Golgi apparatus and sometimes an increase in the frequency of autophagic vacuoles in perikarya.

### Vesicle Frequency in 5-Hydroxy-dopamine Material

As noted in some other adrenergic systems (30, 31), in perikarya and along the axon the larger dense-cored vesicles are more numerous than the smaller dense-cored vesicles. At terminals, dense-cored vesicles at the lower end of the size spectrum predominate.

### Axonal Agranular Reticulum

At all times studied, sacs and tubules of the axonal reticulum are found all along the axon including at terminals (Fig. 2). Serial sections indicate that the sacs and tubules extend for considerable lengths of axon (Figs. 6 a, 6 b) although obviously it was not practical to try to follow a given sac from axon hillock to terminals. As in many other studies (cf. 49) continuities between perikaryal rough ER and smooth ER are encountered. At the endings, the agranular reticulum branches out (Figs. 7 a, 7 b) and in the endings, narrow membrane-delimited tubules of reticulum form an elaborate network (Figs. 2, 16).

For initial characterization of the axonal reticulum, the relative thicknesses (for references, see 71) of Golgi membranes, perikaryal rough ER membranes, plasma membrane, and axonal agranular reticulum membranes were compared. All of these membranes showed "unit" structure (Figs. 30, 31). The average thickness of Golgi and plasma membranes, in these neurons, is approximately 20–25% greater than either perikaryal or axonal ER membranes (the ER membranes were roughly 50 Å thick). As one might expect, because of their close relationship to ER, Golgi membranes show a good deal of variation. The thicker and more intensely stained Golgi membranes used for the comparisons here were selected from the middle Golgi saccules.

### Formation of Dense-Cored Vesicles

Large (700–1,000 Å) dense-cored vesicles found in many perikarya are occasionally seen attached to, as if budding from, membrane systems near the Golgi apparatus (Fig. 10). The very large, chromaffin-reactive, 800–3,500 Å diameter, dense-

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Figure 20   Light micrograph of a 3-day old culture incubated for acid phosphatase activity (90 min). Reaction product is seen in granules, the lysosomes (arrows), which are clustered in the center of the perikarya. Note the peripheral position of the nuclei ($N$). $\times$ 1,500.

Figure 21   Light micrograph of perikarya from a week old culture incubated for acid phosphatase activity (90 min). Reaction product is seen in lysosomes (arrow) distributed around a centrally located nucleus ($N$). $\times$ 1,500.

Figure 22   Light micrograph of an Epon-thick section (ammonium sulfide treatment) from a 3-day old culture incubated for TPPase activity (90 min). Reaction product is seen in the Golgi apparatus ($G$) which is located in the center of a perikaryon. The nucleus ($N$) is located peripherally in the cell. $\times$ 1,700.

Figure 23   Light micrograph of a week old culture incubated in Leskes's medium for G6Pase activity (75 min). Reaction product is seen in nuclear envelope (arrows) in granules similar to those seen in Figs. 20 and 21, and at the cell surface ($S$). Nucleus is seen at $N$. $\times$ 1,600.

Figure 24   Light micrograph of a week old culture incubated for IDPase activity (90 min). Reaction product is seen in the nuclear envelope (arrows). Cytoplasmic reaction product is also present but is difficult to analyze by light microscopy. Nuclei are seen at $N$. $\times$ 1,700.

Figure 25   Light micrograph of a week old culture incubated for TPPase activity (90 min). The Golgi apparatus ($G$) is distributed around a centrally located nucleus ($N$). Light reaction product is also seen in the nucleolus (arrow) (cf. 76). $\times$ 1,500.
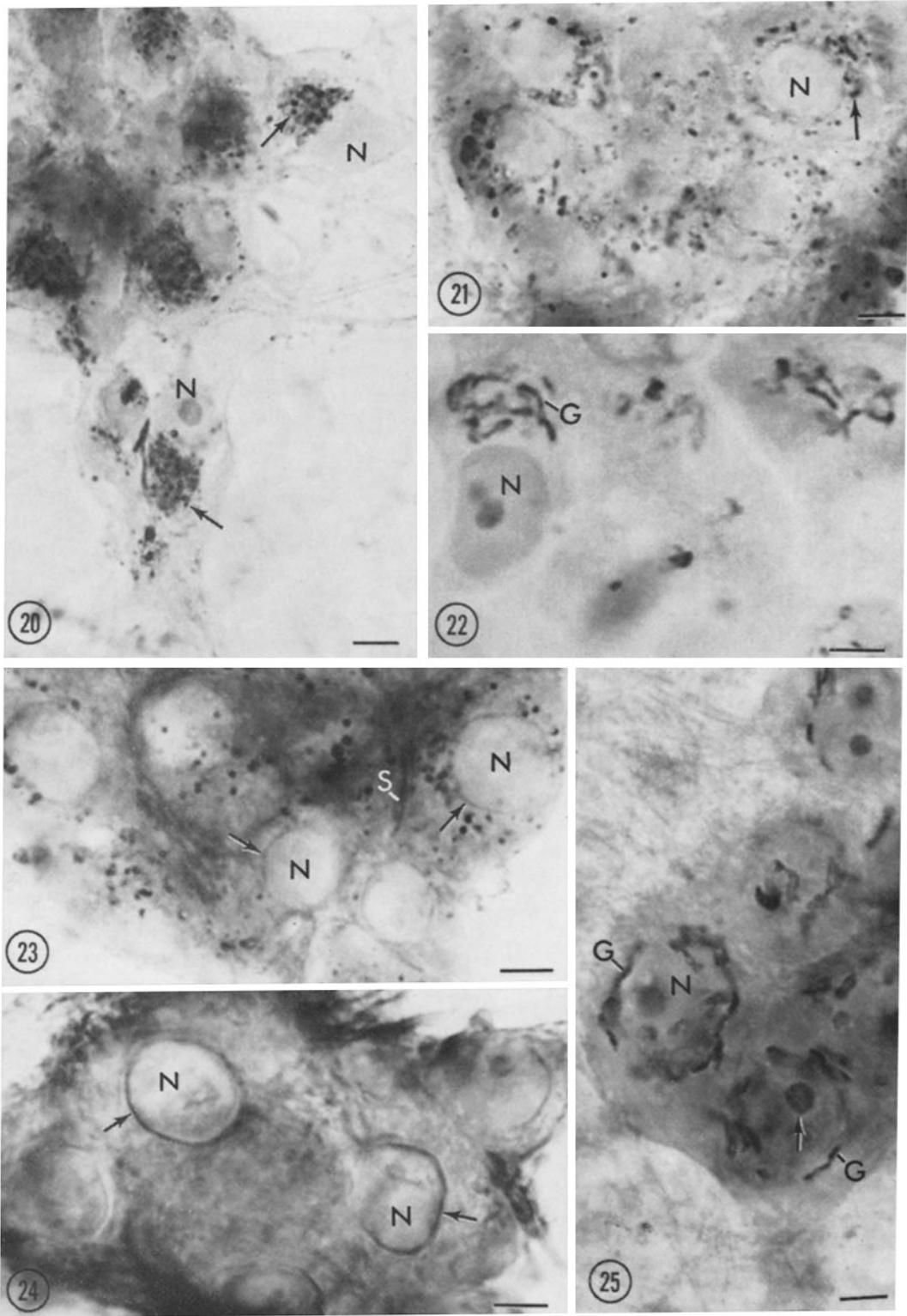
cored vesicles seen in a few cells are also seen as if forming in membrane systems near the Golgi apparatus (Fig. 11). Images which can be interpreted as a splitting of the largest of these vesicles are frequently noted (Fig. 11); obviously such images could also be of fusions of vesicles.

In axons and in terminals, large dense-cored vesicles are regularly seen as if forming from the agranular reticulum (Figs. 7 a, 7 b, 12, 16). Similar observations can readily be made for smaller vesicles in 5-hydroxydopamine-treated material (Figs. 8, 14, 15). Dense material similar in appearance to the vesicle contents is occasionally seen along stretches of the agranular reticulum (Fig. 17). From serial section studies, it is apparent that a small but appreciable fraction of the dense-cored vesicle population in endings is attached to the reticulum.

A preliminary survey of 10-day chick sympathetic ganglia fixed without culturing revealed cells grossly similar to those seen in the cultures, and occasional continuities between the axonal reticulum and dense-cored vesicles were also seen (Fig. 13).

## Enzyme Cytochemistry

Enzyme activities were studied at 1, 3, and most intensively at 7 days in vitro, when the neurons appear most normal by cytological criteria.

### Light Microscopy

Acid phosphatase activity (Figs. 20, 21) with CMP or βGP as substrate is demonstrable in lysosomes and in bodies similar to GERL, the region of Golgi-associated ER and lysosomes described by Novikoff (76, 79) and others (43, 44, 49). G6Pase is seen in bodies similar to those which show acid phosphatase activity as well as in the nuclear envelope (Fig. 23). When 5'-CMP is substituted for G6P as substrate, reaction product is seen only in bodies similar to those which show acid phosphatase activity.

IDPase activity is seen in the nuclear envelope and "diffuse" in the cytoplasm (Fig. 24). As in other studies (49, 75, 77), the Golgi apparatus does show reaction product but it is difficult to distinguish from the reaction product in other cytoplasmic structures. When TPP is substituted for IDP as substrate, enzyme activity is seen only in the Golgi apparatus (Figs. 22, 25) (cf. 49, 77, 79).

With several of these methods reaction product was also seen in structures in the axon bundles, but such reaction product cannot reliably be studied at the low resolution of the light microscope.

### Electron Microscopy

G6PASE: With the electron microscope, G6Pase was found in the agranular reticulum along the axon (Fig. 28) and in some terminals (Fig. 32). In many neurons, reaction product (Fig. 27) is also present in perikaryal ER including the nuclear envelope, Golgi-associated membrane systems, and lysosomes. The cell surface does not show G6Pase activity with our modified Wachstein-Meisel medium, but a little is seen at the surface with the Leskes medium.

If 5'-CMP is used as substrate in the G6Pase medium (pH 6.75), reaction product is seen in occasional lysosomes and in the Golgi-associated membrane systems which show G6Pase activity. No reaction product is found in the axonal reticulum, perikaryal ER, or nuclear envelope.

ACID PHOSPHATASE: Acid phosphatase activity (Fig. 18) with CMP or βGP as substrate was most prominent in lysosomes and in Golgi-associated membrane systems similar to those which show G6Pase activity. These include the systems that Novikoff calls GERL (44, 49, 76, 79) and also some Golgi saccules. Reaction product was sometimes found in perikaryal ER and nuclear envelope and the axonal reticulum (cf. 45, 51). (The acid phosphatase activity in the ER was most extensive with βGP as substrate in Tris buffer.)

IDPASE: IDPase was seen in the axonal agranular reticulum (Fig. 29) and in perikaryal

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FIGURE 26  Portions of two perikarya from a week old culture incubated for IDPase activity (90 min). Extensive reaction product is seen in stacks of ER (E), nuclear envelope (N), and in the Golgi apparatus (G). Light reaction product is also seen at the cell surface (S). Lead stained. × 18,000.

FIGURE 27  Portion of a perikaryon from a week old culture incubated for G6Pase activity (90 min) in our modified Wachstein-Meisel medium. Reaction product is seen in stacks of ER (E) and in the nuclear envelope (N). A mitochondrion is present at M. Lead stained. × 35,000.
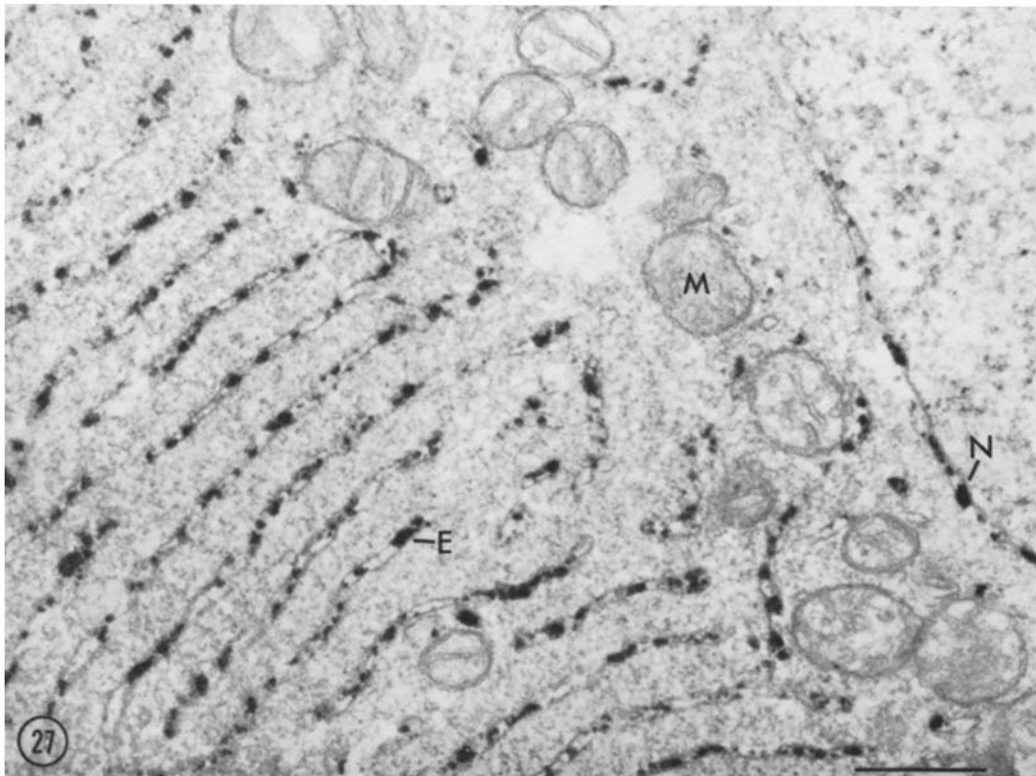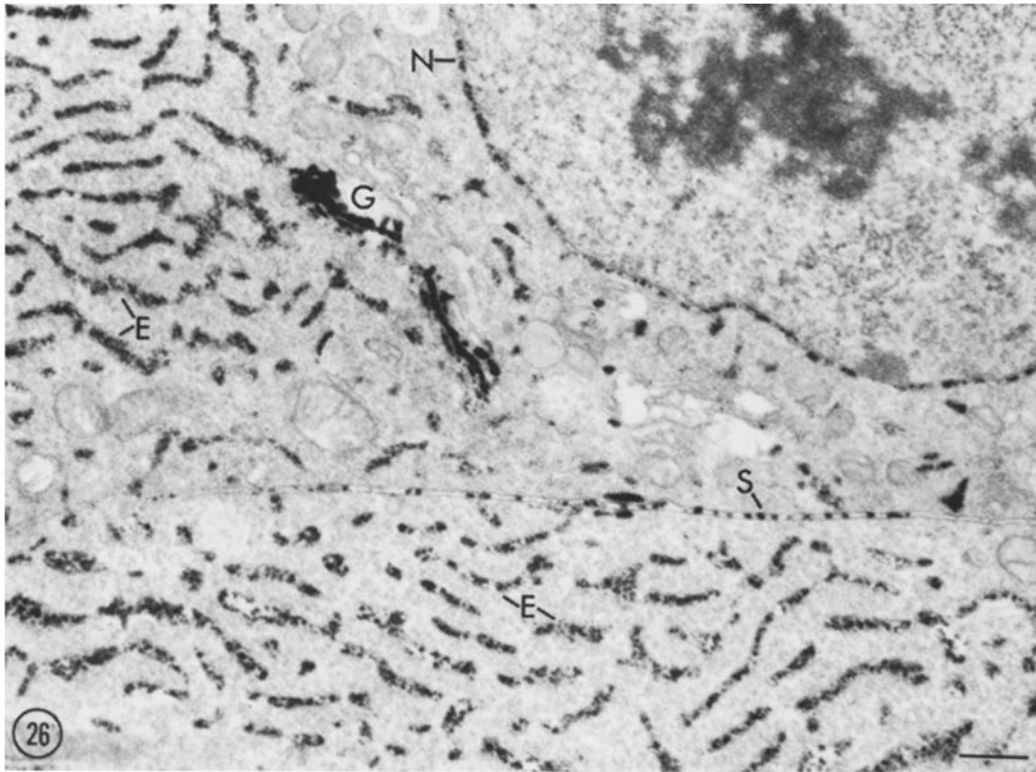
ER including the nuclear envelope of many neurons (Fig. 26); the axonal reaction is less prominent than with G6Pase. Reaction product was also seen in Golgi saccules. We have not studied the Golgi apparatus intensively enough to determine its organization as compared with the IDPase-positive regions studied by others (79). Occasionally, IDPase activity was demonstrable at the perikaryal cell surface (Fig. 26), but it was not seen at the axonal surface. Light reaction product was occasionally seen in GERL (cf. 49).

When TPP is used as substrate in an incubation otherwise identical to the IDPase study, demonstrable enzyme activity is found in portions of the Golgi apparatus similar to those which show IDPase activity (Fig. 19). TPPase activity was found in ER only in extremely rare cases (see 33).

C O N T R O L S : In substrate-free controls, no reaction product for any of the phosphatase enzyme activities studied was found. No reaction product was found when 0.01 M NaF was added to the complete medium in acid phosphatase preparations.

## DISCUSSION

### The Axonal Reticulum

The present study strongly supports the view that the smooth tubules and sacs widely seen in axons are a form of ER. The axonal system shares the ability to split G6P and IDP with perikaryal

ER, and axonal and perikaryal systems both occasionally show modest acid phosphatase activity. Perikaryal and axonal ER are also similar to one another in membrane dimensions (they are thinner than plasma membranes or some membranes of the Golgi apparatus), a feature used by others (see 71 for review) to follow membrane interrelations. Both behave similarly when G6P and IDP are replaced by other substrates; i.e., neither splits CMP at pH 6.75 and neither shows appreciable TPPase activity.

TPPase activity is characteristic of Golgi apparatus; Golgi saccules also split IDP. Membrane configurations resembling the Golgi-associated system named GERL by Novikoff (43, 44, 49, 76, 79) can split G6P and CMP at pH 6.75; this probably reflects the presence of high levels of acid phosphatase or neutral phosphatase activity (cf. 76) and a similar explanation presumably accounts for the ability of the lysosomes to split G6P. It should be borne in mind that increasing evidence supports the view that ER contributes membrane to the Golgi apparatus and associated structures (26, 44, 46, 71, 76, 79, 109), and so one does expect to find similarities between ER and Golgi-associated structures. Further, the significant point for our purposes is that perikaryal and axonal ER resemble one another in cytochemical characteristics; we are not asserting, for example, that neuronal ER does not contain enzymes that might split TPP under conditions different from the ones

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FIGURE 28  Portion of an axon from a week old culture incubated for G6Pase activity as in Fig. 27. Reaction product is seen in a tubule of agranular reticulum (R). Microtubules are seen at T, axonal plasma membrane at P. Lead stained. × 78,000.

FIGURE 29  Portion of an axon from a week old culture incubated for IDPase activity as in Fig. 26. Reaction product is seen in a tubule of agranular reticulum (R). A mitochondrion is seen at M, microtubules at T, and a dense-cored vesicle at D. Stained with uranyl and lead. × 60,000.

FIGURES 30 and 31  Tissue from week old cultured material not specially treated. In Fig. 30 from a perikaryon, the "unit membrane" structure of a cisterna (C) of ribosome-studded (R) ER is most evident at the arrow. Plasma membrane is seen at P and a microtubule at T. Fig. 31 shows portions of axons; extracellular space is seen at E and microtubules at T. The unit membrane structure of several regions of the agranular reticulum is evident at the arrows. A indicates axon plasma membrane. Fig. 30, × 160,000; Fig. 31, × 130,000.

FIGURE 32  Axon terminal from a week old culture incubated for G6Pase activity as in Fig. 27; the thin section was not stained with heavy metals. Reaction product is seen in narrow tubules (R). The structures seen at the arrows are the size and appearance expected for small vesicles containing reaction product; we suspect they are vesicles but it is difficult to rule out the possibility that they are cross sections of tubules. Mitochondrion is seen at M. No reaction product is found in the extracellular space (E). × 48,000.
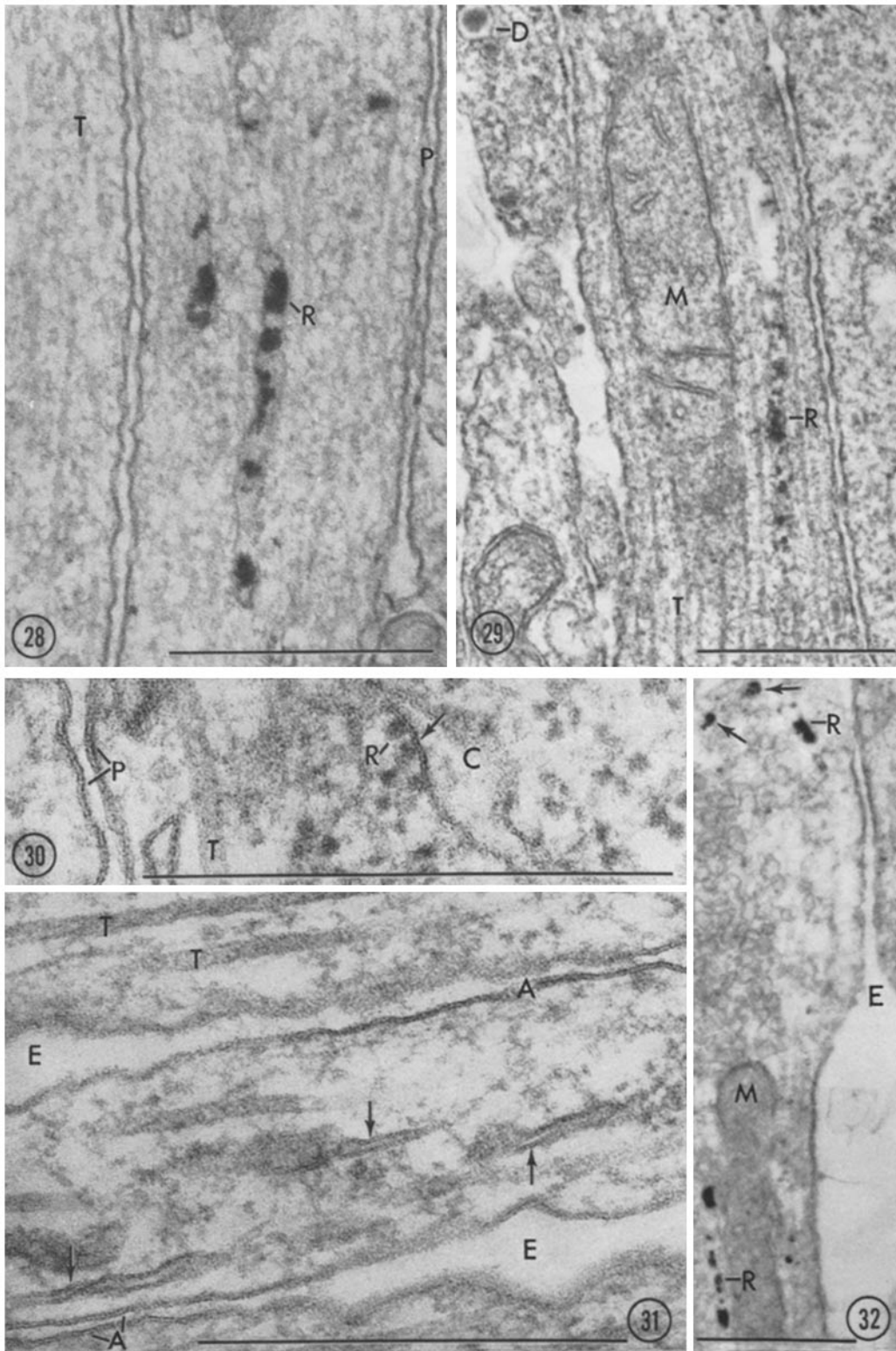
we use (see e.g., 33, 85). A detailed analysis of the phosphatase activities of the Golgi apparatus and associated systems of dorsal root ganglia has recently been published (79).

It may be significant that the axonal and perikaryal reticulum can split G6P and IDP. Nucleoside diphosphatase and G6Pase are useful ER enzyme markers for biochemical and cytochemical studies of hepatocytes (38, 61, 75, 78, 101). We know of no previous demonstration of these activities in the axonal reticulum although G6Pase has been used as a biochemical marker in work on nervous tissue (19, 58) and has been noted in perikarya by light microscopy (100). However, we cannot yet be certain that the enzyme activities we see cytochemically in neurons are the same as those identified by biochemical criteria. (For example the ability of some lysosomes and Golgi-associated sites to split G6P may reflect the presence of acid phosphatase activity but this seems less likely for the ER, in which acid phosphatase is only occasionally seen.) Thus when we refer to G6Pase, strictly speaking we mean simply the ability to split G6P.

We also do not know whether there is a structurally continuous channel of agranular reticulum leading from perikarya to terminals. Individual sacs or tubules do extend over long stretches. Nonetheless, it is still conceivable that large but discontinuous portions of the reticulum are transported down axons from perikarya.

It should be borne in mind that the population of sacs and tubules in axons may be heterogeneous or complex in origin. Tubular structures in axons and terminals do sometimes sequester exogenous tracers (5, 14, 39, 40, 44, 47, 50, 52). Elsewhere (44, 51) we will outline peroxidase studies on the present material and review the evidence for the proposition that membrane can circulate from the Golgi apparatus and axonal reticulum to axon terminals, be used there in synaptic transmission, and subsequently be sequestered by multivesicular bodies or other lysosomes through events that may involve transport of structures back from endings to perikarya (see also 47).

## Catecholamine Localization

Many neurons, including those in cholinergic systems, contain large (700–1,000 Å) dense-cored vesicles (cf. 88). Adrenergic neurons, when appropriately treated, contain dense-cored vesicles ranging in size from 400 to 1,000 Å in diameter

and generally thought of as comprising discrete large and small subpopulations (8, 37). The smaller are presumed to participate in synaptic transmission (cf. 8, 17, 111); the role of the larger type is not known but it certainly has not been ruled out that the larger also participate in transmission (cf. 6, 88). There is no reason, yet, to believe that all adrenergic systems are identical in their vesicle properties. In our cultured neurons, both the larger and smaller dense-cored vesicles behave cytochemically and in pharmacological tests like catecholamine storage vesicles (6, 103); the smaller vesicles are chromaffin reactive when exposed to noradrenaline and nialamide, and cores are prominent only with 5-hydroxydopamine treatments; the larger are chromaffin reactive and contain reserpine-depletable cores.

In different hands, reserpine treatments have produced diverse results on the larger dense-cored vesicles. Some workers (31, 54), as we do, note a loss of core material (the remnant shell may represent residual proteins or other macromolecules), while others (cf. 7, 9) find no change in the large dense-cored vesicles. The reasons for these disparities are obscure. Conceivably, they involve solubility problems (we used water-soluble Serpasil), local concentration effects, or differences in types of dense-cored vesicles. Reserpine may act in a general way on membranes, allowing leakage of catecholamines (84).

## Dense-Cored Vesicles in Axons and Terminals

Our findings strongly suggest that cultured chick sympathetic neurons contain a large population of catecholamine-storing, dense-cored vesicles which arise by budding from the axonal reticulum. Most of our observations concern accumulations of exogenous catecholamines but it seems reasonable to assume that similar mechanisms hold for endogenous catecholamines. To our knowledge, there has been no previous demonstration of the apparent origin of dense-cored vesicles, large or small, from the network of tubules and sacs in axon terminals, although there has been some previous discussion of the reticulum along the axon as a possible source of the larger vesicles (see below). If dense-cored synaptic vesicles are indeed transmitter packets released by exocytosis as much recent work on adrenergic systems implies (94, 108), a supply of new vesicles may be required to keep these systems going. The axonal reticulum could provide an immediate

source of new vesicles. There is not yet general agreement on the lifespan of catecholamine vesicles (see 11, 16, 20 for quite disparate estimates).

Our findings of continuities between agranular reticulum and large dense-cored vesicles support our own preliminary observations (98) and the suggestions of others (90). In studies of compressed nerves (85), images have been noted that were interpreted as reflecting the origin of large dense-cored vesicles from dilated neuronal microtubules; the vesicles did not stain with the chromaffin reaction. In another investigation of embryonic sympathetic material, the origin of the larger vesicles was attributed to agranular reticulum thought to be derived from neuronal microtubules (67). Since microtubules are thin (250 Å) in comparison to section thickness (500–1,000 Å), grazing sections of narrow tubules of axonal reticulum or dense-cored vesicles will often overlap microtubules and, thus, caution is required in interpreting morphological relations of vesicles and microtubules. Furthermore, biochemical studies on hepatocytes support the view derived from microscopy of many cell types that smooth ER derives from rough ER (18).

The relative infrequency of small dense-cored vesicles along the length of some axons (cf. 31, 54) has led to various hypotheses to explain the abundance of such vesicles at terminals. It has been suggested that the larger dense-cored vesicles give rise to the smaller by a process of budding (29, 30), by partial exocytosis of the core contents of larger vesicles followed by endocytosis of smaller vesicles (30, 94), or that the small vesicles arise by formation of small, clear vesicles with subsequent accumulation of cores (67). Our findings suggest that the smaller dense-cored vesicles can also arise directly by budding from the axonal reticulum. The formation of small synaptic vesicles (not dense cored) (2) from the axonal reticulum is also supported by zinc-iodide osmium studies on central nervous system material (2, 97), although this method is relatively unspecific (2), and by morphological studies on preterminal networks in retinal rods (65, 86) and on motor end plates (57).

It should be noted that in our system, as in others, the classification of a particular dense-cored vesicle as "large" or "small" is sometimes difficult. On the basis of size, morphology (e.g., size of core as compared to overall vesicle size), and other properties, there do appear to be at least two dense-cored vesicle populations in the terminals and axons. But in many terminals, one finds a spectrum of vesicles and sometimes only the extremes are clearly differentiable; one finds oneself occasionally tempted to refer to a vesicle as a small large vesicle, or a large small one. The essence of our observations is that structures with sizes and appearances covering most of the vesicle spectrum are seen attached to agranular reticulum in the axons and terminals. The frequency of such continuities, and the results with 5-hydroxydopamine and other agents and cytochemical methods, strongly imply the likelihood that the agranular reticulum is a significant source of catecholamine-storing vesicles. This does not mean that there are no other ways in which such vesicles can form; for example, it is not excluded that a subclass of small dense-cored vesicles can form, directly or indirectly, from the larger ones (29, 30, 94).

As might be expected, we do occasionally encounter what we take to be vesicles showing G6Pase activity in axon terminals (cf. Fig. 32). Unfortunately, this is not frequent enough for us to attach full weight to it; it is quite common in many cell types that structures (such as secretory granules) that bud from the ER or Golgi apparatus lack the cytochemically demonstrable enzymes present in their "parent" membrane systems (see e.g., 81, for other work on nervous tissue, and 24).

The simplest interpretation of these findings is that some materials destined to be packaged into dense-cored vesicles (presumably proteins such as dopamine β-hydroxylase) (19, 94) are transported within ER cisternae from perikarya to packaging sites in axons.

Some of the small, clear vesicles found in the endings may arise through endocytosis (21, 40, 44, 47) since we find that exogenous peroxidase does accumulate in some of the vesicles of the chick endings (51). The possibility that some such vesicles can reaccumulate catecholamines and perhaps be reused for transmission deserves future study (see 40, and review and discussion in 51).

## Perikaryal Dense-Cored Vesicles

As have others (37, 60), we have noted images suggesting the formation of the larger categories of dense-cored vesicles from membrane systems closely associated with the Golgi apparatus (perhaps from GERL). Although numerous small Golgi vesicles are present in our material, these

did not appear to fill with 5-hydroxydopamine and they were not chromaffin reactive. However, small vesicles that show dense cores with permanganate fixation have been noted in other sympathetic perikarya (41). Some workers have found that large dense-cored vesicles (31, 54) predominate over small ones in the proximal portions of ligatured sympathetic nerves under conditions where catecholamines show a similar accumulation (31, 54). This accumulation is colchicine sensitive, presumably implying transport down the axon from perikaryal sites of synthesis by systems in which microtubules are involved (3, 42).

It may turn out that embryonic, cultured, or regenerating systems behave somewhat differently than adult neurons, although hints of phenomena similar to the ones we observe have been obtained by others (25) on adult rats, and the ligation experiments (85, 90) also suggest that the agranular reticulum can be a source of dense-cored vesicles in adult axons. However the simplest explanation for the aggregate of observations is that there are two interrelated pathways for dense-cored vesicle formation, one Golgi associated and one not. This is consistent with studies of hydrolase transport in injured neurons (44, 49) in which lysosomes can be observed to form from Golgi-associated ER and from the axonal reticulum. Also, under some conditions, adrenaline granules of the adrenal medulla can be shown to arise from rough ER (1, 51, 52) as well as from smooth Golgi-associated membrane systems.

That catecholamine-containing vesicles can arise in Golgi-associated membrane systems is also suggested in this study, e.g., the very large (800–3,500 Å) dense-cored vesicles. The cells in which these granules are present may be ganglionic interneurons (cf. 56, 68), which some believe contain dopamine (73).

## Axonal Transport

Since vesicular components such as those associated with synaptic transmission are reportedly transported at rapid rates (cf. 59), our findings may imply that the axonal reticulum is a compartment for relatively fast transport. Acetylcholinesterase, which has been localized in the axonal reticulum (55, 99), is also known to be rapidly transported (for references, see 17, 80). Biochemical studies have indicated that a portion of rapidly transported material is seen in a microsomal fraction (80) or small particulate fraction,

although the purity and details of the content of these preparations are unknown. Biochemical fractionation studies of neuronal catecholamine storage particles (cf. 19, 58) may in part reflect the presence in cell fractions of microsomal derivatives of ER along with fully formed secretory granules. In light of the literature, the present findings strongly hint that heterogeneity of transport rates along axons may reflect the existence of various compartments for transport and that the axonal reticulum deserves considerable further study as a channel for transport of enzymes and other material of importance for nervous function.

## REFERENCES

1. ABRAHAMS, S., and E. HOLTZMAN. 1973. *J. Cell Biol.* **56**:540.
2. AKERT, K., E. KAWARA, and C. SANDRI. 1971. *Prog. Brain Res.* **34**:305.
3. BANKS, P., D. MAYOR, and D. R. TOMLINSON. 1971. *J. Physiol. (Lond.).* **219**:755.
4. BARKA, T., and P. J. ANDERSON. 1962. *J. Histochem. Cytochem.* **10**:741.
5. BIRKS, R. I. 1966. *Ann. N. Y. Acad. Sci.* **135**:8.
6. BLOOM, F. E. 1970. *In* The Neurosciences: Second Study Program. F. O. Schmitt, editor. The Rockefeller University Press, New York. 729.
7. BLOOM, F. E., and R. J. BARNETT. *Nature (Lond.).* **210**:599.
8. BLOOM, F. E., L. L. IVERSEN, and F. O. SCHMITT. 1970. *Neurosci. Res. Program Bull.* **8**:325.
9. BONDAREFF, N. 1965. *Z. Zellforsch. Mikrosk. Anat.* **67**:211.
10. BORNSTEIN, M. B. 1958. *Lab. Invest.* **7**:134.
11. BRIMIJOIN, S. 1972. *J. Neurochem.* **19**:2183.
12. BUNGE, M., R. BUNGE, and E. PETERSON. 1967. *Brain Res.* **6**:728.
13. BURDMAN, J. A. 1968. *J. Neurochem.* **15**:1321.
14. CECCARELLI, B., W. D. HURLBUT, and A. MAURO. 1972. *J. Cell Biol.* **54**:30.

15. Coon, H. C. 1966. *Proc. Natl. Acad. Sci. U. S. A.* **55:**66.

16. Dahlstrom, A. 1969. *In* Cellular Dynamics of the Neuron. S. H. Barondes, editor. Academic Press Inc., New York. 153.

17. Dahlstrom, A. 1971. *Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci.* **261:**325.

18. Dallner, G., P. Siekevitz, and G. E. Palade. 1966. *J. Cell Biol.* **30:**73.

19. de Potter, W. P. 1971. *Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci.* **261:**313.

20. de Potter, W. P., and I. W. Chubb. 1971. *Biochem. J.* **125:**375.

21. Douglas, W., and J. Nagasawa. 1971. *J. Physiol. (Lond.).* **218:**94 P.

22. England, J. M., and M. N. Goldstein. 1969. *J. Cell Sci.* **4:**677.

23. Eranko, O., L. Eranko, C. E. Hill, and G. Burnstock. 1972. *Histochem. J.* **4:**49.

24. Farquhar, M G., D. F. Bainton, M. Bagglioni, and C. de Duve. 1972. *J. Cell Biol.* **54:**141.

25. Fillenz, M. 1970. *Proc. R. Soc. Lond. B Biol. Sci.* **174:**459.

26. Flickinger, C. J. 1969. *J. Cell Biol.* **43:**250.

27. Friend, D. S., and M. G. Farquhar. 1967. *J. Cell Biol.* **35:**337.

28. Fuxe, K., T. Hokfelt, G. Johnsson, and V. Ungerstedt. 1971. *In* Contemporary Research Methods in Neuroanatomy. W. J. H. Nauta and S. Ebbesson, editors. Springer Publishing Co., Inc., New York. 275.

29. Geffen, L. B. 1970. *In* New Aspects of Storage and Release Mechanisms of Catecholamines. H. J. Schümann and G. Kroneberg, editors. Springer Publishing Co. Inc., New York. 39.

30. Geffen, L. B., and B. G. Livett. 1971. *Physiol. Rev.* **51:**98.

31. Geffen, L. B., and A. Ostberg. 1969. *J. Physiol. (Lond.).* **204:**583.

32. Goldfischer, S., E. Essner, and A. B. Novikoff. 1964. *J. Histochem. Cytochem.* **12:**72.

33. Goldfischer, S., E. Essner, and B. Schiller. 1971. *J. Histochem. Cytochem.* **19:**349.

34. Gomori, G. 1953. *In* Microscopic Histochemistry, Principles and Practice. University of Chicago Press, Chicago. 137.

35. Gray, E. G. 1971. *Prog. Brain Res.* **34:**149.

36. Gray, E. G. 1970. *J. Cell Sci.* **7:**189.

37. Grillo, M. A., and S. L. Palay. 1962. *In* Proceedings of the Fifth International Congress for Electron Microscopy. S. S. Breese, editor. Academic Press Inc., New York. 2:U-1.

38. Hers, H. G., J. Berthet, Z. Berthet, and C. de Duve. 1951. *Bull. Soc. Chim. Biol.* **33:**21.

39. Heuser, J. E. 1971. Abstracts of the 11th Annual Meeting of the American Society for Cell Biology. 124.

40. Heuser, J., and T. S. Reese. 1972. *Anat. Rec.* **172:**329. (Abstr.).

41. Hökfelt, T. 1969. *Acta Physiol. Scand.* **76:**427.

42. Hökfelt, T., and A. Dahlstrom. 1971. *Z. Zellforsch. Mikosk. Anat.* **119:**460.

43. Holtzman, E. 1969. *In* Lysosomes in Biology and Pathology. J. T. Dingle and H. Fell, editors. North-Holland Publishing Co., Amsterdam. 1:192.

44. Holtzman, E. 1971. *Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci.* **261:**407.

45. Holtzman, E., S. M. Crain, and E. R. Peterson. 1973. *J. Gen. Physiol.* In press.

46. Holtzman, E., and R. Dominitz. 1968. *J. Histochem. Cytochem.* **16:**320.

47. Holtzman, E., A. R. Freeman, and L. A. Kashner. 1971. *Science (Wash. D. C.).* **173:**733.

48. Holtzman, E., and A. B. Novikoff. 1965. *J. Cell Biol.* **27:**651.

49. Holtzman, E., A. B. Novikoff, and H. Villaverde. 1967. *J. Cell Biol.* **33:**419.

50. Holtzman, E., and E. R. Peterson. 1969. *J. Cell Biol.* **50:**863.

51. Holtzman, E., S. Teichberg, S. Abrahams, E. Citkowitz, S. M. Crain, N. Kawai, and E. R. Peterson. 1973. *J. Histochem. Cytochem.* In press.

52. Holtzman, E., S. Teichberg, S. Abrahams, S. M. Crain, and E. R. Peterson. 1972. *In* Histochemistry and Cytochemistry 1972. Proceedings of the Fourth International Congress for Histochemistry and Cytochemistry. T. Takeuchi and S. Fujita, editors. Japan Soc. Histochem. Cytochem., Kyoto, Japan. 473.

53. Iversen, L. L. 1967. The Uptake and Storage of Noradrenalin in Sympathetic Nerves. Cambridge University Press, London.

54. Kapeller, K., and D. Mayor. 1967. *Proc. R. Soc. Lond. B Biol. Sci.* **167:**282.

55. Kasá, P. 1968. *Nature (Lond.).* **218:**1265.

56. Kebabian, J. W., and P. Greengard. 1971. *Science (Wash. D. C.).* **174:**1346.

57. Korneliussen, H. 1972. *Z. Zellforsch. Mikrosk. Anat.* **130:**28.

58. Largercrantz, H. 1971. *Acta Physiol. Scand. Suppl.* 366.

59. Lasek, R. J. 1970. *Int. Rev. Neurobiol.* **13:**289.

60. Lentz, T. 1969. *Z. Zellforsch. Mikrosk. Anat.* **102:**447.

61. Leskes, A., P. Siekevitz, and G. E. Palade. 1971. *J. Cell Biol.* **49:**264.

62. Lever, J. D., and R. Presley. 1972. *Prog. Brain Res.* **34:**499.

63. Levi-Montalcini, R., and P. U. Angeletti. 1963. *Dev. Biol.* **7**:653.

64. Lieberman, A. R. 1971. *Int. Rev. Neurobiol.* **14**:50.

65. Lovas, B. 1971. *Z. Zellforsch. Mikrosk. Anat.* **121**:341.

66. Luft, J. M. 1961. *J. Biophys. Biochem. Cytol.* **9**:409.

67. Machado, A. B. M. 1971. *Prog. Brain Res.* **34**:171.

68. Matthews, M. R., and E. Raisman. 1969. *J. Anat.* **103**:15.

69. McKenna, O. C., and J. Rosenbluth. 1971. *J. Cell Biol.* **48**:650.

70. Model, P. G., M. B. Bornstein, S. M. Crain, and G. D. Pappas. 1972. *J. Cell Biol.* **49**:362.

71. Morré, D. J., H. H. Mollenhauer, and C. E. Bracker. 1971. *In* Origin and Continuity of Cell Organelles. J. Reinert and H. Ursprung, editors. Springer Publishing Co., Inc., New York. 82.

72. Nichols, B. A., D. F. Bainton, and M. G. Farquhar. 1971. *J. Cell Biol.* **50**:498.

73. Norberg, K. A., M. Ritzen, and U. Under-stedt. 1966. *Acta Physiol. Scand.* **67**:260.

74. Novikoff, A. B. 1963. *Lysosomes Ciba Found. Symp.* 36.

75. Novikoff, A. B. 1965. *In* Intracellular Membranous Structure. S. Seno and E. V. Cowdry, editors. Japan Society for Cell Biology, Okayama. 277.

76. Novikoff, A. B. 1967. *In* The Neuron. H. Hyden, editor. Elsevier Publishing Co., Amsterdam. 255, 319.

77. Novikoff, A. B., and S. Goldfischer. 1961. *Proc. Natl. Acad. Sci. U. S. A.* **47**:802.

78. Novikoff, A. B., and M. Heus. 1963. *J. Biol. Chem.* **238**:710.

79. Novikoff, P., A. B. Novikoff, N. Quintana, and J. Hauw. 1971. *J. Cell Biol.* **50**:859.

80. Ochs, S. 1972. *Science (Wash. D. C.).* **176**:252.

81. Osinchak, J. 1964. *J. Cell Biol.* **20**:35.

82. Palay, S. L. 1958. *Exp. Cell Res.* **5** (Suppl.):275.

83. Palay, S. L., and G. E. Palade. 1955. *J. Biophys. Biochem. Cytol.* **1**:69.

84. Palm, D., H. Grobecker, and I. J. Bak. 1970. *In* New Aspects of Storage and Release Mechanisms of Catecholamines. H. J. Schümann and G. Kroneberg, editors. Springer Publishing Co., Inc., New York. 188.

85. Pellegrino de Iraldi, A., and E. de Robertis. 1970. *In* New Aspects of Storage and Release Mechanisms of Catecholamines. H. J. Schümann and G. Kroneberg, editors. Springer Publishing Co., Inc., New York. 3.

86. Pellegrino de Iraldi, A., and A. M. Suburo. 1971. *Z. Zellforsch. Mikrosk. Anat.* **113**:39.

87. Pelletier, G., and A. B. Novikoff. 1972. *J. Histochem. Cytochem.* **20**:1.

88. Peters, A. F., S. L. Palay, and H. de F. Webster. 1970. The Fine Structure of the Nervous System: The Cells and Their Processes. Hoeber Medical Division of Harper and Row, Publishers, New York.

89. Prasad, K. N. 1970. *J. Cell Biol.* **47**:160 a.

90. Rodriquez-Echandia, E. L., A. Zamora, and R. S. Piezzi. 1970. *Z. Zellforsch. Mikrosk. Anat.* **104**:409.

91. Rosenbluth, J. 1962. *J. Cell Biol.* **13**:405.

92. Sabatini, D. D., K. Bensch, and R. J. Barnett. 1963. *J. Cell Biol.* **17**:19.

93. Siegrist, G., F. de Ribaupierre, M. Dolivo, and C. Rouiller. 1966. *J. Microsc. (Paris).* **5**:791.

94. Smith, A. D. 1971. *Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci.* **261**:363.

95. Smith, D. S. 1971. *Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci.* **261**:395.

96. Sotelo, C., and S. L. Palay. 1971. *Lab. Invest.* **25**:653.

97. Stelzner, D. J. 1971. *Z. Zellforsch. Mikrosk. Anat.* **120**:423.

98. Teichberg, S., E. Holtzman, and J. Abbott. 1971. Abstracts of the 11th Annual Meeting of the American Society Cell Biology. 302.

99. Tennyson, V. C., M. Brzin, and P. Duffy. 1968. *Prog. Brain Res.* **29**:41.

100. Tewari, H. B., and G. H. Bourne. 1963. *J. Histochem. Cytochem.* **11**:121.

101. Tice, L. W., and R. J. Barnett. 1962. *J. Histochem. Cytochem.* **10**:754.

102. Tranzer, J. P., and H. Thoenen. 1967. *Experientia (Basel).* **23**:743.

103. Tranzer, J. P., H. Thoenen, and R. L. Snipes. 1969. *Prog. Brain Res.* **31**:33.

104. van Breeman, V. L., E. Anderson, and J. F. Reger. 1958. *Exp. Cell Res.* **5**(Suppl.):153.

105. Venable, J. H., and R. Coggeshall. 1965. *J. Cell Biol.* **25**:407.

106. Wachstein, M., and E. Meisel. 1956. *J. Histochem. Cytochem.* **4**:592.

107. Watson, M. L. 1958. *J. Biochem. Biophys. Cytol.* **4**:475.

108. Weinshilbaum, R. M., N. B. Thoa, D. G. Johnson, I. J. Kopin, and J. Axelrod. 1971. *Science (Wash. D. C.).* **174**:1349.

109. Whaley, W. G., G. M. Dauwalder, and J. E. Kephart. 1972. *Science (Wash. D. C.).* **175**:596.

110. Whitaker, S., and F. S. La Bella. 1972. *Z. Zellforsch. Mikrosk. Anat.* **125**:1.

111. Wolfe, D. E., L. T. Potter, K. C. Richardson, and J. Axelrod. 1962. *Science (Wash. D. C.).* **138**:440.

112. Woods, R. I. 1969. *J. Physiol. (Lond.).* **203**:35 P.

113. Woods, R. I. 1970. *Proc. R. Soc. Lond. Ser B Biol. Sci.* **176**:63.