ELECTRON MICROSCOPIC LOCALIZATION
OF ACID PHOSPHATASE AND THIAMINE PYROPHOSPHATASE ACTIVITY IN HYPOTHALAMIC NEUROSECRETORY CELLS OF THE RAT

JOSEPH OSINCHAK, Ph.D.

From the Department of Anatomy, Duke University Medical Center, Durham, North Carolina

ABSTRACT

Supraoptic nuclei in the hypothalamus of rats were fixed for the electron microscope by vascular perfusion with solutions of glutaraldehyde followed by post fixation with osmium tetroxide. Cytochemical methods for detection of acid phosphatase and thiamine pyrophosphatase activity have been applied to glutaraldehyde-fixed frozen sections containing the neurosecretory cells. The enzyme activities have been localized to certain Golgi cisternae. Acid phosphatase activity is present in the large (0.4 μ to 1.0 μ) granules or dense bodies which are surrounded by a single limiting membrane; both features characterize these structures as lysosomes. Smaller (0.1 μ) granules also present in the perikarya are generally unreactive towards enzyme activity and resemble in form the neurosecretory granules in the neurohypophysis.

A prevalent finding common to recent electron microscope (EM) studies of neurosecretory cells has been the demonstration of secretion granules varying in size and internal structure within the neuroplasm. Palay (29) has reported the appearance of secretory material, in cells of the preoptic nuclei of goldfish, in the form of dense droplets of two sizes, roughly 0.1 μ and 1.0 μ or larger. Scharrer and Brown (33), and Bern et al. (5), have also described secretion granules in neurosecretory cells of the earthworm, and the leech, cockroach, and frog, respectively.

In mammalian secretory neurons the identification of neurosecretory material is not altogether clear from EM studies thus far. Duncan and Alexander (7), in the rat, could find "little or no specific or obvious evidence of neurosecretion." The numerous vesicles and solid spherules that were observed were not restricted to cells of the supraoptic nucleus, but were also found in other more typical neurons as well. Murakami (18), on the other hand, in his investigation of neurosecretory cells in the hypothalamus of the mouse, has distinguished two kinds of granules, a small (type I, 100 μ to 200 μ) and a large (type II, 400 μ to 600 μ) granule.

Previous EM observations of vertebrate neurosecretion have shown, in general, that the secretion product consists of the smaller (100 μ) granule in the axonal endings of the end-organs or neurohypophyses of mammals (26, 27), birds (6), reptiles (3), and amphibians (10). Palay (29) has pointed out that the small granule is common to most species examined to date with the EM, and suggests that this entity could be the structural counterpart of the neurohypophysial hormones, and that the larger granules or droplets are involved in some other function. In accord with this latter suggestion, Palay also mentions "the morphological similarity between some of the large droplets and lysosomes of other cell types."

The work reported herein represents one aspect
of a more detailed EM study of neurosecretory cells in the hypothalamus of the rat currently in progress. It is an attempt to characterize at least some of the perikaryonal inclusion bodies visible with the EM in mammalian neurosecretory cells.

MATERIALS AND METHODS

Brain tissue for EM study was obtained from eight albino rats fed laboratory diets, whose mean body weight at time of sacrifice was 225 gm. The method of animal preparation and vascular perfusion-fixation used with the majority of animals is essentially similar to that described by Palay et al. (30), except for the fixation vehicle. One animal was sacrificed by decapitation followed by immersion-fixation.

The fixative used in all eight animals was glutaraldehyde. Two modifications of the glutaraldehyde fixative were tried as follows: glutaraldehyde 25 per cent was diluted to 6.5 per cent with 0.1 m phosphate buffer pH 7.2 (32). Glutaraldehyde 25 per cent (20 ml) was also diluted to 5.0 per cent with 0.2 m sodium cacodylate (42.8 gm of Na(CH3)2AsO2.3H2O; 3H2O in 1000 ml) (33 ml) and distilled water (47 ml), this solution having a pH 7.2 (personal communication from Dr. S. J. Holt; see also reference 32). The glutaraldehyde solutions were perfused for 30 to 45 minutes, following which the brain was dissected free from the skull, transversely sectioned with fresh razor blades into 1 to 3 mm slices and immersed in fresh cold fixation fluid; total fixation times were 21/2 and 4 hours. The tissue slices fixed in phosphate-buffered glutaraldehyde were washed overnight or stored in cold 0.1 m phosphate buffer pH 7.2 containing 0.25 m sucrose. The tissue fixed in cacodylate-buffered glutaraldehyde was treated similarly with 0.1 m sodium cacodylate-HCl buffer pH 7.2 containing 0.22 m sucrose. The tissue slices fixed in phosphate-buffered glutaraldehyde were washed overnight or stored in cold 0.1 m phosphate buffer pH 7.2 containing 0.22 m sucrose. The tissue fixed in cacodylate-buffered glutaraldehyde was treated similarly with 0.1 m sodium cacodylate-HCl buffer pH 7.2 containing 0.25 m sucrose.

Following the appropriate buffer wash the tissue slices were then frozen-sectioned at 10 and 40 μ with a Sartorius microtome fitted with a Super Histo-Freeze (Scientific Products, Charlotte, North Carolina). The transverse 40 μ frozen sections were then incubated in a Gomori mori medium for acid phosphatase (11) pH 5.0 for 15 to 30 minutes at 37°C, rinsed in 0.05 m acetate buffer pH 5.0, then in 1.5 per cent acetic acid, and again in the acetate buffer. The phosphate-buffered tissue was then given an additional wash in 0.22 m sucrose preceding the Gomori incubation. Control sections were incubated in an identical but substrate-free medium, or in a complete medium containing 0.01 m NaF to inhibit enzyme activity.

Some of the 40 μ frozen sections were also incubated in a thiamine pyrophosphate medium (TPP) following the procedure outlined by Novikoff and Goldfischer (23). Incubation was performed at 37°C for 5 to 15 minutes (30 minutes for light microscopy). Control sections of all TPP-incubated slices were immersed in a substrate-free medium. Several frozen sections were doubly incubated, first in the acid phosphatase medium (15 to 30 minutes), washed as already described, and then incubated a second time (15 to 30 minutes) in the TPP medium.

The 10 μ frozen sections prepared for light microscopy were incubated in the appropriate medium and then immersed in dilute ammonium sulfide, washed, affixed to gelatinized slides, lightly counterstained with Lillie's hemalum and/or saturated picric acid, dehydrated, and mounted.

In order to be certain that the supraoptic nucleus was being prepared for the EM, areas containing the nucleus were recognized in the 40 μ frozen sections and cut out as small blocks (3 x 3 mm); after incubation these were then postfixed in Palade's osmium tetroxide (26).

The supraoptic nucleus is easily identified in the transverse frozen sections of the brain; the bulk of the nucleus lies lateral to the optic tract just anterior to the posterior limit of the optic chiasma. Usually the lateral edge of the optic tract served as the medial boundary of the small (3 mm) block; this portion of the tract provided additional aid in the identification of the nucleus in the EM. Following postfixation with osmium tetroxide, the tissue blocks were dehydrated in ethyl alcohol, "cleared" in propylene oxide, and embedded in British (Gibco) Araldite (CY-212).

The final embedding medium was poured into a polyethylene beaker as a layer 4 to 5 mm thick, into which the tissues were placed. Following polymerization at 60°C, the Araldite layer or disk containing the tissues can be simply "popped" out of the beaker, the tissues cut free from the disk, thus enabling one to mount the tissue-Araldite block accurately. Reliable orientation can be obtained by affixing the tissue block in desired position to a preshaped Lucite plastic plug (to fit the microtome chuck) with polyester wax. Further trimming can then be undertaken preparatory to fine sectioning. With the above-described technique, only transverse, thin sections of the supraoptic nucleus were cut, thus assuring the correct identification of this nuclear region. The same procedure was used to isolate and identify other areas such as the hypothalamic paraventricular nuclei and regions of the cerebellum.

Both the Porter-Blum and Huxley microtomes fitted with glass knives were used in thin sectioning. Uranium acetate (35) or lead hydroxide (16) were applied to the sections as required. Electron micrographs were taken on a Philips 100-B electron microscope equipped with a Ladd anode and a 25 μ objective aperture, and used at an accelerating voltage of 60 kv. Micrographs were taken at magnifications of 5,000 to 30,000, and were enlarged photographically as desired.
RESULTS

Light Microscopy

Tissue incubated for acid phosphatase: Deposits of the reaction product (lead sulfide), indicating foci of acid phosphatase activity, were found in the neurosecretory cells of the supraoptic nucleus. The reaction product is uniformly dispersed throughout the cytoplasm of the secretory neurons as bodies measuring 0.4 μ to 1.0 μ (Fig. 1). Occasionally the acid phosphatase-containing bodies assume a perinuclear distribution, with some cells containing more reactive dense bodies than others. The reactivity of the neurosecretory cells is in marked contrast to the sparse reaction product displayed in the more typical neurons of surrounding hypothalamic areas. The pericapillary glial cells in the supraoptic nuclear region also show the presence of acid phosphatase activity.

Other areas examined: Discrete acid phosphatase-containing bodies are localized within the neurosecretory cells of the hypothalamic paraventricular nuclei, and in the Purkinje cells of the cerebellum. Nuclear staining was not encountered in any of the sections incubated in Gomori medium, provided that one observed the precautions discussed by Holt (13).

Electron Microscopy

Non-incubated tissue: The secretory neurons of the supraoptic nucleus display a well-developed Golgi apparatus (Fig. 3, G), and contain, as well, parallel-arrayed profiles of rough endoplasmic reticulum (ns) not unlike the highly organized Nissl bodies of ordinary nerve cells (see reference 31).

The perikaryon of the neurosecretory cell contains, in addition to mitochondria (m) and neurofilaments (nf), three kinds of formed elements (see Fig. 3): (a) membrane-bounded, “sac-like” structures (mob) which enclose a number of small vesicles; this type of entity has been observed in oocytes and termed a multivesicular body by Sotelo and Porter (34); (b) large, 0.4 to 1.0 μ, electron-opaque bodies (db), structurally similar to the inclusion bodies found in ordinary nerve cells (see references 15, 31) and secretory nerve cells of other vertebrates (see references 18, 29), as well as to the lysosomes of other cell types; and (c) small, 0.1 μ, granules (nsg) similar in morphology to the neurosecretory granules found in the neurohypophysis of the rat (27, 28) and throughout the hypothalamo-neurohypophysial system (Osinchak, in preparation).

Both the large dense bodies and small granules are limited by a single, smooth membrane about 6 μ thick. In Araldite-embedded, lead hydroxide-stained preparations the large dense bodies display a coarsely granular internum of an electron opacity higher than that of the cytoplasmic matrix, whereas the small granules with finely granulated centra are only faintly stained. In sections doubly stained in lead salts and in solutions of uranyl acetate, both types of formed elements appear to be of equal electron opaqueness.

The small granule has a definite association with the Golgi apparatus (see Fig. 3). Continuity is frequently observed between the Golgi lamellae and the smooth membranes of apparently mature granules. Furthermore, dense aggregations of material, suggestive of granule formation, can be observed within the cisternae of the Golgi network. On the other hand, the large dense bodies are usually dispersed throughout the perikaryon without either direct connection with, or apparent morphological relationship to, any particular cell organelle. Nothing has yet been observed that can be interpreted as intermediate stages between the small granules and the large dense bodies, nor are there transition forms evident between the large dense bodies and the multivesicular bodies. Moreover, no association between the dense bodies and Golgi lamellae comparable to that of the small granules has so far been observed.

In contrast with the uniform appearance presented by the small granule, many structural variations are displayed by the large dense bodies; laminated-membrane inclusions, “ferritin-like” grains, dense spherules, and clear vacuoles are sometimes observed within their limiting membrane. Occasionally, in preparations of certain animals, the dense bodies appear morphologically similar to “old age pigment” or lipofuscin granules in spinal ganglion cells (see reference 8).

Tissue incubated for acid phosphatase: The reaction product indicating the site of acid phosphatase activity, in this case lead phosphate, is readily distinguishable in electron micrographs as an electron-opaque, granular, or rod-shaped precipitate. The lead reaction product has been employed as a lysosomal marker in other cell types (9, 14). It was not surprising, therefore, to discover the reaction product bound to the large...
dense bodies (Fig. 2, db) which, as mentioned previously, bear some structural similarity to the lysosomes in other, non-neural tissue.

EM survey micrographs of low magnification revealed the distribution of reactive dense bodies similar to the pattern of acid phosphatase-positive structures seen in the light microscope. There can be little doubt of the structural identity of the reaction product visible with both optical systems. As is evident in Fig. 2, the pattern of the lead precipitation varies within a neurosecretory cell in that some of the dense bodies are reactive throughout (db-1), whereas others have focal points of precipitation (db-2, arrows) and another is devoid of reaction product (db-3). Note the absence of precipitate in the small granule (nsg-1), and the suggestion of reactivity in another (nsg-2).

Acid phosphatase activity is also localized within the first one or two cisternae of the Golgi apparatus (Fig. 4). In those sections examined thus far, only the most proximal cisternae of a stack, i.e. those closest to the nucleus, contain precipitate; the reaction product is entirely surrounded by smooth membrane. Another element of the Golgi apparatus most consistently reactive for acid phosphatase is the small Golgi vesicle (Fig. 4, gv). Those vesicles near the cisterna (gc) seemingly are more reactive than the more distal vesicles. The mitochondria, Nissl substance or rough endoplasmic reticulum, and nucleus are not reactive for acid phosphatase activity.

No specific reaction product was observed in control sections incubated in either the substrate-free medium or the complete medium containing sodium fluoride. Occasionally during the first trials in which phosphate-buffered glutaraldehyde was used, the incubated sections contain a generalized distribution of fine deposits which, however, are easily distinguishable from the more dense, compact, specific reaction product. This type of artifact probably represents the effects of insufficient washing and hence failing to remove traces of phosphate buffer from the tissues. Use of cacodylate-buffered glutaraldehyde fixative eliminates the necessity of an additional sucrose wash, and does not induce any artificial precipitation.

**Tissue incubated for thiamine pyrophosphatase:** In neurosecretory cells, as in ordinary neurons (see reference 23), the presence of an enzyme acting on TPP was also demonstrated in the cisternae of most stacks of Golgi lamellae. The localization of the reaction product is usually confined to one or two cisternae of Golgi cisternae. Sites of TPP reactivity are restricted to smooth-surfaced elements of the Golgi apparatus, and no reaction product is observed in the large dense bodies. The small granules are also unreactive towards TPP activity except in those cases where the small granules are in close contact with reactive Golgi cisternae. It can be seen in Fig. 5 that the deposition of reaction product around the center of the small granules (arrows) obscures that normally clear annular zone seen in granules at some distance from the Golgi apparatus. It should also be pointed out that in Fig. 5 the increased electron opaqueness of the small granule centers themselves is a result of the uranyl acetate staining applied to the thin section.

**Tissue doubly incubated for acid phosphatase and thiamine pyrophosphatase:**

---

**Figure 1** Transverse frozen section through rat hypothalamus, incubated in Gomori medium for acid phosphatase activity for 30 minutes at 37°C. Deposition of reaction product is prominent in large cytoplasmic bodies (lysosomes) in the secretory neurons of the supraoptic nucleus (son), which lies adjacent to the optic tract (OT). Counterstained with saturated picric acid. X 800.

**Figure 2** Neurosecretory cell, supraoptic nucleus of the rat, incubated for acid phosphatase activity for 15 minutes at 37°C. Two large dense bodies (db-1) display an accumulation of reaction product, whereas others (db-2) are only partially "stained", and another (db-3) is seemingly unreactive. The nucleus (Nc), mitochondria (m), and a small neurosecretory granule (nsg-2) are also unreactive towards enzyme activity. However, note suggestion of reaction product in small neurosecretory granule (nsg-1) near Golgi apparatus (G). Animal decapitated, fixed in glutaraldehyde-cacodylate, and after incubation postfixed in osmium tetroxide. Araldite embedded, lead hydroxide stained. X 75,000.
In those tissue slices doubly incubated, it is possible to observe within the same section the reaction product of both enzymes. The reactive Golgi cisternae are usually lined by precipitate throughout their lengths (Fig. 6), whereas in those sections incubated for acid phosphatase alone, only the terminal segment of a reactive cisterna is outlined by reaction product. This apparent difference in distribution of reaction product suggests the localization of an acid beta-glycerophosphatase within the Golgi lamellae (see also reference 25). However, at this time, our incubation procedures were not intended to test enzyme-substrate specificities, and accordingly, it is conceivable that any number of the nucleoside-diphosphatases which exist in the Golgi apparatus (23) may be responsible for the deposition of reaction product seen in the cisternae.

Although no specific relationship was observed between the Golgi apparatus and the large dense bodies in the doubly incubated material, localization of the reaction product was associated with those small granules in intimate contact with the Golgi cisternae (Gnsg) as is evident in Fig. 7 (arrows) (see also Fig. 6, arrow). Note the absence of precipitate in the "mature" granules (nsg) nearby.

DISCUSSION
The fine structure of the neurosecretory cells in the supraoptic nucleus of normal rats as observed in this study does not essentially differ from that described for similar cells in the mouse (see reference 18) in a report published while this study was in progress. The work reported herein was directed primarily towards differentiating between the formed elements in the perikaryon of the neurosecretory cell by cytochemical methods. It also differs in that special efforts were made to assure the identity of the supraoptic nucleus in the EM.

Electron microscopy of the tissue sections incubated for acid phosphatase activity revealed prominent sites of reactivity in the large dense bodies. According to the terminology of Novikoff (19), such acid phosphatase-positive, single membrane-bounded granules may be defined as lysosomes.

The identification of the large dense body of rat neurosecretory cells as a lysosome precludes it from being considered a specific hormonal product. The evidence that distinguishes it morphologically and functionally from the small granule may be summarized: This particular entity is not present in the axons of the secretory neurons at the level of the supraoptic nucleus, median eminence, or at the terminal endings in the neurohypophysis of the rat (Osinchak, in preparation). No comparable, large granular structure has been identified in the hormone-rich fraction of the neurohypophysis (see reference 12). The large dense body has been shown in this study to possess characteristics of lysosomes which have been identified in a variety of more typical neurons (4, 20, 25), and which would be eliminated by definition, therefore, as the hormonal product distinctive of the secretory neurons in the supraoptic nucleus.

Acid phosphatase and thiamine pyrophosphatase activities have also been localized within the neurosecretory cells to some of the small Golgi vesicles, and one or two cisternae of a Golgi stack; the reaction product is bounded by smooth membrane in each instance. Traces of precipitate have been noted in those small granules in intimate con-
contact with Golgi cisternae, as well as in cisternae which contain small dense granules. These findings may be considered as providing cytochemical evidence for the elaboration of the small neurosecretory granules from the Golgi apparatus, and substantiates the proposals of previous investigators, based primarily on morphological evidence, that the neurosecretory granules arise from Golgi cisternae (see references 5, 29, and 33).

However, an alternative explanation for the association of acid phosphatase-positive Golgi cisternae with the small neurosecretory granules should be considered. Acid phosphatase is generally considered to be lytic in nature, and its presence in reactive cisternae as well as in the small neurosecretory granules in immediate contact could imply a degradative function. It is conceivable, though unlikely, that the small neurosecretory granules are being incorporated into the Golgi cisternae where subsequent hydrolysis of their content ensues.

There are two possible explanations for the localization of acid phosphatase (or non-specific phosphatase) and/or thiamine pyrophosphatase activities within the Golgi apparatus and neighboring small neurosecretory granules: (a) the enzyme(s) may be directly involved in the synthesis, or "condensation," of secretory product (see also reference 24); (b) the enzyme(s) may be entirely unrelated to the neurosecretory process and may be simply "carried over" as membrane-bounded sites of activity into the smooth envelope of the small neurosecretory granule. This latter interpretation derives some support from the finding of aggregates of dense material within completely unreactive cisternae and the "budding off" of small neurosecretory granules from them. These observations, together with the inability to detect signs of either thiamine pyrophosphatase or acid phosphatase activity in the "mature" neurosecretory granules of the nerve axons and terminals in the neurohypophysis, suggest that the activity, when present, is transient and is not part of the permanent nature of the small neurosecretory granule. For these reasons the small neurosecretory granules in the secretory cell perikarya could not appropriately be termed lysosomes.

The possibility of diffusion or spread of reaction activities within the Golgi apparatus and neighboring small neurosecretory granules: (a) the enzyme(s) may be directly involved in the synthesis, or "condensation," of secretory product (see also reference 24); (b) the enzyme(s) may be entirely unrelated to the neurosecretory process and may be simply "carried over" as membrane-bounded sites of activity into the smooth envelope of the small neurosecretory granule. This latter interpretation derives some support from the finding of aggregates of dense material within completely unreactive cisternae and the "budding off" of small neurosecretory granules from them. These observations, together with the inability to detect signs of either thiamine pyrophosphatase or acid phosphatase activity in the "mature" neurosecretory granules of the nerve axons and terminals in the neurohypophysis, suggest that the activity, when present, is transient and is not part of the permanent nature of the small neurosecretory granule. For these reasons the small neurosecretory granules in the secretory cell perikarya could not appropriately be termed lysosomes.

The possibility of diffusion or spread of reaction activities within the Golgi apparatus and neighboring small neurosecretory granules: (a) the enzyme(s) may be directly involved in the synthesis, or "condensation," of secretory product (see also reference 24); (b) the enzyme(s) may be entirely unrelated to the neurosecretory process and may be simply "carried over" as membrane-bounded sites of activity into the smooth envelope of the small neurosecretory granule. This latter interpretation derives some support from the finding of aggregates of dense material within completely unreactive cisternae and the "budding off" of small neurosecretory granules from them. These observations, together with the inability to detect signs of either thiamine pyrophosphatase or acid phosphatase activity in the "mature" neurosecretory granules of the nerve axons and terminals in the neurohypophysis, suggest that the activity, when present, is transient and is not part of the permanent nature of the small neurosecretory granule. For these reasons the small neurosecretory granules in the secretory cell perikarya could not appropriately be termed lysosomes.

The possibility of diffusion or spread of reaction activities within the Golgi apparatus and neighboring small neurosecretory granules: (a) the enzyme(s) may be directly involved in the synthesis, or "condensation," of secretory product (see also reference 24); (b) the enzyme(s) may be entirely unrelated to the neurosecretory process and may be simply "carried over" as membrane-bounded sites of activity into the smooth envelope of the small neurosecretory granule. This latter interpretation derives some support from the finding of aggregates of dense material within completely unreactive cisternae and the "budding off" of small neurosecretory granules from them. These observations, together with the inability to detect signs of either thiamine pyrophosphatase or acid phosphatase activity in the "mature" neurosecretory granules of the nerve axons and terminals in the neurohypophysis, suggest that the activity, when present, is transient and is not part of the permanent nature of the small neurosecretory granule. For these reasons the small neurosecretory granules in the secretory cell perikarya could not appropriately be termed lysosomes.

The possibility of diffusion or spread of reaction activities within the Golgi apparatus and neighboring small neurosecretory granules: (a) the enzyme(s) may be directly involved in the synthesis, or "condensation," of secretory product (see also reference 24); (b) the enzyme(s) may be entirely unrelated to the neurosecretory process and may be simply "carried over" as membrane-bounded sites of activity into the smooth envelope of the small neurosecretory granule. This latter interpretation derives some support from the finding of aggregates of dense material within completely unreactive cisternae and the "budding off" of small neurosecretory granules from them. These observations, together with the inability to detect signs of either thiamine pyrophosphatase or acid phosphatase activity in the "mature" neurosecretory granules of the nerve axons and terminals in the neurohypophysis, suggest that the activity, when present, is transient and is not part of the permanent nature of the small neurosecretory granule. For these reasons the small neurosecretory granules in the secretory cell perikarya could not appropriately be termed lysosomes.

The possibility of diffusion or spread of reaction activities within the Golgi apparatus and neighboring small neurosecretory granules: (a) the enzyme(s) may be directly involved in the synthesis, or "condensation," of secretory product (see also reference 24); (b) the enzyme(s) may be entirely unrelated to the neurosecretory process and may be simply "carried over" as membrane-bounded sites of activity into the smooth envelope of the small neurosecretory granule. This latter interpretation derives some support from the finding of aggregates of dense material within completely unreactive cisternae and the "budding off" of small neurosecretory granules from them. These observations, together with the inability to detect signs of either thiamine pyrophosphatase or acid phosphatase activity in the "mature" neurosecretory granules of the nerve axons and terminals in the neurohypophysis, suggest that the activity, when present, is transient and is not part of the permanent nature of the small neurosecretory granule. For these reasons the small neurosecretory granules in the secretory cell perikarya could not appropriately be termed lysosomes.

The possibility of diffusion or spread of reaction activities within the Golgi apparatus and neighboring small neurosecretory granules: (a) the enzyme(s) may be directly involved in the synthesis, or "condensation," of secretory product (see also reference 24); (b) the enzyme(s) may be entirely unrelated to the neurosecretory process and may be simply "carried over" as membrane-bounded sites of activity into the smooth envelope of the small neurosecretory granule. This latter interpretation derives some support from the finding of aggregates of dense material within completely unreactive cisternae and the "budding off" of small neurosecretory granules from them. These observations, together with the inability to detect signs of either thiamine pyrophosphatase or acid phosphatase activity in the "mature" neurosecretory granules of the nerve axons and terminals in the neurohypophysis, suggest that the activity, when present, is transient and is not part of the permanent nature of the small neurosecretory granule. For these reasons the small neurosecretory granules in the secretory cell perikarya could not appropriately be termed lysosomes.
product precipitate from the more active sites of the Golgi cisternae to closely adjacent structures must also be considered. While this type of uncertainty is especially pertinent to enzyme localization at the EM level, consistent localization of precipitate to circumscribed sites, at minimal times of substrate incubation, is to be regarded as specific and significant. The reactivity of the large dense bodies or lysosomes and those Golgi cisternae positive for acid phosphatase is striking at incubation times of only 5 minutes. When incubation up to 1 hour was employed, a marked spread of non-specific product occurred. Another consequence of longer incubation was the deposition of more lead phosphate at the same reactive sites which tended to obscure the underlying fine structure.

Acid phosphatase activity was not observed throughout the entire population of dense bodies or lysosomes encountered in any particular thin section. Three possibilities may be considered in explanation of this finding: (a) the reaction product is present in all dense bodies but may not have a focal point of localization demonstrable in all planes of section; (b) the enzyme, if it is present at all, is in too low a concentration to be detected by the methods used; (c) the unreactive dense bodies, although morphologically similar to lysosomes, do not possess acid phosphatase activity. In regard to the possibility that there may be two or more classes of lysosomes, Anderson and Song (1), using Barka's method for acid phosphatase, have described a staining pattern in neurons of rat brain which does not correspond to the granular pattern obtained with the Gomori method. In view of this last possibility, supplementary cytochemical methods are now being applied in an attempt to determine the chemical nature of the non-reactive dense bodies.

The origin of the dense bodies or lysosomes remains undetermined at present, as no unequivocal morphological evidence has been obtained which depicts dense body formation in the secretory neurons. Palay (29) has suggested that the "large secretory droplets" in neurosecretory cells of the goldfish (granules similar in size and structure to dense bodies in the rat) may originate from multivesicular bodies. Although this type of transformation may exist, the supporting morphological evidence has not been observed in the secretory neurons of the rat. Novikoff and Essner (21) have supposed that "in liver cells, as in other secretory cells and neurons . . . lysosomes are produced by dilatation and separation of portions of the Golgi cisternae." The localization of acid phosphatase activity within cisternae of the Golgi apparatus as found in this study is indeed consistent with such an assertion (see also reference 25).

It should be pointed out, however, that in the material prepared for both morphological examination and enzyme localization no evidence was found that suggested continuity of Golgi membranes with the limiting membrane of the dense bodies. Structural continuity is readily discernible, on the other hand, with respect to formation of the small neurosecretory granule. Since there is no direct evidence to support the contention that the large dense bodies are formed from any Golgi element, it is difficult at this time, with this material, to relegate the function of lysosomal dense body production to the Golgi apparatus. In the secretory neurons of the rat the finding remains, however, that acid phosphatase activity can be demonstrated in Golgi cisternae as well as in the dense bodies. The possibility that both loci of enzymatic activity are independent is real, as is the alternative that both loci are dependent and related through means undetectable in this study.

An alternative proposal as to the nature and possible origin of lysosomes stems from the study of Ashford and Porter (2) who suggest that lyso-

---

**Figure 8** Perinuclear view of neurosecretory cell. Doubly incubated as described for Fig. 6. Enzyme reaction product is localized in some of the large dense bodies (db) and a structure with a circular profile (ser) surrounded by a smooth surfaced membrane and containing membranous tubules and vesicles. Another circle of unreactive membranes (arrows) is in close spatial relationship to a reactive Golgi cisterna (c). A small neurosecretory granule (nsg) and a mitochondrion (m), although in close apposition to reactive organelles, do not contain traces of reaction product. The nucleus (Ne) of the secretory neuron has a well developed nuclear envelope with frequent fenestrations or nuclear pores (np). Compare cytological preservation of this vascularly perfused specimen to that obtained following decapitation and immersion-fixation as depicted in Fig. 2. Perfusion-fixation with glutaraldehyde-cacodylate, and after incubation postfixed in osmium tetroxide. Araldite embedded, lead hydroxide stained. X 78,000.
somes may represent "foci of physiological autolysis" that are "automatically surrounded by a membrane". Cytoplasmic bodies containing mitochondria, ribosomes, and rough endoplasmic reticulum have been reported (17) in epithelial cells of the small intestine. In our studies, similar cell organelles enclosed by smooth membrane derivatives have also been observed in the neurosecretory cells of normal and experimentally dehydrated rats. In tissues incubated for detection of acid phosphatase activity, similarly structured bodies have been noted (see also Fig. 6) whose outer membranous profile is bounded by reaction-product; whether these complexly structured entities should be termed lysosomes or cytolysomes (see reference 21) is questionable.

In marked contrast to the variety of possible origins for the lysosomes stands the consistent relationship observed between the small neurosecretory granule and the Golgi apparatus; this relationship is not shared by the large dense bodies. The evidence presented in this study indicates, as well, the cytochemical individuality of the perikaryonal inclusions; at least some of the large dense bodies have acid phosphatase activity, while the mature, small neurosecretory granules do not.1 These results extend the obvious morphological differences between formed elements, such as size, electron opaqueness, staining properties, and internal structure. The findings reported here fulfill the attempt to characterize the granular bodies in the secretory neurons of the rat, and corroborate through direct evidence the suggestions of Palay (29), that the small, 0.1 μ, granules "may be considered as the morphological counterpart of" the neurohypophysial hormones, and that the large dense bodies "are concerned with some other function, perhaps with intracellular-metabolic events."

The author is indebted to Dr. Montrose J. Moses for his criticism and helpful advice throughout the course of this work and during the writing of the manuscript.

This work was done in the laboratory of Dr. Moses while the author was a National Defense Education Act predoctoral fellow. Portions have been included in the contents of a thesis submitted to the Graduate School of Duke University in partial fulfillment of requirements for the Ph.D. degree. The laboratory facilities used were supported in part by grants to Dr. Moses from the United States Public Health Service (National Institutes of Health: GM-06753 and GPD-15,640) and the American Cancer Society (E-213).

Received for publication, June 21, 1963.

REFERENCES
24. Novikoff, A. B., Esner, E., Biempica, L., and

1 Novikoff and Esner (22) have recently reported a similar finding in both paraventricular and supraoptic nuclei of the rat.


JOSEPH OSINGHAE Acid Phosphatase and Thiamine Pyrophosphatase Activity

Published April 1, 1964