

LYSOSOMAL PACKAGING IN DIFFERENTIATING AND DEGENERATING ANURAN LATERAL MOTOR COLUMN NEURONS

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ABSTRACT

The role of the Golgi apparatus and the Golgi-endoplasmic reticulum-lysosome complex (GERL) in the genesis of lysosomes was examined in differentiating and degenerating motor neurons of anuran larvae. Acid phosphatase, aryl sulfatase, and thiolacetic acid esterase were utilized as marker enzymes for the lysosomal system, while nucleoside diphosphatase and thiamine pyrophosphatase labeled the inner saccule(s) of the Golgi apparatus. Reduced osmium tetroxide was routinely deposited in the outer Golgi saccule regardless of the state of neuronal maturation. In all young neurons, the disposition of acid hydrolase reaction product paralleled the formation of GERL, with no lytic activity in the Golgi apparatus per se. Hypertrophy of the Golgi apparatus and GERL was observed in the early phases of degeneration, and both organelles apparently exhibit extensive hydrolytic activity. Dense bodies, autophagic vacuoles, and primary lysosomes were found arising from GERL, while the Golgi apparatus may produce primary lysosomal granules during regression. On the other hand, in differentiating neurons, hydrolytic activity was restricted to GERL and an occasional dense body and autophagic vacuole. These studies illustrate a parallelism between the development of GERL and genesis of primary and secondary lysosomes during neuronal cytodifferentiation, and implicate GERL and possibly the Golgi apparatus in lysosomal packaging in degenerating neurons.

INTRODUCTION

Evidence from various sources indicates that a specialized region of smooth endoplasmic reticulum contiguous with the granular endoplasmic reticulum is responsible for the concentration and packaging of several proteins (1). Novikoff has termed this agranular reticulum GERL (Golgi-endoplasmic reticulum-lysosome complex) and considers it to participate in lysosomal packaging (2-5); furthermore, Holtzman et al. (6) have intimated that primary lysosomes arise from GERL. This has been substantiated by Novikoff et al. (1), who further demonstrated the origin of dense bodies in the cisternae of this tubular network by utilizing thick-section cytochemistry. Other investigations variously suggest that GERL packages (a) tyrosinase into coated vesicles (7, 8), (b)

adrenalin-containing granules (9), (c) uniodinated thyroglobulin (10), and (d) peroxidase-positive granules of the rat parotid (11) and possibly of the lacrimal gland (12) as well.

The interrelationship of GERL and the Golgi apparatus with respect to the genesis of lysosomes remains obscure (13). Novikoff and his collaborators (1, 5) emphasize the distinctness of GERL, whereas Whaley et al. (14) and Morré's group (15) would probably incorporate it with the saccules and nearby vesicles that constitute the Golgi apparatus. The purpose of the present study is to provide further support for the identity of GERL as a distinct organelle, while simultaneously demonstrating that in the early phases of neuronal degeneration, both GERL and apparently the Golgi apparatus contribute to lysosomal biogenesis.

MATERIALS AND METHODS

Materials

The following reagents and cytochemical substrates were purchased from Sigma Chemical Co., St. Louis, Mo., except where otherwise specified. (a) Acid phosphatase substrates and reagents: sodium- β -glycerophosphate (grade I); Sigma 104-*p*-nitrophenyl phosphate; cytidine 5'-monophosphate (disodium salt); α -naphthol phosphate; L-(+)-tartrate; and sodium fluoride. (b) Nucleoside diphosphatase substrates: thiamine pyrophosphate (TPPase), and inosine diphosphate (IDPase). (c) aryl sulfatase substrate: *p*-nitrocatechol sulfate. (d) Thiolacetic acid esterase substrate and reagents: thiolacetic acid (Eastman Organic Chemical Co., Rochester, N.Y.); ethyl-*p*-nitrophenyl phosphate (E-600); and (c) diisopropylfluorophosphate (DFP).

Methods

The larval *Rana pipiens* used in this investigation were reared from embryos obtained by artificially induced ovulation (16). When they reached embryonic stage 25 (17), the embryos were placed in aquaria containing 4 l of aerated tap water and kept at $18 \pm 1^\circ\text{C}$ with a constant photoperiod of 12 h of light and 12 h of darkness. The density of the larvae was maintained at 20 per aquarium in order to establish growth independent of crowding influences. All were fed washed spinach or lettuce ad lib. Before fixation for light or electron microscopy, the tadpoles were staged according to the method of Taylor and Kollros (18) and weighed. In addition, their total body measurements and hind limb lengths were recorded.

Morphological Investigations

All larvae were anesthetized with either 2.5% aqueous ethyl carbamate or a 1:3,000 dilution of tricaine methanesulfonate (Sigma Chemical Co.), and the central

nervous system was preserved by vascular perfusion (19). A separatory funnel containing 5–10 ml of Rosenbluth's (20) amphibian saline was hung approximately 3 feet above the anesthetized tadpole. After the insertion of a 27-gauge needle through the ventricle into the conus arteriosus, each tadpole was perfused with saline for 30 s to 1 min and then directly with fresh fixative for 15–20 min.

For histological study, larvae were generally perfused with 10% neutral-buffered formalin prepared from paraformaldehyde (Eastman Organic Chemical Co.) and buffered at pH 7.5 with 0.1 M Sorensen's phosphate buffer. After a 2–3 h delay, the lumbosacral spinal cord was removed and fixed for an additional 1–7 days. Subsequently the tissue was dehydrated, cleared, embedded in paraffin, and serially sectioned at 10 μm . Sectioned material was stained in Ehrlich's acid hematoxylin and counterstained with light green or eosin Y. Frequently sections were also stained with galloycyanine or cresyl violet.

Tadpoles were perfused with either 0.1 M phosphate- or 0.1 M cacodylate-buffered (pH 7.5) 1/3-strength Karnovsky's (21) glutaraldehyde-paraformaldehyde fixative with 0.5% acrolein (Polysciences Inc., Warrington, Pa.) and trace amounts of CaCl_2 for fine structural study. In some instances, the dilute solution was followed by perfusion with full-strength Karnovsky's fixative for an additional 15 min. If no cytochemical tests were to be performed, the tadpole was set aside for 2–3 h before the lateral motor column area was dissected free and placed in fresh fixative for up to 24 h. All tissues were then washed overnight in 0.1 M buffer with 7.5% sucrose (pH 7.5).

Thereafter, the tissues were postfixed in 0.05 M acetate-Veronal-buffered 1% OsO_4 for 1–2 h, rinsed in 0.05 M acetate-Veronal buffer with 5% sucrose, rapidly dehydrated in a series of cold-ethanols and propylene oxide, and embedded in Epon 812 (22). Light gold and silver sections were cut on Porter-Blum MT-1 and -2 microtomes (Ivan Sorvall, Inc., Norwalk, Conn.) and mounted on Formvar-coated 200-mesh or naked 300- and 400-mesh copper grids. The sections were then stained with 2.5% aqueous uranyl acetate and lead citrate and viewed with RCA-EMU 3F or a Siemens 1A electron microscope.

For purposes of orientation, thick sections (0.5–1.0 μm) were routinely cut and stained with either toluidine blue or azur II-methylene blue. Occasionally, sections were affixed to cover slips and dipped for 5–10 s in 1% cresyl violet in a solution of 50% acetone in absolute alcohol, or in Mackay's and Mead's (23) polychrome stain.

Cytochemical Investigations

PREPARATION OF TISSUE: All neural tissue incubated in cytochemical media was fixed in dilute, cacodylate-buffered Karnovsky's fixative (21). After perfu-

sion, the tissue was rapidly removed, fixed for an additional 30–90 min, and then washed overnight in 0.1 M cacodylate buffer (pH 7.5) with 7.5% sucrose before further processing.

Lumbosacral spinal cord was prepared for enzyme localization by three different procedures: (a) Frozen sections (50–80 μm) were obtained with a Harris International microtome cryostat at -20°C ; (b) nonfrozen sections were cut at 20–50 μm on a Smith-Farquhar tissue sectioner and stored in 0.1 M cacodylate buffer with 7.5% sucrose; and (c) 100–150 μm nonfrozen, non-fixed tissue slices were prepared with the Smith-Farquhar tissue sectioner and then fixed in 1/3-strength Karnovsky's (21) medium for 1–3 h and washed overnight. Tissue sections prepared by the latter two techniques yielded the most satisfactory results; in the frozen sections, fine structure was frequently disrupted.

Enzyme Activities

ACID PHOSPHATASE: Some sections were incubated at pH 5.0 for 20 min to 1.5 h in the Barka-Anderson (24) modification of Gomori's medium, with sodium- β -glycerophosphate, p -nitrophenyl phosphate (10 mM), or α -naphthol phosphate (2.5 mM) as substrate. Other tissue sections were incubated with cytidine 5'-monophosphate as the substrate and manganese as the activating ion, according to Novikoff's (25) method. Controls consisted of incubations which either lacked the substrate or included 10 mM NaF or 20 mM L-(+)-tartrate, or were conducted at pH 7.5.

ARYL SULFATASE: Aryl sulfatase was localized by using a modification of Goldfischer's (26) medium containing 10 ml of 6.25 mM p -nitrocatechol sulfate in 0.1 M acetate buffer, pH 5.4, and 3 ml of 8% $\text{Pb}(\text{NO}_3)_2$ or 3 ml of 5% BaCl_2 , plus 7.5% sucrose. After the pH had been adjusted to 5.4 with 0.1 N HCl, sections were incubated for 30–120 min at 25°C , treated with buffered 2% $(\text{NH}_4)_2\text{S}$, and then washed for 1–2 h in 0.1 M acetate-Veronal buffer (pH 5.4) with 7.5% sucrose.

THIOLACETIC ACID ESTERASE: Both frozen and nonfrozen sections were incubated in Bell's and Barnett's (27) medium consisting of 0.05 M thiolacetic acid and 0.01 M lead nitrate in 0.1 M cacodylate buffer (pH 5.5) with 7.5% sucrose. Before this incubation, however, tissue sections were preincubated for 15–30 min in the above medium with 10^{-5} M E-600 or 10^{-4} M diisopropylfluorophosphate (DFP) instead of the substrate. The sections were then rinsed in buffer and incubated in the cytochemical medium for 0.5–1.5 h at 4° or 25°C . Controls consisted of incubating the tissue either without the substrate or in the presence of 0.1 M sodium fluoride (M. Bell, personal communication).

NUCLEOSIDE DIPHOSPHATASE: Nonfrozen sections of spinal cord were incubated in the medium of Novikoff and Goldfischer. Incubations were conducted at 37°C for a 30 to 90-min period with renewal of the media every 30 min. In control incubations, the substrate was omitted from the media.

OSMIUM REDUCTION: Larvae were perfused with an aqueous, 2% OsO_4 warmed to 40°C for 5 min. The spinal cord was then rapidly removed and 100 μm slices were further osmicated at 40°C for 40–50 h.

SUBSEQUENT PROCESSING: After the buffered rinse, all sections were postfixed in 1% OsO_4 buffered with 0.05 M acetate-Veronal buffer (pH 7.4) plus 5% sucrose. Some sections were then stained en bloc for 1 h with 0.5% uranyl acetate in 0.05 M acetate-Veronal buffer (pH 5.5) before dehydration. The tissue was dehydrated and embedded in Epon 812 (22), and thin sections were examined either unstained or lightly stained with lead citrate. Reaction product was frequently visualized in thick sections (0.5 μm) treated for 30 min with 2% $(\text{NH}_4)_2\text{S}$ before staining to convert the colorless lead phosphate deposits to brown lead sulfide. Unconverted thick sections were also viewed in the RCA-EMU 3F electron microscope at 100 kV.

OBSERVATIONS

Lateral motor column (LMC) neurogenesis is separable into three partially overlapping periods (28, 29). The first entails the primary migration of prospective LMC neurons from the mantle into the ventrolateral quadrants of the neural tube and the early growth (stage IV–VIII [20]) of these neuroblasts. In the second period (stages IX–XIV), those neurons which (presumably) fail to make peripheral connections degenerate (histogenetic cell death [28]), while functional neurons are characterized by the organization of their endoplasmic reticulum into Nissl substance and by the appearance of axodendritic and axosomatic synapses within the developing neuropil. An elaboration of Nissl substance and further synaptogenesis typify the tertiary period of neuronal cytodifferentiation (stage XV to juvenile).

Fine Structure of Postmigratory Motor Neurons

By stage V, the primary migration of LMC neuroblasts from the mantle (29, 30) appears to be complete, and a column of relatively undifferentiated cells can be seen in the ventrolateral quadrants of brachial and lumbosacral regions of the neural tube (Figs. 1, 2). These young neurons are uniform in size and structure (Fig. 2). They have large nuclei surrounded by a narrow rim of cytoplasm which contains sparsely distributed rough endoplasmic reticulum, several mitochondria, and generally, one paranuclear Golgi apparatus (Fig. 2). Occasionally elements of endoplasmic reticulum are closely opposed to the neurolemma,

reminiscent of subsurface cisternae (31). Extracellular space between these young neurons is rarely present. However, when the cells are separated, the intervening space is filled with developing neuronal processes (Fig. 2).

Differentiation of GERL

MORPHOLOGY: The appearance of agranular endoplasmic reticulum on the "concave" surface of the Golgi apparatus and its subsequent differentiation is of particular interest with reference to the origin of lysosomes (1-6). Postmigratory neurons (stage V) all possess some unorganized smooth endoplasmic reticulum in the region of the Golgi apparatus (Fig. 3). A proliferation of this endoplasmic reticulum occurs later (stage VI and VII), and the anastomosis of the tubules into polygonal arrays begins about stage VII (Figs. 5, 6) and appears to be completed by stage VIII. At this juncture, the agranular endoplasmic reticulum is predominantly found in the concave surface of the Golgi apparatus (Figs. 7, 8). This series of elaborate membrane rearrangements apparently represents the morphogenesis of GERL (1, 3, 5, 6). Throughout the development of the tubular network, numerous coated and smooth vesicles apparently detach (Figs. 5, inset, 6, 8). Many are acid hydrolase-positive, thus qualifying as primary lysosomes by cytochemical criteria (25, 32; Figs. 6, 8). As the genesis of GERL nears completion, portions of its tubules accrue an electron-dense heterogeneous material similar to that found in dense bodies (Fig. 7). Images of these dense bodies with the long tails (Fig. 7, inset) suggest their origin in elements of GERL which possess such a dense matrix (1, 5, 6, 33, 34).

DIPHOSPHATASE ACTIVITY AND OSMIUM REDUCTION: In postmigratory neurons, reaction product of thiamine pyrophosphatase (TPPase) and inosine diphosphatase (IDPase), classical histochemical markers of the Golgi apparatus (1, 5, 14, 15), is restricted to the inner Golgi saccule(s) (Fig. 3). Even though nucleoside diphosphatase activity is obvious in the Golgi apparatus and some neighboring vesicles, GERL, endoplasmic reticulum, and dense bodies exhibit no evidence of lead phosphate precipitates. Another Golgi marker, osmium (1), prominently stains the outer Golgi saccule adjacent to the transitional endoplasmic reticulum (1, Fig. 4). Like the localization of TPPase and IDPase, little reduced osmium is evident in the endoplasmic reticulum,

GERL, or dense bodies. Moreover, the topographic distribution of these Golgi apparatus indicators remains unaltered throughout subsequent neuronal development.

ACID HYDROLASE ACTIVITY: The distribution of acid hydrolase activity parallels the transformation of GERL in differentiating neurons. When incubated for acid phosphatase activity, the lead phosphate reaction product is observed in GERL, small vesicles apparently emanating from GERL and dense bodies (Fig. 8). Likewise, aryl sulfatase activity predominates in this developing agranular endoplasmic reticulum (Fig. 6) and in some coated vesicles seemingly detaching from it (Fig. 6, inset). The reaction product of aryl sulfatase and thiolacetic acid esterase may be found in dense bodies and in rare autophagic vacuoles as well. During this period the Golgi apparatus reveals no cytochemically detectable lysosomal activity (Figs. 6, 8).

Lysosomal Genesis during Neuronal Degeneration

Hypertrophy of the Golgi apparatus and GERL, together with an accumulation of large dense bodies and autophagic vacuoles, characterize a large class of neurons seen in the LMC at stages IX and X (Figs. 9-17). A smaller number of neurons disclose well-organized endoplasmic reticulum (Nissl substance) and a primitive neuropil (Fig. 22). Evidently at this juncture, cells destined to die accumulate numerous dense bodies and autophagic vacuoles (35), whereas neurons whose axons have supposedly formed peripheral neuromuscular junctions continue to mature (36).

THIN-SECTION CYTOCHEMISTRY: With the proliferation of the Golgi apparatus and nearby GERL, considerable quantities of acid hydrolase reaction product accumulate (Figs. 9-17). Although these areas are morphologically intricate (1), an analysis of transverse (Figs. 10-13) and tangential (Figs. 14, 15) ultrathin sections suggests that the prominent lead precipitates are deposited within the inner Golgi saccules (some of which appear fenestrated) as well as in the anastomosing network of GERL tubules (Figs. 12-15). While acid phosphatase and E-600 resistant esterase activities seem located in both organelles (Figs. 10-13), aryl sulfatase reaction product appears primarily restricted to GERL elements (Figs. 17). Small vesicles harboring acid phosphatase and esterase activity apparently arise from components

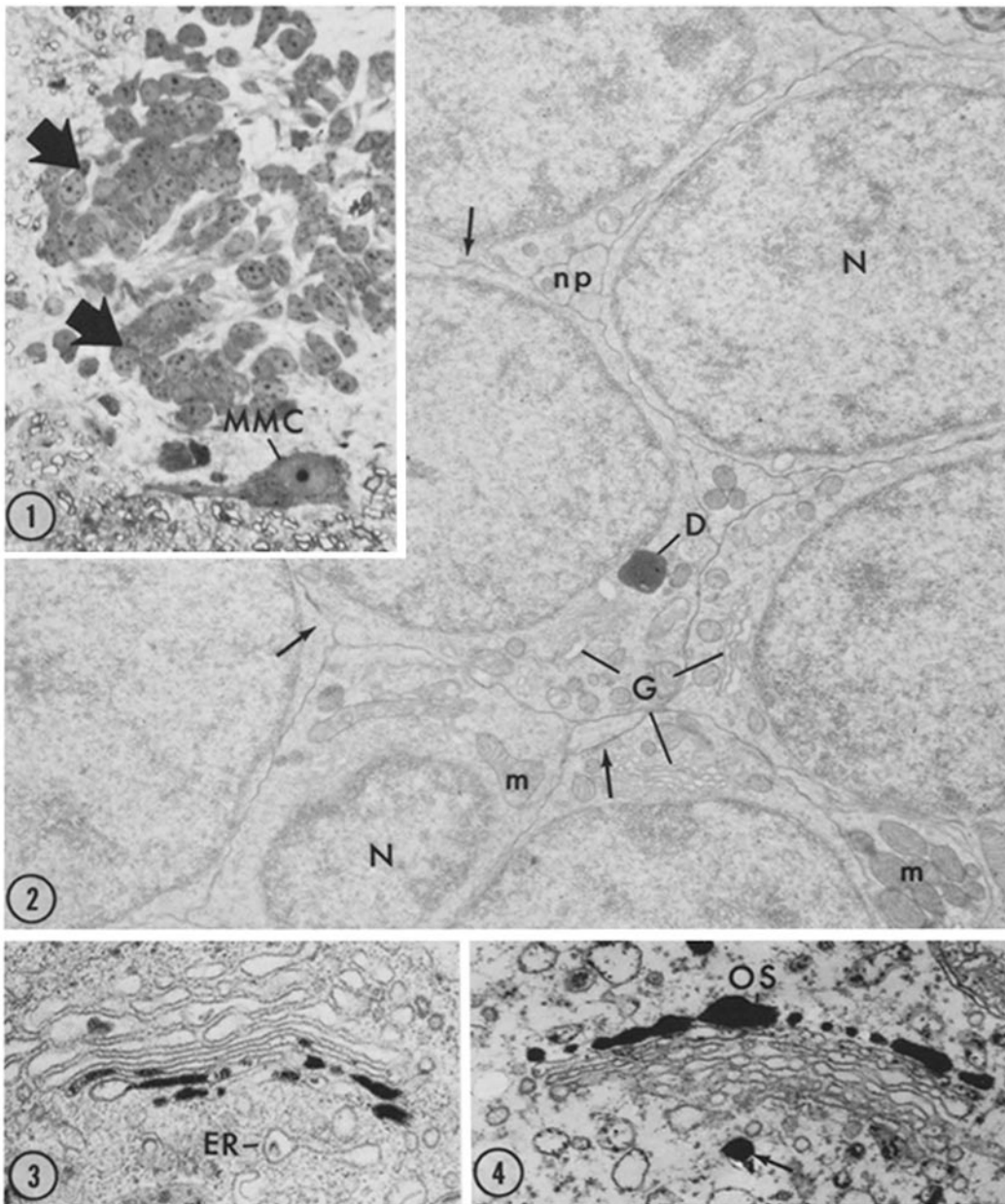


FIGURE 1 Photomicrograph illustrating the packing of young LMC neurons at stage V. Generally the neuroblasts are found in discrete groups of cells (arrowheads). A mesial motor column cell (*MMC*), which innervates the axial musculature, is visible in the ventromedian area. Toluidine blue. $\times 450$.

FIGURE 2 Seven neurons can be identified in a portion of a stage V LMC. They are characterized by a high proportion of free to membrane-bound ribosomes, a paranuclear Golgi complex (*G*), sparsely distributed mitochondria (*m*), and a rare dense body (*D*). Occasionally subsurface cisternae of endoplasmic reticulum (arrows) can be found just under the neurolemma; at other times they are associated with developing neuronal processes (*NP*) passing by the cells. Nucleus (*N*). $\times 10,500$.

FIGURES 3 and 4 In young neurons (stage VI), IDPase reaction product is concentrated within the inner saccule(s) of the Golgi apparatus (Fig. 3). Note the IDPase-negative agranular endoplasmic reticulum (*ER*) in the GERL region (Fig. 3). Reduced OsO_4 consistently impregnates the outer saccule (*OS*), whereas osmium deposits in *ER* (arrow) are variable and infrequent (Fig. 4). Fig. 3, IDPase, 45-min incubation. $\times 49,000$. Fig. 4, 2% OsO_4 , 40 h at 40°C . $\times 40,000$.

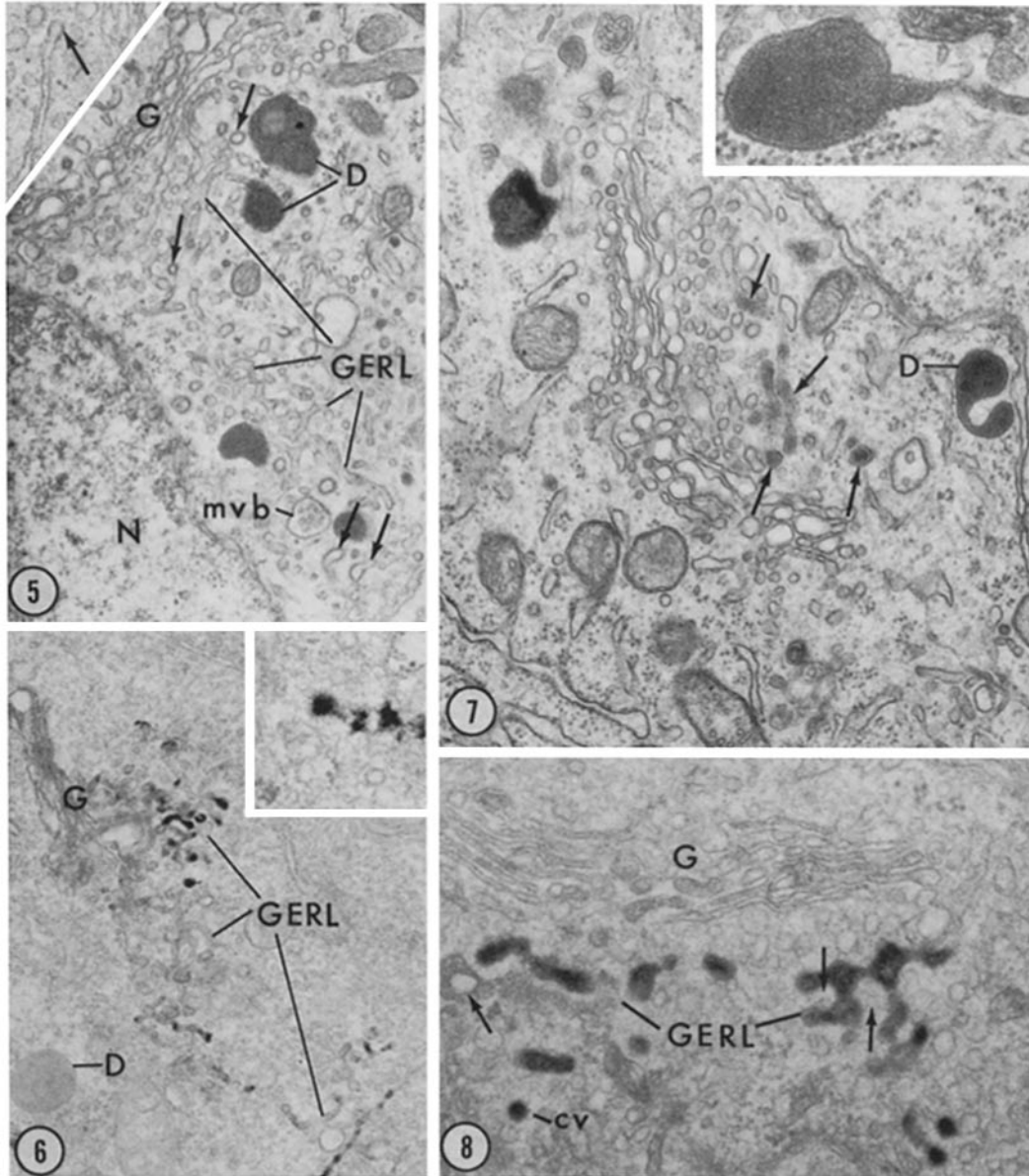
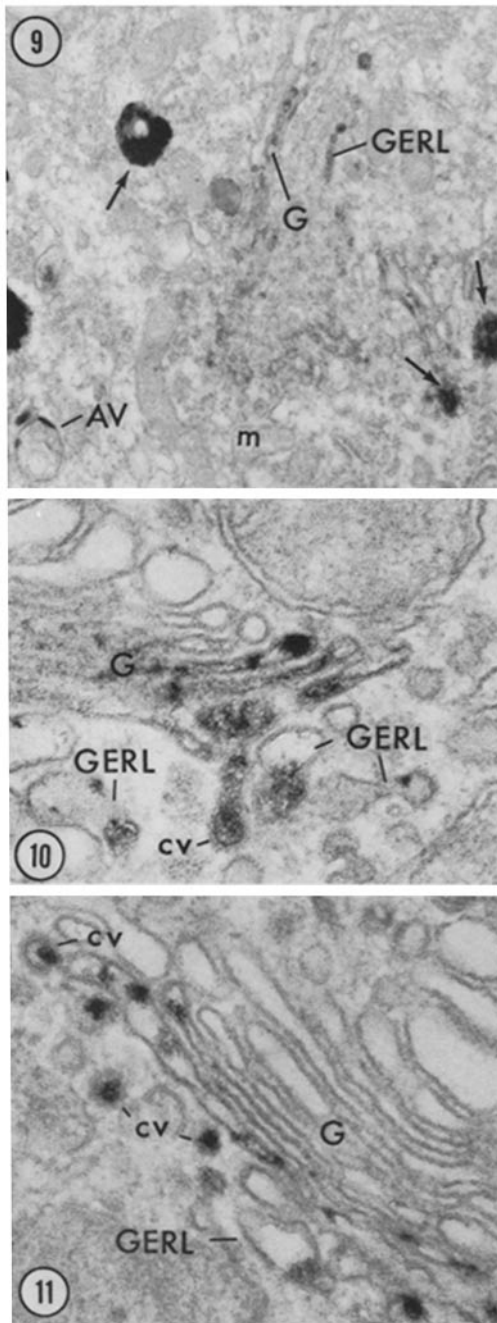


FIGURE 5 In stage VII neurons, large amounts of smooth endoplasmic reticulum begin anastomosing to form GERL. Note the coated vesicles (arrows) apparently arising from the developing GERL. Dense bodies (*D*) and multivesicular bodies (*mvb*) are also visible. Inset, coated vesicles frequently appear to detach (arrow) from GERL as it proliferates. $\times 15,400$; inset $\times 39,500$.

FIGURE 6 Aryl sulfatase reaction product in many portions of developing GERL, but not in the Golgi apparatus (*G*) of stage VII neurons. A large dense body (*D*) at lower left has no barium sulfide reaction product which suggests that heterogeneous classes of lysosomes may exist; or that the prolonged acid wash may have extracted the delicate BaS reaction product. Inset depicts an aryl sulfatase-positive coated vesicle arising from a GERL element. Aryl sulfatase, 80-min incubation with BaCl_2 . $\times 17,400$. Inset, aryl sulfatase, 80-min incubation with $\text{Pb}(\text{NO}_3)_2$. $\times 52,500$.

FIGURE 7 By stage VIII, GERL is more compact and intimately associated with the Golgi apparatus. The distal tubules of GERL (seen here in transverse and longitudinal section) frequently accumulate an electron-dense matrix (arrows) similar to that observed in dense bodies (*D*). The inset depicts the origin of a dense body from the distended end of a GERL tubule. Fig. 7, $\times 36,500$; inset, $\times 46,000$.

FIGURE 8 In stage VIII neurons, acid phosphatase activity is restricted to the compacted GERL and to small coated vesicles (*cv*). Note a hint of the polygonal compartments (arrow) of GERL in this thin section. The Golgi apparatus (*G*) exhibits no acid phosphatase activity in differentiating neurons. β -glycerophosphatase, 40-min incubation. $\times 42,000$.



FIGURES 9-11 Thiolacetic acid esterase reaction product is localized, for the first time, within GERL and the inner saccules of the Golgi apparatus (*G*) during the early phases of neuronal regression (stage X). Dense bodies (arrows) exhibit rather heavy deposits of reaction product, whereas only the limiting membranes of the

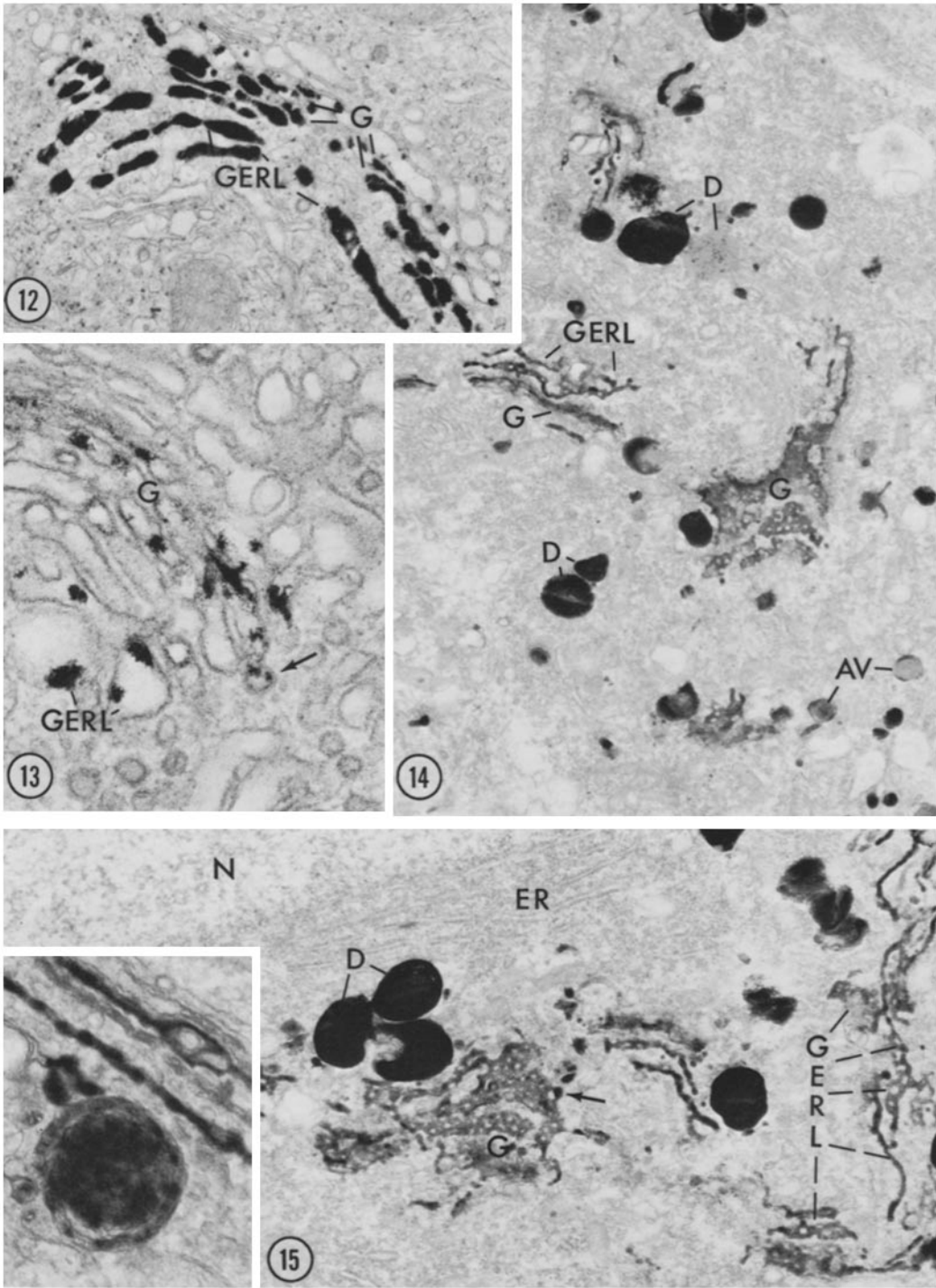
of GERL and possibly the Golgi apparatus (Figs. 10, 11, 13), in contrast to aryl sulfatase-positive vesicles which take their origin from the tubular cisternae of GERL (Fig. 17). In such cells, hydrolytic activity is also visible in an increasing number of dense bodies and autophagic vacuoles (Figs. 9, 14-17) which are probably generated from dilated areas of GERL.

THICK-SECTION CYTOCHEMISTRY: Viewing thick sections also infers that the inner saccules of the Golgi apparatus possess acid phosphatase reaction product (Fig. 18). In face views, acid phosphatase is apparently localized within polygonal arrays which resemble those of the inner Golgi saccule (1, compare Fig. 18 with Fig. 20), as well as in an adjacent fenestrated compartment (Fig. 18). Thus far, neither thick nor thin sections have demonstrated any nucleoside diphosphatase reaction product within this fenestrated element. In some thick sections ($\sim 0.5 \mu\text{m}$), tubules displaying no TPPase reaction product are visualized within the polygonal compartment of the inner Golgi saccule (TPPase-positive, Fig. 20). Images of GERL (1) in thick section illustrate a central cisternal plate from which irregularly anastomosing tubules radiate (Fig. 19). Small vesicles and dense bodies apparently take their origin from these tubules. In such regressing neurons, thick sections reveal that osmium impregnates only the outer elements of the Golgi apparatus and some vesicles nearby (Fig. 21).

Cytochemistry of the Golgi Apparatus and GERL in Maturing Neurons

The differentiating neuron possesses a few randomly scattered dense bodies (Fig. 22) and an extensive Golgi apparatus which reveals no evidence of acid hydrolase activity (Figs. 23-25). GERL is found distributed in a pattern similar to that described by Holtzman et al. (6) and Novikoff et al. (1; Fig. 22, inset). Acid phosphatase and aryl sulfatase (Fig. 25) reaction product is restricted to these

autophagic vacuole (*AV*) possess esterase (Fig. 9). Patchy deposits of reaction product are consistently seen within the inner saccules of the Golgi apparatus (*G*) and GERL (Figs. 10, 11). Frequently, esterase-positive coated vesicles (*cv*) apparently detach from these Golgi saccules (Figs. 10, 11). Fig. 9, thiolacetic acid esterase, 60-min incubation. $\times 20,000$. Fig. 10, thiolacetic acid esterase, 90-min incubation. $\times 99,000$. Fig. 11, thiolacetic acid esterase, 90-min incubation. $\times 99,000$.



GERL membranes, autophagic vacuoles, and dense bodies, while the inner Golgi saccule(s) reveal nucleoside diphosphatase activity (Fig. 22, inset). Thiolacetic acid esterase activity is also observed in dense bodies and in some vesicles and/or tubules, probably, of GERL origin (Figs. 23, 24). Lipofuscin granules (37) become conspicuous in older neurons and usually exhibit acid hydrolase activity in the periphery of the granule (Fig. 25).

DISCUSSION

Development of GERL and the Origin of Lysosomes

The emergence of lysosomal acid hydrolase activity in young neurons parallels the differentiation of GERL (1, 2, 5). It is from this anastomosing membrane system that primary lysosomes (1-6; reviews 33, 34), dense bodies (1, 3, 5, 6, 33, 34), and autophagic vacuoles are apparently derived (1, 3, 32, 34, 35). As the agranular reticulum of the prospective GERL anastomoses, cytochemical demonstration of lytic activity becomes quite vivid (Fig. 6). And as the fusion of these smooth tubules of endoplasmic reticulum proceeds, small smooth and coated vesicles (many exhibiting hydrolytic activity) are generated, whereas dense bodies are doubtless derived from regions of GERL which have amassed an electron-dense material (1, 5, 6; Fig. 7, inset). Autophagic vacuoles probably arise from the tubular elements of GERL or smooth endoplasmic reticulum (5, 6), since they possess acid hydrolase activity and lack nucleoside diphosphatase reaction product (35).

Lysosomal Packaging during Neuronal Degeneration

As simple reflex arcs (stage IX, reference 28) become established, degenerating neurons can be distinguished from those which are differentiating. The regressing cell is characterized by GERL and a Golgi apparatus which are the focal point for the production and accumulation of numerous secondary lysosomes (25, 32). However, unlike normal (1, 2, 4, 5, 33, 34, 37-40) and chromatolytic (6) neurons, degenerating nerve cells exhibit acid hydrolase activity within GERL elements and, possibly, some activity within Golgi saccules. A division of labor seemingly occurs in regressing neurons wherein the inner saccules of the Golgi apparatus may become involved with the production of small primary lysosomes (Figs. 10, 11, 13; references 41, 42), while GERL elements dilate to form large dense bodies (1, 3, 5, 6, 33, 34), generate additional primary lysosomal granules (1-7), and provide limiting membrane for autophagic vacuole formation (5, 33-35).

Under normal circumstances, the GERL concept (1, 5-7, 37) assumes that GERL exists as a unique element of smooth endoplasmic reticulum contiguous with granular endoplasmic reticulum through which lysosomal enzymes are transported (43-46) and packaged, presumably bypassing the Golgi apparatus. However, does the lack of acid hydrolase activity in the Golgi apparatus of differentiating neurons or its tentative presence in regressing neurons merely represent a concentration gradient of lytic enzymes within this organelle? Or does it imply the existence of an alternate route of

FIGURES 12 and 13 Transverse thin sections through the Golgi region (*G*) and GERL apparently reveal acid phosphatase reaction product within both organelles (Figs. 12, 13) of regressing neurons (stage XI). In briefly incubated specimens, lead phosphate precipitates seem associated with both GERL and Golgi (*G*) membranes (Fig. 13) and only on prolonged incubation do they fill the cisternal channels (Fig. 12). Note the reactive coated vesicles (arrow) budding off an apparent Golgi saccule (Fig. 13). Fig. 12, CMPase, 45-min incubation. $\times 27,500$. Fig. 13, CMPase, 20-min incubation. $\times 99,000$.

FIGURE 14 Neuroplasm of a regressing neuron (stage X) illustrates the reactive elements of the vacuolar apparatus (32). Numerous dense bodies (*D*) and autophagic vacuoles (*AV*) accumulate in this cell type. GERL and, probably, Golgi apparatus (*G*) as well, disclose large amounts of acid phosphatase reaction product. β -glycerophosphatase, 50-min incubation. $\times 14,500$.

FIGURE 15 Acid *p*-nitrophenyl phosphatase reaction product is found in the same organelles as that of acid β -glycerophosphatase, CMPase, and α -naphthol phosphatase. Dense bodies (*D*), GERL elements, and possibly fenestrated Golgi saccules (*G*) exhibit heavy deposits of reaction product after an incubation of moderate length. Occasionally, small vesicles (arrow) seemingly detach from the periphery of a Golgi saccule (*G*). Nucleus (*N*), endoplasmic reticulum (*ER*). *p*-nitrophenyl phosphatase, 40-min incubation. Inset depicts a dense body encircled by an element of GERL. *p*-nitrophenyl phosphatase, 40-min incubation. Fig. 15, $\times 20,300$; inset, $\times 60,700$.

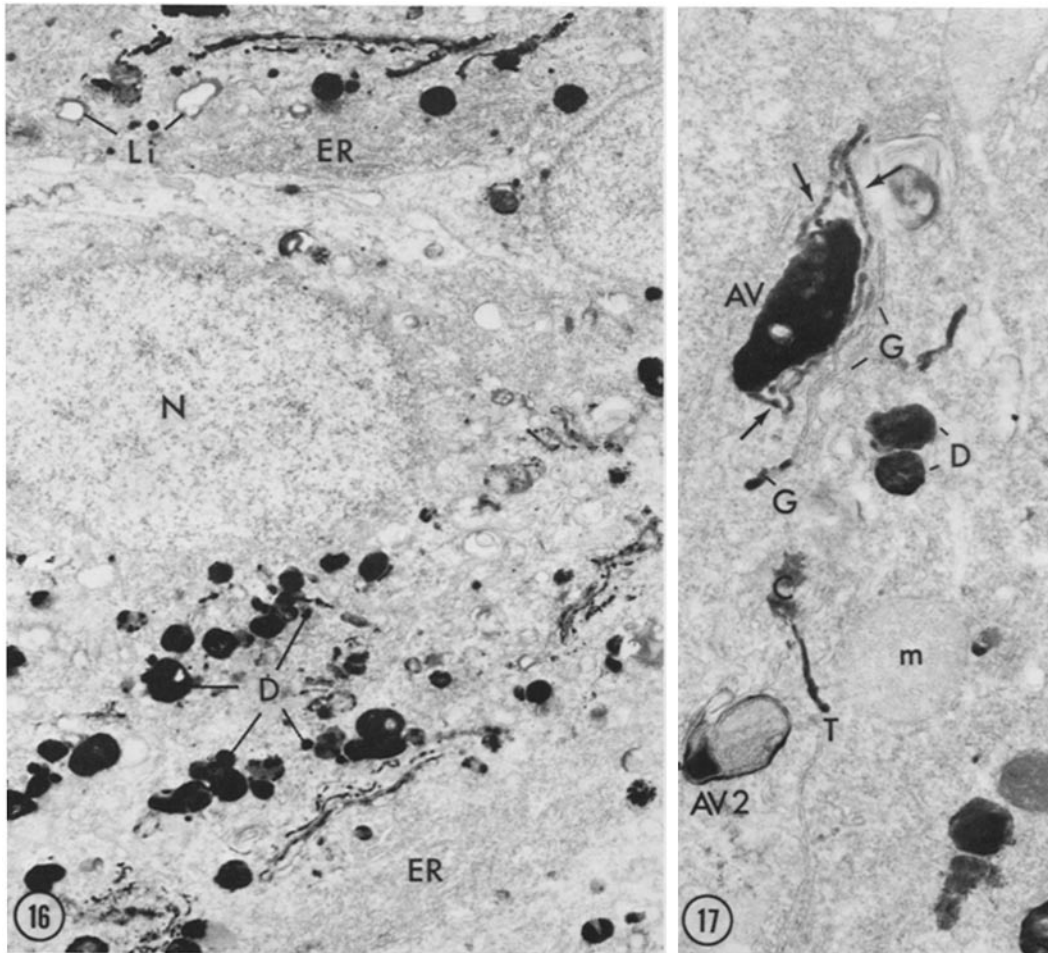
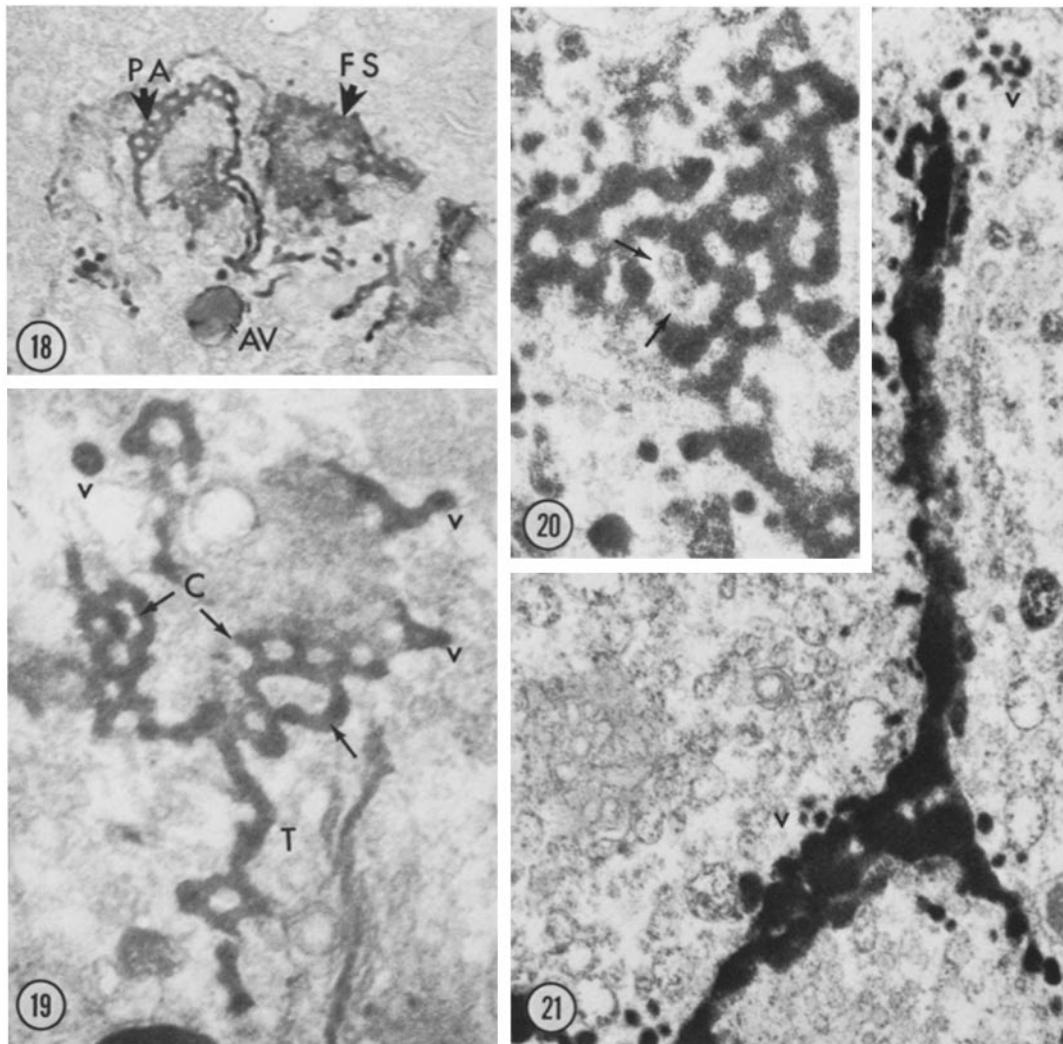


FIGURE 16 The acquisition of dense bodies (*D*) is readily apparent in this degenerating neuron (stage X). In such cells, rough endoplasmic reticulum (*ER*) becomes restricted to the periphery, and the nucleus (*N*) is eccentrically situated. Lipid-like vesicles (*Li*) also accumulate in some degenerating neurons. Acid β -glycerophosphatase, 45-min incubation. $\times 8,400$.

FIGURE 17 Formation of autophagic vacuoles is prominent during neuronal degeneration. Here, aryl sulfatase reactive GERL tubules (arrows) appear in continuity with an autophagic vacuole (*AV*); another vacuole exhibits reaction product within limiting membranes (*AV2*). Golgi saccules (*G*) rarely possess aryl sulfatase activity. Frequently aryl sulfatase reaction product can be found in a GERL tubule (*T*) emanating from its cisternal plate (*C*). Small vesicles detach from these tubules (*T*). Dense bodies (*D*) and mitochondria (*m*). Aryl sulfatase, 90-min incubation with BaCl_2 . $\times 23,200$.

lysosomal packaging? Prolonged incubation for acid phosphatase (2–4 h) and thiolacetic acid esterase (2–5 h) have, thus far, failed to yield any reaction product within the Golgi apparatus of differentiating neurons (stages VIII and XX). Although acid hydrolase concentration below that detectable cytochemically may be present in the Golgi apparatus, it must, as such, reflect only a

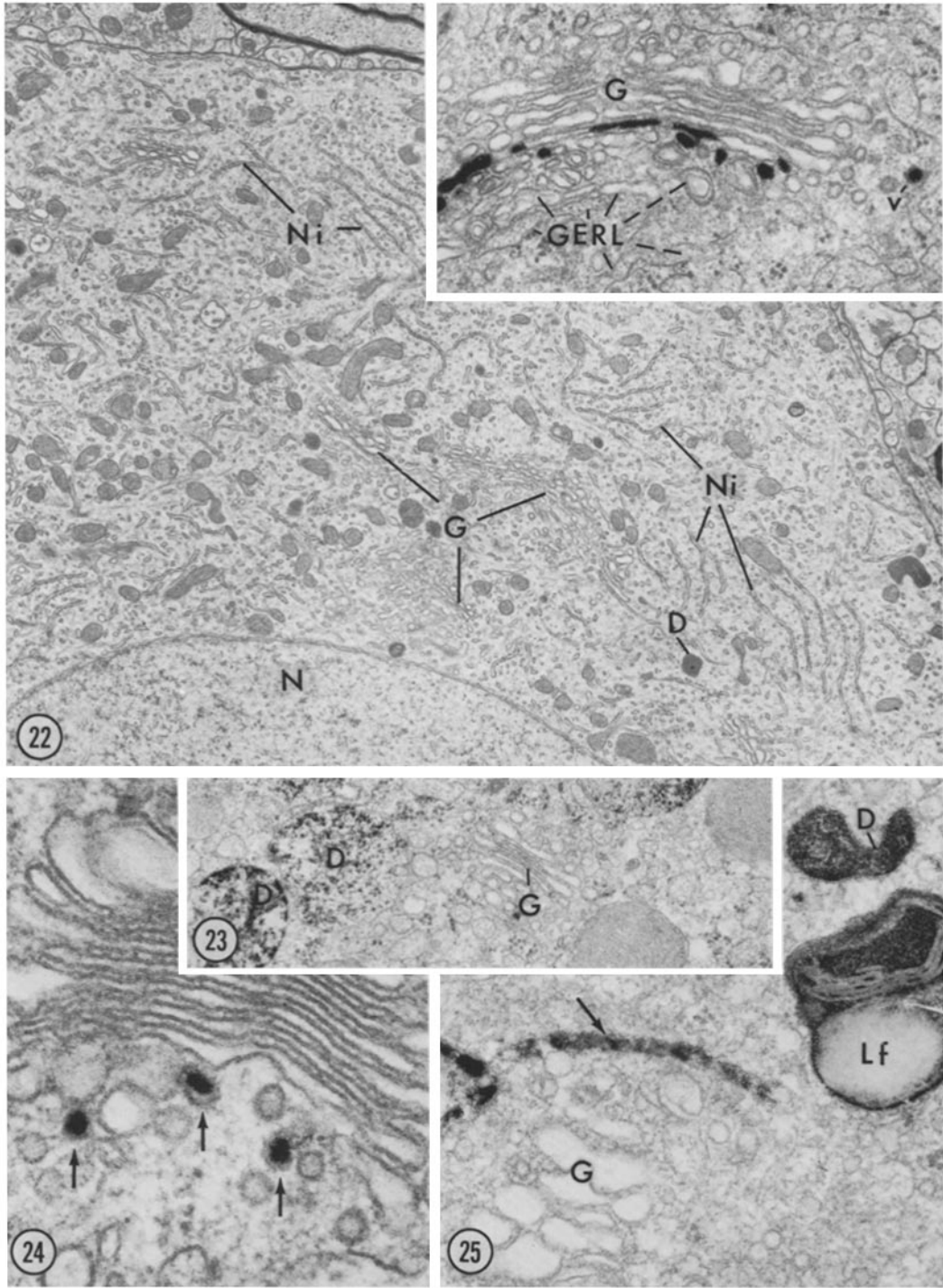
minor portion of the lysosomal complement. It seems conceivable that during the course of neuronal regression the amount of lytic enzyme being processed is of such a magnitude that GERL, alone, cannot package all of it. Consequently some of the hydrolytic enzymes could be channeled into Golgi saccules where they could be packaged into primary lysosomes, as has been demonstrated by



FIGURES 18-21 Thick sections ($0.5 \mu\text{m}$) of regressing neurons (stage XI) incubated for acid phosphatase (Figs. 18, 19), TPPase (Fig. 20), and OsO_4 (Fig. 21). The regular polygonal arrays (PA), representing either the GERL or the inner Golgi saccule, and the adjacent fenestrated compartment (FS) exhibit acid phosphatase reaction product (Fig. 18). Autophagic vacuole (AV). Cisternal (C) and tubular (T) elements are typical of GERL (Fig. 19). The tubules anastomose forming irregularly shaped compartments (arrows), and vesicles (v) seemingly detach from many of these tubules (Fig. 19). Only the inner Golgi saccule (possessing regular polygonal compartments) has TPPase reaction product (Fig. 20). Note the nonreactive, cross-sectioned tubules (arrows)—they may represent GERL tubules (see 1, Fig. 20). Heavy deposits of osmium impregnate the outer Golgi saccules and some vesicles (v) of these cells (Fig. 21). Fig. 18, β -glycerophosphatase, 45-min incubation. $\times 20,000$. Fig. 19, β -glycerophosphatase, 45-min incubation. $\times 58,000$. Fig. 20, TPPase, 40-min incubation. $\times 50,000$. Fig. 21, 2% aqueous OsO_4 , 40-h incubation at 40°C . $\times 48,000$.

Friend and Farquhar (41) in horseradish-peroxidase-perfused rat vas deferens. Furthermore, the preliminary identification of acid phosphatase, thiolacetic acid esterase, and possibly aryl sulfa-

tase within the Golgi apparatus may reflect an adjustment of the vacuolar apparatus (32) in processing lytic enzymes—an adaption similar to that occurring in the packaging of zymogens in carb-



amylcholine-stimulated pancreas (47) or in the monocytic cell line, wherein two types of primary lysosomes are produced during different periods of the cellular life cycle (42).

Novikoff et al. (1) have demonstrated that GERL elements and the inner Golgi saccules interdigitate. If such an intermingling is confirmed between GERL and the inner Golgi saccule during the course of neuronal degeneration (Fig. 20), then it is not improbable that GERL tubules could provide an avenue for the transportation of acid hydrolases to the Golgi apparatus (1, 48). A less likely alternative is the existence of peripheral connections between lysosomal-bearing smooth endoplasmic reticulum and the inner Golgi saccules—similar to transitional endoplasmic reticulum which apparently transports very low-density lipoproteins to the Golgi apparatus in rat liver hepatocytes (49). Such hypothetical pathways require verification by serial thin- and thick-section cytochemistry before they can be considered further.

While hydrolytic activity is widespread within the Golgi-GERL regions of regressing neurons, no acid hydrolase reaction product has been found in the Golgi apparatus of the maturing neuron. All detectable lytic activity is limited to GERL, indicating that the vacuolar apparatus (32) of the functional LMC neuron is cytochemically identical to that of mature motor and sensory neurons of other organisms (1, 2, 4-6, 9, 33, 38). Therefore, the LMC neuron seems able to utilize its Golgi apparatus when the need arises. During the initial period of cell degeneration and after transection of the ventral roots (manuscript in preparation), young neurons apparently exhibit some lysosomal activity within the inner Golgi saccules. In the case of degenerating neurons, the Golgi apparatus appears to be responsible for the generation of

some primary lysosomes, while in the other situation, this aspect of lysosomal biogenesis is not so well documented. In each instance, however, dense bodies and autophagic vacuoles arise from GERL. Yet GERL appears solely responsible for the packaging of lytic enzymes into primary and secondary lysosomes in differentiating and mature neurons (1, 5, 6, 33, 34). Hence LMC neurons, along with epithelial cells of the vas deferens (41), pancreatic acinar cells (47) and monocytes (42), seem capable of altering their mode of packaging protein moieties when stressed or stimulated.

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FIGURE 22 Low-magnification micrograph of a typical stage XVI multipolar motor neuron illustrates its small Nissl bodies (*Ni*), Golgi apparatus (*G*), and scarce dense bodies (*D*). At metamorphic climax (stage XX), a tremendous increase in Nissl substance occurs, producing neurons like those depicted by Price and Porter (50). Inset reveals the localization of TPPase reaction product within the inner Golgi saccule(s) and apparently a small vesicle (*v*) nearby. No activity is evident in GERL. Fig. 22. $\times 10,400$. Inset, TPPase, 40-min incubation. $\times 46,000$.

FIGURES 23 and 24 E-600-resistant esterase activity is restricted to dense bodies (*D*, Fig. 23) and small vesicles and/or GERL tubules (arrow, Fig. 24) in older neurons (stage XX). No esterase reaction product has been discovered in the Golgi apparatus of mature cells. Fig. 23, thiolacetic acid esterase, 50-min incubation. $\times 28,000$. Fig. 24, thiolacetic acid esterase, 70-min incubation. $\times 93,000$.

FIGURE 25 In mature motor neurons (stage XXV), aryl sulfatase reaction product is found exclusively in the tubular components of GERL (arrow), dense bodies (*D*), and lipofuscin granules (*Lf*). In no instance has any lead or barium sulfide precipitates been demonstrated within the Golgi apparatus of these neurons. Aryl sulfatase, 75-min incubation with $Pb(NO_3)_2$. $\times 60,000$.

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