# THE FINE STRUCTURE OF LIPOFUSCIN AGE PIGMENT IN THE NERVOUS SYSTEM OF AGED MICE

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ABSTRACT

An examination of the topographic distribution of lipofuscin pigment granules with the light and electron microscope revealed either smaller and randomly "dispersed" or larger and more complex "clustered" pigment configurations in the cytoplasm of neurons in the dorsal ganglia and ventral spinal cord of 24-month old male mice. Qualitative comparisons revealed no major differences in shape, size, complexity, density, orientation, and cytologic distribution of the pigment bodies in motor and sensory neurons. In general, when the pigment granules were quite numerous within the 2 types of cells, they were smaller in size ($\sim 1\mu$), had a dense homogeneous matrix with few bands or lamellae, and were uniformly distributed throughout the cytoplasm. In contrast, when the pigment configurations were less in number, they were usually larger in size ($\sim 3\mu$), had a more complex internal banded structure, and appeared more localized within the cell. Examination of the bands revealed a repeating pattern of $\sim$70 A. The bands appeared to fuse, forming hexagonal arrays of linear densities intersecting at an angle of approximately 120° in some regions of the pigment bodies. Structural similarities suggested that the striated membranous bands may be composed of phospholipids.

Although conclusive findings regarding the origin, chemical composition, and biochemical significance of lipofuscin granules or age pigment bodies have not been established, the intracellular accumulation of these pigment bodies with increasing age in non-dividing or postmitotically fixed cell populations has been known for a considerable period. As early as 1886, histologists observed that the relative quantity of these pigment granules in nerve cells appeared grossly correlated with the age of human and animal subjects (24). These early morphologic observations were subsequently confirmed by numerous other studies (2, 8, 37). Since it seems reasonable to assume that any extensive intracellular accumulation of these pigment bodies with age may result in significant changes in cellular physiology, several theories of aging have proposed that the deterioration and loss of non-dividing cells may represent one of the most fundamental aspects of biologic aging (4, 10, 29).

Most previously reported histochemical and electron microscope studies have attempted to identify lipofuscin pigment bodies with more specific ultrastructural as well as metabolic constituents in the cytoplasm of various cell populations. At various times such specific cytoplasmic organelles as the mitochondria, Golgi apparatus, endoplasmic reticulum, and lysosomes have been implicated in the histogenesis of lipofuscin in the cell (5, 11, 13, 18, 20, 21). However, more recent and combined ultrastructural and histochemical

observations have provided some convincing evidence that these pigment bodies may be related structurally as well as histochemically to lysosomes (15, 28).

It is recognized that only combined electron microscope and cytochemical studies can provide a more comprehensive clarification of the chemical composition and functional significance of these pigment bodies, and of the exact age at which they may begin to form. However, since the extensive accumulation of lipofuscin observable in old human and animal subjects may be associated more directly with the deterioration of cells, the present electron microscope investigation was undertaken to establish more clearly the gross and submicroscopic morphology and topographic distribution of lipofuscin pigment bodies in neurons of the central and peripheral nervous system of aged mice. Specific aims were as follows: (*a*) to examine the gross and ultrastructural characteristics and topographic distribution of the pigment bodies in neurons of the central and peripheral nervous system of aged mice; (*b*) to establish some morphologic criteria for comparing possible differences in these pigment granules in terms of shape, density, orientation, and distribution in the cytoplasm of motor and sensory neurons; and (*c*) to determine whether some of the previously reported cytochemical characteristics of these pigment bodies can be identified more clearly with some specific ultrastructural constituents revealed by electron microscopy.

## MATERIALS AND METHODS

Since previous studies have indicated that lipofuscin pigment granules may vary in structural and cytochemical complexity among different species, as well as with the sex, age, and condition of the individual subject, only old male subjects from a highly inbred strain of C57Bl/10 mice were used in this study. All of the animals were born and raised in a carefully regulated environmental chamber maintained at 76°F and 52 per cent relative humidity throughout their lifespan. At approximately 24 months of age all subjects were sacrificed by cervical dislocation. Segments of the lumbosacral dorsal ganglia and spinal cord were then removed from a total of 8 different subjects and tissue samples from both the dorsal ganglia and spinal cord of each animal were examined with the light and electron microscopes.

For the histologic observations, representative tissues were fixed for several hours in a mixture of formalin, acetic acid, and methyl alcohol (12). Dehydration was accomplished by direct transfer through several changes of absolute ethyl alcohol. Tissues were cleared in chloroform and embedded in paraffin for sectioning. Slide-mounted sections were stained with 0.25 per cent aqueous cresyl violet or Sudan black B, and counterstained with 1 per cent aqueous neutral Red for visualization of nerve cells with the lipofuscin pigment bodies (27).

Tissues selected for further study by electron microscopy were placed in 4 per cent glutaraldehyde in 0.2 M cacodylate buffer pH 7.2 at 4°C. After fixation (8 to 12 hours), segments of ganglia and spinal cord were trimmed into pieces approximately 1 mm thick and placed for 2 hours in 1 per cent osmium tetroxide in 0.2 M cacodylate buffer (pH 7.2) chilled to 4°C. Tissue blocks were next rinsed in buffer and dehydrated in cold ethanol and propylene oxide for embedding in Maraglas (30). Cells from both stained and unstained sections were examined in a Siemens Elmiskop I.

## OBSERVATIONS

Fig. 1 shows the general topographic distribution of lipofuscin pigment granules in neurons of the dorsal ganglia and ventral spinal cord after staining with Sudan black B, observable with the light microscope. Cells with either large numbers of "dispersed" or a few large "clustered" pigment granules can be identified in the same tissue section. However, in the ganglia, and less frequently in the spinal cord, it was possible to find some cells without pigment bodies. An examination of many histologic sections suggested that each cell may contain only one of the two types of pigment configurations. Neurons of the spinal cord appeared to contain predominantly the clustered form.

Examination of neurons from the dorsal ganglion with electron microscopy revealed numerous dense granular inclusion bodies within the cytoplasm. A survey micrograph of portions of two adjacent ganglion cells shows an example of a fairly uniform distribution of small electron-opaque granules dispersed throughout the cytoplasm (Fig. 2). Such granules measured up to $\sim 1\ \mu$ in diameter and were frequently associated with a small peripherally placed vacuole of low electron opacity. By comparing alternate thick and thin sections of the same material with phase-contrast and electron microscopy, it was possible to identify the electron-opaque granules with the intracellular position of pigment granules observable by light microscopy. At higher magnifications, it was possible to differentiate structures with a homogeneous granular matrix (lysosomes)

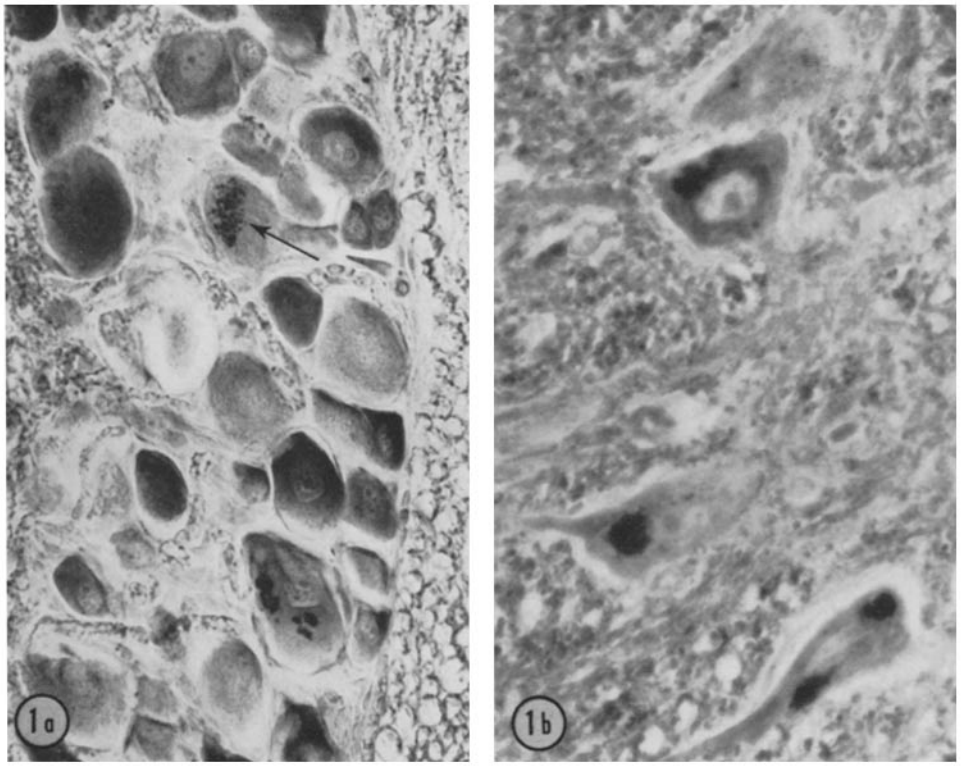



FIGURE 1 Paraffin section of dorsal ganglion (Fig. 1 *a*) and spinal cord (Fig. 1 *b*) from a 24-month old mouse, showing large clustered and smaller dispersed (arrow) granular pigment bodies. Stained with Sudan black B and counterstained with 1 per cent aqueous neutral red. × 350.

from the dense and vacuolated lipofuscin pigment granules as shown in Fig. 3.

Many of the cells examined from sections of dorsal ganglia contained large pigment bodies with a highly complex substructure consisting of many bands and one or more peripherally placed vacuoles. Pigment bodies ranging in size from 2 to 3 $\mu$ in diameter containing closely packed straight and asymmetrically curved bands ~75 A wide are illustrated in Fig. 4. Such pigment bodies were usually not uniform in outline and contained numerous electron-opaque particles associated with the bands. Most of the large banded pigment bodies were concentrated near the nucleus, although an occasional pigment granule was found close to the plasma membrane as shown in Fig. 5. Again, by alternate examination of thick and thin sections, it was possible to demonstrate that the larger banded pigment granules visualized in the electron micrographs were spatially identifiable with the distribution of the clustered pigment forms of light microscopy.

In view of the extremely thin sections used in electron microscopy, it was not possible to determine whether the larger pigment granules represent a single structure or several closely associated banded groups. It was also not possible to reconstruct the spatial relationships between the scattered electron-opaque particles within the pigment granules and the randomly oriented bands. However, from some sections it could be inferred that the dense particles may be formed by a fusion of bands resulting in a highly organized crystalline-like structure. Some bands also revealed a repeating pattern consisting of light and dark lamellae alternating at periods of ~70 A (inset, Fig. 6).

In optimally sectioned material containing pigment bodies visualized at high magnifications, the dense particles may be resolved as parallel linear densities or bands ~80 A wide. At some

locations, the linear densities appeared to be dispersed along two or more axes with their intersection oriented at an angle of approximately 120°. At other locations, the linear bands appeared to have a more vertical orientation with respect to the plane of section, and tubular structures in hexagonal array can be recognized. Such tubules measured ~100 A and ~75 A at the major and minor axes and contained a central cavity of ~50 A (Fig. 7). Arrays of tubular structures were commonly seen within the large banded pigment bodies in cells of the dorsal ganglia and less frequently in the pigment bodies from the anterior horn cells of the spinal cord.

Numerous pigment bodies were also observable in micrographs of neurons from the ventral horn of the spinal cord. The size, complexity, and intracellular distribution of these pigment configurations were again variable throughout the cytoplasm. However, whereas the smaller vacuolated pigment bodies were more uniformly distributed, the larger and more complex bodies associated with one or more vacuoles were usually found in circumscribed perinuclear areas. Examples of two different cells with the small dense pigment granules are shown in Figures 8 and 9. These micrographs indicate also that the small pigment configurations may appear randomly distributed in the cytoplasm, even in close relation to an axosomatic synapse (Fig. 9). In most neurons, numerous typical lysosomes with a homogeneous granular matrix surrounded by a delicate membrane can be seen readily. Occasionally, lysosomes containing one or more bands ~60 A wide with a repeating substructure of ~70 A can be resolved within the matrix (Fig. 10). Structurally, these configurations of lysosomal bands are quite similar in appearance to the band configurations observed in the large clustered pigment bodies.

Another configuration often found in preparations of ganglion cells from aged mice involved large multivesiculate bodies containing from one to as many as twenty small spherical vesicles. Multivesiculate bodies occurred singly or in clusters of 3 or 4 per group and were frequently associated with the Golgi complex and lysosomes (Figs. 11 and 12). Although commonly circular in section, the multivesicular body often had one or more rounded projections from the surface unit membrane, suggesting a possible role in vesicle release or formation. The enclosed vesicles were also similar in appearance to small spherical or oval profiles found in the cytoplasm or enclosed within the vacuoles or between membranes of the Golgi system (Fig. 12). It remains to be determined whether the multivesiculate bodies represent a distinctly new type of granular structure or a modified lysosome.

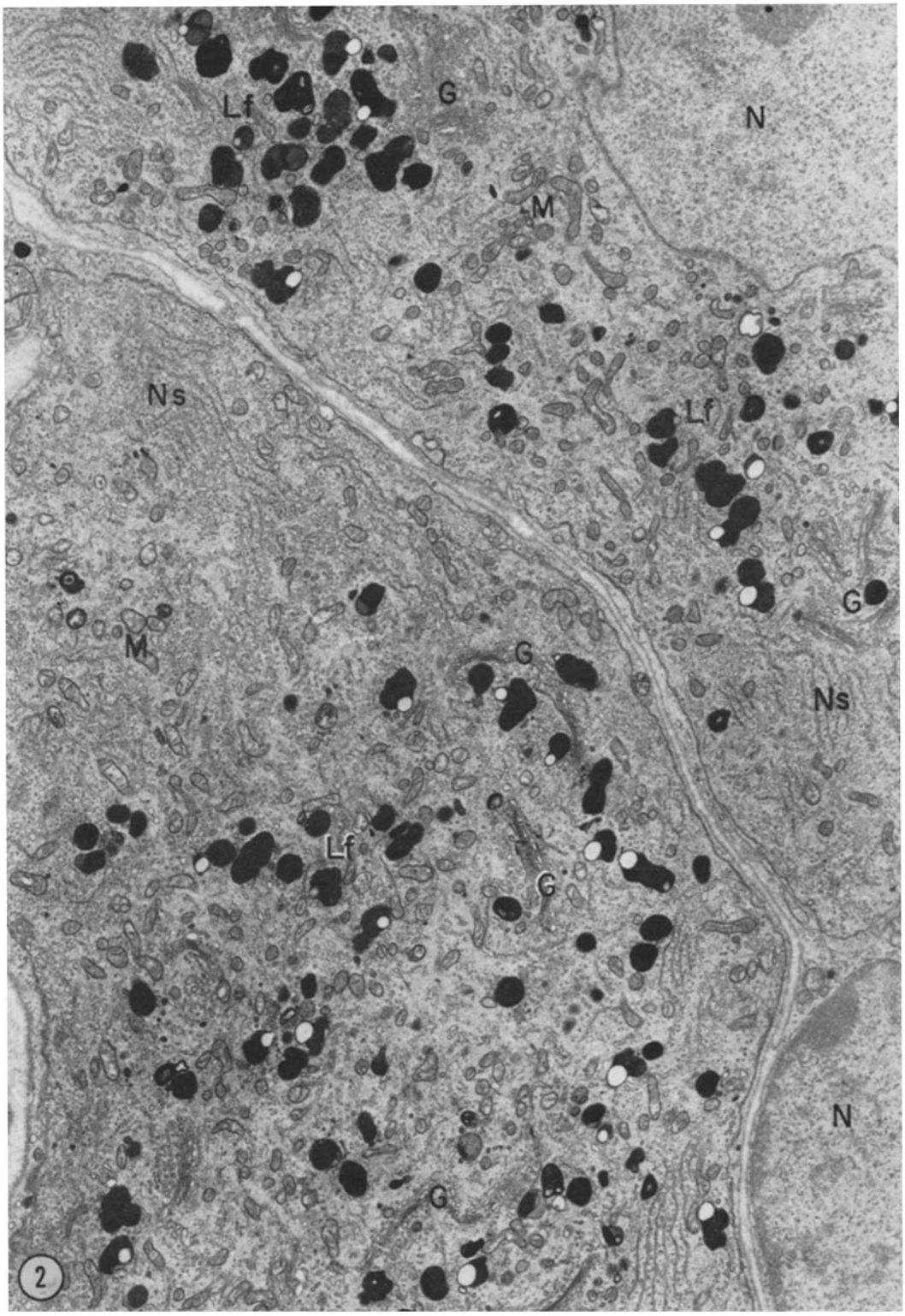
In electron micrographs of neurons from the spinal cord, dense and relatively isolated particulate bodies approximately 3 $\mu$ in diameter were observed with the same characteristic banded substructure already described for the clustered pigment bodies in the dorsal ganglia. Such pigment configurations were associated with a small vacuolar component, contained asymmetrically grouped bands, and were bounded by an easily recognizable unit membrane (Fig. 13).

According to previously reported studies, the ependymal cells of the rodent brain can be identified by the presence of cilia, oval nuclei, whorls of perinuclear filaments, and scattered elements of the granular endoplasmic reticulum (7, 22). In view of the reported presence of supranuclear dense bodies (lysosomes) in the ependyma of the spinal cord, a number of these epithelial cells were examined for lipofuscin. However, the cells examined in this study did not reveal recognizable pigment bodies (Fig. 14). In addition, morphologic entities identifiable as pigment bodies were not observed in extracellular sites, and some electron-opaque material found in non-neuronal cells did not appear to be lipofuscin.

## DISCUSSION

To date, studies of the intracellular accumulation of lipofuscin have dealt principally with various

FIGURE 2 Survey micrograph of portions of 2 ganglion cells and a satellite cell. Many small and vacuolated electron-opaque lipofuscin granules (*Lf*) are distributed in the cytoplasm. The Golgi apparatus (*G*), mitochondria (*M*), Nissl's substance (*Ns*), and the nucleus (*N*) of a neuron (upper right) and a satellite cell (lower right) are illustrated. Lead hydroxide. × 7,500.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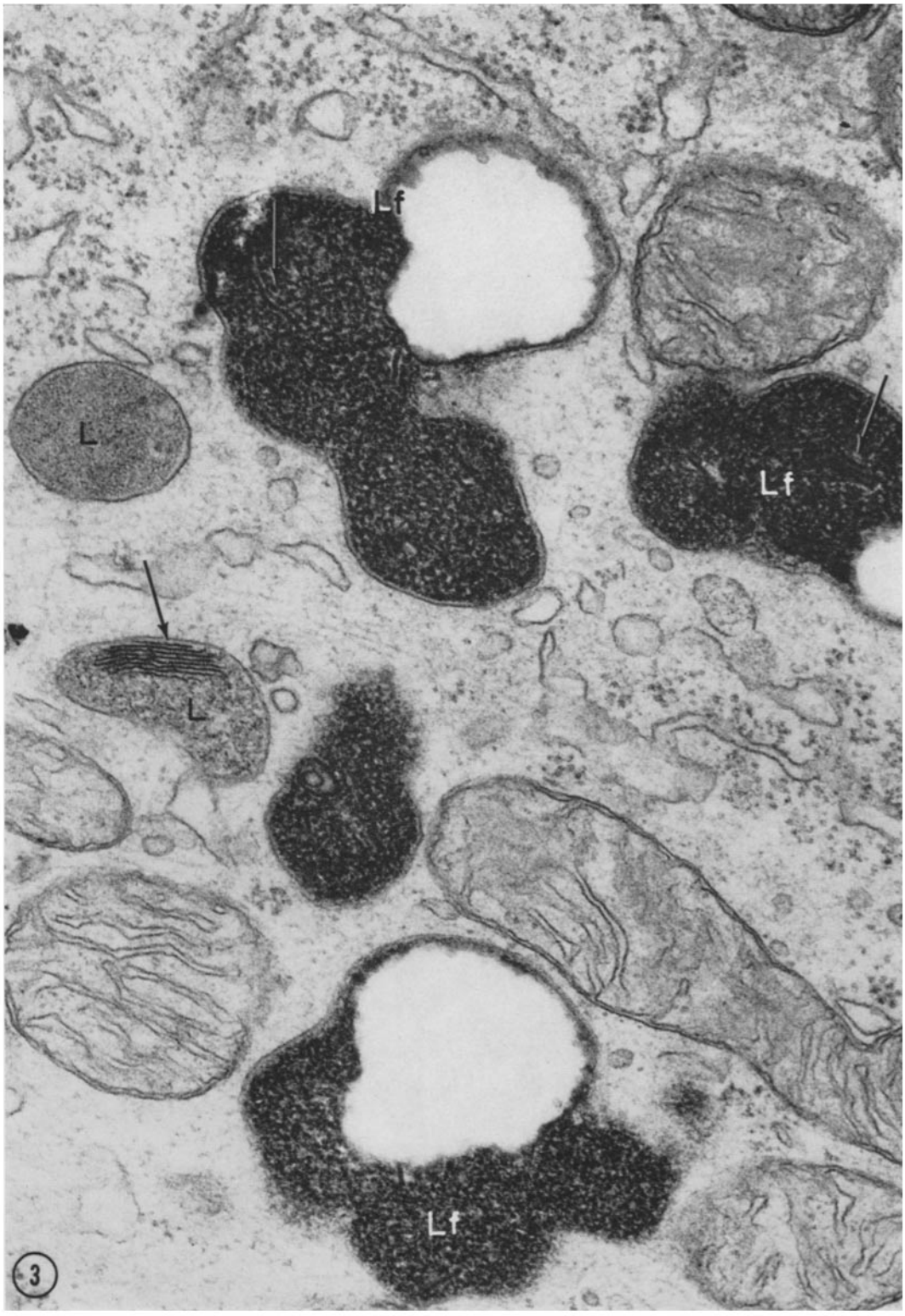
gross morphologic and biochemical properties of the pigment particles in advanced age. Cytochemically, a number of studies have suggested that the formation of lipofuscin pigment in the cytoplasm of neurons with aging may occur in a number of distinct formative stages. In addition, it has also become apparent that the initial deposition and the basic morphologic and biochemical characteristics of this pigment even in old age may vary considerably in different nuclear cell groups of the nervous system and even in similar cells of the same group (6, 16, 21, 34). Regarding the intracellular origin of lipofuscin, it has been suggested that the initial onset may be correlated in part with the appearance of diffusely distributed lipid-rich particles which may either remain uniformly scattered in the cytoplasm or coalesce and concentrate at one end of the cell (19, 36). In a cytochemical study of the aged human brain, possible qualitative correlations among changes in functional activity, extent of lipofuscin deposition, and areas of high oxidative enzyme metabolism have been proposed (16). Studies on the possible sequence of pigment body formation in cells of the cranial nerve nuclei in the guinea pig have also suggested that the more physiologically active neurons may accumulate lipofuscin at a faster rate than metabolically less active cells (37). The present histologic findings are in general accord with these cytochemical findings since some neurons in dorsal ganglia or segments of the spinal cord contained little or no lipofuscin pigment, whereas other adjacent cells contained considerable quantities. In some cells, the pigment bodies filled the entire cytoplasm of the cell.

In terms of shape, size, complexity, density, orientation, and cytologic distribution, qualitative comparisons revealed no major differences in the pigment bodies of motor and sensory neurons. In general, when the pigment granules were quite numerous within the 2 types of cells, they were smaller in size ($\sim 1\ \mu$), had a dense homogeneous matrix with few bands or lamellae, and were uniformly distributed throughout the cytoplasm. In contrast, when the pigment configurations were less in number, they were usually larger in size ($\sim 3\ \mu$), had a more complex internal banded structure, and appeared more localized within the cell. Consequently, the histologic and ultrastructural observations of the present study suggest that the 2 distinct pigment configurations may be observed within the cytoplasm of both sensory and motor neurons in the nervous system.

In attempting to determine whether some of the previously reported cytochemical characteristics of these pigment bodies can be identified more clearly with some specific ultrastructural constituents revealed by electron microscopy, several investigators have pointed out that the two most common properties of lipofuscin in all of the brain areas studied by staining and histochemical reactions are yellow-white fluorescence in near ultraviolet light and intense reactivity to staining with fuchsin (14, 16, 21, 35). Since lysosomes are fluorescent and also share in common with lipofuscin a number of morphologic, histochemical, and cytochemical characteristics, a role for lysosomes in the initial formation or subsequent accumulation of lipofuscin pigment with age has appeared as an obvious possibility (1, 23, 28, 33).

In considering the transformation of the pigment bodies from simpler to the more complex membranous forms, a number of studies have suggested that a progressive increase in the quantity and chemical complexity of the lipid moiety may be a relevant consideration. Formation of complex bands with repeating light and dark striations and hexagonal arrays of crystalline-like structures may represent a particular stage in the transformation of phospholipids. Systems of amorphous (presumably anhydrous) and organized hydrated membranous forms which have been produced *in vitro* from extracted brain phospholipids have a structural similarity to the dense portions of the small granular pigment bodies and the system of bands observed in the large clustered pigment forms of the present study (25, 31, 32). Compara-

FIGURE 3 Electron micrograph of dorsal ganglion cell of 24-month old mouse. Lysosomes (*L*) and early lipofuscin pigment bodies (*Lf*) are visible. Each lysosome and each lipofuscin pigment complex is bounded by a single unit membrane. Arrows indicate distribution of membranes within a lysosome (lamellated residual body) and striated bands in several lipofuscin granules. Lead hydroxide. × 60,000.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3

ble vacuole-contained laminated systems have been observed in electron micrographs of cells known to have a high phospholipid content (9, 26). Similarly, biochemical studies on isolated lipofuscin granules from human heart tissue have indicated that the phospholipids comprise approximately three quarters of the total extractable lipid volume (3). Chromatography of lipid extracts of human cardiac pigment has revealed that the highest specific fluorescence is generally found in the non-phospholipid fraction (17). With these considerations in view, it seems reasonable to conclude that the bulk of lipid material which is neither pigmented nor fluorescent, except possibly for a lecithin fraction, could represent phospholipid organized structurally into striated membranous bands observable with the electron microscope. Finally, whatever the intracellular origin, sequence of transformation, or chemical composition of these so called age pigment bodies, any extensive accumulation of lipofuscin granules with age should affect all aspects of cellular physiology and may ultimately result in the loss of cells. However, any comprehensive interpretation of the functional significance of lipofuscin accumulation in cells with age would require an examination of at least the following structural and cytochemical changes in cell populations from infancy to adulthood and old age: (*a*) a quantitative demonstration of a progressive increase in total lipofuscin pigment concentration or successive enlargement of pigment bodies in cells within various regions of the nervous system; and (*b*) the absolute loss of cells in specific regions of such organs as the nervous system which consist of non-dividing or postmitotically fixed cellular populations in relation to decrements in specific sensory, associative, and motor functions. All of these preceding structural and cytochemical changes in cells with increasing age would have to be examined under carefully controlled genetic, dietary, pathologic, and environmental conditions. These experimental laboratory findings could then be related to concomitant age-correlated alterations in selected neurophysiologic functions as well as to specific age-correlated decrements in behavior. Such unified studies should provide a more inclusive clarification of the functional significance of lipofuscin accumulation in the nervous system in relation to aging.

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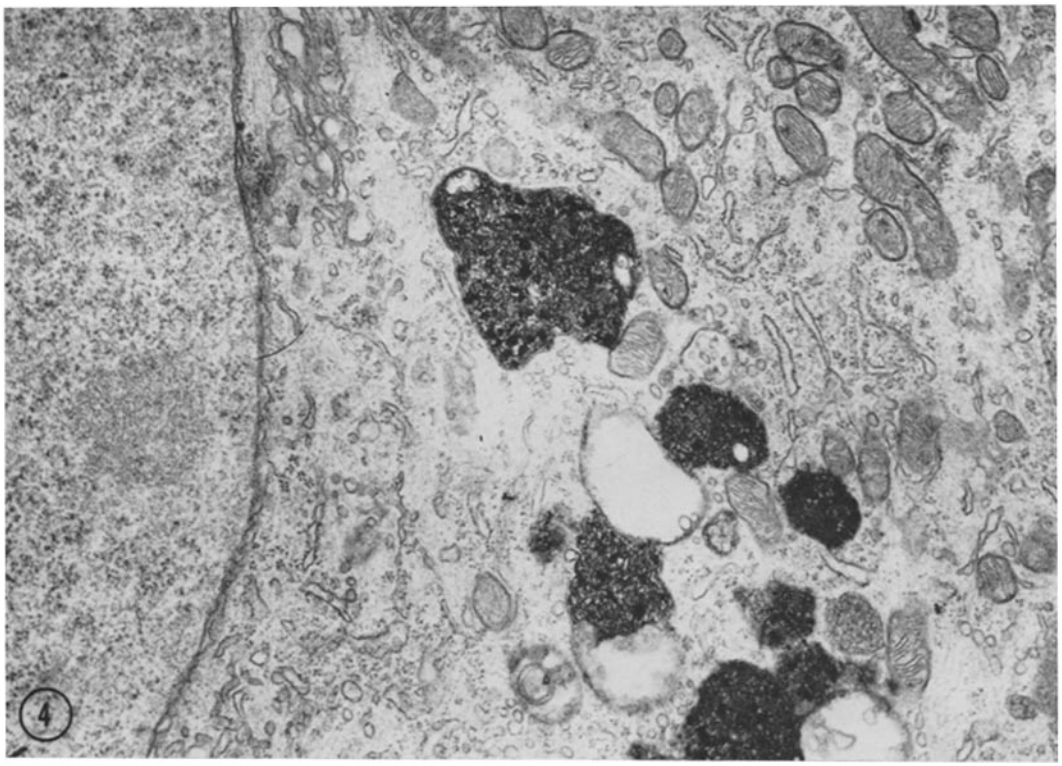
FIGURE 4 Dorsal ganglion cell, 24-month old mouse. Vacuolated lipofuscin pigments are observable near the nucleus. Lead hydroxide. × 21,000.

FIGURE 5 Ganglion cell with a large lipofuscin granule containing bands and dense particles adjacent to a dense body (arrow), plasma membrane, and a satellite cell (*Sc*) with numerous vesicles. Lead hydroxide. × 29,000.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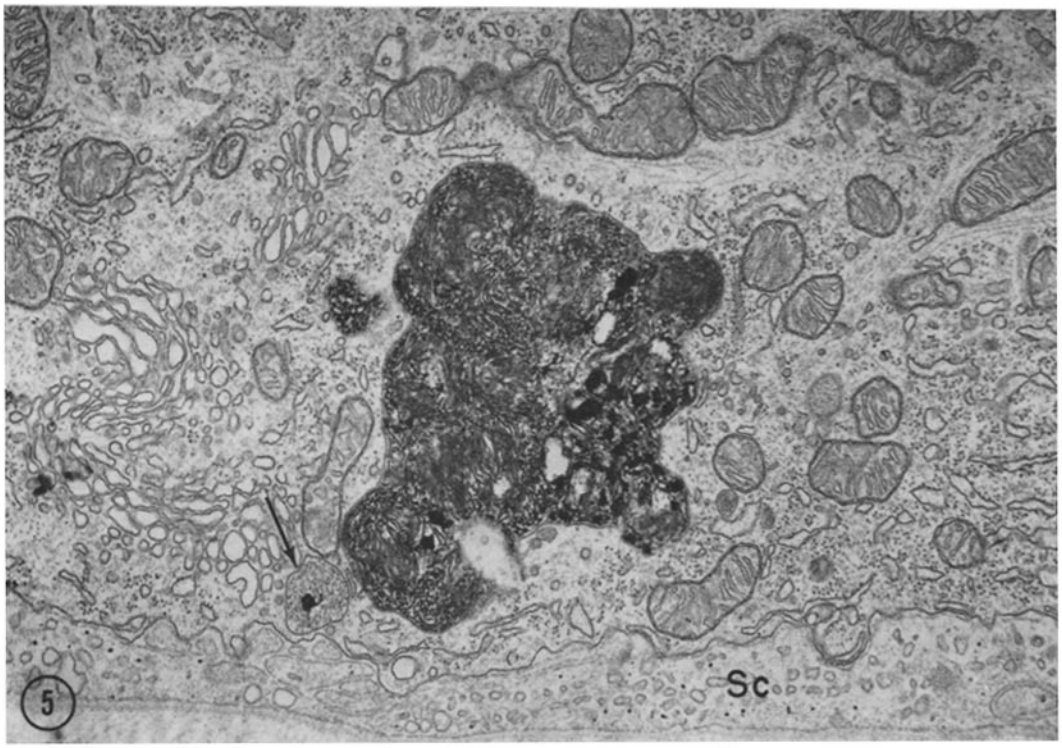
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FIGURE 6 Electron micrograph of a pigment body in a dorsal ganglion cell of a 24-month old mouse. Note close association of dense granules and curved bands ~75 A wide with a "globular" substructure (insert) in some areas (arrows). This could suggest that the crystalline-like particles may be forming by a fusion of bands. A multivesicular body (*Mv*) and a mitochondrion (*M*) are in close proximity to the pigment granule. Lead hydroxide. × 120,000; insert, × 210,000.

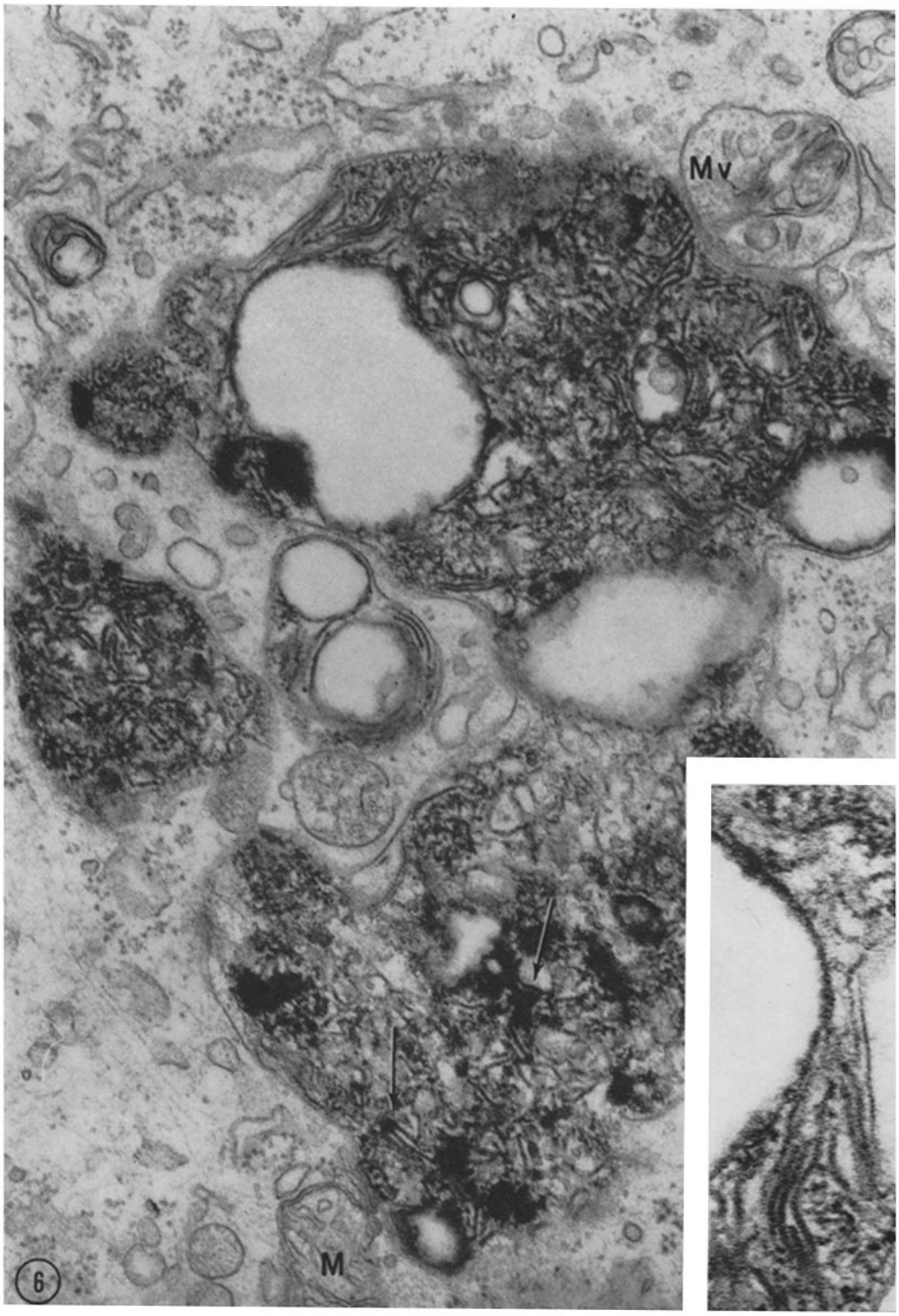
Mv
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FIGURE 7 A portion of lipofuscin pigment body showing bands and electron-opaque particles. Two axes of symmetry are apparent in one particle (*A*), three in another (*B*), intersecting at an angle of approximately 120°. Parallel arrays of bands can be seen in still other particles (*C*). Some of the bands appear as hexagonal tubules ~100 A wide with a central cavity of ~50 A (insert). Lead hydroxide. × 150,000; insert, × 240,000.

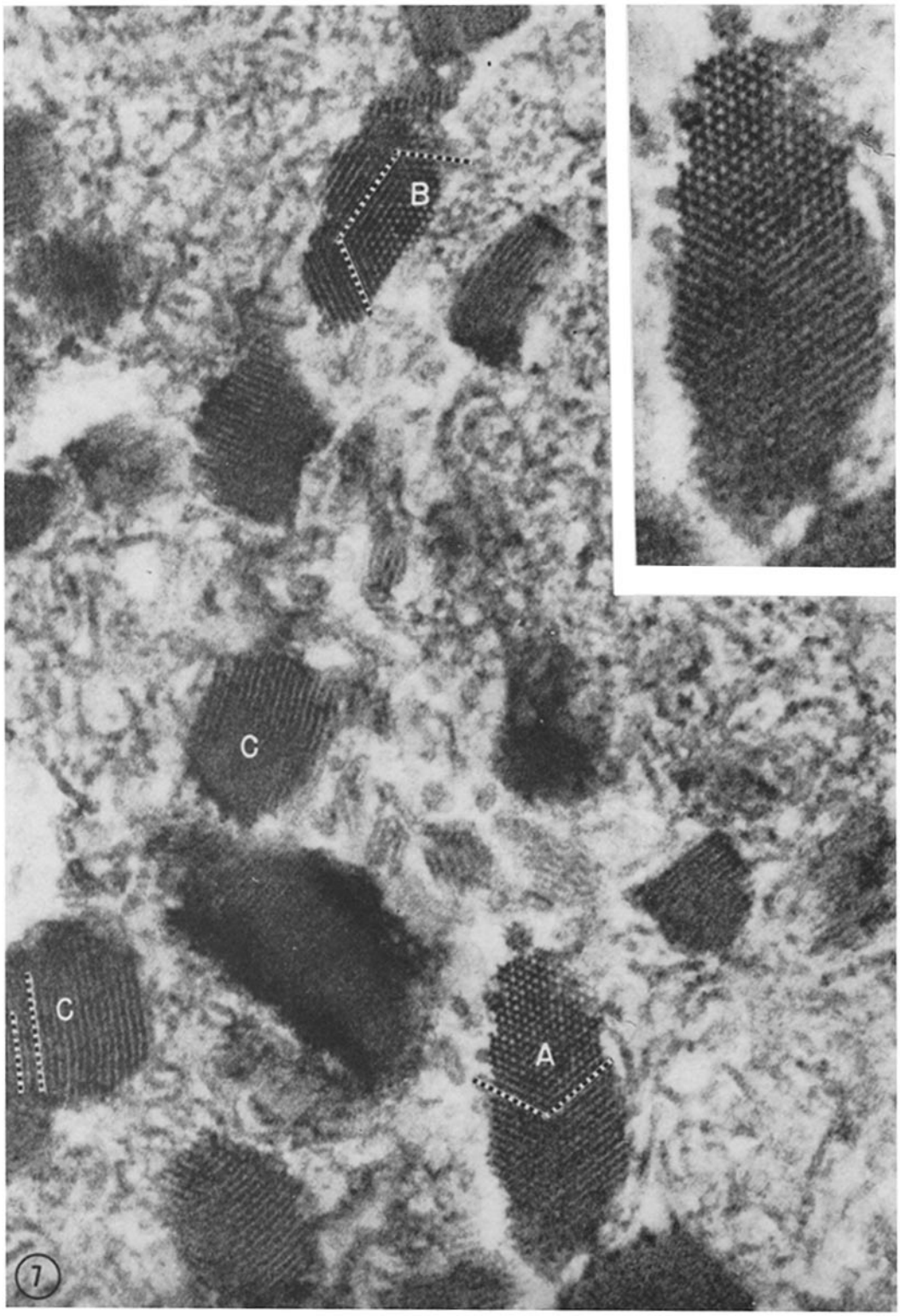
B
C
C
A
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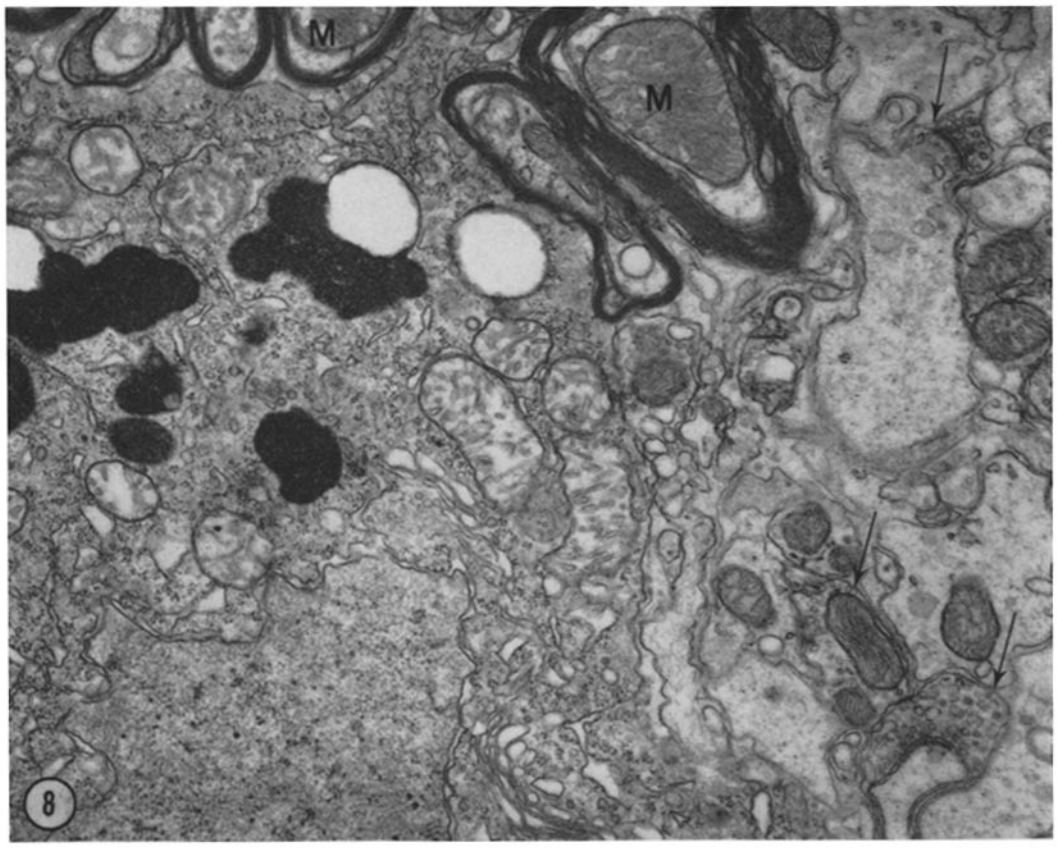
FIGURE 8 Electron micrograph of a portion of an anterior horn cell from the spinal cord of a 24-month old mouse. Several early forms of pigment bodies can be seen distributed in the cytoplasm. Myelinated fibers contain mitochondria (*M*). Synaptic contacts are identified by arrows. Lead hydroxide. × 21,000.

FIGURE 9 Anterior horn cell from the spinal cord, containing early lipofuscin (*Lf*), a lysosome (*L*), and a linear array of cytoplasmic vesicles (*V*). An axo-somatic synapse (arrow) containing synaptic vesicles in the presynaptic component is shown. Lead hydroxide. × 60,000.

FIGURE 10 A lysosome within an anterior horn cell, containing three different forms of electron-opaque material and two striated bands ~60 A wide (arrow). The lysosome is completely surrounded by a single unit membrane. Lead hydroxide. × 160,000.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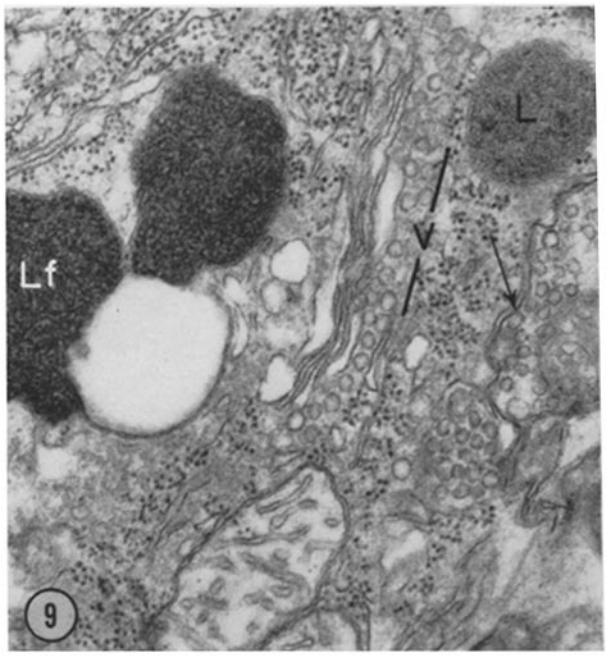
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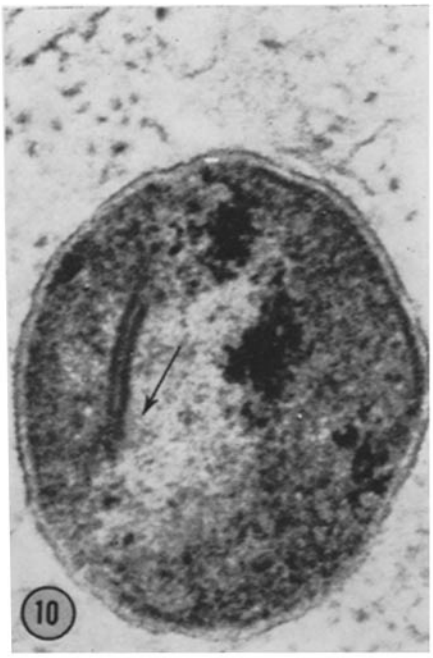
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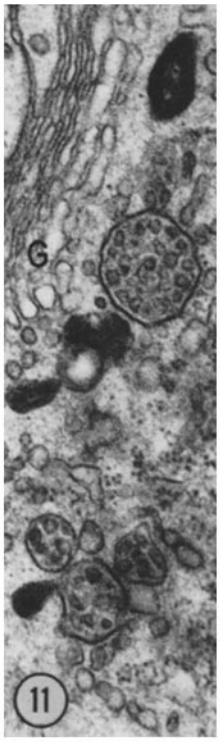
FIGURE 11 Thin section through the anterior horn cell of a 24-month old mouse. Multivesiculate bodies containing spherical vesicles can be observed near the Golgi apparatus (*G*). Lead hydroxide. × 21,500.

FIGURE 12 Golgi area from an anterior horn cell of the spinal cord, containing numerous circular elements (arrows) similar in appearance to the spherical vesicles of a multivesiculate body (*Mv*). A lysosome is present in the lower left corner. Lead hydroxide. × 72,000.

FIGURE 13 An electron micrograph of a large pigment body from an anterior horn cell of a 24-month old mouse. This pigment granule contains an array of straight and curved bands ~75 A wide and is surrounded by a single unit membrane. Lead hydroxide. × 72,000.

FIGURE 14 Portion of an ependymal cell identified by the presence of a ciliary complex (arrow), an oval nucleus containing scattered dense nuclear material, and whorls of perinuclear filaments. Pigment bodies are not apparent in this cell. Lead hydroxide. × 21,500.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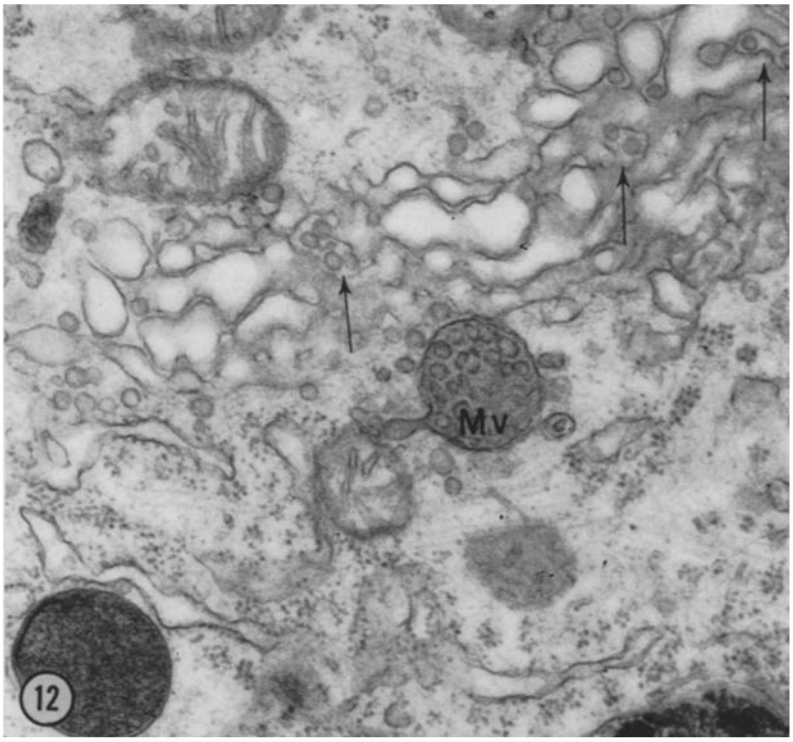
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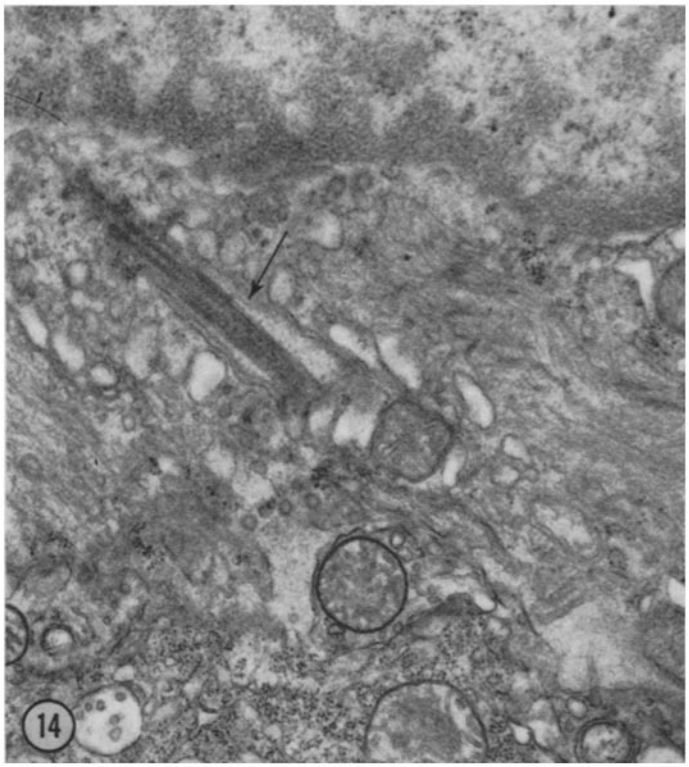
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