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THE OCCURRENCE OF LIPOFUSCIN PIGMENT AS RELATED TO AGING IN THE LUMBAR SPINAL CORD, DORSAL ROOT GANGLIA AND PARAVERTEBRAL GANGLIA OF THE DOG AND PIG.

Iowa State University of Science and Technology,
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# THE OCCURRENCE OF LIPOFUSCIN PIGMENT AS RELATED TO AGING IN THE LUMBAR SPINAL CORD, DORSAL ROOT GANGLIA AND PARAVERTEBRAL GANGLIA OF THE DOG AND PIG

by

Albert Braxton Few, D.V.M.

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Graduate Faculty in Partial Fulfillment of
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DOCTOR OF PHILOSOPHY

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# INTRODUCTION

In view of the controlling influence which the nervous system exercises over all activities of the body, it seems particularly important that age changes in this system should be given primary consideration. According to Andrew (1956), nerve cells are the most highly specialized cells, yet they enjoy the longest life of any cell in the body.

Nerve cells are considered "postmitotic" cells which have, at birth, undergone their last mitotic division and are "fixed" in their condition of inability to divide for the remainder of their lives (O'Leary, 1952). It is for this reason that the old adage is generally true that nerve cells are as old as the individual.

Many neurocytological changes have been associated with the aging process of nerve cells. Among the more common ones are: degeneration and fragmentation of the Golgi apparatus (Andrew, 1939 and Gatenby, 1953), decrease of Nissl substance (Andrew, 1941, Kuntz, 1952 and Bondareff, 1959), alteration of mitochondria including swelling and vacuolization (Payne, 1946, 1949, 1952, and Jayne, 1950), and increase in diameter (Weiss and Lansing, 1953). In addition Gardner (1940) and Andrew (1952) observed vacuolization of the cytoplasm, and Andrew (1938, 1952) described a loss of regularity of the nuclear and cellular outline. A total decrease in the number of cells has been reported (Ellis, 1920, Gardner, 1940,

Brody, 1955, and Bondareff, 1959).

However, of all the neurocytologic changes that have been correlated with aging, the intracytoplasmic accumulation of lipofuscin pigment is perhaps one of the most significant. Lipofuscin is a lipoprotein pigment believed to be composed of many chemically diverse substances. Its significance in regard to function is still uncertain.

It is important to mention that not all nerve cells or groups of cells accumulate lipofuscin pigments, and certainly some cells accumulate lipofuscin deposits at an earlier age and to a greater degree than others. Andrew (1956) described, for certain groups of cells, the occurrence of "reactive" or "defensive" phenomena which seemed to be related to counter-action against the influences which lead to senescence and ultimate destruction of nerve cells. That such counter-action phenomena actually exist seems to be substantiated by Sulkin (1955a), who reported finding no lipofuscin in the inter-mediolateral cell column of the spinal cord of aged dogs. Contrary to this and supporting the observation that some cells do apparently age faster or accumulate lipofuscin earlier, Sulkin (1955a) observed that in the same aged dogs 100% of the ventral horn cells of the spinal cord contained some lipofuscin. Similar findings in the dog and hog brain were described by Whiteford (1964) and Whiteford and Getty (1966). In aged specimens they reported the vagal nuclei to be free of lipofuscin, whereas 80% of the cells in several other brain

stem nuclei were pigmented. In the present study, deposits of lipofuscin were observed in each area studied, which included the ventral horn cells of the lumbar spinal cord, the lumbar dorsal root ganglia, and the lumbar paravertebral ganglia. Deposits of pigment were found in dogs as young as 5 months and in hogs as young as 6 months of age.

In general, studies of age changes in the nerve cells of dogs and hogs have been much less numerous than those dealing with man and other animals. There are a few reports of studies on the dog (Dolley, 1911; Goodpasture, 1918; Harms, 1924; Sulkin and Kuntz, 1952; Sulkin, 1953, 1955a, 1955b; Whiteford, 1964; and Whiteford and Getty, 1966). There are only two reports of age studies on the nervous system of the pig, both dealing with age changes in various brain stem nuclei (Whiteford, 1964, and Whiteford and Getty, 1966). Reports of studies on the pig spinal cord and its associated ganglia have not been found.

The objective of the present study was to further investigate the role of lipofuscin in the aging process of the nervous system of the dog and hog. The specific aims were as follows: (1) to determine if lipofuscin pigments were present in the lumbar spinal cord, dorsal root ganglia and paravertebral (autonomic) ganglia of dogs and pigs; (2) to establish, if it is present, the age at which it first appears, and determine if it increases with age; (3) to examine the different types of neurons and determine if one

kind is more susceptible to age changes than are others; (4) to describe the fine structural morphology of the pigment granule; and (5) to determine more definitively the origin of the pigment granules.

Finally, it is anticipated that this study will be conducted and the results reported in such a manner that they can be compared with other similar studies of the brain, heart, eye, ovary, adrenal gland, thyroid gland and other tissues. Several age studies of these tissues are presently underway in the Department of Veterinary Anatomy, Iowa State University, and since the same animals are being used in most cases, it would be helpful to be able to correlate the results of this study with the findings in the other studies when completed.

# REVIEW OF THE LITERATURE

The literature review covers 6 areas: (1) occurrence and distribution of lipofuscin in the nervous system; (2) nature and origin of lipofuscin; (3) electron microscope studies of lipofuscin; (4) fluorescent microscope studies of lipofuscin; (5) functional significance of lipofuscin in the nerve cell; and (6) chemistry of lipofuscin.

## Occurrence and Distribution of Lipofuscin in the Nervous System

### Occurrence of lipofuscin

As early as 1883, Schulz, a German worker, attempted to prove that pigmentation of human ganglion cells increases with age. A little later, White (1889) determined that human autonomic ganglion cells exhibit pigmentation even more frequently than those of other mammals.

To establish the relationship of intracellular pigment to age in man, Hodge (1894) examined cells of the cerebrum, cerebellum, cervical spinal cord, and first cervical ganglia of individuals ranging in age from birth to senility. He was able to demonstrate considerable amounts of pigment in the senile subject, especially in the cells of the spinal ganglia, but none was evident in individuals at birth.

Later workers have established that the relative amount of pigment is a fairly reliable index of chronological age. Hermann (1952) demonstrated, for example, that in human

sympathetic vagal ganglia the progressive involvement of cells was 7% during the first decade, 8 to 14% during the second decade, 18% during the third decade, 25% in the fourth decade, and 30 to 33% during the fifth decade. Accordingly, Andrew (1941) found no pigment in the large motor cells of the spinal cords of mice that were 40 days old. At middle age (312 days) less than 5% of the cells were pigmented, but in senile mice the accumulation of pigment in the motor cells was conspicuous in every case; indeed, the majority of the cells in old animals had half or more of the volume of cytoplasm filled with pigment. Bailey (1953) reports a similar progressive increase of pigment deposits with advancing years. Pigment was found in the anterior horn cells and sensory ganglion cells of humans in the third decade. By the seventh and eighth decades scarcely an unpigmented ganglion cell could be found. Chu (1954) stated that he found lipofuscin pigment in the anterior horn cells of humans even at 6 years of age. Morrison *et al*. (1959) observed that lipofuscin represented as much as 20% of the volume of ventral horn cells of man in the second decade of life. Only a few cells, however, contained pigment. In one 29-year-old man in this age group, 75% of the volume of cells from Clarke's column was occupied by pigment. Pigment accumulation progressed so that by the ninth decade of life, lipofuscin occupied up to 85% of the volume of most ventral horn cells and cells of Clarke's column.

In the dog, Dolley (1911) described pigment deposits in the Purkinje cells. Goodpasture (1918) found excessive accumulations of yellow pigment in ganglion cells of senile dogs. The ganglion cells of the sympathetic system were especially involved. Harms (1924) found heavy accumulations of pigment in the pyramidal cells of the cerebrum. Sulkin (1955a) described lipofuscin in numerous areas of the nervous systems of dogs over 10 years of age. Some pigment was found in 100% of the spinal cord ventral horn cells. Lesser amounts were found in other areas.

In recent studies of the dog and hog brain (Whiteford, 1964, and Whiteford and Getty, 1966), it was found that lipofuscin was absent in the neurons of animals under one year of age and that it was universally present in old animals. As a result of these studies, the authors concluded that lipofuscin pigment increased with age throughout the life span of the animal and that pigment was the only consistent cellular alteration that could be correlated with age in the specimens studied.

Distribution of lipofuscin

The specificity of aging, and in particular regional differences in lipofuscin distribution among different groups of nerve cells, is a phenomenon that has been known, but poorly understood, for some time. Hodge (1894), for example, demonstrated that the spinal ganglia of man accumulate more pigments than other areas studied. Harms (1924) found heavy

accumulations in the pyramidal cells of the cerebrum of senile dogs, whereas pigment deposition was much less or absent in the Purkinje cells of the cerebellum. Gatenby and Moussa (1951) examined autonomic, thoracic and lumbar chain ganglia of sheep. Pigment granules were found in these areas before they could be demonstrated in the spinal cord or cerebellar and cerebral cortices. Sulkin (1955a), in a group of senile dogs, found the cells of the spinal cord intermediolateral column to be free of pigment whereas, in the same group, 100% of the spinal cord ventral horn cells were pigmented. Generally, Sulkin's survey of the distribution of lipofuscin indicated that efferent neurons have a higher frequency of pigmentation and agreater concentration of pigments than do afferent neurons. Another interesting observation by Sulkin (1955a) was that 8% of the cerebellar Purkinje cells were pigmented whereas 98% of the cerebral cortex pyramidal cells were pigmented. Similarly, Whiteford (1964), in a study of dogs and hogs, found the vagal nuclei to be free of pigment in both species, whereas 80 to 90% of the cells of other brain stem nuclei (hypoglossal, inferior olivary, cochlear, vestibular, mesencephalic nucleus of the trigeminal nerve, tegmental nucleus of von Gudden, oculomotor, and red nucleus) were pigmented. Nandy (1966) also observed in guinea pigs that the largest accumulation of lipofuscin occurred in the neurons of the reticular formation of the brain stem, less in the neurons of the cerebral cortex,

hypothalamic nuclei, and least in the Purkinje cells of the cerebellum, although the cells of the deep cerebellar nuclei showed marked accumulation.

Another striking incidence of specificity of age change in the nervous system was reported by Buttlar-Brentano (1954). She found no lipofuscin deposits in the cells of the nucleus supraopticus or the nucleus paraventricularis of man. Accordingly, Levi (1946) found the ciliary ganglion to be free of pigment in man even at an advanced age.

<u>Basis for distribution pattern in the nervous system</u>

The existence of a "specificity phenomenon" to account for the various distribution differences of lipofuscin in the nervous system is not clearly understood. For those cell groups that maintain a certain resistance to age changes, Andrew (1956) describes the occurrence of certain "reactive" or "defensive" phenomena which seem to be related to counter-action against the influences which lead to senescence and ultimate destruction of the nerve cell. According to Andrew (1956) these "counter-action" mechanisms may be: (1) an increase in the size of the cell as much as 8 to 10 times the size of the cells in younger individuals; (2) a division of the nucleus, whereby the increased surface area for nucleic and cytoplasmic exchanges would place the cell in a better position to survive the metabolic vicissitudes of old age; (3) a division of the nucleoli; and (4) an increased amount of ribonucleotide.

Other possible explanations as to why lipofuscin is variously distributed have been advanced by Vogt and Vogt (1946), Wahren (1957) and Höpker (1951). In their opinion, the specific course of intracellular pigmentation is characteristic for different nuclei. This thesis has been developed by Wahren (1957) who found the onset and intracellular distribution of pigment different in the pallidum, the nucleus tuberomammilaris, and the nucleus tuberolateralis of the hypothalamus. According to Wahren, the large cells of the pallidum were practically free of lipofuscin in the first 3 decades, and only after the age of 70 years did they uniformly contain pigment. In the nucleus tuberomammilaris, lipofuscin appeared in the large cells in the fourth decade and predominated after the age of 60 years, although lipofuscin-free cells could still be found in the sixth decade. In the medium-sized cells of the nucleus tuberolateralis no pigment was found until the third decade, but by the fifth decade, lipofuscin-containing cells predominated, and thereafter no cells free of lipofuscin were found.

The possibility that specific vascular differences may affect the distribution of lipofuscin has been suggested by Andrew (1956) and Sulkin (1958). Sulkin determined that anoxia of any origin could tend to increase the amount of lipofuscin in neurons; therefore, the condition of the vascular tree may have a significant influence on the deposition of lipofuscin.

More recently Whiteford and Getty (1966), in their study of dogs and hogs, concluded that the difference in time of pigment deposition in the various nuclei may be related to the level of function of the nuclear group concerned.

Basis for intracellular distribution patterns

The fact that lipofuscin is variously distributed intracellularly has been of interest to several investigators. Even very early, Mühlmann (1910) observed that the homogeneous distribution of pigment granules in ganglion cells of the guinea pig and man was gradually lost and that specific cell types had specific pigment distributions. In agreement with this is a report by Issidorides and Shanklin (1961), who stated that the size, shape, and distribution of the lipofuscin inclusions were similar in all cells of a given nucleus (cell group). These features were reported to vary widely, however, in the different cell groups, and each cell group in the brain was said to have a characteristic "lipofuscin pattern". Contrary to these views, Höpker (1951) found 5 pigment distribution patterns in the aging cells of one cell group--the dentate nucleus. In studies of dog and hog brains Whiteford (1964) and Whiteford and Getty (1966) described 4 main types of intracellular pigment distributions. These were diffuse, perinuclear, polar or axonal, and bipolar. Their conclusion was that the predominant pattern of pigment distribution was considered to be related more to the age of the animals than to the type of cell group containing it,

although there was some evidence that a particular cell group contained predominantly one pigment distribution pattern at all ages examined.

Factors other than age influencing deposition of lipofuscin

Numerous factors other than age have been reported as influencing the deposition of lipofuscin in different age groups. Studies of environmental factors and their influence on pigment deposition in experimental laboratory animals, including the dog, have been reported by Sulkin and Kuntz (1952), Sulkin (1957, 1958), and Sulkin and Srivanis (1960). Under such conditions as prolonged acetanilid feeding, chronic vitamin E deficiency, and chronic hypoxia, very young rats have been shown to exhibit marked deposition of pigment in nerve cells (Sulkin, 1957). Treatment of unilateral nephrectomized rats with ACTH every other day for 40 days resulted in the deposition of pigment in various parts of the nervous system (Sulkin, 1958; Sulkin and Srivanis, 1960). This deposition was greater than in control rats. Significantly, Strehler (1962) concluded that pigment deposition from environmental alterations may be by an entirely different mechanism than that produced by the aging processes.

Other factors suspected of influencing pigment deposition are peripheral vascular disease, malignancies, and infectious disease (Kuntz, 1934, 1938, 1945; Sulkin, 1953). Sulkin (1953) observed that autonomic ganglion cells of young individuals who had succumbed to chronic diseases in many

instances contained a higher concentration of pigments than those of individuals in older age groups.

However, in the laboratory animals, especially in the dog, where factors of disease, nutrition, and environment can be controlled to some extent, pigmentation of the nerve cells occurs only with senility (Sulkin, 1953).

## Nature and Origin of Lipofuscin

### Nature of lipofuscin

Pilez (1895) and Obersteiner (1903) published reports that a pigment referred to as lipopigment begins to appear during the first 6 years of life. According to Marinesco (1909), there are 2 kinds of pigment granules in ganglion cells namely, black and yellow pigment. Large cells usually lack black pigment, but it is abundant in medium-sized cells. The black pigment is similar to melanin and is found in cells during the early months of life. The yellow pigment stains with dyes used for the demonstration of fatty substances and is generally regarded as a lipopigment. The production and amount of yellow pigment in nerve cells vary according to age, nutritive disturbances, and pathological processes. Its normal production is a slow, progressive process (Marinesco, 1909). Later, Hueck (1912) called this yellow pigment, or one very similar to it, lipofuscin.

Kuntz (1945) observed pigment in autonomic ganglion cells during adult life and reported that they increased with

advancing age. In man, this pigment was, again, mainly of 2 kinds--yellow lipoidal pigment and a darker melanotic pigment, both of which were similar to those described by Marinesco (1909). The lipoidal pigment appeared earlier than the melanotic pigment, but with advancing age the melanotic pigment became predominant, particularly in heavily pigmented cells. Earlier Kuntz (1938) had observed melanotic pigment in preparations of ganglia of the sympathetic trunk and celiac plexus. These tissues were from individuals ranging from 5 weeks to 78 years of age, and nearly all individuals over 30 years of age had melanotic pigment in some ganglion cells. Excessive pigmentation of the autonomic cells occurred in those patients in which death was due to a carcinoma. Truex (1940) has also reported the presence of melanotic pigment in autonomic nerve cells in persons of all ages. Brown (1943, 1944) studied pigments in the substantia nigra and locus coeruleus of the dog and in certain mesencephalic trigeminal nuclei in the dog and cat including the red nucleus, the oculomotor nucleus, and the nucleus of the mesencephalic root of the trigeminal nerve. He concluded that these pigments were melanic in nature and appeared to increase with increasing age of the animal.

Both melanin and lipochrome pigments have been described in sympathetic ganglion cells (Larsell, 1951). According to Larsell, lipochrome was not found in nerve cells at birth, but increased in amount with age. Wolf and Pappenheimer

(1945) pointed out that in the dorsal sensory ganglia the lipochrome pigment is present in addition to melanin, but never in the same cell. Sulkin (1953) attempted to characterize further the nature of the pigment in autonomic nerve cells. He observed pigmented ganglion cells in individuals ranging in age from 7 to 92 years. These data, obtained as a result of histochemical studies, indicated that there were no melanotic pigments in human autonomic ganglion cells, but that the pigments were lipofuscins. Sulkin (1955a, 1955b) later studied aged dogs and reported similar results in the autonomic ganglia; sensory ganglia; cerebellum; pontine nuclei; ventral, dorsal, and intermediolateral cells of the spinal cord; and pyramidal cells of the cerebral cortex. Whiteford (1964) and Whiteford and Getty (1966) reported finding only lipofuscin pigments in several areas of the brain of the dog and pig. They studied the hypoglossal nucleus, dorsal motor nucleus of the vagus nerve, inferior olivary nucleus, vestibular nuclei, trochlear nucleus, oculomotor nucleus, red nucleus, and Purkinje cells of the pig. In addition to these, the cochlear nuclei, the mesencephalic nucleus of the trigeminal nerve, and the tegmental nucleus of von Gudden were studied in the dog. Pigment was reported to be absent in the dorsal motor nucleus of the vagus nerve of the dog and pig and in the Purkinje cells of the dog. Lipofuscin pigment was found in all other areas and it was concluded that it increased in amount with

increasing age.

Origin of lipofuscin

Conclusive findings regarding the origin of lipofuscin pigments or age pigments have not been established. Dolley (1911, 1917) reported pigmentation of nerve cells in senile dogs and attributed its deposition to functional depression of some duration. According to Dolley (1917), the pigment is derived from nuclear substances and represents a transformation of nuclear chromatin. The chromidial substance is extruded from the nucleus during states of functional depression and is transformed into cytoplasmic pigment.

Matzdorff (1948) believes that the cytoplasmic ground substance should be implicated in pigment genesis. He describes the process as beginning with the appearance of fine lipid particles diffusely distributed throughout the cytoplasm. These then increase in size, darken, and clump, the grouping differing with the cell type and producing the variously formed pigment depositions. In its completed form the pigment may be scattered throughout the cytoplasm or concentrated at one pole.

A similar process of pigment genesis has been described on the basis of studies of the nucleus dentatus. According to Höpker (1951), lipofuscin formation begins in the so-called lipophilic center of the cell, generally located near the nucleus, and 5 formative stages may be recognized: (1) fine, spherical particles are found diffusely distributed in the

lipophilic center; (2) lipofuscin formation, still diffuse, extends to the border of the Nissl substance; (3) the particles begin to agglomerate, and the lipofuscin, which formerly could be distinguished only by its double refraction, now assumes a yellowish color, which gradually deepens; (4) as the process continues, the cytoplasm is retracted toward the nucleus so that the cell is almost delimited by lipofuscin on the opposite side; and (5) finally, what once was a cell is represented by an agglomeration of lipofuscin which may be vacuolated.

In the opinion of Wünscher (1957), the aging of cells is considered similar to that of colloid solutions in which, following a decrease in the water-rich phase, there occurs a concentration or precipitation of existing materials (synaresis). This is similar to Sjövall's (1932) finding that lipofuscin represents a dispersed phase of the plasma colloid which tends to decrease in its dispersion and finally to flocculate.

Other cellular organelles have been implicated as contributing to the formation of age pigments in nerve cells. Gatenby and Moussa (1950, 1951) and Gatenby (1953), on the basis of phase microscope studies, have presented the view that pigment granules in autonomic ganglia arise from a transformation of broken-down pieces of Golgi apparatus. Bondareff (1957) supported this view on the basis of studies with the electron microscope (See section on "Electron

Microscope Studies of Lipofuscin Pigment").

Conversely, Hess (1955) presented evidence, also based on electron microscope findings, which indicates that senility pigment originates from degenerate mitochondria. This view was supported by Duncan, Nall and Morales (1960).

de Duve (1959) found that when pigments are deposited in the cell they are first seen within lysosomes, or structures analogous to them. Later as accumulation of pigments proceeds, the entire lysosome may be transformed into a pigment granule. Strehler (1962) stated that it appears probable that lipofuscin is a result of the accumulation and autoxidation of lipid components of lysosomes. Essner and Novikoff (1960) and Barka and Anderson (1962) considered lipofuscin pigments, observed in various organs of senile humans and animals, to be altered lysosomes.

Samorajski _et al_. (1964) concluded that their findings did not establish whether lipofuscin granules were initially deposited, formed or subsequently accumulated within lysosomes with increasing age. However, the concurrent autofluorescence and staining reactions, the increasing ultrastructural complexity and vacuolization, as well as the localization of specific enzymatic reaction products at identical sites, provided convincing evidence for the structural and cytochemical identification of lipofuscin with lysosomes in nerve cells of the old human and animal specimens used in their study.

## Electron Microscope Studies of Lipofuscin

One of the first studies of pigment in neurons with the electron microscope was reported by Beams _et al_. (1952). A hundred or more spinal ganglion cells from approximately 90-day-old rats were examined. Only two were found which contained pigment granules. The granules were reported to be dense spherical bodies concentrated into one region of the cell.

Later Weiss and Lansing (1953) studies morphologic changes in cell structure with age. Observations were made on cells of the anterior pituitary gland of the Swiss Albino mouse, using the electron microscope. It was found that with age the nuclear membrane becomes irregular in outline, the nuclear cortex becomes dense, and the double-membraned system fragments. The most striking age change was found in mitochondria, most of which were 2 to 5 times greater in diameter than those of young cells. They appeared to be vacuolated and less dense, and their cristae were reduced to small stumps. Pigment deposits in the cells were not reported.

Hess and Lansing (1954) and Hess (1955) studied spinal ganglion cells in a group of guinea pigs ranging in age from newborn to senile. In their work with the electron microscope they found some mitochondria which seemed to be degenerating at all ages examined and therefore concluded that mitochondria

do not serve as a good criterion of the stage of the aging process in nerve cells.

Hess (1955) observed that virtually all ganglion cells of senile guinea pigs, despite cell type, contained pigment bodies in varying amounts. The pigment usually tended to form aggregations toward the periphery and frequently occurred more around the entire border of the cell than in its interior. According to Hess (1955), the pigment bodies were characterized by marked electron density and varied in shape from spheroidal to highly irregular bodies. The most frequent appearance was that of a homogeneous mass; however, in some instances the pigment bodies seemed to be composed of a number of vesicles of very low electron density surrounded by a lacework of dense material. In still other instances, the combination of these forms occurred with the pigment body being composed of both solid and vesiculated forms. Beginning pigment forms (Hess, 1955) appeared to occur in a most intimate relation with swollen mitochondria. The mitochondria swelled, their borders became very dense, and the interval folds disappeared, with the interior of the mitochondria assuming a homogeneous, less dense appearance. The pigment granules accumulated in relation to the mitochondria and extended from one pole of the mitochondria. The accumulation of pigment apparently progressed by the formation of a successive series of vesicles which then coalesced to form a pigment body.

Contrary to the views of Hess (1955), Bondareff (1957) concluded on the basis of electron microscope studies of the cervical spinal ganglia of Sprague Dawley rats, that mitochondria are not directly related to the mechanism whereby pigment is formed in senile nerve cells. Studies of electron micrographs led Bondareff to suggest the following mechanism for the genesis of senile pigment: (1) the walls of vesicles and vacuoles of the Golgi complex thicken; in the latter, smaller vesicles form and undergo a similar thickening of their walls; (2) the vesicle walls continue to thicken and the vesicles become particulate; (3) these particulates coalesce, forming larger particular complexes.

On the basis of electron microscope studies of the dorsal root ganglia of 6 mice over one year of age, Duncan et al. (1960) supports the opinion of Hess (1955) that old-age pigment arises as a process of alteration of mitochondria. Hess further describes the pigment bodies as displaying considerable diversity in shape, size, and internal detail. Most conspicuous in the pigment bodies were the dense subparticles situated mainly at the periphery of each mass of pigment. Each of these parts displayed a series of alternate dense and light bands in parallel configurations. In addition many of the bodies contained vesicles of less density.

Essner and Novikoff (1960) reported on the electron microscope studies of lipofuscin pigments in the hepatic cells of a 72-year-old man. Because of their fine structure

and acid phosphatase activities these granules were considered to be lysosomes.

Later, studies of nerve tissue were made to establish the role of lysosomes in the formation of lipofuscin pigment and to describe the fine structure of the lipofuscin age pigment (Samorajski *et al.*, 1964 and Samorajski *et al.*, 1965). In the initial study, Samorajski *et al.* (1964) made electron microscopic examinations of neurons of the lumbosacral dorsal ganglia and spinal cord of 2 humans 70 and 79 years old. Tissues obtained from mice ranging in age from 15 to 28 months were also studied. On the basis of these examinations and concurrent autofluorescent and staining reactions, convincing evidence was found for the structural and cytochemical identification of lipofuscin with lysosomes in nerve cells of aged human and mouse specimens. The electron microscope determinations of the study suggested that the time or stage at which lysosomes may be designated lipofuscin granules may be correlated with the time at which the pigment bodies begin to manifest characteristic membranous substructural configurations.

For a more specific morphologic and cytochemical identification of the role of lysosomes in the formation of the pigment bodies, the enzymatic reaction products of acid phosphatase, E-600 resistant esterase, and acid deoxyribonuclease II activity were localized in neurons by means of electron microscopy. Examination of tissue with the electron

microscope revealed that the selected enzymatic activity was restricted to lysosomes and lipofuscin pigment bodies in dorsal ganglia, the spinal cord, and the liver (Samorajski _et al_., 1964).

Samorajski _et al_. (1964, 1965) described the fine structural morphology of the pigments as being clusters of high density and complex ultrastructure consisting of myelin-like figures arranged in several directions within a single body. The myelin-like figures frequently exhibited a globular substructure of approximately 60 angstroms. Within species comparisons suggested that the pigment bodies appeared more complex and vacuolated in older animals. Both the dense and vacuolated parts were bounded by an easily recognizable single unit membrane. Micrographs indicated that pigments from the ventral horn of the spinal cord and the dorsal root ganglia were very similar in fine structural appearance.

The influence of environment, and in particular the inducement of prolonged periods of stress, is reported to result in marked deposition of pigment in nerve cells (Sulkin, 1957, 1958, and Sulkin and Srivanis, 1960). Accordingly, Rolsten and Samorajski (1966), on the basis of histochemical and ultrastructural studies, determined that there is a progressive accumulation of lipofuscin age pigment in the central nervous system of stressed and non-stressed mice with increasing age.

## Fluorescence Microscope Studies of Lipofuscin

As early as 1911 the fluorescence microscope was used to observe pigmented inclusion bodies in non-dividing cells such as muscle (skeletal and cardiac) and nerve (Stübel, 1911). Hamperl (1934) later confirmed that a wide variety of cells possessed similar fluorescent components. Hydén and Lindström (1950) studied anterior horn cells of the spinal cord from individuals between 16 and 89 years of age. The lipofuscin pigment exhibited a yellowish fluorescence which was not changed or diminished even after treatment of the section by acids, alkalis, chloroform, chloroform-methanol or irradiation.

Issidorides and Shanklin (1961) studied various parts of the brain of 35 individuals varying in age from newborn to 84 years old. Fluorescence studies showed that in all sections the lipofuscin inclusions had a strongly yellowish fluorescence. Even interstitial material in the area of the inferior olive and Purkinje cell zone exhibited fluorescence. The fact that lipofuscin pigments can be readily visualized by means of the fluorescence microscope has been pointed out by Strehler _et al_. (1959) and Strehler (1960). In an unstained section of myocardium from an 86-year-old individual, the age pigment fluoresced bright yellow; the myocardial fibers were dimly fluorescent or green-blue in color (Strehler, 1960).

During an investigation into the intracellular distribution of certain fluorescent drugs, Koenig (1963a) observed

autofluorescent granules in the cytoplasm of neurons and non-neuronal cells of the brain and in parenchymal elements of the liver, kidney and other organs. Their morphologic resemblance to lysosomes was demonstrated by staining for acid phosphatase activity.

According to Koenig (1963b), numerous cytoplasmic inclusions are found in a wide assortment of mammalian tissues, both in fresh squash preparations and in frozen sections of formalin-fixed tissues, which emit a distinctive yellow-orange fluorescence when illuminated by near ultraviolet light. In the spinal cord, brain, and other tissues, these autofluorescent granules closely resemble, in size, shape, number and topographical distribution, particles which give a strong reaction for acid phosphatase. Frozen sections, stained for acid phosphatase activity by Gomori's lead glycerophosphate method, reveal no fluorescent granules other than lipofuscin granules when these contain a negligible enzyme product, *i.e.*, lead sulfide. If the lead phosphate deposited initially as a consequence of enzyme activity is not converted to the opaque sulfide, however, lysosomes remain fluorescent and are simultaneously rendered highly refractile by the presence of colorless lead phosphate. The fluorescent constituents of lysosomes (Koenig, 1962) are associated with the glycolipoprotein matrix of the lipofuscin granules, both being soluble in chloroform-methanol.

Samorajski *et al*. (1964) utilized the fluorescence microscope to examine lipofuscin pigments in the lumbosacral dorsal ganglia and spinal cord of aged humans, mice, and rats. A comparison of neurons from these tissues did not reveal significant species differences when concurrent fluorescence and staining procedures for lipofuscin identification were examined in comparable neurons.

According to Samorajski *et al*. (1964), two types of autofluorescent granules were seen in the cytoplasm of neurons. One type, described as fluorescent cytoplasmic organelles, was found dispersed in the neurons and non-neuronal cells. The size, number, and distribution of these autofluorescent organelles indicated that they represented particles which have been identified as lysosomes. The other fluorescent granules were larger, appeared clustered in the periphery of the cell, and were visible as a very distinctive orange-yellow fluorescence.

Whiteford and Getty (1966), on the basis of examination by fluorescence microscopy, found a considerable number of lipofuscin granules in cranial nuclei of dogs and hogs just one year of age. As a result of this finding, they concluded that it seemed reasonable to postulate that so-called "age change pigments" may also be present at earlier ages, perhaps at 8 months or even 6 months. Thus, it was evident to these investigators that by fluorescence microscopy, granules could

be observed in younger dogs and hogs than could be observed with the Nile blue sulfate stain (Lillie, 1956a), used for most of their determinations.

## Functional Significance of Lipofuscin in the Nerve Cell

The relation of lipofuscin to the functional integrity of the cell containing it has not been clarified. Ranson and Clark (1959) stated that lipofuscin served no useful purpose nor did it appear to interfere in any way with the normal function of the cell. Likewise, Bloom and Fawcett (1962) referred to the pigment as probably a product of normal activity which remains within the protoplasm in a useless although non-injurious capacity. Because of its early appearance in life (as young as 6 years in the human), Chu (1954) stated the lipofuscin has been considered as a product of activity rather than senescence.

Opinions contrary to these views are numerous. According to Altschul (1943), the presence of lipofuscin pigment in nerve cells indicated some change in the individual cells or certain types of cells. This change was described as being either (A) an insufficiency in the metabolism of the cell (incomplete consumption of certain materials); (B) a difficulty in eliminating waste products of normal metabolism; or (C) a replacement of some inactive or absent constituent of cell plasm by an auxiliary material. Altschul (1943) further concluded that pigment in nerve cells seemed to be a

characteristic of "weak" cells. He observed a steady decrease in the lipofuscin content of motor cells starting in the spinal cord and continuing to the motor nuclei of the cranial nerves. This decrease was most abrupt between the nuclei of the facial and abducens nerves.

Murray and Stout (1947) observed cultured, human sympathetic ganglion cells and found that when they contained large amounts of pigment, they never migrated and frequently the nuclei would lose their staining properties. Cells containing lesser amounts moved only short distances. It was concluded from these observations that pigment may be detrimental to normal function either by virtue of its being a rigid mass interfering with the plasticity of the cell or for other reasons not known. Accordingly Sulkin (1953) agrees that normal function of the cell is unlikely when pigmented. He reasoned that cells heavily laden with pigments are deficient in chromidial substance, are usually shrunken, vacuolated, and are often undergoing degeneration. Some of these certainly are cells which are no longer functional (Sulkin, 1953).

More recently Samorajski et al. (1964) explained that when the role of lysosomes in lipofuscin accretion becomes more firmly established, it seems likely that the functional significance of the pigment will become interpretable primarily in terms of possible alterations in normal lysosomal functions. Significant changes in normal cellular physiology

could occur in such organs as the nervous system which consists of postmitotically fixed cellular populations. These physiologic alterations could result in the loss of cells (Samorajski *et al.*, 1965).

Whiteford (1964), on studies of dog and hog brains, interpreted the increased amount of lipofuscin with age and the concomitant alterations in pigment distribution as simply evidence of cellular aging. He further explained, however, that from a purely anatomical point of view, the size and density of lipofuscin accumulations in the neurons of older specimens would be likely to reflect or cause some impaired function.

## Chemistry of Lipofuscin

The complex chemistry of lipofuscin cannot be simplified by reviewing the vast literature or by presenting a detailed description of the histochemical reactions of the pigment in nerve or other tissue. Indeed the chemical composition of lipofuscin is still not completely understood; according to Bondareff (1964), fractionation and collection has proven technically difficult, and adequate analysis of neuronal lipofuscin has yet to be accomplished. There is reason to believe that many chemically diverse substances are known as lipofuscin (Bondareff, 1959).

Chemical composition of lipofuscin

The most complete biochemical analysis reported to date is that of Heidenreich and Siebert (1955), who have isolated and analyzed lipofuscin from the cardiac muscle of old human subjects. The observation that the staining reactions, fluorescence properties, and ultrastructural morphology of cardiac lipofuscin and neuronal lipofuscin are very similar, causes the present investigator to conclude that they are chemically similar, if not identical. With this in mind, the findings of Heidenreich and Siebert (1955) are presented in Table 1.

Histochemistry

According to Barka and Anderson (1962), lipofuscins are generally yellowish-brown, iron negative, fluorescent, basophilic, and PAS-positive. They resist alcoholic dehydration and paraffin embedding, stain with lipid stains, give a positive Schmorl reaction, and reduce silver salts. They give a weak performic acid--Schiff reaction and are occasionally acid-fast.

That lipofuscin pigment is PAS-positive in the dog and man has been shown by several investigators (Sulkin, 1953, 1955a, 1955b; Heidenreich and Siebert, 1955; Issidorides and Shanklin, 1961; Samorajski *et al*., 1964; and Nandy, 1966). According to Sulkin (1955a), lipofuscin in old dogs gives a positive Schiff reaction after prior oxidation with periodic, performic, and peracetic acids, and the periodic acid reaction

Table 1. Chemical properties of cardiac lipofuscin[a]

| Organic Constituents | | Enzymatic Constituents | |
|---|---|---|---|
| Protein | 11.8% N content (Kjeldahl) for lipofuscin. 14.7% N content fat-free residue. | Cathepsin | Activity about ten times smaller than that of pancreas (hemoglobin substrate). |
| Fat | 20% dry weight. | Non-specific Esterases | Activity several hundred times smaller than that of hog kidney or rat liver (phenolphthalein substrate). |
| Amino Acids | Most amino acids characteristic of protein (paper chromatography with casein as standard). | | |

INORGANIC CONSTITUENTS

| Element | Inorganic Constituents in Lipofuscin (%) | Inorganic Constituents in Heart Muscle (%) | Method of Determination |
|---|---|---|---|
| Mg | 0.1-1.0 | 0.056-0.135 | |
| Si | 0.1-1.0 | 0.005-0.013 | |
| Ca | 0.03-0.3 | 0.018-0.068 | |
| Al | 0.01-0.1 | 0.000225 | |
| Cu | 0.001-0.01 | ........... | |
| Fe | 0.01-0.1 | 0.0029-0.0072 | Steinheil quartz spectrograph |
| Mn | 0.0001-0.001 | Trace | |
| Zn | < 0.1 | 0.0033 | |
| Sn | < 0.05 | 0.00535 | |
| Cr | < 0.03 | ........... | |
| Co | < 0.001 | ........... | |
| Ni | < 0.001 | ........... | |
| S | Strong qualitative reaction | ........... | Na-Nitroprusside reaction after sodium reaction |
| P | 0.42 | 0.51-1.06 | Photometrically after ashing |

[a]Heidenreich and Siebert (1955).

is blocked by prior acetylation. The positive reaction for glycogen is eliminated by diastase digestion.

Lipofuscin may be stained with basic dyes such as Nile blue sulfate (Heidenreich and Siebert, 1955; Lillie 1956a, 1956b; Whiteford, 1964; and Nandy, 1966). According to Lillie (1956b), lipofuscins stain with Nile blue by two mechanisms--a fat-solubility one which operates at pH levels below 1.0, and an acid-base mechanism operating at pH levels above 3.0. On the basis of these reactions Lillie (1956a) stained paraffin sections in 0.05 to .01% Nile blue in 1% sulfuric acid, washed them thoroughly in water, and mounted them in aqueous media. The results were that lipofuscins colored deep blue, melanins dark green, myelin and red cells lighter green, and the background pale green. Whiteford (1964) used the Nile blue stain to demonstrate lipofuscin in the brains of dogs and hogs. Other lipid stains such as Sudan black B, Sudan III, IV, V, scarlet red, and neutral red have been used to stain lipofuscin (Sulkin, 1955a; Heidenreich and Siebert, 1955; Issidorides and Shanklin, 1961; Samorajski _et al_., 1964, 1966; and Nandy, 1966). According to Issidorides and Shanklin (1961), the outer layer of the lipofuscin granule takes up Sudan black B; by this method they could observe the actual size of each lipofuscin body. Similar good results were reported with the other lipid stains.

Strehler (1964) stated that lipofuscin is capable of binding a number of basic dyes. On this basis the long Ziehl-Neelson acid-fast stain technique, described by Pearse (1961), has been used to demonstrate lipofuscin. Pearse (1961) indicated that the pigment may not be acid-fast at all times, but any presence of redness is regarded as positive. This is in agreement with Strehler's (1964) observations. Wolf and Pappenheimer (1945), Sulkin (1955a), and Nandy (1966) also reported using a similar Ziehl-Neelson stain to demonstrate the acid fast properties of lipofuscin.

Histoenzymology of lipofuscin

Enzymes present Sulkin (1953) reported that lipofuscin in ganglion cells of man and the dog is high in alkaline phosphatase. In addition to this, other hydrolytic enzymes, including acid phosphatase, have been identified with lipofuscin in nerve tissue (Samorajski *et al*., 1964, and Nandy, 1966). In addition to the hydrolytic enzymes, Nandy (1966) observed that lipofuscin pigments in guinea pigs had strong reactions for succinic, lactic, and glucose-6-phosphate dehydrogenases and cytochrome and monoamine oxidases. Samorajski *et al*. (1964), in addition to acid phosphatase activity, observed positive localization of cathepsin Type-C esterase and acid deoxyribonuclease II at sites identical with lipofuscin pigment in the nervous system of the mouse, rat, and man.

# MATERIALS AND METHODS

## Canine Spinal Cord and Ganglia Specimens

To evaluate the occurrence and nature of lipofuscin in the nervous system of the dog, histologic and electron microscopic examinations were made of neurons of the thoracolumbar spinal cord, dorsal root ganglia, and paravertebral (autonomic) ganglia. All specimens for this study were obtained from dogs reared in the dog colony of the Department of Veterinary Anatomy, Iowa State University. Fifty-seven animals ranging in age from 7 days to 16 years (median age - 16.2 mo.) were utilized, and 50 of these were whelped in the colony; 7 were obtained from other sources.

The dog colony was established in 1952 and is maintained with a population of 44 to 50 pure-bred Beagle hounds. Prior to 1958 a mongrel population was established in the colony, and this served as one source of mongrel dogs used in this study. The colony exists with a minimum of environmental control in order that structural alterations due to growth and aging may be reflected as a "normal" life parameter for the animals.

The diet for these animals after weaning consisted of a dry commercial dog food* which was fed free-choice with a

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*Supplied by Gaines Dog Food Division, General Foods Co., Kankakee, Illinois.

hopper-type self-feeder. The ration consisted of 25% protein, 7% fat, 40% carbohydrate, vitamins, minerals and essential amino acids. One pound of feed supplied 1500 to 1600 calories.* Fresh water was provided by an automatic watering system. There was no evidence of nutritional deficiency in any of the animals studied.

On the basis of routine fecal examinations and observation of the gastro-intestinal system, the colony did not appear to be excessively parasitized at any time during the course of study. Intestinal parasites were controlled by periodic treatment with a commercial anthelmintic and daily cleaning of the animal quarters with steam.

All animals in the colony were vaccinated against canine distemper and infectious canine hepatitis. A solid wooden fence isolated the animal runs and prevented the dogs in the colony from direct contact with outside stray animals. At the time of death, all of the animals appeared to be in good health.

Procine Spinal Cord and Ganglia Specimens

All hogs used in this study were reared at the Swine Nutrition Farm, Iowa State University. Seventy-nine animals ranging in age from 2 days to 7.2 years were utilized. The

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*Feed analysis provided by the Gaines Dog Research Laboratories, Kankakee, Illinois.

median age was 2 years.

The diet of these pigs was the regular diet fed at the Swine Nutrition Farm:

Summer formula: Fresh-chopped alfalfa balancer; 14% protein ration limited to the rate of 0.9 to 2.3 kg. per animal per day; and 3.6 to 4.5 kg. of fresh chopped alfalfa.

Winter formula: Corn silage balancer ration, fed at the rate of 0.9 to 2.3 kg. per animal per day, and 3.6 to 4.5 kg. of a corn silage described as corn-soybean oil meal ration.

Fat content of the diet was 2.5 to 3%; data on specific composition and ingredients were previously described by Johnson *et al*. (1957, 1959).

At the time the pigs were destroyed, they appeared to be normal, healthy subjects exhibiting no clinical evidence of disease.

## Collection and Fixation of Specimens for Light and Fluorescence Microscopy

The animals were killed in the laboratory by electrocution and were exsanguinated by severing the axillary artery and vein. As soon as hemorrhage ceased, the thoracic and abdominal cavities were opened. The thoracolumbar junction of the vertebral column was removed intact and immediately trimmed with a band saw in such a way that the spinal cord and ganglia could be exposed. The dura mater of the spinal cord was removed to allow direct contact of the cord with

the fixative. The tissue was usually placed in the fixative within 45 minutes after death.

Each specimen was fixed at least 48 hours by immersion in 3,000 ml. of 10% buffered neutral formalin prepared by the following method:

| | |
|---|---|
| 40% formaldehyde | 100 ml. |
| Tap water | 900 ml. |
| Sodium phosphate, dibasic | 7 gm. |
| Sodium phosphate, monobasic | 4 gm. |

Processing and Staining Procedures for Light and Fluorescence Microscopy

All tissue blocks were dehydrated, cleared, and embedded in Paraplast* according to the following schedule:

| | |
|---|---|
| Ethanol, 70% | 30 minutes |
| Ethanol, 70% | 30 minutes |
| Ethanol, 95% | 1 hour |
| Ethanol, 95% | 1 hour |
| Ethanol, Absolute | 1 hour |
| Ethanol, Absolute | 1 hour |
| Chloroform, No. 1 | 1 hour |
| Chloroform, No. 2 | 1 hour |
| Chloroform, No. 3 | 1 hour |
| Paraplast, No. 1 | 2 hours |
| Paraplast, No. 2 | 2 hours |

Tissue sections were cut 6 to 8 $\mu$ in thickness on a rotary microtome. Several sections were prepared from each block and in many cases serial sections were prepared for study. Three sections were stained routinely by staining procedures outlined below, and a 4th section was prepared

*Scientific Products, Evanston, Illinois.

for examination with the fluorescence microscope.

The staining procedures routinely used were as follows:

Lillie's Nile Blue Sulfate Method for Lipofuscin

| | |
|---|---|
| 1. Xylol | 2 minutes |
| 2. Xylol | 2 minutes |
| 3. Absolute alcohol | 2 minutes |
| 4. 95% alcohol | 2 minutes |
| 5. 80% alcohol | 2 minutes |
| 6. Distilled water | 5 minutes |
| 7. Nile blue A - 0.5% in 1% $H_2SO_4$ | 20 minutes |
| 8. Wash in running water | 10-20 minutes |
| 9. Mount in gylcerol gelatin | |

Results:

Lipofuscin . . . . . . . . . . . . . . . . . . .blue to green blue
Melanins . . . . . . . . . . . . . . . . . . . .dark green
Erythrocytes . . . . . . . . . . . . . . . . . .greenish yellow
Myelin . . . . . . . . . . . . . . . . . . . . .green to blue
Nuclei . . . . . . . . . . . . . . . . . . . . .poorly stained

Periodic Acid - Schiff for Lipofuscin

The staining solutions were prepared as follows:

1. 0.5% periodic acid ($HIO_4$) in distilled water.

2. Schiff's reagent:

| | |
|---|---|
| a. Basic fuscin | 1.0 gm. |
| b. Distilled water | 200.0 ml. |
| c. 1 N hydrochloric acid | 20.0 ml. |
| d. Anhydrous sodium bisulfite | 1.0 gm. |

Boil 200 ml. of distilled water, add basic fuscin and stir. Cool to 50 C. and filter. Add hydrochloric acid, cool to 25 C. and add sodium bisulfite. Keep in the dark. The fluid takes about 2 days to become orange or straw-colored; it is then ready for use. Shaking with a few grains of activated charcoal will decolorize the solution immediately. Filter and use.

3. Sulfurous acid rinse:

| | |
|---|---|
| a. 10% sodium metabisulfite | 6.0 ml. |
| b. 1 N hydrochloric acid | 5.0 ml. |
| c. Distilled water | 100.0 ml. |

Staining procedure for PAS:

1. Bring sections through xylene and graded alcohols to water.
2. 0.5% Pancreatin* solution** — 20 minutes
3. 0.5% aqueous periodic acid — 5 minutes
4. Rinse in tap and distilled water.
5. Schiff's reagent — 15 minutes
6. Sulfurous acid rinse, 3 changes — 2 minutes(each)
7. Place briefly in running tap water.
8. Dehydrate in graded alcohols.
9. Clear in xylene.
10. Mount in permount.

Results:

Lipofuscin-dark red
Everything else pink to purple

## Long Ziehl-Neelson Acid Fast Method

1. Bring sections through xylene and graded alcohols to water.
2. Stain in carbol fuchsin solution overnight at room temperature.

| | |
|---|---|
| Basic fuchsin | 10.0 gm. |
| Phenol | 50.0 gm. |
| Alcohol | 100.0 ml. |
| Distilled water | 1000.0 ml. |

3. Wash in running water.
4. Differentiate in 1% acid alcohol until cells are just faint pink.
5. Counterstain with 0.5% toluidine blue (if desired).
6. Wash in running water.
7. Dehydrate in alcohols, clear in xylene, and mount in permount.

Results:

Acid fast lipofuscins . . . . . . . . . . . .dark red
Nuclei (if counterstained). . . . . . . . . .dark blue

For examination of the tissue with the fluorescent microscope, the sections were prepared as follows:

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*Matheson, Coleman and Bell, East Rutherford, New Jersey.

**Pancreatin digestion removes glycogen which is also PAS-positive.

| | | |
|---|---|---|
| 1. | Xylol | 2 minutes |
| 2. | Xylol | 2 minutes |
| 3. | Absolute alcohol | 2 minutes |
| 4. | 95% alcohol | 2 minutes |
| 5. | Mount in immersion oil (non-drying, low fluorescence) | |
| 6. | Seal coverslip | |

Examination of sections

The fluorescent preparations were examined with a Bausch and Lomb model PBV5 Dynazoom microscope*, the optics of which included a 10x, 16 mm. 0.25 N.A. objective and a 43x, 4 mm. 0.65 N.A. objective, a 10x compensated eyepiece, and an Abbe 1.30 N.A. condensor fitted with a 12 mm. darkfield stop. The light source consisted of a Bausch and Lomb model 31-33-28-03 fluorescent illuminator fitted with a high pressure mercury arc lamp (No. HBO200). Bausch and Lomb filters were also employed; No. 5-58 exciter filter permitted transmissions in the 400 $m_\mu$ range and a safety filter (No. Y-8 barrier filter) blocked transmissions under 550 $m_\mu$. For the 43x objective the level of excitation was increased by using immersion oil between the slide and the condensor, and refocusing the illuminator.

Photomicrographs of fluorescent preparations were made, employing a Leitz Ortholux microscope** fitted with a 54x, 0.95 N.A. fluorite oil immersion objective and a No. 82 D 1.20 immersion darkfield condensor. A high pressure mercury

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*Bausch and Lomb Incorporated, Rochester, N.Y.

**E. Leitz, Incorporated, New York, N.Y.

burner HBO200 provided illumination. Heat filter No. BG82, blue filter No. BG12, and a K-530 blue-absorbing barrier filter were used. Color transparencies were produced on high speed Ektachrome film (for daylight) which were commercially processed and printed.

Photomicrographs of the stained preparations were also made employing a Leitz Ortholux microscope* fitted with plan-achromatic objectives. Color transparencies produced on Kodachrome II type A Professional film were commercially processed and printed.

## Electron Microscopy Procedures

### Canine and procine specimens

Tissues from 5 dogs and 7 pigs were studied with the electron microscope. The sex, age, weight, breed, and tissues studied are given in Table 2.

### Collection of tissues

The dogs were given a general anesthetic and the selected tissue was removed surgically. A complete dorsal laminectomy provided good exposure of the spinal cord and dorsal root ganglia. A laparotomy approach was usually sufficient to expose the autonomic ganglia. These tissues were usually placed in the fixative within one minute after excision.

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*E. Leitz, Incorporated, New York, N.Y.

Table 2. Sex, age, weight, breed, and tissues of 5 dogs and 7 hogs studied by means of the electron microscope

| Animal | Number | Sex | Age | Weight | Breed | Tissue studied | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Spinal cord | Autonomic ganglia | Dorsal root ganglia |
| Pig | 24 | Male | 62 mo. | 740 lb. | Duroc | | X | |
| Pig | 4512 | Female | 24 mo. | 408 lb. | PC-York-Land.[a] | X | X | X |
| Pig | 19-259 | Stag | 76 mo. | 808 lb. | Hampshire | | X | |
| Pig | 26-258 | Female | 87 mo. | 632 lb. | Hampshire | | X | |
| Pig | 930-259 | Stag | 76 mo. | 860 lb. | Hampshire | | X | |
| Pig | 1350 | Female | 73 mo. | 385 lb. | Landrace | X | X | X |
| Pig | 22-160 | Stag | 71 mo. | 574 lb. | Hampshire | | X | |
| Dog | M37 | Female | 157 mo. | 86 lb. | Golden Ret. | | | X |
| Dog | C1 | Female | 12 mo. | 45 lb. | Mongrel | | | X |
| Dog | C2 | Male | 12 mo. | 35 lb. | Mongrel | | | X |
| Dog | C3 | Female | 12 mo. | 60 lb. | Mongrel | | | X |
| Dog | C4 | Male | 96 mo. | 25 lb. | Dachshund | X | X | X |

[a]PC-York-Land. = 1/2 Poland China - 1/4 Yorkshire - 1/4 Landrace.

Collection of tissues from the hogs, however, proved to be a much more difficult and time-consuming procedure. Surgical excision of a segment of the spinal cord and its associated ganglia from large hogs was not practical without special instruments and equipment. Therefore, subsequent dissection of the tissues from the spinal column of the killed animals caused considerable delay in getting the tissues into the fixative.

It is also well known that nerve cells die and undergo autolysis rather quickly after death; according to Pease (1964), fixation as soon as practical after death is unquestionably desirable, but there is less urgency than was previously supposed. Therefore, to establish a time guideline for this study in regard to the occurrence of autolysis prior to fixation, a preliminary study was initiated to determine approximately how long ganglion cells retain their fine structural morphology after death.

Preliminary studies on autolysis

Small pieces of dorsal root ganglia were fixed at the time of death, 8 minutes after death, 15 minutes, 25 minutes, 45 minutes, and one hour after death. After one hour in cold 6.25% gluteraldehyde in Millonig's phosphate buffer at pH 7.4, these tissues were post-fixed in 1% osmium tetroxide in phosphate buffer solution for 30 minutes. The tissue was embedded in divinylbenzene methacrylate.

After studying the electron micrographs, it was determined that tissue withheld from the fixative for up to 25 minutes (Figure 12) still retained what appeared to be normal fine structural morphology. Tissue fixed at 45 minutes and one hour after death appeared to have undergone autolytic changes (Figure 13). The mitochondria were "blown up" with only remnants of cristae remaining. The nuclear chromatin appeared rather homogeneous, and the nuclear membrane was serrated, with the 2 layers being less distinct (Figure 13). The lipofuscin pigments retained most of their normal fine structural morphology even one hour after death (Figure 13). The "band patterns" of the substructure were, however, less distinct.

Therefore, on the basis of this preliminary study, it was concluded that approximately 25 minutes could elapse between death and the placing of ganglion tissue into the fixative solution. With this interval of time in which to work, the hogs were electrocuted and then exsanguinated by severing the axillary artery and vein. Tissue samples were removed in the same manner described for collection of tissue for light and fluorescence microscopy.

Fixation of specimens

Within one minute after death in the case of the dogs and within 25 minutes after death of the hogs, the tissue was minced into small pieces less than 1 mm. in diameter and placed in the fixative solution. All of the pig tissues were

fixed for 2 hours in 6.25% glutaraldehyde in Millonig's buffer pH 7.4 at a temperature of 4 C. The tissues were then stored in Millonig's buffer for a period of from 1 day to 3 months. Prior to embedding, these tissues were "post-fixed" for 45 minutes in 1% osmium tetroxide in Millonig's buffer at pH 7.4. Tissue blocks were next embedded in divinylbenzene methacrylate.

Most of the dog tissues were processed differently from the hog tissues. Tissues from dogs M37, C1 and C3 were fixed for one hour in 2.5% glutaraldehyde buffered to pH 7.4 with veronal acetate to which 0.045 gm. dextrose/ml. had been added. Tissue from dog C4 was fixed by the same method used for the hog tissue. Tissues from the above animals were embedded in Maraglas, according to the method of Erlandson (1964). The tissue from dog C2 was fixed for one hour in 1% osmium tetroxide buffered to pH 7.4 with veronal acetate (Palade's fixative) to which 0.045 gm. of dextrose/ml. had been added. These blocks of tissue were dehydrated, then embedded in methacrylate.

Buffer solutions

The buffer solution described by Millonig (1962) was prepared as follows:

| | |
|---|---|
| Solution A = $NaH_2PO_4 \cdot H_2O$ | 5.52% |
| Solution B = NaOH | 5.04% |
| Solution C = Glucose | 10.80% |
| Solution D = Solution A | 20.75 ml. |
| Solution B | 4.25 ml. |
| Buffer solution (2 times isotonicity) = Solution D | 22.5 ml. |
| Solution C | 2.5 ml. |

The acetate-veronal buffer stock solution described by Palade (1952) was prepared as follows:

| | |
|---|---|
| Na-veronal (sodium barbital) | 14.714 gm. |
| Na-acetate, crystalline ($NaC_2H_3O_2 \cdot 3H_2O$) | 9.714 gm. |
| Water | 500 ml. |

According to Caulfield's (1957) method for increasing tonicity, the above solution was modified by adding 0.045 gm. sucrose/ml.

Fixatives

The fixative agents used were as follows:

6.25% glutaraldehyde solution

| | |
|---|---|
| Millonig's buffer solution | 50.0 ml. |
| 25% glutaraldehyde | 25.0 ml. |
| NaOH 1 N | adjust to pH 7.4 |
| Distilled water q.s. | 100.0 ml. |

2.5% glutaraldehyde solution

| | |
|---|---|
| 25% glutaraldehyde | 1.0 ml. |
| Acetate-veronal buffer | 2.0 ml. |
| Distilled water | 6.0 ml. |
| HCl 0.1 N | adjust to pH 7.4 |
| Distilled water q.s. | 10.0 ml. |

1% osmium tetroxide solution

| | |
|---|---|
| Millonig's buffer solution | 25.0 ml. |
| 2% osmium tetroxide | 25.0 ml. |
| NaOH 1 N | adjust to pH 7.4 |

Embedding media, dehydration and infiltration

A modification of the divinyl-benzene methacrylate embedding medium described by Kushida (1961) was prepared for use as follows:

| | |
|---|---|
| Benzoyl peroxide | 0.4 gm. |
| Divinylbenzene 55* | 2.5 ml. |

*Dow Chemical Company, Midland, Michigan.

| | |
|---|---|
| Ethyl methacrylate | 19.0 ml. |
| N-butyl methacrylate | 28.5 ml. |

The above solution should be filtered through anhydrous $Na_2SO_4$.

The dehydration and infiltration procedure for methacrylate was as follows:

| | |
|---|---|
| Ethanol, 50% | 5 minutes |
| Ethanol, 50% | 5 minutes |
| Ethanol, 75% | 10 minutes |
| Ethanol, 95% | 15 minutes |
| Ethanol, 100% | 10 minutes |
| Ethanol, 100% | 10 minutes |
| Methacrylate monomer | 15 minutes |
| Methacrylate monomer | 30 minutes |
| Methacrylate monomer | 15 minutes |
| Place tissue in capsule | |
| Polymerize in 60 C. oven | 12-24 hours |

Tissue may be left in 75% ethanol solution for several hours if necessary.

The Maraglas mixture described by Erlandson (1964) was prepared as follows:

| | |
|---|---|
| Maraglas 655* | 36 ml. |
| D.E.R. 732** | 8 ml. |
| Dibutyl phthalate | 5 ml. |
| Benzyl dimethylamine | 1 ml. |

The dehydration and infiltration procedure for Maraglas was as follows:

| | |
|---|---|
| Acetone, 50% | 15 minutes |
| Acetone, 70% | 15 minutes |
| Acetone, 95% | 15 minutes |
| Acetone, 100% (2 changes) | 15 minutes |

---

*Dow Chemical Company, Midland, Michigan.

**Dow epoxy resin, Dow Chemical Company, Midland, Michigan.

| | |
|---|---|
| Acetone + epoxy mixture 1:1 | 45 minutes |
| Epoxy mixture (2 changes) | 1 hour and 2-3 hours |

Embed in gelatin capsules, de-gas, and polymerize in an oven for 17 hours at a temperature of 52 C. The tissue may be left overnight in the epoxy mixture (at 4 C.) before embedding, although this is not necessary (Erlandson, 1964).

Method of sectioning

Thin sections of tissue (silver to pale gold) were cut on a Porter-Blum MT1 microtome with glass knives, and were expanded with xylene in the case of Maraglas, and chloroform in the case of methacrylate sections. The expanded sections were picked up on 300 mesh grids. Some grids were occasionally coated with a thin parlodian film.

Staining solution and procedure

Each of the tissue sections was stained with lead citrate. The stain was prepared by the following method (Reynolds, 1963):

> ...Place 1.33 gm. $Pb(NO_3)_2$,* 1.76 gm. $Na_3(C_6H_5O_7) \cdot 2H_2O$ and 30 ml. distilled water in a 50 ml. volumetric flask. The resultant suspension is shaken vigorously for 1 minute and allowed to stand with intermittent shaking in order to insure complete conversion of lead nitrate to lead citrate. After 30 minutes 8.0 ml. 1 N NaOH** is added, the suspension diluted to 50 ml. with distilled water and mixed by inversion. Lead citrate dissolves and the staining solution is ready for use. The pH of the staining solution was routinely found to be $12.0 \pm 0.1$. Faint turbidity, if present, is usually readily removed by centrifugation.

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*Chemicals of analytical reagent grade were used.

**1 N NaOH was prepared by dilution of 10 N NaOH, carbonate-free. Fisher Scientific Company, New York, New York.

Grids with sections were stained by floating on single drops of staining solution for 3 to 5 minutes. A Petri dish containing a piece of dental wax served as a suitable container. The dental wax may be placed on filter paper moistened with 1 N NaOH. This helps to remove carbon dioxide from the air and thereby reduces the chance of lead carbonate precipitates on the tissue sections. To remove excess staining solution from the tissue sections, they were dipped at least 30 times in 5 different containers of distilled water.

Examination of sections

The stained sections were examined in an Hitachi HU11a electron microscope using the double condensor, with an aperture of $300\mu$. A $30\mu$ or $50\mu$ aperture was used in the objective lens. The microscope was operated at 50 kv.

# RESULTS

## Light and Fluorescence Microscope Findings

Lipofuscin pigments in each of the 3 areas studied were observed in dogs at 5 months of age and hogs at 6 months of age. In both dogs and hogs the pigments were essentially similar in appearance in regard to staining and fluorescence properties. In sections stained by the Nile blue method, lipofuscin pigment appeared as dark blue granules in the cytoplasm (Figures 1-5). With the PAS method, the granules appeared red (Figures 6-7), and with the Ziehl-Neelson stain, the granules inconstantly exhibited varying degrees of acid-fastness. With near-ultraviolet light, the granules exhibited a strong yellow-orange autofluorescence in unstained sections. (Figures 8-11).

The granules in the younger animals (less than 1 year old) were just visible with the light and fluorescence microscope. As the animals increased in age, the granules enlarged and tended to form clusters, sometimes occupying as much as 85% of the cell volume in the older animals. The granules appeared to be larger in the autonomic cells of older hogs than in any other tissue studied and were often up to 3.5$\mu$ in diameter.

The intracytoplasmic location of the pigment granules generally varied with age and the particular cell type. In the younger animals the granules were usually dispersed

and generally observed first near the nucleus. In animals over 1 year of age, the pigment usually appeared as a single mass. In the ventral horn cells this mass was usually observed at one pole, often near the axon hillock (Figures 5, 6, 8, 10). A narrow band of pigment granules frequently extended from the principal mass to partially encompass the nucleus (Figures 5, 8, 10). Various other distribution patterns were observed less frequently. Likewise, in the neurons of the dorsal root ganglia and the autonomic ganglia, the location and pigment patterns varied. In the dorsal root ganglia, the pigment was usually located at the periphery of the cells (Figures 3, 4, 7, 9). Often the peripheral margin was completely outlined with pigment. In the autonomic cells, either a polar or bipolar distribution was observed most frequently (Figures 1, 2).

In some young animals (both dogs and hogs 4 to 12 months of age) atypical, weak-orange autofluorescent particles were observed. These were diffusely distributed in the cytoplasm and were barely visible with the fluorescence microscope. Since these autofluorescent particles were not observed in animals under 4 months of age, they were interpreted to be lysosomes, possibly just beginning to transform into pigment.

The distribution of pigment in the 3 areas of the nervous system is presented (Table 3 and 4) as a percentage of cells containing pigment. Generally, as the percentage of pigmented cells increased with age, so did the amount of pigment.

In the very old animals up to 85% of the volume of some cells was occupied by pigment.

The calculations for the ganglion cells were based upon observations of at least 10 randomly selected fields with the fluorescence microscope at a magnification of 400x. With the cell population being less dense in the spinal cord, 25 or more fields in several sections had to be observed in order to count an equivalent number of ventral horn cells. Serial sections were examined from many specimens, but to count a significant number of cells in serial section for calculating a percentage was impractical and not without error.

Individual observations, given in tabular form (Tables 3 and 4), are divided into 6 progressive age groups for both the dogs and hogs. The first group of each species represents young animals in which no pigment was found. The second group begins with the youngest animals in which lipofuscin was observed. Other groups were designed to reduce the age variability within the groups and to maintain a minimum of at least 6 animals in the smaller groups. The mean and range values for age, weight, and per cent of pigmented cells are given for each group.

The means are summarized for each group in graphic form (Graphs 1, 2). Group comparisons illustrate the progressive pigmentation of cells as the animals aged. This holds true for the 3 cell types in both the dogs and hogs with the following exceptions. In groups 2 and 3 (Table 3 and Graph 1)

the unusually high percentage of pigmented autonomic cells in dog No. B66 causes group 2 to have a slightly higher mean than group 3. In the case of the hogs (Table 4 and Graph 2), the mean percentage of autonomic cells is slightly higher in group 3 than in group 4. This is possibly due to the fact that specimens were not available for examination in some of the younger animals of this group. The anticipated lower values for these animals would have undoubtedly lowered the mean for this group.

Within group comparisons (Graphs 1, 2) illustrate the differential rate of pigmentation in the different cell types. The ventral horn cells or motor cells of the spinal cord are the least resistant to pigmentation of the group studied, and the sensory, dorsal root cells are slightly more resistant; the autonomic cells are the most resistant. This relationship holds true for the dogs (Graph 1) and most groups of hogs, but is not as pronounced in the older age groups. In group 5 of the hogs (Graph 2) the mean percentage of pigmented dorsal root ganglia cells is 96 compared to 91 for the ventral horn cells. In group 6, the mean percentage for the 2 cell types is the same. This can be attributed to a decrease in the differential rate of pigmentation in the different cell types as the animal increases in age and as the percentage of pigmented cells increases. These data suggest that in the senile animals, the resistance to pigmentation is about the same for the

3 cell types studied and that the loss of this resistance is progressive as the animals increase in age.

Though the percentage of cells pigmented was calculated for only the ventral horn cells in the case of the spinal cord, numerous lipofuscin pigment bodies were seen on cursory examination of the dorsal horn cells, cells of Clark's column, and cells of the intermediolateral cell column. There also appeared to be a progressive increase in these areas with age, both in the number of cells pigmented and in the amount of pigment.

Changes other than lipofuscin deposition correlated with age

Lipofuscin pigment was the most consistent cellular alteration that could be correlated with age in the specimens studied. Other changes observed that appeared to be correlated with age included atrophy of the cells, especially the ventral horn cells. This was less evident in the ganglia cells. A marked decrease in the number of cells in the older animals was observed most constantly in the ventral horn of the spinal cord. The commonly reported alterations in nuclear size, shape and cytoplasmic vacuolization were not observed in the present group of specimens.

The most prominent non-cellular change that appeared to be related to age was a decrease in the number of blood vessels in the spinal cord. In the young animals up to 1 year of age, there were numerous blood vessels in the gray areas of the spinal cord, but in the older animals up to and including

Table 3. Incidence of lipofuscin occurring in different areas of the nervous system of 53 dogs ranging in age from 7 days to 192 months (16 years)

| Dog no. | Sex | Age day | Age mo. | Weight lbs. | Breed | Percentage of cells containing lipofuscin: Spinal cord | Dorsal root ganglia | Autonomic ganglia |
|---|---|---|---|---|---|---|---|---|
| **Group 1** | | | | | | | | |
| 061 | M | 7 | | 0.5 | Beagle | 0 | 0 | 0 |
| B34 | M | 14 | | 1.0 | Beagle | 0 | 0 | 0 |
| B45 | M | | 1 | 3 | Beagle | 0 | 0 | 0 |
| B47 | M | | 2 | 3 | Beagle | 0 | 0 | 0 |
| 068 | M | | 2 | 6 | Beagle | 0 | 0 | 0 |
| 072 | F | | 3 | 6 | Beagle | 0 | 0 | 0 |
| 059 | F | | 3 | 5 | Beagle | 0 | 0 | 0 |
| N = 7 Average | | | 2 | 4 | | 0 | 0 | 0 |
| Range | | 7 days - 3 mo. | | 0.5 - 6 | | | | |
| **Group 2** | | | | | | | | |
| 049 | F | | 5 | 15 | Beagle | 1 | 10 | 1 |
| B66 | F | | 6 | 14 | Beagle | 48 | 37 | 8 |
| B59 | F | | 7 | 13 | Beagle | 28 | 3 | 1 |
| B60 | M | | 7 | 20 | Beagle | 32 | 6 | 0 |
| B61 | F | | 7 | 10 | Beagle | 0 | 1 | 0 |
| B48 | F | | 8 | 19 | Beagle | 0 | 1 | 0 |
| B49 | M | | 8 | 28 | Beagle | 8 | 13 | 0 |
| B67 | F | | 9 | 13 | Beagle | 28 | 20 | 0 |
| B53 | M | | 10 | 26 | Beagle | 0 | 0 | 0 |
| B55 | M | | 11 | 30 | Beagle | 30 | 24 | 0 |
| N = 10 Average | | | 8 | 19 | | 17 | 11 | 1 |
| Range | | | 5 - 11 | 10 - 30 | | 0 - 48 | 0 - 37 | 0 - 8 |

Table 3 (Continued)

| Dog no. | Sex | Age mo. | Weight lbs. | Breed | Percentage of cells containing lipofuscin | | |
|---|---|---|---|---|---|---|---|
| | | | | | Spinal cord | Dorsal root ganglia | Autonomic ganglia |
| Group 3 | | | | | | | |
| 1FB | M | 12 | 20 | Beagle | 40 | 15 | 0 |
| 2BB | M | 12 | 15 | Beagle | 68 | 56 | < 1 |
| 2BC | M | 12 | 13 | Beagle | 50 | 30 | < 1 |
| 7BAA | F | 12 | 18 | Beagle | 16 | 28 | 0 |
| 7BAB | F | 12 | 21 | Beagle | 74 | 50 | < 1 |
| 7BAC | F | 12 | 23 | Beagle | 46 | 30 | < 1 |
| 5FC | M | 12 | 16 | Beagle | 60 | 28 | 0 |
| 5FD | M | 12 | 11 | Beagle | 68 | 15 | 0 |
| 5FF | F | 12 | 18 | Beagle | 50 | 34 | < 1 |
| B54 | M | 12 | 20 | Beagle | 76 | 59 | < 1 |
| B56 | M | 12 | 26 | Beagle | 21 | 29 | 0 |
| B57 | M | 12 | 22 | Beagle | 90 | 55 | < 1 |
| B58 | M | 12 | 20 | Beagle | 76 | 69 | < 1 |
| B62 | F | 12 | 16 | Beagle | 54 | 38 | < 1 |
| 2BD | F | 13 | 12 | Beagle | 86 | 34 | < 1 |
| N = 15 | Average | 12 | 18 | | 58 | 38 | < 1 |
| | Range | 12 - 13 | 11 - 26 | | 16 - 90 | 15 - 69 | 0 - < 1 |
| Group 4 | | | | | | | |
| B41 | F | 20 | 35 | Beagle | 80 | 71 | *[a] |
| 21 | M | 25 | 19 | Beagle | 76 | 72 | 46 |
| M31 | M | 30 | 24 | Terrier | 40 | 32 | 3 |
| B36 | M | 41 | 25 | Beagle | 48 | 38 | 10 |
| B65 | F | 43 | 33 | Beagle | 90 | 78 | 69 |
| B51 | F | 50 | 22 | Beagle | 78 | 69 | 56 |
| N = 6 | Average | 35 | 26 | | 69 | 60 | 37 |
| | Range | 20 - 50 | 19 - 35 | | 40 - 90 | 32 - 78 | 3 - 69 |

[a]No specimen available for examination.

Table 3 (Continued)

| Dog no. | Sex | Age mo. | Weight lbs. | Breed | Percentage of cells containing lipofuscin: Spinal cord | Dorsal root ganglia | Autonomic ganglia |
|---|---|---|---|---|---|---|---|
| Group 5 | | | | | | | |
| B43 | F | 93 | 22 | Beagle | 90 | 91 | 83 |
| B44 | F | 93 | 26 | Beagle | 86 | 79 | 66 |
| 7BA | F | 101 | 32 | Beagle | 96 | 81 | 40 |
| B42 | F | 103 | 24 | Beagle | 72 | 75 | 86 |
| M35 | M | 104 | 21 | Terrier | 94 | 77 | 90 |
| 9 | M | 104 | 19 | Beagle | 100 | 88 | 72 |
| B63 | M | 110 | 27 | Beagle | 88 | 89 | 98 |
| M40 | F | 117 | 29 | Dachshund | 90 | 93 | 78 |
| B64 | F | 120 | 24 | Beagle | 96 | 78 | 73 |
| N = 9 Average | | 105 | 25 | | 90 | 83 | 76 |
| Range | | 93 - 120 | 19 - 32 | | 72 - 100 | 75 - 93 | 40 - 98 |
| Group 6 | | | | | | | |
| M38 | F | 125 | 68 | Labrador Ret. | 86 | * | 88 |
| M32 | F | 154 | 42 | Pointer | 82 | 96 | 82 |
| M34 | F | 157 | 19 | Welsh Corgi | 100 | 95 | * |
| M37 | F | 157 | 86 | Golden Ret. | 100 | 98 | 95 |
| M39 | M | 158 | 28 | Welsh Corgi | 94 | 82 | 84 |
| M36 | F | 192 | 42 | Cocker Spaniel | 98 | 86 | 92 |
| N = 6 Average | | 157 | 47 | | 93 | 91 | 88 |
| Range | | 125 - 192 | 19 - 86 | | 82 - 100 | 82 - 98 | 82 - 95 |

Graph 1. Per cent pigmented neurons in the lumbar spinal cord, dorsal root ganglia, and autonomic ganglia of 53 dogs ranging in age from 7 days to 192 months (16 years).

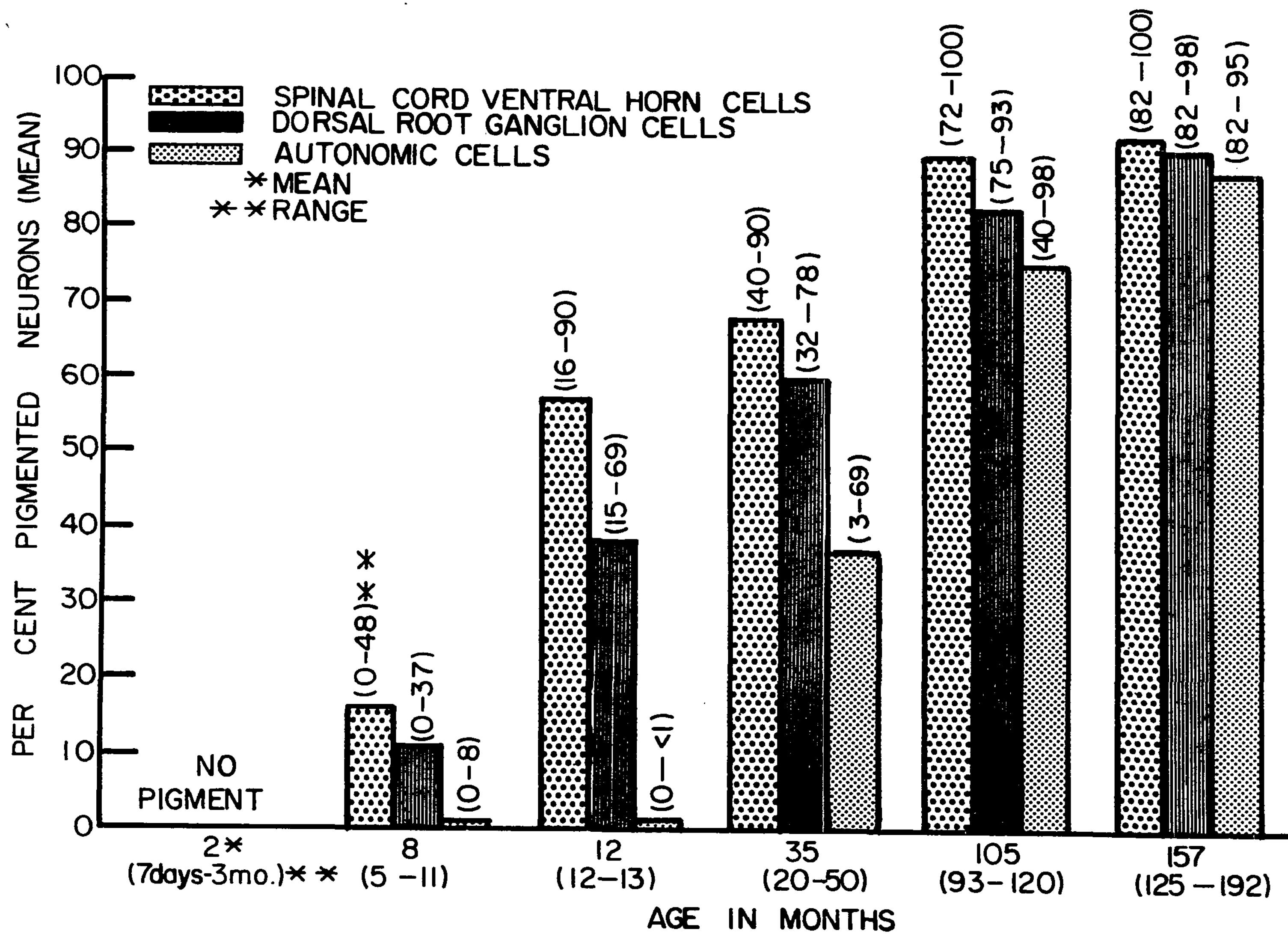

SPINAL CORD VENTRAL HORN CELLS
DORSAL ROOT GANGLION CELLS
AUTONOMIC CELLS
* MEAN
** RANGE
PER CENT PIGMENTED NEURONS (MEAN)
100
90
80
70
60
50
40
30
20
10
0
NO PIGMENT
(0-48)**
(0-37)
(0-8)
(16-90)
(15-69)
(0-<1)
(40-90)
(32-78)
(3-69)
(72-100)
(75-93)
(40-98)
(82-100)
(82-98)
(82-95)
2*
(7days-3mo.)**
8
(5-11)
12
(12-13)
35
(20-50)
105
(93-120)
157
(125-192)
AGE IN MONTHS

Table 4. Incidence of lipofuscin occurring in different areas of the nervous system of 79 hogs ranging in age from 2 days to 87 months (7.2 years)

| Pig no. | Sex[a] | Age day | Age mo. | Weight lbs. | Breed[b] | Percentage of cells containing lipofuscin: Spinal cord | Dorsal root ganglia | Autonomic ganglia |
|---|---|---|---|---|---|---|---|---|
| Group 1 | | | | | | | | |
| 1448B | M | 2 | | 2 | York-Land. | 0 | 0 | 0 |
| 1449B | M | 2 | | 2 | York-Land. | 0 | 0 | 0 |
| 592 | F | | 2 | 36 | PC-York-Land. | 0 | 0 | 0 |
| 627 | F | | 2 | 32 | PC-York-Land. | 0 | 0 | 0 |
| 9753S | F | | 4 | 135 | PC-York-Land. | 0 | 0 | 0 |
| 2250S | F | | 4 | 125 | PC-York-Land. | 0 | 0 | 0 |
| 9713S | F | | 4 | 115 | PC-York-Land. | 0 | 0 | 0 |
| N = 7 Average | | | 2 | 64 | | 0 | 0 | 0 |
| Range | | 2 da. - 4 mo. | | 2 - 135 | | 0 | 0 | 0 |
| Group 2 | | | | | | | | |
| 9310S | F | | 6 | 230 | PC-York-Land. | 0 | 5 | 0 |
| 1292 | F | | 6 | 204 | PC-York-Land. | 8 | 3 | 0 |
| 6022S | F | | 8 | 245 | PC-York-Land. | 30 | 28 | 8 |
| 634S | F | | 8 | 268 | PC-Land. | 16 | 3 | < 1 |
| 2210 | F | | 8 | 277 | York-Land. | 8 | < 1 | < 1 |
| 5903S | F | | 9 | 255 | York-Land. | 14 | 4 | < 1 |
| 2211S | F | | 10 | 396 | York-Land. | 12 | 10 | < 1 |
| 3923 | F | | 10 | 386 | Yorkshire | 14 | 8 | < 1 |
| 9442 | F | | 11 | 345 | York-Land. | 46 | 26 | *[c] |
| 7973 | F | | 12 | 360 | York-Land. | 40 | 26 | 4 |
| N = 10 Average | | | 9 | 297 | | 19 | 11 | ~ 1 |
| Range | | | 6 - 12 | 204 - 396 | | 0 - 46 | < 1 - 28 | 0 - 8 |

[a]Sex is indicated: M = male; F = female; S = stag.

[b]Breed is indicated: York-Land. = 1/2 Yorkshire-1/2 Landrace; PC-York-Land. = 1/2 Poland China-1/4 Yorkshire-1/4 Landrace; PC-Land. = 1/2 Poland China-1/2 Landrace.

[c]No specimen available for examination.

Table 4 (Continued)

| Pig no. | Sex | Age mo. | Weight lbs. | Breed | Percentage of cells containing lipofuscin: Spinal cord | Dorsal root ganglia | Autonomic ganglia |
|---|---|---|---|---|---|---|---|
| Group 3 | | | | | | | |
| 3430S | F | 14 | 383 | York-Land. | 28 | 12 | * |
| 2944S | F | 15 | 450 | York-Land. | 48 | 15 | * |
| 2360S | F | 16 | 389 | York-Land. | 32 | 32 | 29 |
| 2021S | F | 17 | 445 | York-Land. | 82 | 81 | * |
| 4461 | F | 17 | 432 | PC-York-Land. | 100 | 98 | 40 |
| 4475 | F | 18 | 414 | PC-York-Land. | 84 | 95 | 60 |
| 4471 | F | 18 | 393 | PC-York-Land. | 70 | 88 | 35 |
| 4460 | F | 18 | 338 | PC-York-Land. | 90 | 84 | 43 |
| 6333 | F | 19 | 350 | York-Land. | 94 | 68 | * |
| 4491 | F | 23 | 506 | York-Land. | 100 | 100 | 81 |
| 6154 | F | 24 | 368 | York-Land. | 86 | 94 | 40 |
| 4512 | F | 24 | 408 | PC-York-Land. | 60 | 56 | 53 |
| 4496 | F | 24 | 496 | York-Land. | 100 | 99 | 87 |
| 4513 | F | 24 | 367 | PC-York-Land. | 100 | 100 | 72 |
| N = 14 | Average | 19 | 410 | | 77 | 73 | 54 |
| | Range | 14 - 24 | 338 - 506 | | 28 - 100 | 12 - 100 | 29 - 87 |
| Group 4 | | | | | | | |
| 3523 | F | 25 | 574 | York-Land. | 82 | 45 | 38 |
| 3420 | F | 25 | 556 | York-Land. | 80 | 79 | 39 |
| 6153 | F | 25 | 370 | York-Land. | 81 | 75 | 49 |
| 2443 | F | 26 | 430 | York-Land. | 90 | 82 | 61 |
| 2363 | F | 26 | 535 | York-Land. | 92 | 100 | * |
| 2653 | F | 26 | 504 | York-Land. | 72 | 71 | 34 |
| 5912 | F | 26 | 390 | York-Land. | 76 | 69 | 68 |
| 2442 | F | 26 | 486 | York-Land. | 90 | 94 | 64 |
| 2654 | F | 27 | 446 | York-Land. | 96 | 98 | 46 |
| 6024 | F | 27 | 415 | York-Land. | 90 | 93 | 46 |
| 5913 | F | 27 | 440 | York-Land. | 62 | 60 | 47 |

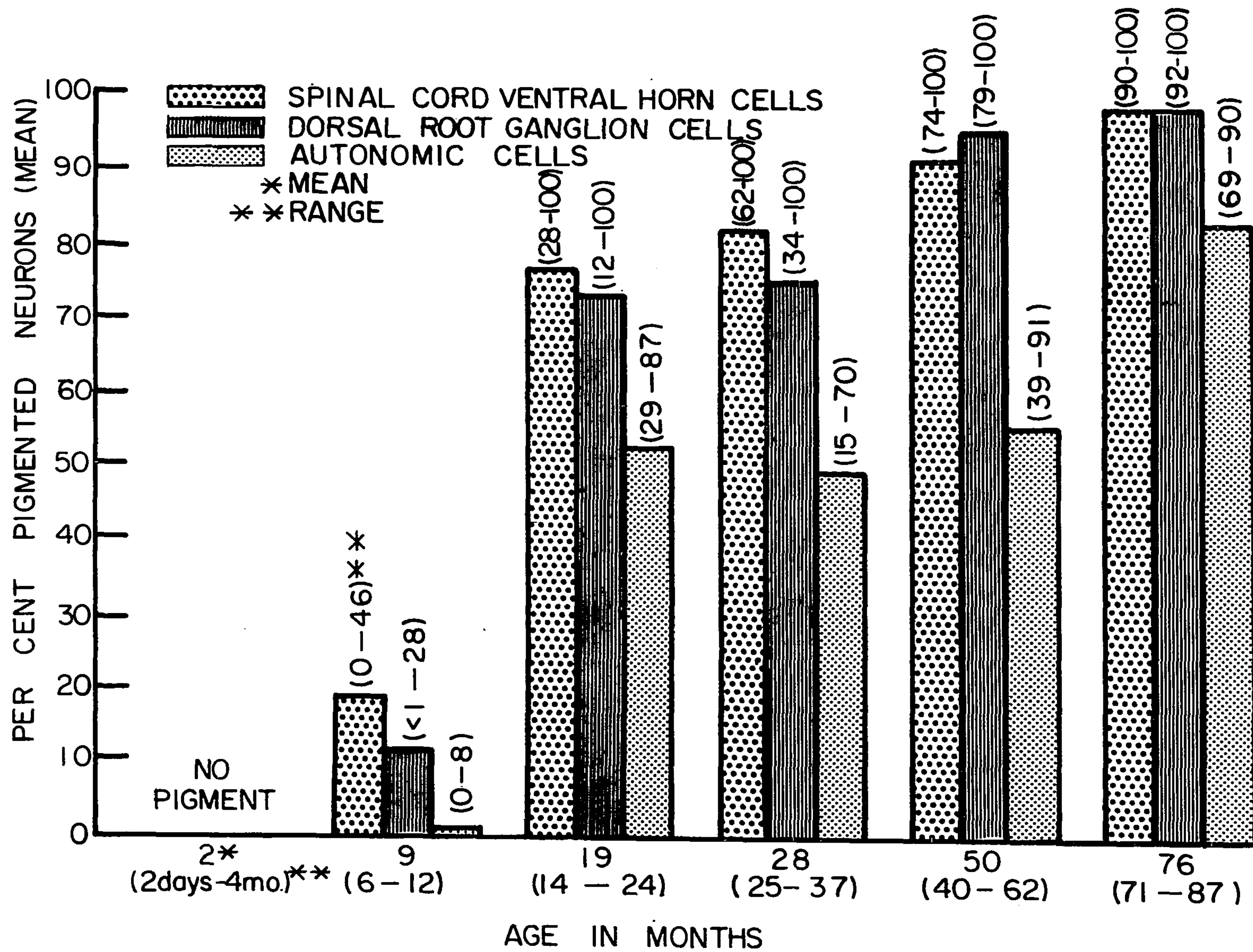
Table 4 (Continued)

| Pig no. | Sex | Age mo. | Weight lbs. | Breed | Percentage of cells containing lipofuscin | | |
|---|---|---|---|---|---|---|---|
| | | | | | Spinal cord | Dorsal root ganglia | Autonomic ganglia |
| Group 4 (Continued) | | | | | | | |
| 6020 | F | 27 | 425 | York-Land. | 78 | 50 | 44 |
| 2651 | F | 27 | 420 | York-Land. | 72 | 71 | 56 |
| 5933 | F | 27 | 420 | York-Land. | 80 | 78 | 23 |
| 2440 | F | 27 | 454 | York-Land. | 92 | 91 | 50 |
| 5931 | F | 27 | 420 | York-Land. | 80 | 84 | 55 |
| 2941 | F | 27 | 438 | York-Land. | 80 | 90 | * |
| 2362 | F | 27 | 495 | York-Land. | 98 | 61 | 34 |
| 2943 | F | 27 | 544 | York-Land. | 88 | 86 | 62 |
| 5910 | F | 28 | 420 | York-Land. | 100 | 86 | 70 |
| 1790 | F | 28 | 506 | York-Land. | 98 | 94 | 15 |
| 1470 | F | 29 | 350 | York-Land. | 76 | 43 | 44 |
| 1424 | F | 29 | 350 | York-Land. | 68 | 69 | * |
| 1573 | F | 29 | 418 | York-Land. | 62 | 34 | * |
| 1472 | F | 29 | 462 | York-Land. | 92 | 97 | 55 |
| 3202 | F | 31 | 386 | York-Land. | 84 | 78 | 47 |
| 3203 | F | 32 | 390 | York-Land. | 78 | 72 | * |
| 3195 | F | 33 | 460 | York-Land. | 70 | 72 | 31 |
| 3196 | F | 33 | 415 | York-Land. | 90 | 62 | 60 |
| 7122 | F | 34 | 505 | York-Land. | 84 | 74 | 61 |
| 1362 | F | 35 | 409 | York-Land. | 84 | 61 | 62 |
| 1361 | F | 37 | 370 | York-Land. | 98 | 93 | 58 |
| N = 32 | Average | 28 | 447 | | 83 | 75 | 49 |
| | Range | 25 - 37 | 350 - 574 | | 62 - 100 | 34 - 100 | 15 - 70 |

Table 4 (Continued)

| Pig no. | Sex | Age mo. | Weight lbs. | Breed | Percentage of cells containing lipofuscin: Spinal cord | Dorsal root ganglia | Autonomic ganglia |
|---|---|---|---|---|---|---|---|
| Group 5 | | | | | | | |
| 4870 | M | 40 | 640 | Poland China | 98 | 99 | 44 |
| 4383 | M | 40 | 640 | Poland China | 74 | 79 | 39 |
| 3652 | M | 44 | 560 | Poland China | 100 | 100 | 91 |
| 4192 | M | 49 | 555 | Poland China | 74 | 95 | 46 |
| 190-10 | F | 54 | 550 | Yorkshire | 100 | 99 | 58 |
| 4110 | M | 55 | 605 | Poland China | 84 | 100 | 45 |
| 392 | F | 55 | 465 | Landrace | 98 | 100 | * |
| 24 | M | 62 | 740 | Duroc | 100 | 99 | 61 |
| N = 8 Average | | 50 | 594 | | 91 | 96 | 55 |
| Range | | 40 - 62 | 465 - 740 | | 74 - 100 | 79 - 100 | 39 - 91 |
| Group 6 | | | | | | | |
| 22-160 | S | 71 | 574 | Hampshire | 98 | 99 | 86 |
| 4583 | F | 72 | 530 | York-Land. | 90 | 100 | 82 |
| 1350 | F | 73 | 385 | Landrace | 100 | 100 | 79 |
| 5815 | F | 75 | 450 | Landrace | 96 | 92 | * |
| 930-259 | S | 76 | 860 | Hampshire | 100 | 98 | 90 |
| 19-259 | S | 76 | 808 | Hampshire | 100 | 100 | 69 |
| 312 | F | 81 | 504 | York-Land. | 100 | 99 | 86 |
| 26-258 | F | 87 | 632 | Hampshire | 100 | 100 | 86 |
| N = 8 Average | | 76 | 593 | | 98 | 98 | 83 |
| Range | | 71 - 87 | 385 - 860 | | 90 - 100 | 92 - 100 | 69 - 90 |

Graph 2. Per cent pigmented neurons in the lumbar spinal cord, dorsal root ganglia, and autonomic ganglia of 79 hogs ranging in age from 2 days to 87 months (7.2 years).



SPINAL CORD VENTRAL HORN CELLS
DORSAL ROOT GANGLION CELLS
AUTONOMIC CELLS
* MEAN
** RANGE
PER CENT PIGMENTED NEURONS (MEAN)
100
90
80
70
60
50
40
30
20
10
0
NO PIGMENT
(0 – 46)**
(<1 – 28)
(0 – 8)
(28-100)
(12 – 100)
(29 – 87)
(62-100)
(34 – 100)
(15 – 70)
(74-100)
(79-100)
(39 – 91)
(90-100)
(92-100)
(69 – 90)
2*
(2days-4mo.)**
9
(6 – 12)
19
(14 – 24)
28
(25 – 37)
50
(40 – 62)
76
(71 – 87)
AGE IN MONTHS

Figure 1. Section of an autonomic ganglion from a 1-year-old dog (No. 5FC) illustrating the normal appearance of non-pigmented cells in the young animals. Nile blue stain. X 250.

Figure 2. Section of an autonomic ganglion from an 8.5-year-old dog (No. M35). Numerous cells contain dark-blue circumscribed lipofuscin pigment deposits, in contrast to no pigment in the 1-year-old animal above (Figure 1). Nile blue stain. X 250.

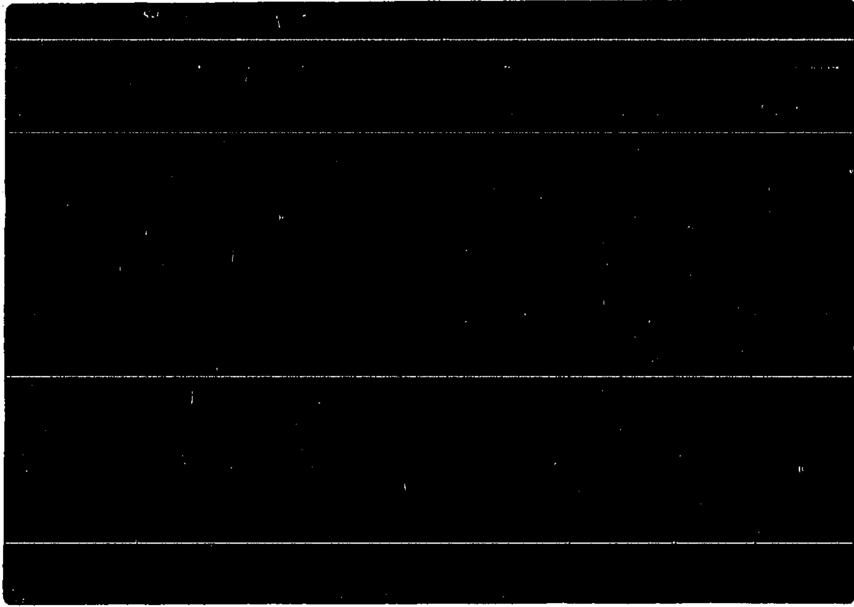
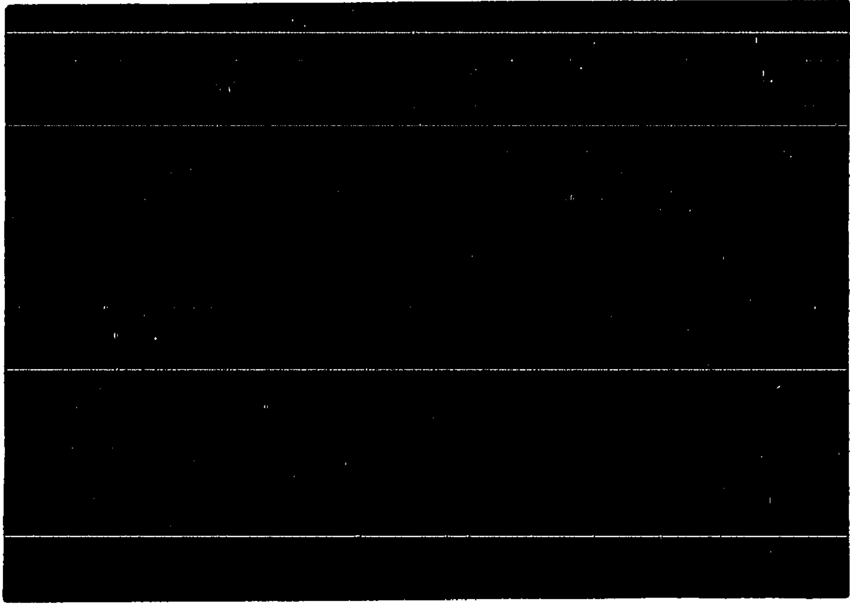


Figure 3. Section of a dorsal root ganglion from a 1-year-old hog (No. 7973). Only a few cells contain a small amount of lipofuscin, located mainly at the periphery of the cell. Nile blue stain. X 250.

Figure 4. Section of a dorsal root ganglion from a 4.5-year-old hog (No. 4110). Numerous cells contain lipofuscin pigment, most of which is located at the periphery of the cells. Nile blue stain. X 250.



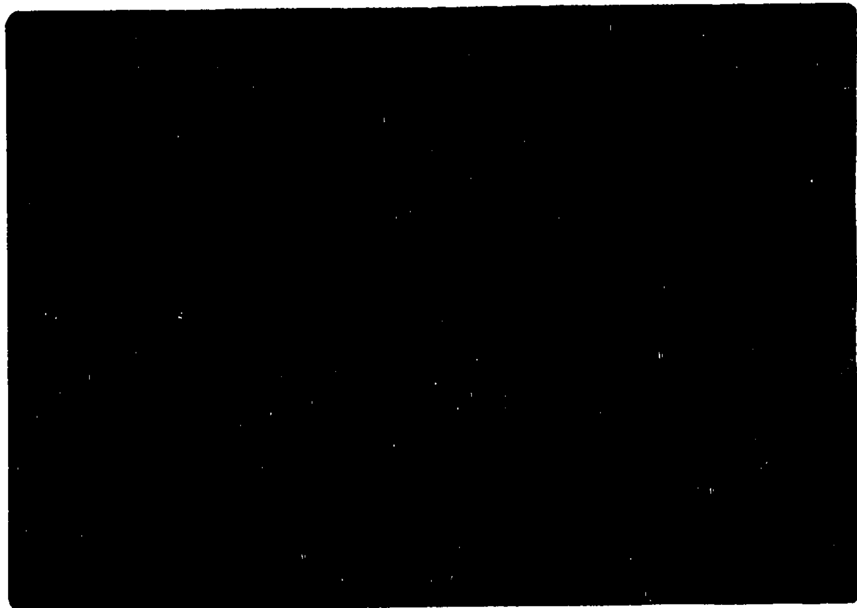


Figure 5. Section of spinal cord ventral horn cells from an 8.5-year-old dog (No. 9). Most of the lipofuscin is located at one pole; however, small deposits are forming at the opposite pole. Nile blue stain. X 250.

Figure 6. Section of spinal cord ventral horn cells from a 4.5-year-old hog (No. 4110). PAS-positive lipofuscin granules are illustrated in both polar and bipolar distributions. PAS stain. X 250.

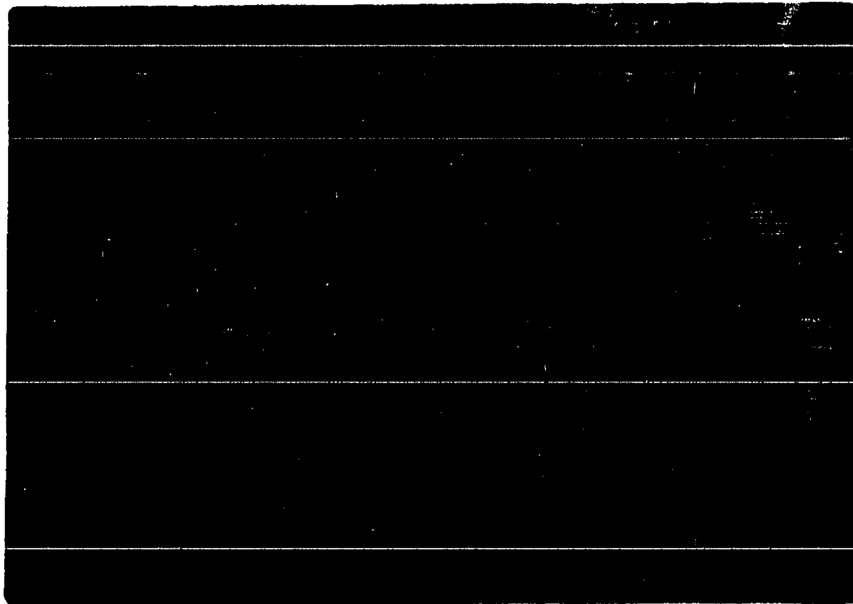


Figure 7. Section of dorsal root ganglion from a 13.2-year-old dog (No. M39) illustrating a large accumulation of PAS-positive lipofuscin granules in one cell and lesser amounts in other cells. PAS stain. X 365.

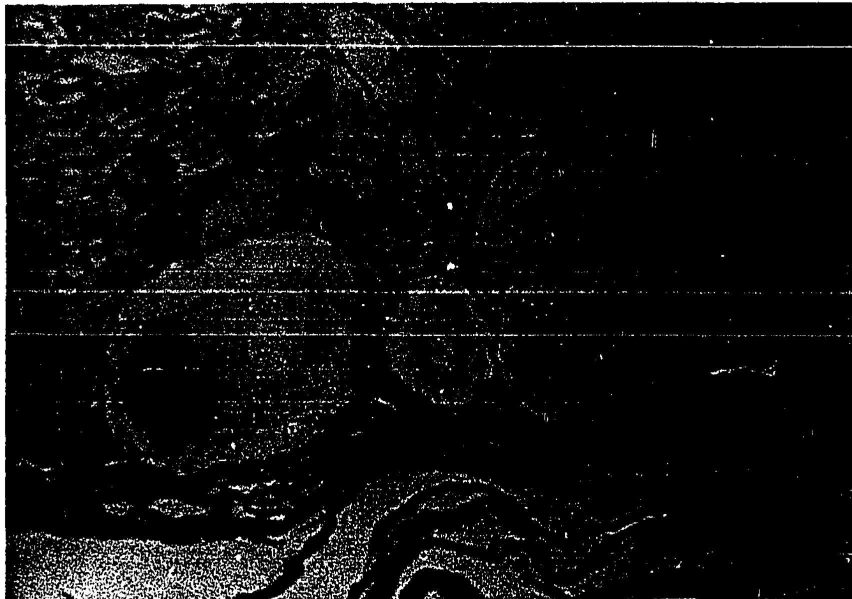


Figure 8. A photomicrograph of a spinal cord ventral horn cell from an 8.5-year-old dog (No. 9) illustrating the yellow-orange autofluorescent lipofuscin pigment. The dark border represents only a space apparently resulting from contraction of the tissue during fixation. X 540.

Figure 9. Section of dorsal root ganglion cells from the same animal as above. The autofluorescent pigment is most often located at the periphery of these cells. X 540.

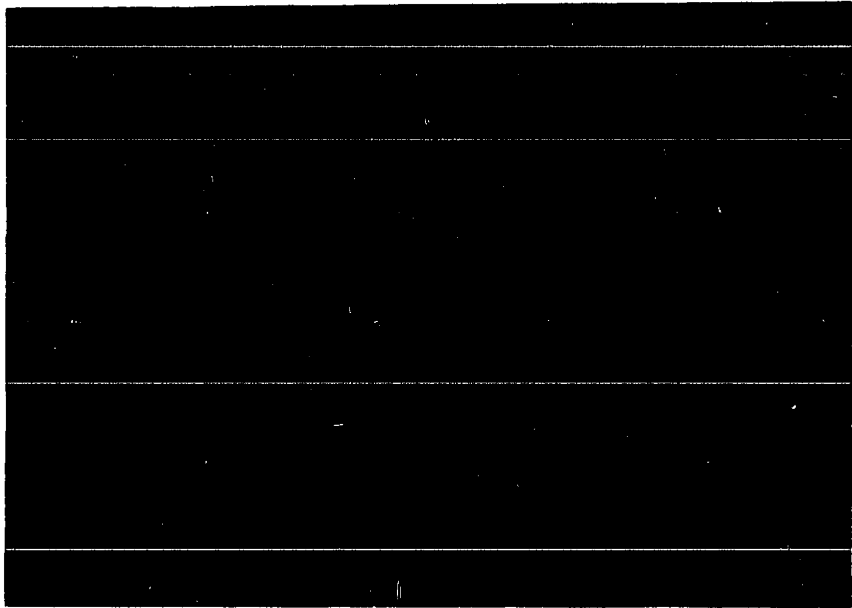
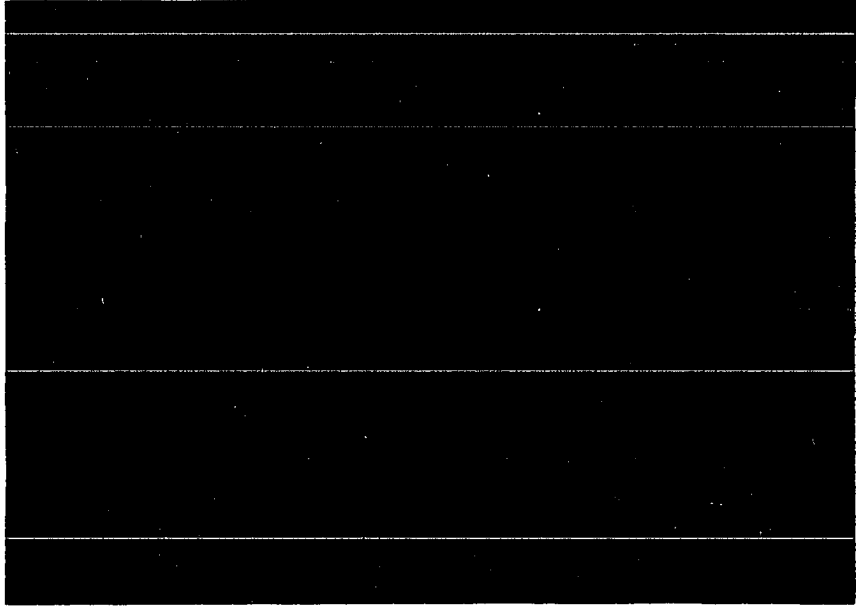
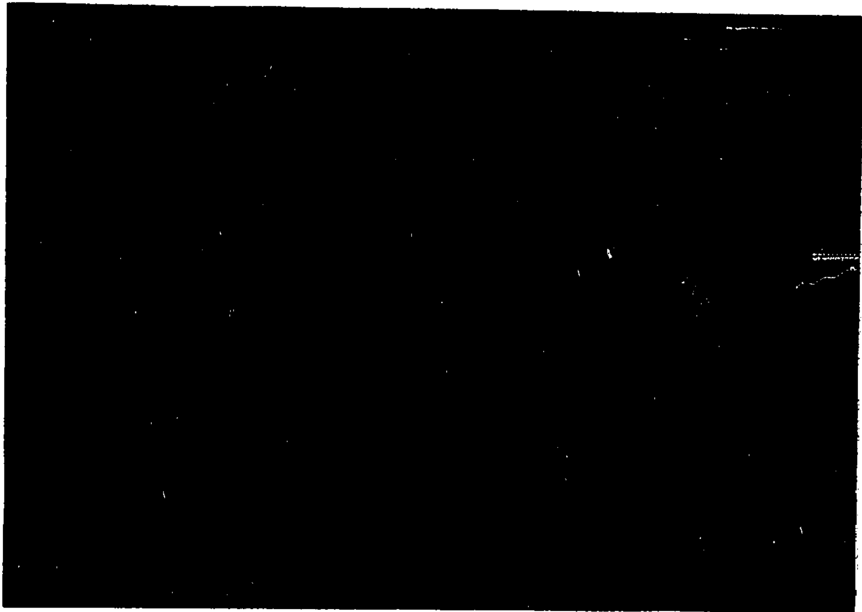


Figure 10. Photomicrograph of a spinal cord ventral horn cell from a 4.5-year-old hog (No. 4110) illustrating yellow-orange autofluorescent lipofuscin pigment. This closely resembles that seen in the dog (Figure 8). X 540.

Figure 11. Section of an autonomic ganglion from the same animal as above. Both polar and bipolar distribution of autofluorescent pigment is illustrated. X 540.





the oldest animals in the study, there was a marked decrease in the number of vessels in the same area. This change was not as obvious in the ganglia.

## Electron Microscope Findings

### Fine structural morphology of lipofuscin

Lipofuscin granules were observed as dense bodies in various shapes and configurations in both dog and hog tissues in each of the 3 areas studied (Figures 12-26). No differences could be detected in the fine structural morphology of lipofuscin in the dog and hog specimens except in the size of pigment bodies; nor were there detectable intraspecies differences in the different cell types studied except in the size of pigment bodies and their pattern of distribution within the cell.

The distribution observed was similar to that found with the light and fluorescence studies. In the spinal cord ventral horn cells the granules were most often observed as a circumscribed mass near the periphery of the cell and in the region of the axon hillock. In the autonomic cells, pigment was observed most frequently in clusters at one or both poles of the nucleus and in the dorsal root ganglia; pigment granules were most often found at the periphery of the cell either in loose or compact clusters. In young animals, however, the pigment usually appeared near the nucleus first (Figure 14), and with age and larger

accumulations eventually became oriented in the manner described above.

The pigment granules generally ranged in size from $\sim 1$ to $3.5\ \mu$ in diameter. Interspecies comparisons indicated that lipofuscin granules in the autonomic cells of the hogs (Figures 18-22) were, on the average, slightly larger than the granules observed in other areas. The pigment bodies of the older animals were generally larger, more complex, more dense and almost always vacuolated.

These vacuoles were very large in the old animals, sometimes more than $1\ \mu$ in diameter (Figures 16, 22), and were usually located near the periphery of the pigment body. They were usually smaller in some lysosomes that exhibited only a coarse, granular matrix (17a) or in pigment granules of younger animals (Figures 14, 23, 24, 26). The vacuoles had a rather dense, single limiting wall (Figure 16), and were an integral part of most pigment bodies. They were usually bound within the pigment complex by the same single-unit membrane (Figures 15, 17a, 20) which was demonstrated inconstantly around the pigment body. In view of the nature of these vacuoles and the fact that they progress in size as pigment bodies become more complex, it may be inferred that they represent lipid bodies, probably altered by ethanol in the dehydration process.

In most of the sections, it was possible to demonstrate

rather normal-appearing lysosomes with a homogeneous granular matrix and bound by a single-unit membrane (Figures 13, 14, 15, 21, 26), but these were observed with decreasing frequency as the animals increased in age and as pigment accumulations increased. Occasionally lysosomes could be seen with part or all of the matrix transformed into a coarse, dense, granular form (Figures 17a, 17b). Also lysosomes containing several bands ~ 50 Å wide with a repeating pattern of alternate light and dark lamellae at periods of ~ 35 Å were observed in the matrix (Figures 16, 21). Structurally, these configurations are almost identical to those seen in the large, more complex pigment bodies. The lysosomes containing only a few band structures were usually ~ 1/2 μ in diameter whereas those with a slightly more complex substructure often were ~ 1 μ in diameter (Figures 16, 17a, 17b, 21, 24).

The most complex of the pigment bodies ranged up to ~ 3.5 μ in diameter and consisted of a substructure of dense bands, dense homogeneous particles, dense granules, and vacuoles as previously described (Figures 13-16, 18-23). The bands were observed to course in all directions to resemble a "fingerprint" (Figures 16, 18, 19-21). Most of the bands appeared to intersect the vacuole, when present, at right angles to its wall (Figures 16, 19, 21, 22, 24, 25). A few appeared to course along the vacuole wall in a circular manner (Figure 16). These dense bands were ~ 100 Å thick

and were separated by bands of low electron density ~ 50 Å wide. Most bands revealed a repeating pattern of alternate light and dark lamellae at periods of ~ 35 Å. From some of the sections it could be inferred that the bands may be formed from the dense, coarse granular matrix observed in the pigment complex and in the altered lysosomes (Figures 18-20, 24-26).

Closely associated with the bands were dense, homogeneous particles (Figures 15, 16, 18-20, 26), which ranged up to ~ 1/2 μ in diameter. Here again, it may be inferred from the sections that a fusion of bands may result in the formation of these deposits. To further substantiate this observation, it appeared that in the oldest animal studied with the electron microscope (Dog No. M37, 13.1 years old), the pigment bodies consisted mainly of dense, homogeneous particles and only a few bands remained (Figure 15). Also tissue from the older hogs (Figures 18-20, 22) generally exhibited fewer bands and more dense particles than did the tissue from the younger hogs (Figure 23).

Occasionally the "band patterns" would blend into a hexagonal "dot pattern" or crystalloid particle ranging up to ~ 1 μ in diameter and generally located centrally in a pigment body (Figure 18). At the margins of the dot pattern, individual bands appeared to blend into its matrix. The matrix of this pattern generally consisted of numerous dense, small particles ~ 75-100 Å in diameter. They appeared to be aligned in parallel rows separated by a space of less electron

density ~ 50 Å wide. Occasionally rows would intersect at slight angles to give a v-shaped appearance. The dot pattern appeared to result from a vertical or tangential section of the band structures. The dense, homogeneous particles, usually associated with the bands, were not observed in the dot pattern.

The mechanism by which pigment bodies originate, enlarge, increase in complexity, and become membrane-bound is not completely understood, but from the micrographs of different age dogs and hogs, it was possible to hypothesize a likely sequence of events in this transformation.

From the initial normal-appearing lysosome (Figures 14, 15, 26), the matrix appeared to change from the homogeneous granular appearance to a dense, coarse granular form (Figures 17a, 17b). A vacuole appeared in the matrix at about this stage (Figures 17a, 24, 25) and a few typical bands were formed from the coarse granules within the matrix (Figures 16, 21, 24, 25). The process appeared to proceed to a more complex pigment body observed in Figures 16, 21 and 23, with more extensive band formation and larger vacuoles. With age and maturation of the pigment body, the bands fused to form dense, homogeneous particles (Figures 18-20, 22, 26), which in the oldest animal occupied most of the pigment body (Figure 15).

From sections of different age animals, it appears that the enlargement process is one of fusion or coalescence.

In the 1-year-old dog (Figure 14) and the 2-year-old hog (Figure 23) there is evidence of fusion of 1 or 2 altered lysosomes into larger pigment bodies. In the older animals (Figures 15, 16, 18-20) it appears that this process has continued to include several of these lysosomal structures into one pigment body. Even before fusion, the initial lysosomes appeared to enlarge as their substructure changed from the normal homogeneous granular matrix (Figure 15, 26) to a more dense, coarse, granular, vacuolated matrix (Figures 15, 16, 17a, 24, 25). Of course the progressive enlargement of the vacuole and the increased band formation contributed to the enlargement process.

The larger pigment bodies, even after the fusion process, appeared to retain a limiting membrane (Figures 15, 20) similar to the single-unit membrane of the initial lysosome (Figures 15, 17a, 17b). The membrane appeared to bind the pigment complex rather loosely in places (Figure 20). It was often difficult to demonstrate and probably represented the original membrane of the lysosomes.

Figure 12. A dorsal root ganglion cell fixed 25 minutes after death. The mitochondria and their substructure (M), the Golgi apparatus (G), the rough endoplasmic reticulum (RER), lysosomes (L), and lipofuscin pigment bodies (Lf) have all retained their normal fine structural morphology. Lead citrate. X 44,000.

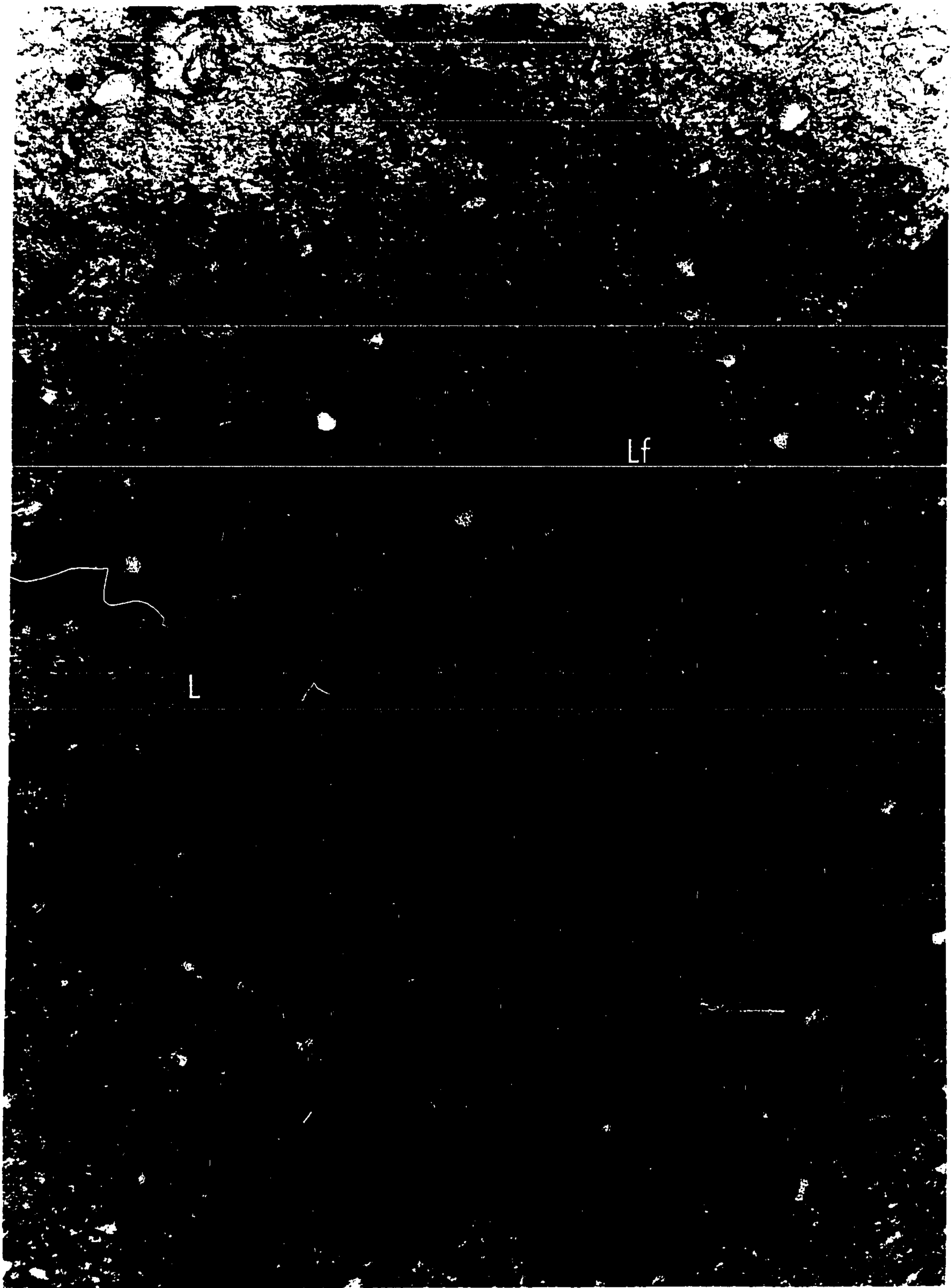

Lf
L

Figure 13. Dorsal root ganglion cell fixed 1 hour after death. The mitochondria (M) have lost their substructure; the nucleus (N) is serrated and the nuclear matrix is more homogeneous than normal; the two layers of the nuclear membrane are indistinct (arrows). The lipofuscin pigment granules (Lf) appear fairly normal with the exception of the band-type substructure which is just slightly visible. The lysosomes (L) and rough endoplasmic reticulum (RER) appear to be intact, however ribosomes appear disassociated from the vesicles. At lower left is part of a satellite cell (SC). Lead citrate. X 33,400.

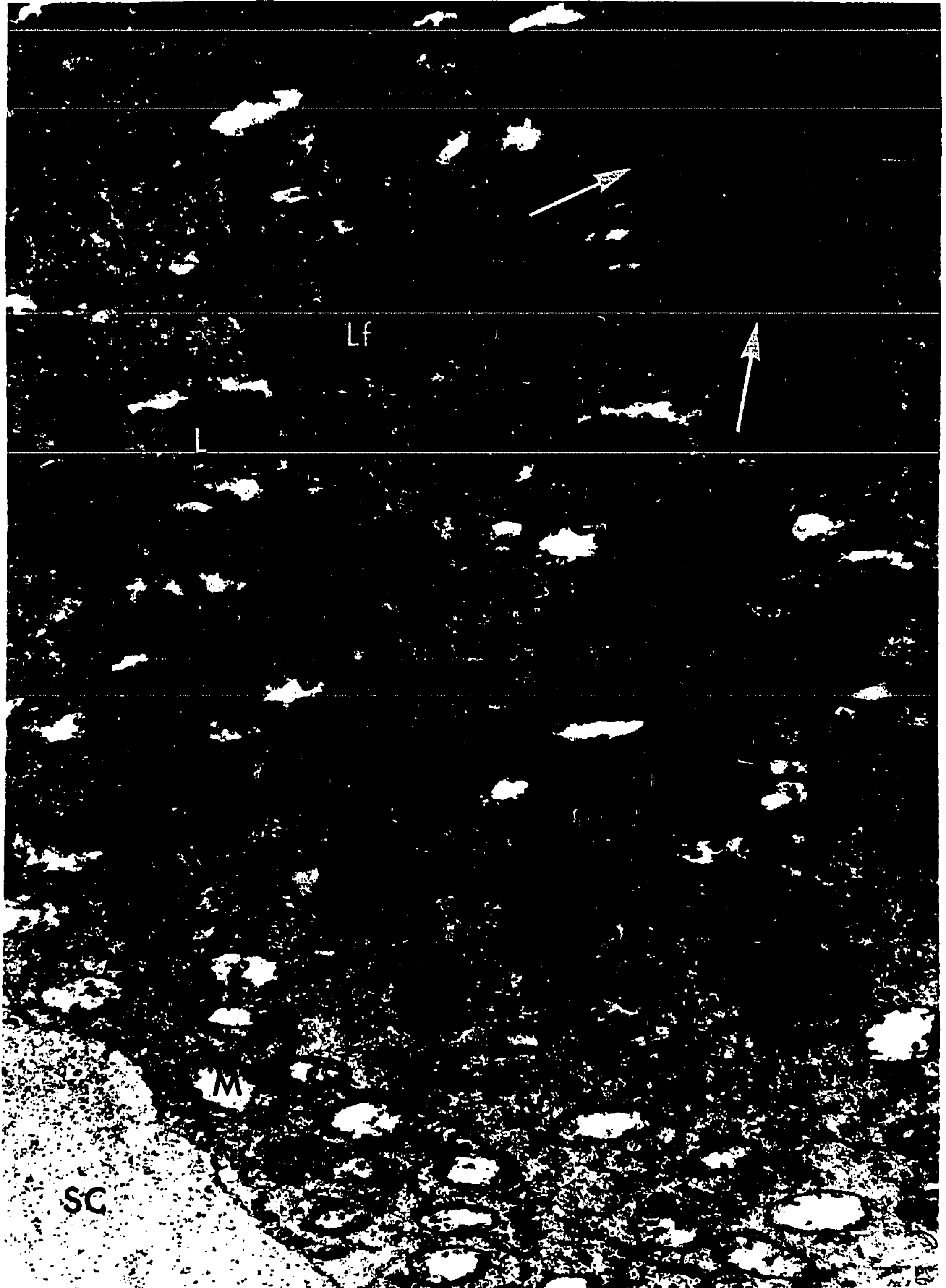

Lf
L
M
SC

Figure 14. Survey micrograph of a dorsal root ganglion cell of a 1-year-old dog (No. C2). Many small and vacuolated electron-opaque lipofuscin granules (Lf) are distributed perinuclearly. The nucleus (N), Golgi apparatus (G), mitochondria (M), and lysosomes (L) are illustrated. Lead citrate. X 22,700.

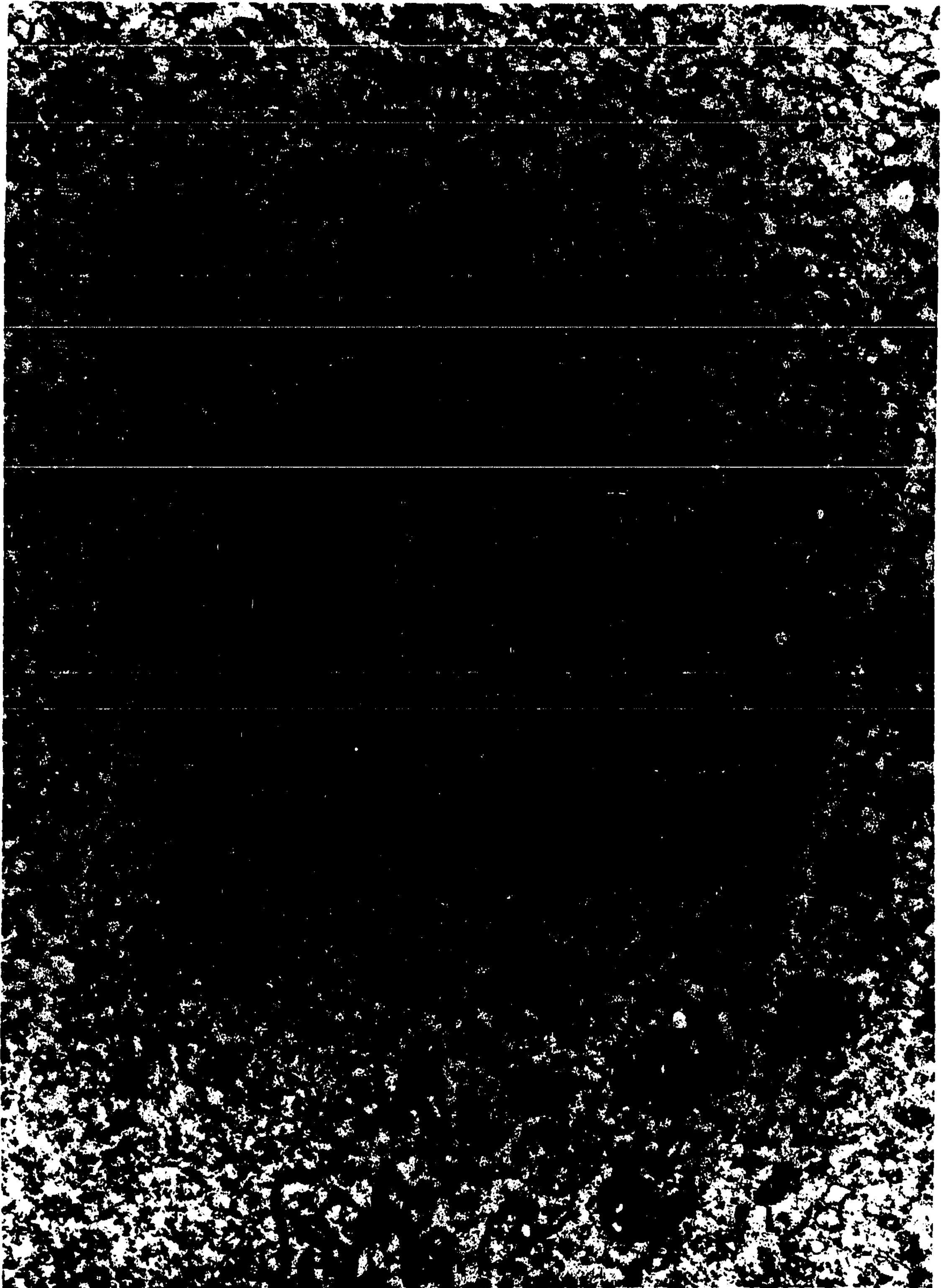


Figure 15. Electron micrograph of a dorsal root ganglion cell of a 13.1-year-old dog (M37) with numerous large, dense lipofuscin pigment bodies (Lf). Large homogeneous, dense particles comprise most of the matrix of these pigment bodies. Each pigment complex is bound by a single unit membrane-like structure (arrows). Lysosomes (L) and mitochondria (M) are illustrated. Lead citrate. X 44,000.

L
Lf
L

Figure 16. Dorsal root ganglion cell of an 8-year-old dog (No. C4) with numerous vacuolated lipofuscin pigment bodies (Lf) with a pronounced banded substructure. Several lysosomes (arrows) appear to be undergoing transition to a more dense, band structure similar to that observed in the larger, more mature pigment bodies. Free ribosomes (R) and Golgi apparatus (G) are illustrated. Lead citrate. X 22,700.

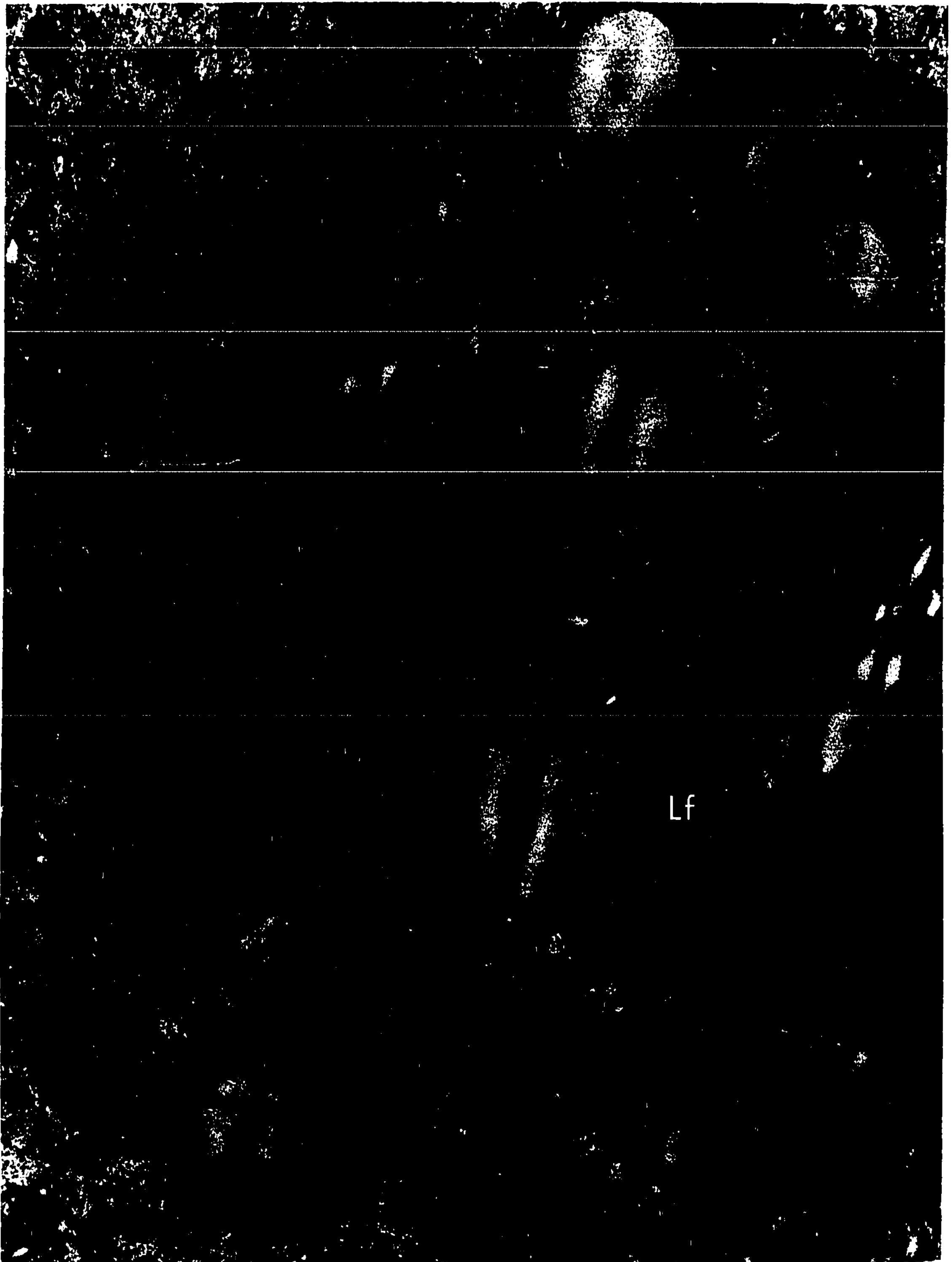
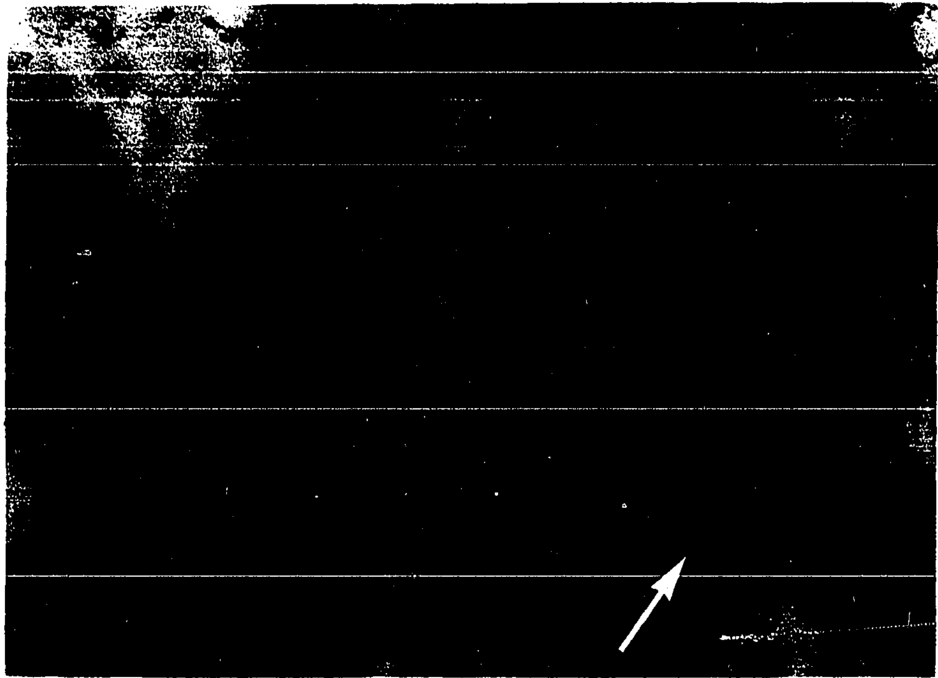

Lf

Figure 17a. Part of the matrix of a single membrane-bound lysosome (dark arrow) is more dense and granular in comparison to the remaining portion. A small vacuole has formed (light arrow). This entire structure probably represents an early pigment form. From an autonomic ganglion of a 5.2-year-old hog (No. 24). Lead citrate. X 68,000.

Figure 17b. Other membrane-bound lysosomes from the same animal and tissue as above are illustrated. The entire matrix of these has changed to a more dense, granular form. Lead citrate. X 68,000.



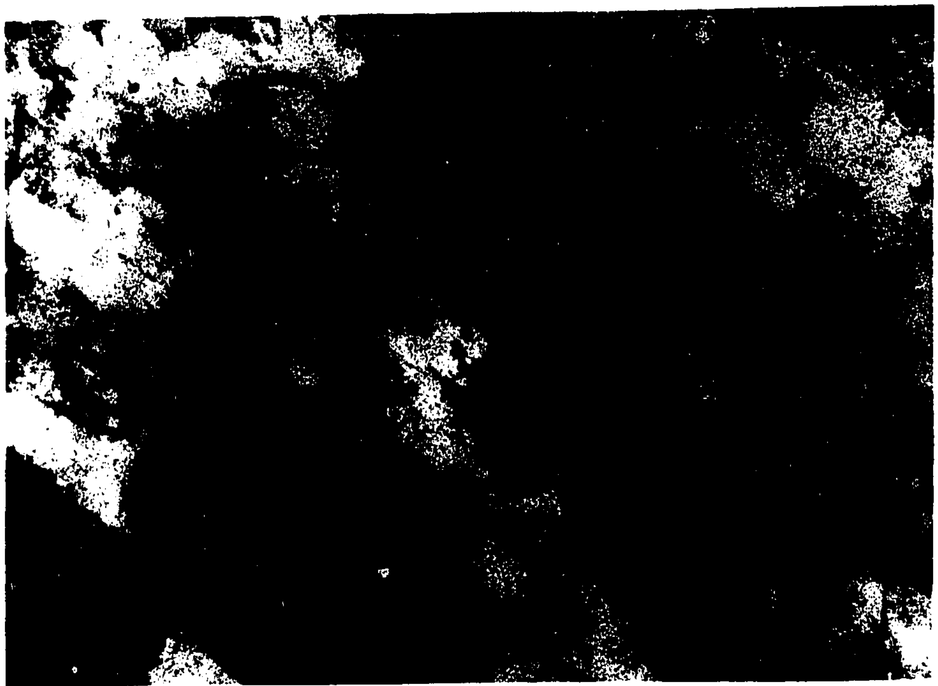


Figure 18. Mature lipofuscin granules in an autonomic ganglion cell of a 6.3-year-old stag hog (No. 19-259). A distinct "band pattern" can be seen. Closely associated with these are very dense, homogeneous particles. The "dot pattern" is also illustrated (arrow and inset). Lead citrate. X 77,500; inset X 152,000.

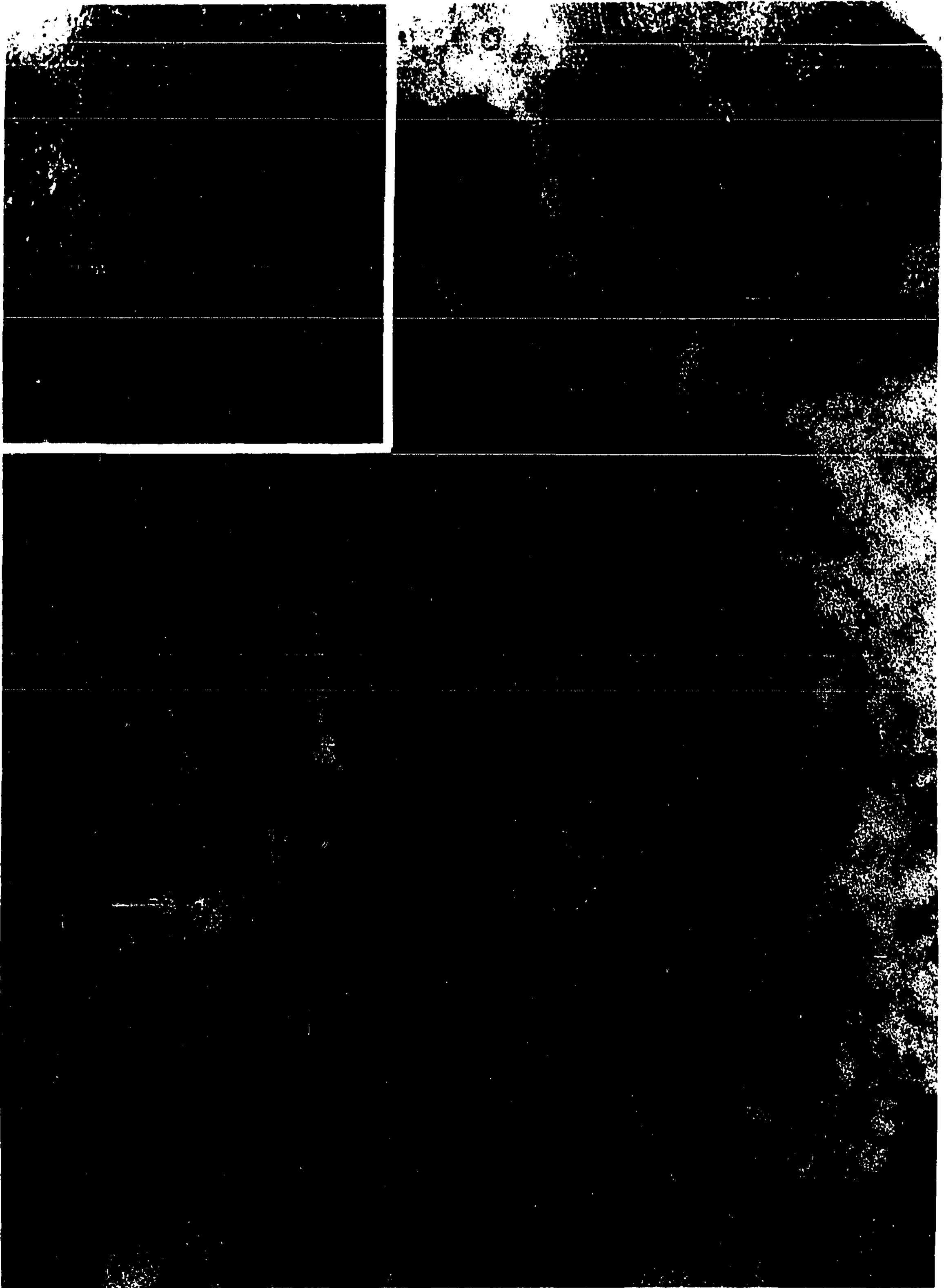


Figure 19. A large lipofuscin pigment body with bands coursing in many directions. Two large vacuoles are illustrated. Tissue is from an autonomic ganglion cell of a 6.3-year-old stag hog (No. 19-259). Lead citrate. X 77,400.

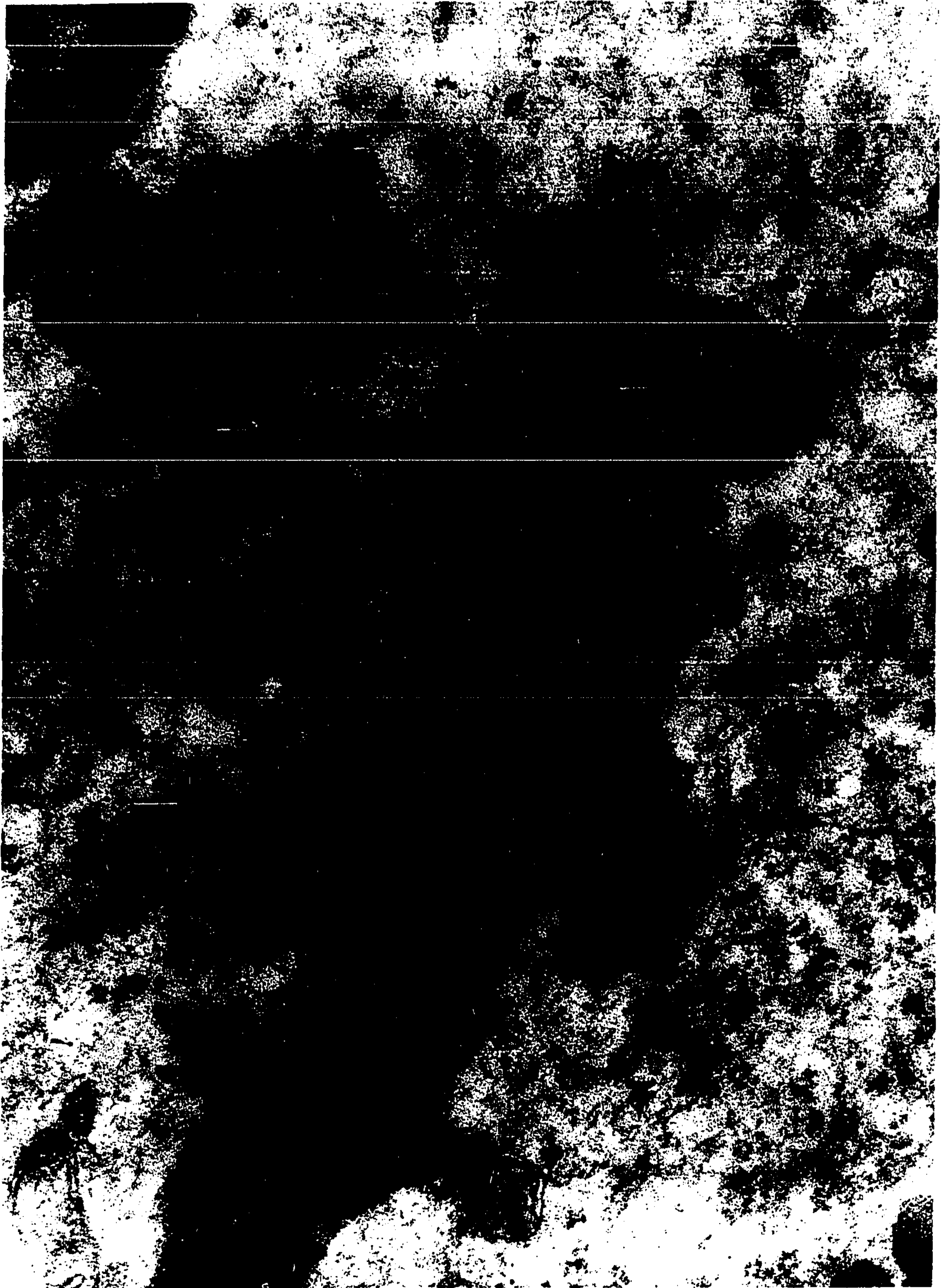


Figure 20. Autonomic ganglion cell from a 6.3-year-old stag hog (No. 19-259). Various shapes and forms of lipofuscin granules, both with and without vacuoles, are demonstrated. Characteristic "band patterns" and dense particles are numerous. The limiting membrane is partly separated from the internal structure of the pigment granules (arrows). Lead citrate. X 68,000.

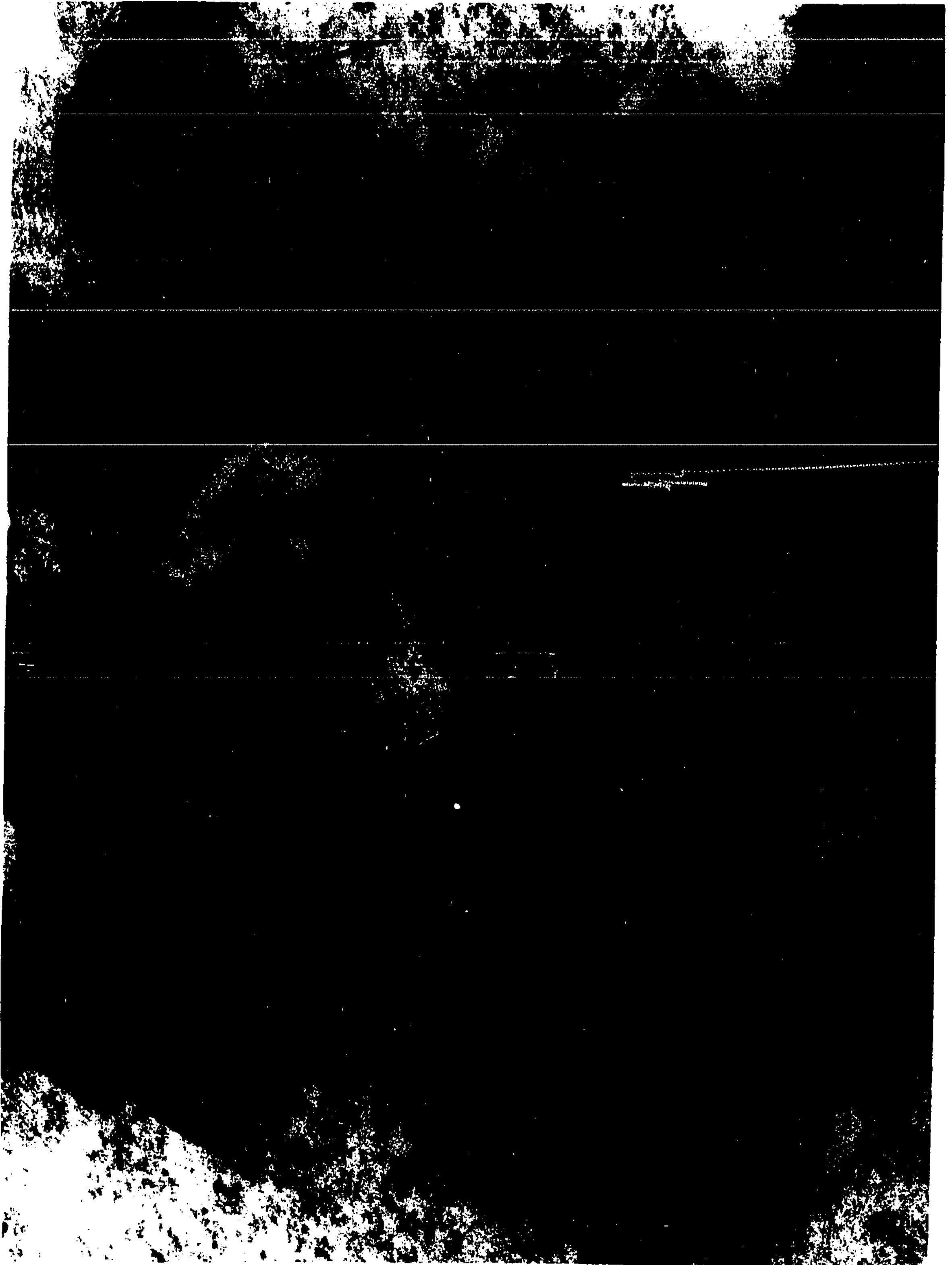


Figure 21. Lipofuscin pigment from an autonomic ganglion cell of a 7.2-year-old sow (No. 26-258). Bands are demonstrated coursing in numerous directions. A small lysosome appears to be developing bands similar to those in the adjacent mature pigment granules (arrow). Lysosomes (L), rough endoplasmic reticulum (RER), and mitochondria (M) are demonstrated. Lead citrate. X 68,000.

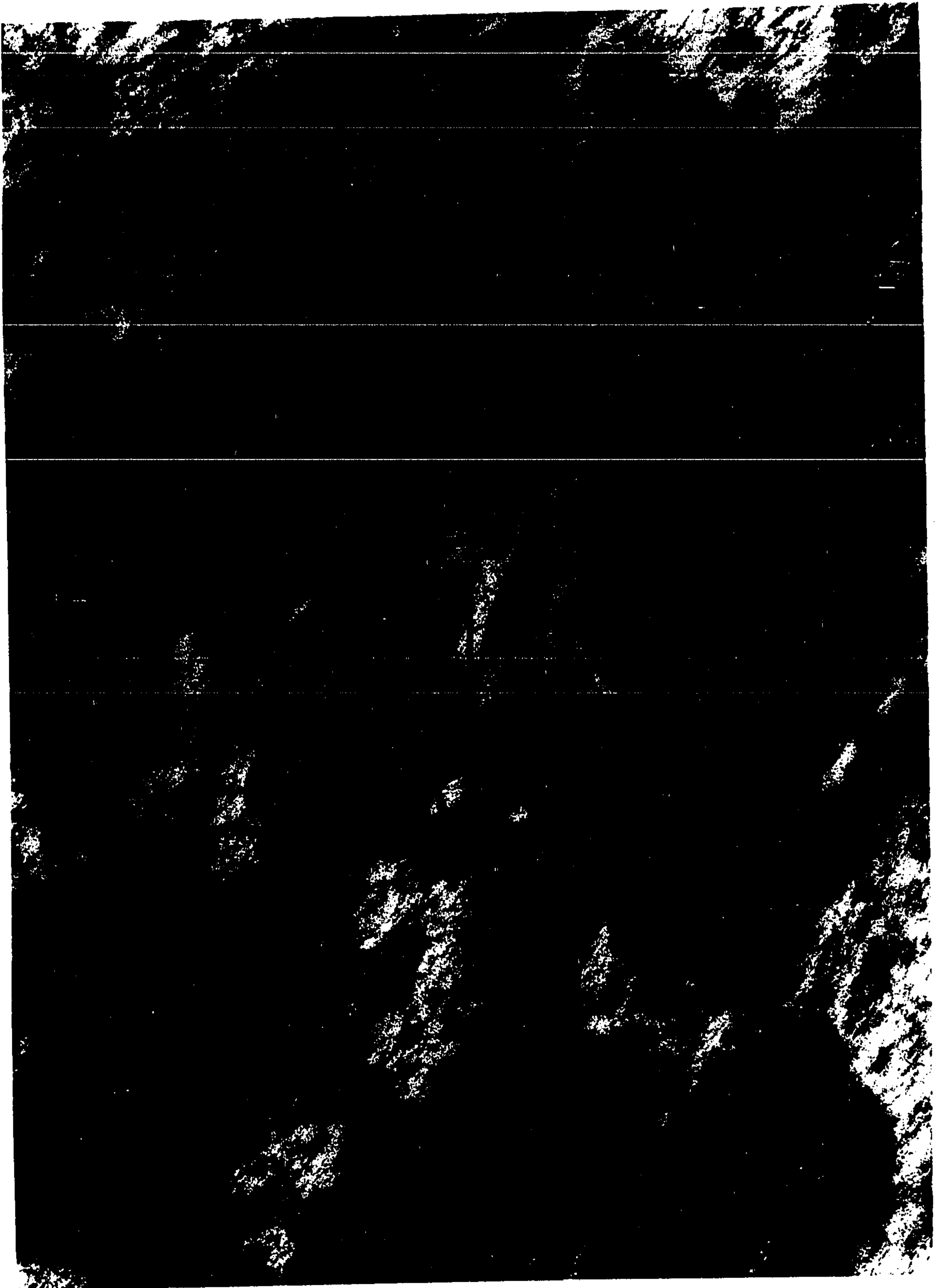


Figure 22. Mature lipofuscin granules in an autonomic ganglion cell from a 7.2-year-old sow (No. 26-258). The vacuoles and band structures with closely associated dense particles are very pronounced. Rough endoplasmic reticulum (RER) and mitochondria (M) are illustrated. Lead citrate. X 77,400.

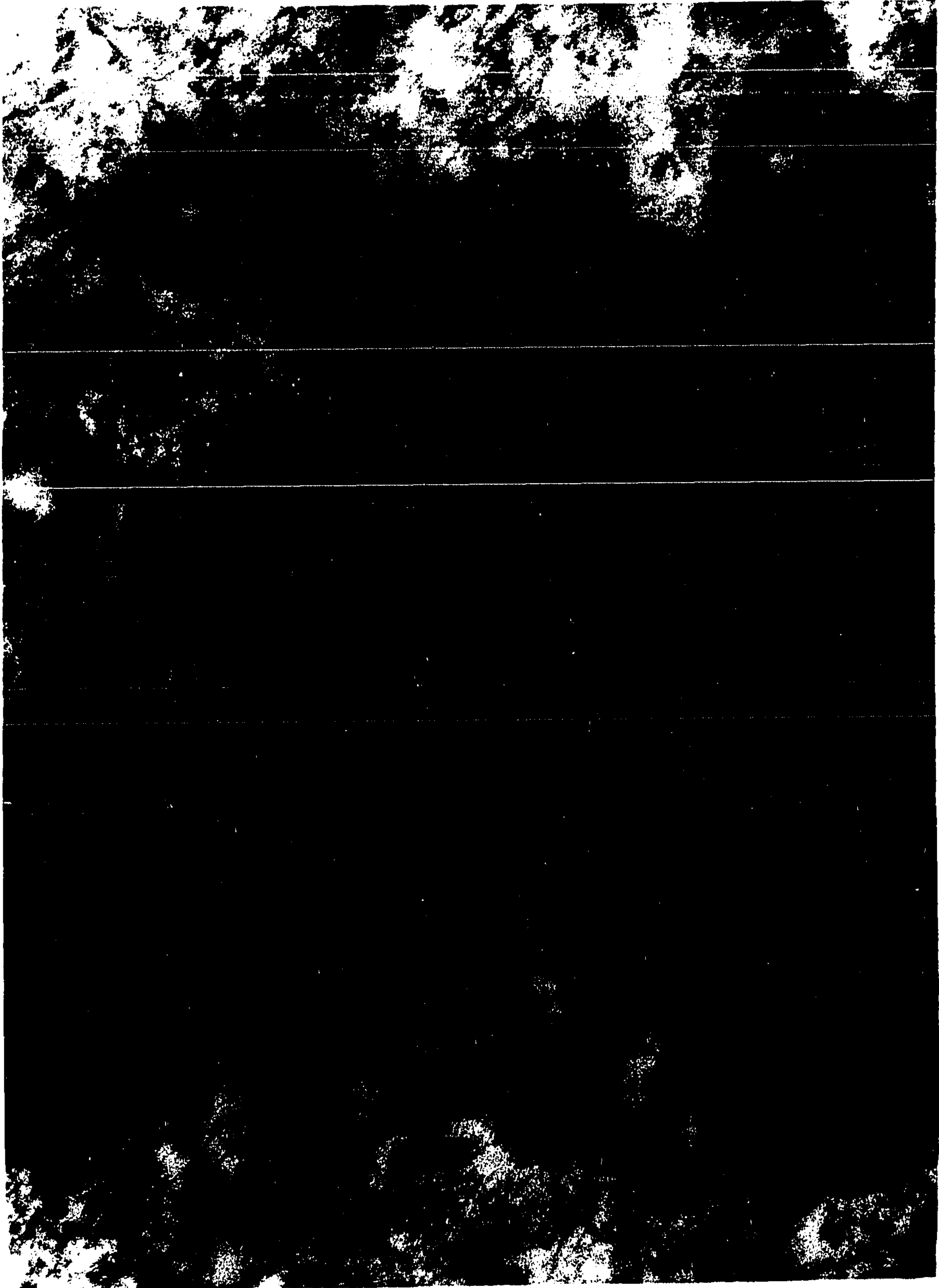


Figure 23. Survey and detail view (inset) of focal accumulation of lipofuscin pigment bodies in a dorsal root ganglion cell of a 2-year-old sow (No. 4512). The typical band patterns and vacuoles are present. The pigment bodies are not as large as in the older animals. Lead citrate. X 77,400; inset X 113,500.

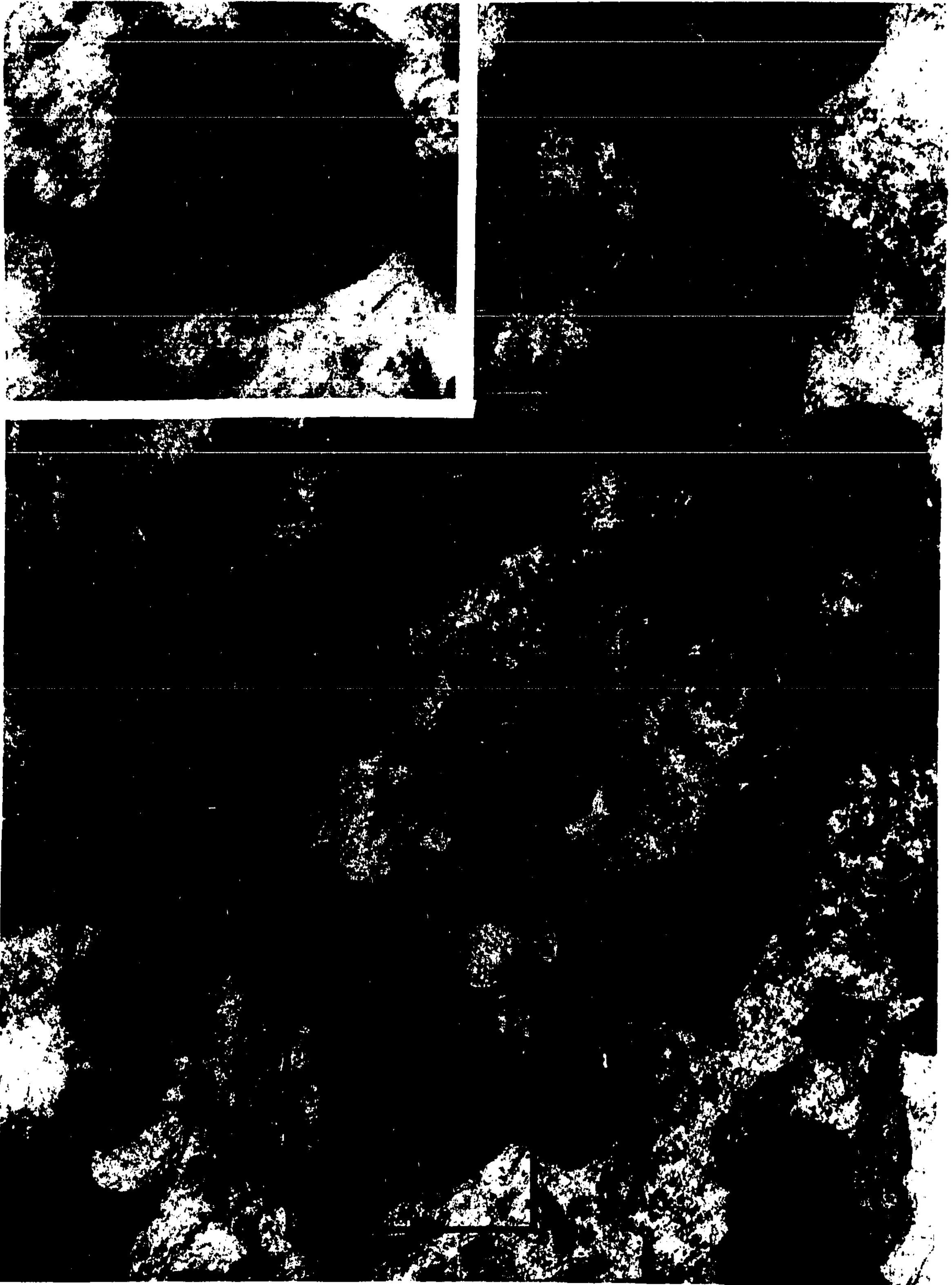
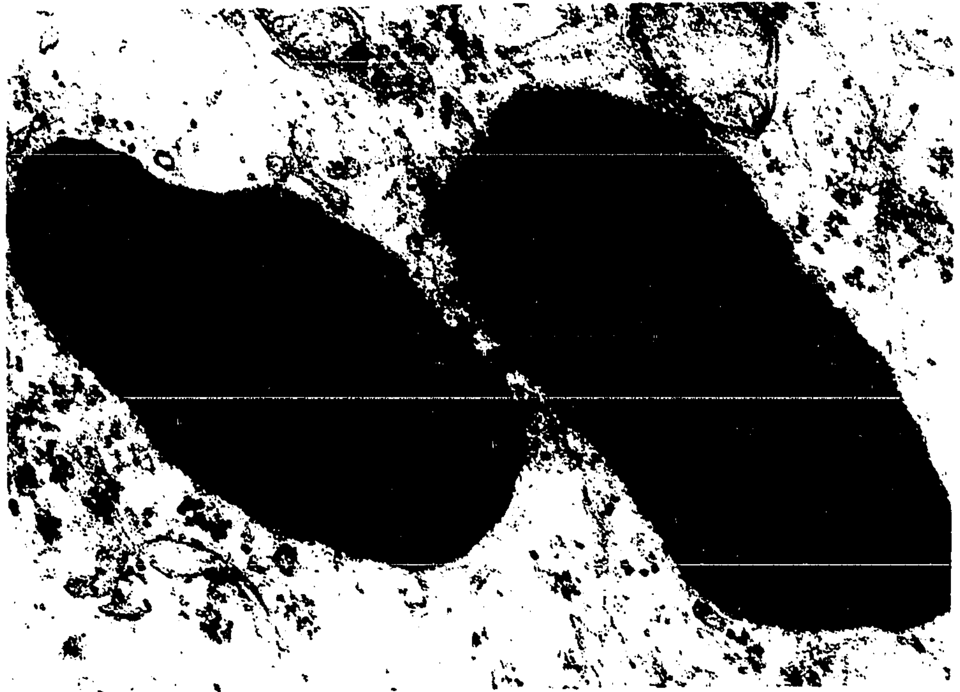


Figure 24. Early form of lipofuscin in a dorsal root ganglion cell from a 2-year-old sow (No. 4512). The matrix of both lysosomes is dense and coarse; a small vacuole and a few bands are forming. Lead citrate. X 113,500.

Figure 25. Pigment from the same tissue and animal as above. Here the bands are more numerous and the matrix is coarser than in the above pigment bodies. Lead citrate. X 113,500.



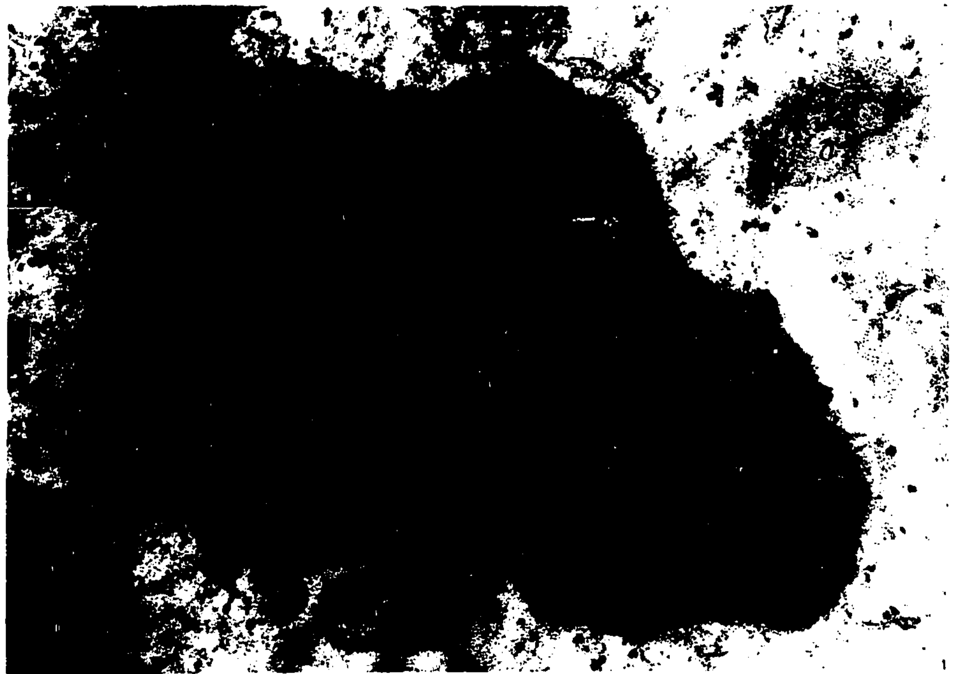
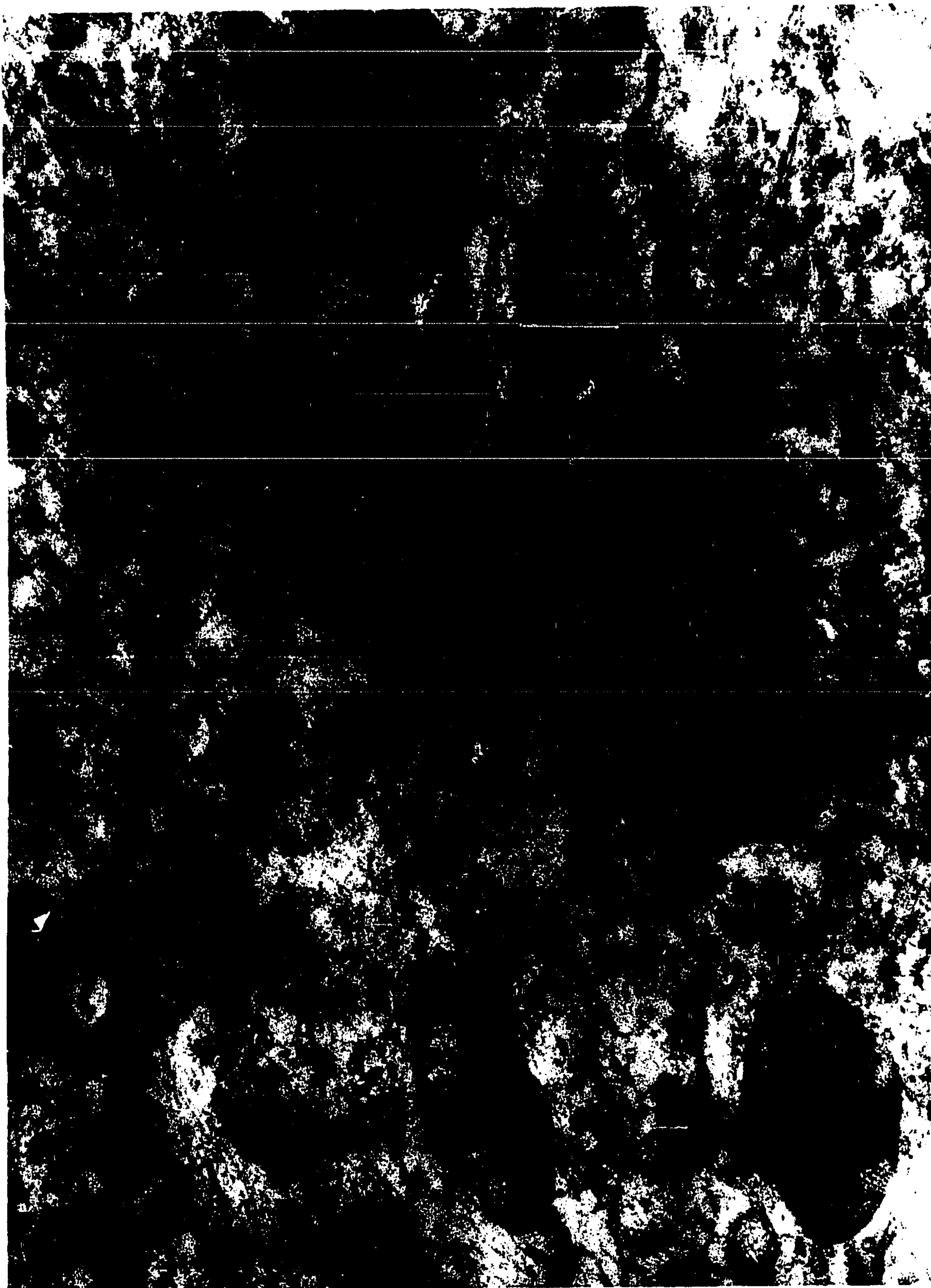


Figure 26. Dorsal root ganglion cell from a 2-year-old sow (No. 4512). The lipofuscin granules exhibit the typical band patterns and in addition the closely associated dense particles not clearly seen in the previous sections from this animal. (Figures 23-25). A lysosome (L), an oblique section of a mitochondrion (M), and rough endoplasmic reticulum (RER) are demonstrated. Lead citrate. X 68,000.



# DISCUSSION

## Occurrence and Distribution of Lipofuscin in the Nervous System of the Dog and the Hog

There are numerous reports in the literature describing the occurrence and distribution of lipofuscin in the nervous system. Most of these have dealt with studies on man, and a few are on dogs. Only one study on the hog brain has been reported (Whiteford, 1964 and Whiteford and Getty, 1966). No reports of studies on lipofuscin or other age changes in the hog spinal cord and its associated ganglia have been found in the literature.

The most extensive studies of lipofuscin in the dog brain, spinal cord, and associated ganglia have been reported by Sulkin (1953, 1955a, 1955b), Whiteford (1964), and Whiteford and Getty (1966). Sulkin (1955a), on the basis of a variety of histochemical procedures, concluded that lipofuscin was widely distributed in the nervous system, but was not found in dogs under 10 years of age. Pigment was demonstrated in every animal over 10 years of age. Whiteford (1964) and Whiteford and Getty (1966) reported lipofuscin, as seen with Nile blue stain, to be widely distributed in the brain of the dog, and the pigment was observed in animals as young as 2 years of age. On cursory examination by fluorescence microscopy, these authors observed pigment in both dogs and hogs at one year of age, and they postulated

that "age change pigments" may be present even at 6 to 8 months of age.

This postulation by the above authors is in accord with the findings of the present study. Lipofuscin, observed with the fluorescence microscope, illuminated with near-ultraviolet light, was observed in small amounts in each of the 3 areas studies at 5 months of age in the dog and 6 months of age in the case of the hog. The observations of Sulkin (1955a) that pigment was not present in his group of animals under 10 years of age, obviously are not in agreement with the findings of the present study, nor do they agree with the findings of Whiteford (1964) and Whiteford and Getty (1966). Even in humans, lipofuscin has been found in the anterior horn cells of children as young as 6 years of age, and moderate amounts were reported in teen-aged individuals (Chu, 1954). In addition, lipochrome pigment was observed in the human spinal ganglia at the 6th year (Pilez, 1895; Obersteiner, 1903).

Regarding the distribution of lipofuscin in various parts of the nervous system in dogs over 10 years of age, Sulkin (1955a) found pigment in 100% of the spinal cord ventral horn cells, 49% of the sensory ganglion cells and 78% of the autonomic ganglion cells. The exact level of the cord at which these determinations were made was not indicated. In the present study the determinations were made at the thora-columbar junction of the spinal cord. In one group of dogs

over 10 years of age (group 6), 93% of the spinal cord ventral horn cells, 91% of the dorsal root ganglion cells (sensory ganglia), and 88% of the autonomic ganglion cells were pigmented. These observations compare favorably with Sulkin's (1955a) findings, except in the case of the sensory ganglia, which had a higher percentage of pigmented cells in the present study. Another significant finding of Sulkin (1955a) was the absence of pigment in the cells of the spinal cord intermediolateral column, the only cell group studied that was free of pigment. In the present study, moderate amounts of pigment were observed in these cells, but the percentage was not determined.

The findings of Sulkin (1955a) led him to conclude that efferent neurons of the somatic type are apparently less resistant to pigmentation than are afferent neurons. This conclusion is substantially in agreement with observations of the present study that the efferent ventral horn cells of the spinal cord were less resistant to pigmentation, whereas the afferent dorsal root ganglion cells exhibited more resistance, and the autonomic cells were the most resistant to pigmentation.

These differences in lipofuscin distribution and accretion among different groups of nerve cells are phenomena that remain to be clarified. The increase of cell size and the division of the nucleus and the nucleoli, which

Andrew (1956) mentions as possible "reactive" or "defensive" phenomena to counteract influences leading to senescence and ultimate destruction of the nerve cell, were not observed in the present study.

Altschul (1943) explained that differences in the distribution of pigment in nerve cells are due to the fact that some cells are characteristically "weak" cells. His explanation was based on the observation of a steady decrease in the accumulation of lipofuscin in motor cells, starting in the spinal cord and continuing to the motor nuclei of the cranial nerves. It appears that further interpretation of these observations is needed for clarification, especially in view of the present studies and those of Sulkin (1955a) and Morrison et al. (1959), which reported that most of the spinal cord ventral horn cells were pigmented in the older specimens. It would appear unlikely that these large motor cells would be classified as "weak" cells.

Sulkin (1955a) described functional differences as a basis for differences in lipofuscin distribution. He concluded that lipofuscin granules were more concentrated in efferent nerve cells than afferent nerve cells. This explanation has received some support from Whiteford and Getty (1966), who concluded that the difference in time of pigment deposition in the various cranial nuclei of the dog and hog may be related to the level of function of the nuclear

group concerned. Certainly, on the basis of findings in the present study, the functional activities of the different cell groups must play a part in the distribution differences.

However, the findings of the present study would also indicate that the factor responsible for differences in lipofuscin distribution is progressively diminished as the animals increase in age. Or simply, the gradation of resistance to pigmentation, as exhibited by some cell groups, is progressively lost with increasing age. This "resistance factor", responsible for delayed pigmentation, could be the blood supply to the different cell groups. This possibility has also been suggested by Andrew (1956) and Sulkin (1958). The observations in the present study that a significant decrease in blood vessels of the spinal cord was noticed as the animals increased in age would tend to account, at least in part, for the large amounts of lipofuscin in the spinal cord ventral horn cells. Similar vascular changes in the ganglia probably occurred but were more difficult to detect without additional special stains. The condition of the vascular system in these same tissues remains a subject for later investigation.

In regard to intracellular distribution of lipofuscin, numerous patterns, including polar, bipolar, perinuclear, diffuse, and peripheral, have been observed both in the present study and others. These different patterns are, according to Vogt and Vogt (1946), Höpker (1951), and

Wahren (1957), specific characteristics of different nuclei or cell groups. However, Whiteford (1964) and Whiteford and Getty (1966) considered the intracellular patterns of distribution to be a function of age. Observations of the present study are substantially in accord with both of the above findings, but the significance of these various distributions, if any, remains to be further clarified in view of the fact that the different patterns described could usually be seen in each of the 3 different cell groups, though one type seemed to predominate for each group.

The fact that both melanin and lipochrome pigments have been described in sympathetic ganglion cells (Larsell, 1951) and in dorsal root ganglia (Wolf and Pappenheimer, 1945) was of major concern in the present study. The Nile blue stain technique by Lillie (1956a) is described as being adequate for the differentiation of melanin and lipofuscin. To observe the staining reaction of both pigments in the same section, sections from the substantia nigra area of a human brain stem were stained with Nile blue. The melanin pigments stained dark green as described by Lillie (1956a), and the lipofuscin stained blue. On subsequent examination of alternate human brain stem sections with the fluorescence microscope, illuminated with near-ultraviolet light, the lipofuscins exhibited a strong yellow-orange autofluorescence, whereas melanin did not exhibit fluorescence. Therefore, on the basis of observations of sections with Nile blue stain,

no melanotic pigments were seen in the cells studied.

Finally, the occurrence of lipofuscin was the most consistent age-associated change observed in the cells studied. Other less consistent alterations included atrophy, especially of the ventral horn cells. The most prominent non-cellular change that appeared to be associated with age was a decrease in the number of blood vessels in the spinal cord.

## Fine Structural Morphology of Lipofuscin

There are a few reports in the literature describing the ultrastructural characteristics of the lipofuscin granule. One of the first reports was by Beams *et al*. (1952). These pigment granules, found in the spinal ganglia, were reported to be dense spherical bodies concentrated into one region of the cell. Hess (1955) further described the pigment granules as having a number of vesicles of low electron density surrounded by a lacework of dense material. Later Samorajski *et al*. (1964, 1965) made detailed studies on the fine structure of lipofuscin in neurons and described the pigments as being clusters of high density and complex ultrastructure consisting of myelin-like figures arranged in several configurations within a single body. The myelin figures or bands were reported to be ~ 75 Å wide. Some bands also had a repeated pattern of light and dark lamellae alternating at periods of ~ 70 Å. Numerous electron-opaque particles were

associated with the bands. The pigment complex was found by a single limiting membrane.

The fine structural features of lipofuscin described by the above authors are almost identical to those observed in the present study. The dense bands observed in the present study were ~ 100 Å thick and separated by bands of low electron density ~ 50 Å wide. A repeated pattern of alternate light and dark lamellae was observed at periods of ~ 35 Å. The small differences in these measurements and those reported by Samorajski et al. (1965) could be attributed to different methods of measurement.

It is interesting to note that lipofuscin-like granules reported in the vascular smooth muscle and juxtaglomerular cells of human kidneys (Biava and West, 1965) have almost identical fine structural characteristics to those observed for lipofuscin in this study and those described in the previous studies by Samorajski et al. (1964, 1965).

According to Biava and West (1965), the dense bands resembled fingerprints; each of the bands was 60 to 80 Å thick, and they were separated by bands of low electron density 30 Å wide. The periodicity of the alternating dense and light bands ranged from 90 to 110 Å. These dimensions conform very closely to those described for lipofuscin in the present study and in a previous study by Samorajski et al. (1965).

The significance and origin of the peculiar band patterns and dot patterns observed in previous studies (Samorajski _et_ _al_., 1964, 1965; Biava and West, 1965) and in the present study are not completely understood. From physical considerations it has been suggested that the band patterns resulted from variable degrees of superimposition and fusion of the images of individual particles, and that the alternating light bands resulted from the apparent fusion of electron lucent spaces within hexagonal complexes of individual particles (Biava and West, 1965).

According to Samorajski _et_ _al_. (1965), the formation of complex bands with repeating light and dark striations and hexagonal arrays of crystalline structures may represent a particular stage in the transformation of phospholipids.

On the basis of structural comparisons, the lipofuscin bands of the present study compare favorably in thickness to lipoprotein layers described by Finean (1961). According to Finean, a lipid bimolecular leaflet would be 40 to 65 Å in thickness and each protein layer 10 Å thick, thus providing a lipoprotein layer which would be expected, in the dehydrated state, to range from 50 to 100 Å in thickness. The different configurations of the bands may be attributed to a possibly helical nature of the protein components.

According to Biava and West (1965), the "dot patterns" resulted from crystalloids cut perpendicular to their axes

and viewed in cross or nearly cross sections. In the present study, the dot pattern appeared to result from a vertical or tangential section of the band structures. A similar observation was made by Samorajski *et al*. (1965).

In the present study, dense, homogeneous particles were closely associated with the bands. These appeared to result from the fusion of one or more bands. Such deterioration of the bands may result from a breakdown of hydrogen bonds which, according to Finean (1961), maintain the helical configurations of proteins. Similar dense particles observed by Biava and West (1965) were described as probably consisting of lipids, proteins and possibly carbohydrates.

The significance of the peripherally situated vacuole, an integral component of many pigment complexes, is not clearly understood. It appeared to form early, as the matrix of the lysosome became coarse and more dense. It tended to increase in size as the pigment body increased in size and complexity, and probably represented lipid droplets that were mostly extracted by etnanol during the dehydration process. The thin, dense wall outlining the vacuole was probably due to precipitation of osmium or lead at the interface between the lipid droplets and surrounding structures. Similar conclusions regarding the nature of these vacuoles were made by Biava and West (1965).

Occasionally a single-unit membrane binding the pigment

complex could be demonstrated. It was usually very distinct and resembled the membrane of normal lysosomes. This membrane could possibly result from a fusion of the original lysosome membrane when one or more lysosomes coalesced, resulting in larger pigment complexes.

In terms of size, the pigment bodies in the autonomic ganglia of the hogs were slightly larger, on the average, than pigment in other areas examined. Other intraspecies and interspecies comparisons in regard to electron density, shape and internal substructure revealed no major differences. Similar conclusions were made by Samorajski et al. (1965) in regard to pigment bodies of the motor and sensory neurons of senile mice.

## Origin of Lipofuscin

A review of the literature indicated that conclusive findings regarding the origin of lipofuscin had not previously been established. The early observations of Dolley (1917) that pigment is derived from nuclear substances has long been discounted and is only of historical interest now. The implication of cytoplasmic ground substance (Matzdorff, 1948), and the concentration or flocculation of plasma colloids (Sjövall, 1932 and Wunscher, 1957) do not appear to be major contributing factors.

The views presented by Gatenby and Moussa (1950, 1951) and Gatenby (1953) that pigment arises from a transformation

of broken-down pieces of Golgi apparatus has received some support. Bondareff (1957), on the basis of electron microscope studies, also concluded that alterations in the Golgi apparatus provide the mechanism for pigment genesis. Other workers (Hess, 1955, Duncan et al., 1960) have, on the basis of electron microscope observations, concluded that old-age pigment arises as a transformation of mitochondria.

In the present study, however, the pigment observed appeared to be more closely identified with lysosomes. This, of course, does not preclude the incorporation of membrane systems or parts of them, or plasma colloids and other cytoplasmic ground substances. In performing the "autophagic" functions of the cell, many different substances may be incorporated into the matrix of lysosomes. On a purely analytical basis, many chemically diverse substances have been isolated in the lipofuscin complex (Heidenreich and Siebert, 1955).

From observations in the present study of electron micrographs, it was possible to hypothesize the following sequence of events in the formation of lipofuscin from lysosomes. (1) The matrix of lysosomes increased in density and became coarse and granular; (2) a small lipid droplet, represented by a vacuole, formed at the periphery of the lysosome; (3) the dense, coarse matrix gave rise to lipoprotein leaflets represented by dense bands; (4) with

increase in age the vacuole increased in size and the bands became more extensive; and (5) the bands fused to form dense, homogeneous particles which were the predominant component of the pigment body in the very old animals.

Other cytochemical and ultrastructural studies have identified lysosomes with the lipofuscin complex. de Duve (1959) was one of the first to suggest that entire lysosomes may be transformed into a pigment granule, and Strehler (1962) suggested that lipofuscin may result from the accumulation and autoxidation of lipid components of lysosomes. Essner and Novikoff (1960) and Barka and Anderson (1962) considered lipofuscin pigments to be altered lysosomes. Samorajski _et al_. (1964) concluded that their findings did not establish definitively whether lipofuscin granules were initially deposited, formed, or subsequently accumulated within lysosomes with increasing age.

Samorajski _et al_. (1964) described two types of fluorescent cytoplasmic organelles dispersed in the neurons and non-neuronal cells of the spinal cord and ganglia when illuminated by near-ultraviolet light. On the basis of size, number, and distribution, one group of autofluorescent particles was identified as lysosomes. The other fluorescent granules were larger, appeared clustered in the periphery of the cell, and were visible as a very distinctive orange-yellow fluorescence. In the present study two different autofluorescent particles were also observed. In young

animals (both dogs and hogs 4 to 12 months of age) numerous small, weak-orange autofluorescent particles were dispersed in the cytoplasm. These were barely visible with the fluorescence microscope. Since these were not observed in the young animals mostly under 4 months of age, they were interpreted as lysosomes, probably just beginning to transform into pigment. The other granules were large (up to 3.5 μ in diameter), exhibited a strong yellow-orange autofluorescence, were often in clusters at one or both poles of the cell or at the periphery, and were seen in all of the older animals. These were interpreted as mature lipofuscin granules.

The observation that lipofuscin appears first near the nucleus has been reported previously. Höpker (1951) concluded that lipofuscin formation begins in the so-called lipophilic center of the cell, generally located near the nucleus. Gatenby and Moussa (1950, 1951) and Gatenby (1953) observed that lipofuscin first forms near the nucleus as a result of transformation of broken-down pieces of Golgi apparatus. Observations in the present study are in accord with these findings concerning the location of the first pigment granules formed, but do not agree with the conclusions of these authors regarding the origin of lipofuscin.

## Significance of Lipofuscin

The opinions on the significance of lipofuscin accumulations in non-replaceable cell lines are numerous and often conflicting. As early as 1911, Dolley concluded that pigment in nerve cells was a product of depression rather than a result of normal or hypernormal activity. Later workers (Bloom and Fawcett, 1962, Ranson and Clark, 1959) referred to the pigment as a product of normal activity that did not interfere in any way with the normal function of the cell.

Other studies have focused attention on the size of pigment accumulations which in the present study and in others (Morrison *et al.*, 1959) often were observed to occupy as much as 85% of the cell volume. In this respect, Sulkin (1953) reasoned that cells heavily laden with pigments are deficient in chromidial substance, are usually shrunken and vacuolated, are often undergoing degeneration, and some are probably no longer functional. In the present study, numerous cells heavily laden with pigment granules were frequently observed. Some of these cells had lost their nuclei and were obviously dead and degenerating. Whether they died because of the large accumulations of lipofuscin or whether death was due to undetected factors could not be determined, but accumulations of lipofuscin did appear to be an obvious possibility. Likewise, Whiteford (1964) concluded that size and density of lipofuscin deposits would likely

reflect or cause some impaired functions. In addition, observations of living cells by Murray and Stout (1947) led them to conclude that a pigment mass could interfere with the plasticity of cells and thereby be detrimental to their normal function.

The observation in the present study that lipofuscin results from altered or transformed lysosomes, and that normal lysosomes are observed less frequently in old animals with large pigment accumulations, would indicate an eventual decrease in normal lysosomal activity which would probably have an adverse effect on the functional activity of the cell. This possibility has also been suggested by Samorajski et al. (1964). Also the possibility, according to Altschul (1943), that lipofuscin pigment in nerve cells indicates a difficulty in eliminating waste products of normal metabolism appears very likely in view of a lysosomal role in pigment genesis.

Therefore, observations made in the present study cannot support the opposing views of Bloom and Fawcett (1962) and Ranson and Clark (1959) that pigment accumulations do not interfere in any way with the normal functions of the cell. The extent to which normal function of the cells or organ is altered remains to be clarified.

Finally, an obvious conclusion regarding the significance of lipofuscin is that, in normal animals, free of disease and abnormal environmental influences, its deposition is a basic biological aging process.

# SUMMARY AND CONCLUSIONS

An investigation was conducted to determine the occurrence of lipofuscin pigment as related to aging in the lumbar spinal cord, dorsal root ganglia and paravertebral (autonomic) ganglia of the dog and hog.

Specimens from 57 dogs ranging in age from 7 days to 16 years (median age - 16.2 mo.) and 79 hogs ranging in age from 2 days to 7.2 years (median age - 2 years) were studied. The animals were from an environment common to the species, and birth records, feed records and the complete past history of the animals were available.

The animals were killed in the laboratory by electrocution. The specimens were removed as quickly as possible and fixed in 10% buffered neutral formalin. Tissues of the dogs for electron microscopy were collected prior to death by surgical means; tissues from the hogs were collected shortly after death. The tissues, with occasional exceptions, were fixed in 6.25% glutaraldehyde in Millonig's buffer at pH 7.4 and "post-fixed" in 1% osmium tetroxide.

Lipofuscin pigment bodies in both dog and hog tissues appeared as dark blue granules, either dispersed or in clusters, in sections stained by Nile blue stain. The granules were PAS-positive and inconstantly exhibited varying degrees of acid-fastness. By these methods, lipofuscin was frequently observed in animals as young as 1 year of age.

Lipofuscin observed with the fluorescence microscope in unstained sections exhibited a strong yellow-orange autofluorescence. By this technique, pigment was found in each of the 3 areas studied at 5 months of age in the case of the dogs and at 6 months of age in the hogs.

The motor ventral horn cells of the spinal cord were the least resistant to pigmentation, and the sensory dorsal root cells were slightly more resistant; the autonomic cells were the most resistant to pigmentation. These differences were progressively reduced with increase in age and generally appeared to be a function of the cell type, the blood supply to that area, and the age of the animal.

The amount of lipofuscin within the cells and the percentage of cells containing lipofuscin increased progressively with age. In many of the older specimens, as much as 85% of the volume of some cells was occupied with lipofuscin, and as many as 100% of the cells were pigmented in some specimens.

The intracellular distribution of lipofuscin generally varied with age and the cell type. In animals 1 year old and younger, the pigment bodies were most often located in a diffuse pattern near the nucleus. In older animals, pigment was most often observed as a single mass near the axon hillock in the case of the ventral horn cells. In the dorsal root ganglia, pigment was most often located at the periphery of the cells, often forming a complete circle; in the autonomic cells, pigment was most often observed as a single mass at

one or both poles of the nucleus.

Lipofuscin pigment observed with the electron microscope in both the dog and hog appeared as variably shaped dense bodies ranging up to 3.5 μ in diameter. The matrix was composed of dense, coarse granules, numerous dense band structures oriented in all directions, dense homogeneous particles, and peripherally situated vacuoles. The dense bands were interpreted to be lipoprotein leaflets, and the dense homogeneous particles appeared to form from the bands, probably as a result of broken hydrogen bonds which bound the helical protein chains. The vacuoles were interpreted to be lipid droplets altered by ethanol in the dehydration process. The internal structure of the pigment bodies usually increased in complexity with age. The distribution of pigment bodies in the cytoplasm was similar to that observed with the light and fluorescence microscopes.

From observations of electron micrographs of animals of different ages, it appeared reasonable to conclude that lipofuscin is of lysosomal origin. The following sequence of events was hypothesized as occurring in the transformation of lysosomes to lipofuscin: (1) the matrix of lysosomes increased in density and became coarse and granular; (2) a small lipid droplet, represented by a vacuole, formed at the periphery of the lysosome; (3) the dense, coarse matrix gave rise to lipoprotein leaflets represented by dense bands; (4) with increase in age the vacuole increased in size and the bands

became more extensive; and (5) the bands fused to form dense homogeneous particles which were the predominant component of the pigment body in the very old animals.

The occurrence of lipofuscin was the most consistent age-associated change observed in the cells studied. The most consistent non-cellular change that appeared to be associated with age was a decrease in the number of blood vessels, especially in the spinal cord. Because of its early appearance in the life of healthy, unstressed animals and the progressive accumulation with age in all animals studied, lipofuscin accumulation was considered to be a basic aging process.

BIBLIOGRAPHY


Altschul, R. 1943. Lipofuscin distribution in the basal ganglia. J. Comp. Neurol. 78: 45-55.

Andrew, W. 1938. Purkinje cell in man from birth to senility. Zeitsch. f. Zellforsch. u. mikr. Anat. 28: 292-304.

Andrew, W. 1939. The Golgi apparatus in the nerve cells of the mouse from youth to senility. Am. J. Anat. 64: 351-375.

Andrew, W. 1941. Cytological changes in senility in the trigeminal ganglion, spinal cord, and brain of the mouse. J. Anat. (London) 75: 406-418.

Andrew, W. 1952. Cellular changes with age. Charles C. Thomas, Springfield, Illinois.

Andrew, W. 1956. Structural alterations with aging in the nervous system. Association for Research in Nervous and Mental Disease Proceedings 35: 129-170.

Bailey, A. A. 1953. Changes with age in the spinal cord. Am. Med. Assoc. Arch. Neurol. and Psychiat. 70: 299-309.

Barka, T. and Anderson, P. J. 1962. Histochemical methods for acid phosphatase using hexazonium pararosanilin as coupler. J. Histochem. Cytochem. 10: 741-753.

Beams, H. W., van Breemen, V. L., Newfang, D. M., and Evans, T. C. 1952. A correlated study on spinal ganglion cells and associated nerve fibers with the light and electron microscopes. J. Comp. Neurol. 96: 249-281.

Biava, C. and West, M. 1965. Lipofuscin-like granules in vascular smooth muscle and juxtaglomerular cells of human kidneys. Am. J. Path. 47: 287-313.

Birrens, James E. 1959. Handbook of aging and the individual. University of Chicago Press, Chicago, Illinois.

Bloom, William and Fawcett, Don W. 1962. Textbook of histology. 8th ed. W. B. Saunders Co., Philadelphia, Pennsylvania.

Bondareff, W. 1957. Genesis of intracellular pigment in the spinal ganglia of senile rats: an electron microscope study. J. Gerontol. 12: 364-369.

Bondareff, W. 1959. Morphology of the aging nervous system. In Birrens, J. E. Handbook of aging and the individual. pp. 136-172. University of Chicago Press, Chicago, Illinois.

Bondareff, W. 1964. Histophysiology of the aging nervous system. In Strehler, B. L. Advances in gerontological research. pp. 1-22. Academic Press, Inc., New York, New York.

Bourne, G. H. 1960. Structural changes in aging. Am. Assoc. Advancement Sci. Publ. 65: 123-136.

Bourne, G. H., ed. 1961. Structural aspects of aging. Hafner Publishing Co., New York, New York.

Brody, H. 1955. Organization of the cerebral cortex. Part III. Study of aging in the human cerebral cortex. J. Comp. Neurol. 102: 511-556.

Brown, James O. 1943. Nuclear pattern of the non-tectal portion of the midbrain and isthmus in the dog and cat. J. Comp. Neurol. 78: 365-405.

Brown, James O. 1944. Pigmentation of certain mesencephalic trigeminal nuclei in the dog and cat. J. Comp. Neurol. 81: 249-253.

Buttlar-Brentano, Karin von. 1954. Zur Lebensgeschichte des Nucleus basalis, tuberomammillaris, supraopticus und paraventricularis unter normalen and pathogenen Bedingungen. J. Hirnforsch. 1: 337-419. Original not available; cited by Birrens, J. 1959. Handbook of aging and the individual. p. 149. University of Chicago Press, Chicago, Illinois.

Caulfield, J. B. 1957. Effects of varying the vehicle for $OsO_4$ in tissue fixation. J. Biophys. and Biochem. Cytol. 3: 827-829.

Chu, L. W. 1954. A cytological study of anterior horn cells isolated from human spinal cord. J. Comp. Neurol. 100: 381-399.

de Duve, C. 1959. Lysosomes: a new group of cytoplasmic particles. In Subcellular particles. pp. 128-159. Am. Physiol. Soc., Washington, D.C.

Dolley, D. H. 1911. Studies on the recuperation of nerve cells after functional activity from youth to senility. J. Med. Res. 24: 309-343.

Dolley, D. H. 1917. The recovery from depression in the Purkinje cell and the decline to senility of depression: with the incidental histogenesis of abnormal pigmentation. J. Comp. Neurol. 28: 465-494.

Dolley, D. H. and Guthrie, F. V. 1918. Pigmentation of nerve cells. J. Med. Res. 39: 123-142.

Duncan, D., Nall, D., and Morales, R. 1960. Observations on the fine structure of old age pigment. J. Gerontol. 15: 366-372.

Ellis, M. S. 1920. Normal for some structural changes in the human cerebellum. J. Comp. Neurol. 32: 1-33.

Erlandson, R. A. 1964. A new maraglas, D.E.R.[R] 732, embedment for electron microscopy. J. Cell Biol. 22: 704-709.

Essner, E. and Novikoff, A. B. 1960. Human hepatocellular pigments and lysosomes. J. Ultrastruct. Research 3: 374-391.

Finean, J. B. 1961. Chemical ultrastructure in living tissue. Charles C. Thomas Co., Springfield, Illinois.

Gardner, F. 1940. Decrease in human neurons with age. Anat. Rec. 77: 529-536.

Gatenby, J. B. 1953. The Golgi apparatus of the living sympathetic ganglion cells of the mouse, photographed by phase contrast microscopy. Roy. Micr. Soc. J. 73: 61-81.

Gatenby, J. B. and Moussa, T. A. 1950. The sympathetic ganglion cell, with sudan black and the Zernike microscope. Roy. Micro. Soc. J. 70: 342-364.

Gatenby, J. B. and Moussa, T. A. 1951. The neuron of the human autonomic system and the so-called senility pigment. J. Physiol. 114: 252-254.

Goodpasture, E. W. 1918. An anatomical study of senescence in dogs, with especial reference to the relation of cellular changes of age to tumors. J. Med. Res. 38: 127-190.

Hamperl, H. 1934. Die Fluorescenzmikoskopie menschlicher Gewebe. Virchows Arch. f. pathol. Anat. 292: 1-51.

Harms, J. W. 1924. Morphologische und experimentalle Untersuchungen an älternden Hunden. Z. anat. Entwicklungsgesch. 71: 319-382.

Heidenreich, O. and Siebert, G. 1955. Untersuchungen an isoliertem, unverändertem Lipofuscin aus Herzmuskulatur. Virchows Arch. f. path. Anat. 327: 112-126.

Hermann, H. 1952. Zusammenfassende Ergenbnisse uber Altersveränderungen am peripheren Nervensystem. Ztschr. Alternsforsch. 6: 197-214. Original not available; cited by Birrens, J. E. 1959. Handbook of aging and the individual. p. 149. University of Chicago Press, Chicago, Illinois.

Hess, A. 1955. The fine structure of young and old spinal ganglia. Anat. Rec. 123: 399-424.

Hess, A. and Lansing, A. I. 1954. The fine structure of young and old spinal ganglia. J. Gerontol. 9: 361.

Hodge, C. F. 1894. Changes in ganglion cells from birth to senile death: observations on man and honey-bees. J. Physiol. (London) 17: 129-134.

Höpker, W. 1951. Das Altern des Nucleus dentatus. Ztschr. Alternsforsch. 5: 256-277.

Hueck, W. 1912. Pigmentstudien. Beitr. Path. Anat. 54: 68-232.

Hydén, H. and Lindström, B. 1950. Microspectrographic studies on the yellow pigment in nerve cells. Discussions Faraday Soc. No. 9: 436-441.

Issidorides, M. and Shanklin, W. M. 1961. Histochemical reactions of cellular inclusions in the human neurone. J. Anat. 95: 151-159.

Jayne, E. P. 1950. Cytochemical studies of age pigments in the human heart. J. Gerontol. 5: 319-325.

Johnson, C. W., Hays, V. W., Speer, V. C., and Catron, D. V. 1959. Thyroprotein for lactating sows. J. Anim. Sci. 18: 1224-1232.

Johnson, C. W., Speer, V. C., Aston, C. C., Culbertson, C. C., and Catron, D. V. 1957. Supplementary plan of nutrition for sows fed corn silage. J. Anim. Sci. 16: 600-606.

Koenig, H. 1962. Histological distribution of brain gangliosides: lysosomes as glycolipoprotein granules. Nature (London) 95: 782-784.

Koenig, H. 1963a. Accumulation of chlorpromazine and reserpine in brain lysosomes. (Abstract) Neurology 13: 356.

Koenig, H. 1963b. The autofluorescence of lysosomes: its value for the identification of lysosomal constituents. J. Histochem. Cytochem. 11: 556-557.

Kuntz, A. 1934. Sympathetic ganglions removed surgically: a histopathologic study. Arch. Surg. 28: 920-935.

Kuntz, A. 1938. Histological variations in autonomic ganglia associated with age and disease. Am. J. Path. 14: 783-796.

Kuntz, A. 1945. Effects of lesions of the autonomic ganglia, associated with age and disease, on the vascular system. Biol. Symp. 11: 101-117.

Kuntz, A. 1952. Aging of the nervous system. J. Gerontol. 7: 439-443.

Kushida, H. 1961. A new embedding method for ultrathin sectioning using a methacrylate resin with three dimensional polymer structure. J. of Electronmicr. 10: 194-197.

Larsell, O. 1951. Anatomy of the nervous system. Appleton-Century-Crofts, New York, New York.

Levi, G. 1946. Accrescimento e senescenza. La Nuova Italia, Florence. Original not available; cited by Andrew, W. 1956. Structural alterations with aging in the nervous system. Association for Research in Nervous and Mental Disease Proceedings 35: 161.

Lillie, R. D. 1956a. Nile blue staining technique for the differentiation of melanin and lipofuscins. Stain Tech. 31: 151-154.

Lillie, R. D. 1956b. Mechanism of Nile blue staining of lipofuscins. J. Histochem. and Cytochem. 4: 377-381.

Marinesco, G. 1909. La Cellule Nerveuse. Doin, Paris, France.

Matzdorff, P. 1948. Grundlagen zur Erforschung des Alterns. Steinkopff, Frankfurt am Main. Original not available; cited by Birrens, J. E. 1959. Handbook of aging and the individual. p. 151. University of Chicago Press, Chicago, Illinois.

Millonig, G. 1962. Further observations on a phosphate buffer for osmium solutions. International Cong. for Electron Microscopy, 5th, Philadelphia, Pennsylvania, Proc. 2: P-8.

Morrison, L. R., Cobb, S., and Bauer, W. 1959. The effect of advancing age upon the human spinal cord. Harvard University Press, Cambridge, Massachusetts.

Mühlmann, M. 1910. Untersuchunger über das dipoide Pigment der Nervenzellen. Virchow's Arch. f. Path. Anat. 211: 155-160.

Murray, M. R. and Stout, A. P. 1947. Adult human sympathetic ganglion cells cultivated _in vitro_. Am. J. Anat. 80: 225-273.

Nandy, K. 1966. Histological and histochemical studies on the lipofuscin pigments in the neurons of senile guinea pigs. (Abstract) Anat. Rec. 154: 393.

Obersteiner, H. 1903. Über das hellgelbe Pigment den nervenzellen und das vorkommen weiterer feitahnlicher korper im centralnervensystem. Arch. Neurol. Inst. Wien Univ. 10: 245-274. Original not available; cited by Brody, H. 1955. Organization of the cerebral cortex. J. Comp. Neurol. 102: 258.

O'Leary, J. L. 1952. Ageing in the nervous system. In Lansing, A. I., ed. Cowdry's problems of ageing. 3rd ed. pp. 223-238. Williams and Wilkins Co., Baltimore, Haryland.

Palade, G. E. 1952. A study of fixation for electron microscopy. J. Exp. Med. 95: 285-298.

Payne, F. 1946. The cellular picture in the anterior pituitary of normal fowls from embryo to old age. Anat. Rec. 96: 77-91.

Payne, F. 1949. Changes in the endocrine glands of fowl with age. J. Gerontol. 4: 193-199.

Payne, F. 1952. Cytological changes in the cells of the pituitary, thyroids, adrenals and sex glands of ageing fowl. In Lansing, A. I., ed. Cowdry's problems of ageing. pp. 381-402. Williams and Wilkins Co., Baltimore, Maryland.

Pearse, A. G. Everson. 1961. Histochemistry, theoretical and applied. 2nd ed. Little, Brown and Co., Boston, Massachusetts.

Pease, D. C. 1964. Histological techniques for electron microscopy. 2nd ed. Academic Press, New York, New York.

Pilez, A. 1895. Beitrag zur lehre von der pigment-wicklung in dem nervenzellen. Arch. Neurol. Inst. Wien Univ. 11: 108-110. Original not available; cited by Brody, H. 1960. Deposition of aging pigment in the human cerebral cortex. J. Gerontol. 15: 258.

Ranson, S. W. and Clark, S. L. 1959. The anatomy of the nervous system. 10th ed. W. B. Saunders Co., Philadelphia, Pennsylvania.

Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17: 208-212.

Rolsten, C. and Samorajski, T. 1966. Environmental stress and biological aging in mice: effects on longevity, CNS lipofuscin, and neuroendocrine ultrastructure. Anat. Rec. 154: 412.

Samorajski, T., Keefe, J. R., and Ordy, J. M. 1964. Intracellular localization of lipofuscin age pigments in the nervous system. J. Gerontol. 19: 262-276.

Samorajski, T., Ordy, J. M., and Keefe, J. R. 1965. The fine structure of lipofuscin age pigment in the nervous system of aged mice. J. Cell Biol. 26: 779-795.

Schulz, R. 1883. Ueber arteficielle, cadaveröse und pathologische Veränderungen des Rückenmarks. Neurol. Centralbl. 11: 529-536.

Sjövall, E. 1932. Åldersförändringarna i centralnervsystemet och deras betydelse. Nord. med. tidskr. 4: 1011-1014.

Strehler, B. L. 1960. Dynamic theories of aging. Am. Assoc. Advancement Sci. Publ. 65: 273-303.

Strehler, B. L. 1962. Time, cells, and aging. Academic Press, New York, New York.

Strehler, B. L. 1964. Advances in gerontological research. Academic Press, New York, New York.

Strehler, B. L., Mark, D. D., Midvan, A. S., and Gee, M. V. 1959. Rate and magnitude of age pigment accumulation in the human myocardium. J. Gerontol. 14: 430-439.

Stübel, H. 1911. Die Fluorezenz tierscher Gewebe in ultra-violetem Licht. Pflug. Arch. ges Physiol. 142: 1-14.

Sulkin, N. M. 1953. Histochemical studies of the pigments in human autonomic ganglion cells. J. Gerontol. 8: 435-445.

Sulkin, N. M. 1955a. The properties and distribution of PAS positive substances in the nervous system of the senile dog. J. Gerontol. 10: 135-144.

Sulkin, N. M. 1955b. Histochemical studies on mucoproteins in nerve cells of the dog. J. Biophysic. and Biochem. Cytol. 1: 459-468.

Sulkin, N. M. 1957. The duration of pigmentation in the nerve cell following prolonged administration of acetanilid. J. Gerontol. 12: 430.

Sulkin, N. M. 1958. The occurrence and duration of "senile" pigments experimentally induced in the nerve cells of the young rat. Anat. Rec. 130: 377-378.

Sulkin, N. M. and Kuntz, A. 1952. Histochemical alterations in autonomic ganglion cells associated with aging. J. Gerontol. 7: 533-543.

Sulkin, N. M. and Srivanis, P. 1960. Experimental production of senile pigments in the nerve cells of young rats. J. Gerontol. 15: 2-9.

Truex, R. C. 1940. Morphological alterations in the Gesserian ganglion cells and their associations with senescence in man. Am. J. Path. 16: 255-268.

Vogt, C. and Vogt, O. 1946. Ageing of nerve cells. Nature (London) 158: 304.

Wahren, W. 1957. Neurohistologischer Beitrag zu Fragen des Alterns. Ztschr. Alternsforsch. 10: 343-357. Original not available; cited by Birrens, J. E., ed. 1959. Handbook of aging and the individual. p. 149. University of Chicago Press, Chicago, Illinois.

Weiss, J. and Lansing, A. I. 1953. Age changes in the fine structure of anterior pituitary of the mouse. Soc. Exp. Biol. and Med. Proc. 82: 460-466.

White, W. H. 1889. Further observations on the histology and function of the mammlian sympathetic ganglia. J. Physiol. 10: 341-357.

Whiteford, R. D. 1964. Distribution of lipofuscin as related to aging in the canine and porcine brain. Unpublished Ph.D. thesis. Library, Iowa State University of Science and Technology, Ames, Iowa.

Whiteford, R. and Getty, R. 1966. Distribution of lipofuscin in the canine and porcine brain as related to aging. J. Gerontol. 21: 31-44.

Wolf, A. and Pappenheimer, A. N. 1945. Occurrence and distribution of acid-fast pigment in the central nervous system. J. Neuropath. and Exp. Neurol. 4: 402-406.

Wünscher, W. 1957. Die Anatomie des alten Gehirns. Ztschr. Alternsforsch. 11: 60-75.

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