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Distribution of lipofuscin as related to aging in the canine and porcine brain

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DISTRIBUTION OF LIPOFUSCIN AS RELATED TO AGING IN THE CANINE AND PORCINE BRAIN

by

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TABLE OF CONTENTS

I. INTRODUCTION

One of the primary objectives of research in the biology of aging is the identification of time-dependent changes in structure and function which contribute to the progressive increase in the probability of death characteristic of aging animals.

For a half century, it has been known that certain tissues of aged humans and other animal species contain golden brown pigment inclusions currently termed lipofuscin or age pigments. Despite an extensive literature, the origin and function, if any, of these pigments remain purely speculative. Very little can be deduced with assurance regarding their chemical structure or their relationship to the normal or abnormal metabolism of the tissues in which they are found.

The present study was undertaken to give further credence to the view that lipofuscin accumulations are, in fact, associated with aging. In this study are described the occurrence and age-wise distribution of lipofuscin pigment in the central nervous system of the dog and pig. Lipofuscin accumulations occurred in clinically healthy subjects and did not seem to be dependent upon sex or breed of animal. The findings agree with previous reports that neuronal pigment was absent in the very young and universally present in older subjects. More interestingly, it is shown that neuronal lipofuscin apparently

increases linearly with age, a parallel observation made by Strehler et al. (1959) for myocardial lipofuscin.

II. REVIEW OF LITERATURE

Among the more reliable intracellular changes occurring during the normal aging of man and other animals is the accumulation of golden-brown, lipid-containing granules called lipofuscin, Bommer (1929), Hamperl (1934), and Hueck (1912). This "age pigment" occurs primarily in non-replaceable cell lines such as cardiac and skeletal muscle, and nerve cells. The origin, deposition, and significance of this pigment has been the subject of study and controversy for nearly a half century. Indeed, Dolley and Guthrie as early as 1918 pointed out that in respect to nerve cell pigment, opinions were most diverse and that there were in fact more opinions than there were investigators. The complexity of the problem is evidenced by the vast literature that has developed during this period.

For the most part, these investigations have dealt with man; studies of age-changes in the nervous system of lower animals were meager, studies on the canine species were few (Dolley (1911), Harms (1924), Kuntz (1938), and Sulkin (1955a, 1955b)), studies on the porcine species were found to be non-existent.

The diversified literature review which follows has been grouped into four parts. The first part deals with the occurrence and distribution of lipofuscin in the nervous system; the second with the histogenesis of the pigment; the

third with its chemical and physical properties. The fourth part deals with the significance of lipofuscin in the aging process.

A. Occurrence and Distribution of Lipofuscin

The presence of lipofuscin in the aging nerve cell was reported by many early investigators. According to White (1889), human autonomic ganglia exhibited pigment much more frequently than those of other animals. Hodge (1894) studied ganglion cells of man from birth to senility and reported that the ganglion cells of senile individuals were largely filled with pigment whereas the neurons of the fetus were not. Pilez (1895) and Obersteiner (1903) described the occurrence and histology of pigment in various parts of the nervous system. Age-changes in the ganglion cells of guinea-pigs and man were studied by Mühlmann (1910). He noted an increase in the number of lipoidal granules in the cell with increasing age and noted further that the homogeneous distribution of the granules was gradually lost and that they gathered in clusters in the neuron to form finally a localized mass which continued to increase in size. Dolley (1911) reported pigmentation of nerve cells of the senile dog and attributed its deposition to functional depression. Harms (1924) studied nerve cells of aged dogs and reported heavy accumulations in the pyramidal cells of the cerebrum and that pigment deposition was much

less or absent in the Perkinje cells of the cerebellum.

Contemporary investigators have reported pigment accumulations in all of the major parts of the nervous system, in many animals including man. Bethe and Fluck (1937) studied the pigment in ganglion cells and showed that the pigment could be distinguished from Nissl substance. Kuntz (1938) and Sulkin (1955a) studied extensively the occurrence, histology, and histochemical characteristics of lipofuscin in the autonomic ganglia in man and senile dogs. Andrew (1941) reported pigment deposition in the trigeminal ganglion, spinal cord, and brain of the mouse. Dixon and Herbertson (1950) and Chu (1954) reported dense aggregations of lipofuscin in the anterior horn cells of the human spinal cord, and Gardner (1940) and Bondareff (1957) reported pigment in the spinal ganglia. Levi (1946) reported pigment deposition in the inferior salivary nucleus in man and noted its uniform distribution at an early age (7th year) and rapid accumulation until about the 30th year. Wilcox (1951) studied a purebred strain of guinea pig and reported pigment deposition in many of the brain stem nuclei. Hopker (1951) investigated the aging process in the dentate nucleus of the cerebellum, reported the presence of pigment and noted that its accumulation affected the form of the cells, and altered the position of the nuclei. Buttlar-Brentano (1954) studied the aging of hypothalamic nuclei; she reported pigment accumulations in

only the nucleus tuberomammilaris and nucleus basalis. D'Angelo et al. (1956) in a study of the staining reactions of lipofuscin in human neurons, demonstrated the presence of the pigment in the motor cortex, various thalamic nuclei, and the supraoptic nucleus. Balthazar (1952) studied the large pyramidal cells of layer V of Area giganticopyramidalis in the human brain and noted the relationship of the granules of lipofuscin to the nuclei of these cells. Kuhlenbeck (1954) noted that pigmentation of the neurons of the rat cerebral cortex was not as conspicuous an occurrence as it was in man. Altschul (1943) described the occurrence and histology of lipofuscin in the basal ganglia of man.

C. and O. Vogt (1946) stated that the sequence of morphological, age-associated changes are characteristically different for each cell type. Wahren (1957) concurred with this observation. He found that in man, the onset and distribution of intracellular pigment differed in the pallidum and in the nucleus tuberomammilaris and nucleus tuberolateralis of the hypothalamus. According to Wahren (1957) the large cells of the pallidum were practically full of lipofuscin by the end of the first three decades, and only after the age of 70 years did they uniformly contain pigment. In the nucleus tuberomammilaris, lipofuscin began to appear in its large cells in the fourth decade and predominated after the age of 60 years. In the medium-sized cells of the nucleus tuberolateralis no

pigment was found up to the third decade, but, by the fifth decade, lipofuscin-containing cells predominated and thereafter, no cells were found free of the pigment. Wahren (1957) also described different intracellular distributions of the pigment. Likewise, Sanides (1957) found that the occurrence of lipofuscin was different in three nuclei of the anygdaloid complex. Hermann (1952) studied the accumulation of lipofuscin in the human sympathetic and vagal ganglia and concluded that the formation and accumulation of this pigment proceded (at least in certain cells) at a steady rate throughout the life-span.

B. Histogenesis of Lipofuscin

Little is known of the histogenesis of lipofuscin. Dolley stated that the pigment is a product of the nucleus; that during prolonged periods of depression, nuclear chromatin was extruded and transformed into cytoplasmic pigment. Höpker (1951), in an excellent study of age-changes in the dentate nucleus of the cerebellum, concluded that lipofuscin was formed in a "lipohilic" center located in the proximity of the nucleus; he also described five recognizable formative stages. Matzdorf (1948) described a similar course of lipofuscin deposition with the exception that no lipophilic center was noted. In this excellent review of aging, Matzdorf (1948) described the process of pigment deposition as beginning with

fine particles of lipofuscin diffusely distributed throughout the cytoplasm. These then increased in size, darkened, and clumped; the latter differing with the cell type. In its completed form, the pigment may be scattered throughout the cytoplasm or clumped at one pole. Bethe and Fluck (1937), in their study on pigment deposition in ganglion cells, concluded that the matrix of lipofuscin was protein and bound on it was a lipoid substance and a yellow pigment. Sjövall (1946) considered lipofuscin to be a dispersed phase of the plasma colloid which tends to decrease in its dispersion and finally to flocculate.

The Golgi complex and mitochondria have also been associated with the origin of lipofuscin. Gatenby et al. (1951) and Gatenby and Moussa (1951) presented the view that pigment granules in autonomic ganglion cells were derived from transformation of broken-down pieces of Golgi material. Bondareff (1957), in an excellent electronmicroscopic study of the genesis of lipofuscin, observed a pigment-vacuole configuration that resembled the Golgi complex in many ways. A mitochondrial origin for lipofuscin has been proposed by Hess (1955) also on the basis of electronmicroscopic studies. In this study, Hess (1955) reported electron dense pigment particles in close morphological association with cytoplasmic vacuoles; he interpreted these vacuoles as mitochondria which had become swollen and vacuolated. Duncan et al. (1960) supported Hess'

views on the mitochondrial origin of lipofuscin.

A short time after the observations of Gatenby and his associates and Hess, a new cytoplasmic organelle, the lysosome was isolated from liver cells by DeDuve and his associates (1959). It seems apparent that these structures are present in all cells (epithelial and mesenchymal), Bloom and Fawcett (1962). The lysosome is surrounded or delimited by a lipoprotein membrane of a single-unit type and contains at least ten hydrolytic enzymes. Of these ten, according to Gedigk and Bontke (1956), Pearse (1961), Novikoff and Essner (1960), Hammerbeck (1960), and Heidenreich and Siebert (1955), three of them (acid phosphotase, esterase, and cathepsin) suggest that lipofuscin is located in the lysosome; in fact, it is entirely possible that lipofuscin is elaborated by this organelle. Bondareff (1957) has stated that when pigment is deposited (or elaborated) in the cell, it is seen first in the lysosome.

C. Physical and Chemical Properties of Lipofuscin

Barka and Anderson (1963) classify as pigments, those substances absorbing light in the visible spectrum and seen microscopically in unstained cells. This broad definition obviously includes substances of different origin, composition, and function. Based on their origin, pigments may be exogenous (arising from outside the animal body) or endo-

genous (arising from within the body). The endogenous pigments, of which lipofuscin is but one, consist of those pigments derived from the break-down of hemoglobin (hemosiderin, hematoidin, and hematin) and those that are of non-hemoglobin origin (melanin and the lipopigments). The latter (the lipopigments) constitute an ill-defined group of pigments, which have in common a yellow-brown coloration and presumed lipid precursors. It is customary to divide these into lipochromes and lipofuscins. The term, lipochrome, is restricted to those pigments containing colored hydrocarbons, principally carotenoids; they are present in vegetables and are found in animals due to accumulation in the tissues following ingestion. Lipofuscin, on the other hand, is thought to be derived from the progressive oxidation of lipids, Pearse (1961).

The chemical constitution of lipofuscin is poorly understood primarily because of technical difficulties encountered in its analysis, which analysis is hampered by the difficulty in making a positive identification of the pigment in homogenates. Also, since the pigment is stained by chemically nonspecific methods, and on the basis of these, is defined as lipofuscin, it is reasonable to believe that many chemically different substances are known as lipofuscin, and one cannot therefore equate lipofuscin of one organ with that of another.

The most recent and thorough biochemical analysis of lipofuscin was that of Heidenreich and Siebert (1955). In

this study, cardiac muscle lipofuscin from aged human subjects was isolated and analyzed. Cardiac muscle was first homogenized centrifuged in a sucrose solution with a discontinuous density gradient. The lipofuscin-containing fraction was repeatedly centrifuged and washed until a black-brown precipitate was obtained. Staining reactions of smears of this material paralleled those obtained from lipofuscin in nervous tissue.

That the Heidenreich-Siebert method could be applied to nervous tissue is apparent but the isolation of a lipoid pigment from whole-brain homogenate would be exceedingly difficult, technically; the presence of melanin in the brain tissue would further compound the difficulty.

The physical properties of lipofuscin are not particularly outstanding. The pigment is usually yellowish-brown, iron-negative, fluorescent, basophilic granules located in cells. They resist alcoholic dehydration and paraffin embedding, and are stained with the usual lipid stains. Stübel (1911) using ultra-violet light observed a dark brown fluorescence in animal hearts. Bommer (1929) employing ultra-violet microscopy noted yellow fluorescent granules in human myocardial fibers. Hamperl (1934) continued and extended Bommer's investigations, pointing out that the fluorescent particles, similar to lipofuscin age pigment, were absent in the hearts of subjects below age 10 and were located at the poles

of the myocardial nuclei when present. Hamperl correlated the apparent color of lipofuscin with the intensity and color of the fluorescent particles, leaving no doubt of their identity. In his opinion, fluorescence microscopy was the most sensitive technique for demonstrating age pigment. Hyden and Lindstrom (1950) using microspectography, have reported absorption peaks at 2600A° and 3750A° and fluorescence emission bands between 4400A° and 4600A° and between 5300A° and 5600A° for human neuronal lipofuscin.

That the staining characteristics of lipofuscin are variable, and that the pigment shows differences according to location was first demonstrated by Hueck (1912), and more recently by Lillie (1956b). Pearse (1961) has postulated that lipofuscin pigment is derived from the oxidation of unsaturated lipids or lipid substances and undergoes characteristic changes as oxidation proceeds. These changes are then reflected in the outcome of the staining reactions. Pigmentation, fluorescence, basophilia and reducing activity increase with progressive oxidation of lipid substances. On the other hand, solubility in lipid solvents and staining with oil-soluble dyes diminishes. The characteristic of acid-fastness is confined to certain stages in the development of lipofuscin. Ceroid and vitamin E deficiency pigment would appear to represent specific stages in the evolution of lipofuscin.

D. Significance of Lipofuscin in the Aging Process

The relation of lipofuscin to the functional integrity of the cell containing it is uncertain, due at least in part, to a surplus of speculation and a dearth of controlled experiments. Predominant in early literature, Dolley (1911) and others, was the view that lipofuscin was detrimental to the cell containing it. Contemporary investigators were unable to agree on the significance of the pigment. Hyden and Lindstrom (1950), Höpker (1951), Ranson and Clark (1959), Bloom and Fawcett (1962) and others stated that lipofuscin was an inert slag product of no metabolic importance. On the other hand, Murray and Stout (1947) in a tissue culture study of human sympathetic ganglion cells observed the deposition of pigment over a period of several weeks. They reported that cells containing large amounts of pigment did not migrate and that their nuclei frequently lost their staining properties; cells containing lesser amounts of pigment would migrate only short distances. These investigators therefore concluded that pigment may be detrimental to the cell by virtue of its being a rigid mass interfering with the plasticity of the cell.

Sulkin and Kuntz (1952) and Sulkin and Scrivanis (1960) challenged the concept that lipofuscin deposition in the neuron is even an evidence of age-change per se. These investigators studied the effect of environment on the deposition of pigment in the nervous system of laboratory animals,

the dog, and man. They found that when the environment had some degree of control (as in the case of laboratory animals) pigment did not develop until the subject was senile. In the case of the dog, where the environment is controlled to a lesser degree, pigment does not occur until old age. Here, however, they found that the accumulation of pigment was much greater than in the laboratory animal. In man, where the environment is even less controlled, pigment was present at almost all ages. These investigators concluded that the deposition of pigment in the nerve cell as a correlate with aging did not appear warranted. Strehler (1962) stated that this view was not necessarily correct; that pigment deposition due to environmental alterations may be by an entirely different mechanism than those produced by aging processes.

The excellent quantitative study of lipofuscin accumulation in the human myocardium by Strehler et al. (1959) represents one of the most useful and significant contributions in recent times. These investigators made four outstanding observations: 1) average pigment concentrations increased linearly at a rate of one-third per cent of the heart volume per decade; 2) pigment concentration was independent of sex, race, cardiac pathology, or the presence or absence of cardiac failure, an observation counter to the conclusion of Sulkin and his associates relative to the effect of extrinsic factors on the deposition of pigment; 3) because of its absence in the

very young, its presence without exception in the aged specimens studied, and its large displacement of myocardial volume, it was concluded that lipofuscin accumulation met the criteria set forth for a basic biological aging process; and 4) relatively large amounts of pigment may well interfere with efficient function of the cells containing it.

III. MATERIALS AND METHODS

A. Canine Brain Specimens

Brain specimens for this study were obtained from dogs reared in the dog colony of the Department of Veterinary Anatomy, Iowa State University. Thirty-seven specimens were utilized; twenty-seven specimens were obtained from dogs whelped in the colony; ten were obtained from other sources. Age, breed, and sex of each subject is shown in Table 1.

Table 1. Age, breed, sex, and number of dogs studied

Age	Breed	Male	Female	Total
14 days	Beagle	1	0	1
1 month	Beagle	1	1	2
2 months	Beagle	1	1	2
10 months	Beagle	1	0	1
11 months	Beagle	1	0	1
12 months	Beagle	9	5	14
20 months	Beagle	0	1	1
30 months	Terrier, Wirehair	1	0	1
2.5 years	Beagle	0	1	1
4.0 years	Beagle	0	1	1
5.0 years	Beagle	2	0	2
7.0 years	Beagle	0	1	1
8.0 years	Terrier	1	0	1
8.0 years	Beagle	0	2	2
10.0 years	Beagle	0	1	1
11.5 years	Terrier	0	1	1
12.0 years	Golden Retreiver	1	0	1
12.9 years	Pointer	0	1	1
13.0 years	Welsh Corgi	0	1	1
		19	18	37

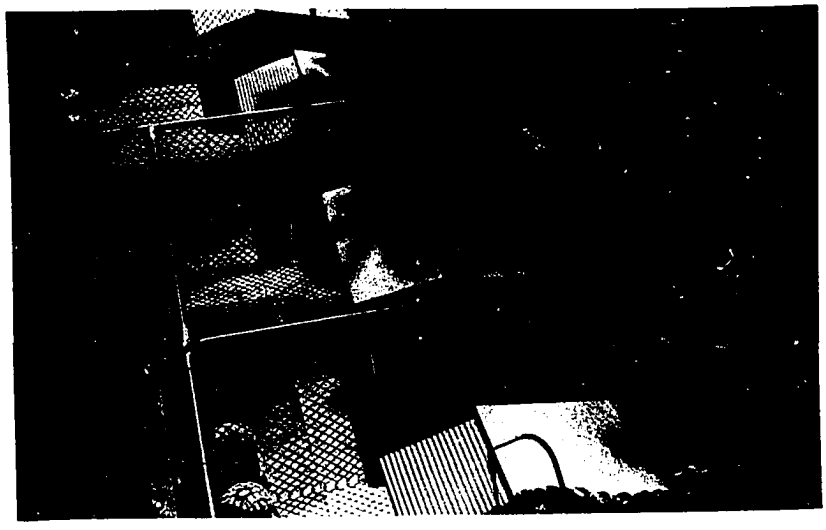
Cammermeyer (1963) has emphasized that an accurate knowledge of the source, age, etc. of specimen material for age-change studies is imperative for valid interpretation of findings. To this end, a brief history of the dog colony of the Department of Veterinary Anatomy is here presented.

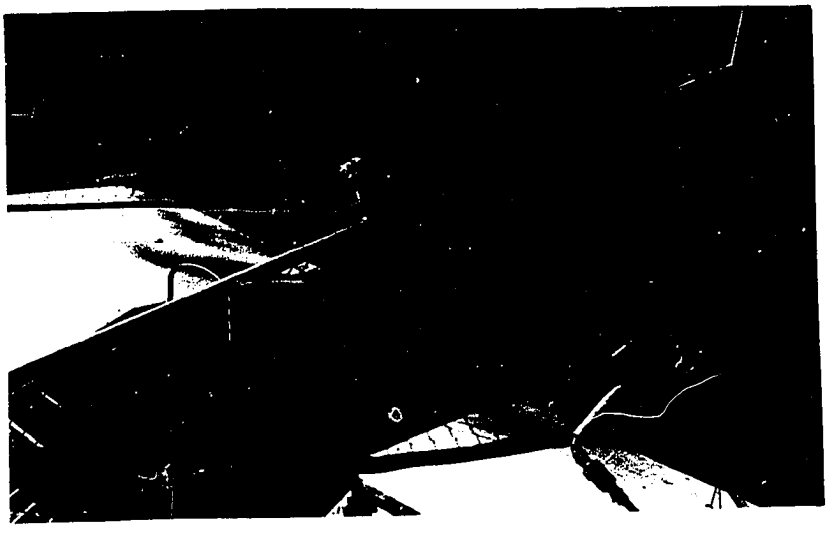
The dog colony (Fig. 1) was established in 1952 to meet the growing need for canine specimens of known chronological age to support gerontological studies then in progress. The initial population consisted of thirty mongrel dogs. The use of mongrels permitted a relatively economical approach to the many problems encountered in establishing a colony of this type. The mongrel population was maintained until a standardized routine of colony management was developed. In 1955, three pure-bred Beagle hounds (two females and one male) were introduced into the colony. At the same time the physical area of the colony was doubled, the space it presently occupies. As the Beagle population increased, the number of mongrel animals was reduced. By 1958, the colony consisted entirely of Beagle hounds. A colony population of forty to fifty animals was maintained.

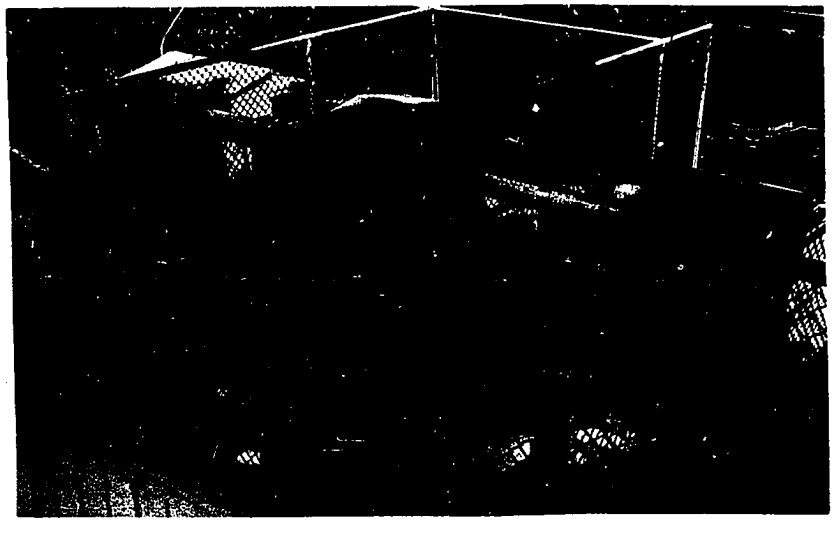
It was decided that the colony should exist with a minimum of environmental control to the end that structural alterations due to growth and aging be reflected as a "normal" life parameter for the colony.

The diet for these animals after weaning consisted of

Fig. 1. Beagle colony, Iowa State University







a dry commercial dog food[1] which was fed free-choice by means of a hopper-type self-feeder. Fresh water was provided by an automatic watering device. There was no evidence of nutritional deficiency during the course of the study. The ration consisted of 25% protein, 7% fat, 40% carbohydrate, plus vitamins, minerals, and essential amino acids. One pound of feed supplied 1500 to 1600 calories.[2]

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

All animals in the colony were vaccinated against canine distemper and infectious canine hepatitis. A solid wooden fence, eight feet high, located five feet from the animal runs, prevented the dogs in the colony from coming in direct contact with stray animals.

There was no clinical evidence of disease at the time the animals were destroyed.

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[1]Supplied by Gaines Dog Food Division, General Foods Co., Kankakee, Illinois.

[2]Feed analysis provided by the Gaines Dog Research Laboratories, Kankakee, Illinois.

B. Porcine Brain Specimens

Porcine brain specimens utilized in this study were obtained from swine reared at the Swine Nutrition Farm, Iowa State University. The animals ranged in age from one to six years. Age, breed, and sex of each subject is shown in Table 2. At the time the pigs were destroyed, they appeared to be normal, healthy subjects exhibiting no clinical evidence of disease.

Table 2. Age, breed, sex, and number of pigs studied

Age	Breed	Male	Female	Total
12 months	Yorkshire-Landrace	0	1	1
2 years	Yorkshire-Landrace	0	4	4
3 years	Yorkshire-Landrace	0	1	1
3 years	Poland China	2	0	2
3 years	Yorkshire	1	0	1
4 years	Poland China	2	0	2
5 years	Yorkshire-Landrace	0	1	1
6 years	Landrace	0	3	3
		5	11	16

C. Collection and Fixation of Brain Specimens

The animals utilized in this study were killed by electrocution. A heavy-duty, rubber-covered electric cable to which had been attached two electrodes was employed. One electrode was attached to the anal mucosa, the other to the

upper lip. Three separate shocks, each of 60 seconds duration were applied from a 110-volt alternating current source.

Immediately after death, the animals were partially exsanguinated by making a generous incision through the skin of the axillary space exposing the axillary artery and vein which were then severed. As soon as frank hemorrhage ceased, the animals were decapitated at the atlano-occipital articulation.

The skin and underlying muscles were next reflected laterally from the dorsal and lateral aspects of the cranium. The calvarium was then removed in the following manner. The temporal bone on one side was trephined to allow insertion of the jaws of a bone ronguer which was then employed to clip away the cranial bones. The calvarium was removed to the level of the orbital cavities rostrally, the zygomatic arches laterally, and the foramen magnum caudally.

The cranial dura was then reflected laterally and the brain carefully lifted from the floor of the cranial vault by inserting the blade of a histological section lifter between the floor of the cranium and the brain and exerting gentle vertical traction. This maneuver exposed the cranial nerves, hypophyseal stalk and blood vessels which were carefully severed; the brain was then removed from the cranial cavity and placed in fixative. In this manner, all brain specimens were removed and placed in fixative within 30

minutes post-mortem.

Each brain was fixed by immersion in 1,000 milliliters of 10 per cent buffered neutral formalin prepared by the following formula:

40 per cent formaldehyde	100 mls.
Tap water	900 mls.
Sodium phosphate, dibasic	7 gms.
Sodium phosphate, monobasic	4 gms.

Whole-brain specimens remained in this fixative at least 48 hours. The brains were then transected and six blocks of tissue were selected according to the points of reference in Fig. 2. The blocks, approximately 10 millimeters in thickness, were chosen; one each from the medulla oblongata, pons, diencephalon, and cerebellum, and two from the mesencephalon.

D. Processing and Staining Procedures

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
Ethanol, Absolute	1 hour
Paraplast #1	1 hour
Paraplast #2	2 hours

Tissue sections were cut 10 to 15 micra in thickness on a rotary microtome. Eight sections were prepared from each block; four were stained routinely by the staining procedures

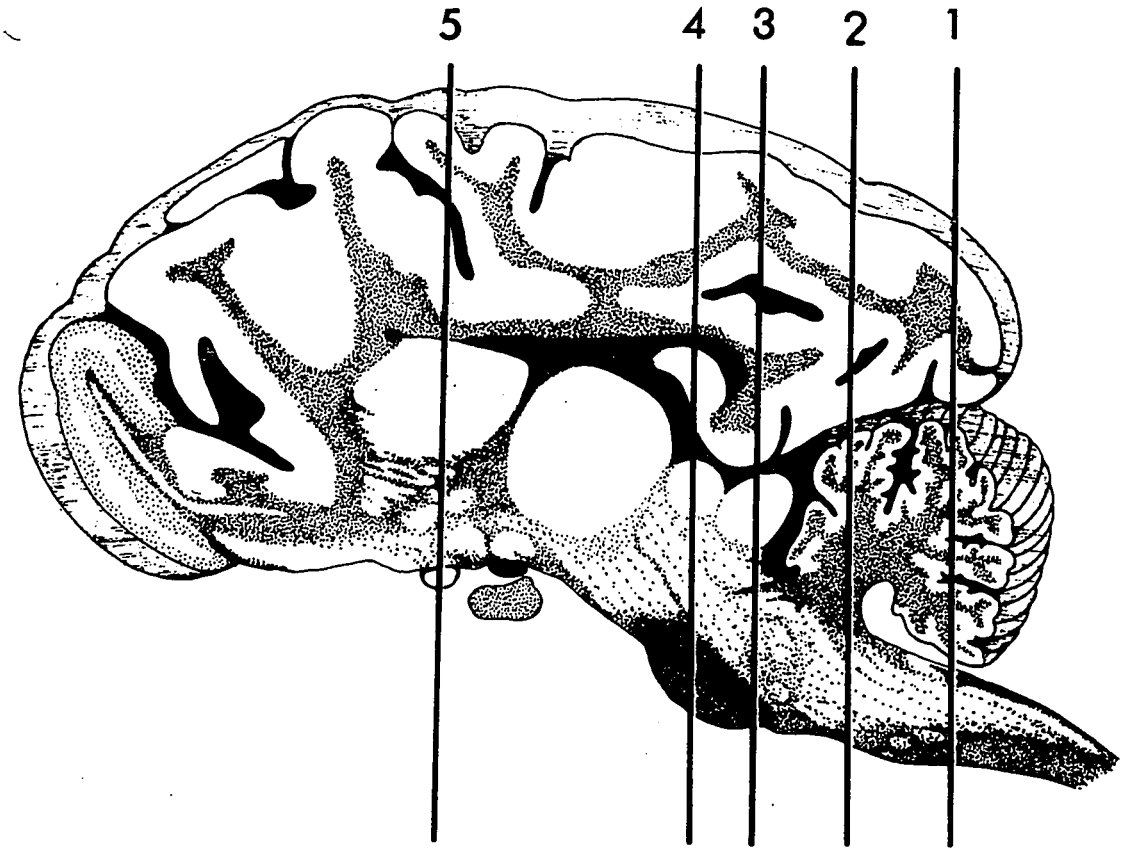


Fig. 2. Sagittal section of canine brain indicating sites of tissue block selection:

1. Medulla oblongata
2. Pons and cerebellum
3. Posterior colliculus
4. Anterior colliculus
5. Diencephalon

outlined below, and four were retained for replacement, evaluation of staining procedures, or possible changes in staining techniques.

Because each of the procedures employed exhibited some difficulty in technique of preparation or execution, the staining procedures for each follows:

Cresyl Violet Stain for Nissl Substance

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. 2% Aqueous cresyl violet	5 minutes
8. Distilled water	Rinse
9. Distilled water	Rinse
10. 70% Alcohol	Rinse (quickly)
11. 95% Alcohol	Rinse (quickly)
12. Xylol-terpinol equal parts	2 minutes
13. Xylol	2 minutes
14. Mount in synthetic resin	

This stain was used to determine the quality and quantity of Nissl substance in the sections.

Cajal's Picro-indigocarmine Trichrome Stain (Castroviejo's Modification)

1. Xylol	2 minutes
2. Xylol	2 minutes
3. Absolute alcohol	1 minute
4. 95% Alcohol	30 seconds
5. 70% Alcohol	30 seconds
6. Distilled water	30 seconds
7. Aceti-fuchsin-formalin*	5 minutes
8. Distilled water	30 seconds
9. Picro-indigocarmine**	5 minutes
10. Distilled water	30 seconds
11. 70% Alcohol	30 seconds
12. 95% Alcohol	30 seconds

13. Absolute alcohol	30 seconds
14. Carbol-xylol	3 minutes
15. Xylol	3 minutes
16. Mount in synthetic medium	

Results:

Epithelium	violet
Connective tissue	blue
Nuclei	red
Erythrocytes	yellow
Muscle	green

*Must be prepared fresh daily (every 4 hours). Time varies from 4 to 9 minutes; check each batch with a "pilot" slide.

**Time varies from 1 to 8 minutes; check with a "pilot" slide.

This stain was used for nuclear studies and routine histological examination.

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 - .05% in 1% H_2SO_4	20 minutes
8. Wash in running water	10-20 minutes
9. Mount in glycerol jelly	

Results:

Lipofuscin	dark-blue to green-blue
Melanins	pale green
Erythrocytes	greenish yellow
Myelin	green to deep blue
Nuclei	poorly stained

E. Visualization of Lipofuscin

Barka and Anderson (1963) noted that the chemical constitution of lipofuscin was poorly understood, and that the pigment was stained by chemically non-specific methods. The Nile blue sulfate method of Lillie (1956a) for demonstrating lipofuscin was employed routinely in this study. Selected sections were examined unstained and by fluorescence microscopy and compared with Nile blue preparations to confirm the presence of lipofuscin (Fig. 3).

The auto-fluorescent pigment was viewed under non-fluorescent oil in a Bausch and Lomb model PBV5 Dynazoom microscope, the optics of which included a 47x,.4mm.,.95N.A. objective, 10x compensated oculars, and a cardiod quartz condenser (1.00 mm. glass 1.05 mm.). The light source consisted of a Bausch and Lomb model 31-33-28-03 fluorescent illuminator fitted with a Bausch and Lomb high pressure mercury arc lamp (No. HB0200). Bausch and Lomb filters were also employed; No. 558 exciter filter permitted high transmissions in the 400 millimicron range and a No. Y-8 barrier filter blocked transmissions above 550 millimicrons.

To avoid an over-abundance of repetitive photomicrographs, three signally different age groups were selected. A five year interval in these ages permitted a more graphic representation of pigment accumulations. The age groups

Fig. 3. Visualization of lipofuscin pigment, 400x

Fluoresence microscopy
Nile blue stain for lipofuscin
Unstained pigment granules

selected were the 2.5 year old specimens, the 8.0 year old specimens, and the 13.0 year old specimens.

Photomicrographs were made employing a Leitz Ortholux microscope fitted with plan-achromatic objectives. Color transparencies were produced on Kodachrome II Professional film which were commercially processed and printed.

IV. OBSERVATIONS

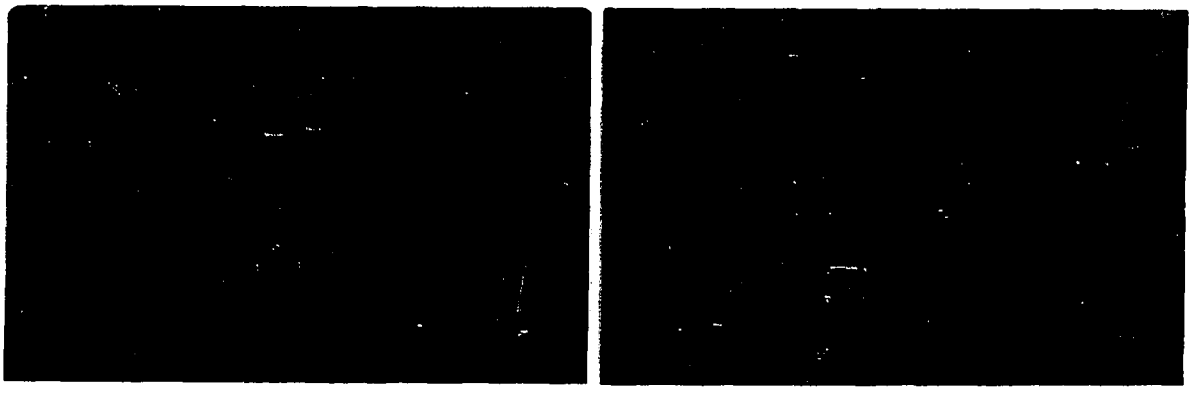
Lipofuscin pigment, stained by the Nile blue method, appeared as dark blue-green intracytoplasmic granules in the cell bodies of neurons. Pigment content of the cells was judged by the intensity of staining and the distribution of the pigment granules within the cells.

For descriptive purposes, it was convenient to group pigment distributions into three categories (Fig. 4): 1) dispersed pigment granules, in which the pigment occurred as discrete particles scattered evenly throughout the cytoplasm, 2) polar or axonal aggregations, in which the pigment was collected at or near the axon hillock. Occasional neurons contained two polar aggregations, one in relation to the axon hillock, and the other in relation to the principal dendrite. These were termed bipolar aggregations, and 3) perinuclear clusters, in which the pigment was concentrated, usually in a crescent-shaped configuration, about the cell nucleus.

A. Occurrence and Distribution of Lipofuscin in the Canine Brain

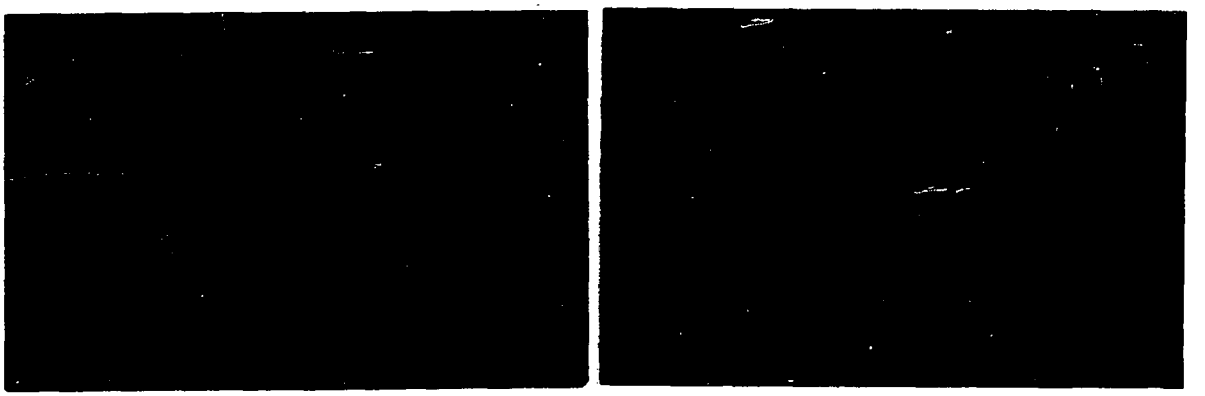
1. Hypoglossal nucleus (Fig. 5)

The neurons of the hypoglossal nucleus exhibited no evidence of pigmentation until the age of 4.0 years. At that age, pigment was present in the region of the axon hillock.



Dispersed pigment granules

Perinuclear pigment clusters



Polar pigment aggregations

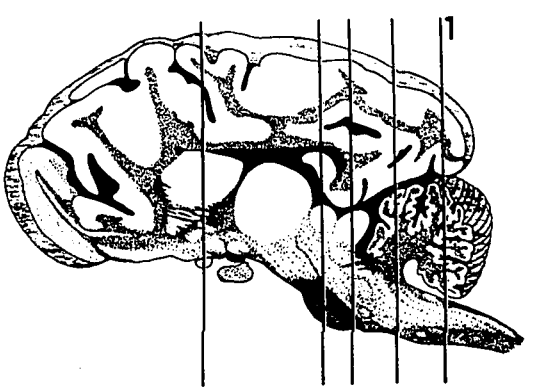
Bipolar pigment aggregations

Fig. 4. Pigment distribution patterns, 400x

Fig. 5. Distribution of lipofuscin pigment in the canine hypoglossal nucleus

Row 2 - 2.5 years of age
Row 3 - 8.0 years of age
Row 4 - 13.0 years of age

Left column - 250x; right column - 400x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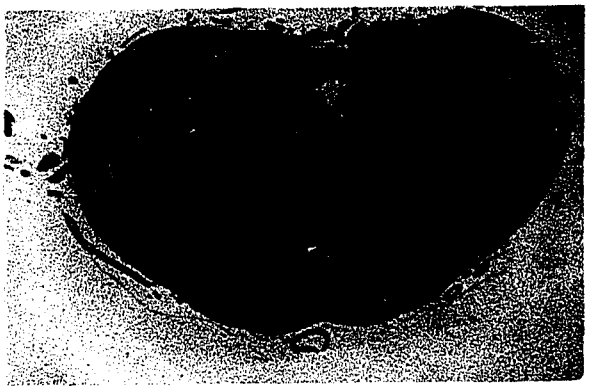


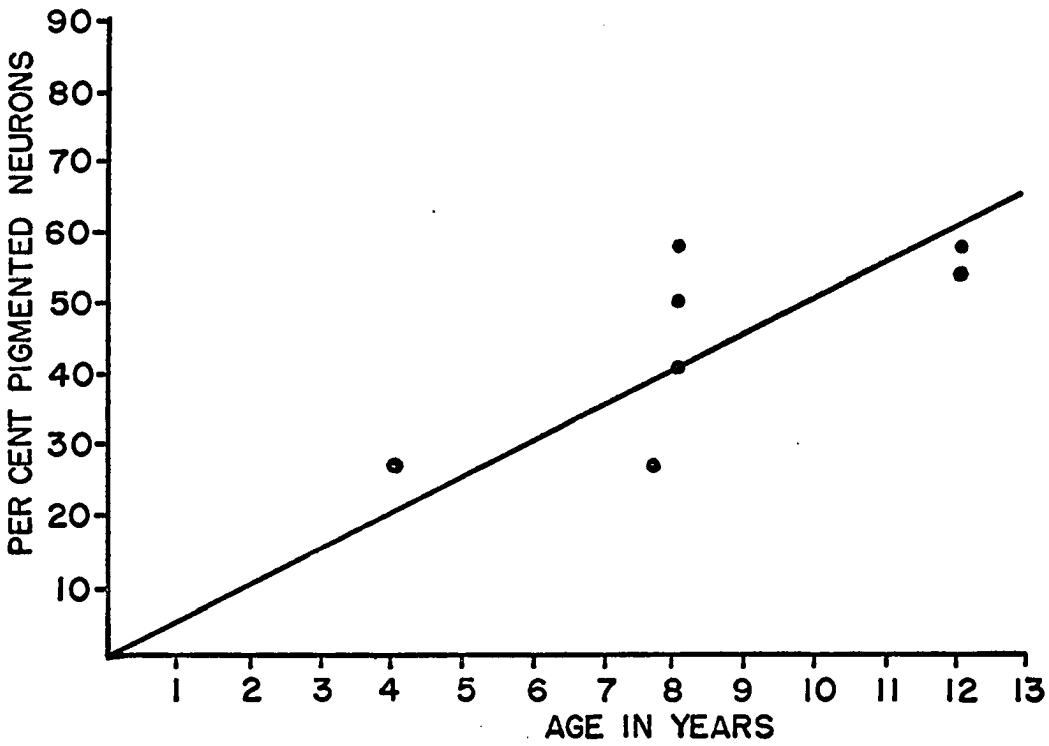


Fig. 6—PER CENT PIGMENTED NEURONS IN THE CANINE HYPOGLOSSAL NUCLEUS

FROM 2 TO 13 YEARS OF AGE

Although it occurred in small amounts, the pigment stained deeply.

Three years later, at 7 years of age, this nucleus exhibited a moderate number of pigmented cells. Pigment accumulations were of the polar type; occasional cells contained discrete pigment granules evenly distributed throughout the cytoplasm.

The cells of the hypoglossal nucleus at 8 year of age did not appear to contain more pigment than at 4 years of age; however, more neurons contained pigment (Fig. 5). Three brain specimens of this age were examined; almost uniformly the pigment was located at or near the axon hillock, as closely packed granules.

The cell bodies of this nucleus examined in two 12 year old specimens contained pigment. The pigment was again located in the region of the axon hillock and occurred as dense, darkly staining amorphous masses. Occasional cells containing disseminated pigment granules were seen.

The hypoglossal nucleus of the 13 year old subject, the oldest speciment available for this study, contained heavily pigmented cells. In all of the cells examined, the pigment was located in relation to the axon hillock and existed as dense, blue-black amorphous deposits.

2. Inferior olivary nucleus (Fig. 7)

The first evidence of pigment accumulation in this nucleus appeared at the age of 4 years; approximately 42 per cent of the cells contained pigment. Pigment accumulations were predominantly loose clusters of granules oriented in a crescent-shaped mass at the periphery of the cell nucleus (perinuclear clusters). Compact clusters of pigment granules were seen frequently in the region of the axon hillock.

The neurons of the inferior olivary nucleus in 7 year old specimens contained moderate amounts of pigment. Approximately equal numbers of cells contained perinuclear clusters, polar aggregations and disseminated pigment granules.

In 8 year old specimens, all of the cell bodies examined in this nucleus contained pigment collections. The pigment was present as closely packed granules near the axon hillock.

The neurons of the inferior olivary nucleus in 12 and 13 year old subjects contained pigment, most of which was located near the periphery of the cell bodies. Perinuclear clusters of pigment granules were a frequent occurrence; no instances of disseminated pigment were noted.

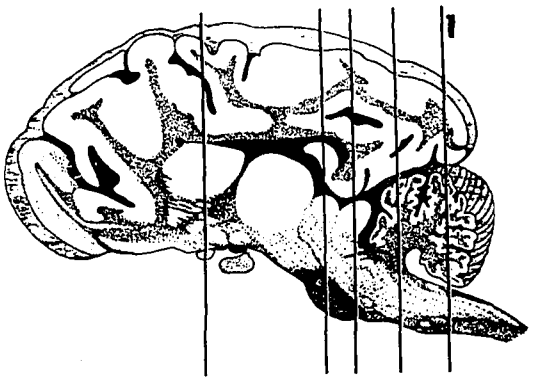
3. Cochlear nuclei (Fig. 9)

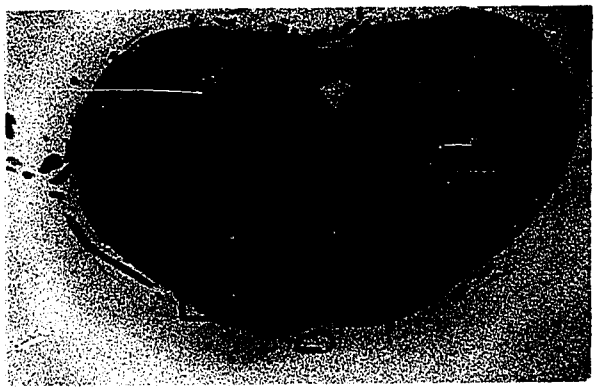
No distinction was made between the cochlear nuclei. These nuclei were free of pigment until the age of 4 years.

Fig. 7. Distribution of lipofuscin pigment in the canine inferior olivary nucleus

Row 2 - 2.5 years of age
Row 3 - 8.0 years of age
Row 4 - 13.0 years of age

Left column - 250x; right column - 400x





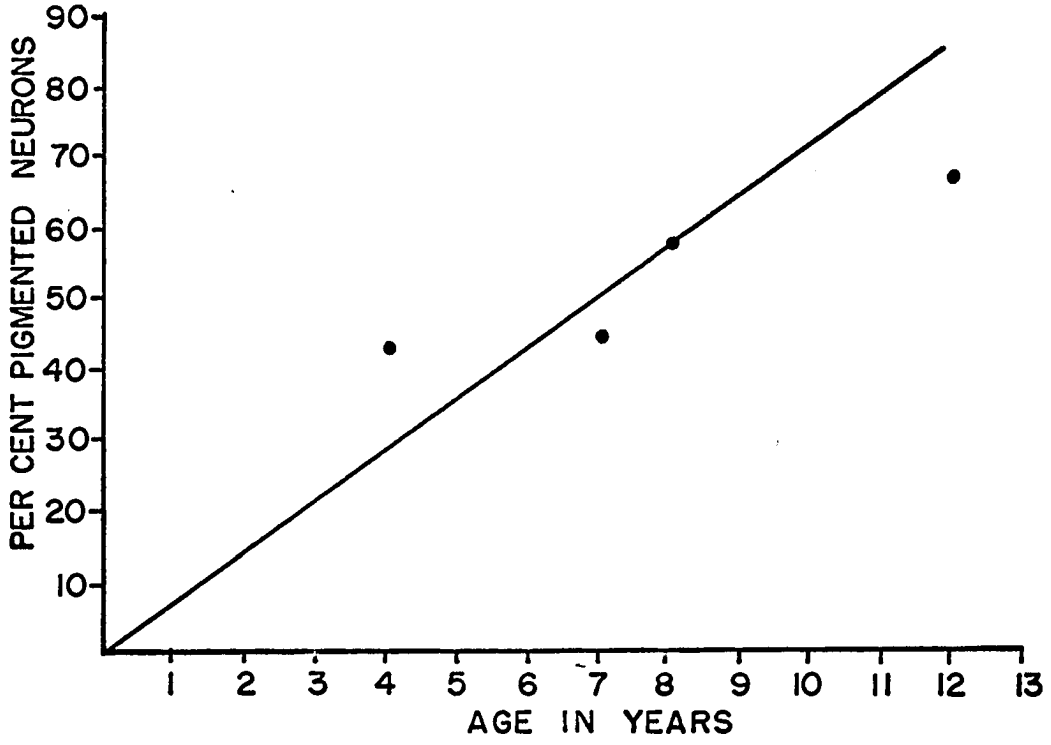


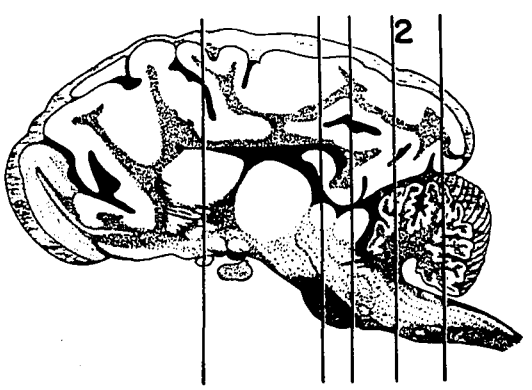
Fig. 8— PER CENT PIGMENTED NEURONS IN THE CANINE INFERIOR OLIVARY NUCLEUS

FROM 2 TO 13 YEARS OF AGE

Fig. 9. Distribution of lipofuscin pigment in the canine cochlear nuclei

Row 2 - 2.5 years of age
Row 3 - 8.0 years of age
Row 4 - 13.0 years of age

Left column - 250x; right column - 400x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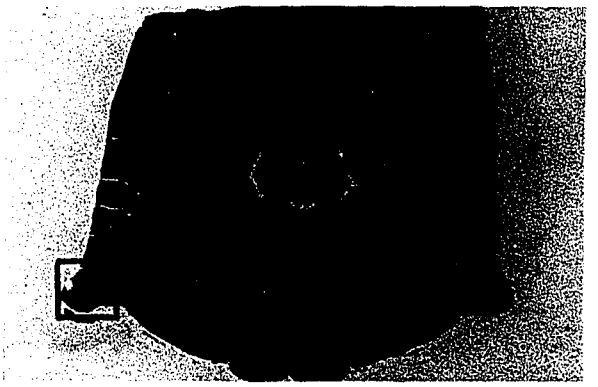


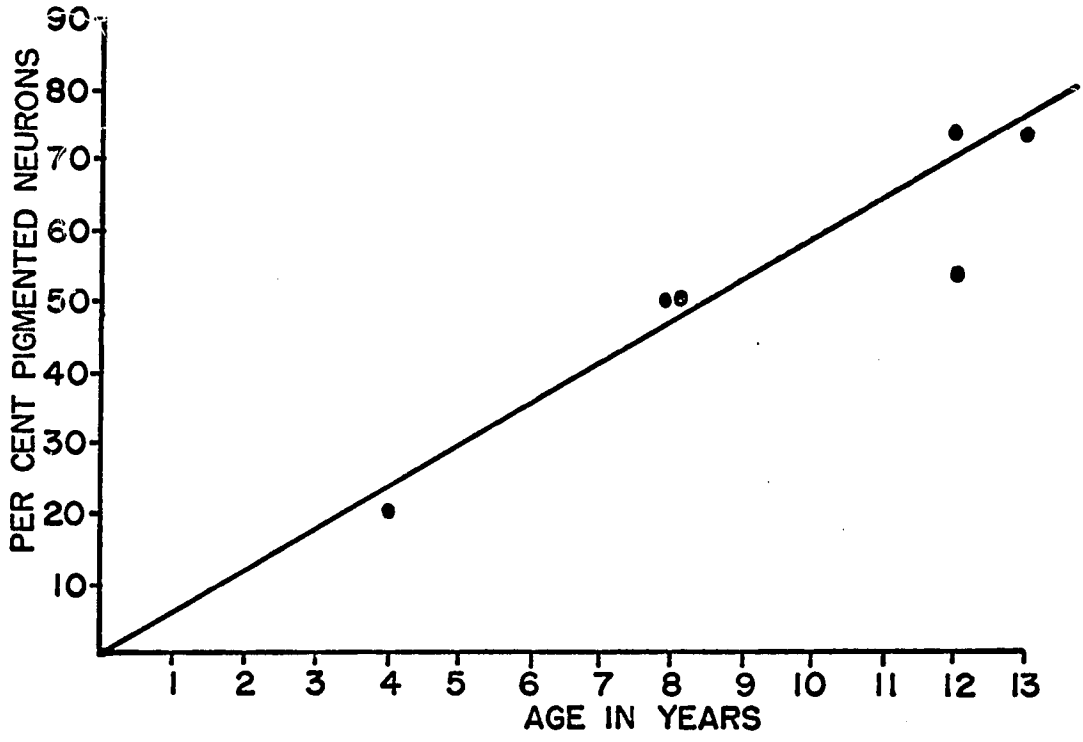


Fig. 10—PER CENT PIGMENTED NEURONS IN THE CANINE COCHLEAR NUCLEUS

FROM 2 TO 13 YEARS OF AGE

Pigment accumulations at that age were moderate; however it was interesting to note that perinuclear clusters of fine, discrete particles were characteristic of these nuclei at that age.

The cochlear nuclei at 7 to 8 years of age were 40 to 50 per cent pigmented. The pigment was concentrated in crescent-shaped masses around the cell nuclei. Occasional neurons contained disseminated pigment granules.

The cell bodies of these nuclei examined in 12 year old subjects contained heavily pigmented cells. The pigment particles were so compact that they lacked a granular texture. The pigment orientation was predominantly perinuclear, however, moderate numbers of cells containing disseminated pigment granules were seen.

While only 70 per cent of the neurons of the cochlear nuclei contained pigment at 13 years of age, the amount of pigment contained in each cell was large. The pigment occurred as dense, amorphous, perinuclear masses.

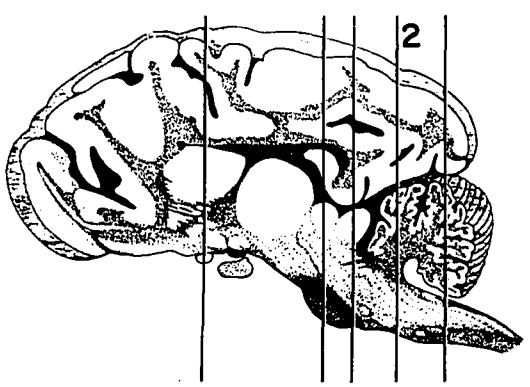
4. Vestibular nuclei (Fig. 11)

No distinction was made between the vestibular nuclei. Pigmented neurons of these nuclear groups were not apparent in the specimens examined until the age of 4 years. At this age pigment was present in the region of the axon hillock. The pigment accumulations were small in amount, occurring in

Fig. 11. Distribution of lipofuscin pigment in the canine vestibular nuclei

Row 2 - 2.5 years of age
Row 3 - 8.0 years of age
Row 4 - 13.0 years of age

Left column - 250x; right column - 400x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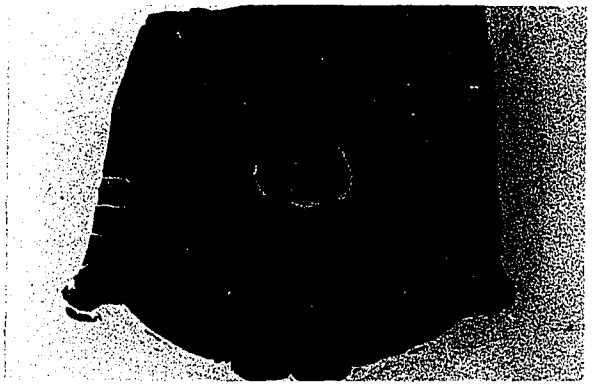
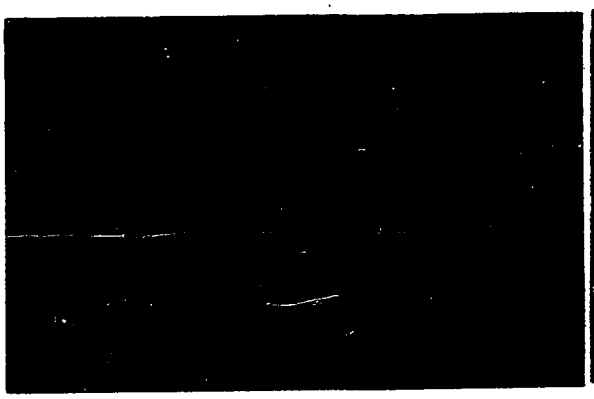




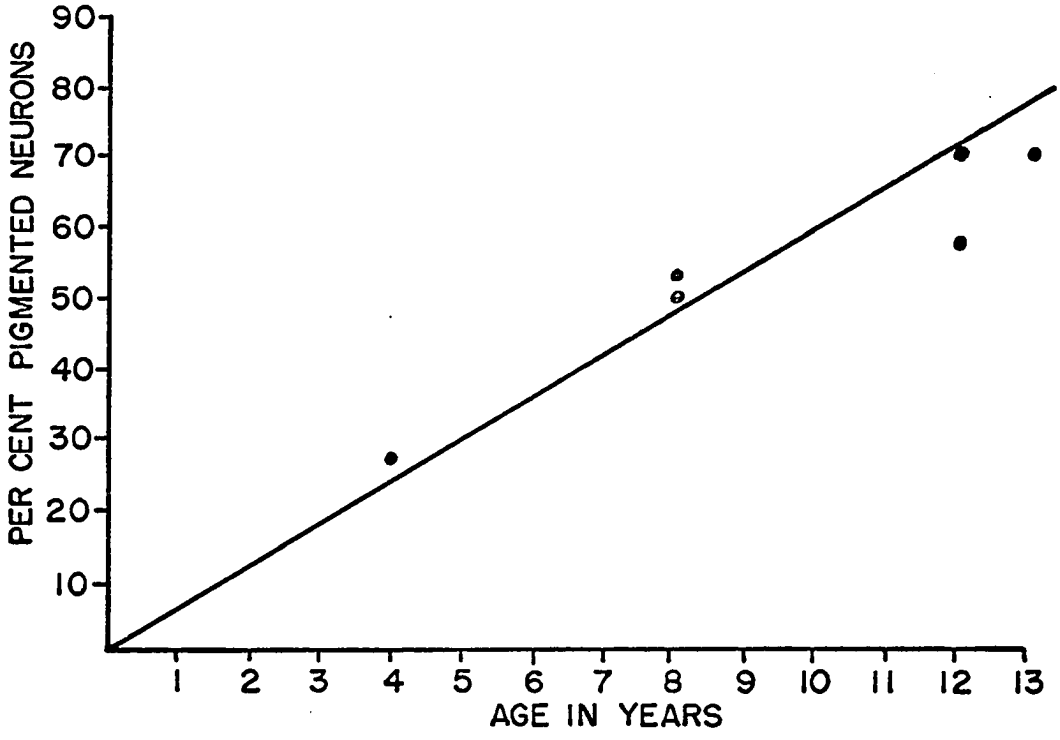


Fig. 12 — PER CENT PIGMENTED NEURONS IN THE CANINE VESTIBULAR NUCLEUS

FROM 2 TO 13 YEARS OF AGE

about 25 per cent of the cell bodies. They existed as fine discrete particles.

The neurons of the vestibular nuclei from 7 and 8 year old subjects were 40 to 50 per cent pigmented. The pigment aggregations were situated near the axon hillock. Occasional neurons contained evenly distributed pigment granules.

The cells of these nuclei from 12 year old subjects presented considerable variation in pigment accumulation and distribution. All neurons examined contained pigment, most of which was located at or near the axon hillock; in these instances, the pigment deposits ranged from dense amorphous masses, to loose granular clusters of varying size. Neurons containing perinuclear clusters were seen frequently as were cells containing disseminated granules. The occasional cell containing bipolar pigment clusters was seen.

The neurons of the vestibular nuclei of 13 year old subjects contained heavy pigment deposits. Pigment distribution was essentially the same as in 12 year old specimens. The predominant pigment distribution was that of axonal clusters of granules and amorphous masses of pigment material. Cell bodies containing perinuclear pigment clusters were seen frequently. Many cells containing dispersed pigment granules were encountered.

5. Mesencephalic nucleus of the trigeminal nerve (Fig. 13)

Pigment was not present in this nucleus until the age of 4 years and then only in negligible amounts. The pigment existed as small polar aggregations. Approximately 4 per cent of the cells contained identifiable amounts of pigment.

The neurons of 7 and 8 year old specimens of this nuclear group did not appear to contain more pigment than at 4 years of age; pigment accumulations were seen in the region of the axon hillock and occurred as small loose clusters. Although the cells did not appear to contain more pigment than at 4 years of age, more cells contained pigment; 4 per cent of the cells containing pigment at 4 years, 12 per cent at 7 years, and 34 per cent at 8 years of age, a three-fold increase in pigment for each time interval.

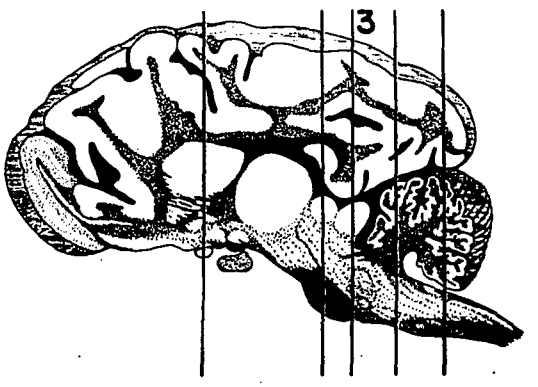
At 12 years of age, the cell bodies of the mesencephalic nucleus of the trigeminal nerve was heavily pigmented. Polar aggregations of pigment predominated. The pigment occurred as dense, amorphous masses. Occasional perinuclear clusters of pigment and pigment-free cells were seen.

Cells of this nucleus at 13 years were heavily pigmented. Dense amorphous masses of pigment in the region of the axon hillock were characteristic at this age.

Fig. 13. Distribution of lipofuscin pigment in the canine mesencephalic nucleus of the trigeminal nerve

Row 2 - 2.5 years of age
Row 3 - 8.0 years of age
Row 4 - 13.0 years of age

Left column - 250x; right column - 400x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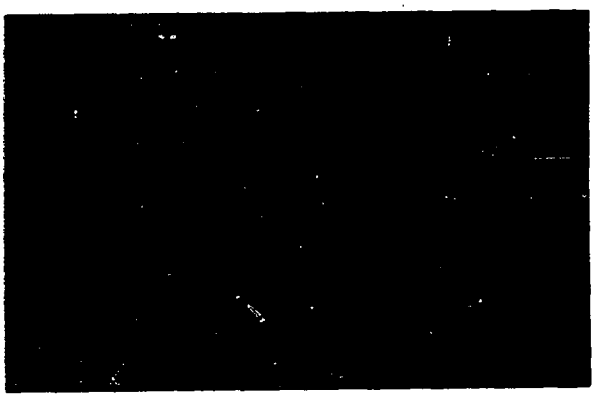


Fig. 14—PER CENT PIGMENTED NEURONS IN THE CANINE MESENCEPHALIC NUCLEUS OF THE TRIGEMINAL NERVE

FROM 2 TO 13 YEARS OF AGE

6. Tegmental nucleus (of von Gudden) (Fig. 15)

No pigment was evident in this nuclear group until the age of 4 years. At that age approximately 4 per cent of the cells contained small accumulations of discrete pigment particles.

Neuronal pigment accumulations in 7 and 8 year old specimens were considered to be heavy due to the small size of the cells. Disseminated pigment particles predominated in 7 year old specimens, while in 8 year old subjects, polar and perinuclear clusters were more evident.

Pigment accumulations in 12 and 13 year old specimens were similar, although more pigment-containing cells were present at 13 years. Axonal and perinuclear clusters of pigment and cells containing disseminated pigment particles were in equal evidence in both age groups.

7. Trochlear nucleus (Fig. 17)

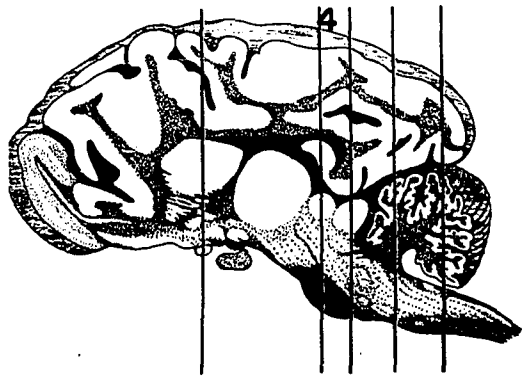
Pigmentation of the neurons of this nucleus was not evident until the age of 4 years. Occasional cells contained small, discrete axonal pigment clusters.

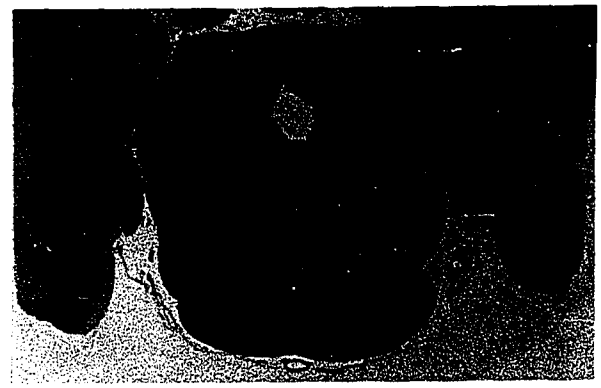
The cell bodies of 7 and 8 year old specimens contained small amounts of pigment which occurred as individual granules evenly dispersed in the cytoplasm. Occasional cells containing perinuclear pigment clusters and small axonal aggregations

Fig. 15. Distribution of lipofuscin pigment in the canine tegmental nucleus

Row 2 - 2.5 years of age
Row 3 - 8.0 years of age
Row 4 - 13.0 years of age

Left column - 250x; right column - 400x





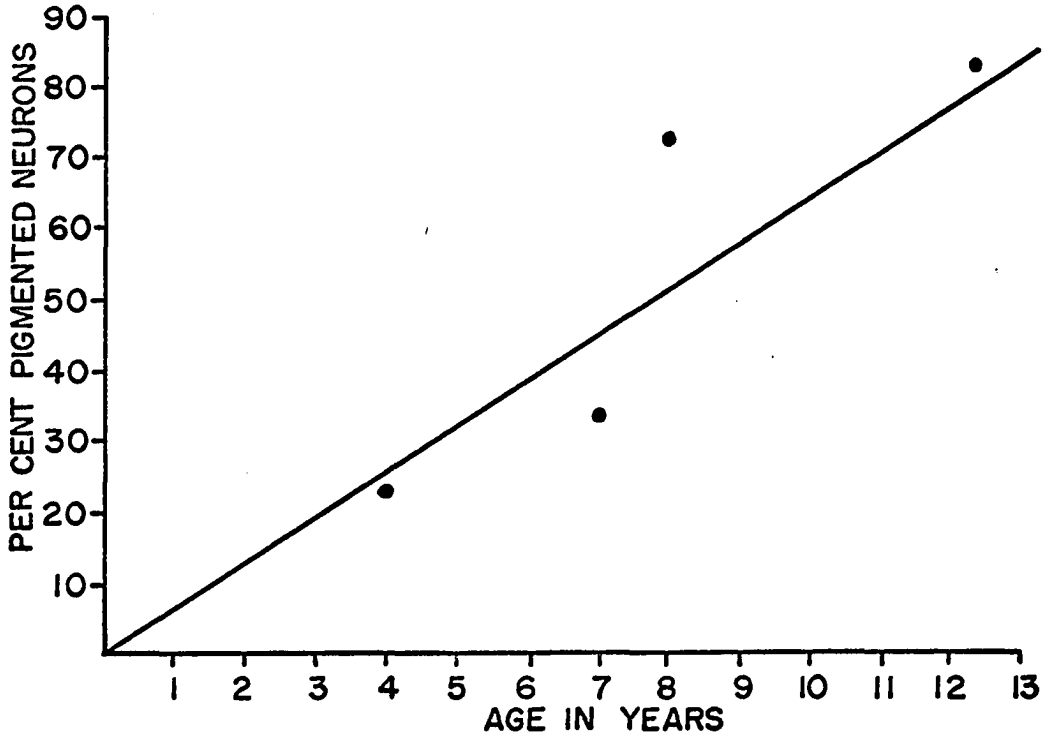


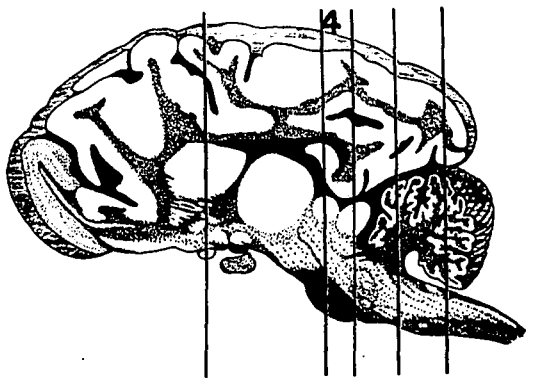
Fig. 16—PER CENT PIGMENTED NEURONS IN THE CANINE TEGMENTAL NUCLEUS

FROM 2 TO 13 YEARS OF AGE

Fig. 17. Distribution of lipofuscin pigment in the canine trochlear nucleus

Row 2 - 2.5 years of age
Row 3 - 8.0 years of age
Row 4 - 13.0 years of age

Left column - 250x; right column - 400x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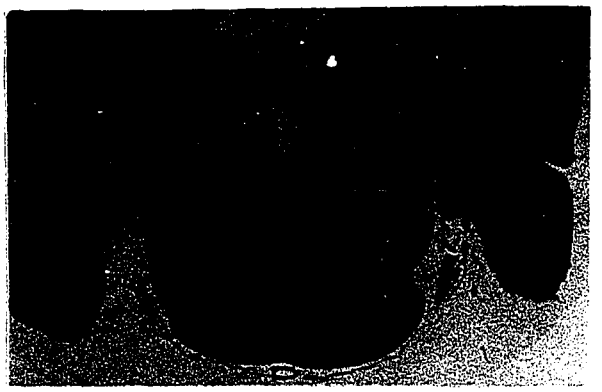
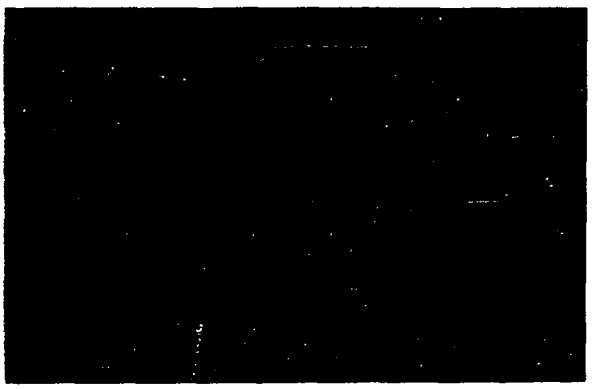




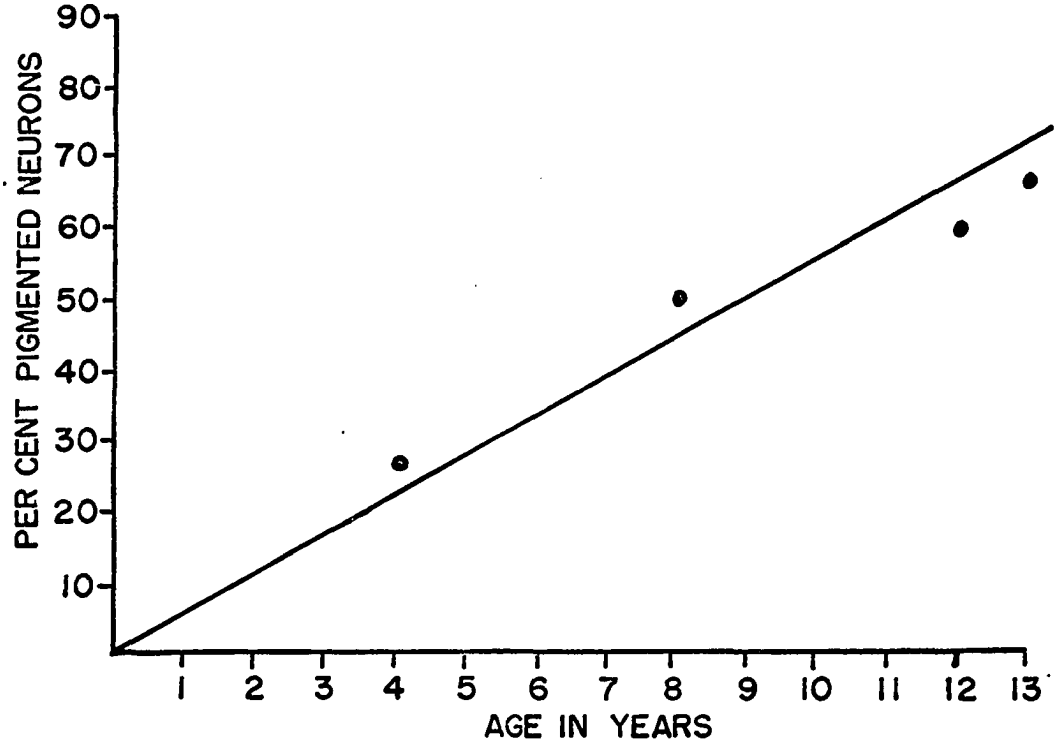


Fig. 18—PER CENT PIGMENTED NEURONS IN THE CANINE TROCHLEAR NUCLEUS

FROM 2 TO 13 YEARS OF AGE

were seen.

Of the nuclei reported in this study, the neurons of the trochlear nucleus in 12 and 13 year old subjects contained less pigment per cell than any other as judged by the presence of individual granules instead of amorphous masses of pigment in the region of the axon hillock, less compact perinuclear clusters, and fine dispersed granules in other cells. Occasional pigment-free neurons were also seen.

8. Oculomotor nucleus (Fig. 19)

Only the main oculomotor nucleus was considered in this study. Pigmented neurons were first detected in specimens of 2.5 years of age (Fig. 19). These pigmented cells were encountered rather frequently; the pigment occurred as small axonal clusters.

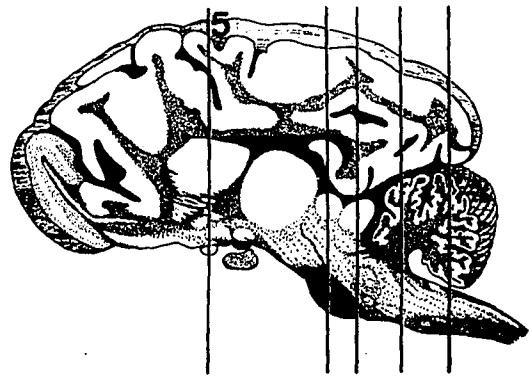
Neuronal pigmentation in this nucleus in 4 year old specimens was present in about 26 per cent of the cells. Of particular note was the absence of axonal and perinuclear pigment collections; instead, the cells contained large, coarse pigment granules which left one with the impression that these granules were on the verge of flocculating.

Eight year old specimens contained 62 per cent pigmented cells. The pigment occurred predominantly as axonal aggregations. Occasional cells contained bipolar pigment accumulations. Cells exhibiting perinuclear pigment clusters were a

Fig. 19. Distribution of lipofuscin pigment in the canine oculomotor nucleus

Row 2 - 2.5 years of age
Row 3 - 8.0 years of age
Row 4 - 13.0 years of age

Left column - 250x; right column - 400x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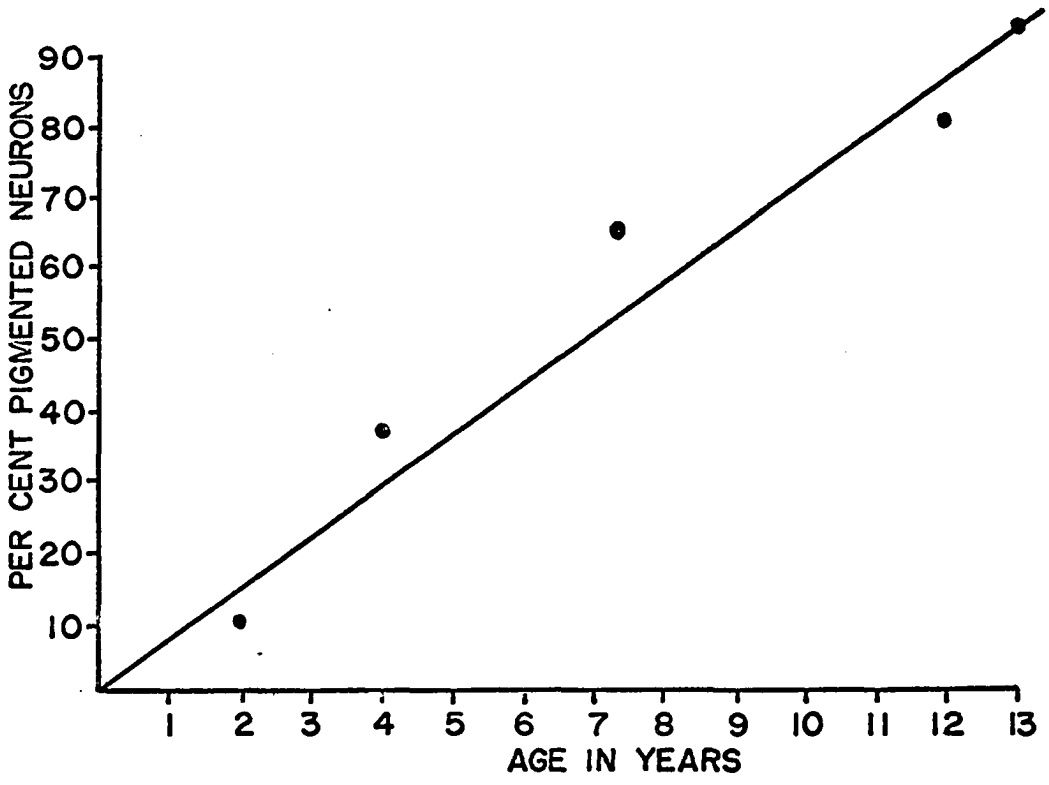


Fig. 20—PER CENT PIGMENTED NEURONS IN THE CANINE RED NUCLEUS

FROM 2 TO 13 YEARS OF AGE

frequent occurrence.

At 12 years of age, the cells of the oculomotor nucleus were heavily pigmented. Seventy-two per cent of the cells contained pigment, which occurred as dense, intensely staining, amorphous masses in the region of the axon hillock. Frequent bipolar pigment deposits were seen.

Virtually all cells of this nucleus contained pigment to some degree by the age of 13 years. The pigment was of the individual, discrete particle type, evenly distributed in the cytoplasm of the cell. Aggregations of particles in the region of the axon hillock were common; occasional bipolar pigment collections were also noted.

9. Red nucleus (Fig. 21)

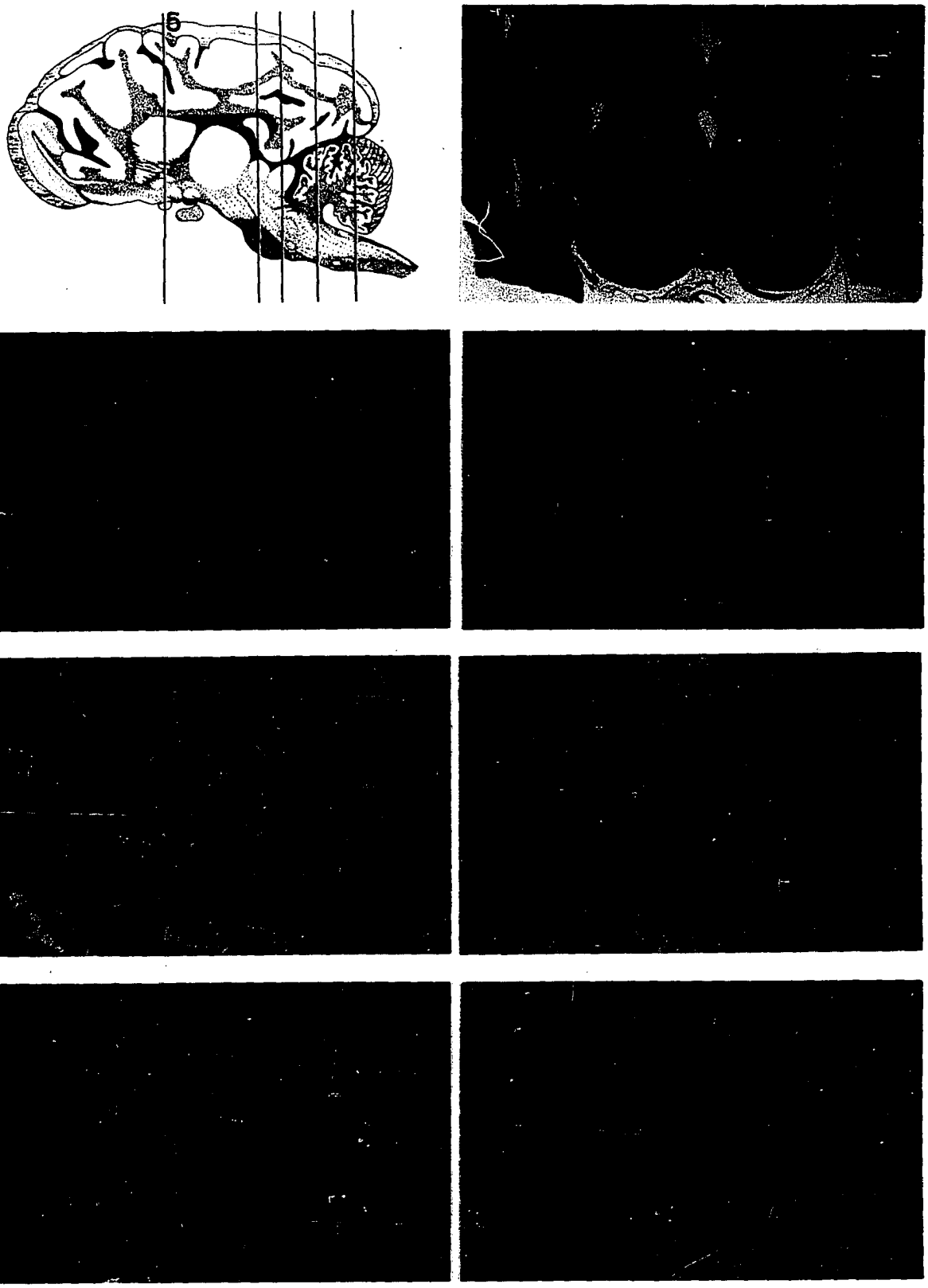
Pigment-containing neurons were first noted at 2.5 years of age. The pigment occurred as discrete particles some of which were of considerable size. The particles were evenly distributed in the cytoplasm in most instances; however, there were numerous cells in which the particles collected in loose aggregations at the axon hillock or in loose perinuclear clusters.

Four year old specimens were considered to be heavily pigmented considering the age of the subject and pigment content of other nuclei in this age group. Pigment was present as axonal collections and disseminated granules about equally.

Fig. 21. Distribution of lipofuscin pigment in the canine red nucleus

Row 2 - 2.5 years of age
Row 3 - 8.0 years of age
Row 4 - 13.0 years of age

Left column - 250x; right column - 400x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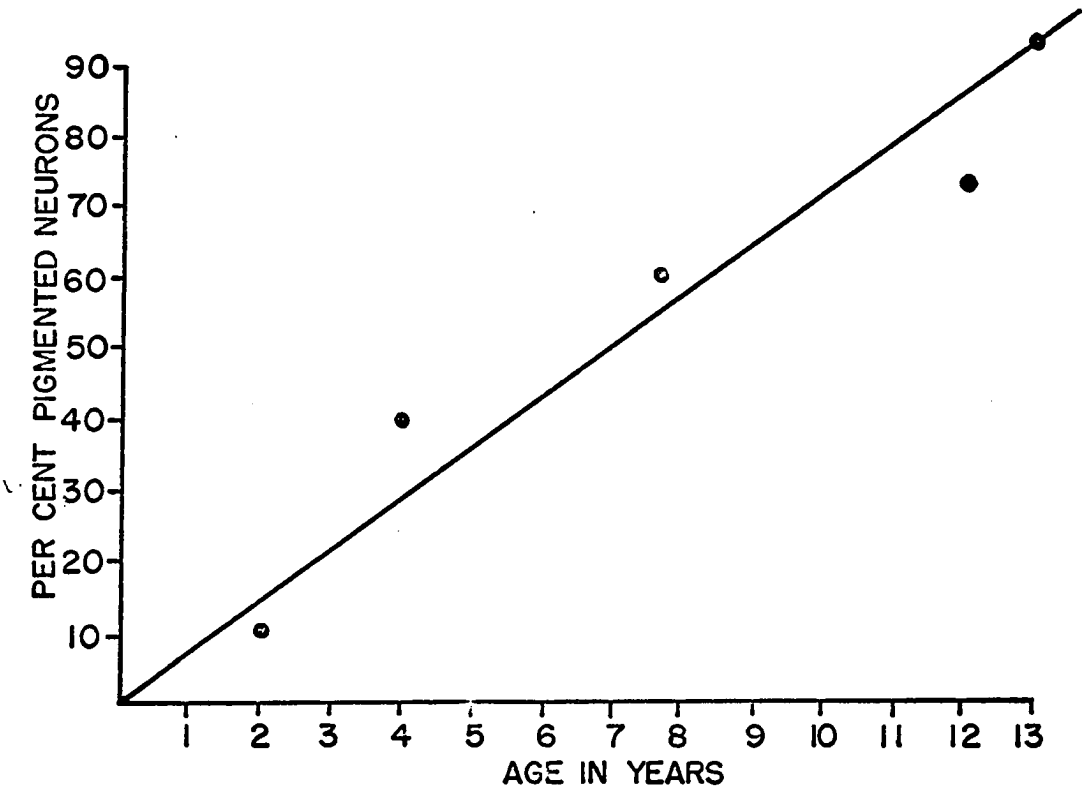


Fig. 22 — PER CENT PIGMENTED NEURONS IN THE CANINE OCULOMOTOR NUCLEUS

FROM 2 TO 13 YEARS OF AGE

Sixty-two per cent of the neurons in the 7 and 8 year old specimens contained pigment, most of which was present as axonal collections. There were, however, numerous cells containing disseminated pigment particles. Occasional cells containing bipolar pigment aggregations were encountered.

Virtually all neurons of the red nucleus of 12 and 13 year old subjects contained large quantities of pigment. Although occasional bipolar pigment accumulations were seen, axonal and perinuclear pigment collections were more common. These clusters consisted of very darkly stained, individual particles. In addition, the cells containing axonal and perinuclear pigment clusters also contained many evenly dispersed particles. The occurrence and distribution of pigment in this nucleus was the most striking of the study.

10. Purkinje' cells of the cerebellum

Lipofuscin pigment was not observed in the Purkinje' cells in specimens examined in this study.

B. Occurrence and Distribution of Lipofuscin in the Porcine Brain

1. Hypoglossal nucleus

The hypoglossal nucleus was free of pigment until the age of 3 years - 4 months at which time pigment was present in moderate amounts. Axonal aggregations were present in the

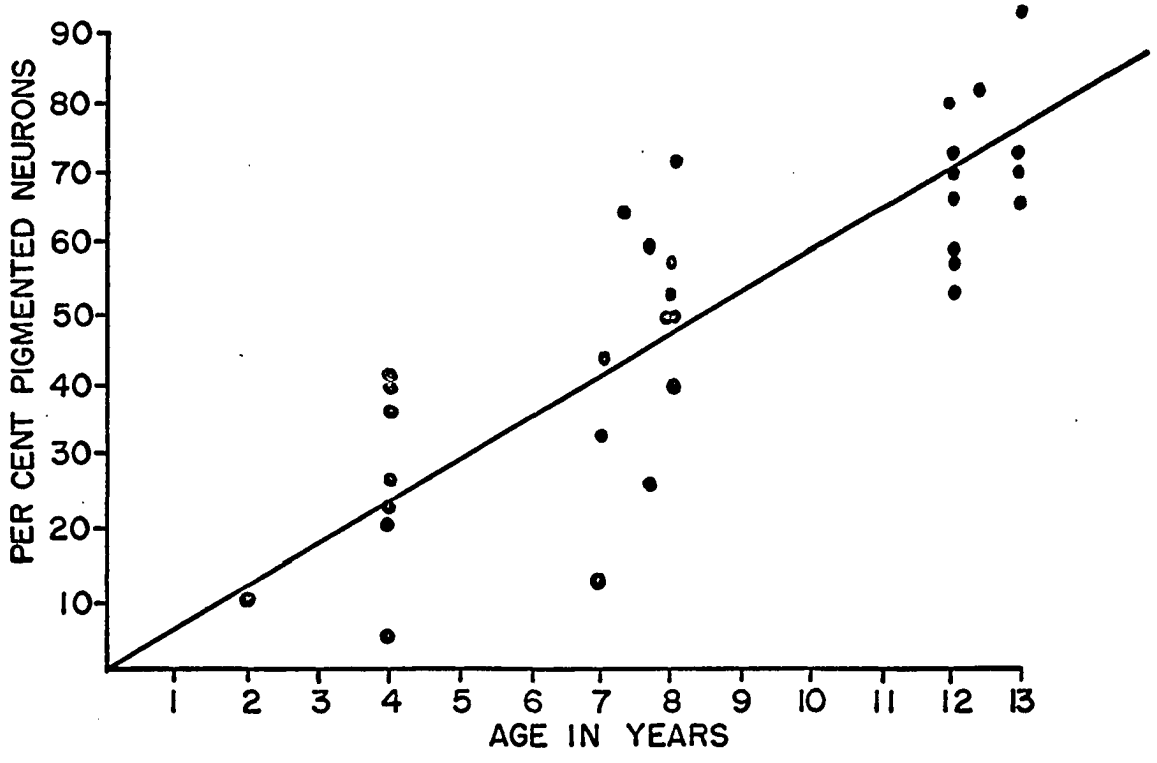


Fig. 23—PER CENT PIGMENTED NEURONS IN EIGHT CANINE BRAIN STEM NUCLEI

FROM 2 TO 13 YEARS OF AGE

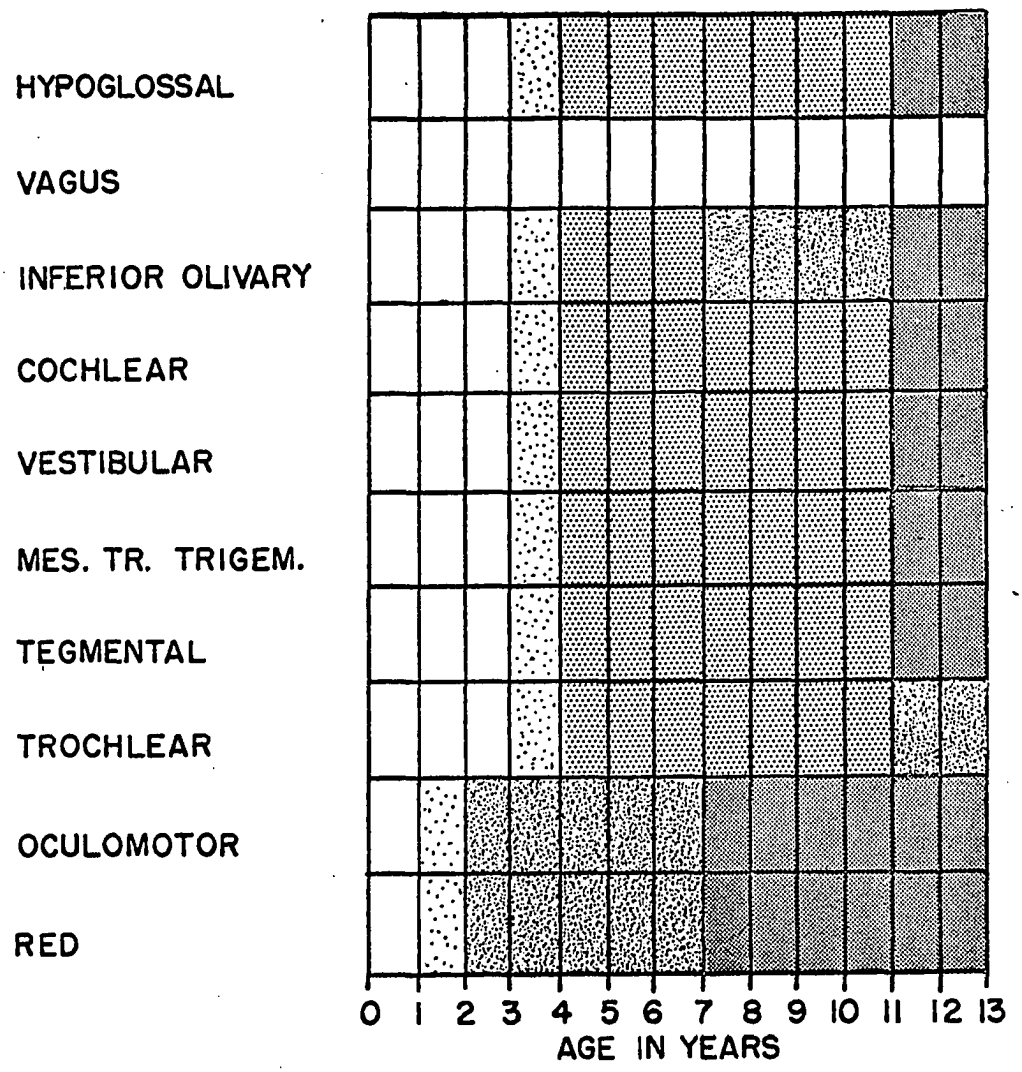


Fig. 24. Occurrence of lipofuscin pigment in ten brain stem nuclei of the canine brain (density of stippling indicates relative degree of pigmentation)

form of loose and compact collections; many cells contained disseminated pigment granules. By the age of 4 years, this nucleus contained large numbers of pigmented cells; the pigment existed as dense, granular, perinuclear clusters. Numerous cells containing axonal aggregations were seen. The hypoglossal nucleus from 6 year old specimens contained many cells which exhibited disseminated pigment particles and numerous cells containing axonal pigment aggregations.

2. Inferior olivary nucleus

Pigment was not evident in the specimens examined until the age of 4 years. Three specimens of this age group contained moderate amounts of polar pigment aggregations. Occasional perinuclear clusters were observed. Two 6 year old specimens of the inferior olivary nucleus were examined. Virtually all cells contained pigment. Approximately equal numbers of cells contained axonal and perinuclear clusters of pigment granules. Many cells contained disseminated pigment particles.

3. Vestibular nuclei

No distinction was made between the vestibular nuclei. Pigment-containing cells of these nuclei were seen at 2 years-3 months of age. Lipofuscin pigment was present in slight amounts as disseminated particles. Occasional cells contained

very small, discrete axonal aggregations. At 3 years-9 months of age, axonal aggregations were more apparent, and by the age of 4 years-1 month, the cells were heavily pigmented. Neurons of these nuclei from 6 year old subjects were heavily pigmented, the pigment occurring as loose and compact perinuclear clusters; occasional axonal collections and many cells containing disseminated pigment granules were observed.

4. Trochlear nucleus

Pigment-containing cells in this nucleus were not apparent until the age of 3 years-8 months. The pigment was not excessive in amount, appearing as fine, discrete particles. The occasional cell appeared to have an increased pigment density about the nucleus. At 4 years-1 month of age, most cells of this nucleus contained disseminated pigment granules; occasional cells contained perinuclear clusters of pigment particles.

5. Oculomotor nucleus

Occasional cells containing small amounts of disseminated pigment granules were observed in this nucleus in 2 year old subjects. By the age of 3 years, virtually all cells of this nucleus contained fine showers of pigment particles; occasional cells contained potential clusters of granules near the axon hillock. Neurons of this nucleus from 4 year old sub-

jects contained moderately heavy accumulations of disseminated pigment. Occasional cells contained loose perinuclear clusters of pigment granules. The oculomotor nucleus of 6 year old subjects was made up of cells, most of which contained large amounts of pigment as individual granules. Moderate numbers of these cells also contained axonal or perinuclear pigment aggregations.

6. Red nucleus

While pigment was absent in 1 year old subjects, considerable numbers of cells contained disseminated pigment granules in subjects 2 years-5 months of age. Frequently, cells containing small, loose axonal pigment collections were encountered. Virtually all neurons of this nucleus in 3 year old specimens contained disseminated pigment granules. There was also a marked tendency to perinuclear clumping in that the disseminated particles appeared to be more concentrated near the periphery of the cell nucleus. There were occasional cells which contained axonal pigment granules.

At the age of 4 years, numerous neurons of the red nucleus contained axonal pigment aggregations; occasional cells contained bipolar pigment collections. Perinuclear clusters were also seen occasionally. The red nucleus of 6 year old specimens was more heavily pigmented than the oculomotor nucleus at that age. Virtually all cells contained

disseminated pigment granules. In addition to disseminated pigment granules, many of these cells also contained perinuclear or axonal pigment collections. Occasional cells contained bipolar pigment aggregations and the occasional pigment-free cell was observed.

7. Purkinje cells of the cerebellum (Fig. 25)

Pigment-containing Purkinje cells were observed in two 4 year old specimens. Twenty-eight per cent of the cells contained scanty amounts of fine polar pigment granules.

Two 6 year old specimens examined contained 44 per cent pigmented cells. The pigment was again scanty in amount and had a polar orientation.

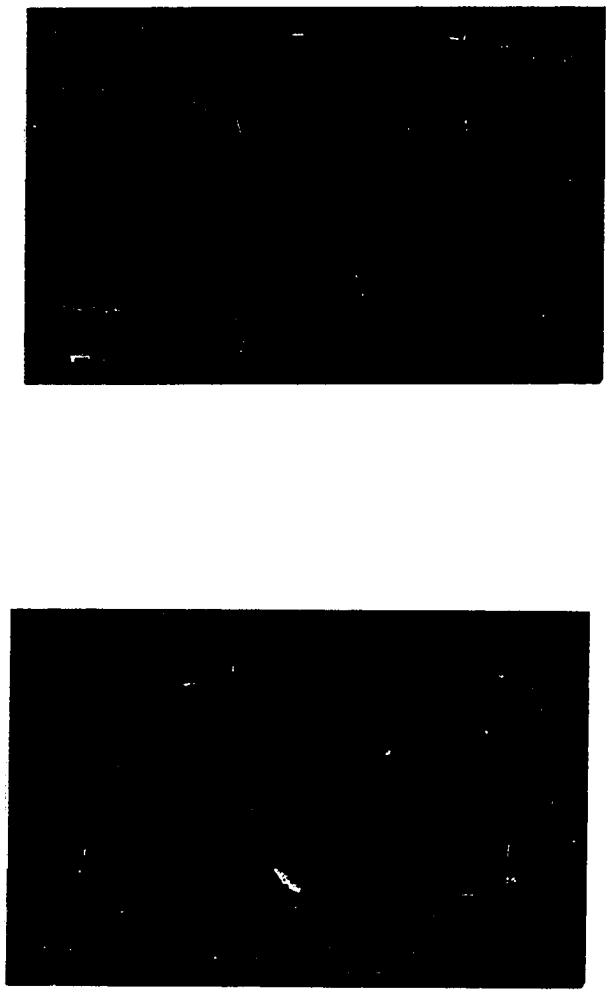


Fig. 25. Pigmented Purkinje cells in the porcine cerebellum, 250x and 1000x

Fig. 26. Lipofuscin deposition in the porcine brain - age: 3 years

Row 1 - Hypoglossal nucleus
Row 2 - Vestibular nucleus
Row 3 - Red nucleus
Row 4 - Main oculomotor nucleus

Left column - 250x; right column - 400x

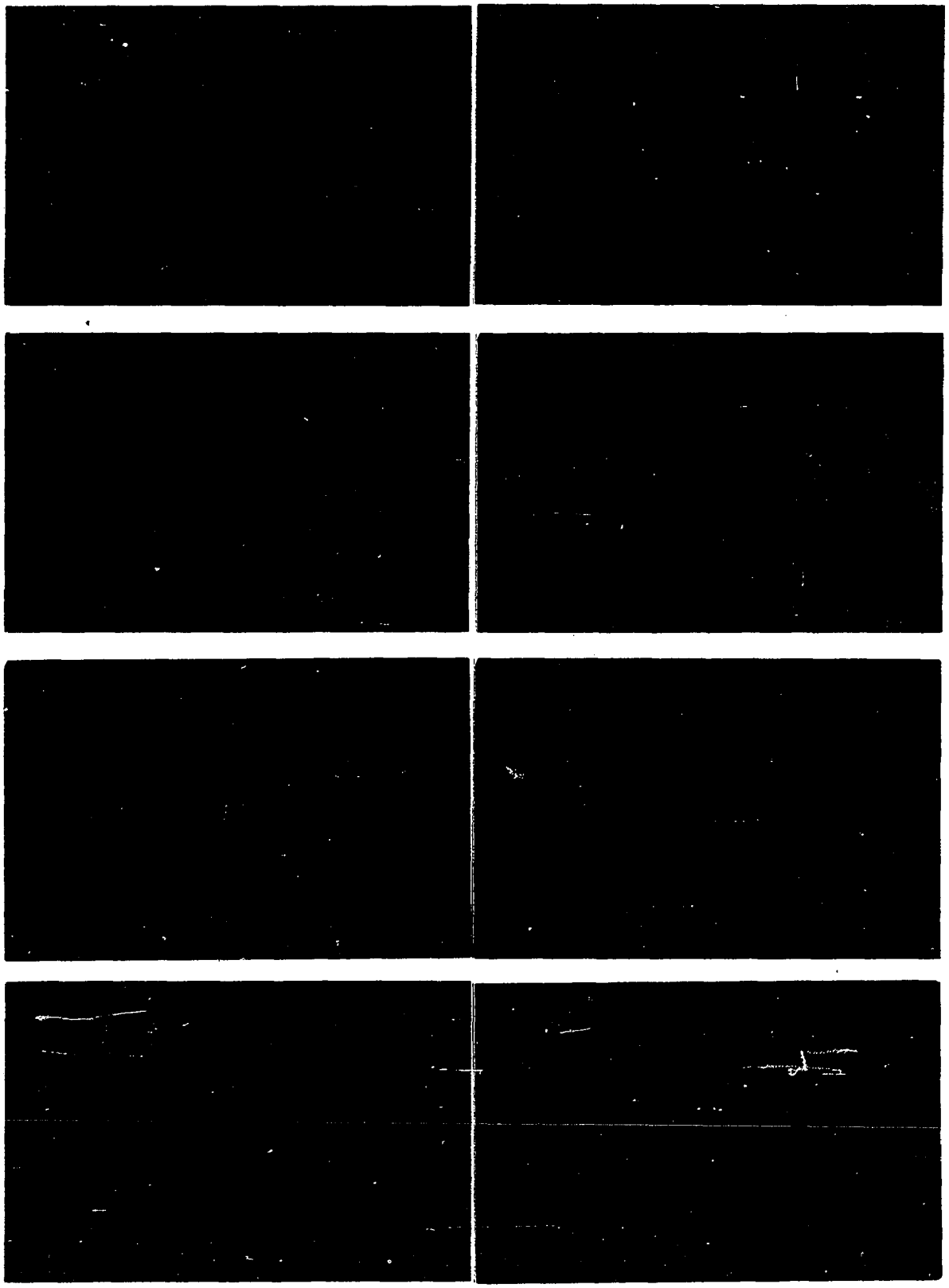
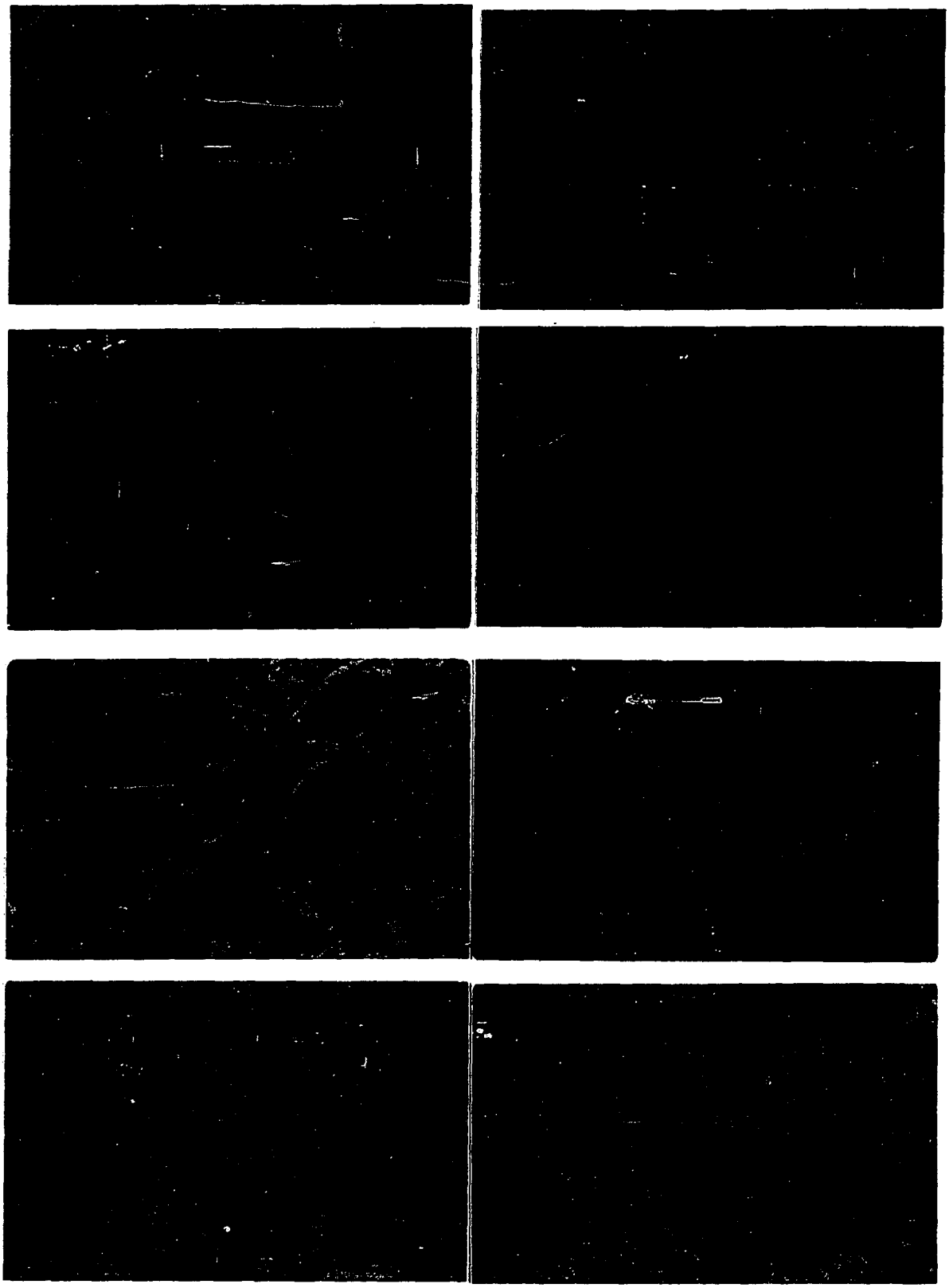


Fig. 27. Lipofuscin deposition in the porcine brain - age: 6 years

Row 1 - Hypoglossal nucleus
Row 2 - Vestibular nucleus
Row 3 - Red nucleus
Row 4 - Main oculomotor nucleus

Left column - 250x; right column - 400x



V. DISCUSSION

A. Occurrence and Distribution of Lipofuscin in the Canine and Porcine Brain

A review of the available literature indicated that age-associated deposition of lipofuscin pigment has been reported in the major parts of the nervous system in many animals including man. On the basis of a detailed study of the occurrence and distribution of lipofuscin pigment in eight brain stem nuclei together with prefunctory examination of other sites of pigment deposition, it was apparent that lipofuscin was widely distributed throughout the aging nervous system of canine and porcine specimens considered in the present study.

Pigment visualized by the methods employed in this study, was not present in significant amounts prior to the age of 2 years. This observation was in agreement with Pilez (1895) and Obersteiner (1903) who observed that pigment was absent in the newborn human. Strehler et al. (1959) stated that lipofuscin in cardiac muscle fibers was absent in the very young, and present without exception in the aged specimens they examined. That lipofuscin deposition may occur at an earlier age cannot be discounted. Munnell (1964) in preliminary studies has observed electron-dense granules resembling lipofuscin in cardian muscle fibers of 8 month old dogs collected from the same series of animals under discussion.

The amount of pigment in the individual neuron appeared to increase from the age of 2 years to 13 years in the dog and from 2 years to 6 years in the pig. Mühlmann (1910) noted this age-wise increase in cellular pigment in studies on ganglion cells of the guinea-pig and man. Brody (1955), in studies of pigment deposition in human cerebral cortical cells, claimed that while the number of cells containing pigment increased with age, the actual number of cells containing large accumulations of pigment was relatively low. On the other hand, Wahren (1957) noted that in man, the cells of the pallidum were practically full of pigment by the end of the third decade.

In the present study, the time of appearance of the pigment was not constant. Among the eight nuclei studied, the red nucleus and the oculomotor nucleus in both the dog and pig exhibited pigment depositions as early as 2 years of age. Pigment accumulations in the remaining nuclei were not observed in significant amounts until 4 years of age. This observation was in agreement with the view of C. and O. Vogt (1946) who stated that the sequence of morphological, age-associated changes were characteristically different for each cell type. Wahren (1957) concurred in this opinion, stating that in man, the onset and distribution of intracellular pigment differed in the pallidum and the nucleus tuberomammillaris and nucleus tuberolateralis of the hypothalamus.

It was observed that the number of cells containing pigment increased with age. One hundred cells of each of the eight nuclei were counted; the number of pigment-containing cells expressed as per cent was plotted against the age of the subjects. It appeared that the deposition of age pigment in the nervous system of the dog bore a linear relationship to age. This observation was in agreement with Strehler et al. (1959), in their study of lipofuscin deposition in aged human cardiac muscle. In their study, they concluded that lipofuscin was increased in cardiac muscle fibers progressively at an approximately mean rate throughout life.

The intracellular distribution of lipofuscin was considered to be of interest and significance. The pigment appeared to have three transient phases of distribution: 1) a dispersed or disseminated phase, in which the pigment granules were evenly distributed throughout the cytoplasm; 2) a perinuclear distribution, in which the granules formed loose or compact clusters, usually crescentic in form, about the cell nucleus; and 3) an axonal or polar distribution in which the pigment collected as loose or dense granules or dense amorphous masses at or near the axon hillock. Certain cells containing this last named pigment distribution possessed a second polar aggregation near the principal dendrite. These were classified as bipolar pigment aggregations. The predominant pattern of pigment distribution was considered to

be related more to the age of the subject 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. The latter was particularly true of the cochlear nuclei which contained perinuclear clusters almost uniformly at all ages and the red nucleus which was outstanding for its moderate numbers of bipolar pigment aggregations.

As a general rule, however, disseminated pigment distributions were most frequent in younger specimens. Seven and 8 year old specimens possessed more cells with perinuclear clusters of pigment, while 12 and 13 year old specimens contained predominantly polar aggregations.

Frequently, cells were encountered which contained disseminated pigment granules in addition to either perinuclear or polar aggregations. Pigment-free cells were encountered in all age groups in both species. Nuclear groups were present which were pigment-free at all ages, viz. the dorsal motor nucleus of the vagus nerve in both the dog and pig, and the Purkinje cells of the canine cerebellum.

The intracellular pigment distributions as described above were in agreement with the findings of Mühlmann (1910) who noted that the homogeneous distribution of pigment granules in ganglion cells of the guinea-pig and man was gradually lost with increasing age and noted certain cell types as having a

specific pigment distribution. Höpker (1951) noted five pigment distribution patterns in the aging cells of the dentate nucleus. Brown (1944) noted the significant numbers of cells exhibiting polar pigment aggregations and reported bipolar pigment collections in the neurons of the red nucleus of canine specimens.

Although the present investigation was concerned only with the deposition of lipofuscin, the possibility that other histologic evidences of aging might be manifest was considered. No overt evidence of nuclear alterations in size, shape, or position were observed; well-fixed sections exhibited apparently intact and undisturbed cell and nuclear outlines; there was no excessive cytoplasmic or nuclear vacuolization.

The findings in this study indicate that lipofuscin accumulations were the only consistent cytological alteration that could be correlated with age. This observation was in agreement with the views of Wilcox (1951) and Strehler (1962).

B. Histogenesis of Lipofuscin

Determination of the primary site of origin of lipofuscin was not within the scope of this investigation; however, a brief summary of the present status of knowledge on the subject will be useful as a preface to discussion of the significance of the pigment in the aging process.

The formation and/or deposition of lipofuscin is not clearly understood. At the subcellular level, several organelles have been implicated; the Golgi apparatus by Gatenby (1951) and Bondareff (1957); the mitochondria by Hess (1955), and Duncan et al. (1960); and more recently, the lysosome by DeDuve (1959) and Samorajski (1964). At the cellular level, employing light microscopy, Mühlmann (1910) noted an increase in the number of lipoidal granules in the neurons with increasing age, observing that the homogenous distribution of the granules was gradually lost and that they gathered in clusters in the cell body to form finally a localized mass which continued to increase in size. Höpker (1951), concluded that lipofuscin was formed in a "lipohilic" center located in proximity to the nucleus and described five formative stages. Matzdorf (1948) described the process of pigment deposition as beginning with fine particles of lipofuscin diffusely distributed throughout the cytoplasm; these then increased in size, darkened, and clumped, the latter differing with the cell type.

C. Significance of Lipofuscin

Irrespective of its primary site of origin, lipofuscin is considered to be a product of cell metabolism and thereby, evidence of cellular activity. That these processes produce, directly or by accident, a series of insoluble and non-

functional side-products or residues which accumulate and mechanically interfere with cellular function has been considered as a possible mechanism of aging.

In the present study, the increase in amount of intracellular pigment with age and the concomitant alterations in pigment distribution were interpreted as an evidence of cellular aging.

It has been shown that the time of pigment deposition depends upon the nuclear group concerned. The main oculomotor nucleus and the red nucleus of the dog and pig contained considerable numbers of pigmented cells as early as 2 years of age, whereas the other nuclei studied contained relatively few, if any, pigmented cells prior to the age of 4 years. This difference in time of pigment deposition was considered to be related to the level of function of the nuclear group concerned. Lower centers of the brain stem are chiefly concerned with autonomic and reflex mechanisms; barring stress conditions, they operate at fairly constant levels of activity. Higher centers, on the other hand, are characterized by varying degrees of voluntary activity and complex integrative modalities and function at increased and usually higher levels of activity. Since lipofuscin collections in cells are considered to be a product of metabolism, it seems reasonable to interpret the time of appearance of lipofuscin as a measure of neuronal function.

The observations of Wilcox (1956) were in agreement with this concept in cause but not in effect. He held the view that the presence of lipofuscin was evidence of low levels of activity and the absence of it was due to high levels of activity. In a study of lipofuscin accumulations in the central nervous system of the guinea-pig, he reported absence of pigment in the cochlear nuclei, a diametrically opposite finding for these nuclei in the canine species in the present investigation. In canine specimens, perinuclear pigment clusters were characteristic of these nuclei. The concept that pigment deposition-time is related to the level of function of a nuclear group is still valid if one considers that the guinea-pig is a lower order of mammal than is the dog. This latter view is supported by the observation of Kuhlenbeck (1954) who noted that pigmentation of cerebral cortical neurons was a less conspicuous occurrence in the rat than in man.

In view of the foregoing discussion, it would be reasonable to consider that three superimposed modalities are operating: 1) lipofuscin is deposited in a cell as a result of metabolic processes, 2) initial pigment deposition occurs in relation to the level of activity characteristic of a nuclear group, and 3) within a cell group, lipofuscin accumulates as a function of time.

It has been shown that lipofuscin pigment was absent in

the neurons of young dogs (except at subcellular levels) and that it was universally present in old subjects. Of particular interest and significance was the finding that the pigment apparently accumulated linearly with respect to age at a steady rate throughout the life-span of the animal. The pigment accumulated in clinically healthy subjects of known chronological age which were selected from an environment common to the subjects concerned. Among the six male and ten female subjects critically studied, pigment accumulations appeared to be independent of sex. Six breeds were represented; pigment accumulations also appeared to be independent of breed.

There is ample evidence in the literature that accumulations of lipofuscin in non-replaceable cell lines is an age-correlated process. That lipofuscin accumulations are a basic biological aging process has been the subject of considerable controversy.

Strehler et al. (1959) derived four tentative criteria of biologic aging. The findings in the present study are substantially in agreement with these criteria as follows:

Universality - The change should occur universally in all old animals of a species and should be essentially absent in the very young. Although the requirements of this criterion cannot be ascertained absolutely since all subjects cannot be tested, the findings in this investigation in general satisfy

the criterion. From this and other studies, Hamperl (1934), and Jayne (1950), there is no reason to doubt the consistency of the effect.

Time dependence - The change should proceed gradually in an individual (and in a population). That this criterion was satisfied was clearly indicated by the constant rate of pigment accumulation after 2 years of age.

Innateness - The change should be a consequence of the action of time on the intrinsic properties of the biological system rather than the result of preventable disease, accident, or pathology. That the requirement of this criterion was met was evident by the age-wise deposition of lipofuscin in clinically healthy subjects of known chronological age which were reared in an environment common to all subjects examined. Sulkin and Scrivanij (1960) reported experimental production of pigment in young dogs and stated that the pigment was not usually seen in this species until approximately 9 years of age. The results of the present investigation are in direct conflict with these observations. It has been shown that lipofuscin was present in at least two nuclei of the canine brain stem as early as 2 years of age and that the occasional pigment-containing cell was seen at younger ages. It would also seem that the time of pigment deposition in different functional types of neurons would have to be considered. The presence of lipofuscin in clinically healthy subjects in

the present study and the lack of correlation of pigment deposition with specific cardiovascular disease and heart failure in the study of lipofuscin deposition in cardiac muscle fibers by Strehler et al. (1959) further satisfy the criterion of innateness.

Deleteriosness - The change should be unfavorable in its effect on the survival capacity of the individual organism in its natural environment. The change should be of such magnitude that it could contribute substantially to the functional debility of an old organ and its host. From a purely anatomical point of view, the size and density of lipofuscin accumulations in the neurons of older specimens in this study would be likely to reflect or cause some impaired function. It has been shown that in older subjects and more active nuclear groups, that lipofuscin collects in the region of the axon hillock; it is entirely conceivable that such a large aggregation of pigment granules could interfere with the propagation of the nerve impulse. The apparent rigidity and insolubility of the pigment was evident in the present study in which several sections of porcine brain material exhibited areas of post-mortem autolysis. The neurons in these areas evidenced varying degrees of autolysis while the pigment contained therein was undisturbed. In adjacent, well-fixed areas, the pigment was visually identical with that in areas of autolysis. Strehler et al. (1959) made this observation

in cardiac muscle fibers collected at varying periods of time between death of the subject and fixation of the tissue. The granules have been reported to be relatively solid, Hyden and Lindstrom (1950), and insoluble, Connor (1928), Jayne (1950) and Pearse (1961). Murray and Stout (1947) in a tissue culture study of the human ganglion cells observed the deposition of pigment over a period of several weeks. They reported that cells containing large amounts of pigment did not migrate and that their nuclei frequently lost their staining properties; cells containing lesser amounts of pigment would migrate only short distances. These investigators concluded that the pigment may be detrimental to the cell by virtue of its being a rigid mass interfering with the plasticity of the cell.

Because of its absence in the very young animal, its presence in the clinically healthy subject, and its large amounts in the neurons of older dogs, it seems that accumulations of lipofuscin meets the criteria of a basic biological aging process.

VI. SUMMARY

An investigation was conducted to determine the occurrence, distribution, and significance of lipofuscin age pigment in the central nervous systems of the dog and pig.

Thirty-eight canine specimens ranging in age from birth to 13 years of age, and 15 pig specimens ranging in age from 1 year to 6 years of age were utilized in the study. Birth records and other vital statistics were obtained for all animals. At the time of death, the animals appeared to be normal, healthy subjects, exhibiting no clinical evidence of disease.

Death was caused by electrocution. Immediately thereafter, each brin was removed from the cranial vault and fixed in 10 per cent neutral buffered formalin. Tissue blocks representing the medulla oblongata, pons, cerebellum, mesencephalon, and diencephalon were embedded in paraffin, sectioned and stained for lipofuscin pigment. Eight brain stem nuclei (the hypoglossal, inferior olivary, cochlear, vestibular, tegmental, trochear, red, and oculomotor) and the cerebellar Purkinje' cells were critically examined.

Lipofuscin age pigment was found to be widely distributed throughout the aging central nervous systems of the canine and porcine specimens examined. Employing light microscopy, pigment was first evident at the age of 2 years in the red and main oculomotor nuclei of the dog and pig. Pigment occurred

at later ages among the other nuclei examined.

The intracytoplasmic pigment granules varied in pattern of distribution, the pattern of which seemed to be related more to the age of the subject than to the functional type of neuron in which it occurred. The amount of pigment contained within the individual cell, and the number of cells containing pigment increased with age and appeared to be independent of breed and sex.

The subjects utilized in this study came from an environment common to all specimens of a species. This factor and the apparent absence of clinically demonstrable disease suggested that lipofuscin pigment was a normal product of cellular metabolism.

The time of initial appearance of the pigment seemed to be related to the functional type of neuron in which it occurred; its continued increase, however, bore a linear relationship to the age of the subject. Pigment was present in large amounts in older subjects; the gradually increasing quantity of which should eventually interfere with normal cellular activity.

Because of its absence in the very young, its presence without exception in the old, its presence in the clinically healthy subject, and its large amounts in aging nervous tissue, it seems that the accumulation of lipofuscin constitutes a basic biological aging process.

VII. CONCLUSIONS

1. Stained by the Nile blue method, lipofuscin pigment appeared as dark, blue-green granules within the cytoplasm of neurons of the canine and porcine brain.
2. A study of brain sections prepared from canine specimens ranging in age from birth to 13 years, and from porcine specimens ranging in age from 1 year to 6 years, indicated that lipofuscin was widely distributed throughout the aging nervous systems of these species.
3. Neuronal lipofuscin granules presented three basic patterns of distribution; these distribution patterns could be correlated with the age of the subject.
4. The pigment was essentially absent in very young subjects, appearing first in significant amounts at the age of 2 years.
5. From the age of 4 years, pigment was consistently present in all specimens examined.
6. The amount of lipofuscin present in the individual neuron, and the number of neurons containing pigment, increased linearly with age at a steady rate.
7. The presence of lipofuscin in clinically healthy subjects suggested that the pigment was a normal product of cell metabolism.
8. The presence of lipofuscin pigment was the only consistent cellular alteration that could be correlated with

age in the specimens studied.

9. The age at which pigment first became apparent varied with different nuclear groups; the variation in time of appearance of pigment was considered to be related to the functional type of neuron concerned.

10. The occurrence and distribution of lipofuscin pigment in the aging canine and porcine central nervous systems conformed in most respects to the criteria set forth for a basic biological aging process.

VIII. BIBLIOGRAPHY

Alpert, M., Jacobwitz, D., and Marks, B. H. 1960. A simple method for the demonstration of lipo-fuchsin pigments. J. Histochem. Cytochem. 8: 153-158.

Altschul, R. 1938. Ueber das segemente "Alterspigment" der Nervenzellen. Virchow's Archiv f. Path. Anat. 301: 275-286.

_____ 1943. Lipofuscin distribution in the basal ganglia. J. Comp. Neurol. 78: 45-57.

Andrew, W. 1936. Nissl substance of the Perkinje' cells in the mouse and rat from birth to senility. Zeitsch. f. Zellforsch. u. mikr. Anat. 25: 583-604.

_____ 1938. Perkinje' cell in man from birth to senility. Zeitsch. f. Zellforsch. u. mikr. Anat. 27: 534-554.

_____ 1939. Golgi apparatus in the nerve cell of the mouse from youth to senility. Am. J. Anat. 64: 351-376.

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

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

_____ 1955. Amitotic division in senile tissues as a possible means of self-preservation. J. Geront. 10: 1-12.

_____ 1956a. Structural alterations with aging in the nervous system. In Moore, J. E., H. H. Merritt, and R. J. Masselink, eds. Neurologic and psychiatric aspects of the disorders of aging. Vol. 35. pp. 129-170. Williams and Wilkins, Baltimore.

_____ 1956b. Mitochondria of the neuron. Rev. Cytol. 5: 147-170.

_____ 1959. Reality of age differences in the nervous system. J. Gerontol. 14: 259-267.

_____ 1961. The aging process and the cell. In Bourne, G. H., ed. Structural aspects of aging. p. 204. Hafner Publishing Co., Inc., New York.

_____ and Andrew, N. V. 1940. Comparison of the changes caused by fatigue and by aging in the cerebral cortex of man. J. Comp. Neurol. 22: 525-533.

Andrew, W., Piper, P., Northrop, R., Trenik, B., and Bjorksten, J. 1962. Observations on age pigment in the hearts of stillborn babies. J. Am. Geriat. Soc. 10: 649-652.

Andrew, W., Shock, N. W., Barrows, C. H., and Yiengst, M. J. 1959. Correlation of age changes in histological and chemical characteristics in some tissues of the rat. J. Gerontol. 14: 405-414.

Balthazar, K. 1949. Anatomie und Lebensgeschichte der Risenzellen und der grossen Pyramidenzellen in der V schicht der Area gigantico-pyramidalis. Nervenarzt. 20: 490. Original not available; cited by Birrens, J. E. 1959. Handbook of aging and the individual. p. 149. University of Chicago Press, Chicago.

_____ 1952. Zur topik der Lipofuscinvolution und Cerebroidspeichrung der grossen Zellen in der V schicht der Area gigantico pyramidalis. J. Nerv. and Mental Dis. 116: 633-645.

Barka, Tibor and Anderson, Paul J. 1963. Histochemistry: theory, practice and bibliography. Hoeber Division, Harper and Row, Inc., New York.

Berg, J. W. 1953. Acid-fastness as a histochemical test. J. Histochem. 1: 436-441.

Bethe, A. and Fluck, M. 1937. Über das gelbe Pigment der Ganglion-zellen, seine kolloid-chemischen und topographischen Beziehungen zu andern Zellstructuren und eine elektive Methode zu seiner Darstellung. Zeitsch. f. Zellforsch. u. mikr. Anat. 27: 211-221.

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

_____, Imus, Henry A., and Windle, William F., ed. 1959. Process of aging in the nervous system. Charles C. Thomas, Springfield, Illinois.

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

Bommer, S. 1929. Weitere untersuchungen über sichtbase floureszenz beim menschen. Acta dermat.-venereol. 66: 391-445. Original not available; cited by Strehler, Bernard L. and Mark, Donald D. 1959. Rate and magnitude of age pigment accumulation in human myocardium. J. Gerontol. 14: 430.

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

_____ 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.

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

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

_____ 1960. Deposition of aging pigment in the human cerebral cortex. J. Gerontol. 15: 258-261.

Broszek, J. and Simonson, E. 1962. Russian research in aging. Geriatrics 17: 464-476.

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.

_____ 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 und 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.

Cammermeyer, Jan. 1963. Cytological manifestation of aging in rabbit and chinchilla braina. J. Gerontol. 18: 41-54.

Chu, L. A. 1954. Cytological study of anterior horn cells isolated from human spinal cord. J. Comp. Neurol. 100: 381-413.

Connor, Charles L. 1928. Studies on lipofuscins. IV. The nature of the pigment in certain organs. Am. J. Path. 4: 298-208.

Cowdry, E. V. 1952. Problems of aging. 3rd ed. Williams and Wilkins Co., Baltimore.

D'Angelo, C., Issiodorides, M., and Shanklin, W. 1956 Comparative study of the staining reactions of granules in the human neuron. J. Comp. Neurol. 106: 487-499.

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

Dixon, K. C. and Herbertson, B. M. 1950. Clusters of granules in human neurones. J. Path. and Bact. 62: 335-339.

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

_____ and Guthrie, F. V. 1918. Pigmentation of nerve cells. J. Med. Res. 34: 123-142.

Duncan, Donald D., Nall, D., and Morales, R. 1960. Observations on the fine structure of old age pigments. J. Gerontol. 15: 568-572.

Einarson, L. 1952. Fluorescent acid-fast substances in the nervous system of adult rats in chronic vitamin-E deficiency. Ugeskrift Leager. 114: 1186-1190. Original not available; cited by D'Angello, Carmine et al. 1956. Comparative study of the staining reactions of granules in the human neuron. J. Comp. Neurol. 106: 494.

Ellis, M. S. 1920-21. Normal for some structural changes in the human cerebellum. J. Comp. Neurol. 32: 1-33.

Evans, H. M. and Schuleman, W. 1914. The action of vital stains belonging to the benzedine group. Science 39: 443-454.

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

Gatenby, J. Bronte. 1951. Neurones of the human autonomic system and the so-called senility pigment. J. Physiol. 114: 252-254.

Gedigk, P. and Bontke, E. 1956. Über den nachweis von hydolytiischen Enzymen in Lipopigmenten. Z. Zellforsch. 44: 495-518.

Hammerbeck, W. 1960. Die Fuscinkorner (Abaukorner) des menschlichen Hersmuskels. Zbl. allg. Path. Anat. 100: 305.

Hamperl, H. 1934. Die fluoreszenzmicroscopiie menschlichen gewebe. Virchow's Arch. f. Path. Anat. 292: 1-51.

Harms, J. W. 1924. Morphologische und experimentelle untersuchungen am altern hunden. Zeitsch. f. Anat. Entwicklungsgesch. 71: 319-382.

Heidenreich, O. and Siebert, G. 1955. Untersuchungen an isolierten, unveranderten Lipofuscin aus Herz muskultar. Virchow's Arch. f. Path. Anat. 327: 112-126.

Hendley, D. D., Mildvan, A. S., Reporter, M. C. and Strehler, B. L. 1963. Properties of isolated human cardiac age pigment. 1. Preparation and physical properties. J. Gerontol. 18: 144-150.

Hermann, H. 1952. Zusammenfassende Ergebnisse über Altersveranderungen 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.

Hess, Arthur. 1955. Fine structure of young and old spinal ganglia. Anat. Rec. 123: 399-425.

Hodge, C. F. 1894-1895. Changes in human ganglion cells from birth to senile death. Observations on man and honeybees. J. Physiol. (London) 17: 129.

Höpker, W. 1951. Das Altern des Nucleus dentatus. Zeitschr. f. Alterforsch. 5: 256. Original not available; cited by Brody, H. Organization of the human cerebral cortex. III. Study of aging in the human cerebral cortex. J. Comp. Neurol. 102: 258. 1955.

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

Humason, Gretchen L. 1962. Animal tissue techniques. W. H. Freeman and Co., San Francisco.

Hyden, H. and Lindstrom, B. 1950. Microspectrographic studies on the yellow pigment in nerve cells. Discussions Faraday Society. 9: 436-441.

Inukai, T. 1928. Loss of Perkinje cells with age in the albino rat. J. Comp. Neurol. 45: 1-33.

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

Kuhlenbeck, H. 1954. Some histologic age changes in the rat's brain and their relationship to comparable changes in the human brain. Confinia Neurologica. 14: 329. Original not available; cited by Birrens, J. E. 1959. Handbook of aging and the individual. p. 149. University of Chicago Press, Chicago.

Kuntz, Albert. 1938. Histological variations in autonomic ganglia and ganglion cells associated with age and disease. Am. J. Path. 14: 783-795.

Levi, G. 1946. Accrescinto e servescenz. La Nuova Italia. Florence. Original not available; cited by Andrew, Wärren. Structural alterations with aging in the nervous system. p. 142. In Moore, J. E., H. H. Merritt, and R. J. Masselink, eds. Neurological and psychiatric aspects of the disorders of aging. Vol. 35. pp. 129-170. Williams and Wilkins, Baltimore.

Lillie, R. D. 1954. Histopathologic technique and practical histochemistry. Blakiston Co., New York.

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

_____ 1956b. Mechanism of Nile blue staining of lipofuscins. J. Histochem. and Cytochem. 4: 377-381.

Lim, Robert K. S., Chan-nao Liu, and Moffitt, Robert L. 1960. Stereotaxic atlas of the dog's brain. Charles C. Thomas, Springfield, Illinois.

Lison, L. 1953. Histochemie et cytochemie animales. Gauther-Villars, Paris.

Luyet, B. 1937. Differential staining for living and dead cells. Science. 85: 106.

Malkoff, D. B. and Strehler, B. L. 1963. Ultrastructure of isolated and in situ human cardiac age pigment. J. Cell Biol. 16: 611-616.

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

Minea, J. 1921. Contribution a l'etude de lesions des celles nerveuses dans la senilite . Arch. de Neurol. 6: 337-340. Original not available; cited by Cowdry, E. V. 1942. Problems of aging. 1st ed. p. 519. Williams and Wilkins, Baltimore.

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

Munnell, J. H. ca. 1965. Age changes in the canine myocardium. Unpublished M.S. thesis. Library, Iowa State University of Science and Technology, Ames.

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

Novikoff, A. B. and Essner, F. 1960. Liver cell: some new approaches to its study. Am. J. Med. 29: 102-131.

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.

Palay, S. L. and Palade, G. E. 1955. Fine structure of neurons. J. Biophys. and Biochem. Cytology. 1: 69-88.

Pappenheimer, A. M. and Victor, J. 1946. "Ceroid" pigment in human tissues. Am. J. Path. 22: 395-413.

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

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, Stephen Walter and Clark, San Lillard. 1959. Anatomy of the nervous system. 10th ed. W. B. Saunders Co., Philadelphia.

Riese, W. 1946. Cerebral cortex in the very old human brain. J. Neuropath. and Exp. Neurol. 5: 160-164.

Roussy, G. and Mosinger, M. 1935. Le pigment jaune de la region thalamo-sous-thalamique. Comptes rend. Soc. Biol. Paris. 117: 1054-1056. Original not available; cited by Altschul, Rudolf. 1943. Lipofuscin distribution in the basal ganglia. J. Comp. Neurol. 78: 47.

Samorajski, Thaddeus, Keefe, J. Richard, and Ordy, J. Mark. 1964. Intracellular localization of lipofuscin age pigment in the nervous system. J. Gerontol. 19: 262-276.

Sanides, F. 1957. Untersuchungen über die histologische Struktur des Mendelkerngebietes. J. Hirnforsch. 3: 56-77. Original not available; cited by Birrens, J. E. 1959. Handbook of aging and the individual. p. 149. University of Chicago Press, Chicago.

Sjövall, E. 1946. Aldersforandringarna i centralnervsystemet och deras betydelse. Nord. med. tidskr. 4: 1011-1014. Original not available; cited by Birrens, J. E. 1959. Handbook of aging and the individual. p. 151. University of Chicago Press, Chicago.

Smallwood, W. M. and Phillips, R. L. 1916. Nuclear size of nerve cells of bees during the life cycle. J. Comp. Neurol. 27: 69-75.

Sobel, H. 1960. Studies on the measurement of aging. Am. Inst. Biol. Sci. Publ. No. 6: 274-278.

Sosa, J. M. 1952. Aging of neurofibrils. J. Gerontol. 7: 191-195.

Stammler, A. 1959. Histochemische Untersuchungen des "ipoiden Pigmentes" in dem Gangkienzellen des Gehirns. Virchow's Archiv. 332: 347-375.

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

_____, Mark, D. D., Mildvan, A. S., and Gees, M. V. 1959. Rate and magnitude of age pigment and accumulation in the human myocardium. J. Gerontol. 14: 430-439.

_____ and Mildvan, A. S. 1962. Studies on the chemical properties of lipo-fuchsin age pigment. In Biological aspects of aging. pp. 174-181. Columbia University Press, New York.

Stübel, H. 1911. Die Fluorezenz tierscher Gewebe in ultravioletem Licht. Pflug. Arch. ges. Physiol. 142: 1-14. Original not available; cited by Strehler, Bernard L., Mark, Donald D., Mildvan, Albert S., and Gee, Malcolm V. 1959. Rate and magnitude of age pigment accumulation in the human myocardium. J. Gerontol. 14: 430.

Sulkin, Norman M. 1953. Histochemical studies of the pigment in human autonomic ganglion cells. J. Gerontol. 8: 435-448.

_____ 1955a. Occurrence, distribution and nature of PAS-positive substances in the nervous system of the senile dog. J. Gerontol. 10: 135-144.

_____ 1955b. Histochemical studies on mucoprotein in nerve cells of the dog. Cytologia. 1: 459-568.

_____ 1961. Aging in the nervous system. In Bourne, G. H., ed. Structural aspects of aging. pp. 201-214. Hafner Publishing Co., New York.

_____ and Kuntz, Albert. 1952. Histochemical alterations in autonomic ganglion cells associated with aging. J. Gerontol. 7: 533-543.

_____ and Srivanij, P. 1960. Experimental production of senile pigments in the nerve cells of young rats. J. Gerontol. 15: 2-9.

Tilney, F. 1928. Aging of the human brain. Bull. New York Acad. Med. 4: 1125-1143.

Tomasch, Joseph. 1962. Human hypoglossal nucleus: a quantitative study. J. Comp. Neurol. 119: 105-111.

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

_____ and Zwemier, R. L. 1942. True fatty degeneration in sensory neurons of the aged. Arch. Neurol. Psychiat. 48: 988-995. Original not available; cited by Bourne, G. H., ed. 1961. Structural aspects of aging. p. 202. Hafner Publishing Co., New York.

Vogt, C. and Vogt, O. 1946. Age changes in neurons. Nature (London). 158: 304.

Wahal, K. M. and Riggs, H. E. 1960. Changes in the brain associated with aging. Arch. Neurol. Psychiat. 2: 151-159.

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.

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

Wilcox, H. H. 1951. Changes accompanying aging in the brains of guinea pigs. (Abstract) J. Gerontol. 6, Supp. 3: 168.

_____ 1956. Changes in nervous system with age. U. S. Public Health Reports. 71: 1179-1184.

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. Original not available; cited by Sulkin, Norman and Scrivani j., Paka. 1960. Experimental production of senile pigment in the nerve cells of young rats. J. Gerontology. 15: 4.

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