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The relationship between lipofuscin and neuromelanin in some sites of the nervous system of the horse*

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Summary. Histochemical and cytochemical features of neuronal pigment in the spinal cord, and in the sympathetic and spinal ganglia of the adult horse have been studied. Light and electron microscopical observations revealed that lipofuscin pigment is present in the spinal ganglia and in the spinal cord, whereas in the sympathetic ganglia both lipofuscin and neuromelanin are present.

The ultrastructural studies showed that lipofuscin granules exhibited a triphasic organization consisting of a matrix, a system of lamellae and one or more lipid vacuoles. Neuromelanin granules have structural features in common with lipofuscin-like granules, except the former types store a highly electron dense material on their matrix. The electron dense material has been identified as melanin by a modification of the Lillie ferrous sulfate reaction applied at the ultrastructural level. From the ultrastructural appearance and cytochemistry of the neuronal pigments in horse it is concluded that neuromelanin should be considered as melanized lipofuscin.

The possibility that the different ratio of lipofuscin to melanin within the same granule is responsible for the peculiar reaction of certain neuromelanin granules to lipophilic dyes, the PAS reaction, a histochemical assay for melanin and exposure to ultraviolet light, is discussed.

Key words: Neurons – Pigments – Spinal cord – Ganglia – Histochemistry

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Introduction

Little information is available which documents the relationship between lipofuscin and neuromelanin, the two pigment types which accumulate within the cytoplasm of neurons in certain areas of the nervous system.

An extensive literature exists on the distribution, origin and chemical nature of lipofuscin. Most investigations were carried out in rodent tissue (Hess 1955; Sulkin and Scrivanij 1960; Kumamoto and Bourne, 1963; Samorajski et al. 1965; Nandy and Bourne 1966; Miyagishi et al. 1967; Sekhon et al. 1969; Nandy 1971), but some reports were on carnivores (Picard et al. 1962), subhuman primates and man (Zeglio 1935; Andrew 1956; Oya 1959; Hyden 1960; Smirnova 1960; Friede 1962; Barden 1970). Histochemical and ultrastructural characteristics of lipofuscin are well known. It demonstrates a strong affinity for lipophilic dyes, and can be stained with Nile Blue sulfate. Lipofuscin deposits exhibit acid fastness and PAS positivity and an additional characteristic of this kind of pigment is its bright yellow orange fluorescence when irradiated by ultraviolet light.

A number of ultrastructural investigations have indicated the similarity between neuronal and extraneuronal lipofuscins (Essner and Novikoff 1960; Radnot and Follman 1974; Ives et al. 1975; Hanzlikowa and Gutmann 1975; Koobs et al. 1978; Miquel et al. 1978; Nickerson, 1979). In both categories a triphasic organization was demonstrated, consisting of an electron dense matrix, an electron lucent component and several geometrically arranged substructures. It is widely accepted that lipofuscin, which is formed within

the neurons of many regions of the central nervous system and in all mammalian species, represents a progressively increasing by-product of the aging process. Conversely the nature of neuromelanin, which is characteristic of neurons found in particular brain regions in man and some, though not all, mammalian species, has remained as yet poorly defined. The claim that neuronal melanin pigment first appears in man by the 5th year, its amount increasing up to as late as 20 years, thereafter remaining quantitatively and qualitatively unchanged is controversial (Levi 1946). In man, subhuman primates and carnivores, melanin pigment occurs only in the neurons of the substantia nigra, red nucleus, locus coeruleus, dorsal motor nucleus of vagus and some hypothalamic nuclei. According to Hyden (1960), only melanin occurs in the sympathetic ganglia in man, however, other workers also reported the presence of melanin mixed with lipofuscin in both sympathetic (Kuntz 1934, 1945) and spinal ganglia (Oya 1959; Smirnova 1960) as well as in the Gasserian ganglion (Smirnova 1960) of man. Within other mammalian species, melanin pigment is found only in the substantia nigra of giraffe (Scherer 1944) and in the substantia nigra and locus coeruleus of horse (Scherer 1944; Russell 1955).

Histochemical investigations carried out at the ultrastructural level by Foley and Baxter (1958) and Kennedy and Zelickson (1963) suggested that the melanin pigment found in human brain differs from the melanin occurring in the skin, hairs and vascular membranes of the eye. These findings have been corroborated by more recent ultrastructural results (Duffy and Tennyson 1965; Moses et al. 1966; Hirosawa 1968; Roy and Wolman 1969; Schwyn et al. 1970; Forno and Alvord 1974) which have pointed to a close similarity between neuromelanin and lipofuscin, rather than extraneuronal melanin. Electron micrographs presented by these workers showed that besides the typical triphasic structure of lipofuscin, neuromelanin displays an additional highly electron dense granular component. On the basis of these findings the concept of an intrinsic difference between extraneuronal and neuronal melanin was established and the term "neuromelanin" coined.

According to histochemical investigations integrated with spectrophotometric and diffractometric observations at the optical level (Barden 1981) neuromelanin is characterized by the presence of both lipofuscin and melanin components. Preliminary morphological and histochemical studies carried out at the optical (Bianchi and Godina 1982, 1983) and ultrastructural level (Merighi and Bianchi 1984) in the horse showed the presence of lipofuscin alone in the neurons of the spinal ganglia and spinal cord; while the pigment in neurons of the ganglia of the sympathetic trunk was found to comprise both lipofuscin and melanin. It seemed therefore of interest to extend our research to a closer characterization of intraneuronal pigments.

Materials and methods

Examinations at the optical level were carried out on tissue from 30 horses ranging from 3 months to 20 years of age. Spinal ganglia, several segments from the spinal cord and ganglia of the sympathetic trunk were collected. Fresh material was fixed in Bouin fluid, 10% formalin,, Zenker-formol and bichromate-formol and Carnoy solution with minimal delay after death. Morphological characteristics and tinctorial properties of the pigment were visualized by the following procedures: unstained paraffin sections were mounted in a low fluorescence mounting medium for examination under ultraviolet light; paraffin or frozen sections were subjected to the following treatments (Pearse 1985): staining with lipophilic dyes (Oil Red, Sudan III and Sudan black B); Lillie's ferrous sulfate reaction; Perl's method for ferric ions; Lillie's sulfuric Nile blue reaction; PAS reaction, often after diastase treatment.

In addition, bleaching with hydrogen peroxide (36 vol %), potassium permanganate, peracetic acid or oxalic acid was performed (Barden 1981).

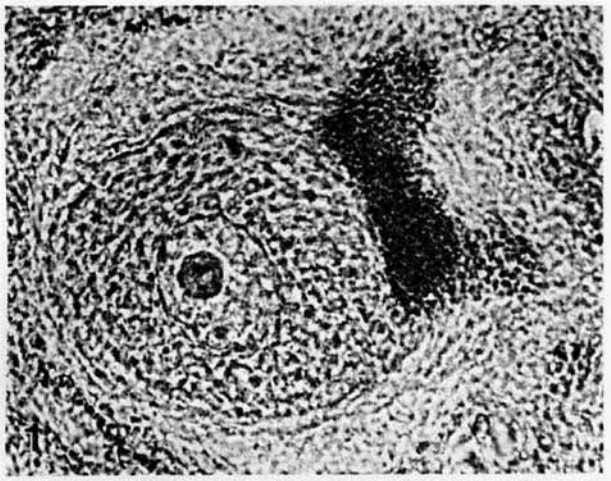
Electron microscopical examination was conducted on spinal ganglia and ganglia of the sympathetic trunk from 10 horses aged from 5 to 20 years. Ultrastructural studies of the pigment occurring in the neurons of the spinal cord were omitted, since at the optical level their characteristics appeared quite comparable to those recorded in the spinal ganglia. Small samples, promptly removed upon the death of the animal were fixed in 0.1 M phosphate-buffered 2.5% glutaraldehyde (pH 7.4), post-fixed in 1% OsO_4 in the same buffer, dehydrated, stained en bloc in 3% uranyl acetate in absolute acetone and embedded in Epon-Araldite. Ultrathin sections, counterstained with lead citrate or Reynolds solution, were examined with a Siemens IA Elmiskop microscope. The following histochemical methods were also used: (a) periodic acid-bismuth (PA-Bi) reaction after Ainsworth et al. (1972) modified by us to use aqueous periodic acid as oxidant; (b) Lillie's ferrous sulfate reaction modified for electron microscopy as follows:

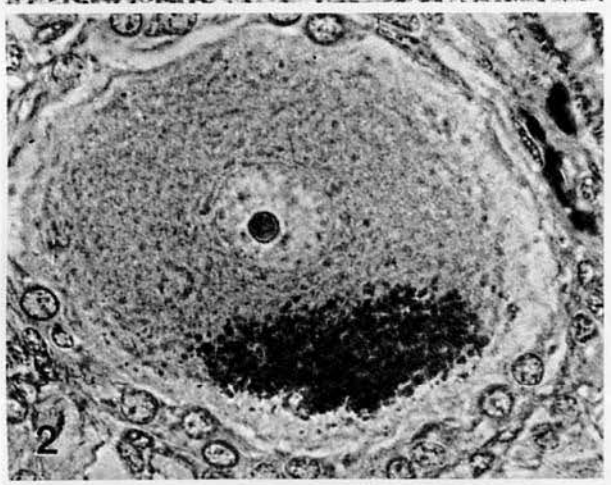
(1) fixation in 0.1 M phosphate-buffered Karnovsky fixative (pH 7.4; Karnovsky 1965) at room temperature for 5 h; (2) treatment with 2.5% ferrous sulfate aqueous solution for 2 h followed by rinses in distilled water; (3) treatment with 1% potassium ferricyanide in 1% aqueous acetic acid for 1 h; (4) wash in 1% aqueous acetic acid; (5) dehydration and embedding as usual. The preparations obtained by means of (a) and (b) were observed without any further contrast reaction.

Results

Size and location of pigment granules

Pigment granules within the neurons of the spinal cord and spinal ganglia were fairly small. They





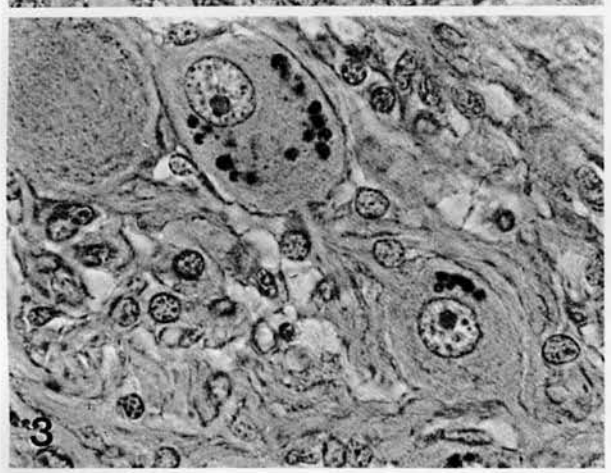


Fig. 1. Spinal cord, 15-year-old horse. Sudan Black ×742

Fig. 2. Spinal ganglion, 15-year-old horse. Sudan Black ×715

Fig. 3. Cranial cervical ganglion, 9-year-old horse. Sudan Black ×730

ranged in size between 0.3 and 1.2 μm. In the former neurons they tend to accumulate into dense clumps at one pole of the cell (Fig. 1), while in the latter they appeared either widely dispersed or clustered into large clumps polarized to the peripheral cytoplasm (Fig. 2). In some neurons the granules were so tightly packed that they masked the cell nucleus. Within the neurons of the ganglia of the sympathetic trunk the granules appeared to form larger masses, globular or ovoid in shape, or they exhibited irregular outlines. They were particularly prominent in neurons of the cranial cervical ganglion, in which they attained and occasionally exceeded 5 μm in diameter (Fig. 3). The granules were largely distributed in a perinuclear pattern. In the other ganglia of the sympathetic trunk they were less prominent, and appeared to be randomly dispersed throughout the cytoplasm.

In serial sections of both spinal and autonomic ganglia from two horses, aged 5 and 15 years respectively, stained by PAS reaction after diastase treatment, pigment granules were found unequivocally present in all of the cells, even though the amounts differed from one neuron to another neuron.

Tinctorial properties and cytochemical characteristics

Light microscopical examination. Under transmitted light and without staining, the pigment occurring within the neurons of the spinal cord, spinal ganglia and sympathetic ganglia from young specimens exhibited a light yellow color. However in young adult animals (4–5 year old) the pigment granules occurring in sympathetic ganglion neurons exhibited a variety of shades such as dark brown and sometimes black; others were pale yellow and indeed some granules were almost entirely colorless.

The pigment granules described below related to adult individuals.

Lipophilic dyes. Sudan black B elicited more selective granule staining and was used preferentially. We observed that in the neurons of the spinal cord and spinal ganglia pigment granules were generally greyish yellow, sometimes tending to brown. Conversely, in the sympathetic ganglia, most obviously in the cranial cervical ganglion, neurons containing colorless or faintly stained granules were found adjacent to neurons exhibiting a range of shades from dark grey to brown and black. It is interesting that paraffin and cryostat sections gave the same result and also that the affinity of the granules for lipidic dyes was unaffected by treatment with lipid solvents (ethanol, benzene, xylene).

Pas reaction. All of the intraneuronal pigment granules in the spinal ganglia and the spinal cord

exhibited a rather uniform reaction, whereas within the sympathetic ganglion neurons, in particular in the cranial cervical ganglion, the granules displayed a variety of shades from bright red to brick red and brown-yellow (the latter being rather similar to the natural color). Once again some granules remained colorless.

Exposure to ultraviolet light. A bright golden yellow fluorescence, typical of lipofuscin, was emitted by the granules within the spinal cord and spinal ganglion neurons (Fig. 4). Conversely, in the sympathetic ganglia, while golden yellow autofluorescent granules were present within most neurons (Fig. 5), at the very start of the irradiation a uniform deep brickred tinge was shown by the pigment granules which were present in some randomly scattered neurons (Fig. 6). In the latter, however, after exposure to ultraviolet irradiation (few min to 24 h according to the material under analysis) a progressively increasing fluorescence was shown by the cytoplasmic granules, the optical properties of which eventually became comparable to those characteristic of the surrounding autofluorescent granules (Fig. 7). This phenomenon was undiminished even when the same preparations, subsequently kept in the dark for varying time intervals up to 3 months, were again exposed to ultraviolet light; the pigment granules exhibited a distinct fluorescence from the beginning of this additional exposure to ultraviolet light.

Melanin histochemical assay. After treatment with Lillie's ferrous sulfate reaction no response was exhibited by the neurons in the spinal cord and spinal ganglia. Conversely, in the sympathetic ganglia, while pigment granules occurring within several neurons remained colorless, in others they appeared faintly grey and in a few cells the granules were distinctly positive to ferrous ion uptake, exhibiting a more or less intense green color (Fig. 8). In the same preparations we observed that under ultraviolet light the intensely green pigment was not fluorescent, a faint fluorescence was shown by the weakly positive cells, whereas the pigment that was entirely negative to the ferrous ion uptake method was highly fluorescent.

The preparations subjected to bleaching with hydrogen peroxide (36 vol%) for 24 h yielded the most reliable results. After this lapse of time, the pigment naturally brownish in visible light appeared entirely colorless. In the same preparations treated with the Lillie's ferrous sulfate method the binding capacity of the pigment for ferrous ion uptake was suppressed. These results indicate that the pigment granules consistently found within the neurons of the spinal ganglia and spinal cord, as well in sympathetic ganglia in horses under 4 years of age, may be considered as typical lipofuscin. Conversely, within the cranial cervical ganglion and to a lesser extent in the other ganglia of the sympathetic trunk from pre-adult or adult specimens, other granules besides those exhibiting typical lipofuscin features, displayed characteristics suggestive of melanin or an intermediate between lipofuscin and melanin.

Electron microscopy

General morphology. Within the neurons of the spinal ganglia, numerous relatively small granules, generally clustered into conspicuous clumps at the cell periphery, were found. They were more or less globular or ovoid in shape, their outline becoming irregular due to the fusion of finer granules. They appeared to contain a relatively homogeneous or granular, moderately electron opaque matrix, and were bound by a limiting membrane which, however, was not always clearly discernible. Within the matrix, one or more electron lucent areas and a number of lamellar systems were visualized (Fig. 9). The electron lucent areas identifiable as lipid vacuoles, may be centrally or eccentrically embedded in the matrix; frequently they protruded from the granule periphery, though still bound by the limiting membrane. The lamellar systems showed a straight or curvilinear configuration, reminiscent of "fingerprints". Each lamellar unit was composed of a slender (22 nm) dark central lamella flanked on

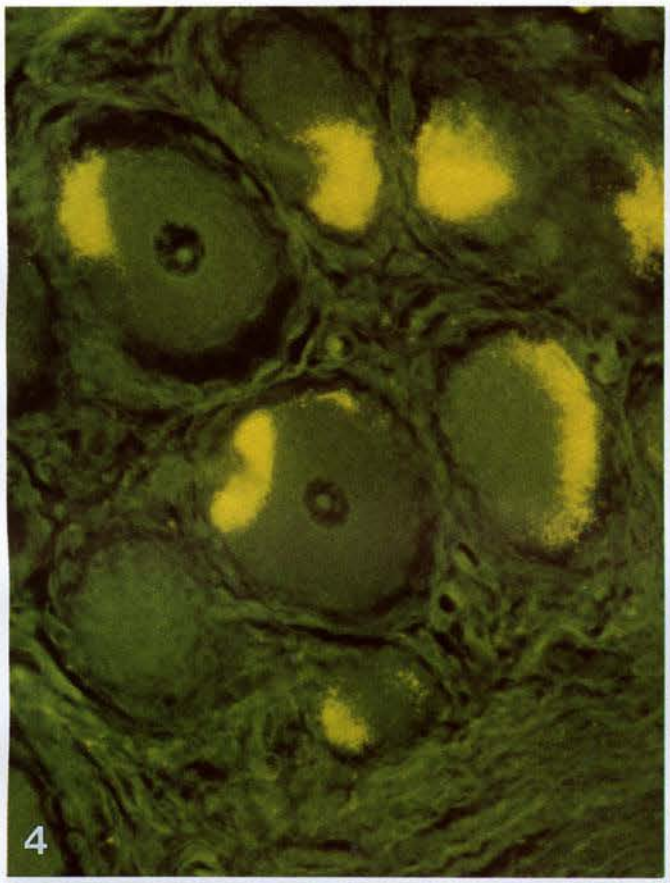
Fig. 4. Spinal ganglion, 15-year-old horse. Ultraviolet light. × 790

Fig. 5. Cranial cervical ganglion, 12-year-old horse. Ultraviolet light. × 790

Fig. 6. Cranial cervical ganglion, 12-year-old horse. Ultraviolet light, at the very start of irradiation, × 790

Fig. 7. The same cell of Fig. 6, 2 h after irradiation with ultraviolet light. × 790

Fig. 8. Cranial cervical ganglion, 18-year-old horse. Lillie ferrous sulfate reaction. × 1100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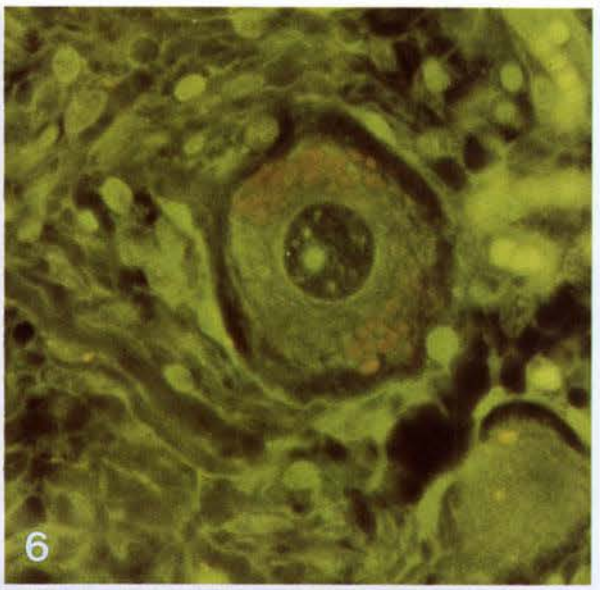
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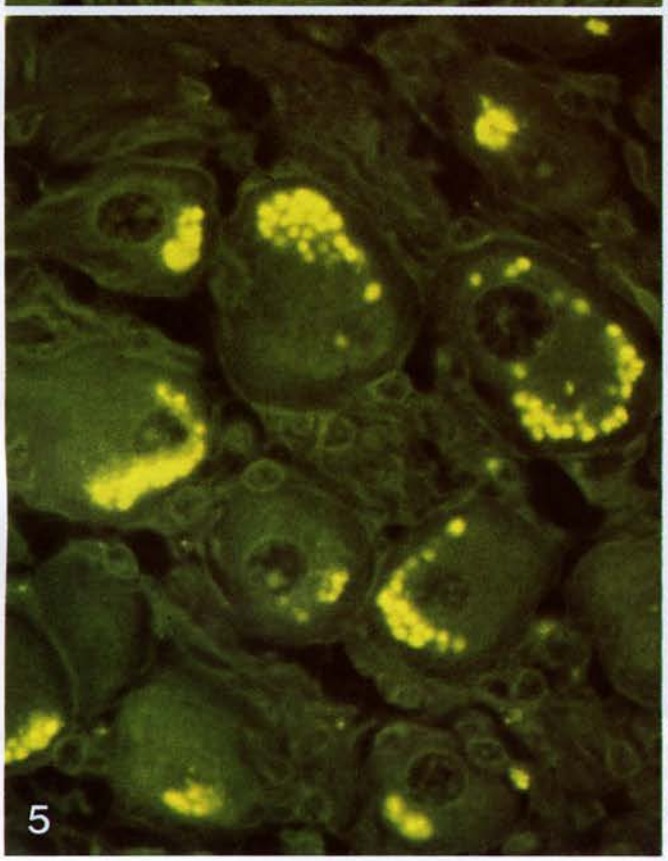
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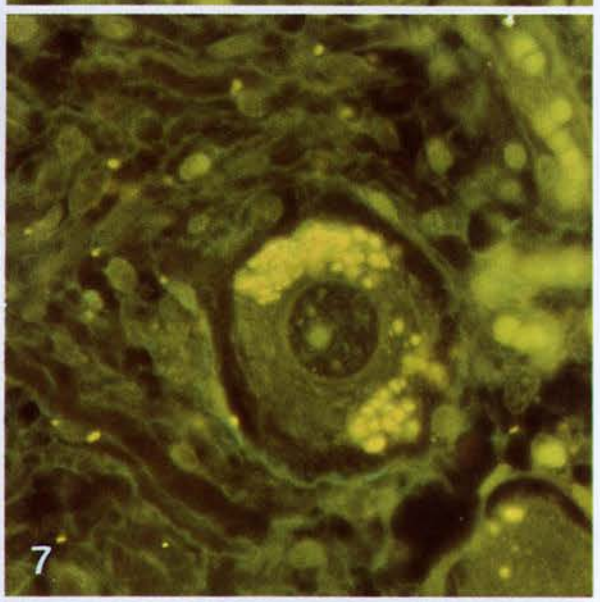
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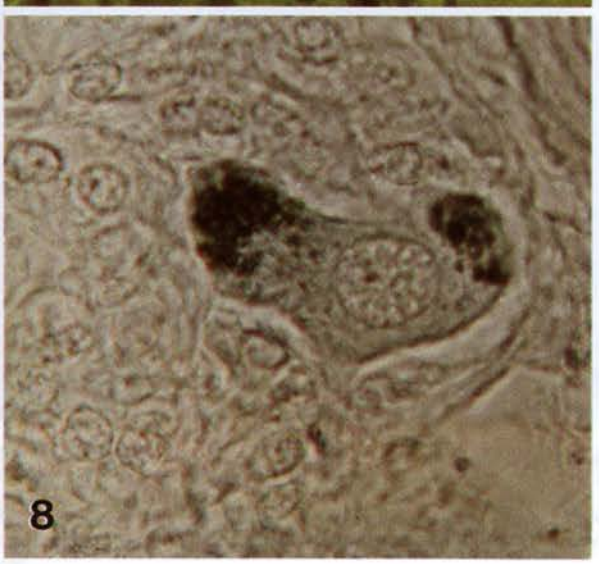
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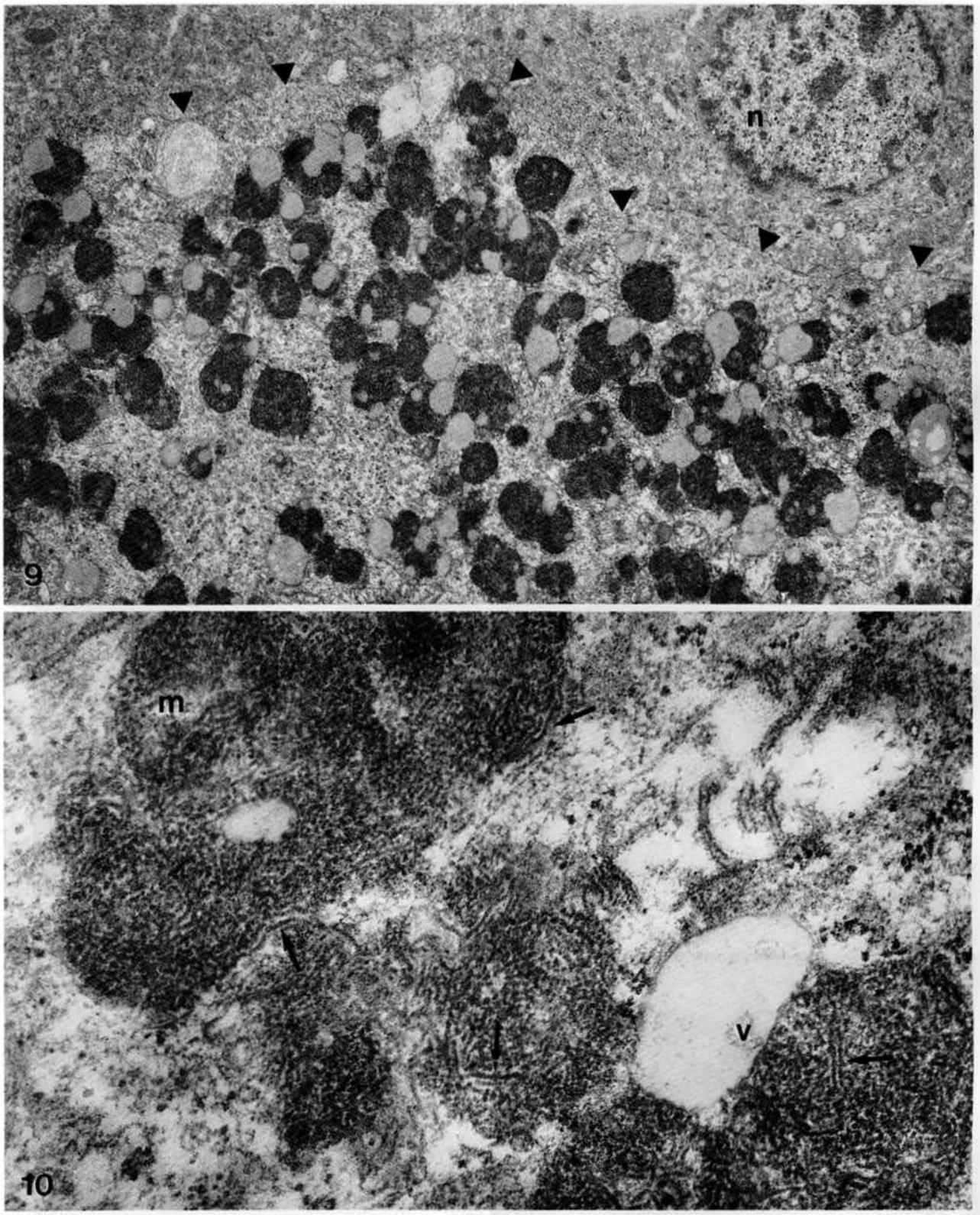


Fig. 9. Low magnification of the lipofuscin pigment in a neuron of the spinal ganglia. *n* nucleus of a satellite cell. *Arrowheads* indicate the border between the satellite cell and the neuron. ×7,750

Fig. 10. High magnification of the lipofuscin pigment in a spinal ganglion neuron. Note the lamellar system. *Arrows* indicate the lamellae in which the fundamental unit of the system is clearly appreciable. *m* matrix; *v* vacuole. ×72,000

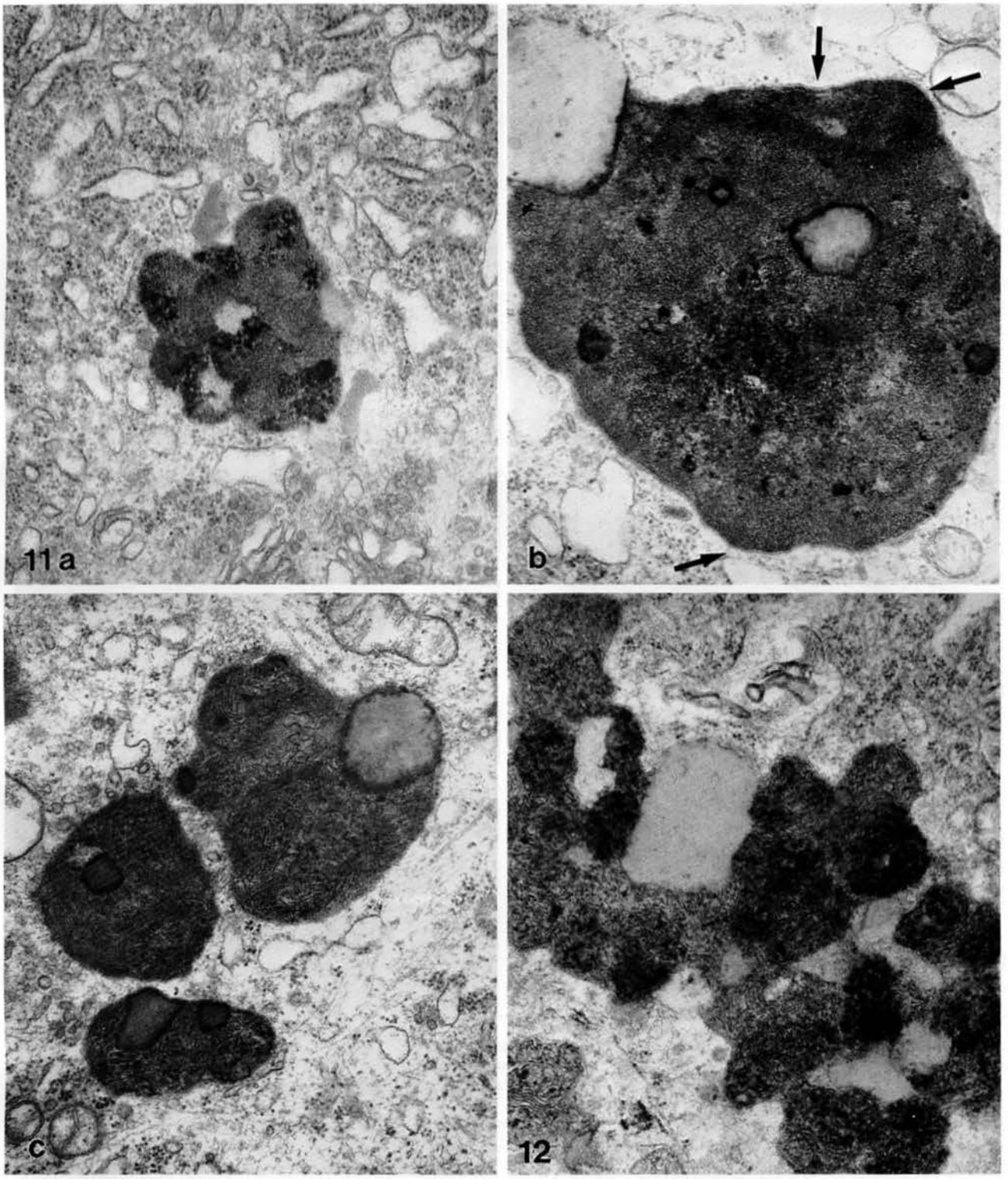
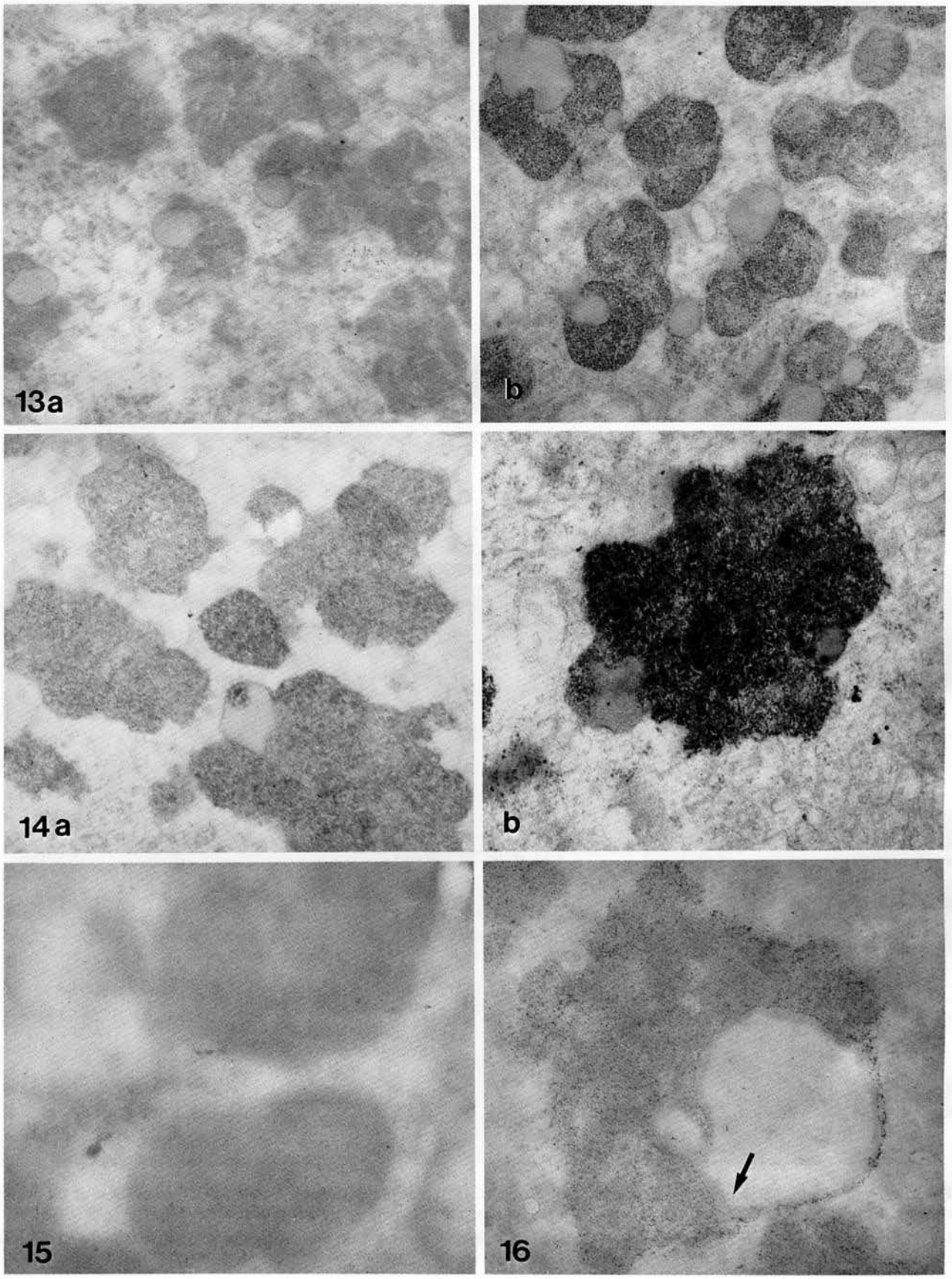


Fig. 11. Neuromelanin pigment in the cranial cervical ganglion. Different morphological aspects of the deposition of the highly electron dense material are shown. In the granule of **b** the limiting membrane is well evident *(arrows).* ×32,000

Fig. 12. Neuromelanin pigment in a neuron of a thoracic sympathetic trunk ganglion. Note the similarity with the granule of Figs. 11 b and c. ×25,000


13a
b
14a
b
15
16

either side by a thicker (45 nm) less electron dense lamella (Fig. 10).

Lamellar systems differed in extent within the same specimen, replacing the matrix almost entirely in some cases, or consisting of a few individual units scattered within the matrix. In the cranial cervical ganglion, pigment granules were less numerous, coarser, and more irregular in outline than those occurring intraneuronally in the spinal ganglia. Not infrequently granule coalescence into more prominent, irregular, often lobular clumps was noted. Generally, the granule limiting membrane was more readily demonstrable. In addition to the granule substructures described in the spinal ganglia (i.e., the matrix, lamellar systems and lipid vacuoles), these granules consistently displayed a highly electron dense particulate component. This material appeared in the form of small clusters randomly scattered through the matrix in some granules, in others as a fine granularity disseminated upon the lamellae, and yet in other granules it formed an electron dense ring around the lipid vacuoles. These various distribution patterns also coexisted within single granules (Fig. 11).

Similar features to those just described in the cranial cervical ganglion were exhibited by the pigment occurring in the neurons of other autonomic ganglia. However, in these granules the electron dense material was less abundant, and the granule type pertaining to the cranial cervical ganglion, represented in Fig. 11a, was never found (see Fig. 12).

Ultrastructural cytochemistry

Periodic acid-bismuth (PA-Bi) reaction. In the spinal ganglia, the areas occupied by the matrix and lamellar systems were seen to be coated by the electron dense bismuth precipitate which sharply demarcated the zones occupied by the lipid vacuoles (Fig. 13). Except for the occasional ribosome, no other cytoplasmic structure yielded a reaction deposit. In ganglia of the sympathetic trunk, notably in the cranial cervical ganglion, the matrix of intraneuronal pigment granules was not so homogeneously loaded with the bismuth precipitate; some negative areas were clearly detectable (Fig. 14). These areas appeared to correspond to those occupied by the additional highly electron dense particulate material typical of neuromelanin granules.

The reaction product was consistently restricted to the matrix and the lamellar systems (i.e. to PAS positive components), while the response of lipid vacuoles to the bismuth reaction was negative.

Lillie's ferrous sulfate reaction. Lillie's ferrous sulfate reaction was never positive in the neurons of the spinal ganglia (Fig. 15). Whereas in those of the cranial cervical ganglion and in other ganglia of the sympathetic trunk, uptake of ferrous ions by the pigment granules occurred, ferrous ions demarcating the lipid vacuoles and in some cases being distributed over the matrix (Fig. 16). The lipid vacuoles were negative. The precipitate exhibited a location corresponding to the sites occupied by the highly electron dense particulate material in conventional micrographs.

Non specific reactions were not shown by any other cell components.

Discussion

Further evidence is presented here for the differential intraneuronal pigmentation between the spinal ganglia and those of the sympathetic trunk in the horse, previously outlined by light and electron microscopic investigations (Bianchi and Godina 1982, 1983; Merighi and Bianchi 1984).

Within the neurons of the spinal ganglia and spinal cord, pigment granules exhibit a more o

Fig. 13. PA-Bi. reaction performed on the lipofuscin pigment of a spinal ganglion neuron. **a** control section (treatment: periodic acid 3 min, no counterstain); **b** positive reaction. The electrondense precipitate fills almost completely the matrix, while the lipid vacuoles remain negative (treatment: periodic acid 3 min, bismuth subnitrate 60 min, no counterstain). ×25,000

Fig. 14. PA-Bi. reaction performed on the neuromelanin pigment of a cranial cervical ganglion neuron. **a** control section. Note the residual electrondensity of the melanin component of the granules (treatment: periodic acid 3 min no counterstain). **b** positive reaction. Note the heterogeneous deposition of the bismuth precipitate on the granules (treatment: periodic acid 3 min, bismuth subnitrate 60 min, no counterstain). ×35,000

Fig. 15. Lillie ferrous sulfate reaction modified for electron microscopy performed on the lipofuscin pigment of a spinal ganglion neuron. Negative reaction (compare with Fig. 16). ×54,000

Fig. 16. Lillie ferrous sulfate reaction modified for electron microscopy performed on the neuromelanin pigment of a cranial cervical ganglion neuron. Positive reaction (compare with Fig. 15). *Arrow* indicates the electroudeuse rim in the lipid vacuole in material not fixed in osmium ×40,000

less deep natural yellowish color, PAS positivity and affinity for lipophilic dyes, but not reactivity with Lillie's ferrous sulfate reaction, Perl's test for ferric ion or Lillie's sulfuric Nile blue test. These properties are unaffected by treatment with hydrogen peroxide (36 vol%), potassium permanganate, peracetic and oxalic acids. These granules are intensely autofluorescent in ultraviolet light.

Electron microscopic examinations revealed that in the spinal ganglia the neuronal pigment granules display a triphasic structure consisting of a matrix, lamellar systems and lipid vacuoles. The cytochemical reaction yielded by the matrix and the lamellar systems make it possible to identify these components as the structure responsible for the PAS positivity shown at the optical level.

According to Moses and coworkers (1966) the existence of an electron lucent inner core within the lipid vacuoles may be due to a neutral lipid component responsible for the affinity for lipophilic dyes.

On the basis of the non-reaction shown by all the granule component to the Lillie's ferrous sulfate technique modified by us for electron microscopy, the absence of melanin can be inferred. Hence, tinctorial properties together with cytochemical and ultrastructural features indicate that the composition of these granules is largely lipofuscin. In the ganglia of the sympathetic trunk, and particularly in the cranial cervical ganglion, pigment granules in unstained sections may appear quite colorless, or may be of a variable yellow, ranging dark yellow to black in ordinary light. They exhibit a variety of tinctorial responses after treatment with lipophilic dyes, adopt different hues when subjected to the PAS reaction, and yield extremely variable responses to the Lillie's ferrous sulfate reaction being either negative or giving rise to an intense dark green to brown and grey precipitate. After bleaching with hydrogen peroxide (36 vol%) the naturally brown-yellow darker granules appear almost colorless. Their binding capacity for ferrous ions uptake is at that time entirely suppressed. At the ultrastructural level, the intraneuronal pigment granules occurring within the ganglia of the sympathetic trunk were found to display, besides the three typical components of lipofuscin (i.e. granular matrix, lamellar systems and lipid vacuoles), a fourth component consisting of a highly electron dense particulate nature, which coated the matrix and the lamellae to a varying extent. This material, positive to the Lillie's ferrous sulfate reaction and negative to the PA-Bi reaction, is considered to represent melanin. The present results are in agreement with those reported by Moses and coworkers (1966) who identified, in the electron dense material of substantia nigra and locus coeruleus, the structures responsible for the argentaffin properties of melanin. On the basis of their response to cytochemical reactions, and irradiation with ultraviolet light, the intraneuronal pigment granules may be classified as follows: (1) granules possessing lipofuscin characteristics, which consistently accumulate within the neurons of spinal ganglia and spinal cord as well as in the sympathetic ganglia of horses under 4 years of age; (2) granules exhibiting melanin properties, and (3) granules in which both lipofuscin and melanin properties are discernible, as recorded in the sympathetic ganglia in pre-adult or adult specimens. This last occurrence lends strong support to the suggestion formerly advanced by us that lipofuscin and melanin may exist within the same neuromelanin granule. The different ratio of melanin to lipofuscin within the same granule may account for both the variability in granule staining and the behavior of some granules after exposure to ultraviolet light.

Notably in the cranial cervical ganglion, neighboring granules were observed to fluoresce immediately upon irradiation whereas others were found which took from a few minutes to several hours for the demonstration of their fluorescence, as if some masking of lipofuscin by melanin had been gradually removed by irradiation. Previous analysis carried out on the pigment granules occurring in the neurons of the cranial cervical ganglion in the horse have shown that the photodynamic effects elicited by irradiation consist of the release of lipofuscin in its autofluorescent form (yellow fluorescence) through a two-step process, in which the earliest is represented by the emission of a photolabile type (orange fluorescence). Accordingly, also taking into account the hypothesis forwarded by several workers that the so-called neuromelanin is actually represented by melanized lipofuscin (cf. Barden 1981), it might be speculated that the photodynamic effect induced by irradiation may set the fluorescent lipofuscin free through some change in the chemical bonds linking the two pigment types (Bianchi et al. 1984).

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