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A histochemical and electron microscopic study on the collagen of nerves in the domestic fowl*

ENRIQUE H. LUQUE, EUSEBIA ANGULO AND GREGORIO S. MONTES

Research Laboratories, Faculty of Veterinary Sciences, National University of La Plata, calle 60 *y* 118, *La Plata* 1900, *Argentina*

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INTRODUCTION

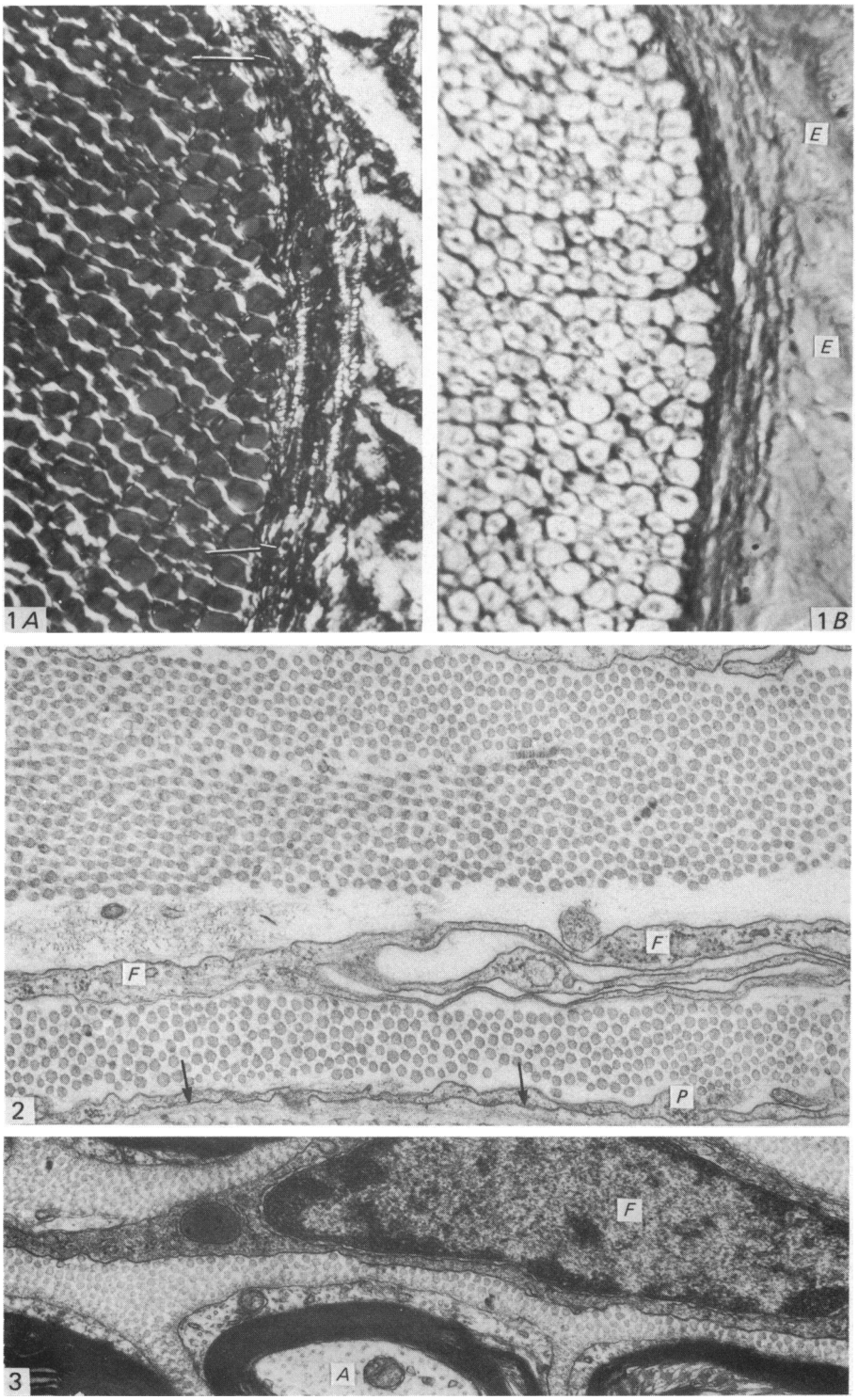
Early electron microscopic studies on the connective tissue of the peripheral nervous system of some mammals have shown that the diameter of the collagen fibrils is larger in the epineurium than in the endoneurium (Thomas, 1963; Gamble, 1964; Gamble & Eames, 1964). A recent report (Junqueira, Montes & Krisztán, 1979*b*), besides studying four mammalian species, extended those initial results to lower classes of vertebrates (namely fish, amphibians, and reptiles), presenting measurements which showed that two distinct populations of collagen fibrils, which could be recognised on the basis of their diameter, are located in different compartments of nerves. In addition, another paper (Montes *et al.* 1980) demonstrated, in the same classes of vertebrates, that the fibrillar component of the *reticular fibres* that surround the axons of nerves was comprised almost exclusively of the thin endoneurial collagen fibrils, whereas the thicker fibrils were localised to the *collagen fibres* of the epineurium.

Based on these observations, it was thought that it would be of interest to investigate whether the nerves of a representative species of the class *Aves* also showed its collagen consisting of two distinct populations located in different compartments. The results presented here demonstrate that collagen distribution in the nerves of birds is very similar to the pattern which was consistently observed in the other vertebrate classes studied. This further evidence strongly reinforces the idea that the structural pattern described represents a general phenomenon in vertebrates.

MATERIALS AND METHODS

Fresh nerves (femoral, sciatic, median, radial and vagus) were obtained from 9 months old White Leghorn domestic fowls (*Gallus gallus domesticus*) that had been killed by decapitation. Specimens were fixed in Bouin's fluid for 20 hours, dehydrated, and embedded in paraffin. Five micrometre sections were studied by either the silver impregnation technique (Montes *et al.* 1980) or the Picrosirius-polarization method (Junqueira, Bignolas & Brentani, 1979*a*). The latter is a specific histochemical method for collagen detection in tissue sections; it is also useful for studying the distribution of collagen Type I (which shows up in the form

* Reprint requests to Dr Angulo.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of thick, strongly birefringent, yellow or red fibres against a dark background) and collagen Type III (which displays a weak birefringence, and is characterised by its thin, greenish fibres).

For electron microscopy, small segments of nerves, approximately 1 mm cubed in size, were fixed in 2 % glutaraldehyde dissolved in 0·15 M phosphate buffer at pH 7·2, followed by post-fixation in 1 % osmium tetroxide dissolved in 0·9 % sodium chloride for one hour. Overnight block staining in 1 % aqueous uranyl acetate was followed by embedding in a polyester resin (Polylite). Sections were cut in an LKB ultratome and were double stained by uranyl acetate and lead citrate. Thin sections were studied and micrographed in a Zeiss EM 9 S electron microscope.

The diameter of cross sectioned collagen fibrils was measured in electron micrographs, enlarged to a convenient size, with a Bausch & Lomb measuring magnifier. A minimum of 300 measurements of fibril diameters was made of the collagen population in both the epi- and endoneurium of each nerve studied. The magnification of the microscope was calibrated with a diffraction grating.

RESULTS

Light microscopy

The outer sheath (epineurium) of all nerves studied by the Picrosirius-polarization method showed thick, bright (strongly birefringent), yellow or red fibres, characteristic of collagen Type I. In contrast, the endo- and perineurium consisted of thin, pale (weakly birefringent), greenish fibres, which are typical of collagen Type III (Fig. 1 A).

When using the silver impregnation technique, characteristically stained, very thin *reticular fibres* were observed in the endo- and perineurium; whereas the *collagen fibres* in the epineurium did not blacken with silver stains (Fig. 1 B). Serial sections, alternately stained by the Picrosirius method and by the silver impregnation technique, clearly showed the correspondence between the *reticular fibres* and the thin, pale, greenish fibres that were localised to the endo- and perineurium (Figs. 1 A, B).

Electron microscopy

Two distinct populations of collagen fibrils, in each nerve, were recognised on the basis of their diameter. One population was present in the epineurium and was

Fig. 1 (A–B). Adjacent serial sections from a chicken sciatic nerve, studied by the Picrosirius-polarization method (A) and a silver impregnation technique (B). × 350. In (A) the epineurium (right hand side) shows thick, strongly birefringent fibres, contrasting with the thinner, weakly birefringent fibres that surround the axons in the endoneurium (at left). Concentric layers display a weak birefringence in the perineurium (arrow). (B) shows the meshwork of black *reticular fibres* in the endo- and perineurium, which contrast with the *collagen fibres* in the epineurium (*E*) that do not blacken with silver stains. There is striking correspondence between the *reticular fibres* in (B) and the thin, weakly birefringent fibres in (A).

Fig. 2. Electron micrograph showing the epineurium of a transverse sectioned chicken vagus nerve. The slender cytoplasmic process from a cell in the outer layer of the perineurium (*P*) shows its characteristic basal lamina (arrows). Cytoplasmic processes from epineurial fibroblasts (*F*) can be seen. Closely packed, thick epineurial collagen fibrils occupy most of the Figure. × 24500.

Fig. 3. Endoneurium, from the same section of the vagus nerve shown in Fig. 2, demonstrates a fibroblast (*F*) and myelinated axons (*A*). Transversely sectioned collagen fibrils occupy the triangular spaces limited by the nerve fibres. Compare these thin endoneurial collagen fibrils with the thicker epineurial fibrils shown at the same magnification in Fig. 2. × 24500.

Table 1. *Average diameter (mean ± standard deviation) of collagen fibrils in the endo- and epineurium of different nerves of the domestic fowl*

Nerve	Epineurium (nm)	Endoneurium (nm)	Epi-/endoneurium ratio
Vagus	56·48 ± 12·25	32·78 ± 3·00	1·72
Radial	61·01 ± 12·93	39·24 ± 6·03	1·55
Median	62·82 ± 10·08	41·60 ± 5·78	1·51
Femoral	54·67 ± 12·72	30·60 ± 3·80	1·79

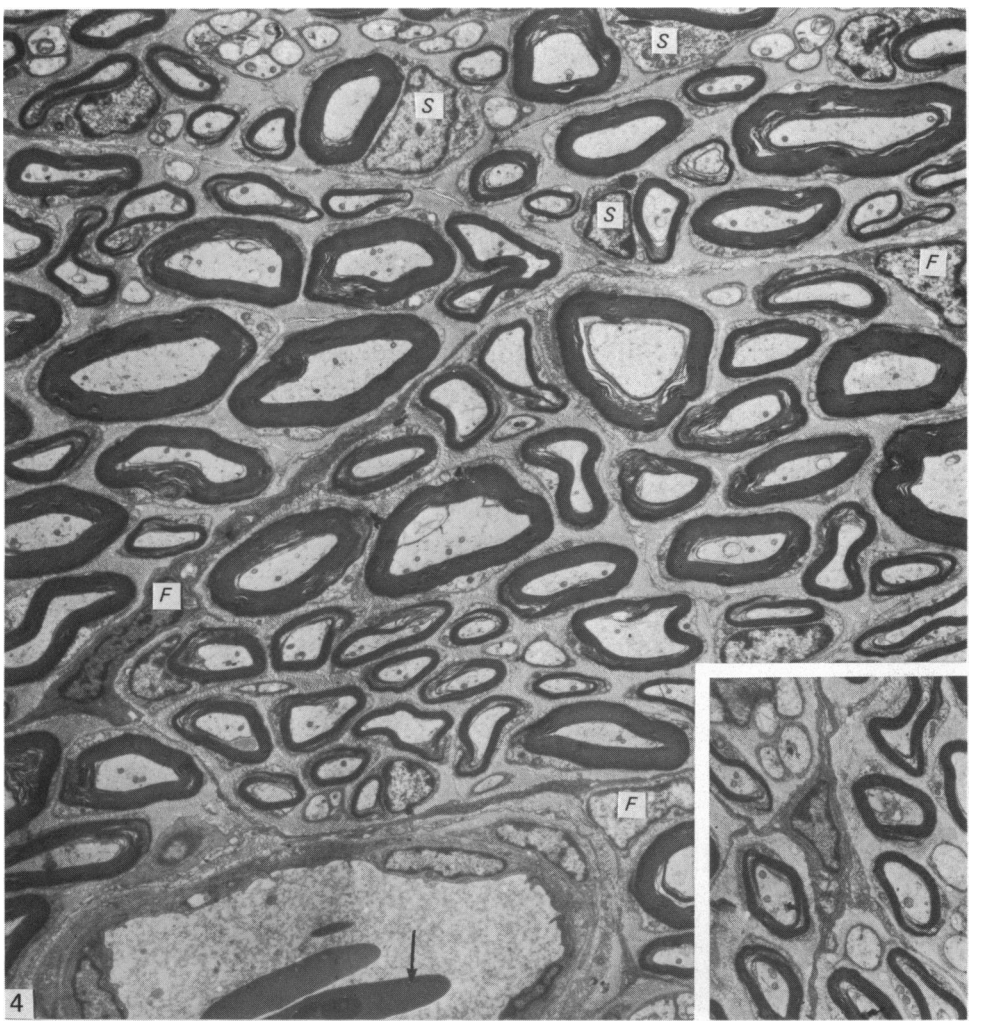

Fig. 4. Electron micrograph of a chicken sciatic nerve showing myelinated and unmyelinated nerve fibres, Schwann cells (*S*) and fibroblasts (*F*). × 4300. The inset shows a typically stellate endoneurial fibroblast with several slender cytoplasmic processes. × 5000.

formed of thick fibrils, with marked variation in diameter, disposed in densely packed blocks (Fig. 2). The second population, composed of loosely arranged thin fibrils of more uniform diameters, was found in the endo- and perineurium (Fig. 3). The difference in collagen fibril diameters described above was consistently observed in all nerves studied, and it is expressed quantitatively in Table 1.

The ultrastructural observations on the connective tissue of avian nerves, in all sites examined, confirm the descriptions available in the literature, as will be discussed below. However, in contrast to the findings in other vertebrate classes (Junqueira *et al.* 1979*b*), fibroblasts and their elongated cytoplasmic processes are fairly abundant in the avian endoneurium (Fig. 4).

DISCUSSION

The present observations regarding the fine structure of avian epi-, peri- and endoneurium agree with the results in other species (Thomas, 1963; Gamble, 1964; Gamble & Eames, 1964; Gray, 1970; Peters, Palay & Webster, 1976; Junqueira *et al.* 1979*b*). Furthermore, the morphometric results now reported are similar to the values available in the papers cited above, for fish, amphibians, reptiles and mammals; thus extending to birds the observation that the diameter of collagen fibrils is larger in the epineurium than in the endo- and perineurium, and strongly suggesting the existence of a general structural pattern occurring in the five main classes of vertebrates.

The histochemical results obtained coincide with the electron microscopic observations, in showing the presence of two distinct collagen populations, segregated into different compartments of the nerve. The *reticular fibres* in the endoneurium displayed a distinct argyrophilia when studied by means of the silver impregnation technique, and showed up as thin, weakly birefringent greenish fibres (characteristic of collagen Type III) when observed by the aid of the Picrosirius-polarization method. In addition, the electron microscopic studies disclosed the presence of thin collagen fibrils in the endoneurium, contrasting with the thicker fibrils that could be localised ultrastructurally to the epineurium where non-argyrophilic, coarse collagen Type I fibres had been characterised by the histochemical methods used. These same two distinct collagen populations have also been recognised, by the same three methods, in the arteries of the five main classes of vertebrates, including birds (Carrasco *et al.* 1981), and in tumours of the peripheral nervous system (Junqueira *et al.* 1981), and these findings are in agreement with immunohistologic (Shellswell, Restall, Duance & Bailey, 1979) and biochemical (Seyer, Kang & Whitaker, 1977) findings that collagens Types I and III co-exist in mammalian nerves.

The histochemical and ultrastructural methods employed in the present report have also been used to study diseased human nerves (Junqueira *et al.* 1980, 1981). The present findings on the collagen in normal avian nerves may provide a basis for the study of its behaviour in diseases that affect the nerves of the domestic fowl, such as Marek's disease (Hofstad, 1978).

The origin of the collagen in the nerve sheaths is still a matter of speculation, but it is generally agreed that the epineurial collagen derives from the abundant fibroblasts present in this layer. Several facts, including the observation that fibroblasts are very rare in the endoneurium of mammalian nerves, are responsible for the current view that Schwann cells are the most probable site of collagen synthesis in the endoneurium (for review, see Montes & Junqueira, 1982). However, the present

observation, that fibroblasts are fairly common in the endoneurium of the domestic fowl, suggests that the collagen production by Schwann cells may still be subject to controversy.

SUMMARY

The collagen in the endoneurium is present as argyrophilic *reticular fibres*, which show up as thin, weakly birefringent greenish fibres when studied by aid of the Picrosirius-polarization method, and are composed of loosely arranged thin collagen fibrils. The epineurium consists of thick, non-argyrophilic *collagen fibres*, which display a strong birefringence of red or yellow colour when studied by the aid of the Picrosirius-polarization method, and consist of closely packed thick collagen fibrils. These characteristics strongly suggest that the *reticular fibres* in the endo- and perineurium are composed mainly of collagen Type III, whereas collagen Type I predominates in the epineurium. The fact that these observations on birds are consistent with the descriptions available in the literature for fish, amphibians, reptiles and mammals, argues in favour of the existence of a uniform structural pattern of collagen distribution that is a general phenomenon in vertebrate nerves.

REFERENCES

CARRASCO, F. H., MONTES, G. S., KRISZTÁN, R. M., SHIGIHARA, K. M., CARNEIRO, J. & JUNQUEIRA, L. C. U. (1981). Comparative morphologic and histochemical studies on the collagen of vertebrate arteries. *Blood Vessels* **18**, 296–302.

GAMBLE, H. J. (1964). Comparative electron-microscopic observations on the connective tissue of peripheral nerves and a spinal nerve root in the rat. *Journal of Anatomy* **98**, 17–25.

GAMBLE, H. J. & EAMES, R. A. (1964). An electron microscope study of the connective tissues of the human peripheral nerve. *Journal of Anatomy* **98**, 655–663.

GRAY, E. G. (1970). The fine structure of nerve. *Comparative Biochemistry and Physiology* **36**, 419–448.

HOFSTAD, M. S. (1978). *Diseases of Poultry*, 7th ed. Ames: Iowa State University Press.

JUNQUEIRA, L. C. U., BIGNOLAS, G. & BRENTANI, R. R. (1979*a*). Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. *Histochemical Journal* **11**, 447–455.

JUNQUEIRA, L. C. U., MONTES, G. S. & KRISZTÁN, R. M. (1979*b*). The collagen of the vertebrate peripheral nervous system. *Cell and Tissue Research* **202**, 453–460.

JUNQUEIRA, L. C. U., MONTES, G. S., ALMEIDA NETO, E., BARROS, C. & TEDESCO-MARCHESE, A. J. (1980). The collagen of permanently damaged nerves in human leprosy. *International Journal of Leprosy* **48**, 291–297.

JUNQUEIRA, L. C. U., MONTES, G. S., KAUPERT, D., SHIGIHARA, K. M., BOLONHANI, T. M. & KRISZTÁN, R. M. (1981). Morphological and histochemical studies on the collagen in neurinomas, neurofibromas, and fibromas. *Journal of Neuropathology and Experimental Neurology* **40**, 123–133.

MONTES, G. S., KRISZTÁN, R. M., SHIGIHARA, K. M., TOKORO, R., MOURÃO, P. A. S. & JUNQUEIRA, L. C. U. (1980). Histochemical and morphological characterization of reticular fibers. *Histochemistry* **65**, 131–141.

MONTES, G. S. & JUNQUEIRA, L. C. U. (1982). Biology of collagen. *Revue canadienne de biologie expérimentale* **41**, 143–156.

PETERS, A., PALAY, S. & WEBSTER, H. (1976). *The Fine Structure of the Nervous System. The Neurons and Supporting Cells.* Philadelphia: Saunders.

SEYER, J. M., KANG, A. H. & WHITAKER, J. N. (1977). The characterization of type I and type III collagens from human peripheral nerve. *Biochimica et biophysica acta* **492**, 415–425.

SHELLSWELL, G. B., RESTALL, D. J., DUANCE, V. C. & BAILEY, A. J. (1979). Identification and differential distribution of collagen types in the central and peripheral nervous systems. *FEBS Letters* **106**, 305–308.

THOMAS, P. K. (1963). The connective tissue of the peripheral nerve: an electron microscope study. *Journal of Anatomy* **97**, 35–44.