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S100 is preferentially distributed in myelin-forming Schwann cells

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Summary

In order to elucidate the role of S100 protein *in vivo* we used postembedding electron microscopical immunocytochemistry with commercially available antibodies to S100, and secondary antibodies conjugated to colloidal gold to determine the distribution of S100 protein in the sciatic nerve and the cervical sympathetic trunk of the rat. We found that S100 immunoreactivity in Schwann cells was differentially distributed. The amount of S100 immunoreactivity in myelinated fibres appeared to correlate directly with the thickness of the myelin sheath formed by the Schwann cell. Unmyelinated fibres were identical to the small myelinated fibres in S100 immunoreactivity. Within the Schwann cells, the immunoreactivity was seen in the nucleus and in the perikaryal cytoplasm, as well as in the Schmidt-Lanterman clefts and in the paranodal loops at the node of Ranvier, but not in the myelin itself. Using these antibodies we did not see reproducible or convincing intra-axonal immunoreactivity in normal nerve.

These results suggest that in Schwann cells S100 expression may be related to axon diameter and degree of myelination. Future studies of S100 expression in development or in response to injury may clarify the role of S100 in Schwann cell biology.

Introduction

The S100 proteins were initially characterized as a group of abundant low molecular weight (10–12 kDa) acidic proteins highly enriched in nervous tissue (Moore, 1965, 1982). The S100 protein is a dimer made up of two subunits, alpha and beta, which are closely related peptides with a 58% homology in amino acid sequences (Isobe & Okuyama, 1981; Isobe *et al.*, 1981). Immunocytochemical studies have shown that S100 is found predominantly in glia in the CNS (Matus & Mughal, 1975; Ludwin *et al.*, 1976; Cocchia, 1981) and in Schwann cells in the peripheral nervous system (PNS) (Cocchia & Michetti, 1981; Stefansson *et al.*, 1982). More recently it has been demonstrated that $S100a_0$, a dimer made up of 2 alpha subunits and which accounts for $< 5\%$ of total S100, is localized principally in neurons, while alpha/beta and beta/beta dimers (S100a and S100b, respectively) are located in glia (Isobe *et al.*, 1984). This differential localization may account for some early reports of S100 immunoreactivity in neurons (Haglid *et al.*, 1974; Hansson *et al.*, 1975).

The S100 proteins are members of a large family of calcium-binding proteins of the EF hand structure (Donato, 1986; Kligman & Hilt, 1988), and detailed studies of their calcium-binding properties and calcium-induced conformational changes have been completed (Baudier, 1988). Because of its similarity to calmodulin, there has been speculation that S100 may play a role in transducing the effects of alterations in intracellular calcium concentration.

In vitro, S100 has been demonstrated to mediate calcium-dependent microtubule dissociation (Baudier *et al.*, 1982; Donato, 1988) and calcium-dependent inhibition of tau phosphorylation (Baudier & Cole, 1988). S100b has been shown to specifically inhibit the phosphorylation of pp80, a major protein kinase C substrate (Kligman & Patel, 1986). But the role of S100 in the nervous system *in vivo* has not been defined yet. S100 is present in the rat nervous system in small amounts at birth and increases rapidly during the first month of life (Herschman *et al.*, 1971; Stewart & Urban, 1972; Hyden & Ronnback, 1975; Donato, 1976; Haglid *et al.*, 1977), suggesting that it may be involved *in vivo* in nervous system development.

In order to gain further knowledge of the role of S100 protein in the PNS, we undertook this electron microscopical histochemical study of S100 localization.

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Materials and methods

Immunocytochemistry

Male Sprague-Dawley rats, 200–250 g, were used in these studies. The animals were perfused through the heart with 100 mM phosphate buffer followed by a mixture of 0.5–2.5% glutaraldehyde and 4% paraformaldehyde in phosphate buffer. The sciatic nerve and the cervical sympathetic trunk were removed, cut into blocks, placed in the same fixative for an additional 2 h, and then embedded in LR White (Polysciences).

Ultrathin sections were exposed to affinity purified polyclonal antiserum to S100 (Dako) diluted 1:300 to 1:1000 for 2 h at room temperature, followed by goat anti-rabbit IgG bound to 15 or 20 nm colloidal gold (E–Y Laboratories) for 1 h at room temperature. The grids were stained for 20 min with uranyl acetate and examined in a JEOL 100–S electron microscope.

We performed two controls for the immunocytochemical reaction. The first control consisted of deletion of the primary antibody. The second control was reaction in an identical manner using preabsorbed primary antibody with 0.5 mg ml^{-1} S100 (Wako).

Quantitative analysis of immunoreactivity

In order to obtain a quantitative estimate of the relationship of gold particle density to fibre type, grids from three different but identically performed experiments were analysed. Each of the grids had been incubated with the primary antibody diluted 1:300 for 2 h followed by secondary antibody conjugated to 15 nm colloidal gold at 1:8 for 1 h. The grids were photographed and printed at × 15 000 magnification on 20 × 25 cm paper. The number of gold particles in each Schwann cell was counted, the myelin thickness measured directly on the photograph, and the area of the Schwann cell computed from the photograph using a computerized digitizer pad (Sigma Scan, Jandel Scientific). All the Schwann cells with visible perikaryal cytoplasm at the level of the nucleus ($n = 149$) were analysed. These included 15 unmyelinated fibres, 53 small myelinated fibres (myelin thickness 0.27 ± 0.09 μm), 24 medium-size myelinated fibres (0.63 ± 0.19 μm), and 57 large myelinated fibres (1.14 ± 0.22 μm). A total of 92 601 gold particles were counted. The density of gold particles (number of particles per 10^9 μm^2) was plotted against myelin thickness and the Pearson correlation computed (SYSTAT).

Western blot

The specificity of the antiserum for S100 in nerve was confirmed by Western blot. Sciatic nerve and cervical sympathetic trunk from similar animals were sonicated with a Polytron (Brinkmann) in 5 mM Tris, 5 mM NaCl (pH 7.3) on ice, centrifuged at 6300 g for 15 min, and the resulting supernatant separated into pellet (membrane) and supernatant (soluble) fractions by centrifugation at 130 000 g for 1 h in an Airfuge (Beckman). The pellet and supernatant fractions were resolved on a 17.5% SDS–polyacrylamide gel using the buffer system of Laemmli (1970) and transferred to nitrocellulose in Towbin buffer (Towbin *et al.*, 1979). The blot was reacted with the primary antibody (1:300) followed by peroxidase-conjugated goat anti-rat IgG (1:200, Cappel), and developed with 4-chloro-1-naphthol as described by Siegel and co-workers (1986). A gel run in parallel was stained with Coomassie Blue.

Results

Antibody characterization

Western blot of the proteins from sciatic nerve demonstrated immunoreactive protein species only in the soluble, supernatant fraction. On these gels S100 alpha and S100 beta were not distinguishable, nor were they distinguishable on 2D gel electrophoresis (not shown). Occasionally, minor immunoreactivity with high M_R components was seen, but these bands were variable and appeared to account for less than 1% of the immunoreactive protein in nerve. The sciatic nerve showed a clear band of immunoreactivity (Fig. 1, lane e) but the sample from the cervical sympathetic trunk shows very little immunoreactivity (Fig. 1, lane d).

Localization of S100 in sciatic nerve

In agreement with previous studies, we found S100 principally in Schwann cells. The immunoreactivity was seen diffusely in the nucleus and cytoplasm of

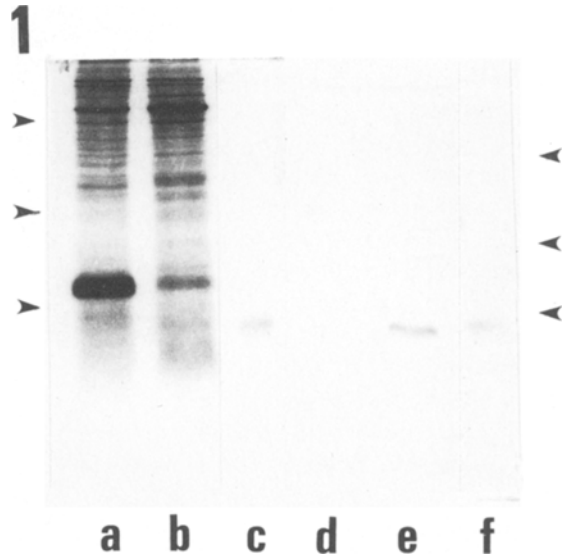


Fig. 1. 17.5% SDS gel stained with Coomassie Blue (a–c) and Western blot of identical samples (d–f). Cervical sympathetic trunk (a and d), sciatic nerve (b and e), and S100 protein (c and f). Molecular weight standards 45K, 30K and 14K (arrowheads).

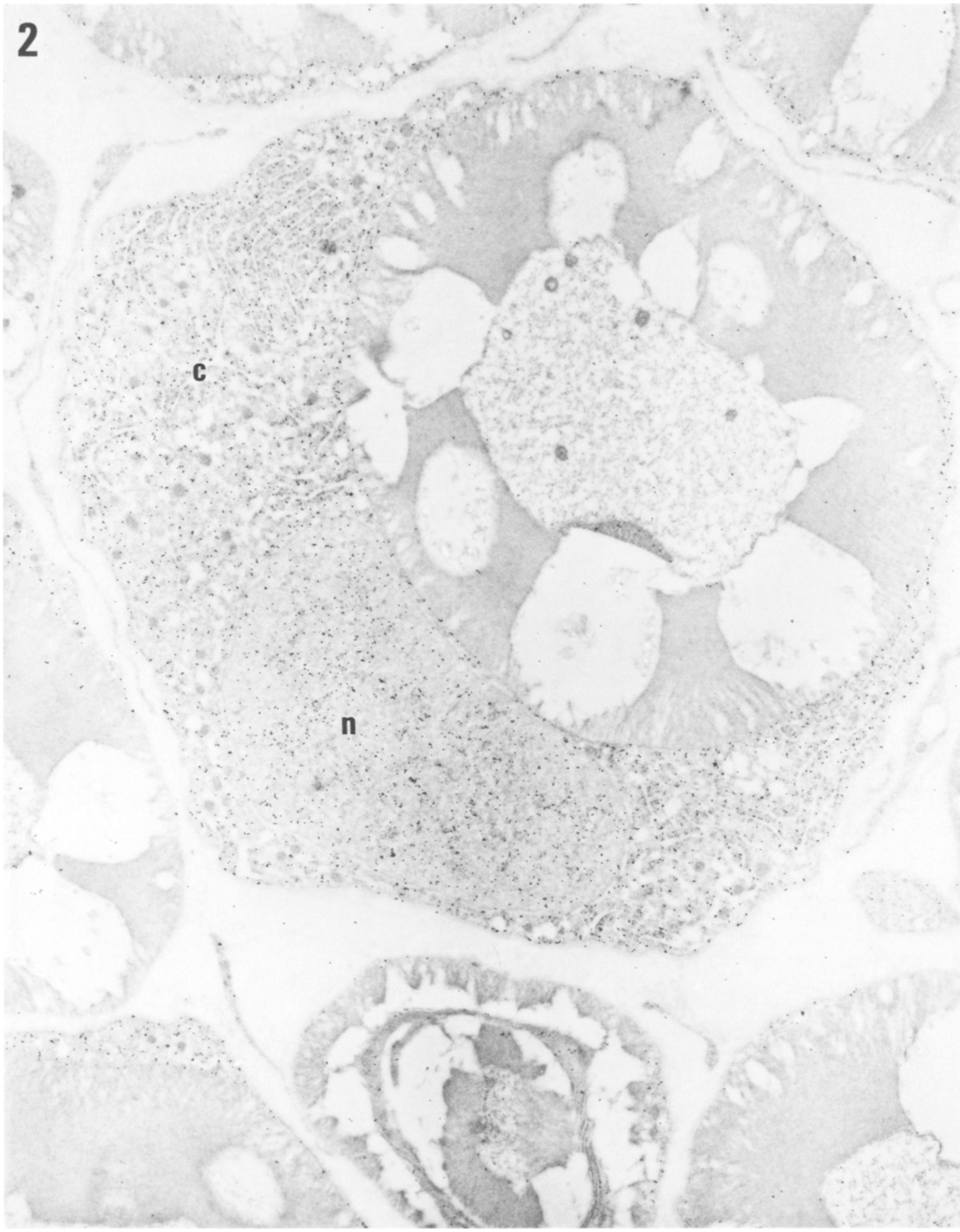


Fig. 2. Cross-section of a large myelinated axon from the sciatic nerve, demonstrating S100 immunoreactivity diffusely through the cytoplasm (c) and nucleus (n). Primary antibody 1:400. × 21 600.

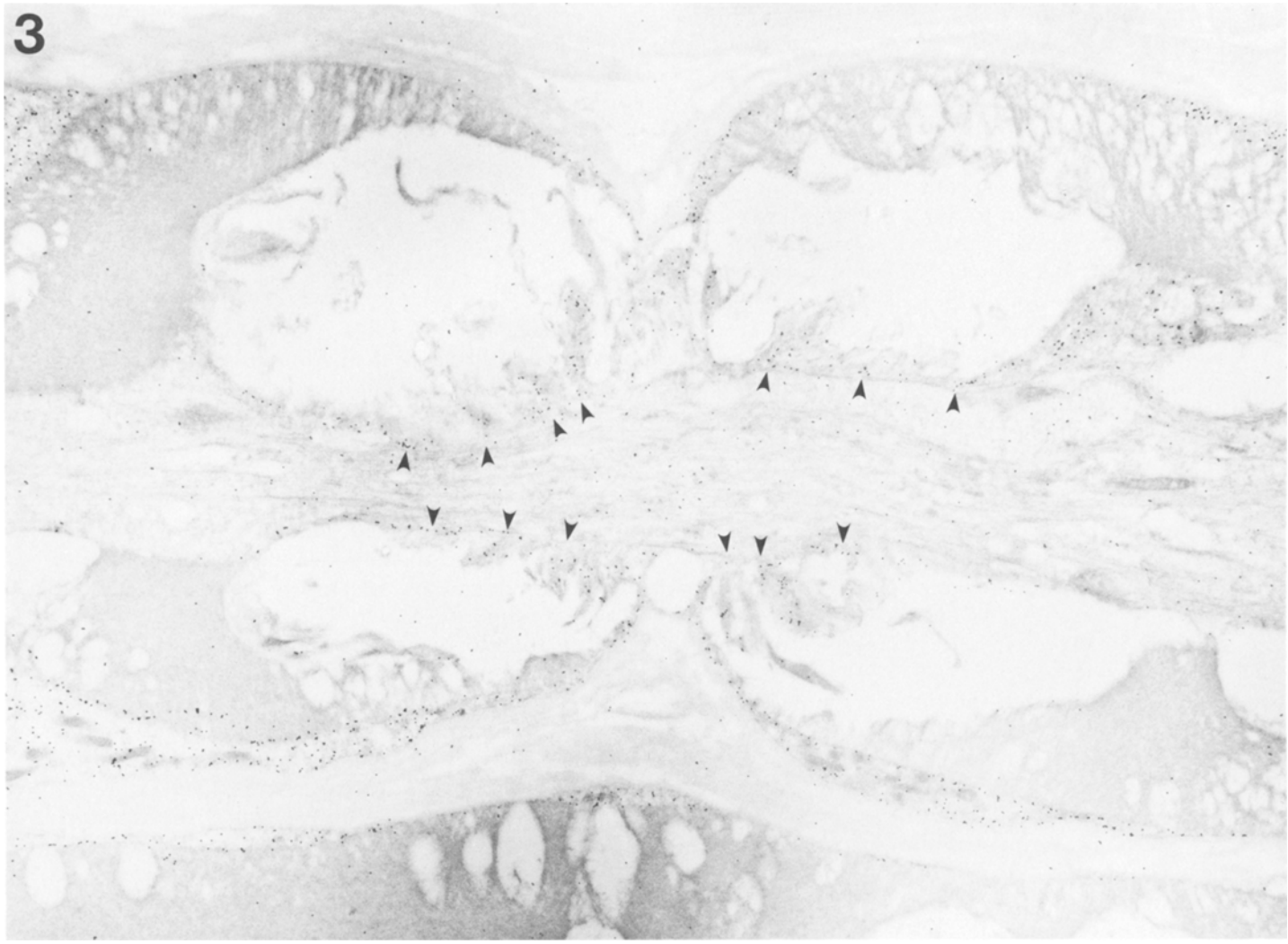


Fig. 3. Longitudinal section of sciatic nerve through a node of Ranvier demonstrating S100 immunoreactivity in the paranodal loops of myelin (arrowheads). Primary antibody 1:300. × 16 200.

these cells (Fig. 2), in the paranodal loops of myelin (Fig. 3) and along the Schmidt-Lanterman clefts (Fig. 4).

We found essentially no S100 immunoreactivity within axons with any of the antibodies we used.

The most important finding was that S100 immunoreactivity appeared to be much more prominent in the cytoplasm of myelin-forming Schwann cells than in those Schwann cells apposed to unmyelinated axons, and a greater density of S100 immunoreactivity was seen in the Schwann cells of large myelinated fibres than in the Schwann cells surrounding small myelinated fibres (Figs 4 and 5). This differential distribution of S100 immunoreactivity on single grids was seen independently of the dilution of the primary antibody and was independent of the concentration of glutaraldehyde used in fixation, although specimens processed in 2.5% glutaraldehyde (Fig. 5A) showed somewhat less immunoreactivity than those processed in 0.5% glutaraldehyde (Fig. 5B).

Controls performed with deletion of the primary antibody (Fig. 6A) or antibody preabsorbed with purified S100 protein (Fig. 6B) showed no colloidal gold deposition.

We also examined the cervical sympathetic trunk which contains principally unmyelinated, and a few small myelinated, fibres. The amount of S100 immunoreactivity in Schwann cells of the cervical sympathetic trunk was low (Fig. 7), comparable to that seen in the Schwann cells of unmyelinated and small myelinated fibres in the sciatic nerve. This is in agreement with the electrophoretic and immunoblot data (Fig. 1).

Quantitative analysis of immunoreactivity

Although immunocytochemistry with colloidal gold is not strictly quantitative, the density of gold particles correlates with the distribution of the antigen in the section when other experimental parameters are held constant. Visual inspection described above suggested that the amount of immunoreactivity correlated with fibre type. In order to demonstrate this correlation, 149 fibres from three identically-performed experiments were analysed for the density of gold particles in Schwann cell cytoplasm, and the results plotted against myelin thickness as described in Materials and methods. The correlation, shown in Fig. 8, is statistically significant ($R = 0.887$, $P < 0.001$). Unmyelinated fibres, not included in Fig. 8, were identical to the smallest myelinated fibres in gold particle density (data not shown).

Discussion

The major finding of this study is that S100 immunoreactivity is predominantly found in myelin-forming Schwann cells, and that the amount of S100 immunoreactivity in the Schwann cell correlates with the thickness of the myelin sheath formed by that cell. Stefansson and co-workers (1982) have previously demonstrated, using light microscopical immunocytochemistry, that S100 is found in Schwann cells in both myelinated and unmyelinated peripheral nerves, but it is not possible to compare the relative amounts of S100 immunoreactivity in the different Schwann cell populations in that study.

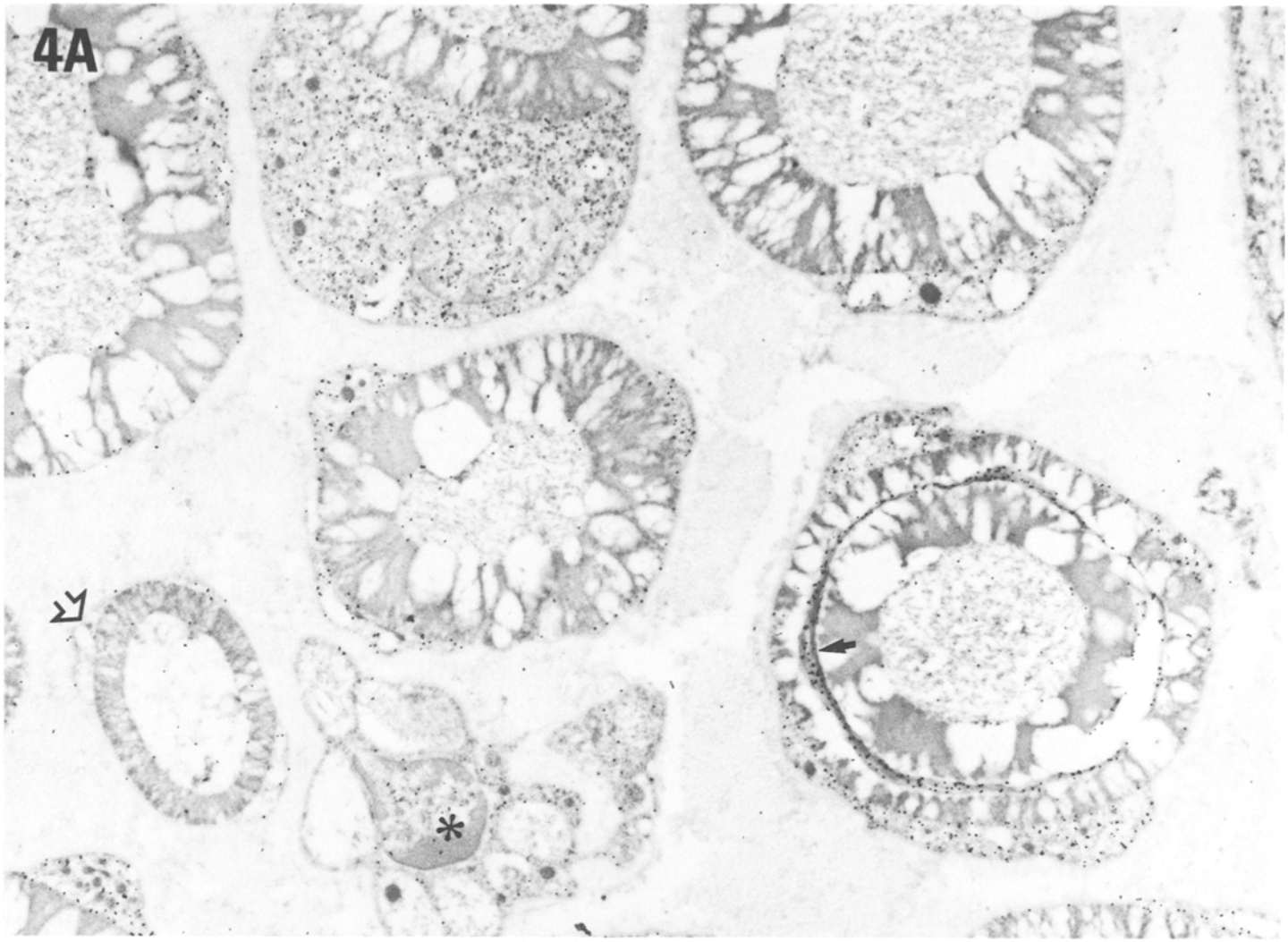
The differential distribution of S100 in cells forming myelin suggests that S100-mediated interactions may be involved in the process of forming or maintaining myelin. Data from *in vitro* studies suggest that glial cells in tissue culture express low levels of S100 immunoreactivity constitutively, but when cultured in the presence of neurons accumulate high levels of S100 (Holton & Western, 1982, 1988). Other investigators have shown that Schwann cells in culture require axonal contact to form myelin (Bunge *et al.*, 1980). The time course of expression of S100 in developing rat nervous system broadly parallels the course of myelination, the amount of S100 increasing rapidly during the first two months of life (Herschman *et al.*, 1971; Stewart & Urban, 1972; Hyden & Ronnback, 1975; Donato, 1976; Haglid *et al.*, 1977). However, a systematic study of the relationship of S100 expression to the time of myelination of specific tracts has not been performed.

Ultrastructural studies suggest that in adipocytes S100 may serve as one of the intracellular protein

Fig. 4. Cross-section of sciatic nerve demonstrating differential distribution of S100 immunoreactivity. Schwann cells surrounding unmyelinated fibres (asterisks) and small myelinated fibres (open arrow) contain less S100 immunoreactivity than adjacent Schwann cells surrounding large myelinated fibres. Immunoreactivity is also seen in the Schmidt-Lanterman cleft (solid arrow). No immunoreactivity is seen in fibroblasts (f). (A) Primary antibody 1:400. × 15 000. (B) Primary antibody 1:400. × 14 400.

Fig. 5. Adjacent axons in cross-section of sciatic nerve (A) and ventral root (B), sectioned through the level of the Schwann cell nucleus, demonstrate the differential distribution of S100 immunoreactivity between the Schwann cell surrounding the large myelinated fibres and those surrounding small myelinated fibres. Cytoplasm (c), nucleus (n), small myelinated fibre (open arrow). (A) Primary antibody 1:700. × 15 000. (B) Primary antibody 1:700. × 16 000.

Fig. 6. Control sections. (A) Omission of the primary antibody. × 18 000. (B) S100 antibody (1:700) preabsorbed by 1.25 mg ml^{-1} S100 protein. × 15 000.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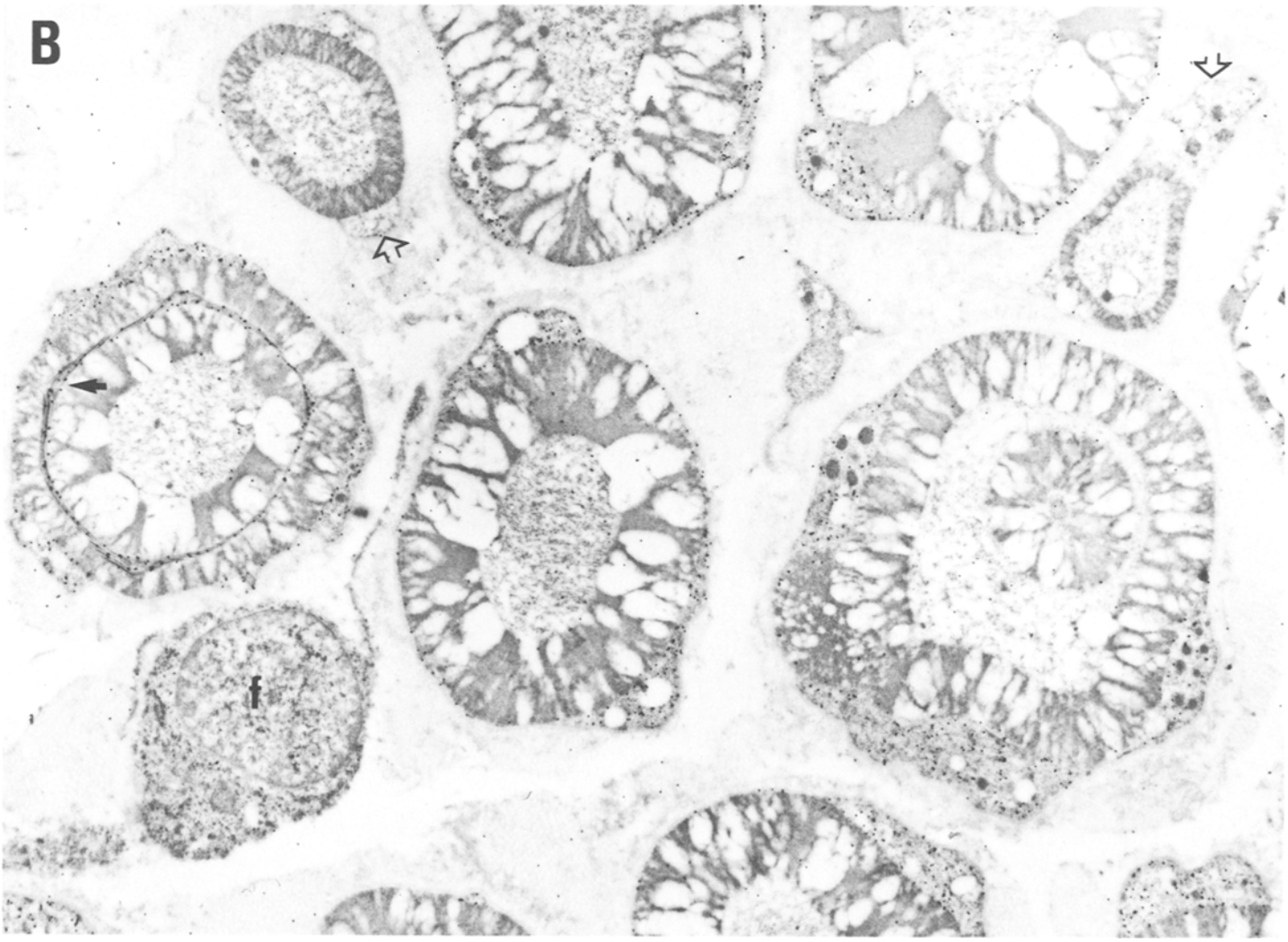
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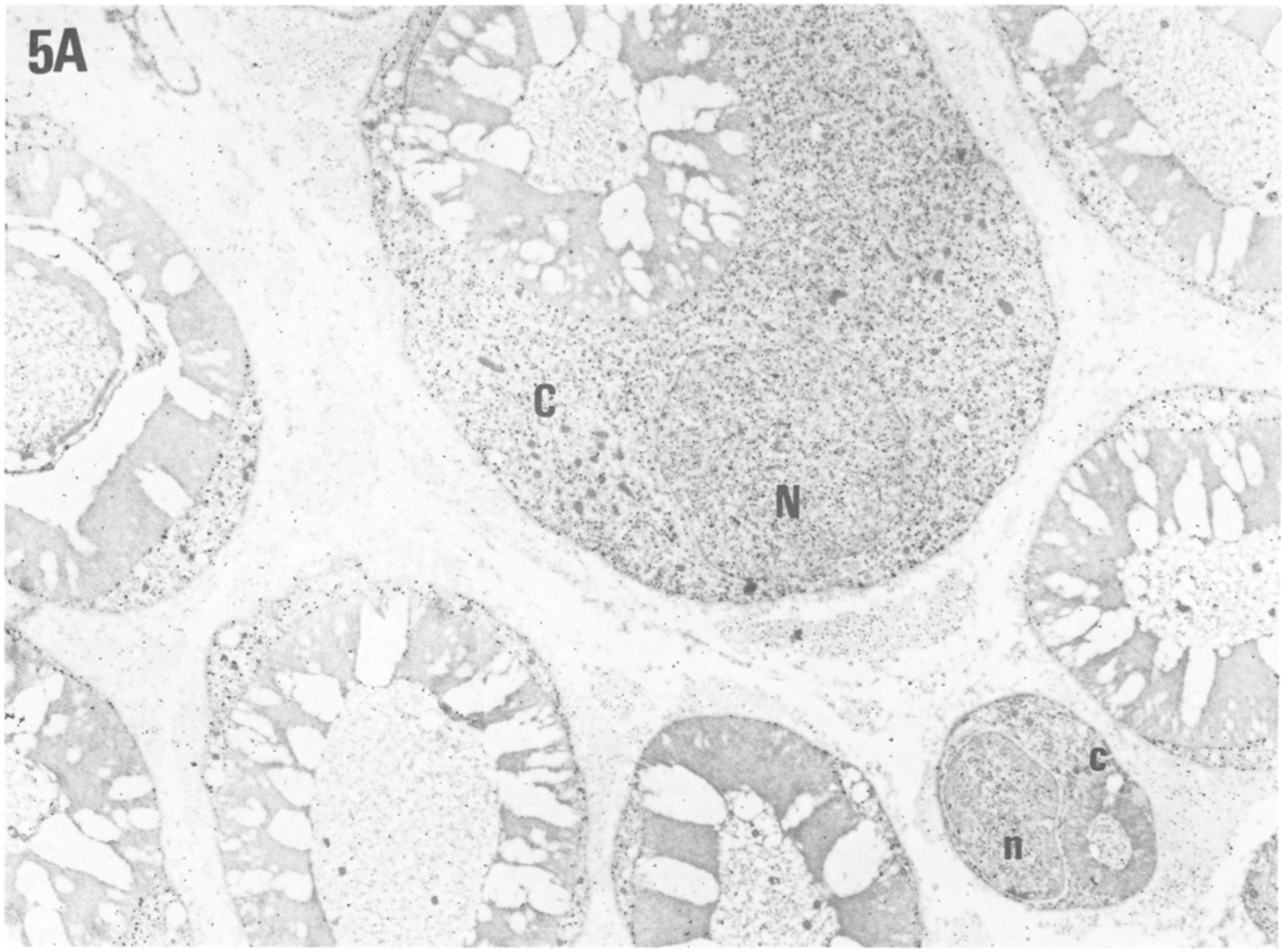
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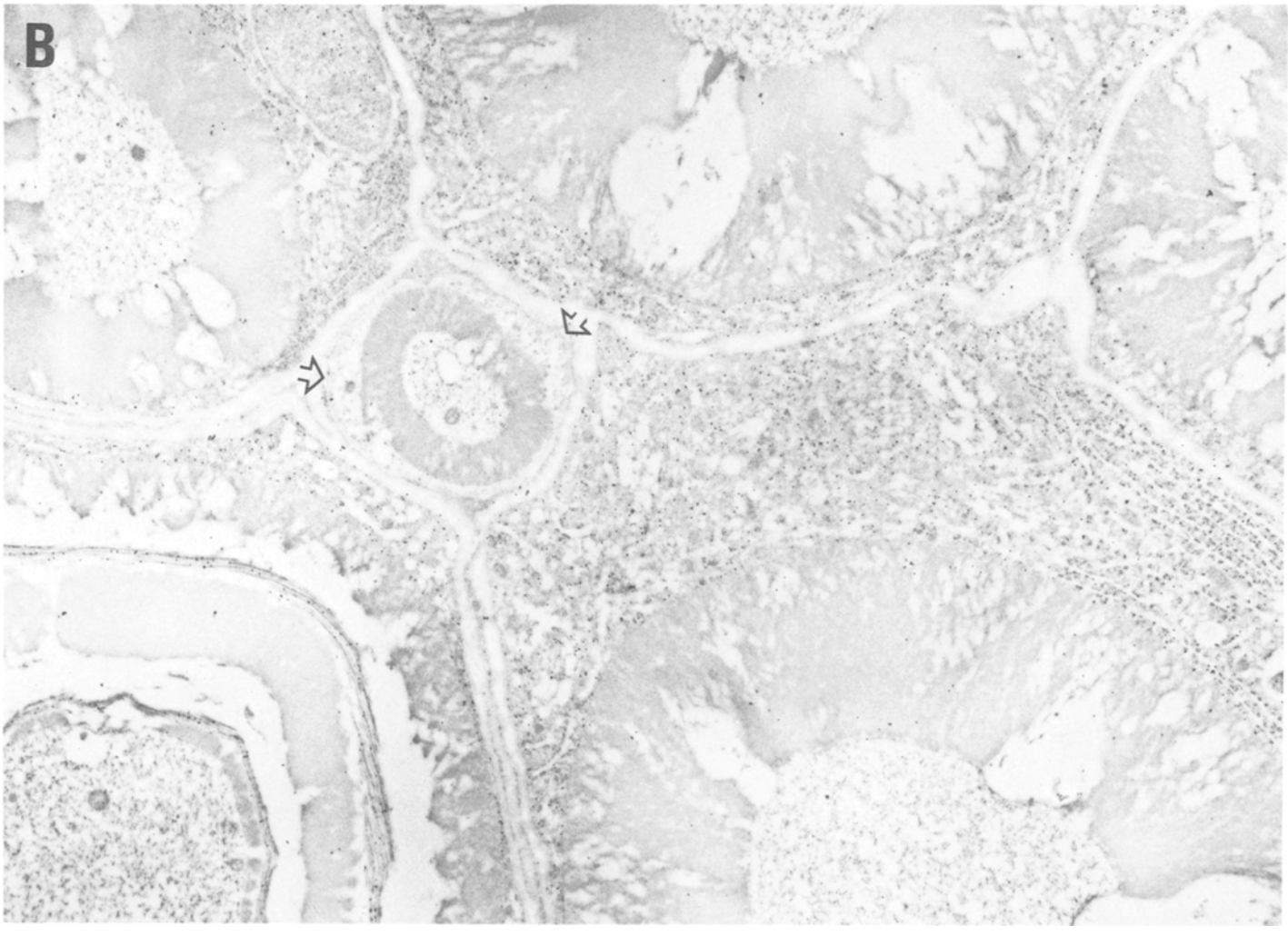
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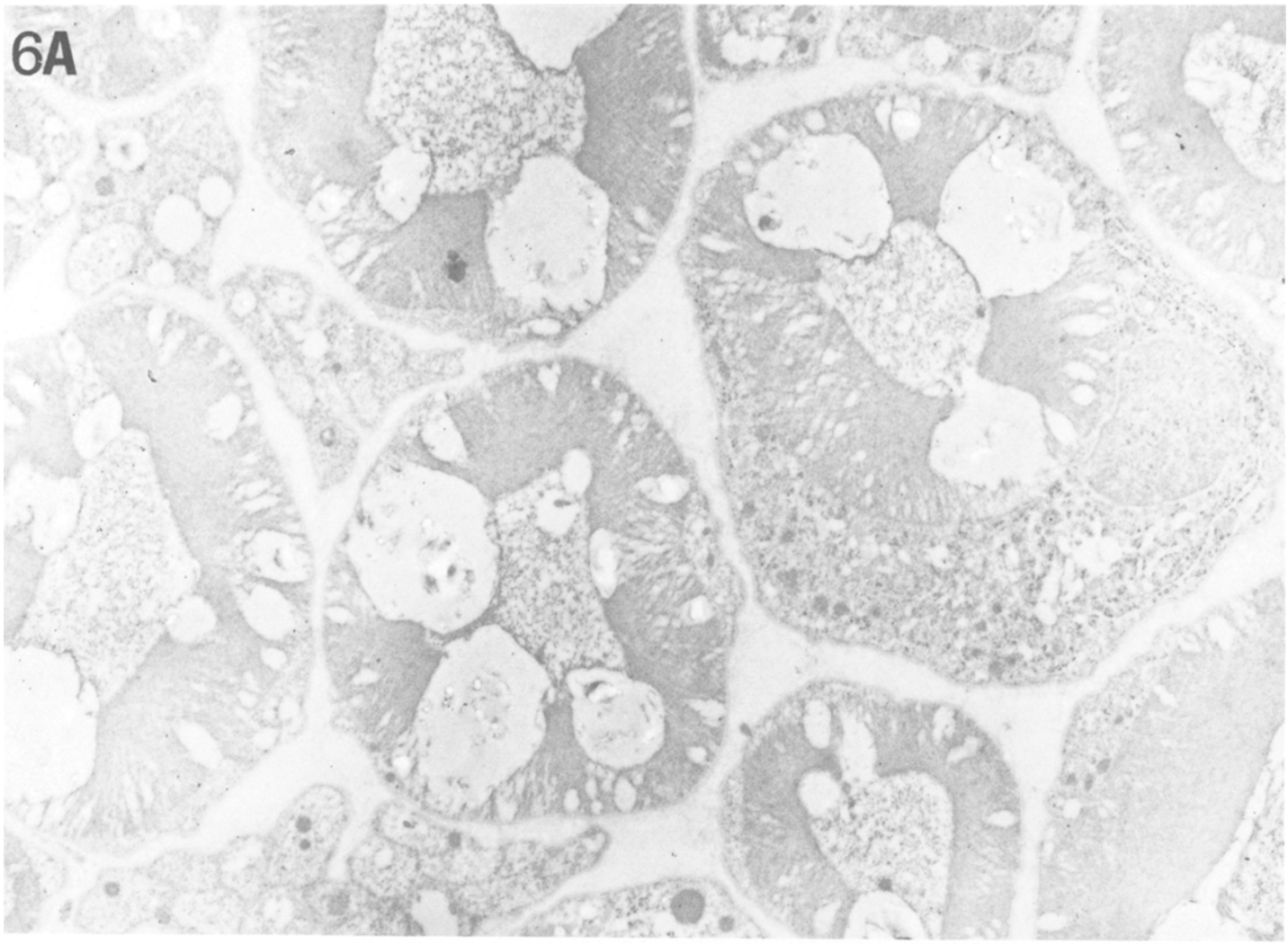
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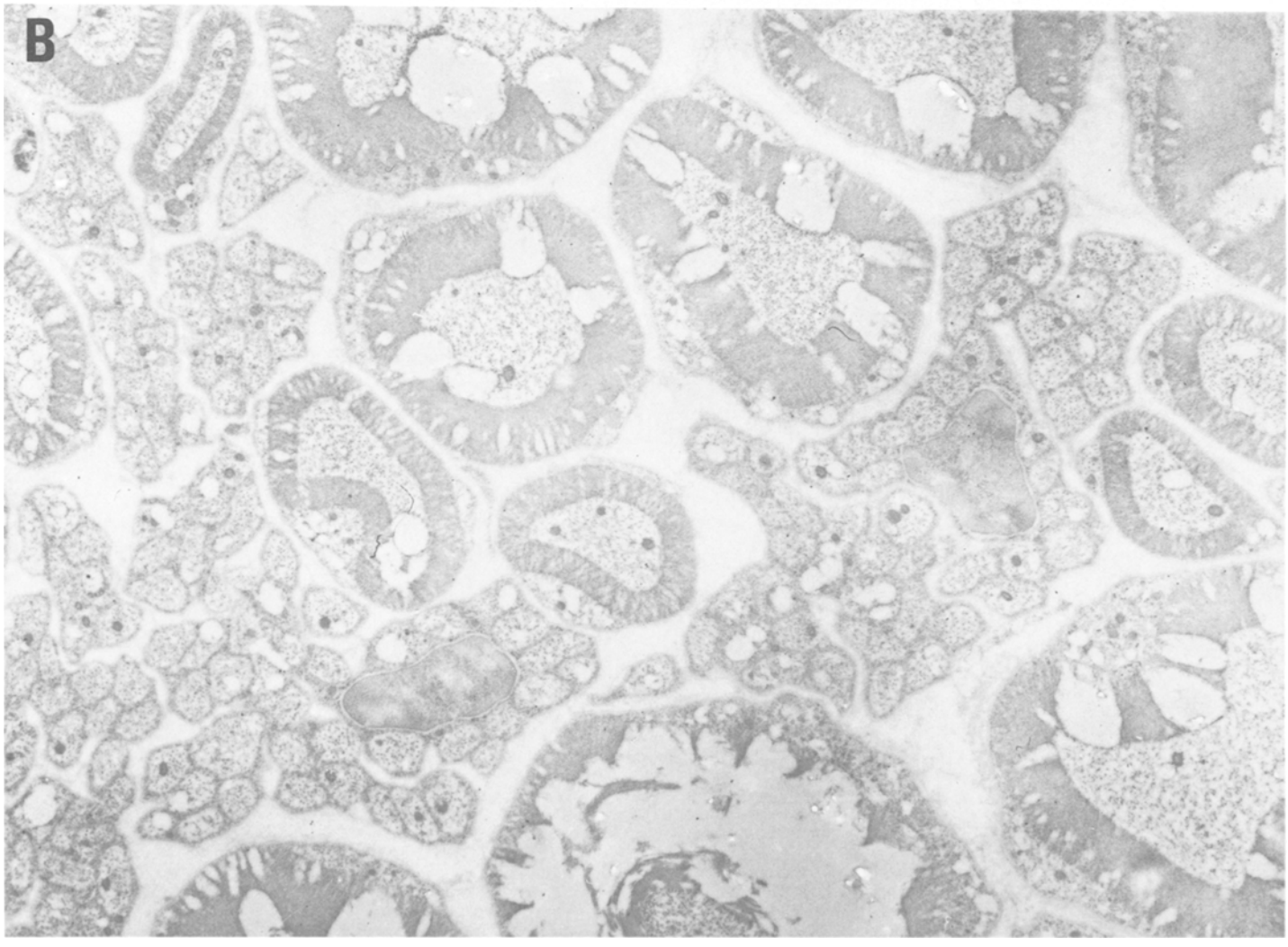
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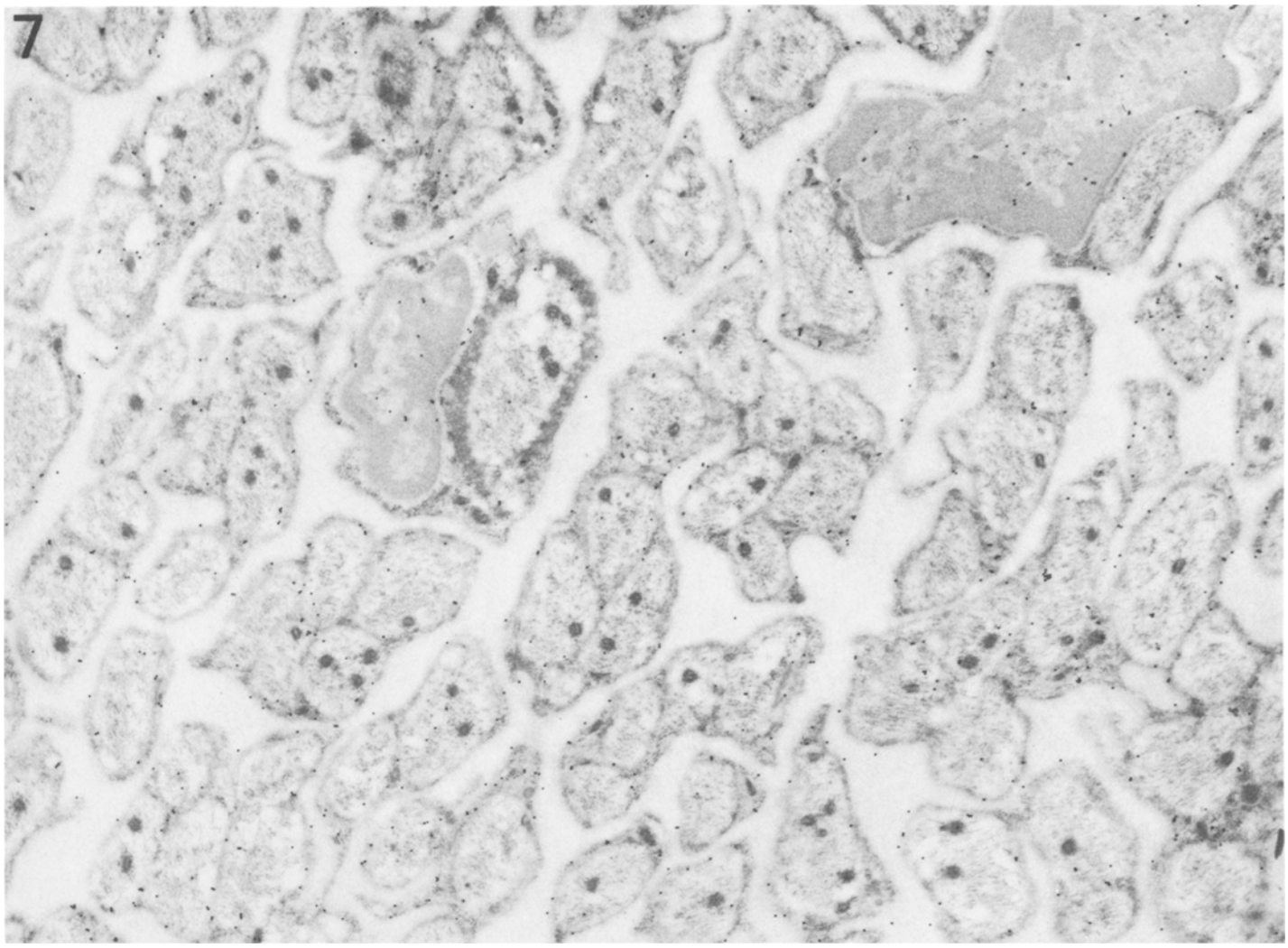

Fig. 7. Cross-section of cervical sympathetic trunk shows unmyelinated and a few very small myelinated fibres with very little S100 immunoreactivity. Primary antibody 1:300. × 26 000.

carriers of free fatty acids (Haimoto *et al.*, 1985). The protein might play a similar role in Schwann cells during myelination. An alternative possibility is that the S100 protein may play a role in the reorganization of microtubules in Schwann cells (Donato *et al.*, 1986; Donato, 1987), which might be required for myelination. These two possibilities are not mutually exclusive.

The localization of S100 in the nucleus of Schwann cells is similar to that previously reported in astrocytes in the CNS (Ludwin *et al.*, 1976; Cocchia, 1981) and supports the biochemical co-localization of S100 protein with nuclei isolated from the cerebral cortex (Michetti *et al.*, 1974). A recent report showed no S100 immunoreactivity in the nucleus of Schwann cells (Spreca *et al.*, 1989). However, that study utilized pre-embedding immunocytochemistry on 80 μm thick sections. It is possible that the different results are due to difficulty in antibody penetration through the nuclear membrane in that preparation. The current study utilized postembedding immunocytochemistry which allows full access of the antiserum to all regions of the sectioned tissue.

We did not find any consistent evidence of S100b immunoreactivity in axons in the mature, steady-state nervous system. While this does not support an active role for S100 as a neurotrophic factor in adult nerve in the steady state (Kligman & Marshak, 1985; Kligman & Hsieh, 1987) the potential role of S100 in development or in recovery from injury is not addressed in this study.

In conclusion, our results suggest that Schwann cell expression of S100 is related to the degree of myelination. We have begun experimental studies to define more clearly the role of S100 in development and in recovery following injury.

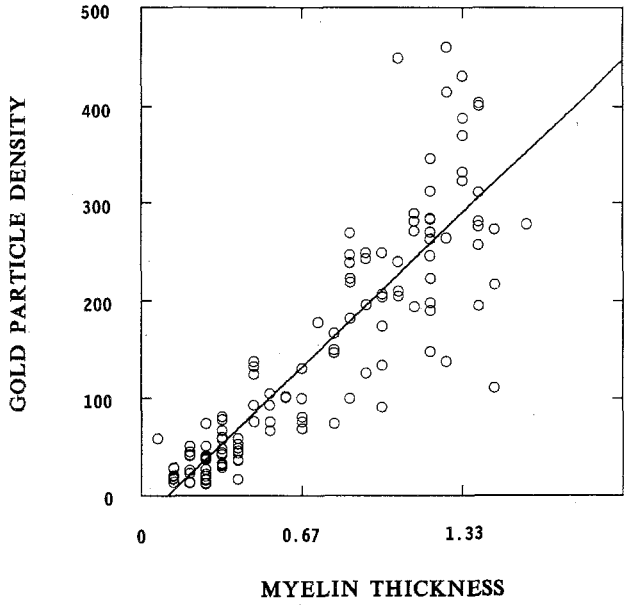

Fig. 8. Density of gold particles (grains per 10^9 μm^2) plotted against myelin thickness (μm). Both measurements are taken from photographs as described in the text. The correlation is statistically significant ($R = 0.887$, $P < 0.001$).

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A preliminary communication of some of these results was presented at meeting of the Society for Neuroscience in October 1989 in Phoenix, Arizona, USA.

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