Immunocytochemical Localization of a Chondroitin Sulfate Proteoglycan in Nervous Tissue. I. Adult Brain, Retina, and Peripheral Nerve

D. A. AQUINO, R. U. MARGOLIS, and R. K. MARGOLIS
Department of Pharmacology, New York University Medical Center, New York 10016; and Department of Pharmacology, State University of New York, Downstate Medical Center, Brooklyn, New York 11203

ABSTRACT Monospecific antibodies were prepared to a previously characterized chondroitin sulfate proteoglycan of brain and used in conjunction with the peroxidase-antiperoxidase technique to localize the proteoglycan by immunoelectron microscopy. The proteoglycan was found to be exclusively intracellular in adult cerebellum, cerebrum, brain stem, and spinal cord. Some neurons and astrocytes (including Golgi epithelial cells and Bergmann fibers) showed strong cytoplasmic staining. Although in the central nervous system there was heavy axoplasmic staining of many myelinated and unmyelinated fibers, not all axons stained. Staining was also seen in retinal neurons and glia (ganglion cells, horizontal cells, and Müller cells), but several central nervous tissue elements were consistently unstained, including Purkinje cells, oligodendrocytes, myelin, optic nerve axons, nerve endings, and synaptic vesicles. In sympathetic ganglion and peripheral nerve there was no staining of neuronal cell bodies, axons, myelin, or Schwann cells, but in sciatic nerve the Schwann cell basal lamina was stained, as was the extracellular matrix surrounding collagen fibrils. Staining was also observed in connective tissue surrounding the trachea and in the lacunae of tracheal hyaline cartilage. These findings are consistent with immunochemical studies demonstrating that antibodies to the chondroitin sulfate proteoglycan of brain also cross-react to various degrees with certain connective tissue proteoglycans.

We have previously described the isolation and properties of a 6.5S chondroitin sulfate proteoglycan from a PBS extract of rat brain (19). This proteoglycan has a relatively high protein content (56% by weight), in addition to 24% glycosaminoglycans (predominantly chondroitin 4-sulfate) and 20% glycoprotein oligosaccharides, including a series of novel *O*-glycosidically linked oligosaccharides that contain mannose at their proximal ends (16, 28). The brain proteoglycan differs from the prototypical cartilage proteoglycans in a number of respects including its higher protein content, smaller monomer size, and its relatively limited ability to interact with hyaluronic acid to produce larger size aggregates.

Biochemical analyses (for reviews, see references 27 and 28) demonstrated the presence of significant amounts of chondroitin sulfate in neuronal cell bodies, axons, and astrocytes (isolated in bulk from brain) as well as in purified nuclei, whereas there is little or no chondroitin sulfate in oligodendroglia, myelin, mitochondria, or nerve endings (synaptosomes). Approximately half of the chondroitin sulfate is found in the soluble fraction after high speed centrifugation of a brain homogenate, and much of the remainder is loosely associated with microsomal membranes from which it is easily extractable by mild washing procedures (19, 29).

When neurons are isolated in bulk from rat brain and lysed by a change in tonicity or pH, 82% of the chondroitin sulfate is released together with >90% of the lactate dehydrogenase (but only 20–25% of the total cell protein and glycoprotein hexosamine; 27). Since the chondroitin sulfate was not removed during the previous washing steps in the cell isolation procedure or by mild trypsinization of the purified neurons, we concluded that a significant portion of the chondroitin sulfate proteoglycan is present as a cytoplasmic (and axoplasmic) component of neurons. However, these biochemical analyses could not provide a very detailed picture of the distribution of chondroitin sulfate among various cell types and subcellular compartments, nor any information relevant

THE JOURNAL OF CELL BIOLOGY · VOLUME 99 SEPTEMBER 1984 1117–1129
© The Rockefeller University Press · 0021-9525/84/09/1117/13 $1.00

to the postulated extracellular localization of chondroitin sulfate proteoglycans in nervous tissue. In the present immunocytochemical studies we have therefore utilized specific antibodies to the proteoglycan to investigate its cellular and tissue distribution at the light and electron microscopic levels, while an accompanying report (2) describes developmental changes in the localization of this proteoglycan in rat cerebellum.

MATERIALS AND METHODS

Isolation of the Chondroitin Sulfate Proteoglycan: The proteoglycan was isolated as previously described (21) from a 140,000 *g* supernatant of adult rat brain (Sprague-Dawley) homogenized in 5 mM PBS, pH 7.2. The supernatant was dialyzed against deionized water, lyophilized, redissolved in 50 mM Tris-HCl buffer (pH 8.25 at 4°C) containing 90 mM NaCl, and applied to a column of DEAE-cellulose previously equilibrated with the same buffer. After stepwise elution with lower ionic strength buffers, the proteoglycan was eluted with 0.5 M NaCl in 50 mM Tris-HCl buffer, pH 8.25. These fractions were pooled, dialyzed against deionized water, lyophilized, redissolved in 0.2 M sodium acetate buffer (pH 5.6), and eluted from a column of Sepharose CL-6B using the same buffer. The purified proteoglycan appeared in the void volume, well separated from a large retarded peak of protein and nucleic acid.

Preparation of IgG and F(ab')$_2$: The purified proteoglycan was dissolved at a concentration of 6.6 mg/ml (wt/vol) in 0.9% NaCl. 0.75 ml of this solution (5 mg of proteoglycan) was mixed with an equal volume of Freund's complete adjuvant (Difco Laboratories Inc., Detroit, MI) and emulsified in an ice bath by probe sonication (10 W, 10 s bursts) until a drop of the emulsion remained undispersed in water. After a preimmune serum specimen was collected, female New Zealand White rabbits were immunized by injecting 0.75 ml of emulsion into each hind leg gluteal muscle. 2 wk after the initial immunization the rabbits were boosted with 2 mg proteoglycan (in 1.5 ml) injected as above. After an additional 2 wk rabbits were again boosted in the same way, except that one-quarter of the innoculum was injected into each thigh and one-half was injected subcutaneously in the interscapular region. 20 ml of blood was obtained from the auricular artery at 1 and 3 wk after the initial immunization, and rabbits were bled-out by cardiac puncture under anesthesia after a total of 5 wk. All serum samples were assayed for antibody titer.

IgG was precipitated from fresh or frozen serum by adding an equal volume of saturated ammonium sulfate at room temperature. After standing for 15 min with occasional mixing, the serum was centrifuged at 25°C for 10 min at 48,000 *g*. The pellet was resuspended in a minimal volume of 50 mM PBS (pH 7.2) containing 0.05% sodium azide, and dialyzed against the same buffer at 4°C.

For preparation of F(ab')$_2$ fragments, the IgG solution was diluted to 25 mg IgG/ml with 0.2 M sodium acetate buffer (pH 4.0) containing 0.1% sodium azide, and dialyzed overnight against the same buffer at 4°C. (IgG concentration was calculated on the basis of $E^{1\%}_{1cm}$ at 280 nm = 14; reference 25). Pepsin (Worthington Biochemical Corp., Freehold, NJ, twice crystallized) was added at a concentration of 0.02 mg/mg IgG, and the solution was stirred for ~24 h at 37°C. The digestion was terminated by adding Tris-base to pH 8.6, followed by stirring for ~20 min. The digest was then dialyzed at 4°C against 50 mM PBS, pH 7.2, containing 0.05% sodium azide. After concentration to 10 ml by pressure ultrafiltration (Amicon Corp., Scientific Systems Div., Danvers, MA, PM30 membrane), the digest was centrifuged for 1 h at 114,000 *g*, and the supernatant was applied to a column (2.5 × 93 cm) of Ultrogel AcA 44 (LKB Instruments, Inc., Gaithersburg, MD) previously equilibrated with 50 mM PBS (pH 7.2) at 4°C containing 0.05% sodium azide. 8-ml fractions were collected and monitored for absorbance at 280 nm.

Fractions containing F(ab')$_2$ fragments were pooled, concentrated by pressure ultrafiltration, and further purified by affinity chromatography on a column of AH-Sepharose 4B (Pharmacia Inc., Piscataway, NJ) to which the purified brain proteoglycan had been coupled using the general procedure of Cambiaso et al. (9). AH-Sepharose 4B was suspended in 50 mM borate-phosphate buffer (pH 8.4) and washed six times, each time separating the washed beads by low speed centrifugation (600 *g* for 10 min). 1 ml of 25% glutaraldehyde diluted with 6 ml of borate-phosphate buffer was added to 3 ml of Sepharose beads (packed by centrifugation). After 15 min of mixing on a Clay Adams Nutator (Clay Adams, Div. of Becton, Dickinson & Co., Parsippany, NJ) at room temperature, the activated Sepharose was washed with the same buffer on a sintered glass filter funnel.

The chondroitin sulfate proteoglycan (1.8 mg protein/ml in borate-phosphate buffer) was centrifuged at 12,000 *g* for 10 min, and the supernatant was added to an equal volume of packed AH-Sepharose 4B. This suspension was mixed for 1 h at room temperature, followed by ~40 h at 4°C. The Sepharose was brought to room temperature, poured into a column (0.9 cm diam), and rinsed with distilled water until no protein was detectable by measuring the absorbance of the effluent at 280 nm. This was followed by additional rinses with 5, 100, and 5 mM Tris-HCl buffer (pH 7.6) containing 0.05% sodium azide. The Sepharose was finally washed with 0.01 M PBS, pH 7.2, containing 0.05% sodium azide.

F(ab')$_2$ fragments were diluted to 10 mg/ml with 50 mM PBS (pH 7.2) containing 0.05% sodium azide, and mixed with an equal volume of settled proteoglycan-Sepharose equilibrated with the same buffer. After ~48 h of mixing at 4°C, the Sepharose was warmed to room temperature and poured into a column (0.9 cm diam). Nonspecifically bound proteins were eluted by sequential rinses with 10 mM PBS (pH 7.2), 1 M NaCl containing 0.1% polyoxyethylene sorbitan monolaurate (Tween 20; Sigma Chemical Co., St. Louis, MO), and 10 mM PBS (pH 7.2), until no protein was detected by measuring the absorbance of the effluent at 280 nm. Specifically bound antibodies were eluted with 0.1 M glycine-HCl buffer (pH 3.0), immediately neutralized to pH 7.0–7.2 with 0.5 N NaOH, pooled, dialyzed at 4°C against 50 mM PBS (pH 7.2), and concentrated by pressure ultrafiltration. All buffers for affinity chromatography and dialysis contained 0.05% sodium azide.

Immunodiffusion and Immunoelectrophoresis: Immunogels were prepared with 0.7% (wt/vol) agarose in 10 mM PBS for immunodiffusion. Gels for immunoelectrophoresis contained 0.6% agarose, 90 mM Tris, 80 mM boric acid, 3 mM disodium EDTA, and 0.05% (wt/vol) sodium azide at pH 8.3. Antibody was added immediately before pouring plates at 52°C. Glass plates (previously coated with 0.2% agarose in distilled water containing 0.05% sodium azide) were usually coated with 0.2 ml of gel per square centimeter.

Immunodiffusion was for 24 h at room temperature in a humidified chamber. Immunoelectrophoresis (either Laurell or crossed) was at constant voltage as indicated in the figure legends. Plates were dried, washed in PBS and deionized water, and finally air-dried before staining with Coomassie Blue (38).

Enzyme-linked Immunosorbent Assay (ELISA)[1]: 5-μg aliquots of the proteoglycan, dissolved in 10 mM PBS containing 0.05% sodium azide, were air-dried in wells of polystyrene microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA: No. 01-010-2201). Unbound antigen was removed by washing the plates for 5 min with PBS containing 0.1% Tween 20. The wells were emptied by inverting the plates, and the washing procedure was repeated once. Residual buffer was aspirated from the wells using polyethylene tubing, after which 20 μl of antibody solution were added to the wells in doubling dilutions (in PBS containing 0.1% Tween 20). After standing for 15 min at room temperature, the plates were washed twice for 1 min with PBS containing 0.1% Tween 20. The buffer was then aspirated before adding 20 μl of peroxidase-conjugated swine IgG anti-rabbit IgG (DAKO, Accurate Chemical and Scientific Corp., Westbury, NY) diluted 20-fold with PBS containing 0.1% Tween 20, or peroxidase-conjugated goat IgG anti-rabbit F(ab')$_2$ (Cappel Laboratories, Cochranville, PA) diluted 320-fold. After standing for 15 min at room temperature the plates were washed twice as before. The buffer was aspirated and 20 μl of phenylenediamine solution was added. The reaction was allowed to develop for 2 h at room temperature in a humidified chamber, after which it was terminated by the addition of 1.2 μl of 4 N HCl. The highest antibody dilution which allowed an observable color development was taken as the ELISA endpoint.

The phenylenediamine solution was prepared by dissolving 2.5 mg of *o*-phenylenediamine (Sigma Chemical Co.) in 0.1 ml of methanol, to which were added 10 ml of 0.1 M phosphate buffer, pH 6.0. Prior to use, 1.6 μl of 50% hydrogen peroxide was added.

Tissue Preparation: Adult Sprague-Dawley rats were anesthestized with sodium pentobarbital (6.5 mg/100 g body wt) and perfused through the left ventricle, first briefly with 0.1 M sodium phosphate buffer (pH 7.4) containing 0.9% NaCl, and then with a solution containing 4% freshly prepared formaldehyde and 0.1% glutaraldehyde in the same buffer. Rats from which retinas were obtained were perfused in three steps: (*a*) sodium phosphate buffer, pH 7.4, as above; (*b*) 4% freshly prepared formaldehyde and 0.1% glutaraldehyde in sodium phosphate buffer, pH 6.5; and (*c*) same aldehydes in sodium phosphate buffer, pH 11 (4). The rats were perfused with ~1 ml of fixative per gram of body weight at a rate of 1–3 ml/min. Tissues of interest were removed and allowed to stand in fixative at room temperature for ~1 h before being placed at 4°C overnight.

Immunofluorescence: Fixed tissue was sectioned to 15-μm thickness with a Vibratome (Oxford Instruments Inc., Columbia, MD), or unfixed tissues were quick-frozen on dry ice and sectioned to 8 μm with a microtome/cryostat, after which sections were mounted on gelatin-coated glass slides. The sections were covered with several drops of affinity-purified antibody solution (0.85 mg

[1] *Abbreviations used in this paper:* ELISA, enzyme-linked immunosorbent assay.

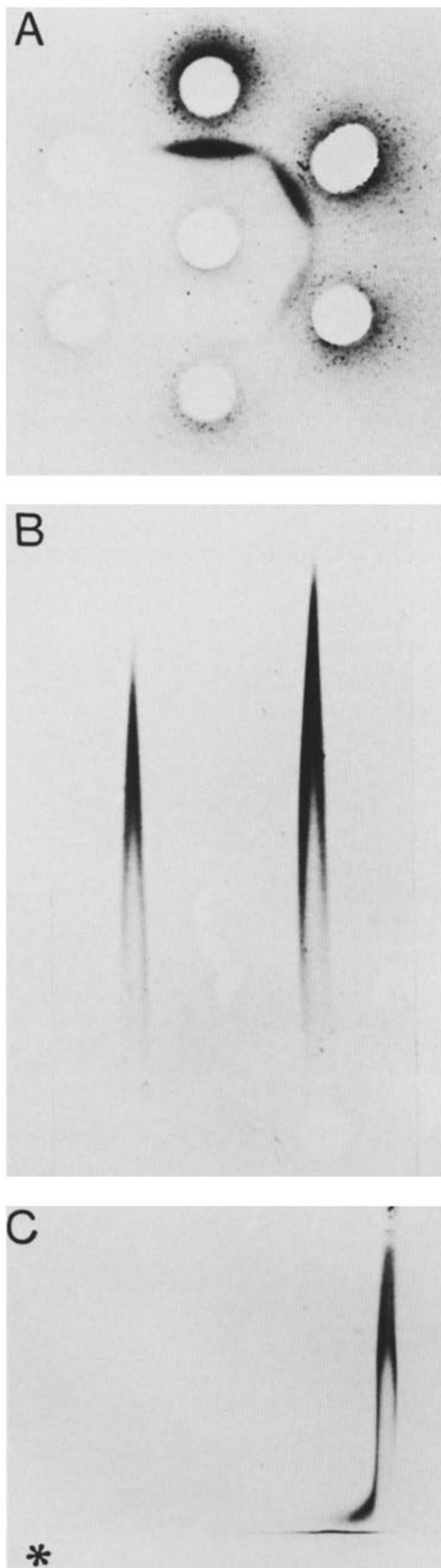

FIGURE 1 (A) Double immunodiffusion of the chondroitin sulfate proteoglycan. 33 μg of proteoglycan was placed in the center well, with doubling dilutions of rabbit antiproteoglycan IgG in surrounding wells. (B) Rocket immunoelectrophoresis (1 V/cm, 18 h) of the proteoglycan (0.41 and 0.83 μg) against IgG. (C) Crossed immunoelectrophoresis of the chondroitin sulfate proteoglycan. Electrophoresis of 3.3 μg of proteoglycan placed at the origin (asterisk) ran initially from left to right at 3 V/cm for 2 h, after which the direction was changed to run from bottom to top against IgG at 1 V/cm for 18 h.

protein/ml of 0.05 M PBS, pH 7.6, containing 0.1% Tween 20), and incubated for 1 h. After three 10-min washes with PBS, the sections were incubated with fluorescein isothiocyanate-conjugated goat IgG anti-rabbit $F(ab')_2$ (Cappel Laboratories) diluted 800-fold with PBS. All incubations with antibodies were at room temperature in a humidified chamber. The sections were washed three times with PBS, and then for 10 min with 0.05 M Tris-buffered saline, pH 7.6. Sections were mounted under glass coverslips in 0.1 M Tris-HCl buffer (pH 8.6) containing 50% glycerol.

Peroxidase-Antiperoxidase Staining: Fixed tissues were sectioned with an Oxford Vibratome to 15 or 20 μm thickness for light or electron microscopy, respectively. Sections were washed with agitation in five changes (10 min each) of 0.1 M sodium phosphate buffer, pH 7.4. After a 30-min incubation with agitation at room temperature in 3% normal swine serum (DAKO, Accurate Chemical and Scientific Corp.), the sections were transferred to a solution of affinity-purified antibodies at a concentration of 7 μg protein/ml for light microscopy or 130 μg protein/ml for electron microscopy. Dilutions were made in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.9% NaCl and 1% normal swine serum. Control sections were incubated with $F(ab')_2$ that was not adsorbed by the affinity column. Sections were incubated with the antibodies for ~40 h at 4°C with agitation in a humidified chamber. They were then treated as follows: (*a*) warmed to room temperature for 2 h; (*b*) washed five times for 10 min each time with agitation in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.9% NaCl; (*c*) incubated with agitation for 30 min at room temperature with swine IgG anti-rabbit IgG (DAKO) diluted 50-fold with Tris-HCl buffer containing 1% normal swine serum; (*d*) washed five times for 10 min as in step (*b*); (*e*) incubated for 30 min at room temperature with a soluble horseradish peroxidase/anti-horseradish peroxidase complex (prepared in rabbits, DAKO) diluted 100-fold in buffer containing 1% normal swine serum; (*f*) washed five times for 10 min; (*g*) incubated for 30 min at room temperature with 0.05% 3,3'-diaminobenzidine and 0.01% hydrogen peroxide in Tris-HCl buffer; and (*h*) washed three times for 10 min in 0.1 M sodium phosphate buffer, pH 7.4.

Sections for light microscopy were mounted on gelatin-coated slides, dehydrated through a series of graded ethanols, cleared with xylene, sealed in Permount (Fisher Scientific Co., Pittsburgh, PA), and examined with a Leitz Ortholux microscope.

For electron microscopy, sections were fixed for 1 h with 1% osmium tetroxide in 0.1 M sodium phosphate buffer, pH 7.4, dehydrated through a series of graded ethanols, infiltrated with EPOX 812 (Ernest F. Fullam, Inc., Latham, NY), and embedded for thin-sectioning using either an LKB Ultrotome V or a Sorvall MT-5000 ultramicrotome (E. I. DuPont de Nemours & Co., Inc., Sorvall Instruments Div., Newton, CT). Tissue was generally not poststained except where specifically indicated, in which case 4% uranyl acetate in 50% methanol was used, followed by 0.2% lead citrate in distilled water. Grids were examined with a JEOL-100S electron microscope.

RESULTS

Characterization of Antibodies

Antibodies raised against the chondroitin sulfate proteoglycan were precipitated from serum, and $F(ab')_2$ fragments were prepared and purified. The purification procedure resulted in an increase in titer of the affinity-purified $F(ab')_2$ fragments of up to 86-fold as compared with the ammonium sulfate precipitated IgG.

The specificity of the antibodies was examined by double immunodiffusion, rocket immunoelectrophoresis, crossed immunoelectrophoresis, and ELISA. Since the antibody titer determined by ELISA plateaued at 5 wk after the initial immunization, rabbits were routinely bled-out at this time. When antibodies obtained 5 wk after the initial immunization were tested against the brain proteoglycan by double immunodiffusion, a single precipitin line resulted. One sharply defined rocket was seen after immunoelectrophoresis. Crossed immunoelectrophoresis also yielded a single rocket, indicating the monospecificity of the antibodies and the absence of antigenic components which are separable from the proteoglycan by electrophoresis (Fig. 1). ($F(ab')_2$ fragments could

not be used for these immunoelectrophoretic studies, since they are less basic than IgG and therefore migrate under our conditions in the same direction as the proteoglycan.)

By ELISA it was shown that the antibodies could be diluted approximately 33,000-fold when assayed against the proteoglycan before a colorimetric endpoint was reached. Furthermore, this dilution is 100–1,000-fold greater than that required for an endpoint when the antibodies were assayed against other fractions obtained in the proteoglycan purification procedure, or against rat plasma proteins or the soluble proteins from whole brain.

When the proteoglycan (1.65 mg/ml) was digested for 1 h at room temperature with chondroitinase ABC (0.04 U/ml of 0.01 M PBS, pH 7.2, containing 0.1% Tween 20) and tested by the immunochemical assays described above, the chondroitinase-treated proteoglycan behaved identically to the native proteoglycan, except that sharper immunoprecipitin lines resulted after chondroitinase treatment. Although the immunochemical reactivity of chondroitin sulfate proteoglycans from cartilage is significantly increased following chondroitinase treatment, which facilitates access of the antibodies to antigenic sites on the protein core, our results are consistent with biochemical data indicating that the chondroitin sulfate polysaccharide chains are less densely spaced on the brain proteoglycan as compared with those of cartilage, and also indicate that the antibodies are not directed against the chondroitin sulfate chains.

Localization of the Proteoglycan

Since the cytoarchitecture of the cerebellum is well documented (35), our studies of adult and immature brain (see following paper) have focused on this region. Other types of nervous and non-nervous tissues, including connective tissue, have also been examined. Our studies demonstrate that in adult rat brain the chondroitin sulfate proteoglycan is present intracellularly in neuronal and astroglial cytoplasm, whereas in early postnatal brain the proteoglycan is predominantly extracellular (primarily in the region of the presumptive white matter). During development there is a gradual shift in local-

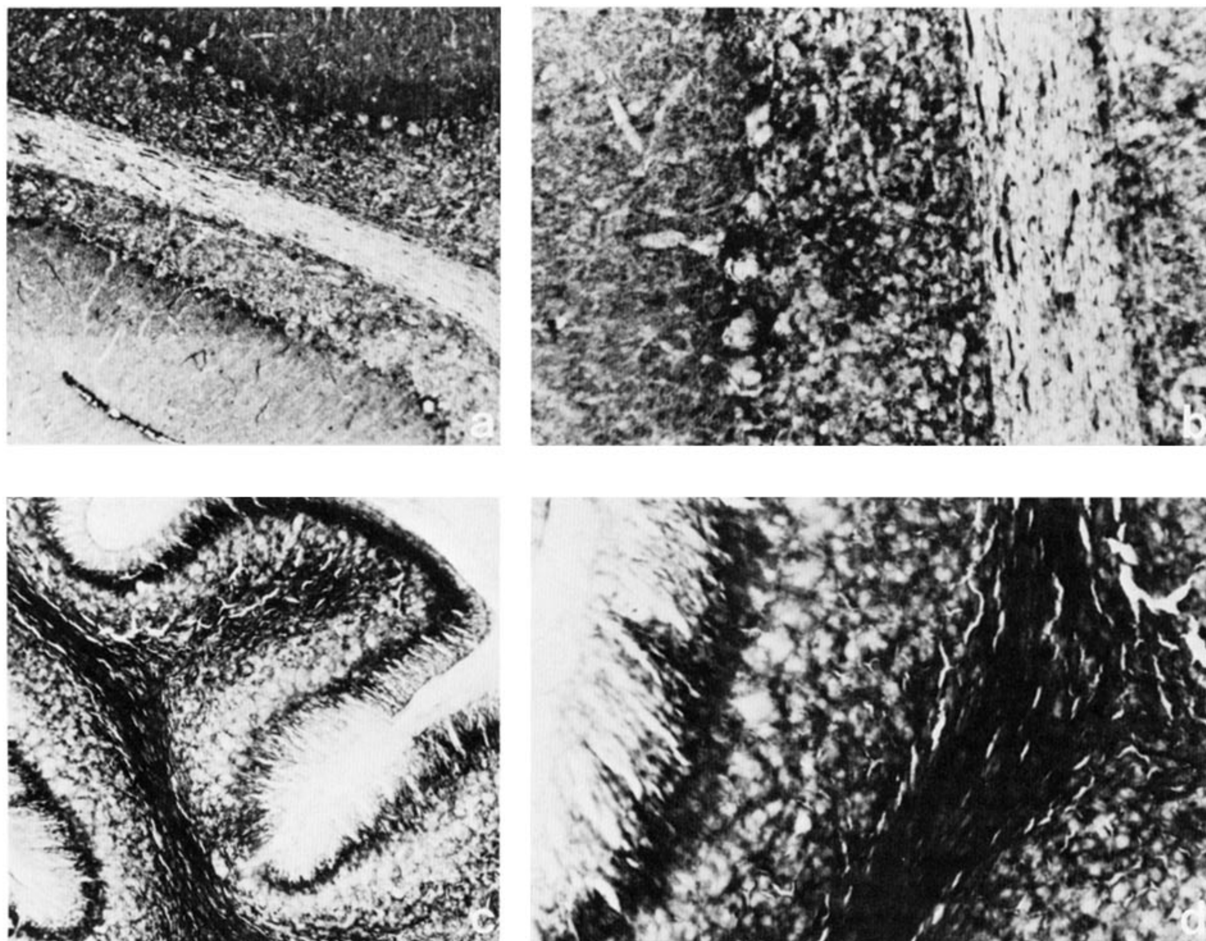

FIGURE 2 Light micrographs of adult (*a* and *b*) and 7-d-old (*c* and *d*) rat cerebellum, stained by the peroxidase-antiperoxidase method. Although the proteoglycan is present throughout the adult cerebellum with the exception of Purkinje cells, the areas of heaviest staining are in the granule cell and molecular layers, and surrounding the Purkinje cell bodies. In the white matter, the axoplasm of some myelinated axons is stained while the myelin itself is unstained. At 7 d of postnatal age, heavy staining is present in the presumptive cerebellar white matter (most axons are not myelinated at this age) and in the molecular layer. Staining extends from the molecular layer to the external granule cell layer, decreasing significantly towards the pia. As in the adult brain, Purkinje cells are not stained. (*a* and *c*) × 40; (*b* and *d*) × 100.

ization from extracellular to intracellular, and its intracellular appearance in astroglia precedes that in neurons (2). A survey of adult rat tissues indicates that while some central nervous tissue elements stain specifically, there is no staining of peripheral nerve axons or sympathetic neurons. Additionally, some connective tissue elements stain in both nervous and non-nervous tissues, indicating the presence of certain similarities between the proteoglycans of nervous and connective tissues.

Nervous Tissue

The localization of the proteoglycan at the light microscopic level in adult and 7-d-old brain is shown in Fig. 2. Results obtained by immunofluorescence staining (Fig. 3) demonstrate a staining pattern in adult brain similar to that seen using the peroxidase-antiperoxidase method.

An unusual finding which was most clearly revealed by electron miroscopy is the intracellular localization of the proteoglycan in the adult cerebellum, cerebrum, brain stem, and spinal cord (Figs. 4–11). Specifically, granule cells of the cerebellum (Fig. 4), cell bodies of cranial nerves located in the dorsal medulla (Fig. 5), and astrocytes (including Golgi epithelial cells and Bergmann fibers; Figs. 6–8) all show strong cytoplasmic staining.

Although throughout the central nervous system there is heavy axoplasmic staining of both myelinated and unmyelinated axons, it is apparent that not all axons are stained (Figs. 9 and 10). However, the axons of the granule cells (i.e., the parallel fibers) stain consistently (Fig. 11). This is in agreement with the heavy staining observed at the light microscopic level since the parallel fibers make up the bulk of the molecular layer. Although nuclei generally do not stain, staining is seen in the nuclei of certain neurons (Figs. 4 and 12). Consistent with the cytoplasmic localization of this proteoglycan in neurons and astroglia of the central nervous system is the intracellular staining which was observed in horizontal cells, ganglion cells, and Müller cells of the retina (Figs. 12–14). Bruch's membrane and the inner limiting membrane of the retina are also stained by antibodies to the chondroitin sulfate proteoglycan (Figs. 13 and 14).

Several central nervous tissue elements are consistently unstained. Among these are Purkinje cells (Figs. 2, *a* and *b*,

FIGURE 4 Adult cerebellum showing granule cells surrounding a cluster of myelinated axons. The characteristic narrow rim of granule cell cytoplasm is stained, and staining is also seen in two of the nuclei. × 18,000.

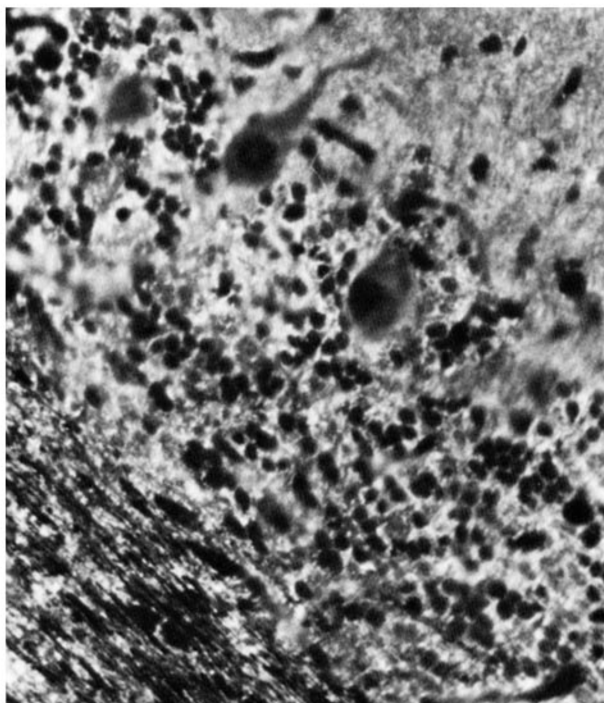

FIGURE 3 Immunofluorescence staining of adult cerebellum, showing unstained Purkinje cells (*upper right*), a reticulum of stained granule cell cytoplasm outlining the large unstained nuclei (*center*), and intra-axonal staining in a longitudinal section of myelinated fibers (*lower left*). × 95.

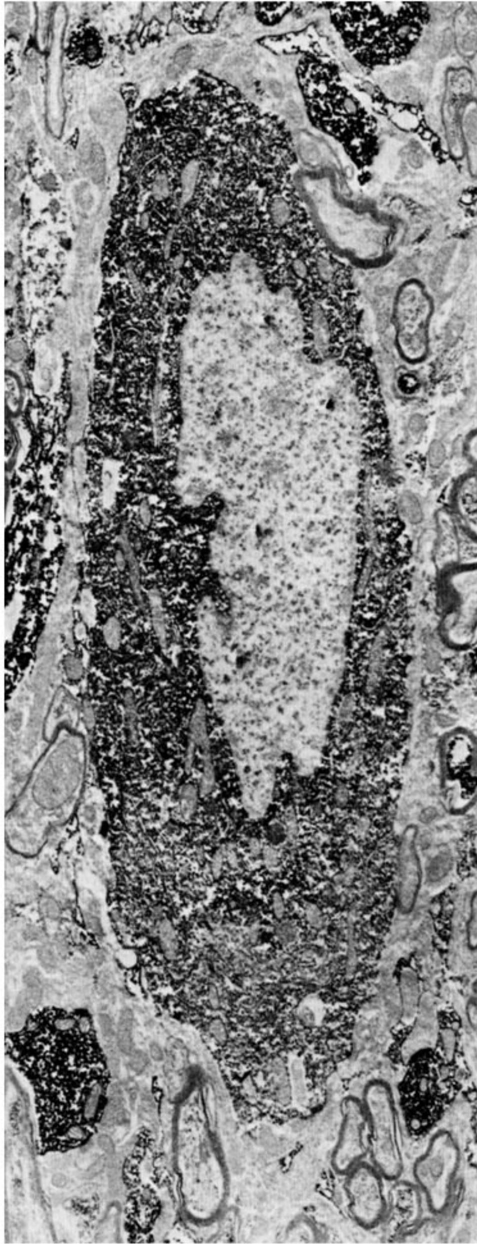

FIGURE 5 Large motor neuron from the dorsal region of the medulla, showing heavy cytoplasmic staining with an unstained nucleus. × 7,500.

8, and 11), oligodendrocytes, myelin (Figs. 2, *a* and *b*, 9, and 10), optic nerve axons, nerve endings (Fig. 10), and synaptic vesicles (Fig. 10). A mixed population of synaptic vesicles isolated from rat brain by the method of De Lorenzo and Freedman (13) was also examined and found not to stain. In axons where the axoplasm is stained next to the node of Ranvier, the nodal region (including the gap substance) remains unstained. Additionaly, we found no evidence for membrane and/or extracellular staining in adult central nervous tissue.

In sympathetic ganglion and sciatic nerve there is no staining of neuronal cell bodies, axons, myelin, or Schwann cells (Figs. 15–17). However, the supporting connective tissue and certain components of the extracellular matrix are stained (see below). Adrenal medulla has a similar staining pattern (i.e., only the connective tissue septa stain, but not the chromaffin cells or chromaffin granules).

Connective and Other Non-nervous Tissues

Antibodies raised against the brain proteoglycan also cross-react to various degrees with the connective tissue and extracellular matrix of certain adult tissues. While the neural elements of sympathetic ganglion, sciatic nerve, and adrenal medulla do not stain, there is heavy staining of connective tissue. In the sympathetic ganglion (Figs. 15 and 16) and adrenal medulla and cortex, the supporting connective tissue septa stain. In sciatic nerve the Schwann cell basal lamina stains, as does the extracellular matrix surrounding the collagen fibrils, although the fibrils themselves do not stain (Fig. 17).

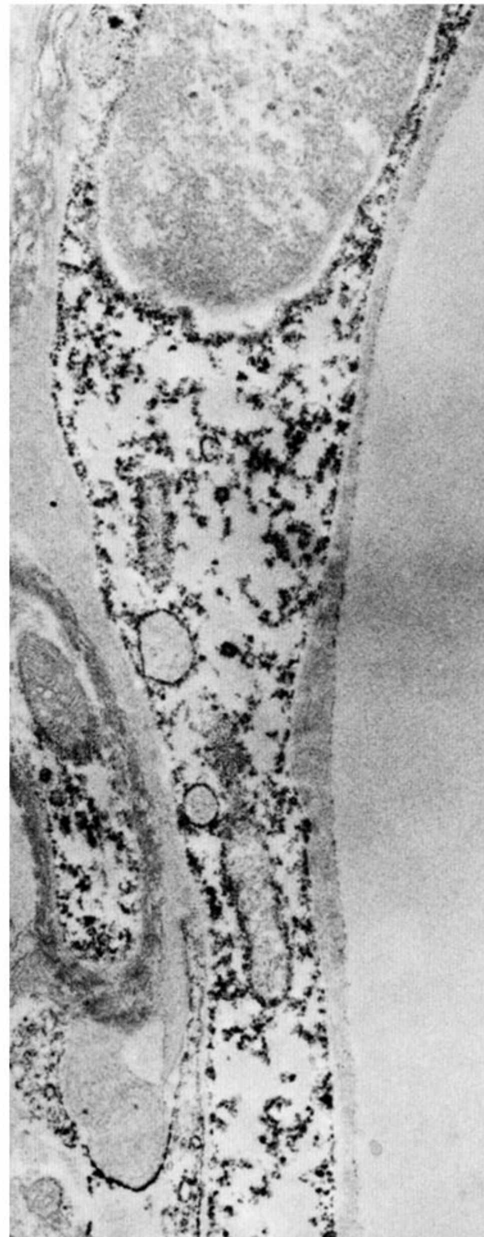

FIGURE 6 Cytoplasmic staining in cerebellar astrocytic end foot apposed to a blood vessel. × 25,000.

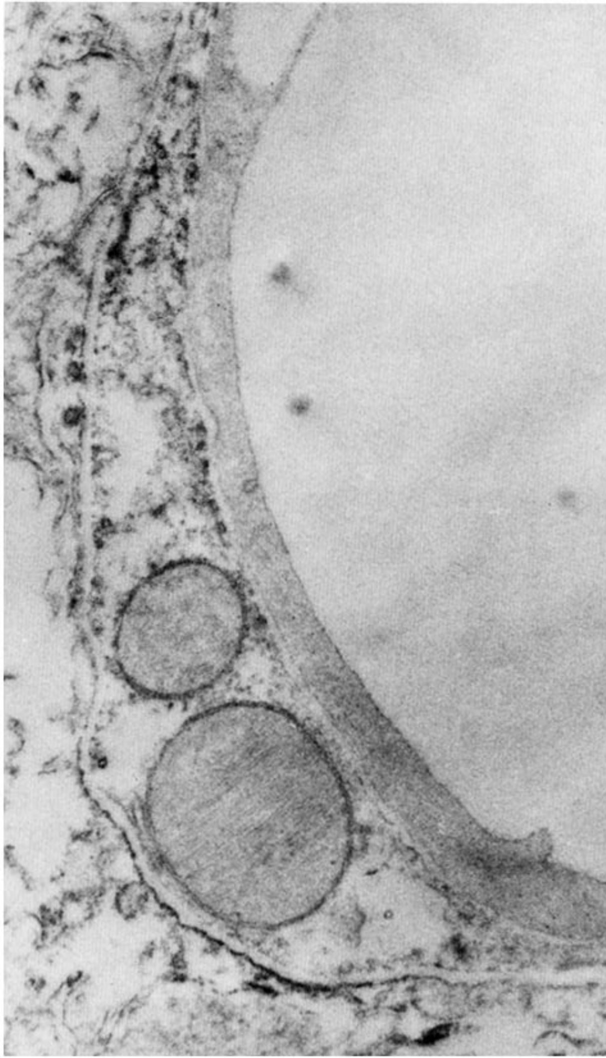

FIGURE 7 Astrocyte similar to that seen in Fig. 6, but incubated with control antibody that was not bound to the proteoglycan affinity column. × 38,000.

In hyaline cartilage of trachea, the lacunae and the surrounding connective tissue capsule are stained (Fig. 18). (The absence of staining in the cartilage matrix is to be expected, since the sections were not treated with chondroitinase ABC to increase permeability to the antibody.) Other tissue components that were found to stain include the pia (Fig. 2*a*) and the basement membrane of choroid plexus and kidney. There is no staining of spinal cord ependyma, liver, or skeletal muscle.

DISCUSSION

There have been several earlier reports concerning the localization of glycosaminoglycans in nervous tissue using electron microscopy (6, 7, 12, 36, 45). Pease demonstrated a uniform layer of phosphotungstic acid-staining material, which he identified as mucopolysaccharide (glycosaminoglycan), separating the pre- and postsynaptic elements of cerebral cortical synapses and in the larger extracellular spaces of the neuropil (36). Studies by Bondareff (6) and Tani and Ametani (45), employing ruthenium red with osmium tetroxide to stain anionic sites in the cerebral cortex, have also been interpreted as demonstrating the presence of extracellular glycosaminoglycans. These conclusions were further supported by Bondareff (7), who found similarly distributed material in tissue fixed by freezing and drying and stained with uranyl acetate.

Castejón and Castejón (12), employing the positively charged coordination compound of osmium and dimethylethylenediamine, Os-DMEDA (42), or Alcian blue with osmium tetroxide (GABOUL impregnation method; reference 3), demonstrated staining in mossy fiber rosettes of the presynaptic axoplasm, surrounding synaptic vesicles, continuous with presynaptic dense projections, and in the synaptic cleft. Results obtained after various enzymatic digestions (e.g., hyaluronidase, neuraminidase, ribonuclease) and carboxymethylation led the authors to conclude that the axoplasmic material of mossy fiber endings is a proteoglycan in which hyaluronic acid and chondroitin 4- and/or 6-sulfate are present.

There is no convincing evidence that the techniques employed by these investigators are specific for glycosaminoglycans, since they would be expected to stain any polyanionic macromolecule including nucleic acids, glycoproteins (especially those containing sialic acid and/or ester sulfate residues), and possibly also gangliosides or acidic proteins. While enzymatic digestion studies provide additional information, they can also be misleading since "specific" glycosidase prep-

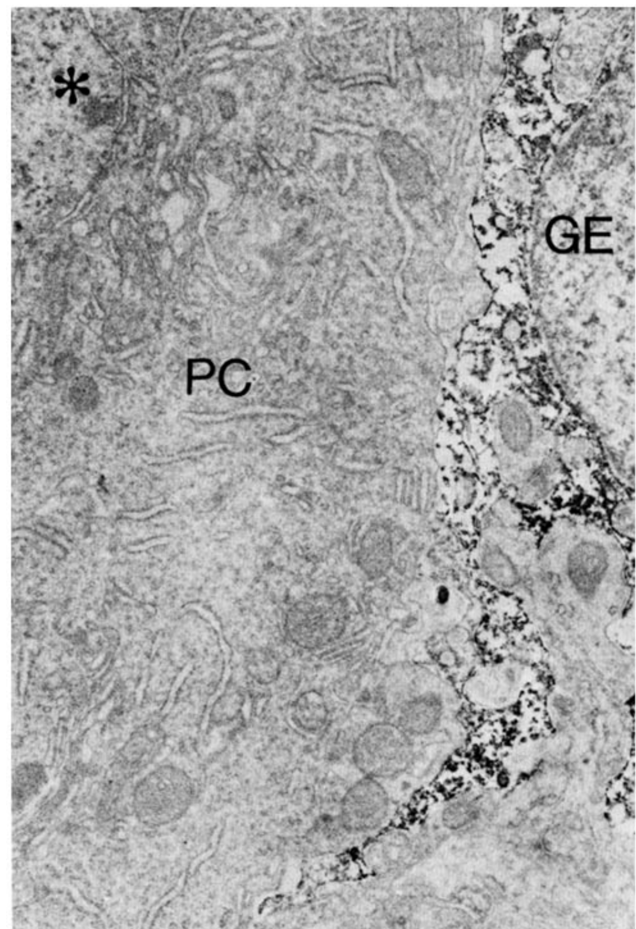

FIGURE 8 Golgi epithelial cell (*GE*), with a process that enwraps a Purkinje cell body (*PC*). The cytoplasm of the Golgi epithelial cell stains heavily and the nucleus remains unstained. (asterisk) Nucleus of Purkinje cell. × 15,000.

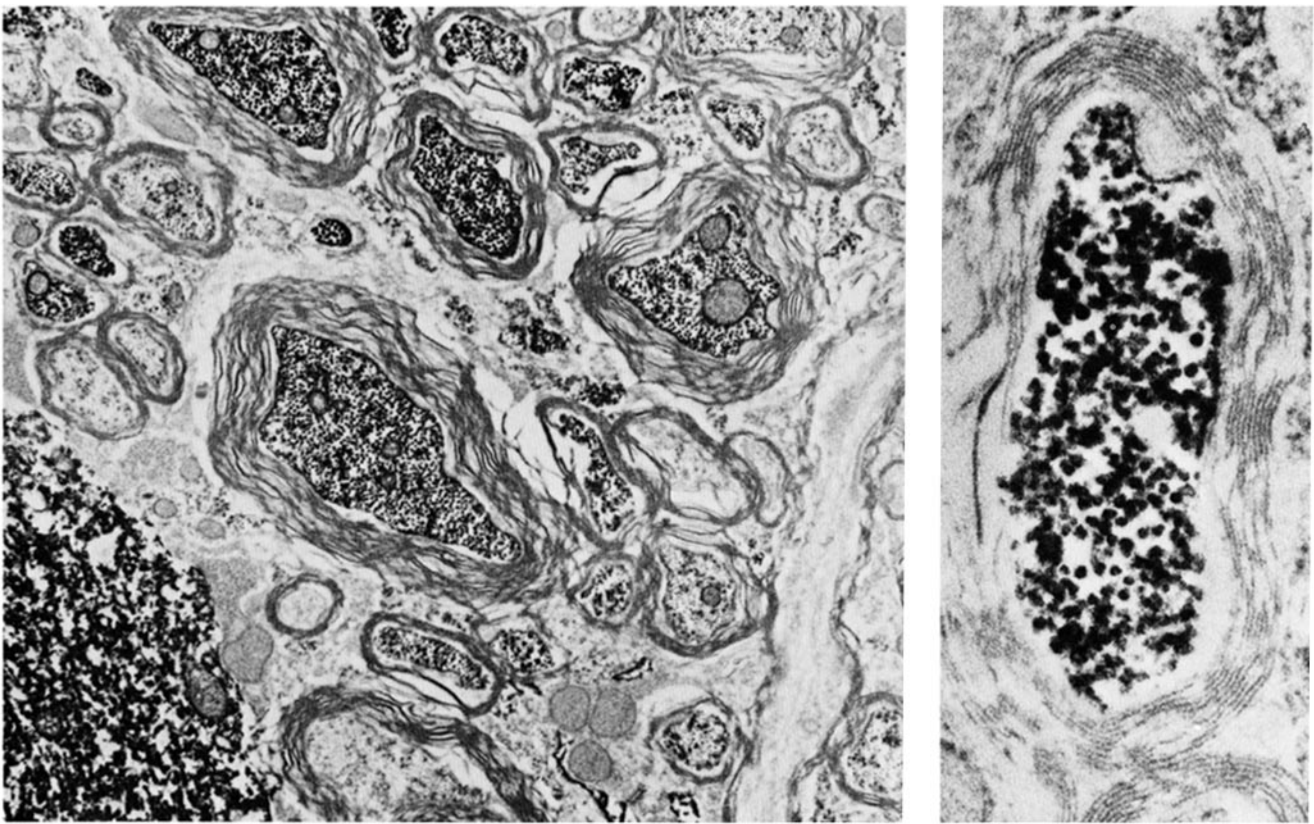

FIGURE 9 Heavy axoplasmic staining and unstained myelin in the dorsal horn of the adult spinal cord (*left*, × 15,000) and cerebellum (*right*, × 60,000).

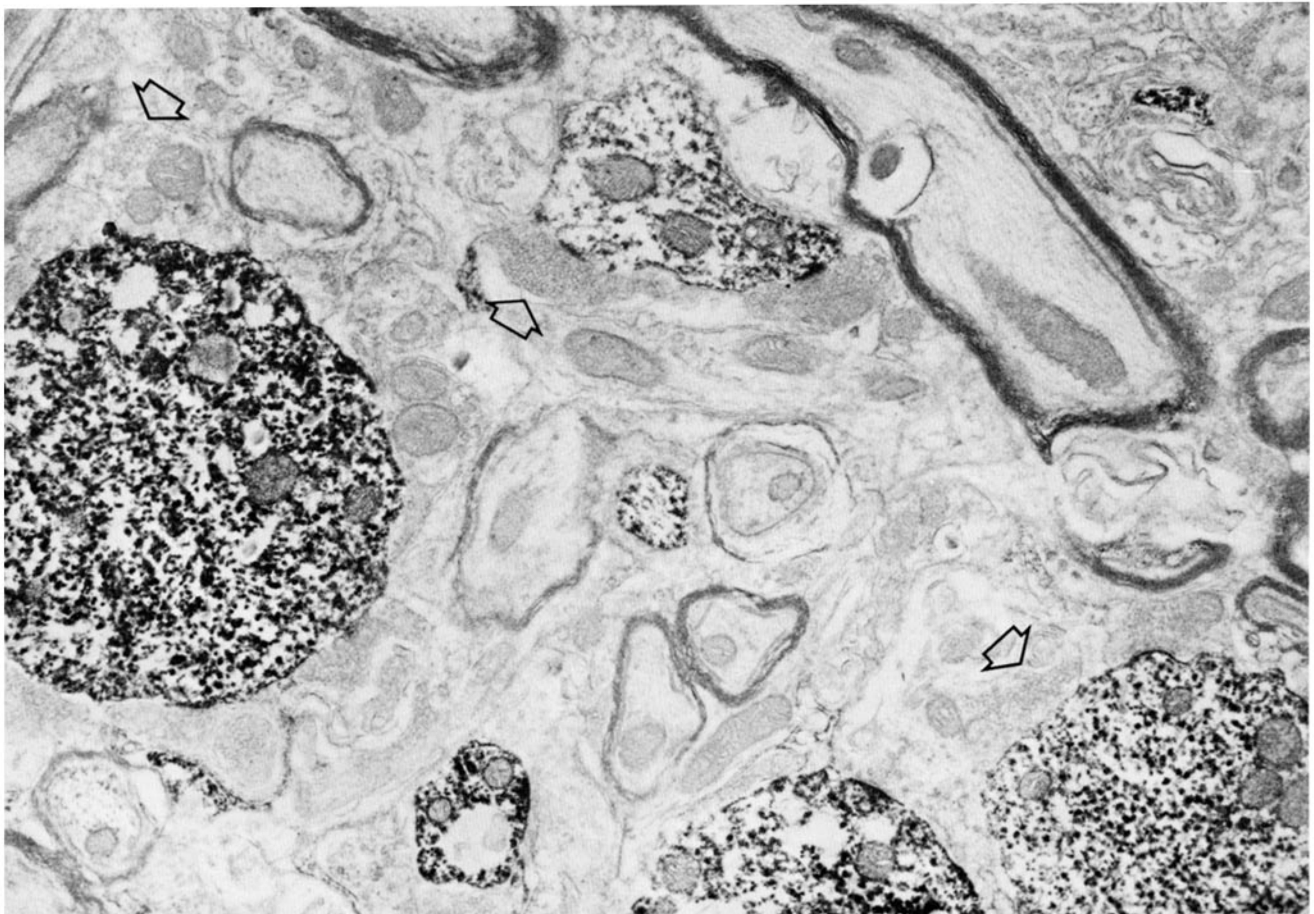

FIGURE 10 Heavy staining of neuronal cytoplasm in ventral horn of the spinal cord. Nerve endings (arrows) containing synaptic vesicles do not stain, and the myelinated axons in this field are also unstained. × 20,000.

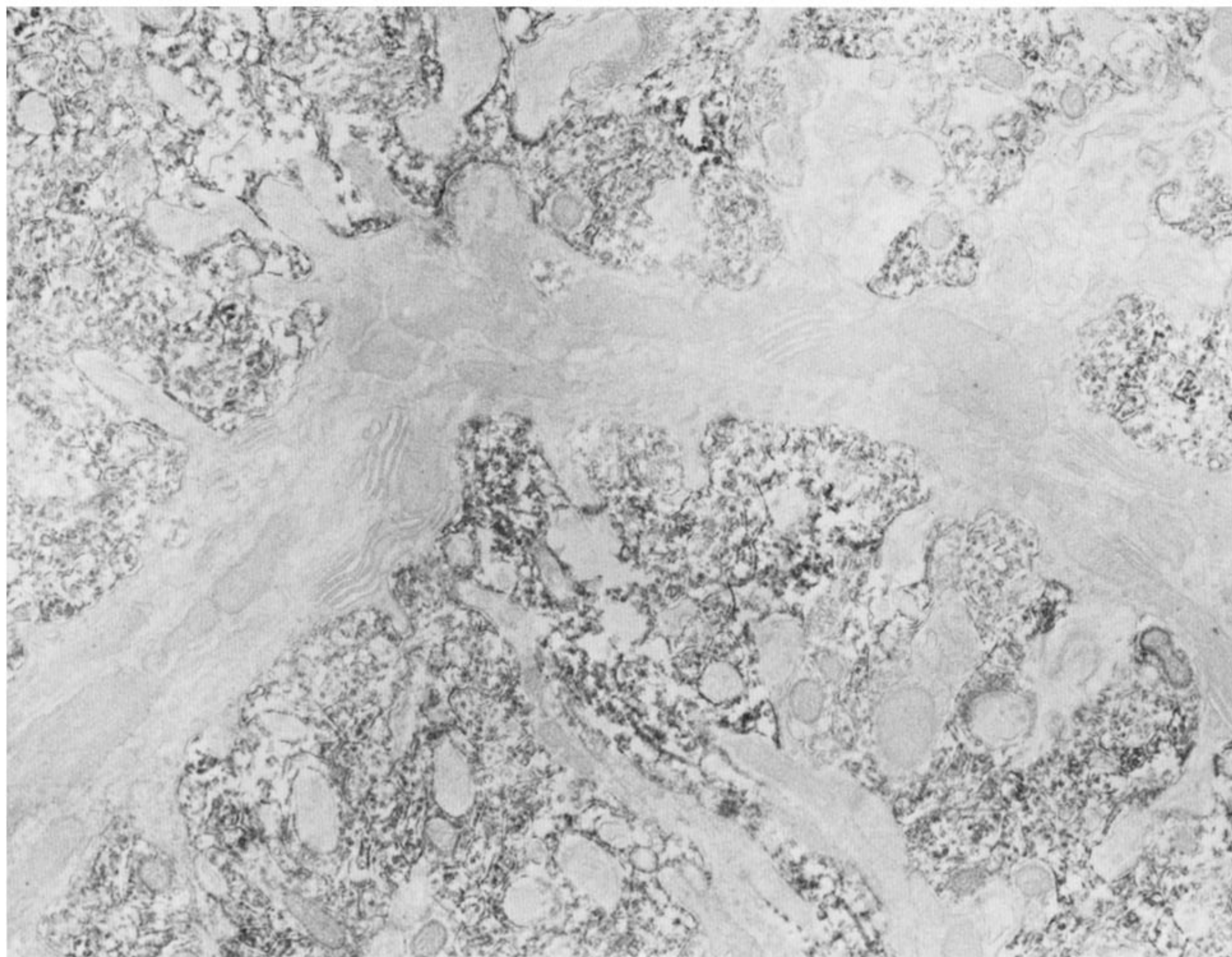

FIGURE 11 Molecular layer of the adult cerebellum. The large, unstained process is a Purkinje cell dendrite having characteristic spines that synapse with clusters of parallel fibers whose axoplasm is heavily stained. × 20,000.

arations are often contaminated with other glycosidases and proteases, a possibility that was not examined by these investigators. Moreover, the changes observed after enzyme treatment are often quite subtle and subjective. Although there are obviously also limitations in the use of immunocytochemical techniques, this method has been successfully employed for the localization of proteoglycans and related components of other tissues (23, 24, 39–41).

Our immunocytochemical study has demonstrated the intracellular localization of a chondroitin sulfate proteoglycan in neurons and astroglia of central nervous tissue, findings that are consistent with those from previous work involving cellular and subcellular fractionation followed by biochemical analysis. Using these latter methods it has also been shown that this glycosaminoglycan is present in rat brain nuclei (26) and bulk-isolated axons (29), but only in small amounts if at all in oligodendroglia (32), myelin, nerve endings and mitochondria (1, 29, 33, 43). These results are all in good agreement with our present observations.

Although strong axoplasmic staining is present in some myelinated and unmyelinated axons, it is apparent that not all axons stain. This selective staining of certain neural elements is also seen in the case of nuclei and neuronal cell bodies. For example, cerebellar granule cells stain intracellularly, whereas Purkinje perikarya are unstained. Similarly, the cytoplasm of retinal ganglion and horizontal cells is stained, but there is no staining of amacrine cells, bipolar cells, or receptor cells (including rods and cones). The basis for this selectivity is not yet clear, but one possible explanation is simply that the proteoglycan is present only in certain cell types or subclasses. It is also possible that in those elements that appear unstained, the proteoglycan is present at a concentration below the limits of detection of our method.

It is known that sulfated glycosaminoglycans are transported in the fast component of axonal flow (for a review, see reference 15). At high magnification the proteoglycan staining observed in axons appears punctate and discrete, whereas staining of neuronal and astroglial cell bodies is more diffuse, less organized, and somewhat clumped. The staining pattern in axons suggests that the proteoglycan may be closely associated with microtubules, which are thought to play a role in axonal transport. However, it is also possible that this staining results from a "condensation" of the proteoglycan around microtubules during tissue processing.

Chondroitin sulfate and smaller amounts of heparan sulfate are present in adrenal chromaffin granules (17, 20, 31), whereas synaptic vesicles isolated from marine electric organ have been reported (11, 44) to primarily contain a sulfated glycosaminoglycan resembling heparan sulfate (occurring in the form of a proteoglycan). We have, however, found no evidence indicating that antibodies to the chondroitin sulfate proteoglycan recognize any component of nerve endings, and

We have previously demonstrated the presence of chondroitin sulfate, together with smaller amounts of hyaluronic acid and heparan sulfate, in astrocyte-enriched fractions prepared from rat and bovine brain (32). However, it should be noted that the chondroitin sulfate proteoglycan of brain that we have isolated and characterized (21) differs considerably from proteoglycans that are reported to be synthesized by "glia-like" cells cultivated from explants of normal human brain, and by cultured glial tumor cells of presumably astrocytic origin (18, 34). It is not surprising that the biochemical properties of these cultures are quite different from those of astrocytes (or oligodendroglia) isolated in bulk from normal brain, insofar as it is impossible to eliminate fibroblasts and endothelial cells (which would be expected to synthesize proteoglycans characteristic of connective tissue) from the explant cultures, and both the "glia-like" and glioma cells frequently lack glial fibrillary acidic protein and contain fibro-

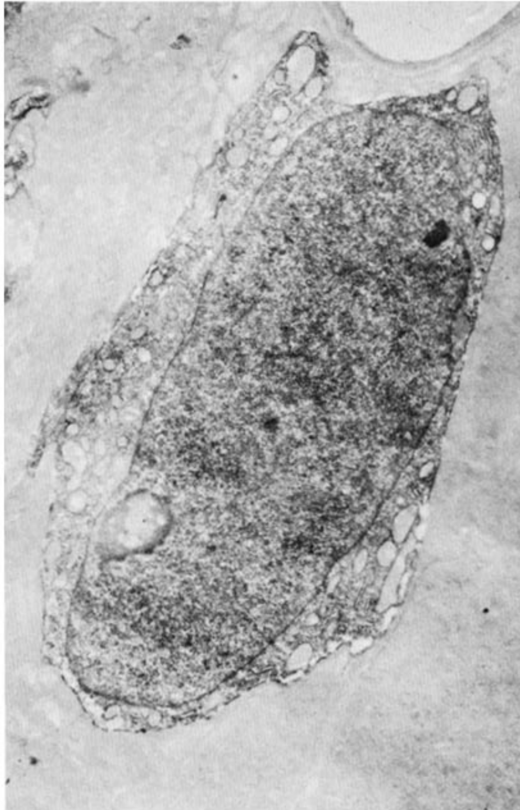

FIGURE 12 Nuclear and cytoplasmic staining of a horizontal cell of the retina. × 8,000.

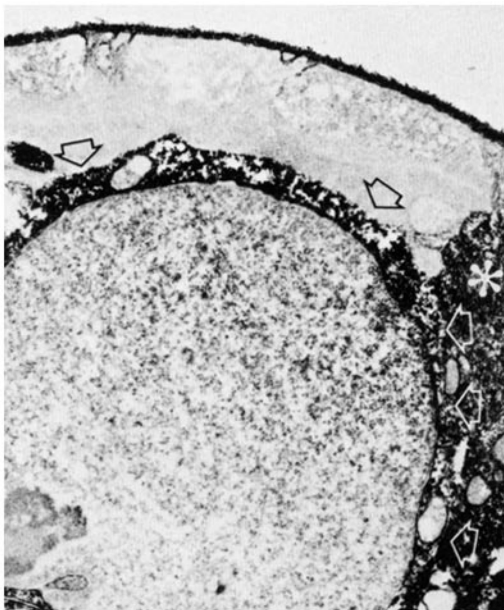

FIGURE 13 Retinal ganglion cell (border indicated by arrows) whose nucleus is unstained whereas its cytoplasm stains intensely. The cytoplasm of a Müller cell radial fiber (asterisk) is also stained, as is the internal limiting membrane. × 8,000.

ELISA studies showed no significant cross-reactivity with either the soluble fraction of bovine chromaffin granules or with large, dense-cored synaptic vesicles from splenic nerve (R. U. Margolis, R. L. Klein, and R. K. Margolis, unpublished results).

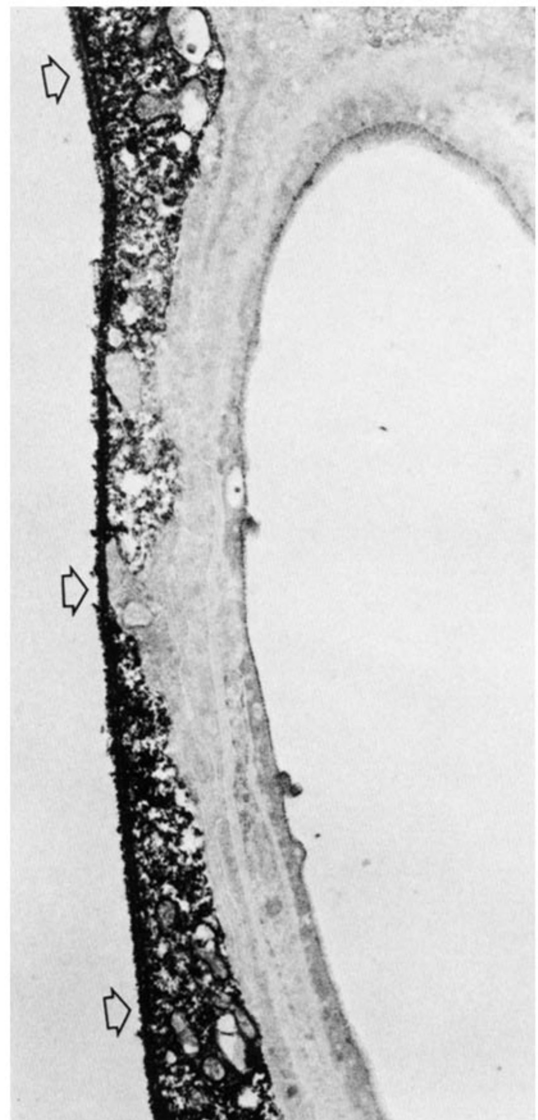

FIGURE 14 Müller cell process adjacent to a blood vessel and terminating at the internal limiting membrane (arrows), which is partly composed of the basement membrane of the Müller cell. The cytoplasm of the Müller cell and the internal limiting membrane both stain heavily. × 10,000.

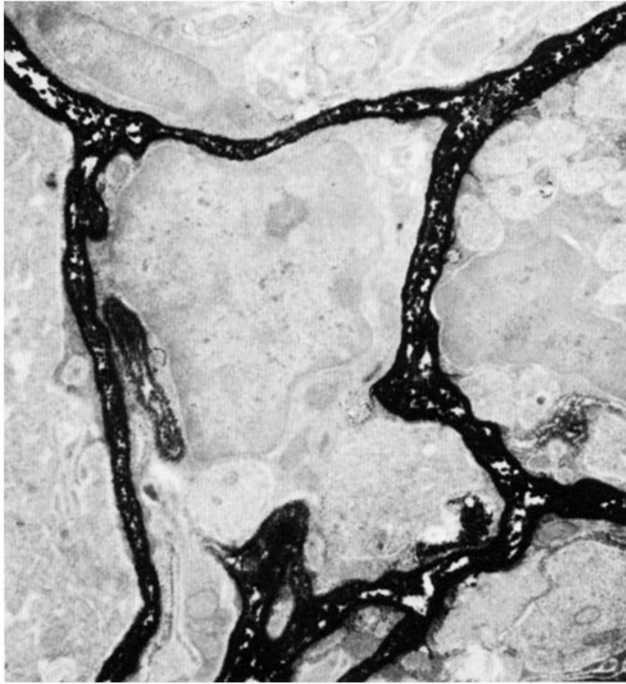

FIGURE 15 Dense staining of connective tissue, and unstained neurons and Schwann cells, in superior cervical ganglion. × 7,500.

nectin, which is not present in normal differentiated astrocytes (5, 37).

Several studies (primarily of peripheral nerve) employing various heavy metal and polycationic stains have found predominantly two areas of staining at the node of Ranvier: over and/or in close proximity to the nodal axolemma, and filling the annular zone surrounding the nodal axolemma between Schwann cell fingers and bound externally by the Schwann cell basal lamina (i.e., the gap substance). It is suspected that the macromolecules involved in nodal staining are acidic glycoproteins containing sialic acid and/or glycosaminoglycans, particularly hyaluronic acid and chondroitin 4- or 6-sulfate (22). These nodal areas, however, are not recognized by antibodies to the chondroitin sulfate proteoglycan in either the central or peripheral nervous system, although our antibodies do recognize the nodal axoplasm of those axons that stain in the central nervous system.

In sciatic nerve, Bunge and Bunge (8) postulated that normal differentiation of the Schwann cell and subsequent myelination require not only contact with nerve fibers, but also contact with a connective tissue matrix or some material associated with that matrix. It is quite possible that a proteoglycan identified by our antibodies, and which is present both in the basal lamina of Schwann cells and surrounding the collagen fibrils, may be involved in this recognition process. Evidence suggesting a similar association was presented by Ebendal (14), and more recently by Carbonetto et al. (10).

On the basis of a number of immunochemical and biochemical criteria, it would appear that our antibodies are monospecific for a chondroitin sulfate proteoglycan that has essentially the same size, charge, density, and chemical composition in both adult and immature brain (see following paper), and that these antibodies do not react with other brain proteins. PAGE of both the intact and chondroitinase-treated [^{35}S]sulfate-labeled proteoglycans, followed by immunoblotting, in each case yielded broad and diffuse bands that were generally uninformative. However, all of the antibody binding coincided with [^{35}S]sulfate-labeled gel regions (detected by fluorography), indicating that the antibody did not recognize other proteins. (In the case of chondroitinase-treated material, a significant portion of [^{35}S]sulfate radioactivity remains with sulfated glycoprotein oligosaccharides present in the chondroitin sulfate proteoglycan of brain [21].) Therefore, though it is clear that our antibodies are specific for a chondroitin sulfate proteoglycan, it is possible that this specificity extends to a number of closely related species that are indistinguishable by the biochemical and immunochemical analyses used in our studies, and some heterogeneity of this type would even appear very likely in view of the known polydispersity of proteoglycans.

It was not unexpected that the antibodies used in this study also stain components of connective and other non-nervous tissues, where staining is generally present in basement membranes or associated with collagen fibrils. For example, the basement membranes of retinal pigment epithelium and the choriocapillaris (components of Bruch's membrane) and the basement membrane of Müller cells (a component of the internal limiting membrane of retina) all stain. These membrane structures also contain collagen fibrils. In addition to the retina, basement membrane staining was seen in Schwann cells, choroid plexus, and kidney. Although in brain these antibodies are specific for a chondroitin sulfate proteoglycan,

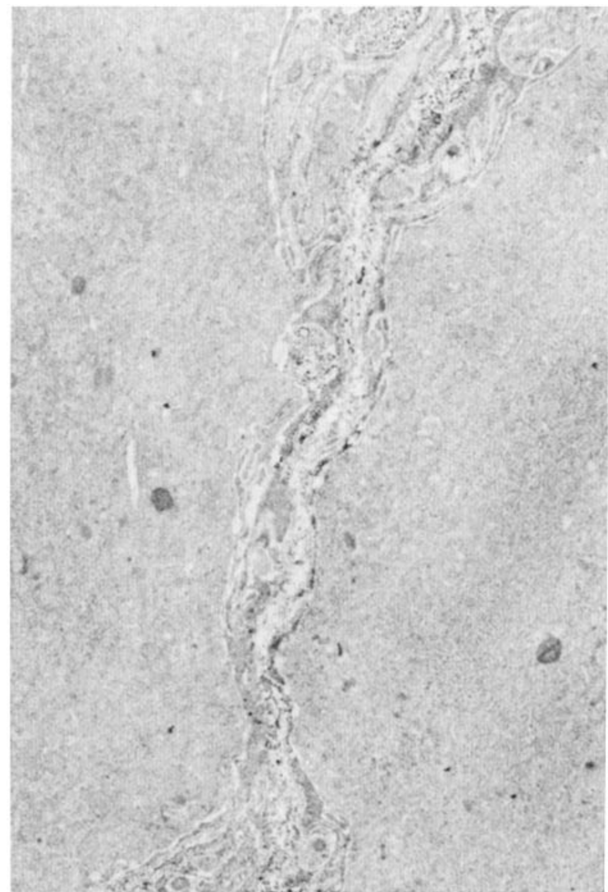

FIGURE 16 Control section of the superior cervical ganglion, which shows two neuronal cell bodies separated by connective tissue. × 7,500.

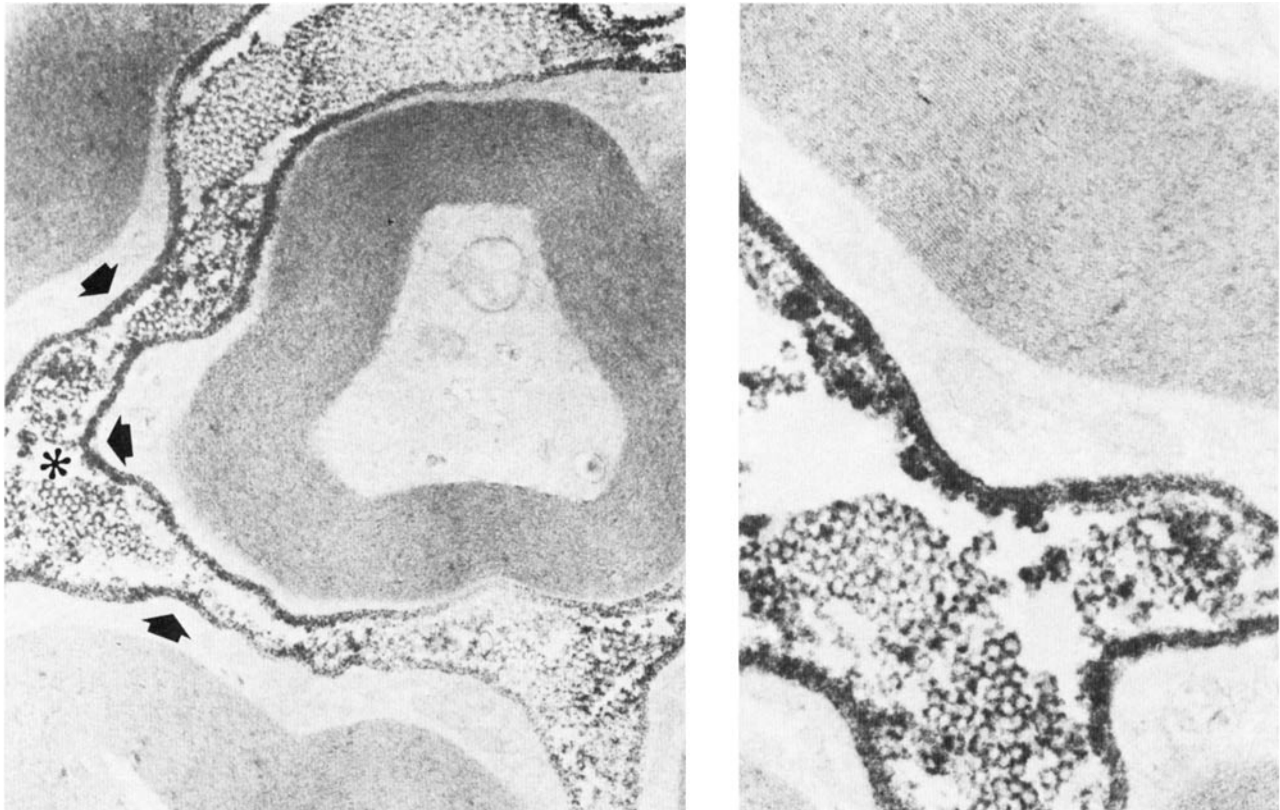

FIGURE 17 Sciatic nerve, showing staining of Schwann cell basal lamina (arrows) and extracellular matrix surrounding collagen fibrils, but no staining of axoplasm, myelin, Schwann cell cytoplasm, or collagen fibrils. (*Left*) × 20,000; (*right*, area marked by asterisk in left panel) × 38,000.

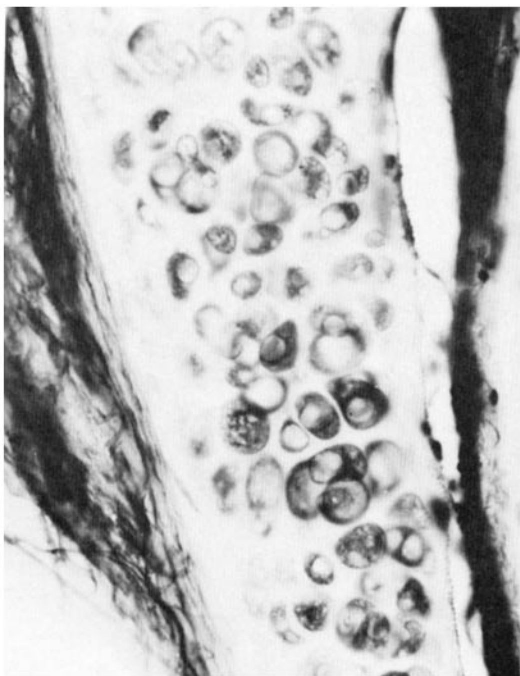

FIGURE 18 Tracheal hyaline cartilage, showing staining of the lacunae and surrounding connective tissue capsule. × 100.

various degrees of immunochemical cross-reactivity are demonstrable between the rat brain proteoglycan and connective tissue proteoglycans, indicating the presence of some similar antigenic determinants (reference 39 and A. R. Poole, D. A. Aquino, and R. U. Margolis, unpublished results). In these studies, antibodies raised against the brain proteoglycan were found to react strongly with bovine nasal septum proteoglycan, weakly with bovine articular cartilage and epiphyseal proteoglycans and rat chondrosarcoma link protein, and not at all with bovine growth plate proteoglycan. Additionally, antibodies raised against bovine articular cartilage proteoglycan and rat chondrosarcoma link protein react with the brain proteoglycan. These results are consistent with the staining of certain connective tissue elements observed in our immunocytochemical studies.

We thank Dr. Carol Mason for her many helpful suggestions, and Dr. A. R. Poole for his guidance concerning immunochemical techniques in the early phases of our work.

This research was supported by grants NS-09348, NS-13876, and MH-00129 from the National Institutes of Health and the National Institute of Mental Health.

Received for publication 14 February 1984, and in revised form 22 May 1984.

REFERENCES

1. Adams, C. W. M., and O. B. Bayliss. 1968. Histochemistry of myelin. VII. Analysis of lipid-protein relationships and absence of acid mucopolysaccharide. *J. Histochem. Cytochem.* 16:119–127.
2. Aquino, D. A., R. U. Margolis, and R. K. Margolis. 1984. Immunocytochemical localization of a chondroitin sulfate proteoglycan in nervous tissue. II. Studies in developing brain. *J. Cell Biol.* 99:1130–1139.
3. Behnke, O., and T. Zelander. 1970. Preservation of intercellular substances by the cationic dye Alcian blue in preparative procedures for electron microscopy. *J. Ultrastruct. Res.* 31:424–438.
4. Berod, A., B. K. Hartman, and J. F. Pujol. 1981. Importance of fixation in immunohistochemistry: use of formaldehyde solutions at variable pH for the localization of tyrosine hydroxylase. *J. Histochem. Cytochem.* 29:844–850.

5. Bigner, D. D., S. H. Bigner, J. Pontén, B. Westermark, M. S. Mahaley, E. Ruoslahti, H. Herschman, L. F. Eng, and C. J. Wikstrand. 1981. Heterogeneity of genotypic and phenotypic characteristics of fifteen permanent cell lines derived from human gliomas. *J. Neuropathol. Exp. Neurol.* 40:201–229.
6. Bondareff, W. 1967. An intercellular substance in rat cerebral cortex: submicroscopic distribution of ruthenium red. *Anat. Rec.* 157:527–536.
7. Bondareff, W. 1967. Demonstration of an intercellular substance in mouse cerebral cortex. *Z. Zellforsch.* 81:366–373.
8. Bunge, R. P., and M. B. Bunge. 1978. Evidence that contact with connective tissue matrix is required for normal interaction between Schwann cells and nerve fibers. *J. Cell Biol.* 78:943–950.
9. Cambiaso, C. L., A. Goffinet, J.-P. Vaerman, and J. F. Heremans. 1975. Glutaraldehyde-activated aminohexyl-derivative of Sepharose 4B as a new versatile immunoabsorbent. *Immunochemistry.* 12:273–278.
10. Carbonetto, S., M. M. Gruver, and D. C. Turner. 1983. Nerve fiber growth in culture on fibronectin, collagen, and glycosaminoglycan substrates. *J. Neurosci.* 3:2324–2335.
11. Carlson, S. S., and R. B. Kelly. 1983. A highly antigenic proteoglycan-like component of cholinergic synaptic vesicles. *J. Biol. Chem.* 258:11082–11091.
12. Castejón, H. V., and O. J. Castejón. 1976. Electron microscopic demonstration of hyaluronidase sensible proteoglycans at the presynaptic area in mouse cerebellar cortex. *Acta Histochem.* 55:300–316.
13. DeLorenzo, R. J., and S. D. Freedman. 1978. Calcium dependent neurotransmitter release and protein phosphorylation in synaptic vesicles. *Biochem. Biophys. Res. Commun.* 80:183–192.
14. Ebendal, T. 1976. The relative roles of contact inhibition and contact guidance in orientation of axons extending on aligned collagen fibrils *in vitro*. *Exp. Cell Res.* 98:159–169.
15. Elam, J. S. 1979. Axonal transport of complex carbohydrates. *In* Complex Carbohydrates of Nervous Tissue. R. U. Margolis and R. K. Margolis, editors. Plenum Press, New York. 235–267.
16. Finne, J., T. Krusius, R. K. Margolis, and R. U. Margolis. 1979. Novel mannitol-containing oligosaccharides obtained by mild alkaline borohydride treatment of a chondroitin sulfate proteoglycan from brain. *J. Biol. Chem.* 254:10295–10300.
17. Geissler, D., A. Martinek, R. U. Margolis, R. K. Margolis, J. A. Skrivanek, R. Ledeen, P. König, and H. Winkler. 1977. Composition and biogenesis of complex carbohydrates of ox adrenal chromaffin granules. *Neuroscience.* 2:685–693.
18. Glimelius, B., B. Norling, B. Westermark, and Å. Wasteson. 1978. Composition and distribution of glycosaminoglycans in cultures of human normal and malignant glial cells. *Biochem. J.* 172:443–456.
19. Kiang, W.-L., C. P. Crockett, R. K. Margolis, and R. U. Margolis. 1978. Glycosaminoglycans and glycoproteins associated with microsomal subfractions of brain and liver. *Biochemistry.* 17:3841–3848.
20. Kiang, W.-L., T. Krusius, J. Finne, R. U. Margolis, and R. K. Margolis. 1982. Glycoproteins and proteoglycans of the chromaffin granule matrix. *J. Biol. Chem.* 257:1651–1659.
21. Kiang, W.-L., R. U. Margolis, and R. K. Margolis. 1981. Fractionation and properties of a chondroitin sulfate proteoglycan and the soluble glycoproteins of brain. *J. Biol. Chem.* 256:10529–10537.
22. Langley, O. K. 1979. Histochemistry of polyanions in peripheral nerve. *In* Complex Carbohydrates of Nervous Tissue. R. U. Margolis and R. K. Margolis, editors. Plenum Press, New York. 193–207.
23. Laurie, G. W., C. P. Leblond, G. R. Martin, and M. H. Silver. 1982. Intracellular localization of basement membrane precursors in the endodermal cells of the rat parietal yolk sac. III. Immunostaining for laminin and its precursors. *J. Histochem. Cytochem.* 30:991–998.
24. Laurie, G. W., C. P. Leblond, and G. R. Martin. 1983. Light microscopic immunolocalization of type IV collagen, laminin, heparan sulfate proteoglycan, and fibronectin in the basement membranes of a variety of rat organs. *Am. J. Anat.* 167:71–82.
25. Little, J. R., and H. Donahue. 1968. Spectral properties of proteins and small molecules of immunological interest. In *Methods Immunol. Immunochem.* 2:343–364.
26. Margolis, R. K., C. P. Crockett, W.-L. Kiang, and R. U. Margolis. 1976. Glycosaminoglycans and glycoproteins associated with rat brain nuclei. *Biochim. Biophys. Acta.* 451:465–469.
27. Margolis, R. K., and R. U. Margolis. 1979. Structure and distribution of glycoproteins and glycosaminoglycans. *In* Complex Carbohydrates of Nervous Tissue. R. U. Margolis and R. K. Margolis, editors. Plenum Press, New York. 45–73.
28. Margolis, R. K., and R. U. Margolis. 1983. Glycoproteins and Proteoglycans. *In* Handbook of Neurochemistry, Vol. 5. A. Lajtha, editor. Plenum Press, New York. Second ed. 177–204.
29. Margolis, R. K., R. U. Margolis, C. Preti, and D. Lai. 1975. Distribution and metabolism of glycoproteins and glycosaminoglycans in subcellular fractions of brain. *Biochemistry.* 14:4797–4804.
30. Margolis, R. K., M. D. Thomas, C. P. Crockett, and R. U. Margolis. 1979. Presence of chondroitin sulfate in the neuronal cytoplasm. *Proc. Natl. Acad. Sci. USA.* 76:1711–1715.
31. Margolis, R. U., and R. K. Margolis. 1973. Isolation of chondroitin sulfate and glycopeptides from chromaffin granules of adrenal medulla. *Biochem. Pharmacol.* 22:2195–2197.
32. Margolis, R. U., and R. K. Margolis. 1974. Distribution and metabolism of mucopolysaccharides and glycoproteins in neuronal perikarya, astrocytes, and oligodendroglia. *Biochemistry.* 13:2849–2852.
33. Matthieu, J.-M., R. H. Quarles, J. F. Poduslo, and R. O. Brady. 1975. [^{35}S]Sulfate incorporation into myelin glycoproteins. I. Central nervous system. *Biochim. Biophys. Acta.* 392:159–166.
34. Norling, B., B. Glimelius, B. Westermark, and Å. Wasteson. 1978. A chondroitin sulfate proteoglycan from human cultured glial cells aggregates with hyaluronic acid. *Biochem. Biophys. Res. Commun.* 84:914–921.
35. Palay, S. L., and V. Chan-Palay. 1974. Cerebellar Cortex: Cytology and Organization. Springer-Verlag, New York. 348 pp.
36. Pease, D. C. 1966. Polysaccharides associated with the exterior surface of epithelial cells: kidney, intestine, brain. *J. Ultrastruct. Res.* 15:555–588.
37. Pontén, J., and B. Westermark. 1978. Properties of human malignant glioma cells *in vitro*. *Med. Biol. (Helsinki).* 56:184–193.
38. Poole, A. R. 1977. Antibodies to enzymes and their uses, with particular reference to lysosomal enzymes. *In* Lysosomes: A Laboratory Handbook. J. T. Dingle, editor. Elsevier/North Holland Biomedical Press, Amsterdam. Second ed. 245–312.
39. Poole, A. R., I. Pidoux, A. Reiner, L. Cöster, and J. R. Hassell. 1982. Mammalian eyes and associated tissues contain molecules that are immunologically related to cartilage proteoglycan and link protein. *J. Cell Biol.* 93:910–920.
40. Poole, A. R., I. Pidoux, A. Reiner, and L. Rosenberg. 1982. An immunoelectron microscope study of the organization of proteoglycan monomer, link protein, and collagen in the matrix of articular cartilage. *J. Cell Biol.* 93:921–937.
41. Poole, A. R., I. Pidoux, A. Reiner, L.-H. Tang, H. Choi, and L. Rosenberg. 1980. Localization of proteoglycan monomer and link protein in the matrix of bovine articular cartilage: an immunohistochemical study. *J. Histochem. Cytochem.* 28:621–635.
42. Seligman, A. M., H. L. Wasserkrug, C. Deb, and J. S. Hanker. 1968. Osmium containing compounds with multiple basic or acidic groups as stains for ultrastructure. *J. Histochem. Cytochem.* 16:87–101.
43. Simpson, D. L., D. R. Thorne, and H. H. Loh. 1976. Sulfated glycoproteins, glycolipids and glycosaminoglycans from synaptic plasma and myelin membranes: isolation and characterization of sulfated glycopeptides. *Biochemistry.* 15:5449–5457.
44. Stadler, H., and G. H. C. Dowe. 1982. Identification of a heparan sulfate-containing proteoglycan as a specific core component of cholinergic synaptic vesicles from *Torpedo marmorata*. *EMBO (Eur. Mol. Biol. Organ.) J.* 1:1381–1384.
45. Tani, E., and T. Ametani. 1971. Extracellular distribution of ruthenium red-positive substance in the cerebral cortex. *J. Ultrastruct. Res.* 34:1–14.