Immunocytochemical Localization of a Chondroitin Sulfate Proteoglycan in Nervous Tissue. II. Studies in Developing Brain

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 In contrast to the intracellular (cytoplasmic) localization of chondroitin sulfate proteoglycans in adult brain (Aquino, D. A., R. U. Margolis, and R. K. Margolis, 1984, *J. Cell Biol.* 99:940–952), immunoelectron microscopic studies in immature (7 d postnatal) rat cerebellum demonstrated almost exclusively extracellular staining in the granule cell and molecular layers. Staining was also extracellular and/or associated with plasma membranes in the region of the presumptive white matter. Axons, which are unmyelinated at this age, generally did not stain, although faint intracellular staining was present in some astrocytes. At 10 and 14 d postnatal there was a significant decrease in extracellular space and staining, and by 21 d distinct cytoplasmic staining of neurons and astrocytes appeared. This intracellular staining further increased by 33 d so as to closely resemble the pattern seen in adult brain. Analyses of the proteoglycans isolated from 7-d-old and adult brain demonstrated that they have essentially identical biochemical compositions, immunochemical reactivity, size, charge, and density. These findings indicate that the antibodies used in this study recognize the same macromolecule in both early postnatal and adult brain, and that the localization of this proteoglycan changes progressively from an extracellular to an intracellular location during brain development.

In the preceding report (2) we have demonstrated by immunoelectron microscopy the intracellular (cytoplasmic) localization of a chondroitin sulfate proteoglycan in neurons and astrocytes of adult rat central nervous tissue. Its functional role in this location is still unknown, and although our immunocytochemical findings were not unexpected on the basis of previous biochemical studies of brain glycosaminoglycans, they are nevertheless quite different from the extracellular matrix or cell surface location of proteoglycans in other tissues (8, 16, 18, 19).

Since the possibility remained that during brain development this proteoglycan might be involved in extracellular processes associated with neural histogenesis and various types of cell-cell interactions, we also examined its localization in rat cerebellum during the period extending from 1 wk to 1 mo of postnatal age. These studies demonstrated that in contrast to the cytoplasmic localization observed in adult brain, the chondroitin sulfate proteoglycan is almost exclusively extracellular in 7-d-old cerebellum, and progressively assumes its final intracellular location in neurons and astrocytes during the succeeding 3 wk.

MATERIALS AND METHODS

The immunochemical and immunocytochemical procedures employed were essentially as described in the preceding paper (2). However, for studies of immature brain (7–33 d postnatal), a two-step fixation procedure was used (3). After perfusion with 0.1 M sodium phosphate buffer (pH 7.4) containing 0.9% NaCl, rats were perfused with 4% freshly prepared formaldehyde and 0.1% glutaraldehyde in sodium phosphate buffer (pH 6.5), followed by the same aldehydes in sodium phosphate buffer, pH 11. Perfusion was carried out at a rate of 1–3 ml/min with ~1 ml of fixative per gram of body weight, and the brains were allowed to stand in fixative for 1 h at room temperature before being placed at 4°C overnight. Sections from immature brain were also stained en bloc for 30 min in the dark at 4°C with 0.5% uranyl acetate in saline after osmication.

Biochemical comparisons of the proteoglycans from 7-d-old and adult brain, involving ion exchange chromatography, gel filtration, and cesium chloride density gradient centrifugation, were carried out using the conditions described in the corresponding figure legends, and compositional analyses were performed as described previously (10).

THE JOURNAL OF CELL BIOLOGY · VOLUME 99 SEPTEMBER 1984 1130-1139

RESULTS

Localization of the Proteoglycan in Immature Brain

$F(ab')_2$ antibodies to the chondroitin sulfate proteoglycan (prepared and characterized as described in the preceding paper) were used in conjunction with the peroxidase-antiperoxidase method to examine the localization of the proteoglycan in developing brain. A survey of cerebellum from 7-, 10-, 14-, 21-, and 33-d-old rats revealed several significant differences from adult brain. The staining pattern observed in 7-d-old cerebellum at the light microscopic level, and its comparison with that of adult cerebellum, can be seen in Fig. 2 of the preceding paper (2). At the electron microscopic level it is apparent that in 7-d-old cerebellum there is very little if any cytoplasmic staining of neurons (Fig. 1), although staining is present intracellularly in some astrocytes (Fig. 2).

Heavy staining is found extracellularly and/or associated with plasma membranes, primarily in the region of the presumptive white matter (Fig. 3), and is also seen in the granule cell and molecular layers (Figs. 1 and 4). Axons, which are unmyelinated at this age, generally do not stain, although there may be light axoplasmic staining in a small proportion of the parallel fibers. In the external granule cell layer, staining is only present extracellularly, primarily in those areas nearest the molecular layer (Fig. 5).

By 10 d the extracellular space has decreased significantly. At this age staining is still present extracellularly (predominantly in the presumptive white matter), but a significant amount is also seen intracellularly in astroglia throughout the cerebellum. The neuronal cytoplasm, however, generally remains unstained.

In the 14-d-old cerebellum some neuronal cell bodies and axons show faint intracellular staining, whereas astroglial staining is equal in intensity to that seen in the adult (Fig. 6). Neuronal staining has increased significantly by 21 d (Figs. 7 and 8), and at 33 d the overall staining pattern and intensity

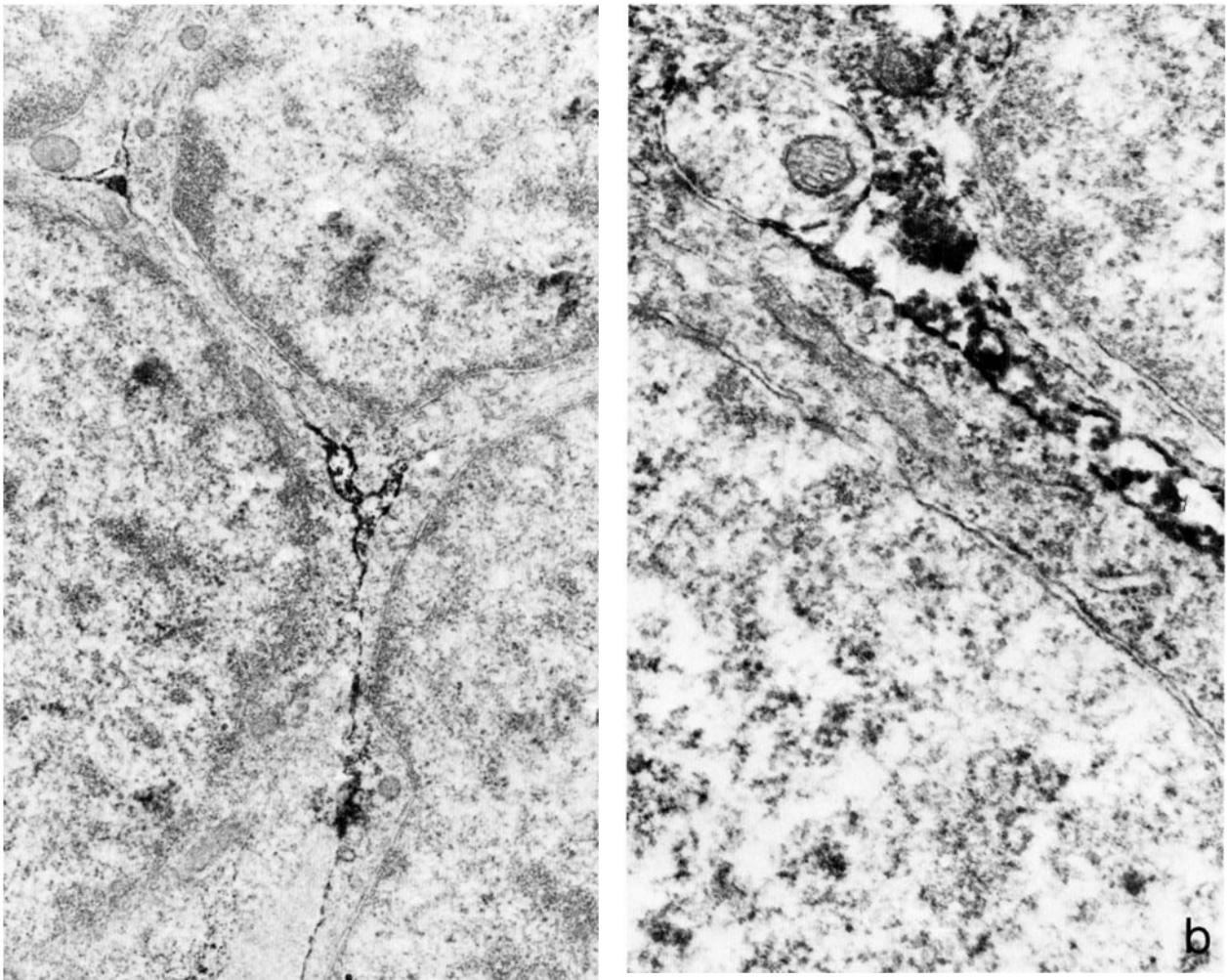

FIGURE 1 (a) Staining between granule cell bodies (which are unstained) in 7-d-old rat cerebellum. × 20,000. (*b*) Similar field from granule cell layer, poststained with uranyl acetate and lead citrate. × 38,000.

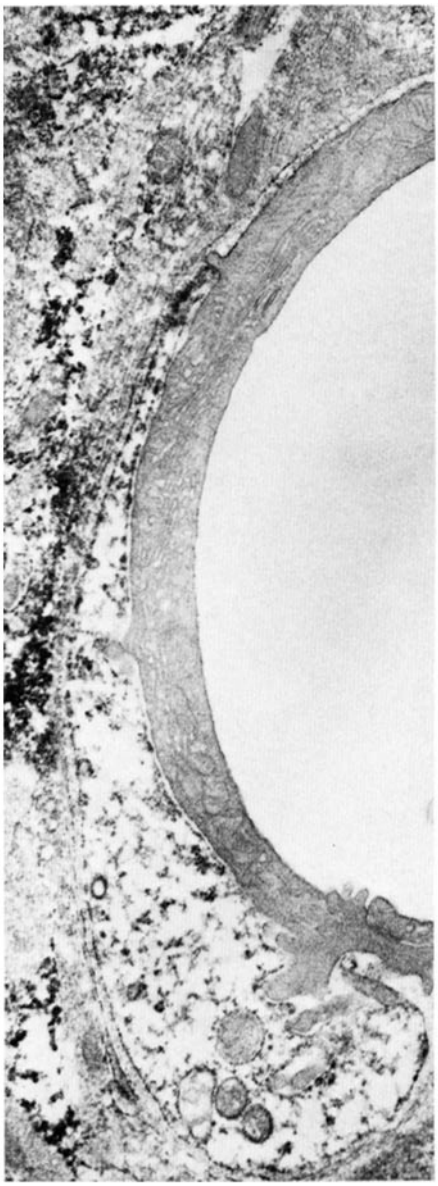

FIGURE 2 Cytoplasmic staining of an astrocyte surrounding a blood vessel in 7-d-old cerebellum. × 20,000.

is similar to that seen in adult brain (Figs. 9–11). However, intracellular staining of granule cells and parallel fibers is often relatively light.

Biochemical Comparison of Proteoglycan from Early Postnatal and Mature Brain

The striking difference in antibody binding that was observed in early postnatal as compared with mature rat brain led us to examine in more detail the biochemical and immunochemical properties of a chondroitin sulfate proteoglycan fraction isolated from the brains of 7-d-old rats, using the same procedure previously described for its isolation from adult brain (10). Since our earlier studies had demonstrated peak levels of chondroitin sulfate at 7 d of age (13), there was good reason to expect that a chondroitin sulfate proteoglycan could be isolated from early postnatal brain.

In fact, this procedure yielded a proteoglycan having proportions of protein and [^{3}H]glucosamine-labeled chondroitin sulfate and glycoprotein oligosaccharides that are almost identical with those previously reported for the chondroitin sulfate proteoglycan from adult rat brain (10). When ^{3}H-labeled proteoglycan from 7-d-old rat brain was mixed with ^{35}S-labeled proteoglycan from adult brain, it was found that the two proteoglycans also have essentially identical charge (demonstrated by gradient elution from DEAE-cellulose, Fig. 12), size (evaluated by gel filtration on Sepharose CL-2B, Fig. 13), and density (measured by CsCl density gradient centrifugation, Fig. 14). The chondroitin sulfate proteoglycans prepared from 7-d-old and adult rat brain also showed the same enzyme-linked immunosorbent assay endpoint when tested with antibody prepared to the adult brain proteoglycans, and were immunoprecipitated to the same extent (74–78%) by this antibody in the presence of Protein A-Sepharose.

FIGURE 3 Staining surrounding unmyelinated axons in the presumptive white matter of 7-d-old cerebellum. × 15,000.

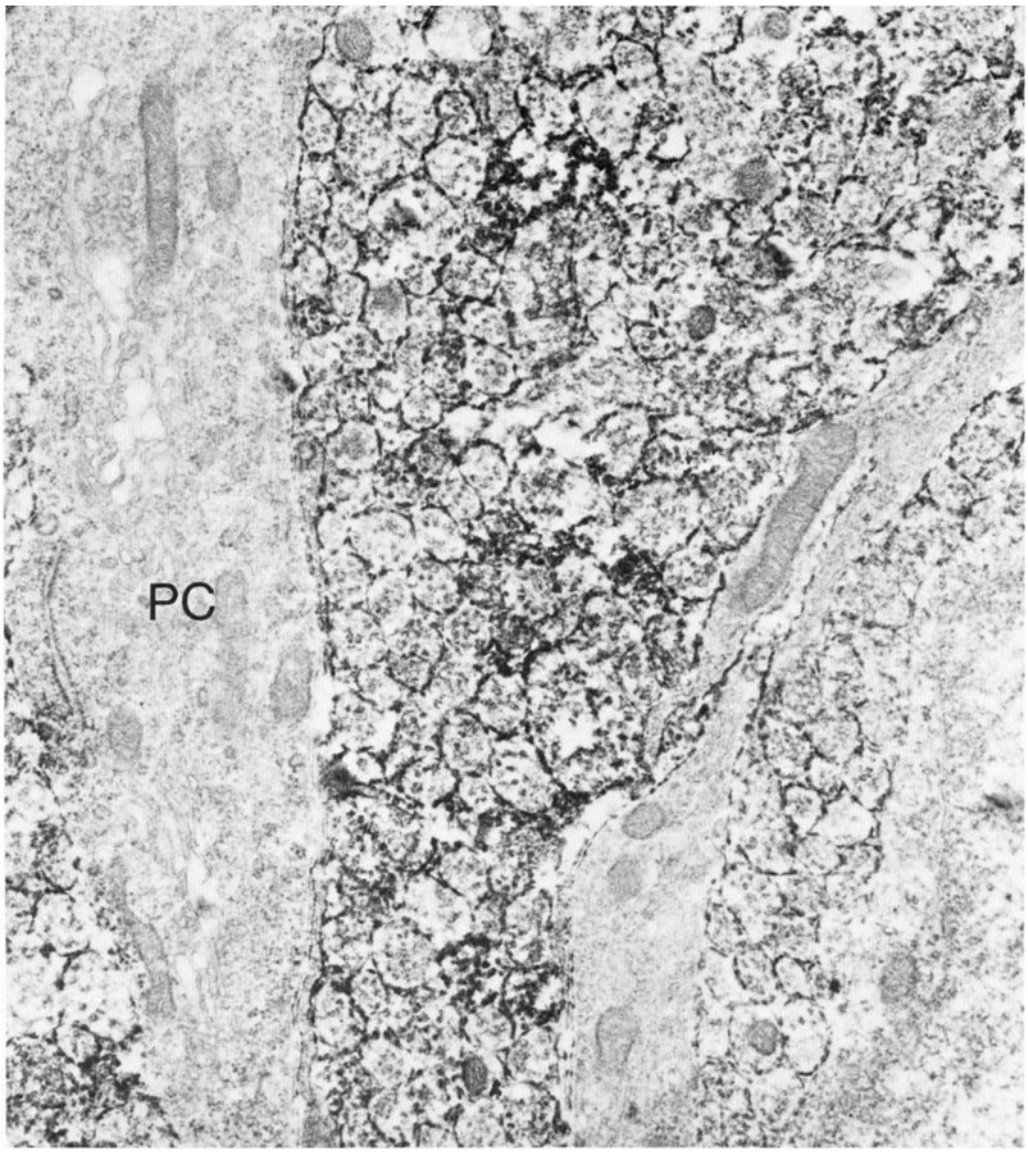

FIGURE 4 Molecular layer of 7-d-old cerebellum, showing heavy staining surrounding the parallel fibers. Purkinje cell dendrite (*PC*) is unstained. × 25,000.

Thus, chondroitin sulfate proteoglycans isolated from early postnatal and adult rat brains both have indistinguishable compositions and physicochemical properties (size, charge, and density) and show the same immunochemical reactivity with antibodies prepared to the adult brain proteoglycan. It would therefore appear that these antibodies are recognizing the same macromolecule at all ages examined in our immunocytochemical study, rather than some other (extracellular) protein of early postnatal brain which fortuitously shares antigenic determinants with the chondroitin sulfate proteoglycan of adult brain.

DISCUSSION

The subject of glycosaminoglycans and proteoglycans in morphogenesis (mostly of non-nervous tissues) has recently been reviewed (22). In rat brain, it was found that the levels of hyaluronic acid, chondroitin sulfate, and heparan sulfate all increased postnatally to reach a peak at 7 d, after which they declined steadily, attaining by 30 d concentrations within 10% of those present in adult brain (13). Since the level of chondroitin sulfate peaks at 7 d postnatal, this age was selected as the first point in our study of possible changes in localization of the proteoglycan during development.

In 7-d-old cerebellum the proteoglycan is present in the extracellular space and in the astroglial cytoplasm (primarily in the region of the presumptive white matter). There is very little, if any, intracellular staining in either neuronal cell bodies or axons. By 10 d, astroglial staining has increased and is present throughout the cerebellum, whereas the extracellular space and its associated staining has decreased significantly. Intracellular staining of astrocytes at 14 d appears to be equal in intensity to that seen in the adult. The first indication of cytoplasmic staining in neurons and myelinated axons also appears at this age. By 21 d significant staining is present in neuronal cell bodies and axons, in addition to that seen in astroglia, and by 33 d the overall staining pattern and intensity closely resemble that of the adult (2). Thus, during development the proteoglycan, which is initially present in

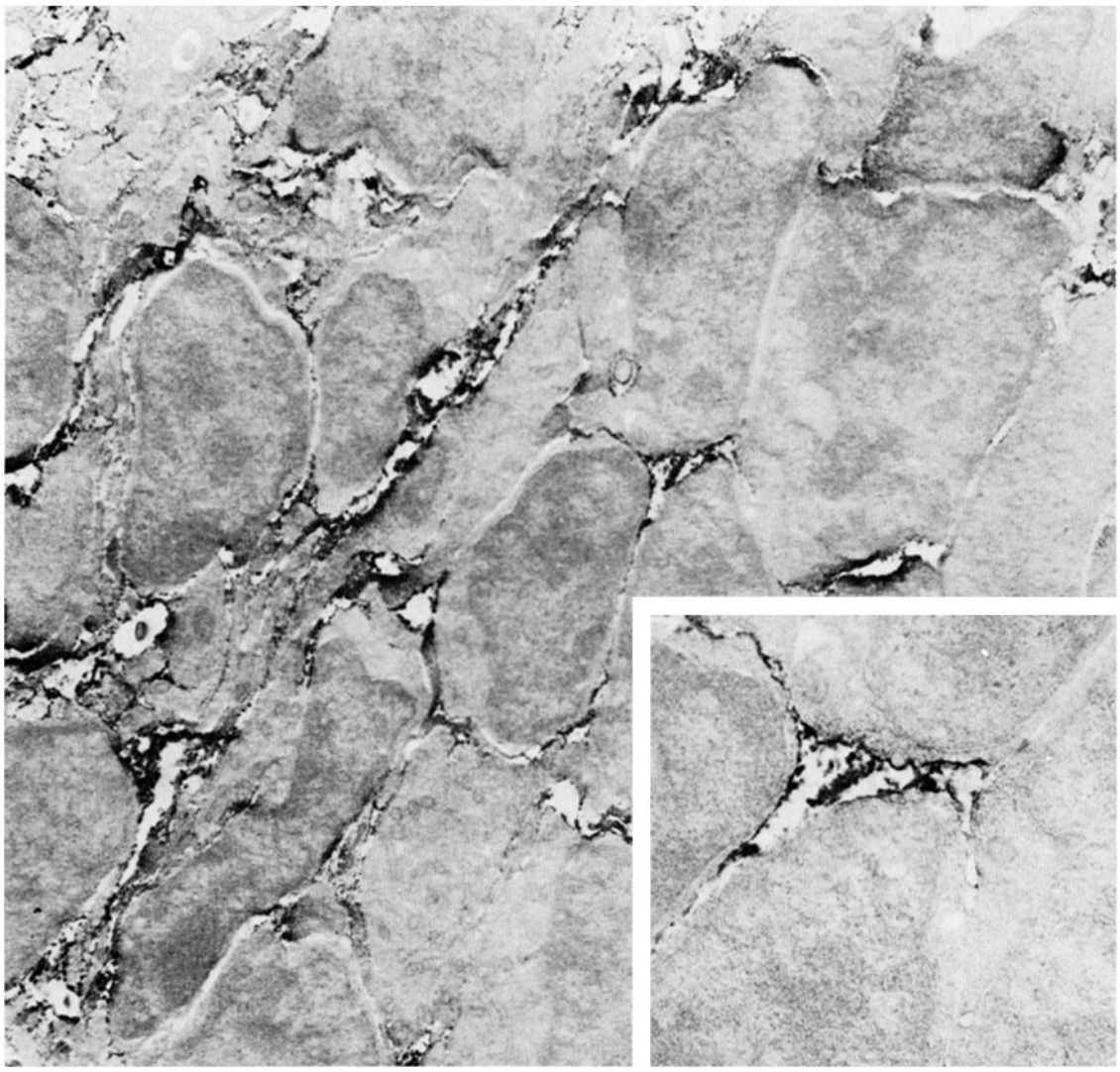

FIGURE 5 Staining surrounding granule cells in the external granule cell layer of 7-d-old cerebellum. × 10,000; (*inset*) × 20,000.

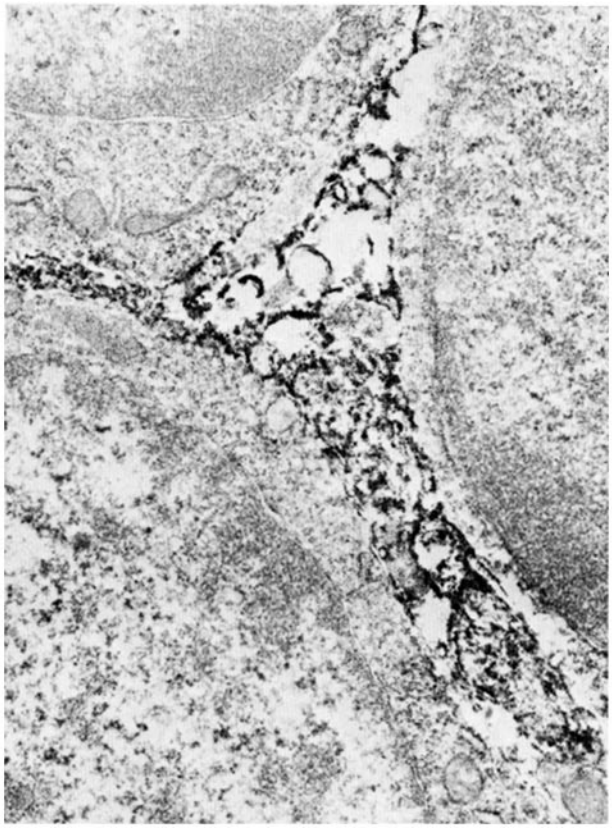

FIGURE 6 Cytoplasmic staining of an astroglial process in 14-d-old cerebellum. The granule cells are unstained. × 20,000.

the extracellular space and in astroglia, gradually becomes an exclusively intracellular (cytoplasmic) component of neurons and astrocytes.

Its intracellular appearance in astroglia generally precedes that in neurons by at least 1 wk. This difference may merely reflect a delay in the onset of synthesis of chondroitin sulfate proteoglycan by the neuronal cell body. However it is also possible that after initial synthesis by glial cells the proteoglycan may be transferred to the neuronal cytoplasm. This latter possibility does not imply that the neuron is unable to synthesize the proteoglycan, but rather that there may be a dynamic interaction between neurons and glia in the regulation of its synthesis. Evidence supporting the transfer of macromolecules to neurons from the intracellular compartment of glia has previously been reported (7, 12, 20).

The apparent change in localization of the chondroitin sulfate proteoglycan during brain development cannot merely be attributed to a lack of specificity of the antibodies, which might recognize two related but different proteoglycans. In such a case one would expect to find both intracellular and extracellular staining at all ages examined, whereas there is only a brief developmental period during which this staining pattern was seen. Moreover, since the antibodies were raised to proteoglycan purified from adult brain, they should not recognize an immunochemically distinct (extracellular) proteoglycan present only in early postnatal brain.

Extracellular space is reported to account for up to 40% of the volume of developing central nervous tissue (5). When compared with tissue fixed optimally by a modification of the method of Karnovsky (9), we observed that the apparent extracellular space in immature cerebellum processed by our

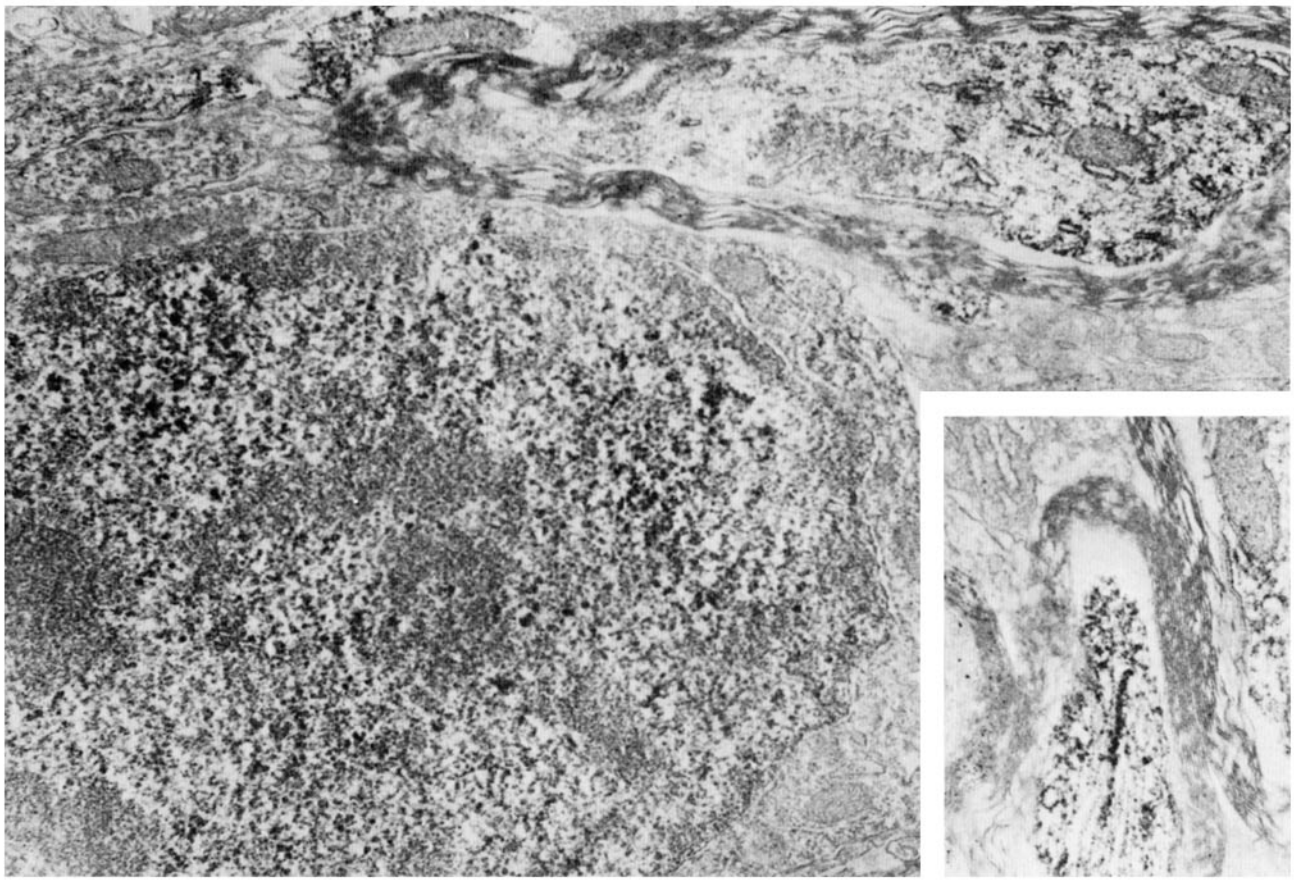

FIGURE 7 Granule cell layer of 21-d-old cerebellum, showing very light staining of neuronal cytoplasm and an adjacent axon. The granule cell nucleus is also stained. × 22,000. (*inset*) Densest axoplasmic staining apparent at this age. × 28,000.

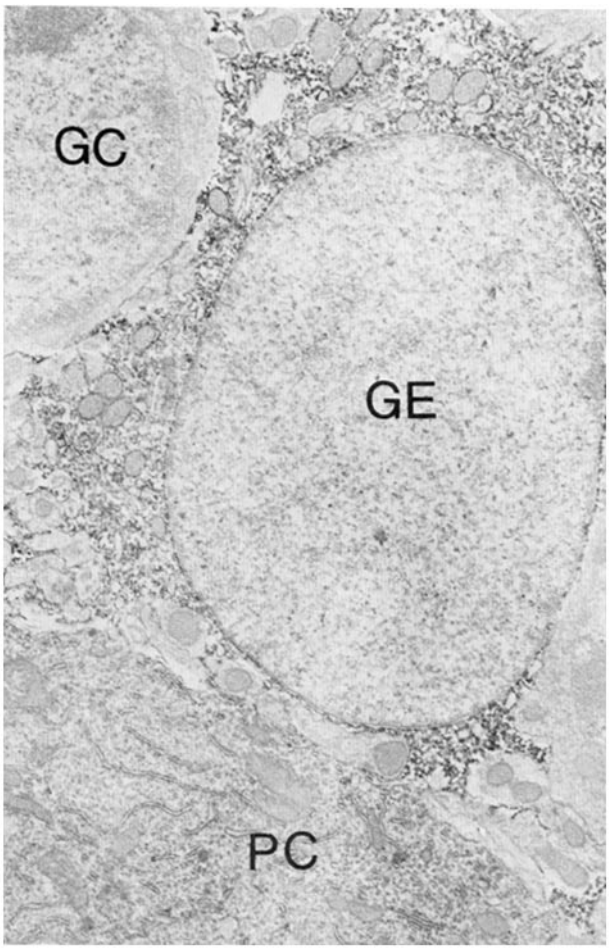

FIGURE 8 Cytoplasmic staining of a Golgi epithelial cell (*GE*) in 21-d-old cerebellum. *PC*, Purkinje cell; *GC*, granule cell. × 10,000.

methods is somewhat increased due to the fixation and handling conditions required for immunoelectron microscopy. This less than optimal preservation reflects the conflicting demands of maintaining adequate ultrastructural morphology and at the same time preserving antigenicity. Since in most cases the plasma membranes are intact and the developmental changes in localization occur gradually and sequentially, it is unlikely that the distribution of the proteoglycan is altered by the fixation conditions employed in this study. Moreover, if the appearance of proteoglycan extracellularly in early postnatal brain were due to some type of "leakage" or extrusion from the cytoplasm, a significant amount of intracellular proteoglycan should also be seen, which was not the case in our studies. Furthermore, application to adult brain of the two-step fixation procedure, which was used to obtain better preservation of immature tissue, did not alter the typical cytoplasmic localization seen in mature brain using the pH 7.4 fixation method.

There has been considerable speculation concerning the possible role of glycosaminoglycans as a component of the extracellular matrix in developing nervous tissue (1, 4, 6, 11, 14, 17, 21, 22). It is generally agreed that one of the more likely functions of extracellular glycosaminoglycans, particularly hyaluronic acid, is to provide a readily penetrable matrix through which neuronal migration and differentiation may take place during brain development. The large domain occupied by the hydrated glycosaminoglycans and their entanglement at even very low concentrations, together with their considerable negative charge density, would all be consistent with these polyanions affecting the movement of ions, unionized compounds, water, and large molecules through the extracellular space. They could therefore influence the transport, rate of diffusion, and distribution of other solutes in solution, as well as act as a molecular sieve or barrier.

The extracellular microenvironment may also be involved in directing axonal extension and cell movement during morphogenesis, in addition to mediating synaptic connections. One indication of this may be evident in the immature cerebellum, where we find that the areas of heaviest staining are in the presumptive white matter (where axons are actively elongating), and extending from the molecular layer up to the external granule cell layer. Staining decreases significantly in the direction of the pia. This latter observation is consistent with an involvement of glycosaminoglycans in mediating cell movement, since the more peripheral external granule cells are relatively stationary, while the innermost ones, which are in direct contact with the proteoglycan, are in the process of migration to the internal granule cell layer.

The possible role of glycosaminoglycans in relation to afferent axon targeting is also discussed in a recent report concerning histochemical studies on the spatial and temporal distribution of extracellular matrix in the developing cerebral

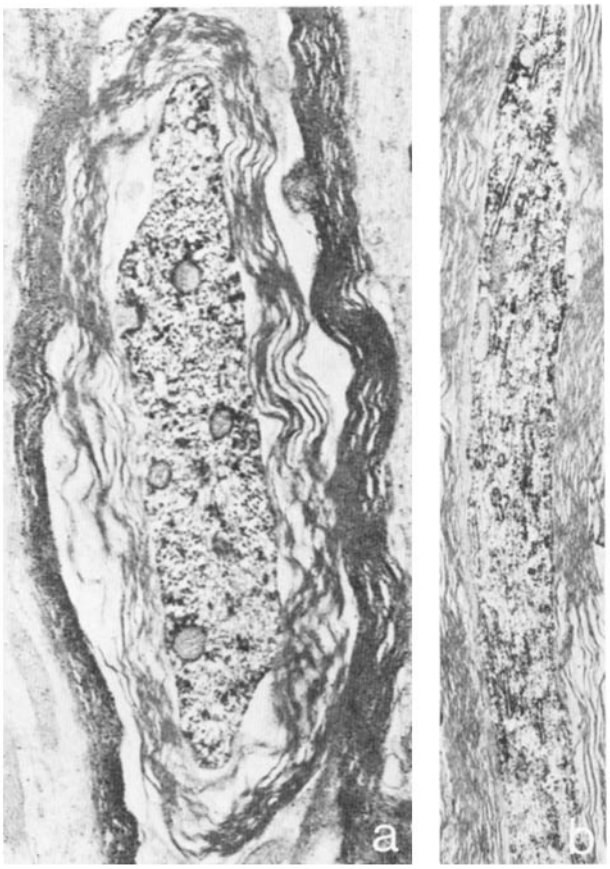

FIGURE 9 (a) Axoplasmic staining of a myelinated axon in 33-d-old cerebellum. × 20,000. (*b*) Longitudinal section of a similar axon. × 16,000.

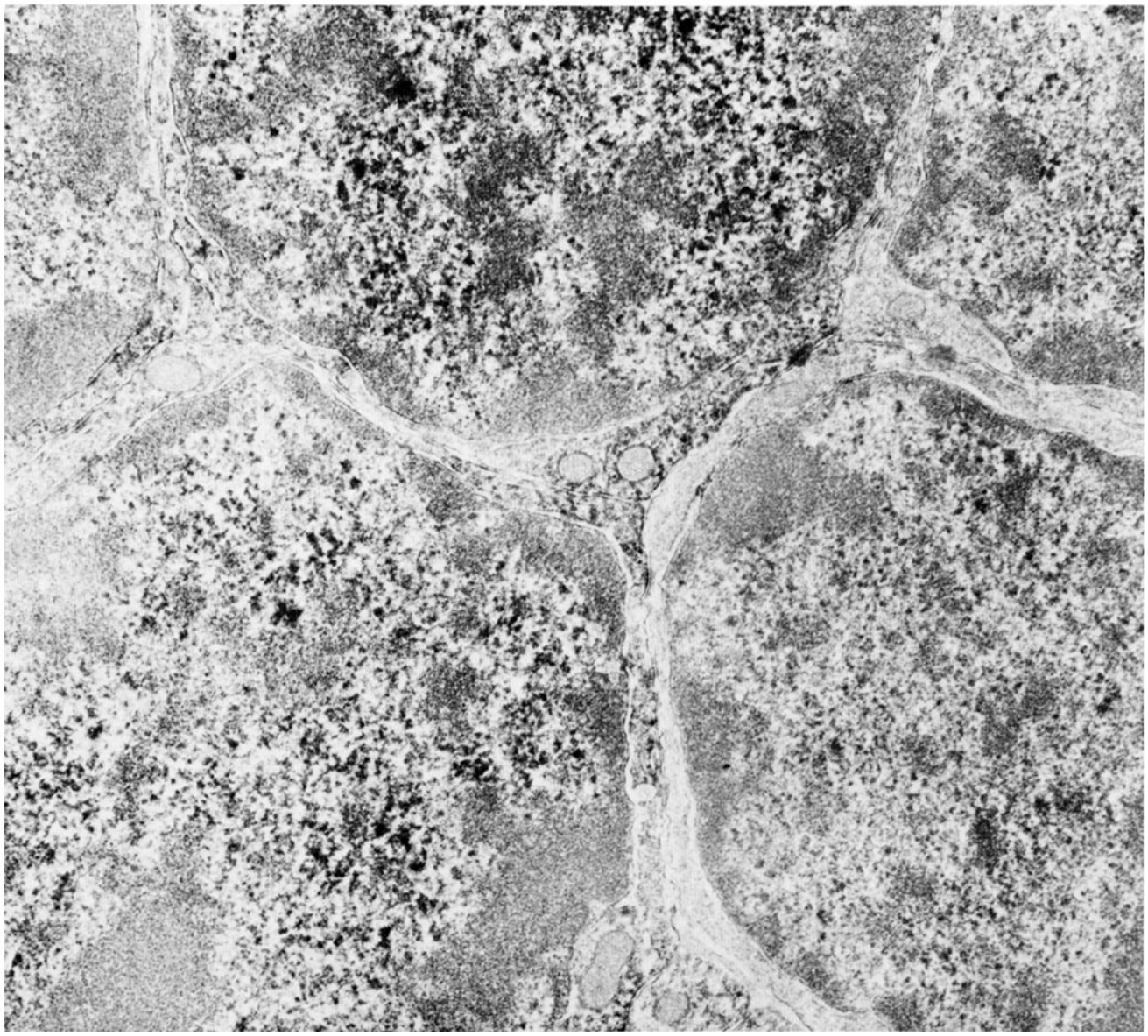

FIGURE 10 Cytoplasmic and nuclear staining in granule cells of 33-d-old cerebellum. × 22,000.

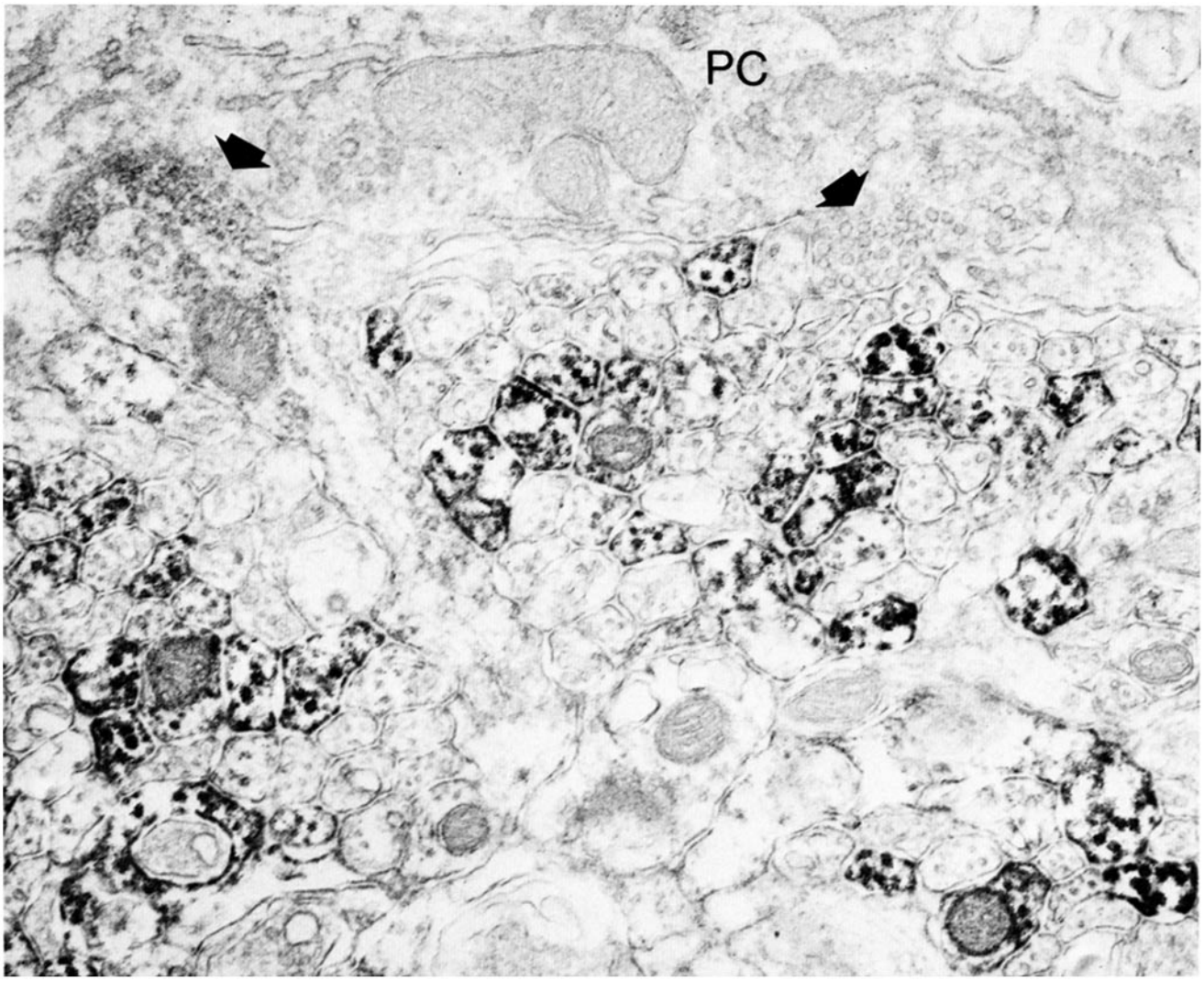

FIGURE 11 Molecular layer of 33-d-old cerebellum, showing heavy axoplasmic staining of some parallel fibers. The Purkinje cell dendrite (*PC*) does not stain. Nerve endings (arrows) containing synaptic vesicles are also unstained. × 42,000.

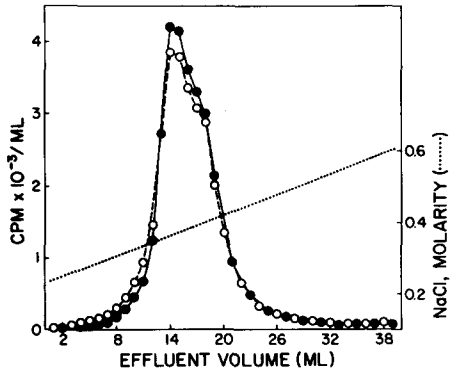

FIGURE 12 Elution of [^{35}S]sulfate-labeled chondroitin sulfate proteoglycan from adult rat brain (●——●) and [^{3}H]glucosamine-labeled proteoglycan from 7-d-old brain (O-----O) from a DEAE-cellulose column (0.5 × 5 cm) using a linear gradient of NaCl (·····) in 50 mM Tris-HCl buffer (pH 8.2). ^{35}S and ^{3}H radioactivity were measured in the combined samples by double label counting, and normalized to the same number of ^{35}S and ^{3}H counts per minute for purposes of comparison. Recovery of radioactivity from the column was 78% for ^{3}H and 86% for ^{35}S.

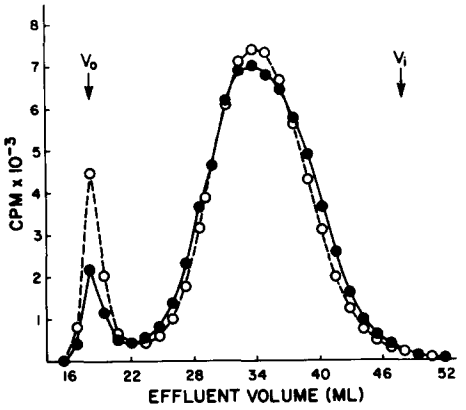

FIGURE 13 Elution of [^{35}S]sulfate-labeled proteoglycan from adult rat brain (●——●) and ^{3}H-labeled proteoglycan from 7-d-old brain (O-----O) from Sepharose CL-2B (0.9 × 65 cm) using 0.2 M sodium acetate buffer, pH 5.6. ^{35}S and ^{3}H counts per minute were normalized as described in the legend to Fig. 12, and recovery of radioactivity was 92% in both cases. The small peak eluting at the void volume represents proteoglycan aggregate (cf. reference 10).

cortex of normal and reeler mutant mice (15). By use of colloidal iron and Alcian blue in conjunction with various enzyme treatments for the ultrastructural visualization of the extracellular matrix, it was concluded that chondroitinase-susceptible glycosaminoglycans are major components of the extracellular matrix in developing mouse cerebral cortex. Colloidal iron-staining material was localized principally in the marginal zone and subplate of normal mice, whereas in reeler mutants, most of the material was found in the outer layers of the cortex.

One can only speculate about why the chondroitin sulfate proteoglycan disappears from the extracellular space of immature brain and appears as a predominantly cytoplasmic component in the adult. However, interactions between various extracellular components are obviously necessary to coordinate the large number of complex and diverse processes that are activated during morphogenesis and differentiation. It is therefore not unlikely that glycosaminoglycans might be involved at this stage of development, and that removal of the chondroitin sulfate proteoglycan from the extracellular

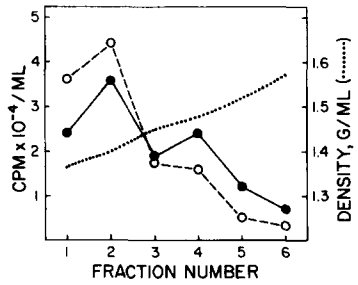

FIGURE 14 Cesium chloride density gradient centrifugation of [^{35}S]-sulfate-labeled proteoglycan from adult brain (●——●) and ^{3}H-labeled proteoglycan from 7-d-old brain (O- - -O). The two proteoglycans were combined and dissolved at a concentration of <1 mg/ml in a solution having a final concentration of 4 M guanidine HCl, 3 M CsCl, and 0.15 M potassium acetate (pH 6.3). Centrifugation was in a fixed angle rotor at 128,000 *g* for 40 h at 5°C. After recording the density of the six fractions, they were dialyzed against distilled water before measuring ^{35}S and ^{3}H radioactivity by double-label counting and normalization as described in the legend to Fig. 12.

space may allow various "adhesive" processes to begin.

We thank Dr. Carol Mason for her invaluable advice and assistance throughout the course of these studies.

This work 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. Anderson, C. B., and S. Meier. 1982. Effect of hyaluronidase treatment on the distribution of cranial neural crest cells in the chick embryo. *J. Exp. Zool.* 221:329–335.
2. Aquino, D. A., R. U. Margolis, and R. K. Margolis. 1984. Immunocytochemical localization of a chondroitin sulfate proteoglycan in nervous tissue. I. Adult brain, retina, and peripheral nerve. *J. Cell Biol.* 99:1117–1129.
3. Berod, A., B. K. Hartman, and J. F. Pujol. 1981. Importance of fixation in immunochemistry: use of formaldehyde solutions at variable pH for the localization of tyrosine hydroxylase. *J. Histochem. Cytochem.* 29:844–850.
4. Bolender, D. L., W. G. Seliger, and R. R. Markwald. 1980. A histochemical analysis of polyanionic compounds found in the extracellular matrix encountered by migrating cephalic neural crest cells. *Anat. Rec.* 196:401–412.
5. Bondareff, W., and J. J. Pysh. 1968. Distribution of the extracellular space during postnatal maturation of rat cerebral cortex. *Anat. Rec.* 160:773–780.
6. Derby, M. A. 1978. Analysis of glycosaminoglycans within the extracellular environments encountered by migrating neural crest cells. *Dev. Biol.* 66:321–336.
7. Fischer, S., and S. Litvak. 1967. The incorporation of microinjected ^{14}C-amino acids into TCA insoluble fractions of the giant axon of the squid. *J. Cell Physiol.* 70:69–74.
8. Hedman, K., J. Christner, I. Julkunen, and A. Vaheri. 1983. Chondroitin sulfate at the plasma membranes of cultured fibroblasts. *J. Cell Biol.* 97:1288–1293.
9. Karnovsky, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. *J. Cell Biol.* 27:137*a*. (Abstr.)
10. 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.
11. Krayanek, S. 1980. Structure and orientation of extracellular matrix in developing chick optic tectum. *Anat. Rec.* 197:95–109.
12. Lasek, R. J., and M. A. Tytell. 1981. Macromolecular transfer from glia to the axon. *J. Exp. Biol.* 95:153–165.
13. Margolis, R. U., R. K. Margolis, L. B. Chang, and C. Preti. 1975. Glycosaminoglycans of brain during development. *Biochemistry.* 14:85–88.
14. Morris, J. E., J. J. Hopwood, and A. Dorfman. 1977. Biosynthesis of glycosaminoglycans in the developing retina. *Dev. Biol.* 58:313–327.
15. Nakanishi, S. 1983. Extracellular matrix during laminar pattern formation of neocortex in normal and reeler mutant mice. *Dev. Biol.* 95:305–316.
16. Oldberg, Å., E. G. Hayman, and E. Ruoslahti. 1981. Isolation of a chondroitin sulfate proteoglycan from a rat yolk sac tumor and immunochemical demonstration of its cell surface localization. *J. Biol. Chem.* 256:10847–10852.
17. Pintar, J. E. 1978. Distribution and synthesis of glycosaminoglycans during quail neural crest morphogenesis. *Dev. Biol.* 67:444–464.
18. 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.
19. 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.
20. Singer, M., and M. M. Salpeter. 1966. The transport of [^{3}H]-L-histidine through the Schwann and myelin sheath into the axon, including a reevaluation of myelin function. *J. Morphol.* 120:281–315.
21. Toole, B. P. 1976. Morphogenetic role of glycosaminoglycans (acid mucopolysaccharides) in brain and other tissues. *In* Neuronal Recognition. S. H. Barondes, editor. Plenum Press, New York. 275–329.
22. Toole, B. P. 1982. Glycosaminoglycans in morphogenesis. *In* Cell Biology of Extracellular Matrix. E. D. Hay, editor. Plenum Press, New York. 259–294.