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Immunocytochemical Localization of (Na^+,K^+)-ATPase in the Goldfish Optic Nerve

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Abstract: Antiserum to the catalytic subunit of goldfish brain (Na^+,K^+)-ATPase has been employed at the electron microscopic level by means of the peroxidase-antiperoxidase immunohistochemical method. In optic nerve, antigenic sites are restricted to the nodes of Ranvier. No reaction product is detected in underlying internodal neurolemma. Outgrowing neurites for cultured retinal explants devoid of glial ensheathment exhibit a continuous distribution of the enzyme subunit. Antibodies against eel electroplax (Na^+,K^+)-ATPase cross-react with the goldfish brain enzyme and show a similar immunocytochemical distribution pattern. **Key Words:** $(Na,^+K^+)$-ATPase—Immunocytochemistry—Ultrastructure—Goldfish visual system. **Schwartz M. et al.** Immunocytochemical localization of (Na^+, K^+)-ATPase in the goldfish optic nerve. *J. Neurochem.* 36, 107–115 (1981).

Since there has been extensive documentation regarding the nature of (Na^+,K^+)-ATPase and its role in cellular ion transport, its localization by cytochemical (DiBona and Mills, 1979), autoradiographic (Ernst and Mills, 1980), and immunochemical techniques (Kyte, 1976) has been of considerable interest. This is particularly true in the nervous system (Guth and Albers, 1974; Stahl and Broderson, 1976; Broderson et al., 1978), where the enzyme plays a critical role in the maintenance of neuronal excitability (Siegel et al., 1981) and is a marker of neuronal differentiation (Bertoni and Siegel, 1978; 1979). Wood et al. (1977) previously reported localization of the enzyme at the node of Ranvier in myelinated optic tract fibers by means of antisera to eel electroplax (Na^+,K^+)-ATPase in a heterologous tissue, the brain of the knifefish. We have recently produced antisera against purified goldfish brain (Na^+,K^+)-ATPase and have demonstrated its specific binding to goldfish optic nerve fibers at the light microscopic level (Schwartz et al., 1980). In the present report, we demonstrate the ultrastructural distribution of the enzyme in the optic nerve as well as in neurites growing out from ganglion cells in retinal explants. Weaker but specific heterologous immunocytochemical binding of anti-eel antibody to goldfish optic nerve is also demonstrated.

MATERIALS AND METHODS

Goldfish (*Carassius auratus*) 6–7 cm in body length, supplied by Ozark Fisheries (Stoutland, Missouri) underwent intraorbital crush of the right optic nerve as previously described (Landreth and Agranoff, 1979).

Antisera against *E. electricus* electroplax (Na^+,K^+)-ATPase were the gift of Dr. D. H. Jean. Preparation of goldfish brain (Na^+,K^+)-ATPase catalytic polypeptide and antisera specific to it have been described previously (Schwartz et al. 1980).

Normal goat serum and peroxidase-rabbit antiperoxidase soluble complex were obtained from Sternberger-Myer Immunocytochemicals, Inc. (Jar-

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Abbreviations used: NGS, Normal goat serum; PAP, Peroxidase-rabbit antiperoxidase soluble complex; PB, Phosphate buffer; HRP, Horseradish peroxidase; BSA, Bovine serum albumin; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-ethanesulfonic acid; 5′Fud R, 5′-Fluorodeoxyuridine.

rettsville, Maryland) and goat anti-rabbit IgG was the product of Miles Laboratories (Elkhart, Indiana).

Purified goat anti-rabbit antibodies were prepared by using an affinity column of Sepharose to which rabbit IgG was coupled. Iodination of the goat anti-rabbit antibodies with $Na^{125}I$ was performed by the chloramine-T method (Hunter, 1973). All procedures, including tissue culture, were performed at room temperature unless specified otherwise.

Preparation of Optic Nerve Sections for Electron Microscopic Immunocytochemistry

Fish were anesthetized and perfused through the heart with phosphate buffer, 0.12 M (PB) containing 4% paraformaldehyde and 0.2% glutaraldehyde for 30 min. The optic nerves were removed and further fixed for 1 h in the same fixative solution, then stored in PB at 4°C overnight. The optic nerves were then embedded in 6% agarose and cut into 80–100-μm slices with an Oxford Vibratome, then transferred into PB and selected for immunocytochemistry.

Explant Cultures

Ten to fourteen days after optic nerve crush, retinas were removed and cut into 500-μm squares as previously described (Landreth and Agranoff, 1979). The retinal explants were placed in culture dishes coated with poly-L-lysine in Leibowitz nutrient medium supplemented with *N*-2-hydroxyethylpiperazine-*N′*-ethane-sulfonic acid (HEPES), pH 7.2 and fetal calf serum in the presence of gentamicin sulfate, 5′-fluorodeoxyuridine (5′Fud R), and uridine (Landreth and Agranoff, 1979). After 6–7 days *in vitro,* cultures were fixed for 90 min in PB containing 2.5% paraformaldehyde and 0.2% glutaraldehyde.

Immunocytochemical Procedures

Fixed cultures or tissue slices were preincubated for 15 min in normal goat serum (NGS) diluted 1:4 in PB. After pre-incubation, the samples were incubated for 2 h with the rabbit primary antisera diluted in PB, then rinsed overnight at 4°C with PB and incubated for 1 h with goat anti-rabbit IgG (320 μg/ml). The samples were rinsed for 3–4 h and then further incubated for 30 min with a solution of 1% NGS containing a soluble complex of peroxidase-rabbit antiperoxidase (3:2) diluted 1:40 (Sternberger, 1974). The samples were then rinsed for 30 min in PB and for an additional 30 min in 0.05 M-Tris-HCl buffer (pH 7.6). The bound peroxidase (HRP) was then reacted with 3,3′-diaminobenzidine (60 mg/100 ml) in the presence of 0.06% hydrogen peroxide in 0.05 M-Tris-HCl buffer. The cultures and the slices were washed for 30 min and then postfixed for 1 h with 2% osmium tetroxide in PB. Next, the specimens were dehydrated through an ethanol series followed by propylene oxide and then embedded in Epon.

Solid Phase Radioimmunoassay

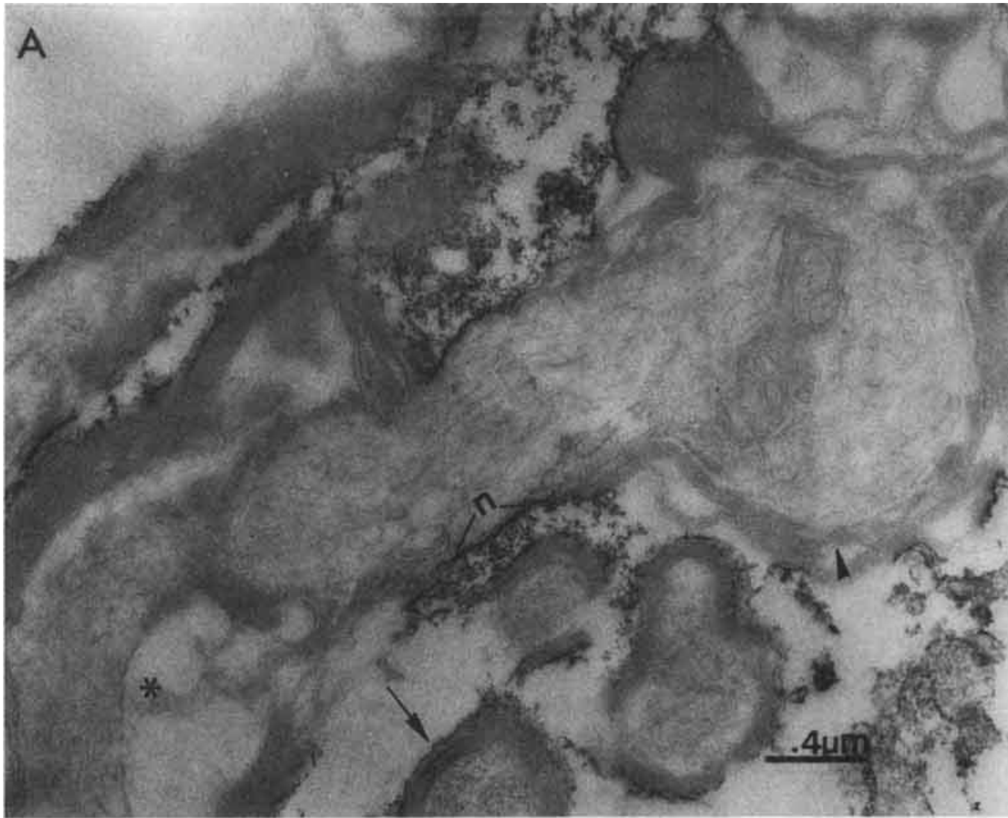
Each well of a flexible microtiter plastic plate was coated for 2 h with 100 μl of goldfish brain microsomes (200 μg protein/ml) enriched in (Na^+,K^+)-ATPase (Schwartz et al., 1980). The plates were then washed with 0.2% BSA (bovine serum albumin) in PB solution. Twenty-five microliters of sera diluted in 0.2% BSA in PB were added to each well of the coated plates. After 1 h of incubation the plates were washed with 0.2% BSA in PB. The binding of the rabbit antisera to the microsomal preparation was determined by the addition of purified ^{125}I-goat anti-rabbit antibodies followed by overnight incubation at 4°C. After extensive washing, the plates were dried and wells were cut out and counted (Schwartz et al., 1978).

RESULTS

Vibratome sections of fixed optic nerves prepared as described in Materials and Methods were treated with rabbit anti-goldfish serum followed by the PAP procedure. HRP staining was seen in superficial sections treated with 1:50 dilutions of the antiserum and was most prominent in the region of the nodes of Ranvier (Fig. 1,A and B). Labeling was also seen along the outer myelin margins of oligodendroglia. There was no apparent staining of internodal axolemma. No staining was found in comparable control sections treated with the same dilution of rabbit preimmune sera (Fig. 1C). Inner myelin layers were unstained even when the external layer was disrupted and pulled away (Fig. 1B). In this instance, questions of antiserum penetration to underlying neurolemma appear obviated, although it remains possible that the myelin separated during a step in the immunocytochemical procedure subsequent to the primary antiserum reaction. When rabbit anti-eel (Na^+,K^+)-ATPase antiserum was used, nodal concentration of reaction product was again seen whereas control sections showed little staining (Fig. 2). The results indicated that there was sufficient cross-reactivity to demonstrate the goldfish optic nerve enzyme. However, the

FIG. 1. Electron micrographs of goldfish optic nerve stained immunocytochemically with antisera (diluted 1:50) against the homologous (Na^+,K^+)-ATPase catalytic subunit. Unless otherwise noted, thin sections shown here and in the other figures were not counterstained with lead and uranyl salts.

A and B: Axolemmal reaction product is restricted to the nodal region (n) and to the outermost lamina of the myelin sheath (arrows). Accumulated product extends into the extracellular space. No staining was observed along internodal axolemmal surfaces, even when the normally adherent myelin sheath was severely disrupted or pulled away from the underlying plasmalemma (asterisk). Note the absence of staining on inner myelin laminae when the outermost layers were disrupted or lost (arrowheads). Although intracellular staining was generally not observed in these preparations (A), sections occasionally showed some product deposition along vesicular elements in the perinodal region (double arrow in B). A, ×36,100; B, ×40,611. **C:** This micrograph demonstrates the absence of immunostaining in control preparations in which preimmune serum was substituted for the primary antiserum. The arrow indicates the nodal region. ×48,400.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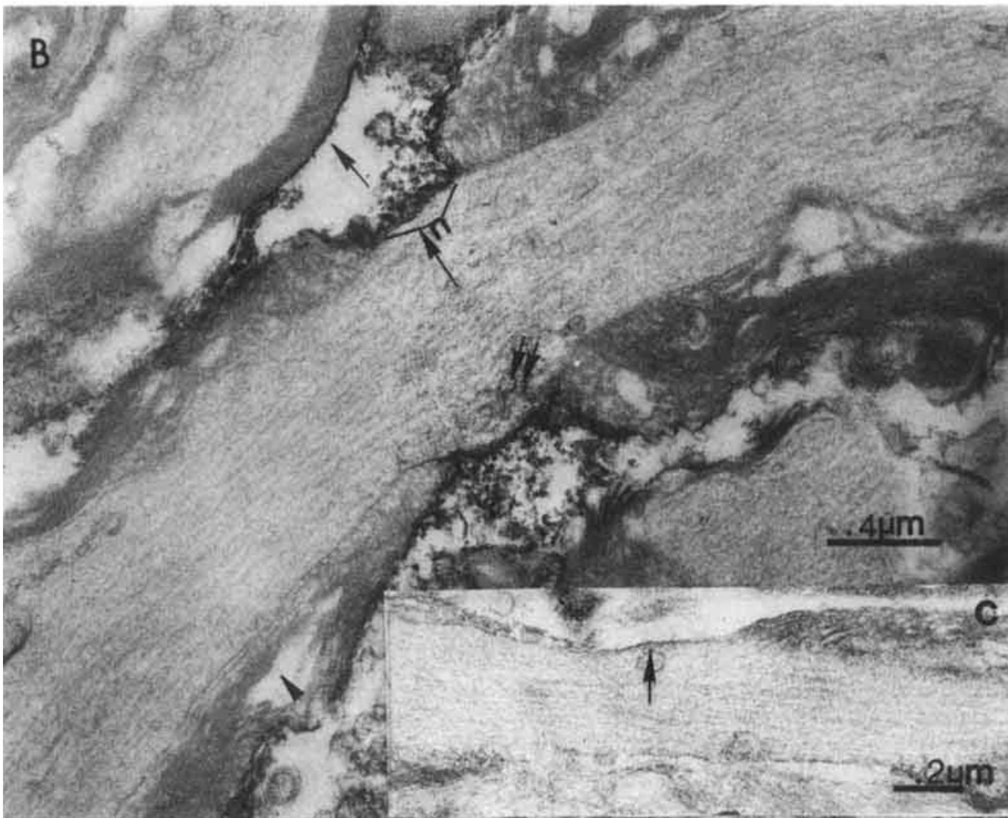
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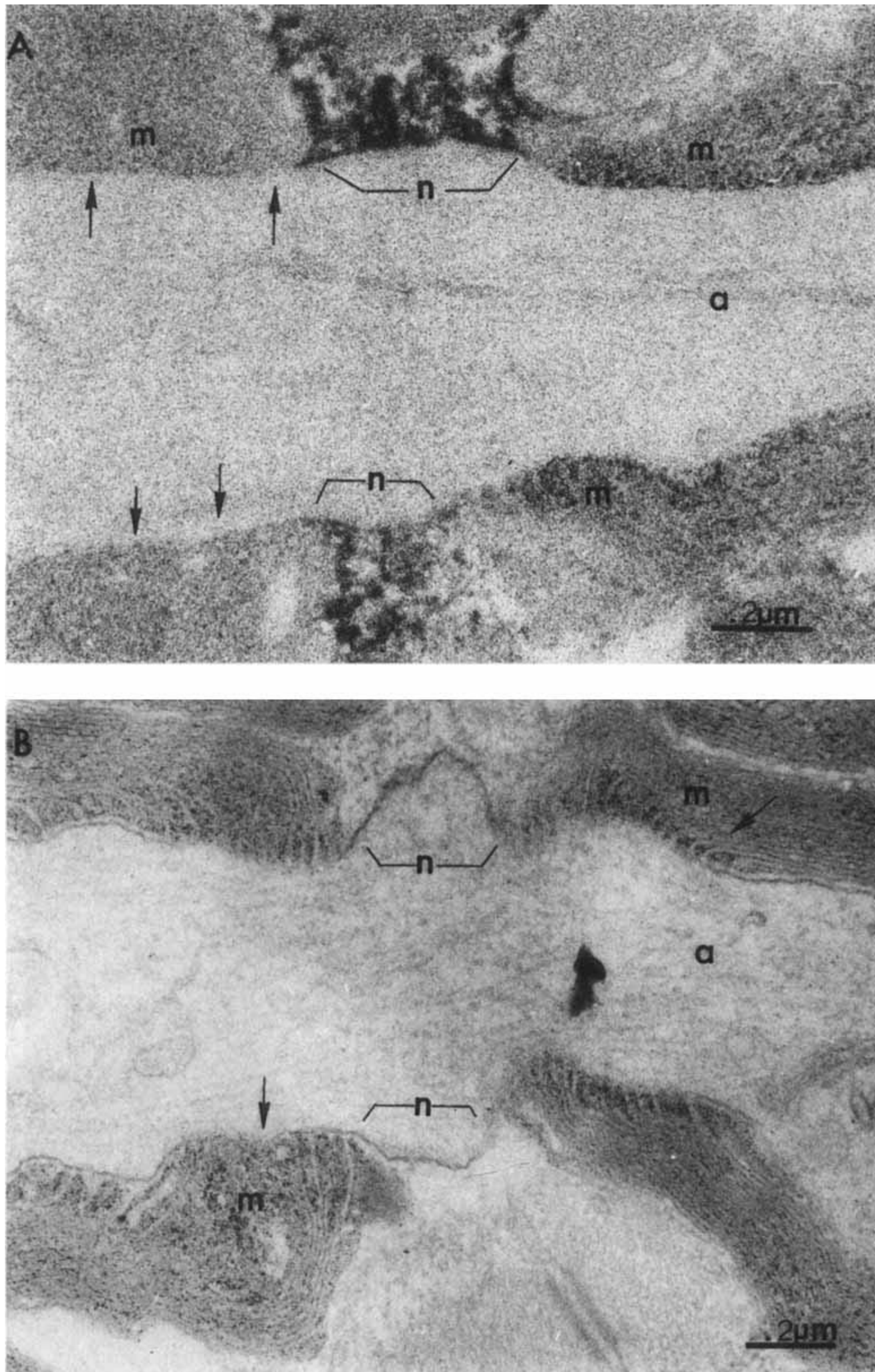

FIG. 2. A: Electron micrograph of goldfish optic nerve stained immunocytochemically with antisera against eel electroplax (Na^+,K^+)-ATPase (diluted 1:20). Dense reaction product is associated with the nodal region (n); adjacent internodal axolemma is devoid of staining (arrows). Staining densities within the myelin laminae (m) are not specific, since they are present in controls. a, axon. ×81,810. **B:** No reaction product is seen in the node (n) when the primary antisera was replaced by preimmune serum. ×70,000.

anti-eel holoenzyme antiserum was immunocytochemically less reactive with the goldfish preparation and therefore was used at a 1:25 dilution.

The cross-reactivity of the antibodies directed against *Electrophorus* electroplax (Na^+,K^+)-ATPase (Lubrol extract) with the goldfish brain enzyme was further established by solid phase radioimmunoassay (Schwartz et al., 1978). Goldfish brain microsomes enriched in (Na^+,K^+)-ATPase was used as the antigenic source. Figure 3 demonstrates the binding curves of the rabbit antisera directed against the goldfish catalytic polypeptide (Schwartz et al., 1980) and of the antisera against Lubrol-solubilized whole eel enzyme and against eel catalytic polypeptide (anti-P96; see Jean et al., 1975). Antisera against the goldfish enzyme led to substantial binding even when diluted 1:1000 whereas the antisera against the eel whole enzyme or the eel catalytic polypeptide could be diluted only 1:100 and 1:10, respectively. The value for nonspecific binding of rabbit preimmune sera was subtracted from those values obtained for each of the specific sera at each respective dilution.

The enzyme was also studied in neurites growing out of retinal explants *in vitro*. Neurites from explant cultures were fixed after 7–10 days of outgrowth *in vitro*. Reaction product was associated with the neurite membranes and was distributed in a continuous fashion (Fig. 4A) whereas the same dilution of preimmune serum indicated little labeling (Fig. 4B). The membrane of the growth cone was also labeled continuously and the pattern was similar to that seen along the neurites (Fig. 5).

It was found that long rinsing times (24 h) are necessary for the removal of the nonspecific labeling. When PAP was excluded from the immunostaining sequence, no labeling was seen, a result that ruled out the possible role of endogenous HRP-like activity in the observed staining.

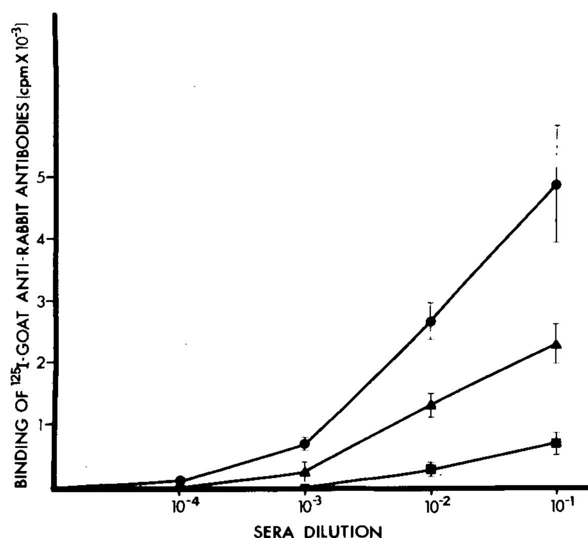

FIG. 3. Binding of ^{125}I-goat anti-rabbit IgG to (Na^+,K^+)-ATPase-enriched goldfish microsomes (200 μg/ml) treated with varying dilutions of rabbit antisera to goldfish catalytic subunit (●), eel electroplax holoenzyme (▲), or to eel catalytic subunit (■).

DISCUSSION

The goldfish visual system has served as the focus of a number of problems in developmental neurobiology including neurogenesis (Rusoff and Easter, 1979), growth, and cell recognition (Gaze, 1970; Jacobson, 1978). Our laboratory has used this preparation for studies on axonal flow (Elam and Agranoff, 1971*a,b;* Heacock and Agranoff, 1977) and concomitants of biochemical regeneration following optic nerve crush (Heacock and Agranoff, 1976; Burrell et al., 1979). We have recently prepared antibodies to goldfish brain (Na^+,K^+)-ATPase and have demonstrated the presence of the enzyme in nerve bundles within the optic nerve and in neurites grown out from retinal explant culture by immunofluorescence microscopy (Schwartz et al., 1979; 1980). Localization is further defined at the electron microscopic level in the present study. In optic nerve sections, we find intense immunocytochemical reaction at the nodes of Ranvier, but in no case along neurolemma underlying internodal myelin (Fig. 1). Even in instances in which myelin is pulled away from the nerve such as is shown in Fig. 1, no reaction product is associated with the exposed neurolemma. Nodal localization of the enzyme would appear reasonable in view of the known function of the enzyme, the nature of saltatory conduction in myelinated fibers (Bostock and Sears, 1977), and other indications of nodal membrane specialization (Langley, 1979; Ritchie, 1979). In neurites grown in tissue culture in the absence of glial investment, continuous labeling of axolemma is seen (Figs. 4 and 5). We hypothesize that the discontinuous neurolemmal distribution seen in adult optic nerve represents actual concentration of the enzyme at the nodes, although it remains possible that the restrictive nature of localization reflects a lack of penetration of immunoreactants through the myelin layers of the optic nerve to reach potential internodal antigenic sites.

The present study (see Fig. 1B) also shows some intracellular reaction for (Na^+,K^+)-ATPase in the region of the node. The finding may be significant, indicating higher levels of enzyme in the nodal region than elsewhere in the adult goldfish nerve axoplasm. The fact that the immunoreactants were clearly present in the axoplasm and did not lead to reaction product along the cytoplasmic side of the paranodal axolemma might also be due to specificity of antibodies for extracellular antigenic sites of the enzyme, or to the rate of diffusion of the im-

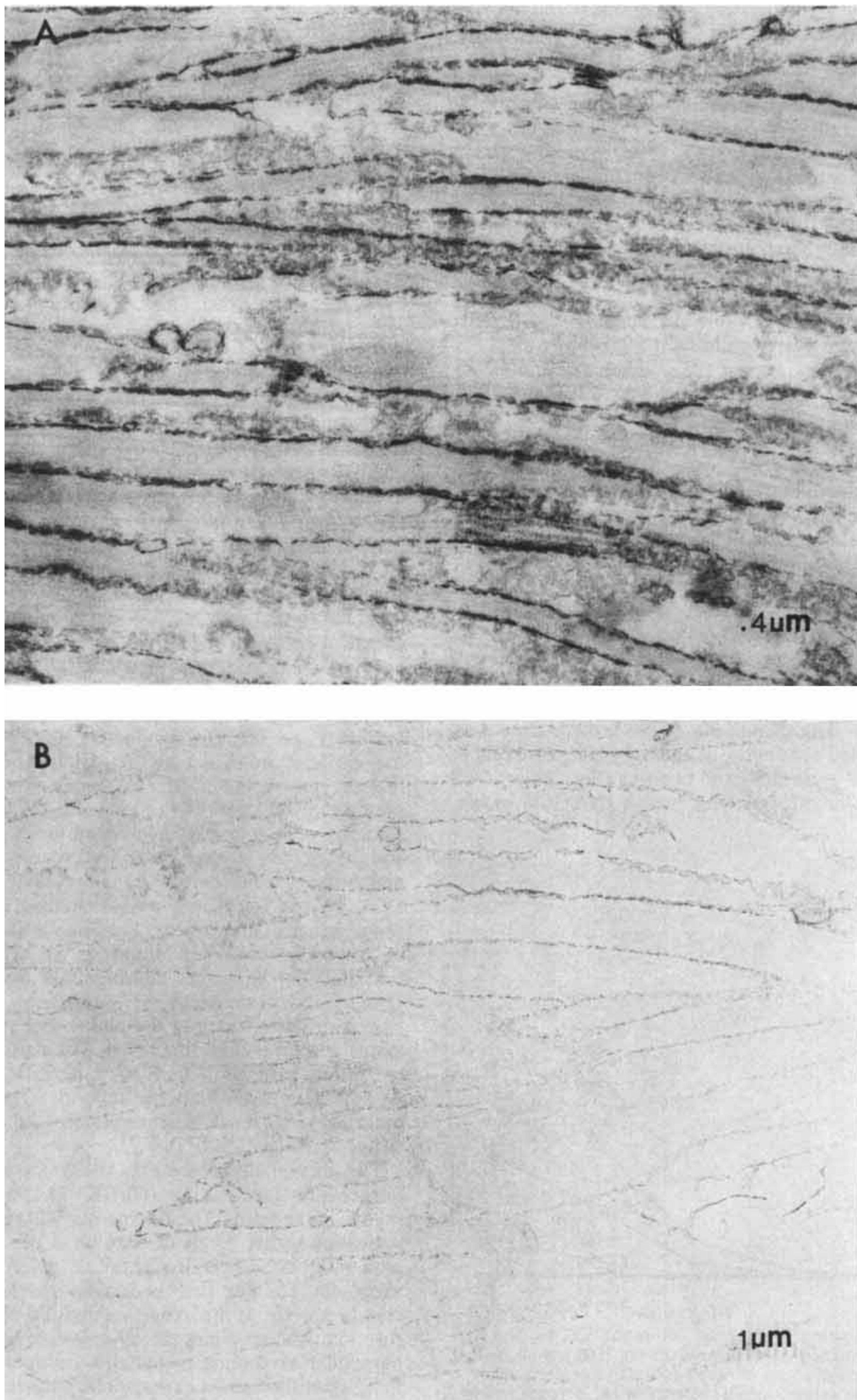

FIG. 4. A: Electron micrograph of neurites stained immunocytochemically with anti-goldfish catalytic subunit. Immunostaining is present uniformly along neurite plasmalemmal surfaces. ×31,870. **B:** When preimmune serum was substituted for the primary antiserum, little immunostaining was present. ×19,170.

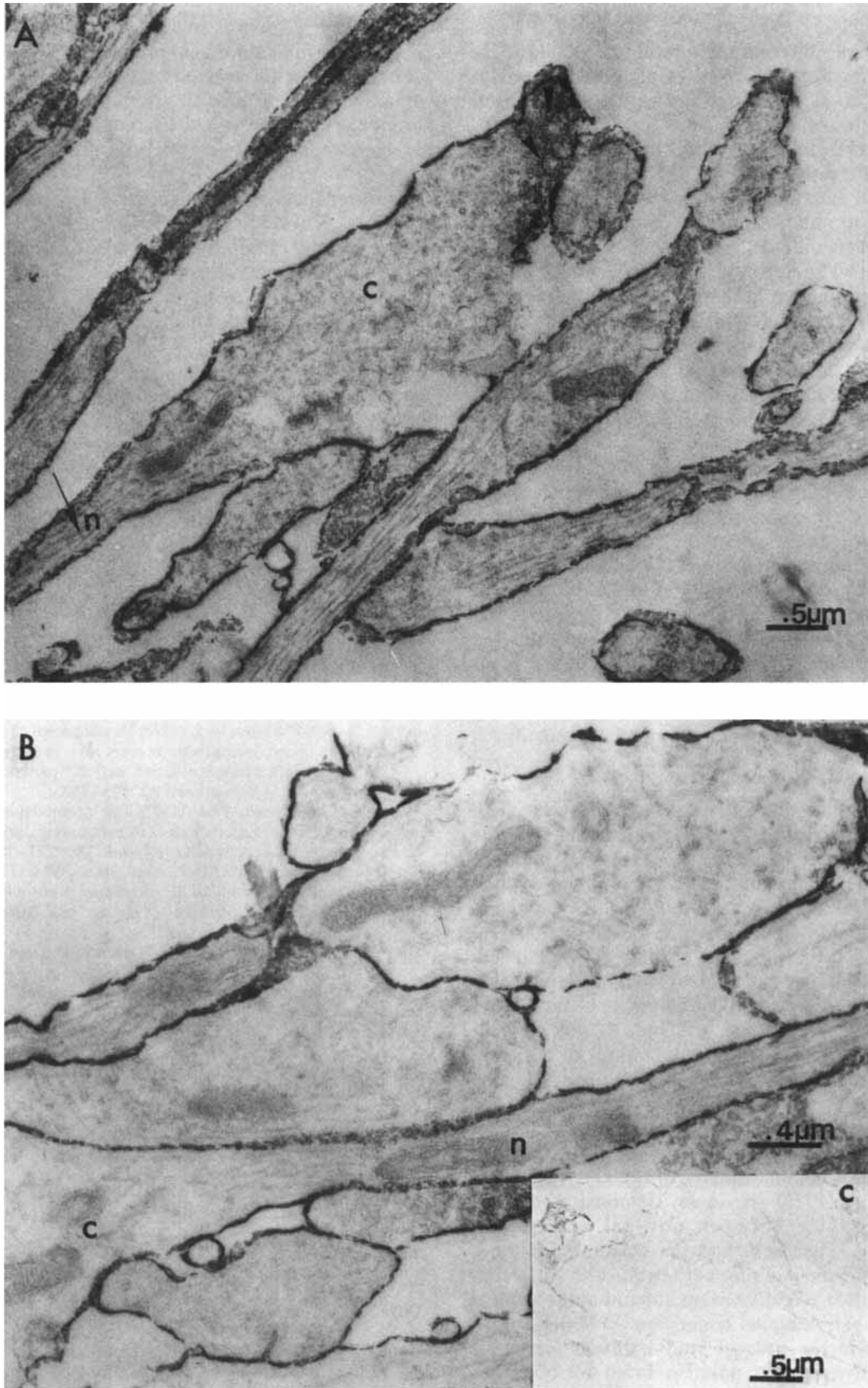

FIG. 5. A: Electron micrographs of neurites and their growth cones. After treatment with antisera against the goldfish catalytic subunit, intense staining is associated uniformly with plasmalemmal surfaces of the neurites (n) and growth cones (c). Note that the microtubules in the neurite (arrow) do not extend into the growth cone and that vesicles are seen at the tip of the partially damaged cone (arrowheads). Some of these vesicles appear stained. This section was counterstained with lead and uranyl salts to enhance contrast. ×19,250. **B:** Immunostaining with antisera against the goldfish catalytic subunit is associated with neurite (n) and growth cone (c) surfaces. ×28,500. **C:** Immunostaining is almost completely absent in controls treated with preimmune serum. ×16,428.

munoreactants through the axoplasm, which might be a limiting factor. Wood et al. (1977) have reported cisternal ATPase in the axoplasm in the region of the node.

Sweadner (1979) has reported that the phosphorylatable polypeptide of (Na^+,K^+)-ATPase derived from mammalian brain (but not from electroplax or non-nervous tissue) could be resolved on 6% polyacrylamide gels into two bands, designated α and $\alpha+$. The method for preparation of the goldfish immunogen in the present study employed 8.75% polyacrylamide gels (Schwartz et al., 1980) and would not distinguish these two proteins. The antisera thus might contain antibodies to both.

The nodal concentration of the enzyme in myelinated nerve (Figs. 1 and 2) and the continuous distribution in neurites (Figs. 4 and 5) were patterns seen with antisera raised against an enzyme devoid of $\alpha+$ (electroplax ATPase) as well as with antisera raised against the homologous brain enzyme, which presumably contains α and $\alpha+$ polypeptides. However, our findings do not permit conclusions concerning the possible localization of $\alpha+$, since there may be immunological cross-reactivity between α and $\alpha+$. Sweadner (1979) has speculated that the additional segment of M_r 2000 unique to $\alpha+$ is an anchor site that binds to nodal neurolemma. In order to distinguish α and $\alpha+$ and to further delineate their functions immunocytochemically, it would be useful to produce antibody directed against the M_r 2000 fraction.

The fact that the antiserum against (Na^+,K^+)-ATPase binds to both nodal neurolemma in the optic nerve and to the entire extent of glia-free neurolemma in explants suggests that the expression of the enzyme does not require the presence of myelin or glia. Whether the glia induce the discontinuity in distribution of the enzyme or whether periodicity develops in the neurons and demarcates sites of subsequent glial attachment is not determined in the present study. There is independent evidence that molecular specializations of the axon membrane at the nodes of Ranvier are not myelination-dependent (Ellisman, 1979). It remains possible that the contrast in distribution of the enzymes in the *in vivo* and *in vitro* preparations is an expression of impenetrability of the myelin.

Jean et al. (1976) previously demonstrated weak cross-reactivity between anti-eel electroplax (Na^+,K^+)-ATPase sera and the eel brain enzyme, using an enzyme inhibition criterion. The antiserum is nevertheless effective as an immunocytochemical stain in a heterologous tissue, the knifefish brain. As shown in the present study, this antiserum is also effective against goldfish brain by both immunocytochemical (Fig. 2) and radioimmunoassay techniques (Fig. 3). The results may indicate the inherent sensitivity of the immunocytochemical and radioimmunoassay approaches. Alternatively, the result may indicate that the rabbit anti-eel antisera contain a specific antibody against the eel electroplax catalytic site with low cross-reactivity to brain enzyme catalytic site in addition to antibodies of high cross-reactivity to other sites on the enzyme.

Further localization of functional components of the (Na^+,K^+)-ATPase will be greatly facilitated by the eventual availability of affinity-purified or monospecific antibodies.

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REFERENCES

Bertoni, J. B. and Siegel, G. J. (1978) Development of (Na^+,K^+)-ATPase in rat cerebrum: Correlation with Na^+-dependent phosphorylation and K^+-paranitrophenylphosphatase. *J. Neurochem.* 31, 1501–1511.

Bertoni, J. B. and Siegel, G. J. (1979) Development of (Na^+,K^+)-ATPase in rat hindbrain: increments in parallel with Na^+-dependent phosphorylation and K^+-paranitrophenylphosphatase. *J. Neurochem.* 32, 573–580.

Bostock, H. and Sears, T. A. (1977) The internodal axon membrane: Electrical excitability and continuous conduction in segmental demyelination. *J. Physiol.* 280, 273–301.

Broderson, S. H., Patton, D. L., and Stahl, W. L. (1978) Fine structural localization of K^+-simulated *p*-nitrophenylphosphatase activity in dendrites of the cerebral cortex. *J. Cell Biol.* 77, R13–R17.

Burrell, H. R., Heacock, A. M., Water, R. D., and Agranoff, B. W. (1979) Increased tubulin messenger RNA in the goldfish retina during optic nerve regeneration. *Brain Res.* 168, 628–632.

DiBona, D. R. and Mills, J. W. (1979) Distribution of Na^+-pump sites in transporting epithelia. *Fed. Proc.* 38, 134–143.

Elam, J. S. and Agranoff, B. W. (1971*a*) Rapid transport of protein in the optic system of the goldfish. *J. Neurochem.* 18, 375–387.

Elam, J. S. and Agranoff, B. W. (1971*b*) Transport of proteins and sulfated mucopolysaccharides in the goldfish visual system. *J. Neurobiol.* 2, 379–390.

Ellisman, M. H. (1979) Molecular specializations of the axon membrane at nodes of Ranvier are not dependent upon myelination. *J. Neurocytol.* 8, 719–735.

Ernst, S. A. and Mills, J. W. (1980) Autoradiographic localization of tritiated ouabain-sensitive sodium pump sites in ion transporting epithelia. *J. Histochem. Cytochem.* 28, 72–77.

Gaze, R. M. (1970) *The Formation of Nerve Connections.* Academic Press, London.

Guth L. and Albers R. W. (1974) Histological demonstration of (Na^+-K^+)-activated adenosine triphosphatase. *J. Histochem. Cytochem.* 22, 320–326.

Heacock, A. M. and Agranoff, B. W. (1976) Enhanced labeling of a retinal protein during regeneration of optic nerve in goldfish. *Proc. Natl. Acad. Sci. USA* 73, 828–832.

Heacock, A. M. and Agranoff, B. W. (1977) Reutilization of pre-

cursors following axonal transport of ^{3}H-proline-labelled protein. *Brain Res.* 122, 243–254.

Hunter, W. M. (1973) In *Handbook of Experimental Immunology* (Weir D. M., ed), Chap. 17. Blackwell Scientific Publications, Oxford.

Jacobson, M. (1978) *Developmental Neurobiology*. Plenum Press, New York.

Jean, D. H., Albers, R. W., and Koval, G. J. (1975) Sodium-potassium activated adenosine triphosphatase of Electrophorus electric organ 10. Immunochemical properties of the lubrol-solubilized enzyme and its constituent polypeptides. *J. Biol. Chem.* **250**, 1035–1040.

Jean, D. H. and Albers, R. W. (1976) Immunochemical studies on the large polypeptide of *Electrophorus* electroplax (Na^+, K^+)-ATPase. *Biochim. Biophys. Acta* **452**, 219–226.

Kyte, J. (1976) Immunoferritin determination of the distribution of (Na^+,K^+)-ATPase over the plasma membranes of renal convoluted tubules. I. Distal segment. *J. Cell Biol.* **68**, 287–303.

Landreth, G. E. and Agranoff, B. W. (1979) Explant culture of adult goldfish retina: A model for the study of CNS regeneration. *Brain Res.* **161**, 39–53.

Langley, O. K. (1979) Histochemistry of polyanions in peripheral nerve, in *Complex Carbohydrates of Nervous Tissue* (Margolis R. U. and Margolis R. K., eds), pp. 193–207.

Ritchie, J. M. (1979) A pharmacological approach to the structure of sodium channels in myelinated axons. *Annu. Rev. Neurosci.* **2**, 341–362.

Rusoff, A. C. and Easter, S. S. (1979) Order in the optic nerve of goldfish. *Soc. Neurosci. Abstr.* **5**, p. 576.

Schwartz, M., Novick, D., Givol, D., and Fuchs, S. (1978) Induction of anti-idiotypic antibodies by immunization with syngeneic spleen cells educated with acetylcholine receptor. *Nature* **273**, 543–546.

Schwartz, M., Feldman, E. L., Jean, D. H., Siegel, G. J., and Agranoff, B. W. (1979) Na-K-ATPase in the regenerating goldfish visual system. *Trans. Am. Soc. Neurochem.* **10**, 233.

Schwartz, M., Siegel, G. J., Chen, N., and Agranoff, B. W. (1980) Goldfish brain (Na^+,K^+)-ATPase: Purification of catalytic polypeptide and production of specific antibodies. *J. Neurochem.* **34**, 1745–1752.

Siegel, G. J., Stahl, W. L., and Swanson, P. D. (1981) Ion transport, in *Basic Neurochemistry*, 3rd Ed. (Siegel G. J., Albers R. W., Agranoff B. W., and Katzman R. K., eds). Little, Brown, Boston, in press.

Stahl, W. L. and Broderson, S. H. (1976) Localization of Na,K-ATPase in brain. *Fed. Proc.* **35**, 1260–1265.

Sternberger, L. A. (1974) *Immunocytochemistry*. Prentice-Hall, Englewood Cliffs, New Jersey.

Sweadner, K. J. (1979) Two molecular forms of (Na^+,K^+)-stimulated ATPase in brain. Separation, and difference in affinity for strophanthidin. *J. Biol. Chem.* **254**, 6060–6067.

Wood, J. G., Jean, D. H., Whitaker, J. N., McLaughlin, B. J., and Albers, R. W. (1977) Immunocytochemical localization of sodium, potassium ATPase in knifefish brain. *J. Neurocytol.* **6**, 571–581.