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THE RETINA AS A BIOCHEMICAL MODEL OF CENTRAL NERVOUS SYSTEM REGENERATION

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ABSTRACT

The visual system of primitive vertebrates has long served as a useful model for the resynthesis of damaged neurons. The prior optic nerve crush imparts a marked tendency on the part of cultured retinal explants to extend neurites. Neurites grown in culture from retinal ganglion cells of the explant have been characterized by lectin-binding and immunohistochemical techniques. Biochemical studies on the retina following optic nerve crush reveal altered RNA and protein metabolism. Results indicate that tubulin mRNA is activated and that new tubulin synthesis is enhanced following the crush. An early and dramatic increase in the rate of nucleotide phosphokinase activity is also seen. The retinal tissue culture data, together with in vivo studies in normal and newly regrown nerve give additional insight into the nature of the regenerative process.

KEYWORDS

Fiber guidance; nerve growth; recognition; specificity; tissue culture; lectin binding; acetylcholine receptor; nucleotide metabolism.

INTRODUCTION

It is probably fair to say that much of our interest in the retina stems from the fact that its ganglion cells send visual information to the brain which can then be processed in a way that is of biological significance to the organism. Important gains have been made in understanding the molecular basis of sensory transduction in the retina, but we can at present say little regarding how its connection to the brain arises during development or how its reconnection following injury is mediated in those species that have this ability. In addition to the intellectual challenge of the problem, there is potentially great practical value in further understanding in what ways those species that cannot support CNS regeneration (including man) differ from those that can. For all of these reasons, the biochemical events that mediate the response of the visual system of lower vertebrates to injury and that lead to recovery of function warrant scrutiny. No attempt will be made here to review exhaustively the vast neurobiological literature that serves as background for this topic. Rather, current findings will be discussed in the

context of viable hypotheses. In vivo studies, primarily electrophysiological and anatomical in nature, will be compared and contrasted with in vitro studies, primarily at the molecular level. Relevant properties of the ganglion cell growth cone are presented and illustrated with current experimental findings from our laboratory.

DEVELOPMENT AND REGENERATION

There are both differences and similarities between de novo development of the visual pathway and regeneration of the adult optic nerve following injury. In both instances, ganglion cells must recognize the correct addresses to which presynaptic terminals must ultimately be assigned. How the ganglion cell fibers are guided to the tectum, however, may be quite different in the two conditions. Within the developing brain, cell replication, migration and axon extension take place concurrently. This is not the case in regeneration, where the pre-existing scaffolding may play an important role in the guidance of regrowing fibers. Significant neuroanatomical and electrophysiological contributions have come from many laboratories (e.g., Weiss, 1966; Sperry, 1966; Gaze, 1970; Jacobson, 1978). In initial experiments, the course of regeneration following axotomy in teleosts and amphibians was studied. Later, various surgical manipulations of both eye and brain were employed to ask more complex questions of the in vivo preparations. The past decade has been marked by extensions and refinements of the basic studies, largely occasioned by the availability of new histochemical tools, such as ^{3}H-labeled proline for anterograde tracing of axonally transported proteins by radioautography (Neale, Neale and Agranoff, 1972; Jones and Hautman, 1978) and of horseradish peroxidase (HRP) (LaVail, 1975; Bunt, Lund and Lund, 1974) primarily for retrograde tracing. It has been demonstrated by combinations of morphological (including histochemical), electrophysiological and behavioral studies, that in the goldfish, regeneration of fibers from an eye from which one half of the retina has been removed results in innervation of only that part of the tectum that would ordinarily have been innervated by the hemi-retina (Jacobson, 1978; Edds and coworkers, 1979). With the passage of time, the optic nerve fibers extend to cover the entire tectum with the partial visual field. Similarly, if an entire retina regenerates to a partially ablated tectum, there is an orderly compression with the net result that the retinal projection is represented on the remaining tectum. One may thus use experimental evidence to conclude that either the retina, the tectum, or the visual system as a single entity exhibit properties of respecification during regeneration. What can be said with some certainty is that the rules for formation and maintenance of connections are dynamic and complex and that there appears to be much inherent redundancy in the mechanisms of specification, since the regenerating subject can overcome a variety of imposed obstacles. While much has been learned from interventive experiments, as this approach becomes more remote from the physiological mechanism of specification, it becomes less likely to shed additional light on its basis.

A somewhat different approach toward understanding the ways in which retinotectal connection and reconnection occur is the consideration of an important hallmark of the growing axon - the growth cone. Both in vivo and in vitro, the growing axon extends itself in a rather smooth translational fashion, while at its extending tip, hair-like filopodia actively thrash about in a seemingly random fashion, sampling its microenvironment in a surround that encompasses several axon diameters. The growth cone may play roles in guidance of fibers to the target region as well as in the process of selective respecification, once initial contact has been made. What is the mechanism by which the growth cone accomplishes its purpose? Does it "taste" or does it "smell" its way to its target? By this we mean, does it sense macromolecular clues at short ranges (e.g., recognition sites on membranes), or does it sense a spatial gradient of a diffusible substance that is operative at great distances? For example, nerve growth factor can be shown to stimulate a vectorial

growth pattern in vitro (Campenot, 1977). Experimental in vivo evidence can be marshalled for either possibility. In favor of the short range argument is the observation that regenerating fibers are guided along tracts of degenerating ones, even if they are inappropriate. An example is found in the regenerating teleost visual system. Axons of the goldfish retina normally cross to reach the contralateral tectum. If one eye of the goldfish is removed, and the other is crushed intraorbitally, so that both tecta have been denervated, a majority of regrowing fibers from the remaining eye (traced by ^{3}H-proline radioautography) regrow to the contralateral optic tectum as expected. However, a significant number of fibers, upon reaching the chiasm, appear to sense degenerating fibers of the enucleated eye, and leave the chiasm along the ipsilateral optic tract to innervate the ipsilateral tectum (Sharma, 1973; Springer and co-workers, 1977). If an optic nerve is crushed and the opposite eye is not disturbed, the ipsilateral tract is not formed. It is reported that following unilateral optic nerve crush in the frog, some regenerating optic nerve fibers will enter the chiasm, cross and grow rostrally, to follow fibers of the opposite eye into the retina (Bohn and Stelzner, 1979). In this instance we must imagine that the regrowing fibers recognize optic nerve by local clues and enter the existing tract, albeit in a reverse and seemingly not useful direction. It has been shown in a number of ways that outgrowing fibers from ectopically placed eyes in developing amphibians will find their way to the tectum (Sharma, 1972; Constantine-Paton and Capranica, 1976). Evidence in this case is seemingly in support of the existence of long-range gradients, but even here there may be elements of short-range recognition: the ectopic optic nerves have been claimed to enter the Rohon-Beard tracts of the spinal cord (Katz and Lasek, 1978; Giorgi and VanderLoos, 1978). If confirmed, the result would suggest that the visual efferent fibers detect a sensory tract "conduit". A recent observation bears directly on this issue. Singer has shown that growing spinal cord fibers in developing Xenopus are preceded by channels in the ependyma (Singer, Nordlander and Egar, 1979). This finding together with those of other laboratories, lends credence to Singer's "blueprint" hypothesis which proposes that the guidance apparatus is the result of neither afferent fiber nor target influences but rather of interposed nonneuronal elements. It remains possible that the channels are the result of an inductive mechanism generated from the advancing growth cone.

The nature of the preexisting trace pathways required for Singer's hypothesis is presently unknown, but its existence may be related to recent studies in which Jacobson traced cell lineage in the developing Xenopus embryo. Injection of HRP into a single cell at the blastomere stage serves as a "fate stain", (Jacobson and Hirose, 1978) since daughter cells bear the same marker. An analogous technique is somatic crossing-over currently being used successfully in studies on Drosophila development (Kauffman, Shymko and Trabent, 1978). The results have altered classical concepts of neurogenesis and may in fact lead to changes in how we view the neuroanatomical organization of the brain. Examination of histological sections of the retina of a tadpole that had been injected with HRP at the two-cell stage reveals the presence of the marker in one half of the embryo's cells. On the labeled side, the brain and most of the eye are also marked. An exception is a small unlabeled ventromedial portion of the retina and diencephalon. Correspondingly, only these structures are labeled on the otherwise unlabeled side. The result indicates that during development, at least two neuroblasts have exchanged position across the midline and that their progeny have given rise to a segment of retinal cells via the optic stalk. Jacobson suggests that the translocation of these "founder" cells results in the establishment of an "incipient chiasma". We can infer from the results that these contralateral progeny send axons back to the brain via trace markers and thus guide neighboring ipsilateral retinal cell axons to the opposite tectum.

Growing nerve fibers tend to form bundles. In Daphnia (Lopesti, Macagno and Levinthal, 1973), the invertebrate ommatidium generates a lead fiber during development

that in turn guides the surrounding axons to the brain. This finding bears resemblance to recent studies in goldfish brain. As the retina continues to grow during adult life, new cells are added at the periphery (Johns, 1977). The new ganglion cell fibers grow toward the optic disc in a spoke-like fashion, unite and travel to the optic tectum in packets (Rusoff and Easter, 1979). Upon reaching the tectum, they then "disembark" and proceed to their various tectal addresses in accordance with the existing two-dimensional map, which may be roughly described as a superimposition of the retinal nasotemporal gradient upon the tectal rostrocaudal axis on the opposite side. The timed basis of optic nerve fascicle organization then reduces the importance of maintenance of neighboring retinal ganglion cell relationships by their axons within the optic nerve in arriving at the final retinotectal map. The result does not preclude the existence of a retinotectal map based on chemical gradients. Nearest neighbor relationships within a given retinal annulus that gives rise to an optic nerve fascicle are in fact preserved. The finding suggests however that the Cartesian x-y coordinate system so favored in speculations regarding retinotectal specification should now be replaced by a system of polar coordinates in which rho, the distance of a ganglion cell from the optic disc, is correlated with the cell's age, while omega expresses its sector.

Developments in cell biology provide insights that begin to point to the kinds of molecular codes and signals that might be required. In terms of their chemical nature, many biochemists favor involvement of glycoproteins and glycolipids, which together constitute the external glycocalyx of the cell (Roseman, 1974). What is the basis of this prejudice? It probably derives partly from our knowledge concerning immunological recognition, a phenomenon in which precise selection mechanisms operate within an immensely diverse population of molecules, and in which carbohydrates figure prominently. In the plant world, symbiotic plants and bacteria seek one another out by means of lectins, proteins whose function is to bind surface carbohydrate moieties. Evidence for the existence of lectins in animal cells has been put forth (Nowak, Haywood and Barondes, 1974). Inferential support comes from *in vivo* experiments in which it can be shown radioautographically that newly synthesized glycoprotein is inserted in the region of the synapse (Bennett and co-workers, 1973), or at the growth cone (Tessler, Autilio-Gambetti, and Gambetti, 1977). Why carbohydrates should be particularly suited for recognition functions may derive from the large variety of possible glycosidic bonds between two sugars. In addition to two or three available functional groups per residue (compared with but one per amino acid in proteins), there is also the possibility of an alpha or beta linkage, branching, etc. Furthermore, while protein synthesis is stringently prescribed in the nucleus, carbohydrate modification of proteins is post-translational, and both glycoproteins and glycolipids could be modified by ectoenzymes, permitting the mediation of intercellular interactions at a distance from the nucleus. These and other arguments support the involvement of carbohydrate-mediated recognition codes in development, but at present, the issue must be regarded as speculative.

IN VITRO STUDIES

Single cells, dissociated from undifferential retina, can reaggregate into vesicles with stratified layers that bear resemblance to the retinal laminae seen *in vivo* (Sheffield and Moscona, 1970). A similar comparison has been proposed between layers seen in reaggregates of cerebellar cells and those seen *in vivo*. Cerebellar cells obtained from a genetically ataxic mouse strain (reeler) fail to exhibit the ability to form the layered appearance (DeLong and Sidman, 1970). These model systems suggest that there may be a role for self-assembly at the cellular level in development. Retinal elements other than ganglion cells possess recognition functions, and dissociated retinal cells do in fact exhibit evidence of temporal and spatial coding. For example, aggregation of retinal cells is blocked by diffusible factors obtained from retinal extracts, the age of which correlates with the age of

retinal cells optimally stimulated to aggregate (Merrell, Gottlieb and Glaser, 1975). Dissociated retinal cells thus can provide useful information that may enhance our eventual understanding of retinotectal coding. A convenient source of cells for such studies is the chick retina since it yields large numbers of viable cells following mild disruptive procedures. After labeling, for example by brief incubation with $^{32}P_i$, probe cells obtained from retinal sectors can be used in binding studies with coated beads or fibers, with monolayer cultures, or with full thicknesses of optic tectum (Marchase, Voxbeck and Roth, 1976). Such experiments have in common that they assume that the dissociated retinal probe cells will express recognition properties that relate to specification in vivo. One could imagine a rather stringent set of rules which would preclude use of this approach for studying retinotectal recognition. It might be, for example, that only fully differentiated ganglion cells recognize tectal sites, that receptors are present only at the growing tip of the axon and perhaps only at a specific time. Furthermore, expression of the recognition site might require the presence of an array of appropriate fibers of neighboring cells. That the rules of retinotectal specificity are more relaxed than outlined above is evident from experimental findings. Binding of cells from various retinal sectors to predicted tectal sites has been reported (Barbera, 1975) and interpreted to reflect a gradient of glycosyl transferases in the retina forming the basis of a chemoaffinity mechanism (Marchase, Vosbeck and Roth, 1976). The importance of these findings to in vivo development awaits extension of the initial observations. It remains uncertain whether dissociated cells can demonstrate properties that reflect recognition or guidance mechanisms. A model which begins to bridge the gap between the cell and the organism is the tissue explant, since some developmental history and structure are preserved in an in vitro system. The seemingly straightforward experiment of putting a piece of retina and tectum together in a culture dish, however, turns out to be an experimentally difficult task. It was noted some time ago by Weiss (1934) that neurites growing out from explants tend to grow into one another regardless of the source the so-called "two-center effect". Metabolic gradients, mechanical stresses within the three-dimensional substratum, etc., may explain the basis of this artifact (Dunn, 1971). Were a retinal explant to invade a tectal explant in vitro in a manner that retained properties of specificity it could be a problem to demonstrate clearly that it had. Up to the present, it has been difficult to label an explant unambiguously in a way that would permit tracing of its fibers within a target explant. The lack of success of this direct approach may also reflect the fact that conditions for optimizing neurite outgrowth in vitro are quite unphysiological and probably unsuitable for the evocation of physiological recognition mechanisms. For example, according to Singer's blueprint hypothesis, neurites are guided by ependymal cells, yet in vitro, we generally grow neurites out on an unstructured substratum - unfamiliar territory. The explant preparation has nevertheless permitted us to characterize some of the properties of the outgrowing neurite, since we can examine its surface and its growing tip in the absence of the contiguous supporting cells present in the optic nerve.

THE EXPLANTED GOLDFISH RETINA

While explants and dissociated primary cell culture are routinely performed with embryonic tissues, our laboratory has been examining outgrowth from explants of adult goldfish retina. Like adult tissues from higher vertebrates, explants from the goldfish retina do not ordinarily support outgrowth, but if a conditioning lesion of the optic nerve is made 1-2 weeks before explantation, we observe excellent outgrowth of neurites. We have demonstrated elsewhere (Johns, Heacock and Agranoff, 1978) that this outgrowth derives from ganglion cells, and that early neurites, seen after 1-2 days in vitro, appear on the edge of the explanted fragment closest to the optic disc of the retina from which they had come (Johns, Yoon and Agranoff, 1978) (Fig. 1).

A marked tendency for neurites from our explants to form fascicles and for the fascicles to grow out onto the substratum in a clockwise fashion is seen (Fig. 2) (Heacock and Agranoff, 1977).

This result has been interpreted to indicate that the outgrowing fibers have a helical nature. Spiralling of fibers can be seen within fascicles (Fig. 3), but it is questionable whether it is characteristic of single neurites grown out from ganglion cells.

Spiralled fascicles may have significance in vivo; the lead fiber and its complement of fibers together form a more coherent cable than the individual neurites, and the spirality assures that mechanical flexion of the nerve will not unravel single fibers. In chick spinal ganglia, fasciculation appears to be mediated by a neuronal protein, the so-called "cell adhesion molecule" (CAM) (Rutishauser, Gall and Edelman, 1978) and it has been proposed that fasciculation may play a role in cell recognition or in fiber guidance. Fasciculation has also been implicated in ganglion cell axon guidance in the mouse retina (Goldberg and Frank, 1979).

Explant culture has also permitted examination of the neurite membrane in the absence of oligodendroglia and other cellular elements. Light microscopic and ultramicroscopic studies with lectins have indicated that alpha-D-mannose, D-galactose and N-acetyl-D-glucosamine are prominent in the neurite membrane surface. It is possible that the galactose is in a glycolipid, while the N-acetyl-D-glucosamine is more likely to be part of a glycoprotein (Feldman, Heacock and Agranoff, 1978).

We have also studied the presence of proteins characteristic of excitable membranes. Antibodies to electroplax Na^+-K^+ ATPase and to acetylcholine receptor (AChR) were available (courtesy of Drs. R.W. Albers, NIH, and S. Fuchs, Weizmann Institute, respectively), and immunohistochemical studies in our laboratory have indicated that goldfish brain can react with each of the rabbit antisera. Immunohistochemical studies both in vivo and in explant culture indicate the presence of the enzyme (Schwartz and co-workers, 1979a) and receptor (Schwartz and co-workers, 1979b) in optic nerve fibers. An HRP-linked indirect method was used to localize the AChR, which appears to be present in optic nerve fibers (Fig. 4).

The presence of ATPase in the nerve membrane was anticipated (Wood and co-workers, 1977), while the presence of AChR was not, since the latter is generally thought to exist in areas of synaptic specialization on the postsynaptic surface. However, AChR has in fact been found in axonal membranes of invertebrates (Marquis, Hilt and Mautner, 1977). The question of its possible role in conduction will have to be considered further. The finding also underlines that care be exercised in interpretations of experiments purported to elucidate the nature of the neurotransmitters of the goldfish retinal ganglion cell (Schechter and co-workers, 1979; Oswald and Freeman, 1977).

The explant has also served for studies on membrane addition during neurite extension. We used a lectin-anti-lectin complex to mark the neurite membrane in a fashion which prevents diffusion of the marker, yet does not block outgrowth. Neurites were tagged with Concanavalin A (Con A) followed by rabbit anti- Con A, then permitted 24 h of additional growth. The preparation was then reacted with fluorescent goat anti-rabbit IgG. By these means we have been able to establish that new membrane originates at the growth cone (Feldman and co-workers, 1979) (Figure 5).

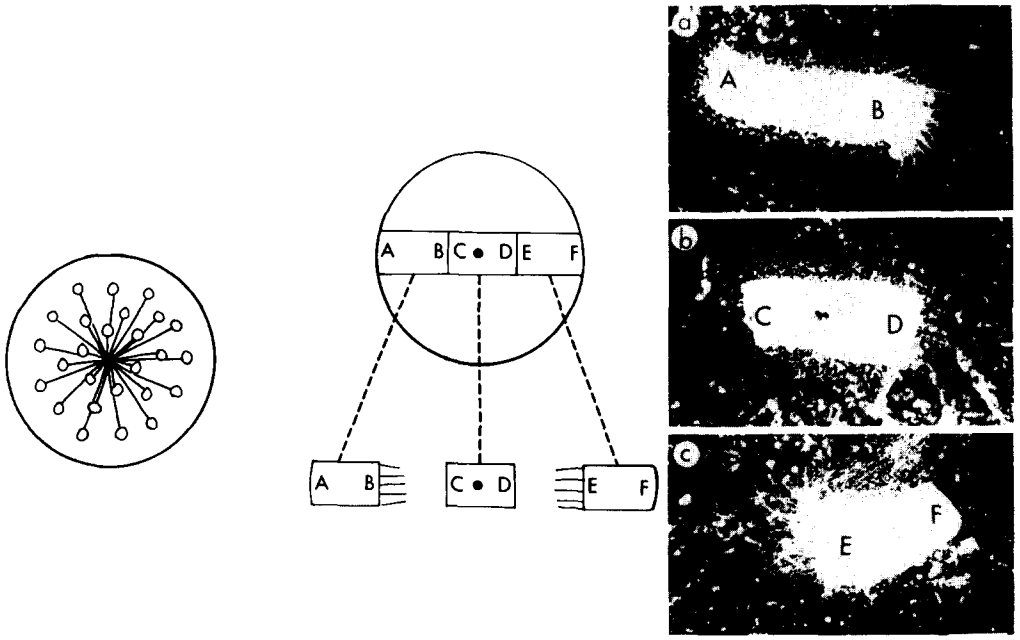

Fig. 1. Directed growth of goldfish retinal neurites. Diagram on left shows radial pattern of optic fibers in the retina. In the center, the retina is shown as it was prepared for explantation. A strip of retina that included the optic disc was divided into three approximately equal pieces and then explanted. If the neurites maintained their centripetal orientation (towards the disc), they would grow out from the ends labeled B and E. The results of such an experiment are illustrated on the right. From Johns, Yoon and Agranoff, 1978.

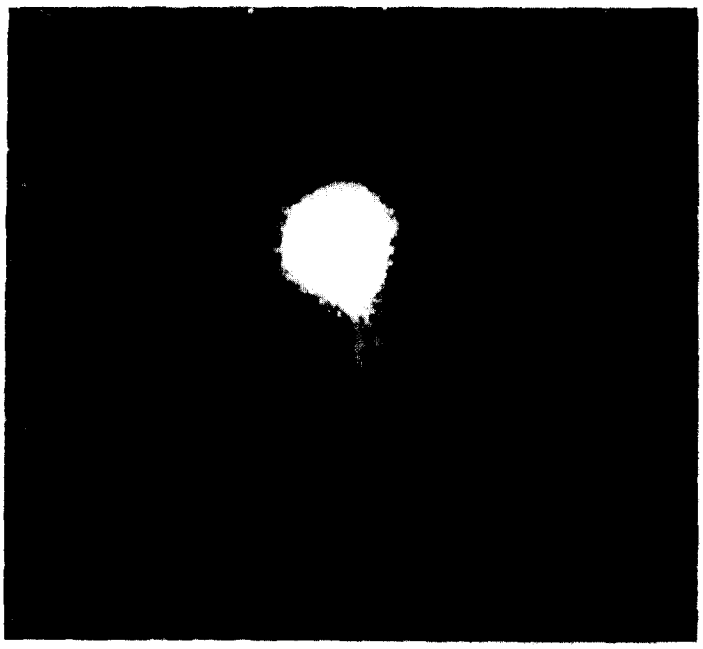

Fig. 2. Clockwise growth of retinal neurites. Dark-field photomicrograph of goldfish retinal explant on a polylysine-coated substratum.

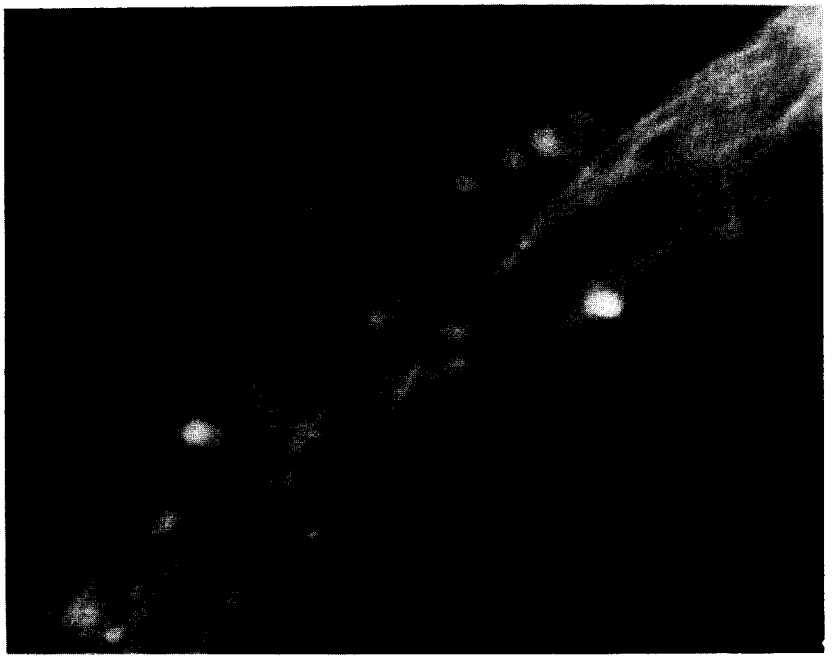

Fig. 3. Spiralling of fibers within a neurite fascicle. Fluorescence photomicrograph of a neurite fascicle stained with the lipophilic dye, dioctadecylindocarbocyanine.

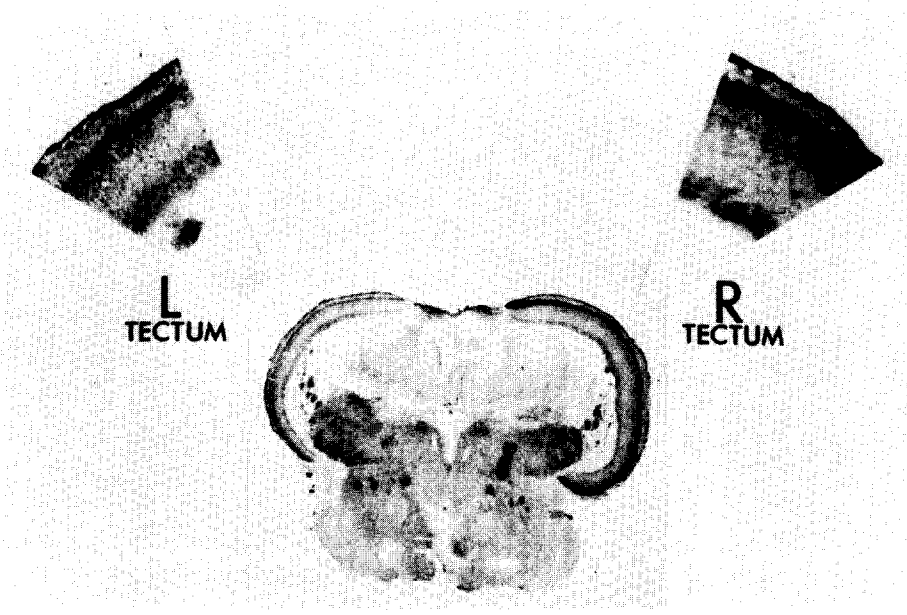

Fig. 4. Effect of eye enucleation on acetylcholine receptor antigenic sites in the tectum. Immunoperoxidase staining for AChR in goldfish brain section 3 months after enucleation of right eye.

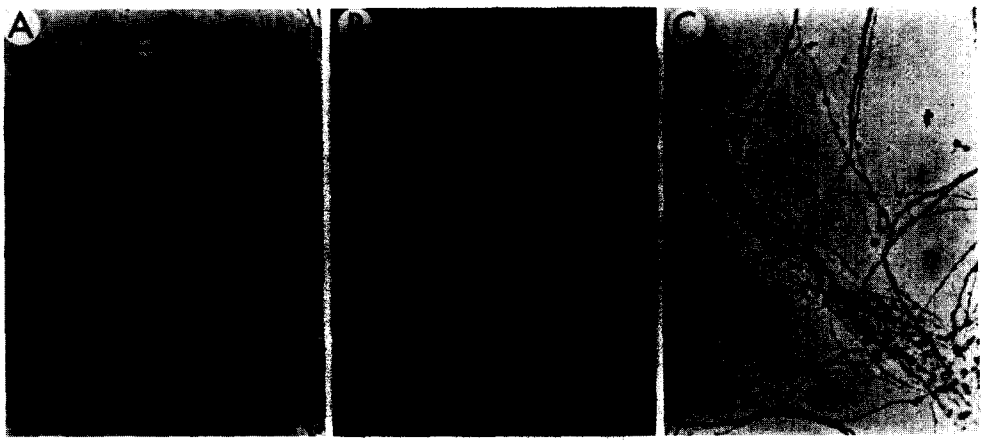

Fig. 5. Lectin binding identifies the site of new membrane addition at the growing tip. Explants were incubated with Con A (25 ug/ml) then anti-Con A (100 ug/ml). Twenty-four hours later the lectin bound portion of the neurite membrane was visualized by fluorescence microscopy following treatment with rhodamine-labeled goat anti-rabbit immunoglobulin (200 ug/ml). Extent of growth was monitored by phase microscopy.
A. Phase photomicrograph of neurites taken at zero time.
B. Fluorescence photomicrograph of same field taken 24 h later.
C. Phase photomicrograph of same field as in B.

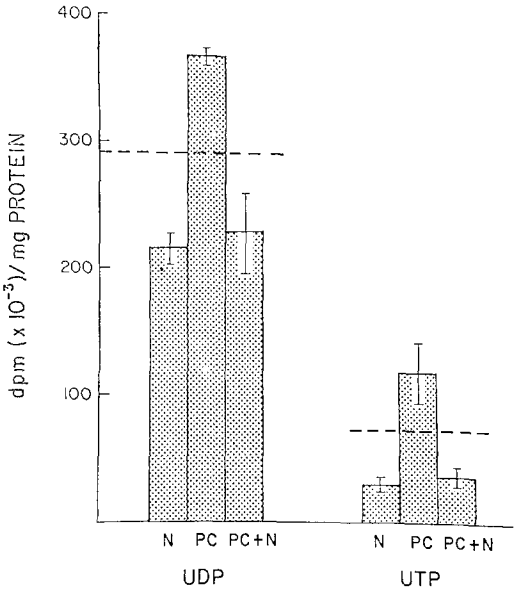

Fig. 6. Characterization of enhanced uridine nucleotide kinase activity in day 3 post-crush post-mitochondrial supernatants, by means of a mixing experiment. Retinal post-mitochondrial supernatants were prepared and aliquots were incubated with 2 uCi of [^{3}H]orotic acid, PPRP and ATP, and an average of 110 ug protein from 3 day PC(post-crush), N (normal) or a mixture of PC and N preparations. The labeling of JDP and UTP was determined by high voltage electrophoresis. Each bar is the average dpm/mg protein ± SEM in each nucleotide from 3 assays of each preparation. PC+N assays containing equal proportions of PC and N enzyme preparations. The dotted line represents the expected dpm/mg protein in UDP or UTP numerical averaging of PC and N preparations. The result suggests that an inhibitory substance is normally present in retina, but is inactive or absent in the retina following optic nerve crush (From Dokas, Burrell and Agranoff, 1979).

Whether the new membrane protein migrates by axonal transport and is inserted at the growth cone or whether post-translational alterations, including carbohydrate addition, occurs at the time of externalization, is not presently known.

BIOCHEMISTRY OF THE RETINA DURING REGENERATION

As stated earlier, higher vertebrates do not regenerate the visual system, and hypotheses to explain the failure include intrinsic differences among species such as inability of the cut axon to initiate the necessary perikaryal repair mechanism in the cell body, and extrinsic mechanisms, such as the formation of an impassable glial scar (Lieberman, 1971; Grafstein, 1975). Some light on biochemical events that accompany axotomy may be shed by experiments in our laboratory in which control retina (from the unoperated eye) has been compared with post-crush retina at various times following the lesion in the goldfish. We found an increase in labeling of tubulin following crush (Heacock and Agranoff, 1976) and more recently, an increase in mRNA for tubulin in post-crush retina (Burrell and coworkers, 1979). The earliest biochemical event that we have thus far detected is an increase in retinal nucleotide phosphorylation that occurs 2-3 days after crush (Dokas, Burrell and Agranoff, 1979). Results in the latter studies are consistent with the hypothesis that an inhibitory substance normally present is reduced following crush (Fig. 6).

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