

EVALUATION OF [³H]PROLINE FOR RADIOAUTOGRAPHIC TRACING OF AXONAL PROJECTIONS IN THE TELEOST VISUAL SYSTEM

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SUMMARY

The efficacy of [³H]proline radioautography for tracing retinal ganglion cell projections to the optic tectum of the jewel fish, *Hemichromis bimaculatus*, has been compared with that of degeneration techniques. There was good agreement between the various methods. Retinal projections to the optic tectum of two other teleosts, the oscar, *Astronotus ocellatus*, and the goldfish, *Carrasius auratus*, were examined radioautographically.

In addition to conventional methods of analysis, radioautograms were scanned in a slit microdensitometer and by an automated isodensity scanning system. Results of studies with the protein synthesis inhibitor, cycloheximide, are compatible with the suggestion that axonally transported proteins labeled with [³H]proline may release diffusible precursors that are reincorporated into protein in adjacent regions.

The possible advantages and limitations of radioautography of [³H]proline-labeled axonally transported protein in brief or extended studies are discussed in terms of the results obtained in the teleost visual system.

INTRODUCTION

Proteins synthesized in neuronal perikarya move within axons to nerve endings by the process of axonal transport⁵³. When nerve cells are provided with radioactive amino acids, their axons and terminals become radiolabeled with these transported proteins⁵¹, whose intracellular distribution in radioautograms constitutes a useful method for tracing neuroanatomical pathways³⁴.

Since the visual system in teleost fishes appears to be completely crossed, it serves as a suitable model for studies of axonal transport^{42,43}. In such studies, labeled amino acids which have been introduced into the eye are incorporated into proteins in retinal ganglion cells and are transported within the optic nerve to brain regions contralateral to the injected eye. Brain regions ipsilateral to the injection site may then be utilized to control for incorporation of labeled precursor that has escaped from the eye and reached the brain from the blood.

When [³H]leucine is used as the precursor, the ratio of labeled protein in the contralateral to that in the ipsilateral tectum is 2:1, indicating the presence of axonally transported protein in the contralateral tectum in addition to systemically labeled protein in both tecta^{23,38}. The degree of 'background' labeling due to the systemic incorporation of [³H]leucine into the brain, however, limits its use for radioautographic studies. Biochemical studies with 18 labeled amino acids indicated that asparagine and proline produced a high ratio of contralateral-ipsilateral tectal radioactivity and would serve as better precursors for radioautography in the nervous system^{17,18}. In subsequent studies, we established the suitability of [³H]proline as a radioautographic tool using the goldfish visual system⁴¹, and determined that the physiological basis of its superiority was its efficient incorporation into protein in the eye as well as its relatively poor incorporation into the brain via the blood⁴⁰.

In the present report, our study of [³H]proline radioautography in the visual system has been extended and validated by comparison with degeneration techniques. The potential error in the radioautographic method attributable to the presence of unincorporated labeled proline in the contralateral optic tectum^{17,40} was investigated by the combined use of intraocular injection (IO) of high specific activity [³H]-proline, and inhibition of protein synthesis with intracranial (IC) injection of cycloheximide (CXM). Since [³H]proline-labeled transported protein has a long apparent half-life⁴⁰, the present report considers the distribution of radioactivity in the brain up to 240 days after injection.

This study was restricted to the teleost visual system, where the neuroanatomy of retinal projections to the brain is of interest from developmental^{21,27} and comparative¹⁵ perspectives. Since the use of [³H]proline has been extended to numerous species^{2,6,8,24,29,34}, the results reported here may, accordingly, have wider relevance.

MATERIALS AND METHODS

Specimens of *Hemichromis bimaculatus* Gill 1862 (jewel fish) and *Astronotus ocellatus* Cuvier 1829 (oscar), weighing 7–11 g, were purchased locally and maintained in 30 gallon tanks at 28 ± 1 °C. Goldfish (*Carassius auratus*) were obtained from Ozark Fisheries (Stoutland, Missouri) and maintained at 20 ± 1 °C. Tricaine methanesulfonate (MS-222, Finquel) was purchased from Ayerst Labs (New York, N.Y.), labeled amino acids from New England Nuclear (Boston, Mass.) and cycloheximide (Actidione, CXM) from Nutritional Biochemical Corporation (Cleveland, Ohio).

Degeneration

Nine specimens of *Hemichromis* were subjected to unilateral enucleation under MS-222 anesthesia (50 mg/liter). After 5–25 days the animals were killed under anesthesia by transcardial perfusion with 10% formalin. The brains were removed, fixed in 10% formalin for at least one week and embedded in gelatin. Frozen sections were cut at 25 μm in transverse, sagittal or horizontal planes, and were subsequently processed by modifications of the Nauta¹⁶ and Fink–Heimer²⁰ procedures for the demonstration of degenerating axoplasm.

Radioautography

Four specimens of *Hemichromis* were processed for radioautography following IO injection of 25 μCi of L-[4,5-³H]proline, 46 Ci/mmol, with a 50 μl Hamilton syringe and 30-gauge needle fitted with a polyethylene sleeve which limited penetration into the eye to 2 mm. The routine injection volume was 5 μl . Aqueous radioactive solutions were evaporated to dryness with nitrogen to remove ³H₂O and redissolved in 0.15 N NaCl immediately before use. Intraocular injections were similarly administered to the specimens of *Carassius* and *Astronotus* used in this study.

Radioautographic procedures were essentially those described by Kopriva and Leblond³². Brains were fixed in Bouin's solution for at least 48 h prior to dehydration and embedding in paraffin. The tissue was sectioned at 8 μm . Kodak NTB3 nuclear track emulsion was diluted 1:1 with distilled water at 40 °C, and slides with sections were dipped into emulsion and dried for approximately 1 h. Slides were stored in light-tight boxes with 10–15 g of Drierite at 4 °C for 7–35 days. The exposed sections were developed in Kodak Dektol for 3 min at 16 °C, washed briefly in 1% acetic acid, fixed in Kodak Fixer for 6 min and in some cases stained with hematoxylin–eosin.

Goldfish were routinely prepared as indicated above; however, in one experiment (see Fig. 4), 12 goldfish were perfused with a solution containing 2% paraformaldehyde, 2.5% glutaraldehyde and 0.05% calcium chloride in 0.14 M sodium cacodylate at pH 7.5. The optic tecta were removed, and a 1 mm thick cross-section was cut midway through each tectum. These slices were immersed in fresh fixative, rinsed, and postfixed in chrome osmium⁹. After *en bloc* staining in uranyl acetate, the tectal slices were dehydrated and cut into quarters before final flat-embedding in Epon. Tectal cross-sections, 1 μm thick, were cut with glass knives using an LKB Ultratome III, and were mounted with heat on glass slides, which were then coated with emulsion, exposed and developed as described above. The dried slides were heated and the sections stained briefly with 0.1% toluidine blue in 1% sodium borate.

Microdensitometry

Bands of silver grains in unstained radioautographs of optic tectum were scanned with a microdensitometer. A Leitz Ortholux binocular microscope was fitted with a densitometer tube and a photomultiplier tube whose output was recorded on a Sargent recorder. A quartz-iodide light source was regulated by a Variac. The

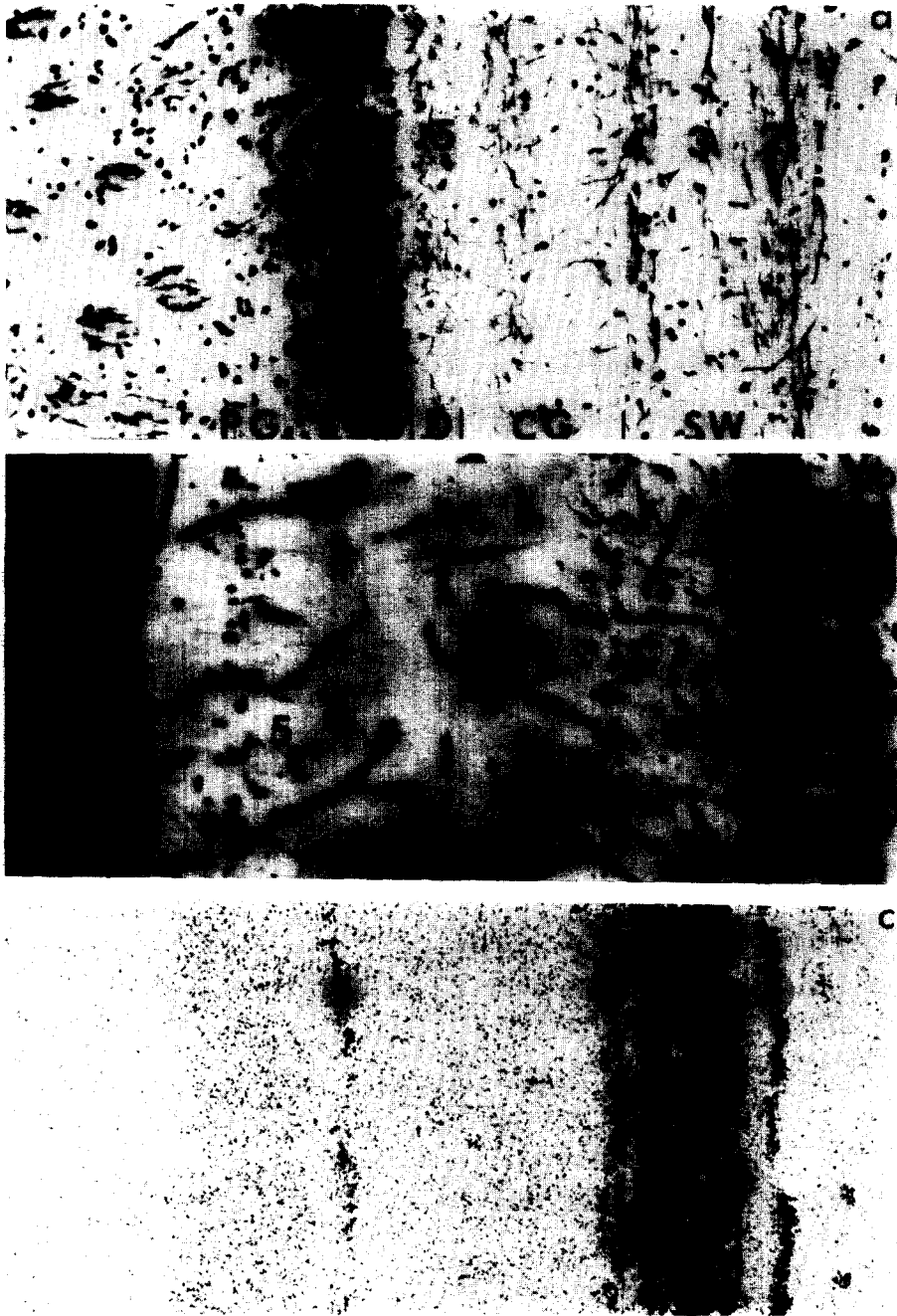


Fig. 1.

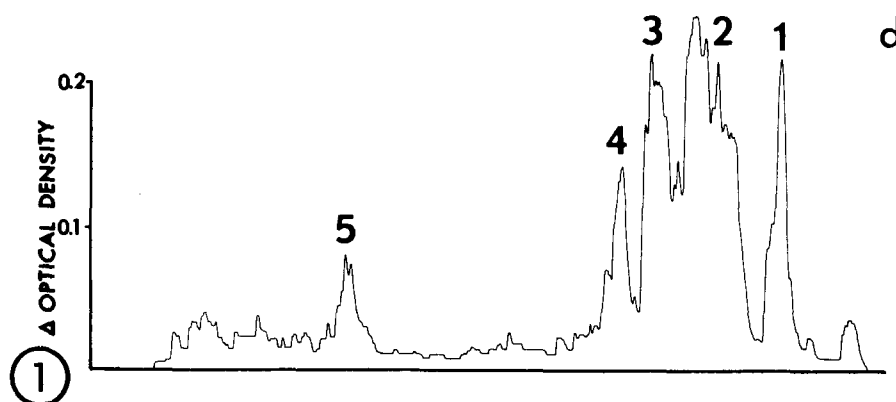


Fig. 1. Transverse sections through the optic tectum of *Hemichromis*. a: Bodian silver stain. b: Fink-Heimer degeneration stain. c: unstained radioautogram. d: microdensitometric scan. c and d were obtained from the same preparation after IO injection of 25 μCi of [^3H]proline; survival time was 24 h. Major tectal strata are as follows: SW, superficial white and gray; CG, central gray; D, deep white; PG, periventricular gray. The fiber components of the optic tract which terminate in the tectum are indicated (1-5). Since the fish selected for these experiments had different tectal thicknesses and these differences were not corrected photographically, the corresponding tectal strata in a, b and c are not in exact register.

densitometer tube held a 100- μm fixed-width slit that scanned the tectal layers by means of a motor-driven stage at a rate of 2.5-10 $\mu\text{m}/\text{sec}$. The tectal area exposed to the photomultiplier was 1-2 $\mu\text{m} \times 30 \mu\text{m}$, with a $\times 40$ objective lens. The isodensity scan was performed on an EDP scanning microscope, courtesy of Photometrics, Inc. (Lexington, Mass.).

Biochemical analysis

Brains were removed, rinsed with saline, and homogenized in distilled water at various intervals after injections with labeled proline. Acid-soluble and insoluble fractions were obtained by a filter paper method³⁷, combusted in a sample oxidizer and radioactivity was measured by liquid scintillation spectrometry⁴⁰. Portions of the homogenate were used to determine the protein content of each sample³⁶. Whole eyes, including lenses, were homogenized using a Brinkman Polytron Type PT-1020. When used, 10 μg of CXM was injected over the brain (IC) in 5 μl of 0.15 *N* NaCl.

RESULTS

The optic tectum of *Hemichromis* is clearly divided into layers which have been described for a number of teleost taxa^{14,35,44,47,50,52}. The nomenclature used in the present description follows the zones and strata of Leghissa³⁵. As the optic tract runs along the surface of the diencephalon and approaches the pretectum, it divides into lateral and medial optic tracts, which terminate in the superficial tectal zone (Fig. 1). In the tectum both tracts stratify into 4 components. The most superficial component (F1) is located at the boundary of the marginal fiber stratum and

terminates there. The second component (F2) forms the major optic fascicles located in the external plexiform stratum of the superficial white and gray zone. These fibers terminate both between the optic fascicles and in a layer immediately deep to the fascicles. The third (F3) and fourth (F4) components are underlain by strata of neuropil which appear to represent their respective terminal fields. It is possible that a given terminal field may receive more than one fiber component. In particular, the terminals of F3 may overlap those of both F2 and F4.

The majority of the retino-tectal fibers reach the optic tectum via the two pathways just described. However, the periventricular gray zone receives direct retinal projections (F5) via two additional pathways. The retinal fibers destined for the dorsal tectal periventricular gray exit the medial optic tract at mid-thalamic levels and course caudally until they reach the level of the posterior commissure where they swing laterally into the deep white zone of the optic tectum. These fibers also appear to contribute to F4. The retinal fibers destined for the ventral tectal periventricular gray exit the optic tract at the point of its division into lateral and medial optic tracts. These fibers course caudally medial to the nucleus rotundus and then turn dorsally to skirt the nucleus corticalis and join the ventral tectal deep white zone. These pathways were followed in both the degeneration stained and radioautographic material, and both types of preparations demonstrated retinal projections throughout the entire rostrocaudal extent of the tectal periventricular gray zone.

The optic tectum of *Hemichromis* is presented after the various preparations in Fig. 1. Degenerating ganglion cell projections were observed (Fig. 1b) in the regions containing the highest density of radioautographic silver grains (Fig. 1c). In this study, the radioautographic method demonstrated superior resolution, as can be seen from the microdensitometric scans (see below) of the exposed silver grains (Fig. 1d).

In preliminary studies with the related cichlid, *Astronotus*, optic tecta were radioautographically analyzed in fish killed 3, 4, 5, 6, 7, 8, 10, 12, 24, 48 or 72 h after IO injection of [^3H]proline. Three hours after injection the only areas with distinct patterns of silver grains were those adjacent to the medial and lateral optic tracts in the rostral tectum. Radioactive material appeared more caudally with time and by 5 h all areas of primary retinal efferent terminals contained radioactivity. This pattern did not change up to 72 h after injection, but the overall grain density increased with the arrival of additional labeled protein. The distribution of radioactivity in *Astronotus* following the arrival of rapidly transported protein indicates a complex pattern of retinal projections to the optic tectum (Fig. 2a and b), similar to that observed in *Hemichromis* (Fig. 1c), and appears more complex than that found in *Carassius*, a cyprinid species of teleost fish (Fig. 5a).

Microdensitometry

Densitometric (optical density) measurements of radioautograms of *Astronotus* optic tectum (Fig. 2c) and grain counts (Fig. 2d) of the same preparations appeared to correlate well (Fig. 2d, inset). The densitometric scan had greater apparent resolution than the grain counts and clearly indicated that the wide band of silver grains over the external plexiform layer of the superficial zone was composed of 3 distinct

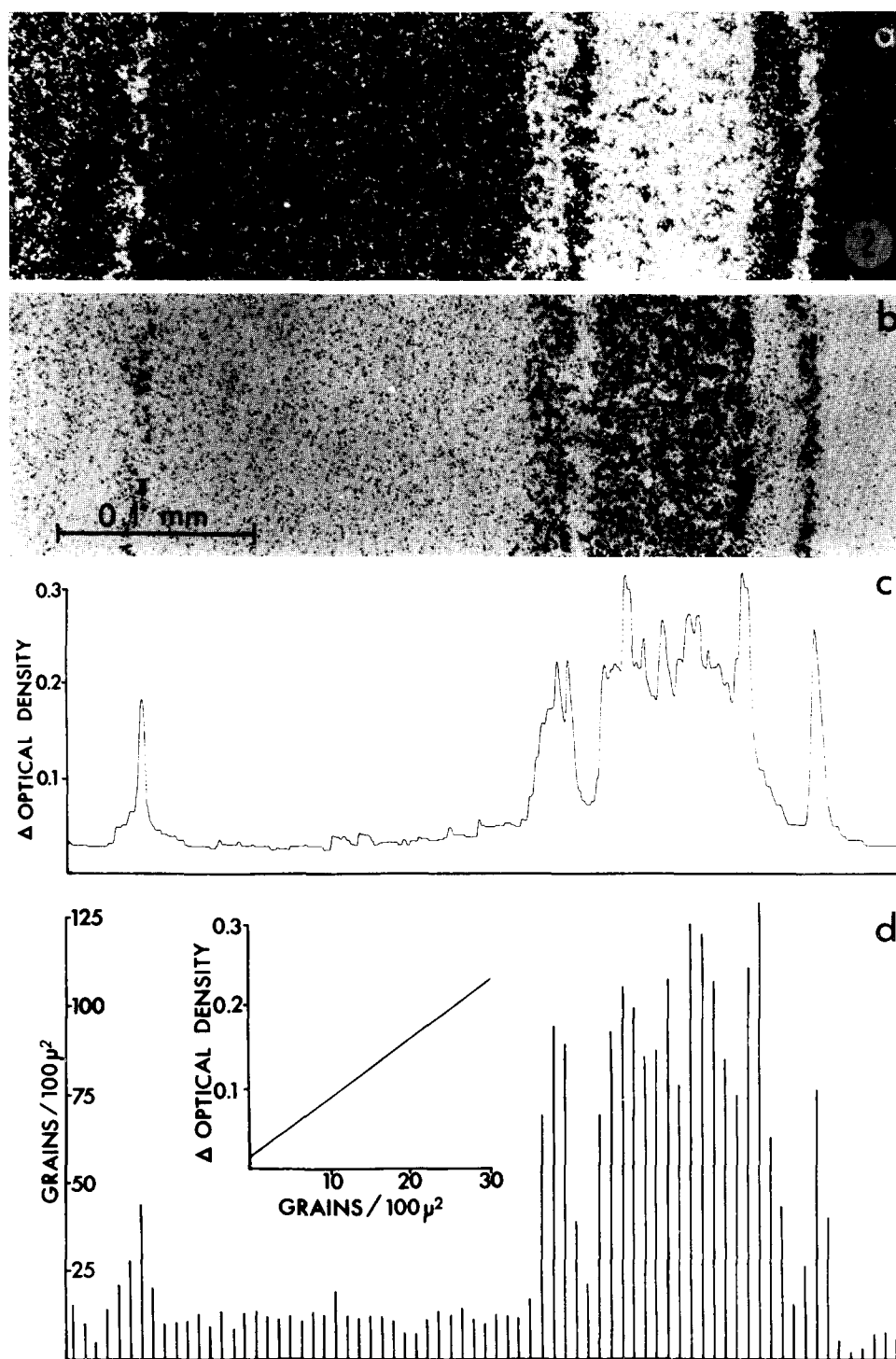


Fig. 2. Radioautograms from an unstained transverse section midway through the contralateral optic tectum of *Astronotus*. a: photomicrograph using dark field optics. b: photomicrograph using bright field optics. c: microdensitometric scan. d: silver grain density. The correlation between optical density and grain density is presented in the inset in d. Survival time for this specimen was 48 h after IO injection of $65 \mu\text{Ci}$ of $[^3\text{H}]\text{proline}$.

terminal fields, as had been suggested by visual inspection. The radioautographic layering pattern was more apparent in isodensity scans of the optic tectum after the arrival of rapidly transported labeled protein (Fig. 3).

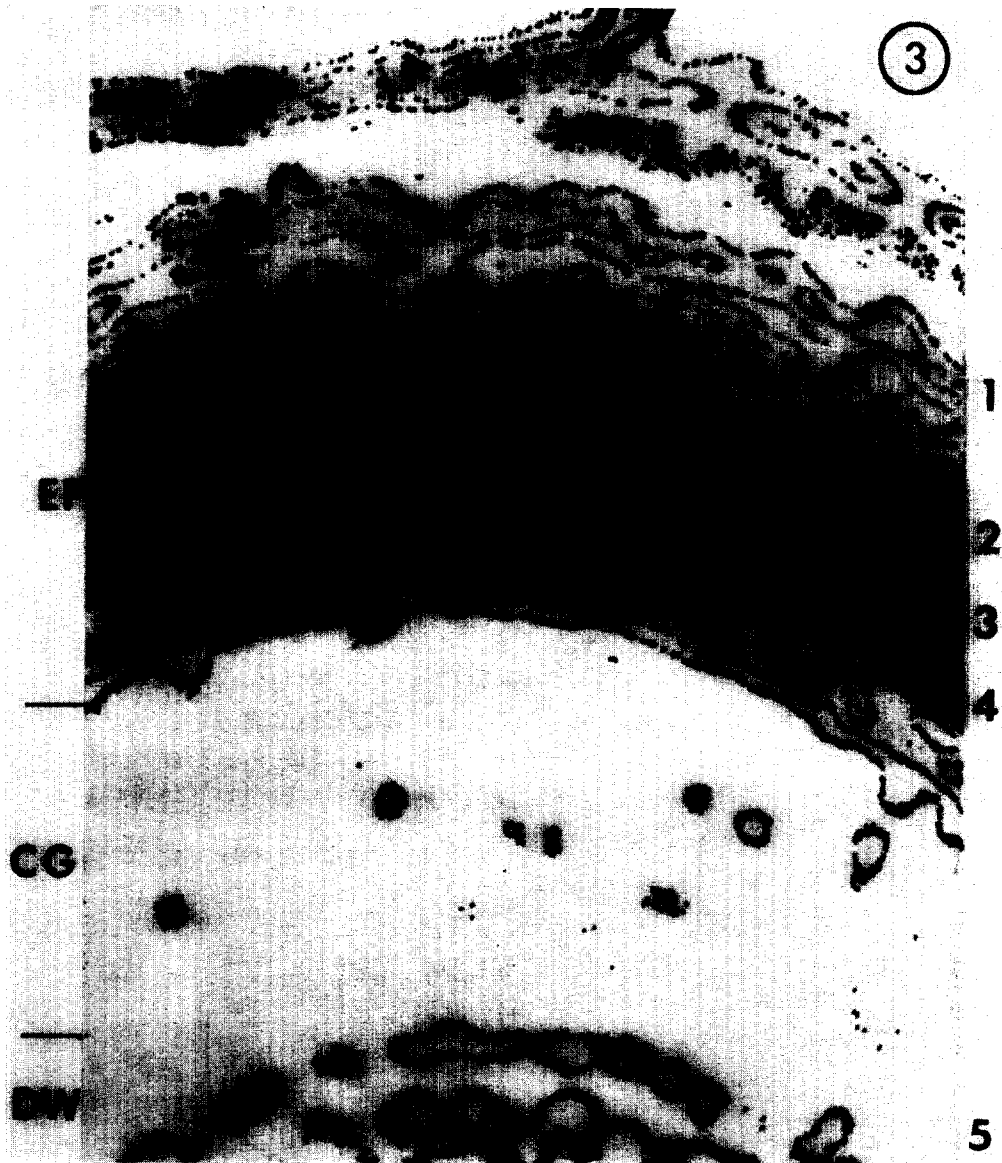


Fig. 3. Isodensity scan of a transverse section through the contralateral optic tectum of *Astronotus*. The isodensity scan is composed of a series of lines of similar density value separated from each other by constant optical density increments. The scan was obtained using an EDP scanning microscope at a magnification of $\times 200$ with a $13 \mu\text{m}$ scanning aperture. Radioautographic grain densities from the unstained section are plotted in 0.04 optical density increments separated by contour lines. Three distinct terminal fields are apparent in the external plexiform (EP) zone. Discrete high density regions are also present in the central gray (CG) and deep white (DW) zones. The preparation was obtained 12 h after IO injection of $65 \mu\text{Ci}$ of $[^3\text{H}]$ proline.

TABLE I

EFFECT OF MULTIPLE INTRACRANIAL CYCLOHEXIMIDE INJECTION ON TECTAL LABELING FOLLOWING AN INTRAOCULAR INJECTION OF [^3H]PROLINE

CXM was administered at 3, 8, 13, and 18 h after injection of 6 μCi of [^3H]proline. Animals were killed 24 h after IO injection and the radioactivity was determined in 3 pools of 8 tecta or eyes. Data are presented \pm the standard error of the mean.

	<i>Radioactivity in protein (10^{-2} disint./min/ tectum)</i>	<i>Specific activity (disint./min/μg protein)</i>	<i>Acid-soluble radioactivity (10^{-2} disint./min/ tectum)</i>	<i>Radioactivity in protein (10^{-6} disint./min/ eye)</i>
Control				
Contralateral	163.8 \pm 7.3	49.30 \pm 0.57	5.74 \pm 0.47	3.65 \pm 0.25
Ipsilateral	3.1 \pm 0.1	0.85 \pm 0.02	2.22 \pm 0.57	
CXM				
Contralateral	111.3 \pm 11.9	32.50 \pm 2.36	11.88 \pm 1.12	3.05 \pm 0.18
Ipsilateral	2.1 \pm 0.3	0.68 \pm 0.07	2.17 \pm 0.41	

CXM inhibition of tectal incorporation

In a preliminary experiment, labeled proline was injected intraperitoneally 15 min, 2.5 h or 4 h after the IC injection of 10 μg of CXM. This dose of the inhibitor was sufficient to reduce incorporation of [^3H]proline into tectal protein by 97% within 15 min after the CXM was administered, and by no less than 84% 4 h later.

A 10 μg dose of CXM was then injected IC 3, 8, 13 and 18 h after IO injection of [^3H]proline. Tectal labeling was determined after 24 h (Table I) in CXM-treated fish and in control fish injected with saline IC. Protein-bound radioactivity in the contralateral tectum of the CXM-treated fish was depressed by 34% compared to control fish, while radioactivity incorporated in the eye decreased by 16%. Since protein synthesis inhibitors do not block rapid axonal transport *per se*³⁸, the decrease in tectal labeling beyond that observed in the eye suggests that a significant portion of the contralateral tectal radioactivity is independent of labeled transported protein synthesized in the eye in the absence of CXM. Since the labeling of ipsilateral protein was only 2% of that in the contralateral tectum, only a fraction of the CXM-blocked labeling in the contralateral optic tectum could be attributed to blood-borne precursor. The increased acid-soluble radioactivity in the CXM-treated contralateral optic tectum further suggests that the antimetabolite interfered with the incorporation of a small, but significant, amount of labeled amino acid in this brain region. In the absence of CXM the acid-soluble radioactivity contralateral to the injected eye was 2-fold greater than on the ipsilateral side.

Radioautographic studies of the optic tectum were performed after IO [^3H]proline with (Fig. 4b) and without (Fig. 4a) multiple IC injections of CXM. Under these conditions, there was a 10% decrease in grain density in the external plexiform region, which contains the highest concentration of optic tract fibers and terminals. In the central gray, periventricular and efferent fiber layers, there was a 40–60%



decrease in grain density. The localized foci of grains (arrows, Fig. 4b) in the central gray region persisted despite CXM administration, and most probably do not represent local incorporation of labeled proline. With the arrival of slowly transported labeled protein in the tectum, a marked increase in grain density over the fascicles of optic tract fibers was observed (Fig. 4c).

Long term studies in the goldfish

Radioautograms were made of optic tecta of fish killed 1, 47, 113, and 240 days after IO injection of [³H]proline (Fig. 5) and grain counts were obtained from brain regions in and near the tectum (Table II). Rapidly transported protein (24 h) primarily labeled the nerve ending regions (Fig. 5a) while after arrival of slowly transported protein (47 days and later) both turnover and redistribution of radioactivity was observed (Fig. 5b). While the grains over the external plexiform layer, a primary synaptic region, decreased dramatically between 47, 113, and 240 days (Fig. 5b, c and d), other tectal strata varied (Table II). In contrast, when the grain densities were normalized against the external plexiform layer, the relative grain density in other tectal regions (Table II, numbers in parentheses), the tectal efferents and the midbrain (torus semicircularis) increased between 47 and 240 days. The absolute grain density increased with time in the valvula of the cerebellum, which borders the optic tectum but remains separated from it by the ventricle.

DISCUSSION

Radioautography of axonally transported proteins as a neuroanatomical tracing method presents several advantages over degeneration methods⁵. The most fundamental one is that the mechanism whereby axonal relationships are traced is a physiological process occurring in an intact system. The method is thus not complicated by neuropathological changes secondary to neuronal degeneration. Furthermore, radioautography clearly distinguishes fibers originating in a region from fibers of passage, since precursors are incorporated significantly only in the cell bodies. The radiochemical method should prove useful in developmental studies in which experimental degeneration is not compatible with the process under investigation.

More practically, this technique saves time. Retinal projections in fish can be adequately labeled as early as 5 h after injection of [³H]proline. The time required for emulsion exposure can be as brief as a few days. In addition, radioautographic methods are consistently reliable, in contrast to the vagaries of degeneration stains.

Fig. 4. Radioautograms of transverse sections through the contralateral optic tectum after IO injection of 140–155 μ Ci of [³H]proline in the goldfish. Shown are 1- μ m thick Epon sections taken from the rostromedial quadrant of the tectum, rich in fascicles of myelinated optic tract fibers. a: 24 h after injection. Rapidly transported labeled protein is concentrated in the layers (EP and S) containing optic tract terminals (t). b: 24 h after injection with CXM administered in multiple IC doses. Grain density in deeper tectal regions decreases under conditions of CXM inhibition, although discrete foci (arrows) persist in the central gray zone. c: 30 days after injection. Grain density increases markedly over the optic tract fiber region (f) with the arrival of slowly transported labeled protein. Toluidine blue stain. MF, marginal fiber; S, external synaptic; EP, external plexiform; CG, central gray; DW, deep white; PV, periventricular zones of the optic tectum.

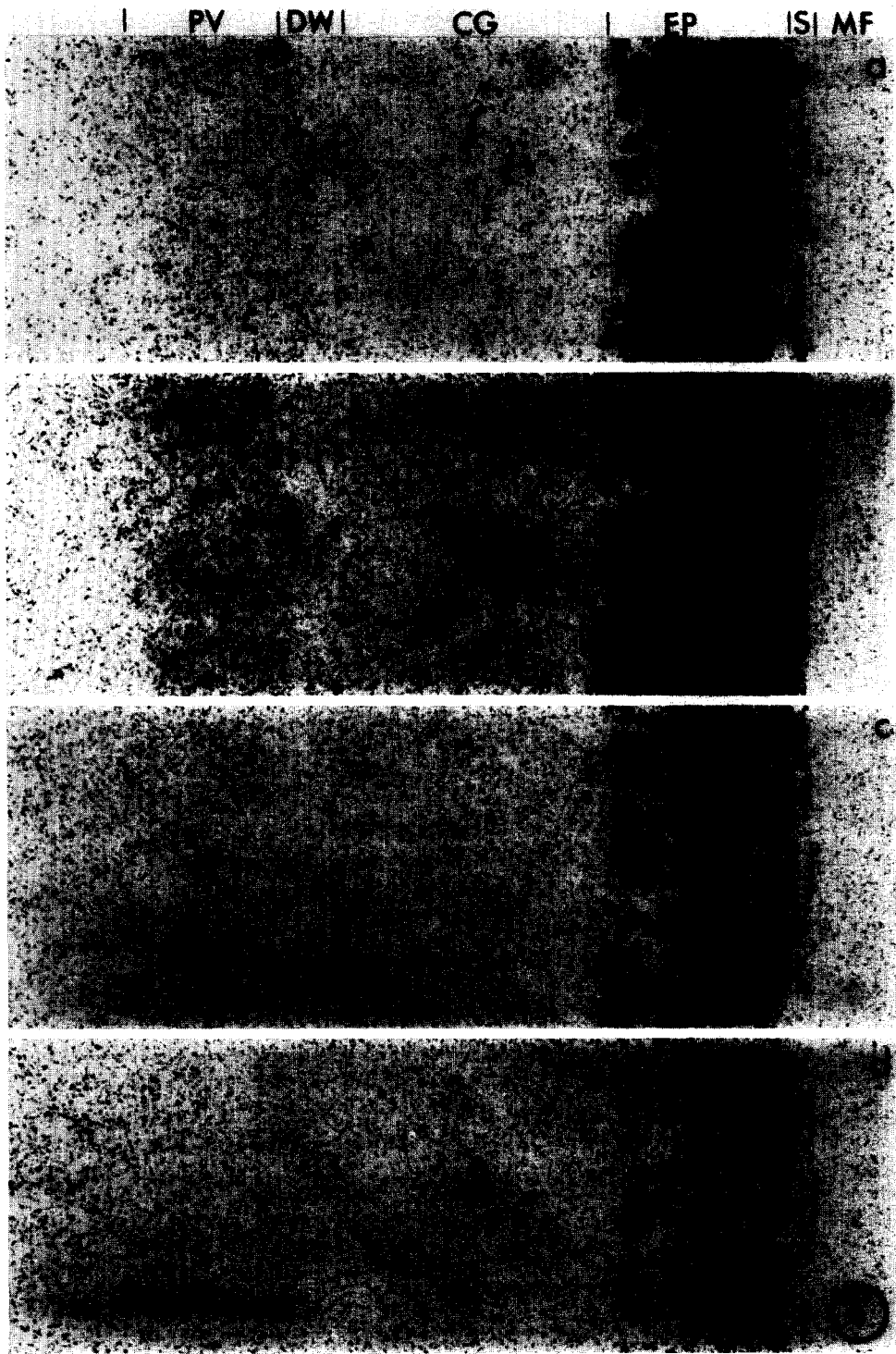


Fig. 5. Radioautograms of unstained transverse sections of the contralateral goldfish optic tectum at various intervals after IO injection of $25 \mu\text{Ci}$ of $[^3\text{H}]$ proline. Shown are mid-tectal regions in which optic tract terminals are dominant and fascicles of myelinated axons are scant in the external plexiform layer. Postinjection survival times are as follows. a: 1 day. b: 47 days. c: 113 days. d: 240 days. MF, marginal fiber; S, external synaptic; EP, external plexiform; CG, central gray; DW, deep white; PV, periventricular strata of the optic tectum.

TABLE II
RADIOAUTOGRAPHIC GRAIN DISTRIBUTION FOLLOWING INTRAOCULAR [³H]PROLINE

Exposed silver grain distribution in various tectal zones and other regions of the goldfish after intraocular injection of 25 μ Ci of [³H]proline. Grain counts were made over: (a) the external plexiform layer, including layers previously referred to as S and X (ref. 41); (b) the central gray, including the internal gray and internal plexiform strata; (c) periventricular gray; (d) periventricular white; (e) torus semicircularis; (f) the granular layer of the valvula and (g) the molecular layer of the valvula. Radioautograms were exposed for 7 days and grains counted on the projection screen of a Zeiss photomicroscope. A 5760 sq. μ m field was counted in each of the specified areas and a minimum of 2600 grains were counted per brain. Six animals were studied at postinjection survival times of 1, 47, and 113 days and 3 were studied after 240 days. The relative grain density = (regional grain density/external plexiform grain density) \times 100 is given in parenthesis. This normalizes the distribution of radioactivity to that in the primary synaptic region of the optic tectum. The torus semicircularis is a mid-brain structure medioventral to the tectum and the valvula is an anterior projection of the cerebellum found dorsal to the torus and medioventral to the tectum. The tectum and torus semicircularis are continuous via the efferent fiber region while a ventricle separates the tectum and valvula.

		<i>Grains per 1000 sq. μm</i>						
	<i>External plexiform</i>	<i>Central gray</i>	<i>Periventricular gray</i>	<i>Efferent fibers</i>	<i>Torus semicircularis</i>	<i>Granular valvula</i>	<i>Molecular valvula</i>	
<i>1 day</i>								
Contralateral	576 (100)	48.6 (8.4)	77.3 (13.4)	25.0 (4.3)	14.1 (2.4)	5.9 (1.0)	6.1 (1.1)	
Ipsilateral	6.1	4.2	6.3	5.7	4.2	4.3	4.3	
<i>47 days</i>								
Contralateral	943 (100)	1.43 (15.2)	190 (21.1)	94.4 (10.1)	31.8 (3.3)	12.7 (1.4)	16.8 (1.8)	
Ipsilateral	7.5	7.8	5.9	9.4	6.6	4.2	4.9	
<i>113 days</i>								
Contralateral	585 (100)	115 (19.6)	135 (23.1)	87.1 (14.9)	45.3 (7.7)	19.1 (3.3)	23.9 (4.1)	
Ipsilateral	7.8	9.9	7.5	6.6	7.3	3.8	7.1	
<i>240 days</i>								
Contralateral	224 (100)	89.0 (39.7)	89.7 (40.0)	62.0 (27.6)	30.2 (4.5)	22.3 (9.9)	25.5 (11.4)	
Ipsilateral	1.8	2.7	3.5	4.2	5.3	1.0	5.2	

The fact that radioautography of proteins is compatible with paraffin embedding facilitates serial sectioning, in contrast to the frozen microtomy recommended for degeneration stain techniques. Having sections in serial order greatly facilitates the tracing of pathways, particularly in small brains in which a given fiber pathway or terminal field may extend for only a few sections.

A limitation of the radioautographic technique is that it does not define fiber tracts with the precision of the Fink–Heimer and Nauta methods, particularly when rapidly transported labeled proteins are examined after brief labeling pulses. Fiber tracts are generally better visualized after labeling with slowly transported protein, but this may require several weeks, and incurs increased precursor reutilization with concomitant loss of definition.

In *Hemichromis*, we have found good agreement between the radioautographic data and those obtained with Fink–Heimer as well as with Nauta degeneration stains. The methods complement each other in determining neuroanatomical relationships, although certain details are more readily apparent in one preparation than in the other. For example, in the radioautograms, a deep projection of the optic nerve is seen which reaches throughout the rostrocaudal extent of the tectum. A number of previous workers^{3,44,48,52} have identified a deep projection, but found it to be restricted in its distribution to the rostral tectum. The more extensive distribution of this projection, which we initially observed in the radioautograms of *Hemichromis*, was confirmed by re-examination of degeneration material. A similar projection has also been observed in radioautograms of *Astronotus*. The laminar arrays in the teleost optic tectum encouraged the use of scanning microdensitometry for quantification. This proved to be a rapid, high resolution method for the identification of layers within the major retinal synaptic region in the cichlid tectum which may be useful for experiments in which physiological variables are manipulated in attempts to alter the anatomical or metabolic relationships among the stratifications.

In the visual system of *Carassius*, we found that the rapid phase of axonal transport preferentially labeled regions of axon terminals while the slow component labeled fiber tracts, observations which have been reported in a number of other studies^{5,23,26,45,46}. The physiological basis and significance of this labeling pattern has been considered in the chick ciliary ganglion by Droz and colleagues^{1,11–13,31} who found that labeled proteins and glycoproteins carried by rapid axonal flow are distributed primarily to nerve endings and are associated with presynaptic membranes and synaptic vesicles whereas the slow component contributes labeled mitochondria and axoplasm. In this system, only 5% of the radioactivity carried by slow flow reached the nerve terminals.

A number of biochemical observations suggest a possible complication in the use of [³H]proline for radioautographic tracing of neuronal pathways, namely that transported labeled proteins may break down *in situ* and provide labeled precursors for reincorporation within neighboring cells. This is exemplified by an experiment (Table 1) in which multiple injections of CXM were administered, beginning 3 h after the IO injection, when the radioactivity in the acid-soluble fraction of the contralateral tectum was increasing, paralleling the arrival of labeled protein. Under these

conditions, CXM led to a reduction in labeling of the contralateral optic tectum. The reduction appears to be greater than could be accounted for as a result of decreased incorporation in the eye together with that due to decreased systemic labeling (estimated from ipsilateral labeling, Table I). Additionally, in radioautographic experiments, CXM decreased the density of silver grains in the tectal regions adjacent to the primary terminal fields. These results are compatible with the proposed breakdown, diffusion and reincorporation of radioactivity.

While it has been reported that free amino acids are not transported axonally in the fish⁷, it remains possible that a small amount of free proline arrives from the eye and is subsequently incorporated into tectal protein. Axonal transport of free leucine has been reported in the cat³⁰, but in the carp very little soluble radioactivity was found in the distal portion of the optic nerve following IO injection of [³H]proline⁷. Nerve-cut experiments in the goldfish suggest that the labeled leucine observed in the optic nerve close to the injected eye has little relationship to the contralateral tectal labeling¹⁰.

If neither axonal transport of free amino acid nor systemic circulation can account for the difference between contralateral and ipsilateral precursor, we are led to conclude that the release of labeled amino acid from breakdown of transported protein is the source of this difference. Such a proposal assumes the existence of rapidly transported proteins with relatively brief half-lives, as has been reported recently in the visual system⁵⁵. Similarly, radiochemical methods for tracing the distribution of [³H]fucose-labeled glycoproteins in the visual system⁴⁹ may be affected by reutilization of labeled fucose in the brain²².

Reincorporation may be a special problem when proline is used in radioautographic studies to follow the axonal transport of labeled protein. In long-term turnover studies, we have found that proline-labeled transported protein has a much longer half-life than leucine-labeled protein. This is probably due to differences in movement of the precursors out of cells, as well as their rates of metabolism. While proline enters the brain from the blood much more poorly than leucine, once in the brain, it remains longer⁴⁰. The rapid breakdown of leucine in nerve endings⁴ may account for this difference. While its apparent lower rate of reutilization would favor the use of leucine for neuroanatomical studies after intracerebral micro-injection, the high background labeling following IO injection renders it less useful than proline.

The radioautographic and biochemical studies reported here with CXM suggest that the antimetabolite may be useful in decreasing reutilization of the labeled amino acid. Droz found that puromycin reduced 'background' labeling in the chick ciliary ganglion by nearly 90% (ref. 13). Other possible ways to reduce proline reutilization include saturation of endogenous amino acid pools with unlabeled precursor following incorporation²² or the administration of inhibitory amino acid analogs²⁸.

The physiological disposition of labeled proline is of interest beyond the question of its precision as a neuroanatomical tool. Proline has been reported to be taken up by synaptosomes *in vitro* by a high affinity uptake mechanism, and to be released by a cation-dependent process³⁹. Additionally, proline competitively displaces

labeled strychnine binding to spinal cord membranes associated with glycine receptors⁵⁶. Künzle has reported what appears to be a selective block of proline uptake by specific neuronal cells in the cat lateral reticular nucleus³³, and more recently in the cerebellum¹⁹. It is possible that specialized cells compete successfully for proline by virtue of a high affinity uptake system, as has been postulated for neurotransmitters and their precursors.

The question has also been raised as to whether undegraded macromolecules can migrate transneuronally, perhaps transsynaptically^{24-26,49}. If this migration indeed occurred, it would be of great significance in understanding cell-cell interactions in the brain. We feel that a more parsimonious interpretation of movement of radioactivity out of primary terminal fields is reutilization of precursor, and that this possibility should be rigorously considered in studies of macromolecular migration. Whatever the mechanism, the release of radioactivity from terminal fields and its subsequent uptake by neighboring cells could serve as a means of identifying the distribution of terminals from the cells upon which they synapse. Thus, Weisel *et al.*⁵⁴ have recently confirmed the report of Grafstein²⁴ that intraocular injection of [³H]-proline in a mammal results in the appearance of radioactivity in the striate cortex. In such studies the extensive reutilization of axonally transported radioactivity may have been exploited in defining the terminal fields of second-order neurons⁵⁴.

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REFERENCES

- 1 BENNETT, G., DIGIAMBERARDINO, L., KOENIG, H. L., AND DROZ, B., Axonal migration of protein and glycoproteins in nerve endings of chicken ciliary ganglion after intracerebral injection of [³H]fucose and [³H]glucosamine, *Brain Research*, 60 (1973) 120-146.
- 2 BONDY, S. C., Axonal transport of macromolecules. I. Protein migration in the central nervous system, *Exp. Brain Res.*, 13 (1971) 127-134.
- 3 CAMPBELL, C. B. G., AND EBBESSON, S. O. E., The optic system of a teleost: *Holocentrus* re-examined, *Brain Behav. Evol.*, 2 (1969) 415-430.
- 4 CHAPLIN, E. R., AND DIAMOND, I., The importance of leucine oxidation in brain, *Trans. Amer. Soc. Neurochem.*, 5 (1974) 167.
- 5 COWAN, W. M., GOTTLIEB, D. I., HENDRICKSON, A. E., PRICE, J. L., AND WOOLSEY, T. A., The autoradiographic demonstration of axonal connections in the central nervous system, *Brain Research*, 37 (1972) 21-51.
- 6 CROOKS, R. F., AND MCCLURE, W. O., The effect of cytochalasin B on fast axoplasmic transport, *Brain Research*, 45 (1972) 643-646.
- 7 CSANYI, V., GERVAI, J., AND LAJTHA, A., Axoplasmic transport of free amino acids, *Brain Research*, 56 (1973) 271-284.

- 8 CUÉNOD, M., MARKO, P., AND NIEDERER, E., Disappearance of particulate tectal protein during optic nerve degeneration in the pigeon, *Brain Research*, 49 (1973) 422-426.
- 9 DALTON, A. J., A chrome-osmium fixative for electron microscopy, *Anat. Rec.*, 121 (1955) 281.
- 10 DIGIAMBERARDINO, L., Independence of the rapid axonal transport of protein from the flow of free amino acids, *Acta neuropath. (Berl.)*, Suppl. V (1971) 132-135.
- 11 DIGIAMBERARDINO, L., BENNETT, G., KOENIG, H. L., AND DROZ, B., Axonal migration of protein and glycoproteins to nerve endings. III. Cell fractionation analysis of chicken ciliary ganglion after intracerebral injection of labeled precursors of proteins and glycoproteins, *Brain Research*, 60 (1973) 143-159.
- 12 DROZ, B., Renewal of synaptic proteins, *Brain Research*, 62 (1973) 383-394.
- 13 DROZ, B., KOENIG, H. L., AND DIGIAMBERARDINO, L., Axonal migration of protein and glycoprotein to nerve endings. I. Radioautographic analysis of the renewal of protein in nerve endings of chicken ciliary ganglion after intracerebral injection of [³H]lysine, *Brain Research*, 60 (1973) 93-127.
- 14 EBBESSON, S. O. E., Retinal projections in two teleost fishes (*Dosanus tau* and *Gymnothorax funebris*): an experimental study with silver impregnation methods, *Brain Behav. Evol.*, 1 (1968) 134-154.
- 15 EBBESSON, S. O. E., Organization of central visual pathways in vertebrates, *Brain Behav. Evol.*, 3 (1970) 178-194.
- 16 EBBESSON, S. O. E., The selective silver-impregnation of degenerating axons and their synaptic endings in non-mammalian species. In W. J. H. NAUTA AND S. O. E. EBBESSON (Eds.), *Contemporary Research Methods in Neuroanatomy*, Springer, N.Y., 1970, pp. 132-152.
- 17 ELAM, J. S., AND AGRANOFF, B. W., Rapid transport of protein in the optic system of the goldfish, *J. Neurochem.*, 18 (1971) 375-387.
- 18 ELAM, J. S., NEALE, E. A., AND AGRANOFF, B. W., Axonal transport in the goldfish visual system, *Acta neuropath. (Berl.)*, Suppl. V (1971) 257-266.
- 19 FELIX, D., AND KÜNZLE, H., Iontophoretic and autoradiographic studies on the role of proline in nervous transmission, *Pflügers Arch. ges. Physiol.*, 350 (1974) 135-144.
- 20 FINK, R. P., AND HEIMER, L., Two methods for selective silver impregnation of degenerating axons and their synaptic endings in the central nervous system, *Brain Research*, 4 (1967) 369-374.
- 21 GAZE, R. M., Neuronal specificity, *Brit. med. Bull.*, 30 (1974) 116-121.
- 22 GLASGOW, M. S., QUARLES, R. H., AND GROLLMAN, S., Metabolism of fucoglycoproteins in the developing rat brain, *Brain Research*, 42 (1972) 129-137.
- 23 GRAFSTEIN, B., Transport of protein by goldfish optic nerve fibers, *Science*, 157 (1967) 196-198.
- 24 GRAFSTEIN, B., Transneuronal transfer of radioactivity in the central nervous system, *Science*, 172 (1971) 177-179.
- 25 GRAFSTEIN, B., AND LAURENO, R., Transport of radioactivity from eye to visual cortex in the mouse, *Exp. Neurol.*, 39 (1973) 44-57.
- 26 HENDRICKSON, A. E., Electron microscopic distribution of axoplasmic transport, *J. comp. Neurol.*, 144 (1972) 381-398.
- 27 JACOBSON, M., AND GAZE, R. M., Selection of appropriate tectal connections by regenerating optic nerve fibers in adult goldfish, *Exp. Neurol.*, 13 (1965) 418-430.
- 28 JOHNSTON, G. A. R., AND BALCAR, V. J., Amino-oxyacetic acid: a relatively non-specific inhibitor of uptake of amino acids and amines by brain and spinal cord, *J. Neurochem.*, 22 (1974) 609-610.
- 29 KARLSSON, J., AND SJÖSTRAND, J., Axonal transport of proteins in retinal ganglion cells. Amino acid incorporation into rapidly transported proteins and distribution of radioactivity to the lateral geniculate body and the superior colliculus, *Brain Research*, 37 (1972) 279-285.
- 30 KIDWAI, A. M., AND OCHS, S., Components of fast and slow phases of axoplasmic flow, *J. Neurochem.*, 16 (1969) 1105-1112.
- 31 KOENIG, H. L., DIGIAMBERARDINO, L., AND BENNETT, G., Renewal of proteins and glycoproteins of synaptic constituents by means of axonal transport, *Brain Research*, 62 (1973) 413-417.
- 32 KOPRIWA, B. M., AND LEBLOND, C. P., Improvements in the coating technique of radioautography, *J. Histochem. Cytochem.*, 10 (1962) 269-284.
- 33 KÜNZLE, H., AND CUÉNOD, M., Differential uptake of [³H]proline and [³H]leucine by neurons: its importance for the autoradiographic tracing of pathways, *Brain Research*, 62 (1973) 213-217.
- 34 LASEK, R., JOSEPH, B. S., AND WHITLOCK, D. G., Evaluation of a radioautographic neuroanatomical tracing method, *Brain Research*, 8 (1968) 319-336.
- 35 LEGHISSA, S., La struttura microscopica e la citoarchitettura del tetto ottico dei pesci teleostei, *Z. Anat. Entwickl.-Gesch.*, 118 (1955) 427-463.

- 36 LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., AND RANDALL, R. J., Protein measurement with the Folin phenol reagent, *J. biol. Chem.*, 193 (1951) 265-275.
- 37 MANS, P. J., AND NOVELLI, G. D., Measurement of the incorporation of radioactive amino acids into protein by a filter paper method, *Arch. Biochem.*, 94 (1961) 48-53.
- 38 MCEWEN, B. S., AND GRAFSTEIN, B., Fast and slow components in axonal transport of protein, *J. Cell Biol.*, 38 (1968) 494-508.
- 39 MULDER, A. H., AND SNYDER, S. H., Potassium-induced release of amino acids from cerebral cortex and spinal cord slices of the rat, *Brain Research*, 76 (1974) 297-308.
- 40 NEALE, J. H., ELAM, J. S., NEALE, E. A., AND AGRANOFF, B. W., Axonal transport and turnover of proline- and leucine-labeled protein in the goldfish visual system, *J. Neurochem.*, 23 (1974) 1045-1055.
- 41 NEALE, J. H., NEALE, E. A., AND AGRANOFF, B. W., Radioautography of the optic tectum of the goldfish after intraocular injection of ^3H -proline, *Science*, 176 (1972) 407-410.
- 42 RAHMANN, H., Darstellung des intraneuronalen proteintransports vom auge in das tectum opticum und die cerebrospinalflüssigkeit von teleostern nach intraocularer injektion von [^3H]-histidin, *Naturwissenschaften*, 7 (1966) 174-175.
- 43 RAHMANN, H., Syntheseort und ferntransport von protein im fischhirn, *Umschau Wissenschaft und Technik*, 9 (1968) 276-277.
- 44 ROTH, R. L., Optic tract projections in representatives of two fresh-water teleost families, *Anat. Rec.*, 163 (1969) 253-254.
- 45 SCHONBACH, J., AND CUÉNOD, M., Axoplasmic migration of protein. A light microscopic autoradiographic study in the avian retino-tectal pathway, *Exp. Brain Res.*, 12 (1971) 275-282.
- 46 SCHONBACH, J., SCHONBACH, C., AND CUÉNOD, M., Rapid phase of axoplasmic flow and synaptic proteins: an electron microscopic autoradiographic study, *J. comp. Neurol.*, 141 (1971) 485-498.
- 47 SCHROEDER, D. M., Some afferent and efferent connections of the optic tectum of a teleost, *Ictalurus*, *Anat. Rec.*, 178 (1974) 458.
- 48 SHARMA, S. C., The retinal projections in the goldfish: an experimental study, *Brain Research*, 39 (1972) 213-223.
- 49 SPECHT, S., AND GRAFSTEIN, B., Accumulation of radioactive protein in mouse cerebral cortex after injection of [^3H]fucose into the eye, *Exp. Neurol.*, 41 (1973) 705-722.
- 50 SPERRY, R. W., Patterning of central synapses in regeneration of the optic nerve in teleosts, *Physiol. Zool.*, 21 (1948) 351-361.
- 51 TAYLOR, C., AND WEISS, P., Demonstration of axonal flow by the movement of tritium labeled protein in mature optic nerve fibers, *Proc. nat. Acad. Sci. (Wash.)*, 54 (1965) 1521-1522.
- 52 VANEGAS, H., AND EBBESSON, S. O. E., Retinal projections in the perch-like teleost, *Engerres plumeri*, *J. comp. Neurol.*, 151 (1973) 331-358.
- 53 WEISS, P., AND HISCOE, H. B., Experiments on the mechanism of nerve growth, *J. exp. Zool.*, 107 (1948) 315-395.
- 54 WIESEL, T. N., HUBEL, D. H., AND LAM, D. M. K., Autoradiographic demonstration of ocular dominance columns in the monkey striate cortex by means of transneuronal transport, *Brain Research*, 79 (1974) 273-279.
- 55 WILLARD, M., COWAN, W. M., AND VAGELOS, P. R., The polypeptide composition of intra-axonally transported protein: evidence for four transport velocities, *Proc. nat. Acad. Sci. (Wash.)*, 71 (1974) 2183-2187.
- 56 YOUNG, A. B., AND SNYDER, S. H., Strychnine binding in rat spinal cord membranes associated with the synaptic glycine receptor: cooperativity of glycine interaction, *Molec. Pharmacol.*, 10 (1974) 790-809.