Modulation of Cell Proliferation in the Goldfish Retina Following Axotomy of Optic Nerve Fibers

by

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Dedication

For my grandparents, Samuel & Rose Henken and Helen & H. David Covel.

I know that they are sharing this simcha with me.

TABLE OF CONTENTS

Abstract

The effects of optic fiber axotomy on cell birth in the retina of the goldfish are examined with tritiated thymidine (^{3}H-TdR) autoradiography. Two types of cells within two nuclear laminae are selectively influenced following optic nerve crush: non-neuronal cells in the ganglion cell/optic fiber layer (GC/OFL) and rod receptor precursors in the outer nuclear layer (ONL). In the GC/OFL, the time course of the changes in cell proliferation as a function of days following axotomy shows an initial increase followed by a gradual decrease when examined 1 day following ^{3}H-TdR injection. One month following injection, no consistent change is seen at the various post-operative periods. In the ONL, axotomy modulates the numbers of labelled rod precursors when examined 1 day following ^{3}H-TdR injection. The time course of these changes appears biphasic: there is an initial increase, followed by a decrease of labelled cells in the axotomized ONL. One month following injection, consistently fewer labelled cells are seen in the axotomized ONL, suggesting that a fraction of these initially labelled cells have undergone further mitotic divisions, diluting the label. The proportion of those cells initially incorporating ^{3}H-TdR that are destined to undergo further cell generation cycles is modulated by axotomy.

Abbreviations

Ci	-Curie
DNA	-deoxyribonucleic acid
EtOH	-ethyl alcohol
g	-gram
GC/OFL	-ganglion cell/optic fiber layers
INL	-inner nuclear layer
IO	-intraocular
IP	-intraperitoneal
IPL	-inner plexiform layer
l	-liter
ln	-natural logarithm
LS	-1 month survival following injection
°C	-degrees Celcius
M	-molar
µCi	-microcurie
µm	-micron
µl	-microliter
mm	-millimeter
mmol	-millimole
MS222	-tricaine methanesulfonate
nm	-nanometer
ON	-optic nerve
ONC	-optic nerve crush

ONL	-outer nuclear layer
OPL	-outer plexiform layer
PBS	-phosphate buffered saline
PE	-pigment epithelium
P.O.	-propylene oxide
rER	-rough endoplasmic reticulum
RNA	-ribonucleic acid
SE	-standard error of the mean
S-phase	-synthesis phase of cell generation cycle
S.A.	-specific activity
SS	-1 day survival following injection
^{3}H-TdR	-tritiated thymidine

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I. GENERAL INTRODUCTION

THE QUESTION

The retinae of vertebrates grow by cell addition. In mammalian and avian systems, cell birth is complete by the time the eye begins to function (Fujita & Horii, 1963; Sidman, 1961). In fish and amphibians, on the other hand, histogenesis in the eye continues throughout life (Hollyfield, 1968; Johns, 1977; Meyer, 1978). Adult teleosts and amphibians also retain the unique capacity to regenerate damaged central nervous system tissue. Specifically, if optic fibers are surgically disconnected, the retinal ganglion cells regrow new axons that then reform functional connections with their target, the optic tectum.

In this thesis I examine whether the axotomy of optic nerve fibers would influence the numbers and types of cells being born in the retina of the goldfish.

HISTOGENESIS IN THE NORMAL RETINA

Müller (1952) studied histogenesis in the retina of the guppy, Lebistes reticulatus, by quantifying the total number of neurons born from hatching (7 mm body length) through adulthood (28 mm body length). During this growth period, he found a seven-fold increase in the number of rods and a three-fold increase in cone, inner nuclear layer, and ganglion cell populations. As the retina enlarges the densities of most cells decrease, with the exception of rod photoreceptors, which maintain a constant density (Johns, 1977; Johns & Easter, 1977; Lyall, 1957; Müller, 1952). Subsequent investigations on histogenesis in the retina have focused on the order in which specific cell types are born and the topographical course of cell production.

Autoradiographic (Hollyfield, 1972; Sharma & Ungar, 1980), light microscopic, and electron microscopic studies (Grun, 1975) have extended and refined the original observations of Müller. It has now been clearly demonstrated that retinal maturation proceeds in a vitreo-sclerad direction, that is, within a given topographical location in the retina, those cells nearest the vitreous humor are produced first, and those farthest away mature last. Tritiated thymidine autoradiography has been used to study the order of production of retinal cells in the killifish (Hollyfield, 1972) and goldfish (Sharma and Ungar, 1980). Using this technique, it was found that ganglion cells are the first cells to cease mitosis in the fundus of the retina. Grun (1975) supported the autoradiographic evidence with electron microscopy by demonstrating that the ganglion cells in a cichlid fish are the first to contain endoplasmic reticulum and the first to produce neurites. As the germinal front progresses, other cells in central retina cease DNA synthesis. Amacrine cells become post-mitotic before other cells of the inner nuclear layer (INL) (Hollyfield, 1972). Sharma and Ungar (1980) could not differentiate between the end of amacrine and bipolar cell production and concluded that both stop cell division concurrently. Horizontal and receptor cells are the last to be produced (Grun, 1975; Hollyfield, 1972; Sharma & Ungar, 1980).

In addition to the general agreement as to the order in which retinal cell types of the laminae are produced, there is also agreement as to the overall topographical course of events involved in retinal histogenesis. Autoradiographic evidence (Johns, 1977, 1982; Meyer, 1978; Sandy & Blaxter, 1980; Sharma & Unger, 1980) and cell count studies (Lyall, 1957; Johns & Easter, 1977) have shown that retinal growth in fish occurs by the accretion of newly formed cells at the margin of the retina. This annulus of germinal cells, which has alternatively been termed the ora serrata, ora terminalis, basal cell zone, or ciliary margin, continues to proliferate throughout the life of the fish to produce all types of new retinal cells. These newly formed cells are thought to differentiate in situ, although some authors suggest that some migration may occur (Johns, 1977). In amphibians, a similar growth pattern involving mitotically active germinal cells at the ciliary margin has been documented (Jacobson, 1968, 1976; Straznicky & Gaze, 1971).

Although there is agreement that the primary source of new retinal cells is the ora terminalis, occasionally it has been noted in passing that, in both juveniles and adults, some mitotic activity is still occurring in regions central to the growth zone. Lyall (1957) notes:

> in two or three retinae I have found a mitosis some distance from the edge of the retina in the outer nuclear-layer, in a region where differentiation is completed and the rods and cones have well-developed outer segments. These mitoses were found in the eyes of fish 1-2 years old, so a few nuclei in the outer nuclear-layer must retain the power to undergo mitosis. (pg. 104)

Similar observations on mitoses or tritiated thymidine incorporation in central retina have been made in other fish (Johns, 1982; Johns & Fernald, 1981; Sandy & Blaxter, 1980; Scholes, 1976), amphibians (Jacobson, 1968; Straznicky & Gaze, 1971), and young chicks (Morris, Wylie & Miles, 1976). The location of these newly formed cells is confined to two laminae, the outer nuclear layer (ONL) (Sandy & Blaxter, 1980; Scholes, 1976) and the ganglion cell/optic fiber layer (GC/OFL)

(Jacobson, 1968; Morris, Wylie & Miles, 1976; Straznicky & Gaze, 1971). The cells in the ONL are thought to be rods (Johns, 1982), while it has been suggested that the labelled cells in more vitread regions are glial or capillary endothelial in nature (Jacobson, 1968). Cell division of non-neuronal elements has been shown to exist in most CNS tissue (See Korr, 1980 for a review).

The birth of cells in the well-differentiated ONL poses more interesting questions than does glial cell activity since cell birth in adult nerve tissue is relatively rare. It is particularly important with regard to the issue of neural connectivity, as presumably these newly-formed rods must make appropriate connections within an already differentiated and functioning retina. Although there is irrefutable evidence that the vast majority of new cells are derived from the germinal peripheral zone, there are enough observations to suggest that the <u>ora terminalis</u> is not the sole source. As a result of the above mentioned hints that cell birth in adult animals occurs in older, differentiated retinal regions in at least two distinct laminae (ONL and GC/OFL), Johns (1982) re-examined cell birth in the retina of juvenile fish. She concluded that, while the circumferential growth zone is the major source of new cells, there are photoreceptor cells born throughout the extent of the retina in both larval and juvenile fish.

The possible origin of the new rods found in central retinal regions is open to speculation. New rods may be formed in the annular growth zone and become displaced by shearing forces (Johns, 1977; Müller, 1952). On the other hand, rods may only be produced in the peripheral germinal zone and then migrate to new positions in more central retina (Johns, 1977; Muller, 1957). Perhaps pigment epithelial cells may give rise to new rods since pigment epithelium has the ability to undergo continuous cell turnover. It could be that other cells in the differentiated retina may be transformed into rod receptors (Bernard, 1900) or new rods may be formed _in situ_ from a germinal cell population scattered throughout the older retinal regions (Johns & Fernald, 1981; Raymond, 1985a, 1985b).

Rod genesis appears to be unique and does not completely conform to standard histogenetic explanations. Locket (1980) examined a deep-sea teleost, which is distinctive in that it has a pure-rod multiple-bank retina when mature. When young, _Chauliodus sloani_ has a single bank of rods, which is formed in a manner similar to that occurring in other fish, but, by maturity, up to five banks of rods have been documented. The increased numbers of rods presumably increases the light-gathering power of these deep-sea teleosts. The ratio of rods to ganglion cells increases from 7:1 in small fish to 200:1 in the largest fish examined. Addition of new rods to form a multiple bank retina cannot proceed strictly by _in situ_ cell birth at the peripheral growth zone. If a shearing process was involved, one would expect a gradient of banks, with central retina having the largest number. This was not observed. If migration from peripheral retina to more central regions occurs, one would expect to see more cell nuclei than outer segments with the difference corresponding to the number of migrating immature cell bodies. This was not found. Migration of pigment epithelial cells would involve a long, tortuous course through the ever-thickening ONL, while recruitment and transformation of INL cells would not be numerically feasible since there are not enough INL cells to account for the increase in the receptor population in this species. The final possibility is that mitotic division occurs in more central regions. This option would require a population of stem or germinal cells scattered throughout the differentiated ONL (or perhaps the INL). This last explanation can be employed to explain the fact that there are no topographical differences in the thickness or number of banks over the extent of the retina. Unfortunately, thymidine autoradiography, which could more directly address this question of the origin of the rods was not applied to this specimen since this deep sea fish cannot survive in the laboratory. Sandy & Blaxter (1980) demonstrated that, in herring and sole, each of which has a pure-cone retina in larval stages and adds rods later in development, the new rods are derived from a population of stem cells located at the border of the ONL and outer plexiform layer (OPL). These results are in agreement with those which Johns (1982) found in the goldfish. Rod cell precursors are formed by mitotic division of a stem cell population scattered throughout the differentiated receptor population in the ONL. In larval and young juvenile fish (1-2 mos.), the annular growth ring was easily discernible. Scattered nuclei tagged with radioactive thymidine were seen in both ONL and INL but, as the fish grew, a larger proportion of dividing cells was found in the ONL in comparison to the INL. This change in proportion of labelled ONL to INL cells led Johns (1977) to postulate that the original location of the rod progenitors is in the INL. The INL, she claimed, contains undifferentiated clusters of neuroepithelial germinal cells that then migrate to the ONL.

Furthermore, she suggested that these labelled progeny are subventricular cells since they divide in place, produce only one kind of neuron, and are active in the late stages of histogenesis. Whether the progenitor cells are left behind in the ONL or in the INL (before migrating to the ONL) as the germinal annulus grows is still to be determined. The fact that there is a population of these cells assuming a "latent" state among the differentiated rod receptors has been clearly demonstrated.

Recently, Fernald and Scholes (1985) have located a zone of exclusive rod neurogenesis in the developing cichlid retina. This rod production zone is composed of specialized neuroblasts concentrated in an annulus in the ONL about 100 um inside the ciliary margin. Using thymidine autoradiography, they also showed that these stem cells migrated outward from more central to peripheral tissue. In addition, as survival time following the thymidine administration increased, this neuroblast population lost the label, suggesting dilution by successive mitotic divisions. They postulate that this dividing population of rod precursors acts to maintain the density of rod cells as the retina grows. These observations suggest that the rod precursor pool is actually a population distinct from the neuroblasts that comprise the original germinal front since their location is separate from that of the <u>ora terminalis</u>, their morphology appears to be unique, and their mitotic kinetics are notably different. The existence of a separate stem cell population appears to be a plausible mechanism for maintaining rod density, although the underlying implications for the formation of new neural connections and plasticity following damage to the system are still unclear.

REGENERATION OF OPTIC FIBERS

When retinal ganglion cell axons are cut or crushed, they grow back along the appropriate pathways to their correct topographical location in the optic tectum. This regrowth and functional reconnection takes between one and two months and has been confirmed behaviorally (Arora & Sperry, 1963; Edwards, Alpert & Grafstein, 1981; Springer & Agranoff, 1977; Springer, Easter & Agranoff, 1977), electrophysiologically (Horder, 1974; Northmore & Masino, 1984; Yoon, 1976) and anatomically (Attardi & Sperry, 1963; Murray & Edwards, 1982; Radel, 1982). Biochemical correlates of optic fiber regeneration have also been described (Grafstein & Murray, 1969; Murray & Grafstein, 1969). Although a continuous process, regeneration can be broken down into discrete events. Following the cut, the distal ends of the axons degenerate. This is followed by regrowth or elongation of the axons concurrent with sprouting of the fibers. The regrowing axons must then locate their appropriate post-synaptic destinations in the target tissue and, finally, synaptic connections must be re-formed.

GANGLION CELL SOMA RESPONSE TO AXOTOMY

Some of the most dynamic morphological changes seen during optic fiber regeneration take place in the ganglion cell soma. This is not surprising since it is the major production site of materials for cell maintenance and axon regrowth. The ultrastructural changes occurring in the cell body, which will be described below, reflect the metabolic alterations and cytoplasmic responses that are necessary for the successful regeneration of the damaged axons.

Between 1 and 4 days following optic tract axotomy the appearance of the ganglion cell soma is similar to an intact cell body although statistical analysis demonstrates that there is an increase in nucleolar volume at this early stage (Murray & Grafstein, 1969). Murray & Forman (1971) examined the time course of structural changes in the ganglion cell soma during regeneration and they found that between 6 and 14 days after axotomy striking changes begin to occur in the cell body. Cellular and nucleolar hypertrophy are evident along with an increase in cytoplasmic basophilia. The swelling of the soma is predominately the result of a marked increase in the free ribosome population, although the number of single profiles of rough endoplasmic reticulum (rER) also increases beginning at about 10 days. During this period the nucleus assumes an eccentric position and the nucleolus appears enlarged and is quite visible. Glial cell bodies in the ganglion cell layer are more frequently found and appear to be closely apposed to the ganglion cell somata. Between 2 and 6 weeks following optic tract cut the ganglion cell soma undergoes further modifications, the most striking perhaps is an increase in both the relative and absolute amounts of rER. The rER is now seen as continuous parallel stacks, and in this distribution, fills most of the cytoplasm. The free ribosome population, grouped in small clusters is still elevated and fills in the gaps between the cisternae, but appears to decline in number towards the end of this period. Groups of neurofilaments and microtubules become visible in the perinuclear cytoplasm. The nucleus remains eccentric and the nucleolus is at its largest. The behavior of the glial elements was not reported during this period. Two months following axotomy, the retinal ganglion cell has begun to recover; cell size and appearance are nearly normal, with the exception of the nucleolus which is still somewhat enlarged. A similar course of events has been documented in the newt (Turner, Delaney & Powell, 1978).

The morphological events occurring in the retinal ganglion cell soma during regeneration directly reflect the changes in protein metabolism that are activated in response to nerve crush. The increased number of free ribosomes seen as early as one week following crush suggest that the first protein changes are slated for use within the cell body, although the possibility exists that early protein production may be implicated in axonal degeneration or may be accumulating in preparation for export to the re-growing axon. The subsequent increase in the amount of rER is associated with enhanced protein production for export to the growing axon tip (Murray & Forman, 1971). These physical changes in the amounts of free ribosomes and rER reflect the changes in protein metabolism which can be visualized through biochemical methods.

In order to examine some of the underlying biosynthetic changes occurring in perikaryal organelles of retinal ganglion cells during regeneration of optic axons, Whitnall & Grafstein (1981, 1982, 1983) looked at tritiated proline incorporation _in vitro_, followed by quantitative electron microscopic autoradiography 6, 14, and 30 days following optic tract injury. They found increased labelling of the nucleus and nucleolus at [illegible] days, but found little, if any change in the amount of rER. Other reports have shown that increases in RNA and protein synthesis begin even earlier at 3 to 5 days following axotomy (Burrell, Dokas & Agranoff, 1978; Heacock & Agranoff, 1976; Murray, 1973). At 14 to 30 days, the absolute volume of rER is seen to increase along with the increase in cell size. Although the relative amount of free ribosomes was greater during all three regenerative periods, the proportion of protein synthesized by the polyribosomes decreased, in contrast to the rER where the total protein synthesized increased during the times examined. Finally, the percentage of total label in the smooth membrane (which is thought to transport material from the rER to the Golgi apparatus) was enhanced, as was the amount of synthesized protein in the Golgi apparatus itself.

The vast majority of the newly synthesized proteins would be logically expected to be slated for export to the growing axon tip (for a review of protein transport see McEwen, Forman & Grafstein, 1971). The synthesis of certain proteins for export from the cell soma is modulated during optic nerve regeneration (Grafstein & Murray, 1969, Murray & Grafstein, 1969). What is unclear is whether selected protein species specified for internal use in the cell body also undergo modification. Heacock & Agranoff (1982) examined protein synthesis in the retina as well as the optic nerve. In their hands those proteins that underwent enhanced production in the retina (eg. tubulin and actin) were the same as those that were later seen to be transported to the tectum. To date, no specific alterations during optic nerve regeneration, which are strictly confined to the retina, have been observed. This does not necessarily mean that they do not exist. Perhaps present techniques are not yet refined enough to detect such small modifications; or possibly the aforementioned changes in protein production are of such magnitude that they mask other smaller shifts that may be occurring.

Since the primary source of the various responses to optic nerve axotomy is the retinal ganglion cell soma, and as the retina is a continuously developing structure in the goldfish, I have chosen to examine if axotomy of retinal ganglion cell axons modulates the normal pattern of cell birth throughout the retina.

II. MATERIALS AND METHODS

Animals

Common goldfish (Carassius auratus) obtained commercially (Mt. Parnell Fisheries, Penn.) were employed for all experiments. They varied in length from six to nine cm and were communally maintained in 100 l aerated aquaria, kept at approximately 22 °C under a 12 hr light/12 hr dark regime. Following surgery, fish were housed in smaller tanks (30-50 l) in groups of five. Animals were fed Tetramin (Hagan Co.) on a daily basis.

Optic Nerve Crush

For all experimental surgeries, fish were anaesthetized with 0.1% tricaine methanesulfonate (MS222; Sigma), sandwiched between two wet sponges and placed into a surgical apparatus. Fresh aerated water was passed through the mouth and gills to keep the animal respirated. For optic nerve crushes, two parallel incisions were made above the ocular orbit, the flap folded back, the eye retracted with straight forceps, and the exposed nerve was gently crushed approximately 1 mm behind the eye with curved forceps. Complete crush was verified in every case by insuring that a transparent segment was visible in the optic nerve sheath, signifying complete transection of the optic fibers. The flap was then replaced and the animal allowed to recover.

Retinal ganglion cell axons exiting from the right eye cross at the chiasm and innervate the left optic tectum. Optic fibers from the left eye innervate the right optic tectum. The decussion at the optic chiasm is complete (Sharma, 1972). Therefore, a unilateral optic nerve crush affects one of the pathways, while leaving the other to serve as an intact control (Figure 1).

Labelling of Proliferating Cell Nuclei

Thymidine is a specific precursor (for deoxyribonucleic acid (DNA)) which is incorporated into DNA in the nuclei of cells during the synthesis (S-)phase of the cell generation cycle. These cells will then undergo mitotic division. Since DNA is stable, the tritium (^{3}H) incorporated into the DNA will remain as a permanent marker unless it is diluted by subsequent mitotic divisions. The nuclei of those cells which have incorporated ^{3}H-TdR can be visualized by autoradiographic methods.

For intraocular injections of ^{3}H-TdR, fish were anaesthetized and placed in the surgical apparatus. The tip of a 10 µl Hamilton syringe was entered into the dorsal most region of the eye at the border between the cornea and eye cup proper. Five µl tritiated thymidine (^{3}H-TdR) in distilled water (1mCi/ml, 6.7 Ci/mmol S.A., New England Nuclear) were injected into the vitreous humor, the syringe was slowly retracted, and the animal was allowed to recover.

Light Microscopic Autoradiography

In preparation for light microscopic autoradiography, fish were heavily anaesthetized with MS222, perfused through the heart with phosphate buffered saline (PBS) and decapitated. Individual eyes were excised, punctured through the cornea, and fixed in Bodian fixative (90 ml 80% ethyl alcohol (EtOH), 10 ml formalin, 10 ml glacial acetic acid) overnight at room temperature. The sclera and choroid coat were then dissected free, the lens and much of the vitreous humor removed and the cleaned retina with pigment epithelium attached was returned to fresh fixative overnight. The following day, the retina was dehydrated (3 x 95% EtOH, 2 x 100% EtOH, 2 x chloroform) and embedded in Paraplast +.

Complete serial sections (16 µm thick) were taken throughout the entire extent of the retina and mounted on subbed slides (5 gm gelatin, 0.5 gm chromium potassium sulfate (chrome alum), 1.0 l distilled water). Slides were air dried overnight and rehydrated through EtOH series the following day to remove the paraffin, in preparation for coating with emulsion. They were then dried overnight in a 40 °C oven. Emulsion was prepared 1:1 in a warm water bath (39 °C; NTB-2 emulsion (Kodak):distilled water) under a sodium lamp. Slides were individually dipped and placed in a rack to dry. The entire rack was placed in a large light-tight box with approximately [illegible]0% humidity for 18 hours. The slides were then loaded into slide boxes (approximately 20 slides/box) with a small amount of desiccant (Drierite), sealed with electrician's tape, wrapped in plastic wrap and again in aluminum foil. The coated slides were exposed in a 4 °C refrigerator for two weeks. At the end of the exposure period, the slides were developed in D-19 (Kodak), washed, fixed (Kodak), and stained with toluidine blue (Fisher Scientific Ltd.) to define cell bodies and thus reveal the laminar organization of the retina. The slides were then dehydrated and coverslipped with Permount

(Fisher Scientific Ltd.) for examination under the light microscope (Zeiss).

Autoradiography Followed by Electron Microscopy

Fish were anaesthetized, perfused, and dissected in the manner described above, using 0.1M cacodylate (CaCo) buffer (pH=7.3) instead of PBS. The retina was removed and immersed in fixative (4% gluteraldehyde, 2.5% paraformaldehyde in 0.1M CaCo buffer; Karnovsky, 1965), at 4 °C for 4 hrs. The retina was cut into small (approximately 1 mm^2) pieces excluding the most peripheral regions, and postfixed in 2% osmium tetroxide for 2 hours, washed and dehydrated through graded EtOH series (50%, 70%, 80%, 90%, 2 x 100%, 2 x 100%:propylene oxide (P.O.), 2 x P.O., P.O.:Epon 812 (Polysciences), 100% Epon 812), embedded in 100% Epon 812, and polymerized in a 60 °C oven for 48 hours.

Thick sections (1.5-2.0 µm) were taken using a glass knife, mounted on subbed slides, and processed as described above for autoradiographic visualization. The only exception was that the exposure time was increased to four weeks to counterbalance for thinness of the section. Slides were stained with toluidine blue and examined under the light microscope. Photographs at 6.3X, 16X, and 40X (objective magnification) were taken of all cells of interest.

Clearly labelled cells from the thick plastic sections were chosen for re-examination under the electron microscope. A fine needle was used to trace the border of the section of interest in order to free the section from the emulsion coat. A small drop of water was placed on the desired section and a razor blade was used to scrape the section free so that it floated on the water droplet. The section was then transferred to a second drop of water on a clean slide, emulsion side down, and a pretrimmed plastic stub in the metal holder was placed on top. This setup was placed on a warm hot plate in order to evaporate the water and secure the section to the stub. Following mounting on the stub, the labelled cell was still visible under the microtome microscope. The block was trimmed and thin serial sections (90 nm) were placed on single-slit, Formvar coated grids, stained in uranyl acetate and lead citrate (Reynolds, 1963), and examined under the electron microscope (Henken & Chernenko, in press).

Data Collection

Serial sections were made throughout the entire extent of the retina in the equatorial plane (Figure 2). The total number of labelled cells was recorded for every fifth serial section, moving from peripheral to more central retina. On average 20 sections were tabulated for each retina with a range of 15 for the smallest to 30 for the largest eye. A cell was considered labelled if at least ten silver grains were visible in the emulsion coat overlying the nucleus. The average background activity was approximately 0.1 silver grains per nucleus. Labelled cells were scored according to their laminar location in one of three retinal categories: photoreceptor layer (outer nuclear layer; ONL), horizontal/amacrine/bipolar/Müller cell layer (inner nuclear layer; INL), or deep layer (retinal ganglion cell layer and optic fiber layer; GC/OFL; See Figure 3).

Preliminary results following intraocular injection of tritiated thymidine illustrated that labelled cells were seen in all nuclear laminae of the retina (see Figure 3). Topographical location was recorded automatically as part of the data collection procedure (see Figure 1). Two extreme retinal locations, at the peripheral margin and the central area around the optic disc were purposely excluded from the analysis. Accurate cell counts using light microscopic autoradiography were impractical in the ora terminalis for two reasons. First, laminar structure could not be defined. Figure 4 shows an example of labelled cells within the ora terminalis in a para-axial section (see Figure 2) through the retina. In contrast to the retinal section shown in Figure 3, it is impossible to distinguish the different layers in the ora terminalis (Figure 4). In the lower regions of the micrograph, the three nuclear layers are identifiable. As one moves from central to peripheral retinal regions (towards the top of the figure), laminar identification becomes progressively more difficult, until in the ora terminalis, it becomes impossible. The second reason for exclusion of the ora terminalis was due to the fact that individual tagged cells could not be distinguished because of the dense sheet of labelled nuclei covering the region. Figure 4B shows an example in which heavy labelling masks the underlying cells. Even if it were possible to define specific laminae, the intensity of the label would obscure any possible identification of the underlying individual cell nuclei.

The second region to be excluded from these studies was the most central portion of the retina. In the central retinal regions the optic fiber layer is relatively thick as all optic fibers, from the most peripheral to the most central, must converge and exit by this route.

As a result of the increased thickness of this region, an estimation of the linear density of labelled cells in the GC/OFL would be misleading. (see Figure 5A). In Figure 5B, an equatorial plane of section of the area near the optic disc is presented. This autoradiograph illustrates the marked increase in the width of the GC/OFL. Cell counts would be exaggerated (if the entire region was included), or subjective (if an arbitrary limit of the region was enforced). To minimize this possible skewing factor, a retinal ring with a circumference of less then 5 mm was excluded from the analysis.

Since serial sectioning proceeded from peripheral to central retina (Figure 2), topographical location was automatically recorded. The circumference of each retinal ring was measured along the inner nuclear layer and this measurement was used to normalize the cell counts into linear densities. These data were then expressed as number of labelled cells per 10 mm. A linear density measurement more accurately reflects the size of the cell population as the area from which the density is taken substantially decreases as one proceeds from peripheral to central retina. Both the axotomized retina and the intact retina for each fish were analyzed and all comparisons made within the same fish.

Data Analysis

The average number of labelled cells per 10 mm was graphed for each section of each retina for each fish. In addition, the circumference examined for each section was graphically represented. The mean linear density of labelled cells/10 mm for each retina in a given fish was calculated by:

$$A = \frac{\sum_{i=1}^{n} a_i}{n} \qquad (1)$$

$$C = \frac{\sum_{i=1}^{n} c_i}{n} \qquad (2)$$

where a_i is the number of labelled cells/10 mm in the i^{th} section of the axotomized retina, c_i is the number of labelled cells/10 mm in the i^{th} section of the contralateral control retina, and i denotes the sequence of equatorial retinal sections in the serial study (see Figure 2). In the cases where intact fish are examined, A refers to the right eye and C refers to the left eye. The mean linear density indicates the overall density of the labelled cell population for each retina.

An equatorial section from the axotomized retina and one from the control retina were considered to be equivalent by virtue of their similar topographical location as determined by their circumferential length (see Figure 2). In those cases where a wedge of retina was lost during processing, or oblique sections were initially taken, the extrapolated perimeter was used in judging equivalent sections. The ratio of cell proliferation (r_i) between the experimental and control retinae for the i^{th} pair of equivalent sections was defined as:

$$r_i = \frac{a_i}{c_i} \qquad (3)$$

The ratios thus obtained [illegible]en the experimental and control sections were recorded for all fish at a given experimental time following axotomy. For any given experimental time following optic nerve crush retinae from three to six fish were examined. The mean value <R> of the ratios of individual pairs of equivalent sections for a given post-operative period was calculated by:

$$\langle R\rangle = \sqrt[n]{r_1 \times r_2 \times r_3 \cdots r_n} \quad (4)$$

$$\ln\langle R\rangle = \frac{\sum_{i=1}^{n} \ln r_i}{n} \quad (4')$$

where ln is the natural logarithm ($\log_e$). All ratios were converted into ln values and the mean value of the ratios of equivalent sections was calculated by Eq.(4'). The ln<R> value was used for statistical analyses.

Analyses of variance (ANOVAs) were conducted to test various hypotheses concerning changes in the linear densities of labelled cells as functions of time following axotomy and topographical location. The values of ln<R> were graphed as a function of time following unilateral optic nerve crush. Standard error bars at each mean were used to represent the relative variability of each ln<R> at each time condition. More detailed comparisions between individual time conditions were made using Student's t-tests. To employ multiple t-tests, the probability of finding significance as a function of the number of comparisons involved must be taken into account. The probability level of .05, therefore, must be divided by the total number of t-tests executed; which is known as Bonferroni's or Dunn's t-test procedure. In no case were more than 30 t-tests performed. Therefore, a reliability level of .001 was considered the appropriate level of acceptance in all statistical analyses involving Student's t-tests.

Figure 1. Diagram of Experimental Procedure

This is a schematic of the paradigm employed for the studies examining the effects of axotomy on cell proliferation observed 1 day (short survival) or 1 month (long survival) following ^{3}H-TdR injection. An optic nerve crush was performed on one eye (axotomized), while the other eye was left intact (control). Because of the near total decussation of the optic fibers at the chiasm, each fish could serve as its own control. At various days following axotomy, ^{3}H-TdR was introduced intraocularly in both eyes. Either one day or one month following injection, the fish was perfused and both eyes processed for light microscopic autoradiographic examination.

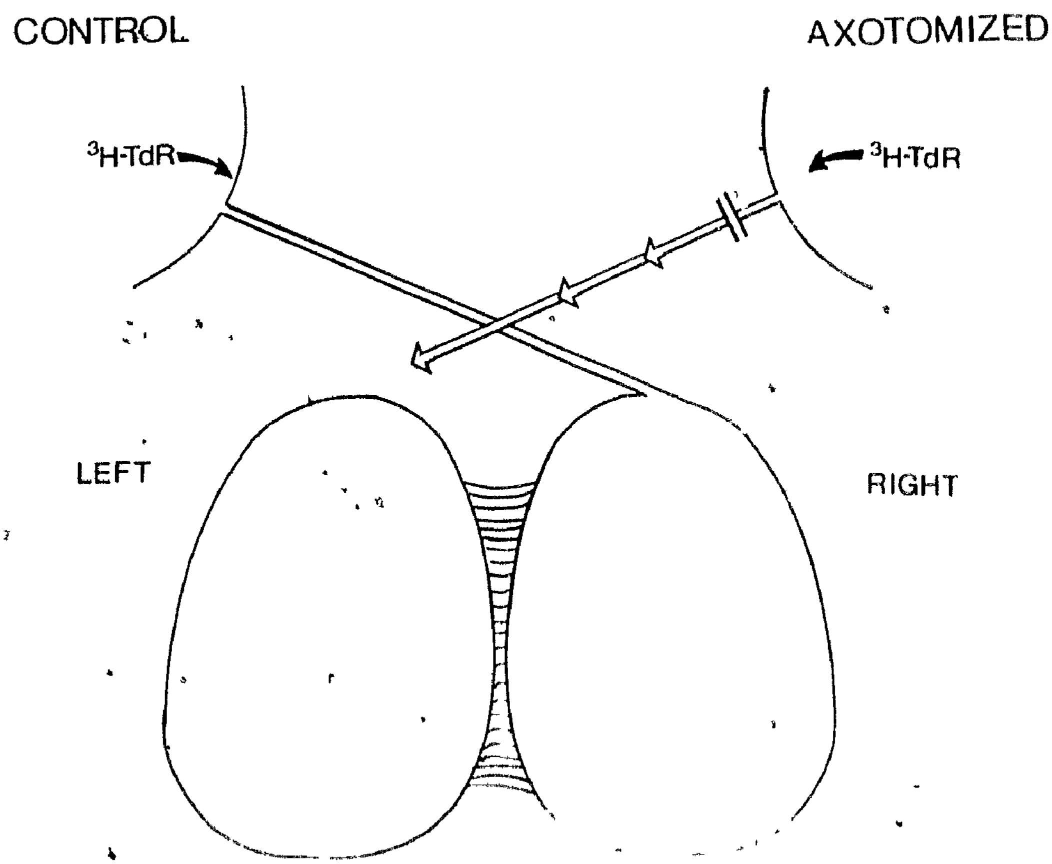

CONTROL
AXOTOMIZED
³H-TdR
³H-TdR
LEFT
RIGHT

Figure 2. Illustration of Planes of Section

This diagram illustrates the two types of sections of retinal tissue that are referred to in this thesis.

A para-axial section is parallel to the optic nerve axis and contains all topographical retinal regions from most peripheral to most central.

An equatorial section is perpendicular to a para-axial section and thus appears as a ring of retinal tissue when examined. Serial equatorial sections were taken throughout the retinal extent, where section i=1 is the most peripheral, and i=n is the most central topographical retinal ring.

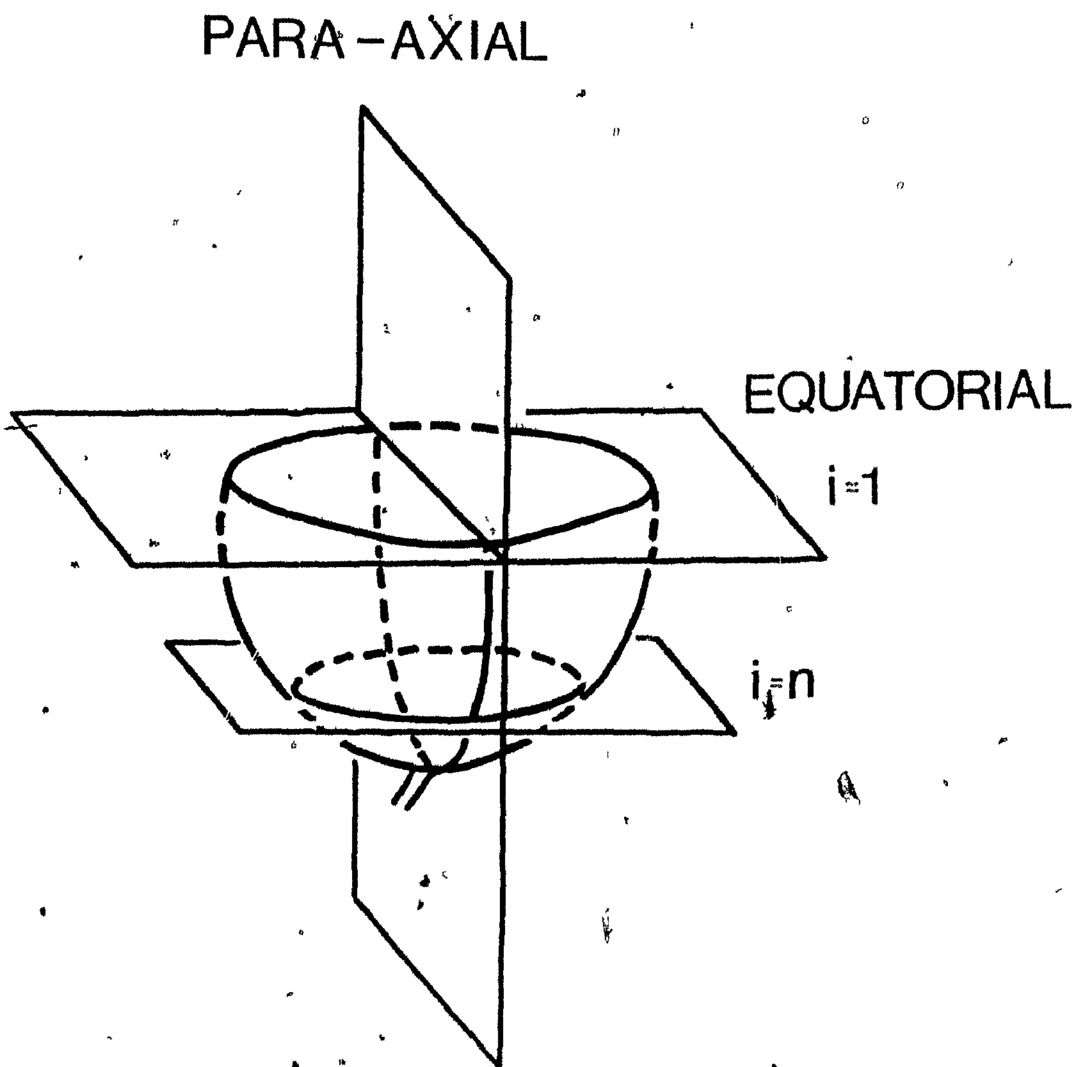

PARA-AXIAL
EQUATORIAL
i=1
i=n

Figure 3. Labelling in Retinal Laminae

Light microscopic autoradiograph of an equatorial section of axotomized retina from a fish that received an unilateral optic nerve crush, 10 days prior to injection of tritiated thymidine. The fish was sacrificed 1 day later. The light autoradiograph illustrates that following intraocular injection of ^{3}H-TdR, labelled cell nuclei are found in all retinal laminae.

Abbreviations: PE- pigment epithelium, ONL- outer nuclear layer (where photoreceptor cell bodies are located), INL- inner nuclear layer (where amacrine, bipolar, horizontal and Muller cell bodies reside), GC:OFL- ganglion cell:optic fiber layer (where retinal ganglion cell somata and glial cell bodies are found). Calibration bar: 100 µm.

Inserts illustrate silver grains located in the emulsion coat overlying the nuclei. Note the density of grains when the fish is sacrificed one day following administration of ^{3}H-TdR. Calibration bar: 25 µm.

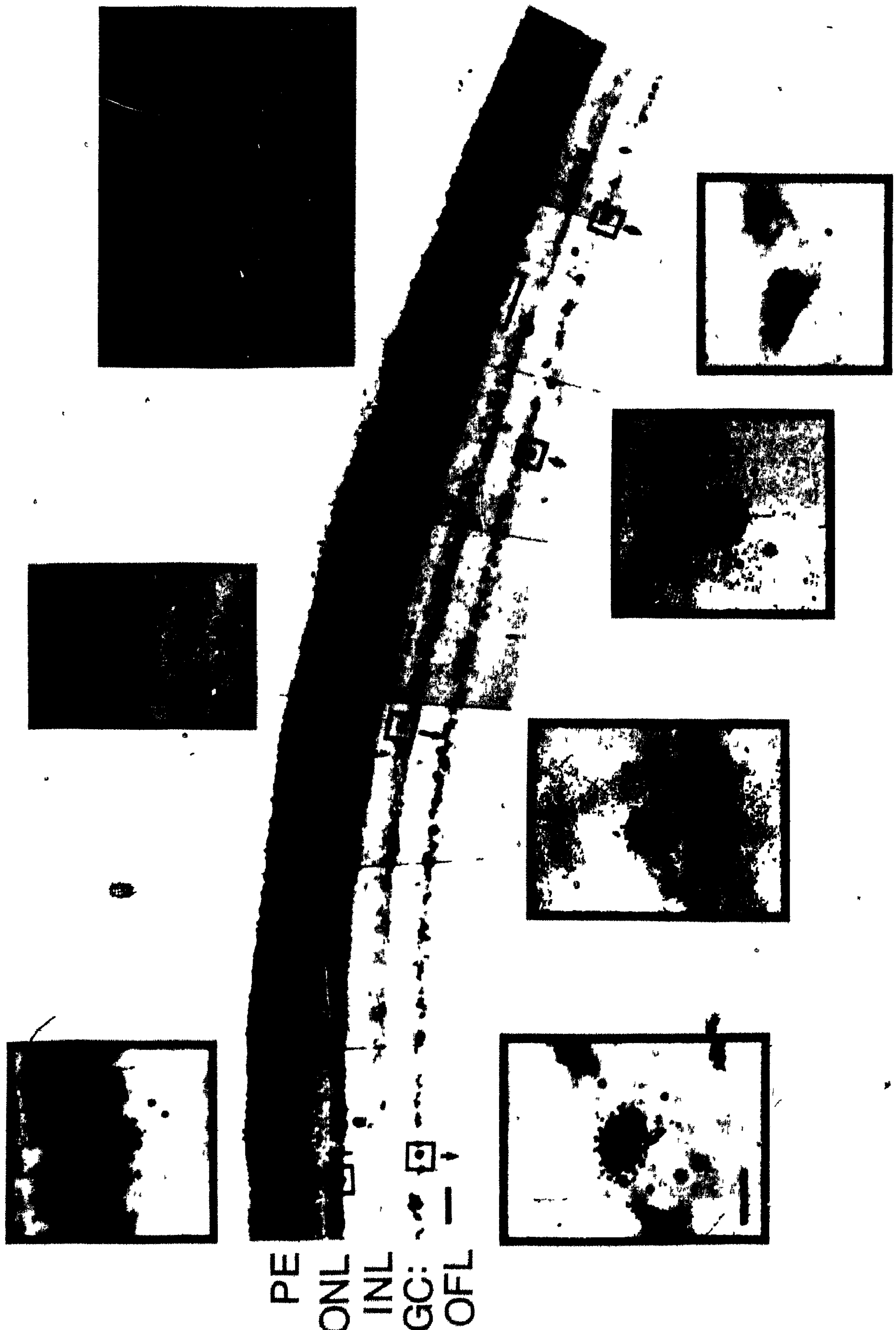

PE
ONL
INL
GC:
OFL

Figure 4. Ora Terminalis- Para-axial View

A. Light microscopic autoradiograph of a para-axial section through the *ora terminalis* and the retinal regions immediately central to it. At the bottom of the page, in the more central portion of the retina, laminar definition is clear. In the more peripheral regions (towards the top of the page), laminar differentiation is not yet complete. Abbreviations are the same as in Figure 3. Calibration bar: 100 µm.

B. Inset of the boxed region seen in A. Note the heavy label obscuring individual cells. Calibration bar: 50 µm.

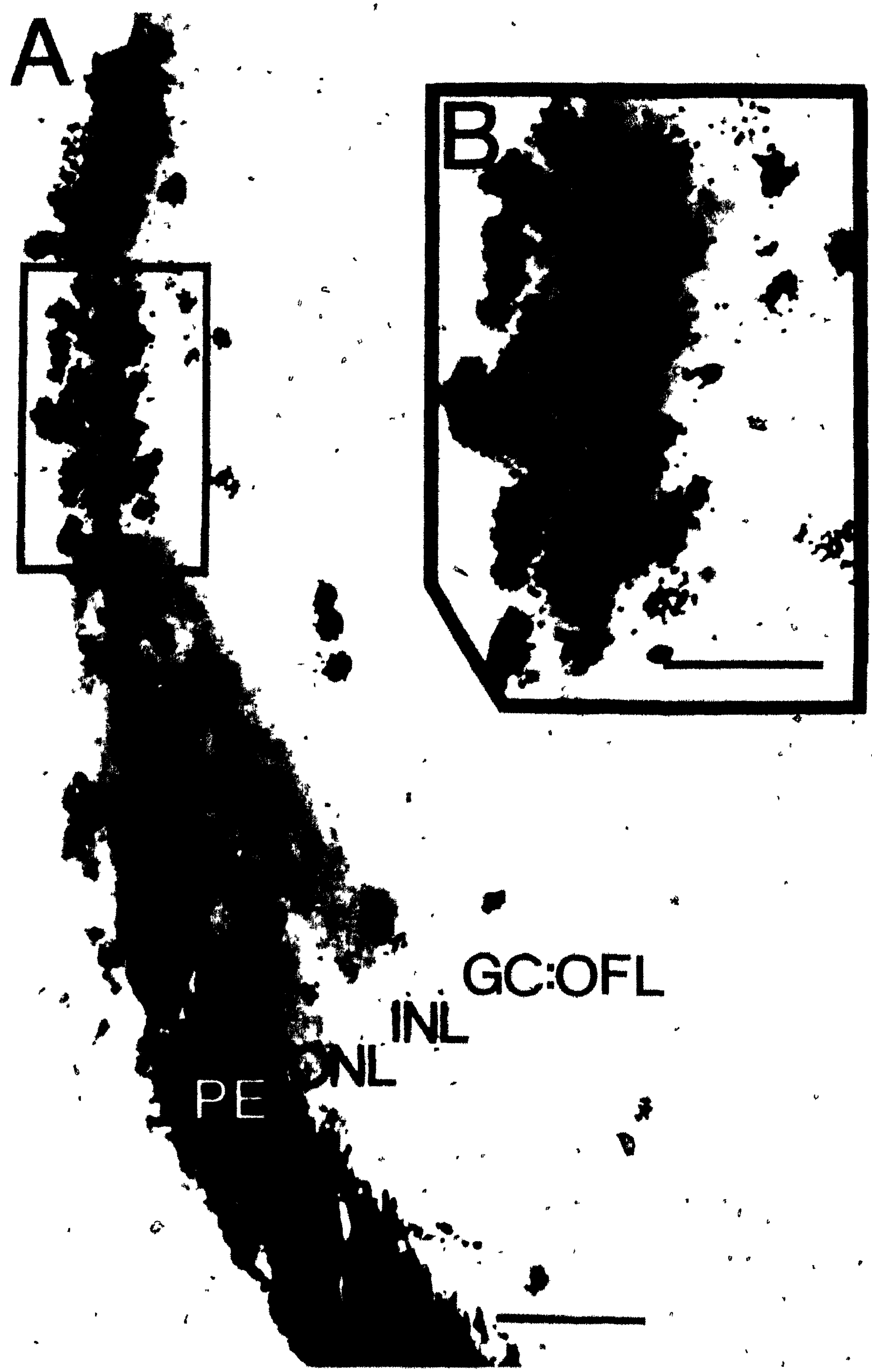

A
B
GC:OFL
INL
ONL
PE

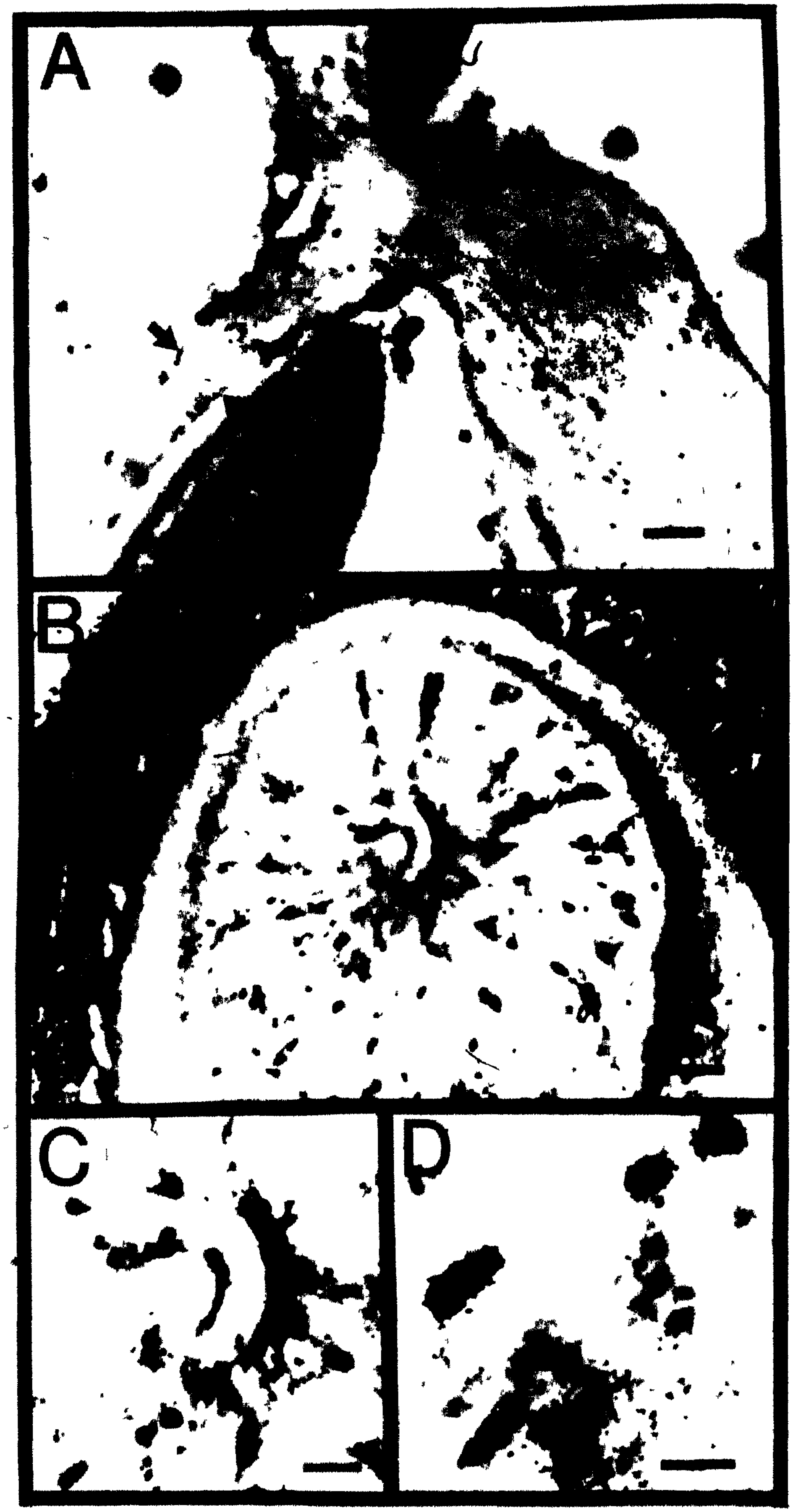
Figure 5. Area Near the Optic Disc- Para-axial and Equatorial Views

A. A para-axial section through the central region of the retina illustrating the thick GC/OFL (between arrows) and the convergence and exit of the optic fibers from the retina. Note the labelled cells in both the GC/OFL and in the optic nerve head. Calibration bar: 100 µm.

B. Equatorial section of the area near the optic disc. Note the width of the GC/OFL and the abundance of labelled nuclei. Calibration bar: 100 µm.

C. Enlargement of the optic disc, illustrating the heavy labelling. Calibration bar: 50 um.

D. Enlargement of individual cells depicting silver grains in the emulsion coat overlying the nuclei. Calibration bar: 25 µm.


A
B
C
D

III. CELL PROLIFERATION IN THE GANGLION CELL AND OPTIC FIBER LAYERS

In this experiment, I quantified normal cell proliferation in the GC/OFL and examined how optic nerve axotomy modulates it at various post-operative periods.

Furthermore, I tested whether the survival time following injection of tritiated thymidine influences the number of labelled cells by quantifying the tagged cell population either 1 day or 1 month following injection.

EFFECTS OF OPTIC FIBER AXOTOMY ON CELL PROLIFERATION IN THE GC/OFL EXAMINED 24 HOURS AFTER THYMIDINE INJECTION

To observe the topographic distribution of labelled cells in the GC/OFL in normal fish, and to illustrate that the two retinae of a single fish do not differ in any appreciable way, four fish received bilateral intraocular injections of ^{3}H-TdR. Furthermore, to insure that physical trauma itself did not affect normal cell proliferation two of these fish received sham optic-nerve crushes. This surgical intrusion mimicked the procedure of nerve crush, without actually injuring the nerve in any way. Fish were sacrificed 24 hours after ^{3}H-TdR administration. All fish used in these experiments ranged between 5 and 7 cm in length from nose to tail base.

In order to examine if axotomy modulates the number of cells that are actively synthesizing DNA in the retina, I performed bilateral intraocular injections of ^{3}H-TdR in seven groups of fish at various post-operative periods: 5, 10, 15, 20, 25, 35, or 50 days following unilateral optic nerve crush. The numbers of fish in each experimental group were 5, 5, 6, 4, 4, 4, and 4, respectively. After waiting 24 hours following the injection, fish were sacrificed and their retinae processed for light autoradiography as described previously.

RESULTS

a. Normal (GC/OFL-N(SS))

Figure 6 shows the patterns of labelling across topographical regions obtained from four intact fish that were sacrificed 24 hours after thymidine injection. In this figure and in all following graphical representations of individual fish, the following conventions are employed. The abscissa represents the serial sections examined, where in biological terms, section 1 is the most peripheral, or the youngest ring of retina (excluding the ora terminalis). The following sections taken at 80 um intervals represent progressively more central regions of the retina. In the upper of each pair of graphs, A-D, the ordinate refers to the linear density of labelled cells, while in the lower graph of each pair, the retinal circumference from which the cell counts were made is presented. As was described above, no section was counted with a circumferential ring of less then 5 mm. The term "short survival" in the figure heading refers to a 1 day survival period following ^{3}H-TdR injection. In later experiments, this will be contrasted with a "long survival" interval, which refers to a survival interval of 30 days. The dashed line represents the right eye and the solid line, the left eye.

Figure 6A illustrates the topographical distribution of labelled cells from a fish that received a sham-right ONC. The mean linear density of labelled cells/10 mm in the GC/OFL of the right retina is 40.4 and that of the left retina is 36.4. There appears to be little variation between the labelling pattern in the two eyes. In Figure 6B the data compiled from a fish that underwent a sham-left ONC, are presented. The mean linear densities of labelled cells/10 mm for this fish are 20.9 for the right and 21.3 for the left retina. This pattern is similar to that seen in 6A, that is, there are relatively consistent densities of labelled cells between the two eyes with a slight increase in the more central topographical regions. Figures 6C and 6D illustrate the labelling distribution found in intact fish, sacrificed 1 day following intraocular thymidine injection. Both appear to have equivalent labelling across most of the retina with the exception of the most central region. At this location, a slight increase in the density of labelled cells is evident. The mean linear densities of labelled cells/10 mm are 8.7 and 14.1 for fish C, and 17.6 and 15.1 for fish D. The slight increase in the numbers of labelled cells in the most central retinal regions is most likely a reflection of the increased thickness of the GC/OFL. Although a retinal ring with less than a 5 mm circumference was excluded, this precaution may not have entirely eliminated the skewing factor of the increasing width of the layer itself.

b. Five Days Following Unilateral Optic Nerve Crush (GC/OFL-D5(SS))

When fish received bilateral intraocular injections of tritiated thymidine 5 days following unilateral axotomy, and the labelling patterns of equivalent sections from both eyes are compared, there is a clear enhancement in the number of labelled cells in the axotomized eye (see Figure 7). In these graphs and in those to follow, the dashed line represents the axotomized eye, while the solid line refers to the intact, control eye of the same fish. In Figure 7A, there is a clear increase in the numbers of cells in the GC/OFL of the axotomized eye as compared with the control eye throughout the topographical gradient. The mean linear density of labelled cells/10 mm in the axotomized eye (A) is 59.1, while only 14.8 labelled cells/10 mm are seen in the control eye (C). Although there is some variation between adjacent sections, the enhanced labelling in the axotomized retina is evident. A similar labelling pattern is seen in another fish as shown by Figure 7B, where A=57.4 and C=12.5. There is also a tendency in toward enhanced labelling in the more central retinal regions. This trend reoccurs to various degrees as can be witnessed in Figures 7C-E. The mean linear densities for these three fish are A=112.9 vs. C=59.0, A=131.9 vs. C=83.3, and A=220.5 vs. C=75.5, respectively. For these three fish, there are clearly more labelled cells in the axotomized GC/OFL of the retina. In addition, enhanced labelling in the central retinal regions is evident. As the equatorial sections become more centrally located, the thickness of the GC/OFL increases because of the increased number of retinal fibers exiting the retina. Associated with this increased fiber density is an increase in the number of non-neuronal elements. Although I attempted to curtail this skewing factor by setting an arbitrary cut off point of a 5 mm circumference, the increased thickness of the GC/OFL is still a relevant point, as can be seen by the increased labelling in the intact retina. It appears, however, that labelling is even more pronounced in the axotomized retina in the central topographical regions.

c. Ten Days Following Unilateral Optic Nerve Crush (GC/OFL-D10(SS))

The results obtained from five fish that had ^{3}H-TdR introduced bilaterally 10 days following unilateral optic nerve crush are presented in Figure 8. The pattern of labelling in the GC/OFL between the two eyes is very similar to that seen on day 5 (GC/OFL-D5(SS)). In all sections, there are more labelled cells in the GC/OFL of the axotomized retina. In Figure 8A, mean linear density for the axotomized GC/OFL (A) is 43.5 and for the intact GC/OFL (C) is 10.5. As one moves from peripheral to central retina, the linear density increases in the axotomized eye. As can be seen in Figures 8B-E, similar patterns emerge in all fish under this experimental regimen. The mean linear densities for these fish are A=34.4 vs. C=15.0, A=111.1 vs. C=23.5, A=30.4 vs. C=10.3, and A=62.8 and C=24.6, respectively.

d. Fifteen Days Following Unilateral Optic Nerve Crush (GC/OFL-D15(SS))

Fifteen days following unilateral ONC, more labelled cells are seen in the axotomized GC/OFL as compared with its intact control, when fish are sacrificed 1 day following administration of ^{3}H-TdR. In Figure 9, the individual data from six fish are presented. For the fish illustrated in Figure 9A, the mean linear density of labelled cells/10 mm in the axotomized GC/OFL is 48.1. In its contralateral control only 27.6 tagged nuclei/10 mm are found. There is some overlap between certain topographical comparisons (eg. sections 1, 5, and 15), but overall the pattern is similar to that seen on D5 and D10, but at a depressed level. Again, there is a clear increase in labelled cell density in the most central regions in the axotomized eye. Figure 9B represents another such fish. The overlap is more pronounced (eg. sections 10, 50, 55, and 60), but the general result remains the same. The mean linear densities for this fish are A=91.3 and C=66.5. The fish represented by the graphs in Figures 9C-F, have similar configurations. In general, there are more tagged nuclei in the axotomized GC/OFL; the mean linear densities of labelled cells/10 mm for these fish are A=42.3 vs. C=18.4, A=48.2 vs. C=28.9, A=37.7 vs. C=24.6, and A=74.7 vs. C=52.4. There are however, certain regions of overlap (eg. Figure 9C, section 10; 9D, sections 5, 10, and 80; 9E, sections 10, and 20; 9F, sections 1, 10, 40, and 45). Overlap was rarely seen at days 5 (GC/OFL-D5(SS)) and 10 (GC/OFL-D10(SS)) following unilateral axotomy. A visual comparison between the labelling patterns of individual fish at D5, D10, and D15 suggests that the differences at D15 are less substantial than that seen at the earlier times. The mean linear density measures support this result. There is still a noticeable increase in labelling density in the most central topographical regions.

e. Twenty Days Following Unilateral Optic Nerve Crush (GC/OFL-D20(SS))

Twenty days following unilateral axotomy, more labelled cells are seen in the axotomized GC/OFL, although the overall pattern between the four fish is not as consistent as for previous groups of fish. In Figure 10A, the first of these four fish is represented. There are clearly more labelled cells in the GC/OFL of the axotomized fish. No overlap is evident between the two eyes. The mean linear density of labelled cells/10 mm for the axotomized GC/OFL is 119.3, while 69.3 labelled cells/10 mm are found in the intact eye. The trend is similar to that seen previously- a clear enhancement in labelling density as one moves from peripheral to central retinal regions. Figure 10B depicts a fish with more overlap between the two retinae. Although there are more labelled cells in the GC/OFL of the axotomized retina (61.0 labelled cells/10 mm) as compared with the intact GC/OFL (52.7 labelled cells/10 mm), this difference is not as pronounced as seen in the fish illustrated in Figure 10A. This fish looks similar in distribution to a normal fish. In Figure 10C, a compromise between the two labelling distributions is evident. The mean linear densities of the axotomized and control layers are A=45.7 and C=21.9. There is a clear upward trend in the more central topographical regions. In Figure 10D, the last fish to be included in this post-operative group is presented. Although there are more labelled cells in the axotomized GC/OFL (45.7 labelled cells/10 mm) as compared with the control GC/OFL (31.1 labelled cells/10 mm), there is some overlap, no increased density of label in central topograhical regions, and in general, this fish appears quite similar in configuration to the fish represented in Figure 10B.

Although there are more pronounced individual differences between the members of this post-operative group, the overall result is similar to that seen in D15 (GC/OFL-D15(SS)). The statistical analyses to be presented in the following sections confirm this observation.

f. Twenty-five Days Following Unilateral Optic Nerve Crush (GC/OFL-D25(SS))

Twenty-five days following unilateral optic nerve crush, labelling differences in the GC/OFL appear to be returning to normal levels. In

Figure 11A, the mean linear densities of labelled cells/10 mm are A=44.8 and C=28.6. There is still a tendency toward a higher degree of labelling in the central topographical areas. In Figure 11B, a reverse trend is seen for the first time- consistently more tagged nuclei are found in the control eye. The mean linear density for each retina is 17.6 (axotomized) and 30.1 (control). Figure 11C represents a fish with no difference in mean linear densities between the two eyes (A=85.4 and C=86.0). The overlap between the two retinae is pronounced, as is the clear increased trend in labelling density from peripheral to central retinal regions in both eyes. In Figure 11D, a less intense-version of 11C is depicted. Overlap between the two eyes is great and the trend toward increased cell labelling as one moves more centrally in the retina is also evident, but to a lesser degree. The linear density measurements for this fish are A=35.2 and C=39.9.

g. Thirty-five Days Following Unilateral Optic Nerve Crush (GC/OFL-D35(SS))

By 35 days following unilateral axotomy, differential labelling between the GC/OFLs of the axotomized and intact retinae have almost disappeared. In Figure 12, four fish are illustrated. As is immediately apparent, there is great overlap between the labelling of the two retinae of a single fish. The only exception is Figure 12B, where there is no overlap, and a consistent tendency for more tagged cells in the axotomized eye. The mean linear densities for this fish are A=26.3 and C=14.7. For the fish presented in 12A, A=9.2 and C=8.8. For fish 12C and 12D, the mean linear densities are A=33.5 vs. C=43.2, and A=28.0 vs. C=24.7, respectively. There does not appear to be any increased tendency for a higher labelling density in more central topographical regions.

h. Fifty Days Following Unilateral Optic Nerve Crush (GC/OFL-D50(SS))

Figure 13 illustrates the topographical labelling pattern in the GC/OFL of fish 50 days following unilateral ONC. When the animals are sacrificed 1 day after administration of ^{3}H-TdR, no differential labelling is seen between the two retinae of a fish in the majority of cases (Figures 13A, 13B, and 13D). The only exception can be seen in Figure 13C. In this case there are more labelled cells in the axotomized GC/OFL, although there is much variability and a considerable amount of overlap. The mean linear densities for this fish are A=74.2 and C=55.2. There is also the tendency for increased labelling in the more central retinal regions. In Figures 13A, 13B, and 13D, the labelling patterns appear almost identical to those seen in normal fish (GC/OFL-n(SS)), (see Figure 6 for comparison). Relatively low labelling is found in both eyes of a single fish. The mean linear densities for these fish are A=7.7 vs. C=10.2, A=27.6 vs. C=32.8 and A=15.5 vs. C=16.4, respectively.

DISCUSSION

In order to test whether there are any differences in the linear densities of labelled cells between the two retinae of normal fish, Student's t-tests were executed on the individual data from four fish. None of these ratios reached statistical significance ($F(1,46)=2.46$, $p>.001$; ($F(1,32)=.020$, $p>.001$; $F(1,54)=8.46$, $p>.001$, and ($F(1,40)=.90$,

$p>.001$, for 6A-D respectively). This result indicates that if intact fish are sacrificed one day following intraocular administration of tritiated thymidine, there is no difference in density of labelled cells between the GC/OFLs of a single fish. Furthermore, surgical intervention does not seem to affect normal labelling patterns. Finally, although differences between fish are relatively high, differences between the two eyes of a single fish are not significant. Therefore, a comparison between a pair of equivalent retinal sections from the same fish is a valid method for analyzing possible modulatory effects of unilateral axotomy of optic fibers on cell proliferation. When each ratio (r_i) between two equivalent i^{th} sections was converted to its ln value ($\ln r_i$) in the intact control group, a total of 90 comparisons yielded a $\ln\langle R\rangle$ value of -0.2039, with a SE of 0.056.

To test the hypothesis that the density of labelled cells varies as a function of time following axotomy, an ANOVA was run across the $\ln\langle R\rangle$ values from the intact and six experimental groups (D5, D10, D15, D20, D25, D35, and D50). The linear density of labelled cells varied with time following unilateral optic nerve crush ($F(7,716)=94.717$, $p<.001$). Individual Student's t-tests were calculated to determine the source of the difference.

A significant enhancement in cell proliferation was seen in the GC/OFL of the axotomized retina as early as 5 days following ONC (see Figure 7). Analysis of 112 observations from five fish showed that $\ln\langle R\rangle=1.0985$ with a SE of 0.053. A similar result was seen 10 days following nerve crush (see Figure 8). The value of $\ln\langle R\rangle$ was 1.0993 with a SE of 0.048. For these five fish, a total of 111 observations were recorded. T-tests revealed no difference between D5 and D10, ($F(1,221)=2.23$, $p>.001$), but both were significantly different from the intact control ($F(1,200)=281.57$, $p<.001$ and $F(1,199)=316.8$, $p<.001$, respectively). By D15 the difference in labelling densities between the axotomized and control retinae had begun to decline. Six fish were examined, resulting in 117 individual $\ln r_1$ values (see Figure 9); by Eq. 4', $\ln\langle R\rangle=0.4872$ with a SE=0.037. Twenty days following axotomy appeared similar to that seen at D15 (see Figure 10). The $\ln\langle R\rangle$ value for the 77 observations on 4 fish was 0.5213, SE=0.064. There were no differences between labelled cell ratios at days 15 and 20, ($F(1,192)=0.24$, $p>.001$). Both, however, were statistically different from normal ($F(1,205)=111.94$, $p<.001$ and $F(1,165)=73.4$, $p<.001$, respectively), and from the results seen on days 5 and 10 (D5 vs D15: $F(1,227)=90.4$, $p<.001$; D10 vs D15: $F(1,226)=103.4$; $p<.001$; D5 vs D20: $F(1,187)=48.7$, $p<.001$; D10 vs D20: $F(1,186)=55.2$, $p<.001$). By D25, labelling in the GC/OFL had appeared to return to normal levels (see Figure 11). Of the four fish analyzed, 73 observations were tabulated. A $\ln\langle R\rangle$ value of -0.0898, SE=0.052 was calculated, which was not different from normal values ($F(1,161)=2.13$, $p>.001$). At 35 days post-surgery, 77 ratios were gathered from four fish, resulting in a $\ln\langle R\rangle$ value of 0.0792, SE=0.073 (see Figure 12). This result was not different from that seen on D25 ($F(1,148)=1.53$, $p>.001$). Finally, at 50 days following axotomy, an $\ln\langle R\rangle$ value of -0.0445, SE=0.067 was calculated (see Figure 13) from four fish. This value was not distinct from that seen on D35, D25, or normal animals ($F(1,142)=1.54$, $p>.001$, $F(1,138)=.29$, $p>.001$, and $F(1,155)=3.35$, $p>.001$, respectively).

In Figure 14A, a summary of these results is presented. The abscissa is marked by days following unilateral optic nerve crush. The right ordinate is the mean value of the ratio (axotomized:control) of labelled cells (<R>) and the left ordinate is the value of ln<R>. In brackets, the number of observations followed by the number of fish examined are presented. Error bars represent the standard error of ln<R>.

The major conclusion from these data is that optic nerve crush affects the normal numbers of cells incorporating ^{3}H-TdR in the GC/OFL layer. The magnitude of the difference is dependent upon the time following axotomy. As early as five days following axotomy, enhanced thymidine incorporation into new DNA is evident in the GC/OFL of the axotomized retina. The enhanced linear density of labelled cells remains high for up to 10 days following axotomy, then gradually returns to normal levels.

The linear density of labelled cells appeared to increase in the more central topographical regions. An additional ANOVA was executed in order to determine whether ln<R> differed significantly as a function of topographic location. Data were grouped into peripheral, middle, and central locations (resulting in three ln<R> values for each fish) and analyzed across times following axotomy. Significant differences were seen between groups at various post-operative periods ($F(7,28)=6.827$, $p<.001$), and between topographical location ($F(2,56)=8.893$, $p<.001$). This difference is a reflection of the increased thickness of the GC/OFL in more central topographical regions. There were no reliable interactions ($F(14,56)=2.368$, $p>.001$).

Absolute identification of the cells involved in these events has not been made, although ultrastructural observations suggest that they are non-neuronal in nature (see Figure 15). Evidence for this claim will be presented in a description of the somal morphology of the labelled cells.

EFFECTS OF OPTIC FIBER AXOTOMY ON CELL PROLIFERATION IN THE GC/OFL EXAMINED 30 DAYS AFTER THYMIDINE INJECTION

To examine what proportion of the initially labelled cells retains the label in normal fish, four fish were injected intraocularly with ^{3}H-TdR and returned to their tank. One month later, they were processed for light microscopic autoradiography as described above. To assess the effect of optic nerve crush on cell proliferation examined 1 month following ^{3}H-TdR injection, bilateral injections were performed on fish at various post-operative periods: 5, 10, 15, 20, 25, or 50 days following unilateral ONC. Four fish were examined in each group, with the exception of 25 days in which 3 fish were recorded. One month later, these fish were sacrificed and processed as described above.

RESULTS

a. Normal (GC/OFL-N(LS))

One month following intraocular thymidine injection, no differences are seen between the two retinae of a single fish. The mean linear densities of the number of labelled cells/10 mm are A=9.0 vs. C=6.1, A=10.7 vs. C=10.6, A=38.6 vs. C=33.1, and A=1.3 vs. C=1.0.

b. Five Days Following Unilateral Optic Nerve Crush (GC/OFL-D5(LS))

Five days following unilateral axotomy, more tagged nuclei are found in the GC/OFL of the axotomized retina, when fish are sacrificed 30 days after ^{3}H-TdR injection. For two of these fish, labelling is low as can be seen by their mean linear densities of A=2.0 vs. C=1.6 and A=2.5 vs. C=1.6. The label appears evenly distributed across topographical regions. In two other fish, the linear densities of labelled cells are slightly higher (A=9.3 and C=3.9). The mean linear density of labelled cells/10 mm is higher in the GC/OFL of both retinae in the peripheral retinal regions and decreases toward the more central regions. In the last fish of this post-operative group, a different topographical trend is evident. In the GC/OFL of the axotomized eye the density of label increases in an almost linear fashion from peripheral to central retina, while in the non-axotomized eye, the density of the labelled cell population appears to remain constant across the topographical gradient. The mean linear densities for this fish are A=57.0 and C=14.9.

c. Ten Days Following Unilateral Optic Nerve Crush (GC/OFL-D10(LS))

The number of labelled cells/10 mm is low in all fish examined ten days following axotomy. Frequently, no labelled nuclei are seen in a section. No topographical gradients are evident. No consistent differences in labelling density between the axotomized and control retinae are seen. The mean linear density measures for these fish are A=2.5 vs. C=5.1, A=0.8 vs. C=0.5, A=4.6 vs. C=8.9, and A=8.9, vs. C=13.6.

d. Fifteen Days Following Unilateral Optic Nerve Crush (GC/OFL-D15(LS))

A similar result to that seen at D10 (GC/OFL-D10(LS)) following unilateral axotomy is found 15 days following crush. In two of the fish examined, labelling is low as is evidenced by mean linear densities of A=1.4 vs. C=1.9 and A=0.9 vs. C=1.2. In these two fish, sections are often devoid of label. In another fish, the density of labelled cells in the GC/OFL of the axotomized eye is 28.1 labelled cells/10 mm, while an equivalent linear density is seen in the GC/OFL of the control (31.5). Both retinae appear to have more label toward the central retinal regions. In the last fish, extremely variable labelling is seen in the GC/OFL of the axotomized eye. For example, in one section, 24.8 labelled cells/10 mm are found, followed by a section in which only 2.9 labelled cells/10 mm are present. For this fish, A=33.1, and C=13.7.

e. Twenty Days Following Unilateral Optic Nerve Crush (GC/OFL-D20(LS))

No consistent labelling pattern is seen between the axotomized and control GC/OFLs across these four fish. No clear topographical gradient is evident. The mean linear densities are A=15.7 vs. C=10.0, A=8.9 vs. C=14.8, A=1.5 vs. C=1.1, and A=8.6 vs. C=17.5.

f. Twenty-five Days Following Unilateral Optic Nerve Crush (GC/OFL-D25(LS))

Although labelled cells are present in every section examined, no consistent patterns are evident within or between fish. In one fish, labelling was linear across all topographical regions, A=3.4 and C=7.2. In another fish, more labelled nuclei are found in the most central regions as compared with the more peripheral regions. More labelled cells are seen in the GC/OFL of the axotomized eye, A=31.2 and C=14.8. In the third fish, more labelled cells are evident in the peripheral retinal regions. Labelling was higher in the control GC/OFL as compared with the axotomized GC/OFL, A=10.3 and C=29.2.

g. Fifty Days Following Unilateral Optic Nerve Crush (GC/OFL-D50(LS))

Low linear densities of labelled cells are found in the four fish examined 50 days following axotomy. Many sections are devoid of labelled cells. There do not appear to be any topographical trends. The mean linear densities for these fish are A=1.8 vs. C=1.1, A=0.8 vs. C=1.2, A=0.8 vs. C=0.8, and A=3.7 vs. C=5.4.

DISCUSSION

To examine whether there was any difference in labelling densities between the two eyes of intact fish, examined 30 days following ^{3}H-TdR injection, Student's t-tests were performed. Linear densities between the two GC/OFLs of the intact fish were not statistically different ($F(1,31)=1.28$, $p>.001$, $F(1,40)=.03$, $p>.001$, $F(1,42)=5.06$, $p>.001$, and $F(1,11)=2.22$, $p>.001$, respectively). There is one qualitative observation that should be noted at this stage. In addition to the lower linear densities seen 1 month following injection (as compared with 1 day following injection), the density of silver grains per nucleus is obviously lighter at the 30 day survival period as compared with the 1 day survival period. The criterion for a labelled cell employed in these studies is 10 silver grains per nucleus. In most cases it was necessary to count all 10 grains to insure that the criterion was met. On the other hand, this is rarely necessary in the 1 day survival group because on average 50-150 grains are visible, often completely masking the nucleus (see Figures 43 and 44).

Optic nerve crush exerts a clear modulatory effect on GC/OFL cell birth when the survival period following thymidine injection is 1 day. One month following ^{3}H-TdR administration, the results are more variable. In order to test whether ln<R> values differed reliably across times following axotomy, an ANOVA was run across the 7 groups (normal, D5, D10, D15, D20, D25, D35, and D50). The results indicated statistically significant differences ($F(6,392)=125.87$, $p<.001$; see Figure 14B) as a function of days following axotomy. The ln<R> value for the 59 observations on four normal fish was 0.0245, SE=0.063. By D5 a significant increase in labelled cell number was found in the axotomized retina (ln<R>=0.7046, SE=0.104) when compared with the intact group, $F(1,114)=31.6$, $p<.001$. Fifty-seven observations from four fish were compiled for this result. From the 55 observed ratios taken from four fish at 10 days post crush, a reversal was seen, with more tagged cells in the control GC/OFL (ln<R>=0.4746, SE=0.093). The ln<R> value found at D10 was significantly different from that seen in intact ($F(1,112)=20.1$, $p<.001$) and D5 ($F(1,110)=70.9$, $p<.001$) fish. At D15, 68 observations from four fish showed that normal values were reached (ln<R>=0.1171, SE=0.096). The ratio of the linear densities seen on D15 was statistically different from that seen either at D5 ($F(1,123)=17.22$, $p<.001$) or D10 ($F(1,121)=19.01$, $p<.001$), but not from that found in intact fish ($F(1,125)=0.61$, $p>.001$). The 67 observations tabulated on D20 showed that more labelled cells were found in the control retina (ln<R>=-0.2451, SE=0.092). This value was distinct from D5

($F(1,122)=46.79$, $p<.001$), but not from normal, D10, or D15 ($F(1,124)=5.48$, $p>.001$, $F(1,120)=2.99$, $p>.001$, and $F(1,113)=4.71$, $p>.001$, respectively). At D25 (61 observations from 3 fish) and D50 (32 observations from 4 fish), similar results to those seen on D20 were found ($\ln\langle R\rangle=-0.2949$, SE=0.160; $\ln\langle R\rangle=-0.1134$, SE=0.129, respectively). Student's t-tests showed no differences between D20 and D25 ($F(1,126)=0.08$, $p>.001$), D20 and D50 ($F(1,97)=0.67$, $p>.001$), or D25 and D50 ($F(1,91)=0.58$, $p>.001$). These results are graphically portrayed in Figure 14B. According to the results obtained from multiple t-tests, normal=D15=D50 and D10=D20=D25=D50, one can reasonably conclude that the only experimental time different from control (and by extrapolation all other groups) is D5.

To test whether labelling densities varied with topographical location, an ANOVA was performed. There was no reliable difference as a result of time following nerve crush ($F(6,20)=2.291$, $p>.001$) when all observed ratios were pooled into topographical location. No difference was seen between topographical regions ($F(2,40)=2.574$, $p>.001$) and no interactions were evident ($F(12,40)=1.032$, $p>.001$).

In addition to the lighter grain density over individual nuclei that is found 1 month following thymidine injection, labelling in this particular layer is sparse and relatively capricious. Many sections are devoid of any labelled cells. The labelling pattern in this lamina is not particularly consistent across the later time intervals. This is manifest in the statistical analyses, as there are no differences found between the later experimental groups. However, an interesting feature is the retention of label at the earliest time examined (Day 5). This suggests the possibility of two distinct populations of cells located in the GC/OFL, one of which becomes post-mitotic shortly after axotomy and one of which undergoes further cell generation cycles. Whether this represents a neuron-glia dichotomy or two types of non-neuronal cell populations is unclear.

Although an ANOVA does not show any difference across topographical domains, there appears to be a tendency toward heavier labelling in the more peripheral retinal regions. This probably reflects activity occurring in the <u>ora terminalis</u> at the time of injection. One month following administration of the radioisotope, those cells originally incorporating the tracer have been displaced by the newer germinal zone. Hence, the older <u>ora terminalis</u>, which is now labelled, occupies a more central position.

ULTRASTRUCTURE OF LABELLED CELLS IN THE GC/OFL

Cells that incorporate the tritium-labelled pyrimidine base, thymidine in the GC/OFL appear to be uniform in character (Figure 15). The nucleus is irregularly shaped and occupies most of the area of the perikaryon leaving relatively little cytoplasm (Figure 15C). The nucleus is composed of densely stained material arranged in granular clusters. There are however, multiple cytoplasm-filled processes giving the overall cell morphology a multipolar appearance (see Figure 15D). These processes are associated with bundles of small (0.05- 0.10 um) diameter fibers passing through the region, often partially encircling them. The cytoplasm contains clusters of free ribosomes and often large (0.5 um) residual bodies are seen. Mitochondria are often found within the plane of section through the cell (see Figure 16).

The above ultrastructural features suggest that these cells are not retinal ganglion cells. Retinal ganglion cells contain a centrally located nucleus, usually round or pear-like in shape. Using routine electron microscopic staining, one can see that the nucleus stains in a granular fashion, but not nearly as densely as the ^{3}H-TdR labelled cells described here. The ganglion cell soma is usually ovoid with a dendrite directed into the IPL. The cytoplasm is sparse and contains rER present as single profiles rather then parallel arrays. Free ribosomes are present but not in large quantities (Murray & Forman, 1971).

The second point, therefore, is that the labelled cells described here are most likely non-neuronal. Murray and Forman (1971) showed that in goldfish, 6-14 days following axotomy, glial cell bodies within the ganglion cell layer are more common. They state, however, that mitotic figures were never seen in the retinal ganglion cell layer. Since thymidine is a DNA precursor and is so avidly incorporated by these cells the last statement is probably a reflection of their sampling procedure and not the actual state of events. Figure 16 illustrates one of these cells captured during nuclear division. This ultrastructural characterization is purely descriptive. To confirm the identity of these cells, immunocytological techniques (eg. glial fibrillary acidic protein (GFAP)) would have to be employed. However, from the cell location, nuclear characteristics, and cytoplasmic constituents, one can argue convincingly that the labelled cells are not retinal ganglion cells.

<u>Figure 6. Topographical Distribution of the Linear Density of Labelled Cells in the GC/OFL- Normal, Short Survival</u>

In this, and all subsequent series of graphs drawn from individual fish, the following conventions apply: The abscissa refers to the section number (i), which in biological terms is the topographical gradient, where section 1 is the most peripheral retinal ring (excluding the <u>ora terminalis</u>) and progressive sections refer to progressively more central tissue. Each fifth section represents a retinal ring approximately 80 um from the previous ring. No equatorial section with a circumference of less than 5 mm is included. The upper ordinate is the average number of labelled cells/10 mm of circumference. The lower ordinate reflects the actual circumference examined (mm). The term "short survival" in the title refers to a 1 Day survival interval following ^{3}H-thymidine injection. In this Figure, the dashed line represents the right eye, while the solid line represents the left eye.

A. This fish received a sham-right optic nerve crush. The left optic nerve was left intact.

B. This fish received a sham-left optic nerve crush.

C. and D. The retinae from two intact fish are depicted in these graphs.

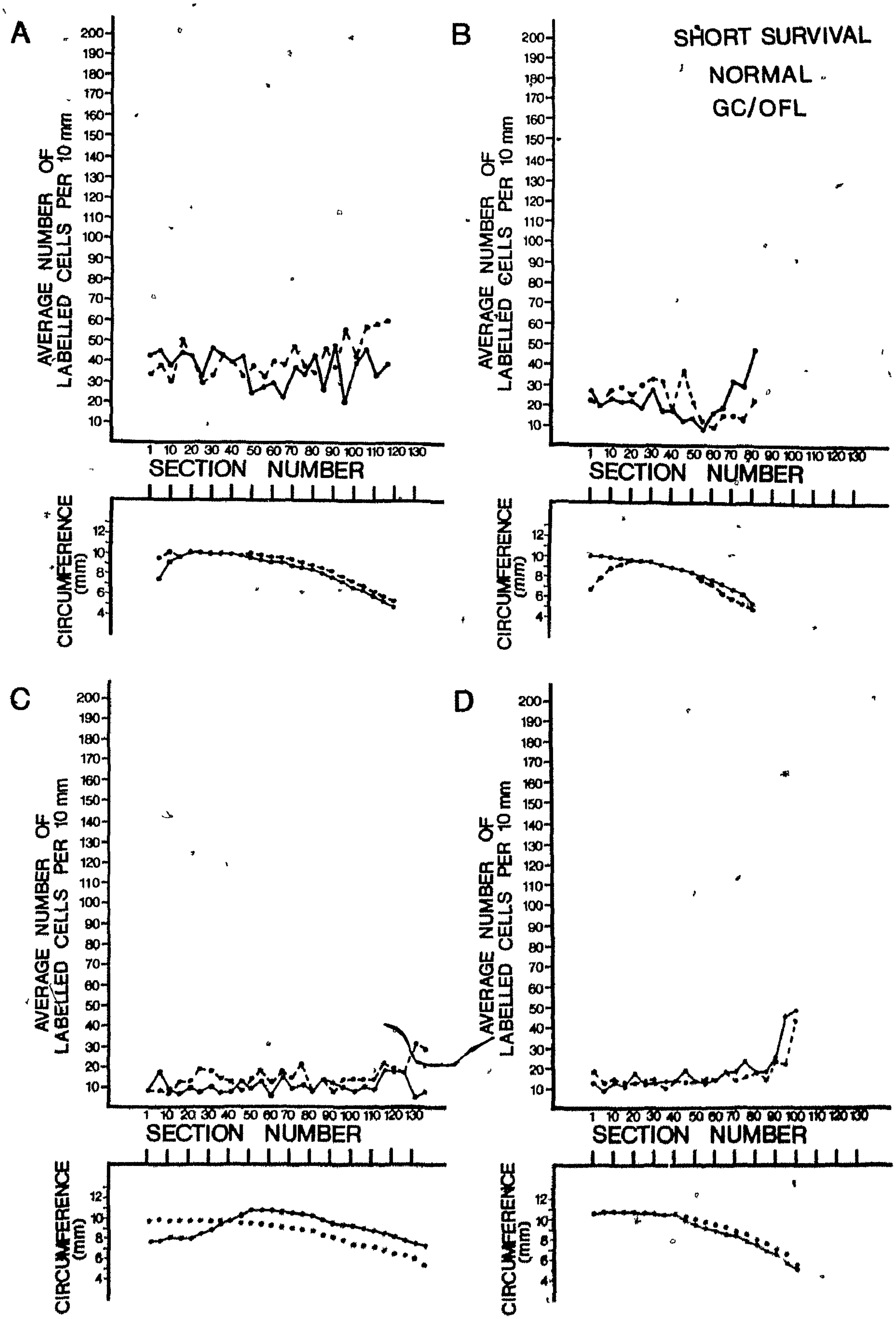
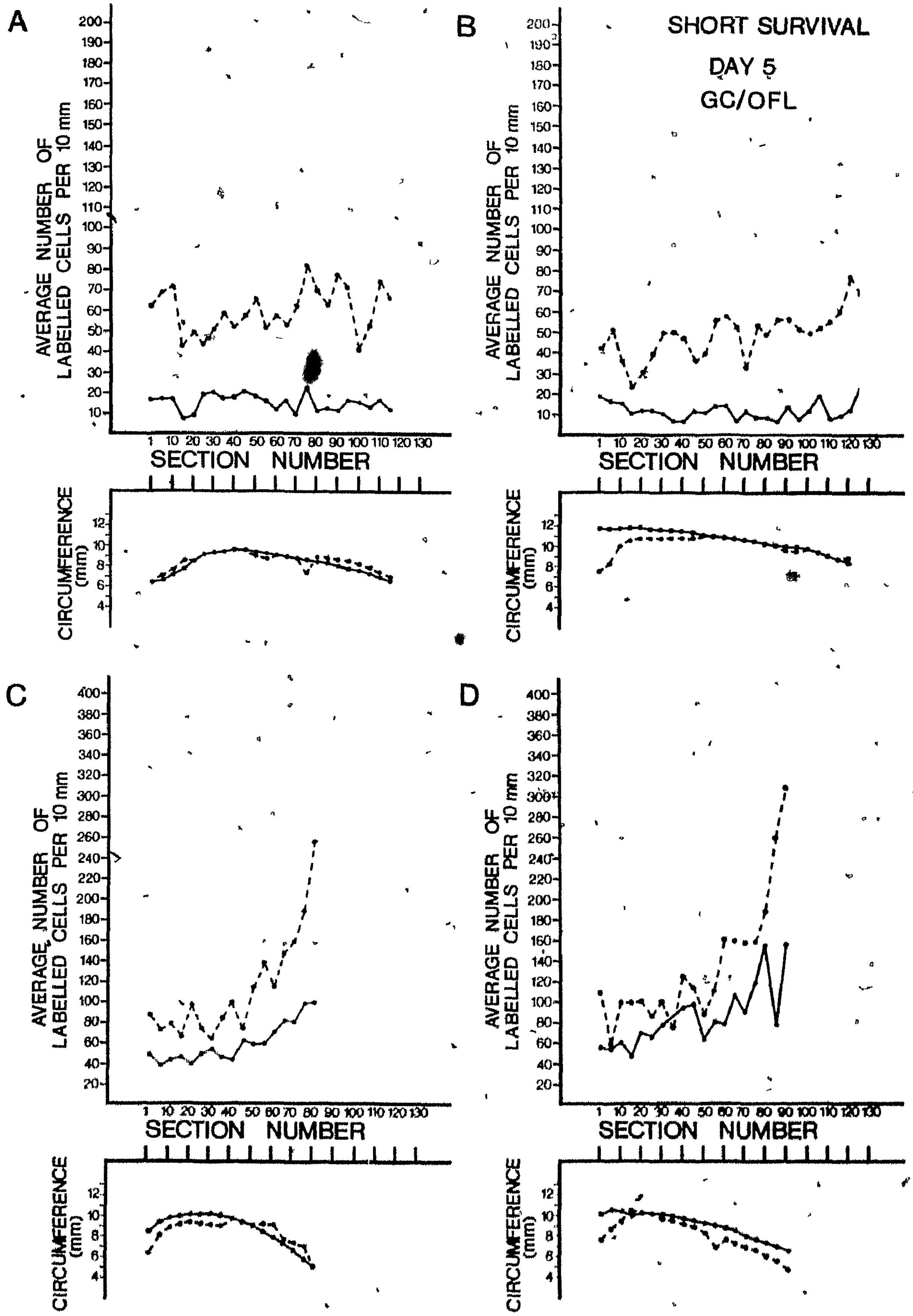
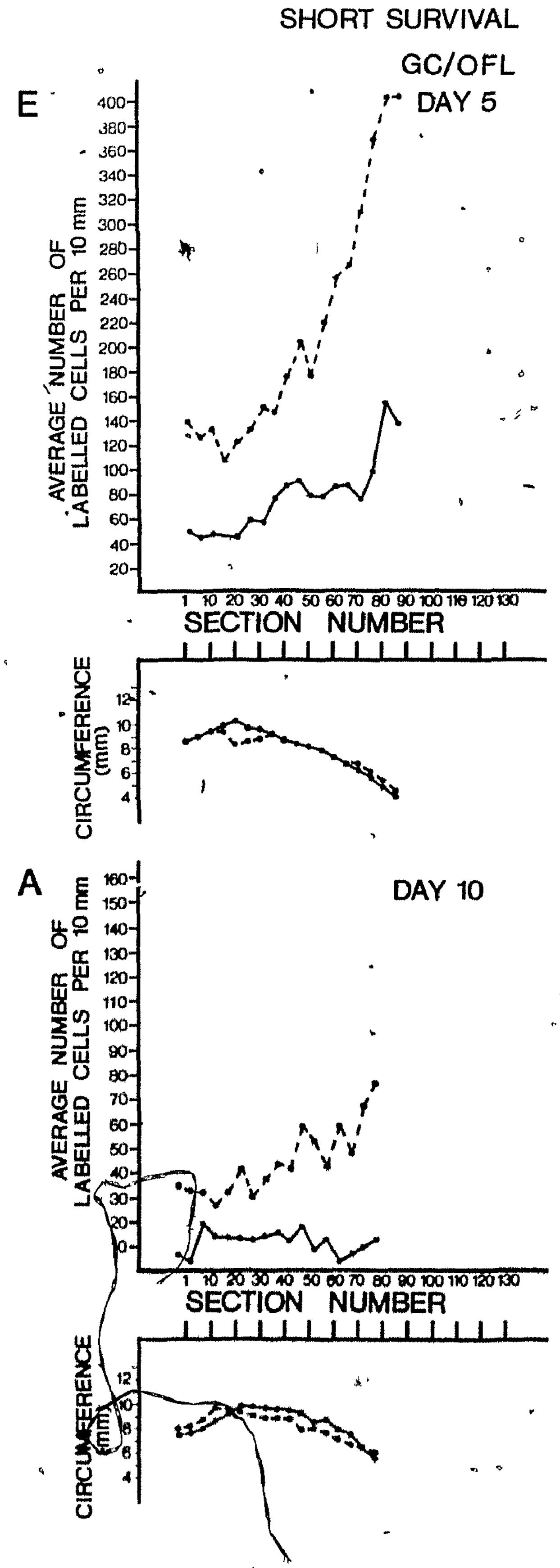

A
B
C
D
SHORT SURVIVAL
NORMAL
GC/OFL
AVERAGE NUMBER OF LABELLED CELLS PER 10 mm
SECTION NUMBER
CIRCUMFERENCE (mm)

Figure 7. Distribution of Labelled Cells in the GC/OFL- (D5, SS)

In these Figures, the conventions described in Figure 6 apply. However, in all cases, the dashed line represents the axotomized eye and the solid line refers to the intact control.

Five fish (A, B, C, D, and E) received unilateral optic nerve crush 5 days before ^{3}H-TdR injection. They were sacrificed 1 day after injection. Note the change in the ordinate for C, D, and E.


A
B
C
D
SHORT SURVIVAL
DAY 5
GC/OFL
AVERAGE NUMBER OF LABELLED CELLS PER 10 mm
SECTION NUMBER
CIRCUMFERENCE (mm)



SHORT SURVIVAL
GC/OFL
E
DAY 5
AVERAGE NUMBER OF LABELLED CELLS PER 10 mm
SECTION NUMBER
CIRCUMFERENCE (mm)
A
DAY 10
AVERAGE NUMBER OF LABELLED CELLS PER 10 mm
SECTION NUMBER
CIRCUMFERENCE (mm)

<u>Figure 8. Distribution of Labelled Cells in the GC/OFL- (D10, SS)</u> A, B, C, D, and E. These fish received bilateral ^{3}H-Tdr injections fifteen days after unilateral optic nerve crush. They were sacrificed 1 day later.

SHORT SURVIVAL
DAY 10
GC/OFL

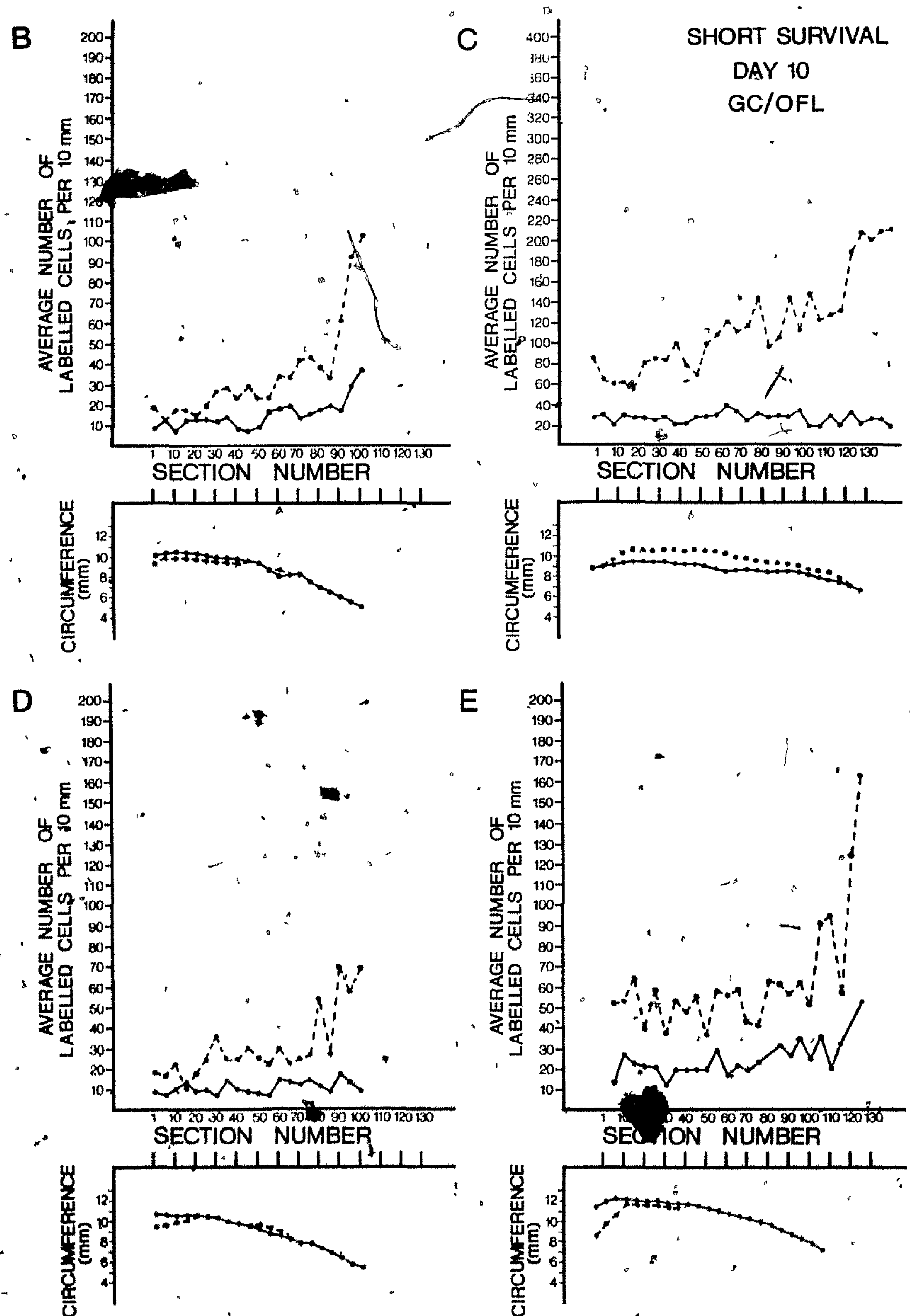
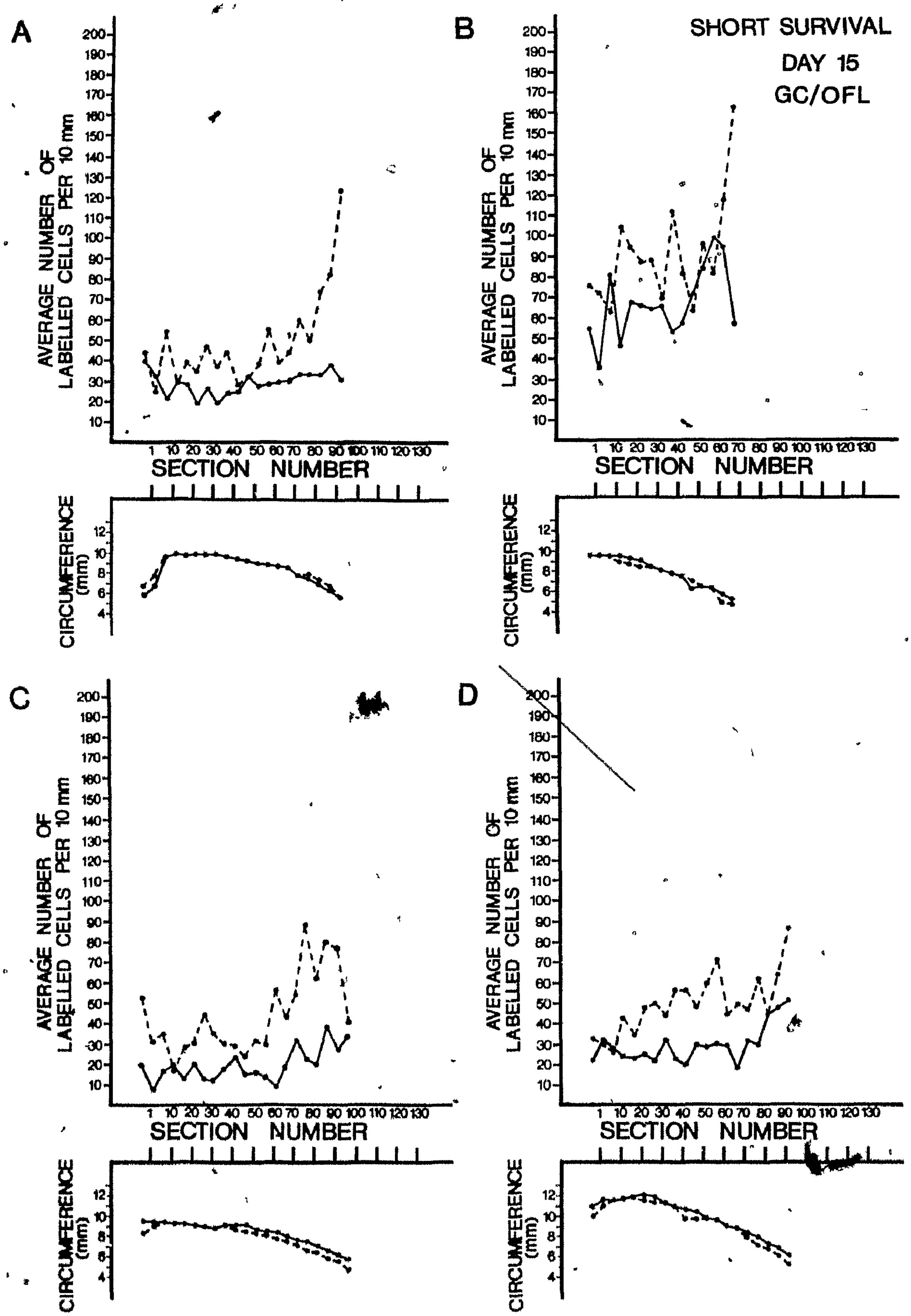


Figure 9. Distribution of Labelled Cells in the GC/OFL- (D15, SS)

A, B, C, D, E, and F. These fish received unilateral optic nerve axotomy 15 days prior to ^{3}H-TdR injection. They were sacrificed 24 hours after the injection.


A
B
C
D
SHORT SURVIVAL
DAY 15
GC/OFL
AVERAGE NUMBER OF LABELLED CELLS PER 10 mm
SECTION NUMBER
CIRCUMFERENCE (mm)

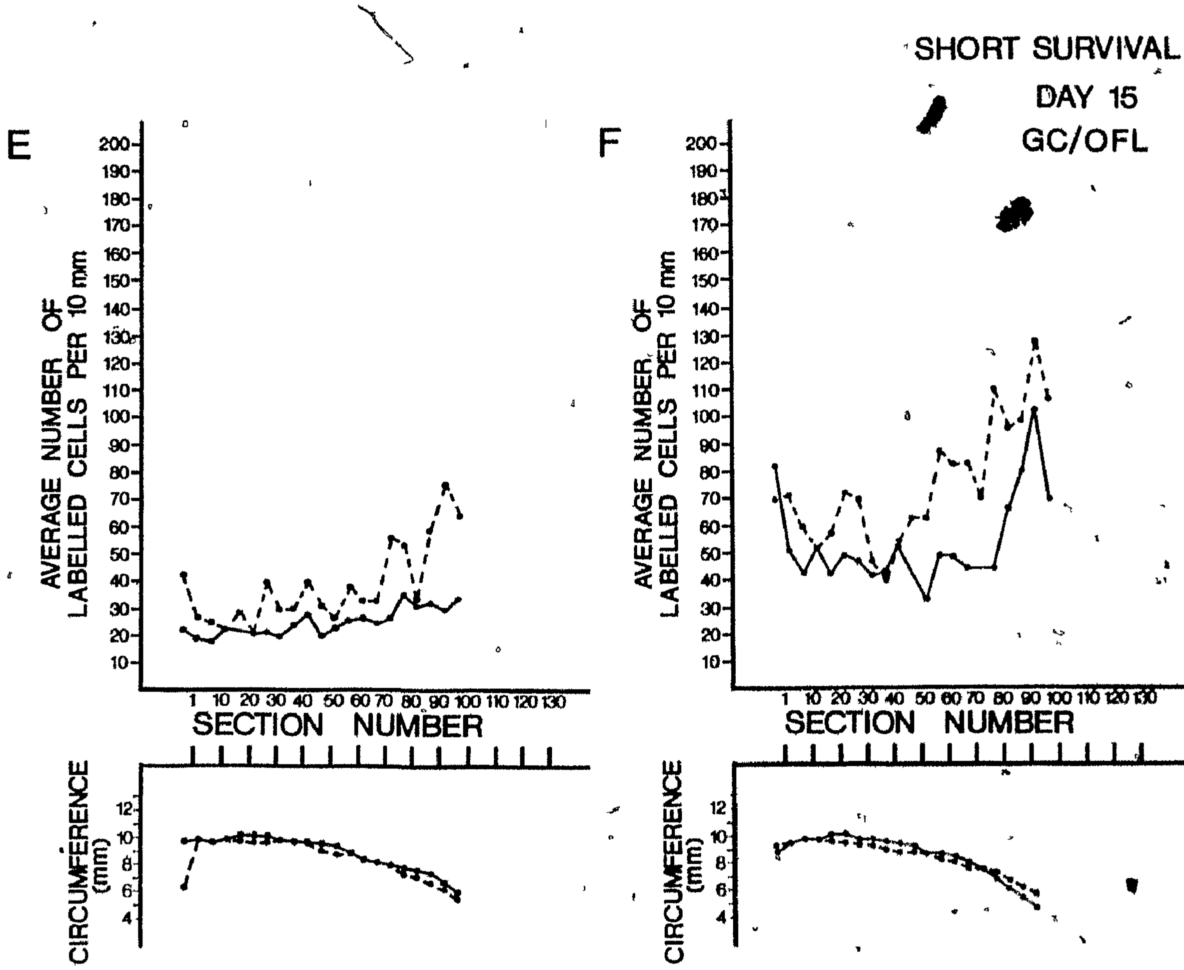

SHORT SURVIVAL
DAY 15
GC/OFL
E
F
AVERAGE NUMBER OF LABELLED CELLS PER 10 mm
SECTION NUMBER
CIRCUMFERENCE (mm)

Figure 10. Distribution of Labelled Cells in the GC/OFL- (D20, SS)

Twenty days following unilateral optic nerve crush, these four fish received bilateral ^{3}H-TdR injections. They were sacrificed 1 day later.

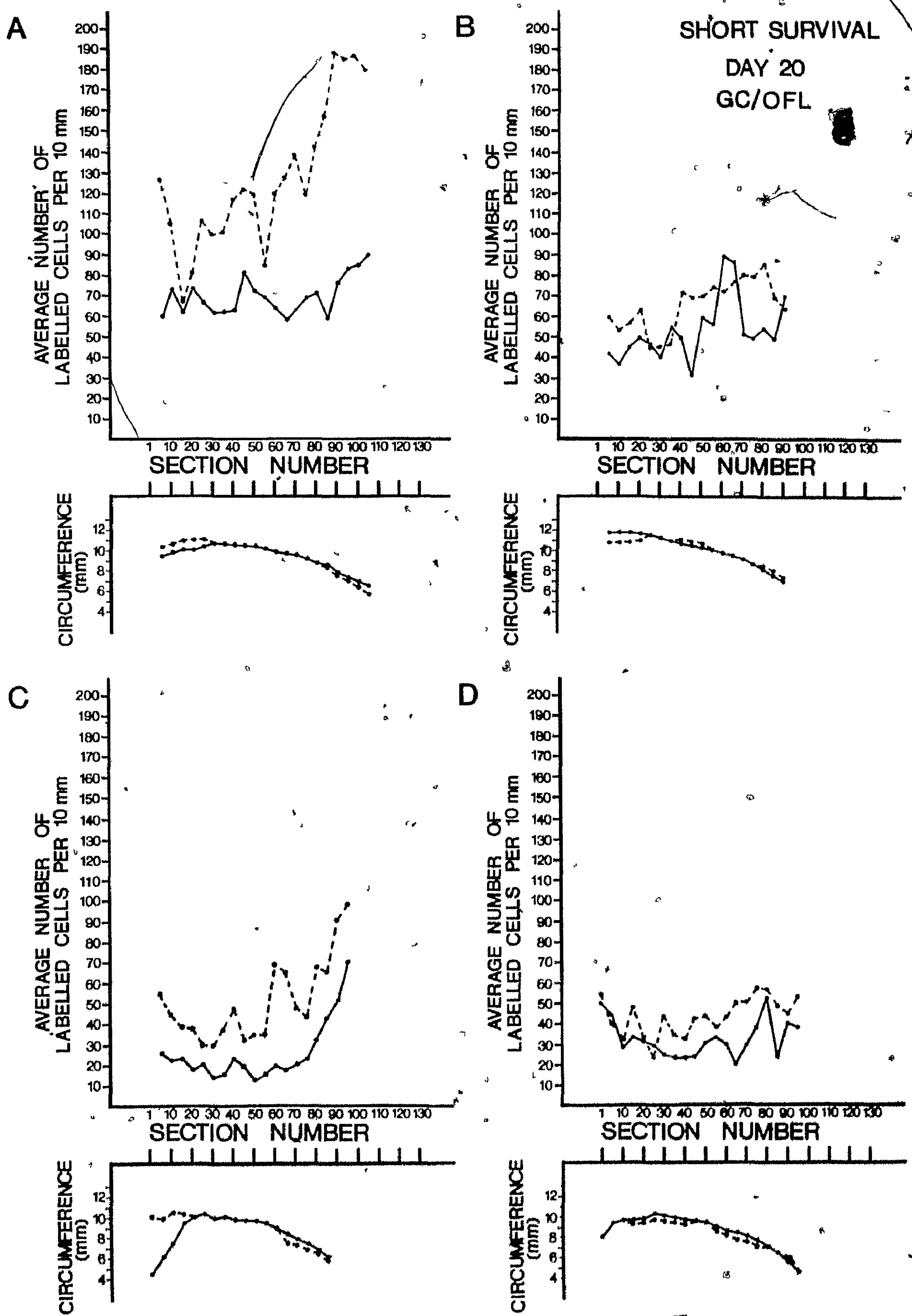

SHORT SURVIVAL
DAY 20
GC/OFL
A
B
C
D
AVERAGE NUMBER OF LABELLED CELLS PER 10 mm
SECTION NUMBER
CIRCUMFERENCE (mm)

Figure 11. Distribution of Labelled Cells in the GC/OFL- (D25, SS)

A, B, C, and D. These fish received bilateral injections of ^{3}H-Tdr twenty-five days after unilateral optic nerve crush. They were sacrificed 1 day later.

SHORT SURVIVAL
DAY 25
GC/OFL

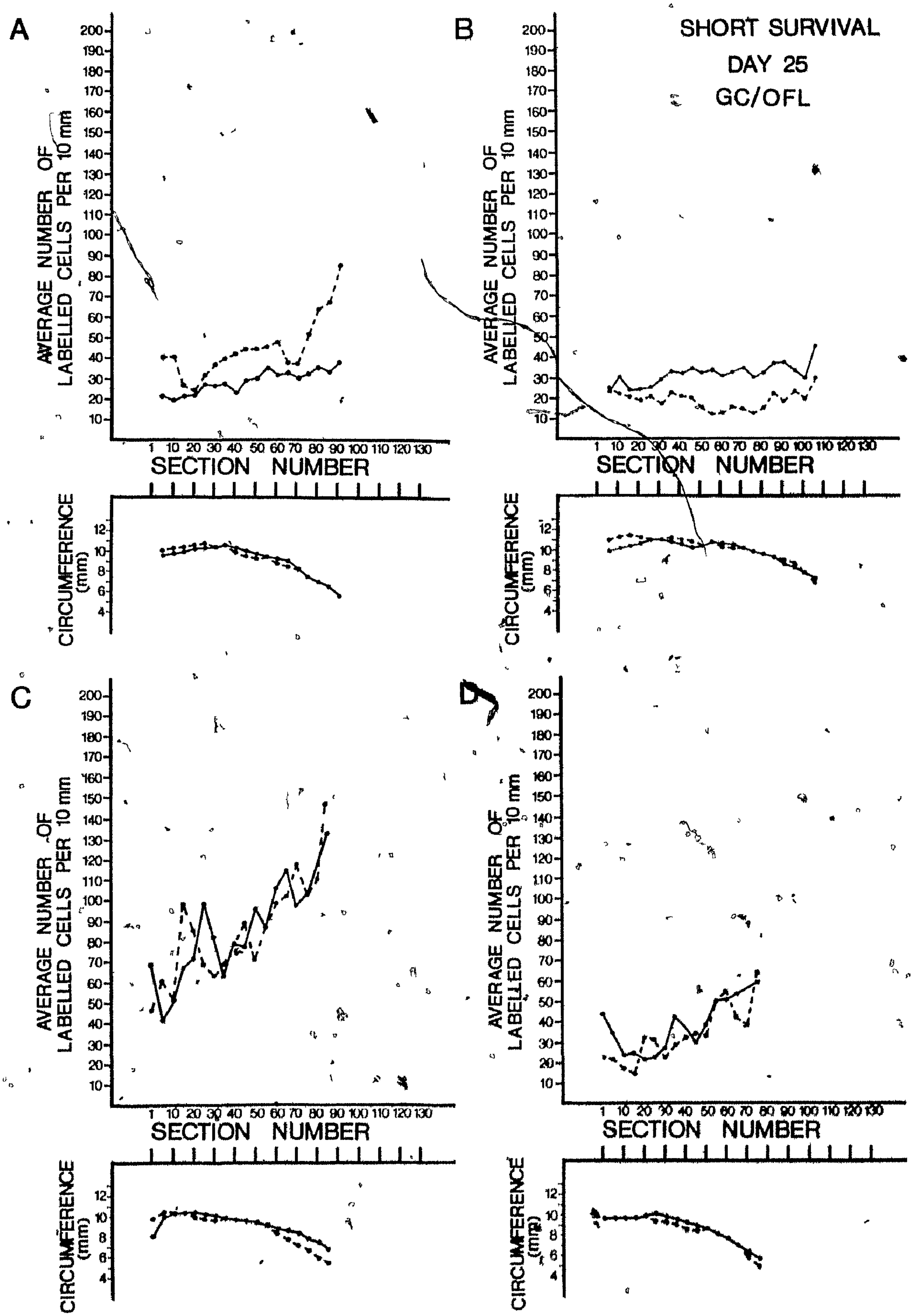
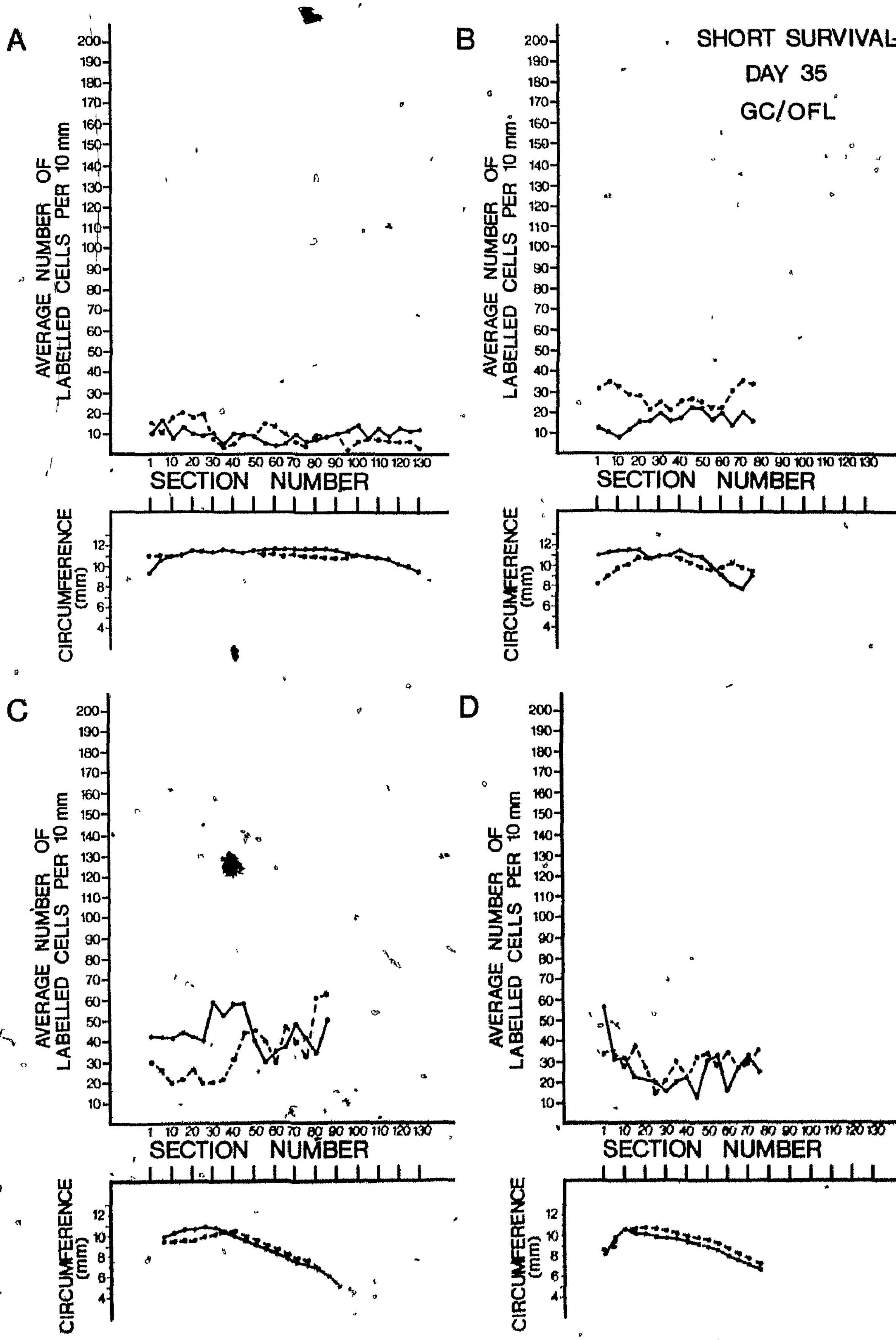


Figure 12. Distribution of Labelled Cells in the GC/OFL- (D35, SS)

Thirty-five days after unilateral optic nerve crush, these fish received bilateral injections of ^{3}H-TdR. They were sacrificed 24 hours later.



SHORT SURVIVAL
DAY 35
GC/OFL
A
B
C
D
AVERAGE NUMBER OF LABELLED CELLS PER 10 mm
SECTION NUMBER
CIRCUMFERENCE (mm)

Figure 13. Distribution of Labelled Cells in the GC/OFL- (D50, SS)

Fifty days after unilateral optic nerve crush, these fish received bilateral injections of ^{3}H-TdR and were processed for light autoradiographic examination 1 day later.

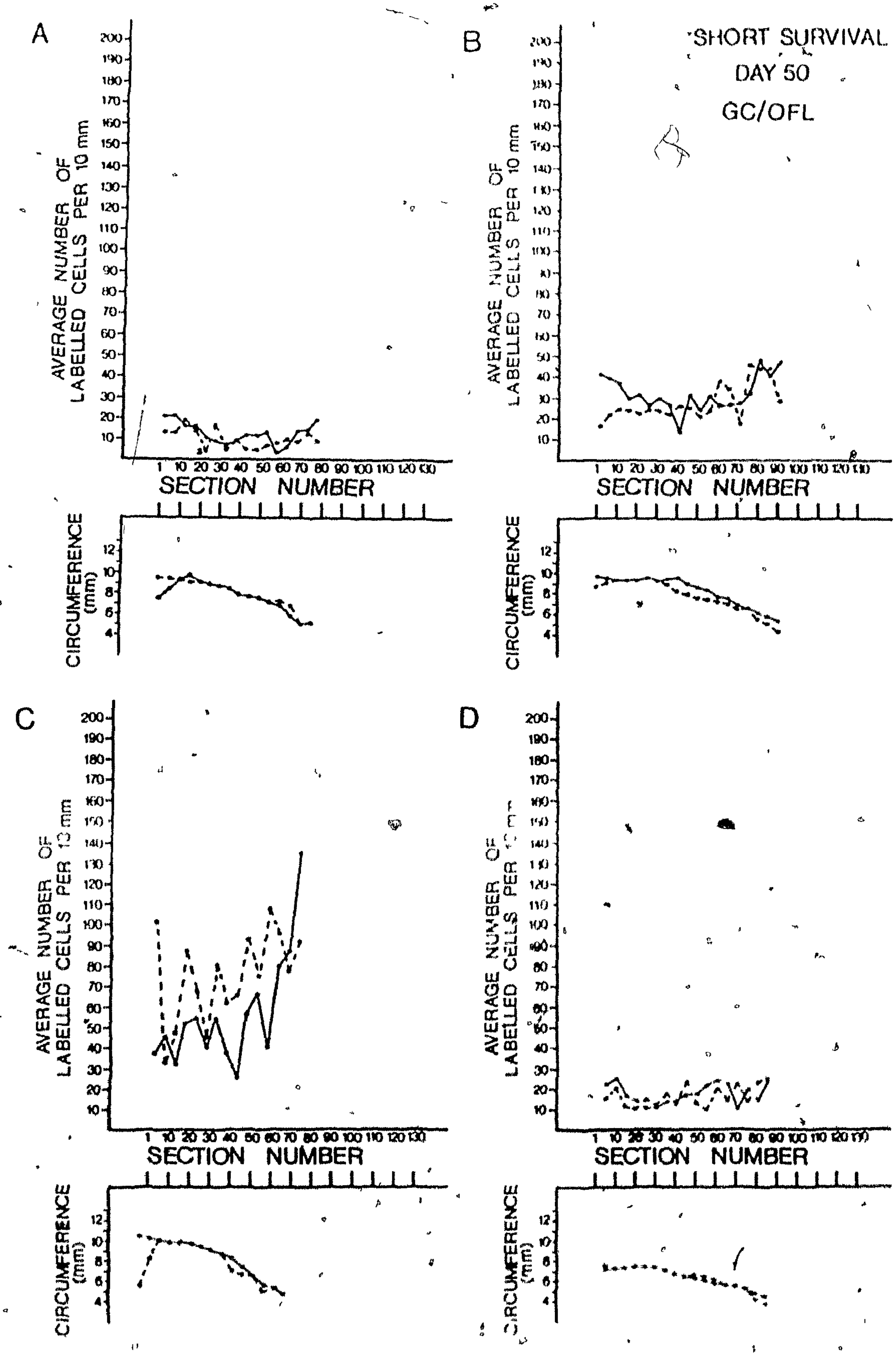

SHORT SURVIVAL
DAY 50
GC/OFL
A
B
C
D
AVERAGE NUMBER OF LABELLED CELLS PER 10 mm
SECTION NUMBER
CIRCUMFERENCE (mm)

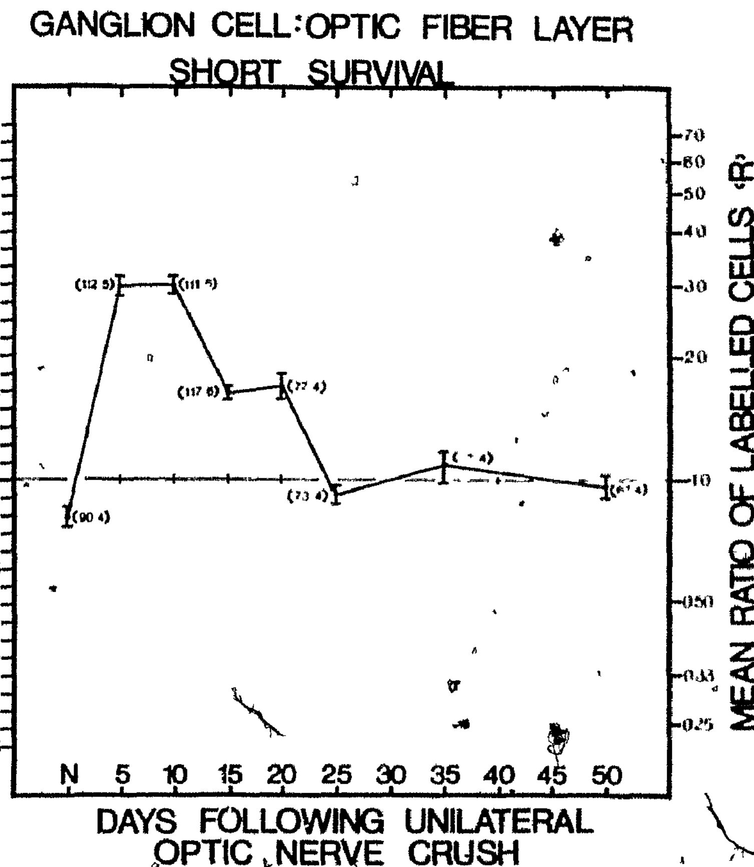
Figure 14. Time Course of Changes in Cell Proliferation in the GC/OFL During Various Post-operative Periods Following Unilateral Optic Nerve Crush

In these Figures, the markings on the abscissa denote the days following unilateral axotomy. The ordinates represent the mean value of the ratio of labelled cells (axotomized:control) (<R>) on the right scale and the natural logarithm of <R> (ln<R>) on the left scale. In brackets are the number of observations compiled for each post-operative period followed by the number of fish from which the individual ratios are taken. The error bars represents the standard error of the ln<R>.

A. 1 Day Following Thymidine Injection: When animals are sacrificed 1 day following injection of tritiated thymidine, there is a clear enhancement in the number of cells incorporating the isotope as early as 5 days following axotomy. This level of labelling remains elevated at D10. At D15 and D20, enhanced labelling is still evident in the axotomized GC/OFL, albeit to a lesser degree. By 25 days and onward, no differences are seen between the two retinae.

B. 30 Days Following Thymidine Injection: When animals are sacrificed 1 month following administration of tritiated thymidine, there is an enhancement in cell labelling in the axotomized GC/OFL 5 days following axotomy. By D10 a reversal is seen, with more tagged nuclei in the control GC/OFL. At 15 days following crush, no differences between the two is evident, although there appears to be decreased labelling in the axotomized retina at D20, D25, and D50. Statistical analyses reveal that at these experimental times the densities of labelled cells are equivalent.

A


GANGLION CELL: OPTIC FIBER LAYER
SHORT SURVIVAL
ln ‹R›
MEAN RATIO OF LABELLED CELLS ‹R›
N 5 10 15 20 25 30 35 40 45 50
DAYS FOLLOWING UNILATERAL OPTIC NERVE CRUSH

B

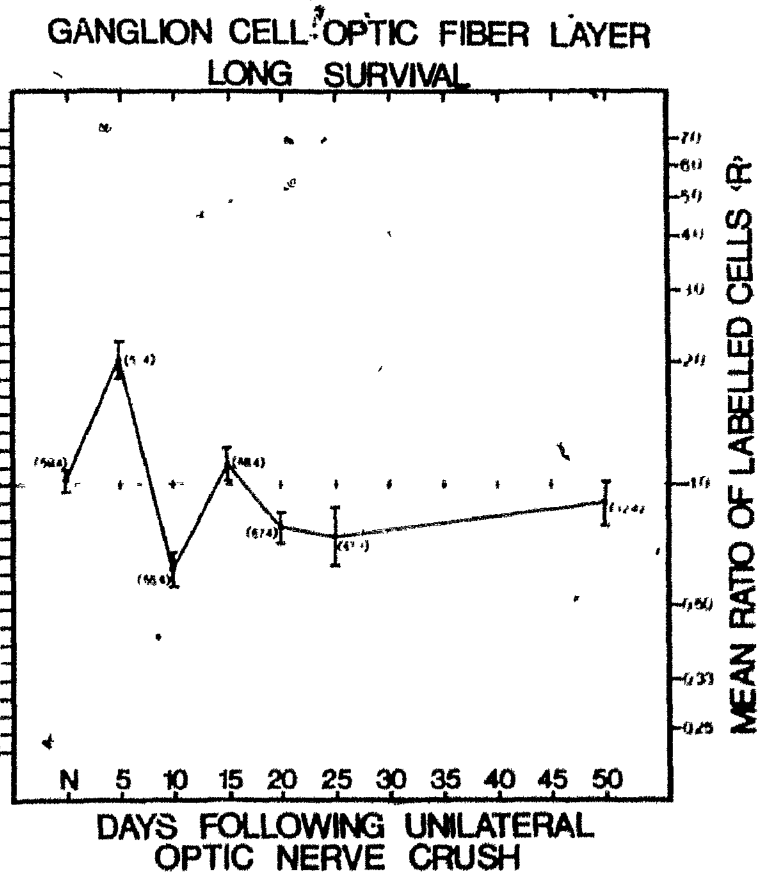

GANGLION CELL: OPTIC FIBER LAYER
LONG SURVIVAL
ln ‹R›
MEAN RATIO OF LABELLED CELLS ‹R›
N 5 10 15 20 25 30 35 40 45 50
DAYS FOLLOWING UNILATERAL OPTIC NERVE CRUSH

Figure 15. Ultrastructure of Labelled Cells in the GC/OFL

A. A light microscopic autoradiograph illustrating the laminar location of a labelled cell (arrow). This fish received a nerve crush 10 days prior to injection with tritiated thymidine, and was sacrificed 1 day later. Calibration bar: 25 μm.

B. Higher power micrograph of the identified cell. Calibration bar: 10 μm.

C. Electron micrograph of the same labelled cell identified in A and B. The nucleus is irregularly shaped and occupies a large proportion of the cell soma. Note the cytoplasmic process near the top of the micrograph. This process appears to surround the small fibers in the region. Calibration bar: 1 μm.

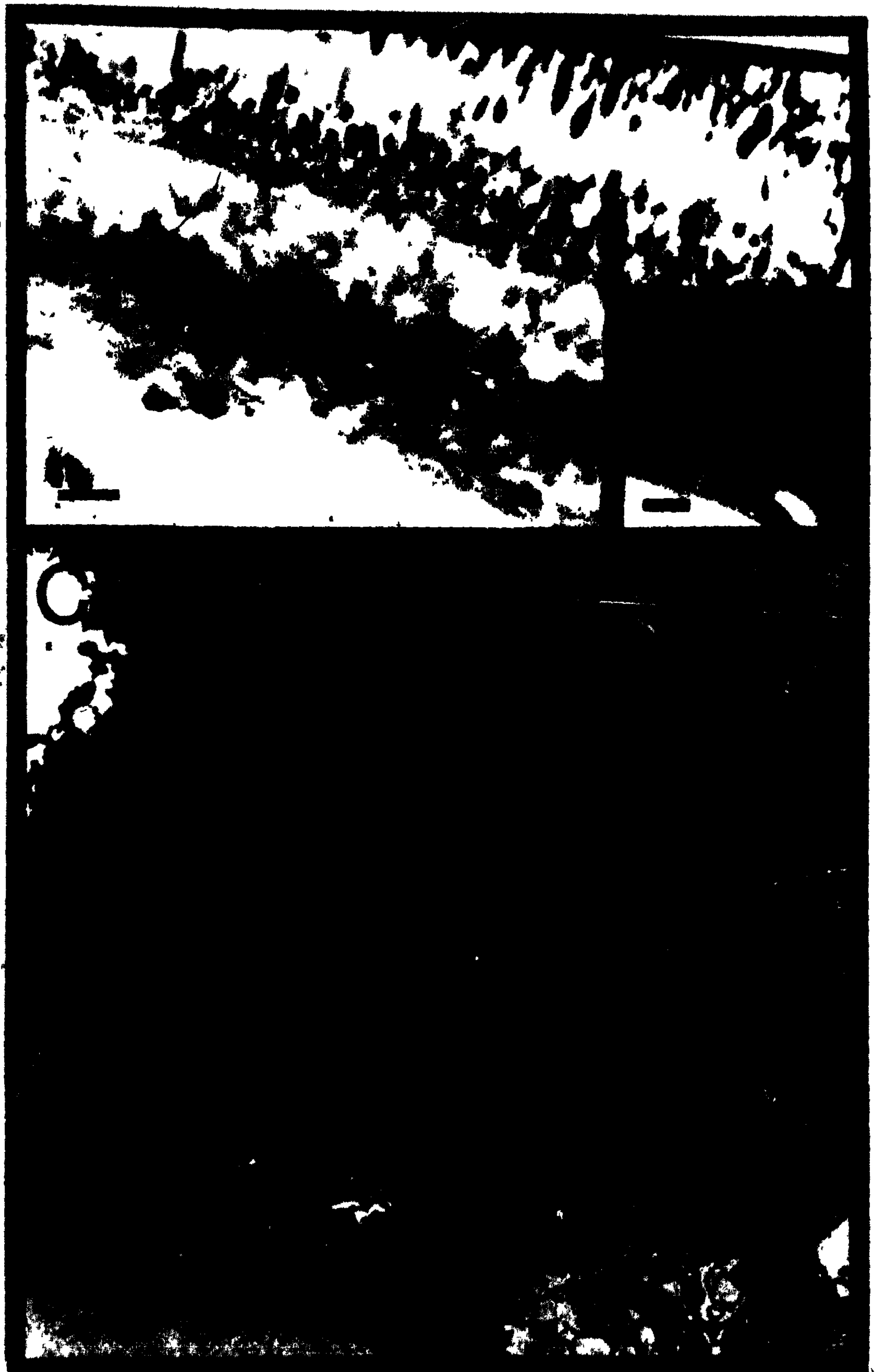


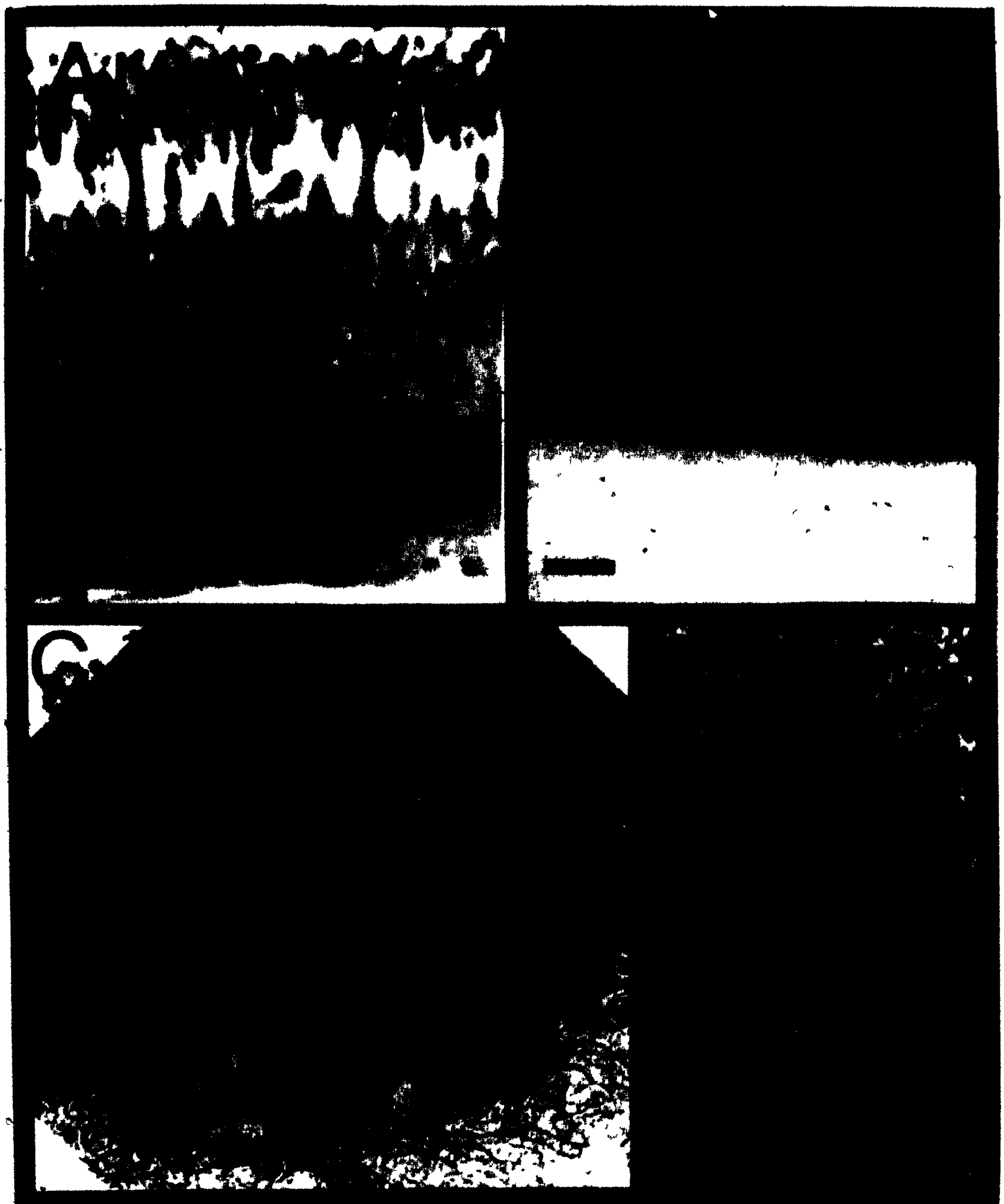
Figure 16. Ultrastructure of Labelled Cells in the GC/OFL

A. An light microscoptic autoradiograph illustrating the location of the tritium tagged cell (arrow). Calibration bar: 25 µm.

B. Higher power micrograph of the labelled cell. Calibration bar: 10 µm.

C. Electron micrograph of the same labelled cell identified in A and B. This cell appears to be caught in the process of nuclear division. Calibration bar: 10 µm.

D. Higher power micrograph of the region delineated by the arrows in C. The arrows emphasize the regions not yet separated. Calibration bar: 0.5 µm.


A
C

IV. CELL PROLIFERATION IN THE RECEPTOR CELL LAYER

Preliminary studies revealed that the GC/OFL was not the only retinal lamina whose normal cell birth pattern appeared to be modulated following optic fiber axotomy. Although there was no apparent modulation of cell proliferation in the INL, ^{3}H-TdR incorporation in the ONL appeared to be selectively affected by axotomy of retinal ganglion cell axons. The pattern of this modulation was examined 1 day or 30 days following injection of ^{3}H-TdR.

EFFECTS OF OPTIC FIBER AXOTOMY ON CELL PROLIFERATION IN THE ONL EXAMINED 24 HOURS AFTER THYMIDINE INJECTION

Four normal fish received intraocular injections of ^{3}H-thymidine and one day later, their retinae were processed for light microscoptic autoradiographic examination as described in general procedures.

Unilateral ONC's were performed on 32 fish. At various post-operative periods: 5, 10, 15, 20, 25, 35, or 50 days later, bilateral ^{3}H-TdR injections were administered. One day following injection, the fish were processed for light microscopic autoradiography as described in general procedures.

Ultrastructural observations were made to characterize the identity of the cells labelled with ^{3}H-TdR.

RESULTS

a. Normal (ONL-N(SS))

In Figure 17, individual data from four fish examined 24 hours following thymidine administration are presented. The graph in 17A represents a fish that underwent a sham-right optic nerve crush.

Although there is some variability, differences between the densities of labelled cells in the receptor layers of the two retinae are minimal. The mean linear densities for this fish are A=40.9 and C=37.9. A less severe version of this distribution is seen in Figure 17B, where this fish received a sham-left optic nerve crush. The labelling is similar between the two retinae as is reflected by the mean linear densities of A=34.5 and C=38.1. The higher overall numbers of labelled cells in the earliest sections are probably indicative of some remnants of the <u>ora terminalis</u>. Although the germinal zone was selectively omitted from cell counts, the first few sections off the block may be oblique with respect to the equatorial axis, and thus contain small regions of the more densely tagged regions. Regardless of the topographical pattern, it is clear that overlap is almost complete between the two eyes of a single fish. In Figures 17C and 17D, two intact fish are depicted. Their mean linear densities are A=12.5 vs. C=13.6 and A=33.1 vs. C=32.8. For these two fish, there is uniform labelling across the entire topographical region. Fluctuations appear minimal. The germinal zone appears to have been entirely excluded.

b. Five Days Following Unilateral Optic Nerve Crush (ONL-D5(SS))

In Figure 18, the individual graphs from 5 fish that received unilateral ONC's followed by ^{3}H-TdR injections and sacrificed 1½ day later, are represented. In general, there do not appear to be any regular trends with regard to labelling patterns within the two eyes of an individual fish, although there are consistent patterns across fish. In Figure 18A, with the exception of the first three sections examined, labelling appears to be quite uniform between the two eyes of this fish. There is a clear decreasing function as one moves from peripheral to central retinal regions. The mean linear densities for this fish are 94.1 (axotomized) and 74.7 (control). The fish represented in Figure 18B has a much greater degree of variability both between sections and between eyes, although there is a tendency for a ~~greater~~ degree of labelling in the axotomized receptor layer. For this fish A=165.5 and C=135.6. The labelling distribution appears linear In Figure 18C, the pattern of labelling across topographical regions is similar to that seen in 18A. In the most central regions examined, more labelled cells/10 mm are present both in the control and axotomized receptor layers. Differences between the two eyes are not apparent (A=119.7 and C=135.3). Again, the graph of the fish represented in 18D resembles the topographical distribution seen in both 18A and 18C- initially high labelling activity, followed by a decreased amount of labelling as one moves from peripheral to central territory. In 18D, however, differential labelling between the two retinae is more pronounced, with a higher proportion of tagged nuclei in the axotomized receptor layer (41.7 labelled cells/10 mm) as compared with the control (32.4 labelled cells/10 mm). In Figure 18E, the final fish in this post-operative group is depicted. There is a high degree of fluctuation both within a single retina across the topographical plane, and between the two retinae. For this fish, A=85.9 and C=96.3.

c. Ten Days Following Unilateral Optic Nerve Crush (ONL-D10(SS))

Ten days following unilateral axotomy is the only post-operative period where consistently more labelled cells are found in the photoreceptor layer of the crushed eye. In Figure 19, the five fish examined at this experimental time are illustrated. In Figure 19A, there is a clear difference in the linear densities of labelled cells between the two retinae. The mean number of labelled cells/10 mm in the axotomized retina is 73.7, while only 22.7/10 mm retinal circumference are located in the control eye. This reflects an enhancement of labelled cells as a result of optic nerve crush in the receptor layer. For this fish, topography does not appear to be a factor in the labelling pattern. In Figure 19B, a similar result is found- consistently more labelled cells in the receptor lamina of the axotomized eye (A=72.5 and C=26.6). The function is U-shaped, with a higher density of tagged nuclei in the more peripheral and central regions examined. For the fish denoted in Figure 19C, there are more labelled cells in the ONL of the axotomized retina. There is no overlap between retinae. This graph illustrates a decreasing curve, with some degree of variability between sections of the same retina. The mean linear densities for this fish are A=87.1 and C=33.5. In Figure 19D, more labelled cells are found in the experimental receptor cell layer (A=110.8 and C=73.1), although there is more overlap than was seen in 19A-C. Note the change in scale on the ordinate for Figure 19D. The function is clearly asymptotic. In 19E, the last fish from this post-operative period is depicted. Greater variability is evident in this graph, but the overall trend is the same- more labelled cells in the ONL of the axotomized eye (A=68.4, C=46.2). There does not appear to be any correlation between labelling density and topographical position.

The fish presented here illustrate the modulatory effect of nerve crush on the photoreceptor lamina of the retina. Ten days following unilateral axotomy is the only post-operative period where consistently more labelled cells are found in the photoreceptor layer of the crushed eye. As will be presented in the next sections, other modulatory trends are evident, but with the opposite polarity.

d. Fifteen Days Following Unilateral Optic Nerve Crush (ONL-D15(SS))

Fifteen days following unilateral axotomy, overall, there appears to be a slight suppression in the number of tagged cells in the crushed eye. In Figure 20A there is a clear asymptotic curve for both eyes; little difference in the density of labelled cells is apparent. The mean linear density for the axotomized eye is 49.3 labelled cells/10 mm, while in the control eye, 55.8 labelled cells/10 mm are seen. In Figures 20B and 20C, minimal differences between the two retinae are evident. There is a small tendency toward more tagged nuclei in the peripheral retinal regions. The mean linear densities are A=25.9 vs. C=31.9 and A=14.9 vs. C=15.4, respectively. The labelling pattern indicated in Figure 20F is irregular. There is a some variability between sections and there seem to be more labelled cells in the nonaxotomized photoreceptor lamina as compared with the axotomized ONL (A=22.2, C=48.1). In Figures 20E and 20F, a clear reversed trend is seen (note the change in scale in the Y-axis). In Figure 20E, the mean number of labelled cells/10 mm in the ONL of the axotomized eye is 64.9. The average number of labelled cells/10 mm in the control side is 108.2.

A clear topographical trend is evident. In Figure 20F, a differential labelling pattern is also evident. Less labelled cells in the ONL of the axotomized retina are present. This curve appears U-shaped in conformation. The mean number of labelled cells in the axotomized eye is 108.7, while in the control eye 185.4 labelled cells per 10 mm is seen. This suppression of labelled cells in the axotomized receptor lamina appears to be consistent for the next 10 days following axotomy.

e. Twenty Days Following Unilateral Optic Nerve Crush (ONL-D20(SS)).

Twenty days following axotomy, more labelled cells are evident in the photoreceptor layer of the intact eye as compared with the photoreceptor layer of the axotomized eye. In Figure 21, the individual data from these four fish are presented. In these graphs it is clear that there is little overlap between the labelling patterns in the two eyes. The mean linear densities for these fish are A=132.6 vs. C=275.9, A=142.2 vs. C=247.8, A=26.6 vs. C=41.6, and A=52.3 vs. C=74.9, for A-D. In Figure 21A, a relatively linear pattern is seen for both eyes. The topographical location does not appear to have a selective influence on labelling pattern. The retinae represented in Figure 21B-D appear to have a higher density of labelled cells in the peripheral retinal regions. As one moves toward more central retina, labelling decreases. The relationship between the intact and axotomized layers remains consistent.

f. Twenty-five Days Following Unilateral Optic Nerve Crush (ONL-D25(SS))

A similar labelling distribution to that seen at D20 (ONL-D20(SS)) is witnessed 25 days following axotomy, when fish are sacrificed 1 day following injection of ^{3}H-TdR. More labelled cells are found in the intact ONL as compared with the axotomized side. In Figure 22, these four fish are represented. In general, more labelled cells are found in the more peripheral retinal regions, although there is an occasional increase in central retina (eg. Figure 22A and 22C). The mean linear densities of the number of labelled cells/10 mm in the axotomized eye as compared with the control eye for these four fish are A=71.2 vs. C=99.7, A=113.5 vs. C=222.5, A=68.2 vs. C=132.0, and A=37.7 vs. C=80.3.

g. Thirty-five Days Following Unilateral Optic Nerve Crush (ONL-D35(SS))

Thirty-five days following unilateral ONC, when fish are bilaterally injected with tritiated thymidine and sacrificed one day later, the resultant labelling pattern in the photoreceptor layers of the two retinae appears like that of an intact animal. In Figure 23, four such fish are presented (see Figure 17 for comparison). Although there is slightly more variability (Figure 23C and 23D), the overall pattern is similar. A higher number of labelled cells is seen in the more peripheral retinal zones; this function decreases as one moves to more central topographical regions. The mean linear densities of the axotomized and control retinae for these four fish are A=17.4 vs. C=19.2, A=44.6 vs. C=39.1, A=108.7 vs. C=146.6, and A=195.0 vs. C=156.5.

h. Fifty Days Following Unilateral Optic Nerve Crush (ONL-D50(SS))

By 50 days following unilateral axotomy, little difference between the labelling pattern in the two retinae are seen. In Figure 24, these fish are illustrated. All display a decreasing or asymptoting function, with close association between the two eyes. For these fish: A=28.2 vs. C=22.1, A=14.5 vs. C=15.9, A=55.4 vs. C=45.8, and A=58.5 vs. C=58.8.

DISCUSSION

The first question I tested is whether there is a difference in cell proliferation between the ONLs of the two retinae of intact fish. In the receptor cell layer of the control fish, no differences were found between the retinae of either the intact or the sham-operated animals. The ln<R> for intact fish was −0.1017, SE=0.061. Individual Student's t-tests between the two retinae of a single fish did not result in significant differences ($F(1,46)=0.28$, $p>.001$, $F(1,40)=0.03$, $p>.001$, $F(1,53)=0.83$, $p>.001$, and $F(1,33)=0.06$, $p>.001$). As was seen in the GC/OFL, ^{3}H-TdR injection and surgical intervention did not differentially affect the numbers of cells which incorporated the tritiated thymidine within an individual fish. Again, however, the variability between fish was evident. There also appeared to be a tendency towards higher overall labelling in the more peripheral retinal regions examined. This is likely carry-over from the ora terminalis.

To test the hypothesis that the density of labelled cells is modulated following unilateral optic nerve crush, an ANOVA was run across experimental groups. In Figure 25A, these results are presented. An analysis of variance resulted in a clear effect of time following crush on the ratios of labelled cells in the receptor laminae ($F(7,716)=66.065$, $p<.001$). Student's t-tests were performed to locate the source of the difference.

Five days following nerve crush, there was a slight increase in the average number of labelled cells/10 mm in the axotomized photoreceptor layer. When the 112 observations gathered from 5 fish were averaged $\ln\langle R\rangle=0.1013$, SE=0.043. Student's t-test showed $F(1,200)=7.78$, $p>.001$ when compared with the intact group. At D10, there was a clear enhancement of labelled cells seen in the axotomized retina, as compared with the contralateral control. In 5 fish, 111 observational ratios were tabulated. The $\ln\langle R\rangle$ value of 0.7636, SE=0.064 was statistically distinct from all other groups (Normal vs D10: $F(1,199)=91.9$, p.001; D5 vs D10: $F(1,221)=73.6$, $p<.001$). Furthermore, this time following nerve crush was the only point at which the numbers of ^{3}H-TdR labelled cells in the photoreceptor layer are higher in the axotomized retina than in the control (see Figure 19). By D15 a clear reversal in labelling patterns was seen (see Figure 20). More tagged nuclei were found in the control receptor lamina then in the equivalent axotomized layer. The $\ln\langle R\rangle$ value for 117 observations on 6 fish was −0.4761, SE=0.058. This value was statistically different from normal ($F(1,205)=19.18$, $p<.001$), D5 ($F(1,227)=63.0$, $p<.001$), and D10 ($F(1,226)=204.8$, $p<.001$). This trend was consistent across the next four experimental windows. At D20, 77 observations were tabulated from 4 fish (see Figure 21). The $\ln\langle R\rangle$ value of −0.5109, SE=0.058 was not different from D15 values ($F(1,192)=0.19$, $p>.001$), but was distinct from normal ($F(1,165)=28.09$, $p<.001$), D5 ($F(1,187)=96.04$, $p<.001$), and D10 ($F(1,186)=244.10$, $p<.001$).

Twenty-five days following axotomy, the suppressed level of labelling was still evident in the axotomized retinal ONL (see Figure 22). Significant differences were seen between D25 and normal ($F(1,161)=55.65$, $p<.001$), D25 and D5 ($F(1,183)=156.25$, $p<.001$), D25 and D10 ($F(1,182)=280.2$, $p<.001$), but not between D25 and either D15 or D20 ($F(1,188)=6.55$, $p>.001$, $F(1,148)=8.29$, $p>.001$). The $\ln\langle R\rangle$ value obtained from 73 observations from 4 fish was -0.6827, $SE=0.042$. Thirty-five days following ONC, $\ln\langle R\rangle=-.2211$, $SE=.081$ which was obtained from 77 observations on 4 fish (see Figure 23). This was not statistically different from normal animals ($F(1,165)=1.42$, $p>.001$). The return to normal levels was confirmed by 50 days following axotomy (see Figure 24). From the 67 observations taken from 4 fish, $\ln\langle R\rangle=0.2097$, $SE=0.072$. This is not different from normal levels ($F(1,155)=10.89$, $p>.001$).

In Figure 25A the mean value of the ratio (axotomized:control) of labelled cells $\langle R\rangle$ and the $\ln\langle R\rangle$ values as a function of the survival period following optic nerve crush are summarized. The overall pattern of labelling appears to be biphasic, with an enhancement of labelling in the axotomized eye up to 10 days, followed by a maximal suppression at about 25 days after axotomy, and a gradual return to control levels by 35 days. These results suggest that axotomy affects the numbers of cells actively synthesizing DNA in the photoreceptor lamina when the animal is sacrificed 1 day following administration of ^{3}H-TdR. Enhancement of thymidine incorporation in the experimental retina occurs about 10 days following axotomy. This increase in cells entering the S-phase of the cell generation cycle is relatively short-lived. By 1 days and onward the number of cells in the axotomized retina is suppressed as compared with the control retina. The decreased incorporation of thymidine in the experimental eye continues for about 2 weeks, with a return to normal levels by about 35 days following axotomy.

The final question tested was whether modulation of cell proliferation varies as a function of topographical location. When these data were grouped into peripheral, middle, and central retinal regions, significant main effects were confirmed across days following axotomy ($F(7,28)=5.561$, $p<.001$). No effect of topographical location ($F(2,56)=.661$, $p>.001$) or interactions between the two ($F(14,56)=.708$, $p>.001$) were found.

One interpretation of this finding is that the precursor pool of germinal cells which is scattered throughout the ONL is triggered by some change taking place in the retina as a result of axotomy. This activation does not take place until around 10 days following nerve crush. Following the initial explosion, the precursor pool is "depleted" in some sense, such that less cells in the axotomized retina are able to enter successfully the S-phase of the cell generation cycle. This depression in the numbers of cells incorporating tritiated thymidine lasts for some period of time (approximately 15-20 days) after which time the precursor pool appears replenished. By 50 days following axotomy, the numbers of labelled cells in both retinae are equal, suggesting that any modulatory effects of optic nerve axotomy on cell birth have ceased.

Preliminary ultrastructural observations of the perikaryal characteristics suggest that these cells are "rod-like" in nature. Ultrastructural evidence for this claim will be presented after a discussion of the events following a 1 month survival interval following injection of tritiated thymidine.

EFFECTS OF OPTIC FIBER AXOTOMY ON CELL PROLIFERATION IN THE ONL EXAMINED 30 DAYS AFTER THYMIDINE INJECTION

In order to examine if cells that incorporate ^{3}H-TdR retain the label, I examined intact fish 30 days following intraocular injection. To examine the effects of axotomy on the labelled cell population after a 30 day survival period, fish received unilateral optic nerve crushes. At various post-operative periods: 5, 10, 15, 20, 25 or 50 days later, both eyes were injected with ^{3}H-TdR. One month later, these animals were processed for light microscopic examination.

RESULTS

a. Normal (ONL-N(LS))

When unoperated fish receive bilateral intraocular injections of tritiated thymidine and are allowed to survive for 1 month, the topographic distribution of labelled cell densities in the receptor layer is nearly identical between retinae. In Figure 26, these data from 4 fish are graphically portrayed. In 3 out of the 4 fish, there is a strong tendency toward more labelled cells in the most peripheral regions examined (see Figures 26A-26C). This levels off in the central retinal territories. In fish D, a low linear density of labelled cells is evident (Figure 26D). Differences between the two receptor layers of individual fish are minimal, illustrating no inherent distinctions between the two eyes using this surgical manipulation. The mean linear densities of labelled cells/10 mm between the two eyes are A=106.1 vs. C=101.4, A=39.6 vs. C=40.8, A=151.6 vs. C=153.5, and A=3.0 vs. C=4.6, respectively.

b. Five Days Following Unilateral Optic Nerve Crush (ONL-D5(LS))

When fish receive a unilateral optic nerve crush, are injected with tritiated thymidine 5 days later, and then sacrificed 1 month following the injection, more labelled cells are seen in the photoreceptor layer of the intact retina. Four fish are presented in Figure 27. For all 4 fish, more labelled nuclei are found in the control ONL, although there is some fluctuation between the retinae of each fish. Overlap between the two eyes is not pronounced. The mean linear densities for these fish are A=48.7 vs. C=81.4, A=16.9 vs. C=30.3, A=18.0 vs. C=26.3, and A=12.2 vs. C=24.2.

c. Ten Days Following Unilateral Optic Nerve Crush (ONL-D10(LS))

In Figure 28, four fish that were sacrificed 1 month following injection of ^{3}H-TdR are portrayed. Axotomy appears to suppress the density of labelled cells as is seen by the solid line. Although there is variability across fish, more labelled cells are present in the photoreceptor layer of the control side than the axotomized side in a single fish. There is some tendency toward increased labelling in the more peripheral regions (eg. Figures 28A, B, and D), with a decreasing function as more central retina is examined. In Figure 28C, a high degree of variability is present, but even in this case, labelling is clearly greater in the ONL of the control retina. The mean linear densities for these four fish are A=106.0 vs. C=193.6, A=87.4 vs. C=129.4, A=42.8 vs. C=64.3, and A=15.7 vs. C=29.0.

d. Fifteen Days Following Unilateral Optic Nerve Crush (ONL-D15(LS))

By 15 days following axotomy, even more labelled cells are seen in the photoreceptor layer of the axotomized eye as compared with the control. In the fish illustrated in Figures 29A-C, this is particularly clear. The mean linear densities are A=34.1 vs. C=91.4, A=25.1 vs. C=97.1, and A=31.2 vs. C=90.2 for these fish. In Figure 29A, labelling in the axotomized eye follows a relatively linear path, while in the control eye, an overall decreasing function is observed. In Figure 29B, the graph is somewhat unique, and reminiscent of the labelling pattern seen in the GC/OFL. Both retinae appear to have an increased number of labelled cells toward the more central regions. There are however, more labelled cells in the non-operated retina. In Figure 29C, more tagged nuclei are evident in the ONL of the unoperated eye. The function for both eyes appears to be decreasing to a minimum value. There is no overlap between the two retinae. In Figure 29D, there is no overlap between the labelling distributions of the two retinae. Although there are more labelled cells in the more peripheral retinal region, this is not as pronounced as that seen in the other fish of this post-operative group. The mean number of labelled cells/10 mm for this fish is 8.4 in the axotomized ONL and 18.0 in the control receptor layer.

e. Twenty Days Following Unilateral Optic Nerve Crush (ONL-D20(LS))

Twenty days following unilateral optic nerve crush, differences in the densities of labelled cells between the ONLs of the retinae are evident when the fish are observed 1 month following thymidine injection. Labelling is suppressed in the photoreceptor layer of the axotomized eye. In Figure 30, four fish that underwent these procedures are presented. The mean linear densities are A=67.6 vs. C=113.2, A=86.6 vs. C=189.0, A=33.4 vs. C=72.1, and A=8.2 vs. C=15.5. Figures 30A and C illustrate the topographical variation in the labelled cell densities in both the axotomized and control receptor layers. Labelling is higher in the more peripheral regions. In Figure 30B, a fish with more variability is presented. In all cases, more tagged nuclei are seen in the control retina. The same holds true for the fish presented in Figure 30D, albeit the topographical distribution appears linear.

f. Twenty-five Days Following Unilateral Optic Nerve Crush (ONL-D25(LS))

As was found as early as 5 days following unilateral optic nerve crush, and was consistent at all other post-operative periods examined, more labelled cells are found in the photoreceptor layer of the control retina as compared with the axotomized retina of the same fish. Twenty-five days following unilateral axotomy, the result is no different. In Figure 31, three fish under this experimental condition are presented. Figure 31A illustrates the changes that occur in the density of the labelled cell population at different topographical locations. More labelled cells are seen in the more peripheral retinal regions in both the intact and axotomized ONLs, but at all locations, with the exception of the most central, a higher density of tagged nuclei is found in the non-axotomized, receptor layer. The linear densities of labelled cells/10 mm in the axotomized receptor layer is 78.2, while almost double this amount is seen in the control (130.1). In Figures 31B and 31C, two fish with less differential labelling are seen. For these fish A=43.8 vs. C=66.1 and A=22.5 vs. C=36.1. Overall, the labelling distributions seen 25 days following axotomy appear to be returning to normal levels.

g. Fifty Days Following Unilateral Optic Nerve Crush (ONL-D50(LS))

By 50 days following unilateral optic nerve crush, labelling between the receptor layers of the axotomized and intact retinae appears to have returned to near normal levels (see Figure 32). Although there appears to be a greater degree of variability between retinae, the overall mean linear densities illustrate no consistent trends (A=14.9 vs. C=20.8, A=35.7 vs. C=30.0, A=25.5 vs. C=18.1, and A=14.6 vs. C=21.9).

DISCUSSION

The first question to be asked is whether the two retinae of the same intact fish have similar densities of labelled cells when examined 30 days following ^{3}H-TdR injection. Student's t-tests confirmed that the linear densities between the two eyes were not significantly different ($F(1,38)=.006$, $p>.001$), $F(1,40)=.004$, $p>.001$, $F(1,42)=.006$, $p>.001$, and $F(1,25)=2.50$, $p>.001$). The ln<R> value for the 74 observations from these 4 fish was −.1198, SE=.006.

The most consistent finding when fish are examined 1 month following ^{3}H-TdR injection is that in the photoreceptor lamina in all experimental cases, more labelled cells are seen in the control eye than in the axotomized eye. Do the suppressed linear densities vary as a function of the time following axotomy when fish are examined 30 days following injection?

An analysis of variance across the days following nerve crush was statistically significant ($F(6,514)=39.878$, $p<.001$). T-tests were used to determine the source of the difference. At 5 days following axotomy, 79 observations were taken from four fish, $\ln\langle R\rangle=-0.6535$, $SE=0.66$ (see Figure 27). This was significantly different from that seen in intact animals ($F(1,151)=34.34$, $p<.001$). A similar $\ln\langle R\rangle$ value was seen at D10 (-0.5502, $SE=0.049$), which is distinct from intact ($F(1,146)=27.25$, $p<.001$), but not from D5 ($F(1,151)=1.66$, $p>.001$) (see Figure 28). Seventy four observations from 4 fish were compiled to form this $\ln\langle R\rangle$ value. Fifteen days following axotomy, a further suppression of the numbers of labelled cells in the axotomized eye was seen (Figure 29). The $\ln\langle R\rangle$ value taken from 85 observation on four fish was -1.1578, $SE=0.064$. The suppressed labelling in the axotomized retina at D15 was significantly different from normal ($F(1,157)=125.66$, $p<.001$), D5 ($F(1,162)=31.47$, $p<.001$), and D10 ($F(1,157)=53.88$, $p<.001$) values. The cell counts for each of these four fish are presented in Figure 29. At Day 20 $\ln\langle R\rangle=-0.7335$, $SE=0.044$, illustrating that less tagged cells were present in the axotomized ONL (see Figure 30). Seventy-eight observations from 4 fish comprised this value. This $\ln\langle R\rangle$ value was neither distinct from D5 ($F(1,155)=1.08$, $p<.001$) nor from D10 ($F(1,159)=7.73$, $p>.001$), but was statistically different from normal

(F(1,150)=60.37, p<.001) and D15 (F(1,161)=28.5, p<.001). At 25 days following axotomy, labelling ratios began to return to near normal levels (see Figure 31). The ln<R> value for this group was -0.4612, SE=0.052, taken from 61 observations from 3 fish. Statistical analyses revealed that D25 was still different from normal (F(1,133)=15.44, p<.001), D15 (F(1,144)=63.04, p<.001, and D20 (F(1,137)=16.08, p<.001) but not from D5 (F(1,138)=5.15, p>.001) or D10 (F(1,133)=1.54, p>.001). By 50 days following axotomy the mean numbers of labelled cells have returned to normal values (ln<R>=-0.0691, SE=0.076). A total of 70 observations were used to form this ln<R> value (Figure 32). When the ln<R> values between normal and D50 fish were compared, the result was not significant (F(1,142)=0.25, p>.001). All other groups were different from D50 (as they were with normal fish) (D50 vs. D5- F(1,147)=35.76, p<.001, D50 vs. D10- (F(1,142)=28.84, p<.001, D50 vs. D15- (F(1,153)=121.0, p<.001, D50 vs. D20- (F(1,146)=59.75, p<.001 and D50 vs. D25 (F(1,129)=17.13, p<.001).

Finally, I tested whether labelling densities in different topographical regions vary with respect to the time following axotomy. An ANOVA confirmed that ln<R> varied as a function of time following axotomy (F(6,20)=11.854, p<.001) when ln<R> values were calculated according to peripheral, middle, and central location. However, no difference was seen between topographical location (F(2,40)=0.206, p>.001) nor were there any reliable interactions (F(12,40)=0.333, p>.001). Although statistical tests do not support a reliable labelling gradient across topographical regions, it is still apparent that labelling is often higher in the more peripheral retinal regions. This probably reflects activity occurring in the <u>ora terminalis</u> at the time of the injection. One month following injection of ^{3}H-TdR, those cells originally incorporating the tracer have been displaced by the newer germinal zone. Hence, the old germinal regions now occupies a more central position, as is witnessed by the topographical distribution of labelled cells in the retina examined 1 month following injection.

In all experimental cases, with the exception of Day 50 where birth rates appear to return to normal, the density of labelled cells in the ONL of the axotomized retina is less than that of the ONL of the intact retina. One interpretation of this result is that there are less visibly labelled cells in the ONL of the axotomized retina because these tagged nuclei have undergone further cell generation cycles, thus diluting the label. The question to be tested, therefore, is do those cells initially in the S-phase of the cell generation cycle undergo further mitotic divisions, diluting the label, and is this modulated by optic nerve axotomy? This question is will be addressed in the final section.

ULTRASTRUCTURE OF LABELLED CELLS IN THE RECEPTOR LAYER

In the photoreceptor layer, those cells labelled with ^{3}H-TdR (see Figure 33) appear to be rod-like in nature. The primary evidence for this interpretation is their distinct location. The ONL of an adult goldfish is four to five cells thick. The nuclei located closest to the OPL have been definitively shown to be rods (Johns & Easter, 1977; Stell & Harosi, 1976). Cone nuclei lie in a single stratum sclerad to the rods. The irregularly shaped nucleus of this cell takes up most of the cell body area (see Figures 33C and D). Their nuclei are stained in a fine granular fashion. Other surrounding rod cells are similar in appearance, but are much more heavily stained. This light staining is probably a result of the active metabolic state of the DNA synthesizing cells. The ultrastructure of the cells presented here compares well with those from other published reports (Johns, 1982; Raymond, 1985a, 1985b). From the location of these cells and especially their similarity to the neighboring population, the cell type described here is a member of the rod receptor family.

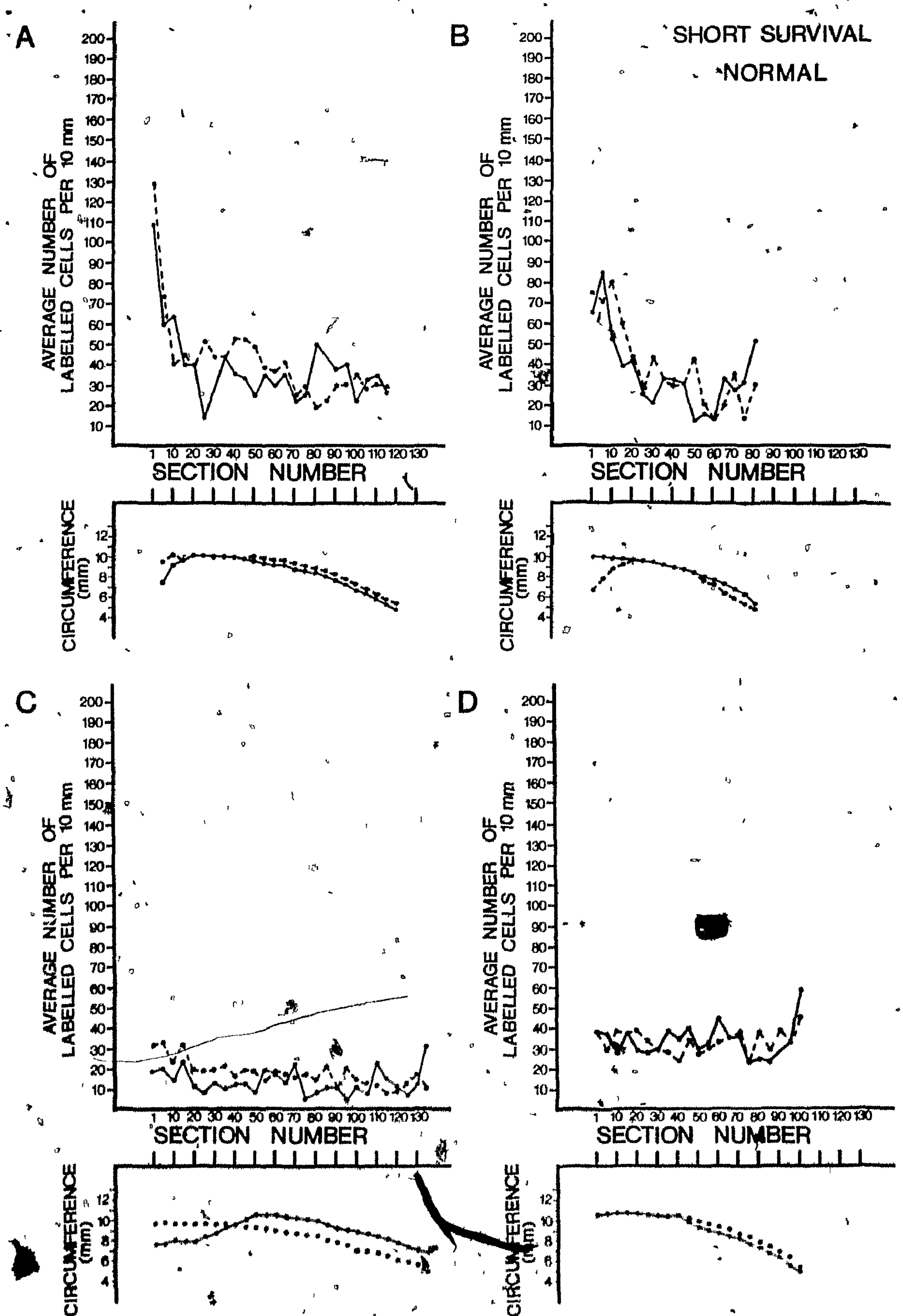
Figure 17. Topographical Distribution of the Linear Density of Labelled Cells in the ONL- Normal, Short Survival

See Figure 6 for description of the conventions employed.

A. This fish received a sham-right optic nerve crush. Both eyes were injected with ^{3}H-TdR and the animal was perfused and sacrificed 24 hours later. Differences between the two eyes are minimal.

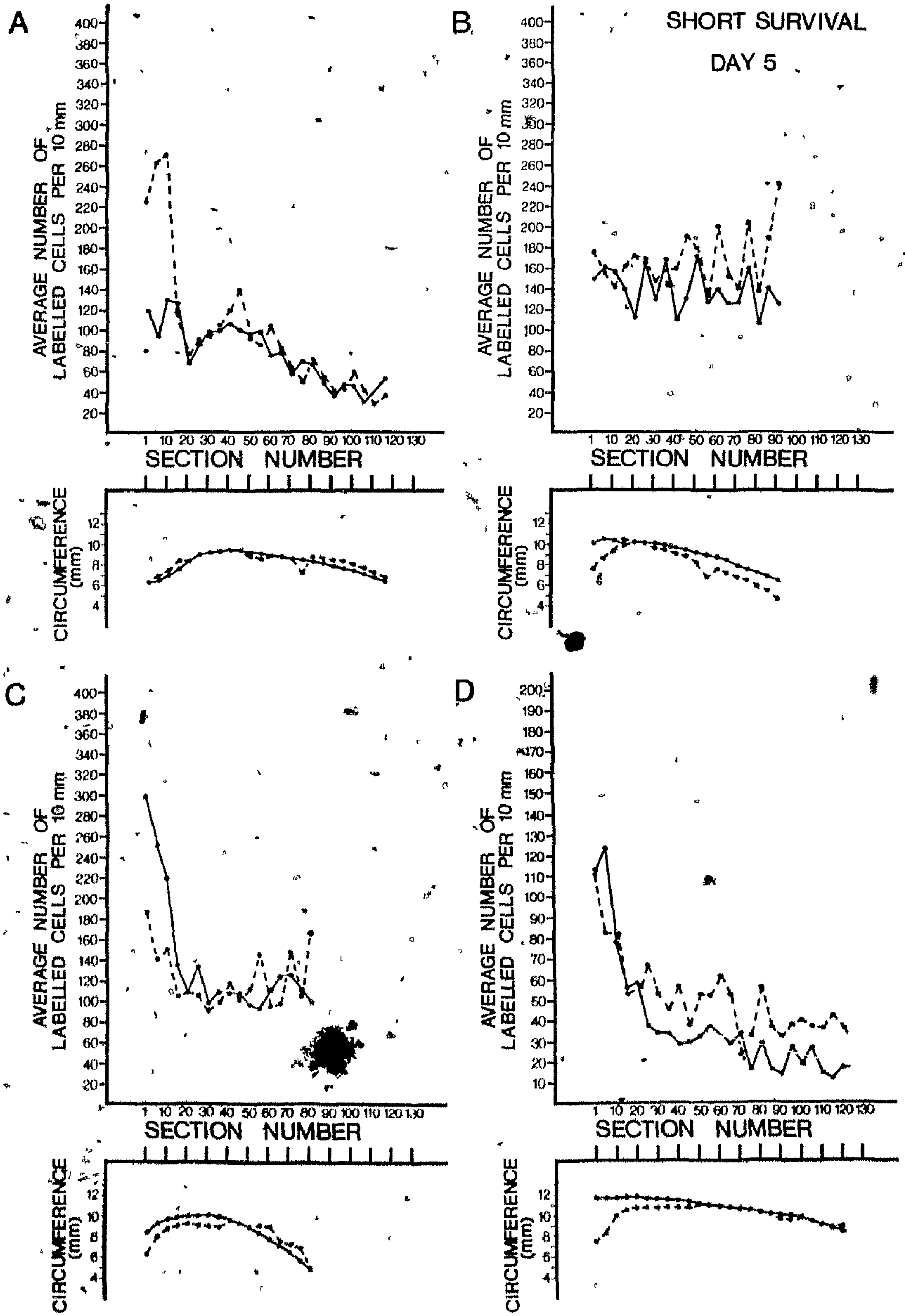
B. This fish received a sham-left optic nerve crush, and the right side was unoperated.

C. and D. These two intact fish received bilateral intraocular injections and were sacrificed 1 day later.



SHORT SURVIVAL
NORMAL
A
B
C
D
AVERAGE NUMBER OF LABELLED CELLS PER 10 mm
SECTION NUMBER
CIRCUMFERENCE (mm)

Figure 18. Distribution of Labelled Cells in the ONL- (D5, SS).
These five fish received unilateral ONCs followed by bilateral ^{3}H-TdR injections five days later. Their retinae were processed for light autoradiography one day following injection.



SHORT SURVIVAL
DAY 5
A
B
C
D
AVERAGE NUMBER OF LABELLED CELLS PER 10 mm
SECTION NUMBER
CIRCUMFERENCE (mm)

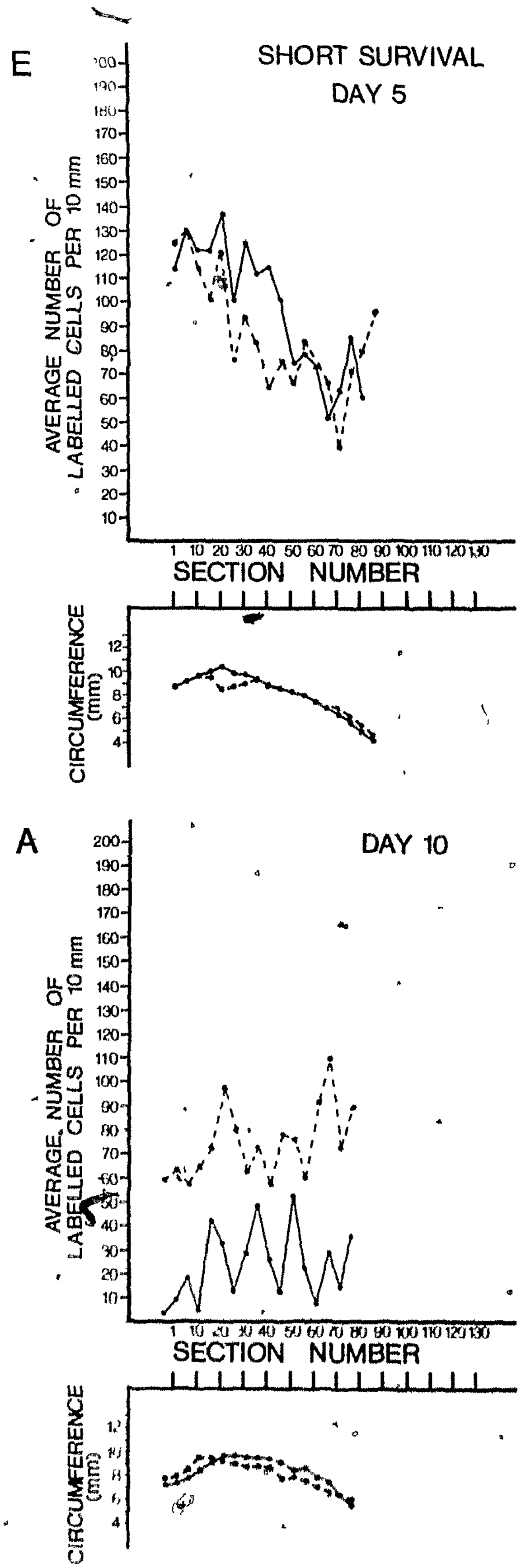

E
SHORT SURVIVAL
DAY 5
AVERAGE NUMBER OF LABELLED CELLS PER 10 mm
SECTION NUMBER
CIRCUMFERENCE (mm)
A
DAY 10
AVERAGE NUMBER OF LABELLED CELLS PER 10 mm
SECTION NUMBER
CIRCUMFERENCE (mm)

Figure 19. Distribution of Labelled Cells in the ONL- (D10, SS)

Unilateral optic nerve axotomy was performed on these six fish. Ten days later they received bilateral ^{3}H-TdR injections and were sacrificed on the following day. Note the change in scale on the ordinate in D.

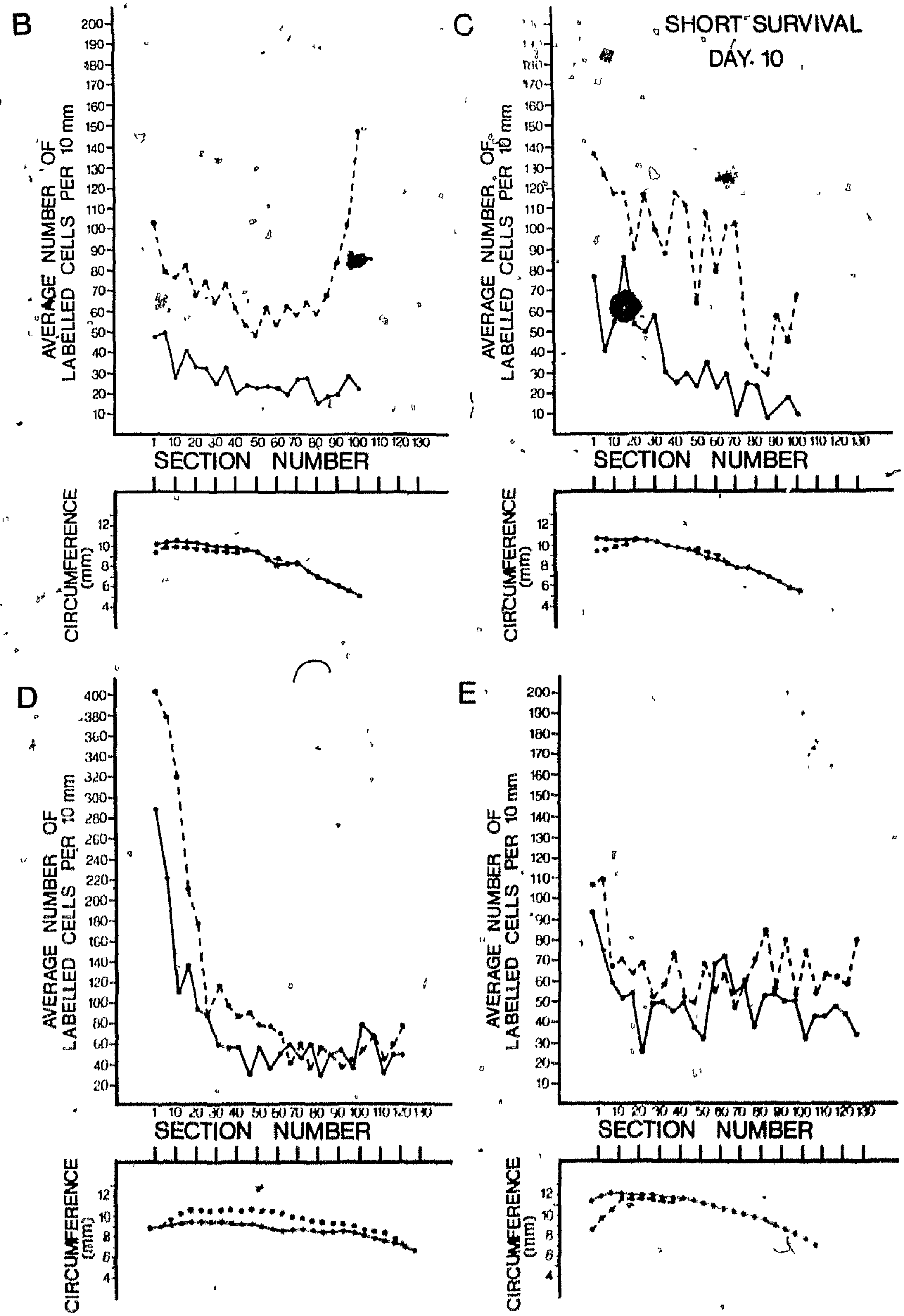
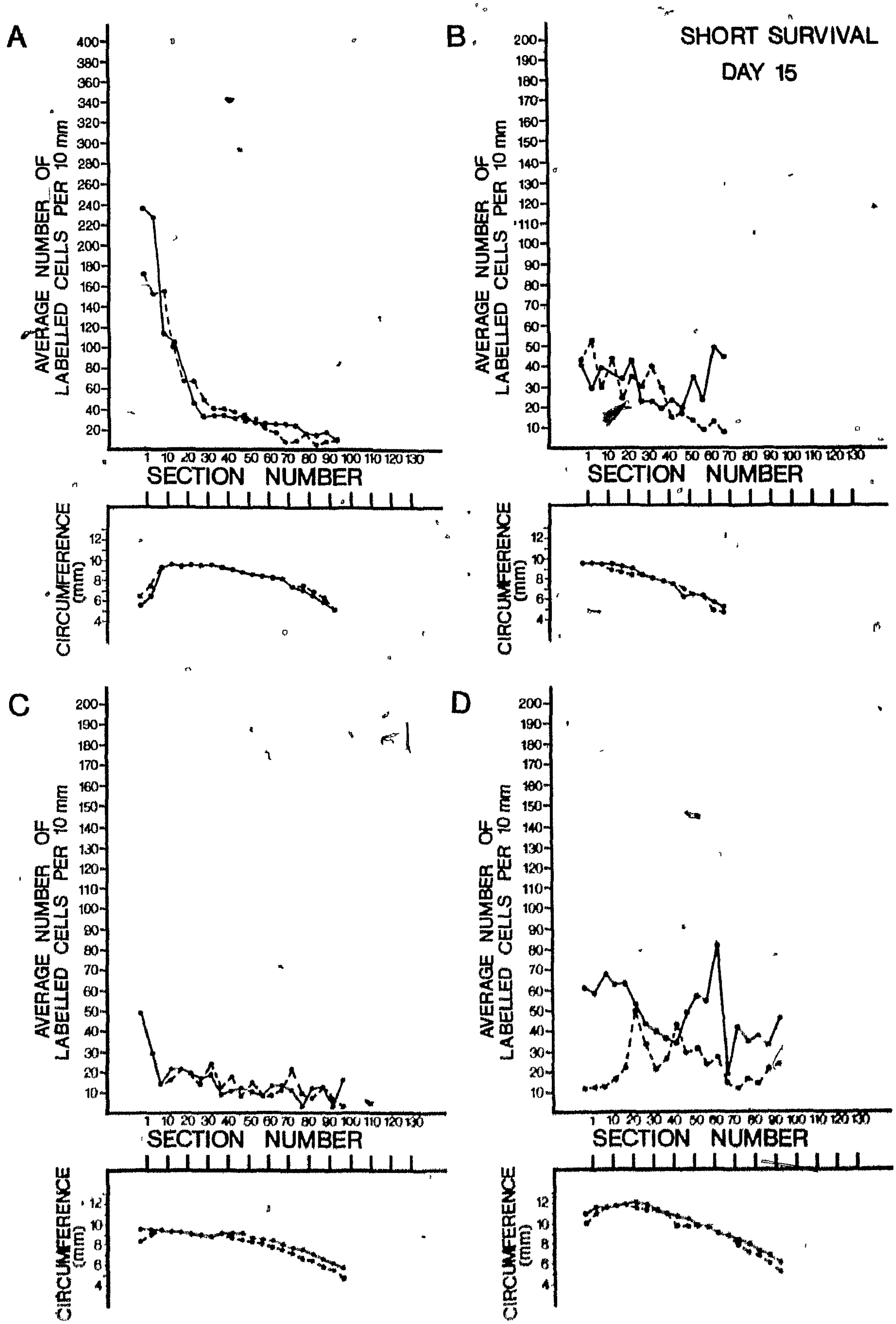

B
C
SHORT SURVIVAL
DAY 10
AVERAGE NUMBER OF LABELLED CELLS PER 10 mm
SECTION NUMBER
CIRCUMFERENCE (mm)
D
E
AVERAGE NUMBER OF LABELLED CELLS PER 10 mm
SECTION NUMBER
CIRCUMFERENCE (mm)

Figure 20. Distribution of Labelled Cells in the ONL- (D15, SS)

A, B, C, D, E, and F. These fish received bilateral ^{3}H-TdR injections fifteen days following unilateral optic nerve crush. Their retinae were processed for light autoradiographic examination 24 hours following injection.


A
B
C
D
SHORT SURVIVAL
DAY 15
AVERAGE NUMBER OF LABELLED CELLS PER 10 mm
SECTION NUMBER
CIRCUMFERENCE (mm)
1 10 20 30 40 50 60 70 80 90 100 110 120 130

SHORT SURVIVAL
DAY 15

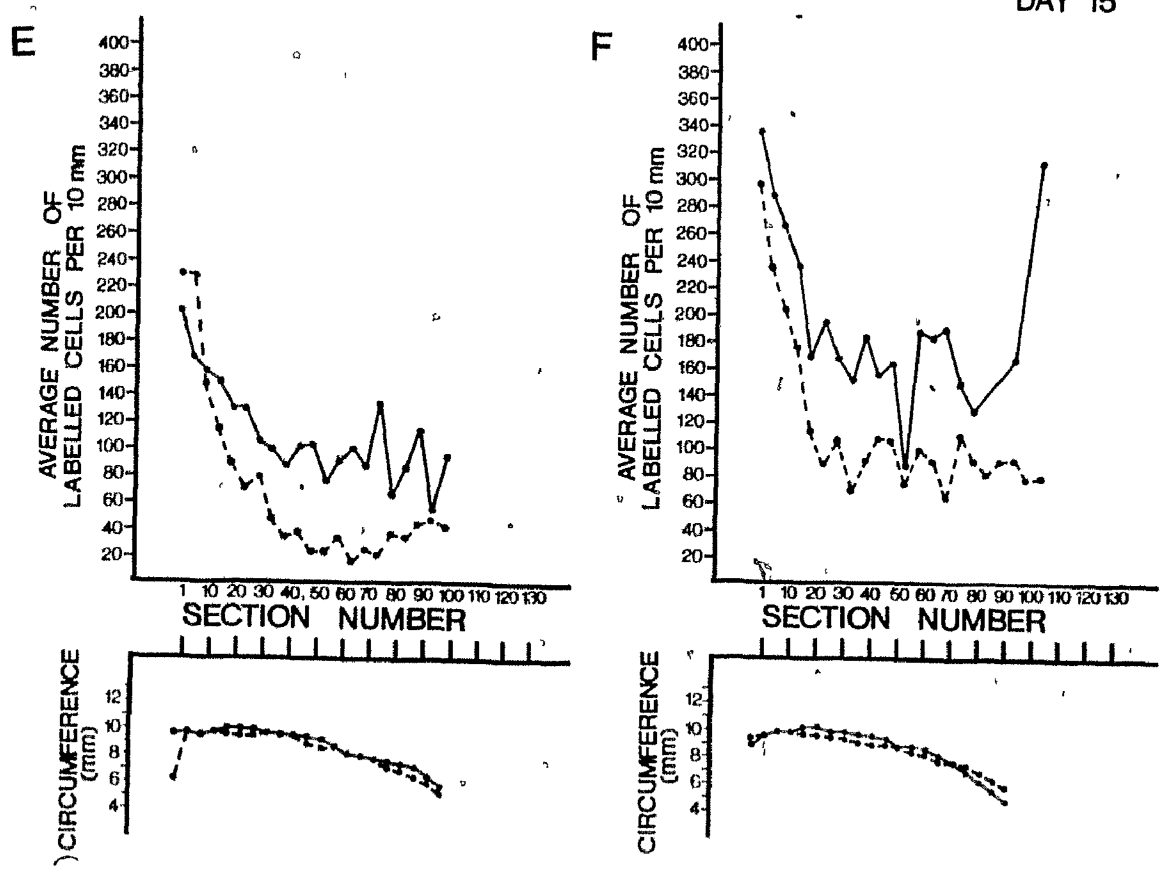


Figure 21. Distribution of Labelled Cells in the ONL- (D20, SS)

Twenty days following unilateral ONC, these fish (A, B, C, and D) received bilateral ^{3}H-TdR injections and were sacrificed 24 hours following injection.

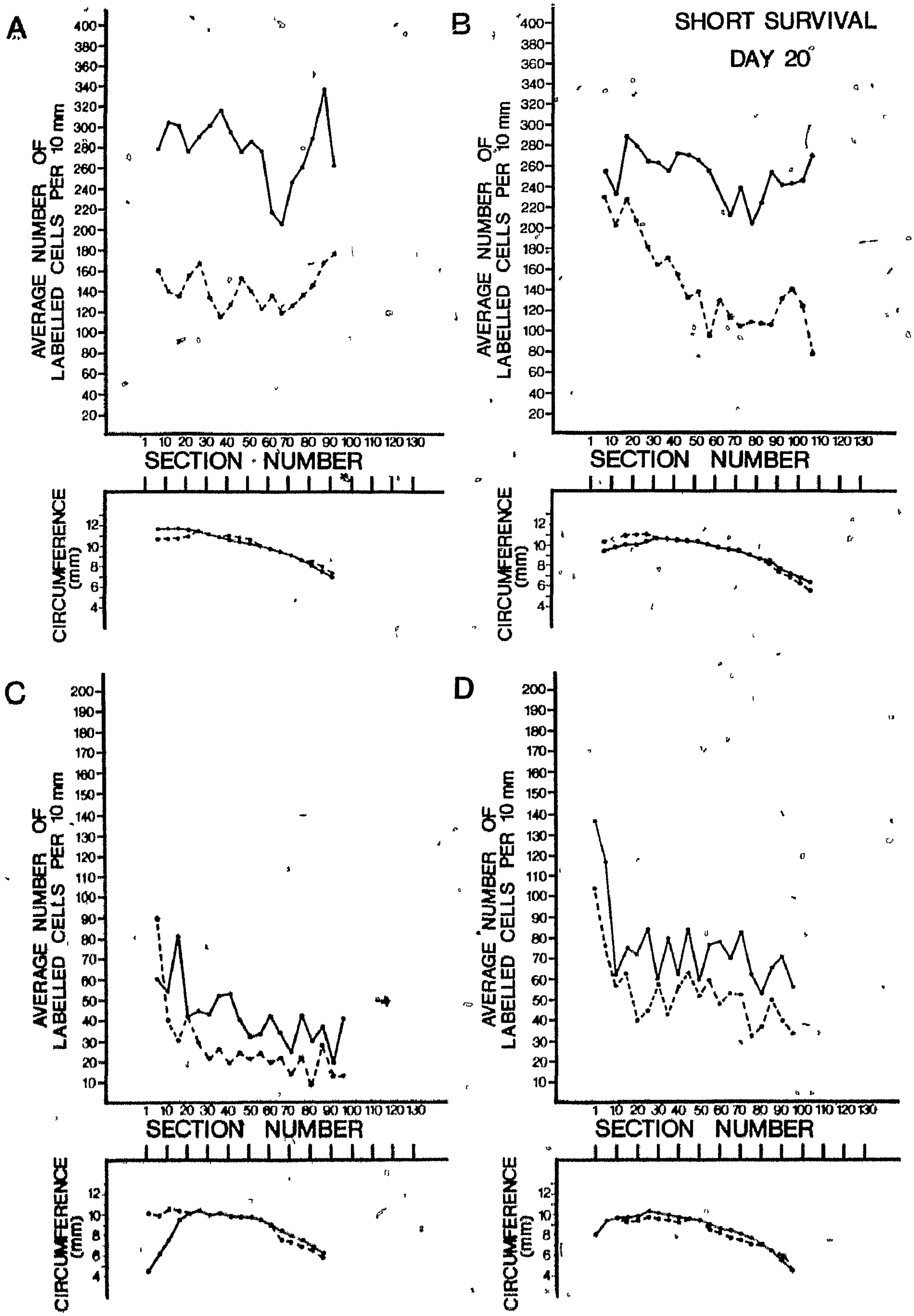

SHORT SURVIVAL
DAY 20
A
B
C
D
AVERAGE NUMBER OF LABELLED CELLS PER 10 mm
SECTION NUMBER
CIRCUMFERENCE (mm)

Figure 22. Distribution of Labelled Cells in the ONL- (D25, SS)

Twenty-five days following unilateral optic nerve crush, these fish received bilateral injections of ^{3}H-TdR. Their retinae were processed for light microscopic autoradiography on the following day. Note the change in scale on the ordinate for A and B.

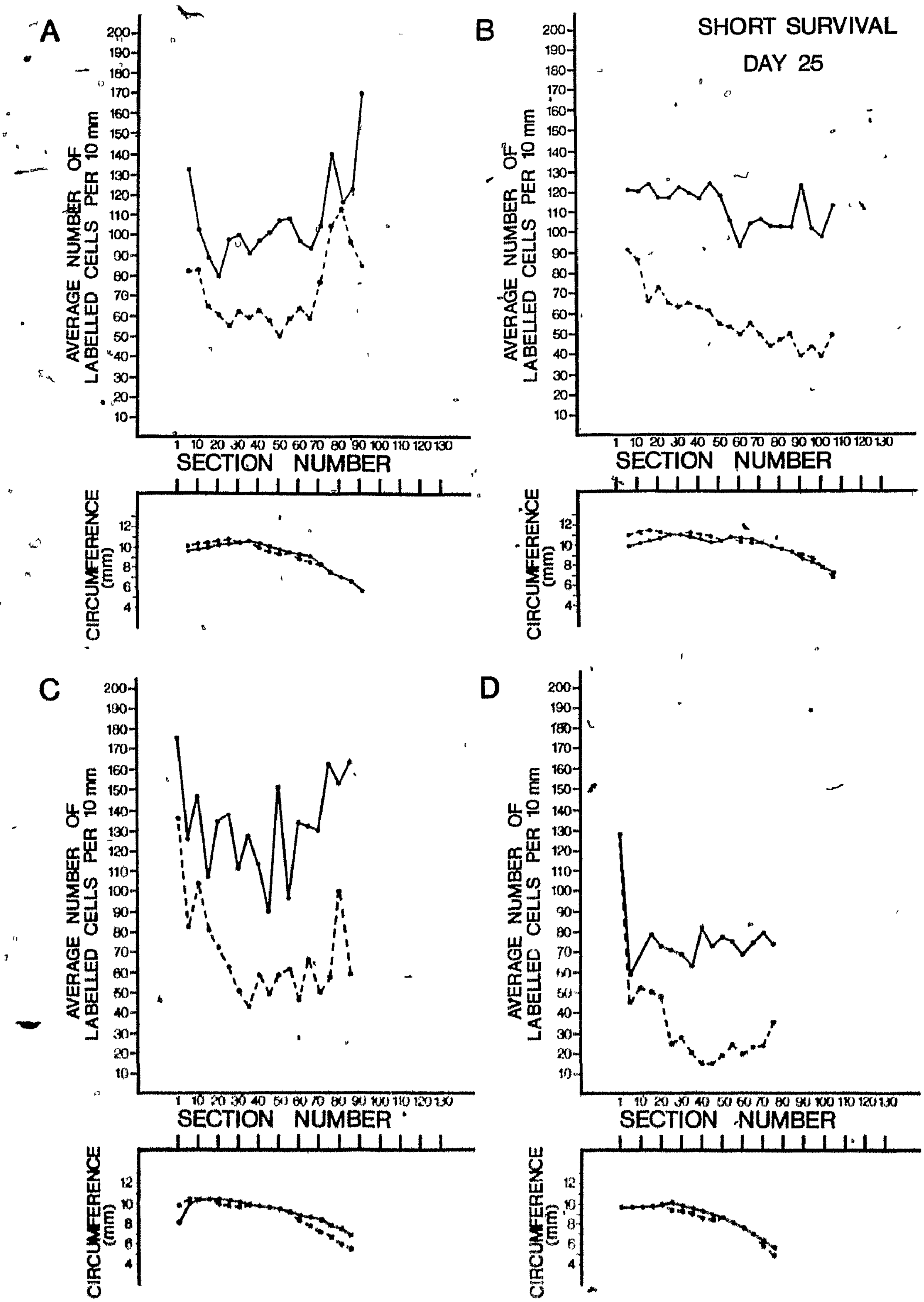

SHORT SURVIVAL
DAY 25
A
B
C
D
AVERAGE NUMBER OF LABELLED CELLS PER 10 mm
SECTION NUMBER
CIRCUMFERENCE (mm)

Figure 23. Distribution of Labelled Cells in the ONL- (D35, SS)

Four fish (A, B, C, and D) received bilateral intraocular injections of ^{3}H-TdR thirty-five days following unilateral optic nerve crush. They were sacrificed 24 hours following the injection. Note the change in scale of the ordinate for C and D.

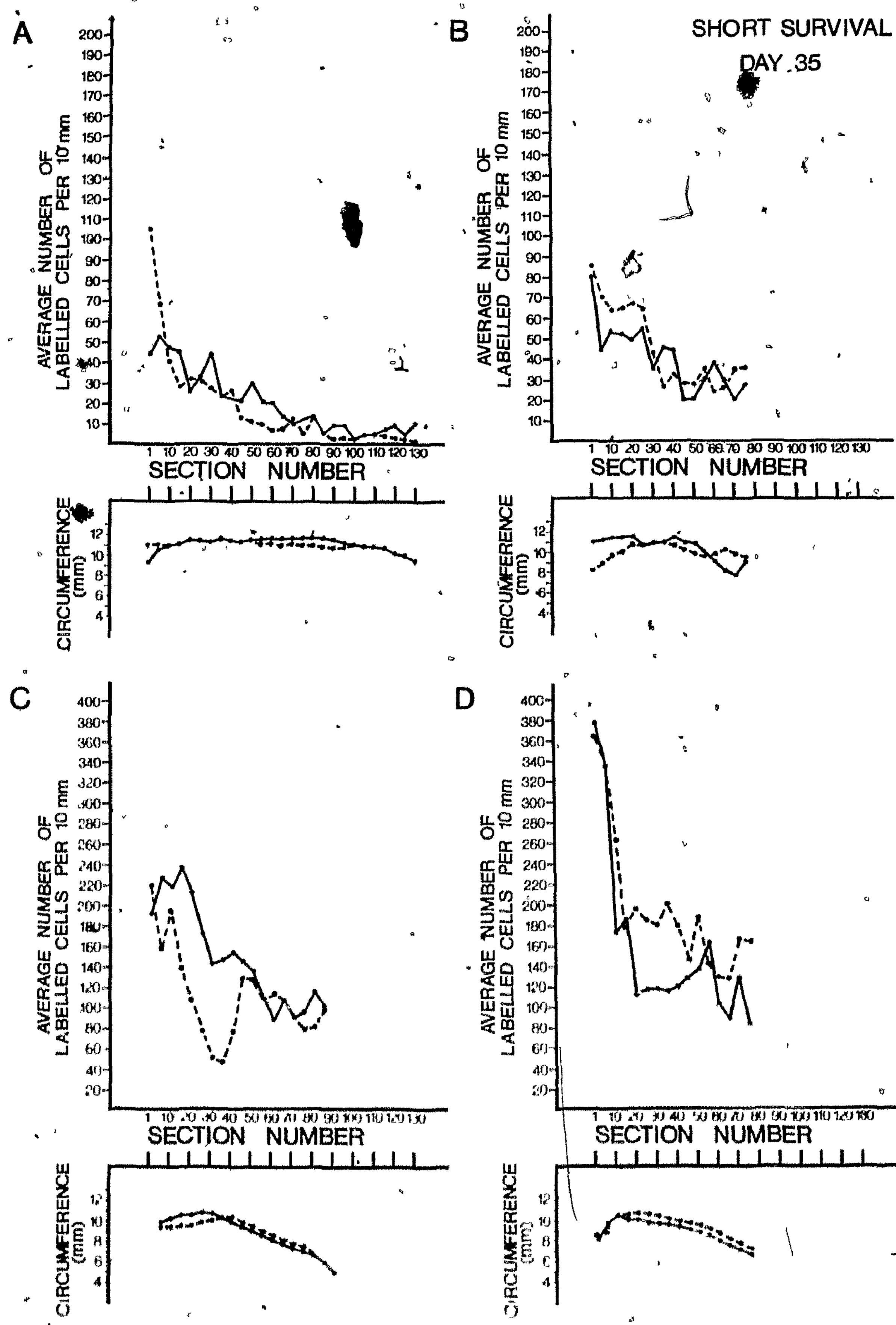
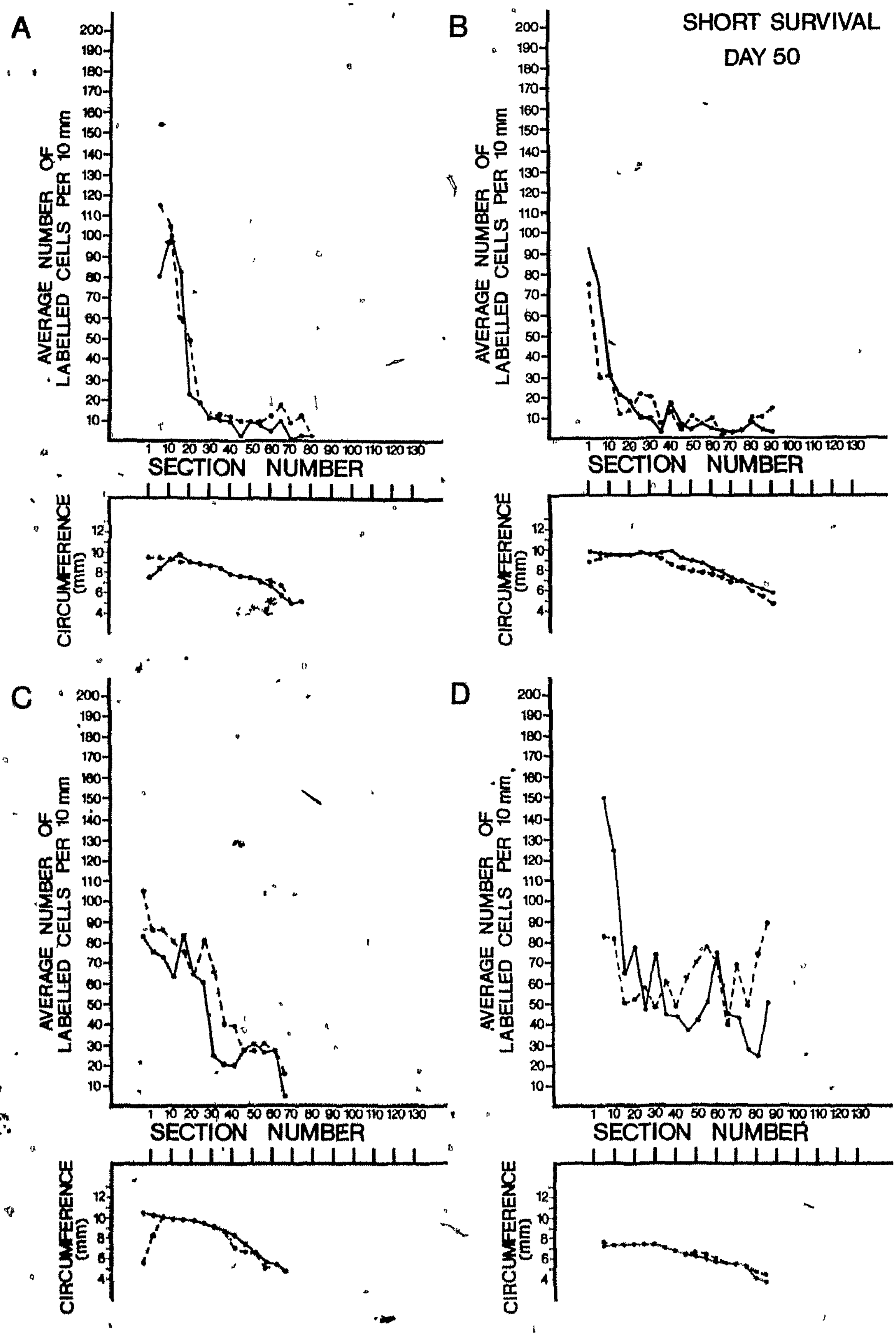

SHORT SURVIVAL
DAY 35
A
B
C
D
AVERAGE NUMBER OF LABELLED CELLS PER 10 mm
SECTION NUMBER
CIRCUMFERENCE (mm)

Figure 24. Distribution of Labelled Cells in the ONL- (D50, SS)

A, B, C, and D. These four fish received bilateral intraocular injections of ^{3}H-TdR fifty days after unilateral ONC. Their retinae were processed for light autoradiographic examination 24 hours after injection.



SHORT SURVIVAL
DAY 50
A
B
C
D
AVERAGE NUMBER OF LABELLED CELLS PER 10 mm
SECTION NUMBER
CIRCUMFERENCE (mm)

Figure 25. Time Course of Changes in Cell Proliferation in the ONL During Various Post-operative Periods Following Unilateral Optic Nerve Crush

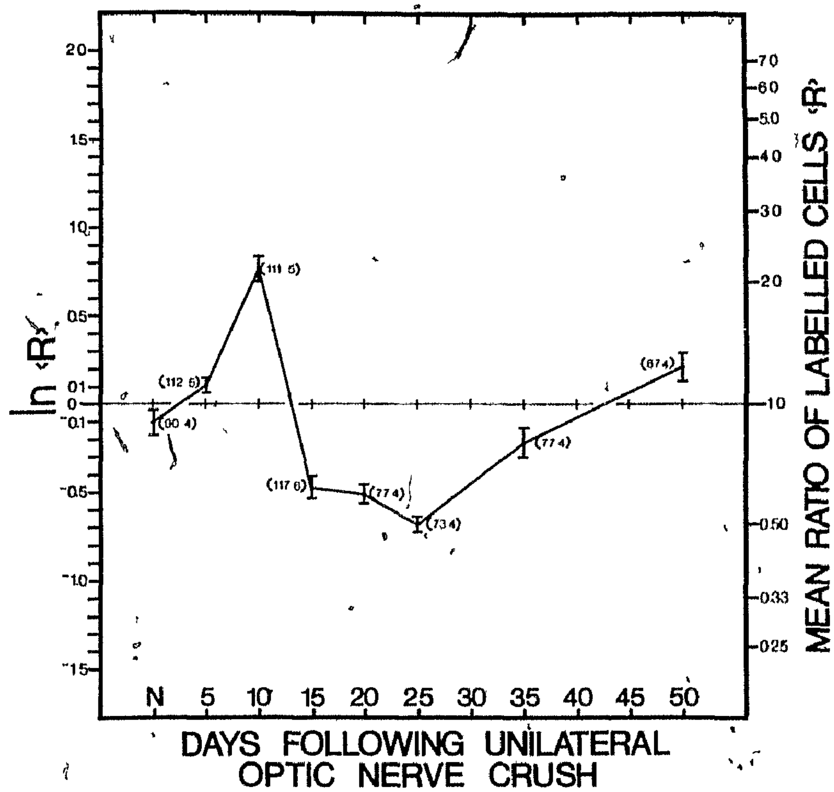
In these Figures, the abscissa denotes the days following unilateral optic nerve axotomy. The ordinates represent the mean value of the ratio of labelled cells (axotomized:control) ($\langle R \rangle$) on the right scale and the natural logarithm of $\langle R \rangle$ ($\ln\langle R \rangle$) on the left scale. The brackets contain the total number of individual ratios, for each post-operative period followed by the number of fish from which the individual ratios are taken. The error bars represent the standard error of the $\ln\langle R \rangle$.

A. 1 Day Following Thymidine Injection: The pattern of differential labelling in the receptor layer when fish are sacrificed 24 hours following thymidine injection is biphasic in conformation. More labelled cells are found in the receptor lamina of the axotomized retina as early as 10 days following unilateral optic nerve crush. By 15 days, a reversal is seen in which less tagged nuclei are evident in the axotomized retina. The suppression of labelled cells remains until about 35 days following axotomy, at which time, labelling between the two retinae returns to normal levels.

B. 30 Days Following Thymidine Injection: The patterns of differential labelling in the ONL when fish are sacrificed 30 days following thymidine injection are illustrated as a function of the days following unilateral optic nerve crush. As early as 5 days following axotomy, there is a suppressed density of labelled cells in the photoreceptor layer of the axotomized eye. At D10, a similar relationship is seen. There is a further suppression 15 days following crush and at D20 and

D25, the differential labelling is still evident, but to a lesser degree. By D50, no difference between labelling patterns is visible, levels similar to those seen in intact fish are evident.

A RECEPTOR CELL LAYER SHORT SURVIVAL



B RECEPTOR CELL LAYER LONG SURVIVAL

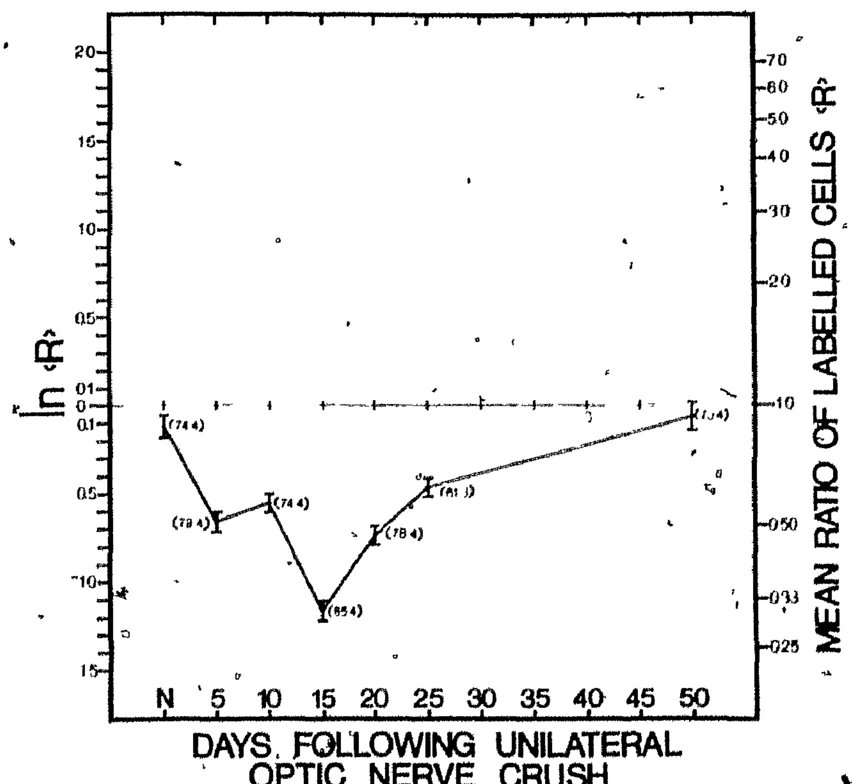
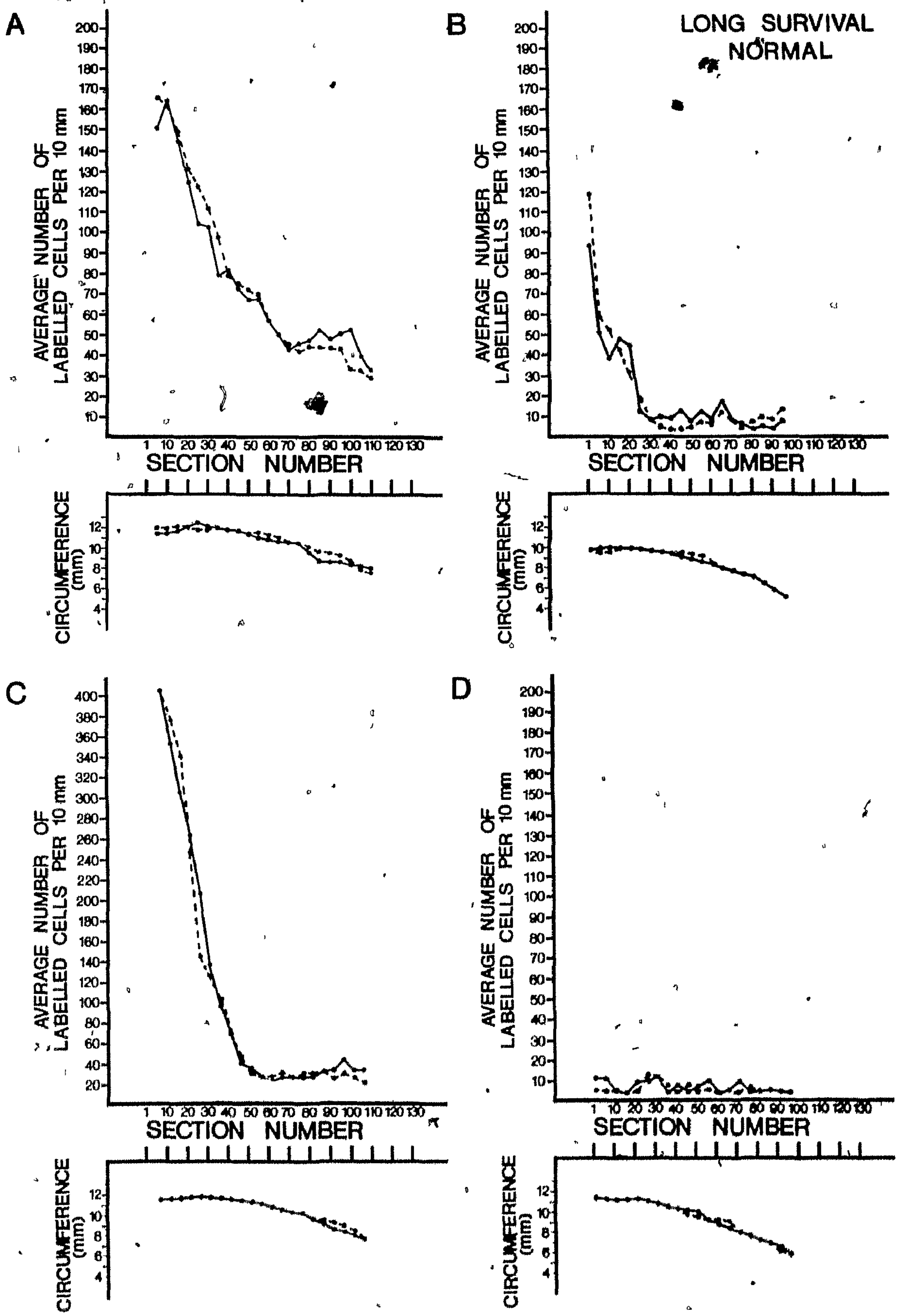


Figure 26. Topographical Distribution of the Linear Density of Labelled Cells in the ONL- Normal, Long Survival

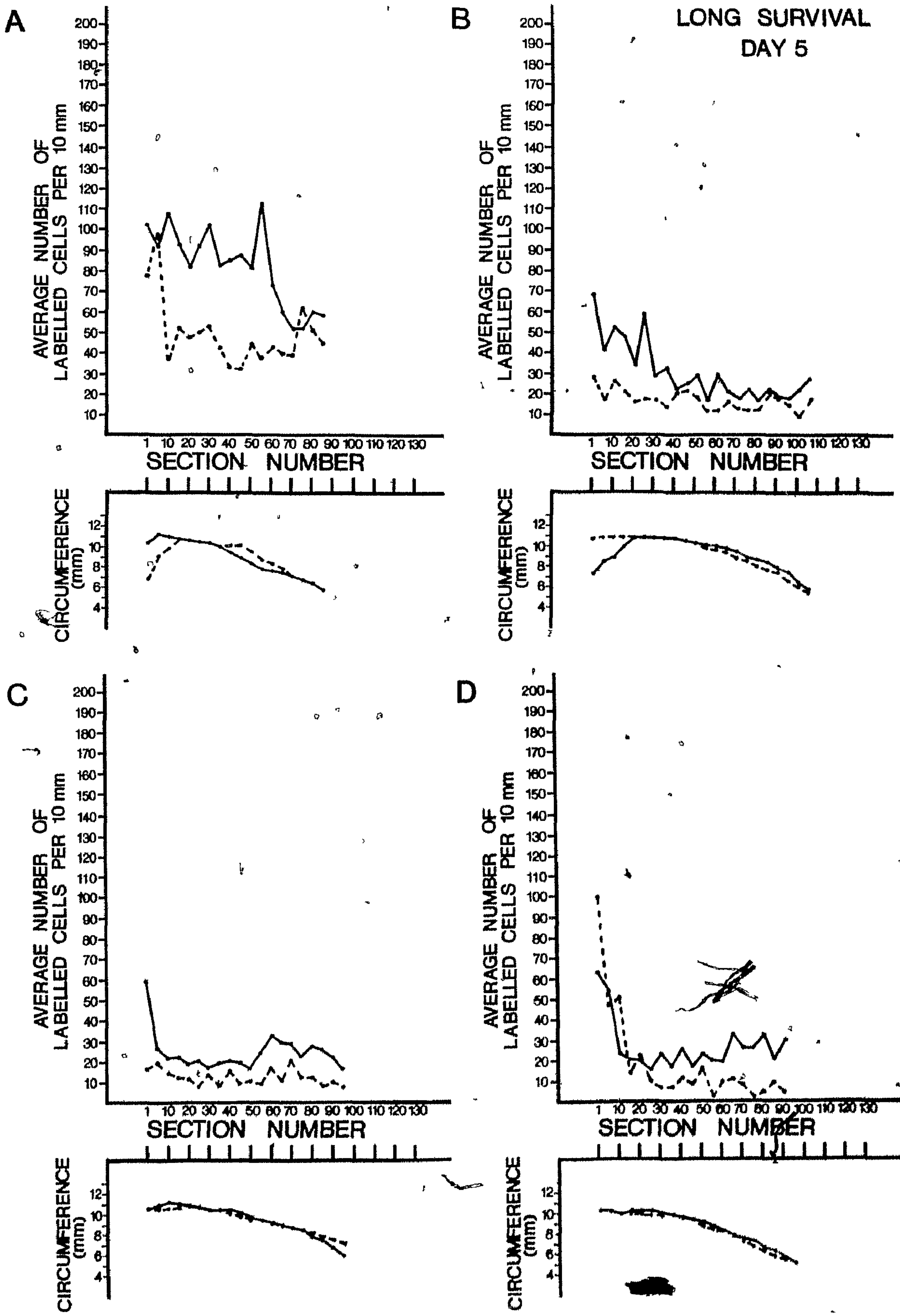
These four fish (A, B, C, and D) received bilateral intraocular injections of ^{3}H-TdR and were sacrificed 30 days later. Note the change in scale on the ordinate for C.



A
B
C
D
LONG SURVIVAL
NORMAL
AVERAGE NUMBER OF LABELLED CELLS PER 10 mm
SECTION NUMBER
CIRCUMFERENCE (mm)

Figure 27. Distribution of Labelled Cells in the ONL- (D5, LS)

A, B, C, and D. These fish received bilateral injections of ^{3}H-TdR five days following unilateral optic nerve crush. Their retinae were processed for light microscopic autoradiography 30 days following injection.


A
B
C
D
LONG SURVIVAL
DAY 5
AVERAGE NUMBER OF LABELLED CELLS PER 10 mm
SECTION NUMBER
CIRCUMFERENCE (mm)

<u>Figure 28. Distribution of Labelled Cells in the ONL- (D10, LS)</u>

Ten days following unilateral optic nerve crush, these fish received bilateral intraocular injections of ^{3}H-TdR. They were sacrificed 1 month following injection. Note the change in scale on the ordinate for A and B.

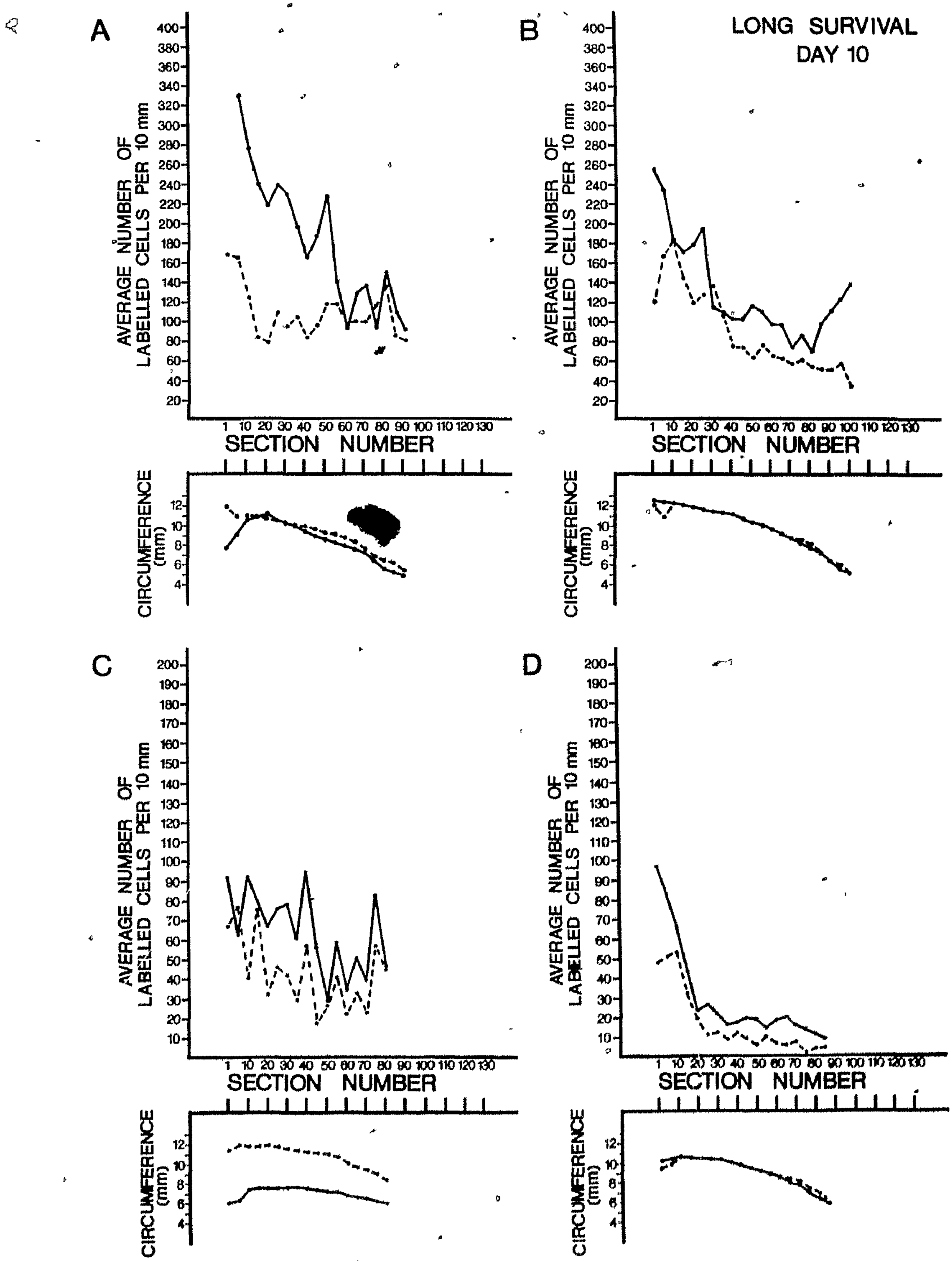
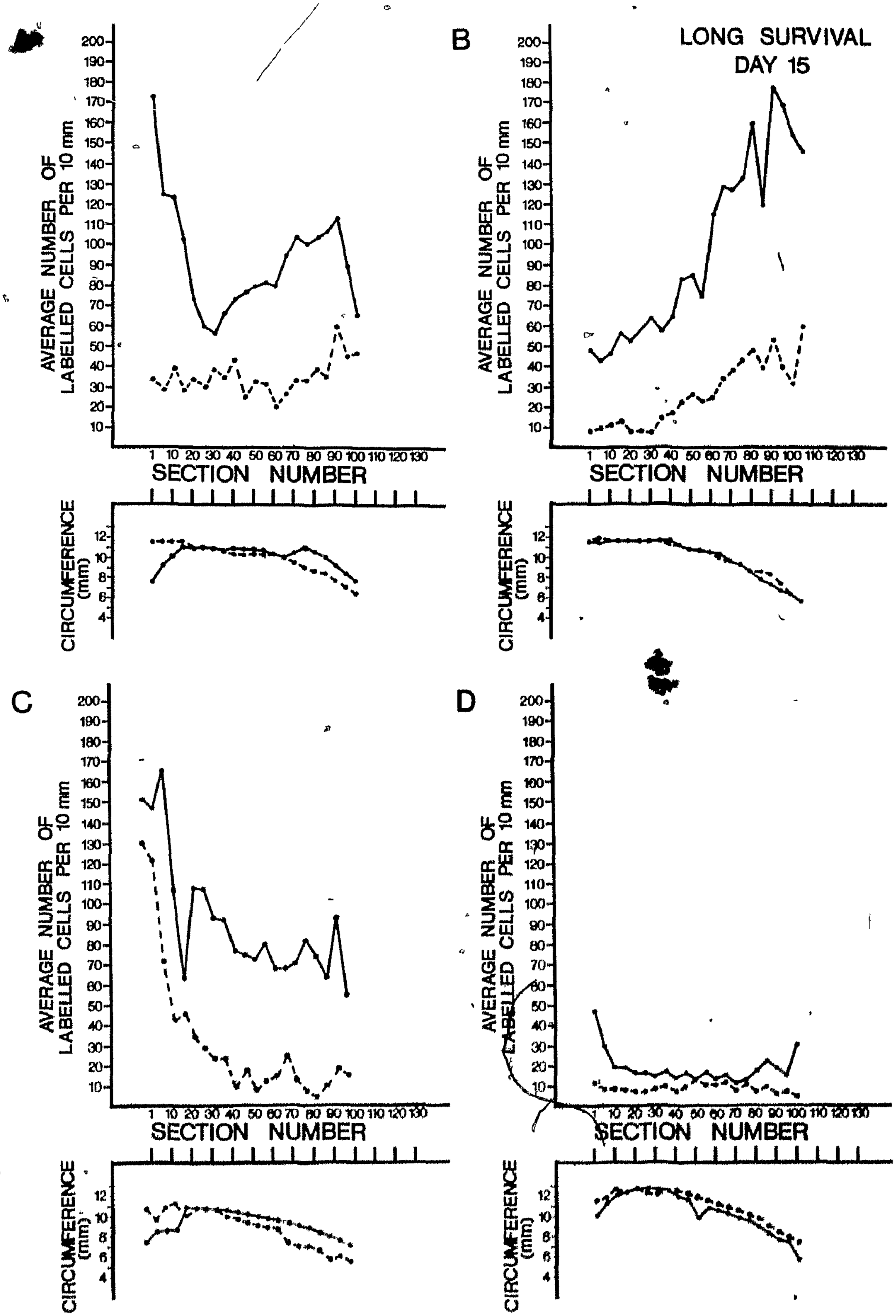

LONG SURVIVAL
DAY 10
A
B
C
D
AVERAGE NUMBER OF LABELLED CELLS PER 10 mm
SECTION NUMBER
CIRCUMFERENCE (mm)

Figure 29. Distribution of Labelled Cells in the ONL- (D15, LS)

Four fish that received bilateral injections of ^{3}H-TdR fifteen days after unilateral ONC are represented. These fish were sacrificed 30 days after the injection.



B
LONG SURVIVAL
DAY 15
C
D
AVERAGE NUMBER OF LABELLED CELLS PER 10 mm
SECTION NUMBER
CIRCUMFERENCE (mm)

Figure 30. Distribution of Labelled Cells in the ONL (D20, LS)

Twenty days following unilateral optic nerve crush, these four fish received bilateral intraocular injections of ^{3}H-TdR and their retinae were processed for light autoradiographic examination 30 days following the injection. Note the change in scale on the ordinate for A and B.

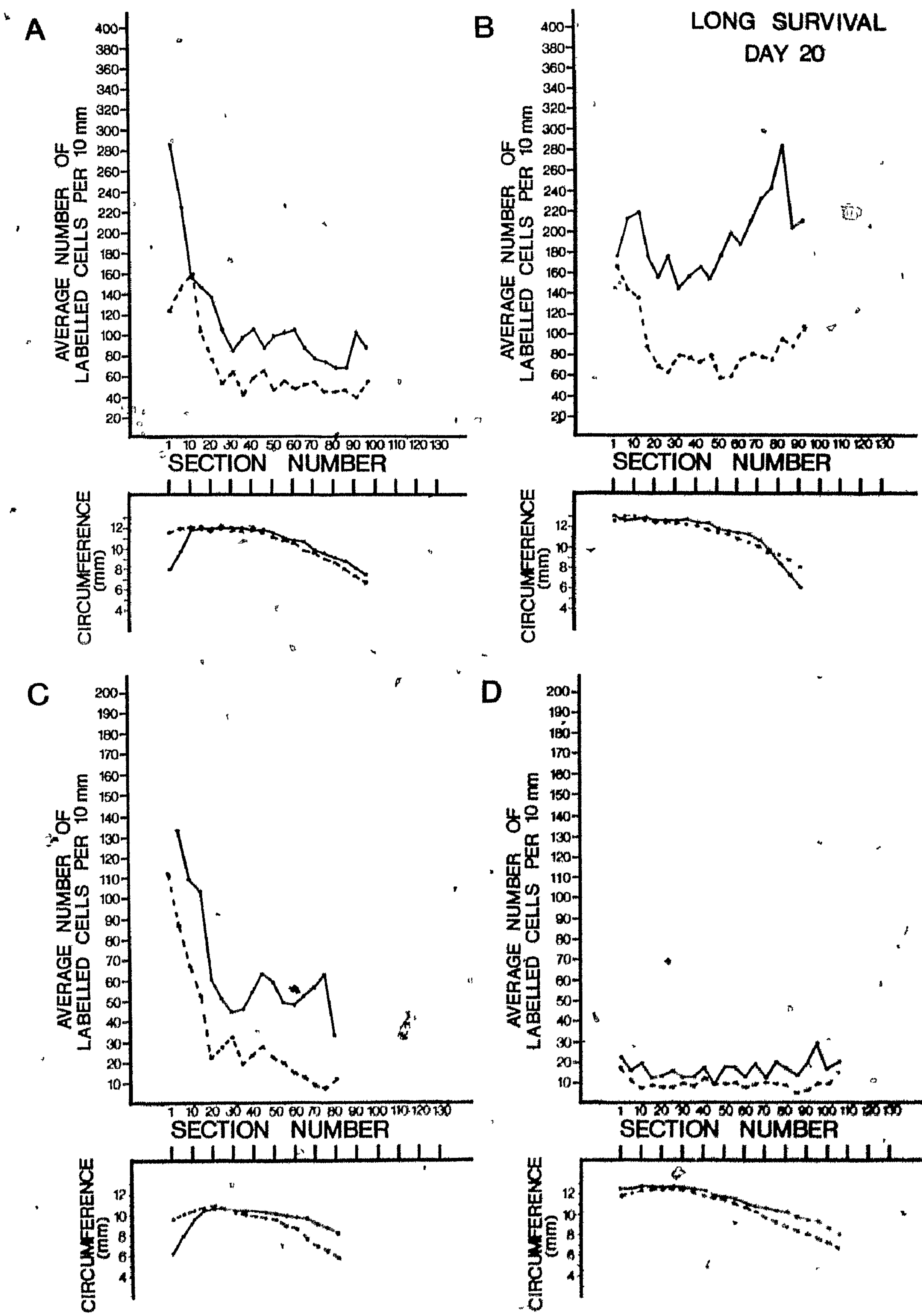

A
B
C
D
LONG SURVIVAL
DAY 20
AVERAGE NUMBER OF LABELLED CELLS PER 10 mm
SECTION NUMBER
CIRCUMFERENCE (mm)

Figure 31. Distribution of Labelled Cells in the ONL- (D25, LS)

A, B, and C. These three fish received bilateral injections of ^{3}H-TdR twenty-five days following unilateral optic nerve crush. They were sacrificed 1 month following injection. Note the change in scale on the ordinate for A. and B.

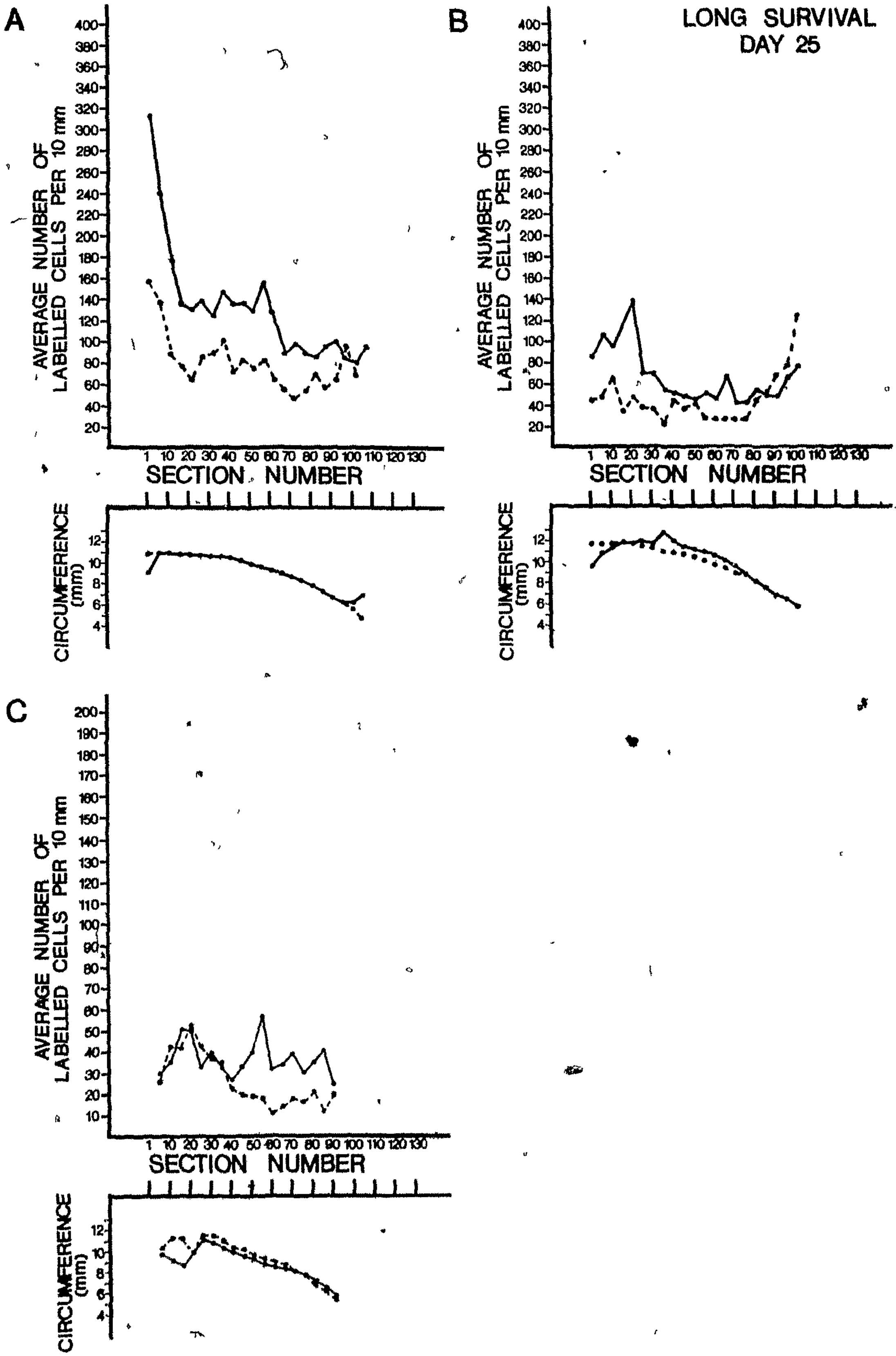
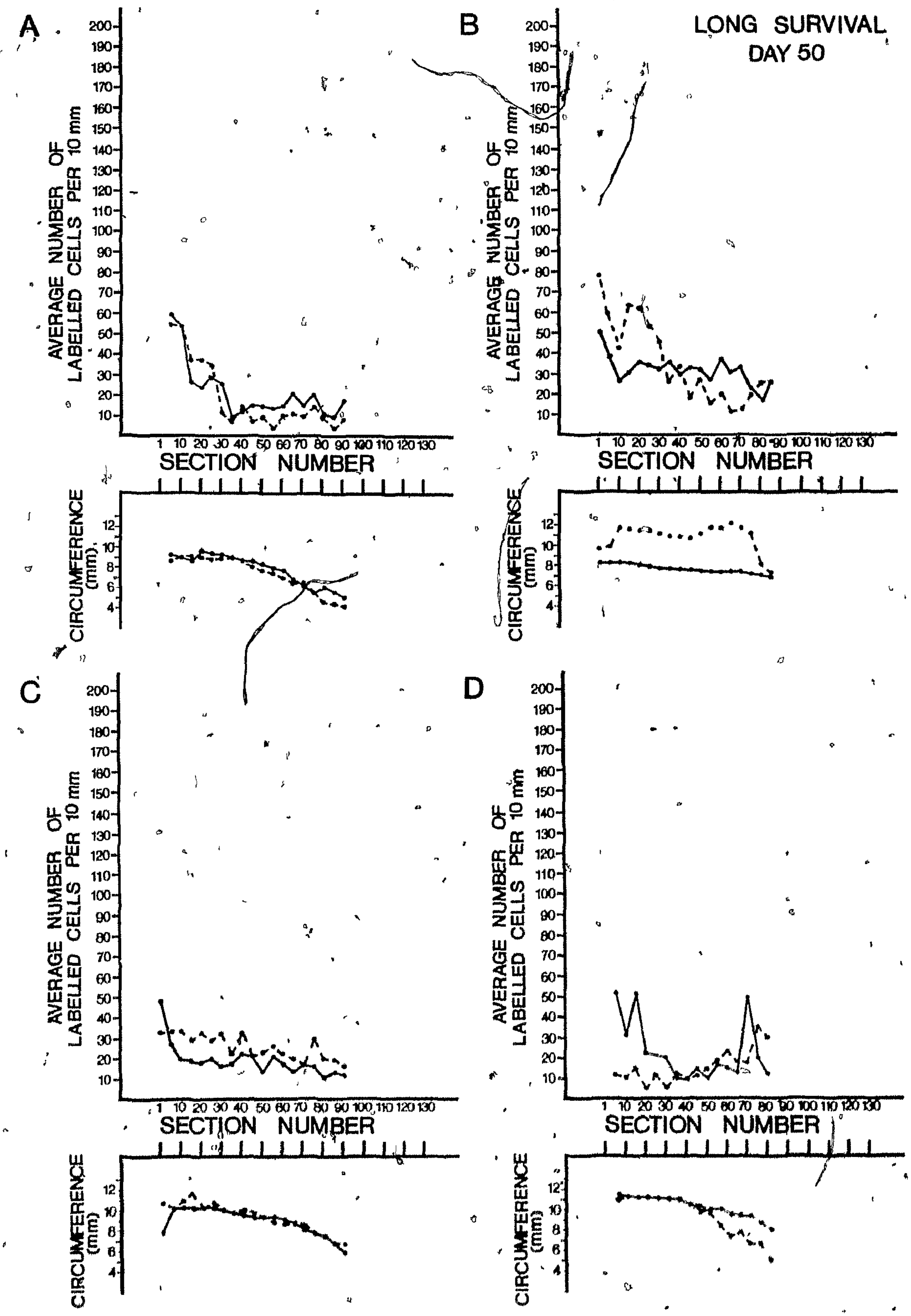

A
B
C
LONG SURVIVAL
DAY 25
AVERAGE NUMBER OF LABELLED CELLS PER 10 mm
SECTION NUMBER
CIRCUMFERENCE (mm)
1 10 20 30 40 50 60 70 80 90 100 110 120 130

Figure 32. Distribution of Labelled Cells in the ONL- (D50, LS)
Fifty days following unilateral optic nerve crush, these fish received bilateral intraocular injections of ^{3}H-TdR and their retinae were processed for light autoradiographic examination 30 days later.



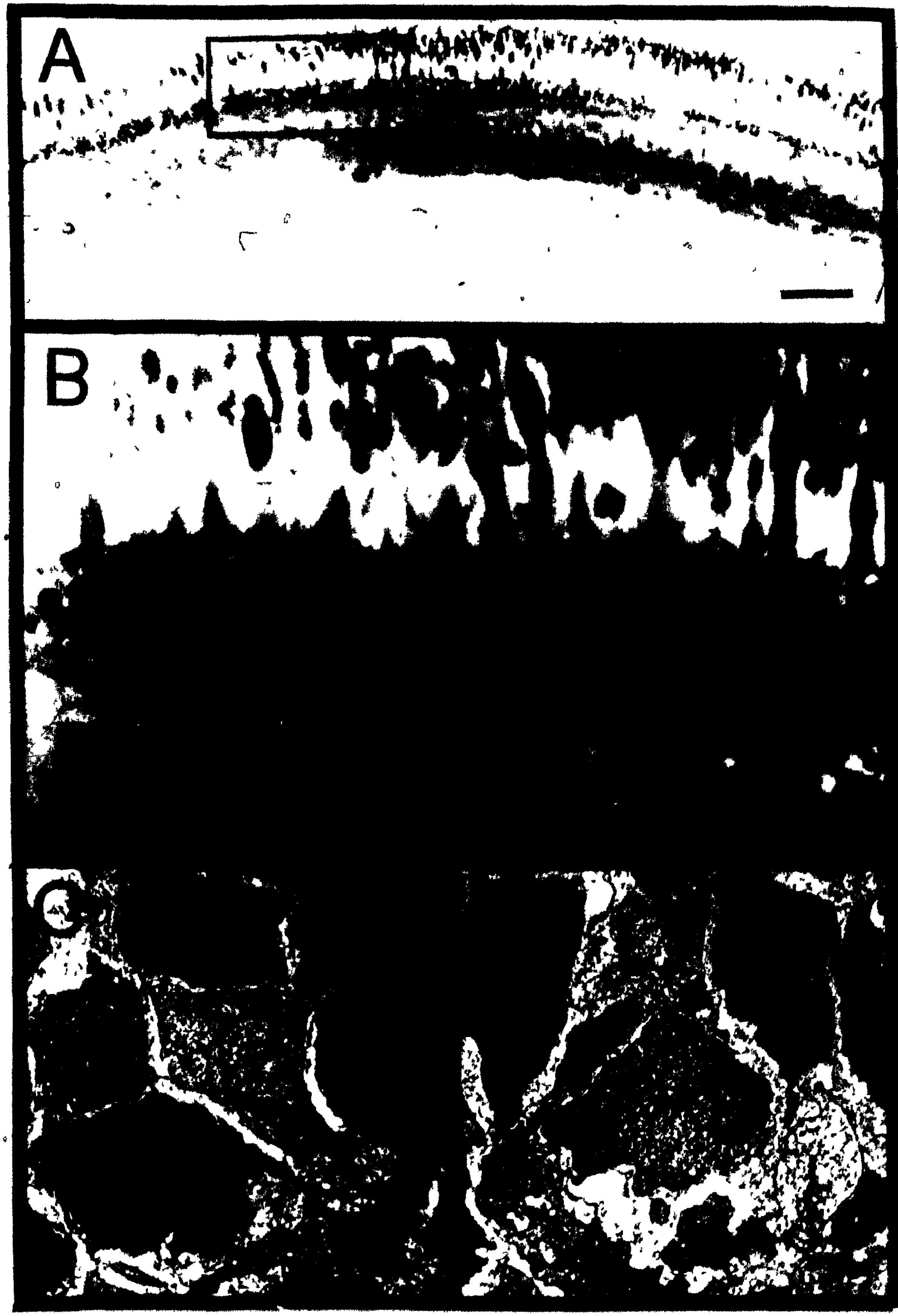
LONG SURVIVAL
DAY 50
A
B
C
D
AVERAGE NUMBER OF LABELLED CELLS PER 10 mm
SECTION NUMBER
CIRCUMFERENCE (mm)

Figure 33. Ultrastructure of Labelled Cells in Receptor Layer

A. Light microscopic autoradiograph of an uncoverslipped plastic section of the retina. In the boxed region, two labelled cells in the ONL are shown. This retina was axotomized, and injected with tritiated thymidine 10 days later. One day following administration of ^{3}H-TdR, the fish was perfused and sacrificed. Calibration bar: 100 µm.

B. Enlargement of the boxed region in A. Note the location of the label (arrows) near to the border of the OPL. Calibration bar: 25 µm.

C. and D. Electron micrograph of the two labelled cells shown in B. The nuclei are irregularly shaped and stain more lightly than the neighboring cells. The granular texture of the nucleus is similar in density and distribution to those cells surrounding it. The differential staining may be a reflection of the metabolic state of the cell. Calibration bars: 1 µm.


A
B
C

V. EFFECTS OF OPTIC FIBER AXOTOMY ON CELL PROLIFERATION IN THE ONL EXAMINED 1 DAY AND 30 DAYS FOLLOWING INJECTION

Cell labelling in the receptor layer is modulated following optic nerve axotomy. When the ONL is examined 1 day following ^{3}H-TdR injection (see Figure 25A), there is an increase in the numbers of rod precursors entering the S-phase of the cell generation cycle at about 10 days following axotomy. By D15, this trend is reversed, as is seen by a decrease in the numbers of labelled cells in the axotomized photoreceptor layer as compared with the control ONL. This relative decrease continues until D35, at which point, no significant difference between the two retinae is evident. These data indicate that axotomy modulates the numbers of photoreceptor stem cells in the S-phase of the generation cycle. The time course of these changes appears biphasic.

When the photoreceptor layer is examined 30 days following injection of ^{3}H-TdR, fewer labelled cells are seen in the receptor layer of the axotomized retina as compared with the control (see Figure 25B). This trend is seen as early as 5 days following crush and is consistent across the next 20 days. By 50 days following axotomy, no differences between the two eyes are evident. The time course of these changes appears monophasic. The latter observation in the receptor lamina 30 days following ^{3}H-TdR injection indicates that changes in labelled cell numbers are dependent upon the observation interval following ^{3}H-TdR injection. This suggests that a fraction of these rod precursor cells initially incorporating ^{3}H-TdR have undergone further mitotic divisions, thus diluting the label. The observation that the grain density over individual nuclei is much lighter in those animals examined one month after injection lends support to this hypothesis (see Figure 43).

The issue to be addressed in this experiment is what proportion of those precursor cells that initially incorporate ^{3}H-TdR into DNA retain the tag. I assessed what percentage of those cells that incorporate ^{3}H-TdR become post-mitotic and what proportion of these cells initially incorporating ^{3}H-TdR continue to undergo further divisions effectively diluting the tag during various post-operative periods after optic nerve axotomy.

PROCEDURE

Bilateral optic nerve crushes were performed on 20 fish. Either 5, 10, 15, 20 or 25 days following surgery both eyes received intraocular injections of ^{3}H-thymidine. One day following injection, one eye was enucleated. This eye was designated as the 24 hour or short survival (SS) side and was processed for light microscopic autoradiography. These fish were then returned to their aquaria. One month following the injection, the remaining eye was removed following anaesthesia and sacrifice of the fish. The 30 day, or long survival (LS) side, was then processed for light autoradiographic examination (see Figure 34). It is useful to note that inherent in this paradigm is the limitation that the fish could not be perfused prior to sacrifice. Using the criteria and general procedures used throughout this thesis, labelled cell populations were quantified and comparisions made between the two retinae of individual fish.

RESULTS

a. Normal (ONL-N(SSvsLS))

When an unoperated fish receives bilateral injections of ^{3}H-TdR and one eye is processed one day following injection and the other eye one month later, approximately 2.3 X as many cells are found in the 24 hour survival eye in all cases. There appears to be a change in labelling density along with topographical location. More labelled cells are found in the more peripheral retinal regions. In Figure 35, four fish are presented. There is no overlap between the two retinae of any single fish. The solid line represents the 1 day survival (SS) retina, while the dashed line represents 1 month survival (LS) following ^{3}H-TdR injection. This convention is employed throughout this experiment. The mean linear densities for each retina are: S=85.7 vs. L=41.6, S=43.3 vs. L=17.3, S=38.5 vs. L=15.5, and S=74.7 vs. L=36.2, where S and L are defined as:

$$S= \frac{\sum_{i=1}^{n} s_i}{n} \qquad (5)$$

$$L= \frac{\sum_{i=1}^{n} l_i}{n} \qquad (6)$$

And s_i is the number of labelled cells/10 mm of the i^{th} section of the 1 day survival (SS) retina and l_i is the number of labelled cells/10 mm of the i^{th} section of the 1 month survival (LS) retina.

b. Five Days Following Unilateral Optic Nerve Crush (ONL-D5(SSvsLS))

Five days following bilateral axotomy, both eyes were injected with ^{3}H-TdR. In the eye that was enucleated 1 day following injection, more labelled cells are evident when compared with the eye that was processed 1 month following injection. Again, a correlation with topographical location is evident. More tagged nuclei are found in the peripheral retinal regions. In Figure 36, the labelling patterns in the receptor layers of four fish are graphically portrayed. In no case is there any overlap between the two eyes. For fish A-D, S=23.9 vs. L=8.0, S=205.7 vs. L=72.0, S=42.5 vs. L=8.9, and S=77.3 vs. L=15.8.

c. Ten Days Following Unilateral Optic Nerve Crush (ONL-D10(SSvsLS))

More individual differences in labelling patterns are found between fish in this post-operative paradigm. In Figure 37A, one of these fish is presented. More tagged nuclei are seen in the peripheral retinal locations and appear to decrease steadily across the topographical gradient. Labelling in the 1 month survival eye appears linear. The mean number of labelled cells/10 mm in the ONL of the SS eye is 43.4, while 7.1 labelled nuclei/10 mm are seen in the LS eye. Figure 37B illustrates another fish in this group. The labelling pattern in the short survival eye appears almost U-shaped in configuration across topographical location. Labelling in the 1 month survival eye appears linear. For this fish, S=84.9 and L=20.2. In Figure 37C, more labelled nuclei are found in the 1 day survival eye as compared with the 1 month (S=38.6 and L=5.8). Labelling in the SS retina appears to increase across the topographical gradient. In the last fish in this experimental group (Figure 37D), more labelled cells are evident in the receptor layer of the SS eye as compared with the LS equivalent (S=18.0 and L=5.0). There is no overlap between the two retinae.

d. Fifteen Days Following Unilateral Optic Nerve Crush (ONL-D15(SSvsLS))

In all fish more labelled cells are found in the 1 day survival ONL (Figure 38). In all cases, labelling in the 1 month survival eye appears linear, while in 3 out of 4 fish (Figure 38A-C), labelling in the SS eye appears U-shaped across the topographical gradient. The fish presented in Figure 38D shows a decreasing function across topographical regions. The mean linear densities for these fish are S=201.3 vs. L=61.4, S=66.1 vs. L=14.0, S=68.4 vs. L=9.7, and S=40.0 vs. L=3.9, respectively.

e. Twenty Days Following Unilateral Optic Nerve Crush (ONL-D20(SSvsLS))

Figure 39 illustrates four fish examined 20 days following bilateral optic nerve crush. Each fish expresses a unique pattern across the topographical gradient, although more labelled cells are found in the SS ONL in all cases. In Figure 39A, both the SS and LS retinae appear U-shaped in configuration. The mean linear density for this fish is S=97.1 vs. L=46.6. In Figure 39B, an asymptoting function is evident in the 1 day survival ONL and is quite variable across sections; the 1 month survival ONL appears almost linear. For this fish S=160.5 and L=36.2. Figure 39C shows a decreasing function across the topographical gradient in the SS retina, and the LS labelling pattern appears linear. The mean linear density for this fish is S=67.6 vs. L=11.3. The last fish in this group has a linear pattern in both eyes across the extent of the retina. For this fish S=1[illegible]4 vs. L=3.4.

f. Twenty-five Days Following Unilateral Optic Nerve Crush (ONL-(D25(SSvsLS))

Labelling is higher in the ONL of fish whose retina was processed 1 day following ^{3}H-TdR injection as compared with 1 month survival in the other eye (Figure 40). Fluctuations between sections are evident in all fish, although there is no overlap between the two retinae. In Figure 40A and B, labelling appears slightly heavier in the more peripheral retinal regions. The mean linear densities for these fish are S=113.7 vs. L=54.1, and S=186.2 vs. L=97.1. In Figure 40C, a slightly increasing function is evident in both eyes, while in Figure 40D, a linear function, with a high degree of variation is illustrated. For these fish, S=366.8 vs. L=223.7, and S=107.2 vs. L=77.6, respectively.

DISCUSSION

Following a single injection of tritiated thymidine, more labelled cells were found in the photoreceptor layer of the 24 hour survival (SS) retina than in the 30 day survival (LS) retina in every case examined. The clear difference in the numbers of labelled cells on the short survival side as compared with the long survival side, indicated that following axotomy a portion of those cells that incorporate tritiated thymidine undergo further divisions, such that 3^H-TdR labelling of their nuclei has become too dilute to be considered as "labelled", when examined 30 days after injection.

In order to test whether the differences in the densities of labelled cells in axotomized and control retinae were statistically reliable across time, the ratio of cell proliferation (r'_i) between the SS and LS retina for the i^{th} pair of equivalent sections was calculated by:

$$r'_i = \frac{s'_i}{l'_i} \qquad (7)$$

The mean value $\langle R' \rangle$ of the ratios of individual pairs of equivalent sections for a given post-operative period was defined as:

$$\langle R' \rangle = \sqrt[n]{r'_1 \times r'_2 \times r'_3 \ldots r'_n} \qquad (8)$$

$$\ln\langle R' \rangle = \frac{\sum_{i=1}^{n} \ln r'_i}{n} \qquad (8')$$

The first question to be asked is whether the difference in the densities of labelled cells between the two retinae of the same fish is statistically significant, when one retina is processed 1 day following injection and the other processed 1 month following injection. Student's t-tests showed that for every intact fish, the densities of labelled cells between the two retinae were reliably different ($F(1,32)=19.89$, $p<.001$, $F(1,34)=66.10$, $p<.001$, $F(1,34)=33.52$, $p<.001$, and $F(1,30)=70.39$, $p<.001$). The $\ln\langle R' \rangle$ value for the 69 observations from these intact fish was .8561, SE=.041.

An analysis of variance was run across the intact and five experimental groups (D5, D10, D15, D20 and D25) and a robust effect was found ($F(5,465)=64.642$, $p<.001$). Individual Student's t-tests were employed to locate the sources of the difference. Since a total of 15 t-tests were run on these data, Bonferroni's modification provides an acceptance level of .003. This level of reliability was employed for interpretation of these results. An ANOVA confirmed the difference between experimental groups when the data were partitioned into topographic location and time following axotomy was the main variable ($F(3,18)=6.03$, $p<.001$). No effects of the location ($F(2,36)=1.206$, $p>.001$) or interactions between the two ($F(10,36)=.949$, $p>.001$) were evident.

In Figure 41, a summary graph of the results to be discussed is illustrated. At 5 days following bilateral nerve crush, the $\ln\langle R'\rangle$ value of labelled cells between the 24 hour and 30 day eye was 1.3795, SE=0.056. Seventy two observational ratios from 4 fish were compiled to achieve this value (Figure 36). When compared with intact animals, a significant difference was found ($F(1,139)=56.25$, $p<.003$). By 10 days following axotomy, the $\ln\langle R'\rangle$ value for four fish was 1.6268, SE=0.060. When this result, compiled from 84 observations, was compared with intact animals, reliable differences were found ($F(1,151)=103.23$, $p<.003$, Figure 37). There were also significant differences between D5 and D10 ($F(1,154)=8.94$, $p<.003$). A similar increased density of number of labelled cells/10 mm in the 1 day following injection retina, as compared with the 30 day following injection retina was seen 15 days following axotomy ($\ln\langle R'\rangle=1.7926$, SE=0.067, Figure 38). Statistical analyses reveal that there was no difference between labelling on D15 and D10 ($F(1,162)=0.154$, $p>.001$). These 80 observations taken from 4 fish at D15 were significantly distinct from normal ($F(1,147)=130.42$, $p<.003$) and D5 ($F(1,150)=21.81$, $p<.003$). Twenty days following axotomy, 83 observations from 4 fish were analyzed (Figure 39); $\ln\langle R'\rangle=1.3189$, SE=0.078. Robust differences were seen between Normal ($F(1,150)=24.60$, $p<.003$), D10 ($F(1,165)=9.86$, $p<.003$, and D15 ($F(1,161)=21.07$, $p<.003$) but not D5 ($F(1,153)=0.38$, $p>.003$). Finally, 25 days following nerve crush, the densities of labelled cells in the two retinae have returned to levels similar to that seen in intact animals, although t-tests show that labelling at all experimental groups are reliably different from D25 (Normal vs. D25- $F(1,150)=37.69$, $p<.003$, D5 vs. D25- $F(1,153)=197.96$, $p<.003$, D10 vs. D25- $F(1,165)=268.3$, $p<.003$, D15 vs. D25- $F(1,161)=302.4$, $p<.003$ and D20 vs. D25- $F(1,164)=83.36$, $p<.003$). Eighty-three observational ratios from 4 fish resulted in $\ln\langle R'\rangle=0.5835$, SE=0.021 (see Figure 40).

These experimental results suggest that optic nerve crush somehow modulates the probability that a cell will undergo further divisions during a given time period, given that the cell has initially incorporated the isotope following injection.

Figure 42 illustrates an equatorial section of retina taken from a fish 10 days following optic nerve crush and examined 1 day following ^{3}H-TdR injection (SS). The density of label overlying the somata of the cells in the photoreceptor layer is heavy, approximately 50-100 grains per nucleus. In Figure 43, the <u>equivalent</u> retinal section from the LS eye of the same fish is presented. Note that the density of silver grains overlying these cell somata is lighter, approximately 12-40 grains per nucleus.

Figure 34. Schematic Diagram of the Procedure for Short vs. Long Survival Following Thymidine Injection

Optic nerve axotomy was performed on both eyes. At selected post-operative periods: 5, 10, 15, 20, or 25 days, both eyes received intraocular injections of tritiated thymidine. One day following injection, one eye was enucleated and processed for light microscopic autoradiography. This retina was designated as the short survival (SS) eye. One month following injection, the fish was sacrificed and the remaining eye was processed for light autoradiographic examination. This retina was designated as the long survival (LS) eye.

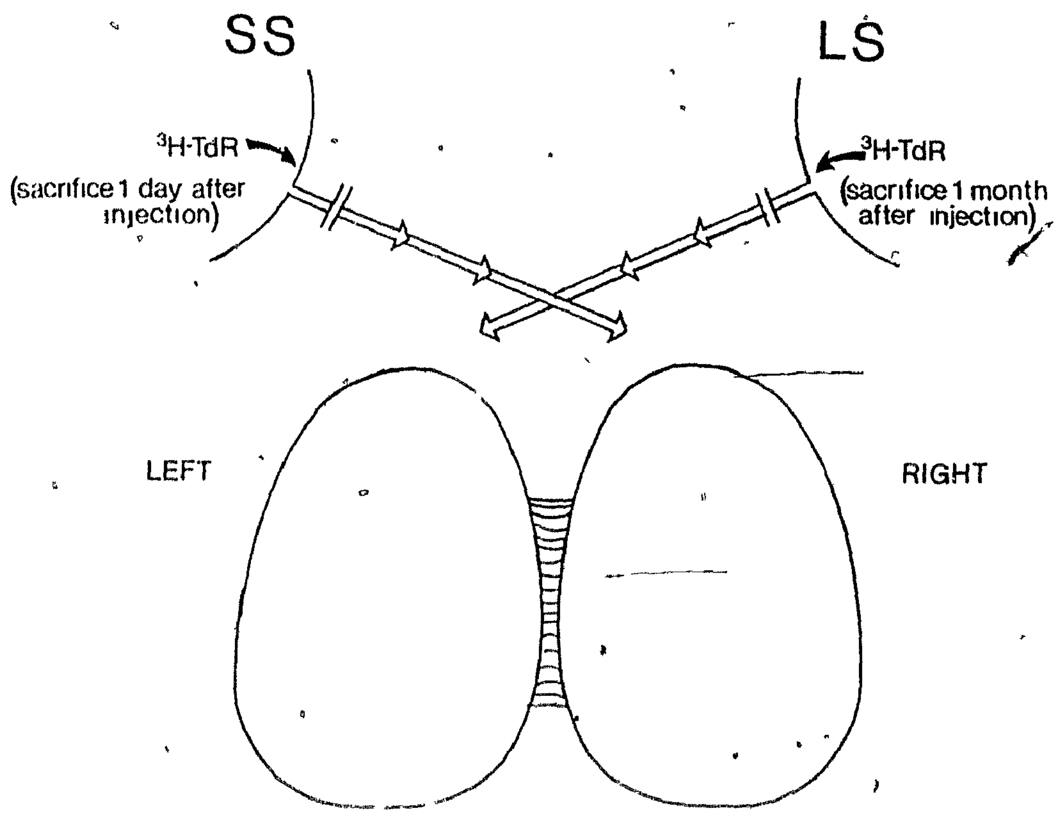
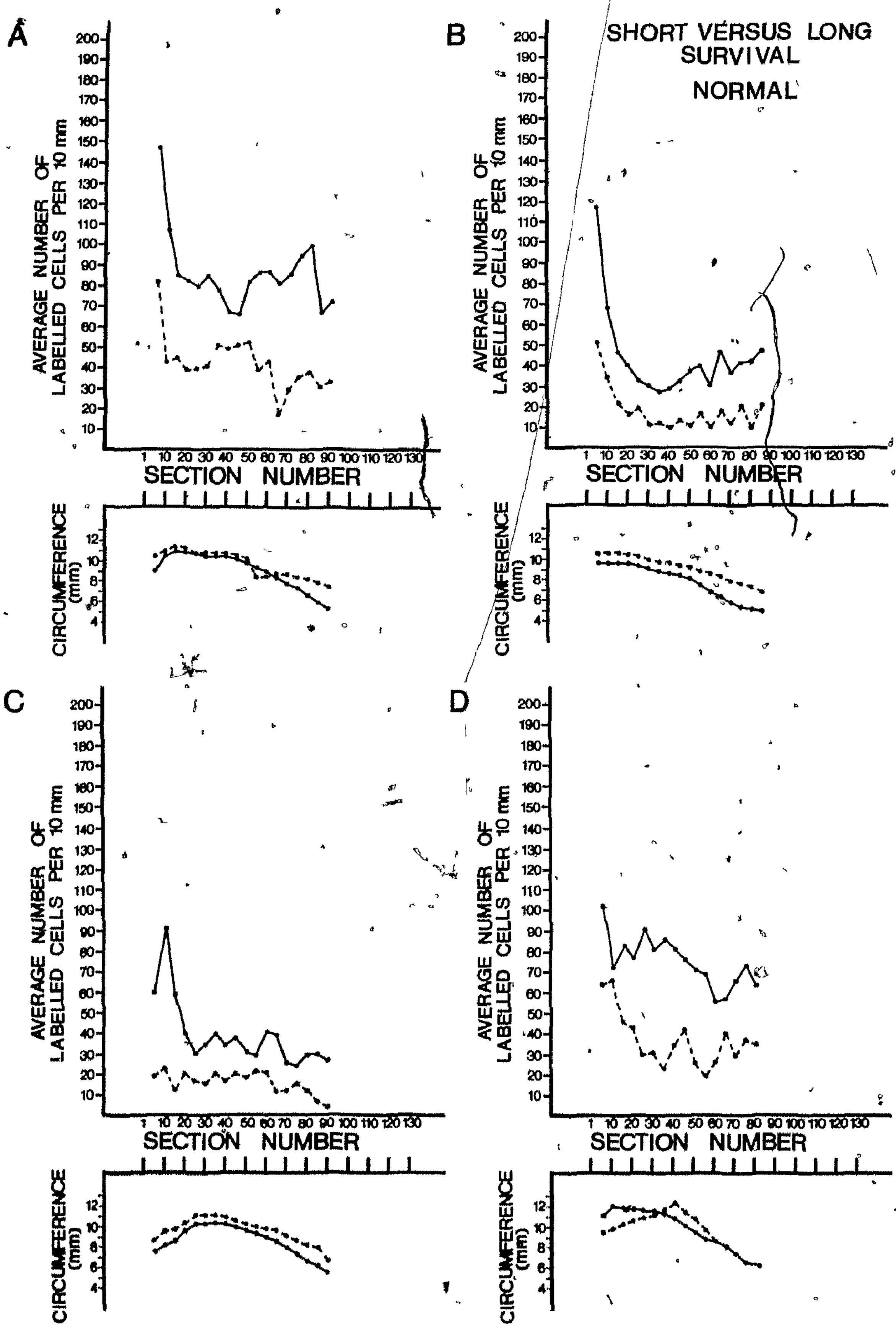

SS
LS
^{3}H-TdR
(sacrifice 1 day after injection)
^{3}H-TdR
(sacrifice 1 month after injection)
LEFT
RIGHT

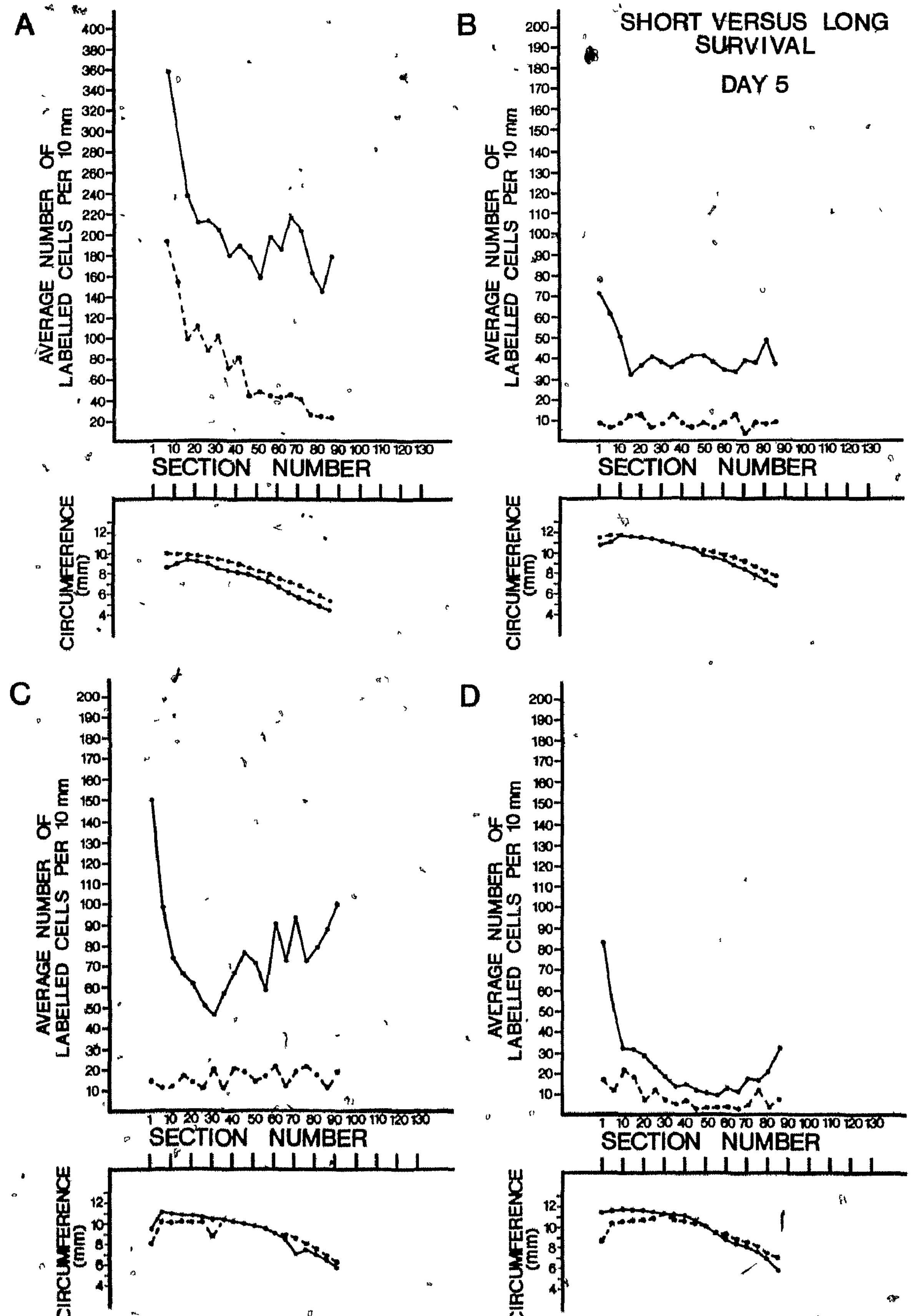
Figure 35. Topographical Distribution of the Linear Density of Labelled Cells in the ONL- Normal, Short vs Long Survival

For the following series of graphs: the solid line represents the eye that was enucleated 1 day following thymidine injection (SS) and the dashed line represents the contralateral eye that was processed 1 month following thymidine injection (LS).



A
B
SHORT VERSUS LONG
SURVIVAL
NORMAL
C
D
AVERAGE NUMBER OF LABELLED CELLS PER 10 mm
SECTION NUMBER
CIRCUMFERENCE (mm)

Figure 36. Distribution of Labelled Cells in the ONL- (D5, SS vs. LS) These fish received bilateral optic nerve axotomy, followed by bilateral intraocular injections of ^{3}H-TdR five days later. One eye was enucleated 24 hours following injection (solid line) and the other eye was processed for light autoradiographic examination 30 days following injection (dashed line).


A
B
SHORT VERSUS LONG SURVIVAL
DAY 5
C
D
AVERAGE NUMBER OF LABELLED CELLS PER 10 mm
SECTION NUMBER
CIRCUMFERENCE (mm)

<u>Figure 37. Distribution of Labelled Cells in the ONL- (D10, SS vs. LS)</u>
Ten days following bilateral optic nerve axotomy, these fish received bilateral intraocular injections of ^{3}H-TdR. One eye was processed for light autoradiographic examination 1 day following injection (SS) and the other eye received the same treatment 1 month following injection (LS).

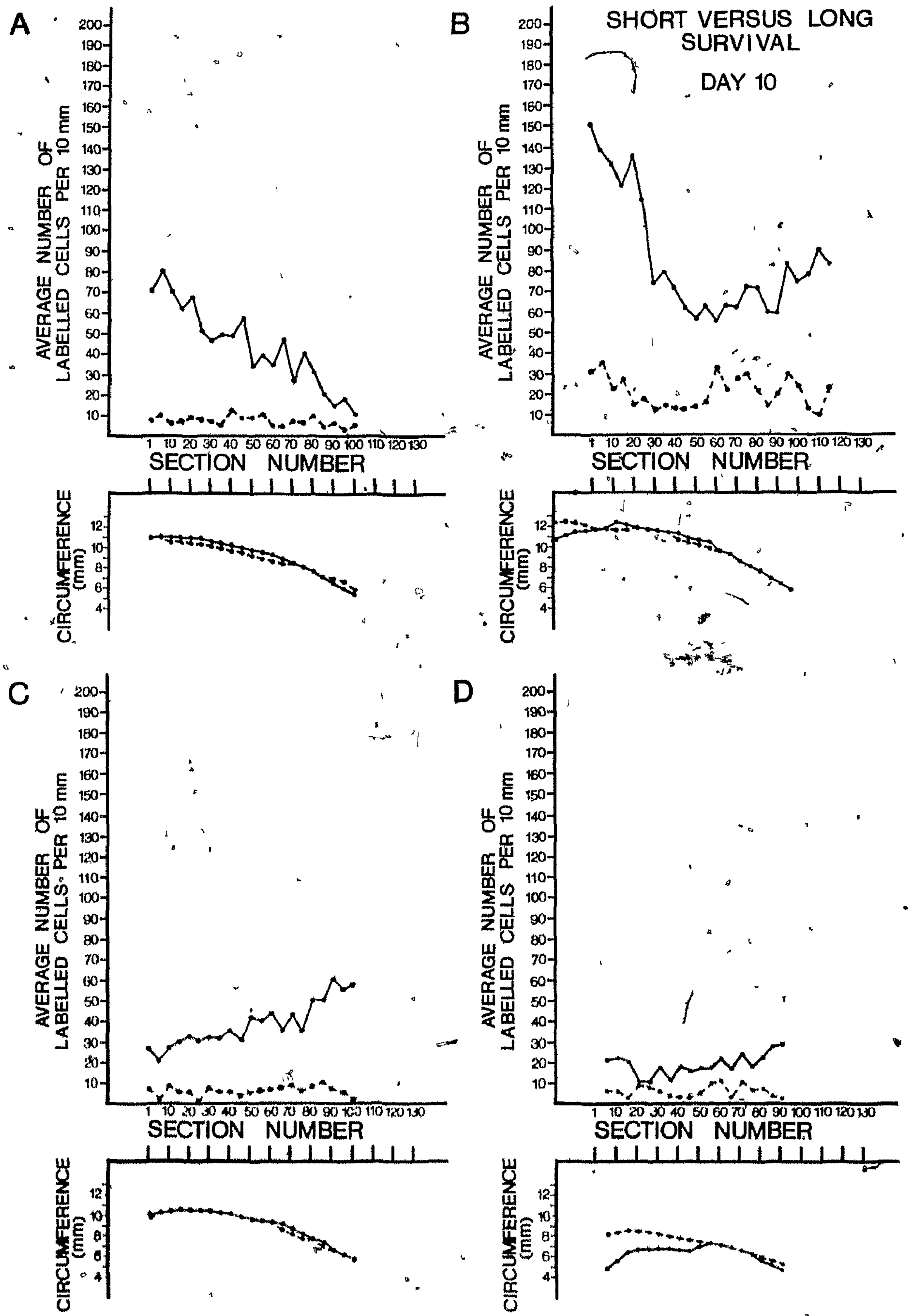

A
B
SHORT VERSUS LONG SURVIVAL
DAY 10
C
D
AVERAGE NUMBER OF LABELLED CELLS PER 10 mm
SECTION NUMBER
CIRCUMFERENCE (mm)

Figure 38. Distribution of Labelled Cells in the ONL- (D15, SS vs. LS)

These fish were received bilateral injections of ^{3}H-TdR fifteen days after bilateral optic nerve axotomy. One day following injection, one eye was enucleated. The other eye was processed for light autoradiographic examination 1 month following injection. Note the change in scale on the ordinate for A and D.

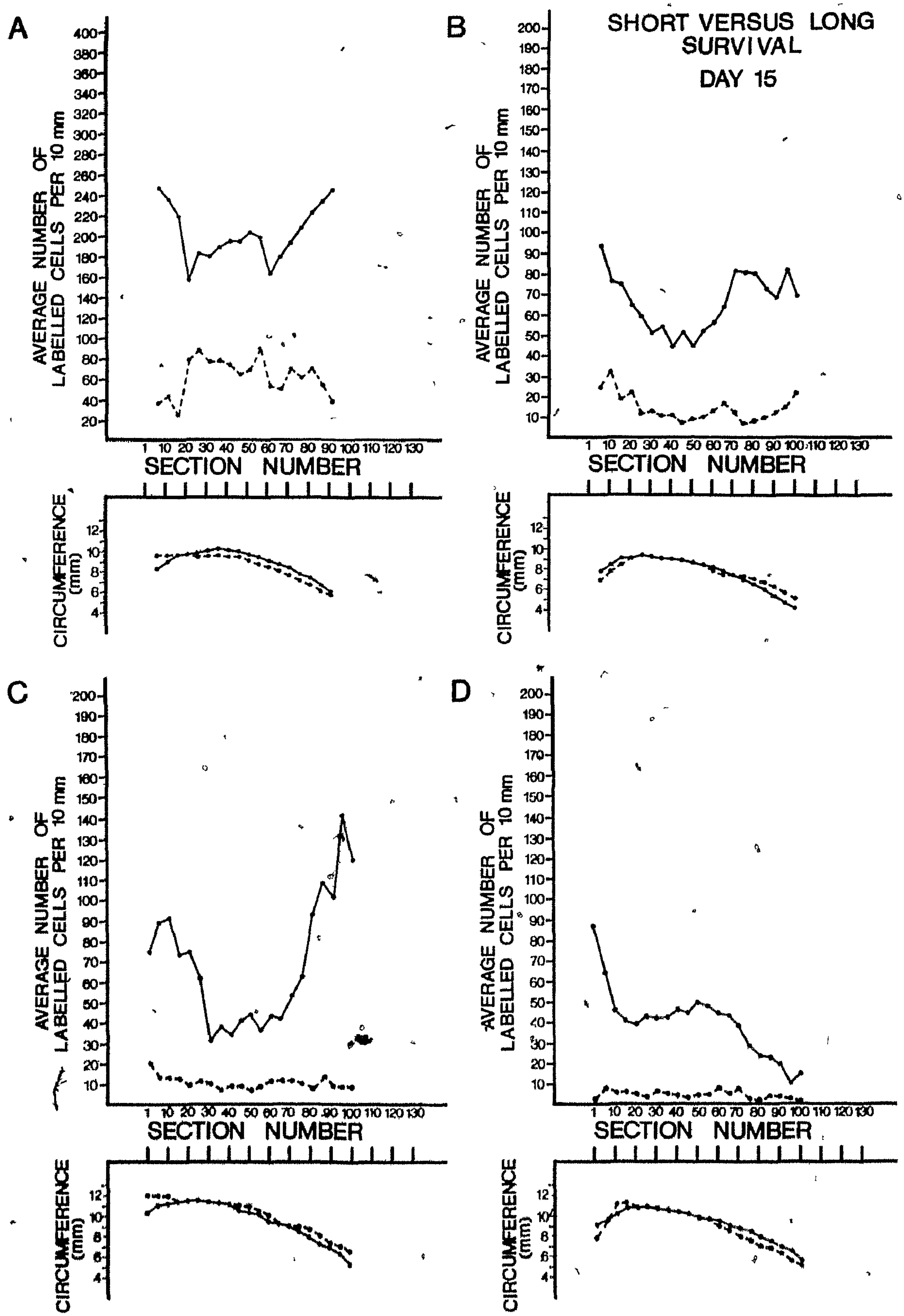

A
B
C
D
SHORT VERSUS LONG SURVIVAL
DAY 15
AVERAGE NUMBER OF LABELLED CELLS PER 10 mm
SECTION NUMBER
CIRCUMFERENCE (mm)

Figure 39. Distribution of Labelled Cells in the ONL- (D20, SS vs. LS)

A, B, C, and D. Bilateral intraocular injections of ^{3}H-TdR were administered twenty days following bilateral ONC. One eye was processed for light autoradiographic examination 24 hours following injection, while the other eye was processed 30 days following injection. Note the change in scale on the ordinate for B.

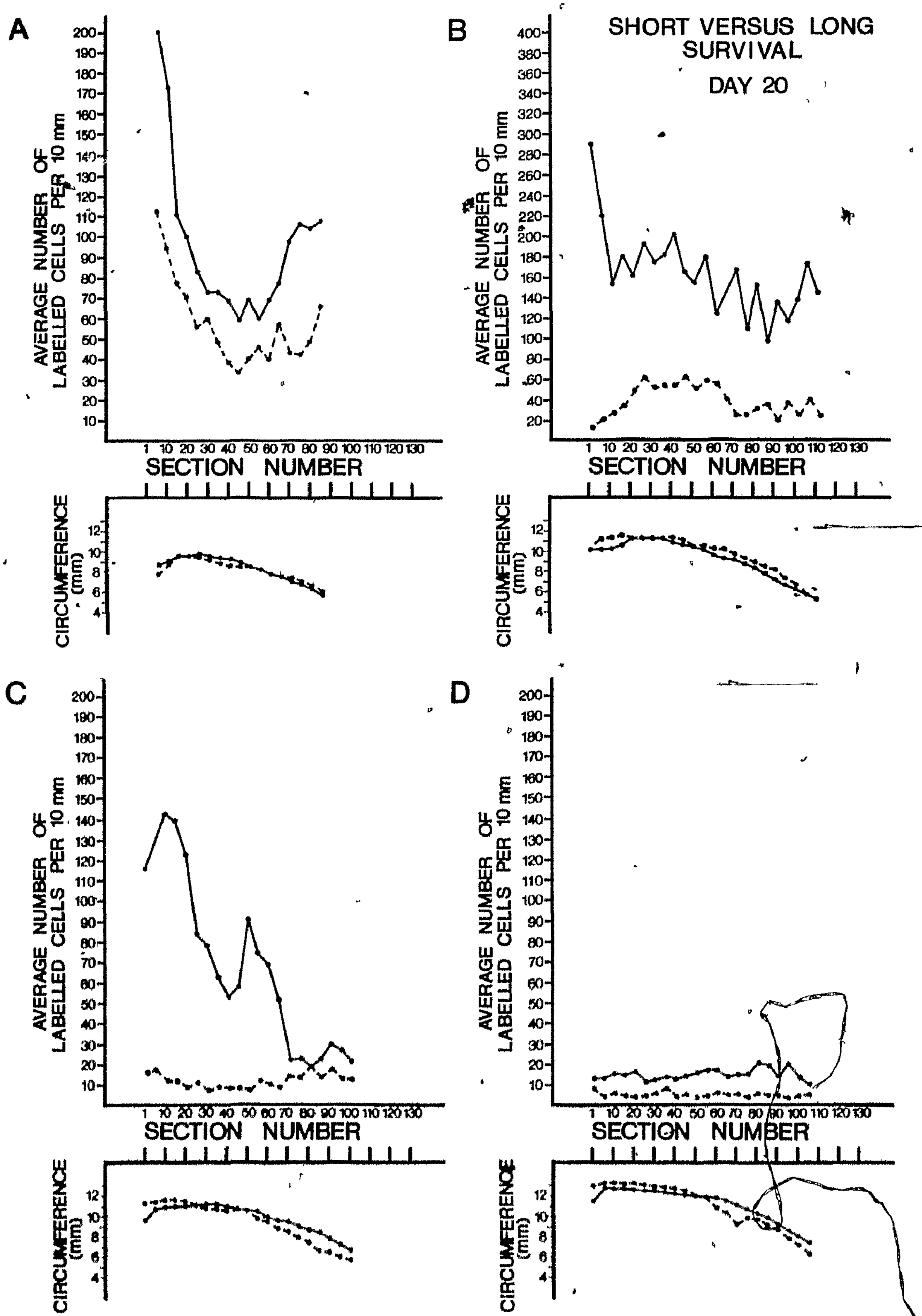
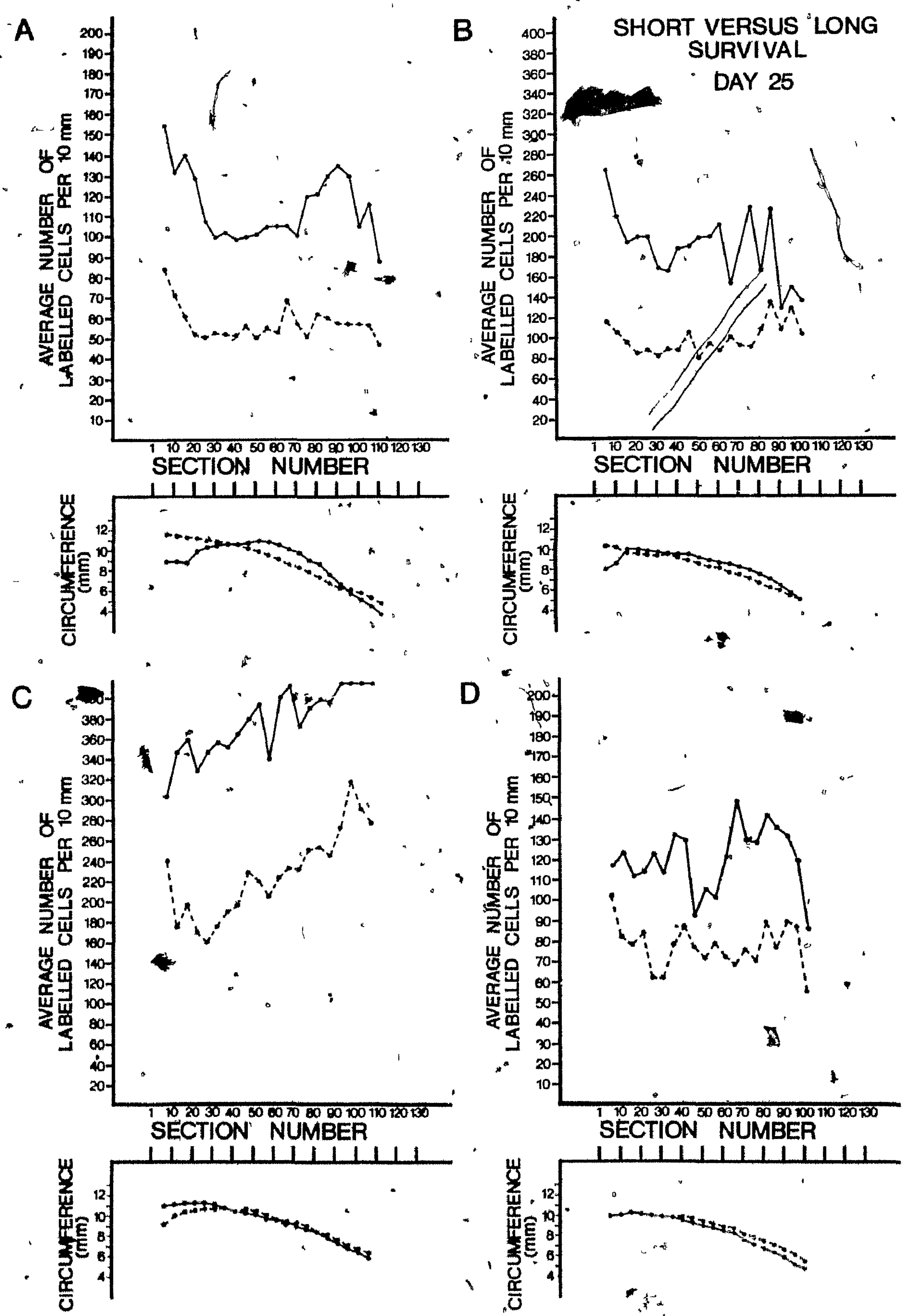

SHORT VERSUS LONG
SURVIVAL
DAY 20
A
B
C
D
AVERAGE NUMBER OF
LABELLED CELLS PER 10 mm
SECTION NUMBER
CIRCUMFERENCE
(mm)
1 10 20 30 40 50 60 70 80 90 100 110 120 130

Figure 40. Distribution of Labelled Cells in the ONL- (D25, SS vs. LS)

A, B, C, and D. Twenty-five days following bilateral optic nerve crush, these fish received bilateral injections of ^{3}H-TdR. One eye was enucleated and processed for light autoradiographic examination 1 day following injection. The other eye was processed 1 month following injection. Note the change in scale on the ordinate in B. and C.


SHORT VERSUS LONG
SURVIVAL
DAY 25
A
B
C
D
AVERAGE NUMBER OF LABELLED CELLS PER 10 mm
SECTION NUMBER
CIRCUMFERENCE (mm)

<u>Figure 41. Time Course of Changes in Cell Proliferation in the ONL During Various Post-operative Periods Following Bilateral Optic Nerve Crush and a 1 Day vs. 1 Month Survival Period Following Injection</u>

In this Figure, the abscissa denotes the days following bilateral optic nerve axotomy. The ordinate represents the mean ratio of cell proliferation (SS:LS) (<R'>) on the right scale and the natural logarithm of <R'> (ln<R'>) on the left scale. The brackets contain the total number of individual ratios, for each post-operative period, followed by the number of fish from which the individual ratios are taken. The error bars represent the standard error of the ln<R'>.

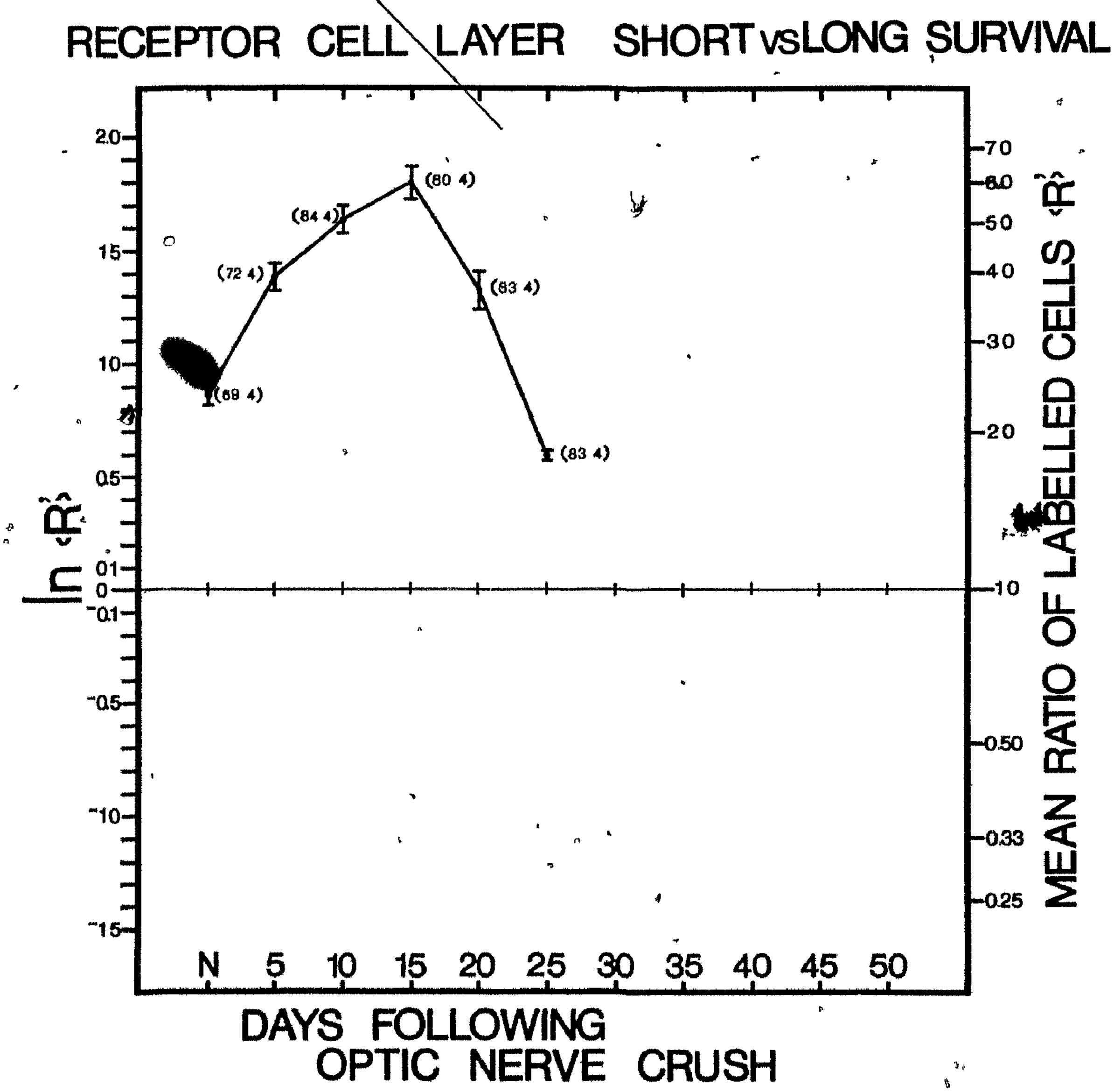

RECEPTOR CELL LAYER SHORT vs LONG SURVIVAL
ln ‹R̂›
MEAN RATIO OF LABELLED CELLS ‹R̂›
DAYS FOLLOWING OPTIC NERVE CRUSH
N
5
10
15
20
25
30
35
40
45
50
(69 4)
(72 4)
(84 4)
(80 4)
(83 4)
(83 4)
2.0
1.5
1.0
0.5
0.1
0
-0.1
-0.5
-1.0
-1.5
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.50
0.33
0.25

Figure 42. Light Microscopic Autoradiograph of Labelled Cells- SS

This fish received bilateral optic nerve crush followed by bilateral injection of ^{3}H-TdR 15 days after surgery. One day later this eye was enucleated. The other eye was processed for light autoradiographic examination 1 month later (see the equivalent equatorial section in Figure 43 for comparison). Abbreviations are as described in Figure 3.

A. Heavily labelled cells in the ONL, INL, and GC:OFL. Calibration bar: 50 µm.

B and C. Higher magnification of individual cells in A. illustrating silver grains in the emulsion coat overlying the nucleus. The labelling is dense in places such that individual grains cannot be distinguished. Calibration bars: 25 µm.

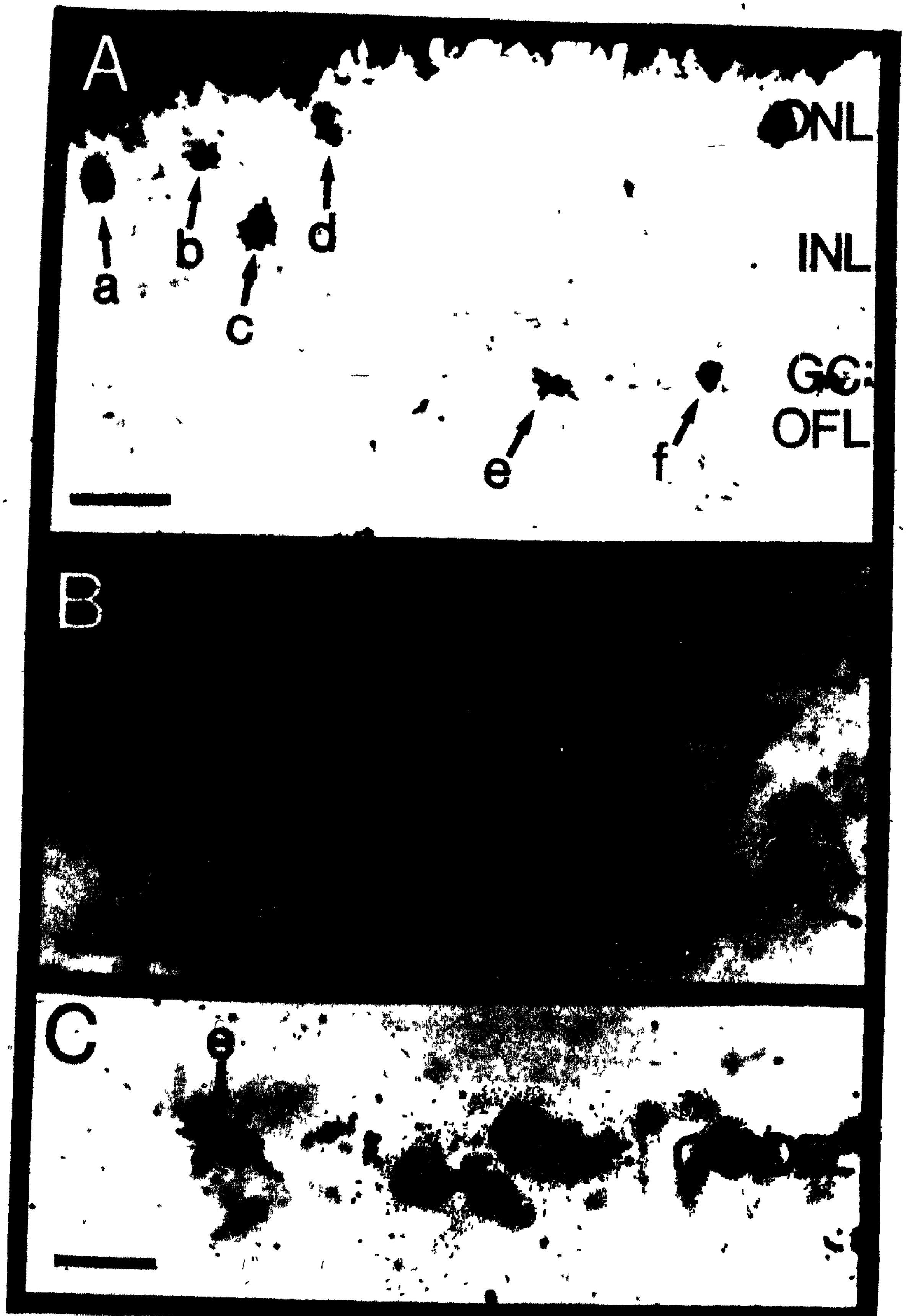
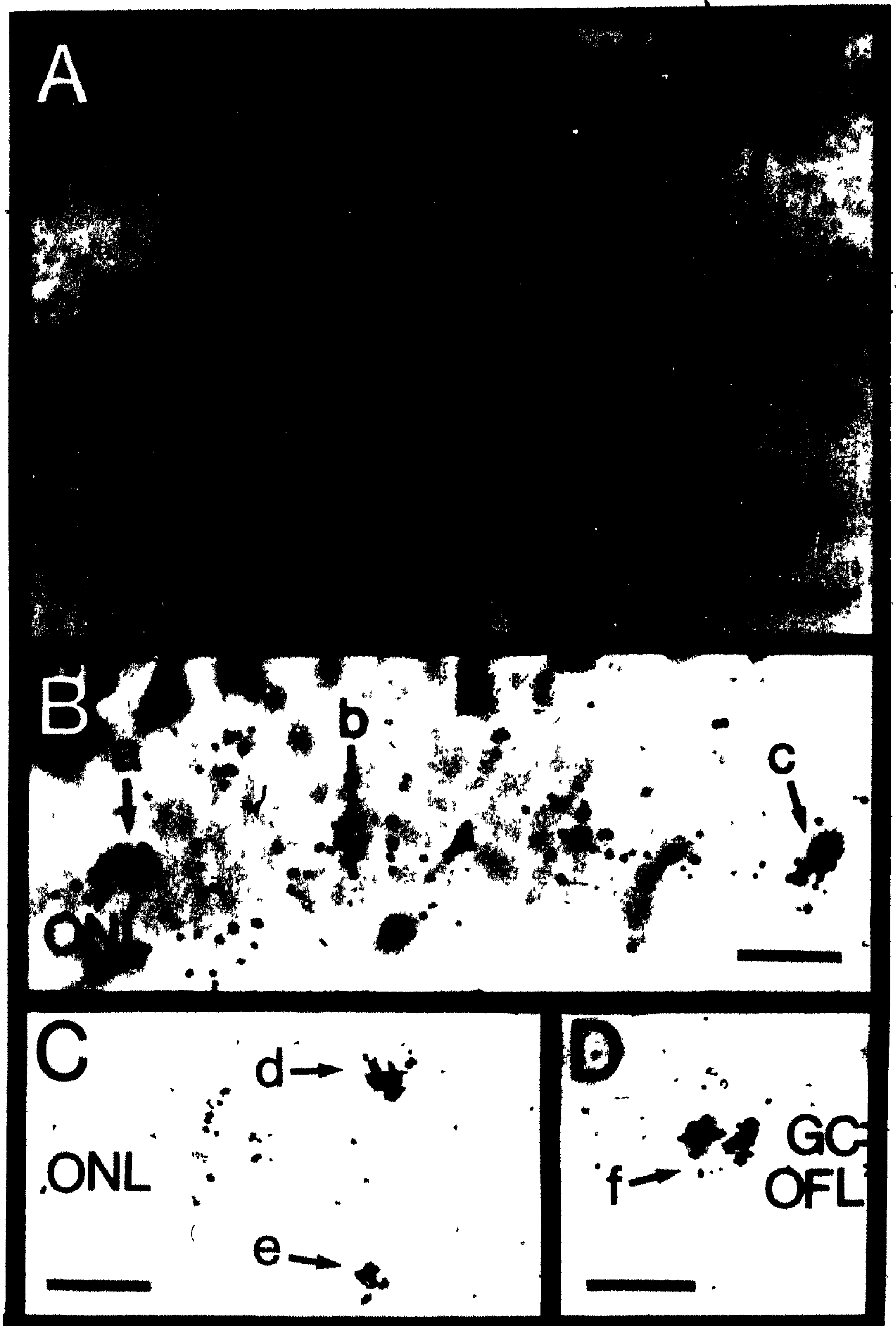

A
ONL
INL
GC:
OFL
a
b
c
d
e
f
B
C
e

Figure 43. Light Microscopic Autoradiograph of Labelled Cells- LS

This equatorial section is from the same fish as was described in Figure 42, however this retina was examined 1 month following injection. The two retinal rings are equivalent according to the criteria described in Materials and Methods. Abbreviations are as described in Figure 3.

A. Labelled cells in the ONL and GC:OFL. Calibration bar: 75 um.

B, C, and D. Higher magnification of individual cells in A. Note that each silver grain in the emulsion coat overlying the nucleus is visible. (Compare with Figure 42 B and C). Calibration bars: 25 µm.


A
B
a
b
c
ONL
C
d
e
ONL
D
f
GC
OFL

VI. GENERAL DISCUSSION

The results of the preceding experiments indicate that axotomy of retinal ganglion cell axons modulates the numbers of cells that enter the S-phase of the cell generation cycle. Only two types of cells within two nuclear laminae appear to be selectively influenced following axotomy; these are the cells (presumably non-neuronal) in the ganglion cell/optic fiber layer and the presumptive rod receptor precursors in the outer nuclear layer.

CHANGES IN CELL BIRTH IN THE GC/OFL

Optic nerve axotomy modulates the number of cells which incorporate ^{3}H-TdR into new DNA in the GC/OFL of the retina of adult goldfish. Five days following unilateral optic nerve crush, when animals are sacrificed 1 day following ^{3}H-TdR injection, there is an enhancement in cell birth in the GC/OFL of the axotomized retina as compared with its control. There is a similar increase in the number of labelled cells ten days following unilateral axotomy. This change in the linear density of labelled cells gradually subsides to normal levels over the next 15 days. There are no differences between the two retinae after 25 days following crush. The time course of the enhancement of cell proliferation following axotomy, presented in Figure 14A, illustrates that axotomy has selectively stimulated cells to enter the S-phase of the cell generation cycle.

One month following administration of ^{3}H-TdR, there is little difference between the linear densities of labelled cells in the axotomized GC/OFL as compared with its contralateral control.

Statistical analyses demonstrate that the only reliable difference is an enhancement in the linear density of labelled cells in the axotomized GC/OFL as compared with its control at D5 following optic nerve crush. Between 10 and 50 days following crush, approximately equal numbers of tagged nuclei are present in the GC/OFL of the axotomized and control retinae. The time course (Figure 14B) of the changes in the linear density of the labelled cell population examined 30 days following injection indicates that axotomy does not modulate the numbers of cells that will retain the label. The only exception to this interpretation is seen five days following unilateral axotomy. The enhanced number of labelled cells at D5 suggests that perhaps there are two populations of cells, one of which selectively becomes post-mitotic five days following axotomy, and one of which undergoes further cell division cycles.

When the time course of the changes in the linear densities of labelled cells of fish that were examined 24 hours following injection (Figure 14A) is compared with fish that were examined 30 days (Figure 14B) following injection, several interpretations can be made. The first is that the time course of changes in the ratios of the linear densities of labelled cells is dependent upon the observation time following ^{3}H-TdR injection. The time course of the changes in cell proliferation shows an initial increase followed by a gradual decrease when examined I day following injection. When they were examined 1 month following injection, however, the ratios of the linear densities of labelled cells between the axotomized and control retinae do not show any consistent change during the post-operative periods (except D5). The second point is that axotomy modulates the numbers of cells initially incorporating ^{3}H-TdR, but does not influence whether they will retain that label, since approximately equal numbers of tagged nuclei are evident between the axotomized and control GC/OFL from 10 to 50 days following axotomy. Since there are uniformly less labelled cells seen 30 days following injection as compared with 1 day following injection, this suggests that most of these cells remain mitotically active. Therefore, this population of cells is probably non-neuronal. The ultrastructural evidence presented in this thesis supports this interpretation. The nuclei of these labelled cells are irregularly shaped and composed of densely stained material arranged in granular clusters. Free ribosomes are abundantly present in the sparce cytoplasm. In contrast, the nuclei of retinal ganglion cells are usually round or pear-shaped and do not stain as densely. The cytoplasm has been shown to contain single profiles of rER and relatively few free ribosomes (Murray & Forman, 1971).

CHANGES IN CELL BIRTH IN THE ONL

Optic nerve axotomy modulates the numbers of cells which incorporate ^{3}H-TdR into new DNA in the photoreceptor layer of the retina of adult goldfish. When cell proliferation is examined one day following injection, more labelled nuclei are found in the ONL of the axotomized retina as contrasted with the normal at about 10 days following crush. This enhancement in cell birth is followed by an abrupt reversal, such that fewer tagged nuclei are visible in the axotomized ONL as compared with the normal from 15 to 25 days after unilateral optic nerve crush. At about 35 days following crush equal linear densities of labelled cells are seen in both retinae. This biphasic time course of the changes in cell birth is represented by the solid line in Figure 44. When cell proliferation is examined 24 hours following injection, the number of cells entering the S-phase of the cell generation cycle is modulated by axotomy in two ways: at ten days following crush there is an enhancement in the numbers of cells born, and between 15 and 25 days following crush, there is a relative suppression in cell birth.

In order to examine whether the cells that have incorporated ^{3}H-TdR when observed 1 day following injection become post-mitotic (retain the tag), or remain mitotic (lose the label), the time course of changes in the numbers of labelled cells was examined 30 days following ^{3}H-TdR injection. At all post-operative intervals (with the exception of 50 days), fewer labelled nuclei were visible in the ONL of the axotomized retina. This monophasic time course of the changes in linear densities of labelled cells is illustrated by the dotted line in Figure 44. The numbers of labelled cells seen 30 days after injection is less in the axotomized ONL at all experimental points (except D50), suggesting that there is a population of cells that undergo further mitotic divisions.

If all of those cells that initially incorporated ^{3}H-TdR had become post-mitotic, then the time course of cell proliferation seen 30 days following injection (dotted line in Figure 44) would have been similar in distribution to that seen 24 hours following injection (solid line in Figure 44). This is clearly not the case and therefore implies that some of the labelled cell population has undergone further cell generation cycles.

This interpretation is confirmed by results from normal fish in which the retinae were examined at different survival times following ^{3}H-TdR injection: one eye was removed 1 day following injection, and the other eye was examined when the fish was sacrificed 1 month following injection. Approximately 2.3X as many cells are found in the retina that was examined 24 hours after injection as compared with the 30 day survival retina, indicating that approximately 68% of the originally labelled cells have lost the tag, presumably because of dilution of the label by successive mitotic divisions.

In the simplest case, let us suppose that if a cell incorporates ^{3}H-TdR, it will either retain the label or continue undergoing further mitotic divisions until the label is no longer detectable (30 days following injection). This probably does not accurately reflect nature, as it precludes the (likely) event that one daughter cell remains labelled while the other daughter cell continues to divide, or that both daughter cells divide and only one of the grand-daughter retains the label. However, it offers a framework within which the following interpretations may be understood. The result obtained in normal fish, therefore, suggests that perhaps there are two populations of cells, one of which retains the label (approximately 32% of the initially labelled population) and the other of which (approximately 68% of the initialy labelled population) continues to undergo further cell generation cycles.

The proportion of cells destined to undergo further cell generation cycles is altered by axotomy. This time course of changes in cell proliferation is depicted by the dashed line in Figure 44, where at various intervals following bilateral optic nerve crush, the retinae of the same fish were examined at 1 day and 30 days following injection. At 5 days following axotomy, approximately 78% of the cells that initially incorporated ^{3}H-TdR are no longer labelled at 30 days following injection. Ten days following crush, 84% of the cells that were tagged by ^{3}H-thymidine 24 hours after injection are no longer visible. A similar proportion to D10 is seen at D15, where approximately 86% of the originally labelled population is no longer detectable by the light autoradiographic technique employed here. Approximately 78% of the cells that were initially labelled at 20 days following axotomy are no longer visible, and by 25 days following axotomy, 65% of them (a similar percentage to that seen in normal fish are no longer labelled. This monophasic time course of changes in the linear densities of cell labelling examined 1 day and 1 month following axotomy illustrates that axotomy has modulated the fraction of cells destined to undergo further cell generation cycles. If axotomy did not modulate the portion of cells undergoing further divisions, one would expect to see a uniform dilution of about 68% (a horizontal line) across days following axotomy.

In summary, optic nerve axotomy appears to affect cell proliferation in the ONL in two ways: First, the number of cells entering the S-phase of the cell generation cycle is influenced and second, the proportion of those cells initially incorporating ^{3}H-TdR into new DNA that will continue through further mitotic divisions is modulated by axotomy.

The photoreceptor cells that respond to axotomy are rod precursors. The primary evidence for this conclusion is the distinct location of their nuclei. As was described in the section on the ultrastructure of these labelled cells, the ONL of the adult goldfish is composed of about five layers of nuclei. The four closest to the OPL are rod nuclei and a single stratum of cone nuclei are located sclerad to the rods. Neither glia nor vascular elements have been reported in the receptor nuclear lamina. Rod precursor cells that have appeared in published micrographs (Johns, 1982; Raymond, 1985a, 1985b) appear similar in location as well as ultrastructural composition to the cells described here.

The results of SS vs LS indicate that axotomy of the optic nerve affects the proportion of labelled cells that will undergo further cell generation cycles. Hence, the further division of those initially labelled cells should result in the ultimate increase, in the rod receptor population in the axotomized retina. There are two ways to test this possibility: 1. Compare rod receptor densities in axotomized and control retinae at various post-operative stages or 2. employ multiple injections of ^{3}H-TdR to label the proliferating population continuously. The second method has been employed by Johns (1982) for normal goldfish.

In normal young juvenile goldfish, Johns (1982) injected ^{3}H-TdR intraperitoneally (IP) and examined one eye 24 hours after injection, and the other eye 30 days following injection. She found 6X as many labelled cells in the 30 day survival eye. When retinae 1 day and 110 days following injection were compared, about 8X as many labelled cells were found in the eye with the longer survival time. This seeming discrepancy with the results presented here can be explained by comparing the procedures used. There appear to be three major differences. First, Johns used young juvenile fish which are generating cells at a much higher rate than adults. In the first two months following hatching, the retinal area increases from 0.05 mm^2 to 1.0 mm^2. Second, because of the initial small size and subsequent rapid growth of the retinae of these fish, accurate definition of the boundaries of the germinal cell zone is difficult. It is likely that the majority of cells included in her analysis were derived from the <u>ora terminalis</u>, rather than from the stem cell population dispersed throughout the mature retina. Third, although the SS eye was removed after a single injection, it is unclear from her procedure whether or not multiple injections were administered before removal of the LS eye. If multiple injections were administered, then the result obtained in normal fish in this study does not conflict with that which Johns reported. If the LS eye was removed following a single injection, then interpretation of the discrepancy is more difficult, but can be accounted for by the differences in the ages of the fish at the time of injection. Since retinal growth is far more rapid between hatching and two months of age, most if not all of those cells incorporating ^{3}H-TdR in the <u>ora terminalis</u> will become post-mitotic. Since the LS eyes were removed at 30 or 110 days following injection, those cells in the germinal zone at the time of injection will be labelled and located in what is now more central retina and thus be included in the analysis.

In the series of experiments reported here, I examined modulation of cell birth following axotomy following a single intraocular injection of ^{3}H-TdR. ^{3}H-TdR, when injected intravenously, remains accessible to S-phase cells for 40-60 minutes (Sidman, 1970), when administered IP, the label "remains in the available concentration for a few hours" (Fujita & Horii, 1963). The availability of the isotope when injected IO is about one hour (Maenza & Harding, 1962; Reddan & Rothstein 1966). If the cell generation cycle is about 18 hours in duration, only a single generation would have the opportunity to incorporate the ^{3}H-TdR into new DNA, using this procedure. If, on average, 50-100 silver grains are visible in the emulsion coat overlying the nucleus following a single injection, and those cells incorporating the tag continue to divide, by the third or fourth generation, the progeny would no longer meet the criteria for labelled cells. From Johns (1982) study, I expected to find increased numbers of labelled cells with increased time following injection in normal fish. This turned out not to be the case, presumably as a result of the differences in experimental protocols. Under the conditions employed here, fewer tagged nuclei were evident in the retina following longer survival times following injection, in normal as well as experimental cases.

In order to interpret properly cell birth in the receptor layer, it will first be necessary to establish the kinetics of its cell generation cycle. The first order of business, therefore, will be to determine a time course for the dilution of the ^{3}H-TdR label after various post-injection periods in normal fish. If the proposed model is correct, one would predict that at shorter survival times following injection, more labelled cells will be present, as the daughter cells of the initially labelled population will still retain the label. Furthermore, the model predicts a decrease in the labelled cell population with time, as the progeny of the initially labelled cells complete more cell generation cycles, diluting the label. Depending on the results from this experiment, the effect of optic fiber axotomy on dilution of the label at various post-injections will be examined.

A further step to confirm the above interpretation will be to label the progeny of the cells that continue to divide and see if the labelled fraction will be modulated at various times following axotomy. By administering multiple injections, the daughter cells of each division will have access to the radioactive pool, thus labelling successive generations. The next experiment, therefore, will be to examine cell birth following bilateral multiple injections in unilaterally axotomized fish at various post-operative intervals. Depending on the survival interval following injections, one would predict the linear density of labelled cells in the ONL of the axotomized retina to be substantially greater then the linear density found in the control retina from 5 to 25 days following axotomy.

FATE OF THE LABELLED CELLS IN THE ONL

In young fish thymidine autoradiography has revealed that approximately 700 cells per mm^2 are added to the ONL per day (Johns, 1982; Pierantoni, Karkhanis & Stell, 1985). However, Kock and Stell (1984) have demonstrated that the average rate of addition of mature rods is only 30 per mm^2 per day. Using the Golgi technique, they examined the bl bipolar cell, a second-order neuron which is known to contact every rod within its dendritic arborization. Their results illustrate that as the dendritic field area increases, by 50%, the number of synapses in normal fish also increases by 50%. The density of rod synapses onto the bl bipolar remains constant at one synapse/11 um^2.

This shows that at least some of the rod precursors ultimately make connections upon differentiated second-order neurons. Stell (personal communication) accounts for this discrepancy in the following way: if only 4% (700 cells/mm^2/day - 30 cells/mm^2/day) of those cells labelled with ^{3}H-TdR achieve the status of mature rods, 96% must either become rod precursors or disappear. At least 4% must become rod precursors (to supply the next generation), but more like 50% are necessary to account for the value of 700 cells per mm^2 per day seen from the autoradiographic data. Stell suggests that the 45% of cells that is unaccounted for degenerates and disappears.

In the work presented here there appear to be at least two populations of rod precursors which incorporate ^{3}H-TdR, one of which retains the label, and the other of which continues to undergo further cycles of cell replication. The proportion of labelled cells that undergoes further cell generation cycles is modulated by axotomy. In light of the report by Kock and Stell (1985) and the proposal by Stell (personal communication), the cells that retain ^{3}H-TdR may be those cells destined to become mature rods, or those cells destined to replenish the precursor population. The fraction of cells that lose the label, presumably because of division by successive mitotic divisions, may in fact be fated to die. Multiple injections of ^{3}H-TdR, followed by a long survival time may help to clarify this issue.

CELL BIRTH VS. CELL DEATH

The mitogenic factors that provide the signal for a cell to undergo further cell generation cycles are unknown. The cell generation cycle is subdivided into two periods: cell-division phase (M-phase) which includes mitosis and cytokinesis, and interphase which is composed of two gap (G-) phases and S-phase. The period between the end of M-phase and the beginning of the DNA synthesis phase is denoted as G_1, while the period between the S-phase and the subsequent M-phase is called G_2. The G_1, S, and G_2 phases take up about 90% of the total cell cycle time. Differences in the length of the cell generation cycle are primarily a result of variations in the length of G_1. Once a cell has passed through G_1, it will complete the cell generation cycle at its normal rate. It has been postulated that there is a restriction point (R) late in G_1, which is the point of no return (Alberts, Bray, Lewis, Raff, Roberts & Watson, 1983).

Alberts et al. (1983) have proposed that a certain threshold amount of some unstable protein (U-protein) must accumulate for the cell to pass through R successfully and trigger the cell to synthesize DNA and divide. According to this hypothesis, any condition that increases the overall protein metabolism should increase the accumulation of the U-protein, and speed up the rate of cell division. Conversely, any condition that reduces the rate of protein synthesis should delay the build up of the U-protein and reduce the rate of cell division.

Murray (1973) and others (Burrell et al., 1978; Whitnall et al., 1981, 1982, 1983) have demonstrated enhanced protein metabolism in the retina as a result of optic nerve axotomy. The increased protein synthesis may initiate production of the U-protein, increasing the rate of cell division at around 10 days following axotomy in the ONL.

Cell division is regulated by a number of mechanisms, including positional signals and density limitations. The decrease in ^{3}H-TdR incorporation in the ONL of the axotomized retina seen at around D15 may be regulated by one, or both, of these feedback controls. Since the density of rod photoreceptor somata remains constant throughout the life of the animal, while the cell populations in the INL and GCL decrease with growth, the mechanism regulating cell birth in the ONL may be based (at least in part) on some density constraint. The reduction in cell birth (at D15) may in reaction to the enhanced proliferative activity seen earlier (D10).

Cell death is another means whereby a cell population can be regulated. Overproduction of new cells, followed by selective degradation can act to limit the population. Although I found no evidence of degeneration in the ONL, it is possible that cell death may be occurring. Perhaps the events occur too quickly to be noticed by my techniques. A careful search using electron microscopy may reveal dying cells. Alternatively, determination of cell densities at various stages following axotomy may give some insight as to whether cell densities decrease after an initial increase.

POSSIBLE ROUTES OF THE "SIGNAL" FOR CELL BIRTH MODULATION

An interesting question resulting from this study is by what routes does the information from the regions of the axotomized ganglion cell body reach the ONL? The swelling of the cell soma in response to axotomy, which is correlated with an enhanced upsurge in protein metabolism, may physically distort, or induce proliferation of cells in surrounding regions. Proliferation of perineuronal glial elements has been documented not only in teleosts (Murray & Forman, 1971), but in mammalian systems as well (Torvik & Skjorten, 1971; Torvik & Soreide;

Watson, 1972). The possibility also exists that those cells which are presynaptic to the axotomized ganglion cells may be affected. Perhaps the overall enhancement of proteins causes a general metabolic upsurge of activity in the retina or perhaps it is one molecular species that may act to modulate normal cell birth in the retina. There are at least three possible routes for the "signal" to take, of which two can be distinguished by further experiments. The "signal" from the ganglion cell layer can reach the ONL via a direct neuronal route, a direct non-neuronal route, or by diffuse dispersion of some general signalling factor. The direct neuronal route would require the signal to be transmitted from the ganglion cell to the cells of the INL and finally to the receptor cell layer. A non-neuronal route could occur via the Muller cells which traverse the entire extent of the retina. The idea of a global diffuse factor is self explanatory. One paradigm for distinguishing "direct" from "global" mechanisms relies on the specificity of retinotectal topography. If one were to sever the lateral optic tract, only the dorsal retina would be axotomized, similarly if a nick were made severing the rostral and caudal tectum, only the nasal portion of the retina would be axotomized. Using either of these general surgical procedures, the pattern of cell birth could be compared between the two regions of the same retina. If differences were evident, then one could conclude that one of the two "direct" signaling mechanisms was involved:

AXOTOMY VS. REGENERATION

Throughout this thesis I have refrained from using the term "regenerate" and have instead chosen the more factual term "axotomize". Although there is irrefutable evidence that the retinal ganglion cell axons regenerate when severed, I have no evidence suggesting that the modulation of cell birth reported here is in anyway related to the regeneration of those fibers, although this is implied. Non-neuronal cell proliferation in the vicinity of the cell body has been shown to occur in response to axotomy in both regenerating (Cova & Aldskogius, 1984; Torvik & Skjorten, 1971; Torvik & Soreide, 1975) and non-regenerating (Torvik & Skjorten, 1971; Torvik & Soreide, 1975) systems. In the goldfish, proliferation of perineuronal glial cells in the ganglion cell layer has been reported following optic tract crush (Forman & Murray, 1971), although this remark was made only in passing. The possibility exists that regeneration of optic fibers is inconsequential and that the important feature is that the retina is undergoing continuous cell birth. It is also possible that regeneration of optic nerve fibers cannot occur in a system that does not have continuous cell birth. In the two cases examining cell birth in the retina as a function of damage to the visual system (Gaze & Watson, 1967; Lombardo, 1972), animals known to have continuous cell birth in the retina were used.

Neurogenesis in the retina of postnatal chick (Morris, Wylie & Miles, 1976), mouse (Carter-Dawson & LaVail, 1979; Sidman, 1961), and kitten (Johns, Rusoff & Dubin, 1979) has been studied with thymidine autoradiography. In all cases, histogenesis is nearly complete by birth, however, a few nuclei incorporated label up to 7 days after hatching in the chick, 5 days after birth in the mouse, and 21 days after birth in the kitten. The labelled nuclei were primarily located at the ciliary margin, however, a few labelled cells were seen in the fundus (Morris, Wylie & Miles, 1976). These systems do not appear to have any regenerative capacity. It would be interesting, therefore, to examine cell birth in the retinae of these young animals following axotomy, providing retinal integrity could be maintained.

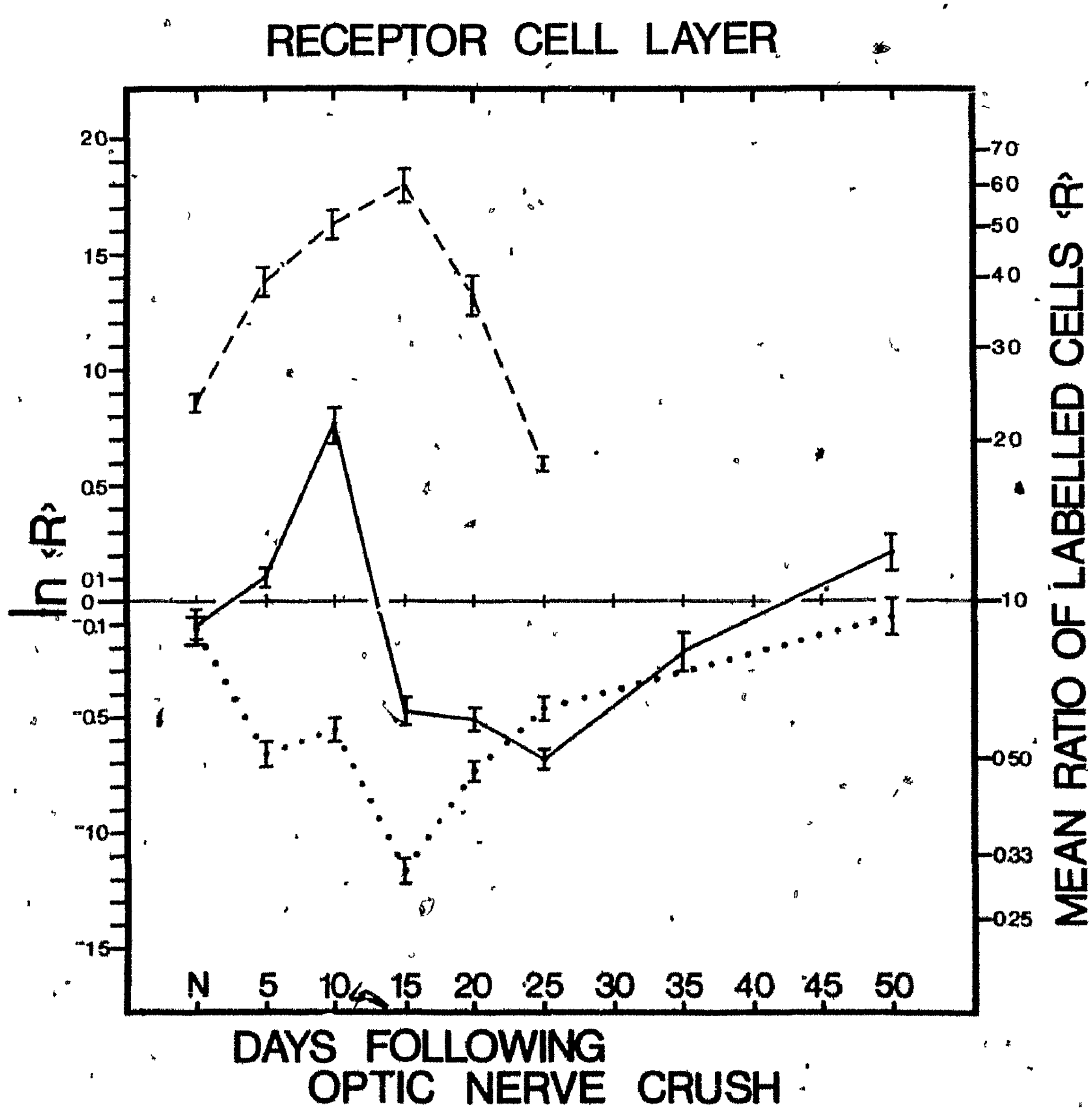
Figure 44. Summary of Time Courses of Changes in Cell Proliferation in the ONL During Various Post-operative Periods Following Optic Nerve Axotomy.

The abscissa denotes the days following optic nerve crush. The ordinate represents the mean value of the ratio of labelled cells on the right scale and the natural logarithm of the mean value of the ratio is presented on the left scale. The error bars represent the standard error of the natural logarithm.

The solid line reflects the time course of changes during various post-operative periods following unilateral optic nerve axotomy when fish are sacrificed 1 day following injection (see Figure 25A).

The dotted line represents the time course of changes during various post-operative periods following unilateral optic nerve axotomy when fish are sacrificed 30 days following injection (see Figure 25B).

The dashed line denotes the time course of changes during various post-operative periods following bilateral axotomy when 1 eye is examined 1 day following injection (SS) as compared with the other eye which is examined 1 month following injection (LS) (see Figure 41).


RECEPTOR CELL LAYER
20
15
10
0.5
0.1
0
-0.1
-0.5
-1.0
-1.5
ln ‹R›
70
60
50
40
30
20
10
0.50
0.33
0.25
MEAN RATIO OF LABELLED CELLS ‹R›
N
5
10
15
20
25
30
35
40
45
50
DAYS FOLLOWING
OPTIC NERVE CRUSH

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