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EXPLANT CULTURE OF THE ADULT GOLDFISH RETINA.

The University of Michigan, Ph.D., 1977
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EXPLANT CULTURE OF THE ADULT GOLDFISH RETINA

by

Gary Eugene Landreth

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Neurosciences)
in The University of Michigan
1977

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ACKNOWLEDGEMENTS

I would like to extend my thanks to the members of my committee for their assistance throughout the course of this work. Their suggestions and criticism were appreciated. I would like to particularly thank Dr. Sam Hicks who tried to convey to me his enthusiasm for neuroscience.

The excellent technical assistance of Marianne Andrews is gratefully acknowledged. She provided a steady supply of operated goldfish and is responsible for the figures appearing in this dissertation.

I also thank Mrs. Gertrude Calloway for preparation of histological material; Dr. Pamela Johns for our numerous discussions and for her assistance with the manuscript; and Eva Feldman for aid in the preparation of this thesis.

Dr. Anne Heacock was unfailing in her support, providing ideas, constructive criticism and fostering an intellectual climate conducive to research. My debt to her is enormous. Her editorial assistance with the manuscript is appreciated.

I would like to express my sincerest appreciation to Dr. Bernard Agranoff for his continued support and guidance. He provided opportunities to broaden my scientific outlook and offered many stimulating ideas and discussions.

Lastly, I would like to thank my wife, Leslie, for her years of understanding and caring. Without her assistance, this work would not have been possible. She spent many hours typing and editing this manuscript, for this I am deeply grateful.

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LIST OF ABBREVIATIONS

BSA	bovine serum albumin
Ci	curie
CNS	central nervous system
cpm	counts per minute
CXM	cycloheximide
°	degrees centigrade
DMSO	dimethylsulfoxide
dpm	disintegrations per minute
EDTA	ethylenedinitrilo-tetraacetic acid
ELM	external limiting membrane
FCS	fetal calf serum
g	gram
h	hour
HEPES	H-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid
INL	inner nuclear layer
IPL	inner plexiform layer
L-15	Leibovitz medium
L-15H	modified L-15 medium
l	liter
MSO	methionine sulfoxide
µCi	microcurie
µg	microgram

µl	microliter
µm	micrometer
mg	milligram
ml	milliliter
mm	millimeter
mM	millimolar
min	minutes
MW	molecular weight
ng	nanogram
NGI	nerve growth index
NGF	nerve growth factor
nm	nanometer
ONL	outer nuclear layer
OPL	outer plexiform layer
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
SDS	sodium dodecyl sulfate
sec	second
S.E.M.	standard error of the mean
TCA	trichloroacetic acid
TEMED	tetramethylethylenediamine

CHAPTER I

GROWTH, REGENERATION AND CULTURE OF NERVOUS TISSUE
A Historical Review

The growth and regeneration of nerve fibers has been a major interest in the study of the nervous system. These topics were first put into the proper perspective with the neuronal theory of Ramon y Cajal (Cajal, 1899). The confirmation of this theory, which has become the central dogma of neurobiology, turned upon the critical experiments of R.G. Harrison (1907, 1910) who was able to conclusively demonstrate, by the application of *in vitro* techniques, that the nerve fiber is an outgrowth of a single cell. The extension of morphological studies to nervous tissue cultivated outside the animal has benefitted our understanding of neuronal function. This review seeks to emphasize the application of tissue culture techniques to the fundamental questions of axonal regeneration and the molecular mechanisms which subserve the formation and extension of nerve processes.

Culture of Nervous Tissue

The first demonstration that living tissue could be maintained and grown *in vitro* was that of Harrison (1907, 1910). If small pieces of neural ectoderm from tadpoles were placed in lymph clots, they would survive for several weeks. The embryonic tissue was observed to

extend nerve fibers into the unorganized clot. The direct visualization of growing nerve fibers revealed an active and motile organ at their tip. The observation of growth cones in the living state confirmed the view of Ramon y Cajal (1899) that they were responsible for the growth of the nerve fiber. Harrison described in great detail the means by which the nerve fiber is extended and emphasized the role of the growth cone in this process. The growth cone was found to have an ameboid movement, extending and retracting small processes, resulting in the elongation of the nerve fiber.

Harrison's work had a far-reaching affect on contemporary scientific thought. The observation of growing nerve fibers was direct support for Ramon y Cajal's neuronal theory. The controversy derived from the conflicting views of Henson and Held that the nervous system was a syncyticium of protoplasmic bridges (reviewed by Harrison, 1910). The latter theory was based upon observations of histological material in which individual neuronal processes were incompletely resolved. This led to the belief that there was continuity between processes of the various neuronal elements.

Harrison emphasized that nerve growth was the result of protoplasmic movement. The similarity in the mode of movement of other tissue cells and the elongation of the nerve fiber was pointed out:

> "It has been shown that the first manifestations of activity observable in the differentiating nerve cell are the same fundamental nature as those found not only in other embryonic cells but also in the protoplasm of the widest variety of organisms. The movement which results in the drawing out of a compact cell into a long filament, the primitive nerve

> fiber, is but a specific form of that general type of movement common to all primitive protoplasm. In studying the specific nerve paths of any organism, we are concerned, therefore, primarily with the laws which govern the direction and intensity of protoplasmic movement, and it is the analysis of these phenomena to which studies of the ontogenetic and regenerative development of the nervous system must now direct their attention." (Harrison, 1910, p. 840)

This view has recently been validated following demonstration of biochemically and morphologically similar cellular components which subserve non-neuronal cell locomotion and nerve elongation (Wessells et al., 1975).

The importance of Harrison's work was not limited to the theoretical questions which it addressed. It set the stage for further investigations on the culture of living tissue in vitro.

Harrison recognized the importance of solid substrata for the growth of tissues in culture. He found that nerve fibers were unable to grow in liquid media alone, but thrived in lymph clots. The improved maintenance of nervous tissue was due to the solid support for the growth of fibers as well as to nutrients contained in the lymph.

Lewis and Lewis (1912 a, b) were able to grow sympathetic ganglia in chick intestinal wall explants for up to 12 days in normal saline. The growth of nerve fibers in a simple balanced salt solution has not been repeated since, and appears to have been forgotten in considerations of tissue culture medium requirements (Murray, 1965). This early work was extended by Matsumoto (1920) who observed, by using vital stains, mitochondria and numerous vacuoles distributed along the fiber and especially in the growth cone.

Ingebrigsten was another pioneer in nervous system tissue culture. He observed extensive neuritic outgrowth from chick cerebrum and cerebellum and described varicose fibers with typical growth cones (1913 a). He attempted to grow a variety of mammalian central and peripheral nervous tissues (1913 b). A significant observation in these studies was that adult tissue generally failed to grow in vitro, while neonatal and embryonic tissue grew well. Furthermore, the younger the donor embryo the more favorable the growth.

The culture of nervous tissue was largely neglected following these initial studies. In a review of tissue culture literature, Lewis and Lewis (1924) could cite no papers on nervous system tissue culture appearing in the decade from 1914 to 1924, aside from those mentioned above. A renewed effort at culture of nervous tissue was begun in the years following World War II, owing largely to the work of M.R. Murray and Pomerat (reviewed by Murray, 1965). In many respects tissue culture of the nervous system did not fulfill its early promise. The difficulty in maintaining the tissue in vitro, coupled with short survival times, even under favorable circumstances, discouraged the extension of the original work beyond descriptive studies.

The application of tissue culture techniques to questions of development and function of the nervous system is clearly a reductionist approach. In employing such an approach interpretations must be advanced cautiously. The experimenter has hopefully removed the tissue from a dynamic environment into a static one in which the

intrinsic properties of the cell can be examined, and in which those attributes which are a characteristic of the neuron itself can be dissociated from influences of the environment. This view is probably not entirely correct. The removal of a cell into a more simple and therefore artificial environment undoubtedly places constraints upon the cell. It may be unable to perform its usual functions by virtue of its isolation, and conversely the absence of its normal milieu may release the expression of traits which are not characteristic of the cell *in situ*. Nevertheless, the ability to maintain living cells under controlled conditions which are directly accessible to experimental manipulations constitutes an important advance in the study of complex tissues. Employment of these techniques has permitted the examination of a variety of issues approachable by no other means.

It has been demonstrated that the behavior of neurons in culture is often representative of their behavior *in vivo*. Early criticism centered around the "abnormal" nature of the culture situation. These objections have been refuted on a number of grounds. The earliest and perhaps the most elegant studies were those of Speidel (1933). His observations on the growth and regeneration of nerve fibers in the tail of the living tadpole exactly paralleled those of Harrison (1910) from the same animal *in vitro*. Cultures of nervous tissue can develop or retain electrophysiological properties which are characteristic of the tissue *in vivo* (Crain, 1966; Crain and Peterson, 1975 a; Deboni et al., 1976). These cultures will generate action potentials in response to iontophoretically applied

neurotransmitters (Peterson and Crain, 1975; Padjen et al., 1975) and are inhibited by the appropriate antagonists (Crain, 1966; Crain and Peterson, 1975 b). Synapse formation in vitro can be demonstrated both morphologically and functionally (Lumsden, 1968; Deboni et al., 1976).

The general inability of mature tissues to survive in vitro is well recognized. This derives in part from the failure of neurons to divide in culture. The most favorable period for explantation is during developmental stages when morphogenesis is nearly complete and differentiation is beginning (Murray, 1971). Adult, differentiated nervous tissue has been successfully cultured in only a few instances.

The first demonstration of prolonged survival of adult neurons in tissue culture was that of Murray and Stout (1947) using human sympathetic ganglia. This report is significant in several respects. Neurons were observed to undergo mitosis. The ability of mature neurons to divide in vitro is entirely restricted to autonomic ganglia and is enhanced by nerve growth factor (NGF) (Lumsden, 1968). In no instance has neuronal cell division been demonstrated to occur in any other nervous tissue cultures (Lumsden, 1968). They also observed active migration of sympathetic ganglion cells away from the explant. This was a novel observation as well, since neurons do not generally migrate in tissue culture.

Costero and Pomerat (1951), and Hogue (1953) were able to cultivate human cortical cells from either cerebrum or cerebellum and

maintain them for several months. Geiger (1963) reported survival and multiplication of adult cortical neurons, although the validity of this work has been criticized on a number of grounds (Murray, 1965).

The culture of amphibian and fish tissues provides another example of the survival in vitro of adult neurons. Vinnikov (1946) reported successful culture of adult carp retina explants. Similarly, Spiedel (1953) observed neuritic and non-neuronal growth from retinal explants of frogs. Both of the above studies appeared only in abstract form and the exact nature of the growth was not detailed. Recently, there have been two reports of viable cultures of adult amphibian peripheral ganglia. Padjen et al. (1975) found continuous neuritic outgrowth in cultures of intact frog sympathetic and spinal ganglia for up to six weeks. These cells were electrophysiologically responsive to ionophoretically applied neurotransmitters. Hill and Burnstock (1975) reported the development of satisfactory culture conditions for the growth of adult frog sympathetic ganglia. This preparation was the subject of extensive morphological and histochemical studies. The frog sympathetic ganglia, like its avian and mammalian counterparts, is highly sensitive to NGF. Deboni and co-workers (1976) were able to culture neurons from adult goldfish brain, either as explant cultures or as single cells. They found that morphologically identifiable neurons would extend neurites and were electrophysiologically active, forming functional synapses with other cells. No dividing neurons were reported. Electron microscopy revealed degenerating synapses on the soma of growing cells, indicating the presence of

differentiated neurons. Other neurons in these cultures may have derived from neuroblasts present in the adult goldfish brain. The teleost brain can regenerate neuronal and glial elements following extensive lesions. These cells derive from matrix, or germinal, zones present in restricted regions of the brain (Richter and Krantz, 1970; Kirsche and Kirsche, 1961). The matrix zones, positioned in periventricular areas, contain undifferentiated cells and are probably related to the continued growth of the brain throughout life. The interpretation advanced by Deboni (1976) is that undifferentiated cells are induced to form extensive processes in vitro, and that some existing differentiated cells are able to regenerate amputated processes. Cultures derived from tissue in which these matrix zones were excluded, grew only glial and other non-neuronal cell types. It was not reported what fraction of the total number of plated cells eventually extended neurites.

The Retina in Tissue Culture

The retina has special advantages which make it an attractive preparation for tissue culture studies (see Lucas, 1965). Champy (1913, 1914) first recognized that the retina's uniform thickness and cellular composition should favor its use as a tissue culture preparation. He described the histological changes occuring in the retina of adult rabbit and tortoise in tissue culture (1913, 1914), including degenerative changes in the photoreceptor cells followed soon after by the loss of other neuronal elements. He was able to maintain them for only a week.

Meyer (1936) successfully cultured embryonic chick retina in plasma clots and reported the growth of nerve fibers from the explants and migration of non-neuronal cells (Jablonski and Meyer, 1938).

Tansley (1933) was able to maintain immature rat retina in culture for periods of up to two weeks. She observed the formation of rosettes within several days of explantation. These consisted of layers of cells organized about a circular membrane, thought to be the external limiting membrane. The cells stratified and appeared to differentiate as in vivo. These formations have been frequently observed in cultures of mammalian retina (Pomerat and Littlejohn, 1956; Hansson and Sourander, 1964; Hild and Callas, 1967; Barr-Nea and Barishak, 1970; LaVail and Hild, 1971). It is of some interest that similar rosettes are found in vivo in congenital malformations of the retina and in retinoblastomas (Lucas, 1965).

The culture of developing rat retina has been the subject of more recent investigations, principally those concerned with the study of retinal differentiation in vitro (Hansson and Sourander, 1964; Hild and Callas, 1967, LaVail and Hild, 1971). The cultured rat retina is observed to organize itself at the cellular level in a manner comparable to its normal development, with the exception of incomplete formation of the plexiform layers. The retinal cells differentiate normally but fail to form synapses. Abundant neuritic growth, thought to be ganglion cell processes, issue from the explants but regress and disintegrate during the second week in culture. These cultures were found to be light-sensitive. Illumination of

the explants resulted in degeneration of the outer nuclear layer after 10 min, and ganglion cells after 30 min (Hansson and Sourander, 1964).

The chick retina has also provided a model for the study of neuronal differentiation in vitro (Barr-Nea and Barishak, 1970; Kim, 1971). In an interesting series of experiments, Moscona (1961) found that if chick neuronal retina cells were enzymatically dissociated they would spontaneously reform histotypically organized aggregates when mixed together.

Vinnikov (1946) claimed to successfully culture retina from a variety of species. He observed neuritic outgrowth which he attributed to retinal ganglion cells. The growth from retinal explants in all cases was oriented or polar i.e., the fibers issued from the side of the explant which was oriented toward the optic disc in situ. He described neurites from carp retina passing through the plasma clot as fascicles and eventually branching, with individual fibers taking their own course. These fibers could reach lengths of up to 1 cm.

Explant Cultures as a Model for Neurobiological Studies

The explant culture is necessarily a heterogeneous population of cell types, containing neuronal and glial cells, as well as various connective and vascular elements. The analysis of neuronal functions is complicated by the anatomical complexity of the tissue fragment. However, its value in the study of nervous system development has been clearly demonstrated. Two examples are pertinent to the present

discussion: the effect of NGF on the development of the sympathetic nervous system and the selective innervation of central nervous system explants *in vitro*.

The effect of NGF on sympathetic and sensory ganglia in the developing animal demonstrate how explant cultures can provide useful models for the study of the mechanisms responsible for growth of individual nerve fibers, as well as to answer more general questions concerning the development of the nervous system.

The stimulation of neuritic outgrowth from chick sympathetic and sensory ganglia by NGF was first demonstrated by Levi-Montalcini and Hamburger (1951). If NGF is added, explant cultures of peripheral ganglia are induced to grow an extensive "halo" of neurites 12-18 h after administration (Levi-Montalcini and Hamburger, 1954; reviewed by Levi-Montalcini, 1968). There is an absolute requirement for NGF in these cultures. If NGF is not available, the neurons rapidly die, generally within 24 h of explantation. The vigorous growth of neurites following NGF stimulation is accompanied by hypertrophy and hyperplasia of the ganglia. This morphological response has served as a sensitive bio-assay for NGF, permitting biochemical studies which led to the purification and isolation of this factor.

The *in vitro* situation is particularly advantageous for the study of developing ganglia since they are thereby freed from a complex hormonal environment. The administration of NGF has numerous metabolic consequences. It results in increased glucose consumption, primarily through stimulation of oxidative pathways (Cohen, 1959).

Lipid synthesis is also increased by approximately the same amount (Liuzzi et al., 1965). However, by blocking DNA-dependent RNA synthesis, the effects could be abolished. The altered biosynthetic capacity is accompanied by changes in cellular morphology. The NGF-enhanced protein synthesis and RNA synthesis is correlated with a massive increase in the number of ribosomes, granular and agranular endoplasmic reticulum and the appearance of prominent Golgi apparatus. There is a two-fold enlargement of the ganglion cell body, and large numbers of microtubules and neurofilaments are found (Levi-Montalcini et al., 1968). A paradox is the absence of a stimulation of tubulin synthesis following NGF treatment (Mizell and Bamburg, 1976), despite the extensive neuritic outgrowth and an increase in the number of microtubules present (Levi-Montalcini et al., 1968; Crain et al., 1968). These results indicate that the ganglia maintains large endogenous pools of the subunit protein. The NGF apparently stimulates the assembly of microtubules rather than net synthesis of tubulin. Levi-Montalcini (1974) has suggested that membrane-bound tubulin may act as binding site and could thus mediate the action of NGF in sympathetic neurons.

The ability of NGF to induce extensive neuritic outgrowth from these ganglia has permitted studies of the mechanism for control of nerve growth (Dunn, 1971, 1973; Ebendal, 1976). These studies indicate that the direction and extent of fiber outgrowth is dependent on competitive interactions of individual fibers and on the orientation of the substratum on which the fibers grow.

In summary, the NGF stimulated growth of sensory and sympathetic ganglia in culture has proven to be a valuable model for examination of the molecular events associated with nerve growth.

Explant cultures of nervous tissue have been used to examine formation of specific neuronal connections and the development of bioelectric activities. This approach has been employed in a series of studies by Crain and co-workers (reviewed by Crain, 1966, 1968). Briefly, explant cultures of fetal rat or mouse tissue are placed in ordered arrays in a culture vessel. The neuritic growth issuing from these tissues can be directly observed to penetrate adjacent explants. Once the explants become connected, electrophysiological recordings are used to demonstrate functional coupling.

When explants of spinal cord with attached dorsal root ganglia were placed in close proximity to explants from brainstem, spinal cord neurites were found to invade the adjacent explant. These connections were found to be electrophysiologically active. Complex bioelectric responses with long latencies were elicited in the brainstem explant following stimulation of the spinal cord. Under similar conditions cerebral cortical explants formed connections with either medulla or spinal cord. The electric coupling of medulla and cerebrum resulted in a complex pattern of activation and inhibition. The medulla could be shown to modify the discharge characteristics of cortical cells as well as elicit cerebral evoked potentials. The electric activity

was shown to be a direct result of coupling by the neurites, since severance of these fibers resulted in loss of transmission between the explants. In some instances they were able to demonstrate coupling of 3 explants, stimulation of a dorsal root ganglia-spinal cord explant resulted in sequential discharges in a medullary explant which in turn was electrically coupled to a forebrain explant (Crain, 1968).

These experiments demonstrate that electrically functional connections can be formed in vitro and retain some of the characteristics observed in situ. They were unable to demonstrate any specificity in the formation of nerve contacts within the explant, nor could they detect any preferential growth of neurites toward specific target tissue.

A difficulty encountered in the above mentioned studies was the inability of dorsal root ganglion cells to send neurites into the spinal cord explants, necessitating removal of the cord and ganglia together, and maintaining them as a single explant. Crain was able to facilitate neuritic growth from dorsal root ganglion by addition of NGF, permitting the separation of the ganglion from the cord and maintenance as independent cultures (Crain and Peterson, 1975 a). Moreover, the reinnervation of the spinal cord by the sensory ganglia resulted in the formation of synaptic contacts in specific regions of the cord normally receiving the sensory fibers. Electrophysiological recordings found primary afferent depolarization (short latency, monosynaptic conduction), rather than the expected diffuse

complex discharge pattern. If, in addition to spinal cord explants, medulla explants were present, the NGF-stimulated dorsal root ganglion sent fibers through the cord to synapse in the brainstem explant. The area of the medulla innervated was found to be in localized zones up to 300 μm in diameter. The electrical response could be partially inhibited by picrotoxin and bicuculline, and was enhanced by γ-aminobutyric acid (Crain and Peterson, 1975 b).

Further studies have shown that not only are fibers attracted to target tissue, but they are repelled by other non-target nervous tissue (Peterson and Crain, 1975). The combination of sensitive electrophysiological techniques and the direct application of pharmacological antagonists have thus permitted the demonstration of regional and functional specificity in the formation of synaptic contacts in vitro.

Regeneration

Conditions under which a neuron regrows an amputated process has drawn sustained interest. Ramon y Cajal (1928), in his early review of this literature, set out a coherent theory regarding the means by which nerves regenerate, and properly separated those facets of the process which are intrinsic to the cell and those which are imposed by the environment. In its main features this theory has survived to the present. Throughout the literature on the regenerating neuron, drawn mainly from work on mammals and other homeotherms, several features are consistent. The neuronal cell body undergoes dramatic

changes following severance of its axon. In peripheral nerves this is followed by regrowth and often reestablishment of functional connections. Functional regeneration of axons within the central nervous system (CNS) has not been conclusively demonstrated to occur in mammals. In mammals regrowth of several axons has been observed for a limited period following axotomy, but circumstances are rarely such that functional connections are reformed. Regeneration in the amphibian and fish CNS constitutes an exception. These topics have been extensively reviewed (Cragg, 1970; Lieberman, 1971, 1974; Grafstein, 1975; Guth, 1975; Torvik, 1976).

The ability to regrow amputated axons is dependent upon a reaction of the cell body to the injury, resulting in initiation or acceleration of synthetic processes necessary to sustain axonal regrowth. The factors which permit, or for that matter prevent, regeneration are incompletely understood.

The literature on regeneration of mammalian peripheral nerves is extensive and often contradictory. Investigations employing this preparation have provided most of what is known concerning the response of the cell body to axotomy. The reaction of the cell body has frequently been termed a "chromatolytic" one. This is inaccurate, as it refers only to a specific aspect of the cellular reaction. Chromatolysis specifically refers to the dispersion and dissolution of Nissl substance resulting in a decreased basophilia (Leiberman, 1971). Hereafter, the resultant changes in the neuronal soma shall be referred to as an "axonal" or "cellular" reaction.

The main features of the axon reaction will be briefly summarized as a preface to subsequent discussions. Following axotomy there is

a swelling of the cell body accompanied by chromatolysis. The nucleus assumes an eccentric position in the cell and may increase in size often exhibiting a folded nuclear membrane. The response of mammalian CNS neurons to axotomy is highly variable, depending upon the types of cells involved, the site of lesion and the age of the animal. Some CNS neurons show no change in response to axotomy, while others demonstrate an axonal reaction typical of peripheral neurons. Some cells rapidly die. It may be generally stated that it has not been possible to find any consistent reason for the failure of mammalian CNS neurons to regenerate. The failure of CNS neurons to regenerate has been variously attributed to the inability of the regrowing fibers to penetrate glial scars, an insufficient cell body response to sustain the anabolic reactions required for fiber regrowth, or the insensitivity of the neuron to trophic factors.

In contrast, the amphibian and fish CNS is an example where extensive regrowth of nervous tissue can be demonstrated (reviewed by Clemente, 1964). Adult teleosts are able to regenerate various CNS structures after ablation or section. Following extirpation of regions of the optic tectum or forebrain the teleost will regenerate the ablated areas (Kirsche and Kirsche, 1961; Segaar, 1965). Spinal cord regeneration has also been demonstrated to occur with complete return of function (Bernstein and Gelderd, 1970). Similar results have been obtained in adult amphibian spinal cord regeneration (Clemente, 1964).

The visual system of these animals has been widely used to study various aspects of regeneration and neuronal specificity. This is a

particularly useful preparation since the retinal ganglion cells lie in an enclosed structure isolated from the rest of the brain and send their central projections into a restricted, yet accessible, region of the midbrain.

The first conclusive demonstration of optic nerve regeneration was that of Matthey (1925, 1926) in the newt (Triturus). Sperry employed the regenerating poikilotherm visual system in a series of studies concerning specific reformation of nerve connections between the eye and tectum (Sperry, 1943, 1944, 1945, 1963; Attardi and Sperry, 1963). The regenerating optic fibers return to their original position on the optic tectum, restoring a normal retinotopic projection. These provocative studies were the forerunners of an extensive series of investigations by Gaze, Jacobson, Yoon and others examining the manner in which specific nerve connections are formed (reviewed by Gaze, 1970; Jacobson, 1970). These studies were not directly concerned with the mechanisms which induce the ganglion cell to regrow an axon, but rather with the resultant axonal growth and the ordering of connections.

The response of the ganglion cell to axotomy in the goldfish has been investigated with both the light and electron microscope (Murray and Grafstein, 1968; Murray and Forman, 1971). In the normal animal the ganglion cell is relatively inconspicious, however, following axotomy they undergo a dramatic change in morphology. At the light microscopic level the cells become highly basophilic, the nucleus takes an eccentric position and the cell enlarges. The earliest

observed change is an increase in the size and number of nucleoli present in the cells as well as in the amount of free ribosomes. The granular endoplasmic reticulum increases rapidly in the first week after optic tract section. The most striking of these changes is the increase in cellular volume, which more than doubles by 28 days (Murray and Grafstein, 1969). Concomitant biochemical changes can be shown to occur. One of the earliest of these is a rapid increase in the amount of radiolabeled uridine incorporated into RNA in the retinal ganglion cells. One week following section of the optic tract uridine incorporation, inferred from radioautographic evidence, had doubled, with the largest increase occuring in the first three days. This elevated level of RNA labeling was sustained for periods of up to a month (Murray, 1973). In a recent study an enhanced uridine uptake was correlated with an increase in the levels of polyadenine containing RNA (Burrell et al., 1977). The increase in RNA biosynthetic activity corresponds well with the observed ultrastructural changes in the cell. Murray has computed that there is a 25-fold increase in nucleolar volume in the first month following optic nerve section yet the net RNA labeling is only doubled.

Protein synthesis may be enhanced in regenerating retinal ganglion cells, as is suggested by radioautographic evidence of increased incorporation of radioactive leucine (Murray and Grafstein, 1969). This increase was first seen four days after section of the optic tract. The amount of labeled precursor incorporated per ganglion cell rises to three times that of controls by 20 days. While

there is an increase in the total amount of radioactive leucine incorporated, the cellular volume is also increasing. If these data are expressed as the number of grains per cross-sectional area of the cell, the regenerating ganglion cell has 40% fewer grains per unit area then comparable controls. Recent data suggest that an enhancement of protein labeling may occur as early as 24 h following optic tract section (Grafstein and Alpert, 1976).

The morphological studies demonstrate that there is a change in the organization of granular endoplasmic reticulum, with a shift toward stacked membrane profiles. This has been taken as an index of an increased synthesis of protein destined for transport away from the cell body. In the goldfish, protein is axonally transported at two different rates, a fast component traveling at 30-70 mm/day and a slow component advancing at approximately 0.5 mm/day (Grafstein and Murray, 1969; Elam and Agranoff, 1971 b). During regeneration the rate of both phases is accelerated. Rapid axonal transport is doubled 36 days following optic tract section. The increased rate is accompanied by a three-fold increase in the total amount of protein carried by this component (Grafstein and Murray, 1969). An increase in slow axonal transport is detectable as early as 6-8 days after axotomy. There is a large (three-fold) increase in both the rate and total amount of protein conveyed by slow transport, comparable to the increase observed with the rapid axonal flow (Grafstein, 1971; Grafstein and Murray, 1969). No qualitative change in the transported proteins have been reported.

Axonal transport is dependent upon the structural integrity of microtubules present in the axon (Smith et al., 1975). The precise role these elements play in the conveyance of cellular materials down the axon is unclear, yet their dissolution halts all transport in the nerve. The regenerating ganglion cell possesses large numbers of these elements (Murray and Forman, 1971). Agranoff and Heacock (1976) have shown that there is a selective enhancement of the labeling of the microtubule subunit protein, tubulin, following crush of the optic nerve. The rise in the incorporation of labeled precursor into this protein parallels the acceleration of axonal transport and occurs at a time when the optic nerve fibers are beginning to regrow.

The foregoing discussion has centered upon changes occuring in the cell body rather than events taking place at the site of the lesion. In the goldfish section of the optic tract results in the formation of bulbous enlargements at the cut ends of the fibers (Grafstein, 1971), similar to Ramon y Cajal's (1928) description of lesion sites in mammalian nerve. After three days, bulbs on the smaller diameter fibers (0.5-1 μm) disappeared and the fibers began to grow. Larger diameter fibers (3-7 μm) retained the enlarged bulb for as long as a month and were not observed to initiate outgrowth. Associated with these changes was the formation of a neuroma containing tangles of fibers and reactive glial cells. The neuroma did not hinder the subsequent regrowth of nerve fibers, but was believed to facilitate the growth of fibers into the degenerating distal optic

nerve stump (Maloney, 1976). One week (or less) following axotomy, bundles of nerve fibers could be found extending across the gap and distributing for several hundred microns in the old optic tract. This regrowth was inhibited by local application of colchicine (Grafstein, 1971).

The optic nerve is largely regrown 30-40 days after retroorbital optic nerve section and mediates functional behavior in the animal (Grafstein and Murray, 1969; Springer and Agranoff, 1977). The biosynthetic capacity of the ganglion cell diminishes over this interval and has returned to normal levels by 60-90 days after optic nerve section.

The axonal reaction is typically induced by axotomy. Recent evidence suggests that section of the axon is not required for the cellular reaction to occur (Schmidt, 1976). In these experiments, one-half of the goldfish retina was removed, denervating half of the optic tectum. If the retinotectal projection was mapped 9-10 months later the retinal fibers were found to have spontaneously shifted their positions to cover the entire tectum. The expansion of the retinal projection was accompanied by cellular hypertrophy of the ganglion cell, an increase in the number of nucleoli and an eccentric nucleus, identical to the cell response following axotomy. Previous explanations invoked to account for the initiation of the axonal reaction have been reviewed by Cragg (1970) and have uniformly required that axotomy or crush occur for the initiation of the cell response. Schmidt's data suggest that more subtle alterations are sufficient to induce the reaction.

In the past few years, retrograde axonal transport has been proposed to mediate the signalling to the cell body (reviewed by Torvik, 1976). According to this theory, interruption of the axon results in transport of material originating at the site of injury to the soma, initiating the axonal reaction (Cragg, 1970), or alternatively loss of trophic substances ordinarily transported to the cell from the terminals. The depletion of these factors following axotomy induces the cellular response (Cragg, 1970). The latter proposal has received some experimental support. If postganglionic fibers from the adult rat superior cervical ganglia are cut, the ganglion cells enlarge and become chromatolytic. The axonal reaction can be prevented by application of NGF to the cut ends of the fibers (West and Bunge, 1976). It was further shown that in neonatal rats, injection of vinblastine (an alkaloid which blocks axonal transport) would result in the rapid death of superior cervical ganglion cells and that this cell death could also be prevented by exogenously supplied NGF (Johnson, 1977). These experiments indicate that retrograde axonal transport carried trophic factors to the cell body from the periphery and that the absence of these factors may initiate the axonal reaction (West and Bunge, 1976). Moreover, these data suggest that in the sympathetic nervous system the trophic factor may be NGF. Hendry et al. (1974) has demonstrated that NGF is taken up at sympathetic nerve terminals and transported to the cell body by a mechanism which is specific for NGF.

There is some evidence that NGF may play a role in CNS regeneration. Glaze and Turner (1976) have reported that regeneration of the newt optic nerve is seriously impaired by administration of antiserum directed against NGF.

The present evidence suggests that the response of the cell body to axotomy is in some instances regulated by trophic factors, presumably operating at transcriptional levels.

A General Theory of Nerve Growth

Any consideration of the growth of neuronal processes is, in the final analysis, concerned with the function of the growth cone. Ramon y Cajal (1899) first described these processes in developing tissue, prompting the suggestion that they were responsible for directing the growth of the nerve. He states:

> "From the functional point of view, the cone of growth may be regarded as a sort of club or battering ram, endowed with exquisite chemical sensitiveness, with rapid amoeboid movements, and with a certain impulsive force, thanks to which it is able to press forward and overcome obstacles met in its way, forcing cellular interstices until it arrives at its destination." (1899, p. 544)

The essential features of Cajal's argument were confirmed by Harrison (1907, 1910). Despite numerous investigations of the growth of individual nerve fibers, few advances were made toward the understanding of nerve growth until electron microscopic studies were initiated to examine the ultrastructure of the nerve fiber and growth cone both *in vivo* (Fox et al., 1970; Tennyson, 1970; Skoff and Hamburger, 1974; Povlishock, 1976) and *in vitro* (Yamada et al., 1971; Bunge, 1973; Pfenninger and Bunge, 1974).

The mechanism by which a neurite advances poses a series of questions concerning the origin, transport and insertion of materials

required for a growing fiber. The growth of the axis cylinder results in a large increase in the amount of axolemmal membrane present. Membrane components could be added either at the cell body, along the length of the fiber or in the growth cone. Hughes (1953) observed that if the growing neurite was severed from its cell body it continued to elongate for several hours, a rather remarkable observation in itself. It has also been observed that once a fiber has grown away from the cell body and subsequently branched into two separate collaterals, the distance between the cell body and the point of bifurcation does not change, while that between the growth cone and the bifurcation does (Bray, 1973 a). If glass or carmine particles are placed on the fiber, they neither move nor change their relationship to one another. Particles in the path of the growth cone or positioned on its upper surface move rapidly in the direction of the cell body and are deposited on the fiber (Bray, 1970). These observations indicated that addition of surface membrane components occurs at the growing tip. A correlation between the ultrastructure of the growth cone and its behavior was suggested by Yamada et al. (1971) and later by Bunge (1973). The high density of membranous vesicles and agranular reticulum, suggests the importance of these structures to the function of the growth cone. It was concluded that since membrane components were undoubtedly being added to the growth cone, these vesicles must serve as the source (Yamada et al., 1971).

Dense-core vesicles (approximately 120 nm in diameter) were prominent occupants of the growth cone and axon. These vesicles have

been described in nearly every study of developing or regenerating nerve fibers (reviewed by Murray, 1976). They have been found in such diverse tissue as human cerebral cortex (Povlishock, 1976) and regenerating Xenopus optic nerve (Reier and Webster, 1971) in vivo as well as in amphibian (Hill and Burnstock, 1976) and mammalian cells in tissue culture (Yamada et al., 1971; Bunge, 1973; Pfenninger and Bunge, 1974). The similarity of these vesicles to both Golgi and smooth endoplasmic reticulum has been noted by several investigators (Tennyson, 1970; Yamada et al., 1971; Bunge, 1973). These vesicles are thought to arise in the growth cone and along the fiber by budding off the smooth endoplasmic reticulum, enclosing electron-dense material present within the reticular structures (Teichberg, 1971; Bunge, 1973). However, this is controversial (Yamada et al., 1971). It is possible that these vesicles derive from the Golgi apparatus in the cell body and are transported to the advancing tip. This class of vesicles is thought to provide new membrane for insertion into the growth cone. [These large dense-core vesicles should not be confused with smaller ones (40-90 nm) which are normally associated with biogenic amine neurotransmitters (Langercrantz, 1976).]

A distinguishing feature of another class of vesicles, in or near the growth cone, was their similarity to the plasma membrane. These are the so-called coated vesicles, which typically arise from pinocytotic activity. This apparent reversal of the postulated flow of vesicular material prompted Bunge (1973) to suggest a mechanism involving recirculation of growth cone membranes. Vesicular material

(dense-core vesicles) is added to the plasma membrane at the growing filopodia, while pinocytotic vesicles are formed in other areas of the growth cone. A similar mechanism was proposed to explain vesicular flux at the frog neuromuscular junction (Heuser and Reese, 1972). The coated vesicles are observed within the fiber and are presumably being transported to the soma. This is an attractive hypothesis since pinocytotic elements could be "sampling" the environment for communication to the cell body and may be related to mechanisms of growth control (Leestma, 1976).

The idea of peripheral membrane insertion is supported by the apparent immaturity of growth cone plasmalemma compared to that of the cell soma or proximal neurite. Close to the cell body the plasmalemma is studded with intramembranous particles (Pfenninger and Bunge, 1974). The particle density diminishes progressively along the fiber toward the growth cone, whose membrane has a very low particle density. The more complex membrane of the soma, inferred from the high density of intramembranous particles, is "older" than that lying more distally. This gradient of particle density suggests a gradual insertion of protein into the plasmalemma in the periphery, although this is not the only possible explanation. The structural disparity would indicate that the fiber should differ in biochemical and functional charateristics along its length.

The peripheral insertion of intramembranous particles into the nerve fiber has received indirect support from a novel series of experiments by Koda and Partlow (1976). They found that red blood cells

bound with Concanavalin A (Con A-RBC) would attach to the plasmalemma membrane of growing neurites and be transported in a retrograde fashion. Concanavalin A is a plant lectin which selectively binds α-methyl glucose residues of glycoproteins or possibly glycolipids in the plasma membrane (Sharon and Lis, 1972). In actively growing neurites only Con A-RBC less than 30 μm from the soma were transported, while non-growing fibers would transport particles from as far as 60 μm. The concept of "retrograde axolemmal transport" provides an explanation for the observed movements of the Con A-RBC as well as supporting Pfenninger and Bunge's (1974) proposal that proteins are inserted into the membrane at peripheral sites. The retrograde flow of membrane proteins may reflect a mechanism by which axolemmal constituents are renewed in the cell body. New protein is added peripherally and is circulated back to the soma where it is turned over. If this is the case then it would be expected that in periods of rapid growth, turnover and consequently retrograde axolemmal flow, would be diminished.

To the extent that studies on growing neurites can be compared with other motile cell types, e.g., fibroblasts, it is of interest that Warren and Glick (1968) could find no difference in the synthesis of new membrane components in growing and non-growing cell populations. The growing cell has a net increase in the amount of membrane present which is due to regulation of other steps in the metabolism of membrane components i.e., there is a decreased turnover of existing membrane. The new membrane is added at the advancing edge of the cell,

moved against the direction of motion over the cell surface, and is reabsorbed in the perinuclear region (Harris and Dunn, 1972). An analogous system may exist in the neurite; as Bray (1973 a, b) points out, there is a net accretion of membrane associated with neuritic outgrowth and vesicular flux in the growth cone.

The growth cone is principally a locomotory organelle. The mechanisms by which it moves has received detailed theoretical treatment, initially by Yamada et al. (1971) and later by Bray (1973 b). It is filled with a filamentous matrix of microfilaments. The organization of the fibrous proteins in the filopodia and the growth cone is apparent in electron micrographs (Yamada et al., 1971). These show polygonal structures which are interconnected forming a dense lattice, and which insert upon the plasma membrane. The polygonal structures are composed of ordered arrays of microfilaments (Yamada et al., 1971). The microfilaments are believed to be contractile elements and chemically similar to muscle actin. Although direct proof has been lacking, inferential evidence is convincing (reviewed by Pollard and Weihing, 1974). Sanger (1975) has recently demonstrated that fluorescein-labeled meromyosin will specifically bind structures in the neuronal growth cone. The distribution of the meromyosin binding parallels the distribution of microfilaments in this structure, as well as a variety of other cell types. This homology is important as the attendant theory is dependent upon its validity.

Yamada et al.(1971) speculated that the fibrous lattice was responsible for the movements of the growth cone, without being more

specific. Bray (1973 b) described a model to account for the movement of the growth cone. The principle feature of his hypothesis is that contractile proteins underlie and are connected to the plasmalemma in an oriented manner, similar to that existing in muscle. Filopodia and other similar membranous extensions of the plasma membrane are about 150 nm in outside diameter. If the thickness of the membrane and the constituents of the filopodia are deducted from that number, a space of approximately 50 nm exists. This 50 nm space is approximately that required for opposed layers of actin and myosin, with one protein present on opposing surfaces of the filopodia and the other protein occupying the central cavity. New membrane is added by the contractile movements forcing vesicles present at the base of the growth cone to the tip of the filopodia where it merges with the plasma membrane.

The movement of an oriented membrane over a constrained area results in the behavior observed in Figure 1. There are two types of discontinuities, a point at which all the "arrows" converge, and another where they diverge. The physical correlate of convergence of arrows results in deformation of the membrane and the formation of vesicles. In the opposite case, the divergence of the arrows results in the formation of filopodia. The arrangement described here would result in a dynamic system. To obtain extension of a growth cone there must be an endogenous source of vesicular material to incorporate into the plasmalemma. These vesicles are provided by a transport system from the cell body. The theory, as it is set forth, has no serious flaws and adequately accounts for the present evidence.

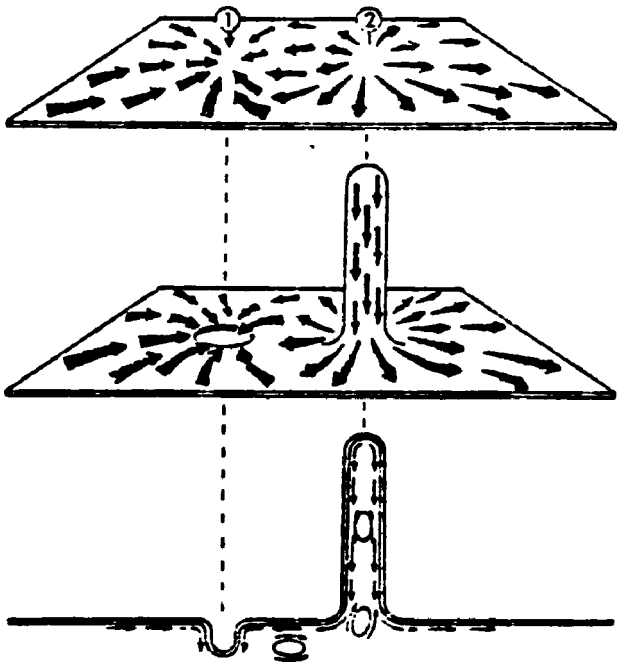


Figure 1 Model for membrane movements in the growth cone The growth cone membrane is postulated to have contractile proteins positioned in an oriented manner on its interior surface. The movement of the membranes by contractile activity results in two types of discontinuities, one where all the arrows converge (1) and where they diverge (2). This causes deformations of the membrane resulting in the formation of vesicles and of filopodia respectively (from Bray, 1973 b).

Interactions and Guidance of Neuronal Processes

The formation of specific nerve connections underlie the entire concept of nervous system organization. The mechanisms by which this may occur have been examined in some detail (reviewed by Jacobson, 1970; Gaze, 1970). Until recently the experimental evidence has centered around the rules which govern the formation of specific connections without dealing with the mechanisms by which they are established. Current concepts suggest that there is a point-to-point specificity in connectivity between two related structures. The "absolute" nature of this specificity is not the main point, but rather that it is possible to somehow interconnect two nervous structures with precision. For example, the formation of specific nerve connections may be concerned with the interaction of individual fibers with one another, the cellular environment and with target cells.

Various mechanisms have been postulated to account for the guidance of neuronal processes. Weiss (1934, 1955) has suggested that contact guidance by an oriented substratum could play a role. There is some evidence for this. Weiss' "two center effect" is an example (1934). In culture, neurites were found to follow aligned fibrin filaments present in plasma clots or aligned collagen substrata (Weiss, 1934; Dunn, 1971; Ebendal, 1976). Recently Ebendal (1977) has shown that in the developing nervous system cell migration and neurite extension are affected by contacts with an oriented extracellular matrix.

Chemotrophic factors have also been suggested to explain directed axonal growth (Weiss, 1955). This theory supposes that there are gradients of diffusable substances to which the neurites will preferentially respond, resulting in directed axonal growth. A modification of this theory has been advanced by Ebendal (1977). He postulates that the diffusable substances become bound to the extracellular matrix in a patterned fashion. In this manner the neurite could be contact guided while at the same time respond to chemotrophic gradients.

Cells in tissue culture are known to secrete proteins, principally as glycoproteins, into the environment (Truding et al., 1975; Lott et al., 1972). Crick (1970) has suggested that the diffusion of these or similar substances in vivo may be responsible for certain events occurring during development. Moreover, Hay (1968) has proposed that inductive phenomena in development may be the result of interactions of glycosyltransferases present on the surface of cells with carbohydrate residues bound to the extracellular matrix. These data, taken together, make Ebendal's hypothesis an attractive one, particularly in view of current theories of neuronal specificity involving specific interactions between surface glycosyltransferases and carbohydrate residues on interacting cells (Shur and Roth, 1975).

Fiber-fiber interactions, despite their obvious importance, have received little attention. The courses of action open to a single nerve fiber in its encounters with other fibers are limited. It can be attracted to another fiber, resulting in the neurites growing

together forming a fascicle; be repelled, resulting in a change in the direction of growth or stopping altogether; or be unaffected, with the fibers crossing one another without influencing each other (Nakajima, 1965).

The contact of an active growth cone with another growth cone or fiber can result in an association of the two (Nakijima, 1965; Nakai, 1960). The selective association of nerve fibers was described in vitro by early investigators (Harrison, 1910; Lewis and Lewis, 1912) and by Speidel (1933) in the living tadpole. The mechanism by which fibers associated into bundles, or fascicles, has been studied in some detail by Nakai (1960). When a growth cone approaches another growth cone or fiber the advancing filipodia touch and attach to the surface. This contact appears to stimulate the formation of "lateral filopodia" which extend from the body of the fiber, often well behind the growth cone. The interactions of the growth cone with the fiber are illustrated in Figure 2 (from Nakai, 1960). The filopodia draw the two fibers together along their length until they adhere. A similar phenomena has been reported to occur in myelinated fibers, with glial cell processes being responsible for the association of fibers into a single fascicle (Deboni et al., 1976).

The environment in which fibers grow, strongly influences their tendency to fasiculate (Weiss, 1955; Dunn, 1971). The ability of neurites to adhere to the substratum on which they are growing together with physical constraints of the substratum affect association of fibers with one another, and the direction in which they grow (Weiss, 1934).



Figure 2 Illustration of nerve fibers forming a fascicle (from Nakai, 1960)

In a highly structured substratum such as a plasma clot or a collagen gel, neurites tend to exist as single fibers, while fibers growing on plastic or glass are highly fasciculated. These observations will be discussed in some detail below.

Neurites growing in culture can often be observed to approach one another then rapidly retract their growth cones, either moving off in another direction or stopping their growth (Nakai, 1959, 1960; Nakijima, 1965). This process has been thought to be analogous to the contact inhibition of growth observed in other cell types (Steinberg, 1973). Dunn (1971) has employed this idea of contact inhibition of neurite extension to explain the radially directed growth of NGF stimulated peripheral ganglia in culture. He suggests that Weiss' theory of contact guidance by oriented environmental structures does not play a substantive role in nerve growth. This conclusion is disputed with recent data suggesting that both contact guidance and contact inhibition are operating in this particular system (Ebendal, 1976, 1977). The relevance of these observations to the situation in vivo is speculative.

We assume that the growing neurite has, in a teleological sense, the goal of forming a synaptic relationship with a target cell. The growth cone is both a chemical and tactile probe of the environment and as such the components which confer specificity must reside in this structure. There are a couple of lines of evidence which may shed light on this function of the growth cone; the demonstration that growth cones from different areas of the nervous system have

dissimilar surface membrane properties (Pfenninger and Pfenninger, 1976), and the observation of specialized regions of the growth cone which may represent the preliminary steps in the formation of a synapse (Vaughn et al., 1976). The growth cone membrane, has only recently received detailed attention, largely as a result of the development of specific membrane probes.

Pfenninger (1976) has used several lectins, specific for different carbohydrates, to examine the growth cone membrane. Neurites derived from explants of various regions of the nervous system were found to have different binding capacities for a given lectin. Moreover, neurites bound the different lectins in characteristic proportions, indicating that the distribution of carbohydrate residues may be distinctive for restricted classes of neurons. This observation is particularly relevant to current theories of neuronal specificity which postulate specific interactions between glycosyltransferases and available carbohydrate residues on interacting membrane surfaces (Roth, 1976).

In a separate study, Wessells (1976) has shown that there is an assymetric distribution of anionic binding sites in the growth cone membrane. The cationic protein, ferritin was found to bind to all regions of the growth cone except at the advancing edge. These studies indicate that the growth cone membrane is regionally specialized, both with respect to the molecular composition of the individual growth cones and between different populations of CNS neurons.

The morphological events which are involved in synapse formation suggest that the growth cone plays a central role in these phenomena. In vivo, the growth cones of embryonic spinal cord are described as having membrane specializations during the period at which synapse formation is known to occur (Vaughn et al., 1976). These electron-dense specializations are restricted to the advancing filopodia. Structural modifications of the growth cone membrane are thought to represent the preliminary steps in synapse formation, or a low affinity phase allowing the neurite to "sample" prospective targets. Membrane specializations have not been reported to occur during synaptogenesis in sympathetic neurons in culture (Rees et al., 1976). Rees et al. (1976) have reported the detailed events that characterize formation of the synapse. The growth cone was observed to become closely apposed to a target neuron soon after its initial contact. Both the growth cone and the post-synaptic cell underwent a series of changes resulting in the formation of pre- and post-synaptic densities and accumulation of synaptic vesicles as the synapse matured.

This review of the literature has attempted to outline the existing data on the growth and regeneration of nerve fibers and to emphasize the contribution of tissue culture techniques. The remainder of this thesis will describe the development of suitable conditions for tissue culture of the adult goldfish retina, and the use of this tissue as a model for the study of central nervous system regeneration. For the visual system, it permits unimpaired access to the growth cone, the presumptive site of both growth and recognition.

CHAPTER II

EXPLANT CULTURE OF ADULT GOLDFISH RETINA

INTRODUCTION

The goldfish visual system has been used in a variety of neurobiological studies. Like other poikilotherms, the goldfish can regenerate its optic nerve following section. The retinal ganglion cell axons, forming the optic nerve, will regrow to form connections to the optic tectum. While experimental manipulation of various parts of the visual system has allowed the elucidation of the rules by which nerve connections are formed, they have not shed much light on the mechanisms which subserve these connections. The goldfish visual system has been characterized with respect to its electrophysiology (Jacobson and Gaze, 1964), anatomy (Leghissa, 1955; Sharma, 1972; Springer and Landreth, 1977) and biochemistry (Grafstein, 1967; Elam and Agranoff, 1971a, 1971b; Neale et al., 1974).

The poikilotherm retina was among the first tissues to be maintained *in vitro*. Champy (1913, 1914) observed histological changes in the tortoise retina after several days in culture. There was no mention of neuritic outgrowth from this tissue. Vinnikov (1946) reported extensive neuritic outgrowth from explant cultures of carp and axolotl. This preliminary report constitutes the only known example of culture of fish retina prior to the work reported here. Speidel (1953) briefly reported on the explant culture of anuran retina, indicating

only that neuritic outgrowth was observed. This approach was not pursued further until the report of Agranoff, Field and Gaze (1976) on explant culture of embryonic Xenopus retina. Xenopus retina survived in culture for several weeks, issuing neurites within a few days of explantation. The neuritic growth was facilitated by crush of the optic nerve several days prior to explantation. In this preparation neurites appeared almost immediately and grew more vigorously than in control cultures in which the optic nerve had not been previously cut. The work with Xenopus retina thus forms the basis for much of the work reported here.

The goals of the present study were to develop satisfactory methods for the preparation of retinal explants from adult goldfish, to determine the optimal conditions for growth of this tissue in vitro and to characterize its growth under these conditions. The preparation offers unique opportunities for the study of regenerative mechanisms under controlled conditions, permitting experimental approaches not previously feasible. Goldfish retinal cultures offer the further advantage of permitting direct visualization of the growth of fibers whose behavior has been well studied in vivo, if not understood. The ultimate intent of these investigations is to develop a model system by which one will someday be able to study neuronal regeneration and recognition.

We report here that the goldfish retina in vitro is capable of undergoing morphological changes normally associated with optic nerve regeneration in vivo. The growth of the neuronal processes has been examined visually and has permitted documentation of the observation

that when the optic nerve is crushed prior to explantation the neuritic growth issuing from the retina is greatly enhanced compared to unoperated retina.

MATERIALS AND METHODS

Common goldfish, Carassius auratus, 6-7 cm in body length, weighing 7-11 g, were obtained from Ozark Fisheries (Stoutland, MO). Fish were maintained in constant light at 21 $\pm$ 1°.

Fetal calf serum (FCS) was from Flow Labs. HEPES (N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid), gentamicin sulfate, poly-DL-ornithine (Type I-B, 15-30,000 MW), and poly-L-lysine (Type VI-B, 15,000 MW) were purchased from Sigma. Inositol was from Nutritional Biochemicals. The synthetic tripeptide L-glycyl-histidyl-lysine was obtained from U.S. Biochemical Corporation. Difco supplied Noble agar. Gelatin was obtained either from Polyscience (purified) or Difco (Bacto). Finquel (MS222), tricane methanesulfonate) was from Ayerst Laboratories.

Thymidine (methyl-^{3}H, 20 Ci/mmole) was purchased from New England Nuclear.

Plastic tissue culture dishes (30 mm) were obtained from Falcon (#3001) and Nunc (Nunclon, Vanguard, Inc.).

The nutrient medium, L-15 (with added glutamine, GIBCO or Flow Labs), was that of Leibovitz (1963). The term "L-15H" refers to L-15 supplemented with 9% fetal calf serum, 20 mM HEPES (pH 7.2) and 100 µg/ml gentamicin sulfate.

HEPES was made up as a 2 M stock solution and the pH adjusted to 7.2 with NaOH. The solution was sterilized either by Millipore filtration or by autoclaving.

Gentamicin stock (10 mg/ml) was filter sterilized and 5 ml aliquots were stored frozen.

To 100 ml of L-15 was added 10 ml of FCS, 1 ml of gentamicin sulfate stock solution (10 mg/ml) and 1 ml of 2 M HEPES. The pH of the resulting solution is slightly acid owing to the antibiotic. Four to six drops of sterile 1 N NaOH was added to bring the pH back to 7.2. L-15H was stored at 4°.

For further studies as indicated, a balanced salt solution was prepared according to Dunlop et al. (1975). The individual ion concentrations were: Na^{+}, 120 mM; K^{+}, 8 mM; Mg^{+2}, 1.3 mM; Ca^{+2}, 2.6 mM; Cl^{-}, 118 mM; SO_4^{-2}, 1.3 mM; PO_4^{-3}, 1.2 mM; Glucose, 12 mM; HEPES, 25 mM. Sterilization was accomplished by Millipore filtration.

Phosphate buffered saline (PBS, pH 7.2) was prepared according to Dulbecco (1954).

Retroorbital Optic Nerve Crush

Goldfish were anesthetized in Finquel. The extraocular muscles were cut with a pair of fine scissors over the postero-dorsal aspect of the eye. The eye was then pulled forward in the orbit exposing the optic nerve. The nerve was crushed with forceps leaving the surrounding dural sheath intact. Care was taken not to damage the artery which runs with the optic nerve. Fish that bled were immediately discarded.

Tectal Ablations

Half-tectal ablations were performed on goldfish previously anesthetized with Finquel. The cranium was opened along the sutures with a sharp scalpel and the bone flap reflected, exposing the brain. The right caudal tectum was directly aspirated following division of the tectum midway between the rostral and caudal poles with a pair of fine scissors. The area was flushed with saline and the bone flap replaced. Tissue adhesive (Ethicon) was applied at its edges.

Preparation of Fish Sera

Serum was obtained from goldfish by intracardiac puncture. A ventral incision was made from the gills to the anus. A heparinized 100 µl pipet was used to rupture the heart. Blood was aspirated and placed in a chilled test tube. The yield from individual fish was quite variable, and ranged from 25 to 200 µl. The blood was allowed to clot, then centrifuged at 2700 X g for 15 min at 4°. To obtain 1 ml of plasma required a minimum of 20 fish. The plasma was removed and sterilized by Millipore filtration.

Histology

Tissue was prepared for histological examination by fixation for a minimum of 48 h in 80% ethanol:formaldehyde:acetic acid (18:1:1) at room temperature. Following fixation the tissue was transfered to 70% ethanol and subsequently dehydrated through a graded ethanol series into butanol. The tissue was embedded in Paraplast and sectioned at 8 µm. Retinal explants were usually cut in cross section, although occasionally horizontal sections were taken. Goldfish eyes

were cut into radial sections. The tissue was stained with Ehrlich's hematoxyalin-eosin or cresyl violet.

Determination of Retinal Ganglion Cell Diameters

Retinal ganglion cells were identified as described in the text. The ganglion cells were measured parallel to the vitreal surface of the retina using an ocular micrometer on a Zeiss Photomicroscope. The final magnification at the ocular was 1562 X, using an 100X oil immension objective. A minimum of 200 measurements were made for each experimental point.

Radioautography

Tritiated thymidine was evaporated to dryness under N_2 and redissolved in L-15H. Each culture dish contained 0.8 µCi of ^{3}H-thymidine in 0.5 ml of nutrient medium. The labeled medium was added 20 h prior to fixation.

Radioautographic procedures were essentially those described by Kopriwa and Leblond (1962). Kodak NTB-2 nuclear track emulsion was diluted 1:1 with distilled water at 40°, and slides with sections were dipped and then dried for approximately 1 h. Slides were stored in light-tight boxes with 10-15 g of Drierite at 4° for 7-35 days. The exposed sections were developed in Kodak Dektol for 3 min at 16°, washed briefly in 1% acetic acid and fixed in Kodak fixer for 6 min.

Collagen Extraction

Collagen was extracted from rat tail tendons by the method of Ehrmann and Gey (1956). Tails from freshly killed rats were frozen.

Prior to dissection, the tails were thawed, immersed in 70% ethanol and transferred to a sterile tray. After the skin was removed, the tails were fractured at successive joints starting at the tip. Individual segments were pulled away from the tail with their attached tendons which were then cut free and held in sterile water. The tendons were transferred through an additional change of sterile water, drained, and placed in a sterile bottle. Dilute acetic acid (0.03 M) was added to the tendons which were extracted at 4°. Approximately 1 g of tendons could be obtained from each tail. An optimal ratio of tendons appeared to be about 2 g of tendons per 150 ml of the dilute acetic acid. After 48 h the bottles were centrifuged at 2700 X g in an International centrifuge for 1 h at room temperature. This generally resulted in a clear liquid phase of approximately 40 ml over a cloudy gel phase. The clear solution was transferred by pipet to a sterile bottle and stored at 4°. A volume of the dilute acetic acid approximately equal to that removed was added to the unsolubilized collagen gel, the contents mixed, and extracted for an additional 24 h at 4°. This mixture was centrifuged again yielding 40-80 ml of clear liquid supernatant fraction. The residue was shaken and held in the cold without any further addition of acetic acid for another 24 h. This solution was centrifuged again, the liquid phase removed and the remainder discarded.

The resulting soluble collagen solution was transferred to sterile polycarbonate centrifuge tubes (30 ml) and centrifuged 1 h at 20,000 X g. The supernatant was carefully decanted into a sterile bottle and stored at 4°. The three fractions generally did not differ

significantly in protein concentration and could be pooled at this point. Collagen obtained under these conditions was normally sterile, and could be stored indefinitely at low pH and ionic strength.

The protein concentration of each fraction was determined by the method of Lowry (1951) using gelatin as a standard. Collagen solutions obtained by this method routinely contained 0.25-0.32% protein.

An aliquot of the collagen solution was dialyzed against water to remove excess acetic acid and other soluble contaminants. The most satisfactory preparations were obtained by dialysis at room temperature of 10 ml of the collagen preparation per liter of water for 24 h. This step is critical for obtaining solutions which will result in good gels. The viscosity of the collagen solution is pH-dependent, therefore increasing periods of dialysis results in progressively more viscous solutions, and conversely the addition of acetic acid results in less viscous solutions.

The method was not uniformly successful. Fresh (unfrozen) tails were unsatisfactory, and not all preparations yielded a clear liquid phase upon centrifugation.

Collagen Gels

Hydrated collagen gels were prepared essentially as described by Elsdale and Bard (1972). The collagen was precipitated to form gels by simultaneously raising the pH and ionic strength. This was accomplished by the addition of a small volume of alkaline electrolyte solution which also brought the collagen concentration to 0.20%. The diluant solution is sufficiently hypertonic that its addition to the soluble

collagen will make the final solution isotonic. For example, 3 ml of 0.25% soluble collagen requires the addition of 0.75 ml of liquid to adjust the protein concentration to 0.20%. The 0.75 ml which is to be added should contain 0.33 ml of 10X saline to adjust the tonicity and 0.42 ml of PBS to raise the pH. Since the protein concentration of different collagen extractions differs, an equilibration solution is prepared with the proper composition for each batch. A concentrated phenol red solution (1 mg/ml) is added to yield a final concentration of 0.01 mg/ml to monitor pH changes.

To form the collagen gels, an appropriate amount of collagen was transferred to a sterile test tube, the equilibration solution added, rapidly mixed and pipetted into 30 mm petri dishes. The collagen mixture (0.40-0.45 ml per dish) was rapidly spread over the bottom by gently tilting and shaking the dish. Since the collagen gels rapidly, usually no more than 6 dishes can be poured at a time. The dishes were allowed to set undisturbed overnight in a humid atmosphere. Prior to use, PBS was carefully added to the gels and allowed to equilibrate a minimum of 2 h. Excess PBS was pipetted off at least 1 h prior to explantation and the nutrient medium added.

Hydrated collagen gels prepared by this method are very fragile and care must be taken not to damage them.

Collagen Films

Collagen films were prepared as described by Bornstein (1958). Collagen in dilute acetic acid from rat tail tendons was diluted to a

final protein concentration of 0.1% with acetic acid (1.5 mM). Phenol red was added to a final concentration of 0.01 mg/ml by addition of an appropriate volume of a 1 mg/ml stock solution. The resulting collagen solution was pipetted into a petri dish, spread over the surface, allowed to stand a few seconds, then drained and the excess collagen removed. The dish was placed in a chamber containing a jar of 1N ammonium hydroxide. The ammonia vapor causes the pH of the collagen to rise, leading to the formation of collagen fibrils. The reaction was monitored by the change in the indicator color. When the pH was 8.5-9.0 (or after approximately 30 sec), the dish was removed and held in a humid chamber overnight. The collagen films are washed with 3 successive changes of sterile water, followed by 3 washes of PBS. Nutrient medium was added to the films at least 1 hour prior to explantation.

The films are mechanically stable and require no special handling.

Polycation-Treated Substrates

Poly-DL-ornithine (PORN) and Poly-L-lysine (PLYS) were prepared as sterile aqueous solutions at a concentration of 20 mg/ml. Solutions for the treatment of substrate surfaces were prepared by dilution of the concentrate to 1 mg/ml in sterile 0.1 M borate buffer, pH 8.4 (Letourneau, 1975a). Both PORN and PLYS gave similar results, however, most of the experiments reported here employed PLYS, as it was the most economical.

Plastic tissue culture dishes were treated by adding 1-2 ml of the polycation-borate solution to each dish. After 24 h at room

temperature the solution was removed and the dishes were washed with 3 successive changes of water followed by 3 changes of PBS (30 min each). Nutrient medium was added after the last PBS wash. Falcon and Nunc tissue culture dishes were used with similar results.

Glass coverslips (Corning, No. 1 1/2) were washed in chromic acid and rinsed for 24 h prior to use in distilled water. The coverslip was either placed in a petri dish or attached to a specially constructed dish as described below. Treatment with polycation-borate solution was as described for the treatment of plastic surfaces.

Interference contrast microscopy requires optically flat surfaces so special dishes were constructed for higher resolution work (Deitch et al., 1973). A 30 mm plastic petri dish had a 15-20 mm hole punched through the bottom. A 22 mm^2 coverslip was placed over the opening and sealed with silicone sealer (Dow-Corning), which was allowed to polymerize overnight. The dish was washed with 3 changes of PBS over a 24 h period, sterilized with ethanol, and the glass was then treated with polycation-borate solution as described for plastic surfaces.

Agar

Agar plates were prepared by dissolving Noble Agar in water and autoclaving the solution for 30 min. While the solution was still hot, 0.6 ml was pipetted into 30 mm plastic petri dishes and allowed to cool. Agar plates were prepared using either a 1% or 2% solution. The dishes were held overnight in humid chambers, then washed with 3 changes of PBS, prior to the addition of nutrient medium.

Gelatin

Gelatin was obtained from either Difco or Polyscience. The Polyscience product was found to result in better growth and was used for most of the studies described here. Gelatin solutions were prepared as 2% or 4% solutions by adding gelatin powder to hot (80-90°) water on a heated stir-plate. The resultant clear solution was poured into a sterile bottle and allowed to gel. Gelatin coated petri dishes were prepared by heating the gelatin until it melted and pipetting 0.4-0.6 ml into a 30 mm plastic petri dish. These dishes could be prepared in advance and stored in humid chambers, following the addition of 2 ml of PBS. These dishes were washed with 3 changes of PBS. Nutrient medium was added at least 1 h before explantation.

Preparation of Retinal Explants from Goldfish Retina

Goldfish whose right optic nerve had been crushed, were dark-adapted for 30-60 min to facilitate separation of the retina from the underlying pigmented epithelium. In the dark-adapted retina the apical processes of the pigmented epithelial cells,which normally envelop the photoreceptor outer segments, are retracted. All subsequent procedures were carried out in a darkened room. The dissection was performed in a dust-free enclosed hood under a Bausch-Lomb dissecting microscope fitted with a narrow beam focused light source. If several eyes were being processed together, care was taken to keep them out of the direct light of the lamp. Normally up to 10 retinas can be processed at once.

The fish were killed by decapitation and both eyes removed. To free the eye, the extraocular muscles were cut and the eye pulled forward in the orbit, exposing the optic nerve. The eye was removed by grasping the nerve with a pair of forceps and pulling the globe free. The eye was transferred to a dish containing PBS and held there until the eyes from several fish had been obtained.

The eyes were decontaminated by immersion in 70% ethanol for 30–45 sec. They were then passed through two changes of sterile PBS to remove any residual alcohol and into a dissecting dish also containing PBS (Figure 3).

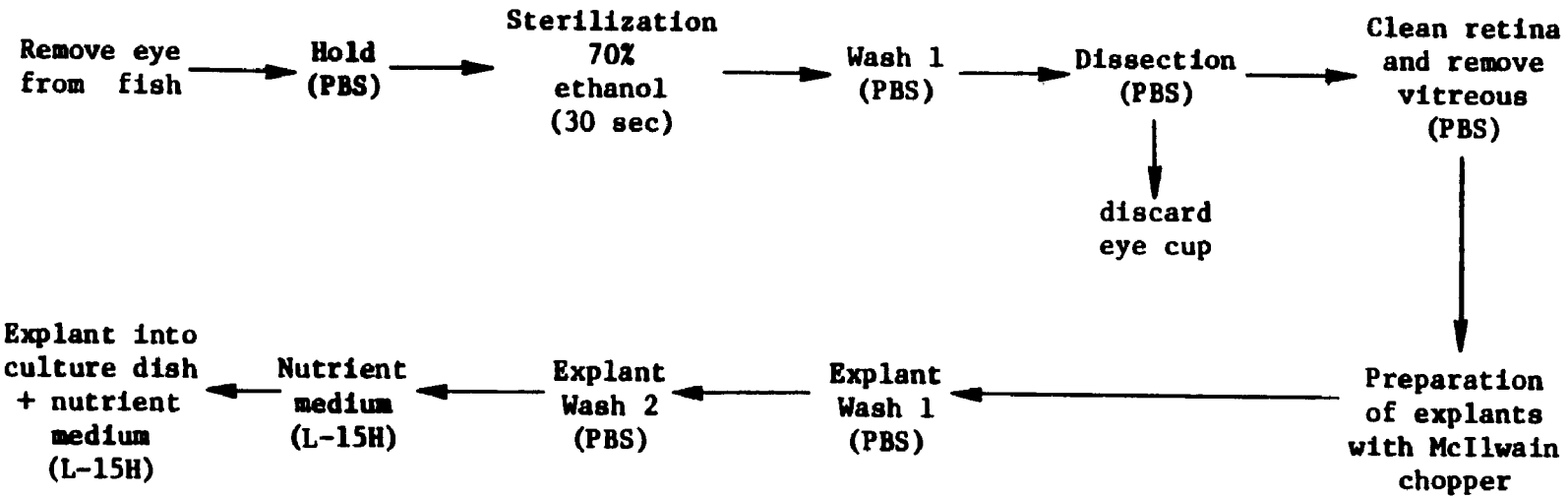
The eye was opened by piercing the cornea with the tip of a pair of sharp forceps. The entire front of the eye, including the anterior chamber and lens was carefully cut away. If the dissection was properly done, the retina should be free at the ciliary margin, though often it remained attached at several points. The retina was removed by inserting the forceps between the retina and the pigmented epithelium and gently teasing them away from one another. Once the retina had been freed, the optic nerve was grasped from the under side of the retina and pulled away from the eye cup.

The retinas were dissected with a variable amount of vitreous humor attached. This material was remarkably difficult to remove. The retinas were transferred to a fresh dish of PBS and an attempt was made to detach as much vitreous as possible with forceps.

In initial experiments the retina was next minced with scissors into pieces about 1 mm^2. This procedure was time-consuming and the yield of fragments suitable for explanting was low. A more satisfactory

Figure 3

Procedure for preparing retinal explant cultures



procedure was subsequently developed, employing a McIlwain tissue chopper (Brinkman). The chopper surface and blade are sterilized with ethanol followed by irradition from a ultraviolet lamp for 10 min. The intact retinas are placed on a sterile polyethylene disc which is mounted on the chopper stage and serves as a cutting surface. The slice thickness is set at 600 μm and the retina sliced. The stage is then rotated 90° and the process repeated. The resulting square pieces are removed from the stage with a pipet and transferred into fresh PBS. The retinal explants are transferred through two changes of PBS to remove debris and finally into nutrient medium. The explants are held in nutrient medium until they can be placed in culture dishes.

The explants are transferred into tissue culture dishes that have been equilibrated with nutrient medium. The explants are sucked into a narrow bore pasteur pipet which is connected to a plugged mouthpiece. Some care is taken in the selection of individual explants. Only those with clean edges and of the proper size are chosen. The tissue is deposited, without touching the pipet tip, onto the substratum. An important factor in the successful growth of these cultures is the amount of medium remaining in this dish. If there is too little the explants dehydrate, while if there is too much they fail to attach and float free in the medium. Each 30 mm culture dish normally contains 16-20 explants. The explant cultures are stored in humid plastic chambers containing moist sponges at room temperature. If the cultures are not properly humidified, they rapidly become hypertonic.

The success of the technique described here is principally due to the use of the McIlwain tissue chopper. This device was employed

earlier in the preparation of cardiac cultures (Paul, 1970) but has not been used extensively for this purpose since. The use of the tissue chopper resulted in uniform pieces of tissue with minimal damage. The high yields of explants of uniform size added a degree of precision which would not have been otherwise possible.

Quantitative Analysis of Nerve Growth

A formidable problem in these studies was that of establishing a method by which the growth of explant cultures could be measured. Two methods were used. In the initial phase of this study neuritic and non-neuritic growth was recorded as simply being either present or absent. Explants with growth which could not be identified with certainty were given a tentative score. On the following day, if the questionable growth persisted, it was confirmed and the tentative score changed to a positive one and counted in the appropriate column for the previous day. Data were expressed as the percentage of explants showing outgrowth. This procedure suffered from the obvious disadvantage of not accurately reflecting the amount of growth present. An explant with a single neurite was scored the same as one with extensive neuritic outgrowth.

While the method was useful it was clear that a more quantitative measure of neuritic growth was required. A procedure was developed for measuring two aspects of the neuritic growth, fiber density and fiber length, from which a neuritic growth index (NGI) was calculated, similar to that described by Mizell and Bamburg (1976). Neurite density was scored on a scale of 0-4; a score of 0 indicating that no

neurites were present at the edge of the explant, while a score of 4 was given for explants with very high fiber densities.

The length of the fibers were initially estimated relative to the known explant width (600 μm on a side). It was, however, more convenient to use an ocular micrometer which measured 530 μm under standard observation conditions (10 X objective, 12.5 X ocular) to measure fiber length. A zone of outgrowth was thus defined as 530 μm. A score of 0.5 was given to explants with an average fiber length of up to one half of a standard zone or 265 μm; a score of 1, 265-530 μm; 2, 530-1060 μm, etc.

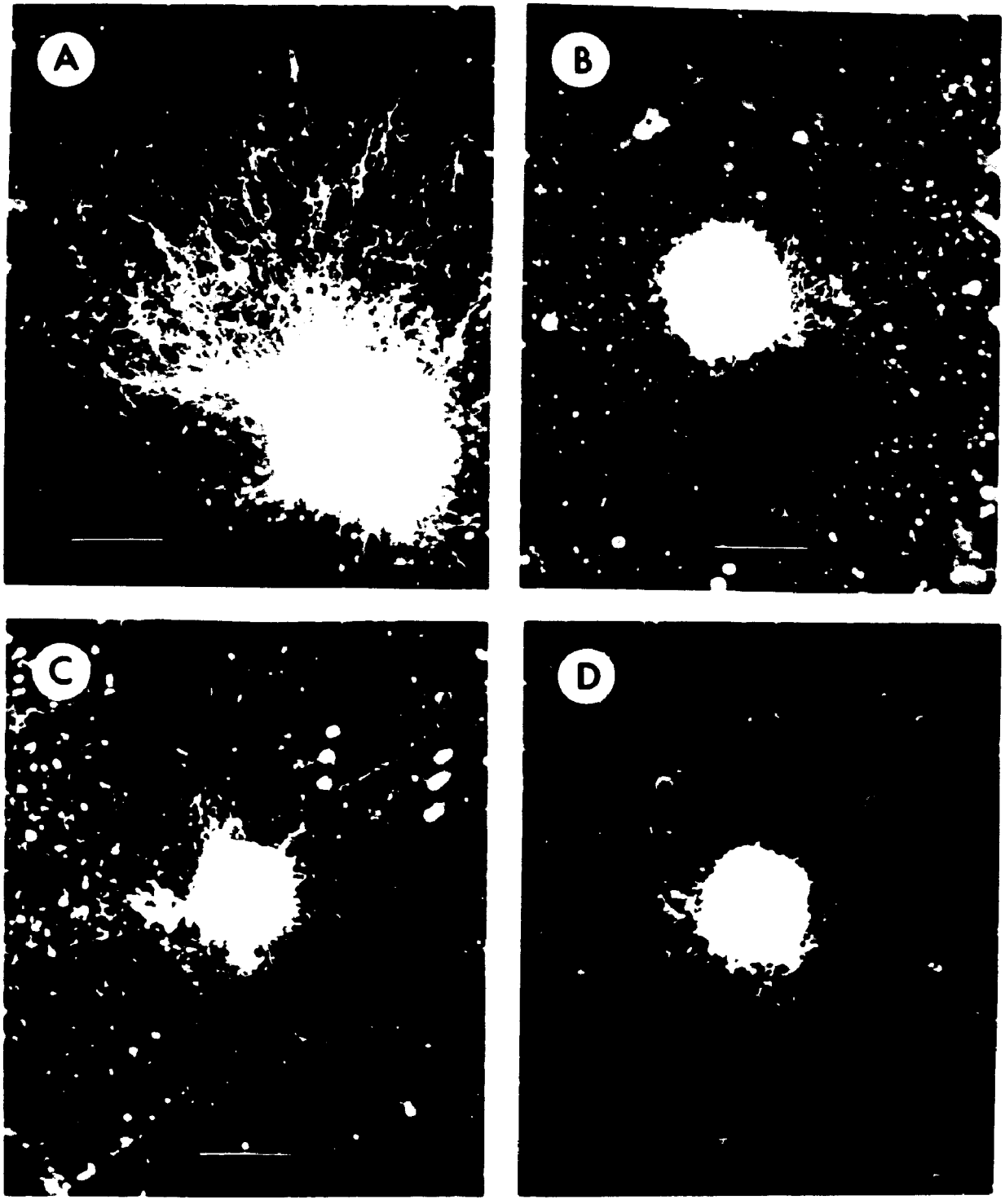
To obtain the NGI for each explant the density score was multiplied by the length score. Individual scores from approximately 50 explants were averaged to obtain the NGI for each experimental group.

In order to facilitate the scoring of fiber densities from individual explants, an atlas was assembled with photographs of explants illustrating growth typical of each density score (Figures 4 and 5).

The scoring procedure was modified somewhat to correct for the different character of growth on polycation-treated plastic compared with collagen. Explants grown on collagen tend to have neurites present as single fibers or in small fascicles, while those grown on polycation-treated plastic are in large fascicles that normally extend longer distances.

Figure 4 Neuritic growth densities on collagen gels
Darkfield micrographs of explants with growth typical of each density score. These are representative examples from an atlas compiled for comparison of neuritic densities in calculating the NGI. Neuritic growth with densities similar to A were assigned a score of 4; B, 3; C, 2 and D, 1. Bar is 500 μm.

Figure 4



The variability in determining the NGI was evaluated by comparing the scores following multiple observations on the same day by a single observer, or by comparing scores obtained by several observers (Table I). Replicate NGI determinations by a single observer were quite close, the scores differing from one another by less than 8%. The scores from multiple observers reflect 20-25% variations in the NGI determination between individuals. In all experiments reported here the neuritic growth index was determined from a single observation by the same observer.

Since the growth on non-neuronal cells was not the principal interest in these studies, the growth was evaluated by a simple 0-4 grading system. A score of 0 was given to explants with no non-neuritic growth visible at the edge of the explant. Non-neuronal cells proliferated after several days in culture, often forming a monolayer extending 1-2 zones from the explant itself. In such cases the explants were given a score of 4.

Figure 5 Neuritic growth densities on polycation-treated plastic substrata

Darkfield micrographs of explants with growth typical of each density score. These are representative examples from an atlas compiled for comparison of neuritic densities in calculating the NGI. Neuritic growth with densities similar to A were assigned a score of 4; B, 3; C, 2 and D, 1. Bar is 500 μm.

Figure 5

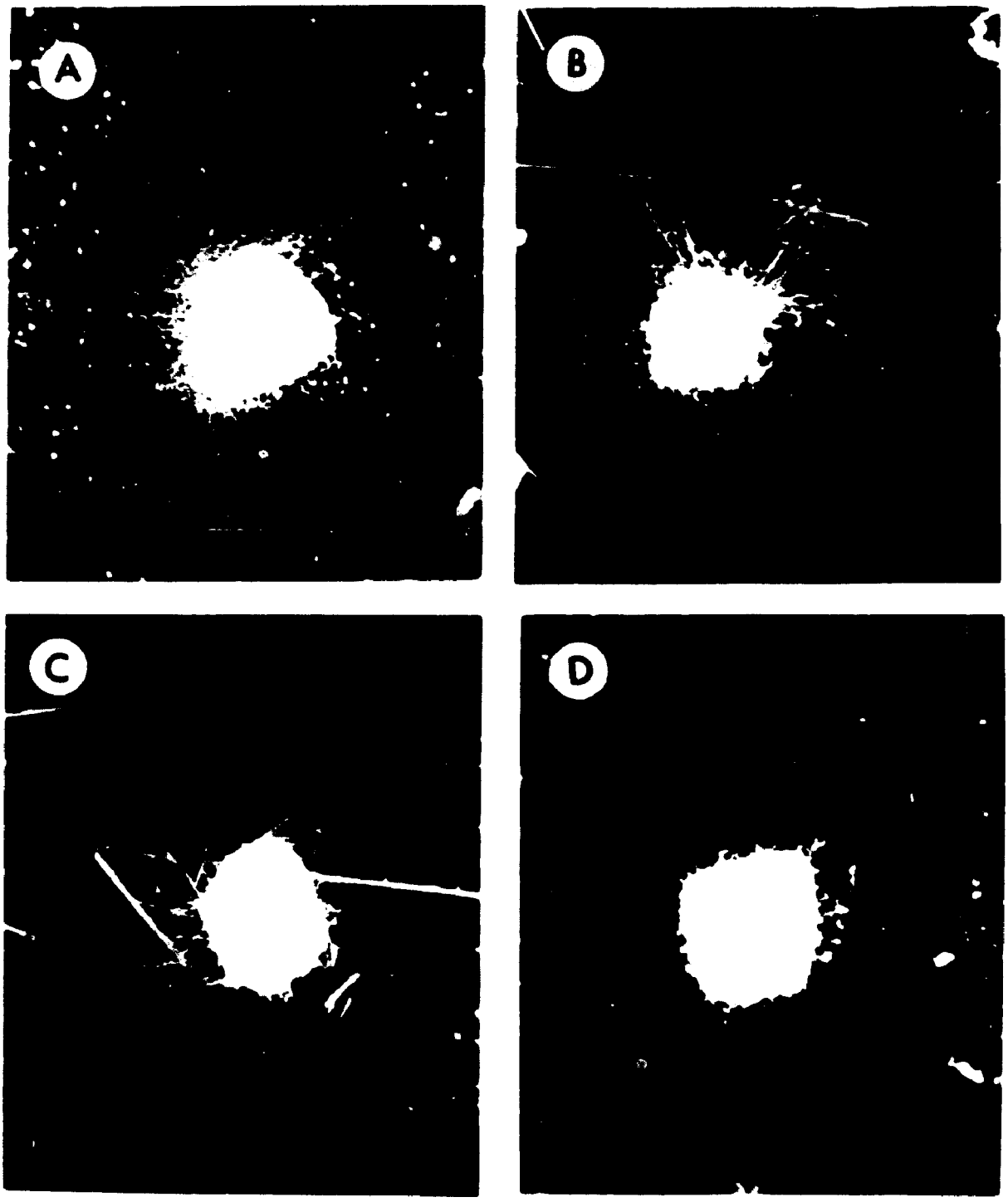


TABLE I

REPRODUCIBILITY OF NGI DETERMINATIONS WITH MULTIPLE OBSERVATIONS, MULTIPLE OBSERVERS

Neuritic Growth Index

Experiment	Observers				
		G.L.		A.H.	E.F.
109 (n=63)	3.77	4.23	4.10		
112 (n=32)	5.81	5.55	5.90	6.54	6.97
116 (n=52)	7.15			5.52	6.82

Explant cultures from post-crush retina were examined after 5 days in culture. The Neuritic Growth Index was calculated by averaging individual explant scores.

PART I

Prior Optic Nerve Crush Facilitates Neuritic Outgrowth from Retinal Explant Cultures

The ability of the poikilotherm to regenerate its optic nerve has provided an experimental model for central nervous system regeneration, as reviewed in the previous chapter. It was recently demonstrated that neuritic outgrowth from explant cultures of embryonic Xenopus retina is facilitated if the optic nerve is cut in situ some time before explantation of the retina (Agranoff et al., 1976). In the goldfish, a similar phenomenon is observed (Landreth and Agranoff, 1976).

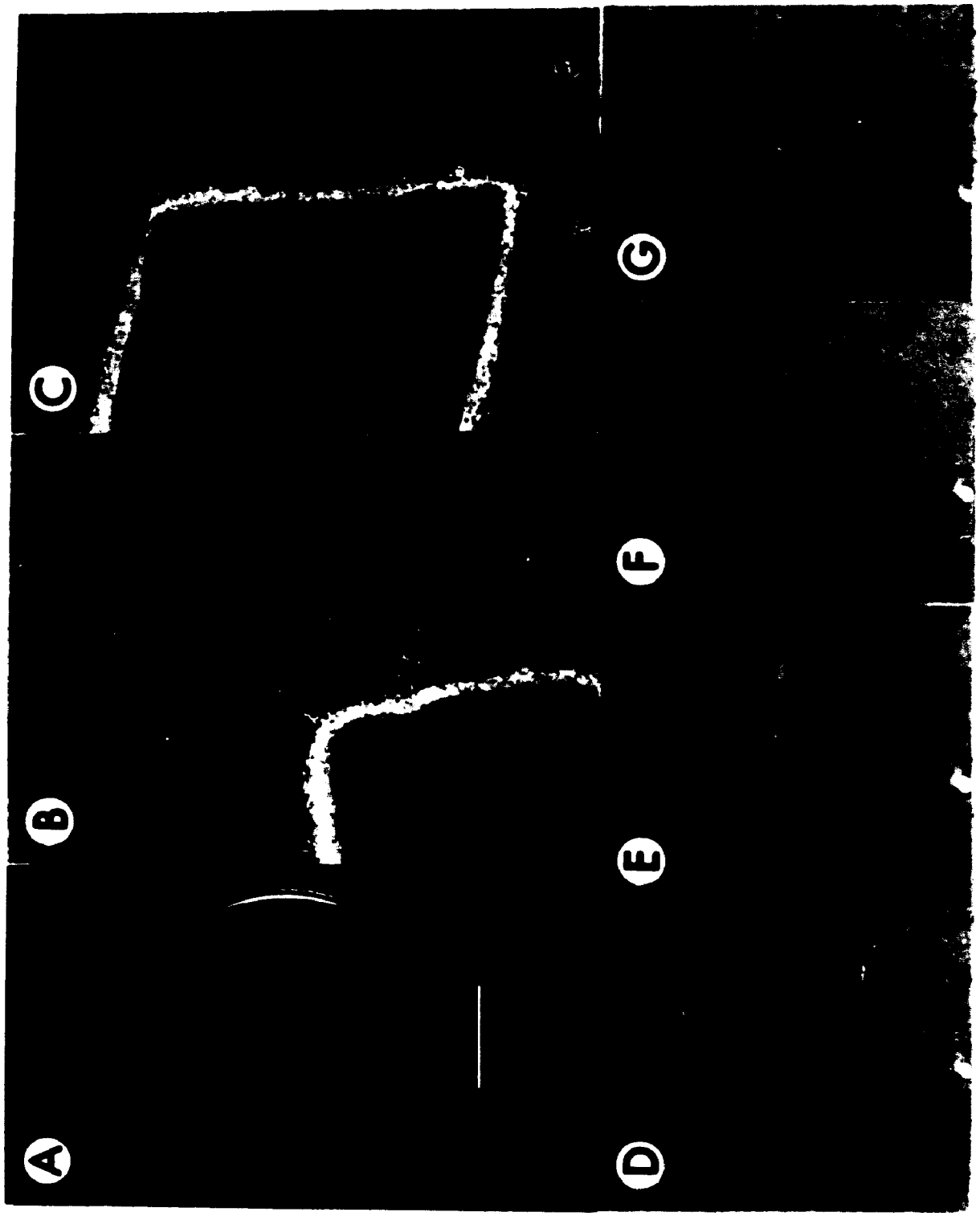
The culture conditions described here permit the maintenance and growth of adult goldfish retinal explants for several weeks. Explants were prepared and grown on hydrated collagen gels with L-15H as the nutrient medium.

Retinas were obtained from fish whose right optic nerve had been crushed 10-14 days prior to explantation. Explants from the opposite (unoperated) eye were used as controls. To avoid confusion, explants from the unoperated left eye will be referred to as unoperated or control retina. Retina from untreated normal fish will be called normal.

There was a striking difference in the growth of control and post-crush retinal cultures. The latter exhibited a vigorous neuritic outgrowth beginning as early as a few hours after explantation (Figure 6b). In explants of control retina, there was a delayed appearance of neurites which were first seen after 2-4 days in culture (Figure 6c).

Figure 6 Phase micrographs of goldfish retinal explants
A: plastic culture dish containing retinal explants prepared with McIlwain tissue chopper, on underlying collagen substratum. Bar = 10 mm. B: phase contrast micrograph of post-crush retina after 3 days in culture. Bar = 100 μm. C: as B, but from control (contralateral, unoperated) retina. D–G: phase contrast micrographs of growth cone of an advancing neurite from post-crush retina after 4 days of explantation, taken at time 0, 30 min, 35 min and 120 min, respectively. Bar = 30 μm.

Figure 6



The fraction of explants from post-crush retina with neuritic growth increased dramatically during the first few days in culture, reaching nearly 90% by 7 days (Figure 7). The development of neuritic growth from control retina was much slower. The fraction of explants exhibiting neurites from the unoperated tissue was always less than that from post-crush retinal explants.

The use of the fraction of explants with neurites as a measure of growth proved insensitive relative to the neuritic growth index (NGI), which allowed more quantitative evaluations to be made. The difference in NGI between the two kinds of explants appeared to be a more valid measure of the large difference in the amount of neuritic outgrowth (Figure 8). The post-crush retinal cultures continued to show increasing NGI values throughout the two-week culture period, while the fraction of explants which demonstrated neuritic outgrowth did not rise significantly after 4-5 days in culture. Post-crush retina had nearly six-fold more neuritic growth than comparable controls after two weeks in vitro using this measure of fiber growth. While the unoperated retina did show an increase in the fraction of explants with neurites over the two-week interval, the neuritic growth was neither as dense nor as extensive, resulting in a lower NGI.

Explants exhibited a non-neuritic, possibly fibroblastic, outgrowth after 2-3 days in culture. Non-neuronal cells migrated from the edge of the explant, extending processes with characteristic ruffled edges and flattening out onto the collagen surface. The non-neuritic outgrowth eventually formed a monolayer around the explant, however, there was considerable variation in the rate at which this outgrowth occurred.

Figure 7 Effect of prior optic nerve crush on the fraction of explants with neuritic outgrowth

Retinal explants were from fish whose optic nerve was crushed 10-14 days previously, or from the unoperated opposite eye (normal). The figure represents data obtained from six separate experiments ($\pm$ S.E.M.). Post-crush; ● (n=251). Control (unoperated) explants; ○ (n=242).

Figure 8 Effect of prior optic nerve crush on growth of neurites from retinal explants

Neuritic growth index was a measure of both the density and length of neurite growth from explants. Retinal explants from fish whose optic nerve was crushed 11-12 days prior to explantation. Post-crush; ● $\pm$ S.E.M. (n=104). Control (unoperated) retinal explants; ○ $\pm$ S.E.M. (n=96).

Figure 7

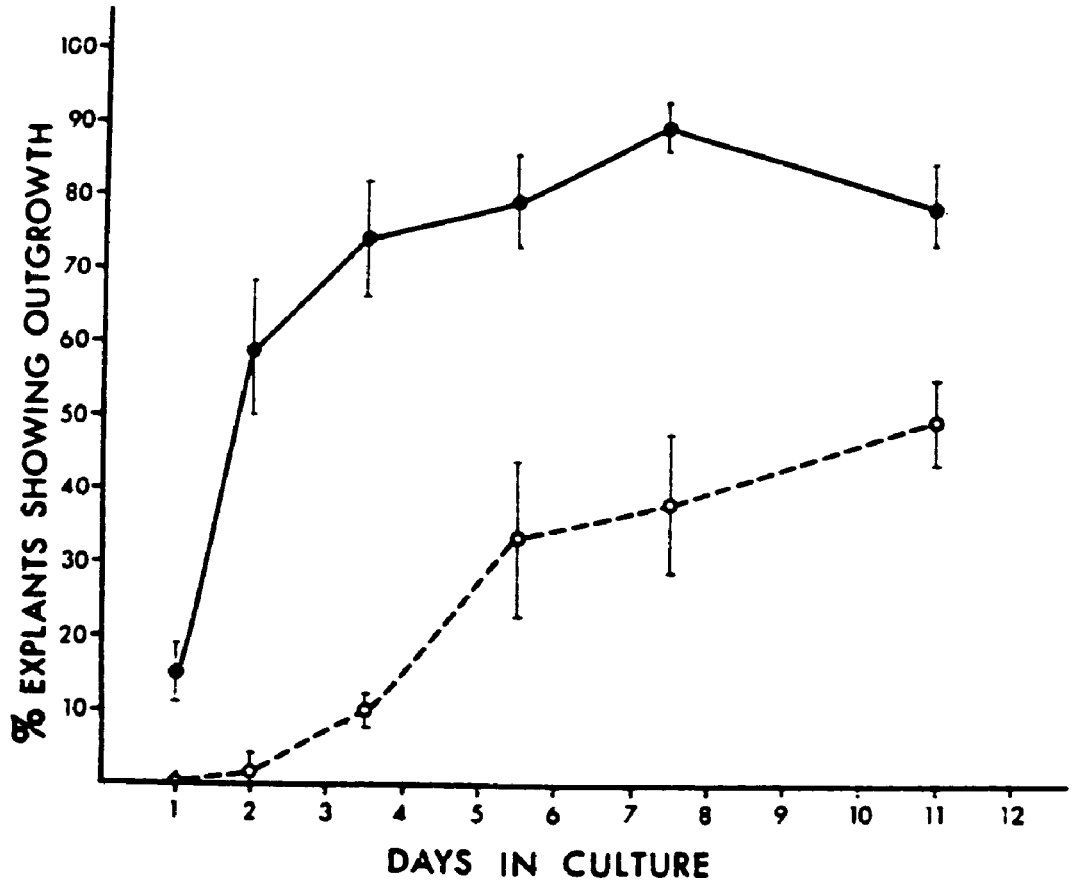
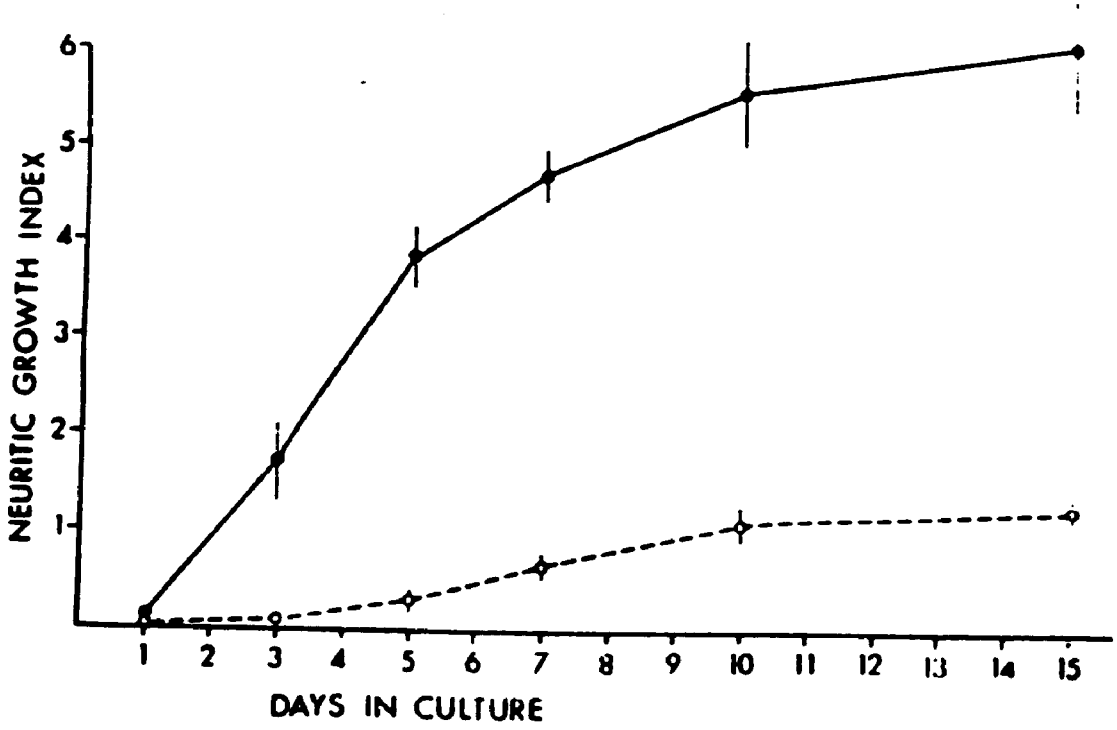


Figure 8



Pigmented epithelial cells, identified by prominent pigment granules, were occasionally observed. Explants from post-crush and control retina showed a small, but significant difference in the fraction of explants exhibiting non-neuritic growth (Figure 9). The more quantitative index for non-neuritic growth gave similar results (Figure 10). Although the difference between post-crush and unoperated cultures was small, control explants had uniformly lower scores. This may be attributed to the generally poorer growth of these explants compared to post-crush retina, as substantially higher fractions of control cultures failed to exhibit any neuritic or non-neuronal growth.

The facilitated outgrowth from retinal explants following optic nerve section in the goldfish strongly suggests that the retinal ganglion cells may be the elements responsible for the neuritic growth. This issue will be addressed in a following section. The observation that control retinas eventually develop neurites supports the hypothesis that outgrowth is a property of the retina, whose activation is stimulated by optic nerve crush and that the process requires several days to occur either *in vivo* or *in vitro*.

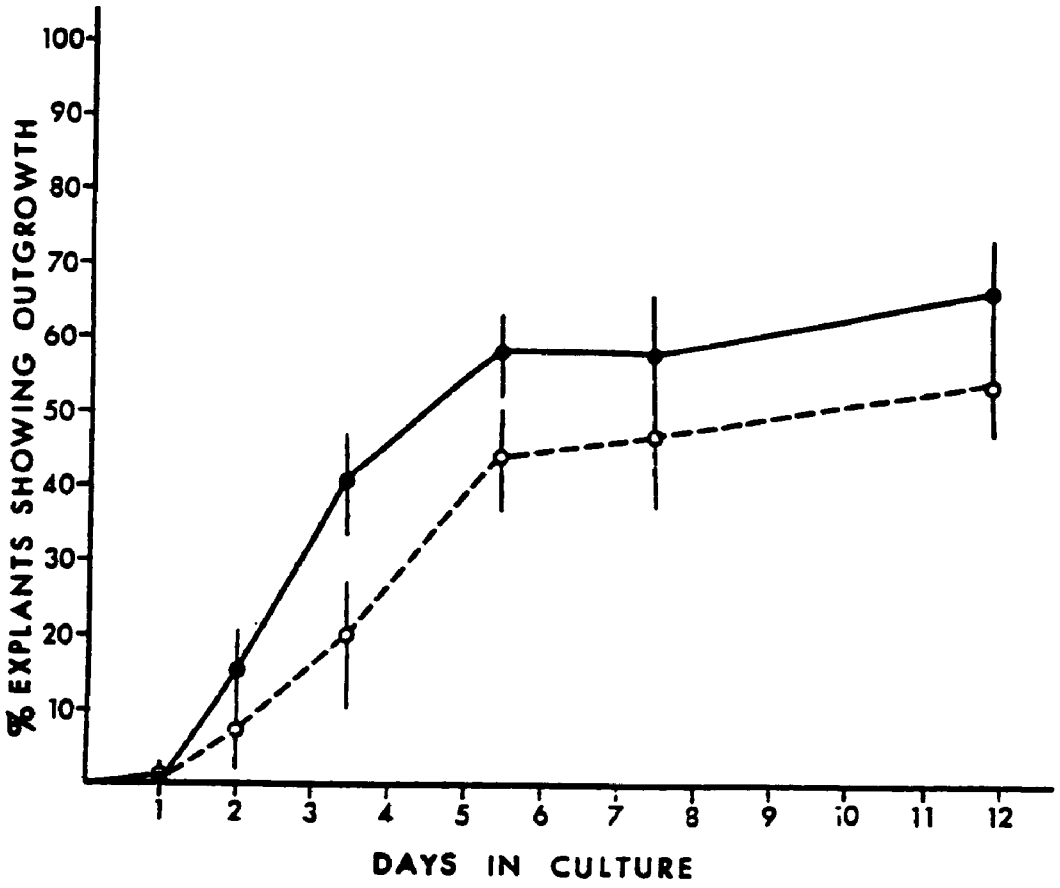


Figure 9 Effect of prior optic nerve crush on the fraction of explants with non-neuronal cellular growth. Explants were from post-crush retina whose optic nerve had been crushed 10-14 days prior to explantation. The figure represents data obtained from six separate experiments ($\pm$ S.E.M.). Post-crush; ● (n=251). Control (unoperated) explants; ○ (n=242).

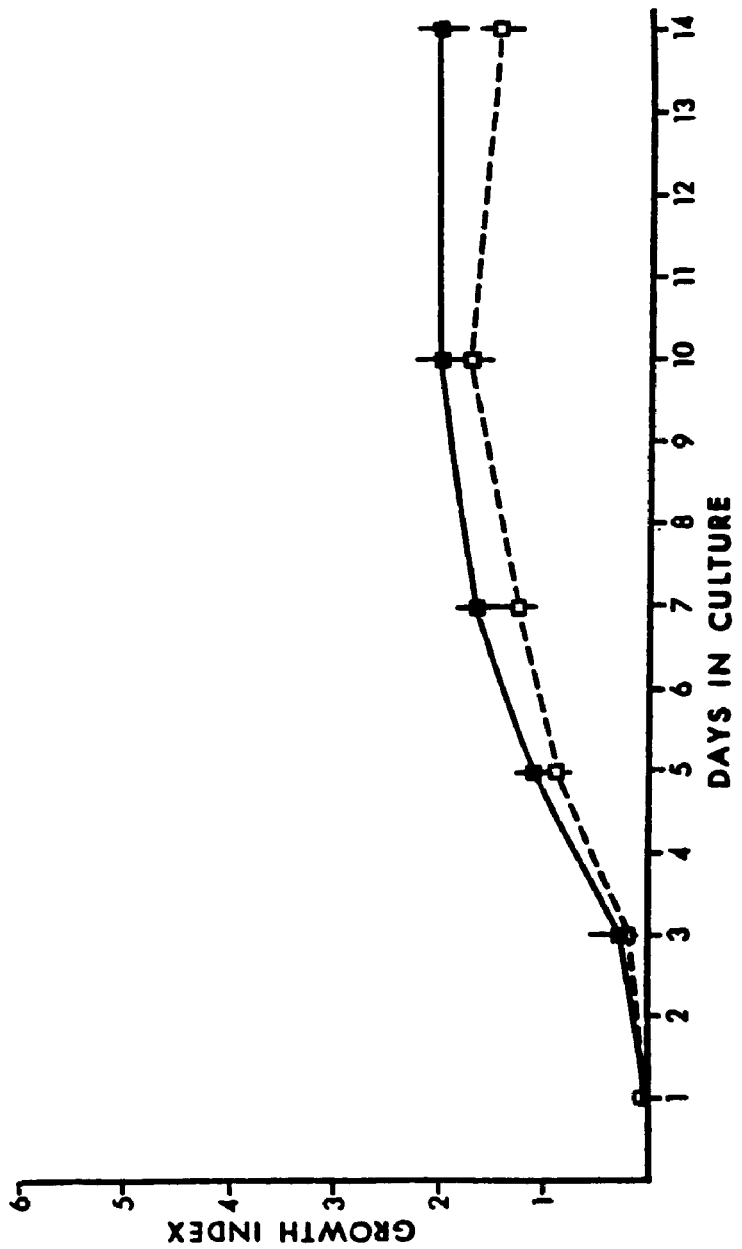


Figure 10 Effect of prior optic nerve crush on growth index of non-neuronal cells from explant cultures of goldfish retina.

The growth index was a measure of the amount of non-neuronal growth associated with retinal explants. Explants were taken from retina 11-12 days following crush of the optic nerve; ■ ± S.E.M. (n=104). Control retinal explants; □ ± S.E.M. (n=96).

PART II

Morphological Changes in Retinal Explant Cultures

Detailed phase- and interference-contrast microscopy of the outgrowing neurites was investigated. At the same time, histological sections of the explants were examined to evaluate possible alterations in the retinal explants. The latter was compared with the histological properties of the normal goldfish retina.

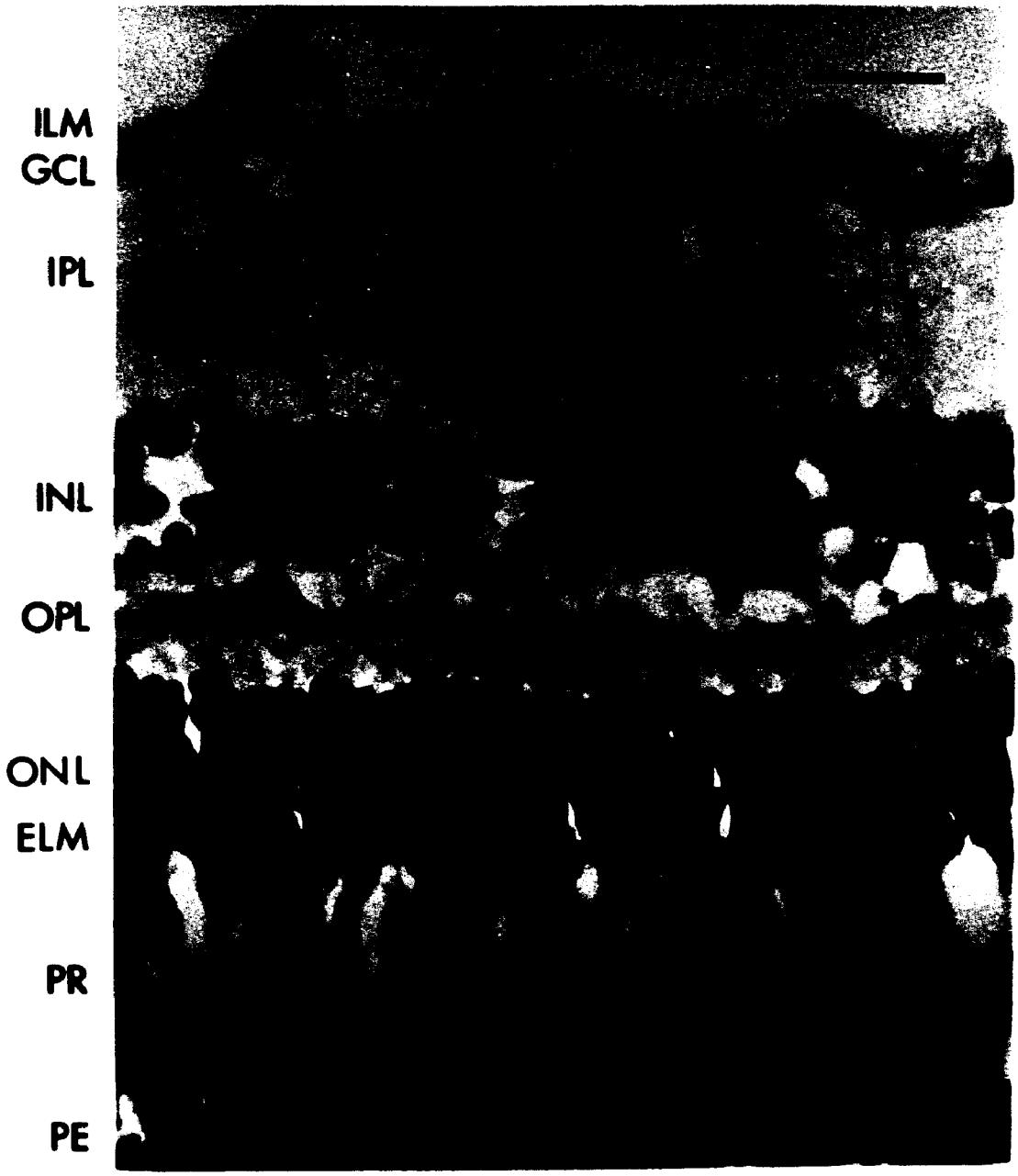
Normal Anatomy of the Goldfish Retina

The retina is distinguished by the regular organization of a limited number of cell types in clearly defined strata (Figure 11). This tissue is remarkable for its structural uniformity across the entire vertebrate radiation. The teleost retina has been described in some detail by Ramon y Cajal (1893) and other workers (reviewed by Rodieck, 1973). It has recently been the subject of a series of ultrastructural studies (Stell et al., 1975).

In the goldfish the rods are the predominant photoreceptor type. The rod outer segments are long fine processes which extend from the retina, and are enveloped by the apical processes of the pigmented epithelium. The cones can be readily distinguished from the rods, as the outer segments are much greater. The cone nuclei extend below the external limiting membrane (ELM). In cresyl-violet stained material the cone nuclei have large, pale nuclei and a small amount of cytoplasm. The outer nuclear layer (ONL) is comprised exclusively of rod nuclei. These cells are ellipsoid in shape and darkly stained.

Figure 11 Normal goldfish retina
Cresyl-violet stained section of normal goldfish retina. Internal limiting membrane, ILM; ganglion cell layer, GCL; inner plexiform layer, IPL; inner nuclear layer, INL; outer plexiform layer, OPL; outer nuclear layer, ONL; external limiting membrane, ELM; photoreceptor cell processes, PR; pigmented epithelium, PE. Bar = 50 μm.

Figure 11



The outer plexiform layer (OPL) is a stratum consisting of the processes of the photoreceptors and those of cells present in the inner nuclear layer. Horizontal cells may be observed in the inner portion of this layer.

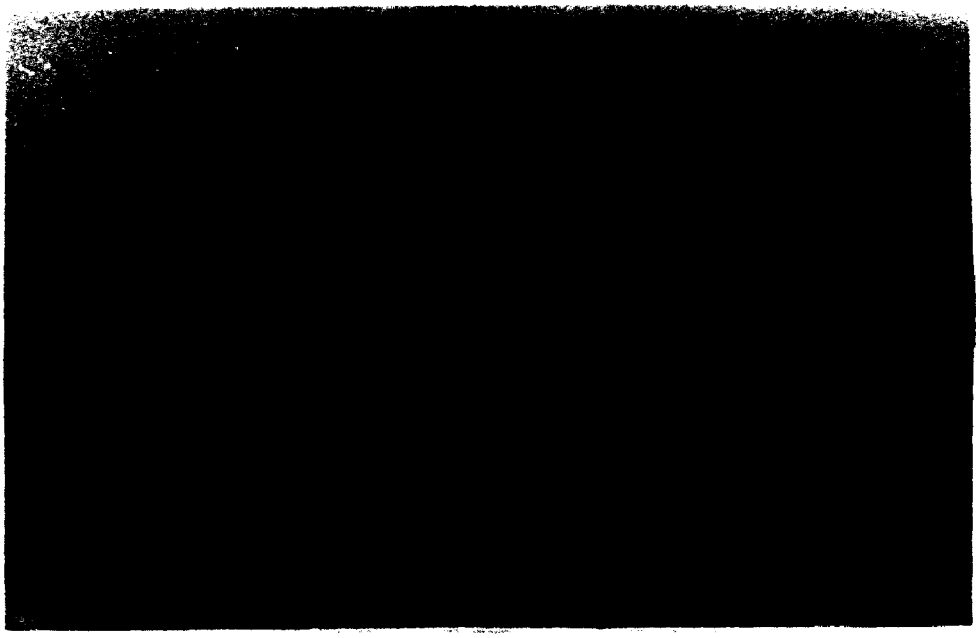
The inner nuclear layer (INL) is occupied by a variety of cell types. The horizontal cells populate the outer portion of the INL sending extensive processes throughout the INL to terminate in or adjacent to the OPL. Bipolar cells extend from the OPL to IPL, and have a darkly staining fusiform appearance. Amacrine cells, as the name implies, have no axons, and are identified by a lightly staining nucleus and pale cytoplasm. These cells normally lie in the inner half of this layer sending processes into the IPL. In the same position are Müller cells, which are neuroglial. These cells have processes that extend from the ELM spanning the entire width of the retina. The inner plexiform layer (IPL) is a plexus containing the processes of ganglion cells which contact ascending fibers from the bipolar and amacrine cells.

The retinal ganglion cells are relatively large cells distributed immediately below the vitreal surface in a single layer (Figure 12 A). In the normal animal these cells are not particularly conspicuous. The retinal ganglion cell has extensive dendritic processes in the OPL, while its axon forms the sole retinal efferent tract. The axons of these cells form the optic nerve, which projects to the optic tectum and diencephalon. Glial elements are also present in this layer. They are identified by a dark staining oval nucleus and their generally

Figure 12 Cresyl-violet stained sections of goldfish retinal ganglion cells

Ganglion cell from the (A) normal retina or from (B) retina 12 days following optic nerve crush. Bar = 5 μm.

Figure 12





smaller size. The ganglion cells are irregularly spaced when viewed in radial sections. This presumably reflects the distribution of vascular elements over the surface of the retina with ganglion cells lying in close proximity to these vessels on the inner surface of the retina (Johns, 1976).

If the optic nerve is sectioned, the ganglion cells undergo the characteristic axonal reaction and begin to regenerate the amputated axon. The ganglion cells initially increase the number and size of nucleoli present, followed by increase in cytoplasmic basophilia (Murray and Grafstein, 1969). The nucleus becomes eccentric and the cell undergoes a dramatic increase in volume. Figure 12 B shows an example of ganglion cells 12 days following section of the optic nerve. The normal ganglion cell is approximately 7.0 μm in diameter. Twelve days following optic nerve crush this has increased to an average of 10.2 μm (Figure 23). The identification of ganglion cells in post-crush retina is unequivocal.

The goldfish retina is avascular, i.e., there are no penetrating vessels. The tissue has no direct contact with vascular elements except along its surface above the optic fiber layer. The retina must thus obtain oxygen and other metabolites from this vascular bed and from the choroid plexus by diffusion.

Growth of Retinal Explant Cultures

The growth of goldfish retina *in vitro* is characterized by two distinct types of growth: neuritic, presumably from neuronal cell types within the explant, and non-neuronal which include fibroblast-

like cells, pigmented epithelial cells and macrophages. These two varieties of retinal elements will be discussed separately.

Neuritic Growth

The term "neurite" is a collective one used to describe neuronal processes in general. Normally its use is restricted to culture situations where an axon cannot be distinguished from a dendrite according to criteria usually employed in such distinctions, i.e., position or trajectory in relation to surrounding tissue.

The neurites from goldfish retinal cultures are thin processes with a number of prominent varicose enlargements along their length. At the tip of the advancing fiber is a growth cone which appears as a conical enlargement whose diameter is several times greater than that of the fiber (Figure 13). The growth cone is observed to constantly change its shape, extending and retracting small finger-like projections. These small processes, termed filopodia or microspikes, advance over the substratum, then retract, or move off the surface and "wave" in the medium, often retracting a moment later. The number of filopodia on each growth cone is variable.

The fiber itself does not generally move once it has attached to the substratum. Occasionally, small filopodia could be observed extending from the fiber well behind the growth cone. These lateral filopodia have been observed by other investigators (Nakai, 1960; Yamada et al., 1971; Bunge, 1973; Hill and Burnstock, 1976) and are thought to promote fasiculation of fibers. Neurites from retinal

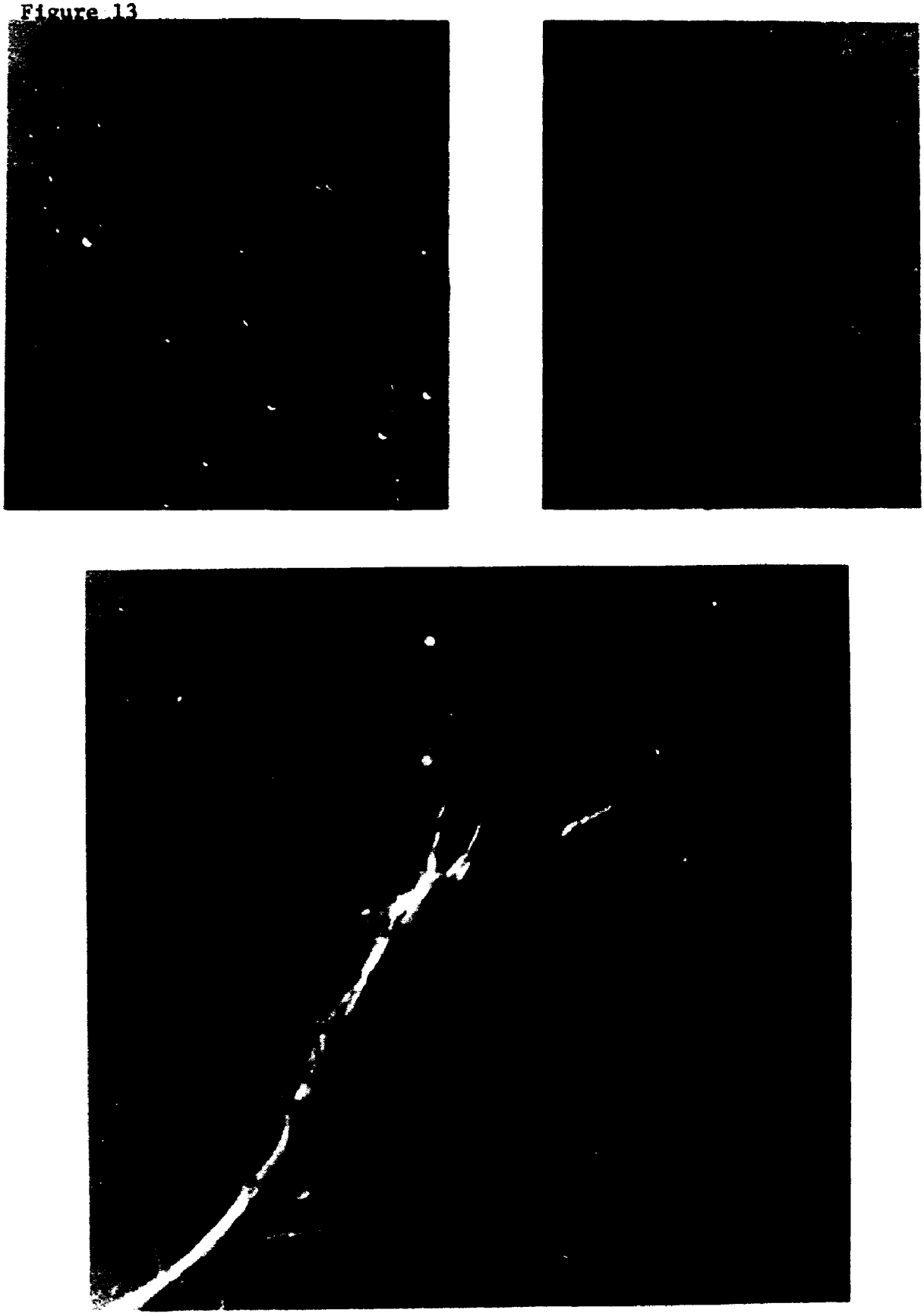
Figure 13 Micrographs of neuritic growth cones

A interference-contrast

B phase contrast

C interference-contrast Bar = 10 μm

Figure 13



explants appear to have a wide range of fiber diameters. It is not clear, however, whether each neuritic extension consists of a single fiber or several fibers which were fasciculated. This uncertainty also complicates the interpretation of bifurcating neurites. In Figure 6 D-G, the neurite is undoubtedly forming separate processes, but whether this represents a divergence of two individual fibers or the branching of a single fiber is uncertain. The question of multiple processes arising from a single fiber is relevant to the observation of Murray (1976). She has found that the number of non-myelinated fibers appearing in the regenerating goldfish optic nerve far exceeded the total number of fibers present normally. She concluded that sprouting was occurring. Whether this is actually the case is an open question.

The post-crush retinal explants can be observed to extend small neurites as early as a few hours after explantation. There is a great deal of variability between explants in the initiation of neuritic growth, that is, the first observation of neurites appearing at the edge of the explant. The growth from post-crush retinal explants is characterized by a rapid outgrowth of neurites. The neurites will routinely extend 1000-1500 μm from the explants during the first 10 days in culture. In control tissue, neurite elongation generally does not exceed 1 mm with the average fiber length being on the order of 500 μm. The growth of retinal cultures is shown in Figures 14 and 15.

If the distribution of NGI scores are examined (Figure 16) it is clear that all explants do not grow equally well. With increasing

Figure 14 Phase micrographs of a typical post-crush retinal explant after various periods in culture

The explants were photographed after A, 3 days; B, 5 days; C, 7 days; D, 10 days *in vitro*. Explants were grown on collagen gels.

Bar = 100 μm.

Figure 14

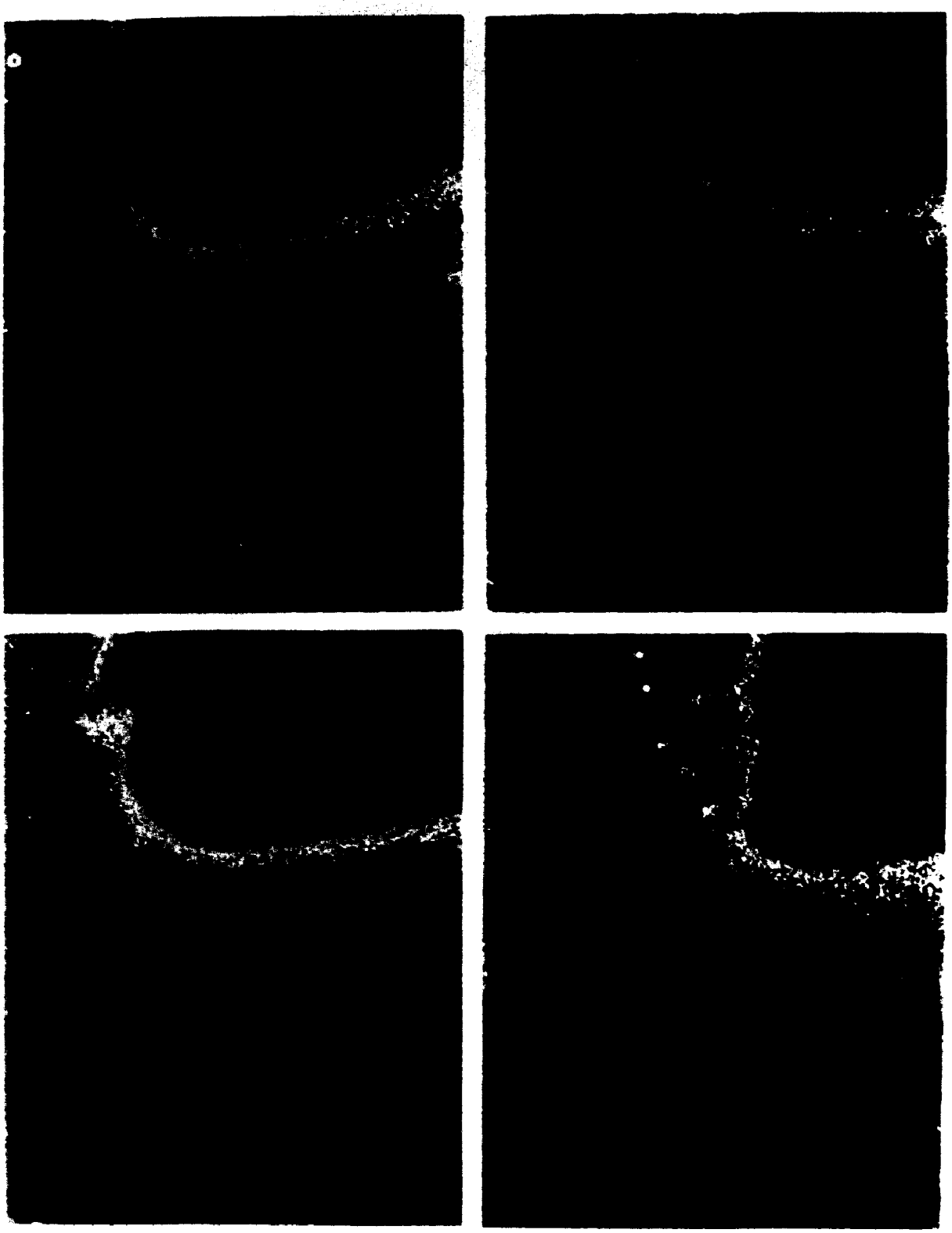


Figure 15 Phase micrographs of unoperated retinal explants after various periods in culture

The growth illustrated is typical of that observed in normal and unoperated retinal explants after A, 5 days; B, 7 days; C, 10 days *in vitro*. Explants were grown on collagen gels. Bar = 100 μm.

Figure 15

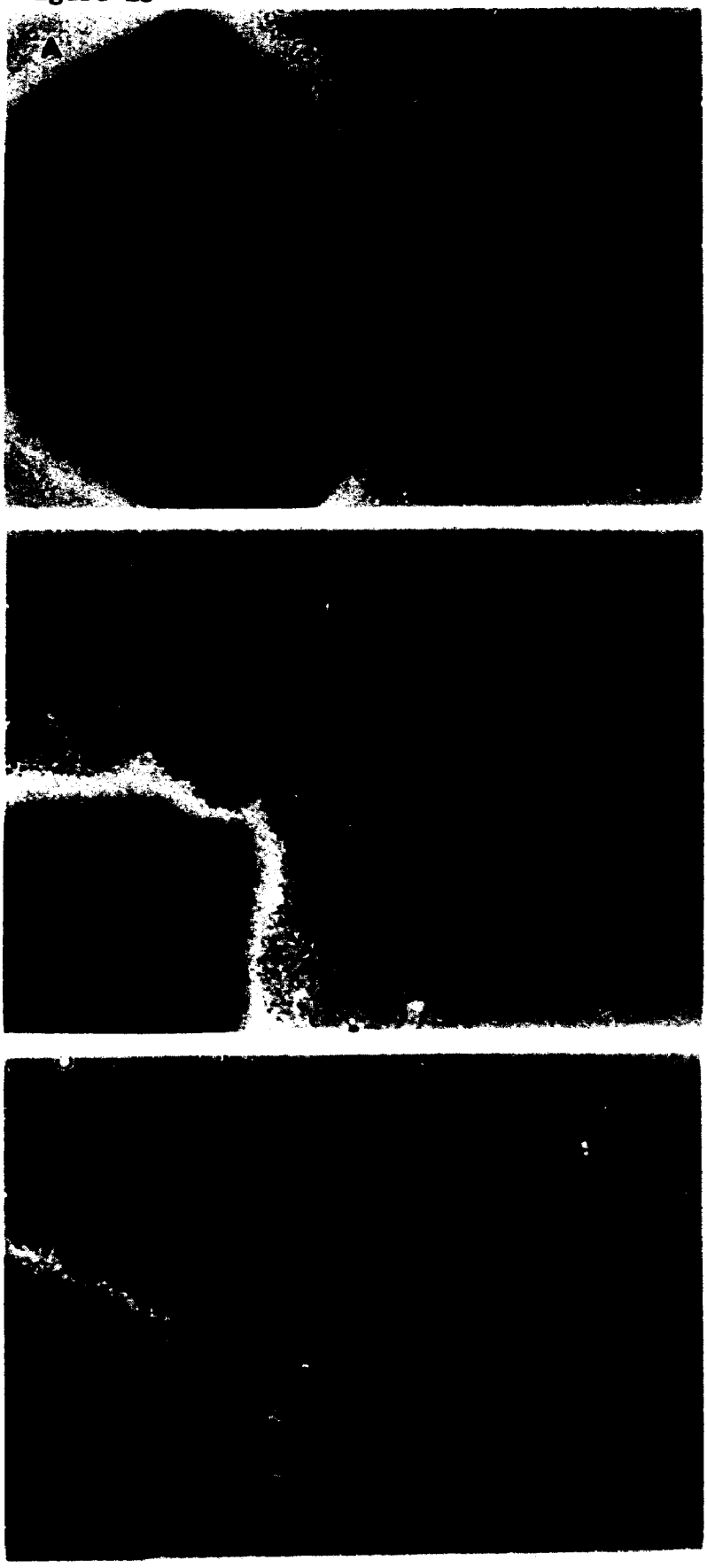
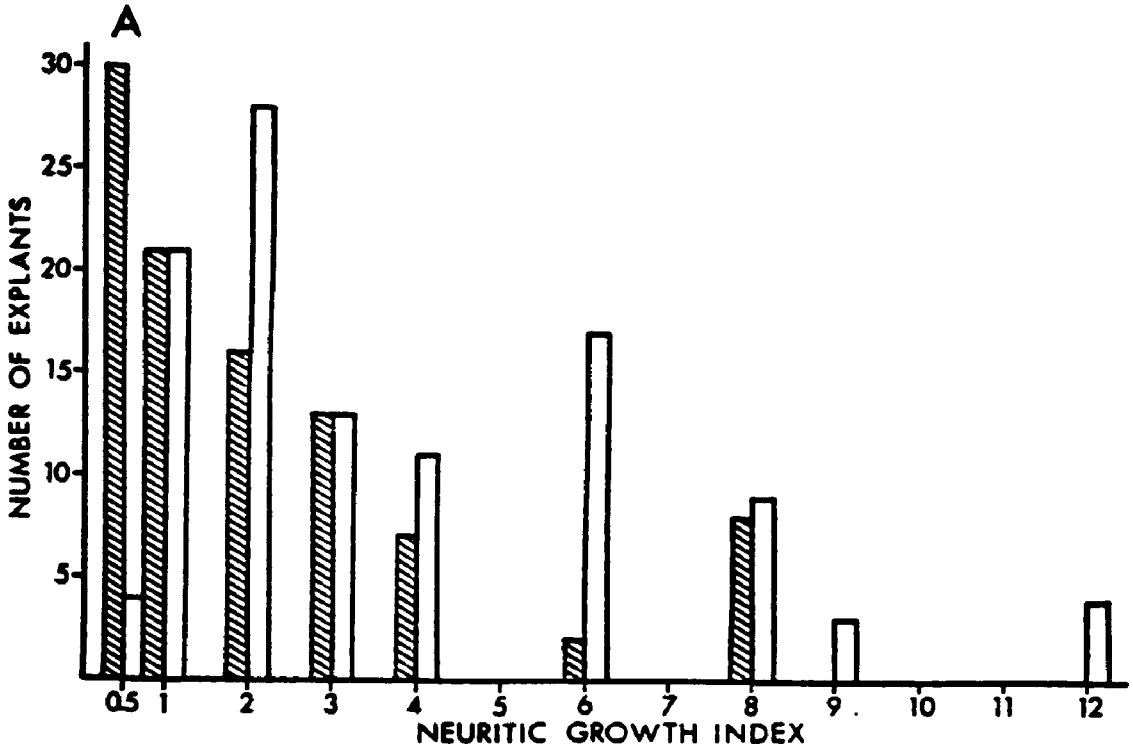


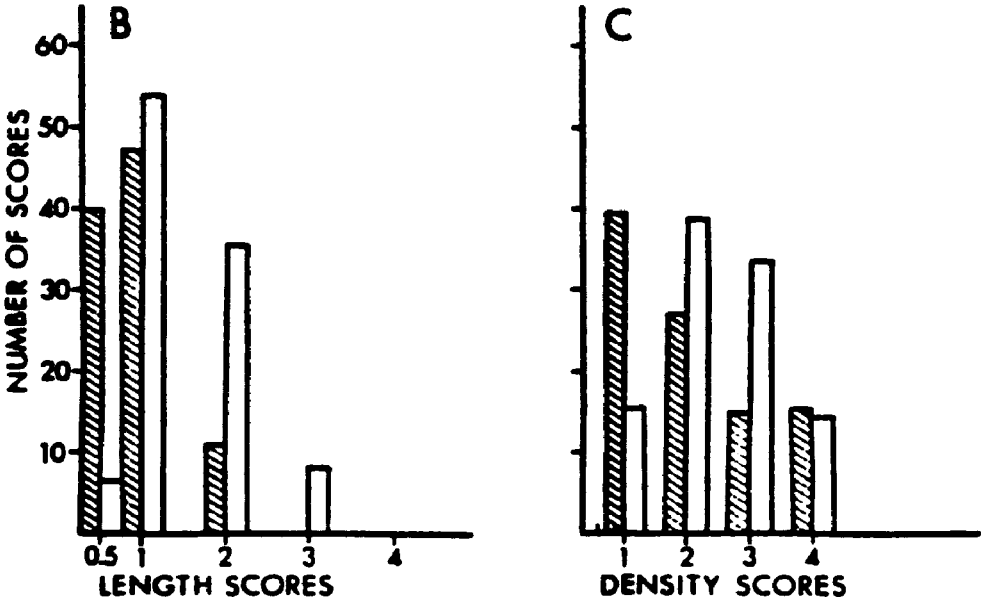
Figure 16 Histogram of the distribution of neuritic growth index scores of individual explants

A. The NGI was determined on culture day 3 (hatched bars) or day 5 (open bars) from post-crush retinal explants. The number of explants having a given NGI score is plotted. Mean NGI score for explants after 3 days *in vitro* is 1.65; after 5 days, 3.90.

B,C. The NGI scores are computed as the product of separate length and density scores. The distribution of each is shown on day 3 (hatched bars) and on day 5 (open bars) *in vitro*. The number of explants having a given NGI score is plotted. Mean NGI score for explants after 3 days *in vitro* is 1.65; after 5 days, 3.90.

Figure 16


A
NUMBER OF EXPLANTS
30
25
20
15
10
5
0.5 1 2 3 4 5 6 7 8 9 10 11 12
NEURITIC GROWTH INDEX


B
NUMBER OF SCORES
60
50
40
30
20
10
0.5 1 2 3 4
LENGTH SCORES
C
1 2 3 4
DENSITY SCORES

periods in culture the mean NGI score increases (Figure 8), yet the distribution about the mean is wide. Examination of neurite length and density measurements, which are used to calculate the NGI, indicate that both parameters change with increasing periods in culture (Figure 16).

The most vigorous growth from post-crush retinal explants occurs in the first week in culture. After 10 days, growth cones are less frequently encountered. The NGI shows a generally uniform increase following the initial phase of growth. Explants reach a stationary phase after about 7 days in culture, after which only minimal increases in NGI occur, although they retain their healthy appearance.

The growth of adult goldfish retinal explants differs from that of Xenopus in several respects. Significant numbers of neurites issuing from the Xenopus post-crush retina did not appear before culture day 3, or until day 5 in control retina. The fraction of post-crush explants with neuritic outgrowth increased with longer periods in culture in a manner similar to that in goldfish, but was delayed by approximately two days. Control retinal cultures from Xenopus demonstrate the same delay relative to goldfish cultures, but once neurites appear, the number of explants exhibiting neuritic outgrowth rises more rapidly in the former (Agranoff et al., 1976).

In goldfish retinal explants the rate of neuritic growth during the first week in culture at room temperature was 25-30 μm/h, determined from direct measurement of neurite extension or from sequential photographs. This rate was significantly more rapid than that seen with Xenopus retinal explants maintained under similar conditions.

Neurites issuing from goldfish explants were generally longer than those from Xenopus. The Xenopus neurites from control retinas were found to generally remain confined to the non-neuronal cell "lawn" surrounding the explant, except in those cases when neuritic outgrowth preceded the appearance of non-neuronal cells. This preferential contact with non-neuronal cells was not observed with goldfish neurites.

The morphology of the neurites from the two types of retinal explants was quite different. Goldfish neurites, from both control and post-crush retina, were characterized by slender beaded processes. Xenopus neurites were uniformly larger in diameter with no conspicuous varicosities. Active growth cones were observed at the advancing tips in both preparations. The varicosities observed in culture may be related to those described in the regenerating goldfish optic nerve in vivo by Murray (1976) who found them to be filled with dense-core vesicles. The dense-core vesicles, as discussed previously, probably subserve fiber elongation. It is interesting that Ramon y Cajal described the optic fibers in the normal teleost retina as having "numerous varicose swellings" (1893). The formation of local enlargements of the growing nerve fiber in vivo has been attributed to the nerve encountering local obstructions, or to fibers crossing one another, leaving an enlarged area on the side proximal to the obstacle (Speidel, 1933).

When the explants are grown on collagen, neuritic growth is typically, but by no means always, restricted to a single side of the

explant and extends radially away from the tissue (Figure 17). The observation of growth issuing from restricted regions of the explants may reflect the polarity of the tissue with respect to its site of origin in the eye (Vinnikov, 1946). Fibers which are regrowing may thus respond to positional information within the explant and exit at a position normally oriented toward the optic disc. Explants which are grown on polycation-treated surfaces do not show this effect to as great an extent.

Neurites growing from explants on collagen gels normally extend directly away from the tissue. The paths of individual fibers often take tortuous routes, moving freely in the 3-diminsional substratum (Figure 17), however, the tendency is to grow radially. Neurites grown on collagen films also tend to exist as single fibers or in small fascicles.

Dunn (1971) has noted that when sympathetic ganglia are grown under conditions which prevent fasciculation, the nerve fibers appear to be repelled by one another resulting in relatively evenly spaced radial outgrowth. The goldfish retinal fibers, either on collagen or polycation-treated substrates, show no strong repulsive characteristics, although this has not been extensively examined. There is a great deal of crossing of individual fibers and small fascicles without a consistent pattern of interaction.

The manner in which neurites on a polycation-treated surface grow from the explant is quite different from that on collagen. These differences will be treated in detail in the following section.

Figure 17 Neuritic growth from post-crush retinal explant after 3 days in culture

Extensive neuritic growth extends radially from post-crush retinal explant grown on a collagen gel. The outgrowth can be observed to issue from a single side of the explant.

Figure 17





Non-neuronal Cells

The growth of non-neuronal cells from the retinal explant cultures occurs after several days *in vitro*. The general classification "non-neuronal" derives from the assumption that they, unlike neuronal cells, are able to migrate *in vitro* (Lumsden, 1968; Luduena and Wessells, 1973). The identification of specific cell types is difficult due to their variable morphology under different culture conditions. An actively migrating cell looks quite different from the same cell that is not migrating. The cells observed under the conditions described here have been tentatively identified on the basis of their similarity to those in a standard atlas of vertebrate cells in tissue culture (Rose, 1970).

Fibroblast-like Cells

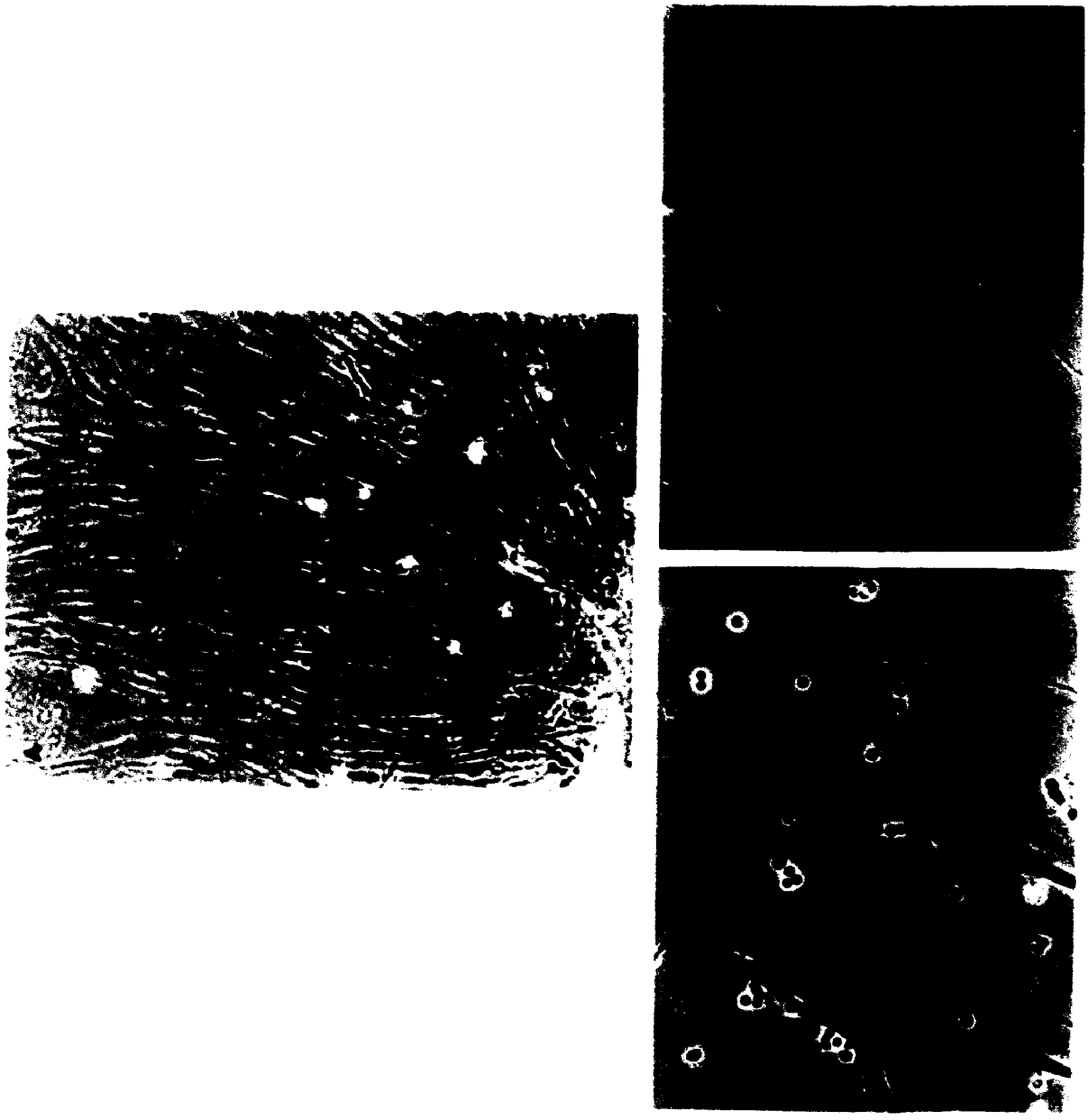
Fibroblast-like cells are the predominant non-neuronal cell type present in these cultures, generally comprising 95-100% of the total non-neuronal cell population. These cells were the first to appear at the edge of the explant, generally after 2-3 days in culture (Figure 14). The cells appeared phase-gray and were variable in shape (Figure 18).

Nuclei with one or multiple nucleoli were readily visible, and occasionally binucleate cells were observed. Putative fibroblasts migrated by extending filopodia with characteristic ruffled edges. During active migration these cells were spindle-shaped and tended to migrate radially from the explant. When the cell density around the explant was high, the cells tended to appear more uniform.

Figure 18 Growth of fibroblast-like cells from retinal explant cultures

(A) Extensive growth of fibroblast-like cells from retinal explant culture forming a monolayer. Bar = 100 μm.
Phase (B) and interference-contrast (C) micrographs of same field showing morphology of typical fibroblast-like cells present in these cultures. Bar = 50 μm.

Figure 18



The number of fibroblast-like cells emerging from an individual explant was quite variable. There was no consistent difference in either their rate of migration or in the number of cells associated with normal compared to post-crush retinal explants.

The distribution of these cells about the explant show no regular pattern. They emerge from all sides of the explant. They proliferate quite rapidly and after a few days in culture surround the explant, eventually forming a monolayer (Figure 18 A). A confluent layer of cells can extend as far as 1-1.5 mm away from the explant. Routinely, fibroblast-like cells are localized within 500 μm of the tissue after 10 days in culture.

These cells appear to derive almost exclusively from the tissue lying on the vitreal surface of the retina including the vasculature and possibly parts of the optic fiber layer. When this tissue becomes separated from the retina during explantation, fibroblast-like cells proliferate very rapidly. The corresponding bit of denuded retina demonstrates much less of this type of growth.

Melanocytes

Melanocytes, or pigmented epithelial cells,were occasionally observed. The number of these cells in explant cultures was related to the ease with which the retina was removed from the eye cup. If the fish was not fully dark-adapted, retinal pigmented epithelium was pulled away still attached to the rod outer segments. These

cells survived in culture and were identified by their prominent pigment granules. Melanocytes were similar to fibroblast-like cells in their morphology and migration pattern.

Macrophages

Macrophages were occasionally observed in retinal explant cultures, generally, but not exclusively, associated with post-crush explants. They have a very characteristic morphology and appearance. These cells are typically much darker than the other non-neuronal cells under phase microscopy. Occasionally, they may appear refractile. The nucleus is not normally visible. Macrophages, during active migration, assume an elongated Y or V shape (Figure 19) and are thereby clearly distinguished from other cell types. They are not present in the majority of explants and in no case do they comprise more than 5% of the total non-neuronal cells present.

Other Cell Types

In many cultures non-neuronal cells appear which cannot be readily identified as belonging to one of the above groups. Whether this represents a different population of cells or a variant of those already described is unclear. These unclassified cell types are rarely encountered and never constitute more than 1% of the total non-neuronal cells associated with the retinal explants.

Figure 19 Phase micrograph of macrophages migrating from retinal explant culture Bar = 50 µm.

Figure 20 Phase micrograph of neurite in contact with fibroblast-like cell Bar = 50 µm.

Figure 19



Figure 20



Interaction of Neurites with Non-Neuronal Cells

Neurites are often observed to contact migrating non-neuronal cells, apparently on a random basis. Neurites will contact a fibroblast-like cell at one or more places along its upper surface where it appears to adhere (Figure 20). The neurite will sometimes move off the cell at a later time and continue to elongate. There are no apparent changes in the non-neuronal cell as a result of this contact.

Histology of Retinal Explant Cultures

The retinal explants have been examined histologically 1, 3, 5, 8, and 12 days following explantation onto collagen gels. An experimental group consisted of 3 culture dishes each containing 10-16 explants. Both unoperated and post-crush retinal explants undergo similar changes in response to increasing periods in culture, with the exception of one cell type, the ganglion cell. The reactions common to both types of explants will be described, followed by a discussion of the ganglion cell response.

Retinal explants after 1 day in culture, show a dramatic alteration in the morphology of cells in the INL and ONL (Figures 21 A and 22A). The most obvious change is the large number of pyknotic cells present in both nuclear strata. Pyknotic cells are characterized by a small hyperchromatic nucleus with little or no cytoplasm present. The majority of the rod nuclei, which comprise the ONL, have lost their ellipsoid shape, and are rounded and darkly stained. A similar situation exists in the INL where perhaps 50% of the cells appear

Figure 21 Cresyl-violet stained sections of post-crush retinal explants after various periods in culture

Post-crush retinal explants were grown on collagen gels and fixed after A, 1 day; B, 3 days; C, 5 days; D, 12 days *in vitro*.

Bar = 20 μm.

Figure 21

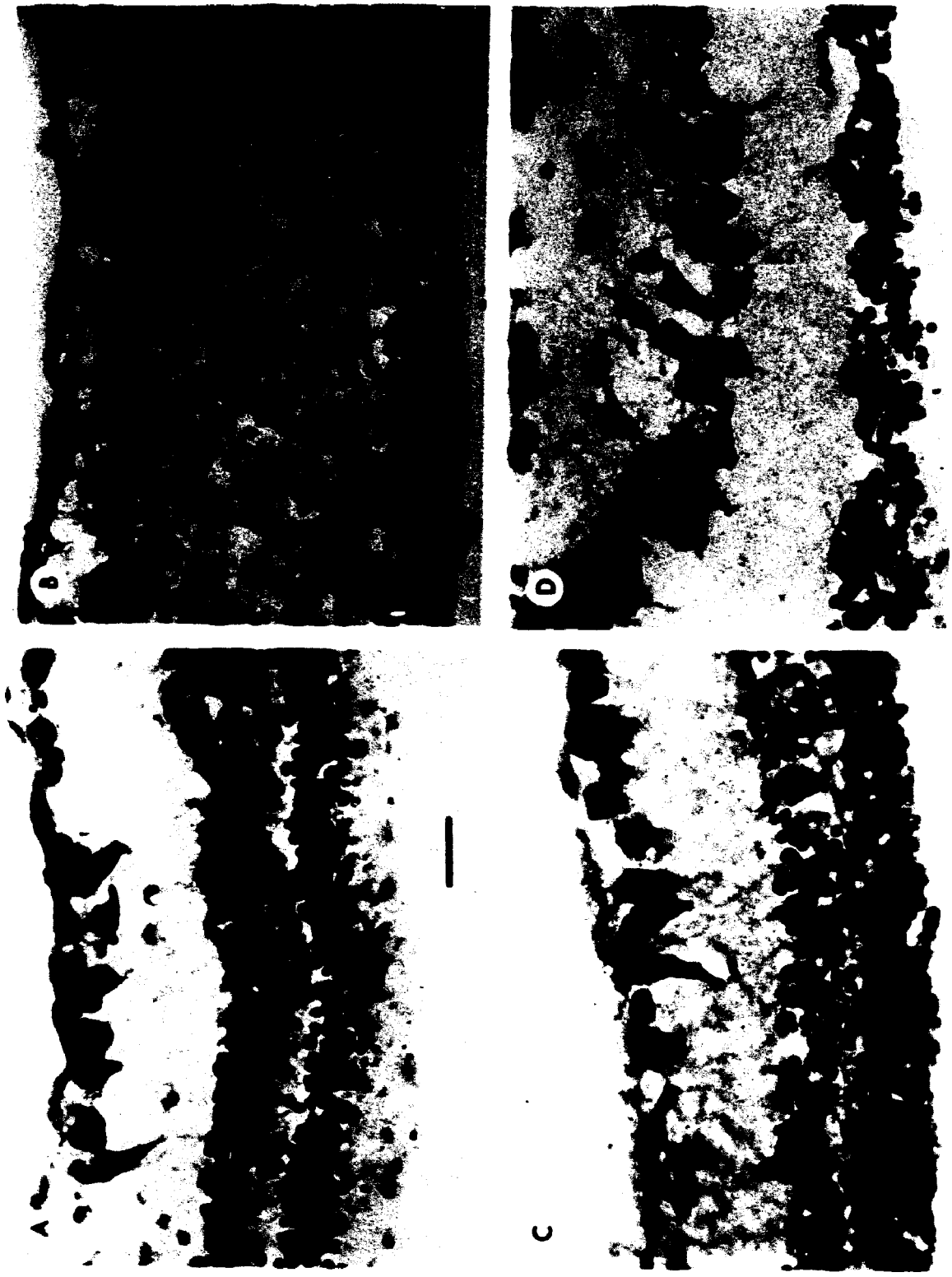
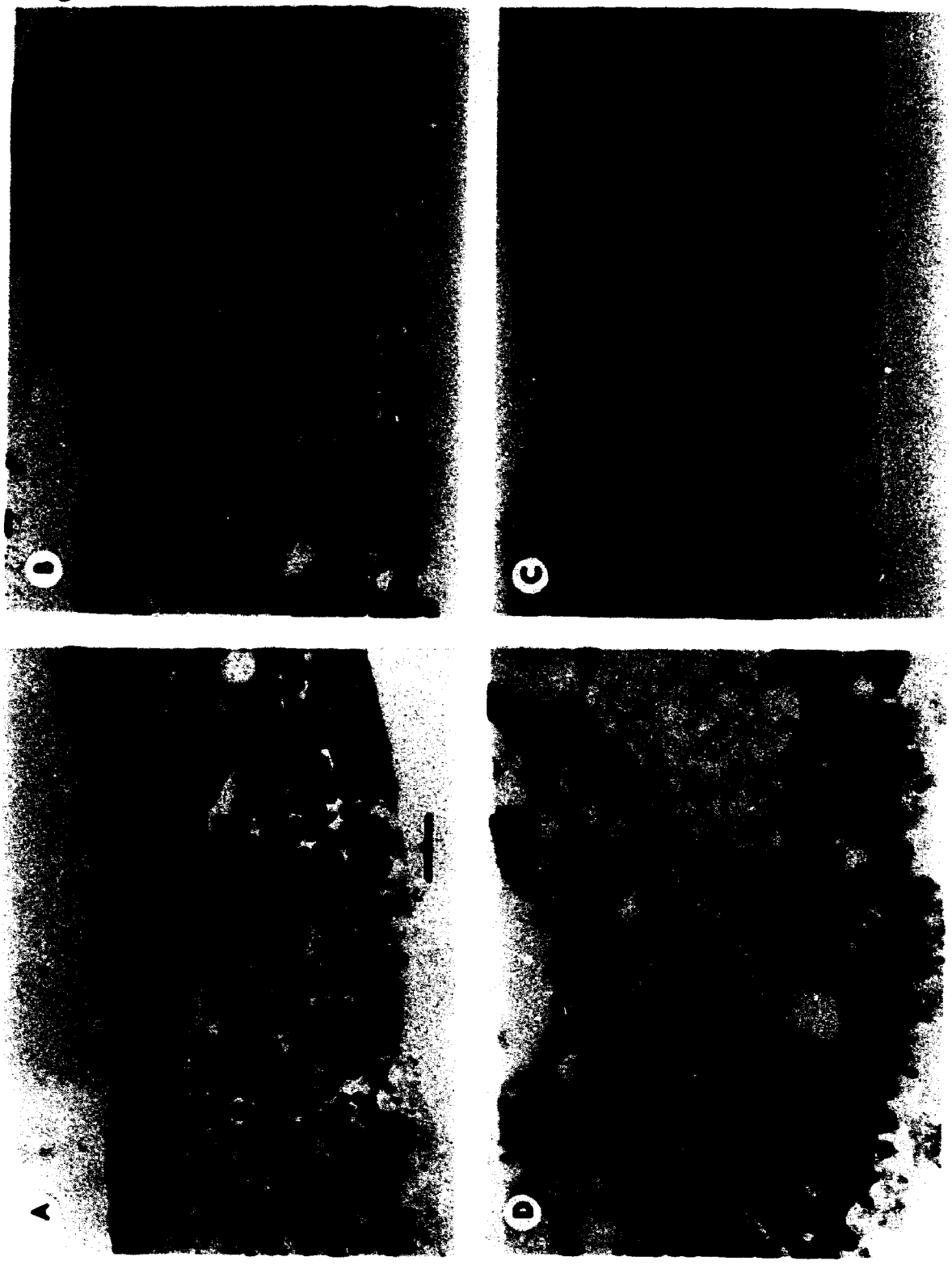


Figure 22 Cresyl-violet stained sections of unoperated retinal explants after various periods in culture

Unoperated retinal explants were grown on collagen gels and fixed after A, 1 day; B, 3 days; C, 5 days; D, 12 days *in vitro*.

Bar = 20 μm.

Figure 22



pyknotic. The INL contains a heterogeneous population of cells. It has not been possible to distinguish the survival or loss of a specific cell type with any certainty. Cone nuclei are present in many explants, but rod and cone outer segments have not been observed.

Three or five days following explantation, the retinal cultures have undergone a progressive deterioration of the ONL and INL (Figures 21 B,C and 22 B,C). The rod nuclei in the ONL are all pyknotic and the total number of cells in this stratum has diminished. In the INL, more than 80% of the cells appear pyknotic after three days in vitro. This fraction increases with lengthening periods in vitro. There is also a net decrease in the number of cells in this layer. The dying cells in the INL appear to change their position in the explant, invading the OPL and INL. The result of this process is that the OPL loses its definition. In the INL, some cells clearly survive. They are characterized by a pale nucleus and abundant cytoplasm. These cells constitute less than 5% of the original number of cells in this stratum. Their position in the inner part of the INL adjacent to the IPL as well as their morphology suggest they may be amacrine or Müller cells. The most striking feature of the three and five day old cultures is that the dying cells have collected toward the outer (photoreceptor) side of the retinal explant, lying at the lower border of the ONL.

Twelve days following explantation, the loss of cells from the INL and ONL is readily apparent, with only a fraction of those cells remaining (Figures 21 D and 22 D). The morphology of the cells populating the nuclear stratum has not changed in the interval from 5 to 12 days.

The OPL has disappeared with the merging of the two nuclear layers. A small number of viable cells persist immediately below the IPL as described previously. These cells appear more prominent than before, principally due to the loss of surrounding cells. There is some spongiform degeneration throughout the explant which was not present at earlier times. The IPL is largely intact, and often, darkly staining Müller cell processes can be observed running radially through this area extending to the vitreal surface.

The ganglion cells, in both unoperated and post-crush retinal explants, appear healthy and in their original position throughout the culture period. Ganglion cells in post-crush explants, as in vivo, demonstrate the characteristic cellular hypertrophy and basophilia associated with axonal regeneration. These cells do not increase their size any further with increasing periods in vitro.

The ganglion cells in normal retinal explants were found to undergo hypertrophy in culture (Figure 23). The increase in cellular diameter is accompanied by all the morphological changes associated with the axonal reaction which occurs in vivo. The cells undergo a nearly linear increase in cellular diameter with lengthening periods in culture. After 12 days in culture, they are comparable in size to post-crush ganglion cells either in vivo or in vitro.

Deterioration of Retinal Explant Cultures

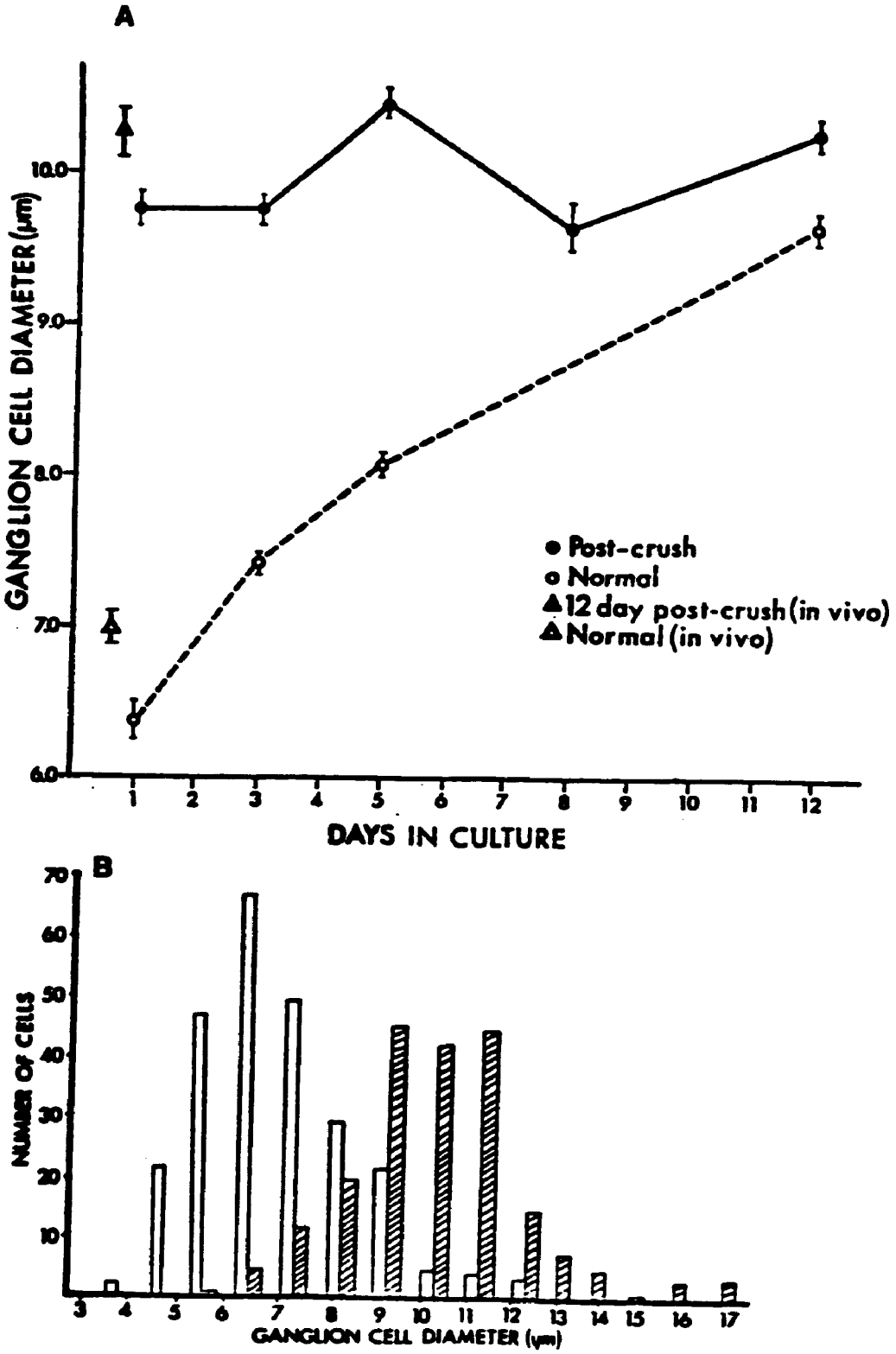
After about two weeks in culture, the explants are observed to deteriorate. The varicosities present along the length of the neurite

Figure 23 Ganglion cell diameter of normal and post-crush retinal explants

A. The ganglion cell diameter was measured in retina from normal fish 12 days following optic nerve crush in situ. Ganglion cell diameter in normal (unoperated) and 12 day post-crush retinal explants was determined on successive days in culture. A minimum of 200 measurements was made for each experimental point ($\pm$ S.E.M.).

B. Distribution of ganglion cell diameters in the normal goldfish (open bars, n=250) or 12 days following optic nerve crush (hatched bars, n=207) in vivo.

Figure 23


A
GANGLION CELL DIAMETER (μm)
10.0
9.0
8.0
7.0
6.0
1 2 3 4 5 6 7 8 9 10 11 12
DAYS IN CULTURE
● Post-crush
○ Normal
▲ 12 day post-crush (in vivo)
△ Normal (in vivo)
B
NUMBER OF CELLS
70
60
50
40
30
20
10
3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
GANGLION CELL DIAMETER (μm)

became increasingly prominent. The fibers have a tendency to become detached from the substratum except at their tips, and growth cones have virtually disappeared. Eventually the neurites begin to disintegrate leaving only the larger fascicles intact near the explant. After 3-4 weeks *in vitro*, the neurites have largely disappeared. The non-neuronal cells become increasingly vacuolated throughout the culture period, particularly during the third week in culture. They become round and refractile with some eventually detaching from the substratum. The non-neuronal cells often persist for several days following the disappearance of the neurites. In some cultures on polycation-treated surfaces, these cells remain viable for as long as a month after explantation. Even after long culture periods, there are only rare instances of bacterial and fungal contamination.

Thymidine Labeling Studies

The incorporation of ^{3}H-thymidine into retinal explant cultures was evaluated by radioautography. The cultures were labeled for 20 h with 0.8 µCi ^{3}H-thymidine per culture dish.

The growth of non-neuronal cells from explant cultures was a characteristic feature of this tissue in culture. The cells were observed to migrate, eventually forming a monolayer around the explant. The labeling studies were performed to determine if these cells were proliferating or simply migrating from the explant onto the substratum. Additionally, it was of interest to see if there was any mitotic activity within the explant itself.

Inspection of unoperated and post-crush retinal explants after 1, 3, 5, 8 and 12 days in culture indicated considerable variability in the number and distribution of labeled cells. There was no apparent difference between normal and post-crush explants as a function of time *in vitro*. The detection of labeled non-neuronal cells migrating out from the explant was hampered by poor preservation of the substratum around the explant in paraffin sections. In favorable material, labeled cells could be observed in the monolayer of non-neuronal cells surrounding the explant (Figure 24). In the explant itself, there was a small number of thymidine-labeled cells distributed in the INL, ONL and optic fiber layer. The identity of these cells is uncertain, but they are unlikely to be neural elements, since the germinal cell zone at the ciliary margin is not generally included in the tissue explanted.

Cell types that are known to divide in cultures of nervous tissue include fibroblasts, macrophages and glial elements. Fibroblast-like cells are the predominant cell type which is observed to migrate from the retina *in vitro*. Fibroblasts in the retina would be largely restricted to the connective tissue of the vasculature and the outer limiting membrane on the vitreal surface. The presence of labeled cells in the monolayer surrounding the explant indicate that these cells are undergoing division. Macrophages are also occasionally observed to move away from the explant in culture. These cells are known to divide, principally at the site of inflammation *in vivo*, or in this case, probably within the retina itself (Macleod, 1969).

Figure 24 Radioautograph of ^{3}H-thymidine labeled retinal explant after 12 days *in vitro*

Retinal explants were incubated with 0.8 µCi of ^{3}H-thymidine for 20 h prior to fixation. Labeled cells are present in the monolayer of non-neuronal cells surrounding the explant and within the explant itself (arrows). Bar = 100 µm.

Figure 24



Neuroglial cells may be responsible for the thymidine labeling. Microglia will divide and serve as phagocytes, however, they would presumably be located among the neural elements of the retina (Watson, 1974). Oligodendroglia are present in the optic fiber layer particularly near the optic disc. Whether these cells will divide under the circumstances described here is unknown. The observation of mitotic figures in Müller cells was noted by Champy (1914) in rabbit and tortoise retinal explants, suggesting that similar events could occur in the goldfish retina in culture. Mitotic figures were not observed in our explants.

In examining the histology of retinal explant culture, the overall impression is that a very substantial fraction of the cells in this tissue do not survive in culture. It is, in fact, fortunate that the cells of interest are the sole survivors of the culture situation. While the degeneration of retinal cells is not necessarily a desirable feature of the explant cultures, it simplifies the analysis of the growth associated with this tissue.

The successful maintenance of the retinal ganglion cells is the most significant feature of the explant cultures. The hypertrophy of retinal ganglion cells from control retina indicates that not only are these cells capable of surviving in culture, they are able to undergo all the morphological changes associated with cells regenerating their axons.

PART III

Optimization of Culture Conditions

A major goal of this study was to develop satisfactory conditions for the culture of goldfish retinal explants and reduce the variability in the outgrowth from this tissue. A systematic study of the several factors that may affect the explant growth was undertaken. The initial studies were performed using conditions that were found to support growth of Xenopus retina in vitro (Agranoff et al., 1976). These were subsequently modified to meet the requirements of goldfish retina.

Substrata

The selection of a suitable substratum for the culture of goldfish retinal explants required that it support vigorous neuritic outgrowth, allow firm attachment of retinal explants and be optically suitable for observation by phase microscopy. Additionally, the substratum must be relatively easy to prepare and consistent in its quality.

Initial efforts were directed at obtaining growth on commercially available substrata. Post-crush retinal explants were grown in culture dishes containing agar or gelatin. The results are shown in Figure 25 and 26. Agar, a mixture of complex polysaccharides, at a concentration of 1 or 2% supported little growth of either neurites or non-neuronal cells.

Figure 25 Neuritic growth on gelatin and agar substrata
The fraction of post-crush retinal explants with neuritic outgrowth on substrata composed of: 2% gelatin ● , 4% gelatin ⊖ , and 1% agar ○ .

Figure 26 Growth of non-neuronal cells on gelatin and agar substrata
The fraction of post-crush retinal explants with non-neuronal cells present on substrata composed of: 2% gelatin ● , 4% gelatin ⊖ , and 1% agar ○ .

Figure 25

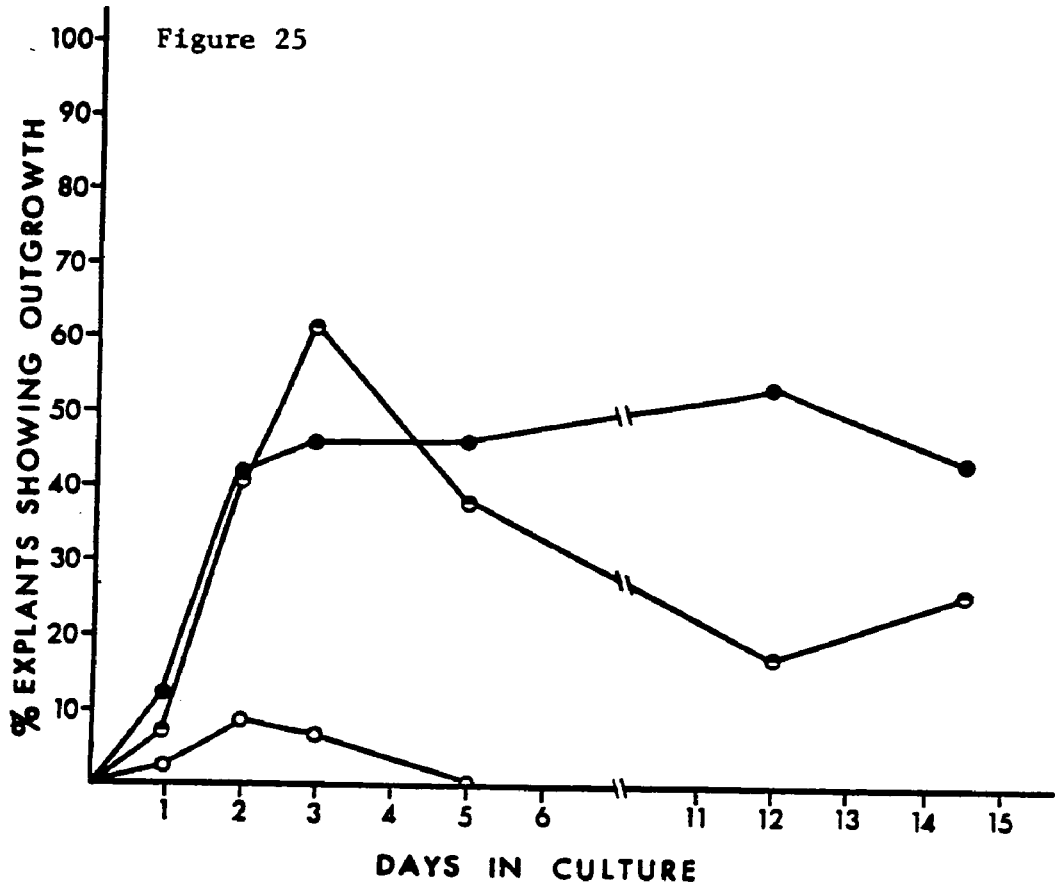
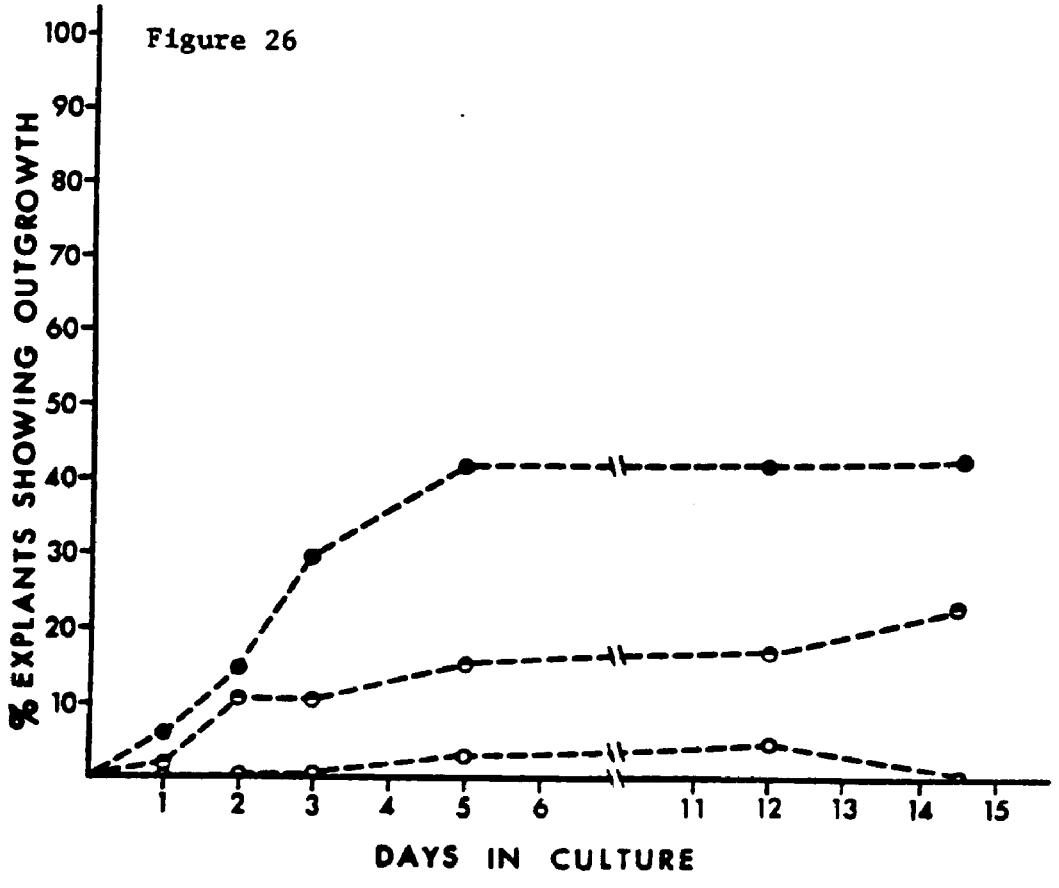


Figure 26



Gelatin, a denatured form of collagen, was prepared at concentrations of 2% or 4%. The optical qualities of this substratum were good and its preparation simple. Neurites (Figure 25) and non-neuronal cells (Figure 26) grew well once the explant had become attached to the gelatin surface. The principal difficulty with this substratum was the inability of substantial numbers of explants to attach firmly. The soft surface of the 2% gelatin substratum was superior to that of 4% gelatin in this regard.

Fibrous collagen as a mechanical support for tissue cultures has been extensively used since the report of Ehrmann and Gey (1956) and of Bornstein (1958). Two different types of collagen substrata were employed in studies reported here, a hydrated collagen gel (Elsdale and Bard, 1972) and a collagen film (Bornstein, 1958).

Collagen gels have proved to be a much superior substratum than 2% gelatin (Figure 27). Neuritic growth is present in a substantially larger percentage of the explants grown on collagen. There appears to be little difference in the fraction of explants with non-neuronal growth between the two substrata throughout the culture period.

The growth of retinal explants on collagen films does not differ from that on collagen gels (Figure 28). The film is a thin coating of collagen fibrils attached to the surface of the plastic.

While the outgrowth from retinal explants is described on a number of different substrata, the majority of studies reported here were performed using collagen gels.

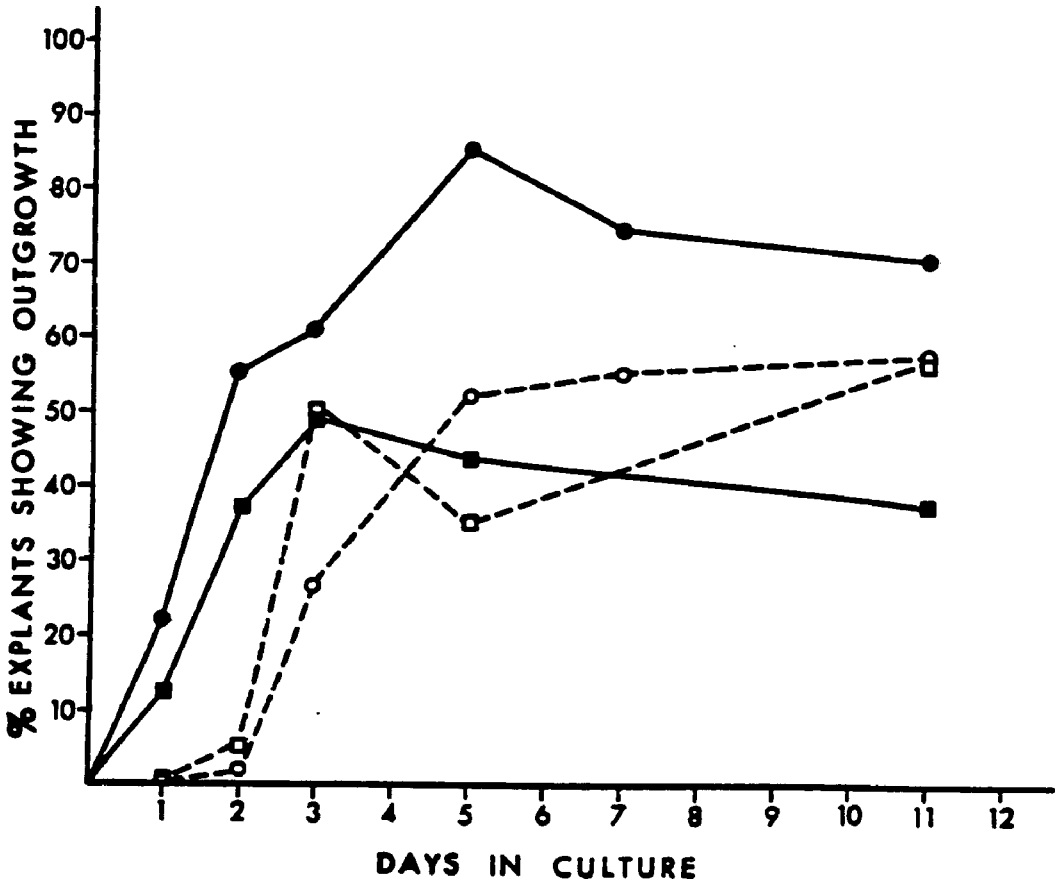


Figure 27 Comparison of growth on collagen and gelatin substrata

The fraction of post-crush retinal explants possessing neuritic growth on 0.2% collagen ● or 2% gelatin ■ is plotted with solid lines. Non-neuronal cellular growth on 0.2% collagen gels ○ or 2% gelatin □ is plotted with broken lines.

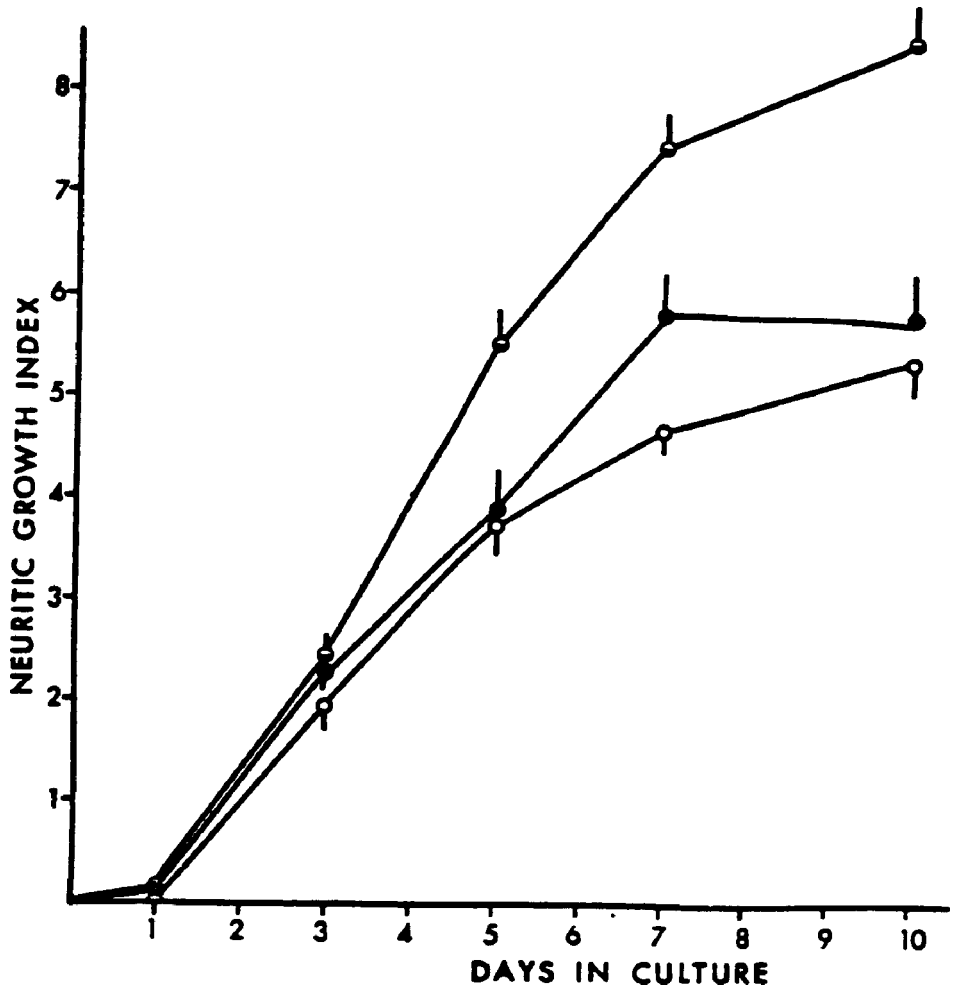


Figure 28 Comparison of neuritic growth on various substrata The NGI of post-crush retinal explants grown on 0.2% collagen gels ● , 0.1% collagen films ○ or on PLYS-treated plastic substrata ⊖ was computed by averaging individual explant scores.

The preparation of soluble collagen is a modification of Ehrmann and Gey's procedure (1956). The published method has not been uniformly successful in our hands. The modified procedure is detailed in Methods. The dialysis of the resulting collagen solution was a critical step and was closely controlled. A two-fold increase in the acetic acid concentration over that used by Bornstein allowed for better control of the viscosity in the dialysis. The final viscosity of the soluble collagen must be sufficient to allow pipetting of the solution. Undialyzed collagen will not support growth of retinal explants. This is not due to the presence of acetic acid but to toxic soluble components extracted from the rat tail tendons which are removed during the dialysis. The length of the dialysis required to remove toxic soluble contaminants must be balanced against the increasing viscosity.

A variety of artifical substrata were tried with no success. Plastic tissue culture dishes from either Falcon or Nunc did not support neuritic growth and only occasionally were fibroblast-like cells observed. Unsuccessful attempts were also made to grow explants on fluorocarbon-treated glass or plastic (tetrafluoroethylene polymer). The failure to obtain growth on these substrata may be due to the net negative charge present at their surface.

Polycation-treated surfaces have been demonstrated to promote cell-substratum adhesions and growth in culture (Leiberman and Ove, 1958; Letourneau, 1975a; Macieira-Coelho and Avrameas, 1972; McKeehan and Ham, 1976; Mazia et al., 1975; Mousson and Maurer, 1976; Stulting and Berke, 1973; Yavin and Yavin, 1972). When retinas were

explanted onto polylysine (PLYS) or polyornithine (PORN)-treated glass or plastic surfaces neuritic outgrowth was markedly better than that observed on collagen substrata (Figure 28). The NGI values reflect a generally higher density of neurites, however, the greater length of the fibers can account for much of the increase. The neurites grown on polycation-treated substrata were more evenly distributed around the explant and were normally present in large fascicles (Figure 29). There was no difference in the amount of non-neuronal growth associated with explants grown on PLYS compared to collagen.

Initial efforts to obtain growth from retinal explants on polycation-treated surfaces were successful only with glass coverslips. This met our immediate need for optically flat surfaces required for interference-contrast microscopy. Similar studies with plastic tissue culture dishes were only marginally successful. Later studies, using methods described by Letourneau (1975a), resulted in excellent adhesion and growth of the explants on polycation-treated surfaces (Heacock, A., unpublished observations). The reason for the initial failure to obtain satisfactory growth was probably due to inadequate washing procedures to remove residual polycation remaining in the dish, since basic polymers seriously inhibit growth when present in the culture medium (McKeehan and Ham, 1976). Polylysine was the polycation used almost exclusively in the present studies and was not significantly different from PORN in promoting adhesion and growth from retinal explant cultures.

Figure 29 Neuritic growth from a post-crush retinal explant grown on PLYS-treated plastic substratum

The explant was photographed after 5 days in culture. Bar = 100 μm.

Figure 29



Media

The selection of a nutrient medium for goldfish retinal explants required that it support the maintenance and growth of this tissue for extended periods in culture. Wolf and Quimby (1969) have discussed media requirements which are peculiar to fish. Tissue from freshwater fish can be grown in nutrient media designed for mammalian systems without modifications. Inorganic ion concentrations in serum differ little between teleosts and mammals, allowing the use of mammalian-type salines as orginally compounded. Hank's balanced salt solution has been the most commonly used. The osmolarity of most mammalian media is approximately 300 mOsm. This is similar to that of fish sera (Prosser, 1961; Levine and Musallam, 1964; Wolf and Quimby, 1969). Deboni and co-workers (1976) have found cultures from goldfish brain will grow in Eagle's minimum essential medium (MEM) or Medium 199.

Leibovitz's medium (L-15, 1963) was chosen principally for its ability to be in free exchange with the atmosphere. This obviates the need for CO_2 equilibrated incubators required for bicarbonate buffering systems and avoids the large fluctuations of pH which accompany their use. The medium, as compounded by Leibovitz, uses Hank's formulation of inorganic salts, and vitamins at concentrations reported by Eagle (1959). The buffering capacity of the medium is largely due to high concentrations of amino acids, some of which are present as the free bases, especially arginine. Glucose, which is the principle energy source in most culture media, has been replaced by high levels of galactose (900 mg/ml). Galactose in the presence

of alanine and pyruvate will adequately substitute for glucose with only small amounts of lactic acid being formed (Eagle et al., 1958).

Retinal explant cultures were maintained in air without changing the medium for up to two weeks. Over a period of 24 h the pH of the L-15 would rise from 7.2 to nearly 8.0. The drift of the pH was controlled by the addition of 20 mM HEPES (pH 7.2), which provided excellent maintenance of pH over extended intervals. Neither the elevated pH nor the presence of HEPES had any effect on the growth of the cultures (data not shown).

The use of an organic buffer offers several advantages over the traditional bicarbonate systems (Eagle, 1971; Fisk and Pathak, 1969; Itagaki and Kimura, 1974). Bicarbonate has a pKa of 6.1 which is well below the physiological pH range required for tissue cultures. Good et al. (1966) developed a series of ethanesulfonic acid buffers with optimal buffering capacities in the ranges normally employed in biological systems. These buffers are non-toxic and do not require a controlled atmosphere.

Organic buffers have been reported to have some adverse effects. Pfeiffer and Eagle (1976) have reported that ethanesulfonic acid buffers affect the synthesis of the nervous system specific protein, S-100. They suggest the use of these buffers with nervous tissues should be approached cautiously due to the structural similarity of these compounds with taurine, a putative neurotransmitter and α - flupenthixol, an anti-psychotic drug. In hamster trachael cultures (Daniel and Wolf, 1975), HEPES inhibited the incorporation of labeled

glucosamine into glycoproteins. If goldfish retinal cultures were incubated with ^{3}H-glucosamine in L-15 or L-15H there was no difference in the incorporation of radioactivity into acid soluble or acid insoluble material (data not shown).

Minimal sterile precautions were taken in preparing the tissue, yet only rarely were cultures contaminated. Gentamicin was used at concentrations of 100 µg/ml, a level which Agranoff et al. (1976) found was effective with a similar preparation from Xenopus. Laskey (1970), in a survey of antibiotics, found that gentamicin was bacteriocidal at concentrations above 50 µg/ml, while toxicity did not occur until concentrations above 400 µg/ml were reached. The antibiotics of choice in most tissue cultures are penicillin and streptomycin; however, these were not effective against the gram-negative bacteria encountered in these and similar cultures. Fungal contamination of cultures occured sporadically and could generally be traced to poor sterile technique in handling the cultures, or to exposure of an open culture dish to the atmosphere admitting air-borne spores.

Effect of Serum on Growth of Retinal Explant Cultures

The use of fetal calf serum as a supplement to nutrient medium is a common practice in tissue culture. Since it had been previously shown that FCS was required for neuritic growth from Xenopus retina, initial studies with goldfish included 9% FCS.

The amount of serum required for optimal growth of retinal explant cultures was examined by altering the concentration of FCS present in the culture medium. In L-15H which had not been

supplemented with FCS, the growth of neurites was poor (Figure 30). The NGI values for these cultures suggests a high dependency on FCS. Non-neuronal growth in L-15H minus FCS was also diminished compared to serum-supplemented cultures. The optimal concentration for support of neuritic growth is approximately 10% (Figure 31). Goldfish retina clearly grows less well in 20% FCS. Elevated serum concentrations are known to be toxic to some tissues in culture (Schneider, 1973). If explants are grown in a simple balanced salt solution (Dunlop et al., 1975) essentially no growth is observed. Cultures grown in unsupplemented medium consistently have large numbers of explants which remain suspended in the medium and never attach to the substratum. The low NGI values observed in these cultures is due in part to the failure of a significant fraction of the explants to adhere well enough to extend neuritic or non-neuritic processes.Those which do attach are easily dislodged. The extent to which neuritic outgrowth is affected by nutritive deficiencies compared to poor attachment cannot be determined from the NGI, since firm attachment is a prerequisite for the initiation of neuritic growth. Presumably both factors are operating.

Retinal cultures explanted in medium containing homologous fish serum failed to grow. Medium was prepared containing 5% FCS and 5% goldfish serum. In this medium the cultures rapidly deteriorated and became necrotic. Control cultures containing only 5% FCS grew well. Other workers have reported satisfactory growth from fish tissues in homologous or heterologous fish sera (Wolf and Quimby, 1969), however, Clem (1961) found it to inhibit the growth of a line of fish epithelial cells. Fryer et al. (1965) have found homologous sera to be

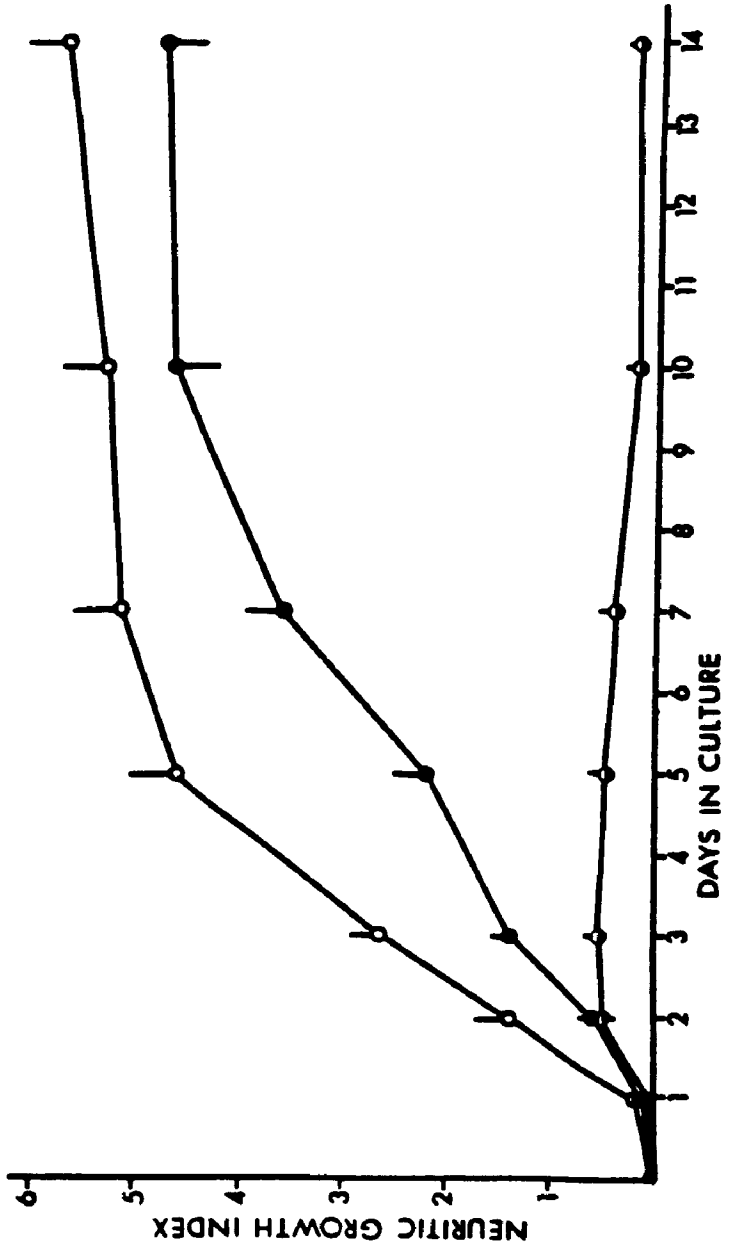


Figure 30 Effect of different fetal calf serum concentrations on neuritic growth from retinal explant cultures

Post-crush retinal cultures were grown in L-15H with different fetal calf serum supplements. The neuritic growth was evaluated on day 7 in culture. Retinal explants were cultured in 20% FCS (●), 9% FCS (○), or in serum-deleted medium (◐) ± S.E.M.

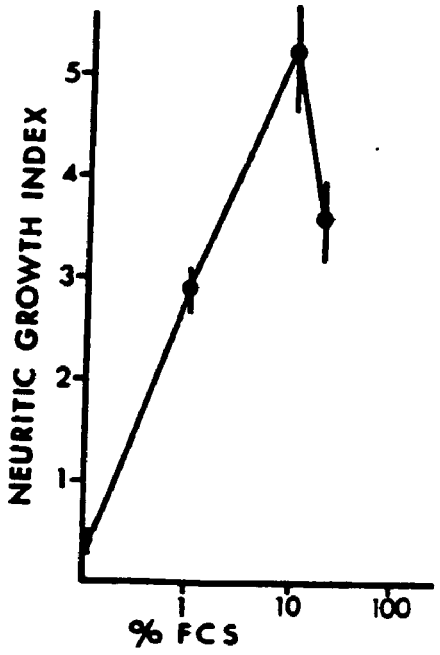


Figure 31 Effect of fetal calf serum on neuritic growth of post-crush retinal explant cultures

The neuritic growth index was measured on culture day 7 and is plotted against the fractional supplement of fetal calf serum ($\pm$ S.E.M.).

highly toxic to various cultures from fish embryo.

Explants grown in FCS-depleted media (L-15H or Dunlop's medium) characteristically had a fibrous material associated with them (Figure 32). The fibrous material, presumably derived from the explant as a response to absence of serum, may be related to a diminished level of protein in the environment. Typically, the extracellular material consisted of very long slender fibers which appeared to be attached at the liquid-air interface. These fibers could often reach 2 mm in length and commonly formed a dense mat around the explant. The fibrous material appeared within 24 h of explantation and had largely disappeared by 3 days. The fibers did not resemble neurites and were not mistaken for them due to their small caliber. The fibrous material was present in both unoperated and post-crush retinal cultures.

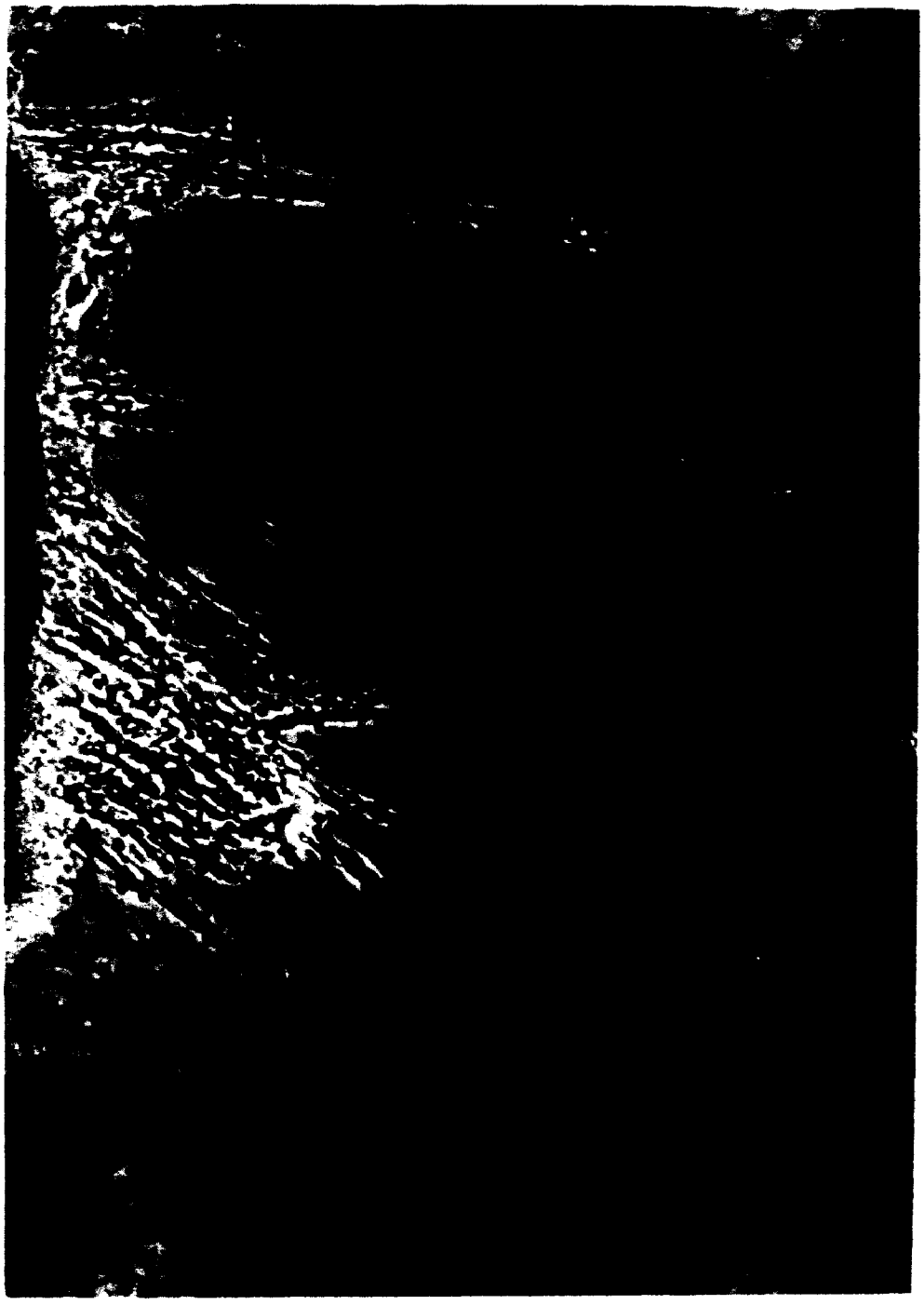
The effect of the tripeptide L-glycyl-histidyl-glycine on growth of retinal explant cultures was examined in order to determine if it might substitute for FCS in these cultures, allowing maintenance in a completely defined medium.

Pickart and Thaler (1973) isolated and purified a factor from normal human serum which would stimulate nucleic acid and protein synthesis at nanomolar concentrations in liver cells. The factor was identified as a tripeptide, glycyl-histidyl-lysine. The synthetic tripeptide was equally effective (Pickart et al., 1973). Sensenbrenner et al. (1975) have examined the effect of this tripeptide on differentiating chick neuroblasts. They found that if the synthetic tripeptide was added to cell dissociates (in the presence of limiting serum concentrations i.e. 1% FCS) neuroblasts would differentiate,

Figure 32 Fibrous material from retinal explant cultures in FCS-deleted medium

The fibrous material illustrated here appeared within 24 h following explantation and generally disappeared after 3 days in culture. Bar = 100 μm.

Figure 32



while glial growth was inhibited. The tripeptide alone was insufficient for maintenance of these cultures for extended periods.

The growth of goldfish retinal cultures in tripeptide-supplemented medium are shown in Table II. Post-crush retinal explants cultured in serum-deleted L-15H medium grow poorly. If tripeptide was added in concentrations from 20 ng/ml to 100 µg/ml the already poor neuritic growth appeared to be further diminished. Non-neuronal growth was unaffected. A separate series of cultures (amino acid-supplemented) were grown in L-15H minus FCS, and 0.29 mM with respect to glycine, histidine and lysine, which was comparable to the molar concentration of the tripeptide at 100 µg/ml. There is a slight inhibition of growth in amino acid supplemented media. The L-15H is 2.7 mM, 1.6 mM, and 0.5 mM respectively in each of the above amino acids making it unlikely that any affect of the tripeptide may have been due to its hydrolysis into its constituent amino acids. It should be noted that at these low levels of growth the difference in NGI cannot be considered significant due to the inherent error in the scoring methods.

Retinal explants grown with limiting concentration of FCS (1%) will grow neurites and non-neuronal cells, albeit at low levels. The 1% FCS is considered to be a maintenance concentration. If failure to obtain a stimulation in FCS deleted medium was due to the poor condition of the retinal explants, then properly maintained explants should be expected to show an effect. Tripeptide seriously inhibited the neuritic growth in these cultures while having no effect on the growth of non-neuronal cells.

TABLE II

EFFECT OF THE TRIPEPTIDE L-GLYCYL-HISTIDYL-LYSINE ON GROWTH OF POST-CRUSH RETINAL EXPLANT CULTURES

Medium	FCS deleted		1% FCS	
	Neuritic	Non-neuronal	Neuritic	Non-neuronal
Control	0.72 (0.12)*	0.35 (0.08)	2.91 (0.19)	0.94 (0.23)
Amino acid supplemented	0.36 (0.09)	0.30 (0.08)	-	-
100 µg/ml Tripeptide	0.54 (0.10)	0.48 (0.12)	-	-
200 ng/ml	0.05 (0.02)	0.06 (0.04)	1.17 (0.27)	0.78 (0.24)
20 ng/ml	0.21 (0.06)	0.22 (0.08)	1.32 (0.36)	1.00 (0.24)

* ± S.E.M.

The growth of neurites and non-neuronal cells was measured in post-crush retinal cultures grown in L-15H with 1% FCS or in FCS-deleted medium after 7 days *in vitro* on collagen gels. The synthetic tripeptide was added at concentrations of 100 µg/ml, 200 ng/ml and 20 ng/ml. Control cultures were grown in L-15H with the serum concentration indicated. Amino acid supplemented cultures contained 0.29 mM of each of the constituent amino acids.

Inositol

In the course of studies on the effect of inositol on microtubule stabilization (Chapter III) it was found that cultures containing high levels of inositol in the nutrient medium grew better than comparable controls. Inositol has been demonstrated to be an essential nutrient for the maintenance and growth of cells in tissue culture (Eagle et al., 1955, 1956). When myoinositol (10 mM) was added to retinal explant cultures, neuritic growth was enhanced (Table III). The difference in neuritic growth between inositol-supplemented and unsupplemented medium was small but reproducible. Non-neuronal cell growth was not consistently improved. Eagle (1957), in a survey of inositol requirements for a number of cell lines, found maximal growth in occur at 1-10 μM depending upon the strain tested. The inositol concentration in L-15H is 11 μM, well within the range reported by Eagle to support maximal growth.

The stimulatory effect of inositol in this system may be related to the high galactose levels (5 mM) present in the L-15H. Stewart et al. (1968, 1969) have reported that rats fed galactose-enriched diets show a 60% reduction in the amount of free inositol present in peripheral nerve. This reduction is not due to conversion of inositol to glucose or inhibition of its synthesis from glucose. The depressed free inositol concentration, in the presence of elevated galactose, may be due to competition for similar carriers for transport across the cell membrane (Stewart, 1969). There is no direct evidence for this, however (Johnstone and Sung, 1967). Cultured cells are capable of inositol synthesis, yet they still require that it be supplied

TABLE III

EFFECT OF 10 mM INOSITOL ON GROWTH FROM POST-CRUSH RETINAL CULTURES

	Substrate	Collagen gel			PLYS		
	Days in culture	3	5	7	3	5	7
Neuritic Growth Index:	Control	2.3 (0.4)	3.85 (0.4)	5.0 (0.4)	3.3 (0.4)	5.1 (0.5)	6.8 (0.7)
	10 mM Inositol	2.9 (0.4)	4.8 (0.4)	5.7 (0.4)	5.3 (0.5)	8.1 (0.8)	8.8 (0.7)
Growth Index, Non-neuronal cells:	Control	0.7 (0.1)	1.1 (0.1)	1.8 (0.2)	0.1 (0.1)	0.9 (0.1)	1.3 (0.2)
	10 mM Inositol	0.9 (0.1)	1.3 (0.2)	1.5 (0.2)	0.5 (0.1)	1.2 (0.2)	2.1 (0.2)

Retinal explant cultures were grown in L-15H supplemented with 10 mM myoinositol either on collagen gels or on PLYS-treated plastic substrata. The growth of neurites and non-neuronal cells was evaluated on day 3, 5 and 7 in culture ($\pm$ S.E.M.).

exogenously (Eagle et al., 1960). The magnitude of the enhancement of neuritic growth from retinal explant cultures would argue that the inositol level present in L-15H was sufficient to largely offset any antagonistic effect of high galactose levels.

Post-Crush Interval

Experiments were performed to establish at what point following crush of the optic nerve maximal neuritic outgrowth would occur. The enhanced neuritic growth from post-crush retinal explants was compared to that obtained from explants from the unoperated (opposite) eye and from the eyes of untreated normal fish.

Each experimental point (Figure 33) represents a minimum of 50 explants from 3 separate culture dishes. Explants were grown on collagen gels. The post-crush interval is that period from the crush of the optic nerve to explantation of the retina. The NGI represents only the amount of neuritic growth present following 7 days in culture, and does not reflect the delay in initiation of neurites from cultures obtained at the various post-crush intervals. The cultures explanted after short post-crush intervals (1-2 days) did not exhibit neurites until 5 days *in vitro*. Retina from fish crushed 3-5 days prior to explantation did not issue neurites before culture day 3, while 7 and 12 day post-crush explants generally grew neurites within 24 h following explantation. If the data from the different culture periods are plotted, their values relative to one another are the same as shown in Figure 33, only the absolute NGI values change.

There was no clear difference in the neuritic growth from post-crush and control cultures until 5 days following optic nerve crush.

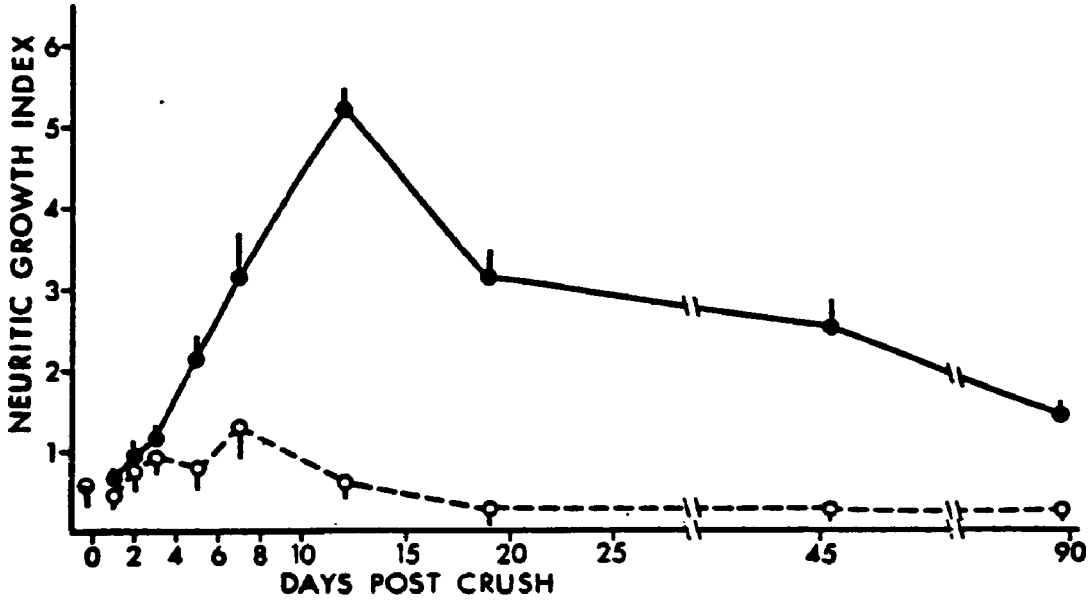


Figure 33 The effect of varying the post-crush interval on neuritic growth index on post-crush and normal retinal explants

The interval from crush of the optic nerve in explantation of goldfish retina was varied and the NGI determined on day 7 in culture. Explants were taken from post-crush retina (● ± S.E.M.) and from the unoperated opposite eye (O ± S.E.M.). Data from normal (untreated) fish are plotted at 0 time. The 12 day point represents pooled data from fish whose optic nerve had been crushed 11 or 12 days prior to explantation.

The difference increased rapidly reaching a maximum in the interval 11-12 days post-crush. The 12 day point represents pooled data from that period. The NGI values progressively diminished as the post-crush interval increased. The difference between post-crush and control explants was still apparent after 89 days.

Some cultures were examined as late as 110 days following optic nerve crush. In these cultures, post-crush retinal explants often issued a few fibers (generally less than a dozen) soon after explantation, but no further neuritic growth was observed. After several days in culture, post-crush explants were indistinguishable from unoperated explants.

The variation in the growth of control explants was problematic. Initial observations suggested that neuritic growth from the opposite (unoperated) eye was greater than that of retinal cultures taken from normal untreated fish. When further experiments were performed no consistent difference could be demonstrated (see Part IV). Two difficulties complicated the analysis. Firstly, the NGI scores involved were on the order of one. Quantitatively, this does not represent very much neuritic growth. At these growth levels, the NGI as a quantitative measure has questionable significance. A change from 0.5 to 1.0 NGI, while doubling the growth index is only a minimal increase in the amount of neuritic growth present. This deficiency in the scoring method derives from the need to weight the lower end of the scale to include explants which exhibit neuritic growth, even if it represents a very small number of neurites. Secondly, the biological variability in this system is quite high. This problem is illustrated by distribution of NGI scores in the preceding section (Figure 16). We have attempted to control for this by using large experimental groups.

Temperature

The dependence of neuritic outgrowth on the temperature at which the cultures were incubated was investigated with post-crush retinal explants grown on collagen gels. The cultures were held in humid chambers which were kept at constant temperature by circulating water baths. The percentage of explants which grew neurites or non-neuronal cells was measured over the first four days in culture. These experiments were performed early in the study before the development of the more sensitive NGI.

The growth of neurites and non-neuronal cells from retinal explants was temperature dependent over the range from 15°-30° (Figure 34). The elevated temperature results in a larger fraction of the explants exhibiting neurites, or non-neuronal cell growth. If the average percentage of explants over the 0-4 day interval is plotted against temperature (Figure 35),the increase is nearly linear over the range 15° to 30°.

These experiments were hampered by technical difficulties in maintaining the explants at temperatures greater than ambient. Despite efforts to maintain a humid environment, the cultures incubated at 30° and 35°, rapidly became dehydrated through evaporation. The resulting hypertonicity frequently caused the explants to deteriorate and neuritic processes to disappear. Although nutrient medium was changed daily this was insufficient to keep the cultures alive for long intervals.

Cultures incubated at 30° could be maintained for nearly a week, after which they rapidly deteriorated. During the first few days

Figure 34 Effect of temperature on the fraction of retinal explants with outgrowth

Post-crush retinal explant cultures were grown on collagen gels at 35°, ⊖ ; 30° O ; 22° ● ; or 15° ◒ in humid chambers. A, neuritic growth; B, non-neuronal growth.

Figure 35 Effect of temperature on the average percentage of retinal explants with outgrowth

The average percentage of explants without growth during the first four days in culture was computed from data shown in Figure 34.

Neuritic growth ● . Non-neuronal growth O .

Figure 34

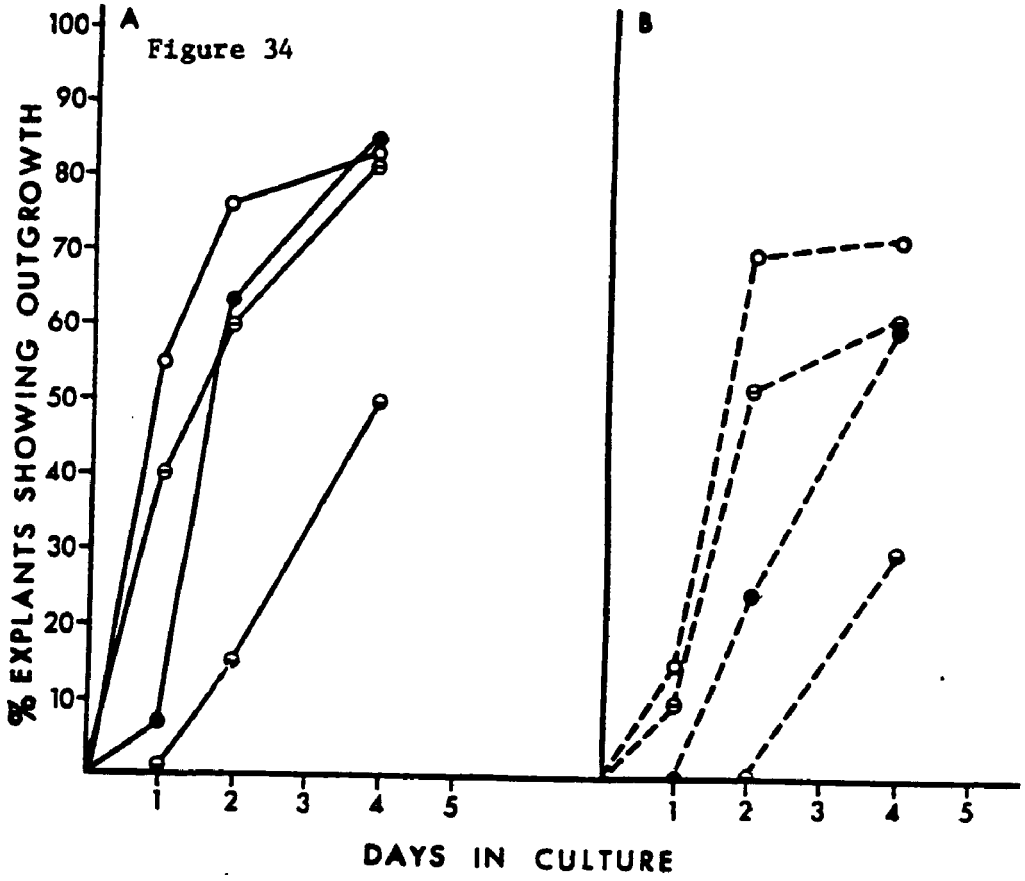
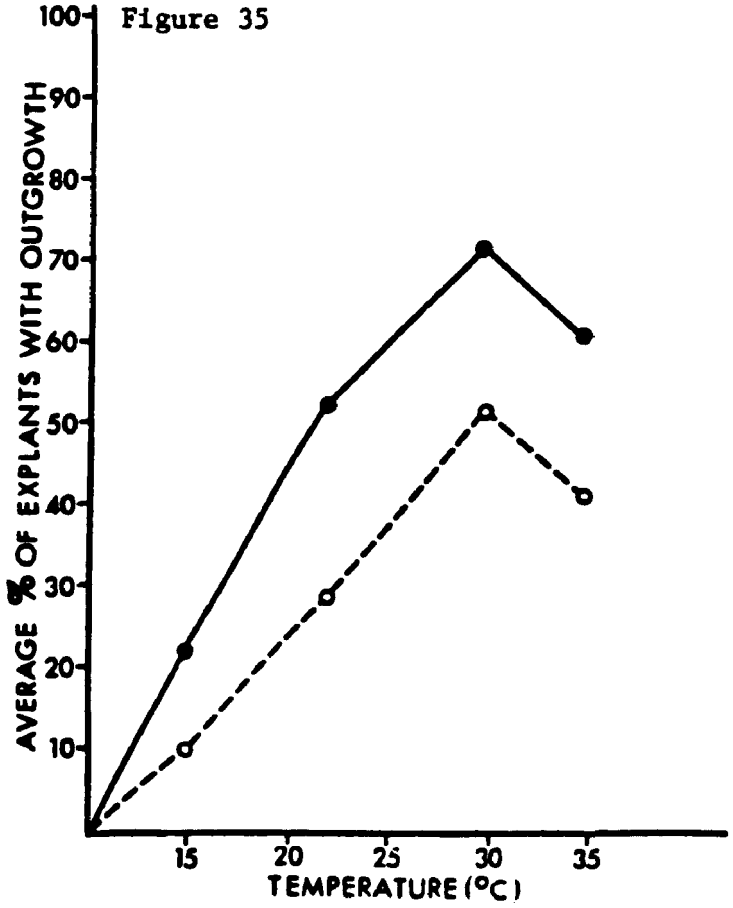


Figure 35



in vitro these explants grew well. This was not the case with cultures kept at 35°. Retinal explants did not survive at this temperature. Some explants grown at 35° initially grew a small number of neurites, but the explant rapidly died. This can be partially attributed to the hypertonic medium, however, even with frequent medium changes, the cultures did not look healthy.

Regional Differences in Growth of Goldfish Retina in Culture

The ability of different regions of the goldfish retina to support neuritic growth was examined in studies involving the dissection of the retina into defined areas.

The goldfish retina grows throughout life by adding cells at the ciliary margin, with chronologically "older" cells lying progressively closer to the optic disc. It was of interest to investigate the growth of peripheral retina compared to more central regions. The outer 1-2 mm of a whole goldfish retina was separated from the central retina with iridectomy scissors, and each prepared for explantation. The two groups were explanted into separate dishes on collagen gels. Peripheral retina from fish whose optic nerve had been crushed 15 days previously had nearly twice as much neuritic growth as the corresponding central retina explants (Figure 36).

If the data are expressed as the percentage of explants showing neuritic outgrowth (Figure 37), two features are apparent. The post-crush retinal explants, from either region, grow better than comparable explants from unoperated tissue, which is consistent with the original observation detailed in Part I. Secondly, the fraction of explants

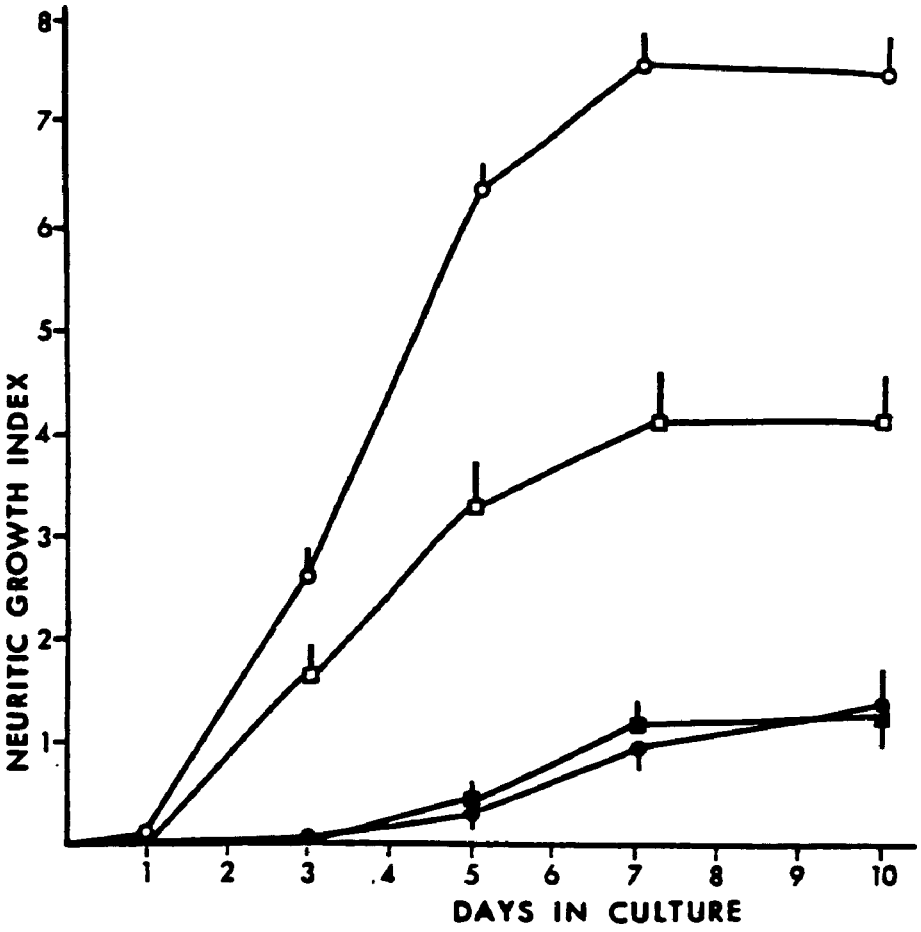


Figure 36 Neuritic growth from central compared to peripheral retinal explants

The NGI of explants obtained from post-crush and normal retina was measured. Explants were prepared from central or peripheral retinal regions. Post-crush retina: peripheral explants, O ; central explants, □ . Unoperated retina: peripheral explants, ● ; central explants ■ ($\pm$ S.E.M.).

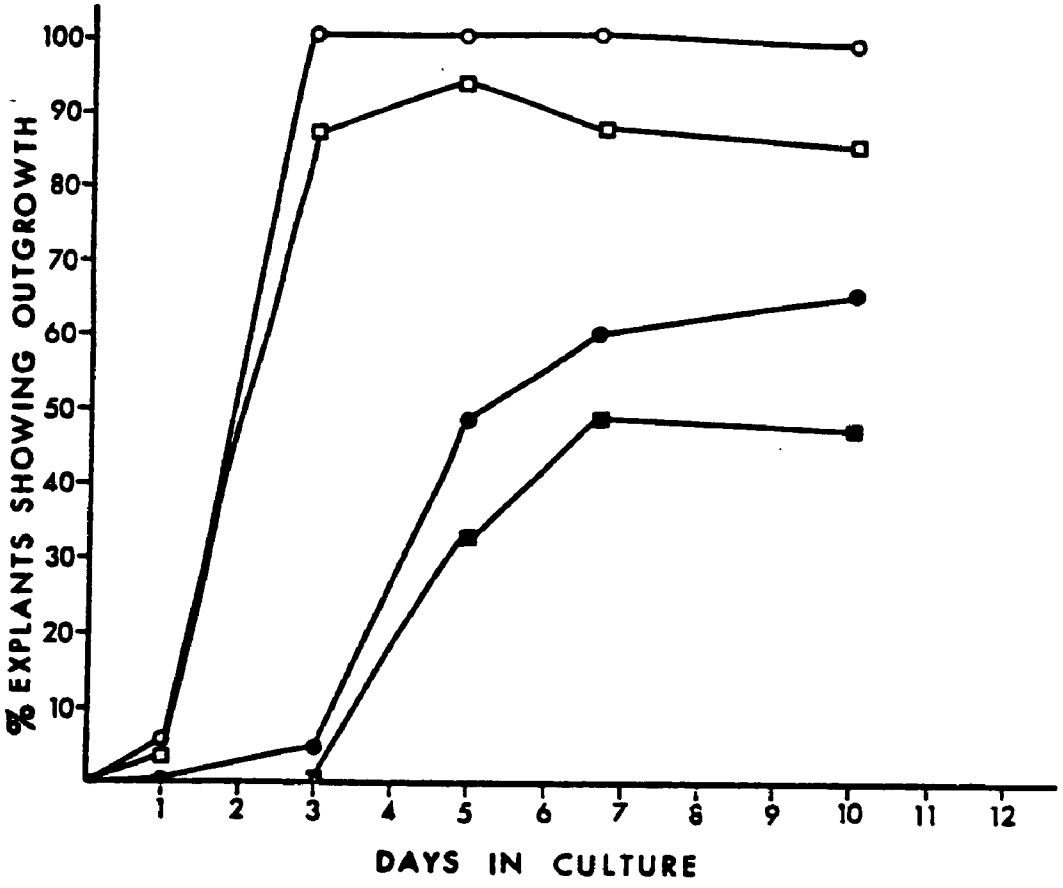


Figure 37 The fraction of explants with neuritic growth from central compared to peripheral retinal explants

The fraction of explants exhibiting neuritic growth from central and peripheral regions of the retina obtained from both post-crush and unoperated retina were determined after the indicated periods in culture. Post-crush retina: peripheral explants, ○ ; central explants, □ . Unoperated retina: peripheral explants, ● ; central explants, ■ .

demonstrating neuritic growth is larger in peripheral retinal explants than from central explants despite whether the explants are taken from post-crush or unoperated retina.

The large difference in the NGI values from the two regions could possibly be due to a small number of explants having exceptionally high NGI values, causing the elevation in the mean score. When the distribution of scores was examined (Figure 38), the peripheral explant NGI scores showed a similar shape but shifted to higher NGI scores compared to central explants. These data indicate that explants obtained from different regions of the retina differ in their ability to grow neurites.

Non-neuronal cellular growth demonstrates a similar pattern (Figure 39). The growth index of peripheral retina, either from unoperated or post-crush explants is higher than comparable explants from more central regions. The uniformly higher indices for post-crush explants here, as with the neurites, in part reflects the larger fraction of explants which will eventually exhibit growth (Figure 40).

The unoperated retina showed no difference between central and peripheral retina in their ability to grow neurites. The events which lead to the enhanced growth from peripheral explants in post-crush retina either are not operating in unoperated explants or were too small to be detected with the present scoring methods (Figure 36).

A separate study involved the dividing of the retina into dorsal and ventral halves and explanting the retina in separate dishes. Since the retina is a symmetrical structure, some means was required to indicate its original orientation in the eye. This was accomplished by puncturing the retina at one of its poles with a micropipet filled with

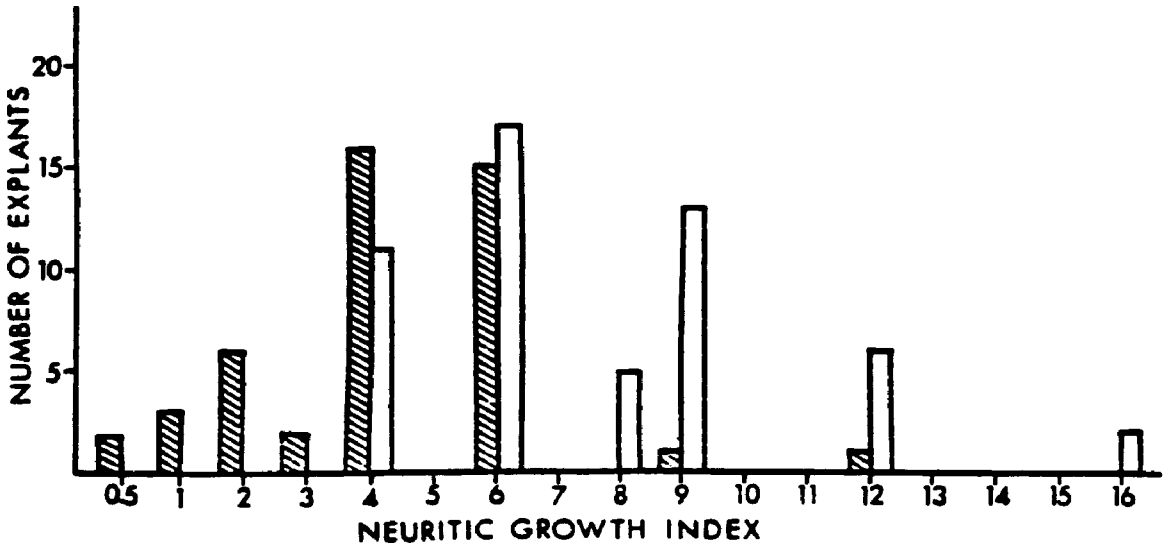


Figure 38 Distribution of NGI scores from central and peripheral retinal explants

Post-crush explant cultures from central and peripheral retina were grown on collagen gels. The distribution of NGI values was determined on day 5 in culture. The number of peripheral explants with a given NGI score are shown with open bars. Central explants are shown with hatched bars.

Figure 39 Non-neuronal growth from central compared to peripheral retinal explants

The growth index of non-neuronal cells was measured from cultures of post-crush and unoperated retina. Explants were prepared from central or peripheral retinal regions. Post-crush retina: peripheral explants, ○ ; central explants □ . Unoperated retina: peripheral explants ● ; central explants ■ , ± S.E.M.

Figure 40 The fraction of explants with non-neuronal growth from central compared to peripheral retinal explants

The fraction of explants exhibiting non-neuronal growth from central and peripheral regions of the retina obtained from both post-crush and unoperated retina were determined after the indicated periods in culture. Post-crush retina: peripheral explants, ○ ; central explants, □ . Unoperated retina: peripheral explants, ● ; central explants, ■ .

Figure 39

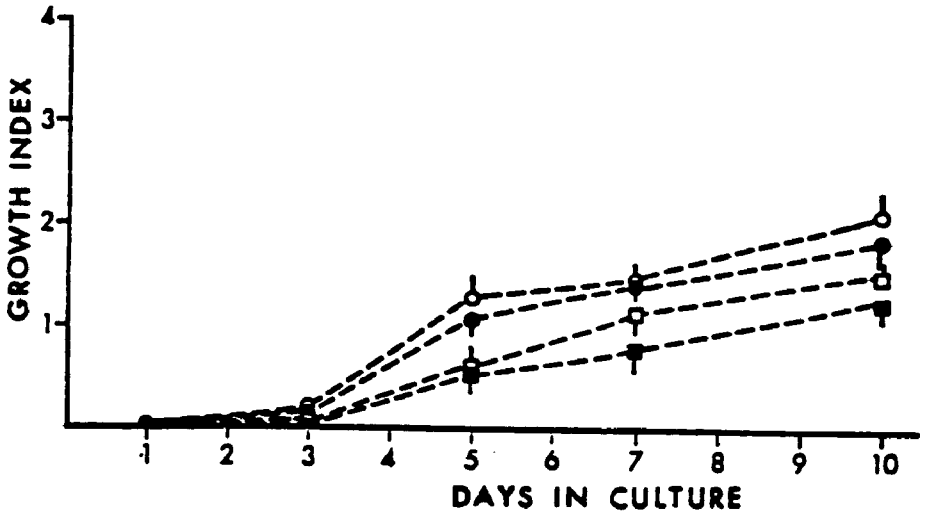
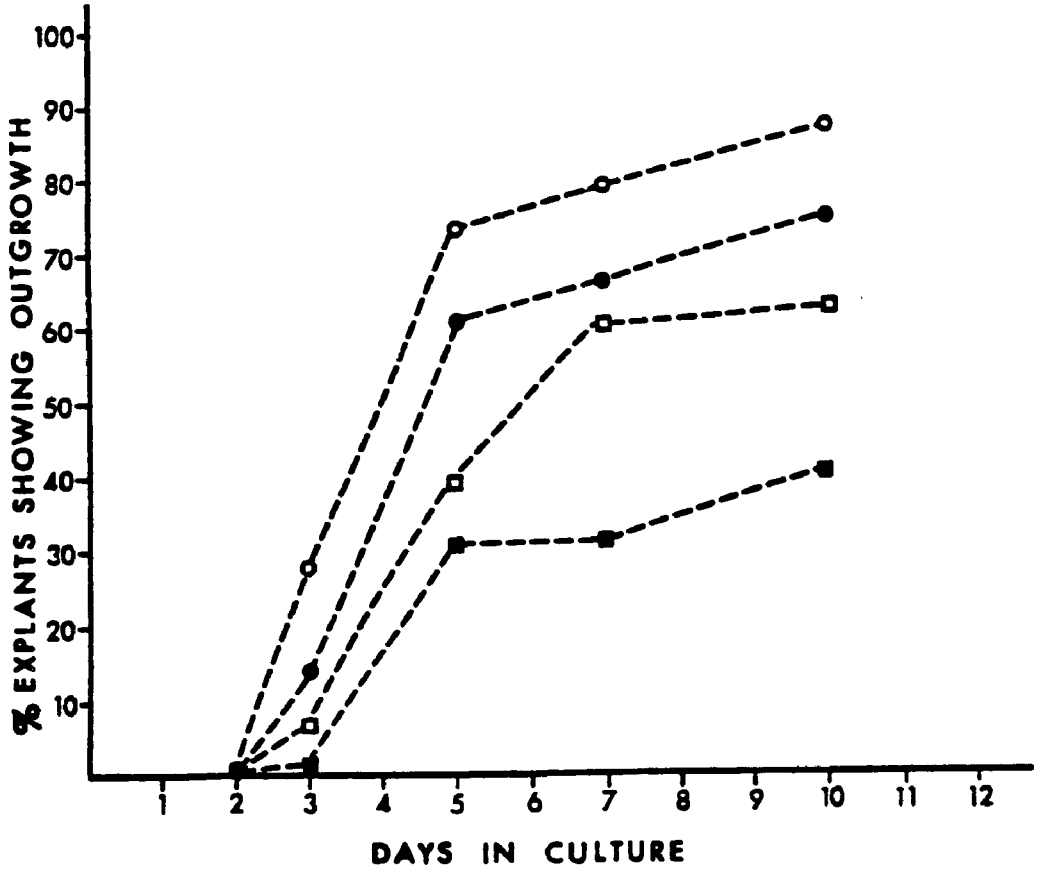


Figure 40



methylene blue (1% aqueous solution), leaving a small blue spot. The retina was then removed from the eye cup and later divided into the appropriate halves. The neuritic growth from the two retinal halves was identical as measured by the NGI or as the fraction of explants showing outgrowth (Figures 41 and 42). Growth of non-neuronal cells also did not differ in dorsal or ventral retinal explants.

A comparison was made of the growth from dorsal and ventral retina on the strength of data obtained in the developing chick which indicated differences between the two hemiretina. Cells from dorsal and ventral (but not nasal and temporal) retina could be distinguished by their preferential adhesion to one another in culture (Barbera et al., 1973; Gottlieb et al., 1976).

Figure 41 Growth from explants taken from dorsal and ventral retina
The neuritic growth from post-crush explants obtained from dorsal, ● and ventral, ■ retina was measured. The non-neuronal growth index, plotted on the same axis was determined from the same dorsal, ○ and ventral retinal, □ explants ± S.E.M.

Figure 42 The fraction of explants with outgrowth from dorsal compared to ventral retinal explants
The fraction of explants with neuritic growth from dorsal, ● and ventral, ■ retina were determined after the indicated periods in culture. Non-neuronal growth was measured from the same explants; dorsal ○ , ventral □ .

Figure 41

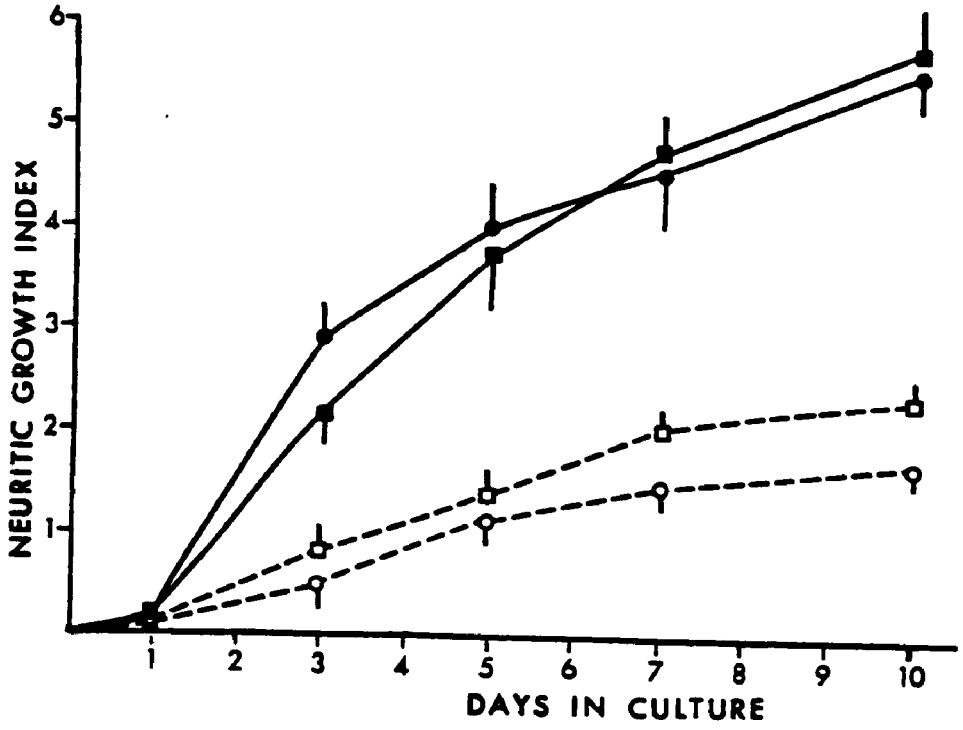
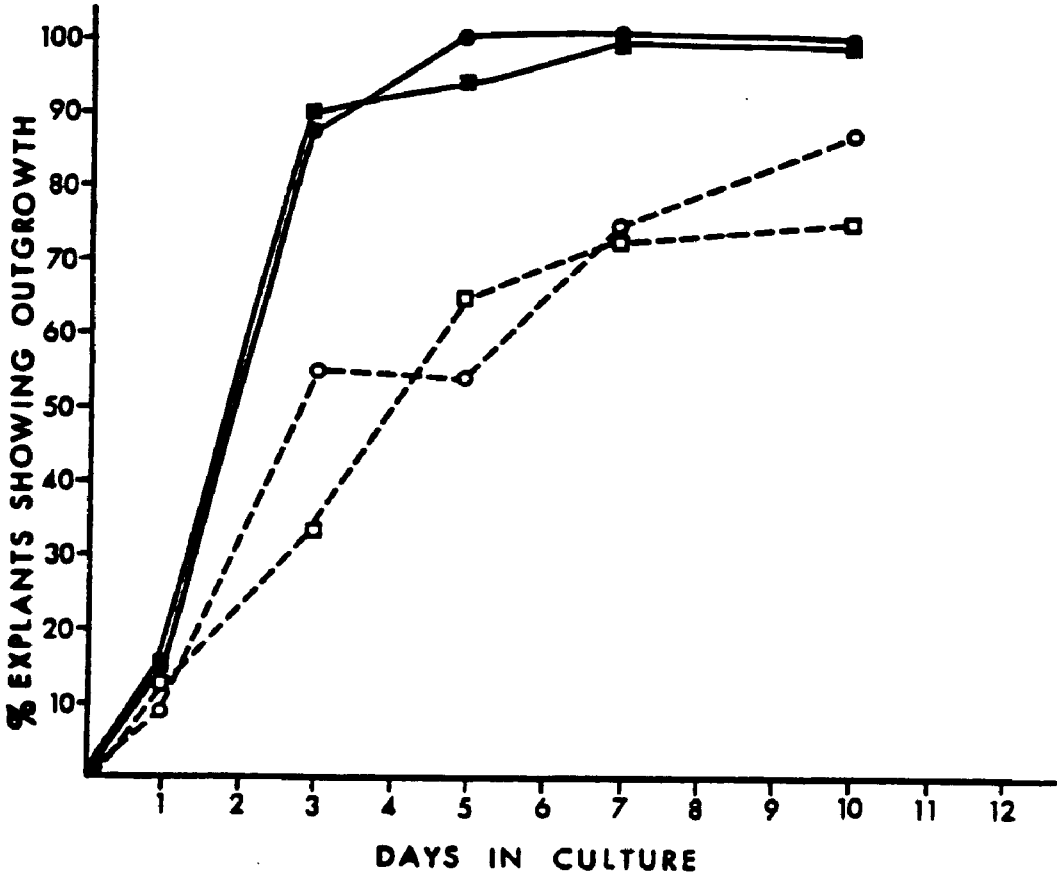


Figure 42



PART IV

Role of Diffusible Factors in Stimulation of Neuritic Growth from Retinal Explant Cultures

The mechanisms that induce the reinitiation of axonal growth following axotomy can be approached in tissue culture in a manner not practicable with *in vivo* studies. In the studies reported here, the role of diffusible factors in stimulating the regrowth of axons was examined from two experimental approaches: the use of surgical manipulation to selectively axotomize cells in a restricted area of a single retina, and the culture of control and experimental explants in the same culture dish. These studies were prompted by the observation of an apparent stimulation of neuritic growth in the unoperated eye during the first 72 h following crush of the opposite optic nerve (Figure 33).

The suggestion that there may have been changes in unoperated retina following optic nerve crush of the other eye is not without precedent. In a variety of paired nervous structures, axotomy or trauma to one side produces metabolic and morphologic changes in the symmetric contralateral structure. The best known example of this is sympathetic ophthalmoplegia (Sorsby, 1972). A penetrating wound to the eye can result in inflammation and loss of the opposite uninvolved eye 1-3 months following the initial injury. The mechanism by which this occurs is not understood.

Such observations are not new. Greenman (1913) reported that the section of peroneal nerve on one side resulted in morphological changes

in both the severed nerve and its uninvolved contralateral counterpart. The uninvolved nerve experienced a decrease in mean fiber diameter. Nittono (1935) found that if a peripheral branch of the right trigeminal nerve was cut, degenerating and regenerating fibers were found on the left side.

Austin and co-workers (1975; Kaye et al., 1975) found following axotomy of the nodose ganglion there were increases in acid phosphatase and carbamoyl phosphate synthetase activity on the cut side and similar, but smaller changes on the uncut side. Hamberger and Sjostrand (1966) reported similar alterations of succinate dehydrogenase activities following section of a single hypoglossal nerve. Enzyme activity of neurons in the hypoglossal nucleus on the operated side was elevated 600% over normal controls, while the unoperated side was increased 350%.

These studies point to unexplained changes in related, but uninvolved structures as a result of axotomy. Blood-borne diffusible factors have been suggested to explain these phenomena (Cragg, 1970), although there is no direct evidence for this in the nervous system.

Other tissues are capable of responding to blood-borne factors which appear as a result of injury, for example, the liver will regenerate after partial hepatectomy (Bucher, 1963). Regeneration is induced by a hormone in the blood which stimulates DNA and protein synthesis (Paul et al., 1972). This factor is not present in the normal animal and is produced in response to removal of part of the liver.

The growth and differentiation of the nervous system is known to be affected by several soluble factors present in the developing animal.

These agents have been found to act principally on embryonic tissue either *in vivo* or *in vitro*. The best studied of these is nerve growth factor (NGF), (reviewed by Levi-Montalcini, 1968). NGF is known to act principally on sympathetic nervous system, although central effects have been reported (Merrell et al., 1975).

Two separate factors have been reported to differentially affect cells in the central nervous system. Lim (1974, 1976) has investigated a soluble protein from adult brain that will induce the differentiation of glial cells *in vitro*. The transformation results in morphological differentiation into astrocyte-like cells. This is accompanied by biochemical differentiation indicated by an increase in glial-specific S-100 protein and elevated cAMP concentrations. The "Glial Maturation Factor" is reported to have no effect on neuroblasts, and is not present in immature brains.

Monard has described a glial cell-derived factor that induces morphological differentiation in neuroblastoma cultures (Monard et al., 1973). This factor is distinct from NGF (Monard, 1975), and is believed to play a role in central nervous system development.

The balance of the data indicate that the various growth factors which have been investigated are likely to be principally concerned with the developing nervous system rather than regenerative processes. It remains possible, however, that similar factors may act on regenerating neurons (West and Bunge, 1976).

The studies described below were designed to examine the possible effects of diffusible factors which may be present in the blood stream

or elaborated by the retina itself in response to optic nerve crush. The hemiaxotomized retina is an ideal preparation for this type of experiment. It is possible to cut the optic fibers from ganglion cells in restricted regions of the retina; thus within the same organ, half the ganglion cells are undergoing an axonal reaction while the remaining half are unaffected. If the ganglion cells whose axons have been severed elaborate diffusable factors intended to facilitate neuritic growth from the organ as a whole, then the uninvolved ganglion cells may be expected to exhibit enhanced neuritic growth.

The nasal half of the left goldfish retina was axotomized by ablation of the caudal half of the right optic tectum. The ganglion cells in the nasal hemiretina are clearly hypertrophied 12 days following surgery, while those in the temporal hemiretina appear normal.

Explants were prepared from these animals 7 or 12 days following surgery. The left eye was removed and divided into nasal and temporal halves and each was prepared for explantation on collagen gels. The explants from nasal and temporal hemiretina were cultured in the same or separate dishes.

Nasal (axotomized) retina exhibited vigorous neuritic outgrowth while temporal retina grew neurites poorly (Figure 43). The difference in neuritic growth from these tissues is similar to that observed when post-crush retinal explants were compared with those from the unoperated opposite eye (Figure 8). The data shown in Figure 43 is from retina explanted 7 days following surgery. Similar results were obtained from cultures explanted 12 days after surgery. Non-neuronal

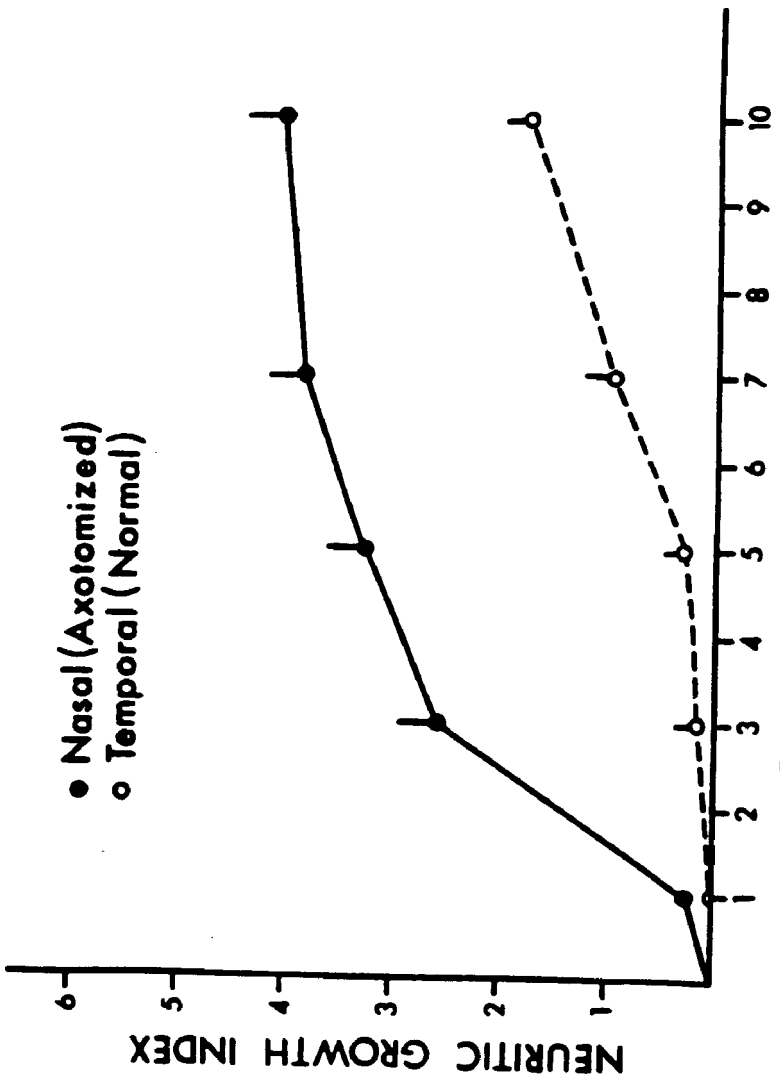


Figure 43 Neuritic growth from explants of hemiaxotomized retina
The right caudal tectum was removed from goldfish and 7 days later the retina was removed and divided into nasal and temporal hemiretina. Explants from each group were cultured in the same or different culture dishes.

cellular growth from nasal retina did not differ from temporal retina.

A parallel series of experiments were performed to illustrate the selective stimulation of neuritic growth from the nasal hemiretina in another way. The left retina from fish that had had the right caudal half-tectum removed 12 days previously was cut into 300 μm strips across the nasotemporal axis and carefully placed in a culture dish. Neurites grew exclusively from the axotomized hemiretina during first few days in culture (Figure 44). Neuritic growth from this preparation was less extensive than that from the 600 μm^2 explant cultures. Non-neuronal cells tended to migrate from the periphery of the retinal strip before appearing at other areas of the retina. This was a consistent observation during the first 3-5 days in vitro, after that period non-neuronal cells migrated away from the retina along its length.

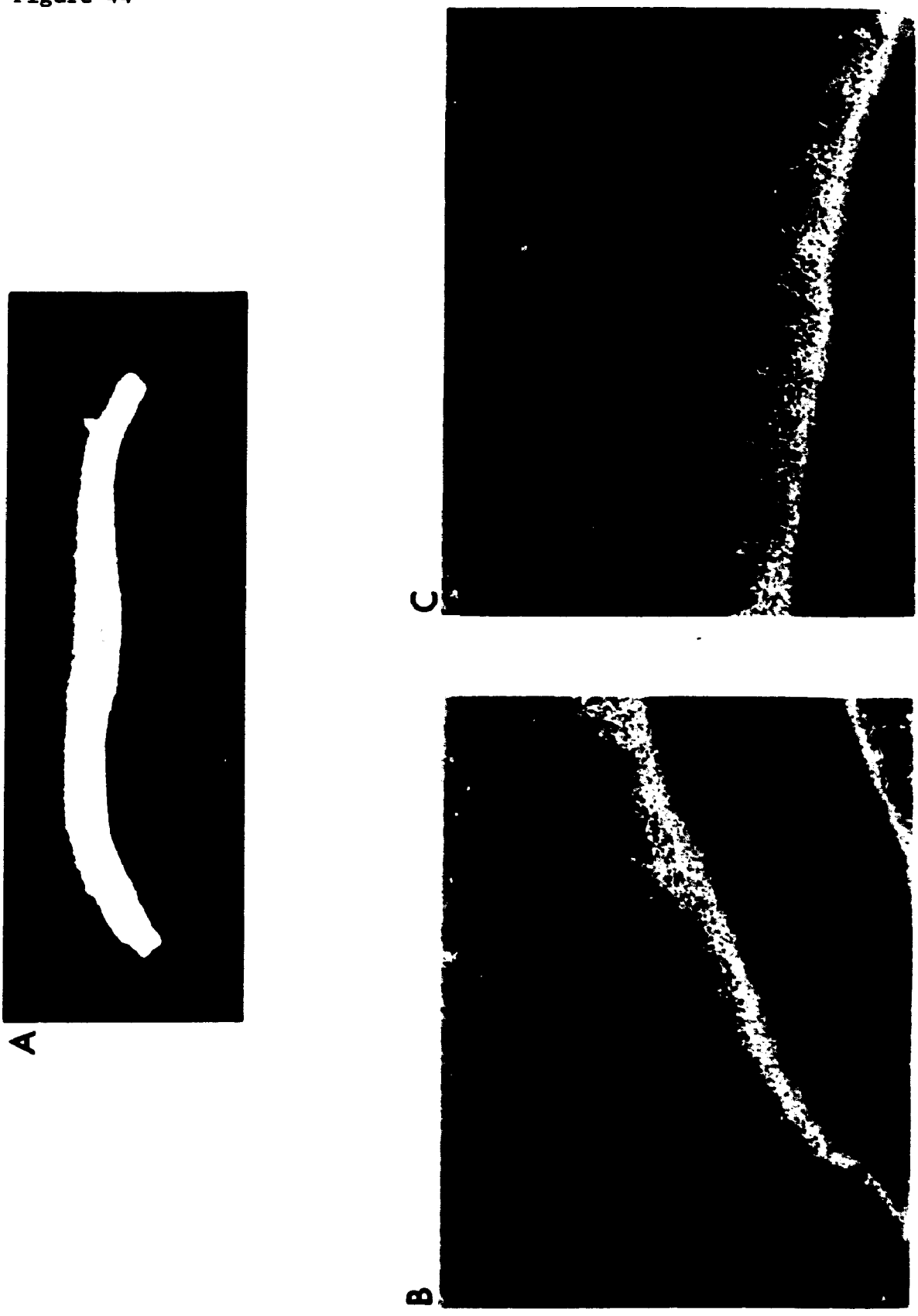
These data indicate that if diffusable factors are elaborated by axotomized ganglion cells they have no effect on the morphology of their neighbors in the opposite hemiretina, nor do they facilitate neuritic growth in that tissue. This supports the view that the axonal reaction is an intrinsic property of the ganglion cell.

The observation that at early post-crush intervals (24-72 h) the retinal explants from the opposite unoperated eye grew better than normal controls, and equally well as post-crush explants, suggested that crushing the optic nerve affected neuritic growth from the unoperated retina (Figure 33). If crushing of the optic nerve does result in changes in the opposite eye, then it is likely to be mediated through diffusible factors, although this is not the only possible

Figure 44 Neuritic growth from radial sections of hemiaxotomized goldfish retina

The right caudal tectum was removed from goldfish 7 days prior to explantation. The left retina was sliced into 300 µm strips parallel to the nasotemporal axis and explanted onto collagen gels. A. Retinal slice. Bar = 100 µm. Neuritic growth from the nasal (axotomized) hemiretina is compared to the B. temporal hemiretina after 2 days in culture.

Figure 44



explanation. It should be clarified that only during the first 72 h after optic nerve crush were these changes suspected. Longer post-crush intervals did not show a great deal of variation in NGI values from retina obtained from the opposite eye.

In the search for the putative growth factors, three experimental approaches were tried; the use of hemi-axotomized retina, culture of post-crush and normal retina in the same vessel and the supplementation of the nutrient medium with serum obtained from animals 24 h following optic nerve crush.

To test the possibility that trauma to the optic nerve results in facilitated neuritic outgrowth from the unoperated eye, the left retina was taken from fish whose right caudal tectum had been removed 24 or 72 h previously. Explants from nasal and temporal hemiretina were grown in separate dishes, as were control cultures from normal, untreated fish. If the surgery resulted in the stimulation of neuritic growth from uninvolved ganglion cells in the temporal hemiretina these explants should differ from explants obtained from normal fish in the amount of neuritic growth present. This was not the case. Temporal retinal explants grew no better than normal controls, and had a lower NGI than nasal cultures (Table IV). There is no evidence from these data to support the view that stimulatory factors are elaborated from axotomized retina.

In a separate series of experiments retinal explants from normal fish were grown in the same culture dish with post-crush retinal explants or explants from the unoperated eye. The latter tissues were obtained from retina taken from fish whose optic nerve had been crushed

TABLE IV

GROWTH FROM HEMIAXOTOMIZED RETINA AT EARLY POST-OPERATIVE INTERVALS

	Neuritic Growth Index	
Time following surgery	24 h	72 h
Nasal	1.28 (0.13)	0.82 (0.10)
Temporal	0.84 (0.11)	0.32 (0.05)

The right caudal tectum was removed from fish, denervating the nasal hemiretina. Twenty-four or 72 h later, the fish were killed and explants taken from both the hemiretina and cultured in separate dishes. The NGI was determined on culture day 7 ($\pm$ S.E.M.). Retinal explants from normal untreated fish had an NGI value of 0.63 $\pm$ 0.09 after 7 days *in vitro*.

24 or 72 h previously. These experiments were designed to discriminate between retinally derived stimulatory factors and blood-borne factors originating at other sites in the body. Neither the presence of post-crush, nor unoperated retinal explants had any effect on neuritic growth from normal tissue (Table V). Here, as with the experiments of hemiaxotomized retina, there was no demonstrable effect of any stimulatory factors on neuritic growth.

The stimulation of neuritic growth in the unoperated retina as a result of injury to the opposite eye could presumably be mediated via the blood. To investigate this possibility, blood was taken from fish 24 h after optic nerve crush, or from normal fish. The culture medium was supplemented with 5% fish serum and 5% FCS. However, as discussed previously, retinal explants failed to grow in the presence of 5% fish serum. The presence of a stimulatory factor in the blood could not be demonstrated by this method.

The data reported here are consistant with the thesis that the regrowth of nerve fibers is dependent upon mechanisms which are intrinsic to the cell and are not due to stimulation by extrinsic factors. Within the limits of the present scoring method, no diffusible factor which facilitates neuritic growth could be detected.

TABLE V

EFFECT OF CULTURING RETINA FROM FISH WHOSE OPTIC NERVE HAD BEEN CRUSHED IN SAME CULTURE DISH WITH NORMAL RETINAL EXPLANTS

		Neuritic Growth Index	
		Retinal explants from operated fish	Retinal explants from normal fish
A	post-crush	1.03 (0.12)	0.37 (0.09)
	unoperated	0.85 (0.10)	0.43 (0.11)
B	post-crush	0.54 (0.09)	0.24 (0.04)
	unoperated	0.57 (0.10)	0.25 (0.05)

Retina from fish whose right optic nerve had been crushed 24 or 72 h previously was explanted onto collagen gels. The culture dish was divided by a line drawn across the bottom of the dish, 8-12 retinal explants from post-crush or unoperated retina were placed on one side, and retinal explants from normal, untreated fish on the opposite side. The NGI was determined after 7 days in culture. Cultures from normal fish cultured alone had an NGI value of 0.63 ± 0.09 on day 7 in vitro. Experimental tissue was taken from animals whose optic nerve had been crushed. A, 24 h; B, 72 h, previously ($\pm$ S.E.M.).

DISCUSSION

The maintenance and growth of the goldfish retina in explant culture is a simple matter requiring no special equipment or techniques. This is largely due to the ability of the poikilotherm tissue to grow at room temperature, the selection of an air-equilibrated nutrient medium and an effective antibiotic.

The goldfish eye is a particularly advantageous preparations, in that it encloses the retina and other ocular structures in a thick scleral covering. This situation makes it possible to sterilize an otherwise highly contaminated tissue with a rather harsh method.

The extensive literature on the goldfish visual system and the retina's reproducibly good growth in vitro make this an attractive preparation for studies of nerve growth and regeneration. Champy (1913, 1914) pointed out the advantage of retina as an organ culture preparation noting that it is essentially a tissue "slice" of uniform thickness. Even in mammalian retina the thickness does not exceed 1 mm, allowing unimpeded exchange of gases and nutrients. The goldfish retina has several unique features which may facilitate its survival in vitro. The fish retina is avascular, that is, it has no penetrating blood vessels. The cells of the retina receive oxygen and nutrients by diffusion from vessels in the choroid and from vessels lying on the vitreal surface. Since these cells do not require intimate contact with vascular elements, it is unlikely that the culture situation imposes any hardship in this respect. The reliance of the teleost

retina (*in vivo*) on diffusion to rid itself of metabolic wastes suggests that the retinal cells will tolerate wide variations in the composition of the microenvironment, particularly during periods of intense metabolic activity. This may account for the pH insensitivity of this tissue *in vitro*.

Substrata

Retinal explant cultures have been found to grow well on collagen or polycation-treated substrata. Collagen supports vigorous neuritic growth and has been the principal substratum employed in these studies. Polycation-treated substrata were found to be much superior to collagen in promoting neuritic growth.

The mechanism by which the polycation polymer enhances cellular adhesion is thought to be through electrostatic interactions of a negatively charged cell surface with a positively charged amino group in the polymer. The plasma membrane carries a net negative charge (Weiss, 1970) which can be demonstrated to interact with polylysine (Wessells et al., 1976). If the free amino groups are modified by reaction with dansyl chloride, cells will no longer attach to treated surfaces (Yavin and Yavin, 1972). Experiments with amino acid polymers possessing negatively charged functional groups did not enhance cellular attachment (Yavin and Yavin, 1972; McKeehan and Ham, 1976).

Polycationic proteins obtained from natural sources, e.g. protamine and histone work as well as synthetic polymers in promoting non-neuronal cell attachment to treated substrata (Yavin and Yavin, 1972; McKeehan and Ham, 1976). Polylysine has been the principal

polymer employed in studies of cell adhesivity.

Polycation-treated surfaces for culture of goldfish retinal explants have met all the criteria initially set out. Most importantly, the growth of neurites is measurably better than that on alternative substrata (Heacock, A., unpublished observations). The use of these surfaces for retinal explant culture facilitates biochemical analysis by obviating the need for collagen which interferes in protein determinations.

Polycations are known to affect cellular metabolism by a variety of mechanisms. The ability of these polymers to facilitate cell growth and adhesion has been postulated to be related to increased permability of cell membranes in the presence of basic polymers (Macieira-Coelho and Avrameas, 1972). Moreover, polycations have been demonstrated to enhance the uptake of extracellular proteins (Ryser and Hancock, 1965), the uptake of polynucleotides (Schell, 1974) and facilitate the polymerization of microtubules (Erickson and Voter, 1976). The enhanced growth of retinal cultures on polycation-treated substrata is not likely to be due any intracellular action the polycations may have, as it has been demonstrated that all of the polycation present is bound to the plastic substrata (Yavin and Yavin, 1972). The basic polymers are cytotoxic to some cells (McKeehan and Ham, 1976).

The characteristics of the substratum on which the cultures grow, influences the degree of interaction between fibers, and consequently the extent to which neurites will collect into fascicles. When cultures were grown on polycation-treated surfaces neurites gathered into

large fascicles, while those grown on collagen were largely present as single fibers or in small bundles. Polycation-treated glass or plastic surfaces present no physical barrier to the interaction of individual neurites. The fibers are likely to be predisposed to cooperative interactions, since in vivo, they fasciculate readily and coalesce to form the optic nerve. Perhaps it should not be surprising that when no constraints are placed on the growth of a homogeneous population of fibers in vitro they will also tend to collect and grow together.

The physical characteristics of the collagen constrain the movements and interactions of the neurites. The collagen gels are porous enough that the fibers grow into the matrix and occupy the interstices between the collagen fibrils. The tendency of neurites to exist as single fibers or in small aggregates may be due to restrictive spatial characteristics of the collagen gel which inhibit association of fibers into fascicles. It is also possible that the neurites may preferentially adhere to the collagen fibrils, thus preventing interactions with one another. In vivo, the extracellular matrix, of which collagen is the major component, is believed to have a role in the guidance of growing axons (Weiss, 1955, Ebendal, 1976, 1977).

Current theories of neuronal specificity postulate that glycoproteins and glycosyltransferases confer positional specificity in the nervous system (Roth, 1976). The presence of a glycosylated collagen matrix has been thought to have a role in inductive phenomena in development (Hay, 1973) and may facilitate neurite adhesion and

interaction with collagen fibers.

The degree to which adhesivity to the substratum affects the extension of neuronal processes and cell migration is incompletely understood. The observation of these cells in tissue culture has allowed the examination of the factors which affect migratory processes. Neural cells in vitro generally do not migrate per se; that is, the cell body is immobile while neuritic processes are advanced over the substratum. Fibroblasts and other non-neuronal cell types migrate by moving the entire cell. While the behavior of the neuronal and non-neuronal cell types is different, similar events are thought to be occuring at the advancing edge of each. The adhesion of cells to the substratum is an energy-dependent phenomenon (Klebe, 1975; MeKeehan and Ham, 1976). The strength of these adhesions govern the movement of cellular processes. Non-neuronal cells will fail to migrate on surfaces to which they cannot adhere, for instance, agar (Strassman et al., 1973). When they are confronted with progressively more adhesive substrata, they preferentially move toward those with which they form the firmest attachments (Harris, 1972; Gail and Boone, 1972; Letourneau and Wessells, 1974). On very adhesive surfaces, non-neuronal cells are unable to move (Gail and Boone, 1972; Letourneau and Wessells, 1974).

The extension of neuronal processes differs in several important respects from that of non-neuronal cells. Neuritic extension can occur under conditions where adhesion is poor (Strassman et al., 1973) but the rate of neurite extension is enhanced by stronger attachment

(Letourneau, 1975a, 1975b). The dissimilar response of neurites and non-neuronal cells to substrates of variable adhesivity is undoubtedly related to the requirement of non-neuronal cells to break contact with the surfaces in order to migrate. Neurites remain adherent while growth and movement occur only at the advancing growth cone. This differential adhesivity is postulated to have a role in the formation of specific nerve connections (Letourneau, 1975a).

Role of Serum in Tissue Culture

The use of various sera to supplement synthetic media is a standard procedure in most cell cultures. Only a few cell lines have been grown and maintained in serum-deficient medium. While serum has been the predominent supplement of choice, other biological fluids have been used as well, e.g., chick embryo extract, lymph, ascites fluid, amniotic fluid, etc. Fetal calf serum is the most commonly used of the available sera due to its low toxicity and growth stimulating properties. The exact nature of the contribution of serum to the maintenance of tissues in culture is unclear.

In tissue culture, serum can generally be characterized as affecting cellular attachment or cellular nutrition and metabolism. The distinction between these two is not always clear. Serum components which facilitate growth by either mechanism are often referred to as growth factors. The initial discussion of serum growth factors will be limited to those which affect cellular adhesion, the latter discussion to those compounds which can be demonstrated to act on cellular metabolism.

The ability of serum to facilitate the attachment of cells to glass substrata was observed in early studies of human cell lines (Sato et al., 1957). The component responsible for the adhesion was found to reside in the α-globulin fraction. Further fractionation of normal sera resulted in the two separate reports of the identity of this factor. Leiberman and Ove (1958) reported that a single protein present in the α-globulin fraction would promote cell adhesion. Fisher et al. (1958), in an independent study, reported that the glycoprotein, fetuin, was the responsible agent and was distinct from the factor isolated by Lieberman on the basis of solubility and electrophoretic behavior. Fetuin has a galactosyltransferase activity which is believed to be responsible for the facilitation of cellular adhesion (Shur and Roth, 1975).

A recent study has shown that the addition of calf serum to tissue culture dishes results in eight major serum proteins binding to the plastic surface (Culp and Buniel, 1976). A protein co-migrating on SDS-polyacrylamide gel electrophoresis with bovine serum albumin (BSA) was the principal bound protein. Whether the attachment of cells to the plastic substrata is due to the proteins non-specifically altering the physical properties of the surface or is the result of cellular interaction with specific classes of the bound proteins has not been determined. Mizell and Bamburg (1976) were able to grow chick sensory ganglion on BSA-treated plastic surfaces while other substrata were unable to support growth (Lumsden, 1968).

Although numerous studies have implicated a variety of serum components as the active factors in stimulation of cellular growth and proliferation, the issue is far from resolved. Serum components are known to stimulate a variety of synthetic processes (Martin et al., 1969), with the responsible factors localized in protein fractions (Waymouth, 1967; Puck et al., 1968) or small molecular weight compounds (Rechler et al., 1976; Hayashi and Sato, 1976; Pickart and Thaler, 1973). The discussion here will focus on those serum components which may be relevant to our experimental system.

The suggestion that serum provides hormones necessary for the growth of cells in vitro is not a new one (see Temin, 1972). Sato and co-workers have recently reported that a combination of hormones will substitute for calf serum in supporting the growth of rat pituitary cells in defined medium. If thyrotropin-releasing hormone, triiodothyronine, transferrin and somatomedin were added to cultures, growth is commensurate with that in serum-supplemented medium. The pituitary cultures may be somewhat atypical in hormonal requirements. However, they suggest that the main function of serum is to provide essential hormones (Hayashi and Sato, 1976).

The stimulation of DNA synthesis and cell proliferation in some cell lines has been found to be directly proportional to the fetal calf serum concentration (Dulbecco, 1970). The stimulatory activity resides in a group of small peptides denoted as "multiplication stimulating activity." These peptides bind the same receptor on the cell surface as insulin. Both the serum peptides and insulin stimulate

^{3}H-thymidine incorporation in fibroblasts (Rechler et al., 1976). It is of some interest that NGF shares structural similarities with insulin, although NGF has not been found in fetal calf serum (Oger,1971).

The tripeptide isolated from human serum by Pickart and Thaler (1973) has been discussed previously. The failure to obtain enhanced growth with the synthetic tripeptide in goldfish retinal cultures may reflect a specificity of its stimulatory effect for mammalian tissue. The mechanism of the apparent inhibition of neuritic growth in goldfish retinal explants caused by the tripeptide is unknown.

Temperature

The data presented here have indicated that goldfish retina will grow over a wide temperature range. Fish tissues in culture have been reported to grow over a temperature range of 5° to 40°, and goldfish tissues from 20° to 37° (McKenzie and Stephenson, 1973; Wolf, 1973; Wolf and Quimby, 1969). The temperature range which fish explant cultures will tolerate is reported to be related to the environmental temperature range of the animal (Stephenson, 1968). Presumably by acclimating the donor to a specified temperature the tissues could be grown at a temperature of choice and the rate of growth controlled experimentally.

The inability of explants to grow at elevated temperatures may be related to the rapid temperature change. If goldfish are maintained at 21°, a sudden alteration of the water temperature to 35° is fatal. It is possible that the physiological requirements necessary for

growth at higher temperatures cannot be immediately met. Goldfish can be kept at 35° if the temperature is raised incrementally over several days (Springer and Agranoff, 1977).

Morphological Changes in Retinal Explants

Goldfish retinal explants undergo a series of morphological changes after being placed in culture. The explantation of this tissue results in the degeneration of a majority of cells which have processes that are intrinsic to the retina, while the cells that form the efferent tract appear to be preserved. Despite long periods in vitro, certain anatomical relationships are maintained while others are lost. Champy (1914) described similar histological changes in retinal explant cultures from the adult rabbit and tortoise. In those cultures the rod nuclei became pyknotic within 24 h following explantation. This was followed by pyknosis of cells in the INL. The bipolar cells survived longer than other cells in this strata. Ganglion cells did not degenerate as rapidly as the other neurons in the retina. These cells persisted as long as four days, well after the death of all other neural elements. Müller cells survived and were observed to undergo mitosis. These observations are entirely consistent with the results reported here.

In the goldfish retinal explants, the rapid deterioration of the rod nuclei may be a consequence of the damage to the photoreceptor outer segments occuring during the dissection. The degree to which these elements are damaged is uncertain. Large numbers of rod outer

segments are found in the dissection medium, suggesting that extensive damage to these processes is occurring as a result of the handling it receives during the removal from the eye cup and in transferring it through the various washes. Nevertheless, rod outer segments and cone mosaics can often be observed in those explants which have the photoreceptor side apposed to the substratum. Scanning electron micrographs of the explants show rod outer segments present after several days in vitro (Heacock, A. and P. Johns, unpublished observations).

The cells in the INL which persist in the retinal explants are tentatively identified as amacrine or Müller cells. This is based on their position at the outer border of the IPL and their morphology, taking into consideration that the culture situation alters conventional morphological criteria normally employed in identification.

The IPL remains intact throughout the culture period. This may be due to extensive ganglion cell dendrites distributed in this stratum as well as processes from the surviving INL cells. Amacrine and Müller cells both send processes into the OPL, although their contribution to the stability of the IPL is small since only a few of these cells persist. The loss of the OPL as a distinct stratum after a week in culture is a result of mixing of the cells present in INL and ONL. There are two possible explanations for this phenomenon: the OPL has lost its structural integrity and has collapsed, or cells are migrating through the tissue and collecting at the outer edge of the ONL. The structure of the OPL has undoubtedly been undermined by the degeneration of most of the cells extending processes into this

layer. The collapse of this layer would account for the observed cell distribution, but this is unlikely to be the case, since had this stratum been weakened to the point of collapse, spongiform degeneration would have been apparent.

The explant has two possible orientations, either the photoreceptor side or the ganglion cell side can be apposed to the substratum. The dying cells collect at the photoreceptor side regardless of the orientation of the explant. Were the combination of the cell layers due primarily to structural collapse of the OPL, it would have been unlikely to occur against gravitational forces.

The initial conclusion, after examination of this material was that dying cells were migrating into the ONL. The concentration of dead or dying cells in this stratum of the explant was associated with a drop in the number of these cells within the explant, presumably due to phagocytotic activity. This observation is not unprecedented. During normal development of the frog retina some cells die and migrate to the photoreceptor side of the retina where they are sloughed off or phagocytosed (Glucksman, 1965). If cell death is induced by irridiation a similar migration is observed, the dying cells congregate at the outer edge of the retina (Glucksman, 1940; Glucksman and Tansley, 1936). The migration of cells away from the INL is a more attractive hypothesis, since those cells which survive in the INL maintain their relative position in the explant, lying at the outer margin of the IPL and on the inner border of the layer of degenerating cells.

In the present experiments the cells of origin of the neurites have not been directly demonstrated, although the results strongly suggest that they arise from the retinal ganglion cells. The rapid appearance and vigorous neuritic outgrowth from the retinal cultures following prior optic nerve section suggests that the ganglion cells may be responsible. There is no reason to believe that optic nerve section would facilitate neuritic growth of the intrinsic retinal cells. Were these cells to issue neurites, they would not be expected to appear before 3-5 days in vitro. This is the minimum interval required for the growth of neurites from normal explant cultures.

Of the three retinal cell types which form extensive processes, the ganglion cell, the horizontal cell and the interplexiform cell, the latter two are intrinsic to the retina and send processes only short distances. The length of the neurites seen in culture also militates against them being the source of the neuritic growth.

The most compelling evidence that the ganglion cells are the source of the neurites is the observation that after several days in culture they are the only group of cells surviving in both normal and post-crush explants. The ganglion cells remain healthy during the period in which nearly all of the intrinsic retinal cells are dying.

The small number of INL cells which persist in these cultures could form neurites. These cells have been tentatively identified as either amacrine or Müller cells. Amacrine cells, as the name implies, have no axons in vivo so it is unlikely that they could

account for the neuritic outgrowth. Müller cells are glial elements.

The morphology of the ganglion cell can be directly correlated with the retinal explant's ability to grow neurites. The marked hypertrophy of these cells in the post-crush retina and the rapid appearance of neurites indicate that the ganglion cells are mediating the neuritic outgrowth. The response of the normal retina in culture provides more substantive evidence, since the hypertrophy of the ganglion cells can be directly correlated with the growth of neurites from this tissue.

The conclusion that the ganglion cells are the cells of origin of the neurites can be made with some certainty. While no single piece of evidence is definitive proof, the data when taken together, support such a conclusion. This problem awaits satisfactory histological and experimental techniques to demonstrate continuity between the neurites and ganglion cells.

Why then do all neuronal elements, save one, die during the first few days in culture? A satisfactory explanation can be made to account for the present evidence based on the behavior of adult tissue in vitro and cellular mechanisms of regeneration.

It can be generally stated that adult neurons do not survive in vitro. The reasons for this are obscure. The degeneration of the photoreceptor nuclei in the ONL and the loss of the other cell types occupying the INL are not atypical responses, and are entirely consistent with the balance of data existing on culture of adult nervous tissue. The anomalous result is the survival of the ganglion cells.

The ability of the ganglion cell to regrow its axon following section and the survival of these cells in culture are not unrelated. Regeneration represents a flexibility which permits a recapitulation of some of the events which occurred during development (Price and Porter, 1972; Griffith and Lavelle, 1971). While I would hesitate to describe this as dedifferentiation, certain features of the process are directly analogous, e.g., growth of axonal processes. The axotomized ganglion cell shares with embryonic and differentiating cells an enhanced biosynthetic capacity which may be responsible for the selective survival of these cells in retinal explant cultures.

The studies of Grafstein and Murray on the response of the goldfish retinal ganglion cell to axotomy, demonstrate morphological and biochemical changes which parallel the ability of the retina to grow neurites in culture. Enhanced protein synthesis required for the regrowth of the optic nerve can be demonstrated as early as 24 h following optic nerve section and steadily increases for more than three weeks (Grafstein and Murray, 1969; Grafstein and Alpert, 1976). Similarily, RNA labeling is maximally stimulated within 7 days, corresponding with an increase in the number of nucleoli present and a rise in the amount of free ribosomes (Murray and Grafstein, 1969; Murray, 1973). The predominant structural elements of the axon are microtubules, which are also intimately involved with axonal transport. The growth of the neurite is dependent upon assembly of microtubules for mechanical stabilization of the fiber and axonal flow for continuous supply of material to the growing tip. Following optic nerve crush <u>in vivo</u>, the rate of rapid axonal flow rapidly increases, doubling over 14 days. The slow phase of axonal flow is also accelerated

as soon as 6-8 days following section of the optic nerve (Grafstein, 1971). The synthesis of the microtubule subunit protein, tubulin, is selectively enhanced (Heacock and Agranoff, 1976). The increase in tubulin synthesis parallels that of the rate of axonal flow, beginning at about 5 days and steadily increasing for another 10 days.

The optimal post-crush interval for the growth of neurites from goldfish retinas in culture is approximately 12 days. The enhancement of neuritic growth due to section of the optic nerve is first demonstrable after 5 days. Similarly, normal (unoperated) retinal explants first issue neurites on or about culture day 5. This lag period corresponds well with the enhancement of macromolecular synthesis in the ganglion cell, suggesting that the normal ganglion cell does not possess sufficient endogenous stores of cellular constituents to support neuritic outgrowth and approximately 5 days are required to activate cellular mechanisms required for initiation of a new axon. The activation of the molecular events which subserve axonal regeneration are believed to occur at transcriptional levels. The diminished ability of the retinal explants to issue neurites at post-crush intervals greater than 12 days may reflect the decrease in biosynthetic activities in the ganglion cell (Figure 33). The ganglion cell *in vivo* shows an enhanced macromolecular synthesis which decays, returning to normal levels 30-90 days later. The period over which the synthetic processes of the cell is facilitated is sufficient for regrowth of the optic nerve and restoration of functional behavior. The decreased biosynthetic capacity of the axotomized retinal ganglion cell after several weeks *in vivo* may be responsible for the diminished ability of the retinal explants to issue neurites *in vitro*.

Since both normal and post-crush explants will grow neurites, why then does normal retina never achieve NGI values comparable to post-crush retina after sufficiently long periods in culture? Histologically after 12 days *in vitro*, the post-crush and normal retina are indistinguishable. The normal ganglion cells hypertrophy to the same degree as those from post-crush retina. Two explanations can be offered, neither of which is entirely satisfactory.

It is possible that the culture conditions may not be optimal for the prolonged maintenance of goldfish *in vitro*. Essential nutrients or co-factors that are required for mammalian cells may differ from those of goldfish. The growth of neurites from post-crush retinal cultures occurs largely during the first week in culture. Only modest increases in NGI occurring in the subsequent week, with death ensuing after or during the third week in culture. The ganglion cells may only have sufficient stores of endogenous nutrients to sustain growth for a period of several days, following which synthetic processes cannot support vigorous neuritic extension. By the time normal ganglion cells have engaged the cellular machinery required for axonal regrowth, the conditions imposed by the culture situation become limiting, resulting in poor neuritic growth. The continued hypertrophy of retinal ganglion cells in the normal retina argue against this. It is unlikely that the processes which subserve cellular hypertrophy are entirely distinct from those required for neuritic growth.

Another explanation for the disparity in neuritic growth between the normal and post-crush retinal explants may relate to the adult origin of these cells. A case has been made for the selective survival of ganglion cells on the basis of their response to axotomy. While we

believe this is true, these are nevertheless adult cells. The factors which are responsible for the degeneration of the intrinsic retinal cells may be operating in the ganglion cells as well, and their ability to regenerate only delays the process. Observation of neuritic growth from embryonic Xenopus retinal cultures support this view (Agranoff et al., 1976). The unoperated normal eye, after a lag period, generally has as much neuritic growth after two weeks in culture as the post-crush retinal cultures. This suggests that the embryonic ganglion cells survive and are capable of vigorous neuritic outgrowth even after long periods in vitro.

The wide variation in NGI values of individual retinal explants has been a continuing difficulty. The source of this variability has only been partially evaluated. The present evidence indicates that the region of the retina from which the explant is taken, affects the ability to grow neurites. The disparity in growth from central and peripheral regions of post-crush retinal explants may be related to the manner by which the goldfish retina grows. New cells are continually being added at the ora serrata throughout the life of the fish. This results in the youngest cells being positioned at the periphery while older cells lie toward the optic disc. The facilitated neuritic growth from peripheral retinal explants is consistent with the observation that regenerative responses to axotomy are much more vigorous in the young (but not neonatal) animal compared to adults (Lieberman, 1974). To attribute the better growth of peripheral retina to this "younger" population of cells is conjecture based solely upon the pattern of growth in the fish retina.

The great biological variability of goldfish is also recognized. In most experiments reported here explants were taken from retinas of more than one fish. The difference between individual animals in the ability of the retina to grow neurites may cause some scatter in the distribution of NGI scores. However, this is not the only explanation. Explants taken from a single organ also show wide variation in the amount of neuritic growth which develops.

There is no final solution to the problem of variability in neuritic growth between individual explants. It complicates experimental analysis and requires that large numbers of explants be used in each experimental group. This problem is particularly troublesome in evaluating cultures with low NGI scores, for example, neuritic growth from normal retinal explants. The NGI is not sensitive enough to permit valid comparisons to be made at low NGI values. The difficulty lies not with NGI as a measure of neuritic growth, but with the variation in neuritic growth from individual retinal explants. The difference in neuritic growth between explants at low NGI values can frequently be a factor of six. There is no advantage to a more sensitive assay when the biological variability would preclude any firm conclusions being drawn from such data. The NGI as a quantitative measure of neuritic growth is not ideal. The evaluation of the growth from the retinal explants is dependent upon subjective judgments, a situation that is less than desirable when a quantitative assay is required. The index has been sufficient for the goals of the present study; that is, to provide an accurate, rapid and reproducible

measure of neuritic growth from retinal explant cultures. We have attempted to minimize the variation inherent in this type of analysis by using large numbers of explants in each experimental group and comparing neuritic growth with a standard atlas containing photographs illustrating growth characteristic of each scoring catagory.

The procedures that have been adopted are similar to others reported in the literature for sensory ganglia (Mizell and Bamburg, 1976; Levi-Montalcini, 1968).

The primary intent of these studies was to develop an in vitro model for the study of nerve growth and particularly as it applies to regeneration of the central nervous system. This study has demonstrated that the morphological changes that occur as a result of optic nerve section in vivo can occur in vitro. Normal retinal explants, after a sufficient period in culture, demonstrate the two morphological criteria which distinguish ganglion cells in the post-crush retina; cellular hypertrophy accompanied by increased cytoplasmic basophilia, and neuritic outgrowth.

The rapid appearance of neurites from the post-crush retina in culture is only the visualization of the axonal regrowth which was occurring in the animal at the time of explantation. The vigorous growth of neurites from post-crush retina offers a unique opportunity to examine in vitro fibers whose behavior in vivo has been well studied. Moreover, these fibers should be predisposed to cooperative interactions since they derive from a homogeneous population of cells

which normally coalesce to form the optic nerve. The study of these interfiber interactions is facilitated by the absence of myelinated fibers, and at least during the first days in culture the absence of other non-neuronal cell types.

The use of tissue culture to examine the selective formation of connections between two neuronal populations represents a major step toward understanding the molecular mechanisms which are responsible for these processes. Recent studies have examined this selective affinity at several levels. Bunge (1975) has described the formation of synaptic contacts at the ultrasturctural level, while Glaser and co-workers have employed biochemical techniques to study complementary surface membrane characteristics between different regional populations of retinal cells (Gottleib et al., 1976; Merrell and Glaser, 1973). Crain has described the selective innervation of spinal cord explants by dorsal root ganglia in culture (Peterson and Crain, 1975).

The culture of the goldfish visual system has reached a point where studies directed toward examination of the mechanisms subserving the formation of retinotectal connections are feasible and remain only to be executed. It is possible that the relevance of the present work may in the end be related to the furtherance of studies of neuronal specificity <u>in vitro</u>. The work of Deboni (1976) on explant and single cell culture of goldfish brain, including the optic tectum, sets the stage for such studies. It is possible that this type of approach may bridge the conceptual gap separating the biochemical experiments of Glaser from physiological studies of retinotectal specificity.

CHAPTER III

METABOLIC AND INTERVENTIVE STUDIES

INTRODUCTION

A major objective of this study was to develop a model system for the study of nerve growth and regeneration which is amenable to biochemical manipulation and analysis as well as direct morphological observation. The use of explant cultures permits metabolic and interventive studies on an isolated tissue, which has the further advantage that drugs or radioactive precursors can be administered at known concentrations.

In studies described here two drugs which affect the fibrous proteins of the neurite were examined. Cytochalasin B is known to selectively disrupt microfilaments, while colchicine prevents the assembly of microtubules. Both microfilaments and microtubules are major structural elements of the axon. The effects of these drugs on neuritic growth and morphology will be described.

Metabolic studies, involving the labeling of retinal proteins were performed. Of particular interest was the labeling of tubulin, the microtubule subunit protein, in post-crush retinal explants which are in the process of actively growing out neurites. In the course of these studies, a labeled protein was found in the culture medium. Subsequent experiments determined that the incorporation of radioactivity into this protein was the result of non-specific binding of labeled precursor to bovine serum albumin.

MATERIALS AND METHODS

Materials

Cytochalasin B, (B Grade) was purchased from Calbiochem. Colchicine and dimethylsulfoxide (DMSO) were from Sigma. Radioactive amino acids, L-(methyl-^{3}H)-methionine (14.6 Ci/mmole), L-(^{35}S)-methionine (407 Ci/mmole) and L-(4-5-^{3}H)-leucine (57 Ci/mmole) were obtained from New England Nuclear.

Sodium dodecyl sulfate (SDS, electrophoresis purity) was supplied by BioRad. Reagents for polyacrylamide gels were obtained from the following sources: acrylamide and bis-acrylamide, Eastman Chemicals; urea, Mallinckrodt and tetraethylmethylenediamine (TEMED), Sigma.

Cycloheximide (Actidione) was obtained from Nutritional Biochemicals. Purified bovine serum albumin (crystallized, Pentex) was from Worthington. RNase and ovalbumin were from Sigma. Triton X-100 was purchased from Rohm and Haas. Nonidet P-40 was from BDH Chemicals.

Methods

Explant cultures from adult goldfish retina were prepared as previously described and grown in L-15H on collagen gels or films, or on polylysine-treated plastic surfaces. The right optic nerve of these fish had been crushed 15 days prior to explantation. Explants from the opposite (unoperated, left) eye served as controls.

Labeling of retinal protein

Retinal explant cultures were routinely incubated with labeled precursors for 24 h at room temperature from culture day 4 through 5. In those experiments in which shorter incubation periods were used, labeling occurred on day 5 in culture.

The labeled methionine was evaporated to dryness under N_2 and redissolved in the incubation medium of Dunlop et al. (1975, described in Chapter II). Post-crush retinal explants received 50 µCi of ^{35}S-methionine, adjusted to the same specific activity as that of the ^{3}H-methionine which was given to control explants. The incubation was terminated by removal of the incubation medium, followed by washing the cultures twice with unlabeled Dunlop's medium containing 2 mM methionine. The explants were mechanically dislodged from the substratum and transferred to a glass-to-glass homogenizer. Explants from post-crush and normal retina were combined and homogenized in 0.01 M sodium phosphate buffer (pH 7.1) containing 1 mM EDTA. Normally, an unlabeled goldfish retina was homogenized together with the explants to act as carrier. The homogenate was centrifuged at 100,000 X g for an hour at 4°. The resulting supernatant, containing soluble proteins, was made 0.2% in SDS and dialyzed overnight against one liter of a 0.2% SDS solution.

The pellet was resuspended in 0.01 M sodium phosphate buffer (pH 7.1) containing 0.2% Nonidet P-40 and homogenized. The homogenate was allowed to stand at 4° for 30 min prior to centrifugation for 1 h at 100,000 X g. The resulting supernatant was made 0.2% in SDS and

dialyzed as described above. The pelleted material remaining after the second centrifugation was resuspended and then homogenized in 0.2% SDS. The homogenate was centrifuged at 100,000 X g for one hour and the supernatant treated as described previously. The pellet was discarded.

The dialyzed supernatants were lyophilized and then resuspended in a small volume of 0.1 M sodium phosphate buffer (pH 7.1) containing 1 mM EDTA. This solution was heated at 80° for 5 min. Urea was then added to a final concentration of 8 M. Mercaptoethanol was added to a final concentration of 1%. This sample was then directly applied to polyacrylamide gels for electrophoresis following the addition of 20 µl of 0.033% bromophenyl blue tracking dye.

In those experiments in which the proteins present in labeled incubation medium was examined by polyacrylamide gel electrophoresis (PAGE), the medium which was removed from the cultures was dialyzed for 36 h against three changes of water at 4°. The non-dialyzable material was lyophilized and resuspended in a small volume of 0.1 M sodium phosphate buffer (pH 7.1) containing 1 mM EDTA and 1% SDS. This solution was heated at 80° for 5 min. Urea was added to a concentration of 8 M, the solution made 1% in mercaptoethanol and tracking dye added. This sample was then subjected to PAGE.

Urea Treatment of Dialyzed Medium

A volume of dialyzed incubation medium was brought to a final concentration of 8 M in urea, 1% in mercaptoethanol and either directly applied to filter paper discs or placed in a dialysis bag and dialyzed against water at 4° for 24 h.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gels were prepared by the method of Weber and Osborn (1969), modified by the addition of urea (Heacock and Agranoff, 1976). Glass tubes 12 cm long having an inside diameter of 5 mm were filled to a height of 10.5 cm with a solution containing 5% acrylamide, 0.2% bisacrylamide, 0.1 M sodium phosphate buffer (pH 7.1), 1.4 mM TEMED and 0.003% ammonium persulfate. The gels were allowed to stand overnight then prerun 30 min at 6 mA per tube prior to addition of the sample. The running buffer was 0.1 M sodium phosphate buffer (pH 7.1) containing 0.1% SDS. The protein samples (100-300 µg protein) were separated by PAGE at 6 mA per tube until the tracking dye had reached the bottom of the gel. The gels were fixed in methanol:acetic acid: water (6:3:1) for 2 h at 37°, then stained with 1% amido black or cut into 1 mm slices for the determination of radioactivity. Polyacrylamide gel slices were dissolved by incubating each slice at 60° in 0.4 ml of 30% hydrogen peroxide overnight. Toluene-based scintillant (10 ml) and 0.1 ml glacial acetic acid were added and the radioactivity determined. Molecular weights were estimated by calibration, using bovine serum albumin (BSA, 68,000 MW), ovalbumin (45,000 MW) and RNase (13,600 MW) as standards.

Chromatographic Procedures

A Sephadex G-50 column was prepared (1.2 cm X 45 cm) and the sample was applied, then eluted with water. Material eluting with the void volume was discarded and three 20 ml fractions collected. The ion exchange resin (Dowex 50-W, 200-400 mesh) was poured into a 0.7 cm X 7 cm column and rinsed with water until the eluant was at neutral pH. The fractions from the Sephadex column were then applied to the ion exchange resin and the column washed with water. The sample was eluted with 1N NH_4OH. The NH_4OH eluant was lyophilized, redissolved in small volume of water and analyzed by paper chromatography.

Descending paper chromatography was carried out in a solvent system of butanol:acetic acid:water (4:1:1:v/v) for 18-24 h at room temperature. Samples were applied to Whatman #1 filter paper with carrier methionine. Methionine and methionine sulfoxide standards were run in separate lanes, and visualized with ninhydrin. Chromatograms with radioactive samples to be measured were cut into 2 cm strips, beginning 1 cm above the origin, placed in individual scintillation vials and radioactivity measured.

Trichloroacetic acid (TCA)-precipitable radioactivity was determined by a modification of the method of Mans and Novelli (1961). An aliquot of the sample was applied to 4 cm discs of Whatman #2 filter paper and allowed to dry. The discs were washed twice, singly or together in cold 10% TCA (10 ml per disc) on a rotary shaker for five minutes. An aliquot of the first TCA wash was counted for determination of TCA-soluble radioactivity. The discs were incubated in an

additional 10 ml TCA for 30 min at 80°. The samples were lipid extracted with two, 5 min washes of ether-ethanol (4:1, 10 ml per disc) and finally washed with ether and air-dried. Tritium labeled samples were oxidized in a Packard Tricarb 306 sample oxidizer. For samples labeled with ^{35}S, the discs were cut into quarters and placed into a scintillation vial containing 1 ml of Soluene (Packard). The vial was heated to 60° for 1 h before scintillant was added. Protein was determined by the method of Lowry et al. (1951).

Scintillation Counting

Radioactivity was determined in a Packard Tricarb (3 channel) scintillation spectrophotometer. Counting efficiency was evaluated by comparison with an external standard. All samples were counted in a toluene-based scintillation mixture containing 26% Triton X-100 (v/v). Each vial contained 10 ml of scintillant, having 50 mg of 2, 5-diphenyl-oxazole (PPO) and 1 mg of 1, 4 bis-2-(4-methyl-5-phenoxazolyl)-benzene (POPOP).

Tritium was measured with an efficiency of approximately 17%, ^{35}S with an efficiency of 65% when both isotopes were measured simultaneously. The ^{35}S counts appearing in the tritium channel were approximately 10%. Samples containing tritium alone were counted in a tritium channel with an efficiency of 25-30%.

PART I

Interventive Studies

Effect of Cytochalasin B on Neurites and Non-neuronal cells

Cytochalsin B is a fungal metabolite which will disrupt the 4-6 nm microfilaments present within the cell (Yamada et al., 1971). The effect of cytochalasin B on neuritic and non-neuronal elements of retinal explant cultures was examined by phase and interference contrast microscopy.

Post-crush retinal cultures were grown on polylysine-treated plastic or glass surfaces and photographed at various intervals following administration of the drug. Cytochalasin B was dissolved in DMSO and stored at 4°. The drug was diluted to 10 µg/ml with L-15H and added to the culture dish.

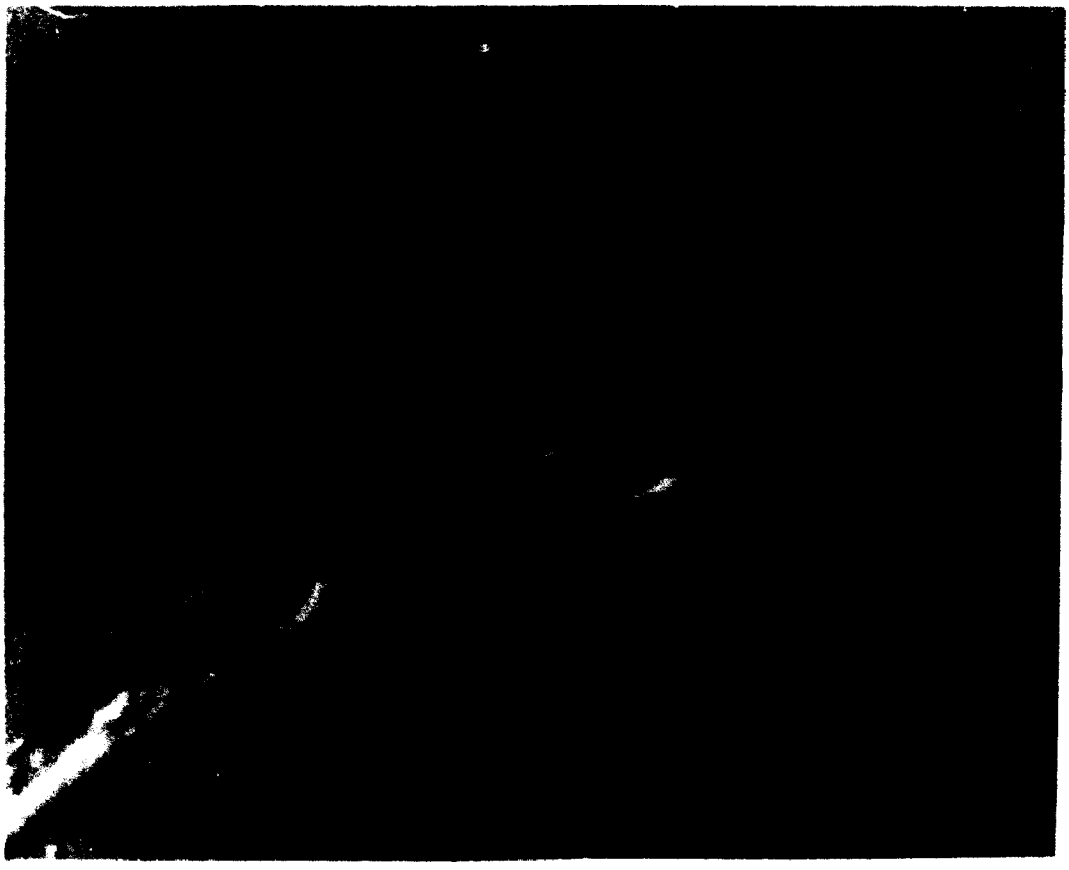
Neuritic growth was arrested within a few minutes following addition of the drug. Cytochalasin B caused the cessation of movement of the growth cone; existing filopodia did not advance and were subsequently retracted. The growth cone rapidly lost its normal appearance and flattened out on the surface of the substratum. The "wilting" of the growth cone can be observed in Figure 45. The neuritic shaft was unaffected.

Non-neuronal cells normally appear raised with even contours by interference-contrast microscopy, becoming thinner at the periphery. Ruffled membranes can often be observed at the advancing edge. Cytochalasin B caused a collapse of the plasma membrane and stopped

Figure 45 Interference contrast micrographs of the effect of cytochalasin B on neuritic growth cones

Cytochalasin B was added to retinal explant cultures at a concentration of 10 µg/ml in L-15H (A) T=0 (B) T=40 min following administration of the drug. Bar = 10 µm.





ruffling activity almost immediately. The appearance of these cells is shown in Figure 46. The nucleus looks like a mound over which the plasma membrane is draped. Some filopodia retain their original position but are flattened, while others are retracted.

The effect of cytochalasin B is reversible. If the drug is removed and fresh medium added, growth cone and non-neuronal cells recover and assume their normal appearance after 1-3 h. DMSO (1%) alone had no effect.

Effect of Colchicine on Neurites from Retinal Explant Cultures

Colchicine is known to inhibit the polymerization of microtubules by binding to the subunit protein, tubulin. Microtubules are the predominant structural element of the axon, consequently the disruption of these structures results in profound changes of axonal morphology and halts neurite elongation. The effect of this drug on neurites from goldfish retinal explants was examined.

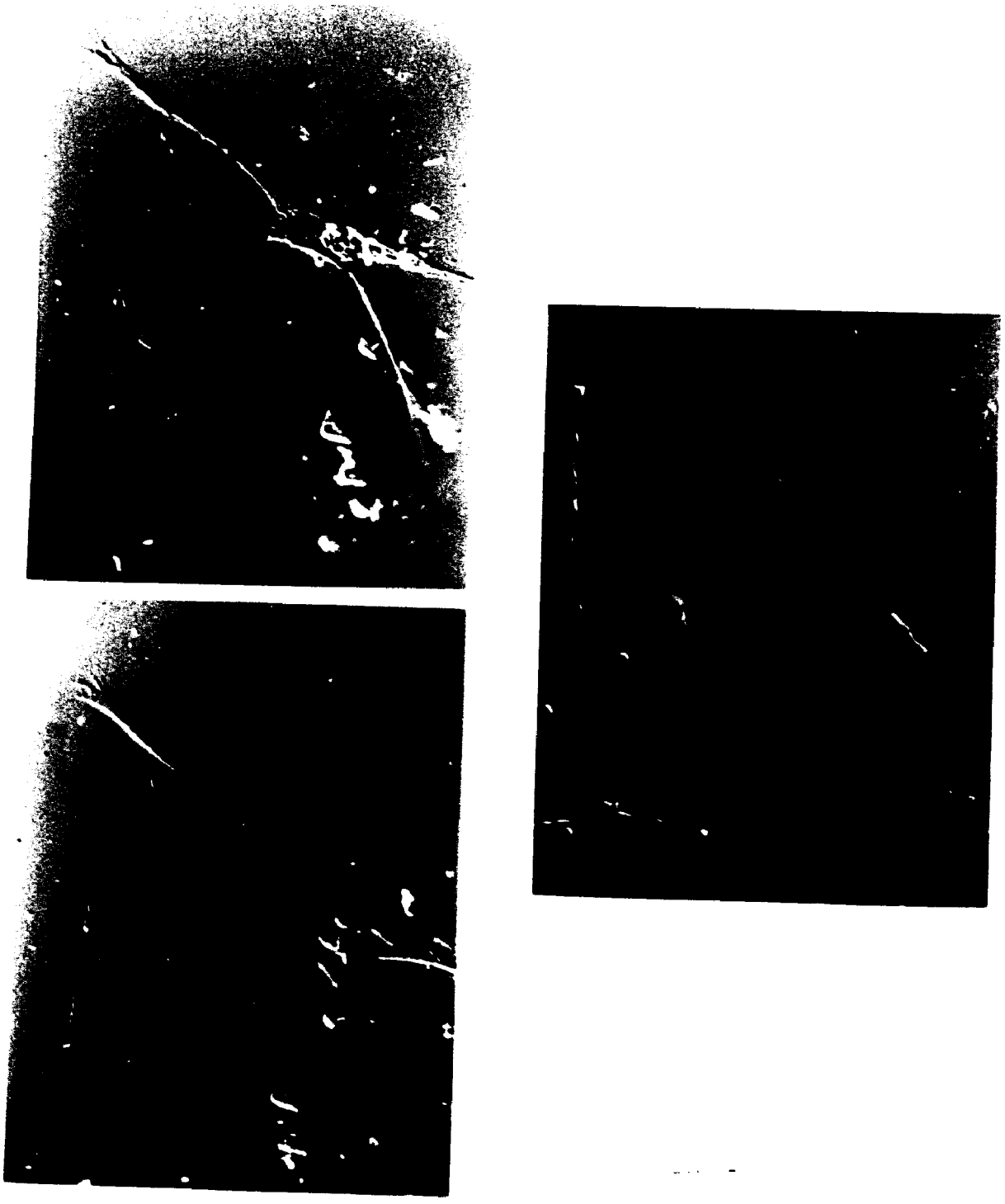
Effect of Colchicine on Morphology of Individual Neurites

The effect of colchicine on the morphology of individual neurites or small fascicles from post-crush retinal explant cultures was examined by phase microscopy. Explant cultures were grown on collagen gels or on polylysine-treated plastic substrata for several days in nutrient medium prior to administration of the drug. Colchicine was added to the nutrient medium at a concentration of 0.05 µg/ml (1.25×10^{-7} M). The appearance of the neurites was slightly different

Figure 46 Interference contrast micrographs of the effect of cytochalasin B on non-neuronal cells

Cytochalasin B was added to retinal explant cultures at a concentration of 10 µg/ml in L-15 H (A) T=0 (B) T=15 min following administration of the drug. Bar = 50 µm. (C) Non-neuronal cell after 30 min of exposure to cytochalasin B.

Figure 46

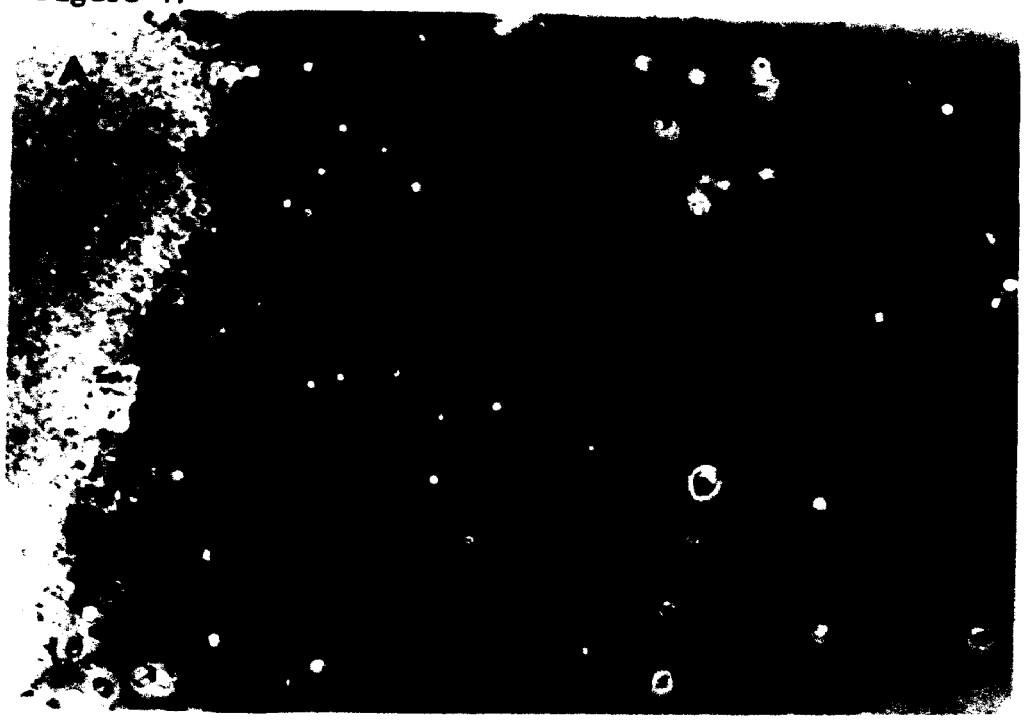


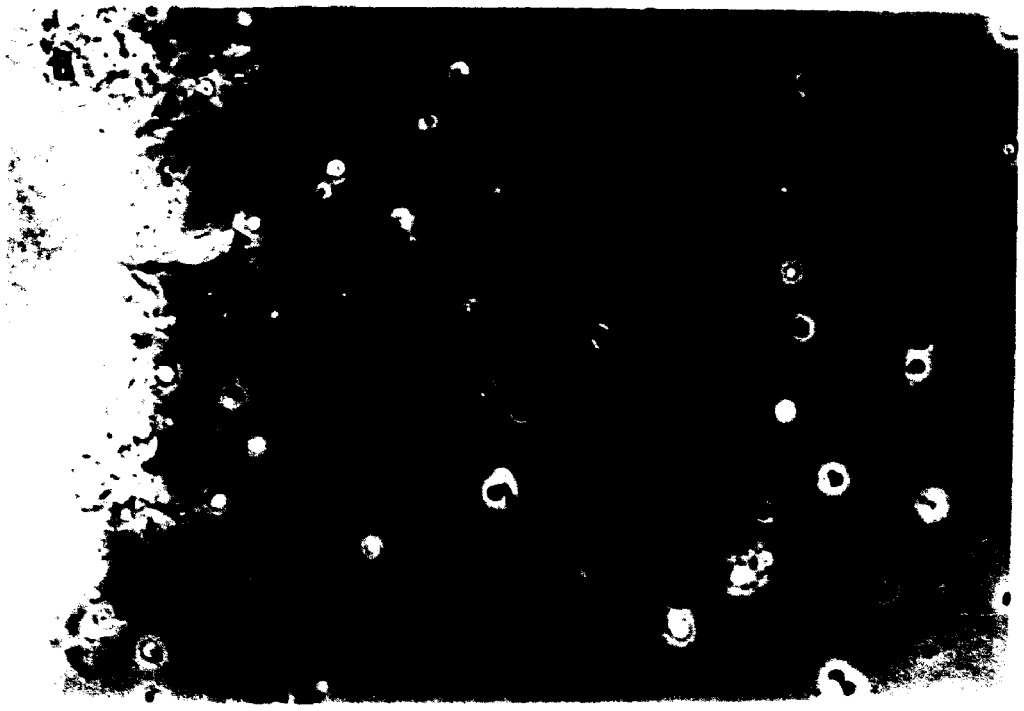
on the two types of substrata. Neurites grown on collagen gels showed a conspicuous narrowing of the fiber diameter within 6 h following administration of the drug. The varicosities that are normally present became more prominent. At early intervals, the fibers continued to elongate and the growth cones appeared normal. After 6-8 h, neurite extension ceased and growth cones were less frequently encountered. The fibers became progressively more narrow and by 24 h they were difficult to see even at high magnifications. The varicosities appear to increase in size during this period. Eventually the varicosities become the only means of visualizing the course of the fiber. Fibers were not observed to retract, however, and the majority disintegrated after prolonged (48-72 h) treatment with colchicine.

Neurites grown on polylysine-treated plastic showed essentially the same changes as those on collagen gels (Figure 47). The first change to be observed was the increasing prominence of the varicosities, with the fiber itself appearing more phase-gray and narrow. Following 24 h of colchicine treatment, the above-mentioned changes became more pronounced. If colchicine was removed from the cultures following 8 h of treatment, the effect was only partially reversible. Some of the axons present during the drug treatment did not recover even after 5 days, and eventually disintegrated. However, 72 h or later, following removal of the drug, new neurites could be observed to grow from the explant, along with regrowth of some of the existing neurites.

Figure 47 Effect of colchicine on neurites
Colchicine was added to retinal cultures at a concentration of 0.05 µg/ml (1.25×10^{-7}M) (A) T=0, (B) T=21.5 h following addition of the drug. Bar = 50 µm.

Figure 47





Inhibition of Neuritic Extension by Colchicine

Post-crush retinal explants were cultured in polylysine-treated plastic dishes in nutrient medium containing various concentrations of colchicine. The NGI was determined after 5 days in culture. Colchicine inhibits the initiation of neuritic growth from retinal explants in a dose-dependent manner over a concentration range of 1.25×10^{-8} M to 5×10^{-8} M (Figure 48). Complete inhibition of neuritic growth was obtained at the higher concentration.

Inositol has been demonstrated to antagonize the effect of colchicine. The addition of colchicine to cultures of dividing rat fibroblasts will result in metaphase arrest of mitosis (Murray et al., 1951). If myoinositol is added to the culture medium the mitotic arrest is largely prevented. The antagonism is specific to myoinositol, since other sugars including various inositol isomers have no effect (Murray et al., 1951; Benitz et al., 1954).

Experiments were performed to determine if inositol would antagonize the colchicine-induced morphological changes in neurites from retinal explant cultures. Colchicine was added to the nutrient medium at the minimum concentration found to inhibit neuritic growth from post-crush retinal explant cultures (0.02 µg/ml, 5×10^{-8} M). Other groups of cultures received nutrient medium containing colchicine and 1 mM or 10 mM myoinositol. Inositol did not prevent the inhibition of neuritic growth caused by colchicine at either concentration (Figure 49). Lumicolchicine, the photooxidation product of colchicine, does not bind tubulin, nor does it inhibit neuritic growth when added

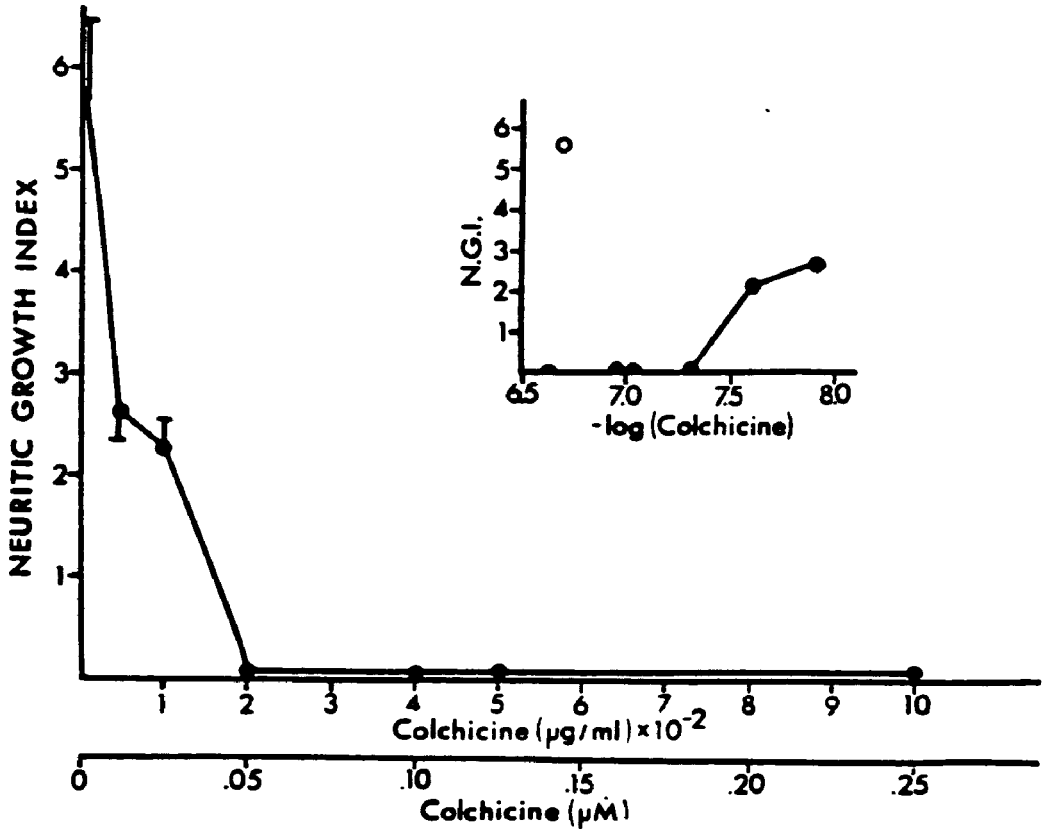


Figure 48 Effect of colchicine concentration on neuritic growth index of post-crush retinal cultures

Post-crush retinal explants were cultured in L-15H containing the indicated colchicine concentration. The NGI was measured on day 5 in culture (± S.E.M.). Inset: the same data plotted as -log [colchicine], O , control cultures grown in L-15H.

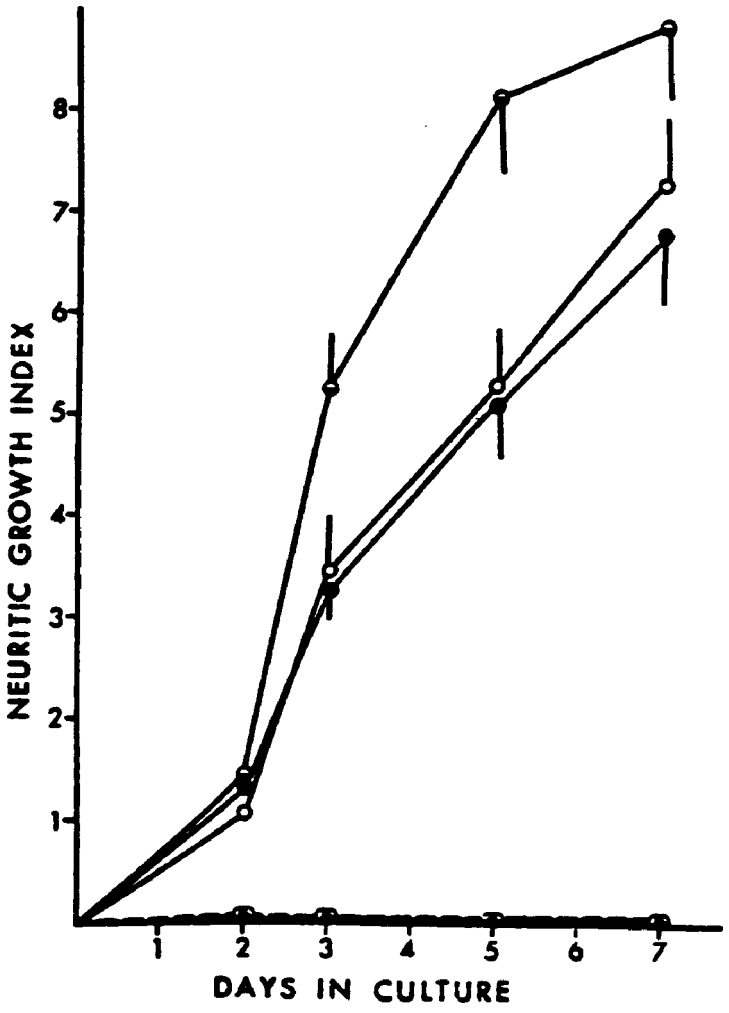


Figure 49 Effect of inositol, lumicolchicine and colchicine on the NGI of post-crush retinal explant cultures

Post-crush retinal explant cultures were grown in L-15H ○ or L-15H containing 10 mM myoinositol ⊖ , or 0.02 µg/ml lumicolchicine ● , (solid lines, ± S.E.M.). To test if inositol would antagonize the inhibition of neuritic growth by colchicine, colchicine (0.02 µg/ml) and 1 mM ○ or 10 mM ⊖ myoinositol was added to explant cultures (broken lines).

at equivalent concentrations. Control cultures receiving only 10 mM inositol have higher NGI values than comparable controls grown in L-15H alone.

The conditions employed in the present studies investigating inositol antagonism of the effect of colchicine were more sensitive than those used by other investigators. Previous studies in which antagonism was observed employed colchicine concentrations of 10^{-2} M to 10^{-3} M (Murray et al., 1951; Benitz et al., 1954). The inositol levels used in experiments reported here were comparable to those of the previous investigators, while colchicine concentrations were several orders of magnitude less. Inositol has not been shown to antagonize the effect of colchicine on axonal microtubules.

The apparent enhancement of neuritic growth in control cultures containing 10 mM inositol was an unexpected finding. This facilitation of fiber outgrowth may reflect stabilization of microtubules or the action of inositol as a nutrient or co-factor.

PART II

Metabolic Studies

Labeling of Retinal Proteins

The incorporation of radioactive methionine into proteins of goldfish retinal explants was examined following fractionation of these proteins by polyacrylamide gel electrophoresis. Of particular interest were the soluble proteins. Heacock and Agranoff (1976) have shown a selective enhancement in the labeling of a single soluble protein, tubulin, following optic nerve crush in the goldfish. The purpose of this study was to extend the previous work to retinal explant cultures and additionally, to examine insoluble proteins extractable by detergent treatment.

Initial studies were directed toward analysis of soluble proteins using a double label technique. Explants from normal or post-crush retina were incubated with 50 μCi of ^{3}H or ^{35}S-methionine respectively, for 24 h. The combined soluble proteins were separated by PAGE and the radioactivity in each gel slice was determined. The ratio of ^{35}S dpm to ^{3}H dpm is a sensitive measure of the differential incorporation of methionine into protein of post-crush compared to normal retinal explants. An increase in the ^{35}S dpm to ^{3}H dpm ratio indicates increased incorporation of methionine into retinal proteins in the post-crush explants. The position of the proteins in the gel, following SDS-PAGE, reflects the molecular weight of the proteins. Thus the molecular weight of the specific protein species which have enhanced

or diminished incorporation of methionine can be identified.

The results of the first experiment are shown in Figure 50. The incorporation of methionine in soluble retinal proteins of post-crush and normal explants is similar, with the exception of a class of proteins having an approximate molecular weight of 50,000-60,000. A purified tubulin sample run on a separate gel co-migrated with these highly labeled proteins. The ratio of ^{35}S dpm to ^{3}H dpm is elevated in this region (Figure 50 B) reflecting selectively increased incorporation of methionine into these proteins in the post-crush retinal explants. This labeling pattern was similar to that seen by Heacock and Agranoff (1976) following incubations of whole retina in vitro. The "noise" in the regions of the gel showing low incorporation reflect statistical error in counting low activity samples.

Efforts to replicate these findings were unsuccessful. In four repetitions of this experiment the enhanced labeling of retinal protein from post-crush explants could not be obtained. The results of a typical negative experiment are shown in Figure 51. The distribution of the radioactivity across the gel appears to show increased ^{35}S - labeling in the 50,000-60,000 MW region, however, the ratio of ^{35}S dpm does not reflect this. While there may be changes among the 50,000 - 60,000 MW protein species, other changes are occurring as well. The ratio is quite variable as a result. Any enhancement of tubulin synthesis is masked by a general increase in protein synthesis.

Control experiments were carried out using two identical groups of normal retinal explant cultures after 5 days in vitro. The

Figure 50 Comparison of soluble protein labeling on SDS-urea PAGE of normal and post-crush retinal explants

Retinal explants grown on collagen gels were labeled with 50 μCi of ^{3}H or ^{35}S-methionine. Post-crush retinal cultures received ^{35}S-labeled precursor while normal explants were given ^{3}H-methionine. Incubations were in Dunlop's medium. The specific activity of the ^{35}S-methionine was adjusted to that of the ^{3}H-methionine (14.6 Ci/mmole) and incubated for 24 h between culture day 4 and 5. Each gel contained 250-300 μg of protein. The distribution of labeled soluble protein is shown in (A) ^{35}S-labeled protein (solid line), ^{3}H-labeled proteins (broken line) (B) normalized ratio of ^{35}S dpm/^{3}H dpm.

Figure 50

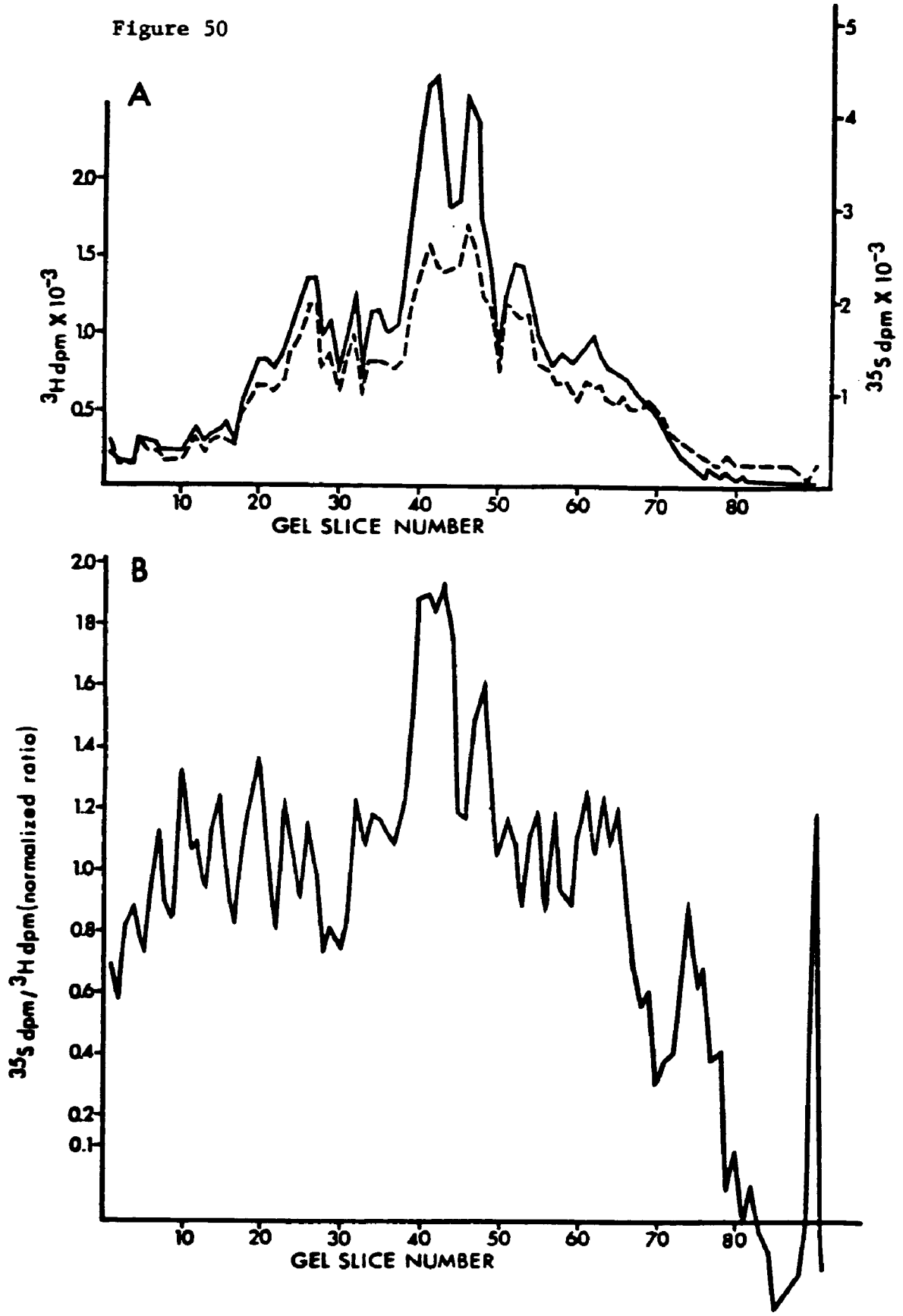
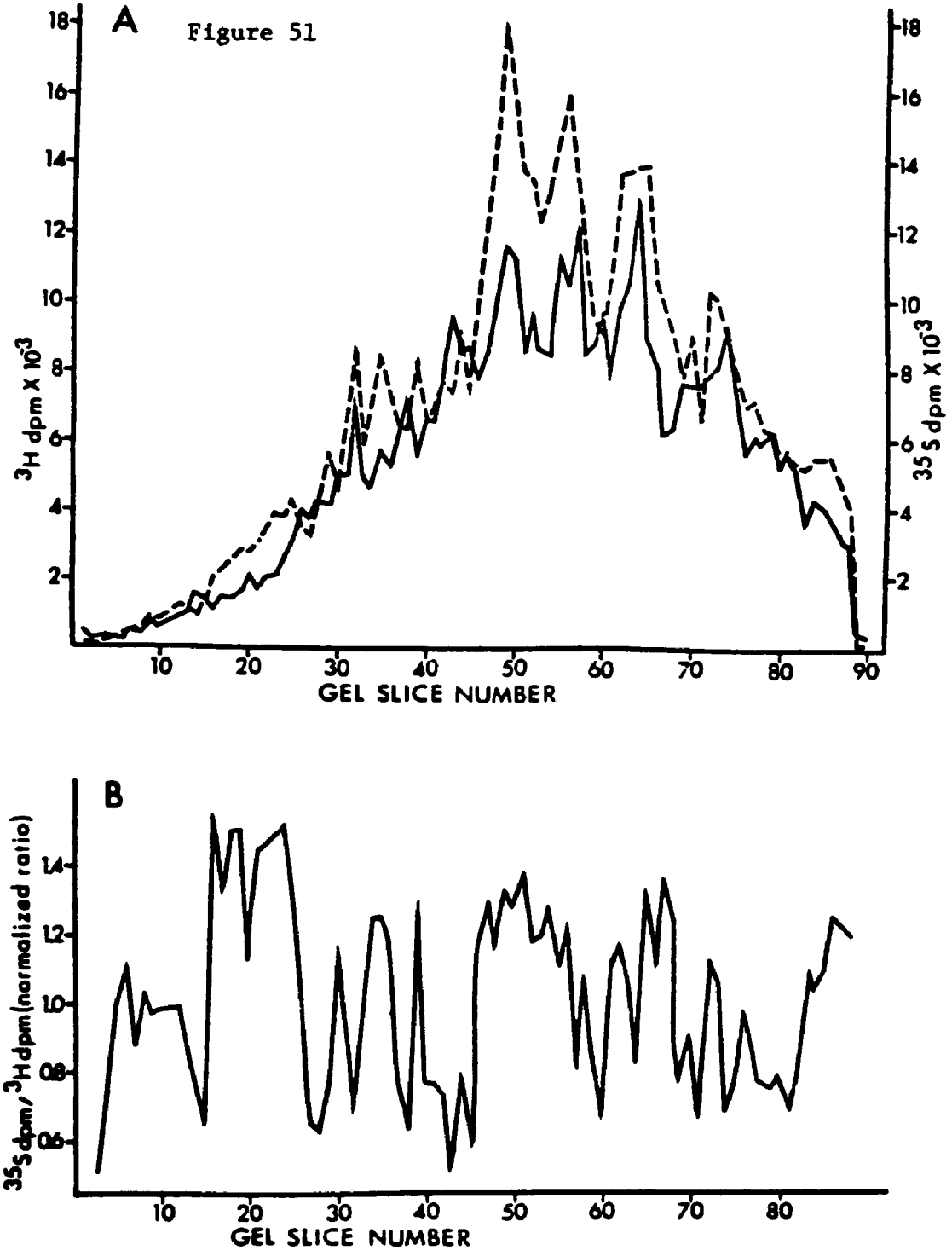

A
^{3}H dpm X 10^{-3}
^{35}S dpm X 10^{-3}
GEL SLICE NUMBER
B
^{35}S dpm / ^{3}H dpm (normalized ratio)
GEL SLICE NUMBER

Figure 51 Comparison of soluble protein labeling on SDS-urea PAGE of normal and post-crush retinal explants

Retinal explants grown on collagen films were labeled with 50 µCi of ^{3}H or ^{35}S-methionine. Post-crush retinal cultures received ^{35}S-labeled precursor while normal explants were given ^{3}H-methionine. Incubations (24 h) were in Dunlop's medium between culture day 4 and 5 (see Figure 50). (A) The labeling pattern of soluble proteins, (^{35}S-labeled proteins, broken line; ^{3}H-labeled proteins, solid line). (B) Normalized ratio of ^{35}S dpm/^{3}H dpm.


A
Figure 51
^{3}H dpm X 10^{-3}
^{35}S dpm X 10^{-3}
GEL SLICE NUMBER
B
^{35}S dpm/^{3}H dpm (normalized ratio)
GEL SLICE NUMBER

distribution of labeled soluble protein was nearly identical, resulting in little fluctuation of the ^{35}S dpm/^{3}H dpm ratio (Figure 52), ruling out artifactual variation resulting from use of the double-label technique.

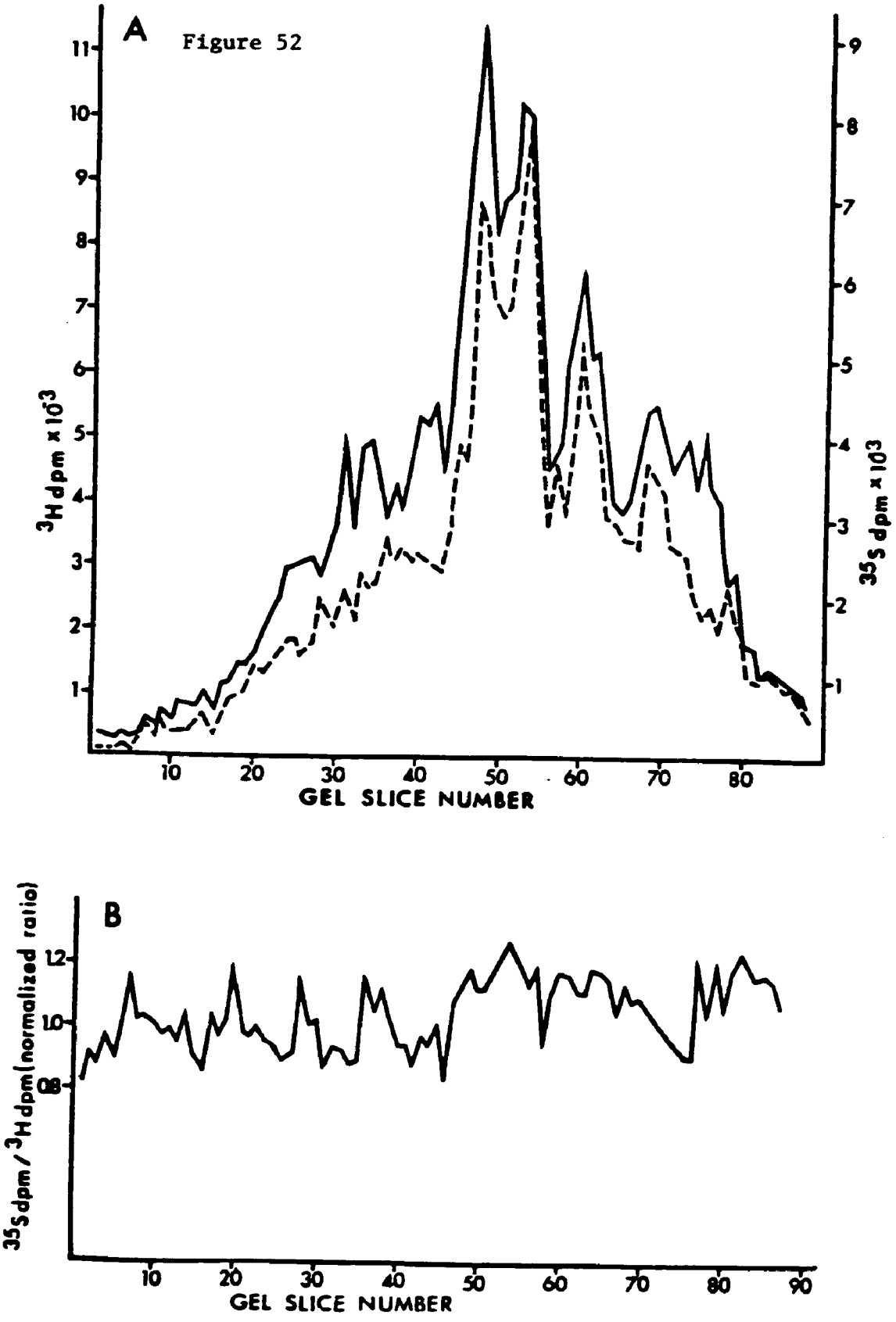
Various steps were taken to reproduce the original findings, among them a briefer labeling period. If the incubation period is shortened to 6 or even 1 h (the same incubation time used by Heacock and Agranoff) there were no significant differences in either the labeling pattern or the ^{35}S dpm/^{3}H dpm ratio in various protein species compared with explants incubated for 24 h with labeled precursor.

Experiments comparing normal retinal explants at different intervals following explantation were run in parallel with those comparing post-crush and normal retinal explants described above. The labeling of soluble proteins in normal retinal explants after 5 days in culture was compared to that of normal retinal explants following 12 days in vitro. The rationale for these studies was that the known morphological changes in the normal retinal explant with lengthening periods in vitro might be reflected in alteration of protein synthesis. Two groups of normal retinal explant cultures were incubated for 24 h with radioactive methionine. One group was given ^{35}S-methionine on culture day 4. The other received ^{3}H-methionine on culture day 11. No major difference in methionine labeled soluble proteins could be detected between the two groups of normal explants following PAGE (Figure 53). The ^{35}S dpm/^{3}H dpm ratio, while not as variable as the

Figure 52 Comparison of soluble protein labeling on SDS-urea PAGE of normal retinal explants

Two identical groups of normal retinal cultures were labeled with 50 µCi of ^{3}H or ^{35}S-labeled methionine for 24 h between culture day 4 and 5. Incubation was in Dunlop's medium (see Figure 50).
(A) The labeling pattern of soluble retinal protein (^{35}S-labeled proteins, solid line; ^{3}H-labeled proteins, broken line).
(B) Normalized ratio ^{35}S dpm/^{3}H dpm.

Figure 52


A
B
^{3}H dpm x 10^3
^{35}S dpm x 10^3
GEL SLICE NUMBER
^{35}S dpm / ^{3}H dpm (normalized ratio)
GEL SLICE NUMBER

post-crush vs normal gel, did show some variation (Figure 53 B). This result indicates that biosynthesis of proteins in normal explants was not measurably affected in the interval from 5 to 12 days in vitro, despite the loss of large numbers of intrinsic retinal cells during the culture period.

The labeling of proteins present in the high speed pellet was investigated by solubilization of these proteins with detergents. Sequential extractions were performed, first with Nonidet P-40 followed by SDS. The extracted protein fractions were analyzed separately by PAGE. Nonidet P-40, a nonionic detergent, has been shown to extract tubulin-like protein from membrane fractions (Bhattacharyya and Wolff, 1975). The distribution of methionine-labeled proteins from post-crush and normal retinal explants solubilized with 0.2% Nonidet P-40 showed no major differences following PAGE (Figure 54). There is no striking alteration in the ^{35}S dpm/^{3}H dpm ratio (Figure 54 B). Parallel experiments comparing normal retinal explants after 5 and 12 days in culture gave similar results.

Proteins remaining in the pellet following Nonidet P-40 extraction were solubilized in 0.2% SDS. The distribution of radioactivity in these gels looked very similar to that of the Nonidet P-40 extracted material. No differences were observed between normal and post-crush retinal protein nor between normal explants after 5 or 12 days in vitro (data not shown).

In summary, following an extensive survey of methionine-labeled protein from retinal explants, no reproducible differences were found

Figure 53 Comparison of soluble protein labeling on SDS-urea PAGE of normal retinal explants after 5 and 12 days in culture

Normal retinal explants grown on collagen films for 5 or 12 days were labeled with 50 µCi of ^{35}S or ^{3}H-methionine respectively. Incubation was in Dunlop's medium for 24 h. (A) The labeling pattern of soluble retinal proteins (^{35}S-labeled proteins, broken line; ^{3}H-labeled proteins, solid line). (B) Normalized ratio ^{35}S dpm/^{3}H dpm.

Figure 53

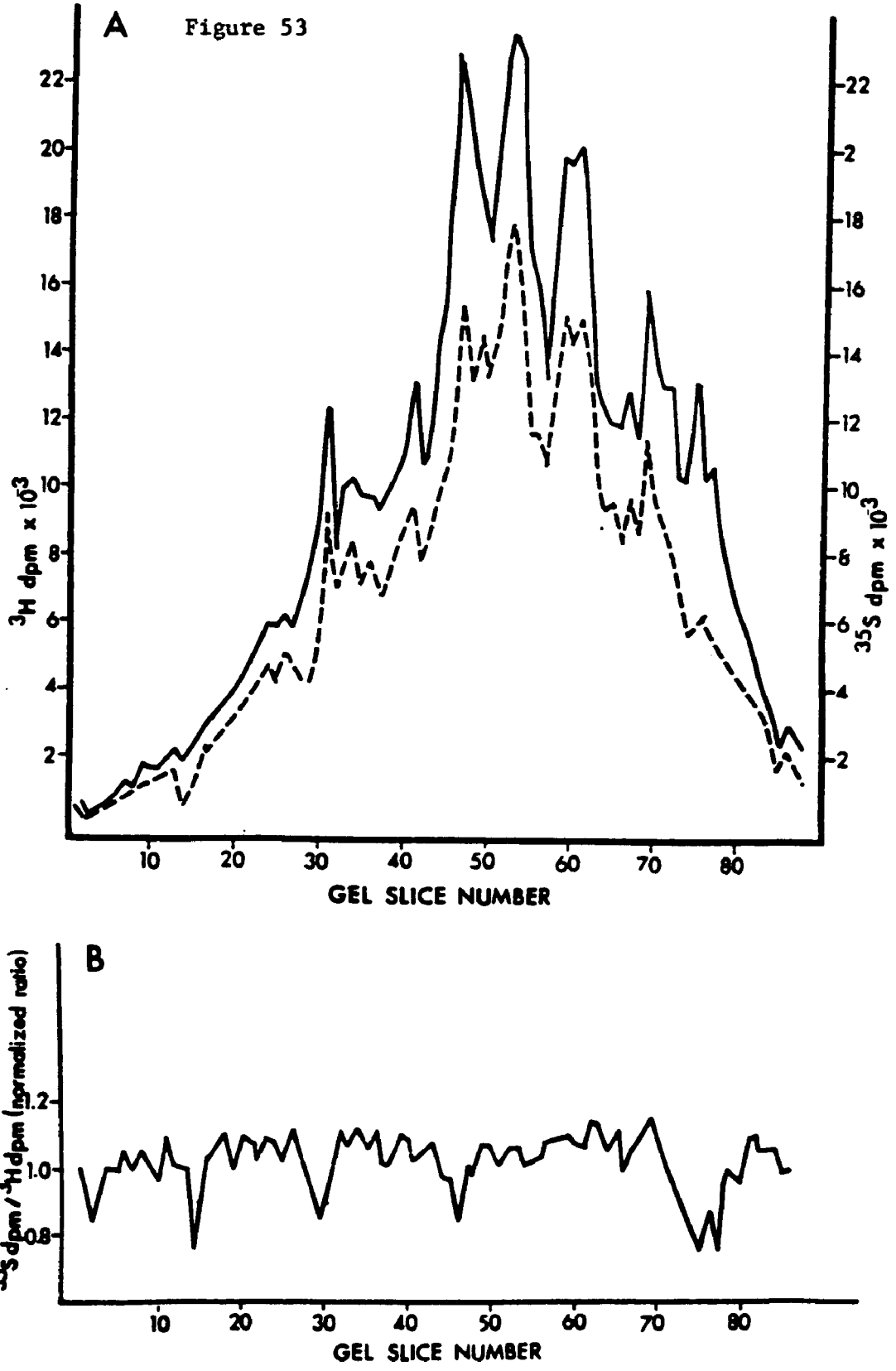
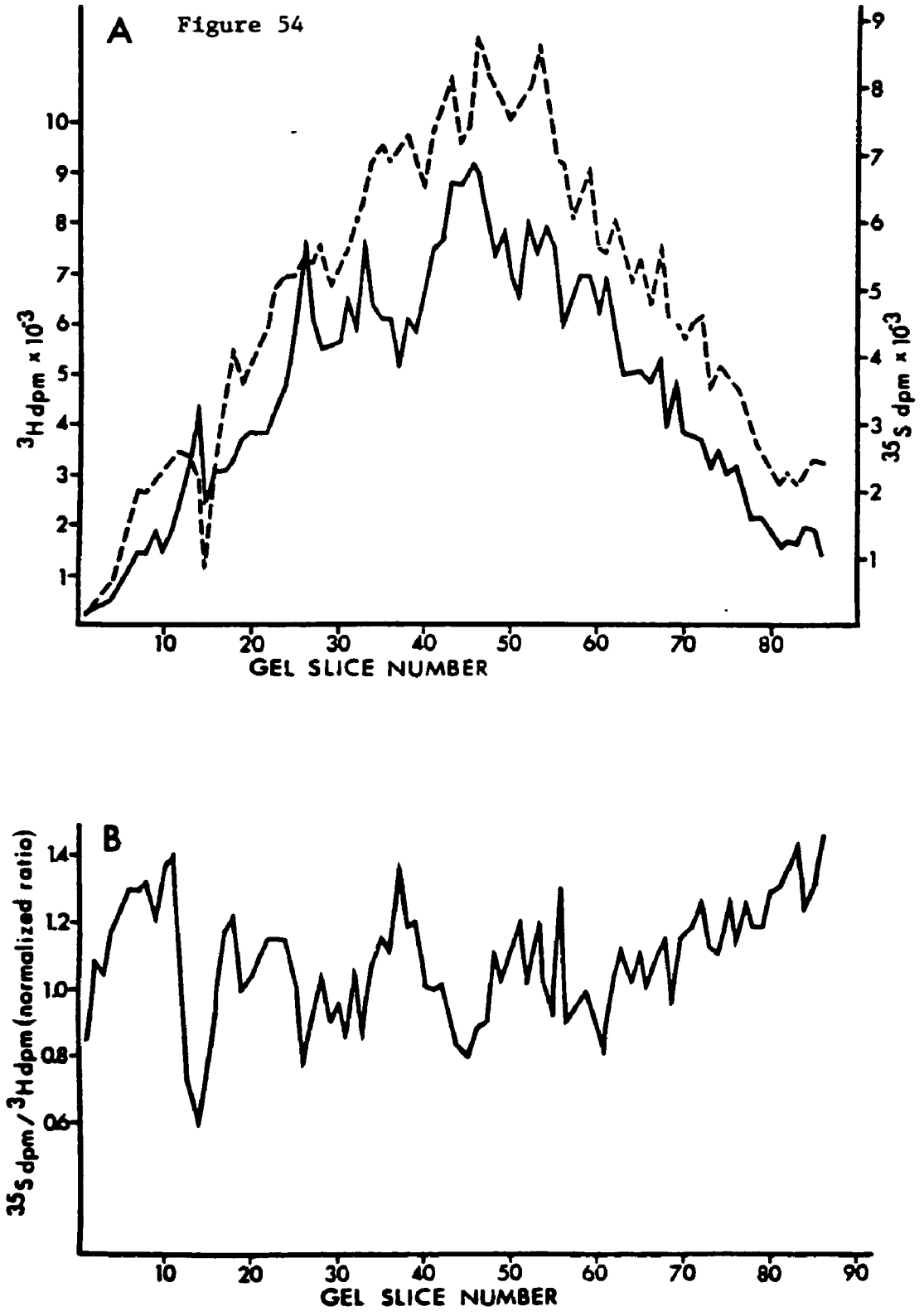

A
^{3}H dpm x 10^{-3}
^{35}S dpm x 10^{-3}
GEL SLICE NUMBER
B
^{35}S dpm / ^{3}H dpm (normalized ratio)
GEL SLICE NUMBER

Figure 54 Comparison of Nonidet P-40 extracted proteins on SDS-urea PAGE of normal and post-crush retinal explants

Retinal explants grown on collagen films were labeled with 50 µCi of ^{3}H or ^{35}S-methionine. Post-crush retinal cultures received ^{35}S-labeled precursor while ^{3}H-methionine was given to normal explants. Incubations were for 24 h between culture day 4 and 5. The high-speed pellet was extracted with 0.2% Nonidet P-40 and subjected to PAGE. Each gel contained 250-300 µg of protein. (A) The distribution of labeled proteins solubilized with Nonidet P-40, (^{35}S-labeled proteins, broken line; ^{3}H-labeled proteins, solid line). (B) Normalized ^{35}S dpm/^{3}H dpm ratio.

Figure 54


A
^{3}H dpm x 10^{-3}
^{35}S dpm x 10^{-3}
GEL SLICE NUMBER
B
^{35}S dpm / ^{3}H dpm (normalized ratio)
GEL SLICE NUMBER

among the experimental groups. The findings with the soluble protein fractions are problematic. Examination of the data from the various experiments demonstrates a broad band of radioactivity in the 50,000-60,000 MW region of the gel. Tubulin, however, comprises only a fraction of the highly labeled proteins.

The inability to detect preferential labeling in the 55,000 MW region of the gel contrasts with the finding of Heacock and Agranoff (1976) with whole goldfish retina incubated *in vitro*. The result cannot be attributed to the long incubation used, since experiments as brief as 1 h were also negative. In fact, experiments with brief labeling periods showed less difference between the two groups than did comparable studies with longer incubations.

Binding of Methionine to Bovine Serum Albumin

This study was initiated in an effort to determine if the goldfish retinal explants elaborated protein into the culture medium. Investigations with various cell lines (Truding et al., 1975) and organ cultures (Lott et al., 1972) have detected proteins (principally glycoproteins) appearing in the culture medium. It has been suggested that the elaboration of protein is the result of normal metabolic turnover of surface membranes (Warren and Glick, 1968) or of specific secretion of soluble proteins (Schubert, 1973). In the course of protein labeling experiments, radioactivity was observed in the incubation medium, which migrated as a single band on PAGE and with bovine serum albumin (BSA). Efforts were made to determine the manner in which this protein was labeled.

Protein Labeling Experiments

Retinal explant cultures from normal retina and retina whose optic nerve had been crushed 15 days prior to explantation were grown on collagen films as described previously. Incubation medium containing 50 µCi/ml (14.6 Ci/mmole) of either ^{3}H or ^{35}S-methionine was added to the cultures after removal of the nutrient medium. Normal retinal cultures received ^{3}H-methionine while post-crush cultures were incubated with ^{35}S-labeled precursor. The cultures were incubated at room temperature for 24 h. The incubation medium was removed, dialyzed against distilled water, lyophilized and subjected to SDS-urea PAGE. The radioactivity, with either precursor, appeared as a single labeled peak with an apparent molecular weight of 65,000-70,000 (Figure 55). The protein staining pattern was identical to that obtained with FCS. If this region was excised, cut into 1 mm sections and counted, the radioactivity with both isotopes was found to be coincident with the dye, and identical to that of BSA. Since FCS contains albumin as its major protein, the results suggested either that the radioactivity was associated with BSA derived from the calf serum, or that the explant was elaborating a protein of the same molecular weight. Since the explants had been grown in nutrient medium supplemented with 9% fetal calf serum on collagen films, a small volume of the nutrient medium was undoubtedly trapped in the collagen at the time the culture medium was exchanged with BSA-free incubation medium containing labeled precursor. It was calculated that if only 50-100 µl of the nutrient medium were trapped by the

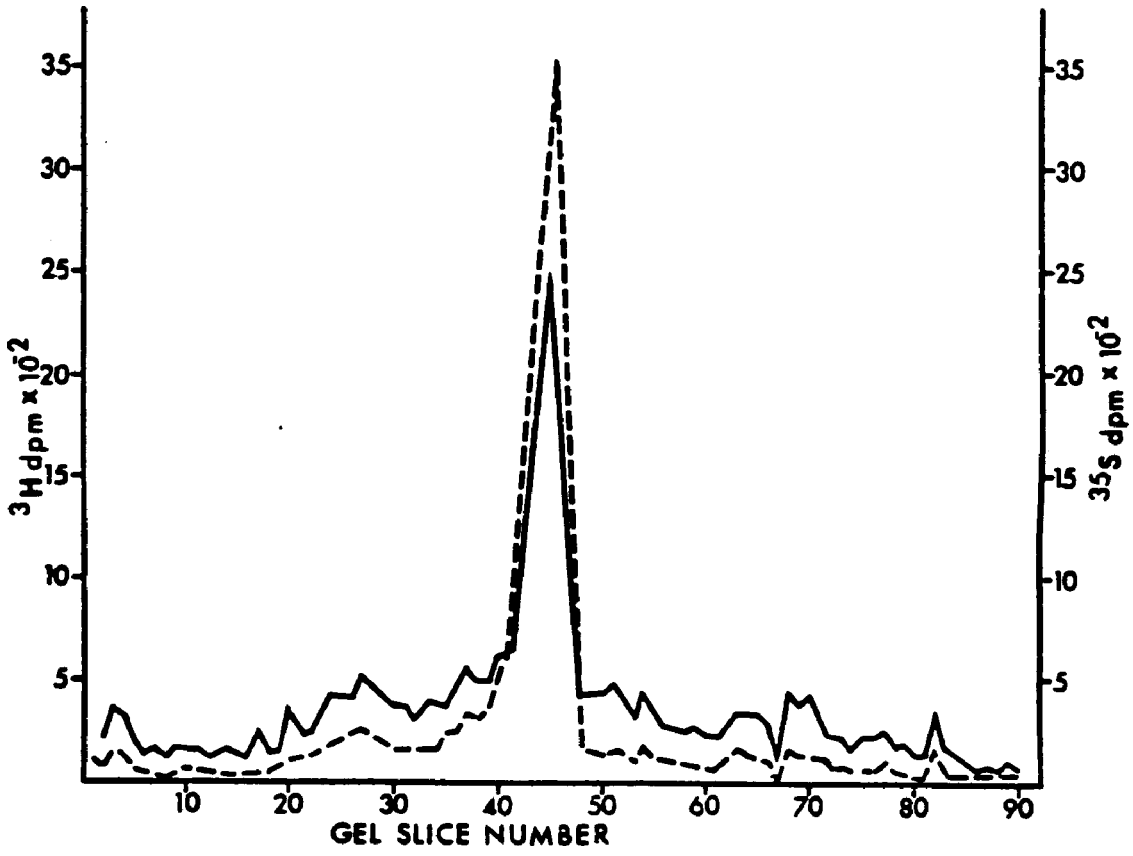


Figure 55 Polyacrylamide gel electrophoresis of incubation medium from retinal explant cultures

SDS-urea polyacrylamide gels containing both ^{35}S and ^{3}H-labeled material from post-crush and normal retinal explants respectively. ^{3}H-methionine (45 µCi, 14.6 Ci/mmole) was incubated with explants for 24 h at room temperature. Incubation medium was dialyzed, lyophilized, and PAGE carried out.

collagen, it could account for the amount of albumin-like material found in the incubation medium.

If, however, the labeling of the BSA-like protein was the result of protein synthesis by the explants and the subsequent secretion of protein into the medium, inhibition of protein synthesis should abolish the associated radioactivity. The addition of cycloheximide (10 µg/ml) to the incubation medium along with the ^{3}H-methionine inhibited the incorporation of TCA-precipitable radioactivity in the explants by 94%. However, the labeling of the BSA-like protein in the medium was unaffected (Figure 56 A). This suggested that de novo protein synthesis by the explant could not be the source of labeled protein appearing in the medium.

The dependence of the labeling on the presence of FCS was examined by extensive washing of the cultures prior to incubation to remove any residual FCS retained by the collagen. Electrophoresis of the incubation medium showed that in these samples, the labeling in the 68,000 MW region of the gel was absent. Incubation of a control culture which was not washed, resulted in a single labeled medium peak similar to that observed in the previous experiment (Figure 56 B), indicating that the labeling of the 68,000 MW protein was dependent upon the presence of serum proteins.

The collagen film, although extensively washed prior to explantation, could possibly be contributing soluble factors or an enzymatic activity which are necessary for the labeling of proteins in the medium. A polylysine-treated tissue culture dish was used to grow the explants (Figure 56 A) controlling for any effect the collagen

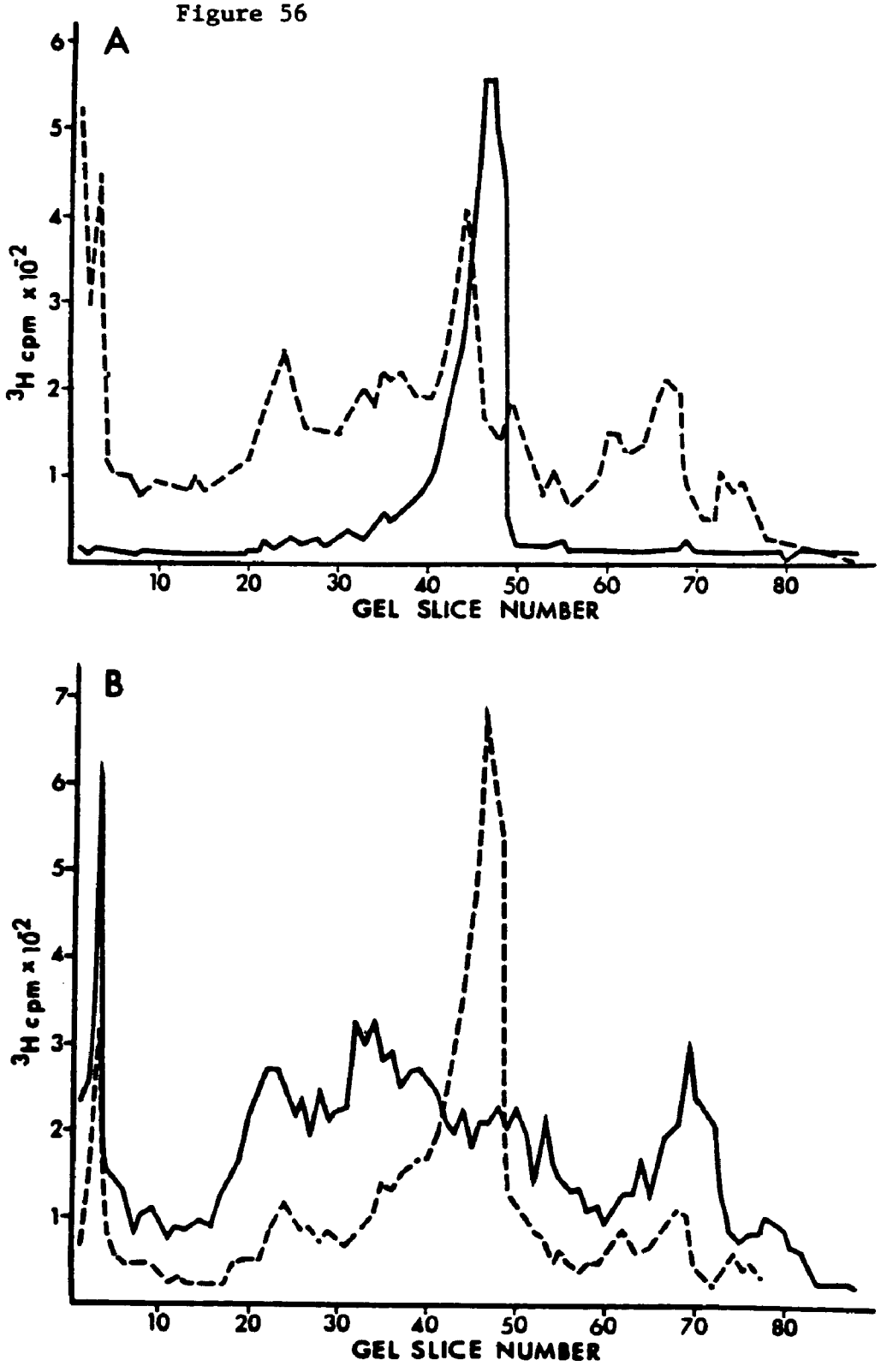
Figure 56 Comparison of radioactive proteins in the incubation medium of retinal explant cultures

Post-crush retinal explant cultures were incubated with 45 µCi of ^{3}H-methionine (14.6 Ci/mmole) for 24 h between culture day 4 and 5 at room temperature. The labeled proteins appearing in the incubation medium were subjected to PAGE.

(A) Retinal explants were grown on PLYS-treated plastic and incubated Dunlop's medium containing labeled methionine (broken line). Other explants were grown on collagen films and incubated in the same medium supplemented with 10 µg/ml CXM (solid line).

(B) Retinal explants were grown on collagen films and incubated in Dunlop's medium containing labeled methionine. The incubation medium was added immediately upon removal of the nutrient medium (no washing of substratum, broken line) or after three washes with unlabeled incubation medium prior to the addition of the labeled precursor (solid line).

Figure 56



may have had in these experiments. The plastic surface should not retain serum from the nutrient medium to the same extent as collagen, and there should be correspondingly less serum protein remaining in the dish prior to the addition of incubation medium. If the radioactivity appearing on the gel is related to the amount of serum protein present, then an intermediate level of labeling should be observed. Moreover, if the labeling is dependent upon the presence of collagen the radioactivity associated with the 68,000 MW band should be eliminated. The results showed significant labeling in the 68,000 MW region of the gel, indicating that labeling does occur in the absence of collagen, yet the amount of radioactivity associated with this peak was not as great as in cultures in which collagen was used as the substratum.

It was of some interest to see if other amino acids would label BSA, particularly leucine, since this amino acid has been shown to be enzymatically added to the N-terminal residue of BSA (Leibowitz and Soffer, 1971). In this experiment, 1% FCS was added to the explant incubation medium. Leucine did not significantly label the 68,000 MW region of the gel even at four times the specific activity of ^{3}H-methionine (Figure 57). The small amount of radioactivity found was, however, principally associated with BSA (Figure 57).

A control was finally run in the absence of explants. Dunlop's medium with 1% FCS was incubated in a test tube with 50 µCi of labeled precursor for 24 h, then processed for gel electrophoresis. The resulting distribution of radioactivity on acrylamide gels was almost

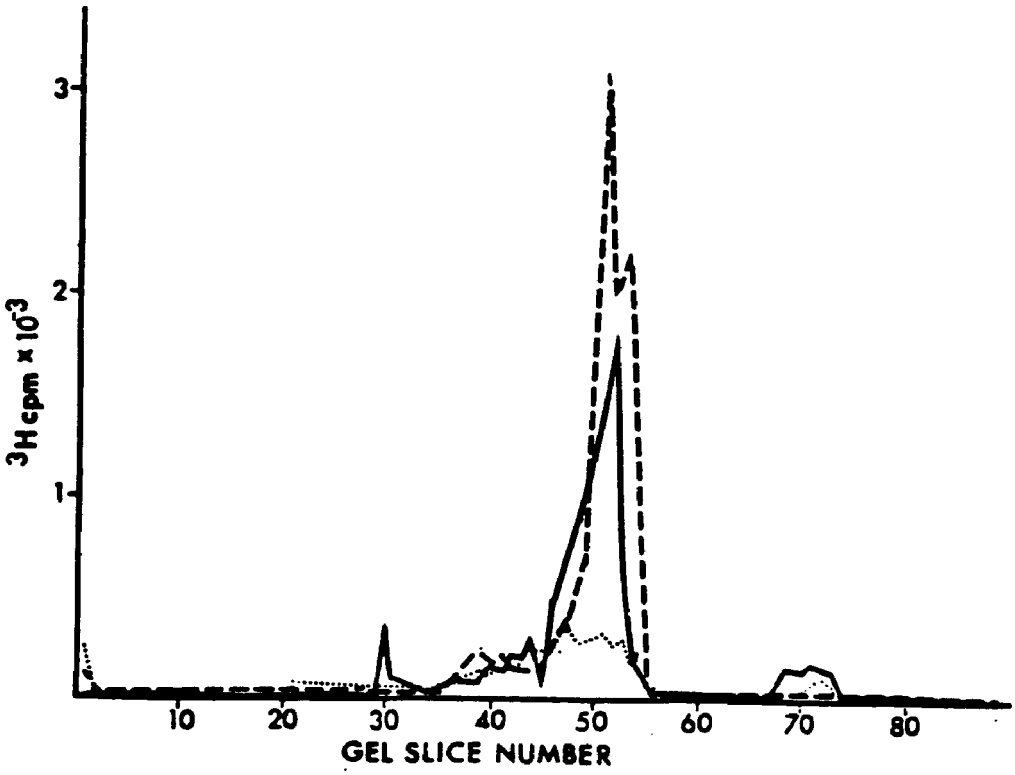


Figure 57 Comparison of ^{3}H-leucine and ^{3}H-methionine-labeled proteins present in the incubation medium

Post-crush retinal explants grown on collagen films were incubated for 24 h between culture day 4 and 5 in Dunlop's medium containing 50 µCi of ^{3}H-leucine (57 Ci/mmole, dotted line) or ^{3}H-methionine (14.6 Ci/mmole, solid line) and 1% FCS. The proteins were separated by PAGE. A control incubation was carried out by simply incubating the labeled methionine in Dunlop's medium containing 1% FCS in the absence of retinal cultures (broken line).

completely restricted to the 68,000 MW region (Figure 57). The amount of label associated with the BSA was significantly greater than the comparable experiment with retinal explants incubated in medium which had no additional FCS present, other than that retained by the collagen.

The time course of labeling of serum proteins is shown in Figure 58. The increase in TCA-precipitable radioactivity was linear for the first 5 h, and maximal after 10 h.

The foregoing results suggested that ^{3}H-methionine was associated almost exclusively with serum albumin. If the binding was an intrinsic property of the albumin molecule and not dependent upon other serum factors, then commercial bovine serum albumin should give similar results. The protein concentration in 1% FCS solution was 520 µg/ml of which an estimated 75% was albumin. Dunlop's medium was supplemented with 1% FCS or with BSA at a concentration of 390 µm/ml. To examine the possibility that binding of methionine was not unique to BSA, a different protein, ovalbumin, was employed under identical conditions.

Fetal calf serum per µg protein has nearly twice as much acid-precipitable radioactivity bound as the commercial BSA preparation (Table VI). Dialysis against water removes a larger fraction of this radioactivity from the FCS suggesting that a significant amount of low affinity binding was occuring. The specific activities of FCS compared to BSA are approximately equal following dialysis. The TCA-precipitable radioactivity associated with ovalbumin was almost completely removed by dialysis.

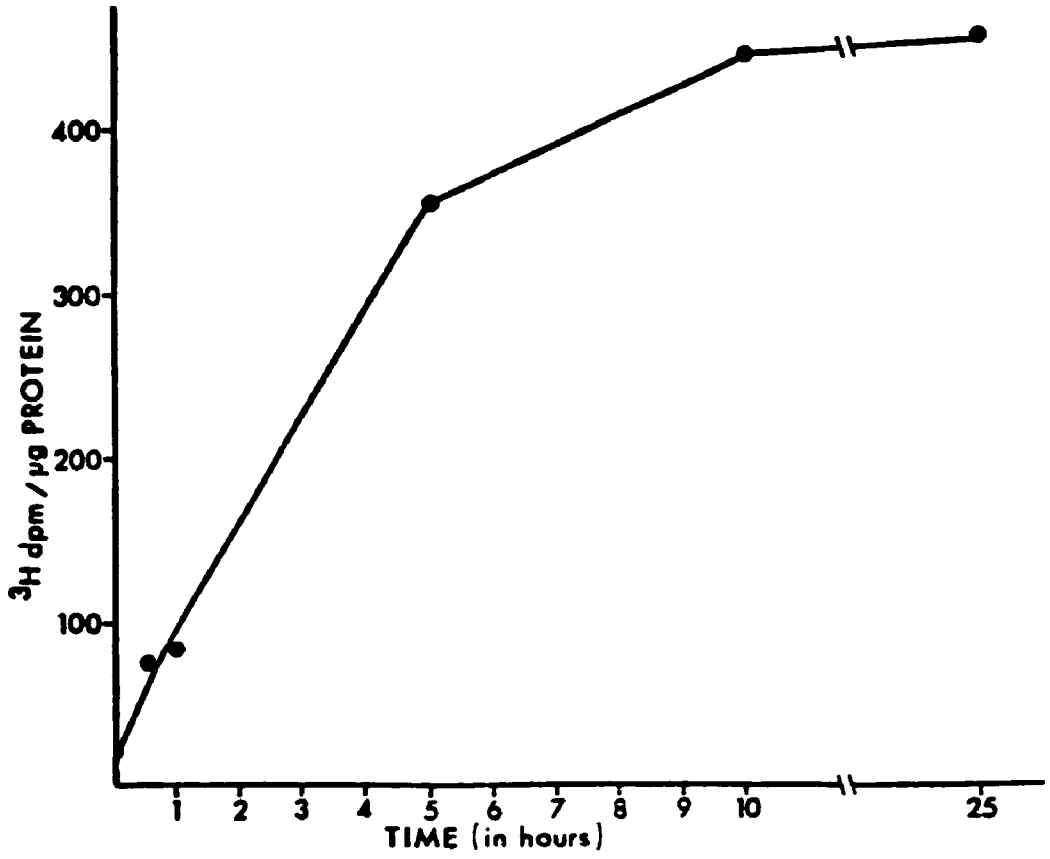


Figure 58 Time course of incorporation of radioactivity into TCA-precipitable material

Dunlop's medium containing 1% FCS and 24 µCi ^{3}H-methionine was incubated at room temperature. Aliquots were removed at indicated intervals and total TCA-precipitable radioactivity determined.

TABLE VI

COMPARISON OF TCA-PRECIPITABLE RADIOACTIVITY ASSOCIATED WITH FETAL CALF SERUM, BOVINE SERUM ALBUMIN AND OVALBUMIN

	TCA-Precipitable Radioactivity					
	Incubation Medium		After Dialysis		Urea-Treated	
	dpm/ml X 10^{-3}	dpm/µg protein	dpm/ml X 10^{-3}	dpm/µg protein	dpm/ml X 10^{-3}	dpm/µg protein
FCS	127.6	245	60.7	116	7.3	14
BSA	58.7	150	48.0	123	4.7	12
Ovalbumin	23.0	59	2.3	6	1.4	4

The fetal calf serum sample was a 1% solution containing 520 µg protein/ml in Dunlop's medium. BSA and ovalbumin were each present at concentrations of 390 µg/ml in the same medium. ^{3}H-methionine was added (24 µCi, 14.6 Ci/mmole) and incubated 24 h at room temperature. The total TCA-precipitable radioactivity in the incubation medium was directly determined as described in the text. The incubation medium was then dialyzed 36 h and TCA-precipitable radioactivity of the non-dialyzable material determined. The dialyzed solution was made 8 M in urea, 1% in mercaptoethanol, heated 5 min at 80°, then TCA-precipitated and radioactivity measured.

The nature of the binding was examined by treating the non-dialyzable material with urea followed by TCA precipitation of the protein (Table VI). If the binding was the result of covalent linkage, this treatment should not affect the amount of radioactivity found in the acid-insoluble material. If the non-dialyzable material is made 8 M in urea and 1% in mercaptoethanol, TCA-precipitable radioactivity is almost completely lost. It was possible that the combined TCA-urea treatment caused the loss of radioactivity either by direct loss of ^{3}H-methyl group from methionine or by denaturation of albumin and release of ^{3}H-methionine. The former possibility was ruled out by similar results using ^{35}S-methionine under the same conditions.

To test whether the release of ^{3}H-labeled precursor was the result of the combined urea-TCA treatment, a separate experiment was performed using urea alone, followed by dialysis to remove any released radioactivity. The radioactivity remaining after dialysis was determined by counting aliquots of the non-dialyzable material. FCS with bound ^{3}H-methionine was dialyzed 36 h in the cold against water. The non-dialyzable material was made 8 M in urea and 1% in mercaptoethanol and dialyzed for a further 24 h at 4°. Under these conditions, over 90% of associated radioactivity was removed, indicating that denaturation of the protein by urea was sufficient to release the labeled precursor.

The incubation medium in all of these studies was supplemented with 0.1 mg/ml gentamicin sulfate. This antibiotic has been reported (Cukier et al., 1974) but not confirmed (Wennberg and Rasmussen, 1975)

to displace bilirubin from serum albumin. When gentamicin was deleted from the incubation medium it did not significantly change the amount of TCA-precipitable ^{3}H-methionine bound to serum proteins.

At equilibrum, one mole of methionine is bound per 2000-5000 moles of BSA. This, however, assumes that all of the associated radioactivity was present as methionine. In all the labeled methionine preparations there was substantial contamination by methionine sulfoxide (MSO). Methionine is spontaneously oxidized in solution, so that the longer the storage period the greater the fraction of the radioactivity present as MSO. The contamination by MSO in the experiments reported here varied from 5-44% of the total radioactivity. The acid-precipitable radioactivity could be the result of binding of methionine, MSO or both. The bound label was separated from the urea-treated albumin by column chromatography on Sephadex G-50 followed by passage through Dowex 50-W (to remove the urea), the recovered radioactivity was chromatographed on paper. Seventy-one percent of the radioactivity co-migrated with methionine sulfoxide and 16% with methionine. Whether this represents actual binding of MSO to BSA or the oxidation of methionine to MSO during the chromatographic procedures was uncertain.

Studies to determine whether MSO, methionine or both were binding to BSA were performed (Table VII). The labeled methionine preparation used in these experiments had 41% of the radioactivity present as methionine and 44% as MSO. Methionine was separated from MSO by paper chromatography. Fifty μCi of each precursor was added to 1 ml

Table VII

Labeled precursors were added to 1 ml of Dunlop's medium containing 1 mg/ml of BSA. Fifty μCi of each of the precursors were added at a specific activity of 14.6 Ci/mmole and incubated for 24 h at room temperature. TCA-precipitable radioactivity was determined as described in Methods. Aliquots of the incubation medium were taken and acid precipitable radioactivity was measured, the remainder was dialyzed against 2 changes of 0.1 N NaOH in the cold for 24 h. An aliquot of the non-dialyzable material was removed for measurement of TCA-precipitable radioactivity. The non-dialyzable material was then made 2 M in urea, and TCA-precipitable radioactivity of this solution was determined.

The labeled methionine and methionine sulfoxide (MSO) mixture was the result of spontaneous oxidation of ^{3}H-methionine to the sulfoxide during storage. The mixture contained 41% methionine and 44% methionine sulfoxide. Purified preparations of these compounds were obtained by paper chromatography of the mixture, the appropriate areas of the chromatogram were cut out and the precursor eluted with 70% ethanol, all in a nitrogen atmosphere.

TABLE VII

COMPARISON OF TCA-PRECIPITABLE RADIOACTIVITY BOUND TO BOVINE SERUM ALBUMIN FOLLOWING INCUBATION WITH TRITIATED, METHIONINE, METHIONINE SULFOXIDE AND LEUCINE

	TCA-Precipitable Radioactivity					
	Incubation Medium		After Dialysis in 0.1 N NaOH		After Treatment with 2 M urea	
	dpm/ml X 10^{-3}	dpm/µg protein	dpm/ml X 10^{-3}	dpm/µg protein	dpm/ml X 10^{-3}	dpm/µg protein
Methionine + Methionine sulfoxide Mixture	909	909	8.1	8.1	2.4	2.4
Methionine	408	408	-	-	-	-
Methionine sulfoxide	222	222	4.8	4.8	1.3	1.3
Leucine	38	38	25.9	25.9	9.1	9.1

of Dunlop's medium containing 1 mg/ml BSA and incubated at room temperature for 24 h. Tritiated leucine was diluted to the same specific activity as the methionine and incubated under the same conditons. The acid-insoluble radioactivity bound to BSA due to methionine was nearly twice that of MSO, while the total bound radioactivity of the methionine and MSO mixture was greater than that of both of the compounds combined. This disparity in the acid-precipitable radioactivity may be accounted for by loss of methionine to MSO in the purified preparation during the 24 h incubation. When an aliquot of the incubation medium was rechromatographed, only 60% of the radioactivity could be recovered as methionine, while 11% of the precursor was present as MSO, with the remaining radioactivity distributed over the chromatogram. The purified MSO preparation was found to be 94% MSO by chromatography. Leucine was bound to BSA poorly, having only approximately 10% of the radioactivity bound compared to methionine.

If another aliquot of the incubation medium was dialyzed against two changes of 0.1 N NaOH for 24 h at 4°, nearly all (98%) of the radioactivity bound to BSA was removed. Leucine was not as effectively removed by this treatment.

If the remaining solution was made 2 M in urea there was a further reduction in the amount of acid-insoluble radioactivity.

If methionine is the predominant species binding to BSA, then a reduction in the specific activity of the precursor should result in a proportional decrease in the amount of radioactivity bound. It was also of interest to determine if tryptophan, the only other

amino acid known to selectively bind to BSA, would antagonize methionine binding. Similarly, oleic acid and other non-esterified fatty acids are known to bind to BSA and will competitively inhibit the binding of tryptophan. Tritiated methionine (25 μCi, 90% as methionine) was added to 1 ml Dunlop's medium containing 1 mg/ml BSA. Methionine and tryptophan were added at concentrations of 2, 20 or 200 μM. Oleic acid was present at 7, 15 and 30 μM, which corresponds to a molar ratio to BSA of 0.5, 1 and 2 respectively. The results are shown in Table VIII. Tryptophan had no effect on methionine binding to BSA. Oleic acid inhibited the association of methionine with BSA reducing the amount of radioactivity bound by half. There was no concentration dependence of this inhibition.

Dilution of the labeled methionine with unlabeled precursor did not result in a significant reduction in the amount of radioactivity bound. There is a small, concentration-dependent decrease in the total amount of acid insoluble radioactivity, however, much less than would be expected if methionine was the major species being bound. The chemical amount of labeled methionine added is 1.6 μM, the 200 μM sample represents a greater than 100-fold dilution, yet the binding is inhibited by only 20%.

TABLE VIII

EFFECT OF METHIONINE, TRYPTOPHAN AND OLEIC ACID ON BINDING OF ^{3}H-METHIONINE TO BSA

	TCA-Precipitable Radioactivity					
	Methionine		Tryptophan		Oleic Acid	
μM concentration	dpm/ml X 10^{-2}	dpm/μg protein	dpm/ml X 10^{-2}	dpm/μg protein	dpm/ml X 10^{-2}	dpm/μg protein
Control 0	1033	103				
2	938	94	964	96		
20	845	85	984	98		
200	811	81	990	99		
7					530	53
15					455	45
30					470	47

Dunlop's medium containing 1 mg/ml of BSA was incubated for 12 h with 24 μCi (15 Ci/mmole, 90% of radioactivity present as methionine) of ^{3}H-methionine at room temperature. Methionine, tryptophan and oleic acid were added at the concentrations indicated.. TCA-precipitable radioactivity was determined as described in the text.

DISCUSSION

The goldfish retinal explant culture provides a useful model for biochemical investigations of nerve growth and regeneration. The use of interventive techniques, as in the two examples reported here, permit a chemical dissection of the process of nerve growth. The ability of drugs to disrupt specific structural elements, or to preferentially inhibit or facilitate selective aspects of cellular metabolism allow the precise determination of their contribution to processes involved in nerve growth.

Cytochalasin B

Cytochalasin B disrupts the microfilamentous network or lattice present in the growth cone and beneath the plasma membrane (Yamada et al., 1970, 1971; Spooner, 1971). Ultrastructural studies of the neuritic growth cone and of locomotory cell types have shown disorganization of the microfilamentous network following treatment with the drug (Yamada et al., 1971). The microfilament network normally has a regular and oriented appearance. Cytochalasin B disrupts this organization without completely destroying it. Some local areas appear unaffected while in other areas the microfilaments collapse, forming densly packed aggregates. Cytochalasin B has no demonstrable effect on any other cellular structures. The selective disruption of the microfilamentous network and the arrest of cell movement has led to the suggestion that the microfilaments were responsible for

motile activity of the plasma membrane required for cellular locomotion and axon elongation.

The subunit protein of the 5 nm microfilaments, is similar to actin. Actin-like and myosin-like proteins have been isolated from a number of non-muscle tissues, including brain (Berl et al., 1973). In cultured neurons and fibroblasts the distribution of actin-like protein can be shown to correspond with that of the microfilamentous network. Fluorescein-labeled muscle heavy meromyosin will specifically bind actin-like proteins. This binding is highest in the growth cone and at the advancing edges of fibroblasts and other non-neuronal cells (Sanger, 1975). The interaction of actin and myosin-like proteins are believed to be responsible for the contracile activity required for cell locomotion, axon elongation, pinocytosis and neurotransmitter release (Allison, 1973).

The molecular mechanisms by which cytochalasin B affects microfilament structure is unknown. The drug is believed to prevent the interaction of the subunit proteins, however, there is no direct evidence for this (Spooner et al., 1971). The reassembly of the microfilamentous network following removal of the drug is unaffected by cycloheximide (Spooner et al., 1971). The restoration of the normal morphology suggests that the rearrangements underlying the formation of an organized microfilamentous network do not require newly synthesized proteins.

Cytochalasin B is known to have effects at the cell surface aside from its action on microfilaments. It has been shown to inhibit

the transport of glucose and glucosamine across the cell membrane (Zigmond and Hirsch, 1972) while amino acid transport and protein synthesis are not altered by the drug (Yamada et al., 1971; Zigmond and Hirsch, 1972). Cytochalasin B has been shown to bind to cells (Lin et al., 1973) and is localized in plasma membrane and microsomal fractions (Mayhew et al., 1974).

The studies reported here are consistent with known effects of the drug. The rapid inhibition of neurite elongation and non-neuronal cell locomotion was accompanied by morphological changes associated with loss of microfilamentous structures.

Colchicine

The effects of colchicine on neurite elongation and morphology in retinal explant cultures are comparable to similar studies by other investigators (Seeds, 1970; Daniels, 1972, 1973, 1975; Yamada et al., 1970, 1971; Strassman, 1973).

Fibers from single dorsal root ganglion cells fail to elongate and eventually retract following the administration of colchicine. This response is dose-dependent and reversible. The inhibition of neuritic extension is not immediate, the fiber will continue to elongate for the first 20-30 min following addition of the drug to the culture medium. After 6 h there is 45% reduction in the number of microtubules present in the neurite (Daniels, 1973). There was a direct correlation between the reduction in the number of microtubules and the inhibition of neurite extension. Yamada et al.(1971) using two-fold higher colchicine concentrations, found no microtubules present after 18 h. This loss of the microtubules caused the fiber

to become progressively narrower with varicose enlargements appearing along its length. The varicosities were found to contain dense-core vesicles and neurofilament bundles (Daniels, 1973). Colchicine was not observed to affect other organelles or fibrous proteins (Yamada et al., 1971)

Daniels (1972) found that neurite extension from dorsal root ganglion cells was inhibited at colchicine concentrations of 2.4 X 10^{-8} M to 1.2 X 10^{-7} M, with fiber retraction occurring at the higher end of the range. The goldfish neurites were affected at significantly lower concentrations. Inhibition was clearly occurring at colchicine concentrations of 1.25 X 10^{-8} M. Grafstein (1971) has reported that colchicine will prevent regrowth of the goldfish optic nerve if applied directly to the cut end.

Microtubules are linear polymers of the subunit protein, tubulin, and provide structural support for the axon. These elements are known to be involved in a variety of cellular phenomena such as the maintenance of cell shape, secretion and movement of cytoplasmic components (reviewed by Burnside, 1975). In neurons, the integrity of the microtubules is essential for intraaxonal transport, although their precise role is unclear (Smith et al., 1975; Wooten et al., 1975).

Much of what is known about microtubule function has been derived from studies with agents such as colchicine, colcemid and vinblastine which interfere with the polymerization of the subunits.

Colchicine, the best studied of these drugs, binds to the tubulin dimers, composed of two non-identical tubulin subunits of 54,000 and

56,000 daltons respectively. This binding is characterized by its high affinity and long half life (36 h, Garland and Teller, 1975). In the cell, an equilibrium exists between free tubulin pools and the polymerized protein. The binding of colchicine to the free tubulin shifts the equilibrium, favoring disassembly of microtubules.

Several agents are known to stabilize microtubular structures such as DMSO (Jacob and Herschler, 1975) and D_2O (Stadler and Franke, 1972), presumably by altering the microenvironment preventing the dissolution of intact microtubules. These agents will also enhance neuritic outgrowth (Murray and Benitz, 1967; Schmitt, 1976). Cyclic AMP stimulates neurite formation from chick sensory ganglion, and partially reverses the effect of colchicine (Roisen et al., 1975). This effect is probably due to facilitated assembly from the existing subunit pool or stabilization of the intact microtubules.

Labeling of Retinal Proteins

The morphological properties of the retinal explant cultures suggested that there might be detectable biochemical differences between post-crush and normal explants. On day 5 in culture post-crush retinal explants were actively growing neurites and possessed hypertrophied ganglion cells. The ganglion cells of the normal retinal explants were considerably smaller. The failure to demonstrate any reproducible differences in incorporation of labeled precursors into retinal protein was therefore unexpected, particularly in view of the report of Heacock and Agranoff (1976) demonstrating

an enhanced biosynthetic capacity of the ganglion cells from post-crush retina. A five day interval after explantation was chosen somewhat arbitrarily, based principally on the abundant neuritic outgrowth present after this period from post-crush explants. A shorter interval may have yielded bigger differences. The ganglion cells in normal retinal explants have begun to hypertrophy by day 5 in culture, so the synthetic processes required for axonal regrowth may have already been accelerated. While enlargement of the ganglion cells was small, the synthesis of the various proteins could be increased to levels comparable to post-crush retina. The cellular hypertrophy may be an unreliable index of increased biosynthetic capacity, since an increase in cellular volume could represent a previous net accretion of cellular materials either by increased synthesis, by decreased degradation, or both. These considerations can perhaps explain the difference in the present finding with those of Heacock and Agranoff (1976). As Seeds has pointed out, neuritic outgrowth need not be accompanied by preferential synthesis of tubulin (Morgan and Seeds, 1975). It is possible that the tissue culture conditions are not optimal for tubulin biosynthesis compared to the situation in vivo.

Hier et al. (1972) reported spurious results which occurred in the measurement of colchicine binding activity in the presence of rat tail collagen. They found that when cultures of dorsal root ganglia were grown on collagen, colchicine binding activity was lost following 37,000 X g centrifugation. They concluded that tubulin formed an insoluble complex with the collagen which was precipitated during

centrifugation. The retinal explant cultures used in the present study were grown on collagen substrata, so it is possible that the soluble proteins recovered after high speed centrifugation do not represent the total soluble tubulin originally present in the explant; this could explain the failure to find enhanced labeling of tubulin in soluble fractions. If a tubulin-collagen complex was precipitated, the detergent extraction might have been expected to solubilize the proteins. The absence of a selectively labeled protein of the tubulin class in these extracts may reflect the insolubility of the complex, or it may have been masked by large amounts of detergent solubilized protein co-migrating with tubulin.

The issue of whether do novo synthesis of microtubule protein is required for neuritic growth is unresolved and is the subject of some controversy. It is clear that microtubule assembly from preformed subunits and neurite elongation can occur for a short time in the absence of protein synthesis (Seeds et al., 1970). Conversely, the inhibition of neurite elongation and microtubule assembly does not affect tubulin synthesis (Hier et al., 1972). Studies of NGF stimulated neuritic growth from sympathetic ganglia (Hier et al., 1972; Stockel et al., 1974) and human neuroblastoma (Kolber et al., 1974) have demonstrated enhanced synthesis of tubulin based on a colchicine binding assay. Conflicting evidence was presented by Yamada and Wessells (1971). They could detect no increase in tubulin levels in dorsal root ganglia following NGF treatment. A common feature of all of these studies is the use of a single time point colchicine assay

as a measure of tubulin concentrations. This assay has been criticized on technical grounds (Morgan and Seeds, 1975; Mizell and Bamburg, 1975). Subsequent studies, using an improved time-decay assay, could demonstrate neither increased chemical amounts of tubulin nor increased colchicine binding activity in NGF stimulated peripheral ganglia. Similarly, the morphological differentation of several neuroblastoma cell lines is not accompanied by alteration of tubulin concentrations or the rate of tubulin synthesis (Morgan and Seeds, 1975; Mizell and Bamburg, 1975; Schmitt et al., 1976). The differentiated cells have large numbers of intact microtubules which are absent in the undifferentiated cells, indicating that microtubules are assembled from preformed subunit pools. In these cells, the regulation of neurite growth is more closely related to the assembly of microtubules rather than regulation of tubulin synthesis.

In developing brain the situation is somewhat different. Tubulin comprises a substantially higher proportion of total soluble protein in developing animals compared with adults, suggesting a preferential synthesis of tubulin during maturation (Schmitt et al., 1977), as has indeed been seen in incubations of regenerating goldfish retina (Heacock and Agranoff, 1976).

Binding of Methionine to BSA

The initial observation of a single labeled protein appearing in the incubation medium of retinal explant cultures following the administration of radioactive methionine suggested that the explants may be secreting protein into the medium. The CXM-insensitive labeling

occurring with retinal explant cultures was conclusive evidence that incorporation of radioactive methionine was not due to ribosomal protein synthesis.

The migration of the labeled medium protein on polyacrylamide gels containing 8 M urea and SDS initially led to the question of whether the label was covalently bound to protein. The persistence of the labeling in the presence of CXM suggested a post-translational modification of an existing protein, catalyzed either by the explants or the serum. There are several reports in the literature documenting non-ribosomal mechanisms for the incorporation of labeled amino acids into protein.

Dunlop et al. (1974) reported CXM and puromycin-insensitive incorporation of amino acids into TCA-precipitable protein in brain slices, which was not removed even following repeated TCA washes. They tested four different amino acids with similar results. If ^{14}C-lysine was used as a precursor in the presence of CXM, protein hydrolysates showed only a small fraction of the radioactivity present as lysine. This probably represents transfer of the labeled carbon into other metabolites. Cycloheximide-insensitive labeling was greater when tritiated precursors were used. It should be noted that these studies were carried out using relatively high precursor concentrations (0.01-5 mM).

Incorporation of radioactive amino acids into high speed supernatants have been described in a number of preparations (reviewed by Barra et al., 1973). The incorporation of arginine into protein has

been studied extensively by Soffer in rabbit liver (1970). The reaction is dependent on the presence of Arg-tRNA and soluble enzyme. The enzyme Arg-tRNA:protein transferase will quantitatively transfer arginine to the N-terminal residue of the protein. The reaction is totally dependent upon the presence of an appropriate acceptor protein, in this case BSA. In later studies, small peptides with a terminal glutamate or aspartate residue were found to work equally well (Soffer, 1973 b). Methionine did not inhibit the arginine transfer reaction, nor would it substitute as an acceptor when present as the N-terminal residue. A similar tRNA:protein tranferase has been described in E.coli which is specific for addition of leucine and phenylalanine (Leibowitz and Soffer, 1971; Soffer, 1973 b.)

Caputto and co-workers have found that tyrosine and phenylalanine will be incorporated into a single protein, tubulin, by a ribonuclease-insensitive mechanism (Barra et al., 1973a; 1973b; 1974). These amino acids are added to the carboxyl-terminal glutamate residue (Arce et al., 1975). The responsible enzyme, tyrosyltubulin ligase, has an absolute requirement for ATP and Mg^{+2} and has a molecular weight of approximately 150,000 daltons. The ligase is reported to act only on the α subunit of tubulin (Raybin and Flavin, 1975). Tyrosyltubulin ligase will also catalyze the addition of dopamine to α tubulin, the incorporation of both phenylalanine and dopamine by this enzyme is on the order of 5% that of tyrosine (Deanin and Gordon, 1976).

These studies were particularly relevant to the protein labeling observed in our system, especially those of Soffer, in which BSA was

used as an acceptor. The observation that the reaction would occur in the absence of tissue did not rule out enzymatic mechanisms as the source of the labeling. Fetal calf serum is an undefined mixture of proteins and soluble components and is known to have a variety of enzyme activities. It is possible that it may possess all the necessary factors required for the incorporation of amino acids, although it has never been reported. While it remained plausible that methionine may have been incorporated into TCA-precipitable material by these mechanisms, methionine has not been reported to act as a substrate for ligases. The most conclusive evidence against such a mechanism was the incorporation of labeled methionine into acid precipitates in a solution containing only BSA. BSA is not known to possess any enzymatic activity.

Methionine typically undergoes reactions which cleave the methyl group and transfer it to various acceptors. Tritiated methionine used in these studies was labeled in the methyl group and could have participated in transfer reactions to BSA, were the appropriate co-factors present. This mechanism could not account for the results, as ^{35}S-labeled methionine was as effective as tritium-labeled precursor in labeling BSA.

Methionine is readily oxidized to methionine sulfoxide (MSO) in solution. In all the labeled preparations used in this study, MSO was a significant contaminant. A pertinent question was whether the radioactivity bound was methionine or its oxidized derivative. When the bound radioactivity is removed from the protein and separated

chromatographically, 70% of radioactivity is associated with MSO. Whether this represents the actual fraction of label bound as MSO or reflects oxidation of methionine during chromatographic procedures, is unclear. Subsequent experiments did not completely clarify this issue. The results indicate that both methionine and MSO can directly bind to BSA. Methionine bound nearly twice as well as MSO. The binding of radioactive precursor to BSA occurs in a molar ratio of 1 to 2000-5000, i.e., only a very small fraction of the albumin molecules bind the labeled precursors. The amount of radioactivity bound in no case exceeds 1%, so it is unlikely that the concentration of methionine or MSO is limiting. From data presented in Table VII, it was inferred that 60-70% of the bound label could be attributed to methionine. The disparity in these results and those of the isotope dilution experiments (Table VIII), are problematic. The isotope dilution resulted in only a small (10-20%) loss of TCA-precipitable radioactivity with a 10-100 fold reduction of the specific activity of the labeled methionine. This method is more reliable and must be considered a more accurate estimation of actual binding of methionine to BSA. In the former experiments, only 60% of the administered radioactivity could be recovered as methionine. The possibility that contaminants present in the labeled methionine preparation are binding cannot be excluded. Since the total amount of material bound is small, a contaminant present at very low concentrations could account for some of the labeling. If a significant fraction of the bound radioactivity were attributable to a labeled contaminant, then the recovery of 87% of the radioactivity released from the BSA, following urea treatment as methionine or MSO, must be explained. It is possible that the contaminants

may co-migrate with methionine or MSO in the solvent system used for paper chromatography.

The time course of incorporation of labeled precursor in a 1% FCS solution suggests that the binding is saturable. If there are a limited number of sites which will bind the radiolabeled molecules then the binding must occur with only a very small sub-population of the BSA molecules. Alternatively another protein of approximately the same molecular weight may be responsible for the incorporation of radioactivity. If this is the case then the commercial BSA preparation must contain this protein as a contaminant.

If methionine is non-specifically bound to albumin and is removed by urea and mercaptoethanol, why then did the radioactivity migrate with the protein in SDS-urea gels? Those samples which were prepared for electrophoresis contained 1% SDS. The detergent may have sequestered the methionine in the micelle, masking it from the urea.

Serum albumin is known to bind a variety of agents in the blood: bilirubin, fatty acids, steriods, drugs and amino acids. Albumin binds these substances, many of them sparingly soluble in aqueous solutions, and transports them to the relevant tissues. The molecules bound to albumin constitutes a physiological "reservoir", and are particularly important in the pharmacology of a number of drugs (Kock-Weser, 1976). Tryptophan is the only amino acid which has been demonstrated to specifically bind to serum albumin (McMenamy, 1957). This amino acid binds at a single hydrophobic site on albumin in a stereospecific manner and is inhibited in the presence of nonesterified fatty acids (McMenamy, 1958). Tryptophan did not affect the binding of labeled precursor to BSA. Oleic acid, however, reduced the acid insoluble radioactivity by

50%. Fatty acids are known to bind to BSA at several sites of the molecule and thus may have non-specifically inhibited the association of labeled compounds with the protein.

These studies have demonstrated the tight binding of a small molecule, either methionine, methionine sulfoxide, or possibly a contaminant, to bovine serum albumin. The use of labeled methionine in studies where it may possibly bind protein should be closely controlled to avoid artifactual incorporation of radioactivity into non-dialyzable and acid insoluble fractions. The CXM-insensitive incorporation of labeled amino acid in the study of Dunlop et al. (1974), could represent the type of non-specific binding encountered here. Henry et al. (1964) have reported that MSO will non-specifically bind to soluble proteins. It is disturbing that ovalbumin will retain significant amounts of radioacitvity following TCA-precipitation. Even though the label can be dialyzed away, it could result in artifically high levels of apparent incorporation. Labeling studies using serum-supplemented incubation medium should be viewed with some caution, particularly when amino acid precursors are used (Lott, 1972; Chiavugi, 1973).

The goldfish retinal culture constitutes one of the few known explicit examples of regenerating neuronal processes in vitro. The distinction between actual regeneration of neuronal processes and regrowth of an axon during development should be clarified. Most of the work with other nervous tissue in culture has undoubtedly been dealing with axonal regrowth, but these tissues are largely derived from developing animals. It is, therefore, not clear to what degree

a given observation represents regeneration or differentiation. Perhaps these are artificial distinctions, and processes subserving regeneration do not differ significantly from those occurring during development. Nevertheless, the goldfish retinal ganglion cells are clearly differentiated neurons which are amenable to biochemical manipulation and analysis. The regrowth of these cells in vitro may allow the determination of the molecular mechanisms which initiate and sustain axonal regeneration. The vigorous neuritic outgrowth obtained from post-crush retinal cultures permits the examination of general questions concerning biochemical characteristics of growing axons.

Preliminary attempts have shown that it may be possible to obtain a purified neurite preparation by removing the body of the explant after several days in culture, leaving the neurites attached to the substratum. This fraction would be ideal for biochemical analysis of the neurites to determine changes that occur with maturation in vitro.

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