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GROWTH OF THE ADULT GOLDFISH RETINA

by
Pamela Ann Raymond Johns

A dissertation submitted in partial fulfillment
of the requirements for the degree of
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PREFACE

This thesis is concerned with the growth of the nervous system during postembryonic life. In particular, I will focus on the problem of neurogenesis, the formation of nerve cells. Postnatal neurogenesis is especially interesting since any addition of new neurons during postembryonic life must occur in conjunction with the neural activities necessary to produce the behavioral and metabolic responses required by the functioning organism in its interactions with the environment. These two processes, neurogenesis and neural activity, are usually thought of as temporally distinct and mutually exclusive, the former being the province of the embryonic nervous system and the latter the concern of the postembryonic animal. Several instances of postnatal addition of neurons have, however, been documented.

Chapter I will first review the process of histogenesis of the embryonic nervous system as it was classically and is currently understood, and then describe the extension of this process into postnatal life, where it has been found. The literature on this topic divides itself into two general categories, on both systematic and mechanistic grounds. In mammals, postnatal neurogenesis has been postulated as a mechanism involved in learning, or modification of behavior dependent on experience. In amphibians and

teleost fish, postnatal neurogenesis is thought to be related to growth and regeneration of the nervous system. In Chapter II, I will present evidence based on cell counts in animals of different ages, that growth of the eye in adult goldfish is in part a result of the continued addition of retinal neurons. Chapter III describes the results of an experimental technique, ^3H -thymidine radioautography, which was used to determine the source and fate of these new retinal neurons. Finally, the implications of the retinal growth and cell addition will be discussed in terms of the anatomy and physiology of the neuronal connections in the visual system of the growing adult goldfish.

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LIST OF ABBREVIATIONS

<u>df</u>	degrees of freedom
ELM	external limiting membrane
<u>F</u>	the variance ratio
GC	ganglion cell
³ H	tritium
ILM	inner limiting membrane
INL	inner nuclear layer
m.p.	melting point
ONL	outer nuclear layer
<u>p</u>	probability
PRE	pigmented retinal epithelium
<u>r</u> ²	coefficient of determination
S.D.	standard deviation
S.E.M.	standard error of the mean
<u>t</u>	Student's t-statistic
μCi	microcuries

CHAPTER I

HISTOGENESIS OF THE CENTRAL NERVOUS SYSTEM:

A Review of the Literature

The development of the vertebrate central nervous system is a field which has been dominated by the ideas and theories of a few influential scientists. Dogmas have often arisen even in the face of conflicting evidence; the "reign of the ruling theory" (Chamberlain, 1965) is an apt description of the intellectual environment in neuroembryology from the late 19th century until recent times. The following discussion will trace the rise and fall of several of the more pervasive and influential theories.

EMBRYONIC STAGES

The classical interpretation of histogenesis of the nervous system is based on the descriptions of His (1889, 1904; reviewed by Jacobson, 1970). During the early stages of embryonic development, the vertebrate central nervous system is composed of a neural tube containing an inner, cell-rich mantle layer and an outer cell-poor marginal region (Fig. 1). Early workers agreed that all of the mitotic figures in the neuropithelium, were confined to the

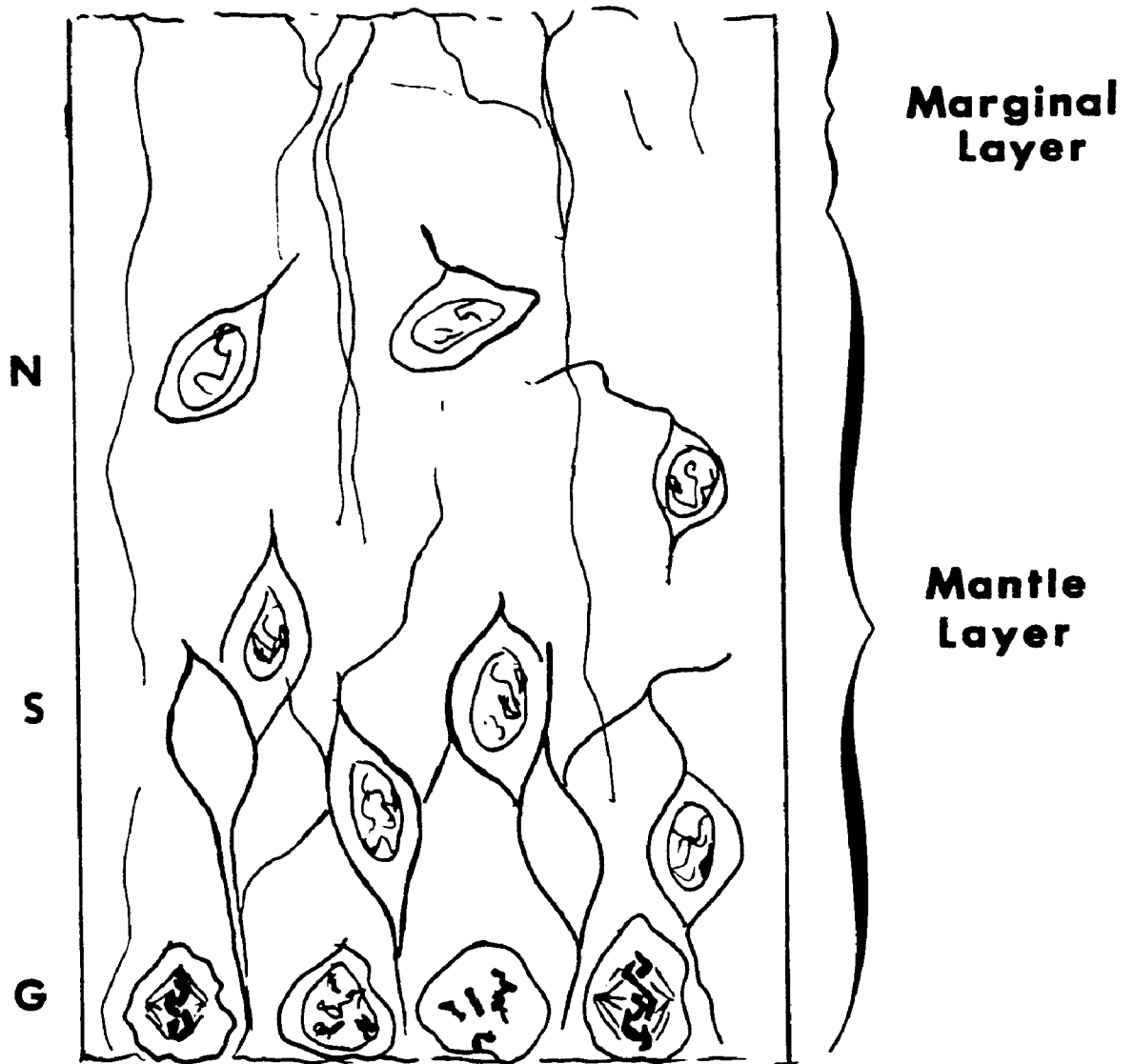


Fig. 1. Classical view of the neural tube. The early neuroepithelium is divided into two layers: an outer, cell-poor marginal layer and an inner, cell-rich mantle layer. The three cell types in the mantle layer include the germinal cells, G; the spongioblasts, S; and the neuroblasts, N.

cells lining the lumen of the neural tube (Ramón y Cajal, 1909-11). Based on this observation, His categorized the cells in the early embryonic vertebrate neuroepithelium into three types: 1) an innermost layer of proliferating germinal cells which divided mitotically at the ventricular surface to generate 2) spongioblasts and 3) neuroblasts, post-mitotic cells giving rise to neuroglia and neurons, respectively. His thought that the glia cell precursors, the spongioblasts, formed a syncytial mass directly above the germinal cells, through which the newly formed neuroblasts migrated into the overlying mantle layer. A diagrammatic representation of these ideas is shown in Fig. 1. This view of the embryonic central nervous system was universally accepted until the advent of modern cytological techniques, including electron microscopy and radioautography, which allowed a more accurate definition of cellular morphology and mitotic activity. Even before these new methods were developed, however, evidence appeared in the literature which directly contradicted His' theories, but the conflicting information was almost universally ignored.

F. C. Sauer (1935a) studied histological sections of chick and pig embryos and concluded that the germinal cells and the spongioblasts of His were not separate cell types, but represented two phases in the life cycle of a single neuroepithelial cell. Sauer's ideas were based on careful observations which showed that the germinal cells could not be traced through the complete mitotic cycle;

specifically the stage between late telophase and early prophase was missing. The cytological appearance of the spongioblasts corresponded to this missing stage. Restriction of mitosis to the region adjacent to the lumen is a common feature of multilayered epithelia (Sauer, 1936). Sauer therefore concluded that the cells in the developing neural tube constituted a pseudostratified neuroepithelium whose nuclei underwent migration toward the ventricular surface to divide and away from it during interphase (Fig. 2). Sauer also showed (1935b) that the spongioblasts of the neuroepithelium were distinct entities and not a syncytium; the anastomoses between the cells which often made them appear syncytial were an artifact of poor fixation.

Sauer was not the first to disagree with His' interpretations. Schaper, a contemporary of His, proposed a theory of neurogenesis essentially the same as Sauer's (Schaper, 1897 a,b). Using histological methods similar to those of His and making essentially the same observations as His, Schaper concluded that the spongioblasts and germinal cells were really one type of cell at different stages in the mitotic cycle when at different levels in the neuroepithelium. Why this theory, which has since proved to be the correct one, was ignored until Sauer (1935a) confirmed it, one can only guess. Jacobson (1970) speculates that it was the influence of Ramón y Cajal who favored His' interpretation which lead to its prominence, although careful

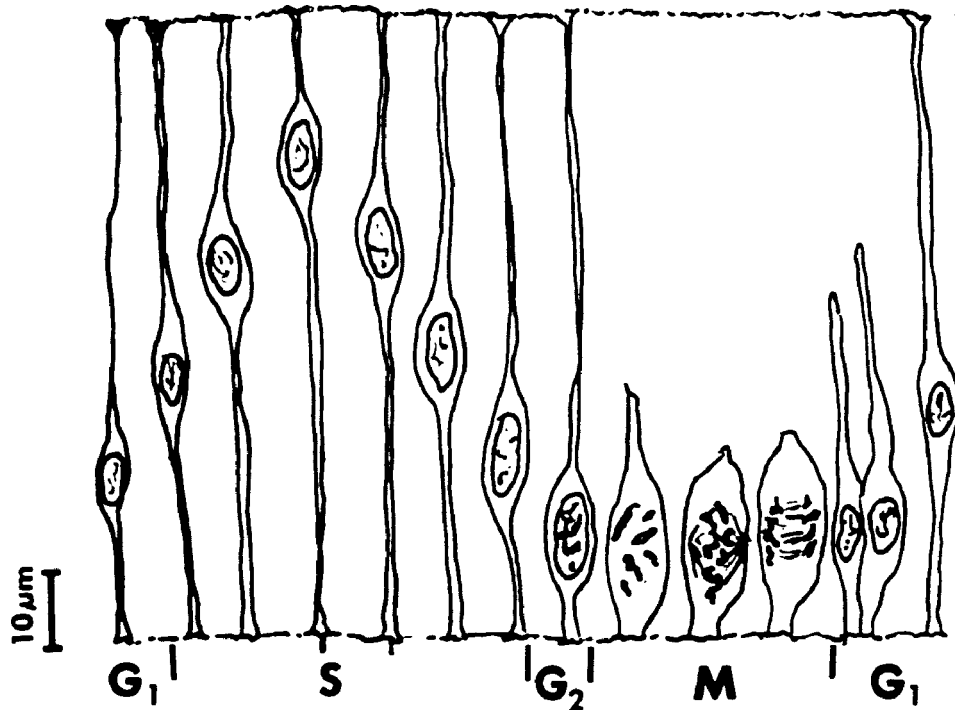


Fig. 2. The neuroepithelial germinal cell and interkinetic nuclear migration. The nucleus of the neuroepithelial cell is located at different levels in the early neural tube depending on its stage in the mitotic cycle. During interphase, G₁, the cell extends processes to both the pial and the ventricular surface. During the phase of DNA synthesis, S, the nucleus migrates toward the pial surface. During G₂ the nucleus migrates to the ventricular surface; it loses its pial attachment, rounds up, and divides mitotically, M. (Adapted from F. C. Sauer, 1936, and Jacobson, 1970.)

analysis of the theory reveals a major flaw which was never fully explained by its proponents: the relatively small number of spongioblasts present in the early neural tube could not possibly be the sole precursors of all of the neuroglia in the adult nervous system without increasing their numbers through proliferation.

Sauer's conclusions, although not accepted immediately, have been amply verified subsequently using the modern technique of ^3H -thymidine radioautography. This technique involves injection of tritiated thymidine, a metabolic precursor of DNA, into the embryo or pregnant female (Messier and Leblond, 1960; Sidman, 1970). As shown schematically in Fig. 3, the label is incorporated exclusively into cells synthesizing DNA in preparation for cell division, although there are some exceptions: 1) a duplication of the genome without subsequent mitosis, leading to tetraploidy, has been found in a few cells in the nervous system, notably the Purkinje cells of the cerebellum (Lapham, 1968) and 2) metabolic DNA synthesis in differentiated cells not involved with mitosis has also been suggested as a possible source of cell labeling (Pelc, 1963). In the vast majority of cases, however, incorporation of ^3H -thymidine has been shown to be associated with the synthetic phase of the mitotic cycle (Mahler, 1972; Hopwood et al., 1975). Following DNA synthesis the labeled cells divide and if their progeny shortly thereafter leave the mitotic cycle and differentiate, their nuclei will remain labeled (Hughes, 1959; Sidman, 1970).

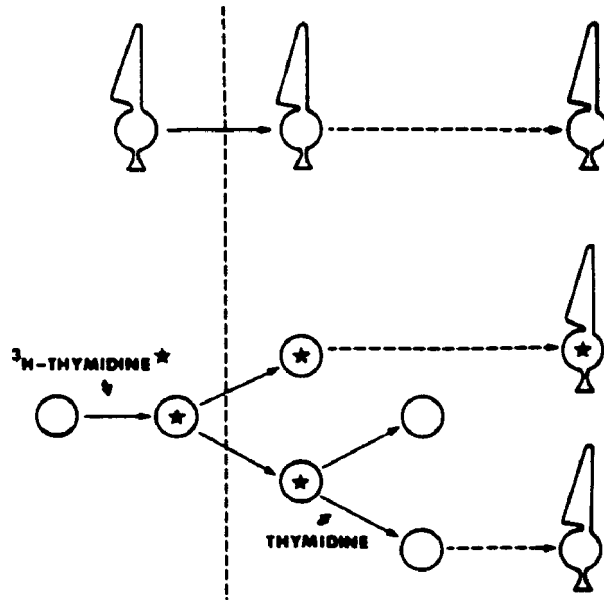


Fig. 3. Schematic view of the technique of ^3H -thymidine radioautography.

The differentiated cells at the top of the diagram, represented here as photoreceptor elements, do not incorporate ^3H -thymidine. Proliferating cells, at the bottom, incorporate the label (*) during the DNA synthetic phase of the mitotic cycle and then divide. If the daughter cell shortly thereafter ceases mitotic activity and differentiates, its nucleus will remain labeled. If the daughter cell continues to divide after labeled thymidine is no longer present in the metabolic pool its label will be progressively diluted, and it will be unlabeled when it eventually differentiates. The vertical dashed line represents the end of ^3H -thymidine availability.

If the cells continue to synthesize DNA when ^3H -thymidine, is no longer available in the metabolic pool, their label will be progressively diluted by further cell division. Thus if the animal is sacrificed shortly after administering the ^3H -thymidine, and its tissues examined radioautographically, only the mitotically active cells will be labeled. If the animal is allowed to live for a longer period of time, the cells now labeled will be those which left the mitotic cycle and differentiated soon after the label was given (Fig. 3). This technique is a powerful tool which has been used to great advantage in determining the kinetics of the cell cycle and the time of origin of specific cell types in the nervous system (Altman, 1968; Jacobson, 1970; Sidman, 1970).

Two different methods have been used to determine the time of origin of different cell types. Both involve injecting ^3H -thymidine at an early stage and allowing the animal to survive until a later developmental stage. The first (Sidman, 1960, 1970) is explicit in the above description of radioautography; following a pulse of ^3H -thymidine, the cells most heavily labeled at a later time will be those which were born, i.e. differentiated, very near the time at which the label was injected. The second method, cumulative labeling (Fujita, 1963), arose in part fortuitously as a consequence of the inability to obtain pulse labeling in the chick embryo. Whereas exogenous thymidine administered to mammals is cleared from the blood within 30 minutes

(Potter, 1959) it remains continuously available to embryonic chick tissues when injected into the yolk sac. The time of origin of a given cell type in the chick can be determined from the first stage at which ^3H -thymidine is no longer incorporated by those cells; the cells born near the injection time will be those which are unlabeled at later stages.

Several investigators have used radioautographic methods to study histogenesis in the embryonic nervous systems of the chick (M. E. Sauer, 1959; Fujita, 1962) and the mouse (Sidman et al., 1959). These studies have all confirmed the theory put forth by Schaper (1897a,b) and F. C. Sauer (1935a). The germinal cells and the spongioblasts of His are not separate cells, but are merely separate stages in the mitotic cycle of a single cell type, called a matrix cell (Fujita, 1962), a primitive ependymal cell (Sidman et al., 1959) or a neuroepithelial germinal cell (Jacobson, 1970). Fujita (1963) has shown that these cells form a homogeneous population with regard to mitotic activity. His conclusions were based on kinetic studies in the 6-day chick embryo mesencephalon. Following injection of ^3H -thymidine into the yolk sac, the percentage of cells labeled in the neuroepithelium increases linearly until all of the cells are uniformly labeled. This indicates that the germinal cells are a homogeneous population in random phases of the mitotic cycle.

Using Golgi staining methods or serial reconstruction of cells with transmission electron microscopy (Stensaas and

Stensaas, 1968; Hinds and Hinds, 1974) and more recently using scanning electron microscopy (Hattori and Fujita, 1974; Seymour and Berry, 1975) the neuroepithelial germinal cell in the early neural tube has been shown to be a pseudostratified columnar epithelial cell with a variable morphology depending on its stage in the cell cycle (Fig. 2), just as Sauer described (1935a). The cell always retains a connection to the ventricular surface, and during interphase it also sends a process to the pial surface (Stensaas and Stensaas, 1968; Hinds and Hinds, 1974; Hattori and Fujita, 1974). Its nucleus is located at a variable level in the neuroepithelium during interphase; during the S phase of the mitotic cycle, when DNA is synthesized, the nucleus is located near the pial surface (Sidman et al., 1959; M. E. Sauer, 1959; Fujita, 1962, 1963). Subsequently, the nucleus descends, and the distal cytoplasmic process is withdrawn or pinched off (Hinds and Hinds, 1974). When the nucleus reaches the ventricular surface the cell rounds up and divides (Hinds and Hinds, 1974; Seymour and Berry, 1975).

This picture of the early development of the central nervous system involving stratification of the cell cycle in both time and space through the mechanism of interkinetic nuclear migration is now widely accepted (Jacobson, 1970). Unfortunately, it is based entirely on studies of chick (M. E. Sauer, 1959; Fujita, 1962, 1963; Hattori and Fujita, 1974) and mammalian (Sidman et al., 1959; Stensaas and Stensaas, 1968; Hinds and Hinds, 1974; Hattori and Fujita,

1974; Seymour and Berry, 1975) embryos. Surprisingly, only a few studies have been done on the early amphibian neuroepithelial cells (McKeehan, 1966; Glücksmann, 1940, 1965); they reveal no evidence of interkinetic nuclear migration. Mitotic figures are seen throughout the neuroepithelium in the youngest embryonic stages corresponding to those in the chick and mammal in which mitotic figures are seen only at the ventricular surface. Why the non-amphibian neuroepithelial cells exhibit interkinetic nuclear migration is not known for certain. It is not a unique property of neural tissue since it occurs in other epithelia as well (Sauer, 1936). The lack of nuclear migration in the amphibian neuroepithelium may be merely a consequence of the large amounts of yolk present in the cytoplasm of these cells (Glücksmann, 1940; Dixon and Cronly-Dillon, 1972). Glücksmann (1965) suggests that the amount of intercellular space is the important parameter. The rapid utilization of yolk by the embryonic amphibian cells decreases their volume and creates more intercellular space; the cells have room to round up and divide in situ. Later, as the yolk becomes more completely resorbed, the amphibian neuroepithelial cells become more tightly packed, as are those in the chick and mammalian embryos, and at these stages mitotic figures are confined even in amphibians to the only available free surface at the ventricular edge. That the yolk in the embryonic cells is the critical factor is supported by the observation that in embryonic teleost neuroepithelial cells which,

similar to the mammalian and avian cells, contain no yolk, mitosis is restricted to the ventricular surface from the earliest stages (Hollyfield, 1972).

Despite the acceptance of these new theories, His' terminology has lingered in the literature, even in papers dealing directly with interkinetic nuclear migration (Stensaas and Stensaas, 1968). The confusion which arose from combining old terms with new ideas prompted several investigators to attempt to establish a uniform, unambiguous terminology which is unfortunately not yet universally acknowledged (Boulder Committee, 1970). They described five stages in the development of the vertebrate central nervous system (Fig. 4). The first stage (Fig. 4A) is the one discussed above. Rather than "matrix cells" or "primitive ependymal cells" or "germinal cells", they have proposed the term "ventricular cells" for the proliferating cells of the early neuroepithelium. The ventricular zone is designated as the region to which their nuclear migration is confined. These ventricular cells give rise ultimately to all of the neurons and neuroglia in the central nervous system. The pial surface of the ventricular zone is soon overlain with a cell-free marginal zone (Fig. 4B) which contains the cytoplasmic processes, but not the nuclei, of the ventricular cells.

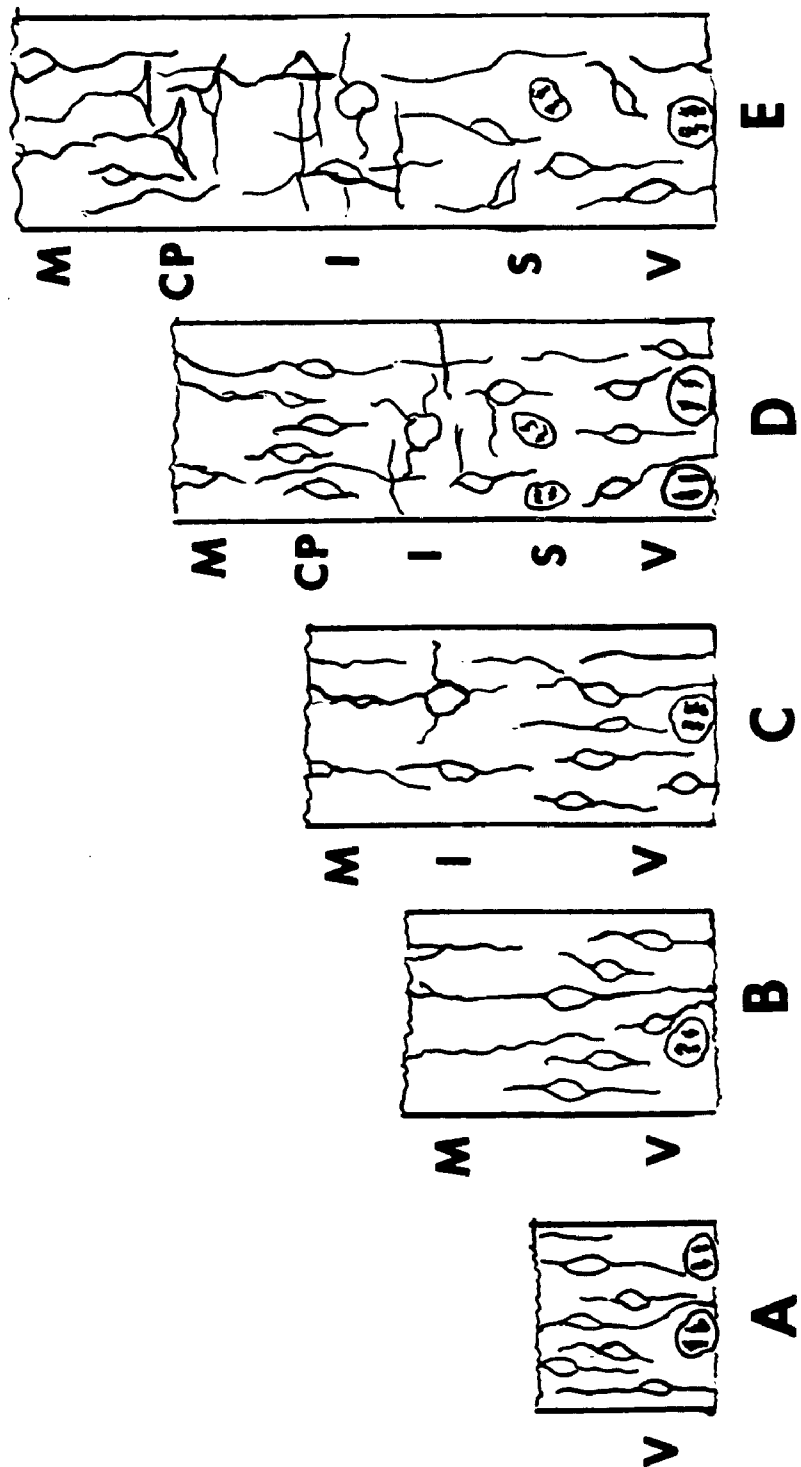
The next developmental stage involves the formation of the intermediate zone (Fig. 4C), formerly known as the mantle layer. Some of the progeny of the ventricular cells lose

Fig. 4. Stages of vertebrate neurogenesis.

- A, the early neural tube composed of a ventricular zone; V, with mitotically active ventricular cells.
- B, formation of the marginal layer, M, from which the nuclei of the ventricular cells are excluded.
- C, formation of neuroblasts and development of the intermediate zone, I.
- D, formation of the cortical plate, CP, by further outward migration of neuroblasts. Formation of the subventricular zone, S, containing proliferating cells which do not exhibit interkinetic nuclear migration.
- E, further maturation of the neural tube.

(Adapted from the Boulder Committee, 1970.)

4



their attachment at the ventricular surface and migrate through the ventricular zone to the base of the marginal zone (Stensaas and Stensaas, 1968; Hattori and Fujita, 1974; Hinds and Hinds, 1974). These cells are called neuroblasts, and are presumed to be the precursors of the neurons. The suffix "blast" is somewhat inappropriate because these cells are post-mitotic and do not incorporate ^3H -thymidine (Fujita, 1974); they are therefore more appropriately called young or immature neurons. Describing their movement through the ventricular zone as migration is also somewhat misleading since they are not really ameboid; rather the nucleus moves or is pulled along behind a previously formed process (Morest, 1969a, 1970) which is in contact with the now spiny processes of the ventricular cells (Hattori and Fujita, 1974). Hattori and Fujita equate the processes of the ventricular cells with the elongated, radially oriented spiny fibers which had been observed much earlier in the embryonic central nervous system using the Golgi technique. These early workers could not trace the cells of origin but assumed them to be a type of glia because of the similarity between the spiny fibers and the ependymal cell processes of the adult central nervous system (Ramón y Cajal, 1909-11). Hattori and Fujita (1974) suggest that guidance of the migrating young neuroblasts may be an important second function of the ventricular cells in addition to their acknowledged role as progenitors of the neurons and glial cells.

In some brain regions, notably the cerebrum and cerebellum, some of the neuroblasts of the intermediate zone undergo further outward migration to form a specialized layer of cells, a cortical plate, at the junction of the marginal layer (Fig. 4D). The cells of the ventricular zone are eventually depleted, and in the adult all that remains is a single layer of ependymal cells (glia) lining the ventricular surface (Fig. 4E).

The early formation of young neurons in the embryonic nervous system has suggested a scheme of histogenesis which is now widely accepted (Jacobson, 1970; Fujita, 1964; Fujita et al., 1966; Morest, 1969b). According to the formulation by Fujita and co-workers, stage I involves ventricular cell proliferation; in stage II, ventricular cells are devoted to the production of neuroblasts; neuroblast production ceases and glioblasts are produced exclusively in stage III. This scheme has been further refined by the observation that the first formed neuroblasts usually give rise to the large, long-axoned macroneurons (Golgi type I), while the later formed neuroblasts differentiate into the small, short-axoned, Golgi type II microneurons (Altman, 1969c; Morest, 1969b; Jacobson, 1970). A scheme involving such a rigid ordering and sequential production of different cell types is supported by evidence from several systems, particularly the cerebellum (Ramón y Cajal, 1929; Miale and Sidman, 1961), but it tends to obscure at least two important phenomena. The first involves regional differences in rates

of development even within a given structure such as the cerebellum (Altman, 1969b), the retina (Straznicky and Gaze, 1971), or the optic tectum (Straznicky and Gaze, 1972). Proliferation and differentiation are patterned in both time and space; such regional differences coupled with secondary migrations of neuroblasts away from their sites of origin are some of the major morphogenic forces in the developing vertebrate nervous system (Berquist and Kallén, 1954; Hamburger, 1956). A scheme which says that macroneurons are produced before microneurons and glial cells are formed last, tends to obscure important variations.

The second oversight which is encouraged by strict adherence to Fujita's theory of cell formation is the contribution of the last major layer to be formed in the developing neural wall, the subventricular zone (Fig. 4D). The subventricular cells continue to proliferate; unlike their progenitors the ventricular cells, these nuclei do not migrate but divide in situ. They are the source of the mitotic figures which can be seen in the so-called mantle layer during later stages of histogenesis (Schaper, 1897a,b). Both neurons and glia are produced from these cells which are thus appropriately called neuroblasts and glioblasts. This zone is extremely variable in form, duration and purpose (Kershman, 1938). It is transient and vestigial in some regions, but in others, such as the cerebral hemispheres, it persists into adulthood as the subependymal layer (Allen, 1912; Globus and Kulenbeck, 1944;

Smart, 1961; Altman, 1969c). Schaper (1897a,b) described these cells, calling them "indifferent cells", and acknowledged their contribution to the later stages of neurogenesis and gliogenesis. He also recognized their potential as a latent source of proliferating cells perhaps important in regenerative processes in the postembryonic brain, a subject to be discussed later. A summary of the stages of histogenesis in the vertebrate nervous system as described above is shown in Fig. 5.

The persistence of the subventricular layer of proliferating cells into adult life is the first example of the phenomenon of postnatal histogenesis which will be the subject of the remainder of this work. Although postnatal growth of the brain occurs in the neonatal mammal (Altman et al., 1968) and even in the adult non-mammalian brain (Packard, 1972), it is usually attributed to growth and maturation of the dendritic processes and myelination of the axons of the nerve cells (Eayrs and Goodhead, 1959; Jacobson, 1970). The idea that cells continue to divide in the brains of adult animals has not been generally accepted, since mitotic figures could rarely be found (Penfield, 1932a). Konigsmark (1970), for example, gives a table of the definitive number of neurons in the adult brain of several species in which such counts have been made. The constancy of neurons, or more accurately, their gradual depletion through cell death in adult animals is a widely held belief (Prestige, 1974). It has long been recognized, however,

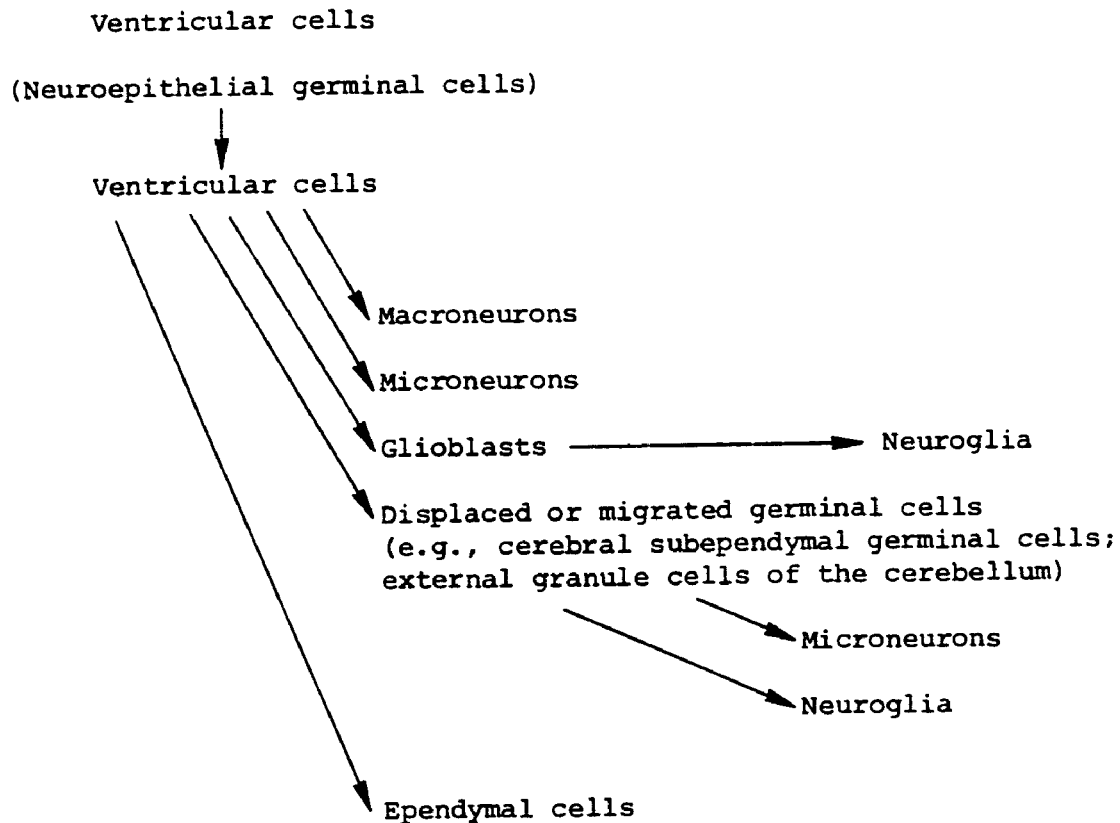


Fig. 5. Histogenesis of the nervous system involving sequential formation of different cell types.

(Adapted from Jacobson, 1970.)

that glial cells retain the capacity to proliferate in response to injury of various sorts, and an abundant literature exists on this subject.

POSTNATAL GLIOGENESIS

Before discussing the literature dealing with postnatal gliogenesis it is necessary to review briefly the morphology and functions of the various neuroglial elements in the adult vertebrate brain. The following summary is based on a review of this subject by Watson (1974). There are two general types of glia in the central nervous system, the macroglia and the microglia. The latter are not important in the context of the present discussion, since they have been shown to be of mesodermal, not ectodermal, origin. They are invaders in the nervous system, wandering leucocytes which have escaped from the blood to perform their phagocytic function in the brain. Of the "true" neuroglia, the ependymal cells and the astrocytes are typically epithelial in nature. The ependymal cells line the ventricular surfaces of the brain and spinal cord, and the astrocytes interpose themselves between nervous tissues and blood vessels. They both form basal laminae in conjunction with mesenchyme and are joined by gap junctions. The third type of neuroglia, the oligodendrocyte, is atypical. It is found primarily in association with fiber tracts where it participates in the formation of myelin.

Following damage to a nerve (Watson, 1965; Sjöstrand,

1965, 1966; Gaze and Watson, 1968) or the spinal cord (Adrian and Walker, 1962; Friede and Johnstone, 1967); removal of an eye (Skoff and Vaughn, 1971); lesion of certain brain regions (Altman, 1962; Konigsmark and Sidman, 1963; Cavanagh, 1970; Mori, 1972); damage of the retina (Laqua and Machemer, 1975); during certain types of physiological stress such as dehydration (Murray, 1968); and in certain pathological states (Penfield, 1932a; Kimberlin and Anger, 1969) increased numbers of glial cells and increased local incorporation of ^3H -thymidine, indicating proliferative activity, have been observed in a number of vertebrate species. A possible but trivial, explanation for the appearance of labeled glial cells in the region around a lesion is that they are microglia, escaped blood macrophages, which have been attracted to the site to phagocytize the degenerating debris. Since microglia are blood elements, their incorporation of ^3H -thymidine is not evidence for postnatal gliogenesis in the brain. Many of these injury-induced labeled cells have indeed been identified as microglia (Sjöstrand, 1966; Mori, 1972), but some studies (Adrian and Walker, 1962; Konigsmark and Sidman, 1963; Skoff and Vaughn, 1971) have controlled for leucocytic infiltration by pre-labeling these cells before making the lesion. Not as many labeled cells were seen when only the microglia were labeled as there were when the ^3H -thymidine was injected following surgery. Thus some of the proliferating cells are

neuroglia intrinsic to the brain, which were stimulated to divide by the trauma.

Even more intriguing is recent evidence showing that gliogenesis is a normal function of the adult brain (Bryans, 1959; Hain et al., 1961; Altman, 1963; Altman and Das, 1965a; Vaughn, 1969). The failure of previous investigators to detect mitotic figures in the normal adult brain has been attributed, like the anastomoses between the spongioblasts which made them appear syncytial to His and his colleagues, to the poor fixation techniques available at the time (Cavanagh and Lewis, 1969). Although a few reports of mitotic activity in the adult brain did exist in the literature (Allen, 1912), Bryans (1959) was the first to use colchicine in an attempt to demonstrate mitotic figures in the adult brain. Colchicine is a mitotic inhibitor which arrests dividing cells at metaphase (Taylor, 1965) and has, since 1960, frequently been used in studies of central nervous system development to enhance the detectability of mitotic figures (review by Jacobson, 1970). Bryans (1959) counted mitotic figures in frontal sections through the forebrains of one-year-old rats at the level of the optic chiasma. He found that the numbers of mitotic figures in the subependymal layer was increased eight times over control levels at 4 hours following administration of colchicine. Although the numbers of mitotic figures he saw was not impressive (10 in 10 sections), his results have since been verified repeatedly using ^3H -thymidine radioautography

(Messier et al., 1958; Hain et al., 1961; Smart, 1961; Smart and Leblond, 1961; Adrian and Walker, 1962; Altman, 1963; Altman and Das, 1965).

Most of these studies have followed Bryan's lead and have enumerated labeled cells in sections through the forebrain of adult mice or rats, particularly at the level of the optic chiasma. Much of the proliferative activity in this region is in the subependymal layer, a part of the subventricular zone of the embryonic brain which persists around the anterior pole of the lateral ventricles in adult mammals (Opalski, 1933; Globus and Kulenbeck, 1944). The subependymal cells can be thought of as "embryonic rests" since their morphology is undifferentiated (Globus and Kulenbeck, 1944) and their metabolism, dependent on anaerobic glycolysis, is typical of fetal tissues in general (Shimizu et al., 1957; Benjamins and McKhann, 1972).

In addition to the subependymal cells, labeled cells are also seen scattered throughout the forebrain, especially in the corpus callosum and other fiber tracts (Dalton et al., 1968; Altman, 1963). Some of the cells which incorporate ^3H -thymidine are cytologically similar to the subependymal cells, and therefore probably glioblasts (Smart and Leblond, 1961; Smart 1961; Paterson et al., 1973), but others appear to be fully differentiated neuroglia (Messier et al., 1958; Altman, 1963; Hommes and Leblond, 1967; Mori and Leblond, 1969, 1970; Korr et al., 1973, 1975). Some of the studies purporting to show mitotic activity in

differentiated glial cells used paraffin histology with standard nuclear stains, whereas the established criteria for identifying glial cell types are based primarily on the morphology of their cytoplasm. Correlations do exist, however, between the nuclear morphology seen in paraffin sections and the definitive cytoplasmic characteristics shown with the gold and silver impregnation techniques (Penfield, 1932b; Smart and Leblond, 1961), but the identification of neuroglia in these studies must be considered tentative. Specific glial stains, the gold chloride sublimate method of Cajal for astrocytes and the del Rio Hortega silver carbonate stain for microglia, have been used in combination with radioautography (Hommes and Leblond, 1967) or in conjunction with electron microscopic radioautography (Mori and Leblond, 1969, 1970) to provide unequivocal evidence that mature glial cells in the brain of the adult rat do in fact incorporate ^3H -thymidine and divide mitotically. Mitotic activity in differentiated cells is not unique to neuroglia; differentiated cardiac cells have also been shown to incorporate ^3H -thymidine and divide (Goldstein et al., 1974).

The purpose of the mitotic activity in glioblasts and neuroglia in the adult nervous system remains unclear. It is apparently not necessarily associated with a growth process since it continues unabated in thyroidectomized mice whose brains cease growing (Hain et al., 1961). Smart (1961) concluded that the mitotic activity of the glioblasts in the subependymal layer in the adult rat is abortive in that

it contributes no new cells to the surrounding brain substance. Although in the 3-day-old rat, the subependymal layer produced cells which migrated into the overlying cortex and could be identified by the persistent labeling of their nuclei, in the adult no such migration was found. Since pyknotic nuclei seen in the subependymal layer were approximately equal in number to the mitotic figures, Smart (1961) concluded that degeneration was the fate of most of the progeny of the proliferating subependymal cells in the adult. Smart's conclusion may be suspect, however, since Pannese and Ferannini (1967) have shown that abnormally high numbers of pyknotic nuclei may occur in mammalian brains even under the best of circumstances when immersion rather than perfusion fixation is used; Smart used the former.

Death is not the fate of the proliferating glioblasts outside of the subependymal layer in either the newborn or the adult rat (Smart and Leblond, 1961). A few hours after injecting ^3H -thymidine, most of the label was found over glioblasts, cells with small or medium sized, darkly staining nuclei. These cells differentiated into oligodendrocytes, as evidenced by an increase in the number of labeled oligodendrocytes at 7 days following the injection, with a concomitant decrease in the number of labeled glioblasts. From 14 to 32 days, labeled astrocytes appeared and gradually increased in number. Since transitional forms were seen between oligodendrocytes and astrocytes, the authors concluded that the oligodendrocytes and not the

glioblasts were the precursors of the astrocytes. Although the identifications of glial cell types must again be presumed tentative due to the inadequacies of the stains used, their conclusion that these two types of neuroglial cell may be successive stages of a single cell type is supported by previous cytological studies which also demonstrated transitional forms (Ramön-Moliner, 1958).

Smart and Leblond (1961) suggested that the terminal stage in the life history of this multifunctional glial cell is death, since the number of labeled astrocytes decreased slowly from 32 to 84 days following injection. Their conclusion, however, is based on an observed decrease in the average number of labeled cells in a transverse section through the forebrain, but they made no correction for changes in total brain volume over the period investigated. Since the rat brain is increasing in size during this time (Sugita, 1917; Altman et al., 1968) a decrease in the number of labeled cells in a given section may merely reflect a decrease in their density due to brain growth, rather than to a loss of these cells through death. Their conclusion is supported however, by biochemical evidence showing that the total content of DNA in the rat brain does not increase beyond 14 days of age (Mandel and Bieth, 1952; Enesco, unpublished work cited by Smart and Leblond, 1961).

Dalton et al., (1968) and Marēs et al., (1975) have also concluded that the neuroglia in the adult brain represent a population of cells which are turning-over, or

constantly renewing themselves. Their conclusion was based on calculations which indicated that more glia were being produced than could be accounted for by the growth of the brain. Many problems, particularly with regard to sampling methods and assumptions needed in the calculations, make these conclusions somewhat tentative. Moreover, this scheme does not explain the mitotic activity in mature oligodendrocytes and astrocytes (Hommes and Leblond, 1967; Mori and Leblond, 1969, 1970).

In summary, although mitosis continues in the undifferentiated cells of the subependymal layer in the adult mammalian forebrain, it may be abortive (Smart, 1961), functioning only as potential source of cells for regenerative (Schaper, 1897a,b) or pathological (Globus and Kulenbeck, 1944) processes. Scattered cells throughout the brain, both glioblasts and mature glia, also continue to divide mitotically and it has been suggested that the glial population of the adult brain is not stable but in a state of constant renewal (Smart and Leblond, 1961; Dalton et al., 1968; Marěs et al., 1975).

POSTNATAL NEUROGENESIS IN MAMMALS

Whereas postnatal gliogenesis has been amply demonstrated, if not fully understood, neurogenesis is considered to be predominately a prenatal phenomenon. A few earlier studies had purported to show ^3H -thymidine incorporation by scattered differentiated neurons in the cerebral cortex of

adult mice, rats, and cats (Messier et al., 1958; Altman, 1962, 1963), but they were plagued by two problems: 1) the difficulty of distinguishing between neurons and glia in nuclear stained, paraffin embedded tissue, and 2) the limits of resolution of the radioautographic method which made it difficult to distinguish between label originating in closely associated perineuronal glia and the neuronal nucleus itself.

It was recognized very early, however, that certain cells in the mammalian cerebellar cortex are of post-natal origin. During fetal life a group of proliferating cells from the ventricular zone of the fourth ventricle, from a region called the rhombic lip, migrate to a position near the pial surface of the developing cerebellar plate, where they form the transient external granular layer of Obersteiner (Obersteiner, 1883). The cells of this layer are mitotically active, and do not show interkinetic nuclear migration (Fujita et al., 1966). They are thus similar to the subventricular cells; both can be thought of as a secondary germinative zone. With time, the external granular layer disappears, its cells giving rise to neurons and glia of the internal granular and molecular layers of the cerebellar cortex (Ramón y Cajal, 1929; Schaper, 1894; Miale and Sidman, 1961). Radioautography has shown that most of the internal granule cells are formed during the first few weeks postnatally in the rat (Altman, 1972) and mouse (Miale and Sidman, 1961; Fujita et al., 1966; Fujita, 1967).

Although the maturity of the cerebellum at birth varies depending on the species, some cerebellar neurogenesis occurs postnatally even in precocial mammals such as the guinea pig (Altman and Das, 1967). In man, the external granular layer persists up to 20 months of age (Raaf and Kernohan, 1944). In the adult mammalian cerebellum no evidence of the external granular layer remains, and it is assumed that neurogenesis in the cerebellum ceases with its demise (Fujita et al., 1966).

Postnatal neurogenesis also occurs in certain other, less well-studied, brain regions (reviewed by Altman, 1970). For example, radioautographic studies have shown that most of the granule cells of the dentate gyrus in the rat are of postnatal origin, although their source is controversial. Altman and co-workers (Altman and Das, 1965b, 1966; Altman, 1966) believe that these granule cells originate from cells formed by the subependymal layer of the lateral ventricle which migrate across the fimbria into the granular layer of the dentate gyrus. Schlessinger et al., (1975) find that locally proliferating neuroblasts within the dentate gyrus are the source of the granule cells. Both agree, however, that most of these neurons are born postnatally.

The subependymal layer has been discussed previously in conjunction with postnatal gliogenesis. During embryonic development its homologue, the subventricular zone, is a source of both glia and neurons. In the forebrain, the subependymal layer persists into adult life especially around

the anterior wall of the lateral ventricles and extending rostrally into the olfactory bulb, in man (Opalski, 1933; Globus and Kulenbeck, 1944) and other mammals (Kirsche, 1967; Lewis, 1968). Does it continue to produce both glia and neurons even in the adult?

Globus and Kulenbeck (1944) describe the subependymal layer as a source of indifferent cells, including glioblasts, neuroblasts, and a hypothetical bipotential mother cell. The authors suggest that the subependymal layer is pathologically significant in that it provides a source of proliferative cells which may contribute to brain tumors in the adult. Likewise, the cells of the external granular layer in the cerebellum have been suggested as a source of cells in certain tumors of the cerebellum (Kershman, 1938). Smart (1961) described two cell types in the adult mouse subependymal layer, one light and the other dark when stained with hematoxylin; he suggested that the former is a glioblast and the latter a neuroblast based on the cytological appearance of transitional forms between the immature and differentiated cell types. Although ^3H -thymidine radioautography indicated that these cells were mitotically active, he concluded, in agreement with previous workers, that the mitosis was abortive; the cells died instead of contributing either to the glial or the neuronal population.

Altman reached a very different conclusion based on radioautographic studies of the subependymal layer in the infant (Altman, 1966; Altman and Das, 1966) and adult rat

(Altman, 1969c). In the infant rat, cell proliferation is brisk along the entire extent of the subependymal layer from the anterior horn of the lateral ventricle rostrally along its extension into the olfactory ventricle. With time the mitotic activity is gradually restricted rostrally so that by day 30 after birth only the subependymal cells in the anterior wall of the lateral ventricle continue to proliferate. In rats injected with ^3H -thymidine on day 30 and killed at various times from 1 to 150 days later, he could trace labeled cells migrating rostrally through the subependymal layer from the lateral ventricle into the olfactory bulb and then radially out of the subependymal layer into the internal granular layer and glomerular layer of the olfactory bulb. Altman (1969c) also distinguished two cytologically distinct cell types in the subependymal layer: a lighter and a darker nucleated variety. In sagittal sections, the darker type was spindle shaped with its long axis oriented parallel to the direction of cell migration. In contrast to Smart (1961) who concluded that the lighter type was a neuroblast and the darker one a glioblast, Altman interpreted the lighter one as a stationary, proliferative element and the darker one as the migratory phase of the same cell. Accordingly, Altman refers to the subependymal layer in the olfactory bulb of the 30 day and older rat as a "rostral migratory stream" of cells instead of as a subependyma, since it contains only migratory, not proliferative, elements. The fate of these migrating cells in

the olfactory bulb was not definitively established; they have been tentatively identified as both granule cells, the neurons of the internal granular layer of the olfactory bulb, and glia, but the histological procedures used did not allow precise distinctions to be made between neurons and glia (Altman, 1969c). If they do contribute to both glial and neuronal populations, then subependymal cells in the adult rat must be both glioblasts and neuroblasts, or perhaps bipotential mother cells giving rise to both (Kerschman, 1938; Globus and Kulenbeck, 1944).

The final example of mammalian postnatal neurogenesis to be discussed occurs in the retina but only in a few species. The retina is formed embryonically in all vertebrates from an outpocketing of the lateral wall of the developing prosencephalon, and is at first composed of a homogeneous population of proliferating ventricular cells much like those in the rest of the nervous system (Glücksmann, 1965; Hinds and Hinds, 1974; Mann, 1969; O'Rahilly, 1975). In all vertebrate species which have been examined, the first sign of differentiation is the separation of a group of cells on the inner retinal surface. These cells are no longer mitotically active, and their position on the inner surface, the side of the neuroepithelium opposite to the former ventricular surface, indicates that they are homologous to the young neurons of the intermediate layer of the neural tube (Fig. 4C). These young neurons differentiate into retinal ganglion cells whose axons exit the eye via the

optic nerve, forming the primary visual afferents to the brain (Noel, 1958; Sidman, 1960; Fujita and Horii, 1963; Denham, 1967; Jacobson, 1968; Goldberg and Coulombre, 1972; Hollyfield, 1972; Hollenberg and Spira, 1972; Kahn, 1973, 1974; Hinds and Hinds, 1974; O'Railly, 1975). The remaining neurons in the retina, including the photoreceptors at its outer surface and the interneurons, the horizontal, bipolar, and amacrine cells, are formed next in a variable order depending on the species. The time of origin of the ependymoglia element in the retina, the Müller cell, is in dispute (Bhattacharjee and Sanyal, 1975). In a general way then, retinal histogenesis is in accord with the sequential theory of neurogenesis (Fig. 5); the ganglion cells (macroneurons) are the first to differentiate and the other retinal neurons (microneurons) are formed later. The times of origin of the various cell types are not entirely distinct and non-overlapping, however, so that at any one time all of the different cell types are forming simultaneously (Sidman, 1960). This point will be discussed more fully later.

At birth, the retina in rodents is at a primitive stage of differentiation. Only the ganglion cells and some of the amacrine cells have begun to withdraw from the cell cycle. All of the remaining microneurons are formed during the first few weeks postnatally (mouse: Sidman, 1960; rat: Denham, 1967). In other species the early postembryonic retina is more highly differentiated, and its cells are

already stratified into the three layers characteristic of the adult retina (Coulombre, 1955; Fujita and Horii, 1963; Jacobson, 1968; Hollyfield, 1972; Hollenberg and Spira, 1973; Kahn, 1973, 1974; O'Railly, 1975). Rodents are the only group in which all or most of the retinal micro-neurons are of postnatal origin.

Several conclusions emerge from the above studies of mammalian postnatal neurogenesis. First, all of the structures in which postnatal neurogenesis has been demonstrated, the cerebellum, the dentate gyrus of the hippocampus, the olfactory bulb, and the retina, are highly organized with different neuronal types restricted to specific lamina (Ramón y Cajal, 1909-1911). This makes the identification of these postnatally forming microneurons much easier; elsewhere in the brain they are more scattered and are difficult to distinguish from large astrocytes (Altman, 1970). If they could be identified using techniques compatible with radioautography, more of them might also prove to be postnatal in origin.

A second characteristic common to all of the postnatally proliferating brain regions is that they have only one type of macroneuron: the Purkinje cell in the cerebellum, the pyramidal cell in the dentate gyrus, the mitral cell in the olfactory bulb, and the ganglion cell in the retina. All of these macroneurons form prenatally, while only certain microneurons are born after birth. What is the significance of the late formation of the microneurons in these regions?

The period of postnatal cell formation in the cerebellum is restricted; and its duration seems to be correlated with the period of maturation of motor abilities. In the precocial guinea pig, which possesses nearly adult-like motor skills at birth, postnatal addition of granule cells is limited in extent and duration, whereas in altricial mammals such as man, addition of granule cells continues for up to 2 years, throughout the period of acquisition of both basic and refined motor coordination (Raaf and Kernoham, 1944; Altman and Das, 1967; Altman, 1969, 1970). The function of the granule cells is that of an interneuron; they modulate the activity of the Purkinje cells with which they synapse (Eccles et al., 1967). Although the Purkinje cells are formed prenatally their dendritic structure is still in the process of differentiation during the period of granule cell production and differentiation (Addison, 1911; Altman, 1972b). The newly formed granule cells move from their site of origin in the external granular layer on the surface of the cerebellar cortex, through the layer of Purkinje cells to the internal granular layer at the base of the cortex. Ramón y Cajal (1929) postulated that the growth and maturation of Purkinje cell dendrites was stimulated by interactions with the migrating granule cells and by the formation of their axons, the parallel fibers, Sidman (1968) pointed out that in several types of genetic mutations in the mouse in which the cerebellar granule cells die or fail to migrate, the development of the Purkinje cells

is abnormal. The primary genetic defect in these mutants is variable. In some cases it has been postulated that granule cell death is secondary to their failure to form synapses due to death of or abnormalities in the Purkinje cells, whereas in other mutants anomalies in Purkinje cell maturation are secondary to the failure of granule cells to migrate (Rakic and Sidman, 1973). Whatever the causal relationships, interactions between granule cells and Purkinje cells are probably essential to the normal development of both. The importance of this interrelationship was also demonstrated by Altman (1975b,c,d) using specific timed dosages of X-irradiation to selectively destroy the proliferating external granule cells in rats. The loss of granule cells was accompanied by abnormalities in Purkinje cell development.

Altman (1967, 1969, 1970) has suggested that the significance of the postnatal origin of the cerebellar granule cells is to allow environmental influences to modify the development of the Purkinje-granule cell connections; such modifications might be manifest in the learning and fine tuning of motor skills. In support of this notion, he has evidence that the destruction of granule cells with X-irradiation interferes with the development of motor coordination in rats (Altman, 1970). As infants, the irradiated rats displayed tremor and ataxia; although these symptoms disappeared in adulthood, various behavioral tests indicated persistent locomotor deficiencies. In particular,

the animals showed a decrease in spontaneous activity and were handicapped in tasks requiring motor skills such as rope climbing. The severity of the effects was correlated with the magnitude of the irradiation dose. Of course, since the granule cells were destroyed in these animals, the experiments do not provide evidence for environmental influences on their differentiation, but merely serve to indicate that the Purkinje-granule cell interaction is probably involved in skilled motor behavior.

The postnatal neurogenesis in the mammalian fore-brain, which continues even in the adult, is more difficult to interpret since the functions of the various structures involved are not nearly as well understood as in the cerebellum. If microneurons are thought of as associative elements, as Ramón y Cajal (1909-11) first suggested, their late origin may be involved in the acquisition and retention of learned behaviors, by analogy with the cerebellum (Altman, 1967, 1969c, 1970). Current theories usually invoke more or less stable specific changes in functional synaptic connectivity as the mechanism of learning and memory (Hebb, 1949), and it has been suggested that these changes may represent a continuation of the dynamic processes which are involved in the embryological development of the brain and the formation of specific synaptic connections (Altman, 1967; Mark, 1970). The continued proliferation of micro-neurons in the adult mammalian forebrain may provide a source of cells whose synaptic connections are dependent on

the individual experiences of the animal (Altman, 1967, 1969c, 1970). In support of this idea, Altman and his colleagues suggest that alterations in the rearing environment, such as training and "enrichment", may affect the magnitude of cell proliferation in the forebrain subependymal layer in young rats. This suggestion was based on measurements of the size of the cerebral cortex which revealed that growth in length, but not in width, of the cerebral cortex, continues even in adult rats (Altman et al., 1968). The amount of growth was greater in the more experienced rats than in their less experienced littermates (Altman, 1970). Growth of the forebrain in this dimension correlates with the caudorostral direction of migration of the cells produced postnatally in the subependymal layer and destined for the olfactory bulb (Altman, 1969c), but the authors did not demonstrate that the increased length of the cerebrum in the experienced rats was due to increased cell proliferation. Earlier studies (Altman and Das, 1964) which did show a higher proportion of labeled cells in the cerebral cortex of the experienced rats after injection of ^3H -thymidine at 4 1/2 months of age were interpreted as evidence of increased glial proliferation, since most of the cells were small and located in fiber tracts.

Similar studies by Rosenweig et al. (1971) in which rats were reared in "enriched" environments, consisting of large cages containing several rats and an abundance of playthings which were changed daily, resulted in increased

weight and thickness of the cerebral cortex, increased neuronal size and metabolic activity and increased numbers of neuroglial cells in the enriched rats compared with their "impoverished" littermates reared in isolation in standard laboratory cages. Greenough and his colleagues have extended these studies both anatomically and behaviorally (reviewed by Greenough, 1975). Rats and mice reared in what they describe as a "complex" environment, again involving increased social and sensory stimulation combined with freedom to interact with the environment, seem to possess superior learning abilities. Quantitative Golgi studies indicated that cortical neurons in the enriched rats had more higher-order dendritic branches (Volkmar and Greenough, 1972). Globus et al. (1973) showed that the density of synapses on individual dendrites remained constant or even increased in the enriched rats, implying that the actual number of synapses per cortical cell increased. In contrast to Altman's work on cell proliferation in the olfactory bulb (1970), in the above studies the most marked effects of rearing environment occurred in the occipital lobe, and involved structural changes associated with synaptic connectivity rather than proliferative changes related to neuronal numbers. The evidence for involvement of postnatal microneurons in a learning process is therefore very weak, and the idea must be considered only a tentative hypothesis.

The postnatal origin of microneurons in the rodent

retina certainly cannot be related to an environmentally or functionally dependent learning process since the eyes of the neonates are closed, and the animals are visually inactive. Indeed, the mechanism of formation of the retinal microneurons is different; they are produced from primary germinative cells in the ventricular zone and not from secondary, migrated neuroblasts as are their counterparts in the forebrain and cerebellum. Moreover, this phenomenon is exclusively a property of rodents, unlike the other two examples which are more general in occurrence. In this case the most likely explanation is that the time of birth is an arbitrary point with respect to the development and maturation of the mammalian retina.

In summary, mammalian postnatal neurogenesis involving exclusively microneurons is consistent with the following scheme. Macroneurons are formed prenatally from the primary ventricular zone and send out axons which grow independently to a specific target in a way that is genetically fixed and determined (Ramón y Cajal, 1929; Sperry, 1963; Morest, 1969b; Jacobson, 1970). These cells provide a basic skeleton of neuronal connections into which are added the micro-neurons, or interneurons, associative elements whose function is to modulate and refine the activity of the macroneurons (Ramón y Cajal, 1909-11). Those born postnatally in the cerebellum, dentate gyrus, and olfactory bulb originate from a secondary, subventricular zone and migrate through the developing dendritic processes of the

macroneurons, spinning out an axon behind them as they move (Ramón y Cajal, 1909-11; Miale and Sidman, 1961; Angevine, 1965; Hinds, 1968; Altman, 1972a, 1975a). This difference in the mechanism of axon formation, along with the difference in time of origin of these microneurons, may reflect a differential ability to respond to exogenous stimuli, indicative of a greater range of potential synaptic connections, with the ultimate connectivity being determined by individual experiences.

The above discussion of postnatal histogenesis has been restricted almost entirely to work on the mammalian nervous system; this reflects the bias which exists in the current literature. The most informative studies have used the technique of ^3H -thymidine radioautography to locate mitotically active cells and to trace their fate. Two problems which have frequently arisen in these studies are the difficulty of distinguishing between different cell types, neurons versus glia and one type of glia cell versus another, and conversely, the failure to recognize a single cell type throughout its various morphological and developmental stages. From a position which denied the presence of mitotic activity in adult mammalian brains, the consensus of opinion has evolved to include first gliogenesis and finally a specific and restricted amount of neurogenesis as part of the normal activity of the adult brain. The only postnatally formed neurons which have been identified are small, short-axoned microneurons; the only brain regions

in which neurogenesis apparently continues even in the adult are associated with the forebrain sub-ependymal layer. It has been proposed that the significance of the postnatal origin of these cells be related to a learning process.

POSTNATAL NEUROGENESIS AND GROWTH

Growth of the brain occurs postnatally in all vertebrates, but it is usually attributed to increases in the volume of the neuropil and in the number of glial cells (Eayrs and Goodhead, 1959; Altman, 1970; Jacobson, 1970). The possibility that some of the growth is due to addition of new neurons has rarely been considered. Kirsche (1967) has examined transverse sections through the brains of 26 species of vertebrates, including teleost fish, amphibians, reptiles, birds, and mammals of various ages and has found regions in the telencephalon in all of them resembling the embryonic ventricular zone, the "zona germinativa dorsalis et ventralis", even up to 5 years of age in the domestic chicken, for example. These areas consist of layers of small, tightly packed, densely staining, undifferentiated cells which are mitotically active and are probably capable of producing both neurons and glia. In addition to these forebrain germinative zones, in the non-mammalian species there are similar restricted areas of increased mitotic activity in the diencephalon, mesencephalon, and metencephalon. In particular, three "matrixzonen" in the adult telostean optic tectum have received much recent attention

in the German literature. The term "matrixzonen" is derived from Fujita's study (1964) on the matrix layer of the embryonic chick optic tectum with which the adult teleostean matrix zones are thought to be homologous (Rahmann, 1968; Richter and Krantz, 1970). There are three matrix zones in the teleost optic tectum: 1) a dorsal matrix zone at the mediodorsal boundary of the tectum adjacent to the ventricle, 2) a ventral matrix zone at the latero-ventral edge of the tectum, and 3) a caudal matrix zone which is the posterior continuation of the first two at the caudal pole of the tectum (Richter and Krantz, 1970). Cells in these three zones are mitotically active as indicated by their incorporation of ^3H -thymidine (Rahmann, 1968; Richter and Krantz, 1970). The proliferation capacity decreases with age, however, and the labeling index (percentage of cells labeled) is smaller in older animals (Richter and Krantz, 1970).

That these proliferating cells are capable of producing both neurons of all types, not just microneurons, as well as glia is supported by evidence from regeneration studies (reviewed by Kirsche, 1966; and Bernstein, 1970).¹ Following tectal lesions of variable severity in juvenile crucian carp (Carassius carassius) (Kirsche, 1960; Kirsche

¹The term "regeneration" in the context of this discussion refers exclusively to regeneration involving production of new cells and reconstitution of a damaged neural structure, and is not meant to refer to regeneration of nerve fibers following more selective lesions which directly affect only axons and leave their perikarya intact.

and Kirsche, 1961), in a related cyprinid, the moderlieschen (Leucaspius delineatus) (Richter, 1965), and in adult guppies (Lebistes reticulatus) (Richter, 1968), more or less complete restoration of tectal structure was demonstrated after several months. The tectal regeneration was shown to be dependent on cells in the matrix zones, since regeneration did not occur if these regions were removed (Kirsche, 1960; Kirsche and Kirsche, 1961). The new cells in the regenerate were produced as a result of enhanced proliferative activity in the matrix zones induced by the trauma (Richter, 1968). Following a punctate tectal lesion in adult Lebistes reticulatus, cells produced by the matrix zones migrate into the wound region and displace the fibrotic and connective tissue material which had accumulated there immediately after the lesion (Richter, 1968). After 175 days, an almost complete restitution of tectal cytoarchitecture was found in some of the adult fish although the regenerative response was much more vigorous in juvenile fish (Kirsche, 1960; Kirsche and Kirsche, 1961; Richter, 1965). Whether the proliferative activity in the normal animal is responsible for the production of new cells, or whether it serves merely as a potential source of cells in case of damage, is not known. None of the radioautographic studies which demonstrated the capacity of the matrix cells to divide in the intact animal were of sufficient duration to define the ultimate fate of the labeled cells. It is known, however, that the brains of most adult fish continue to grow

along with the rest of the body throughout life (Brown, 1957; Packard, 1972), and these germinative regions may be contributing new cells to such a growth process.

The telencephalon of adult teleosts also has extensive regenerative capacities which can lead to restitution of structure and return of function following surgical lesions (Segaar, 1965). Again, regeneration is more vigorous in juvenile fish; Marón (1963) observed nearly perfect regeneration at 60 to 120 days following complete ablation of the telencephalon in 4-day-old Lebistes reticulatus. Segaar (1965) demonstrated regeneration, although not complete, in adult sticklebacks (Gasterosteus aculeatus) following ablation of the lateral region of one telencephalic hemisphere. Regeneration in the telencephalon is associated with an increased proliferative activity in the ependymal layer (former ventricular zone) which in teleosts extends over the medial, dorsal, and lateral surfaces of the telencephalic hemispheres (Nieuwenhuys, 1962). Again, it is not known whether the proliferative activity induced by trauma is also associated with growth of the telencephalon, although labeled cells are found in the telencephalon following injection of ^3H -thymidine in the intact animal (Rahmann, 1968).

Postnatal regeneration of central nervous tissue including both brain and retina, reaches its most dramatic level in larval and post-metamorphic amphibians (Terry, 1956; Jordan, 1958; L. Stone, 1959; Keefe, 1973a,b;

Levine, 1965). In amphibians, the capacity for regeneration is clearly associated with a continuing growth process since much of the development of the nervous system in terms of neuronal addition and synaptic differentiation takes place in larval stages and may continue after metamorphosis (Jacobson, 1962; Hollyfield, 1968; Straznicky and Gaze, 1971, 1972; Wilson, 1971; Fisher, 1972; Lázár, 1973; Chung et al., 1975; Currie and Cowan, 1975).

Continued addition of neurons associated with growth of the adult animal has also been demonstrated in certain invertebrates. For example, mitotic figures have been found in neuroblast cells in some adult, post-pupal, insect brains (Panov, 1957, 1960; Edwards, 1969) and in insect eyes (Schoeller, 1964), although in other insects the neuroblasts were found to degenerate midway through pupation (Nordlander and Edwards, 1969). Unlike the situation in vertebrates, neuroblasts in insects are easily distinguished from glial precursors, so that cell division in these cells potentially represents the formation of neurons. Another example of invertebrate postnatal neurogenesis occurs in the molluscan brain. The total number of cells, counted in tissue homogenates of the brain, increased with growth in juvenile and adult Octopus vulgaris (Guiditta et al., 1971; Packard, 1972; Guiditta and Pozzo, 1974). Although no attempt was made in these studies to distinguish between neurons and glial cells, the magnitude of the increase, from 4×10^7 to 17×10^7 cells, makes it likely that both

were added. In summary, the evidence for postnatal neurogenesis in invertebrates is very suggestive, but not fully substantiated.

Finally, returning to teleost fish, continued growth, involving neuronal addition in the adult animal has been clearly demonstrated in the retinas of adult guppies (Lebistes reticulatus) (Müller, 1952), trout (Salmo trutta) (Lyall, 1957), and herring (Clupea harengus) (Blaxter and Jones, 1967). The early stages of retinal histogenesis have been discussed briefly above, and are similar in all vertebrate species. During the earliest stages, all of the cells in the presumptive retinal epithelium are mitotically active. Differentiation begins in the central regions, with the withdrawal of the young ganglion cells from the mitotic cycle, and as differentiation progresses, mitotic figures become increasingly more restricted to peripheral regions (Sidman, 1960; Denham, 1967; Straznicky and Gaze, 1971; Kahn, 1973). Eventually the germinative zone is confined to the outermost edge of the retina, the ora terminalis (Jacobson, 1968, 1976; Straznicky and Gaze, 1971; Morris et al., 1976), where it is also located in adult fish (Müller, 1952; Lyall, 1957; Blaxter and Jones, 1967). The neuronal addition involves all cells including ganglion cells (Müller, 1952), the retinal macroneurons, and is therefore a growth process in which whole, functional "units" are added to an already complete neural structure.

As described above, neurogenesis in the vertebrate retina involves two distinctly different patterns of cell addition. The earliest retinal neurons are formed by delamination of the primitive retinal epithelium into three separate strata, the first formed being the ganglion cell layer. This stratification process begins in the center of the retinal anlage concurrently with the cessation of cell division and the commencement of cell differentiation. Once the germinative cells have been restricted to the ora terminalis, further cell production takes place by concentric apposition of new neurons at the retinal margin (Müller, 1952; Staznicky and Gaze, 1971; Jacobson, 1976). During this latter phase, the ganglion cell macroneurons are not formed before the retinal microneurons, since all cell types are being produced simultaneously. Here the scheme of sequential neuronal production (Fig. 5) breaks down, and this is one reason for the above caution against too rigid an adherence to this theory.

Not only fish eyes, but the eyes of other vertebrates also grow postnatally. In humans, for example, the diameter of the eye increases 1.8 times from birth to adult size (Mann, 1969). Increases in the size of the young neurons and the formation of cell processes in the plexiform and fiber layers, contribute to retinal growth, along with the secretion of the vitreous, a jelly-like matrix inside of the eye, which tends to increase the size of the eye by distension (Weiss, 1949; Coulombre, 1955). Generally,

postnatal growth of the eye is thought to involve only these processes and not increases in cell number (Coulombre, 1955; Ali, 1964; Mann, 1969).

The possible contribution of a marginal germinative zone is not usually considered, but the relation between total retinal cell number and age has been determined in only a few species of fish and larval amphibians. For instance, Coulombre (1955) states that the chick retina contains its definitive complement of cells at 15 days of incubation, but Morris et al. (1976) have recently shown with repeated injections of ^3H -thymidine during the first week post-hatching that some cells at the ora terminalis of the chick retina continue to incorporate thymidine and are presumably still mitotically active. Such proliferating cells, when they have been noted in the postnatal retina, have usually been dismissed as being too few in number to make any significant contribution to the overall complement of retinal cells in the adult. The potential hazard in such an assumption was clearly illustrated by Straznicky and Gaze (1971) in their analysis of retinal growth in larval toads (Xenopus laevis). At stage 30, cell proliferation is confined to the retinal margins, and previous workers (Jacobson, 1968) had assumed that most of the cells of the adult retina were already formed by this time. At this stage the retina is very small and has only about 20 ganglion cells across its equator in a vertical section through the maximal diameter of the eye (Straznicky and Gaze, 1971).

At metamorphosis, there are more than 200 ganglion cells across the equator in a comparable section, so clearly the vast majority of cells which are found in the adult Xenopus eye were formed by the small number of proliferating cells at the margin. Injections of ^3H -thymidine indicate that cell proliferation continues for an undetermined length of time after metamorphosis in these animals. I suggest that a more careful analysis involving radioautography and quantification of cell numbers may reveal that the "insignificant" peripheral germinative region in the retinas of other species may be playing a larger role than expected in postnatal retinal neurogenesis.

Two general conclusions emerge from the above discussion of non-mammalian postnatal neurogenesis. First, in contrast to the mammalian case, neurogenesis in juvenile and adult teleost fish and amphibians is not restricted to a specific class of neurons, but involves all neuronal types. It is not associated with acquisition of a specific function, but rather reflects growth of the brain in the sense of addition of all functional components. The orderly scheme shown in Fig. 5 which seemed to explain the pattern of histogenesis in the mammalian nervous system does not apply in the teleost and amphibian retinas, since production of all types of cells continues here, and probably in the brain as well. Are we forced to conclude that the basic mechanism of neurogenesis in mammals is fundamentally different from that in teleost fish and amphibians?

Probably not; the second characteristic of non-mammalian postnatal neurogenesis offers a clue to the resolution of this conflict. The production of cells does not continue throughout a given neural structure, but is spatially restricted to a specific area, always at its border; recall for example, the germinative region at the ora terminalis of the retina and the matrix zones in the optic tectum. Moreover, these regions are homologous to the embryonic ventricular zone, the primary germinative region, rather than to the secondary, migrated subventricular zone. The only criteria for distinguishing between the ventricular and subventricular zones are anatomical; the former is adjacent to the ventricle, and its cells exhibit interkinetic nuclear migration, whereas the cells of the latter have migrated away from the ventricular surface, and their nuclei divide in situ. Functionally, however, these two classes of germinative cells may be distinct in that those in the ventricular zone produce macroneurons, microneurons, and glia, while the subventricular cells seem to be restricted to microneurons and glia. If this is the case, and the only evidence to support it is a negative result--the lack of any example of macroneuron production by the subventricular zone--then the difference between mammals and fish may only be in the relative importance and persistence of these two germinative zones.

In conclusion, this review of the literature has been an attempt to compile the existing work on the subject

of postembryonic histogenesis in the central nervous system, to relate it to the processes occurring embryonically, and to incorporate it into the framework of a functioning organism. This last point, neuronal addition concurrent with functional activity, is the one which is, to me, the most exciting. How does an animal grow and add new structure to a functioning nervous system, the activity of which is uniquely and specifically dependent on the precision of its anatomical structure? One important condition seems to be the separation of the proliferating compartment from the functioning one: in all of the examples discussed, the external granular layer of the cerebellum, the forebrain subependymal layer, the tectal matrix zones, and the ora terminalis of the retina, cell proliferation occurs in a region adjacent to, or even displaced from, the ultimate site of differentiation of the new cells. It is fair to say, however, that in no case has this dichotomy between cell genesis and function been solved, or even completely defined. The teleost fish retina offers the best hope for such an analysis for several reasons. First, the configuration of the proliferative compartment is geometrically simple: an annulus. Second, the structure of the differentiated compartment is extremely well organized, with separate cell types confined to strictly demarcated strata, thus removing some of the burden and uncertainty in their identification, categorization, and quantification. Finally, the physiology of the retina is probably the best understood

of any neuronal, certainly of any sensory, structure in the vertebrate central nervous system, thus allowing specific predictions to be made concerning the functional effects of continued neuronal addition. The remainder of this thesis is devoted to an analysis of the growth of the adult goldfish retina, and the implications of that growth to the visual physiology and anatomy.

CHAPTER II

GROWTH OF THE ADULT GOLDFISH RETINA:

Increase in Size and Cell Number

INTRODUCTION

Although it is generally acknowledged that vertebrate eyes continue to grow for some period of time post-embryonically, the mechanism of this growth has not been studied in detail. Neurogenesis is normally considered an embryonic phenomenon, and so postnatal increases in the area of the neural retina are usually attributed to vaguely defined forces tending to stretch the eyeball, often thought to be associated with vitreal secretion and the development of intraocular pressure (Weiss, 1949; Coulombre, 1955; Ali, 1964; Mann, 1969). Although direct evidence for this suggestion is lacking, the idea that the retina grows by stretching is consistent with two observations: during juvenile or larval growth in several species, the layers of retinal cell nuclei become thinner (teleost fish: Müller, 1952; Ali, 1964; chick: Coulombre, 1955; rabbit: Noel, 1958; human: Mann, 1969), or the density of retinal cells decreases without a thinning of the cell layers (marine mammals: Pütter, 1902; teleost fish: Müller, 1952;

Lyall, 1957a; O'Connel, 1963; Ali, 1964; Ahlbert, 1968). There are two problems with this interpretation of retinal growth. First, in some cases not all of the retinal cell layers decrease in thickness to the same extent. In the human retina, for example, Mann (1969) states that the inner nuclear layer in the paracentral region decreases from 8 rows of nuclei in the late fetal period to 4-5 rows in the adult, whereas the outer nuclear layer during this time remains 6-7 cells thick. Moreover, Hollyfield (1968) has shown that the inner nuclear layer actually increases in thickness during larval growth in Xenopus laevis, while the thickness of the other retinal layers remains constant. Clearly, a passive stretching mechanism is inconsistent with these observations.

Second, in no case in which retinal expansion has been postulated as the sole mechanism of growth has an attempt been made to quantify the number of cells in the retina as a function of age and size. Such an analysis has been done in only a few species of teleost fish and in one amphibian, and in all cases the number of retinal cells increased with growth (Xenopus laevis: Wilson, 1971; Lebistes reticulatus: Müller, 1952; Salmo trutta: 1957a; Clupea harengus: Blaxter and Jones, 1967).

The retinal cells which have received the most attention in these quantitative growth studies are the ganglion cells and their axons, the optic nerve fibers, and the photoreceptor cells, in particular the cones. Several

studies have attempted to enumerate the optic nerve fibers in various vertebrate species; Bruesch and Arey (1942) reviewed the early literature and added a list of their own estimations of the number of optic nerve fibers in 33 species. The optic nerve is a reasonable choice for a quantitative study of retinal elements since in it all of the ganglion cell axons are gathered into a compact bundle, where they can be easily counted in transverse sections. The overwhelming majority of these studies, however, have been done only on adult animals, usually in conjunction with inter-specific comparisons of visual ability; very few studies have used this method of analysis for ontogenetic comparisons.

One notable exception is the work by Wilson (1971) on retinal growth in larval and adult Xenopus laevis. She counted every optic fiber in electron micrographic montages of a transverse section through the optic nerves of 14 animals from larval stage 35 to adult. In addition, she determined the total number of ganglion cells from samples of cell density taken at various points in the retina. The number of fibers in the optic nerve was approximately equal to the estimated total number of ganglion cells, and the magnitude of both increased with growth for up to 6 months after metamorphosis. Several important points emerge from this study in addition to the obvious one that retinal growth in larval and juvenile Xenopus includes ganglion cell addition. First, only rarely has an attempt

been made to demonstrate that the number of optic nerve fibers accurately reflects the number of retinal ganglion cells, although a one-to-one correspondence may be lacking. For example, retinal efferents, fibers from the brain going to the retina via the optic nerve, have been demonstrated in some vertebrates, most convincingly in birds (McGill et al., 1966; Cowan, 1970), and they are suspected in fish (Witkovsky, 1971; Sandeman and Rosenthal, 1974). Their presence would result in an overestimate of the number of optic nerve fibers if they could not be distinguished from the ganglion cell axons. An erroneously high estimate of the number of ganglion cells would also result if their axons branched in the optic nerve.

Another precaution that Wilson (1971) emphasized is the necessity of using electron microscopy. Maturana (1959) and Tapp (1974) found that the values given by Bruesch and Arey (1942) for the number of optic nerve fibers in amphibians and fish were much too low, primarily because most or all of the small, unmyelinated fibers, whose diameters are usually less than 1 μm , cannot be resolved with the light microscope. Wilson (1971) pointed out that the use of electron microscopy is particularly critical when counting optic nerve fibers in young growing animals since the fibers are even smaller than in the adult, and more are unmyelinated. In Xenopus for example, the process of myelination continues until well after the adult complement of fibers is attained. Thus the number of optic fibers may

be a useful indicator of retinal ganglion cell addition, providing electron microscopy is used, but its validity must be considered suspect unless confirmed by comparisons with intraretinal ganglion cell counts.

The other retinal element which has received the most quantitative attention is the cone, particularly those in teleost fish. The cones in most fish are arranged in a regular, repeating mosaic, best seen in tangential sections through the retina (Eigenmann and Shafer, 1900; Engström, 1960, 1963). This regular pattern greatly simplifies the analysis of cell density. Lyall (1957a) used the cone mosaic to analyze the postembryonic growth of the trout retina (Salmo trutta) over a tenfold increase in eye diameter. She concluded that both rods and cones are added to the trout retina during growth, although she did not actually calculate cell numbers. For the cones, her conclusion is based on measurements of the size of the mosaic unit in small and large eyes. The dimensions of the mosaic unit increase linearly as the eye grows, and the cones themselves increase in size, but the mosaic unit only doubles in size over a fivefold increase in the diameter of the eye. The combined increase in area of the original complement of mosaic units is insufficient to entirely account for the increase in retinal area, so the total number of mosaic units must increase. It must be noted that inferences about retinal area in this study were based solely on measurements of eye diameter, and it was assumed that the

retinal dimensions increased proportionately with growth of the eye. In other teleosts the retina in fact grows faster than the eye itself (Müller, 1952).

Several other investigators have studied the cone mosaic and its relation to growth of the teleost eye, not with reference to cell addition, however, but to changes in visual acuity. The density of cone elements is thought to define a physiological lower limit of visual acuity in terms of the minimum separable angle which can be detected; this angle is usually considered to be the distance between two cones which when stimulated are separated by one unstimulated cone (Tamura, 1957). O'Connell (1963) calculated cone densities for 23 specimens of Pacific sardine (Sardinops caerulea) and 3 specimens of Pacific mackerel (Pneumatophorus diego) of different sizes. The cones in Sardinops are not distributed uniformly throughout the retina but are more concentrated in the ventrotemporal quadrant of the eye. This circumscribed region of maximal cone density constitutes an area temporalis from which rods are virtually excluded. This pattern of photoreceptor distribution is consistent with similar areas in other vertebrate retinas and represents a specialization for increased visual acuity (Walls, 1942). Although Pneumatophorus lacks a well defined area, the cone density also reaches a maximum in the temporal quadrant of the retina. The maximum cone density in both of these species decreases with increasing size of the eye, but as Lyall (1975a) showed for the trout, the magnitude

of the decrease in cell density is less than magnitude of the increase in retinal area. O'Connell (1963) did not directly measure retinal size, but used lens diameter as an index of growth, so again inferences about retinal areas are based on the assumption of proportionate growth; this has been confirmed in other species of fish (Müller, 1952; Easter, Johns, and Baumann, 1977). Even if the assumption of proportionate lens/retinal growth can be accepted, O'Connell's work suffers from another problem. His conclusions regarding cell numbers relied only on the maximum cone density, and he did not consider the possibility that changes in the density distribution with growth might affect the total cell number.

The most comprehensive studies of postembryonic growth of the teleost retina have been done by Müller (1952) and Blaxter and Jones (1967). Müller studied guppies (Lebistes reticulatus) from 7 to 28mm in body length. He determined the density of not only the cones, but also the rods, ganglion cells, and inner nuclear cells, in median radial sections through the dorsoventral or nasotemporal axis of the eye. In agreement with the studies mentioned previously, Müller found that the density of the cones decreased with growth, as did the density of the ganglion cells and the inner nuclear layer cells. From measurements of the retinal area, he calculated that the total number of cells increased with growth despite their decrease in density. The retinal area increased from about 0.5 to 3.7 mm², while the total

number of cones (based on average densities) increased from 94,000 to 290,000; the ganglion cells from 42,000 to 146,000; and the inner nuclear layer cells from 340,000 to 1,120,000. The rod density, in contrast to the other cells, remained constant or even increased with growth, and the total number of rods increased correspondingly from 50,000 to 340,000.

Blaxter and Jones (1967) studied the larval and post-metamorphic development of the retina in the herring (Clupea harengus). The larval herring retina contains only one type of photoreceptor element, which they identified as cones based on electron microscopic examination of ultra-structure. They calculated the number of cones as a function of body length, but stated that their values are only approximate. Based on the average cone densities, they found that the total number of cones increased from 20,000 to 300,000 in the unspecialized retina and from 30,000 to 450,000 in the area, from hatching to an adult length of 300 mm. During metamorphosis, rods appear in all retinal regions except the specialized area temporalis in the herring (Blaxter and Jones, 1967) and in other species of teleost fish (Blaxter, 1975), so clearly they are being added postembryonically. Lyall, (1957a,b) also noted that the proportion of rods increases in the trout retina during growth.

The functional significance of postembryonic neuronal addition is more easily determined for the retina than for

most other neural structures, since the physiology of vision is relatively well understood. Teleost fish offer the best example of continued retinal neurogenesis reported in the literature. Of the many species of teleosts, goldfish (Carassius auratus) have been studied most extensively by visual physiologists, psychophysicists, anatomists, and developmental neurobiologists interested in the mechanism of formation of specific synaptic connections. It is therefore a most appropriate choice for an investigation of postembryonic neurogenesis in which the goal is to understand the integration of growth and cell addition with the ongoing functional activity of the retina. In the following study, I will demonstrate with cell counts that neurogenesis continues in the retina of adult goldfish at least through four years of age. Chapter III deals with the source of the new cells and the geometry of the growth process. The implications of this continued growth with regard to neuronal connections and visual physiology will be discussed.

METHODS

Goldfish (Carassius auratus), Comets, were obtained from Ozark Fisheries, Stoutland, Missouri. They were kept in aerated aquaria at room temperature (18-22°C), and fed dry commercial fish food (TetraMin). The fish ranged in size from 4 to 23 cm in total length.

Histology. Smaller fish were fixed in toto; larger animals were decapitated and their heads fixed in Bouins'

for at least 48 hours. The cornea was punctured to allow the fixative to penetrate to the retina. Following fixation, the eyes were removed, and a notch was cut in the iris at the dorsal pole of the eye for purposes of orientation. In most cases, the lens was removed from the eye at this stage. The tissue was rinsed overnight in 50% ethanol, and then dehydrated through a graded series of ethanol-butanol mixtures to 100% butanol. The eyes were embedded in paraffin (Paraplast, m.p. 61°C) either whole or after being cut into nine pieces. Sections were cut at 5, 8, or 10 μ m radially through the dorsoventral or nasotemporal axis of the intact eyes, or, in the case of the eyes which had been cut into pieces, tangential to the convex surface of each piece. The sections were stained with Ehrlich's hematoxylin and eosin, cresyl violet, gallocyenin, or Foot's modification of Masson's trichrome. In some cases, the dark melanin-like pigments in the pigmented retinal epithelium were oxidized with a 1-5% solution of potassium permanganate and bleached in 2% oxalic acid prior to staining. The sections were dehydrated, cleared with xylene, and mounted in Permount.

Retinal cell counts - radial sections. Retinal cells were identified by criteria to be described in the Results. In radial sections cut horizontally (nasotemporal axis) or vertically (dorsoventral axis), cells were counted in one to three sections through the center of the eye. The retinal length was measured with an ocular micrometer along the

crescent-shaped section from one margin, or ora terminalis, to the other. The maximum error in this measurement was estimated at 3% (± 0.15 mm or less). The number of each type of cell counted ranged from about 100 to 1200 per section. The counts were done at a magnification of 450 X or, more often, 900 X using an oil immersion objective. Two counting methods were used. One involved determining the number of cells in a given length of retina and correcting the counts for split nuclei using the Abercrombie (1946) correction method as modified by Konigsmark (1970). The cell density per mm^2 of retinal surface was calculated based on the length of the segment counted and the section thickness. This method was used for counting the cones, which are tightly packed in a layer one cell thick. For the cells in layers more than one cell thick, the rod nuclei and the inner nuclear layer cells, the line sampling technique described by Trowell and Westgarth (1959) was used. This method involves counting the number of nuclei which touch a line oriented perpendicular to the retinal layers. The line is then moved, the count taken again, and so on. The average number of nuclei per line and the average diameter of the nuclei are then used to determine the cell density D, according to the following formula:

$$D = \frac{x}{a}, \text{ where } a = r(2t + \pi r)$$

r = average nuclear radius

t = section thickness

x = average number of nuclei per line

The ganglion cells were counted using either of these two methods.

The retinal area was determined from the maximum retinal length. The geometry of the retinal surface was determined from frozen sections through goldfish eyes as described previously (Easter, Johns and Baumann, 1977). The goldfish retina is approximately hemispherical in shape and subtends 185° throughout the range of sizes used in these studies. The retinal surface area, A , can be calculated from the solid of revolution defined by an arc, $\theta = 185/2 = 92.5^\circ$ in subtense, from a circle of radius R , according to the formula:

$$A = \int_0^{92.5} 2\pi R^2 \sin\theta d\theta = 2\pi R^2 [-\cos\theta]_0^{92.5}$$

The radius, R , of the circle is related to the length of the arc, ℓ (the retinal length), by the following:

$$\frac{185}{360} = \frac{\ell}{2\pi R}.$$

$$R = 0.31\ell, \text{ so } R^2 = 0.10\ell^2.$$

$$\text{Thus, } A = 0.64\ell^2.$$

Retinal cell counts - tangential sections. Both eyes from 3 fish, 7.1, 13.0, and 23.3 cm in length, were cut into 9 pieces representing the dorsal, dorsonasal, nasal, nasoventral, ventral, ventrotemporal, temporal, temporo-dorsal, and central regions of the retina. Serial, $5 \mu\text{m}$, sections were cut tangential to the convex surface of each piece. Camera lucida drawings were made of selected sections through the ganglion cell layer and the layer of

cone nuclei at 900 X using an oil immersion objective. The density of ganglion cells was determined from the drawings in areas ranging from 0.0012 to 0.00275 mm² in each of the 9 retinal regions. The cone density was determined likewise in areas ranging from 0.0007 to 0.0039 mm².

RESULTS

Size and Age. Most species of teleost fish continue to grow throughout life, unlike mammals which cease growing in adulthood (M. Brown, 1957). This study is an analysis of retinal cell numbers in goldfish as a function of size; it is based on the assumption that differences in size are related to age and reflect a growth process. The possibility exists, however, that such differences could be due to polymorphism in body size rather than to growth. The assumption that larger fish are older was tested using a standard ichthyological technique for determining the age of fish. The results of this analysis are presented in the Appendix. Although the growth rate of goldfish is variable, as it is in all fish (M. Brown, 1957), in general larger fish are older.

Growth of the eye. The goldfish eye grows along with the body, as shown in Fig. 6. The diameter of the eye was measured in situ through the nasotemporal axis with a hand micrometer. It increased threefold over a fivefold increase in body length. The growth of the eye is thus negatively allometric with respect to the growth of the body; that is,

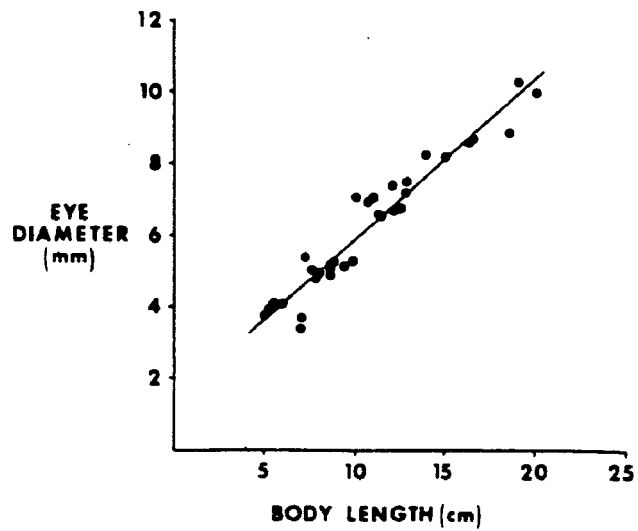


Fig. 6. The relation between eye diameter and body length. Each point is the mean eye diameter of one fish. The least squares linear regression drawn through the points is:

$$\text{Eye diameter (mm)} = 0.45 [\text{body length (cm)}] + 1.39,$$

$$r^2 = 0.94.$$

the eyes of large fish are relatively smaller, compared to the overall body size, than are the eyes of small fish. Some of the scatter in Fig. 6 is probably due to differences in the ages of the fish (see the Appendix). Variability in the length of the tail among fish of the same standard body length probably also contributes to the scatter.

Determination of the amount of histological shrinkage.

Retinal dimensions were corrected for a linear histological shrinkage of 30%, determined as follows. Eyes were removed from anesthetized goldfish and frozen in an acetone-dry ice mixture as described previously (Easter, Johns and Baumann, 1977). The frozen eyes were sectioned radially with a sliding microtome, and the retinal length, defined in the same way as for the histological sections, was determined from photographs of the block face. The retinal lengths measured from the frozen tissue were compared with those measured in the paraffin-embedded material as shown in Fig. 7. The measurements from the frozen sections were systematically above those from the paraffin-embedded tissue; the amount of displacement indicates the extent of the shrinkage due to the histological processing. The average amount of shrinkage was determined by grouping the data into 4 bins with respect to body length: 4-8, 8-12, 12-16, and 16-20 cm. The mean retinal lengths of the frozen and embedded tissue for each group are indicated by the

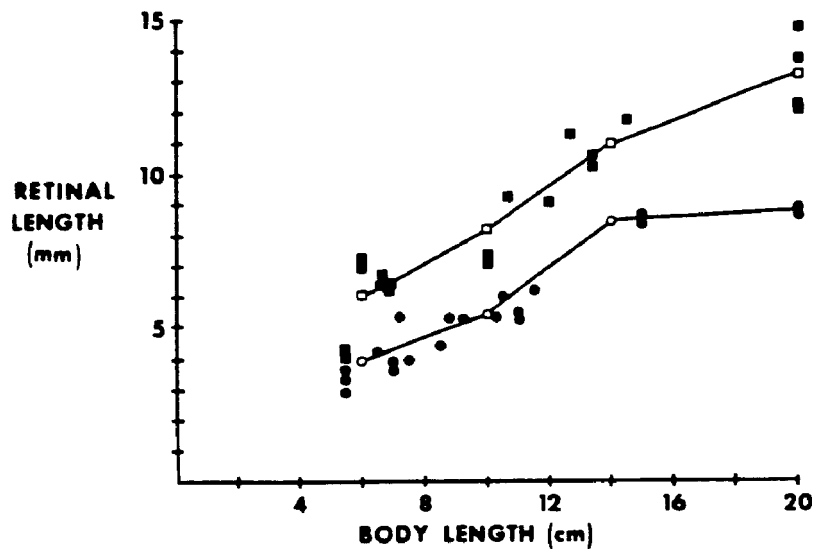


Fig. 7. The magnitude of the histological shrinkage. The retinal lengths measured in frozen, sectioned eyes are given by the squares, and the retinal lengths measured in histological preparations are given by the circles. The data were grouped into four bins with respect to body lengths, 4-8, 8-12, 12-16 and 16-20 cm, and the average retinal lengths in each bin are given by the open symbols connected by lines.

open circles connected by lines in Fig. 7. The average difference between the two means for each group was 30%.

Growth of the retina. The retinal area was calculated from the corrected retinal length, using the formula given in the Methods. Fig. 8 shows that the retinal area at the vitreal surface increased linearly from 12 to 120 mm² as the fish increased from 4 to 20 cm in length. Some of these points were calculated from retinal lengths measured in histological sections, and others are from frozen sections (Easter, Johns, and Baumann, 1977). Some of the scatter in the data is due to a slight asymmetry in the goldfish retina. In 7 fish in which both retinas were sectioned, one through the nasotemporal and the other through the dorsoventral axis, the retinal length in the nasotemporal axis was slightly greater than in the dorsoventral axis, by about 4%.

Identification of cell types. The appearance of the goldfish retina in radial sections is shown in Fig. 9. The structure of the retina is surprisingly similar in all vertebrates, and was described in detail by Ramón y Cajal (1892) and Polyak (1957). In goldfish, the retinal cells and their processes are confined to well-defined strata; the various retinal layers from the proximal (vitreal) to the distal (pigmented retinal epithelial) surface shown in Fig. 9 are: the internal limiting membrane (ILM), the optic fiber layer (OFL), the ganglion cell layer (GCL), the inner plexiform layer (IPL), the inner nuclear layer (INL), the

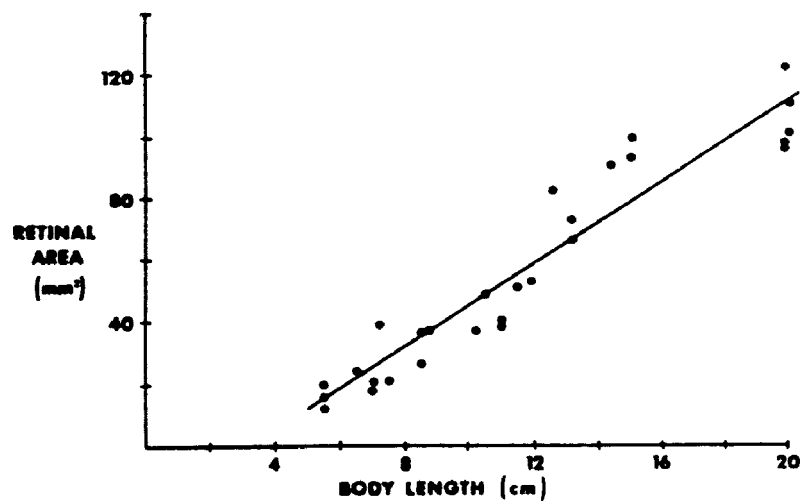


Fig. 8. The increase in retinal area with growth. The retinal area at the vitreal surface, is given as a function of body length. Each point is one retina. The least squares linear regression drawn through the points is:

$$\text{Retinal area (mm}^2\text{)} = 6.74 [\text{body length (cm)}] - 22.63,$$

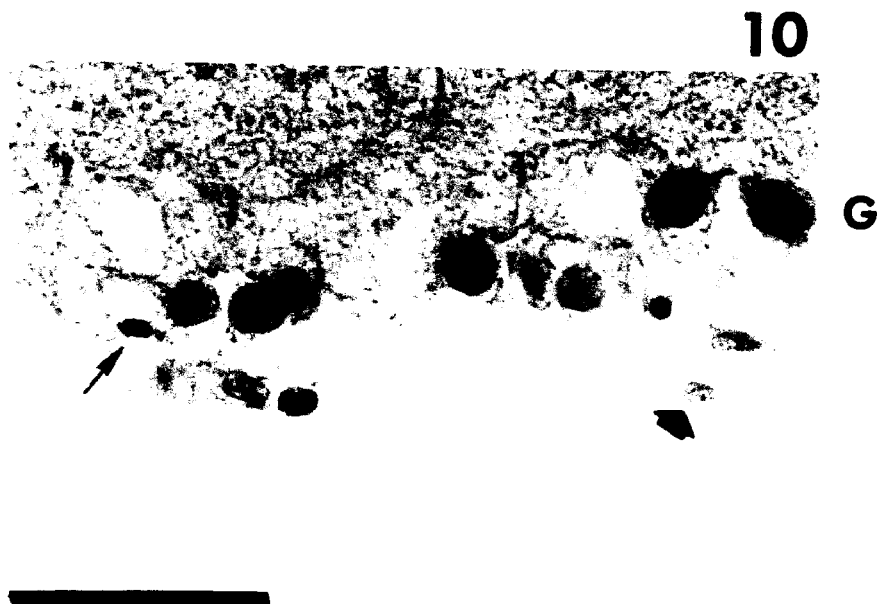
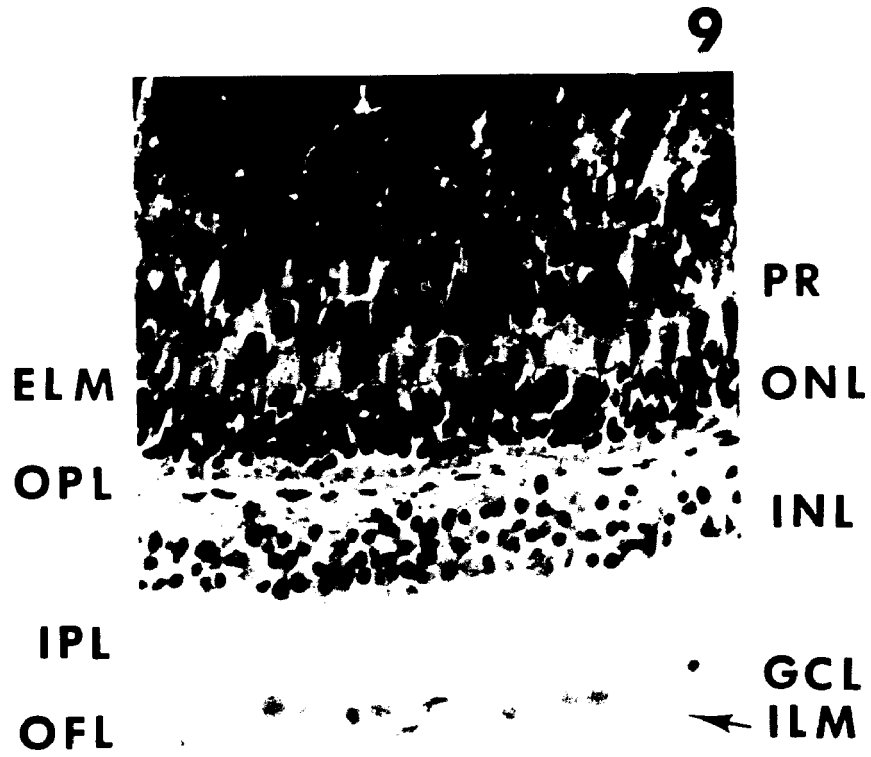
$$r^2 = 0.91.$$

Fig. 9. The goldfish retina.

This is a radial section through a goldfish retina. Internal limiting membrane, ILM; optic fiber layer, OFL; 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. Foot-Masson trichrome, section thickness: 5 μm . Calibration bar: 50 μm . The calibration bars in this and subsequent figures reflect dimensions in the histological preparations, uncorrected for shrinkage.

Fig. 10. The ganglion cell layer.

Ganglion cells, G; glia, small arrow; endothelial cell, large arrow. Galloccyanin, 5 μm . Calibration bar: 25 μm .



outer plexiform layer (OPL), the outer nuclear layer (ONL), the external limiting membrane (ELM), and the layer of photoreceptor cell processes (PR).

Ganglion cells were identified on the basis of their position in the ganglion cell layer, and their cytological characteristics: a relatively large, round, pale nucleus, 7.0-8.5 μm in diameter, surrounded by an irregularly-shaped ring of basophilic cytoplasm. The range of nuclear diameters given here and below indicates the increase in cell size which accompanies growth of the retina; the cells in larger fish are bigger. In all of the goldfish examined, the ganglion cells were arranged in a layer a single cell thick throughout the retina. Other cells in the ganglion cell layer included neuroglial cells and vascular elements. The neuroglia were smaller than the ganglion cells, with a round or elliptical, dark nucleus, often containing irregular clumps of chromatin, and with little visible cytoplasm. Endothelial cells formed the walls of the blood vessels on the vitreal surface. They had pale, elongated, irregularly-shaped nuclei, and were often seen in association with groups of nucleated erythrocytes. Fig. 10 is a photomicrograph showing the various types of cells in the ganglion cell layer.

The distributions of the ganglion cells and the glia in the ganglion cell and optic fiber layers in a representative radial section through the optic disk in a 10.5 cm fish are shown by the histograms in Fig. 11. The number of cells

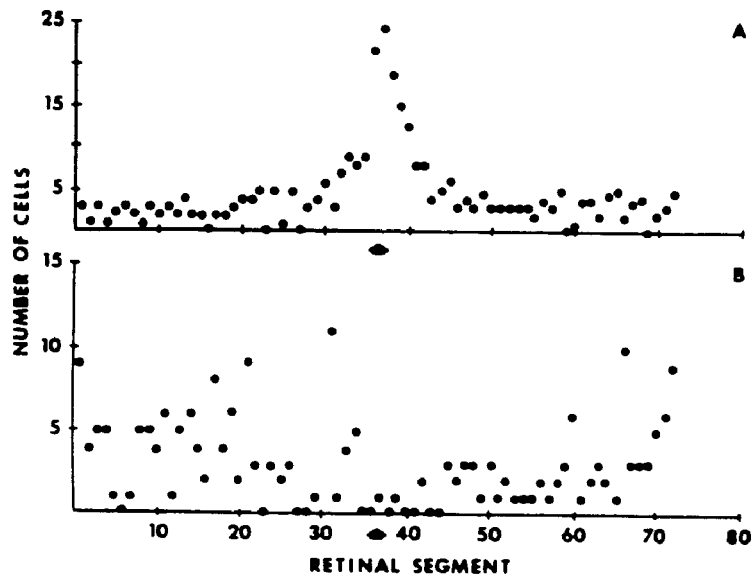


Fig. 11 Distribution of cells in the ganglion cell and optic fiber layers.

The number of glial cells in each successive 128.6 μm segment across the retina is given in A and the number of ganglion cells in B. The position of the optic disk is indicated by the arrows. The fish was 10.5 cm in length. Dorsal retina is to the left and ventral is to the right on the abscissa.

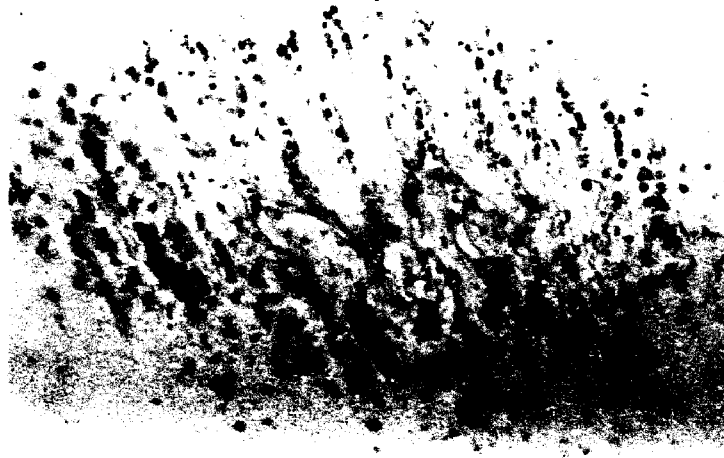
in successive 128.6 μm segments across the retina is plotted on the ordinate; the position of the optic disk is indicated by an arrow. The frequency of neuroglial cells was very low everywhere except at the optic disk where their numbers increased sharply (Fig. 11A). This sharp maximum reflects their close association with the optic fibers which have gathered there to exit the retina (Van Buren, 1963; Goldberg and Coulombre, 1972; Watson, 1974). The ganglion cells were virtually excluded from the optic disk, and their density varied non-systematically throughout the rest of the retina (Fig. 11B). The often striking difference in ganglion cell density between adjacent retinal segments was due to their irregular spacing: in radial sections a string of two or more ganglion cells was sometimes followed by a length of retina completely devoid of them (see Figs. 9 and 10). This clumped distribution is best seen in tangential sections through the ganglion cell layer, such as in the photomicrograph in Fig. 12. Groups of ganglion cells were partitioned between rows of vitreal blood vessels which coursed radially over the retinal surface to converge upon the optic disk; there they joined and exited the retina along with the optic fibers (Fig. 13). This segregation of ganglion cells into columns by the blood vessels was probably responsible for their irregular distributions in Fig. 11B.

To be certain that the ganglion cells were correctly identified, their chromatolytic response to optic nerve

Fig. 12. The relation between ganglion cells and blood vessels. This section was cut tangentially through the layer of blood vessels on the vitreal surface. The ganglion cells are at a slightly different level and can be seen surrounding the vessels. Note that the cells are segregated into columns between adjacent vessels. Foot-Masson, 5 μm . Calibration bar: 50 μm .

Fig. 13. The optic disk. This is a tangential section through the optic disk showing the associated retinal artery as it exits the eye (arrow). Foot-Masson, 5 μm . Calibration bar: 100 μm .

12



13



section was observed. "Chromatolysis" is a term used to describe the morphological changes occurring in a neuronal soma in response to damage of its axon (Cragg, 1970; Engh and Schofield, 1974; Grafstein, 1975). Although it was classically defined for the mammalian nervous system, the characteristics of the axonal reaction in goldfish retinal ganglion cells following optic nerve section are similar to chromatolysis and even more dramatic. These changes were described in detail by Murray and Grafstein (1969) and consist of cellular hypertrophy, nuclear eccentricity, increased prominence of nucleoli, and a markedly increased cytoplasmic basophilia. This response develops over a period of several days, and reaches a maximum at 21-28 days following optic nerve section. Some examples of chromatolytic ganglion cells are shown in Fig. 14A, together with normal ganglion cells for comparison (Fig. 14B).

The chromatolytic response was used to answer two questions: are all of the cells in the ganglion cell layer which have the cytological appearance described above and shown in Fig. 10 really ganglion cells; that is, do they all send an axon into the optic nerve? Conversely, are all ganglion cells located in the ganglion cell layer? The second question is important since several anatomists have described "displaced ganglion cells" with soma located in the inner plexiform or inner nuclear layer (Ramón y Cajal, 1892; in fish: de Testa, 1966; Stell and Witkovsky, 1973). To answer these questions the right optic nerve of a

12.5 cm fish was crushed intraorbitally, and the animal was killed 28 days later. The retina was sectioned radially through the nasotemporal axis, and all of the chromatolytic cells in every 10th section were counted. A total of 5282 chromatolytic cells was counted, and all of the cells which could be identified as ganglion cells showed evidence of chromatolysis. In addition, 54 chromatolytic cells, or about 1% of the total, were found distal to the ganglion cell layer, and thus represented displaced ganglion cells, an example of which is shown in Fig. 15. These displaced cells could not be easily identified in normal tissue with paraffin histology, but they represented such a small fraction of the ganglion cell population that they were disregarded in the analysis of ganglion cell numbers below.

The inner nuclear layer contains the nuclei of several different cells including amacrine, bipolar, and Müller cells. The horizontal cells are displaced slightly into the outer plexiform layer in goldfish. The nuclear morphology seen in paraffin embedded, nuclear stained sections varies somewhat among the different cell types, but a continuous gradation in cytological appearances often made positive identifications difficult. In general, the amacrine cells were located closest to the inner plexiform layer and resembled the ganglion cells with a round, pale nucleus, 7.0-8.5 μm in diameter, depending on the size of the fish. The bipolar cells were located throughout the

Fig. 14. A comparison of normal and chromatolytic ganglion cells.
A, chromatolytic ganglion cells,
B, normal ganglion cells.
Gallocyanin, 5 μm . Calibration bar: 25 μm .

Fig. 15. A displaced ganglion cell.
The arrow indicates a displaced ganglion cell, located at
the border of the inner nuclear layer. Gallocyanin, 5 μm .
Calibration bar: 25 μm .

14



B



15



inner nuclear layer; they also had a round, but smaller, nucleus 3.5-5.5 μm in diameter, which stained darkly and homogeneously. The Müller cells are glial elements which have processes extending both distally and proximally through the entire width of the retina. Their nuclei were usually slightly elongated radially and irregularly shaped, 4.5-7.0 μm in diameter; they could be identified with certainty in favorable sections which included all or part of their radial processes (Fig. 16). The horizontal cell nuclei were located at the border between the inner nuclear layer and the outer plexiform layer. They were also elongated, but in the tangential direction; their nuclei were up to 14.5 μm or more in length and irregular in shape.

This classification of inner nuclear cell types is not rigid. Villegas and Villegas (1963) and Naka and Carraway (1975) have questioned the above classification in the fish retina based on biochemical, morphological, and physiological evidence. The latter believe that there may be several more distinct types of cells in the goldfish inner nuclear layer, and have even abandoned the term "amacrine" cell.

The outer nuclear layer proximal to the ELM contained the nuclei of the rods, which are dark, sometimes spindle shaped, with a diameter of 3.5-4.5 μm . The nuclei of the cones were located distal to the rod nuclei and penetrated through the ELM. They were ellipsoid in shape, 6.5-8.5 μm in diameter, and pale, with fine, granular chromatin. The

Fig. 16. The inner nuclear layer.
Müller cell, M; amacrine cell, A; bipolar cell, B; horizontal cell, H. Gallocyanin, 5 μm . Calibration bar:
25 μm .

Fig. 19. Ganglion cells in tangential section.
This is a tangential section through the ganglion cell layer in the center of the retina. Foot-Masson, 5 μm .
Calibration bar: 50 μm .

16



19



distal processes of the cones consisted of a contractile myoid, an inner segment of ellipsoid, and an outer segment containing the visual pigment. The distal processes of the rods had the same three components, but they were small (2 μm or less in diameter) and difficult to see in these histological preparations. The average diameter of the cone ellipsoids increased from about 8 μm in the small fish to about 11 μm in the larger fish.

The above descriptions of cellular morphology seen in paraffin sections correspond with those given for retinal cells in other teleosts by Müller (1952), Lyall (1957b) and O'Connell (1963). The cellular dimensions were corrected for a linear histological shrinkage of 30% and were measured parallel to the retinal layers.

Cell death as well as cell proliferation has been implicated in the embryonic development of the retina (Glücksman, 1940, 1965; Silver and Hughes, 1975), and so a careful search was made for pyknotic nuclei in the adult goldfish retina. Dying cells can be distinguished by several cytological criteria (Glücksman, 1930; Bessis, 1964): cytoplasmic edema and vacuolation, a round and shrunken nucleus with a homogeneous, dense chromatin, nuclear fragmentation and necrotaxis, a peculiar force of attraction which causes dying cells to clump together. No evidence of cell death was seen in the goldfish retina.

Retinal cell density. The object in counting retinal cells was to determine their total numbers as a function of

size, and by inference, age. The total number of cells can be calculated from the cell density and retinal area, but if the cell density is not homogeneous, the density distribution throughout the retina must be defined in order to correctly estimate the total cell number. Two methods were used to quantify the density distribution in the goldfish retina. First, the eyes from 3 fish, 7.0, 11.5, and 20.0 cm in length, were sectioned radially, one through the nasotemporal axis and the other through the dorsoventral axis in each fish. All of the ganglion cells and cones were counted in three sections through the center of the eye, and the average number of cells in each successive 857 μm segment across the retina was determined for each eye. The means $\pm$ 2 S.D. are plotted for the ganglion cells in Fig. 17 and for the cones in Fig. 18; there was no obvious systematic relation between cell spacing (#/unit length) and retinal position. Table I summarized the results of the statistical test which was used to determine whether any small but systematic variations in cell spacing existed. The size of the fish and the eye used (right, RE, or left, LE) is given in the first column. The ganglion cell data are listed in the upper half and the cone data in the lower half. The mean cell spacing in one half of each retina was compared with the mean cell spacing in the other half of the same retina using Student's t test; nasal was compared to temporal and dorsal to ventral. The two means are shown in the second column, where M_1 is the mean number of

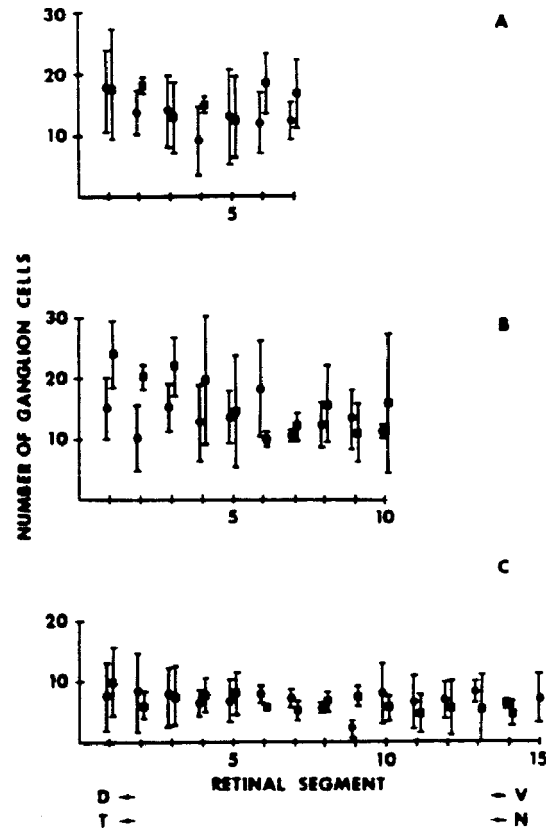


Fig. 17. Ganglion cell distribution in radial sections. The average number of ganglion cells ± 2 S.D. in successive 857 μm segments is plotted for the nasotemporal (circles) and dorsoventral (squares) axis of the retina. The orientation of the sections is indicated below: dorsal, D; temporal, T; ventral, V; and nasal, N. The data in A are from a 7.0 cm fish, in B from an 11.5 cm, and C from a 20.0 cm fish.

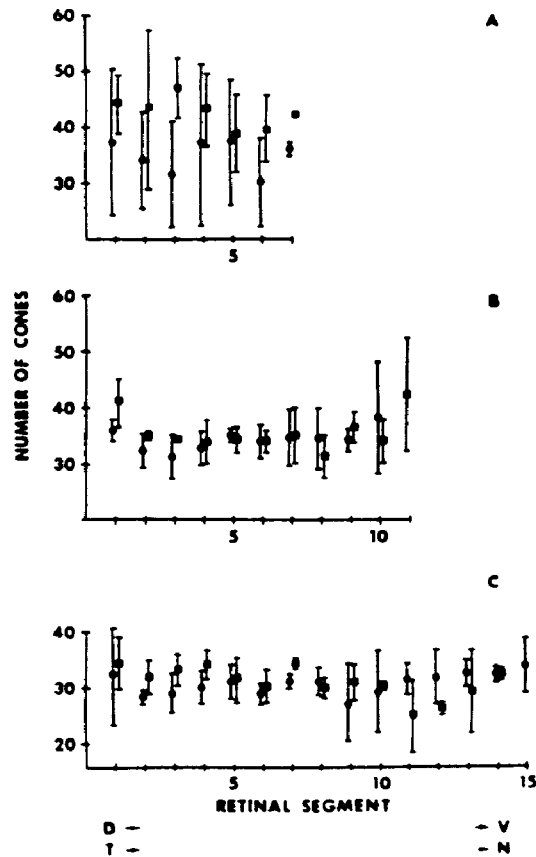


Fig. 18. Cone distribution in radial sections. The average number of cones ± 2 S.D. in successive 857 μ m segments is plotted for the nasotemporal (circles) and dorsoventral (squares) axis of the retina. The orientation of the sections is indicated below: dorsal, D; temporal, T; ventral, V; and nasal, M. The data in A are from a 7.0 cm fish, in B from an 11.5 cm, and C from a 20.0 cm fish.

TABLE I
GANGLION CELL AND CONE DENSITIES IN RADIAL SECTIONS

<u>Ganglion Cells</u>		M_1/M_2	$\underline{t}$	$\underline{df}$	$\underline{P}$	Section Plane
A.	7 cm, LE	13.9/12.5	0.96	19	>0.1	DV
	7 cm, RE	15.8/16.5	-0.48	19	>0.1	NT
B.	11.5 cm, LE	13.1/13.5	-0.32	27	>0.1	DV
	11.5 cm, RE	13.5/20.1	-4.17	29	<0.001**	T>N
C.	20 cm, LE	7.4/6.8	-0.92	43	>0.1	DV
	20 cm, RE	5.9/7.1	-2.08	40	>0.05	NT
<u>Cones</u>						
A.	7 cm, LE	35.5/34.4	0.49	19	>0.1	DV
	7 cm, RE	40.1/44.7	-3.09	19	<0.01*	T>N
B.	11.5 cm, LE	34.1/35.1	-1.00	29	>0.1	DV
	11.5 cm, RE	35.8/35.2	0.49	30	>0.1	NT
C.	20 cm, LE	30.4/31.6	-1.67	43	>0.1	DV
	20 cm, RE	29.6/32.9	-3.95	41	<0.001**	T>N

cells/857 μm in the dorsal or nasal half and M_2 in the ventral or temporal half. The value of " $\underline{t}$ " is given in the next column, followed by the degrees of freedom, $\underline{df} = n - 2$, where n is the number of samples. The probability that the two means came from the same population, i.e. that the cell density is homogeneous across the retina, is given in the fourth column; those which show significant (*) or highly significant (**) deviations from homogeneity are indicated. The last column gives the plane of section, dorsoventral (DV) or nasotemporal (NT), and where a significant difference was found the order is shown. In three eyes a significant or highly significant difference was found between cell densities in the two retinal halves, one involving the ganglion cells and the other two the cones. In all cases the discrepancy occurred in the right eye, sectioned through nasotemporal axis; the greater density was always in the temporal half. The differences were small, however, amounting to less than 30%. A comparison of the means in the dorsal and ventral halves in the left eye of the same fish reveals that in these cases the cell density was greater in the temporal retina than elsewhere.

The cell densities shown in Figs. 17 and 18 and Table I are limited to two perpendicular transects, one through the nasotemporal and the other through the dorsoventral axis of the retina. To sample the cell density in other retinal regions, both retinas from three more fish

were sectioned tangentially at 9 different points in each. A photomicrograph of a tangential section through the ganglion cell layer is shown in Fig. 19. All of the ganglion cells in the entire width of the ganglion cell layer were usually included in one tangential section, and this is the section which was drawn. The density of ganglion cells in 8 of the retinal regions in the three fish are shown in the polar plots in Fig. 20. The average density in comparable segments from the two eyes in each fish is given by the length of the ray whose direction indicates the retinal region in which the density was sampled--dorsal is up and nasal is to the right. The end points of adjacent rays are connected. The cell density in the ninth region in the center of the retina is not shown in these plots. In some cases the proximity of the optic disk interfered with the density measurements in this region; in those cases in which measurements were possible they were close to the mean of the other regions. Homogeneity of ganglion cell density would result in regular octagonal figures (dotted lines) in these polar plots; although the shapes in Fig. 20 are not regular octagons, they do not seem to be systematically misshapen. The tendency for an increased density in the temporal half found in some of the retinas sectioned radially was not seen in any of these fish.

In contrast to the ganglion cell layer, several serial tangential sections were needed to traverse the extent of the outer and inner segments and nuclei of the

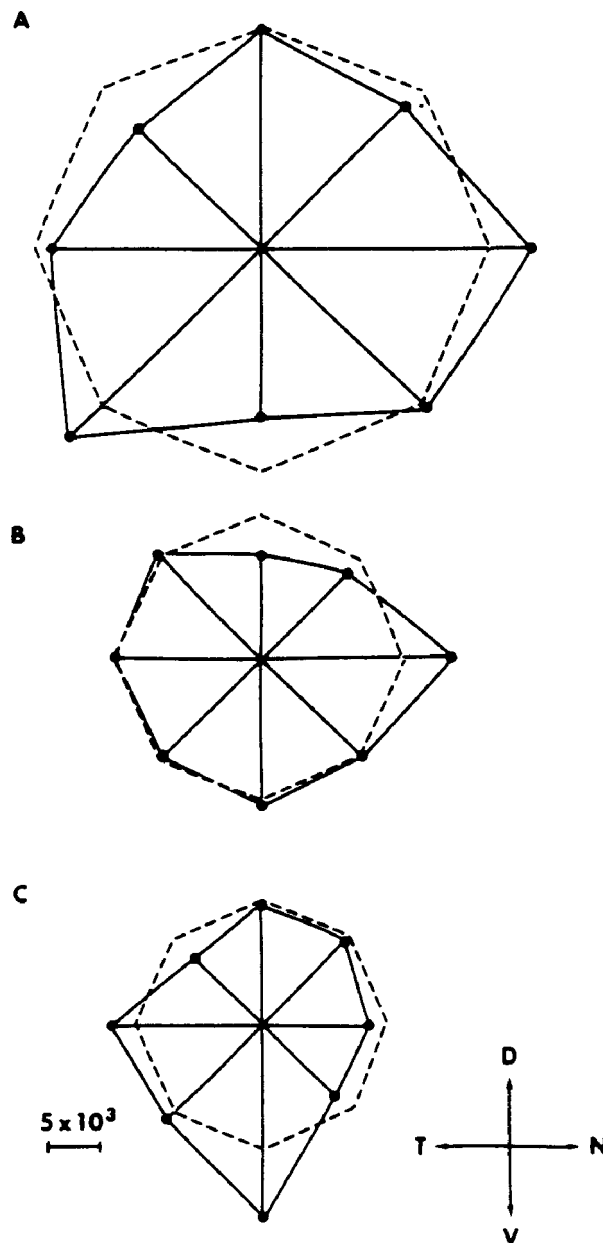


Fig. 20. Ganglion cell density in tangential sections. The length of each ray indicates the mean ganglion cell density ($\#/\text{mm}^2$ retinal surface); a scale is given in the lower left. The direction of the ray indicates the location of the retinal sample as shown in the lower right: dorsal, D; ventral, V; nasal, N; and temporal, T. The upper graph, A is from a fish 7.1 cm in length; B is from a fish 13.0 cm in length; and C is from a fish 23.3 cm long. The dashed lines are regular octagons, representing the mean density.

cones. The distal processes of the cones in the goldfish are variable in length (Stell and Lightfoot, 1975; Marc and Sperling, 1976; Stell and Hárosi, 1976), and therefore not all of them were contained in the same tangential section. Photomicrographs of serial tangential sections through the cones are shown in Fig. 21. The mosaic pattern often found in teleost fish cones is evident in the goldfish, as previously described by Engstrom (1960); one mosaic unit is circled in Fig. 21C. Preliminary estimations of cone density revealed that the most accurate counts were obtained from the section through the cone nuclei; this was due to the variability in the lengths of their distal processes. Polar plots of cone densities in 8 retinal regions in three fish are shown in Fig. 22; these were created in the same way as for the ganglion cells. The cone plots are more nearly regular octagons than are the ganglion cell plots. No evidence of an increased density in the temporal retina was seen.

These results indicate that the cell density is nearly homogeneous throughout the goldfish retina. A slight tendency for an increased density in the temporal retina was not found consistently in all retinas. Although only the ganglion cells and cones were analyzed in detail, the density of the other retinal cells, the rods and the inner nuclear layer cells, followed a similar pattern.

Age-related changes in cell density and number. For the analysis of retinal cell numbers as a function of size,

Fig. 21. Cones in tangential section.
These are serial sections from the temporal region of the retina. The layer of cone nuclei is shown in F; one mosaic unit is circled in C. The darkest profiles are the inner segments. Foot-Masson, 5 μm .
Calibration bar: 50 μm .

21

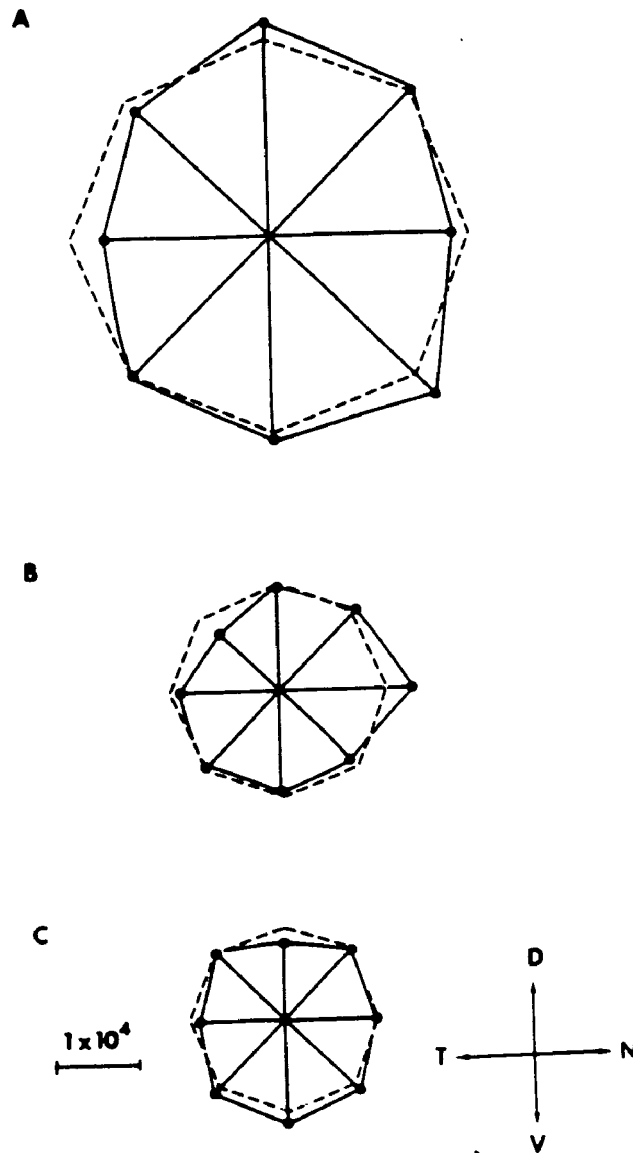


Fig. 22. Cone density in tangential sections.
Description as in Fig. 20

median radial sections from 25 goldfish eyes were used. Rods, inner nuclear layer cells and ganglion cells were counted in 20 retinas using the line sampling technique, and cones were counted in 21 retinas using an Abercrombie method. Counts were made at regular intervals across one entire section, through the center of the eye. The retinas used for cone counts included 16 of those used for counts of the other cells and 5 in which only cones were analyzed. Cones were not counted in 4 of the retinas in which the other cells were analyzed, due to inadequacies in the staining of the photoreceptor layer. Cells in the inner nuclear layer were counted together, except the horizontal cells, which were sufficiently distinct to allow accurate identification; their densities were then combined with that of the other inner nuclear layer cells. The inner nuclear layer cells which were tallied together, the bipolar, amacrine, and Müller cells, also varied somewhat in diameter, from about 4.5 to 8.5 μm . As a result these counts were not as accurate as were those for the more homogeneous cell populations, the rods and ganglion cells. The magnitude of this error in the inner nuclear layer counts may have been up to 20-30%.

The retinas of a small (7 cm) and a large (20 cm) fish are compared in Fig. 23. The retina between the ELM and the ILM increased in thickness from about 135 μm to 175 μm during growth, primarily due to increases in the thickness of the inner plexiform and nuclear layers. The

Fig. 23. Comparison of the retinas of small and large fish.
A, 7 cm fish,
B, 20 cm fish.
Gallocyanin - eosin, 5 μm , bleached sections.
Calibration bar: 100 μm .

23

B



layer of rod nuclei, remained relatively constant in thickness and density, while the cell density in the inner nuclear layer decreased dramatically. These sizes of the cells also increased with growth. Fig. 24 is a semi-logarithmic plot of cell density ($\#/mm^2$ of retinal surface) versus retinal length. Retinal length was used as the size parameter since it covaried more tightly with retinal cell counts than did body length. This graph indicates that the density of inner nuclear cells, cones, and ganglion cells decreased with growth, but the rod density remained constant or even increased.

The density can also be expressed with reference to visual angle in degrees². The density of retinal cells per visual degree² was calculated from the retinal magnification factor (μm retinal surface/degree visual angle). As the goldfish eye grows, the extent of the retinal field remains constant at 185.3° , and the retinal magnification factor, increases from about 35 to $80 \mu m/\text{degrees}$ (Easter, Johns, and Baumann, 1977). The number of cells/visual degree², given in Fig. 25, increased with growth. This means that despite the spreading apart of retinal cells with growth, the number of cells upon which an image of a given size was projected increased.

A change in the number of cones per visual degree is thought to be associated with a change in the visual acuity. According to the formulation by Tamura (1957) the

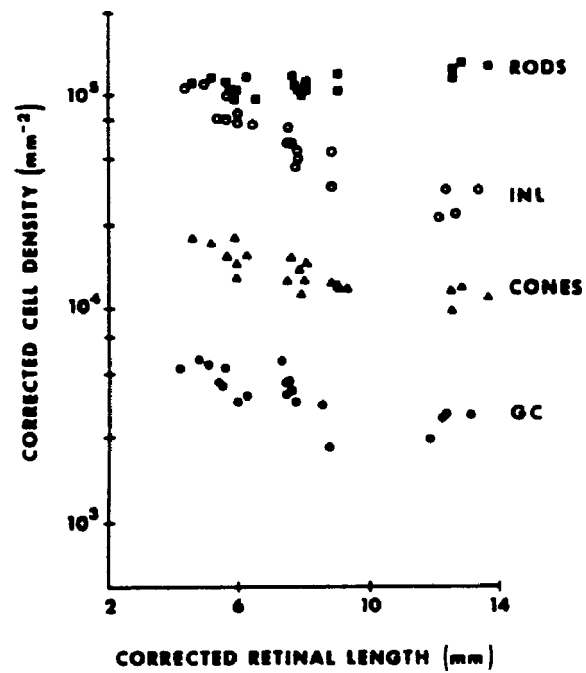


Fig. 24. Retinal cell density as a function of retinal length. The density of rods (squares), inner nuclear layer cells (open circles, INL), cones (triangles), and ganglion cells (filled circles, GC) is given in $\#/mm^2$. Each point is one retina. The retinal length was measured along the ILM for the ganglion cells, along the ELM for the rods and cones; the mean retinal length was used for the inner nuclear layer cells.

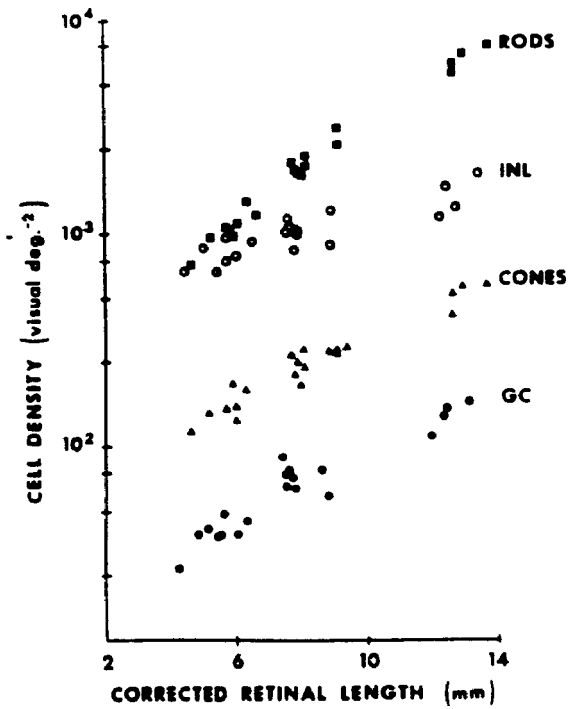


Fig. 25. Retinal cell density as a function of visual angle. These are the same data as in Fig. 24, plotted in terms of $\#/ \text{visual degree}^2$, as described in the text.

"minimum separable angle," α , in radians is:

$$\alpha = \frac{1}{F} \frac{0.1 (1.0 + 0.25) \times 2}{n}$$

where F = focal length of the lens (mm)
 n = # cones/0.01 mm²

The factor "0.25" is a correction for an histological shrinkage of 25%; the "2" selects alternate cones in a retinal segment 0.1 mm in length. Since the shrinkage determined for the goldfish retina using the histological procedures described above was 30%, the formula becomes:

$$\alpha = \frac{1}{F} \frac{0.1 (1.0 + 0.30) \times 2}{n}$$

The focal length, F , was, estimated from the empirical relation between retinal length and the distance from the center of the lens, to the ELM as described previously (Easter, Johns, and Baumann, 1977):

$$F = 2.46 \times \text{lens radius (mm)},$$

where lens radius = $0.12 [\text{retinal length (mm)}] + 0.135$.

This estimation of "F" assumes that the goldfish eye is emmetropic as shown by Charman and Tucker (1973) and Schwassmann (1975), and that the image is focused at the level of the photoreceptors, as Tamura (1975) verified. The calculated visual acuity is given in Fig. 26 as a function of retinal length; it increased, i.e. the minimum separable angle decreased, from 50' to 18' with growth.

To calculate the total number of cells in the retina, the area was determined from the retinal length. Since the retina is a curved surface of finite thickness, the outer

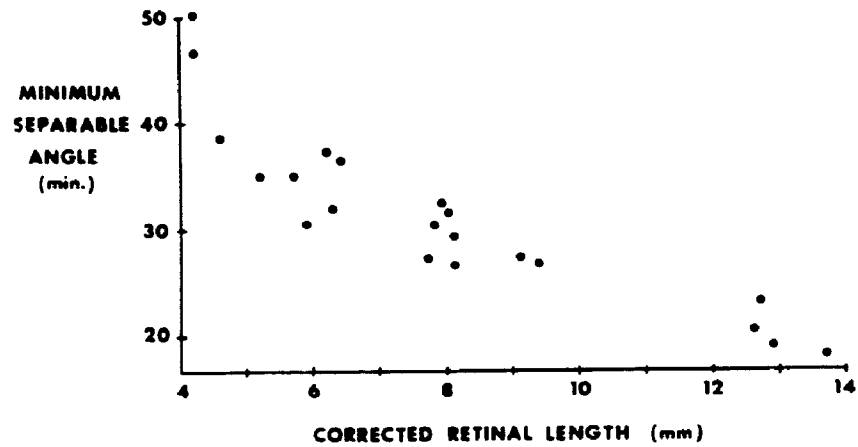


Fig. 26. Calculated visual acuity as a function of retinal length. The minimum separable angle was calculated as described in the text. Each point is one retina. The retinal length measured along the ELM is given by the abscissa.

layers were longer than the inner layers. The area of the retinal surface at the ILM was less than at the ELM by about 5 - 15%, with the smaller eyes showing the greatest discrepancy. Accordingly, the retinal length was measured at two depths in the retina, along the ILM and along the ELM, and the retinal area calculated from the ELM length was used to determine the total number of rods and cones; the ILM length was used for the ganglion cells, and the average of the two was used for the inner nuclear layer cells. The total cell numbers as a function of retinal length are given in Fig. 27. Despite the decrease in density of all but the rods, the total number of all cells increased with growth: the ganglion cells increased from 60,000 to 350,000; the inner nuclear layer cells from 1,500,000 to 4,000,000; the cones from 250,000 to 1,400,000; and the rods from 1,500,000 to 15,000,000.

Since the density of the rods was constant while that of all the other cells decreased, there were relatively more rods in a larger retina than in a smaller one. This is shown in Fig. 28 in which the mean ratio of rods, inner nuclear layer cells, and cones per ganglion cell is given for three ranges of retinal length: 4-7, 7-10, and 10-14 mm. While the ratio of cones to ganglion cells remained constant at about 4:1, the number of inner nuclear cells per ganglion cell decreased from 20:1 to 10:1, but the number of rods per ganglion cell increased from 24:1 to 40:1.

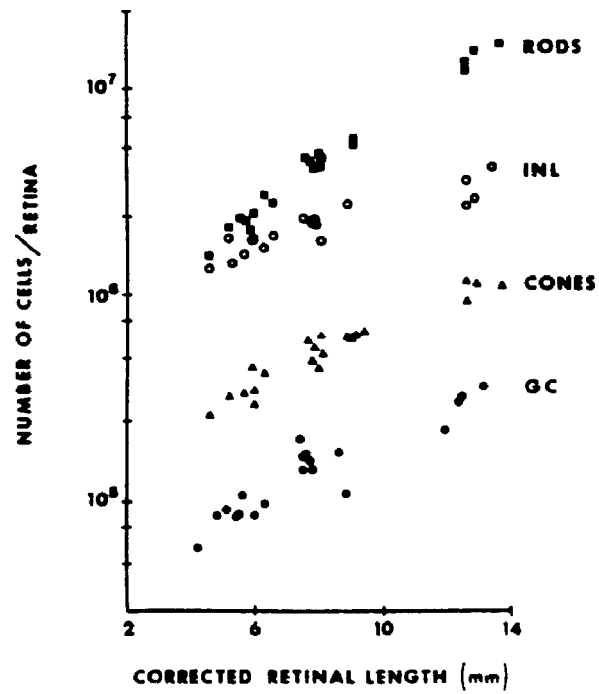
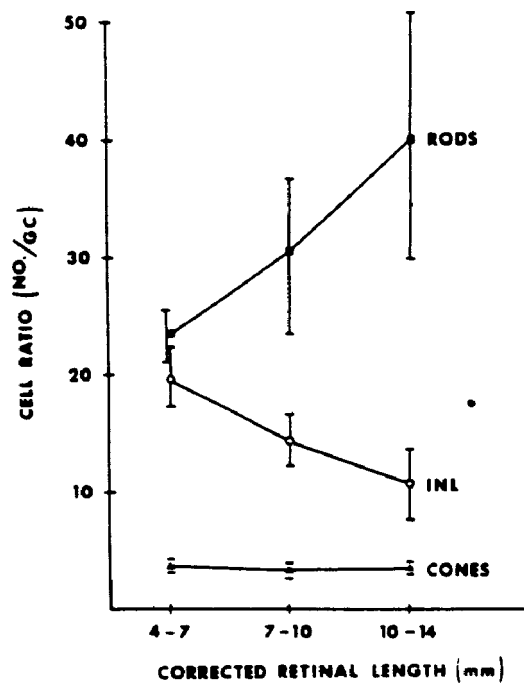


Fig. 27. Total number of retinal cells as a function of retinal length. The number of rods (squares), inner nuclear layer cells (open circles, INL), cones (triangles), and ganglion cells (dots, GC) was calculated from the cell density and retinal area. Each point is one retina.



Figs. 28. Cell ratios as a function of retinal length. The number of rods (squares), inner nuclear layer cells (open circles, INL), and cones (triangles) per ganglion cell (GC) was calculated from the cell densities. The data were grouped into three bins with respect to retinal length, and the mean ratios ± 2 S.E.M. are plotted.

The decrease in the INL:GC ratio indicates that the rate of decrease in density of cells with growth of the retina was higher in the inner nuclear layer than in the other retinal layers. Due to the greater inaccuracy of the cell counts from the inner nuclear layer the ratio in the small eye may be as low as 14:1 and in the large eye as high as 13:1, so the conclusion is weakened. If the decrease in the INL cell ratio is real, it implies either a loss of cells from the inner nuclear layer through cell death or migration out of the layer, or a differential addition of cells to the other layers. The inner nuclear layer is composed of at least four cell types, and if the first alternative is correct, the cell loss may be confined to only one or more of these. Accordingly, cells were categorized in the inner nuclear layer in median radial sections from two retinas, one from a small fish (7 cm) and the other from a large fish (20 cm). Based on the cytological characteristics given above, "bipolar cells" accounted for 70.6% of the inner nuclear layer cells in the small retina, "amacrine cells" were 11.8%, "horizontal cells" were 8.2% and "Müller cells" were 9.4%. In the large eye the corresponding values were 52.0%, 21.7%, and 10.6%. Although the exact values of these percentages are not considered definitive, their relative magnitudes are probably significant. Since the "bipolar cells" were the only ones which decreased in relative frequency, they were probably responsible for the cell loss in the inner nuclear layer. It must be

emphasized that the identification of these cells was only tentative; all that can be said with certainty is that the inner nuclear layer in larger fish has relatively fewer cells with small, dark, round, homogeneous nuclei.

DISCUSSION

The major conclusions of this study are four: 1) the adult goldfish retina grows, 2) one component of the retinal growth can be attributed to cellular hypertrophy, 3) the hypertrophic component of retinal growth is insufficient to account for the entire increase in retinal area, so the total number of cells must increase, and 4) in contrast to the other cells, the rods do not decrease in density, and so must be added in even greater numbers during growth.

Growth of the adult goldfish. Adult goldfish continue to grow. Although sexual maturity is difficult to establish, goldfish have been observed spawning at one year and in captivity usually reach maximum fertility at three years (M. Brown, 1975). Since growth is indeterminate in most fish, a division of the life cycle into juvenile and adult stages is not as meaningful as it is in those animals which cease growing as adults. Based on an age analysis (see the Appendix) most if not all of the fish studied here were adult; those which were not were distinct only in their lack of sexual maturity.

The eye also grows in adult goldfish, albeit at a slower rate than the body. A negatively allometric relation

between growth of the eye and the body in teleost fish has been reported previously (Müller, 1952; Lyall, 1957a). In the goldfish, intraretinal measures were more highly correlated than body-retina relations, and Ali (1963) described a similar situation in growing Atlantic salmon (Salmo salar). This is probably related to the tendency for the teleost eye to continue growing when the growth of the body is inhibited, as discussed in the Appendix.

Retinal cell density. The estimation of cell numbers was greatly aided by the homogeneity of cell density throughout the goldfish retina. Previous investigators had also remarked on the uniformity of cells in this retina. Hester (1968) found that the density of cones was greater, but only slightly so, in the dorsotemporal quadrant of the retina in goldfish 10-15 cm in length; the maximum density ($2.2 \times 10^4/\text{mm}^2$) was not even twice the minimum ($1.4 \times 10^4/\text{mm}^2$). Schellart (1973) also indicated that the density of cones and rods might be slightly greater in the dorsotemporal quadrant in fish 18-21 cm in length; the average cone density was $1.4 \times 10^4/\text{mm}^2$, and the rod density averaged $1.4 \times 10^5/\text{mm}^2$. Schellart found no systematic variation in ganglion cell density ($2.6 \times 10^3/\text{mm}^2$) or inner nuclear layer cell density ($2.8 \times 10^4/\text{mm}^2$). Stell and Hárosi (1976) found that the densities of the rods and cones in 15 cm fish were $1.25\text{--}1.75 \times 10^5/\text{mm}$ and $1\text{--}2 \times 10^4/\text{mm}^2$, respectively. Dawson et al. (1971) measured cone densities in the central retina in fish weighing 150-300 g, much more than any of the fish

studied here. They found a cone density of $2.1 \times 10^4/\text{mm}^2$, and a rod: cone ratio of 5:1. Verrier (1928) counted rods, cones and ganglion cells in the dorsal half of goldfish retinas, and found nearly a 1:1 ratio between rods and cones, and a 1.5:1 ratio of cones to ganglion cells. Her values are clearly suspect, however, since even casual observations indicate that there are many more rods than cones in the goldfish retina. Vilter (1947) found that the rods and cones in a closely related species, the carp (Cyprinus carpio), were slightly more dense in the ventral retina. All of the above values of cell density in goldfish are in reasonable agreement, when the decrease in density with growth is taken into account (Fig. 24).

There are at least five different types of cones in the goldfish retina, including one type of double cone and three types of single cones, (Stell and Harosi, 1976), all of which have been found in other teleost retinas (Wünder, 1925; Müller, 1952; Engström, 1960, 1963; Scholes, 1975). In many of the previous studies, single and double cones were counted separately, and a pair of double cones was considered as one element. Although tightly opposed along the length of their inner segments, each member of the double cone pair has a nucleus and appears to be a complete cell (Stell and Hárosi, 1976). There is as yet no structural or functional evidence to suggest that the double cone acts as a single unit (Engström, 1953). Their origin is also a mystery, although most authors favor the

hypothesis that double cones are formed by fusion of two single cones (Eigenmann and Shafer, 1900; Müller, 1952; Lyall, 1957b; Ahlbert, 1968). Perhaps related to this is the report by Stell and Hárosi (1976) of occasional single cones which were closely apposed along part of their length, but not as extensively as the double cones. Since there are no sound reasons for considering a double cone as a single unit, and because of the possibility of confusion between double cones and contiguous single cones, especially in radial sections, each cone element was counted separately in the present study. In the goldfish, double cone pairs outnumber single cones by between 1.5 and 2:1 (Hester, 1968; Stell and Hárosi, 1976), and so the values of cone density cited above have been modified by multiplying the double cone density by two and adding the single cone density to allow direct comparisons with those found here. No attempt was made in this study to categorize the various cone types.

The homogeneity of cells in the goldfish retina is unusual; almost all species of fish and other vertebrates which have been examined show some variation in retinal cell density associated with a region specialized for increased visual acuity, an area or a fovea (Walls, 1942; Polyak, 1957; fish: Tamura, 1957; O'Connell, 1963; Ahlbert, 1968; reptiles: K. Brown, 1969; birds: Fite and Rosenfield-Wessels, 1975; mammals: Van Buren, 1963; J. Stone, 1965; Hughes and Whitteridge, 1973; Ogden, 1975). The small increase in density in the ventrotemporal quadrant present

in some goldfish, has little effect on the calculation of total cell numbers. If we assume that the cell density in one entire quadrant is 30% greater than in the rest of the retina, which is certainly an overestimate, then the total cell number is increased by only 7.5% over the value calculated assuming a homogeneous density with a value equal to the lower density.

Changes in cell density with growth. The cell density in the goldfish retina in general decreases with growth of the eye as the cells increase in size. In the goldfish retina, as in other species, the rods do not decrease in density at all and so account for a larger fraction of the total cell complement in larger eyes. In contrast, the cells in the inner nuclear layer become even sparser than the cones and ganglion cells. These two changes indicate that the retina is not a passive participant in growth by stretching or expanding, but its cells participate actively in some sort of rearrangement.

The decrease in the relative numbers of cells in the inner nuclear layer could be explained either by a differential loss of cells or by an augmentation of the other cell populations. No evidence of cell death or pyknotic nuclei was seen in the inner nuclear layer, but the proportion of cells with small, dark nuclei, and a homogeneous chromatin decreased. Addition of cells to the other retinal layers would not alter the cell proportions within the inner nucleus layer, so the more likely explanation is that cells

are somehow lost. The morphology of the cell which is apparently lost is similar to that typically ascribed to the bipolar cells; whether they are indeed bipolars cannot be determined from paraffin sections, but requires more sophisticated techniques such as Golgi staining and electron microscopy. Where these cells go if they are not dying remains a mystery; some of the possibilities will be discussed later in Chapter III.

The increase in the proportion of rods during retinal growth, also found in some larval amphibians and in other teleosts (Bernard, 1900; Glücksmann, 1940; Müller, 1952; Lyall, 1957a; Blaxter and Jones, 1967; Blaxter, 1975), is puzzling in view of the widely held belief that cell proliferation is restricted to the retinal margin in these animals. Several hypotheses have been suggested; cells might be added to the central retina via migration from the mitotically active periphery or by transformation of other cells into rods. These possibilities, and others, will be discussed further in Chapter III, which deals directly with the problem of the source of the new retinal cells.

Functional implications of retinal cell addition.

One possible reason for maintaining the rod density constant during growth is suggested from functional considerations. Rods are the photoreceptor elements responsible for detecting weak stimuli at low levels of ambient illumination (Walls, 1942; Brindley, 1970). The sensitivity of the eye, the ability to accomplish this detection task, is directly

related to the amount of active visual pigment interposed in the path of the incoming quanta of light (Pirenne, 1962; Brindley, 1970). If we assume that the effective pigment density of individual rods does not change with growth, then the density of rods must be maintained if the sensitivity of the eye is to be unaffected. Unfortunately, there are no data, either physiological or behavioral, concerning the relation between scotopic sensitivity and growth in teleost fish.

In contrast to the rods the other photoreceptor elements, the cones, decrease in density with growth. The cones are responsible for vision at photopic light levels (Walls, 1942; Pirenne, 1962), and play a role in determining the visual acuity. The hypothetical relation between cone density and acuity described in the Results assumes that the information from adjacent cone elements is preserved intact, that their signals do not converge in the retina. This has been demonstrated only in the primate fovea, however (Polyak, 1947; Kolb, 1970). Moreover, Westheimer (1975) has shown that in humans the theoretical physiological limit is actually exceeded psychophysically under some conditions, a phenomenon he calls "hyperacuity." Therefore, the calculation of minimum separable angle based on cone density is only theoretical and needs experimental verification to be meaningful behaviorally. With those precautions in mind, it can be shown that despite the decrease in cone density, the calculated visual acuity

increases with growth in teleost fish. Decreases in cone density with growth are counteracted by increases in the optical capacity of a larger eye (Müller, 1952; O'Connell, 1963). A larger lens has a greater resolving power: the image it forms is larger. If no cones were added, and if the visual field remained constant in size, then growth of the eye accompanied by a decrease in cone density and a proportional increase in the size of the lens, would result in no change in the calculated visual acuity. In the goldfish, however, growth of the eye is accompanied by an increase in the calculated visual acuity from 50' to 18' minimum separable angle. These calculated values agree surprisingly well with the physiological measurement of 17' obtained by Schwassmann (1975) using single unit recordings in the optic tectum of goldfish about 14 cm in length, with the behavioral value of 17' found in goldfish using line gratings (Wilkinson, 1972, cited by Schwassmann, 1975), and with the value of 24' which Hester (1968) calculated based on his estimation of the cone density in goldfish about 12 cm in length. The only behavioral evidence for increases in visual acuity with growth in teleost fish was provided by Baerends et al. (1960) in their study of a cichlid (Aequidens portalegrensis from 3 to 11.5 cm in length. Hester (1968) also showed that the contrast threshold, the ability to distinguish an object from the background, decreases with growth in goldfish, but this measure is difficult to relate to acuity.

Several other authors have concluded that both the acuity and the number of cones increase with growth in teleost fish (Lyall, 1957a; Tamura, 1957; O'Connell, 1963), but only Müller (1952) actually measured the retina and the lens and showed that they grew proportionately. The others did not even directly measure retinal size, but assumed it to be proportional to lens or eye diameter. This assumption is critical to the implication that the number of cones and the visual acuity both increase with growth, as shown in Fig. 29. The upper diagram (A) represents retinal growth with an increase in the total number of cones (from 9 to 18 cells in this simplified scheme) and an increase in the retinal field (from 90° to 180°) resulting in no change in visual acuity (most easily thought of as 1 cone/ 10°). Fig. 29B shows growth with a constant retinal field (180°) and no change in cone number resulting in no change in acuity (1 cone/ 20°). Fig. 29C reflects the situation found in guppies (Müller, 1952) and goldfish (Easter, Johns and Baumann, 1977, and the present study). Growth of the retina with an increase in both acuity (1 cone/ 20° to 1/ 10°) and in the total number of cones (from 9 to 18) is possible only if the retinal field remains constant (180°) or decreases. Any substantial decrease in the retinal field, however, would result in an increase in the cone density, which was never observed.

The effects on visual acuity can be extended to include retinal cells other than the cones. Since the extent

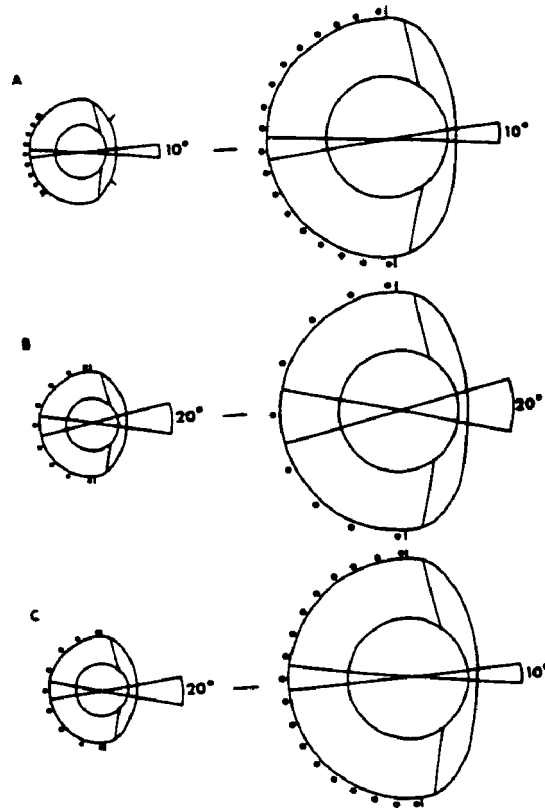


Fig. 29. The relation between cell number, retinal field, and visual acuity.

These are schematic diagrams of goldfish eyes. Cones are represented by dots; the extent of the retinal field is indicated by the short segments perpendicular to the outer surface of the eyes. The visual angle subtended by one cone in each eye is indicated by the rays projecting through the center of the lens, which is represented by the large circle.

of the visual field remains constant during growth, the retinal magnification factor increases (Easter, Johns and Baumann, 1977). Because of this the receptive field of a given retinal cell, the region of space or the retinal locus in which a stimulus provokes a response, must increase in size from, for example, 350 to 800 μm if the angular subtense of the receptive field is to remain constant at 10° (Fig. 30A). If the cell does not increase the size of its retinal locus, the subtense of its receptive field will decrease from 10 to 4.4° (Fig. 30B). If the cell follows the former course, the size of its dendritic tree must increase (J. Brown, 1965; Pomeranz and Chung, 1970; Pomeranz, 1972) which probably implies the formation of new synaptic connections. There is as yet no physiological evidence to suggest which strategy the retinal cells follow, but Fischer (1973) has described an interesting situation in the cat retina which may be parallel to the ontogenetic changes in the goldfish. In the area centralis of the cat, the ganglion cells are smaller and more densely packed than in the periphery; correspondingly, the receptive fields of central cells are smaller than those of peripheral cells. Nevertheless, the overlap between receptive fields remains constant in spite of these differences in size; that is, a small spot of light imaged on the retina stimulates about 35 ganglion cells no matter where it is located. If a similar strategy is followed by the growing goldfish retina, then the size of the receptive fields, measured in μm on the

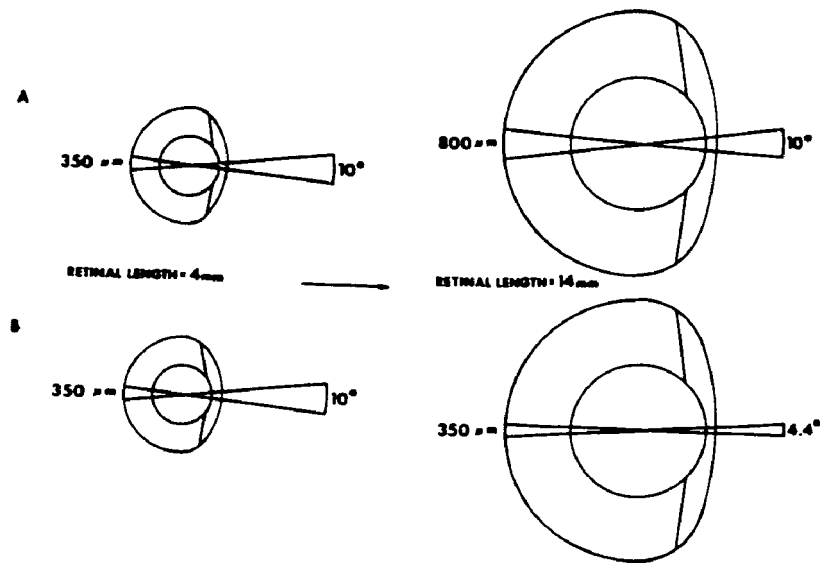


Fig. 30. Changes in receptive field size with retinal growth. These are schematic diagrams as in Fig. 29. The size of the receptive field of one cell in each eye is indicated by the rays projecting through the center of the lens; its magnitude is given in terms of retinal distance (μm) and visual angle (degrees).

retina, must increase. Although this implies the formation of new synaptic connections within the retina, such a mechanism would be conservative with respect to the brain, since activity in a certain number of fibers would always signal a stimulus of the same size regardless of age.

Effects on central synaptic connections. Special attention was paid in this study to the identification of ganglion cells. Ganglion cell addition during postembryonic growth is particularly interesting since their axons synapse in the brain. This implies that new synapses are being formed not only within the retina, but in the higher-order visual centers as well. Therefore, both the retina and the brain must carry out their normal activities associated with visual function while simultaneously adjusting to an ever increasing number of neurons and synaptic connections. Just how this integration of physiology and development is accomplished is completely unknown; it is a challenging problem worthy of further investigation.

SUMMARY

1. The adult goldfish retina grows. The growth involves both hypertrophy and hyperplasia; the total number of cells of all types increases.

2. Cell density is homogeneous throughout the goldfish retina. The cones, inner nuclear layer cells, and ganglion cells decrease in density during growth, but the

rod density remains constant, resulting in an increased proportion of rods in larger eyes.

3. Since the extent of the retinal field is constant, and new cones are added, the number of cones per visual degree increases during growth. This results in a decrease in the calculated minimum separable angle, implying that the visual acuity may increase.

4. The change in the retinal magnification factor implies that the size of the receptive field of individual cells must either increase in area on the retina or decrease in visual angle subtended. The former implies the formation of new synaptic connections by already fully differentiated cells.

5. The addition of ganglion cells implies that new functional connections are being made not only within the retina, but between the retina and the brain as well.

CHAPTER III

GROWTH OF THE ADULT GOLDFISH RETINA:

Source of the New Cells and Geometry of the Growth Process

INTRODUCTION

Chapter II presented direct evidence for cell addition during retinal growth in adult goldfish based on cell counts in animals of different sizes and ages, but the source of the new cells could not be determined from the normal material. To understand fully the growth process it is necessary to determine not only the place of origin of the new cells, but also their final destination and site of differentiation; these may not be the same.

Following an initial early embryonic period during which cells proliferate throughout the developing neural retina, mitotic activity becomes restricted to a narrow region at the extreme margin of the retina in all vertebrates which have been examined (fish: Müller, 1952, chick: Coulombre, 1955; mouse: Sidman, 1960; amphibian: Straznicky and Gaze, 1971). The separation of the retina into a central differentiated region and a marginal proliferative zone was first suggested by histological observations of mitotic figures (Müller, 1952; Coulombre, 1955; Lyall, 1957a;

Blaxter and Jones, 1967; Mann, 1969) and later confirmed with ^3H -thymidine radioautography (Sidman, 1960; Denham, 1967, Jacobson, 1968, 1976; Hollyfield, 1968, 1972; Straznicky and Gaze, 1971; Morris et al., 1976). The reason for compartmentation of functional and developmental activities into separate areas of the retina is not known; its significance may be of a universal nature since spatial restriction of the proliferating component is common in growing tissues (Weiss, 1949).

At its simplest, cell proliferation at the retinal margin would result in the appositional addition of concentric rings of new retinal cells; radioautographic evidence from larval amphibians has confirmed this simple interpretation (Straznicky and Gaze, 1971; Gaze and Watson, 1968; Jacobson, 1976). This does not preclude the possibility that in some species retinal growth might involve regions other than the periphery. Indeed, during postembryonic growth in certain teleost fish and amphibians the relative proportions of some retinal cells, the rods and/or inner nuclear layer cells, increase in central retinal regions which are devoid of mitotic activity. To explain this, several workers have suggested that another type of retinal cell, either undifferentiated or even differentiated, is transformed into a rod (Bernard, 1900; Lyall, 1957a,b; Blaxter and Jones, 1967), or that cells migrate into the central regions from the mitotically active periphery (Glücksmann, 1940; Hollyfield, 1968).

Although some of the cells produced in the marginal germinative zone may migrate into the central retina, not all of them do; some remain and differentiate in situ.

For example, cones and ganglion cells appear to be added exclusively at the retinal margins on the basis of morphological (Glücksmann, 1940; Lyall, 1957a) and radioautographical (Jacobson, 1968, 1976; Hollyfield, 1968; Straznicky and Gaze, 1971) evidence in fish and amphibians. In summary, retinal growth in teleost fish and larval amphibians may involve at least three different mechanisms:

- 1) appositional addition of concentric annuli of new retina, including all layers and cell types, and differential addition of some cells to central regions via
- 2) migration of undifferentiated precursors from the peripheral germinative zone or
- 3) transformation of one type of differentiated cell into another.

One other source of new retinal cells has been suggested, not in association with development, but with retinal regeneration. Following removal of or damage to the retina during early embryonic development the pigmented epithelial cells are capable of regenerating new retinal tissue (Coulombre and Coulombre, 1965). This capacity is lost early in development in avian and mammalian retinas, and after metamorphosis in anuran amphibians, but is retained in most adult urodeles (reviewed by Keefe, 1973a). Following section of the optic nerve and its accompanying blood vessels in adult newts (Triturus cristatus,

Notophthalmus viridescens, and Triturus cristatus carnifex) the retina degenerates. It is later reconstituted in part from proliferation of pigmented epithelial cells at the posterior pole (central region of the retina) and in part from cells produced by the ora serrata (terminalis) and pars ciliaris retinae at the anterior, or peripheral, margin (Gaze and Watson, 1968; Keefe, 1973a,b; Levine, 1975). These authors based their conclusions on radioautographic analyses using ^3H -thymidine to label the newly forming retinal cells. Unlike newts, the retina in teleost fish does not degenerate when the optic nerve is cut; instead the retinal ganglion cells already present regenerate a new optic nerve (Sperry, 1955; Murray and Grafstein, 1969). Regeneration of the neural retina in teleost fish has not been studied in detail, although it has been noted in the goldfish following retinal lesions (Attardi and Sperry, 1963; Schmidt, 1976). Although the pigmented retinal epithelium has never been implicated in postembryonic retinal growth it must be considered as a possible source of cells, particularly in central regions, since at least a latent capacity to form retinal cells exists.

The following longitudinal growth study employing ^3H -thymidine radioautography in adult goldfish was undertaken to determine the source of the new cells, the mechanism of addition of rods to the central retina, and the overall geometry of the retinal growth.

METHODS

Goldfish (Carassius auratus), Comets, 5-7 cm in overall length; were purchased from Ozard Fisheries, Stoutland, Missouri. They were kept in standard aerated aquaria at room temperatures (18-22°C). The tanks were exposed to natural light as well as to normal laboratory illumination. The fish were fed twice daily whenever possible, and always at least once a day, with dry commercial fish food (TetraMin Staple Fish Food) supplemented with live brine shrimp during the first 3 months and with live Daphnia during the remainder of the experiment.

Experimental groups. The fish were divided into five groups. The first group of 20 fish was injected intraocularly (IO) with ^3H -thymidine (specific activity, 50.8 $\mu\text{Ci}/\text{mmole}$, New England Nuclear, Boston, Massachusetts). A one microliter syringe (Hamilton, Reno, Nevada) with a 30-gauge needle was used to inject the isotope into the right eye of each fish: 0.25 μCi in 0.25 μl in the first injection, and 0.1 μCi in 0.1 μl in a second injection 24 hours later. Ninety-six hours after the first injection, 6 of the 20 fish were anesthetized by immersion in 0.05% tricaine methanesulfonate and their left eyes (uninjected) were removed (group IO-X). The eyes of the remaining 14 fish were left intact (group IO).

The next group of 15 fish (group IP) were injected intraperitoneally with four doses of ^3H -thymidine. The fish were weighed and injected with 10 $\mu\text{Ci}/\text{g.b.w.}$ (15-35 μCi),

followed by three more injections of 10 μ Ci separated by 12 hour intervals. The total dose was approximately 25 μ ci/g.b.w.

Another group of 15 fish (group TdR) was injected intraperitoneally with non-radioactive thymidine according to the same dosage schedule as the IP fish. The total amount of thymidine, labeled or unlabeled, injected into each IP and TdR fish was approximately 12 μ g/g.b.w.

The final control group of 15 fish (group NC) was injected intraperitoneally with 0.9% NaCl according to the same schedule as the IP and TdR fish. The volume of the first injection was 25 μ l, and 10 μ l was given in each of the three subsequent injections. The injections given to fish in the last three groups contained approximately the same total volume - 55 μ l. All injections were done between 8-10 AM and 8-10 PM, and all fish were injected during the same 48 hour period.

Histological and radioautographical procedures. The fish were weighed and their lengths measured; the diameters of their eyes were measured through the nasotemporal axis with a hand micrometer. The fish were then killed by decapitation. Their heads were fixed 'in Bouins' for 1 week; a hole was cut in the top of the skull and the cornea was punctured to allow the fixative to penetrate to the retina and brain. One or more fish from each group were killed at 12, 24, and 36 hours, 2, 4, 28, 84, 168, and 336 days following the first injection. The fish killed during the first 2 days had

received less than the full schedule of injections. All of the fish, including those killed and those allowed to continue growing were weighed and their lengths measured at 28, 84, 168, and 336 days.

Following fixation the tissue was rinsed overnight in 50% ethanol, dehydrated through a graded series of ethanol-butanol mixtures to 100% butanol, and embedded in paraffin (Paraplast, m.p. 61°C). Sections were cut at 5 μ m radially through the nasotemporal or dorsoventral axis of the eye, and mounted on albuminized slides.

The slides from the IO and IP fish were processed for radioautography according to the technique of Kopriwa and Leblond (1962). The sections were rehydrated, and the slides allowed to air-dry for 2-3 hours. Some of the slides were stained with gallocyanine - chrome alum for 4 hours at 45° C prior to drying. The pigment in the eye was oxidized in some of the slides with 1-5% potassium permanganate and bleached in 2% oxalic acid. The dry slides were dipped into Kodak NTB-2 nuclear emulsion diluted 1:1 or 2:1 with distilled water, dried, and stored at 4°C for 2-5 weeks in light-proof boxes containing a packet of Drierite. The radioautograms were developed in 1:1 Dektol (Eastman Kodak, Rochester, New York) for 3-4.5 minutes at 14-16°C, rinsed in distilled water, fixed in two changes of Kodak Fixer, 3-5 minutes each, and then rinsed in running tap water for at least 20 minutes. The slides which had not been stained previously were post-stained through the emulsion with

Ehrlich's hematoxylin. All slides were then counterstained with eosin, dehydrated, cleared in xylene, and coverslips were mounted with Permount. The slides from the TdR and NC fish were processed in the same way, omitting the radio-autographic steps.

Measurements of retinal size and cell number. Retinal cells were counted in median radial sections through the center of the eye as described in Chapter II. Rods, inner nuclear layer cells, and ganglion cells were counted using the line sampling technique of Trowell and Westgarth (1959), and cones were counted using a modified Abercrombie method (Konigsmark, 1970). The maximum retinal length was measured and the retinal area calculated as described in Chapter II.

The circumference (C) at the ora terminalis was calculated according to the following formula:

$$C = 2\pi R \cos(2.5^\circ), \text{ where } R = 3.31\ell$$

ℓ = retinal length

The factor " $\cos(2.5^\circ)$ " reflects the fact that the retina is not a hemisphere, but subtends approximately 185° ; the cosine of 2.5° is 0.9991, and so it contributes insignificantly to the actual calculation of C. It is included in the above formula only for geometrical correctness. This formula was also used to calculate the circumference of circles parallel to the ora at other specific locations in the retina.

All retinal dimensions were corrected for an histological shrinkage of 30%, determined as described in Chapter II.

RESULTS

Intergroup comparisons of growth. Individual growth rates were remarkably variable (see the Appendix for a discussion of this point), but no differences were found among the average growth rates for the five groups of fish. This is illustrated in Fig. 31. The mean body lengths $\pm$ 2 S.E.M. for each group of fish at each time point are given. All confidence intervals overlapped extensively leading up to the conclusion that growth was not affected by the injections, or by the enucleations in the IO-X fish. The increased variability in the body lengths at 336 days was a result of the differences in individual growth rates. The average length at the start of the experiment was 5.6 cm, and at the conclusion (336 days later) it was 10.1 cm. The average eye diameter increased from 3.9 to 5.6 mm.

Interpretation of the radioautograms. The rationale behind the radioautographic approach is as follows (also see Fig. 3): at short survival times following injection of ^3H -thymidine the cells labeled will be those which are actively dividing. At longer intervals following injection, the progeny of the cells which ceased dividing to differentiate will be labeled. Those which were labeled during their terminal mitotic phase will be heavily labeled at later times; those which continued to divide for several more cycles will be less and less heavily labeled until

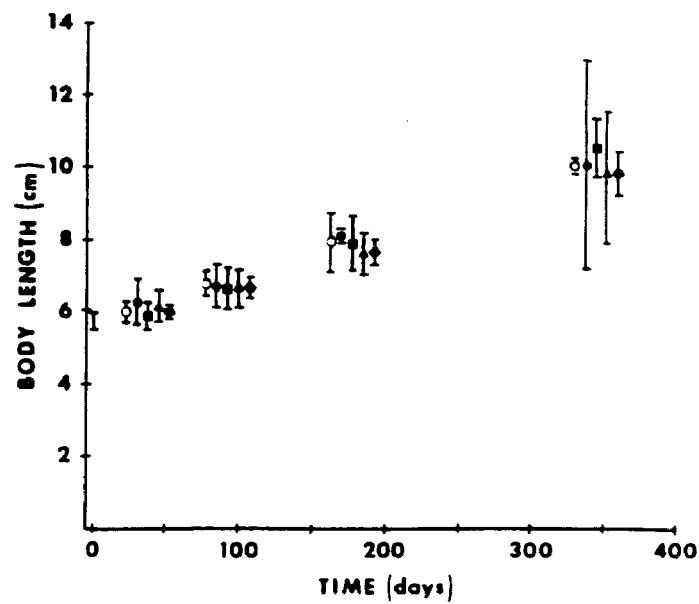


Fig. 31. Growth of the fish.
The mean body length $\pm$ 2 S.E.M. is plotted as a function of time post-injection. IO fish, open circles; IO-X fish, filled circles; IP fish, squares; TdR fish, triangles; NC fish, diamonds.

finally the label is diluted to background levels and is no longer detectable.

For the purposes of the following descriptions, a cell was considered heavily labeled if the grain density over the nucleus was so high that individual grains could not be resolved. The minimum number of silver grains overlying a nucleus which was considered sufficient for classification as a labeled cell was 4-5 grains.

Radioautograms at short survival times (12 hours - 4 days). Following intraocular or intraperitoneal injections of ^3H -thymidine most labeled retinal cells were found at the ora terminalis as shown in the photomicrographs in Fig. 32. Twelve hours following a single injection, usually only one or two cells were labeled at each retinal edge in radial sections; the number of labeled cells per section in general increased both with increasing time and continuing injections. This is shown quantitatively in Fig. 33 in which the mean number of labeled cells at each retinal margin ± 2 S.E.M. is plotted versus time post-injection. The number of labeled cells was counted in 14-17 sets of 3 radial sections, 10 μm apart, at intervals of 200 μm across the entire eye. Each point is the average at each edge of one retina. In the IP fish, both eyes were sectioned, one through the nasotemporal axis and the other through the dorsoventral axis. Only the right (injected) eyes from the IO fish were used. In a few fish the injections were not as successful, and only a light level of labeling was found; these were not plotted

Fig. 32. Radioautograms at short survival times.

A, a labeled cell at the ora terminalis in an IO fish killed at 12 hours following a single injection of ^3H -thymidine.

B, labeled cells at the ora terminalis in an IP fish killed 4 days after the beginning of a series of injections of ^3H -thymidine.

Hematoxylin and eosin, 5 μm . Calibration bar: 50 μm .

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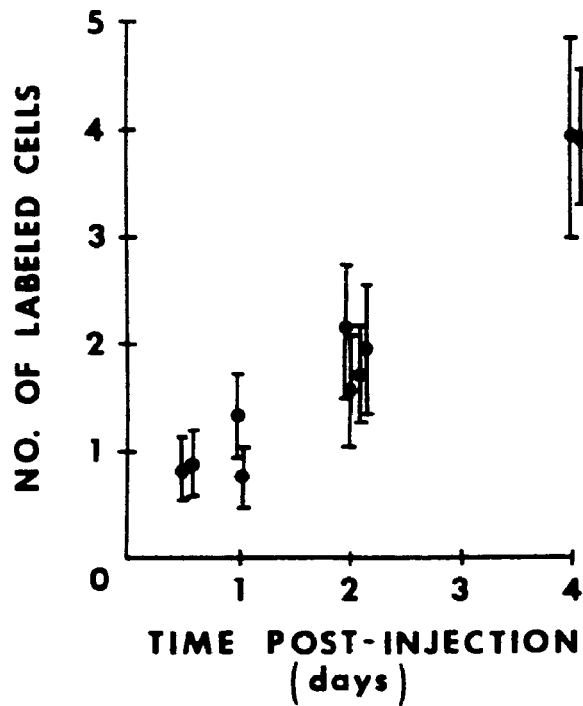


Fig. 33. Increase in the number of labeled cells with time. The mean number of labeled cells per section at each retinal edge ± 2 S.E.M. is plotted as a function of time post-injection. Each point is one retina. The retinas at 0.5 and 4 days are from the right eyes of IO fish, and those at 1 and 2 days are from IP fish.

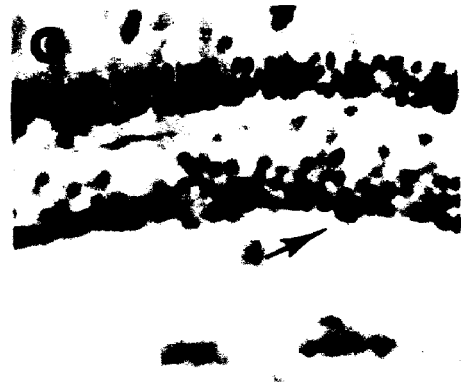
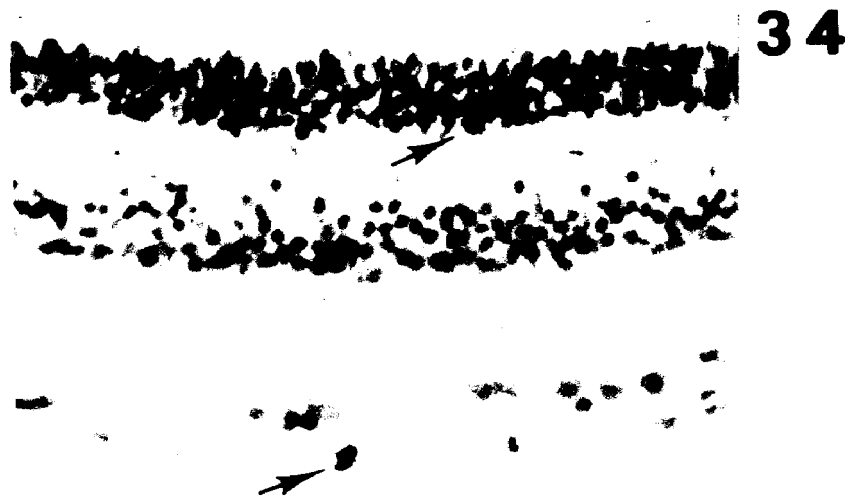
in Fig. 33. A large number of labeled cells were seen in the vicinity of the ora terminalis in one IP fish killed at 12 hours following a single injection. It was suspected that these cells were vascular elements rather than retinal cells based on their close association with neighboring blood vessels; this fish was also not included in Fig. 33.

Occasional heavily labeled cells were also found scattered throughout the retina, particularly in the IO fish. They were most prevalent in the optic fiber layer (Fig. 34A), particularly near the optic disk (Fig. 34B), but were also found in other retinal layers (Fig. 34A,C). The morphology of their nuclei could not be distinguished due to the high density of overlying silver grains, but they were probably glial elements. Those in the optic fiber layer were undoubtedly macroglia or vascular endothelial cells, and the others may have been phagocytic microglia, escaped blood macrophages. Cells with the nuclear morphology characteristic of macrophages were sometimes seen scattered throughout the normal retina (Fig. 35). Another reason for believing that these scattered labeled cells were glia was that they did not make any permanent contribution to the retinal cell population, since they could not be found at the later time intervals.

In the IP, but not in the IO, radioautograms, labeled cells were found throughout the pigmented retinal epithelium (PRE) particularly in those sections which were bleached (Fig. 36). In the unbleached sections, they were

Fig. 34. Scattered labeled cells at short survival times.
A, labeled cells in the optic fiber and outer nuclear layer (arrows),
B, labeled cells near the optic disc (arrows),
C, a labeled cell in the inner nuclear layer (arrow).
Hematoxylin and eosin, 5 μm . Calibration bar: 50 μm .

Fig. 35. A macrophage cell in the retina.
The arrow indicates a cell at the border of the outer nuclear and outer plexiform layers with the nuclear morphology typical of a macrophage. Foot-Masson, 5 μm .
Calibration bar: 25 μm .



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difficult to detect since the radioautographic silver grains were similar in appearance to the dark pigment granules in these cells. Vascular elements associated with the choroid were also labeled in the bleached, IP radioautograms (Fig. 36).

Radioautograms at longer survival times - (28 - 336 days). Labeled cells were not found anywhere in the central retina or in the PRE after 28 days. The only cells still labeled were in the peripheral retina, displaced progressively from the ora terminalis with increasing survival times. Fig. 37 indicates that the labeled cells in the ganglion cell and inner nuclear layers and the labeled cone nuclei were confined to a circumscribed area of retina in a band oriented perpendicularly to the layers of the retina. The most heavily labeled cells were at the edge of the band furthest from the ora terminalis, forming a sharp boundary between labeled and unlabeled retina. Less heavily labeled cells extended for a short distance toward the periphery, but the edge of the band on this side was not as distinct. Cells both peripheral and central to this region were unlabeled.

In the retina as a whole, the labeled cells formed a complete annulus surrounding the retina near its peripheral edge. Fig. 38 is a radioautogram from a section, cut near the edge of the eye, which passed tangentially through the strip of labeled cells. The collection of

Fig. 36. Labeled cells in the pigmented retinal epithelium.
Labeled pigment epithelial cells (PRE) in an IP
fish killed at 2 days following the beginning of the
injection series.

Fig. 38. Continuity of the labeled annulus.
A section cut tangentially to the band of labeled cells in
an IP fish killed at 336 days.
Calibration bar: 50 μ m

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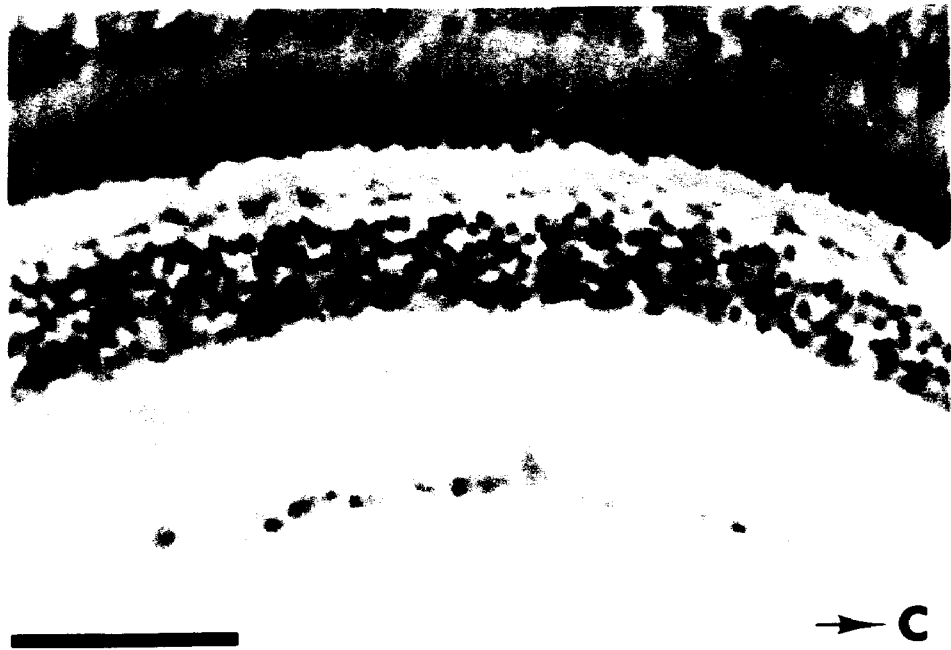
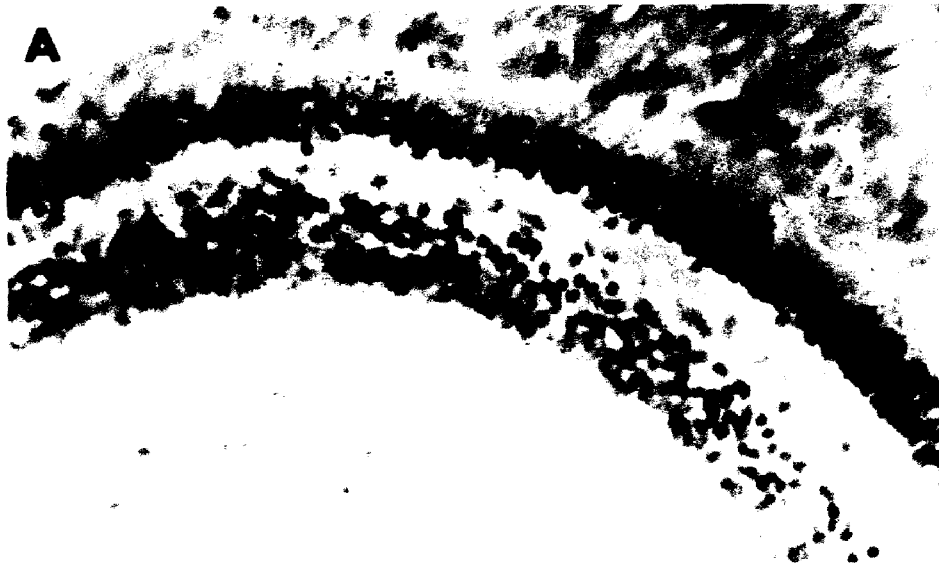


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Fig. 37. Radioautograms at long survival times.
A, labeled cells in the retina of an IOX fish killed at
168 days,
B, an IP fish killed at 336 days.
The arrow at the bottom points toward the central retina (C).
Hematoxylin and eosin, 5 μm . Calibration bar: 50 μm .

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labeled cells in this photomicrograph helps to illustrate the continuity of the labeled annulus.

In contrast to the rest of the retinal cells, the labeled rod nuclei in the outer nuclear layer were not confined to this narrow strip of retina. Labeled rods spread inward toward the center for some distance beyond the most central, heavily labeled cells in the inner nuclear layer (Fig. 37). At no time, however, were labeled rod nuclei found in the center of the retina. The labeled rods were distributed very diffusely, and the distance which they spread centrally was quite variable from section to section within a given eye. In one fish killed at 168 days whose retina was particularly well labeled, the mean distance from the central edge of the labeled band to the most central labeled rod was 500 μm in the nasal half and 400 μm in the temporal half. The maximum distances were 700 and 950 μm , respectively.

The results described above are shown schematically in Fig. 39. On the left are representations of radioautograms of radial retinal sections at 1, 84, and 336 days following the beginning of label injection. The filled circles represent labeled cells. On the right, the regions containing labeled cells are enlarged; again labeled cells are represented by circles in which the degree of shading reflects the intensity of the label. Labeled cells in all three retinal layers are shown--ganglion cells (G), inner nuclear layer cells (I), and rods (R) and cones (C) in the

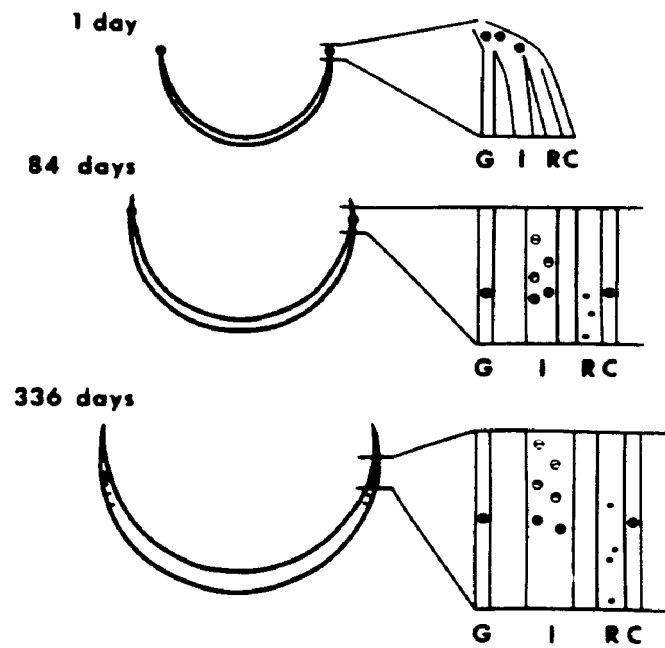


Fig. 39. Schematic of radioautographic results. Radial sections through the retina are represented diagrammatically on the right for 1, 84, and 336 days post-injection. The regions containing labeled cells are enlarged at the left. The symbols represent labeled cells: the degree of shading reflects the intensity of labeling. Ganglion cell layer, G; inner nuclear layer, I; layer of rod nuclei, R; layer of cone nuclei, C.

outer nuclear layer. Whereas the cells labeled in all other layers were confined to a restricted region of retina on each side, labeled rods spread inward toward, but not all the way to, the center of the retina.

Cytological characteristics of the germinal zone.

The radioautograms indicated that cell proliferation leading to the formation of new retinal neurons occurred only at the extreme periphery of the goldfish retina. This region can therefore be called a germinal zone. In normal histological preparations, this region showed several characteristics consistent with this interpretation of its activities, cell production and differentiation. Most striking and obvious were the changes in cone morphology. The cones at the peripheral edge of the retina were small; at the ora terminalis their distal processes were completely lacking (Fig. 40A). There was a gradient of increasing size and development of the cone distal processes extending some 100-200 μm inward to a point where the cones were fully differentiated and indistinguishable from those in the central retina (Fig. 40C).

At the ora terminalis the three nuclear layers merged together, and the plexiform layers were obliterated, and the nuclear morphology of the cells became nonspecific. These undifferentiated nuclei were the ones which were labeled in the radioautograms at 0-4 days. Farther, peripherally, the retinal cells thinned to a single layer and merged with the cuboidal cells of the iris epithelium

- Fig. 40. The germinal zone.
- A, the retinal margin in a 7 cm fish; ora terminalis, OT.
 - B, the distal processes of the cones at the retinal margin (arrow),
 - C, the same retina as in B, but in a more central region,
 - D, the retinal margin in an 11.5 cm fish.
- A-C, Foot-Masson, 5 μm .
 D, Hematoxylin and eosin, 5 μm . Calibration bar: in A and D, 50 μm ; in B and C, 25 μm .

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(Fig. 40A), which were also sometimes labeled in the short-term radioautograms.

In the peripheral germinal zone of the retina where the cones were developing, the inner nuclear layer was thicker and the density of its cells was increased by 25-30%. Categorization of these cells based on differences in nuclear morphology which was possible in the more central regions (see Chapter II) became impossible here. Although the horizontal cells were still displaced distally from the rest, their nuclei were squatter and less elongated.

In contrast to the inner nuclear layer, the outer nuclear layer containing the rod nuclei became thinner at the margin and the cell density decreased by 20-30%. Cells in the ganglion cell layer at the extreme margin were sometimes more dense, on the average by about 15%, but this varied from section to section. It was sometimes difficult to identify cells at the margin which were clearly in the ganglion cell layer because of the nondescript nature of their nuclei.

All of the above characteristics of the germinal zone were found not only in the small fish, but in large fish as well (Fig. 40D).

Geometry of the retinal growth. The amount of new retina added during growth was determined by measuring the size of the retinal region peripheral to the band of labeled cells in the inner nuclear layer. The distance from the central edge of the band (the sharp boundary

formed by the most heavily labeled cells) to the point where the inner plexiform layer ended at the ora terminalis was measured in radioautograms from 10 retinas. Three retinas were from fish killed at 84 days, one sectioned through the nasotemporal axis and two through the dorsoventral axis; three retinas were from fish at 168 days, one sectioned through the nasotemporal axis and two through the dorsoventral axis; four retinas were from fish at 336 days, two sectioned through each axis. This distance was measured at each retinal edge in 8-11 sections spaced at regular intervals of 400 μm through the central 90-110° of each eye, using an ocular micrometer and a magnification of 450 X. Sections farther toward the edge of the eye could not be used because they began to cut tangentially through the retinal margin, distorting the distances being measured. These distances were averaged separately for the two retinal edges, and the means, defined as the retinal increments, for each eye are plotted in Fig. 41A, B, and C for the fish killed at 84, 168, and 336 days, respectively. A different symbol is used for each retina within a graph, and a line is drawn connecting the means at each retinal pole. Two mean retinal increments were obtained from each retina measured, one from the nasal pole and the other from the temporal pole, or one from the dorsal and the other from the ventral pole, depending on the axis of sectioning. Note that the nasal values are plotted twice.

The two 336 day fish whose retinas were sectioned in

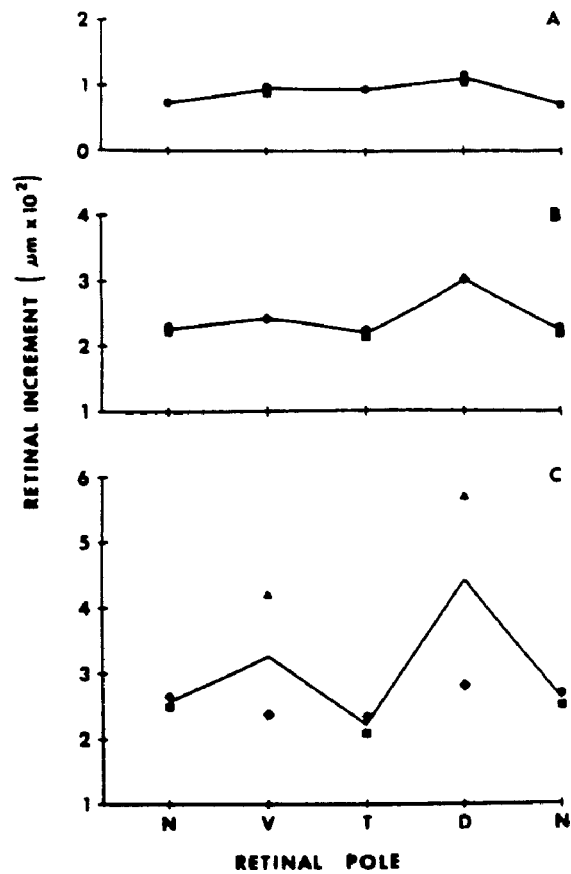


Fig. 41. The amount of new retina added during growth of the eye. The mean retinal increment (see text) at each margin at 84 days (A), 168 days (B), and 336 days (C). A different symbol is used for each retina within a graph. The values for the nasal increments are plotted twice. Nasal, N; ventral, V; temporal, T; dorsal, D. Lines are drawn connecting the points, when only one retina, or the means when two retinas were used, at each retinal pole.

the dorsoventral axis (Fig. 41C) had very different growth rates, and the retinal increments differed correspondingly. The one with the larger retinal increment (triangles) had a retinal length of 9.0 mm, and the other (diamonds) had a retina only 7.9 mm in length.

Two conclusions can be drawn from Fig. 41. First, the distance between the band of labeled cells and the ora terminalis increased with time following the injection. This is consistent with the interpretation of peripheral cell addition. An estimate of the amount of new retina added peripherally was obtained for each fish from the average of the two retinal increments (nasal and temporal or dorsal and ventral). This value was taken as the height of a trapezoid whose two parallel sides were the circumference of the retina at the ora terminalis and at the central edge of the labeled annulus, respectively. The curvature of the retinal surface was ignored in this estimation. The average area of new retina at each post-injection interval is given in Table II, column 2: the amount of retina added in 84 days was 1.08 mm^2 ; by 168 days, 3.35 mm^2 of new retina had been added and by 336 days, 4.98 mm^2 . The increase in the size of the entire retina was also calculated from the average retinal lengths at each time point (Table II, column 3) and is given in column 4. The size of the "old" retina originally present, which was central to the band of labeled cells at later times, was determined by subtracting the area of new retina from the

TABLE II

GEOMETRY OF THE RETINAL GROWTH

Time post- injection (days)	Area of new retina (mm ²)	Ave. Retinal length (mm)	Area of entire retina (mm ²)	Area of "old" retina (mm ²)	% increase in area of "old" retina
0	0	5.2	17.8	17.8	-
84	1.08	5.8	22.2	21.1	18.7
168	3.35	7.3	35.2	31.9	79.2
336	4.98	8.0	42.2	37.2	109.0

area of the entire retina. These values are given in column 5. The size of the "old" retina increased with time: 18.7% after 84 days, 79.2% after 168 days, and 109.0% after 336 days. The contribution of the new retina to the increase in the overall size of the retina after 336 days of growth was 20%, and the retinal expansion accounted for the remaining 80% of the growth.

A second conclusion suggested by Fig. 41 is that new retina was not added symmetrically around the circumference. In all cases, the amount of dorsal retina added was greater. This is shown more quantitatively in Fig. 42. The ratio of the mean retinal increment at the dorsal pole to the retinal increment at the ventral pole in each eye is given by the squares in Fig. 42, and the ratio of the nasal to the temporal by the circles. In general, more retina was added dorsally than ventrally, and more nasally than temporally, at least at the longer time intervals. Statistical evaluation with Student's t test showed that in two cases (*), one nasotemporal and the other dorsoventral, the mean retinal increments at the two edges were highly significantly different from one another ($p < 0.001$).

The asymmetry in retinal growth resulted in a shift of the retinal axis, defined as the line connecting the center of the lens to the center of the nearly hemispherical retina (Easter, Johns, and Baumann, 1977). The magnitude of this shift was calculated for the two retinas in which the asymmetry of growth was significant. For the one sectioned

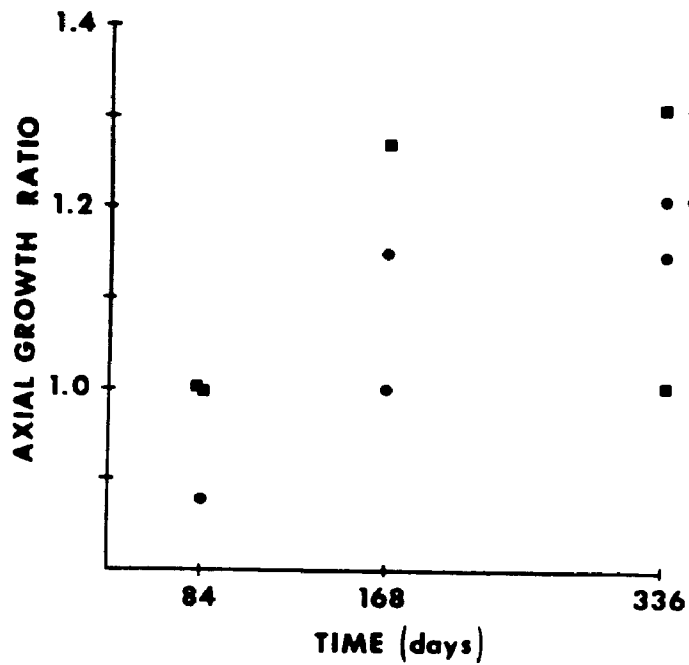


Fig. 42. Asymmetry in retinal cell addition. The axial growth ratio (see text) as a function of time post-injection. Each point is one retina. Ratio of dorsal to ventral increments, squares; ratio of nasal to temporal increments, filled circles. Two of the ratios (*) differed highly significantly ($p < 0.001$) from 1.0 ($t = 4.47$, $df = 17$ for the dorsal-to-ventral ratio; $t = 3.88$, $df = 22$ for the nasal-to-temporal ratio).

through the dorsoventral axis, the difference between dorsal and ventral retinal increments was 135 μm ; thus the retinal center had shifted 67.5 μm dorsally, which corresponded in this eye to 1.4° . For the other eye, sectioned through the nasotemporal axis, the difference in retinal increments was 42.9 μm . The retinal center had therefore moved 21.5 μm or 0.9° toward the nasal pole. These values represent the maximum amount of shift observed over 336 days.

Retinal growth by cell addition. The total number of retinal cells as a function of retinal length is shown in Fig. 43. The points cluster, indicating that the experimental procedures, injections of ^3H -thymidine or non-radioactive thymidine or enucleation, had no effect on retinal growth by cell addition.

In Fig. 44 these same data are plotted as a function of time post-injection. The points now scatter widely due to the variability in individual growth rates. An estimate of the rate of retinal cell addition was obtained by dividing the mean cell numbers at each interval by the time post-injection; it ranged from 2,700 to 13,600 cells per day.

A better estimate of the rate of retinal cell addition resulted from a comparison of cell numbers in the right and left eyes of the IO-X fish. The left eyes of these fish were removed at the beginning of the experiment and the right eyes were removed when the fish were killed 84, 168, or 336 days later. Table III gives the number of

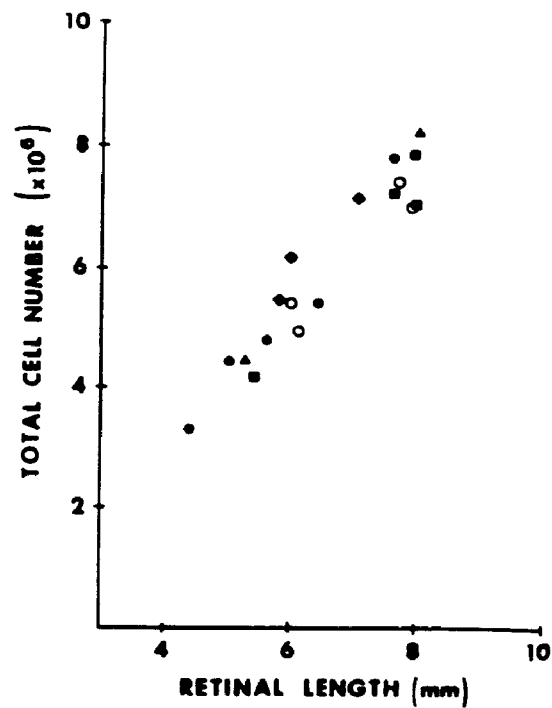


Fig. 43. The relation between cell number and retinal length. The total number of cells in the retina is plotted as a function of the retinal length. Each point represents one retina. The symbols are as in Fig. 32.

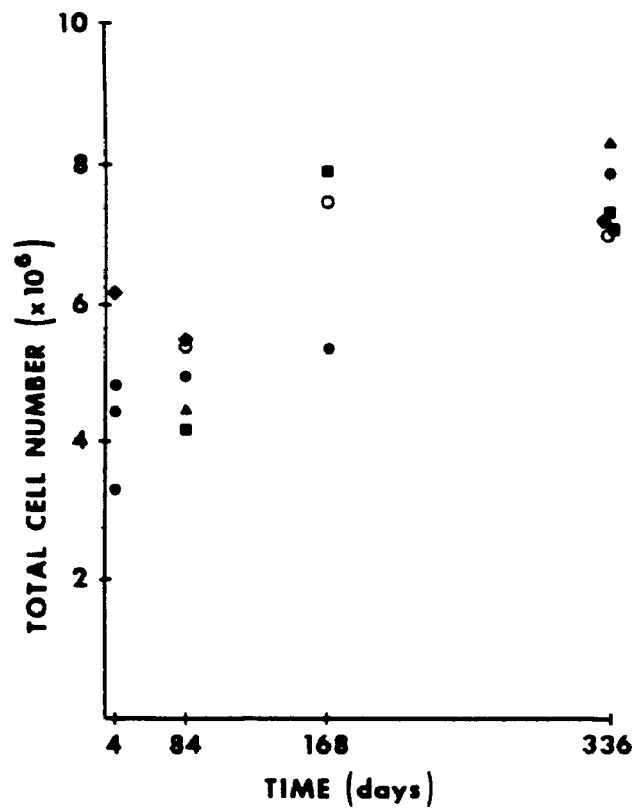


Fig. 44. The relation between cell number and time post-injection. These are the same data as in Fig. 43, replotted as a function of the time (days) post-injection at which the fish was killed.

TABLE III

RATE OF CELL ADDITION

Fish	Days	# Rods ($\times 10^6$)	Rate ($\times 10^3$ /day)	# INL ($\times 10^6$)	Rate ($\times 10^3$ /day)	# Cones ($\times 10^5$)	Rate ($\times 10^3$ /day)	# GL ($\times 10^5$)	Rate ($\times 10^3$ /day)
1A LE	4	1.57		1.38		2.74		0.60	
1A RE	84	2.45	10.9	1.94	7.0	4.63	2.36	0.85	0.32
1D LE	4	2.10		1.90		3.36		0.87	
1D RE	168	2.68	3.50	2.01	0.66	6.03	1.63	0.995	0.07
1F LE	4	2.14		2.10		4.59		1.45	
1F RE	336	4.60	7.38	2.38	0.83	6.27	0.51	1.98	0.34
Mean rates ($\times 10^3$ /day)			7.30		2.80		1.50		0.25

rods, inner nuclear layer cells, cones, and ganglion cells in the right and left eyes of three of these fish. The rate of cell addition was determined by dividing the difference in cell number between right and left eyes by the number of days of growth. The mean rates, given at the bottom of Table III are 7,300 rods per day, 2,800 inner nuclear layer cells per day, 1,500 cones per day, and 250 ganglion cells per day, for a grand total of 11,800 cells per day. This value is within the range obtained in the more crude estimate given above.

DISCUSSION

Possible problems associated with the radioautographic technique. The technique of ^3H thymidine radioautography has proven invaluable in studies of cell proliferation (reviewed by Wimber, 1963; Sidman, 1970). Exogenous thymidine is incorporated into the nuclei of cells synthesizing DNA during the S-phase of the mitotic cycle (Walker and Leblond, 1958). The cells which become labeled nearly always go on to divide mitotically, although some exceptions have been found. For example, in amputated, denervated adult amphibian limbs (Ambystoma mexicana) DNA synthesis is stimulated without accompanying cell division (Tassava et al., 1974; Mescher and Tassava, 1975). In most instances, however, labeling with ^3H -thymidine is associated with mitotic activity (Sidman, 1970). The best demonstration of this association is the appearance of

labeled mitotic figures at a specific interval following injection, determined by the kinetics of the cell cycle (Wimber, 1963). In the adult goldfish retina mitotic figures were not seen using the normal histological procedures described. This is not considered a serious problem however, since mitotic figures have been described at the ora terminalis in younger, presumably more rapidly growing teleost fish (Müller, 1952; Lyall, 1957a; Blaxter and Jones, 1967. Mitotic figures are notoriously more difficult to find than the number of labeled cells might indicate since the length of time devoted to the actual mitotic process is such a small fraction of the entire cell cycle (Hughes et al., 1958). Moreover, the cell cycle lengthens with age while the portion devoted to mitosis remains constant, so in older animals it is even more difficult to catch a dividing cell (Graham and Morgan, 1966; Leshner et al., 1961; Schultze et al., 1974).

There are several possible problems which may influence the results or the interpretation of cell labeling with ^3H -thymidine. Dobson and Cooper (1971) have shown that substantial amounts of label from catabolized exogenous thymidine can be incorporated into polysaccharide molecules by mouse liver cells in vitro; Silver (1976) has recently described an in vivo situation which may result from a similar process. During early mammalian ocular morphogenesis, large numbers of cells in the ventral retina die after dividing mitotically (Glücksman, 1965; Silver and

Hughes, 1973). Silver (1976) has shown that if these cells are labeled with ^3H -thymidine, a non-specific but relatively high level of label remains in the ventral retina after the necrotic phase. Presumably this is due to recycling of the tritium through metabolic breakdown and reincorporation. The best precaution against false positives due to metabolic incorporation is a careful attention to the distribution of the silver grains; they should be restricted to cell nuclei. The resolution of light microscopic radioautography is sufficient to allow this distinction to be made, because the beta particles emitted by tritium are energetically so weak that 90% of them are absorbed within $1\text{ }\mu\text{m}$ (Fitzgerald et al., 1951). Another possible source of labeling not associated with DNA synthesis and mitotic activity is restricted to the nucleus and involves DNA repair (Pelc, 1963); generally this is of such an insignificant level that it is undetectable radioautographically.

Both excess thymidine and high levels of radioactivity can have profound effects on cell division. Excess thymidine, in concentrations $> 0.1\text{ mM}$, applied to cells dividing in culture, will arrest them in S-phase (Thomas and Lingwood, 1975). Lewis (1968b) noted that heavy doses of ^3H -thymidine (10-50 μCi injected intraventricularly in adult rats) resulted in many abnormal nuclei around the injection site. Although the possibility of effects due to mechanical trauma were not entirely ruled out, the damage seemed to be associated with mitotic activity since the number of abnormal

nuclei peaked at 8 hours and again at 30 hours, an interval close to the cycle time of these cells. Lewis attributed these effects to radiological damage. In the present study, a high specific activity of ^3H -thymidine was used to minimize both the total volume of the injection and the absolute amount of thymidine injected, while still obtaining a high level of tritium labeling. The TdR and NC fish served as controls for radiotoxicity and the effects of excess thymidine. No difference was found in either the overall growth rate or the rate of retinal cell addition between these fish and the experimental groups injected with ^3H -thymidine. I am therefore confident that the radioautograms accurately reflect the normal growth process.

Multiple injections of ^3H -thymidine were given in order to maximize the number of cells labeled. The pulse time following single intraperitoneal injections, the length of time in which a significant level of the precursor circulates before being cleared from the blood, is on the order of one half hour in mammals (Wimber, 1963), but it has only rarely been measured in fish or amphibians. Hay and Fischman (1961) measured a pulse time of about 3 hours after a small injection (5 μCi) in adult newts; Rafferty (1967) found that 20% of the label was present in the serum at 24 hours following injection in frogs; Schwassmann (personal communication) found a significant level of label remaining in the plasma 4-12 hours after an intraperitoneal injection of 3-15 $\mu\text{Ci/g.b.w.}$ in a teleost, opaleyes

(Girella nigricans). Clearly, the endogenous precursor is available for a longer period of time in these poikilothermic vertebrates than in mammals. The injection interval of 12 hours used for the IP fish was judged to be sufficiently short that ^3H -thymidine was probably almost continuously available to the neural tissues. There is no information on the length of time thymidine remains in the vitreous and is available to the retina following an intraocular injection, but it is undoubtedly longer than the systemic pulse. An injection interval of 24 hours was arbitrarily chosen for the IO animals.

Cell labeling not associated with retinal cell addition. Cells were labeled at short times following ^3H -thymidine injection in addition to those at the ora teminalis. Some of these were scattered throughout the retina in both the IO and the IP fish. They were identified as glial or vascular elements on the basis of their retinal position and lack of persistence in the radioautograms prepared after longer survival times. Similar labeled cells found in larval Xenopus (Jacobson 1976) and newly-hatched chick (Morris et al., 1976) retinas were also identified as glial and vascular cells.

Another class of labeled cells were found in the PRE, but only in the IP fish. These cells were labeled all across the eye in radioautograms prepared at 0-4 days post-injection. Since pigment epithelial cells are capable of regenerating neural retina in adult newts (Keefe, 1973a,b)

it was thought that these cells might be responsible for the increase in the number of rods in central retinal regions. This proved incompatible with other observations, however; in the radioautograms from longer survival times the label in these cells had been diluted by further cell divisions with no evidence of cell migration proximally into the neural retina. The pigmented retinal epithelium can therefore be categorized as a population of cells which are continuously turning-over.

The lack of labeling in the pigmented retinal epithelium in the IO fish implies that a diffusion barrier exists, preventing the thymidine which was injected into the vitreous from penetrating to the extra-retinal layers. A possible candidate for such a barrier is the external limiting membrane (ELM), formed by glial and photoreceptor cell processes which are tightly coupled together at this level (Cohen, 1963). Despite the difference in labeling patterns between the IO and IP fish at short survival times, at longer intervals, radioautograms from the two groups were indistinguishable. This provides further evidence that the pigment epithelial cells make no cellular contribution to the neural retina during normal growth.

The germinal zone. The restriction of retinal cell labeling to the extreme margin of the retina at the ora terminalis confirms all previous studies on postembryonic retinal cell proliferation. In addition to the evidence from radioautography, several morphological characteristics

of this region are consistent with its designation as a germinal zone (Glücksmann, 1940). The cells at the ora terminalis have an undifferentiated morphology in adult goldfish as they do in other species such as adult amphibians (L. Stone, 1959). Müller (1952) described, and this study confirmed, a gradient of differentiation of the cone distal processes beginning with short, bud-like projections from the ELM at the retinal edge and progressing to complete differentiation a short distance centrally.

Certain characteristics of the cone mosaic at the peripheral edge have also been attributed to a lack of differentiation. In several species of teleost fish, the cones at the periphery are arranged in parallel rows, either of double cones or of double and single cones in alternating rows, oriented perpendicularly to the margin. Farther centrally the mosaic is transformed into the square pattern typical of teleost fish in which four pairs of double cones form a square with or without single cones at the center and/or corners (Müller, 1952; Lyall, 1957b; Borwein and Hollenberg, 1973). The row pattern is considered the less differentiated (Engström, 1963) since in some species, for example the pike-perch (Lucioperca lucioperca), only the row pattern is present in the larvae (Baburina, 1961) whereas the adult pattern is the square-type (Ahlbert, 1968). The conclusion from both normal morphology and radioautography is that the germinal zone in the adult teleost retina is restricted to the margin.

The hypertrophic component of retinal growth. In addition to providing evidence for a germinal zone in the adult goldfish, the radioautograms were useful for quantifying the hypertrophic component of the growth. The annulus of labeled cells, particularly those in the inner nuclear layer, provided a marker for the original position of the ora terminalis at the time of the injection. The distribution of label over the cells, with those most heavily labeled at the central edge, and the contiguity of labeled cells in the inner nuclear layer, ganglion cell layer, and layer of cone nuclei, are both consistent with the interpretation that these cells were born at the ora and differentiated in situ. Unlabeled cells central to them were part of the original retina, whereas the unlabeled cells more peripherally were added later.

The labeled annulus was used to estimate the size of the "old" retina after various intervals; these were compared with the size at the beginning of the experiment to yield an estimate of the amount of hypertrophy. Of course, each retina could be measured at only one time point, so conclusions were of necessity based on the assumption that all of the retinas were approximately the same size at the beginning. Since the fish were approximately the same length, their retinas were at least similar in size (see Fig. 8, Chapter II). The area of the "old" retina increased by more than 100% over 336 days. This is consistent with the decrease in cell density of the cones, ganglion cells,

and inner nuclear layer cells during growth (see Chapter II). These results imply that the retina does indeed "stretch" as Weiss (1949), Coulombre (1955), and Ali (1964) suggested, but could not prove, since they had no way of knowing which parts of the retina were "old" and which new.

A small fraction of the increase in area of the "old" retina was due to a process other than active expansion. Given that new retina is added concentrically at the margins, and that the retina maintains its hemispherical shape during growth (Easter, Johns, and Baumann, 1977), some increase in area would result from the requisite flattening of the "old" retina. Imagine trying to modify the peel of half of an orange to make it look like half of a grapefruit. The orange peel would have to be cut radially, wedge shaped pieces inserted, and then a strip of additional peel added around the circular, and now longer, cut edge. Those wedges represent an increase in the area of the "old" orange. Analogously, the magnitude of the increase in the area required to flatten the "old" retina, given the amount of new retina added over 336 days, was only 5%. Since the actual increase was 95% more than this, the retina must grow by stretching, as does a balloon when filled with air.

The above discussion applied only to the ganglion cells, cones, and inner nuclear layer cells. Both the radio-autograms and the cell counts (Chapter II) showed that the rods clearly did not follow the same pattern as the other cells. The rods were the only cells whose density remained

constant with growth, and they were the only cells with labeled nuclei not confined to the narrow zone or annulus of other labeled cells. Following is a discussion of several hypotheses which might explain the apparent addition of rods to the central retina, and an evaluation of their relative merits in light of the radioautographic results.

Central addition of rods - an evaluation of the hypotheses. There are four major ways in which rods might be added to the central retina. These will be discussed in turn, and evaluated in terms of their predictive value for the radioautographic results.

1. Mitotic division throughout the retina.

The easiest way for the population of rods to increase would be by in situ mitotic division. This hypothesis can be eliminated immediately based on all previous work and on the results of this study. Fig. 45A is a schematic diagram showing the appearance of radioautograms at short and long survival times as predicted by the in situ mitosis hypothesis. For simplicity, the labeled rods are indicated by the dots outside of the semi-circular line and all of the other retinal cells are on the inside of the line. Although the radioautograms at short survival times looked similar to the diagram on the left -- scattered labeled cells were found in both the PRE and retina -- the radioautograms at longer survival times did not look like the diagram on the right -- the scattered labeled cells did not persist. We can therefore eliminate this hypothesis.

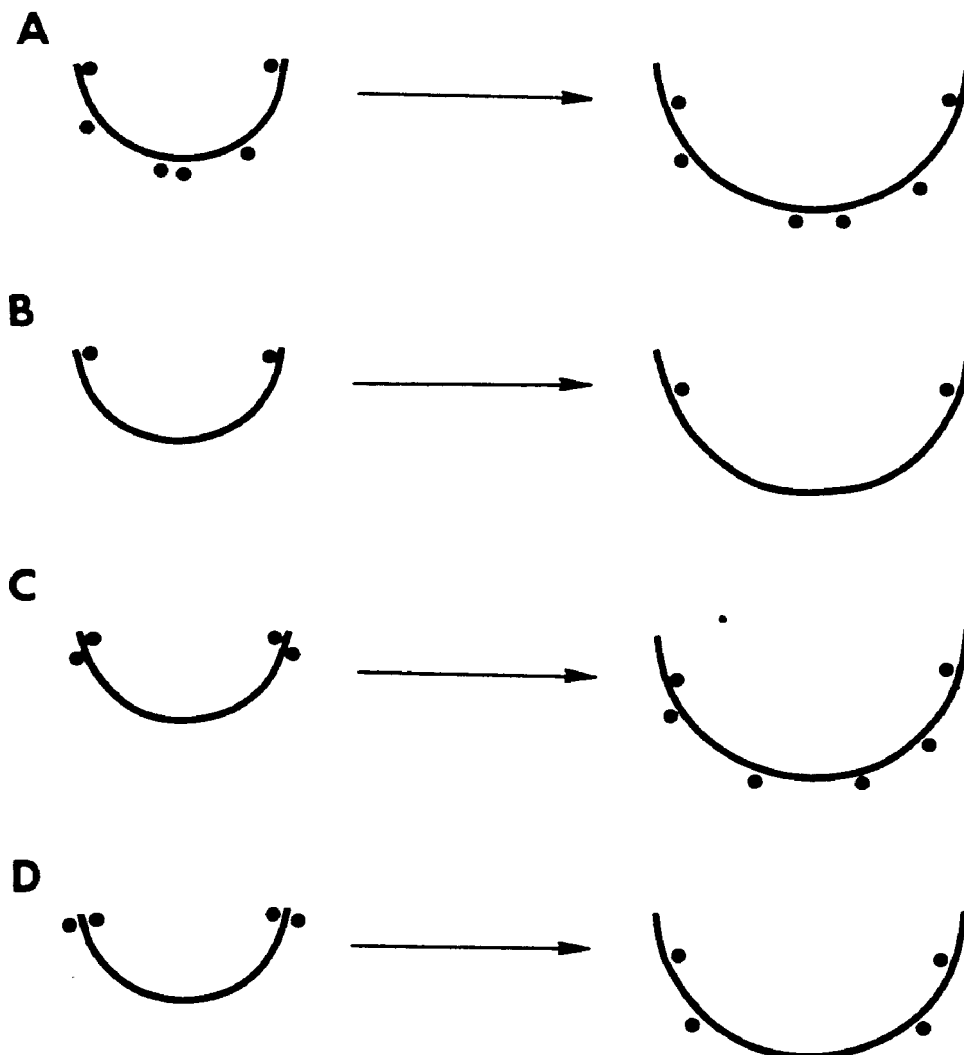


Fig. 45. Hypotheses concerning central addition of rods. These are schematic drawings of the appearance of radioautograms at short (left) and long (right) survival times as predicted by each of the four hypotheses. The dots represent labeled cells: rods on the outside and the other retinal cells on the inside of the semi-circular line.

A, in situ mitosis,
 B, transformation,
 C, cell migration,
 D, retinal shearing.

2. Transformation

The rod density might be maintained via transformation of other cells into rods. This idea was suggested as early as 1900 by Bernard based on his studies of photoreceptor development in the amphibian retina. He observed that the relative number of rods increased during larval growth, and suggested that these new cells were created from inner nuclear layer cells migrating distally through the outer plexiform layer into the outer nuclear layer, transiently becoming cones, and finally evolving into rods. His evidence was purely cytological, and based on the appearance of various intermediate cell types and supposed transitional forms. Lyall (1957a,b) also proposed that cones in the larval and adult trout retina are transformed into rods. She observed that the cone mosaic in the young trout consisted of four pairs of double cones arranged in a square with a single cone in the center and four additional single cones at each corner, whereas in the adult retina, the additional single cones were missing. The pattern of change-over mimicked the earlier, embryonic pattern of differentiation characteristic of all vertebrate retinas, in that it began in the center and progressed toward the periphery. Concomitant with the loss of the additional single cones was an increase in the proportion of rods, and she concluded that the cones were transformed into rods. Lyall (1957b) presented a light micrograph of a possible transitional element; Blaxter and Jones (1967) saw similar structures in the larval herring

retina, but only in the area temporalis, which never acquires rods. This casts some doubt on Lyall's interpretation.

Another possible source of rods is the inner nuclear layer. Both in the herring (Blaxter and Jones, 1967) and in the goldfish (Chapter II) the relative proportion of inner nuclear layer cells decreased with growth. If these cells were not dying they might have been migrating into the outer nuclear layer and becoming rods. The numbers argue against their being the sole source of new rods, however, since not as many cells were lost from the inner nuclear layer as were added to the rod population.

These cells were identified by Blaxter and Jones (1967) as bipolar cells, and their morphology in the goldfish was consistent with this interpretation, but positive identification would require Golgi staining or electron microscopy. It is possible that they were really undifferentiated cells which were being stored in the inner nuclear layer until needed by the outer nuclear layer. If a pool of undifferentiated cells existed in the inner nuclear layer, it should be detectable with electron microscopy. Several ultrastructural characteristics have been associated with the differentiation of neurons (reviewed by La Velle and La Velle, 1970), and specifically with retinal neurons (Smelser et al., 1974; Grün, 1975). If new rods were being formed in the central retina, then rods in various stages of differentiation must also exist. The mature rod has a

complex and easily recognizable morphology. The outer segment is particularly characteristic with its vertical stacks of disk-shaped, membranous sacks of visual pigment (Cohen, 1963). If nuclei could be found in the outer nuclear layer which lacked a distal process, it would be evidence for central addition of rods. Again light microscopic studies do not provide sufficient detail to detect such undifferentiated cells; electron microscopic studies are needed.

The radioautographic results have nothing to offer concerning this hypothesis (Fig. 45B). Changes taking place without an intervening cell division step cannot be detected with this method. We must therefore consider this hypothesis to be still tenable.

3. Cell migration.

New rods or their precursors might be born at the margins and migrate into the central regions. There is a precedent for such cell migration in the amphibian retina. Glücksmann (1940) observed cells migrating from the periphery into the inner nuclear layer during larval growth in tadpoles (Rana temporaria), and Hollyfield (1968) confirmed these results radioautographically in Rana pipiens. The migration hypothesis predicts that the radioautograms in the goldfish would look like Fig. 45C, if this were the mechanism of rod addition. Again the diagram on the right is inconsistent with the data--labeled rods were never found

in the center of the retina. The cell migration hypothesis is therefore ruled out.

4. Retinal shearing.

The density of rods could be maintained if they were immune to the retinal expansion occurring in the other layers. This hypothesis was first suggested by Müller (1952) based on data in the guppy retina which was qualitatively similar to the results presented in Chapter II. Müller's hypothesis predicts radioautograms which would look like Fig. 45D. All of the retinal cells would be added exclusively at the margin and differentiate in situ, but with time, the labeled rods would be displaced centrally relative to the other labeled cells. Although it might be argued that this is merely a modification of the cell migration hypothesis, it is conceptually different in at least two ways. First with regard to which cells are moving. Cell migration as it was presented in the third hypothesis applies to individual rods moving actively toward the center of the retina through a network of other cells and their processes. Glücksmann (1940) detected these moving cells in the amphibian retina by their streamlined shape (Glücksmann, 1930). The hypothesis in Fig. 45D, on the other hand, implies that it is the rest of the retinal cells which are moving peripherally in association with the retinal expansion, whereas the rods are the static elements. Secondly, the intercellular relations among the rods differ in the two hypotheses. The migration hypothesis requires that nearest

neighbors change, whereas the shearing hypothesis predicts that the rods adjacent to one another in the small eye will be the same as those in the large eye.

Fig. 45D is the closest to what was actually found in the goldfish radioautograms, but requires a slight modification to be consistent with the data. The labeled rods did not all remain together, but spread diffusely inward from the zone of labeled cells in the other layers (Figs. 37 and 39). If we imagine that the rods are not entirely immune to the centrifugal force created by the retinal expansion, some being influenced more than others, then the spreading apart of the labeled rods is explained. If this is indeed the correct interpretation, none of the rods should have been peripheral to the labeled band, which they were not, and none should have been located farther centrally than the former position of the ora terminalis. This second condition is difficult to evaluate; the extent of the annulus of labeled rods could not be easily measured since they were so diffuse, and the former position of the ora could not be predicted exactly in any one retina. Based on average values, however, the data are consistent with this idea. In one fish killed at 168 days the distance from the labeled band of cells in the inner nuclear layer to the most central rod was, at the maximum, 700 - 950 μm . The mean retinal length at the beginning of the experiment was 5.2 mm, and the mean length of the "old" retina at 168 days was 6.9 mm, so the former position of the ora was

approximately $\frac{6.9 - 5.2}{2} = 0.85$ mm, or 850 μ m, central to the labeled band. This value is certainly within the range of the distances measured above, and is consistent with the idea that the labeled rods were spread out by their varying degrees of susceptibility to the shearing forces.

This modification of the shearing hypothesis allows for movement within the layer of rods; this in part negates the distinction between cell migration and shearing, since adjacent rods would not be fixed in relation to one another. The other distinction remains, however; the active components are the retinal cells participating in the expansion, and not the rods.

One other observation tends to support the shearing hypothesis. The rods at the extreme edge of the retina were less dense in the goldfish, as in other teleosts (Vilter, 1947; Müller, 1952), almost as if the cell proliferation could not quite keep pace with the shearing force tending to pull them peripherally. The other cells, particularly the cones, were more dense at the margin in the goldfish, and again in other species (Ahlbert, 1968), as if the cell proliferation were working in conjunction with the forces of retinal expansion tending to push these cells toward the periphery.

In summary, I believe that the apparent central addition of rods during growth of the goldfish retina is not really differential cell addition at all, but the result of many or most of the rods resisting the hypertrophic component

of the retinal growth. This resistance leads to a shearing strain between the retinal cell layers resulting in a shift in the relative positions of the rods with respect to the other retinal cells.

Implications of the shearing between retinal layers.

Although some of the rods are pulled peripherally by the retinal expansion, others remain behind, and the post-synaptic cells (bipolars and horizontals) adjacent to them and with which they form synapses may not be the same after growth. This inference is based on the radial orientation and short length of the rod axons in the fish retina (Ramón y Cajal, 1892; Stell, 1967; Stell and Hárosi, 1976; Scholes, 1975). If the rod were to maintain its contacts with the same horizontal and bipolar cells, its axon would have to be pulled peripherally along with them, while its nucleus remained behind. The only examples of vertebrate photoreceptors with slanting axons are both cones: 1) a rare type of cone in teleosts (Scholes, 1975; Stell and Hárosi, 1976), and 2) the cones in the central fovea of the primate retina (Ramón y Cajal, 1909-11; Polyak, 1957; Kolb, 1970; Missotten, 1974). If the rod axon is not pulled peripherally, the dendrites of the postsynaptic cells would have to be displaced centrally; this has not been seen (Ramón y Cajal, 1892; Stell, 1967; Scholes, 1975). The lack of oblique rod axons and displaced dendritic trees in the goldfish implies that the synaptic connections in the central retina are constantly being rewired to accommodate the retinal shearing.

Implications of peripheral cell addition. The radioautograms revealed that new retinal tissue was added appositionally in the goldfish, in other species.

In the trout for example, the uniformity of the cone mosaic throughout the retina during growth implies that cone addition must occur only at the periphery, since cone addition elsewhere would disrupt the regularity of the pattern (Lyall, 1957a). In Xenopus, ^3H -thymidine radioautography has shown that all cell addition is restricted to the periphery during postembryonic growth (Straznicky and Gaze, 1971; Jacobson, 1976). Appositional addition has a specific effect on the spatial and topographical properties of the cells already present in the retina. As Fig. 46 indicates, the result of this type of growth is the progressive displacement of receptive field positions toward the center of the visual field. A given cell which in the small eye reported on information in the periphery would gradually be stimulated by objects more and more centrally located. This means that the spatial information relayed to the brain by an individual ganglion cell changes with growth. How the brain accomplishes the required reinterpretation of the retinal input is not known, but it may involve rewiring of central synaptic connections.

Quantitative aspects of retinal cell addition. Straznicky and Gaze (1971) found that during larval growth in Xenopus, more retinal cells were added dorsally than ventrally, whereas Jacobson (1976) found just the opposite.

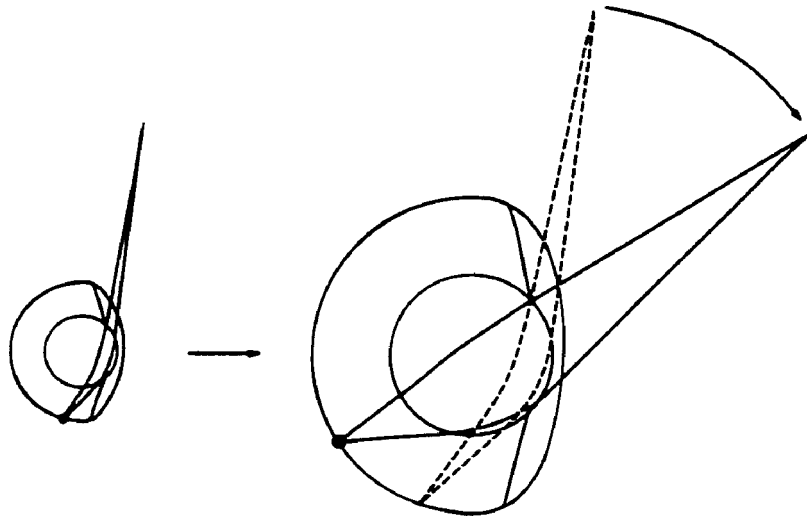


Fig. 46. Changes in receptive field positions with retinal growth. The position of the receptive field of a cell (dot) is indicated by rays entering at each pupillary margin. The receptive field position of the same cell after the eye has grown is indicated by the solid rays and its former position by the dashed rays.

The results in goldfish are similar to those of the previous authors. The more rapid cell addition at the nasal and especially at the dorsal margins may be related to the position of the optic disk, which is located in the opposite, ventrotemporal quadrant in both the goldfish and in Xenopus. The asymmetry in cell addition produced a slight dorsonasal shift in the retinal axis away from the position of the optic disk.

The rate of retinal cell addition in the adult goldfish is striking: several thousand cells are added each day. Approximately 250 of these are ganglion cells; this is comparable to the rate found by Jacobson (1976) in larval and postmetamorphic Xenopus, which ranged from 120-500/day depending on the stage. Because only one size of goldfish was studied, nothing can be inferred about changes in the rate of cell addition with age in this species, although both Müller (1952) and Lyall (1957a) found that the rate of cell addition decreased with age in other teleosts.

The relation between retinal and tectal growth. Most of the new ganglion cells will send their axons to the optic tectum. The projection from retina to tectum is topographically organized; the dorsal retina is represented in the lateral tectum, the ventral retina in the medial tectum, the temporal retina in the rostral tectum, and the nasal retina in the caudal tectum (Jacobson and Gaze, 1964; Schwassmann and Kruger, 1965). As Straznicky and Gaze (1972) pointed out, the simplest way for the tectum to grow

would be to add new cells all around its margins, thereby assuring the newly arriving ganglion cell axons of new tectal cells with which to synapse. Logic does not prevail, however since the amphibian tectum grows in a rostro-lateral to caudomedial direction (Kolross, 1953; Straznicky and Gaze, 1972; Lázár, 1973; Currie and Cowan, 1974).

Preliminary observations in the adult goldfish indicate that tectal neurogenesis is also restricted in this species to a medial region located mostly in the caudal tectum. In radioautograms the labeled cells which apparently are the source of the new tectal neurons, at least those in the periventricular layer, are located at the medial border near the ventricle and probably also in the torus longitudinalis. This corresponds to the dorsal and caudal matrix zones described by Richter and Kranz (1970) in the teleost optic tectum, which are associated with tectal regeneration (Kirsche, 1960; Kirsche and Kirsche, 1961). My preliminary observations suggest that these matrix zones are also responsible for growth of the tectum in the normal animal. If new tectal cells are added only to the caudomedial tectum, then the new ganglion cells from the dorsal and temporal retinal edges will not find new tectal cells with which to synapse, but must form connections with tectal cells already innervated. The retinotectal projection in the adult goldfish, as in larval Xenopus, is probably in a constant state of flux, with synaptic connections being

made, broken, and remade as the map shifts toward the site of tectal neurogenesis at the caudomedial pole.

Implications for neuronal plasticity. The optic nerves in fish and amphibians regenerate when severed, reconstituting the topographical retinotectal map (Sperry, 1955; Attardi and Sperry, 1963; Jacobson and Gaze, 1965). Sperry's pioneering studies and his theory of neuronal specificity have inspired numerous studies directed toward the problem of how these specific connections are formed (reviewed by Jacobson, 1970; Hunt and Jacobson, 1974). Sperry proposed that during development, retinal and tectal cells acquire individual cytochemical specificities dependent on their position, and that central connections are made based on matching affinities between retinal and tectal loci (Sperry, 1951). Later work involving surgical manipulations of the nervous system in embryonic and larval amphibians and adult goldfish showed that the point-to-point matching of retinal and tectal cells was not as rigid as Sperry had postulated. Certain orderly modifications of the map resulted when size disparities were produced between the retina and the tectum, or when composite, double-nasal or double-temporal retinas were created (reviewed by Gaze and Keating, 1972; Hunt and Jacobson, 1974). These experimentally-induced manifestations of "plasticity" are strikingly similar in nature to the processes postulated to occur during normal growth in larval Xenopus and adult goldfish as a consequence of the geometrical differences in their modes of growth.

Meyer and Sperry (1973, 1974) have attempted to show that retinotectal plasticity is associated only with growing systems capable of "embryonic type regulative processes", since it apparently does not occur in adult tree frogs (Hyla regilla) whose tectal development presumably ceases at metamorphosis. The evidence for the latter assumption is very slim; no radioautographic studies of retinal or tectal development have been done on this species. Nevertheless, their hypothesis is intellectually satisfying because it tends to support the existence of a fundamental link between development and regeneration. The well-known observation that recovery of function following lesions of the central nervous system is more complete in young animals than in older ones (reviewed by Rosner, 1974) also tends to support this idea that synaptic plasticity is dependent on embryological or developmental properties.

The other possible significant implication of changing synaptic connections in the growing adult goldfish central nervous system is related to learning and memory. The most widely held theory of learning and memory postulates changes in functional synaptic connections as the underlying mechanism (Hebb, 1949; Mark, 1970). If the retinotectal projection in goldfish is not hard-wired but in a constant state of flux, it may provide a useful model for functional alterations in synaptic connectivity. Although the techniques for studying dynamic properties of synapses over the required periods of time have only begun to be developed in

invertebrate systems (reviewed by Kandel and Spencer, 1968), some morphological and physiological evidence for synaptic remodeling in vertebrates is beginning to appear (reviewed by Cragg, 1974). Sotelo and Palay (1971) reported that altered axons and axon terminals with changes characteristic of degeneration are commonly found in normal animals, for example in the lateral vestibular nucleus of the rat, and may reflect a normal process of synaptic plasticity. Any relation to learning and memory is as yet only speculative, however.

The most intriguing aspect in all of the above is that whatever the mechanism behind synaptic plasticity, in the postembryonic organism it must be accomplished without interfering with the normal functional activity. Embryonic development is in itself a tremendously intricate and well-controlled process, and the simultaneous needs of a functioning animal only add yet another level of complexity to what seems like an already overwhelming task.

SUMMARY

1. Retinal cell addition in adult goldfish is restricted to the margins. The rate of cell addition varies, but averages more than 10,000 cells per day.

2. Cell addition is asymmetric; more cells are added to the dorsal than to the ventral and slightly more to the nasal than to the temporal retina.

3. Growth also results from expansion; the retina increases in size via hypertrophy. The rods do not participate in this expansion, and a shearing stress is thereby created between the retinal layers.

4. Synaptic connections between the rods and other retinal cells may be changing throughout the retina as a consequence of the retinal shearing.

5. The appositional addition of ganglion cells results in a shift in the receptive field positions of individual cells, requiring the brain to reinterpret the messages it receives from the retinal cells.

6. Cell addition in the optic tectum is probably not circumferential; the topography of the retinotectal projection requires that some of the newly arriving retinal axons form synapses in tectal regions which are not actively growing, implying that previously formed synaptic connections must be altered to accommodate the new fibers.

7. The synaptic changes required by the normal growth processes in the adult goldfish visual system may provide a model for neuroplasticity as it is expressed in both regeneration and the process of learning and memory.

APPENDIX

RELATION BETWEEN AGE AND SIZE IN THE GOLDFISH

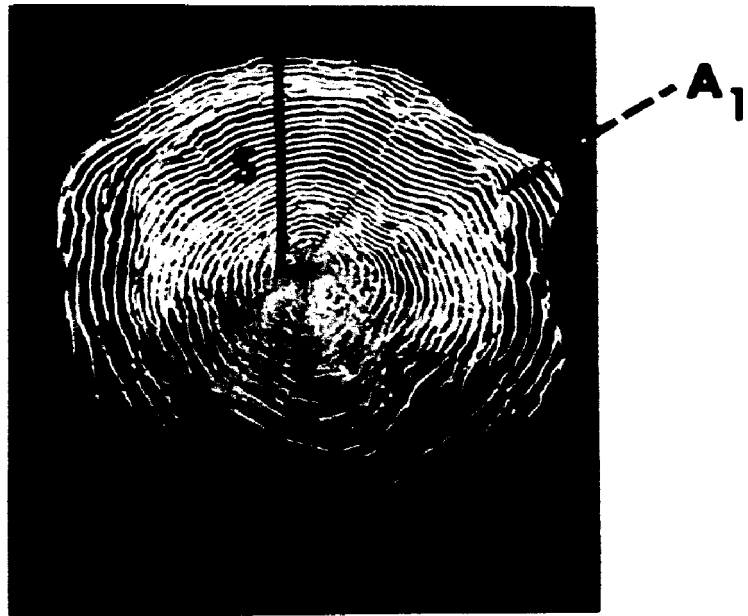
For the analysis of retinal cell numbers in goldfish which was presented in Chapter II, fish of the same strain, but of various sizes were purchased from one commercial supplier, and it was assumed that differences in size reflected differences in age and represented growth of the adult animal. To test the assumption that larger fish are older, the ages of 101 fish were determined using the scale method described by Lager (1956) and Miller (1966). Two or more scales were removed from each fish from a standard region dorsal to the lateral line and just posterior to the opercular opening on the left side of the body. The scales were preserved in 70% ethanol until they could be analyzed using a rear-projection scale reader with a magnification of 40 X (courtesy of Dr. P. Webb, Department of Natural Resources, University of Michigan). The scales and other body structures in fish, especially in those species native to temperate climatic zones, grow roughly concentrically at a non-uniform rate. This produces visible rings, or annuli, on the scales; each successive annulus represents one year of growth, and the age of the fish can be determined simply by counting these growth rings.

The method of analysis used to determine the relation between age and body size in goldfish is described by Miller (1966). The anterior scale radius, the distance from the center, or focus, of the scale to the edge which was formerly attached to the skin, was measured on each scale. The outer, exposed edge of the scale was not used for this measurement since it is often damaged or worn. Scales which showed any structural abnormalities were discarded. In addition to the scale radius, the distance from the focus to each growth ring was measured. The distance to the first annulus represents the radius of the scale when the fish was one-year-old; the distance from the focus to the second annulus is equivalent to the scale radius at two years of age, etc. Two scales from each fish were measured, and the mean scale radius and distance to each annulus was determined. The structure of a goldfish scale is shown in the photomicrograph in Fig. 47. The anterior scale radius, S , and the distance between the focus and the first annulus, A_1 , are indicated.

The values of scale radii are plotted in Fig. 48 as a function of the size of the fish, and the least squares linear regression is drawn through the points. The mean distance from the focus to the first annulus for all scales measured was determined, as were the mean distances to the second, third, and fourth annuli. Since these values represent the average scale radii at one, two, three, and four years of age, the regression equation for scale radius on

Fig. 47. A goldfish scale.
This is a dark field photomicrograph of a scale.
An arrow indicates the annular growth ring (A_1 , see text), and the anterior scale radius (S) is shown by the line segment. Calibration bar: 1 mm.

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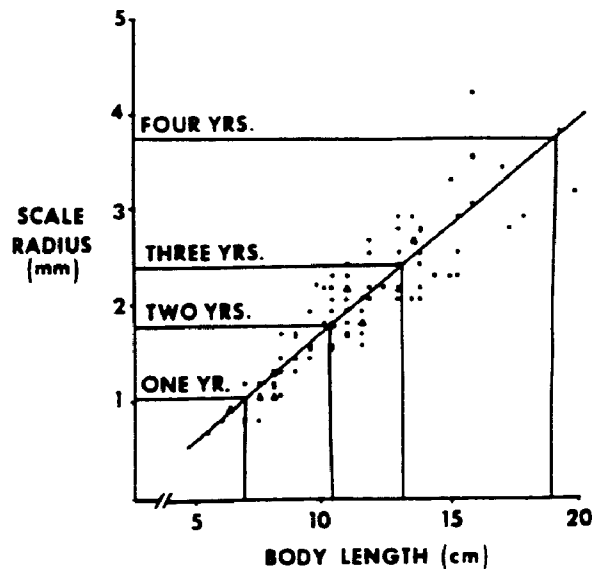


Fig. 48. Age analysis based on scale measurements. The average scale radius in mm is given as a function of body length, and the least squares linear regression is drawn through the points:

$$\text{Scale radius (mm)} = 0.215 [\text{body length (cm)}] - 0.45, r^2 = 0.82.$$

The average distance between the center or focus of the scale and the first annulus is indicated by the horizontal line labeled "one yr." and represents the scale radius of a one-year-old fish; the regression line gives the corresponding body length. The scale radii and body lengths of two-, three-, and four-year-old fish are also shown.

body length can be used to determine the corresponding average size of goldfish at each age. The mean distances from the focus to each annulus ± 2 S.E.M. are $A_1 = 1.1 \pm 0.10$; $A_2 = 1.8 \pm 0.22$; $A_3 = 2.5 \pm 0.44$; and $A_4 = 3.7 \pm 0.38$ mm. These mean annular distances are indicated in Fig. 48 by the four horizontal lines labeled one, two, three, and four years. The regression equation of scale radius on body length gives 7.2 cm as the average body length of one-year-old fish. Similarly, the average lengths of fish two, three, and four years old are 10.5, 13.7, and 19.3 cm, respectively.

The growth rate in fish is extremely variable, and depends on many factors including temperature, availability of food, lighting conditions, seasonal and reproductive variables, and degree of crowding (M. Brown, 1957). In a group of fish of the same species which are initially all the same size, a rapid dispersion in sizes occurs with time (see Fig. 32). This has been attributed to species-specific crowding factors, hormones produced by the largest fish which inhibit growth and reproduction in conspecifics (Rose, 1959); such chemicals have been isolated from the water in which a high density of goldfish were living (Pfuderer, 1974). As a result, the relation between size and age is not an exact one; some fish grow much more rapidly than others. Although in general larger goldfish are older, some fish were found in the above analysis which were less than one year old based on their scale structure,

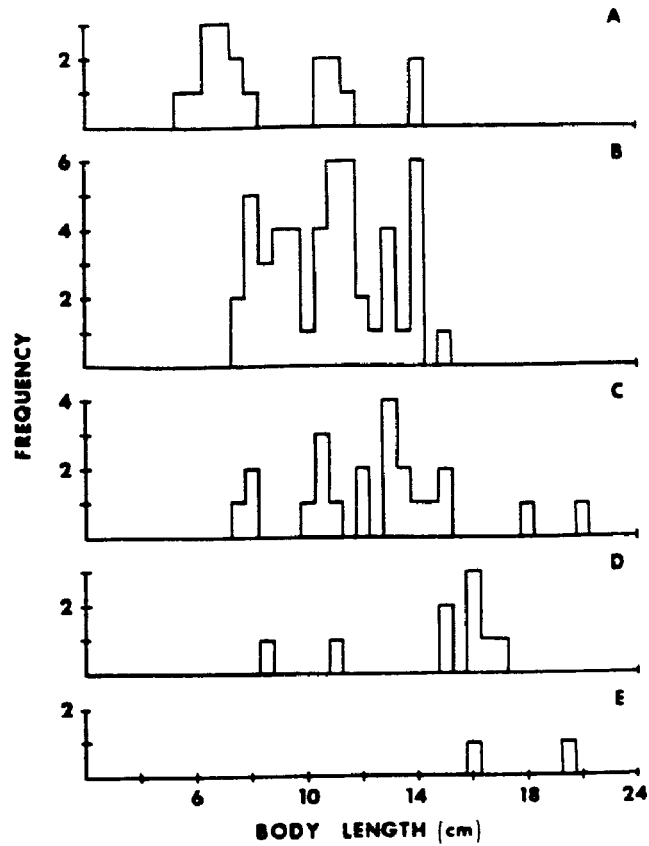


Fig. 49. The variability in the size of fish of the same age. The 101 fish were grouped according to the number of annuli on their scales: less than one, A; one, B; two, C; three, D; or four, E. The number of fish in each 0.5 cm interval of body length is plotted.

but which were as large as the average three-year-old fish. This is illustrated in Fig. 49 in which the 101 fish used in the scale analysis have been grouped into five classes depending on the number of annuli on their scales: 0,1,2,3, or 4. These histograms indicate the number of fish of various lengths in each of the five classes. The greatest range of sizes occurs in the group of two-year-old fish (Fig. 49C) and all of the groups overlap extensively; there are not two distinct populations of "small-type" and "large-type" goldfish. The assumption that large fish are older versions of small fish is supported.

The variability in growth rate in teleost fish was also demonstrated by Müller (1952) in his study of the guppy (Lebistes reticulatus). He found that some fish were 10 mm long at 15 days post-hatching, whereas other fish did not reach that size until 90 or even 120 days. Müller stated that the relative diameter of the eye (percentage of body length) was larger in older guppies than in younger ones of the same size (see his Fig. 19a,b, page 291). In the experiment described in Chapter III, a group of fish which were initially approximately the same size (5-7 cm) were allowed to grow for up to 336 days. The variability in individual growth rates characteristic of fish was observed in these; some grew much more rapidly than others. Although the age of the fish at the beginning of the experiment was not known, comparisons between eye size and body size during the 336 day interval showed the same phenomenon that Müller observed:

older fish have larger eyes. Table IV demonstrates this point. Fish of five different lengths (6.0, 6.5, 7.0, 7.5, and 9.0 cm), selected because several of the same length found at two or more of the five time points sampled (0-4, 28, 84, 168, and 336 days) were grouped according to "age" (time post-injection) and size. The mean diameter of all eyes in each group is given in the table; the number of eyes in each group is in parentheses. The values of eye diameter in a given row increase from left to right; for example the fish 7.0 cm in length (row 3) have an average eye diameter of 4.1 mm at 0-4 days (column 1), 4.8 mm at 84 days (column 3), and 5.4 mm at 168 days (column 4). The only exception to this rule is in the last row; the 9.0 cm fish had larger eyes at 168 than at 336 days. This influence of age on eye size independent of body length undoubtedly contributed in part to the variability in the relation between eye diameter and body length in Fig. 6. In some unknown way the eye is partially immune from the constraints which limit the rate of growth of the body. The good correspondence between retinal cell number and retinal length in Fig. 27 implies that both retinal cell proliferation ocular growth are controlled by factors intrinsic or at least specific to the eye.

TABLE IV
RELATION BETWEEN AGE, SIZE, AND EYE DIAMETER

Body Length (cm)	Time at Sacrifice (days)			
	0-4	28	84	168
6.0	4.1 (n=6)	4.6 (n=6)	5.3 (n=2)	-
6.5	4.2 (n=6)	-	4.9 (n=4)	-
7.0	4.2 (n=2)	-	4.8 (n=8)	5.4 (n=2)
7.5	-	-	4.9 (n=2)	5.3 (n=2)
910	-	-	-	5.2 (n=2)
				5.1 (n=4)

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