# ENZYME-HISTOCHEMICAL ANALYSIS OF RETINAL DEVELOPMENT IN THE MOUSE

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE
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**JOSOBANTA BHATTACHARJEE**
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PROMOTOR : PROF. DR. J. MOLL

COREFERENTEN : PROF. DR. W. J. VAN DOORENMAALEN
PROF. DR. P. VAN DUIJN

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# I. GENERAL INTRODUCTION

Development of neuronal specificity remains one of the major enigmas in contemporary neurobiology. The embryonic neuroepithelium, derived from the surface ectoderm due to the inductive influence of the chorda-mesoderm, acquires a highly complex differentiation when neuronal and glial cells appear and establish specific structural relationships. Arising as a protrusion of the diencephalon during ontogeny, the retina presents the same anatomical and physiological problems as do other parts of the central nervous system. In the retina a spatial separation of both the perikarya and the cell processes into distinct nuclear and synaptic layers is effected during histogenesis. This makes the retina a very suitable material for studying vertebrate neurogenesis and therefore the developing retina has been a preferred target of analysis through various approaches.

Information about the structural integration of the optic nerve with the retina (Corti, 1850), transretinal organization of the radial fibres (Müller, 1851) and the replacement of Treviranus' hypothesis of vitreal location of the photoreceptors by photoreceptive function of the bacillary layer (Müller, 1853) led to a first comprehensive description and functional interpretation of retinal structure (Kolliker, 1854; Müller, 1854). This provided a basis for developmental study of the retina and using routine histological methods Babuchin published his observations on retinal development in 1863.

The discovery of the silver impregnation technique by Golgi (1873, 1878) and of methylene blue staining by Ehrlich (1885, 1886) marked the beginning of neurohisto-

logical methods. These two methods and their various modifications have been applied extensively on the adult, young and developing retina of several vertebrate species (Tartefuri, 1888; Dogiel, 1888a, b; Cajal, 1888, 1892, 1894, 1896, 1919; Kallius, 1894; Krause, 1894; Neumeyer, 1897; Marenghi, 1901; Greeff, 1900). Cajal (1892, 1929) in reviewing these findings as well as his own comprehensive study on the development of the mouse, rabbit, calf and chick retina gave an elaborate description of the development of different retinal cells. However his observations were restricted to the stages when the ganglion cells and the inner plexiform layer have already differentiated. His attempts to stain the retina of younger embryos were unsuccessful. Since then, application of the silver impregnation method has provided more information on the retinal structure (Kolmer, 1930; Polyak, 1941, 1957; Boycott & Dowling, 1969; Dowling & Werblin, 1969; Shkolnik-Yarros, 1971; Ogden, 1974) but studies on the developing retina by such methods (Morest, 1970) remained restricted to relatively late stages.

Turning our attention to the earliest stages of retinal development the following can be said. The cells of the embryonic neuroepithelium of course go through a period of repeated mitosis. With respect to this early phase of development the following question has become important: Are different cell lines established which give rise to distinct neuronal and glial cell types or do all cells remain developmentally equivalent till cessation of division. No definite answer to this question have been found (Jacobson, 1970). However, morphological differentiation of a cell, at least of the neuronal cells, is manifest after mitosis has stopped. Autoradiography, after administration of thymidine - $H^3$, a DNA precursor, at different stages of development, has been used to trace the time of origin of the different cells in the developing retina of mice (Sidman, 1961), chick (Fujita &

Horii, 1963; Kahn, 1973, 1974), frog (Hollyfield, 1968), xenopus (Jacobson, 1968; Hollyfield, 1971) and killifish (Hollyfield, 1972). Precise information has been obtained about the time of origin of certain retinal cells e.g, the ganglion and photoreceptor cells, due to the circumstances that their perikarya are separated in distinct layers. In the case of the horizontal and Müller cells in mice, however, the problem of cell identification in routine histological preparations makes similar observations difficult as pointed out by Sidman (1961). Commenting on this Fujita & Horii (1963) discussed the difficulty of correct designations of cells within the mixed population of amacrine, Müller, bipolar and horizontal cells prior to the separation of the nuclear layers and advocated application of selective staining to distinguish the different cell types. Similarly Sidman (1970) emphasized the need for correlation of silver impregnation and autoradiographic data but stressed that the techniques are mutually incompatible. Furthermore, since the labelled cells are identified after they have attained their definitive form and location, autoradiographic data do not provide information about the events intervening between the time of cessation of mitosis and the separation of retinal layers.

The present study is an attempt to bridge this gap in our understanding of the process of neurogenesis in the retina. Differentiation of the various retinal cells from the embryonic neuroepithelium must necessarily be reflected in changes of metabolic rate, appearance or concentration of enzymes, or in the products of specific metabolic pathways. A histochemical approach demonstrating some of these changes in cells remaining in situ seemed to be of particular advantage, since the biochemical changes in a differentiating cell can not be ascertained using in vitro biochemical techniques whitout affecting its structural orientation in respect to other cells.

About the histochemical approach Shen (1958) pointedly remarked "it thus enables one to see as it were, both the topography of a forest and the characteristics of individual trees".

We have followed histochemical changes in the localization of several different enzymes in the developing retina of mice from the embryonic optic cup to the adult stage. The main purpose is to trace the developmental sequence of retinal cells using histochemically detectable enzymatic features as indices of cytodifferentiation and to study the interrelation of these cells in the course of histogenesis of the retinal layers.

For references see end of chapter III.

# II. CYTOARCHITECTURE OF THE RETINA OF THE MOUSE

The retina is one of the best studied parts of the central nervous system. Cytoarchitectural features of the retina have been studied and described in great detail ( Polyak, 1941; 1957 ), but the observations are largely based on the primate retina. Comparative studies have established that the organization of the retinal layers is the same in all vertebrates, but that differences, mainly in the relative proportion of constituent cells, are present ( Walls, 1942; Prince, 1956 ).

The specific cytoarchitectural organization of the mouse retina, which has the same constituent cell types as other mammals, is schematically presented in Fig. 1 and a brief description follows.

The pigment epithelium is a single layer of flattened cuboidal cells at the outer circumference of the retina. These cells contain in pigmented strains numerous pigment granules which give it a dark appearance. The outer surface facing Bruch's membrane has numerous short infoldings while the inner surface, facing the receptors, shows many elongated microvilli projecting into the optic ventricle and between the receptor outer segments.

The neural retina is composed of five types of neuronal cells and one type of neuroglial cell. The perikarya and the processes of the cells are arranged in concentric layers.

1. Bacillary layer : It is composed of the inner and outer segments of the photoreceptor cells which in mice and rats are primarily composed of rods with a few rudimentary cone cells ( Droz, 1963 ). Villous projections from Müller cells are present between the rods.

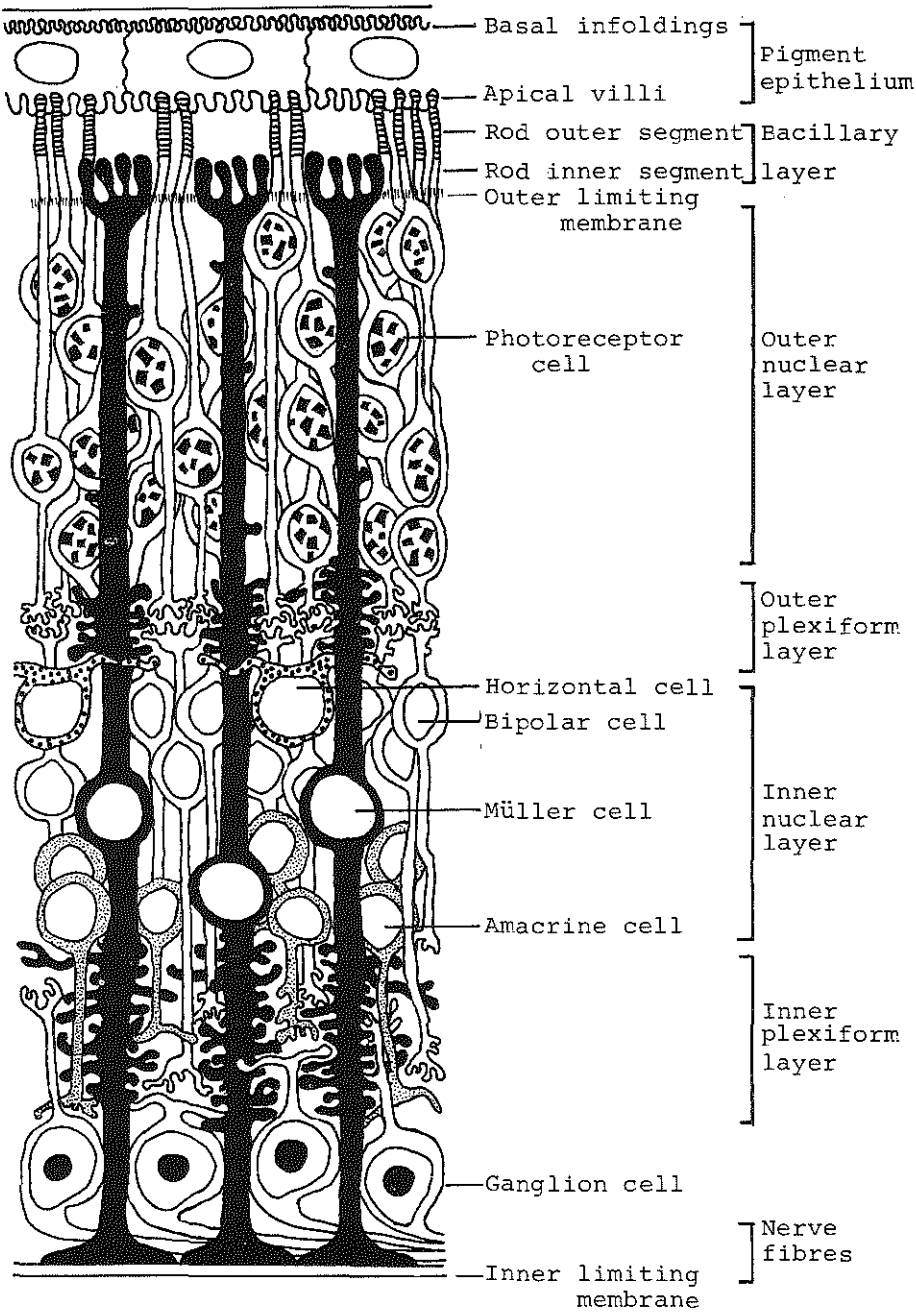



Fig. 1. Schematic diagram showing the different retinal cell-types and their organization in the retina of the adult mouse.

2. Outer limiting membrane : The region at the base of the receptor inner segments appears under the light microscope as an extremely thin fenestrated membrane scleral to the outer nuclear layer. However electron microscopic studies have shown that it is not a true membrane but is produced by a series of terminal bars or desmosomes uniting the cell membranes of the photoreceptors and the Müller cells ( Sjöstrand, 1958; Yamada et al., 1958;Missotten, 1960; Cohen, 1960; Fine, 1961; Fine & Zimmerman, 1962, 1963; Spitznas, 1970 ).

3. Outer nuclear layer : This layer is relatively thicker in mice than in animals with a predominantly cone or duplex retina ( Cajal, 1892; Prince, 1956 ) and contains 10 - 12 rows of nuclei of the photoreceptor cells invested in thin perinuclear cytoplasm.

4. Outer plexiform layer : This thin layer represents the synaptic zone between the photoreceptor terminals, the bipolar cell dendrites and the scleral expansion of the horizontal cells. It also contains the lateral processes branching off from the passing radial fibres of Müller cells.

5. Inner nuclear layer : This layer is formed by the perikarya of 5 - 7 rows of cells arranged in 4 ill defined zones. a) The outer zone, is occupied by the perikarya of the horizontal cells interspersed between the outermost row of bipolars. The horizontal cells are relatively fewer in number and are larger than the bipolar cells. The scleral processes of the horizontal cells extend horizontally ( vide name ) in the outer plexiform layer and synapse with the photoreceptor terminals and bipolar cell dendrites ( Yamada & Ishikawa, 1965; Dowling, Brown & Major, 1966; Olney, 1968; Sjöstrand, 1969, Dowling & Werblin, 1969; Fisher & Boycott, 1974 ) and possibly mediate lateral interaction in the outer plexiform layer ( Baylor, Fourtes & O'Bryan, 1971; Baylor, 1974 ). b) The outer intermediate zone contains 2 - 3 rows of oval shaped bipolar cell

perikarya. These appear identical and presumably are the common rod bipolar variety ( Cajal, 1892 ) comparable to the mop ( rod ) bipolars described by Polyak ( 1941 ) in the primate retina. c) The inner intermediate zone can be distinguished by the presence of the perikarya of some of the Müller cells which are wedged between bipolar and amacrine cells as observed in other species ( Inomata, 1965; Dowling, 1970; Hogan, Alvarado & Weddell, 1971 ). The radial fibres of the Müller cells traverse the entire thickness of the retina and lateral processes extend into the plexiform layers. d) The inner zone is formed by 2 - 3 rows of amacrine cells, perikarya of some of the Müller cells and of a few displaced ganglion cells located in the innermost row of this zone. The amacrines are round cells with a thick rim of perinuclear cytoplasm. Two types of amacrine cells can be identified in the mouse. They resemble the diffuse and the stratified varieties described in other species. The diffuse amacrines have tufted expansions that extend throughout the depth of the inner plexiform layer while the processes of the stratified type spread laterally at definite levels ( Cajal, 1892; Polyak, 1941; Boycott & Dowling, 1970; Gallego, 1971 ).

6. Inner plexiform layer : This synaptic zone is formed by the processes of the amacrines, axons of bipolars and dendrites of the ganglion cells, together with the processes of Müller cells. The zone shows horizontal striations.

7. Ganglion cell layer : This layer contains a single row of ganglion cells. These are usually spherical and at least two morphological types, large and small can be distinguished besides some cells of intermediate size. The ganglion cells are similar to those reported by Cajal ( 1892 ) in different mammalian species.

8. Layer of nerve fibres : This layer is formed by the axons of the ganglion cells which converge towards the optic nerve and also contains the inner ends of the

Müller cells.

9. Inner limiting membrane : The vitreal border of the retina shows a basal membrane produced by the adjoining inner endfeet of the Müller cells ( Polyak, 1941; Wolff, 1961; Wolter, 1961; Cohen, 1961; Uga & Smelser, 1973 ).

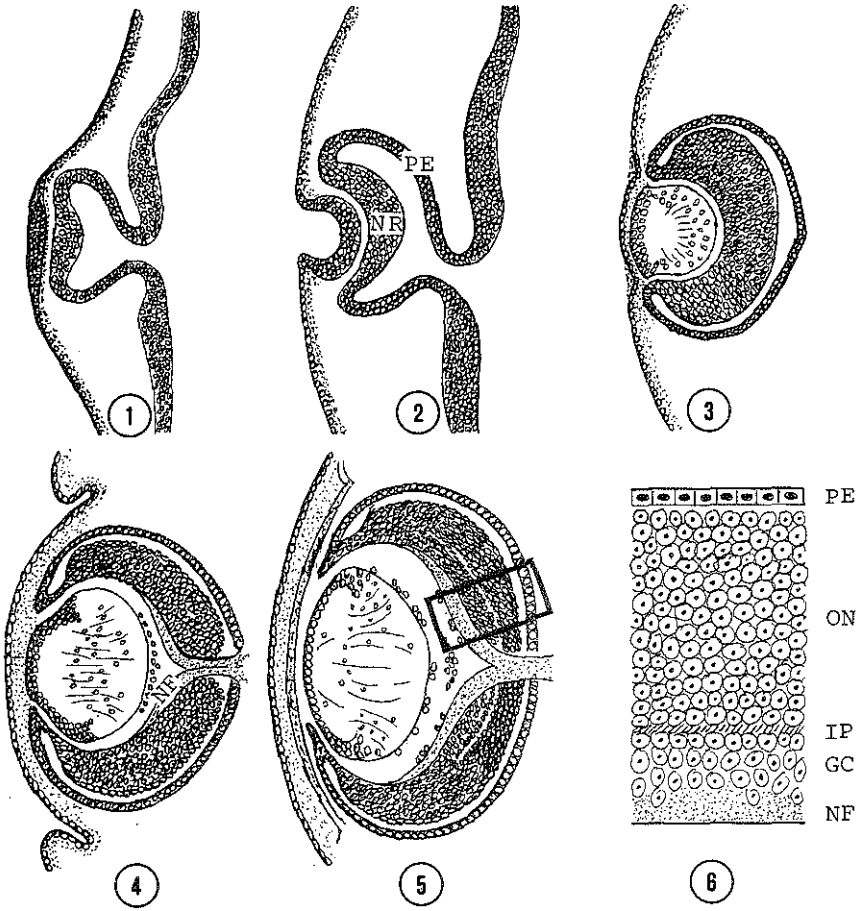
Macrophages, originating from the vascular monocytes, have been demonstrated ( Sanyal, 1972 ) in the mouse retina and it has been suggested that they are comparable to the microglia described by Rio-Hortega ( 1932 ).

For references see end of chapter III.

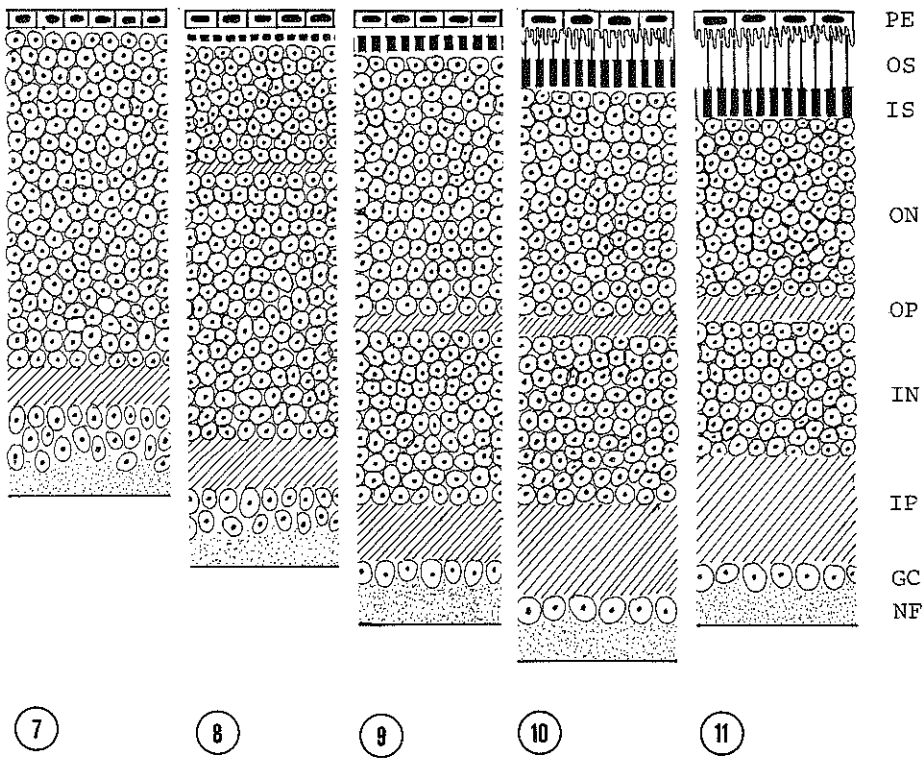
# III. HISTOMORPHOLOGICAL CHANGES DURING RETINAL DEVELOPMENT IN THE MOUSE

As a basis for our enzyme-histochemical studies on the developing retina we give here a brief description of the morphological changes during retinal development in the Balb/cHeA strain of mouse, used throughout this study. (Figs. 1-11). Our observations are in agreement with the earlier studies of Pye & Rhodin (1970) on prenatal development, and of Tanseley (1951, 1954), Sorsby et al. (1954), Noell (1958), Caley et al. (1972) and Sanyal and Bal (1973) on postnatal development in different strains of mice. Available descriptions of the developmental stages of the mouse (Rugh, 1968; Theiler, 1972) also provide information on the stages of eye development.

The earliest differentiation of the neuro-ectoderm which eventually develops into the retina is visible in the 7 somite embryo at 8 days of embryonic life. At this stage two barely noticeable dents at each side of the rostral end of the neural plate mark the beginning of the optic sulci, which deepen into two pits called foveolae opticae (Walls, 1942; Duke-Elder & Cook, 1963) At 8.5 days of gestation, when the neural folds begin to approach each other to close the neural tube, the foveolae opticae rotate, forming a pair of protrusions of the lateral diencephalic wall, which expand to form the optic vesicles (Fig.1). When the anterior neuropore closes at 9 days, the optic vesicles reach the overlying ectoderm and induce the formation of the lens placode. Simultaneously the invagination of the optic vesicle, leading to the formation of a deep lens pocket takes place (Fig. 2). At 10 days, a two layered optic cup is formed (Fig. 3).




Figs. 1-6. Schematic representation of retinal development in the mouse during the prenatal period. Fig. 1. 8.5 days of gestation, Fig. 2. 9 days, Fig. 3. 10 days, Fig. 4. 13 days, Fig. 5. 16 days, Fig. 6. Magnified view of the area shown in the rectangle in Fig.5. PE, pigment epithelium; NR, neural retina; ON, outer neuroblastic layer; IP, inner plexiform layer; GC, ganglion cells; NF, layer of nerve fibres.




Figs. 7-11. Schematic representation of retinal development in the mouse during the postnatal period. Fig. 7. Newborn, Fig. 8. 4 days, Fig. 9. 7 days, Fig. 10. 14 days, Fig. 11. 26 days.
PE, pigment epithelium; OS, rod outer segment; IS, rod inner segment; ON, outer nuclear layer; OP, outer plexiform layer; IN, inner nuclear layer; IP, inner plexiform layer; GC, ganglion cells; NF, layer of nerve fibres.

The outer layer of the optic cup, the presumptive pigment epithelium, consists of 1-2 rows of columnar cells, while 6-7 rows of neuroblasts in the inner layer represent the presumptive neural retina. The cells of the neural retina rapidly proliferate and by 13 days the nerve fibre layer can be distinguished in the fundus area (Fig. 4). At 16 days of embryonic life, a hair line gap, the prospective inner plexiform layer appears at the inner level of the neural retina and separates the presumptive ganglion cells from the outer layer of neuroblasts (Figs. 5, 6). The pigment epithelium consists at this stage of a single row of flattened cells.

The animals are born after 19 days of gestation. In the newborn mouse, the inner plexiform layer has grown in thickness and the ganglion cells are clearly recognized. Two to 3 rows of cells close to the outer side of the inner plexiform layer appear morphologically different and can be recognized as amacrine cells. At 4 days after birth the retina shows a further sign of differentiation when a poorly defined outer plexiform layer appears in the outer part of the neuroblastic layer (Fig. 8). This is the begining of separationof the inner and outer nuclear layers. Although formation of photoreceptor inner segments is known from electron microscopic studies to have already started at birth, they are first seen with light optics at this stage. As the outer plexiform layer increases in thickness, migrating photoreceptor nuclei segregate into the outer nuclear layer around 6-8 days (Sanyal & Bal, 1973). This, together with the addition of cells at the margin of the retina where mitosis continues for a few days after birth (Sidman, 1961), results in a considerable increase in thickness of the outer nuclear layer (Fig. 9). The rod inner segments increase markedly in length and developing outer segments can be distinguished at 10 days. The eye opens around 12 days after birth and then an electroretinographic response can first be obtained (Noell,

1965). Between this period and the time of weaning, usually at about 4 weeks, a linear increase in growth of the rod outer segment takes place (LaVail, 1973). The adult stage is finally reached by an increase in thickness and complexity of the synaptic knit-work in the inner plexiform layer accompanied by a relative decrease of the thickness of the outer and inner nuclear layers and of the ganglion cell layer (Figs. 9-11). Simultaneously the retina expands along with the growth of the eyeball (Caley, 1972).

## REFERENCES


BABUCHIN, A. (1863).: Beiträge zur Entwicklungsgeschichte des Auges, besonders der Retina. Würzb. Naturw. Zeitschr. 4, 71-90.

BAYLOR, D.A. (1970).: Lateral interactions between vertebrate photoreceptors. Fed. Proceedings. 33, 1074-1077.

BAYLOR, D.A., FOURTES, M.G.F. & O'BRYAN, P.M. (1971).: Receptive fields of cone in the retina of the turtle. J. Physiol. (London). 214, 265-294.

BOYCOTT, B.B. & DOWLING, J.E. (1969).: Organization of the primate retina : Light microscopy. Phil. Trans. R.Soc. London B. 255, 109-184.

CAJAL, S. RAMON Y (1888).: Morfología y connexiones de los elementos de la retina de las aves. Rev. Trimestral Histol. Nor. Patol. Mayo.

CAJAL, S. RAMON Y. (1892).: La retina de los teleosteos y algunas observaciones sobre la de los vertebrades superiores. Ann. Soc. Esp. Hist. Nat., Madrid 21, 281-305.

CAJAL, S. RAMON Y. (1892).: The structure of the retina (Transl. S.A. Thorpe & M. Glickstein, 1972). C.C. Thomas. Illinois.

CAJAL, S. RAMON Y. (1894).: Die Retina der Wirbeltiere. Untersuchungen mit der Golgi-Cajal'schen Chromsilbermethod und der Ehrlich'schen Methylenblaufärbung. J.F. Bergman. Wiesbaden.

CAJAL, S. RAMON Y. (1896).: Nouvelles contributions à l'étude histologique de la rétine et à la question des anastomoses des prolongements protoplasmiques. J. Anat. Physiol. Paris. 32, 481-543.

CAJAL, S. RAMON Y. (1919).: La desorientación inicial de las neuronas retinianas de axon corto (Algunos hechos favorables a la concepción neurotrópica). Trab. Lab. Invest. biol., Univ. Madrid, 17, 65 - 86.

CAJAL, S. RAMON, Y. (1929).: Studies on vertebrate neurogenesis (Transl. L. Guth, 1960). C.C. Thomas. Illinois.

CALEY, D.W., JOHNSON, C. & LIEBELT, R.A. (1972).: The postnatal development of the retina in the normal and rodless CBA mouse : a light and electron microscopic study. Am. J. Anat. 133, 179-211.

COHEN, A.I. (1960).: The ultrastructure of the rods of mouse retina. Am. J. Anat. 107, 23-48.

COHEN, A.I. (1961).: Electron microscopic observation of the internal limiting membrane and optic fiber layer of the retina of the rhesus monkey (M. mulatta). Am. J. Anat. 108, 179-198.

CORTI, A. (1850).: Beitrag zur Anatomie der Retina. Arch. f. Anat., Physiol., u. Wissensch. Med. (J. Müll). pp. 273-275.

DOGIEL, A.S. (1888a).: Uber das Verhalten der nervösen Elemente in der Retina der Ganoiden, Reptilien, Vögel und Saügetiere. Anat.Anz. 3, 133-143.

DOGIEL, A.S. (1888b).: Uber die nervösen Elemente in der Netzhaut der Amphibien und Vögel. Anat. Anz. 3, 342-347.

DOWLING, J.E. (1970).: Organization of vertebrate retinas. Invest. Opthalmol. 7, 655-680.

DOWLING, J.E., BROWN, J.E. & MAJOR, D. (1966).: Synapses of horizontal cells in rabbit and cat retinas. Science 153, 1639-1641.

DOWLING, J.E. & WERBLIN, F.S. (1969).: Organization of retina of the mudpuppy. J. Neurophysiol. 32, 315-338.

DROZ, B. (1963).: Dynamic condition of proteins in the visual cells of rats and mice as shown by radioautography with labelled amino acids. Anat. Rec. 145, 157-167.

DUKE-ELDER, SIR. W. STEWART. & COOK, C. (1963).: System of opthalmology Vol. III. Normal & abnormal development. Henry Kimpton. London.

EHRLICH, P. (1885).: Zur biologischen Verwertung des Methylenblau. Centralbl. Med. Wiss. 23, 113.

EHRLICH, P. (1886).: Uber die Methylenblaureaktion der lebenden Nervensubstanz. Biol. Centralbl. 6, 214-224.

FINE, B.S. (1961).: Limiting membranes of the sensory retina and pigment epithelium. Arch. Opthalmol. 66, 847-860.

FINE, B.S. & ZIMMERMAN, L.E. (1962).: Müller cells and the "middle limiting membrane" of the human retina. Invest. Opthalmol. 1, 304-326.

FINE, B.S. & ZIMMERMAN, L.E. (1963).: Observations on the rod and cone layer of the human retina. Invest. Ophthalmol. 2, 446 - 459.

FISHER, S.K. & BOYCOTT, B.B. (1974).: Synaptic connections made by horizontal cells within the outer plexiform layer of the retina of the cat and the rabbit. Proc. R. Soc. London B. 186, 317-331.

FUJITA, S. & HORII, M. (1963).: Analysis of cytogenesis in chick retina by tritiated thymidine autoradiography. Arch. Histol. Jap. 23, 359-366.

GALLEGO, A. (1971).: Horizontal and amacrine cells in mammal's retina. Vision Res. Suppl. 3, 33-60.

GOLGI, C. (1873).: Sulla struttura della sostanza grigia dell cervello. Gazz. Med. Ital. Lombarda 33, 244-246.

GOLGI, C. (1878).: Un nuovo processo di tecnica microscopia . R.C. Inst. Lombardo. Sci. 2nd Ser. 12,5.

GREEFF,R.(1900). Die mikroskopische Anatomie des Sehnerven und der Netzhaut. In:Handbuch der gesamten Augenheilkunde. 2te Aufl. Bd.1. pp.1-212. W. Engelman. Leipzig

HOGAN, M.J., ALVARADO, J.A. & WEDDELL, J.E. (1971).: Histology of the human eye. W.B. Saunders Co. Philadelphia.

HOLLYFIELD, J.G. (1968).: Differential addition of cells to the retina in Rana pipiens tadpoles. Develop. Biol. 18, 164-179.

HOLLYFIELD, J.G. (1971).: Differential growth of the neural retina in Xenopus laevis larvae. Develop. Biol. 24, 264-286.

HOLLYFIELD, J.G. (1972). Histogenesis of the retina in the killifish, Fundulus heteroclitus. J. Comp. Neurol. 144, 373-379.

INOMATA, H. (1965).: Electron microscopic observations on Müller's fiber in the human retina. Acta Soc. Opthalmol. Jap. 69, 2133-2143.

JACOBSON, M. (1968).: Cessation of DNA synthesis in retinal ganglion cells correlated with the time of specification of their central connections. Develop. Biol. 17, 219 - 232.

JACOBSON, M. (1970).: Developmental neurobiology. Holt, Reinhert & Winston. New York.

KAHN, A.J. (1973).: Ganglion cell formation in the chick retina. Brain Res. 63, 285-290.

KAHN, A.J. (1974). An autoradiographic analysis of the time of appearance of neurons in the developing chick neural retina. Develop. Biol. 38, 30-40.

KALLIUS, E. (1894).: Untersuchungen über die Netzhaut der Saügetiere. Anat. Heften 3, 307-318.

KOLLIKER, A. (1854).: Mikroskopische Anatomie oder Gewebelehre des Menschen, Vol. 2, Part 2, pp. 605-648. Engelmann. Leipzig.

KOLMER, W. (1930).: Zur Kenntnis der Auges der Primaten Zeits. f. d. Ges. Anat. Abt.1, Bd. 93, 679-722.

KRAUSE, W. (1894).: Die Retina. V. Die Retina der Vögel. Int. Monatss. Anat. Physiol. 11, 1-66, 69-122.

LaVAIL, M.M. (1973).: Kinetics of rod outer segment renewal in the developing mouse retina. J. Cell Biol. 58, 650-661.

MARENGHI, G. (1901). Contributo alla fina organizzazione della retina. Boll. Soc. Med.-Chir. Pavia, 1-33.

MISSOTTEN, L. (1960).: Etude des bâtonnets de la rétine humaine en microscope électronique. Opthalmologica 140, 200-214.

MOREST, D.K. (1970).: The pattern of neurogenesis in the retina of the rat. Z. Anat. Entwickel.-Gesch. 131, 45-67.

MÜLLER, H. (1851).: Zur Histologie der Netzhaut. Z. Wiss. Zool. 3, 234-237.

MÜLLER, H. (1853).: Ueber einige Verhaltnisse der Netzhaut bei Menschen und Tieren. Verhandl. d. phys. med. Gesellsch. Würzburg 3, 336-351.

MÜLLER, H. (1854).: Ueber die entoptische Wahrnehmung der Netzhautgefasse,insbesondere als Beweismittel für die Lichtperception durch die nach hinten gelegenen Netzhautelemente. Verhandl. d. phys. med. Gesellsch. Würzburg 5, 411 - 437.

NEUMAYER, L. ( 1897 ) : Der feinere Bau der Selachier Retina. Arch. f. mikr. Anat. 48, 83 - 92.

NOELL, W.K. ( 1958 ). Differentiation, metabolic organization and viability of the visual cells. Arch. Ophthalmol. 60, 702 - 733.

NOELL, W.K. ( 1965 ) : Aspects of experimental and hereditary retinal degeneration. In : Biochemistry of the retina ( Ed. C.N. Graymore ), pp. 51 - 72. Academic Press. London.

OGDEN, T.E. ( 1974 ) : The morphology of retinal neurons of the owl monkey. J. Comp. Neurol. 153, 399 - 428.

OLNEY, J.W. ( 1968 ) : An electronmicroscopic study of synapse formation, receptor outer segment development, and other aspects of developing mouse retina. Invest. Ophthalmol. 7, 250 - 268.

PEI, Y.F. & RHODIN, J.A.G. ( 1970 ) : The prenatal development of the mouse eye. Anat. Rec. 168,105 - 126.

POLYAK, S.L. ( 1941 ) : The retina. The University Press. Chicago.

POLYAK, S.L. ( 1957 ) : The vertebrate visual system. The University Press. Chicago.

PRINCE, J.H. ( 1956 ) : Comparative anatomy of the eye. C.C. Thomas. Illinois.

RIO-HORTEGA, P. DEL ( 1932 ) : Microglia. In : Cytology and cellular pathology of the nervous system ( Ed. W. Penfield ), Vol. II, pp. 482 - 534. Hoever. New York.

RUGH, R. ( 1968 ) : The mouse - its reproduction and development. Burgess Publishing Co. Minneapolis.

SANYAL, S. ( 1972 ) : Changes of lysosomal enzymes during hereditary degeneration and histogenesis of retina in mice. II. Localization of N-Acetyl-$\beta$-Glucosaminidase in macrophages. Histochemie 29, 28 - 36.

SANYAL, S. & BAL, A.K. ( 1973 ) : Comparative light and electronmicroscopic study of retinal histogenesis in normal and rd mutant mice. Z. Anat. Entwickl. Gesch. 142, 219 - 238.

SHEN, S.C. ( 1958 ) : Changes in enzymatic patterns during development. In : The chemical basis of development ( Eds. W.D. McElroy & B. Glass ), pp. 416 - 432. John Hopkins Press. Baltimore.

SHKOLNIK-YARROS, E.G. ( 1971 ) : Neurons of the cat retina. Vision Res. 11, 7 - 26.

SIDMAN, R.L. ( 1961 ) : Histogenesis of mouse retina studied with thymidine-$H^3$. In : The structure of the eye ( Ed. G.K. Smelser ), pp. 487 - 506. Academic Press. London.

SIDMAN, R.L. ( 1970 ) : Autoradiographic methods and principles for study of the nervous system with thymidine-$H^3$. In : Contemporary research methods in neuroanatomy ( Eds. W.J.H. Nauta & S.O.E. Ebbesson ), pp. 252 - 274. Springer Verlag. Berlin.

SJÖSTRAND, F.S. ( 1958 ) : Ultrastructure of retinal rod synapses of the guineapig eye as revealed by three dimensional reconstructions from serial section. J. Ultrastructure Res. 2, 122 - 170.

SJÖSTRAND, F.S. ( 1969 ) : The outer plexiform layer and the neural organization of the retina. In : The retina : Morphology, structure and clinical characteristics ( Eds. B.R. Straatsma, M.O. Hall, R.A. Allen & F. Crescitelli ), pp. 63 - 100. University of California Press. Los Angeles.

SORSBY, A., KOLLER, P.C., ATTFIELD, M., DAVEY, J.B. & LUCAS, D.R. ( 1954 ). Retinal dystrophy in the mouse : histological and genetic aspects. J. Exp. Zool. 125, 171 - 197.

SPITZNAS, M. ( 1970 ) : Zur Feinstruktur der sog : Membrana limitans externa der menschlichen Retina. Graefes Arch. Ophthalmol. 180, 44 - 60.

TANSELEY, K. ( 1951 ). Hereditary degeneration of the mouse retina. Brit. J. Ophthalmol. 35, 573 - 582.

TANSELEY, K. ( 1954 ). An inherited retinal degeneration in the mouse. J. Heredity 45, 123 - 127.

TARTEFURI, F. ( 1887 ) : Sull' anatomia della retina. Int. Monatss. Anat. Physiol. 4, 421 - 441.

THEILER, K. ( 1972 ) : The house mouse : Developmental and normal stages from fertilization to 4 weeks of age. Springer Verlag. Berlin.

UGA, S. & SMELSER, G.K. ( 1973 ) : Electron microscopic study of the development of retinal Müllerian cells. Invest. Ophthalmol. 12, 295 - 307.

WALLS, G.L. ( 1942 ) : The vertebrate eye and its adaptive radiation. Hafner Publishing Co. New York. 1967.

WOLFF, E. ( 1961 ) : Anatomy of the eye and orbit. Ed. 5, pp. 391. W.B. Saunders Co. Philadelphia.

WOLTER, J.R. ( 1961 ) : Silver carbonate techniques for the demonstration of ocular histology. In : The structure of the eye ( Ed. G.K. Smelser ), pp. 117 - 138. Academic Press. London.

YAMADA, E., TOKUYASUM, K. & IWAKI, S. ( 1958 ) : The fine structure of retina studied with electron microscope. III. Human retina. J. Kurume Med. Assoc. 2, 1979.

YAMADA, E. & ISHIKAWA, T. ( 1965 ). The fine structure of the horizontal cells in some vertebrate retina. Cold Spring Harbor Symp. Quant. Biol. 30, 383 - 392.

# IV. DEVELOPMENTAL ORIGIN AND EARLY DIFFERENTIATION OF RETINAL MÜLLER CELLS IN MICE

J. BHATTACHARJEE AND S. SANYAL

Department of Anatomy, Faculty of Medicine,
Erasmus University Rotterdam,
Rotterdam. The Netherlands

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## INTRODUCTION

Although the literature on the developmental cytology of the retina is considerable, information about the origin and initial differentiation of Müller cells is scanty and often contradictory. Müller identified these cells as the most important, if not the only, glial elements of the retina in 1851. Cajal ( 1892 ) noted the first appearance of Müller cells as elements scattered throughout the retina in mouse embryos of about 15 mm length, a stage at which the ganglion cells could also be recognized. The observations of Berkow & Patz ( 1964 ) on the developmental histochemistry of oxidative enzymes and of Morest ( 1970 ) on the rapid Golgi impregnated retina of the rat indicate relatively late appearance of Müller cells. However, recent electron microscopic studies by Uga & Smelser ( 1973 ) in the rabbit and by Kuwabara & Weidman ( 1974 ) in the rat have shown that Müller cells are the earliest among the retinal cells to differentiate.

Lack of identifiable characteristics prior to their attaining definitive form and location clearly is an impediment in analysing the early events of Müller cell differentiation. In a histochemical study on the developmental changes of enzymes with esterase activity in the retina of mice, it was observed that the initial differentiation of cells, identifiable as presumptive Müller cells, was characterized by a localized and transient activity of non-specific esterase. This report describes the origin and change of these cells, visualized by the non-specific esterase reaction, during the prenatal development of the retina in mice.

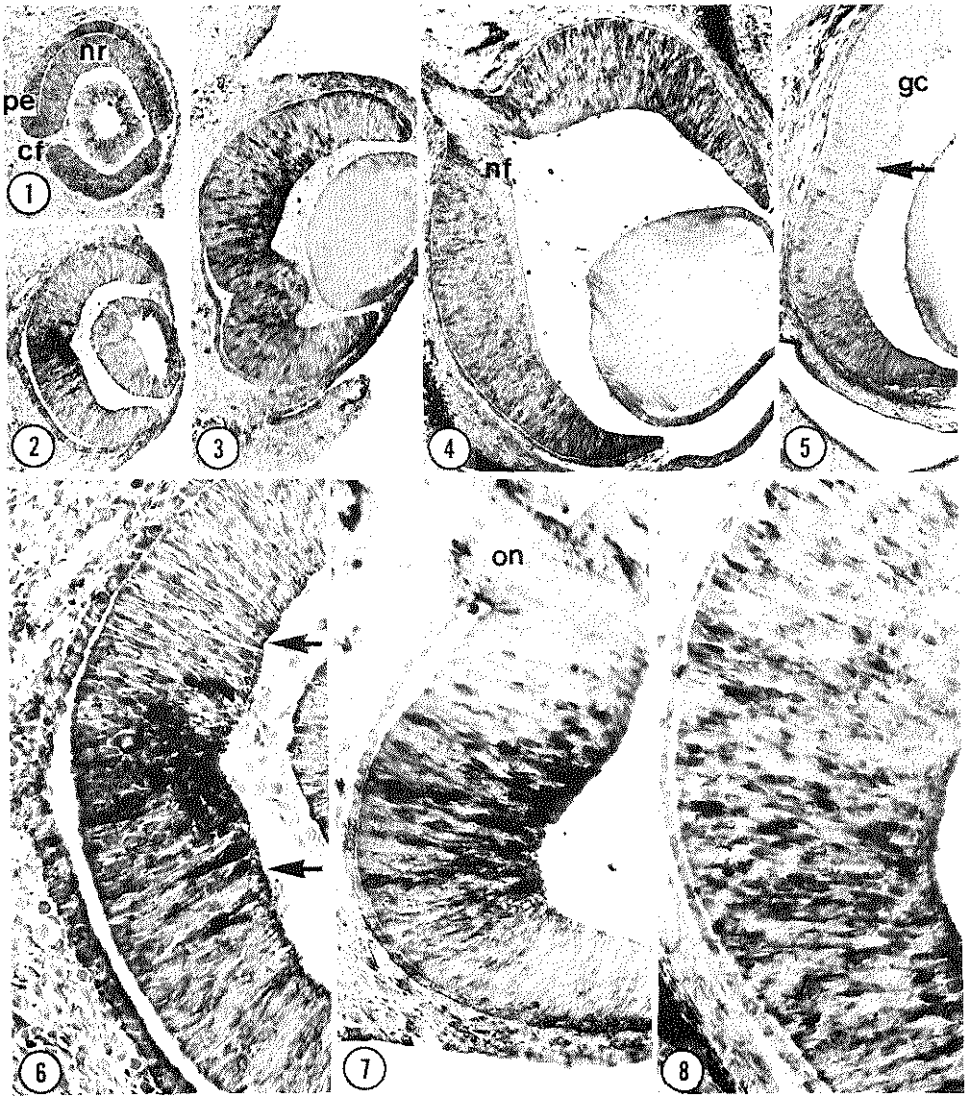
## MATERIAL AND METHODS

Balb/cHeA mice were used throughout this study. Sexually mature females and males were allowed to mate overnight and the presence of vaginal plugs in the morning marked the beginning of pregnancy; the next day was considered as day 1. Starting on day 10 of gestation females were killed at daily intervals until birth. The embryos were removed from the uterus and immediately immersed in physiological saline. The entire heads, or the eye regions, were dissected out and fixed overnight in ice-cold fixative ( 4% paraformaldehyde in 0.067 M phosphate buffer at pH 7.2 and 7.5% sucrose ). The material was stored in gum-sucrose ( Holt, 1959 ) for up to 5 days before sectioning in a cryostat at 10 µm. Non-specific esterase was localized according to the method of Barka & Anderson ( 1963 ). α-naphthyl acetate ( Sigma ) was used as substrate at pH 6.5 with hexazotized pararosanilin as coupler. The sections were incubated at 37° C for 20 minutes and were counterstained with methyl green. Specific substrates and inhibitors were used according to the directions of Pearse ( 1972 ) to confirm the histochemical reaction as due to non-specific esterase activity.

## RESULTS

The optic vesicle is formed in the mouse embryo around day 9 of gestation and by day 10 develops into a two-layered optic cup connected with the diencephalon by the optic stalk. The choroid fissure is then still open ( Rugh, 1968; Pei & Rhodin, 1970; Theiler, 1972 ). At this stage the cells of the inner layer, the presumptive neural retina, do not show any noticeable activity for non-specific esterase or any morphological evidence of differentiation ( Fig. 1 ).

On day 11, the choroid fissure has closed and non-specific esterase activity is now seen in a very few cells




Photomicrographs of cryostat sections of developing mouse retina showing changes of non-specific esterase activity in Müller cells.

Fig. 1. Optic cup of day 10 embryo; cells in the neural retina (nr) and pigment epithelium (pe) do not show any esterase activity. cf, choroid fissure. x 100.

Fig. 2. Optic cup at 11 days; note esterase activity in cells near the vitreal border of the central retina. x 100.

Fig. 3. 13 days; section of the optic cup lateral to the point of entry of the optic nerve. Note growth of the optic cup and thickness of the neural retina along with the appearance of esterase-positive cells in the fundus. x100.

Fig. 4. 15 days; cells with high esterase activity are seen near the ora. Cells with reduced activity are scattered throughout the retina. nf, layer of nerve fibres. x 100.

Fig. 5. 16 days; esterase activity is seen near the ora while activity has disappeared from the fundus. Note presence of the layer of ganglion cells (gc) and the inner plexiform layer ( arrow ). x 100.

Fig. 6. Magnified view of 11 day retina ( Fig. 2 ). Note esterase activity along the inner limiting membrane ( arrow ). x 400.

Fig. 7. 13 days; part of section passing through the optic nerve (on). Most intensely stained cells are located along the vitreal border. Cells near the optic nerve show reduced activity. x 400.

Fig. 8. 15 days; part of retina shown in Fig. 5. Note cells with reduced esterase activity scattered throughout the depth of the retina. x 400.

located on the vitreal side near the optic stalk ( Fig. 2 ). These cells are elongated with external and internal processes oriented perpendicular to the retinal surface ( Fig. 6 ). They are considered to be Müller cells at the beginning of their differentiation ( see discussion ). At the same time, esterase activity of moderate intensity has appeared along the vitreal border of the retina close to the inner limiting membrane. Some necrotic cells with clumps of esterase-positive material are also seen in the centre of the retina adjacent to the optic stalk.

On day 13, the number of esterase-positive cells has increased markedly. Such cells are seen as far forward as half of the distance from the centre of the optic cup to the ora ( Fig. 3 ). As cells with esterase activity appear in peripheral regions the area immediately adjacent to the optic nerve, where the emergence of the layer of nerve fibres is now discernible, shows considerably reduced enzyme activity ( Fig. 7 ). Although nearly all cells which are most intensely stained are located on the vitreal border, many cells with a varying intensity of esterase activity are seen more externally with a few close to the scleral border of the neural retina, thus producing a characteristic radial orientation ( Figs. 3, 7 ). It appears that the perikarya of these cells acquire the esterase activity while located in the vitreal position, and migrate in the scleral direction, losing some of the activity on the way. By day 15, the most intensely stained cells are located in the more peripheral part of the retina, close to the ora, and cells with esterase activity of varying but reduced intensity are scattered uniformly throughout the fundus ( Figs. 4, 8 ). The layer of nerve fibres has now expanded into the peripheral area, but no further progress of development is recognizable. Along with the growth of the optic cup from 11 days onwards, a sudden increase in the thickness of the neural retina is recorded and is most pronounced in that part of the central

retina where esterase-positive cells have appeared.

On day 16, the esterase activity of cells has considerably declined ( Fig. 5 ). In the fundus, towards the optic nerve, the cells appear uniformly negative, but some cells with faint activity are still found scattered in the peripheral part, including the ora. Besides the extension of the layer of nerve fibres throughout the central part of the retina, further advance in the histogenesis of the retinal layers is seen in the separation of the layer of ganglion cells along with the emergence of the inner plexiform layer. Gradually the changes hitherto observed in the fundus extend into the ora, and the esterase activity in the remaining scattered population of cells disappears around the time of birth. The margin of the optic cup which develops into the iris and ciliary body continues to show a high level of esterase activity.

## DISCUSSION

The identification of the esterase-positive cells as Müller cells is based on two previous findings. Müller cells are the first type of cells to differentiate in the rabbit retina; this has been demonstrated by the early appearance of cells with smooth endoplasmic reticulum, characteristic of mature Müller cells ( Uga & Smelser, 1973 ). The earliest cells with these organelles appeared in the vitreal border on the 14th prenatal day which is developmentally equivalent to the 11th prenatal day of mice, when the esterase-positive cells first appear at the same site. The Müller cells subsequently migrate with the growth of the retina, and this leads to their scattered distribution ( which Cajal (1892) observed with the silver method in 15 mm mouse embryo, approximately corresponding to the 15 or 16 day old embryos used in our study ).

Migration presumably involves only the perikarya, including the outer ends of the cells, while the inner ends

remain fixed to the vitreal surface, since the inner limiting membrane is considered to be formed by the Müller cell endfeet and has been shown to be a basement membrane continuously present from the optic vesicle stage ( Cohen, 1961 ). It is tempting to speculate, in this context, whether position along the vitreal border predetermines the presumptive Müller cell population of the retina in the way positional information has been proposed to control cellular differentiation in many instances ( Wolpert, 1969 ).

The functions generally attributed to Müller cells in the adult retina are the provision of mechanical support in the form of a framework to the retinal neurons ( Cajal, 1904; Polyak, 1941 ), of nutritive and metabolic support as evidenced by high concentration of glycogen ( Shimizu & Maeda, 1953; Kuwabara & Cogan, 1961 ) and several enzymes ( Cogan & Kuwabara, 1959; Kuwabara & Cogan, 1960 ) and of insulation of neuronal networks ( Polyak, 1956 ). Early detection of Müller cells at the light microscopic level provides an opportunity to observe their appearance in relation to the progress of development in the whole retina. The initial phase of differentiation of Müller cells, marked by transient esterase activity, advances as a concentric wave from the centre to the periphery. A rapid growth of the neural retina occurs almost immediately and is followed by differentiation and separation of the inner retinal layers as the perikarya of the Müller cells migrate towards more outer levels. The sequence of these changes suggests that early acquisition of some of the metabolic functions might be important in promoting growth of the retinal cells. Further, outward migration of the perikarya is a part of the process that results in formation of the radial fibres which constitute the glial framework. The early development of this structure suggests that it might act as a substratum for the neuronal precursor cells which show repeated to and fro movement along the scleral-vitreal

axis in the course of the cell cycle and subsequent differentiation ( Sidman, 1961 ). Furthermore, active participation of Müller cells in the separation of the receptor and the bipolar cells has been suggested by Meller ( 1964 ) and Meller & Glees ( 1965 ) on the basis of electron microscopic observation on 9 day old chick embryos. It has been earlier reported ( Coulombre, 1955 ) that, in the chick retina, Müller cells differentiate around 4 - 5 days of incubation, which is developmentally comparable to the 11 - 12 day stage in mice. Thus a spatio-temporal relationship between differentiation of Müller cells and histogenetic separation of the neuronal layers appears to be a regular feature of retinal development. Experiments with other enzyme markers and autoradiography are in progress to observe retinal histogenesis during the postnatal period when the outer retinal layers differentiate, and the Müller cell perikarya become finally located in the inner nuclear layer.


## SUMMARY

During the prenatal development of the retina in mice Müller cells at the initial stage of differentiation show a high level of histochemically detectable non-specific esterase activity. These are the first of the retinal cell types to differentiate and appear at the 11th day of gestation along the vitreal border in the central retina. As development proceeds they appear in more peripheral areas and their perikarya migrate outwardly and become scattered throughout the depth of the retina. Appearance of presumptive Müller cells is followed by rapid growth of the retina and differentiation of the inner retinal layers. With the progress of histogenesis from the central to the peripheral areas the esterase activity in the Müller cells gradually diminishes. The possible significance of early differentiation of Müller cells in promoting

growth and histogenesis of retina is discussed.

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REFERENCES

BARKA, T. & ANDERSON, P.J. ( 1963 ). Histochemistry, Theory, Practice and Bibliography. New York : Hoeber.

BERKOW, J.W. & PATZ, A. ( 1964 ). Developmental histochemistry of the rat eye. Investigative Ophthalmology 3, 22 - 33.

CAJAL, S. RAMON Y. ( 1892 ). The structure of the retina ( English translation, 1972 ). Springfield : C.C. Thomas.

CAJAL, S. RAMON Y. ( 1904 ). Histologie du système nerveux de l'homme et des vertébrés ( French edition, 1955 ). Madrid : Instituto Ramon Y. Cajal.

COGAN, D.G. & KUWABARA, T. ( 1959 ). Tetrazolium studies on the retina. II. Substrate dependent patterns. Journal of Histochemistry and Cytochemistry 7, 334 - 341.

COHEN, A.I. ( 1961 ). Electron microscopic observations of the developing mouse eye. I. Basement membrane during early development and lens formation. Developmental Biology 3, 22 - 33.

COULOMBRE, A.J. ( 1955 ). Correlation of structural and biochemical changes in the developing retina of the chick. American Journal of Anatomy 96, 153 - 189.

HOLT, S.J. ( 1959 ). Factors governing the validity of staining methods for enzymes, and their bearing upon the Gomori acid phosphatase technique. Experimental Cell Research Suppl. 7, 1 - 27.

KUWABARA, T. & COGAN, D.G. ( 1960 ). Tetrazolium studies on the retina. III. Activity of metabolic intermediates and miscellaneous substrates. Journal of Histochemistry and Cytochemistry 7, 214 - 223.

KUWABARA, T. & COGAN, D.G. ( 1961 ). Retinal glycogen. Archives of Ophthalmology 66, 680 - 688.

KUWABARA, T. & WEIDMAN, T.A. ( 1974 ). Development of the prenatal rat retina. Investigative Ophthalmology 13, 725 - 739.

MELLER, K. ( 1964 ). Elektronenmikroskopische Befunde zur Differenzierung der Rezeptorzellen und Bipolarzellen der Retina und ihrer synaptischen Verbindungen. Zeitschrift für Zellforschung und mikroskopische Anatomie 64, 733 - 750.

MELLER, K. & Glees, P. ( 1965 ). The differentiation of neuroglia-Müller -cells in the retina of the chick. Zeitschrift für Zellforschung und mikroskopische Anatomie 66, 321 - 332.

MOREST, D.K. ( 1970 ). The pattern of neurogenesis in the retina of the rat. Zeitschrift für Anatomie und Entwickelungsgeschichte 131, 45 - 67.

PEARSE, A.G.E. (1972 ). Histochemistry, Theoretical and Applied, 3rd ed., vol. 2. London : Churchill Livingstone.

PEI, Y.F. & RHODIN, J.A.G. ( 1970 ). The prenatal development of the mouse eye. Anatomical Record 168, 105 - 126.

POLYAK, S.L. ( 1941 ). The retina. Chicago : The University Press.

POLYAK, S.L. ( 1956 ). The vertebrate visual system. Chicago : The University Press.

RUGH, R. ( 1968 ). The mouse - its reproduction and development. Minneapolis : Burgess Publishing Co.

SHIMIZU, N. & MAEDA, S. ( 1953 ). Histochemical studies on glycogen of the retina. Anatomical Record 116, 427 - 434.

SIDMAN, R.L. ( 1961 ). Histogenesis of mouse retina studied with thymidine-$H^3$. In : The Structure of the Eye ( Ed. G.K. Smelser ), pp. 487 - 506. New York : Academic Press.

THEILER, K. ( 1972 ). The House Mouse. Berlin : Springer Verlag.

UGA, S. & SMELSER, G.K. ( 1973 ). Electron microscopic study of the development of retinal Müllerian cells. Investigative Ophthalmology 12, 295 - 307.

WOLPERT, L. ( 1969 ). Positional information and the spatial pattern of cellular differentiation. Journal of Theoretical Biology 25, 1 - 47.

# V. DEVELOPMENTAL CHANGES OF CARBONIC ANHYDRASE IN THE RETINA OF THE MOUSE : HISTOCHEMICAL STUDY

J. BHATTACHARJEE

Department of Anatomy, Faculty of Medicine,
Erasmus University Rotterdam,
Rotterdam. The Netherlands

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Synopsis. In the optic cup of early mouse embryos, carbonic anhydrase activity is seen only in the pigment epithelial cells. During the late prenatal period the enzyme appears in the Müller cells, first in the perikarya. During the postnatal development, the enzyme activity appears in the radial processes of the Müller cells starting at the vitreal border, along with the separation of the outer retinal layers. This is followed by increased activity in the plexiform layers, presumably in the lateral processes. Carbonic anhydrase localization, in pigment epithelial and Müller cells attains adult pattern at about 16 days corresponding with the initiation of retinal function.

## Introduction

The localization of the enzyme carbonic anhydrase (E.C. 4.2.1.1.) in the pigment epithelial and the retinal layers of several vertebrate species (Korhonen & Korhonen, 1965; Leder, 1966; P. Bhattacherjee, 1971) has invoked considerable interest. Electron microscopic study of Musser & Rosen (1973) has shown that the enzyme in the neural retina is localized in the Müller cells and its presence in the different retinal layers is due to the extension of processes from these cells. Recent observations on the developing retina of rabbit (Uga & Smelser, 1973), rat (Kuwabara & Weidman, 1974) and mouse (Bhattacharjee & Sanyal, 1975) have shown that Müller cells are the earliest retinal cells to differentiate. This report deals with the changes in the histochemical localization of carbonic anhydrase in the pigment epithelial and Müller cells during differentiation of the retinal layers from the embryonic optic cup to the adult stage.

## Materials and methods

Albino mice of Balb/cHeA strain were used in all cases. Sexually mature females and males were caged together and the pregnant females detected by the presence of vaginal plugs, were isolated on the following morning. Females were killed after 10, 11, 13 and 16 days of gestation and the entire heads or pieces of the head region of the embryos including the eyes were dissected out and fixed for 2 hours in ice-cold fixative containing 2% paraformaldehyde, 1.5% glutaldehyde, 7.5% sucrose in 0.067M phosphate buffer at pH 7.2. The newborn and animals of 5, 7, 11, 16 and 26 days of age were killed by cervical dislocation and the eyes were immediately removed and immersed in the fixative as above. After an hour the cornea and the lens were removed and fixation was continued for another hour. This fixation schedule was preferred after initial trial with unfixed tissues and tissues fixed for various lengths of time in different concentrations of formaldehyde and glutaraldehyde. Unfixed tissues gave qualitatively comparable results but loss of enzyme activity through diffusion (Lönnerholm, 1974) and damage of the retinal layers affected the final picture. The materials were washed overnight in gum-sucrose (Holt, 1959) and were then frozen rapidly and sectioned at 10 µm in a Pearse-Slee cryostat operating at -45°C. Sections were either picked up on millipore filters (25 µm thick, pore size 0.45 µm) or handled free floating.

Carbonic anhydrase was localized according to the procedure of Hansson (1967). The details about the specificity of this method and the reactions involved have been discussed by several authors (P. Bhattacherjee, 1971; Rosen & Musser, 1972; Lightfoot & Cassidy, 1973; Cassidy & Lightfoot, 1974; Rosen, 1974; Lönnerholm, 1974). The incubation media contained 1 ml 0.1M $CoSO_4$, 5 ml 0.067M $KH_2PO_4$, 5 ml 0.5M $H_2SO_4$, mixed together and added to a

solution containing 560 mg of $NaHCO_3$ in 40 ml $H_2O$. The pH of the medium was 5.8 immediatly after mixing. The sections were floated on the media for 8-10 minutes at room temperature (20°C), rinsed quickly in phosphate buffered saline (pH 5.9) and treated in 2% ammonium sulphide for visualizing the reaction sites. The sections were washed in $H_2O$ and were mounted on slides with glycerinejelly (Gurr) or entellan (Merck). Inhibition by acetazolamide (Sigma) was used as control for enzymatic reaction. Complete inhibition of visible reaction was recorded in 1 X $10^{-6}$M concentration.

## Results

### Localization of carbonic anhydrase in the retina of adult mouse

In the retina of the adult mouse, carbonic anhydrase is present only in the pigment epithelium and Müller cells (Korhonen & Korhonen, 1965) and the histochemical localization resembles the observations in other species (Leder, 1966; P. Bhattacherjee, 1971; Musser & Rosen, 1973). In the pigment epithelium, intense carbonic anhydrase activity is seen along the scleral border which forms the basal infoldings facing Bruch's membrane and in the vitreal

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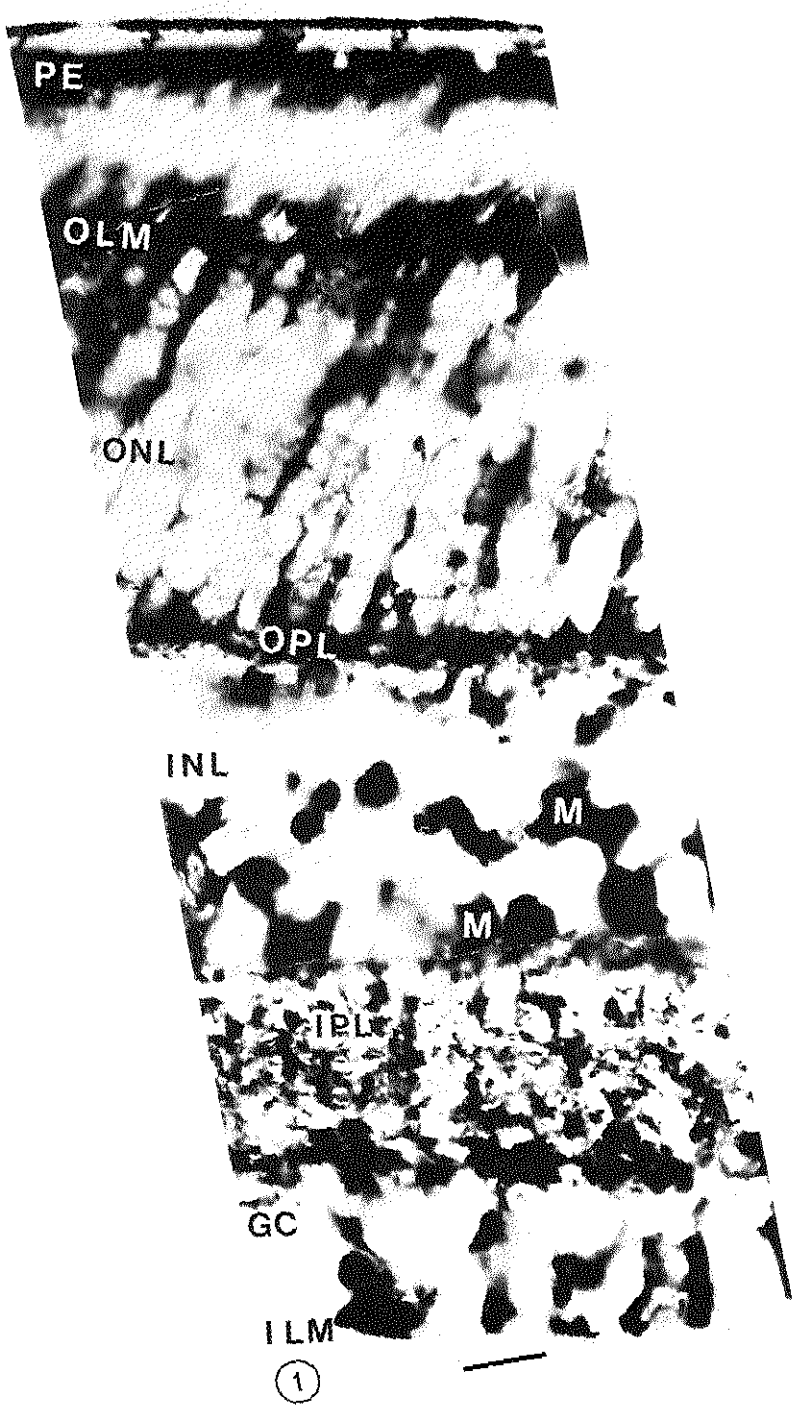
Figure 1. Montage of microphotographs of retina from adult mouse showing distribution of carbonic anhydrase activity in the pigment epithelium (PE) and in the Müller cell perikarya (M) and processes in the neural retina. OLM, outer limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GC, ganglion cells; ILM, inner limiting membrane. Bar= 10 µm.


PE
OLM
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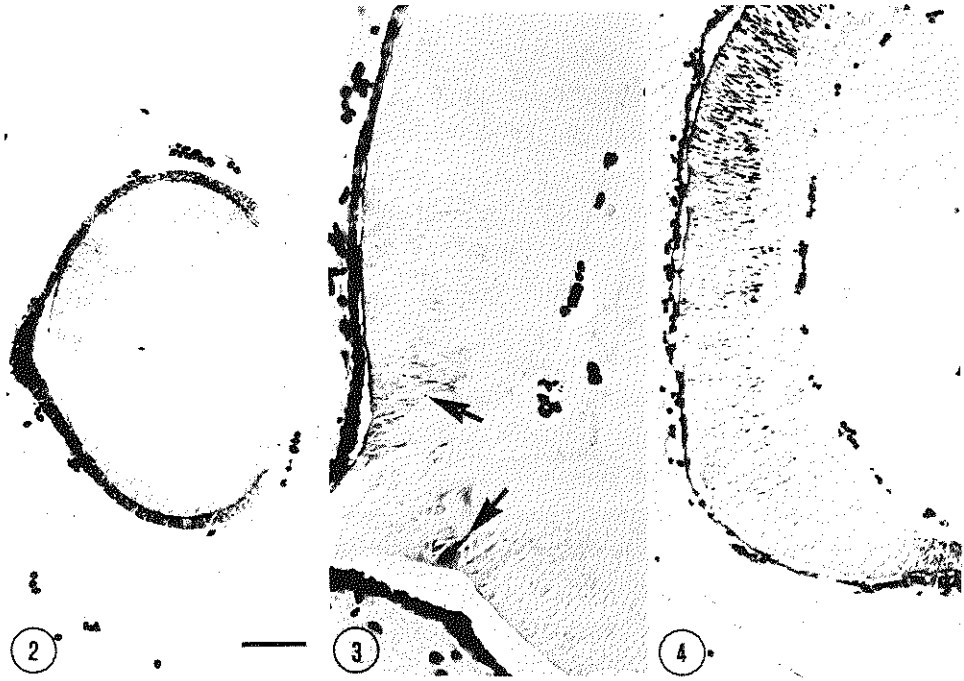
side which forms long villous processes projecting into the optic ventricle and interdigitating between the rod outer segments (Fig. 1). Enzyme activity is also seen along the lateral cell junctions but the central cytoplasmic part is negative.

Carbonic anhydrase activity in the different layers of the neural retina in the mouse is considered to be localized in Müller cells of which the perikarya located in the inner nuclear layer are completely stained. Intense enzyme activity around the outer limiting membrane extending into the optic ventricle and between the rod inner segments represent the apical part including the villous projection of the Müller cells, collectively producing the characteristic fibre basket (Polyak, 1941) pattern. In the outer nuclear layer a large number of stained obliquely oriented processes can be seen. These constitute the external part of the radial processes. The inner processes can be traced running across the inner plexiform layer and between the ganglion cells finally forming umbrellar expansions at their basal end facing the vitreous and in contact with the inner limiting membrane. The parts of the radial processes located within the inner nuclear layer show comparatively less staining. High carbonic anhydrase activity and the pattern of localization in the outer and inner plexiform layers suggest staining of the lateral processes of Müller cells.

Changes in the localization of carbonic anhydrase during prenatal development

The optic cup at 11 days of gestation consists of an outer layer of pigment epithelium containing 1-2 rows of columnar cells and an inner thick layer of neuroblasts, the presumptive neural retina. At this stage, a moderate activity of carbonic anhydrase is seen in the fundal area of the pigment epithelium but the reaction is weak towards




Figures 2-4. Changes in the localization of carbonic anhydrase in mouse retina during prenatal development. Bar = 50 µm.
Figure 2. Optic cup at 11 days, activity is seen only in the pigment epithelial cells.
Figure 3. At 13 days, pigment epithelial cells show increased activity. In the neural retina faint activity is seen in a few cells (arrow) near the optic nerve.
Figure 4. At 16 days, many presumptive Müller cells with high carbonic anhydrase activity are seen scattered in the fundal retina.

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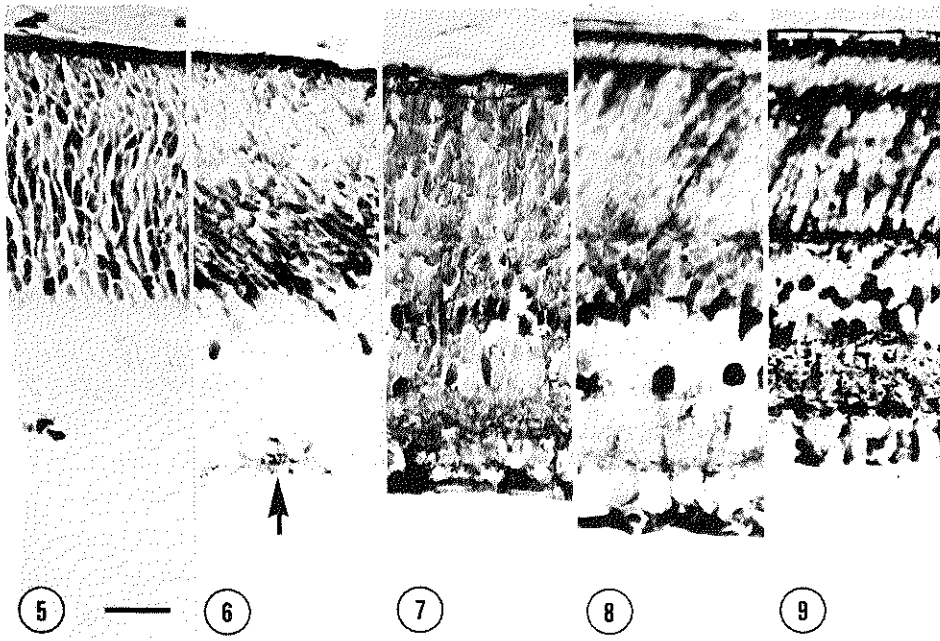
the peripheral areas (Fig. 2). The reaction product is evenly distributed in the cytoplasm around the nuclei but the intervening areas between the adjacent cells appear unstained. No enzyme activity is seen in the neural retina. At 13 days, the optic cup has grown in size. Carbonic anhydrase activity has appeared in the peripheral cells of the pigment epithelium. In the neural retina, faint reac-

tion is seen in a very few cells on either side of the optic nerve (Fig. 3). At 16 days (Fig. 4), the pigment epithelium consists of a single row of flattened cuboidal cells which are completely filled with reaction product. The retina, in the fundal region, has differentiated into an inner layer of ganglion cells, outer layer of neuroblasts and an incipient inner plexiform layer in between. In the outer layer of neuroblasts a large number of carbonic anhydrase positive cells are seen randomly distributed throughout the fundal region. The enzyme activity is seen only in the perikarya. On the basis of subsequent developmental changes, these cells can be easily identified as developing Müller cells. The presumptive Müller cells in the peripheral retina do not show any activity of the enzyme at this stage but the cells in the margin of the optic cup wich differentiate into iris show high carbonic anhydrase activity (Fig. 4, lower right corner).

Changes in the localization of carbonic anhydrase during postnatal development

In the newborn mouse, the retina has increased in thickness and the inner plexiform layer is well developed. The entire population of Müller cells now show carbonic anhydrase activity as stained cells are seen in the peripheral retina in the same frequency as in the fundus. However, many of these cells seem to have moved inwards from the outermost levels (Fig. 5). At 5 days, the outer plexiform layer has appeared in the fundal retina as a very thin horizontal fissure separating the inner and outer nuclear layers. At the same time the perikarya of Müller cells located in the outer retina have moved into the inner nuclear layer and no carbonic anhydrase positive cells are seen in the outer nuclear layer. While some of Müller cells remain at the innermost border of the inner nuclear layer many of them seem to have migrated outward producing a thick band




Figures 5-9. Changes in the localization of carbonic anhydrase activity in mouse retina during postnatal development. Bar = 30 μm.

Figure 5. Newborn, enzyme activity in Müller cells is seen only in the perikarya scattered in the undifferentiated layer of neuroblasts, note fewer stained cells in the scleral zone.

Figure 6. 5 days, note accumulation of Müller cell perikarya in the inner nuclear layer and appearance of activity along the inner limiting membrane (arrow).

Figure 7. 7 days, Müller cell perikarya arranged in their definitive location, note appearance of enzyme activity in the inner plexiform layer and increased activity along the inner limiting membrane.

Figure 8. 11 days, in the pigment epithelium the enzyme activity is seen in the vitreal and scleral surfaces. Note increased activity in the outer limiting membrane and appearance of activity in the outer nuclear layer and the outer plexiform layer.

Figure 9. 26 days, note increased enzyme activity in pigment epithelium and in Müller cell processes at all levels of the neural retina.

of Müller cell perikarya in the middle of this layer (Fig. 6). Except for the appearance of discrete staining near the inner limiting membrane, presumably in the end feet, carbonic anhydrase activity is still seen only within the perikarya. At 7 days, along with growth and separation of the retinal layers, the perikarya of Müller cell reach their final location (Fig. 7). These are mostly located in a single row along the middle of the inner nuclear layer and a few are located among the amacrine cells occupying the innermost border of this layer. Carbonic anhydrase activity along the inner limiting membrane is much increased and staining of moderate intensity is seen in the inner plexiform layer suggesting appearance of enzyme activity in the internal processes of Müller cells. In the outer retina very faint reaction is seen in the outer plexiform layer and along the outer limiting membrane. In the pigment epithelium, compared to the uniformly dark reaction seen at earlier stages, areas of cytoplasm now show reduced staining. At 11 days, a remarkable increase of carbonic anhydrase activity is seen in the outer retina and is most pronounced along the outer limiting membrane (Fig. 8). Some of the radial processes in the outer nuclear layer are moderately stained and the outer plexiform layer also shows increased staining. A differential distribution of the enzyme activity is seen in the pigment epithelium. Intense staining is recorded along the vitreal border, overlying the developing receptor segments; faint activity has appeared along the basal membrane at the scleral border but the intervening cytoplasm is completely negative. From this stage, the enzyme activity at all levels, increases rapidly and by 16 days resembles the pattern of localization seen in the adults (Fig. 9).

## Discussion

Carbonic anhydrase catalyzes the hydration of metabolic $CO_2$ into $HCO_3^-$ and facilitates exchange for plasma $Cl^-$ and $Na^+$ ions. Specific occurence of this enzyme in the glial cells of the brain is an indication of the special role of these cells in providing metabolic support maintaining the ionic milieu of the neurons (Giacobini, 1962). In retina, this function is shared by the pigment epithelial and Müller cells (Korhonen & Korhonen, 1965; Lasansky, 1965). Developmentally, these two cell-types show considerable difference in the time of appearance and changes in the intra-cellular distribution of carbonic anhydrase. In the embryonic optic cup, the pigment epithelial cells are, for several days, the only site of carbonic anhydrase activity, the neural retinal cells being completely negative. This suggests early metabolic dependence of the neural retina on the pigment epithelium and might be one of the reasons of developmental failure of isolated neural retina (Hollyfield & Witkovsky, 1974). The Müller cells, on the other hand, originate near the vitreal border, migrate outward and become scattered throughout the depth of the rapidly growing retina during the 11th and 15th days of gestation (Bhattacharjee & Sanyal, 1975). Carbonic anhydrase first appears in the perikarya of these cells around 16 days and permits a close follow-up of developmental changes in these cells in course of the histogenesis of retinal layers. The first noticeable change is the gradual shifting of the Müller cell perikarya towards their definitive position in the inner nuclear layer simultaneously with its separation from the outer nuclear layer. Enzyme activity begins to appear in the radial processes at this stage and progresses from the vitreal to the scleral end and finally, in the lateral processes as evidenced by the increased activity in the plexiform layers. As carbonic anhydrase activity appears in the Müller

cell processes, the pigment epithelium shows a gradual shift of the enzyme activity towards the scleral and vitreal border corresponding with the change in the distribution of mitochondria and rapid growth of their basal infoldings and apical microvilli (Moyer, 1969; Braekvelt & Hollenberg, 1970). Finally, a marked increase of carbonic anhydrase activity occurs at all sites and is most noticeable near the outer limiting membrane and the vitreal border of the pigment epithelium corresponding with the functional maturation of the retina as seen in growth of the rod outer segments (LaVail, 1973; Sanyal & Bal, 1973) and development of electroretinogram response (Noell, 1965) of which the b wave is known to be generated by the Müller cells (Miller & Dowling, 1970)..

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References

BHATTACHARJEE, J. & SANYAL, S. (1975). Developmental origin and early differentiation of retinal Müller cells in mice. J. Anat. (In press).

BHATTACHERJEE, P. (1971). Distribution of carbonic anhydrase in the rabbit eye as demonstrated histochemically. Exp.Eye Res. 12, 356-359.

BRAEKVELT, C. R. & HOLLENBERG, M.J. (1970). Development

of the retinal pigment epithelium, choriocapillaris and Bruch's membrane in the albino rat. Exp.Eye Res. 9, 124-131.

CASSIDY, M.M. & LIGHTFOOT, F.G. (1974). Carbonic anhydrase In Electron microscopy of enzymes, Principles and methods. Vol. 3 (ed. Hayat, M.A.) pp. 77-108. New York: Van Nostrand Reinhold Co.

GIACOBINI, E. (1962). A cytochemical study of the localization of carbonic anhydrase in the nervous system. J.Neurochem. 9, 169-177.

HANSSON, H.P.J. (1967). Histochemical demonstration of carbonic anhydrase activity. Histochemie 11, 112-128.

HOLLYFIELD, J.G. & WITKOVSKY, P. (1974). Pigmented retinal epithelium involvement in photoreceptor development and function. J.Exp.Zool. 189, 357-378.

HOLT, S.J. (1959). Factors governing the validity of staining methods for enzymes and their bearing upon the Gomori acid phosphatase technique. Exp. Cell Res. Suppl. 7, 1-27.

KORHONEN, E. & KORHONEN, L.K. (1965). Histochemical demonstration of carbonic anhydrase activity in the eyes of rat and mouse. Acta Opthalmol. 43, 475-481.

KUWABARA,T. & WEIDMAN, T.A. (1974). Development of the prenatal rat retina. Inv.Opthalmol. 13, 725-739.

LASANSKY, A. (1965). Functional implications of structural findings in retinal glial cells. Progress in Brain Res. 15, 48-72.

LaVAIL, M.M. (1973). Kinetics of rod outer segment renewal in the developing mouse retina. J.Cell Biol. 58, 650-661.

LEDER, O. (1966). Die Verteilung von carboanhydrase im Rattenauge. Pflügers Archiv 287, 351-356.

LIGHTFOOT, F.G. & CASSIDY, M.M. (1973). Characteristics of the staining reaction for carbonic anhydrase in light and electron histochemistry. J.Histochem.Cytochem. 21, 402.

LÖNNERHOLM, G. (1974). Carbonic anhydrase histochemistry, a critical study of Hansson's cobalt-phosphate method. Acta Physiologica scand. Suppl. 418, 1-43.

MILLER, R.F. & DOWLING, J.E. (1970). Intracellular response of the Müller cells of the mudpuppy retina. J.Neurophysiol. 33, 324-341.

MOYER, F.H. (1969). Development, structure and function of the retinal pigment epithelium. In The retina: Morphology, function and clinical characteristics (eds. Straatsma, B.R.; Hall, M.C.; Allen, R.A. and Crescitelli, F.) pp. 1-30. Los Angeles: University of California Press.

MUSSER, G.L. & Rosen, S. (1973). Localization of carbonic anhydrase activity in vertebrate retina. Exp. Eye Res. 15, 105-119.

NOELL, W. (1965). Aspects of experimental and hereditary retinal degeneration. In: Biochemistry of the retina (ed. Graymore, C.M.), pp. 51-72. London: Academic Press.

POLYAK, S.L. (1941). The retina. Chicago: The University Press.

ROSEN, S. (1974). Carbonic anhydrase - Alternative method. In Electron microscopy of enzymes, Principles and methods. Vol. 3 (ed. Hayat, M.A.) pp. 109-134. New York: Van Nostrand Reinhold Co.

ROSEN, S. & MUSSER, G.L. (1972). Observations on the specificity of newer histochemical methods for the demonstration of carbonic anhydrase activity. J.Histochem.Cytochem. 20, 951-954.

SANYAL, S. & BAL, A.K. (1973). Comparative light and electron microscopic study of retinal histogenesis in normal and rd mutant mice. Z.Anat.Entwickl.-Gesch. 142, 219-238.

UGA, S. & SMELSER, G.K. (1973). Electron microscopic study of the development of retinal Müllerian cells. Inv. Opthalmol. 12, 295-307.

# VI. SEQUENTIAL DIFFERENTIATION OF RETINAL CELLS IN THE MOUSE STUDIED BY DIAPHORASE STAINING

JOSOBANTA BHATTACHARJEE

Department of Anatomy, Faculty of Medicine,

Erasmus University Rotterdam,

Rotterdam. The Netherlands

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# INTRODUCTION

Presence of high levels of DPN and TPN linked dehydrogenases in the retina has been reported in histochemical studies based on the tetrazolium reaction (Wislocki & Sidman, 1954; Cogan & Kuwabara, 1959; Kuwabara, Cogan, Futterman & Kinoshita, 1959; Berkow & Patz, 1961a, b; Eranko, Niemi & Merenmies, 1961; Pearse, 1961; Niemi & Merenmies, 1963). Since the reaction depends on the activities of flavoprotein intermediates called diaphorases (Farber, Steinberg & Dunlap, 1956; Nachlas, Walker & Seligman, 1958; Scarpelli, Hess & Pearse, 1958; Cascarano & Zweifach, 1959), the positive sites are considered to represent substrate dependent dehydrogenase with accompaying diaphorase activity (Pearse, 1961). Although phenazine methosulphate as an artificial electron carrier has been used to bypass the diaphorase tetrazolium route, enzyme activity distinct from diaphorases has only been demonstrated in the retina in the case of succinic (Berkow & Patz, 1961b) and of lactic dehydrogenase Graymore & Kissun, 1969). In the developing retina of the rat, several dehydrogenases as well as diaphorases with nearly identical localization have been demonstrated to appear very early and precede the morphological differentiation of the retina (Berkow & Patz, 1964).

It appears that precise cytological localization of such enzymes (see material and methods) will permit early identification of differentiating retinal cells and will also enable study of their changes in localization which lead to the characteristic stratified organization of the retina. To this end, this report describes in detail the changing pattern of DPN and TPN diaphorase stained cells in the developing retina of the mouse, from the embryonic optic cup to the adult stage.

## MATERIAL AND METHODS

Albino mice of the Balb/cHeA strain were used throughout this study. The method of collection of the embryonic materials has been described earlier (Bhattacharjee & Sanyal, 1975.). Pregnant females were killed at daily intervals from day 10 till day 17 of pregnancy. The entire heads or pieces of the head region of the embryos including the eyes were dissected out and fixed for 30 minutes in ice-cold fixative, 4% paraformaldehyde and 7.5% sucrose in 0.067M phosphate buffer at pH 7.2. In addition, newborns and animals of 4, 7, 11, 14 and 26 days of age were used. They were killed by cervical dislocation and the eyes were immersed in the fixative described above. Then the cornea and lens were removed. Fixation was carried out over a total period of 15 minutes at 4°C. Use of unfixed tissues resulted in less and diffuse enzyme activity at comparable sites and also caused considerable damage to the retinal cytoarchitecture. Brief fixation at low temperature (Novikoff & Masek, 1958; Walker & Seligman, 1963; Pearse, 1972; Spector, 1975) permitted precise cytological localization of the enzymes without apparent loss in activity. Following fixation the tissues were washed overnight in gum-sucrose (Holt, 1959) and sectioned in a Pearse-Slee cryostat at 10 µm. Sections were collected on gelatin coated slides and stored at -20°C (Farber et al; 1956) for maximally 15 days. No detectable loss in enzyme activity could be demonstrated during this period.

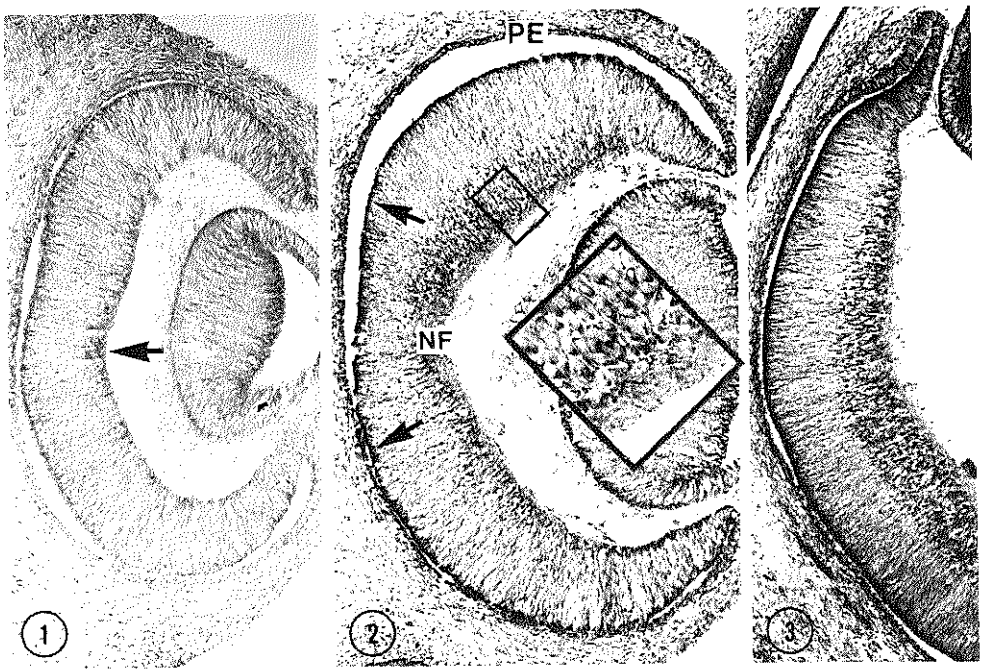
Before staining, the sections were thawed at room temperature (20°C) and allowed to dry. The DPN and TPN diaphorases were localized according to Burstone (1962), with reduced nicotinamide adenine dinucleotide (BDH) and reduced nicotinamide adenine dinucleotide phosphate (Boehringer) as substrates, with nitro blue tetrazolium (BDH) as electron acceptor and with 0.2M phosphate buffer

and 0.1M veronal acetate buffer for the respective enzymes at pH 7.4. The incubation was performed at 37°C for 15-20 minutes. Sections were rinsed in $H_2O$ and mounted in glycerine jelly (Gurr) without counterstaining.

Control sections, either incubated in media lacking substrates or preheated to 100°C in a humid chamber and then incubated in complete media, were negative. Addition of 1 X $10^{-3}$M KCN to the media or pretreatment of the sections in cold acetone (4°C) produced no difference in the staining reaction.

## RESULTS

The pattern of distribution of the two diaphorases is the same unless stated otherwise. The DPN diaphorase reaction appears however more intense and granular than the TPN diaphorase. Diaphorase activity first appears in the day 12 embryos, when a few cells at the centre of the vitreal margin of the optic cup show a faint reaction (Fig. 1). Faint staining indicating the outer limiting membrane is also seen. At day 13, a sharp increase in diaphorase activity is seen at different levels of the retina (Fig. 2). In the fundus area, particulate diaphorase activity is seen in a few rows of cells facing the vitreal border and corresponding in position to the developing ganglion cells (Hinds & Hinds, 1974). In the immediate vicinity of these cells, the appearance of a thin layer of nerve fibres with moderate staining is also discernible. More stained cells are seen at the nasal side of the optic cup than at the other side. Along the outer limiting membrane, diaphorase activity is very much increased and appears as a dark band between the outermost row of nuclei and the optic ventricle. As development proceeds and as the optic cup grows in size, more cells with diaphorase activity are located at the vitreal side of the retina and they gradually extend to the ora.




Figs. 1-3. Photomicrographs of cryostat sections of the embryonic retina showing changes in the activity of diaphorases. X 150

Fig. 1. Optic cup at 12 days, TPN diaphorase; note faint activity in a few cells near the vitreal border (arrow) and along the outer limiting membrane.

Fig. 2. 13 days, DPN diaphorase; note increased activity along the outer limiting membrane (arrow) and in the cells of the innermost layer, more pronounced in the nasal side of the retina. Moderate activity is seen in the pigment epithelium (PE) and layer of nerve fibres (NF). Inset, magnified view of area shown in rectangle; diaphorase activity is localized in the vitreal side of the cells.

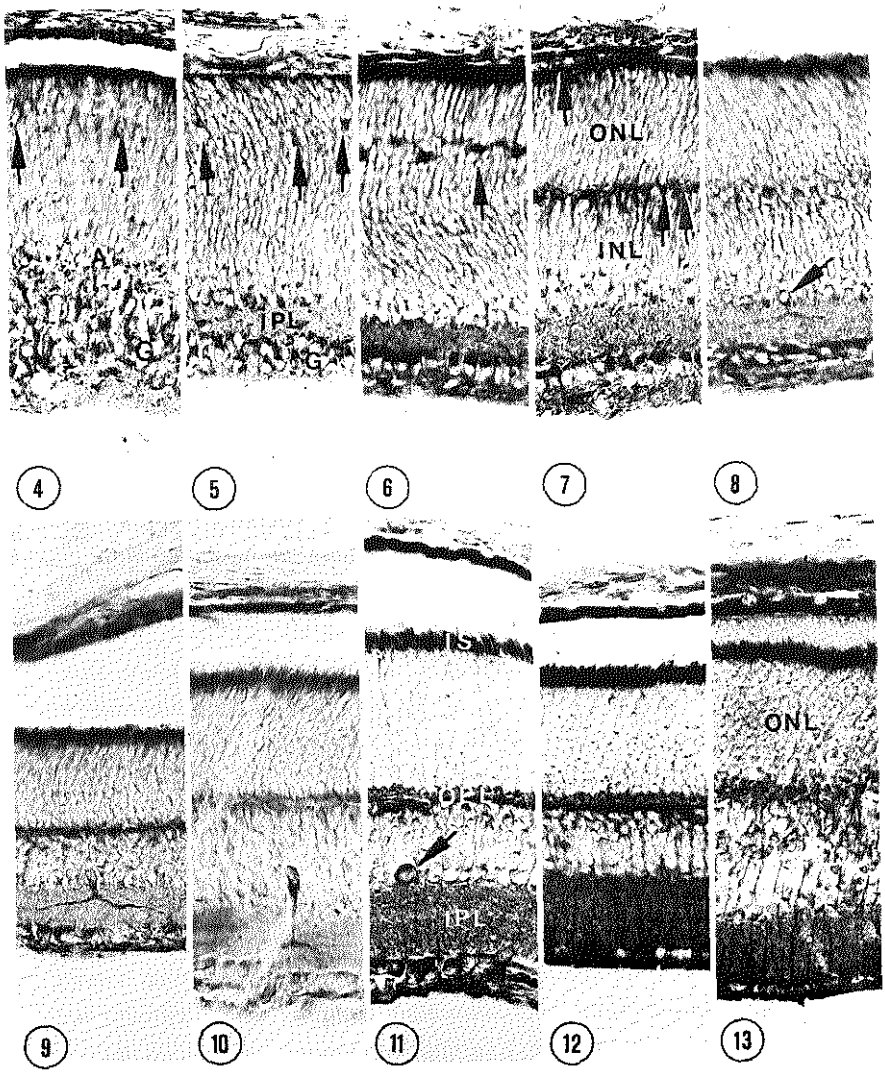
Fig. 3. Part of retina at 16 days, showing appearance of DPN diaphorase positive cells in the ora.

At this stage, the staining of these cells is localized at the vitreal side of the perikarya, facing the layer of nerve fibres (Figs. 2 and 4), i.e., at the axon hillock of these cells. At day 16, a dense layer of cells with diaphorase activity is seen throughout the retina extending upto the ora (Fig. 3). In the fundus area, rudiments of the inner plexiform layer have appeared and separate the inner rows of diaphorase positive cells in what is now readly recognized as the layer of the ganglion cells (Fig. 4) from the other, more outer retinal cells. On the scleral side of the developing inner plexiform layer 1-2 rows of cells showing moderate diaphorase activity in the vitreal side of their perikarya can be recognized as amacrine cells at early stage of differentiation At the scleral side, diaphorase activity along the outer limiting membrane is also increased.

At birth, the layer of ganglion cells is completely separated (Fig. 5) along with the growth of the inner plexiform layer. The ganglion cells show increased activity which is now localized at the scleral side of the perikarya In the outer level of the retina a row of evenly spaced cells extending up to the ora now show moderate activity. The outer plexiform layer develops at a later period along these cells when their identification as horizontal cells become apparent. A few cells showing diffuse staining but similar configuration and location could already be seen in the 16 day old embryos (Fig. 4). At 4 days, the ganglion cells have grown in size and show increased activity. Most of the amacrine cells do not show any change. However, intense reaction for both diaphorases can be localized in a few large amacrine cells scattered in the inner row of the inner nuclear layer. The horizontal cells have grown in size and show intense reaction (Fig. 6). At the outer margin of the developing horizontal cells an irregular line marking the appearance of the outer plexiform layer can be distinguished.

The processes of these cells show more staining with TPN than with DPN and are aligned horizontally along the presumptive outer plexiform layer. Along with the appearance of staining in the rod inner segments, reaction around the outer limiting membrane is very much increased. At 7 days, the outer plexiform layer is well developed and is located at a more inner level where moderate activity is localized (Fig. 7). Increased activity is now localized within the growing rod inner segments but the activity in the inner side of the outer limiting membrane is considerably reduced. In cells, identifiable as bipolar cells on the outermost levels of the inner nuclear layer, diaphorase activity has now appeared in the scleral side of the perikarya. No significant change in the localization of diaphorase activity is seen in the inner retina except in a few isolated amacrine cells. The vitreal side of the perikarya of these cells as well as the stout descending processes which branch and often traverse a great distance in the inner plexiform layer now show moderate reaction for TPN diaphorase while DPN diaphorase activity is seen only in the perikarya. The stained processes of these amacrine cells are seen to arborize at two different levels of the inner plexiform layer. Those cells which are located at the innermost row of the inner nuclear layer show stout descending processes and branch at a relatively outer level in the inner plexiform layer (Figs. 8,9) while some of these cells with perikarya located at more outer level, have long processes and branch at a more inner level (Fig. 10). At 11 days, the ganglion cells appear round and cells of different sizes can be distinguished. The nerve fibre layer shows dense precipitation of diformazan. The activity in the amacrine cells is except in the large ones, very much reduced (fig. 9). In the photoreceptor layer, developing outer segments are discernible but show no reaction.

In the retina of 14 day old mice an overall increase




Figs. 4-13. Photomicrographs of the central parts of retina of albino mice showing diaphorase activity at different stages of development. X 400

Fig. 4. Embryonic retina at 16 days, DPN diaphorase. Note rudiments of inner plexiform layer with heavily stained ganglion cells (G) in the innerside and moderately stained amacrine cells on the outerside (A). Faint activity appears in a few cells in the outer retina (arrow).

Fig. 5. Newborn, DPN diaphorase. Note distinct inner plexiform

layer (IPL). Activity in the ganglion cells (G) is seen in the scleral side of the perikarya. Sparsely distributed cells in the outer retina show moderate activity (arrow).

Fig. 6. 4 days after birth, DPN diaphorase. Note appearance of outer plexiform layer along the row of horizontal cells showing intense staining (arrow), increased activity near the outer limiting membrane and in the ganglion cells.

Fig. 7. 7 days, DPN diaphorase. Note activity in the developing rod inner segments (arrow). Outer plexiform layer (double arrow) has moved inward, with the growth of the outer nuclear layer (ONL). Bipolar cells in the outer rows of the inner nuclear layer (INL) shows diaphorase activity.

Fig. 8. 7 days, TPN diaphorase. Note reduced staining, positive reaction in large amacrine cell (arrow).

Fig. 9. 11 days, TPN diaphorase. Note increased staining in large amacrine cell with short descending process bifurcating in the inner plexiform layer.

Fig. 10. 14 days, TPN diaphorase activity in a large amacrine cell with a long descending process bifurcating at a different level.

Fig. 11. 14 days, DPN diaphorase. Note accumulation of activity in the rod inner segments (IS) and outer plexiform layer(OPL). Staining of the Müller cell processes in the inner plexiform layer (IPL) is first seen at this stage. Note increased activity in the ganglion cell and along the inner limiting membrane. Arrow points to a displaced ganglion cell.

Fig. 12. 26 days, DPN diaphorase. Note high activity at all levels of the retina.

Fig. 13. 26 days, TPN diaphorase. Note intense staining in the Müller cell processes which can be followed from the inner limiting membrane to their perikarya in the inner nuclear layer. Faint activity in the outer nuclear layer (ONL) is presumably located in the outer processes of the Müller cells.

in the activity of DPN diaphorase is seen. In the inner plexiform layer a large number of stained vertical processes has appeared (Fig. 11); the distal ends of these seem to be in connection with the cell bodies occupying the middle of the inner nuclear layer and corresponding in position and morphology to the Müller cells (Bhattacharjee, in press). These processes terminate proximally in a densely stained area along the inner limiting membrane. The staining is probably located in the adjoining basal endfeet of the Müller cell processes reported to have high concentration of mitochondria (Rasmussen, 1975). The bipolar cells show increased activity in the scleral side of the perikarya which collectively appear as a dark band. The outer plexiform layer has continued to widen and coarse diformazan granules are localized along its outer margin (Fig. 11). The rod inner segments are well developed and show intense reaction. Located among the inner row of amacrine cells, the large amacrine cells described earlier show increased TPN diaphorase activity (Fig. 10). At 26 days, the activity in the inner plexiform layer is considerably increased and dense reaction in the inner processes of the Müller cells is evident (Fig. 12). In the outer nuclear layer sparsely distributed diformazan granules, as well as faintly stained vertical processes are seen (Fig. 13) and seem to represent the external processes of the Müller cells.

In the pigment epithelium, moderate activity appears at day 13 of gestation (Fig. 2). From 16 days the activity gradually increases reaching adult pattern around day 14 after birth. At the initial stages of development the activity is localized in the perinuclear cytoplasm but with the gradual increase in the surface area dense precipitation of diformazan covers the entire epithelium except for the nuclei (Fig. 12).

## DISCUSSION

Differential localization of the diaphorase activity in the developing retina of the mouse permits identification of several retinal cell types at a very early stage

Ganglion cells: First appearance of diaphorase activity in the 12-13 day old mouse embryos in the presumptive ganglion cells near the vitreal border and along the outer limiting membrane is similar to the observation of Berkow and Patz (1964) in the rat and confirms the postulates of these authors that patterns of enzyme localization appear before morphological differentiation.

In mice, since we have followed the developmental stages at close intervals, it can be said that ganglion cells differentiate from 12-16 days of embryonic life. As most of the ganglion cells become postmitotic during this time (Sidman, 1961), acquisition of diaphorase activity can be considered to be an immediate expression of enzymatic differentiation. Change of intracellular site of diaphorase activity from the scleral to the vitreal side probably coincidies with the periods of axonal and dentritic growth respectively. This is indicated by the timing of the growth of the layer of nerve fibres and the inner plexiform layer. Therefore, this change in enzyme localization is likely to be related to the site of energy requirement of the cells.

Amacrine Cells : The amacrine cells with discrete activity in their perikarya, could be detected when their position in the innermost level of the inner nuclear layer is demarcated by the appearance of the inner plexiform layer. However, the possibility remains that these cells differentiate simultaneously with the ganglion cells as suggested by Cajal (1892). A further differentiation within the population of the amacrine cells could be followed from around 7 days after birth. This observation is based on the localization of TPN diaphorase activity, in the

processes and in the perikarya. A variety of large amacrine cells, of which the long processes run horizontally within the inner plexiform layer at two distinct levels, could be identified. The cells morphologically resemble the unistratified amacrine cells described in various other vertebrate species (Cajal, 1892; Castro, 1966; Boycott & Dowling, 1969; Gallego, 1971). Existence of sublayers in the inner plexiform layer as a result of synaptic contacts between various ganglion, amacrine and bipolar cells at several different strata have been described (Cajal, 1892; Polyak, 1941; Shen, Greenfield & Boell, 1956; Shen, 1958). In the mouse, synaptic contacts in the inner plexiform layer start developing from day 10 after birth (Olney, 1968) and at the completion of development five acetylcholinesterase positive bands can be recognized (Bhattacharjee & Sanyal. in press). Therefore it appears that the differentiation among the amacrine cells and the growth of the processes in distinct strata precedes synaptic development.

Horizontal cells: Very little information is available about the early developmental stages of the horizontal cells. Cajal (1929) demonstrated the appearance of these cells in 1 day old mice and speculated about their possible earlier origin. In our study, horizontal cells marked by increasing diaphorase activity could be detected first in the 16 day old embryos and have appeared throughout the retina, in the newborn mouse, when they are already positioned in a line where the outer plexiform layer later develops as the horizontal processes grow out. The outer plexiform layer along with the horizontal cells gradually shifts inwards as the perikarya of the photoreceptor cells are segregated in the outer nuclear layer (Sanyal & Bal, 1973). Thus the differentiation of the horizontal cells seems to be an initiating factor in the development of the outer plexiform layer where the photoreceptor terminals and the bipolar cell dendrites synapse..

Photoreceptor cells: Early differentiation of the photoreceptor cells is also marked by diaphorase activity which first appears in the embryonic retina along the outer limiting membrane, simultaneously with the differentiation of the ganglion cells as previously observed in the rat (Berkow & Patz, 1964). Indication of early differentiation of the photoreceptor cells is in contrast to the general assumption, based upon the late appearance of receptor elements, that these are the last retinal cells to differentiate. However, electron microscopic observations have shown the presence of rudimentary inner segments and accumulation of mitochondria along the outer limiting membrane in the newborn mouse (Olney, 1968; Caley et al, 1972; Sanyal & Bal, 1973). Early appearance and increasing diaphorase activity in the photoreceptor cells of the embryonic retina thus seem to reflect very early growth and accumulation of mitochondria preceeding the formation of the inner segments.

Bipolar cells: Diaphorase activity in the bipolar cells appears relatively late and coincides with the time of synaptic development in the outer plexiform layer starting from day 7 after birth (Olney, 1968).

Müller cells: In contrast to the other retinal sites, diaphorase activity appears in the Müller cells at day 14 after birth when histogenesis of the retinal layers is already completed and appears to coincide with the initiation of retinal function.

## SUMMARY

During retinal development of mice, the early stages of differentiation of ganglion, amacrine and horizontal cells were characterized by high diaphorase activity in the perikarya. The reaction in the ganglion cells which start differentiation at day 12 of gestation when the

layer of nerve fibres is developing was first localized near the axonal end; later in the period of dentritic growth it shifted to the scleral side. The amacrine cells were first detected on day 16 and showed a transient activity at the initial stage of their differentiation. A variety of large amacrine cells with long bifurcating processes appeared at day 7 after birth and showed particularly high TPN diaphorase activity. The horizontal cells could be followed from the 16th day of embryonic life. Their activity increased during the period of formation of the outer plexiform layer.

Along the outer limiting membrane appearance of diaphorase activity was marked from the day 13 of embryonic life and seemed to accumulate later in the rod inner segments. Activity in the bipolar cells first appeared at day 7 after birth and increased thereafter coinciding with the period of synaptic development in the outer plexiform layer. Activity in the Müller cells appeared around 14 days after birth and was most pronounced in the inner processes and basal end feet.

The sequential differentiation of retinal cells and their interrelation during hisotgenesis of the retina are discussed.

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REFERENCES

BHATTACHARJEE, J. Developmental changes of carbonic anhydrase in the retina of the mouse: Histochemical study. Histochemical Journal (in press).

BHATTACHARJEE, J. & SANYAL, S. Developmental changes of esterases in the retina of the mouse : Histochemical study. Histochemistry (in press).

BHATTACHARJEE, J. & SANYAL, S. (1975). Developmental origin and early differentiation of retinal Müller cells in mice. Journal of Anatomy 120 (in Press).

BERKOW, J.W. & PATZ, A. (1961a). Histochemistry of the retina I. Introduction and methods. Archives of Opthalmology 65, 820-827.

BERKOW, J.W. & PATZ, A. (1961b). Histochemistry of the retina. II. Use of phenazine methosulphate to demonstrate succinoxidase system. Archives of Opthalmology 65, 828-831.

BERKOW, J.W. & PATZ, A. (1964). Developmental histochemistry of the rat eye. Investigative Opthalmology 3, 22-33.

BOYCOTT, B.B. & DOWLING, J.E. (1969). Organization of the primate retina : Light microscopy. Philosophical Transactions of the Royal Society of London B. 255, 109-184.

BURSTONE, M.S. (1962). Enzyme Histochemistry and its application in the Study of Neoplasms. New York: Academic Press.

CAJAL, S. RAMON Y. (1892). The structure of the retina (Translated by S.A. Thorpe & M. Glickstein, 1972). Springfield : C.C. Thomas.

CAJAL, S. RAMON Y. (1929). Studies on vertebrate neurogenesis (Translated by L.Guth, 1960). Springfield: C.C. Thomas.

CALEY, D.W., JOHNSON, C. & LIEBELT, R.A. (1972). The postnatal development of the retina in the normal and rodless CBA mouse: A Light and electron microscopic

study. American Journal of Anatomy 133, 179-212.

CASCARANO, J. & ZWEIFACH, B.W. (1959). Factors influencing the histochemical demonstration of co-enzyme dependent dehydrogenase and diaphorases. Journal of Biophysical and Biochemical Cytology 5, 309-317.

CASTRO, G. DE OLIVEIRA. (1966). Branching pattern of amacrine cell processes. Nature 212, 832-834.

COGAN, D.G. & KUWABARA, T. (1959). Tetrazolium studies on the retina. II Substrate dependent pattern. Journal of Histochemistry and Cytochemistry 7, 334-341.

ERANKO, O., NIEMI, M. & MERENMIES, E. (1961). Histochemical observations on esterases and oxidative enzymes of the retina. In The Structure of the eye (Ed. G.K. Smelser), pp. 159-171. New York: Academic Press.

FARBER, E. STEINBERG, W.H. & DUNLAP, C.E. (1956). Histochemical localization of specific oxidative enzymes I. Tetrazolium stains for diphosphopyridine and triphosphopyridine nucleotide diaphorase. Journal of Histochemistry and Cytochemistry 4, 254-265.

GALLEGO. A. (1971). Horizontal and amacrine cells in mammal's retina. Vision Research Suppl. 3, 33-60.

GRAYMORE, C.N. & KISSUN, R.D. (1969). Use of phenazine methosulphate (PMS)in the histochemical localization of lactic acid dehydrogenase (LDH) in the retina. Experimental Eye Research 8, 375-378.

HINDS, J.W. & HINDS, P.L. (1974). Early ganglion cell differentiation in the mouse retina : An electron microscopic analysis utilizing serial sections. Developmental Biology 37, 381-416.

HOLT, S.J. (1959). Factors governing the validity of staining methods for enzymes and their bearing upon the Gomori acid phosphatase technique. Experimental Cell Research Suppl. 7, 1-27.

KUWABARA, T. & COGAN, D.G. (1959). Tetrazolium studies on the retina I. Introduction and technique. Journal of Histochemistry and Cytochemistry 7, 329-333.

KUWABARA, T., COGAN, D.G., FUTTERMAN, S., & KINOSHITA, J.H. (1959). Dehydrogenases in the retina and Müller ler's fibres. Journal of Histochemistry and Cytochemistry 7, 67-68.

NACHLAS, M.M., WALKER, D.G. & SELIGMAN, A.M. (1958). A histochemical method for the demonstration of diphosphopyridine nucleotide diaphorase. Journal of Biophysical and Biochemical Cytology 4, 29-36.

NIEMI, M. & MERENMIES, E. (1961). Cytochemical localization of the oxidative enzyme system in the retina. I. Diaphorase and dehydrogenases. Journal of Neurohistochemistry 6, 200-205.

NOVIKOFF, A.B. & MASEK, B. (1958). Survival of lactic dehydrogenase and DPN-diaphorase activities after formol-calcium fixation, Journal of Histochemistry and Cytochemistry 6, 217.

OLNEY, J.W. (1968). An electron microscopic study of synapse formation, receptor outer segment development, and other aspects of developing mouse retina. Investigative Opthalmology 7, 250-268.

PEARSE, A.G.E. (1961). Localization of oxidative enzymes in rat and chick retina in various physiological condition. In The structure of the eye (Ed. G.K. Smelser), pp. 53-72. New York : Academic Press.

PEARSE, A.G.E. (1972 ). Histochemistry, Theoretical and applied, 3rd ed., vol. 2. London : Churchill Livingstone.

POLYAK, S.L. (1941). The Retina. Chicago: The University Press.

RASMUSSEN, K.E. (1975). A morphometric study of Müller cell in rod and cone retinas with and without retinal vessels. Experimental Eye Research 20, 151-166.

SANYAL, S. & BAL, A.K. (1973). Comparative light and electron microscopic study of retinal histogenesis in normal and rd mutant mice. Zeitschrift für Anatomie und Entwickelungsgeschicte 142, 219-239.

SCARPELLI, D.G., HESS, R. & PEARSE, A.G.E. (1958). The cytochemical localization of oxidative enzymes. I. Diphosphopyridine nucleotide diaphorase and triphosphopyridine nucleotide diaphorase. Journal of Biophysical and Biochemical Cytology 4, 747-751.

SHEN, S.C. (1958). Changes in enzymatic patterns during development. In The Chemical basis of development (Eds. W.D. McElroy & B. Glass), pp. 416-432. Baltimore : John Hopkins Press.

SHEN, S.C., GREENFIELD, P. & BOELL, E.J. (1956). Localization of acetylcholinesterase in chick retina during histogenesis. Journal of Comparative Neurology 106, 433-461.

SIDMAN, R.L. (1961). Histogenesis of mouse retina studied with thymidine-$H^3$ In The Structure of the Eye. (Ed.G.K. Smelser), pp. 487-506. New York : Academic Press.

SPECTOR, G.J. (1975). The ultrastructural cytochemistry of lactic dehydrogenase, succinic dehydrogenase, dihydro-nicotinamide adenine dinucleotide diaphorase and cytochrome oxidase activities in hair cell mitochondria of the guineapig cochlea. Journal of Histochemistry and Cytochemistry 23, 216-234.

WALKER, D.G. & SELIGMAN, A.M. (1963). The use of formalin fixation in the cytochemical demonstration of succinic and DPN- and TPN- dependant dehydrogenases in mitochondria. Journal of cell Biology 16. 455-469.

WISLOCKI, G.B. & SIDMAN, R.L. (1954). The chemical morphology of the retina. Journal of Comparative Neurology 101, 53-91.

# VII. DEVELOPMENTAL CHANGES OF ESTERASES IN THE RETINA OF THE MOUSE : HISTOCHEMICAL STUDY

J. BHATTACHARJEE AND S. SANYAL

Department of Anatomy, Faculty of Medicine,
Erasmus University Rotterdam,
Rotterdam. The Netherlands

Histochemistry ( in press )

Summary. Using specific substrates and inhibitors, the presence of acetylcholinesterase (AChE), carboxylesterase (B-esterase) and arylesterase (A-esterase) has been histochemically studied in the developing mouse retina.

In the neural retina the perikarya of the Müller cells, ganglion cells and horizontal cells showed high B-esterase activity at the initial stages of their development. In the Müller cells, a second period of B-esterase activity, coinciding with the time of formation of their lateral processes, was found.

AChE first appeared in the ganglion cells at birth and increased thereafter. With the progress of synaptic development, AChE activity in the ganglion cell perikarya was reduced while the enzyme rapidly accumulated in the inner plexiform layer. AChE was also localized in the horizontal cells at the initial stage of their development and completely disappeared thereafter. AChE activity was localized in the outer plexiform layer at the time of weaning.

A-esterase was detected in the ganglion cells only and appeared to be lysosomal.

In the pigment epithelium only B-esterase was present. The enzyme first appeared in the late prenatal period and rapidly increased after birth.

## Introduction

Esterase activity can be demonstrated with a number of substrates and accordingly comprises several enzymes. Comparison of relative stainability with different substrates and the use of varous inhibitors permit a characterization of the reaction products as due to acetylcholinesterase, cholinesterase, or nonspecific esterase A, B, or C. The Neuronal

and glial components of the vertebrate retina, stratified into distinct layers, show a very characteristic staining pattern for several of these enzymes (Koelle, Wolfland, Friedenwald and Allen, 1952; Eränkö, Niemi and Merenmies, 1961; Esilä, 1963; Nichols and Koelle, 1968). In the developing retina, specific changes of acetylcholinesterase in the course of synaptogenesis in the chick (Shen, Greenfield and Boell, 1956; Shen, 1958), rabbit (Raviola and Raviola, 1962) and rat (Wolff, 1969; Spira, 1974) and of nonspecific esterase during the early differentation of the Müller cells in mice (Bhattacharjee and Sanyal, 1975) have been found.

In this study, changes in the activity of the various esterases during the histogenesis of the retina in the mouse have been followed from the earliest stage of optic cup formation till the functional maturation of the retinal cells.

## Materials and Methods

Albino mice of the Balb/cHeA strain were used throughout this study. The method of obtaining and processing of the embryonic materials has been described earlier (Bhattacharjee and Sanyal, 1975). Newborn and older animals were killed by cervical dislocation and the eyes were immediately removed to ice cold fixative: 4% paraformaldehyde and 7.5% sucrose in 0.067M phosphate buffer at pH 7.2. After one hour the cornea and lens were removed and the open eyeballs were fixed overnight in the same fixative. The materials were washed for 24 hours in gum-sucrose (Holt, 1959) and were sectioned in a cryostat at 10 µm. The sections were mounted on gelatin coated slides, air dried and were either used immediately or stored in a refrigerator up to 7 days.

Cholinesterases were localized according to the procedure of Karnovsky and Roots (1964) with acetylthiocholine-

iodide (BDH) and butyrothiocholineiodide (BDH) as substrates at pH 6.0. Incubation for 90 minutes at 37°C was found to be optimal. Nonspecific esterase was localized according to Barka and Anderson (1963) using $\alpha$-napthyl acetate (Sigma) as substrate and hexazotized pararosaniline as coupler at pH 6.5. The sections were incubated for 20 minutes at 37°C.

Eserine (BDH) and diethyl-p-nitrophenylphosphate (E600, Sigma) were used to discriminate between the cholinesterase and nonspecific esterase activity according to Pearse (1972). 15,-bis- (4-allyldimethylammoniumphenyl) pentan-3-one dibromide (BW284C51, Wellcome) and tetraisopropylpyrophosphoramide (iso-OMPA, Koch-Light) were used to determine the nature of cholinesterase involved (Silver, 1974).

## Results

Characterization of retinal esterases - Based on observations with different substrates and inhibitors three different esterases were identified in the mouse retina.

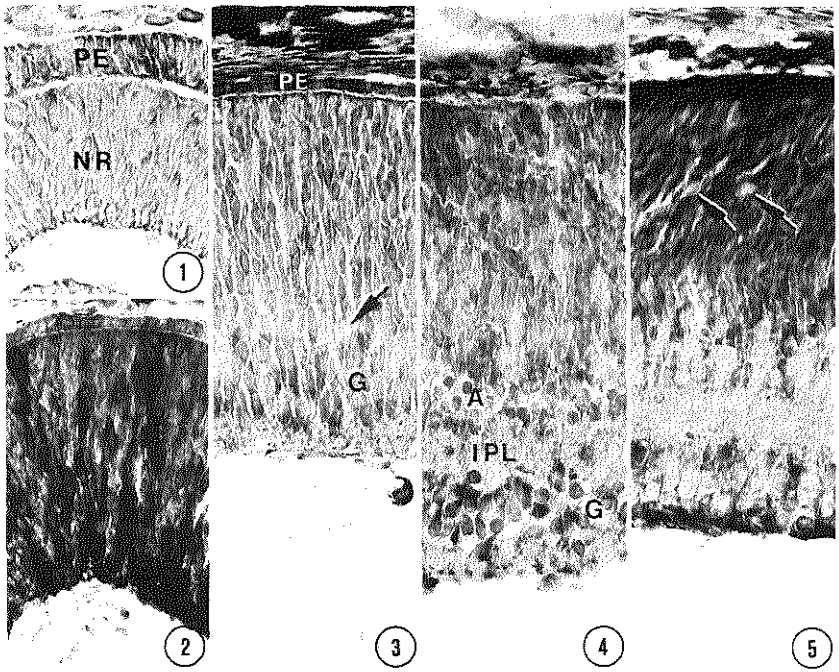
1. Acetylcholinesterase (E.C. 3.1.1.7., AChE) has been localized in the ganglion cells, horizontal cells, inner plexiform layer and outer plexiform layer at different stages of development. This enzyme hydrolyses $\alpha$-napthyl acetate and acetylthiocholineiodide but the staining reaction with both substrates is completely inhibited after preincubation in 0.1 $\mu$M E600 and preincubation and incubation with 1 $\mu$M BW284C51. The intensity of staining remains unaffected after treatment with 0.2mM iso-OMPA, a specific inhibitor of cholinesterase (E.C. 3.1.1.8., also called pseudocholinesterase). This latter enzyme seems to be absent in the mouse retina as no reaction could be obtained with butyrothiocholineiodide even after prolonged incubation.

2. Arylesterase (E.C. 3.1.1.2., A-esterase), generally considered as organophosphate resistant, has been localized only in the ganglion cells with $\alpha$-napthyl acetate after preincubation and incubation with 10 µM E600.
3. Carboxylesterase (E.C. 3.1.1.1., B-esterase) accounts for most of the nonspecific esterase activity in the retina and is identified by its resistance to 0.1 µM E600 and sensitivity to 10 µM E600.

Acetylesterase (E.C. 3.1.1.6., C-esterase) usually identified by its activation with 0.1mM PCMB (p-Chloromercuribenzoate) after preincubation with 10 µM E600 was not found in the mouse retina.

Developmental changes of retinal esterases - The optic cup is formed in the mouse embryo around 9-10 days of gestation. Its outer layer forms the pigment epithelium and the inner layer differentiates into the various cell components of the neural retina. The cellular sites of various esterases and their changes in the course of development are presented in Table 1. Up to 10 days, no activity of any of the esterases could be detected (Fig. 1). At 11 days, a few presumptive Müller cells, located in the vitreal border of the optic cup (Bhattacharjee and Sanyal, 1975), first show B-esterase activity. At 13 days, many such cells with high B-esterase activity are found scattered in the fundus area (fig. 2) and at 15 days have also appeared in the ora. At 16 days, rudiments of the inner plexiform layer can be seen (unstained) and the ganglion cells show faint B-esterase activity (Fig. 3). The Müller cells, on the other hand, then become completely negative except in the ora, where diminished activity still persists.

In the newborn mice, the neural retina has grown in thickness and the inner plexiform layer is well developed. The perikarya of ganglion cells show increased activity of B-esterase (Fig. 4) and now also contain AChE. The enzyme is diffusely localized in the cytoplasm




Figs. 1-5. Distribution of esterases during early histogenesis of mouse retina. $\alpha$-napthyl acetate, counterstained with methyl green x 400.

Fig. 1. Retina at 10 days of gestation. Note absence of reaction in the pigment epithelium (PE) and neural retina (NR).

Fig. 2. At 13 days of gestation the presumtive Müller cells show high esterase activity.

Fig. 3. 16 days, note loss of esterase activity in Müller cells, emergence of the inner plexiform layer (arrow). Developing ganglion cells (G) and pigment epithelial cells (PE) show faint reaction.

Fig. 4. Newborn. Note appearance of amacrine cells (A), widening of the inner plexiform layer (IPL) and increased esterase activity in ganglion cells.

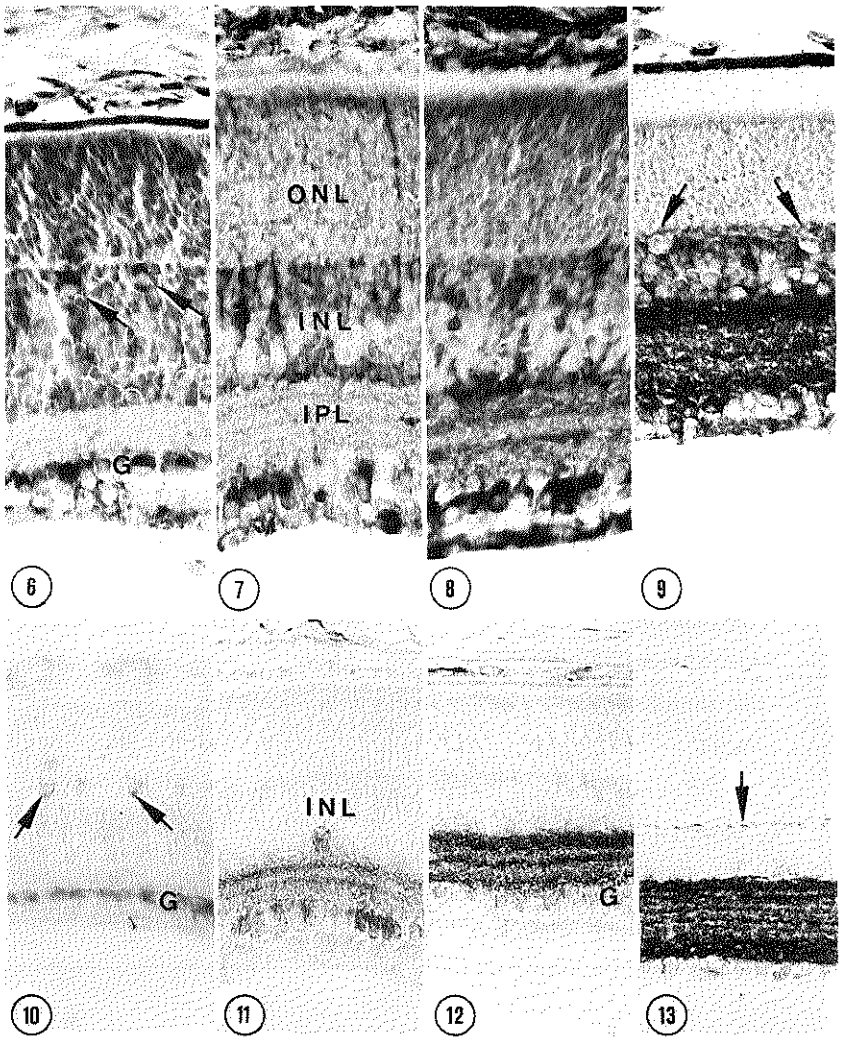
Fig. 5. 4th postnatal day. Pigment epithelium showing strong activity. Developing horizontal cells (arrow) are first discernible and show faint reaction.

Table 1. Changes in the histochemical localization of esterases in the developing retina of mouse

| Site of activity | Enzyme present | Prenatal period Days after mating | | | Postnatal period Days after birth | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 11 | 13 | 16 | 0 | 4 | 6 | 10 | 12 | 26 |
| NF | B-Esterase | | + | + | + | + | + | + | + | + |
| GC | AChE | | | - | + | + | ++ | ++ | + | + |
| | A-Esterase | | | - | - | - | - | - | + | + |
| | B-Esterase | | | + | ++ | ++ | +++ | +++ | +++ | ++ |
| IP | AChE | | | - | - | - | - | + | ++ | +++ |
| MC | B-Esterase | ++ | +++ | + | - | - | ++ | +++ | ++ | ++ |
| HC | AChE | | | | | + | ++ | + | - | - |
| | B-Esterase | | | | | + | +++ | ++ | + | - |
| OP | AChE | | | | | - | - | - | - | ++ |
| RI | B-Esterase | | | | | + | + | + | + | + |
| PE | B-Esterase | - | - | + | ++ | ++ | +++ | +++ | +++ | +++ |

Abbreviations : NF, layer of nerve fibres; GC, ganglion cells; IP, inner plexiform layer; MC, Müller cells; HC, horizontal cells; OP, outer plexiform layer; RI, rod inner segments; PE, pigment epithelium.

Symbols : -, negative staining; +, faint; ++, moderate; +++, intense staining.




Figs. 6-9. Distribution of esterases at different stages of postnatal development; $\alpha$-napthyl acetate counterstained with methyl green X 400.

Fig. 6. 6 days after birth, horizontal cells (arrow) and ganglion cells (G) show increased activity.

Fig. 7. 10 days, Müller cell perikarya in the inner nuclear layer (INL) show intense reaction; activity is also seen in external processes in the outer nuclear layer (ONL). Two faintly stained horizontal bands appear in the inner plexiform layer (IPL).

as observed in the rat (Spira, 1974). At 4 days after birth, simultaneously with the appearance of the outer plexiform layer, a number of cells lying closely apposed to it on the vitreal side become distinguishable by faint activity of B-esterase (Fig. 5) and AChE. Similar cells could also be detected in the newborn mice by prolonged incubation with $\alpha$-napthyl acetate. At 6 days, the perikarya of these cells are enlarged and show increased activity of both the enzymes (Fig. 6 and 10) and are easily identifiable as horizontal cells. In the layer of ganglion cells, a few larger cells with intense B-esterase activity can be distinguished at this stage among cells of smaller sizes with relatively less staining.

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Fig. 8. 12 days, note increased staining of the bands in the inner plexiform layer, Müller cell processes are no longer visible but the perikaryal activity persists.

Fig. 9. 26 days. The activity in the inner plexiform layer is greatly increased. Horizontal cells (arrow) are completely negative.

Figs. 10-13. Distribution of acetylcholinesterase at different stages of postnatal development, acetyl-thiocholineiodide, without counterstaining, X 300.

Fig. 10. 6 days, note staining in horizontal (arrows) and ganglion cells (G).

Fig. 11. 10 days. Two parallel bands are seen in the inner plexiform layer. A displaced ganglion cell is recognized in the inner nuclear layer (INL).

Fig. 12. 12 days. Note increased staining of the bands in inner plexiform and reduced activity in ganglion cells (G).

Fig. 13. 26 days. Maximum level of activity in the inner plexiform layer where between two heavily stained bands three faint bands can be traced. Note discrete activity along the outer plexiform layer (arrow).

At 10 days, an indication of synaptic development in the inner plexiform layer is seen in the appearance of AChE activity localized in two distinct bands ( Fig. 11 ); at the same time, the smaller ganglion cells still show perikaryal staining. A few cells showing similar staining properties are seen at the innermost level of the inner nuclear layer among the perikarya of amacrine cells which are completely devoid of enzyme activity. Absence of AChE in the amacrine cells of the mouse is in keeping with previous observation in other species with primarily rod retina ( Esilä, 1963; Nichols & Koelle, 1968; Spira, 1974 ). The perikarya of Müller cells show intense B-esterase activity and some of the radial processes are also stained ( Fig. 7 ). At 12 days, AChE activity in the inner plexiform layer is increased and the staining appears in three distinct bands ( Fig. 12 ). The horizontal cells do not show any AChE activity and their B-esterase is also reduced. Appearance of faint A-esterase activity in the ganglion cells, more pronounced in the larger ones,can be detected at this stage. The staining is localized in a dense mass close to the nuclei. From this stage on, a progressive increase of AChE activity is recorded in the inner plexiform layer and at the time of weaning ( 26 days ), five intensely stained bands are seen ( Fig. 13 ). Some AChE activity has also appeared in the outer plexiform layer. B-esterase activity in the ganglion cells is reduced and has completely disappeared from the horizontal cells ( Fig. 9 ). Rod inner segments, which are first seen at 4 days, show faint B-esterase activity throughout the period of development.

In the pigment epithelium, only B-esterase could be detected and the enzyme first appears around the 16th day of embryonic life. The activity increases at birth and attains maximum level around 6 - 8 days ( Fig. 6 ).

## Discussion

The first appearance of a differential esterase activity within a uniformly non-reactive and otherwise homogeneous population of cells can be considered as an expression of differentiation. The histochemical changes of specific esterases observed in the developing retina of mice fall into two categories. Besides progressive changes leading to the characteristic adult pattern, transient presence of esterases has been localized in certain cells in specific periods of development. High B-esterase activity marks the initial phase of differentiation of the Müller cells which originate in the vitreal border and migrating outward lose their enzyme activity. The significance of the early developmental changes in the Müller cells has already been discussed ( Bhattacharjee and Sanyal, 1975 ). At the initial stage of development, the ganglion cells show - first - B-esterase and shortly afterwards AChE activity. As the perikarya of the ganglion cells grow in size these enzymes are rapidly increased. During this stage, appearance of some large ganglion cells with relatively more enzyme activity and of a few similar cells in the innermost level of the inner nuclear layer is striking and these cells resemble the giant and displaced ganglion cells observed in the retina of many other species ( Cajal, 1892; Polyak, 1941; Shen et al., 1956; Shkolnik-Yarros, 1971; Stell and Witkovsky, 1973; Anctil & Ali, 1974 ). The origin and early developmental changes of the horizontal cells are also marked by B-esterase and AChE activity. The enzyme activities rapidly increase as the cell perikarya grow and assume their definitive position along the inner aspect of the developing outer plexiform layer.

Renewed B-esterase activity in the Müller cells coincides with the time of increased carbonic anhydrase activity in the outer and inner plexiform layers indicating

growth of the lateral processes ( Bhattacharjee, in press ). Near the outer plexiform layer, where synaptic contacts are established earlier than in the inner plexiform layer ( Olney, 1968; Weidman & Kuwabara, 1968 ), a rapid loss of B-esterase and AChE activity of the horizontal cells is recorded. AChE activity, which appears later in the outer plexiform layer is possibly located in the processes of the horizontal cells ( Dickson, Flumerfelt, Hollenberg & Gwyn, 1971 ).

Next, the appearance of AChE positive bands in the inner plexiform layer and their progressive increase in number and intensity in staining represent increasing arborization and synaptic contacts between the processes of various types of ganglion, bipolar and amacrine cells. Concomitant reduction of AChE in the perikarya of the ganglion cells suggests a transfer of enzyme from the site of synthesis to the dendritic terminals. But the possibility of other sources of AChE in the inner plexiform layer must be considered as Spira ( 1974 ) has demonstrated the presence of this enzyme in almost every cell process in this layer. Furthermore, the existence of cholinergic centrifugal fibres as a source of retinal AChE has been suggested ( Lewis & Shute, 1965 ) but the issue remains controversial as far as the mammalian retina is concerned ( Nichols & Koelle, 1968 ).

A-esterase has been detected only in the ganglion cells and the distribution resembles that of acid phosphatase and N-Acetyl-β-glucosaminidase ( Sanyal, 1970; 1972 ). Therefore this enzyme seems to be lysosomal, as organophosphate resistant esterase has been demonstrated in the lysosomes of other tissues as well ( Holt, 1963 ). Its absence in other retinal cells is an indication of lysosomal heterogeneity among the retinal cells.

In the retina of the adult mice, a relatively high B-esterase activity is maintained in the pigment epithelial and the Müller cells which are known to play a special role in the metabolic support of the neuronal cells ( Lasansky,1965).

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## References

Anctil, M., Ali, M.A. : Giant ganglion cells in the retina of the hammerhead shark ( Sphyrna lewini ). Vision Res. 14, 903 - 904 ( 1974 ).

Barka, T., Anderson, P.J. : Histochemistry, Theory, Practice and Bibliography. New York. Hoever 1963.

Bhattacharjee, J. : Developmental changes of carbonic anhydrase in the retina of the mouse : Histochemical study. Histochem. J. ( in press ).

Bhattacharjee, J., Sanyal, S. : Developmental origin and early differentiation of retinal Müller cells in mice. J. Anat. 120, in press ( 1975 ).

Cajal, S. Ramon Y. : The structure of the retina ( 1892 ). Transl. by S.A. Thorpe and M. Glickstein. Springfield, Ill. Thomas 1972.

Dickson, D.H., Flumerfelt, B.A., Hollenberg, M.J., Gwyn, D.G. : Ultrastructural localization of cholinesterase activity in the outer plexiform layer of the newt retina. Brain Res. 35, 299 - 303 ( 1971 ).

Eränkö, O., Niemi, M., Merenmies, E. : Histochemical observation on esterases and oxidative enzymes of the retina. In : The structure of the eye, edit. G.K. Smelser, p. 159 - 171. New York: Academic Press 1961.

Esilä, R. : Histochemical and electrophoretic properties of cholinesterases and nonspecific esterases in the retina of some mammals including man. Acta Ophthalmol. Suppl. 77, 1 - 113 ( 1963 ).

Holt, S.J. : Factors governing the validity of staining methods for enzymes and their bearing upon the Gomori acid phosphatase technique. Exp. Cell Res. Suppl. 7, 1 - 27 ( 1959 ).

Holt, S.J. : Some observation on the occurrence and nature of esterase in lysosomes. In : Ciba foundation symposium, Lysososmes, p. 114 - 120. London : Churchill 1963.

Karnovsky, M.J., Roots, L. : A direct coloring thiocholine method for cholinesterases. J. Histochem. Cytochem. 12, 219 - 221 ( 1964 ).

Koelle, G.B., Wolfland, L., Friendenwald, J.S., Allen, R.A. : Localization of specific cholinesterase in ocular tissues of the cat. Amer. J. Ophthalmol. 35, 1080 - 1084 ( 1952 ).

Lasansky, A. : Functional implications of structural findings in glial cells. Progress in Brain Res. 15, 48 - 72 ( 1965 ).

Lewis, P.R., Shute, C.C.D. : Fine localization of acetylcholinesterase in the optic nerve and retina of the rat. J. Physiol. ( London ) 180, 8P - 10P ( 1965 ).

Nichols, C.W., Koelle, G.B. : Comparison of the localization of acetylcholinesterase and nonspecific cholinesterase activities in mammalian and avian retina. J. Comp. Neurol. 133, 1 - 16 ( 1968 ).

Olney, J.W. : An electron microscopic study of synapse formation, receptor outer segment development, and other aspects of developing mouse retina. Invest. Ophthalmol. 7, 250 - 268 ( 1968 ).

Pearse, A.G.E. : Histichemistry theoretical and applied, 3rd ed. Vol. II. London : Churchill 1972.

Polyak, S.L. : The retina. Chicago : The University Press 1941.

Raviola, E., Raviola, G. : Richerche istochemiche sulla retina di conglio nel corso dello sviluppo postnatale. Z. Zellforsch. 56, 552 - 572 ( 1962 ).

Sanyal, S. : Changes of lysosomal enzymes during hereditary degeneration and histogenesis of retina in mice. I. Acid phosphatase visualized by azo dye and lead nitrate methods. Histochemie 23, 207 - 219 ( 1970 ).

Sanyal, S. : Changes of lysosomal enzymes during hereditary degeneration and histogenesis of retina in mice. II. Localization of N-Acetyl-β-glucosaminidase in macrophages. Histochemie 29, 28 - 36 ( 1972 ).

Shen, S.C. : Changes in enzymatic patterns during development. In : A symposium on the chemical basis of development, edit. W.D. McElroy and B. Glass, p. 416 - 432. Baltimore : John Hopkins Press 1958.

Shen, S.C., Greenfield, P., Boell, E.J. : Localization of acetylcholinesterase in chick retina during histogenesis. J. Comp. Neurol. 106, 433 - 461 ( 1956 ).

Shkolnik-Yarros, E.G. : Neurons of the cat retina. Vision Res. 11, 7 - 26 ( 1971 ).

Silver, A. : The biology of cholinesterases. Amsterdam : North Holland Publishing Co. 1974.

Spira, A.W. : Ultrastructural localization of cholinesterase activity in the developing rat retina. J. Histochem. Cytochem. 22, 668 - 680 ( 1974 ).

Stell, W.K., Witkovsky, P. : Retinal structure in the smooth dogfish, Mustellus canis : General description and light microscopy of giant ganglion cells. J. Comp. Neurol 148, 1 - 32 ( 1973 ).

Weidman, T.A., Kuwabara, T. : Postnatal development of rat retina. Arch. Ophthalmol. 79, 470 - 484 ( 1968 ).

Wolff, H.H. : Über die Entwicklung des Enzymmusters der Rattenretina. Histochemie 17, 11 - 29 ( 1969 ).

# VIII. GENERAL DISCUSSION

The pattern of neurogenesis in the retina, emerging from the findings in the foregoing chapters shows three aspects, which may be discussed separately: 1. the sequence of development of the various retinal cells. 2. possible interrelations in retinal histogenesis. 3. the enzymatic differentiation of the retina.

## The sequence of development of the retinal cells

The first sign of differentiation among the cells of the neural retina was seen at 11 days of embryonic life when a small batch of elongated cells could be distinguished by their reaction for nonspecific esterase (B-esterase) near the centre of the vitreal margin of the optic cup. From their morphology and location (Cajal, 1892; Uga & Smelser, 1973; Kuwabara & Weidman,1974) these cells were identified as Müller cells. Evidence derived from our studies with other enzymes suggest that these are the earliest of the retinal cells to differentiate. As the retina increases in size, the differentiating Müller cells migrate outward and then occupies different levels of the neural retina including the ora. While increasing numbers of Müller cells differentiate, we found at 12 days, a very few cells close to the centre of the vitreal border that could be distinguished by diaphorase activity and in our view represent the earliest batch of differentiating ganglion cells. Within the next 24 hours, when more ganglion cells have appeared at the vitreal side of the retina, Müller cells are seen, seemingly spread randomly throughout the depth of the retina. Although it has been reported

by Coulombre (1955) that ganglion cells and Müller cells differentiate concurrently, an earlier origin of ganglion cells has been indicated by autoradiographic studies in several species (Fujita & Horii, 1963; Jacobson, 1968; Hollyfield, 1972; Kahn, 1973, 1974) including mice (Sidman 1961).

The difference in the time of appearance of the esterase and diaphorase positive cells, as well as their differential location in the retina at later stages of development, appear to provide clear evidence that differentiation of Müller cells precedes in mice that of ganglion cells. This is in agreement with the sequence of differentiation demonstrated by electron microscopy in the retina of the rabbit (Uga & Smelser, 1973) and the rat (Kuwabara & Weidman, 1974). Moreover, the period during which in mice the ganglion cells become postmitotic (Sidman, 1961) coincides precisely with the period of ganglion cell differentiation as determined by diaphorase activity. Since autoradiographic studies could not determine when the Müller cells become postmitotic it has been speculated that they divide throughout life (Kahn, 1974). Attempts have been made in this laboratory (Sanyal & Battacharjee, unpublished) to label and locate Müller cells by autoradiography after staining cryostat sections of embryonic retina from thymidine-$H^3$ injected mice for nonspecific esterase. When the animals were injected at day 10 or 11 of gestation, labelling was too slight to provide any clear answer. In contrast, if animals were injected at day 12 or 13 of gestation, all the esterase positive cells were clearly unlabelled, suggesting that the cells had stopped dividing. Moreover visual estimation of the number of esterase stained Müller cells at day 15 of gestation and comparison of this number with the number of carbonic anhydrase positive cells in the retina of adult mice suggested that the entire population of Müller cells is produced between 11-15 days of embryonic development. In case of the ganglion cells the comparable

period seemed to span the period between days 12 and 16 of gestation.

The precise localization of the amacrine cells prior to the separation of the ganglion cell layer proved difficult since both cell types show diaphorase activity at the initial stage of differentiation. However, comparison of the position of the ganglion cells and amacrine cells at day 16 of gestation with that of the mass of the diaphorase positive cells seen at day 13, indicated that some of the amacrine cells had already appeared at day 13. Thus, it is reasonable to conclude that differentiation of amacrine cells closely follows the differentiation of the ganglion cells. This conclusion is in line with the autoradiographic findings in mice (Sidman, 1961) and chick (Fujita & Horii, 1963; Kahn, 1974).

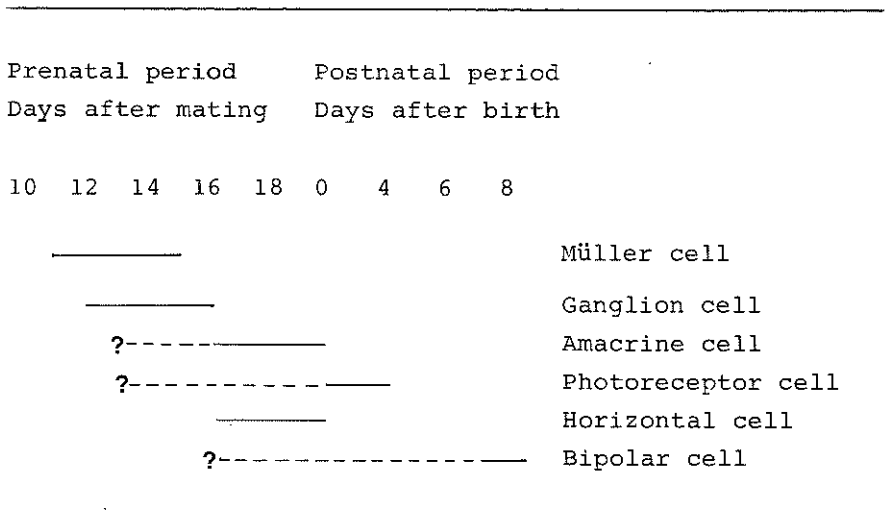
The first indication of the phororeceptor development appeared around day 4 after birth from the emergence of the rudiments of mitochondria-studded receptor inner segments (de Robertis, 1956), when a high diaphorase activity was also observed in these structures. On the other hand the progressive accumulation of formazan granules around the outer limiting membrane, starting from day 13 of gestation is suggestive of an earlier differentiation of these cells. It appears that marshalling of mitochondria at the prospective site of receptor development is part of the process of differentiation of the visual cells. Since autoradiographic data indicate that some of the photoreceptor cells become postmitotic relatively early, while others continue to divide (Sidman, 1961), it is reasonable to conclude that increasing diaphorase activity in the presumptive receptor site is the resultof a gradual increase in the number of cells starting to differentiate, as well as of mitochondrial accumulation in individual cells.

The location of horizontal cells along the inner margin of the outer plexiform layer (Polyak, 1941; Walls, 1942; Prince, 1956; Dowling, 1970) provides an essential

guideline for their identification. In the mouse retina, where the earliest rudiment of the outer plexiform layer is only visible from day 4 after birth, these cells could be located by their diaphorase activity as early as day 16 of embryonic life, i.e. after the differentiation of the photoreceptor cells.

Most of the bipolar cells seem to differentiate in mice postnatally (Sidman, 1961), and probably are the last of the retinal cells to differentiate in the chick (Kahn, 1974). This has been suggested by autoradiographic data. In the present study, these cells could first be identified by their diaphorase activity at day 7 after birth, when the inner and outer nuclear layers were already separated completely.However it can not be exluded that these cells start differentiating earlier but that this does not invlolve the enzymes demonstrated in this study.

Summing up the data mentioned so far the time scale of differentiation of the various retinal cells in mice can be presented by the following tentative diagram.



## Possible interrelations in retinal histogenesis

The development in the retina of a complex cytoarchitectural pattern out of a thin layer of undifferentiated cells necessarily involves complex cellular interactions, that for a long time have defied analysis. The early identification in the course of retinal histogenesis of different cell-types by localizing differential activity of enzymes enabled us to study the spatio-temporal changes of the cells leading to the characteristic adult organization.

The earliest sign of a structural differentiation of the retina is evident from day 13 of gestation at the centre of the vitreal border of the optic cup, where a layer of nerve fibres starts developing due to the growth of the axons of the ganglion cells from day 13 of gestation. The gradual displacement in opposite directions of the ganglion and amacrine cell perikarya, starting from around day 16 of gestation, marks the beginning of the formation of the inner plexiform layer and the consequent separation of the ganglion cell layer. What causes these cells to move apart is not known, but the localization of diaphorase activity at the vitreal tip of the amacrine cells, which appears at the same time, suggests that it is at this time that the processes of these cells start developing and that this might be related to the first development of the inner plexiform layer.

The separation of the photoreceptor cell perikarya in the outer nuclear layer and the bipolar cell perikarya in the inner nuclear layer is effected by the emergence of the outer plexiform layer with which the horizontal cells are intimately associated. The horizontal cells, which have their perikarya located in the outermost level of the inner nuclear layer and which send their horizontal processes in the outer plexiform layer, make synaptic contacts in this layer with both the photoreceptor and bipolar terminals (Yamada & Ishikawa, 1965; Dowling, et.

al., 1966; Dowling & Werblin, 1969; Sjöstrand, 1969; Fisher & Boycott, 1974). In the mouse, such synapses are first detectable at day 7 after birth (Olney, 1968); but the horizontal cells have already appeared at day 16 of gestation and at the time of birth they are aligned in a row that clearly determines the initial position of the outer plexiform layer, which gradually moves inwards along with the perikarya of the horizontal cells to their definitive position observed at 7 days. Therefore, it appears likely that the horizontal cells play an important role in the histogenesis of the outer retinal layers.

The Müller cells, the vertical processes of which traverse the entire depth of the retina seem to play a key role in the histomorphological differentiation of the retina. The early phase of development of these cells, when radial peocesses are formed, is marked by vitreoscleral migration of their perikarya. This movement immediately precedes the emergence of the early differentiation of the neurons. The role of these retinal glial elements at this stage may be one of providing guidance for migrating neurons in the way Bergman's glia of the cerebellar cortex aids in the migration of the granule cell nuerons (Rakic, 1971, 1972; Rakic & Sidman, 1973 a,b; Sotelo & Changeux, 1974). The second phase of the development of the Müller cells appears to be initiated after the settlement of their perikarya in the inner nuclear layer. The lateral processes of the Müller cells which occupy neuronal interspaces both in the nuclear layers (Polyak, 1941; Inomata, 1965; Hogan et al, 1971) and in the plexiform layers (Villegas, 1960, 1961; Meller & Glees, 1965; Sjöstrand, 1969; Leure-Dupree, 1974), are formed, as suggested by the localization of carbonic anhydrase, after the separation of the nuclear layers and at the time of synaptic development.

## The enzymatic differentiation of the retina

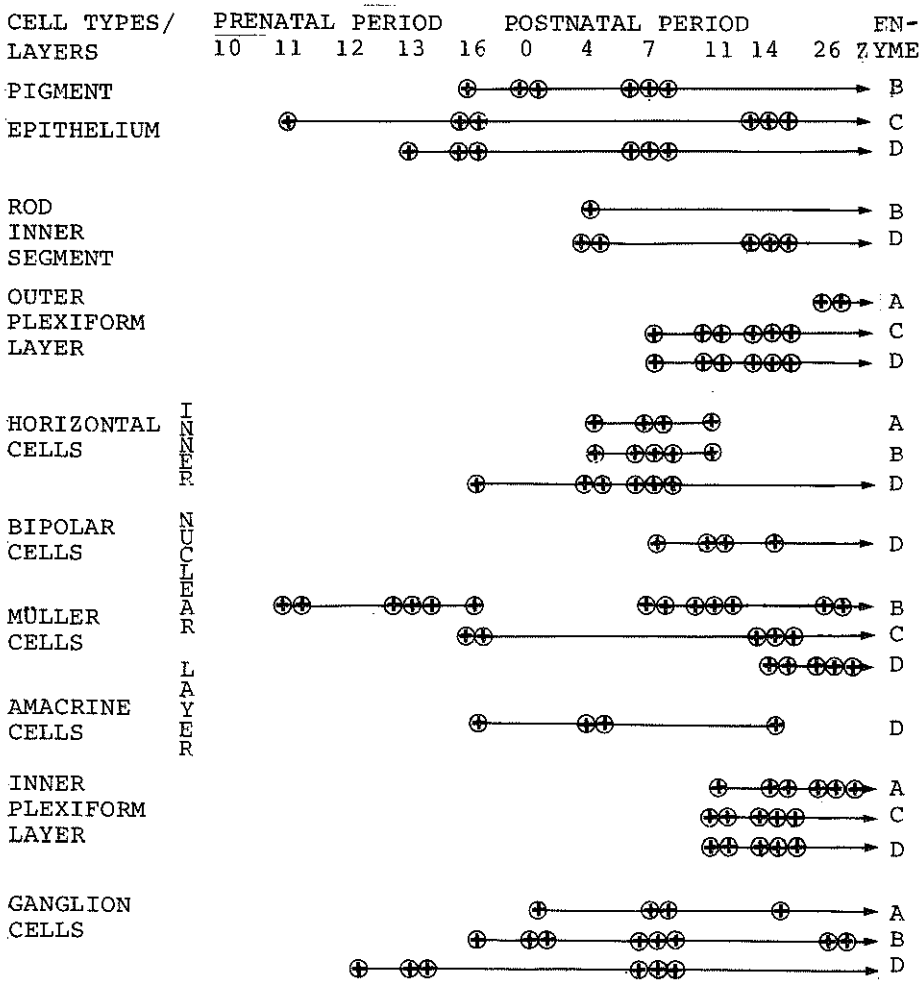
The chronology of appearance and distribution of the various enzymes observed in the developing retinal cells in the present study, is summarized in Table 1.

The earliest enzymatic differentiation of the retina begins simultaneously in the pigment epithelium and in the neural retina at day 11 of gestation. The pigment epithelium then shows activity of carbonic anhydrase, an enzyme considered to regulate the transport of ions (Giacobini, 1962; Hansson, 1968; Carter, 1972; Rosen & Friedley, 1973; Lönnerholm, 1974). In the neural retina it is the earliest batch of Müller cells which becomes demonstrable first; it is marked by non specific esterase to which a function in growth and development has been atributed (Mendel et. al., 1953; Smith & Wagenknecht, 1959). As the pigment epithelial cells continue to grow, the activity of carbonic anhydrase increases, while B-esterase and diaphorase activity also appear and rise progressively, reaching the adult pattern between 7-14 days of postnatal development. The period of increased enzyme activity coincides with that of rapid growth in the basal infoldings and apical villi (Moyer, 1969), and with an increased metabolic activity following the initiation of visual function around day 14 after birth (Noell, 1965).

In the Müller cells, the initial stage of differentiation is characterized by a transient B-esterase activity. This is followed by the appearance of carbonic anhydrase which steadily increases and reaches the adult level after day 14 after birth when the diaphorase activity also appears. B-esterase reappears at day 7 after birth.

The discrete localization of diaphorase activity first at the axon hillock of the ganglion cells during the time of formation of the layer of nerve fibres but later shifting towards the dendritic base has been interpreted by us as an indication of increased energy

TABLE 1




Abbreviations

A, Acetylcholinesterase
B, B-Esterase
C, Carbonic anhydrase
D, Diaphorase

Symbols

⊕ Slight activity
⊕⊕ Moderate activity
⊕⊕⊕ Intense activity
► No further change

requirement at the respective sites of the cells. By day 7 after birth, the ganglion cells reach their maximum activity for all the enzymes which have been found in perikarya of these cells. The levels of acetylcholinesterase and B-esterase decline in the ensuing period. The loss of acetylcholinesterase is presumably associated with the transport of the enzymes to the dendritic terminals (Dale, 1955; Koelle & Steiner, 1956; Nichols & Koelle, 1968;), as evident from the appearance of acetylcholinesterase containing bands in the inner plexiform layer. The gradual increase in the number of acetylcholinesterase positive bands from the initial two bands at day 10 to five bands at day 26 suggests that the synaptic connections between the ganglion, bipolar and amacrine cells continue to form after initiation of function at 12-14 days.

Although the early development of horizontal cells is marked by the activities of diaphorase, nonspecific esterase and acetylcholinesterase, which appear in this order, the highest activity of these three enzymes is not evident until about 7 days after birth when growth of their perikarya is visible and growth of their processes probably occurs as well. However, the activity of these enzymes, diaphorases excepted, dissappears in the course of development. The appearance of acetylcholinesterase in the outer plexiform layer around the time of weaning, possibly indicates an activity in the horizontal cell processes as observed in other species (Dickson, 1971) and is suggestive of their role in spatial summation and lateral interaction (Witkovsky & Dowling, 1969, Hibbard, 1971; Baylor, Fuortes & O'bryan, 1971; Baylor, 1974).

The amacrine and bipolar cells show positive reactions for diaphorases only. Whereas the amacrines show enzyme activity from around day 16 of gestation to day 11 after birth , the activity of the bipolars becomes first detectable at day 7 after birth and persists throughout life.

By comparing the differences in time of appearance and the concentrations of various enzymes (See Table 1), a significant correspondence between enzyme activities and structural and functional differentiation of the retina emerges. Three stages in retinal development seem to be marked by enhanced enzyme activities. The retina at day 16 of gestation is marked by the appearance of diaphorases in the amacrine and horizontal cells, B-esterase in ganglion cells and carbonic anhydrase activity in the Müller cells. At day 7 after birth, at least from the enzyme histochemical point of view, all retinal cells are differentiated and their spatial separation also is complete. The horizontal and ganglion cells then show maximum activities for acetylcholinesterase, B-esterase and diaphorase whereas the appearance of diaphorase in bipolar cells and reappearance of B-esterase in Müller cells further characterize this stage. At the last stage of retinal differentiation, starting at around 14 days after birth, a significant increase in the activity of several enzymes can be recorded. This phase thus coincides with the time of eye opening and the beginning of visual function as indicated by the appearance of the electro-retinogram response (Noell, 1965).

Finally, the present findings, clearly indicate that the enzymatic differentiation of most of the retinal cells precedes their morphological differentiation, as detectable with routine histological or neurohistological methods Whereas, with routine histological methods the early differentiation of ganglion and horizontal cells is detectable respectively at day 16 of gestation and day 7 after birth, i.e. after the formation of the inner and outer plexiform layers their identification is possible histochemically as early as day 12 of gestation in case of the ganglion cells and day 16 of gestation in case of the horizontals. Similary, in contrast to the general assumption that Müller cells differentiate late in

development, very early differentiation of these cells could be observed histochemically. Hence, the enzyme histochemical methods seem useful in studying the development and cytodifferentiation of the retina and have also been of help in elucidation of the cellular interrelations involved in retinal histogenensis, the final goal of this investigation.

## REFERERENCES


BAYLOR, D.A. ( 1974 ) : Lateral interactions between vertebrate photoreceptors. Fed. Proceedings 33, 1074 - 1077.

BAYLOR, D.A., FOURTES, M.G.F. & O'BRYAN, P.M. ( 1971 ) : Receptive fields of cones in the retina of the turtle. J. Physiol., London 214, 265 - 294.

CAJAL, RAYMON Y. ( 1892 ) : The structure of the retina ( Transl. S.A. Thorpe & M. Glickstein, 1972 ). C.C. Thomas. Illinois.

CARTER, M.J. ( 1972 ): Carbonic anhydrase : Isoenzymes, properties, distribution and functional significance. Biol. Rev. 47, 465 - 513.

COULOMBRE, A.J. ( 1955 ) : Correlation of structural and biochemical changes in the developing retina of the chick. Am. J. Anat. 96, 153 - 189.

DALE, H.H. ( 1955 ): Functional transmission of nervous effects by chemical agents. Proc. Mayo Clin. 30, 5 - 20.

DE ROBERTIS, E. ( 1956 ): Morphogenesis of the retinal rods. An electron microscopic study. J. Biophys. Biochem. Cytol. 2. Suppl. 4, 209 - 218.

DICKSON, D.H., FLUMERFELT, B.A., HOLLENBERG, M.J. & GWYN, D.G. ( 1971 ) : Ultrastructural localization of cholinesterase activity in the outer plexiform layer of the newt retina. Brain Research 35, 299 - 303.

DOWLING, J.E. ( 1970 ) : Organization of vertebrate retinas. Invest. Ophthalmol. 9, 655 - 680.

DOWLING, J.E., BROWN, J.E. & MAJOR, D. ( 1966 ) : Synapses of horizontal cells in rabbit and cat retinas. Science 153, 1639 - 1641.

DOWLING, J.E. & WERBLIN, F.S. ( 1969 ): Organization of retina of the mudpuppy. J. Neurophysiol. 32, 315 - 338.

FISHER, S.K. & BOYCOTT, B.B. ( 1974 ) : Synaptic connections made by horizontal cells within the outer plexiform layer of the retina of the cat and the rabbit. Proc. R. Soc. London B. 186, 317 - 331.

FUJITA, S. & HORII, M. ( 1963 ) : Analysis of cytogenesis in chick retina by tritiated thymidine autoradiography. Arch. Histol. Jap. 23, 359 - 366.

GIACOBINI, E. ( 1962 ) : A cytochemical study of the localization of carbonic anhydrase in the nervous system. J. Neurochem. 9, 169 - 177.

HANSSON, H.P.J. ( 1968 ) : Histochemical demonstration of carbonic anhydrase activity in some epithelia noted for active transport. Acta Physiol. Scand. 73, 427 - 434.

HIBBARD, E. ( 1971 ) : Grid patterns in the retinal organization of the chichlid fish Astronotus ocellatus. Exp. Eye Res. 12, 175 - 180.

HOGAN, M.J., ALVARADO, J.A. & WEDDELL, J.E. ( 1971 ) : Histology of the human eye. W.B. Saunders, Philadelphia.

HOLLYFIELD, J.G. ( 1972 ) : Histogenesis of the retina in the killifish, Fundulus heteroclitus. J. Comp. Neurol. 144, 373 - 379.

INOMATA, H. ( 1965 ) : Electron microscopic observations on Müller's fibres in the human retina. Acta Soc. Ophthalmol. Jap. 69, 2133 - 2143.

JACOBSON, M. ( 1968 ) : Cessation of DNA synthesis in retinal ganglion cells correlated with the time of specification of their central connections. Develop. Biol.17,219 - 232.

KAHN, A.J. ( 1973 ) : Ganglion cell formation in the chick retina. Brain Research 63, 285 - 290.

KAHN, A.J. ( 1974 ) : An autoradiographic analysis of the time of appearance of neurons in the developing chick neural retina. Develop. Biol. 38, 30 - 40.

KOELLE, G.B. & STEINER, E.C. ( 1956 ) : The cerebral distributions of a tertiary and a quaternary anti-cholinesterase agent following intravenous and intraventricular injection. J. Pharmacol. Exptl. Therap. 118, 420 - 434.

KUWABARA, T. & WEIDMAN, T.A. ( 1974 ) : Development of the prenatal rat retina. Invest. Ophthalmol. 13, 725-739.

LEURE-DUPREE, A.E. ( 1974 ) : Observations on the synaptic organization of the retina of the albino rat : a light and electron microscopic study. J. Comp. Neurol. 153, 149 - 178.

LÖNNERHOLM, G. ( 1974 ) : Carbonic anhydrase in the cornea. Acta Physiol. Scand. 90, 143 - 152.

MELLER, K. & GLEES, P. ( 1965 ) : The differentiation of neuroglia-Müller cells in the retina of the chick. Z. Zellforsch. Mikros. Anat. 66, 321 - 332.

MENDEL, B. MYERS, D.K., UYLDERT, I.E., RUYS, A.C. & De BRUYN, W.M. ( 1953): Ali-esterase inhibitors and growth. Brit. J. Ophthalmol. 8, 217 - 224.

MOYER, F.H. ( 1969 ) : Development, structure and function of the retinal pigmented epithelium. In : The retina: Morphology, structure and clinical characteristics ( Eds. B.R. Straatsma, M.O. Hall, R.A. Allen & F. Crescitelli ) pp. 1 - 30. University of California Press. Los Angeles.

NICHOLS, C.W. & KOELLE, G.B. ( 1968 ). Comparison of the localization of acetylcholinesterase and non-specific cholinesterase activities in mammalian and avian retinas. J. Comp. Neurol. 133, 1 - 16.

NOELL, W.K.( 1965 ) : Aspects of experimental and hereditary retinal degeneration. In : Biochemistry of the retina ( Ed. C.N. Graymore ) pp. 51 - 72. Academic Press, London.

OLNEY, J.W. ( 1968 ) : An electron microscopic study of synapse formation, receptor outer segment development, and other aspects of developing mouse retina. Invest. Ophthalmol. 7, 250 - 268.

POLYAK, S.L. ( 1941 ) : The retina. The University Press. Chicago.

PRINCE, J.H. ( 1956 ). Comparative anatomy of the eye. C. C. Thomas. Illinois.

RAKIC, P. ( 1971 ) : Neuron-glia relationship during granule cell migration in developing cerebellar cortex. A Golgi and electron microscopic study in Macacus rhesus. J. Comp. Neurol. 141, 283 - 312.

RAKIC, P. ( 1972 ) : Mode of cell migration to the superficial layers of fetal monkey neocortex. J. Comp. Neurol. 145, 61 - 84.

RAKIC, P. & SIDMAN, R.L. ( 1973 ) : Sequence of developmental abnormalities leading to granule cell deficit in cerebellar cortex of weaver mutant mice. J. Comp. Neurol. 152, 103 - 132.

RAKIC, P. & SIDMAN, R.L. ( 1973 ) : Weaver mutant mouse cerebellum : defective neuronal migration secondary to abnormality to Bergman glia. Proc. Nat. Acad. Sci. ( Wash. ) 70, 240 - 244.

ROSEN, S. & FRIEDLEY, N.J. ( 1973 ) : Carbonic anhydrase activity in Rana pipiens skin : Biochemical and histochemical analysis. Histochemie 36, 1 - 4.

SIDMAN, R.L. ( 1961 ) : Histogenesis of mouse retina studied with thymidine-$H^3$. In : The structure of the eye ( Ed. G.K. Smelser ). pp. 487 - 506. Academic Press. London.

SJÖSTRAND, F.S. ( 1969 ) : The outer plexiform layer and the neural organization of the retina. In : The retina: Morphology, structure, and clinical characteristics ( Eds. B.R. Straatsma, M.O. Hall, R.A. Allen & F. Crescitelli ). pp. 63 -100. University of California Press. Los Angeles.

SMITH, E.H. & WAGENKNECHT, A.C. ( 1959 ) : The ovicidal action of organophosphate insecticides. Cand. J. Biochem. 37, 1135 - 1144.

SOTELO, C. & CHANGEUX, J.P. ( 1974 ) : Bergman fiber and granule cell migration in the cerebellum of homozygous weaver mutant mouse. Brain Research 77, 484 - 491.

UGA, S. & SMELSER, G.K. ( 1973 ) : Electron microscopic study of the development of retinal Müllerian cells. Invest. Ophthalmol. 12, 295 - 307.

VILLEGAS, G.M. ( 1960 ) : Electron microscopy of the vertebrate retina. J. Gen. Physiol. 43, Suppl. 15 - 44.

VILLEGAS, G.M. ( 1961 ) : Comparative ultrastructure of the retina in fish, monkey and man. Freiburg Symposium ( 1960 ) : The visual system, pp. 3 - 13. Springer Verlag. Berlin.

WALLS, G.L. ( 1942 ) : The vertebrate eye and its adaptive radiation. Hafner Publishing Co. New York. 1967.

WITKOVSKY, P. & DOWLING, J.E. ( 1969 ) : Synaptic relationships in the plexiform layers of carp retina. Zeitschrift für Zellforschung und mikroskopische Anatomie 100, 60 - 82.

YAMADA, E. & ISHIKAWA, T. ( 1965 ) : The fine structure of the horizontal cells in some vertebrate retina. Cold Spring Harbor Symp. Quant. Biol. 30, 383 - 392.

## SUMMARY

The purpose of this investigation was to study the sequence of cell differentiation in the developing retina of the mouse using histochemically detectable enzymatic differences as parameters of differentiation. In addition, the interrelation of the differentiating cells during histogenesis was studied.

Changes in the histochemical localization of different esterases, carbonic anhydrase, DPN and TPN diaphorases have been followed from the optic cup stage up to the time of weaning.

At day 10 of gestation, when the development of the optic cup is just completed, no localized reaction was obtained for any of the enzymes.

At day 11, Müller cells identifiable due to a high non-specific esterase activity, were first seen, localised along the vitreal border of the central retina. Between day 11 and day 15 the perikarya of the Müller cells migrated outward, became scattered throughout the depth of the retina and started to lose their esterase activity. At day 16, the perikarya of the Müller cells showed carbonic anhydrase activity which increased gradually as they shifted to their definitive position in the inner nuclear layer during the period up to 7 days after birth.

Ganglion cells appeared between 12 and 16 days of gestation at the innermost level of the retina and were marked by DPN and TPN diaphorase activity localized at the axon hillocks of the cells. The emergence of the inner plexiform layer, starting from day 16, separated the layer of the ganglion cells from deeper layers. Simultaneously, diaphorase activity moved to the dendritic side of the cells. A further differentiation of the ganglion cells is the appearance of a non specific esterase at day 16 of gestation and of acetylcholinesterase (AChE) at the time of birth. The activities of all these enzymes

increased along with the growth of the perikarya and ganglion cells of different sizes could be recognized.

The cells located scleral to the ganglion cells differentiated into amacrine cells. They are positioned on the outer side of the inner plexiform layer and showed a transient diaphorase activity at the initial stage of development.

Progressively increasing diaphorase activity along the outer limiting membrane from day 13 of gestation onward characterized the initiation of photoreceptor differentiation. The staining was later localized in the rod inner segments.

The horizontal cells differentiated between day 16 of gestation and the time of birth; they were characterized by diaphorase, non specific esterase and AChE which appeared in this order. At day 4 after birth these cells were sparsely distributed in a single row at an outer level of the retina, where the outer plexiform layer is being formend and horizontal processes start emerging. Afterwards the outer plexiform layer along with the horizontal cells seemed to shift inwards as the outer nuclear layer increased in thickness. The horizontal cells seem to play an important role in determining the site of the outer plexiform layer where the photoreceptor and bipolar cells synapse.

The bipolar cells were the last of the retinal cells to differentiate. These cells are characterized by an increasing diaphorase activity starting at day 7 after birth when the nuclear layers are already completely separated.

During the development of the synapse in the outer plexiform layer, starting from day 7 after birth, increasing activities of diaphorases, carbonic anhydrase and finally of AChE were observed in this zone, whereas the activities of AChE and non specific esterase completely disappeared from the horizontal cell perikarya.

A rapid increase of AChE, carbonic anhydrase and diaphorases was seen in the inner plexiform layer starting from days 10-11 after birth and coincided with the time of synaptic development in this zone. During the same time the AChE activity in the ganglion cell perikarya become very much reduced.

The increased carbonic anhydrase activity in both plexiform layers seemed to be due to growth of the lateral processes of the Müller cells of which the perikarya and the radial processes also showed increased activity.

Enzymatic differentiation of the pigment epithelial cells was indicated by the appearance of carbonic anhydrase at day 11, of diaphorases at day 13 and of non specific esterase at day 16 of gestation. The activities of all the enzymes increased progressively and reached adult level during 7-14 days after birth.

With the enzyme histochemical methods applied, different retinal cells in mice could be identified much earlier than has been possible with routine histological and neurohistological methods. Therefore, development of these cells could be traced to earlier stages of retinal histogenesis. This will aid in elucidation of the causative factors involved in retinal histogenesis.

## SAMENVATTING

Het doel van dit onderzoek was bestudering van de volgorde van differentiatie van de verschillende cel types tijdens de ontwikkeling van de retina van de muis, Als parameter van differentiatie werd gebruikt het voorkomen van histochemisch aantoonbare veranderingen in enzym patroon. Tevens werd de onderlinge afhankelijkheid van de zich differentierende cellen tijdens de histogenese bestudeerd.

Veranderingen in de histochemische localisatie van verschillende esterases, van carbo-anhydrase en van DPN en TPN diaphorases werden gevolgd van het stadium van de oogbeker tot het moment van spenen.

Op dag 10 van de zwangerschap, wanneer de vorming van de oogbeker juist voltooid is, kan nog geen gelocaliseerde reactie voor één van de enzymen verkregen worden.

Op dag 11 werden de Müller cellen, welke geïdentificeerd konden worden dankzij een hoge niet-specifieke esterase activiteit het eerst waargenomen: zij kwamen voor aan de inwendige zijde van het centrale deel van de retina. Op dag 15 waren zij in het periphere deel van de retina verschenen. Gedurende de periode van 11-15 dagen migreerden de Müller cellen naar de sclerale zijde van de retina, raakten verspreid over de gehele diepte van de retina terwijl hun esterase activiteit daalde. Op dag 16 vertoonden de perikarya van de Müller cellen carbo-anhydrase activiteit, welke geleidelijk toenam, terwijl de cellen zich verplaatsten naar hun definitieve ligging in de inwendige korrellaag; dit gedurende de periode tot 7 dagen na de geboorte.

De ganglion cellen verschenen tussen de 12e en 16e zwangerschapsdag in het binnenste gebied van de retina en werden gekenmerkt door DPN en TPN diaphorase activiteit in het gebied van de axon-heuvel. Het verschijnen van de inwendige vezellaag, vanaf dag 16, scheidde de ganglion

cel laag van de diepere lagen. Gelijktijdig verplaatste de diaphorase activiteit zich naar de dendrieten-zijde van de cel. Op een verdere differentiatie van de ganglion cellen wees verschijnen op dag 16 van niet-specifieke esterase en van acetyl-cholinesterase (AChE) ten tijde van de geboorte. De activiteit van al deze enzymen nam toe parallel met de groei van de perikarya. Verder konden ganglioncellen van verschillende grootte onderscheiden worden.

De cellen, gelegen aan de sclerale zijde van de ganglion cellen differentieerden zich tot amacrine cellen. Deze zijn gelegen bij de buitenzijde van de inwendige vezellaag en vertoonden in de eerste phase van hun ontwikkeling voorbijgaand diaphorase activiteit.

Een geleidelijk, vanaf de 13e zwangerschapsdag toenemende diaphorase activiteit bij de membrana limitans externa was karakteristiek voor het begin van de differentiatie van de photoreceptoren. Deze activiteit bleek zich later in de binnenste segmenten van de kegeltjes te bevinden.

De horizontale cellen differentieerden zich tussen de 16e zwangerschapsdag en de geboorte en vertoonden diaphorase, niet-specifieke esterase en AChE, welke in deze volgorde verschenen. Op dag 4 na de geboorte waren deze cellen spaarzaam verdeeld in een enkele rij in een periphere zone van de retina, waar de uitwendige vezellaag gevormd wordt en de horizontale uitlopers verschijnen. In een latere phase verplaatsen de uitwendige vezellaag en de horizontale cellen zich tesamen naar een meer centraal gelegen gebied van de retina; dit in samenhang met een toenemende dikte van de uitwendige korrellaag. De horizontale cellen lijken een belangrijke rol te spelen bij het tot stand komen van de plaats van de uitwendige vezellaag, waar de photoreceptoren en de bipolaire cellen synapteren.

De bipolaire cellen bleken de retina cellen te zijn, welke zich het laatst differentieren. Zij kenmerken zich

door toenemende diaphorase activiteit, welke verscheen op dag 7 na de geboorte, wanneer de korrellagen reeds volledig gescheiden zijn.

In de uitwendige vezellaag werd, tijdens de ontwikkeling van de synapsen vanaf dag 7 na de geboorte een toenemende activiteit gevonden van diaphorases, carbo-anhydrase en tenslotte van AChE, gelijktijdig met een volledige verdwijnen van de activiteiten van AChE en niet-specifieke esterase uit de perikarya van de horizontale cellen.

Een snelle toename van AChE, carbo-anhydrase en diaphorase werd vanaf dag 11-12 na de geboorte in de inwendige vezellaag gelijktijdig met de vorming van synapsen in deze zone. In dezelfde periode trad een sterke daling op van de AChE activiteit in de perikarya van de ganglion cellen.

De toenemende carbo-anhydrase activiteit in beide vezellagen leek een gevolg te zijn van het uitgroeien van laterale uitlopers van de Müller cellen, waarvan de perikarya en radiale uitlopers eveneeens een toegenomen activiteit lieten zien.

De enzymatische differentiatie van de pigment epitheel cellen werd gekenmerkt door het verschijnen van carbo-anhydrase activiteit op dag 11, diaphorase op dag 13 en niet-specifieke esterase op dag 15 van de zwangerschap. De activiteit van al deze enzymen nam geleidelijk toe en bereikte een volwassen niveau in de periode van de 7e tot de 14e dag na de geboorte.

Met de gebruikte histochemische methodes konden de verschillende celsoorten van de muizenretina aanzienlijk vroeger geidentificeerd worden dan met de standaard histologische en neurohistologische technieken. Dientengevolge kon de ontwikkeling van deze cellen tot vroegere ontwikkelingsstadia terugvervolgd worden. Dit zal een bijdrage kunnen leveren bij het onderzoek naar de causale factoren, welke ten grondslag liggen aan de histogenese van de retina.

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## CURRICULUM VITAE

Born in Calcutta on the 1st of March, 1942, the author received his high school education from Bengali Higher English School, Gauhati and matriculated in 1956.

Having passed the Intermediate, and Bachelor of Science examination with honours in zoology from Cotton College, Gauhati, under Gauhati University, he was enrolled in the Presidency College, Calcutta under Calcutta University in 1960 for the study of M.Sc in zoology and comparative anatomy and recieved the degree in 1962 with thesis paper in developmental biology. In 1963, he joined Cotton College, Gauhati, as lecturer in zoology and is presently on study leave.

He joined the Department of Anatomy, of the medical faculty of the Erasmus University Rotterdam in July 1973 on a fellowship granted by the Stichting Fonds, Medische Faculteit Rotterdam and completed the present study there.