PHYSIOLOGICAL AND HISTOCHEMICAL STUDIES ON THE VISION OF SOME FISHES

ABSTRACT

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DEPEARTMENT OF ZOOLOGY

A THESIS SUBMITTED IN FULFILMENT FOR THE REQUIREMENT OF THE DEGREE OF
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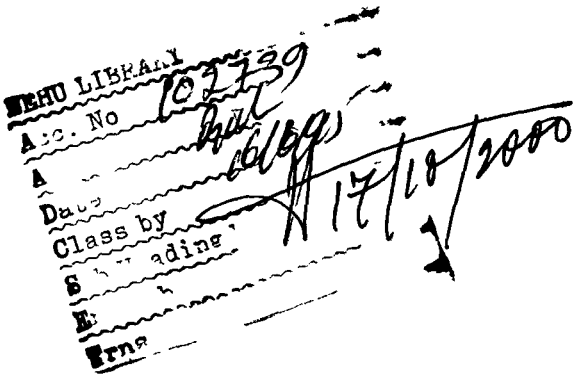

ABSTRACT

In the present investigation, certain physiological, histochemical as well as some biochemical studies on the eyes of common carp, Cyprinus carpio, catfish, Clarias batrachus and a marine fish pomfret, Stromateus argenteus, have been performed, which are summarized as follows:

(1) The protein-polysaccharide complex, acid mucopolysaccharide components in the cornea and lens of the above fishes have detected histochemically. The mucopolysaccharides have also been extracted from these tissues and an attempt has been made to analyze some sugar fractions by paper chromatography following acid hydrolysis of the crude extract.

The nature of the extracted mucopolysaccharides has been investigated by paper electrophoresis in various buffer systems and their Rf values have been compared with some standard mucopolysaccharides such as, chondroitin sulfate A, B, C, heparan sulfate and keratan sulfate. It has been found that all the mucopolysaccharides behave like sulfated mucopolysaccharides in the electrophoretic field. The significance of the occurrence of this important constituent in connection to the vision of fish has been discussed.

(2) Report on the enzymology on fish ocular system is very scanty and an attempt has been made to study the sodium-potassium

activated adenosine triphosphatase ($Na^+ - K^+$ - ATPase) histochemically in the cornea and lens of Cyprinus and Clarias.

(3) A study on the significance of reducing substances, particularly of ascorbic acid, in energy generation in cornea and lens of Cyprinus and Clarias has been attempted. Histochemical detection as well as biochemical estimation of ascorbic acid has been performed spectrophotometrically. It is known that ascorbic acid in the biological system not only occurs as free form (AA) but also occurs in bound form as ascorbigen (ASG). It is also known that ascorbic acid is continually acted upon by a number of oxidizing enzymes. Further, Chinoy (1967,1969) has reported that in living system exogenous as well as endogenously added ascorbic acid forms a complex with macromolecules like proteins and nucleic acids. Isherwood and Mapson (1962) have suggested that in a tissue, the actual concentration of ascorbic acid represents the excess, formed in synthesis over that used in the cornea and lens has been studied by simultaneous estimation of (i) free form of ascorbic acid, (ii) bound form of ascorbic acid or ascorbigen (ASG), (iii) enzymic utilization of ascorbic acid (AAU) and (iv) complexing of ascorbic acid with macromolecules.

(4) An investigation on the retinal pigment (melanin) migration in Cyprinus and Clarias eye has been performed. It is

known that the pigments act as screening substance and the migration is under the control of various photic stimulations such as dark and light and is also affected by other factors.

Effects of 5-HT, colchicine and cyclic-AMP on the movement of retinal pigment in the dark adapted eyes of Cyprinus and Clarias have been investigated. It has been found by comparing with controlled light adapted eyes that these substances stimulate pigment dispersion simulating the light adapted state. Even a lower concentration of 0.3 mM of colchicine stimulated the pigment dispersion similar to light adapted state.

Moreover, a preliminary study on the effect of light and dark conditions on the neurosecretory system of Cyprinus and Clarias has been performed in view of the suggestion of Veron (1973) regarding the influence of neurosecretion on pigment migration. Ali (1964) also suggested that there might be some relationship between hormone and light and dark adaptations. It has been found that during dark adaptation a large accumulation of neurosecretory granules takes place while in the light adapted state there is a significant reduction in the neurosecretory substances in the neurosecretory cells of diencephalon of brain presumably due to axonal transport.

(5) It is known that some pigments fluoresces in the presence of U-V irradiation in dark and one such fluorescent compound is pteridine. This pigment has been extracted from the cornea and lens of Cyprinus, Clarias and Stromateus and analyzed by paper chromatography. The chromatograms yielded strong fluorescence following their examination under U-V light in dark. The possible roles of pteridine in the ocular structures have been discussed in terms of vision.

(6) An attempt has been made to study the visual pigments from the photoreceptor cells such as rods and cones. The visual pigment has been analyzed spectrophotometrically following extraction from the retina of Cyprinus and Clarias. The retinal extract contained presumably a bleached product or photoproduct ranging between 400-404 nm in both the fishes, similar to a product (400 nm) obtained in the retina of crucian carp by Reuter (1973).

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Dedicated to the
Memory of my Parents.

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SUPERVISORS' CERTIFICATE

We certify that the thesis entitled,"Physiological and Histochemical Studies on the Vision of some Fishes" submitted by Mr.M.K.Deb for the degree of Doctor of Philosophy of the North-Eastern Hill University, Shillong, embodies the record of original investigation carried out by him under our supervision. He has been duly registered and the thesis presented here is worthy of being considered for the award of the Ph.D. degree. This work has not been submitted for any degree of any other university.

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Shillong
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Mrinal K. Deb.
(Mrinal K. Deb)

C O N T E N T S

LIST OF TABLES

LIST OF FIGURES

GENERAL INTRODUCTION

Life and light are synonymous. Photoresponse is a universal phenomenon in living organisms. Light is effectively utilized by the diverse animals to fulfill their basic biological demands. Perceptible light is a form of radiant energy which acts both as a wave and a particle.

Solar radiation is the ultimate source of energy for almost all life. When a photon strikes and interacts with particles of matter, it sends an electron into a higher energy level or excited state (Hoar, 1983).

Life exists and operates on a relatively narrow band of the electromagnetic spectrum which extends from the cosmic and gamma rays with wavelengths of only a ten-billionth of a centimeter to the radio waves which may be miles in length. Within this broad expanse of radiant energy, there is a narrow band which we call "light" because of the sensation which it creates when it falls on the retina of human eye. Its wavelengths extend from 380 to 760 nanometers (nm) with extreme limits of 310 to 1050 nm in very intense artificial sources. Photoreception in all animals is almost covered by the extreme human range (Hoar, 1983).

The visual process in all animals is governed by the same physical rules for adjustment in a particular ecological niche. The light activates some photosensitive molecules ultimately leading to image formation.

The significant photochemical processes of life depend on different pigmented molecules which absorb radiant energy to initiate vital biochemical and physiological processes. The visual pigments which are carotenoids generate electronic power through a molecular rearrangement causing a nerve impulse (Hoar, 1983) for vision.

The visual process involves three steps: first is the optical stage when an image of the outside world is projected on the retina being aided by cornea and lens; second is the transduction stage when the photosensitive visual cells absorb photons and respond by generating electrical signals; and third is the physiological stage when these primary signals are analysed. Possibly there is a fourth stage that marks the conscious awareness of visual display.

Thus, vision is a complex and integrated process of reflection, refraction, selective absorption and a psychomatic process to see the objects.

The fish eye inspite of its gross similarity with the vertebrates is unique per se owing to its remarkable adaptability. The aquatic medium poses problems in light reception because of its effects of scattering, depth etc. Even closely related species living under different conditions may have quite different visual adaptations.

One of the most striking differences between aerial and aquatic vision is in the field of view. Photoreception in aquatic medium depends upon reflection and refraction of light at air-water interface. The cornea is practically useless in aquatic medium in fishes but effectively compensated by the lens. The fishes also exhibit remarkable retinal adaptations during migration and reaching to the climax of colour vision in addition to photopic and scotopic vision. According to Wagner and Ali (1974), the visual strategies employed by fishes in different environments resemble vertebrates in some and differ in others.

The detailed anatomy and adaptations of eyes has been described by Walls (1942). Valuable reviews of the structure and functions of fish eyes have been prepared by Brett (1957); Nicol (1963) and Munz (1971).

The fish eye is a more or less flattened, fluid-filled, hollow organ built on the same basic vertebrate plan. The eye consists of normal complement of six oculomotor muscles. Its wall is composed of three general layers variously modified in different regions to perform different functions.

The outermost layer is sclera which is composed of tough connective tissue giving rigidity and shape to the eye. The sclera is usually cartilagenous but fibrous in cyclostomes and often calcified in elasmobranchs (Munz, 1971). The sclera is anteriorly modified into a flattend and optically clear

cornea with little or no refraction since its refractive index is almost similar to that of water. The fish eye is myopic in air due to added refraction. The cornea in many fishes have transparent and protective "adipose-eyelids" (Munz, 1971).

The lens is usually spherical and protrudes through the pupil ensuing wide field of view because of the absence of corneal refraction. Accommodation is achieved by the small movement of the lens. The lens, in teleosts, is pulled backward by retractor muscle while in elasmobranchs it is pulled forward by protractor muscle.

The lens or cornea of many fishes contains pigments which filter out ultraviolet radiation, probably improving visual acuity (Kennedy and Milkman, 1956; Denton, 1957; Motais, 1957). Little is known about aqueous or vitreous humors. The consistency of vitreous humor in different species varies from a liquid to firm gel (Munz, 1971).

The intermediate vascular and complex layer of choroid lies immediately beneath the sclera. The choroid has combined functions of retinal nourishment and absorbing stray light or reflect back through the retina by a tapetum lucidum increasing the visual acuity. The retina, like other nervous tissue has a high oxygen consumption which is actively secreted by the specialized "choroid gland". Wittenberg and Wittenberg (1962), have shown that the choroid gland actively secretes oxygen to fulfil the high oxygen demand of the retina. The innermost part

of the choroid, lying just behind the retina is modified into a choriocapillary structure. In most teleosts, but not in elasmobranchs, the choroid projects through the optic cleft into the posterior chamber of the eye, as the richly vascular pigmented falciform process (Hanyu, 1959) having a nutritive function.

The photosensitive layer, the retina of fishes is constructed according to the general vertebrate plan. The innermost layer of retina consists of various relatively transparent neuronal and glial cells. Light passes through these to the photoreceptor or visual cells (Munz, 1971). The visual cells of lampreys are usually of two morphologically distinct types, but their affinities with rods and cones of other vertebrates are not certain (Walls, 1942). Elasmobranchs and teleosts each depart from the familiar duplex pattern of vertebrates, but in different ways. Most elasmobranchs are thought to have pure rod retinae, but some sharks (Mustelus, Lamna, Squantina, etc.) and rays (Myliobatis, and Dasyatus) are reported to have cones as well as rods (Walls, 1942; Tamura and Niwa, 1967). But teleosts typically possess both rods and cones. In addition to the ordinary single cones, they also have peculiar visual cells called "twin cones". These differ from the double cones found in most vertebrates, is that the "twin" in each cell pair are morphologically similar and fused longitudinally (Munz, 1971). Certain teleosts, in which a tapetum is well developed, lack single cones (Tamura, 1957; O'Connell, 1963). Cones are entirely

absent in many deep sea fishes (Munz, 1971) with one well established exception Omosudis, which has an almost pure-cone retina (Munk, 1965b).

A typical feature, probably of teleosts is that the visual cells are not uniformly distributed over the retina. Commonly there is a specialized temporal "area" or "area centralis" where cones are more numerous. This area may contain twin cones alone (e.g. anchovies which lack single cones) or both twin and single cones (O'Connell, 1963). A cone rich area also occurs in the shark, Mustelus. Further retinal specialization is achieved by the formation of a retinal pit or fovea containing only single cones, overlying the area of more numerous visual cells to increase visual acuity as the light directly falls on the photoreceptors. Blood vessels are typically absent from the fovea. Foveae are usually present near the temporal (posterior) border of the retina. Foveae are rare in teleosts. They have been described in 20 littoral marine species (Kahmann, 1936; Barron and Verrier, 1951). Foveae have also been reported in fresh water species of Fundulus Umbridus (Prince, 1956). However, pure-rod retinae also have been reported in several genera of deep-sea fishes, e.g. Bathylagus (Vilter, 1954a,b; Munk, 1966), Scopelosaurus and Searsia (Marshall, 1966), Platyroctegen (Munk, 1966).

The refractive index of cornea is almost same to that of water and ocular humors in fishes. Hence, the lens or dioptric

apparatus is almost entirely responsible for refraction and image formation by the way of concentrating light on the photoreceptor cells. Pumphrey (1961), has shown that the spherical lens has highest refractive index at the centre which gradually decreases towards the periphary and has no spherical aberration to ensure uninterrupted image formation.

In fishes sharp image on the retina for both near and far objects is formed by altering the distance between the lens and the retina unlike higher vertebrates (e.g. man) where this is done by changing the curvature of the lens. This capacity is referred to as accomod-ation, which is already mentioned. The eyes of cyclostomes and teleosts are generally focused for near vision at rest and must be actively accomodated for distance vision. In these animals the lens is said to be moved backward to focus on distant objects (Pumphrey, 1961). But Munz (1971), reports several exceptions and reviewed recent developments. Elasmobranchs, amphibians and snakes move the lens forward to adjust for near vision.

In addition to accomodation, several other curious devices can have somewhat the same result, but without requiring any active mechanism (Walls, 1942). Munz (1971) summarize them as follows:

(1) A pinhole produces a fairly sharp image regardless of the distance from the object and to the retina. When light

adapted, such elasmobranchs as Scyliorhinus and Raja have a pupil with a very small aperture.

(2) A "ramp" retina (slanted or tilted), which is tilted away from the lens, could simultaneously have in focus images of objects located at different distances. In Raja, the upper portion of the retina is farther from the lens than the lower portion. Objects nearby on the ocean bottom could therefore, be in focus at the same time as distant objects located above the animal.

(3) The fact that the outer segments of the visual cells have considerable length means that objects at various distances would be equally in (or out of) focus; presumably this has more to do with increasing sensitivity than with accomodation.

(4) Another structural modification is to have the eye permanently set for vision at two particularly useful distances; an analogy of bifocal spectacles, suggested by Walls (1942). The well known example is "four-eyed" fish, Anableps, which swims at the surface with its eyes partly out of water. The pupil of the light adapted eye is divided horizontally by flaps of the iris. Objects in air are imaged on the ventral part of the retina, those in water on the dorsal part. The two parts of retina are specialized for their different functions Walls, 1942; Schwassman and Kruger, 1965).

The world is alive both day and night. Both the arthropods and vertebrates show several distinctive retinal specializations associated with daily activity rhythms (Hoar, 1983). Several adaptations for light (photopic) and dark (scotopic) vision which are accomplished by changing the intensity of light at the receptor site. The most common feature of light and dark adaptations is the change of the concentration of visual pigment and modification in neuronal interaction (Munz, 1971). During vertebrate evolution there has been a progressive development of pupillary responses as an alternative and better mechanism for controlling retinal illumination. Only a few fishes have a variable pupil diameters and in these the response is slow. In these fishes, apparently, the iris responds directly to light intensity, whereas in higher forms the response, which is fast and effective, is mediated by way of nervous reflex arcs (McCauley, 1971). The shape of the aperture (pupil) may be circular in some species or slit-shaped in others depending on the requirement of the light of the animal.

In the great majority of teleosts the pupillary control of light intensity is replaced by retinomotor or photomechanical movements. Two kinds of such responses have been described: (i) the dispersion or concentration of masking of pigment within and around the retinal cells (Hoar, 1983); and (ii) the expansion and contraction of the rods and cones in response to change in light (Ali, 1975). Pigment migration have been studied in many

invertebrates as well as in all groups of sub-mammalian vertebrates. It is, in Arthropods, is more striking in superposition eyes but also occurs in apposition eyes. Pigment migration is regulated by hormones in Crustaceans (Highnam and Hills, 1977) but not well understood in insects. They may depend on nerves (Goldsmith and Bernard, 1974).

Retinal pigment migration in vertebrates is rapid and pronounced in the teleosts, anurans and birds but slow and slight in turtles and crocodiles; absent in snakes and mammals.

A mirror or tapetum lucidum, a curious example of pigment migration to increase the total retinal illumination by the presence of light reflecting substances in nocturnal forms, including members of all vertebrate classes, usually present in the pigment layer of choroid. The tapetum was first reported in elasmobranchs. Walls (1942), described several morphologically distinct types but only three are common in fishes. A retinal tapetum occurs in the pigment epithelium of many freshwater fishes. The epithelial cells contain particles or crystals of the reflective substance guanine. Melanin is present in the same cells and migrates normally, occluding the tapetum in bright light. A nonocclusible tapetum of the same type was said to occur in pelagic deep-sea teleosts (Walls, 1942). The pigment cells of the choroid seem to behave as independent effectors which are sensitive to light (Nicol, 1965b).

Visual adaptation in response to a particular habitat is spectacular in deep-sea forms. Ever since Brauer's treatise (1908) revealed the startling diversity of ocular structures in deep-sea fishes, authors have speculated on their functional significance and has been reviewed by Marshall, (1954).

Photoreception in deep-sea fishes is enhanced by many specialized ocular features. Among the deep-sea fishes, some teleosts, in addition to pure rod retina have rod outer segments arranged in several distinct layers (Munk, 1963,1966) while the elasmobranchs may have well developed tapetum in the absence of black pigment from the back of the eye (Denton and Nicol, 1964). Brauer (1908); Walls (1942); and Munk (1966) have reported the presence of tubular and elongated eyes with large lenses in a number of teleosts for a better binocular vision and even have an accessory retina touching the lens at the back of the eye. These adaptations are extremely helpful in the dark abyssmal depth. Munk (1964a,1965a), has examined the degeneration of eyes in fishes from great depths and has conclusively proved that the Ipnops with transparent bony plates covering the orbital area which were described as photophores (Walls, 1942) or modified eyes, though lacking cornea and lens are actually eyes with rod cells and optic nerve. Another peculiar fish (Bathylychnops), has dorsally directed primary eyes as well as ventrally directed secondary globe overhanging the jaws. The two globes are conti-

nuous but a flap keeps light from passing between them. The retina of the secondary globe is diverticulum of the main retina (Pearcy et al. 1965; Munk, 1966).

A series of photochemical events lead to the image formation with the help of visual pigments. These are carotenoid pigments. The carotenoid pigments of animals are either dissolved in the tissue fats or chemically combined with specific proteins;in either form, they play a part in animals. As prosthetic groups of proteins, two of them can form the highly specialized molecular machines which are the basis of photoreception. A bichrome extracted from frog retinas was used in the first studies of visual pigments. Visual pigments have now been investigated in representatives of each of the major phyla with highly specialized eyes. In all cases, the active pigment is an aldehyde of vitamin A, known as RETINAL, combined with protein OPSIN. Vitamin A is an alcohol, often referred to as RETINOL; the aldehyde (retinaldehyde) is RETINAL or, less frequently, RETINENE (Hoar, 1983).

The terminal segment of each rod contains the photosensitive pigment rhodopsin or visual purple. When this substance absorbs a sufficient amount of light energy, it splits into its constituent parts, opsin and transretinene. Presumably, similar photochemical processes take place in cones, and one photosensitive substance, iodopsin, has been identified. In

order for colour discrimination to take place it is postulated that three different types of cones must be present (McCauley, 1971).

The mammals, birds and almost all of the marine fishes were found to have rhodopsin, while the first fresh water fishes studied possessed porphyropsin. Of even greater interest was the observation that several groups of vertebrates which make a profound change in habitat during their life cycle show associated changes in their rhodopsin-porphyropsin system (Hoar, 1983).

Relatively few groups of animals possess colour vision. Among the vertebrates, hue discrimination has been demonstrated with certainity in primates, birds, lizards, turtles, frogs and teleost fishes. It is associated with bright light vision, foveae with rich areas of cones, and eyes with good mechanisms for accomodation (Hoar, 1983).

Though, much work has been done on induced breeding and reproductive biology of fishes in India (Chaudhuri, 1960, 1963); Alikunhi (1966); and Jhingran (1975); but very little attention has been given to the physiology of vision in fishes. Since the pioneering work of Walls (1942) on the functional anatomy of eyes of fishes, valuable reviews of the structure and functions of the eyes of fishes have appeared (Brett, 1957; Nicol, 1963). Munz (1971), has reviewed the structure of the eye, role of lens in

accomodation, role of visual pigments in image formation and during light and dark adaptation. But, inspite of a large amount of works (Rawal and Rao, 1977,1983), in India, there are many areas yet to be studied.

For example, the cornea which consists autochthonous layer, scleral and dermal layers; the sclera and the lens are modified parts of the general body wall, has not been studied in detail with regard to their chemical composition with special reference to different ecological (fresh water and marine) niches. The other components of the eye, such as, acid mucopolysaccharide which is a basic constituent of cornea and lens require further investigation in fishes; retina, aqueous and vitreous humors have not been studied in detail. Besides, the physiology of colour vision, fluorescent compounds, role of various elements have not been studied in fishes in general and in Indian fishes in particular.

Moreover, enzymic activity, such as, ATP-ase, which has been studied in other vertebrates but very little work has been done in the lens and cornea of fishes. Although much work has been carried out on pigment migration but neuro-endocrinological control of the same requires a fresh approach.

Another area which deserves attention, is the role of fluorescent compounds in the eye structure, since these might play a significant role in visual physiology.

In light of the major role of lens in the vision of fishes, more detailed study from the structural stand point is required.

Keeping this in view, a study on the physiology and histochemistry of eye of some fishes from freshwater including air-breathing and marine habitats have been undertaken.

The fishes that have been chosen for the present investigation, are the common carp (Cyprinus carpio), an air-breathing cat fish (Clarias batrachus) both from freshwater habitat and pomfret (Stromateus argenteus), from marine habitat.

The aim of the research programme is to obtain further informations and to compare some aspects of vision in fishes from various ecological niches by physiological and histochemical methods.

C H A P T E R I

HISTOCHEMICAL ANALYSIS OF THE CORNEA AND LENS :

ACID MUCOPOLYSACCHARIDES

INTRODUCTION

There is a great adaptive variation among fishes in the minute structure and visual capacity of the organ. Thus, the cornea in fishes is the structure for light entry while the lens plays the exclusive role of refraction.

Out of the three tunics in vertebrate eye, the dense fibrous sclera in all cases is replaced by a peculiar transparent connective tissue window, the cornea. The circular cornea in fishes, unlike higher vertebrates is almost flat without any effective refraction since the optical density of this tissue is almost the same to water.

The cornea is modified integument to serve the optical purpose. The cornea proper (Stroma, Substantia propria) is externally lined by an epithelium of several layers of cells of different sizes and shapes. The cells of superficial layers are of squamous type. The inner layer called the endothelium is of very thin single layer of cells.

The substantia propria is a tough transparent membrane consisting of a number of flattened lamellae composed of modified connective tissue fibres continuous with those of sclera and lying in a mucoprotein ground substance (Willmer, 1966). The cornea owes its transparency to the precise spacing and arrangement of the collagen fibrils that make up its substance

(Lythgoe, 1979). But the exact bio-chemical mechanism of the transparency is not clear.

What does distinguish the cornea from other tissues is its particular anatomical position. This necessitates, among other things, that the reactions that take place in it can operate at temperatures below that of the blood; it must share the flexibility with the outer layers of the skin and perhaps, the epithelial lining of the lungs and air passages. Most striking is the absence of any blood vessels except around its extreme periphery. This poses problems in respect of the nutrition of the tissue (Maurice and Riley, 1970).

Chemically, the cornea is chiefly composed of proteins and carbohydrates. The nature of cellular and extra cellular proteins of the cornea is so different that each of the anatomical layers must be considered separately (Maurice and Riley, 1970).

The corneal epithelium, like the epidermis sheds cells constantly from the outer surface and replaces them from the rapidly dividing basal layer (Maurice and Riley, 1970). Electron microscopic studies by Jakus (1964); Pedler (1962) revealed the presence of well organized tonofibrils in the cytoplasm of the flattened epithelial cells. Kawerau and Ott (1961), reported the high level of soluble proteins in the epithelium.

Maurice and Riley (1970) observed, that the stroma contains about 94% of protein and greater part of this is collagen which is organized into fibril. The size of the fibrils and their regular spacing in the ground substance is essential for the transparency of the cornea. The arrangement of collagen fibrils in regular and parallel patterns has been proposed to depend on reactions with the soluble protein-polysaccharides, chondroitin-sulfate proteins (Mathews, 1965). It is suggested that the acid polsaccharides are distributed along a fibrous protein, attached only by a short length of the polysaccharide chain, so that the remainder of the chain is free. This part, with a considerable negative charge, can combine with the cationic sites of the collagen fibrils. In this way several fibrils can be attached, firmly and over well-regulated distances, to a single non-collagen protein. Links to the same collagen fibrils from other glycoprotein units would result in the formation of a stable structure, resembling a crystal lattice.

The endothelial layer consisting of a single sheet of cells 2-5 μ thick, is not easily analyzed (Maurice and Riley, 1970). It secretes a basement membrane, Descemet's membrane which is several times thicker than itself and probably continues to grow throughout life (Salzmann, 1912). This layer consists of a collagenous framework (Jakus, 1956). Maurice and Riley (1970), observed that the Descemet's membrane is

extremely resistant to chemical and enzymetic actions because of the unusual association of protein with high content of carbohydrates.

The chemical composition of the various layers and the biochemical reaction that takes place in them, do not appear to differ in any major respect from those in other tissues of the body. However, the mucopolysaccharide components of the connective tissue present a distinctive pattern, and there is a transport mechanism resident in the endothelium whose mode of action is at present not clear but which may prove to be unusual. These features are part of the mechanism that maintains the proper hydration and submicroscopic structure of the tissue upon which its transparency depends (Maurice and Riley, 1970).

The lens on the other hand plays a vital role in the process of image formation and accomodation. The crystalline lens according to Campbell (1967) is a bi-convex, transparent structure which is enclosed in a highly elastic non-cellular capsule of varying thickness. The capsule is formed by the single-layered epithelial cell below it. The substance of the lens consists of a series of ribbon-like fibres which arise from the equatorial region and are actually greatly elongated epithelial cells. The fibriller cells proceed from the equator towards the lens centre. The transparent lens possesses a regularity of cellular arrangement very similar in some ways

to that of a cornea (Kuck, 1970). The lens of the fish eye is a firm transparent ball made up of non-collagenous protein. The lens has a high refractive index (1.67), the only refracting and image forming structure. The fish lens is not homogeneous but has an actual refractive index from 1.53 at the centre to 1.33 near the outside. (Lagler et al. 1977).

Crystallines are the principal soluble proteins present in the eye-lens. They are sub-divided into four main immunologically distinct families, commonly called α , β , γ and δ . With the exception of the δ -crystallins, which is characteristic of only birds and reptiles, all families are found in all vertebrates (Piatigorsky, 1984). Each class is composed of multiple polypeptides with its own primary structure (Basaglia, 1989).

The chief non-soluble lens proteins is albuminoid (Krause, 1933). The albuminoid is a mixture (Waley, 1965a) and can be extracted to give glycoproteins (Dische et al., 1962). According to Dische (1965a), the growth and differentiation of the lens is controlled by influencing the systhesis of albuminoid by the glycoproteins.

MATERIALS AND METHODS

The eyes, enucleated from the live animals were fixed in 10% buffered formalin until they were used.

Histological studies of eyes has been carried out by routine paraffin embedding technique-Microtome sections of 7-8 μ thick were used.

Histochemistry

Histochemical studies has been performed by employing Mallory's triple connective tissue stain (Pantin method, 1946), Masson's trichrome stain (Gurr modification 1956), Delafield haematoxylin stain (Carlton, 1947), in connection to the differential staining properties of various eye tissues.

Detection of Mucopolysaccharides (MPS)

The histological and histochemical methods employed for the study of mucopolysaccharides are as follows:

(1) Staining Method - The stains used for the detection of mucopolysaccharides are - Basic fuchsin, Aldehyde fuchsin and Toluidine blue (Humason, 1971).

Examination of Stained Sections - The "metachromatic" reaction given by a cationic dye, such as Toluidine blue is distinguished by its ability to survive alcoholic degradation (Curran, 1964). However, some authors (Cejkova and Brettschneider, 1969; Cejkova and Bolkova, 1974) have shown that histochemical demonstration of MPS in cornea is extremely dependent on the state

of hydration. This has been found true by us, where some wet sections as well as ethanol washed sections were studied.

(1) Extraction of Acid Mucopolysaccharides (AMPS) - Crude AMPS fraction from the tissues has been prepared with proteolytic enzyme, as described by Dietrich et al. (1977).

Previously defatted and dried tissues (100 µg to 1g) with acetone was suspended in 20 ml of 0.05M Tris-HCl buffer, pH 8.0. To this mixture 10 mg of trypsin was added and incubated at 37°C for 24 hrs. With a few drops of toluene forming a layer at the surface. After incubation the pH of the mixture was brought to 11.0 with conc. NaOH and maintained for 6 hrs. at room temperature. The pH was then brought to 6.0 with HCl and the mixture was centrifuged. To the supernatant, 0.1 ml of 2M NaCl and 2 vol. of alcohol were added and kept overnight at 5°C. The precipitate formed was collected by centrifugation and dried. The resultant powder was resuspended in 1 ml of 0.05M sodium acetate, pH 6.5, and DNAse and RNAse (1 mg of each) were added to the solution. The solution was again incubated for 24 hrs at 37°C with a layer of toluene. After incubation, 0.1 ml of 2M NaCl and 2 vol. of ethanol were added, to the solution and kept overnight at 5°C. The precipitate formed was collected by centrifugation and dried. The resulting powder was dissolved in 0.5 ml of water, heated at 100°C

for 2 min., any residue present was removed by centrifugation and the supernatant was analyzed.

(3) Chromatography - The extracted AMP was hydrolysed with 6N HCl at 100°C for 10-12 hrs. The acid hydrolysate was then evaporated to dryness. The dried residue was then dissolved in 0.5 ml of water and spotted on Whatman No. 1 paper and circular paper chromatogram run using butanol : acetic acid : water (4:1:1, v/v) as solvent (Giri and Nigam, 1954). The chromatogram was developed with silver nitrate (0.1 ml of saturated solution in 20 ml of acetone) and sodium hydroxide (0.5 gm NaOH in 25 ml of rectified spirit), as suggested by Trevelyan et al. (1950). The chromatogram was washed in 6N NH_4OH for 10 minutes and then washed in running tap water for 10 minutes and dried in room temperature.

(4) The electrophoresis of the AMPs was carried out by applying streaks of the samples on Whatman No. 1 paper strips using different electrolyte solutions.

(a) 0.1M Phosphate, pH 6.6, at 4v/cm for 8-10 hrs.

(b) 0.1M Barium acetate, pH 8, at 50v for 2-4 hrs.

(c) 0.1M Cupric acetate, pH 5.3, at 100v for 1.5-2 hrs.

(d) 0.1M Zinc acetate, pH 6.5, at 4v/cm for 16-18 hrs.

The paper strips, after removal from the electrophoresis unit, were dried at room temperature and stained with toluidine

blue (0.04% toluidine blue in 80% acetone). The staining of the strips were followed by 2-3 rinsing in 0.1% acetic acid and then 2-3 times with water respectively. The strips were then dried in room temperature.

OBSERVATIONS

Histochemistry

Examination of corneal histological sections of the common carp (Cyprinus carpio) and catfish, (Clarias batrachus), reveals that the stroma is externally lined by epithelium and internally by endothelium like other vertebrates. But the corneal epithelial layer of Stromateus argenteus could not be retained intact for its thinness. The stroma of common carp and cat fish reveals that it is formed of loosely arranged, fine collagen fibrils along the plane of the cornea while in Pomfret the fibriller arrangement in the stroma is more compact and thick. The corneal sections showed strong affinity to aniline blue when stained with Mallory's triple connective tissue stain as in other connective tissue and collagen (Figs. 1, 2 and 3).

The lens sections of above fishes upon staining with Mallory's stain gave orange colour (Figs. 4,5 and 6).

The retinal visual cell layer, on the other hand, showed lilac colour and the neuronal and glial cells exhibited varying shades of blue and light yellow colour (Figs. 7,8 and 9).

EXPLANATION TO FIGURES

Fig. 1. Transverse section through the cornea of common carp, Cyprinus carpio, stained with Mallory's triple stain x 500.

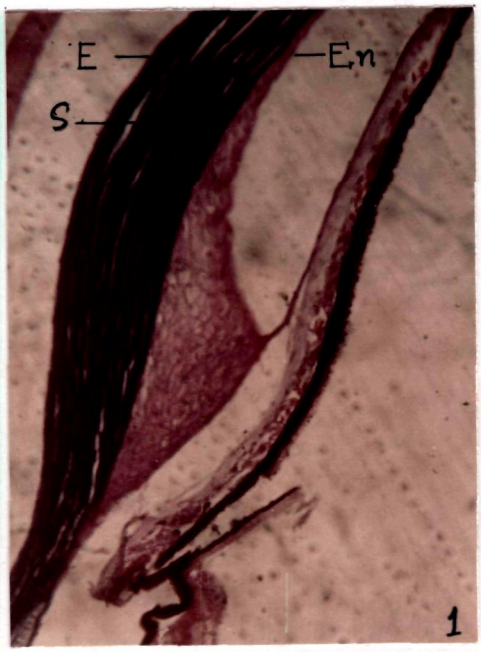
Fig. 2. T/S through the cornea of catfish, Clarias batrachus, stained with Mallory's triple stain x 375.

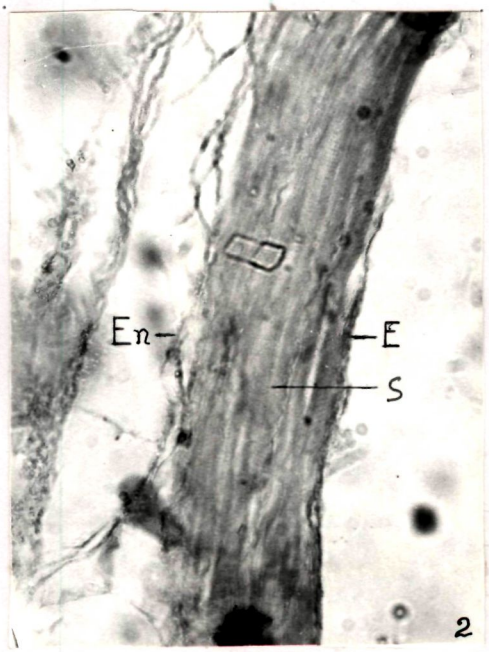
Fig. 3. T/S through the cornea of pomfret, Stromateus argenteus, stained with Mallory's triple stain x 500.

E, Epithelium;

En, Endothelium

S, Stroma


E
En
S
1


En
E
S
2

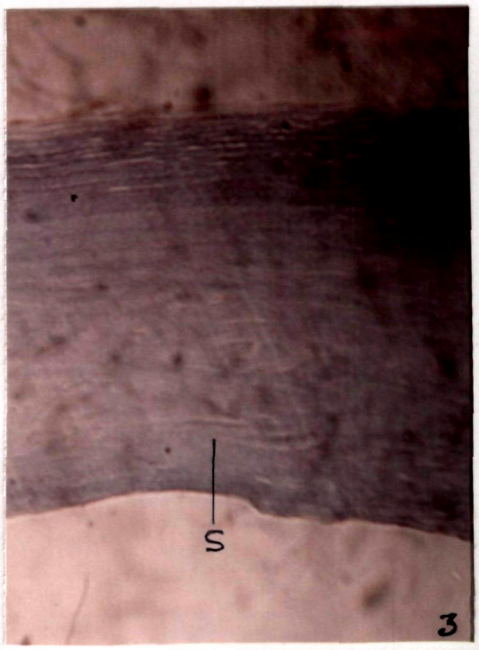

S
3

Table 1

Results of Staining Reactions and Histochemical Tests Obtained with the Corneae of the Common Carp, Cyprinus carpio; catfish, Clarias batrachus, and pomfret, Stromateus argenteus

Stains and Tests	References	Cornea		
		Epithelium	Stroma	Endothelium
Mallory's triple stain	Pantin (1946)	Blue	Blue	Blue
Masson's trichrome stain	Carr (1956)	Green	Green	Green
Delafield haematoxyline	Carlton (1947)	Violet	Violet	Violet
Biuret test	Serra (1946)	++	++	++
Millon's test	Baker (1956)	+	++	+
Xanthoproteic test	Pears (1961)	+	++	+
Nile blue	Lillie (1956)	++	+	++
Sudan III	Baker (1956)	++	+	++

\+ Moderately positive

++ Intensely positive

EXPLANATION TO FIGURES

Fig. 4. T/S through the lens of *Cyprinus carpio*, stained with Mallory's triple stain x 500.

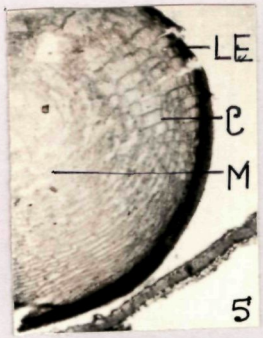
Fig. 5. T/S through the lens of *Clarias batrachus*, stained with Mallory's triple stain x 150.

Fig. 6. T/S through the lens of *Stromateus argenteus* stained with Mallory's triple stain x 375.

C, Cortex. LE, Lens epithelium,

M, Medulla


C
LE
M
4


LE
C
M
5

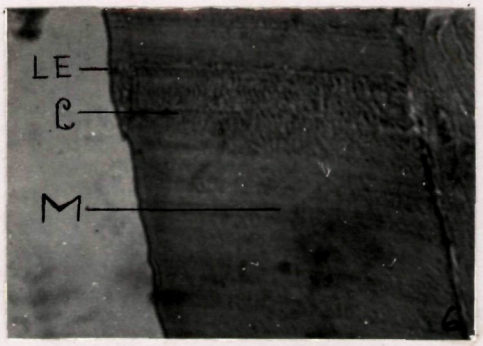

LE
C
M
6

Table 2

Results of Staining Reactions and Histochemical Tests Obtained with Lenticular tissue of C. carpio, C. batrachus and S. argenteus

Stains and tests	Reference	Lens tissue
Mallory's triple stain	Pantin (1946)	Orange
Masson's trichrome stain	Gurr (1956)	Green
Delafield haematoxyline	Carlton (1947)	Pink
Biuret test	Serra (1946)	++
Millon's test	Baker (1956)	++
Xanthoproteic test	Pears (1961)	++
Nile blue	Lillie (1956)	++
Sudan III	Baker (1956)	++

++ Intensely positive

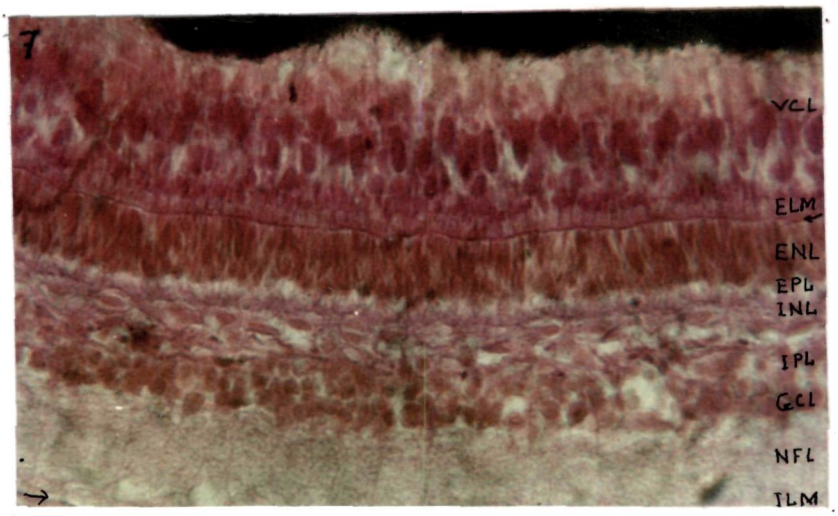
EXPLANATION TO FIGURES

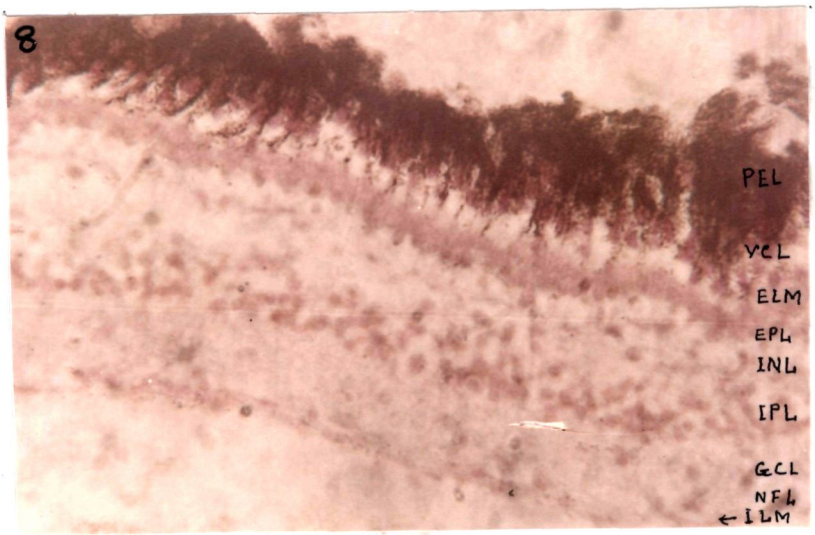
Fig. 7. T/S through retina of Cyprinus carpio, stained with Mallory's triple stain x 500.

Fig. 8. T/S through retina of Clarias batrachus, stained with Mallory's triple stain x 500.

Fig. 9. T/S through retina of Stromateus argenteus, stained with Mallory's triple stain x 500.

PE, Pigment epithelium. VCL, Visual Cell layer. ELM, External limiting membrane. ENL, External nuclear layer. EPL, External plexiform layer. INL, Internal nuclear layer. IPL, Internal plexiform layer. GCL, Ganglion cell layer. NFL, Nerve fibre layer. ILM, Internal limiting membrane. C, Cone. R, Rod. TC, Twin Cone.


7
VCL
ELM
ENL
EPL
INL
IPL
GCL
NFL
ILM


8
PEL
VCL
ELM
EPL
INL
IPL
GCL
NFL
ILM

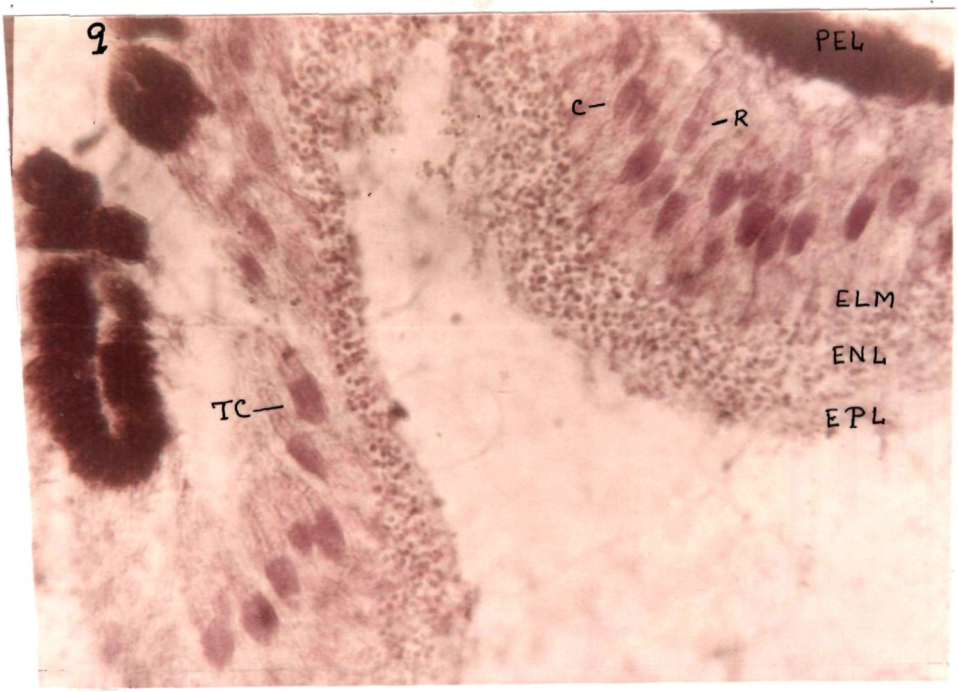

9
PEL
C
R
ELM
ENL
EPL
TC

Table 3

Results of Staining Reactions and Histochemical Tests Obtained with Retinal Tissue of *C. carpio*, *C. batrachus* and *S. argenteus*

Stains and tests	References	Retina	
		Visual cell layer	Neural and glial cell layer
Mallory's triple stain	Pantin (1946)	Lilac	Light blue to light violet
Masson's trichrome stain	Gurr (1956)	Violet	Violet
Delafield haematoxiline	Carlton (1947)	Pink	Light to deep violet
Biuret test	Serra (1946)	+	+
Millon's test	Baker (1956)	++	++
Xanthoproteic test	Pears (1961)	++	++
Nile blue	Lillie (1956)	+	+
Sudan III	Baker (1956)	++	+

\+ Moderately positive

++ Intensely positive

The corneal, lenticular and retinal layers when subjected to Biuret, Millon's and Xanthoproteic tests gave intense positive reactions indicating the presence of protein.

When the sections were treated with Nile blue and Sudan III for lipid they reacted positively.

For the detection of polysaccharide complex, corneal sections when treated with 0.5% Basic fuchsin responded positively in all three fishes showing purple colour. Similarly, when the sections were stained with Aldehyde fuchsin, the corneal layers became purple in colour. The corneal stroma, upon staining with Toluidine blue became purple in colour (Figs. 10, 11 and 12) showing "metachromasia" (Table 4). The reactions indicate the presence of AMPs, mostly sulphated.

The lens tissue of the three fishes gave purple colour with basic fuchsin, but the reaction in the cortical region was intense compared to medullary part which was lighter. Staining with Aldehyde fuchsin, the lens cortex gave strong reaction than the medulla. The cortical part of the lens tissues were slightly "metachromatic" while medullary zones were "orthrochromatic" (Table 4). The lens thus contains very little AMPS (Figs. 13, 14 and 15).

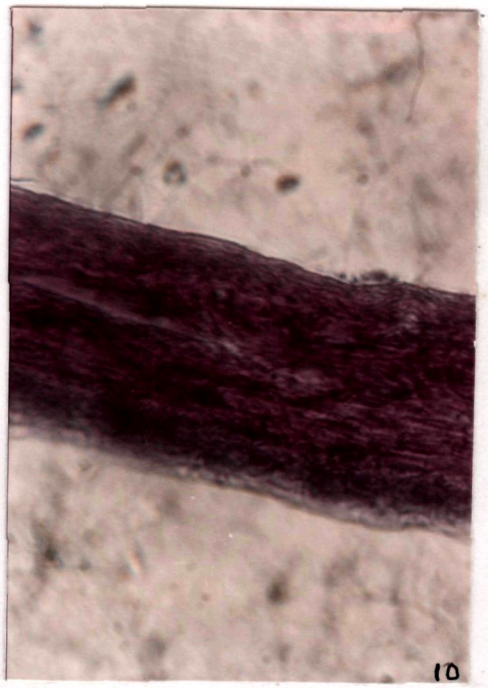
Retinal layers responded variously. The neuronal and glial cells stained deeply showing a pink colour while the visual cell layers were lighter in reaction. Staining with

EXPLANATION TO FIGURES

Fig. 10. T/S of cornea of *Cyprinus carpio*, showing "metachromasia" upon staining with Toluidine blue x 500.

Fig. 11. T/S of cornea of *Clarias batrachus*, showing "metachromasia" upon staining with Toluidine blue x 500,

Fig. 12. T/S of cornea of *Stromateus argenteus*, showing "metachromasia" upon staining with Toluidine blue x 500.



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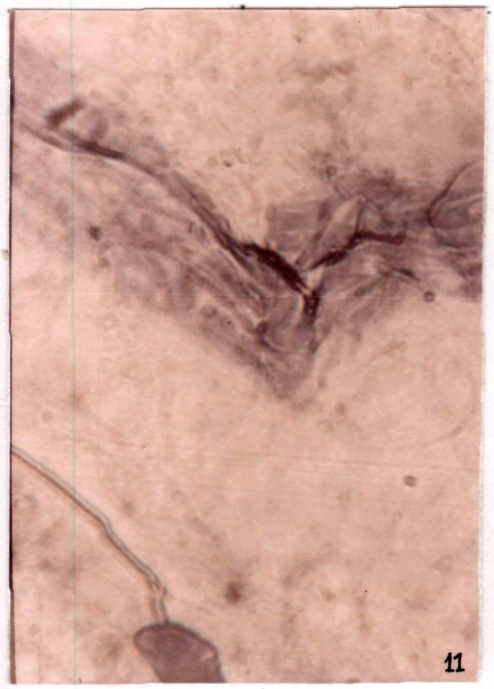

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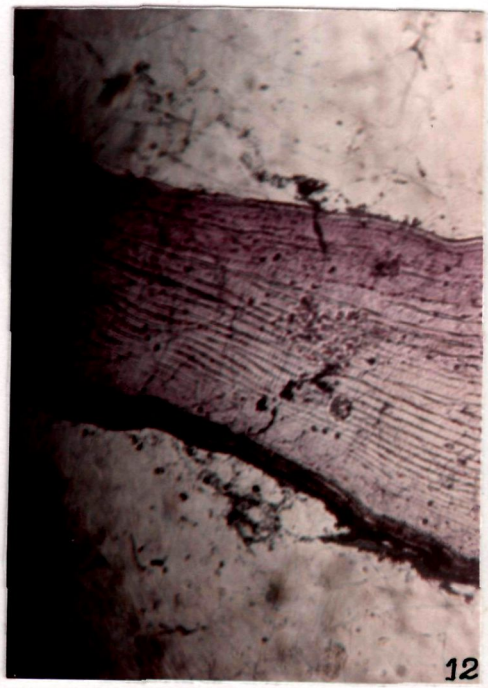


12

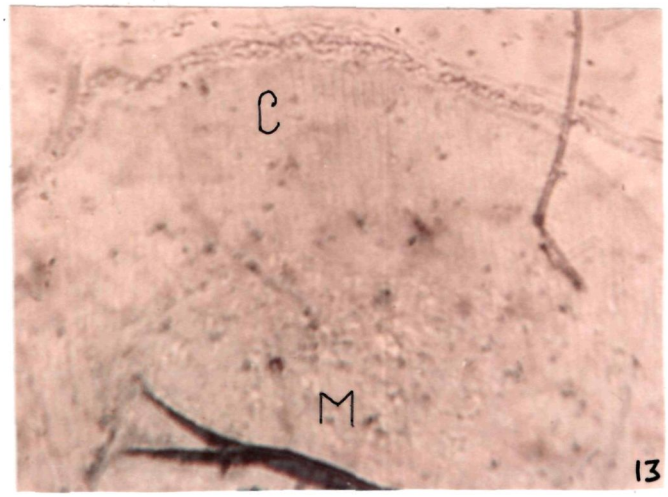
EXPLANATION TO FIGURES

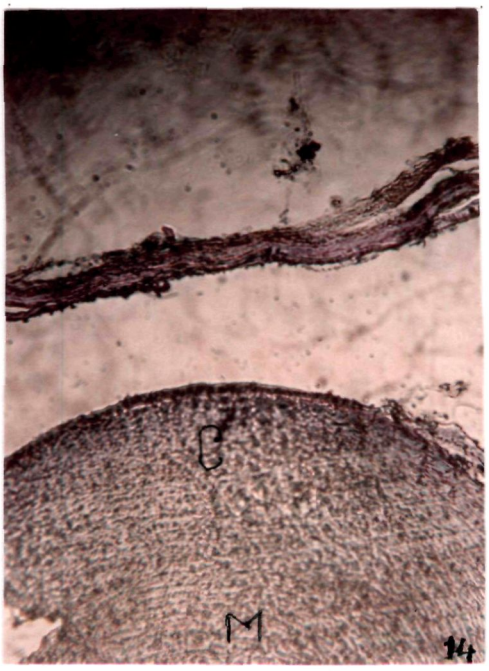
Fig. 13. T/S of lens of *Cyprinus carpio*, upon staining with Toluidine blue x 500.

Fig. 14. T/S of lens of *Clarias batrachus*, upon staining with Toluidine blue x 500.

Fig. 15. T/S of lens of *Stromateus argenteus*, upon staining with Toluidine blue x 500.

C, Cortex (Metachromatic)
M, Medulla (Orthochromatic)


C
M
13


C
M
14

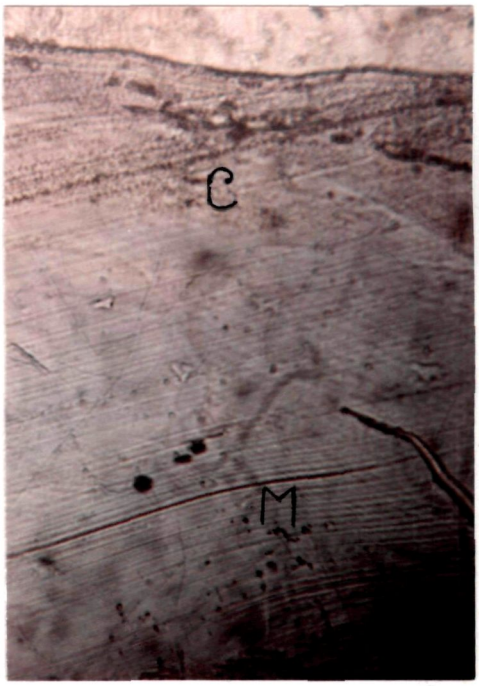

C
M

Table 4

Results of Staining Reactions and Histochemical Tests for the Detection of Acid Mucopolysaccharides in the eyes of C. carpio, C. batrachus and S. argenteus.

Stains and tests	References	Cornea	Lens	
			Cortex	Medulla
Basic fuchsin	Stempien (1962)	deep red	deep red	red
Aldehyde fuchsin	Cameron and Steele (1959)	Purple	Purple	light purple
Toluidine Blue	Pearse (1961)	Violet (Meta-chromatic)	Light violet (Slightly metachro-matic)	Bluish (Ortho-chromatic)
PAS - test	McManus and Mowry (1960)	+++	++	+
Alcien Blue	Luna (1968)	+++	++	+

\+ Slightly positive

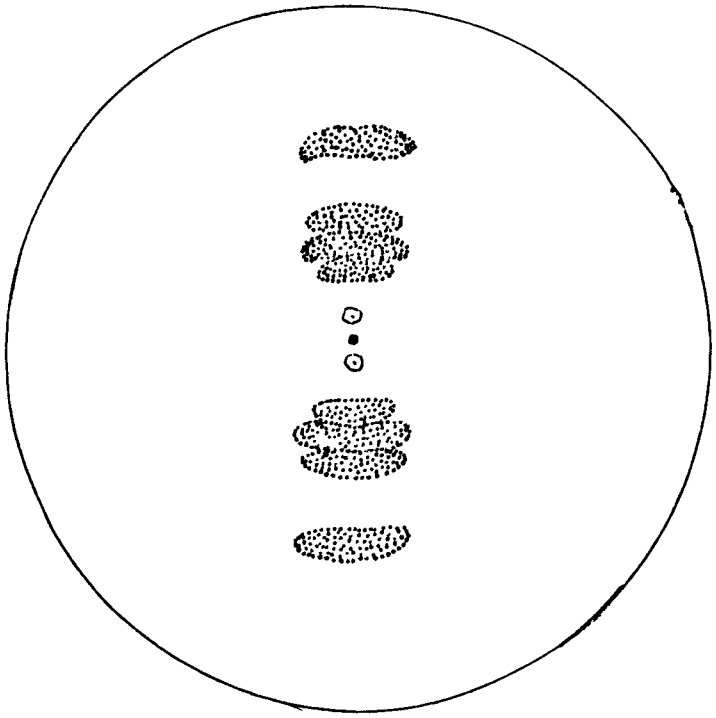
++ Moderately positive

+++ Intensely positive

EXPLANATION TO FIGURES

Fig. 16. Circular paper chromatogram of the sugar components of the corneal mucopolysaccharides of Cyprinus carpio

Solvent : Butanol : Acetic acid : water
(4 : 1 : 1, v/v)

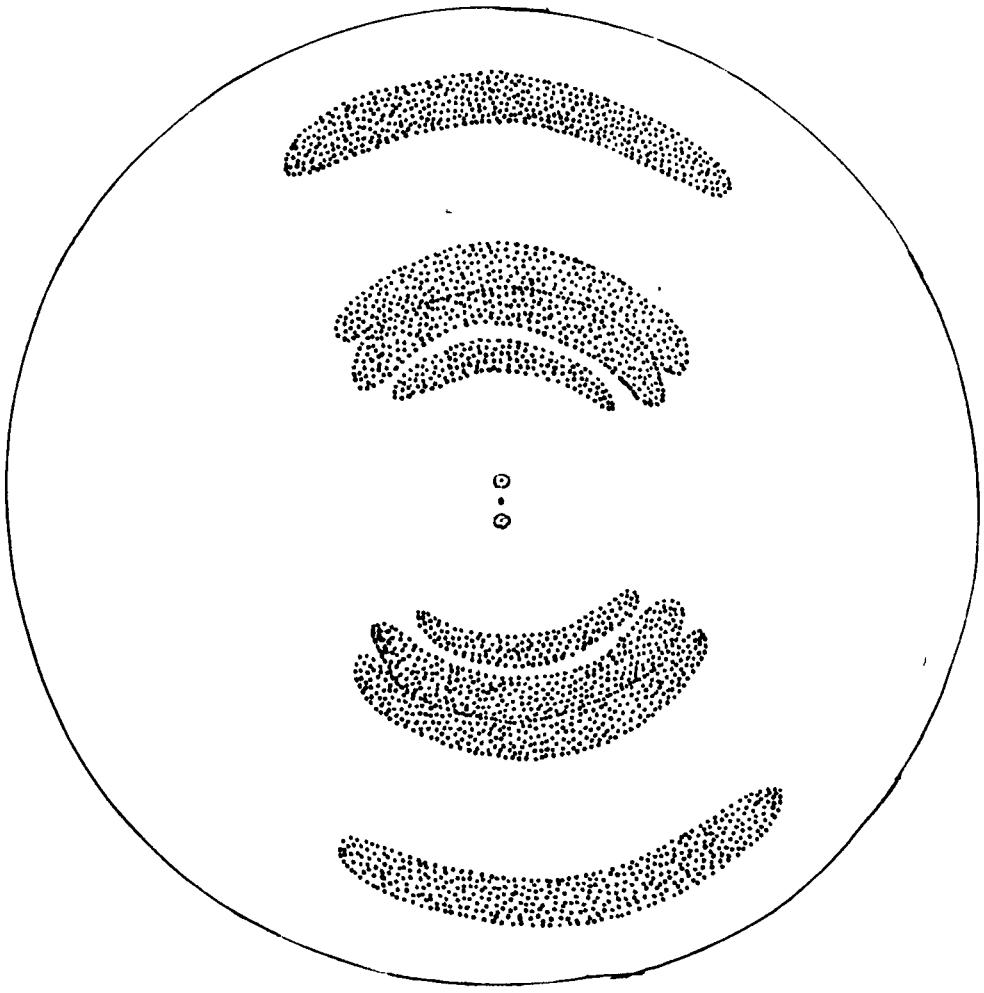


EXPLANATION TO FIGURES

Fig. 17 Circular paper chromatogram of sugar components of the corneal mucopolysaccharides of *Clarias batrachus*

Solvent : Butanol : Acetic Acid : Water

(4 : 1 : 1, v/v)

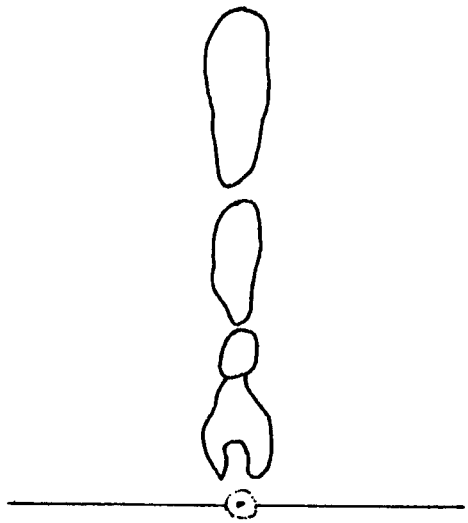


EXPLANATION TO FIGURES

Fig. 18 Ascending paper chromatogram of the sugar components of the corneal mucopolysaccharides of *Stromateus argenteus*

Solvent : Butanol : Acetic acid : water

(4 : 1 : 1, v/v)

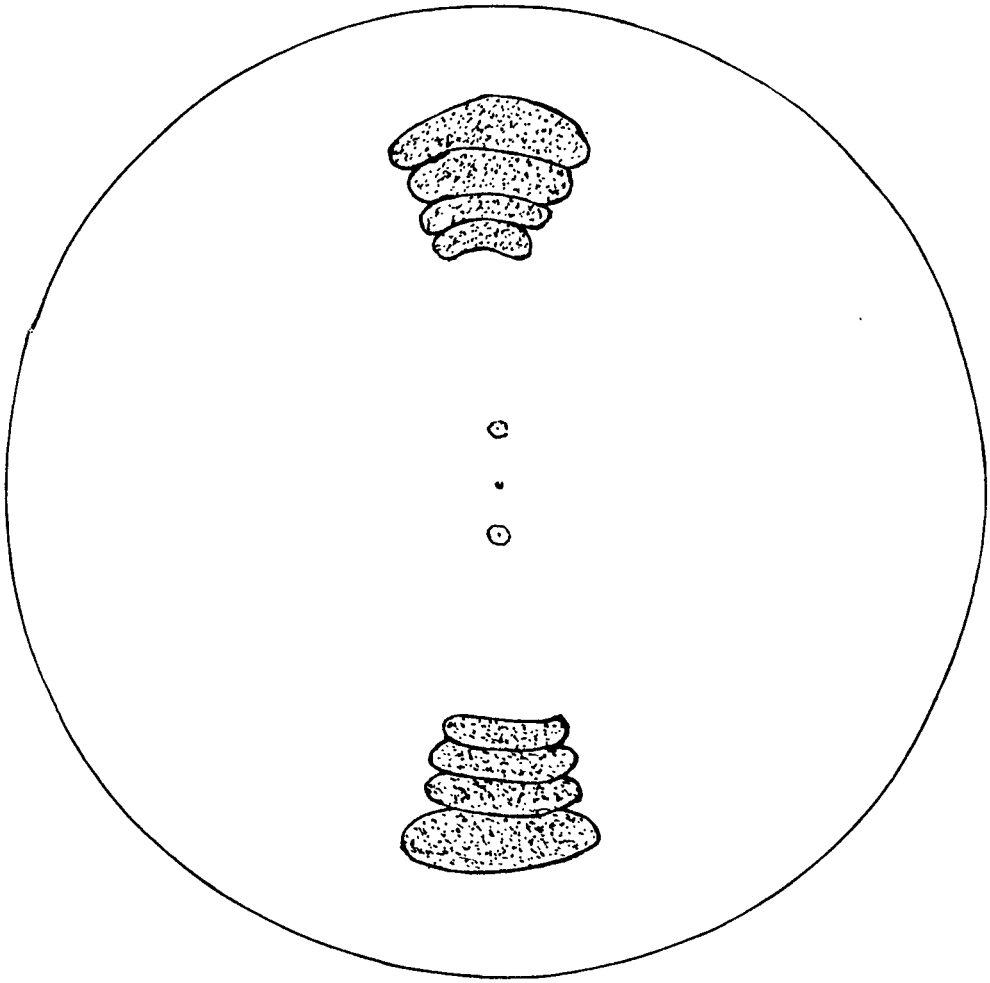


EXPLANATION TO FIGURES

Fig. 19. Circular paper chromatogram of the sugar components of the lens mucopolysaccharides of *C. carpio*.

Solvent : Butanol : Acetic acid : water

(4 : 1 : 1, v/v)

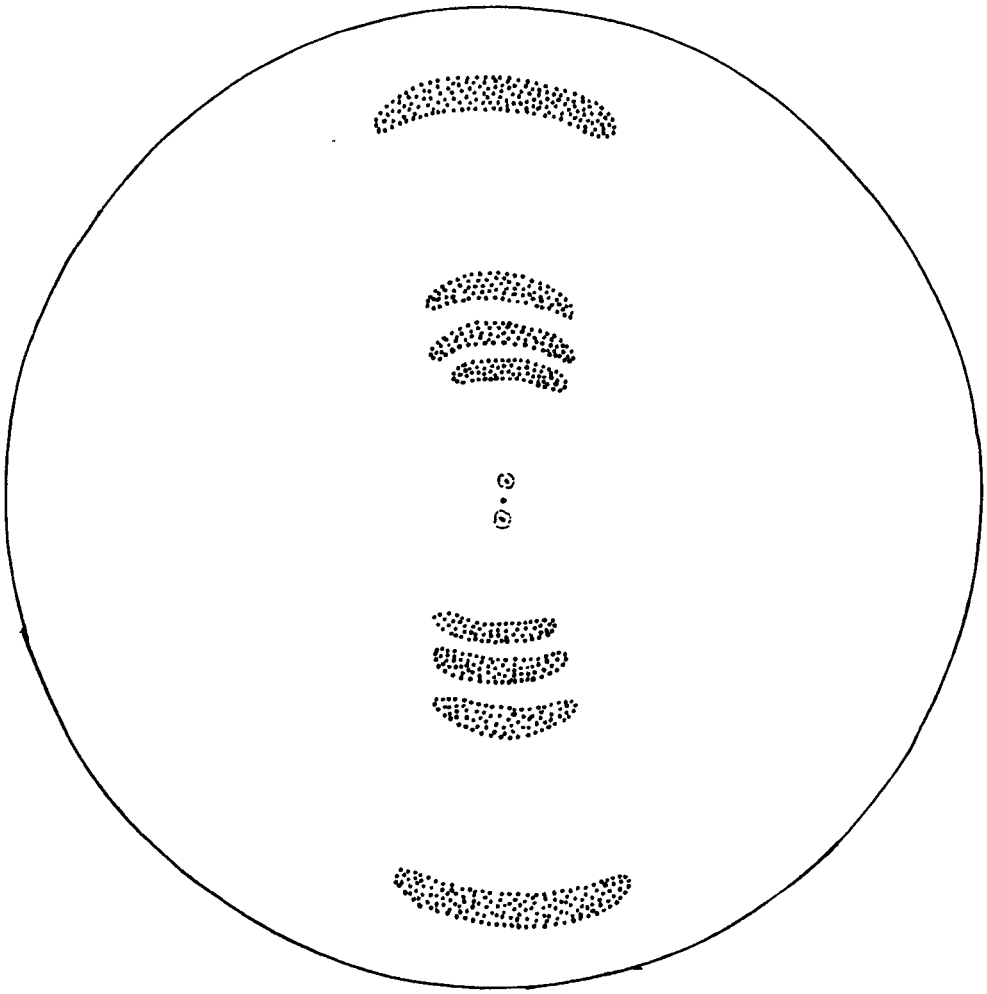


EXPLANATION TO FIGURES

Fig. 20. Circular paper chromatogram of the sugar components of the lens mucopolysaccharides of *C. batrachus*.

Solvent : Butanol : Acetic Acid : Water

(4 : 1 : 1, v/v)



EXPLANATION TO FIGURES

Fig. 21. Ascending paper chromatogram of the sugar components of the lens mucopolysaccharides of *S. argenteus*.

EXPLANATION TO FIGURES

Fig. 22. The electrophoretic movement pattern of the crude mucopolysaccharide (MP) extract from the cornea of Cyprinus carpio, as compared with the movement pattern of some standard mucopolysaccharides (MPs).

Ch A - Chondroitin sulfate A = Chondroitin 4 - sulfate

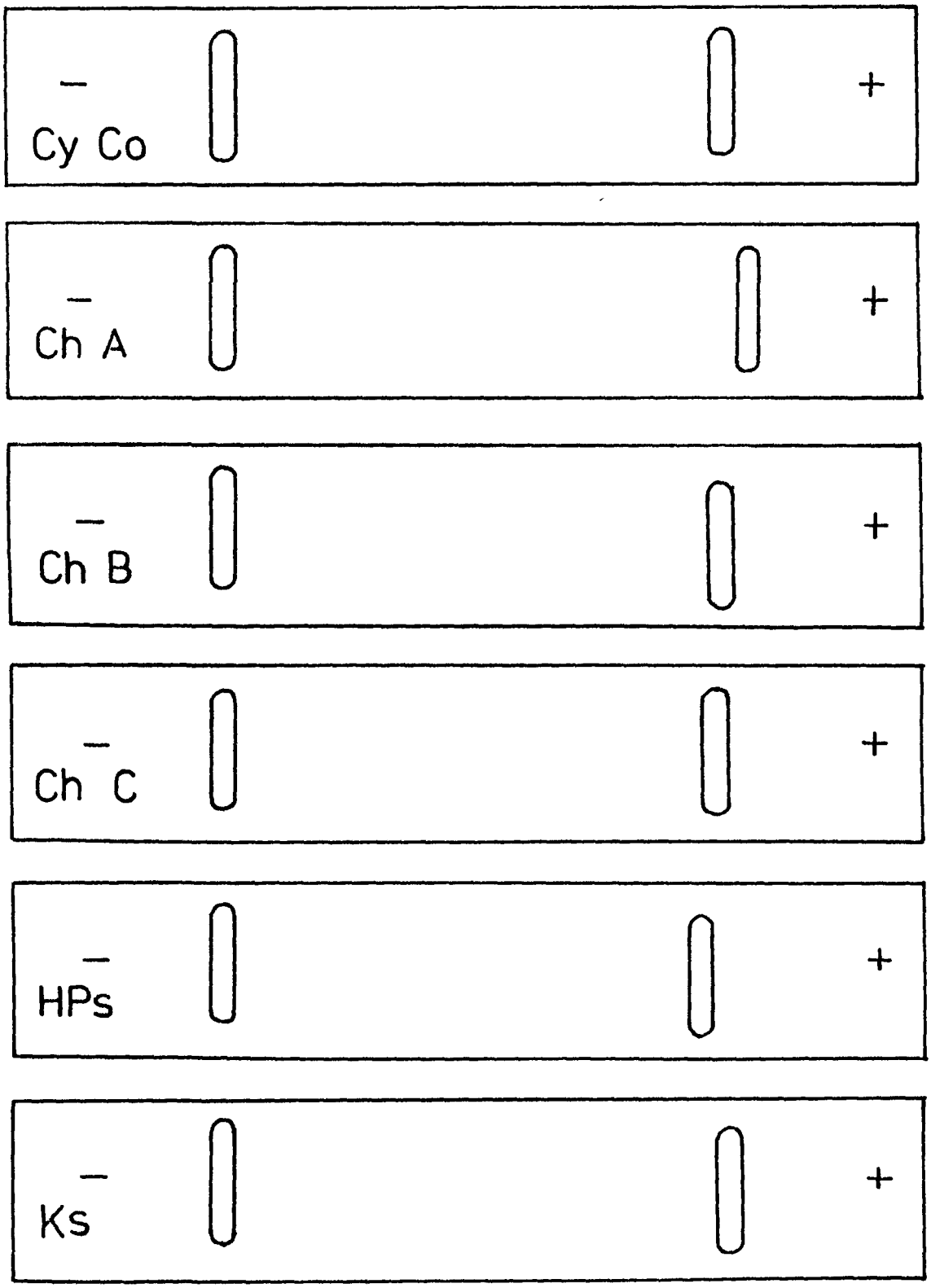
Ch B - Chondroitin sulfate B = Dermatan sulfate

Ch C - Chondroitin sulfate C = Chondroitin 6 - sulfate

HPs - Heparan sulfate

Ks - Keratan sulfate

CyCo - Cyprinus cornea

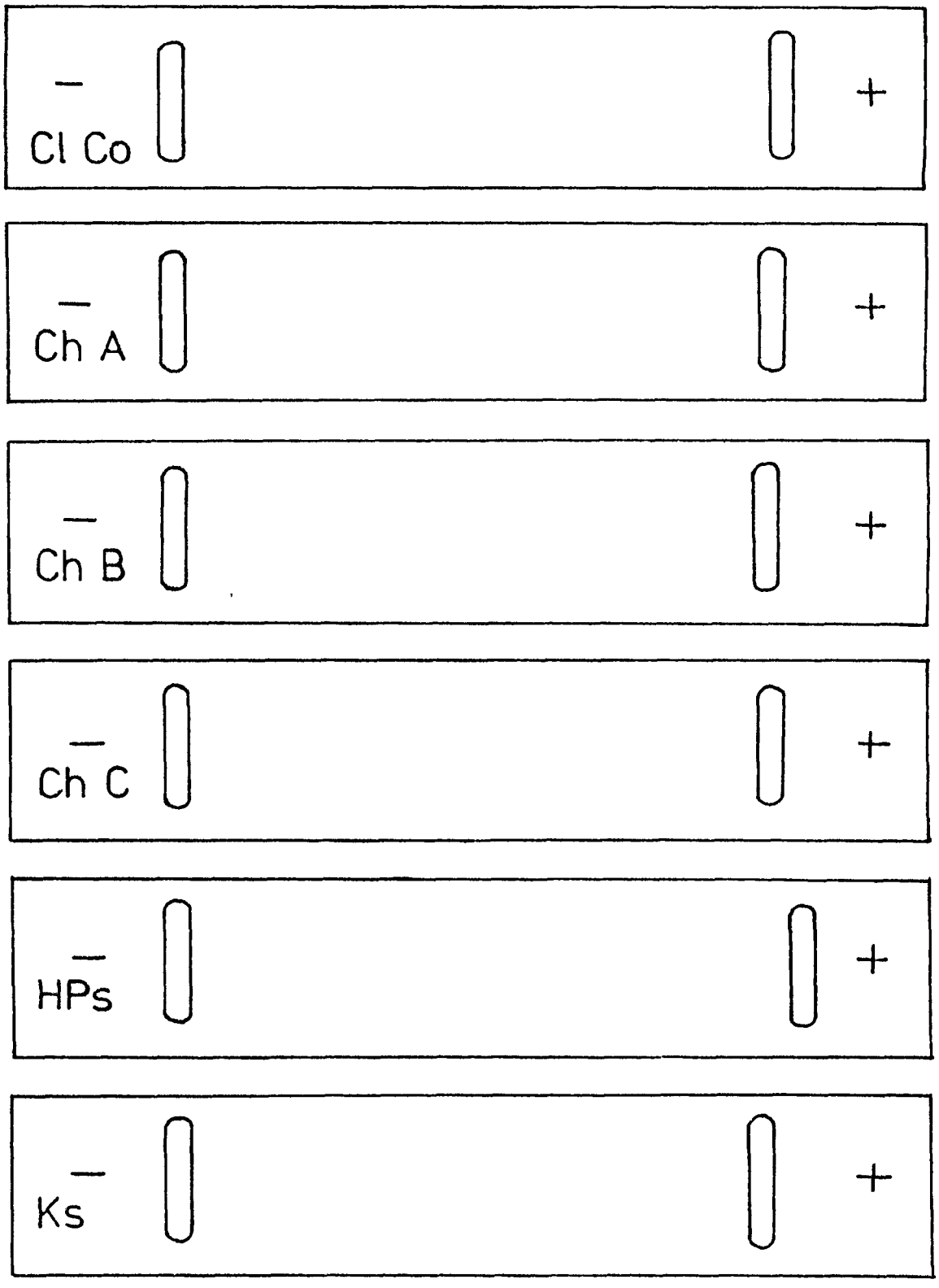

– Cy Co +
– Ch A +
– Ch B +
– Ch C +
– HPs +
– Ks +

EXPLANATION TO FIGURES

Fig. 23. The electrophoretic movement pattern of the crude MP extract from the cornea of *Clarias batrachus*, as compared with the movement pattern of some standard MPs.

(Other details as in Fig. 22)

Cl Co *Clarias* cornes

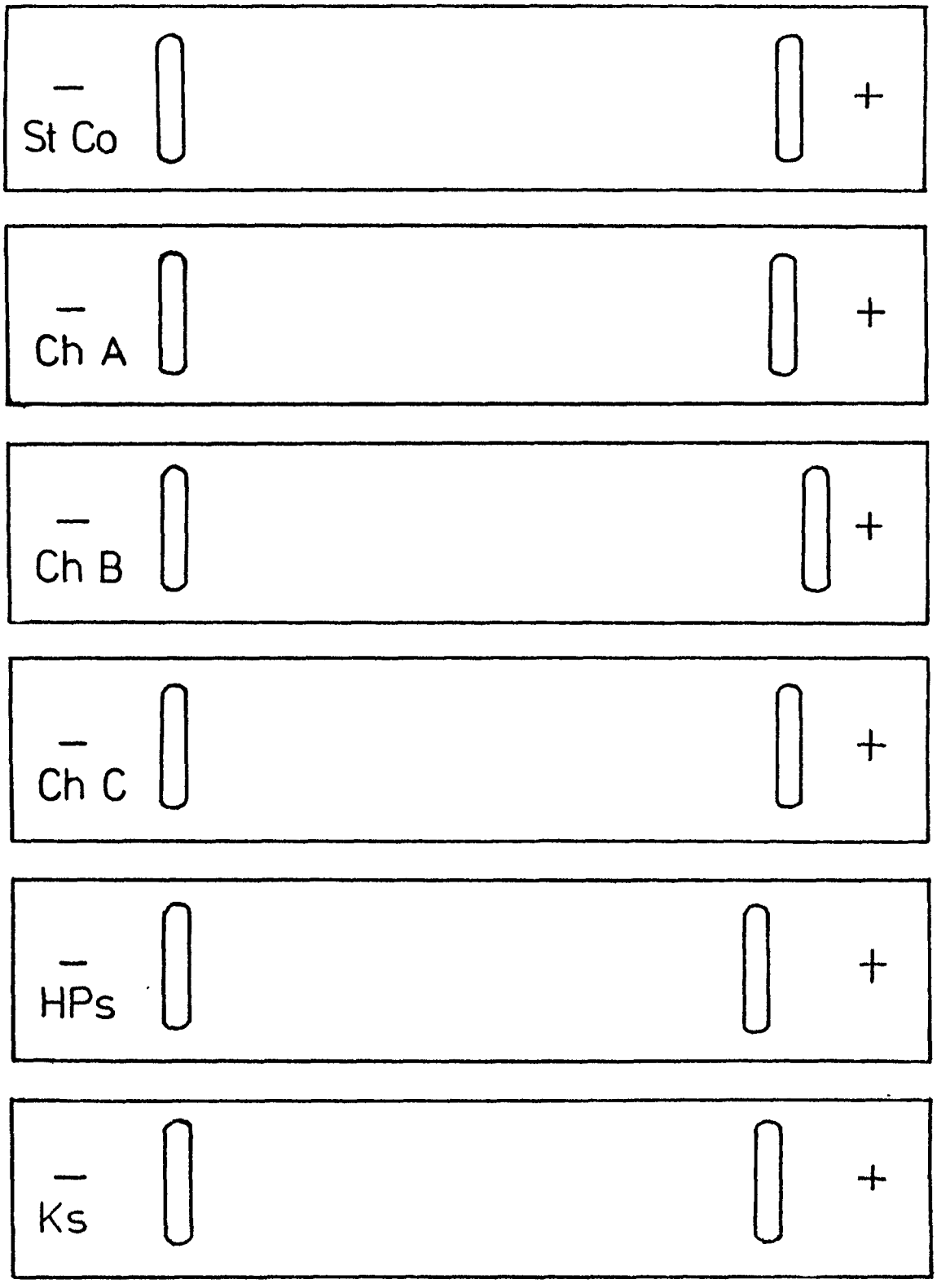

—
Cl Co
+
—
Ch A
+
—
Ch B
+
—
Ch C
+
—
HPs
+
—
Ks
+

EXPLANATION TO FIGURES

Fig. 24 The electrophoretic movement pattern of the crude MPs extract from the cornea of Stromateus argenteus, as compared with the movement pattern of some standard MPs.

(Other details as in Fig. 22)

St Co - Stromateus cornea

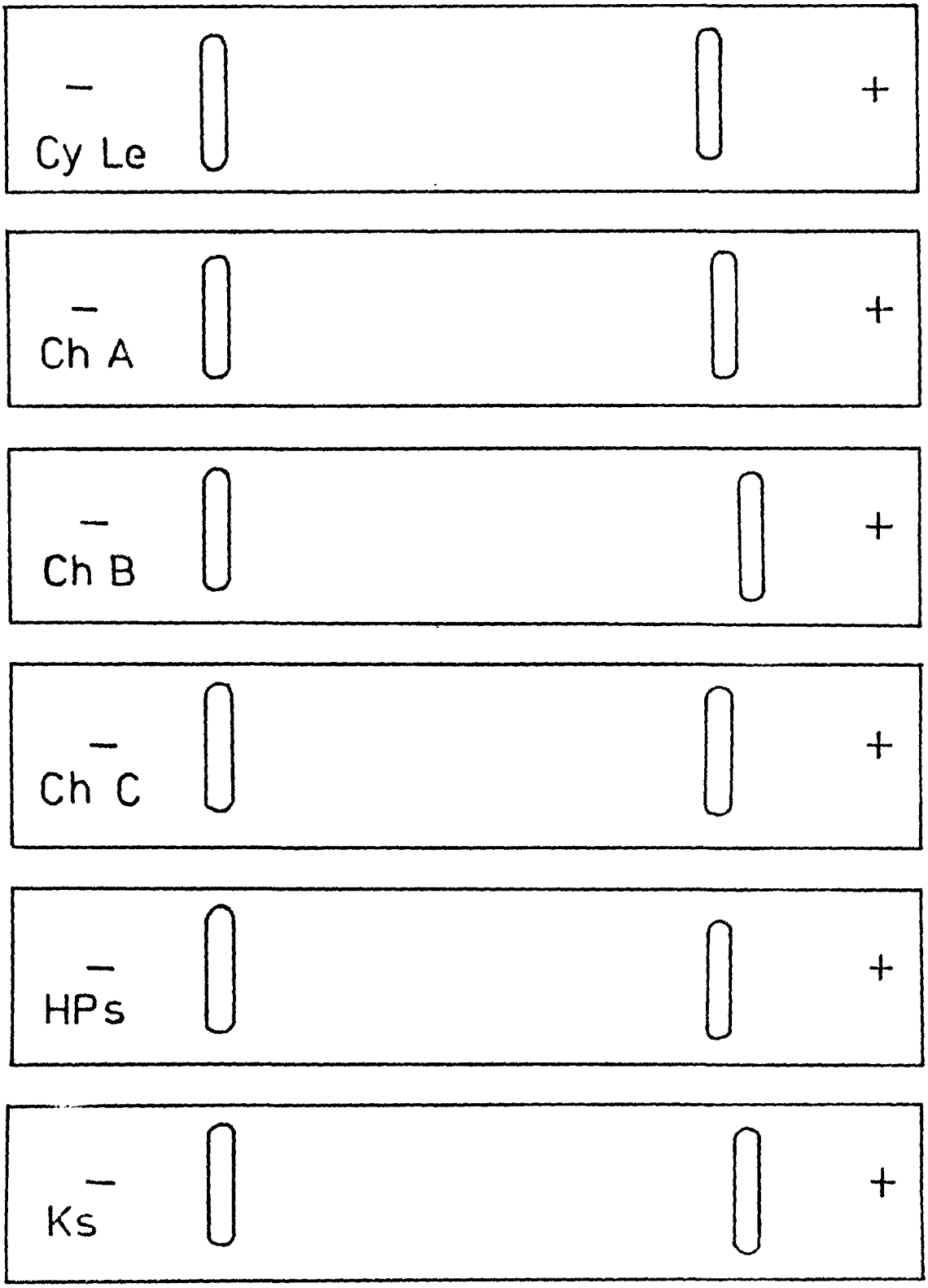

St Co
Ch A
Ch B
Ch C
HPs
Ks
–
+

EXPLANATION TO FIGURES

Fig. 25. The electrophoretic movement pattern of the crude MPS extract from the lens of *Cyprinus carpio*, as compared with the movement pattern of some standard MPS.

(Other details as in Fig. 22).

Cy Le - *Cyprinus* lens

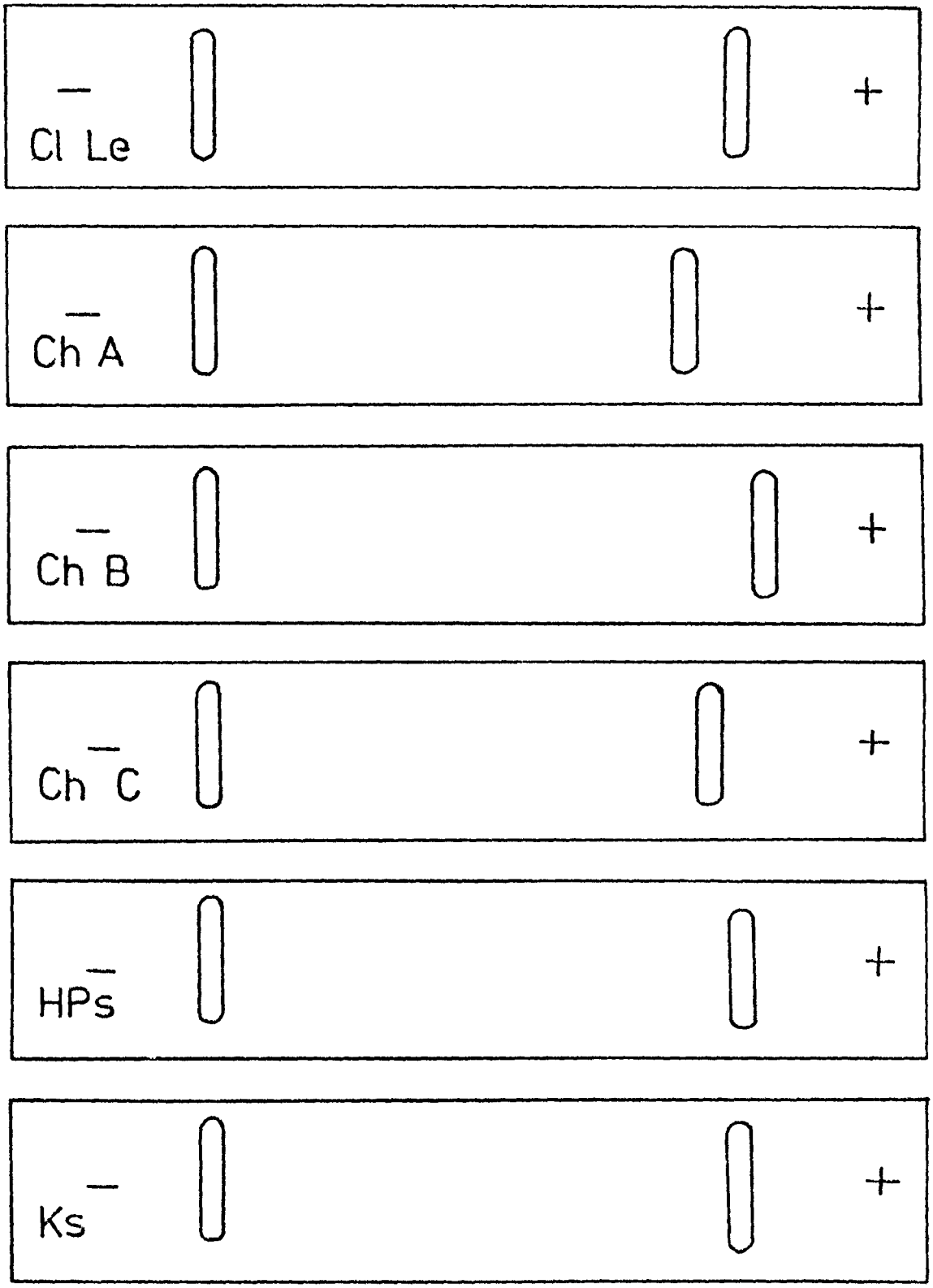

–
Cy Le
+
–
Ch A
+
–
Ch B
+
–
Ch C
+
–
HPs
+
–
Ks
+

EXPLANATION TO FIGURES

Fig. 26 The electrophoretic movement pattern of the crude MP extract from the lens of *Clarias batrachus*, as compared with the movement pattern of some standard MPS.

(Other details as in Fig. 22)

Cl le - *Clarias* lens

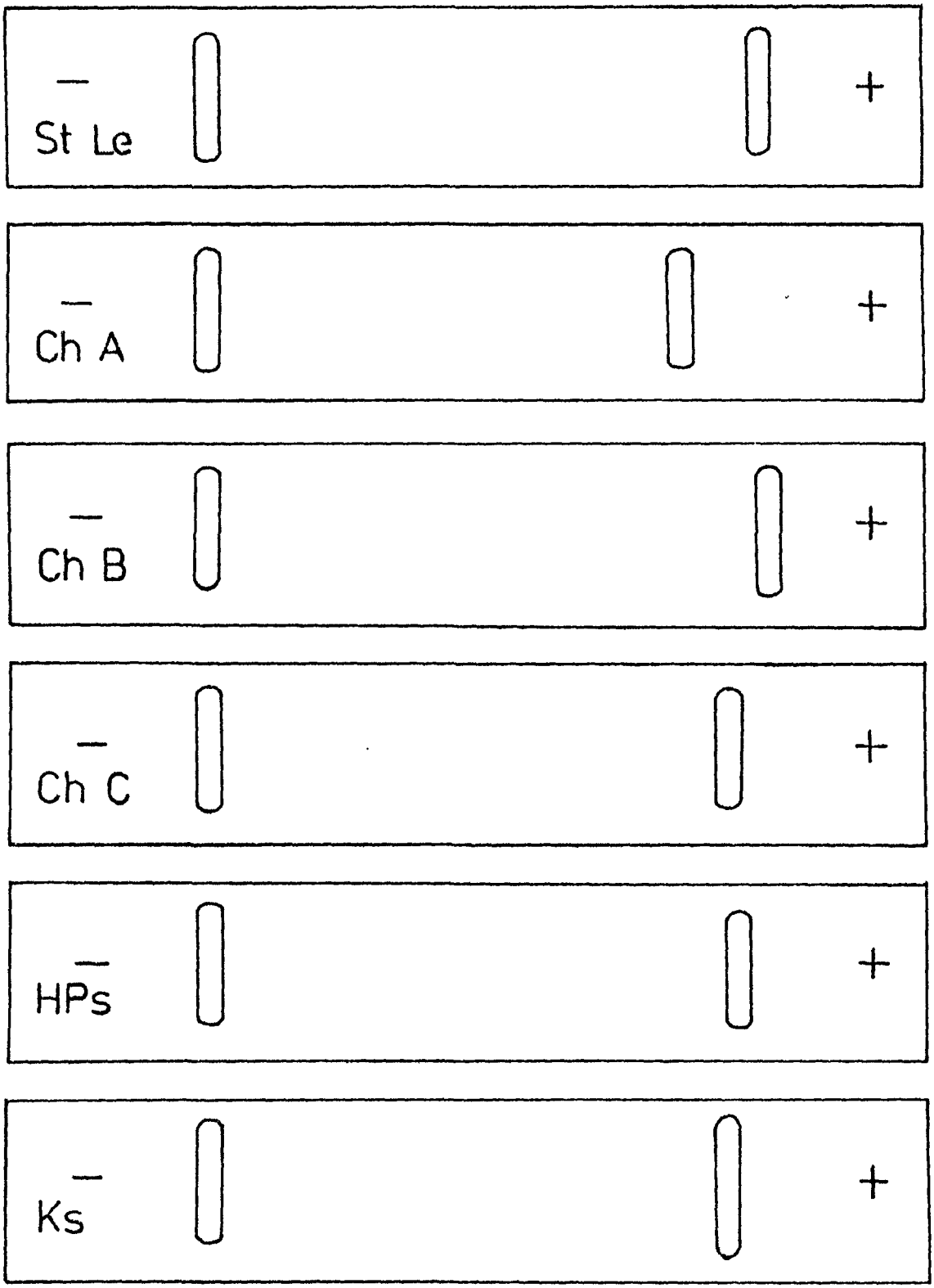

Cl Le
Ch A
Ch B
Ch C
HPs
Ks
+

EXPLANATION TO FIGURES

Fig. 27 The electrophoretic movement pattern of the crude MP extract from the lens of *Stromateus argenteus*, as compared with the movement pattern of some standard MPS.

(Other details as in Fig. 22).

St le - *Stromateus* lens


St Le
Ch A
Ch B
Ch C
HPs
Ks

Aldehyde fuchsin, the retinal layers responded by giving a faint violet colour.

The visual cell layer of retina responded orthochromatically but the neuronal and glial elements yielded "metachromasia". The above stain suggests the presence of AMPS, mostly in the neuro-glial layers of retina.

Chromatography

The chromatographic analysis of the AMPS from the cornea and lens of the eyes of Cyprinus carpio, Clarias batrachus and Stromateus argenteus indicates the presence of the sugars - glucose, galactose, mannose and xylose (Figs. 16,17,18,19, 20 and 21).

Electrophoresis

Electrophoretic mobilities of the crude extracts of AMPS from the corneae and lenses of Cyprinus carpio, Clarias batrachus and Stromateus argenteus, when compared with several standard AMPS in different buffer systems indicate that the major components of AMPS belong to the group of sulphated AMPS (Figs. 22,23,24,25,26 and 27).

DISCUSSION

The foregoing observations reveal the fact that the corneal and lenticular tissues of Cyprinus carpio, Clarias batrachus and Stromateus argenteus contain AMPs in general but sulphated mucopolysaccharides in particular.

The term "mucopolysaccharide" has originally been coined by Meyer (1938) to those substances with similar physico-chemical properties, isolated from connective tissues. With the increased understanding of the biochemistry of these substances in recent times, many of these substances are variously termed as glycoproteins, mucoproteins, mucopolysaccharides or acid mucopolysaccharides. The term "Glycoseaminoglycans", was also in vogue of late. But this terminology fails to distinguish bacterial polysaccharides and antibiotics containing amino sugars. Hence, "acid mucopolysaccharides (AMPs) and sulphated mucopolysaccharides (SMPs) appear to provide an adequate description and have the further advantage of continuous use (Jaques, 1977).

AMPs are polydisperse compounds composed of amino sugars with a uronic acid and may have sulfate radicals. The mucopolysaccharides are of following varieties:

1. Non-sulfated mucopolysaccharide-hyaluronic acid
2. SMP : Sulfated mucopolysaccharides:

(a) Chondroitins:

(i) Ch A : Chondroitin Sulfate A = Chondroitin 4 - sulfate;

(ii) Ch B : Chondroitin Sulfate B = Dermatan sulfate;

(iii) Ch C : Chondroitin Sulfate C = Chondroitin 6-sulfate.

(b) Heparitins:

Hpt A : Heparitin Sulfate A
Hpt B : Heparitin Sulfate B
Hpt C : Heparitin Sulfate C

(c) KS : Keratan Sulfate = Keratosulfate

(d) Heparin.

The amino sugar of these heteropolysaccharides is glucosamine in heparins, heparitins, keratosulfate and hyaluronic acid and galactoseamine in chondroitins while it is uronic acid (chiefly iudoronic acid) in heparin, heparitins C and D, Chondroitin B and glucuronic acid in other compounds except keratosulfate where it is replaced by galactose. Although heparin belongs to the group of mucopolysaccharide but it is usually absent in connective tissue. However, in some biological situations, the same function exercised by heparins in some cells or species seems to be performed by chondroitins in other cells or species (e.g. in basophilic leucocytes and mast cells)(Jaques, 1975).

Mucopolysaccharides have been detected in many vertebrate and invertebrate tissues and their biological significance has also been studied by different authors.

AMPs havebeen isolated from aorta of man (Barnes and Partridge, 1968; Barenson et al., 1966), pig (Wagh and Roberts, 1972), cow (Radhakrishnamurthy and Berenson, 1973), horse, sheep (Robert, Robert and Robert, 1970), elephant (McCullagh et al., 1973) and chicken (Keeley et al., 1969).

Rich AMPs content of skins in some vertebrates like, cattle (Bowes et al., 1958; Wolff et al., 1971), rat (Furthmayer and Timpl, 1970) and rabbit (Timpl et al., 1969; Borel et al., 1970) have been reported.

They have also been detected in cartilage of some vertebrates such as in ox by Schubert and Hamerman (1968); Enomoto et al., (1966), Janado and Dunstone (1972); Vittur et al., (1972a,b).

Denizot (1970) has studied AMPs in the ampullary sense organs of some weakly electric fishes.

The organic matrix of bone is reported to be the richest source of AMPs among the various tissues of vertebrates (Harring, 1972). In addition to these, brain of some mammals like, rat are found to contain a rich amount of AMPs (DiBenedetta et al., 1969; Margolis and Margolis, 1970).

Again, Jaques (1977) opines that heparin has received much more attention than the other sulfated mucopolysaccharides.

Rubin and Howard (1950) have observed association of AMPs with the calcification of bones and cartilages. Mathews (1959) and OOsawa (1971), opines that one of the characteristic properties of mucopolysaccharides is the selective association or binding with small inorganic cations, especially H^+, Na^+ and Ca^{++} and also with cationic groups of macromolecules. Farber and Schubert (1957) has shown that in chondroitin-sulfate, percentage binding of Ca^{++} is greater than that of Na^+.

Urist et al. (1968) has also found a small preference for binding Ca^{++} over Na^+ in chondroitin sulfate. Mathews (1975), thus have rightly suggested that these substances act as a store for Ca^{++} in cartilage tissue and that is why have specific roles in tissue calcification.

Some very important roles of AMPs, in "water-binding" and maintenance of tissue osmotic pressure has also been reported (Ogston and Wells, 1972; Wells, 1973b). According to Ogston (1970), the role of AMPs on tissue osmotic pressure is not only by influencing the water balance but also by introducing excess swelling pressure which is balanced by an internal structural resistance. Ogston and Wells (1972) and Wells (1973b) has also suggested the role of AMPs in maintaining mechanical flexibility and elasticity of tissues. AMPs may also have some functions in controlling of metabolism of cells, movement of metabolites on the basis of their rather specific chemical structure (Jeanloz,

(1970). Kobayashi and Pedrini (1973) have suggested that AMPs have a major role in structural organization of intracellular matrix. According to them, AMPs may be involved in electrostatic and steric interactions with other macromolecules of the matrix, such as, collagen and elastin.

Compared to extensive amount of works on the AMPs of various vertebrate tissues, the knowledge on invertebrate AMPs is scanty. Hunt and co-workers (1970) have detected a glucan sulfate-peptide component from mucin of marine snail, Buccinum undatum. Mathews (1975), has detected chondroitin sulfate-like substance from cranial cartilage of L. opalescens.

AMPs has also been reported in arthropods, specially hyaluronic acid has been detected in various glands, like, salivary glands (Vadgama and Kamat, 1971) and dermal glands (Baldwin and Salthouse, 1959). Its occurrence has also been reported in brain, ganglia and imaginal disc of the house fly Musca domestica by Mustafa and Kamat (1970). SMPs have been reported in Locusta migratoria by Ashhurt and Costin (1971).

Some very interesting roles of AMPs, specially in arthropodan cuticle has been reported by Meenakshi and Scheer (1959) and Sundara Rajulu (1969) in terms of calcification of the cuticle of Hemigrapsus nudus and Cingalobolous bungnioni respectively. Krishnan (1965) has suggested that AMPs may be associated with -S-S-bonding of the cuticle in the scorpion, Palamneus swammerdami.

The extreme flexibility of the intersegmental cuticles of the queen of Odontotermes Obesus has been considred to be due to the occurence of AMPs (Sannasi, 1969). This conclusion is based on the fact that AMPs are said to possess high water binding capacities (Ogston, 1966a; Laurent et al., 1969; and Katchalsky, 1964).

Pathogenic role of AMPs has also been reported by various workers. Matsuoka et al., (1982), has observed that AMPs are responsible for dermal thickening in acromegalic patients. Age related decreased synthesis of proteochondroitin sulfate in the costal cartilage of old rats by mechanism involving a reduced activity of xylosyltransferase has been reported by Wolf et al. (1982). According to Shahnaz and Glaser (1982), there exists an association in the accumulation of glycosaminoglycans (GAG) and fibronectin (a large external transformation sensitive protein). This association is affected by dimethyl sulfoxide and may help in further studies and treatment of the genetic disease, mucopolysaccharidoses, caused due to increased accumulation of GAG. In the light of above findings it can be assumed that there certainly exist some roles of AMPs in the visual process of the fishes Cyprinus carpio, Clarias batrachus and Stromateus argentius) from different habitats, since the occurence of AMPs and their visual significance have been reported in ocular tissues of various vertebrates and invertebrates by various workers.

Moczar and Moczar (1973) and Anseth (1961a) have reported the occurence of AMPs in the corneal stroma of squids. The cornea in squids unlike the analogous tissue of vertebrate eye is a direct continuation of the skin. Balazs et al. (1959) have found high concentration of hexuronic acid and hexosamine in the vitreous body of the squid eye.

The biochemistry of the cornea and other tissues have throughly been reviewed by Balazs (1965). AMPs have been detected in the corneas of elasmobranchs (Balazs, 1965), and in twelve vertebrates including teleosts (Anseth, 1961a).

The other ocular tissues where AMPs have been reported are vitreous body (Balazs, 1965; Berman and Voaden, 1970); Bleckmann, 1984); aqueous and ciliary body (Cole, 1970; Schachtschabel et al. 1977), interstitial matrix surrounding the photoreceptor cells of cattle (Berman and Bach, 1968; Berman, 1969); interphotoreceptor matrix of vertebrates (Rolich, 1970) and sclera of ox (Robert and Robert, 1967).

AMPs have been also reported in the compound eyes of some insects, such as Periplanata americana, Belostoma sp. (Dey, 1976); Musca domestica, Apis cerana indica (Dey, 1980) and in certain crustaceans like Palaemon sp.; Limulus polyphemus (Dey, Raghu Varman and Michael, 1978).

Thus, with foregoing account in view, it can be assumed that AMPs in the ocular tissues, like other vertebrates, play

some vital roles in the visual life of the fishes from different ecological conditions. The cornea being only the most important external structure of the eye has drawn special attention of various authors in terms of its visual physiology and adaptation.

Cornea

It is already known that bulk of the cornea of vertebrate eye is the stroma, which functions as a supporting structure and is adapted for the transmission of a high percentage of incident light of visible wave-lengths (Maurice, 1969). Anseth and Fransson (1970), have found that during chick corneal development, the occurence of highly sulfated KS is associated with the rise in transparency of stroma. They have also suggested that stromal transparency is correlated with the presence of the normal proportions of KS and Ch A.

The transparency of cornea is based on the state of hydration of the tissue. Payrau et al., (1967) observed that corneal stroma of most vertebrates, including mammals, birds, and teleost imbibe water whenever it has free access. According to Maurice and Riley (1970), oedema of cornea leads to disorganization of its structure and loss of transparency but dehydration does not appear to have serious optical effect. Moczar et al., (1969); Moczar and Moczar (1970, 1972), on the basis of their studies on non-swelling properties of elasmabranch cornea supposed that high content of mannose in the structural proteins

is responsible for the non-swelling. According to other authors, the AMPs of the corneal stroma are mainly responsible for the dehydration properties of this tissue (Heringa et al., 1940; Hedbys, 1961, 1963). They explain the mechanism of the corneal swelling on the basis of electrostatic repulsion between acidic groups of these macromolecules. It is now known that the pH value is a decisive factor for the taking up of water by the cornea (Cejkova and Brettschneider, 1969).

The protein polysaccharide complex provide more stable and specific configuration within the molecule than electrostatic linkage could (Maurice, 1970). Further, several authors have demonstrated the synthesis of AMPs by corneal epithelial and stromal cells (Anseth and Fransson, 1969; Trelstad et al., 1974; Gnadinger and Hubner, 1975).

Both hydration and dehydration is a limiting factor in aquatic (fresh and marine water) medium. We can thus reasonably assume that corneal AMPs is chiefly responsible for maintaining the corneal structure in relation to its environment. This can be verified through the fact that the cornea of Pomfret is more interoven fasciculate structure than the other two fishes - carp and the cat fish. The Pomfret corneal tissue does not permit excessive hydration.

A role of AMPs in the fish cornea can also be attributed on the basis of the fact that AMPs can act as selective ion

barrier (Jeanloz, 1970). The protective role of AMPs in the cornea has been shown by Robert and Robert (1967).

Maurice and Riley (1970), have rightly pointed out that impermeability of the outer surface must be of greater moment in fish eyes but this subject seems to have been little investigated. The outer surface prevents the loss of metabolites (Hermann and Love, 1959; Hermann, 1961) and incorporate sulfate in the polysaccharide of stroma (Pasternak et al., (1963) and Honda (1965).

The three layers of the cornea are largely self-sufficient with regard to their glucose metabolism and energy supplies. In the case of synthetic processes, there may be some dependence on the enzymes systems of other layers for intermediate metabolites, but this has not been demonstrated conclusively (Maurice and Riley, 1970).

Lens Protein-Polysaccharide Complex

Volumes of works have been performed on the crystalline proteins and their roles on the transparency of the lens (Morener, 1894; Spector, 1964, 1965; Spector et al., 1968; Kuck, 1970); various biochemical aspects and cataract formation (Kuck, 1970; Kinoshita, 1965; Rawal and Rao, 1978,1980,1983), evolutionary conservatism of lens proteins (Zigler and Sidbury, 1977), species specificity of crystallines in fish along with other

correlations such as environmental conditions, visual acuity and age (Basaglia and Callegarini, 1987b; 1988, 1989).

But relatively very little work has been done on glycoproteins of vertebrates lens in general and fish lens in particular.

Kuck (1970) observes that the sole function of the lens is to refract the image bearing light beam in a controllable fashion while remaining perfectly transparent. The chief function of lens in vision is accomodation which involves two factors: deformability and refractivity. The former depends upon the fine structure, i.e. the configuration of lens fibres and the means used to weld them into a medium which is optically homogeneous but which however, still consists of discrete fibres which can slide over each other in the process of streatching or contracting. Deformability from this view point involves the properties of the glycoproteins, the cement lubricant substance which is located at the cell surface. This observation has been confirmed by electron microscopic observations (Jakus, 1964; Wanko and Gavin, 1959; Cohen, 1965; Kuwabara, unpublished). Refractivity, on the other hand, is dependent on the major lens proteins: the soluble crystallines and albuminoid (Maurice, 1970) which give a relatively high refractive index to the lens.

The lens contains very little glycoprotein (Kuck, 1970). Krause (1933) found 0.84% "mucoprotein" in the 1 year old bovine

lens. Bellows (1944) believed this was extracellular and would serve as a cement substance between individual fibres to give the loose bonding necessary for the optical functions of the lens. Dische (1965a) rendered an additional function from a developmental aspect. It is evident that the function of the glycoproteins gives them importance much greater than one might infer from the small quantity present (150-200 µg/g wet wt. in lens cortex of cows 1½ - 2½ years old; 90 µg/g in lens nucleus (Dische et al. 1962).

The glycoprotein of the lens appear to belong to the group of sialoglycosaminoglycans and contains glucosamine, galactose, glucose, mannose, fucose and derivatives of neuraminic acid. The cortex contains much of the glycoprotein but the nucleus contains a small amount of glycoprotein. Furthermore, the cortex in young and old cattle and rabbits is richer in glycoprotein than the nucleus, leading to the conclusion suggested by Morner's experiment that it is localized at the interfibriller surfaces rather than within the lens (Kuck,1970).

Another facet of the topical distribution which is even more interesting and significant is the comparison of the polar cortex with the equatorial cortex. The latter not only has more glycoprotein, but that present is relatively richer in neuramic acid (Kuck, 1970). Dische (1965a) believes that the abundance of the hydrophilic type at the equator is related to the high rate of growth and differentiation there. This

explanation has been suggested generally as one role of glycoproteins in the lens, that they control the differentiation of cells and fibres which vary so remarkably between different parts of the lens.

Thus, on the basis of the above reports, similar roles can be adduced to the fish lenticular glycoproteins following our detection of the same in fish lens.

Moreover, electron microscopic observation has revealed that prolonged starvation in a cat fish, Heteropneustes fossilis, (Bloch) caused severe lenticular aberrations including the loss of connection between the fibres and the equatorial cortex was most effected compared to the polar cortex. The important constituents like, glycosaminoglycans may be effected by the deficiency of essential nutrients. (Deb et al. 1989).

In this context, it is worthwhile to mention that vitamin A has been shown to be involved in the synthesis of SMPs. It is presumed that retinol or an active form of it or a retinoic acid releases an enzyme(s) required for the synthesi of AMPs. Wolf and Varandani (1960) have reported that deficiency of vitamin A in the culture of cartilage tissue blocks the synthesis of AMPs. Occurrence of AMPs in the retinal layer has been reported by Rohlich (1970); Marchase et al. (1982) and Verner et al. (1987). We have also observed the

metachromatic reaction in the retinal layer of fish, thereby indicating the presence of AMPs in retina. But our attempt to study the relationship between vitamin A and the synthesis of AMPs failed due to non-availability of required isotopes and other constraints. Thus it would be of extreme significance to study the above relationship.

CHAPTER II

ADENOSINE TRIPHOSPHATASE (ATPase) OF CORNEA AND LENS

INTRODUCTION

The plasma membrane regulates and maintains an ionic difference as well as an electrical potential between the cell and the medium throughout the life to maintain a satisfactory intracellular physiological condition, such as the osmotic pressure that play an important role in the life of the cell. The plasma membrane contains some thirty enzymes, out of which Adenosine triphosphatase (ATPase) is one of the most important because of its role in ion transfer across the cell membrane (De Robertis et al. 1970).

ATPase has been shown to be intimately associated with the phenomenon of photoreception (Langer, 1964,1974; Weber and Schorrath, 1971; Drujan and Ali 1972; Bownds et al. 1972; Macgregore and Matschinsky, 1986) and there are firm indications that ATPase has an important role in phosphorylation.

Relatively little information is available so far about the mechanism by which photolysis of rhodopsin leads to the nervous excitation. Three main hypotheses have been proposed to explain this excitatory mechanism, that is, how one quantum of light by reacting with a single rhodopsin molecule results in nervous stimulation. The hypotheses being i) the enzyme hypothesis; ii) the solid state hypothesis; and iii) the ionic hypothesis.

The enzyme hypothesis holds that rhodopsin is a pro-enzyme which upon absorption of light is converted into an active enzyme catalyzing an electrochemical process leading to visual excitation.

McConnell and Scarpelli (1963) have claimed that rhodopsin is an ATPase where retenene is a co-factor, activated by light. However, Bonting and Bangham (1966) found that the experimental evidence of this claim is faulty and neither Mg^{2+} activated nor the $Na^{+} - K^{+} - Mg^{2+}$ activated ATPase can be identical with rhodopsin.

The solid state hypothesis postulates that a solid state of energy transfer by resonance or photoconduction might take place following the light absorption by the rod outer segments. However, Hagins and Jennings (1960), failed to find any evidence in support of the energy transfer resonance in frog rods.

The ionic hypothesis holds that there is a movement of ions across the photoreceptor membrane following visual excitation, just as it occurs in a neuron after excitation. Hagins *et al.* (1962) have observed that action of light on photoreceptor cell produces a local inflow of current within 7 μ m from the site of light absorption. This receptor current consists of an influx of Na^{+} ions into the rod outer segments, due to a local

light induced increase in Na^+ permeability as well as an efflux of K^+ ions.

Light stimulated potentials has also been observed in invertebrate photoreceptor cells of Limulus (Stieve, 1965); cray fish (Eguchi, 1965); squid (Hagins, 1965), barnacle (Brown et al. 1969) and the honey bee (Fulpius and Baumann, 1969) as a direct consequence of an increased Na^+ permeability through the photoreceptor membrane.

SeKoguti (1960) and Bonting et al. (1963) have detected the presence of $Na^+ - K^+$ activated ATPase in the rod outer segments which lends further support to the ionic hypothesis, since this enzyme system has been implicated in the process of repolarization of nerve, muscle and electric organ (Bonting et al. 1963).

The ionic hypothesis thus holds that maintenance of ionic gradients across the photoreceptor membrane is important for visual excitation and this gradient of ions is regulated by the enzyme, adenosine triphosphatase (ATPase).

The ATPase activity has also been reported in other ocular tissues. SeKoguti (1960) have observed high activity of this enzyme in cattle retina and outer segments who also found that the enzyme activity is stimulated by the addition of K^+ in the Na^+ containing assay medium. Bonting et al. (1963,1964) found high activity of this enzyme in retina,

choroid, sclera, iris, lens and vitreous of man and cat. Further, altered ATPase activity in pathogenic retina has been reported by Macgregore and Matschinsky (1986).

Maeda and Sakaguchi (1965) have reported the presence of N^+ and K^+ activated ATPase in the corneal endothelium. It also occurs in the epithelium and in the nerve fibres of the stromal tissue of the cornea (Maurice and Riley, 1970; Tervo and Palkama, 1975; Midelfart, 1987). The activity of this enzyme has been demonstrated in the ciliary body by Riley (1964), Palkama and Uusitalo (1970), Harkonen et al. (1972). Kinoshita et al. (1961), Kinsey et al. (1965), Harris and Becker (1965), Palva and Palkama (1974) have demonstrated the ATPase activity in the lens.

The available informations show that most of the studies on ATPase has been carried out in higher vertebrates (e.g. cat, rat, rabbit and bovine tissues). Reports on the ATPase activity in the fish ocular tissues, such as cornea and lens are lacking. However, activities of this enzyme in the non-ocular tissues of fishes have been reported (Kamiya 1967; Giles and Vanstone, 1976; Jhonston and Cheverie, 1985) in terms of their osmotic adaptation.

Keeping this in view, an attempt has been made mainly to localize the ouabain sensitive $Na^+ - K^+$ activated ATPase enzyme system with a specific histochemical method in the

corneae and lenses of the common carp (C. carpio) and the cat fish (C. batrachus). So far as is known there are no reports on the histochemical localization of $Na^+ - K^+$ ATPase activity in the ocular tissues of fishes. The enzyme activity of these tissues has been demonstrated by the original Wachstein-Meisel lead trapping technique (1957), as modified by McClurkin (1964), which has further been optimized and made highly specific by Palkama and Uusitalo (1968,1970). The effects of omission of each of the activators Na^+, K^+ and Mg^{2+} has been investigated with special reference to the epithelial localization. Further, this highly specific methodology equips us with a scope to study its relevance in fish eye structures.

MATERIALS AND METHODS

The animals were killed by decapitation and the eyes were removed carefully and immersed in the pre-incubating solutions. The eyeball was then carefully opened by dissecting along the sclero-corneal junction. The quickly separated and extracted corneae and lenses were then kept separately in the cooled pre-incubating media for cleaning. Ouabain was used as a specific inhibitor of $Na^+ - K^+$ activated ATPase system and acted as control medium.

(1) Pre-incubation - The corneae and lenses were pre-incubated for 1 hr. at 4°C in the following media:

Solution A - This medium contained 0.2 M-Tris-HCl buffer, pH 7.2.

Solution B - 3×10^{-4}M ouabain was added to the above solution for its inhibitory effect on the enzyme.

(2) Fixation - The tissues were then fixed in 2.5% gluteraldehyde diluted in Tris-HCl buffer, pH 7.2 for 20-30 min. at 4°C.

(3) Rinsing - The tissues were then rinsed in the above mentioned buffer solution for 4 hrs. at 4°C.

(4) Incubation - The tissues were then transferred to the incubation medium. This medium contained 3m M Tris-ATP as substrate, 3m M-$Pb(NO_3)_2$ as precipitating agent, 3m M-$MgSO_4$,

70m M-NaCl, 70m M-KCl as activating agents and 0.2M-Tris-HCl buffer, pH 7.8. All these chemicals were dissolved in Tris-HCl buffer, pH 7.8.

The pH of the medium was always adjusted to 7.2 *before incubation. Incubation was performed for 30 mins.* at 37°C.

(5) Rinsing - The tissues were then rinsed in 0.33M sucrose solution.

(6) Staining - The specimens were then stained in ammonium sulphide (1:100) for 1 min.

The corneal tissues were mounted in glycerol jelly on slides for observation. A portion of the corneal stroma was exposed by removing the epithelium in some cases.

The lens requires some preparations before the whole mount observations of the epithelium could be made. The whole mount preparation was carried out under a dissecting microscope. An incision was made on the lens capsule which was pulled downwards with a very fine needle (since the lenses in fishes are quite small particularly of the cat fish, having a size of about 0.01 cmm) and the body of the lens was carefully lifted out. The isolated capsule with the adhering epithelium was then transferred on a glass slide and mounted in glycerol jelly.

When required, the intact lenses were post-fixed in 2.5-3.5% gluteraldehyde buffer solution. The lenses were then frozen and 20 µm sections were cut with a cryomicrotome. The sections were then briefly rinsed in distilled water and mounted in glycerol jelly on a slide.

Control Studies

In control experiments, in addition to above mentioned ouabain addition in the pre-incubation medium (Solution B) for its inhibitory effect on $Na^+ - K^+$ - ATPase activity, effect on the enzyme activity of the cornea and lens due to the omission of some other essential co-factors from the incubation medium were also investigated. Following are the co-factor(s) those omitted from the respective medium.

(i) Na^+ and K^+, omitted from the incubation medium;

(ii) Na^+, K^+ and Mg^{2+} were omitted from the medium;

(iii) Only Na^+, omitted from the medium;

(iv) K^+, omitted from the medium;

(v) Mg^{2+}, omitted from the medium;

(vi) The substrate ATP was omitted from the medium;

(vii) The precipitant,lead,omitted medium.

Effect of Temperature

The tissues were pre-heated (60-65 °C) for 1 hr in order to exclude the artifactal precipitates due to non-enzymatic hydrolysis of ATP.

OBSERVATIONS

The $Na^+ - K^+$ ATPase reaction has been observed in the adjoining epithelial cell membranes of the whole mount preparations of corneae and lenses of carp and cat fish. The positive reaction was indicated by a dark brown colour while no reaction was visible inside the cell. However, a light brown cytoplasmic colour due to background staining and a heavy staining of the nucleus was observed especially when the concentration of Mg^{2+} or K^+ was not optimal but this is not indicative of any enzyme activity. Estimation of $Na^+ - K^+$ - ATPase activity has been further confirmed by the addition of ouabain in one pre-incubation solution (Solution B). The inhibitory effect of ouabain was observed by the absence of any brown colour.

Corneal Reaction

The pre-incubated solution without the inhibitor (Solution A) yielded a positive reaction in the corneal epithelial (Figs. 28 and 29) and endothelial cells (Figs. 32 and 33), but very little or no reaction was visible when the tissue was pre-incubated with ouabain (Solution B) (Figs. 30,31,34 and 35). The cells in the epithelium and endothelium are loosely arranged. The stroma in both cases was negative confirming the enzyme activity in the epithelial cells.

In the control experiments marked inhibition in enzyme activity could be seen in the absence of activators Na^+ and K^+ both in corneal epithelium and endothelium (Figs. 36,37,38 and 39) in both the fishes. There was complete absence of reaction when Na^+, K^+ and Mg^{2+} were omitted from the medium; so also in case of omission of ATP, Mg^{2+} and lead from the respective medium.

Pre-heating of the tissues failed to produce any positive result even when incubated in the complete medium.

Reaction in Lens

The lens consists of a single layered epithelium immediately below the capsule. The enzyme activity is restricted only in the epithelial cell borders (Figs. 40 and 41) in the whole mount preparation when pre-incubated in the solution A, while it is negative (Figs. 42 and 43) when pre-incubated in presence of ouabain (Solution B). The capsule as well as the lens fibres were negative in enzyme activity.

The results of the control experiments are almost similar to the observations made in connection to the above mentioned corneal reactions (Figs. 44 and 45).

Our findings, thus support the theory of Tormey and Diamond (1967) that the active transport site is between the adjacent cells, as has been observed in both cornea and lens by us.

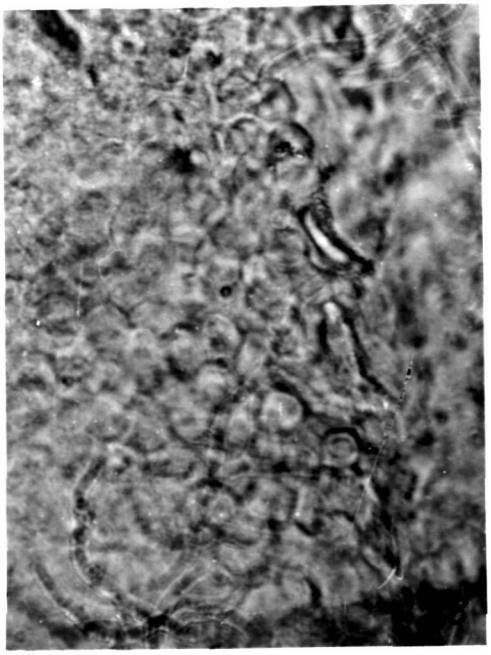
EXPLANATION TO FIGURES

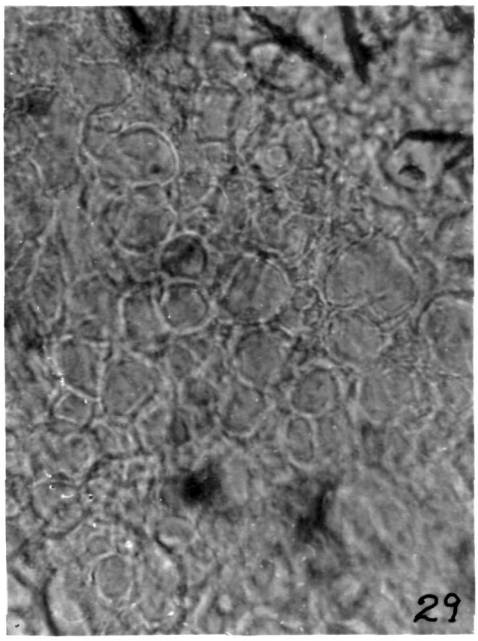
Fig. 28. A whole mount preparation of corneal epithelium of Cyprinus carpio incubated with Na^+, K^+ and Mg^{2+} in the ATP-Pb medium showing Na^+-K^+-ATPase reaction in the epithelial cell membranes. No reaction is seen inside the cells x 500.

Fig. 29. A similar preparation of corneal epithelium of Clarias batrachus as in Fig. 28, showing ATPase activity in the cell membranes x 500.

Fig. 30. Similar preparation of corneal epithelium of C. carpio as in Fig. 28 but preincubated in the presence of 3×10^{-4}M ouabain demonstrating the inhibition of ATPase activity x 500.

Fig. 31. Similar preparation of corneal epithelium of C. batrachus as in Fig. 30, demonstrating ouabain sensitive ATPase activity x 500.

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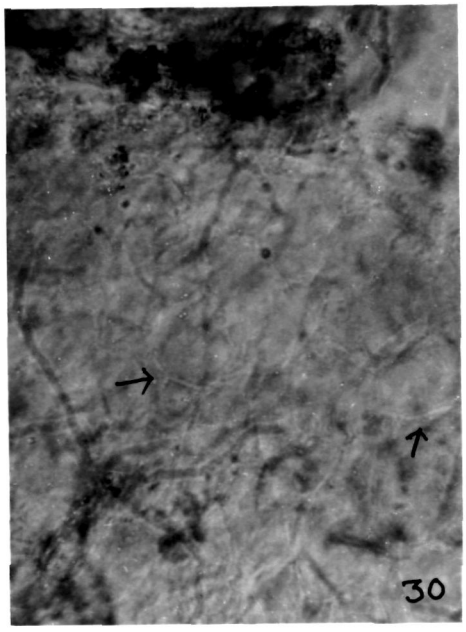
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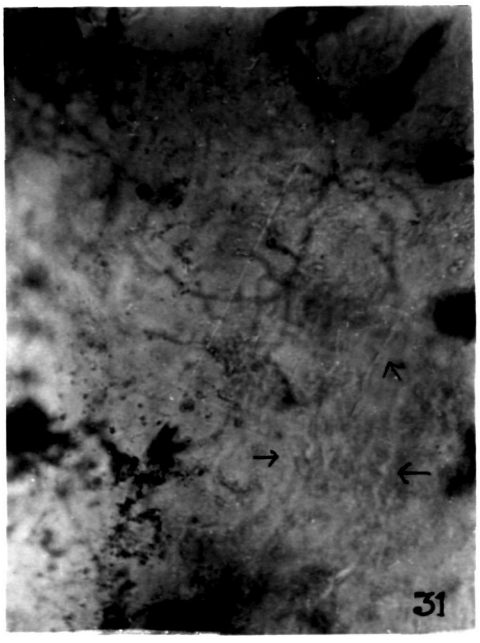

31

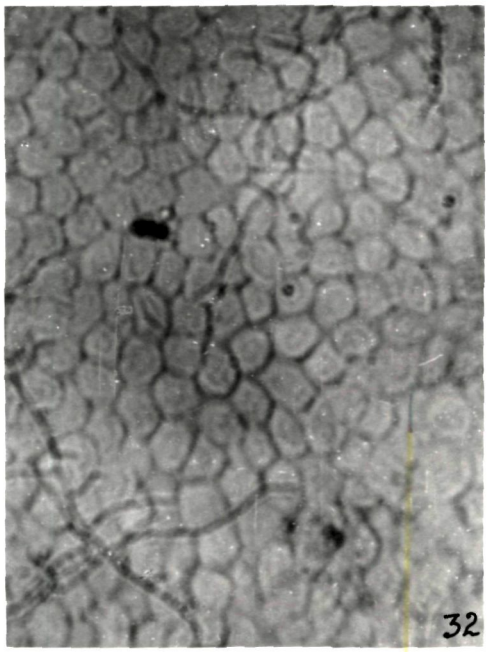
EXPLANATION TO FIGURES

Fig. 32 Whole mount preparation of corneal endothelium of C. carpio incubated with Na^+, K^+ and Mg^{2+} in the ATP-Pb medium showing Na^+-K^+-ATPase reaction in the endothelial cell membranes x 500.

Fig. 33 A similar preparation of corneal endothelium as in Fig. 32, of C. batrachus showing ATPase activity in the cell membranes. x 500.

Fig. 34 Similar preparation of corneal endothelium of C. carpio as in Fig. 32 but preincubated in the presence of 3×10^{-4}M ouabain demonstrating the inhibition of ATPase activity x 500.

Fig. 35 Similar preparation of corneal endothelium of C. batrachus as in Fig. 34 demonstrating ouabain sensitive ATPase activity x 500.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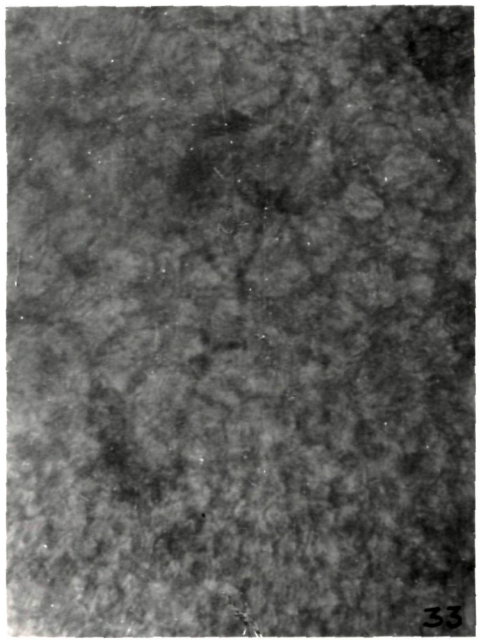
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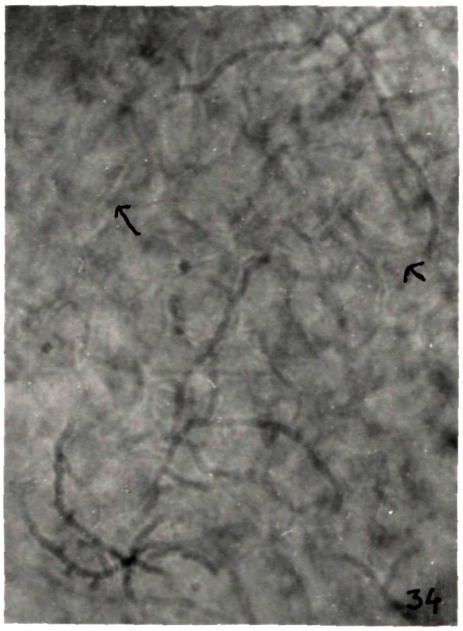
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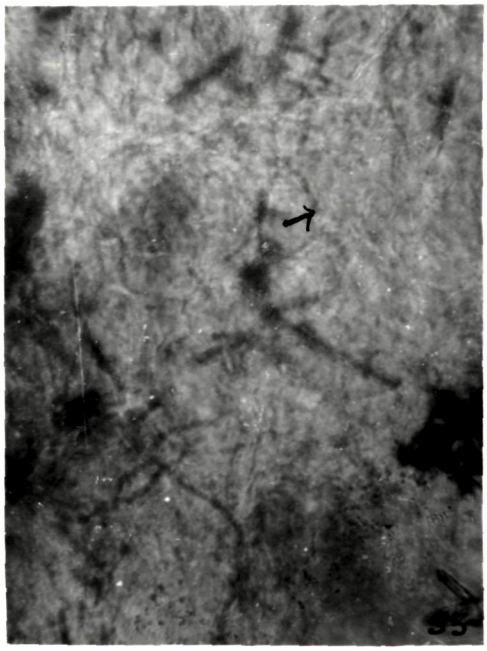
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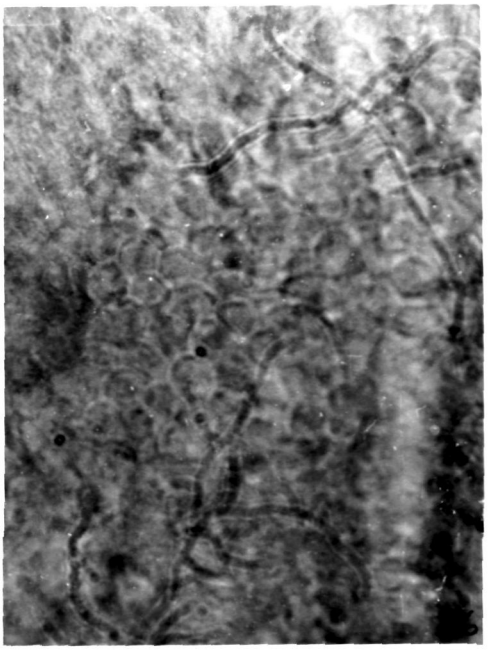
EXPLANATION TO FIGURES

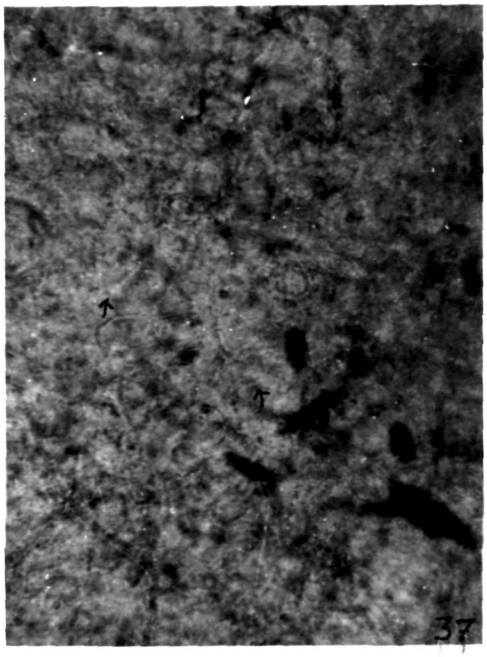
Fig. 36 Whole mount preparation of corneal epithelium of C. carpio, incubated without the activators Na^+ and K^+ showing marked inhibition of ATPase activity x 500.

Fig. 37 Corneal epithelium of C. batrachus, prepared as in Fig. 36 to demonstrate inhibition of ATPase activity x 500.

Fig. 38 Corneal endothelium of C. carpio, prepared as in Fig. 36, demonstrating inhibition of ATPase activity x 500.

Fig. 39 Corneal endothelium of C. batrachus prepared as in Fig. 36, demonstrating inhibition of ATPase activity x 500.

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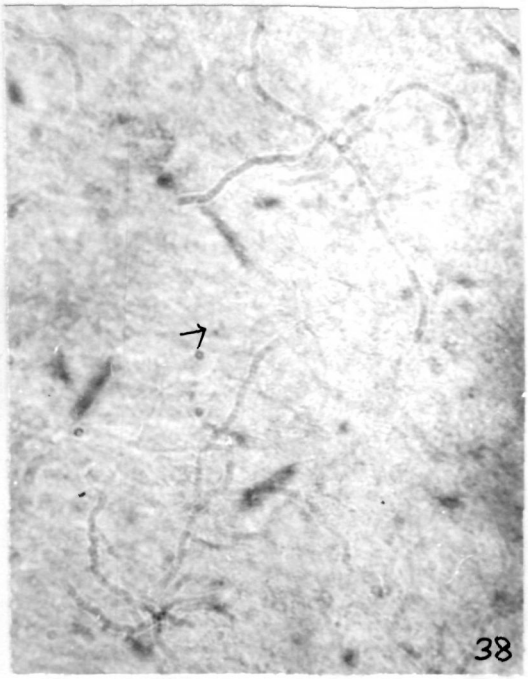
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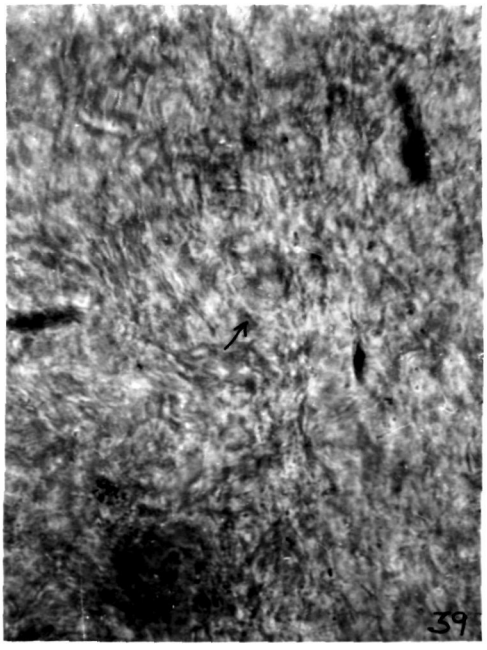
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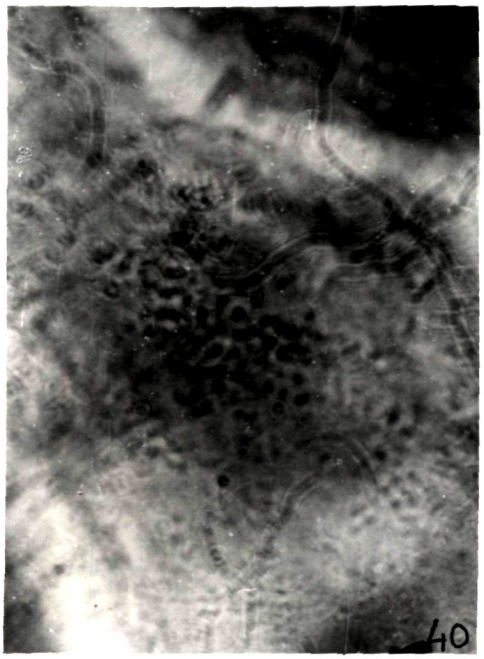
EXPLANATION TO FIGURES

Fig. 40 Whole mount preparation of lens epithelium of C. carpio, incubated with Na^+, K^+ and Mg^{2+} in the ATP-Pb medium showing Na^+-K^+-ATPase reaction in the epithelial cell membranes. No activity is visible inside the cell. A weak background staining of some nuclei is visible x 125.

Fig. 41 A similar preparation of lens epithelium of C. batrachus as in Fig. 40 to demonstrate Na^+-K^+ ATPase activity x 500.

Fig. 42 Similar preparation of lens epithelium of C. carpio as in Fig. 40 but pre-incubated in the presence of 3×10^{-4}M ouabain demonstrating the inhibition of ATPase activity x 125.

Fig. 43 A preparation of lens epithelium of C. batrachus, similar to Fig. 42 demonstrating ouabain sensitive ATPase activity x 500.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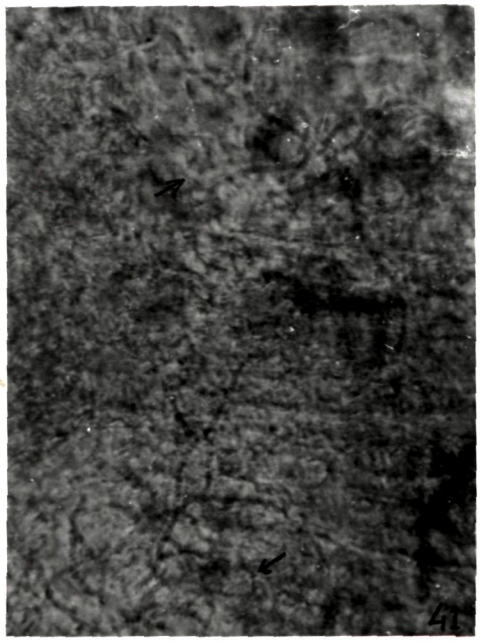
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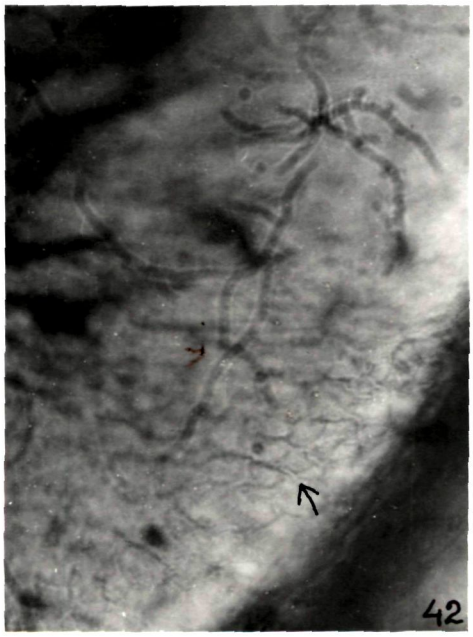
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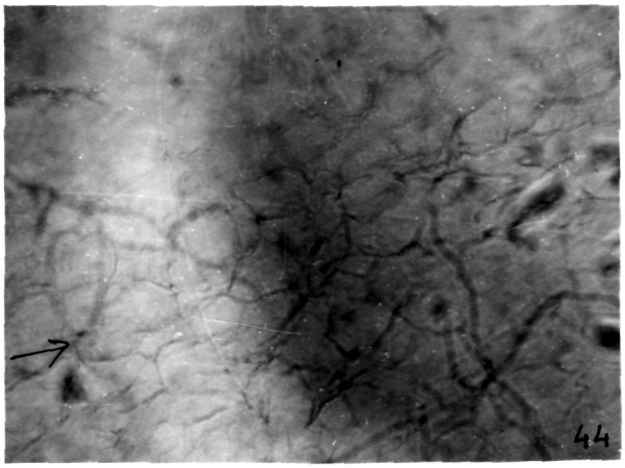
EXPLANATION TO FIGURES

Fig. 44 Whole mount preparation of lens epithelium of C. carpio, incubated without the activators Na^+ and K^+, showing marked inhibition of ATPase activity x 500

Fig. 45 Lens epithelium of C. batrachus prepared as in Fig. 44, demonstrating inhibition of ATPase activity x 125.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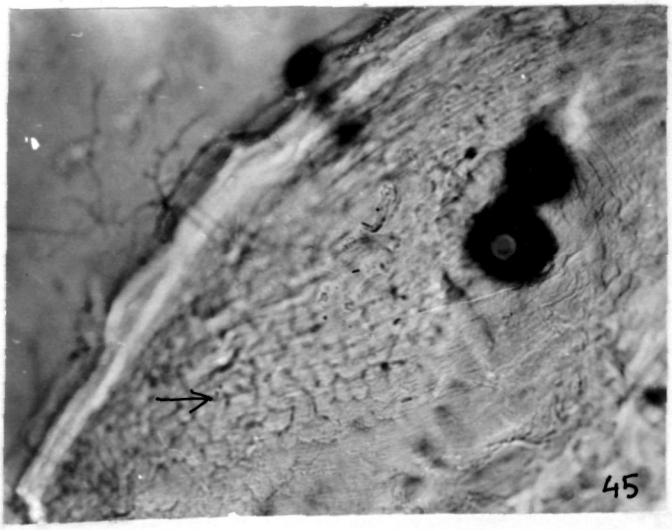
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DISCUSSION

The results obtained indicate that the histochemical properties of the $Na^+ - K^+$ - ATPase activity in the corneal and lens epithelium has marked resemblance to the enzyme activity found in the rat cornea (Tervo and Palkama, 1974), in the rat lens (Palva, 1980) as well as in the ciliary ep'thelium of rabbit (Harkonen, Palkama and Uusitalo, 1972; Palkama and Uusitalo, 1970). This enzyme has also been detected in the epithelium, stroma and endothelium of rabbit by Maeda and Sakaguchi (1965).

$Na^+ - K^+$ - ouabain sensitive ATPase (Na^+-K^+-ATP) has been implicated in the active Na^+-K^+ transp^rt system of a variety of biological membrane (Skou, 1965; Post et al. 1960; Bonting, Carvaggio and Hawkins, 1963). The enzyme is specially inhibited by cardiac glycoside and ouabain. The ATPase has been considered as sodium-potassium "pump enzyme". The Na^+-K^+ pump nature of ATPase is presumed on the fact that Na^+-K^+-ATPase leads to the hydrolysis of ATP resulting in the movement of sodium and potassium against their electro chemical gradients (Skou, 1972). The hydrolysis of ATP occurs on intracellular side using OH^- from the inside and H^+ from the outside liberating phosphate (Mitchell, 1961). Albert (1967) has postulated that ATP-ATPase-enzyme-substrat complex may act as a true carrier mechanism binding internal sodium and releasing it outside the membrane. A similar but

reverse mechanism is postulated for K^+. In the absence of active cation pump the cell would swell and burst due to internal osmotic pressure (Lehninger, 1970).

The $Na^+ - K^+$ - activated ATPase enzyme system occurs in nearly all cell membrances over which a gradient for sodium and potassium exists (Bonting, 1970). The outer medulla of the kidney consists of highest concentration of Na^+-K^+-ATPase (Hendler et al. 1971). The activity has been observed more particularly in the peritubular cell membranes of the ascending limb of the Henle's loop in accordance with the postulated role in active cation transport (Schmidt and Dubach, 1969). Bonting et al. (1964a) have reported that a major part of the Na^+-K^+-ATPase activity is localized in rod outer segments. Frank and Goldsmith (1965) have reported similar findings in the isolated pig outer segments. The high activity of this enzyme in the rod outer segments led Bonting and Bangham (1967) to formulate their "Cation Channel" hypothesis for the visual process. Considering high activity of this enzyme, Bonting and Bangham (1967) have assumed that the enzyme is not only located in the rod outer segment membrane but also in the rod sac membranes. Earlier, Scarpelli and Graig (1963), have histochemically detected the presence of ATPase activity in rod sac membrane.

Moreover, the Na^+-K^+ stimulated ATPase activity is rich in the membranes of the excitable cells such as brain, nerve, muscle and the electric organ of the electric eel as well as in the Na^+-transporting tissues. It has also been found that concentrations of Na^+ and K^+ are required for the maximal stimulation of the ATPase activity (Lehninger, 1970).

The Na^+-K^+ activated ATPase in the corneal epithelium, endothelium and nerve fibres of the stromal layers is presumed to play some part in transport mechanism (Maeda and Sakaguchi, 1965; Tervo, 1975). According to Maurice and Riley (1970), corneal hydration and thickness is controlled by the low permeability of the surface membranes and by an active pump mechanism that actively pumps out the fluid that enters and a cation-dependent ATPase system one of the pump mechanisms involved in the regulation of corneal water balance. But there has been a great deal of disagreement over the precise localization and character of the mechanism controlling corneal hydration (Tervo and Palkama, 1975).

The exact role of Na^+-K^+-ATPase in the corneal epithelium is still partially open to question. The epithelium, according to Mishima and Kudo (1967) plays no role in pump mechanism but offers high resistance to the movement of all ionic substances (Cogan et al., 1944; Maurice, 1968b).

An active inward Na^+-transport mechanism to the stroma has been identified in rabbit corneal epithelium (Donn et al., 1959). A sodium dependent mechanism pumping chloride ions from the aqueous humour to the tear film has also been shown to exist in the frog cornea (Zadunaisky and Lande, 1971). Klyce et al. (1973) have, moreover shown that the rabbit corneal epithelium not only pumps Na^+-ions from the tear film towards the stroma but also chloride ions in the opposite direction when such stimulators as epinephrine and cAMP are present. However, it seems possible that, definite variations between different species may exist in the pumping mechanisms of the corneas (Ehlers, 1973). Green (1969) suggests that the epithelium is chiefly responsible for the normal hydration of the cornea.

The endothelium on the other hand, is more permeable to ions than the epithelium (Maurice, 1961) and is directly concerned with the transport mechanism (Takahasi, 1967). Maurice (1972) considers that the endothelium is the chief site for the active pumping process and the permeability of the endothelium also seems to influence the corneal water balance and this has led Maurice (1972) to advance the "pump-leak" hypothesis, which proposes that the endothelium pump sodium and water into the anterior chamber against hydrostatic pressure, which causes a passive leak of water and electrolytes through the endothelium towards the stroma.

Friedman (1973) has proposed that the corneal water balance is exclusively based on a passive mechanism according to which the osmotic properties of the tear film and the permeability of the endothelium would be the main regulatory systems. The endothelial pumping mechanism needs sodium and bicarbonate (Dikstein and Maurice, 1972; Hodson, 1971) and absence of these activators causes a fall in the transendothelial potential difference (Fischbarg, 1973). Davson (1949) proposed that the endothelium is essential in maintaining normal corneal thickness and transparency.

Harris and Nordquist (1955); Hara (1965) have shown that changes in the ionic balance causes swelling of the cornea. Langham and Kostalnik, (1965), demonstrated that ouabain causes a decrease in the corneal Na^+-K^+-ATPase activity and obviates the temperature reversal phenomenon. Thus, it seems that the cation pump aids in the process of maintenance of the ionic balance. The presence of Na^+-K^+-ATPase activity may have many metabolic functions (Bonting, 1965) in the fish cornea and the active cation transport system seems to posses many properties similar to epithelial Na^+-K^+-ATPase (Bonting, 1965, 1970; Bonting et al., 1963) and may be in the fish cornea as well.

In the metabolism of the lens, the presence of Na^+-K^+-ATPase activity has been found to have many basic

metabolic functions (Bonting, 1965). It has also been suggested that Na^+-K^+ activated ATPase is mainly concerned with cation transport (Kinsey and Reddy, 1965; Bonting, 1965). Bonting (1965) assumes that one of these ions (potassium) is required for protein synthesis of lens.

The histochemical localization of the Na^+-K^+-ATPase on the epithelial cell membrane supports the general opinion that the active cation transport mechanism of the lens is located in the epithelium and that the ion movement through the capsule is caused by passive diffusion (Bonting, 1965; Kinsey and Reddy, 1965).

The energy required for the active cation transport is supplied by high energy phosphate bond mainly derived from anaerobic glycolysis (Kinoshita et. al., 1961; Mandel and Klethi, 1962). The cation pump thus regulates the normal volume of lens by actively extruding sodium from it. Conversly, when the enzyme of the membrane is inhibited or denatured, the potassium concentration of the lens is decreased with consequent increase of sodium resulting in the increase of water content of the lens leading to loss of transparency.

The roles of Na^+-K^+-ATPase enzyme system, as shown by various authors in higher vertebrates appears to play

similar roles in fish ocular tissues. Further, we believe that this enzyme system requires to be more efficient in the aquatic medium.

Moreover, ATPase in the epithelial layers of cornea and lens of fishes also requires Na^+, K^+ and Mg^{2+} for activation and its sensitivity to ouabain inhibition appears to fulfill all the criteria of the enzyme as described by Skou (1964).

In this context, we like to mention that though the ATPase system maintains the cationic balance in the ocular tissues, it would be worthwhile to study the relationship between Na^+-K^+-ATPase activity and mucopolysaccharides, if any, since the latter also plays some role in ionic balance.

C H A P T E R III

ASCORBIC ACID IN THE CORNEA AND LENS

INTRODUCTION

The visual process and ability are largely influenced by the generation of energy in the photoreceptor organs of animals. The oxidative energy generation in the biological system is chiefly accomplished through oxidation-reduction reactions. Goldschmidt (1924) has shown that the energy generation in the vertebrate photoreceptors, particularly in the lens is affected by the process of reduction, i.e. removal of hydrogen and for which a reducing factor should essentially be present in the visual system. Glick et al. (1936); Bellows and Rosner (1936); Rosner et al. (1938); Henkes (1946) have reported the presence of two such reducing agents such as ascorbic acid and glutathione in the lens of some vertebrates.

Pirie (1946) has reported that the concentration of ascorbic acid is approximately 1.8 μ moles per gm. wet wt. of the cornea of ox. Oxidation-reduction role of ascorbic acid in the calf corneal epithelium has been reported by Anderson and Spector (1971) and in bovines and rabbits (Kulhmen and Resnick, 1959). The high concentration of ascorbic acid in corneal epithelium shows that it is generally more concentrated in cells than in body fluids (Maurice and Riley, 1970). Heath (1962) has reported the presence of ascorbic acid in the lens of rat, dog, guinea pig, rabbit,

sheep, frog, pig, man, cow and horse. It has also been reported in the aqueous humour of rabbit (Muller and Buschke, 1934; Podesta and Baucke, 1938), ox (Muller and Buschke, 1934; Podesta and Baucke, 1938; Vladesco and Stefanesco, 1939; Johnson, 1936), man (Muller and Buschke, 1934; Purcell et al. 1954), monkey (Kinsey and Jackson, 1949), guinea pig (Muller and Buschke, 1934; Podesta and Baucke, 1938; Johnson, 1936); sheep (Vladesco and Stefanesco, 1939; Podesta and Bauchke, 1938), cat (Langham, 1950), rat (Muller and Buschke, 1934) and frog (Muller and Buschke, 1934).

The occurrence of ascorbic acid has been reported in the vitreous body (Balazs, 1961; Reddy and Kinsey, 1960; Naumann 1959 ; Krueger et al. 1959) and also in retina (Heath, 1962; Heath et al. 1962b; Heath and Fiddick, 1964, 1965a,b; Fiddick and Heath, 1966) of several vertebrates.

Heath and Fiddick, 1964; 1965a,b; Pirie 1965; Kinoshita, 1964; Henkes, 1946 studied the importance of ascorbic acid in visual function of animals following the detection of high ascorbic acid level in various ocular tissues of vertebrates.

Rawal and Rao (1977) have studied the importance of ascorbic acid in visual physiology of various vertebrates such as amphibian (Rana hexadactyla, Bufo melanostictus) birds (Columba livia) and mammals (Rattus norvegicus, Cavia porcel-

lus, Tophozous longimanus). The valuable observations of them was that the animals like amphibians which live in well-illuminated areas contained low level of ascorbic acid in the lens while those living in shaded or less lighted area have comparatively higher amount of ascorbic acid in their lens. Among the mammals, the nocturnal bat have comparatively higher amount of ascorbic acid in the lens. Ascorbic acid in higher forms shows degradation excepting birds, where it is lower than amphibians and fishes and higher than reptiles and mammals. According to Sharma (1989), ascorbic acid is unusually high in the primate eye. The level of ascorbic acid is higher in the lens and aqueous humour in diurnal mammals than nocturnal ones.

A gradual decrease, however, in the concentration of ascorbic acid in the lens has been observed as the animal becomes old. In spite of the wide variations in the concentration of this vitamin between species, the level has been found to be greatly reduced in the aging lens of all the species investigated so far, like, the rat and cow differ in lens-size, life span and average ascorbic acid content but both the animals show similar drops in ascorbic acid concentration in the aging lens (Kuck, 1961). However, a decrease in normal level of ascorbic acid in tissues is a sign of physiological stress (Wedemeyer, 1964), due to pollution (Chatterjee and Pal, 1975; Mauck, et al. 1978), infection (Lewin, 1974) or disease (Wilson, 1974).

In fishes, a limited number of reports available on the level of ascorbic acid in the tissues only (Rudra, 1936a; Saha 1939; Ikeda et al. 1963; Raghubanshi and Swarup, 1978; Halver et al., 1975; Agarwal and Mahajan, 1980), but no special attention on ascorbic acid level and its role in visual process has been given on fish ocular tissues excepting Rawal and Rao (1977), who studied the importance of the ascorbic acid in the lens of some fishes, such as mullet (Ophiocephalus punctatus), cat fish (Saccobranchus fossilis) and barbus (Barbus pinnuratus). Agarwal and Mahajan (1980), have studied the comparative tissue ascorbic acid level in four major carps (Labeo rohita, Labeo calbasu, Catla catla, Cirrhina mrigala). The ascorbic acid level was found to be highest in the spleen in the four species, followed by the anterior (adrenal) kidney, gonads, liver, renal kidney, brain and/or eye. A seasonal variation in the ascorbic acid level of tissues has also been reported in Notopterus notopterus by the same authors.

But where fishes are concerned the physiological role of vitamin C has not been elucidated excepting Halver et al. (1975), who reported the high concentration of ascorbic acid in head kidney. Role of ascorbic acid in gonodal functions has been reported (Kucen and Cavazos, 1958; Luitwak-Mann, 1958; Chatterjee, 1967; Horning, 1975).

Dietary deficiency symptoms causing various distortions of vertebral column has been reported by Poston (1967).

The commonly known deficiency state in fishes is "broken back syndrome" is characterized by fractured dislocation of the spine and cartilage, impaired collagen formation, depigmented areas, poor growth and mortality (Bauernfeind, 1982).

Keeping the above in view, histochemical and biochemical studies have been undertaken in the cornea and lens of the carp, Cyprinus carpio and the cat fish, Clarias batrachus.

MATERIALS AND METHODS

Histochemistry

The histochemical tests employed for the detection of ascorbic acid is following the method of Bacchus (1950). The eyes are carefully separated from the decapitated fish and immersed in the dark for 30 minutes in 5% silver nitrate with two drops of acetic acid per ml. at 56°C. The tissues are thoroughly washed in several changes of distilled water for 30 minutes and then treated in 5% sodium thiosulphate for another 30 minutes and again washed in distilled water and transfered to 70% alcohol. Dehydration, clearing and infiltration are performed in dark or subdued light. The materials are sectioned, mounted on slides and followed the usual methods of histological preparations. The sections were counterstained in eosin.

Biochemical detection (Spectrophotometric detection)

The modified method of Chinoy et al. (1976) has been followed for the detection of ascorbic acid. Ascorbic acid, it is known, that in biological materials not only exists in free form (AA) but also occurs in bound form or ascorbigen (ASG) (Guha and Paul, 1936; Sengupta and Guha, 1938). It is a well known fact that ascorbic acid is continually acted upon by a number of oxidizing enzymes. These include a specific

peroxidase catalyzing the formation of its free radical-(FR)-monodehydroascorbic acid (MDHA) (Gurevich, 1963). Further, Chinoy (1967,1969) has reported that in a living system exogenous as well as endogenously added ascorbic acid forms a complex with macromolecules like proteins and nucleic acids. Isherwood and Mapson (1962) have suggested that the actual concentration of ascorbic acid in a tissue represents the excess formed in synthesis over that used in metabolism. Thus, according to them any study regarding the concentration of ascorbic acid in free form alone may lead to an inaccurate inference. Taking all these in consideration ascorbic acid turn over in the cornea and lens have been estimated by simultaneous determination of (i) free form of ascorbic acid (AA); (ii) bound form of ascorbic acid (ASG); (iii) enzymatic utilization of ascorbic acid (AAU); and (iv) complexing of ascorbic acid with macromolecules (AA-MM).

Reagents

(1) Metaphosphoric Acid (HPO_3)

(i) 3% w/v solution (0.275M)

(ii) 15% w/v solution (1.375M)

The solutions were refrigerated at 3°C.

(2) Buffer Solutions

(i) Buffer Solution A (0.5M) - 10.55g of citric acid dissolved in 1N NaOH and the volume is made upto 100 ml. (pH 4.8).

(ii) Buffer Solution B (1.5M) - 31.65g of citric acid dissolved in 3N NaOH and the volume is made upto 100 ml. (pH 4.8).

(3) Buffered HPO_3

Three volumes of 3% HPO_3 is mixed with one volume of buffer solution A (pH 3.6). Buffered HPO_3 is prepared fresh every time.

(4) Standard Ascorbic Acid Solution

10 mg. of ascorbic acid is dissolved in glass distilled (double) water saturated with CO_2. The volume of the solution is made upto 100 ml and stored in an amber-coloured bottle at 3-5°C. Ascorbic acid solution is always prepared fresh.

(5) Standard Dye Solution - 10 mg of 2,6-dichlorophenol-indophenol (BDH) is dissolved in de-ionised water at 80°C, cooled and the volume is made upto 200 ml.

Standard Curve

Ascorbic acid solutions of concentrations ranging from 10 µ g to 100 µ g/ml are prepared from a stock solution by diluting with CO_2-saturated glass distilled water to the required concentration.

1 ml aliquot of each AA solution is mixed with 1 ml. of buffered HPO_3 (pH 3.6) solution (pH remains stable following the addition of ascorbic acid due to high buffering capacity) and 5 ml of standard dye solution and the reading is taken in a Beckmen Spectrophotometer at 500nm. The readings are taken for each AA solution and a graph is prepared by plotting the values.

Extraction of the Materials (Preparation of the tissues)

The eyes are separated from the decapitated fish and the cornea and lens are quickly separated from the eyes.

The weighed tissue is placed in a mortar, covered with 1-2 ml. of cold CO_2 saturated distilled water and quickly homogenized with a pinch of purified silica. The contents are transferred to a test tube. The mortar and pastel are rinsed 2-3 times with 1-2 ml. of cold CO_2-DW, transferred to the test tube and the volume is made upto 12 ml with CO_2-saturated distilled water.

The homogenate is divided into three parts:

(i) 4 ml for the estimation of AA;

(ii) 4 ml for the estimation of ASG; and

(iii) 4 ml for the estimation of AAU and MM complex.

Determination of Ascorbic Acid (AA)

4 ml of cooled buffered HPO_3 is added to 4 ml of the original homogenate and after thorough mixing the solution is

filtered. Now 2 ml of the filtrate is diluted with 5 ml of distilled water and the scale of the spectrophotometer is adjusted to "0" for the turbidity factor. 5 ml of standard dye solution is added to another 2 ml of the same filtrate and the reading is noted.

Calculation

The concentration of ascorbic acid is 1 ml of the original extract is calculated as follows: As 2 ml each aliquot contains 1 ml of the original homogenate, the concentration of ascorbic acid/gm fresh weight of the material is:-

$$A = \frac{a \cdot v}{W} \times 1000$$

where, A = AA content of the sample in μ g/g fresh weight.

a = AA in μ g/ml of the original homogenate

v = Total volume of the original homogenate

and W = Weight of the sample taken for analysis (in mg).

Determination of Ascorbigen (ASG)

2 ml of 15% HPO_3 is added to 4 ml of the original homogenate and the mixture is kept in a water-bath at 75°C for 15 minutes for hydrolyzing ascorbigen. After cooling, the system is buffered at pH 3.6 by adding 2 ml of the buffer solution B, thus increasing the volume of the mixture to 8.0 ml and filtered. Then the spectrophotometric reading is recorded as in free ascorbic acid.

Concentration of ascorbic acid in 2 ml of the buffered hydrolysed extract is determined from the standard graph. The value of free ascorbic acid in 1 ml of the homogenate, which has been determined previously, is subtracted from it to obtain the ascorbic acid equivalent of ascorbigen in 1 ml of the homogenate.

Ascorbigen content per gm fresh wt. of tissue is calculated as follows:-

$$B = \frac{V(b-a)}{W} \times 1000$$

where B = Ascorbic acid equivalent of ascorbigen in μ g per gm. fresh weight of the sample

b = Ascorbic acid (μg) in 2 ml of buffered hydrolyzed extract

a = Ascorbic acid (μg) per ml of the original homogenate

V = Total volume of the original homogenate

W = Weight of the sample taken for analysis (in mg).

Ascorbic acid utilization (AAU) and ascorbic acid-macromolecule complexing (AA-MM complex)

4 ml of ascorbic acid solution (100 μg/ml) are added to 4 ml of the original homogenate. The mixture is incubated at 30 $\pm$ 2°C with thorough shaking in every 10 min. The mixture is filtered after incubation and 3 ml of the filtrate are taken out separately for the analysis of AAU-MM complex respectively.

Determination of AAU

3 ml of buffered HPO_3 are added to 3 ml of the filtered and incubated solution, and ascorbic acid content is estimated. The ascorbic acid content of 2 ml aliquot of the buffered solution (i.e. 0.5 ml of the original homogenate) is determined from the standard graph. The value of ascorbic acid (in μ g) thus obtained is multiplied by 2 to obtain the value of ascorbic acid per 1 ml of the original homogenate left unutilized after incubation.

The calculation of AAU per gm. fresh weight is as follows:

$$C = \frac{V\ (a + 100) - 2c}{W} \times 1000$$

where, C = Ascorbic acid in μ g utilized per gm. fresh weight during a given period of incubation

c = Amount of ascorbic acid in μ g left over in 2 ml of the buffered-incubated solution (i.e. 0.5 ml of the original homogenate).

a = Ascorbic acid in μ g/ml of the original homogenate.

V = Total volume of the original homogenate

W = Weight of the sample taken for analysis (in mg).

Determination of AA-MM complex

At the end of the incubation period 2 ml of 15% HPO_3 are added to 4 ml aliquot and the mixture is hydrolyzed.

After cooling, the mixture is buffered at pH 3.6 by adding 2 ml of buffer solution B bringing the total volume to 8 ml and the complexing of ascorbic acid is determined as in ascorbigen. The ascorbic acid content in the aliquot is estimated from the standard graph and is multiplied by 2. Subtracting the value of ascorbic acid left over in 1 ml of the original homogenate before hydrolysis from the above value gives the amount of ascorbic acid released by hydrolysis of AA-MM complex (in μ g/ml). The amount of ascorbic acid complexing with macromolecules per gm. fresh weight has been calculated as follows:

$$D = \frac{2v\ (d-c)}{W} \times 1000$$

where, D = μ g of ascorbic acid released from the complex per gm. fresh weight of the tissue

d = μ g of ascorbic acid in 2 ml of the incubated hydrolyzed buffered solution (i.e. i.. 0.5 ml of the original homogenate)

V = Total volume of the original homogenate

c = Amount of ascorbic acid in μ-g left over in 2 ml of the buffered-incubated solution (i.e. 0.5 ml of the original homogenate)

W = Weight of the sample taken for analysis.

OBSERVATIONS

The principle of the histochemical tests for the detection of ascorbic acid in biological system based on the fact that silver nitrate reduces ascorbic acid in tissue sections and produces a characteristic pattern of black granules scattered in the region, where ascorbic acid is present. The efficiency of the method, however, has been criticised by Barnett and Fisher (1943) which has been mainly answered by Bourne (1944).

Barnett and Bourne (1941), on the basis of the available evidences have stated that in vertebrates it is "justifiable to assume that the reactions observed are unlikely to be due to reducing substances other than ascorbic acid".

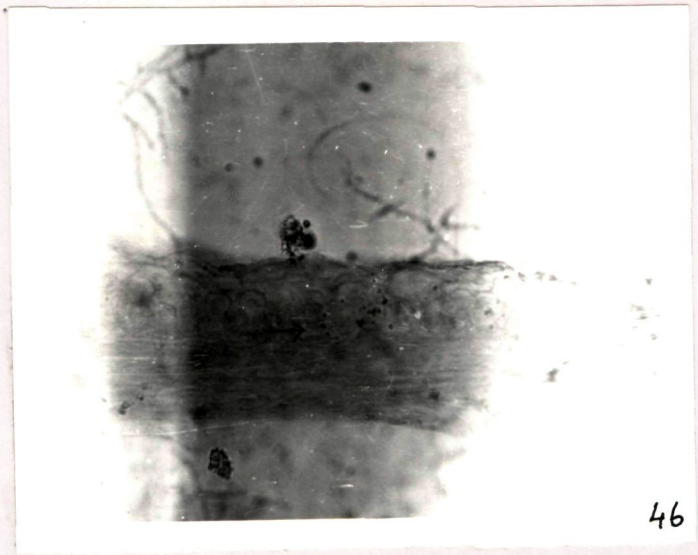
In the present study, the corneal epithelium and endothelium of Cyprinus carpio and Clarias batrachus, gave ascorbic positive granules but no reaction has been observed in the corneal stroma. Presence of ascorbic acid positive granules confirm their presence only in epithelium and endothelium. However, these granules are very limited in both the layers. (Figs. 46 and 47).

The lens, on the other hand, showed strong reaction histochemically. The ascorbic acid granules are more abun-

EXPLANATION TO FIGURES

Fig. 46 Ascorbic acid positive granules in the cornea of *Cyprinus carpio* x 500

Fig. 47 Ascorbic acid positive granules in the cornea of *Clarias batrachus* x 500



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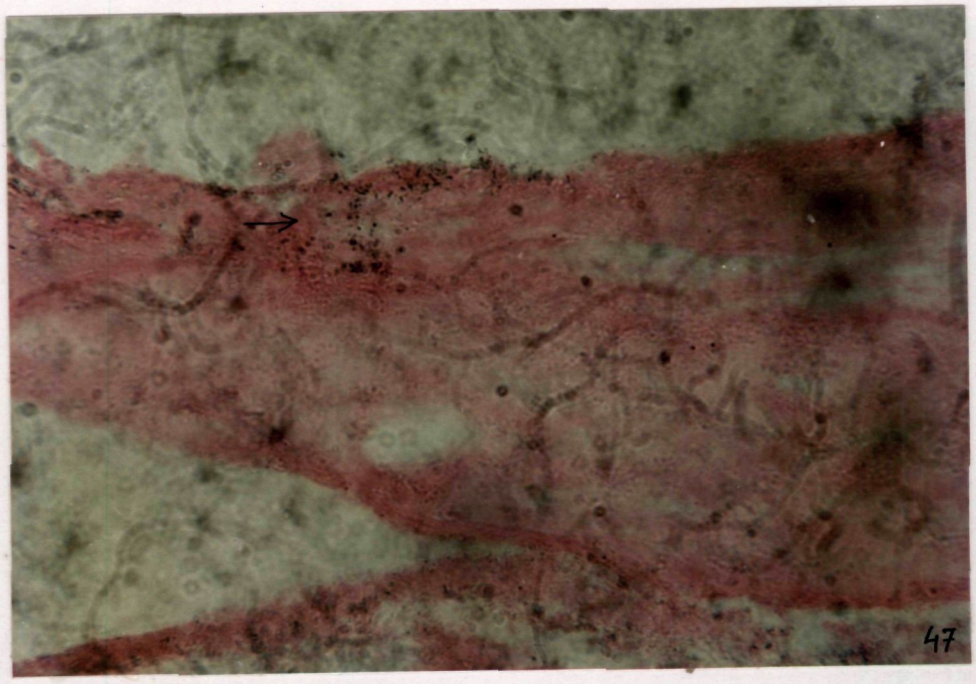

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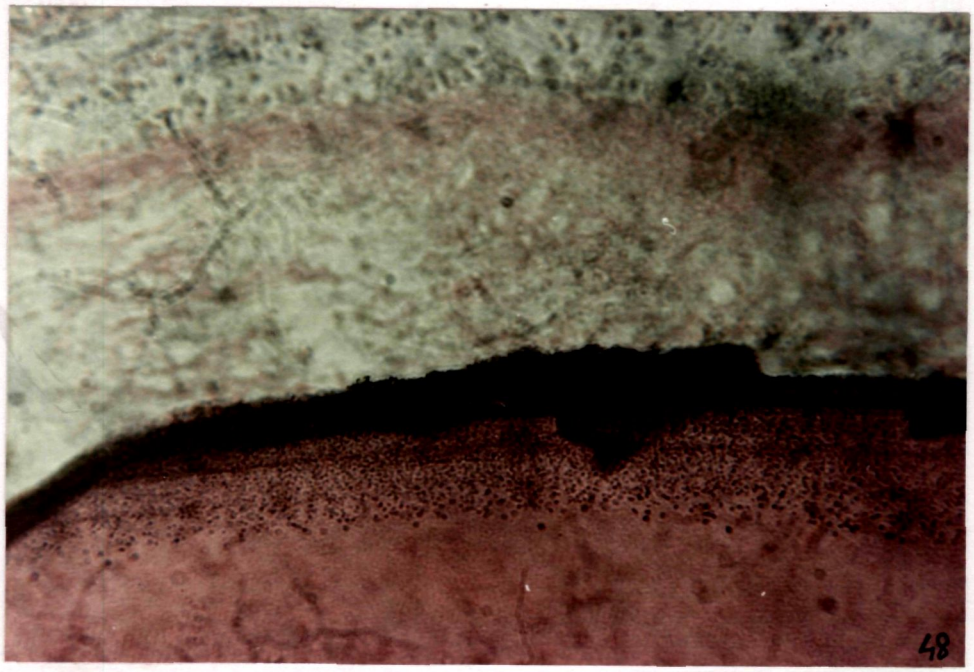
dant in the anterior half of the critical portion of the lens compared to the posterior half in both the fishes (Figs. 48 and 49). The histochemical detection has been further confirmed by bio-chemical estimation of ascorbic acid in the cornea and lens which has been carried out separately. The ascorbic acid turn over (free form of ascorbic acid or AA, bound form of ascorbic acid or ASG, enzymatic utilization of ascorbic acid or AAU and association of ascorbic acid with macromolecules or AA-MM complex) in the cornea and lens of carp and cat fish (Tables 5 and 6).

The major source of error in studying the ascorbic acid concentration in any biological system is the auto-oxidation of ascorbic acid. This auto-oxidation has been prevented by a fairly simple method as described by Chinoy et al. (1976), in which glass distilled water saturated with CO_2 has been used for extraction and preparation of ascorbic acid solution. Chinoy et al. (1975) while studying the auto-oxidation of ascorbic acid in relation to time have found that auto-oxidation is rapid in glass distilled water kept at 30°C. But when glass distilled water cooled to 3-5°C after boiling is used, it has been observed that the rate of auto-oxidation is slowed down, which is noted upto 180 mins. The glass-distilled water when saturated with CO_2, the auto-oxidation is completely checked at both 30°C and 3-5°C.

EXPLANATION TO FIGURES

Fig. 48 Ascorbic acid positive granules in the cortical region of the lens of C. carpio x 500

Fig. 49 Ascorbic acid positive granules in the capsular surface of the lens of C. batrachus x 500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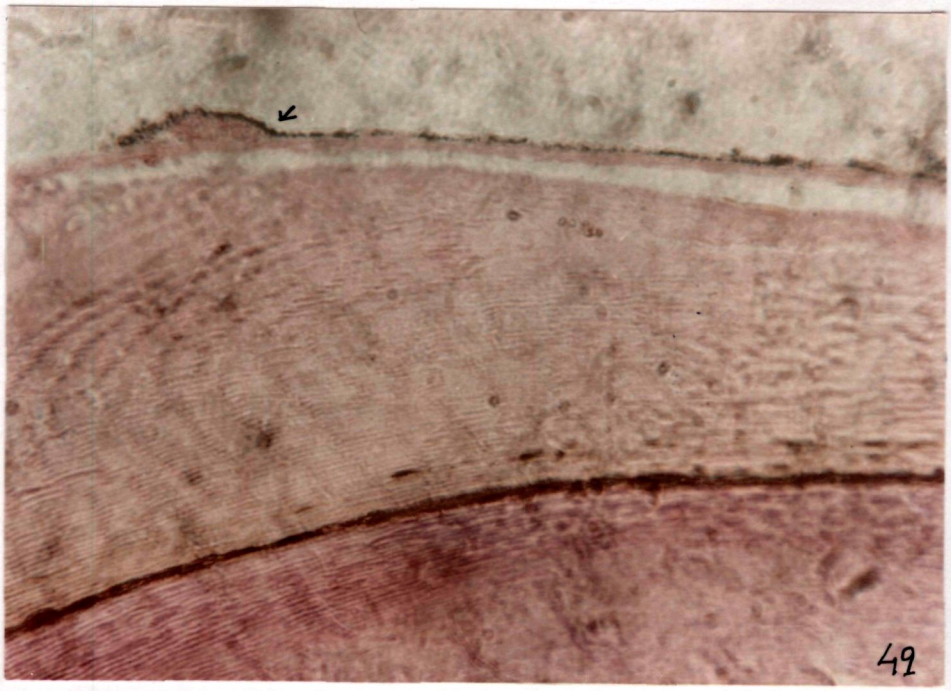
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Table 5

Ascorbic acid (free form, bound form, enzymic utilization and association with macromoles) turn over in the cornea of the Cyprinus carpio and Clarias batrachus

Different forms of ascorbic acid	Fish (1) C. carpio (2) C. batrachus	Concentration (mg/gm)
Free form (AA)	(1) carp cornea	0.102
	(2) cat fish cornea	0.643
Bound form (Ascorbigen)	(1) carp cornea	0.105
	(2) cat fish cornea	0.63
Enzymic utilization (AAU)	(1) carp cornea	1.75
	(2) cat fish cornea	4.81
Association with macromolecules (AA-MM complex)	(1) carp cornea	1.48
	(2) cat fish cornea	1.52

Table 6

Ascorbic acid (free form, bound form, enzymic utilization and association with macromolecules) turn over in the lens of the Cyprinus carpio, and Clarias batrachus

Different forms of ascorbic acid	Fish (1) C. carpio (2) C. batrachus	Concentration mg/gm
Free form (AA)	(1) carp lens	0.138
	(2) cat fish lens	0.334
Bound form (Ascorbigen)		
	(1) carp lens	0.042
	(2) cat fish lens	0.36
Enzymic utilization	(1) carp lens	0.982
	(2) cat fish lens	6.22
Association with macromolecules (AA-MM complex)	(1) carp lens	0.295
	(2) cat fish lens	1.61

Another probability of error is the estimation of ascorbic is due to the instability of the dye, 2,6-dichlorophenol indophenol. This dye solution is very much unstable at low pH, a characteristic of indophenol dyes. These dyes at lower pH decompose to give quinones and aminophenols (Karrer, 1950).

Chinoy et al. (1976) have reported that unbuffered HPO_3 decolourises the dye rapidly and the rate of decolourisation is proportional to the concentration of HPO_3 present in the system. We could effectively stabilize the dye by buffering it with citrate-NaOH buffer at a pH 3.6 in our study.

Another difficulty is faced in the case of ascorbic acid turn over, is that, during the determination of ascorbigen and AA-MM complex, hydrolysis with metaphosphoric acid is carried out at 75°C for 15 minutes. Heating at such a high temperature there is always a possibility of loss of ascorbic acid. In the present study the loss could be effectively checked by the use of 15% metaphosphoric acid in the system.

Chinoy et al. (1976) while studying the effect of metaphosphoric acid on the stability of ascorbic acid during hydrolysis have suggested that 15% or higher concentration of metaphosphoric acid (HPO_3) efficiently checks the loss of

ascorbic acid during heating at 80°C upto 15 minutes. While HPO_3 is used to check the loss of ascorbic acid, the system is buffered to a pH 3.6 before the dye is added to it. When 15% metaphosphoric acid is used, the buffer solution B (cf. Materials and Methods) effectively brings the pH level to 3.6 when mixed in a ratio of 1:1

Finally, the interfering substances other than ascorbic acid is checked by carrying out the estimation in a strong acid medium. Thus it can be inferred that the dye reducing property observed in the corneal and lens homogenates of cyprinus and clarias is most likely to be due the presence of ascorbic acid in these tissues.

DISCUSSION

The physiological roles of ascorbic acid have not yet been described in a manner that is scientifically satisfactory. The presence of ascorbic acid in all eucaryote organisms suggests fundamental roles that are not understood (Seib and Tolbert, 1982).

However, the detection of ascorbic acid in the cornea and lens of fish in the present investigation confirms the earlier findings of ascorbic acid level by various authors, as already mentioned in the fish eye (Rudra, 1936a; Saha, 1939; Ikeda et al., 1963; Raghubanshi and Swarup, 1978; Halver et. al. 1975; Agarwal and Mahajan, 1980). Thus, ascorbic acid may be equally important in the visual physiology of fish like other vertebrates. Again, the role of ascorbic acid in vertebrate corneal tissue is least studied compared to lens.

Various roles have been attribured to ascorbic acid by a number of workers. Ascorbic acid is one of the most important sugar acids, a very unstable compound and readily undergoes oxidation to dehydroascorbic acid and all higher species appear to employ ascorbic acid as a co-factor in certain specific enzymatic reactions (Lehninger, 1972). It has been postulated that monodehydroascorbic acid, a stable and a free radical anion, is the intermediate in the oxidation of

ascorbic acid by a metal ion. Stability of this radical anion and its conversion to dehydroascorbic acid and ascorbic acid helps to explain the antioxidant role that ascorbic acid plays in biological systems (Seib and Tolbert, 1982).

The requirement of ascorbic acid in the formation of connective tissue has been reported by Seib and Tolbert (1982) and its role in collagen formation by Mayes, (1988). Ascorbic acid is essential for the formation of intercellular cement and the imperfect formation of cement substance leads to the defective synthesis of connective tissue. Imperfect formation of the cementing substance is caused by the failure in collagen synthesis due to a deficiency in the enzyme proline hydrolase which converts proline to hydroxyproline (Herper et al. 1979). Electron microscopic observations have revealed cellular atrophy and damage of nerve cells due to hypovitaminosis C (Sulkin and Sulkin, 1975). Fiddick and Heath (1967) have observed the bound form of ascorbic acid in the guinea pig adrenals where several protein fractions are bound to ascorbic acid.

The eye takes up its ascorbic acid by an energy dependent active transport mechanism (Nicola et al. 1968; Sharma et al. 1964) because the ability to synthesize ascorbic acid is absent in insects, invertebrates, fishes, and certain bats and birds (Chaudhuri and Chatterjee, 1969; Chaterjee et al. 1975). It has also been shown that ascorbic acid is taken up by

several tissues by an energy dependent and Na^+ sensitive process (Omaye et al. 1982) and possibly it also plays some role in the active transport of ascorbate across the ciliary epithelium (Cole, 1970).

According to Heath (1962), the ocular tissues which are exposed to ultraviolet light have high content of ascorbic acid. Pirie (1946) has shown that ascorbic acid content is higher in the corneal epithelium than stroma in ox and rabbit and the concentration of glutathione has also shown to be higher in the ox epithelium than in the stroma which originate endogenously (Hermann and Moses, 1945). According to Maurice and Riley (1970) the cornea seems to behave like other collagen rich structures in scurvy. It has been observed that ascorbic acid reduces the ulceration of cornea following alkali induced burn in rabbit (Pfister and Paterson, 1977; Pfister et al. 1978).

One of the important functions, that is the ability of glucose metabolism by corneal epithelium of bovines (Kinoshita et al. 1955) and rabbits (Kuhlman and Resnik, 1959) through hexose monophosphate shunt has been demonstrated (Kinoshita, 1964). The reoxidation of the reduced nicotinamide adenine dinucleotide (NADPH) produced during the above process is of fundamental importance. The role of ascorbic acid in the oxidation of NADPH has been established by Anderson and Spector

(1971). The authors have shown that oxidation of NADPH to NADP is accomplished through ascorbic acid and glutathione oxidation-reduction system with consequent production of hydrogen peroxide (H_2O_2) in calf cornea and the process is catalyzed by two enzymes, such as dehydroascorbic acid reductase and glutathione peroxidase.

Buck and Zadunaisky (1975) have suggested a very important function of ascorbic acid in stimulating ion transport through the inhibition of 3,5-cyclic AMP phosphodiesterase in the corneal epithelium and other ocular tissues of frog and rabbits. They have experimentally found that ascorbic acid increases the short circuit current of the isolated cornea of frog and rabbit. It has also been demonstrated by Buck and Zadunaisky (1975) that addition of 10mM ascorbic acid supresses the 37% of the phosphodiesterase activity in the corneal epithelium of frog but which was not affected by pH. This inhibitory effect of ascorbic acid has also been reported in the corneal epithelium of toad and rabbit (Buck and Zadunaisky, 1975).

The inhibitory effect of ascorbic acid on the 3,5-cyclic phosphodiesterase activity causes an increase in the cyclic AMP in the corneal epithelium (Buck and Zadunaisky, 1975; Lewin, 1973). This increase in the cyclic AMP content in the corneal epithelium possibly enhances active ion transport across the cornea.

The lens contains a high concentration of ascorbic acid in the cortex than the nucleus and the level falls rapidly in the deficiency state (Mclaren, 1970). Pirie (1962) and Horning (1975) have suggested that ascorbic acid is associated with the metabolism of lens. It prevents in vitro light induced damage to the lens cation pump in rat eyes (Varma et al. 1979). Kinsey and Jackson (1949) relates the high concentration of ascorbate in the eye with the regression of the hyaloid system and this might account for the interspecies differences. While Heath (1962) observed that high concentration of ascorbic acid in the lens than aqueous humour is necessary to maintain the normal levels of ascorbic acid in the aqueous end vitreous humours and the cornea. The high concentration of ascorbic acid in the ocular tissue is maintained by an active transport of ascorbate from the plasma across blood/ aqueous barrier (Sharma, 1989).

The importance of ascorbic acid in vision is that the maintenance of relatively high content of ascorbic acid in the eyes than other tissues during deficiency. Hughes et al. (1971) have observed that the brain and the ocular lens retain a high ascorbic acid level even in the absence of dietary source of this vitamin while other tissues show total depletion. The authors, in their experiment, maintained guinea pigs in an ascorbic acid free diet for 14 days and which followed

the estimation of ascorbic acid level in various tissues. This revealed that fall of ascorbic acid content was 1% in the spleen, 4% in the adrenals and less than 1% in the aorta respectively compared to their initial content. On the contrary, the corresponding values for brain and eye lens have been found to be 24% and 28% respectively. This difference of ascorbic acid concentration between the eye lens and other tissues may be due to an efficient mechanism operating in the eye which assures retention or uptake of this vitamin in the eye than other organs. Hughes et al. (1971) have further suggested that the function of ascorbate maybe of very importance to the eye and that might account for the maintenance of the level of the vitamin in the eye as long as possible. This may explain the experiment of Baker (1946) in which it was observed that in vitro cultured lens loss all the detectable ascorbic acid after 10 days but its transparency was retained even after 21 days of culture. Besides the lens, aqueous humour of the eye of various animals retain a high concentration of ascorbic acid by a mechanism of active transport from blood (Kinsey, 1947; Barany and Langham, 1955).

The lens maintains a high level of reducing agents such as ascorbate and glutathione to maintain a high energy demand (Goldschmidt, 1924). The high concentration of ascorbic acid suggests that in addition to some co-enzymatic functions ascorbate may also modulate some non-enzymatic metabolic

reactions in the lens. The most probable action of ascorbate is as reducing agent preventing unwanted reactions initiated by O_2 and free radicals either photochemically or under ambient non-photochemical condition. It has also been suggested that ascorbate terminates the propagation of various free radical reactions in oxidation, photolysis and radiolysis (Sharma, 1989).

Kinoshita (1964) has reviewed some of the biochemical reactions involving glutathione and ascorbic acid which may be relevant to lens metabolism. It is known that the pentose phosphate pathway is main source of energy of the lens where $NADP^+$ is made available for the enzymes of the pathway through the respiratory link between ascorbic acid and glutathione.

In this context, it is worth mentioning that, Rawal and Rao (1977) in their experiment have shown that fishes living in the upper strata (ophiocephalus and barbus) of the water have lower amount of ascorbic acid and glutathione in their lens than the fishes living at the bottom or deeper layer of water (catfish). The authors suggest that the relatively high concentration of ascorbic acid and glutathione in the normal lens of some forms is due to their high energy demand and significant for the maintenance of lens transparency as suggested by Daisley, 1955.

In this context, it is worth to mention that in our studies we have also found a high content of ascorbic acid in the lens of the bottom dwelling cat fish (Clarias batrachus) than the lens of the carp which is not strictly a bottom dweller. Thus, our observation corroborates the important finding of Rawal and Rao (1977). Similarly, it is of interest to note that the ascorbate concentration of the cornea of cat fish is much higher than the carp cornea as we have found in our investigation.

In our investigation, while studying the utilization of ascorbic acid it has been observed that a portion of ascorbic acid of the aliquot incubated for AAU forms complexes, presumably with macromolecules instead of being oxidized. The bound ascorbate, however can be recovered by hydrolysing with metaphosphoric acid. This complexing ability of ascorbic acid, most probably is responsible for the formation of bound form of ascorbic acid or ascorbigen (Sengupta and Guha, 1938). Such complexing may lead to the formation of charge transfer complexes which has also been reported to exist in biological system very frequently, and they are known to take part actively in the energy transfer process (Szent-Gyorgyi, 1960).

Szent-Gyorgyi (1960) has suggested for the first time the importance of these charge-transfer complexes in biological system. Since his report, complexing of ascorbic acid with macromolecules has been reported from time to time (Chinoy,

1967,1969; Chinoy et al., 1971, 1973, 1974). Bonner (1957) has reported that ascorbic acid is acted upon by a number of enzymes which oxidise ascorbic acid directly or indirectly and over and above this oxidation, it forms complexes with macromolecules, such as proteins and nucleic acid.

Considering the above reports, it is reasonable to assume that the ascorbic acid might be equally significant in the visual process of the fishes. Even the high content of ascorbic acid in cornea as well as in lens in the bottom dwelling forms might help in some way or other in the scotopic adaptation.

C H A P T E R IV

PIGMENT MIGRATION

INTRODUCTION

Vision is a special somatic efferent sense and for maximum efficiency of vision it is necessary for the eyes to be adapted to various stages of illumination. This adaptation is aided by the movement of screening pigments and the migration of screening pigment in the eyes is a characteristic feature. The pigment of the visual system is melanin, produced in the melanaphores, the best known of all pigment cells or chromatophores (Bagnara et al. 1978). The term melanin is a generic one that actually includes the black or brown eumelanins and the phaeomelanins that are yellow or red in colour (Prota, 1980). Melanin is the structural pigment of the skin, hair, many feathers, scales and eye.

The biological significance of melanin, is that they absorb light throughout the visible spectrum as well as in the U-V spectrum range. Melanin granules prevent reflection of transmitted light (Lythgoe, 1979). This pigment is the essential component in the retinal layer preventing reflection. The retinal pigment granules, melanin in the pigment epithelial cells exhibit distinct movements during light and dark adaptations and thereby help in the process of visual adaptations in various photic levels.

Several different methods of dark and light adaptations are known; some methods are characteristic of particular animal

groups, although a particular species normally possesses more than one method. The various adaptive methods can be divided into three classes: (1) optical regulation of the light reaching the visual pigment (melanin) through pupil; (2) absorption by the visual pigment; and (3) neural process (Lythgoe, 1979).

The melanin granules of the pigment epithelium and the rods and cones themselves frequently move in response to changes in light intensity in such a way that particular cell types are shielded from unwanted light. The changes are collectively known as retinomotor or photomechanical movements. Phylogenitically they appear to be older than the pupillary movements and usually one or the other mechanism is well developed in a particular class of animal (Lythgoe, 1979).

Retinomotor movements are best developed in birds, fishes and frogs, the phenomenon was first studied separately by Boll (1877) and Kuhne (1877) and has been reviewed by Blaxter (1970) and Ali (1971,1975). Though the underlying mechanisms are not known but both the rods and cones and the pigment granules within the epithelial cells of the pigment epithelium may move in response to changing light intensity. The myoids and ellipsoids of rods and cones contract and elongate in response to change in light intensity. This movement is almost coincident with the migration of pigment granules within the cells of the pigment epithelium (Lythgoe, 1979). In

dark adapted state the pigment granules within the epithelial cells surrounding the rods and cones move to the back of the retina and the cones move in the direction of the retinal pigment epithelium. At the same time, the rod myoids may contract resulting the movement of rods away from the pigment epithelium. During light adapted state the pigment granules are dispersed fairly evenly through the pigment epithelium and the rod myoids expand so that pigment granules surround the rod outer segments (Anctil et al. 1980; Klyne and Ali, 1980).

The movements of the rods, cones and retinal pigment granules are not exactly synchronized and in some cases either pigment migration or rod and cone migration proceeds in the absence of the other. The movements during dark adaptation are slow and require times of the order of an hour to complete (Walcott, 1975) while the light adaptation is much faster and proceeds rapidly. The most familiar components of light and dark adaptations are, changes in concentration of visual pigments and modifications in neuronal interaction (Munz, 1971).

The neural process is probably the most important and least understood mechanism involved in dark and light adaptations. During the complete state of full light adapted to the fully dark adapted retina, the neural organization of the retina changes. The state of dark adaptation involves rods, much summation and without any colour vision while the light

adaptation involves cones, little summation and colour vision. The initial stages of adaptations are extremely rapid completing in less than one fifth of a second. The later stages of adaptation proceed more slowly. Perhaps the whole neural organization from brightness to darkness takes place within about 30 mins. (Lythgoe, 1979) In spite of pronounced positional changes in the retinal pigment and visual cells in dark and light there is difference in the results in photochemical movement due to photic stimulation in teleosts (Ali, 1959, 1964; Detwiler, 1943; Arey, 1916).

In this connection, it is of interest to note that Kakcheyev (1943) formulated the hypothesis of nervous control in the process of dark adaptation. Ali (1964a) suggested that pigment migration may be influenced by hormones. Bagnara and Hadley (1969) believes that in all probability intermedin is the agent in melanophoric response in the skin of fishes. A number of suggestions as to the involvement of ions in the pigment migration have been proposed (Freeman et al. 1968). According to Veerdonk (1962) and Novales (1959) transmembrane ion induces pigment migration through intermedin.

Further, retinomotor responses can be influenced by colchicine (Anctil et al. 1979), cyclic AMP (Bitensky et al. (1973) and 5-hydroxytryptamine (Kato et al. 1982; Allen and Burnside, 1986).

Effect of photopic and scotopic states on neurosecretion and its role on pigment migration has been suggested in insects (Veron, 1973; Dey, 1980). Although various roles of neurosecretion in fish have been described but reports on the relationship between light and dark states and neurosecretion are lacking.

Keeping above in view, the effect of colchicine, cAMP and 5-HT in the pigment migration in the retina and effect of light and dark on neurosecretory cells have been investigated in Cyprinus carpio and Clarias batrachus.

However, the above experiments could not be performed in marine fish, pomfret,due to non-availability of live ones.

MATERIALS AND METHODS

Studies on Light and Dark Adaptations

To study the movement of retinal pigment granules in light- and dark-adapted states, two groups of experimental fishes were selected. One group has been kept in sunlight for about 3 hrs. for light adaptation. After that period, the fishes are decapitated and eyes fixed in 10% buffered formalin. Routine histological preparations are carried out for paraffin embedding methods and 7-8 μ thick sections are cut for microscopic preparations.

The other group of fishes has been dark-adapted for the same period as the light-adaptation experiment. The eyes are similarly fixed and after necessary preparations 7-8 μ microtome sections were cut. All preparations were carried out in dark and the eyes were kept in light-tight vials since in some enucleated dark-adapted eyes pigment migration may take place when exposed to light (Ali, 1964).

Effect of Light and Dark on Neurosecretion

To examine the influence of light and dark on the neurosecretory system of Cyprinus and Clarias, the fishes are adapted in light and dark for about 3 hrs.

After the required period of adaptations the specimens were decapitated and fixed in alcoholic Bouin's fixative for 24 hrs and a hole was done on the skull for the penetration of the fixative. After that the brain was taken out and again fixed in the fixative for another 24 hrs. Routine histological methods were followed and 10 μ thick sections were cut. The sections were stained in paraldehyde-fuchsin-one-step trichrome (Gabe, 1966).

Effect of 5-HT (Sigma), cyclic AMP (Sigma) and Colchicine

To study the effect of 5-HT, cAMP and colchicine on retinal pigment (melanin) on the eyes of Cyprinus and Clarias, each of the above drugs was administered intraocularly by microsyringe in dark adapted animals only.

Concentrations of 5-HT and cAMP being 0.8 mM each and the concentration of colchicine being 0.3 mM. Each of the substances were dissolved in cold-blooded ringer solution.

OBSERVATIONS

The position of the pigment granules during light- and dark-adaptations in carpio and catfish are shown in Figs. 50, 51, 52 and 53 respectively. However, all the parts of retina do not respond equally.

Effects of Light and Dark on Neurosecretory System

It has been observed in both the dark adapted fishes (carpio and catfish), a large accumulation of compact and purple neurosecretory substances in the neurosecretory pericarya. (Figs. 54 and 55).

Conversely, in the light adapted fishes, a significant reduction of the neurosecretory materials has been observed presumably due to axonal transport (Figs. 56 and 57).

Effect of 5-HT, cAMP and Colchicine

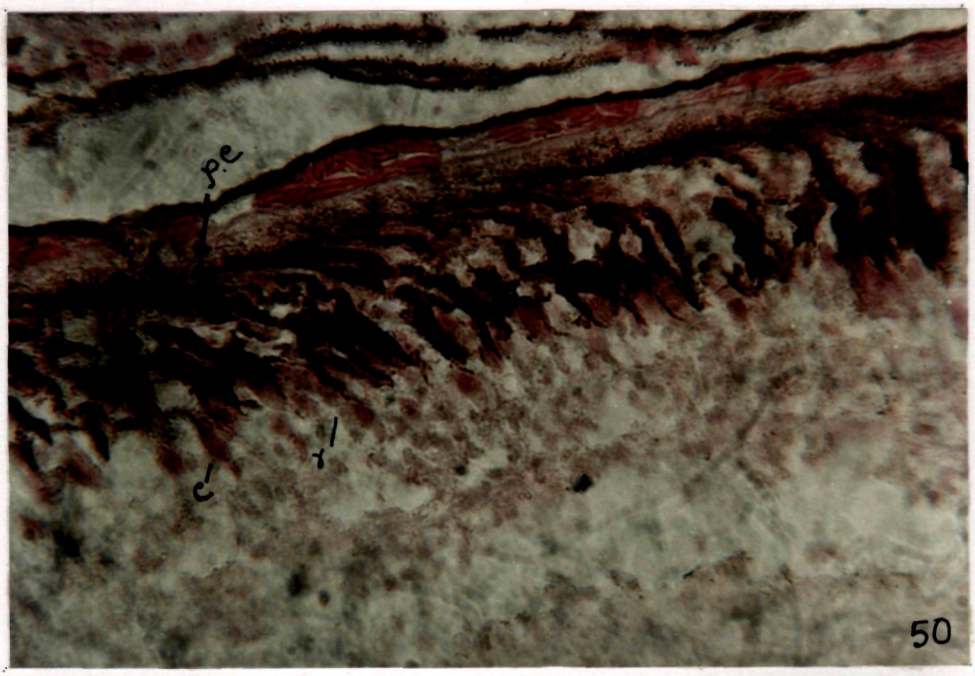
Light microscopic studies of paraffin sections of dark adapted eyes reveals that in all cases almost complete migration (dispersion) of retinal pigment granules similar to controlled light adapted eyes. It seems that pigment dispersion is more intense in the treated eyes (Figs. 58,59,60,61, 62 and 63) and total masking of the visual cells could be seen.

EXPLANATION TO FIGURES

Fig. 50 Light adapted (control) retina of *Cyprinus carpio*, showing pigment dispersion and movement of cones away from the pigment epithelium x 500.

Fig. 51 Dark adapted (control) retina of *C. carpio*, showing pigment concentration and exposed rod outer segment x 500.

C, cone. r, rod. PC, pigment epithelium. P, pigment. ros, rod outer segment.



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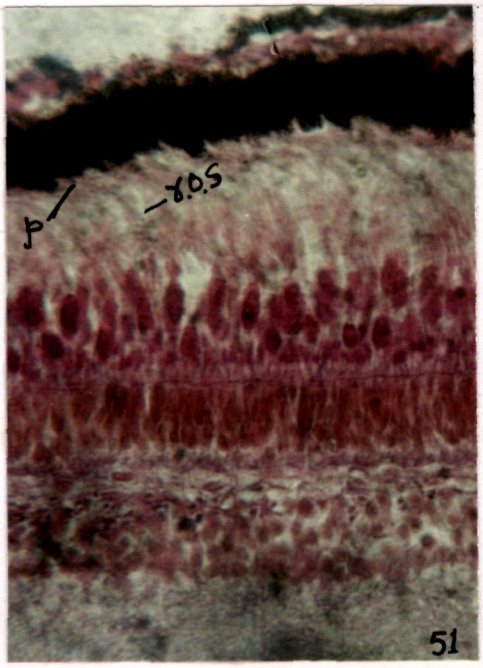

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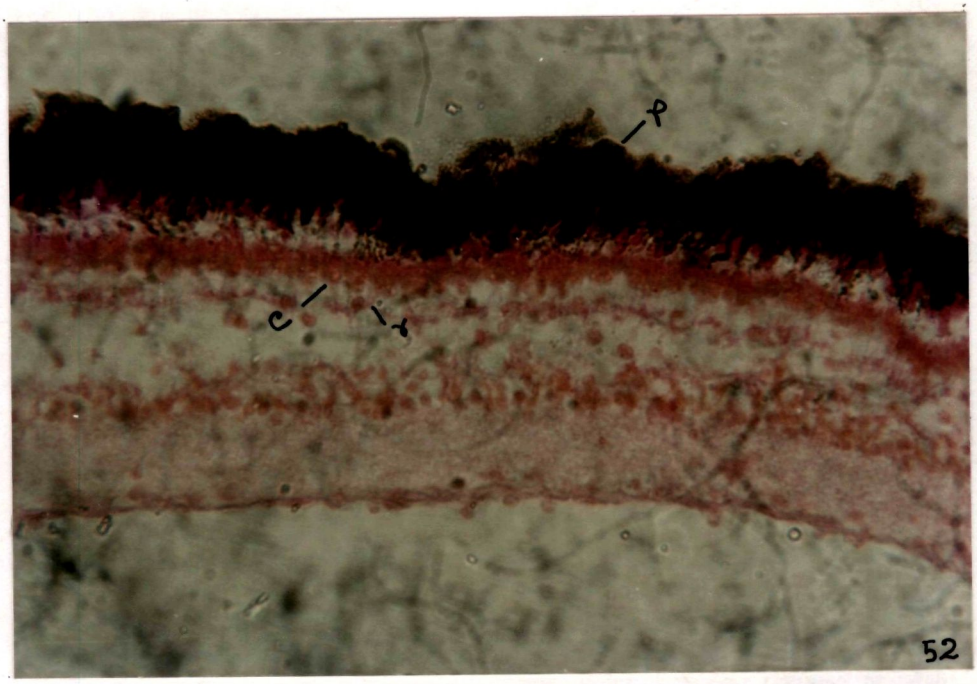
EXPLANATION TO FIGURES

Fig. 52 Light adapted (control) retina of *Clarias batrachus* x 500.

Fig. 53 Dark adapted (control) retina of *C. batrachus* x 500.

(Other details as in Figs. 50 and 51)



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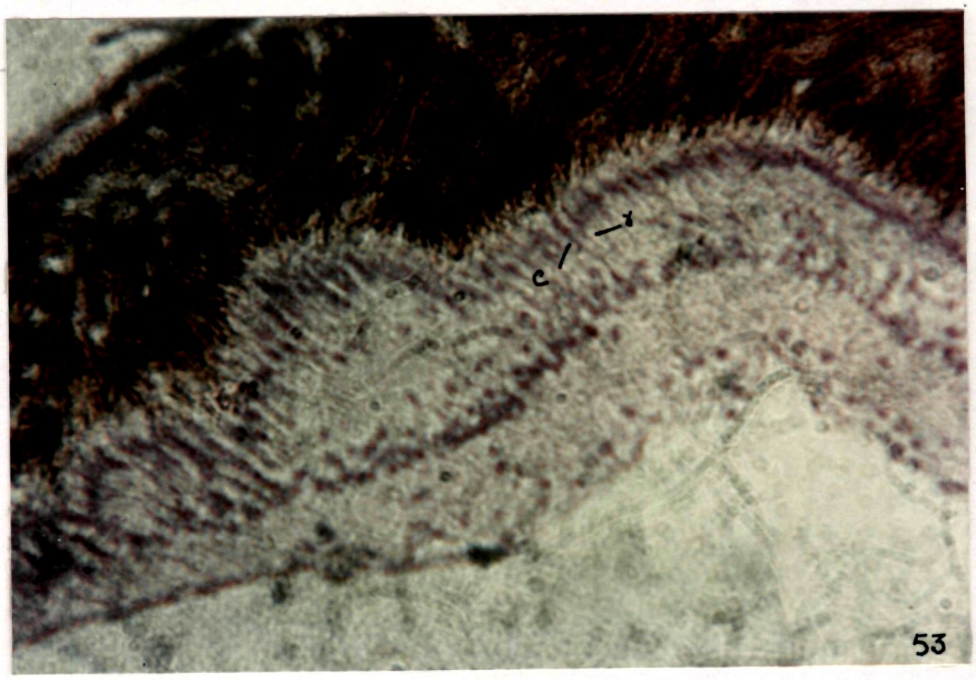

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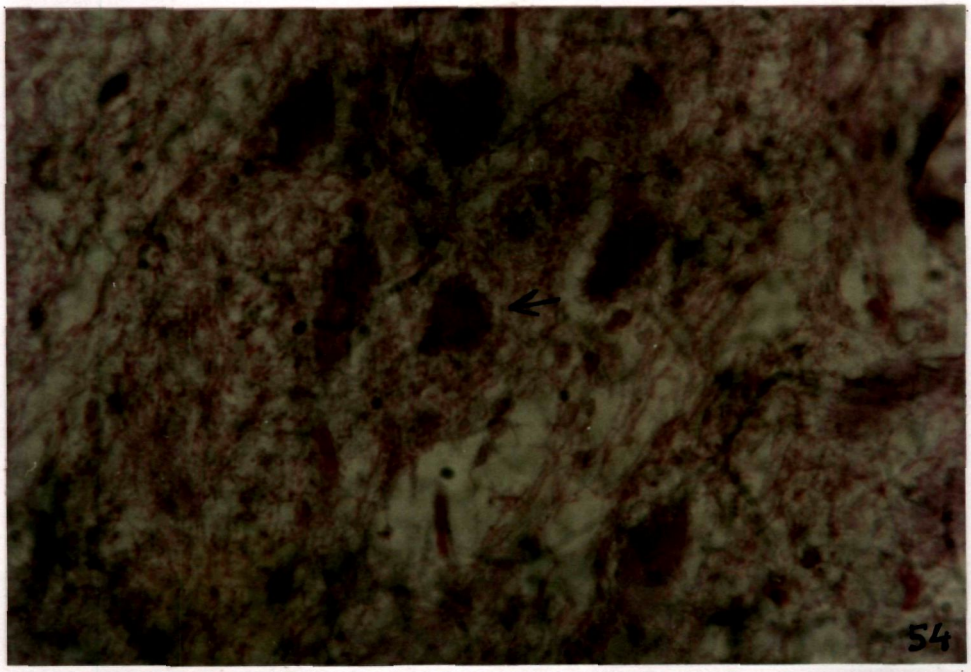
EXPLANATION TO FIGURES

Fig. 54 Sagittal section through the diencephlon of dark adapted *Cyprinus carpio*, showing large accumulation of neurosecretory materials in the neurosecretory cells x 1250.

Fig. 55 Sagittal section through the diencephalon of dark adapted *Clarias batrachus*, showing large accumulation of neurosecretory materials in the neurosecretory cells x 500.

NSC - Neurosecretory cell.



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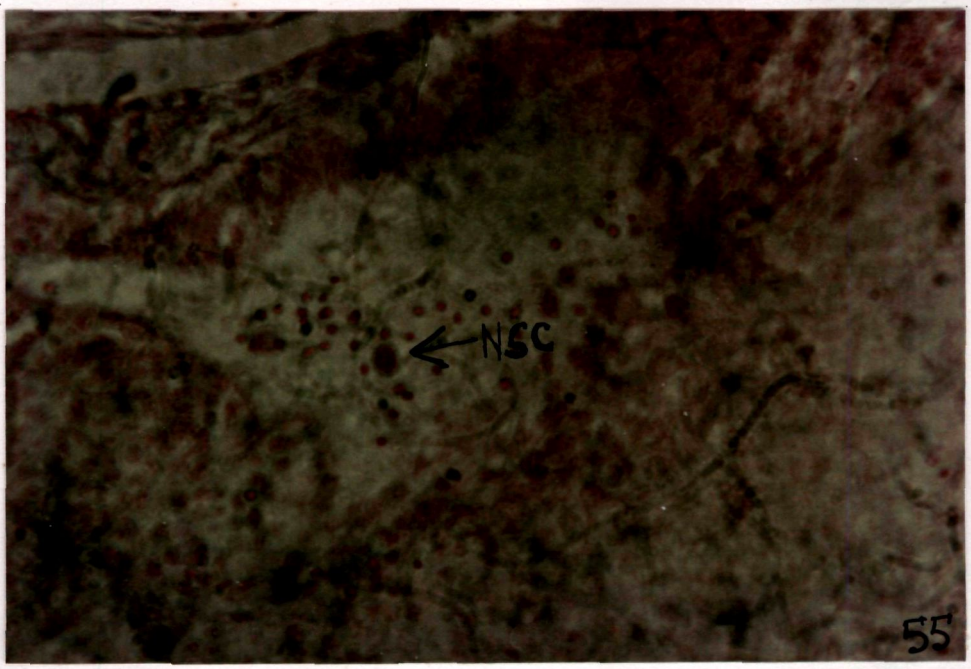

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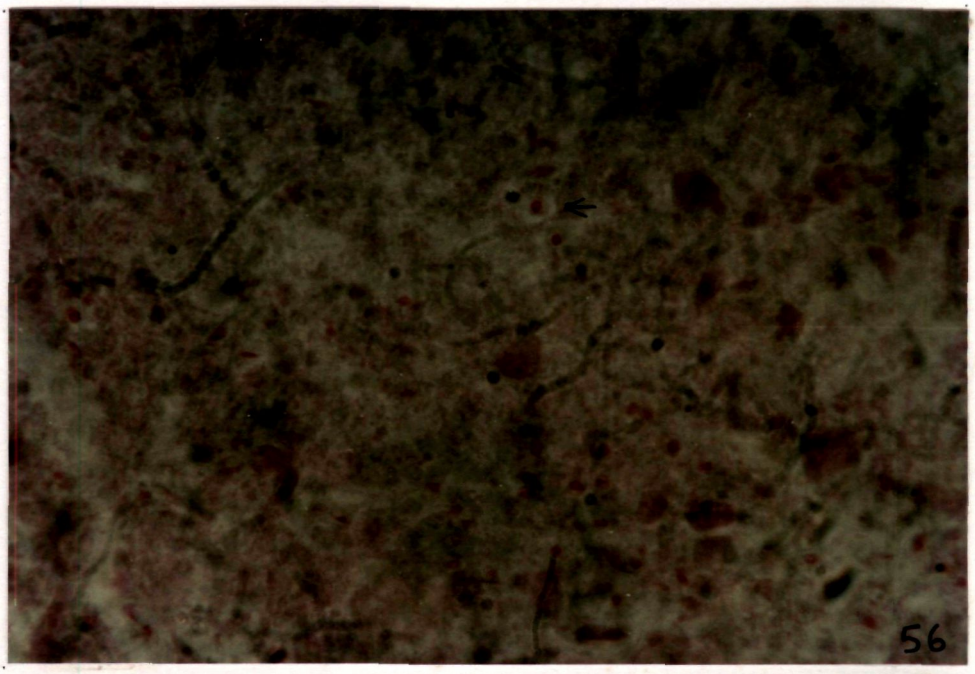
EXPLANATION TO FIGURES

Fig. 56 Sagittal section through the diencephalon of light adapted *Cyprinus carpio*, showing reduced neurosecretory contents in the cells x 500.

Fig. 57 Sagittal section through the diencephalon of light adapted *Clarias batrachus*, showing reduced neurosecretory contents in the cells x 500.



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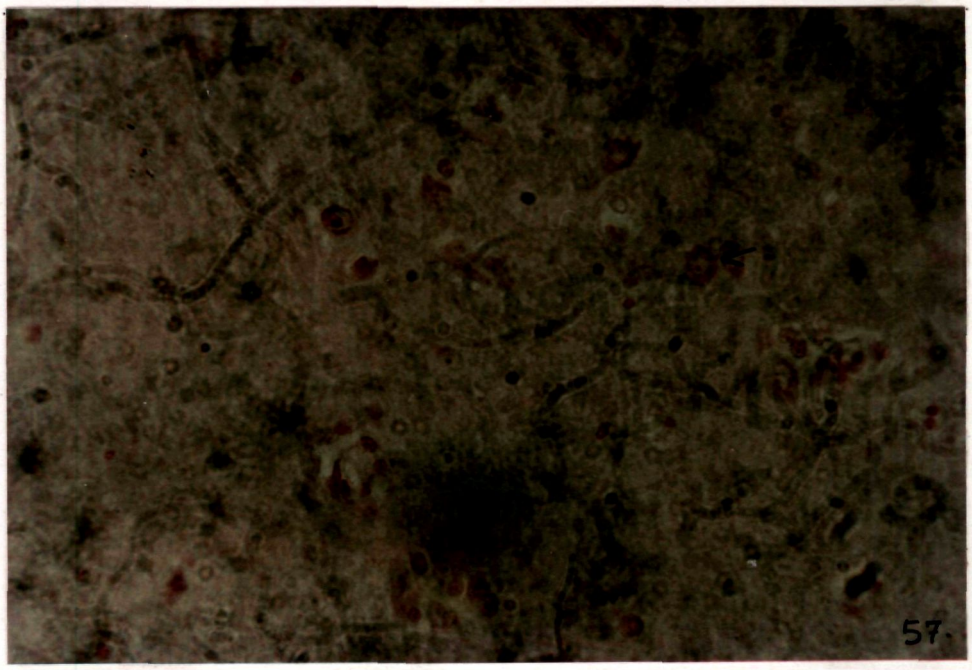


57.

EXPLANATION TO FIGURES

Fig. 58 Effect of 5-HT on dark adapted retina of C. carpio, showing pigment dispersion similar to light adapted state x 500.

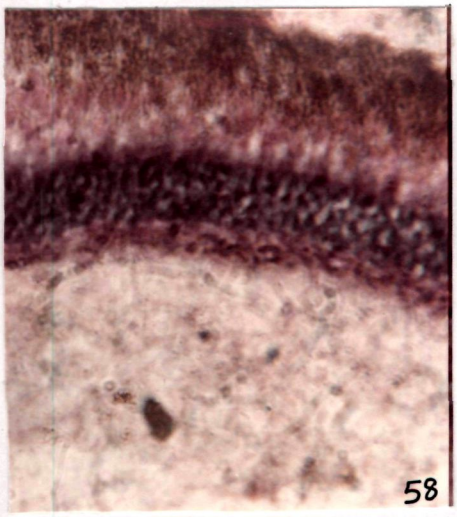
Fig. 59 Effect of 5-HT on dark adapted retina of C. batrachus, showing pigment dispersion similar to light adapted state x 500.

Fig. 60 Effect of cAMP on dark adapted retina of C. carpio, showing pigment dispersion similar to light adapted state x 500.

Fig. 61 Effect of cAMP on dark adapted retina of C. batrachus, showing pigment dispersion similar to light adapted state x 500.

Fig. 62 Effect of colchicine on dark adapted retina of C. carpio, showing pigment dispersion similar to light adapted state x 500.

Fig. 63 Effect of colchicine on dark adapted retina of C. batrachus, showing pigment dispersion similar to light adapted state x 500.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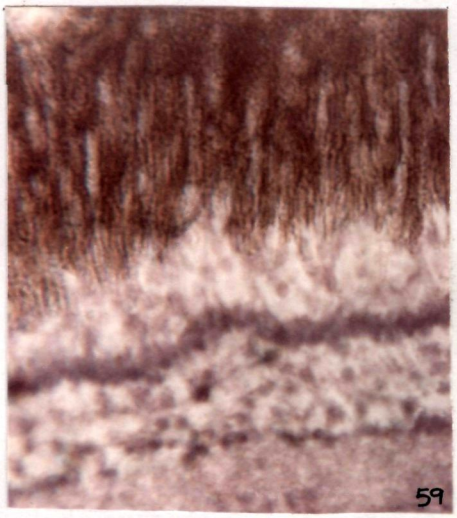
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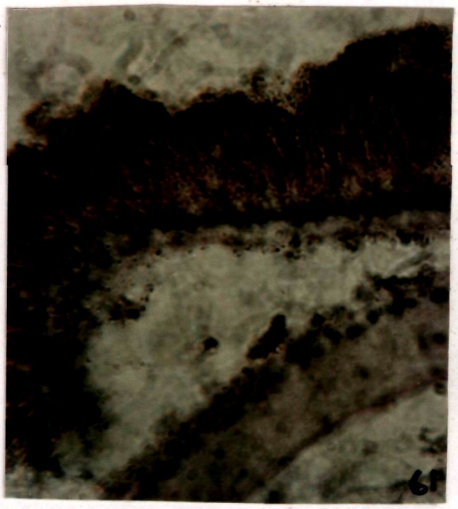
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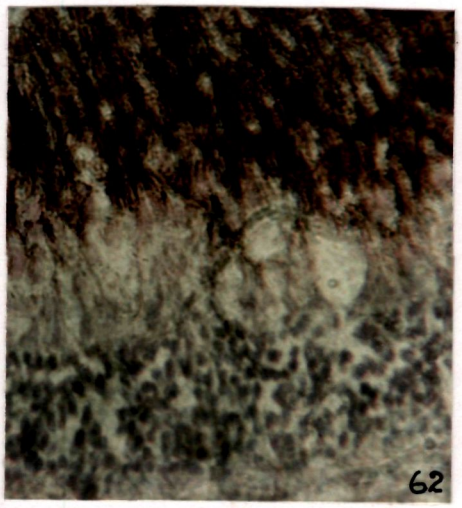
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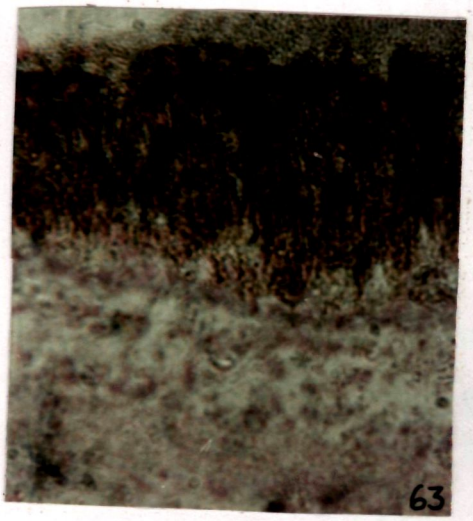
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DISCUSSION

The basic physiology of the photoreceptor system has been adapted in many different ways to varied habits and habitats. The two basic adaptive features being (1) the changes in length and shape of photoreceptors (rods and cones) that are brought by changes in the lighting conditions of the environment (Ferrero et al., 1979); and (2) the dispersion or concentration of pigment granules (Ali, 1975b).

The arrhythmic eye, which performs well over a wide range of light intensities, has several adaptive mechanisms associated with activity during both day and night. These are primary devices for controlling the amount of illumination which reaches the photosensitive cells. The maximum available light should impinge on the receptor during the night, but during the day when illumination is adequate the significant problem is the resolution of pictures, that is, the retinal elements or small groups of the cells must be excited separately by different points from the picture during the day, but at night acuity is sacrified for sensitivity and light is collected from many angles to excite the receptor cells. This adaptive mechanism depends on several photomechanical or retinomotor responses involving rapid changes in the pigment distribution and the action of contractile elements in the iris and/or the retina (Hoar, 1987).

Adomian and Sjostrand (1975) on the basis of their observation of elongation of microtubules in the cat fish retina, concluded that microtubles must be assembled and disassembled in connection with myoid elongation and shortening. Burnside (1976,1978) and Warren and Burnside (1978) demonstrated the role of microtubules and microfilaments in retinomotor responses. The authors found actin- and myosin like filaments are responsible for cone contraction of some marine teleosts. Disappearance of the microtubles following colchicine induced block of cone elongation suggests that microtubules mediate cone elongation through a sliding mechanism (Warren and Burnside, 1978). While Anctil et al., 1979), have shown that rod contraction is mediated by microtubules but not elongation and suggested that there may exist inter- and intraspecific differences in retinomotor (rod versus cone) mechanism in lower vertebrates. However, Walls (1942) commented on the confusion surrounding mechanisms controlling photomechanical movements.

The retina respond to light and signals its presence and pattern to the brain through the optic nerve. The signals from individual rods, cones or retinular cells are not transmitted to the brain in isolation from its neighbours but the signals from neighbouring visual cells may interact with each other, they may be added together so that their sensitivity is the sum of their receptive areas or they may inhibit each other

(Pirenne, 1967). However, it is likely that the reception of a single photon by a vertebrate rod is potentially enough to trigger a response (Ashmore and Falk, 1976). There are topographical differences within the retina itself where some types of rod or cone may respond by moving while others do not even within a same species (Wall, 1942; Tansley, 1965). Neural summation seems to be less developed in cones (Wall, 1942) and some predator fishes have also adapted their cone for low light intensities (Munz and McFarland, 1977).

Inherent, in the transmission of information is that various salient features of the pattern are abstracted by the neural retina and transmitted inwards to the brain. Exactly how this is done is presently an active area of research (Lythgoe, 1979).

Among the vertebrates, retinal pigment migration is rapid and extensive in the teleosts, anurans and birds while it is slow and less marked or slight in turtles and crocodilians and is absent in snakes and mammals (Walls, 1942). During dark the melanin pigment granules contract and move away towards the back of the retina, thus exposing the rods and cones; in light the pigment granules disperse through the receptor layer and outer layer of the retina and surrounds the rod tip.

But, the mechanism by which the pigment granules alter their position in response to photic stimulation is not clearly understood and a number suggestions have been put forwarded. For example, Kinosita (1963) has discussed the role of electrochemical changes causing melanin migration in fish melanophores; ionic exchange between cell exterior and interior (Lerner and Takahashi, 1956) and the importance of intracellular Ca^{2+} level in pigment migration has been suggested by Ishibashi (1957). Role of microtubules in pigment aggregation and dispersion in the scale/fish has also been proposed by Wikswo and Novales, 1969).

As for neuroendocrinological control of pigment migration in fishes, it has been suggested that pigment migration is either controlled by nerves or hormones or both (Fujii, 1969). The two hormone hypothesis of Enami (1955), assuming two antagonistic principles, e.g. melanocyte stimulating hormone (MSH) which disperses melanin and melanocyte concentrating hormone (MCH) has not been accepted universally. Baker (1963,1968a) suggested that MSH is solely responsible for both pigment concentration and dispersion in fish melanophore. In all probability intermedin is the actual hypophyseal agent involved in melanophoric response in fishes (Bagnara and Hadley, 1969), though Chavin (1956) emphasized the role of ACTH in melanophoric response of fishes.

Even, the light sensitivity of the diencephalon in blinded minnows was first reported by von Frisch (1911), indicating the role of nervous control of pigment migration. Teleosts possess both nervous as well as hormonal control over their skin melanophores, where the former plays the major role (Davson, 1970). But Osborn (1938) has shown that in cat fish both hormonal and nervous factors are responsible for pigment dispersion. According to Parker (1948) the melanophore activity is under the control of double innervation where concentrating is adrenergic and dispersion is cholinergic. Ali (1964a) in his experiment, restrained and anaesthesized goldfish and then exposed one eye to bright light. The rods and cones of the dark adapted eye did not move, but the retinal pigment melanin expanded partially. This led Ali to suggest that pigment migration may be influenced by hormones.

The nervous control in melanin dispersion and aggregation is mediated by the release of transmitter substance by nerves in the skin of fishes (Fujii, 1969).

On the basis of the above, it can be assumed that there could be some relationship between visual adaptation and neurosecretory mechanism.

As for the general significance of neurosecretion, Scharrer's concept (1952b), that neurosecretory cells represent a connecting link between the nervous system and endocrine glands,

is widely accepted. Their essential role is to transmit stimuli received from the nervous system to endocrine glands (Gabe, 1966). The neurosecretory cells respond to stimuli despite their glandular activity and it seems reasonable that neurosecretion plays an essential part in maintaining equilibrium between the organism and its surroundings. There is also a general relationship between hypothalamo-neurohypophyseal neurosecretion and general adaptation (Gabe, 1966). The secretion is elaborated also during alarm stimuli (Ames and van Dyke, 1952).

In fish, relationship between neurosecretory peptides and osmoregulation-salt balance and reproduction (Perks, 1969) has been established. Depletion or accumulation of neurosecretory products in the neurohypophysis during stress has been observed (Leatherland, 1967). Maetz (1963) pointed out the importance of neurosecretion to avoid stress in fish. However, reports on the relationship between vision and neurosecretion are lacking.

Keeping this in view an attempt has been made in the present investigation and a distinct effect of light or dark on the neurosecretory system has been observed. In the dark adapted fish there is a large accumulation of neurosecretory products whereas in the light adapted ones there is considerable reduction of the material in the hypothalamic region of the brain. During light adaptation, presumably axonal transport of neurosecretory materials take place. It may be due to the fact that during

dark the rate of discharge of the neurosecretory material is much slower while in light adapted forms synthesis of neurosecretory material is slower but discharge is faster. Thus, it can be suggested that the accumulation and discharge of the materials in response to photopic and scotopic stimulation is for visual adaptations.

Generally, there are two basic types of neurosecretory products. One class includes those neurotransmitters with a low molecular weight such as catecholamines (dopamine, adrenaline and nor-adrenaline) or biogenic amines like 5-hydroxytryptamine (5-HT) or serotonin. The other class is characterized by compounds of relatively high molecular weight such as proteins or peptides (neuropeptides).

It is well established that catecholamines and the biogenic amines like 5-HT are released instantaneously for rapid physiological phenomena or adaptations. It has also been shown that light adaptation occurs at a very faster rate in animals while dark adaptation is a slower process. Thus in any attempt to study the effect of neurosecretion in pigment migration in the vertebrate eye, the involvement of catecholamines or biogenic amines, like 5-HT seems to be important.

With this view, the effect of 5-HT on the migration of pigment granules of retina in fish eye has been tested. It has b found following the administration of 5-HT that it induces complete pigment dispersion like light adapted state in the dark

adapted eyes of both the fishes. In this connection it can be mentioned that the role of 5-HT in inducing light adapted condition in the dark adapted retina of green fish is effected by the release of dopamine by 5-HT. These observations strongly suggest that 5-HT induced dopamine, acts as extracellular messanger directly inducing light adapted cone retinomotor movement (Allen and Burnside, 1986). Kato et al. (1982) has also shown Ca^{2+} dependent 5-HT stimulates dopamine release in carp retina. However, 5-HT has been reported to have melanin aggregating action (Scheline, 1963; Scott, 1965).

In addition to this role of a physiological activator such as cyclic 3,5-adenosine monophosphate (cAMP) has been tried. We have observed that cAMP triggers pigment migration as in light adapted state in dark-adapted eyes. It has been reported that cAMP activates the physiological process characteristic of a particular effector cell which also acts as a regulatory agent in all animal cells (Bonner, 1971; Robison et al., 1971) and as intracellular messanger (Robison et al., 1971). cAMP also triggers the specific response of the cell (Vander et al. 1980). Level of cyclic nucleotide such as cAMP has been shown to differ between light- and dark-adapted retina of ground squirrel (Citellus tridecemlineatus) by DeVries et al., (1982). According to Bagnara and Hadley (1969), the effect of intermedin is mimicked by cAMP by expanding melanophores in amphibians (Bagnara and Hadley, 1969).

Further, effect of an alkaloid, colchine has been studied in the present investigation. Pigment dispersion in the dark adapted eye is induced similar to light light-treated state. Wilsow and Novales (1969) found that colchicine disperse melanosomes of Fundulus scale. Colchicine inhibits cone myoid elongation and rod myoid contraction in trout retina (Anctil et al., 1979). The important cytoskeletal elements, microtubules are disrupted by colchicine (Margulis, 1973). The microtubules are important motile processes concerned with intracellular transport nerve cells, the flow of pigment granules in chromatophores and the secretion of glands (Hoar, 1987).

It seems that the visual system and the neurosecretory system as well, is effected by dark and light condition. These together respond to photic stimulation in a co-ordinated manner, since vision is a special exteroceptive sense (Buchanan, 1957), postulated that neurosecretory materials are discharged under the influence of exteroceptive or visceral impulses and is transported through the hypophyseal portal system to the anterior hypophysis where it contributes to the synthesis and release of anterior lobe hormone. (Palay, 1953)

A photoreceptor is a light trap converting radiant energy into nerve impulses. The metabolic machinery such as mitochondria and associated organelles, in addition to, routine activities of the cell, assemble or generate chromoproteins and produce

transmitters which effect the synapse. The photoreceptive cells are metabolically very active, for chromoproteins are subject to destruction by light (Young, 1970) and transmitters must be steadily passed into synaptic vesicle (Hoar, 1987).

CHAPTER V

FLUORESCENT COMPOUNDS OF THE EYE

INTRODUCTION

Pigmented compounds are found in animals at all levels in phylogeny where some of the pigments possess characteristic property of fluorescence. These pigments are biochemically extremely varied, ranging from the almost ubiquitous melanins, carotenoids and pteridines to the less common quinones and flavins (Needham, 1974; Kennedy, 1979). Amongst these pigments pterin-type is a fluorescent substance (Matsumoto et al., 1960) also known as pteridine.

Fluorescent compounds absorbs and convert high frequency light energy to lower frequency. The fluorescent pigments originate in chromatophores or pigment cells. Chromatophores are particularly prevalent among cold-blooded vertebrates with principal pigmentary activities of scattering or reflecting light (Bagnara et al. 1978) and also act as filters (Lythgoe, 1979). It has been shown that the pteridines in the fresh water fish, amphibians and reptiles are found characteristically in high concentration in chromatophore cells termed as xanthopores or erythropores. The bright colour of these cells has been shown actually to be imparted by pteridines either solely or together with other kinds of pigments such as carotenoids (Matsumoto, 1965a,b; Hama, 1963; Obika and Bagnara, 1964).

Pteridines constitute a variety of compounds having the molecular structure of pyrimido [4,5-b] pyrazine (Purrman 1940). Each pteridine has a common structure of either 2-amino-4-hydroxy-

pteridine or 2,4-dihydroxypteridine and is customarily called pterine or lumazine, respectively. The term pteridine comes from the Greek word "pteros", which means wings or feathers (Wieland and Schopf, 1925; Schopf and Becker, 1936; Wieland and Purrman, 1940; Schopf and Reichert, 1941). These pigments named so because of their discovery in the wings of butterflies for the first time by Hopkins (1889). Purrman (1940) reported that the pteridines have a structural similarity to that of purines, imidazo [4,5-d] pyrimidine. Chemical similarity between pteridines and purines has been established by Albert (1957) and Cresswell et al., 1965) and the metabolic similarity between purines and pteridines has been shown by Weygand and Waldschmidt, 1955; Aaronson and Rodriguez, 1958; McNutt, 1964. Various workers have shown that naturally occurring pteridines are synthesized from purine nucleot(s)ides (Weygand et al., 1964; Kidder and Dewey, 1968; Sugiura and Goto, 1968).

On the basis of these findings, close relationship between purines and pteridines with regard to the fundamental synthetic pathway and general chemical properties such as solubility, chromatographic behaviour and susceptibility to enzymes have been reported by some authors (Buchanan et al., 1948; Albert, 1954; Weygand et al., 1961; Stackhouse, 1966).

Since the discovery of the chemical structure of some naturally occurring pteridines such as leucopterin, xanthopterin, iso-xanthopterin (Purrman , 1940a,b; 1941), numerous pteridine

derivatives have been reported from the biological system. They may occur either as conjugated or unconjugated, mono- or polymers, oxidized or reduced, phosphorylated or as glucoside.

Ultrastructural studies indicate that pteridines are localized in pterinosomes (pteridine containing organelles) and in carotenoid vesicles (Bagnara and Taylor, 1970) which are deposited in the crystals that make up reflecting plates (Bagnara et al., 1988).

The colour of pteridines vary from the white leucopterin to the yellow sepiapterines or to the red dorsopterins (Bagnara, 1966). Sepiapterines comprise yellowish sepiapterine and isosepiapterine while red dorsopterines include dorsopterine, isodorsopterine and neodorsopterine. The colourless or leucopterines are generally divided into two groups such as blue or violet blue or violet fluorescent. They include biopterine, rana-chrome 3, xanthopterine and isoxanthopterine (Fujii, 1969). It has been suggested that the vastly different pigment cells are related to each other due to their similar origin from neural crest and can transform from one kind to another particularly in fishes and amphibians due to their common origin (Bagnara, 1988).

Pteridines and their derivatives have been reported to perform a variety of functions in the biological system.

Large amounts of pteridines are located in the brightly coloured pigment cells found in the skin, scale, pigment epithelium-choroid-layer and peritonium while exceedingly small amounts exist in tissues devoid of pigment cells (Hama et al., 1960a; Obika, 1963; Matsumoto, 1965a,b; Matsumoto and Obike, 1968; Matsumoto et al., 1969).

They have been reported from the eyes of some insects such as Drosophila, Calliphora, Ephestia etc. (Gregg and Smucker, 1964; Viscontini and Stierlin, 1961-1963) and are reported to perform the functions of screening pigments.

Most investigators concerned with animal pigmentation including pteridine have concentrated their efforts on the skin. But report on the occurrence of pteridine in the vertebrate ocular system is scarce. Pirie and Simpson (1946) reported a pteridine compound which they suggested to be xanthopterine in the choroid of Squalus acanthius. A fluorescent pteridine product was reported in the primate lens by Francois, Rabaey and Recoules (1961). Presence of pteridine has been reported in bovine and rabbit lenses, and in the cornea and retina of bovines (Cremer-Bartles, 1962). Pteridine has been detected in the iris of some birds (Oliphant, 1988).

With this view a study has been performed on the corneae and lenses of Cyprinus, Clarias and Stromateus with regards to the occurrence of pteridines and their possible functions have been discussed.

MATERIALS AND METHODS

Solubility Test

The principle of this method is based on the differential solubility of each pigment in various solvent system. Pteridines are easily soluble in aqueous alkaline solutions such as dilute ammonia (1% v/v) but are not readily soluble in organic solvents such as acetone, absolute ethanol, benzene, carbon tetrachloride, ether, petroleum ether etc. (Matsumoto et al., 1971).

The eye tissues (lens and cornea), for solubility test are fixed in a mixture of chloroform-ethanol (2:1 v/v) for more than two hours. The tissue thus prepared is embeded in paraffin following two changes in absolute ethanol and benzene. Relatively better tissue fixation has been obtained with a fixative containing 2 parts of formaldehyde or gluteraldehyde : 7 parts of 90% ethanol and 1 part of acetic acid. In this fixation, the dehydration is started with 95% ethanol and routine histological method is followed. The specimens thus prepared are examined after treatment with carbon tetrachloride, benzene, chloroform, ethyl ether or petroleum ether, for more than two hours in room temperature.

Chromatography

Separation and identification of pteridines from corneae and lenses have been carried out by paper chromatography. Paper

chromatography is the most commonly used technique because of its simplicity which also yields excellent separation (Matsumoto et al., 1971). Chromatography has been carried out with Whatman No. 1 filter paper in dark or in dim light because pteridines are photolabile. According to Matsumoto et al. (1971) light may cause the decomposition of sepiapterin, biopterin and most of 6-substituted pteridines into 6-carboxypterine and other unidentified pteridines. For chromatographic analysis fresh tissue samples have been either directly applied to the chromatogram or after suitable extraction using the following methods:

(1) Squash technique - This quick and convenient method has been developed by Hadorn and Mitchel (1951). After careful dissection the tissue samples-cornea and lenses were squashed directly on the chromatographic paper by firmly pressing with a clean microscopic slide. The squashed materials are then dried with an electric drier. The chromatogram is then developed in n-propanol : 1% (or 7%) ammonia.

(2) Quick extraction - It has been suggested by Matsumoto et al., (1971) that chromatographic separation of pteridines could be achieved from the extract itself. Here the cornea and lenses were separated from the eye and cut into small pieces and approximately 10 mg of each tissue sample was treated in a small glass vial with a few drops of absolute alcohol : 1% ammonia mixture (7 : 3 v/v). The sample is then warmed in a water-bath

at 70°C. The extract thus prepared is then applied to filter paper with the aid of a glass capillary tube. Chromatogram was run in a mixture of n-propanol : 1% (or 7%) ammonia (2:1 v/v).

Extraction with ethanol (Awapara's method) - This is a slightly modified extraction method of Awapara. The material was homogenized with a volume of absolute ethanol 20 times that of the fresh tissue in a glass homogenizer. The homogenates were diluted with re-distilled water to give a 70% ethanol solution. The ethanol solution was heated three times in an 80°C water bath for 20 minutes and then after adjusting its volume by adding absolute ethanol, it was centrifuged at 3000 rev./min. for 5 min. to remove proteins. The resulting clear supernatant was mixed with chloroform 3 times its volume and again centrifuged for 20 minutes at 3000 rev./min. The aqueous layer containing the fluorescent substances was separated from the mixture of chloroform-ethanol. The obtained extract was concentrated to 0.5 ml in vacuum and then chromatographed.

Extraction with trichloroacetic acid - For extraction, the tissue samples were homogenized 10% (w/v) TCA and extracted with a volume equal to the fresh tissue weight, in a water-bath at 70°C for 20 min. TCA was removed from the supernatant by washing 6 times with one-half the volume of ethyl ether, since TCA is an unfavourable contaminant in chromatography. The ether layer which was

separated by centrifugation was discarded at each washing to obtain finally an aqueous extract having an approximate pH of 3.5. The extract was then reduced to 0.5-1.0 ml *in vacuo*. The remaining ether in the extract was removed by treating them in a water-bath at 55°C.

Extraction by hydrochloric acid-hydrolysis - The materials were homogenized in a volume of 0.1N hydrochloric acid 20 times their fresh weight and hydrolysed for 24 hrs at 60°C. The resulting solution was then neutralized with ammonia and was then centrifuged. The supernatant is concentrated in a desiccator to analyse by paper chromatography.

One- and two-dimensional chromatography and rechromatography - The extracts were applied to the filter paper in the form of a spot for both one- and two-dimensional chromatography and in a narrow streak for rechromatography. Every paper chromatogram was subjected to one-dimensional separation first. According to Matsumoto *et al.* (1971), some pteridines overlap one another and for this they have stressed the importance of two-dimensional separation. Both ascending and descending techniques were tried in one-dimensional chromatography. The ascending technique was preferred for its better separation of pteridines in general, while descending method is suitable for the separation of pteridines with lower Rf values (Matsumoto *et al.*, 1971).

In the present investigation the ascending technique was carried out several times simultaneously with squash technique. The chromatogram was dried in the dark after each development. The combination for rechromatography was one run with n-propanol : 1% ammonia (2:1% v/v) and a second with iso-propanol : 2% w/v ammonium acetate.

In two-dimensional chromatography, pteridines first separated in one direction were subjected to further separation through the second development, made at an angle perpendicular to the first.

The position of pteridines were determined by examining the chromatogram with UV lamp and measuring the Rf values.

OBSERVATION AND DISCUSSION

The chromatograms following the examination under uv light reveal that the corneae of Cyprinus and Clarias contains highly photolabile fluorescent pteridines.

The carp cornea contains pterine and two unknown components while the lens possesses isoxanthopterine, cyprino-purple and one unknown fraction (Fig. 64).

The corneal pteridine compounds of catfish being 6-hydroxmethylpteridine and cyprino-purple while only one unknown component could be detected in the lens (Fig. 65).

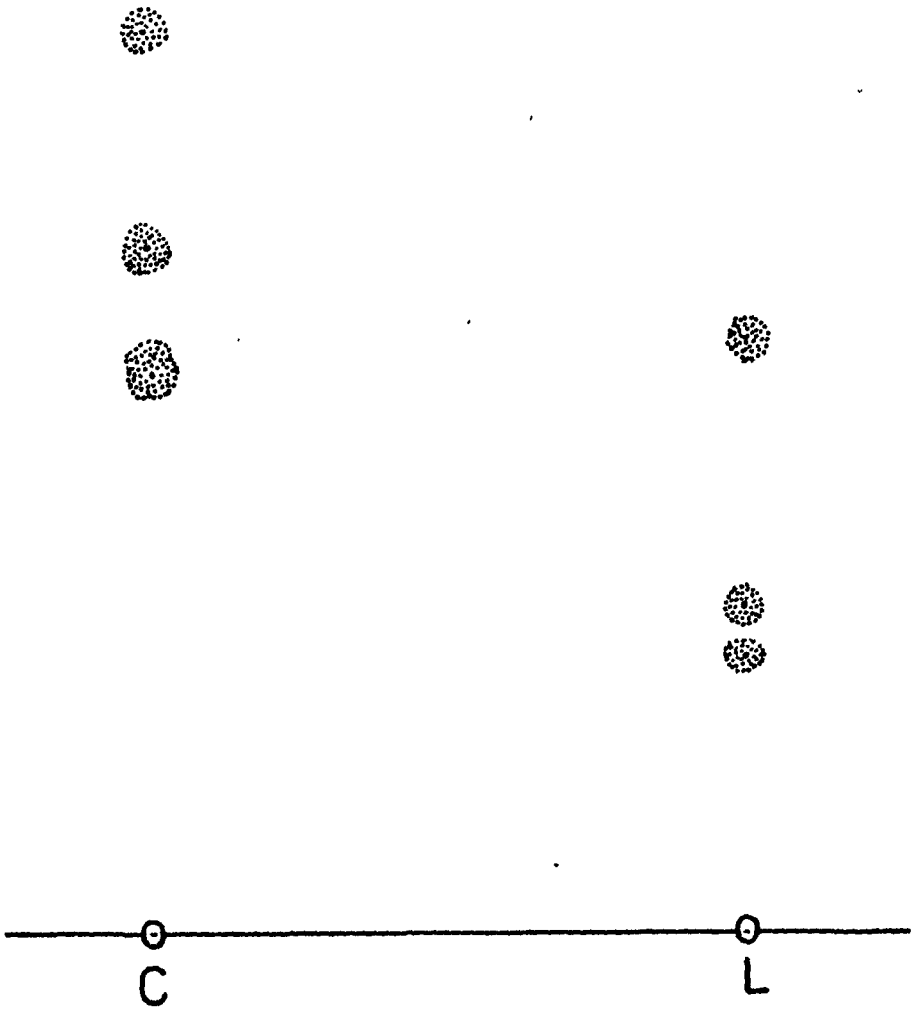
To have some knowledge regarding the retinal fluorescent compounds, the retinal extract of carp (Cyprinus) also was studied since melanophores too contain considerable amount of colourless pteridines (Hama, 1963; Matsumoto, 1965b). The chromatogram upon examination under uv light exhibited various pteridine compounds such as carboxypteridine, 7-hydroxybiopterine and two unknown fractions.

The cornea and lens of the marine fish Stromateus contains different fluorescent pteridine compounds with high Rf values, most of which are unknown in both the tissues, only one component in the lens appears to be pterine while none could be identified from cornea. It is worth to note that the cornea consists of atleast six (6) pteridine components as against three (3) fractions in the lens (Fig. 66).

EXPLANATION TO FIGURES

Fig. 64 Chromatogram showing the pteridine components in the cornea and lens of *Cyprinus carpio*.

C, cornea. L, lens.
Solvent : n-propanol : 1% ammonia (2:1w/v)

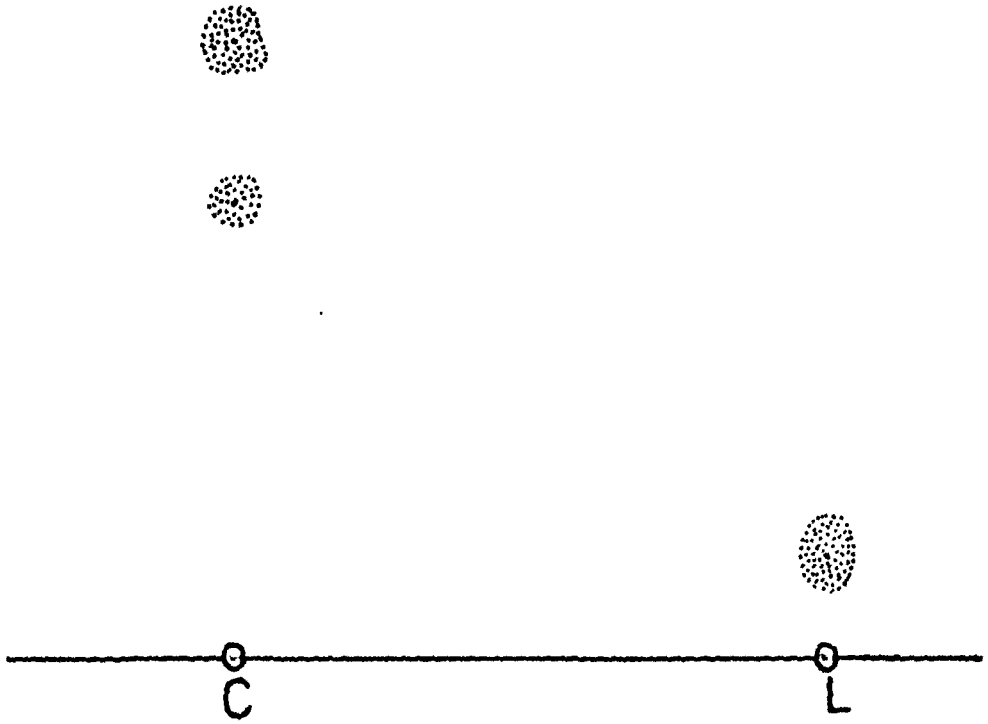

C
L

EXPLANATION TO FIGURES

Fig. 65 Chromatogram showing the pteridine components in the cornea and lens of *Clarias batrachus*

C, cornea. L, lens.

Solvent : n-propanol : 1% ammonia (2:1 v/v)


C
L

EXPLANATION TO FIGURES

Fig. 66 Chromatogram showing the pteridine components in the cornea and lens of *Stromateus argenteus*

c, cornea. L, lens.

Solvent : n-propanol : 1% ammonia

(2 : 1 v/v)

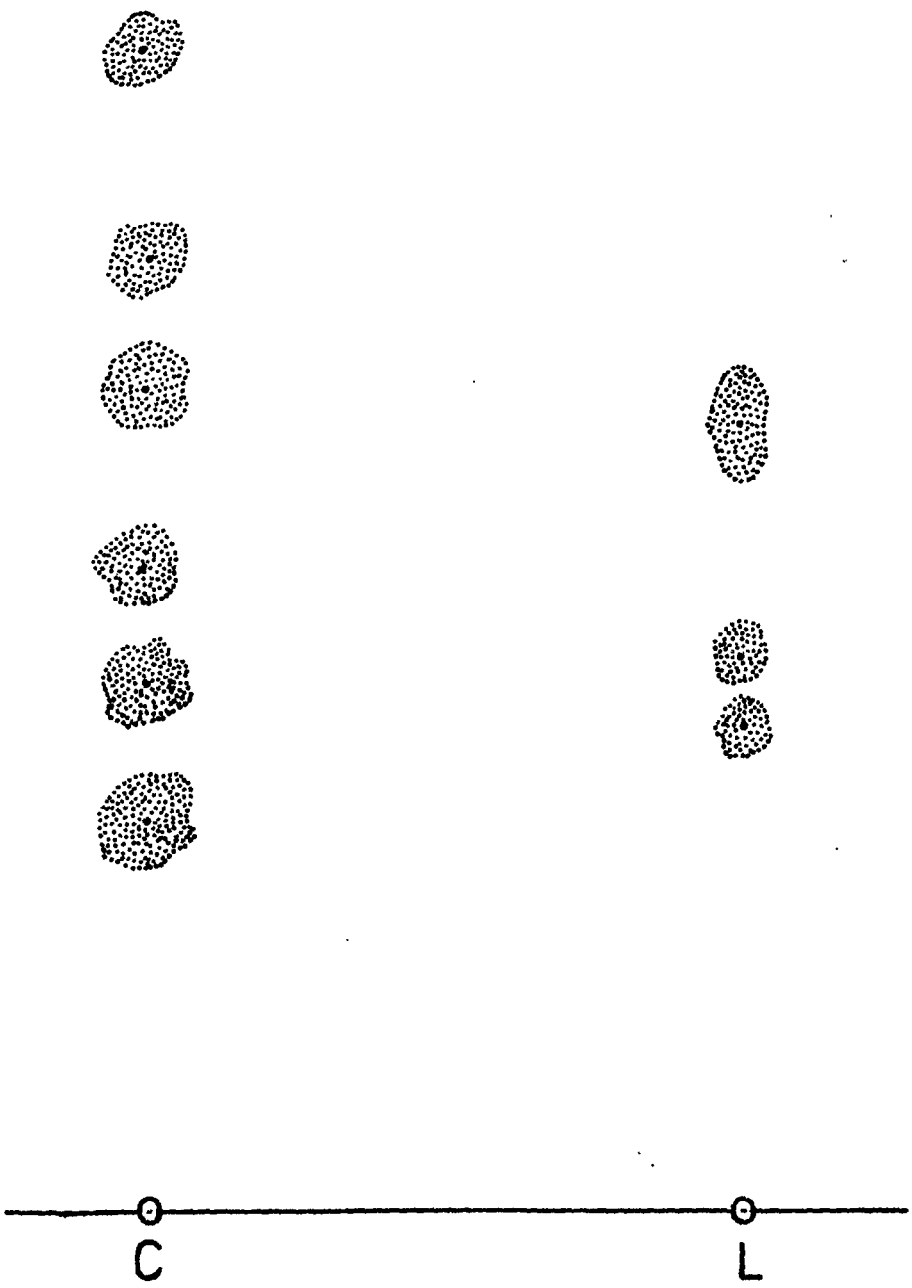

C
L

Table 7

Paper chromatographic detection of Pteridines in corneae and lens of Cyprinus, Clarias and Stromateus. Solvent : n-propanol : 1% NH_3 (2:1 v/v)

Fish	Ocular Tissue	Fluorescence colour	Rf value	Identification (Tentative)
Cyprinus	Cornea	Blue	0.44	Pterine
		Blue	0.55	Unknown
		Violet	0.67	Unknown
	Lens	Violet	0.23	Isoxanthopterin
		Violet	0.48	Cyprino-purple
		Blue	0.26	Unknown
	Retina	Greenish-yellow	0.11	Unknown
		Blue	0.17	Carboxpteridine
		Blue	0.29	7-Hydroxybiopterin
		Violet	0.34	Unknown
Clarias	Cornea	Blue	0.32	6-Hydroxymethylpteridine
		Violet	0.46	Cyprino-purple
		Violet	0.69	Unknown
Stromateus	Cornea	Yellow	0.29	Unknown
		Violet	0.41	Unknown
		Blue	0.51	Unknown
		Violet	0.62	Unknown
		Violet	0.68	Unknown
		Violet	0.9	Unknown
	Lens	Yellow	0.38	Unknown
		Blue	0.44	Pterine
		Violet	0.61	Unknown

However, all these pteridines which have been identified in these fishes are only on the basis of Rf values, hence tentative (Table 7). In this connection, it is worth mentioning that all the tissues of the eye in all cases, some photo-stable yellow fractions, probably carotenoid, could be detected as has been described by McFall-Ngai (1986).

Pteridines appear to be universal occurrence in living organisms (Hoar, 1987). It is known that pteridines and its derivatives perform a variety of functions in the living system. The conjugated pteridines are known to function as principal therapeutics for acute leukemy (Handschumacher and Welch, 1960). The conjugated pteridines act as coenzymes in the enzymatic synthesis of inosinic acid, serine, methionine etc. (Blackley, (1969). An important vitamin folic acid is pterine containing compound. Another important vitamin riboflavin seems to be closely related to the pteridines in its biosynthesis (Forrest, 1962).

It has been suggested that certain reduced pteridines such as sepiapterin, dihydrobiopterin and tetrahydrofolic acid take part in the hydroxylation of phenylalanine to tyrosine (Kaufman, 1969; Matsubara et al., 1966). Pteridines are also reported to be associated with lipid synthesis (Kidder and Dewey, 1963) and other physiological functions (Ziegler and Ziegler, 1965). However, amongst all the functions cited above,

the most significant role played by pteridines is that they act as pigment in the biological system. In this context, it is worth to mention that, in vertebrates pteridine synthesis is an autonomous xanthopore event (Bagner, 1983) and ptridines play significant roles as potential regulator of cellular events (Frost and Bagnara, 1979).

It is also of significance to mention that iridophores has been shown to contain pteridine (Bagnara et al., 1988). Iridophores are cells that participate in pigmentary phenomenon by virtue of their physical properties. They utilize crystalline deposits of purines in organelles called reflecting platelets that produce structural colours by means of an orderly arrangement of these organelles within the cell. Thus, the reflecting platelets may be elements used in reflection, light scattering, diffraction and interference (Menter et al., 1979).

During the last 40 years, pteridine has been reported in a variety of animals from lepidopterans to higher vertebrates, it has now become evident that these occur as red, yellow or orange pigments. Pteridines have been detected in the eyes of certain insects such as Drosophila (Viscontini et al., 1957, 1958 1959a,b; Gregg and Smucker, 1966), Calliphora (Ziegler, 1961c), Ephestia (Hadorn and Kuhn, 1953; Viscontini and Stierlin, 1961-1963) and some other insects, and in the integumentary pigment cells of lower vertebrates including fishes (Kaufman, 1959a;

Matsumoto et al., 1960, 1968, 1969, Zieglar, 1964; Matsumoto, 1965a,b; Hama et al., 1965); amphibians (Gunder, 1955; Hama and Obika, 1960, Obika, 1963; Bagnara, 1961, Richards and Bagnara, 1967) and reptiles (Hama and Fukuda, 1964).

Contrary to the above, reports on the occurrence of pteridine in ocular system are very few. Pteridine has been reported in the choroid of eyes of Squalus by Pirie and Simpson (1946), in the primate lens (Francois et al., 1961), in bovine and rabbit lenses (Cremer-Bartels, 1962).

The most distinguishing features of pteridines is their fluorescence under uv irradiation which range from violet to red depending upon concentration, pH and molecular species (Rauen and Stamm, 1952; Uyeda and Rabinowitz, 1963). The characteristic fluorescence of most of the pteridines has long been employed to detect these photolabile compounds.

According to Walls and Judd (1933a,b) yellowish pigment is an effective intraocular filter for blue light. The filters prevent the highly dispersive violet ray of the spectrum and thus increase the visual acuity. The pigments also protect the lens from near uv (Kuck Jr., 1970). It has been supposed that the fluorescence in the young human lens is meant to protect the retina against uv light. A protective fluorescent pigment, in addition to absorbing uv light, converts the light to a less harmful wave length by fluorescence (Kuck Jr., 1970). The total

light emitted in a fluorescent system always contains less energy than the light absorbed but it is possible for the emitted light to be brighter than the incident light of long wavelength (Clayton, 1970). The energy for emission of light in fluorescence comes from light of shorter wavelength (Lythgoe, 1979). McFall-Ngai et al., (1986), have suggested that the lens pigmentation may function to increase visual acuity by reducing chromatic aberration, glare and any scattering that may be caused by shorter wavelength visible light.

Nearly all of the pigments are so called cut-off filters, that is, wavelengths shorter than a certain value are absorbed whilst the longer wavelengths are transmitted or reflected (Lythgoe, 1979).

Cremer-Bartles (1962) described a photosensitive fluorescent substance, apparently a pteridine which occurs in the cornea, lens and retina. The behaviour of this substance in taking up phosphate suggests the interesting hypothesis that it may be a photodynamic substance in a system by which light energy is trapped in the lens for a useful purpose involving phosphate transfer. The investigator has also shown that the photo-labile fluorescent compound is synthesized in lens when incubated in light but decreased when incubated in dark. The compound is extremely photo-labile in a highly purified lens extract.

Lens fluorescence varies widely in various species (Kuck Jr., 1970) as well as within the same species (Matsumoto

et al., 1971). It has been shown that despite their common occurrence in a variety of species, each species exhibits more or less specific pteridine pattern in which each component is clearly defined (Matsumoto et al., 1971).

The significance of occurrence of pteridines in the compound eyes as screening pigments has been suggested by a number of authors (Goldsmith, 1958a,b; Langer and Thorell, 1966).

All these reports suggest that pteridines may play some significant roles in the visual system of fishes. But, as already mentioned that studies of pteridines in the vertebrate as well as in fish eyes have not been carried out much, and thus it entails further studies on the pteridine compounds in vertebrates in general and specifically in fishes.

C H A P T E R VI

VISUAL PIGMENTS

INTRODUCTION

The primary processes of photoreception takes place in two types of photosensitive visual cells - rods and cones in the retina. These elongated cells are oriented roughly in the direction of the incoming light. The chief site of light absorption is outer segments of the photoreceptor cells those contain the photosensitive visual pigments. The visual pigments absorb radiation of those wavelengths useful for vision (400-700nm) (Bridges, 1970).

The basis of photoreception is carotenoproteins or visual pigments. Visual pigments have been investigated in representative of each of the major phyla with specialized eyes. The active pigment in all cases is an aldehyde of vitamin A known as retinal or retinene combined with a protein, opsin. The aldehyde of vitamin A_1 with one single bond is $retinal_1$ or $retinene_1$ or rhodopsin results oxidatively from vitamin A_1 (alcohol with one double bond) and the aldehyde with two double bonds is $retinal_2$ or $retinene_2$ or porphyropsin originate from vitamin A_2 (alcohol with two double bonds) following its oxidation. $Retinal_1$ is also converted oxidatively to $retinal_2$ (Hoar, 1987).

Although there are only two known retinals, many different visual pigments have been categorized on the basis of their absorption and action spectra; the absorption maxima (λ_{max}) of

the known pigments of A_1 series range from 430 to 562 nm and from 510-620 nm in the A_2 series (Dartnall and Tansley, 1963). The differences are presumably caused by the opsin components of the molecule. However, a completely satisfactory explanation of the differences has not yet been found (Wald, 1960a; Dartnall and Tansley, 1963).

The literature on visual pigments is vast. Wald (1947, 1953) proposed the classical theory regarding rod visual pigments, the essential feature of the theory is that marine and terrestrial vertebrates have the visual pigment called rhodopsin and fresh water vertebrates have the more red-sensitive visual pigment called porphyropsin (Wald, 1936a,b; 1937,1938). The chemical basis of this dichotomy is that the rhodopsin has a prosthetic group or chromophore based on $retinene_1$ (aldehyde form of vitamin A_1 or retinol) while the prosthetic group or chromophore of porphyropsin is $retinene_2$ (aldehyde form of vitamin A_2 or dehydroretinol)(Wald, 1935a,b; 1939a,b). But critical analysis has revealed that the fresh water fishes contain a mixture of rhodopsin and porphyropsin (Dartnall, 1962), both of which differ in the chromophore (retinal or 3-dehydroretinal) and form either rhodopsin or porphyropsin by forming a bond with the same opsin (Beatty, 1984). The relative proportion of the two pigments even within a given species often varies, for example, on migration between the sea and freshwater (Wald, 1957; Beatty, 1966) with lighting conditions (Dartnall *et al.*, 1961; Bridges, 1965b).

Multiple rod pigments in a retina results from the combination of retinal or 3-dehydroretinal with different rod opsins (Beatty, 1984). However, multiple rod pigments are unknown excepting a few species (Munz and McFarland, 1975). On the other hand, many species of fish have dual rod pigments by combining retinal and 3-dehydroretinal with a species specific rod opsin to form a rhodopsin-porphyropsin pair (Bridges, 1972; Beatty, 1975). Furthermore, microspectrophotometry (MSP) has revealed that two pigments are present in the same rod (Liebman, 1972; Lowe and Dartnall, 1976; Tsin et al., 1981).

Though freshwater fish may have solely porphyropsins, only rhodopsin or mixture of the two visual pigments (Beatty, 1984), but there is evidence that a greater proportion of temperature zone freshwater fish has only porphropsin compared to tropical species. However, at least 50% of the species from each zone has a paired-visual pigment system (Schwanzara, 1967). Numerous studies of several paired-pigment freshwater and diadromous species have revealed that the scotopic visual system is not fixed but is highly labile and undergoes pronounced changes in the proportions of rhodopsin and porphyropsin in response to specific extrinsic and intrinsic factors (Beatty, 1984).

The visual pigment could be manipulated experimentally by altering the light environment. Seasonally, the highest proportion of rhodopsin occurred in summer and for porphyropsin,

it was winter, in the rudd (Sacardinus erythrophthalmus). Rudd kept in darkness had increased porphyropsin while those in bright outdoor light had increased rhodopsin (Dartnall et al., 1961).

It has been shown by Reuter (1973) that photoactive rhodopsin in isolated frog retina is converted to metarhodopsin II (λ_{max} 380 nm) and this in turn slowly converts into another 380 nm intermediate and a 470 nm pigment. Following Weale (1967), Baumann (1967) named the 470 nm pigment metarhodopsin III (but Wald, 1968, termed it pararhodopsin) and identified the 380 nm product as free retinal.

The freshwater fish crucian carp (Carassius carassius) retinal rods contain a pure victim A_2-based visual pigment porphyropsin with λ_{max} 522 nm (Dartnall, 1962; Bridges, 1967). This pigment upon bleaching yields only one long-lived yellow photoproduct (390-400 nm) which during the next few hours undergoes a slow decay to vitamin A_2 (3-dehydroretinol) and opsin in a temperature range of 7-22°C (Reuter, 1973).

No metarhodopsin III was observed in the retina of the fish, like frog retina. This difference between the bleaching patterns of crucian carp porphyropsin and frog rhodopsin does not, however, originate from the different chromophores, but rather from some characteristic of the opsin or peculiarity of the rod outer segments of this fish (Reuter, 1973). Donner (1973) has

established a link between dark adaptation and decay of the 400 nm photoproduct or metarhodopsin II.

Though ambhibians, freshwater, euryhaline and diadromous fishes contain a mixture of rhodopsin porphyropsin depending upon the habitat, life cycle etc. but bottom dwelling fishes mostly contain porphyropsin in their retina. However, pure porphyropsin type of eyes are rare among teleosts (mostly catfishes and centrarichids) (Hoar, 1987).

Wealths of reports of the visual pigments are available from various parts of the world but there is practically no report on visual pigments in this sub-continent, presumably due to various analytical constraints.

Keeping above in mind and despite the lack of adequate analytical facilities, an attempt has been made to study the visual pigments of common carp (Cyprinus carpio) and catfish (Clarias batrachus) both of which are bottom dwellers.

MATERIALS AND METHODS

Collection of Specimen

The carps (Cyprinus) were collected live from Umium lake (av. depth about 1.5 meters, with a water temperature ranging from 10°-12°C) during winter and early spring and transported to the laboratory in shallow plastic cages. The catfishes (Clarias) were collected from the market and kept in shallow cages in the laboratory.

In case of carps two groups such as fingerlings and adults and only adults of catfish were sampled for retinal pigment analysis. Some of the fishes were reared in laboratory for some days before extraction is made while some others were utilized immediately after collection.

Preparation of retinal extracts

The fish were dark adapted for about three hours and killed by decapitation in dark. Eunucleation and separation of retinas were performed under dim red light without any filter system. The retinae then hardened in 4% potassium alum solution (Beatty, 1966) and kept overnight in deep freeze. Some eyes were also kept overnight in light-proof vials in deep freeze for visual pigment regeneration (McFall-Ngai et al., 1986), then followed the usual process of extraction.

Following morning, the frozen retinae were thawed and the alum solution was decanted after light centrifugation. Then the retinae were washed twice or thrice with double distilled water and the supernatant again being discarded after light centrifugation (Beatty, 1966). The retinae, then either washed with phosphate buffer (pH 7.4) (Crescitelli and Liu, 1985; McFall-Ngai et al., 1986) in some studies or washed in McIlvaine's acid buffer (pH 4.6) (Muntz and Northmore, 1971).

Extraction of visual pigment in certain samples was carried out in freshly prepared 2% digitonin in phosphate buffer (pH 7.4) (McFall-Ngai et al., 1986). In some other sets, extraction was carried out in freshly prepared 3% aqueous digitonin solution (Muntz and Northmore, 1971). Overnight extraction was performed in room temperature for all sample. Multiple extraction was undertaken to extract all pigments in some cases.

Aliquots from each group was analyzed by spectrophotometric measurement (Jasco UVI DEC 610) either with the addition of 0.05 ml each of 10% by vol. sodium borate solution and 0.2M hydroxylamine followed by centrifugation at 17,000 rev./min. for 20 mins. and the supernantant thus obtained was analyzed (Muntz and Northmore, 1971) in some samples, while in other samples spectral absorbance was recorded without the addition of the above (McFall-Ngai 1986). The pH in all cases was maintained between 6.8-7.4 and the analysis was carried out in room temperature (16°-18°C).

In this connection, it is to be pointed out that all manipulations of retinae and recordings were performed in subdued light or twilight condition instead of dark room but in light proof containers.

RESULTS AND DISCUSSIONS

The results of our repeated studies, despite the limitations in analytical techniques indicate the presence of a product ranging between 400-404 nm (Figs. 67 A & B) in both the fishes (Cyprinus and Clarias). A similar product of bleaching of 400 nm has been found in crucian carp (Carassius carassius) retina by Reuter (1973) but the significance of which is yet to be ascertained.

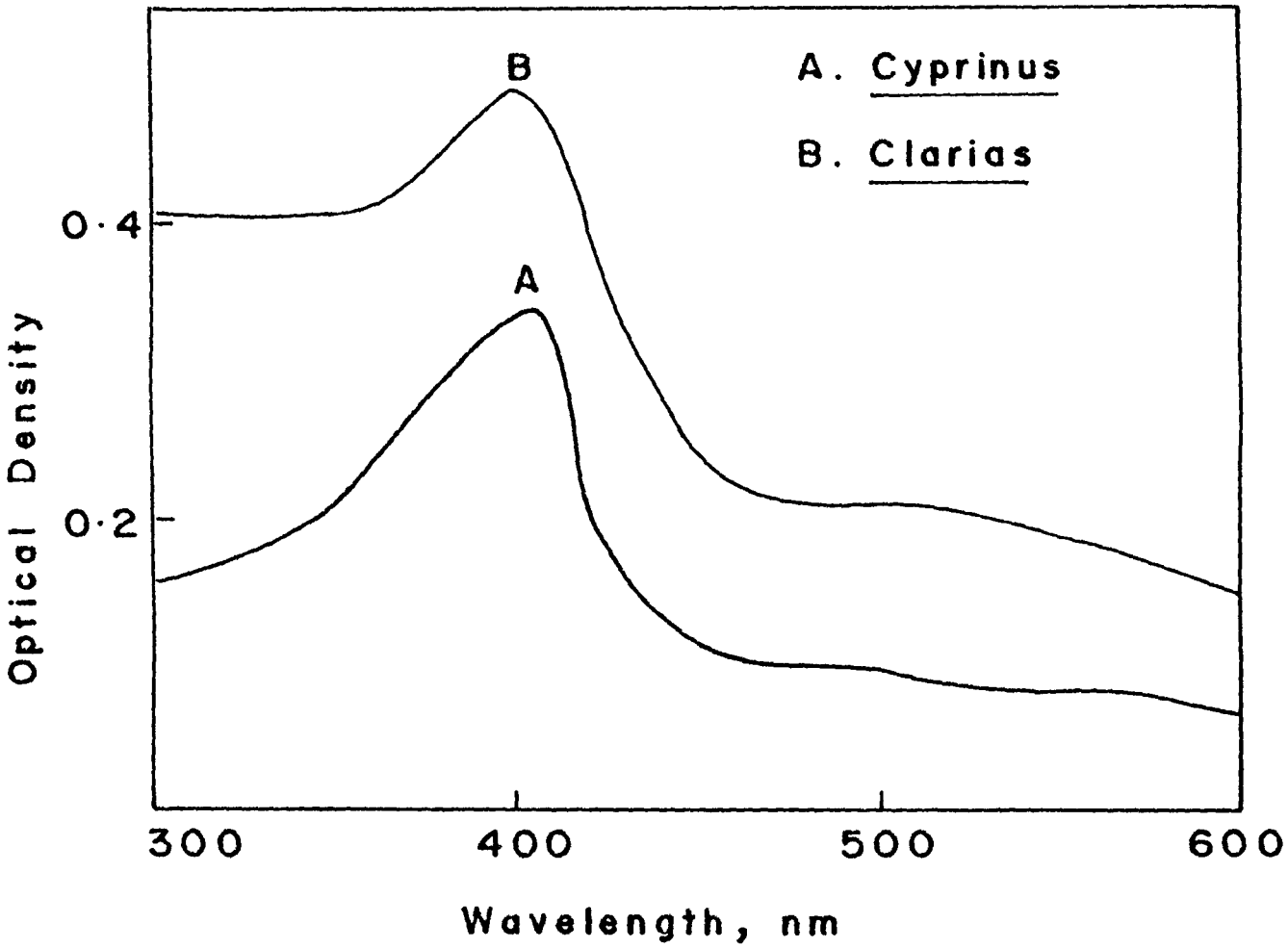
It is well known that the light sensitive materials that absorbs light to initiate nerve impulses are chemically very closely related both in vertebrates and invertebrates. These are visual pigments and occupy a pivotal role in visual physiology (Lythgoe, 1979). The visual pigments based on retinene$_1$ as rhodopsin and those based on retinene$_2$ as porphyropsin, this is on the basis of their origin in rods or cones (Muntz, and Northmore, 1971).

A_1-based pigments, usually paired with A_2-based pigment occur in surface-living fish whereas fish that live on the bottom usually have A_2-based pigment alone. For example, the British species of carp (Cyprinus carpio) yield a single pigment only based on vitamin A_2 (Dartnall and Lythgoe, 1965) like other bottom dwelling and feeding fishes.

EXPLANATION TO FIGURES

Fig. 67 Absorbance curves (A and B) of the visual pigments (photoproducts) in the retinae of:

A - Common carp, *Cyprinus carpio*, and
B - Catfish, *Clarias batrachus*


B
A
A. Cyprinus
B. Clarias
0·4
0·2
Optical Density
300
400
500
600
Wavelength, nm

Muntz (1973) while studying visual pigment of four species of siluroid fishes (catfishes) found a pronounced absorption maxima at short wavelengths (between 420 and 440 nm) which was photostable and did not contribute to difference spectra. According to Dartnall (1968), the A_2-based pigments are less photosensitive than A_1-based pigments.

The spectral characteristics of water vary widely and in many cases the maximal absorption of the visual pigments of fishes coincides with the wavelengths of the light that prevails in their environment (Denton and Warren, 1956, 1957; Munz 1957, 1964). According to Wald (1960a) all visual pigments are based on 11-cis retinaldehyde or its 3-dehydro derivative in conjunction with the appropriate opsin and all -trans isomers emerging during photic bleaching. The absorption band of retinaldehyde is centred at about 380 nm and that of 3-dehydroretinaldehyde at 400 nm. From each arises a series of pigments due to the interactions between retinaldehyde and opsin and thus there exist many rhodopsins and prophyropsins (Bridges, 1970).

As to the long lasting 400 nm photoproduct in the crucian carp retina, it corresponds to the absorption of metarhodopsin II and also to the free retinal$_2$ (Donner, 1973). However, a positive identification of this substance with metaporphyropsin II or free retinal$_2$ could not be made since the photoproduct did not get converted immediately to other pigments upon irradiation to near u-v light but a small fraction of the

400 nm product converted to porphyropsin and isoporphyropsin while most of it decayed to vitamin A_2 (Reuter, 1973). According to Donner (1973) all the bleached porphyropsin molecule in crucian carp occur as the 400 nm product and presumably causes all rod desensitization.

Wald (1973) and Donner (1973) suggested that this 400 nm pigment in the isolated retina is converted finally into retinol$_2$ and into porphyropsin in living animal or open eye. Again, the 400 nm product has been termed as "free" 3-dehydroretinal by Reuter (1973) while Abrahamson (1973) and Bonting (1973) did not consider the 400 nm intermediate as retinal$_2$ but a true analog of metarhodopsin II (380 nm) and a 386 nm is 3-dehydroretinal oxime (Beatty, 1984).

It has been suggested that the 380 nm product or photoproduct (in the frog retina) in general are regulating dark adaptation (Donner and Reuter, 1966,1967,1968). The authors also concluded that there exist a casual relationship between rod sensitivity and the presence of M II (metarhodopsin II) or M II - like photoproducts.

The rhodopsin-porphyropsin ratio in many fishes changes seasonally, the proportion of porphyropsin being higher in winter than in summer (Allen et al., 1973), this is because of change in the seasonal light intensities (Dartnall et al., 1961). Diet can affect the visual pigments (Jacquest and Beatty, 1972). The proportion of these two pigments also vary on salinity of

the environment (Wald, 1957; Beatty, 1966), age of the fish, administration of thyroid hormone (Beatty, 1969; Allen, 1971), prolactine (Jacquest and Beatty, 1972, Allen, 1977).

In principle, any opsin can combine with either retinene and in this way a pair of visual pigments can be formed (Munz, 1971). The spectral absorption of the visual pigments is influenced depending on either chromophoric groups (retinal or dehydroretinal) or on the linkage between the chromophore and the opsin. There may be a score of opsin types, each of which can bind to either retinal or dehydroretinal. Thus, about 40 different visual pigment species can be obtained (Lythgoe, 1979). Moreover, microspectrophotometry has extended the range of λ_{max} values of the retinal-based visual pigments in fish at least from 420 to 565 nm and that of the 3-dehydroretinal-based visual pigments at least from 455 to 625 nm (Loew and Lythgoe, 1978; Levine and MacNichol, 1979).

Despite considerable speculations no completely satisfactory theory has yet been advanced as to the functional significance of these variations (Muntz, 1973).

In fishes and in many other animals as well (Muntz, 1971), a wide variety of spectral sensitivity curves may be obtained depending upon the experimental conditions. The visual system of the animal apparently analyses incoming information in different ways according to circumstances, which means that

the data obtained under one set of conditions cannot be used with any confidence when the conditions are changed (Northmore and Muntz, 1973). In this connection, it is worth to mention that the site of our collection falls under sub-tropical, high rainfall and humid climatic area.

Thus, it seems from the above that the 400-404 nm product which has been obtained in the present investigation could be a photoproduct. Since, there is no report on the fish visual pigments in this country, hence it is difficult to conclude anything and requires further studies.

GENERAL DISCUSSION

Photoreceptor organs though differ morphologically but operate as transducer of light energy into membrane potential or nervous action. The photoreceptors, the eyes of fishes are of considerable interest in elucidating visual system of vertebrates. The fishes are active day and night and their eyes are curiously adjusted to habitat, activity rhythm and behaviour (Hoar, 1987).

Moreover, teleost fishes are capable of orienting themselves with respect to polarized light, since light becomes polarized in the aquatic medium (Waterman, 1975b; Brines and Gould, 1979). The occurrence of this ability indicates that the visual machinery has the capability to acquire sensitivity to the plane of polarization and signifies an evolutionary advantage (Hoar, 1987). This suggests the existence of a polarized light analyser within the eye of the fish. Thus to understand the functioning of the eye in response to light wave, it is important to study its structure and bio-chemistry.

In the present investigation, some attempts have been made to study certain aspects of fish ocular system histochemically and biochemically and some interesting observations have been made.

While studying histochemistry and biochemistry of the cornea and lens of common carp Cyprinus carpio, catfish, Clarias batrachus and pomfret, Stromateus argenteus, a significant

component such as protein-polysaccharide complex, "acid mucopolysaccharide" has been detected. Mucopolysaccharides have been detected in various tissues and a number of roles have been assigned to them.

Amongst the ocular tissues much importance has been given on corneal mucopolysaccharides for its polydisperity and chemical heterogeneity and thus unique amongst all connective tissues (Maurice and Riley, 1970). Relationship between mucopolysaccharides and macromolecular composition and swelling nature of cornea of elasmobranchs and teleosts as well as other vertebrates and invertebrates have been studied by various authors (Robert and Schillinger, 1967; Moczar and Moczar, 1970c, 1973; Moczar et al., 1969a,b; Cejkova and Bolkova, 1970, 1973 and 1974). Moczar and Moczar (1973) have shown the evolutionary changes of macromules in cornea in relation to differentiated connective tissue. Smelser and Chen (1955) studied hydrophilic nature of mucopolysaccharides in carp and mammalian cornea. Mayer et al., (1953); Laurent and Anseth (1961) studied polydisperity and chemical heterogeneity of mucopolysaccharides in ox cornea. Robert et al., (1965b) with a view to elucidate chemical nature of cornea isolated various fractions of mucopolysaccharides in calf and rabbit cornea.

Anseth (1961a) studied the swelling property of cornea of some teleosts and other vertebrates in relation to glucosamine and galactosamine content and suggested that glucosamine and galactosamine ratio might be the critical factor in fish cornea which show limited swelling compared to mammals. In view of intimate relationship between acid polysaccharides and proteins as well as hexosamine synthesis in developing tissues, it may help to understand the role of polysaccharides and glycoproteins in determining the different structure of connective tissues (Maurice and Riley, 1970).

The protein polysaccharide complex is present at the interfibriler surfaces of the lens (Kuck Jr., 1970) and also control the growth and differentiation of cells and fibres in different parts of the lens (Dische, 1965c). Mucopolysaccharides serve as interfibriller cement substances in the lens necessary for optical functions (Bellows, 1944).

Murray (1988) has suggested that glycosaminoglycans are the structural molecules of collagen, serves as lubricant and protective agents; they transport minerals and trace elements. These are also responsible for cell to cell attachment and communication. They also appear to serve as receptors and carriers for macromolecules, regulate cell growth and have effects on protein synthesis and intra-nuclear functions.

All of the glycosaminoglycans are polyanions, since they have acidic sulfate or carboxyl groups of uronic acids present throughout their structures. Many of their functions result from this particular characteristic. Moreover, the binding between glycosaminoglycans and other extracellular macromolecules is electrostatic in character because of its polyanionic nature. This binding contributes significantly to the structural organization of connective tissue matrix (Murray, 1988). Thus, it is reasonable to presume that mucopolysaccharides in these ocular structure may play some significant roles in the visual physiology of the fishes. Since in the present investigation, studies on the various aspects of fish vision have been performed, it has not been possible to study the mucopolysaccharides alone of the fishes in details. Thus, it would be of great significance, if the mucopolysaccharides are studied in details and to find out whether the function is mechanical or optical or both. Further, it would be even more important if the relationship between vitamin A and mucopolysaccharides synthesis is investigated which could not be studied in the present investigation.

Another interesting observation made on the cornea and lens of fish is the study of the enzyme adenosine triphosphatase (ATPase). This enzyme enhances the metabolism to supply energy for visual purpose through cation transport which also maintains ionic balance and has many metabolic

functions (Bonting, 1965,1969 and 1970). The Na^+-K^+ activated ATPase maintains the water balance or hydration of the ocular tissues (Dikstein and Maurice, 1969) and thus maintain their transparency. The role of Na^+ - K^+ stimulated ATPase of cell membrane in active cation transport has been well established (Skou, 1965). Thus, it is clear that this enzyme might be responsible in the visual process through cation transport by the way of influencing osmoregulation.

It is known that ATPase system is responsible for glucose transfer, hence it would be worthwhile to study if there exists any relationship between this enzyme and muco-polysaccharide synthesis.

In the present investigation, detection of ascorbic acid in the cornea and lens of fish, is another important aspect. Ascorbic acid, as already mentioned in the relevant section, may play some vital role in energy generation in the fish photoreceptors. In addition to this, which can be mentioned here is that ascorbic acid may have some role in mucopolysaccharide synthesis which is present in the cornea and lens. Evidence for and against ascorbate stimulation of sulfated glycosaminoglycans are found in the literature. Human skin fibroblasts in tissue culture produce more sulfated polysaccharides in the presence of ascorbate (Dische, 1947).

Kafoed and Robertson (1966), on the other hand, have suggested that ascorbate is not required for chondroitin sulfate synthesis in the tracheal cartilage of guineapig. Lavietes (1971) has shown that the presence of ascorbate in the culture medium does effect the distribution of chondroitin sulfate synthesized.

With these reports in view, it can be suggested that, it will be of significance to study whether there is any such relationship between mucopolysaccharides and ascorbate in the fish ocular system. However, role of ascorbic acid in collagen synthesis is established and collagen is an essential constituent of cornea where mucopolysaccharides remains in association with collagen.

An attempt has been made in the present investigation to study the retinal pigment (melanin) migration in response to various factors. It has been observed that intraocular administration of 5-hydroxytryptamine, colchicine, and cyclic AMP in dark adapted eyes stimulate pigment migration similar to light adapted state. In addition to this, it has been observed that neurosecretion may play some significant role in pigment migration leading to visual adaptation during various photic stimuli. But, inspite of all these it is not possible to predict definitely which factor is responsible or how this is effected.

Fluorescent substances in the fish eyes is another aspect where some interesting observations have been made.

Presence of the fluorescent compound, pteridine in the eyes of Cyprinus, Clarias and Stromateus is significant in terms of visual phenomenon and certainly serve as screening pigments to filter the harmful ultraviolet rays.

Another significant attempt in the present investigation is the study of visual pigment (retinene) in Cyprinus and Clarias. In this connection, an important observation in Indian context has been made by extracting a pigment (400-404 nm) in both the fishes. But nothing is possible to conclude regarding the nature of the pigment. However, this attempt is the starting point in this direction for future investigations.

Considering all these, it can be concluded that much research still remains to be done before one can completely understand how eyes are structured for accomplishing such a complicated physiological phenomenon of vision in different ecological nisches.

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