A BIOCHEMICAL AND PHYSIOLOGICAL STUDY ON SOME VISUAL MECHANISMS IN THE BUTTERFLY, *Pieris brassicae* AND THE MOTH, *Philosamia ricini*.

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BY

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CERTIFICATE

This is to certify that the thesis entitled "**A biochemical and physiological study on some visual mechanisms in the butterfly, *Pieris brassicae* and the moth, *Philosamia ricini***" submitted to Nagaland University in fulfilment of the requirements for the degree of Doctor of Philosophy in Zoology, is an original research work carried out by Ms. SENTIMENLA, Registration No. 236/2006 under my supervision and guidance.

Further, certified that no part of this thesis has been submitted anywhere for any other research degree.

Place: (BENDANG. AO)

Date: Supervisor

DECLARATION

I, Ms. SENTIMENLA, hereby declare that the subject matter of this thesis is the record of work done by me, that the contents of this thesis did not form the basis of award of any previous degree to me, or to the best of my knowledge, to anybody else, and that the thesis has not been submitted by me for any research degree in any other university/Institute.

This is being submitted to Nagaland University for the degree of Doctor of Philosophy in Zoology.

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GENERAL INTRODUCTION

Vision is a photo-physiological phenomenon by means of which an organism sees objects around it. The two most important factors involved in the process are light and photoreceptors - the organs which are capable of absorbing light. The electromagnetic spectrum extends from the cosmic and gamma rays with wavelength of only a ten-billionth of a centimetre to the radio waves, which may be miles in length and light is a narrow band within this, with wavelengths extending from 380nm to 760nm with extreme limits of 310 to 1050 nm in very intense artificial sources. Photoreception in all animals is covered by or within the extreme human range (Hoars, 1987).

The eyes are photoreceptor organs capable of absorbing light and also act as transducers by converting the energy of electromagnetic radiation into nervous impulses. In the animal kingdom, there are varieties of photoreceptor organs - the most primitive type of photoreceptor being the eye spot of *Euglena*, capable of detecting only the presence or absence of light, while more complex ones are found in metazoans which are capable of forming images on the photoreceptor surface.

Among the photoreceptor organs of animals, the compound eye of arthropods has attracted many vision physiologists because of its unique structural organization. They are image - forming eyes capable of determining the plane of polarized light. The lens (refractive elements) focus light from the objects, forming an inverted image on the retinal surface and the animals can use such information for orientation or directed locomotion. Thus vision can also be defined as the perception of light.

Insects perceive light through three classes of sensory organs. *viz*: (i) compound eyes, (ii) stemmata such as those present in primitive eyes of beetle

(Coleoptera), sawfly (Hymenoptera), Neuroptera, Trichoptera and catepillar (Lepitoptera larvae), and (iii) ocelli which are present on the dorsal aspects of the head of many adult insects. Ocelli and stemmata are also called simple eyes.

Compound eyes are prominent features of adult insects although they are reduced or absent in parasitic forms, many soil insects and in some species that live in very dark places such as caves. The eyes occupy a fairly large portion of the surface of insect's heads facilitating a rather wide field of vision. The compound eyes of adult insects are packed together as a hexagonal array, with each individual facet and its underlying ommatidial unit aimed at a different spot in the visual field. The eyeless (ey) gene is responsible for production of the compound eye of insects. A homologous gene in mammals performs the same function (Halder *et. al.* 1995).

The basic unit of compound eyes is the ommatidium. Ommatidia vary in size and number among insects groups. For example, dragon flies have thousands of ommatidia numbering between 10,000 to 28,000 or more in each compound eye, while the worker of the ant species *Pomera punctatissima* has only one ommatidium in each eye (Wolken, 1968). The size of ommatidia also vary, usually measuring about 5 to 40 microns in diameter among species and even within a single compound eye. In some dragon flies, the dorsal units are considerably larger than the ventral ones. The external appearance of the eye thus reveals the exact location in each ommatidium as indicated by the individual facets.

Compound eyes or ommatidia consist of a lens system, a retina and underlying optic ganglia. The lens system is called the "dioptric" and is

composed of a cornea and underlying crystalline cone structure that together make up the optical part of ommatidia, and conduct light into the centre of the optical axis of each ommatidium. Cornea is the transparent area of the cuticle and is a specialized part of the insect cuticle. It is more or less bi-convex and is cast off during each act of ecdysis (Imms, 1957). If the crystalline cone is replaced by a cuticular invagination such as in lampreys and beetles, it is termed as "eucone"of pseudocone eye.

Immediately behind the crystalline cone are the longitudinal sensory elements or the "retinula cells". The number of retinula cells is not constant in all species. In many ommatidia, the number of retinula cell is 8 *e.g. Apis* sp, in certain moths they are 10 to 12 in number (Imms, 1957), in certain cases the number is reduced to 6 or 7. The retinula cells collectively secrete an internal light - trapping rod like structure known as rhabdom consisting of a dense array of microvillar membranes.The portion of the rhabdom contributed by each retinula cell is known as rhabdomere. Rhabdomeres consists of thousands of closely packed tubules which are about 500^0A in diameter and one micron long. They are aligned at right angles to the long axis of the rhabdomeric microvilli. Either the rhabdomeres are positioned to form a contiguous central rhabdom called as "closed" rhabdom such as in honey bee, or separated in an open structure as in Drosophila.

In addition, the ommatidia also contain a number of densely pigmented cells known as "primary iris cells" which surround the crystalline cone and cornea. Primary iris cell and the retina cell in turn are surrounded by a second group of elongated pigmented cells, known as 'secondary iris cells". These pigmented cells serve to isolate one ommatidium from the next. They also act

as cylindrical diaphragms for adaptation to light and dark conditions, which is accomplished by pigment migration in specialized cells alongside the ommatidia.

Compound eyes are commonly categorized as either “apposition” eyes or “superposition eyes”. Apposition eyes are also known as photopic eyes and characteristically seen in diurnal insects. Apposition eyes are the most common type and are present among all the apterygota and exopterygota (ametabolous and hemimetabolous insects). The holometabolous - the so called advanced order of insects, possess apposition compound eyes, or specialization from these. The rhabdom is long and thin and lies directly beneath or against the crystalline cone. A double layered sheath of pigment cells surrounds each ommatidium, and thus it is optically isolated from its neighbour. Light falling upon the lens of an ommatidium can only reach the rhabdomeres of that ommatidium. During light-dark adaptation there is little or no longitudinal migration of the screening pigments. But in some cases there is retinal movement of the screening pigments around the rhabdom due to change in the condition of illumination (Walcott, 1974). There is no separation in the corneal layer and the photoreceptors in apposition eyes. The crystalline cone couples the lens and the photoreceptors in the apposition eyes.

Superposition or scotopic eyes are those in which short and broad rhabdoms lie some distance away from the crystalline cone. A marked longitudinal migration of the screening pigment during light-dark adaptation is a characteristic feature of this type of eye. During conditions of bright illumination, the pigment granules migrate inwards and extend the full length of the ommatidium. At dark, however, the pigments move upwards and

condense distally between the crystalline cones. There is a clear space between the two units in superposition eyes. In superposition eyes the space between the corneal lines and the photoreceptors is transversed by crystalline tracts. These are thought to act as wave guides that direct light to the photoreceptors. Some authors use the expression “clear zone eyes” for the superposition eyes. Superposition eyes are found in nocturnal and crepuscular insects (Dethier, 1963). Night flying species like beetles and moths, for example have compound eyes with larger ommatidia or wider clear zones than daytime-active species (Caveney and Mcintyre, 1981; Jander and Jander, 2002; Moser *et. al.* 2004).

Some higher Dipterans have “pseudocone eyes”. Crystalline cones do not occur in pseudocone eyes, and optical coupling is by means of a gelatinous substance that is contained in a two celled structure. Still another arrangement is found in some apposition eyes that lack solid cones or gelatinous pseudocones. In these “acone” eyes flat transparent cells are found in place of the cones.

Light sensitivity of insects vary according to the state of light adaptation or dark adaptation of the eyes. In bright light, the eyes are less sensitive to light, and the eye is regarded as light adapted. Maximum sensitivity occurs in darker conditions, when the eyes are fully dark adapted. Some insect’s eyes can change light sensitivity by about three orders of magnitude within a few minutes of changing light conditions (Autrum, 1981).

There are a number of mechanisms of adaptation to light and dark conditions. One mechanism relates to biochemistry where the visual pigment is broken down by interaction with light. In daylight the breakdown rate can

equal or exceed the replacement rate. This leads to decreased light sensitivity, and partly explains light adaptation. In the dark, the visual pigment accommodates, and the insects become dark adapted.

A second mechanism of adaptation is the movement of screening pigments, found in the retinula cells. In light-adapted eyes, granules of screening pigments move and surround the rhabdom. These pigments absorb light, and they have the effect of optically isolating individual ommatidia. Moreover the endoplasmic reticulum of dark-adapted eyes forms large, clear vesicles which form a clear space around the rhabdom, so that a greater amount of light falls upon the rhabdom.

Stemmata can consist of clear cuticle shaped as an optical lens above a single ommatidium-like photoreceptor structure or alternatively the stemmata can have a large number of photoreceptors. The larvae of holometabolous insects have stemmata on the side of the head capsule. Many larvae have a single stemma (singular of stemmata) on either side, but the number can be as many as six on the side. Stemmata generally do not produce clear images, but most caterpillars can discriminate some shapes and they can orient themselves with respect to boundaries.

Ocelli occur in larvae of hemimetabolous insects and in nearly all adults. Although there are variations in structure, a typical ocellus has a single lens that is usually rather thickened. Rhabdomers of several cells combine to form a number of rhabdomeres. Most ocelli feature a large number, often hundreds of retinula cells. Axons project from the retinula cells through the basement membrane and they end in a synaptic plexus behind the eye. Light cause a sustained depolarization of the retinula cells, however the biological

roles of ocelli remain unknown. It is generally agreed that ocelli allow only poor, if any perception of forms. In some orthoptera the ocelli are active in orientation to the light source.

Dorsal ocellli are constructed according to the same basic pattern as stemmata, except that the photoreceptors are much larger in number. The number of ocelli varies among different insects, with an elaborate retinal structure and also appears to have diverse functions *e.g.* they contribute to flight steering.

To explain the function of the compound eye of arthropods, Johannes Muller (1829) formulated the classical theory known as “Mosaic theory”. According to this theory, each ommatidium receives light from its own lens. Light falling on the lens of the ommatidium cannot reach the adjacent one because of the presence of pigment sheath. Thus, an image of the limited part of the visual field is produced by which each ommatidium and the entire eye forms the image from the reports from individual ommatidia.

It is seen from a review of previous works on the subject, that there is a need of information on certain aspects of arthropod vision. To give an example, the lens which covers the ommatidium of the compound eye is a modified cuticle. It is well known that the nature of the cuticle of insects and other arthropods varies in different regions of the body which perform various types of functions (Richards, 1972). It is also evident that the chemical nature of cuticles involved in various functions may vary (Wiglesworth, 1965). Since lens performs a special optical function of conducting light rays to the rhabdomeres besides playing a general defensive role, it is expected that the lens-cuticle may differ in chemical nature and ultra structure from the cuticle

covering the other part of the body. But the chemical composition of the lens in relation to its special function has not been studied in detail. In a similar way there are many other aspects worth studying.

Substances with similar physico chemical properties isolated from connective tissue are termed as mucopolysaccharides (Meyer, 1938). They are of two main classes - neutral mucopolysaccharides and acid mucopolysaccharides. The latter may be further of sulphated or non sulphated types (Meyer, 1938; Jaques, 1977). Acid mucopolysaccharides have been detected in many vertebrate and invertebrate tissues (Berenson *et. al.* 1966; Wagh and Roberts, 1972; Radhakrishnamurthy and Berenson, 1973; McCullagh *et. al*. 1973; Dey, 1980 *etc*.), in the eyes of fishes, and birds (Deb, 1990, and Bendang, 1998), and also in the compound eyes of some insects, such as *Musca domestica*, *Apis cerana* and *Periplanata americana* (Dey, 1980). In addition, various authors have also reported on their biological significance, for instance, their role in the structural organization of intercellular matrix (Kobayashi and Pedrini, 1973), movement of metabolites (Jeanloz, 1970), maintenance of tissue osmotic pressure (Ogston and Wells, 1972), protection of cornea (Robert and Robert, 1967), maintenance of corneal structure, transparency and function (Anseth and Fransson, 1970; Takahashi *et. al.* 1993; Freund *et. al.* 1995; Funderburgh *et. al.* 1996; Fischberg, 1997). In case of the lens Roquomore *et. al.* (1992) have suggested that the vertebrate lens crystallins are responsible for establishing the remarkable optical properties of the lens. Lampi *et. al*. (1997) and Feng *et. al.* (2000) have reported that major portions of the human lens is crystalline. In

the view of the above, the present work attempt to find the presence of acid mucopolysaccharides in the two insects and its role with regards to vision .

An interesting work undertaken in the present study is the integration and the co-ordination of vision and neural processes. It is well established that rods and cones as well as visual pigments move in a well-coordinated manner in response to, or as adaptation to different photic and physiological states (Barlow, 1972; Lythgoe, 1979; Lamb, 1981 and 1990; Tamura *et. al.* 1989, 1991; Donner *et. al.* 1998; Thomas, and Lamb, 1999; Fain *et. al.* 2001). These movements may be due to electro chemical changes, perhaps mediated through intracellular Ca^{++} (Ishibashi, 1957; Kinosita, 1963; Matthews and Fain, 2001), or may be due to ionic exchanges between the cell exterior and interior (Lerner and Takahashi, 1956), or may be due to the involvement of microtubules (Novales, 1959; Warren and Burnside, 1978). Bendang *et. al.* (2004) have shown that colchicine reverses the dispersion pattern of pigment granules, thereby proving that microtubules are involved. The role of hypothalamic neurosecretion in integrating various physiological events is well documented (Geris *et. al* . 2002; Sharp and Sreekumar, 2002) and many other workers have suggested a co-ordination between neural and hormonal mechanism in lower vertebrates (Osborn, 1938; Scharrer, 1952; Ali, 1964; Fujii, 1969; Davson, 1970). Thus the present work attempts to find a connecting link between the neurosecretory status in the neurosecretory cells of the brain and the physiological adaptation of the eyes.

Fluorescent compounds *i.e.* the pterines and pteridines appear to be of universal occurrence and perform a variety of functions in biological systems such as absorbing and converting high frequency light energy to that of a

lower frequency. They originate in pigment cells or *chromatophores*. These pigments are usually bright in colour, either alone, or together with other kinds of pigments such as carotenoids (Matsumoto, 1965). They function in scattering of reflected light (Bagnara *et. al.* 1978) and also act as filters (Lythgoe, 1979). Most investigators concerned with animal pigmentation have concentrated their effects on the skin and scales (Matsumoto, 1965; Matsumoto *et. al.* 1971; Melber and Schmidt, 1992 and Zieglar *et. al.* 2000). Pterines and pteridines have also been reported in the eyes of some insects such as *Drosophila* (Gregg and Smucker, 1965), *Musca* and *Apis* (Dey, 1980). McFall Ngai *et. al.* (1986) have suggested that lens pigmentation of the deep sea hatchet fish *Argyropelus affinis* may function to increase visual acuity by reducing chromatic aberration, glare and scattering that may be caused by shorter wavelength visible light. Pteridines have been detected in the eyes of birds where these fluorescent compounds act as screening pigments to filter the harmful ultra violet rays and thus protect the eyes (Oliphant, 1988 and Bendang, 1998). In view of the above reports, the present work attempts to study the florescent compounds of the eye and their role in visual physiology.

The visual process is greatly influenced by the generation of energy in the photoreceptor organs of animals. Energy generation is an important process in the biological system and in animals it is chiefly accomplished through oxidation-reduction reactions. This necessitates the presence of a reducing factor, which, in biological system may be ascorbic acid or glutathione (Heath and Fiddick, 1965b; Dey and Raghuvarman, 1984a). Ascorbic acid, otherwise known as vitamin C, is an antiscorbuitic, water-soluble and heat labile compound. It is one of the most important sugar acids,

readily undergoing oxidation to dehydroascrorbic acid, and all higher species appear to employ ascorbic acid as a co-factor in certain specific reactions (Lehninger, 1970). In ocular tissues, even in the absence of a dietary source, a high level of ascorbic is maintained, while other tissue shows total depletion. Thus, Rawal and Rao (1977) opined that this might be to sustain or maintain a high-energy demand, and also to modulate some co-enzymatic as well as non-enzymatic reactions. It is also postulated that ascorbic acid plays an important antioxidant role in biological systems through its conversion to mono-dehydroascorbic acid and dehydroascorbic acid (Seib and Tolbert, 1982). It is also suggested that ascorbic acid checks the propagation of various free radical reactions (Sharma, 1989), and may act as a UV filter shielding internal eye structures from radiation damage (Ringvold *et. al.* 2000). Insects, invertebrates, fishes, certain bats and birds cannot synthesize ascorbic acid, and therefore, the eye takes up ascorbic acid by an energy-dependent active transport mechanism, which is a Na^+/K^+-ATPase (Nicola *et. al.* 1968). Thus the present work attempts to ascertain the presence or absence of ascorbic acid, its probable functions in vision, and differences if any, in between the two insects.

CHAPTER I

HISTOCHEMICAL AND BIOCHEMICAL ANALYSIS OF ACID MUCOPOLYSACCHARIDES IN THE EYES

INTRODUCTION

The term "mucopolysaccharides" was coined by Meyer (1938) for those substances isolated from connective tissues and having similar physico-chemical properties. With increased understanding of their biochemistry in recent times, terms like "glycoproteins", "mucoproteins", "glucosaminoglycans" were also used, but they failed to distinguish between bacterial polysaccharides and amino sugars containing antibiotics.

Glycosaminoglycans (GAGs) are long, unbranched polysaccharide chains composed of repeating disaccharide units. The repeating unit consists of a hexose (six-carbon sugar) or a hexuronic acid, linked to a hexosamine (six-carbon sugar containing nitrogen). When most glycosaminoglycan chains are synthesized, they are covalently linked at their reducing end to core proteins, thus forming proteoglycans (PGS)- the exception is the GAG hyaluronan, which is uniquely synthesized without a protein core and is spun out by enzymes at the end surface directly into the extracellular space (Spicer *et. al*. 2002; Toole, 2001, 2004). They may have specific biological functions conferred upon them by specific sequences within the carbohydrate chain (Carney, 1994).

The term "mucopolysaccharides" was introduced to describe 2-amino-2-deoxyhexose containing polysaccharide of animal origin and occurring either as free polysaccharide or as their protein derivatives (Kennedy and White, 1983). Mucopolysaccharides are of two main classes *i.e.* (i) those that are neutral and (ii) those that contain uronic acid. Acid mucopolysaccharide (AMPs) comes under the second class. Carney (1994) had indicated that glycosaminoglycans may have specific biological functions conferred upon

them because of specific sequences within the carbohydrate chain. “Glycosaminoglycan” is the systematic name for the carbohydrate residues which form linear chains of alternating acidic and basic monosaccharides. The basic units are usually N-acetylated and sometimes N-sulfated, while the acidic units are sometimes O-sulfated (Kennedy and White, 1983).

Acid mucopolysaccharides (AMPs) may be further sulfated or non sulfated *e.g.* chondroitin sulfate and hyaluronic acid respectively. These terms *i.e*. AMPs and SMPs (Sulfated mucopolysaccharides) appear to provide an adequate description and also have the added advantage of continuous uses (Jaques, 1977). The different types of AMPS are as follows

1. Non-sulfated mucopolysaccharides e.g. Hyaluronic acid or Hyaluronan
2. Sulfated mucopolysaccharides

i.Chondroitins

a. Chondroitin sulfate A (ChS A) or Chondroitin 4-sulfate.

b. Chondroitin sulfate B (ChS B) or Dermatan sulfate.

c. Chondroitin sulfate C (ChS C) or chondroitin 6-sulfate.

ii Heparitins

Heparitin sulfate A

Heparitin sulfate B

Heparitin sulfate C

iii. Keratins

Keratan sulfate/Kerato sulfate

iv. Heparins

It is to be noted here that although heparins belong to the group of mucopolysaccharides, they are usually absent in connective tissues. However, in some biological situations the function performed by heparins in some cells or species seem to be performed by chondroitins in other cells or species as in basophilic leucocytes and mast cells (Jaques, 1977).

Members of acid mucopolysaccharide types vary in the type of hexosamine, hexose or hexuronic acid they contain *e.g.* glucuronic acid, iduronic acid, galactose, galactosamine, glucosamine etc. They also vary in the geometry of the glycosidic linkage, molecular weight, binding energy of the components and in functional properties.

It is to be noted that glycosaminoglycan always comes within the mucopolysaccharides category irrespective of the ways in which the term has been used, and it is now known that glycosaminoglycans are attached covalently to proteins. Therefore, AMPs actually refer to glycosaminoglycans of a proteoglycan plus, sometimes a few amino acid unit.

Glycoproteins or proteoglycans also have a protein backbone, but the carbohydrate (which are glycosaminoglycans) are in the forms of liner chains with regularly alternating basic monosaccharides (2-amino-2-deoxy-D-galactose, 2-amino-2-glucose) and acidic monosaccharides (D-glucoronic acid, L-iduronic acid). The basic units are usually N-acetylated and sometimes N-sulfated, while the acidic units are sometimes 0-sulfated. These compounds are the only source of hexuronic acids in animals, and occur in nearly all parts of mammalian bodies and to a lesser extend in fish and bacteria. According to Kennedy and White (1983), they are amongst the essential building blocks of the macromolecular frame work of connective and other tissues.

Studies on the vertebrate mucopolysaccharides are extensive. For instance, AMPs have been isolated from aorta of man (Berenson *et. al*. 1966; Barnes and Partridge, 1968), pig (Wagh and Roberts, 1972), cow (Radhakrishnamurthy and Berenson, 1973, and Funderburgh *et. al.* 1991), horse and sheep (Robert *et. al*. 1970), elephant (Mc Cullagh *et. al*. 1973), chicken (Bowes *et. al.* 1958; Kelley *et. al.* 1969), cattle (Wolff *et. al.* 1971), rabbit (Timple *et. al*. 1969), human articular cartilage (Huckerby *et. al.* 1999), bovine tracheal cartilage (Huckerby and Lauder, 2000), cartilages of fishes (Enomoto e*t. al.* 1966), human placenta (Achur *et. al.* 2002, 2008), the ampullary sense organs of some weakly electric fishes (Denizot, 1970), bovine nasal cartilages (Janado and Dunstone, 1972) on the brain of rat (DiBenedetta *et. al.* 1969 and Margolis and Margolis, 1970).

Among the various tissues of vertebrate, where mucopolysaccharides have been detected, the organic matrix of bone are reported to be the richest source (Herring, 1972). In addition to these, brain of some mammals like the rat are found to contain rich amount of some mucopolysaccharides. (DiBenedetta *et. al.* 1969; Margolis and Margolis, 1970).

Compared to extensive amount of work on the AMPs of various vertebrate tissues, works on invertebrate AMPs is scanty. Hunt and co-workers (1970) have detected a glucan sulphate peptide component in mucin of marine snail, *Buccinum undatum*; Matthews (1975) has detected a chondroitin sulphate like substance from cranial cartilage of *Locusta opalescens*; Raghuvarman *et. al.* (1998) from the body cuticle of the Peripatus *Typhiloperipatus wildoni*; Vadgama and Kamat (1971), on various glands like salivary glands; Baldwin and Salthouse (1959) on dermal glands,

Mustafa and Kamat, (1970) in the connective tissue of brain, ganglia, and imaginal disc of the housefly, *Musca domestica*; Ashhurt and Costin, (1971 a,b) have reported sulfated mucopolysaccharides in the connective tissue surrounding the ejaculatory duct and in the ganglia of the locust, *Locusta migrateria*. Ashhurt and Richards, (1964) have also detected mucopolysaccharides in the connective tissue, surrounding the nerve cord of pupa of the wax moth, *Galleria mellonella.*

Their presence in the visual system of vertebrates are also well documented. For example, they have been reported in the bovine cornea (Coster *et.al*. 1987; Funderburgh *et.al*. 1996; Corpuz *et. al*. 1996; Plaas *et. al*. 2001; Achur *et. al*. 2004; and Conrad *et. al*. 2010), in the eye of rabbit (Yue *et. al*. 1984; Lutjen Drecoll, 1990; Fitzsimmons *et. al*. 1992; Takahashi *et. al*. 1993; Goes *et. al*. 1999; Kato *et. al.* 1999), in chick cornea (Conrad *et. al*. 1977; Li *et. al*. 1992; Mc Adams and McLoon 1995), human and rabbit cornea (Freund *et al*. 1995; Tai *et. al*. 1997), in calf lens capsule (Mohan and Spiro 1991), and in the corneal stroma of squid (Anseth, 1961 and Moozar and Moozar, 1973).

Other visual apparatus where AMPs have been reported are in the cornea of elasmobranchs (Balazs, 1965), vitreous body of the eye of squids (Balazs *et. al.* 1965), in aqueous and ciliary body (Cole, 1970; Schachtschabel *et. al.* 1977), interstitial matrix surrounding the photoreceptor cell of the cattle (Berman and Bach, 1968; Berman, 1969), inter photoreceptor matrix of vertebrate (Rolich, 1970), sclera of ox (Robert and Robert, 1967) *etc*.

In the case of insects, AMPs have also been reported in the compounds eyes of *Periplaneta americana*, *Belostoma* sp (Dey, 1976),

Paelemon sp, *Limunus polyphorus* and Macrobrachium birmanicum (Dey *et.al.* 1978) *Musca domestica, Apis cerena indica* (Dey, 1980)

The present study thus is an attempt to analyze the compound eyes of the two insects concerned with regards to the occurrence of AMPs and their possible roles in vision.

MATERIALS AND METHODS

Eyes were separated from the live insects and fixed in 10% buffered formalin until they were used.

Histochemical study: The tissues were embedded in paraffin and 8 µ thick sections were cut by microtome. The section were stained with Toluidine blue and Alcian blue (Humason, 1971) for detection of mucopolysaccharides.

Biochemical study according to Dietrich *et. al.* (1977).

Extraction: Fresh eyes (1gm) were defatted in cold acetone for 3 hours and dried. Tissues were then homogenized and suspended in 20 ml of 0.05M Tris-Hcl buffer (pH 8). To the mixture, 10 mg of trypsin was added and then a few drops of toluene were added forming a layer at the surface, and incubated at 37^0C for 24 hours. After incubation, pH of the mixture was brought to 11 with Conc. NaOH and maintained for 6 hours at room temperature. Then the pH was brought to 6 by the addition of Hcl and then mixture was centrifuged for 15 minutes at 3000rpm. To the supernatant, 0.1 ml of 2M NaCl and 2 volume of ethanol were added and kept overnight at 5^0C. The mixture was centrifuged for 15 minutes at 3000 rpm and the precipitate collected and dried. The

resultant powder was re-suspended in 1 ml of 0.05M sodium acetate (pH 6.5) along with 1 mg of DNAase and RNAase. The solution was again incubated for 24 hours at 37^0C with a layer of toluene. After incubation, 0.1 ml of 2M Nacl and 2 volumes of ethanol were added to the solution and kept overnight at 5^0C. It was then centrifuged for 15 minutes at 3000 rpm and precipitate was collected and dried. The resultant powder was dissolved in 0.5 ml of water, heated at 100^0C for 2 minutes and analyzed by paper chromatography and electrophoresis.

Chromatography: The extracted acid mucopolysaccharides were hydrolyzed with 6N HCl at 100^0C for 12 hours. The acid hydrolysate was then evaporated to dryness. The dried residue was then dissolved in 0.5 ml of distilled water and spotted in whatman No 1 filter paper and ascending paper chromatograms run using butanol , acetic acid and water in the ratio of 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 of NaOH in 25 ml of rectified spirit) as suggested by Trevelyan *et. al.* (1950). The chromatogram was then washed in 6N ammonia hydroxide for 10 minutes and then washed in running water and dried at room temperature.

Electrophoresis: Electrophoresis of the acid mucopolysaccharides was carried out by applying streaks of the samples on Whatman No.1 paper strips using 0.1M phosphate buffer (pH 6.6) at 4v/cm for 8 hours. After removal from the electrophorectic apparatus, the paper strips were dried at room

temperature and stained with Toluidine blue (0.04% in 80% acetone). The staining of the strips was followed by 2-3 rinsing in 0.1% acetic acid and then 2-3times in H_2O. The strips were then dried at room temperature.

OBSERVATIONS

Histochemical: Compound eyes of insects include the lens system, a retina and underlying optic ganglia. Lens is a modified cuticle and is composed of the cornea and underlying crystalline cone. Immediately behind the crystalline cone are the longitudinal sensory elements or the retinula cells. The inner sides of the retinula cells collectively secrete an internal light trapping rod like structure known as rhabdom.

Lens cuticle of the butterfly, *Pieris brassicae*: The histological preparations of the lens cuticle reacted positively when stained with Toluidine blue and Alcian blue. When the sections of the eyes are stained with Toluidine blue, cornea and the crystalline cone became purple in color showing metachromasia (Photoplate 1). This reaction indicates the presence of acid mucopolysaccharides. The region of the rhabdom was orthochromatic *i.e.* blue in colour. Rhabdom region is devoid of acid mucopolysaccharides. When the eyes were stained with Alcian blue, the lens and crystalline cone gave purple colour (Photoplate 2) which indicates the presence of acid mucopolysaccharides.

Lens cuticle of moth, *Philosamia ricini*: When the sections are stained with Toluidine blue, cornea as well as the crystalline cone became purple in colour

(Photoplate 3) showing the presence of mucopolysaccharides. The more intense reactions were observed towards the corneal lens. The rhabdom region however gave a blue colour reaction in the presence of toluidine blue *i.e*. the region is orthochromatic (Photoplate 4). When the eyes were stained with alcain blue the corneal lens and crystalline cone became purple in colour and the rhabdom became blue in colour.

Biochemical:

Chromatographic analysis of the acid mucopolysaccharide extract showed the presence of three sugars *viz* lactose, galactose and xylose in case of *Pieris brassicae* and presence of three sugars *viz* galactose, xylose and rhamnose in case of *Philosamia ricini* (Figure 1&2; Table 1).

Electrophorectic movement pattern of the crude extracts of the acid mucopolysaccharides from the eyes of *Pieris brassicae* and *Philosamia ricini*, when compared with several standard acid mucopolysaccharides showed that the mucopolysaccharides extracted resembles more of chondroitin 4-sulfate (Figure 3 & 4; Table 2).

PHOTOPLATES 1 & 2

Photo plate 1: T. S. of the eye of *Pieris brassicae* showing metachromasia with toluidine blue stain (400X).

Co (Crystalline cone): Purple colour: mucopolysaccharides present;

Rh (Rhabdom): Blue colour: mucopolysaccharides absent.

Photo plate 2: T. S. of the eye of *Pieris brassicae* showing metachromasia with alcian blue stain (400X).

Other details as in photo plate 1.

PHOTOPLATES 3 & 4

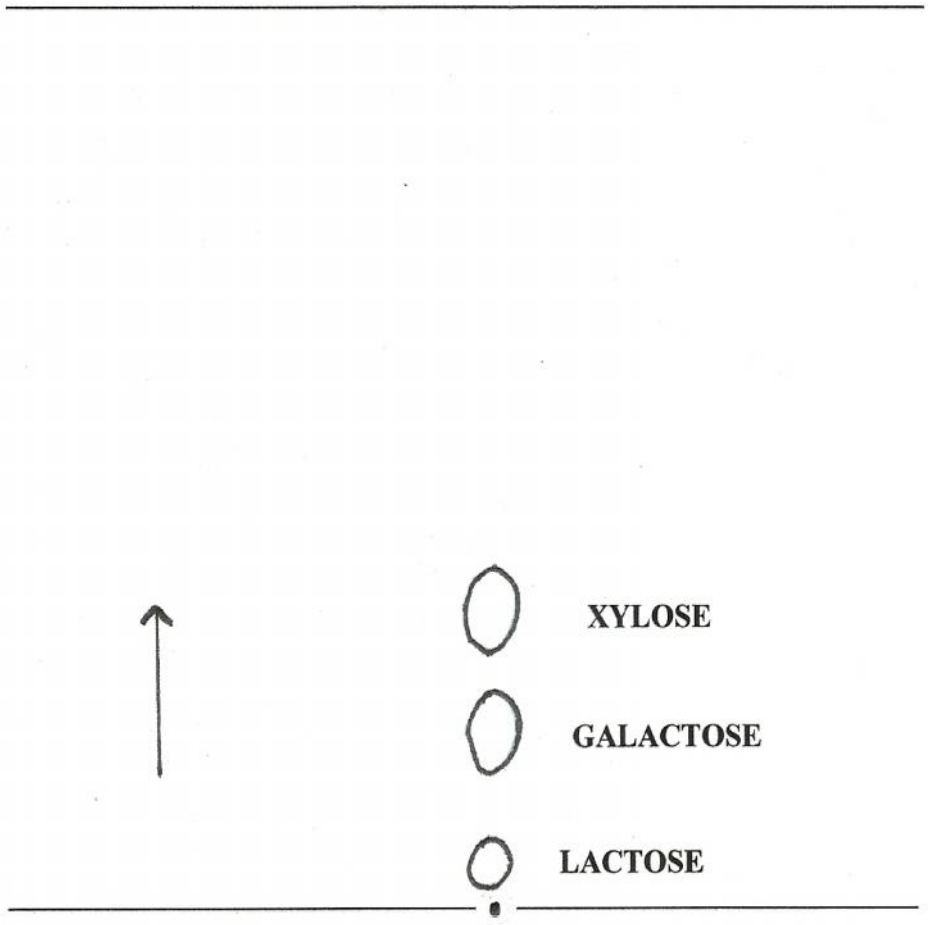
Photo plate 3: T. S. of the eye of *Philosamia ricini* showing metachromasia with toluidine blue stain (400X).

Co (Crystalline cone): Purple colour: mucopolysaccharides present;

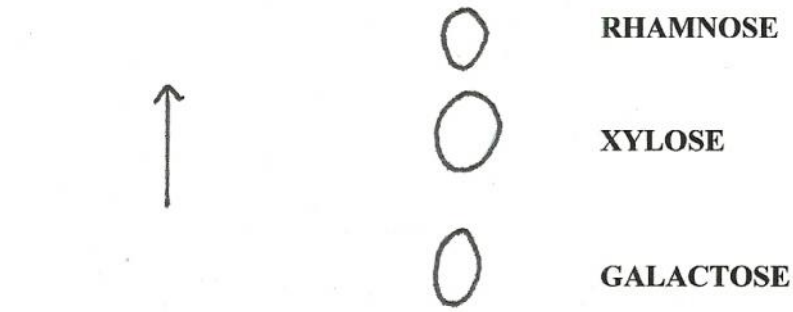
Rh (Rhabdom): Blue colour: mucopolysaccharides absent.

Photo plate 4: T. S. of the eye of *Philosamia ricini* showing metachromasia with alcian blue stain (400X).

Other details as in photo plate 3.



SUGAR COMPONENTS (BUTTERFLY)



SUGAR COMPONENTS (MOTH)

CHONDROITIN-4-SULFATE

ACID MYCOPOLY SACCHARIDES (BUTTERFLY)

CHONDROITIN-4-SULFATE

ACID MYCOPOLY SACCHARIDES (MOTH)

Table 1: Ascending paper chromatogram of sugar components of the butterfly, *Pieris brassicae* and the moth, *Philosamia ricini*.

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

Insect	Rf value	Identification
Butterfly, *Pieris brassicae*	0.05	Lactose
	0.18	Galactose
	0.33	Xylose
Moth, *Philosamia ricini*	0.16	Galactose
	0.33	Xylose
	0.43	Rhamnose

Ascending Paper chromatogram of some standard sugar components

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

Sugar	Rf value
Raffinose	0.03
Lactose	0.05
Glucose	0.10
Sucrose	0.13
Galactose	0.18
Mannose	0.25
Fructose	0.28
Xylose	0.34
Ribose	0.38

Table 2: Paper electrophorectic movement patterns of the crude mucopolysaccharides from the eyes of butterfly, *Pieris brassicae* and moth, *Philosamia ricini.*

Solvent: Phosphate buffer (pH 6.5)

Insect	Distance travelled (cms)	Acid mucopolysaccharide type
Butterfly, *Pieris brassicae*	6.4	Chondroitin 4-sulfate
Moth, *Philosamia ricini*	6.8	Chondroitin 4-sulfate

Paper electrophorectic movement patterns of some standard mucopolysaccharides. Solvent: Phosphate buffer (pH 6.5)

Standard mucopolysaccharides	Distance travelled (cms)
Heparin	5.5
Chondroitin 4-sulfate	6.6
Heparan sulfate	7.2
Chondroitin 6-sulfate	7.6
Keratan sulfate	8.7
Dermatan sulfate	10

DISCUSSION

Acid mucopolysaccharides play several important physiological roles owing to their capacity to bind and hold water. They serve as natural lubricants in the joints, impart elasticity to connective tissue, and as a component of cartilage and ligaments, are involved in support and motor functions, and also have bactericidal properties. Dysfunction in the mucopolysaccharide metabolism leads to a change in the composition of connective tissue and of the body fluids, resulting in diseases such as collagenosis, mucoplysaccharidosis, and rheumatism. Their dysfunction is also correlated with aging.

Vertebrates produce multiple chondroitin sulphate proteoglycans that play important roles in development and tissues mechanics. In the nematode, *Caenorhabditis elagans*, the chondroitin chains lack sulphate but nevertheless play essential roles in embryonic development and vulval morphogenesis (Olson *et. al.* 2006). Corneal stromal glycosaminoglycans bind together and thus may influence keratocytes and nerve growth in cornea (Cornard *et. al*. 2010). Glycosaminoglycans also play a central role in the physiological maintenance of trabecular meshwork as seen in the eyes of rabbits and humans (Yue *et. al.* 1984 and Cavallotti *et. al*. 2004).

Glycosaminoglycans and their core proteins have important physiological and homeostatic roles *e.g*. during inflammation and the immune response (Park *et. al*. 2001; Li *et. al*. 2002 and Wang *et. al*. 2005). Bulow and Hobert (2006) also suggested that glycosaminoglycans may provide a specific code that contributes to the correct development of a multicellular

organism. They influence many biological functions, including cell division, differentiation, signal transduction, adhesion, migration, peripheral nerve extension or regeneration, and responses to growth factors (Miao *et. al*. 1996; Groves *et. al*. 2005; Manton *et. al.* 2007; Fthenou *et. al*. 2006, 2008). Chondroitin and dermatan proteoglycans have attracted much attention as inhibitors of axon growth and have been shown to be important components of the glial scar that prevents axon regeneration (Rhodes & Fawcett 2004).

The role of mucopolysaccharides in pathogenecity has been widely reviewed. They are responsible for dermal thickening in acromegalic patients (Matsuoka *et. al.* 1982), involved in inborn errors of metabolism and/ or storage disorders (Matalon *et. al*. 1974a; Hall *et. al.* 1978; Neufeld and Fratantoni 1970; Mc Kusick *et. al.* 1978), maintenance of retinal structure and neural tube closure Knobloch syndrome (Sertie *et. al*. 2000), treatment of diabetic nephropathy (Gombaro and VanDerWoude, 2000), calcification of bones (Rubin and Howard, 1950).

Matthews (1959) and Oosawa (1971) have suggested that one of the characteristic property of mucopolysaccharides is the selective association or binding with small inorganic cations, especially H^+, Na^+, and Ca^{++}, and also with cationic groups of macromolecules. In these regard, Farber and Schubert (1957) have shown that the percentage of chondroitin-sulphate binding of Ca^{++} is greater than that of Na^+. Urist *et. al.* (1968) have also found a small preference for binding Ca^{++}over Na^+ in chondroitin sulphate. Matthews (1975) thus suggested that these substance act as a store for Ca^{++} in cartilage tissue and that is why have specific roles have in tissue-calcification.

Mucopolysaccharides have been reported to play important roles in "water binding" and maintenance of tissue osmotic pressure (Ogston and Wells, 1972; Wells 1973 b). 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) have also suggested the role of AMPs in maintaining mechanical flexibility and elasticity of tissues. AMPs may also have some functions in controlling metabolism of cells, and 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.

Some roles of AMPs, especially in arthropodan cuticle have been reported by Meenakshi and Scheer (1959) and Sundara Rajulu (1969) in terms of calcification of the cuticle of *Hemigrapsus mudus* and *Cingalabolus bungnioni* respectively. Krishnan (1965) has suggested that AMPs may be associated with –S-S- bonding of the cuticle in the scorpion, *Palaemoneus swammerdami.* Sannasi (1969) reported that extreme flexibility of the intersegmental cuticles of the queen of *Odontotermes obesus* to be due to the occurrence of acid mucopolysaccharides. He based his conclusion on the fact that acid mucopolysaccharides are said to possess high water binding capacities (Ogston, 1966a and Katchalsky, 1964).

Thus it can be observed that there certainly exist some roles of AMPs in the visual process of insects, since their occurrence and their visual significance have been reported in the ocular tissues of various vertebrates and some invertebrates by various workers. Since the occurrence of acid mucopolysaccharides is not a general feature of the arthropod cuticle and it occurs in some special types of cuticle where it performs some special functions (Meenakshi and Scheer, 1959; Sundara Rajulu, 1969; Krishnan, 1965; Sannasi, 1969 and Raghuvarman *et. al*. 1998), it is reasonable to presume that the specific occurrence of mucopolysaccharides in the lens cuticle and the crystalline cone may have a bearing on the visual system of the insects, examined in present study. Keeping the above account in view it is possible to assume a role of AMPs in the lens-cuticle of insects.

The lens-cuticle as already stated, besides playing a general defensive role, performs a special optical function of conducting light rays to the inner rhabdomere. It is possible to presume that the transparency of the lens-cuticle, which is more than that of other types of cuticle (*e.g.* body cuticle), may be affected by the occurrence of mucopolysaccharides. The basis of this presumption is that the transparency of the cornea of the vertebrates has been reported to be affected by mucopolysaccharides (Anseth and Fransson, 1970). It is known that the bulk of 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 length (Maurice, 1969). Anseth and Fransson (1970) have found that during chick corneal development, the occurrence of a highly sulfated keratan sulfate is associated with the rise in transparency of stroma. They have also suggested that stromal

transparency is correlated with the presence of normal proportions of keratan sulfate and chondroitin 4-sulfate. Funderburgh *et. al*. (1996) have reported that keratan proteoglycans are the major proteoglycans of the bovine cornea and secreted by keratocytes in the corneal stroma and they are thought to play an important role in corneal structure and physiology, particularly in the maintenance of corneal transparency. Takahashi *et. al*. (1993) have also reported that keratan sulfate and dermatan sulfate proteoglycans are associated with collagen in fetal rabbit cornea. Freund *et. al*. (1995) also reported that the presence of AMPs in human and rabbit cornea is related to transparency. Blochberger, *et. a*l. (1992), has reported that corneal keratan sulfate proteoglycans contribute to corneal transparency in chick.

Transparency of the corneal stroma depends partially on the degree of spatial order of its collagen fibrils which are narrow in diameter and closely packed in a regular array (Maurice, 1957; Cox *et. al*. 1970; Benedek, 1971; McCally and Farrell 1990 and Bron, 2001). Mc Adams and McLoon (1995) have shown that retinal axons grow in the presence of chondroitin sulphate and keratan sulfate proteoglycans and that these proteoglycans helps in developing chick visual pathway.

Payrau *et. al.* (1967) observed that the transparency of the cornea is based on the stake of hydration of tissue. They based this on the fact that the corneal stroma of most vertebrates, including mammals, birds and teleosts absorb water wherever free water is accessible. In contrast, according to Maurice and Riley (1970) odema of the cornea leads to disorganization of its structure and less transparency, but dehydration does not appear to have serious optical affects. Maurice (1972) suggested that the presence of AMPs in

the cornea is mainly responsible for the dehydration properties of the tissue and hence transparency. This has been supported by workers like Hedbys (1961, 1963); Kikkawa and Hirayama (1970); Bettelheim and Plessy (1975); Lee and Wilson (1981) and Castoro *et. al*. (1988).

Many studies that focused on corneal swelling behavior noticed a gradual decrease in swelling from posterior to anterior side (Van Horn *et. al*. 1975; Bettelheim and Plessy 1975; Castoro *et. al.* 1988 and Cristol *et. al*. 1992). This process was thought to be related to the organization of the collagen lamellae and to the presence of different types of proteoglycans. In the posterior part, keratan sulfate, a more hydrophilic proteoglycan is prevalent, whereas in the anterior part dermatan sulfate, a much less hydrophylic proteoglycan, is present (Bettelheim and Plessy 1975; Castoro *et.al*. 1988). Muller *et. al*. (2001) studied the differential behavior of the anterior and posterior stroma during corneal swelling and drew an interesting conclusion that it is the high negative charge of the glycosaminoglycan components of the proteoglycans that is responsible for the corneal swelling. They also suggested that the structural stability of the anterior stroma under condition of extreme hydration imply an important role for this zone in the maintenance of corneal curvature and that this stability is determined by the tight interweave of the stromal lamellae.

The non- swelling properties of elasmobranchs cornea is supposed to be due to the high mannose content in their structural proteins (Moozar *et. al.* 1969b; Moozar and Moozar, 1972). On the other hand, the mechanism of corneal swelling is due to electrostatic repulsion between acidic groups of the macromolecules *i.e* mucopolysaccharide.

It is now known that the pH value is a decisive factor for the taking of water by the cornea (Cejkova and Brettschneider, 1969). The protein polysaccharides complex provides a more stable and specific configuration within the molecules than electro-static linkage could (Maurice, 1970). For the cornea to remain transparent, it is essential that an active mechanism counter the natural tendency of the stroma to increase its hydration, swelling and opacity. It is well-established that one of the corneal limiting cell layers *i.e*., the corneal endothelium, transports fluid at a substantial rate and that this transport is essential to maintain normal stromal hydration (Maurice, 1972; Candia, 1976; Candia and Zamudio, 1995; Narula *et. al*. 1992; Bonanno *et. al*. 1989 and Yang *et. al*. 2000). Anseth and Fransson (1969) had demonstrated the synthesis of AMPs by corneal epithelial and stromal cells, and that they are important in maintaining the corneal structure in relation to its environment. Deb and Raghuvarman (1994) have also observed that glucosaminoglycans are essential for the maintenance of corneal structure and function.

Acid mucopolysaccharides thus detected in the compound eyes of the butterfly, *pieris brassicae* and the moth, *Philosamia ricini* may play an important role in visual excitation, when light rays pass through the outer epicuticle to the inner endocuticular region (crystalline cone) - the sites of AMPs due to the fact that they act as a selective ion barrier (Jeanloz, 1970). It may also be noted that they are present not only in the corneal lens but also in the crystalline cone, which are in close connection with the inner rhabdomeres (the actual sites of photochemical reactions), the products of which may depolarize the membrane of the retinula cells and initiate impulse

CHAPTER II

STUDIES ON LIGHT AND DARK ADAPTATIONS

formation (Wigglesworth, 1957). Further, mucopolysaccharides may play role in increasing transparency of lens-cuticle. In this context, it is worth mentioning that during corneal development of vertebrates, rise in transparency of stroma was found to be associated with occurrence of mucopolysaccharides (Anseth and Fransson, 1970).

INTRODUCTION

Vision is a special somatic efferent sense. It is necessary for the eye to be adapted to various stages of illumination for maximum efficiency and a characteristic feature of this adaptation is the movement or migration of screening pigments in the eye.

The visual pigments of all invertebrate, including insects, crustaceans and squids are all rhodopsins. The biological significance of rhodopsin is that they absorb light through the visible spectrum as well as the UV spectrum or range. Lythgoe (1979), showed that the rhodopsin granules prevent reflection of transmitted light. The maximum absorption value of different rhodopsins range from about 345nm (ultra violet) to as high as 610nm (red) (Autrum, 1981; Arikawa, 1999). The absorbing properties of any given rhodopsin are related to the disposition of changed opsin groups around the chromotophore.

The retinal pigment granules (rhodopsin) in the pigment epithelial cells exhibit distinct movement during light and dark adaptation, and thereby help in the process of visual adaptations in various photic levels. Several methods of dark and light adaptations are known- some methods being characteristic of particular groups, although a particular species normally possesses more than one method. According to Lythgoe (1979), the various adaptive methods can be divided into three classes *i.e.* (i) optical regulation of the light reaching the visual pigment through the pupil (ii) absorption by the visual pigment and (iii) neural processes

The pigment granules as well as the rods and cones themselves, move in response to changes in light intensity in such a way that particular cell type are shielded from unwanted light. These changes are collectively known as

retinomotor or photomechanical movements. The phenomenon of retinomotor movement was first studied separately by Boll (1877) and Kuhne (1877) and has been reviewed by Blaxter (1970). Other notable works are those by Ali (1971), Ali and Wagner1975, Munz (1971), Walcott (1975), Lythgoe (1979), Ferrero *et. al*. (1979), Anctil *et. al*. (1980), Klyne and Ali (1980), Kunz (1980), Fernald (1982, 1988), Kaneko *et. al*. (1981), Burnside and Nagle (1983), Teranishi *et. al*. (1983), Weiler and Wagner (1984), kurz-Isler and Wolburg (1986, 1988), Dey *et. al*. (1994). Both arthropods and vertebrates show several distinctive retinal specializations associated with several daily activity rhythms (Hoars, 1987). Several adaptations for light (photopic) and dark (scotopic) vision are accomplished by changing the intensity of light at the receptor site and the most common feature of light and dark adaptations is change in concentration of visual pigments and modification in neural interaction (Munz, 1971).

Pigment migration has been studied in many invertebrates as well as in all groups of sub-mammalian vertebrates. In arthropods it is more striking in superposition eyes but also occur in apposition eyes. Retinal pigment migration in vertebrate is rapid and pronounced in fishes, frogs and birds but slow and slight in turtles and crocodiles and absent in snakes and mammals. Pigment migration is regulated by hormones in crustacean (Highnam and Hills, 1979), but not well understood in insects and it may depend on nerves (Goldsmith and Bernard, 1974).

The physiological basis of adaptation in the visual system has been studied by Barlow (1972), Pugh (1975), Autrum (1981), Shapley and Enroth-Cugell (1984), Lamb (1981 and 1990), Tamura *et. al*. (1989, 1991),

Lamb and Pugh (1992), Hood and Birch (1993,1994), Donner *et. al*. (1998), Thomas and Lamb (1997, 1998 a,b, 1999), Pianta and Kalloniatis (2000), Paupoo *et. al*. (2000), Fain *et. al*. (2001), Moser *et. al*. (2004) *etc*.

In insects, the pigments of the iris move in response to changing photic conditions, and are also otherwise known as screening pigments or accessory pigments. The most widely distributed amongst these pigments are the "ommochromes". They may be of different types - ommatin, ommin and ommidin being the most important. Ommatin can be further of three types, out of which xanthomatin is the only one found in the eyes, as seen in case of *Calliphora* and related flies, where it is the principal pigment. Ommin on the other hand is the most widely distributed of the ommochromes in the compound eyes of other insects. It differs from ommatin in respect of a higher molecular weight as well as reduced tendency towards auto-oxidation (Wigglesworth, 1965). Ommidin is closely related to ommin and is chiefly found in the eye of orthopterans. These pigments move in response to changes in light intensity *i.e.* in relation to light and dark conditions, but other anatomical changes during light and dark adaptations are not similar in all insects.

In the apposition type of compound eyes, the major portion of the retinular cells *i.e.* up to one-third of its width from the rhabdom, is filled up by elongated large vesicles of the palisade (sacks of endoplasmic reticulum)- the pigment granule occurring between the vesicles of the palisade as well as in the peripheral cell-cytoplasm (Kirschfeld and Franceschini, 1969). In dark-adapted condition, the palisade surrounds the rhabdom, while during light-adapted state, the palisade is reduced to a great extent. In addition the pigment

of the retinula cells move toward the rhabdomeres as seen in *Musca* (Kirschfeld and Franceschini, 1969).

Light and dark adaptations are associated with the longitudinal movement of pigment granules in the retinula and pigment cells of lepidopteran compound eyes, as also in fireflies, while in the beetles (example, *Dytiscus*) it results in the movement of some retinula cell bodies (Walcott, 1969). Another interesting features of light-dark adaptation is the extensive changes in the shape of the cone cells along with their movement with the crystalline tract and rhabdom of the retinula cells - a typical example being seen in hemipterans (Walcott, 1974).

Studies on pigment migration in compound eyes of arthropods started as long back as in 1889 through the pioneering works of Exner (1889), yet the mechanism is still open to conjecture, even though much work is being done in this field. Some prominent workers like Day (1941), have found that in moths subjected to cold, narcosis, CO_2 or even injury, the pigments move and assume a state similar to that of light-adapted condition. Similarly Stavenga (1977) has shown that in praying mantis and katydids, the circadian movement of the pigment is influenced by temperature. He found that a light-adapted state can be achieved during dark via cooling.

In the dragonflies, *Austrolestes annulosus* and *Ischnura heterostecta*, pigment migration is associated with pronounced colour changes that resemble that of their epidermal chromatophores (Veron, 1973). During light phase the pigment is concentrated around the base of the crystalline cone as a layer of Tyndall (blue) bodies to produce a bright blue colour, but during dark phase the pigment migrate distally and this disrupts the Tyndall effect, and the eyes

turn grey-brown in colour. Thus, pigment migration and epidermal chromatophores are under similar environmental and physiological control (Veron, 1973).

Other notable works are those by Kitamato *et. al* 1998; Kelber and Pfaff (1999), Kinoshita *et. al*. (1999), Kinoshita and Arikawa (2000), Hardice (2001), Kelber *et. al*. (2003) *etc* who have worked on colour vision of honey bees, butterfly, nocturnal hawkmoths and fruitflies. Studies on the reflection of butterflies and bumblebee ommatidia with respect to angular and spectral sensitivity have been carried out by Stavenga (2000a, 2000b, 2003a,b, and 2004) and Skorupski (2007). In crustacean eyes the phenomenon of light and dark adaptation have been studied by Meyer-Rochow (2001), in insects by Meyer-Rochow (1974), Keskinen and Meyer-Rochow (2004), Meyer-Rochow and Mishra (2007), *etc*. An interesting work has also been done by Lau and Meyer-Rochow (2006) and Lau *et.al*. (2007) on sexual dimorphism of the compound eyes with respect to light and dark adaptation in coleopterans and lepidopteran.

A point to be noted here is that movements of the pigment granules and also the rods and cones are not synchronized, and in some cases either type can occur in the absence of the other. But an unalterable fact is that movements during dark adaptations are much slower, requiring times to the order of an hour to complete (Walcott, 1975).

That these movement must be integrated with the nervous system is an accepted fact, but its mechanism is poorly understood. Kakcheyev (1943) had put forward the hypothesis that dark adaptation is under nervous control. Similarly, Veron (1973) and Dey (1980), have suggested that neurosecretion

and pigment migration are affected by photopic and scotopic states. This has been further confirmed through the works of Deb (1990) and Bendang (1998), who also attributed photopic and scotopic states on the phenomena of neurosecretion and pigment migration in fishes and birds respectively.

It is now known that dark adaptation involves rods and much summation, while light adaptation involves cones and much lesser summation. Moreover, the time taken for the different stages of adaptation is different *i.e.*, the initial stages of adaptation are quite fast (less than one-fifth of a second), while the later stages are less faster. In this regard, Lythgoe (1979) has suggested that perhaps the whole neural organisational changes *i.e* from light to dark and *vice versa* is accomplished within thirty minutes.

Ali (1964 a), has suggested the possibility of hormones in influencing pigment migration, and Bagnara and Hadley (1969), believed that in all probability it is via intermedin. Other workers like Novales (1959), Van de veerdonk (1962) and Freeman *et. al.* (1968), *etc* suggested that ions are involved in pigment migration - these (transmembrane) ions acting through intermedin. Thus, Fujii (1969) suggested that both nerves and hormones, either alone or in tandem may be involved in pigment migration.

Different responses induced in the retina by different illuminations are signalled or transmitted to the brain (via the optic nerve) - individual signals from neighbouring cells interacting and adding up, so that their sensitivity is the sum of their respective areas (Pirenne, 1967), but exactly how, is an ongoing area of research (Lythgoe, 1979).

Von Frisch (1911), first reported the role of nervous control of pigment migration in blinded minnows. He showed that the diencephalon was

sensitive to light. Scharrer (1952b) put forward the concept of neurosecretory cells forming a link between the endocrine glands and the nervous system. But reports on the relationship between vision and neurosecretion in insects is scanty. Therefore an attempt has been made to study the effect of light and dark on the neurosecretory system. This is because an accumulation and discharge of secretory materials in the brain (optic lobe) of the two insects in response to light and dark condition is expected.

Another important aspect that has been undertaken is on the role of neurosecretory products. The role of hypothalamic neurosecretion in integrating various physiological events is well-documented (Geris *et. al*. 2002; Sharp and Sreekumar, 2002) . It is known that biogenic amines are released instaneously for rapid physiological adaptation to light and dark condition. In addition the role of catecholamines, alkaloids and secondary messenger have been reported to be involved in visual systems (Scheline, 1963; Scott, 1965; Bonner, 1971; Bitensky *et. al*. 1973; Robinson *et. al*. 1971; Anctil *et. al*. 1979; Vander *et. al*. 1980; Devries *et. al*. 1982; Hasegama and Cahill, 1999; Kato *et. al*. 1982; Burnside *et.al*. 1982; Allen and Burnside, 1986; Koumenis *et. al*. 1995) *etc.*

Thus the involvement of serotonin or 5-Hydroxytryptamine, cyclic AMP and colchicine in the visual process have been investigated in the present work.

MATERIALS AND METHODS

Adaptation to light and dark conditions: Two groups of experimental insects, each containing about 20 insects were selected. One group was kept in light

for three hours for light adaptation, while the other group was kept in darkness for the same period for dark adaptation. After the completion of three hours the insects of both the groups, were decapitated and the eyes quickly removed and fixed in alcoholic Bouin's fluid. The next day the eyes were washed in distilled water, then routine histological preparation were carried out by paraffin embedding method and 8 µ thick sections cut for microscopic preparations and study. The sections were stained in paraldehyde fuchsin-one step tichrome (Gabe, 1966). In the case of the dark adapted eyes, all the preparations were carried out in light-proof vials, since pigment migration might take place when exposed to light (Ali, 1964).

Effect of light and dark on neurosecretion: Two groups of insect, each containing about 20 insects were adapted in light and dark for three hours each. After the required period of adaptation, the insects were decapitated immediately and the heads fixed in alcoholic bouin's fluid and a hole was done on the head for penetration of the fixative and kept for 24 hours. The brain was then taken out and again fixed in the fixative for another 24 hours. Then routine histological preparation were carried out for paraffin embedding method and 8 µ thick sections cut . The sections were stained in Paraldehyde fuchsin- one step tichrome (Gabe,1966).

*Effect of 5-hydroxy tryptamine, cyclic AMP and colchicine***:** Three groups of insects of ten each were adapted in complete darkness for three hours. After three hours, the insect were given topical application of 0.1ml of 5-hydroxytryptamine, cyclic AMP and colchicine respectively. Each of the

drugs were dissolved in insect's Ringer's solution. The concentrations of 5-hydroxytryptamine, cyclic AMP were 0.8mM each while the concentration of colchicine was 0.3mM. One hour after topical application of drugs, the eyes were dissected out and fixed in alcoholic Bouin's fluid. Then routine histological preparations were made and 8 micron thick sections were cut. The sections were stained in paraldehyde fuchsin-one step tichrome (Gabe,1966).

OBSERVATIONS

Adaptation to light and dark conditions: In the light adapted state, there was radial movement of pigment granules whereas in the dark adapted state, there was peripheral distribution of the pigment granules. However all parts of retina did not respond equally (Photoplates 5-8).

Effect of light and dark on neurosecretory system: When both the insects are dark adapted there was a large accumulation of neurosecretory material in the form of compact neurosecretory granules as evident from the positive reaction to Paraldehyde- fuchsin stain (Photoplates 10 & 12). In contrast to this, when the insects were light adapted, it was observed that there is a significant reduction of the neurosecretory material (Photoplates 9 & 11), presumably due to the axonal transport of neurosecretory material. The cytoplasm now appearing lightly stained, and the shape of the cell becoming slightly irregular.

Effect of 5-hydroxytryptamine, cyclic AMP and colchicine: Light microscopic studies of paraffin sections of dark- adapted eyes revealed that in all cases, almost complete migration (dispersion) of retinal granules similar to

controlled light adapted eye occured *i.e* after treatment with 5-hydroxytryptamine, cyclic AMP and colchicine. There were no marked differences in retinal pigment granule dispersion between groups receiving 5-HT, cAMP and colchicines (Photoplates 13-18).

Photoplates 5&6

Photo plate 5: T. S. of the light adapted eye of *Pieris brassicae* showing radial distribution of pigment granules (100X).
Co (Crystalline cone); **Rh** (Rhabdom)

Photo plate 6: T. S. of dark adapted eye of *Pieris brassicae* showing peripheral distribution of pigment granules (100X).
Other details as in photo plate 5.

Photoplates 7&8

Photo plate 7: T. S. of light adapted eye of *Philosamia ricini* showing radial distribution of pigment granules (100X).
Co (Crystalline cone); **Rh** (Rhabdom)

Photo plate 8: T. S. of dark adapted eye of *Philosamia ricini* showing peripheral distribution of pigment granules (100X).
Other details as in photo plate 7.

Photoplates 9&10

Photo plate 9: T. S. of light adapted brain of *Pieris brassicae* showing reduced neurosecretory content in the neurosecretory cells (100X).

NSC (Neurosecretory cells)

Photo plate 10: T. S. of dark adapted brain of *Pieris brassicae* showing large accumulation of neurosecretory material in the form of compact neurosecretory granules in the neurosecretory cells (100X).

Other details as in photo plate 9.

Photoplates 11&12

Photo plate 11: T. S. of light adapted brain of *Philosamia ricini* showing reduced neurosecretory contents in the neurosecretory cells (100X).
NSC (Neurosecretory cells)

Photo plate 12: T. S. of dark adapted brain of *Philosamia ricini* showing large accumulation of neurosecretory materials in the form of compact granules in the neurosecretory cells (100X).
Other details as in photo plate 11.

Photoplates 13,14 &15

Photo plate 13: T. S. of dark adapted eye of *Pieris brassicae* treated with 5-hydroxytryptamine showing radial movement of pigment granules similar to that seen in light adapted state (100X) .
Co (Crystalline cone); **Rh** (Rhabdom)

Photo plate 14: T. S. of dark adapted eye of *Pieris brassicae* treated with cyclic AMP showing radial movement of pigment granules similar to that seen in light adapted state (100X).
Other details as in photo plate 13.

Photo plate 15: T. S. of dark adapted eye of *Pieris brassicae* treated with colchicine showing radial movement of pigment granules similar to that seen in light adapted state (100X).
Other details as in photo plate 13.

Photoplates 16,17 &18

Photo plate 16: T. S. of dark adapted eye of *Philosamia ricini* treated with 5-hydroxytryptamine showing radial movement of pigment granules similar to that seen in light adapted state (100X).
Co (Crystalline cone); **Rh** (Rhabdom)

Photo plate 17: T. S. of dark adapted eye of *Philosamia ricini* treated with cyclic AMP showing radial movement of pigment granules similar to that seen in light adapted state (100X).
Other details as in photo plate 16.

Photo plate 18: T. S. of dark adapted eye of *Philosamia ricini* treated with colchicine showing radial movement of pigment granules similar to that seen in light adapted state (100X).
Other details as in photo plate 16

DISCUSSION

The basic physiology of the photoreceptor system has been adapted in many different ways to varied habits and habitats. Two basic adaptive features being (i) the change in the length and shape of photoreceptors that are brought by changes in the lighting conditions of the environment (Ferrero *et. al*. 1979) and (ii) the dispersion or concentration of pigment granules (Ali, 1964).

The compound eye which performs well over a wide range of light intensities has several adaptive mechanisms associated with activity during both day and night. These adaptive mechanisms are the primary devices for controlling the amount of illumination which reaches the photosensitive cells. They depend on several photo-mechanical or retinomotor responses involving rapid changes in the pigment distribution, and the action of contractile elements in the lens and in the retina (Hoars, 1987).

Light conditions induce topographical differences within the retina where some type of retinal cells may respond by moving, while some remain in place, even within the same species (Walls, 1942; Tansley, 1965). At night the maximum available light should impinge on the receptors, but where illumination is adequate, another significant problem arises with regard to resolution of pictures because the retinal elements or small groups of cells must be excited separately from different parts of the objects. At night acuity is sacrificed for sensitivity and light is collected from many angles to excite the receptor cells.

Most of the diurnal insects have apposition eyes in which radial movement of pigment granules occurs. On illumination, small pigment granules move towards the rhabdomere, and assemble in the immediate

neighbourhood of the rhabdomere, constituting a longitudinal pupil. The optical formation of this pigment migration and other photochemical effects is presumably to control the amount of light in the photoreceptor organelle *i.e.* the rhabdomere.

Pronounced longitudinal pigment migrations are characteristic features of nocturnal or crepuscular species with superposition eye. The superposition eye is common in insects and crustacean that live in dim habitats or lead a nocturnal life (Nilsson, 1989). The clear zone in the superposition eye allows light that enters the eye through many facets to be focused on more or less one single photoreceptor in the retina (Land, 1981; Stavenga, 2006). In this way superposition eyes have the potential to collect more light than apposition eyes without having to sacrifice resolving power.

The pigment granules near the rhabdomere lead to two effects *viz* (i) it tends to increase the refractive index of the surrounding zone (negating or reducing the total internal reflection and intensifying the surrounding) and (ii) absorb a fraction of the light coming from the organelle. According to Stavenga (1974), the rhabdomere acts as an optical wave guide owing to its high refractive index as compared to its surrounding medium. The rhabdomere/organelle propagates a set amount of energy and this is a function of the difference in refractive indices between itself and its surroundings, as also of its physical dimensions and of its physical dimension and wavelength.

But the mechanism by which the pigment granules alter their position in relation to change in the condition of illumination is not clearly understood and a number of suggestions have been put forward. For example Lerner and Takahashi (1956) have discussed the role of ionic exchange between cell

exterior and interior with regard to pigment migration. Ishibashi (1957), Eugenio (1988) and Matthews and Fain (2001) suggested the importance of intracellular calcium ion level in pigment migration and Fain *et. al*. (2001) reported that change of free Ca^{++} is believed to have a variety of effects in the transduction mechanism. Kinoshita (1963) put forward the view that electro-chemical changes causes melanin migration in fish melanophores; Wiksow and Novales (1969) commented on the role of microtubules in pigment aggregation and dispersion in the scale of fishes.

Pigment migration in the compound eyes of insects is a rapid process. The palisade in *Locusta* requires fifteen minutes of dark adaptation to develop fully (Walcott, 1974). In the cockroach *Periplaneta americana*, where both palisade and pigment granular changes have been observed, anatomical light adaptation requires ten minutes of exposure to light (Butler, 1971).

Adomian and Sjostrand (1975) showed that microtubules must be assembled and disassembled in connections with myoid elongation and contraction. The role of microtubules and microfilaments in retinomotor responses was demonstrated by Warren and Burnside (1978). They found that actin and myosin like-filament are responsible for cone contraction of some marine teleosts. Further the disappearance of the microtubules following colchicine-induced block of cone elongation suggests that microtubules mediate cone elongation through a sliding mechanism. Similarly Anctil *et. al.* (1979) have shown that rod contraction is mediated by microtubules, but not elongation, and suggested that there may exist inter and intra-specific differences in retinomotor (rods versus cones) mechanisms in lower vertebrates.

Different light conditions induce responses in the retina, which then signals the pattern to the brain via the optic nerve. The signals from the individual rods, cones or retinular cells are not transmitted to the brain in isolation from its neighbour, but the signals from neighbouring visual cells may interact with each other, and may be added up together so that their sensitivity is the sum of their respective 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). In this respect Walls (1942) has commented on the confusion surrounding the mechanism that controls photochemical movements.

There are topographical differences within the retina itself, where some types of rods or cones may respond by moving, while others do not move, even within the same species (Walls 1942; Tansley 1965). Munz and Mc Farland (1977) have reported that some predactor fishes have also adapted their cones for low light intensities.

Pigment migration is either controlled by nerves or hormones or both (Fujii, 1969). In this connection, an earlier work by Enami (1955) can be correlated. Enami (1955) proposed a two hormone hypothesis which assumed two antagonistic principles. An example is melanocytes stimulating hormone (MSH) and melanocyte containing hormone (MCH) which are involved in melanin dispersion and concentration. But this hypothesis is not universally accepted, because in fish melanophores MSH is solely responsible for both pigment concentration and dispersion.

Ali (1964a) also suggested that hormones may influence pigment migration. This was based on the fact that when goldfish were dark adapted,

the rods did not move, but the retinal pigment partially expanded. In all probability, intermedin is the actual hypophyseal agent involved in melanophore responses in fishes (Bagnara and Hadley, 1969), though Chavin (1956) emphasised the role of ACTH in melanophore response of fishes.

The role of nervous control of pigment migration was revealed when Von Frisch (1911) reported that the diencephalon of blinded minnows is sensitive to light. Davson (1970) has shown that teleosts possess both nervous as well as hormonal control over their melanophores whereas the former plays the major role. But Osborn (1938) has shown that in catfish both hormonal and nervous factors are equally responsible for pigment dispersion. Works in this regard are those by Day (1941), Fujii (1969), Dey (1980), Deb (1990), Bendang (1998) and Bendang *et. al*. (2004). *etc*.

Veron (1974) opined that in dragonflies, the eye pigment cells act as independent effectors during proximal migration, but the control of the distal migration appears to be complex. Distal migration is reduced significantly in the eyes, as well as in the chromatophores of decapitated insects. Another observation was that in both the pigment cells of the eyes and the epidermal chromatophores, distal pigment migration occurs at similar rates. Thus, the close relationship between visual adaptation and a neurosecretory mechanism is evident.

That the neurosecretory cells represent a connecting link between the nervous system and the endocrine glands has been proposed by Scharrer (1952b). The neurosecretory cells respond to stimuli despite their glandular activity. *i.e*. they receive stimuli from the nervous system and transmit it to endocrine glands. Gabe (1966) had also shown that there is a general

relationship between hypothalamo-neurohypophyseal secretion and general adaptation. Ames and Van Dyke (1952) also showed that neurosecretion is elaborate during alarm stimuli. Therefore, it is logical to assume that neurosecretion is involved in the maintenance of equilibrium of an organism with its surroundings via adaptive processes.

The role of hypothalamic neurosecretion in integrating various physiological events is well documented (Geris *et. al.* 2002 and Sharp and Sreekumar 2002), however reports on the relationship between vision and neurosecretion in insects are lacking. In the present experiment, dark-adapted insects showed large accumulation of neurosecretory material, whereas in light-adapted ones, the quantity was distinctly reduced. This may be due to the fact that rate of discharge is slower in dark, while in light, apart from a faster discharge, the production of secretory material is slower. Presumably, during light, axonal transport of neurosecretory material takes place. Thus, accumulation and discharge of the material may be an optical or visual adaption in response to photic and scotopic condition.

There are, generally two types or classes of neurosecretory products *viz*, (i) neurotransmitters with low molecular weights such as dopamine, 5-Hydroxytraptamine, adrenaline and nor-adrenaline (catecholamines) and (ii) those with relatively high molecular weights such as neuropeptides (biogenic amines). They are responsible for physiological phenomena requiring instantaneous liberation of the neurosecretory products.

In the present experiment the effect of these products have been tested. It has been seen that on application of 5-HT to dark adapted eyes, a reversal to a light-adapted state is seen in both the insects. It can be mentioned that 5-HT

directly affects pigment migration or via release of dopamine. The 5-HT induced dopamine acts as an extracellular messenger to induce a light-adapted cone retinomotor movement (Allen and Burnside, 1986). In this connection, 5-HT has been reported to have melanin aggregating action (Scheline, 1963; Scott, 1965). Moreover, Kato *et. al.* (1982) showed that Ca^{++} dependent 5-HT stimulated dopamine release a carp retina. Desai (1996) also showed that 5-HT induces alterations in the kidney of the fowl *Gallus domesticus*.

Another physiological activator *i.e.* cyclic 3, 5-adenosine monophosphate (cAMP) has been taken up for study in the present work. It is well established that it acts as an intracellular messenger (Robinson *et. al*. 1971), as a regulatory agent in all animals cells (Bonner, 1971), triggers specific responses of the cells (Vander *et. al.* 1980), mimics the effect of intermedin by expanding melanophores (Bagnara and Hadley, 1969). It is also known to play an important role in the visual system. Some notable works are by De Vries *et. al.* (1982) on ground squirrel retinas, Burnside *et. al*. (1982) on photic adaptations of retinomotor movements, Hasegawa and Cahill (1999), on entrainment of retinal photoreceptors, Rey and Burnside (1999) on cone myoid elongation *etc*. In the present experiment it has been shown that cAMP triggers pigment migration similar to light adapted state when applied to dark-adapted eyes of the two insects.

Apart from this, the effect of colchicine, an alkaloid, has also been tested to see its role in the visual adaptation of the two insects. This has been taken up based on reports of its involvement in the visual adaptational process of other animals. On application of colchicine to dark-adapted eyes of the insects, a reversal to that of a light-adapted state has been observed. This

observation is in consonance with the findings of Miller and Cawthon (1974) who found this same effects in pigment granule movement of *Limulus* retinular cells. Colchicine has been reported to disperse melanosomes of the scales of *Fundulus* (Wiksow and Novales, 1969) and also inhibits cone myoid elongation and rod myoid contraction (Anctil *et. al.* 1979).

The rationale behind this is that, the microtubules are important motile processes concerned with intracellular transport in nerve cells, gland secretion and pigment flow of chromatophores (Hoars 1987). Thus Shelanski and Taylor (1968) had concluded that the radially oriented microtubules of retinular cells mediate light and dark induced pigment migration. This was also corroborated by Margulis (1973), who showed that microtubules are dispersed by colchicine. Colchicine specifically binds the tubulin protein of the microtubules subunit (Shelanski and Taylor, 1968).

A photoreceptor is a light trap that that converts radiant energy into nerve impulses. The metabolic activity of the photoreceptor cells is due to mitochondria and other associated organelles, which generate chromoproteins and transmitters affecting synapse. The chromoproteins are subject to destruction by light (Young, 1970) and therefore the transmitters must be steadily passed into synaptic vesicles (Hoars, 1987).

Thus, photopic stimulation results in a co-ordinated response of the visual and the neurosecretory systems. In this connections, Buchanan (1957) postulated that since vision is a special exteroceptive sense, it is logical that neurosecretory materials are discharged under the influence of exteroceptive or visual impulses, which are then transported through the hypophyseal portal

system to the interior hypophysis. In the hypophysis this leads to the synthesis and release of anterior lobe hormone (Palay, 1953).

CHAPTER III

FLUORESCENT COMPOUNDS OF THE EYES

INTRODUCTION

Fluorescent compounds absorb and convert high frequency light energy to lower frequency. They originate in chromatophores or pigment cells, and help in scattering of reflected light (Bagnara *et. al.* 1978), and also act as filters (Lythgoe, 1979). According to Matsumoto (1965a, b), Hama (1963), Obika and Bagnara (1964), *etc*, fluorescent compounds are usually bright in colour which have been actually shown to be imparted by pteridines either solely, or together with other kinds of pigments such as carotenoids.

Pteridines or pterins (Ferre *et. al*. 1991) are naturally occurring, highly fluorescent compounds which represent one of the families of pigmentary colours of insect cuticle, but some of them are also important eye pigments (Chapman, 1969). The first pteridine compounds were isolated and described as xanthopterin by Wieland and Schopf (1925) and as leucopterin by Schopf and Wieland (1926).

The structure of pteridines was reported first by Purrmann (1940). They have a molecular structure of Pyrimido {4, 5-b} Pyrazine. Pteridines are soluble in water and closely related to purines and flavins. Each pteridine consists of a pyrimidine and a Pyrazine ring system. There are two groups of pteridines - pterins which are derivatives of 2 - amino - 4 – oxodihydroxy - pteridine and lumazines which are derivatives of 2 - 4 – dihydroxypteridine (Pfleiderer, 1992). The term pteridine is derived from the Greek word “Pteros’’,n which means wings or feathers (Wieland and Schopf, 1925 and Schopf and Reichert, 1941). These pigments are so named because they were isolated first in the butterfly wings by Hopkins in 1889.

Both coloured and colourless pteridines are present in the chromatophores (Hama, 1963). Dorsopterines (including dorsopterine, isodorsopterine and neodorsopterine) are red, sepiapterines (including sepiapterine and isosepiapterines) are yellowish, while leucopterines are almost colourless. Leucopterines are generally divided into two groups, *i.e.* (i) the blue or violet-blue and (ii) violet fluorescent. The colourless pteridines includes biopterines, rana-chrome 3, xanthopterine and isoxanthopterine (Fujii, 1969). According to Bagnara (1983), the vastly different pigments cells are related to each other due to their similar origin from the neural crest, and can transform from one kind to another, particularly in fishes and amphibians due to their common origin. Hama *et. al.* (1960 a), Obika (1963), Matsumoto (1965a,b), Matsumoto and Obika (1968), and Matsumoto *et. al.* (1969), reported that the brightly coloured pigment cells of the skin, scales, pigment-epithelium-choroid-layer and peritonium contain large amount of pteridines while pigment less tissues have small amount of pteridines.

Hama (1963) and Matsumoto (1965a,b) reported an intimate association of pteridines with yellowish or red pigmentation, emphasizing that coloured pteridines actually serve as functional pigments of both xanthophores and erythophores. The coloured pteridines are usually accompanied by an array of colourless pteridines. Pteridines are primarily contained in the pterinosomes (Matsumoto, 1965a). Melanophores also seem to contain a considerable amount of colourless pteridines comprising xanthopterine, isoxanthpterin, and biopterin (Hama, 1963; Matsumoto, 1965b).

Much attention has recently been directed to the presence of pteridines in the chromatophores of fishes, amphibians, reptiles and mammals. Matsumoto (1965b) reported the presence of pteridines in the early larval stages of cyprinid fish which are responsible for integumentary colour. He found that both larval and adult xanthophore contain pteridines as well as carotenoids and that at least in larval stages sepiapterins act as the actual colouring agent in xanthophores. Matsumoto *et. al*. (1968; 1969; 1971) also reported the presence of pteridines in fishes, Hama and obika (1960), Obika, (1963), Bagnara (1961) and Richards and Bagnara (1967) reported in amphibians; Hama and Fukada (1964), Matsumoto *et. al*. (1971) in reptiles, Rembold *et. al.* (1969) in mammals, Ziegler *et. al*. (2000) also studied the pteridine pathway in the Zebrafish, *Danio rerio.*

In insects pteridines have been identified in some holometabolous (Fuzeau-Braesch, 1972) as well as in hemimatabolous species, in particular, *Oncopeltus fasciatus* (Forrest *et. al.* 1966), *Pyrrhocoris apterus* (Socha and Nemec, 1992), and *Dysdercus cardinalis*, *Dysdercus intermedius* and *Dysdercus nigrifasciatus* (Melber and Schmidt, 1992, 1994, and 1997). Pteridines are also found in *Sallatoria* (Filshie *et. al.* 1975), Homoptera (Banks and Cameron, 1973), Lepitoptera (Descimon, 1975a, b; Shields, 1987) and Diptera (Harmsen, 1970a; Schwinck and Mancini, 1973; Cerioni *et. al*. 1975). They concentrated on the presences of pteridines mostly in skin and scales. Nemec *et. al*. (2003) analysed pteridine-like pigment in the migratory locust, *Locusta migratoria migratorioides*. Mclntyre *et. al*. (1995), reported the presence of pteridine in the head capsule of adult house fly *Musca domestica.*

Reports of the occurrence of pteridines in the eyes of insects are very limited. Pteridines have been detected in the eyes such as *Drosophila* (Gregg and Smucker, 1966), and *Musca* and *Apis* (Dey, 1980). Pteridines have also been reported in vertebrate ocular system. Pteridines have been detected in human beings and some mammals by Raghuvarman *et. al.* (1998). They have also been reported in the choroid of eyes of *Squalus* by Pirie and Simpson (1946), in bovine and rabbit lenses (Cramer-Bartles, 1962) and in the cornea and lenses of *Cyprinus carpio, Clarias batrachus* and *Stromateu argenteus* (Deb, 1990) and in birds, by Oliphant (1988), and Bendang (1998).

In view of the above reports, a study has been made in the compound eye of insects with regards to the occurrence of pteridines and their possible functions in the eyes.

MATERIALS AND METHODS

Paper chromatography has been used for the separation and identification of pteridines and pterines from the eyes of the insects. Paper chromatography has been chosen because it is simple yet yields excellent separation (Matsumato, *et. al.* 1971). Chromatography was carried out with Whatman No 1 paper in dark or dim light because they are photo labile and light cause the decomposition of sepiapterine, lecopterine, and most of 6-substituted pteridines into 6-carboxypterine and other unidentified pteridines (Matsumoto *et. al.,* 1971).

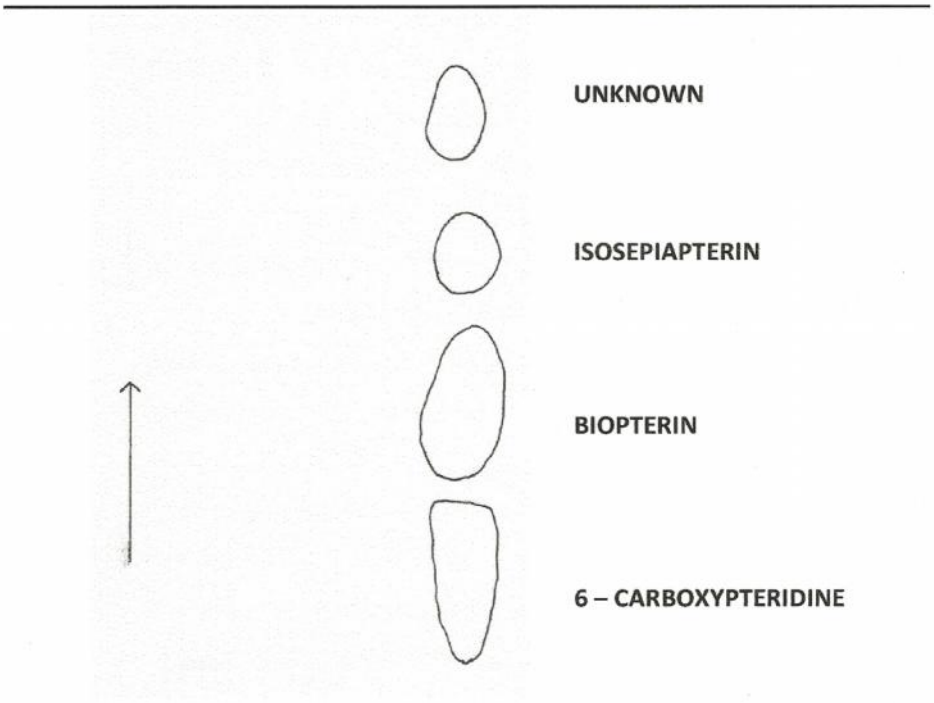
Squash technique: This technique was developed by Hadorn and Mitchel (1951). In this method, after careful dissection, the fresh tissue samples were

squashed directly on the Whatman No 1 chromatographic paper by firmly pressing between two clean microscopic cover slides. The squashed sample were then dried with an electric dryer and the chromatogram run in a solvent made up of n-propanol and 7% ammonia in the ratio 2:1 v/v and dried in dark.

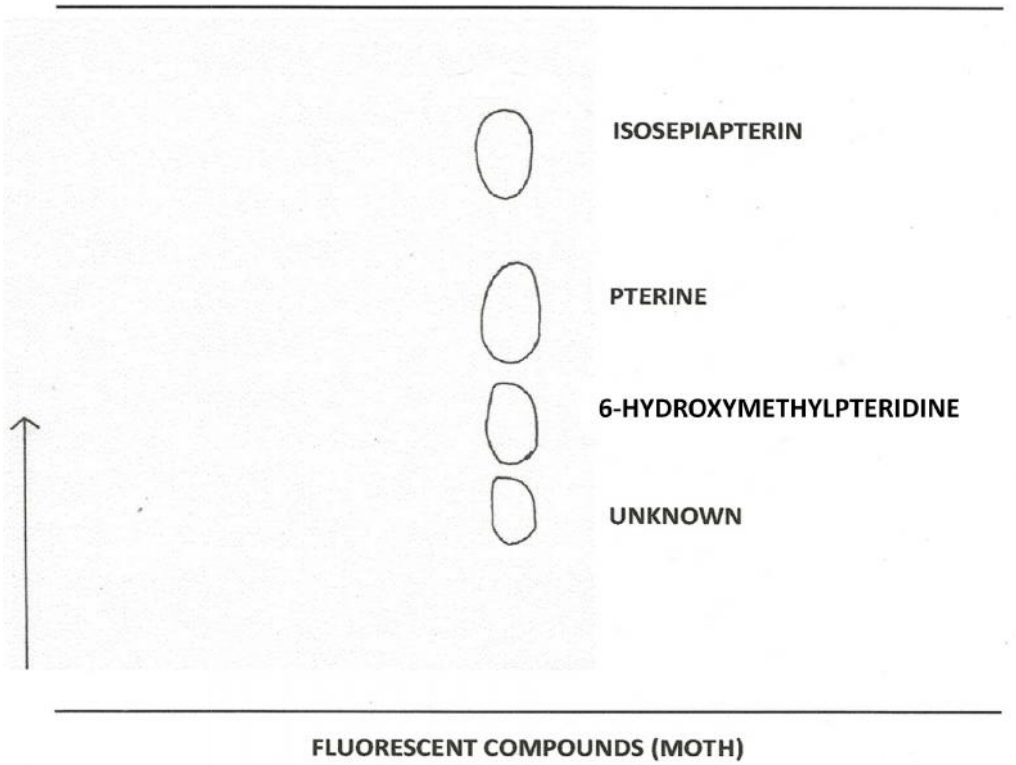
The position and colour of the pteridines were determined by examining the chromatograms under UV lamps and the Rf value of the pteridines were compared with those of the known standard pteridines.

OBSERVATIONS

Examination of chromatograms under UV light reveals that the eyes of the two insects contain highly photolabile fluorescent compounds. Eyes of the butterfly, *Pieris brassicae* contains 6-carboxypteridine, biopterin, isosepiapterin and an unknown compound with yellow fluorescence, while the eyes of the moth, *Philosamia ricini* contains 6-hydroxymethylpteridine, pterin, isosepiapterin, and an unknown compound with blue fluorescence (Figures 5 & 6). All these pteridines and pterine in the two insects were tentatively identified according to their Rf-value and fluorescent colours that they display under UV-illumination (Table 3), and as compared with standards. The spots were recorded on transparent chromatographic paper. The Rf values were obtained by direct measurement.



FLUORESCENT COMPOUNDS (BUTTERFLY)



FLUORESCENT COMPOUNDS (MOTH)

Table 3: Paper chromatographic detection of pteridines and pterines in the butterfly, *Pieris brassicae* and the moth, *Philosamia ricini*.
Solvent: n-propanol: 7% ammonia (2: 1 v/v)

Insect	Fluorescence	Rf value	Identification
Butterfly, *Pieris brassicae*	Blue	0.19	6-Carboxypteridine
	Blue	0.41	Biopterin
	Yellow	0.65	Isosepiapterin
	Yellow	0.81	Unknown
Moth, *Philosamia ricini*	Blue	0.23	Unknown
	Blue	0.35	6-Hydroxymethylpteridine
	Blue	0.45	Pterine
	Yellow	0.65	Isosepiapterin

Ascending paper chromatogram of some standard fluorescent compounds.
Solvent : n-propanol : 7% ammonia (2:1 v/v)

Fluorescence	Rf value	Compound
Violet	0.22	Isoxanthopterin
Violet	0.30	7-Hydroxybiopterin
Violet	0.47	Cyprino-purple
Blue	0.17	6-Carboxypteridine
Blue	0.34	6-Hydroxymethylpteridine
Blue	0.40	Biopterine
Blue	0.44	Pterine
Yellow	0.42	Sepiapterin
Yellow	0.65	Isosepiapterin

DISCUSSION

Pteridines appear to be of universal occurrence in living organisms (Hoars, 1987), and play several important physiological roles. They are co-factors for hydroxylation of phenylalanine to 3, 4-dihydroxyphenylalanine (DOPA), which is the precursor of melanin. They are also cofactors of the enzymes involved in ommochrome synthesis. The auto-chelating potency of simple pteridines enables the formation of products of low solubility under physiological conditions. These products are a part of the general nitrogen pool and a means of inactivation and deposition of toxic products of nitrogen metabolism. They can be deposited in the cuticle and play a role as well in signalling (Harmsen, 1966; Melber and Schmidt, 1997). The redox potential of some pteridines indicates that they play a role in cellular electron transport (Rembold, 1975). Pteridines are important metabolically as co-factors of enzymes associated with growth and differentiation and they act as controlling agents (Chapman, 1969). Pteridines are required as co-factors of many enzymes (Ziegler and Harmsen,1969), and with purines serve as storage forms of excretory products (Harmsen,1966).

According to Uyeda and Rabinowitz (1963), the most important function of pteridines is their fluorescence under UV irradiation which range from violet to red depending upon concentration, pH and molecular species. The characteristic property of fluorescence of most of the pteridines has been used for detection of these photolabile compounds.

Goldsmith (1958a,b), and Langer and Thorell (1966), reported that pteridines act as screening pigments in compound eyes. Most pigments are the so-called cut off filters which absorb wavelengths shorter than certain

values, while the longer wave lengths are transmitted or reflected (Lythgoe, 1979). In *Papilio xuthus*, for example, a UV-A filter (presumably 3-hydroxyretinol) has been shown to shift to spectral sensitivity of certain photoreceptors of the compound eye from the UV-A to the violet region of the spectrum (Arikawa *et. al*. 1999). Mazza, *et. al*. (2010) reported that ommatidia of *Caliothrips phaseoli* compound eye is highly fluorescent when exposed to UV-A of wave length longer than 330nm. According to them this fluorescent compound acts as an internal filter preventing radiation less than 330nm from reaching the photoreceptor cell.

A yellowish pigment has been detected by Walls and Judd (1933), in the vertebrate eye which acts as an effective intraocular filter for blue light. These filters prevent the highly dispersive violet rays of the spectrum thus increasing visual acuity. These pigments also protect the lens from the near UV wave length waves by converting the light to a less harmful wave length by fluorescence (Kuck Jr, 1970). Kuck Jr (1970) and Matsumoto *et. al.* (1971) have reported that lens fluorescence varies widely in various species, and also within the same species and despite their common occurrence in a variety of species, each species exhibits more or less specific pteridine patterns in which each component is clearly defined. It has also been reported 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 wave length (Clayton, 1971). The

energy for emission of light in fluorescence comes from light of shorter wavelength (Lythgoe, 1979).

Cramer-Bartles (1962) isolated a light sensitive fluorescent substance from rabbit and bovine lenses and thought that it may be pteridine. They further suggested that these pteridine-like substances may have a protective role against light-induced effects in the lens and retina. In birds, Oliphant (1988) and Bendang (1988) have detected pteridine in the eyes where the fluorescent compounds act as screening pigments to filter the harmful ultra violet rays and thus protect the eyes. Mc Fall-Ngai *et. al.* (1988) and Deb (1990) detected pteridines in the eyes which play a role in the visual system of fishes and also function to increase visual acuity by reducing chromatic aberration, glare and scattering that may be caused by shorter wavelength visible light. Collier and Zigman (1987) found that lens pigments (pteridines) absorb short wave radiation and serve as a filter to promote visual quality by decreasing chromatic aberration and glare. Lens pigments also protect the retina against damage resulting from the high energy constant of the UV and short wave length blue radiation. Werten *et. al.* (2000) also reported on a cellular retinal binding protein becoming an eye lens ultra violet filter. Rao and Cotlier (1985), reported that pteridine compounds such as neopterin, sepiapterine and biopterin are synthesised by lens, retina and ciliary body-iris of rat and human and these light sensitive pteridines may protect eye tissues against the effects of sunlight in addition to their role in the hydroxylation of aromatic amines. Fluorescent compounds like neopterin are used as biochemical markers of immune activity like in inflammatory diseases. Neopterin is also used in the evaluation of medical conditions as in infectious

diseases, oncology, rheumatology, transplantology, transfulogy, cardiology, neurology *etc* (Nazar and Jankowoska, 2011)

It is also known that in visual systems of vertebrates (Bowmaker, 1977; Neumeyer and Jager, 1985; Partridge, 1989; Kawamuro *et. al*. 1997; Hart *et. al*. 1999; Dyer, 2001), and invertebrates (Ribi, 1979; Arikawa and Stavenga 1997; Arikawa *et. al*. 1999; Marshall and Oberwinkler, 1999; Cronin *et. al*. 2001), internal filters play an important role in sharpening the spectral sensitivities of otherwise broadband photoreceptors (Stavenga, 2002a,b; Stavenga and Arikawa, 2006).

All these reports suggest that pteridines may play a significant role in the visual system of insect eyes.

CHAPTER IV

HISTOCHEMICAL LOCALIZATION OF ASCORBIC ACID IN TH EYES

INTRODUCTION

The visual process is greatly influenced by the generation of energy in the photoreceptor organs of animals. Energy generation in the biological systems 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 this a reducing factor is necessary in the visual system. Many investigator such as Rosner *et. al.* (1938), Pirie (1965), Heath and Fiddick (1966), Dey and Raghuvarman (1984a) and Kern and Zolot (1987) have revealed two such reducing agents ascorbic acid and glutathione in the eyes of animals.

Ascorbic acid was first isolated in 1928 by Szent Gyorgyi. Purified ascorbic acid is a crystalline compound with an amprical formula of $C_6H_8O_6$ and a molecular weight of 176.1. Ascorbic acid, otherwise known as vitamin C is an antiscorbutic, water soluble and heat labile compound. It is one of the most important sugar acids, readily undergoing oxidation to dehydroascorbic acid, and all higher species appear to employ ascorbic acid as a co-factor in certain specific reactions (Lehninger, 1970). It has been postulated that monodehydroascorbic acid, a stable and 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 plays in biological systems (Seib and Tolbert, 1982).

Ascorbic acid is found throughout the eye of many species in concentrations that are relatively high to most other tissues (Birch and Dann

1933; Pirie and Van Heyningen, 1956; Heath, 1962; Kodama *et. al.* 1985; Reiss *et. al.* 1986). The variation of ascorbic acid concentrations in different ocular tissues is species dependent with the variation being greatest for the aqueous humor and the least for the retina. Concentrations upto 3mmol/L have been reported in the aqueous humor and the lens. Cow, man and horse have a concentration of around 1mmol/L (Garland, 1991).

Reiss *et. al*. (1986), analysed the aqueous humor of diurnal and nocturnal mammals. Diurnal mammals, such as the humans, antelope, tree shrew and rhesus monkey, have aqueous concentrations of ascorbic acid 20-40 times higher than in plasma. In nocturnal species such as the slow loris, fruit bat, cat and owl monkey, ascorbic acid concentrations are similar in the aqueous humor and plasma. The correlation between animal behaviour and aqueous humor ascorbate concentration is further obvious from studies on two closely related species of spiny mice by Koskela *et.al*. in 1989.

They found that, of the two closely related species of spiny mice, the diurnal species *Acomys rassatus* has an ascorbic acid concentration in aqueous humor that is 35 times higher than that of the nocturnal species *Acomys cahirinus.* This 35 fold difference in ascorbic acid concentration in these two closely related species may be an adaptation of the eye to protect itself from intense solar radiation. Kronfeld (1952) has also shown that ascorbic acid is found in aqueous humor in concentration 25 times that in plasma in other species, including humans.

Pirie (1946), first reported that the corneal epithelium of rabbits and oxen contain very high concentrations of ascorbic acid. The observations were consistent with the notion that ascorbate enters the aqueous humor, diffuses

through the endothelium into the stroma, and then is concentrated by the epithelial cells. Linner (1951), found that the ciliary body of rabbit cleaved its plasma of ascorbic acid and pumped it into the posterior chamber along with newly formed aqueous humor. This idea was also supported by the work of Reim *et. al.* (1978), who found the concentration of ascorbate in the corneal epithelium of rabbits to be as high as 2 mg/gm wet weight, eight times the concentration in the aqueous humor. Sharma (1989), also reported high concentrations of ascorbic acid in the primate eye. This efficient process is carried out by a sodium-ascorbate co-transporter in the ciliary body (Socci and Delamere, 1988).

Excellent reviews of ascorbic acid metabolism in the eyes have been published by Rose and Bode (1991) and by Delamere (1996). Ringvold et. *al*. (1998), measured ascorbate in the corneal epithelium of different animals and found the highest concentrations in diurnal species that encounter the highest environmental levels of ultraviolet radiation. Ringvold (1980,1996) also reported that ascorbic acid is an excellent absorber of UV radiation between 280 and 310 nm and that it has an absorption curve that roughly matches the absorption curves of protein and nucleic acids in this region of the spectrum. Pitts and Tredici (1971) showed that the absorption spectrum of ascorbic acid is the inverted action spectrum of UV damage to the cornea. These findings, coupled with the finding that diurnal animals have the highest concentrations of ascorbic acid in the anterior chamber (Reiss *et. al*. 1986; Ringvold *et. al*. 1998; Brubaker *et. al*. 2000) *etc* suggest that the eye is able, either through evolution or physiological adaption, to create its own “sunscreen” to protect itself from the deleterious effects of ambient UV radiation.

Muller *et. al*. (1934), found that ascorbic acid of both the aqueous humor and lens of cattle and rabbit decreased with age. Kuck (1961), reported that a gradual decrease in the concentration of ascorbic acid in the lens has seen observed as the animal becomes old. In spite of the wide variations in the concentration of ascorbic acid between tissues and species, the levels was found to be greatly reduced in the ageing lens of all the species investigated so far. Though the rat and cow differ in lens-size and life span, the average ascorbic acid content between both the animals show similar drops in ascorbic acid concentration as they age. However, a decrease in the normal levels of ascorbic acid in tissues is also due to other factors like physiological stress, pollution, infection, diseases and seasons (Lewin 1974; Chatterjee and Pal, 1975; Mauck *et. al.* 1978; Agarwal and Mahajan, 1980).

Ascorbic acid is supplied to the eye from the plasma. It is transported across the blood aqueous barrier by the ciliary body into the aqueous humor (Friedenwald *et. al*. 1944; Kinsey *et. al*. 1947; and DiMattio, 1989). It is generally thought the aqueous humor serves as a source of ascorbic acid for all the other ocular tissues. Accumulation of ascorbic acid in the isolated ciliary body-iris of guinea pig, a diurnal species, occurs by an energy-dependent carrier-mediated process (Becker, 1967; Delamere and Williams, 1987). Studies show that ascorbic acid transport across isolated rabbit ciliary epithelium is unidirectional with uptake being an active Na^+-dependent, carrier-mediated process, whereas efflux is by passive diffusion (Chu and Candia, 1988; Socci and Delamere, 1988). This active transport results in a concentration of ascorbic acid in the aqueous humor. The ciliary epithelium of the rat has the capacity to synthesize ascorbic acid and it is not actively

transported to aqueous humor, but enters by passive diffusion down a concentration gradient. This results in a higher concentration than in the plasma (Ringvold, 1975; Delamere and Williams, 1987; DiMattio, 1989).

Helbig *et. al*. (1990) showed that pigmented ciliary epithelial cells of bovines have two mechanisms for transport *i.e.* active and passive processes, which allows an efficient entry of both oxidized and reduced ascorbate. Bode and Rose (1991) have also shown that the ciliary body contains an NADPH and GSH-dependent activity similar to dehydroascorbate reductase. They opined that these may participate in ascorbic acid recycling.

Khatami *et. al.* (1986) reported that transport of ascorbate into cultured retinal pericytes was a carrier-mediated, facilitated diffusion process with no accumulation of ascorbic acid. Transport was not sodium or energy dependent but was still inhibited by glucose. Studies on the transport of ascorbate into all ocular tissues or cells suggest a close relationship between glucose and ascorbate transport.

Retinal pigment epithelial cells primarily transport the reduced form of ascorbic acid (Bohmer *et. al.* 2001). Dehydroascorbic acid has a much higher Km for transport and will inhibit transport of ascorbic acid to some extent (Khatami *et. al.* 1986). Corneal endothelial cells take up dehydroascorbic acid much faster than ascorbic acid, but uptake of both forms is inhibited by metabolism (Rose *et. al.* 1991). These results suggest that dehydroascorbic and ascorbic acid may be transported by the same carrier system.

Kern and Zolot (1987) concluded from earlier studies on bovine, humans and guinea pig lenses that dehydroascorbate enters by carrier-mediated, facilitated diffusion, while ascorbic acid was not taken up to any

significant extent. In addition, transport was not energy or sodium dependent and uptake was inhibited by cytochalasin B, a compound known to inhibit uptake of D-glucose. This and results of other inhibitor studies indicate that transporters of dehydroascorbic acid and glucose are somewhat related. Accumulation of ascorbate against a concentration gradient was explained in terms of the subsequent reduction of the dehydroascorbate to the lens diffusible ascorbic acid after transport into the lens (Kern and Zolot, 1987). This relation was thought to be by glutathione, which is present in lens at concentrations several times that of ascorbate. A dehydroascorbate reductase described in some ocular tissues is another mechanism that may be involved in reduction of oxidised ascorbate and maintenance of reduced ascorbate, but this activity has not yet been reported in the lens (Bode and Rose 1991; Di Mattio, 1989).

A review of literature on the subject reveals that studies have been confined mainly to the vertebrate eye. No major attempt has been made so far in the photoreceptor of invertebrates, especially of the insects. Some notable works in this respect are those by Joly (1940), who reported the occurrence of ascorbic acid in the blood of the queen termite, *Bellicositermes natalensis*, Haydak and Vivino (1943) in the honey bee *Apis mellifera.* Giroud and Rakoto-Ratsimamango (1936), reported that in the muscle of *Dystiscus,* a very high amount of ascorbic acid, about three times higher than that of vertebrate muscles is present. Day, (1949) has detected ascorbic acid in various number of arthropods such as the silverfish, *Ctenolepisma longicaudata,* the cockroach, *Blatella germanica,* worms of termite, *Nasutitermes exitiosus,* larvae and adult of the mealworm *Tenebrio molitor,*

adult of the flour beetle, *Tribolium confusum*, larvae, pupae and adults of the bowfly, *Lucilia cuprina* wield, larvae and adult of potato moth, *Gnorimoschema operculella*, larvae of the cloth moth *Tineola visellilla hummel* and workers of the honey bee, *Apis mellifera* by means of histochemical test. He detected ascorbic acid in the dermal tissues, blood, circulatory system, alimentary canal, excretory system, storage tissue, muscle tissue, respiratory tissue, nervous tissue, glandular tissue, organs of intermediary tissue, and reproductive system of those arthropods. But in spite of all these, it seems that little attempt has been made on the compound eyes of insects, apart from the work Dey and Raghuvarman (1984a,b) on some insects.

With these in view a histochemical study has been performed on the compound eyes of the butterfly, *Pieris brassicae* and moth, *Philosamia ricini* to ascertain the presence and the possible roles of ascorbic acid in visual physiology.

MATERIALS AND METHODS

Histochemical method (according to Bacchus, 1950): The eyes of insects were separated from the live insects and immersed in 5% silver nitrate with 2 drops of acetic acid per ml at 56^0C for 30 minutes in the darkness. The tissue was then thoroughly washed in several changes of dH_2O for 30 mins and treated with 5% sodium thiosulfate for 30 mins. Again washed in dH_2O and transferred to 70% alcohol. This was then followed by dehydration, clearing and infiltration all in the dark or subdued light .The materials were then

sectioned, mounted on slides following routine histological methods and sections stained with Toluidine blue and counter stained with eosin.

OBSERVATIONS

The principle of the histochemical tests for the detection of ascorbic acid in the biological system is based on the fact that silver nitrate reduces ascorbic acid in tissue sections and produces a characteristics pattern of black granules scattered in the regions where ascorbic acid is present. In the present study rhabdom regions of both butterfly, *Pieris brassicae* and moth, *Philosamia ricini* gave positive reaction *i.e* reveals the presence of dark ascorbic acid granules but no reactions have been observed in the corneal lens which is a modified cuticle. The granules are more dense in the case of the butterfly (Photoplates 19 & 20).

Photoplates 19&20

Photo plate 19: Ascorbic acid granules in the eye of *Pieris brassicae* (400X).

Co (Crystalline cone): No granules present;

Rh (Rhabdom): Dark granules present

Photo plate 20: Ascorbic acid granules in the eye of *Philosamia ricini* (400X).

Co (Crystalline cone): No granules present;

Rh (Rhabdom): Dark granules present

DISCUSSION

The physiological role of ascorbic acid have not yet been described in a manner that is scientifically satisfactory. The presence of ascorbic acid in all eukaryotic organisms suggests fundamental roles, even though in many cases the exact role of ascorbic acid is still not clear. It has been suggested that the most important role of ascorbic acid in cells may not yet be known (Seib and Tolbert, 1982; Englard and Seifter, 1986) or it is only that of a reductant (Padh, 1990) .

The presence of ascorbic acid in each of the ocular tissues certainly argues for an important function. The high concentration of ascorbic acid in ocular tissues combined with its well-known properties as a strong reductant and scavenger of radicals (Bielski, 1982) such as superoxide have been used as arguments that the major function of ascorbic acid in the eye is that of a protector against oxidative damage, particularly light induced damage. Only a few studies have addressed other possible function of ascorbic acid in the eye, functions that are certain to be important and that may be unique for each ocular tissue. The importance of ascorbic acid in vision is indicated by the maintenance of relatively high content of ascorbic acid in the eyes during deficiency, while other tissues show total depletion.

Since the first report of significant amounts of ascorbate in the aqueous humor (Harris, 1933), high concentrations have been observed in many parts of the eye (Heath, 1962), with peak values in the corneal epithelium (Pirie, 1946; Reim *et. al.* 1978). The ascorbate content is higher in diurnal than in nocturnal mammals, both in the aqueous humor (Ringvold, 1980; Reiss *et. al*. 1986; Koskela *et.al.* 1989), and in the corneal epithelium (Ringvold *et. al.*

1998). The amount of ascorbate in different ocular compartments seems adjusted to the suggested ambient radiation dose at each particular level, and from these observations it has been deduced that the ascorbate acts as a UV filter protecting the eye from radiation damage. Reiss *et. al.* (1986), reported that ascorbic acid concentration is known to be very high in the aqueous humor of humans and most animals. They examined the aqueous humor from 22 species of mammals to determine the range of levels and to see if there was a correlation with behaviour. They found a wide range of ascorbic acid levels with most of the animals considered to be diurnal having higher ascorbic levels than the nocturnal ones, and suggested that ascorbic acid in the aqueous humor may play a protective role in those animals who are most exposed to light. Koskela *et. al.* (1989), reported that diurnal mammals have a very high concentration of ascorbic acid in aqueous humor whereas nocturnal ones do not. It has been suggested that high concentration of ascorbic acid is an adaptation similar to pigmentation of the skin that permits the eye to withstand intense solar radiation. Varma (1991), also reported that diurnal animals have much higher levels of ascorbic acid in the aqueous humor than nocturnal ones, suggesting a protective role of the acid against tissue photo-oxidation. This high concentration in the aqueous humor is maintained by an active uptake of ascorbic acid by iris and ciliary body (Chu and Candia, 1988).

During recent years, some experimental support has been presented for this hypothesis. Reddy *et. al*. (1998) compared the effect of UV radiation on DNA strand breaks in the lens epithelium of rat and guinea pig and concluded that high levels of ascorbate in the aqueous humor of diurnal animals may protect the lens against UV radiation under physiological conditions.

However, Williams and Delamere (1986), have pointed out that the lack of antioxidant protection due to low ascorbate in the nocturnal aqueous humor might be compensated for by the high activity of a peroxidase enzyme. Brubaker *et. al.* (2000), also reported that high concentration ascorbic acid could serve to protect the deeper layers of the cornea from radiation damage, such as the basal epithelium layer, the stromal keratocytes, and the corneal endothelium. Ascorbic acid could carry out an energy-absorbing function for the central area of the cornea, a function that can be carried out by melanin pigment in the interpalpebral region of the limbus, an area that is often pigmented, especially in darker races. Ringvold (1980) and Ringvold *et. al*. (2000) reported that the central corneal epithelium covering the pupillary area of the bovine eye has the highest ascorbate concentration, and this ascorbate may act as UV filter shielding internal eye structures from radiation damage.

Giblin *et. al*. (1984), demonstrated a direct correlation between ascorbic acid and hydrogen peroxide levels in the aqueous humor. Barros *et. al.* (2003) have opined that ascorbic acid has a role in the maintenance of the antioxidant state of the eye. It is not clear what role ascorbic acid might play in protecting the cornea from radiation. However, if ascorbate is evenly distributed throughout the corneal epithelium, it alone would absorb 77% of the incident radiation at wavelengths likely to be most dangerous to the genetic material of the basal layer. Ascorbate could also protect the epithelium of the lens because before reaching the lens, 99.96% of radiation at 260 nm would have been absorbed by ascorbate in the intervening structures.

Most studies on the role of ascorbic acid in retina and lens have focused on the protective effects of the molecules. Ringvold (1980) proposed

that it provides protection against ultraviolet irradiation. Ascorbic acid clearly provides protection against light-induced loss of retinal pigment epithelial cells and photoreceptor cells (TsoMom *and* Woodford, 1983; Organisciak *et. al*. 1985; Organisciak *et. al*. 1990), and in the lens, ascorbic acid prevents the riboflavin-mediated, light-induced damage to the cation pump (Varma *et. al.* 1979; Varma and Richards *et. al.* 1988), and decreases the photoperoxidation of the membranes. Varma *et. al.* (1982), also suggested that that it protects the crystalline lens from photoperoxidation and helps to prevent cataracts. Supplementation of guinea pig diets with ascorbic acid appeared to decrease ultraviolet and heat-induced damage to lens protein (Blondin *et. al.* 1986; Tsao *et. al.* 1990). Rose *et. al.* (1998), suggested that ascorbic acid, because of its high concentration in the eye, is thought to be a primary substrate in ocular protection.

Boyd and Campbell (1950), Levinson *et. al*. (1976), Pfister and Paterson (1977) as well as pfister *et. al.* (1978) reported that ascorbic acid reduces the ulceration of cornea following alkali induced burn in rabbit. It effects the metabolism of arachidonic acid in the iris, ciliary body and cornea. Birch and Dann (1933) and Schwatrz and Leinfelder (1955) have ascribed the role of ascorbic acid in redox systems. It has been postulated that ascorbic acid operates as a redox system in ocular tissues and is linked to the activity of the hexose monophosphate shunt, thus contributing to the maintenance of reduced pyridine nucleotide levels (Reddy, 1971; Varma *et. al.* 1987). The pentose pathway is the main source of energy in the lens and cornea, where $NADP^+$ is made available for the enzymes of the pathway through the respiratory link between the reducing factors. The oxidation of NADPH and

$NADP^+$ is accomplished through ascorbic acid and glutathione oxidation-reduction systems, catalysed by two enzymes dehydroascorbic acid reductase and glutathione peroxidise, with consequent production of hydrogen peroxide. (Anderson and Spector, 1971)

Numerous studies in many cell types and tissues have defined roles for ascorbic acid in protein and catecholamine biosynthesis, in collagen, lipids and iron metabolism, in hormone activation, and as an antioxidant (Englard and Seifter 1986; Padh, 1990 and Niki, 1991) and as an inhibitor of polymorphonuclear leucocyte activity (William *et. al.* 1984). Ascorbic acid has a role in recycling vitamin E in membrane (Tappel, 1968; Packer *et. al.* 1979) and also interact with selinum (Cupp *et. al.* 1989). Ascorbic acid decreases the membrane damage found in lenses of diabetic rats (Linklater *et. al.* 1990). Brewitt and Clark (1990) *in vitro* studies indicated an important role of ascorbic acid in lens development and maintenance of transparency during development. Ascorbic acid is also thought to rid the lens of oxygen, thus decreasing the probability of oxidative injury (Pirie, 1965; Eaton, 1990). Electron microscopy observations have revealed cellular atrophy and damage of nerve cells due to hypovitaminosis C (Sulkin and Sulkin, 1975). This has also been corroborated by Malik *et. al.* (1995).

Chatterjee (1973) and Chatterjee *et. al.* (1975) reported that insects, invertebrate, fishes and certain bats and birds cannot synthesis ascorbic acid, and consequently the eye takes up ascorbic acid by an energy-dependent active transport mechanism (Nicola *et. al.* 1968). According to Chatterjee *et. al*. (1975), and Sharma (1989), the high level of ascorbic acid in ocular tissues is maintained by an active transport of ascorbate from the plasma

across the blood or aqueous barriers and this stimulates ion transport by inhibiting the 3,5-cyclic AMP phosphodiesterase activity, which consequently leads to increase in the level of cyclic AMP (Buck and Zadunaisky, 1975). This high concentration of ascorbic acid in the ocular tissues may be to maintain a high-energy demand, and also to modulate some co-enzymatic, as well as non-enzymatic reactions (Rawal and Rao, 1977). Omaje *et. al.* (1982) have also reported that ascorbic acid is also taken up by several tissues by an energy-dependent and Na^+ sensitive process, which according to Cole (1970), might also possibility play some role in the active transport of ascorbate across the ciliary epithelium. Recently Bohmer *et. al.* (2001) have reported that transport of ascorbic acid requires a Na^+/K^+ATPase.

All these reports give some ideas regarding the way in which ascorbic acid may play some roles in the photoreceptor of vertebrates. Now the compound eyes of arthropods, as already mentioned, are somewhat different from the vertebrate eye, as far as the structure is concerned. But in spite of this difference, it seems that chemically, and also functionally, both the arthropod and vertebrate eyes are more or less similar. As for example, it has been reported that, the arthropod compound eyes use vitamin A and retinene-complex for the visual pigment chemistry as is done in vertebrates. In addition to that the rhabdomere microtubules of arthropods are very similar to that of vertebrate retina rod outer segments sacs (Wolken, 1968).

Thus, it is reasonable to presume that the functions which have been suggested for ascorbic acid in the photoreceptor of vertebrate may similarly also be applicable to the compound eyes of arthropods. It is reasonable to assume that ascorbic acid might be equally significant in the visual processes

of insects. Even the high content of ascorbic acid in the aqueous humor, cornea as well as in the lens of nocturnal and diurnal forms might help in some way or other in adaptation. Taking into consideration all the above mentioned reports and correlating the findings of the present study, it can be reasonably assumed that ascorbic acid might be equally significant in the visual processes of the two insects studied.

GENERAL DISCUSSION

Photoreceptor organs though differing morphologically operate as transducers of light energy into membrane potential. The photoreceptors of the compound eye of insects are of considerable interest in elucidating the visual system of animals. Compound eyes are image - forming eyes which are particularly efficient in detecting movements in their total visual field. In addition, many arthropods exhibit orientation relative to the direction of polarized light, which suggest the existence of a polarized light analyzer in the eyes. Thus to understand the functioning of the compound eye in response to light stimulus, it is important to study its structure and biochemistry.

In the present investigation, certain physiological and histochemical studies have been performed in the compound eyes of the butterfly, *Pieris brassicae* and the moth, *Philosamia ricini,* and some interesting observations have been made.

In the lens cuticle of the eyes of *Pieris brassicae* and *Philosamia ricini*, a protein-carbohydrate complex, *i.e.* acid mucopolysaccharides has been detected by histochemical and biochemical methods. Three sugars *i.e.* lactose, galactose and xylose were present in *Pieris brassicae* while galactose, xylose and rhamnose were present in *Philosamia ricini*. Moreover the mucopolysaccharides extracted from the compound eyes resemble more of chondroitin 4-sulphate in terms of Rf- value.

The acid mucopolysaccharides may play an important role in the visual excitation due to the fact that they act as selective ion barriers. It may be noted that they are present not only in the corneal lens but also in the crystalline cone, which are in close association with the inner rhabdomeres - the actual sites of photochemical reactions, the products of which depolarize the

membrane of the reticular cells and initiate impulse formation. Further mucopolysaccharides may play a role in the transparency and elasticity of lens cuticle. In this context, it is mentioned that during corneal development of vertebrates, rise in transparency of stroma was found to be associated with the occurrence of mucopolysaccharides.

Retinal pigment migration in response to various physiological states has been studied in the present work. In the light adapted insects, there is radial movement of pigment granules whereas in the dark adapted state, there is peripheral distribution of the pigment granules. Moreover it has been observed that topical administration of 5-hydroxytryptamine, colchicine and cyclic AMP in the dark-adapted eyes stimulate pigment migration similar to that of light adapted state. Almost complete migration (dispersion) of retinal granules similar to controlled light adapted eyes occur. It appears that pigment migration is more intense in the treated eyes and total masking of visual cell can be seen. This may be stimulated due to dopamine release which acts as intracellular messenger and by disturbing the microtubules.

In addition to this, a study on the effect of light and darkness on neurosecretion of insects has been performed. It has been observed that, both in butterfly, *Pieris brassicae* and moth, *Philosamia ricini*, when the insects are dark adapted there is a large accumulation of neurosecretory material in the form of compact neurosecretory granules. In contrast to this, when the insects are light adapted, it has been observed that there is a significant reduction of the neurosecretory material presumably due to the axonal transport of neurosecretory material. The cytoplasm now appears lightly stained, and the

shape of the cell becomes slightly irregular. But in spite of all these it is not possible to state definitely which factor is responsible or how it is affected.

Fluorescent substances in the insect eyes is another aspect where some interesting observations have been made. It has been known that the compound eyes of the insects show blue, blue-green or yellow fluorescence when irradiated with UV light. In the present study pteridines have been extracted from the compound eyes. The eyes of *Pieris brassicae* contains 6-carboxypteridine, biopterin, isosepiapterin and an unknown compound with yellow fluorescence, while the eyes of *Philosamia ricini* contains 6-hydroxymethylpteridine, pterin, isosepiapterin, and an unknown compound with blue fluorescence. Presence of the fluorescent compounds - pteridines and pterines, in the eyes of the two insects is significant in terms of visual phenomenon because they serve as screening pigment to filter the harmful ultraviolet rays.

Another important aspect of the present investigation is the study of ascorbic acid in the eyes of the two insects. The rhabdom regions of both *Pieris brassicae* and *Philosamia ricini* revealed the presence of dark ascorbic acid granules but no ascorbic acid was observed in the corneal lens which is a modified cuticle. Ascorbic acid, as already mentioned, might play an important role in energy generation of the insects photoreceptors. Ascorbic acid may also have some role in synthesis of mucopolysaccharides which are specifically present in the lens cuticle. According to Mayes (1988) its role in collagen synthesis is well established, and collagen is an essential constituent of cornea, where collagen and mucopolysaccharides are closely associated.

Therefore, its role in mucopolysaccharides synthesis will be a worthwhile area of study.

Thus it can be concluded that AMPs, ascorbic acid, fluorescent compounds like pteridines all have significant roles in the physiology of vision coupled with neurosecretory mechanisms in the two insects studied. But further research work is necessary for comprehensive understanding of the phenomenon of vision.

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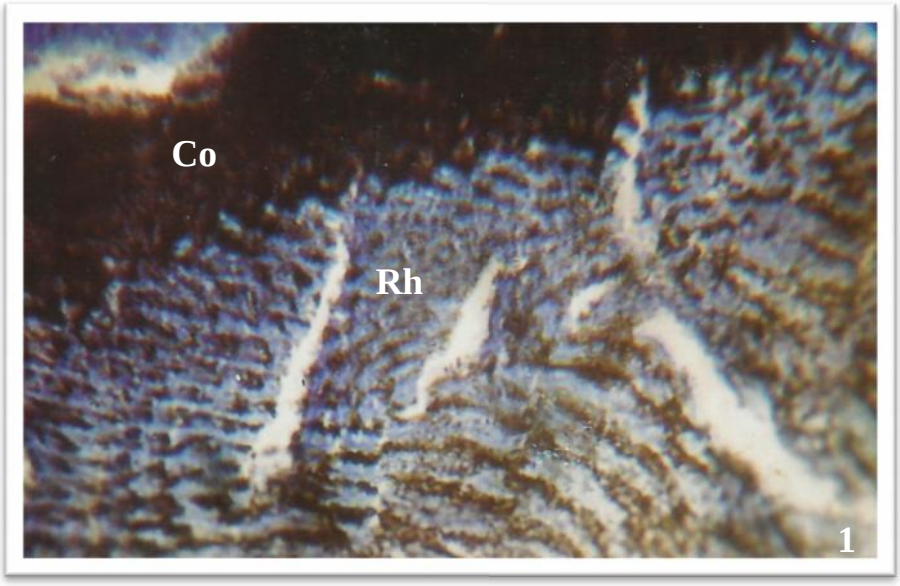
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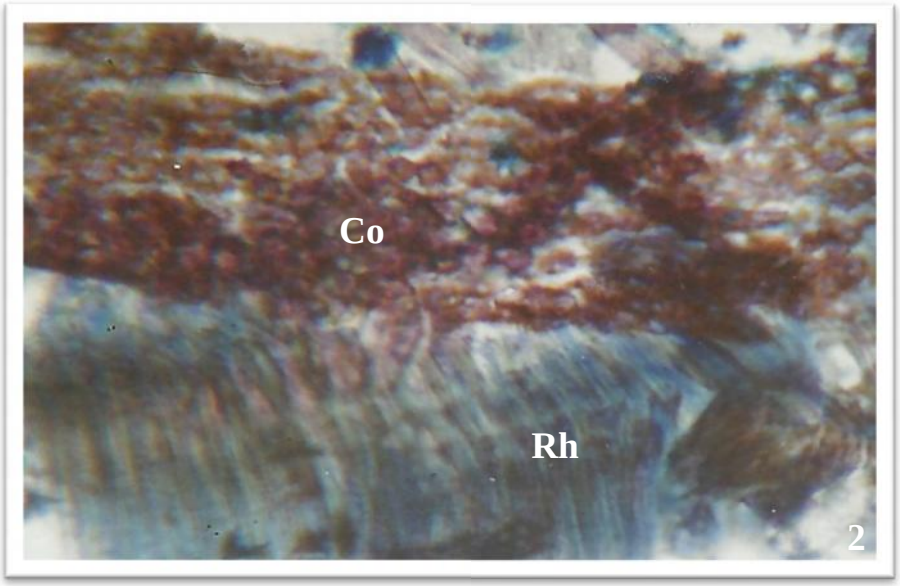
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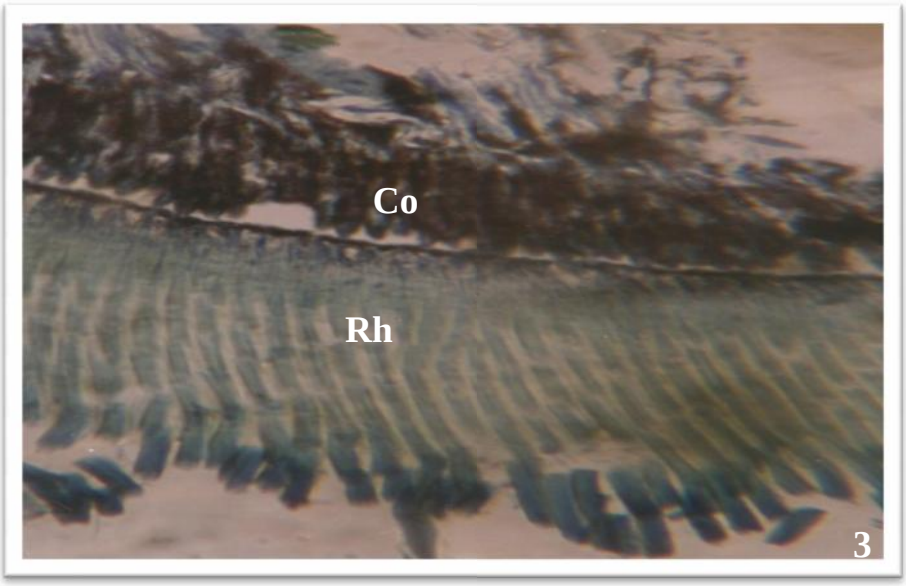
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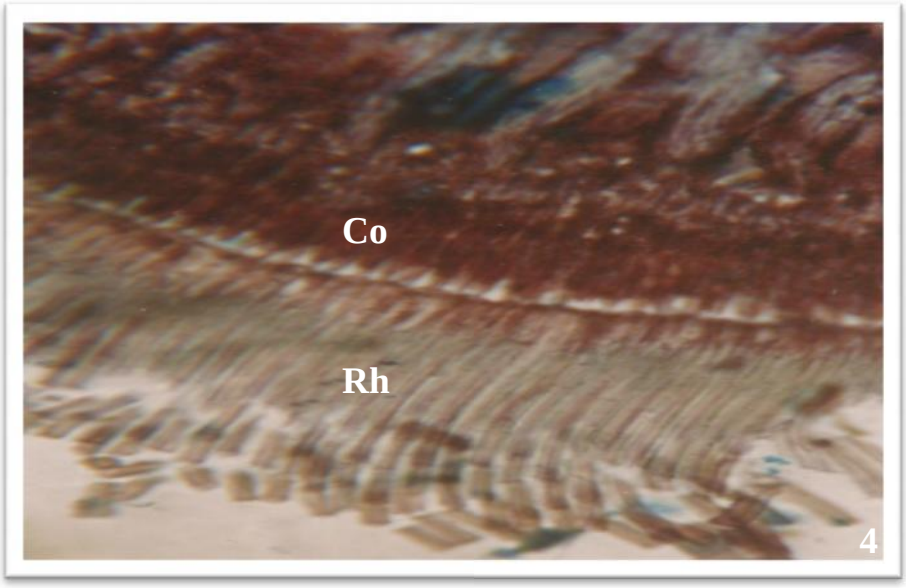
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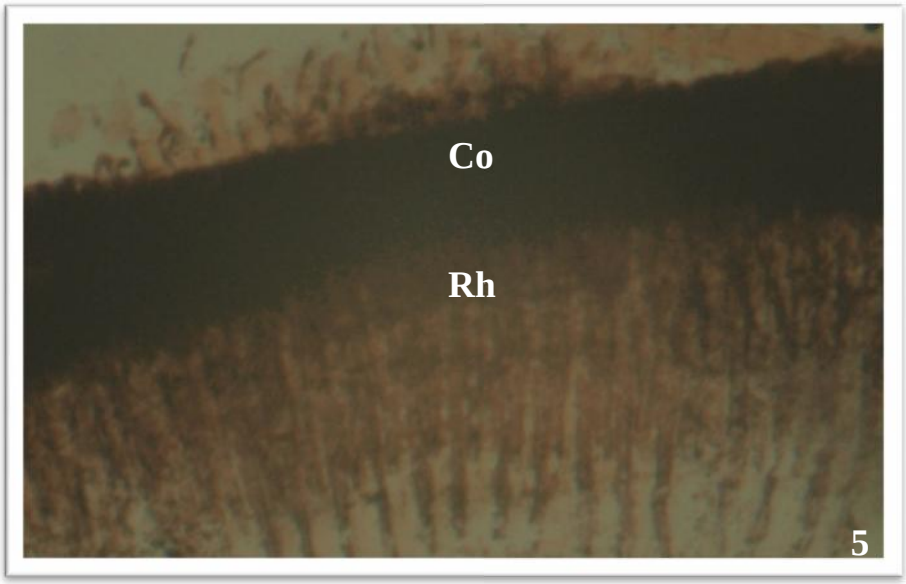
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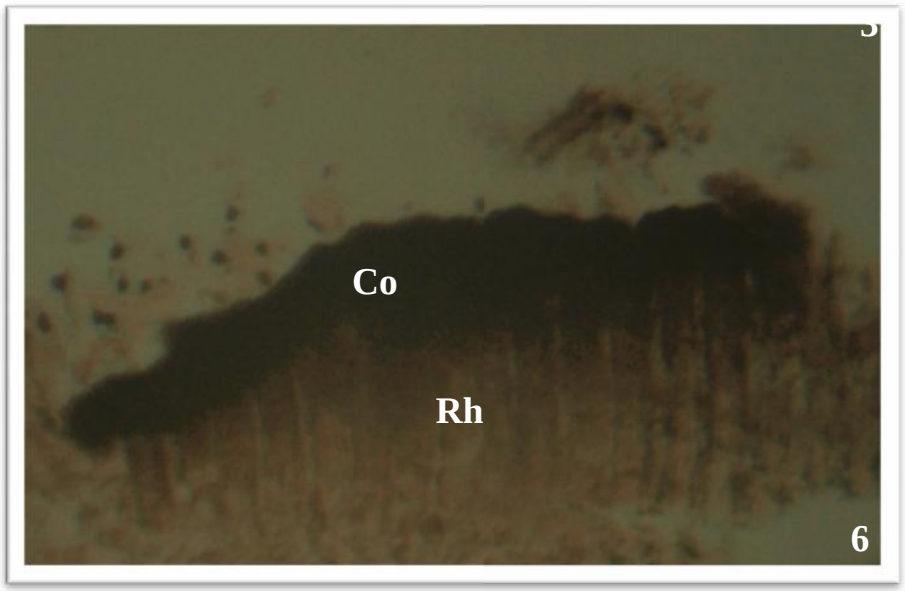
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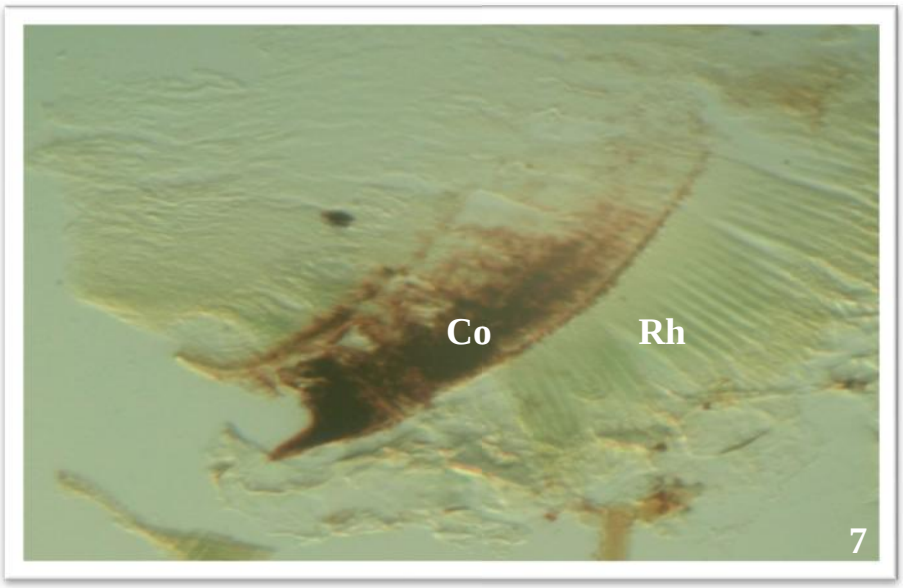
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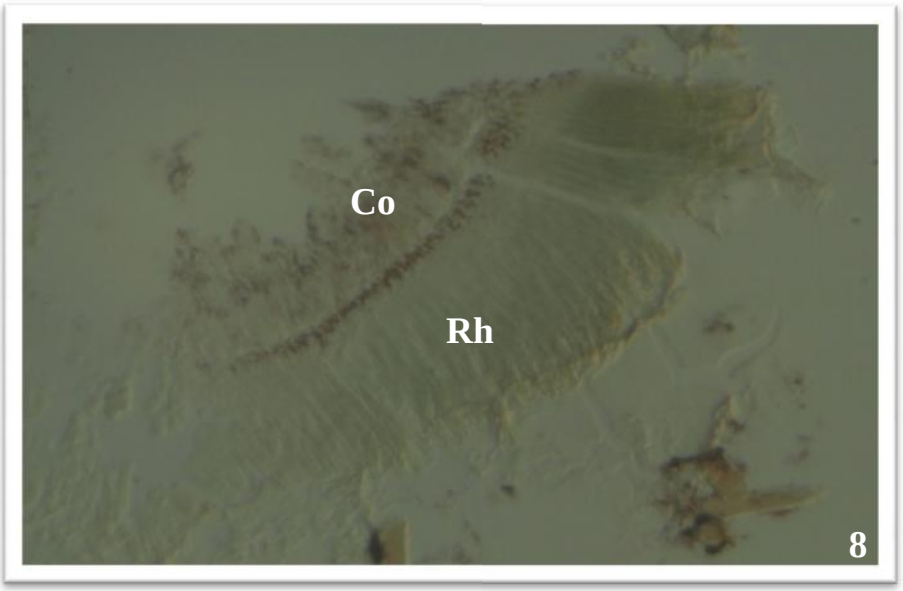
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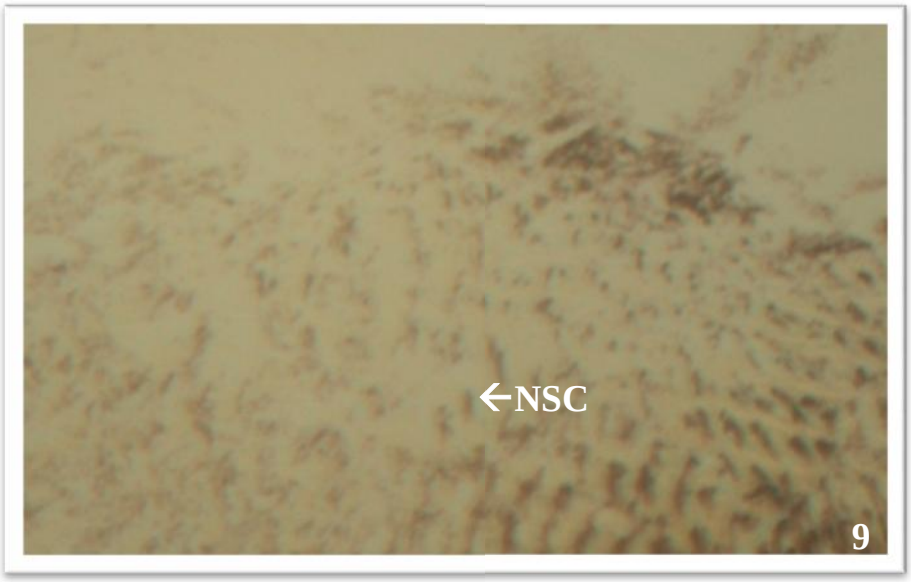
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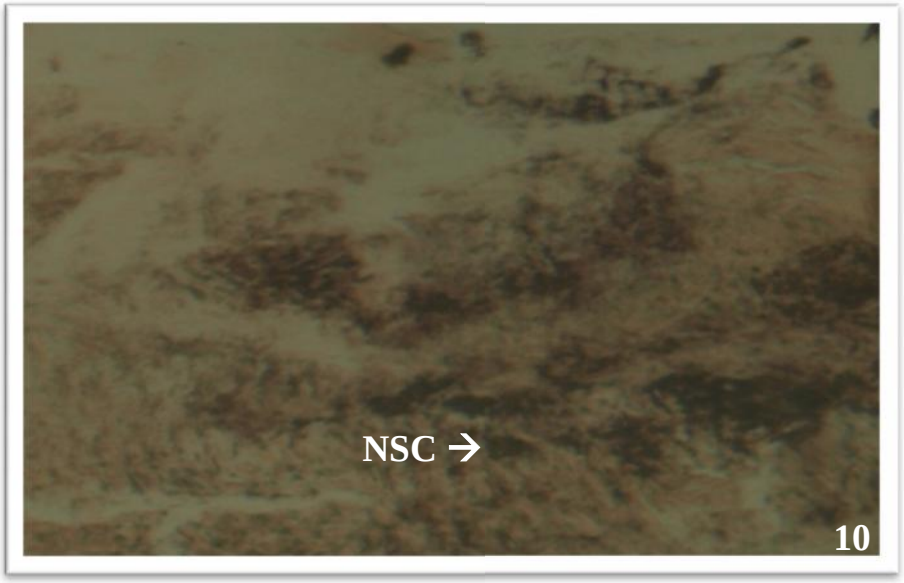
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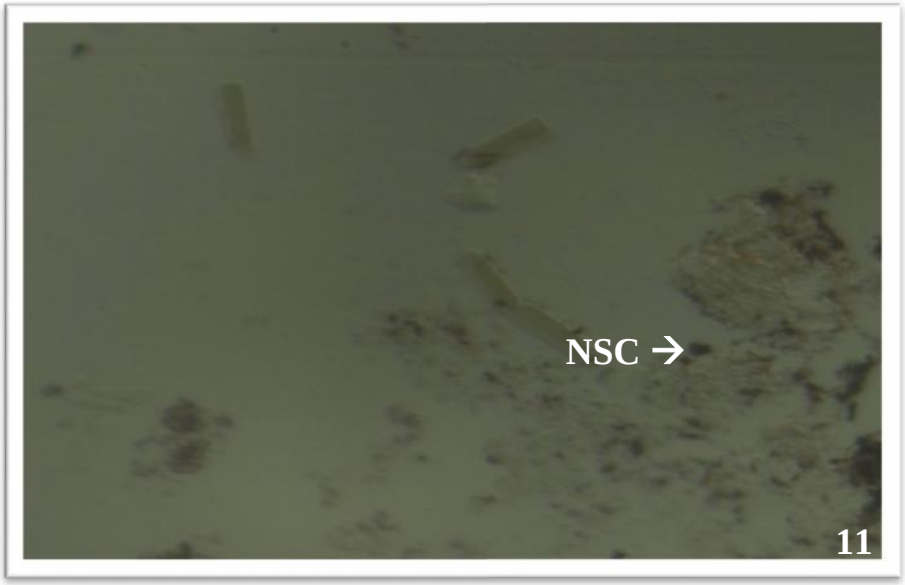
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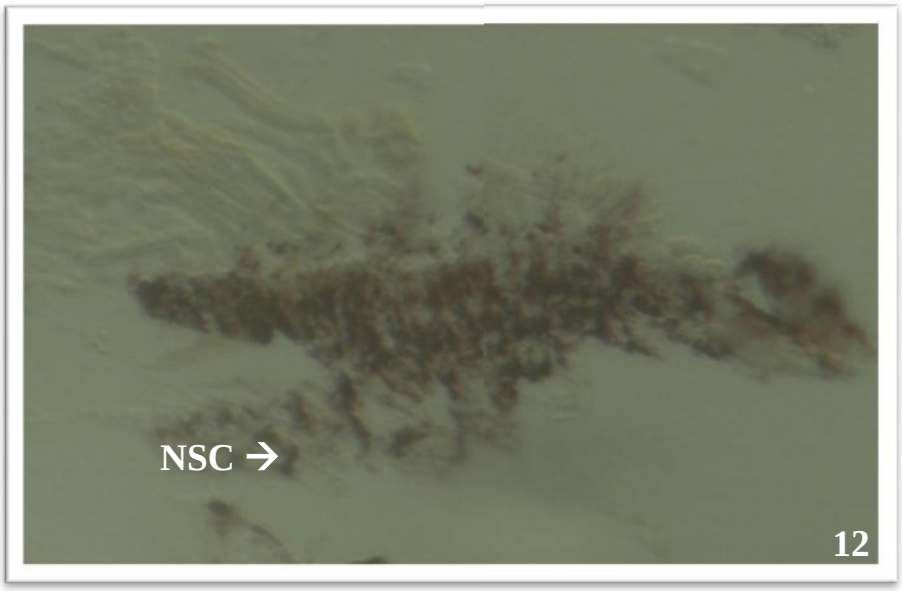
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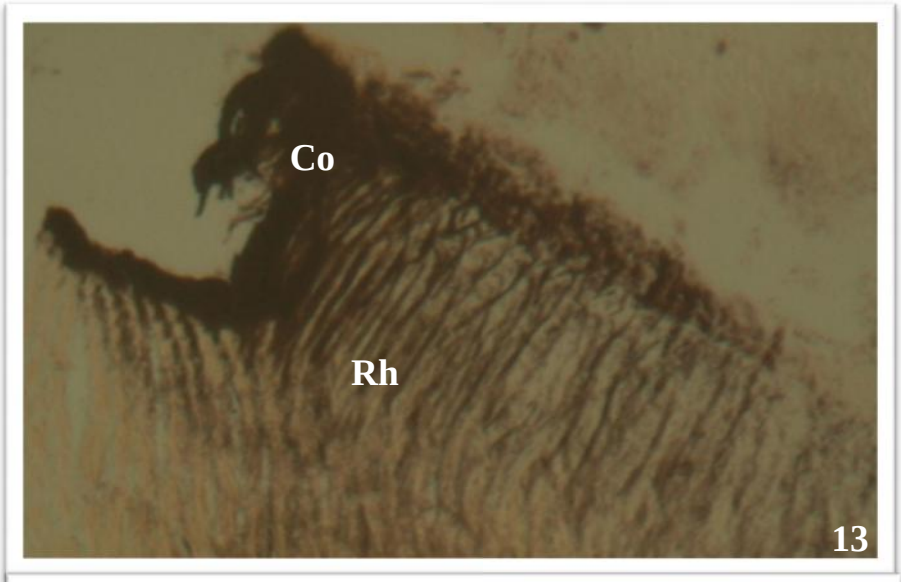
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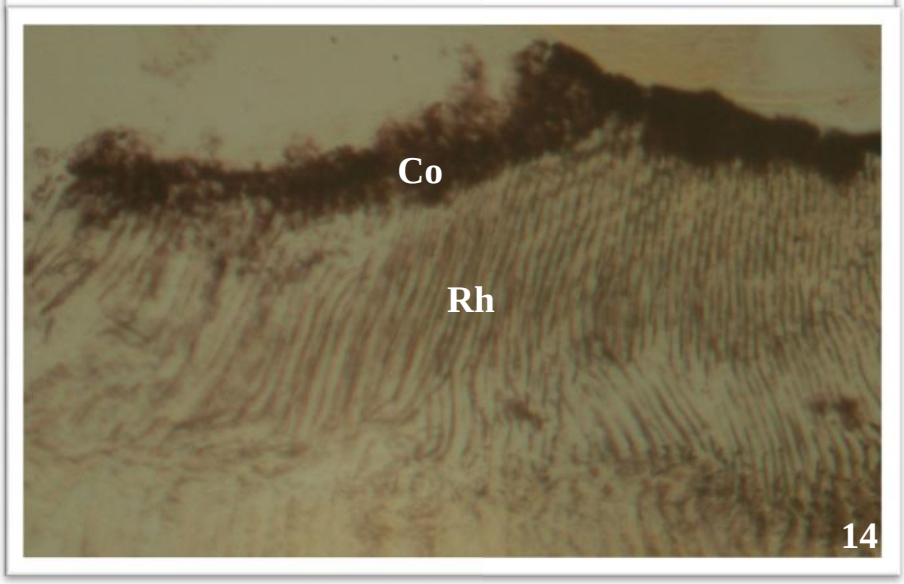
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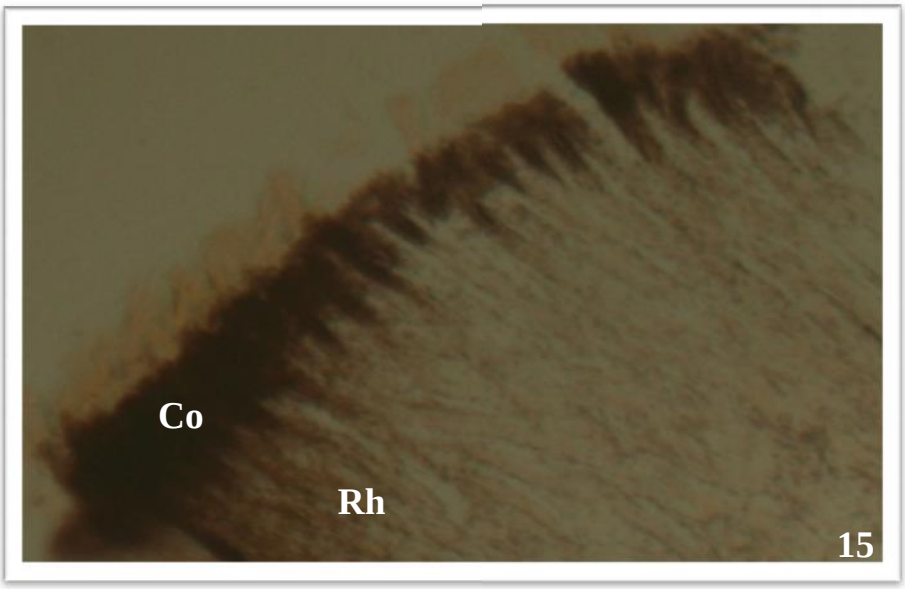
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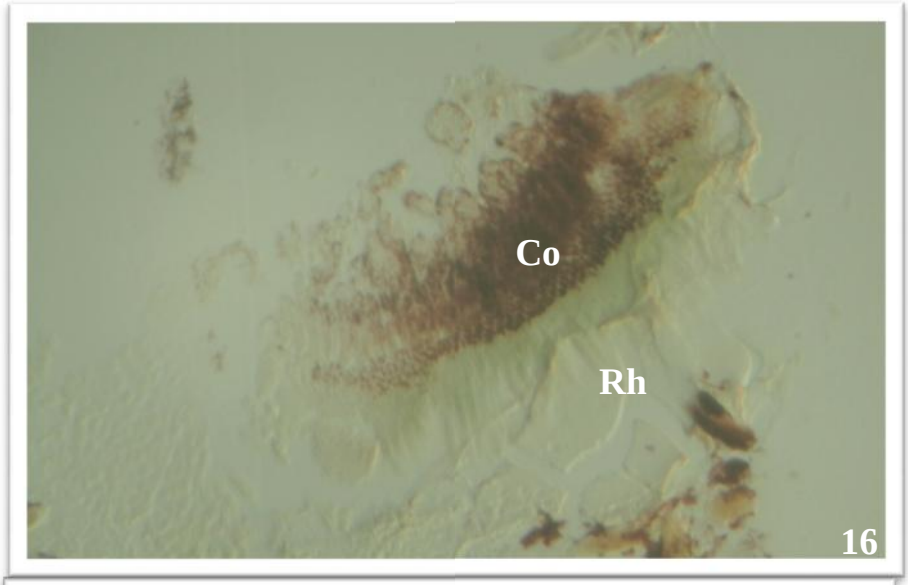
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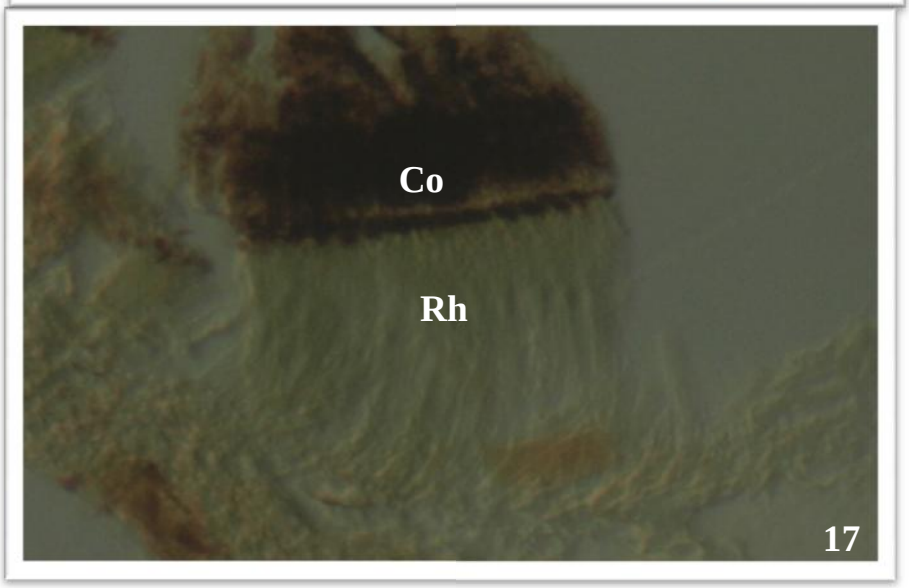
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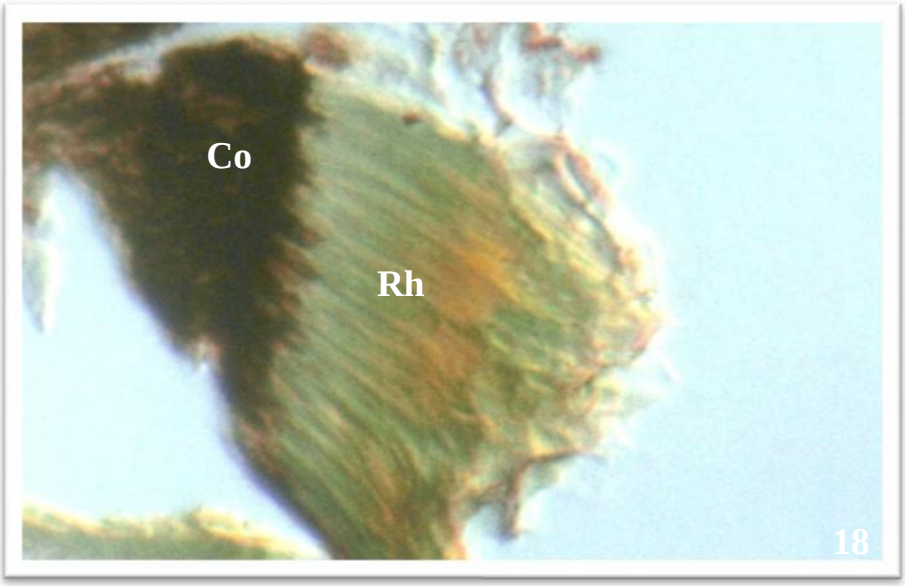
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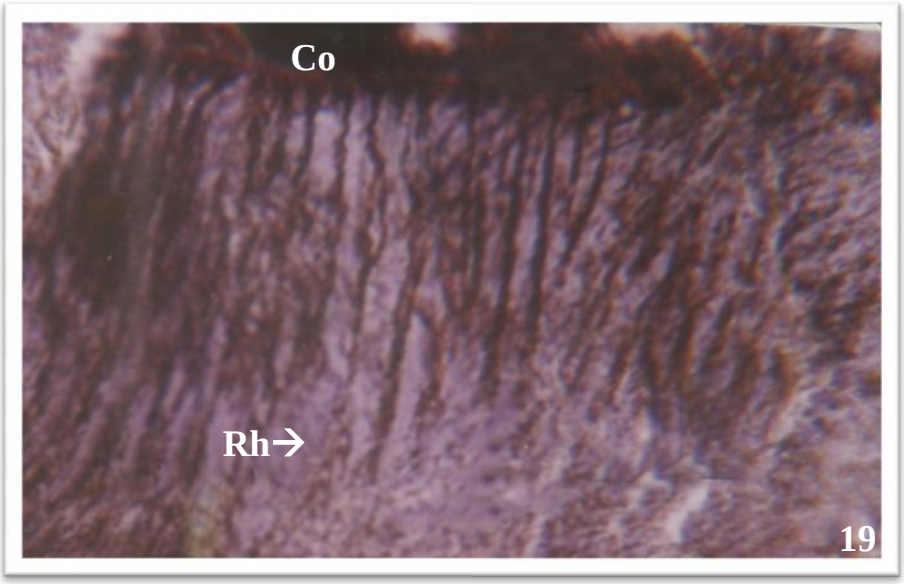
Co
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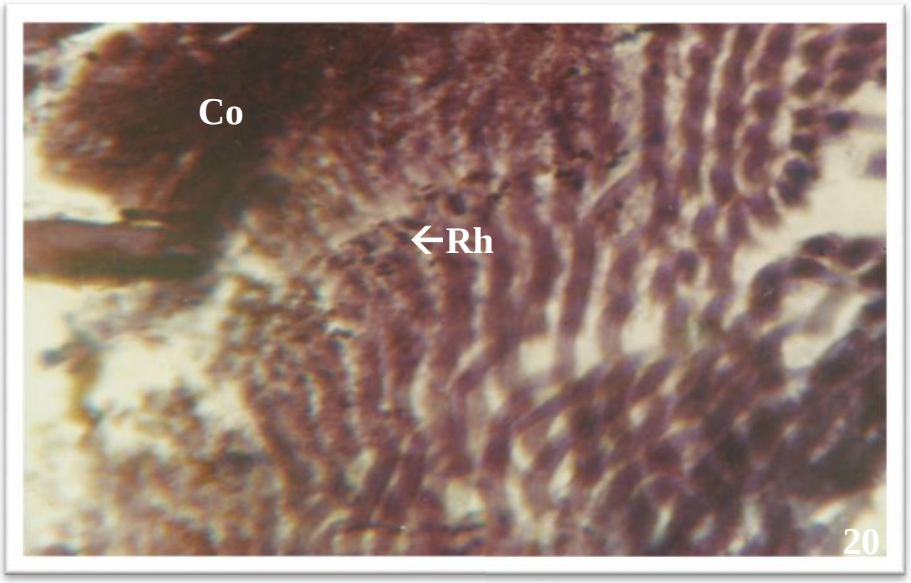
Co
←Rh
20