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Acid mucopolysaccharides in the eyes of the butterfly, *Pieris brassicae* and the moth, *Philosamia ricini*

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ABSTRACT:

Mucopolysaccharides were detected by histochemical methods in the crystalline cones of both the butterfly (*Pieris brassicae*) and the moth (*Philosamia ricini*) commonly known as large cabbage white and eri silk moth respectively, but they were absent in the rhabdome part of both the insects. The mucopolysaccharides were extracted by biochemical method and the subsequent electrophoretic analysis revealed that they were similar to chondroitin 4 – sulfate. Moreover, chromatographic analysis revealed different sugar components in the eyes of the two insects. It is concluded that acid mucopolysaccharides have structural and other physiological roles in the visual apparatus but no part in light and dark or photoperiodic adaptations.

Keywords:
Mucopolysaccharides, Rhabdome.

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INTRODUCTION

Kennedy and White (1983) introduced the term "mucopolysaccharides" to describe 2-amino-2-deoxyhexose containing polysaccharides of animal origin and occurring either as free polysaccharides or as their protein derivative. They can be those that contain uronic acid and those that are neutral. Acid mucopolysaccharides (AMPs) come under the second class. Acid mucopolysaccharides (AMPs) may be further sulphated (SMP) or non sulphated *e.g.*, chondroitin sulphate and hyaluronic acid respectively. These terms *i.e.*, AMPs and SMPs (sulphated acid mucopolysaccharides) appear to provide an adequate description and also have the added advantage of continuous use (Jaques, 1977).

Meyer (1938) coined the term "mucopolysaccharides" to include all substances with similar physico-chemical properties isolated from connective tissues. Later on, the terms "glycosaminoglycans" "glycoproteins" and "mucoproteins" were used, but they failed to distinguish between bacterial polysaccharides and antibiotics containing amino sugars. But these terms are still found in literature.

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.

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).

It is to be noted that glycosaminoglycans always come 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 units.

Presence of acid mucopolysaccharides in the visual system of vertebrates are 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), in 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 apparatuses 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), *Palaemon* sp, *Limulus polyphemus* and *Macrobrachium birmanicum* (Dey *et al.,* 1978), *Musca domestica*, *Apis cerana indica* (Dey, 1980)

Acid mucopolysaccharides play several important physiological roles owing to their capacity to bind and hold water (Ogston, 1970; Ogston and Wells, 1972; Wells, 1973b). They serve as natural lubricants in the joints, impart elasticity to connective tissue, and are a component of cartilage and ligaments. They are also involved in support and motor functions, and also have bactericidal properties. It is also known that many diseases such as collagenosis, mucoplysaccharidosis, and rheumatism *etc* which are correlated with aging, are also a result of disorders in mucopolysaccharides metabolism which lead to compositional changes of connective tissue and of the body fluids.

With this view a study was done in the compound eye of the insects *viz.,* butterfly, *Pieris brassicae* and moth, *Philosamia ricini* with regards to the occurrence of acid mucopolysaccharides, and their possible functions in the eyes have been discussed.

MATERIALS AND METHODS

The eyes were separated from 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 three hours and dried. The 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°C for 24 hours. After incubation, pH of the mixture was brought to 11 with Conc. NaOH and maintained for six hours at room temperature. Then the pH was brought to 6 by the addition of HCl and the mixture was centrifuged for 15 minutes at 3000rpm. To the supernatant, 0.1 ml of 2M NaCl and two volumes of ethanol were added and kept overnight at 5°C. The mixture was centrifuged for 15 minutes at 3000 rpm and the precipitate was 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°C with a layer of toluene. After incubation, 0.1 ml of 2M Nacl and two volumes of ethanol were added to the solution and kept overnight at 5°C. It was then centrifuged for fifteen minutes at 3000 rpm and precipitate was collected and dried. The resultant powder was dissolved in 0.5 ml of water, heated at 100°C for two minutes and analyzed by paper chromatography and electrophoresis.

Chromatography*:*

The extract was hydrolyzed with 6N HCl at 100°C 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 were 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 ammonium hydroxide for 10 minutes and then washed in running water and dried at room temperature.

Electrophoresis:

This was according to the method as described by Leitner and Kerby, (1954). Streaks of the acid mucopolysaccharide samples were applied 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 2-3 times in H_2O. The strips were then dried at room temperature.

OBSERVATIONS

Histochemical observations:

Lens cuticle of the butterfly, *Pieris brassicae*:

When the sections of the eyes were stained with toluidine blue, the cornea and crystalline cone became purple in color showing metachromasia (Photoplate 1) *i.e.*, indicating the presence of acid mucopolysaccharides, while the region of the rhabdom was orthochromatic (blue in colour) and therefore devoid of acid mucopolysaccharides. Similarly, when the eyes were stained with alcian blue, the lens and crystalline cone became purple in colour (Photoplate 2) which indicates the presence of acid mucopolysaccharides. (Fig 1)

Lens cuticle of the moth, *Philosamia ricini*:

When the sections were stained with toluidine blue, the cornea as well as 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 *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 indicating the presence of AMPs, while the rhabdom became blue in colour which indicates absence of AMPs. (Fig 2)

Biochemical observations:

Chromatographic analysis of the acid mucopolysaccharides extract showed the presence of three sugars *viz* lactose, galactose and xylose in case of *Pieris brassicae* and galactose, xylose and rhamnose in the case of *Philosamia ricini* (Figure 3 and 4; Table 1 and 2).

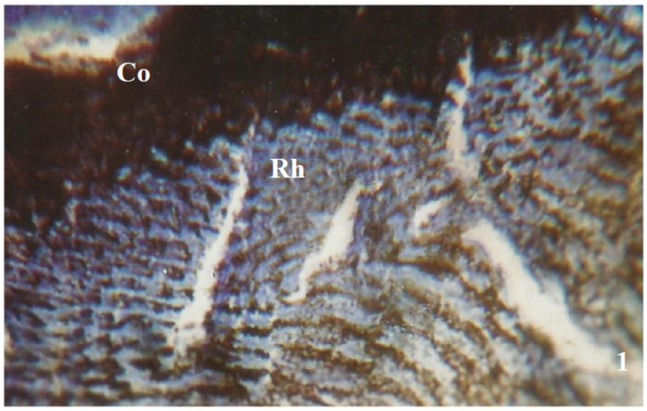
Electrophorectic movement pattern of the crude extracts of the acid mucopolysaccharides from the eyes of *Pieris brassicae* and *Philosamia ricini,* when compared with several standard acid mucopolysaccharides showed that the mucopolysaccharides extracted resemble chondroitin 4-sulfate (Figure 5 and 6; Table 3 and 4).

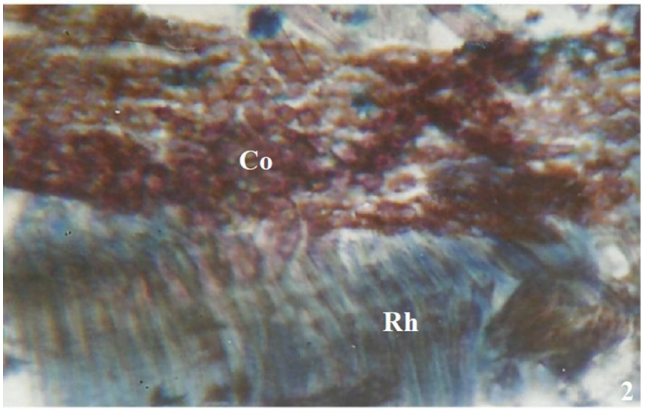
DISCUSSION

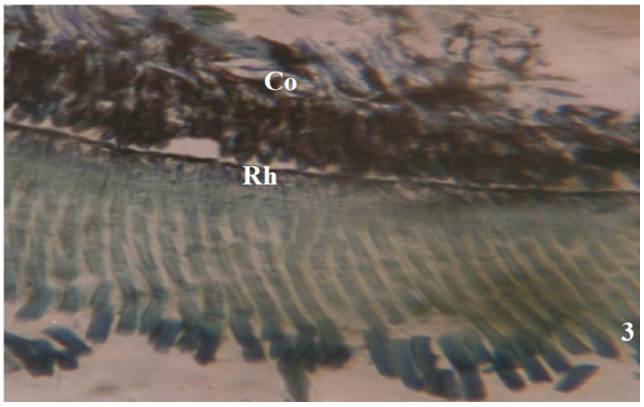
Several workers like Miao *et al*., (1996), Groves *et al*., (2005), Manton *et al.,* (2007), Fthenou *et al*., (2006, 2008) *etc*. have studied the influence of glycosaminoglycans on cell division, differentiation, responses to growth factors, adhesion, migration, peripheral nerve extension or regeneration and signal transduction. In this regard, Bulow and Hobert, (2006) are of the opinion that the correct development of a multicellular organism is *via* a specific code contributed by the glycosaminoglycans.

In the case of the visual apparatus, they play a central role in the physiological maintenance of trabecular meshwork in the eyes (Yue *et al.,* 1984 and Cavallotti *et al*., 2004). They may also have a role in influencing keratocytes and nerve growth in corneal stroma because of their ability to bind together (Cornard *et al*., 2010). They, and their core proteins also have important physiological and homeostatic roles *e.g.* during inflammation and immune response (Park *et al*., 2001; Li *et al*., 2002; Wang *et al*., 2005).

AMPs influence tissue osmotic pressure not only by influencing the water balance, but also by introducing excess swelling pressure which is balanced by an internal structural resistance (Ogston, 1970). Moreover, AMPs play important roles in “water binding” and maintenance of tissue osmotic pressure (Ogston and Wells, 1972). Payrau *et al.,* (1967) observed that the transparency of the cornea is based on the state of hydration of tissue. They based this on the fact that the corneal stroma of most vertebrates, including mammals, birds and teleosts







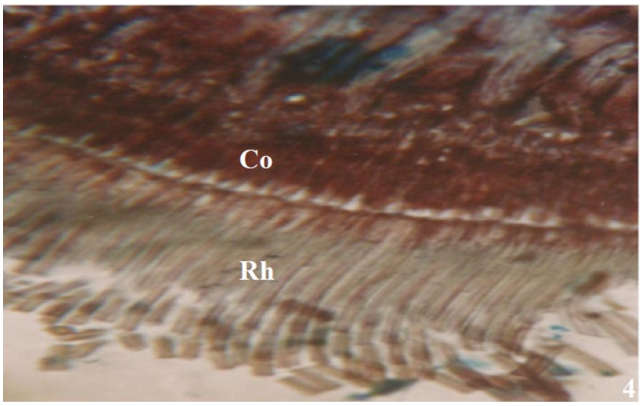


Fig 1. Histochemical observations of Lens cuticle of the butterfly, *Pieris brassicae*

Fig 2. Histochemical observations of Lens cuticle of the moth, *Philosamia ricini*

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 is supported by workers like Hedbys (1961, 1963); Kikkawa and Hirayama (1970); Bettelheim and Plessy (1975); Lee and Wilson (1981) and Castoro *et al*., (1988).

AMPs have also been suggested to play a major role in the structural organization of intracellular matrix *via* electrostatic and steric interactions with other macromolecules of the matrix, such as collagen and elastin (Kobayashi and Pedrini, 1973). Similarly, Ogston and Wells, (1972) have suggested that AMPs help in the maintenance of mechanical flexibility and elasticity of tissues. Ogston, (1966a) and Katchalsky, (1964) have shown that acid mucopolysaccharides possess high water binding capacities.

Multiple types of chondroitin sulphate proteoglycans are seen in vertebrates and they greatly influence development and tissue mechanics. For example, the chondroitin chains in the nematode *Caenorhabditis elegans* are not sulphated, but are nevertheless essential for embryonic development and vulval morphogenesis (Olson *et al.,* 2006). Chondroitin and dermatan proteoglycans have also been the subject of much interest as inhibitors of axon growth and have been shown to be important components of the glial scar that prevents axon regeneration (Rhodes and Fawcett, 2004).

The role of mucopolysaccharides in pathogenicity has been widely reviewed. For instance, they are responsible for calcification of bones (Rubin and Howard, 1950), dermal thickening in acromegalic patients (Matsuoka *et al.,* 1982), involved in inborn

Table 1: Ascending paper chromatogram of sugar components of the butterfly, *Pieris brassicae* and the moth, *Philosamia ricini*. (Solvent used is butanol, acetic acid and water in the ratio of 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

Table 2: Ascending Paper chromatogram of some standard sugar components. (Solvent used is butanol, acetic acid and water in the ratio of 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

errors of metabolism and/ or storage disorders (Matalon *et al*., 1974a; Hall *et al.,* 1978; Neufeld and Fratantoni, 1970; McKusick *et al.,* 1978), maintenance of retinal structure and neural tube closure in Knobloch syndrome (Sertie *et al*., 2000) and treatment of diabetic nephropathy (Gambaro and Van Der Woude, 2000).

Matthews (1959) and Oosawa (1971) have suggested that one of the characteristic properties of mucopolysaccharides is the selective association or binding with small inorganic cations, especially H^+, Na^+, and Ca^{++}, and also with cationic groups of macromolecules. In these regard, Farber and Schubert (1957) and Urist *et al.,* (1968) have also found a small preference for binding Ca^{++} over Na^+ in chondroitin sulphate. Matthews (1975) thus suggested that these substances act as a store for Ca^{++} in cartilage tissue and that is the reason for their specific roles in tissue-calcification. 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 nudus* and *Cingalobolus bugnioni* respectively. Krishnan (1965) has suggested that AMPs may be associated with -S-S- bonding of the cuticle in the scorpion *Palaemonetes swammerdami.*

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 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. 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 (Anseth and Fransson, 1970). Similarly, Freund *et al.,* (1995) also reported that the presence of AMPs in human and rabbit cornea is related to transparency. 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 rise in the transparency of stroma. They have also suggested that stromal transparency is correlated with the presence of normal

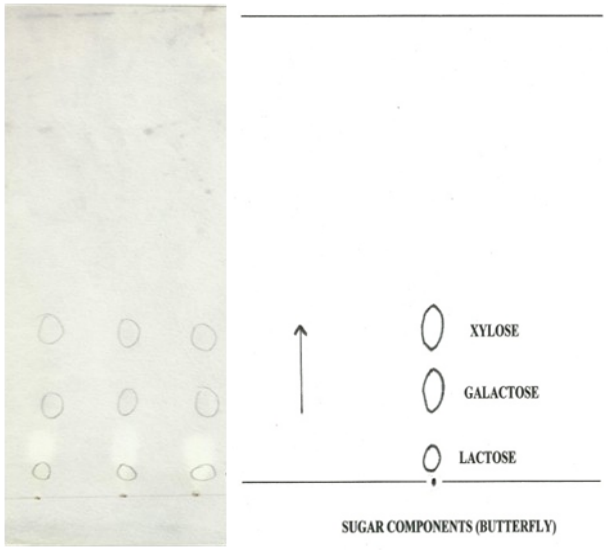


Fig. 3: Ascending paper chromatogram showing the sugar components of the mucopolysaccharides from the eye of the butterfly, *Pieris brassicae.*

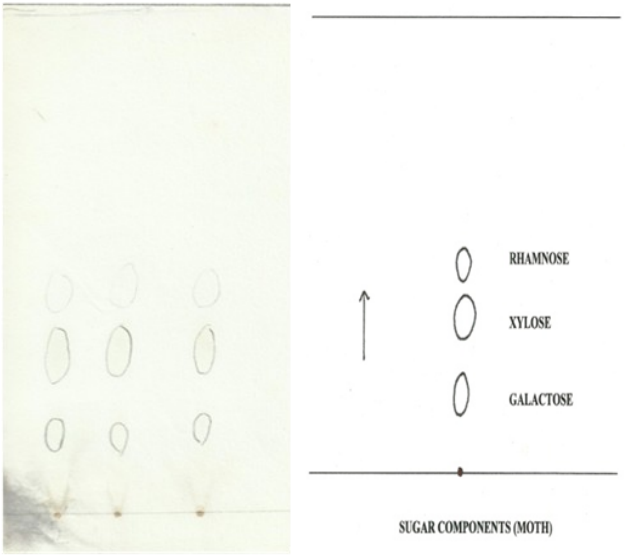


Fig. 4: Ascending paper chromatogram showing the sugar components of the mucopolysaccharides from the eye of the moth *Philosamia ricini.*

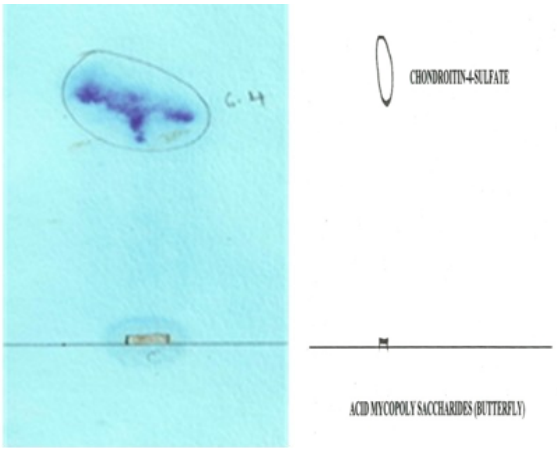


Fig. 5: Paper electrophorectic movement patterns of the crude mucopolysaccharides from the eyes of the butterfly, *Pieris brassicae.*

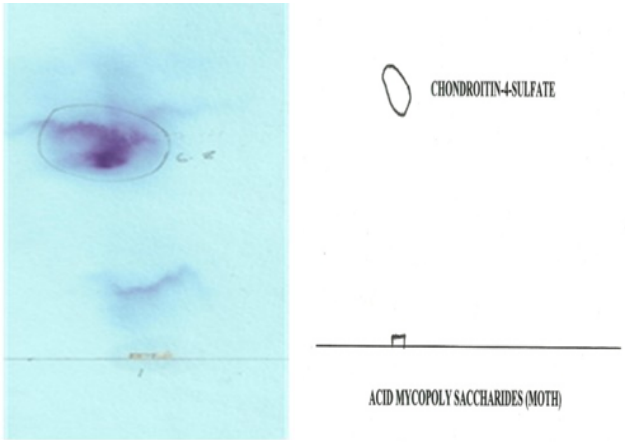


Fig. 6: Paper electrophorectic movement pattern of the crude mucopolysaccharides from the eyes of the moth *Philosamia ricini*

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. Blochberger *et al*., (1992), has reported that corneal keratan sulfate proteoglycans contribute to corneal transparency in chick. Takahashi *et al*., (1993) have also reported that keratan sulfate and dermatan sulfate proteoglycans are associated with collagen in foetal rabbit cornea. 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; Mc Cally and Farrell, 1990 and Bron, 2001). Mc Adams and Mc Loon (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.

Many studies that focused on corneal swelling behavior have noted a gradual decrease in swelling from the posterior to anterior side (Van Horn *et al*., 1975;

Table 3: Paper electrophorectic movement patterns of the crude mucopolysaccharides from the eyes of the butterfly, *Pieris brassicae* and the moth, *Philosamia ricini.* (Solvent used is phosphate buffer of pH 6.5)

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

Table 4: Paper electrophorectic movement patterns of some standard mucopolysaccharides. (Solvent used is phosphate buffer of 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	10.0

Bettelheim and Plessy 1975; Castoro *et al.,* 1988 and Cristol *et al*., 1992) and this was thought to be related to the organization of the collagen lamellae and 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 hydrophilic proteoglycan, is present (Bettelheim and Plessy 1975; Castoro *et al.* 1988). An interesting conclusion was drawn by Muller *et al*., (2001) while studying the differential behaviour of the anterior and posterior stroma during corneal swelling, that it is the high negative charge of the glycosaminoglycan components of the proteoglycans that is responsible for the corneal swelling due to electrostatic repulsion between acidic groups. 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.

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 polysaccharide complex provides a more stable and specific configuration within the molecules than electro-static linkage could. 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 may be noted here that the non - swelling properties of elasmobranch cornea is supposed to be due to the high mannose content in their structural proteins (Moozar and Moozar, 1972).

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 glycosaminoglycans 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 formation (Wigglesworth, 1965). Further, mucopolysaccharides may play a 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).

It is thus concluded that AMPs do indeed play various roles in the physiology of vision, but no photoperiodic adaptational mechanisms can be attributed to them.

CONCLUSIONS

The present investigation revealed that mucopolysaccharides are present in the ocular tissues (crystalline cones, but absent in the rhabdome) of both the insects studied *i.e.*, *Pieris brassicae*, and *Philosamia ricini*. Moreover, the analysis of sugar components show that the ocular tissues of both the insects have similar sugars – galactose and xylose, except for two different sugar components *i.e.*, lactose (in *Pieris brassicae*) and rhamnose (in *Philosamia ricini*), but no definitive conclusion can be drawn on the matter of this difference pending further studies. It is thus concluded that acid mucopolysaccharides have structural and other physiological roles in the visual apparatus but no part in light and dark or photoperiodic adaptations.

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