CHAPTER IV

HISTOCHEMICAL LOCALIZATION OF ASCORBIC ACID IN THE 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 empirical 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 been 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 Raghuvanman (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°C for 30 minutes in the darkness. The tissue was then thoroughly washed in several changes of dH₂O for 30 mins and treated with 5% sodium thiosulfate for 30 mins. Again washed in dH₂O 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. 1986).
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 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 selenum (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.