CHAPTER III

ASCORBIC ACID IN THE CORNEA AND LENS
INTRODUCTION

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

Pirie (1946) has reported that the concentration of ascorbic acid is approximately 1.8 μ moles per gm. wet wt. of the cornea of ox. Oxidation-reduction role of ascorbic acid in the calf corneal epithelium has been reported by Anderson and Spector (1971) and in bovines and rabbits (Kulhmen and Resnick, 1959). The high concentration of ascorbic acid in corneal epithelium shows that it is generally more concentrated in cells than in body fluids (Maurice and Riley, 1970). Heath (1962) has reported the presence of ascorbic acid in the lens of rat, dog, guinea pig, rabbit,
sheep, frog, pig, man, cow and horse. It has also been reported in the aqueous humour of rabbit (Muller and Buschke, 1934; Podesta and Baucke, 1938), ox (Muller and Buschke, 1934; Podesta and Baucke, 1938; Vladesco and Stefanescu, 1939; Johnson, 1936), man (Muller and Buschke, 1934; Purcell et al., 1954), monkey (Kinsey and Jackson, 1949), guinea pig (Muller and Buschke, 1934; Podesta and Baucke, 1938; Johnson, 1936); sheep (Vladesco and Stefanescu, 1939; Podesta and Baucke, 1938), cat (Langham, 1950), rat (Muller and Buschke, 1934) and frog (Muller and Buschke, 1934).

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

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

Rawal and Rao (1977) have studied the importance of ascorbic acid in visual physiology of various vertebrates such as amphibian (*Rana hexadactyla*, *Bufo melanostictus*) birds (*Columba livia*) and mammals (*Rattus norvegicus*, *Cavia porcel-
lus, *Tophozous longimanus*). The valuable observations of them was that the animals like amphibians which live in well-illuminated areas contained low level of ascorbic acid in the lens while those living in shaded or less lighted area have comparatively higher amount of ascorbic acid in their lens. Among the mammals, the nocturnal bat have comparatively higher amount of ascorbic acid in the lens. Ascorbic acid in higher forms shows degradation excepting birds, where it is lower than amphibians and fishes and higher than reptiles and mammals. According to Sharma (1989), ascorbic acid is unusually high in the primate eye. The level of ascorbic acid is higher in the lens and aqueous humour in diurnal mammals than nocturnal ones.

A gradual decrease, however, in the concentration of ascorbic acid in the lens has been observed as the animal becomes old. In spite of the wide variations in the concentration of this vitamin between species, the level has been found to be greatly reduced in the aging lens of all the species investigated so far, like, the rat and cow differ in lens-size, life span and average ascorbic acid content but both the animals show similar drops in ascorbic acid concentration in the aging lens (Kuck, 1961). However, a decrease in normal level of ascorbic acid in tissues is a sign of physiological stress (Wedemeyer, 1964), due to pollution (Chatterjee and Pal, 1975; Mauck, et al. 1978), infection (Lewin, 1974) or disease (Wilson, 1974).
In fishes, a limited number of reports available on the level of ascorbic acid in the tissues only (Rudra, 1936a; Saha 1939; Ikeda et al. 1963; Raghubanshi and Swarup, 1978; Halver et al., 1975; Agarwal and Mahajan, 1980), but no special attention on ascorbic acid level and its role in visual process has been given on fish ocular tissues excepting Rawal and Rao (1977), who studied the importance of the ascorbic acid in the lens of some fishes, such as mullet (Ophiocephalus punctatus), cat fish (Saccobranchus fossilis) and barbus (Barbus pinnatus). Agarwal and Mahajan (1980), have studied the comparative tissue ascorbic acid level in four major carps (Labeo rohita, Labeo calbasu, Catla catla, Cirrhina mrigala). The ascorbic acid level was found to be highest in the spleen in the four species, followed by the anterior (adrenal) kidney, gonads, liver, renal kidney, brain and/or eye. A seasonal variation in the ascorbic acid level of tissues has also been reported in Notopterus notopterus by the same authors.

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

Dietary deficiency symptoms causing various distortions of vertebral column has been reported by Poston (1967).
The commonly known deficiency state in fishes is "broken back syndrome" is characterized by fractured dislocation of the spine and cartilage, impaired collagen formation, depigmented areas, poor growth and mortality (Bauernfeind, 1982).

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

Histochemistry

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

Biochemical detection (Spectrophotometric detection)

The modified method of Chinoy et al. (1976) has been followed for the detection of ascorbic acid. Ascorbic acid, it is known, that in biological materials not only exists in free form (AA) but also occurs in bound form or ascorbigen (ASG) (Guha and Paul, 1936; Sengupta and Guha, 1938). It is a well known fact that ascorbic acid is continually acted upon by a number of oxidizing enzymes. These include a specific
peroxidase catalyzing the formation of its free radical-(FR)-monodehydroascorbic acid (MDHA) (Gurevich, 1963). Further, Chinoy (1967, 1969) has reported that in a living system exogenous as well as endogenously added ascorbic acid forms a complex with macromolecules like proteins and nucleic acids. Isherwood and Mapson (1962) have suggested that the actual concentration of ascorbic acid in a tissue represents the excess formed in synthesis over that used in metabolism. Thus, according to them any study regarding the concentration of ascorbic acid in free form alone may lead to an inaccurate inference. Taking all these in consideration ascorbic acid turn over in the cornea and lens have been estimated by simultaneous determination of (i) free form of ascorbic acid (AA); (ii) bound form of ascorbic acid (ASG); (iii) enzymatic utilization of ascorbic acid (AAU); and (iv) complexing of ascorbic acid with macromolecules (AA-MM).

Reagents

(1) Metaphosphoric Acid (HPO$_3^-$)

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

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

The solutions were refrigerated at 3°C.

(2) Buffer Solutions

(i) Buffer Solution A (0.5M) - 10.55g of citric acid dissolved in 1N NaOH and the volume is made upto 100 ml. (pH 4.8).
(ii) **Buffer Solution B (1M)** - 31.65g of citric acid dissolved in 3N NaOH and the volume is made upto 100 ml. (pH 4.8).

(3) **Buffered HPO**₃

Three volumes of 3% HPO₃ is mixed with one volume of buffer solution A (pH 3.6). Buffered HPO₃ is prepared fresh every time.

(4) **Standard Ascorbic Acid Solution**

10 mg of ascorbic acid is dissolved in glass distilled (double) water saturated with CO₂. The volume of the solution is made upto 100 ml and stored in an amber-coloured bottle at 3-5°C. Ascorbic acid solution is always prepared fresh.

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

**Standard Curve**

Ascorbic acid solutions of concentrations ranging from 10μg to 100μg/ml are prepared from a stock solution by diluting with CO₂-saturated glass distilled water to the required concentration.
1 ml aliquot of each AA solution is mixed with 1 ml. of buffered HPO₃ (pH 3.6) solution (pH remains stable following the addition of ascorbic acid due to high buffering capacity) and 5 ml of standard dye solution and the reading is taken in a Beckmen Spectrophotometer at 500nm. The readings are taken for each AA solution and a graph is prepared by plotting the values.

Extraction of the Materials (Preparation of the tissues)

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

The weighed tissue is placed in a mortar, covered with 1-2 ml. of cold CO₂ saturated distilled water and quickly homogenized with a pinch of purified silica. The contents are transferred to a test tube. The mortar and pastel are rinsed 2-3 times with 1-2 ml. of cold CO₂-DW, transferred to the test tube and the volume is made upto 12 ml with CO₂-saturated distilled water.

The homogenate is divided into three parts:

(i) 4 ml for the estimation of AA;
(ii) 4 ml for the estimation of ASG; and
(iii) 4 ml for the estimation of AAU and MM complex.

Determination of Ascorbic Acid (AA)

4 ml of cooled buffered HPO₃ is added to 4 ml of the original homogenate and after thorough mixing the solution is
filtered. Now 2 ml of the filtrate is diluted with 5 ml of distilled water and the scale of the spectrophotometer is adjusted to "0" for the turbidity factor. 5 ml of standard dye solution is added to another 2 ml of the same filtrate and the reading is noted.

**Calculation**

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

\[ A = \frac{a \cdot V}{W} \times 1000 \]

where, \( A \) = AA content of the sample in \( \mu \) g/g fresh weight.

\( a \) = AA in \( \mu \) g/ml of the original homogenate
\( V \) = Total volume of the original homogenate
\( W \) = Weight of the sample taken for analysis (in mg).

**Determination of Ascorbigen (ASG)**

2 ml of 15% \( \text{HPO}_3 \) is added to 4 ml of the original homogenate and the mixture is kept in a water-bath at 75°C for 15 minutes for hydrolyzing ascorbigen. After cooling, the system is buffered at pH 3.6 by adding 2 ml of the buffer solution B, thus increasing the volume of the mixture to 8.0 ml and filtered. Then the spectrophotometric reading is recorded as in free ascorbic acid.
Concentration of ascorbic acid in 2 ml of the buffered hydrolysed extract is determined from the standard graph. The value of free ascorbic acid in 1 ml of the homogenate, which has been determined previously, is subtracted from it to obtain the ascorbic acid equivalent of ascorbigen in 1 ml of the homogenate.

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

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

where

- \( B \) = Ascorbic acid equivalent of ascorbigen in \( \mu g \) per gm. fresh weight of the sample
- \( b \) = Ascorbic acid (\( \mu g \)) in 2 ml of buffered hydrolyzed extract
- \( a \) = Ascorbic acid (\( \mu g \)) per ml of the original homogenate
- \( V \) = Total volume of the original homogenate
- \( W \) = Weight of the sample taken for analysis (in mg).

**Ascorbic acid utilization (AAU) and ascorbic acid-macro-molecule complexing (AA-MM complex)**

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

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

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

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

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

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

\(a\) = Ascorbic acid in \(\mu g/ml\) of the original homogenate.

\(V\) = Total volume of the original homogenate

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

Determination of AA-MM complex

At the end of the incubation period 2 ml of 15\% HPO\textsubscript{3} are added to 4 ml aliquot and the mixture is hydrolyzed.
After cooling, the mixture is buffered at pH 3.6 by adding 2 ml of buffer solution B bringing the total volume to 8 ml and the complexing of ascorbic acid is determined as in ascorbigen. The ascorbic acid content in the aliquot is estimated from the standard graph and is multiplied by 2. Subtracting the value of ascorbic acid left over in 1 ml of the original homogenate before hydrolysis from the above value gives the amount of ascorbic acid released by hydrolysis of AA-MM complex (in μ g/ml). The amount of ascorbic acid complexing with macromolecules per gm. fresh weight has been calculated as follows:

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

where, 
- \(D\) = μ g of ascorbic acid released from the complex per gm. fresh weight of the tissue
- \(d\) = μ g of ascorbic acid in 2 ml of the incubated hydrolyzed buffered solution (i.e. 1.0.5 ml of the original homogenate)
- \(V\) = Total volume of the original homogenate
- \(c\) = Amount of ascorbic acid in μ-g left over in 2 ml of the buffered-incubated solution (i.e. 0.5 ml of the original homogenate)
- \(W\) = Weight of the sample taken for analysis.
OBSERVATIONS

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

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

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

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

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

Fig. 47  Ascorbic acid positive granules in the cornea of *Clarias batrachus*  
         x 500
dant in the anterior half of the critical portion of the lens compared to the posterior half in both the fishes (Figs. 48 and 49). The histochemical detection has been further confirmed by biochemical estimation of ascorbic acid in the cornea and lens which has been carried out separately. The ascorbic acid turnover (free form of ascorbic acid or AA, bound form of ascorbic acid or ASG, enzymatic utilization of ascorbic acid or AAU and association of ascorbic acid with macromolecules or AA-MM complex) in the cornea and lens of carp and cat fish (Tables 5 and 6).

The major source of error in studying the ascorbic acid concentration in any biological system is the auto-oxidation of ascorbic acid. This auto-oxidation has been prevented by a fairly simple method as described by Chinoy et al. (1976), in which glass distilled water saturated with CO₂ has been used for extraction and preparation of ascorbic acid solution. Chinoy et al. (1975) while studying the auto-oxidation of ascorbic acid in relation to time have found that auto-oxidation is rapid in glass distilled water kept at 30°C. But when glass distilled water cooled to 3-5°C after boiling is used, it has been observed that the rate of auto-oxidation is slowed down, which is noted upto 180 mins. The glass-distilled water when saturated with CO₂, the auto-oxidation is completely checked at both 30°C and 3-5°C.
EXPLANATION TO FIGURES

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

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

Ascorbic acid (free form, bound form, enzymic utilization and association with macromolecules) turnover in the cornea of the *Cyprinus carpio* and *Clarias batrachus*

<table>
<thead>
<tr>
<th>Different forms of ascorbic acid</th>
<th>Fish</th>
<th>Concentration (mg/gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free form (AA)</td>
<td>(1) <em>C. carpio</em> carp cornea</td>
<td>0.102</td>
</tr>
<tr>
<td></td>
<td>(2) <em>C. carpio</em> cat fish cornea</td>
<td>0.643</td>
</tr>
<tr>
<td>Bound form (Ascorbigen)</td>
<td>(1) <em>C. carpio</em> carp cornea</td>
<td>0.105</td>
</tr>
<tr>
<td></td>
<td>(2) <em>C. carpio</em> cat fish cornea</td>
<td>0.63</td>
</tr>
<tr>
<td>Enzymic utilization (AAU)</td>
<td>(1) <em>C. carpio</em> carp cornea</td>
<td>1.75</td>
</tr>
<tr>
<td></td>
<td>(2) <em>C. carpio</em> cat fish cornea</td>
<td>4.81</td>
</tr>
<tr>
<td>Association with macromolecules</td>
<td>(1) <em>C. carpio</em> carp cornea</td>
<td>1.48</td>
</tr>
<tr>
<td>(AA-MM complex)</td>
<td>(2) <em>C. carpio</em> cat fish cornea</td>
<td>1.52</td>
</tr>
</tbody>
</table>
Table 6

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

<table>
<thead>
<tr>
<th>Different forms of ascorbic acid</th>
<th>Fish</th>
<th>Concentration (mg/gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(1) <em>C. carpio</em></td>
<td></td>
</tr>
<tr>
<td>Free form (AA)</td>
<td>(1) carp lens</td>
<td>0.138</td>
</tr>
<tr>
<td></td>
<td>(2) cat fish lens</td>
<td>0.334</td>
</tr>
<tr>
<td>Bound form (Ascorbigen)</td>
<td>(1) carp lens</td>
<td>0.042</td>
</tr>
<tr>
<td></td>
<td>(2) cat fish lens</td>
<td>0.36</td>
</tr>
<tr>
<td>Enzymic utilization</td>
<td>(1) carp lens</td>
<td>0.982</td>
</tr>
<tr>
<td></td>
<td>(2) cat fish lens</td>
<td>6.22</td>
</tr>
<tr>
<td>Association with macromolecules</td>
<td>(1) carp lens</td>
<td>0.295</td>
</tr>
<tr>
<td>(AA-MM complex)</td>
<td>(2) cat fish lens</td>
<td>1.61</td>
</tr>
</tbody>
</table>
Another probability of error is the estimation of ascorbic is due to the instability of the dye, 2,6-dichloro-phenol indophenol. This dye solution is very much unstable at low pH, a characteristic of indophenol dyes. These dyes at lower pH decompose to give quinones and aminophenols (Karrer, 1950).

Chinoy et al. (1976) have reported that unbuffered HPO₄ decolourises the dye rapidly and the rate of decolourisation is proportional to the concentration of HPO₄ present in the system. We could effectively stabilise the dye by buffering it with citrate-NaOH buffer at a pH 3.6 in our study.

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

Chinoy et al. (1976) while studying the effect of metaphosphoric acid on the stability of ascorbic acid during hydrolysis have suggested that 15% or higher concentration of metaphosphoric acid (HPO₄) efficiently checks the loss of
ascorbic acid during heating at 80°C up to 15 minutes. While HPO₃ is used to check the loss of ascorbic acid, the system is buffered to a pH 3.6 before the dye is added to it. When 15% metaphosphoric acid is used, the buffer solution B (cf. Materials and Methods) effectively brings the pH level to 3.6 when mixed in a ratio of 1:1.

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

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

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

Various roles have been attributed to ascorbic acid by a number of workers. Ascorbic acid is one of the most important sugar acids, a very unstable compound and readily undergoes oxidation to dehydroascorbic acid and all higher species appear to employ ascorbic acid as a co-factor in certain specific enzymatic reactions (Lehninger, 1972). It has been postulated that monodehydroascorbic acid, a stable and a free radical anion, is the intermediate in the oxidation of
ascorbic acid by a metal ion. Stability of this radical anion and its conversion to dehydroascorbic acid and ascorbic acid helps to explain the antioxidant role that ascorbic acid plays in biological systems (Seib and Tolbert, 1982).

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

The eye takes up its ascorbic acid by an energy dependent active transport mechanism (Nicola et al. 1968; Sharma et al. 1964) because the ability to synthesize ascorbic acid is absent in insects, invertebrates, fishes, and certain bats and birds (Chaudhuri and Chatterjee, 1969; Chaterjee et al. 1975). It has also been shown that ascorbic acid is taken up by
several tissues by an energy dependent and Na\(^+\) sensitive process (Omaye et al. 1982) and possibly it also plays some role in the active transport of ascorbate across the ciliary epithelium (Cole, 1970).

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

One of the important functions, that is the ability of glucose metabolism by corneal epithelium of bovines (Kinoshita et al. 1955) and rabbits (Kuhlman and Resnik, 1959) through hexose monophosphate shunt has been demonstrated (Kinoshita, 1964). The reoxidation of the reduced nicotinamide adenine dinucleotide (NADPH) produced during the above process is of fundamental importance. The role of ascorbic acid in the oxidation of NADPH has been established by Anderson and Spector
(1971). The authors have shown that oxidation of NADPH to NADP is accomplished through ascorbic acid and glutathione oxidation-reduction system with consequent production of hydrogen peroxide \((H_2O_2)\) in calf cornea and the process is catalyzed by two enzymes, such as dehydroascorbic acid reductase and glutathione peroxidase.

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

The inhibitory effect of ascorbic acid on the 3,5-cyclic phosphodiesterase activity causes an increase in the cyclic AMP in the corneal epithelium (Buck and Zadunaisky, 1975; Lewin, 1973). This increase in the cyclic AMP content in the corneal epithelium possibly enhances active ion transport across the cornea.
The lens contains a high concentration of ascorbic acid in the cortex than the nucleus and the level falls rapidly in the deficiency state (McLaren, 1970). Pirie (1962) and Horning (1975) have suggested that ascorbic acid is associated with the metabolism of lens. It prevents in vitro light induced damage to the lens cation pump in rat eyes (Varma et al., 1979). Kinsey and Jackson (1949) relates the high concentration of ascorbate in the eye with the regression of the hyaloid system and this might account for the interspecies differences. While Heath (1962) observed that high concentration of ascorbic acid in the lens than aqueous humour is necessary to maintain the normal levels of ascorbic acid in the aqueous end vitreous humours and the cornea. The high concentration of ascorbic acid in the ocular tissue is maintained by an active transport of ascorbate from the plasma across blood/aqueous barrier (Sharpe, 1989).

The importance of ascorbic acid in vision is that the maintenance of relatively high content of ascorbic acid in the eyes than other tissues during deficiency. Hughes et al. (1971) have observed that the brain and the ocular lens retain a high ascorbic acid level even in the absence of dietary source of this vitamin while other tissues show total depletion. The authors, in their experiment, maintained guinea pigs in an ascorbic acid free diet for 14 days and which followed
the estimation of ascorbic acid level in various tissues. This revealed that fall of ascorbic acid content was 1% in the spleen, 4% in the adrenals and less than 1% in the aorta respectively compared to their initial content. On the contrary, the corresponding values for brain and eye lens have been found to be 24% and 28% respectively. This difference of ascorbic acid concentration between the eye lens and other tissues may be due to an efficient mechanism operating in the eye which assures retention or uptake of this vitamin in the eye than other organs. Hughes et al. (1971) have further suggested that the function of ascorbate maybe of very importance to the eye and that might account for the maintenance of the level of the vitamin in the eye as long as possible. This may explain the experiment of Baker (1946) in which it was observed that in vitro cultured lens loss all the detectable ascorbic acid after 10 days but its transparency was retained even after 21 days of culture. Besides the lens, aqueous humour of the eye of various animals retain a high concentration of ascorbic acid by a mechanism of active transport from blood (Kinsey, 1947; Barany and Langham, 1955).

The lens maintains a high level of reducing agents such as ascorbate and glutathione to maintain a high energy demand (Goldschmidt, 1924). The high concentration of ascorbic acid suggests that in addition to some co-enzymatic functions ascorbate may also modulate some non-enzymatic metabolic
reactions in the lens. The most probable action of ascorbate is as reducing agent preventing unwanted reactions initiated by $O_2$ and free radicals either photochemically or under ambient non-photochemical condition. It has also been suggested that ascorbate terminates the propagation of various free radical reactions in oxidation, photolysis and radiolysis (Sharma, 1989).

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

In this context, it is worth mentioning that, Rawal and Rao (1977) in their experiment have shown that fishes living in the upper strata (ophiocephalus and barbus) of the water have lower amount of ascorbic acid and glutathione in their lens than the fishes living at the bottom or deeper layer of water (catfish). The authors suggest that the relatively high concentration of ascorbic acid and glutathione in the normal lens of some forms is due to their high energy demand and significant for the maintenance of lens transparency as suggested by Daisley, 1955.
In this context, it is worth to mention that in our studies we have also found a high content of ascorbic acid in the lens of the bottom dwelling cat fish (*Clarias batrachus*) than the lens of the carp which is not strictly a bottom dweller. Thus, our observation corroborates the important finding of Rawal and Rao (1977). Similarly, it is of interest to note that the ascorbate concentration of the cornea of cat fish is much higher than the carp cornea as we have found in our investigation.

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

Szent-Gyorgyi (1960) has suggested for the first time the importance of these charge-transfer complexes in biological system. Since his report, complexing of ascorbic acid with macromolecules has been reported from time to time (Chinoy,
1967, 1969; Chinoy et al., 1971, 1973, 1974). Bonner (1957) has reported that ascorbic acid is acted upon by a number of enzymes which oxidise ascorbic acid directly or indirectly and over and above this oxidation, it forms complexes with macromolecules, such as proteins and nucleic acid.

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