CHAPTER II

ADENOSINE TRIPHOSPHATASE (ATPase) OF CORNEA AND LENS
INTRODUCTION

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

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

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

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

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

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

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

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

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

The ATPase activity has also been reported in other ocular tissues. Sekoguti (1960) have observed high activity of this enzyme in cattle retina and outer segments who also found that the enzyme activity is stimulated by the addition of K\(^+\) in the Na\(^+\) containing assay medium. Bonting et al. (1963, 1964) found high activity of this enzyme in retina,
choroid, sclera, iris, lens and vitreous of man and cat. Further, altered ATPase activity in pathogenic retina has been reported by Macgregore and Matschinsky (1986).

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

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

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

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

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

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

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

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

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

(4) **Incubation** - The tissues were then transferred to the incubation medium. This medium contained 3m M Tris-ATP as substrate, 3m M-Pb(NO\(_3\))\(_2\) as precipitating agent, 3m M-MgSO\(_4\).

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

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

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

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

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

The lens requires some preparations before the whole mount observations of the epithelium could be made. The whole mount preparation was carried out under a dissecting microscope. An incision was made on the lens capsule which was pulled downwards with a very fine needle (since the lenses in fishes are quite small particularly of the cat fish, having a size of about 0.01 cmm) and the body of the lens was carefully lifted out. The isolated capsule with the adhering epithelium was then transferred on a glass slide and mounted in glycerol jelly.
When required, the intact lenses were post-fixed in 2.5-3.5% gluteraldehyde buffer solution. The lenses were then frozen and 20 μm sections were cut with a cryomicrotome. The sections were then briefly rinsed in distilled water and mounted in glycerol jelly on a slide.

Control Studies

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

(i) Na\(^+\) and K\(^+\), omitted from the incubation medium;
(ii) Na\(^+\), K\(^+\) and Mg\(^{2+}\) were omitted from the medium;
(iii) Only Na\(^+\), omitted from the medium;
(iv) K\(^+\), omitted from the medium;
(v) Mg\(^{2+}\), omitted from the medium;
(vi) The substrate ATP was omitted from the medium;
(vii) The precipitant, lead, omitted medium.

Effect of Temperature

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

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

Corneal Reaction

The pre-incubated solution without the inhibitor (Solution A) yielded a positive reaction in the corneal epithelial (Figs. 28 and 29) and endothelial cells (Figs. 32 and 33), but very little or no reaction was visible when the tissue was pre-incubated with ouabain (Solution B) (Figs. 30, 31, 34 and 35). The cells in the epithelium and endothelium are loosely arranged. The stroma in both cases was negative confirming the enzyme activity in the epithelial cells.
In the control experiments marked inhibition in enzyme activity could be seen in the absence of activators Na\(^+\) and K\(^+\) both in corneal epithelium and endothelium (Figs. 36, 37, 38 and 39) in both the fishes. There was complete absence of reaction when Na\(^+\), K\(^+\) and Mg\(^{2+}\) were omitted from the medium, so also in case of omission of ATP, Mg\(^{2+}\) and lead from the respective medium.

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

**Reaction in Lens**

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Na$^+$ - K$^+$ - ouabain sensitive ATPase (Na$^+$-K$^+$-ATP) has been implicated in the active Na$^+$-K$^+$ transport system of a variety of biological membrane (Skou, 1965; Post et al. 1960; Bonting, Carvaggio and Hawkins, 1963). The enzyme is specially inhibited by cardiac glycoside and ouabain. The ATPase has been considered as sodium-potassium "pump enzyme". The Na$^+$-K$^+$ pump nature of ATPase is presumed on the fact that Na$^+$-K$^+$-ATPase leads to the hydrolysis of ATP resulting in the movement of sodium and potassium against their electrochemical gradients (Skou, 1972). The hydrolysis of ATP occurs on intracellular side using OH$^-$ from the inside and H$^+$ from the outside liberating phosphate (Mitchell, 1961).

Albert (1967) has postulated that ATP-ATPase-enzyme-substrate complex may act as a true carrier mechanism binding internal sodium and releasing it outside the membrane. A similar but
reverse mechanism is postulated for $K^+$. In the absence of active cation pump the cell would swell and burst due to internal osmotic pressure (Lehninger, 1970).

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

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

The exact role of Na\(^+\)-K\(^+\)-ATPase in the corneal epithelium is still partially open to question. The epithelium, according to Mishima and Kudo (1967) plays no role in pump mechanism but offers high resistance to the movement of all ionic substances (Cogan et al., 1944; Maurice, 1968b).
An active inward Na\(^+\)-transport mechanism to the stroma has been identified in rabbit corneal epithelium (Donn et al., 1959). A sodium dependent mechanism pumping chloride ions from the aqueous humour to the tear film has also been shown to exist in the frog cornea (Zadunaisky and Lande, 1971). Klyce et al. (1973) have, moreover shown that the rabbit corneal epithelium not only pumps Na\(^+\)-ions from the tear film towards the stroma but also chloride ions in the opposite direction when such stimulators as epinephrine and cAMP are present. However, it seems possible that, definite variations between different species may exist in the pumping mechanisms of the corneas (Ehlers, 1973). Green (1969) suggests that the epithelium is chiefly responsible for the normal hydration of the cornea.

The endothelium on the other hand, is more permeable to ions than the epithelium (Maurice, 1961) and is directly concerned with the transport mechanism (Takahasi, 1967). Maurice (1972) considers that the endothelium is the chief site for the active pumping process and the permeability of the endothelium also seems to influence the corneal water balance and this has led Maurice (1972) to advance the "pump-leak" hypothesis, which proposes that the endothelium pump sodium and water into the anterior chamber against hydrostatic pressure, which causes a passive leak of water and electrolytes through the endothelium towards the stroma.
Friedman (1973) has proposed that the corneal water balance is exclusively based on a passive mechanism according to which the osmotic properties of the tear film and the permeability of the endothelium would be the main regulatory systems. The endothelial pumping mechanism needs sodium and bicarbonate (Dikstein and Maurice, 1972; Hodson, 1971) and absence of these activators causes a fall in the transendothelial potential difference (Fischbarg, 1973). Davson (1949) proposed that the endothelium is essential in maintaining normal corneal thickness and transparency.

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

In the metabolism of the lens, the presence of Na⁺-K⁺-ATPase activity has been found to have many basic
metabolic functions (Bonting, 1965). It has also been suggested that Na\(^+\)-K\(^+\) activated ATPase is mainly concerned with cation transport (Kinsey and Reddy, 1965; Bonting, 1965). Bonting (1965) assumes that one of these ions (potassium) is required for protein synthesis of lens.

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

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

The roles of Na\(^+\)-K\(^+\)-ATPase enzyme system, as shown by various authors in higher vertebrates appears to play
similar roles in fish ocular tissues. Further, we believe that this enzyme system requires to be more efficient in the aquatic medium.

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

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