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John Lambert Ubels

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J R Hoffman

Major professor

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A COMPARATIVE STUDY OF THE EFFECT OF HYPERBARIC OXYGEN ON THE ACTIVITY OF SODIUM-POTASSIUM ACTIVATED ADENOSINE TRIPHOSPHATASE IN THE RETINA

by

John Lambert Ubels

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ABSTRACT

A COMPARATIVE STUDY OF THE EFFECT OF HYPERBARIC OXYGEN ON THE ACTIVITY OF SODIUM-POTASSIUM ACTIVATED ADENOSINE TRIPHOSPHATASE IN THE RETINA

by

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Hyperbaric oxygen is known to be toxic to the mammalian retina, attenuating or abolishing the electroretinogram (ERG). The activity of sodium-potassium activated adenosine triphosphatase (Na^+-K^+ ATPase) is essential for retinal function. Therefore experiments were designed to determine whether the inhibition of Na^+-K^+ ATPase might, in part, be responsible for the effect of hyperbaric oxygen on the electrical activity of the retina.

Representatives of different classes of animals, the rainbow

trout (Salmo gairdneri), the frog (Rana pipiens), the Long-Evans rat (Rattus rattus) and the bovine (Bos taurus) were used since it is known that there are class differences in susceptibility to oxygen toxicity. Homogenates of retina and intact retinas in tissue culture medium were exposed to pure oxygen at 3800 and 11,600 mm Hg.

Trout retina Na^+-K^+ ATPase is inhibited slightly (approximately 10%) when homogenates are exposed to hyperbaric oxygen at 22C and 37C while no effect on enzyme activity was seen at 12.5C.

Exposure of intact trout retinas to hyperbaric oxygen at 14C had no significant effect on Na^+-K^+ ATPase activity. The teleost retina is adapted to high oxygen tensions generated by the choroidal counter current multiplier and is capable of withstanding oxygen tensions well above those normally encountered. Exposure of intact trout retinas to hyperbaric oxygen at 23C resulted in a 15-20% decrease in Na^+-K^+ ATPase activity.

Exposure of intact frog retinas to hyperbaric oxygen under the conditions used in this study has no effect on Na^+-K^+ ATPase activity. These data indicate that a decrease in sodium pump activity is probably not involved in the attenuation of the frog ERG by hyperbaric oxygen.

Bovine and rat retina Na^+-K^+ ATPase is inhibited when homogenates of retina are exposed to hyperbaric oxygen. Exposure of intact rat retinas to hyperbaric oxygen resulted in a 50-66% decrease in Na^+-K^+ ATPase activity. Based on current

understanding of the function of the vertebrate retina and its dependence on the sodium pump for normal activity this decrease in enzyme activity should be adequate to cause major decreases in the retina's responsiveness to light. The data presented in this report on Na^+-K^+ ATPase in addition to data from previous studies concerning the effect of hypserbaric oxygen on oxygen consumption, LDH activity and the ERG indicate that the teleost retina is highly resistant to oxygen toxicity, that the amphibian retina is intermediate to the teleost and the mammal in its susceptibility to oxygen toxicity and that the mammalian retina is highly susceptible to attack by active oxygen.

It is concluded that inhibition of Na^+-K^+ ATPase is a contributing factor in the toxic effect of high oxygen tensions on the mammalian retina.

DEDICATION

Dedicated to my loving wife, Jan, in gratitude for her patience, support and encouragement.

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I wish to thank my major advisor, Dr. Jack Hoffert, for his guidance, encouragment and support over the past five years. I am deeply appreciative of the opportunities for professional advancement which he provided by allowing me to assist him in the preparation of several research publications and making it possible for me to attend several scientific meetings. Above all, I thank him for his close friendship. I wish to thank Dr. P.O. Fromm for introducing me to the Physiology Department, for his constant interest in my work and for providing valuable teaching experience by allowing me to present several lectures in Physiology 401. I also appreciate the advice and guidance of my other committee members, Dr. Lynne Weaver, Dr. William Frantz and Dr. Ralph Pax. Dr. William Jackson (Doctor Bill) is to be thanked for his helpful suggestions concerning statistical analysis and for assisting in the derivation of equations.

A very special word of thanks goes to Mrs. Esther Brenke. Her patient and expert technical assistance had a direct effect on the success of this project and she also spent many hours typing the first draft of this dissertation.

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From the greater strength at vivacity of the flame of a candle in this pure air, it may be conjectured that it might be peculiarly salutary to the lungs in certain morbid cases when the common air would not be sufficient to carry off the phlogistic putrid effluvium fast enough. But perhaps we may also infer from these experiments that, though pure dephlogisticated air might be very useful as a medicine it might not be proper for us in the usual healthy state of the body; for, as a candle burn out much faster in dephlogisticated air than in common air, so we might, as may be said, live out too fast and the animal powers be too soon exhausted in this pure kind of air. A moralist at least may say that the air which nature has provided for us is as good as we deserve.

Priestly, 1775

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INTRODUCTION

The toxicity of hyperbaric oxygen to biological systems has curtailed its use in medicine, aviation, space flight and diving. In cellular metabolism highly reactive oxygen free radicals (such as superoxide radical, $O_2^{\cdot -}$) are produced along the pathway of univalent oxygen reduction. Normally these toxic free radicals are scavenged by the antioxidant systems of the cell, however, if the partial pressure of oxygen (P_{O_2}) is raised above that normally encountered by the cell the rate of production of free radicals is accelerated and the antioxidant mechanisms of the cell may be saturated. This allows the free radicals to engage in lipid peroxidation and enzyme inactivation, which leads to the structural and metabolic derangements known as oxygen toxicity. The most familiar symptoms of oxygen toxicity in man and other animals are convulsions and pulmonary edema. These effects have as their primary basis the toxicity at the cellular level mentioned above and therefore most research concerning oxygen toxicity is being conducted at the cellular level.

The toxic effect of oxygen on the retina has been of interest since the early 1950's when it was shown that retrolental fibroplasia (RLF) in premature infants is caused by their exposure to pure oxygen in incubators (Patz, Hoeck and De La Cruz, 1952). It is well known that the time to the onset of oxygen toxicity is inversely related to the P_{O_2} and therefore it has been possible to prevent many cases of RLF by reduced oxygen exposure times and reduction of the P_{O_2} to the minimum

required to maintain adequate blood oxygenation (Kingman, 1977).

Although RLF primarily involves the retinal vasculature, the direct effects of high oxygen tension on the metabolism of retinal cells and on their ability to respond to light is also an important consideration. Exposure of rabbits to hyperbaric oxygen (HBO) causes irreversible structural changes in the retina and these changes are preceded by attenuation and eventual disappearance of the electroretinogram (ERG) (Noell, 1962). It is also known that hyperbaric oxygen abolishes the ERG recorded from isolated retinas of frogs and rats. This indicates that oxygen has a direct effect on retinal cells apart from any changes mediated by the cardiovascular and respiratory effects of oxygen toxicity (Ubels, 1976).

The purpose of the present study was to investigate the effect of hyperbaric oxygen on the retinal enzyme sodium-potassium activated adenosine triphosphatase (Na^+-K^+ ATPase). The activity of this enzyme is required for retinal function since application of ouabain which inhibits Na^+-K^+ ATPase to the isolated retina abolishes the light response (Frank and Goldsmith, 1967; Sillman, Ito and Tomita, 1969).

Na^+-K^+ ATPase is a lipid and sulfhydryl group dependent enzyme. Enzymes of this type are particularly susceptible to attack by active oxygen through lipid peroxidation and disulfide bond formation.

It has been shown that exposure of brain slices and frog skin to hyperbaric oxygen reduces the rate of active transport of Na^+ and K^+ in these tissues (Kaplan and Stein, 1957a; Cymerman

and Gottlieb, 1970). This information led to the hypothesis that inhibition of the sodium pump in retinal cells due to attack by active oxygen on the Na^+-K^+ ATPase system may contribute to the observed effect of hyperbaric oxygen on the ERG. The idea that the metabolic pump is susceptible to attack by active oxygen has been supported by a recent report that exposure to superoxide radical inhibits Rb^+ uptake by lens epithelium (Varma, Kumar and Richards, 1979). The pump is capable of transporting Rb^+ as well as K^+ (Skou, 1967) and therefore inhibition of Rb^+ transport by $O_2^{\cdot -}$ exposure suggests inhibition of Na^+-K^+ ATPase by this free radical.

Previous studies of ocular oxygen toxicity have shown that the rainbow trout retina is resistant to oxygen toxicity as determined from studies of oxygen consumption, lactate dehydrogenase (LDH) activity and the electroretinogram (ERG) (Baeyens, Hoffert and Fromm, 1973, 1974; Ubels, Hoffert and Fromm, 1977). This resistance appears to be an adaptation to the unusually high P_{O_2} (>400 mm Hg) to which the teleost retina is normally exposed (Fairbanks, Hoffert and Fromm, 1969). This high P_{O_2} is generated by the counter-current oxygen multiplier of the choroidal rete mirabile.

Similar studies showed that LDH activity of frog retina and oxygen consumption and LDH activity of dog retina are decreased by exposure of retinal tissue to hyperbaric oxygen. The effect of oxygen on frog and rat retina electrical activity has been cited above.

A comparative study of the effect of hyperbaric oxygen on

the activity of the enzyme Na^+-K^+ ATPase was conducted using retinal tissue from rainbow trout (Salmo gairdneri), frogs (Rana pipiens) and Long-Evans rats (Rattus rattus). Results of these experiments are presented and discussed in relationship to previous studies of ocular oxygen toxicity in these animals, and in relationship to the role of Na^+-K^+ ATPase in retinal function.

LITERATURE REVIEW

Sodium-Potassium Activated Adenosine Triphosphatase (Na^+-K^+ ATPase).

Discovery and Characteristics

It is well known that most cells have a low internal sodium concentration and a high internal potassium concentration and that the opposite conditions exist in the extracellular fluid. This results in a chemical gradient for both of these ions. Although the permeability of the cell membrane to these ions is quite low, especially for sodium (0.01 of the permeability for K^+), Na^+ tends to leak into the cell and K^+ out of the cell down their respective gradients. The action potential in most nerve and muscle cells is the result of a rapid increase in Na^+ conductance resulting in the rapid influx of Na^+, followed by an increase in K^+ conductance and movement of K^+ out of the cell during the recovery period.

These ionic fluxes, the leak in the resting state and the rapid movement during neural and muscular activity, would lead to a decay of the ionic gradients described above if it were not for an active transport system which extrudes sodium from the cell and transports potassium into the cell. An early report of this process was given by Hodgkin and Keynes (1955) who described the transport of Na^+ and K^+ in giant axons from Sepia and Loligo.

Since the transport of these ions takes place against a chemical gradient they postulated that metabolic energy must be required for the process. They showed that dinitrophenol (DNP), cyanide and low temperature reduced the extrusion of Na^+ and the uptake of potassium but had no effect on passive movements of these ions. They also showed that removal of K^+ from the extracellular fluid reduced the efflux of Na^+. Later Hodgkin and Keynes (1956) showed that increasing the internal Na^+ concentration by microinjection of Na^+ into a giant axon increased the rate of active transport of Na^+ out of the cell.

The experiments of Hodgkin and Keynes indicated that transport of Na^+ and K^+ requires ATP as an energy source since DNP and cyanide uncouple oxidative phosphorylaton and thus reduce the cell's ATP supply. They also showed that the rate of active transport was influenced by levels of extracellular K^+ and intracellular Na^+. Another study by Abood and Gerard (1954) had demonstrated the presence of an ATPase in the sheath of giant axons. Based on this information Skou (1957) investigated the effects of the cations magnesium, sodium and potassium on the membrane ATPase of peripheral nerves from crab legs. He found that there is an absolute requirement for Mg^{++} for enzyme activity and that the activity of the enzyme responds to changes in Na^+ and K^+ concentrations in ways that correspond to the response of the rate of active transport to changes in $[Na^+]$ and $[K^+]$, as observed by Hodgkin and Keynes (1955, 1956). Thus Skou suggested that this ATPase was involved in the active transport of sodium from the nerve fiber.

This work by Skou (1957) led to many studies on Na^+-K^+ ATPase by other investigators resulting in a major review article by Skou (1965) on the enzymatic basis of Na^+ and K^+ active transport.

Skou listed eight requirements which an enzymatic transport system should fulfill if it is to be identified as the system responsible for active transport of Na^+ and K^+ across the cell membrane. The requirements are as follows:

It should

1) be located in the cell membrane
2) have an affinity for Na^+ that is higher than for K^+ at a site located on the inside of the cell membrane
3) have an affinity for K^+ that is higher than for Na^+ at a site located on the outside of the membrane
4) contain an enzyme system that can catalyse the hydrolysis of ATP and thus convert energy from ATP into a movement of cations
5) be capable of hydrolysing ATP at a rate dependent on concentraton of Na^+ inside the cell and also on concentration of K^+ outside the cell
6) be found in all cells where active linked transport of Na^+ and K^+ occurs
7) show a close correlation between the effect of cardiac glycosides on cation transport in the intact cell and their effect on the enzyme system
8) have the same quantitative relation to Na^+ and K^+ as the

transport system of the intact cell.

A large volume of literature indicates that Na^+-K^+ ATPase fulfills these requirements. This evidence is briefly summarized below and specific references may be obtained by referring to the original review article (Skou, 1965). Although at the time of this review evidence did not limit the presence of Na^+-K^+ ATPase to the cell membrane it was definitely shown to be abundant in that location. Studies using red blood cells indicated that the affinity of the enzyme for Na^+ is in fact higher inside the cell membrane while the K^+ affinity is greater on the outside of the membrane. The use of ITP, UTP, and GTP as substrates instead of ATP showed that the enzyme requires ATP as a substrate since little or no transport occurs and the enzyme is not activated when ITP, GTP and UTP are supplied as energy sources.

Na^+-K^+ ATPase activity is found in a wide variety of tissues including red blood cells, brain, nerve axons, kidney, muscle, liver, intestinal mucosal, electric organ, frog skin, ciliary body, lens, retina, thyroid and toad bladder. All of these tissues exhibit high rates of active transport.

Schatzmann (1953) had shown that active transport of sodium can be inhibited by the cardiac glycosides. Skou's review presented evidence that cardiac glycosides also inhibit the activity of Na^+-K^+ ATPase and that there is a close correlation between the effect of cardiac glycosides on active transport and the effects on Na^+-K^+ ATPase activity, with respect to effective dose and the effects of different cardiac glycosides. The ratios

of Na^+ and K^+ needed to activate the enzyme were also shown to be correlated with the numbers of Na^+ and K^+ ions transported per ATP hydrolysed. Although for many years it has been accepted that Na^+-K^+ ATPase is the active transporter of Na^+ and K^+, proof was given when it was shown that purified Na^+-K^+ ATPase incorporated into single bilayer phospholipid vesicles transports Na^+ and K^+ in the presence of internal Na^+, Mg^{++} and ATP and external K^+ (Hilden, Rhee and Hokin, 1974; Hilden and Hokin, 1975; Korenbrot, 1977). The process is ouabain sensitive and the ratio of Na^+ transported out to K^+ transported in per ATP hydrolysed is 1.43:1 which agrees well with the Na:K ratio of 3:2 which was reported for red blood cells by Garrahan and Glynn (1967).

Since 1965 a vast amount of work on Na^+-K^+ ATPase and the sodium pump has appeared in the literature and has been reviewed repeatedly (Whittam and Wheeler, 1970; Dahl and Hokin, 1964; Schwartz, Lindenmayer and Allen, 1975; Glynn and Karlish, 1975; Korenbrot, 1977; Wallick, Lane and Schwartz, 1979). Most of these studies have been concerned with clarification of the chemical structure, mechanism of action and interaction with cardiac glycosides of Na^+-K^+ ATPase.

Structure and Lipid Requirement

Analysis of purified Na^+-K^+ ATPase from several sources including dog kidney outer medulla, eel electroplax and shark rectal gland indicates that the enzyme is made up of two protein subunits. The larger subunit is the catalytic protein and has a molecular weight of about 95,000. A smaller glycoprotein with a molecular weight of 45,000 is also present in purified enzyme

fractions. The function of this protein is unclear, however, it appears to be linked to the catalytic protein forming a monomer which is linked to another like monomer forming a Na^+-K^+ ATPase dimer with a molecular weight of 280,000 (Glynn and Karlish, 1975; Wallick, et al. 1979).

Little is known about the amino acid sequence of Na^+-K^+ ATPase or even the exact relationship of the protein subunits to each other and to the function of the enzyme. Work from Hokin's laboratory (Nishigaki, Chen and Hokin, 1974) indicates that in the presence of Mg^{++} and Na^+ the catalytic protein is phosphorylated by ATP at the beta-carboxyl group of an aspartic acid residue. There is also evidence that there is an essential tyrosine at the active site since modification of tyrosine inhibits the enzyme activity (Wallick, et al., 1979). A sulfhydryl group is also present at the active site (Skou, 1963; Wallick, et al., 1979).

In the membrane, Na^+-K^+ ATPase is associated with phospholipids and the presence of these lipids is an absolute requirement for enzymatic activity (Korenbrot, 1977). The molar ratio of phospholipid:protein is 120:1 in Na^+-K^+ ATPase purified from Squalus rectal gland (Perrone, et al., 1975). The role of these lipids in enzymatic activity and the specificity of these lipids is not known, however, it appears that the phospholipids must be negatively charged (Wallick, et al., 1979).

Reaction Mechanism of Na^+-K^+ ATPase

Details of the reaction mechanism of Na^+-K^+ ATPase are not yet known. It is generally understood that enzymatic activity requires the presence of Mg^{++}, Na^+ and ATP inside of the cell and

K^+ outside the cell membrane. Ouabain exhibits its inhibitory effect only when present outside the cell membrane.

Skou (1963) originally suggested that the hydrolysis of ATP in the process of Na^+ and K^+ transport did not require the phosphorylation of the Na^+-K^+ ATPase. Phosphoenzymes are indeed formed, however, as shown in many studies (reviewed by Glynn and Karlish, 1975; Wallick et al., 1979). These studies indicated that ATP phosphorylates the enzyme in the presence of Mg^{++} and Na^+ and that the enzyme is subsequently dephosphorylated in the presence of K^+. Fahn, Koval and Albers (1966) presented the following scheme which included two intermediate forms of phosphoenzyme

$$E_1 \xrightarrow[Mg^{+2}+Na^+]{ATP} E_1P \xrightarrow{Mg^{+2}} E_2P \xrightarrow{K^+} E_2 + P_i \qquad (1)$$

(ADP is released from E_2P; E_2 returns to E_1)

where E_1 and E_2 represent two forms of the enzyme.

This scheme does not adequately describe the steady state hydrolysis of ATP and work by Wang, Lindenmeyer and Schwartz (1977) indicates that there may be as many as 14 forms of the active enzyme and that more than one of these can break down to release inorganic phosphate (P_i). During this process Na^+ and K^+ are bound to the enzyme and Wang et al. (1977) suggest formation of the complex $E\text{-}Na_x^+\text{-}K_y^+$ where x=2 or 3 and y=1 or 2 respectively.

The mechanism by which the Na^+ and K^+ ions are moved across the membrane, that is, the spatial and temporal aspects of the pump, is not yet known. "Simultaneous" and "sequential" models

have been proposed (Hoffman, 1975). Both models propose a protein which bridges the membrane. The simultaneous model suggests that Na^+ and K^+ are bound at the same time on the inside and outside of the cell membrane respectively. Phosphorylation by ATP causes a conformational change in the enzyme, perhaps a rotation of the molecule within the membrane which causes the movement of the ions to opposite sides of the membrane. As K^+ passes through the membrane the enzyme is dephosphorylated which causes a change in the affinity of the cation binding sites, resulting in the release of the ions.

The sequential model is similar except that the cation binding sites are present only on one side of the membrane. Sodium binds inside the cell and phosphorylation causes the molecule to shift Na^+ to the outside where the binding site's ion affinity changes and Na^+ is released. This is followed by binding of K^+ and dephosphorylation as K^+ passes through the membrane. No experimental evidence gives strong support to either model. The simultaneous model is not likely for structural reasons (Glynn and Karlish, 1975) and the fact that an $E\text{-}Na_x^+\text{-}K_y^+$ complex is known to exist (Wang et al., 1977) calls the sequential model into question. Neither model explains the 3:2 Na:K transport ratio.

The enzyme $Na^+\text{-}K^+$ ATPase is still an enigma. Since it was first described by Skou in 1957, thousands of papers have been published concerning this enzyme. The review articles over this period of time however, are very similar to each other in content. It is known that $Na^+\text{-}K^+$ ATPase is the Na-K pump but the details of its chemistry and mechanism of action are still largely unknown.

The Relationship of the Na^+-K^+ Pump to the Membrane Potential.

The Goldman constant field equation predicts the membrane potential of a cell with the assumption that there is no net ionic movement across the membrane. The imbalance of the Na^+ and K^+ concentrations between the intracellular and extracellular fluids leads to a continuous influx of Na^+ and efflux of K^+. The imbalance of K^+ ($[K^+]_i > [K^+]_o$) is the prime determinant of the membrane potential (E_m) in most nerve and muscle cells so that the E_m is very near the K^+ equilibrium potential (E_K). This is known from experiments which show that Em decreases as $[K^+]_o$ increases. The membrane potential in most nerve and muscle cells has very little dependence on Na^+ since the membrane's permeability to Na^+ is so low ($P_{Na}=0.01\ P_K$). These are well known physiological principles which are briefly reviewed here because of their importance in the understanding of the Na-K pump's role in the determination of the cell's membrane potential.

The activity of the sodium pump in transporting K^+ into the cell establishes and maintains the concentration gradient for K^+ across the cell membrane. The pump, therefore, is important in long term maintenance of the membrane potential by keeping the $[K^+]_i$ well above the $[K^+]_o$. If the pump is inhibited the E_m slowly decreases as $[K^+]_i$ decreases (Thomas, 1972).

The results of early studies of the active transport of Na^+ and K^+ led investigators to question whether there might be an electrogenic component to the sodium pump, that is, whether the activity of the pump is in part responsible for the membrane potential. Originally the pump was proposed as an electrogenic

pump which transported only Na^+, but the observation that the pump exchanged Na^+ for K^+ brought the idea of an electrogenic pump into disfavor since it was assumed that the Na:K exchange ratio was 1:1 and thus that the pump was electroneutral. Hodgkin and Keynes (1955) recorded no change in membrane potential when the squid giant axon was treated with metabolic inhibitors giving further evidence for an electroneutral pump.

Although they did not recognize it as such, Ritchie and Straub (1957) presented evidence for an electrogenic pump in a study of the post-tetanic hyperpolarization (PTHP) in non-medullated nerve fibers. The increase in internal Na^+ during tetany stimulates the pump and this increased rate of pumping causes hyperpolarization during recovery. Ritchie and Straub thought that the PTHP was due to depletion of K^+ during increased pump activity. Later studies, however, confirmed that the PTHP is the result of the activity of an electrogenic pump (Straub, 1961).

Subsequent data collected by various authors using mollusc neurons, giant axons, and skeletal, smooth and cardiac muscle have provided ample data that the pump can be electrogenic. (See Thomas 1972 for references). Many of these studies have used sodium loading techniques in which the pump is inhibited by cooling the cell and removing K^+ from the external medium. Restoration of pump activity by replacement of K^+ and warming the neuron or muscle cell results in a dramatic hyperpolarization as pump activity is stimulated above normal levels by the elevated Na^+. The electrogenicity is caused by a coupling ratio of Na:K greater than 1, probably 3:2, as in the case of red blood cells. (Garrahan and

Glynn, 1967).

Given that the pump is electrogenic, what is the magnitude of its contribution to the membrane potential in the steady state? Mullins and Noda (1963) derived an equation based on the Nernst equation and the constant field equation which allows the calculation of the contribution of the electrogenic pump to the steady state resting membrane potential:

$$E_m = \frac{RT}{F} \ln \frac{rK_o + bNa_o}{rK_i + bNa_i} \qquad (2)$$

where r = the coupling ratio of the pump, b = Na permeability/K permeability, K_o, K_i, Na_o, Na_i are the internal and external ionic concentrations. For a neutral pump r = 1 and for a pump with a coupling ratio of 3Na:2K, r = 1.5. Thomas (1972) has shown that for a frog muscle or squid axon where b = 0.01 and r = 1.5 the contribution of the pump to the resting membrane potential is only 3 mv, which explains why Hodgkin and Keynes (1955) saw little effect of metabolic inhibitors on the membrane potential. In certain cells such as vertebrate photoreceptors which have a very high Na^+ permeability the pump can be shown to be responsible for as much as 50% of the membrane potential (see discussion).

Equation 2 allows comparison of an electrogenic pump to a neutral or inhibited pump. From this may be derived an equation which gives the maximum possible steady state contribution of an electrogenic pump to the membrane potential: (Thomas, 1972)

$$E_p = \frac{RT}{F} \ln \frac{rK_o + bNa_o}{rK_i} - \frac{RT}{F} \ln \frac{K_o + bNa_o}{K_i} \quad (3a)$$

$$= \frac{RT}{F} \ln \frac{1}{r} \cdot \frac{rK_o + bNa_o}{K_o + bNa_o} \quad (3b)$$

where Ep is the pump contribution to Em. Since for most cells bNa_i is much less than rK_i it has been eliminated. The first term of equation 3a predicts E_m with an electrogenic pump and the second term predicts Em based on constant field theory. The difference (E_p) is the contribution of the pump to E_m. The simplification of the equation (3b) shows that for any r, the maximum contribution of the pump to E_m is (RT/F ln 1/r), and for r = 1.5 this equals 10 mv. This describes the steady state and any stimulation of the pump may result in a larger hyperpolarization.

In summary, there is much evidence that the Na^+, K^+ pump is electrogenic. For long term regulation the pump is important in maintaining high $[K^+]_i$ and low $[Na^+]_i$. During periods of pump stimulation the pump may contribute significantly to the membrane potential. For most cells in the steady state the contribution of the pump to the resting membrane potential is only a few millivolts. In cells with a low resting membrane potential and high Na^+ permeability the pump may contribute a major portion of the resting membrane potential. Kerkut and York (1971) and Thomas (1972) have presented thorough reviews of the evidence for the electrogenicity of the Na^+-K^+ pump.

Na^+-K^+ ATPase in the Retina-

Its Location and Importance in Retinal Function.

The discovery of Na^+-K^+ ATPase in crab nerves by Skou (1957) led to a long series of papers on this enzyme by Bonting and his co-workers. In the first of these publications Bonting, Simon and Hawkins (1961) described the distribution of Na^+-K^+ ATPase in 36 different tissues of the cat. This study was the first to report the presence of Na^+-K^+ ATPase activity in the retina and the assay method used indicated that 72% of the total retinal ATPase activity is Na^+-K^+ activated. The same study showed that Na_+-K_+ ATPase is also present in the human retina accounting for 85% of the total ATPase activity. McConnel and Scarpelli (1963) suggested that rhodopsin is an ATPase with retinene acting both as a light trap and cofactor for the enzyme. This idea was refuted by Bonting, Caravaggio and Canady (1964) who showed that light does not have a direct effect on Na^+-K^+ ATPase activity in homogenates of bovine, rabbit and frog retinas. This was later confirmed by Frank and Goldsmith (1965) using pig retina.

In the studies cited above (Bonting et al. 1964, Frank and Goldsmith, 1965) the authors used preparations of isolated rod outer segments and the neural retina and found Na^+-K^+ ATPase activity in both fractions. Later papers (Frank and Goldsmith 1967, Hemminki 1974, 1975) also reported the presence of Na^+-K^+ ATPase activity in the plasma membrane and disc membranes of the rod outer segment. At the same time however this idea was disputed by electrophysiological (Zuckerman, 1973) and osmotic studies of

rod outer segments (Korenbrot and Cone, 1972) which indicated that the pump is located in the inner segments rather than the outer segments. The studies which indicated the presence of Na^+-K^+ ATPase in rod outer segment were done using crude rod outer segment fractions. Further studies by Berman and co-workers (Berman and Azimova, 1976; Berman, Azimova and Gribakin 1977) using highly purified subfractions of the crude rod outer segments preparations treated with a detergent which solublizes "latent" Na^+-K^+ ATPase, indicated that a negligible amount of Na^+-K^+ ATPase is present in the rod outer segment and they suggested that the Na^+-K^+ ATPase activity in rod outer segment detected in previous studies was due to contamination of the rod outer segment fractions with membranes from the inner parts of the retina. It is now well accepted that Na^+-K^+ ATPase is absent from the photoreceptor outer segments and that Na^+-K^+ pump is located in the photoreceptor inner segments and the neural retina.

Having established the presence and location of Na^+-K^+ ATPase in the retina, what is the pump's role in the function of the retina? Frank and Goldsmith (1967) showed that activity of the pump is essential for normal retinal function. Application of ouabain to the receptor surface of the isolated frog retina abolishes the b-wave of the ERG within four minutes and after seven minutes the retina does not respond to light (a-wave abolished). Application of ouabain to the vitreal surface attenuates the b-wave in less than a minute. Honda (1972a; 1972b) showed that ouabain also abolishes the ERG of the isolated rabbit retina. An important finding by Frank and Goldsmith (1967) was that less potent

inhibitors of Na^+-K^+ ATPase such as hexahydroscillaren A, and 17-α-cymarin also have a lesser effect on the ERG indicating a direct relationship between the activity of the pump and the ability of the retina to respond to light. Hamasaki (1963) had shown that replacement with choline of 95% of the Na^+ in Ringer solution bathing an isolated frog retina abolishes the ERG indicating that high extracellular Na^+ is necessary for generation of the ERG. Frank and Goldsmith (1967) confirmed this and showed further that if the retina is exposed to ouabain in low Na^+ Ringer's solution the ERG can be transiently restored by replacement of the low Na^+ Ringer's solution with normal Ringer's solution. They suggested that low Na^+ Ringer's solution in some way protects the retina from the effects of ouabain. Sillman, Ito and Tomita (1969) presented an explanation for this phenomenon in a study of the mass receptor potential of the isolated frog retina in which the b-wave had been suppressed by aspartate. They showed that the amplitude of the receptor potential is directly related to the log of the external Na^+ concentration and that if the retina is depleted of sodium by exposure to low sodium Ringer's solution and then exposed to ouabain in a low Na^+ solution the receptor potential can be transiently restored by exposing the retina to a normal Na^+ Ringer's solution containing ouabain.

Thus, the activity of the pump is required for maintenence of a Na^+ gradient across the cell membrane. If the pump is inhibited by ouabain, Na^+ leaks into the cell and as the intracellular sodium concentration increases, the amplitude of the response of the retina to light is decreased. The activity of the pump is not

directly involved in the light response as shown by the experiments in which leakage of Na^+ into the cell was prevented by decreasing extracellular Na^+ concentration during exposure to ouabain. In this case the pump is inhibited but a response to light can be transiently restored if the Na^+ gradient is reestablished by increasing the extracellular Na^+ concentration.

Prior to the above study by Sillman, et al. (1969), Toyoda, Nosaki and Tomita (1969) had shown that the hyperpolarization of the vertebrate photoreceptor in response to light is due to an increase in membrane resistance. Penn and Hagins (1969) had shown that in the dark a current flows extracellularly between the inner and outer segments of the photoreceptor. On the basis of these studies Sillman et al. (1969) proposed that the receptor potential is due to a change in the sodium flux across the membrane. This could be accounted for in one of two ways. Light could stimulate the metabolic pump to increase flux of Na^+ out of the cell, or light could decrease permeability of the membrane to sodium influx causing hyperpolarization of the photoreceptor. The first possibility was rejected since their experiment in which the Na^+ was replaced in the extracellular fluid after the pump was inhibited in the presence of low sodium, showed that the photoreceptor is capable of responding to light as long as a sodium gradient across the membrane is maintained. The function of the pump would be to maintain this gradient. Yoshikami and Hagins (1970) showed that the dark current which flows between the inner and outer segments is in fact carried by Na^+ ions and that light reduces this current by a decrease in the permeability of the outer

segment to Na^+. Importantly, they showed that in isolated retina the dark current is abolished by ouabain within two minutes and that if the Na^+ gradient is artificially reestablished by perfusing the retina with high Na^+ Ringer's solution the gradient decays in 60 seconds. Studies of the osmotic characteristics of rod outer segments showed that they have a high permeability to Na^+ in the dark and that this permeability is reduced in the light (Korenbrot and Cone, 1972). Based on the above studies (Toyoda et al., 1969; Sillman et al., 1969; Penn and Hagins, 1969; Yoshikami and Hagins, 1970), Korenbrot and Cone (1972) proposed a model which clearly shows the importance of Na^+-K^+ ATPase in photoreceptor function. The outer segment is highly permeable to Na^+ in the dark which results in a constant influx of Na^+ raising the membrane potential toward the equilibrium potential for Na^+. An electrogenic Na^+-K^+ exchange pump (Zuckerman, 1973) located in the inner segment (which has a high sodium resistance) constantly pumps sodium out of the cell resulting in the flow of current between inner and outer segments which is recorded in the dark. During illumination the Na^+ resistance of outer segment increases, the Na^+ influx decreases and the membrane is dominated by its K^+ permeability and hyperpolarizes. If the pump is stopped by inhibition of Na^+-K^+ ATPase (as by ouabain) Na^+ will continue to flow into the cell at the outer segment until the ionic gradient has decayed which will result in a loss of the photoresponse. The importance of Na^+-K^+ ATPase in the photoreceptor then is obvious since the pump is essential to the maintenance of a Na^+ gradient across the photoreceptor membrane. Because of the unusually high permeability

of the outer segment to Na^+ in the dark, the photoresponse is rapidly abolished by inhibition of the pump (see Figure 13).

The mechanism of action of the pump has not been worked out as completely for the inner retina as it has for the photoreceptors. The activity of Na^+-K^+ ATPase is important in maintaining the responsiveness of these cells as indicated by the studies of Frank and Goldsmith (1967) and Honda (1972a, 1972b) who showed that the b-wave is abolished before the a-wave when ouabain is applied to the retina. Intraretinal recording has shown that the b-wave originates in the inner nuclear layer (Brown, 1968). Intracellular recording shows that the only cells which respond to photostimulation with potentials corresponding to the b-wave are the Müller (glial) cells (Miller and Dowling, 1970). The Müller cells are not involved in signal transmission in the retina but act as potassium electrodes responding to increases in K^+ ion concentration in the extracellular fluid (Miller, 1973). The bipolar cells may make a major contribution to the changes in extracellular potassium (Armington, 1974) however, other cells are probably also involved. Tomita (1976) points out that although Muller cells do make a significant contribution to the b-wave the question whether it is exclusively of Müller cell origin is still unanswered. Recently Saito, Kendo and Toyoda (1979) have studied the ionic mechanisms of on-center bipolar cells in the carp retina. Bipolar cells which receive predominantly cone input show an increase in membrane resistance during depolarization and a reversal potential at -63 mv indicating that the depolarization is due to a decrease in K^+ and/or Cl^- conductance.

Rod dominated bipolar cells show a decrease in membrane resistance during depolarization and have a reversal potential at +29 mv indicating that these cells depolarize due to an increase in Na^+ conductance. The ionic gradients which lead to these ionic fluxes must be maintained by a metabolic pump and thus inhibition of the Na^+-K^+ ATPase may lead to a reduction in the response of these cells.

Oxygen Toxicity.

The gross effects of oxygen toxicity on the respiratory, cardiovascular and central nervous systems of animals such as pulmonary edema, cardiac arrhythmia and convulsions are well known and have been previously reviewed (Ubels, 1976). The toxic effects of oxygen are the result of derangements at the cellular level and the effects of oxygen on enzyme systems were described in detail by Haugaard and his associates (Stadie, Riggs and Haugaard, 1945; Haugaard, 1946; Horn and Haugaard, 1966; Haugaard, 1968). The early work on oxygen toxicity was primarily descriptive and there was little understanding of the primary mechanism of oxygen toxicity, that is, how high oxygen tensions actually cause cellular damage. It was known that cellular damage by irradiation is caused by the generation of free radicals such as $H^\cdot$ and $OH^\cdot$. Gerschman, et al. (1954) observed that many of the effects of oxygen poisoning are similar to those of x-irradiation and suggested that these two sources of cellular damage have a mechanism in common, namely the generation of oxidizing free radicals. They showed experimentally that the effects of irradiation are increased under conditions of

high oxygen tension. Haugaard (1968) also suggested the involvement of free radicals in the peroxidation of lipids and inactivation of sulfhydryl (SH) enzymes observed during oxygen toxicity.

Oxygen Free Radicals.

Evidence for the involvement of oxygen free radicals in biological systems was given by McCord and Fridovich (1969) who showed that an enzyme which catalyses the dismutation of the primary oxygen free radical, superoxide anion ($O_2^{\cdot-}$) is present in cells. This enzyme which they called superoxide dismutase (SOD) was shown by them to be identical to a copper containing enzyme known as erythrocuprein which had previously been found in erythrocytes and other cells, but had no known function. The discovery of this enzyme was a fortuitous accident which arose from studies of the reduction of cytochrome c by $O_2^{\cdot-}$. The reaction was inhibited by addition of carbonic anhydrase and this inhibition was shown to be due not to the action of carbonic anhydrase but to the contamination of the carbonic anhydrase by superoxide dismutase (McCord and Fridovich, 1977). The presence of superoxide dismutase in cells gave strong evidence that oxygen free radicals are produced in biological systems and that superoxide dismutase is present to protect against the harmful reactions of these free radicals with biological molecules.

In the following discussion of the involvement of free radicals in oxygen toxicity it is important to bear in mind that under normal conditions oxygen free radicals are produced in the cell as byproducts of normal metabolic reactions. Superoxide

dismutase and other antioxidants are present to scavenge these free radicals and render them harmless to the cell. Oxygen toxicity occurs when the amount of oxygen available to the cell becomes so high that the protective mechanisms are inundated and can no longer keep up with the production of free radicals. These reactive species then build up and attack the structural and metabolic components of cells.

A discussion of the reactions of $O_2^{\cdot-}$ and its involvement in oxygen toxicity requires an explanation of why such a free radical can be formed from molecular O_2. Fridovich (1977a) has presented a particularly lucid explanation of this phenomenon. Molecular oxygen is paramagnetic which is unusual for a gas. Each oxygen atom has six outer shell electrons, giving 12 electrons in the outer shells of the oxygen molecule. Of these electrons 10 are paired in five orbitals. The remaining two electrons are in different orbitals and have parallel spins. This results in spin restriction which means that a reductant which can offer an electron pair will not react because, as stated by the Pauli exclusion principle, two electrons with parallel spins cannot occupy the same orbital. The reduction of O_2 to H_2O requires four electrons and spin restriction favors a univalent pathway of reduction with the production of highly reactive free radical intermediates. This situation has been largely circumvented by the cytochrome oxidase system which catalyses the tetravalent reduction of O_2 to H_2O and handles most of the oxygen which is reduced in the cell. Significant amounts of oxygen can pass through a univalent pathway however, as much as 17% in the bacterium Streptococcus

faecalis (Fridovich, 1978).

As stated previously, the primary oxygen free radical is superoxide anion which is formed when an electron is added to O_2 forming $O_2^{-\cdot}$. Several reactions of $O_2^{-\cdot}$ are then possible. Superoxide radical may spontaneously dismutate forming hydrogen peroxide and singlet oxygen:

$$O_2^{-\cdot} + O_2^{-\cdot} + 2H^+ \longrightarrow H_2O_2 + {}^1O_2^* \qquad (4)$$

Singlet oxygen ($^1O_2^*$) is ground state oxygen in which an electron has been excited to a higher energy level. The resulting spin inversion yields the highly reactive singlet oxygen. The importance of this reaction in biological systems has been questioned (Fee and Valentine, 1977) however, it is known that $^1O_2^*$ is involved in lipid peroxidation and that its effects can be prevented by the $^1O_2^*$ scavenger 1,3 diphenylisobenzofuran (Pederson and Aust, 1973).

Superoxide radical can also react with H_2O_2 in a reaction known as the Haber-Weiss reaction which takes place in the presence of iron salts (Haber and Weiss, 1934):

$$O_2^{-\cdot} + H_2O_2 \longrightarrow O_2 + OH^- + OH^{\cdot} \qquad (5)$$

The hydroxyl free radical ($OH^{\cdot}$) produced in this reaction is the most powerful oxidant known and will attack most any organic

biomolecule (Fridovich, 1977a). Fee and Valentine (1977) have also questioned the importance of this reaction in cells based on purely physical chemical considerations since there is no chemical evidence for the direct reduction of H_2O_2 by $O_2^{\cdot-}$. It has been observed however in biochemical systems that when $O_2^{\cdot-}$ and H_2O_2 are generated by a system such as a xanthine-xanthine oxidase that a strong oxidant with the properties of $OH^{\cdot}$ is generated. The effects of $OH^{\cdot}$ such as oxidation of cytochrome c can be inhibited by $OH^{\cdot}$ scavengers such as ethanol (Cohen, 1977). The presence of iron-containing salts and proteins in the cell should favor the occurrence of the Haber-Weiss reaction in cells given an abundance of $O_2^{\cdot-}$ and H_2O_2.

The third important reaction of $O_2^{\cdot-}$ in the cell is the enzymatic dismutation catalysed by superoxide dismutase:

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \xrightarrow{SOD} O_2 + H_2O_2 \qquad (6)$$

Superoxide dismutase activity, while eliminating $O_2^{\cdot-}$, results in the production of H_2O_2 which itself is a highly reactive species. The levels of H_2O_2 are kept low in the cell by catalase and peroxidase enzymes (Fridovich, 1975). These enzymes reduce H_2O_2 to H_2O by the following reactions:

$$H_2O_2 + H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2 \qquad (7)$$

$$H_2O_2 + H_2R \xrightarrow{\text{peroxidase}} 2H_2O + R \qquad (8)$$

The peroxidase enzymes require the presence of a reductant (R) such as glutathione or ascorbic acid.

Given that $O_2^{\cdot-}$ and the other intermediates of the univalent reduction of oxygen are responsible for oxygen toxicity, where is $O_2^{\cdot-}$ produced in living cells? The autooxidation of several biologically important compounds is known to result in the production of $O_2^{\cdot-}$. These include reduced flavins, hydroquinones, catecholamines, hemoproteins and reduced ferridoxins (Misra and Fridovich, 1972a; 1972b; Fridovich, 1977b). This production of $O_2^{\cdot-}$ has been observed in vitro and it is assumed that similar reactions take place in the cell under conditions of hyperoxia. Several cytosolic enzymes such as xanthine oxidase and aldehyde oxidase are known to produce $O_2^{\cdot-}$ (Fridovich, 1978).

The mitochondrion is the cellular organelle primarily responsible for the reduction of oxygen. In the mitochondrial membrane are the components of the electron transport redox chain as well as the enzymes of the tricarboxylic acid cycle which produces NADH. Small amounts of H_2O_2, which appear to be produced from $O_2^{\cdot-}$, can be detected in metabolizing preparations of mitochondria. Under specialized conditions using submitochondrial particles it can be shown that $O_2^{\cdot-}$ can be generated by reduction of O_2 by cytochrome b_{566} (Flohe, et al., 1977). A more likely site for production of $O_2^{\cdot-}$ in the mitochondrion is NADH dehydrogenase.

Several of the flavin dehydrogenases are known to produce $O_2^{\cdot-}$

(Fridovich, 1978) and NADH dehydrogenase is a flavin dehydrogenase located in the mitochondrial membrane which is capable of reducing O_2 to $O_2^{-\cdot}$ (Gutman, Singer and Beinert, 1972). This $O_2^{-\cdot}$ can in turn inhibit NADH dehydrogenase by oxidation of SH groups (Tyler, 1975). Since this enzyme catalyses the transfer of electrons from NADH at the beginning of the electron transport chain, inhibition of this enzyme would stop the process of electron transport. Superoxide radical and H_2O_2 are of no known benefit to mitochondria (Flohe, et al., 1977) and the mitochondria are normally protected from these reactive species by superoxide dismutase and glutathione peroxidase (Tyler, 1975).

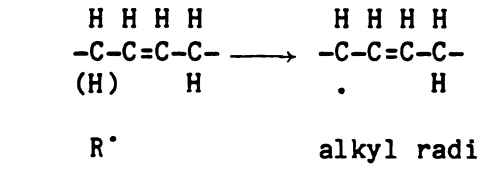
In summary, oxygen free radicals are produced in the cell due to univalent reduction of O_2. Several biological reactions normally produce these free radicals in the cell and the cell possesses protective mechanisms which prevent the interference of these free radicals with the cell's metabolic processes. Under conditions of hyperoxia however, these protective mechanisms are overwhelmed and free radicals accumulate causing cellular damage.

Lipid Peroxidation and Protein Damage.

The basis of most of the damage caused by free radicals in oxygen toxicity appears to be the peroxidation of lipids (Haugaard, 1968; Tappel, 1973). The lipid peroxidation products in turn can attack other lipids and proteins, although to a certain extent, oxygen free radicals can also attack proteins and mucopolysaccharides directly.

The biological membranes which make up the walls of cells, mitochondria and other organelles are made up of bilayers of

polyunsaturated fatty acids. The double bond character of these lipid molecules makes them particularly susceptible to attack by free radicals because the presence of carbon-carbon double bonds in the molecule weakens the carbon-hydrogen bond of the "alpha-methylene" carbons adjacent to the double bond. The weakening of the bond makes these "allylic" hydrogens particularly labile to abstraction by free radicals as shown below (Demopoulos, 1973):



(9)

$R^{\cdot}$ alkyl radical

The lipid free radicals formed can go on to react further with other polyunsaturated fatty acid molecules. This chain reaction, which takes place in the presence of oxygen, has been described by Feeney and Berman (1976). The lipid peroxidation chain reaction is begun by attack of a free radical ($R^{\cdot}$) on a lipid molecule (LH) forming a lipid free radical ($L^{\cdot}$):

$$LH + R^{\cdot} \longrightarrow L^{\cdot} + RH \qquad (10)$$

In the presence of oxygen the chain reaction is propagated resulting in formation of lipid peroxide radical ($LOO^{\cdot}$):

$$L^{\cdot} + O_2 \longrightarrow LOO^{\cdot} \qquad (11)$$

The $LOO^{\cdot}$ is highly reactive and further propagates the chain

reaction by attacking adjacent polyunsaturated fatty acid molecules:

$$LOO^{\bullet} + LH \longrightarrow LOOH + L^{\bullet} \qquad (12)$$

The $L^{\bullet}$ radical can react further with O_2 and the lipid hydroperoxide (LOOH) can also enter into the autooxidation chain reaction.

This chain reaction can be terminated by the following reactions:

$$L^{\bullet} + L^{\bullet} \longrightarrow$$
$$L^{\bullet} + LOO^{\bullet} \longrightarrow \quad \text{non-radical products} \qquad (13)$$
$$LOO^{\bullet} + LOO^{\bullet} \longrightarrow$$

however, a more damaging reaction can also take place, namely the breakdown of $LOO^{\bullet}$ into malonaldehyde ($O=(CH)_2=CHOH$) which can cause cross-linking, through Schiff bases, of free amino acids of protein and nucleic acids. This is an especially important reaction considering the number of membrane bound proteins embedded in the lipid bilayers of cell and mitochondrial membranes. Chio and Tappel (1969) suggest the following mechanisms of intramolecular and intermolecular enzyme inactivation by malonaldehyde:

Intramolecular:

$$O=(CH_2)=CHOH + \text{enzyme}\begin{matrix}\diagup \text{NH2} \\ \diagdown \text{NH}_2\end{matrix} \longrightarrow \text{enzyme}\begin{matrix}\diagup \text{NH=CH} \\ \diagdown \text{NH=CH}\end{matrix} \qquad (14)$$

Intermolecular:

malonaldehyde + 2 enzyme $\longrightarrow$ enzyme-NH-CH=CH-CH=N-enzyme (15)

It is easy to see how active sites may be blocked and how protein polymerization may occur through this mechanism.

Kellogg and Fridovich (1975, 1977) have studied the effects of oxygen free radicals on linolinate, liposomes, and red blood cells, using the xanthine-xanthine oxidase system which generates $O_2^{-\cdot}$ and H_2O_2. They observed peroxidation of the linolinate and liposomes and lysis of the blood cells. The cell lysis apparently was due to changes in the permeability of the cell membrane caused by lipid peroxidation. It was proposed in these papers that the lipids were attacked by $OH^{\cdot}$ and $^1O_2^*$ produced by the reaction:

$$O_2^{-\cdot} + H_2O_2 \longrightarrow OH^- + OH^{\cdot} + {}^1O_2^* \qquad (16)$$

The suggestion that $^1O_2^*$ is involved in lipid peroxidation is reasonable. Superoxide radical is highly soluble in aqueous solutions but not in lipids. The C=C bonds of polyunsaturated fatty acids are buried deep in the hydrophobic portion of the membrane and would be protected from $O_2^{-\cdot}$. The highly reactive $^1O_2^*$ however, is very soluble in lipid and thus could easily enter the membrane. Free radicals have also been shown to attack the lipids of mitochondrial and lysosomal membranes (Tappel, 1973). This causes swelling and lysis of the mitochondria and rupture of the lysosomes. Lysosomal membranes are peroxidized more slowly than other membranes due to their low lipid content, however, when they do break down, hydrolytic enzymes are released which cause generalized digestion of cellular contents, further explaining the

cellular breakdown observed in severe oxygen toxicity.

The mechanism of enzyme inactivation by malonaldehyde has been described above. Chio and Tappel (1969) have stated that sulfhydryl (SH) group containing enzymes are particularly susceptible to attack by lipid free radicals. Another important factor in the inactivation of membrane bound enzymes is the intimate association of some of these proteins with phospholipids. The presence of these lipids is required for enzymatic activity and modification of this relationship due to lipid peroxidation may have deleterious effects on the function of these enzymes (Demopoulos, 1973; Korenbrot, 1977).

Since free radicals are produced in cells under normal conditions, protective mechanisms against lipid peroxidation are also present in cells. Glutathione peroxidase reacts with LOOH to form LOH which is harmless to the cell (Feeney and Berman, 1976). More importantly, tocopherol (Vitamin E) is a first line of defense against lipid peroxidation (Tappel, 1965). This vitamin appears to function by offering hydrogen ions for abstraction by free radicals in preference to the allylic hydrogens of lipids. It has been shown that Vitamin E deficient animals are more susceptible to oxygen toxicity (Block, 1977).

As stated earlier, oxygen toxicity occurs when the supply of oxygen overwhelms these protective mechanisms. In addition to increased oxygen free radical production, hyperbaric oxygen can also promote the reaction:

$$L^{\cdot} + O_2 \longrightarrow LOO^{\cdot} \qquad (17)$$

increasing the rate of the free radical chain reaction.

Ocular Oxygen Toxicity

Effects of High Oxygen Tension on Blood Vessels, Lens and Cornea.

It has been known for many years that the ocular tissues, especially the retinal blood vessels and retina, are highly susceptible to oxygen toxicity (Nichols and Lambertsen, 1969). The main emphasis of the present study is the effect of oxygen on the retina, however the effect of oxygen on the retinal blood vessels will be covered briefly because of its historical and clinical importance.

The appearance of the disease retrolental fibroplasia (RLF) in the early 1940's provided the impetus for all work concerning the toxic effects of oxygen on the eye. This disease which is found in premature infants is characterized by irreversible constriction of the retinal arteries and cessation of growth of the retinal vessels. This is followed after removal to room air by neovascularization in the retinal periphery, and proliferation of fibrous tissue in the retina which eventually breaks through into the vitreous humor.

The retina eventually detaches and this, in combination with the proliferation of fibrous tissue, results in blindness. The search for the etiology of the disease revealed that it was caused by the pure oxygen to which premature infants were often exposed (Patz et al., 1952; Ashton, Ward and Serpell, 1953; Patz, 1965). Since this discovery the use of oxygen has been greatly curtailed.

Oxygen tensions used and exposure times have been lowered, however, RLF remains a problem in premature infants suffering from respiratory distress syndrome to whom oxygen must be administered (Patz, 1976; Kingman, 1977; Shahinian and Malachoski, 1978). In the mature retina hyperbaric oxygen causes reversible vasoconstriction (Anderson and Saltzman, 1965).

Hyperbaric oxygen and superoxide radical also have toxic effects on the cornea and lens. In guinea pigs, exposure to O_2 at 3-5 atm results in thinning of the corneal endothelium, and pycnosis and loss of lens epithelium nuclei (Nichols et al., 1972). Superoxide radical can be produced photochemically and it has recently been suggested that superoxide radical produced by this mechanism may be responsible for light induced cataract formation. Bhuyan, Bhuyan and Podos (1979) showed that cataracts can be induced by exposure of the lens to $O_2^{\bar{\cdot}}$, H_2O_2 and $OH^{\cdot}$ and that these lenses show elevated malondialdehyde levels, indicating lipid peroxidaton, as well as altered membrane permeabiilty. Superoxide dismutase, catalase, and ascorbate protect against light induced cataract formation indicating that the process is $O_2^{\bar{\cdot}}$ and H_2O_2 mediated (Varma et al., 1979). The aqueous humor of diurnal animals is high in ascorbate and the work cited above indicates that this ascorbate may protect the lens against photochemically produced $O_2^{\bar{\cdot}}$. Nocturnal animals have low ascorbate levels in the aqueous humor (Varma et al, 1979) and it is an interesting observation that domestic dogs which in the wild state are noctural, have a high incidence of cataract in old age.

Effects of High Oxygen Tension and Active Oxygen on the Retina.

The original work on the effect of high oxygen tension on the retina was done by Noell. Histological examination of retinas from rabbits exposed to O_2 at 760 mm Hg for 48 hr and 600 mm Hg for 100 hr revealed degeneration of 70% of the photoreceptors (Noell, 1955; 1962). Exposure of the animals to oxygen at 1262 mm Hg resulted in the death of 100% of the visual cells in 24 hr. Examination of rabbit retinas four days after exposure to O_2 at 5320 mm Hg for 4 hr showed that the outer nuclear layer was reduced in thickness and that pycnotic nuclei were present. A few days later the visual cells had disappeared by autolysis, however the ganglion and bipolar cells appeared normal (Noell, 1962). Bresnick (1970) essentially repeated Noell's work and showed by electron microscopy that ultrastructural changes appear in the visual cells before the changes are visible by light microscopy.

Hyperbaric oxygen is also toxic to the metabolic and enzymatic processes of the retina. Baeyens et al. (1973) showed that exposure of dog retina to oxygen at 1470 mm Hg for 24 hr caused a 37% decrease in the rate of oxidative carbohydrate metabolism as compared to control (O_2 at 154 mm Hg). Activity of lactate dehydrogenase is also decreased by exposure of frog and canine retinal tissue to oxygen pressures ranging from 740 mm Hg to 1470 mm Hg (Baeyens and Hoffert, 1972; Baeyens et al., 1974).

The electroretinogram which measures the massed electrical response of the retina to light has long been used as an indicator of the functional integrity of the retina. Noell (1955; 1962)

first reported that the ERG of the rabbit is attenuated by exposure of the animal to high oxygen tensions. Under the same conditions used for his histological studies, the ERG was abolished before the deterioration of the visual cells occurred. At an oxygen pressure of 5320 mm Hg the ERG b-wave was abolished in 43 min. Bridges (1966) exposed rabbits to oxygen tensions ranging from 1900-5320 mm Hg and noted an attenuation of both the a-wave and b-wave. The time to disappearance of the ERG was an inverse function of pressure with the a-wave lasting longer than the b-wave. Ubels et al. (1977) exposed isolated retinas to O_2 at 3800 mm Hg. This study showed that the frog ERG is abolished by 6 hr exposure to hyperbaric oxygen and that the rat ERG is abolished in 90 minutes. The importance of this study is that oxygen can have a direct effect on the electrical activity of the retina apart from its effects on the respiratory and cardiovascular systems.

As in other systems the toxic effect of oxygen in the retina is due to the formation of oxygen free radicals and it appears that most of the effects described above can be explained by the effects of lipid peroxidation on cellular function. As stated previously both ionizing radiation and visible light can cause free radical formation. Noell (1962) noted that the histological and electrophysiological effects of oxygen on the rabbit eye were very similar to the effects of x-irradiaton. He (Noell et al., 1966) also showed that exposure of rats to constant light causes breakdown of the photoreceptors and postulated that this light damage was due to "photosensitized oxidations" which lead to lipid peroxidation. Kagan et al. (1973), using frog retinas showed that

light exposure does in fact cause lipid peroxidation in the retina. The generation of free radicals leading to the formation of lipid peroxides appears to be a photodynamic effect related to the presence of rhodopsin since the effect was greater in the outer segments than in the neural retina. An identical effect was observed when retinas were exposed to chemically ($FeSO_4$ + ascorbate) generated free radicals (Kagan et al., 1975). Chemically induced lipid peroxidation also leads to a decrease in the ERG amplitude similar to that seen under hyperbaric oxygen (Shvedova et al., 1979). Yagi and Ohishi (1977) showed that the visual cell damage and ERG attenuation caused by exposure of retinas to hyperbaric oxygen *in vivo* and *in vitro* is associated with the increased amounts of lipid peroxide in the tissue.

It has been shown that after lipid peroxidation in photoreceptors rhodopsin is more easily extracted from rod outer segments and that lipid peroxidation weakens lipid-protein and lipid-lipid bonds in the membrane (Novikov et al., 1975). This has important implications for the electrical activity of the retina in terms of membrane permeability and pump activity as will be emphasized in the discussion.

The Resistance of Teleost Ocular Tissues to Oxygen Toxicity.

The teleost retina is normally enveloped by oxygen tensions in excess of 400 mm Hg (Wittenberg and Wittenberg, 1962; Fairbanks et al., 1969; Hoffert and Ubels, 1979b). Extended exposure to such high oxygen tensions is toxic to the retinas of other animals. These high oxygen tensions are generated by the counter current oxygen multiplier known as the choroidal *rete mirabile*. The rete

is capable of a 10 fold or greater concentration of oxygen since the P_{O_2} of rainbow trout (Salmo gairdneri) blood is 45-60 mm Hg while the P_{O_2} at the retina is about 650 mm Hg as recently shown by Hoffert and Ubels (1979b). Electroretinographic studies in vivo and in vitro, and a study of retinomotor activity have shown that the trout retina is dependent on these high oxygen tensions for normal function (Fonner, Hoffert and Fromm, 1973; Hoffert and Ubels, 1979a, 1979b, 1979c).

A mechanism for oxygen concentration has been proposed (Fairbanks et al., 1974) which depends on the enzyme carbonic anhydrase (CA). Erythrocyte CA in the choriocapillaries hydrates CO_2, producing hydrogen ions and releasing O_2 into the plasma by the Bohr and Root effects. In the retina, bicarbonate neutralizes lactic acid and CA dehydrates H_2CO_3 forming CO_2 which diffuses into the choriocapillaries again raising the P_{O_2} of the venous blood by the Bohr and Root effects. Oxygen diffuses from the venous side of the rete into the arterial side and is carried to the retina. In the venous side of the rete CA, which is probably located in the walls of the vessels, hydrates CO_2 thereby preventing short circuiting of the system by diffusion of CO_2 into the arterioles. The use of the CA inhibitors, acetazolamide and CL-11,366, showed that this short circuiting does in fact occur when CA is inhibited (Fairbanks et al., 1974; Fonner et al., 1973). Histochemical studies have shown that high levels of CA may be present in the retial vessels (Eldred, 1975). The histochemical technique used in this study has, however, come under criticism.

The presence of high P_{O_2} at the retina suggests that the

ocular tissues of teleosts may be resistant to oxygen toxicity. Several studies of the effect of hyperbaric oxygen on metabolism, LDH activity and the ERG in the teleost retina have indicated that this is the case. Baeyens et al. (1973) showed that trout retinal metabolism is enhanced by exposure to hyperbaric oxygen. Incubation of retinal tissue under 100% O_2 at 154 mm Hg, 400 mm Hg and 1470 mm Hg for 24 hr causes a linear increase in metabolic rate with rising P_{O_2}. Metabolism of lens, cornea and retina of the white grunt (Haemulon plumieri) is enhanced by exposure to O_2 at 3040 mm Hg (Hoffert, Baeyens and Fromm, 1973).

In general the enzyme LDH is inhibited by molecular oxygen, however trout retinal LDH activity is not affected by exposure to oxygen at 740 mm Hg (Baeyens and Hoffert, 1972) indicating that there is a mechanism which prevents oxidation of the enzyme. Trout retina LDH activity is in fact enhanced by exposure to oxygen at 1470 mm Hg for 24 hr (Baeyens et al., 1974). The ERG recorded from trout and goldfish retinas is unchanged after exposure to oxygen at 3800 mm Hg for 6 hr (Ubels et al., 1977).

This evidence indicates that the teleost has developed a mechanism of resistance to ocular oxygen toxicity. The dependence of the teleost retina on high P_{O_2} is probably related to the fact that it has an avascular retina and therefore the whole retina depends on O_2 diffusion from the choroid.

Substances which protect against oxygen toxicity by scavenging active oxygen species have been described. It is expected that these substances may be present in elevated concentrations in the teleost retina. Studies of superoxide dismutase levels in trout

retina and choroid have begun in Hoffert's laboratory, however, no data comparing the amount of superoxide dismutase found in the trout retina compared to retinas of other species is available. It should be noted that trout brain and liver are not resistant to oxygen toxicity (Baeyens et al., 1973; 1974).

Effect of Hyperbaric Oxygen on
Neural Activity and Ion Transport.

In addition to the effects of oxygen on electrical activity in the retina, oxygen also has toxic effects on the rest of the nervous system. The convulsive effects of hyperbaric oxygen on the brain are well known. Central nervous system toxicity occurs in man after only a few minutes of oxygen breathing at 3040 mm Hg (Saltzman, 1967) and mice convulse within 30 min during exposure to O_2 at 3800 mm Hg (Hoffert, Baeyens and Fromm, 1975).

Peripheral nerves are also subject to the toxic effects of oxygen. Perot and Stein (1956; 1959) showed that conduction in frog sciatic nerve is blocked after 4 hr exposure to O_2 at 9880 mm Hg (13 atm) and that cat ulnar nerve is much more sensitive to oxygen than frog nerve with conduction blockade occurring in 3 hr at 3040 mm Hg (4 atm). They showed that there is an inverse relationship between time to conduction blockade and oxygen pressure and that with increasing stimulation frequency, time to conduction blockade is decreased. Later work by Cymerman and Gottlieb (1970) using frog sciatic nerve gave similar results. They also showed that hyperbaric oxygen causes a decrease in action potential amplitude, conduction velocity and rheobase.

Since maintenance of ionic gradients for sodium and potassium through active transport of the ions is essential for normal neural activity the observation that hyperbaric oxygen interferes with normal neural activity lead to several studies of the effects of hyperbaric oxygen on sodium and potassium content and transport in

various tissues. Kaplan and Stein (1957a) exposed slices of guinea pig brain cortex to oxygen at pressures ranging from 760-8360 mm Hg and showed that the potassium content of the slices decreased and the sodium content increased. The magnitude of these changes is directly related to the oxygen pressure. In vivo studies showed that brain sodium content increased as a result of exposure of guinea pigs to oxygen at 5016 mm Hg for 2 hr (Kaplan and Stein, 1957b). These results suggest that hyperbaric oxygen may affect the active transport mechanism for these ions. At the time of these studies the concept of an ion pump was developing, however, the mechanism of the pump (Na^+-K^+ ATPase) was not yet understood. However, they suggested a possible effect of oxygen on enzymes providing energy for active transport.

Joanny, Corriol and Brue (1970) exposed guinea pig cerebral cortex to oxygen at 2280-7600 mm Hg (3-10 atm) and found that intracellular potassium decreased and intracellular sodium increased as a function of pressure. They also showed increased levels of lipid peroxides and found that ATP levels decreased slightly while inorganic phosphate increased. It was suggested that changes in mitochondrial and plasma membrane permeability due to lipid peroxidation could lead to the movements of potassium and sodium which were observed.

In addition to the evidence from studies on nerve tissue that hyperbaric oxygen may be toxic to sodium and potassium transport, direct measurements of the effect of oxygen on sodium transport have been made using frog skin and toad bladders which actively transport sodium from mucosa to serosa. Falsetti (1959) made bags

from the skin of frog hind legs, filled them with Ringer's solution and immersed the bag in Ringer's solution. After 24 hr exposure to O_2 at 6060-9120 mm Hg (8-12 atm) the sodium content of the Ringer's solution in the bags was significantly lower than control indicating that exposure to hyperbaric oxygen inhibited transport of sodium from the environment. Gottlieb and Cymerman (1970) using a preparation of frog skin similar to that of Ussing and Zerahn (1949) showed a decrease in potential difference and short circuit current and an increase in resistance during hyperbaric oxygenation under pressure ranging from 3800-26600 mm Hg (5-40 atm). Oxygen at 3800 mm Hg also decreases sodium transport through toad urinary bladder causing a decrease in short circuit current (Miller, Hall et al., 1976; Miller and Mendoza, 1978).

Effects of Hyperbaric Oxygen on Na^+-K^+ ATPase.

Gottlieb and Cymerman (1970) and Miller, Hall et al. (1976) suggested inhibition of Na^+-K^+ ATPase as a possible cause of the changes in sodium transport observed with hyperbaric oxygenation. Kovachich and Haugaard (1976) have also suggested a toxic effect of oxygen on the sodium pump in cortical brain slices. It is well known that oxygen toxicity involves lipid peroxidation. An increase in membrane permeability alone due to disruption of membrane lipids could result in increased movement of sodium into the cell and potassium out of the cell. If the pump were not affected by oxygen the resultant increase in intracellular sodium could stimulate pump activity. On the other hand, however, it is highly unlikely that the pump would be unaffected by oxygen since

it is known that there is an absolute requirement for the phospholipids which are associated with Na^+-K^+ ATPase. Peroxidation of these lipids or a direct attack of free radicals on the enzyme itself, since it is known to be sulfhydryl dependent (Skou, 1963), could lead to the decreases in ion transport which have been observed following hyperbaric oxygenation. Changes in membrane permeability and inhibition of the pump, of course, are not mutually exclusive as explanations for the toxic effect of hyperbaric oxygen on cellular ionic composition.

Gottlieb and co-workers (Koehler and Gottlieb, 1972; Gottlieb, Koehler and Rhodes, 1976; Hemrick and Gottlieb, 1977; Gottlieb et al, 1977) have studied the effects of hyperbaric oxygen on Na^+-K^+ ATPase from rat intestinal mucosa, rat brain, bovine brain and bovine cardiac muscle. These reports are inconclusive and confusing. The effects of oxygen on the enzyme vary, with slight inhibition occurring at low pressures, stimulation occurring at high pressures. The papers are also of little value since the techniques used are questionable and data are presented only as percent change from control with no values given for enzyme activity. These reports will be discussed in greater detail below.

MATERIALS AND METHODS

Experimental Animals

Rainbow trout (Salmo gairdneri) weighing 150-200 g were obtained from Midwest Fish Farming Enterprises (Harrison, MI). They were maintained in fiberglass tanks at 12±1C. Dechlorinated tap water flowed continously through the tanks and was aerated by compressed air filtered through activated charcoal. The photoperiod was 16L:8D.

Long-Evans rats (Rattus rattus) weighing 150-200 g were obtained from Charles River Breeding Laboratories (Wilmington, MA). They were kept under natural lighting conditions or in a room with a 14L:10D photoperiod. Long-Evans rats were chosen rather than the commonly used albino Sprague-Dawley strain, since their eyes are normally pigmented. All previous studies from this laboratory on ocular oxygen toxicity have been done using normally pigmented animals (Baeyens et al., 1973, 1974; Hoffert et al., 1973; Ubels et al., 1977). This is important since the retina of the albino is exposed to high levels of light due to lack of protection by the melanin of the pigment epithelium. As cited previously, light damage to the retina often resembles the effects of oyxgen toxicity in that it is mediated by free radical formation. Thus, investigators studying the eyes of albino animals may be dealing with abnormal, damaged retinas. Albinos often suffer from visual problems (Creel, O'Donnell and Witkop,

1978) and Weidner (1976) has shown that there are marked differences between the ERG's of albino and pigmented rats.

Bovine (Bos taurus) eyes were obtained at slaughter from the Michigan State University Meat Laboratory. The eyes were placed on ice and the retinas were removed within 1 hr after enucleation.

Frogs (Rana pipiens) were obtained from Nasco Biologic Co. (Ft. Atkinson, WS). They were held at 15C in a moist environment. The photoperiod was 16L:8D.

Tissue Preparation

Review of Methods.

For all experiments the retinas were removed from the eye without the pigmented epithelium. In order to do this it was necessary, when using trout, to dark adapt the animals because these animals exhibit retinomotor activity (Hoffert and Ubels, 1979c). In the light the rods are extended and the outer segments interdigitate with the apical processes of the pigmented epithelium so that the two tissues adhere to one another. In the dark-adapted state the rods contract and the pigment epithelium separates easily from the retina. Since mammalian eyes do not exhibit retinomotor activity dark-adaptation does not facilitate removal of the pigmented epithelium.

The cornea, lens and iris were removed from the eye, the attachment of the optic nerve to the retina was severed and the retina was gently removed from the eye.

The tissue was homogenized by the method originally used by Bonting et al. (1961) which simply requires homogenization of the

retina in cold distilled water. Since Bonting's original study other methods of Na^+-K^+ ATPase preparation have been developed which serve to increase the specific activity of the preparation. The simplest of these is homogenization of the tissue in a 0.25 M sucrose solution containing EDTA, buffered with an organic buffer such as Tris or histidine (Jampol and Epstein, 1970; Kawada, Taylor and Barker, 1969, 1975; Miller, Kinter et al., 1976). Although the buffer system of this preparation may tend to stabilize the enzyme, the high osmolality of the solution would not aid in disruption of the cells and therefore the advantage of this type of preparation is questionable. Many investigators carry this method a step farther achieving partial purification of the enzyme through preparation of a microsomal fraction by differential centrifugation. The detergent Na-deoxycholate is also added in some cases to aid in solubilization of the enzyme (Bonting et al, 1964; Weaver, Akera and Brody, 1977; Nechay and Saunders, 1978; Lagerspetz and Senius, 1979). It should be noted here that Korenbrot (1977) warns that care should be taken when treating Na^+-K^+ ATPase preparations with detergents lest activity be lost due to interference of the detergent with the lipids associated with the enzyme.

More elaborate procedures are used to obtain highly purified enzyme from these microsomal preparations. These have been described by Nakao et al. (1965), Akera and Brody (1969) and notably by workers from L.E. Hokin's laboratory (Perrone et al., 1975; Dixon and Hokin, 1978). Such preparations are usually obtained from large amounts of tissue high in Na^+-K^+ ATPase such

as brain, eel electroplax and shark rectal gland and are usually used in biochemical work such as studies of binding kinetics of drugs, e.g. ouabain (Akera, 1971), and determination of the chemical structure of the enzyme (Perrone et al., 1975). These preparations approach 95% purity; however preparations of this type were not desirable in the present study.

The microsomal preparations described above are often used in studies involving biochemical characterization of Na^+-K^+ ATPase (Kawada et al., 1975) or in studies of the direct effects of exogenous agents such as digitalis (Weaver et al., 1977), lead (Nechay and Sanders, 1978) or DDE (2,2-bis-p-chlorophenyl 2,2,2-trichlorethane) (Miller, Kinter et al., 1976) on Na^+-K^+ ATPase.

This method of prepartion was deemed unnecessary for the present study since, as described earlier, molecular oxygen (O_2) itself has no toxic effect on biological systems. All of the enzyme systems responsible for the production of such species as superoxide radical, singlet oxygen and hydrogen peroxide should be present during exposure of tissues to hyperbaric oxygen. Thus a crude homogenate containing the mitochondria and all of the cytosolic oxidase enzymes was used in this study. Distilled water was used as a homogenization medium rather than a sucrose buffer since it has the advantage of osmotically lysing the cells and raising the specific activity of the enzyme since the inner and outer surfaces of the membrane fragments are exposed to the incubation medium.

The use of crude, distilled water homogenates is also

desirable when working with retinas since the amount of tissue available is minimal. Recently published studies of Na^+-K^+ ATPase of rat retina and pigmented epithelium have used the same method of preparation used in the present study (Winkler and Riley, 1977; Riley et al, 1978).

Protocol

In the initial studies, in which trout and bovine retinas were used, the retinas of several animals were pooled and homogenized using a motor driven teflon tissue homogenizer. The retinas of 15 trout were homogenized in 20 ml of ice cold distilled water (wt/volume unknown due to adherence of vitreous humor to retina). The homogenate was divided among several serum vials each holding about 2 ml and was then quickly frozen on dry ince, lyophilized and stored at -4C. For each experiment the lyophilized tissue was reconstituted with distilled water using 5 mg dry tissue/ml, giving a protein concentration of 1.92 ± 0.04 mg protein/ml (determined by the method of Lowry et al., 1951; see Appendix I).

Bovine retinas were homogenized using 1 g wet weight/10 ml distilled water and lyophilized. The dry tissue was reconstituted using 2.0 mg dry tissue/ml, giving a protein concentration of 1.3 ± 0.08 mg protein/ml.

This method of tissue preparation proved to be somewhat unsatisfactory since pooling of the retinas of several animals did not allow comparisons among animals. Therefore after the initial trout experiments (homogenates exposed to O_2 at 3800 mm Hg at 37C) retinas were no longer pooled. Each retina was

homogenized individually in 4 ml of cold distilled water and placed in a serum vial. The homogenate was frozen on dry ice and lyophilized. The retinas were reconstituted in 3.5 ml distilled H_2O giving a protein concentration of 1.8±0.03 mg protein/ml which was comparable to the protein concentration used in the initial experiments.

An attempt was made with trout retinas to dispense with the lyophilization step of the preparation procedure and simply measure ATPase activity immediately after homogenizing the retina, as was possible when using rat retinas (see below). Na^+-K^+ ATPase activity using this method was very low and in several cases could not be measured. It appears that freezing the homogenate and later reconstituting the freeze dried tissue lyses the cells more completely.

In the final series of trout experiments, intact retinas rather than homogenates were exposed to hyperbaric oxygen and ATPase activity was measured immediately after homogenization of the retina. Freezing the homogenate quickly in a dry ice-ethanol bath and followed by immediate thawing increased the specific activity to acceptable levels (Na^+-K^+ ATPase activity 50% of the total ATPase activity, as compared to 75% for lyophilized tissue). Schwartz (1962) showed that storage of rat cardiac muscle ATPase at -5C for several days increased the Na^+-K^+ ATPase:Mg-ATPase ratio because of a decrease in Mg-ATPase activity. Frog retinas were also prepared by this method after exposure to oxygen. Each retina was homogenized in 1.7 ml water giving a protein concentration of 0.772 mg/ml.

A paper by Winkler and Riley (1977) which indicated that Na^+-K^+ ATPase activity could easily be measured in single rat retinas led to the use of the rat retina for this project rather than the bovine retina. Their method of tissue preparation was adopted and comparable enzyme activities were obtained. The two retinas of a single rat were homogenized in 3.5 ml of water for experiments in which homogenates were exposed to hyperbaric oxygen. Individual retinas were homogenized in 1.7 ml of water after exposure of intact retinas to hyperbaric oxygen. The protein concentration was 0.60 ± 0.01 mg/ml. Rat retina homogenates were never frozen.

ATPase Assay

The assay for ATPase activity was similar to methods found in the literature (Bonting et al., 1961; Winkler and Riley, 1977) and is based on the determination of inorganic phosphate (P_i) liberated from ATP.

The assay was carried out in 8 ml test tubes. First 0.1 ml homogenate was added to 0.8 ml assay medium, either Medium I or Medium II, and preincubated for 10 minutes to allow association between ions and enzyme. The compositions of Medium I and II are shown in Table 1. The reaction was started by addition of 0.1 ml of a solution containing disodium ATP (Sigma grade I, Vanadium free, Sigma, St. Louis, MO) and $MgCl_2$ dissolved in 50 mM Tris, pH 7.5. After 20 min (10 min for trout and bovine at 37C and 5 min for purified canine kidney Na^+-K^+ ATPase, because of the high activity of these tissues) the reaction was stopped with 3 ml of

ice-cold color reagent (pH=1.0) and the tubes were placed on ice for 5 minutes. After centrifugation at 2000 g for 15 minutes the supernatant was read within 30 min at 700 nm on a Beckman DBG spectrophotometer (Beckman Instruments, Fullerton, CA) or a Coleman Junior II Model 6-35 spectrophotometer (Coleman Instrument, Maywood, IL). All determinations were done in duplicate.

Table 1. Composition of assay media.

	Medium I	Medium II*
NaCl	100 mM	100 mM
KCl	10 mM	---
EDTA	0.1 mM	0.1 mM
Tris#	50 mM, pH 7.5	50 mM, pH 7.5

*Ouabain 0.1 mM (Sigma) was also added to Medium II in some experiments but had no effect on enzyme activity indicating that the absence of K^+ in Medium II was sufficient to inhibit Na^+-K^+ ATPase activity.

#Trisma Base (Sigma) and Tris HCl (Sigma) were blended to give the desired pH at any given assay temperature.

Principle of Assay Procedure

Na^+-K^+ ATPase activity requires the presence of Na^+, K^+ and Mg^{+2}. Unpurified preparations, such as the homogenates used in this study, also contain ATPase enzymes which depend only on Mg^{+2} for activity. The total ATPase activity (Na^+-K^+ ATPase + Mg ATPase) was measured with assay Medium I and only the Mg^{+2} dependent activity was measured in the presence of assay Medium

II since K^+ was absent. Thus subtracting the ATPase activity obtained with Medium II from the activity obtained with Medium I gives the Na^+-K^+ ATPase activity.

Principle of Inorganic Phosphate Determination

The color reagent used in the assay procedure consisted of 1% ammonium molybdate in 1.5N H_2SO_4 in which ferrous sulfate (4g/100 ml) was dissolved shortly before use. This color reagent served two purposes; it stopped the ATPase reaction by denaturing the protein due to its low pH (pH=1.0) and it reacted with P_i to give a blue color proportional to the amount of P_i present. The use of color reagent to stop the reaction has also been reported by other authors (Miller, Kinter et al., 1976; Winkler and Riley, 1977) and eliminates a step in the procedure compared to the older method which used trichloroacetic acid to stop the reaction followed by addition of color reagent (Bonting et al., 1961).

The principle of the P_i determination is the reaction of phosphorous with molybdate to form phosphomolybdic acid. In the presence of a reducing agent such as ferrous sulfate or alpha-amino-naphtholsulfonic acid (Fiske and Subbarow, 1925) a blue color develops which is proportional to the amount of P_i present.

The P_i present in the assay tubes was determined from a standard curve. A standard solution was prepared by dissolving 175.2 mg KH_2PO_4 in 100 ml of distilled water giving a phosphorous concentration of 40 mg/100 ml. A standard curve was prepared from this solution by preparing a set of tubes containing 0.0, 0.5, 1.0, 2.0 and 4.0 mg/100 ml phosphorous. This was done by

first adding 0.1 ml of water or a known phosphorous solution (5,10,20, 40 mg phosphorus/100 ml) to 0.8 ml of assay Medium I. During the assay procedure these tubes also received 0.1 ml of ATP-Mg solution and were treated identically to the ATPase assay tubes but received no enzyme. Thus, addition of 0.1 ml of ATP solution to the standard tubes not only brought the P_i in these tubes to the desired concentration by dilution but also served as an internal correction for non-enzymatic ATP hydrolysis. The tubes which received 0.1 ml of distilled water served as blanks against which all other tubes were read.

ATP Concentration and Enzyme Kinetics

Most ATPase assay techniques reported in the literature use ATP concentrations ranging from 2-5 mM with Mg^{+2} concentration equal to the ATP concentration (Bonting et al, 1961; Winkler and Riley, 1977; Weaver et al., 1977). ATP at 5 mM was used in most of the experiments reported in this study.

In some experiments ATP concentrations ranging from 0.25 to 10 mM were used in order to generate kinetics curves (Lineweaver-Burke plots) from which K_m and V_{max} for the enzyme could be calculated.

Experimental Protocol

Two types of experiments were conducted to determine the effect of hyperbaric oxygen on retinal Na^+-K^+ ATPase. In one series of experiments retinal homogenates were exposed to oxygen. A homogenate of the retina was prepared and 1 ml aliquots of this homogenate were placed in each of two 20 ml beakers. The remainder of the homogenate was saved for protein determination.

One of these beakers was designated as control and was placed in a sealed, moist container under air at 740 mm Hg (PO_2=154 mm Hg).

The other beaker was placed in a Bethlehem Environmental Chamber (Model H-70-A, Bethlehem, PA) which was flushed with O_2 and pressurized. The control chamber and hyperbaric chamber were placed in an incubator which could be warmed or cooled to the desired temperature. After exposure to O_2 for 4 hr the homogenates were removed from the chambers and immediately analyzed for ATPase activity.

In a second series of experiments intact retinas were exposed to hyperbaric oxygen. Both retinas were removed from the animal. One retina was placed in 1 ml of Tissue Culture Medium 199 (TC 199) (DIFCO, Detroit, MI) at pH 7.5 in one section of a 4 chambered petri dish designated control (air, 740 mm Hg) and the other was placed in TC 199 in a similar petri dish which was placed in the hyperbaric chamber. Since the petri dishes had four chambers the retinas of four animals could be placed in each dish. After exposure to O_2 the retinas were removed from the chambers, homogenized and analyzed for ATPase activity.

Similar experiments were conducted using nitrogen at high pressure to separate possible effects of high pressure from the effects of oxygen on the enzymes.

EXPERIMENTAL RESULTS AND DISCUSSION

Experiments on the Effect of Hyperbaric Oxygen on Na^+-K^+ ATPase Activity of Retina Homogenates

Trout Experiments.

The trout is a poikilothermic animal and therefore the characteristics of its retinal Na^+-K^+ ATPase were studied at several different temperatures, 12.5C, 22C and 37C. The rainbow trout is a cold water fish preferring water below 15C. Studies from our laboratory (Hoffert and Ubels, 1979a, 1979b) indicate that the electrical activity of the trout retina is adversely affected by temperatures above 20C. The Na^+-K^+ ATPase from trout retina appears to function quite well at temperatures as high as 37C as shown by Lineweaver-Burke plots of the relationship of enzyme activity to substrate concentration (Figures 1,2 and 3) The Q_{10} plot is linear and the value for Q_{10} (1.734) is within the expected range for enzymatically catalysed reactions (Figure 4).

The effect of hyperbaric oxygen on Na^+-K^+ ATPase of homogenates of trout retina are shown in Tables 2-6. Homogenates were exposed to oxygen for 4 hr at 3800 or 11,600 mm Hg. Exposures and assays were carried out at 12.5C, 22C or 37C. The 4 hr exposures and pressures of 3800 mm Hg were chosen based on a previous study of the effect of oxygen on the ERG (Ubels, 1976). The higher pressure was used to study the effect of increasing

Figure 1. Lineweaver-Burke plot of trout retina Na^+-K^+ ATPase activity at 14.5C.
Vmax = 8.09 µmol P_i/mg protein/hr
Km = 1.25 mM ATP
x ± SE n = 3
r = 0.863
[ATP] = 0.25 - 15.0 mM

Figure 2. Lineweaver-Burke plot of trout retina Na^+-K^+ ATPase activity at 22C.
Vmax = 14.47 µmol P_i/mg protein/hr
Km = 1.84 mM ATP
x ± SE n = 3
r = 0.994
[ATP] = 0.25 - 15.0 mM

Figure 3. Lineweaver-Burke plot of trout retina Na^+-K^+ ATPase activity at 37C.
Vmax = 38.34 µmol P_i/mg protein/hr
Km = 3.49 mM ATP
x ± SE n = 3
r = 0.903
[ATP] = 0.25 - 10.0 mM

Figure 4. Q_{10} for trout retina Na^+-K^+ ATPase.
Solid line shows Q_{10} plot of enzyme activity at a substrate concentration of 5 mM ATP. Q_{10} = 1.734 (calculated using data from Tables 2-6).

Dashed line shows Q_{10} for Vmax as determined from Figures 1,2, and 3.
Q_{10} Vmax = 1.997

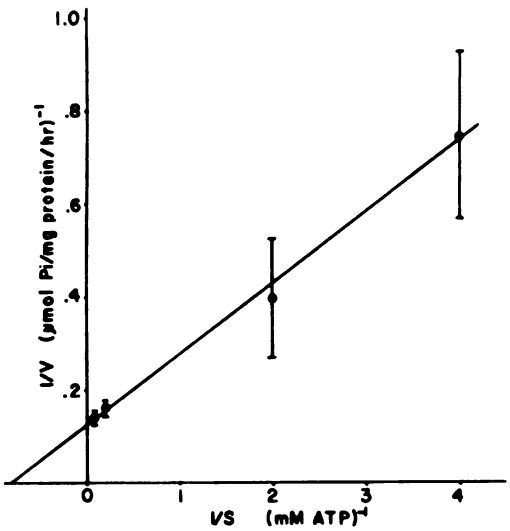


Figure 1

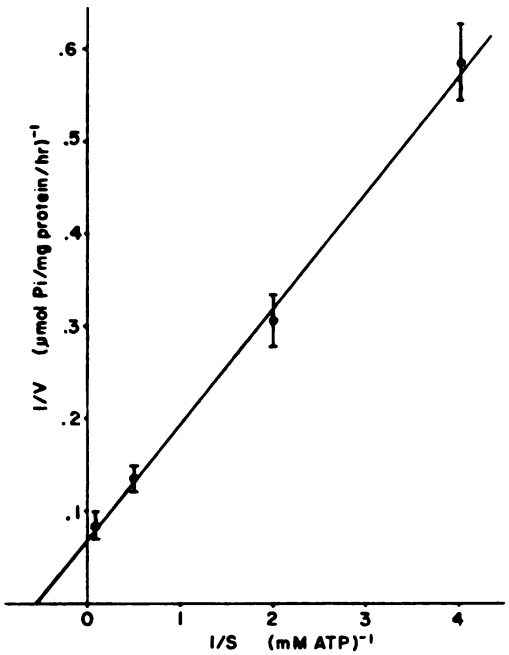


Figure 2

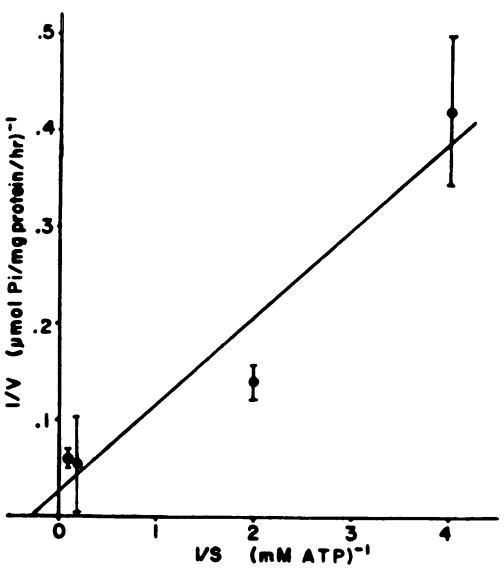


Figure 3

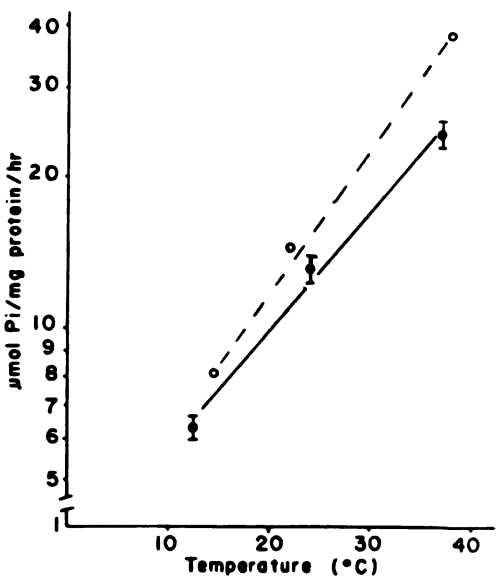


Figure 4

oxygen tension. The pressure 11,600 mm Hg is near the upper limit of safety for the system used in this study.

The time required for development of symptoms of oxygen toxicity is dependent on the P_{O_2}. That is, the higher the P_{O_2} the shorter the time required to reach a given level of oxygen toxicity. This has been shown in numerous studies of which Bridges (1966) work on the ERG and Gottlieb and Cymerman's (1970) work on frog skin are examples. Animals would usually not be exposed to O_2 at pressures as high as those used in this study but might be exposed to oxygen pressures of 1 or 2 atm for longer periods of time. Pressures as high as those used in this study are therefore used as a matter of convenience since any toxic effects will be seen in a shorter period of time than at low pressures. This is especially important when isolated tissues are used. Control preparations were exposed to air at 740 mm Hg (P_{O_2} = 154 mm Hg) for 4 hr. All experiments were paired, with tissue from the same animal exposed to oxygen and air simultaneously. The paired t-test, split-plot analysis of variance (ANOVA) and Student-Newman-Kuels test were used for statistical analyses.

It is not possible to make comparisons of the control trout retina Na^+-K^+ ATPase activity obtained in this study to other studies since there are no reports in the literature for teleost retina Na^+-K^+ ATPase activity. The activity obtained at 22C is comparable to that obtained by Bonting et al. (1964) for frog retina. The percentage of the total ATPase activity which is activated by Na^+ and K^+ was 75% and is also in the range observed

in other studies of retinas of several species (Bonting et al., 1961).

Several ATP concentrations were used in the assay for Na^+-K^+ ATPase activity because the original intention was to analyse the inhibition of the ATPase by oxygen in terms of Michaelis-Menten kinetics. However, further study showed that the type of enzyme inhibition involved in oxygen toxicity should not be treated by Michaelis-Menten principles, which assume the formation of enzyme-inhibitor and enzyme-substrate-inhibitor complexes which are rapidly established and easily reversible (Lehninger, 1975). Inhibition of enzymes by active oxygen involves enzyme modification (disruption of lipid-enzyme relationships, formation of disulfide bonds). This may not be reversible and the decrease in enzyme activity takes place over a period of time and becomes more severe with prolonged exposure to hyperbaric oxygen as a greater proportion of enzyme molecules in the system are attacked by free radicals. Therefore, rather than plotting the relationship between Na^+-K^+ ATPase activity and ATP concentration as a Lineweaver-Burke plot, a plot of enzyme activity versus substrate concentration was used. These curves were analysed by the split-plot ANOVA which allowed statistical analysis of (1) the relationship between Na^+-K^+ ATPase and ATP concentration, (2) the effect of hyperbaric oxygen on Na^+-K^+ ATPase activity and (3) via the interaction term, the effect of oxygen on the response of the Na^+-K^+ ATPase to increasing substrate concentration.

It should be noted that Lineweaver-Burke plots were generated for control activity at all temperatures used. These

plots indicated that the Na^+-K^+ ATPase activity at an ATP concentration of 5 mM is very close to V_{max}, that is, 5 mM ATP is a saturating substrate concentration.

Hyperbaric oxygens had no effect on Na^+-K^+ ATPase activity at 12.5C, the trout's preferred environmental temperature (Tables 2 and 3; Figures 5 and 6). The increase in enzyme activity with increasing ATP concentration is significant. The same conclusions can be drawn for exposure of trout retina homogenates to O_2 at 3800 mm Hg at 23C (Table 4; Figure 7). Exposure to O_2 at 11,600 mm Hg and 23C however did cause a slight but significant decrease in Na^+-K^+ ATPase activity (Table 5 and Figure 8) indicating that an increase in temperature may make the enzyme more susceptible to inhibition at higher oxygen pressures. Exposure of homogenate to O_2 at 3800 mm Hg at 37C did not cause decreases in enzyme activity at all ATP concentrations used (Table 5), however a split-plot ANOVA of the curves presented in Figure 9 indicates a general decrease in enzyme activity due to hyperbaric oxygen exposure. The relationship of the enzyme activity to ATP concentration is significant and was not affected by hyperbaric oxygen.

These data generally indicate that there is little effect on Na^+-K^+ ATPase activity when homogenates of trout retina are exposed to hyperbaric oxygen. This is in agreement with previous studies which show that teleost ocular tissues are highly resistant to oxygen toxicity (Baeyens et al., 1973, 1974; Hoffert et al., 1973; Ubels et al., 1977).

Exposure of homogenates to nitrogen under the same

Table 2. Na^+-K^+ ATPase activity of trout retina homogenates exposed to O_2 at 3800 mm Hg for 4 hr at 12.5C.

	[ATP] 1mM	2mM	5mM
Control	4.94 ± 0.37	5.54 ± 0.35	6.63 ± 0.53
HBO	4.73 ± 0.24	5.13 ± 0.38	5.67 ± 0.53

Means not underscored by the same line (within groups) are significantly different ($p \leq 0.05$) n = 6

No significant difference between HBO and control

Na^+-K^+ ATPase activity 75.6% of total ATPase activity

No significant effect of O_2 on Mg^{+2} ATPase

Enzyme activity expressed as μmol P_i/mg protein/hr

Table 3. Na^+-K^+ ATPase activity of trout retina homegenates exposed to O_2 at 11,600 mm Hg for 4 hr at 12.5C.

	[ATP] 1mM	2mM	5mM
Control	4.99 ± 0.19	5.73 ± 0.23	6.05 ± 0.46
HBO	4.45 ± 0.03	5.16 ± 0.40	5.95 ± 0.61

Means not underscored by the same line (within groups) are significantly different($p \leq 0.05$) n = 6

No significant difference between HBO and control

Na^+-k^+ ATPase activity 75.6% of total ATPase activity

No significant effect of O_2 on Mg^{+2} - ATPase

Enzyme activity expressed as μmol P_i/mg protein/hr

Figure 5. Effect of O_2 at 3800 mm Hg on the response of trout retina Na^+-K^+ ATPase to increasing substrate concentration at 12.5C. Data plotted from Table 2.

Control - solid line and closed circles

HBO - dashed line and open circles

The interaction term of the split-plot ANOVA is not significant indicating no effect of HBO on the shape of the response curve.

$p \leq 0.05$

$x \pm SE \quad n = 6$

Figure 6. Effect of O_2 at 11,600 mm Hg on the response of trout retina Na^+-K^+ ATPase to increasing substrate concentration at 12.5C. Data plotted from Table 3.

Control - solid line and closed circles

HBO - dashed lines and open circles

The interaction term of the split-plot ANOVA is not significant indicating no effect of HBO on the shape of the response curve.

$p \leq 0.05$

$x \pm SE \quad n = 6$

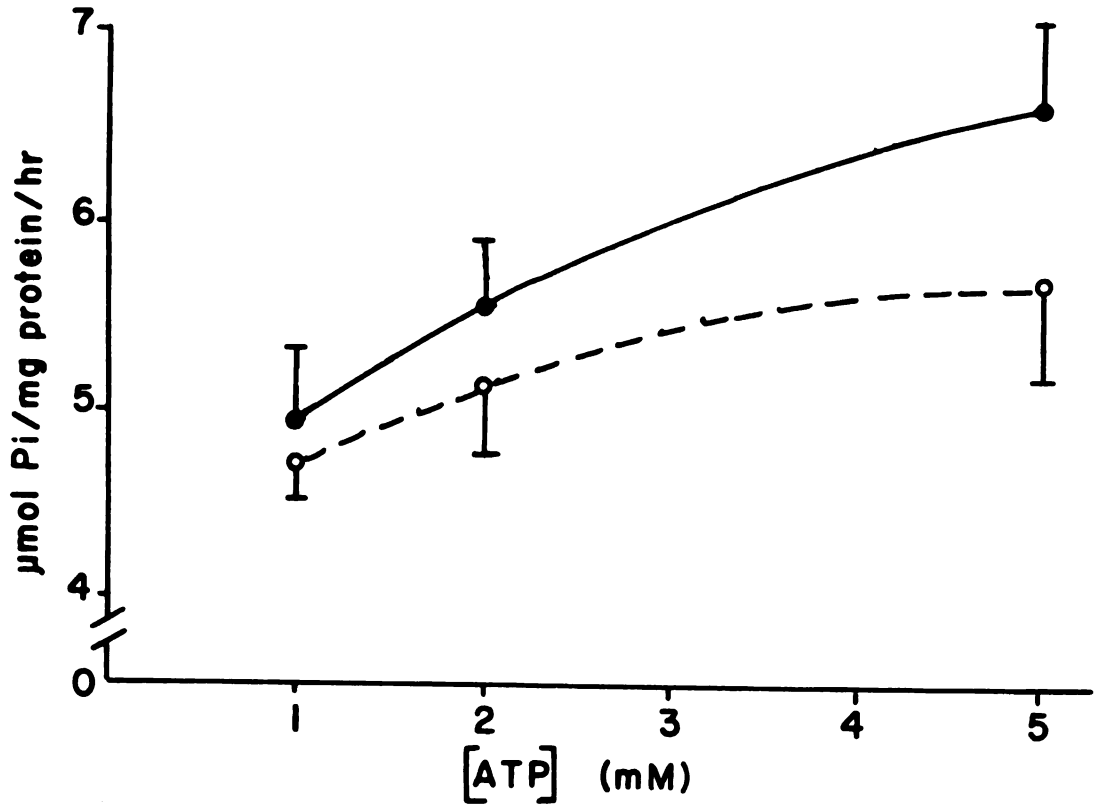


Figure 5

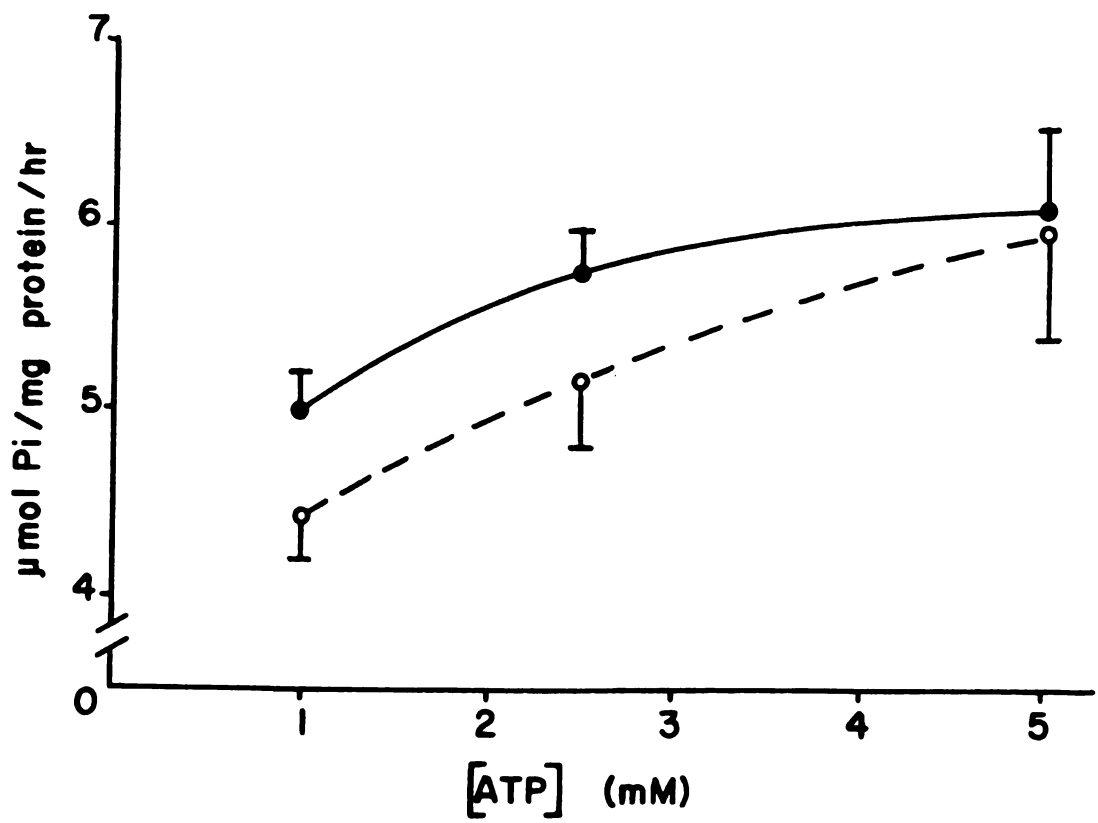


Figure 6

Table 4. Na^+-K^+ ATPase activity of trout retina homogenates exposed to O_2 at 3800 mm Hg for 4 hr at 23C.

	[ATP] 1mM	2mM	5mM
Control	8.23 ± 0.68	11.54 ± 0.83	13.53 ± 0.92
HBO	8.38 ± 0.64	11.24 ± 0.70	12.93 ± 0.56

Means not underscored by the same line (within groups) are significantly different ($p \leq 0.05$). n = 6

No significant differences between HBO and control

Na^+-K^+ ATPase activity 70.6% of total ATPase activity

No significant effect of O_2 on Mg^{+2} ATPase

Enzyme activity expressed as μmol P_i/mg protein/hr

Table 5. Na^+-K^+ ATPase activity of trout retina homogenates exposed to O_2 at 11,600 mm Hg for 4 hr at 23C.

	[ATP] 1mM	2mM	5mM
Control	6.82 ± 0.53	8.47 ± 0.62	12.89 ± 0.34
HBO	6.00 ± 0.59*	7.32 ± 0.81*	11.76 ± 0.50*

Means not underscored by the same line (within groups) are significantly different ($p \leq 0.05$)

*HBO different than control ($p \leq 0.05$)

n = 9, 10, 6 at 1mM, 2mM, 5mM respectively

Na^+-K^+ ATPase activity 66.0% of total ATPase activity

Mg^{+2} ATPase activity significantly decreased

Enzyme activity expressed as μmol P_i/mg protein/hr

Figure 7. Effect of O_2 at 3800 mm Hg on the response of trout retina Na^+-K^+ ATPase to increasing substrate concentration at 23C. Data plotted from Table 4.

Control - solid lines and closed circles

HBO - dashed lines and open circles

Enzyme activity is dependent on substrate concentration.

The interaction term of the split-plot ANOVA is not significant indicating no effect of HBO on the shape of the response curve.

$p \leq 0.05$

$x \pm SE \quad n = 6$

Figure 8. Effect of O_2 at 11,600 mm Hg on the response of trout retina Na^+-K^+ ATPase to increasing substrate concentration at 23C. Data plotted from Table 5.

Control - solid lines and closed circles

HBO - dashed lines and open circles

Enzyme activity is dependent on substrate concentration.

The interaction term of the split-plot ANOVA is not significant indicating no effect of HBO on the shape of the response curve.

$p \leq 0.05$

$x \pm SE$

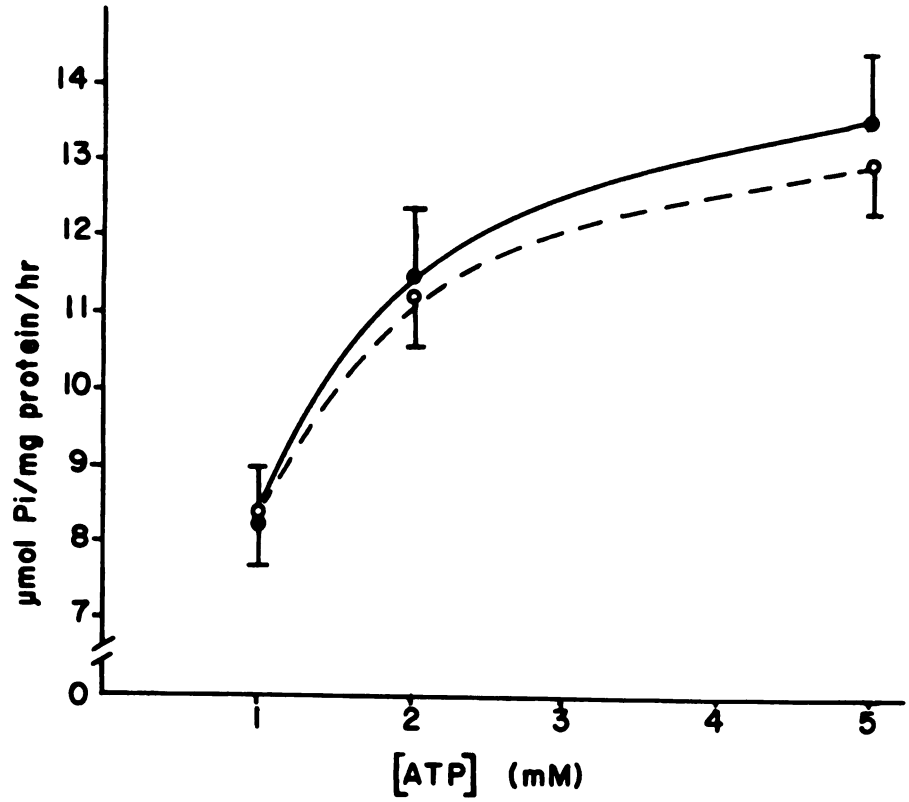


Figure 7

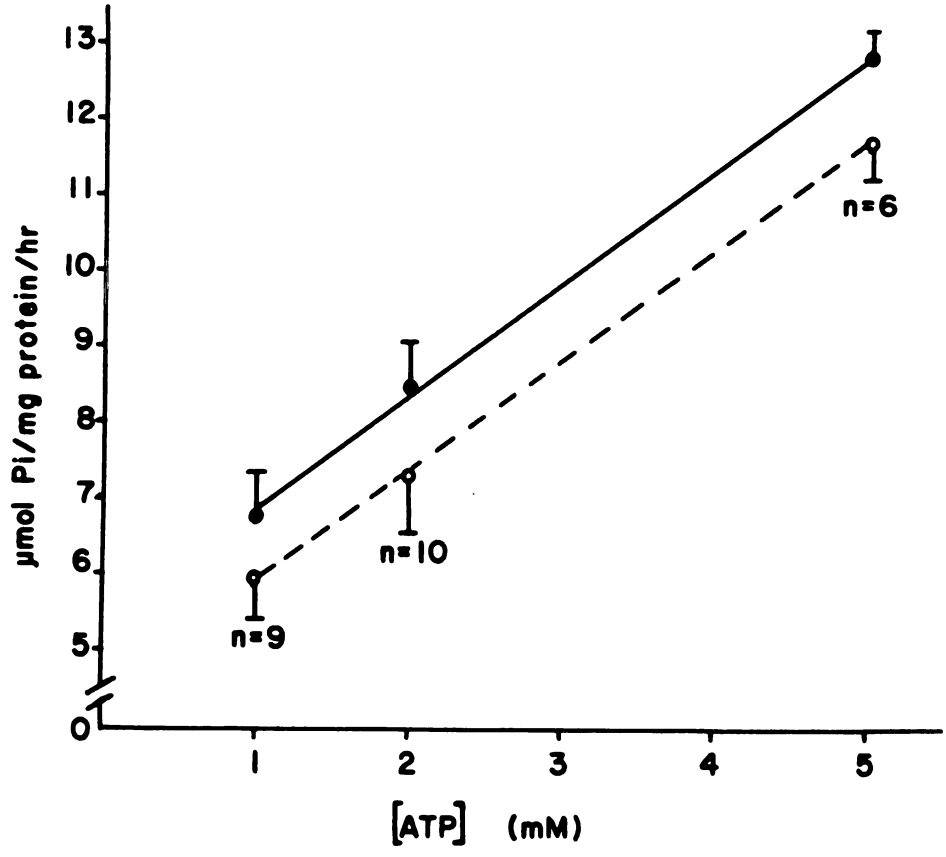


Figure 8

Table 6. Na^+-K^+ ATPase activity of trout retina homogenates exposed to O_2 at 3800 mm Hg for 4 hr at 37C.

	[ATP]		
	1mM	2mM	5mM
Control	15.66 ± 0.74	21.08 ± 1.01	24.42 ± 1.41
HBO	13.88 ± 1.01	16.55 ± 0.98*	22.71 ± 1.20

All means within groups are significantly different ($p \leq 0.05$)
n = 9

*HBO different from control ($p \leq 0.05$)

Na^+-K^+ ATPase activity 65.1% of total ATPase activity

No significant effect of O_2 on Mg^{+2} ATPase

Enzyme activity expressed as μmol P_i/mg/hr

Figure 9. Effect of O_2 at 3800 mm Hg on the response of trout retina Na^+-K^+ ATPase to increasing substrate concentration at 37C. Data plotted from Table 6.

Control - solid lines and closed circles

HBO - dashed lines and open circles

Enzyme activity is reduced by exposure to HBO.

The interaction term of the split plot ANOVA is not significant indicating no effect of HBO on the shape of the response curve.

$p \leq 0.05$

$x \pm SE \quad n = 9$

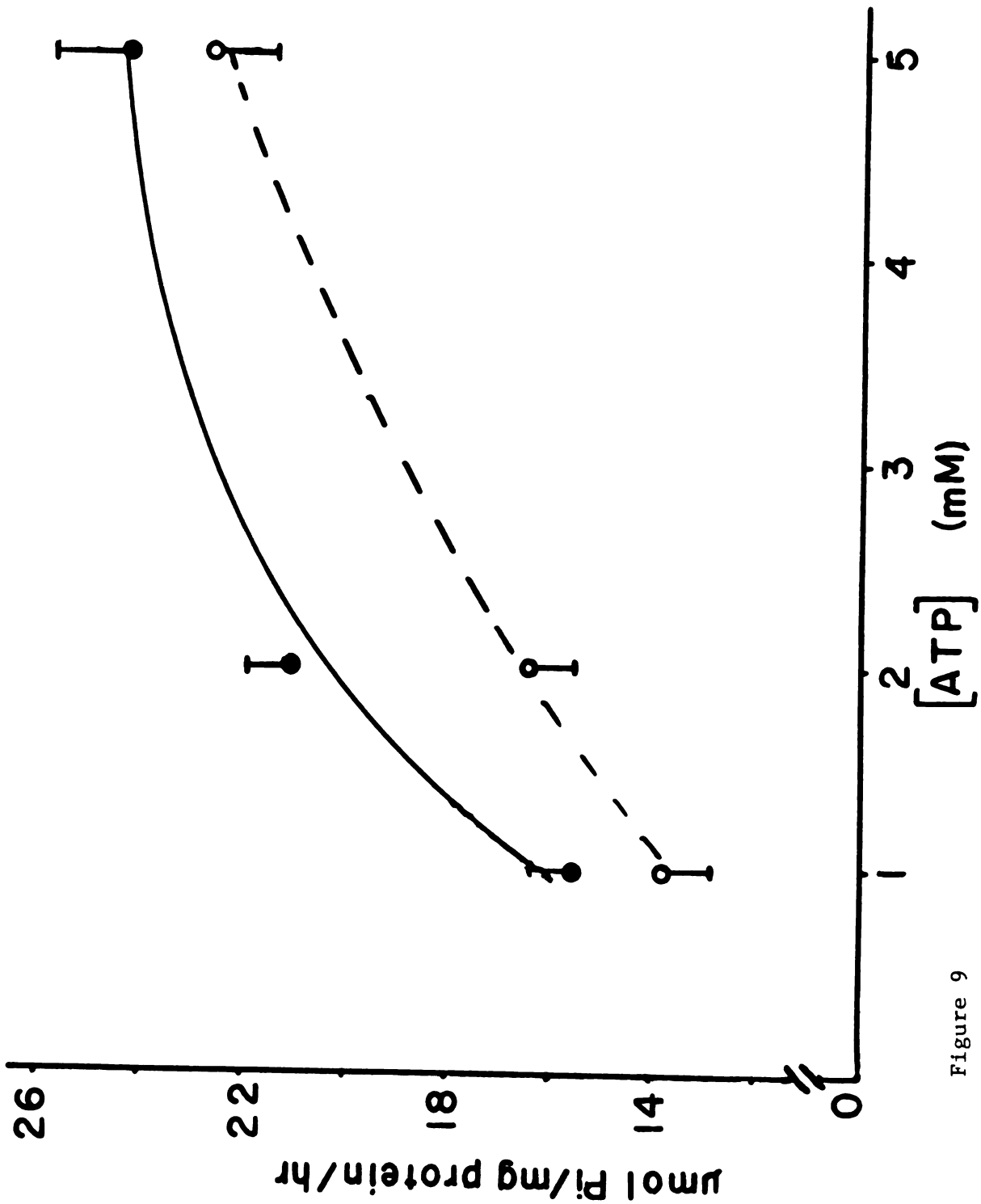


Figure 9

conditions used for the oxygen experiments showed that high pressure in itself causes no decrease in ATPase activity (Appendix IV). The question of direct effects of pressure on enzyme systems will be discussed in greater detail in a subsequent section.

Bovine Experiments

A short series of experiments was conducted using bovine retinal homogenates. The homogenates were exposed to oxygen (3800 mm Hg) and assayed for ATPase activity at 37C. Experiments using bovine retina were discontinued in favor of the use of rat retina as a representative mammalian tissue. The bovine data indicates a decrease in Na^+-K^+ ATPase activity following exposure to hyperbaric oxygen (Table 7). Control enzyme activity shows no correlation with substrate concentration. In agreement with many previous studies of the effect of oxygen on mammalian tissues, Na^+-K^+ ATPase from bovine retina is inhibited when retinal homogenates are exposed to hyperbaric oxygen.

Table 7. Na^+-K^+ ATPase activity of bovine retina homogenates exposed to O_2 at 3800 mm Hg for 4 hr at 37C.

	[ATP]		
	1mM	2mM	5mM
Control	12.07 ± 2.18	10.57 ± 1.56	13.70 ± 1.44
HBO	5.64 ± 1.53*	6.49 ± 0.61*	10.17 ± 0.70*

*Significant difference ($p \leq 0.05$. Wicoxon signed-rank test) n=6

Na^+-K^+ ATPase activity 51.5% of toal ATPase activity

No significant effect of O_2 on Mg-ATPase

Enzyme activity expressed as μmol P_i/mg protien/hr

Rat Experiments

Homogenates of rat retina were exposed to oxygen at 3800 mm Hg and 11,600 mm Hg for 4 hr at 37C. Controls were exposed to air at 740 mm Hg (PO_2 = 154 mm Hg). All experiments were paired and the split-plot analysis of variance and Student-Newman-Kuels test were used for data analyses. Levels of ATPase activity in this study agree well with values for rat retina reported by Winkler and Riley (1976).

Data presented in Tables 8 and 9 show that Na^+-K^+ ATPase activity is not affected by O_2 at 3800 mm Hg but decreases significantly when the oxygen tension is increased to 11,600 mm Hg. These data are plotted in Figures 10 and 11. The interaction term in the split-plot ANOVA is not significant indicating that the response of the enzyme to increasing substrate concentration is not affected by hyperbaric oxygen.

The percentage decrease in enzyme activity (30% at 5mM ATP) is greater for rat retina than for trout retina. This was expected since mammalian tissues are believed to be more susceptible to oxygen toxicity.

Experiments using nitrogen showed that high pressure has no effect on Na^+-K^+ ATPase activity of rat retina homogenates (Appendix IV).

Table 8. Na^+-K^+ ATPase activity of rat retina homogenates exposed to O_2 at 3800 mm Hg for 4 hr at 37C.

	[ATP]		
	1mM	2mM	5mM
Control	9.71 ± 1.12	9.70 ± 0.84	9.99 ± 1.04
HBO	7.67 ± 1.83	9.67 ± 1.65	10.78 ± 1.60

No significant differences within groups ($p \leq 0.05$) n = 6

No significant differences between HBO and control ($p \leq 0.05$)

Na^+-K^+ ATPase activity 52.5% of total ATPase activity

No significant effect of O_2 on Mg^{+2} ATPase

Enzyme activity expressed as µmol P_i/mg protein/hr

Table 9. Na^+-K^+ ATPase activity of rat retina homogenates exposed to O_2 at 11,600 mm Hg for 4 hr at 37C.

	[ATP]		
	1mM	2mM	5mM
Control	7.93 ± 0.47	8.91 ± 0.35	10.06 ± 0.61
HBO	6.34 ± 0.56*	6.59 ± 0.23*	7.04 ± 0.12*
	n=6	n=10	n=6

Means not underscored by the same line (within groups) are significantly different ($p \leq 0.05$)

*HBO different than control ($p \leq 0.05$)

Na^+-K^+ ATPase activity 52.5% of total ATPase activity

No significant effect of O_2 on Mg^{+2} ATPase

Enzyme activity expressed as µmol P_i/mg protein/hr

Figure 10. Effect of O_2 at 3800 mm Hg on the response of rat retina Na^+-K^+ ATPase to increasing substrate concentration at 37C. Data plotted from Table 8.

Control - solid lines and closed circles

HBO - dashed lines nd open circles

The interaction term of the split plot ANOVA is not significant indicating no effect of HBO on the shape of the response curve.

$p \leq 0.05$

$x \pm SE \quad n = 6$

Figure 11. Effect of O_2 at 11,600 mm Hg on the response of rat retina Na^+-K^+ ATPase to increasing substrate concentration. Data plotted from Table 9.

Control - solid lines and closed circles

HBO - dashed lines and open circles

Enzyme activity is dependent on substrate concentration.

Enzyme activity is significantly reduced by exposure to HBO.

The interaction term of the split-plot ANOVA is not significant indicating no effect of HBO on the shape of the response curve.

$p \leq 0.05$

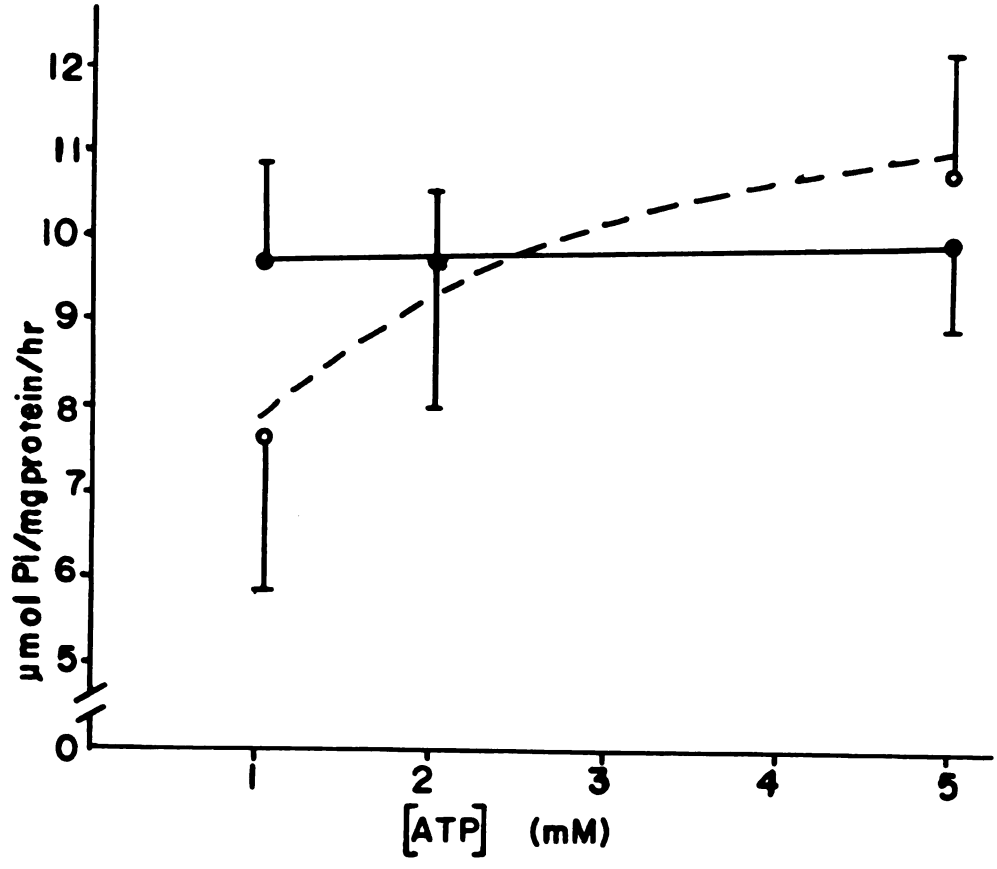


Figure 10

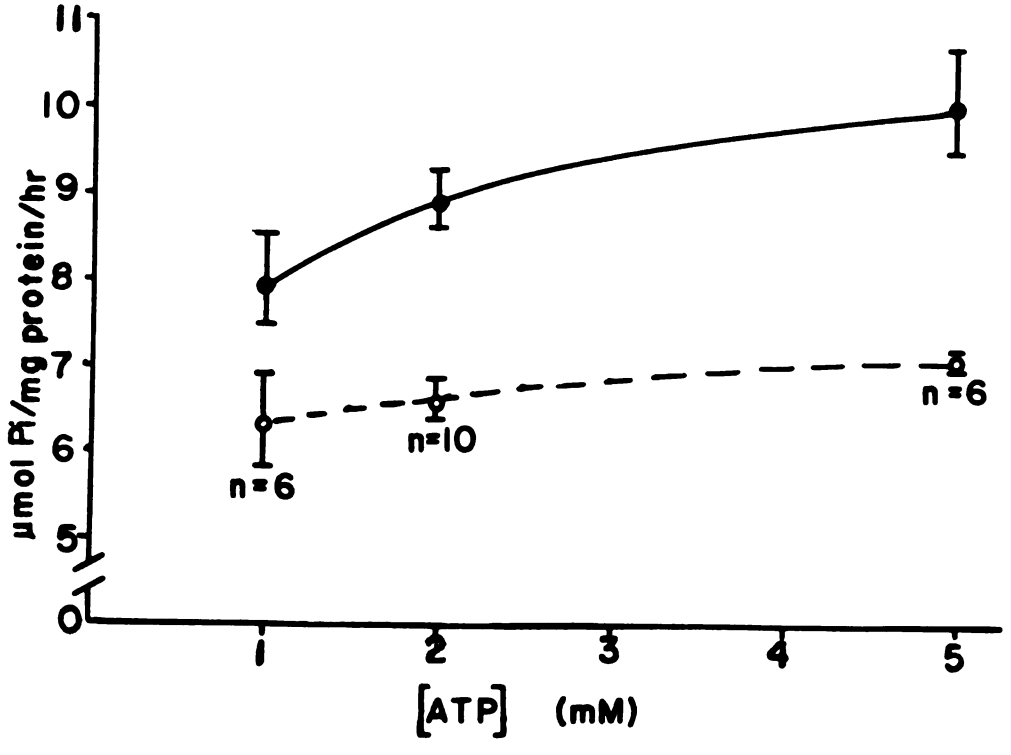


Figure 11

Canine Kidney Na^+-K^+ ATPase Experiments

An additional experiment was conducted to show that in the absence of superoxide radical producing enzyme systems oxygen has no effect on enzyme activity. Purified canine kidney Na^+-K^+ ATPase (prepared by the method of Nakao et al, 1965) was purchased from Sigma (St. Louis, MO) and reconstituted with 50 mM Tris buffer at pH 7.4. The homogenate was exposed to O_2 or N_2 at 3800 mm Hg at 37C for 4 hr after which ATPase activity was measured. No changes in Na^+-K^+ ATPase activity were observed as a result of either O_2 or N_2 exposure (Table 10). This supports the choice of the use of crude homogenates in this study since in the absence of superoxide producing systems hyperbaric oxygen has no effect on enzyme activity. This is to be expected because of the low reactivity of molecular oxygen (Fridovich, 1977b).

Other studies of the effect of hyperbaric oxygen on enzyme activity have also shown that when highly purified enzyme is exposed to oxygen no decrease in enzyme activity is seen, while enzyme activity is inhibited when tissue slices or crude homogenates are exposed to oxygen (Davies and Davies, 1965).

Xanthine-Xanthine Oxidase Experiments

Since it is known that oxygen toxicity is due to the effects of increased cellular levels of highly reactive forms of oxygen such as superoxide radical ($O_2^{\cdot-}$) and singlet oxygen ($^1O_2^*$), a study was conducted to determine whether Na^+-K^+ ATPase is subject to direct attack by chemically produced superoxide. Such experiments are commonly used to show that enzymes or physiological processes are susceptible to attack by active oxygen (McCord and Fridovich,

1969; Kagan et al., 1975; Kellogg and Fridovich, 1977).

The breakdown of xanthine to uric acid, which is catalysed by xanthine oxidase, produces superoxide radicals by the following reaction:

$$\text{Xanthine} + H_2O + O_2 \xrightarrow{\text{Xanthine Oxidase}} \text{urine acid} + O_2^{\cdot -} \quad (18)$$

(Fridovich, 1970). This system was chosen as a source of $O_2^{\cdot -}$ to test the susceptibility of Na^+-K^+ ATPase to attack by active oxygen.

Lyophylized canine kidney ATPase was reconstituted in 100 mM xanthine and buffered to pH 7.4 with Tris. The protein concentration of this homogenate was 0.5 mg/ml. A 1.0 ml aliquot of this solution was designated control and received 0.5 ml water. A second 1.0 ml aliquot of ATPase solution received 0.5 ml of xanthine oxidase (Sigma) solution which had been diluted 150 µl/50 ml water. This solution was allowed to incubate at room temperature and ATPase activity was measured at 37C after 10, 17, or 22 min of exposure to the xanthine-xanthine oxidase system. This activity was compared to the activity of the Na^+-K^+ ATPase of the control solution. Results were analysed by a randomized complete block ANOVA (Tables 11-14).

Similar experiments were performed using homogenates of trout and rat retinas. Trout experiments were conducted at 12.5C and 22C and the homogenates were exposed to the xanthine oxidase reaction for 20 minutes. Rat retina homogenates were exposed to the xanthine oxidase reaction for 20 minutes at 22C and Na^+-K^+ ATPase

activity was measured at 37C. Results were analysed by the paired t-test.

Canine kidney Na^+-K^+ ATPase activity was significantly decreased by exposure to the superoxide producing system. Enzyme activity decreased 13% during 10 min of exposure to the xanthine oxidase reaction and reached a maximum of 20% inhibition in 17 min (Table 11). The Na^+-K^+ ATPase system thus appears to be susceptible to attack by active oxygen. This decrease in activity is probably due either to peroxidation of the lipids associated with the ATPase enzyme, and/or direct attack of active oxygen on the enzyme.

Exposure of trout and rat retina homogenates to the xanthine oxidase system did not decrease Na^+-K^+ ATPase activity (Tables 12,13,14). It is possible that the levels of antioxidants present in these homogenates were sufficient to protect the enzyme from the superoxide produced by the xanthine oxidase reaction.

Table 10. Activity of purified Na^+-K^+ ATPase from canine kidney exposed to O_2 or N_2 at 3800 mm Hg for 4 hr at 37C.

	Gas	
	O_2	N_2
Control	11.32 ± 0.83	11.38 ± 0.82
Experimental	11.32 ± 1.16	11.70 ± 0.99

No significant differences ($p \leq 0.05$, Paired t-test) n = 5

Enzyme activity expressed as μmol P_i/mg protein/hr [ATP] = 5 mM

Table 11. Effect of $O_2^{\cdot -}$ produced by xanthine oxidase on activity of Na^+-K^+ ATPase purified from canine kidney.

	Exposure Time		
	10 min	17 min	22 min
Control	47.71 ± 3.43	45.29 ± 3.47	35.38 ± 3.65
Expt	41.67 ± 3.75*	36.07 ± 3.51*	28.82 ± 4.01*

*Significant difference ($p \leq 0.05$, ANOVA) n = 6

Enzyme activity expressed as μmol P_i/mg protein/hr [ATP] = 5 mM

Enzyme activity also decreased significantly over 22 min period

Table 12. Na^+-K^+ ATPase activity of trout retina homogenates exposed for 20 min to $O_2^{\cdot -}$ produced by xanthine oxidase at 12.5C.

		ATPase	
	Total	Mg^{+2}	Na^+-K^+
Control	7.17 ± 0.25	1.56 ± 0.18	5.60 ± 0.18
Expt	7.42 ± 0.31	1.73 ± 0.25	5.69 ± 0.22

No significant differences ($p \leq 0.05$, paired t-test) n = 6

Enzyme activity expressed as μmol P_i/mg protein/hr [ATP] = t mM

Table 13. Na^+-K^+ ATPase activity of trout retina homogenates exposed for 20 min to $O_2^{\cdot -}$ produced by xanthine oxidase at 22C.

	Total	ATPase Mg^{+2}	Na^+-K^+
Control	13.38 ± 2.14	3.04 ± 0.22	10.34 ± 0.29
Expt	15.63 ± 2.83*	13.19 ± 0.16*	11.72 ± 0.30*

*Significant difference ($p \leq 0.05$, paired t-test) n = 6

Enzyme activity expressed as μmol P_i/mg protein/hr [ATP] = 5 mM

Table 14. Na^+-K^+ ATPase activity of rat retina homogenates exposed for 20 min to $O_2^{\cdot -}$ produced by xanthine oxidase.

	Total	ATPase Mg^{+2}	Na^+-K^+
Control	14.37 ± 0.90	6.90 ± 0.85	7.47 ± 1.70
Expt	21.11 ± 0.90*	13.79 ± 0.69*	7.31 ± 0.73

*Significant difference ($p \leq 0.05$, paired t-test) n = 6

Enzyme activity expressed as μmol P_i/mg protein/hr [ATP] = 5mM

Summary of Homogenate Experiments

These experiments indicate that Na^+-K^+ ATPase from trout retina is resistant to oxygen toxicity since only a 10-15% reduction in enzyme activity occurred under the rather rigorous conditions of exposure to an oxygen pressure of 11,600 mm Hg at 22C, a temperature well above the trout's preferred environmental temperature. Negative results, however, may indicate that the experimental method being used is not adequate to produce the desired effect. In the present study this raised the question, "Do distilled water homogenates of retina metabolize at a normal rate or is the metabolism so low that significant amounts of active oxygen are not being produced, resulting in the observed lack of an effect?" The pH of these homogenates was in the range 7.4-7.6. The metabolic rate of trout retina homogenates was measured at 22C in a YSI Oxygen Monitor, Model 15 (Yellow Springs, Ohio) and oxygen consumption was 6.12+0.8 ul O_2/mg protein/hr (n=9). This is comparable to a value of 4.49 ul O_2/mg protein/hr at 15C reported by Baeyens et al. (1973) and showed that the lack of an oxygen effect on these retinas was not due to the low metabolic rate of the tissue.

The mammalian data indicate a decrease in Na^+-K^+ ATPase activity of retina homogenates following hyperbaric oxygen exposure. A 25-50% decrease in activity was observed when bovine retinas were exposed to hyperbaric oxygen at 3800 mm Hg and at an oxygen pressure of 11,600 mm Hg a 20-30% decrease in Na^+-K^+ ATPase activity of rat retina was observed. This decrease was expected because of previous work, however the method of oxygen exposure

which had been chosen still was not considered to be totally satisfactory. A more physiological set of conditions was desired and therefore, since the results of the homogenate studies were encouraging, further experiments were conducted during which intact retinas in tissue culture medium were exposed to hyperbaric oxygen.

Exposure of Intact Retinas to Hyperbaric Oxygen

Trout Experiments

Intact trout retinas were placed in TC 199 tissue culture medium (Difco, Detroit, MI) and exposed in the dark to oxygen at 3800 mm Hg and 11,600 mm Hg for 4 hr at 14C and 23C. After exposure the retinas were immediately prepared as described previously and the ATPase activity of the homogenate was assayed at 14C or 23C in the presence of 5 mM ATP. Under control conditions Na^+-K^+ ATPase activity was nearly 50% lower than that obtained with lyophilized tissues and comprised only 45% of the total ATPase activity, however, these results were accepted and experiments were continued.

Exposure of retinas to hyperbaric oyxgen at 14C (within the trout's preferred environmental temperature range) had no effect on Na^+-K^+ ATPase activity (Tables 15 and 16).

When retinas were exposed to hyperbaric oxygen at 23C, an increase in the susceptibility of the Na^+-K^+ ATPase system to attack by active oxygen was observed. Enzyme activity was significantly reduced by exposure to oxygen at both 3800 and 11,600 mm Hg (Tables 17 and 18). This effect was not seen when homogenates were exposed to O_2 at 3800 mm Hg and 23C. This is probably due to the fact that the intact retina, supplied with nutrients by the TC 199, has a higher metabolic rate at 23C which would lead to increased production of active oxygen in the presence of hyperbaric oxygen. At this abnormally high temperature the retina's antioxidant compounds may not be capable of handling the increased active oxygen load, leading to

Table 15. ATPase activity of trout retinas exposed to O_2 at 3800 mm Hg for 4 hr at 14C.

	ATPase		
	Total	Mg^{+2}	Na^{+}-K^{+}
Control	8.79 ± 0.30	4.78 ± 0.21	3.84 ± 0.17
HBO	8.75 ± 0.22	4.98 ± 0.20	3.76 ± 0.21

No significant differences ($p \leq 0.05$, paired t-test) n = 8

Enzyme activity expressed as µmol P_i/mg protein/hr [ATP] = 5 mM

Table 16. ATPase activity of trout retinas exposed to O_2 at 11,600 mm Hg for 4 hr at 14C.

	ATPase		
	Total	Mg^{+2}	Na^{+}-K^{+}
Control	8.86 ± 0.56	4.84 ± 0.39	4.02 ± 0.20
HBO	8.64 ± 0.32	4.92 ± 0.28	3.72 ± 0.11

No significant differences ($p \leq 0.05$, paired t-test) n = 8

Enzyme activity expressed as µmol P_i/mg protein/hr [ATP] = 5 mM

Table 17. ATPase activity of trout retinas exposed to O_2 at 3800 mm Hg for 4 hr at 23C.

	ATPase		
	Total	Mg^{+2}	Na^{+}-K^{+}
Control	13.73 ± 1.31	7.02 ± 0.84	6.71 ± 0.55
HBO	11.31 ± 1.36*	5.86 ± 0.92*	5.39 ± 0.52*

*Significant difference ($p \leq 0.05$, paired t-test) n = 8

Enzyme activity expressed as μmol P_i/mg protein/hr [ATP] = 5 mM

Table 18. ATPase activity of trout retinas exposed to O_2 at 11,600 mm Hg for 4 hr at 23C.

	ATPase		
	Total	Mg^{+2}	Na^{+}-K^{+}
Control	14.93 ± 0.59	7.81 ± 0.39	7.12 ± 0.35
HBO	14.46 ± 0.63	8.34 ± 0.44	6.11 ± 0.28

*Significant difference ($p \leq 0.05$, paired t-test) n = 12

Enzyme activity expressed as μmol P_i/mg/hr [ATP] = 5mM

increases in cellular levels of active oxygen and attack of these species on lipids and enzymes.

High pressure nitrogen had no significant effect on Na^+-K^+ ATPase activity at 23C (Appendix IV). Since no effect of oxygen was seen at 14C and since no effect of high pressure on homogenates was observed, it was considered unnecessary to expose intact retinas to high pressure N_2 at 14C.

Summary of Trout Retina Experiments

Although a decrease in Na^+-K^+ ATPase activity was observed at 23C this decrease was slight, only 14-20%, and this minor reduction in pump activity would be unlikely to have a significant effect on the function of the retinal cells. Since at the trout's preferred environmental temperature no effect of oxygen was observed, these results, in addition to evidence previously presented from metabolic (Baeyens et al., 1973; Hoffert et al., 1973), enzymatic (Baeyens et al., 1974) and electrophysiological (Ubels et al, 1977) studies indicate that the normally hyperoxic tissues of the trout retina are highly resistant to oxygen toxicity. The precise mechanism of this resistance to oxygen toxicity is as yet unknown. It is suggested that the trout retina contains elevated levels of antioxidants. Since all of the oxygen supply to the trout retina is by diffusion from the choroid an oxygen gradient exists between the choroid and the vitreous humor. Measurements of this gradient are now being made in Hoffert's laboratory. Eldred (1979) recently has studied superoxide dismutase in the trout retina to test the hypothesis that elevated levels of this enzyme are present in the retina and that a gradient for superoxide dismutase exists within the retina paralleling the expected oxygen gradient. Although he was able to confirm the presence of superoxide dismutase in the trout retina Eldred is unwilling to state that such a gradient exists. Further studies of superoxide dismutase and other antioxidants will be undertaken in Hoffert's laboratory and it is expected that comparative studies will show

that increased levels of these compounds are found in the teleost retina compared to retinas of other animals.

Frog Experiments

All frog experiments reported were conducted using intact retinas. Several experiments were attempted using homogenates, as reported for trout and rat, however the results of these experiments were unsatisfactory since the ATPase activity was very low and results were inconsistent. At the time when these experiments were conducted the difficulties were thought to be related to the fact that winter frogs were being used and further experiments were postponed until summer frogs could be obtained.

Before the experiments were resumed an abstract appeared which reported that at pH 7.5 the Na^+-K^+ ATPase activity of frog pigmented epithelium measured using Tris as a buffer is only 50% of the activity obtained when imidazole is used as a buffer (Ostwald and Steinberg, 1979).

Therefore, when experiments were resumed, the Tris in the incubation medium was replaced by 50 mM imidazole buffer, adjusted to pH 7.5. Retinas were exposed in the dark to O_2 at 22C at 3800 or 11,600 mm Hg for 4 hr. Controls were exposed to air at 740 mm Hg for 4 hr. One series of experiments was conducted at 37C using O_2 at 3800 mm Hg. Since the results of the trout and rat studies showed no effect of N_2 at high pressure on Na^+-K^+ ATPase, no experiments were conducted on frog retinas using high pressure N_2.

After exposure to oxygen the retinas were prepared as previously described and ATPase activity was measured at 22C or

37C in the presence of 5 mM ATP. Na^+-K^+ ATPase activity was about 60% of the total ATPase activity at 22C. This is comparable to the data of Bonting et al. (1964) who reported a value of 65% for frog retina. The Na^+-K^+ ATPase activity is lower than that of trout retina at 22C and rat retina at 37C. Winkler (personal comunication) also measured low Na^+-K^+ ATPase activity in frog retina.

At 22C no significant changes in ATPase activity were observed as a result of exposure to hyperbaric oxygen (Tables 19 and 20). At 37C Na^+-K^+ ATPase activity was only 33% of total ATPase activity and no effect of hyperbaric oxygen was observed (Table 21).

Summary of Frog Retina Experiments

These data indicate that a decrease in Na^+-K^+ ATPase activity may not have been involved in the decline of the ERG observed during hyperbaric oxygen exposure in a previous study (Ubels et al., 1977). In that study the ERG amplitude had begun to decline after 4 hr of exposure to O_2 at 3800 mm Hg while in the present study no effect on Na^+-K^+ ATPase activity was observed after 4 hr exposure to O_2 at 3800 and 11,600 mm Hg. It should be noted however that there was a difference between the experimental conditions of these two studies. In the present studies isolated retinas were placed in a nutrient tissue culture medium, while in the electrophysiological studies ERG's were recorded from eye cups. This may have affected the system's susceptibility to oxygen toxicity.

This study, in addition to data from previous studies

Table 19. ATPase activity of frog retinas exposed to O_2 at 3800 mm Hg for 4 hr at 22C.

	Total	ATPase Mg^{+2}	Na^+-K^+
Control	6.63 ± 0.42	2.89 ± 0.38	3.74 ± 0.28
HBO	6.35 ± 0.47	2.74 ± 0.24	3.61 ± 0.28

No significant differences ($p \leq 0.05$, paired t-test) n = 7

Enzyme activity expressed as μmol P_i/mg protein/hr [ATP] = 5 mM

Table 20. ATPase activity of frog retinas exposed to O_2 at 11,600 mm Hg for 4 hr at 22C.

	Total	ATPase Mg^{+2}	Na^+-K^+
Control	5.27 ± 0.43	2.03 ± 0.45	3.24 ± 0.24
HBO	4.74 ± 0.55	2.17 ± 0.40	2.90 ± 0.17

No significant differences ($p \leq 0.05$, paired t-test) n = 7

Enzyme activity expressed as μmol P_i/mg protein/hr [ATP] = 5 mM

Table 21. ATPase activity of frog retinas exposed to O_2 at 3800 mm Hg for 4 hr at 37C.

	ATPase		
	Total	Mg^{+2}	Na^{+}-K^{+}
Control	14.31 ± 0.43	9.40 ± 0.38	4.91 ± 0.46
HBO	13.18 ± 0.47	8.91 ± 0.40	4.27 ± 0.26

No significant differences ($p \leq 0.05$, paired t-test) n = 8

Enzyme activity expressed as μmol P_i/mg protein/hr [ATP] = 5 mM

indicates that the amphibian (frog) retina is intermediate to the teleost and mammalian retinas in its susceptibility to oxygen toxicity. All studies of teleost retina have shown resistance to oxygen toxicity (Baeyens et al., 1973, 1974; Hoffert et al., 1973; Ubels et al., 1977) while studies of the frog retina have shown that lactate dehydrogenase (Baeyens et al., 1974) activity and ERG (Ubels et al., 1977) amplitude are reduced by hyperbaric oxygen while oxygen consumption (Baeyens et al., 1973) and Na^{+}-K^{+} ATPase activity are not affected. Since in mammals (dogs and rats) all of these systems are inhibited by hyperbaric oxygen (Baeyens et al., 1973, 1974; Ubels et al., 1977) while the frog shows a variable response, it is clear that the frog retina is less susceptible to oxygen toxicity than the mammalian retina but is not adapted for resistance to toxicity to the degree seen in the teleost.

Rat Retina Experiments

Intact rat retinas were placed in TC 199 at pH 7.5 and exposed to oxygen in the dark at 3800 mm Hg or 11,600 mm Hg at 37C for 4 hr or 2 hr. Controls were exposed to air at 740 mm Hg. Under control conditions Na^{+}-K^{+} ATPase activity was again equal to or slightly higher than that reported by Winkler and Riley (1976). After incubation in TC 199 the Mg^{+2} ATPase activity was slightly elevated compared to activity in homogenates so that Na^{+}-K^{+} ATPase was only 45% of the total activity rather than 53%, as reported for the homogenate experiments.

Exposure of rat retinas to oxygen at 3800 mm Hg for 4 hr resulted in a 48% decrease in Na^{+}-K^{+} ATPase activity while

exposure to oxygen at 11,600 mm Hg for 4 hr resulted in a 66% decrease in Na^+-K^+ ATPase activity (Tables 22 and 23). Figure 12 illustrates the dose response effect of O_2 at partial pressures of 154 mm Hg (P_{O_2} of air at 740 mm Hg), 3800 mm Hg and 11,600 mm Hg.

Exposure to oxygen for 2 hr caused a slight stimulation of activity at 3800 mm Hg but this effect was not observed at 11,600 mm Hg (Tables 24 and 25).

High pressure, exerted by nitrogen at 11,600 mm Hg had no effect on ATPase activity (Appendix IV).

Placing retinas in tissue culture medium provided more favorable conditions for retinal metabolism and, it appears, also resulted in increased production of active oxygen species as shown by the increased toxic effect of oxygen observed under these conditions as compared to exposure of homogenates to hyperbaric oxygen. The results again show that mammalian tissues are highly susceptible to oxygen toxicity.

Table 22. ATPase activity of rat retinas exposed to O_2 at 3800 mm Hg for 4 hr at 37C.

	ATPase		
	Total	Mg^{+2}	Na^{+}-K^{+}
Control	17.90 ± 1.80	10.35 ± 1.40	7.56 ± 0.45
HBO	10.98 ± 2.42*	7.03 ± 1.69*	3.94 ± 0.76*

*Significant difference ($p \leq 0.05$, paired t-test) n = 8

Enzyme activity expressed as μmol P_i/mg protein/hr [ATP] = 5 mM

Table 23. ATPase activity of rat retinas exposed to O_2 at 11,600 mm Hg for 4 hr at 37C.

	ATPase		
	Total	Mg^{+2}	Na^{+}-K^{+}
Control	24.32 ± 1.30	17.40 ± 0.95	7.77 ± 0.59
HBO	18.78 ± 1.35*	15.56 ± 1.22	2.67 ± 0.51*

*Significant difference ($p \leq 0.05$, paired t-test) n = 8

Enzyme activity expressed as μmol P_i/mg protein/hr [ATP] = 5 mM

Table 24. ATPase activity of rat retina exposed to O_2 at 3800 mm Hg for 2 hr at 37C.

	Total	ATPase Mg^{+2}	Na^+-K^+
Control	20.97 ± 1.89	10.25 ± 1.20	10.71 ± 0.93
HBO	23.99 ± 0.97	10.46 ± 0.66	13.54 ± 0.70*

*Significant difference ($p \leq 0.05$, paired t-test) n = 7

Enzyme activity expressed as µmol P_i/mg protein/hr [ATP] = 5 mM

Table 25. ATPase activity of rat retinas exposed to O_2 at 11,600 mm Hg for 2 hr at 37C.

	Total	ATPase Mg^{+2}	Na^+-K^+
Control	23.14 ± 1.09	12.94 ± 1.18	10.20 ± 0.47
HBO	22.25 ± 1.32	12.22 ± 1.00	10.03 ± 0.50

No significant differences ($p \leq 0.05$, paired t-test) n = 6

Enzyme activity expressed as µmol P_i/mg protein/hr [ATP] = 5 mM

Figure 12. The effect of increasing oxygen tension on rat retina Na^+-K^+ ATPase activity. Retinas were exposed to air or pure O_2 for 4 hrs at 37C in tissue cluture medium at pH 7.5. This graph is derived from data in Tables 22 and 23.

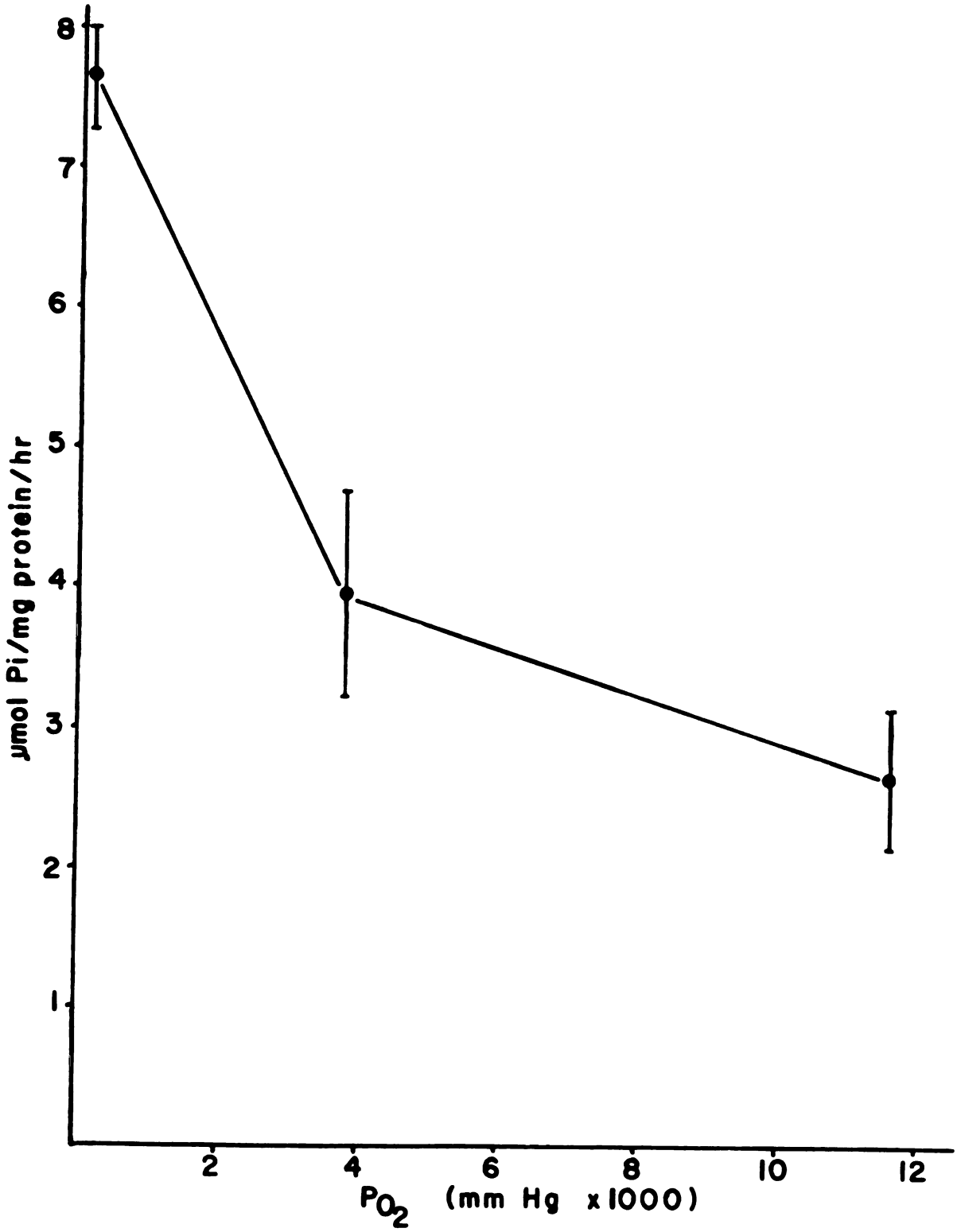


Figure 12

Mechanism of Inhibition of Na^+-K^+ ATPase by Active Oxygen

The inhibition of Na^+-K^+ ATPase may occur via direct attack of active oxygen on essential SH groups on the enzymes. Na^+-K^+ ATPase requires an SH group for normal function and can be inhibited by SH inhibitors such as N-ethyl-maleimide and para-chloromethylbenzoate (Skou, 1965). It is more likely however that the inhibition is due to lipid peroxidation. As discussed in the Literature Review, lipid peroxidation probably is the most important mechanism of oxygen toxicity. The phospholipids associated with Na^+-K^+ ATPase are essential for its function (Korenbrot, 1977) and peroxidation of the lipids could lead to direct attack by the lipid peroxidation products on the enzyme or change the relationship between the lipids and the protein. Chio and Tappel (1969) showed that SH group containing proteins are particularly susceptible to attack by lipid free radicals. This attack leads to polymerization via formation of disulfide bonds and causes inactivation of the enzyme. Since Na^+-K^+ ATPase is a SH dependent enzyme, this mechanism could be involved in the observed effect of hyperbaric oxygen on the Na^+-K^+ ATPase in rat retina.

A study by Sun (1972) supports the idea that the relationship between the enzyme and its associated lipids is altered. He exposed synaptosomal Na^+-K^+ ATPase isolated from squirrel monkey cerebral cortex to lipoxigenase and H_2O_2 in order to cause lipid peroxidation in synaptosomal membranes. This lipid peroxidation resulted in a 58% decrease in Na^+-K^+ ATPase activity. Treatment of the synaptosomal Na^+-K^+ ATPase with

peroxidized lenolenic acid or peroxidized oleic acid at concentrations much higher than that found in the treated membranes, however had no effect on ATPase activity. These results indicated that there may be no direct effect of lipid peroxide on enzyme structure but rather that the structural integrity of the cell membrane is changed by lipid peroxidation. It was also shown that after peroxidation of membrane lipids the affinity of the enzyme for K^+ was reduced. Therefore, Sun (1972) suggested that the relationship between membrane lipids and Na^+-K^+ ATPase is altered by lipid peroxidation thus interfering with the steric changes in the enzyme which occur during K^+ transport.

A Critique of Previous Studies of the Effect of Hyperbaric Oxygen on Na^+-K^+ ATPase.

It is necessary that the results presented here, which indicate an inhibition of Na^+-K^+ ATPase by hyperbaric oxygen, be compared to work done by Gottlieb and co-workers (Koehler and Gottlieb, 1972; Gottlieb et al., 1976; Hemrick and Gottlieb, 1977; Gottlieb et al., 1977) who studied effects of oxygen and other gases on Na^+-K^+ ATPase. Their experiments stemed from the same premise as the present study, that Na^+-K^+ ATPase should be particularly susceptible to inhibition by hyperbaric oxygen since it is a SH dependent lipoprotein. The effect of high pressure of oxygen, nitrogen and helium on Na^+-K^+ ATPase of rat intestinal mucosa, bovine cardiac muscle and bovine, mouse and rat brain are reported in their papers. The methods of tissue preparation and

the ATPase assay used by Gottlieb are similar to the methods used in the present study however the Na^+-K^+ ATPase reaction was carried out under hyperbaric conditions over a period of 2 hr. Gottlieb's data differ greatly from the data of this study. Pressures in the range of 2-70 atm were used and the data plotted as percent of control (1 atm air) activity versus pressure. Complex curves which show enzyme activation in some pressure ranges and inhibition in other ranges are computer fitted to the data. Gottlieb's data are summarized here, as described in the original papers. Koehler and Gottlieb (1972) state that O_2 inhibits intestinal Na^+-K^+ ATPase slightly at 1 atm and at 7-9 atm but activates it at pressures above 10 atm. The effects of N_2 and helium are similar; N_2 having no inhibitory effect below 8 atm and causing a 20% inhibition above this pressure. A slight stimulatory effect is ascribed to He in the lower pressure range (1-8 atm). Bovine cardiac muscle Na^+-K^+ ATPase (Gottlieb et al., 1976) behaved differently under O_2. It was inhibited by 30% at 2 atm and activated by 140% at 3 atm. Activity dropped to normal at 5 atm and gradually increased up to 21 atm. Helium over a range of 1-21 atm activated cardiac Na^+-K^+ ATPase with the highest activity (120% activation) occurring at 8 atm. Bovine brain Na^+-K^+ ATPase (Hemrick and Gottlieb, 1977) is inhibited (30%) by O_2 at 2 atm and stimulated (75%) at 3-4 atm. N_2 stimulates at 1-3 atm and inhibits at 4 atm. Helium stimulates at 1-2 atm and has no effect at 3-4 atm. Gottlieb et al. (1977) state that mouse brain Na^+-K^+ ATPase is inhibited by O_2 at 2 atm and stimulated at 3 atm. It is impossible to judge the validity

of the data in these papers since the specific activity of the enzyme preparation is never stated and no indication of the variability of the data is given (i.e., no standard errors on the curves). Also, the authors state that only 2-4 experiments were run at each pressure. Gottlieb and co-workers find it difficult to explain the effects of O_2, N_2 and Helium at high pressure on Na^+-K^+ ATPase which they observed, especially in view of the fact that oxygen has a marked inhibitory effect on other enzymes which have been studied (Haugaard, 1968). They discuss such things as effects of diatomic versus monoatomic gases, differences in molecular volume and differences in thermodynamic activity of the gases in an attempt to explain the data. They assume that helium is totally inert and do what they call a "pressure compensated analysis" to show that Na^+-K^+ ATPase is sensitive to pressure per se. This is highly questionable since studies of the effect of high hydrostatic pressure on biological systems and enzymes, including Na^+-K^+ ATPase show that changes occur only at extremely high pressures, e.g., pressures in excess of 300 atm (Zimmerman, 1970; Moon, 1975). Pressures used by Gottlieb fall well below this range.

The data presented in this dissertation differ greatly from the data cited above. Although no alternative explanation of Gottlieb's data will be presented here since it is impossible to judge the validity of the data, some reasons for the differences between these studies and the present study can be suggested.

There is a major difference in methods between the studies. In Gottlieb's work the ATPase reaction was run under hyperbaricq

conditions over a period of 2 hr, while in the present study homogenates and intact retinas were first exposed to O_2 or N_2 and ATPase activity was measured after decompression. Allowing the ATPase reaction to continue for 2 hr is a questionable procedure since the reaction rate may vary over this period of time due to substrate depletion and, in the case of Gottlieb's work, changes in enzyme activity as a result of hyperbaric oxygen exposure. Preliminary work in the present study showed that Na^+-K^+ ATPase activity of rat retina decreases over time if the reaction is stopped after 10, 20 or 30 min of incubation. Such changes may have had an effect of Gottlieb's data. The method of the present study, especially when intact retinas are used, should give a more accurate picture of the effects of oxygen especially since the retina is in a more physiological situation metabolically when exposed to O_2 as an intact retina in tissue culture medium. This method also makes it possible to run the ATPase reaction for a shorter period of time.

Gottlieb exposed his homogenates to experimental gases for only 2 hr while in the present study a 4 hr exposure was used for most experiments. It should be noted that in one experiment of the present study when rat retina was exposed to O_2 at 3800 mm Hg for only 2 hr an increase in Na^+-K^+ ATPase activity was observed (Table 24). However, this increase was not observed at 11,600 mm Hg (Table 25).

The unlikely possibility exists that Na^+-K^+ ATPase from retina may behave differently than Na^+-K^+ ATPase from brain, heart and intestinal mucosa when exposed to hyperbaric oxygen and

nitrogen. There is much evidence that Na^+-K^+ ATPase from many different tissues and animals has basically the same properties and, as shown in this study, canine kidney Na^+-K^+ ATPase is inhibited by $O_2^{\cdot-}$. The data presented in this dissertation conforms more closely to the expected results of exposure of a tissue to hyperbaric oxyen both in terms of the animals used in this study (Baeyens et al., 1973; Hoffert et al., 1973; Ubels et al., 1977) and in terms of the expected effect of oxygen on an enzyme (Haugaard, 1946; Haugaard, 1968; Baeyens et al., 1974). Therefore more confidence may be placed in the results of this study than in the confusing and unexpected results of Gottlieb's work on Na^+-K^+ ATPase.

The Effects of High Hydrostatic Pressure on Biological Systems

When oxygen toxicity is discussed with those not familiar with this field of study the question is often raised concerning the direct effects of pressure, *per se*, on biological systems, since hyperbaric oxygen exposure also involves the exertion of high pressure on the animal or tissue under study. Possible pressure effects were controlled for in the present study by the use of hyperbaric nitrogen.

The effect of high hydrostatic pressure is an important consideration in the study of the adaptation of abyssal animals to life in the ocean depths, since these animals are found at depths of over 1500 meters where pressures are well over 100 atm. The effect of hydrostatic pressure is also of interest in deep diving by humans but these dives seldom exceed about 330 meters

where the pressure is about 32 atm. As will be shown, this is well below the pressures which affect biological systems significantly.

As stated above, the question of the effects of high pressure is also raised concerning experiments of the type described in this dissertation. Studies conducted aboard the research vessel Alpha Helix have demonstrated the differences in response to high pressure between enzymes of abyssal and surface dwelling fish. Moon (1975) studied the effect of high hydrostatic pressure on gill Na^+-K^+ ATPase of the teleost (Antimora rostrata) which is found at depths where the pressure is 200-500 atm and the coho salmon (Onchorhynchus kisutch) a surface dwelling species. At a hydrostatic pressure of 5000 psi, Na^+-K^+ ATPase activity of Antimora gill was stimulated while ATPase activity of salmon gill was reduced. Above this pressure the ATPase activity of both species decreased but that of Antimora was always greater than that of the salmon.

Hochachka, Storey and Baldwin (1975) studied gill citrate synthase of Antimora and compared it to citrate synthase from porcine liver. This enzyme is under feedback inhibitory control from ATP and GTP. It was shown in this study that the response of the Antimora enzyme to increasing levels of ATP is identical at 1 atm and 680 atm, while at 680 atm the feedback effect of ATP on porcine citrate synthase is greatly reduced. Thus it has been shown that Antimora is well adapted to life at high hydrostatic pressure as compared to animals not normally exposed to these pressures.

Other studies have also demonstrated effects of high hydrostatic pressure on cell division, nerve and muscle excitability, cell permeability and oxygen consumption (Murakami, 1970). High pressure causes cells to divide abnormally, causes muscles and nerves to depolarize, decreases the voltage across frog skin. Oxygen consumption of muscle, kidney and brain is increased below 1000 atm and inhibited above this pressure. It is important to emphasize that 1400 psi (100 atm) is considered to be a low pressure in these types of studies. Pressures below this are not used because no effect is seen. The theory behind these effects of pressure are beyond the scope of this dissertation.

It is obvious that pressure, per se, is not an important factor in the experiments presented in this dissertation since the pressure range used ($\leq$ 15 atm) is well below the range where significant pressure effects are observed. The experiments using high pressure N_2 were conducted to confirm this.

Implications of a Decrease in Sodium Pump Activity for Retinal Function

When a statistically significant effect is observed in any physiology research project it is important to consider whether or not it is physiologically significant. This is especially important in the present study since Hodgkin and Keynes (1955) observed that inhibition of the sodium pump has very little effect on the membrane potential of squid giant axon and that the nerve will respond with action potentials for more than 2 hr after poisoning of the pump. It will be shown, however, that the cells of the retina are unique, compared to other excitable cells, in their dependence on the function of the sodium pump. Any discussion of the physiological significance of the decrease in trout retina Na^+-K^+ ATPase at 23C would be largely academic since it was shown that at the trout's preferred environmental temperature the retina is resistant to oxygen toxicity. The decrease in Na^+-K^+ ATPase activity in the retina of the rat requires more extensive discussion.

The Photoreceptor

In the dark, the vertebrate photoreceptor is depolarized and light causes hyperpolarization. Penn and Hagins (1969) showed that in the dark current flows radially along the photoreceptor from the inner segment to the outer segment (Figure 13). This current is carried by Na^+ ions (Sillman et al., 1969; Pinto and Ostroy, 1978) and flows into the outer segment, which has a high Na^+ conductance in the dark (Korenbrot and Cone, 1972). Light causes an increase in the resistance (decreased Na^+ conductance)

of the outer segment membrane which varies logarithmically with the amount of light absorbed. This increase in resistance causes a decrease in the dark current, resulting in the hyperpolarizing response to light typical of the vertebrate photoreceptor (Toyoda et al., 1969; Korenbrot and Cone, 1972). A sodium-potassium pump located in the inner segment membrane pumps out the sodium thus maintaining the low internal sodium concentration of the cell and contributing to the dark current (Korenbrot and Cone, 1972; Zuckerman, 1973). A diagram and circuit model of the photoreceptor are shown in Figures 13 and 14.

This situation gives the photoreceptor several characteristics which have a direct bearing on the interpretation of the results of this study. The high Na^+ conductance of the outer segment results in a low resting (dark) membrane potential. Most studies report dark membrane potentials in the range of -10 to -30 mv. This may be explained by the following general relationship (see, Eckert and Randall, 1978); where I = current, g = conductance, E_{Na} = sodium equilibrium potential, E_K = potassium equilibrium potential, E_m = membrane potential, E_{rev} = reversal potential for light response.

$$I_K = gK\ (E_m - E_K) \tag{19}$$

$$I_{Na} = g_{Na}\ (E_m - E_{Na}) \tag{20}$$

When,

$$E_m = E_{rev} \tag{21}$$

$$-I_K = I_{Na} \tag{22}$$

From equations 1 and 2

$$g_K(E_m - E_K) = g_{Na}(E_m - E_{Na}) \tag{23}$$

If equation 23 is solved for E_m the membrane potential for any g_K and g_{Na} can be predicted.

$$E_m = \frac{g_K}{g_{Na} + g_K} E_K + \frac{g_{Na}}{g_{Na} + g_K} E_{Na} \tag{24}$$

For most cells $g_{Na} << g_K$ and thus $E_m = E_K$. However for the photoreceptor $g_{Na} = g_K$ (Werblin, 1975; Pinto and Ostroy, 1978) and, thus E_m lies about halfway between E_K and E_{Na}, near E_{rev}. Werblin (1975) reported that in the Necturus rod at E_m = -10 to -15 mv, E_{rev} = +10 mv. This equation (24) also explains the hyperpolarization which occurs in the light, since as g_{Na} decreases, E_m approaches E_K.

As stated above, the g_{Na} of the outer segment is high. Zuckerman (1973) calculated that about 10^8 to 10^9 Na^+/sec cross the membrane in the frog rod and Werblin measured a dark current of 0.2 nA in Necturus.

In the face of such a high sodium influx the sodium pump becomes very important in maintaining a sodium gradient across the cell membrane, and according to Zuckerman's data (1973) this would

Figure 13. Model of the rod photoreceptor. The pump is located near the junction of the inner and outer segments. The Na^+ current is reduced in the light, leading to hyperpolarization of the membrane (After Korenbrot and Cone, 1972).

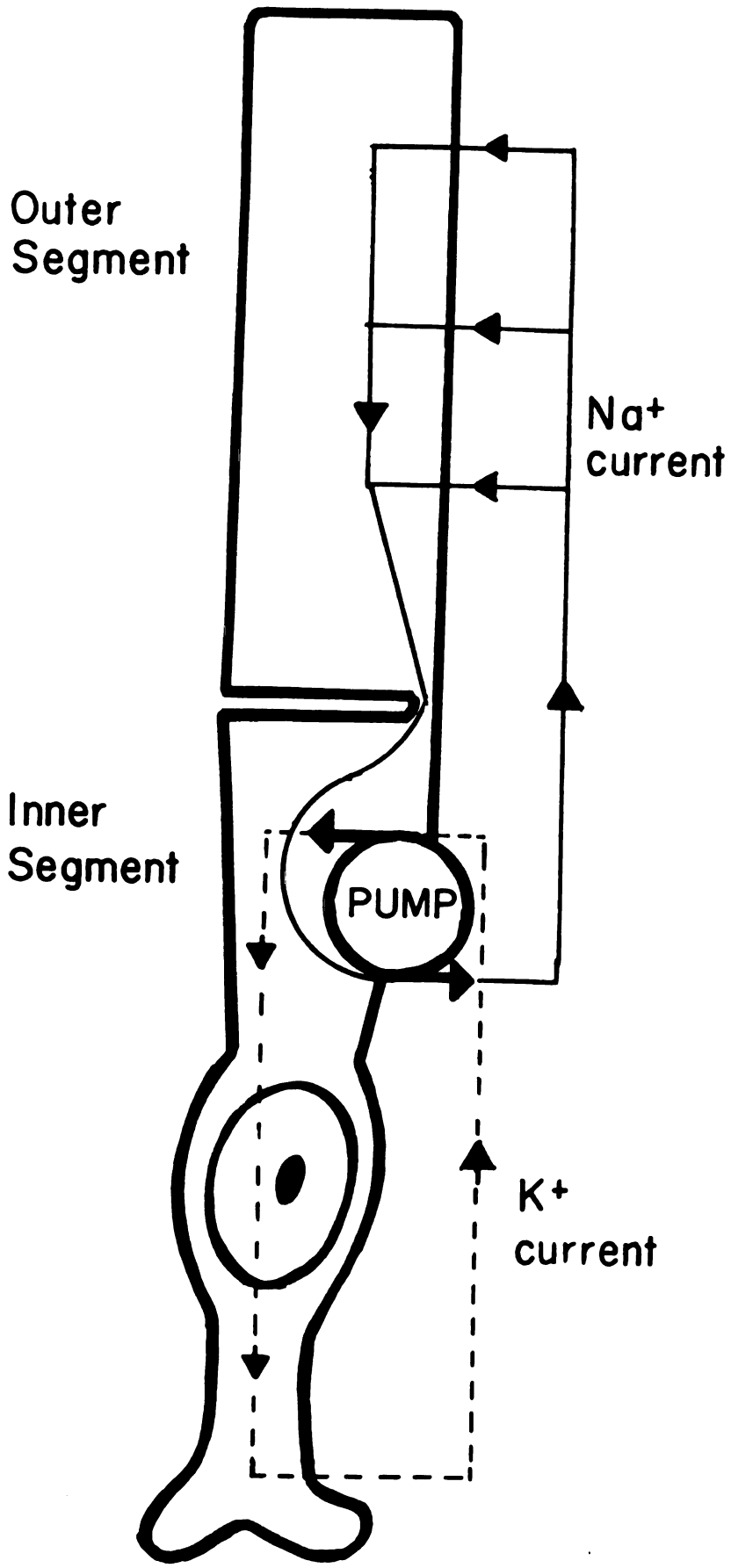


Figure 13

Figure 14. Circuit diagram for the photoreceptor. A variable Na^+ resistance which increases in response to light is located in the outer segment membrane. The Na^+-K^+ ATPase is coupled and the Na:K coupling ratio is Probably 3:2.

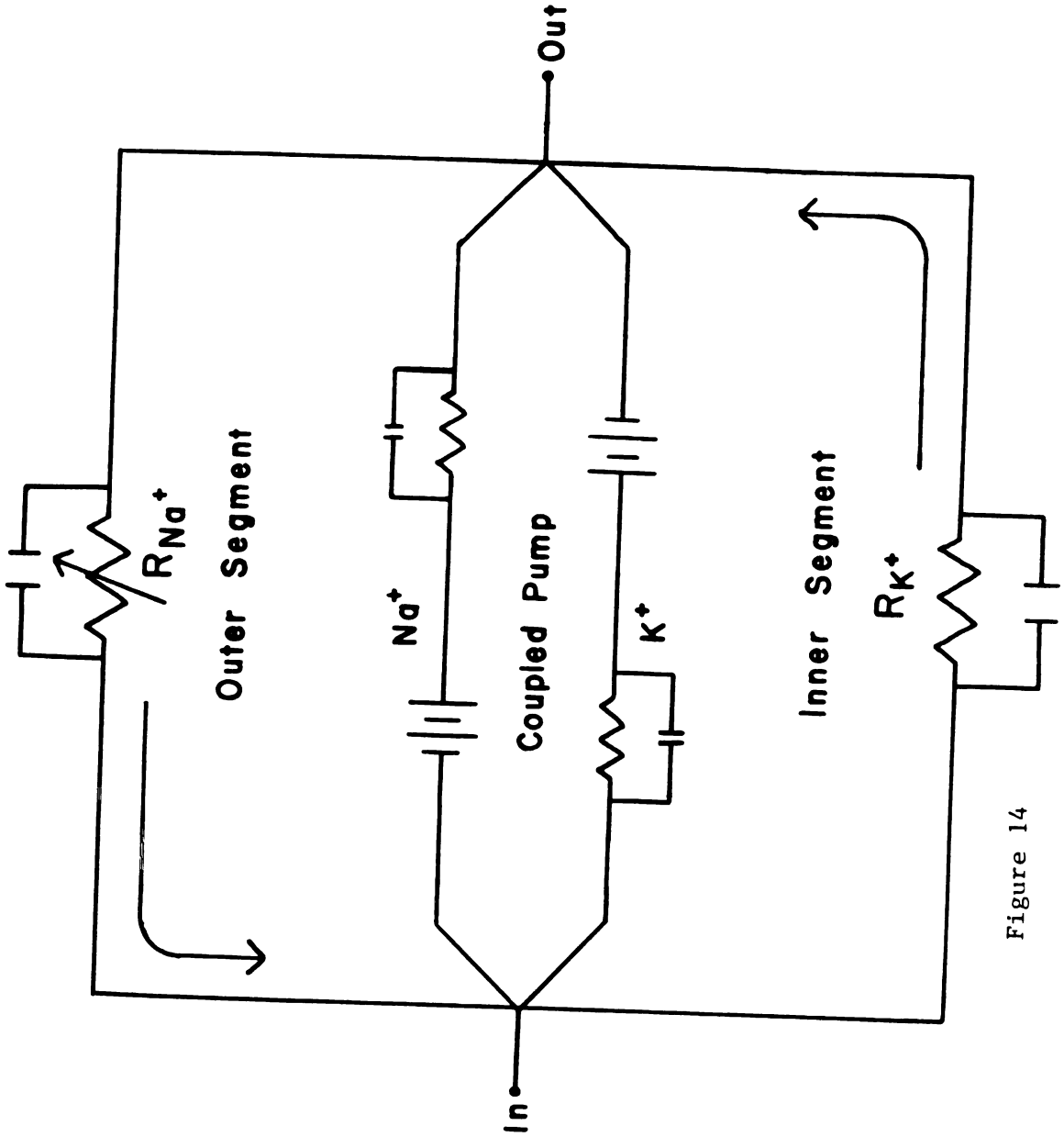


Figure 14

mean a pumping rate of 10^8 - 10^9 Na^+/sec out of the cell. Zuckerman and Weiter (1979) have recently reported that inhibition of the pump decreases retinal oxygen consumption by 64-83% and that exposure to saturating light reduces oxygen consumption by 56%. This decrease in oxygen consumption in the light is not due to pump inhibition, but is the result of decreased pump activity in the face of decreased Na^+ influx in the light. They measured the sodium dependent oxygen consumption in light and darkness and concluded that over 50% of the retinal O_2 consumption in the dark is used for sodium transport by the photoreceptors.

The pump in the inner segment exchanges Na^+ for K^+. To fit the model in Figure 13 it need not be electrogenic. Zuckerman (1973), however, pointed out that at its high rate of activity in the photoreceptor even a slight increase of Na^+:K^+ exchange ratio above 1.0 would result in transfer of a considerable amount of charge across the membrane. Zuckerman showed that it is in fact electrogenic since a major portion of the dark current is abolished and the inward current at the outer segment is dramatically reduced within 60 sec by application of ouabain. The passive (osmotic) component of the dark current is abolished within 25 min with a time constant of 5.5 min. This explains the previous data of Frank and Goldsmith (1967) who showed that the ERG is abolished by ouabain with a similar time course.

Since the pump is electrogenic it is also possible to calculate the pump's contribution to the resting membrane potential of the cell. As shown in the Literature Review, the electrogenic pump contributes very little to the resting membrane potential in a

cell with a low g_{Na}.

An electrogenic pump may make a significant contribution to the resting potential (E_m) in a cell with a high g_{Na}.

Using the equation of Thomas (1972)

$$E_m = \frac{RT}{F} \ln \frac{rK_o + bNa_o}{rK_i + bNa_i} \qquad (25)$$

where r = Na:K pump ratio and b = ratio of Na:K permeability. E_m is calculated using the following ionic concentrations: K_o = 2.5 mM, K_i = 140 mM, Na_o = 120 mM, Na_i = 10 mM. For r = 1.5 and b = 1, E_m = -14.5 mv. This is within the range reported for Gekko rods (Toyoda et al, 1969) and Necturus rods (Werblin, 1975). If r = 1, which describes the situation for a neutral or inhibited pump, E_m decreases to -5.1 mv. This means that 65% of the resting E_m in the dark is due to pump activity. Reducing b to 0.5 changes E_m to -22.4 mv when r = 1.5. If r is reduced to 1.0, E_m decreases to -13.1 (42% pump contribution) illustrating the effect of decreased g_{Na} on the membrane potential and its electrogenic component. The decrease in membrane potential upon pump inhibition will have an immediate effect on the receptor potential since it has been shown that depolarization of the photoreceptor results in decreased receptor potential amplitude at a given stimulus intensity (Toyoda et al., 1969; Werblin, 1975).

Based on the above information, assuming that the decrease in Na^+-K^+ ATPase activity observed in this study is equally distributed among all retinal cells, a 66% decrease in pump activity should result in a 66% decrease in the electrogenic

component of the membrane potential, assuming that resistance remains constant. For example, if the membrane potential of the cell in the dark is -15 mv and 10 mv of this potenital is contributed by the pump, then a 66% decrease in pump activity would result in a drop in the membrane potential to about -8.4 mv. The receptor potential is logarithmically related to light intensity, brighter lights causing larger potentials. Since depolarization of the photoreceptor results in decreased receptor potential amplitude, a drop in membrane potential from -15 mv to -8.4 mv would cause decreased retinal sensitivity to light because the response to a light of a given intensity would be less at E_m = -8.4 mv than at E_m = -15 mv. The above discussion is highly theoretical and no experimental data concerning the effect of pump inhibition by hyperbaric oxygen on photoreceptor membrane potential are available at this time. Such experiments are feasible using intracellular recording techniques, however it is expected that the data would be highly variable since the membrane potentials of photoreceptors and other retinal cells are quite variable, being determined not only by the characteristics of the cell itself, such as ionic permeability and pump activity, but also by inputs from horizontal cells and other photoreceptors.

Data are available on the effects of hyperbaric oxygen on the membrane potential of skeletal muscle cells (Hoffert and Ubels, unpublished data; see Appendix III). In this study skeletal muscle samples were placed in TC 199 and exposed to air or hyperbaric oxygen at 3800 mm Hg as were the retinas in the present study. Exposure to hyperbaric oxygen caused significant decreases in

membrane potential in rat diaphragm and Xenopus gastrocnemius. A small, but not significant, decrease was observed in frog (Rana pipiens) gastrocnemius. There was no change in effective membrane resistance. Exposure to 10^{-3}M ouabain for one hour also caused a significant decrease in membrane potential in rat diaphragm and frog (Rana) gastrocnemius. This work indicates that membrane potential can be altered by exposure of cells to hyperbaric oxygen or by pump inhibition, although the two cannot necessarily be correlated on the basis of the data. This preliminary work indicates that a study of the effect of hyperbaric oxygen on membrane potentials in the retina should be pursued. As stated above, membrane potentials of retinal cells can be quite variable. If this should prove to be a problem, a study of the effect of hyperbaric oxygen on the dark current and photocurrent, as measured by Zuckerman (1973), should yield valuable information on the effect of oxygen on the retina.

The generation of the receptor potential is dependent upon the maintenance of a high external Na^+ concentration with respect to the internal Na^+ concentration and the maintenance of a high internal K^+ concentration (Sillman et al, 1969). The extracellular fluid volume is rather low in the retina, especially in the photoreceptor layer where the outer segments and apical processes of the pigmented epithelium are tightly packed. Extracellular K^+ concentration therefore can vary significantly and these changes in K^+ concentration can affect the membrane potentials of the Müller cells (Miller, 1973) and the pigmented epithelial cells (Oakley and Green, 1976). Activity of the pump can cause a decrease in

extracellular K^+ in the retina (Oakley, Flaming and Brown, 1979). Pump activity maintains a zero net flux of Na^+ and K^+ across the membrane and any reduction in pumping rate will cause a net flux of these ions into and out of the cell, leading to an increase in the intracellular Na^+ concentration and depletion of intracellular K^+. The time course of this change in concentration may be described by the following equations (derived with the aid of W.F. Jackson):

$$\frac{dNa_i}{dt} = \frac{J^{Na}_{Net}}{V} \tag{26}$$

$$\frac{dK_i}{dt} = \frac{J^{K}_{Net}}{V} \tag{27}$$

where Na_i = intracellular sodium concentration, K_i = intracellular potassium concentration, J^{Na}_{Net} = net sodium flux, J^{K}_{Net} = net potassium flux, V = cell volume, t = time.

Since both ions are transported by the pump and since the binding of one ion to the ATPase affects the binding of the other, these equations must be solved simultaneously. The change in concentration is not a simple exponential, as when a substance is infused at constant rate into a known volume, since the net ionic flux changes over time as a function of changes in the concentration and electrical gradients. This introduces a non-linearity into the equations 26 and 27 so that J^{Na}_{Net} and J^{K}_{Net} must be calculated as a function of ionic concentration, membrane potential and pump rate. J^{Na}_{Net} and J^{K}_{Net} may be calculated by the following equations:

$$J_{Net}^{Na} = K_{diff}\,(Na_o - Na_i) + K_{elec}\,\frac{RT}{F}\,\ln\frac{rK_o + Na_o}{rK_i + Na_i} - \frac{J_{pump}\,Na_i}{K_M^{Na} + Na_i} \tag{27}$$

$$J_{Net}^{K} = K_{diff}\,(K_i - K_o) + K_{elec}\,\frac{RT}{F}\,\ln\frac{rK_o + Na_o}{rK_i + Na_i} - r\,\frac{J_{pump}\,Na_i}{K_M^{Na} + Na_i} \tag{28}$$

where K_o = extracellular potassium concentration; Na_o = extracellular sodium concentration; K_{diff} = diffusion transfer capacity; K_{elec} = electrical mobility; K_M^{Na} = Michaelis constant for the pump; J_{pump} = pump rate, or ionic flux generated by the pump; and r = Na:K ratio.

If J_{Net}^{Na} and J_{Net}^{K} can be calculated for any set of conditions then, as shown in equations 29 and 30 Na_i and K_i can be calculated at any time, $(t+\Delta t)$,

$$Na_i(t + \Delta t) = Na_i(t) + t\frac{J_{Net}^{Na}}{V} \tag{29}$$

$$K_i(t + \Delta t) = K_i(t) + t\frac{J_{Net}^{K}}{V} \tag{30}$$

where t = initial time and Δt = some time increment.

The first two terms of equations 27 and 28 determine the influx of Na^+ and efflux of K^+ due to concentration and electrical gradients. There is a pump rate, J_{pump}, at which the third term of the equation will be equal in magnitude to the sum of the first two terms and the net ionic flux will be zero. If the pump is then inhibited so the J_{pump} decreases, a net ionic flux will ensue and the internal ionic concentrations will change. This will continue until a new steady state level for the new pump rate is reached. It is clear that any decrease in pump rate will lead to a net ionic flux and cause depolarization of the photoreceptor. It is expected that the decrease in Na^+-K^+ ATPase activity of the magnitude seen in this study would have a significant effect on the intracellular levels of Na^+ and K^+, decreasing the gradients for these ions across the cell membrane and reducing the photoreceptor's ability to respond to light.

The situation is more complex than that described above and the equations presented probably underestimate the effects of Na^+-K^+ ATPase inhibition by oxygen on intracellular ionic concentrations. This is true for three reasons. First, the simplest way of using these equations is to assume a step decrease in pump activity (J_{pump}). In actuality, oxygen toxicity develops over a period of time with the magnitude of the inhibition continuously increasing with time.

Second, it is assumed in the third term of equations 27 and 28 that the inhibited pump is capable of responding to increasing Na_i. If the enzyme is capable of responding to increasing Na_i the magnitude of the third term of the equation will increase as

Na_i increases, reducing the net ionic flux. If the mechanism of inhibition by active oxygen is such that the enzyme does not respond to increasing Na_i, the net ionic flux will be higher than that predicted by the equations for any level of pump inhibition.

Third, the equations assume an infinite sink for K^+ ions. As stated previously K_o can vary significantly in the receptor layer of the retina. This means that, in the face of pump inhibition, K^+ may equilibrate across the membrane more quickly than predicted by the equation. Over a long period of time (several hours) an infinite sink for the K^+ which leaks from the cell is probably a valid assumption, especially in the highly vascularized mammalian retina.

The Neural Retina

The preceding discussion has dealt only with the photoreceptor. The effect of a decrease in Na^+-K^+ ATPase activity on the cells of the neural retina, such as horizontal and bipolar cells must also be considered. The b-wave of the ERG is abolished by ouabain (Frank and Goldsmith, 1967). This response to ouabain occurs before the abolishment of the a-wave indicating that there is a direct effect of pump inhibition on the cells of the neural retina which is not mediated by inhibition of Na^+-K^+ ATPase in the photoreceptors.

The interconnections among the cells of the neural retina are very complex and the ionic mechanisms which govern the function of the horizontal and bipolar cells have only recently been explained (Waloga and Pak, 1978; Saito et al., 1979). Since there are several types of bipolar cells with different ionic

mechanisms, the horizontal cell which is less complex has been chosen as an example in a discussion of the effect of Na^{+}-K^{+} ATPase inhibition on the neural retina. Like photoreceptors, horizontal cells have low resting membrane potentials in the dark, ranging from -15 to -40 mv, and light causes these cells to hyperpolarize (Waloga and Pak, 1978). Dowling and Ripps (1973) showed that application of Mg^{+2} to the retina also causes the horizontal cell to hyperpolarize. The Mg^{+2} inhibits release of neurotransmitter from the photoreceptors, which continually release a depolarizing neurotransmitter in the dark. The release of this neurotransmitter is also decreased when the photoreceptor hyperpolarizes in the light and the horizontal cell responds to this decrease in neurotransmitter release by hyperpolarization. Waloga and Pak (1978) showed that the membrane potential of the horizontal cell is dependent on external Na^{+}. The depolarizing neurotransmitter released by the photoreceptors in the dark increases the Na^{+} conductance of the horizontal cell membrane leading to a low resting potential. With a decrease in the amount of transmitter released in the light, sodium conductance decreases and the cell hyperpolarizes.

As in the photoreceptor, a high sodium conductance would require a high rate of pump activity in order to maintain a low internal sodium concentration. It is suggested that inhibition of the pump in the horizontal cells in the presence of high sodium conductance would result in a rapid equilibration of sodium across the cell membrane and a loss of the cell's responsiveness to input from other cells.

Concluding Statement Concerning the Toxicity of Oxygen to the Mammalian Retina

The results of this study provide additional evidence that, in comparison to lower vertebrates, the mammalian retina is highly susceptible to oxygen toxicity. It has been shown by several studies that oxygen at high partial pressure affects that cells of the retina directly apart from the well known effects of oxygen on the retinal vasculature.

From electroretinographic studies (Bridges; 1966; Ubels et al., 1977) it is known that hyperbaric oxygen reduces the ability of the retina to respond to light. This toxic effect of oxygen involves the inhibition of enzymes required for oxidative carbohydrate metabolism in the mammalian retina (Baeyens et al., 1973) and, as shown in the present study, the inhibition of Na^+-K^+ ATPase, an enzyme directly involved in maintaining the sensitivity of the retina to light.

Attenuation of the ERG is accompanied by an increase in levels of lipid peroxidation products in the retina (Yagi and Ohishi, 1977). Since lipids are probably the primary target for attack by active oxygen, it is likely that the inhibition of membrane bound enzymes, such as those cited above, is mediated by lipid peroxidation. This also suggests that changes in membrane permeability and interference with rhodopsin chemistry may also be involved in retinal oxygen toxicity.

It is concluded that the inhibition of Na^+-K^+ ATPase is involved in retinal oxygen toxicity. Inhibition of this enzyme should have a marked effect on the ability of the retina to

respond to light in view of the extreme sensitivity of the retinal cells to changes in their ionic environment and in view of the high sodium conductance of cells such as photoreceptors and horizontal cells in the dark.

The accumulation of evidence for a direct toxic effect of high oxygen tensions on the function of retinal cells is of clincal importance. The oxygen pressures used in the present study are far higher than those which would be present in the clinical situation. Data collected using high oxygen pressures is, however, clinically relevant since, as stated earlier, at lower pressures (1-2 atm) symptoms of oxygen toxicity simply develop over a longer period of time than at higher pressures. It is expected that at a P_{O_2} of 760 mm Hg enzymatic derangements would be observed in less than 2 days since Noell (1962) has shown that O_2 at 760 mm Hg caused degeneration of rabbit photoreceptors in 48 hr. The P_{O_2} and exposure time which result in retrolental fibroplasia are highly variable. Severe cases have been observed after 34 days exposure to increased P_{O_2} (814 hr, O_2 < 300 mm Hg; 20 hr, O_2 > 300 mm Hg) while milder cases have been observed in less than 3 days (Shahinian and Malachowski, 1978).

Thus, it is clear that increased oxygen tension can cause gross evidence of ocular oxygen toxicity within a few days. It is expected that enzymatic processes would be affected within a shorter period of time. This indicates that extreme caution should be exercised in the clinical administration of oxygen, particularly to the premature infant whose eyes are known to be especially susceptible to oxygen toxicity.

SUMMARY

1. Experiments conducted by exposing homogenates of retina to hyperbaric oxygen indicated that bovine and rat retina Na^+-K^+ ATPase is inhibited by exposure to hyperbaric oxygen. Trout retina Na^+-K^+ ATPase is also inhibited slightly (approximately 10%) when homogenates are exposed to hyperbaric oxygen at 22C.

2. It was confirmed that Na^+-K^+ ATPase is susceptible to attack by superoxide radicals ($O_2^{\cdot -}$) as shown by experiments in which purified canine kidney Na^+-K^+ ATPase was inhibited by exposure to a xanthine-xanthine oxidase system which generates $O_2^{\cdot -}$. Exposure of purified canine kidney Na^+-K^+ ATPase to hyperbaric oxygen had no effect on enzyme activity indicating that O_2 in itself has no effect on the enzyme and that the cellular processes capable of producing oxygen free radicals must be present in order for oxygen to have a toxic effect.

3. Exposure of retinal tissue to N_2 at high pressure has no effect on Na^+-K^+ ATPase activity indicating that in the pressure range used in this study (3800-11,600 mm Hg), pressure <u>per se</u> has no direct effect on enzyme activity.

4. Exposure of intact trout retinas to hyperbaric oxygen at 14C has no significant effect on Na^+-K^+ ATPase activity. This resistance to oxygen toxicity is in agreement with previous studies of the effect of hyperbaric oxygen on the trout retina. The teleost retina is adapted to high oxygen tensions generated by the choroidal counter current multiplier and is capable of withstanding oxygen tensions well above those normally encountered. This adaptation to to oxygern exposure is probably mediated by the presence of high levels of antioxidant compounds in the retina.

5. Exposure of trout retinas to hyperbaric oxygen at 23C results in a 15-20% decrease in Na^+-K^+ ATPase activity. An increase in temperature may increase the production of oxygen free radicals, and enzymes and lipids may be more labile to attack by active oxygen at this temperature since 23C is well above the trout's preferred environmental temperature range.

6. Exposure of intact frog retinas to hyperbaric oxygen under the conditions used in this study has no effect on Na^+-K^+ ATPase activity. These data indicate that a decrease in sodium pump activity is probable not involved in the attenuation of the frog ERG by hyperbaric oxygen.

7. Exposure of intact rat retinas to hyperbaric oxygen resulted in a 50-66% decrease in Na^+-K^+ ATPase activity. Based on current understanding of the function of the vertebrate retina and its dependence on the sodium pump for normal activity this decrease in enzyme activity should be adequate to cause major decreases in the retina's responsiveness to light.

8. The results of this project provide additional data for an ongoing comparative study of the toxicity of oxygen to the vertebrate retina. The data presented in this report concerning Na^+-K^+ ATPase in addition to data from previous studies of oxygen consumption, LDH activity and the ERG indicate (1) that the teleost retina is highly resistant to oxygen toxicity, (2) that the amphibian retina is intermediate to the mammal and the teleost in its susceptibility to oxygen toxicity, and (3) that the mammalian retina is highly susceptible to attack by active oxygen.

9. It is concluded that inhibition of Na^+-K^+ ATPase is a contributing factor in the toxic effect of high oxygen tensions on the mammalian retina.

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APPENDICES

APPENDIX I

Lowry Method for Protien Determination

Principle

Tyrosine and tryptophan in proteins react with Folin's phenol reagent to give a blue color which is read photometrically

Reagents

A. Lowry A

1.	Sodium carbonate (anhydrous)	60.0 g
2.	Sodium hydroxide (pellets)	12.0 g
3.	Sodium or potassium tartrate	0.6 g
4.	Distilled H_2O to make	3000.0 ml

B. Lowry B

1.	Copper sulfate solution ($CuSo_4.5H_2O$)	0.5 g%

C. Lowry C (prepared fresh daily)

1.	Lowry A	50 parts
2.	Lowry B	1 part

D. Phenol reagent according to Folin Ciocalteu

1.	Phenol Reagent-concentrate	1 part
2.	Distilled H_2O	1 part

E. Protein Standard 8.0 g% (Dade Reagents Inc., Miami, Fl Lot No. PRS0406)

1. Dilute with 300 ml distilled H_2O to give

   800 g/ml

Concentrations of protein standards used for determination of standard curve: 0, 20, 40, 60, 80 and 160 g/ml.

Procedure

1. 1 ml of protein solution (standard or unknown) added to 5 ml of Lowry C.
2. Incubate 20 min at room temperature.
3. 0.5 ml phenol reagent jetted in for rapid mixing.
4. Incubate 1/2 hr at room temperature (20-22C), mix occasionally.
5. Read at 660 m .

APPENDIX II

Composition of Modified Medium 199 (Earle's Base) in mg/l

NaCl	6800.0	Na_2 tocopherol PO_4	0.01
KCl	400.0	p-Aminobenzoic acid	0.05
$Mg_2SO_4.7H_2O$	200.0	L-Cystine	20.00
$NaHOP_4.2H_2O$	—	L-Tyrosine	40.00
$NaH_2PO_4.H_2O$	125.00	L-Cysteine HCL	0.1
KH_2PO_4	--	Adenine Sulfate	10.0
Glucose	1000.0	Guanine HCl	0.30
Phenol red	20.0	Xanthine	0.30
$CaCl_2$ (anhyd.)	200.0	Hypoxanthine	0.30
$NaHCO_3$	1250.0	Uracil	0.30
L-Arginine HCl	70.0	Cholesterol	0.20
L-Histidine HCl	20.0	Tween 80	20.0
L-Lysine	70.0	ATP	10.0
DL-Tryptophan	20.0	Adenylic acid	0.20
DL-Phenylalanine	50.0	D-2-Desoxyribose	0.50
DL-Methionine	30.0	D-Ribose	0.50
DL-Serine	50.0	Choline Cl	0.50
DL-Threonine	60.0		
DL-Leucine	120.0		
DL-Isoleucine	40.0		
DL-Valine	50.0		
DL-Glutamic acid	150.0		
DL-Aspartic acid	60.0		
DL-Alpha-Alanine	50.0		
L-Proline	40.0		
L-Hydroxyproline	10.0		
Glycine	50.0		
L-Glutamine	100.0		
Sodium acetate	50.0		
Thymine	0.30		
Thiamin HCl	0.01		
Pyridoxine Hcl	0.025		
Riboflavin	0.010		
Pyridoxal HCl	0.025		
Niacin	0.250		
Niacinamide	0.025		
Ca Pantothenate	0.010		
i-Inositol	0.050		
Ascorbic acid	0.050		
Folic acid	0.010		
Ferric nitrate.$9H_2O$	0.010		
Biotin	0.010		
Menadione	0.010		
Glutathione	0.050		
Vitamin A	0.10		
Calciferol	0.10		

APPENDIX III

EFFECT OF HYPERBARIC OXYGEN AND OUABAIN ON RESTING MEMBRANE POTENTIAL IN SKELETAL MUSCLE

(J.R. Hoffert and J.L. Ubels)

This study was related to the study of the effect of hyperbaric oxygen on Na^+-K^+ ATPase in retina. It was intended to investigate possible effects of hyperbaric oxygen and ouabain on the membrane potential of skeletal muscle and served as a preliminary study to future studies in which intracellular recording techniques will be applied to retinal cells.

Rat diaphragm and frog (Xenopus laevis and Rana pipiens) gastrocnemius muscles were isolated and placed in nutrient Ringer's solution. They were exposed to O_2 at 740 mm Hg (control) or 3800 mm Hg for the time periods indicated in Table 26. After removal from the exposure chambers membrane potentials and resistance were measured. Muscles were also exposed to 10^{-3}M ousabain in Ringer solution for 1 hr after which membrane potentials were recorded.

Electrodes were pulled from 1.0 mm OD, 0.58 mm ID microfilament, capillary glass (AM Systems, Toledo, OH) and filled with 3 M KCl. Membrane potentials and resistance were recorded using a WPI-701 microprobe preamplifier (WP Instruments, New Haven, CN) and a Tektronix storage oscilloscopy (Beaverton, OR). Date collected are shown in Tables 26 and 27 and are discussed in the section of this dissertation entitled "Implications of a Decrease in Na^+-K^+ ATPase Activity for Retinal Function".

Table 26. Effect of hyperbaric oxygen on membrane potential and resistance.

Animal	Time (hr)	Potential (mv) Control	Potential (mv) HBO	Resistance (Mohms) Control	Resistance (Mohms) HBO	Temp. (C)
Rat	1	-63.3±2.6(24)	-38.2±2.8(24)*	19.5±2.4(24)	16.6±1.7(24)	38
Xenopus	4	-58.6±1.3(82)	-44.0±1.7(82)*			22
Rana	4	-65.0±1.6(70)	-60.7±2.](67)	14.9±1.6(70)	13.1±1.7(67)	22

$\bar{X}$±SE(N)

*Significant difference ($p \leq 0.05$)

Table 27. Effect of ouabain on membrane potential.

Animal	Potential (mv) Control	Potential (mv) Ouabain
Rat	-57.2±1.4(33)	-31.2±1.6(33)*
Rana	-74.0±2.7(32)	-45.2±2.0(29)*

$\bar{X}$±SE(N)

*Significant difference ($p \leq 0.05$)

APPENDIX IV

Effect of High Pressure

Table 28. The effect of Nitrogen at high pressure on ATPase activity of retina homogenates.

Animal	Temp.	Gas and Pressure	Total	ATPase Mg	Na-K
Trout	14C	Control	10.65 ± 0.60	2.74 ± 0.66	7.84 ± 0.37
		N-740	10.92 ± 0.84	2.71 ± 0.59	8.20 ± 0.46
		N-3800	11.29 ± 0.64	2.80 ± 0.55	8.47 ± 0.20
	14C	Control	6.89 ± 0.34	1.09 ± 0.22	5.79 ± 0.21
		N-740	6.76 ± 0.30	0.98 ± 0.15	5.79 ± 0.28
		N-11600	6.86 ± 0.32	1.01 ± 0.16	5.86 ± 0.27
	22C	Control	15.59 ± 0.26	4.04 ± 0.51	11.55 ± 0.65
		N-740	16.09 ± 0.55	3.91 ± 0.63	12.25 ± 0.85
		N-3800	16.61 ± 0.37	4.27 ± 0.62	12.41 ± 0.74
	22C	Control	11.36 ± 0.70	2.11 ± 0.29	9.25 ± 0.29
		N-740	10.80 ± 0.94	1.89 ± 0.29	8.91 ± 0.76
		N-11600	10.58 ± 1.14	1.97 ± 0.29	8.61 ± 0.91
		Control	35.62 ± 0.99	9.28 ± 0.55	26.34 ± 0.72
	37C	N-740	36.49 ± 3.18	9.44 ± 1.16	27.05 ± 2.12
		N-3800	36.68 ± 0.95	9.61 ± 0.66	27.06 ± 0.72
Rat	37C	Control	17.73 ± 1.11	8.30 ± 0.55	9.44 ± 0.57
		N-740	19.82 ± 0.91	9.30 ± 0.80	10.78 ± 0.22
		N-3800	19.99 ± 1.15	9.16 ± 0.73	10.83 ± 0.68
	37C	Control	13.03 ± 1.03	5.97 ± 0.78	7.05 ± 0.34
		N-740	13.89 ± 1.56	6.18 ± 1.02	7.72 ± 0.78
		N-11600	13.72 ± 0.78	5.84 ± 0.88	7.87 ± 0.62

*Significantly different from control, within group ($p \leq 0.05$)

Pressures in mm Hg

Control = Air at 740 mm Hg

Enzyme activity expressed as μmol P_i/mg Protein/hr

Table 29. The effect of nitrogen at high pressure on ATPase activity of intact retinas.

Animal	Temp	Gas and Pressure	ATPase Total	ATPase Mg	ATPase Na-K
Trout	23C	Control	15.80 ± 0.38	9.52 ± 0.27	6.28 ± 0.35
		N-3800	16.53 ± 0.30	9.82 ± 0.30	6.71 ± 0.27
		Control	14.49 ± 0.40	7.99 ± 0.24	6.50 ± 0.37
		N-11600	15.24 ± 0.39	8.45 ± 0.29*	6.78 ± 0.27
Rat	37C	Control	24.79 ± 0.96	14.01 ± 1.26	10.78 ± 0.32
		N-11600	25.41 ± 3.53	13.94 ± 2.46	11.47 ± 1.15

*Significant difference within group ($p \leq 0.05$)

Trout n = 8; Rat n = 5

Pressures in mm Hg

Enzyme activity expressed as μmol P_i/mg protein/hr