# COMPARATIVE ASPECTS OF OXYGEN TOXICITY

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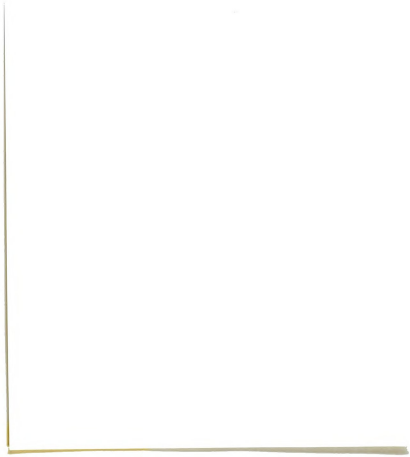

ABSTRACT

COMPARATIVE ASPECTS OF OXYGEN TOXICITY

By

Dennis A. Baeyens

Evidence from *in vitro* as well as *in vivo* experiments has strongly indicated that increased tensions of oxygen produce alterations of cellular metabolism. The metabolic changes eventually lead to disturbances of cellular function sufficiently great to produce the symptoms of oxygen toxicity seen in the intact organism. At present the mechanism of oxygen poisoning and the nature of cellular resistance to the toxic action of oxygen are not well understood.

Numerous enzymes containing sulfhydryl groups have been shown to be reversibly inhibited on exposure to oxygen. The mechanism of this inhibition is believed to be an oxygen induced formation of disulfide bridges resulting in enzyme inactivation.

Most of the studies dealing with oxygen toxicity have utilized homeotherms and there are only a few studies dealing with the toxic effects of oxygen on poikilotherms. Cells vary greatly in their resistance to oxygen toxicity, but the reason for this phenomenon is not well understood.

Dennis A. Baeyens

Oxygen toxicity was investigated in the retina, brain and liver of a teleost, amphibian and mammal. The teleost retina was chosen as the focal point of the study because in vivo it is enveloped by oxygen tensions in excess of 400 mm Hg; such oxygen tensions have been demonstrated to be inhibitory to certain other tissues. The influence of oxygen on tissue metabolism was investigated by measuring lactate dehydrogenase (LDH) activity to determine the effects of oxygen on glycolysis. Effects of oxygen were also investigated by quantitating oxygen consumption of tissue slices after exposure to hyperbaric oxygen and comparing these results with tissues exposed to room air. Thus by measuring oxygen consumption it was possible to quantitate the effects of oxygen on tricarboxylic acid cycle (TCA) activity. By measuring the activity of the glycolytic pathway and the TCA cycle, it was possible to determine at what points in carbohydrate metabolism oxygen exerted its effect. Attempts were also made to correlate oxygen induced changes in LDH activity with changes in the migratory pattern of LDH isozymes. Lineweaver-Burk plots were drawn to determine the effect of oxygen on the Michaelis-Menten kinetics of LDH.

The teleost retina showed an increased oxygen consumption after both 4 and 24 hrs exposure to hyperbaric oxygen ($P_{O_2}$ = 1470 mm Hg) compared to room air exposures ($P_{O_2}$ = 154 mm Hg). This indicates that the availability of oxygen is

rate limiting and that oxygen does not inhibit any essential respiratory enzymes in this tissue. The oxygen consumption of the teleost retina is not maximal at the normally encountered *in vivo* oxygen tension of 400 mm Hg but continued to increase with increasing oxygen tensions.

The amphibian retina showed no change in oxygen consumption upon exposure to high oxygen tensions. Thus the rate of oxidative retinal metabolism in the amphibian is not limited by availability of oxygen or the inhibition of any associated enzymes.

Mammalian retinas exposed to elevated oxygen tensions demonstrated marked decreases in oxidative metabolism mediated through inhibition of one or more essential enzymes of carbohydrate metabolism.

In the three species both hepatic and brain tissue demonstrated marked deleterious metabolic effects upon exposure to elevated oxygen tensions. It was also noted that the higher the metabolic rate of a tissue the more susceptible it is to oxygen toxicity.

Most cases of decreased LDH activity were not associated with changes in tertiary structure, intermolecular disulfide bridge formation or isoelectric points. These observations are based on the inability of oxygen to change the electrophoretic mobilities of LDH isozymes.

Michaelis-Menten kinetics showed that the LDH from mammalian brain and liver tissue responded to oxygen exposure

by decreasing the potential for enzyme-substrate complex formation.

A mechanism is proposed for the countercurrent diffusional accumulation of reduced substances in the vicinity of the teleost retina.

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A THESIS

Submitted to
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Dedicated to the memory of

my grandparents

Marie and Gustaff Baeyens

and

Everett L. Kinzer

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# INTRODUCTION

It has been established that oxygen at pressures only slightly in excess of 150 mm Hg produce adverse effects in intact organisms (Robinson et al., 1969). It has furthermore been demonstrated in a number of studies that oxygen may exert its toxic effect directly at the cellular level independent of any vascular changes that might occur in response to elevated oxygen tensions. Most cellular processes require a certain, rather narrowly defined, oxygen tension to function at peak efficiency. Investigations into the toxic effects of oxygen are currently, therefore, being conducted at the level of the enzymes. Mechanisms by which oxygen controls metabolic processes may be elucidated through such enzymatic studies. In this study, oxygen toxicity is defined as the influence of oxygen on enzyme activity. Most studies dealing with the toxic effects of oxygen have been carried out utilizing intact homeotherms or homeothermic tissues. There is a noticeable paucity of information in the literature concerning the toxic effects of oxygen on poikilotherms.

Studies of the toxic action of oxygen at elevated pressure on animals and man may be of considerable practical

importance. Oxygen toxicity is a real danger under all conditions in which man is exposed to concentrations of oxygen greater than that present in air (150 mm Hg). This is particularly true for the conditions of hyperbaric oxygenation used in medical practice, underwater work, and the space program. A better understanding of oxygen toxicity may be of importance for the safe application of oxygen at pressure in excess of 150 mm Hg.

In studies dealing with oxygen toxicity, in vitro procedures have the advantage that oxygen tensions to which the tissue slices are exposed can be carefully controlled. Intact cells can be studied under conditions at which changes in the rate of diffusion of oxygen, caused by vasomotion, cannot influence the results. Investigations involving in vitro methods have revealed that oxygen, at a partial pressure only slightly higher than that present in air, inhibits metabolic reactions in many types of cells (Cooper, Burt, and Wilson, 1958). These investigations have shown that the cellular concentrations of oxygen necessary to produce oxygen toxicity are below those needed to cause lung damage and also lower than those needed to produce central nervous system derangements in vivo.

Studies of oxygen poisoning in vitro have provided strong evidence for the hypothesis that oxidation of sulfhydryl groups of important tissue constituents plays a critical role in the

production of the symptoms of oxygen toxicity *in vivo*. Haugaard (1968) speculated that the inactivation of the sulfhydryl enzymes by oxygen was due to the formation of a reversible disulfide configuration. Several enzymes involved in glycolysis, as well as the tricarboxylic acid cycle (TCA) which are dependent on sulfhydryl groups for activity have been shown to be inhibited by oxygen. Retinal succinic dehydrogenase, an important enzyme of the TCA cycle was irreversibly inhibited by exposure of rabbits to oxygen at 1 atm for 24 hours (Shaw and Leon, 1970). Inhibition of enzymes involved in oxidative carbohydrate metabolism would cause a decrease in oxygen consumption of tissues. Horn, Haugaard, and Haugaard (1965) found that glyceraldehyde phosphate dehydrogenase was the only enzyme in the reactions from fructose-diphosphate to lactate that was rapidly inactivated by oxygen at 1 atm. There is some controversy in the literature concerning the susceptibility of lactate dehydrogenase (LDH), a glycolytic enzyme, to oxygen toxicity. Lactate dehydrogenase of yeast, however, was found by Armstrong, Coates, and Morton (1960) to be so easily inactivated in air that it was difficult to purify. They concluded that oxygen caused inactivation through the formation of intramolecular disulfide bonds with concomitant change in tertiary structure.

The teleost retina was chosen as a focal point for this study because it is one of the few animal tissues that functions normally in the presence of oxygen tensions in excess

of 450 mm Hg (Fairbanks, Hoffert, and Fromm, 1969). These high oxygen tensions are maintained by a countercurrent multiplying system in choriocapillaries located posterior to the retina of the trout eye. The possibility exists, therefore, that the enzyme systems of the teleost retina are refractory to a certain degree to the influence of high oxygen tensions, a phenomenon that may also be characteristic of the retinal enzymes in various other species. The oxygen tensions in the vicinity of the mammalian and amphibian retina which have no countercurrent multiplying system are no higher than those encountered in arterial blood (80-100 mm Hg). Several neural and non-neural mammalian tissues have been found to be susceptible to oxygen toxicity. In vitro studies involving tissues of cold-blooded animals, in particular the teleost retina, may provide some insight to an explanation of oxygen toxicity at the cellular level.

The objectives of this study were to investigate the following questions concerning oxygen toxicity:

1. Are the TCA cycle enzymes of the teleost retina resistant to oxygen toxicity?
2. Do the enzymes of the TCA cycle in retinas of other species, not normally exposed to elevated oxygen tensions in vivo, differ in their sensitivity to oxygen?
3. Does LDH, a glycolytic enzyme, of the teleost retina differ in sensitivity to oxygen as compared to the

LDH of retinas normally exposed to oxygen tensions not in excess of 150 mm Hg?

4. Will lowering of teleost retinal $P_{O_2}$ change the LDH sensitivity to oxygen?

5. Do other neural and non-neural teleost tissues differ from the retina in response to elevated oxygen tensions?

6. How do neural and non-neural tissues from other species respond to elevated oxygen tensions?

7. What is the relationship between temperature and oxygen toxicity?

# LITERATURE REVIEW

## Inhibitory Effects of Oxygen on Tissue Metabolism: In Vitro Studies

There is a great deal of evidence that respiration of tissue slices is depressed by oxygen at partial pressures above 1 atm. This inhibitory effect on cellular metabolism has been demonstrated to occur in a number of tissues, with brain probably being the most sensitive (Stadie, Riggs and Haugaard, 1945). Tissue homogenates have also been found to decrease their oxygen uptake following oxygen exposure and with such preparations inhibitory effects were observed at lower tensions of oxygen than with tissue slices (Elliott and Libet, 1942).

Haugaard, Hess and Itskovitz (1957) showed that the oxidation of glucose and pyruvate by heart homogenates was inhibited by 1 atm of oxygen compared to controls in air or in 7% $O_2$-93% $N_2$. This inhibitory effect was slow in onset and, with glucose as substrate, did not appear before 45-60 min after the start of the incubation. In other experiments by Horn *et al.* (1965) it was observed that metabolic reactions in the heart homogenate, other than glucose utilization or oxygen uptake, were altered much faster.

High oxygen tensions inhibited glyceraldehyde phosphate dehydrogenase and consequently caused marked changes in concentrations of glycolytic intermediates, lactate, and ATP. These metabolic effects were observed after less than 15 min of incubation, before there was any evidence of inhibition of glucose utilization or the rate of oxygen uptake. Thus it appears that when the effect of an elevated pressure of oxygen on metabolism is measured, one or more enzymatic steps may be markedly inhibited without it being reflected in any significant change in the rate of respiration or substrate utilization.

Experiments by Thomas, Neptune and Sudduth (1963) have shown that inhibition of carbohydrate metabolism by oxygen in vitro can occur rapidly in brain preparations. These investigators observed that after a 30 min incubation at 5 atm oxygen the production of radioactive $CO_2$ from $^{14}C$-glucose in brain homogenates was depressed to 20-30% of control values at 1 atm. Since the production of $^{14}CO_2$ from labeled pyruvate was also markedly and rapidly depressed by 5 atm oxygen, they concluded that the toxic effect of oxygen was caused mostly by an inhibition of pyruvate oxidation. The oxidation of α-oxyglutarate by brain mitochondria was also shown to be inhibited by high oxygen pressures.

The tissue culture technique has advantages in studies dealing with oxygen toxicity in that oxygen tensions can be carefully controlled and intact cells can be studied under

conditions at which changes in the rate of diffusion of oxygen, caused by vasomotion, do not influence the results. Investigations employing tissue culture techniques have revealed that oxygen, at a tension only slightly higher than that present in air, inhibits cell division and growth and influences metabolic reactions in many types of cells (Cooper et al., 1958). Tensions of oxygen lower than 0.2 atm ($P_{O_2}$ = 152 mm Hg) have been shown to be toxic to mammalian liver and kidney cells (Kieler, 1957). Fisher (1960) observed the growth of L-strain fibroblasts and noted that, when the tension of oxygen in the gas phase was increased to 26 vol% ($P_{O_2}$ = 198 mm Hg), there was a delay in the onset of growth that was longer, the higher the concentration of oxygen, and with 50% oxygen at 1 atm ($P_{O_2}$ = 380 mm Hg) the delay was as long as 24 hrs. These experiments are important because they demonstrate that the cellular $P_{O_2}$ necessary to produce oxygen toxicity in vitro is below that needed to cause lung damage in animals ($P_{O_2}$ = 266 mm Hg) and far smaller than the tension of oxygen at which central nervous system derangements occur in vivo (Bond, Jordan and Allred, 1967).

Except for cells exposed directly to the ambient gas, the tissues in an animal during hyperbaric oxygenation have an oxygen tension lower than that of the inspired gas. When the toxic effects of oxygen are studied in cell suspensions it is not necessary, therefore, to utilize pressures of

oxygen much above 1 atm. In studies involving the effect of oxygen tension on fibroblasts Fisher (1960) observed that the rate of respiration was only slightly depressed at tensions of oxygen that greatly affected growth. He concluded that some mechanisms concerned with cell multiplication were more sensitive to oxygen than reactions concerned with energy metabolism. The same conclusion was also reached by Rueckert and Mueller (1960), who found that the growth of HeLa cells was inhibited by tensions of oxygen greater than 340 mm Hg at 1 atm. Brosemer and Rutter (1961) found that a strain of fibroblast showed an inhibition of DNA synthesis by 95% $O_2$ at 1 atm as measured by the rate of incorporation of $^{32}P$ into DNA. An interference with nucleic acid metabolism was also observed in the damaging effects of 1 atm $O_2$ on the development of frog embryos (Rosenbaum, 1960). Finally, Heppleston and Simnett (1964) reported the appearance of degenerative changes in cells from different mouse tissues cultured in vitro after exposure to 1 atm of oxygen.

A number of studies dealing with the problem of oxygen toxicity have been carried out with intact tissue preparations. Bean and Bohr (1938) demonstrated that rabbit pyloric sphincter muscle suspended in Tyrode solution began immediately to relax when the oxygen pressure was increased to 5 atm. They proposed that the action of high oxygen pressures on muscle function was caused by what they called "hyperoxic anoxia", which resulted from inhibition of tissue dehydrogenases.

Riggs (1945) confirmed the findings of Bean and Bohr in experiments in which the rabbit pyloric sphincter muscle preparations were exposed to 8 atm of oxygen. Under these conditions the rate of relaxation under high oxygen pressure was extremely rapid and essentially complete in 20 minutes. After the pressure was lowered to 1 atm, the tonus returned to the original level and the process could be repeated at least three times with the same preparation. These experiments on smooth muscle demonstrate a rapid and reversible effect of oxygen at high pressure that may be a reflection of a distinct, localized biochemical alteration in the cell. Experiments dealing with intact frog muscle showed that in the presence of 8-35 atm of oxygen, respiration as measured by $CO_2$ production, was greatly inhibited (Cass, 1947). Finally, Falsetti (1959) observed that sodium transport across frog skin was inhibited about 40% in 8 atm of oxygen compared to a control in room air at atmospheric pressure.

## Enzyme Inhibition by Oxygen

It has been established that oxygen is capable of inactivating a number of enzymes (Bean, 1964) and that respiration of brain homogenates and slices is markedly depressed during hyperbaric oxygenation (Mann and Quastel, 1946). Dickens (1946) and Stadie *et al*. (1945) concluded that although some enzymes were resistant to oxygen toxicity, many

others were easily inhibited by oxygen at high pressures or even by oxygen at the partial pressure present in air. Among the enzymes particularly susceptible to oxygen toxicity were those containing essential sulfhydryl groups (Haugaard, 1946).

Despite conclusive demonstrations of enzyme inhibition by oxygen, there has until recently been a reluctance to conclude that the inhibitory effects of oxygen on metabolism are the direct cause of the symptoms of oxygen toxicity in the intact animal (Haugaard, 1968). There are primarily two reasons for this. First, inactivation of enzymes in vitro seemed to be too slow in onset to account for the symptoms of oxygen toxicity that occurred so rapidly in the intact animal. Second, there had been no conclusive demonstration that any enzyme was inhibited during exposure of an animal to oxygen at an elevated pressure. Recent experiments have shown that the earlier views were too cautious. Inhibitory effects of oxygen on enzyme activity have been demonstrated after short-time exposure of tissue preparations to relatively low oxygen tensions (Haugaard, 1968). Furthermore, evidence has shown that some enzyme reactions are inhibited in vivo during hyperbaric oxygenation (Shaw and Leon, 1970).

One group of enzymes was found to be especially sensitive to the inhibitory action of oxygen, the so-called sulfhydryl or SH enzymes (Haugaard, 1946). These enzymes are characterized by their inactivation by reagents such as

iodosobenzoate, iodoacetate, p-chloromercuribenzoate, and organic arsenicals (Bacq, 1946). All these substances tend to oxidize or combine with SH groups on the enzyme. Experiments on the inhibitory effects of oxygen on enzymes indicate strongly that oxygen should be included in this group of substances (Barron, 1955).

Hellerman, Perkins and Clark (1932) were the first to show that oxygen at a partial pressure of 159 mm Hg inactivated the sulfhydryl-containing enzyme, urease, in vitro. Partial or complete restoration of the activity of the inhibited enzyme could be obtained by incubation with reducing agents such as cysteine or reduced glutathione. Sizer and Tytell (1941) measured the activity of crystalline urease in the presence and absence of various reducing agents and determined at the same time the oxidation-reduction potential of the medium with platinum electrodes. They observed that the activity of urease tended to be a continuous function of the oxidation-reduction potential over a wide range. In the case of the urease preparations the enzyme activity increased with increased reduction of the enzyme.

In early work on the effect of elevated pressures of oxygen on enzymes it was shown that the sensitivity to oxygen of different sulfhydryl-containing dehydrogenases varied widely (Davies and Davies, 1965). One such enzyme, 3-phosphoglyceraldehyde dehydrogenase, an important glycolytic enzyme, was shown to be inhibited by oxidizing agents (Rapkine, 1938).

Dickens and Stadie (1946) observed that this enzyme in brain tissue was inactivated on exposure to high pressures of oxygen. Horn et al. (1965) in studies dealing with the effect of oxygen tension on the metabolism of fructose-diphosphate and glucose by heart homogenates, demonstrated that 3-phosphoglyceraldehyde dehydrogenase was inhibited by oxygen at 1 atm within 10 min. In spite of the fact that other glycolytic enzymes have been shown to contain essential sulfhydryl groups, the glyceraldehyde phosphate dehydrogenase reaction was the only one of the steps from fructose-diphosphate to lactate that was rapidly inactivated by oxygen at 1 atm. Furthermore, the toxic effect of oxygen was reversible since addition of cysteine or reduced glutathione caused immediate restoration of enzyme activity. Another glycolytic enzyme, lactate dehydrogenase (LDH), was found by Bach, Dixon and Zerfas (1946) to be inactivated on exposure to air. The LDH of bakers' yeast when purified under nitrogen was also found to be inactivated by air (Armstrong et al., 1960). These results raise the possibility that glycolysis may be inhibited during exposure to elevated pressures of oxygen in vivo.

Various enzymes associated with the TCA cycle are also sensitive to the toxic effects of oxygen. Succinic dehydrogenase has been shown to be inactivated by oxygen in vitro (Stadie, Riggs and Haugaard, 1944). They showed that this

enzyme, after oxygen inactivation, returned to full activity upon incubation with cysteine or reduced glutathione. When succinate was added in high concentration the enzyme was protected from inhibition by oxygen. This raised the possibility that protection of sulfhydryl groups in enzymes by their substrates may play a role in the defense of the cell against the influence of oxygen and other oxidizing agents.

In the past one of the reasons for the reluctance to accept the fact that enzyme inactivation could account for the symptoms of oxygen toxicity *in vivo* was the slowness of onset of the inhibitory effects of elevated tensions of oxygen *in vitro*. Stadie, Riggs and Haugaard (1945) found that xanthine oxidase activity in liver homogenates was inhibited within minutes during incubation in 100% oxygen at 1 atm. Haugaard (1965) found inhibitory effects of 1 atm of oxygen on ATP formation during oxidation of α-oxyglutarate by brain homogenates within 15 min. Thomas *et al.* (1963) noted a marked inhibition of α-oxyglutarate and pyruvate oxidation during short-term incubation of brain homogenates at 5 atm $O_2$, and Chance, Jamieson and Coles (1965) observed that on exposure of liver mitochondria to 12 atm of oxygen the energy-dependent reduction of nicotinamide adenine nucleotide was inhibited after less than 1 min. Thus, there is considerable evidence that enzymes can be inactivated *in vitro* within the time that symptoms of oxygen toxicity appear *in vivo*. Davies and Davies (1965) have published a review

article presenting a table listing the enzymes that have been shown to be inhibited by oxygen, as well as enzymes that have been reported to be insensitive to oxygen inactivation.

## Oxygen Toxicity in the Intact Animal: In Vivo Studies

An inhibition of hydrolytic enzymes by oxygen was demonstrated *in vivo* (Rosenbaum, Wittner and Wertheimer, 1966). These workers exposed the protozoa *Paramecium caudata* to 100% oxygen at 3 atm for varying time periods and subsequently determined the proteolytic activity of homogenates of the organisms with hemoglobin as substrate. Within 2 hrs the enzymatic activity was inhibited about 90%. High pressures of nitrogen had no effect.

There have been relatively few experiments done with intact animals that provide significant information about the cellular mechanisms of oxygen toxicity. Allen (1961) noted great changes in the development of the vascular system during the first 4 days of incubation of fertilized hen eggs at 1 atm of oxygen. It has become increasingly evident that oxygen at an elevated tension, in addition to causing vasoconstriction and damage to blood vessels in the eye (retrolental fibroplasia), also has a deleterious effect on the retina (Nichols and Lambertsen, 1969). Noell (1959) experimented with the effects of oxygen on the activity of the rod

cells of the mammalian eye. With electrodes in contact with the cornea of white rabbits, he measured the electroretinogram. This important manifestation of visual cell activity was found to be profoundly influenced by oxygen tension. Continued exposure of rabbits to oxygen at 1 atm resulted in severe attenuation or disappearance of the electroretinogram. With pressures of oxygen above 1 atm the effects were more marked and at 7 atm $O_2$ the electroretinogram completely disappeared after 40 min. Studies of the toxic action of oxygen in the eye have been reviewed by Nichols and Lambertson (1969).

Jamieson, Ladner and Van Den Brenk (1963) demonstrated tissue damage and metabolic changes in the lungs of rats exposed to 5 atm of oxygen. The metabolic changes consisted of a progressive lowering of the activity of succinic dehydrogenase with the time of exposure to oxygen. The importance of these experiments lies in the demonstration of metabolic changes that are concomitant with or precede the tissue damage caused by oxygen.

Dolezal, Vorel and Andel (1962) measured glucose, lactate, and pyruvate in the blood of white rats exposed to 1 atm of oxygen for long periods of time. The blood glucose level rose and the concentration of lactate and pyruvate decreased significantly during the breathing of oxygen at 1 atm. Wegicki et al. (1966) measured blood levels of lactate in dogs exercising at 3 atm of oxygen and found a decrease in lactate production that they explained as an inhibition of glycolysis.

These experiments provide evidence that glycolysis may be depressed in certain tissues during exposure to elevated pressures of oxygen. Inhibition of glycolysis *in vivo* may lead to changes in the oxidation-reduction states of the pyridine nucleotides in the cell and to disturbances of cell function.

Metabolic actions of oxygen in rats exposed to a pressure of oxygen only slightly higher than that of air were reported by Bond *et al.* (1967). The animals lived in chambers filled with oxygen at about 0.35 atm. At different times up to 90 days, they were killed and tissues taken for analysis of coenzyme A. In brain and liver the concentration of this coenzyme decreased and reached a minimum (for brain 52% of the initial value) after 30 days. It would be interesting to know whether a decrease in tissue coenzyme A concentration occurs during acute exposure of animals to high oxygen tensions.

Sanders *et al.* (1966) quantitated metabolic changes in rat tissues after exposure of the animals to elevated pressures of oxygen. They found a significant decrease in the ATP content of brain, liver, and kidney after 1.5 hr at 5 atm $O_2$. Brain ATP decreased after breathing 1 atm oxygen for 2 hr. Finally, Hall and Sanders (1966) observed a large increase in soluble nitrogen in homogenates of brain, liver, and kidney obtained from rats exposed to 5 atm $O_2$. Changes in unbound cathepsin and acid phosphatase, enzymes associated

with lysosomes, led the authors to postulate that destruction of lysosomes may be of importance in oxygen toxicity.

## Proposed Mechanisms of Oxygen Toxicity

Upon short treatment with mild oxidizing agents, including molecular oxygen, the inactivation of the hydrolytic enzymes is considered to involve the formation of the disulfide form of the enzyme according to the equation:

$$\underset{\text{(active)}}{2\ \text{EnSH}} \underset{\text{reduction}}{\overset{\text{oxidation}}{\rightleftharpoons}} \underset{\text{(inactive)}}{\text{EnS-SEn}} + 2(\text{H})$$

where: En = enzyme

More prolonged exposure to oxygen could lead to irreversible changes probably associated with further oxidation of the sulfur atoms (Hellerman, 1937). Hellerman proposed that the kinetics of several hydrolytic and other processes *in vitro* as well as *in vivo* may be controlled by reversible oxidations and reductions of thiol and disulfide groups of certain enzymes.

The oxidation by oxygen of SH groups to disulfide (S-S) linkages within the enzyme molecule require that the sulfhydryl groups be present in reasonably close juxtaposition (Haugaard, 1968). This could involve two SH groups situated close together in the same peptide chain, as in 3-phosphoglyceraldehyde dehydrogenase. In this enzyme two cysteine

residues are separated by three non-sulfur amino acids and disulfide formation would lead to formation of a ring within a peptide chain.

There are cases in which a ring cannot easily be formed between SH groups from cysteine residues in the same chain (LDH). It is possible that SH groups from two different peptide chains may be close enough for interchain S-S groups to be formed by oxidation with molecular oxygen. Finally, in many enzymes SH groups are sufficiently far apart so that oxidation to the disulfide by oxygen is extremely slow or impossible, except by dimerization between enzyme molecules. In some enzymes only one cysteine residue is present and in such molecules the formation of S-S bridges by oxidation can only occur between two molecules (Haugaard, 1968).

Another mechanism that may be involved in the oxidation of enzyme sulfhydryl groups by molecular oxygen is free radical formation caused by oxidation of an enzyme SH group and an SH group from a low molecular weight sulfhydryl compound. After free radical formation the enzyme and sulfhydryl compound could combine according to the following equation:

$$EnSH + HSR + \tfrac{1}{2}O_2 \rightleftharpoons En\text{-}S\text{-}S\text{-}R + H_2O$$

According to this reaction, the addition of a sulfhydryl compound to an enzyme may tend to accelerate rather than decrease

the rate of inactivation of the enzyme by oxygen (Haugaard, 1968).

A final mechanism of oxygen inactivation of enzymes is an oxidation of low molecular weight sulfhydryl compounds in the environment of the enzyme followed by mixed disulfide formation (Eldjarn, 1965) by the following reactions:

$$2RSH + \tfrac{1}{2}O_2 \rightleftharpoons RSSH + H_2O$$

$$En\text{-}SH + RSSH \rightleftharpoons En\text{-}S\text{-}S\text{-}R + RSH$$

Eldjarn's work has shown that a number of enzymes can become inactivated by incubation with cystine or oxidized glutathione. This proposed mechanism could lead to oxygen inactivation of enzymes in which the sulfhydryl groups are not in close juxtaposition.

In general, sulfhydryl groups in enzymes vary widely in reactivity toward all reagents that react with these groups including oxidizing agents, substances that form mercaptides, alkylating agents, and molecular oxygen. Even within an individual enzyme there may be sulfhydryl groups that vary widely in reactivity toward molecular oxygen (Webb, 1966). There are probably many factors involved in the differential activity of enzyme sulfhydryl groups including: juxtaposition to other SH groups, degree of ionization, extent of hydrogen bonding, and situation in the interior and exterior of the protein molecule. Haugaard (1968) presents an excellent review article in which the proposed mechanisms of oxygen toxicity are thoroughly discussed.

## EXPERIMENTAL RATIONALE

The experiments were designed to study oxygen toxicity in selected teleost, amphibian, and mammalian tissues. Oxygen toxicity was investigated at the cellular level and is defined in this study as the inhibition of selected sulfhydryl containing enzymes involved in carbohydrate metabolism. The teleost retina was chosen as the nucleus of the study because *in vivo* it is enveloped by oxygen tensions in excess of 400 mm Hg; such a $P_{O_2}$ has been shown to be inhibitory to certain other tissues. The retinas of other vertebrate classes do not encounter oxygen tensions in excess of those in arterial blood. Amphibian and mammalian retinas were employed, therefore, to compare the effects of elevated oxygen tensions on the metabolism of these tissues with the teleost retina.

Studies involving the exposure of tissues and tissue homogenates to elevated oxygen tensions were divided into two arbitrary categories: 1) Normobaric oxygen studies involved exposures to oxygen at tensions ranging between 0 and 740 mm Hg; 2) Hyperbaric studies involved all exposures to oxygen at tensions in excess of 740 mm Hg. In general normobaric studies involved total pressures of less than 1 atm, whereas hyperbaric studies involved pressures in excess of 1 atm.

Oxygen toxicity was investigated by two methods: 1) The influence of oxygen was investigated by measuring enzyme (LDH) activity of tissue homogenates after exposure to hyperbaric and normobaric oxygen and nitrogen, and 2) Effects of oxygen were investigated by quantitating oxygen consumption of tissue slices after exposure to hyperbaric oxygen (1470 mm Hg) and comparing these results with the oxygen consumption of tissues exposed to 154 mm Hg $P_{O_2}$.

Lactate dehydrogenase activity was used as an index of glycolysis. The activity of the TCA cycle was quantitated by measuring oxygen consumption of the tissues. Thus by measuring the activity of the glycolytic pathway, as well as the TCA cycle, it was possible to determine at what points in carbohydrate catabolism oxygen exerted its effect. One must be aware of the fact that changes in TCA cycle activity (as measured by changes in oxygen consumption) are not limited entirely to modifications of TCA cycle enzyme activity. The possibility exists that oxygen may inhibit enzymes of the EMP pathway, decreasing the formation of pyruvate which would result in a concomitant decrease in TCA cycle activity.

Attempts were made to correlate changes in LDH activity with changes in the migratory pattern of LDH isozymes after exposure to normobaric (740 mm Hg or less) and hyperbaric (740 mm Hg or greater) oxygen and nitrogen. Changes in isozyme patterns could result from intermolecular dimerization or from changes in the isoelectric point of the isozymes which

in turn result in changes in tertiary structure brought about by nitrogen or oxygen.

Hyperbaric oxygen studies were investigated in the brain of the rainbow trout (teleost), frog (amphibian), and dog (mammal) in order to ascertain whether oxygen exerted effects on these tissues similar to those found in the retina. Liver tissue from the three species was studied to determine if oxygen exerted similar effects on a non-neural tissue. Through the utilization of both homeotherms and poikilotherms, it was possible to investigate the correlation between body temperature and metabolism, as influenced by oxygen.

# MATERIALS AND METHODS

## Experimental Animals

Rainbow trout (Salmo gairdneri) were donated by the Michigan Department of Natural Resources at Grayling, Michigan. The 2-2$\frac{1}{2}$ year old trout selected were between 23 and 28 cm in length and weighed from 380-480 g. The fish were maintained in fiberglass tanks in a constant temperature room at 15±1°C. Dechlorinated tap water was kept constantly flowing into the tanks and was aerated with compressed air filtered through activated charcoal. The photo-period consisted of 15 hrs of light and 9 hrs darkness. Frogs (Rana pipiens) 15-20 cm long were obtained from the Mogul-ED Corp., Oshkosh, Wis., and maintained under the same conditions as the fish. Dogs (Canis familiaris) weighing approximately 15-18 kgs were obtained through the Center for Laboratory Animal Resources at Michigan State University.

## Initial Tissue Preparation

Trout were killed by cervical dislocation while frogs were double pithed. Dogs were anesthetized with sodium pentabarbitol. In all cases the tissues were removed immediately

after the animal was killed. All surgical procedures used in tissue isolation were carried out using sterile techniques. The eyes were extracted from their orbits by severing the ocular muscles and optic nerves. The eyes were then immediately immersed in a petri dish filled with sterile phosphate buffered saline (PBS) (Appendix A). While holding the eye with rat tooth forceps, a small incision was made into the periphery of the cornea with the blade of an iris scissors, the incision being continued along the circumference of the cornea parallel to the iris. After removing the cornea, the lens was lifted out of the aqueous humor with small curved forceps. Next, the optic nerve stump was firmly grasped with iris forceps while a small circular incision was made into the posterior surface of the sclera to one side of the optic nerve. Eye dressing forceps were carefully inserted into the incision and the retina and choroid were worked free from the sclera by gentle scraping. The retina, choroid and vitreous body were gently removed anteriorly from the eye and placed in sterile PBS solution. After removal from the PSB solution the tissues were blotted dry on sterile No. 1 Whatman filter paper. During the blotting process as much of the vitreous body was removed as was possible without damaging the retinal cells and the choroid was separated from the retina by careful dissection. In these studies an attempt was made to retain the pigmented epithelium on the retina.

To obtain liver tissue from the fish an incision was made from the base of the operculum along the mid-ventral line. After the liver was exposed a piece of tissue was removed. In the amphibian the liver was exposed by a mid-ventral incision. Dog hepatic tissue was obtained by making a mid-ventral incision approximately 10 cm just caudal to the rib cage with a scalpel. Rat tooth forceps were used to grasp a portion of the liver while a piece was excised. After removal from the animal the samples of liver from the three species were placed in sterile PBS and were trimmed with iris scissors to obtain wet weights approximating 220 mgs.

The brain of the fish and frog were exposed by using a heavy duty serated scissors to cut through the skull cap. Pieces of brain tissue were removed using rat tooth forceps. In the case of the dog, a scalpel was used to cut through the skin and muscle overlying the skull. A 2.5 $cm^2$ piece of the skull was removed by means of a Striker saw exposing the dura mater. The dura and pia mater were pierced with a scalpel and a piece of the cerebral cortex was removed with rat tooth forceps. Upon removal from the animals all brain tissue samples were placed in sterile PBS and were trimmed with iris scissors to a weight of approximately 140 mgs. All initial tissue preparations were completed in less than 15 minutes. Further tissue preparation involving slicing, homogenizing or mincing were performed as the individual experiments warranted.

## Oxygen Consumption Studies

### Tissue Preparation and Gassing Procedure

Pieces of retina, liver, and brain of the teleost, amphibian and mammal were isolated under sterile conditions. Retinas were removed by the procedure outlined previously, and trout and frog retinas were cut into approximately three equal portions while in the case of the dog three pieces of comparable size were used. Approximately 2x2x0.5 mm pieces of brain tissue were removed. The liver was sliced by means of a Model 7120A Stadie-Riggs Tissue Slicer (Arthur H. Thomas Co., Philadelphia, Pa.) into pieces not exceeding 0.2 mm in thickness.

Four pieces of each tissue were placed in 90 mm disposable sterile plastic petri dishes containing 3 ml of culture media. For the amphibian and teleost tissue the incubation media was TC 199 (Grand Island Biological Co., Grand Island, N.Y.) (Appendix A) containing 50 mg% glucose. In the case of mammalian tissues the incubations were performed in Pucks Minimal Essential Media (Grand Island Biological Co., Grand Island, N.Y.) with antibiotics added (Appendix A).

One-half of the uncovered petri dishes were placed in a water-saturated, 5.6 liter capacity, aluminum environmental pressure chamber (No. A603A, National Presto Ind., Inc., Eau Claire, Wis.). The teleost and amphibian tissues were incubated for 4 or 24 hours at 15°C and mammalian tissues for

24 hours at 15 or 37°C. The environmental chambers were thoroughly flushed with the incubation gases prior to the start of each incubation. Gases consisted of room air with the pressure adjusted to obtain $P_{O_2}$'s of 154 and 400 mm Hg and pure oxygen at pressures of 1470 mm Hg.

Assay Procedure

Oxygen consumption was measured using a YSI Model 53 Biological Oxygen Monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio) in conjunction with a Beckman 10 in. strip chart recorder. The tissues were assayed in 3 ml of Mammalian Krebs Saline Medium saturated with room air and containing 2.94 g/liter $NaHCO_3$ (Appendix A). During the assay procedure the temperature of the Biological Oxygen Monitor was maintained by means of a constant temperature water bath at 15±0.1°C or 37±0.1°C (Haake 70, Polyscience Corp., Evanston, Ill.). Oxygen utilization by the tissues was measured by recording a linear change in $P_{O_2}$ over a 10 minute period. Calculations of oxygen consumption were based on the solubility coefficient of oxygen in Ringer solution (0.0340 ml $O_2$/ml fluid at 1 atm at 15°C; Umbreit, Burris and Stauffer, 1964). Prior to oxygen consumption measurements the pieces of brain tissue from each petri dish were homogenized in 15 ml of the Krebs-Ringer bicarbonate media and 3 ml of the homogenate was placed in the apparatus. The pieces of retina and liver were placed directly into 3 ml

of Krebs-Ringer bicarbonate media in the apparatus. The oxygen consumption of the tissues was expressed as microliters of oxygen consumed per hour per milligram protein with all values corrected to standard temperature and pressure dry (STPD). The average barometric pressure used in the calculations was 735.46±0.74 mm Hg based on 23 observations. Protein determinations (Appendix B) were done by the method of Lowry (Oyama and Eagle, 1956).

## Lactate Dehydrogenase Isozyme Studies

### Tissue Preparation and Gassing Procedure

Retina, liver and brain tissue from the teleost, amphibian and mammal were employed in these experiments. The amount of tissue used for each preparation consisted of either two retinas, approximately 220 mg piece of liver, or approximately 140 mg fragment of brain tissue. For the teleost each tissue was sonified in 5 ml of Eagle Minimum Essential Medium (Grand Island Biological Co., Grand Island, N.Y., Appendix A). In the case of the amphibian each tissue was sonified in 5 ml of TC 199. Mammalian tissues were sonified in 5 ml of Pucks Minimal Essential Media (Appendix A). After sonification each tissue homogenate was divided in half and placed in two 90 mm diameter plastic disposable petri dishes.

The two aliquots of each tissue preparation were placed in the humidified aluminum environmental pressure chambers

and one sample being exposed to hyperbaric oxygen (1470 mm Hg) while the other sample was exposed to hyperbaric nitrogen (1470 mm Hg). All tissues were incubated for 24 hrs at 15°C.

Electrophoretic Procedure

The disc electrophoretic apparatus was constructed from two 17x12.5x7.5 cm polyethelene trays. The glass tubes (0.5 cm in diameter and approximately 9.0 cm long) were placed in two rows of seven holes each with the electrodes aligned in the middle (Figure 1). The anode and cathode, which were placed in the lower and upper bath, respectively, were 14.5 cm long, 2.0 cm diameter carbon rods. The power was supplied by a Vokam 2541 Constant Current Power Supply (Consolidated Laboratories Inc., Chicago Heights, Ill.).

The method of Ornstein and Davis (1962) was used in preparing the gels and tubes (Appendix C). The individual lengths of the separating, stacking and sample gels were 4.8, 1.0, and 1.0 cm, respectively. After incubation the tissue homogenates were mixed with the sample gel in a 1:10 ratio (v/v). Immediately after polymerization the isozymes were separated electrophoretically in glycine buffer (pH 8.4) at a constant current of 50 ma for approximately 5 hrs at 4°C. Bromphenol blue was added to the upper bath before the current was applied to mark the movement of the buffer front. Immediately following separation the gel columns were removed and stained.

FIGURE 1.--Polyacrylamide gel disc electrophoresis apparatus used for separating lactate dehydrogenase isozymes.

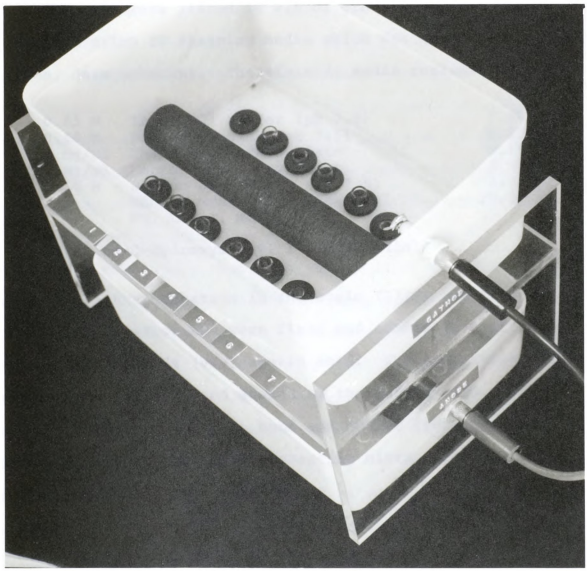

FIGURE 1

Staining Procedure

The gels were placed in 2.5 ml glass tubes containing 1 ml of a nitro BT staining media which was specific for LDH (Allen, date unknown). The staining media consisted of:

| | | |
|---|---|---|
| 0.05 M | Tris*-HCl buffer (pH 7.5) | 7.50 ml |
| 0.50 M | Sodium cyanide (pH 7.5) | 1.25 ml |
| 2 mg/ml | Phenazine methosulfate | 0.15 ml |
| 2 mg/ml | Nitro Blue tetrazolium | 3.50 ml |
| 0.50 M | Sodium dl-lactate (pH 7.5) | 3.00 ml |
| | NAD (A Grade-Sigma Chemical Co., St. Louis, Mo.) | 10.00 mg |

*2-Amino-2-Hydroxymethyl 1-3-Propandiol

After a 0.5 hr incubation in the stain (37°C) the gels were rinsed in tap water and were fixed and stored in 5 ml test tubes containing 7% (v/v) acetic acid. The test tubes were sealed with Parafilm and stored in the dark at 4°C. The migratory distance of each band and buffer front were determined with the aid of a dissecting microscope so that Rf values could be calculated.

## Lactate Dehydrogenase - Hyperbaric Studies

### Tissue Preparation and Gassing Procedure

The experiments involved the retina, liver, and brain of the teleost, amphibian and mammal. All tissues upon isolation were placed in thick-walled glass sonification tubes containing deoxygenated phosphate buffer (Appendix A). The teleost preparations consisted of two retinas placed in 10 ml

of phosphate buffer, an approximately 220 mg piece of liver placed in 10 ml of phosphate buffer, and an approximately 140 mg piece of brain tissue in 5 ml of phosphate buffer. In the case of the amphibian, 4 retinas were placed in 5 ml of phosphate buffer; liver and brain (weights comparable to the teleost tissues) were placed in 5 and 3 ml of phosphate buffer respectively. The mammalian preparations employed the same amount of tissue and the same dilutions as were used in the teleost preparations.

The tissues were then homogenized using a Sonifier Cell Disrupter fitted with a micro tip (Heat Systems Co., Melville, L.I., N.Y.). Samples of the homogenate (0.1 ml) were transferred to 5 ml glass vials by means of disposable prothrombin pipettes (Scientific Products, Evanston, Ill.). The remaining homogenates were sealed and kept frozen at -20°C for protein determinations.

The 5 ml vials were placed in water-saturated aluminum environmental pressure chambers. One-half of the vials containing each tissue preparation were exposed to hyperbaric nitrogen (1470 mm Hg); the other half were exposed to hyperbaric oxygen (1470 mm Hg). All tissue samples were incubated for 24 hours at 15°C.

## Lactate Dehydrogenase Assay Procedure

LDH activity was assayed according to the method of Worthington (Worthington Biochemical Corp., Freehold, N.J.).

Using a Beckman DB-G recording spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) the change in absorbancy produced by the conversion of $NADH_2$ to NAD was measured. The measurements were made at a wave length of 340 millimicrons (mμ) and a slit width of 1.0 mm. The change in absorbancy was recorded on a Beckman (10 inch) Potentiometric Recorder. Water was circulated through the cuvette holder from a constant temperature water bath. The assay medium, consisting of 0.1 ml $NADH_2$ (0.0027 M, pH 8.0; Grade III-Sigma Chemical Co., St. Louis, Mo.), 2.7 ml phosphate buffer (0.03 M, pH 7.4) and 0.1 ml sodium pyruvate (0.01 M, pH 7.0) was maintained in the constant temperature water bath at 15°C. At time zero 2.9 ml of the medium was added to an incubation vial containing the enzyme and vigorously mixed. The mixture was then immediately placed in a quartz cuvette and its changing optical density compared to a blank (assay medium plus 0.1 ml phosphate buffer) and recorded on a 10 in. Beckman Potentiometric Recorder.

Changes in absorbancy were linear over a 0.5 minute period, with linearity over this range being obtained by adjusting the enzyme concentration. Enzyme activity was expressed as change in absorbancy/min/mg protein. Statistical analysis to determine the significant difference in enzyme activity between nitrogen (control) and oxygen (experimental) incubations was carried out by employing a 2-way analysis of

variance. Values considered significant in this study have a calculated P value of 0.05 or less.

## Lactate Dehydrogenase - Normobaric Studies

### Tissue Preparation

Using the procedure previously outlined the retinas from two teleost or amphibian eyes upon isolation were immediately removed to a glove bag (Instruments for Research and Industry, Cheltenham, Pa.) containing an atmosphere of 100% nitrogen. Retinas were then homogenized in 10 ml of deoxygenated phosphate buffer (0.03 M, pH 7.4; Appendix A) using a 2 ml capacity ground glass tissue homogenizer, and the homogenate was transferred to a 15 ml glass stoppered centrifuge tube. The homogenate was centrifuged under a nitrogen atmosphere and the clear supernatant was used directly in the case of the amphibian and diluted 1:5 with the phosphate buffer in the case of the teleost. Under a nitrogen atmosphere in the glove bag, 0.1 ml of the supernatant was placed in each 15 ml incubation tube, making a total of 30 incubation tubes. Each incubation tube was then sealed by means of an injection stopper through which were inserted two 20-gauge needles with attached stopcocks (Figure 2). One stopcock needle assembly served to admit gas which was directed to the bottom of the incubation tube by PE 90 tubing and the other needle was utilized for gas outflow.

FIGURE 2.--Water displacement spirometer used in normobaric lactate dehydrogenase studies.

A. Rubber gas bag for nitrogen
B. Rubber gas bag for oxygen
C. 3-way glass stopcock
D. 100 ml graduated spirometer
E. Leveling bulb for spirometer
F. Leveling bulb for gas mixing
G. Gas mixing chamber
H. Incubation tube

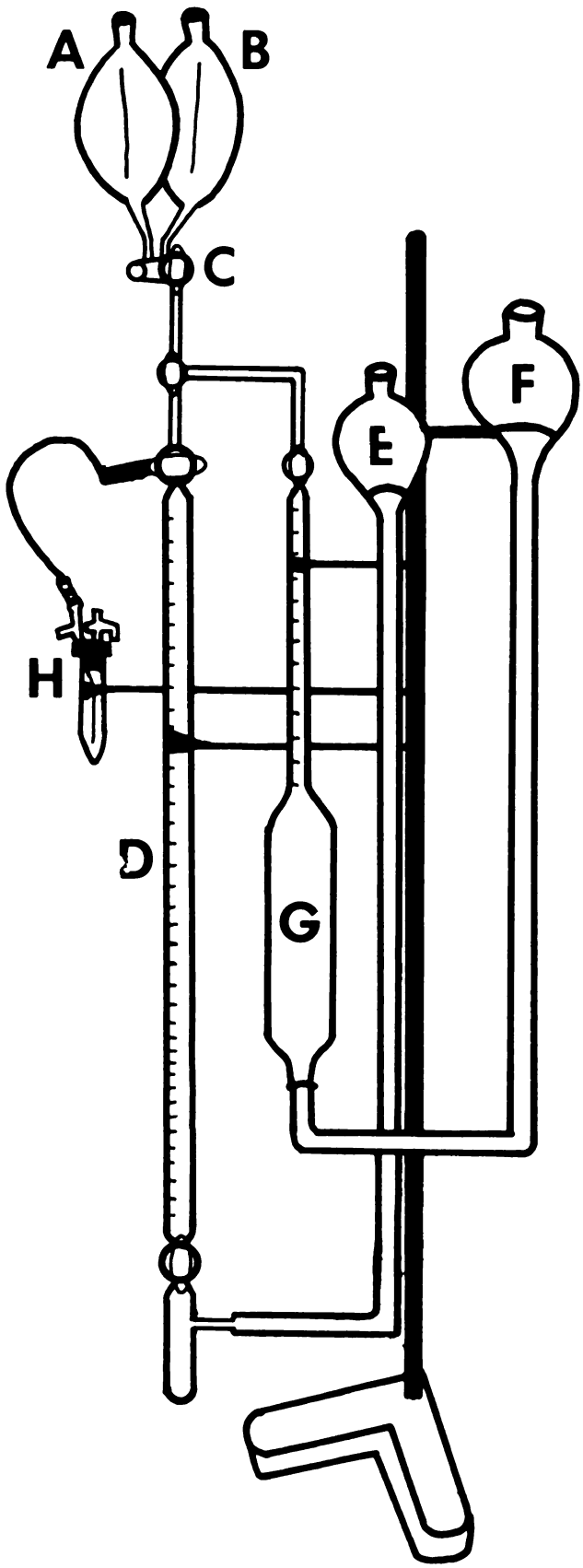


FIGURE 2

Gassing Procedure

Gas mixtures were made by means of a water displacement spirometer having a capacity of 100 ml (Figure 2). Gases were mixed to contain 0, 148, 296, 444, 592, or 740 mm Hg $P_{O_2}$ in nitrogen. Five incubation tubes were each gassed with 100 ml of gas at each $O_2$ tension, and, with stopcocks closed, were incubated at 15°C for 24 hours while being slowly rotated parallel to their long axes using a Multi-Purpose Rotator (Scientific Industries, Inc., Springfield, Mass.).

Lactate Dehydrogenase Assay Procedure

Lactate dehydrogenase activity was assayed as described for the hyperbaric LDH studies. The change in absorbancy, produced by the conversion of $NADH_2$ to NAD, was linear from 0.5 to 1.5 minutes with linearity over this range being obtained by adjusting the enzyme concentration. Enzymatic activity was expressed as change in absorbancy/min/mg protein. Protein determinations were done by the method of Lowry (Oyama and Eagle, 1956). The enzyme activity (ordinate) as related to $O_2$ tensions (abscissa) was determined by fitting a regression line using the method of least squares.

In a parallel experiment, acetazolamide (Diamox, Lederle Laboratories, Pearl River, N.Y.), a potent inhibitor of carbonic anhydrase, was administered ip to fish every third day for two weeks at a dose of 0.5 mg/kg body weight and the retinas were removed 24 hours after the last injection.

Lactate dehydrogenase activity was then assayed by the procedure previously described.

## Lactate Dehydrogenase Kinetics

### Preparation and LDH Assay Procedure

Homogenates as described for disc electrophoresis were prepared from mammalian brain and liver. One-half the aliquots were incubated in hyperbaric nitrogen ($P_{N_2}$ = 1470 mm Hg) and the others were incubated in hyperbaric oxygen ($P_{O_2}$ = 1470 mm Hg) for 24 hours at 15°C. Using a Beckman DB-G spectrophotometer the change in absorbancy produced by the conversion of $NADH_2$ to NAD was measured. The measurements were made at a wave length of 340 mμ and a slit width of 1.0 mm. The change in absorbancy was recorded on a 10 inch Beckman Potentiometric Recorder. Water was kept circulating through the spectrophotometer from a constant temperature water bath (15°C). The reaction mixtures were kept in the same water bath. The reaction mixture consisted of

| | | |
|---|---|---|
| 0.0340 M | Phosphate buffer (pH 7.4; Appendix A) | 2.7 ml |
| 0.0027 M | $NADH_2$ | 0.1 ml |
| Selected | Sodium pyruvate solution (pH 7.0) | 0.1 ml |
| | Tissue homogenate (enzyme source) | 0.1 ml |
| | (0.1 ml phosphate buffer was substituted for the enzyme in the case of the reagent blank) | |

The reaction was initiated by rapidly mixing the pre-incubated enzyme with the substrate, coenzyme and buffer and

immediately recording the decrease in absorbancy for three minutes.

Kinetic Assay

Five different pyruvate concentrations were used ranging from $10^{-2}$M to $10^{-4}$M. Lineweaver-Burk plots were drawn by plotting the reciprocals of the change in absorbancy (ordinate) against the reciprocals of the substrate concentration (abscissa). The $K_m$ values were determined from the X intercepts ( $-\frac{1}{K_m}$ ) and the V max values were determined from the Y intercepts ( $\frac{1}{Vmax}$ ).

# RESULTS

## Introduction

Elucidating the influences of oxygen on tissue metabolism was done by measuring both oxygen consumption and LDH activity. Oxygen consumption is assumed to reflect TCA cycle activity, which in turn may itself be influenced by the supply of intermediates produced by the EMP pathway. Lactate dehydrogenase activity will reflect the potential for the conversion of pyruvate to lactate (glycolysis). Additional investigations into the influence of oxygen on LDH were made through analysis of electrophoretic mobilities and Michaelis-Menten kinetics.

In each experiment a single preparation was divided into two aliquots, one being the control which was exposed to the more reducing atmosphere, i.e., pure nitrogen or room air. The experimental aliquot was exposed to the more oxidizing atmosphere consisting of oxygen tensions in excess of 159 mm Hg. All pressures used were absolute pressures measured at 880 ft above sea level. Gas volumes were corrected to STPD. All lines were fitted by the method of least squares while analysis of variance and the Student's t test were used to test the differences between the experimental

and control means. In all statistical tests in this work the fiducial limits were set at P = 0.05.

A summary of the various media employed in these experiments is given below; chemical compositions are found in Appendix A.

| Experiment | Animal | Medium Employed |
|---|---|---|
| Oxygen Consumption | Fish | TC 199 |
| | Frog | TC 199 |
| | Dog | Pucks Minimal Essential Medium |
| LDH Isozymes | Fish | Eagle Minimal Essential Medium |
| | Frog | TC 199 |
| | Dog | Pucks Minimal Essential Medium |
| LDH Activity (normo and hyperbaric) | Fish | Deoxygenated Phosphate Buffer |
| | Frog | Deoxygenated Phosphate Buffer |
| | Dog | Deoxygenated Phosphate Buffer |
| LDH Kinetics | Dog | Deoxygenated Phosphate Buffer |

## Oxygen Consumption Studies

### Effects of Elevated Oxygen Tensions on the Retina

Oxygen consumption determinations were made on teleost, amphibian and mammalian retinas. Pieces of retina were exposed to a gaseous environment of oxygen ($P_{O_2}$ 1470 mm Hg) or room air ($P_{O_2}$ 154 mm Hg) for 24 hours at 15°C. There were no apparent changes in the physical appearance of the retinas after incubation, however in some cases, the medium was grayish in color after 24 hours, probably due to a detachment of the pigmented epithelium from the retina.

Marked species differences were noted in the response of retinal tissue to hyperbaric oxygen tensions (Table 1). The rainbow trout retinas showed an increased oxygen consumption when exposed to high $P_{O_2}$'s over trout retinas exposed to room air. The results of oxygen exposure and air exposure were statistically different. There was no statistical difference in the metabolic rate of frog retinas exposed to oxygen tensions of 154 mm Hg and 1470 mm Hg. Dog retinal tissue demonstrated a significantly decreased rate of metabolism when exposed to hyperbaric oxygen at both 15 and 37°C.

## Effects of Increasing Oxygen Tensions on the Teleost Retina

Teleost retinal metabolism under different oxygen environments was tested utilizing an incubation period of 24 hours at 15°C. In this group of experiments, the oxygen consumption of three aliquots of trout retinal tissue was measured after 24 hours exposure to three oxygen tensions, viz. 154, 400 (a tension normally encountered *in vivo*) and 1470 mm Hg. The mean oxygen consumption was found to increase with increasing oxygen tensions (Table 2). It was, furthermore, observed that oxygen consumption increased in a linear fashion with increased $P_{O_2}$ as shown in Figure 3. The results of the 154 $P_{O_2}$ and 400 $P_{O_2}$ experiments were not statistically different, whereas there was a statistical difference between the 154 $P_{O_2}$ and 1470 $P_{O_2}$ experiments as

TABLE 1.--The oxygen consumption of teleost, amphibian and mammalian retinas as influenced by hyperbaric oxygen. Metabolic determinations were made after 24 hrs at 15°C and were expressed in µl of oxygen/hr/mg protein corrected to STPD.

| Species | Partial Pressures of Oxygen | | % |
|---|---|---|---|
| | 154 mm Hg | 1470 mm Hg | Change |
| Teleost (Trout) | 2.57±0.230(20) | 3.29±0.289(20)* | +28.0 |
| Amphibian (Frog) | 4.88±0.559(19) | 4.84±0.741(17) | - 0.72 |
| Mammal (Dog) | 3.56±0.426(18) | 2.40±0.152(18)* | -32.5 |

Mean ± S.E.(N)

*Significant difference in oxygen consumption at the 5% level.

TABLE 2.--The oxygen consumption of the teleost retina as influenced by oxygen tension. Metabolic determinations were made after 24 hr incubations at 15°C and were expressed in terms of µl of oxygen/hr/mg protein corrected to STPD.

| Partial Pressures of Oxygen | | |
|---|---|---|
| 154 mm Hg | 400 mm Hg | 1470 mm Hg |
| 2.81±0.252(15) | 3.18±0.223(15) | 4.78±0.450(15) |

Mean ± S.E.(N)

Results of the 154 $P_{O_2}$ and 400 $P_{O_2}$ experiments were not statistically different at the $P < 0.05$ level. Results between the 154 $P_{O_2}$ and 1470 $P_{O_2}$ experiments as well as the 400 $P_{O_2}$ and 1470 $P_{O_2}$ experiments were statistically different at the $P < 0.05$ level.

FIGURE 3.--The influence of 24 hr exposures to various oxygen tensions on retinal metabolism. Measurements were made at an oxygen tension of 154 mm Hg. See Table 1 for statistical details.

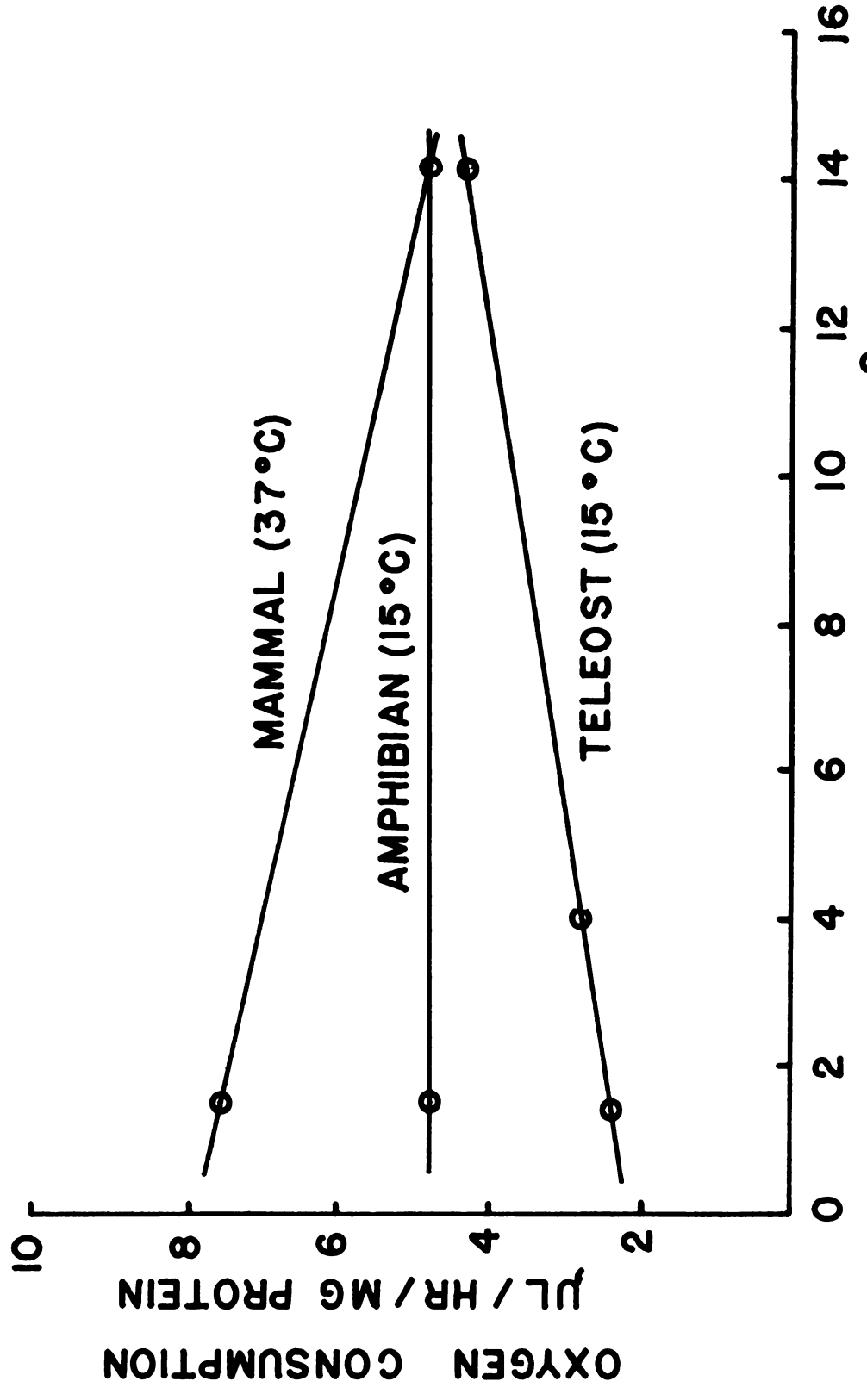
OXYGEN CONSUMPTION
μL / HR / MG PROTEIN
10
8
6
4
2
0
2
4
6
8
10
12
14
16
$P_{O_2}$ (MM HG X $10^2$)
MAMMAL (37°C)
AMPHIBIAN (15°C)
TELEOST (15°C)

FIGURE 3

well as the 400 $P_{O_2}$ and 1470 $P_{O_2}$ experiments. The response of frog and dog retinal tissue to the range of oxygen tension encountered in this experiment are also presented in Figure 3.

Effects of Elevated Oxygen Tensions on the Brain and Liver

Oxygen consumption measurements were made on the brain and liver of the teleost, amphibian and mammal. The gaseous environment consisted of oxygen ($P_{O_2}$ 1470 mm Hg) or air ($P_{O_2}$ 154 mm Hg). Both brain and liver tissue from the three species maintained their metabolic viability after the 24 hour incubations at 15°C. Samples of brain and liver tissue from the trout and the frog showed a significant decrease in oxygen consumption when exposed for 24 hrs to a $P_{O_2}$ of 1470 mm Hg at 15°C (Table 3). Dog liver also demonstrated a significant decrease in the rate of metabolism when exposed to hyperbaric oxygen, but in the case of dog brain, the decrease in the rate of metabolism following exposure to hyperbaric oxygen was not significant. Percent changes between hyperbaric oxygen and room air data are also presented in Table 3, negative values being indicative of oxygen toxicity.

Time Course of Oxygen Toxicity

The time course needed for oxygen to exert its inhibitory metabolic effects was investigated by exposing trout tissues to hyperbaric oxygen for 4 hrs and comparing the results to

TABLE 3.--The oxygen consumption of brain and liver tissue from the teleost, amphibian and mammal as influenced by hyperbaric oxygen. Metabolic determinations were made after 24 hrs at 15°C and were expressed in terms of µl of oxygen/hr/mg protein corrected to STPD.

| | Partial Pressures of Oxygen | | % |
|---|---|---|---|
| | 154 mm Hg | 1470 mm Hg | Change |
| Teleost (Trout) | | | |
| Brain | 1.99±0.295(20) | 1.43±0.145(20)* | -28.0 |
| Liver | 3.05±0.407(20) | 1.94±0.139(20)* | -37.0 |
| Amphibian (Frog) | | | |
| Brain | 5.72±0.646(15) | 3.33±0.301(16)* | -41.8 |
| Liver | 3.22±0.328(20) | 2.40±0.167(20)* | -25.6 |
| Mammal (Dog) | | | |
| Brain | 1.39±0.152(18) | 1.21±0.128(16) | -12.9 |
| Liver | 1.80±0.130(18) | 1.27±0.144(18)* | -29.4 |

Mean ± S.E.(N)

*Significant difference in oxygen consumption at the 5% level.

24 hr exposure studies. All trout tissues incubated at 15°C in hyperbaric oxygen for only 4 hours showed an increase in mean oxygen consumption over that which occurred at a $P_{O_2}$ of 154 mm Hg (Table 4). In the case of the retina the increased oxygen consumption after 24 hour hyperbaric exposures was slightly greater than the increase after 4 hrs hyperbaric exposures. It is apparent that in the brain and liver of the trout an exposure period in excess of 4 hours is necessary for elevated oxygen tensions to exert an inhibitory effect on metabolism.

## Effect of Temperature on Oxygen Toxicity

The effect of temperature on oxygen toxicity was studied only with mammalian retina, brain and liver. Tissues were incubated for 24 hours at 37°C for comparison with the results obtained from 24 hour incubation at 15°C. The gaseous environments were the same as those employed in previous oxygen consumption experiments. At 37°C antibiotics were added to the tissue preparations to prevent bacterial growth. With dog retinal tissue metabolic inhibition was virtually the same at both 15 and 37°C; hence, oxygen toxicity in the mammalian retina appears to be independent of temperature (Table 5). Dog brain tissue showed a decrease in metabolism upon hyperbaric oxygen exposure only at the higher temperature, whereas, the liver gave indications of oxygen toxicity at both temperatures, but the mean percent decrease in

TABLE 4.--The oxygen consumption of selected teleost tissues as influenced by hyperbaric oxygen and time of exposure. Metabolic determinations were made at 15°C and expressed in terms of µl of oxygen/hr/mg protein corrected to STPD.

| Tissue | Exposure (hrs) | Partial Pressures of Oxygen 154 mm Hg | 1470 mm Hg | % Change |
|---|---|---|---|---|
| Retina | 4 | 4.49±0.383(12) | 5.63±0.342(12)* | +25.5 |
| | 24 | 2.57±0.230(20) | 3.29±0.289(20)* | +28.0 |
| Brain | 4 | 3.53±0.462(12) | 4.69±0.281(12)* | +32.8 |
| | 24 | 1.99±0.295(20) | 1.43±0.145(20)* | -28.0 |
| Liver | 4 | 2.99±0.265(12) | 3.33±0.272(12) | +11.3 |
| | 24 | 3.05±0.407(20) | 1.94±0.139(20)* | -37.0 |

Mean ± S.E.(N)

*Significant difference in oxygen consumption at the 5% level.

TABLE 5.--The oxygen consumption of selected mammalian tissues as influenced by hyperbaric oxygen and temperature. Metabolic determinations were made after 24 hr incubations and were expressed in terms of µl of oxygen/hr/mg protein corrected to STPD.

| Tissue | Temp. °C | Partial Pressures of Oxygen | | % |
|---|---|---|---|---|
| | | 154 mm Hg | 1470 mm Hg | Change |
| Retina | 15 | 3.56±0.426(18) | 2.40±0.152(18)* | -32.5 |
| Retina | 37 | 7.61±1.421(16) | 4.82±0.791(16)* | -36.6 |
| Brain | 15 | 1.39±0.152(18) | 1.21±0.128(16) | -12.9 |
| Brain | 37 | 4.47±0.849(16) | 1.92±0.365(16)* | -57.0 |
| Liver | 15 | 1.80±0.130(18) | 1.27±0.144(18)* | -29.4 |
| Liver | 37 | 8.30±1.196(16) | 3.05±0.443(16)* | -63.3 |

Mean ± S.E.(N)

*Significant difference in oxygen consumption at the 5% level.

metabolism at 15°C was only one-half that which occurred at 37°C. A summary of the effects of hyperbaric oxygen tension on tissue oxygen consumption at 15°C for 24 hours of the three species studied is given in Figure 4.

## Lactate Dehydrogenase Isozymes Studies

Experiments were designed to investigate the effect of hyperbaric oxygen (1470 mm Hg) and hyperbaric nitrogen (1470 mm Hg) on the electrophoretic properties of LDH isozymes from the retina, brain and liver of the teleost, amphibian and mammal. In all cases tissue homogenates were exposed to the incubation gas for 24 hrs at 15°C prior to determination of electrophoretic mobilities.

### Effect of Oxygen on the Electrophoretic Properties of Retinal Lactate Dehydrogenase Isozymes

The teleost retina showed six bands of LDH activity after exposure to oxygen as well as nitrogen (Figure 5). Although the Rf values were the same upon exposure to either gas, the individual isozymes appeared more distinct and darker after nitrogen exposure. There was only one LDH isozyme in the amphibian retina and its physical properties, except for a slight lightening in intensity after oxygen, were not altered after exposure to either gas. The mammalian retina, after exposure to hyperbaric oxygen, showed four LDH isozymes,

FIGURE 4.--The metabolic influence of hyperbaric oxygen exposure (24 hrs, 1470 mm Hg $P_{O_2}$, 15°C) compared to 154 mm Hg oxygen exposure on retina, brain and liver. T(teleost - trout) A(amphibian - frog) M(mammal - dog). Plotted are mean ± S.E. See Tables 1 and 3 for statistical details.

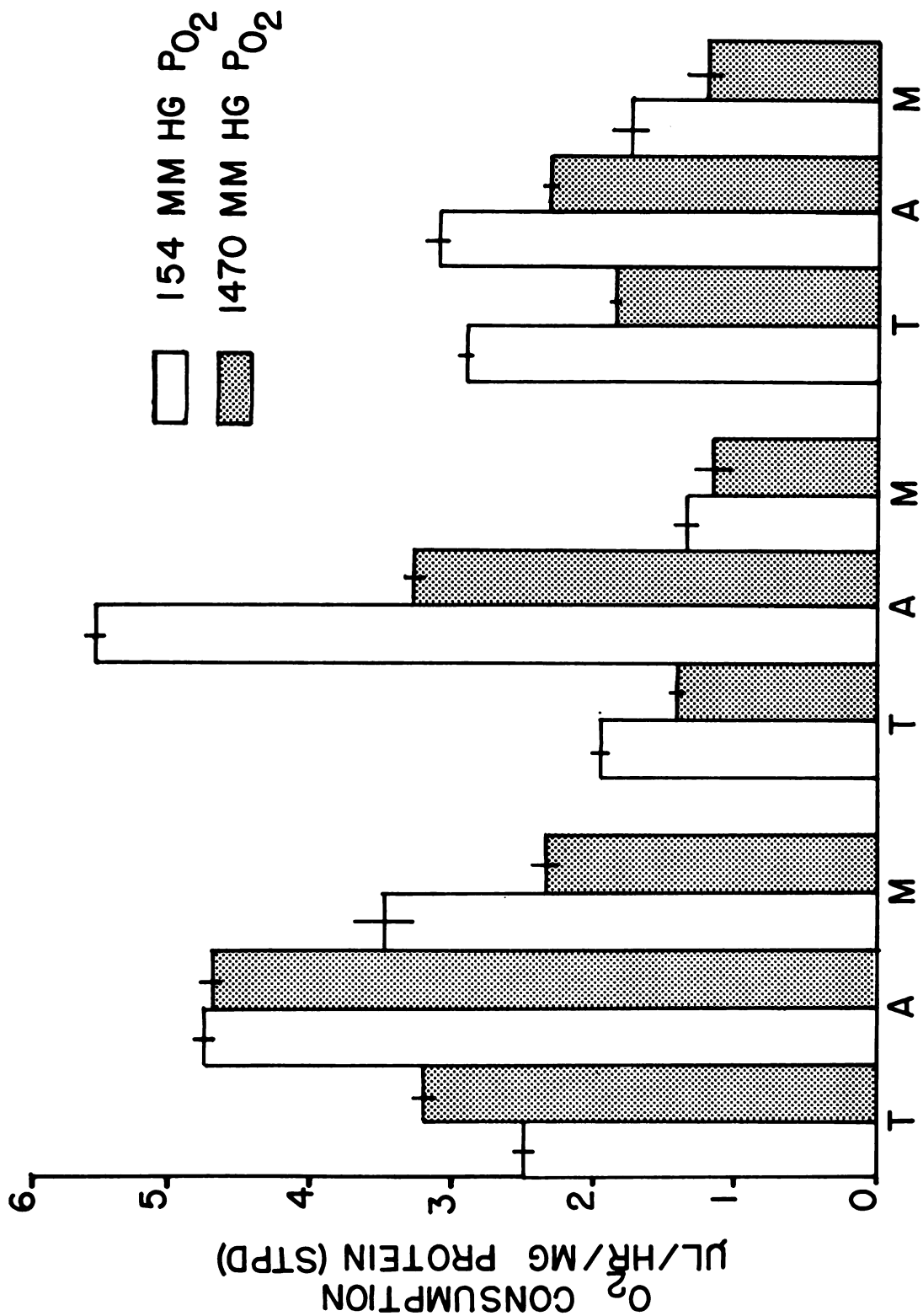
154 MM HG $P_{O_2}$
1470 MM HG $P_{O_2}$
$O_2$ CONSUMPTION
µL/HR/MG PROTEIN (STPD)
0
1
2
3
4
5
6
T
A
M
RETINA
BRAIN
LIVER

FIGURE 4

FIGURE 5.--Retinal lactate dehydrogenase isozymes from the teleost (T), amphibian (A) and mammal (M) after 24 hr exposures to hyperbaric nitrogen (N) or oxygen (O) at 15°C. The upper figures are photographs of the gel stained for lactate dehydrogenase. The lower figures are schematic sketches of the columns which have been adjusted so the distance traveled by the buffer front (lower end of tube) is the same in all cases. The lower figures are based on four or more individual observations.

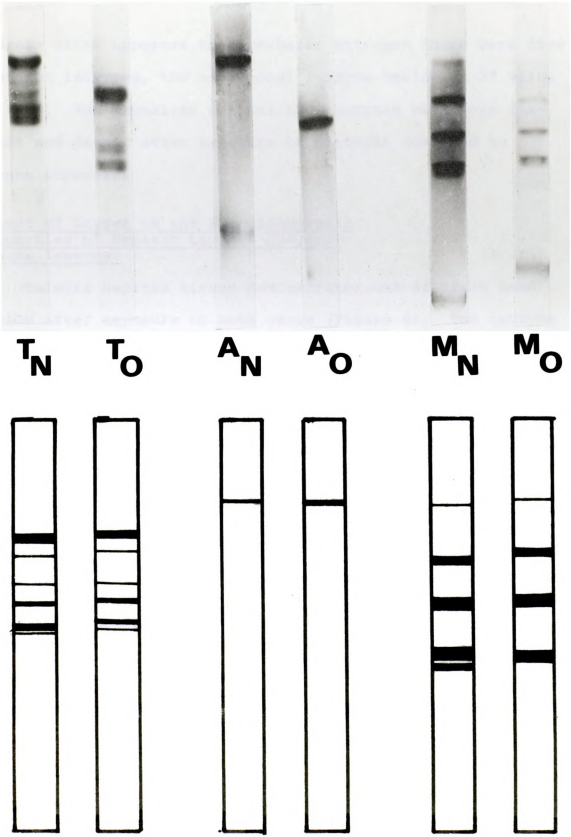


FIGURE 5

whereas after exposure to hyperbaric nitrogen there were five distinct isozymes, the additional isozyme having an Rf value of 0.60. The mammalian retinal LDH isozymes were more distinct and darker after exposure to nitrogen compared to oxygen exposure.

Effect of Oxygen on the Electrophoretic Properties of Hepatic Lactate Dehydrogenase Isozymes

Teleost hepatic tissue demonstrated one distinct band of LDH after exposure to both gases (Figure 6). The isozyme after exposure to oxygen had an Rf value of 0.40 and had a band thickness of 1 mm. After exposure to nitrogen the Rf value was 0.38 and the band had increased to 2 mm in thickness. Amphibian hepatic tissue also showed one distinct band of LDH after exposure to hyperbaric oxygen and nitrogen. The Rf value after exposure to oxygen was 0.185 and the band was 3 mm thick. After nitrogen exposure the Rf value was 0.13 and the band was 5 mm thick. Mammalian liver tissue showed four bands of LDH activity after exposure to either gas. The electrophoretic mobilities of mammalian liver tissue were identical after exposure to both gases. In all cases, liver tissue exhibited a single hemoglobin isozyme but its electrophoretic mobility was not altered by oxygen or nitrogen exposure.

FIGURE 6.--Hepatic lactate dehydrogenase isozymes from the teleost (T), amphibian (A) and mammal (M) after 24 hr exposures to hyperbaric nitrogen (N) or oxygen (O) at 15°C. The upper figures are photographs of the gel stained for lactate dehydrogenase. The lower figures are schematic sketches of the columns which have been adjusted so the distance traveled by the buffer front (lower end of tube) is the same in all cases. The lower figures are based on four or more individual observations.

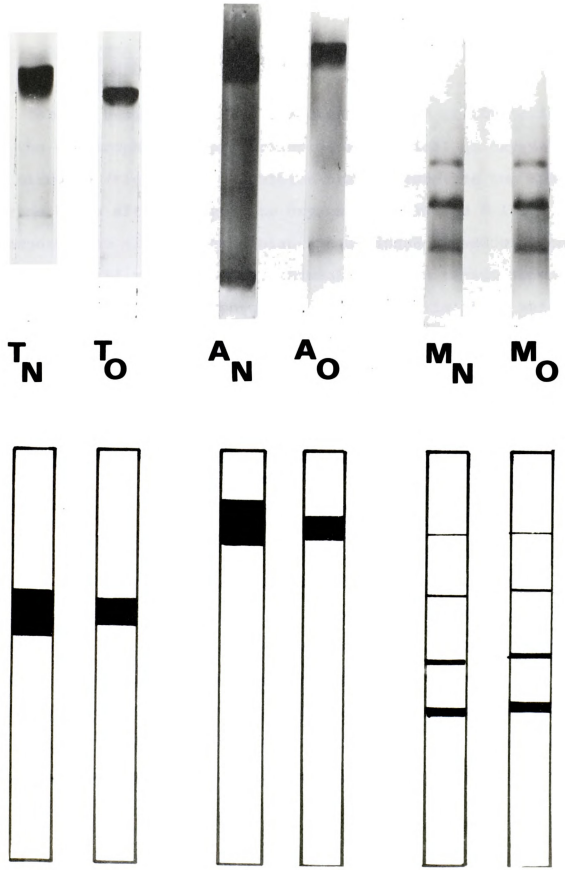


FIGURE 6

## Effect of Oxygen on the Electrophoretic Properties of Brain Lactate Dehydrogenase Isozymes

Teleost brain tissue had one distinct band of LDH activity and the electrophoretic properties were identical in oxygen and nitrogen (Figure 7). Amphibian brain tissue had two LDH isozyme bands after exposure to oxygen (Rf 0.15 and 0.19). On exposure to nitrogen amphibian brain tissue showed only one band with an Rf value of 0.20. Mammalian brain tissue showed six LDH isozymes after exposure to nitrogen while there were only five bands after exposure to oxygen. Table 6 gives Rf values after exposure to hyperbaric oxygen and hyperbaric nitrogen in the three tissues of the three species studied.

## Hyperbaric Lactate Dehydrogenase Studies

Experiments were conducted to measure LDH activity in the retina, brain and liver of the teleost, amphibian and mammal. Tissue homogenates (0.1 ml) were exposed to hyperbaric gas environments consisting of oxygen ($P_{O_2}$ 1470 mm Hg) or nitrogen ($P_{N_2}$ 1470 mm Hg) for 24 hrs at 15°C. Incubation periods of 24 hrs were employed because LDH retained its activity (a pilot study revealed no statistical difference in LDH activity between 12 and 24 hr incubations) and when exposed to oxygen there was sufficient time for enzymatic inhibition.

FIGURE 7.--Brain lactate dehydrogenase isozymes from the teleost (T), amphibian (A) and mammal (M) after 24 hr exposures to hyperbaric nitrogen (N) or oxygen (O) at 15°C. The upper figures are photographs of the gel stained for lactate dehydrogenase. The lower figures are schematic sketches of the columns which have been adjusted so the distance traveled by the buffer front (lower end of tube) is the same in all cases. The lower figures are based on four or more individual observations.

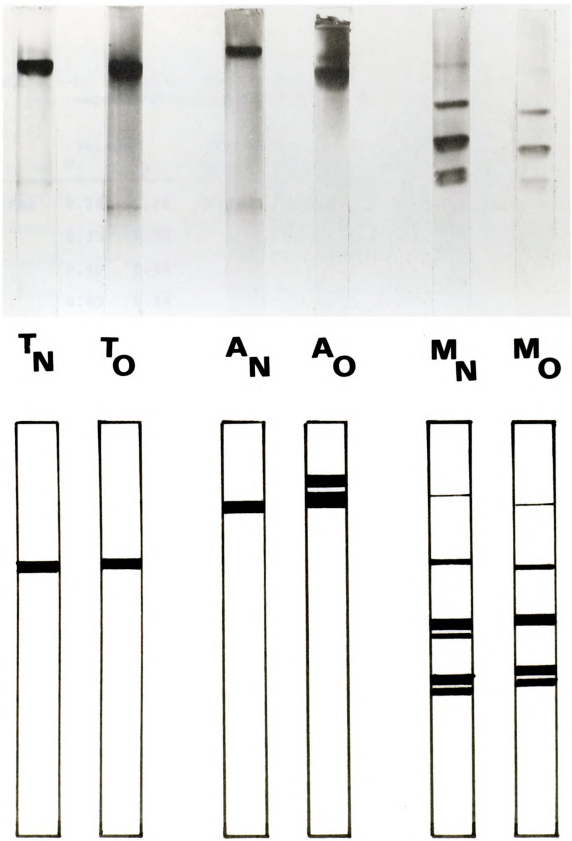


FIGURE 7

TABLE 6.--Rf values of lactate dehydrogenase isozymes separated in polyacrylamide gel columns.

| | Teleost | | Amphibian | | Mammal | |
|---|---|---|---|---|---|---|
| | $N_2$ | $O_2$ | $N_2$ | $O_2$ | $N_2$ | $O_2$ |
| Retina | 0.28 | 0.28 | 0.21 | 0.21 | 0.21 | 0.20 |
| | 0.33 | 0.32 | | | 0.34 | 0.32 |
| | 0.40 | 0.40 | | | 0.45 | 0.44 |
| | 0.45 | 0.44 | | | 0.56 | 0.58 |
| | 0.51 | 0.49 | | | 0.60 | |
| | 0.52 | 0.51 | | | | |
| Liver | 0.38 | 0.40 | 0.13 | 0.18 | 0.20 | 0.20 |
| | | | | | 0.35 | 0.34 |
| | | | | | 0.51 | 0.49 |
| | | | | | 0.62 | 0.62 |
| Brain | 0.35 | 0.34 | | 0.14 | 0.17 | 0.20 |
| | | | 0.20 | 0.19 | 0.34 | 0.36 |
| | | | | | 0.49 | 0.47 |
| | | | | | 0.51 | |
| | | | | | 0.62 | 0.60 |
| | | | | | 0.65 | 0.63 |

## Effect of Elevated Oxygen Tensions on Retinal Lactate Dehydrogenase

Species differences were apparent in the response of retinal tissue to hyperbaric oxygen tensions (Table 7). After 24 hr exposures teleost retinal LDH activity was greater in hyperbaric oxygen than hyperbaric nitrogen. The differences in LDH activity between oxygen and nitrogen exposures were significant at the 5% level. Both amphibian and mammalian retinal LDH activity decreased after hyperbaric oxygen exposures.

## Effect of Elevated Oxygen Tensions on Brain and Hepatic Lactate Dehydrogenase

Exposure of liver tissue of the three species to hyperbaric oxygen for 24 hrs resulted in a significant decrease in LDH activity (Table 8). In the case of brain tissue the teleost and mammal demonstrated a significant decrease in LDH activity after exposure to oxygen; however, the decrease in amphibian brain LDH activity was not significant (Table 9). A summary of the effects of hyperbaric oxygen and hyperbaric nitrogen on LDH activity of the three species studied is given in Figure 8.

## Normobaric Lactate Dehydrogenase Studies

Experiments were done to determine the effect of normobaric ($P_{O_2}$'s less than 760 mm Hg) oxygen exposures on teleost

TABLE 7.--Retinal lactate dehydrogenase activity as influenced by hyperbaric nitrogen and oxygen. LDH activity determinations were made after 24 hrs at 15°C and were expressed in terms of change in absorbancy/min/mg protein.

| | $P_{N_2}$ 1470 mm Hg | $P_{O_2}$ 1470 mm Hg | % Change |
|---|---|---|---|
| Teleost (Trout) | 0.165±0.005(20) | 0.174±0.003(20)* | + 5.5 |
| Amphibian (Frog) | 0.294±0.037(20) | 0.219±0.023(20)* | -25.5 |
| Mammal (Dog) | 0.127±0.007(20) | 0.087±0.007(20)* | -31.5 |

Mean ± S.E.(N)

*Significant difference in LDH activity at the 5% level.

TABLE 8.--Hepatic lactate dehydrogenase activity as influenced by hyperbaric nitrogen and oxygen. LDH activity determinations were made after 24 hrs at 15°C and were expressed in terms of change in absorbancy/min/mg protein.

| | $P_{N_2}$ 1470 mm Hg | $P_{O_2}$ 1470 mm Hg | % Change |
|---|---|---|---|
| Teleost (Trout) | 0.182±0.008(20) | 0.154±0.007(20)* | -15.4 |
| Amphibian (Frog) | 0.057±0.003(20) | 0.045±0.002(20)* | -21.1 |
| Mammal (Dog) | 0.054±0.004(20) | 0.045±0.005(20)* | -16.7 |

Mean ± S.E.(N)

*Significant difference in LDH activity at the 5% level.

TABLE 9.--Brain lactate dehydrogenase activity as influenced by hyperbaric nitrogen and oxygen. LDH activity determinations were made after 24 hrs at 15°C and were expressed in terms of change in absorbancy/min/mg protein.

| | $P_{N_2}$ 1470 mm Hg | $P_{O_2}$ 1470 mm Hg | % Change |
|---|---|---|---|
| Teleost (Trout) | 0.130±0.005(20) | 0.119±0.004(20)* | -17.7 |
| Amphibian (Frog) | 0.439±0.022(20) | 0.423±0.011(20) | - 3.6 |
| Mammal (Dog) | 0.084±0.002(20) | 0.064±0.002(20)* | -23.8 |

Mean ± S.E.(N)

*Significant difference in LDH activity at the 5% level.

FIGURE 8.--Lactate dehydrogenase activity (change in absorbancy/min/mg protein) in the retina, brain and liver as influenced by 24 hr exposure to hyperbaric nitrogen or hyperbaric oxygen at 15°C. T(teleost-trout) A(amphibian-frog) M(mammal-dog). Plotted are mean ± S.E. Refer to Tables 7, 8 and 9 for statistical details.

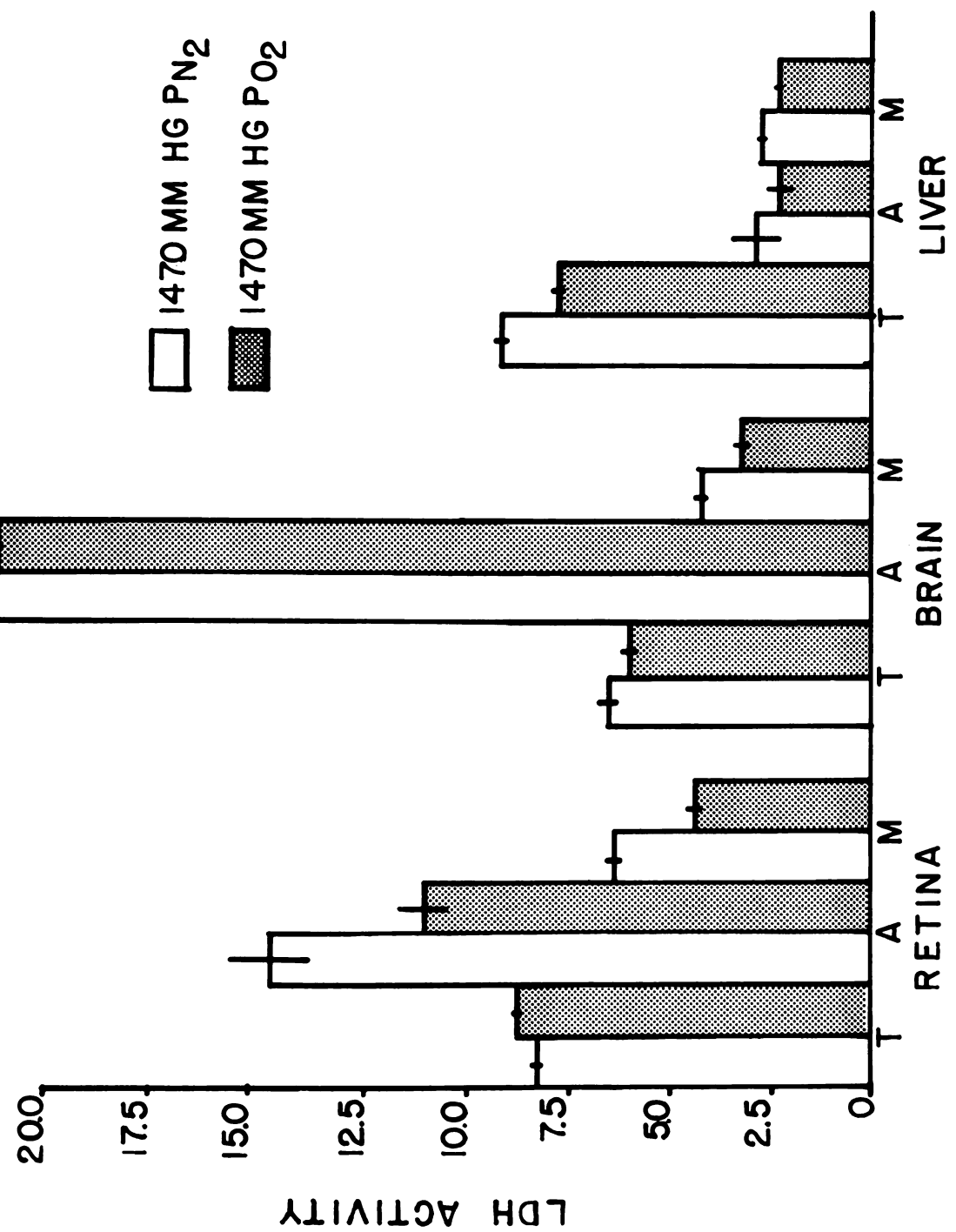
1470 MM HG $P_{N_2}$
1470 MM HG $P_{O_2}$
LDH ACTIVITY
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0
T
A
M
RETINA
BRAIN
LIVER

FIGURE 8

and amphibian retinal LDH. Lactate dehydrogenase activity was measured in the teleost retinal homogenates after an incubation period of 24 hrs at 15°C. An incubation period of 24 hrs was found to be optimal in that teleost retinal LDH retained its activity in an anaerobic environment and when exposed to oxygen allowed sufficient time for enzymatic inhibition (Table 10; Figure 9). The incubation gases employed in the normobaric studies were mixed to contain 0, 148, 296, 444, 592 or 740 mm Hg $P_{O_2}$ in nitrogen. It was observed that after 24 hr incubations at oxygen pressures less than 1 atm teleost retinal LDH activity was some 8 times higher than after 24 hr exposures to gases at 2 atm in the hyperbaric studies. Teleost retinal LDH exhibited no significant change in activity over the entire range of oxygen tensions employed in the normobaric experiment.

Effect of Acetazolamide Administration on Teleost Retinal Lactate Dehydrogenase

The acetazolamide (Diamox) treated teleost retinas exhibited higher LDH activity throughout the entire range of oxygen tensions than did the untreated teleost retinas. The results shown in Table 10 for 'a', the Y intercept, are an indication of enzyme activity in an anaerobic environment. The 'a' values were significantly different at a $P < 0.01$ between the Diamox-treated and untreated teleost retinas. In contrast to the untreated teleost retinas there was a

TABLE 10.--Retinal lactate dehydrogenase activity at 15°C as a function of $P_{O_2}$. Regression line determined by the method of least squares; Y represents enzyme activity and X represents oxygen tension (Y = a + bX).

| | a | b | $S_b$ | $r_{xy}$ | N |
|---|---|---|---|---|---|
| Teleost | 1.2480 | + $1.00 \times 10^{-5}$ | $\pm 1.530 \times 10^{-4}$ | +0.0073 | 83 |
| Amphibian | 2.1965 | $-32.04 \times 10^{-5}$* | $\pm 1.729 \times 10^{-4}$ | -0.2046* | 120 |
| Teleost (Diamox) | 2.2699 | $-28.65 \times 10^{-5}$* | $\pm 1.431 \times 10^{-4}$ | -0.2172* | 83 |

a = Y intercept (change in absorbancy/min/mg protein)

b = Slope of the regression line

$S_b$ = Standard error of the slope

$r_{xy}$ = Coefficient of correlation between X and Y

N = Number of observations

*Significantly different from zero at $P < 0.05$.

FIGURE 9.--Regression lines showing lactate dehydrogenase activity (change in absorbancy/min/mg protein) plotted against oxygen tensions in mm Hg in the Diamox-treated (0.5 mg/Kg) teleost and the untreated teleost. See Table 10 for further details.

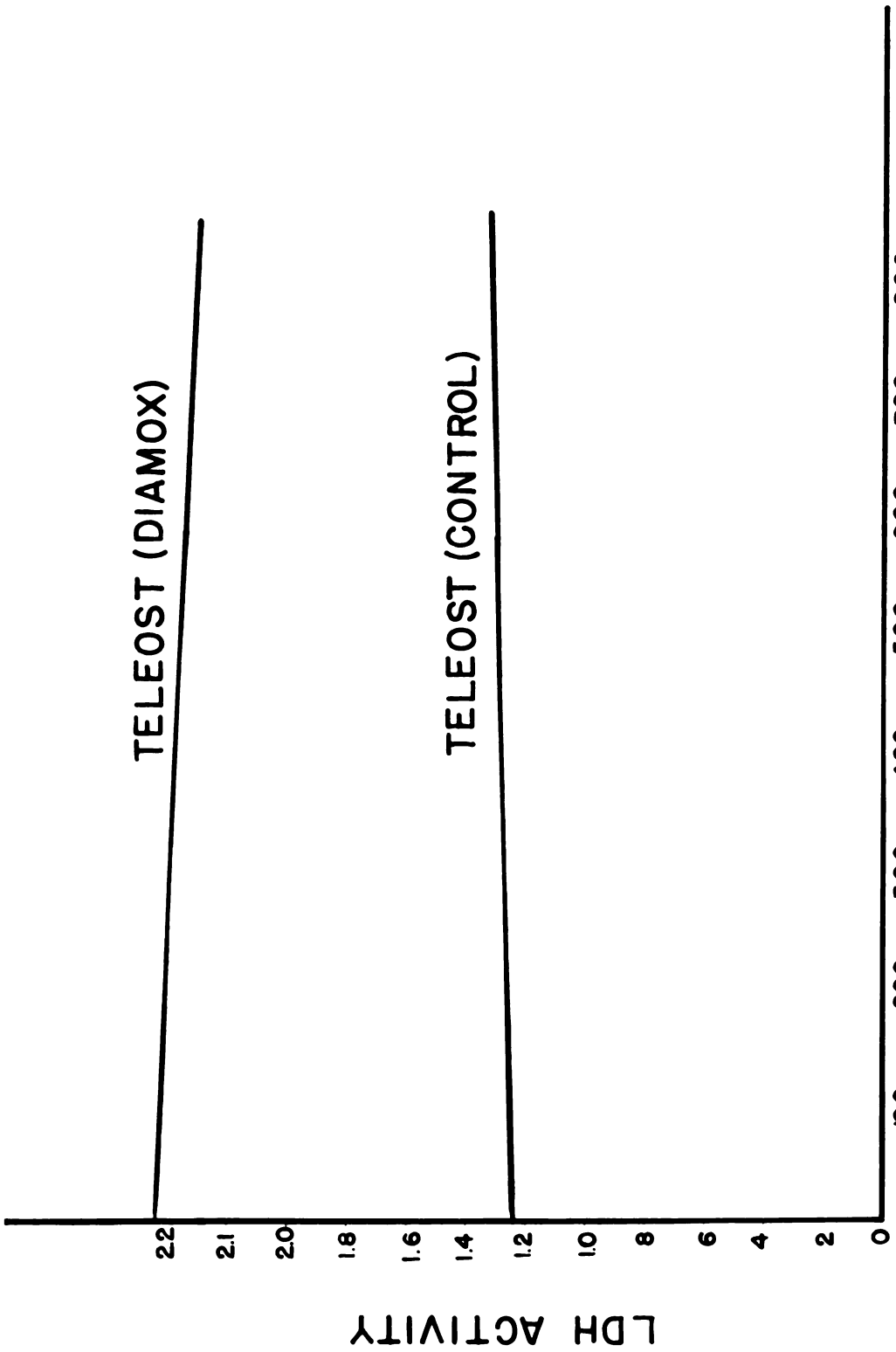
TELEOST (DIAMOX)
TELEOST (CONTROL)
LDH ACTIVITY
2.2
2.1
2.0
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
0
100
200
300
400
500
600
700
800
PO2 (MM HG)

FIGURE 9

decrease in enzyme activity in the Diamox-treated fish with increasing oxygen tensions as shown by the negative slope of the regression line (Figure 9). Retinal LDH activity following Diamox treatment was 7.8% less at 740 mm Hg $P_{O_2}$ than at 0 mm Hg $P_{O_2}$. The decrease in activity with increasing $P_{O_2}$ was significant at a $P < 0.05$.

Amphibian Retinal Lactate Dehydrogenase

As was the case with the teleost retina an incubation period of 24 hrs was found to be optimal in that amphibian retinal LDH retained its activity in an anaerobic environment and allowed oxygen sufficient time for enzymatic inhibition. Lactate dehydrogenase activity in the amphibian retina was nearly 8 times greater after normobaric oxygen exposures than after hyperbaric oxygen exposures. Similar to the Diamox-treated teleost retinas there was a progressive decrease in amphibian retinal LDH activity with increasing oxygen tensions as shown by the negative slope of the regression line (Figure 10; Table 10). Enzyme activity in the amphibian decreased 10.8% when a $P_{O_2}$ of 0 mm Hg was increased to a $P_{O_2}$ of 740 mm Hg. The decrease in LDH activity with increasing $P_{O_2}$ was significant at a $P < 0.05$.

## Lactate Dehydrogenase Kinetics

Utilizing a standard Lineweaver-Burk plot, where the reciprocal of the substrate concentration is plotted against

FIGURE 10.--Regression line showing amphibian retinal lactate dehydrogenase activity (change in absorbancy/min/mg protein) plotted against oxygen tensions in mm Hg. See Table 10 for further details.

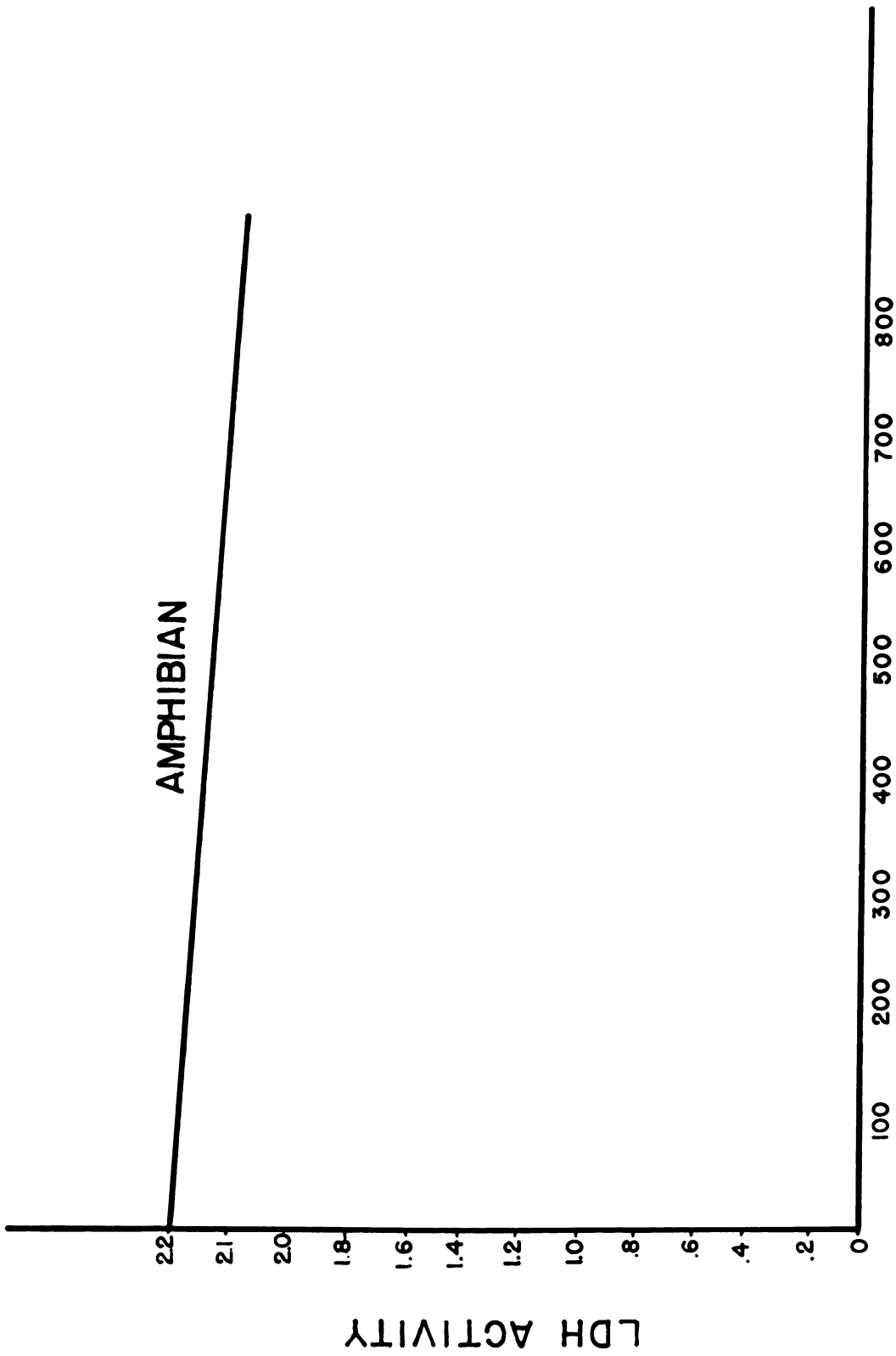
AMPHIBIAN
LDH ACTIVITY
2.2
2.1
2.0
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
0
100
200
300
400
500
600
700
800
$PO_2$ (MM HG)

FIGURE 10

the reciprocal of the reaction velocity, LDH kinetics were investigated. The point at which the regression line crosses the Y intercept is the reciprocal of the maximal velocity of the enzymatic reaction. This value (Vmax) represents the enzyme activity when the substrate concentration is maximal, i.e., the enzyme is completely saturated with substrate. At the point where the regression line crosses the abscissa the reciprocal of the Michaelis-Menten constant (Km) is found. The value Km represents the dissociation constant of the enzyme-substrate complex.

Studies were done utilizing homogenates of mammalian liver and brain to determine if LDH kinetics were altered by exposure to hyperbaric oxygen (1470 mm Hg) for 24 hrs at 15°C. Control homogenates were exposed to hyperbaric nitrogen under identical conditions. Data of these studies are summarized in Figures 11 and 12 and Table 11. Oxygen exposure in both liver and brain resulted in an increased slope indicative of a decreased activity. All slopes in Figures 11 and 12 were significantly different from zero at $P < 0.005$. Similarly oxygen treatment resulted in an increase in both Km and Vmax in liver and brain. Statistical evaluation of differences in Km values have presented problems for which the consultation services of both the Computer Center and the Department of Statistics and Probability at Michigan State University have been used. In the author's experience published data of these types are not available in the

FIGURE 11.--Lineweaver-Burk plot of lactate dehydrogenase from dog liver.

A is oxygen treated enzyme
B is nitrogen treated enzyme
V is reaction velocity (ΔA/min/mg protein)
S is substrate concentration in moles
Y-intercept is 1/Vmax
X-intercept is -(1/Km)

Regression analyses are given in Table 11.

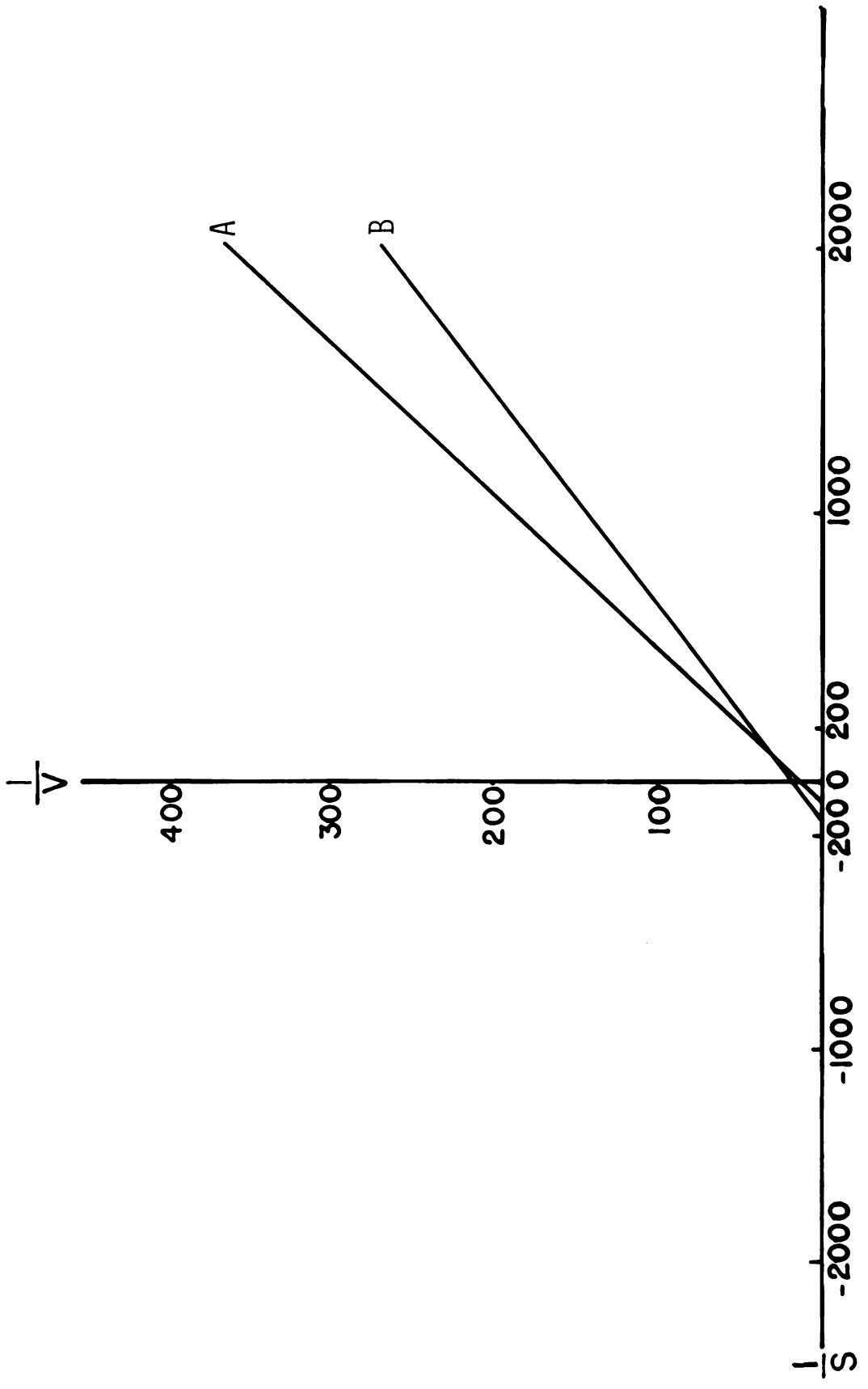


FIGURE 11

FIGURE 12.--Lineweaver-Burk plot of lactate dehydrogenase from dog brain.

A is oxygen treated enzyme
B is nitrogen treated enzyme
V is reaction velocity (ΔA/min/mg protein)
S is substrate concentration in moles
Y-intercept is 1/Vmax
X-intercept is -(1/Km)

Regression analyses are given in Table 11.

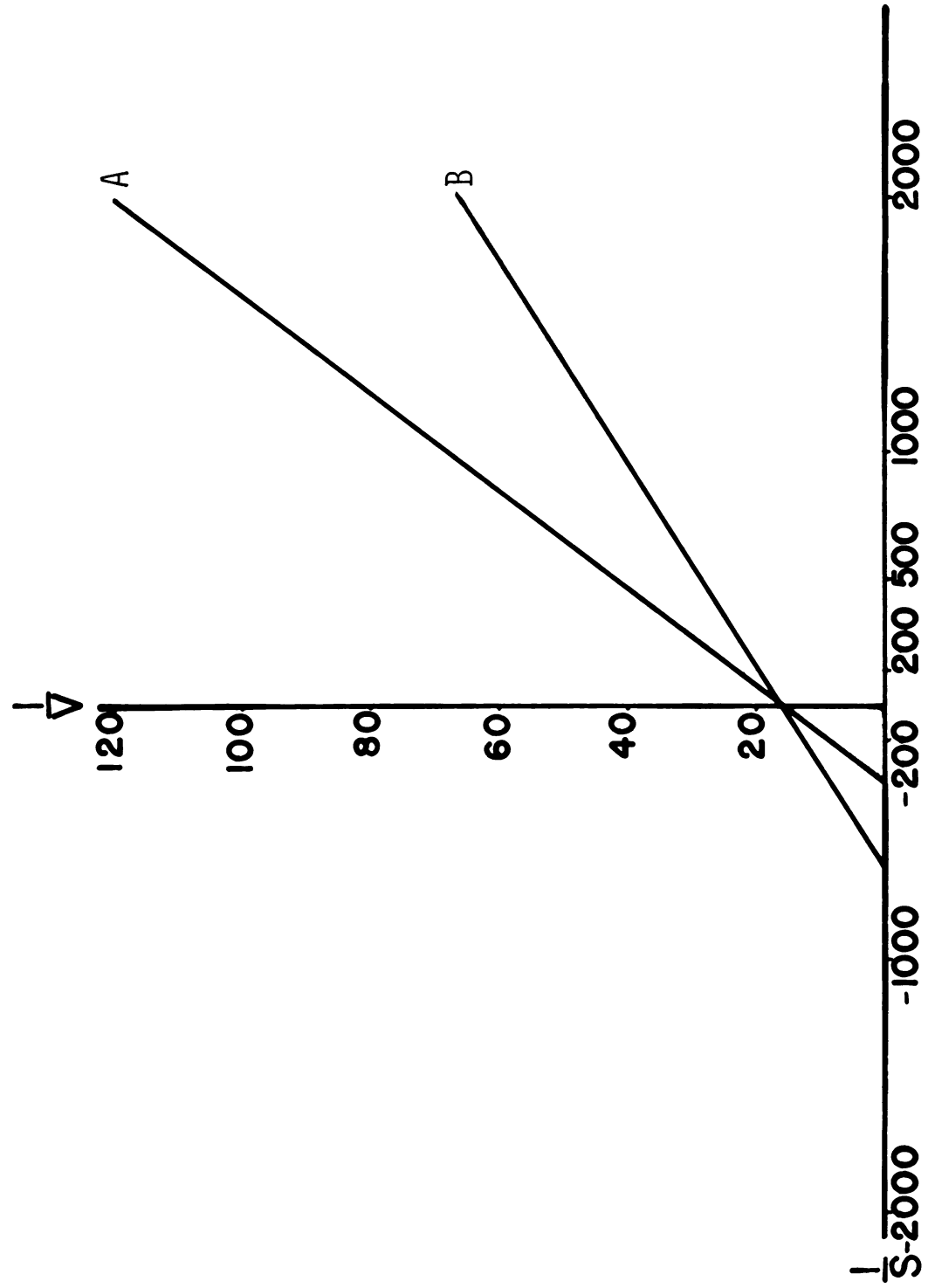


FIGURE 12

TABLE 11.--Results of Lineweaver-Burk plots based on least squares regression analysis of mammalian lactate dehydrogenase after 24 hr incubation at 15°C and 1470 mm Hg.

| Tissue | Gas Used | N | a | b | r | Km | Vmax |
|---|---|---|---|---|---|---|---|
| Liver | $O_2$ | 25 | 13.47 | 0.1728 | 0.844 + | $12.83 \times 10^{-3}$ | $74.3 \times 10^{-3}$ |
| Liver | $N_2$ | 25 | 21.92 | 0.1198 | 0.828 + | $5.47 \times 10^{-3}$ | $45.6 \times 10^{-3}$ |
| Brain | $O_2$ | 25 | 9.91 | 0.0558 | 0.960 + | $5.62 \times 10^{-3}$ | $100.9 \times 10^{-3}$ |
| Brain | $N_2$ | 25 | 14.51 | 0.0266 | 0.878 + | $1.38 \times 10^{-3}$ | $68.9 \times 10^{-3}$ |

a = Y intercept (change in absorbancy/min/mg protein)

b = Slope of regression line

r = Coefficient of correlation

N = Number of observations

literature. Personal communication with Dr. James Stapleton, Chairman of the Department of Statistics and Probability, has supplied statistical solutions to the Michaelis-Menten kinetic data which are currently being prepared for publication by him.

# DISCUSSION

All mammalian species and nearly all vertebrates possess an extensively developed intraretinal vascularization. Only fresh water and marine teleosts possess an avascular retina. Retinas in general are characterized by having a high oxygen demand; therefore, it would seem that the lack of retinal vascularization in the teleost might lead to difficulties in oxygen supply to this tissue. Wittenberg and Wittenberg (1962) have demonstrated that marine teleosts can concentrate oxygen behind the retina to partial pressures above environmental water oxygen tensions, and that the magnitude of this ability is correlated with the anatomical development of the choroidal rete mirabile.

Using a micro oxygen polarographic electrode it was demonstrated that oxygen tensions of 400 mm Hg occurred behind the retina of the rainbow trout, a fresh water teleost, these $P_{O_2}$'s were some 20 times higher than those associated with arterial blood ($P_{O_2}$ 25 mm Hg) and 3.5 times those of the environmental water, i.e., 150 mm Hg (Fairbanks *et al.*, 1969). It was surmised that the high oxygen tensions in the vicinity of the trout retina were evidence for an oxygen concentrating mechanism in the eye. In no other class of

vertebrates have oxygen tensions exceeding arterial $P_{O_2}$'s been observed in the region of the retina.

Acetazolamide (Diamox) is a highly specific inhibitor of carbonic anhydrase (Brodie, 1965). In a critical experiment it was observed that after ip administration of 0.05 mg/kg Diamox to rainbow trout the ocular oxygen tension behind the retina and in the vitreous body was reduced to a value not significantly different from arterial blood oxygen tensions (25 mm Hg) and remained depressed for over 24 hrs (Fairbanks et al., 1969). It was concluded that carbonic anhydrase was, therefore, essential for the generation of high oxygen tensions.

## Oxygen Consumption Studies

In the retina, as in other neural tissue, the oxidation of glucose to $CO_2$ and water provides the primary source of metabolic energy. Thus a decrease in oxygen consumption would be the result of inactivation of one or more critical enzymes of carbohydrate metabolism. In the case of the teleost retina exposure to oxygen tensions of 700 mm Hg caused a decrease in lactic acid production after 3 hrs (Baeyens, Hoffert and Fromm, 1971). In the present study the substantial increase in oxygen consumption of the teleost retina following exposure to hyperbaric oxygen tensions (Table 1) indicates that enzymes of the Embden-Meyerhof-Parnas

(EMP) pathway and TCA cycle are refractory to the toxic actions of oxygen. The oxygen-induced decrease in lactic acid production by the teleost retina noted by Baeyens et al. (1971) may result from a channeling of EMP intermediates into an accelerated TCA cycle.

It is clear that the metabolism of the teleost retina is limited by the availability of oxygen (Figure 3). The oxygen consumption of the teleost retina was not maximal at the normally encountered in vivo oxygen tension of 400 mm Hg, but increased in a linear fashion with increasing oxygen tensions up to 1470 mm Hg. These data demonstrate conclusively that the high in vivo oxygen tensions do not exert an inhibitory effect on teleost retinal oxidative metabolism and, in fact, may actually be necessary for a level of metabolism commensurate with normal visual function.

In the amphibian retina, exposure to hyperbaric oxygen tensions caused no decrease in activity of any rate limiting enzyme associated with the oxidation of glucose. In contrast to the teleost retina, the oxygen consumption of the amphibian retina was not increased by exposure to elevated oxygen tensions indicating that the supply of oxygen as a substrate is not rate limiting. In general $Q_{10}$ values associated with thermochemical (enzymatic) reactions range from 2 to 3. If one assumes a $Q_{10}$ of 2.5 for the enzymes of the TCA cycle in the amphibian retina, which were not affected by 24 hr exposure to hyperbaric oxygen, it is clear

that the amphibian retina normally operates at a metabolic rate in excess of either the teleost retina or the mammalian retina. It is possible that high oxygen tensions caused some inhibition of various enzymes involved in the carbohydrate metabolism of the amphibian retina. The amount of inhibition of these enzymes, however, could not have been of sufficient magnitude to result in a decrease in the total energy production.

The mammalian retina clearly demonstrated the toxic effects of hyperbaric oxygen. There was a 32.5% decrease in oxygen consumption of mammalian retinas after exposure to a $P_{O_2}$ of 1470 mm Hg for 24 hrs at 15°C compared to control retinas exposed to 154 mm Hg $P_{O_2}$ under the same conditions. If one makes the assumption that aerobic glycolysis remains the same or is decreased, the deleterious effects of hyperbaric oxygen must be mediated through the inhibition of one or more enzymes involved in oxidative carbohydrate metabolism.

Liver and brain tissue were inhibited by hyperbaric oxygen in the teleost, amphibian and mammal after 24 hr exposures. That oxygen exerts a toxic effect on brain tissue in the teleost indicates that not all teleost neural tissue is equally sensitive to oxygen and that the teleost retina is indeed unique in this respect. Using a manometric technique Elliott and Libet (1942) found that respiration of cat brain slices fell 55% after 3 hrs exposure to 1 atm of pure oxygen at 37°C. The results of the present study indicate

that the oxidative metabolism of the teleost brain actually increased after exposure to oxygen tensions at 2 atm for 4 hrs. Thus, for short-time exposures it can be concluded that the teleost brain appears to be more refractory to the toxic action of oxygen than is the cat brain. A decreased sensitivity of the teleost brain to oxygen toxicity was still apparent after 24 hrs exposure. The response (decrease in mean oxygen consumption) of the dog brain (37°C) and the frog brain (15°C) was nearly twice that of the teleost brain (15°C) after 24 hrs exposure to hyperbaric oxygen. Even though teleost brain tissue may show a relatively low sensitivity to hyperbaric oxygen, teleost retinal tissue appears to be unique in its capability of responding to elevated oxygen by increasing its metabolic activity.

After a two week exposure of white rats to 100% oxygen at 1/3 atm the activity of the hexose monophosphate shunt in the liver was almost doubled (Gorman et al., 1971). The mechanism responsible for the increase in pentosephosphate activity was postulated to be an oxygen mediated inactivation of one or more of the enzymes of the TCA cycle. Our results show that the oxygen consumption of the liver in all three species was inhibited after 24 hrs exposure to hyperbaric oxygen (Table 3). These data confirm the results that the TCA cycle of the liver is inhibited by elevated tensions of oxygen not only in homeotherms but also in poikilotherms.

Popovic, Gerschman and Gilbert (1964) exposed normal and hibernating ground squirrels to pure oxygen at 6 atm and found that survival times were much longer in the hibernating animals. They concluded that the decreased rate of metabolism which resulted from the lowering of body temperature provided the protection against oxygen toxicity. In only one study has the effect of temperature on oxygen toxicity in poikilotherms been investigated. It was found that in the sand dollar, an echinoderm, oxygen toxicity was alleviated as the temperature of the organisms was lowered (Rosenbaum and Wittner, 1960). Our results indicate that, in general, the higher the metabolic rate of a tissue the greater its susceptibility to oxygen toxicity. Furthermore, the susceptibility of a given tissue to the toxic effects of oxygen is also lowered when the metabolism of that tissue is suppressed by decreasing temperature.

## Lactate Dehydrogenase

### Lactate Dehydrogenase Isozymes

Isozymes are enzymes which exist in multiple forms, all performing the same functions generally at different rates. Isozymes differ somewhat in chemical composition so that they are separable electrophoretically. Lactate dehydrogenase is found in several electrophoretically distinct fractions. Each of the electrophoretic species of lactate

dehydrogenase is a tetramer consisting of two polypeptide chain units, H and M, present in different proportions. The lactate dehydrogenase isozymes differ in many properties: catalytic activity, amino acid composition, sensitivity to heat and immunological responses (Giese, 1968).

Different proportions of the various isozymes are produced in cells of differentiating tissues during development of an organism. Isozymes of lactate dehydrogenase with a high content of H are most active at low pyruvate concentrations and are inhibited by high concentrations of pyruvate. Such isozymes are found in cardiac muscle. The properties of the isozyme high in H would have a tendency to channel lactic acid into the aerobic reactions of the TCA cycle rather than to the formation of lactic acid, a regulation of adaptive significance to cardiac muscle. Lactate dehydrogenases with a high M content remain active at high pyruvate concentrations and therefore channel the EMP pathway toward formation of lactic acid. These lactate dehydrogenase isozymes are found in skeletal muscle cells. They also represent regulations of adaptive value because skeletal muscle cells undergo periods of intense activity during which they go into oxygen debt and at such times lactic acid formation permits rapid release of energy (Cantarow and Schepartz, 1967).

Isozymes may form in response to stresses upon cells. Fish exposed to low temperature acclimate in such a manner

that the respiratory rate increases at the low temperature over the initial rate at that temperature. Isozymes have been identified in the muscle cells of such fish and it has also been noticed that the energy of activation for some of the reactions involved in metabolic activity is decreased. The change in respiratory rate is the result of the development of isozymes with decreased energies of activation (Hochachka, 1967).

Enzyme activity is not easily quantitated by observing the staining reactions of the component isozymes. Electrophoresis, however, becomes important for detecting gross changes in activity which will be reflected through a change in band width or a change in the staining reaction. Furthermore, only isozymes that have catalytic activity can be identified by electrophoretic techniques.

In the present experiments exposure to cell homogenates to oxygen might result in the following changes: 1) Synthesis of a new isozyme. The probability of this occurring is highly unlikely since the homogenates employed in these experiments represent a highly inefficient system for the synthesis of protein. 2) There is the possibility of one ixozyme combining with another isozyme resulting in the formation of a new isozyme. This could only happen in tissues in which there are two or more isozymes present. 3) Treatment with oxygen may result in dimerization between sulfhydryl groups from different molecules causing changes in molecular

weight. Changes in molecular weight would result in changes in the molecular sieving properties with concomitant changes in electrophoretic migratory patterns. 4) The most likely possibility is that oxygen exposure will cause a change in electrical charge with a concomitant change in tertiary structure. A change in electrical charge would result in a new isoelectric point which would be reflected through a change in Rf value. A change of this type may or may not affect the active site on the enzyme. 5) Finally, it is possible that the active site can be affected by oxygen treatment without a concurrent change in the isoelectric point of the isozyme.

Hyperbaric Lactate Dehydrogenase - Activity and Isozyme Studies

Measurements of lactate dehydrogenase activity allowed an index for the quantitation of glycolysis occurring in the tissues. In the case of the teleost retina, LDH activity increased after exposure to hyperbaric oxygen compared to controls subjected to hyperbaric nitrogen (Table 7 and Figure 8). Upon examination of the teleost retinal LDH isozymes it is apparent that oxygen had no effect on the electrophoretic mobilities. Since there are no apparent differences in the electrophoretic properties of the isozymes, the increase in teleost retinal LDH activity after oxygen exposure can not be explained by a changed isoelectric point or a change in secondary or tertiary structure. There was only a slight

increase in teleost retinal LDH activity (5.5%) and a change this small would be difficult to detect through the semi-quantitative electrophoretic technique. Treatment of the amphibian retina with oxygen resulted in a significant reduction in LDH activity but the electrophoretic mobility of the single isozyme appeared to be unaffected. The only observable change in physical properties was a lightening of the band after oxygen exposure which is indicative of decreased enzyme activity. The mammalian retina also demonstrated a significant decrease in LDH activity after oxygen treatment. Upon examination of mammalian retinal isozymes, after oxygen exposure, one can see a reduction in activity of each isozyme, with one isozyme completely disappearing (Rf = 0.60). Thus it appears that oxygen exerts its deleterious effect on mammalian retinal LDH at the active site of the molecule, resulting in complete inactivation of one isozyme.

Teleost and amphibian hepatic LDH activities were significantly decreased after oxygen exposure (Table 8). The decreased LDH activities were reflected in the isozyme patterns where oxygen exposure resulted in a decreased staining reaction and a decreased band width. The decreased mammalian hepatic LDH activity after oxygen treatment could not be detected through a change in physical properties of the isozymes.

In the case of the teleost brain, LDH activity decreased after oxygen exposure (Table 9) but there were no changes in

the electrophoretic properties of the single isozyme. The amphibian brain was the only tissue studied in which the LDH activity was insensitive to hyperbaric oxygen. After oxygen exposure there was the appearance of a new isozyme band (0.14). The possibility exists that the new band represents the synthesis of a new isozyme. A more plausible explanation for the appearance of the new band, however, is that exposure to oxygen resulted in dimerization by disulfide bridge formation between sulfhydryl groups from different molecules causing molecular weight changes. A change in molecular weight could result in an altered electrophoretic migratory pattern caused by a sieving effect of the separating gels. The decreased LDH activity of mammalian brain tissue after oxygen exposure was reflected through a decreased staining reaction of all the isozymes.

From the isozyme studies it can be concluded that oxygen exerts its effect on enzyme activity in different ways. One cannot predict the susceptibility of a tissue to oxygen toxicity on the basis of isozyme distributions. Even though similar isozymes were found in the various tissues, there was no particular isozyme that exemplified oxygen inactivation in all tissues. In general, oxygen inactivation of LDH occurs equally in all isozymes from a given tissue. Oxygen inactivation in most cases did not result in dimerization or gross changes in tertiary structure but most likely reflected a

subtle change at the active site or an associated allosteric site which is not sufficient to change the electrophoretic mobility. Only in the case of the amphibian brain was a new molecular species formed upon exposure to oxygen.

Lactate Dehydrogenase Kinetics

Evidence presented has shown that LDH exposed to high oxygen tensions has resulted in a decreased catalytic activity. The isozymes studied, however, have failed to elucidate a conclusive explanation for this decreased activity. Consequently, a preliminary study was undertaken utilizing an entirely new approach to the study of the influence of oxygen on enzyme activity. Enzyme homogenates of tissues showing pronounced oxygen inhibition were chosen (dog liver and brain) and the Michaelis-Menten kinetics of these enzymes were examined through the use of Lineweaver-Burk plots.

In both liver and brain LDH treatment with oxygen resulted in an increased Km (Figures 11 and 12). The value Km represents the dissociation constant for the following reaction:

$$E + S \rightleftharpoons ES$$

where E = enzyme

S = substrate

ES = enzyme-substrate complex

An increase in Km, which was observed after oxygen exposure, reflects a shift in the reaction to the left. One of the

apparent modes, therefore, by which oxygen influences the kinetic properties of LDH is to decrease the affinity of the enzyme for substrate and hence decrease the rate of product formation.

It is clear from Table 11 that Vmax is slightly greater in the case of the oxygen-treated enzyme. The value Vmax represents the maximum rate of product formation by an enzyme when that enzyme is completely saturated with substrate. Since this condition can never exist because of substrate inhibition, Vmax is only a hypothetical reaction velocity. Values of Vmax represent the kinetic activity of the enzyme when enzyme-substrate complex formation is not a rate limiting factor, because the substrate would have to be present in infinitely high concentrations. It is apparent from the fact that Vmax is slightly greater following oxygen treatment that the ability of enzyme to form product is not impaired with and in fact may be slightly enhanced following oxygen treatment.

## Normobaric Lactate Dehydrogenase and Acetazolamide Studies

The inhibition of glycolysis by high oxygen tensions is known as the Pasteur effect and this phenomenon has long been associated with the mammalian retina (Cohen and Noell, 1959). Aerobic glycolysis was confirmed in the teleost retina in spite of associated high oxygen tensions (Baeyens *et al.*, 1971). It was further observed that more glucose was

utilized (41.5%) and lactic acid produced (33%) under an anaerobic environment than an aerobic environment, thus verifying the Pasteur effect in the teleost retina. The Pasteur effect, however, was more prominent in the mammalian retina (Cohen and Noell, 1959) than in the teleost retina. In the case of the teleost retina elevated oxygen tensions had less effect on lactic acid production indicating that teleost retinal LDH is more refractory to elevated oxygen tensions than is mammalian retinal LDH. The insensitivity of teleost retinal LDH to oxygen at tensions less than 1 atm is confirmed in Figure 9.

Assuming that in general LDH is inhibited by molecular oxygen as a result of the formation of disulfide bonds and noting from Table 10 that teleost LDH activity is independent of oxygen tension allows one to conclude that teleost retinal LDH is protected in part from the elevated oxygen tension occurring *in vivo*. The amphibian retina, like the mammalian retina, does not have oxygen tensions that exceed those of arterial blood and one would not expect amphibian retinal LDH to be resistant to high oxygen tensions as was apparently the case for teleost retinal LDH. The significance of aerobic glycolysis to the vertebrate retina is in some doubt except in the case of the teleost retina where the lactic acid produced by aerobic glycolysis is an essential component of the countercurrent multiplier for the generation of high oxygen tensions (Fairbanks *et al.*, 1969).

In the present experiment Diamox administration to trout resulted in complete blindness within 24 hrs due to the loss of the high oxygen tension at the retina. It is assumed that inhibition of carbonic anhydrase by the administration of Diamox has no direct effect on LDH activity, other than that mediated by the concurrent reduction in the teleost ocular oxygen tensions. After chronic Diamox administration (1) LDH activity of the retina increased over control values and (2) there was an inhibition of LDH activity with increasing oxygen tensions. The increased LDH activity in the teleost retina after Diamox may indicate that the resulting retinal hypoxia stimulates the synthesis of new LDH. Secondly, during the prolonged hypoxic state (two weeks) the sulfhydryl groups may be slowly reduced thereby increasing the enzyme activity. This increased activity (55%) is represented in Table 10 by 'a' which is the predicted value for the Y intercept representing the *in vitro* enzyme activity at an oxygen tension of zero.

If hypoxia, after Diamox administration, results in the synthesis of new teleost retinal LDH the newly formed molecules behave differently in the presence of oxygen in that enzyme activity decreases with increasing oxygen tensions. The newly formed enzyme might be partially made up of an isozyme which is selectively sensitive to oxygen. Another possible explanation is the reoxidation of the previously reduced

sulfhydryl groups on exposure to molecular oxygen resulting in decreased enzyme activity.

## Proposed Mechanisms for the Protection of Teleost Retinal Enzymes from Oxygen Toxicity

That teleost retinal LDH does not decrease in activity with increasing oxygen tension seems to indicate that there is some mechanism preventing the oxidation of the enzyme. *In vivo* and *in vitro* various sulfhydryl compounds have a moderating effect on oxygen toxicity. One such compound, reduced glutathione, provides significant protection against oxygen toxicity in mice subjected to hyperbaric oxygenation (Gerschman, Gilbert and Caccamise, 1958). The delay that exists at all tensions of oxygen before symptoms of toxicity appear indicates that oxidation-reduction buffering systems exist in the cell. If this is true, glutathione is an important substance to consider for a role in keeping the oxidation-reduction potential constant. It may be oxidized by molecular oxygen, but the oxidized form can be rapidly reduced by enzymes in the cell.

There are two possible outside sources of reduced substances which may protect the teleost retina against the toxic effects of oxygen *in vivo*. The first source consists of reduced compounds coming from the lens. Glutathione as well as ascorbic acid are both present in relatively high

concentrations in the normal lens (Lerman, 1964). Furthermore, only a small fraction of the total glutathione is present in the oxidized form (about 3%) the remainder being in the reduced form. It is possible that reduced glutathione and ascorbic acid could diffuse through the vitreous body to the retina and thereby protect the teleost retina against the toxic effect of oxygen in vivo. A second and more plausible source of reduced compounds may come from the retina by the vascular system. This is especially important in the case of the teleost retina where the possibility exists for a countercurrent diffusion of reduced substances. A schematic representation of the situation as it exists in the teleost retina is given in Figure 13. The situation existing in the amphibian and mammalian retina has also been presented in Figure 13 for comparison. In the choroid of the teleost retina there are capillaries lying in close juxtaposition (choriocapillaris). The possibility exists that the reduced by-products of teleost retinal metabolism diffuse out of the retina into the choriocapillaris. In the choriocapillaris reduced compounds can pass from the venous side to the arterial side by a process of countercurrent diffusion. This would allow for a continual maintenance of high concentrations of the reduced substances in the vicinity of the teleost retina. The mammalian and amphibian retinas have a double circulation. They receive a direct source of blood

FIGURE 13.--Retinal vascularization as related to countercurrent diffusion in various vertebrates. Diagram on the left represents the teleost. Diagram on the right represents the amphibian or mammal. The vitreous body (V), retina (R) and choroid (C) are shown for both retinas. The vasculature consists of the ophthalmic artery (OA), ophthalmic vein (OV), choriocapillaris (CC), retinal artery (RA) and retinal vein (RV). Arrows represent the movement of reduced material (R-SH) with a scheme for concentration at the choriocapillaris in the teleost.

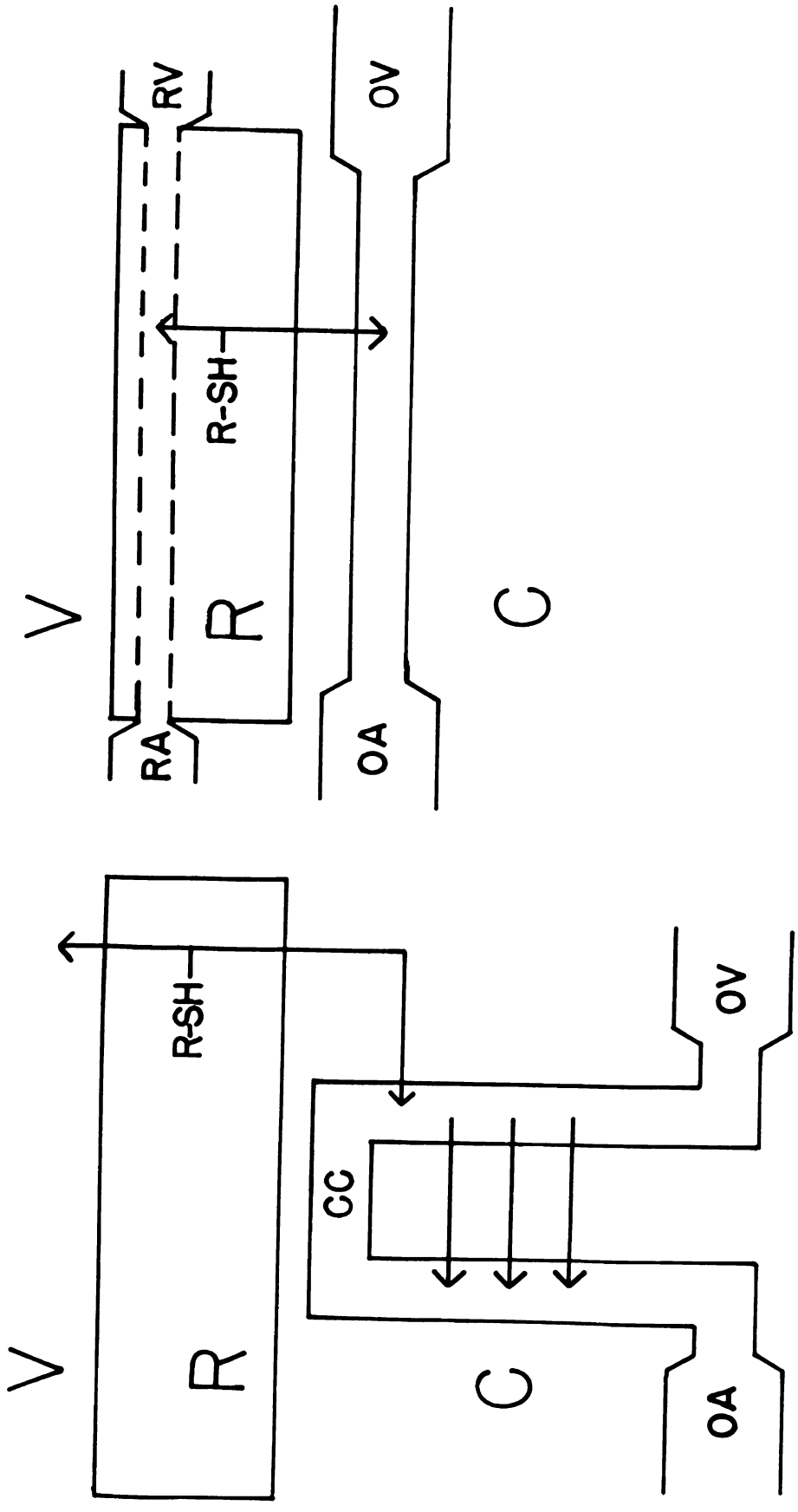


FIGURE 13

through the ophthalmic artery into the choroidal circulation. Thus reduced substances from the retina are likely to be "washed out" into either the retinal capillaries or the choroidal capillaries. Since the choroidal capillaries of the mammalian and amphibian retina do not lie in juxtaposition the possibility of a countercurrent diffusion of reduced substances does not exist.

The idea of a countercurrent diffusion of reduced substances in the teleost retina fits in well with the data obtained from the Diamox studies. After Diamox administration teleost retinal LDH activity nearly doubled indicating an increase in the reduced form of the enzyme. With oxygen treatment, however, there was a decrease in LDH activity with increasing oxygen tension. This fact points to the conclusion that the choriocapillaris, by the mechanism of countercurrent diffusion of reduced substances, is necessary to maintain the retina in a reduced and viable state.

## SUMMARY AND CONCLUSIONS

1. Teleost retinas showed an increased oxygen consumption after 4 (25.5%) and 24 hrs (28%) exposures to hyperbaric oxygen compared to room air exposures. This is interpreted as indicating that the availability of oxygen is rate limiting and that oxygen does not inhibit any essential respiratory enzymes in this tissue.

2. Exposure of amphibian retinas to high oxygen tensions resulted in no change in oxygen consumption which is consistent with the hypothesis that the rate of oxidative retinal metabolism is not limited by availability of oxygen or the inhibition of any associated enzymes.

3. Mammalian retinas exposed to similar elevated oxygen tensions demonstrated marked decreases in oxidative metabolism mediated through inhibition of one or more essential enzymes of carbohydrate metabolism.

4. In the three species studied both hepatic and brain tissue demonstrated marked deleterious metabolic effects upon exposure to elevated oxygen tension for 24 hrs. Therefore, of all the tissues in the various species studied the teleost retina is unique in its response to high oxygen tensions.

5. In general, it was noted that the higher the metabolic rate of the tissue the more susceptible it is to oxygen toxicity. When the temperature is decreased there is a lowered susceptibility to oxygen toxicity mediated through the decreased metabolism.

6. The oxidative metabolism of the teleost retina is limited by the availability of oxygen. The oxygen consumption of the teleost retina was not maximal at the normally encountered *in vivo* oxygen tension of 400 mm Hg but continued to increase in a linear fashion with increasing oxygen tensions up to 1470 mm Hg.

7. Exposure periods in excess of 4 hrs are necessary for high oxygen tensions to exert an inhibitory effect on the oxidative metabolism of liver and brain in the teleost.

8. The influence of oxygen on lactate dehydrogenase structure was investigated through electrophoretic techniques. In general, most cases of decreased enzyme activity were not associated with changes in tertiary structure, intermolecular disulfide bridge formation or isoelectric points. These observations are based on the inability of oxygen to change the electrophoretic mobilities.

9. Teleost retinal LDH does not decrease in activity with increasing oxygen tensions, indicating that there is some protective agent or mechanism preventing the inactivation of the enzyme. After hypoxia induced by Diamox, teleost

retinal LDH increased in activity and demonstrated oxygen toxicity. The phenomena are explained through the synthesis of new LDH made up of an isozyme sensitive to oxygen, or the reoxidation of previously reduced sulfhydryl groups on exposure to molecular oxygen.

10. All other tissues studied showed some inhibition of LDH activity upon treatment with oxygen. It is hypothesized that the decreased LDH activity is caused by the formation of a disulfide configuration at the active and/or allosteric site of the enzyme.

11. Mammalian hepatic and brain tissue demonstrated altered LDH kinetics after oxygen treatment. The slope of the Lineweaver-Burk plots was increased indicative of decreased enzyme activity. There was also an increased Km value signifying a decreased affinity of the enzyme for substrate resulting in a decreased rate of product formation after oxygen exposure.

12. Hypotheses are presented for the protection of the teleost retina against the toxic effects of oxygen in vivo. A mechanism is proposed for the countercurrent diffusional accumulation of reduced substances in the vicinity of the teleost retina.

## RECOMMENDATIONS FOR FURTHER STUDY

1. To quantitatively measure the free sulfhydryl groups in enzymes before and after exposure to oxygen. A measurement of this type would be paramount to the elucidation of the cellular mechanisms of oxygen toxicity.
2. Further analysis of the Michaelis-Menten kinetics of sulfhydryl-containing enzymes through the use of Lineweaver-Burk plots may lead to valuable information concerning the influence of oxygen at the enzymatic level.
3. A measurement of the oxidation-reduction potential of the interstitual fluid around the teleost retina may be valuable in leading to an explanation of the protection afforded the teleost retina against the toxic effects of oxygen and the role of the countercurrent diffusion system on the generation of a reduced environment.

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# APPENDICES

# APPENDIX A

## Incubation Solutions

Composition of Phosphate Buffered Saline (PBS)*

| | |
|---|---|
| NaCl | 8.00 g/liter |
| KCl | 0.20 g/liter |
| $Na_2HOP_4$ (Anhydrous) | 1.15 g/liter |
| $KH_2PO_4H_2O$ | 0.20 g/liter |
| $CaCl_2$ | 0.10 g/liter |
| $MgCl_2.6H_2O$ | 0.19 g/liter |
| Glucose | 1.00 g/liter |

*(Grand Island Biological Co., Grand Island, N. Y.)

Composition of Modified
Mammalian Krebs Saline Medium

| | |
|---|---|
| NaCl | 6.9 g/liter |
| KCl | 0.345 g/liter |
| $CaCl_2$ | 0.282 g/liter |
| $NaHCO_3$ | 2.94 g/liter |
| $MgSO_4.7H_2O$ | 0.294 g/liter |
| $KH_2PO_4$ | 0.162 g/liter |
| pH | 7.60 |
| Osmolarity | 289.25 mOsm/kg |

## Composition of Modified Medium 199 (Earle's Base) in mg/L*

| | | | |
|---|---|---|---|
| NaCl | 6800.0 | $Na_2$ α tocopherol $PO_4$ | 0.010 |
| KCl | 400.0 | p-Aminobenzoic acid | 0.050 |
| $MgSO_4.7H_2O$ | 200.0 | L-Cystine | 20.0 |
| $Na_2HPO_4.2H_2O$ | -- | L-Tyrosine | 40.0 |
| $NaH_2PO_4.H_2O$ | 125.0 | 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 | 0.010 | | |
| Pyridoxine HCl | 0.0250 | | |
| Riboflavin | 0.010 | | |
| Pyridoxal HCl | 0.0250 | | |
| Niacin | 0.0250 | | |
| Niacinamide | 0.0250 | | |
| Ca Pantothenate | 0.010 | | |
| i-Inositol | 0.050 | | |
| Ascorbic acid | 0.050 | | |
| Folic acid | 0.010 | | |
| Ferric nitrate.$9H_2O$ | 0.10 | | |
| Biotin | 0.010 | | |
| Menadione | 0.010 | | |
| Glutathione | 0.050 | | |
| Vitamin A | 0.10 | | |
| Calciferol | 0.10 | | |

*(Grand Island Biological Co., Grand Island, N. Y.)
**Trademark of Atlas Powder Co.

## Composition of Puck's Medium given in mg/L*

| | |
|---|---|
| NaCl | 7400.0 |
| KCl | 285.0 |
| $Na_2HPO_4.7H_2O$ | 290.0 |
| $MgSO_4.7H_2O$ | 154.0 |
| $CaCl_2.2H_2O$ | 16.0 |
| $KH_2PO_4$ | 83.0 |
| Glucose | 1100.0 |
| L-Arginine HCl | 38.0 |
| L-Histidine HCl | 38.0 |
| L-Lysine HCl | 80.0 |
| L-Tryptophan | 20.0 |
| β-Phenyl-L-alanine | 25.0 |
| L-Methionine | 25.0 |
| L-Threonine | 38.0 |
| L-Leucine | 25.0 |
| DL-Isoleucine | 25.0 |
| DL-Valine | 50.0 |
| L-Glutamic acid | 75.0 |
| L-Aspartic acid | 30.0 |
| L-Proline | 25.0 |
| Glycine | 100.0 |
| L-Glutamine | 200.0 |
| L-Tyrosine | 40.0 |
| L-Cystine | 8.0 |
| Hypoxanthine | 25.0 |
| Thiamine HCl | 5.0 |
| Riboflavin | 0.50 |
| Pyridoxine HCl | 0.50 |
| Folic acid | 0.10 |
| Biotin | 0.10 |
| Choline | 3.0 |
| Ca pantothenate | 3.0 |
| Niacinamide | 3.0 |
| i-Inositol | 1.0 |
| Phenol red | 5.0 |
| $NaHCO_3$ | 1200.0 |

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## Antibiotics

500,000 units Nystatin-Fungacide
500,000 units Penicillin
100,000 units Streptomycin Sulfate
add to 100 ml of medium

Composition of Minimum Essential Medium (Eagle) given in mg/L*

| | |
|---|---|
| NaCl | 6800.0 |
| KCl | 400.0 |
| $NaH_2PO_4.H_2O$ | 140.0 |
| $MgSO_4.7H_2O$ | 200.0 |
| $CaCl_2$ (anhyd.) | 200.0 |
| Glucose | 1000.0 |
| L-Arginine | 105.0 |
| L-Cystine | 24.0 |
| L-Histidine | 31.0 |
| L-Valine | 46.0 |
| Choline Cl | 1.0 |
| Folic acid | 1.0 |
| i-Inositol | 2.0 |
| Nicotinamide | 1.0 |
| D-Ca pantothenate | 1.0 |
| L-Glutamine | 292.0 |
| L-Isoleucine | 52.50 |
| L-Leucine | 52.40 |
| L-Lysine | 58.0 |
| L-Methionine | 15.0 |
| L-Phenylalanine | 32.0 |
| L-Threonine | 48.0 |
| L-Tryptophan | 10.0 |
| L-Tyrosine | 36.0 |
| Pyridoxal HCl | 1.0 |
| Riboflavin | 0.10 |
| Thiamine HCl | 1.0 |
| Phenol red | 10.0 |
| $NaHCO_3$ | 2200.0 |

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Phosphate Buffer (0.034 M)

| | |
|---|---|
| $Na_2HPO_4$ | 3.81 g |
| $NaH_2PO_4.H_2O$ | 0.905 g |
| Distilled $H_2O$ to make 1 liter | |
| pH (adjusted with NaOH or HCl) | 7.40 |

# APPENDIX B

## Lowry Method for Protein 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 | 3,000.0 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 (Central Scientific Co.) | 1 part |
| 2. Distilled $H_2O$ | 1 part |

E. Protein Standard 8.0 g% (Dade Reagents Inc., Miami, Fla. Lot No. PRS-406)

1. Dilute with 100 ml distilled $H_2O$ to give 800 µg/ml

Concentrations of protein standards used for determination of standard curve: 0, 20, 40, 60, 80 and 160 µg/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 ½hr at room temperature (20-22°C), mix occasionally.

5. Read at 660 mμ.

# APPENDIX C

## Electrophoresis

Stock Solutions (Stored at 4°C - warmed to room temperature before use)

| | |
|---|---|
| A. 1N HCl | 48 ml |
| 2-Amino-2-Hydroxymethyl 1-3-Propandiol (Tris) | 36.3 g |
| N,N,N',N' Tetramethylethylene-diamine (temed) | 0.23 ml |
| $H_2O$ to make (pH 8.8-9.0) | 100 ml |
| B. 1N HCl | 48 ml |
| Tris | 5.98 g |
| Temed | 0.46 ml |
| $H_2O$ to make (pH 6.6-6.8) | 100 ml |
| C. Acrylamide | 28.0 g |
| N,N'-Methylene bis acrylamide (Bis) | 0.735 g |
| $H_2O$ to make | 100 ml |
| D. Acrylamide | 10.0 g. |
| Bis | 2.5 g |
| $H_2O$ to make | 100 ml |
| E. Riboflavin 4.0 mg per 100 ml $H_2O$ | |
| F. Sucrose | 40.0 g |
| $H_2O$ to make | 100 ml |
| G. Ammonium persulfate (unstable reagent - make fresh daily) | 0.14 g |
| $H_2O$ to make | 100 ml |

Buffer

| | |
|---|---|
| Tris | 3.0 g |
| Glycine | 14.4 g |
| $H_2O$ to make | 100 ml |

Tracking Dye

Bromphenol Blue solution 0.005 g per 100 ml $H_2O$

Working Solutions

Separating Gel Solution - Lower Gel

1 part A
2 parts C
1 part $H_2O$
4 parts G
(pH 8.8-9.0)

Stacking Gel Solution

1 part B
2 parts D
1 part E
4 parts F or distilled $H_2O$
(pH 6.6-6.8)

Tube Preparation

The separating gel solution was prepared immediately prior to use and 1.0 ml put in each glass tube. A layer of water was then gently placed on top of the gel solution. Polymerization took 40 minutes at room temperature. After polymerization the water was removed and the stacking gel, 0.2 ml, put in each tube and then water layered. Polymerization took 20 minutes under fluorescent light.

The sample gel was prepared by mixing the supernatant from the tissue homogenate with the stacking gel in an approximately 1:5 ratio. 0.1 ml to 0.2 ml of this mixture was then added to the tubes. Fluorescent light was again needed for polymerization. In many cases, due to the dilution and protein content of the sample, the gel failed to polymerize properly. This was not a problem in separating the LDH isozymes.

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