THE IMPORTANCE OF THE ENZYME CARBONIC ANHYDRASE IN OCULAR OXYGEN CONCENTRATION IN THE RAINBOW TROUT (SALMO GAIRDNERI)

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
MICHAEL B. FAIRBANKS
1970

LIBRARY
Michigan State
University

This is to certify that the

thesis entitled

THE IMPORTANCE OF THE ENZYME CARBONIC ANHYDRASE IN OCULAR OXYGEN CONCENTRATION IN THE RAINBOW TROUT (Salmo gairdneri)

presented by

Michael Bruce Fairbanks

has been accepted towards fulfillment of the requirements for

Ph.D. degree in Physiology

Jack R Hoffert
Major professor

Date September 15, 1970

O-169

BINDING BY
HOAG & SONS'
BOOK BINDERY INC.
LIBRARY BINDERS
SPRINGPORT, MICHIGAN

ABSTRACT

THE IMPORTANCE OF THE ENZYME CARBONIC ANHYDRASE IN OCULAR OXYGEN CONCENTRATION IN THE RAINBOW TROUT (SALMO GAIRDNERI)

By

Michael B. Fairbanks

Oxygen is concentrated by the choroidal rete in the eye of the rainbow trout (Salmo gairdneri) to levels in excess of arterial blood and environmental water oxygen tension (P_{O_2}) through countercurrent diffusion multiplication of a single concentrating effect for O_2. This mechanism is completely inhibited by the administration of 50 mg/kg of acetazolamide (Diamox), a carbonic anhydrase inhibitor. Since this dose level of acetazolamide inhibits carbonic anhydrase in all tissues containing the enzyme, an uncertainty exists as to the relative importance of pseudobranch, choroidal rete, red blood cell and retinal carbonic anhydrase in ocular O_2 concentration.

To clarify this point selective inhibition of carbonic anhydrase in these four tissues was achieved using CL-11,366. The

effects of this inhibitor on ocular O_2 concentration were monitored using a micro oxygen polarographic electrode while verification of carbonic anhydrase inhibition was achieved utilizing an electrometric assay technique. The O_2 electrode was used in determining (1) the O_2 profile in the eye, (2) the rapidity of O_2 depletion (or reconcentration) in the region of the retina after blocking (or reestablishing) the arterial blood supply to the choroidal rete and (3) the measurement of O_2 profiles and maximum ocular P_{O_2} in fish chronically treated with carbonic anhydrase inhibitors or subjected to bilateral pseudobranchectomy. Histochemical localization of carbonic anhydrase in the choroidal rete by the CoS technique was attempted; and electrophoretic studies of carbonic anhydrase isozymes from the pseudobranch, choroidal rete, red blood cells and retina were made.

O_2 profile measurements in the eye show a very steep P_{O_2} gradient from the region of the retina, 446 ± 47.7 mm Hg, to the anterior chamber of the eye, 20 ± 4.4 mm Hg. The P_{O_2} in the vitreous is also low, indicating that either the retina consumes O_2 as rapidly as it is concentrated or the ocular humors act as diffusion barriers to O_2.

Chronic treatment of fish with CL-11,366 (0.6 mg/kg/day) results in a change in the O_2 profile, with higher P_{O_2} values being

found in the aqueous and vitreous humors. These results, coupled with indications of histopathology and an apparent decreased viscosity of the ocular humors, suggest a lowered O_2 consumption by the retina or a breakdown in the O_2 diffusion barrier presented by the ocular humors. Fish chronically treated with acetazolamide (50 mg/kg) or bilaterally pseudobranchectomized have a significantly lower ($p < 0.001$) maximum ocular P_{O_2}, 62 ± 5.7 mm Hg and 14 ± 4.0 mm Hg, respectively, when compared to the controls, 476 ± 35.7 mm Hg. Extensive retinal histopathology was noted in both groups of fish.

Ligation of the afferent pseudobranch artery, which blocks the arterial blood supply to the choroidal rete, results in a rapid depletion of O_2 from the region of the retina; the P_{O_2} dropped to half of the average maximum value, 430 ± 82.3 mm Hg (6) within 71 sec. After removing the ligature O_2 was reconcentrated to within 5 per cent of the initial value within 20 min.

Administration, intraperitoneally, of CL-11,366 at a dose of 2.5 mg/kg resulted in an even more rapid depletion of O_2 than did ligation of the afferent pseudobranch artery. This was attributed to a rapid arrest of the ocular O_2 concentrating mechanism through inhibition of choroidal rete carbonic anhydrase, and not a change in circulation time.

Michael B. Fairbanks

A hypothesis is given for the role of retinal, choroidal rete and red blood cell carbonic anhydrase in the ocular O_2 concentrating mechanism. Electrophoretic, histological and carbonic anhydrase inhibitor studies suggest that the pseudobranch may be the source of choroidal rete carbonic anhydrase and that this enzyme appears to be located on the surface of the endothelial cells in the rete capillaries.

THE IMPORTANCE OF THE ENZYME CARBONIC ANHYDRASE IN OCULAR OXYGEN CONCENTRATION IN THE RAINBOW TROUT (SALMO GAIRDNERI)

By

Michael B. Fairbanks

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Physiology

1970

667210

DEDICATION

To my mother, whose selfless dedication to her children is an inspiration, and to my wife, for her patience and understanding throughout my college education.

ACKNOWLEDGMENTS

The author wishes to express his thanks and appreciation to Dr. J. R. Hoffert for his guidance and support throughout this study and for his aid in the preparation of this dissertation. Special recognition to Dr. Hoffert is also given for the photographic work.

It is also a pleasure to acknowledge the assistance of Mrs. Esther Brenke in the histological and electrophoretic work described in this dissertation. Mrs. Brenke and my wife Geri are to be thanked for their patience in the typing of the rough drafts of this thesis.

I would also like to express my gratitude to the members of my committee, Drs. Frantz, Fromm, Heisey and Reineke, for their critical review of the manuscript and their suggestions for its improvement.

The author is also indebted to the National Institutes of Health and the National Heart and Lung Institute for the support of this work through Grant EY-00009 VIS and the Predoctoral Traineeship HE 05873-02, respectively.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

FIGURE Page

INTRODUCTION

Oxygen concentration in the eyes of teleosts is a physiological phenomenon that has enjoyed little publicity even though it is unique in the animal kingdom. Wittenberg and Wittenberg (1962) were the first to observe an ocular O_2 concentrating ability among marine teleosts, and seven years later (Fairbanks, Hoffert and Fromm, 1969) these observations were advanced to include the fresh water teleost, Salmo gairdneri. The latter authors showed that this concentrating ability was not only dependent on a functional choroidal gland (a rete mirabile situated within the choroid layer of the eye, immediately behind the retina) but also on the enzyme carbonic anhydrase (CA). The fact that this enzyme is specific for the catalysis of either CO_2 hydration or H_2CO_3 dehydration implies that its function in the O_2 concentrating mechanism of the eye is through regulation of these reversible reactions between CO_2 and H_2O. Fairbanks et al. (1969) hypothesized that the role of CA in ocular O_2 concentration was to insure that the single concentrating effect (an essential feature of the countercurrent diffusion multiplication theory for O_2 concentration) would take place at the proper time.

They reasoned that red blood cell CA was most essential, but retinal, choroidal and pseudobranch CA may also play vital roles. The main goal of this research was to determine the validity of the above hypothesis through selective inhibition of CA and to attempt to define the roles of the other sources of CA possibly involved in O_2 concentration. It was hoped that any clarification of the action of CA on build-up of ocular P_{O_2} would also shed light on the function of this enzyme in the O_2 concentrating mechanism of the swim bladder.

LITERATURE REVIEW

The generation of superatmospheric oxygen tensions (i.e., oxygen tensions above 150 mm Hg at one atmosphere and 21% oxygen) occurs in two different organs and for two very different purposes in the teleost. The first, and the most widely studied, involves the regulation or maintenance of neutral buoyancy through the "secretion" of gaseous oxygen (and other gases) into the lumen of the swim bladder of fishes (Denton, 1961; Fänge, 1966); and the second involves the supply of dissolved O_2 to the very metabolically active but avascular teleost retina by generating a high P_{O_2} within the choroid layer of the eye (Wittenberg and Wittenberg, 1962; Fairbanks et al., 1969). In spite of this wide disparity of purpose, the mechanism of oxygen concentration in the two systems is believed to be quite similar.

Both systems have a rete mirabile. Scholander (1954) has elegantly described this structure in the swim bladder, while Barnett (1951) has studied it in the choroidal gland of the eye. The artery supplying the rete of both the eye and swim bladder divides into many branches which in turn split up into an enormous number of arterial

capillaries. These capillaries run parallel to one another the length of the rete before reuniting to form several main branches which exit the rete and go on to form a capillary bed, (the choriocapillaris network of the choroid proper in the eye) or the capillary bed in the gas gland of the swim bladder. These capillaries unite into a few main vessels and return the blood to the venous side of the rete, whereupon they then divide into an enormous number of parallel vessels. These venous capillaries run the length of the rete, intercalated with the arterial capillaries, before reuniting to form several main branches and finally a single vein. Blood flow in the arterial capillaries is countercurrent to the flow in the parallel venous capillaries. It was this anatomical arrangement of vessels in the swim bladder which led to the development of the theory for O_2 concentration involving countercurrent diffusion multiplication of a single concentrating effect (Kuhn et al., 1963). Functionally, the two mechanisms are similar in that both are inhibited by CA inhibitors, as shown by Fänge (1953) in the case of the swim bladder and by Fairbanks et al. (1969) in the case of the eye.

Carbonic Anhydrase and Countercurrent Diffusion Multiplication of Oxygen

Countercurrent diffusion multiplication of O_2 as originally described by Kuhn et al. (1963) begins with the delivery of arterial

blood to the capillary bed interposed between the arterial and venous capillaries of the rete. This capillary bed is adjacent to very metabolically active tissue (the gas gland of the swim bladder and the retina of the eye), and CO_2 is added to the blood. Upon entering the red blood cell the CO_2 will effect a P_{O_2} increase in the blood returning to the venous side of the rete, through the Bohr shift (a shift to the right in the O_2 dissociation curve) and/or the Root shift (a decrease in the HbO_2 binding capacity). The consequence of this single concentrating effect is that the blood in the venous side of the rete will now have a greater P_{O_2} than that in the arterial side, creating a gradient for the diffusion of O_2 from the venous to arterial capillaries. This O_2-enriched blood returns to the capillary bed connecting the two sides of the rete, and the sequence of events is repeated. The continuation of this cycle leads to the concentration of O_2 in the swim bladder and in the eye, and the magnitude of this concentrating ability is in part determined by the surface area available for O_2 diffusion across the rete capillaries. Krogh (1929) found that the swim bladder retia of the eel (Anguilla vulgaris) contain 88,000 venous and 116,000 arterial capillaries with an aggregate length of 352 and 464 meters, respectively. The capillary surface area was 106 cm^2 for the venous side and 105 cm^2 for the arterial side, even though the total volume of the retia was only 0.064 cm^3.

The ratio of total diffusion area to rete volume was some 1,700 cm^2/cm^3, compared to a ratio of about 100 cm^2/cm^3 for the alveolar diffusion area and volume of the human lung (Steen, 1963). The distance between the two blood streams in the rete of the longnosed eel (Synaphobranchus pinnatus) averages 1.5 μ, a distance approximately equal to the distance from the alveolar air space to the capillary lumen of the mammalian lung (Scholander, 1954). Scholander has also noted that each arterial capillary is surrounded by four venous capillaries, resulting in a checkerboard arrangement of capillaries which Scholander suggests ". . . is the geometrical arrangement which gives the maximal exchange between the afferent and efferent capillaries. . . ." In this unique pattern the entire surface area of each afferent (arterial) capillary in the rete is available for diffusion exchange with the efferent (venous) capillaries. Even in the presence of this unique anatomical specialization for the facilitation of gas exchange by diffusion, efficient concentration of O_2 in the eye and swim bladder would be difficult if the single concentrating effect did not occur at the correct place. For maximal efficiency of countercurrent diffusion multiplication the single concentrating effect should occur as the recently acidified blood traverses the capillary bed between the two sides of the rete. If this is not the case, and the P_{O_2} increase occurs at some point after the blood has

entered the venous side, the surface area and time available for diffusion of O_2 will be reduced.

This line of reasoning prompted Fairbanks et al. (1969) to hypothesize that the inhibitory action of acetazolamide on the O_2 concentrating mechanism was "en raison de" its inhibition of the single concentrating effect through inhibition of the Bohr or Root shift. Berg and Steen (1968) have indicated that acetazolamide increases the time of the Root-off shift of eel blood at 20°C from a normal half-time of 0.05 sec to at least 30 sec. The Bohr shift should be similarly affected, since both reactions are a result of increased formation of hydrogen ions in red blood cells, which is blocked by inhibition of RBC carbonic anhydrase. By slowing down the formation of H^+ from CO_2 hydration and subsequent dissociation of H_2CO_3, the single concentrating effect is rendered ineffective by CA inhibitors; and consequently the oxygen concentrating mechanism is inhibited. This hypothesis ignores the possibility that pseudobranch CA may function in concentrating O_2 in the eye. The pseudobranch contains a very high concentration of this enzyme in relation to other tissues of the teleost (Maetz, 1953; Hoffert, 1966), and the only arterial blood supply to the choroidal gland is by way of the efferent pseudobranch artery. Copeland (1951) found that bilateral pseudobranchectomy results in the apparent inability of the killfish

(Fundulus heteroclitus) to refill its swim bladder with gases. Fänge (1953) observed that generalized CA inhibition destroys a fish's ability to refill its swim bladder with gases; thus it may be inferred that pseudobranch CA may play an active role in the concentration of O_2 in the swim bladder and, if so, it would be quite natural to assume a similar role in the eye. However, it must be mentioned that Maetz (1956) found that pseudobranchectomy had no effect on the swim bladder refilling mechanism of the perch (Perca fluviatilis).

In addition to RBC and pseudobranch CA, gas gland CA and rete CA are present in the swim bladder, as well as retinal CA and choroidal gland CA in the eye (Maetz, 1956). Unfortunately Maetz' work on CA and O_2 concentration in the swim bladder has been overlooked by most researchers of swim bladder physiology. Even though the two studies were independent of one another, the role of CA in the generation of high oxygen tensions in the swim bladder as described by Maetz (see below) is very similar to that suggested by Fairbanks et al. (1969) for CA in the eye.

Ball, Strittmatter and Cooper (1955) have shown that the swim bladder gas gland, like the mammalian retina, does not show the Pasteur effect but continues to produce lactic acid in the presence of 70 - 90% O_2 at one atmosphere. Recently D'Aoust (1970) has shown an absence of any Pasteur effect even in the presence of 51 atm

of O_2 and he found very little production of CO_2 by the gas gland, which indicates that citric acid cycle enzymes and oxidative phosphorylation are very weak in this tissue. Apparently, then, the metabolic acid responsible for the single concentrating effect in the swim bladder is lactic acid, but how does this slowly diffusable acid move from its intercellular location in the gas gland into the RBC's circulating through the gas gland capillary bed?

Maetz (see Figure 1) has suggested that gas gland CA facilitates movement of acid by catalyzing the formation of rapidly diffusable CO_2 from lactic acid and blood HCO_3^-. The CO_2 may then diffuse into erythrocytes to effect O_2 dissociation through the Bohr or Root shift. However, this increased plasma CO_2 tension could also result in diffusion of CO_2 from the venous to the arterial side of the rete, which in effect would result in a short circuiting caused by a premature release of O_2 from Hb in the arterial side of the rete. The O_2 would then diffuse to the venous side and be carried away instead of being concentrated. To counteract this possibility Maetz has suggested that in the endothelial cells of the rete an "HCl secretory unit" incorporating rete CA secretes HCl toward the venous side of the rete, thus preventing CO_2 from diffusing into the arterial rete capillaries (Figure 1).

FIGURE 1. --Possible mechanism for the secretion of gas into the swim bladder. (Adapted from Maetz, 1956)

A. Swim bladder
B. Gas gland--The H^+ from lactic acid combines with HCO_3^- from the plasma. The H_2CO_3 formed is rapidly dehydrated in the presence of gas gland CA (carbonic anhydrase) to give CO_2 and H_2O.
C. Gas gland capillary bed--CO_2 from the gas gland enters the red blood cells within this capillary network and elicits the Bohr or Root shift, releasing O_2 from hemoglobin.
D. Arterial capillary of the swim bladder rete
E. Endothelial cells of the swim bladder rete--Rete CA prevents diffusion of CO_2 from the venous to arterial side by catalyzing in the hydration of CO_2. The H_2CO_3 formed dissociates to HCO_3^- and H^+, with the HCO_3^- passively diffusing into the arterial side of the rete. The H^+ moves into the venous side of the rete by an energy requiring process, either by the active pumping of H^+ itself or by following the active pumping of Cl^-. This constitutes the "HCl secretory unit" postulated by Maetz.
F. Venous capillary of swim bladder rete

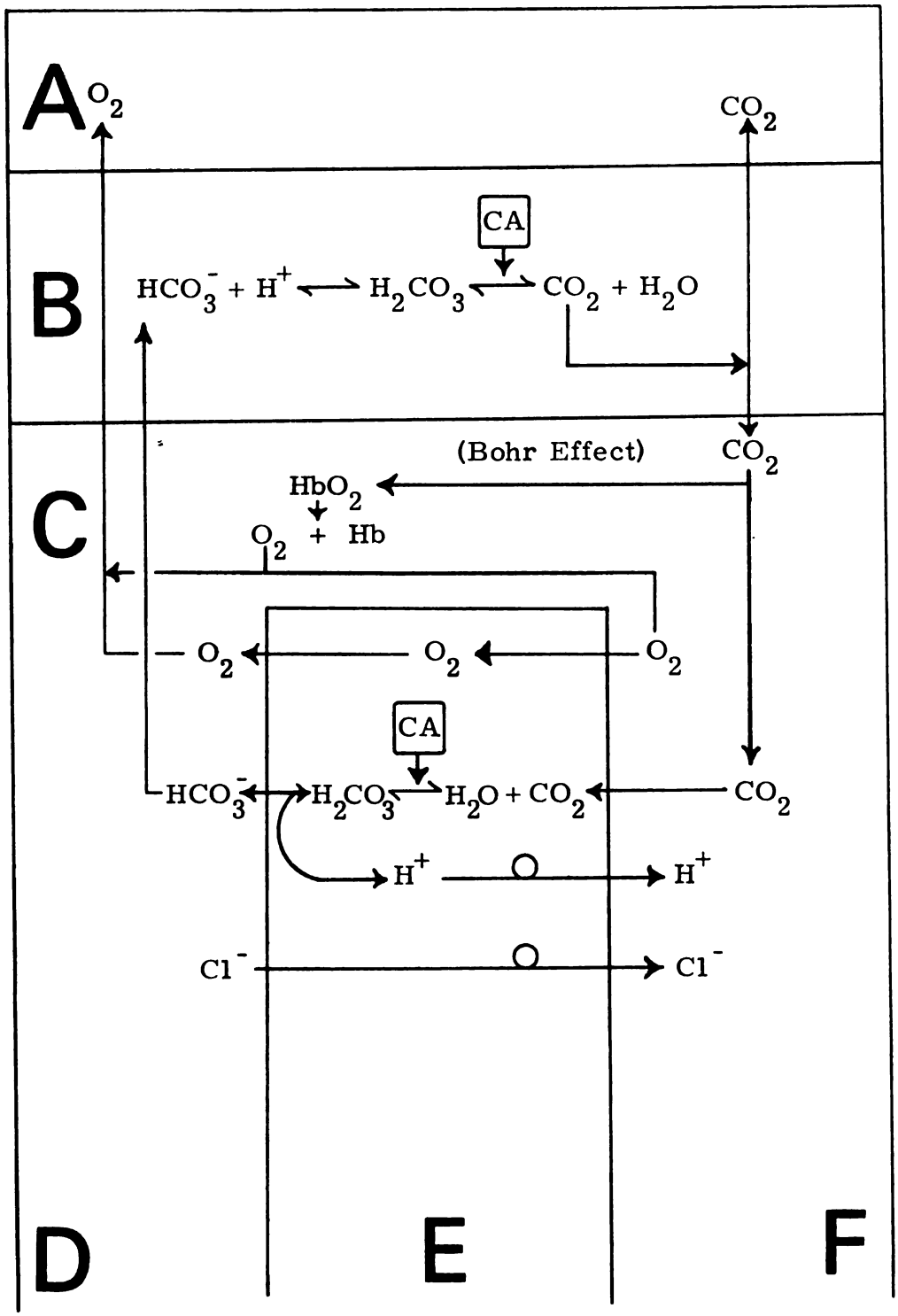

A
O_2
CO_2
B
CA
$HCO_3^- + H^+ \rightleftharpoons H_2CO_3 \rightleftharpoons CO_2 + H_2O$
C
(Bohr Effect)
CO_2
HbO_2
O_2 + Hb
O_2
O_2
O_2
O_2
CA
HCO_3^-
H_2CO_3
$H_2O + CO_2$
CO_2
H^+
H^+
Cl^-
Cl^-
D
E
F

FIGURE 1

In the eye, retinal CA and choroidal rete CA probably serve a function analogous to that of gas gland CA and rete CA in the swim bladder. Bakker (1939) suggested that retinal CA is an intracellular pH regulator, important in the neutralization of lactic acid formed during aerobic glycolysis. It is postulated that H^+ from lactic acid combines with blood HCO_3^- to form H_2CO_3 which, in the presence of retinal CA, is rapidly dissociated to CO_2 and H_2O. The more diffusible CO_2 then goes on to elicit the Bohr or Root shift in the choriocapillaris, while choroidal rete CA prevents concentration of CO_2 in the eye by catalyzing CO_2 hydration in the rete (Figure 1). The H^+ formed from dissociation of H_2CO_3 is secreted into the rete venous blood. To date, no experimental evidence exists which unequivocally demonstrates a "HCl secretory unit" in either the swim bladder or eye rete capillaries.

In summary, there are four sources of CA in the swim bladder and the eye: (1) Red blood cell CA, which catalyzes the formation of H_2CO_3 from CO_2 and facilitates the Bohr and/or Root shift, (2) pseudobranch CA, whose role is as yet undetermined, (3) gas gland and retinal CA, which acts as an intracellular pH regulator by catalyzing the formation of the rapidly diffusible CO_2 from H^+ and (4) swim bladder and choroidal rete CA, which prevents short circuiting of the rete by prohibiting the diffusion of CO_2 from the venous to the arterial side of the rete.

Determination of Carbonic Anhydrase Activity in Tissues

Davis (1963) has recently reviewed the numerous methods in use for determining the presence and kinetic behavior of CA in tissues. All methods depend on either (1) the evolution or absorption of CO_2 or (2) a pH change. The electrometric method of Wilbur and Anderson (1948) was used in the series of studies described in this thesis because it is fast, simple, and avoids the possible interference of ocular pigment as would be present in the colorimetric methods. This electrometric method involves the mixing (at 0°C) of veronal buffer with an amount of CO_2 - saturated water, which will cause an increase of the H^+ concentration and consequent drop in pH of the buffer. For an uncatalyzed reaction the pH drops from 8.3 to 6.3 within a time period (T_0) of 100 - 120 sec. This time is compared to the rate of pH change (T), over the same pH range and temperature in the presence of enzyme. Units of activity per mg tissue are expressed as:

$$\frac{T_0 - T}{Tx \text{ mg tissue used}} \qquad (1)$$

Carbonic anhydrase can also be identified in tissue by a histochemical method (Kurata, 1953; see Hansson [1967] for the most recent modification). In this method freeze dried sections are

floated on a fresh mixture of acid $CoSO_4$ (pH 5.8) and $NaHCO_3$. Exposure of the solution to the atmosphere results in the loss of CO_2 and consequent consumption of HCO_3^- and $CO_3^=$ with the production of OH^- (see reaction 2).

$$CO_3^= + H^+ \rightleftharpoons HCO_3^- + H^+ \rightleftharpoons H_2CO_3 \overset{CA}{\rightleftharpoons} CO_2 + H_2O \qquad (2)$$

$$\begin{array}{cc} + & + \\ OH^- & OH^- \\ \downarrow\uparrow & \downarrow\uparrow \\ H_2O & H_2O \end{array}$$

The net result is a production of OH^- ions and an increase in the pH of the solution. The concentration of OH^- ions will eventually exceed the solubility product of $Co(OH)_2$ and precipitation of this compound will occur. In regions where CA is present, CO_2 evolution and OH^- production will occur at a significantly faster rate, as will the precipitation of $Co(OH)_2$ and its conversion to a visible black CoS by dipping the sections in $(NH_4)_2S$. The selectivity of this reaction for CA has been shown by the fact that it is inhibited in the presence of acetazolamide (4mM) in the incubation media as well as by the fact that acetazolamide in vivo (20 mg/kg) in mice 30 min before killing also inhibits the staining.

Extensive work on the electrophoretic separation and characterization of isozymes of CA from various tissues, most

notably the erythrocytes, has become an important method for investigating the numerous actions of this enzyme (Maren, 1967). Separated erythrocyte CA isozymes show close similarities in general properties such as molecular weight and zinc content, but their catalytic activity (hydration of CO_2) is remarkably different. Although a study of the kinetic characteristics of fish CA was not a primary aim of the present thesis, some preliminary work was done on the electrophoretic similarities and/or differences of the carbonic anhydrases involved in ocular O_2 concentration.

MATERIALS AND METHODS

Introduction

The ability of the fish eye to concentrate oxygen has been shown to be completely inhibited by acetazolamide (Diamox) (Fairbanks et al., 1969), but the dose of acetazolamide given inhibited all of the CA which could possibly play a role in ocular O_2 concentration. A major goal of the present study was to administer graded doses of CA inhibitors to achieve selective inhibition of CA. The effects on the O_2 concentrating mechanism were monitored using a micro polarographic O_2 electrode, and verification of CA inhibition was accomplished by electrometric assays for changes in enzyme activity.

Histological examination and O_2 tension measurements were made on eyes of fish exposed to chronic CA inhibition and therefore chronic inhibition of the O_2 concentrating mechanism as well as from fish which had undergone bilateral pseudobranchectomy and therefore disruption of choroidal rete blood flow. Comparison of the results gives an indication of the importance of the O_2 concentrating mechanism and of choroidal blood flow to retinal

integrity. O_2 profile studies in the fish eye, utilizing the O_2 electrode, were made to determine the region of high ocular O_2.

Injections of Microfil (a microvascular injection compound) was used to outline the vascular blood supply to the choroidal gland. These observations were utilized in studies on O_2 decay and reconcentration in the eye which involved ligation of vessels previously localized by the Microfil technique.

Histochemical location of CA in the choroidal gland and determination of the electrophoretic mobility of the various carbonic anhydrases were used as a possible means of determining the source of choroidal gland CA.

Animals

Rainbow trout (Salmo gairdneri) were obtained from the Michigan State Department of Natural Resources Hatchery at Grayling, Michigan, where they were kept in uncovered three feet deep raceways. The fish were transported to the Michigan State University Campus in a galvanized metal tank, insulated to maintain a constant temperature during transport and fitted with an agitator for aeration. The campus holding facilities consisted of a cold box, capable of maintaining a temperature of 13 ± 0.5°C and fiberglass-lined wooden tanks equipped with a continuous flow of aerated water

which had been previously treated by passing it through a charcoal filter that removed iron and chlorine. The fish were fed commercial trout pellets (Zeigler Bros. Feed Mills Inc., Gardners, Penn.) once weekly and maintained under a photoperiod of 16 hours light and 8 hours darkness. For the majority of the experiments 75-125g fish were used, but in those requiring serial blood samples 200-300g fish were used.

Ocular Oxygen Tension Measurements

Apparatus

A complete description of the construction, characterization and calibration of the micro oxygen polarographic electrode has been previously given (Fairbanks, 1968). A description of the electrode circuit and of the constant temperature chamber (15°C) used in the O_2 tension measurements was also given. During the ocular P_{O_2} determinations the fish was restrained on its side in a small plastic water-filled chamber (24 × 7 × 8cm) by passing an 18 gauge needle through both the upper and lower jaw. Aerated water directed through a rubber catheter, placed in the fish's mouth, was pumped over the gills at a rate of about 150 ml/min. In the majority of the experiments the water contained MS-222 (Tricane Methane Sulfonate, Crescent Research Chemicals, 120 West 5th Avenue, Scottsdale,

Arizona). The concentration of MS-222 in the perfusion water was increased to a level (ca.1:25,000) where tail movement by the fish was minimal but opercular movement was still evident, although weakened by the anesthetic.

Oxygen Profile Studies

A perforation was made in the cornea along the midline and posterior to the lens using a 22 gauge hypodermic needle. A Brinkmann micromanipulator (Model HS-11, Brinkmann Instruments, Cantiague Road, Westbury, N.Y.) was used to lower the electrode through the hole and down towards the back of the eye in 0.1-1.0 mm steps. After each advance the electrode was allowed to come into equilibrium with the P_{O_2} in the region of the electrode tip. The electrode was lowered in this fashion, starting from the anterior chamber, until the tip came in contact with the back of the eye, as noted by a precipitous drop in the P_{O_2} recording or a slight movement of the globe back into the socket caused by the electrode pressing against the back of the eye.

In a few experiments the fish was placed in an upright position with the restraining needle passed through the nostrils. A perforation was made in the upper region of the globe at a point distal to the corneal side of the corneal-scleral junction (limbal

region). The electrode could be lowered through this hole and manipulated to place the tip in the anterior chamber. In this manner P_{O_2} measurements in the anterior chamber were made without puncturing the cornea.

Effects of Carbonic Anhydrase Inhibitors on Ocular P_{O_2}

The electrode was lowered into the eye in the manner previously described until the region of maximum P_{O_2} was located. The fish was then left undisturbed for a minimum of 5 min to insure a stable preparation (i.e., one in which the recorded P_{O_2} value remained constant, indicating the ocular O_2 concentrating mechanism was not interfered with by the presence of the electrode), after which 0.5 ml of a carbonic anhydrase inhibitor (Diamox or CL-11,366) or control solution (CL-13,850 or Ringer solution) was injected intraperitoneally (IP). See Appendix I for drug preparation and chemistry. In some instances this resulted in a disturbance of the electrode position in the eye and consequent change in recorded P_{O_2}. However, after removal of the needle used for drug injection the recorded value quickly returned to the preinjection level. Changes in ocular P_{O_2} resulting from CA inhibition were monitored for periods of time ranging from 15-180 min, after which the fish was removed from the bath, a blood sample taken, the fish killed and tissues taken for CA assay (pseudobranch, retina and choroid). See

Carbonic Anhydrase Assay section for method of preparing the tissue for CA analysis.

Ocular Oxygen Decay and Reconcentration

Fish were removed from the cold box and placed in a pail of water containing MS-222. After they had reached a stage of deep anesthesia (as indicated by the lack of a righting reflex or opercular movement), they were removed from the pail and the afferent pseudobranch artery on one side occluded by cauterization. Next, using a sharpened pithing needle, the holes were made in the opposite operculum on either side of the afferent pseudobranch artery. A thread was looped through the two holes and the fish returned to fresh water in the cold box. Examination of the fish five minutes later showed that they were swimming around and exhibited no adverse effects from their short stay out of water (less than two min) or from the operative procedures. The next day the fish were prepared for P_{O_2} measurements with the region of maximum P_{O_2} located in the eye contralateral to the cauterized pseudobranch artery. The thread looped around the ipsilateral pseudobranch artery was tied down on a short (ca. 1.0 cm) piece of gum rubber tubing, occluding that artery and preventing the flow of blood to the choroidal gland. The resulting decay in ocular P_{O_2} was monitored,

and after the O_2 was depleted the pseudobranch artery ligature was removed and reconcentration of O_2 in the eye monitored.

Ocular P_{O_2} After Chronic Carbonic Anhydrase Inhibition and Bilateral Pseudobranchectomy

Five fish were given daily IP injections for 14 days of a 5 mg% (w/v) solution of acetazolamide at a dose level of 5 mg/100g body wt. On the 15th day intraocular P_{O_2} measurements were made, and a blood sample taken to check the plasma for the presence of acetazolamide (see Determination of Plasma Carbonic Anhydrase Inhibitor). The fish were killed, by severing the spinal cord with a heavy scissors, and the eyes and pseudobranchs removed and placed in buffered formalin. Following fixation the tissues were dehydrated, embedded in Paraplast (Arthur H. Thomas Co., Philadelphia, Pa.), blocked, sectioned at 8 microns (μ), stained with hematoxylin and eosin, mounted in Permount (Fisher Scientific Co., Fair Lawn, New Jersey) and cover-slipped. Another group of five fish received daily IP injections of a 0.45 mg% (w/v) solution of CL-11,366 at a dose of 0.06 mg/100g body wt for 14 days, after which the fish were processed in the same manner as described for the acetazolamide-treated fish.

In another group of five fish bilateral pseudobranchectomies were performed by scraping out the pseudobranchs of anesthetized

fish with a cautery iron. The fish were observed over a three month period after which ocular P_{O_2} measurements were made and the eyes saved for histological examination.

Carbonic Anhydrase Assays

Apparatus

The method of Wilbur and Anderson (1948) was used to determine relative CA concentrations in pseudobranchs, retinas, choroids and RBC's of control fish and fish treated with CA inhibitors. The apparatus for the assay consisted of a water bath of $\frac{3}{8}$ in. clear acrylic plastic (50 × 27 × 23cm) filled with water maintained at 1.0 ± 0.5°C with a refrigeration coil and stirrer and containing one 250 ml mariotte bottle of CO_2 - saturated distilled water produced by bubbling CO_2 into the water through an air stone (see Figure 2). A flow meter in series with the gas cylinder and mariotte bottle permitted the investigator to set the flow at the same level each day. The CO_2 was bubbled through the distilled H_2O for 1 hr before starting the assay to insure complete saturation. Another 250 ml mariotte bottle contained pH 8.2 veronal buffer which was made by dissolving 0.879g of reagent grade Barbital (Fisher Scientific Co., Fair Lawn, N.J.) and 2.268g of Sodium Barbital in 500 ml of distilled H_2O. The pH of the buffer was adjusted to 8.2 at 1.0°C using

FIGURE 2. --Water bath containing reagents and pH electrode chamber used in the assay for carbonic anhydrase in the tissues involved in ocular oxygen concentration.

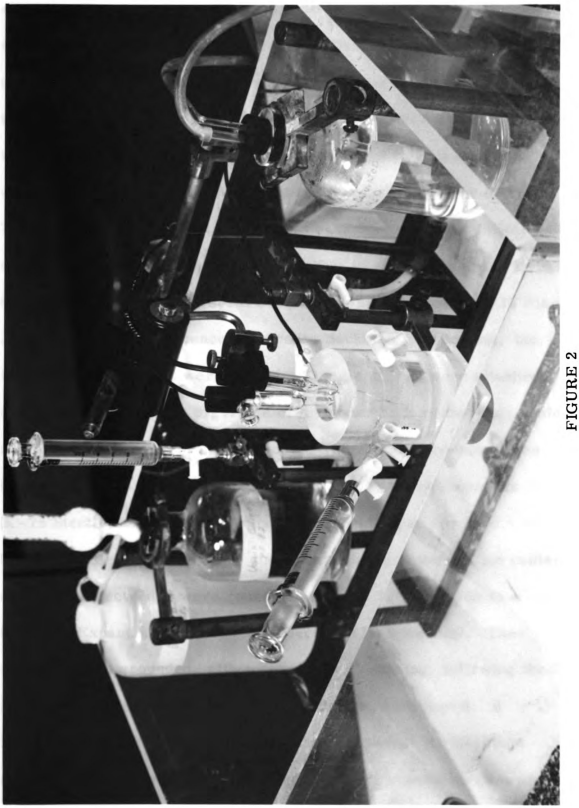


FIGURE 2

4N HCl. The water bath also contained two plastic squeeze bottles, one filled with pH 7.12 Beckman 3501 buffer and the other with distilled H_2O for rinse purposes. All solutions used in the assay were kept in either the water bath or in a Thermos containing ice water so that their temperature was kept at 1.0°C. An electrode chamber constructed from a clear acrylic-plastic cylinder (height 7.3cm, dia 5.1cm) was fastened to a brass frame. The cylinder had a center well (4.0cm deep and 2.5cm dia) into which standard Beckman electrodes (41252 General Purpose Glass Electrode; 41239 Fiber Junction Calomel Reference Electrode; Beckman Instruments, Inc., Fullerton, California) were placed. The electrodes were attached to a swing arm fixed to the frame of the water bath so that they could be easily removed from the chamber. The chamber contained two inlet ports (1/16 in. dia), fitted with three-way teflon stopcocks (K-75 Sterile Stopcocks, Pharmaseal Laboratories, Glendale, Calif.), drilled at a 45° angle and flush with the bottom of the center well. The electrodes were connected through 12 inch leads to a Beckman Expandomatic pH meter (Beckman Instruments). The meter was not grounded, although solution grounding, following the procedure outlined in the Beckman manual, was employed. A 3.7M solution served as the electrolyte (in place of the Beckman electrolyte) in the reference electrode to avoid supersaturation and

clogging of the fiber junction at the low temperature. The meter and electrodes were standardized at 1.0°C with the standard buffer each day that assays were run. The standardization was checked with each run by noting the equilibrium pH of the veronal buffer (see below).

To begin an assay 0.2 ml of blank (distilled water) or solution containing enzyme was added to the center well from above. A 5 ml Luer-lok glass syringe (B-D Yale: Becton, Dickerson and Co., Rutherford, N.J.) was partially filled with 2.5 ml of veronal buffer from the stock mariotte bottle and this sample introduced into the center well through one of the inlet ports. The pH meter was set in the read position and a second glass syringe used to obtain 2.0 ml of CO_2 saturated water while the electrodes were coming into equilibrium with the buffer solution (ca. 30 sec). The calibration was checked by noting the equilibrium pH and the CO_2 water forcibly injected into the center well of the chamber through the second inlet port. The forcible injection of the CO_2 water provided good mixing of the three solutions, as seen by the fact that substitution of a dye solution (0.1% [w/v] methylene blue) for the CO_2 water resulted in a homogeneous mixture. When the pH of the mixture dropped to 8.1 an electric stop clock (Model No. K15140, A. W. Haydon Co., Waterbury, Conn.) was turned on and then turned off when the pH read 6.3. The elapsed time was recorded to the nearest 0.1 of a sec.

Three successive runs were made for each sample and averaged; in between runs the electrodes and electrode chamber were washed with distilled water. Suction from a vacuum pump was used to remove the sample and rinse water from the cell while the electrodes were wiped with gauze pads.

Preparation of Tissues

Blood samples (0.5 - 1.0 ml) were collected in heparinized syringes and immediately transferred to the test tubes for 5 minutes of centrifugation (Clinical Centrifuge, Model CL, International Equipment Co., Needham Heights, Mass.). After centrifugation, the plasma layer was removed and placed in the freezer, while the packed RBC's were resuspended in 5-10 cc of sterile Ringer solution (Abbott Laboratories, North Chicago, Ill.) and recentrifuged. The sample was washed and centrifuged once more before 0.1 ml of packed RBC's were pipetted into a thick-walled sonifying test tube containing 2 ml of frozen sterile Ringer solution. Simultaneously with the preparation of the RBC's the pseudobranchs, retinas, and choroids of the fish were removed, blotted dry and weighed to the nearest 0.1 mg on a Mettler Balance (Mettler Macro Balance, Model No. B5, Mettler Instrument Corp., 20 Nassau Street, Princeton, N.J.). Next, the tissues were placed on 2.0 ml of

frozen Ringer solution in sonifying tubes, and after the solution had begun to melt the cells were disrupted by sonification (Bronson Sonifier, Model No. W1850, Melville, N.Y.). By beginning the sonification before all the Ringer solution had melted, the cells could be disrupted before the temperature had risen to a point which would inhibit the enzyme. As long as the tubes still felt cool after sonification, it was assumed that the temperature of the homogenate had not risen to a point which could inhibit the enzyme. Finally the homogenates were transferred to small test tubes (1.0 × 7.5cm), stoppered, and placed in the freezer until CA analysis.

Sonification was used as the method for preparing the tissue homogenates since it appeared to be the best available. Some objection might be raised to this method, because of the fact that Roughton and Booth (1946) have found that shaking inhibits or inactivates purified preparations of CA during analysis by the manometric technique. However, in that no mention has been made of this inhibition occurring in a crude preparation of the enzyme, it may be, as suggested by Roughton and Booth, that during the process of purification an enzyme stabilizer had been removed, resulting in the inhibition seen during the analysis by the manometric technique.

Carbonic Anhydrase Assay of Perfused Tissue

Fish were killed by severing the spinal cord with a heavy scissors, the abdominal region was opened to expose the heart and a section of PE-50 polyethylene tubing (Clay Adams Inc., New York, N.Y.) was inserted into the conus arteriosus through a slit in the ventricle. Ten ml of heparinized Ringer solution (a volume sufficient to flush the blood vascular system) was forced slowly through the canulae, after which pseudobranchs, retinas and choroids were removed and prepared for CA assay in the manner just described.

Movement of CL-11,366 Into Tissues

Control blood samples (0.5 ml) were drawn from a group of 12 fish into heparinized syringes. One hour later the fish were given IP injections of CL-11,366 (0.45 mg/ml) at a dose of 2.5 mg/kg. Post injection blood samples were taken at 2, 4, 8, and 15 min, with three fish per group. The blood was processed as before for CA analysis and the plasma samples saved to check for the presence of CL-11,366.

A second group of 15 fish also received IP injections of CL-11,366 (0.45 mg/ml) at a dose of 2.5 mg/kg. These fish were

divided into subgroups of three and killed (by severing the spinal cord) at 1.5, 3, 6, 10, and 15 min post injection. The pseudobranchs, retinas and choroids were removed and processed for CA analysis as previously described.

Determination of Plasma Carbonic Anhydrase Inhibitor

Plasma from control and treated fish was assayed for the presence of inhibitor. To estimate the inhibitory activity present, four runs were made: (1) a blank or distilled H_2O, (2) an RBC enzyme solution, (3) an RBC enzyme solution plus plasma, and (4) a blank plus plasma to check for the presence of enzyme in the plasma. The enzyme acceleration (A_1) in the absence of the plasma was expressed as:

$$A_1 = \frac{T_0 - T_{RBC}}{(T_{RBC}) \times (\mu l \text{ RBC used})} \quad (3)$$

where T_0 is equal to the time of the blank run and T_{RBC} is equal to the time of the run of the RBC enzyme solution. The enzyme acceleration (A_2) in the presence of the plasma was expressed as:

$$A_2 = \frac{T_0 - T_p}{T_p \times (\mu l \text{ RBC used})} \quad (4)$$

where T_p equals the time of the run of the RBC enzyme solution in the presence of 0.1 ml plasma. The per cent inhibition by the plasma is equal to:

$$\frac{A_1 - A_2 \times 100}{A_1} \qquad (5)$$

Histochemistry of the Choroid Rete

The fish were killed and the blood vascular system flushed as previously described. The choroid retia were then removed and sectioned at 10-20 μ using an International Model CTD Microtome-Cryostat (International Equipment Co., Needham Heights, Mass.). The fresh sections were stained following the procedure of Hansson (1967), as outlined in Appendix II.

Carbonic Anhydrase Disc Electrophoresis

Tissues

Pseudobranchs, choroids, retinas, and RBC's were prepared in the same manner as described for the CA assays.

Apparatus

The disc electrophoresis apparatus (Figure 3) as described by Fonner (1968) consists of two polyethylene trays (17 × 12.5 ×

FIGURE 3. --Disc electrophoretic apparatus used for separating carbonic anhydrase isozymes.

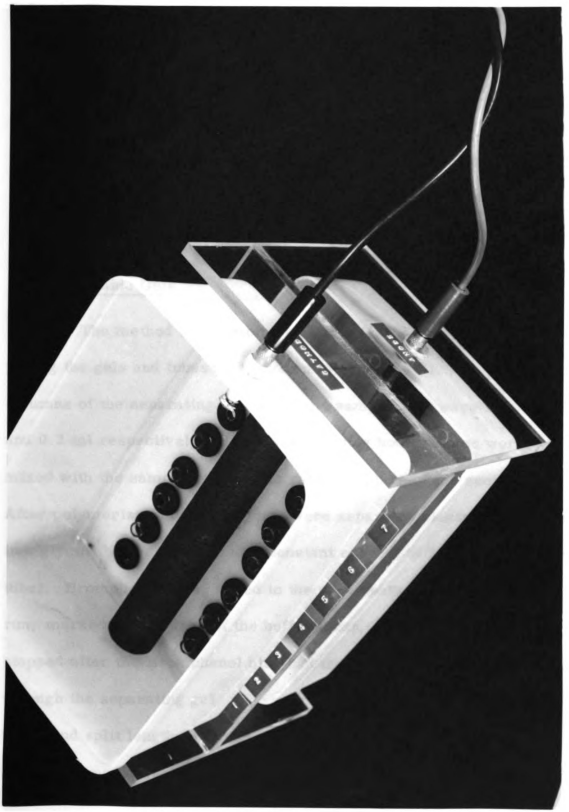


FIGURE 3

7.5cm). Two rows, of seven holes each, in the top tray contain rubber gaskets and are used to hold the polyacrylamide gel tubes (0.5 × 8.0cm). The electrodes (carbon cylinders 2.0 × 14.5cm) are aligned in the middle of the trays with the cathode in the top and the anode in the bottom tray. A Heathkit Variable Voltage Regulated Power Supply (Model P33, Benton Harbor, Mich.) provided the current for the isozyme separations.

Polyacrylamide Gels

The method of Ornstein and Davis (1962) was used in preparing the gels and tubes and is given in Appendix III. The individual columns of the separating, stacking and sample gels were 1.0, 0.2, and 0.2 ml respectively. The CA-containing homogenates were mixed with the sample gel in a 1:1 ratio, in place of the sucrose. After polymerization the isozymes were separated electrophoretically in a glycine buffer (pH 8.4) at a constant current of 70 mA (5 mA per tube). Bromphenol blue, added to the upper bath at the start of the run, marked the advance of the buffer front. Electrophoresis was stopped after the Bromphenol blue had moved approximately 1.5 inches through the separating gel. The gels were then removed from the tubes and split lengthwise with a scalpel. One half of each gel was stained immediately for CA according to the method of Hodgen and

Gomes (1969) and the other half incubated for one hour in a 10^{-4}M solution of acetazolamide before staining (see Appendix III for outline of staining procedure).

Anatomy

Fish were killed and perfused with heparinized Ringer solution as described previously. A Microfil solution (Canton Bio. Medical Products, P.O. 154, Swarthmore, Pa.) consisting of 7 parts MV112, 9 parts MV Diluent and 0.5 parts MV curing agent was injected into the fish through the truncus arteriosus. A volume and pressure sufficient to fill the vasculature of the fish was used, after which the fish was placed in the refrigerator (4°C) until polymerization of the silicone rubber injection compound was complete (ca. 1 hour). The heads were then removed from the fish, fixed in Carnoy's fixative for 24 hours, washed for 12 hours in tap water and dehydrated in successive ethyl alcohol solutions (70, 95, 100%, 12 hrs each). When working with the eyes alone, dehydration was preceded by 12 hours of bleaching in 3% H_2O_2. After dehydration the tissues were cleared in benzene and then placed in Methyl salicylate, which replaced the benzene. The blood vascular pattern of the eyes and pseudobranchs was easily visualized in these preparations by the presence of the white silicone rubber.

Blood Flow Rate in the Presence of CL-11, 366

Fish were placed in the plastic tray previously described, ventral side up and with the restraining needle passed through the nostrils. The water pumped over the gills contained MS-222 in a concentration of 1:25, 000. The plastic tray was situated so that the head of the fish was beneath an untraviolet lamp. Intraperitoneal injections of 0. 5 ml of CL-11, 366 (0. 45 mg/ml) or 0. 5 ml of Ringer solution were given and then three minutes post injection the fish received a bolus of sodium fluorescein (0. 2 ml of a 2 gm% solution) in the tail vein. A stop clock was started, utilizing a foot switch, at the time of the fluorescein injection, and stopped at the first appearance of fluorescein in the pseudobranch. The fluorescein circulation times were recorded to the nearest 0. 01 sec and the times after IP injection of CL-11, 366 compared to those after IP injection of Ringer solution.

RESULTS

Oxygen Profile Studies

Shown in Figures 4 and 5 are the results obtained upon electrode penetration into the eyes of six fish. In general, as the electrode is lowered into the eye the P_{O_2} remains relatively constant until about midway into the eye. With further penetration of the electrode there is either (1) a steady increase in the ocular P_{O_2} up to a maximum (Figure 4) or (2) a much more gradual increase until the recorded P_{O_2} suddenly shows a very rapid increase to the maximum (Figure 5). This maximal P_{O_2} is apparently located in the region of the choroidal rete in that further penetration of the electrode gave rise to a decline of the recorded P_{O_2} and a slight movement of the eye back into the socket, indicating that the electrode is pressing against the posterior scleral coat. In some cases, after the O_2 profile studies were complete and the fish killed the eye was removed and a measurement made of the anterior-posterior diameter. This value was within 1.0 mm of the value determined from the O_2 profile studies, assuming that the region of maximal P_{O_2} is located at the back of the eye.

FIGURE 4. --Per cent maximum P_{O_2} vs. electrode depth. At zero depth the electrode tip was in the anterior chamber and at 7.0-8.0 mm it was at the back of the eye. The numbers in parentheses are the maximum P_{O_2} (mm Hg) values recorded while the dotted lines indicate the decline of the recorded P_{O_2} seen when the electrode was lowered beyond the region of maximum P_{O_2}.

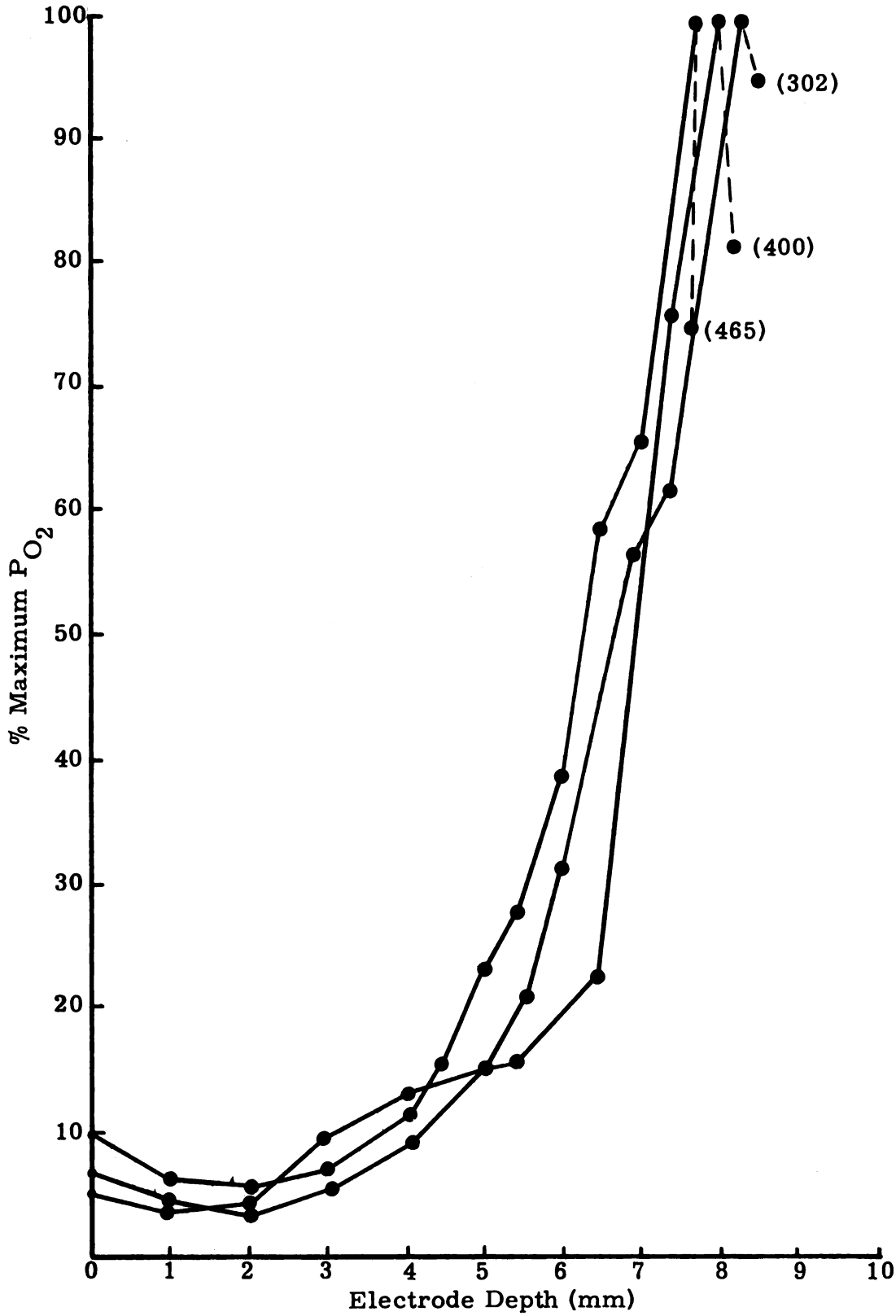

100
90
80
70
60
50
40
30
20
10
% Maximum P_{O_2}
0
1
2
3
4
5
6
7
8
9
10
Electrode Depth (mm)
(302)
(400)
(465)

FIGURE 4

FIGURE 5.--Per cent maximum P_{O_2} vs. electrode depth. At zero depth the electrode tip was in the anterior chamber and at 7.0-8.0 mm it was at the back of the eye. The numbers in parentheses are the maximum P_{O_2} (mm Hg) values recorded while the dotted lines indicate the decline of the recorded P_{O_2} seen when the electrode was lowered beyond the region of maximum P_{O_2}.

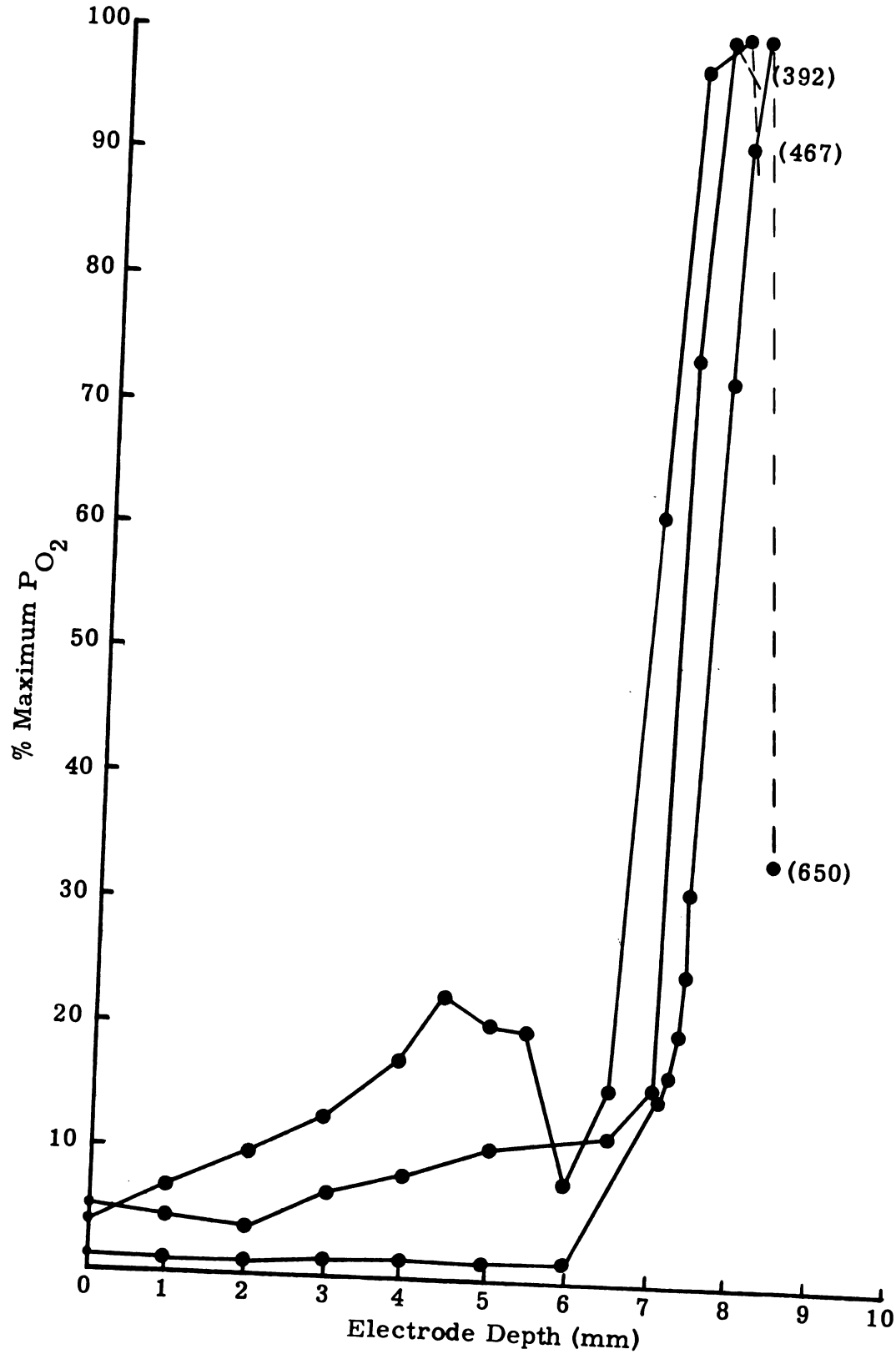

100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
% Maximum P_{O_2}
Electrode Depth (mm)
(392)
(467)
(650)

FIGURE 5

Anterior chamber P_{O_2} measurements were made without puncturing the cornea using the procedure described in the Materials and Methods section. The average P_{O_2} of four observations was 21 ± 3.5 mm Hg, a value comparable to the average anterior chamber P_{O_2} of 20 ± 4.4 mm Hg for the six fish represented in Figures 4 and 5.

Oxygen Decay and Reconcentration

Data on the rate of ocular P_{O_2} decay following occlusion of the afferent pseudobranch artery are given in Figure 6. The rapidity of retinal oxygen consumption is indicated by the short $T\frac{1}{2}$ ($\bar{x}$ = 71 sec) where the $T\frac{1}{2}$ is defined as that time at which the recorded P_{O_2} reached 50% of its initial value. Reconcentration of the high ocular P_{O_2} does occur when the ligature is removed, although it is a much slower event than O_2 decay (Figure 7). In fish number 6, in which the afferent pseudobranch artery remained tied for 30 min, the reconcentrating mechanism showed little evidence of being functional 20 min after the ligature was removed. These results have some interesting implications contrary to the theory of Parry and Holliday (1961) and will receive further consideration in the discussion.

FIGURE 6.--Ocular oxygen decay curve as a consequence of ligating the afferent pseudobranch artery. The per cent of the average initial P_{O_2} from 6 fish (430 ± 82.3 mm Hg) is plotted against time (secs) after afferent pseudobranch artery ligation. Zero time represents the time at which the vessel was first ligated. The vertical bars on the curve represent the SE of the mean.

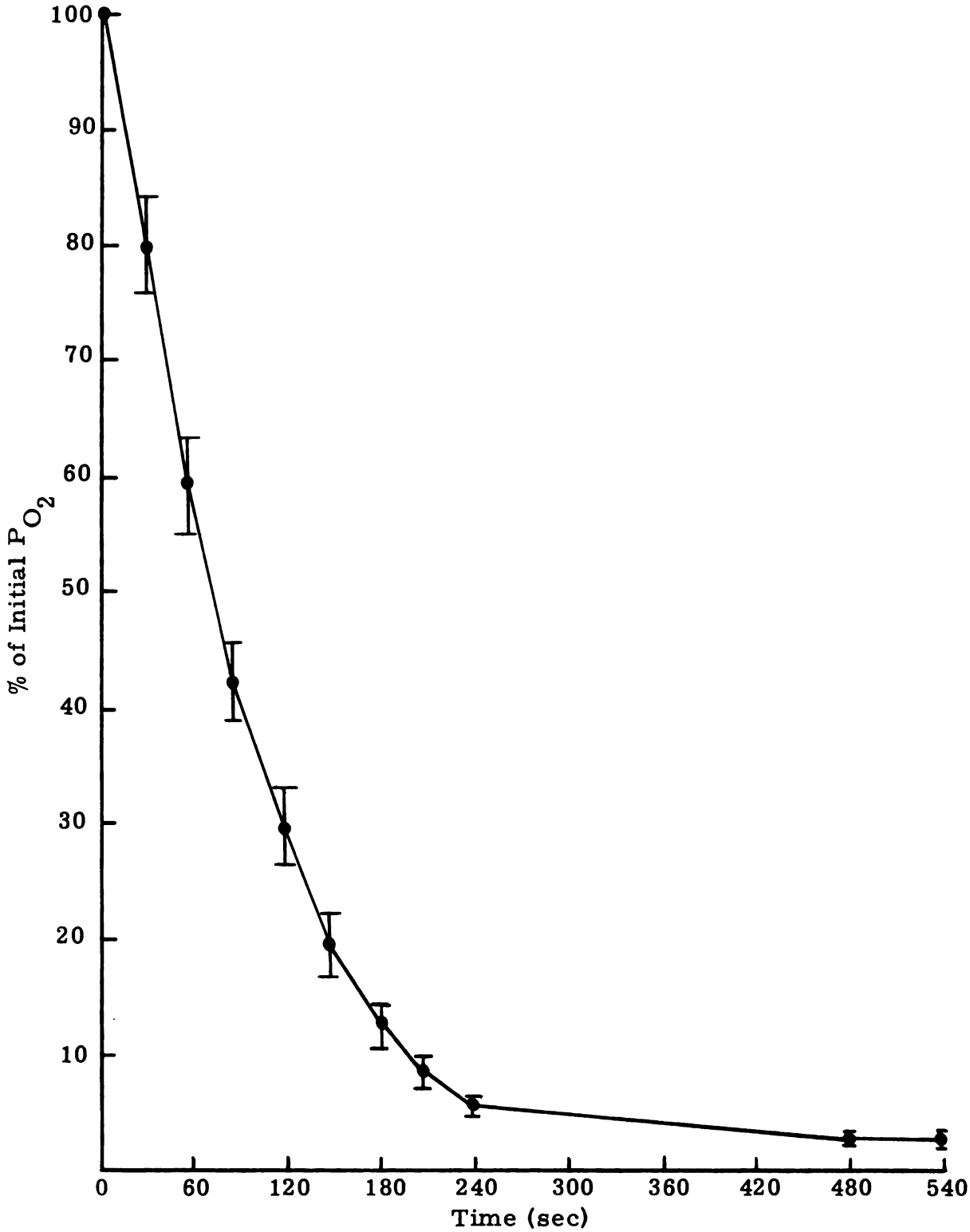

100
90
80
70
60
50
40
30
20
10
% of Initial P_{O_2}
0
60
120
180
240
300
360
420
480
540
Time (sec)

FIGURE 6

FIGURE 7.--Ocular oxygen reconcentration after removal of the afferent pseudobranch artery ligature. The average ocular P_{O_2} from five fish prior to ligation of the pseudobranch artery was 512 ± 95.5 mm Hg. After removal of the pseudobranch artery ligature, reconcentration of O_2 in the eye began immediately and is plotted as the per cent of the initial average value vs. time (min) after removal of the ligature. The points are the means while the vertical bars represent the SE from five fish. The time delay between ligation and release of the ligature was 12-15 min except for fish 6, in which the delay was 30 min.

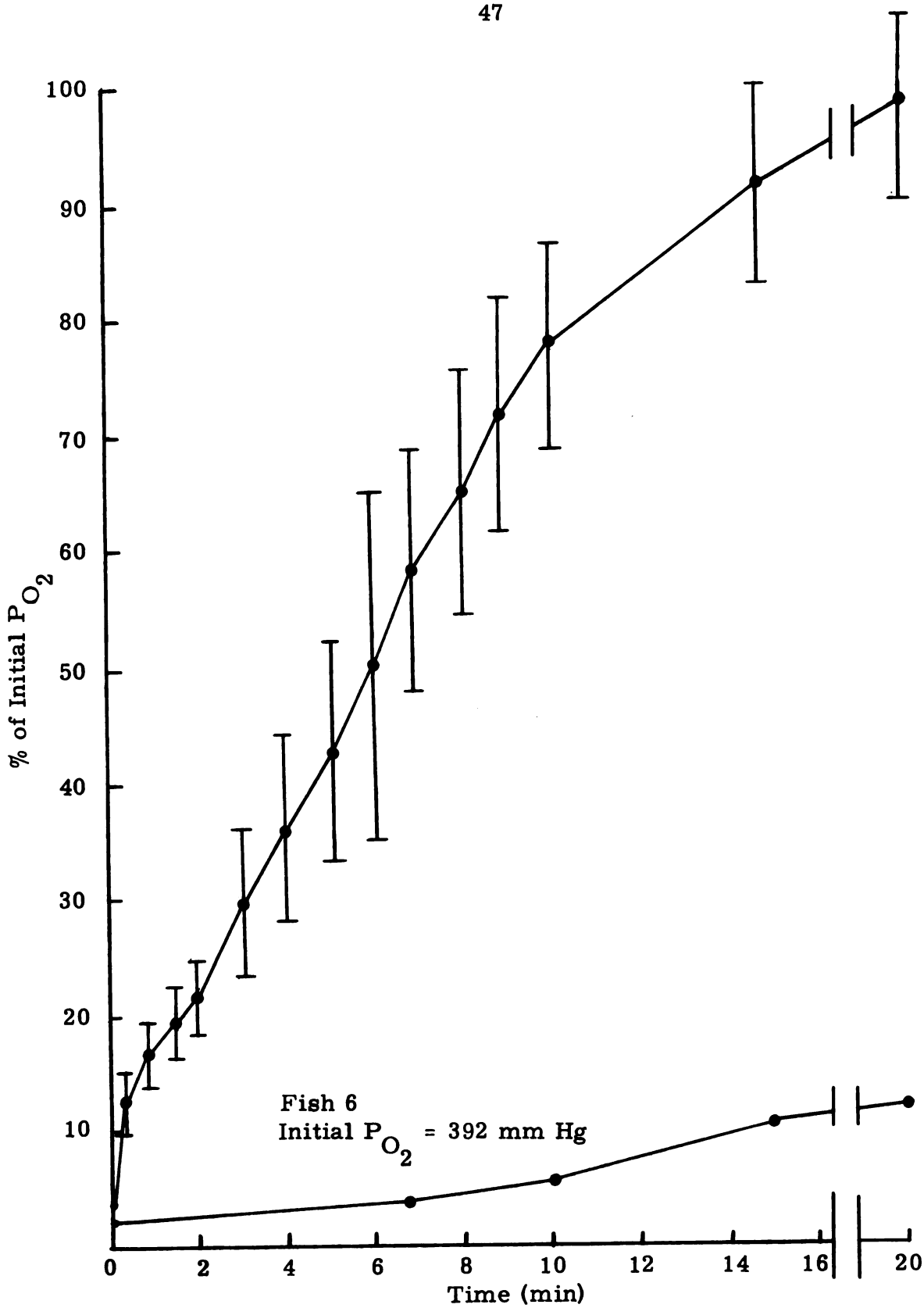

100
90
80
70
60
50
40
30
20
10
% of Initial P_{O_2}
Fish 6
Initial P_{O_2} = 392 mm Hg
0
2
4
6
8
10
12
14
16
20
Time (min)

FIGURE 7

Carbonic Anhydrase and Oxygen Concentration

Concentrations of CA in pseudobranch, choroid, retina, and RBC of control and experimental fish are given in Table 1. The pseudobranchs from fish that have been perfused with 10 ml of heparinized Ringer solution prior to collection of tissues for assay had lower average concentrations (although not significant at $\alpha < 0.05$) than controls, since perfusion removed the red blood cells and consequently the red blood cell CA activity. Similar results were expected for the choroid layer of the eye, since it is a highly vascular structure, but unexplainably the opposite was true. The high activity in the choroid must be due to the presence of the enzyme in choroidal tissue and not to that present in RBC. The lower value for the retinas of the perfused fish is not significantly different from that for controls.

Also included in Table 1 are the individual CA concentrations found in fish that received CA inhibitors or a control solution (CL-13,850). The drug CL-13,850 is a structural analogue of of acetazolamide but has no CA inhibitory activity (Appendix I). In addition, Figures 8-11 illustrate the effects which these drugs had on the ocular O_2 concentrating mechanism.

The rapid "depletion" of O_2 following CL-11,366 injection was a surprising event. Initially it was thought that a maximum P_{O_2}

TABLE 1. --Comparison of the carbonic anhydrase concentrations (units/μg wet weight) found in the tissues which may be involved in ocular oxygen concentration. Comparison of the activity from the control and Ringer solution perfused fish allow the extimation of carbonic anhydrase activity in the pseudobranch and choroid which is due to the presence of red blood cells. The remainder of the table represents a survey of various carbonic anhydrase inhibitors as to their inhibitory activity in the four tissues in relation to dose level and duration of action.

Fish	Treatment	Dose mg/kg	Sample[a] Time (min)	CA Activity Units/μg Tissue			
				Pseudobranch	Choroid	Retina[b]	RBC
1-11	Control	----	----	249.3 ± 47.18*	501.6 ± 32.70	107.0 ± 19.42	133.8 ± 38.50
12-17	Perfused	----	----	178.5 ± 22.33	589.3 ± 48.99	86.5 ± 16.77	----
21	CL-11,366	2.37	22.0	128.0	59.0	23.8	86.0
22	CL-11,366	2.45	8.0	137.0	169.0	66.8	291.0
23	CL-11,366	2.50	17.0	146.0	536.0	22.3	52.0
25	CL-11,366	2.32	12.0	175.0	74.0	56.4	81.0
26	CL-11,366	2.78	13.5	37.0	11.0	27.7	64.0
28	CL-11,366	2.08	182.0	75.0	23.0	15.8	68.0
$\bar{X}$	----	2.41	----	116.3	145.3	35.4	107.0
29	CL-11,366	0.51	172.0	212.0	27.0	34.1	594.0
30	CL-11,366	0.27	86.0	208.0	26.0	67.6	381.0
31	CL-11,366	0.33	90.0	446.0	400.0	70.2	379.0
$\bar{X}$	----	0.37	----	288.6	131.0	57.3	451.3
35	Na Salt CL-11,366	2.50	55.0	75.2	143.8	243.4	171.0
27	Acetazolamide	20.9	11.0	12.0	5.0	5.6	5.0
37	CL-13,850	24.8	48.0	64.0	877.0	125.0	198.8

* mean ± SE

[a] Blood drawn (min) post injection of inhibitor.

[b] Pigment layer lost.

FIGURE 8.--Ocular oxygen depletion curves of fish given IP injections of CL-11,366 (0.5 ml of a 0.45 mg/ml solution). The numbers in parentheses next to the fish number are the initial P_{O_2} values. The curve is a plot of the per cent of this initial value vs. the time (min) after injection of CL-11,366. The numbers above the oxygen depletion curves represent the electrode depth, in the eye, with the first number on the left representing the depth at which the maximum P_{O_2} was initially found. Fish 22 jumped and broke the electrode, preventing completion of the experiment. Dose levels for individual fish can be found in Table 1.

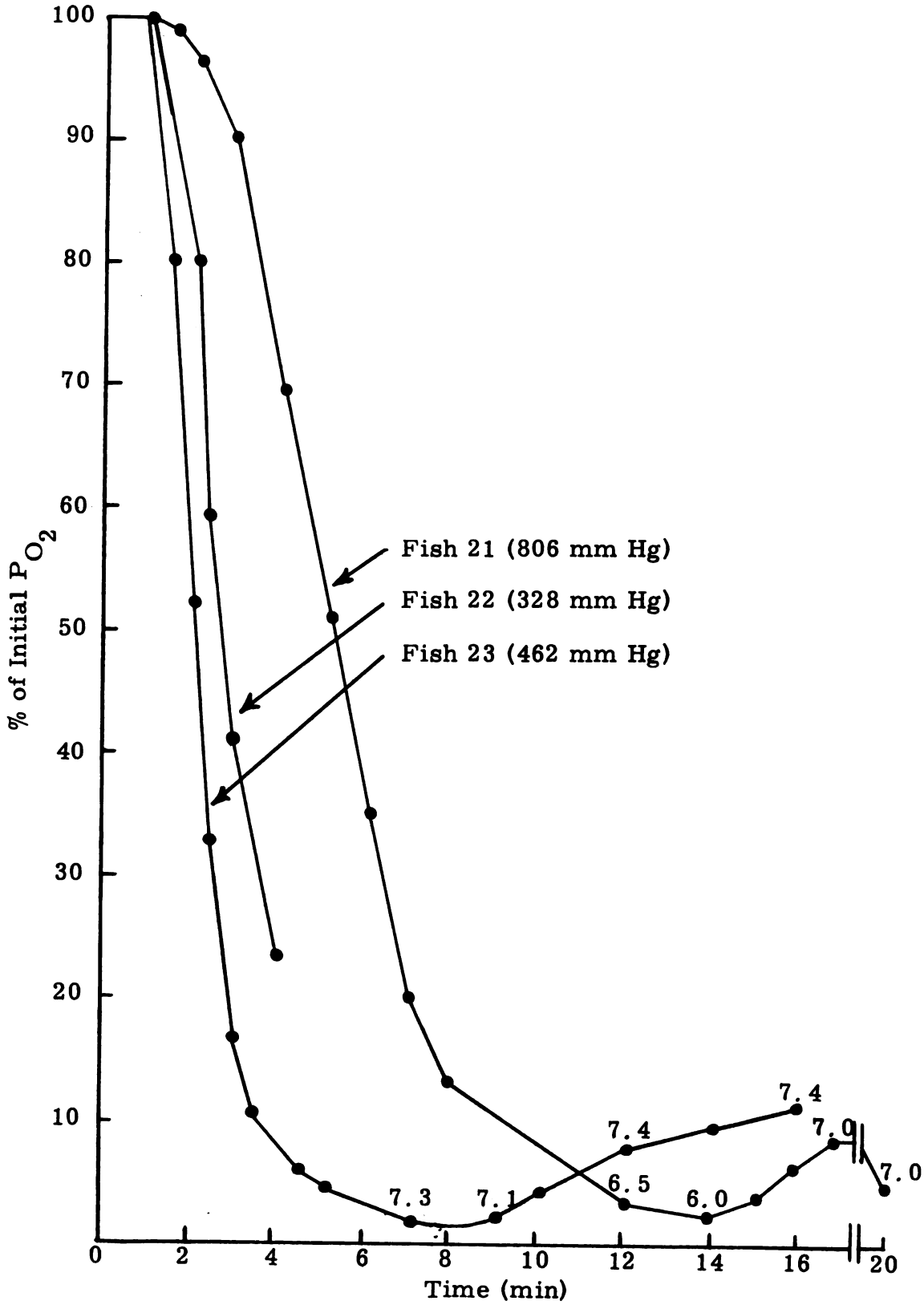

100
90
80
70
60
50
40
30
20
10
0
2
4
6
8
10
12
14
16
20
% of Initial P_{O_2}
Time (min)
Fish 21 (806 mm Hg)
Fish 22 (328 mm Hg)
Fish 23 (462 mm Hg)
7.4
7.4
7.0
7.0
6.5
6.0
7.3
7.1

FIGURE 8

FIGURE 9. --Ocular oxygen depletion curves of fish given IP injections of CL-11,366 (0.5 ml of a 0.45 mg/ml solution). The numbers in parentheses next to the fish number are the initial P_{O_2} values. The curve is a plot of the per cent of the initial value vs. the time (min) after injection of CL-11,366. Fish 27 received 0.5 ml of a 4.0 mg/ml solution of acetazolamide. Dose levels for the individual fish can be found in Table 1. The dotted line represents the electrode response time in the eye of a normal fish. The maximum ocular P_{O_2} (644 mm Hg) was located and then the electrode raised rapidly 5.0 mm, removing the tip from the region of high O_2.

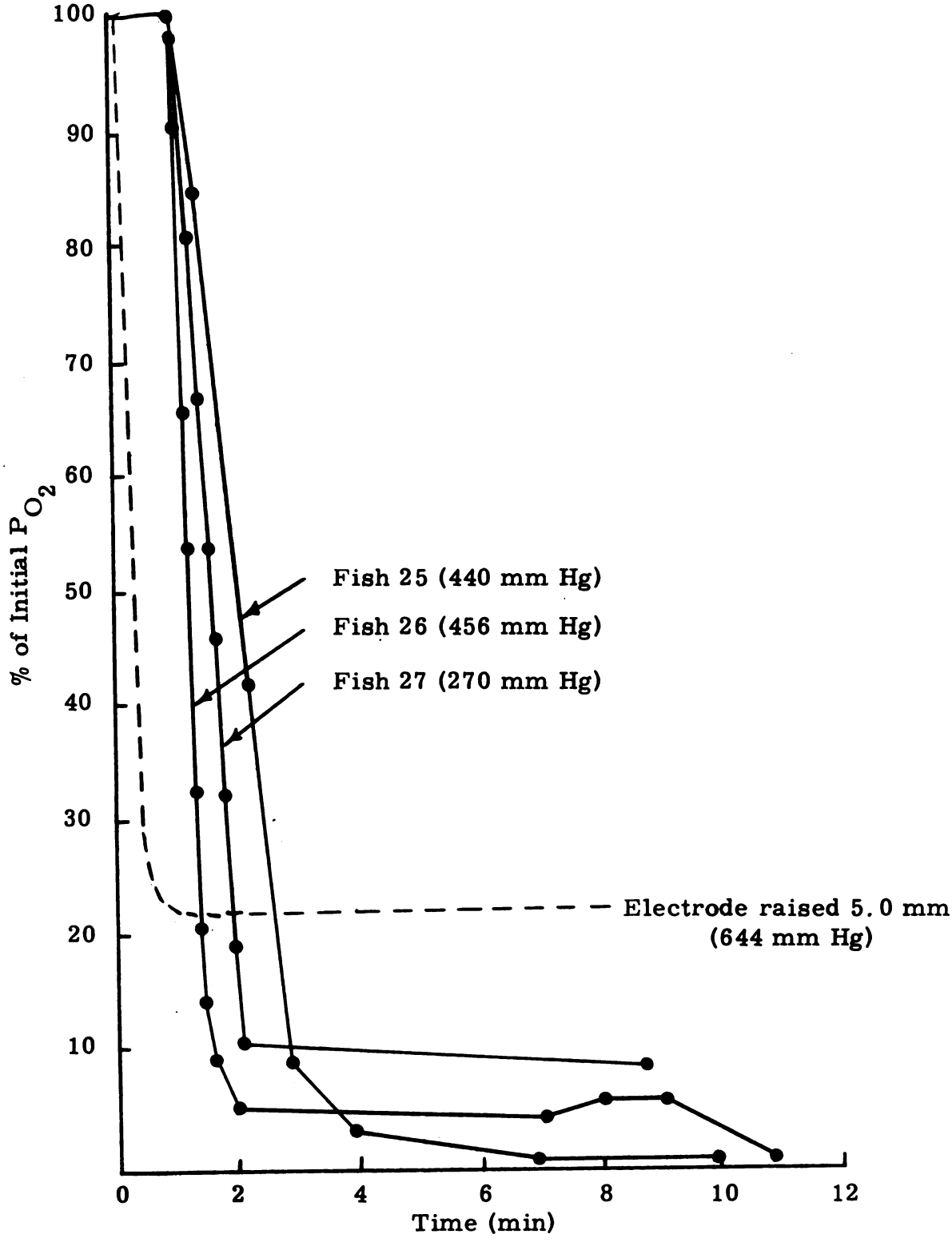

100
90
80
70
60
50
40
30
20
10
% of Initial P_{O_2}
Fish 25 (440 mm Hg)
Fish 26 (456 mm Hg)
Fish 27 (270 mm Hg)
Electrode raised 5.0 mm
(644 mm Hg)
0
2
4
6
8
10
12
Time (min)

FIGURE 9

FIGURE 10.--Ocular oxygen depletion curves of fish given IP injections of CL-11,366. The numbers in parentheses next to the fish number are the initial P_{O_2} values. The curve is a plot of the per cent of the initial P_{O_2} vs. time (min) after injection of CL-11,366. Fish 28 received 0.5 ml of a 0.45 mg/ml solution, while Fish 29 received 0.5 ml of a 0.10 mg/ml solution. Dose levels are given in Table 1.

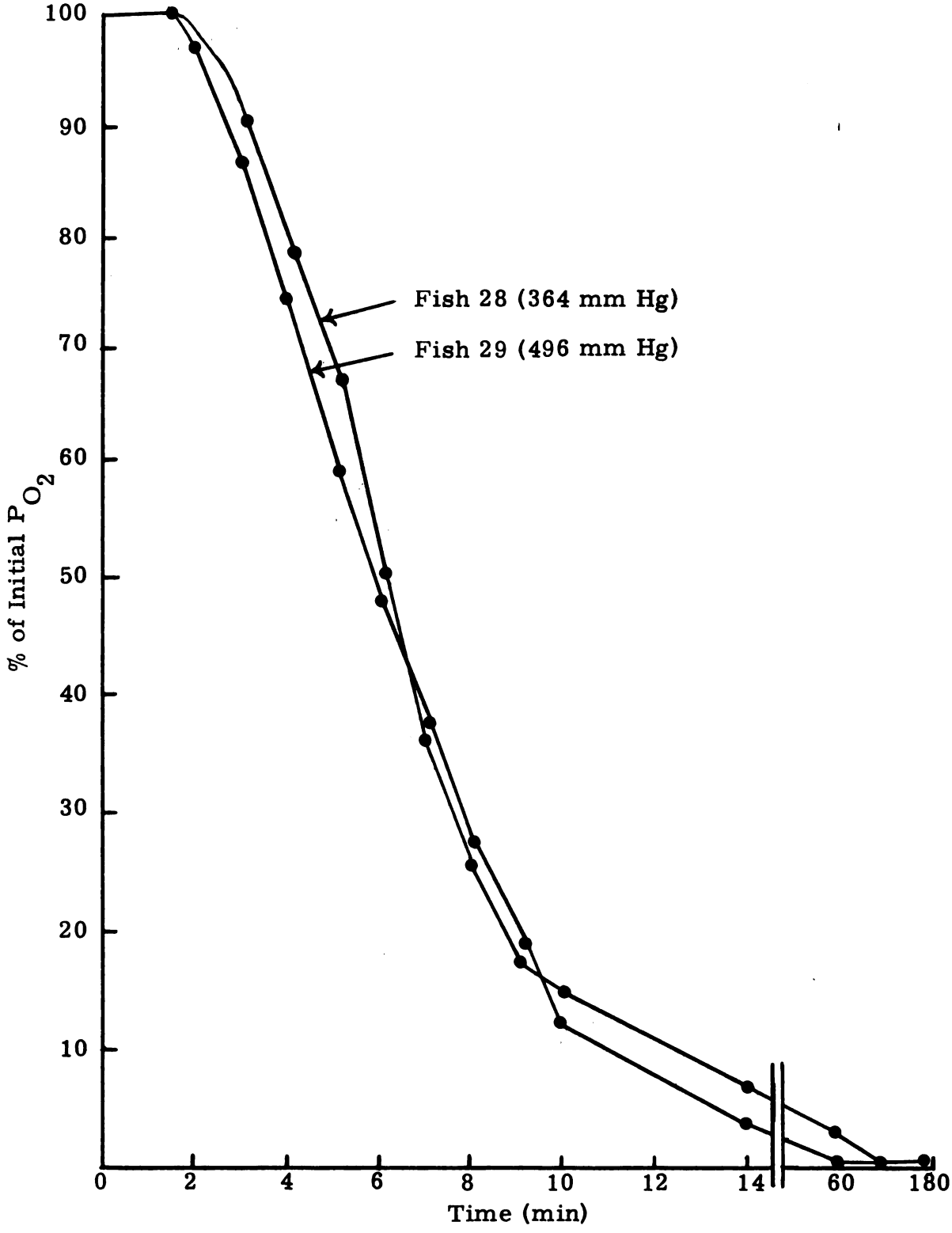

100
90
80
70
60
50
40
30
20
10
% of Initial P_{O_2}
Fish 28 (364 mm Hg)
Fish 29 (496 mm Hg)
0
2
4
6
8
10
12
14
60
180
Time (min)

FIGURE 10

FIGURE 11. --Ocular oxygen depletion curves of fish given IP injections of CL-11, 366. The numbers in parentheses next to the fish number are the initial P_{O_2} values The curve is a plot of the per cent of the initial P_{O_2} vs. time (min) after injection of CL-11, 366. Fish 30 and 31 received 0.5 ml of a 0.045 mg/ml solution of CL-11, 366, while Fish 35 received 0.5 ml of a 0.45 mg/ml solution of the Na salt of CL-11, 366. The individual doses are given in Table 1.

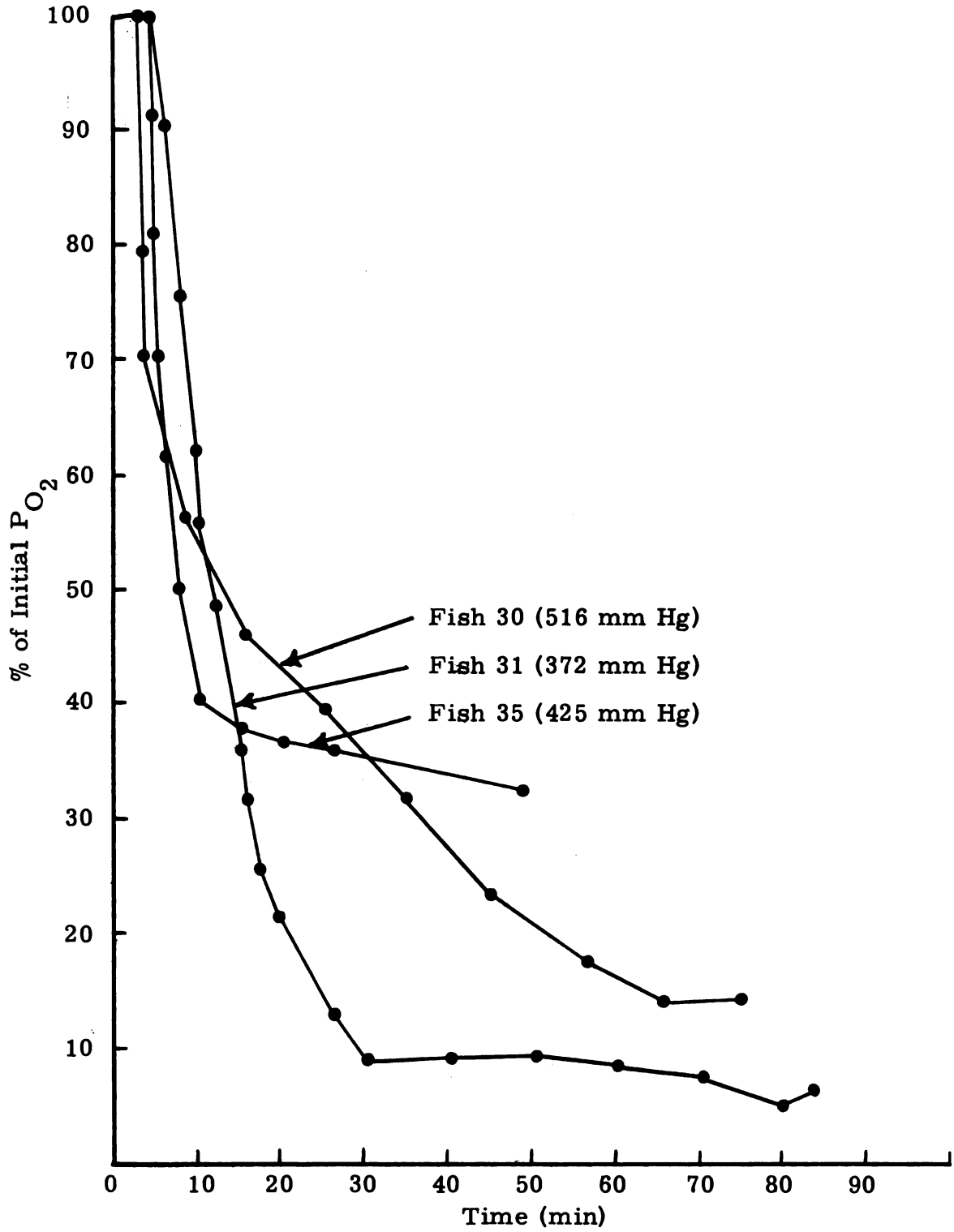

100
90
80
70
60
50
40
30
20
10
% of Initial P_{O_2}
Fish 30 (516 mm Hg)
Fish 31 (372 mm Hg)
Fish 35 (425 mm Hg)
0
10
20
30
40
50
60
70
80
90
Time (min)

FIGURE 11

could be located in the eye, after which the fish could be removed from the water bath, given a 1 mg/kg intravenous dose of CL-11,366, placed back in the bath and the maximum P_{O_2} value relocated. Because of its low pk (pk_a = 3.2) and high plasma binding (> 90% in most species for a dose of 1 mg/kg; Maren, 1967), CL-11,366 penetrates red cells slowly (Holder and Hays, 1965). Therefore it was expected that the maximum P_{O_2} following drug injection could be relocated, and after a longer delay inhibition of the O_2 concentrating mechanism would be noted. However, in two of three fish treated in this manner, no region of high O_2 could be relocated after drug injection, while in the third a "submaximal" region was located and the O_2 in this area was depleted in a short time. For this reason IP rather than intravenous injections were used, and again the results were surprising. A higher dose was used initially (ca. 2.5 mg/kg) on the assumption that the uptake of the drug from the peritoneal cavity would be slow. That this was not the case is illustrated in Figures 8 and 9, where the effect was seen 1-2 minutes post injection ($T\frac{1}{2}$ = 30-210 sec). Thus, in some cases this "depletion" of O_2 was even more rapid than that which occurred after afferent pseudobranch artery occlusion.

An indication of electrode response time in a normal eye is given in Figure 9. The maximum P_{O_2} was located, and then the

electrode was raised 5.0 mm as rapidly as possible using the micromanipulator. This removed the electrode tip from the area of O_2 concentration. Since the recorded response was more rapid ($T \frac{1}{2}$ = 22.0 sec) than any seen after drug administration, it is believed that the response time of the electrode was sufficient to follow the O_2 decay subsequent to administration of CA inhibitors.

Some of the variability in the rate of O_2 depletion in fish which received more than 2 mg/kg of CL-11,366 may have been due to the fact that no attention was paid to when the fish were last fed. The slower depletion seen in Fish 21 (Figure 8) and Fish 28 (Figure 10) may have been due to slower absorption of the drug due to the fed state of the fish. The rapid absorption of CL-11,366 in the other fish may be explained by the fact that it was administered in the acid form (see Appendix I for drug preparation). As such, the solution injected had a pH of 3.3, so that most of the injected drug was in the nonionized form and could therefore pass into the blood stream quite rapidly, where it would become ionized. When a Na salt of CL-11,366 was prepared and administered IP (Fish 35) at a dose of 2.5 mg/kg, the O_2 decay was much slower and incomplete after 45 min (Figure 11) when compared to fish receiving comparable doses (Figures 8 and 9). The O_2 decay was not due to the acidity of the solution administered, since an IP injection of 0.1N HCl (pH 1.5)

had no effect on the ocular O_2 concentrating mechanism (Figure 12), nor did 0.5 cc of Ringer solution alone (Figure 12). Figures 10 and 11 show a dose-response effect of CL-11,366, with the lowest doses giving the longest delay before onset and completion of inhibition of the O_2 concentrating mechanism. In addition, the fish showed little evidence of regaining their ability to concentrate O_2 for up to 3 hours after drug administration, probably because the choroidal rete CA remained inhibited during this time (Table 1). The reader is cautioned against interpreting the lowered CA activity in the retina as being due to inhibition by CL-11,366. The apparent inhibition of retinal CA shown in Table 1 can be explained by the loss of the pigment layer. Normally, when the retina is removed for analysis of CA activity the pigment layer remains attached, but this is not the case with fish that have been treated with a CA inhibitor. In these fish the pigment cells apparently contract to the extent that they are no longer in contact with the retina. A similar observation involving pigment contraction is commonly made on fish that have been placed in the dark for a long period of time (Maetz, 1956). The lower activity for retinal CA in the fish treated with the inhibitor therefore reflects loss of the pigment cells which contain CA.

As an additional control experiment the effects of acetazolamide (Diamox) and CL-13,850 were compared. CL-13,850 is

FIGURE 12. --Per cent of initial ocular P_{O_2} vs. time (min) after IP injection of control solutions. Values in parentheses are the initial maximum ocular P_{O_2}. Fish 24 received 0.5 ml of 0.1N HCl (pH 1.5); Fish 37 received 0.5 ml of a 5.0 mg/ml solution of CL-13,850. The dose level for this fish is given in Table 1.

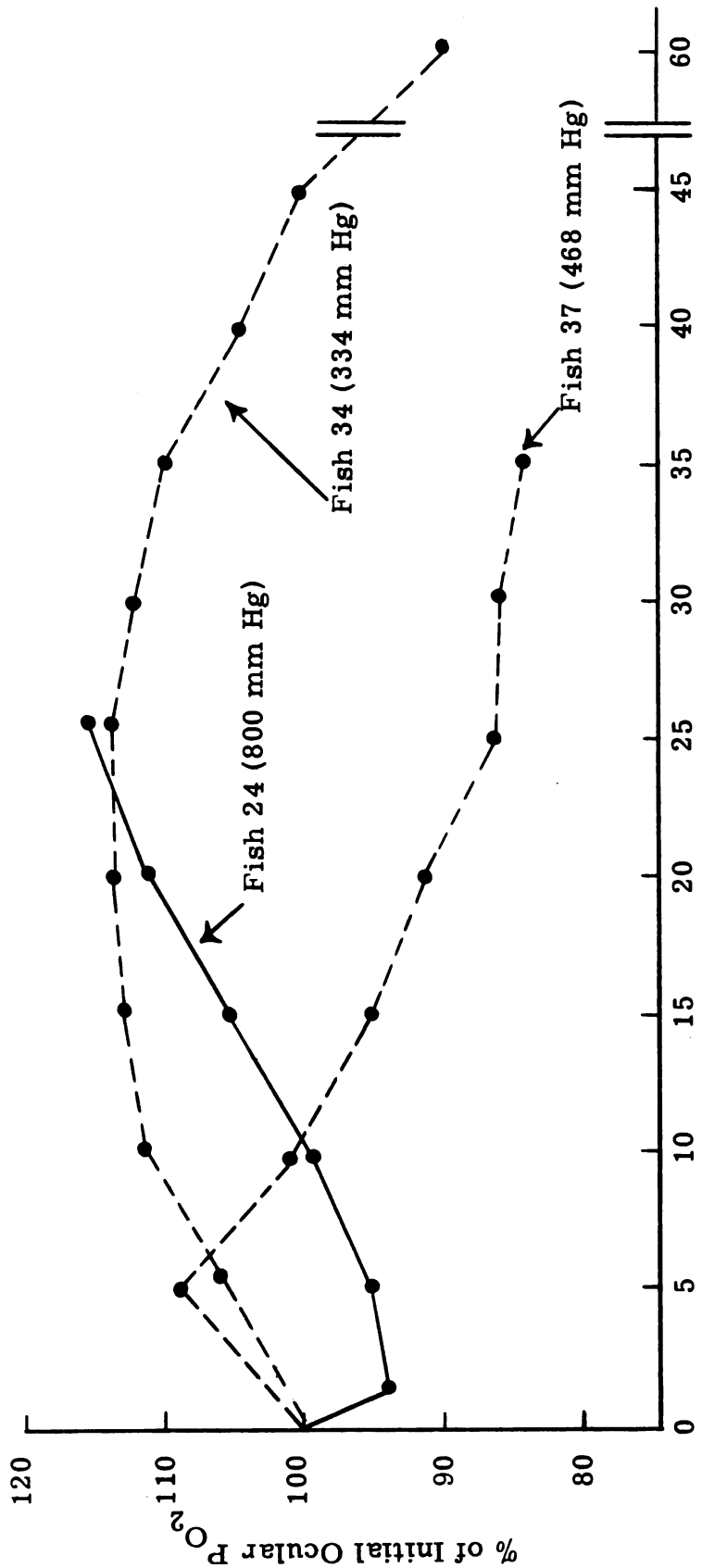

% of Initial Ocular P_{O_2}
120
110
100
90
80
0
5
10
15
20
25
30
35
40
45
60
Time (min)
Fish 24 (800 mm Hg)
Fish 34 (334 mm Hg)
Fish 37 (468 mm Hg)

FIGURE 12

a structural analogue of acetazolamide (see Appendix I) but has no carbonic anhydrase inhibitory activity (Gray et al., 1957). Fish 27 (Figure 9) received 20.9 mg acetazolamide/kg while Fish 37 received 24.8 mg CL-13,850/kg. With acetazolamide, inhibition of the O_2 concentrating mechanism was complete within 2 min after drug administration, while CL-13,850 had little (if any) effect on O_2 concentration. In a second fish (not illustrated) treated with CL-13,850 (21.6 mg/kg) there was an apparent depletion of O_2, with the P_{O_2} dropping to 9.9% of the initial value 38 min post injection. However, when the electrode was lowered 0.1 mm, the recorded P_{O_2} rose to 23.2% of the initial value and finally to 92.6% of the initial value when lowered 0.3 mm further (in 0.1 mm steps). Since the P_{O_2} began to drop immediately after the injection, the apparent O_2 depletion was no doubt an artifact caused by a change in electrode position. On the other hand, the O_2 depletion seen after administration of CL-11,366 and acetazolamide (Figures 13 and 14) is not an artifact of electrode displacement. In these two fish the region of maximal P_{O_2} was located, after which the fish were given 0.5 ml of inhibitor. After complete arrest of the ocular O_2 concentrating mechanism, the electrode was lowered further into the eye, in a stepwise fashion, until the displacement of the ocular globe, due to the electrode pressing against the posterior sclera, was very

FIGURE 13. --Ocular oxygen depletion curve after administration of 0.5 ml of a 0.45 mg/ml solution of CL-11,366 (Fish wt = 105g). The initial P_{O_2} is given in parentheses next to the fish number. The curve is a plot of this initial value vs. time (min) after injection of CL-11,366. After oxygen depletion was complete the electrode was lowered further into the eye from its initial depth of 8.1 mm.

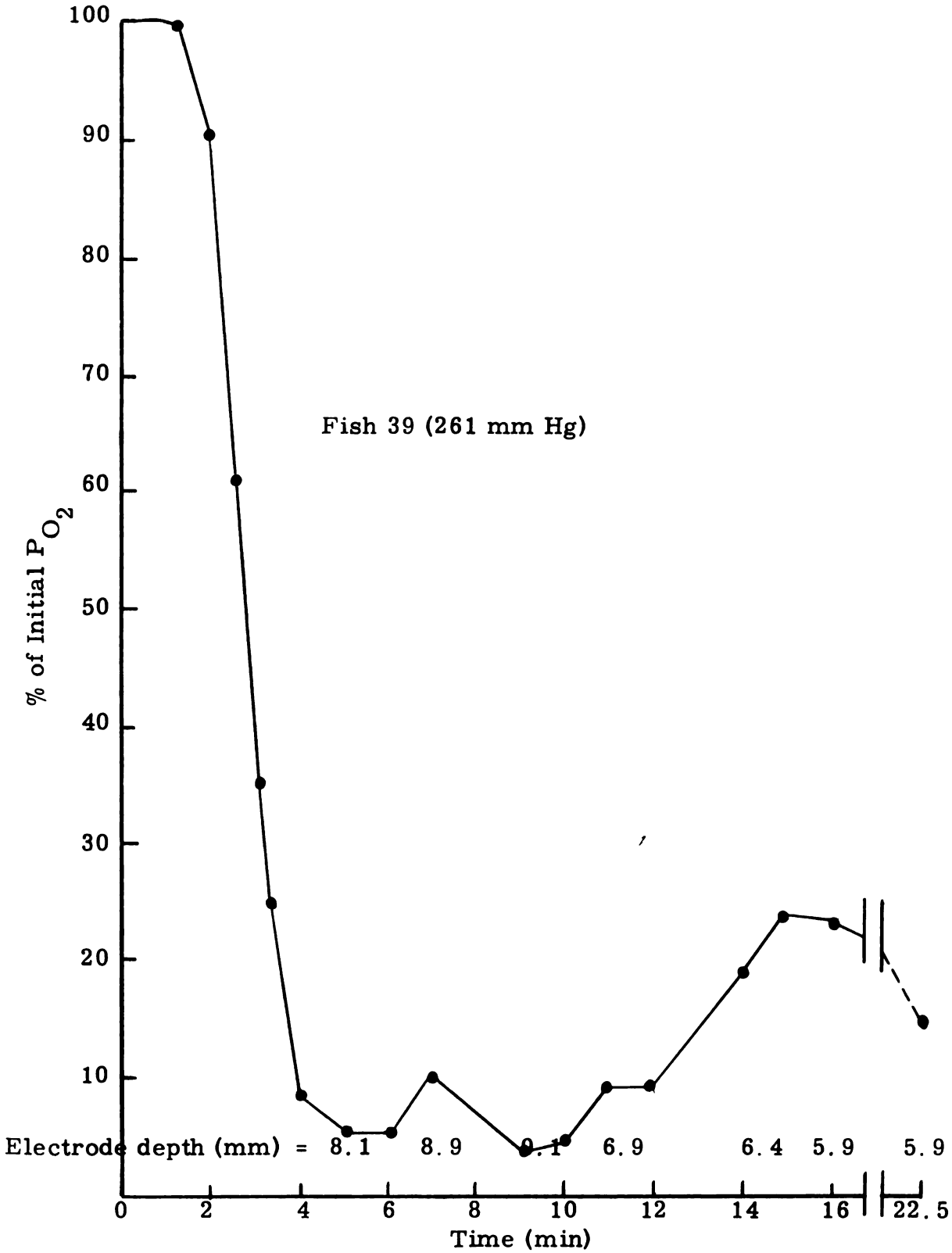

Fish 39 (261 mm Hg)
% of Initial P_{O_2}
100
90
80
70
60
50
40
30
20
10
Electrode depth (mm) = 8.1 8.9 9.1 6.9 6.4 5.9 5.9
0
2
4
6
8
10
12
14
16
22.5
Time (min)

FIGURE 13

FIGURE 14. --Ocular oxygen depletion curve after administration of 0.5 ml of a 2.5 mg/ml solution of acetazolamide (Fish wt = 116g). The initial P_{O_2} is given in parentheses next to the fish number. The curve is a plot of this initial value vs. time (min) after injection of acetazolamide After oxygen depletion was complete the electrode was lowered further into the eye from its initial depth of 7.5 mm.

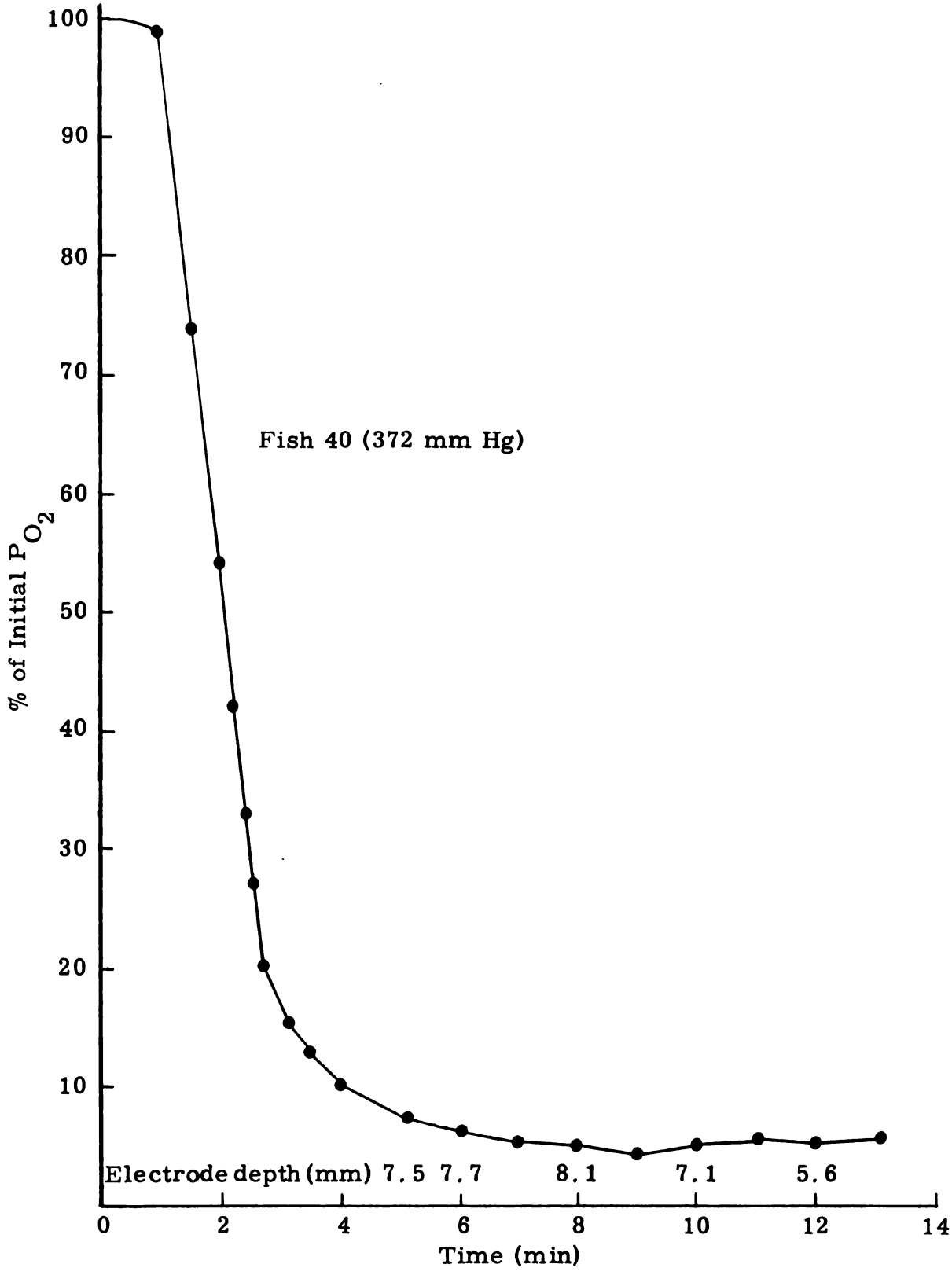

100
90
80
70
60
50
40
30
20
10
% of Initial P_{O_2}
Fish 40 (372 mm Hg)
Electrode depth (mm) 7.5 7.7 8.1 7.1 5.6
0
2
4
6
8
10
12
14
Time (min)

FIGURE 14

evident. In both fish this procedure did not locate a region of high O_2.

Even though the carbonic anhydrase inhibitors used in this study have no reported pharmacological effect other than those that result from CA inhibition, the rapid depletion of O_2 observed after drug administration prompted the author to look for a possible effect on circulation. The circulation time (Table 2) was measured as described in the Materials and Methods section and was the same for the control and CL-11,366-treated fish. The technique utilized in these measurements is admittedly unrefined but should have been sufficient to pick up any large increase or decrease in circulation time, which the author believes would have been necessary to cause the rapid ocular O_2 depletion.

Movement of CL-11,366 Into Tissues

Since O_2 depletion begins within minutes after a 2.5 mg/kg IP dose of CL-11,366, it was necessary to determine CA concentrations in the tissues involved in O_2 concentration within minutes after CL-11,366 administration (Table 3). There is no clear cut inhibition of CA in any of the tissues until 6 min post injection, but the inhibitor (CL-11,366) is present in the plasma within 2 min after an IP injection of 2.5 mg/kg (Table 4, Column $I_T - I_C$). In this experiment, red

TABLE 2. -- Circulation time of fluorescein from the tail vein to the pseudobranch in Ringer solution and CL-11,366-treated fish. The time between injection of fluorescein into the tail vein and its first appearance in the pseudobranch was noted. The fluorescein injections were made 3 min after administration of 0.5 ml of Ringers or 0.5 ml of a 0.45 mg/ml solution of CL-11,366. Each fish received a bolus of sodium fluorescein (0.2 ml of a 2 gm% solution) in the tail vein.

Fish	Fish Wt. (g)	Treatment	Circulation Time (sec)
1	61	0.5 ml Ringers IP	11.60
2	93		18.87
3	87		22.49
4	67		14.31
5	77		15.09
$\bar{X}$	76.2 ± 6.34 (5)		16.47 ± 1.90 (5)
6	91	0.5 ml CL-11,366 IP	11.19
7	95		15.63
8	62		21.47
9	60		16.34
10	101		11.54
$\bar{X}$	81.8 ± 8.65 (5)		15.23 ± 1.87 (5)

mean ± SE (n)

TABLE 3. -- Carbonic anhydrase concentrations (units/μg wet weight) found in the pseudobranch, retina, and choroid of CL-11,366-treated fish (2.5 mg/kg). The time column in this table refers to the time in minutes at which the fish were killed after the IP injections of CL-11,366.

Time	n[a]	Pseudobranch	Choroid	Retina + Pigment Layer
0	11[b]	249.4 ± 47.18	501.6 ± 32.70	107.0 ± 19.42
1.5	3	185.3 ± 26.18	520.7 ± 64.37	85.1 ± 14.47
3.0	3	155.3 ± 10.97	498.7 ± 95.23	124.2 ± 12.06
6.0	3	117.3 ± 17.56[c]	265.0 ± 66.73[e]	104.2 ± 25.48
10.0	3	140.1 ± 30.45	247.8 ± 39.62[f]	86.3 ± 26.18
15.0	3	92.3 ± 11.02[d]	82.7 ± 21.31[f]	81.5 ± 14.37

mean ± SE (n)

[a] n = number of observations.

[b] Zero time data taken from control data in Table 1.

[c] $0.025 < P > 0.02$ Control vs. treated, t-test.

[d] $0.01 < P > 0.005$ Control vs. treated, t-test.

[e] $0.01 < P > 0.005$ Control vs. treated, t-test.

[f] $P < 0.001$ Control vs. treated, t-test.

TABLE 4. -- Change in red blood cell carbonic anhydrase activity after intraperitoneal administration of 2.5 mg/kg of CL-11,366. Control blood samples were taken from the fish and then a second sample was removed at 2, 4, 8 and 15 min after administration of CL-11,366.

Fish	RBC_c	RBC_T	$\frac{RBC_c - RBC_T}{RBC_c} \times 100$	I_C(%)	I_T(%)	$I_T - I_C$
1A	65.0	117.0 (2 min)	+23.5	- 23.1	-95.4	- 72.3
2A	79.8	80.4 (2min)	+ 0.8	- 25.6	-69.9	- 44.3
3A	87.7	91.1 (2 min)	+ 3.9	- 19.4	-96.1	- 76.7
$\bar{X}$	75.5	96.1	+ 9.4	- 22.7	-87.1	- 64.4
4A	275.2	195.2 (4 min)	-29.0	+ 32.1	-24.8	- 66.9
5A	114.0	88.4 (4 min)	-22.4	- 5.4	-99.3	- 83.9
6A	239.6	289.1 (4 min)	+20.6	- 23.4	-27.1	- 3.7
$\bar{X}$	209.6	132.9	-10.2	+ 1.1	-50.4	- 51.5
7A	476.3	390.4 (8 min)	-18.0	+ 31.4	-86.6	-118.0
8A	453.7	420.4 (8 min)	- 7.9	+149.0	-98.2	-247.2
9A	663.5	629.0 (8 min)	- 5.2	+189.0	-97.2	-286.2
$\bar{X}$	520.1	491.0	-10.4	+123.1	-94.0	-217.1
10A	59.9	53.5 (15 min)	-12.0	- 30.3	-68.6	- 38.3
11A	62.0	47.4 (15 min)	-30.8	- 37.6	-94.3	- 56.7
12A	91.8	88.2 (15 min)	- 4.1	+ 33.4	-71.4	-105.3
$\bar{X}$	63.0	71.2	-15.6	- 11.5	-78.1	- 66.8

RBC_c = Red blood cell CA activity (units/μl packed RBC) of control sample.

RBC_T = Red blood cell CA activity (units/μl packed RBC) after CL-11,366.

I_C = Per cent difference between red blood cell CA activity of the control sample (RBC_C) and red blood cell CA activity of a sample from the same source but in the presence of 0.1 ml of plasma. The + sign indicates activation while the - sign indicates inhibition.

I_T = Per cent difference between red blood cell CA activity of the control sample (RBC_C) and red blood cell CA activity of a sample from the same source but in the presence of 0.1 ml of plasma from the CL-11,366-treated fish.

blood cell CA activity of the zero time sample (before administration of CL-11,366) was first determined (RBC_C), and then the activity was measured in the presence of either 0.1 ml of the zero time plasma or 0.1 ml of plasma from a blood sample from the same fish taken 2, 4, 8 or 15 mins after drug administration. The per cent change in the red blood cell CA activity in the presence of the zero time plasma sample (T_C) estimates the amount of endogenous CA inhibitor (or activator) present in the plasma sample. The data obtained also indicates the presence of an exogenous CA inhibitor (CL-11,366) in the plasma as soon as 2 min after the IP injection, since I_T (CA inhibitory activity of the plasma samples from the CL-11,366-treated fish) is always greater than I_C. The indication of a plasma CA activator in the zero time samples was an unexpected result, but has been reported previously (Van Goor, 1949). This subject will receive further consideration in the discussion section.

Even though CL-11,366 rapidly enters the plasma after the IP injection, there is no significant decrease in the RBC CA activity by 15 min post injection (RBC_T). It might be argued that this result reflects a washing out of the inhibitor (in the two washings made during the preparation of the RBC's for CA analysis) rather than an inability of CL-11,366 to enter the red cell this rapidly. Washing the RBC's in this study, as well as in the others, is justified because

if the cells were not washed there would be an error due to plasma trapping in the centrifuged samples; and secondly, Maren et al. (1960) has shown that no detectable washout of CL-11,366 bound to red blood cell CA occurs even after three washings.

Bilateral Pseudobranchectomy and Chronic Carbonic Anhydrase Inhibition

Bilateral Pseudobranchectomy

Data on the ocular P_{O_2} found in fish 3 months after bilateral pseudobranchectomy or 2 weeks of chronic CA inhibition are given in Table 5. In the bilaterally pseudobranchectomized fish chromatophore expansion was noted 30 min after the operation and proceeded to the point of apparent maximal expansion, as shown by the fact that the fish became almost black, and remained so for the duration of the observation period (3 months). Other than the darkening there were no grossly observable changes in the fish, except for the fact that they gave every indication of being blind. For example, when the fish were fed they initially ignored the presence of the food and did not come to the surface to take the floating pellets as is the normal behavior. However, after the food had settled to the bottom of the tank and had been in the water for about a minute, the fish became quite excited and began to swim in an almost vertical

TABLE 5. -- Maximum ocular P_{O_2} values found in control, bilaterally pseudobranchectomized and chronic acetazolamide and CL-11,366-treated fish. For more complete data on treatment of these fish, see Methods section.

Treatment	n[b]	Maximum Ocular P_{O_2} (mm Hg)	n[b]	I_o(%)[d]
Control[a]	24	467 ± 35.7	-	---
Pseudobranchectomy	6	14 ± 4.0[c]	-	---
Acetazolamide	8	62 ± 5.7[c]	4	86.7 ± 2.41
CL-11,366	6	323 ± 77.1	2	8.8 ± 6.00

[a]The control data is the average maximum P_{O_2} found in the fish used in the studies on oxygen depletion.

[b]n = number of observations.

[c]$P < 0.001$ (Control vs. bilateral pseudobranchectomy or control vs. acetazolamide; t-test).

[d]I_o = Estimate of plasma CA inhibitor. Blood samples were taken from the fish after the ocular P_{O_2} measurement and assayed for CA activity. The samples were then assayed in the presence of 0.1 ml of plasma from the treated fish and the % inhibition calculated as previously described. From the % inhibition it appears that acetazolamide is still in the plasma while CL-11,366 has been excreted.

position with their noses at the bottom of the tank until they touched a pellet of food, which was immediately swallowed.

The retinas of these blind fish were almost totally degenerated, except perhaps for the pigment layer (Figure 15b). The optic nerve appears normal, which is not surprising since it receives its blood supply from the retinal artery, a branch of the internal carotid (Barnett, 1951). The choroidal gland of the pseudobranchectomized fish (not shown) retained most of its anatomical integrity and contained some apparently normal RBC's. In all probability these cells came from the lentiform body found in the central part of the choriocapillaris and whose capillaries ". . . freely communicate with the capillaries fed via the choroidal gland . . ." (Barnett, 1951). Even if this is the case, O_2 concentration would not occur, since there would be no countercurrent blood flow through the choroidal rete. The ocular P_{O_2} found at the back of the eye in pseudobranchectomized fish must (because of the absence of arterial choroidal rete flow) be related to circulation in the lentiform body or falciform process, both of which are supplied with blood from the retinal artery.

Acetazolamide

Chronic administration of acetazolamide (5 mg/100g) significantly lowered the ocular P_{O_2}. The fish also exhibited

FIGURE 15a. --Photomicrograph of the retina from a fish treated with CL-11,366 (0.6 mg/kg/day) for 14 days. From the pigment layer up:

1. Pigment layer in complete contracted state
2. Rod and cone layer; appears elongated due to pigment contraction
3. Nuclei of rods and cones; appear normal
4. Outer plexiform layer; appears normal
5. Bipolar cell layer; showing extensive loss of cells
6. Inner plexiform layer
7. Ganglion cell layer
8. Nerve fiber layer; reduced or absent (× 266)

FIGURE 15b. --Photomicrograph of the back of the eye from a bilaterally pseudobranchectomized fish. The structure in the upper left hand corner is the normal optic nerve, while the lens fills the lower right hand corner. Note the complete absence of the retina in this section. (× 133)

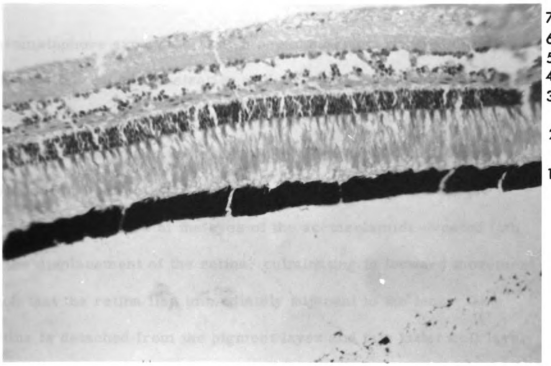


FIGURE 15a

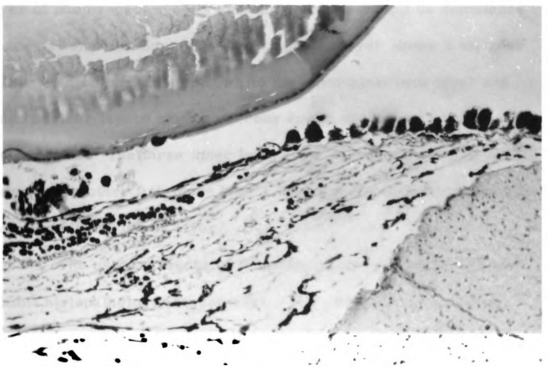


FIGURE 15b

chromatophore expansion, which became evident approximately 1 hour after the administration of the drug and was maximal within 2 hours. The chromatophores remained expanded for the duration of the study, although these fish did not become as dark as the pseudobranchectomized fish. Histologically, (Figure 16b) the most notable feature of the eyes of the acetazolamide-treated fish is the displacement of the retina, culminating in forward movement such that the retina lies immediately adjacent to the lens. The retina is detached from the pigment layer and this latter cell layer is quite contracted compared to the normal state (Figure 16a). All of the outer layers of the retina appear abnormal. The outermost layer of photoreceptor cells are clumped and exhibit no semblance of normal organization. The outer nuclear layer shows a circular rather than oblong cell type, while the outer plexiform layer and inner nuclear layers show numerous empty spaces with apparent loss of cells. The three inner layers are essentially normal in this view, but in other fish these layers have become completely detached from the inner nuclear layer.

The pseudobranchs of the acetazolamide-treated fish also exhibit histopathology (Figure 17b). The gland loses its normal compact appearance, apparently because of contraction of the cytoplasm in the acidophilic cells, resulting in clear regions bordering

FIGURE 16a. -- Photomicrograph of a normal fish retina. From the pigment layer up:

1. Pigment layer
2. Rod and cone layer
3. Nuclei of rods and cones
4. Outer plexiform layer
5. Bipolar cell layer
6. Inner plexiform layer
7. Ganglion cell layer
8. Nerve fiber layer (× 266)

FIGURE 16b. -- Photomicrograph of the retina from a fish treated with acetazolamide (50 mg/kg/day) for 14 days. From the pigment layer up:

1. Pigment layer; contracted
2. Rod and cone layer; detached from pigment layer, cells clumped
3. Nuclei or rod and cones; rounded rather than oblong
4. Outer plexiform layer
5. Bipolar cell layer
6. Inner plexiform layer
7. Ganglion cell layer
8. Nerve fiber layer (× 266)

FIGURE 16a

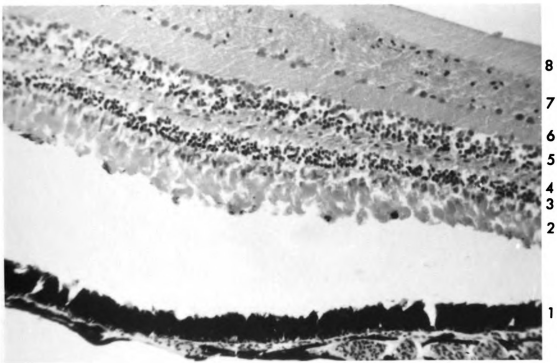


FIGURE 16b

FIGURE 17a. -- Photomicrograph of normal pseudobranch showing rows of acidophilic cells (light staining) lining capillaries filled with red blood cells (dark staining nuclei). (× 266)

FIGURE 17b. -- Photomicrograph of pseudobranch from a fish chronically treated with acetazolamide (50 mg/kg/day). Note disorientation of normal compact structure with numerous empty spaces between or within the acidophilic cells. (× 266)

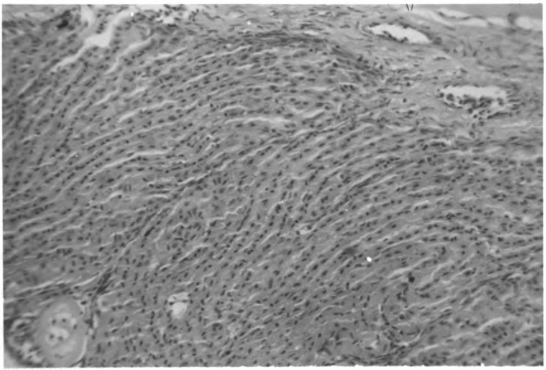


FIGURE 17a

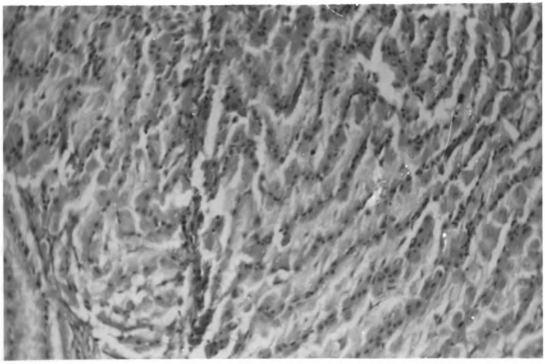


FIGURE 17b

the cytoplasm. In addition, the cytoplasm that is present is not as acidophilic as in the normal pseudobranch cell (Figure 17a).

CL-11,366 Treatment

P_{O_2} levels in the eyes of the CL-11,366-treated fish show no evidence of inhibition of the O_2 concentrating mechanism in the eye when the ocular P_{O_2} is measured 24 hr after administration of the last dose of drug. However, this must be due to excretion of the drug and reconcentration prior to the P_{O_2} measurements. This is in agreement with the fact that no evidence could be found for CL-11,366 in the plasma 24 hrs after the last dose, while there was evidence for acetazolamide being present (Table 5). In addition, O_2 depletion was demonstrated at this dose level. In other words, after the P_{O_2} values in both eyes were determined on the 15th day, one fish was given an IP injection of CL-11,366 (0.6 mg/kg) and O_2 depletion to 8.7% of the initial value observed within 30 min (Figure 18). For the first two days of CL-11,366 administration the fish showed a chromatophore reaction (darkening of the integument); but this lasted for only a few hours, and by the third day no such reaction was observable.

With CL-11,366, retinal displacement was observed; but even though contraction of the pigment layer is evident, it was not

FIGURE 18.--Ocular oxygen depletion curve of a chronic CL-11,366-treated fish. The fish was given daily injections of CL-11,366 (0.6 mg/kg/day) for 14 days and the ocular P_{O_2} determined 24 hrs after the last injection. The maximum ocular P_{O_2} at this time was found to be 230 mm Hg. The fish was then given another injection of CL-11,366 (0.6 mg/kg) and ocular oxygen depletion resulted, indicating that the dose level used in the chronic study was sufficient to cause daily inhibition of the oxygen concentrating mechanism. The per cent of the initial ocular P_{O_2} (230 mm Hg) is plotted against the time (min) after CL-11,366 administration.

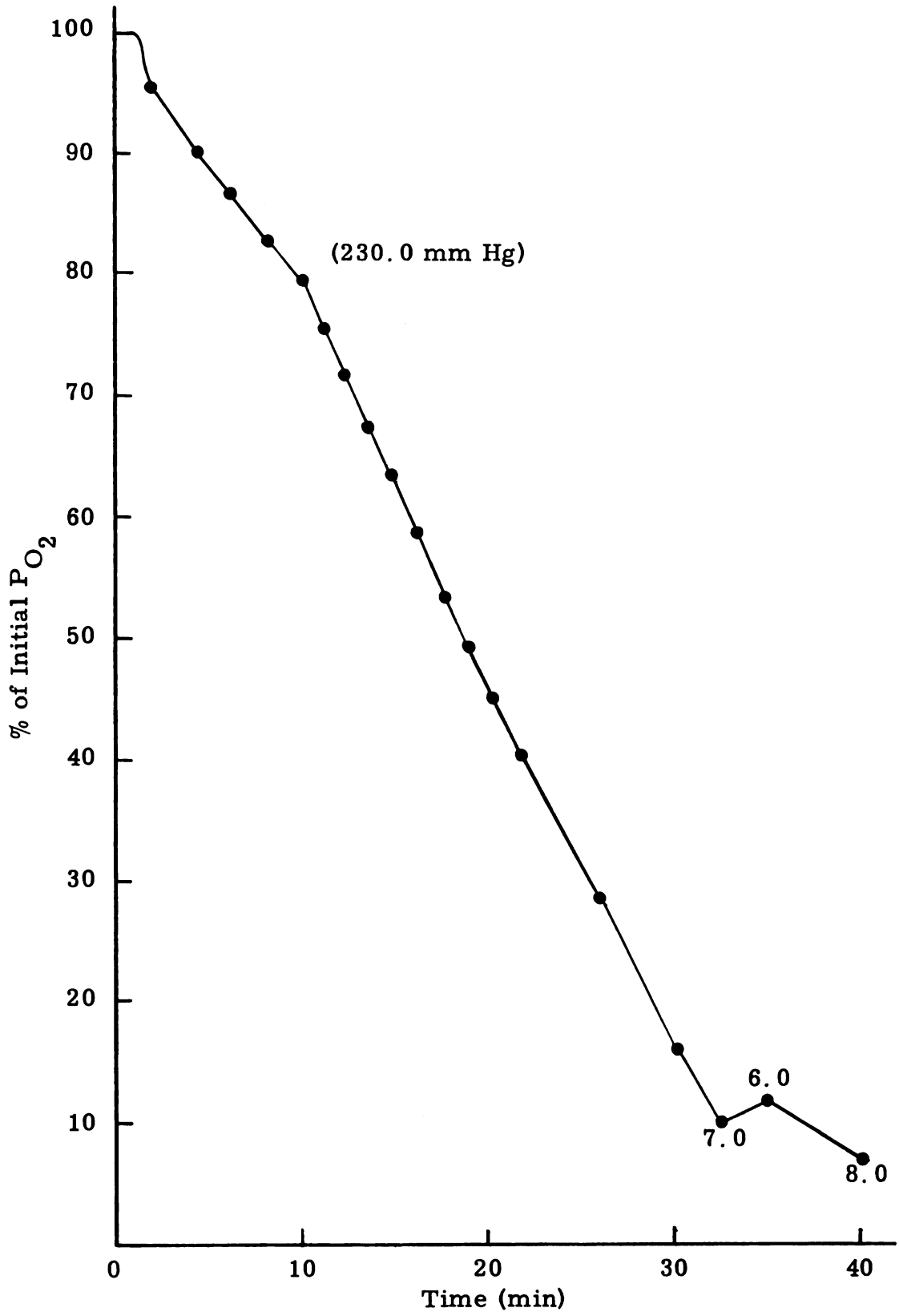

100
90
80
70
60
50
40
30
20
10
% of Initial P_{O_2}
(230.0 mm Hg)
6.0
7.0
8.0
0
10
20
30
40
Time (min)

FIGURE 18

detached from the retina. The photoreceptor layer appeared broader, but this no doubt is the result of pigment layer contraction. The inner nuclear layer is sparsely populated with cells, but it is not certain whether this is pathological or a sectioning artifact. It was very difficult to obtain good sections of the retinas from these fish and those treated with acetazolamide. In fact, the retina could be said to be "brittle," as it was shattered in most sections, with pieces distributed throughout the posterior chamber. The pseudobranchs of these fish were similar to the acetazolamide fish, although the pathology was not nearly as extensive.

Quite commonly in acetazolamide and CL-11,366-treated fish but never in controls, when the initial hole to allow insertion of the O_2 electrode was made in the cornea, there was a collapse of the cornea. Also, some of the CL-11,366-treated fish showed very different O_2 profiles than those found in the normal fish (Table 6), with high P_{O_2} values found throughout both the anterior and vitreous chamber in contrast to the low values found in the control fish.

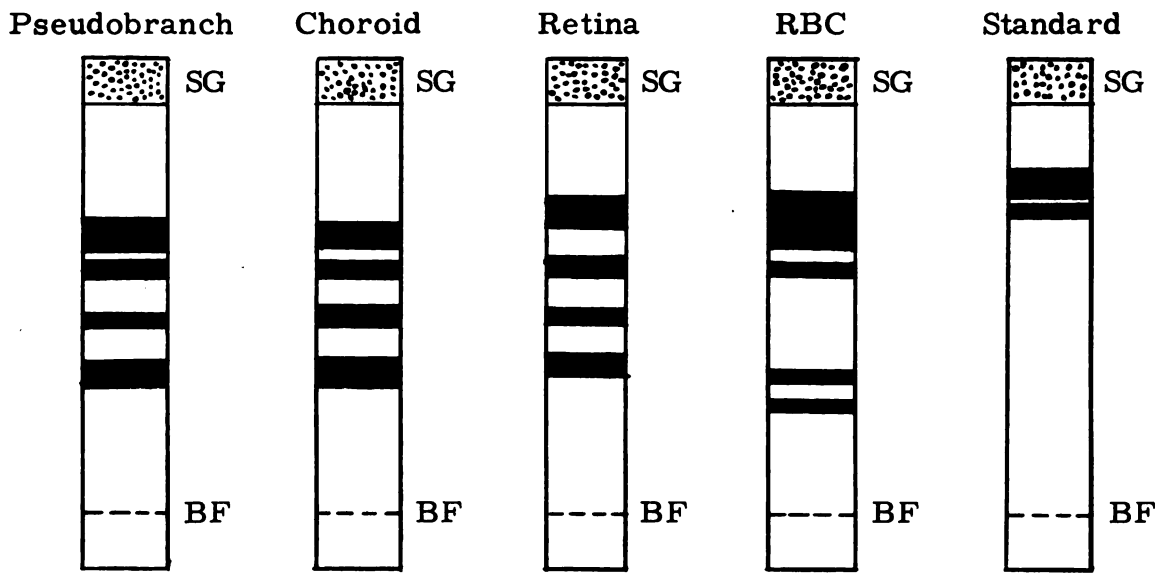
Carbonic Anhydrase Electrophoresis

Drawings of CA isozyme bands obtained from electrophoresis of the tissue samples and CA standard are presented in Figure 19. The bands are drawn to scale with respect to width, but no attempt

TABLE 6. --Electrode depth vs. ocular oxygen tension in CL-11,366-treated fish. Fish received 0.6 mg/kg/day of a 0.45 mg/ml solution of CL-11,366 for 14 days. Oxygen tension measurements were made 24 hrs after the last injection.

Electrode Depth (mm)	% of Maximum P_{O_2}					
	Fish A		Fish B		Fish C	
	Right Eye	Left Eye	Right Eye	Left Eye	Right Eye	Left Eye
Anterior Chamber	11.9	60.9	71.4	3.2	8.3	54.6
1.0	10.2	73.0	62.9	3.9	5.9	60.6
2.0	9.9	76.5	85.7	3.0	4.0	66.6
3.0	9.6	80.0	100.0	11.6	3.3	58.8
4.0	27.7	84.4	94.3	23.6	4.2	63.6
5.0	79.1	86.1	80.0	41.5	12.8	71.5
5.5	89.3	90.4	-----	-----	25.2	-----
6.0	96.6	100.0	34.3	82.1	34.1	77.6
6.5	100.0	95.7	-----	92.4	40.8	-----
7.0	-----	-----	-----	54.1	44.8	87.9
7.5	-----	-----	-----	-----	68.8	-----
8.5	-----	-----	-----	-----	100.0	100.0
8.5	-----	-----	-----	-----	46.6	42.4
Maximum P_{O_2} (mm Hg)	354	230	140	407	674	165

FIGURE 19. --Carbonic anhydrase isozymes separated in polyacrylamide gel columns. Bands are drawn schematically as to width and distance traveled but are not representative of staining density.



SG = spacer gel

BF = buffer front

Standard--Purified, bovine erythrocyte CA (Sigma Chemical Co., St. Louis, Mo.)

was made to represent the relative staining density of each band. The time of this particular run was 80 min at 5 mA/tube, with the control one-half of the gels incubated for 1 hour in 10^{-4}M acetazolamide before staining. Identical bands were found for the choroid and pseudobranch samples, whereas the RBC, retinal, and CA standard bands were located at different sites in the gel. Even though control gels were incubated in 10^{-4}M acetazolamide (a concentration 1000 times greater than that used by Hodgen and Gomes, 1969), we were unable to show any inhibition of staining. When sections of the gels that contained the stainable regions were cut out, homogenized, and assayed for CA activity, definite activity was found in the standard gel but not in sections from the sample gels. In fact, when using homogenates from these latter sections the time for the pH change from 8.1-6.3 in the electrometric assay for CA was longer than that for the distilled H_2O blank.

Rete Histochemistry

Figure 20b shows the positive staining of the rete capillaries for CA, while 20a shows the absence of staining in the sections incubated in acetazolamide (10^{-6}M) prior to staining. Disorganization of rete structure, as evident in the photomicrographs, was unavoidable in that each section had to be handled a number of times

FIGURE 20a. --Photomicrograph of choroidal rete incubated in 10^{-6}M acetazolamide showing absence of staining for CA. (× 133)

FIGURE 20b. --Photomicrograph of choroidal rete showing positive CoS stain (black areas) for CA.

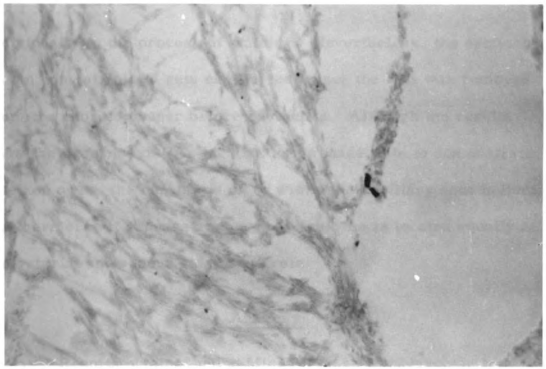


FIGURE 20a

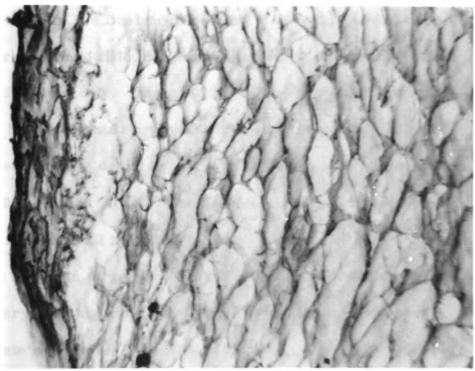


FIGURE 20b

individually in the process of staining. Nevertheless, the sections shown consist only of rete capillaries, since the rete was removed from the choroid proper before sectioning. Although the results show the presence of CA, localization is inadequate to demonstrate whether or not the enzyme is on or within the capillary endothelium. Furthermore, it appears as though the enzyme is located equally on the arterial and venous side of the rete.

Anatomy

Results obtained using Microfil as an intravascular injection compound for the purpose of tracing the vascular pattern in the eye and pseudobranch were very pleasing from the standpoint of the aesthetic nature of the final preparations. Microfil proved to be very easy to work with and filled the capillary beds of the pseudobranch and choroid quite nicely. The anatomy of the choroidal rete, as illustrated by the injected preparations shown in Figures 21 and 22, has been described in detail by many previous authors (Allis, 1908; Barnett, 1951; Maetz, 1956). The low power view of the choroid gland shows its overall horseshoe shape, with the optic nerve in the center (not shown in this view) and the opthalmic or efferent pseudobranch artery dividing into the two main branches which go on to form the rete capillaries. The close up view (Figure 21b) of a

FIGURE 21a. --Photomicrograph of the horesehoe shaped choroidal rete filled with Microfil, as viewed under low power of dissecting scope. This preparation is a view of the back of the eye after removal of the sclera. The large vessel on the inner margin of the horseshoe is the opthalmic or efferent pseudobranch artery which divided into the numerous arterial capillaries forming the compact choroidal rete. Vessels coming off the rete are part of the choriocapillaris network formed by the reuniting of the rete capillaries. (× 5)

FIGURE 21b. --Photomicrograph of choroidal rete filled with Microfil as viewed under high power of dissecting scope. Note the numerous fine arterial capillaries of the rete. The large globular structures are artifacts resulting from rupturing of the blood vessels. (× 25)

FIGURE 21a

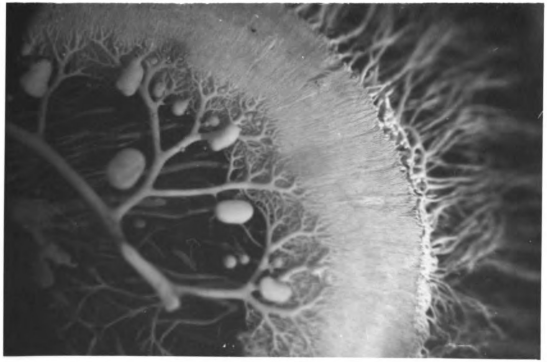


FIGURE 21b

FIGURE 22a. --Photomicrograph of a choroidal rete whose arterial capillaries were filled with Microfil (black spots). The venous capillaries surround the arterial capillaries and in many instances contain red blood cells. (× 266)

FIGURE 22b. --Photograph of a Microfil injected fish showing the pseudobranchs and communicating artery. This is an anterior view of the back of the head following the removal of the lower jaw and gills. The dorsal aspect of the head is on the right side of the photograph. The left pseudobranch is in the upper portion of the photograph, and the right pseudobranch is located in the lower half. The efferent pseudobranch arteries are clearly visible and are joined by the communicating artery which is located in the center of the photograph. The right afferent pseudobranch artery was cauterized previous to injection of the Microfil so that filling of the efferent side of this pseudobranch was by way of the left pseudobranch and the communicating artery.

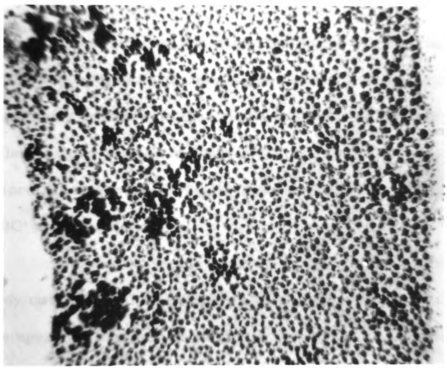


FIGURE 22a

FIGURE 22b

H_2O_2-bleached preparation shows the numerous arterial capillaries of the rete and the formation of the choriocapillaris as the arterial capillaries reunite. In these preparations only the arterial side is filled, as can be seen in Figure 22a. The black areas are filled with Microfil and in between these regions venous capillaries (containing RBC's) can be seen.

Figure 22b is a view of the back of the fish's head with the body cut away. The pseudobranchs are situated anterio-dorsally in the opercular cavity and in this preparation can be seen to have a vascular connection through the communicating artery. Previous to Microfil injection the right afferent pseudobranch artery was cauterized in this fish so that filling of the right pseudobranch with Microfil is by way of the left pseudobranch and communicating artery. The exposed side of the pseudobranch consists of the efferent filamental arteries. Although the presence of the communicating artery has been described previously (Maetz, 1956), Fairbanks et al. (1969) were unaware of its existence.

In this latter study, Fairbanks et al. (1969) found that after unilateral pseudobranchectomy the O_2 tension in the ipsilateral eye was significantly lower than the normal value but still higher than the P_{O_2} of arterial blood, indicating the retention of an oxygen concentrating mechanism. Unaware of the communicating artery, these

authors felt that the O_2 concentrating mechanism present after unilateral pseudobranchectomy must have been due to circulation through the lentiform body, a small rete mirabile in the choroid layer of the eye which supplies blood to the central region of the choriocapillaris. It now appears more probable that their result indicates O_2 concentration from blood supplied to the choroid rete from the contralateral pseudobranch, via the communicating artery, rather than O_2 concentration by the lentiform body. In fact, the inadequacy of the lentiform body as a source of nutrients to the retina is indicated by the results obtained after bilateral pseudobranchectomy, a procedure which destroys the functioning of the choroid rete but should not damage the lentiform body. In the bilaterally pseudobranchectomized fish the retina is completely degenerated (Figure 15b).

DISCUSSION

Oxygen Profile

Studies presented in this thesis indicate that in the eye of the normal rainbow trout there is a steep O_2 tension gradient across the cornea, from approximately 150 mm Hg in the water surrounding the fish to about 20 mm Hg in the anterior chamber of the eye. The gradient is reversed from the anterior chamber to the back of the eye in the region of the choroid, where average P_{O_2} values in excess of 450 mm Hg have been recorded. In some fish, this P_{O_2} gradient within the eye shows a gradual increase (Figure 4), while in others the gradient is very steep with low P_{O_2} values being recorded until the tip of the oxygen electrode reaches the retina at the back of the eye, whereupon a very high O_2 tension is recorded (Figure 5). These profiles are in contrast to those seen in fish chronically treated with the CA inhibitor CL-11,366, wherein one finds high O_2 tensions throughout the eye (Table 6) although the maximum ocular P_{O_2} is still found at the back of the eye. This result might reflect a change in the physical properties of the ocular humors. Normally the vitreous exists in a gel state while the acueous is fluid but very

viscous, approximately ten times more viscous than dog aqueous humor (Hoffert and Fromm, 1969). This high viscosity of the aqueous and the gel state of the vitreous may act as a diffusion barrier to O_2, preventing dissipation of the high O_2 tensions generated at the retina. During O_2 tension measurements of both normal and treated fish it was necessary to make a perforation in the cornea, through which the O_2 electrode was inserted. Apparently because of the very viscous nature of the fish aqueous, this perforation did not result in a large loss of ocular fluids and consequent collapse of the cornea even though the intraocular pressure would favor this sequence of events. The intraocular pressure in the rainbow trout is about 5 mm Hg above atmospheric (Hoffert, 1966), thus one could expect to see a loss of aqueous humor after the cornea was perforated with a collapse or change in the normal curvature of the cornea. Although this was not the case in the normal fish, it was seen in the fish chronically treated with CA inhibitors. It is believed that this indicates a physical change had taken place in the aqueous humor of these fish, resulting in a lowered viscosity, which could be offered as an explanation for the different O_2 profiles found in the CL-11,366-treated fish. The lowered viscosity could alter the diffusion characteristics of the ocular humors to O_2 and should facilitate diffusion into the posterior and anterior chambers.

An alternative explanation for the difference in O_2 tension profiles of control and CL-11,366-treated fish is that chronic treatment with CL-11,366 causes some destruction of the retina, resulting in a change in the O_2 consumption of this metabolically active tissue. In other words, the reason for the normal O_2 tension profile is that the retina consumes O_2 at a rate equivalent to the amount being supplied by diffusion from the choriocapillaris so that little O_2 diffuses into the vitreous or aqueous. After 14 days of treatment with CL-11,366, the O_2 consumption pattern of the retina changes, due to some pathology which has not been positively identified histologically, while the choroidal rete continues to concentrate O_2 at its normal rate. The O_2 concentrated, rather than being consumed by the retina, diffuses out into the posterior or even anterior chambers of the eye. Vindication of this hypothesis awaits O_2 consumption studies on control and acetazolamide-treated retinas, as well as ERG (electroretinogram) studies to determine the level of O_2 concentration by the choroidal rete which is necessary to sustain normal retinal function.

In a previous study, Hoffert, Fairbanks and Fromm (1970) found changes in the O_2 profiles of lake trout eyes (similar to those just described for the CL-11,366-treated fish). These changes were associated with severe ocular pathology which characteristically did

not involve the retina, although edema formation in the choroid and an actual forward displacement of the choroidal rete was a common finding. However, in the present study, even though retinal detachment was observed in the acetazolamide and CL-11,366-treated fish, no edema or change in the anatomical positioning of the choroidal rete was noted. On the other hand, there was an apparent decrease in the viscosity in the lake trout aqueous humor, as was the case with chronic CA inhibition, so that changes in the O_2 diffusion characteristics may also have taken place in the aqueous and vitreous humors of the lake trout eyes.

The retinal ocular pathology in fish associated with chronic CA inhibition has been noted previously (Maetz, 1956) and is the only reported instance in which pathology does occur at the dose levels utilized (50 mg/kg/day of acetazolamide and 0.6 mg/kg/day of CL-11,366). In fact rats have survived 900 mg/kg/day of acetazolamide or 260 mg/kg/day of CL-11,366 in their diet for up to 6 months without any observable pathological changes (Maren, 1967). Therefore, the ocular pathology in fish is a special case and deserves further consideration.

Maetz (1956) has shown that CA inhibition in the perch brings about a decrease in retinal and vitreous body pH by as much as 1.0 unit and that with the highest dose of inhibitor (100 mg/kg

acetazolamide) the pH decrease lasts beyond 48 hours. He suggested that the retinal pathology observed in these fish might be due to the pH change which would have its origin in the inhibition of retinal CA and consequent loss of the ability to rapidly neutralize retinal lactic acid. The present author is in partial agreement with this supposition but would suggest that inhibition of choroidal rete CA is more influential in the development of retinal pathology than is inhibition of retinal CA. The basis for this belief is that the O_2 concentrating mechanism of the eye can be arrested through inhibition of choroidal rete CA alone (see below), and inhibition of this mechanism will place the retina in an anoxic state. The retina, being neural tissue, is no doubt quite susceptible to anoxia. Therefore, during the chronic acetazolamide treatment (50 mg/kg/day) the retina is placed in a continuous state (15 days) of abnormally low oxygen, which might be the sole cause of the retinal pathology which develops. These fish show the same darkening reaction (chromatophore expansion) that is seen in the bilaterally pseudobranchectomized fish, a reaction that is probably indicative of retinal damage and a break in the neural link between light and the neural control of chromatophore expansion.

In addition to O_2 debt, the pH change as reported by Maetz (1956) will occur. In fact, this pH change may be a function of the anoxic state, since anaerobic lactic acid production is greater than

aerobic lactic acid production in the fish retina (Baeyens, 1970). As a result, not only will the neutralization of lactic acid be slower because of inhibition of retinal CA, but there will be more of it that requires neutralization.

In the fish chronically treated with CL-11,366 (0.6 mg/kg/day) there was no persistent darkening reaction, nor did the retina exhibit the degree of histopathology observed in the acetazolamide-treated fish. The fish did show a darkening for a few hours after each dose, but even this response was observed for only the first two days of the daily injections. This is surprising in light of the fact that even though the drug is rapidly excreted ($T\ \frac{1}{2}$ in plasma of 20 min for dogs), the retinas of these fish were subjected to daily periods of anoxia of at least 2-3 hrs (Figure 18). At the dose level of CL-11,366 used in this chronic study, it is doubtful that retinal CA was ever inhibited, based on the rapid excretion of the drug and the data obtained during the acute studies at this same dose level which showed lack of retinal CA inhibition (Table 1). This would mean that retinal CA was available for neutralization of lactic acid produced anaerobically and might have prevented irreversible retinal damage during the intervals of anoxia caused by daily inhibition of choroidal rete CA.

Chronic administration of acetazolamide (500 mg 2× daily) is currently advocated as a treatment for glaucoma in humans.

Even in light of the fact that to this author's knowledge there have been no reported incidents of ocular pathology in man associated with chronic acetazolamide treatment, a re-evaluation of the toxicity of this drug appears warranted from the observations presented here and by Maetz (1956). For example, the following questions require clarification: (1) Do pH changes take place in the retina and vitreous of mammals treated with acetazolamide? (2) If so, are these changes detrimental to retinal metabolism and visual function? (3) Can the chemical changes which take place in the vitreous and aqueous, if allowed to persist for a prolonged period of time, upset the ocular milieu intérieur to the extent that pathological changes will occur in the ocular tissues?

Oxygen Decay and Reconcentration

In the Literature Review the similarities between O_2 concentration in the eye and swim bladder were pointed out, while an important difference was omitted for discussion at this time. In the swim bladder gas concentration involves the "secretion" of gases into a gas-filled bladder which represents 7.5% of the total volume of the fish in some species (Maetz, 1956). Consequently, because of the large volume, when the swim bladder is experimentally emptied or a weight attached to a fish, the process of gas "secretion"

aimed at regaining neutral buoyancy is a slow process and may take days before completion (Fänge, 1966). On the other hand, O_2 concentration in the eye involves the "secretion" of O_2 into a liquid medium (the plasma and interstitial fluid). The fish used in the O_2 reconcentration experiments (Figure 7) had an average weight of approximately 100 g, and the wet weight of the choroid was probably less than 100 mg. Thus, assuming (1) that the blood volume of the choroid is 0.1 ml, (2) that the hematocrit is 50%, (3) that the hemoglobin O_2 carrying capacity of rainbow trout blood at 15°C is 14.0 vol% (Irving, 1941) and (4) that the O_2 solubility coefficient of fish plasma is 0.04 μl/ml/mm Hg P_{O_2} (Scholander, 1954), we can calculate the ml of O_2 which would have to be "secreted" to generate an O_2 tension at the retina of 500 mm Hg. At a retinal P_{O_2} of 500 mm Hg the Hb in the choroidal rete would be one hundred per cent saturated and therefore would contain 7 μl of O_2 based on the following calculation:

$$\begin{bmatrix}\text{Vol of choroidal}\\ \text{rete blood}\\ \text{0.1 ml}\end{bmatrix} \times \begin{bmatrix}\text{Hematocrit}\\ \text{0.5}\end{bmatrix} \times \begin{bmatrix}O_2 \text{ carrying capacity}\\ \text{14 ml/100 ml}\end{bmatrix} = \begin{matrix}\text{0.07 ml or}\\ \text{7.0 } \mu\text{l}\end{matrix}$$

In addition, 1.0 μl of O_2 is dissolved in the plasma based on the following calculation:

$$\begin{bmatrix}\text{Vol of choroidal}\\ \text{rete plasma}\\ 0.05\ \text{ml}\end{bmatrix} \times \begin{bmatrix}\text{Retinal } P_{O_2}\\ 500\ \text{mm Hg}\end{bmatrix} \times \begin{bmatrix}O_2\ \text{solubility}\\ 0.04\ \mu\text{l/ml/mm Hg}\end{bmatrix} = 1.0\ \mu\text{l}$$

Therefore the generation of the high ocular P_{O_2} at the retina requires the "secretion" of only 8 μl of O_2, whereas 7.5 ml would be required to refill an emptied swim bladder of a 100 g fish. Thus it is not surprising that O_2 reconcentration in the eye occurs rapidly, and it would be even more rapid if the retina was not consuming O_2 during the reconcentration period. The small volume of O_2 present in the rete also indicates why O_2 decay, after afferent pseudobranch artery occlusion, is so rapid. For example, Lindeman (1943) has reported retinal O_2 consumption data for the shiner (Notemigonus) of 1.25 μl/100 mg (wet wt)/min. If this data is applied to the trout retina, which for a 100 g fish has a wet wt of about 200 mg, the O_2 in the region of the retina in the example given above would be depleted in 3-4 minutes, a time which is comparable to the data given in Figure 6.

At this time it would be informative to review the rapid O_2 decay seen in fish given large doses of acetazolamide or CL-11,366. Following injection, the time interval between the onset and completion of O_2 decay was as short as 1.0 min (Figure 9), even more rapid than O_2 decay after tying off the afferent pseudobranch artery (Figure 6). This could perhaps be produced by a large increase in

the rate of blood flow through the rete which would severely limit the time available for O_2 diffusion and facilitate "washing" out the O_2. The results obtained from the fluorescein circulation times give no indication of such a change (Table 2). On the other hand, the rapid decay which did occur indicates that there was a removal of O_2 from the region of the retina, which seems only to be explained by a sudden inhibition of the choroidal rete O_2 concentrating mechanism.

This data on O_2 decay and reconcentration has some added implications contrary to the hypothesis of Parry and Holliday (1960) in which they suggested an endocrine function for the pseudobranch. These authors found that if ligatures were placed around both afferent pseudobranch arteries of brown trout, the fish would begin to darken (chromatophore expansion) within 5 minutes, and complete chromatophore expansion was evident in 12 - 15 min. If the ligature was released immediately after darkening had taken place, the chromatophores returned to a contracted state within 30 min; this change began to take place within 10 min of releasing the ligature. If the ligature was left on for a longer period (about 1 hr), the color reversal was either slow, or in some cases absent. These authors postulated that the pseudobranch secretes a substance "P" responsible for chromatophore contraction, and when circulation in the pseudobranch was shut down the fish darkened because substance "P" had been

removed from the systemic circulation. The fish returned to normal coloration after circulation through the pseudobranch is restored. If, however, the afferent pseudobranch artery remains tied off for about an hour, cell death occurred in the pseudobranch, resulting in the destruction of this gland's ability to produce and secrete substance "P." In support of their hypothesis Parry and Holliday showed that extracts of the pseudobranch could bring about a paling of fish that had been pseudobranchectomized and dark for 6 months. The fish turned a light grey color 3-4 hr after intraperitoneal or intramuscular injections of a 1 ml extract from two pseudobranchs. The paling was a temporary response, as the fish became dark again within 24 hr.

A more logical explanation of Parry and Holliday's results (excluding those involving injection of the extract) follows: Within minutes after the afferent pseudobranch arteries are tied off, the ischemic retina has consumed all the O_2 in the region of the choroid (Figure 5) and the anoxic condition causes a blackout or loss of visual function. This would be analogous to blackout in man resulting from intraocular ischemia produced by raising intraocular pressure above ophthalmic artery pressure and consequently shutting off retinal circulation, such as is seen during positive G acceleration (Anderson, 1968). Such a state can also be produced by pressing on the eyeball with the finger, in which case a moderate amount of

pressure produces blackout in a matter of seconds. In fish, with the loss of visual function the link between light and chromatophore control is broken and the fish darkens. After the afferent pseudobranch artery ligatures are removed and O_2 reconcentration begins, retinal function is restored and the fish begins to pale. However, if the ligature is left on for a long period of time, O_2 reconcentration is a slower event, as is restoration of the natural coloration, and may not occur at all if the ischemic state is prolonged to the point where irreversible damage to the retina occurs. In retrospect, it is surprising that any of the retinas in Parry and Holliday's work survived the 1 hr of afferent pseudobranch artery ligation. That the O_2 would be depleted in a matter of minutes has already been stated, but one would also expect to find the glycogen stores rapidly depleted, so that even anaerobic glycolysis as an energy source for the retina would be nonfunctional in a short period of time.

With respect to their results on injection of pseudobranch extracts, the present author has found that administration of purified CA to pseudobranchectomized fish will also cause a paling after a long delay (4-6 hr). In that the pseudobranch contains a high concentration of CA, one might be led to believe that this enzyme is the substance "P" described by Parry and Holliday. However, the results obtained by this author, and also those obtained by Parry and

Holliday, were quite variable, with some fish paling after injection of the purified CA or pseudobranch extract, and others not. Also, because of the long delay between injections and paling, it might be argued that this response is in some way linked with a foreign protein reaction. It is well known that trauma to fish elicits a chromatophore expansion, but perhaps the reverse is true in fish whose chromatophores are already maximally expanded.

Carbonic Anhydrase and Ocular Oxygen Concentration

Intraperitoneal administration of the CA inhibitor (CL-11, 366) at a dose level of 2.5 mg/kg to rainbow trout results in a sudden and dramatic arrest of the ocular O_2 concentrating mechanism, even though no significant CA inhibition can be detected in the tissues involved in ocular O_2 concentration coincident with this arrest. This is particularly surprising in view of the fact that in mammals, carbonic anhydrase inhibition is not physiologically recognizable until 99% of the tissue enzyme is inhibited (Maren, 1963). Maren's explanation for these findings deals with the concentration of CA found in mammalian tissues. In the kidney and pancreas CA is found at a concentration 500-1000 times greater than needed for the maximal physiological reaction rates, associated with this enzyme,

and similar results have been reported for stomach and red blood cell CA.

Contrary to this, it has been stated (Maren, 1967) that CA activity of fish blood is about 2% that of mammals, and quite possibly the enzyme is not present in excess in fish tissues as it is in mammals. If this is the case, then the effects seen in the eye could be due to inhibition of an undetectable (using the present methods) amount of CA. Another possibility, since the inhibitor does appear in the plasma coincident with the arrest of ocular O_2 concentration, is that a plasma CA activator is inhibited. This latter hypothesis deserves further investigation.

If arrest of the ocular O_2 concentrating mechanism is coincident with inhibition of an undetectable amount of CA, one must decide in which of the four tissues possibly involved in ocular O_2 concentration this inhibition is most likely to occur. At a physiological pH of 7.4, CL-11,366 is 99.99% ionized, and at a dose level of 2.5 mg/kg more than 90% is bound to plasma proteins. These two characteristics of the inhibitor limit the rate of its diffusion into tissues (Holder and Hayes, 1965). In the present experiments it was shown that when the fish were given 2.5 mg/kg (IP) of CL-11,366 in the acid form, the drug was rapidly assimilated into the plasma

(Table 4). However, once in the plasma, the drug would become ionized and bound to the plasma proteins. Its rate of diffusion into the red blood cells, choroidal rete, pseudobranch and retina would depend on the concentration in the plasma and the diffusional barriers (i.e., biological membranes) through which it would have to pass. Thus it is not surprising that the retina, being an avascular tissue, exhibited little inhibition of CA even 15 min after IP injection of CL-11,366 (Table 3). In other experiments, some apparent inhibition of retinal CA did occur (Table 1), but as previously explained, this was probably due to loss of the pigment layer. The pseudobranch is a very vascular tissue, but diffusion of the inhibitor into its CA containing acidophilic cells would be slowed by the presence of a number of membranes (i.e., those of the capillary endothelium as well as the cellular and basement membranes of the acidophilic cells). In contrast, diffusion of the inhibitor into red blood cells or choroidal rete capillaries would be limited by only the red blood cell membrane or the outer membrane of the endothelial cells of the rete capillaries, respectively. The CA of these latter two tissues should have shown the earliest evidence of inhibition. The results listed in Tables 3 and 4 are in partial agreement with this reasoning. Some inhibition of pseudobranch CA is evident. In part this could be due to diffusion of CL-11,366 into the acidophilic cells.

It may also have been due to the fact that CL-11,366 was present in the plasma. When the pseudobranchs were removed the still contained a certain amount of whole blood. Sonification of this tissue released the CA from the acidophilic cells, placing it in contact with the CL-11,366 in the plasma of the residue blood. The inhibition of pseudobranch CA referred to in Table 3 could therefore be an overestimate of the amount of inhibitor which diffused into the acidophilic cells of the pseudobranch. Likewise, the inhibition of choroidal rete CA may be an overestimate, since this tissue was also filled with blood when it was removed from the fish for the assay of CA activity. The degree of CA inhibition from CL-11,366 in the residue plasma of these two tissues should be approximately the same, since the blood volume of the pseudobranch and choroid is comparable (Hoffert, 1966). Nevertheless, the inhibition of choroidal rete CA is much greater than the inhibition of pseudobranch CA, indicating a substantial degree of inhibition above that due to the presence of CL-11,366 in the residue plasma. This latter inhibition reflects binding of CL-11,366 to choroidal rete CA, signifying that the CA in this tissue is quite susceptible to inhibition at the dose level used. In contrast, red blood cell CA shows little evidence of being inhibited even 15 min after injection of the drug (Table 4). This difference in *in vivo* susceptibility of choroidal rete and red blood cell CA to

CL-11,366 was not expected, since both tissues are exposed to the same concentration of inhibitor in the plasma. However, if choroidal rete CA is postulated to be on, rather than within, the endothelial cells of the rete capillaries, then this difference in susceptibility is easily explainable. Histochemical evidence for the presence of CA in the choroidal rete has been given (Figure 20b), but this technique was inadequate to determine the exact location of the enzyme (i.e., whether it is on or within the endothelial cells). The rapid arrest of the ocular O_2 concentrating mechanism does, however, imply that this enzyme is exposed to the plasma CL-11,366. Indirect evidence therefore points to choroidal rete CA being located on the surface of the rete capillary endothelial cells and also as the enzyme whose inhibition results in the rapid arrest of the O_2 concentrating mechanism. This in no way negates the importance of the red blood cell CA as the agent assuring that the Bohr or Root shift takes place at the proper time, as hypothesized by Fairbanks et al. (1969). On the contrary, it implies that more than one CA is essential to ocular O_2 concentration in the fish. Figure 23 illustrates the role of three carbonic anhydrases (retinal, RBC and choroidal rete) in O_2 concentration.

As stated previously, retinal CA is necessary for the rapid neutralization of lactic acid but probably does not enter directly into

FIGURE 23. --Scheme for the role of retinal, red blood cell and choroidal rete carbonic anhydrase in ocular oxygen concentration.

A. Retina. Retinal carbonic anhydrase facilitates the neutralization of lactic acid through catalization of the dehydration of H_2CO_3, thus assuring that the reaction proceeds to the right.
B. Choriocapillaris containing nucleated red blood cell. Red blood cell carbonic anhydrase catalyzes the hydration of CO_2 diffused from the retina to raise the intracellular H^+ concentration and facilitate the occurrence of the Bohr or Root shift before choriocapillaris blood returns to venous side of the rete.
C. Arterial capillary of choroidal rete
D. Endothelial wall separating the arterial and venous rete capillaries and containing a Cl^- - HCO_3^- pump facilitating the return of HCO_3^- to the retina where it can be reutilized for neutralizing.
E. Venous rete capillary containing carbonic anhydrase on the surface of the endothelial cell. Catalyzation of the hydration of CO_2 prevents diffusion of this gas into the arterial side of the rete where it could cause a short-circuiting of the O_2 concentrating mechanism.

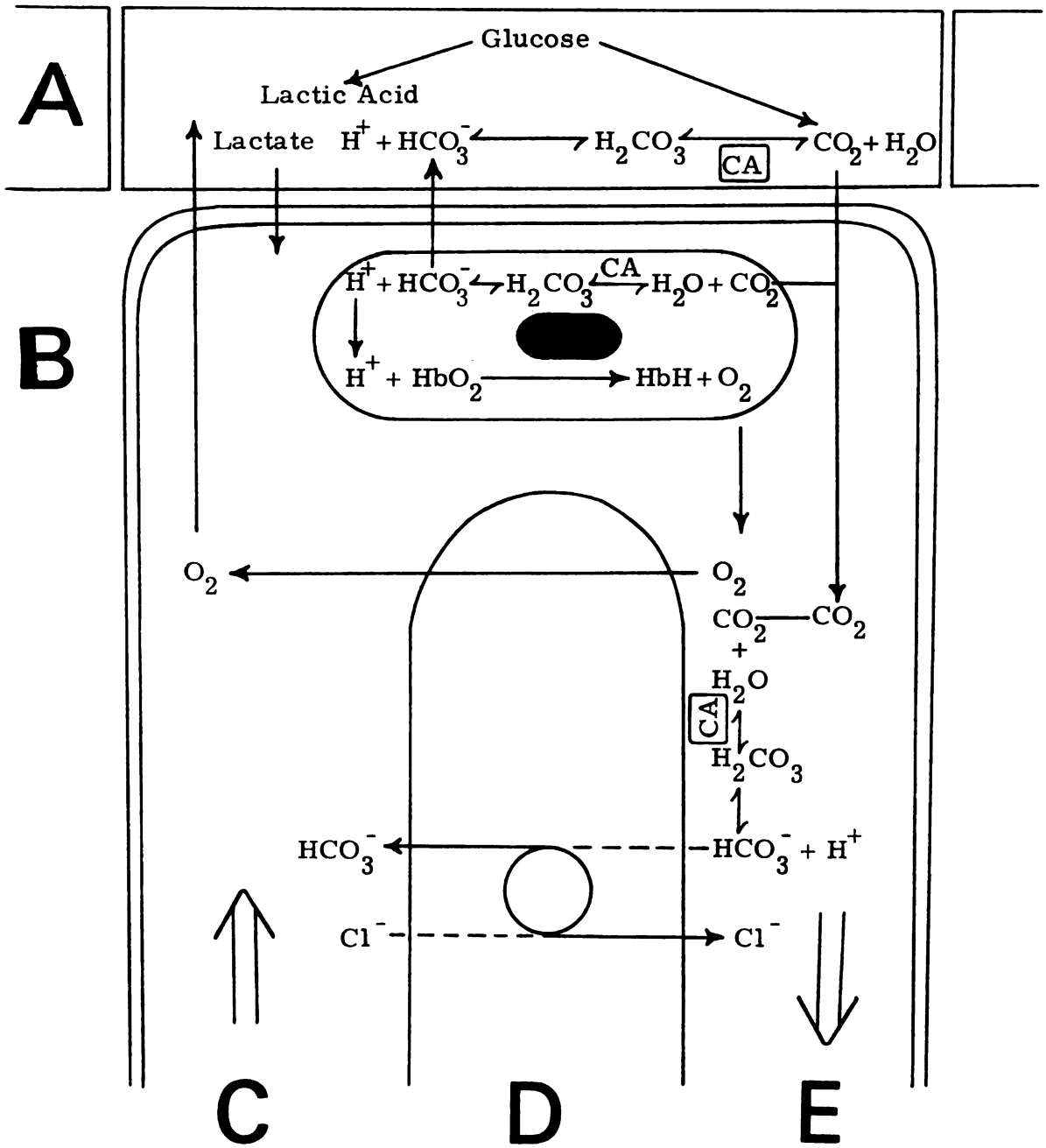

A
Glucose
Lactic Acid
Lactate
$H^+ + HCO_3^-$
H_2CO_3
CA
$CO_2 + H_2O$
B
$H^+ + HCO_3^- \rightleftharpoons H_2CO_3 \overset{CA}{\rightleftharpoons} H_2O + CO_2$
$H^+ + HbO_2 \rightarrow HbH + O_2$
O_2
O_2
CO_2
CO_2
+
H_2O
CA
H_2CO_3
HCO_3^-
$HCO_3^- + H^+$
Cl^-
Cl^-
C
D
E

FIGURE 23

the O_2 concentrating mechanism. The CO_2 formed in the retina from neutralization of lactic acid, as well as that coming from complete catabolism of glucose by way of the Krebs Cycle, diffuses into the plasma and red blood cells within the choriocapillaris. The CO_2 that enters the red blood cells is rapidly hydrated, in the presence of red blood cell CA. Dissociation of H_2CO_3 leads to the Bohr or Root shift while the blood is still in the choriocapillaris, consequently increasing the P_{O_2} of the blood entering the venous side of the rete. With the Bohr or Root shift occurring in the choriocapillaris, the rete concentrates O_2 efficiently since the entire surface area of the rete is available for O_2 diffusion to the arterial side.

Not all retinal CO_2 enters the red blood cells; some remains in the plasma, raising the P_{CO_2} on the venous side of the rete higher than that on the arterial side and therefore favoring the diffusion of CO_2 into the arterial rete blood. Such a condition would be detrimental to the O_2 concentrating mechanism of the rete in that an increase in the P_{CO_2} of the arterial rete blood could lead to a premature single concentrating effect and virtual short-circuiting of the rete. With the P_{O_2} increase occurring on the arterial side, O_2 would diffuse to the venous side and, rather than being concentrated, would be carried away from the rete in the venous blood.

To prevent this short-circuiting, CA is located on the endothelial cells of the rete capillaries (probably venous) and catalyzes the hydration of CO_2 carried in the rete venous blood. The H_2CO_3 formed dissociates to H^+ and HCO_3^-, with the HCO_3^- diffusing into the arterial blood and being returned to the retina where it can be reutilized in the neutralization of lactic acid. According to Maetz (1956), these reactions occur within the endothelial cells of the rete and the H^+ formed moves unidirectionally into the venous side. In contrast, in the present hypothesis CA is located on the surface of the endothelial cells and the reactions take place in the venous blood. The H ion never enters the rete endothelium. Movement of HCO_3^- to the arterial side is active and coupled with the active transport of Cl^- into the venous blood. This Cl^- - HCO_3^- pump is only an adjunct of the O_2 concentrating mechanism, significant because it re-cycles HCO_3^- for the neutralization of lactic acid in the retina.

Apart from the ocular O_2 concentrating mechanism, this postulated Cl^- - HCO_3^- pump in the choroidal rete may function in the formation of fish aqueous humor. In mammals, the ciliary process is responsible for aqueous humor formation. However, this tissue is absent in fish, and the site of aqueous humor secretion has not yet been determined. Hoffert (personal communication) believes

that the aqueous humor of the fish eye is continuous with a thin layer of fluid vitreous bordering the retina, a fact which enhances the possibility that a secretory process in the choroidal rete is responsible for aqueous humor formation in the fish.

Source of Choroidal Rete Carbonic Anhydrase

The possible presence of CA on the surface of the endothelial cells of the rete capillaries is a unique situation. Is this enzyme produced by specialized endothelial cells of the rete, or is it produced at some other site and transported (via the blood) to the choroidal rete capillaries? If the enzyme is indeed located on the surface of the endothelial cells, as implied from the inhibitor studies, then production and transport from some other site becomes a very real possibility. The most likely source of the enzyme, considering the latter situation to be true, is the pseudobranch, a gland that has been shown to contain large amounts of the enzyme. Furthermore, Copeland and Dalton (1959) have presented electronmicroscopic evidence showing that the acidophilic cells of the pseudobranch contain a highly specialized tubular form of endoplasmic reticulum associated with numerous mitochondria and the vascular border of the cell. They concluded that the pseudobranch is secretory in nature and probably involves the enzyme CA.

Even though the electrophoretic studies presented in this thesis are inconclusive, the specificity of the stain used suggests that the localized bands in the polyacrylamide gels do represent isozymes of CA. If such is the case, the fact that the bands for pseudobranch and choroid isozymes are exactly the same, but differ from those for the other tissues, may be used as evidence for the pseudobranch being the source of choroidal rete CA. Also in studies involving chronic inhibition of CA, histological examination of the pseudobranchs from both acetazolamide and CL-11,366-treated fish revealed apparent degenerative changes (Figure 17b), even though the concentration of CL-11,366 was insufficient to cause inhibition of pseudobranch CA. Rather than degenerative changes, these findings may indicate an exhaustive holocrine secretion by the pseudobranch in response to drug-induced arrest of the ocular O_2 concentrating mechanism. That is, some feedback mechanism from the eye may direct the pseudobranch to produce and secrete CA in an attempt to restore the O_2 concentrating mechanism. The author is prompted to this speculation by the intricate anatomical relationship between the pseudobranch and choroidal gland, including a communicating artery which appears to be further refinement in assuring a ready supply of pseudobranch CA to both eyes. Laurent and Dunel (1966) have described nerve fibers which terminate on the acidophilic cells

of the pseudobranch, although there is no evidence as yet that these fibers represent the terminal link in a reflex arc coordinating ocular O_2 tension and the release of pseudobranch CA.

Contrary to this hypothesis, Maetz (1956) has concluded that the pseudobranch is not a source of CA for the eye. He was unable to find any difference in the CA content of afferent and efferent pseudobranch blood. However, it would be worthwhile to duplicate his experiment in fish whose O_2 concentrating mechanism was inhibited, in which case secretion of pseudobranch CA would be called for and detectable amounts might possibly be found in the efferent blood of the pseudobranch.

Any consideration of CA on the endothelial surface of capillaries must also include the possibility of an endogenous CA inhibitor in the plasma (Booth, 1938). Even though an inhibitor is present in some mammals, a general interest in this area has not developed because of the weak nature of the inhibitor and the fact that it is not present in primates or birds (Maren, 1967). The present studies indicated the possibility of an endogenous CA inhibitor in rainbow trout plasma, but they also suggested the presence of an activator (Table 4). Carbonic anhydrase activators have also been found in the plasma of mammals (Van Goor, 1948), although some authors believe they are artifacts of experimental design (Clark and Perrin, 1951).

The presence of an endogenous CA inhibitor in fish plasma could be detrimental to the ocular O_2 concentrating mechanism described in this work, either by acting on CA released by the pseudobranch or by acting on CA on the surface of the endothelial cells of the rete capillaries. On the other hand, an endogenous CA activator in fish plasma might facilitate the O_2 concentrating mechanism in some manner. Further work in this area is necessary for the positive identification of an inhibitor or activator.

SUMMARY AND CONCLUSIONS

1. Oxygen tension measurements in six rainbow trout eyes indicate a steep P_{O_2} gradient across the cornea, from approximately 150.0 mm Hg in the surrounding water to 20.5 ± 4.4 mm Hg in the anterior chamber. This gradient is reversed from the front to the back of the eye, with P_{O_2} values of 446 ± 47.7 mm Hg found in the region of the choroidal rete. Apparently little O_2 diffuses into the posterior or anterior chambers of the eye, either because the retina consumes it as rapidly as it is concentrated or because the vitreous and aqueous humor act as a diffusion barrier.

2. In fish treated for 2 weeks with the CA inhibitor CL-11,366 at a dose of 0.6 mg/kg/day, the average O_2 tension measured at the back of six eyes was 323 ± 77.1 mm Hg and was not significantly different from the mean of 24 control values (467 ± 35.7 mm Hg). The P_{O_2} in the anterior chamber of six eyes (90 ± 14.7 mm Hg) was significantly different ($p < 0.005$) from the mean of six control observations (20.5 ± 4.4 mm Hg). The

fact that the P_{O_2} in these fish was higher throughout both the anterior and posterior chambers may indicate that the retina is consuming less O_2 and/or that the permeability of the aqueous and vitreous humors to O_2 has changed. These statements are compatible with the observation of retinal histopathology and apparent decreased viscosity of the ocular humors in these fish.

3. After ligating the afferent pseudobranch artery of six fish, consequently blocking the arterial blood supply to the choroidal rete, the P_{O_2} in the region of the retina dropped to 50 per cent of the value before ligation (430 ± 82.3 mm Hg) within 71 sec. The P_{O_2} decrease continued to 5 per cent of the initial value in 4 min; this decrease can be attributed to retinal O_2 consumption.

4. If the afferent pseudobranch artery ligature is removed within 15 min, reconcentration of O_2 will take place and will reach 95 per cent of the value determined before ligation within 20 min. On the other hand, if the ligature is left on for a longer period of time (30 min), O_2 reconcentration is a slower process and may not occur if the retina is irreversibly damaged, thereby not producing sufficient acid metabolites for the "single concentrating effect."

5. After administration of the CA inhibitors CL-11,366 (2.5 mg/kg) and acetazolamide (20 mg/kg), the O_2 concentrating mechanism in the eye is rapidly inhibited (1-2 min). In some cases O_2 depletion is even more rapid than that seen after ligation of the afferent pseudobranch artery and therefore indicative of O_2 being "washed out" of the region of O_2 concentration after the mechanism for concentration is inhibited. This rapid removal of O_2 from the back of the eye cannot be attributed to a change in circulation brought about by administration of the inhibitors.

6. Even though the CA inhibitor can be found in the plasma coincident with arrest of the O_2 concentrating mechanism, significant inhibition ($\alpha < 0.05$) of CA in the tissues possibly involved in the O_2 concentrating mechanism is not evident until 6 min after administration of the drug, with only the choroidal rete CA showing a high degree of inhibition.

7. Carbonic anhydrase in the choroidal rete has been demonstrated both enzymatically and histochemically. Evidence for the functional location and possible source of choroidal rete CA comes from: (1) the rapid arrest of the ocular O_2 concentrating mechanism after drug administration, (2) electrophoresis studies of CA and (3) from chronic CA inhibition studies indicating a

holocrine secretion by the pseudobranch. These results suggest that choroidal rete CA may be located on the surface of the endothelial cells of the rete capillaries and may come from the pseudobranch.

8. Evidence is given for the possible presence of CA inhibitors and activators in the plasma of the rainbow trout. The physiological significance of these substances remains questionable.

9. Bilateral pseudobranchectomy results in complete degeneration of the retina, indicating the importance of the choroidal rete in the maintenance of retinal integrity. The average ocular P_{O_2} of six fish (14 ± 4.0 mm Hg) is significantly lower than the average from 24 control fish (467 ± 35.7 mm Hg) at $p < 0.001$. In that the lentiform body is still present for the exchange of metabolic products, the major cause of retinal degeneration may be attributed to disruption of the choroidal rete's ability to concentrate O_2 through interruption of the arterial blood supply to this structure.

10. Chronic treatment of fish with acetazolamide (50 mg/kg/day) also results in a significant lowering of the ocular P_{O_2}, 62 ± 5.7 mm Hg for eight observations with $p < 0.001$, while chronic treatment with CL-11,366 (0.6 mg/kg/day) does not.

However, because of the rapid excretion of CL-11,366, reconcentration had probably occurred prior to measurement of the ocular P_{O_2}, which took place 24 hrs after the last administration of the drug on the 14th day. Histological damage to the retina and pseudobranch is seen in both groups of fish, although it is more evident with the acetazolamide treatment. Retinal damage is attributed to the long periods of anoxia brought about by administration of the CA inhibitors.

11. A hypothesis is given for the involvement of CA from the retina, red blood cells and choroidal rete in ocular O_2 concentration in the rainbow trout.

LITERATURE CITED

LITERATURE CITED

Allis, F. P. 1908. The Pseudobranch and Carotid Arteries in the Gnathostome Fishes. Zool. Jahrb. Anat., 27:103-198.

Anderson, B., Jr. 1968. Ocular Effects of Changes in Oxygen and Carbon Dioxide Tension. Tr. Am. Ophth. Soc., 66:423-474.

Baeyens, D. A. 1970. Aerobic and Anerobic Glycolysis in the Retina of a Teleost (Salmo gairdneri). M.S. Thesis, Michigan State University. East Lansing, Michigan.

Bakker, A. 1939. Ueber Kohlensäureanhydrase in Normalen und kataraktösen Linsen. Graefer. Arch. Ophthalmal., 140: 543-552.

Ball, E., C. F. Strittmatter and O. Cooper. 1955. Metabolic Studies on the Gas Gland of the Swimbladder. Biol. Bull., 108:1-17.

Barnett, C. H. 1951. The Structure and Function of the Choroidal Gland of Teleostean Fish. J. Anat., 85:113-119.

Berg, T., and J. B. Steen. 1968. The Mechanism of Oxygen Concentration in the Swimbladder of the Eel. J. Physiol., 195:631-638.

Booth, V. H. 1938. The Carbonic Anhydrase Inhibitor in Serum. J. Physiol., 91:474-489.

Clark, A. M., and D. D. Perrin. 1951. A Re-investigation of the Question of Activators of Carbonic Anhydrase. Biochem. J., 48:495-503.

Copeland, D. E. 1951. Function of Glandular Pseudobranch in Teleosts. Amer. J. Physiol., 167:775 (abstract).

Copeland, D..E., and A. J. Dalton. 1959. An Association between Mitochondria and the Endoplasmic Reticulum in Cells of the Pseudobranch Gland of a Teleost. J. Biophys. Biochem. Cyt., 5:393-395.

D'Aoust, B. G. 1970. The Role of Lactic Acid in Gas Secretion in the Teleost Swimbladder. Comp. Biochem. Physiol., 32: 637-668.

Davenport, H. W. 1966. Physiology of the Digestive tract. Year Book Medical Publishers Inc., 35 East Wacker Drive, Chicago, 230p.

Davis, R. P. 1963. The Measurements of CA Activity, p. 307-328. In Glick, D. (Ed.), Methods of Biochemical Analysis, Vol. XI. Interscience, New York.

Denton, E. J. 1961. The Buoyancy of Fish and Cephalopods. Progr. Biophys., 11:177-234.

Fairbanks, M. B. 1968. Characterization of the Normal Ocular Oxygen Tension in the Eye of Rainbow Trout (Salmo gairdneri) using a Micro Oxygen Polarographic Electrode. M.S. Thesis, Michigan State University, East Lansing, Michigan.

Fairbanks, M. B., J. R. Hoffert and P. O. Fromm. 1969. The Dependence of the Oxygen-concentrating Mechanism of the Teleost Eye (Salmo gairdneri) on the Enzyme Carbonic Anhydrase. J. Gen. Physiol., 54:203-211.

Fänge, R. 1953. Mechanisms of Gas Transport in the Euphysoclist Swimbladder. ACTA Physiol. Scand., 30(110):1-133.

Fänge, R. 1966. Physiology of the Swimbladder. Physiol. Rev., 46:299-322.

Fonner, D. B. 1968. An Investigation of the Metabolic Pathways of Glucose and their Possible Regulation by Lactate Dehydrogenase in the Ocular Tissues of Rainbow Trout (Salmo gairdneri). M.S. Thesis, Michigan State University. East Lansing, Michigan.

Gray, W. D., T. H. Maren, G. M. Sisson and F. H. Smith. 1957. C.A. Inhibition VII. C.A. Inhibition and Anticonvulsant Effect. J. Pharm. Expt. Therap., 121:160-170.

Hansson, H. P. J. 1967. Histochemical Demonstration of Carbonic Anhydrase Activity. Histochemie, 11:112-128.

Hoffert, J. R. 1966. Observations on Ocular Fluid Dynamics and Carbonic Anhydrase in Tissues of Lake Trout (Salvelinus namaycush). Comp. Biochem. Physiol., 17:107-114.

Hoffert, J. R., and P. O. Fromm. 1969. Viscous Properties of Teleost Aqueous Humor. Comp. Biochem. Physiol., 28: 1411-1417.

Hoffert, J. R., M. B. Fairbanks and P. O. Fromm. 1970. Ocular Oxygen Concentration Accompanying Severe Chronic Ophthalmic Pathology in the Lake Trout (Salvelinus namaycush). Comp. Biochem. Physiol., Accepted for Publication 8/24/70.

Hodgen, G. D., and W. R. Gomes. 1969. Staining Carbonic Anhydrase Isoenzyme Bands in Polyacrylamide Gel. Stain. Technol., 44:303-304.

Holder, L. B., and S. L. Hayes. 1965. Diffusion of Sulfonamides in Aqueous Buffers and into Red Cells. Mol. Pharmacol., 1:266-279.

Irving, L. 1941. Temperature and O_2 Affinity of Fish Blood. Biol. Bull., 80:1-17.

Krogh, A. 1929. The Anatomy and Physiology of Capillaries. Yale University Press, New Haven, 422p.

Kuhn, W., A. Ramel, H. J. Kuhn and E. Marte. 1963. The Filling Mechanism of the Swimbladder. Experientia, 19:497-552.

Kurata, Y. 1953. Histochemical Demonstration of Carbonic Anhydrase Activity. Stain. Technol., 28:231-233.

Laurent, P., and S. Dunel. 1966. Récherches sur L'innervation de la pseudobranchie des téléostéens. Arch. Anat. Microscop. Morphol. Exp., 54:633-656.

Lindeman, V. F. 1943. A Comparative Study of the Oxygen Consumption of the Vertebrate Retina, with Special Reference to the Nucleoprotoplasmic Ratio. Am. J. Physiol., 139: 9-16.

Maren, T. H. 1963. The Relation between Enzyme Inhibition and Physiological Responses in the Carbonic Anhydrase System. J. Pharmacol. Exptl. Therap., 139:140-153.

Maren, T. H. 1967. Carbonic Anhydrase Chemistry, Physiology and Inhibition. Physiol. Rev., 47:595-781.

Maren, T. H., B. Robinson, R. F. Palmer and M. E. Griffith. 1960. The Binding of Aromatic Sulfonamides to Erythrocytes. Biochem. Pharmacol., 6:21-46.

Maetz, J. 1953. L'anhydrase carbonique dans deux téléostéens varions. Comparison des activités anhydrasiques chez Perca et Serranus. Compt. Rend. Soc. Biol., 147:204-206.

Maetz, J. 1956. Le Role Biologique de L'anhydrase Carbonique chez Quelques Téléostéens. Bull. Biol. France Belgique Suppl. XL 1-129.

Ornstein, L., and B. J. Davis. 1962. Disc Electrophoresis. Distillation Product Industries, Rochester, New York.

Parry, G., and F. G. T. Holliday. 1960. An Experimental Analysis of the Function of the Pseudobranch in Teleosts. J. Gen. Biol., 37:344-354.

Roughton, F. J. W., and V. H. Booth. 1946. The Manometric Determination of the Activity of Carbonic Anhydrase under Various Conditions. Biochem. J., 40:309-319.

Scholander, P. F. 1954. Secretion of Gases against High Pressures in the Swimbladder of Deep Sea Fishes. II The Rete Mirabile. Biol. Bull., 107:260-277.

Steen, J. B. 1963. The Physiology of the Swimbladder in the Eel, Anguilla vulgaris. III The Mechanism of Gas Secretion. ACTA Physiol. Scand., 59:221-241.

Travis, D. M., C. Wiley, B. R. Nechay and T. H. Maren. 1964. Selective Renal Carbonic Anhydrase Inhibition without Respiratory Effect: Pharmacology of 2-Benzensulfonamido-1, 3, 4, thiadiazole-5-sulfonamide (CL-11, 366). J. Pharmacol. Exptl. Therap., 143:383-394.

Van Goor, H. 1949. Carbonic Anhydrase, its Properties, Distribution and Significance for Carbon Dioxide Transport. Enzymologia, 13:73-164.

Wilbur, K. M., and N. G. Anderson. 1948. Electrometric and Colorimetric Determination of Carbonic Anhydrase. J. Biol. Chem., 176:147-154.

Wistrand, P. J., J. A. Rawls and T. H. Maren. 1960. Sulphonamide Carbonic Anhydrase Inhibitors and Intra-Ocular Pressure in Rabbits. ACTA Pharmacol. et Toxicol., 17:337-355.

Wittenberg, J. B., and B. A. Wittenberg. 1962. Active Secretion of Oxygen into the Eye of Fish. Nature, 194:106-107.

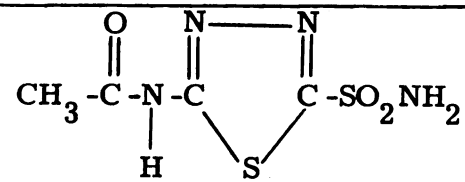
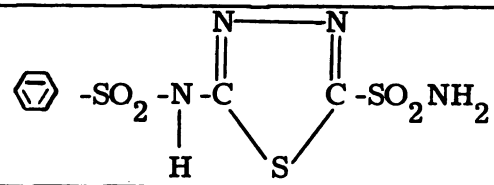
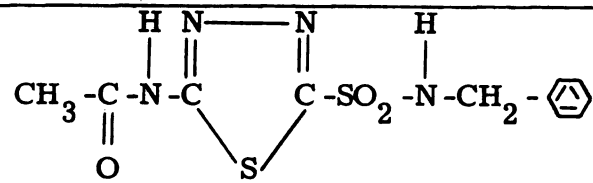
APPENDICES

APPENDIX I

DRUG CHEMISTRY, PREPARATION, AND SOURCE

APPENDIX I

TABLE 7. --Drug chemistry.

Structure	Name - Symbol (Molecular wt)	pK_A [a]	% Ionized pH 7.4 [b]	% Plasma Bound [b]		Solubility in H_2O [c]
				Dog	Man	
a 	Acetazolamide (222)	7.4, 9.1	50	50	95	45 mg%
a 	CL-11,366 (330)	3.2, 9.0	99.994	92	95	50 mg%
d 	CL-13,850 (278)	——	——	——	——	——

[a] Maren et al., 1960.

[b] Holder and Hayes, 1965.

[c] Maren, 1967.

[d] Gray et al., 1957.

Drug Preparation

CL-11, 366 (2-Benzenesulfonamido-1, 3, 4 thiodiazole-5-sulfonamide). The acid form of the drug was prepared by dissolving 0.45 ml of CL-11, 366 in 1 ml of sterile Ringer solution (Abbott Laboratories, North Chicago, Ill.). The stock solution was administered at this concentration to the fish receiving the highest dose of inhibitor and diluted to the appropriate concentration with sterile Ringer solution before administration to the fish receiving the lower doses.

Preparation of the Na salt of CL-11, 366 was achieved by adding approximately 1.2 moles NaOH per mole of drug (Maren, 1967). The pH of the acid preparation was 3.30 and that of the Na salt was 9.20.

Acetazolamide (2-Acetamido-1, 3, 1, thiadiazole-5-sulfonamide) was supplied as the Na salt. Dilutions of the drug were made with sterile Ringer solution. The pH of the solution was 8.85.

CL-13, 850 (2-Acetamido-1, 3, 4-thiadiazole-5-N-t-butyl sulfonamide). Prepared as the Na salt by addition of 1.2 moles of NaOH per mole of drug. The pH of the solution was 8.80.

Source of Drugs

Lederle Laboratories, Pearl River, New York 10965

APPENDIX II

HISTOCHEMICAL STAINING OF CARBONIC ANHYDRASE

APPENDIX II

HISTOCHEMICAL STAINING OF CARBONIC ANHYDRASE

Composition of Staining Media

Solution I	1 ml of 0.1M $CoSO_4$ 6 ml of 0.5M H_2SO_4 1 to 10 ml of 0.067M KH_2PO_4 Distilled H_2O to 17 ml
Solution II	1.875 gm%(w/v) $NaHCO_2$ (freshly prepared)
Blackening Solution	0.5 gm% $(NH_4)_2S$
Rinsing Solution	Phosphate (6.7×10^{-4}M) buffered saline (0.9%) pH 5.9

Staining Procedure (Hansson, 1967)

1. Pour solution II (40 ml) over solution I (17 ml) in a Petri dish at room temperature. Float the 10-20 μ tissue sections on the surface of the media, making sure that the sections do not dip under the surface of the medium. Time of incubation--2 min.

2. Float sections on rinsing solution (3 min).

3. Float sections on blackening solution (3 min).

4. Rinse in three successive dishes of rinsing solution (1 min in each).

5. Dry sections onto slides 5 min at 37°C. One drop of Carnoy's fixative was placed over the sections prior to drying to insure good adhesion of the sections to the slide during counterstaining.

6. Sections may be counterstained in nuclear fast red or haemotoxylin and eosin.

7. Mount sections in Permount.

8. For control sections, Solution II contained 10^{-6}M acetazolamide.

APPENDIX III

ELECTROPHORESIS

APPENDIX III

ELECTROPHORESIS

Stock Solutions

(A) 1N HCl 48 ml
2-Amino-2-Hydroxymethyl 1-3-Propandiol (Tris) 36.3 gm
N, N, N', N' Tetramethylethylene-diamine (Temed) 0.23 ml
H_2O to make 100 ml
(pH 8.8-9.0)

(B) 1N HCl 48 ml
Tris 5.98 gm
Temed 0.46 ml
H_2O to make 100 ml
(pH 6.6-6.8)

(C) Acrylamide 28.0 gm
N, N'-Methylene bis acrylamide (Bis) 0.735 gm
H_2O to make 100 ml

(D) Acrylamide 10.0 gm
Bis 2.5 gm
H_2O to make 100 ml

(E) Riboflavin 4.0 mg per 100 ml H_2O

(F) Sucrose 40.0 gm
H_2O to make 100 ml

(G) Ammonium persulfate 0.14 gm
H_2O to make 100 ml

Buffer

Tris 3.0 gm
Glycine 14.4 gm
H_2O to make 1 liter

Tracking Dye

Bromphenol Blue solution 0.005 gm per 100 ml H_2O

Working Solutions

Separating Gel Solution--Lower Gel

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

Stacking Gel Solution

1 part (B)
2 parts (D)
1 part (E)
4 parts (F)
(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 a layer of water placed on the top. Polymerization took 20 minutes under fluorescent light.

The sample gel was prepared by substituting the tissue homogenate for the sucrose (solution F) in the stacking gel. 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 CA isozymes.

Gel Staining

After electrophoresis the gels were removed from their columns and split lengthwise with a razor blade. One half of each was stained for CA according to the following procedure (Hodgen and Gomes, 1969).

1. Incubation for 1 hr in a solution made by dissolving 3.0 gm of $NaHCO_3$ in a mixture of 90 ml of 0.01M Na_2SO_4, 8 ml of 0.1M $CoSO_4$ and 2 ml of 0.05M H_2SO_4.
2. After incubation, two rinses in distilled H_2O.
3. Submersion in 5.0 ml of distilled H_2O in 15 × 80 mm test tubes, after which 0.15 ml of 20% (w/v) $(NH_4)_2S$ was added to each tube. The tubes were stoppered, inverted twice and the gels removed after 3 min.

4. After two rinses in distilled water the gels were submerged in 7% acetic acid for a 48-72 hr destaining period.

The other half of each gel was incubated for 1 hr in 10^{-4}M acetazolamide prior to staining.

MICHIGAN STATE UNIVERSITY LIBRARIES
3 1293 03056 3112