ELECTROPHYSIOLOGICAL EVIDENCE FOR THE NECESSITY OF THE COUNTER CURRENT OXYGEN MULTIPLIER MECHANISM IN MAINTAINING TELEOST VISION

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presented by

Douglas Brian Fonner

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ABSTRACT

ELECTROPHYSIOLOGICAL EVIDENCE FOR THE NECESSITY OF THE COUNTER CURRENT OXYGEN MULTIPLIER MECHANISM IN MAINTAINING TELEOST VISION

By

Douglas B. Fonner

The teleost eye has evolved a unique method of providing sufficient O_2 to its retina. This involves the generation of a superatmospheric oxygen tension (P_{O_2}) by the choroidal gland posterior to the retina. As in the secretion of O_2 into the lumen of the teleost swim bladder, the generation of a superatmospheric P_{O_2} in the teleost eye is dependent on carbonic anhydrase. The necessity of the oxygen concentrating mechanism for maintaining vision in the rainbow trout was determined by administering acetazolamide, a potent inhibitor of this enzyme. This drug was found to have a rapid and adverse effect on both the electroretinogram and the visually evoked potential from the optic tectum. *In vitro* experiments confirmed that in contrast to the frog, a P_{O_2} at arterial levels would be insufficient to maintain the functional integrity of the trout retina. In addition, *in vitro* preparations were

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employed to show that acetazolamide does not exert a direct effect on the retina that could account for the *in vivo* results.

Although the hypoxic period lasts for a minimum of 24 hr after a single injection of acetazolamide, the retina shows a remarkable ability to recover from this adverse condition. After acetazolamide treatment there is a large increase in retinal lactic acid, indicative of an increase in glycolysis. This factor, along with the continued blood flow through the choroid and the low environmental temperature (15°C) at which the trout were maintained, probably account for the ability of the trout retina to recover from the chronic hypoxia.

The DC potential across the trout retina could be altered by either an hypoxia produced by shutting off the water flow across the gills or by administering acetazolamide. During the retinal hypoxia produced by blocking the oxygen concentrating mechanism the transretinal DC potential was found to be sensitive to changes in ambient illumination. A general hypothesis was presented which could account for this phenomenon.

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ELECTROPHYSIOLOGICAL EVIDENCE FOR THE NECESSITY OF THE COUNTER CURRENT OXYGEN MULTIPLIER MECHANISM IN MAINTAINING TELEOST VISION

By

Douglas Brian Fonner

A DISSERTATION

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

DOCTOR OF PHILOSOPHY

Department of Physiology

1973

To Jill and Stacy

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INTRODUCTION

One of the primary characteristics of the vertebrate retina is its high rate of respiratory activity. In order to meet this metabolic demand many species have developed an extensive retinal vascular system. For instance, there is an arrangement of capillary nets within the human retina and in the frog a layer of superficial vessels lies between the retina and the vitreous body (Michaelson, 1954). With the exception of the eel, all vertebrates also have a choroidal circulation behind the retina. The importance of this circulation seems to vary according to the extent of the retinal vascular network.

In addition, in certain teleosts there is a special structure within the choroid called the choroidal gland. Until a few years ago the primary purpose of the choroidal gland was assumed to be the regulation of the intraocular pressure at varying hydrostatic pressures and/or the regulation of blood circulation and biochemical metabolism (Davson, 1962).

It wasn't realized until 1962 that an additional function of the choroidal gland might be the secretion of O_2 into the retina. Wittenberg and Wittenberg (1962)

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reported that the oxygen tension (P_{O_2}) at the surface of the retina in many marine teleosts was considerably above the environmental P_{O_2}. Fairbanks, Hoffert and Fromm (1969) reported that a similar phenomenon occurred in fresh water teleosts. Using micropolarographic oxygen electrodes, they found that the average P_{O_2} at the surface of the rainbow trout retina was 445 mm Hg or approximately 15 times arterial levels.

Wittenberg and Wittenberg (1962) and Fairbanks *et al.* (1969) attributed the high P_{O_2} to the ability of the choroidal gland to concentrate O_2. Fairbanks *et al.* (1969) reported that this oxygen concentrating mechanism was critically dependent upon the enzyme carbonic anhydrase.

Until the present time the functional significance of this high ocular P_{O_2} has not been extensively examined. It was the purpose of this study to answer the following questions in order to further understand the physiology of the teleost eye.

1. What are the acute and chronic effects on the rainbow trout visual system when the ocular superatmospheric P_{O_2} is reduced to arterial levels?
2. Does the trout retina require a higher P_{O_2} in order to function than retinas from other poikilotherms lacking the oxygen concentrating mechamism?

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3. Is there any change in glucose metabolism by the trout retina when the high P_{O_2} is reduced to arterial levels?

Psychophysical and electrophysiological techniques are the two generally accepted methods of appraising the visual system in laboratory animals. The psychophysical technique, however, has two major drawbacks: (1) the animal must undergo a preconditioning period in which it is trained to respond to a specific photostimulus and (2) the site or nature of any impairment to the visual system cannot be readily resolved. These limitations are not encountered by electrophysiological recordings since they can be made from specific structures and a preconditioning period is not required. As the primary interest in this study centered on the superatmospheric P_{O_2} at the retina, the electroretinogram (ERG) was employed as the monitor of the functional integrity of this tissue and acetazolamide, a specific inhibitor of carbonic anhydrase, was employed to block the oxygen concentrating mechanism. The effect on the ERG of blocking the oxygen concentrating mechanism was then compared to the effect of experimentally induced hypoxia. Changes in the ERG were also correlated with changes observed in the pigmentation characteristics of the fish as well as the visually evoked potential recorded from the optic tectum.

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In vitro studies were carried out to ascertain the minimum P_{O_2} required to sustain the functional integrity of the trout retina. The trout responses were compared with those from the retina of the frog, which lacks the ocular oxygen concentrating mechanism.

If the oxygen concentrating mechanism is required in order to prevent retinal hypoxia, the major change expected in glucose metabolism after blocking this mechanism would be an increase in retinal glycolysis. As an index of this glycolytic activity, both retinal lactic acid and lactate dehydrogenase activity were measured before and after the administration of acetazolamide.

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LITERATURE REVIEW

General Aspects of Retinal Metabolism

Glucose metabolism in the adult mammalian retina appears to be almost exclusively by way of glycolysis and the tricarboxylic acid (TCA) cycle. Indeed, the outstanding characteristic of mammalian retinal metabolism is its capacity for aerobic glycolysis as well as a marked Pasteur effect. In addition, there is evidence that the glycolytic pathway is associated with tissue maintenance and O_2 utilization with the visual cycle (Cohen and Noell, 1965).

The distribution of enzymes for glucose metabolism within a retina is in certain species related to its vascularity. In an attempt to define the cellular processes responsible for the high metabolic activity, Strominger and Lowry (1955) and Lowry, Roberts and Lewis (1956) dissected the retina into its various layers and did enzymatic studies on each layer. They found that where malate dehydrogenase (MDH) activity (indicative of the TCA cycle) was high, lactate dehydrogenase (LDH) activity was low. Conversely, there was more LDH and less MDH activity in the inner layers of the avascular rabbit retina than in the vascular monkey

retina. The outer reticular layer, which is avascular in both rabbit and monkey, was rich in LDH in the two species.

There have been few published reports regarding glucose metabolism in the teleost retina. deVincentiis (1951) reported that little lactic acid was produced by the retina of the marine teleosts *Scorpaena scrofa* and *Schylliorhinus* in the presence of 100 percent O_2. On the other hand, Baeyens (1970) found that lactic acid production by the *in vitro* rainbow trout retina at 700 mm Hg P_{O_2} was only 33 percent less than under anaerobic conditions. Based on the kinetic characteristics of the rainbow trout retinal LDH, Fonner (1968) concluded that this tissue probably depends primarily on the TCA cycle for energy rather than on aerobic glycolysis.

Santamaris *et al.* (1971) observed that O_2 consumption by the fish retina (*Eugerres plumieri*) is approximately the same as that of the mammalian retina and that the photoreceptors account for 40 percent of the total consumption. These authors also calculated the O_2 distribution in the teleost retina using the cylinder model of Krogh. Considering: (1) the O_2 consumption of the tissue; (2) the O_2 diffusion coefficient of the tissue; (3) the arterio-venous O_2 tension gradient; and (4) the intercapillary distance to the retina, they concluded that a P_{O_2} at normal arterial

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levels at the choroid-retinal interface would be insufficient to maintain retinal metabolism.

Oxygen Concentrating Mechanism

Oxygen tensions greater than the environmental P_{O_2} appear in two different organs and for two different functions in the teleost. The most widely studied of these two involves the regulation of buoyancy through the secretion of O_2 (and other gases) into the lumen of the swim bladder of fishes (Denton, 1961; Fänge, 1966). The second reported occurrence of superatmospheric P_{O_2} is in the choroid layer of the eye behind the retina (Wittenberg and Wittenberg, 1962; Fairbanks *et al.*, 1969). In both systems the concentrating of O_2 appears to rely on a rete mirabile and requires the enzyme carbonic anhydrase (Fänge, 1953; Fairbanks *et al.*, 1969).

The ocular rete mirabile is the choroidal gland which is a horseshoe-shaped structure overhanging the optic nerve at the back of the eye (Fig. 1). Its sole blood supply is through the efferent pseudobranch artery (ophthalmic artery) which comes directly from the pseudobranch (Barnett, 1951). After entering the sclera the artery divides into two branches which lie along the inner border of each limb of the horseshoe-shaped gland. Clusters of large capillaries arise almost directly from the artery and parallel one another

FIGURE 1.--Frontal view of the back of the eye showing the vascular patterns of the choroidal gland and lentiform body of the teleostean fish (after Barnett, 1951).

AC	Arterial capillaries
CH	Choroid (choriocapillaris network)
CG	Choroidal gland
LB	Lentiform body
OA	Ophthalmic artery
ON	Optic nerve
OVS	Ophthalmic venous sinus
RA	Retinal artery
RV	Retinal vein
RVS	Retinal venous sinus
VC	Venous capillaries
VCV	Ventral choroidal vein
SC	Sclera

across the width of the gland to its periphery and connect with the choriocapillaris network.

Blood is returned from the choriocapillaris vessels by venous capillaries which enter the periphery of the choroidal gland and traverse it to open into the ophthalmic venous sinus. This is drained by the ophthalmic vein. Within the choroidal gland venous capillaries are sandwiched between the arterial capillaries and blood flow in the venous capillaries is counter current to the arterial capillaries. A similar arrangement for blood vessels in the gas gland of the swim bladder led to the theory of the counter current diffusion multiplication of O_2 by a single concentrating effect (Kuhn *et al.*, 1963).

Counter current diffusion multiplication of O_2 as described by Kuhn *et al.* (1963) and as it would apply to the eye, begins with the delivery of arterial blood to the choriocapillaris network interposed between the arterial and venous capillaries of the choroidal gland. CO_2 from the retina is added to the blood as it traverses the choriocapillaris and quickly diffuses into the red blood cell. 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), this CO_2 will produce an increase in the blood P_{O_2}. The consequence of this is that the blood in the venous side of the rete will now have a higher P_{O_2}

than that in the arterial side, creating a diffusion gradient for O_2 from the venous to arterial side of the rete. This O_2-enriched arterial blood returns to the choriocapillaris where the sequence of events is repeated, leading to the concentration of O_2 within the choroid.

The critical role of carbonic anhydrase (CA) in the oxygen concentrating mechanism was first revealed in 1953 when Fänge reported that generalized CA inhibition destroys the ability of fish to refill their swim bladders with gas. Similarly, Fairbanks (1970) reported that acetazolamide, as well as other inhibitors of CA, caused a rapid drop in the ocular P_{O_2} measured at the retina with micropolarographic O_2 electrodes. Three locations have been proposed where CA may play a critical role in O_2 concentration.

1. Within the red blood cell CA catalyzes the reversible hydration of CO_2 to form carbonic acid which dissociates into hydrogen and bicarbonate ions.

$$H_2O + CO_2 \overset{CA}{\rightleftharpoons} H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

   The hydrogen ions then produce the Bohr and Root shifts and a release of O_2 into the choriocapillaris.

2. Within the retina, CA may participate in the regulation of the pH of the tissue by removing hydrogen ions from acid metabolites, e.g., lactic acid.

$$\text{Lactate}^- + H^+ + HCO_3^- \rightleftharpoons H_2CO_3 \overset{CA}{\rightleftharpoons} H_2O + CO_2$$

The CO_2 produced by this reaction then diffuses to the capillaries to produce the Bohr and Root shifts as previously shown.

3. Carbonic anhydrase on or within the cells lining the venous capillaries of the rete may prevent the diffusion of CO_2 from the venous to arterial side by converting the CO_2 to HCO_3^- and slowly diffusible H^+ (see above). CO_2 diffusing from the venous to the arterial capillaries would theoretically "short circuit" the oxygen concentrating mechanism by causing a premature release of O_2.

The three sites of action of CA are summarized in Figure 2 and for a further description and explanation the reader is referred to Fairbanks (1970).

Effect of Carbonic Anhydrase Inhibition on the Teleost Eye

Fish injected with acetazolamide begin to darken within several minutes (Fairbanks, 1970). Noting that the treated animals had difficulty in locating food, Fairbanks concluded that these fish were blind and the darkening was due to the visual-chromatophore reflex. Maetz (1956) and

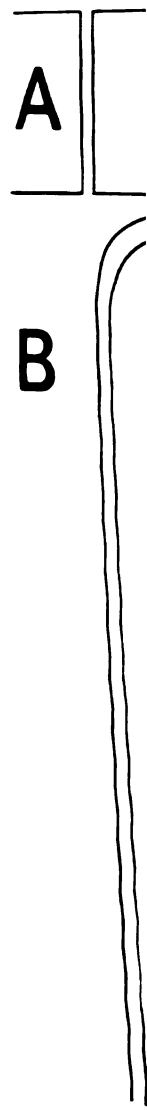


FIGURE 2.--Scheme for the role of retinal, red blood cell and choroidal rete carbonic anhydrase in ocular oxygen concentration in the teleost (from Fairbanks, 1970).

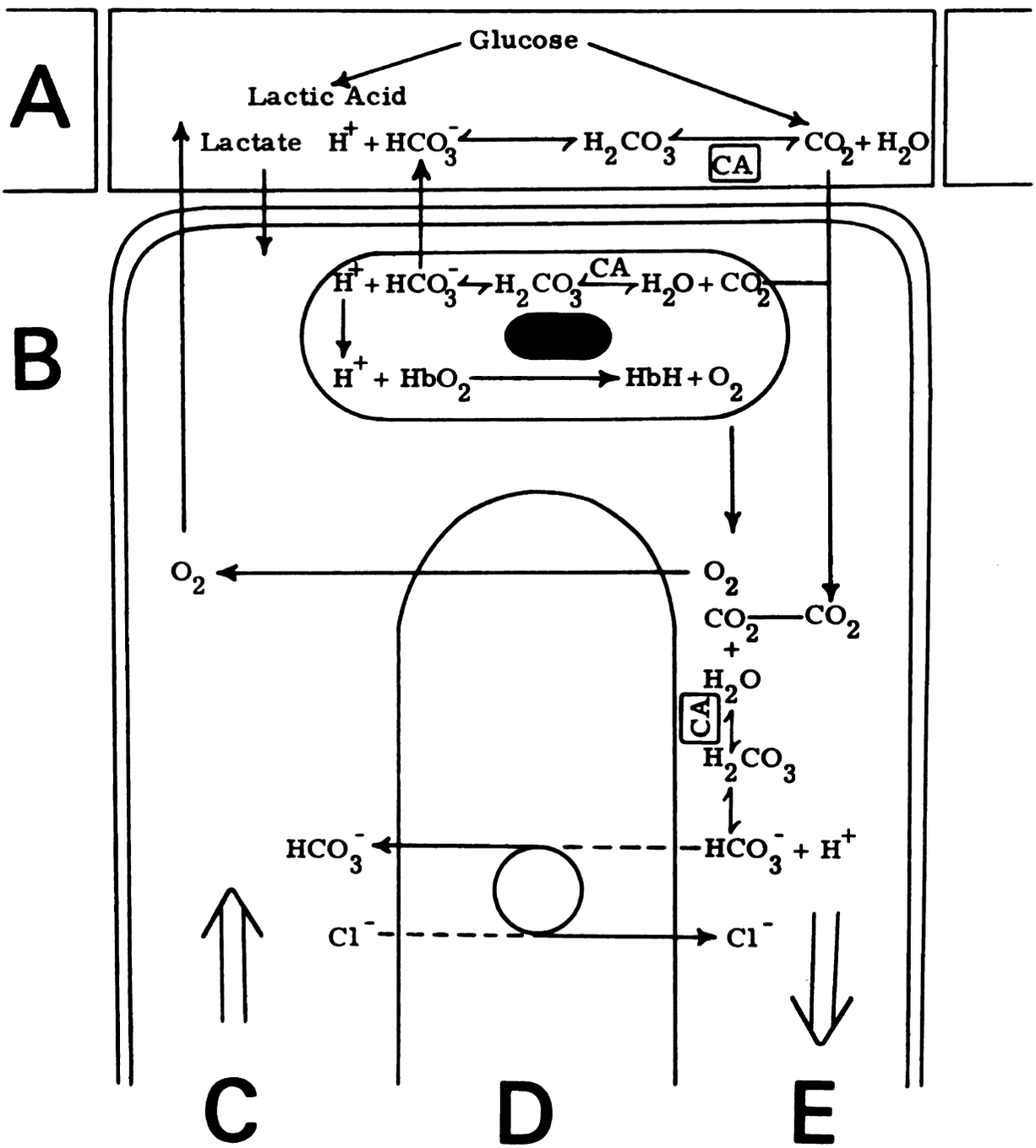
A. Retina. Retinal carbonic anhydrase facilitates the neutralization of lactic acid through catalyzation of the dehydration of H_2CO_3, thus assuring that the reaction proceeds to the right.

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 lactic acid.

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
$H^+ + HCO_3^- \leftrightarrow H_2CO_3 \overset{CA}{\leftrightarrow} H_2O + CO_2$
$H^+ + HbO_2 \rightarrow HbH + O_2$
O_2
O_2
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+
H_2O
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H_2CO_3
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$HCO_3^- + H^+$
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FIGURE 2

Fairbanks (1970) have also shown that the chronic administration of CA inhibitors causes an ocular pathology in teleosts, characterized by retinal detachment and a decrease in aqueous humor viscosity. Maetz also noted that the retinal and vitreous pH decreased by as much as 1.0 unit following CA inhibition.

Electroretinogram and Slow Oscillatory Potentials

The discovery of the electroretinogram (ERG) in 1865 by Holmgren initiated the use of electrophysiological methods for studying visual systems (Brown, 1968). The ERG is elicited by photostimulation and recorded with a pair of electrodes placed effectively across the retina. By convention the active electrode is placed on the vitreal side and the reference electrode on the receptor side. In practice this can be most readily achieved by placing the active electrode either on the corneal surface or within the vitreous body and the reference electrode elsewhere on the head.

The vertebrate ERG is a complex response which is characterized by a negative potential (a-wave) followed by a positive potential (b-wave). In some species the positive b-wave is followed by another slow positive potential termed the c-wave. The complexity of the vertebrate ERG

indicates that it consists of a number of separate components, each of which consists of the summed activity of many cells of a given class which respond in synchrony to photostimulation (Brown, 1968). While this offers the advantage of a readily recorded response from several classes of retinal cells, this same complexity has been the biggest hindrance in its use as a tool in electrophysiological studies of the retina.

The components of the ERG were analyzed in the early 1930's in terms of their disappearance under ether anesthesia (Granit, 1963). According to Granit's analysis, the ERG consists of three primary components, which he termed PI, PII and PIII, in order of their disappearance under the anesthesia. In accordance with the previous terminology, the PI component gives rise to the c-wave, PII to the b-wave and PIII to the a-wave.

In the last two decades, microelectrodes and pharmacological studies have been used extensively to examine the cellular origins of these components. The results of these studies have not always been in agreement with each other due to differences in techniques and in the criteria used in the localization of the ERG components (Murakami and Sasaki, 1968). There is a general consensus, however, that the c-wave (PI) originates in the pigmented epithelium. Noell (1954) has shown that sodium iodate

selectively abolishes the c-wave as it destroys the pigmented epithelial cells. Also, Brown and Wiesel (1961) have recorded intracellularly from the pigmented epithelium and their records show a c-wave of reversed polarity with respect to the extracellular c-wave. Although the available evidence indicates the pigmented epithelium as the probable c-wave generator, the functional significance of this wave remains unknown.

Brown (1968) and Brown and Watanabe (1962a) have provided evidence that the leading edge of the a-wave (PIII) is generated by the receptors. Brown and Watanabe (1962b) measured the PIII voltage-depth profile using micropipettes and localized the maximum PIII amplitude near the outer segments of the receptors. The presence of subcomponents that contribute to PIII have been demonstrated in the frog by Murakami and Kaneko (1966) and in the cat and rabbit by Pautler, Murakami and Nosaki (1968). These workers suggested that these subcomponents, termed the distal and proximal PIII, may arise from separate retinal structures. In an analogous manner the a-wave is often recorded as a duplex wave. According to the standard nomenclature these early and late a-waves are termed a_1 and a_2 respectively. There is considerable controversy as to the origin and significance of these two subcomponents. Clinicians who use the ERG generally describe the duplex a-wave in terms of

cone and rod responses. According to this interpretation, cone potentials generate the a_1-wave and rod potentials generate the a_2-wave (Brunette, 1972). Brunette (1972) reports that the a_1- and a_2-waves are the result of a small separate positive potential which splits the a-wave into two portions. Yonemura and Hatta (1966) found that a_1 was maximal at the level of the receptors and a_2 maximum in the bipolar layer and thus attributed the origin of the a-wave to these two respective sites. Fatechand (1971) concluded that the frog a_2 can be generated by either stimulating rods or cones and probably originates in the amacrine cell layer while the first subcomponent (a_1) has its source in the receptors.

Numerous investigators have shown that the cellular origin of the b-wave (PII) lies in the inner nuclear layer (Brown, 1968). Using a difficult technique of intracellular recording and cellular marking by dye injection, Miller and Dowling (1970) reported that the only cell type producing graded potentials of the same duration in the vicinity where the b-wave is believed to arise is the Müller cell. They hypothesized that the graded depolarization of the Müller cells is caused by the K^+ released from the bipolar cells in response to photostimulation. If this theory is correct then the b-wave can be used as a measure of bipolar cell activity although it is not produced directly by these cells.

As far as it is known ganglion cells do not contribute to any component of the ERG. This can probably be attributed to the fact that when the retina is photostimulated, the ganglion cells respond with asynchronous action potentials (spikes) rather than with graded synchronous potentials which can be recorded in the ERG.

In addition to the ERG, other slow oscillatory potentials can often be recorded across the eye. In contrast to the ERG, these potentials have been relatively ignored by investigators. One of the principle reasons for this lack of research is that they do not appear to be directly involved in the visual process. Research has also been hampered because several different loci and cell types seem to be responsible for their generation.

The pigmented epithelium, in addition to being the source of the c-wave, may also be responsible for a monophasic potential similar to the c-wave, although of much larger magnitude, observed in the rabbit when azide is injected intravenously (Noell, 1952). Prior treatment of the animals with sodium iodate, a drug which selectively destroys the pigmented epithelium, abolishes the azide response. Hanawa, Kuge and Matsumura (1967) have shown, however, that the isolated frog retina, even though devoid of its pigmented epithelium, can generate an oscillatory potential when azide is applied to the media bathing the receptor side.

The ERG as a Monitor of the Functional Integrity of the Retina

In the future the ERG should become increasingly important as an aid in research and diagnostic investigations involving the regina. At present, however, when using the ERG as an index of the functional integrity of the retina, one must view the results with some caution. In man, an increase in the intraocular pressure causes retinal hypoxia and a condition commonly termed "blackout," i.e., loss of vision. Noell (1951) reported that this "blackout" period occurs prior to a significant decline in the b-wave, indicating that the hypoxia affects a cell response not recorded in the ERG (e.g., the ganglion cells). In contrast, in the monkey, cat and rabbit, optic nerve potentials could still be recorded after the b-wave was abolished by an increase in intraocular pressure. Thus after the elimination of a component from the ERG the retina may still transmit visual information to the brain. By the same notion, the ability to record a normal ERG does not necessarily imply a completely intact visual pathway.

MATERIALS AND METHODS

Experimental Animals

Rainbow trout *(Salmo gairdneri)* (100-215 g) and lake trout *(Salvelinus namaycush)* (25-30 g), supplied by the Michigan Department of Natural Resources and the U.S. Bureau of Sport Fisheries and Wildlife, were maintained in the laboratory on a 14 hr light (60 foot candles measured at the surface of the water with a Weston light meter, Weston Electrical Instrument Corp., Newark, N.J.): 10 hr dark photoperiod. The fish were maintained in fiberglass tanks in a constant temperature room at 15±1°C. Dechlorinated tap water continuously flowing into the tanks was aerated with compressed air filtered through activated charcoal. Frogs *(Rana pipiens)* 15-20 cm long were obtained from Mogul-ED Corp. (Oshkosh, Wisc.) and maintained under the same environmental conditions as the fish except that they were not submerged in water.

In Vivo ERG Recordings from Rainbow Trout

In initial studies, 2-4 units of tubocurarine chloride (Eli Lilly and Co., Indianapolis, Ind.) injected

intraperitoneally (IP) were employed to immobilize the animals during the recording procedure. Although ERGs could be recorded from these animals for several hours, there is some criticism in the use of curare in animal experiments since this drug by itself has no anesthetic properties. For this reason MS-222 (Tricaine methanesulfonate, Ayerst Labs. Inc., N.Y., N.Y.) anesthesia was utilized in one series of experiments but pilot studies have shown that this drug has an adverse effect on the rainbow trout ERG. The most routinely used method to immobilize the animals was single pithing. A heavy pithing needle was inserted through a soft region of the skull approximately 3 mm behind the eyes. The needle was then pointed caudally and pushed into the cranial cavity and rapidly rotated several times until the animal became flacid and the rhythmic operculum movements ceased.

After immobilizing the animal with either curare or by single pithing, it was positioned on its side in a water-filled plastic chamber (24 x 7 x 8 cm) so that the eye under observation was above the surface of the water. A 13-gauge needle was inserted through both the upper and lower jaws and anchored on each side of the chamber to hold the fish in place and provide a stable preparation for recording (Fig. 3). The water-filled chamber was placed within a light-tight, electrically shielded cage and aerated water

FIGURE 3.--*In vivo* holding chamber showing the position of the trout and electrodes during an ERG recording session.

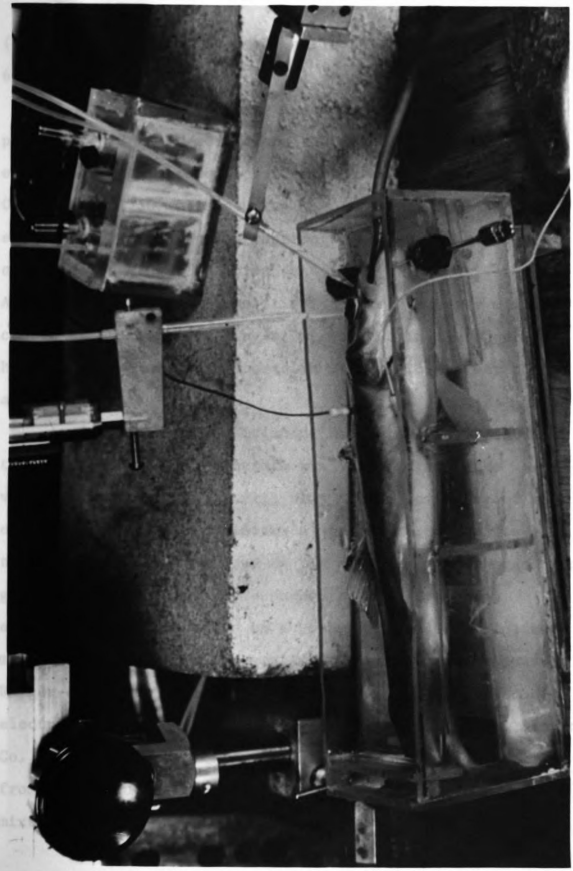

FIGURE 3

(13-15°C) was pumped over the gills at a rate of 625 ml/min.

The electrodes used in all experiments were hand pulled from 3 mm ID soft glass tubing. The tip diameters of the active and reference electrodes were approximately 0.75 mm and 1.50 mm respectively. The lower half of the electrodes were filled by suction with a warm mixture of cold blooded Ringer solution containing 4 g% agar (see Appendix I for media composition). After the agar mixture cooled and solidified, Ringers was placed in the upper half and the electrodes stored in Ringers to prevent the agar from drying.

Subsequent to positioning the animal for recording, the active recording electrode was inserted into the vitreous body through a small slit in the periphery of the cornea. The reference electrode was positioned on the tissue at the anterior edge of the orbit and the preparation grounded through the needle anchoring the fish. The electrodes were connected to a preamplifier (Grass Instruments, Model P16 AC/DC preamplifier, Quincy, Mass.) by Ringer-4 g% agar filled bridges and saturated KCl-calomel electrodes (S-30080-17 miniature calomel cells, E. H. Sargent Co., Detroit, Mich.) (Fig. 4). The agar bridges were made from PE 240 tubing and filled by suction with the same agar mixture used in the electrodes.

FIGURE 4.--Schematic diagram of the *in vivo* recording circuitry. The electrodes were led to the P16 DC preamplifier by way of agar bridges and calomel electrodes. The sweep of the oscilloscope was synchronized with the photostimulus delivered with the PS-2 photostimulator at 1 min intervals.

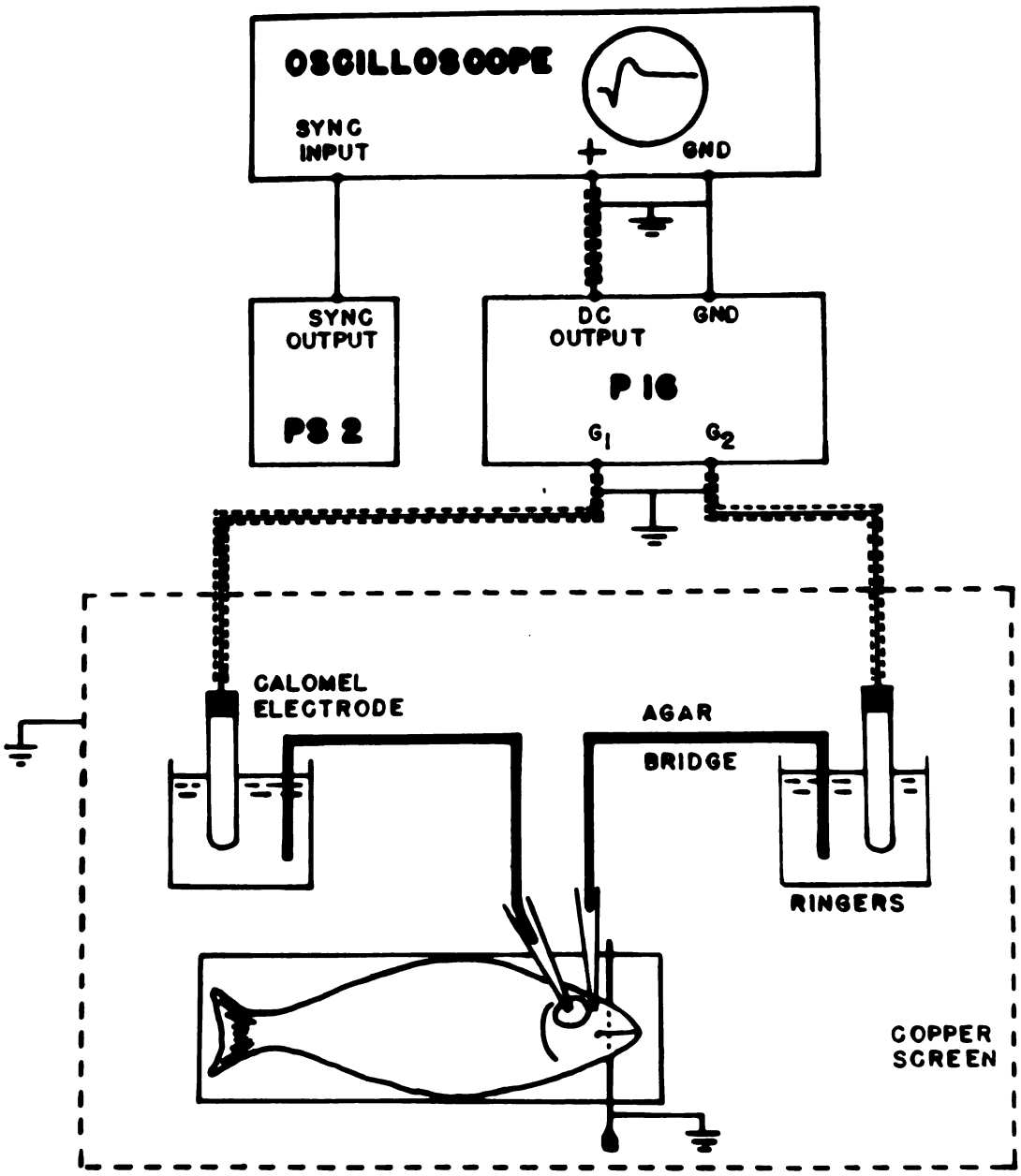
OSCILLOSCOPE
SYNC INPUT
+
GND
SYNC OUTPUT
PS 2
DC OUTPUT
GND
P 16
G1
G2
CALOMEL ELECTRODE
AGAR BRIDGE
RINGERS
COPPER SCREEN

FIGURE 4

The Grass P16 AC/DC preamplifier has independent capacitive-coupled (AC) and direct-coupled (DC) outputs. Due to the slow response of the c-wave of the ERG, the DC output was used for ERG recordings. The upper frequency response was adjusted by the rise time switch and was normally set at 1 msec (1/2 amplitude at 325 c/s). A storage oscilloscope (Tektronix Instruments, Model R5031, Dual Beam Storage Oscilloscope, Beaverton, Oregon) in the DC-coupled mode was used to display the ERGs which were photographed with a 35 mm camera. The instrumentation was calibrated with the internal calibration signal in the P16 preamplifier.

Evoked Potential Recording

To record the visually evoked potential from the optic tectum (VETP), the animal was first anesthetized in water containing MS-222 (1:15,000) and then placed in the plastic holding chamber in an upright position with the skull and both eyes out of the water. A small section of the skin and muscle overlying the skull was removed and a hole (3-5 mm diameter) was made in the skull. The underlying tissue was gently removed to expose the left optic lobe and a small piece of cotton soaked with Ringers placed on it. This cotton prevented tissue dehydration and served as an electrical junction between the optic tectum and electrode.

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The electrodes used in this study were similar to those previously described. The active electrode made contact with the piece of cotton on the surface of the optic lobe and the reference electrode was placed on the forehead, 1-2 cm from the edge of the surgical opening. During the VETP recording the animal was immobilized with tubocurare. This was administered (2-4 units, IP) after exposing the optic lobe and following recovery from MS-222 anesthesia.

The electrodes were connected in series to the Grass P16 preamplifier and the storage oscilloscope. The DC output of the preamplifier was utilized and the oscilloscope AC-coupled. The upper frequency response was limited by the preamplifier with the rise time set at 1 msec and the lower frequency response by the oscilloscope (time constant 100 msec, i.e., time for a signal to decay by 1/e from its initial value).

In those experiments in which the VETP and the ERG were recorded simultaneously, the ERG recording electrodes were connected directly to the second channel of the AC-coupled oscilloscope.

Slow DC Potential Changes

During the course of the ERG studies it was frequently observed that different experimental conditions elicited slow DC potential changes which could not be

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conveniently recorded by the oscilloscope. In order to follow these slow potentials, the oscilloscope was replaced in several experiments by a pen recorder (Mosely, Model 7100B strip chart recorder, Pasadena, Calif.) connected to the DC output of the P16 preamplifier. The two recording electrodes were the same type as used in the *in vivo* ERG studies. One electrode was inserted into the vitreous and the other into the tissue between sclera and eye socket. Prior to this the two electrodes were placed in a beaker of Ringers and the small resting potential balanced out using the "balance control" available on the preamplifier. This was repeated after the recording period to check for any drift within the recording instruments.

Photostimulation

Photostimulation was provided by a Grass Instruments PS-2 Photostimulator whose lamp housing was mounted 40 cm above the eye. The photostimulation furnished by this instrument is a 10 μsec flash whose maximal peak intensity is 1.5×10^6 candlepower (Appendix II). Through adjustments on the instrument the intensity could be decreased by 1.2 logarithmic units. Except where noted otherwise, the ERGs were elicited with the instrument set at its maximal intensity. Photostimulation was given automatically at 1 min intervals for the duration of each recording session.

Methods of Producing Retinal Hypoxia

Retinal hypoxia was produced in fish by one of two methods. An ischemic hypoxia was produced by occluding the afferent pseudobranch artery. Fish were initially anesthetized with MS-222, removed from the water and the left afferent pseudobranch artery cauterized. A piece of thread was then looped through the operculum and around the afferent pseudobranch artery on the right side of the animal. Following this procedure curare was administered and the animal placed in the holding chamber. After positioning the electrodes for recording the ERG from the right eye, the animal was dark adapted for 60 min. After this control period the thread looped around the right afferent pseudobranch artery was tied down on a short piece (ca. 1.0 cm) of gum rubber tubing, occluding that artery and preventing blood flow to the right choroidal gland. The ERGs were then monitored for the next 20 min.

In a similar experiment, the ability of the retina to recover from ischemic hypoxia was determined by releasing the ligature after a period of 12 min. In this experiment, the animals were pithed and the ERGs recorded from the right eye while in the light adapted state (ambient illumination of 15 foot candles). The recovery of the a- and b-waves of the ERG were followed for 35 min

after releasing the ligature around the right afferent pseudobranch artery.

Retinal nonischemic hypoxia was produced by shutting off the aerated water flow across the gills for 4 min. This procedure produced an asphyxic condition in the fish, since the operculum movements had previously been eliminated when the animal was immobilized with curare or by single pithing.

Acetazolamide Experiments

Both the acute and chronic effects of acetazolamide were examined. In the acute studies the animal was immobilized, placed in the holding chamber, the appropriate electrodes positioned, and an IP catheter inserted for later drug administration. The dose of acetazolamide administered (IP) in this and all other studies was 10 mg/fish (ca. 5.0-10.0 mg/100 g body weight). In examining the chronic effect of carbonic anhydrase (CA) inhibition on the teleost ERG, two different experimental procedures were employed. In the first procedure, the drug was given to a group of fish on the first day. On each succeeding day thereafter, several fish were selected randomly from this group and their ERGs recorded as previously described.

Because of the acute nature of the recording procedure employed in the above experiment, it was impossible

to follow the ERG of any respective fish on succeeding days. For this reason a modification in the recording technique was made, whereby MS-222 was used as an anesthetic and a wick electrode utilized to record the ERGs. In this latter experiment, animals were initially injected with a single dose of acetazolamide and the ERGs of each animal recorded on a daily basis over the next two weeks. The animal was placed for 5-8 min in water containing MS-222 (1:15,000), positioned obliquely on its side in the recording chamber, and wick electrodes placed on the cornea and forehead. These electrodes were similar to those previously used except that a small piece of cotton was tied to their tips. The ERG was recorded in the light adapted state immediately after the trace on the oscilloscope was stabilized. Difficulty was often encountered in making these recordings because of interference from opercular movements. Immediately after obtaining a suitable recording the animal was returned to its holding tank.

In Vitro ERG Recordings

Lake trout and frogs were dark adapted for 24 hrs. The animals were decapitated and one of the eyes immediately removed under subdued white light. The cornea and lens were removed after sectioning along the corneal-scleral junction. The posterior eye cup was cut in half and the

retina from one of the halves freed of any attached choroid and pigment layer by blotting with filter paper. During this procedure the tissue was kept moistened with the incubation media used in this experiment.

The incubating chamber (Fig. 5) was constructed from ¼" plexiglass and consisted of two compartments separated by a 4.0 mm diameter hole. These two compartments were clamped together with four 3" x 1/8" bolts and wing nuts, allowing them to be assembled within a few seconds. After the retina was dissected free it was positioned over a 4.0 mm diameter hole placed in the center of a 1 cm diameter circular piece of filter paper. The filter paper and retina were then positioned over the 4.0 mm hole, thus the retina formed a membrane separating the compartments. The two compartments were filled simultaneously with 15 ml media, whose formula is given in Appendix I. After filling, the chamber was transferred to a constant temperature water bath (14±0.5°C) within a light tight electrically shielded cage and O_2 vigorously bubbled into the media. A Beckman O_2 electrode was introduced into the media on the receptor side for monitoring P_{O_2} (Appendix III). The retina was stimulated as before with the exception that the intensity of the stimulator was reduced by 1.2 logarithmic units. In some of the lake trout recordings the a-wave was the predominate component of the ERG. In these preparations a

FIGURE 5.--Apparatus for recording the electroretinogram from the isolated frog and trout retinas. A section of a retina was placed across the opening separating the two compartments of the *in vitro* chamber. The active electrode was placed in the media bathing the vitreal side (V) and the reference electrode into the media on the receptor side (R). During the control portion of the experiment O_2 was bubbled into the media in both compartments. The PO_2 was monitored with a Beckman PO_2 electrode in the media bathing the receptor side.

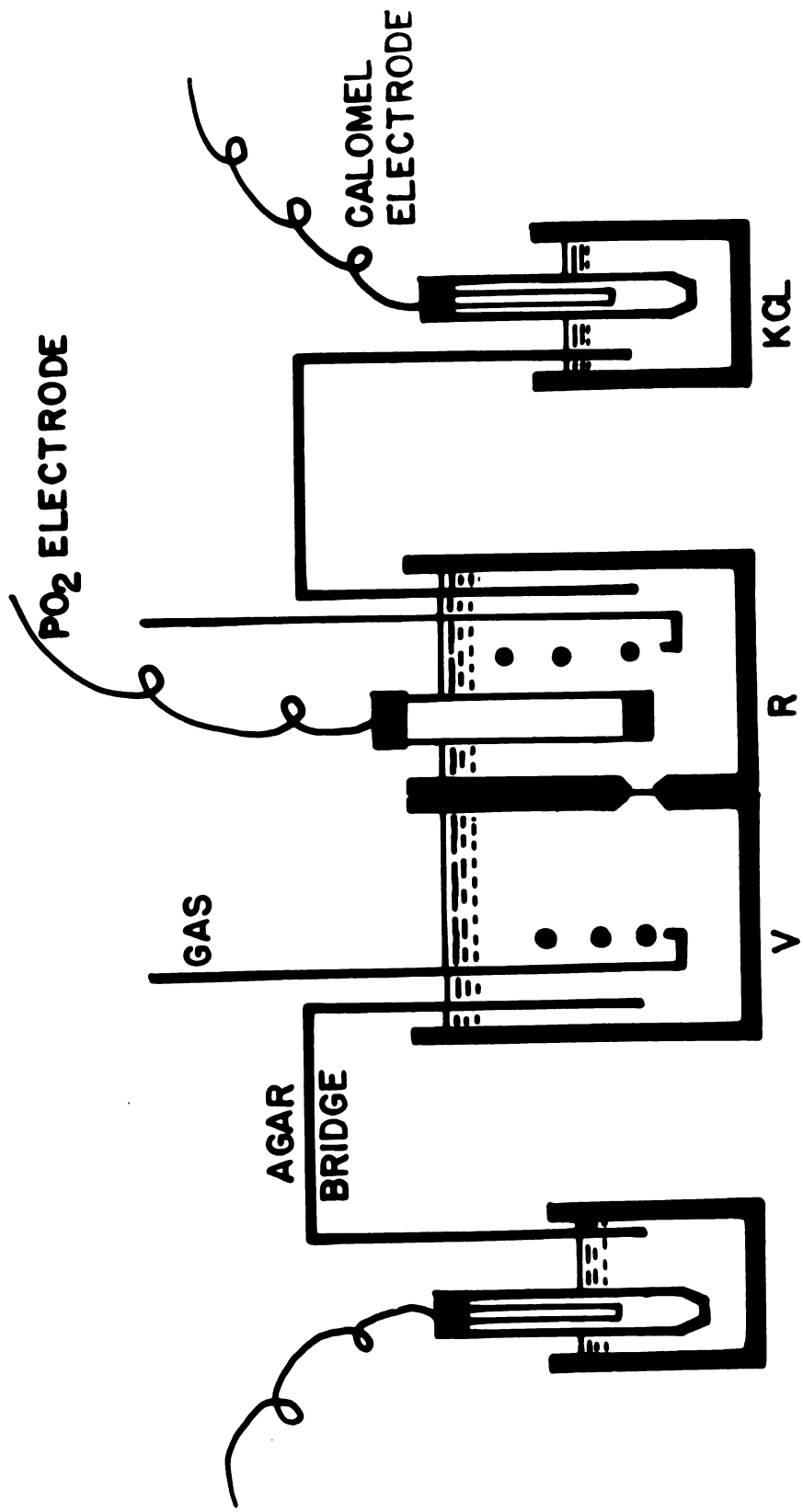


FIGURE 5

more conventional appearing ERG was recorded after inserting a red plexiglass filter (700 mμ) on the stimulator to further reduce its intensity by another logarithmic unit (see Appendix II) and thereby reduce the amplitude of the a-wave. The same recording circuitry was used as in the *in vivo* system and by convention, the active recording electrode was placed in the vitreous side.

After a control period of 60 min during which O_2 was continuously bubbled through both compartments of the incubation chamber, the P_{O_2} was reduced by bubbling N_2 into the media. Both the P_{O_2} and ERG were monitored at 1 min intervals throughout the experiment. In several experiments after the b-wave was abolished by hypoxia, O_2 was once again bubbled through the media and the ERG allowed to recover for a period of 40-60 min. After recovery 0.5 mg acetazolamide was added to the media bathing both sides of the retina and the ERGs monitored over the next 15 min.

Lactate Dehydrogenase Activity

The Wacker method (Wacker, Ulmer and Vallee, 1956) was employed for determining LDH activity. Retinas were removed from fish and placed in 5.0 ml phosphate buffer (0.1 M, pH 8.8) maintained in an ice bath. Immediately after removal the tissues were homogenized with a Branson Sonifier (Melville, N.Y.). The homogenate was centrifuged

at 2000 rpm for 10 min and the supernatant removed and immediately placed in an ice bath.

A Beckman DB-G (dual beam-grating) spectrophotometer (Fullerton, Calif.) measured the change in absorbance (ΔA) produced by the conversion of nicotinamide adenine dinucleotide (NAD) to its reduced form (NADH).

$$\text{Lactic acid} + \text{NAD} \xrightarrow{\text{LDH}} \text{Pyruvate} + \text{NADH}$$

The measurements were made at a wave length of 340 millimicrons and a slit width of 1.0 mm. The (ΔA) was recorded on a Mosely strip chart recorder. Temperature within the spectrophotometer was regulated by circulating water through the sample compartment coolant unit. The reaction mixture minus the tissue homogenate was kept in this same water bath ($15.0 \pm 0.5°C$). The reaction mixture consisted of

0.100 M	Phosphate buffer (pH 8.8)	1.5 ml
0.005 M	NAD	0.3 ml
0.160 M	Sodium dl-lactate (adjusted to pH 8.0)	1.0 ml

The supernatant of the tissue homogenate was diluted 1:5 with the phosphate buffer and a 0.2 ml aliquot of this diluted homogenate added to the sample cell along with the above mixture and vigorously shaken. This was then immediately placed in the spectrophotometer and the change in absorbance (read against the reference blank) followed for

3 min. The reference blank consisted of the above mixture, but with phosphate buffer substituted for the sodium lactate. The unused tissue homogenate was frozen for later protein determination.

The LDH activity are expressed as units of LDH activity per mg protein, with a unit of activity being defined as a 0.001 ΔA per minute under the defined conditions.

Lactic Acid and Protein Determinations

Retinas were homogenized in 5 ml of a 2% H_2SO_4 solution (2 ml concentrated H_2SO_4 diluted to 100 ml with distilled water). Immediately after homogenization two 0.1 ml aliquots were removed for protein determinations and two 0.5 ml samples were added to 2.5 ml of 10% trichloroacetic acid (TCA). The latter were centrifuged and lactic acid determined in the protein free supernatant. Venous blood lactic acid was determined by withdrawing a blood sample from the caudal vein just prior to or immediately after pithing the animal. This was diluted 1:10 with 10% TCA, centrifuged and the lactic acid determined in the supernatant by the method of Barker and Summerson (1941). Standards having concentrations of 0, 2, 4 and 8 mg% lactate were prepared from distilled water and a 40 mg% lactic acid standard solution (Sigma Chemical Co., St. Louis, Mo.).

Protein was assayed by a modified colorimetric method of Lowry (Oyama and Eagle, 1956). Protein standards were prepared from an 800 µg/ml standard (Dade Reagents, Inc., Miami, Fla., Lot No. PRS-406) to give a standard curve ranging from 0 µg/ml to 160 µg/ml. The tissue homogenates were then diluted with enough distilled water to give a protein concentration within this range.

RESULTS

In Vivo Trout ERG

The normal light adapted rainbow trout ERG is composed of two primary components (Fig. 6). By convention the negative portion of the ERG immediately following photostimulation is termed the a-wave and the following positive component the b-wave. As in other animals, two smaller wavelets, a_1 and a_2, are sometimes superimposed on the a-wave (Fig. 7b). With dark adaptation the b-wave increases in amplitude (measured from the resting potential) during the first 40-50 min (Fig. 8). The a-wave is larger immediately after the background light is eliminated but declines to a plateau after 10 min. In addition to the increase in the amplitudes of the a- and b-waves, their temporal aspect changes during dark adaptation. The elapsed time from the moment of stimulation to the peak of each component, the implicit time, increases after dark adaptation (Fig. 6).

The other principal difference between the ERG of the light and dark adapted eye is the appearance of the positive c-wave (Fig. 6). This wave occurs after the

FIGURE 6.--Normal rainbow trout ERG recordings.

A. ERG elicited with a short (ca. 10 μsec), high intensity photostimulus and a background illumination of 15 foot candles. The light adapted trout ERG consists of a negative a-wave (left reference point) followed by a positive b-wave (right reference point).

   Calibration: 100 μV and 100 msec.

B. ERG elicited with the same photostimulus but after 60 min dark adaptation. The dark adapted trout ERG, in addition to the a- and b-waves (left and center reference points respectively) is characterized by a slow positive c-wave (right reference point).

   Calibration: 200 μV and 100 msec.

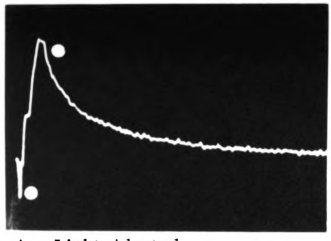
C. ERG recorded from the same dark adapted eye but elicited with a photostimulus 1.2 logarithmic units below that previously used.

   Calibration: 200 μV and 100 msec.

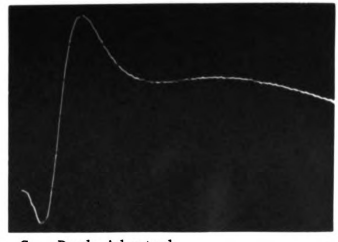
D. Superimposed photographs of two ERGs, one recorded in the light adapted state (LA) and the other after dark adaptation (DA) (ca. 65 min). The same photostimulus intensities were used to elicit both ERGs.

   Calibration (LA): 100 μV and 20 msec.

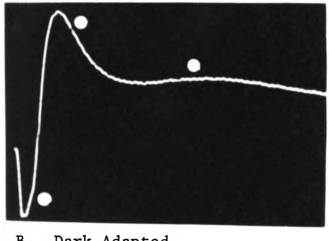
   Calibration (DA): 200 μV and 20 msec.



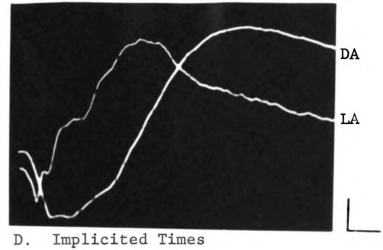
A. Light Adapted



C. Dark Adapted



B. Dark Adapted



D. Implicited Times

FIGURE 6

FIGURE 7a.--A single a-wave recording from a rainbow trout showing only one discernible peak. Recorded after 60 min dark adaptation.

Calibration: 50 μV and 10 msec.

FIGURE 7b.--A double a-wave recording from a rainbow trout showing two clearly discernible peaks, a_1 and a_2. Recorded after 60 min dark adaptation. By convention the first peak of the double a-wave is termed a_1 and the second peak a_2.

Calibration: 50 μV and 10 msec.

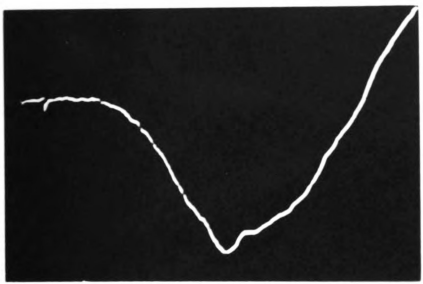


FIGURE 7a.

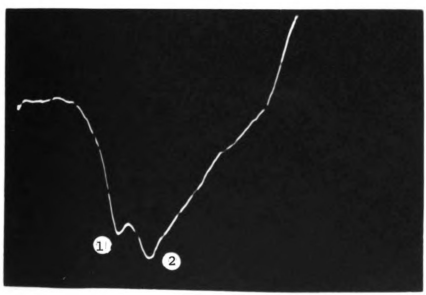


FIGURE 7b.

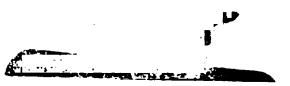


FIGURE 8.--The effect of dark adaptation on the amplitudes of the a- and b-waves. The amplitudes for both the a- and b-waves were measured from the resting potential and are plotted as their average value vs. time (min) of dark adaptation. The points are the means while the vertical bars represent the S.E. from 5 trout.

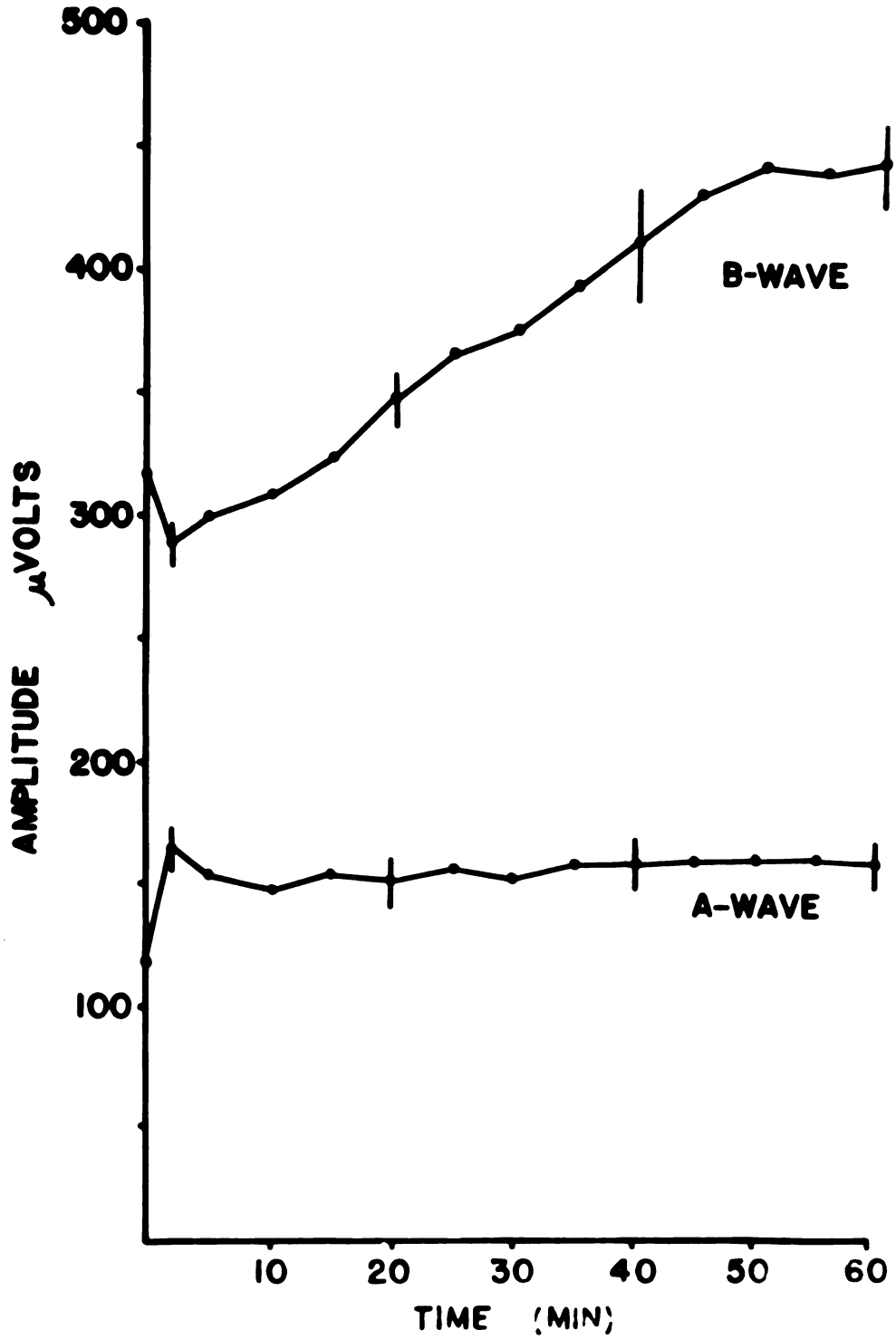

500
400
300
200
100
AMPLITUDE μVOLTS
B-WAVE
A-WAVE
10
20
30
40
50
60
TIME (MIN)

FIGURE 8

b-wave and can only be recorded from the eye after dark adaptation.

In the intensity range of the photostimuli utilized in these experiments, a reduction of the intensity by 1.2 logarithmic units produced a decrease in the a-wave while the b-wave remained relatively unaffected (Fig. 6). A red plexiglass filter placed on the lamp housing was found to produce a similar preferential decrease in the amplitude of the a-wave. This characteristic of the ERG was utilized in the *in vitro* experiments to elicit a more conventional appearing ERG that was otherwise dominated by the negative component.

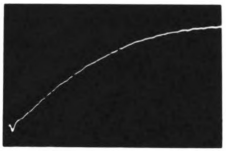
Effect of Hypoxia on the Trout ERG

The effect of ligating the ipsilateral afferent pseudobranch artery on the teleost ERG is shown in Fig. 9. The contralateral afferent pseudobranch artery was cauterized prior to the experiment to prevent blood from flowing through the communicating artery from the contralateral side to the eye under observation. The first change in the ERG during this form of retinal ischemic hypoxia is an increase in the c-wave. This occurs within 1 min after ligation and is accompanied by a reduction and subsequent abolishment of the b-wave. The decline in the amplitude of the b-wave is rapid and disappears within 3-4 min after

FIGURE 9.--ERGs of the rainbow trout retina during ischemic hypoxia produced by occluding the afferent pseudobranch arteries. The 0 min trace is a control recording taken after 60 min of dark adaptation.

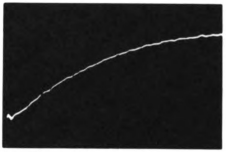
Calibration: 100 μV and 500 msec.

0 min Control



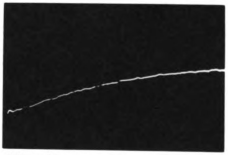
4 min

1 min



5 min

2 min



10 min

3 min

FIGURE 9

ligating the artery. The c-wave reaches its maximum amplitude by the second or third minute and then declines.

The a-wave is not as predictable in its response as are the other two waves. In this instance (Fig. 9) the a-wave began to decline in amplitude 3-4 min after ligation and disappeared within another minute or two. In other instances the a-wave could still be recorded after 10 min and in one animal it was present 20 min after ligation.

The necessity of occluding both afferent pseudobranch arteries in order to assure retinal hypoxia in either eye is demonstrated in Fig. 10. In this fish, the right afferent pseudobranch artery was cauterized while that on the left side was left intact. Immediately following cauterization, the b-wave recorded from the right eye declined, but was still recordable after 100 min. The stability of the ERG under these conditions was no doubt due to the continued blood flow through the communicating artery connecting the right and left afferent pseudobranch arteries.

The interruption of the flow of aerated water across the gills produces a change in the ERG similar to that of ligating the afferent pseudobranch artery (Fig. 11). The b-wave is abolished, the c-wave becomes large in relation to its control value, and the amplitude of the a-wave declines but is still recordable after 4 min.

FIGURE 10.--ERGs of the rainbow trout retina after cauterization of the ipsilateral afferent pseudobranch artery. The contralateral afferent pseudobranch artery was left intact. The 0 min trace is a control recording after 60 min of dark adaptation.

Calibration: 200 μV and 50 msec.

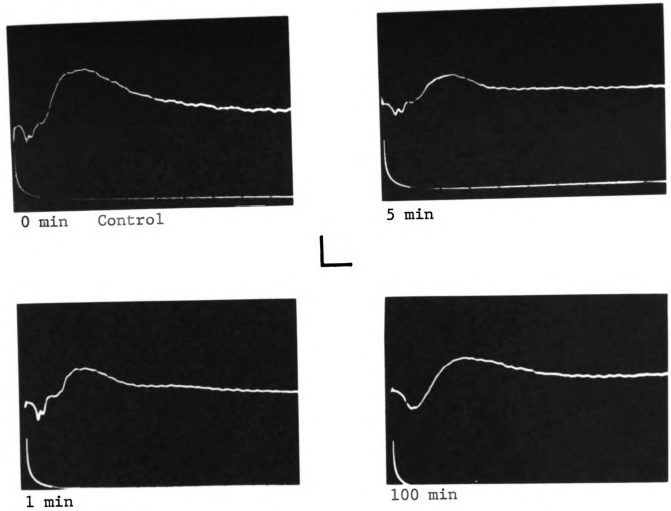
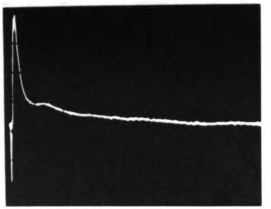


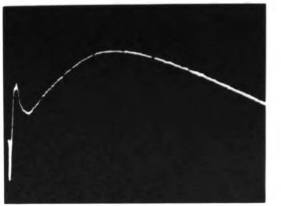
FIGURE 10

FIGURE 11.--Effect of non-ischemic hypoxia on the trout ERG. The hypoxia was produced by shutting off the water flow across the gills. The 0 min trace recorded after 60 min dark adaptation.

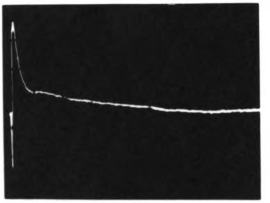
Calibration: 100 μV and 500 msec.



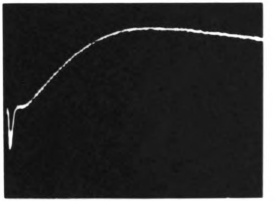
0 min Control



3 min



2 min



4 min

FIGURE 11

Recovery of the ERG Following Ischemic Hypoxia

The data on the rate of recovery of the a- and b-waves following a 12 min ligation of the afferent pseudobranch artery are given in Fig. 12. The amplitudes of the a- and b-waves are expressed as a percentage of their control value before ligation and are plotted as a function of time. Although there is regeneration of the a- and b-waves, it is a slow process requiring up to 30 min for complete recovery. The a-wave begins to recover within 1-2 min whereas the b-wave is not recordable until 6-10 min after the release of the ligature. Recovery of the b-wave is relatively linear over the next 20 min. The decline in the a-wave seen after the fourth minute is probably the result of the summation of the negative a-wave and the beginning of the recovery of the positive b-wave.

Effect of Acetazolamide on the Trout ERG

Within 3 min after an IP injection of acetazolamide there is an increase in the amplitude of the c-wave and simultaneous decrease in the b-wave (Fig. 13), which is abolished by the fifth minute. The c-wave reaches its maximum amplitude 15-20 min after injection and thereafter slowly declines with time. The a-wave increases in

FIGURE 12.--Recovery of the a- and b-waves following 12 min of ischemic hypoxia produced by occluding the afferent pseudobranch arteries. Amplitudes for the a- and b-waves (measured from the resting potential) are plotted as a percentage of the initial average value vs. time (min) after removal of the ligature. The points are the means while the vertical bars represent the S.E. from 5 fish.

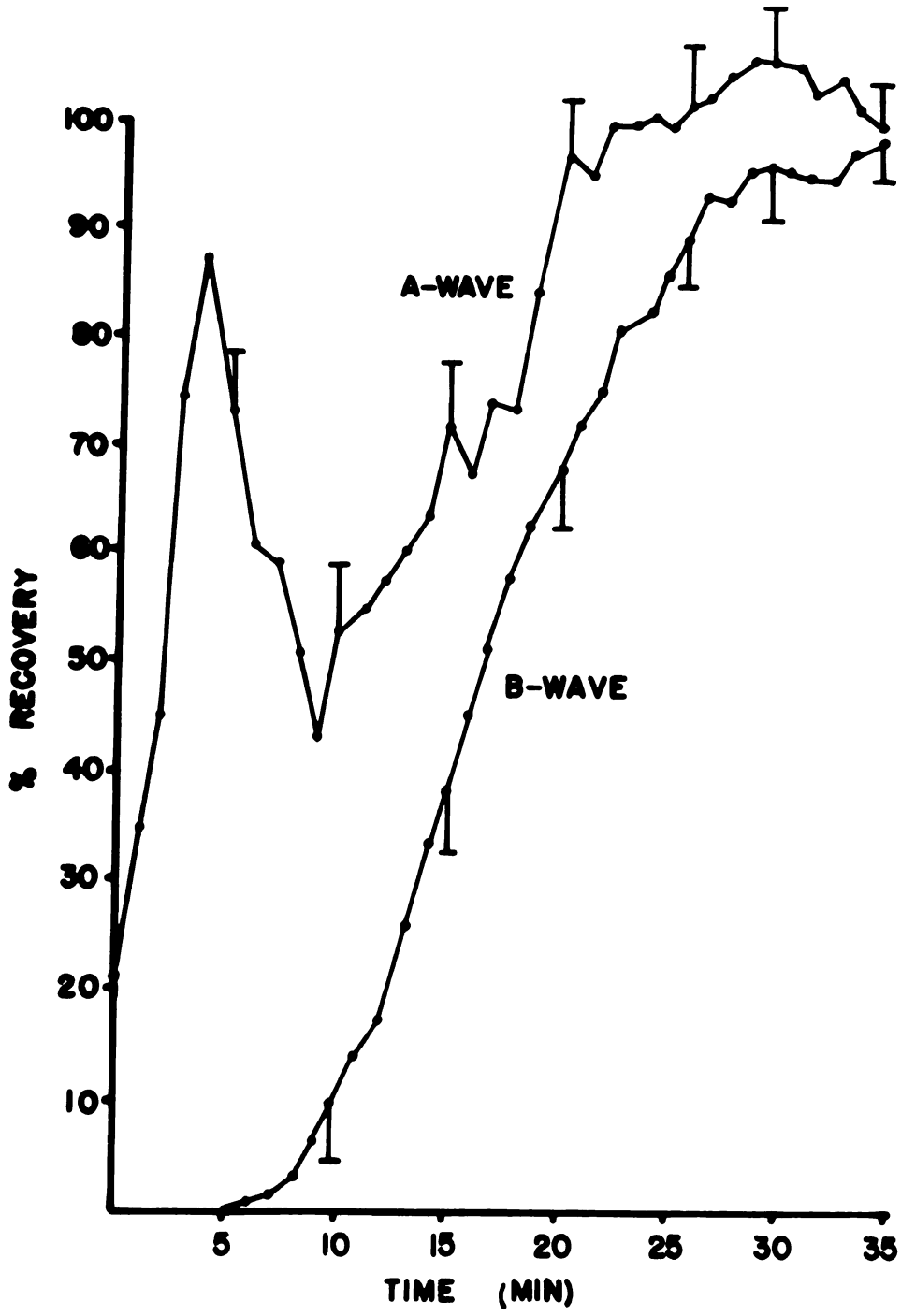

100
90
80
70
60
50
40
30
20
10
% RECOVERY
A-WAVE
B-WAVE
5
10
15
20
25
30
35
TIME (MIN)

FIGURE 12

FIGURE 13.--ERGs of a dark-adapted trout retina following an injection of acetazolamide (10 mg). The 0 min trace was recorded after 60 min dark adaptation.

Calibration (0-5 min): 100 μV and 500 msec.

Calibration (10-60 min): 200 μV and 500 msec.

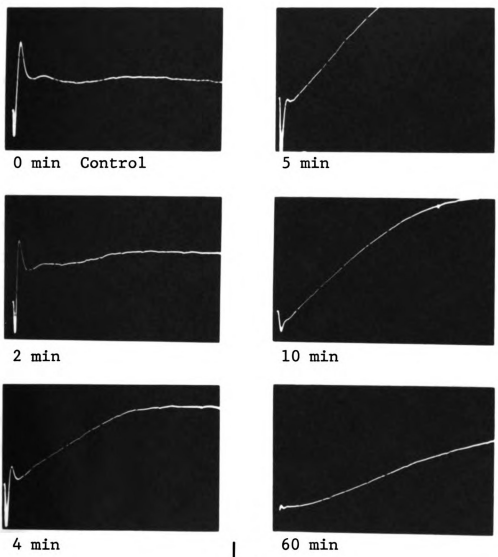


FIGURE 13

amplitude 3-5 min after injection and then declines. As is the case with the c-wave, the a-wave can be recorded long after the abolishment of the b-wave.

Interrupting the water flow across the gills after acetazolamide administration produces a further enhancement of the c-wave, while the subsequent resumption of water flow causes the c-wave to decline in amplitude (Fig. 14).

Acetazolamide and Its Effects on the Primary Evoked Potential Recorded from the Optic Tectum

Within minutes after an IP injection of acetazolamide a profound effect on the VETP is observed (Fig. 15). Three minutes after the drug is administered the VETP is reduced and disappears 2 min later. In this and similar recordings from other fish another potential is recorded after the disappearance of the VETP. But this potential is transitory and always disappears within 20 min after drug administration. In animals in which the VETP and ERG are recorded simultaneously the abolishment of the VETP is closely correlated with the disappearance of the b-wave from the ERG (Fig. 16).

Chronic Effect of Acetazolamide on the Trout ERG

Retinas in trout treated with acetazolamide show an ability to recover from the adverse effects of the drug.

FIGURE 14.--Effect of hypoxia on the trout c-wave.

The rainbow trout ERG recorded 19 min after administering acetazolamide (A). Immediately after recording this ERG, the water flow across the gills was shut off. Three minutes later a small increase in the c-wave was observed (B), which declined only slightly over the next 5 min (C). After this 5 min period, the water flow was again resumed across the gills. Within 1 min after resuming water flow, there was a significant decline in the c-wave (D) which continued over the next 2 min (E). Repeating the previous procedure of shutting off the water flow caused a large increase in the c-wave recorded 1 min later (F).

Calibration: 200 μV and 500 msec.

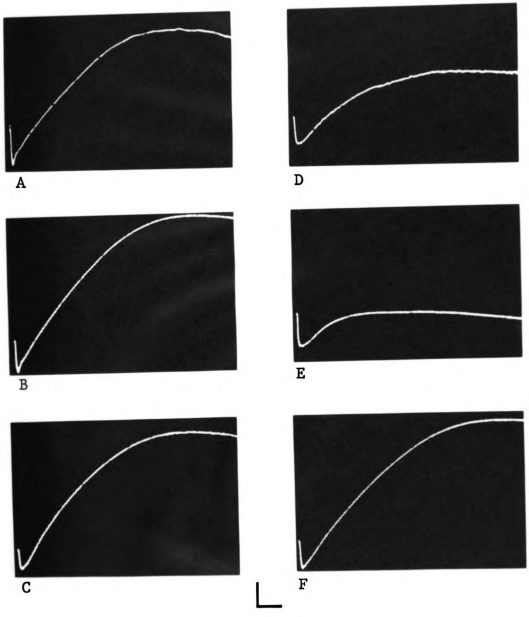
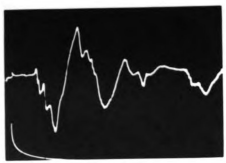


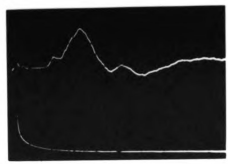
FIGURE 14

FIGURE 15.--Visually evoked potentials (VETP) following an injection of acetazolamide (10 mg). The 0 min trace was recorded after 20 min dark adaptation.

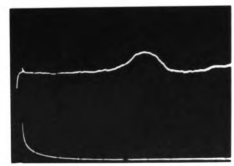
Calibration: 100 μV and 20 msec.



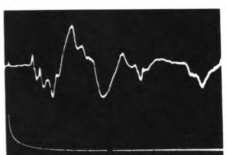
0 min Control



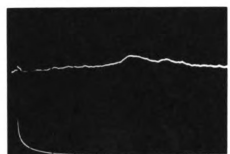
4 min



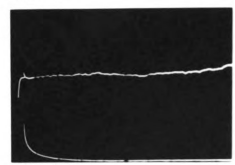
15 min



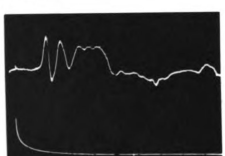
2 min



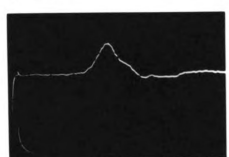
5 min



21 min



3 min



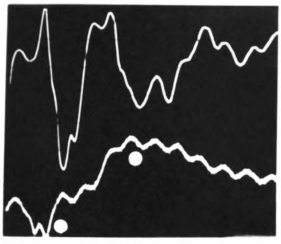
11 min

FIGURE 15

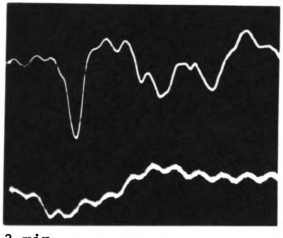
FIGURE 16.--Visually evoked potentials (VETP) and ERGs following an injection of acetazolamide (10 mg). The 0 min trace was recorded after 20 min dark adaptation. The rapid low amplitude oscillation in both traces is 60 c/s interference. The white reference points, from left to right, refer respectively to the a- and b-waves of the ERG.

Calibration (VETP): 200 μV and 20 msec.

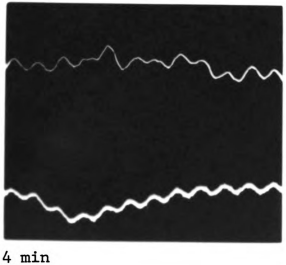
Calibration (ERG): 100 μV and 20 msec.



0 min Control



3 min



4 min

FIGURE 16

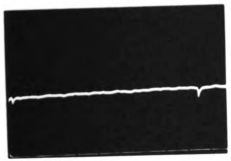
In one experiment a group of 19 fish were given a single dose (IP) of the inhibitor. From this group three to four fish were selected daily in a random manner over the next 5 days. The dark adapted ERGs recorded from those animals selected 24 hrs later were found to lack a discernible ERG (Fig. 17). By the second day an a- and c-wave was present in one fish, whereas, the responses from the other three animals were identical to the ones recorded on the first day. On the third day a b-wave was recorded from one of the animals and by the fourth day two of the four animals examined had a recordable b-wave. The recovery was more complete by the fifth day as the ERGs of the three remaining fish all possessed a b-wave. It should be noted that the ERGs in Fig. 17 were from the animal showing the greatest degree of recovery for any given day.

In a similar experiment four rainbow trout were injected with acetazolamide, but in this study the recovery of the ERGs was followed in the same animals for up to 14 days. In three of the fish, the ERGs were found to reappear over the next several days (Fig. 18). In addition to the ERGs the VETPs were recorded in two of the animals 14 days after treatment. This response could not be detected in the animal lacking an ERG.

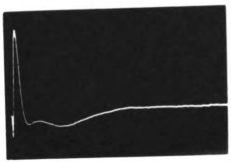
A close correlation exists between the ability to record a b-wave and the degree of chromatophore contraction.

FIGURE 17.--The ERGs recorded from rainbow trout following a single dose (10 mg) of acetazolamide on day zero. The recordings are from animals which had the greatest degree of recovery on any given day. By the fifth day the three animals had ERGs characterized by a-, b- and c-waves.

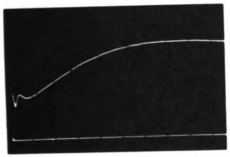
Calibration: 100 μV and 500 msec.



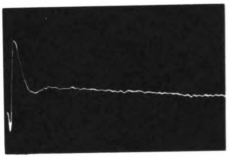
Day 1



Day 4



Day 2



Day 5

Day 3

FIGURE 17

FIGURE 18.--The ERGs recorded from a representative rainbow trout before and after a single dose of acetazolamide (10 mg) administered on day 0. The animal was anesthetized with MS-222 and the ERGs recorded in the light adapted state with wick electrodes. The last recording is the visually evoked potential (VETP) recorded from the optic tectum on day 14.

Calibration (ERG): 100 μV and 50 msec.

Calibration (VETP): 20 μV and 20 msec.

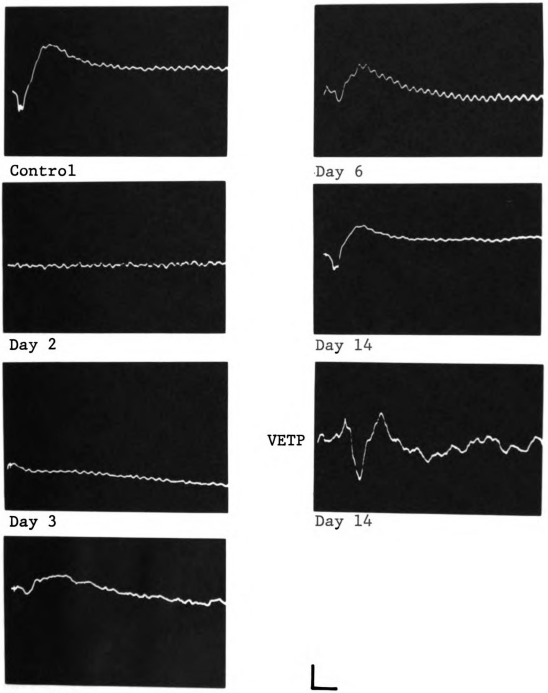


FIGURE 18

In both of the above studies it was found that as long as a fish remains dark the animal has an ERG lacking the b-wave.

Slow DC Potential Changes

In the normal animal, light or dark adaptation and photostimulation did not significantly alter the transretinal DC potential, or produce slow potential changes other than those normally associated with the ERG (Fig. 19). However, 1-2 min after shutting off the water flow across the gills there is a slow change in the potential with the vitreal side becoming more negative. This occurs in both light and dark adapted eyes and upon resumption of the water flow the transretinal potential returns to its resting level.

The administration of acetazolamide (IP) produces a similar slow change in the potential. The increase in vitreal negativity reaches its maximum 4-6 min after drug administration and thereafter a slow positive shift in the transretinal potential is observed. In contrast to the normal animal the cessation of background light causes a large transitory negative potential in the acetazolamide treated fish. The reinstatement of steady illumination produces a transient shift in the opposite direction, with the vitreous becoming more positive. The onset of this

FIGURE 19.--Typical response of the transretinal DC potential to illumination, hypoxia, and acetazolamide. Observations similar to these were made in 4 other animals.

A. The effect of ambient illumination on the DC potential across the normal rainbow trout eye.

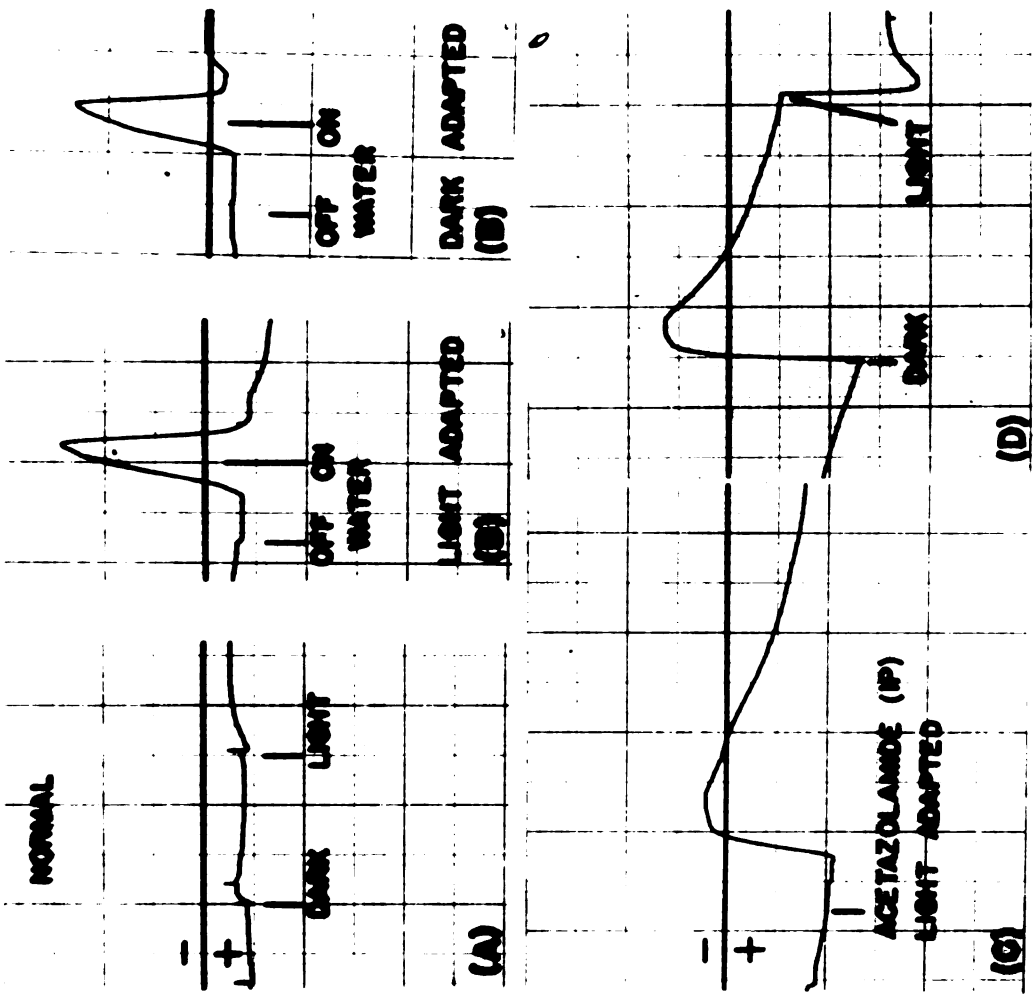
B. Shifts in the DC potential in the light and dark adapted eye as a result of hypoxia. The hypoxia was induced by shutting off the water flow across the gills for 4 min.

C. The effect of acetazolamide (10 mg IP) on the DC potential across the light adapted eye.

D. The effect of ambient illumination on the DC potential across the eye of a rainbow trout treated with acetazolamide.

Calibration: 2.0 mV and 5.0 min. Dark horizontal line represents the isoelectric potential. Active electrode was placed in the vitreous body.

FIGURE 19


NORMAL
DARK
LIGHT
(A)
OFF ON
WATER
LIGHT ADAPTED
(B)
OFF ON
WATER
DARK ADAPTED
(D)
ACETAZOLAMIDE (IP)
LIGHT ADAPTED
(C)
DARK
LIGHT
(D)

positive shift in the transretinal potential has the same time course as the onset of the large slow c-wave of the ERG elicited by a single short photostimulus.

Lactic Acid Content and Lactate Dehydrogenase Activity of the Retina after Acetazolamide Administration

Figure 20a shows the lactic acid concentration of the retina in normal and acetazolamide treated fish. Within 1.25 hours after injection (IP) there is a significant rise in the retinal lactic acid which reached a maximum value after 48 hr, declined thereafter and was at the normal pretreatment level in 7 days.

In preparing the retina for these assays, most, if not all, of the choroid was included in the analysis. For this reason, blood lactic acid was determined to insure that the observed changes in lactic acid concentration were not simply a reflection of altered blood lactic acid. As is apparent from the means and standard errors (Fig. 20b), there is a large variability in blood lactic acid from animal to animal. The blood lactic acid of fish injected with acetazolamide 1.25 hrs previously is higher than the control group but in the fish treated 24 hrs previously it is lower than the control animals.

The analysis of retinal LDH activity (0.001ΔA/min/mg protein) revealed that there is no difference between

FIGURE 20a.--The lactic acid concentration of the retina in the normal (N) and acetazolamide treated rainbow trout. The values on the abscissa refer to the elapsed time between acetazolamide administration and when the tissues were taken for the assays. Plotted are the means and vertical bars represent the S.E. of the means. The values in parenthesis refer to the number of observations utilized in each assay.

FIGURE 20b.--Blood lactic acid in normal (N) and acetazolamide treated rainbow trout. The values on the abscissa refer to the elapsed time between acetazolamide administration and when the blood samples were taken for lactic acid determinations. Blood lactic acid is expressed as mg lactic acid/100 ml whole blood. Plotted are the means and the vertical bars represent the S.E. of the means.

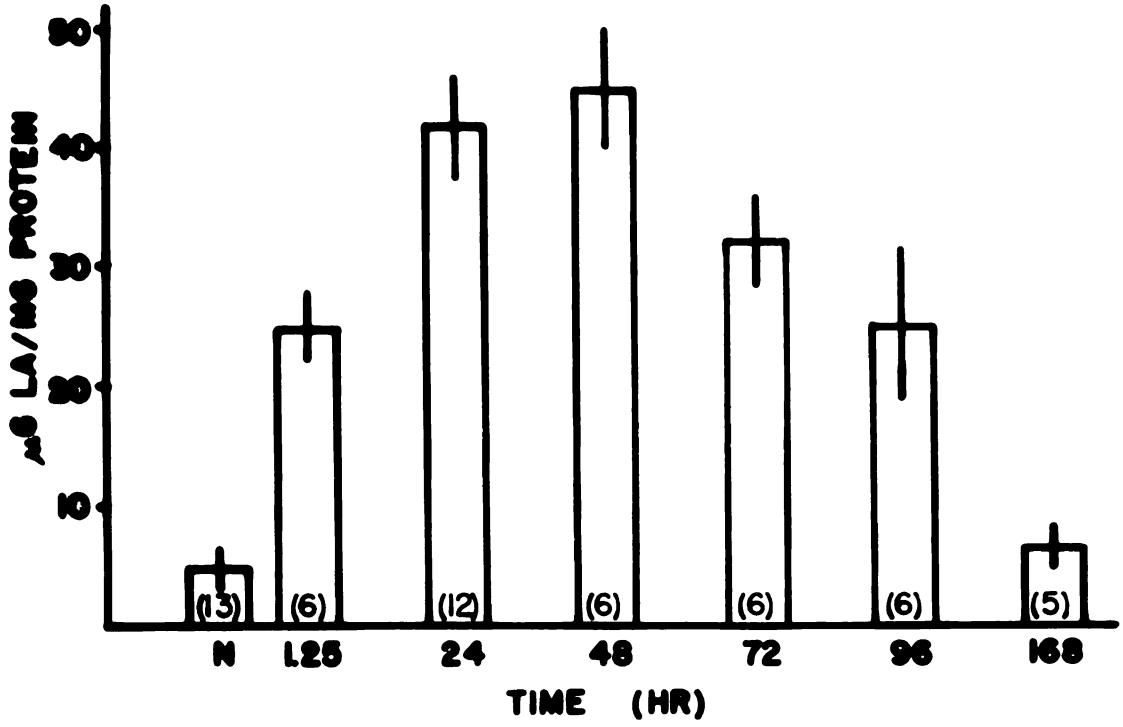


FIGURE 20a.

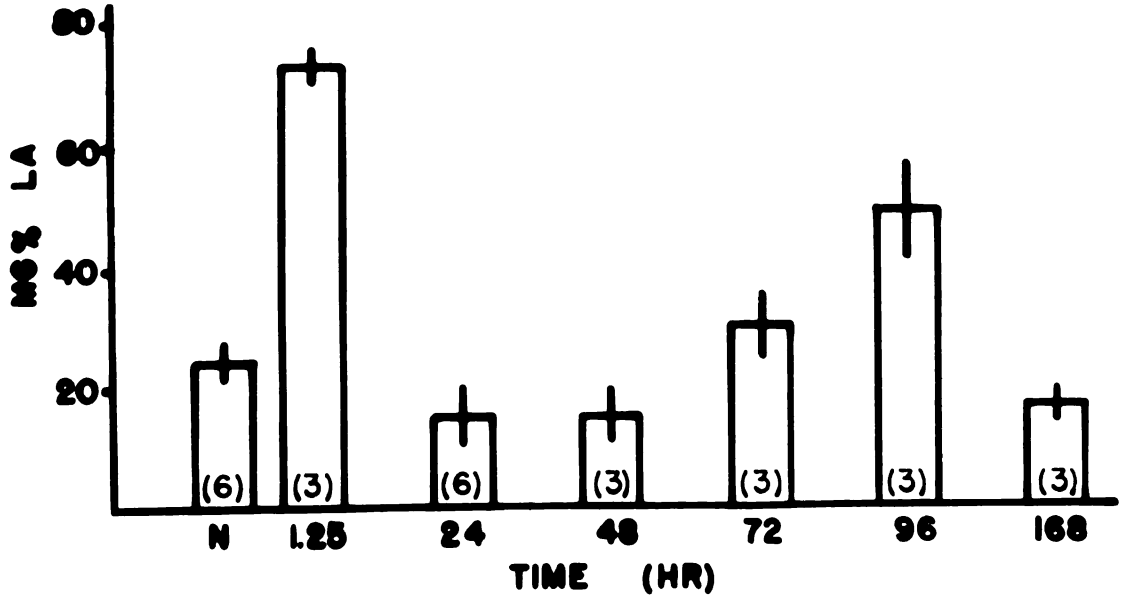


FIGURE 20b.

non-treated animals, 257±10 (mean±SE), and those injected 24 hrs previously with acetazolamide, 256±10.

ERGs of the *In Vitro* Retinal Preparations

In the *in vitro* preparation it was generally more difficult to obtain a suitable response from the teleost than the frog retinas. In many cases the teleost ERG was either lacking or consisted only of the negative a-wave. In those retinas however, in which both the a- and b-wave were initially recorded, the ERGs remained stable for several hours. In contrast, little difficulty was encountered in recording the a- and b-waves from the frog retinas.

Figure 21 shows the lake trout and frog ERGs respectively as recorded *in vitro* at different levels of P_{O_2}. Since the pigmented epithelium (presumed source of the c-wave) was removed during tissue preparation, the *in vitro* ERGs are characterized by an a- and b-wave only. As in the *in vivo* study, the principal effect of hypoxia is the attenuation of the b-wave. In Figure 22 the percent of the maximum response of the b-wave for the teleost and frog are both plotted as a function of time. In these studies the ERGs would remain steady until the P_{O_2} dropped to a critical level. Thereafter the b-wave would decline

FIGURE 21a.--Lake trout ERGs recorded *in vitro* at different P_{O_2}s. These recordings lack the c-wave due to the removal of the pigmented epithelium during tissue preparation.

Calibration: 50 μV and 200 msec.

FIGURE 21b.--*In vitro* frog ERGs recorded at different P_{O_2}s.

Calibration: 100 μV and 200 msec.

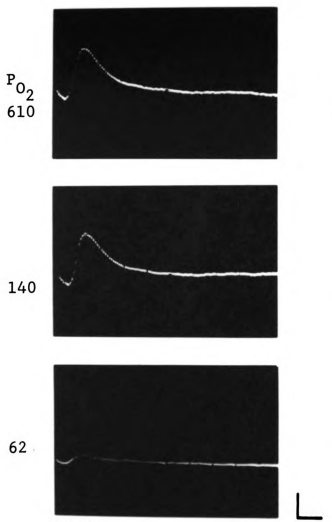


FIGURE 21a.

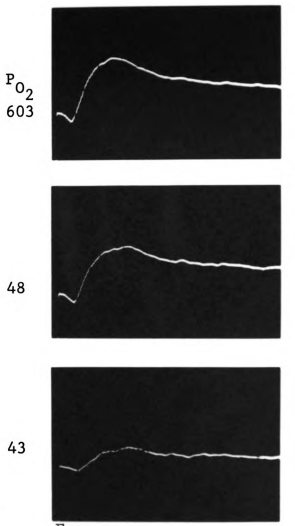


FIGURE 21b.

FIGURE 22.--The percentage of the control response of the b-wave amplitude as recorded *in vitro* with decreasing P_{O_2} produced by bubbling N_2 into the media. The P_{O_2} and ERG were monitored at 1 min intervals and the plots represent an average of five observations each. The b-wave response lines were fitted and the slopes calculated by the method of least squares, where $y = a + bx$. Frog: $a = 181.04$, $b = -4.82\%/min$, correlation coefficient (r_{xy}) = 0.84, N = 5. Trout: $a = 342.52$, $b = -18.34\%/min$, $r_{xy} = 0.90$, N = 5. The slopes of these two responses were significantly different from each other at the 0.01 per cent level.

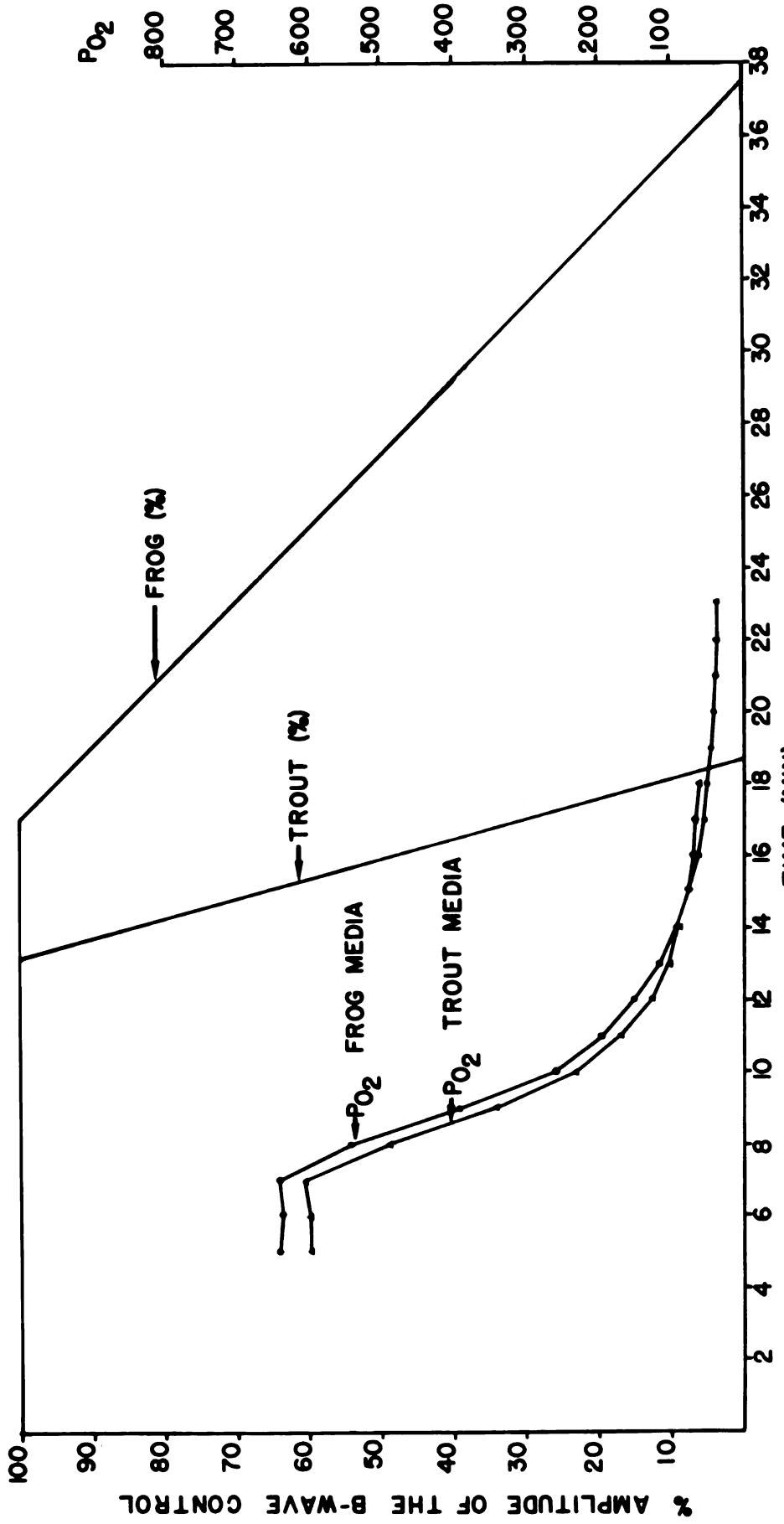

% AMPLITUDE OF THE B-WAVE CONTROL
TIME (MIN)
P_{O_2}
FROG (%)
TROUT (%)
FROG MEDIA
TROUT MEDIA
P_{O_2}
P_{O_2}

FIGURE 22

in a relatively linear manner, with the teleost b-wave declining both earlier and at a faster rate than that of the frog. From the slope of the lines (fitted by the method of least squares) it was determined that the fish b-wave declines at a rate of 18.34%/min after the P_{O_2} drops below 100 mm Hg, whereas, the frog b-wave declines 4.82%/min after the P_{O_2} drops below 50 mm Hg.

After the recovery from hypoxia these same preparations were used to determine the effects of acetazolamide on the *in vitro* trout retina. In contrast to the *in vivo* results, acetazolamide (0.5 mg added to the 15 ml of media bathing each side of the retina) was found to have no effect on the ERG.

DISCUSSION

In this study, the ERG was used as a monitor of the functional integrity of the teleost retina. As such, the interpretations of the results are based on what is currently known or at least the best available evidence concerning the cellular sources of the ERG. The a-, b-, and c-waves will be considered photoreceptor, inner nuclear layer, and pigmented epithelium responses respectively. While the rainbow trout a-wave was often recorded with two discernible peaks (a_1 and a_2) (Fig. 7), for the sake of clarity the discussion in this section will consider the a-wave as a single component. Preliminary experiments established that the rainbow trout ERGs recorded in these studies resembled those from other vertebrates having retinas composed of a mixture of rods and cones (Brown, 1968). In the light adapted state, the ERG recorded tends to be like that of a predominately cone ERG, consisting only of the a- and b-waves. With dark adaptation the slow positive c-wave appears, which is characteristic of the rod eyes (Fig 6). The other major developments with dark adaptation are an increase in the amplitude and implicit times of the a- and b-waves (Fig. 6). These are

characteristics common to other vertebrate ERGs (Brunette, 1969; Gramoni and Ali, 1970; and Burian, 1970). While the mechanisms responsible for the increased implicit times are not known, Auerbach and Burian (1955) report that it may be related to the fact that the rod response or primary response of the dark adapted eye is inherently slower than the cone response which is the primary response of the light adapted eye.

Effect of Hypoxia on the Trout a- and b-Waves

In the following discussion the term "non-ischemic hypoxia" will be used to refer to the hypoxia produced by interrupting the water flow across the gills and "ischemic hypoxia" to the hypoxia brought on by ligating the afferent pseudobranch artery. This latter term should be clarified since there is an additional blood supply to the teleost retina by way of the retinal artery (Barnett, 1951). This artery, which arises from the internal carotid artery, sends branches to the extraocular muscles, optic nerve, retractor lentis muscle and the choriocapillaris. The relative contribution to the total blood supply of the choriocapillaris by way of the retinal artery remains unknown. But considering the anatomical aspects, it is this author's belief that it probably supplies only a small percentage of the total blood supply.

The rapid reduction of the b-wave brought on by either ligating the afferent pseudobranch artery (Fig. 9) or shutting off the water flow (Fig. 11) illustrates the susceptibility of the rainbow trout retina to even short periods of hypoxia. The results of these studies and those of other workers are in agreement that the b-wave is the first component to disappear during hypoxia. Winkler (1972) reported that the a-wave recorded from the isolated rat retina remained stable even throughout 75 min of hypoxia, whereas, the b-wave was abolished within 5 min. Similarly, Tazawa and Seaman (1972) found that while the a-wave was still present in the isolated bovine eye after 5 min of anoxia, the b-wave was absent. In the intact cat, the occlusion of the aorta causes the b-wave to disappear within 38-61 seconds, but a negative potential (a-wave) can be recorded for up to 7 min later (Horsten and Winkelman, 1957).

A factor in these studies which may contribute to the relative stability of the a-wave is the flow of blood within the choriocapillaris. This flow is not interrupted in the non-ischemic hypoxia and in the ischemic hypoxia the choriocapillaris receives a residual blood supply by way of the retinal artery. The source of the a-wave, the photoreceptors, lies in close proximity to the choriocapillaris. These cells would therefore have more exposure to the blood

supply and the available O_2 than the inner nuclear layer which gives rise to the b-wave.

The recovery period for the ERG after 12 min of ischemic hypoxia is relatively long (20 min for the a-wave and 30 min for the b-wave) (Fig. 12). Fairbanks (1970), however, has shown that although the superatmospheric P_{O_2} begins to be regenerated within 1 min after releasing a ligature around the afferent pseudobranch artery, complete regeneration (95% recovery) requires up to 20 min. This is approximately the same amount of time required for the complete regeneration of the a-wave. The longer time required for the b-wave may again be partially attributed to the fact that the inner nuclear layer is more distal to the choriocapillaris than the photoreceptors.

Effect of Hypoxia on the Trout c-Wave--Future Considerations

In contrast to the a- and b-waves, the c-wave has often been omitted from the analysis of the ERG in studies by other investigators on the effects of hypoxia on the retina. Where it has been included it was found to decline at approximately the same rate as that of the a-wave (Tazawa and Seaman, 1972; O'Rourke and Berghoffer, 1968). In the dark adapted rainbow trout, however, an increase in the c-wave always accompanied the initial stages of hypoxia

(Figs. 9, 11 and 14). Indeed, a large c-wave recorded in the control stage of an experiment was used as a criterion to discard that preparation as these ERGs tended to decline with time.

Since there is little information concerning the c-wave, other than its source being the pigmented epithelium, future consideration should be given to the mode of generation of this component. Theoretically the c-wave could be generated by one of two methods: 1) Within the pigmented epithelium the photons could directly alter the conductance properties of the cellular membrane. This would result in a change in the cellular potential in a manner analogous to that which occurs in the photoreceptors. 2) A relationship between the photoreceptors and pigmented epithelium may exist analogous to the bipolar-Müller cell relationship which generates the b-wave (i.e., some ion released from the photoreceptors during photostimulation could alter the cellular potential of the pigmented epithelium). Support for the latter proposal comes from two sources: 1) the spectral response curve for the c-wave and rhodopsin are nearly identical (Granit and Munsterhjelm, 1937); and 2) the potential produced as a result of direct photostimulation of the pigmented epithelium, freed of the retina, is several orders of magnitude faster than that of the c-wave (Brown and Gage, 1966). While either proposal may form the basis

for a working hypothesis for the origin of the c-wave, such a hypothesis must take into account the fact that the c-wave can only be recorded after the elimination of the ambient illumination; and second, hypoxia accentuates the amplitude of the c-wave. The reader is referred to the section below, Effect of Hypoxia on the Slow DC Potentials, for a further discussion of this subject.

Another phenomenon, whose relationship to the c-wave is unknown, is that during dark adaptation the pigmented epithelial cells expand and during light adaptation they contract. The effect that this expansion or contraction has on the conductance properties of the pigmented epithelium should be evaluated to determine if this could influence the amplitude of the c-wave.

Chromatophore Expansion and Vision

The pseudobranch has been recognized as important in controlling the chromatophores in teleosts. This has been attributed to an endocrine function by the pseudobranch. Parry and Holliday (1960) found that if ligatures were placed around both afferent pseudobranch arteries of brown trout, the fish would begin to darken within 5 min, and complete chromatophore expansion was evident 12-15 min later. If the ligatures were

released at this time the chromatophores returned to a contracted state within 30 min. These authors postulated that the pseudobranch secretes a substance "P" responsible for chromatophore contraction and when circulation to the pseudobranch was occluded, the fish darkened because substance "P" had been removed from the circulation. Fairbanks (1970) proposed that this darkening reaction might be linked with a visual response due to a decreased retinal P_{O_2}. The results of this study support this latter premise. The darkening and recovery periods observed by Parry and Holliday are remarkably similar to the decay and recovery periods for the b-wave (Fig. 9 and 12). Since the disappearance of the b-wave can be correlated with the disappearance of the VETP, a more plausible explanation for the results obtained by Parry and Holliday is that when the afferent pseudobranch arteries are ligated vision is lost within a few minutes, producing an expansion of the chromatophores through the visual-chromatophore reflex. After releasing the ligatures, vision gradually returns, and causes a concurrent contraction of the chromatophores.

The Necessity of the Oxygen Concentrating Mechanism for Maintaining Trout Vision

The importance of the oxygen concentrating mechanism in maintaining vision in the trout is clearly demonstrated

by the results of these studies. Fairbanks (1970) reported that the ocular P_{O_2} starts to decline within 1 min after acetazolamide injection (IP) and is only 15 percent of its original value after 3 min. A similar dosage (IP) of acetazolamide causes a reduction in the b-wave within 3 min and its abolishment by the fifth minute (Fig. 13). Simultaneous recordings of both the VETP and ERG reveal that the disappearance of the VETP is concurrent with the loss of the b-wave (Figs. 15 and 16). Thus in these experiments, the b-wave can be used as a good indicator of the loss of visual function in the animal. The effect of acetazolamide on the trout ERG is similar to that of both ischemic and non-ischemic hypoxia. However, in the ischemic hypoxia experiments the a- and c-waves were not maintained to the same degree as that following acetazolamide administration. This can be explained by the fact that the inhibitor does not interrupt retinal blood flow (Fairbanks, 1970). While the residual P_{O_2} (presumed to be at arterial levels) offers some protection to the mechanisms within the pigmented epithelium and photoreceptors responsible for the generation of the a- and c-waves, it would be of negligible importance to the inner nuclear layer. Santamaris *et al.* (1971) have shown that the photoreceptors are responsible for nearly 40% of the total O_2 consumption of the teleost *Eugerres plumieri* retina. Thus the abolishment of the high

O_2 gradient (from the choroid to the vitreal side of the retina) and the high rate of O_2 consumption by the photoreceptors (interposed between the choroid and the rest of the retina) would quickly lead to a hypoxic condition within the inner nuclear layer.

Possible Direct Effect of Acetazolamide on the Retina

Since the rainbow trout retina contains CA (Fairbanks, 1970), a reasonable argument could be put forth that acetazolamide was producing its action on the ERG by directly affecting the retina. However, the available evidence is against this hypothesis. Although large doses of acetazolamide may have an effect on the central nervous system (producing drowsiness in some patients, Goodman and Gilman, 1965), to this author's knowledge, it has never been shown to directly affect the vertebrate ERG. In fact, the drug is often given clinically to patients (250-1000 mg daily) for the management of glaucoma. This evidence and the fact that acetazolamide did not affect the teleost ERG recorded from the isolated lake trout retina, confirms that the drug affects the oxygen concentrating mechanism rather than acting directly on the retina.

The results of these studies thus indicate that the teleost retina is unique among neural tissues in that an hypoxic condition can be produced specifically within

the tissue by drug administration. The teleost retina may therefore be ideally suited for future *in vivo* studies on hypoxia where neither the interruption of tissue blood flow nor general asphyxia are desirable.

Minimum Oxygen Tension Required for Maintaining Trout Vision

The presence of the oxygen multiplier mechanism and its importance, as demonstrated above, in maintaining the ERG strongly indicate that the trout arterial P_{O_2} is insufficient to sustain vision. The *in vitro* experiments verified this since the very minimum P_{O_2} required to maintain the trout ERG was 100 mm Hg or approximately 3 times the arterial P_{O_2} (Figs. 21 and 22). In these experiments the trout retina was compared to the frog retina which lacks an oxygen concentrating mechanism. In the frog experiments the P_{O_2} had to drop below 50 mm Hg, the approximate arterial P_{O_2}, before the ERG was affected.

Recovery of the Trout Retina from Chronic Hypoxia

The trout ERG disappears after acetazolamide administration as a result of the hypoxia brought on by abolishing the oxygen concentrating mechanism. Although the time necessary to regenerate the superatmospheric P_{O_2} posterior to the retina after a single injection of acetazolamide is

not precisely known, it is probably at least 24-48 hrs. This is based on evidence obtained by Fairbanks *et al.* (1969) who found that fish given acetazolamide (5 mg/100 g) had an ocular P_{O_2} of only 25 mm Hg 24 hrs after treatment. In addition, studies on the regeneration of oxygen in the teleost swim bladder, a process requiring the enzyme carbonic anhydrase, revealed that even 48 hrs after a fish is given acetazolamide there is only a slight regeneration of the bladder oxygen (Valerien, 1957). These data agree with the results of this study since a normal ERG was never recorded in animals treated 24 hr previously with acetazolamide. Thereafter on succeeding days, ERGs could be recorded in various stages of recovery.

Protection of the Retina During Chronic Hypoxia

In view of the fact that even short periods of hypoxia are often detrimental to neural tissue, it is surprising that the trout retina can survive the chronic hypoxia imposed by blocking the oxygen concentrating mechanism.

There are several factors which may offer some protection to the retina during this long hypoxic period. Ironically, one factor may be acetazolamide itself. Anderson (1968) has shown that prior treatment of human subjects with acetazolamide delayed the onset of blackout brought

on by an increase in intraocular pressure. These results were attributed to the fact that acetazolamide causes an increase in tissue CO_2 and this acts as a vasodilator to effect an increase in blood flow to the retina. The fact that a similar protection against blackout can be achieved by breathing a high CO_2-air mixture supports this hypothesis. Fairbanks (1970), however, reported that CA inhibition does not affect choroidal blood flow in the teleost. It is doubtful though that the technique he employed, i.e., circulation times for fluorescein from the caudal vein to the choroid, is refined enough to accurately measure increases in blood flow at the capillary level. If such an increase should occur, it would facilitate both the delivery of glucose and O_2 to the retina and removal of metabolic waste products. According to Ames and Gurian (1963), the accumulation of these products (i.e., lactic acid, CO_2) may be a critical factor which limits the *in vivo* retina to survive even relatively short periods of hypoxia. In *in vitro* preparations where such accumulations do not occur the isolated rat retina is able to recover from periods of anoxia lasting up to 30 min (Winkler, 1972).

A second factor which may contribute to the protection of the retina is temperature. There are numerous reports on the fact that lower temperatures increase the survival time of tissues exposed to anoxic conditions.

This protection is based on the well known relationship between temperature and tissue metabolism and it is for this reason that hypothermia is often employed in surgery when blood flow to the CNS must be interrupted for any period of time. With regard to the eye, Sickel (1966) reported that the greatest prolongation of viability of isolated incubated rabbit retina was when the experiments were carried out at room temperatures. A similar observation was made by Bock, Bornschein and Hommer (1964), who found that the survival time of the rabbit eye was longer at 20°C than at 37°C. While these two factors, temperature and a possible increase in blood flow, may be important in acute hypoxic conditions, it is difficult to see how they could adequately account for the recovery from such a long hypoxic period as that observed in the trout retina after CA inhibition.

The critical factor protecting the retina during chronic hypoxia may be that the rainbow trout retina can carry on a high rate of glycolysis. Hoffert and Fromm (1970) and Baeyens, Hoffert and Fromm (1971) reported that rainbow trout retinas incubated *in vitro* convert over 60 percent of the glucose metabolized to lactic acid. Retinas from rainbow trout treated for two weeks with acetazolamide show a slight increase in this glycolysis (Hoffert and Fromm, 1972) and an increase in LDH activity

(Baeyens, 1972). It is also interesting to note that the ability of the rainbow trout retina to reduce O_2 is only slightly impaired during the two weeks of acetazolamide treatment (Hoffert and Fromm, 1972). This indicates that even after two weeks of hypoxia, the metabolic pathways for the metabolism of glucose remain intact. The data on the accumulation of ocular lactic acid after acetazolamide injection support the hypothesis that glycolysis plays an important role in protecting the retina during this chronic hypoxia. Retinas removed from rainbow trout treated 1.25 hrs previously had a significantly higher lactic acid content than the controls (Fig. 20a). This remained at an elevated level for the next two days and then declined on a daily basis. The fact that LDH activity did not increase within the first 24 hrs indicates that the increase in lactic acid cannot be attributed to a net synthesis in LDH. Rather the increase is probably the result of a Pasteur effect. Baeyens (1970) has shown that such an effect is possible, since the rainbow trout retina utilizes more glucose (41.5%) and produces more lactic acid (33%) under anaerobic conditions than under aerobic conditions.

Although blood lactic acid was routinely determined in these animals, it is difficult to see how the amount of lactic acid within the choroidal blood could markedly affect the lactic acid assay. Based on the total protein

content and the lactic acid concentration/mg protein, the normal retina of these animals contained an average of 75 µg lactic acid. The blood volume of the choroid was estimated by Fairbanks (1970) to be 0.1 ml but this is probably a considerable overestimate. Blood volume measurement of the retina and its attached choroid by the radiochromate method indicates that the volume is in the order of 0.01-0.015 ml (Walker, 1973). Even at a concentration of 100 mg% blood lactic acid, this amounts to only a maximum of 15 µg lactic acid that can be attributed to the blood contained within the choroid.

Effect of Hypoxia on the Slow DC Potential Changes

Hypoxia normally causes a negative drift (cornea becoming more electronegative) in the potential recorded across the vertebrate eye *in vivo* (Granit, 1963). This is in agreement with the results of this study, as within a few minutes after shutting off the water flow across the gills, the potential increased in negativity (Fig. 19). Noell (1952), however, reported that in the rabbit artificial respiration with 99.5% N_2 induced a slow positive potential during the first 3 min of its action before rapidly declining to zero. The sudden interruption of this anoxia by oxygen in its initial stages produced a sharp positive increase in the potential. A positive potential

similar to this was observed in the trout when water flow was resumed across the gills after 4 min. Whereas in the normal trout, illumination had little effect on the steady potential recorded across the eye, after abolishing the high P_{O_2}, steady illumination caused a transient positive potential and darkness elicited a transient negative potential.

The interpretation of the results of this study is difficult due to the complexity of the retina and the fact that several sites are apparently involved in the generation of the transretinal DC potential. According to Noell (1963), the pigmented epithelium is the source of a major fraction of this potential, and it is generated by the active transport of K^+ into the retina proper. However, Hanawa, Kuge and Matsumura (1967) have shown that slow oscillating DC potentials are produced by the retina *in vitro* even when devoid of its pigmented epithelium. Since ouabain, azide or high K^+ in the media bathing the receptor side of the retina were effective in initiating the oscillating potentials, these authors proposed that they were generated by the scleral portion of the Müller cells.

Without further supportive evidence it would be premature at this point to offer a very detailed discussion of the mode of generation of the slow oscillations in the

transretinal potential observed in this study. However, a hypothesis can be put forth which could form the basis for future investigations into this area. Ambient illumination is known to affect the conductance properties of the outer segments of the photoreceptors, causing the cellular membrane to become more permeable to sodium with decreasing illumination (Sillman, Ito and Tomita, 1969). While there is no current evidence, it is possible that there is a simultaneous alteration in the conductance properties for other ions across the membrane. Any such change in conductance, unless opposed by active transport systems, would result in the alteration of the extracellular ionic composition. For instance, if an adjacent cell (i.e., pigmented epithelium) behaved as a K^+ electrode, a change in the extracellular K^+ concentration would change the potential recorded across the membrane of that particular cell. Such a phenomenon has been proposed for the generation of the b-wave by the Müller cells (Miller and Dowling, 1970). It could be argued that in the normal state, active pumps (in the photoreceptors, pigmented epithelium, and the sclera portion of the Müller cells) prevent any large change in the extracellular ionic composition in the region between the pigmented epithelium and external limiting membrane (formed by the Müller cells). However, during hypoxia the activity of these pumps is reduced. Any

subsequent alteration of the conductance properties of the photoreceptors, as a result of changing the ambient illumination, would result in a net flux of ions and produce a change in the ionic composition of the extracellular fluid. As predicted by the Goldman equation, this would change the potential across the cellular membrane of the pigmented epithelium (proximal to the photoreceptors) and/or across the external limiting membrane. Such a phenomenon would explain why the effect on the transretinal DC potential in response to ambient illumination is of a large magnitude in the hypoxic retina compared to the response observed in the normal state (assuming, of course, that the pigmented epithelium and external limiting membrane are the sources of this potential).

It is this author's belief that the transretinal potential is linked with the mechanism that generates the large c-wave in the hypoxic retina. This is based on the fact that with repetitive photostimuli the resultant c-waves summate with the net effect being a positive shift in the transretinal DC potential similar to that produced by steady illumination.

Concluding Statements

The teleost eye has evolved a unique method of providing sufficient O_2 to its retina. This involves the

generation of a superatmospheric P_{O_2} by the choroidal gland posterior to the retina. Since this oxygen concentrating mechanism requires carbonic anhydrase, the necessity of this mechanism for maintaining vision was demonstrated by administering acetazolamide, a potent inhibitor of this enzyme. This drug was found to have a rapid and adverse effect on both the ERG and VETP. *In vitro* experiments confirmed that in contrast to the frog, a P_{O_2} at arterial levels would be insufficient to maintain the functional integrity of the trout retina. In addition, *in vitro* preparations have shown that acetazolamide does not exert a direct effect on the retina which could account for the *in vivo* results.

Although the hypoxic period lasts for a minimum of 24 hr after a single injection of acetazolamide, the trout retina shows a remarkable ability to recover from this adverse condition. Several factors probably contribute to the protection of the retina during this chronic hypoxia including the continued retinal blood flow, the low environmental temperature (15°C), and a large increase in glycolysis. The evidence for this latter factor is that after acetazolamide treatment there is a large increase in the retinal lactic acid. Blood lactic acid determinations revealed that the increase in retinal lactic acid could not be accounted for by an increase in blood lactic

acid. This correlates with Cohen and Noell's (1965) observation regarding metabolism in the mammalian retina. These authors found that while aerobic metabolism was important in maintaining vision, anaerobic metabolism was required for proper tissue maintenance.

In addition to the ERG studies, slow transretinal DC potentials were examined. The transretinal DC potential could be altered by either an hypoxia produced by shutting off the water flow across the gills or by administering acetazolamide. During the retinal hypoxia produced by blocking the oxygen concentrating mechanism the transretinal DC potential was found to be sensitive to changes in ambient illumination. While a general hypothesis was presented which could account for this phenomenon, the specific ions and structures involved in its generation must await further evidence.

SUMMARY

1. The normal rainbow trout ERG is typical of a vertebrate having a retina composed of a mixture of rods and cones. In the light adapted state the ERG is characterized by the a- and b-waves and with dark adaptation there is the appearance of the slow c-wave.

2. The rainbow trout ERG was found to be very sensitive to hypoxia. In the initial stage of hypoxia there is an increase in the amplitude of the c-wave and simultaneous decline in the b-wave. One to two minutes later the a- and c-waves also begin to decline in amplitude.

3. The administration of acetazolamide to the rainbow trout causes an alteration in its ERG which resembles the hypoxic response.

4. Acetazolamide does not affect the ERG recorded from the isolated teleost retina.

5. Because of the similarity of the hypoxic and *in vivo* acetazolamide responses and the lack of a direct effect by the drug on the retina, it was concluded that

acetazolamide produces its effect on the ERG by abolishing the oxygen concentrating mechanism.

6. *In vitro* experiments demonstrated that in contrast to the frog, a P_{O_2} at arterial levels (~ 30 mm Hg) would not support the rainbow trout ERG.

7. The abolishment of the b-wave after acetazolamide administration can be correlated with the abolishment of the VETP and vision.

8. Even though the retinal hypoxia lasts for a minimum of 24 hr after acetazolamide administration, the sources responsible for the generation of the ERG are not permanently damaged.

9. In the normal rainbow trout retina the lactic acid concentration is 5 µg/mg protein. Shortly after an IP injection of acetazolamide (1.25 hrs) there is a dramatic rise in the ocular lactic acid (25 µg/mg protein). This remains at an elevated level for the next 2 days and thereafter declines on a daily basis.

10. Retinal LDH activity 24 hrs after acetazolamide administration was found to be not significantly different from the controls.

11. Hypoxia alters the transretinal DC potential causing the vitreal side to become more negative.

12. Acetazolamide produces a transient negative shift in the transretinal DC potential. This is presumably the result of retinal hypoxia brought on by abolishing the oxygen concentrating mechanism. During this hypoxic period the transretinal DC potential is more sensitive to different light and dark conditions than in the normal state.

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APPENDICES

APPENDIX I

MEDIA COMPOSITION

Cold Blooded Ringers

		mM
NaCl	6.40 g	111.0
CaCl	0.20	1.8
KCl	0.10	1.3
H_2O	to 1 liter	

In Vitro Media

		mM
NaCl	6.40 g	110.0
KCl	0.15	2.0
$NaHCO_3$	0.42	5.0
$CaCl_2$	0.20	1.8
$MgSO_4 \cdot 7H_2O$	1.20	5.0
Glucose	3.60	20.0
H_2O	to 1 liter	

APPENDIX II

ILLUMINATION

Photostimulus

Ambient illumination values reported in the text were measured with a Weston light meter. Due to the frequency response limitations of this instrument, this and similar light meters cannot be used to measure the illumination of a photostimulus generated by the Grass PS2 photostimulator. According to Grass Instruments, the maximal peak intensity of the PS2 is 1.5×10^6 candelas (candle power). Since each candela emits 4π lumens (Seliger and McElroy, 1965), this amounts to a peak intensity of $6.0\pi \times 10^6$ lumens.

Provided the light source is either a pinpoint or a uniformly distributed source, the illumination at a given distance from the source can be calculated from its intensity. If the PS2 lamp is assumed to be a pinpoint light source, then at 1.31 ft (40 cm) the peak illumination would be

$$\frac{6.0\pi \times 10^6 \text{ lumens}}{4\pi(1.31)^2 \text{ ft}^2} = 0.87 \times 10^6 \text{ foot candles}$$

Conversely, if the lamp of the PS2 is assumed to be a uniformly distributed light source, then the intensity is independent of the distance; hence the illumination at the surface of the eye would be

$$\frac{6.0\pi \times 10^6 \text{ lumens}}{\text{ft}^2} = 6.0\pi \times 10^6 \text{ foot candles}$$

Because reflectors cause lamps to have properties intermediate between those of a pinpoint source and a uniformly distributed source (Smith and Hanawalt, 1969), neither of the above assumptions is valid. The actual illumination is therefore between 0.87×10^6 and $6.0\pi \times 10^6$ foot candles.

Red Filter

The red filter used in some of the trout *in vitro* ERG recordings was made from red plexiglass and its peak transmission was at 700 mμ This was determined by placing a small section of the plexiglass in a Beckman DB-G spectrophotometer (Beckman Instruments, Fullerton, Calif.) and scanning its spectrum. Using the Weston light meter and an incandescent light source it was determined that the filter decreased total transmitted light by 1.0 logarithmic unit.

APPENDIX III

IN VITRO RETINAL PREPARATION

In preliminary studies it was observed that bubbling N_2 into the media bathing the receptor side and O_2 into the vitreal side caused an immediate decrease in the amplitude of the b-wave (Fig. 23). When the gases were reversed the b-wave recovered within a few minutes. For this reason, the P_{O_2} in the media bathing the receptor side was monitored rather than that of the vitreal side. The inability of O_2 on the vitreal side to support the mechanisms responsible for the generation of the ERG can be attributed to one of two factors. 1) A barrier (i.e., the internal limiting membrane) prevents the diffusion of O_2 from the vitreal to the receptor side of the retina. 2) The photoreceptors, which initiate the chain of events leading to the generation of the ERG, receive insufficient O_2 because of the high O_2 consumption by the inner portions of the retina (i.e., bipolar and ganglion cells). Further studies will be necessary to clarify the contribution that each of these factors has in affecting the availability of oxygen to the retina.

FIGURE 23.--Effect on the lake trout b-wave recorded *in vitro* of bubbling N_2 and O_2 into the media bathing the receptor and vitreal sides of the retina. During the control portion of the experiment (A), pure O_2 was bubbled vigorously into the media on the receptor side and pure N_2 into the media on the vitreal side. The gases were then switched (B) for 14 min, producing a rapid decline in the b-wave. The b-wave showed considerable recovery when O_2 was again bubbled into the media bathing the receptor side (C).

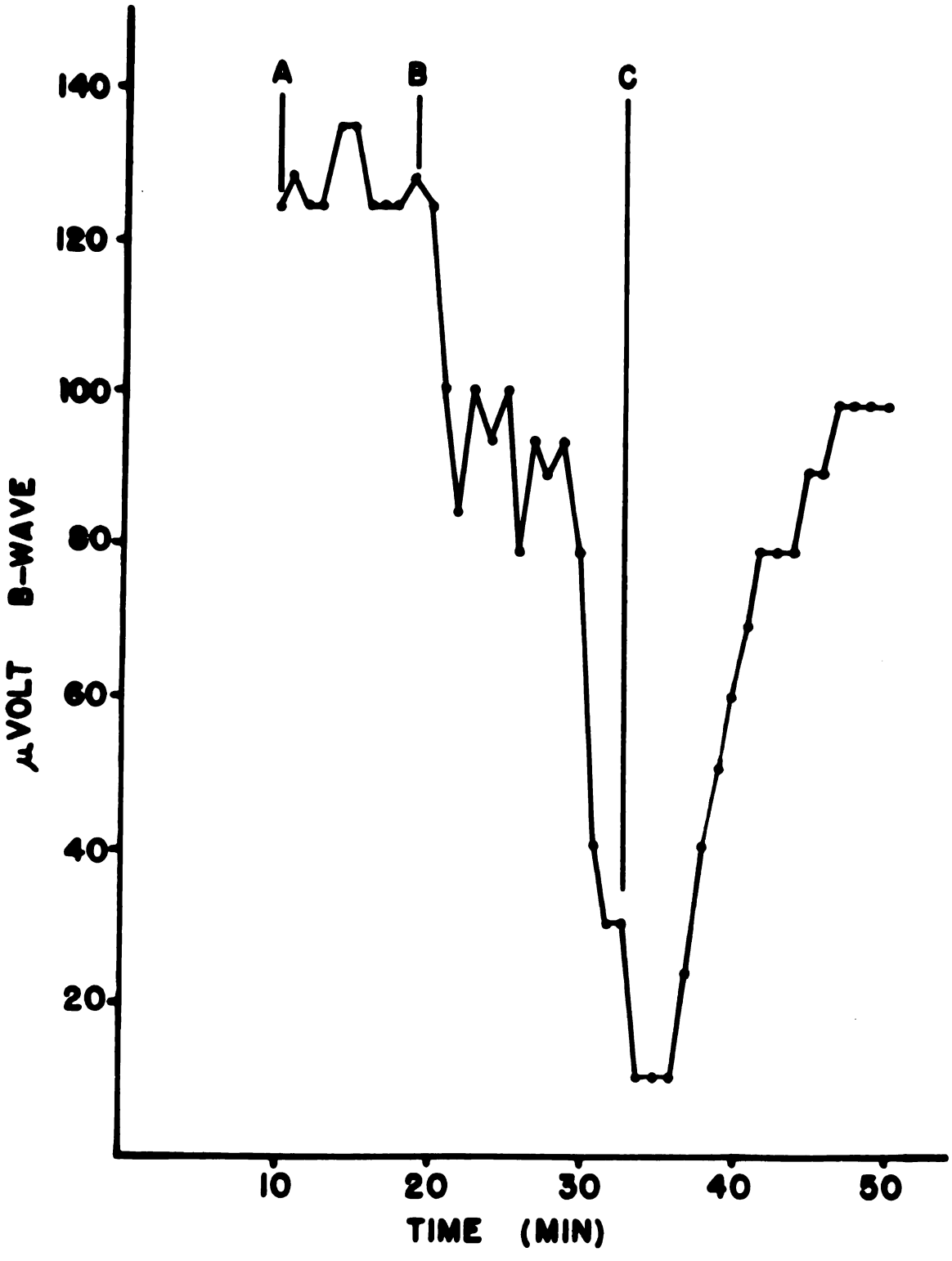

FIGURE 23