Short Circuiting of the Ocular Oxygen Concentrating Mechanism in the Teleost *Salmo gairdneri* Using Carbonic Anhydrase Inhibitors

MICHAEL B. FAIRBANKS, J. RUSSELL HOFFERT, and PAUL O. FROMM

From the Department of Physiology, Michigan State University, East Lansing, Michigan 48824. Dr. Fairbanks' present address is the Department of Biology, Central Michigan University, Mt. Pleasant, Michigan 48849.

ABSTRACT Ocular oxygen concentration by the process of counter current multiplication in rainbow trout (*Salmo gairdneri*) was rapidly suppressed after intraperitoneal injections of the carbonic anhydrase inhibitor CL-11,366. The rapidity with which this drug acted suggested a short circuiting of the choroidal rete mirabile. A comparison was made between the time after injection of inhibitor at which oxygen concentrating ability was lost to the time after injection of inhibitor at which its presence in red blood cells, choroidal rete, pseudobranch, and retinal tissue was first noted. A scheme for the possible role of carbonic anhydrase from each of these tissues in the process of ocular oxygen concentration is given.

INTRODUCTION

Concentration of oxygen in the fish eye is associated with the presence of a choroidal rete mirabile (Wittenberg and Wittenberg [1]) and is dependent on the enzyme carbonic anhydrase (CA) (Fairbanks et al. [2]). We believe that the choroidal rete concentrates oxygen by counter current multiplication similar to that proposed for gas concentration in the swim bladder of fishes (Kuhn et al. [3]; Scholander [4]), a process which is also dependent on carbonic anhydrase (Fange [5]; Maetz [6]).

With previous investigations of the role of carbonic anhydrase in ocular tissue (Fairbanks et al. [2]) or swim bladder (Fange [5]; Maetz [6]) oxygen concentration, inhibitors were used in concentrations sufficient to suppress activity of this enzyme in all tissues possibly having a role in the concentrating mechanism. The pseudobranch may secrete carbonic anhydrase to be used

in gas concentration by the swim bladder (Copeland [7]) or "retinal gas metabolism" (Leiner [8]). The retia of the swim bladder or eye have carbonic anhydrase which may prevent short circuiting of the rete (Maetz [6]). Evidence indicates that carbonic anhydrase of the gas gland of the swim bladder or eye may neutralize lactic acid and produce highly diffusable CO_2, the possible acidifying agent for rapid promotion of the single concentrating effect (Maetz [6]; Fairbanks et al. [2]). Red blood cell (RBC) carbonic anhydrase may be necessary for the proper timing of the single concentrating effect (Berg and Steen [9]; Forster and Steen [10]).

In an attempt to clarify the role of one or more of these sources of carbonic anhydrase in the process of ocular oxygen concentration we selected an inhibitor of low diffusivity, CL-11,366 (2-benzenesulfonamido-1,3,4 thiodiazole-5-sulfonamide) (Maren [11]; Travis et al. [12]). After injection of this drug the onset and time-course of changes in the ocular oxygen concentrating ability were monitored and correlated with the appearance of the drug in the tissues having a possible role in oxygen concentration. Detection of the inhibitor in one or more of these tissues, coincident with an inhibition of the concentrating mechanism, would support the concept that tissue carbonic anhydrase was essential to the concentrating mechanism.

MATERIALS AND METHODS

Rainbow trout (*Salmo gairdneri*) 100–150 g were obtained from the Michigan Department of Natural Resources. The fish were kept in laboratory aquarium facilities at a temperature of 13 ± 0.5°C with 16 h light per day.

Ocular oxygen tension measurements were made at 13 ± 0.5°C using a micropolarographic electrode (Fairbanks et al. [2]). The electrode had a 10-μm tip diameter platinum cathode and was insensitive to pH or salinity over the physiological range; electrode response time was about 99% full response within 2 min. The fish was restrained on its side in a plastic water-filled trough with aerated water pumped over the gills at an approximate flow of 150 ml/min. The water contained MS-222 (tricane methane sulfonate, Crescent Research Chemical, Scottsdale, Ariz.) at a concentration which kept the fish lightly anesthetized (ca. 1:25,000).

The acid form of CL-11,366 was prepared by dissolving 0.45 mg in 1 ml of physiological saline. The sodium salt of the drug was prepared by adding 1.2 mol of NaOH to each mole of CL-11,366. Some fish were given the CA inhibitor acetazolamide (Diamox, Lederle Laboratories, Pearl River, N. Y.) which was supplied as the sodium salt. The controls received the sodium salt of CL-13,850 (2-acetamide-1,3,4-thiodiazole-S-L-butylsulfonamide), a structural analogue of acetazolamide which has no CA inhibitory activity (Maren [11]).

To determine the effect of inhibitors on ocular oxygen concentrating ability the tip of the oxygen electrode was inserted through a hole made in the cornea with a 22-gauge needle. The hole was located along the midline, slightly posterior to the lens. The electrode was lowered with a micromanipulator until the sensing tip was in a region of maximum oxygen tension (P_{O_2}) at the back of the eye (see the ocular oxygen

tension profile curve, Fig. 1). After waiting a minimum of 5 min to see if the presence of the electrode had any effect on the oxygen concentrating ability, 0.5 ml of inhibitor or control solution was injected intraperitoneally (i.p.). The ocular P_{O_2} was monitored until it declined to below the average arterial blood P_{O_2} indicating complete inhibition or, in experimental controls, until we were certain that the drug had no effect on the oxygen concentrating mechanism.

After determining the concentration of CL-11,366 which produced a rapid inhibition of the concentrating mechanism (approximately 2.5 mg/kg) a second group of

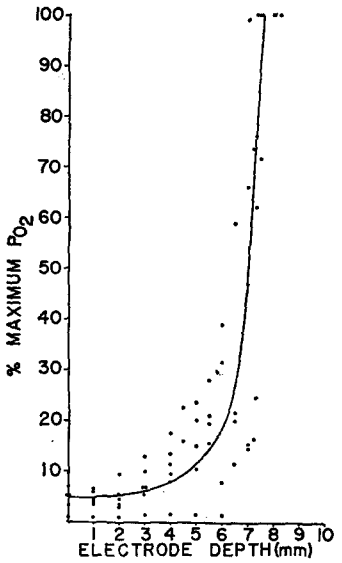

FIGURE 1. Ocular oxygen tension profile, a localization of the region of maximum ocular P_{O_2}. The oxygen electrode was lowered into the eye in 0.5–1.0-mm steps using a micromanipulator. The maximum P_{O_2} (446 mm Hg) was found in a narrow band at the back of the eye. Further advancement of the electrode resulted in a decrease of the recorded P_{O_2} and eventual movement of the eye in the eye socket as the electrode pressed against the back of the eye.

fish were used to determine in which tissue(s) the inhibitor first appeared and whether this was coincident with inhibition of the concentrating mechanism. The method of Maren [13] was used to determine the concentration of inhibitor in the tissues. Twenty fish were given 2.5 mg/kg i.p. injections of CL-11,366. At 2, 4, 6, and 10 min postinjection five fish were killed by cervical section and samples of the pseudobranch, choroidal rete, retina, and RBC's were removed and weighed. Homogenates of the tissues were prepared, placed in a boiling water bath for 5 min to destroy endogenous CA and assayed for the presence and concentration of CL-11,366. Plasma samples were also checked for CL-11,366.

The rapidity with which the inhibitors worked suggested that they may be affecting blood flow through the choroidal rete. To investigate this possibility we examined both the decline of ocular P_{O_2} after ligation of the ophthalmic artery and the fluorescein

circulation time immediately after injection of inhibitor. The opthalmic arteries (efferent pseudobranch vessels) going to the choroidal retia are not in communication with any neighboring vessels but are themselves united by the commissure artery at the level of the parasphenoid bone (Maetz [6]). Before determining the effect of ligation of the ophthalmic artery the commissure artery was cauterized to prevent blood flow from the contralateral pseudobranch. To ligate the ophthalmic artery holes were placed through the opercular flap on either side of the vessel and a thread looped through the holes to surround the vessel. With a small piece of gum rubber tubing placed in the loop, the thread could be tightened down on the tubing to clamp off the vessel without damaging it. Decline in ocular Po_2 after occlusion of the ophthalmic artery was monitored as indicated above.

To determine the effect of inhibitors on the fluorescein circulation time fish were restrained as for Po_2 measurements. Inhibitor (CL-11,366, 2.5 mg/kg) or physiological saline was injected i.p. at a volume of 0.5 ml; 3 min later the fish received a bolus of sodium fluorescein (0.2 ml of a 2 g % solution) in the caudal vein. The elapsed time between injection and appearance of fluorescein in the pseudobranch was determined visually to the nearest 0.1 s utilizing ultraviolet light.

RESULTS

The onset and completeness of inhibition of the O_2 concentrating mechanism was dose related and apparently dependent on the ionization state of the inhibitor at the time of injection (Fig. 2 A,B,C). The acid form of CL-11,366 at an approximate dose of 2.5 mg/kg acted as rapidly to inhibit oxygen concentration as did the sodium salt of acetazolamide given at a dose of 20 mg/kg (Fig. 2 A and B). However, when CL-11,366 as the sodium salt was given at a dose of 2.5 mg/kg the onset and degree of inhibition was noticeably less than that produced by the acid form of the drug (Fig. 2 C). This difference is probably due to the pK_a and the ionization state of the drugs administered.

CL-11,366 has a pK_a of 3.2 (Maren [11]). The pH of the acid preparation was 3.3 while that of the Na salt was 9.2, thus the latter preparation contained more drug in the ionized form. Since the drug was administered i.p. its rate of absorption was in part dependent on the ionized fraction at the site of absorption; i.e., the greater the degree of ionization the slower the rate of absorption. This may explain the difference in inhibitory activity of the acid and Na salt preparations. Acetazolamide, with a pK_a of 7.4, would be only partially ionized at the pH at which it was given (8.8) and therefore readily absorbed even though it was given as the Na salt. The control drug, CL-13,850, (25 mg/kg) was without effect on the ocular oxygen concentrating mechanism.

Choroidal retia and pseudobranch tissues were the first to show the accumulation of the inhibitor CL-11,366 after a 2.5 mg/kg i.p. injection of this drug (Table I). The drug was also present in the plasma as early as 2 min after injection but there was no clear-cut inhibition of CA in red blood cells until 6

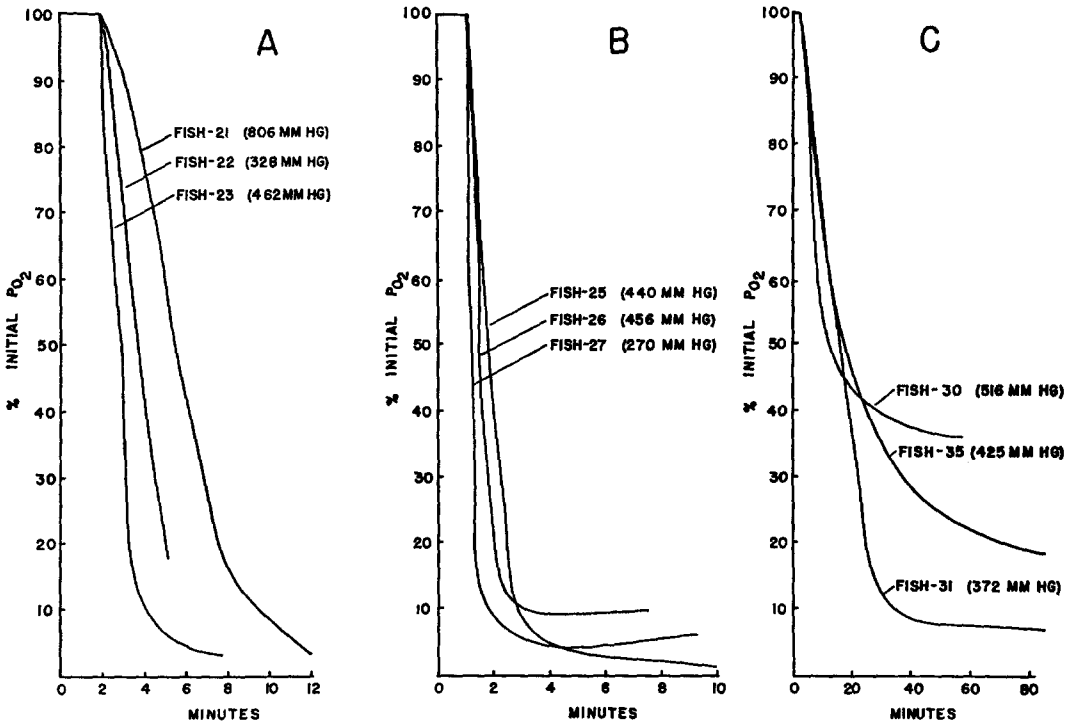

FIGURE 2. Time-courses of P_{O_2} changes after i.p. injections of CL-11,366. The numbers in parenthesis are the initial P_{O_2} values. (A) Fish 21, 22, and 23 received 0.5 ml of a 0.45 mg/ml solution of CL-11,366, producing respective dose levels of 2.37, 2.45, and 2.5 mg/kg. (B) Fish 25 and 26 received 0.5 ml of a 0.45 mg/ml solution of CL-11,366. Fish 27 received 0.5 ml of a 4.0 mg/ml solution of acetazolamide. Respective dose levels of drugs were 2.32, 2.78, and 20.9 mg/kg. (C) Fish 30 and 31 were given 0.5 ml of a 0.045 mg/ml solution of CL-11,366. Fish 35 receives 0.5 ml of a 0.45 mg/ml solution of the Na^+ salt of CL-11,366. Respective dose levels were 0.27, 0.33, and 2.50 mg/kg. Note expanded time scale.

TABLE I

CONCENTRATION OF INHIBITOR (CL-11,366)

Concentration of inhibitor (CL-11,366) in tissues possibly involved with ocular oxygen concentration at times 2, 4, 6, and 10 min postinjection of 2.5 mg/kg of inhibitor. The acid form of the inhibitor was given i.p. at a volume of 0.5 ml.

Time post-injection	*N*	Concentration of CL-11,366				
		Pseudobranch	Choroidal rete	Retina	RBC	Plasma
min			*μg/g tissue*		*μg/ml*	
2	5	9.2	8.0	0	1.1	3.0
4	5	1.8	10.5	0.4	0.9	2.3
6	5	5.0	11.0	0	0.8	2.4
10	5	0	12.3	0.3	0.2	1.7

min postinjection. Only in the choroidal rete was there evidence of retention and progressive accumulation of the inhibitor.

Stasis of blood flow to the choroid rete by ligation of the ophthalmic artery caused an expected immediate decrease in ocular Po_2 (Fig. 2). However, the time required for depletion of ocular Po_2 from 100 to 50% after ligation (Fig. 3) was greater than the time required for a similar depletion starting at the onset (about 90 s postinjection) of inhibition with the drugs (Fig. 2 A and B). The time for half-maximal effect (after onset) in the case of ligation was 72 s (Fig. 3) while the time after CL-11,366 administration averaged about 30 s (Fig. 2 B). It was also found that the circulation time for fluorescein

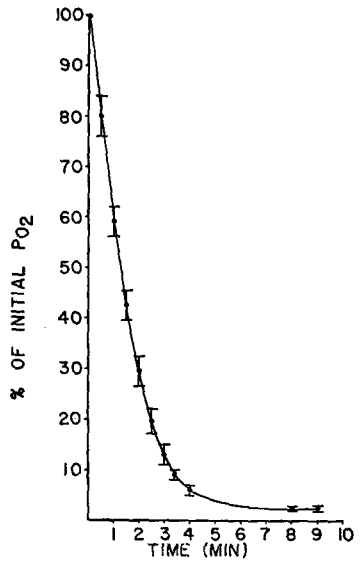

FIGURE 3. Time-course for the decrease in the ocular Po_2 after ligation of the ophthalmic artery (efferent pseudobranch vessel). We attribute the Po_2 decrease to retinal utilization of available oxygen.

TABLE II

CIRCULATION TIME OF FLUORESCEIN

Circulation time of fluorescein from the caudal vein to the pseudobranch in Ringer solution and CL-11,366-treated fish. Each fish received a bolus of sodium fluorescein (0.2 ml of a 2 g% solution) in the caudal vein 3 min after treatment.

Treatment	Circulation time
	s
0.5 ml Ringer i.p.	16.47±1.90 (5)
0.5 ml CL-11,366 i.p. (0.45 mg/ml)	15.23±1.87 (5)

Mean ± SE (*N*)

from the caudal vein to pseudobranch was similar in controls and CL-11,366-treated fish (Table II). The only vascular supply to the choroidal rete is the ophthalmic artery (the efferent pseudobranch vessel). In view of the speed of action and the absence of an effect of the drug on circulation time, the CA inhibitors appear to affect the oxygen concentrating mechanism by some means other than an alteration in blood flow.

DISCUSSION

The ocular oxygen concentrating mechanism of rainbow trout and ocular Po_2 was suppressed after i.p. injection of CA inhibitors. Within 90 s after administration of the acid form of CL-11,366 (2.5 mg/kg) effective suppression was noted. The ocular oxygen concentrating mechanism and ocular Po_2 were likewise suppressed after ligation of the ophthalmic artery. In the latter case the drop in ocular Po_2 was presumably due to the rapid utilization of available oxygen by the metabolically active retinal tissue, however, the rate of depletion of Po_2 was less than that seen after injection of CA inhibitors. Although we have no direct evidence that blood flow between the pseudobranch and choroidal rete was affected by CL-11,366, the more rapid rate of CA inhibition with the drug is interpreted as an indication that its mode of action on this system is not mediated through an alteration (decrease) in rete blood flow. We have shown that this drug has no effect on the time required for transport of fluorescein from the caudal vein to the pseudobranch. If the drug had restricted flow in the efferent pseudobranch vessel (the ophthalmic artery) it follows that the time required for transport of fluorescein as observed would also have been affected. The absence of an effect on the circulation is not surprising since there is no evidence that at the concentrations given the CA inhibitors used have any effect on physiological systems apart from inhibition of CA-dependent activities (Maren [11]).

Oxygen depletion after ligation of the efferent pseudobranch artery is most probably due to the rapid oxygen consumption of the retina and assuming that the presence of inhibitor in a tissue indicates inhibition of CA in that tissue, our results indicate that after drug administration the rapid suppression of the oxygen concentrating mechanism is due to inhibition of pseudobranch and/or choroidal rete CA.

Copeland [7] has suggested that the pseudobranch secretes CA which is transported to and is involved in the filling of the swim (gas) bladder with O_2 and CO_2. Since the pseudobranch vasculature is in series with the choroidal rete any CA secreted by this gland would also be available for the oxygen concentrating mechanism in the eye. More recently Maetz [6] was unable to duplicate Copeland's results. He reported no evidence of a difference in the concentration of CA in afferent and efferent blood going to or from the pseudobranch and concluded that the gland does not secrete CA. In light of Maetz'

results it is difficult to attribute rapid suppression of the ocular oxygen concentrating mechanism to inhibition of pseudobranch CA. In addition, even though we found CL-11,366 in the pseudobranch concurrent with the early suppression of oxygen concentration, none was detected 10 min after injection and, at this time, the concentrating mechanism remained suppressed (Table I and Fig. 2 B).

There is some evidence in support of the idea that inhibition of choroidal rete CA is responsible for suppression of the ocular concentrating mechanism. The inhibitor CL-11,366 suppressed ocular oxygen concentration and was found to accumulate in choroidal tissue but not in RBC's or retinal tissue. Electron micrographs of the swim bladder rete mirabile have revealed definite structural differences between arterial and venous rete capillaries (Jasinski and Kilarski [14]; Fawcett and Wittenberg [15]). The arterial rete endothelium is exceptionally thick while venous capillaries are characterized by smaller endothelial cells of irregular thickness with occasional pores between the cells. One of us (M. B. F.) has likewise noted in the rainbow trout choroidal rete that the arteriole endothelium is thicker than the venous capillary endothelium (unpublished observations). Perhaps the endothelium of choroidal rete capillaries is more permeable to the drug than the erythrocyte cell membrane or retinal cells and this could lead to rapid and effective inhibition of choroidal rete CA. Previously it was believed that in fish the erythrocyte CA was the most susceptible to inhibition by CL-11,366 (Maren [11]).

Alternatively, the rete carbonic anhydrase could be bound to the luminal wall of the capillary, exposing the enzyme to inhibitor which would account for the relative ease of its inhibition. If the enzyme is located on the luminal surface of the endothelium its source could be the pseudobranch as suggested by Leiner [8] and (indirectly) by the work of Copeland [7].

Based on this discussion and previous work (Fairbanks et al. [2]) our concept of the role of CA (especially choroidal rete CA) in the ocular oxygen concentrating mechanism is as follows: Retinal CA catalyzes the dissociation of carbonic acid which results from the neutralization of retinal lactic acid with bicarbonate. The CO_2 produced diffuses into vessels in the choriocapillaris adjacent to the retina and some enters RBC's where it is rapidly hydrated when RBC CA is present. This gives rise to the Bohr and Root effects leading to the single concentrating effect, an increase in plasma P_{O_2}. The remaining fraction of retinal CO_2 causes an increase in venous blood P_{CO_2} providing a gradient for the diffusion of CO_2 from the venous to the arterial side of the rete. This movement of CO_2, we believe, is prevented by the action of choroidal rete CA. If it were allowed to occur it could cause a premature single concentrating effect. The rete would then act as a *counter current exchanger*, oxygen would then diffuse from the afferent to the efferent vessels, completely by

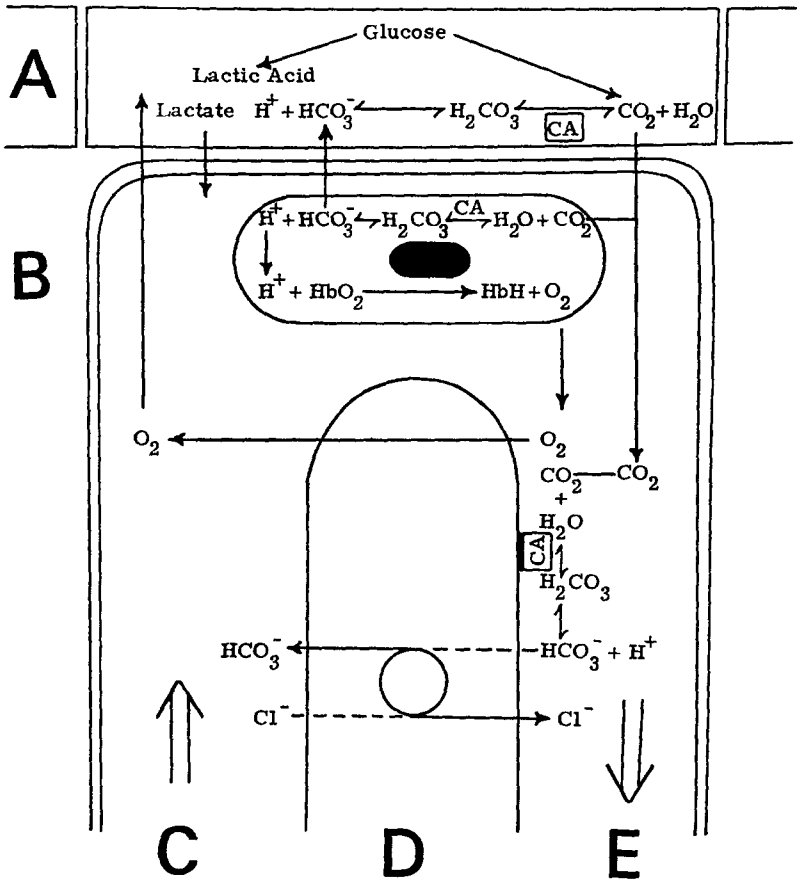

FIGURE 4. Scheme for the role of retinal, red blood cell, and choroidal rete carbonic anhydrase in ocular oxygen concentration. (A) Retina. Retinal carbonic anhydrase facilitates the neutralization of lactic acid through catalyzation of the dehydration of H_2CO_3, thus assuring that the reaction proceeds 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 facilitates 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.

passing the choriocapillaris, and would be very rapidly carried away from the eye.

Prevention of the aforementioned short circuiting would occur if the choroidal rete CA was located on the luminal surface or within the endothelial

cells of the venous rete. The carbonic acid formed from hydration of CO_2 would dissociate to H^+ and HCO_3^- with the bicarbonate moving to the arterial rete where it would be made available for the neutralization of more retinal lactic acid. Maetz [6] has suggested such a role for swim bladder rete CA. He visualized the hydration of CO_2 as occurring within the endothelial cells with the H^+ moving unidirectionally into the venous rete and the HCO_3^- to the arterial rete in exchange for chloride. We suggest, because of the rapidity of suppression of the ocular oxygen concentrating mechanism, that the rete CA is located on the luminal surface of the endothelial cells with the reactions taking place in the venous blood. Diffusion of H^+ into the rete endothelium would not occur and movement of HCO_3^- to the arterial rete is again in exchange for Cl^- and may actually be coupled with active Cl^- transport. This chloride-bicarbonate pump would serve as an important adjunct to the O_2 concentrating mechanism being of significance mainly because of the need to recycle bicarbonate for the neutralization of lactic acid in the retina.

Apart from the ocular oxygen concentrating mechanism the chloride-bicarbonate pump in the choroidal rete could be of some importance in the formation of ocular humors. In mammals, the ciliary body is responsible for aqueous humor formation but this structure is absent in eyes of teleosts and the site of aqueous humor formation has not yet been determined (see discussion by Zadunaisky [16], [17]). One of us (J. R. H.) has found that the aqueous humor of the fish eye is continuous with a thin layer of fluid between the retina and the vitreous body which indicates that the choroidal rete may participate in fish aqueous humor formation.

The authors gratefully acknowledge Mrs. Esther Brenke for her technical assistance. We would also like to thank Dr. J. M. Smith, Jr. of Lederle Laboratories, American Cyanamid Company for his kind donation of the carbonic anhydrase inhibitors.

This research was supported in part by Grant EY-00009VIS from National Eye Institute (USPHS) and by a predoctoral traineeship from the National Heart and Lung Institute to Dr. Fairbanks (HE 05873-02).

Received for publication 10 May 1974.

REFERENCES

1. WITTENBERG, J. B., and B. A. WITTENBERG. 1962. Active secretion of oxygen into the eye of fish. *Nature (Lond.)*. **194**:106.
2. FAIRBANKS, M. B., J. R. HOFFERT, and P. O. FROMM. 1969. The dependence of the oxygen-concentrating mechanism of the teleost eye (*Salmo gairdneri*) on the enzyme carbonic anhydrase. *J. Gen. Physiol.*, **54**:203.
3. KUHN, W., A. RAMEL, H. J. KUHN, and E. MARTE. 1963. The filling mechanism of the swim bladder. *Experientia (Basel)*. **19**:497.
4. SCHOLANDER, P. F. 1954. Secretion of gases against high pressures in the swim bladder rete of deep sea fishes. II. The rete mirabile. *Biol. Bull. (Woods Hole)*. **107**:260.
5. FANGE, R. 1953. Mechanism of gas transport in the Euphysoclist swim bladder. *Acta Physiol. Scand.* **30**:1.

6. MAETZ, J. 1956. Le role biologique de l'anhydrase carbonique chez quelques teleosteens. *Bull. Biol. Fr. Belg.* **XL**:1. (Suppl.).
7. COPELAND, D. E. 1951. Function of glandular pseudobranch in teleosts. *Am. J. Physiol.* **167**:775. (Abstr.).
8. LEINER, M. 1938. Die Augen Keimendrüse (Pseudobranchie) der Knochen Fische. Experimentelle Untersuchenger über ihre physiologische Bedeutung. *Z. Vgl. Physiol.* **26**: 416.
9. BERG, T., and J. B. STEEN. 1968. The mechanism of oxygen concentration in the swim bladder of the eel. *J. Physiol. (Lond.).* **195**:631.
10. FORSTER, R. E., and J. B. STEEN. 1968. Rate limiting processes in the Bohr shift in human red cells. *J. Physiol. (Lond.).* **196**:541.
11. MAREN, T. H. 1967. Carbonic Anhydrase chemistry. Physiology and inhibition. *Physiol. Rev.* **47**:595.
12. TRAVIS, D. M., C. WILEY, B. R. NECHAY, and T. H. MAREN. 1964. Selective renal carbonic anhydrase inhibition without respiratory effect: Pharmacology of 2-benzensulfonamido-1,3,4,thiadiazol-5-sulfonamide (CL-11,366). *J. Pharmacol. Exp. Ther.* **143**:383.
13. MAREN, T. H. 1960. A simplified micromethod for the determination of carbonic anhydrase and its inhibitors. *J. Pharmacol. Exp. Ther.* **130**:26.
14. JASINSKI, A., and W. KILARSKI. 1971. Capillaries in the rete mirable and in the gas gland of the swim bladder in fishes, *Perca fluviatilis L.* and *Misgurnus fossilis L.* An electron microscopic study. *Acta Anat.* **78**:210.
15. FAWCETT, D., and J. B. WITTENBERG. 1959. The fine structure of capillaries in the rete mirabile of the swim bladder of *Opsanus tau. Anat. Rec.* **133**:274.
16. ZADUNAISKY, J. A. 1972. The electrolyte content, osmolarity, and site of secretion of the aqueous humor in two teleost fishes (*Carassius auratus* and *Diplodus sargus*). *Exp. Eye Res.* **14**:99.
17. ZADUNAISKY, J. A. (1973). The hypotonic aqueous humor of teleost fishes. *Exp. Eye Res.* **16**:397.