Teleost Retinal Metabolism as Affected by Acetazolamide[1] (36298)

J. Russell Hoffert and Paul O. Fromm
Department of Physiology, Michigan State University, East Lansing, Michigan 48823

An elaborate countercurrent diffusion multiplier (rete mirabile) constitutes the major portion of the choroid body and choriocapillaris contained in the choroid layer of the teleost eye. The rete plays a key role in the generation of superatmospheric oxygen tensions in the choroid and the avascular retina depends upon the diffusion of molecular oxygen from the choroid to supply its metabolic demands (1, 2). The importance of the enzyme, carbonic anhydrase, to the countercurrent multiplication system was originally identified by Fairbanks *et al.* (1). Reports from our laboratory (4–6) indicate that the teleost retina is a very active tissue metabolically that produces copious amounts of acid metabolites in the form of carbon dioxide and lactic acid. These metabolites are believed to diffuse from the retina into the choriocapillaris and the area surrounding the arterial and venous capillaries of the rete mirabile. CO_2 from the retina and/or that resulting from neutralization of lactic acid effectively causes a rapid Root and/or Bohr shift resulting in dissociation of oxyhemoglobin and release of molecular oxygen. Oxygen tensions as high as 800 mm Hg have been recorded but the average is around 400 mm Hg which is some 20 times higher than the average arterial oxygen tension (1). The precipitous drop in ocular P_{O_2} after administration of acetazolamide is believed to be due to a selective inhibition of carbonic anhydrase located on the venous side of the rete and does not involve inhibition of carbonic anhydrase located in the retina or erythrocytes.

Much of the evidence for the role of carbonic anhydrase in generation of high oxygen tensions has come from experiments utilizing acetazolamide as an enzyme inhibitor. The effect of this drug might be directly on retinal tissue or it could be by way of its inhibitory effect on retinal carbonic anhydrase. The purpose of the present study was to determine whether or not acetazolamide could directly affect retinal metabolism sufficiently to alter the production of acid metabolites which would in turn affect the oxygen-concentrating system of the choroid rete mirable.

Materials and Methods. The Michigan Department of Natural Resources hatchery at Grayling, MI supplied the rainbow trout (*Salmo gairdneri*) weighing 211.12 ± 9.39 g used in this experiment. The fish were held at 14 ± 0.5° with a 16:8 L:D photoperiod. The fish were killed by cervical dislocation and retinas immediately removed and placed in a large volume of a Ringer solution which contained neither bicarbonate nor glucose. While in this solution the choroid and vitreous body were removed and the retinas cut into three sections, each of which was then placed in a 10-ml Luer-lok glass syringe. The syringe, previously cooled to 15°, contained a glass bead (volume = 0.116 ml/bead) and was fitted with a male–female-tipped gas-tight stopcock.

The incubation media used was Mammalian Krebs saline (7) with 2.856 g $NaHCO_3$/liter and glucose added. It had an osmolarity of 178 mOsm/kg and a pH of 7.6 at 15° when equilibrated with 5% CO_2, 21% O_2, and 74% N_2. The syringe was flushed with the gas mixture before filling with media. Next, 10 ml of gas-saturated media were transferred anaerobically into the syringe taking care not to trap any gas bubbles. The syringes were then placed on a slowly rotating mixer positioned at an angle from

[1] This research was supported in part by U.S. Public Health Service grant (EY 00009 VIS) from the National Eye Institute, U.S. Public Health Service.

horizontal such that the glass bead effectively mixed the incubation media. The incubations were carried out at 15° for 3 hr unless otherwise indicated.

A Radiometer (BMS 3, Radiometer Co., Copenhagen) thermostatically regulated at 37° was used for measurement of pH, P_{O_2}, and P_{CO_2}. A media blank, containing no retinal tissue, was transferred anaerobically to the BMS 3 and used to determine the initial oxygen concentration (m*M*/liter). In a similar fashion, the oxygen concentration of media containing experimental tissues was determined. Calculations of O_2 concentration were based on the solubility coefficient of oxygen in Ringer solution (0.0340 ml O_2/ml fluid at 1 atm at 15°) provided by Umbreit *et al.* (8). Q_{O_2} values were expressed as μM_{O_2} consumed/hr/g protein. It was impossible to blot retinal tissue dry because of it's low cohesiveness, therefore, total protein was used as a measure of metabolic mass. After P_{O_2} determinations, the remaining media and tissue (7 ml) were homogenized (Bronson Sonifier, Heat Systems Co., Melville, L.I., NY) and aliquots taken for duplicate determinations of lactic acid by the method of Barker and Summerson (9) and protein by the Lowry method (10). Acetazolamide (Diamox, Lederle Laboratories, Pearl River, NY) was administered by ip injection to some fish at a dose of 50 mg/kg every 2 days for 2 weeks. In the *in vitro* studies, acetazolamide was added to the incubation media to give a concentration of 10 mg/100 ml.

Results. Conditions used in these incubation studies differed in several respects from those that occur *in vitro* but many of the discrepancies were unavoidable due to instrumentation difficulties involving the Radiometer. The response times of the P_{CO_2} and P_{O_2} electrodes at 15° precluded doing measurements at this temperature; thus, the tissues were incubated at 15° but pH and gas tension determinations were made at 37°, the temperature which is optimal for response of the blood–gas electodes. Data on the pH, P_{CO_2}, and P_{O_2} of the incubation media at 37° are presented in Table I. When the pH value of 7.4 at 37° is corrected back to 15° (11)

TABLE I. Analysis of Retinal Q_{O_2} and Incubation Media vs. Time at 15° and in Media Containing 22 mg/100 ml Glucose.[a]

	Media (0 hr)	1st hr	2nd hr	3rd hr	4th hr
pH[b]	7.389 ± 0.009 (56)	7.333 ± 0.008 (67)	7.305 ± 0.007 (61)	7.324 ± 0.006 (114)	7.274 ± 0.028 (6)
P_{O_2} (mm Hg)[b]	182.7 ± 1.568 (36)	152.0 ± 1.673 (61)	130.8 ± 2.295 (61)	108.7 ± 3.012 (61)	107.8 ± 16.843 (6)
P_{CO_2} (mm Hg)[b]	44.4 ± 1.233 (47)	49.8 ± 0.939 (61)	54.9 ± 1.022 (61)	53.7 ± 0.773 (109)	56.3 ± 3.275 (6)
Q_{O_2} (m*M* O_2/hr/*g* protein)		0.085 ± 0.005 (61)	0.055 ± 0.003 (59)	0.051 ± 0.003 (61)	0.047 ± 0.009 (6)

[a] Mean ± SE (observations).
[b] Measured at 37° after anaerobically warming from 15°. Average retinal protein = 4.91 ± 0.204 (79) mg/syringe.

TABLE II. Retinal Q_{O_2} and Lactate Production at 15° with 10 mg/100 ml Acetazolamide Added to Incubation Media (*in vitro* studies) or 50 mg/kg Acetazolamide Injected ip for 2 Weeks (*in vivo*).

	Q_{O_2} (m*M*/hr/g protein)[a]	Lactic acid production (m*M*/hr/g protein)[a]
Control	0.084 ± 0.006 (53)	0.205 ± 0.014 (53)
Acetazolamide		
in vitro	0.100 ± 0.007 (24)[b]	0.186 ± 0.016 (22)
in vivo	0.067 ± 0.003 (30)[c]	0.237 ± 0.015 (30)

[a] Mean ± SE (number of observations).
[b] Significantly different at 10% level.
[c] Significantly different at 1% level.

the calculated value is 7.6 which is nearly identical to the normal pH of trout blood at that temperature. The pH of the media decreased slightly during the 4-hr incubation period but remained within the range considered normal for rainbow trout arterial blood.

The P_{O_2} of the media when adjusted to 15° was 38 mm Hg which is considerably lower than the oxygen tension which is believed to occur immediately posterior to the retina of intact trout eyes. We have no data for the actual intraretinal P_{O_2} but because of the generation of high P_{O_2} in the choriocapillaris, it is probable that the P_{O_2} in the media used is somewhat below those encountered *in vivo* even though it is quite similar to that of arterial blood (20–40 mm Hg). This level of oxygen was chosen because of the fact that when samples of the media, which were initially equilibrated to higher oxygen tensions, were anaerobically warmed from 15 to 37° gas came out of solution. To obtain reliable data on oxygen consumption the gas had to remain in solution under the experimental conditions.

When the P_{CO_2} of the media was adjusted from 37 to 15° it was 14.4 mm Hg which is somewhat higher than that encountered *in vivo* (8 mm Hg). This tension of CO_2 was used because our Radiometer will not record P_{CO_2} values lower than 8 mm Hg. The relatively high P_{CO_2} and the bicarbonate content of the media provided a very effective buffer system for the incubations. It has been reported that a bicarbonate buffer system is to be preferred over a phosphate system in studies of mammalian retinal metabolism.

As indicated in Table I, pH showed a slight but insignificant decrease during incubation. P_{O_2} decreased during the first 3 hr of incubation and Q_{O_2} decreased significantly during the second hour of incubation but was relatively constant thereafter. P_{CO_2} increased during the first 2 hr but was nearly unchanged during the remainder of the incubation period. Data for the first 3 hr were expressed as a parabolic function ($Y = aX^b$) and correlation coefficients determined. Slopes for both Q_{O_2} and P_{O_2} verus time were significantly different from zero but the correlation coefficient of P_{O_2} vs. Q_{O_2} was not significant ($r = 0.3269$) which indicates an independence of these two variables. The data presented in Table I were obtained with 22 mg/100 ml glucose in the incubation media which is somewhat below the normal blood glucose level of trout (40–60 mg/100 ml).

The Q_{O_2} values given in Table II were obtained over a 3-hr period with 111 mg/100 ml glucose in the incubation media. Under these conditions the average Q_{O_2} was not statistically different from the value for the first hour of incubation at low glucose levels but was significantly higher (at 1% level) than the Q_{O_2} values for hours 2, 3, and 4 (Table I).

Rainbow trout liver tissue incubated with 111 mg/100 ml glucose consumed less oxygen [0.048 ± 0.001 (21)] and produced less lactate [0.015 ± 0.002 (21)] than the retina. We have assumed that all oxygen consumed was used in the oxidation of glucose by the tricarboxylic acid (TCA) cycle and that the only other metabolite resulting from

glucose utilization was lactic acid. The trout liver consumed 0.015 m*M* glucose/hr/g protein with 52% being oxidized by the TCA cycle and 48% utilized by aerobic glycolysis.

Administration of acetazolamide to trout retinas *in vitro* caused a drop in glucose utilization from 0.116 to 0.109 m*M* glucose/hr/g protein, whereas, when given *in vivo* there was an increase to 0.129. When given *in vitro* TCA cycle activity was apparently stimulated so that some 15% of the total glucose utilized was by this pathway, up from 12.4% by control retinas. When given *in vivo* acetazolamide caused a very significant decrease in TCA cycle activity such that only 8.6% of the glucose was utilized in this manner (Table II).

Effects of acetazolamide on aerobic glycolysis were somewhat less conclusive. *In vitro* administration of the drug tended to depress aerobic glycolysis, whereas, *in vivo* administration tended to increase it. Differences from control values were not statistically significant due to the variability of the data. Each case taken individually indicates that the two metabolic pathways were inversely affected by acetazolamide.

Discussion. Analysis of data from *in vitro* incubations of trout retinas indicates that the decreases in P_{O_2} and Q_{O_2} during the 4-hr period are statistically independent of one another. Thus decreased P_{O_2} does not appear to be a limiting factor for Q_{O_2}. It can be shown by calculation that at high glucose levels some 0.0017 m*M* of glucose would be consumed by 5 mg of retinal tissue during a 3-hr period. The syringe with the low glucose media contained a total of 0.028 m*M* glucose which is more than 10 times the calculated consumption of 5 mg of retinal tissue. The total amount of glucose available in these experiments was not a rate-limiting factor for retinal metabolism but the rate of diffusion of glucose into retinal tissue could have been. This idea is supported by the observation that when glucose was increased to 111 mg/100 ml in the incubation media, retinal Q_{O_2} for 3 hr was nearly identical to that for the first hour of incubation with the low-glucose media.

As stated above, the aim of this study was to determine if acetazolamide had any direct action on the retina and if so whether this action could affect the oxygen-concentrating mechanism in the trout eye. Data presented above indicates that injection of the drug into trout does indeed cause a decrease in retinal oxygen consumption (Q_{O_2}) when measured under *in vitro* conditions. However, due to the fact that more glucose was metabolized by aerobic glycolysis under these conditions, total production of acid metabolites was increased.

When acetazolamide was administered to trout retinal tissue *in vitro* somewhat different results were obtained. The drug caused an increase in oxygen consumption, a decrease in aerobic glycolysis, a decline in glucose utilization but the total production of acid metabolites was comparable to that of controls. Kutchai (12), in *in vitro* studies using the swimbladder of *Anguilla anguilla*, found that acetazolamide markedly reduced the rate of lactate release. These results coincide with our findings of a decreased lactate production in the case of the teleost retina.

In unpublished observations from our laboratory we have shown that chronic administration of acetazolamide to trout produces a marked histopathology of the retina. It could be argued that the effects of acetazolamide given over a 2-week period in these experiments, were the result of general histopathological changes in retinal tissue rather than a direct effect of the drug on the metabolic pathways *per se*. Certainly the effects noted were not due solely to inhibition of retinal carbonic anhydrase. It must be assumed that when acetazolamide was administered *in vitro* the effect on retinal metabolism was a direct one and not mediated through any general pathological deterioration of the tissue.

If the effects of acetazolamide in the intact trout eye had been the same as those noted when the drug was given *in vitro*, we believe that the increase in oxygen consumption (with no change in the production of acid metabolites) would have been inadequate to account for a very rapid decrease in

ocular P_{O_2} which has been noted after administration of the drug (3). The effects of the drug when administered ip could in no way aid in the depletion of ocular P_{O_2} after drug administration. In fact, the heretofore unknown direct effect of acetazolamide on retinal metabolism in the latter case would tend to hinder a rapid depletion of P_{O_2} in the teleost eye.

It could be argued, however, that chronic treatment of fish by ip injection or acute *in vitro* treatment of teleost retinas with acetazolamide could affect the ocular oxygen concentrating mechanism in the following manner. Acetazolamide could cause a preferential shift in retinal metabolism to the formation of lactic acid and if retinal carbonic anhydrase was simultaneously inhibited the total production of highly diffusible CO_2 by the retina would be decreased. The net decrease in CO_2 production could result in a deficiency in the amount of CO_2 needed for H^+ production in erythrocytes adequate to release oxygen from oxyhemoglobin in the choriocapillaris.

Summary. Trout retinas were incubated in air-tight syringes at 15°. Values for the P_{O_2} of the incubation medium and the Q_{O_2} of retinal tissue varied independently indicating that P_{O_2} was not a rate-limiting factor for Q_{O_2}. When compared to controls, retinas from trout treated with acetazolamide had a decreased Q_{O_2} an increase in aerobic glycolysis, and an over-all increase in production of acid metabolites. Retinas treated with the drug *in vitro* showed a decrease in Q_{O_2}, decrease in aerobic glycolysis, and a decline in glucose utilization but no decrease in total production of acid metabolites. The relationship between the effects of acetazolamide on retinal metabolism and the depletion of ocular oxygen tensions after administration of the drug are discussed.

The technical assistance of Mrs. Esther Brenke is acknowledged.

1. Fairbanks, M. B., Hoffert, J. R., and Fromm, P. O., J. Gen. Physiol. **54**, 203 (1969).

2. Wittenberg, J. B., and Wittenberg, B. A., Nature (London) **194**, 106 (1962).

3. Fairbanks, M. B., Hoffert, J. R., and Fromm, P. O., Submitted to J. Gen. Physiol. October, 1971.

4. Fonner, D. B., Hoffert, J. R., and Fromm, P. O., Comp. Biochem. Physiol. **31**, 175 (1969).

5. Hoffert, J. R., and Fromm, P. O., Exp. Eye Res. **10**, 263 (1970).

6. Baeyens, D. A., Hoffert, J. R., and Fromm, P. O., Proc. Soc. Exp. Biol. Med. **137**, 740 (1971).

7. Krebs, H., Biochim. Biophys. Acta **4**, 249 (1950).

8. Umbreit, W. H., Burris, R. H., and Stauffer, J. F., "Manometric Techniques," 305 pp. Burgess, Minneapolis, MI (1964).

9. Barker, S. B., and Summerson, W. H., J. Biol. Chem. **138**, 535 (1941).

10. Oyama, V. I., and Eagle, H., Proc. Soc. Exp. Biol. Med. **91**, 305 (1956).

11. Severinghaus, J. W., J. Appl. Physiol. **21**, 3 (1966).

12. Kutachi, H., Comp. Biochem. Physiol. **39A**, 357 (1971).

Received Oct. 18, 1971. P.S.E.B.M., 1972, Vol. 139.