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Oxygen delivery to the fish eye: Root effect as crucial factor for elevated retinal P_{O_2}

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Summary

Although the retina has one of the highest metabolic rates among tissues, certain teleost fishes lack any vascular supply to this organ which, in combination with the overall thickness of the organ, results in extremely long diffusion distances. As the only way to compensate for these obstacles, oxygen partial pressure (P_{O_2}) in the eyes of such fish is elevated far above atmospheric values. Although not supported by any direct evidence, the enhancement of P_{O_2} is considered to be related to the Root effect, the release upon acidification of Hb-bound O_2 into physical dissolution, possibly supported by counter-current multiplication similar to the loop of Henle.

The present study evaluates the magnitude of intraocular P_{O_2} enhancement under tightly controlled physiological conditions, to directly confirm the involvement of the Root effect on intraocular P_{O_2} in the retina of rainbow trout *Oncorhynchus mykiss*. Intraocular P_{O_2} was determined with special polarographic microelectrodes inserted into the eye. P_{O_2} profiles established *in vivo* by driving electrodes through the entire retina yielded average P_{O_2} values between 10 mmHg (1.3 kPa) at the inner retinal surface and 382 mmHg (50.9 kPa) close to the outer retinal limit (Bruch's membrane). According to estimates on the basis of the diffusion distances determined from sections of the retina (~436 μm at the site of P_{O_2} measurement) and literature data on specific oxygen consumption, the *in vivo* determined values would be sufficient to cover the oxygen demand of the retina with some safety margin.

For a clear and direct in-tissue-test as to the involvement of the Root effect, an isolated *in vitro* eye preparation was established in order to avoid the problem of indirect blood supply to the eye from the dorsal aorta only *via* the pseudobranch, a hemibranch thought to modulate blood composition before entry of the eye. Any humoral effects (e.g. catecholamines) were eliminated by perfusing isolated eyes successively with standardized red blood cell (RBC) suspensions in Ringer, using trout (with Root) and human (lacking any Root effect) RBC suspension. To optimize perfusate conditions for maximal Root effect, the Root effect of trout RBCs was determined *in vitro via* graded acidification of individual samples equilibrated with standardized gas mixtures. During perfusion with trout RBC, P_{O_2} at the outer retinal limit was 99 mmHg (13.2 kPa), but fell by a factor of 3.3 upon perfusion with human RBC in spite of higher total oxygen content (T_{O_2} 2.8 for trout *vs* 3.9 mmol l^{-1} for human RBC). Upon reperfusion with trout RBC, P_{O_2} was restored immediately to the original value. This regularly observed pattern indicated a highly significant difference (P=0.003) between perfusion with trout (with Root effect; high retinal P_{O_2}) and perfusion with human (no Root effect; low retinal P_{O_2}) RBC suspension, thus clearly demonstrating that the Root effect is directly involved and a crucial prerequisite for the enhancement of P_{O_2} in the retina of the teleost eye.

Key words: rainbow trout, *Oncorhynchus mykiss*, ocular oxygen partial pressure, avascular retina.

Introduction

Oxygen (O_2) transfer to the site of consumption within tissues takes place in animals generally by simple diffusion along a diffusion gradient. Thus, oxygen partial pressure (P_{O_2}) in the tissues is expected to be lower than environmental P_{O_2}. This general rule, however, does not apply to a few known instances in teleost fish species. P_{O_2} is extremely high in the swimbladder of deep swimming fish (Nielsen and Munk, 1964), and P_{O_2} values in the eyeballs of several teleost species have also been reported to exceed atmospheric P_{O_2} values (Wittenberg and Wittenberg, 1961, 1962; Fairbanks et al., 1969). While secretion of O_2 into the swimbladder serves the adjustment of buoyancy but is metabolically irrelevant, the extraordinary feature of superatmospheric P_{O_2} values in the eye is thought to compensate for the special conditions of O_2 delivery to retinal tissues. Passive diffusion of O_2 to meet the demands of thick and completely avascular retinae in some teleosts is possible only by establishing a high P_{O_2} diffusion gradient (Wittenberg and Wittenberg, 1974). This task is aggravated by the retina possessing one of the highest metabolic activities among tissues (Anderson, 1968; Yu and Cringle, 2001).

P_{O_2} can be elevated by acidification of blood possessing a Root effect (Root, 1931; Root and Irving, 1943; Brittain, 1987; Pelster and Randall, 1998). With lowering of pH, the amount of haemoglobin-associated O_2 in the blood of some teleost fish is reduced and O_2 transferred into physical dissolution, which may result in considerable elevation of P_{O_2} on the basis of the low aqueous O_2 solubility. This primary effect of P_{O_2} elevation may suffice to produce the elevated P_{O_2} values observed in teleost eyes, but is too small to adequately pressurize the swimbladder in deeper waters. Even if the whole amount of O_2 bound to haemoglobin (Hb) in the arterial blood of a teleost fish (about 5 mmol l^{-1}) could be released upon acidification, P_{O_2} and thus hydrostatic pressure would rise by only about 2800 mmHg (373 kPa).

Any elevation of P_{O_2} in excess of that produced by the single-pass Root effect is thought to be brought about in the swimbladder by a counter-current blood vessel arrangement, the rete mirabile. The primary effect of P_{O_2} enhancement through the Root effect and acidification by the gas gland at the tip of the vessel system can be boosted by back-diffusion of blood gases, metabolites and possibly also HCO_3^- from the venous capillaries into the arterial vessels of the rete mirabile or *vice versa* (Kobayashi et al., 1989, 1990), greatly enhancing the initial effect by counter-current multiplication (Kuhn et al., 1963), but also minimizing gas and metabolite loss from the location, similar to the conditions in Henle's loop of the kidney. Purportedly, the choroid rete mirabile underlying teleost retinae has a similar function in elevating ocular P_{O_2}.

Lack of alternative mechanisms makes the Root effect the most likely candidate to be responsible for initial elevation of P_{O_2}. This notion is supported by the *in vitro* demonstration of reduced O_2-carrying capacity of Hb at low pH and high P_{O_2} (Root, 1931; Root and Irving, 1943; Scholander and van Dam, 1954; Hamann, 1990; Pelster and Weber, 1990), the pattern of distribution of the Root effect almost exclusively to teleost fish with swimbladder gas secretion and high P_{O_2} values in the eye (Farmer et al., 1979; Pelster and Weber, 1991; Pelster and Randall, 1998), the evolutionary co-development of certain morphological and physiological traits deemed essential for gas secretion (Berenbrink et al., 2005), and by the observation of higher venous than arterial P_{O_2} values in the eel gas gland (Steen, 1963; Kobayashi et al., 1990). All these data, however, represent indirect evidence; direct confirmation of the postulated chain of mechanisms is still lacking.

The basis for an evaluation of the mechanistic process in the eye of teleosts is even scarcer than for the swimbladder. Apart from a few studies in trout on the dependence of high ocular P_{O_2} on carbonic anhydrase activity (Fairbanks et al., 1969, 1974; Hoffert and Fromm, 1973) and the relevance of high P_{O_2} for vision (Fonner et al., 1973; Hoffert and Ubels, 1979) little evidence is available. Moreover, reported control values (arterial pH and P_{O_2}) much below physiological ranges raise questions as to the conditions of experimental animals or employed methods (Fairbanks et al., 1969, 1974).

The lack of relevant direct evidence as to the involved mechanisms may reflect difficulties in accessing the supply vessel of the teleost eye (Waser and Heisler, 2004), but may also be related to the complicating factor of indirect blood supply of the teleost eye from the dorsal aorta (DA) *via* pseudobranchial artery, pseudobranch and ophthalmic artery (Müller, 1839). The function of the interconnected gill-like pseudobranch is still unknown (Bridges et al., 1998; Kern et al., 2002; for a review, see Laurent and Dunel-Erb, 1984), although 'a role for vision' (Müller, 1839) and 'a role in altering blood chemistry to support oxygen secretion in the eye' have been postulated (Bridges et al., 1998). Following these suggestions, key parameters in the ophthalmic artery blood carrying the ocular supply may deviate significantly from the DA site.

This study is aimed at analysing the intraretinal conditions of O_2 supply, in particular at evaluating intraretinal P_{O_2} of rainbow trout under controlled physiological conditions, as well as at a direct test of the contribution of the Root effect for ocular P_{O_2} enhancement. For this purpose a number of experimental series were conducted: (1) measurement of retinal diffusion pathways, (2) *in vivo* determination of intraretinal P_{O_2} with particular emphasis on blood gas and acid–base homoiostasis, and on eventual effects of intraocular hydrostatic pressure changes, (3) determination of the Root effect in erythrocyte suspensions applied for *in vitro* perfusion, (4) evaluation of the contribution of the Root effect for high intraretinal P_{O_2} by direct (eliminating the pseudobranch) *in vitro* perfusion of isolated eyes with erythrocyte suspensions possessing (trout) or lacking (human) a Root effect.

The presented data are regarded as a first step towards a closer elucidation of mechanisms involved in the elevation of ocular P_{O_2} in some teleost fish species.

Materials and methods

Experimental animals

Specimens of rainbow trout *Oncorhynchus mykiss* Walbaum 1792 of either sex (mass range 197–774 g; 420±130 g, mean ± S.D.; fork length 27–42 cm; 33.2±3.5 cm, mean ± S.D.; N=52) were obtained from a local hatchery (Umweltbundesamt, Berlin, Germany) and kept in large glass aquaria for at least 4 weeks before experimentation. The aquarium water was maintained between 12 and 17°C, recirculated through biological filters and supplemented with a steady flow of tapwater, dechlorinated in activated charcoal columns. The animals were fed daily on 0.5% (w/w) commercial trout pellets and kept at a fixed daily light regime of 11 h:1 h:11 h:1 h (light:transition:dark:transition). Animals were transferred to the laboratory lightly anaesthetized (25 mg l^{-1}, 1:40000 w/v, neutralized MS222, 3-aminobenzoic acid ethyl ester methanesulfonate; Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) in aerated thermostatted water. All animal experiments were carried out in accordance with local regulations (G0294/96).

Procedures

For surgical preparations, fish were suspended on an

operating rack and the gills were irrigated with recirculated, air-equilibrated tapwater thermostatted to 15°C. Deep anaesthesia was induced and maintained by addition of 60–80 mg l^{-1} MS222. A tapered polyethylene catheter (PE60, o.d.=1.2 mm, Portex, Hythe, Kent, England), filled with heparinized trout Ringer solution (in mmol l^{-1}: NaCl 150, KCl 4, $CaCl_2$ 1.3, $MgCl_2$ 1.2, D(+)-glucose 7.5, $NaHCO_3$ 5, heparin 125 i.u. ml^{-1}), was inserted into the DA by a modified Seldinger technique, similar to the general approach of Soivio et al. (1975), Holeton et al. (1983) and Waser and Heisler (2004). Following catheter implantation, the trout were transferred to an aquarium and allowed to recover from surgery for at least 16 h before experimentation. Within the aquarium, the fish were slightly confined to submerged opaque cylinders, leaving catheters freely accessible for blood sampling.

Reference blood samples were taken from well-recovered, conscious and resting animals for determination of arterial pH and P_{O_2} (BMS 3 Mk II, Radiometer, Copenhagen, Denmark). For experimentation, animals were then anaesthetized (MS222; 60–80 mg l^{-1}) and returned to the operating rack. The gills were irrigated with thermostatted, well-aerated anaesthetic-containing water throughout the course of the experiment. DA blood pressure and heart rate (HR) were continuously monitored (P23AA, Statham, Hato Rey, Puerto Rico), arterial pH and P_{O_2} were determined repeatedly. During the experiment arterial pH was maintained essentially constant at 7.9 by changes in carbon dioxide partial pressure (P_{CO_2}) of the gill irrigation water between 0.25 and 5 mmHg (0.033–0.67 kPa; Mass Flow Controllers, MKS Instruments Deutschland GmbH, München, Germany).

Retinal morphometry

In order to evaluate retinal diffusion distances, one eye ball from each of three trout were enucleated and sectioned horizontally around the limbus. After removal of lenses, the eye-cups were fixed by exposure to 2.5% glutaraldehyde in Ringer solution for several days and dehydrated for each several days in three successive baths of 80% ethanol. After embedding in paraffin, the eyes were halved by median section. Only a few slices of 6 μm each were cut from the halving interface, ensuring perpendicular cuts of the retina. Microscopic analysis of individual layer thickness as well as total retinal thickness was performed at numerous sites spanning the entire retinal arc. Tissue shrinkage due to paraffin embedding was corrected for by dividing through a factor of 0.74 (Weibel, 1979).

Oxygen microelectrodes: construction, calibration and characteristics

Polarographic O_2 microelectrodes for determination of intraocular P_{O_2} were constructed following the general approach of Whalen et al. (1967) and Linsenmeier and Yancey (1987). Single-barrelled borosilicate capillaries (GC100-10, Clark Electrochemical, Pangbourne, Reading, UK) were pulled to fine tip diameters (<5 μm; Model P-97, Sutter Instruments, Novato, CA/USA). A thin bar of low-melting alloy (47.2°C; Whalen et al., 1967) was inserted into the pulled capillary and gently heated until completely molten. The fluid alloy was pushed towards the tip of the electrode under microscopic control, taking care to leave a metal-free recess in the tip of the glass capillary. The recess provides a diffusional resistance, greatly reducing O_2 consumption and diminishing stirring-induced spurious current signals (Schneiderman and Goldstick, 1978). The recessed metal electrode was connected by a soldered-on copper wire (fixed in the rear capillary aperture by quick bond resin) to a power supply for electrolytic plating. A thin layer of gold was electro-plated to the metal surface in the capillary recess after filling the tip completely with the plating solution (200 mmol l^{-1} ammonium citrate with 5% $K[Au(CN)_2]$, pH 6.3) by application of 1.5 V for about 10–30 min between electrode lead and a secondary platinum electrode in the plating solution. After plating, electrodes were soaked in deionized water for at least 24 h and stored dry; before use each electrode was checked microscopically and some arbitrarily selected specimens from each production batch were tested electronically. The resulting P_{O_2} microelectrodes were characterized physically as having tip diameters of less than 5 μm and an average recess of 77±20 μm (mean ± S.D., N=42).

After establishing an individual polarogram (current *vs* voltage), the microelectrodes were polarized in the plateau range of the relationship (usually at about –800 mV). The very low current signals (in the fA to pA range) were converted into voltage signals using special head stages, incorporating customised electronic circuitry on the basis of low bias-current operational amplifiers (OPA128JM, Burr Brown, Darmstadt, Germany). Teflon-coated silver wires (Gi 1106, 0.37/0.45 mm; Advent ResearchMaterials, Eynsham Oxon, England, UK), chlorinated at the exposed tip, served as reference electrodes. The electrode chains were calibrated at the experimental temperature (15°C) in isotonic saline solution (0.9% NaCl) or trout Ringer solution, equilibrated with gases of known P_{O_2} values ranging from 0 to 760 mmHg (101 kPa; gases provided by precision gas mixing pumps; Type 1 M 303/a-F, Wösthoff GmbH, Bochum, Germany).

Calibration of zero intersect and sensitivity was performed for each individual electrode immediately before experimentation. Sensitivity of the gold-plated sensor averaged 173±82 fA $mmHg^{-1}$ (mean ± S.D., N=78). The linearity in the range of P_{O_2} from 0 to 760 mmHg (0–101 kPa) was 0.99987 (S.D.=0.00023, N=15) in terms of the average correlation coefficient. Following repeated exposure to tissues, the electrodes showed a slight decrease in sensitivity, probably due to masking part of the catalytic metal surface by contamination with proteins or nucleotides. Linearity and zero intersect current, however, remained essentially unaffected during any one experiment. There was no analytical quality degradation during long-term operation; the lifetime of P_{O_2} microelectrodes was limited only by physical destruction of the tip during experimentation.

The P_{O_2} sensor was insensitive to metabolic and respiratory changes in pH from 5.8 to 8.8. As expected, the current signal

was sensitive to temperature changes, rising by about 1% per °C (range 10–35°C), although not quite as much as reported for other polarographic O_2 sensors (Gnaiger and Forstner, 1983). Since ionic strength is a modulator of electrode sensitivity (determined as –0.05 mmHg per 1 mmol l^{-1} of ionic strength, range 75–1200 mmol l^{-1}) the electrodes were exclusively calibrated in solutions resembling extracellular fluid of trout. Calibrations and checks were generally conducted at the experimental temperature of 15°C.

Intraocular hydrostatic pressure

Introduction of electrodes into the eye for the purpose of P_{O_2} measurement may well disrupt the intraocular pressure (IOP) regime, effecting local changes in perfusion and thus P_{O_2}. This possibility was checked out by direct measurement of IOP during determination of intraocular P_{O_2}. After induction of anaesthesia as described above, the anterior chamber of the eye was punctured with a 0.4 mm hypodermic needle. IOP was recorded by means of a pressure transducer (P230b, Statham, Hato Rey, Puerto Rico) connected to the hypodermic *via* PE-tubing (Portex, Hythe, Kent, England, UK), taking care to completely fill the pressure pathway with physiological fluid. After reading the IOP for 15 min, further preparations required for determination of intraocular P_{O_2} (see below) were conducted in order to directly correlate eventual changes in ocular P_{O_2} with impacts on IOP and *vice versa*.

Intraretinal P_{O_2}*:* in vivo *determination*

After induction of anaesthesia as described above, cornea and iris were punctured ventro-laterally just inside the limbus, using a 1.5 mm diameter hypodermic needle. The needle was replaced by a guide for the P_{O_2} electrode made of 1.5 mm diameter stainless steel tube, which remained in place throughout the experiment. P_{O_2} microelectrodes were threaded through the guiding tube and advanced with the tip close to the retina, with visual check of the position through a binocular operating microscope (Carl Zeiss, Jena, Germany) in combination with an ophthalmoscopic lens (Super Pupil XL, 132 dpt, Biomicroscopy lens JH0987, Volk Optical Inc., Mentor, Ohio, USA). The electrodes were advanced towards and into the retina and reproducibly positioned (0.1 μm), using a step motor-driven 3-axis micromanipulator (HS 6, Märzhäuser, Wetzlar, Germany) in combination with a digital programmable electronic driving unit (N. Heisler and H. Slama, unpublished). A chlorinated silver wire (see above) inserted into the dorsal muscle behind the head served as a reference for P_{O_2} microelectrodes.

Profiles of P_{O_2} were recorded in the range of the posterior pole of the eyeball, slightly anterior to the optic disk. After inserting the electrode into the eye to just above the retina (at 800 μm s^{-1}), the electrode was gradually inserted into the retina in preprogrammed steps of 25 to 100 μm (at 3200 μm s^{-1}; the step magnitude depended on the extent of the preceding change in P_{O_2}), each time awaiting stable readings of electrode current until P_{O_2} readings levelled off during further advancement. It was assumed that the tip had then reached or passed Bruch's membrane. The electrode was then gradually withdrawn, applying the reverse of the advancement profile, and the return P_{O_2} profile was recorded.

Intraretinal P_{O_2}*:* in vitro *experiments*

Enucleation of eyes

After establishment of normal pH and P_{O_2} in appropriately anaesthetized specimens (see above), the conjunctiva were cut and removed from the eye. Covering bones and muscle mass from ventral and temporal sectors of the orbita as well as the eye muscles were severed and completely removed. After carefully exposing optic nerve as well as ophthalmic artery and vein by removal of the suspending adipose tissue, two ligatures for later use were threaded under the ophthalmic artery, proximal at the entry into the orbita and distal directly at the eye cup.

After inserting a custom-made suspending holder under the eye cup, a preformed catheter attached to the holder was quickly inserted into the ophthalmic artery to supply the eye and the ligatures were tightened around catheter/artery as well as around the cut-off artery at the orbital entry point. Perfusion of the eye with trout erythrocytes (red blood cells, RBC) suspension started immediately after, limiting ischaemia of the eye to less than 60 s. After sectioning optic nerve and ophthalmic vein the eye was removed from the orbita. During perfusion, the eye surface was kept hydrated by irrigation with water thermostatted to 15°C.

Perfusion

Isolated eyes were perfused with erythrocyte suspensions (see below) rather than full blood in order to avoid any direct or indirect effects of chatecholamines and other humoral factors on the release of O_2 from the carrier (Hb). Suspensions were supplied to the ophthalmic artery by a peristaltic pump (Type IP-4, Ismatec, Wertheim-Mondfeld, Germany) at the flow rate of 180 μl min^{-1}, previously determined *in vivo* in the afferent pseudobranchial artery (Waser and Heisler, 2004). A miniature bubble trap in the inflow path immediately before the eye served for elimination of gas bubbles from the perfusate. Vascular occlusions in eye capillaries by micro-clots and cell aggregations were prevented by passing the perfusate through a 40 μm mesh filter (Polyester 07-40/25, Bückmann, Mönchengladbach, Germany; Mesh holder: Swinnex 13 mm, Millipore, Eschborn, Germany). Perfusion pressure was monitored by a transducer T-connected to the catheter leading into the ophthalmic artery (P23AA, Statham, Hato Rey, Puerto Rico).

Preparation of erythrocyte suspensions

Trout blood pooled from several individuals and human blood (human transfusion blood supplied by Charité, Berlin, Germany) was centrifuged, plasma and white blood cells removed and the RBCs were three times washed in trout Ringer solution (in mmol l^{-1}: NaCl 146.6, KCl 4, $CaCl_2$ 1.3, $MgCl_2$ 1.2, D(+)-glucose 7.5, $NaHCO_3$ 5.4, sodium pyruvate 3,

polyvinylpyrrolidone 0.5% (w/v), heparin 50 i.u. ml^{-1}), before being resuspended and stored overnight at 4°C. The washing procedure (3×) was repeated next morning before resuspension to the nominal haematocrit (Hct) used during experimentation (0.20). The resulting suspensions were conditioned for the experiment by at least 45 min equilibration at 15°C in rotating 100 ml round bottom glas flasks (Farhi, 1965) with the experimental gas of 0.27% CO_2 in air [P_{CO_2}: 2.0 mmHg (0.27 kPa), P_{O_2}: 156.2 mmHg (20.8 kPa)] prepared by Wösthoff gas mixing pumps. Glucose transfer into the cells was facilitated by addition of 10 u l^{-1} insulin (Insuman Rapid, Hoechst Marion Roussel, Bad Soden, Germany; Pelster et al., 1989).

Perfusates

For an evaluation of the role of the Root effect for complete O_2 supply of the trout retina, eyes were perfused with two different species of red blood cells: trout cells, having a pronounced Root effect and thus capable of massive O_2 release into physical solubility upon acidification, and human erythrocytes lacking any effect of O_2 release upon acidification at high P_{O_2}. The following perfusates were utilized. 'Tr', trout RBCs in trout Ringer, pH about 7.48 (start of the steep range of the Root effect curve), Hb saturation high (~91%, Root effect less than 15%; 'H', human RBCs in trout Ringer, pH about 7.16, Hb saturation high (100%), no Root effect.

As a reference for full Hb oxygenation (100% saturation, 0% Root effect), a control suspension of trout RBCs in trout Ringer ('TrC', pH>8) was prepared and concomitantly equilibrated.

Determination of intraocular P_{O_2}

Initial preparations for determination of intraocular P_{O_2} were identical to those described above (*in vivo* conditions). For the isolated eyes, however, the metal suspending eye holder served as a reference for the P_{O_2} microelectrode. Reference readings of P_{O_2} were acquired always during perfusion with trout RBC suspensions (Tr; pH about 7.48; see above). P_{O_2} microelectrodes were advanced into the retina until P_{O_2} attained a maximal level. The electrode was then left in position for the remainder of the experiment, during which the response in P_{O_2} was recorded during alternating perfusion with trout and human RBC suspensions. In a few occasions, eyes were also flushed with trout Ringer solution.

Determination of the Root effect in vitro

The relationship between changes in extracellular pH and the release of Hb-associated O_2 into physical dissolution was determined in erythrocyte suspensions identical to those used for perfusion experiments. Individual samples from the same preparation of erythrocyte suspension were adjusted to pH over the range 6.0–8.5, either by changes in P_{CO_2} of the equilibrating gas, in plasma [HCO_3^-], or by addition of each 200 μl of HCl of the required concentration. Gases with specified P_{CO_2} (0.033% to 7.13%) were produced by mixing air with CO_2 by Wösthoff gas mixing pumps.

Series 1: relationship between pH and total O_2 *concentration at constant (high)* P_{O_2}

After adjustment of pH and re-equilibration, the relevant variables of the suspension were determined by application of appropriate techniques: pH (BMS 3 MkII; Radiometer, Copenhagen, Denmark), total O_2 content (T_{O_2}; OxyConAnalyzer, Department of Anatomy and Physiology, University of Tasmania, Australia) and Hct (centrifugation in glass capillaries for 3 min at 14 980 ***g***). T_{O_2} of acidified samples relative to the O_2 capacity (at pH >8) was taken as quantitation of the Root-effect. Hb-bound O_2 concentration ($[O_2]_{Hb}$) was plotted against pH and approximated by a sigmoidal curve fit (Eqn 1 and Fig. 4). The obtained relationship is expressed by Eqn 1:

$$f(x) = \frac{\left(10^{\frac{x-i}{s}}\right)\max + \min}{10^{\frac{x-i}{s}} + 1}$$

$$\text{specifically: } [O_2]_{Hb} = \frac{\left(10^{\frac{pH-7.36}{0.33}}\right)3.31 + 1.37}{10^{\frac{pH-7.36}{0.33}} + 1}, \qquad (1)$$

where x = pH, i = pH at point of inflection, s = slope, max = maximal O_2 content and min = minimal O_2 content.

Series 2: elevation of P_{O_2} *upon anaerobic acidification*

Trout and human RBC suspensions, prepared with trout Ringer containing 20 or 24 mmol l^{-1} [HCO_3^-], respectively, were adjusted to Hb tetramer concentration ([Hb_4]) of 0.5 mmol l^{-1} (Hct approx. 0.10) and equilibrated with a gas of P_{CO_2}=2.2 mmHg (0.29 kPA) and P_{O_2} of about 150 mmHg (20 kPa), resulting in an initial pH of about 8.2. A lower Hct (adjusting [Hb] for higher precision) than employed for perfusion media was chosen to limit P_{O_2} to less than 1 atm following acidification. The initial pH of individual samples was reduced under anaerobic conditions by addition to each one of 100 mmol l^{-1} acetic acid graded in volume from 0–160 μl g^{-1} RBC suspension. After mixing for 30 s with an enclosed metal sphere, P_{O_2} and pH of the acidified samples were determined.

Data acquisition and analysis

Data were recorded on a standard IBM-compatible PC with an analog/digital converter board (DAS 1602, Keithley Instruments Inc., Taunton, MA, USA), using customized Test Point runtime modules (TestPoint 3.0, Capital Equipment Corporation, Billerica, Ma, USA). Data were analyzed using SigmaPlot 4.01, SigmaStat 2.03 (SPSS Software, München, Germany), 'R' (www.r-project.org), and StarOffice 5.2 (Sun Microsystems, Berlin, Germany). Data are presented as average ± standard deviation (S.D.); levels of statistical significance were determined by Student's *t*-test unless otherwise noted.

Results

Morphometry of the trout retina

Trout retina reflects the typical vertebrate pattern of layer arrangement (Fig. 1), but lacks any vascular supply. The thickness of individual layers are presented in Fig. 1. The overall thickness of the retina depends on the location: thickness increases from the anterior margin (~200 μm) to the posterior regions in the range of the optic nerve (~500 μm, Fig. 2), with an average of 407 μm (Fig. 1). The local variability is somewhat related to the density of photoreceptors, reflected in the thickness of the neuronal layers PRL and GCL+NFL (Fig. 2B,C), with the NFL appearing particularly extended close to the optic nerve. In contrast, the nuclear and plexiform layers were found more uniform throughout the retina. The whole retina completely lacks blood vessels, and the inner surface is not supplemented by hyaloid vessels often found in species without intraretinal vascularization. This pattern is in vast contrast to the European eel *Anguilla anguilla*, for example, with its clear intraretinal vascularization (Fig. 1, insert).

Oxygen profiles were measured in the posterior part of the trout retina. In order not to affect correct estimate of the average representative for this site by values of largely different thickness from other areas of the retina, the thinner anterior parts (25% of all data) and six exceptionally high values obtained close to the optic nerve with an extremely thick NFL layer were not included into the data pool for averaging. The posterior retina thickness was accordingly estimated to be 436±75 μm (*N*=95 measurement points, in 3 eyes of three fishes).

Experiments in vivo

Variables of homoiostasis

In concious, resting trout, arterial pH and P_{O_2} averaged 7.89±0.11 (*N*=41) and 102±19 mmHg (13.6±2.5 kPa; *N*=39), respectively. During anaesthesia and artificial gill irrigation, arterial P_{O_2} was hardly affected (99±21 mmHg, 13.2±2.8 kPa; *N*=38, respectively). Arterial plasma pH (8.02±0.12, *N*=41) was slightly but significantly higher ($P<0.001$) than in non-anaesthetized controls. DA blood pressure and HR of anaesthetized trout averaged 28±6.8 mmHg (3.7±0.9 kPa; *N*=52) and 73±9.6 beats min^{-1} (*N*=51), respectively.

Intraocular hydrostatic pressure (IOP)

IOP measured in the anterior eye chamber was 4.9±0.56 mmHg (0.65±0.07 kPa; *N*=8). After puncturing the eye, inserting the guiding tube and P_{O_2} electrode, IOP was not significantly different (4.6±1.06 mmHg, 0.61±0.14 kPa; *N*=8; P=0.58, paired Student's *t*-test). Evidently, insertion of the guiding tube with electrode into the eye effectively sealed the corneal puncture, thus supporting maintenance of constant IOP. The magnitude of IOP obtained by this study is identical to the value reported by Hoffert (1966), whereas other species

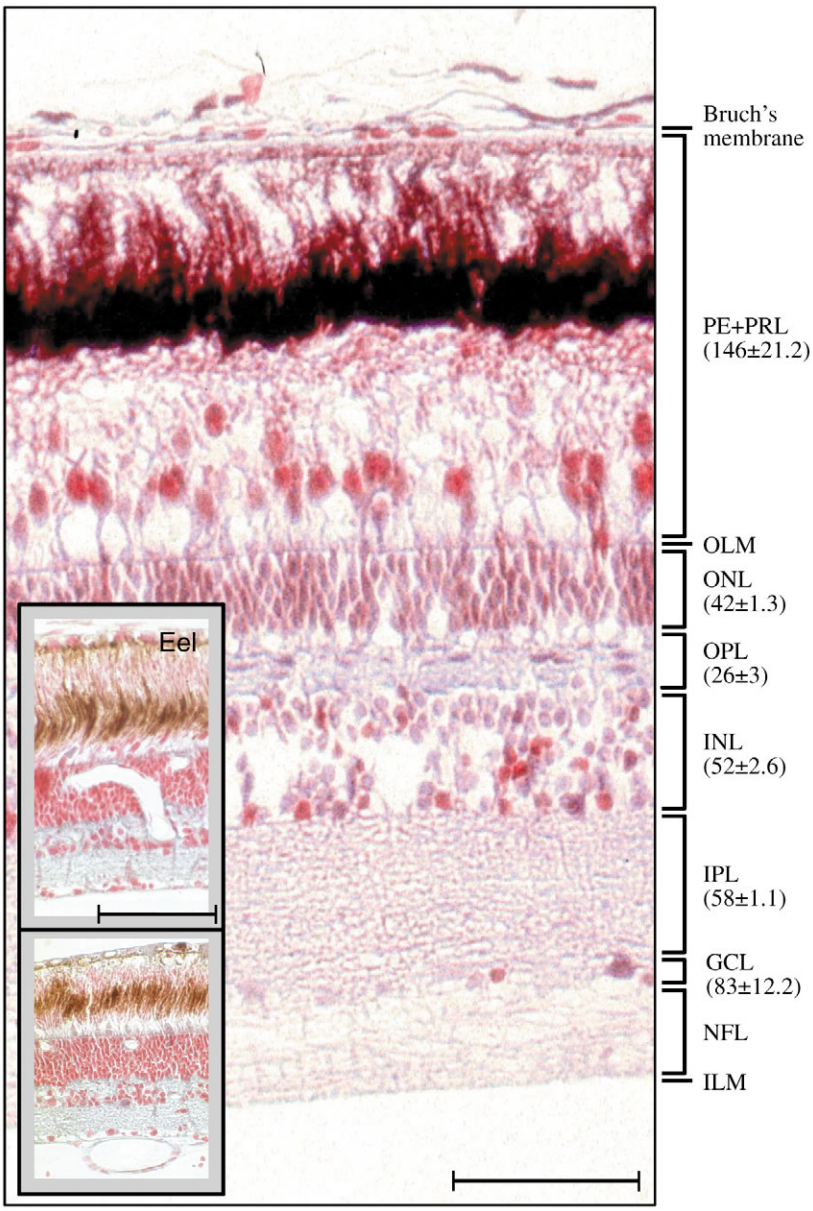


Fig. 1. Microphotograph of trout retina section (PE+PRL, pigment epithelium and photoreceptor layer; OLM, outer limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; NFL, nerve fibre layer; ILM, inner limiting membrane). The trout retina is completely devoid of blood vessels. Numbers denote thickness ± S.D. (*N*=3) in μm of the respective layers. For GCL and NFL the combined thickness of the two layers is given. Insert: Microphotographs of eel retina as an example of a teleost retina with intraretinal vascularization. Large blood vessels visible on ILM reach into the retina to OLM. Bars, 100 μm, 50 μm (insert).

maintain specific, mostly higher values, e.g. *Mustelus canis* (7.8 mmHg; Nicol, 1989) and *Salvelinus namaycush* (13.2 mmHg; Hoffert, 1966).

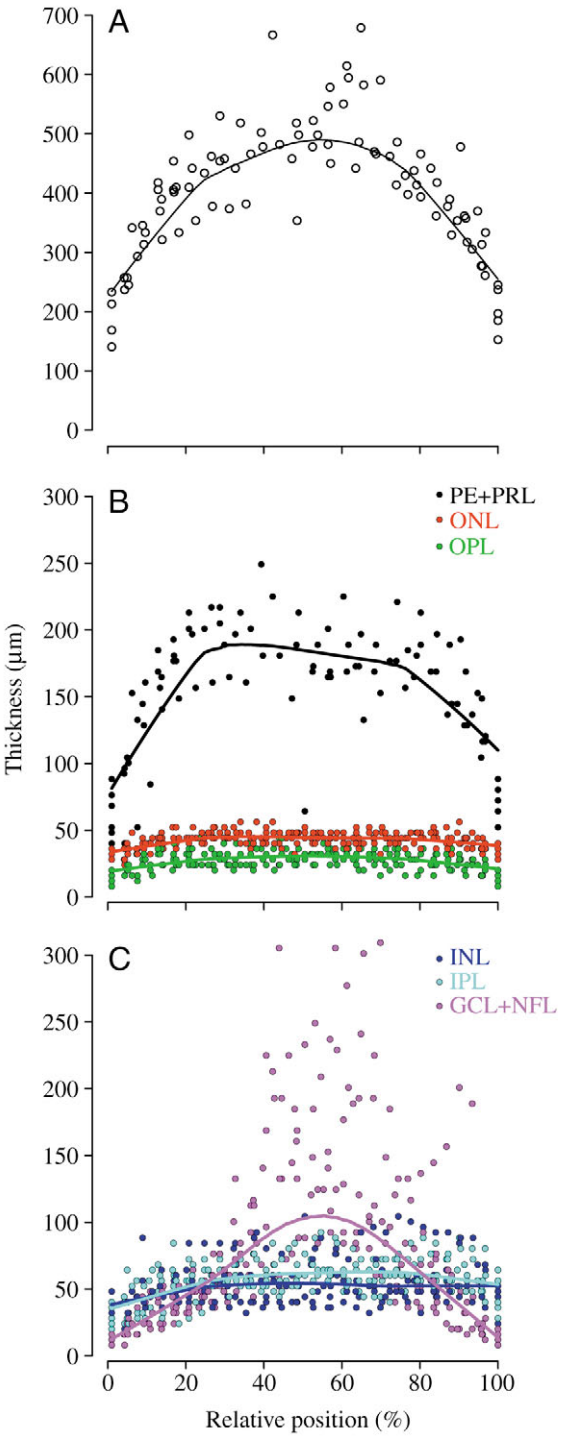


Fig. 2. Thickness of retina and individual layers. (A) Thickness of the whole retina (Bruch's membrane to ILM). (B,C) Thickness of individual layers (B): PE+PRL, ONL, OPL; (C): INL, IPL, GCL+NFL. Position of measurement (*x*-axis) is presented as relative; the axis spans the entire semicircular arc formed by the retina (for abbreviations, see Fig. 1).

Intraretinal P_{O_2}

Intraretinal P_{O_2} was minimal at the interface between vitreous humor and retina (ILM, see Fig. 1), averaging 10±21 mmHg (1.33±2.8 kPa; *N*=23; Fig. 3). Traversing the retina, P_{O_2} rose to 382±143 mmHg (50.9±19.1 kPa; *N*=23) in the region of Bruch's membrane (Fig. 3). P_{O_2} was significantly different among ILM, arterial blood, and at Bruch's membrane ($P<0.05$; Kruskall–Wallis one-way ANOVA, pairwise multiple comparison procedure: Dunn's method). The electrode path driven between minimal and maximal P_{O_2} averaged 433±106 μm (*N*=23; Fig. 3).

In vitro *experiments*

Root effect in trout erythrocyte suspensions

The maximum T_{O_2} (100% Hb saturation, 0% 'Root on') of alkaline (pH 8.0–8.5) trout RBC suspension (Hct=0.20) was 3.31 mmol l^{-1} (Fig. 4). Minimum To_2 (100% 'Root on') at acidic pH (6.0–6.8) was 1.37 mmol l^{-1}, 41% of the maximum T_{O_2}. A sigmoidal curve fit on the raw data, characterized by a curve inflection at pH 7.36 and T_{O_2} of 2.34 mmol l^{-1} (at 50% 'Root on'), resulted in a correlation coefficient r=0.962. According to the curve fit, Root effect was 10% activated at pH 7.67 and 90% activated at pH 7.03.

P_{O_2} *upon anaerobic acidification of RBC suspensions*

Under anaerobic conditions, graded acidification of trout RBC suspensions resulted in an enhancement of P_{O_2} from 156 mmHg (equilibration P_{O_2}) at pH 8.1 to a maximum of 449 mmHg (59.9 kPa) at pH 6.4. Similar to the O_2 content series, the data could be well fitted by a sigmoidal curve (r=0.949; Fig. 4B). Identical treatment of human RBC suspension did not result in any significant change in P_{O_2} (blue symbols, Fig. 4B). The correlation coefficient of the linear regression fitted to the measured values was r=0.055 (Fig. 4B).

Parameters of perfusates

Physiological parameters determined during perfusion in the RBC suspensions are listed in Table 1. The pH of human RBC suspensions was significantly lower than the pH of trout RBC suspension (P=0.0026), although both suspensions had been prepared and treated identically. Also, human RBC suspensions exhibited a significantly higher T_{O_2} than trout RBC suspension (P=0.0029, cf. Fig. 4A), due to the higher cell Hb concentration of human erythrocytes. T_{O_2} of trout RBC suspension 'Tr' (pH=7.48±0.04, *N*=3) was 0.91±0.079 (*N*=3) as compared to the concomitantly equilibrated control trout RBC suspensions ('TrC') at a more alkaline pH (>8.0).

Intraretinal P_{O_2} *upon perfusion with trout RBC* vs *human RBC*

Hydrostatic perfusion pressure at the entry to the ophthalmic artery catheter was the same (P=0.9031) for trout and human RBC suspensions (Hct 0.20), but was much lower for pure Ringer solution (cf. Table 1). A large part of the hydrostatic pressure measured at that site was related to the pressure drop according to the flow resistance of the catheter itself (trout

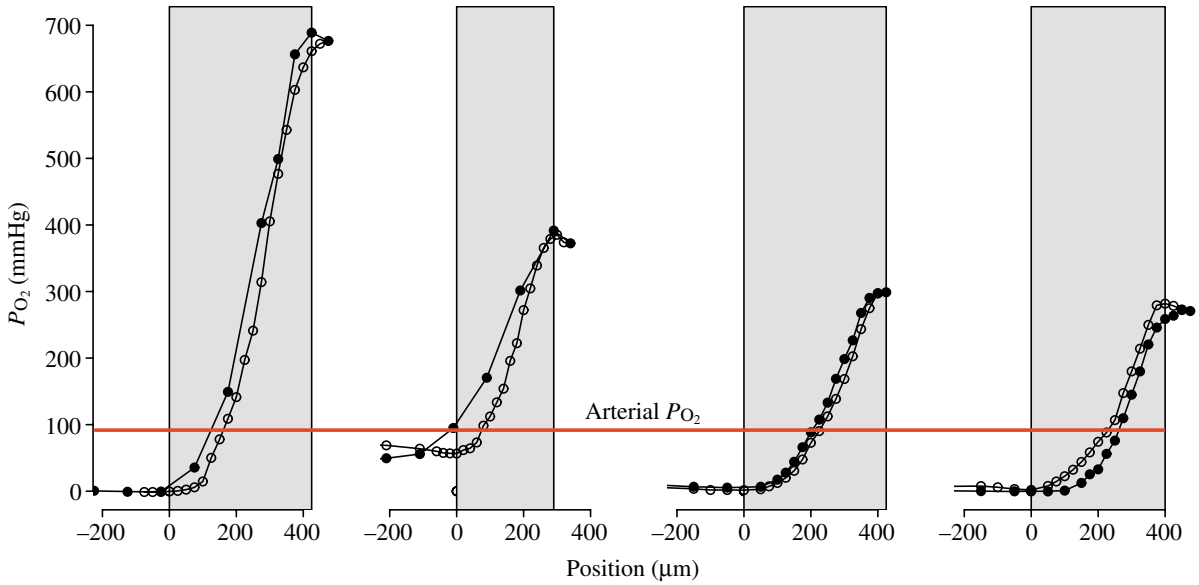
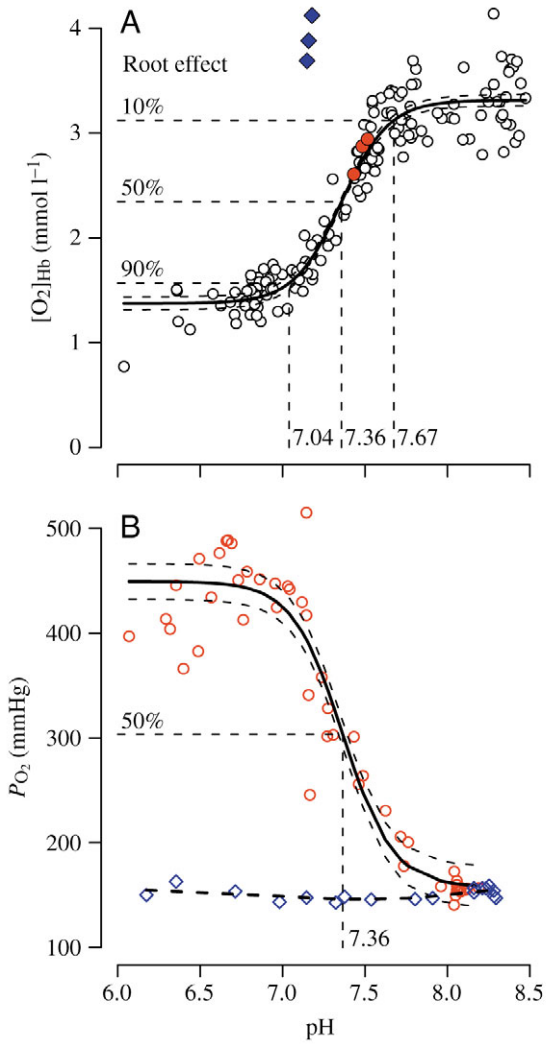


Fig. 3. Selection of P_{O_2} profiles in the trout retina. Position at 0 μm corresponds to the inner surface of the retina (ILM). Shaded areas depict range of lowest to highest P_{O_2}. Electrodes were advanced into (closed symbols) and withdrawn out of (open symbols) the retina, resulting in duplicate recording lines. The red horizontal line indicates arterial P_{O_2} (1 mmHg=133.3 Pa).



RBC, 60 mmHg/8.0 kPa; human RBC, 67 mmHg/8.9 kPa; Ringer, 42 mmHg/5.6 kPa). Thus, net tissue perfusion pressure (equivalent to the vascular resistance) was only 43 mmHg (5.7 kPa) for trout RBC, 37 mmHg (4.9 kPa) for human RBC, and 16 mmHg (2.1 kPa) for Ringer, respectively. When *in vitro* perfusion was switched from trout RBC suspension (Root effect) to human RBC suspension (lacking a Root effect), intraretinal P_{O_2} was largely and significantly reduced by a factor of 3.3 (Fig. 5; P=0.003) and fell even further during perfusion with Ringer solution (Table 1).

Discussion

Experimentation in order to elucidate the mechanisms involved in elevating intraocular P_{O_2} above atmospheric values is most complicated due to limited physical access to the site of mechanisms. This is related to the anatomical conditions with blood supply of the eye provided from the DA only through the rudimentary hemibranch designated as pseudobranch, but is further complicated by a vascular connection (the ‘commissura’) between the two collateral flow

Fig. 4. Root-effect of red blood cell (RBC) suspensions. (A) Haemoglobin-bound oxygen concentration *vs* pH. pH values at ‘Root on’ of 90%, 50% and 10% are indicated. RBC suspensions utilized for *in vitro* perfusion of isolated eyes: red circles (trout) and blue diamonds (human). Correlation coefficient of fitted line: r=0.962. (B) P_{O_2} *vs* pH of anaerobically acidified samples of trout (red circles) and human (blue diamonds) RBC suspensions. The pH value at 50% Root-effect is indicated by broken lines. Correlation coefficients of fitted lines: r=0.949 (trout RBC samples) and r=0.055 (human RBC samples). Confidence intervals (95%) to the fitted sigmoidal curves are indicated by broken lines.

Table 1. *Parameters of the media used for perfusion of isolated eyes*

	Trout RBC suspension	Human RBC suspension	Ringer solution
Haematocrit	0.20±0.01	0.20±0.01	–
P_{CO_2}eq (mmHg)	2,0	2,0	ND
pH	7.48±0.04	7.16±0.02	approx. 7.5
$[HCO_3^-]$ (mmol l^{-1})	2.6±0.16	1.1±0.10	5,4
$[O_2]_{Hb}$ (mmol l^{-1})	2.8±0.16	3.9±0.23	–
P_{perf} (mmHg)	103±10.9	104±7.5	58±2.0
Net tissue P_{perf} (mmHg)	43	37	16
Intraretinal P_{O_2} (mmHg)	99±1.6	30±3.5	20±10.7

Values are means ± S.D. (N=3). ND, not determined.
P_{CO_2}eq, equilibration CO_2 partial pressure; P_{perf}, perfusion pressure.

paths (Waser and Heisler, 2004). Literature studies on elevated P_{O_2} in the eye of teleosts have always dealt with the combined serial arrangement of pseudobranch and eye, rendering differentiation of contributing mechanisms in those two organs difficult if not impossible. Severe disturbances of homoiostasis with respect to pH, $[HCO_3^-]$ or P_{O_2} in the arterial supply, as often prevailing in literature studies (Fairbanks et al., 1969, 1974) may have further compromised elucidation of the complex pattern.

In order to avoid any influence of non-physiological conditions, large efforts were taken in the course of the present study to maintain normal blood homoiostasis. The average values measured (P_{O_2} 99 mmHg, 13.2 kPa; DA blood pressure 28 mmHg, 3.73 kPa; HR 74 beats min^{-1}) are well within the reported range of normal values of conscious, free swimming trout (Tetens and Christensen, 1987; Playle et al., 1990; Wood et al., 1996; Bernier and Perry, 1999; Perry et al., 1999), despite the necessity for long-term anaesthesia imposed by the experimental approach and the related loss of respiratory activity. The gill surface had to be irrigated for respiratory gas exchange, which evidently did allow sufficient transfer of O_2, but at the same time resulted in some hypocapnia that could only incompletely be compensated by addition of CO_2 to the inspired water. This is probably related to heterogeneous distribution of blood and water flow at the gas exchange surface, which can hardly be avoided with present techniques. However, the induced marginal alkaline shift in pH is considered much less disturbing to animal homoiostasis than excessive acid shifts in pH resulting from anaerobiosis induced by extremely low arterial P_{O_2} values, as often found in earlier studies on high intraocular P_{O_2} values (e.g. Fairbanks et al., 1969, 1974; Hoffert and Ubels, 1979; see also below).

Intraretinal P_{O_2} in vivo

Intraretinal P_{O_2} (P_{retO_2}) measured in anaesthetized trout with well preserved homoiostatic conditions were almost four times as high as simultaneously recorded arterial P_{O_2} (Pa_{O_2}; P_{retO_2}, 382 mmHg, 50.9 kPa, average of highest recordings/path profile, *vs* Pa_{O_2}, 99 mmHg, 13.2 kPa). This partial pressure is sufficient to completely satisfy the demand of the thick and avascular retina by passive diffusion, as demonstrated by comparative considerations on the O_2 supply in the well-vascularized human retina with maximal diffusion distances of 142 μm (Chase, 1982). Since P_{O_2} at Bruch's membrane is four times higher than human blood P_{O_2} (380 *vs* 90 mmHg, 50.6 *vs* 12 kPa) and the lower temperature T in trout can be expected to reduce metabolic rate by another factor of at least 4 (ΔT approx. 20°C, $(Q_{10})^2$ 4–16), the diffusion path deduced from the conditions in humans (142 μm×$\sqrt{16}$) provides a safe margin of 568 μm depth of O_2 entry.

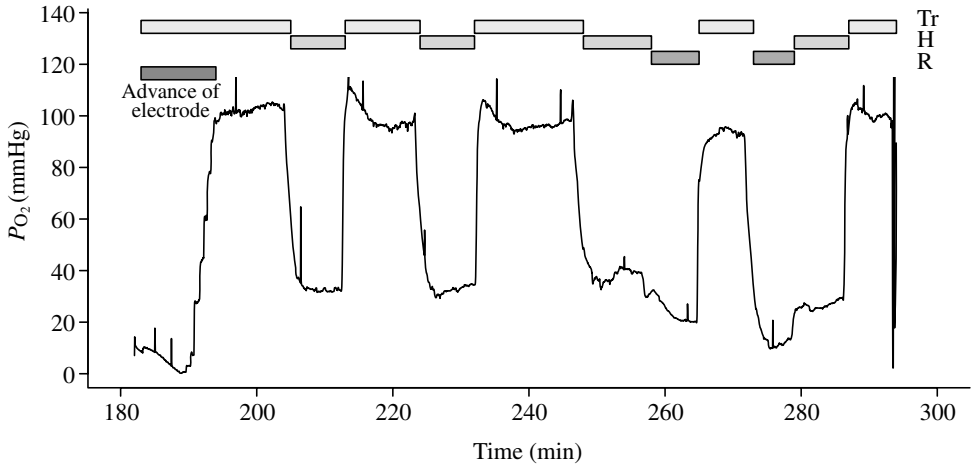


Fig. 5. Time course of selected *in vitro* determination of intraocular P_{O_2}. Periods of perfusion with different RBC suspensions (Tr, trout; H, human; R, Ringer solution) are indicated by grey bars above the graph. Initial increase in P_{O_2} was due to the advance of the microelectrode into the retina.

The magnitude of the present intraretinal P_{O_2} data is in good agreement with relevant literature data (Fairbanks et al., 1969, 1974; Hoffert and Ubels, 1979; Pratt and Hoffert, 1982; Desrochers et al., 1985), although in previous experiments little attention was paid to maintaining the general physiological conditions of the animals. Evidently, the regulatory process for P_{O_2} in the eye is capable of compensating for even largely non-physiological border conditions of the homoiostatic system as expressed by extremely low arterial P_{O_2} and pH values (Pa_{O_2}: 20 mmHg, Fairbanks et al., 1969; 13 mmHg, Hoffert and Ubels, 1979; pHa: 7.22–7.62, Hoffert and Ubels, 1979).

Undoubtedly, a low pH of the blood before entry into the eye will lower the amount of O_2 available by activation of the Root effect, providing only 25% at pH 7.2 and about 84% at 7.6 of the full Root capacity at pH 8 (cf. Fig. 4). However, even more than by the cited low pH values the amount of O_2 available for release through activation of the Root effect will be reduced by extremely low arterial P_{O_2} values (20 and 13 mmHg, 2.7 and 1.7 kPa, respectively; Fairbanks et al., 1969; Hoffert and Ubels, 1979), allowing for only minor oxygenation (20% and 12%, respectively) of trout Hb (Randall, 1970). As a matter of speculation, the interposed pseudobranch, already attributed 'a role for vision' by Müller (1839), may be capable of 'altering the blood chemistry' in order to adjust the threshold for the onset of the Root effect (Bridges et al., 1998), a notion recently supported by experiments on isolated pseudobranchial cells showing an acidifying effect (Kern et al., 2002). But even if the pseudobranch was actually capable of alkalinizing the blood during passage before entry into the eye, for the lack of additional O_2 on the flow path to the eye the correction of the O_2 binding characteristics by the pseudobranch would not result in an enhanced amount of Hb-bound O_2 to be released by activation of the Root effect in the retina.

On the basis of present knowledge, the above adverse homoiostatic conditions could only be offset if the choroid rete mirabile actually supported elevation of P_{O_2} by counter-current multiplication. This mechanism is suitable for largely reducing the amount of O_2/unit of time required to maintain high P_{O_2} values in the retina and thus render blood border conditions less important. Although the contribution of any of the mentioned mechanisms to the regulation of retinal P_{O_2} cannot be quantified to date, it becomes quite clear in face of the above data how robust and insensitive to fluctuations of arterial parameters this regulatory chain must be.

Root effect in vitro

Although generally determined as a function of the extracellular (plasma) pH, the Root effect is clearly a function of pH directly at the Hb substrate. Intracellular pH of trout red blood cells, however, is not a direct function of extracellular pH, but is affected by catecholamines (c.f. Nikinmaa and Salama, 1998) and possibly other humoral factors. In order to avoid such effects on pH_i and also complications with vasopressive substances carried by plasma, the present study has utilized red blood cell suspensions rather than full blood for the *in vitro* perfusion experiments. This decision has made necessary the determination of the Root effect for the special RBC preparations used.

The Root effect of RBC suspensions (Hct 0.20) equilibrated with high P_{O_2} at various pH is characterized by the expected sigmoidal relationship between T_{O_2} and pH_e, with the maximal release by full activation of the Root effect of about 60% of the total O_2 capacity (Fig. 4A). Total release of the difference between maximal (3.31) and minimal (1.37 mmol l^{-1}) O_2 by full Root activation will accordingly transfer 1.94 mmol O_2 l^{-1} blood into physical dissolution, equivalent to an additional P_{O_2} of 1093 mmHg (145.7 kPa) (on the basis of αO_2=1.7745 μmol l^{-1} $mmHg^{-1}$ for human plasma at 15°C; Boutilier et al., 1984). Complete activation of the Root effect will accordingly result in supersaturation of the blood, which may persist *in vivo* for a limited vascular range with laminar flow and lack of condensation points, also because the formation of gas bubbles is counteracted by the extremely high bubble pressures at small radii (LaPlace's Law).

During determination of P_{O_2} upon closed system acidification *in vitro* (cf. Fig. 4B), supersaturation occurring after activation of the Root effect is not maintained at atmospheric pressure, due to the vigorous shaking applied and thus turbulent flow and cavitation within the samples. The higher than atmospheric sum of partial pressures then leads to the establishment of a gas phase and a new distribution of gases between aqueous and gaseous compartments. With such an *in vitro* system, full activation of the Root effect will theoretically produce a P_{O_2} of 442 mmHg (58.9 kPa), which is in very good agreement with the measured P_{O_2} of acidified trout RBC suspension (449 mmHg, 59.9 kPa).

Full activation of the Root effect actually may not be required for O_2 supply to the retina *in vivo*. The measured retinal P_{O_2} values average only about one third (382 mmHg, 50.9 kPa) of the theoretical P_{O_2} upon maximal Root activation, and with the additional enhancement of retinal P_{O_2} by counter-current multiplication the required fraction of Root activation will be even smaller. Nevertheless, teleost fish may live close to the limit with respect to gas embolism. To date, nothing is known as to the correlation between water pressure on fish and retinal P_{O_2}.

In vitro *eye perfusion: the Root effect as a crucial factor for elevated retinal* P_{O_2}

In vitro perfusion of the trout eye was chosen as an experimental model in order to completely eliminate external factors such as the pseudobranch activity from the process of P_{O_2} enhancement in the retina. This approach allowed for extensive and immediate change of perfusate quality and direct identification of factors responsible for observed effects. Aside from these important experimental advantages the preparation certainly carries a number of problems generally involved in extracorporeal perfusion studies.

Perfusion of the organ at an adequate rate is one of the most important prerequisites for maintenance of tissue function. In

the course of the present study the eyes were perfused at the rate determined in a unilateral pseudobranchial artery (Waser and Heisler, 2004). Blood supply *in vivo* is from the DA through the pseudobranch to the ophthalmic artery, with no significant arterial vessels arising from this path to other tissues, except the bilateral connection of the 'commissura' (cf. Waser and Heisler, 2004). During steady state conditions, significant flow through the 'commissura' cannot be expected because of same pressure conditions on both sides of the visual blood supply system, but part of the pseudobranchial inflow may have been diverted from the eye path into the secondary circulatory system of the pseudobranch, being directly returned to the *sinus venosus*. However, on the basis of typical flow rates for the secondary circulatory system (cf. Ishimatsu et al., 1988, Iwama et al., 1993, Heisler, 1993), any possible misestimate for ocular blood flow has to be considered small.

The perfusion pressure as a second prominent characteristic of tissue blood supply was maintained essentially constant during the experiment, in particular between perfusion with trout RBC and human RBC suspensions, and was accordingly not correlated with establishment of different retinal P_{O_2} values. Perfusion pressure was also rather constant as a function of time, indicating good stability of the preparation. The absolute values of perfusion pressure were higher as compared to normal arterial blood pressure in trout [net tissue perfusion pressure of 43 mmHg (5.7 kPa) for trout RBC and 37 mmHg (4.9 kPa) for human RBC suspensions *vs* 28 mmHg (3.7 kPa) average blood pressure in the DA; see above]. The normal hydrostatic pressure *in vivo* in the ophthalmic artery has to be expected to be even smaller than in the DA, due to the flow resistance of the pseudobranch connected in series in the blood supply path.

The perfusion pressure elevated in comparison with *in vivo* blood pressure may be related to release of vasopressive activity due to haemolysis in the tonometer, the perfusion pump and other constituents of the perfusion system, or to the lack of vasodilators in the normal *in vivo* blood supply, but may also reflect microembolism of the vascular bed. Filtering of the perfusate through 40 μm mesh width may not have been sufficient to prevent occlusion of capillary vessels with smaller emboli. In particular, small clots may have been produced during the short time (1 min) of ischaemia before perfusion of the organ was initiated through the catheter inserted into the ophthalmic artery. Another possibility may be related to immunological differences. Although no direct incompatibility has been observed, and naturally the plasma factors were eliminated during preparation of perfusates, membrane proteins may interact with the endothelium of capillary vessels. However, the tendency to lower perfusion pressure with human erythrocytes renders this factor unlikely.

Regardless of the mechanism, partial occlusion of the vascular bed may have led to the relatively low intraretinal P_{O_2} values registered during *in vitro* perfusion (99 mmHg, 13.2 kPa *vs* 382 mmHg, 50.9 kPa *in vivo*). P_{O_2} enhancement may have been hampered by a reduced overall area of P_{O_2} enhancement just outside the retina or by a generally reduced O_2 supply as compared to normal conditions *in vivo*. Also an overall reduction of retinal O_2 consumption (due to the lack of central neural connection or by damage of neural retinal cells during ischaemia) and thus less demand for high P_{O_2} at the entry of the diffusion path cannot be excluded at present.

Independent of the reduced level of absolute P_{O_2} values, the immediate and direct response to perfusion with trout *vs* human RBC suspensions clearly indicates the crucial role of the Root effect for retinal O_2 supply. High P_{O_2} (about 100 mmHg, 13.3 kPa) during perfusion with trout RBCs was promptly reduced by a factor of 3.3 upon perfusion with human RBCs (to about 30 mmHg, 4 kPa) and was as promptly returned to the high initial value, when perfusion was switched back to trout RBCs with Root effect (cf. Fig. 5). This response was achieved only on the basis of O_2 release by the Root effect, regardless of the absolute amount of O_2 bound to Hb in the suspension (2.8 mmol l^{-1} in trout RBCs *vs* 3.9 mmol l^{-1} in human RBCs). O_2-loaded but Root-effect-lacking human RBCs actually present little advantage over pure Ringer solution with respect to the produced intraocular P_{O_2} (cf. Fig. 5). These data accordingly represent the first direct demonstration of the involvement of the Root effect for the enhancement of P_{O_2} in the teleost eye.

Conclusions

(1) Estimates of the depth of O_2 entry on the basis of measured retinal diffusion pathways indicate that elevated ocular P_{O_2} values are indispensable for the transfer of O_2 by simple diffusion across the thick avascular retina.

(2) The *in vitro* Root capacity of trout RBC suspensions is capable of providing enough O_2 for the establishment of the retinal tissue P_{O_2} gradients and does not necessarily require counter-current enhancement.

(3) Direct in-tissue-tests utilizing Root-effect-containing trout erythrocytes contrasted with Root-effect-lacking human erythrocytes clearly demonstrated that the Root effect is directly involved and an indispensable prerequisite for any enhancement of P_{O_2} in the retina of the teleost eye.

List of symbols and abbreviations

DA	dorsal aorta
H	human RBC suspension used for perfusion
Hb	haemoglobin
$[Hb_4]$	Hb tetramer concentration
Hct	haematocrit
HR	heart rate
IOP	intraocular pressure
$[O_2]_{Hb}$	Hb-bound O_2
pHa	arterial pH
pH_e	extracellular (plasma) pH
pH_i	intracellular pH
P_{O_2}	oxygen partial pressure
P_{retO_2}	retinal P_{O_2}
Pa_{O_2}	arterial P_{O_2}
RBC	red blood cell

T_{O_2}	total oxygen content
Tr	trout RBC suspension used for perfusion
TrC	trout RBC 'control' suspension

Retinal layers:

GCL	ganglion cell layer
ILM	inner limiting membrane
INL	inner nuclear layer
IPL	inner plexiform layer
NFL	nerve fibre layer
OLM	outer limiting membrane
ONL	outer nuclear layer
OPL	outer plexiform layer
PE	pigment epithelium
PRL	photoreceptor layer

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