Oxygen Consumption of the Rabbit Cornea

Daniel M. Harvitt and Joseph A. Bonanno

PURPOSE. Tear oxygen tension beneath contact lenses, measured in a previous study, was found to be lower than mathematical model predictions. In this study, the authors developed a phosphorescence-based technique for measuring corneal O₂ consumption (Q O₂) to determine whether errors in previous determinations of Q O₂ could explain the discrepancy between measured and predicted tear oxygen tension.

METHODS. Corneal oxygen consumption is measured using the phosphorescence quenching of Pd-meso-tetra-(4-carboxyphenyl)porphine by oxygen. Oxygen tension of a pH 7.5 Ringer's solution is measured in an airtight chamber that holds an excised rabbit cornea, and Q O₂ is calculated from the decreased PO₂ in the stirred chamber solution. Q O₂ rates are measured for the whole trephined rabbit cornea with the epithelium, the endothelium, or both, removed, which makes possible the estimation of the consumption rates of the epithelium, stroma, and endothelium.

RESULTS. Control experiments indicated that the cornea Q O₂ was constant for 3 hours and that exposure to 2,4-dinitrophenol increased Q O₂. Mean Q O₂ rates (microliters of O₂/cm² per hour) were: whole cornea, Q O₂ = 7.53; epithelium, Q O₂ = 3.73; stroma, Q O₂ = 2.97; and endothelium, Q O₂ = 0.86.

CONCLUSIONS. Phosphorescence-based measurements can be used to determine the Q O₂ of the component layers of the cornea. Estimates of Q O₂ in this study were similar to previous measurements, indicating that the discrepancy of tear PO₂ measures and model predictions are not a result of errors in the Q O₂ measurements at pH 7.5. (Invest Ophtalmol Vis Sci. 1998;39:444-448)

In a previous study,¹ we presented a method of direct non-invasive measurement of tear oxygen tension beneath gas-permeable contact lenses in the rabbit, which was based on the principle of phosphorescence quenching. These in vivo measurements were lower than the tear oxygen tensions predicted by mathematical models. The model predictions of the oxygen distribution across the cornea and contact lens incorporated the geometry of the cornea and contact lens, the PO₂

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Oxygen Measurement

Oxygen measurements were based on the quenching of the phosphorescent Pd-coproporphyrin dye in the excited state by oxygen. The theory and application of this technique have been discussed in detail elsewhere.¹,⁹,¹⁰ Briefly, the Stern-Volmer relationship relates the phosphorescence lifetime, τ, to the oxygen concentration, [O₂] as follows:

\[ \frac{\tau}{\tau_o} = 1 + \frac{\tau_o}{k_q [O_2]} \]

where \( \tau_o \) is the lifetime in the absence of quencher and \( k_q \) is the quenching constant. These phosphorescence-based oxygen measurements require the capture of a phosphorescent intensity decay profile to calculate the lifetime. The lifetime is then converted to oxygen concentration using equation 1, where \( \tau_o \) is the decay rate constant measured under zero

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FIGURE 1. Schematic of the phosphorescence measurement system. The light source is a xenon flash triggered by a counter-timer board under program control using a personal computer. The same trigger initiates flash discharge and data collection. The flash emission is band pass filtered and focused onto a bifurcated fiber optic bundle. The common end of the fiber optic bundle delivers the flash to the corneal chamber and collects the resultant phosphorescent emission. This emission is filtered and directed to a photomultiplier. The signal is amplified and digitized at 1 MHz using a 12-bit, analog-to-digital converter. A stir bar rotating at 2 cycles/second is separated from the cornea by a mesh disc. Further details of the instrumentation are given in Harvitt and Bonanno.1

Oxygen conditions, and \( k_0 \) (345 mm Hg\(^{-1}\)/second) is obtained in calibration experiments, in which \( r \) is measured at different known Po\(_2\) levels.1,2 Equation 1 predicts that the phosphorescence lifetime is inversely proportional to the oxygen concentration; therefore, a low Po\(_2\) level results in a long phosphorescence lifetime, and a higher Po\(_2\) level produces a shorter lifetime.

Figure 1 shows a schematic diagram of the corneal chamber phosphorescence measurement system, which is a simple adaptation of the tear oxygen measurement system.1 A fiber optic bundle, positioned 6 mm from the chamber, delivers an excitation flash (<4 usec; 539 ± 23 nm) and collects the phosphorescence emission (>645 nm) of the dye at 1 MHz for 2 msec. The chamber was airtight and constructed to hold a freshly excised cornea immersed in 2 ml Ringer’s solution with 20 \( \mu \)M Pd-coproporphyrin at 34°C. Solutions were prepared fresh, filtered, and kept in sterile containers until use. To keep the solution well mixed to avoid oxygen gradients, a stir bar was included in the chamber and separated from the cornea by a mesh disc. The stir bar was driven by a rotating magnet beneath the chamber (120 cycles/minute). Two oxygen tension measurements, the average of eight phosphorescence decays each, are made every 3 to 5 minutes over a 30- to 60-minute interval. When oxygen tension in the chamber fell beneath approximately 40 mm Hg, the chamber was emptied and refilled with fresh air-bubbled solution. When Po\(_2\) fell beneath 40 mm Hg at the anterior and posterior corneal boundaries, the corneal consumption rate decreased, because the central regions of the stroma became anoxic.11 The change in chamber oxygen concentration per unit of time was determined by linear regression of the oxygen tension as a function of time and is proportional to the corneal oxygen consumption rate. Oxygen consumption was expressed in microliters of O\(_2\)/cm\(^2\) per hour to make comparisons to previous studies. From Hitchman,12 there is 0.02775 l O\(_2\)/atm per liter solution at 35°C. Oxygen consumption was then calculated using this solubility value, the measured change in chamber oxygen tension per unit time, the 1.99-cm\(^3\) volume of solution in the chamber, and a 0.64-cm\(^2\) corneal area, which underestimates the increase in area caused by curvature to facilitate comparisons with other estimates of \( Q_{O2} \). Repeated series of measurements were made to ensure that the consumption rate is constant and not increasing because of possible contamination. When the chamber was filled with only dye solution (no cornea) that had been bubbled with 100% nitrogen, phosphorescence decay values were constant over 2 hours, indicating that the chamber was airtight.

All experiments used New Zealand White rabbits, handled according to the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Enucleated eyes were taken from rabbits immediately after the rabbits were killed for unrelated experiments. The rabbits weighed between 2.1 kg and 2.5 kg and were killed by a lethal intravenous injection (120 mg/kg) of sodium pentobarbital in the marginal ear vein. The eyes were removed with the lids intact to protect the corneal epithelium from damage. Eyes were transported and stored in Ringer’s solution at 4°C, and the experiments were completed within 12 hours of enucleation. The procedure for dissection of the cornea was to cut through the eye at the equator, remove the lid and the crystalline lens, and trephine the cornea (9-mm diameter) against a lucite block. The cornea was rinsed in Ringer’s solution (pH 7.5), and the remnants of the iris were removed. In pilot experiments, the corneas were fixed and stained with hematoxylin and eosin to...
examine the integrity of the cellular layers. Removing the lens, but leaving the ciliary body and iris intact before trephining the cornea, preserved the integrity of the endothelium.

To determine the oxygen consumption rate of the component layers of the cornea, the endothelial and/or the epithelial layers were removed. The epithelium was removed by first pulling back the lids and holding the globe while repeatedly scraping the epithelium with a scalpel blade. In pilot experiments, after \( Q_{O2} \) measurements, complete removal of the epithelium was confirmed with Mayer’s hematoxylin solution and eosin staining. The endothelium was easily removed by removing the ciliary body and iris and rubbing with a cotton swab before trephining the cornea. The same fixing and staining regimen indicated that the epithelial and endothelial layers were not damaged by the dissection of the cornea during those experiments in which the layers were not removed. Experiments to determine the epithelial and endothelial oxygen consumption rates were conducted with pairs of corneas. The consumption rates of the component layers are calculated as follows:

\[
Q_{\text{epi}} = Q_{\text{whole cornea}} - Q_{\text{dc-epithelialized cornea}} \\
Q_{\text{stroma}} = Q_{\text{dc-epithelialized and de-endothelialized cornea}} \\
Q_{\text{endo}} = Q_{\text{dc-epithelialized cornea}} - Q_{\text{stroma}} 
\]

**RESULTS**

Figure 2A depicts an experiment in which the whole cornea oxygen consumption rate was measured four times during a 4-hour period. \( Q_{O2} (\mu l O_2/cm^2 \text{ per hour}) \) was calculated to be 6.76 for trial 1, 6.10 for trial 2, 7.18 for trial 3, and 7.03 for trial 4. This indicates that there was little deterioration of the tissue in 4 hours. Corneas bathed in 10-fold higher and lower concentrations of the Pd-coproporphyrin phosphorescent dye gave similar \( Q_{O2} \) values (data not shown).

To corroborate the validity of the technique, DNP was added to the bathing solution. DNP uncouples oxidative phosphorylation; oxygen is consumed without adenosine triphosphate production, thereby increasing the oxygen consumption rate. In Figure 2B, adding DNP to the chamber of Ringer’s solution demonstrated this effect. In the first two series of measurements, the excised cornea was placed in control pH 7.5 Ringer’s solution, and the oxygen consumption rate, which is proportional to the slope of the chamber oxygen tension versus the time regression slope, was approximately equal. The bathing solution for the next three measurement series then included 50.0 \( \mu M \) DNP, and the consumption rate increased by more than 70%.

In addition to experiments to determine the consumption rate of the whole cornea (Fig. 2A), the epithelial and endothelial layers were removed to estimate the consumption rates of the epithelial, stromal, and endothelial layers. In the experiment shown in Figure 3A, the epithelium was removed from one cornea and the other cornea was left intact. The difference in rates between these two paired corneas gave the oxygen consumption of the epithelium. Similarly, in the experiment shown in Figure 3B, the epithelium was removed from one cornea and the epithelium and endothelium were removed from the other cornea. The difference in rates between these two paired corneas gave the oxygen consumption of the endothelium. The data from these experiments, together with polarographic-based measurement data, are summarized in Table 1.

**DISCUSSION**

The oxygen consumption rates of the excised rabbit cornea and its major layers, the epithelium, stroma, and endothelium, were assessed using an in vitro measurement system. Oxygen consumption was found to remain constant for at least 4 hours (Fig. 2A). By removing the epithelium, endothelium, or both, and measuring the consumption rate of the remaining corneal tissue (Fig. 3), the rates of oxygen consumption were calculated for each layer of the cornea (Table 1). These values, especially for the whole cornea, stroma, and epithelium, are similar to previous measurements.
The advantage of the phosphorescence system is that it is noninvasive: There is no mechanical interference of the tissue by the measurement technique. Furthermore, unlike oxygen electrode measurements of corneal $Q_{O_2}$, phosphorescence measurements do not require a knowledge of tissue oxygen permeability and do not consume oxygen. This technique has been used extensively to measure the $P_{O_2}$ of tissue, cells, or mitochondrial suspensions with no indications of interference with metabolic activity. Here, we varied the dye concentration more than 3 log units and found no change in corneal oxygen consumption rates. The dye is bound to albumin and is not thought to react with corneal proteins. The steady state oxygen tensions achieved during contact lens wear and the consistent corneal oxygen metabolic rate over 4 hours also indicate that there is no short-term interaction between dye and metabolism. One disadvantage of the present phosphorescence measurement system is the limited resolution at high-oxygen concentrations, at which the phosphorescence lifetimes are short and difficult to separate from background autophosphorescence. To some extent, these problems may be overcome in the future with improved dyes, electronics, and imaging systems, but the present system was adequate for the principal objectives of this study. The polarographic electrode is advantageous because it may be applied to humans and it is relatively quick and inexpensive. It has, therefore, often been used for estimating the amount of oxygen beneath contact lenses using the equivalent oxygen percentage technique and in studies that compare corneal oxygen uptake rates. Polarographic-based measurements of corneal oxygen consumption, such as those performed by Freeman, are limited because there may be damage to the tissue and the consumption rate is estimated using oxygen uptake rates at either surface of the excised cornea using mathematical models of diffusion. Variables that may affect these readings include the amount of pressure applied to the electrode, temperature fluctuations, instability of the electrode itself, estimates of corneal layer thickness and transmissibility that go into the calculations of $Q_{O_2}$, and extrapolation of the results of the experiments performed at room temperature to physiological temperatures.

In this study, the oxygen tension at the anterior and posterior boundaries were equal because the cornea was bathed in a mixed solution. This is different from the in vivo case, in which the aqueous humor $P_{O_2}$ and tear $P_{O_2}$ were at different levels. The experiments also may take place in higher $P_{O_2}$ than in the physiological case. This is especially true for the endothelium, in which the boundary $P_{O_2}$ has been estimated to be as low as 15. This should not matter because the consumption rate is constant across the range of chamber $P_{O_2}$ used in these experiments. If the consumption rate was signifi-

### Table 1. Oxygen Consumption of the Rabbit Cornea Expressed as Microliters of $O_2$/cm$^2$ per Hour

<table>
<thead>
<tr>
<th>Author</th>
<th>Method</th>
<th>Whole Cornea</th>
<th>Epithelium</th>
<th>Stroma</th>
<th>Endothelium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present study</td>
<td>Phosphorescence</td>
<td>7.53 ± 0.51</td>
<td>3.73*</td>
<td>2.97 ± 0.51</td>
<td>0.86*</td>
</tr>
<tr>
<td>Freeman 1972</td>
<td>Electrode</td>
<td>9.54</td>
<td>3.85</td>
<td>3.68</td>
<td>2.05</td>
</tr>
<tr>
<td>Riley 1969</td>
<td>Electrode</td>
<td>10.4</td>
<td>3.95</td>
<td>5.78</td>
<td>0.84</td>
</tr>
<tr>
<td>Takahashi 1966</td>
<td>Electrode, pH 9.0</td>
<td>2.74</td>
<td>7.7</td>
<td>1.9</td>
<td>0.71</td>
</tr>
<tr>
<td>Langham 1952</td>
<td>Warburg</td>
<td>8.1</td>
<td>7.7</td>
<td>1.9</td>
<td>0.71</td>
</tr>
</tbody>
</table>

*Calculated using equations 2 and the $Q_{O_2}$ for the deepithelialized cornea. 3.83 ± 0.29 (n = 14).
cantly affected by the oxygen tension, the data would not be linear at higher oxygen levels as they are in Figures 2 and 3.

Superficial stromal keratocyte function is impaired within approximately 30 minutes of epithelium removal, and epithelial injury may induce keratocyte apoptosis. This may result in an underestimation of the stroma consumption rate and an overestimation of the epithelium consumption rate ($Q_{epi} = Q_{whole cornea} - Q_{de-epithelialized cornea}$). The most important of these values, in terms of our models, are the epithelial rates. However, the measured consumption rates were still less than those produced by Freeman, and it is doubtful that the underestimation is significant.

Table 1 summarizes the oxygen consumption rates of the rabbit cornea as determined by this study and by other investigators. For the whole cornea, the consumption rates are in general agreement, ranging from 7.5 to 10.4 μl O2/cm2 per hour. For the epithelium, again the rates are close, with the exception of Langham's 1952 Warburg consumption experiments and Takahashi's measurements at pH 9.0. The stroma rates vary significantly between studies, from 1.9 to 5.9 μl O2/cm2 per hour, but the present value of 3.0 μl O2/cm2 per hour falls within the range. The range of values for the stroma oxygen consumption may be a result of the variation in stroma thickness because these consumption rates are expressed in terms of surface area and stroma thickness, and oxygen consumption increases with rabbit age and weight. The present experiments did not control for rabbit size (except that the weights of the rabbits were within the range). The weight of the dried corneas was consistent (average weight, 11.23 mg [± 0.23 SE]). Finally, the endothelium consumption rates range from 0.71 to 2.05 μl O2/cm2 per hour, but the present finding of 0.86 μl O2/cm2 per hour agrees with Riley's measurement of 0.84% and Langham's value of 0.71 μl O2/cm2 per hour. There are several plausible explanations for the discrepancy with Freeman's data.

First, the endothelium is a very thin (approximately 5 μm) layer, and its total oxygen consumption is small and difficult to measure with either electrode-based measurements or the present methods. In the experiments described here, the endothelium oxygen consumption is calculated by the difference between the stroma (2.97 ± 0.53, n = 5) and a deep epithelialized cornea (3.83 ± 0.29, n = 14). Inspecting the standard error and sample sizes for these measurements leads to the conclusion that the estimate for the endothelium consumption rate is not precise. However, for the purpose of this study (the investigation of the discrepancy between tear oxygen measurements and mathematical model predictions), further experiments to improve confidence in our endothelium estimation were not warranted because the endothelium oxygen consumption rate has a minimal impact on the model predictions of tear oxygen.

References