mechanism could lead to one or more forms of retinal degeneration.

Key words: actin, photoreceptor, cilium, retina, immuno-cytochemistry

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References


Permeability of the Isolated Dog Retinal Pigment Epithelium to Carboxyfluorescein

Shunji Tsuboi* and Jonathan E. Pederson

Outward (retina to choroid) and inward (choroid to retina) permeabilities of carboxyfluorescein and fluorescein in the isolated dog retinal pigment epithelium (RPE)-choroid were determined. Outward permeability was 9 and 47 times larger than inward permeability for carboxyfluorescein and fluorescein, respectively. The outward permeability of carboxyfluorescein was seven times lower than that of fluorescein, whereas there was no statistical difference between the inward permeabilities. Carboxyfluorescein is thus distinguished from fluorescein by its low affinity to the outwardly directed organic anion transport system. $10^{-4}$ M probenecid caused >98% inhibition of the outward transport of $6 \times 10^{-3}$ M carboxyfluorescein and $6 \times 10^{-4}$ M fluorescein. Invest Ophthalmol Vis Sci 27:1767–1770, 1986

Carboxyfluorescein shares many similarities with fluorescein, but is 1,000 times less lipid soluble than fluorescein. This difference is exemplified by fluorescence microscopy, where intravascular fluorescein, but not carboxyfluorescein, can be identified intracellularly in the retinal pigment epithelium (RPE) and ciliary epithelium.1,2 The transmembrane permeability across the RPE may not be as dissimilar, since vitreous fluorophotometry in normal and diabetic rats has shown that the inward permeabilities of carboxyfluorescein and fluorescein are the same.3 Carboxyfluorescein is potentially useful in ophthalmic research, and its permeability characteristics across the blood–retinal barrier (BRB) deserve further study.

Tsuboi et al have shown that, in the isolated dog RPE-choroid, there is an active outward (retina to choroid) transport of fluorescein.4 In the present study, the outward and inward (choroid to retina) permeabilities of carboxyfluorescein are compared with those of fluorescein in the isolated dog RPE-choroid. In addition,
the effectiveness of probenecid as an inhibitor of fluorescein and carboxyfluorescein transport is studied.

**Materials and Methods.** The details of the experimental protocol are described in a previous report. Briefly, the RPE-choroid was prepared from 20–30 kg adult dogs immediately after sacrifice. All procedures conformed to the ARVO Resolution on the Use of Animals in Research. The isolated RPE-choroid was placed on a piece of stretched nylon net, and clamped gently between Ussing-type chambers (5 ml each), with fenestrations 0.2 cm² in area. The tissue was bathed with buffered HEPES (pH = 7.4, 285 mOsm/kg H₂O) at 37°C. This solution has been shown to be useful in membrane transport studies in dogs.

All experiments were done under an open-circuit condition. The transepithelial potential difference across the RPE-choroid was continuously monitored by a recorder connected with calomel electrodes through an automatic voltage clamp device. The transepithelial resistance was calculated periodically from the transepithelial potential difference and short-circuit current by Ohm’s law, and any tissues with a resistance lower than 100 ohm-cm² at the beginning of the experiment were discarded.

6-carboxyfluorescein (Eastman Kodak Co, Rochester, NY) and fluorescein sodium (Alcon Laboratories Inc, Fort Worth, TX) solutions were used. Carboxyfluorescein was not repurified. A 10 µl aliquot of carboxyfluorescein or fluorescein solution was added either to the solution facing the retinal side or to the solution facing the choroidal side of the tissue. Each tissue was used to measure either inward or outward permeability of one tracer. The final concentrations of the fluorescent tracers in the bathing solutions were as follows: for inward permeability, 3 × 10⁻⁴ M (both tracers), for outward permeability, 6 × 10⁻⁶ M (both tracers) and 6 × 10⁻⁵ M (carboxyfluorescein). These concentrations were chosen because of the large difference in inward and outward permeabilities, and because of the differences in permeabilities between carboxyfluorescein and fluorescein. Each of the bathing solutions was continuously mixed by 95% O₂ and 5% CO₂ gas bubbling. After the concentration became uniform (10 min), a 100 µl sample was taken from the solution in the opposite chamber (sampling side) every 15 min for about 60–90 min. Each sample was replaced by an equal amount of HEPES-buffered solution. Fifty microliters of 10⁻² M probenecid (Sigma Chemical Co., St. Louis, MO) was then added to each chamber to yield a final concentration of 10⁻⁴ M. Sampling was continued for 60–90 min.

The concentration of fluorescent tracer in the sample was measured by a flash fluorophotometer with a computer which automatically calculated the concentration from standard curves of known fluorescence. The standard curves were linear from 2 × 10⁻⁹–1 × 10⁻⁶ g/ml for carboxyfluorescein, and from 1 × 10⁻⁹–5 × 10⁻⁷ for fluorescein. The rate of appearance of tracer as a function of time was determined using linear regression. Outward and inward permeabilities were then calculated by Fick’s first law.

The percent inhibition of fluorescein or carboxyfluorescein transport by probenecid was calculated from the following formula:

\[
\% \text{ inhibition} = \left(1 - \frac{P_{\text{out}*} - P_{\text{in}*}}{P_{\text{out}} - P_{\text{in}}}ight) \times 100,
\]

where \(P_{\text{out}} - P_{\text{in}}\) = outward minus inward permeabilities without probenecid, and \(P_{\text{out}*} - P_{\text{in}*}\) = with probenecid.

**Results.** At a concentration of 6 × 10⁻⁶ M carboxyfluorescein, the outward flux was found to be too small to yield meaningful data. Therefore, 6 × 10⁻⁵ M was used to determine the outward permeability of carboxyfluorescein. This problem was not encountered with fluorescein.

Carboxyfluorescein or fluorescein appearing in the sampling side increased linearly as a function of time during the experiment, while the concentration of fluorescent tracers in the control side was constant. With the administration of 10⁻⁴ M probenecid, outward flux of tracers markedly decreased, whereas inward flux increased somewhat. The potential difference across the RPE-choroid gradually decreased about 10–30% after probenecid was added to the bathing solution. The potential difference and tissue resistance were monitored throughout the procedure and decreased about 10% by the termination of the experiment. Figures 1 and 2 show typical data.
show a representative result for carboxyfluorescein permeability.

Table 1 summarizes the outward and inward permeability of carboxyfluorescein and fluorescein with or without $10^{-4}$ M probenecid. Without probenecid, the outward permeability was significantly larger than the inward permeability for both carboxyfluorescein and fluorescein. However, the outward permeability of carboxyfluorescein was significantly less than that of fluorescein ($P < 0.001$), while there was no statistical difference between the inward permeabilities of carboxyfluorescein and fluorescein. With $10^{-4}$ M probenecid, there was no statistical difference between any of the four categories of outward and inward permeabilities of carboxyfluorescein and fluorescein.

$10^{-4}$ M probenecid caused a 98% transport inhibition of $6 \times 10^{-6}$ M fluorescein across the RPE-choroid and a 100% transport inhibition of $6 \times 10^{-5}$ M carboxyfluorescein. Since other concentrations were not tested, the maximal tracer concentration that still yields complete inhibition by $10^{-4}$ M probenecid is unknown.

### Discussion

In the presence of probenecid, and in the absence of large volume fluxes across the RPE-choroid, the inward permeability represents the diffusional permeability of the membrane. The present study with $10^{-4}$ M probenecid reveals that the diffusional permeability of the RPE-choroid to either carboxyfluorescein or fluorescein is about $1.7 \times 10^{-6}$ cm/sec. This finding strongly suggests that the lipid solubility of these tracers is of minimal importance in their diffusion across the RPE-choroid. A previous study using intravenous carboxyfluorescein and fluorescein in rats showed greater intracellular penetration of fluorescein than carboxyfluorescein in the retinal outer segments and in the ciliary epithelium. However, the present study in dog RPE-choroid shows little difference in transcellular movement of carboxyfluorescein vs. fluorescein, suggesting the importance of a paracellular pathway for passive tracer movement. In the absence of probenecid, the inward permeability is reduced, presumably due to outward transport counteracting inward diffusion.

A striking difference is noted between the outward permeabilities of carboxyfluorescein and fluorescein in the absence of probenecid. It is known that fluorescein is transported by an active carrier-mediated transport in the rabbit anterior uvea and dog RPE-choroid. The smaller outward permeability of carboxyfluorescein implies that carboxyfluorescein has lower affinity to the carrier in the cell membrane than fluorescein. Carboxyfluorescein is thus advantageous in clinical vitreous fluorophotometry where pretreatment with probenecid is uncommon. Table 2 lists comparable values for inward and outward fluorescein permeability in other species. Published values vary by more than one order of magnitude.

In a previous study of vitreous fluorophotometry in monkeys, the various components of fluorescein loss from the vitreous cavity were quantitated, namely diffusion, fluorescein transport, and solvent drag (flow). The accuracy of the results depended on the assumption that probenecid completely inhibited fluorescein transport. The present study confirms that probenecid is capable of virtually complete inhibition of either fluorescein transfer.

### Table 1. Outward and inward RPE permeability

<table>
<thead>
<tr>
<th>Tracer</th>
<th>Outward (n)</th>
<th>Inward (n)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboxyfluorescein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>$46.7 \pm 7.7$* (6)</td>
<td>$5.4 \pm 0.4$ (5)</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>$10^{-4}$ M probenecid</td>
<td>$16.3 \pm 2.6$ (6)</td>
<td>$16.7 \pm 1.8$ (6)</td>
<td>ns</td>
</tr>
<tr>
<td>Fluorescein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>$320 \pm 24$* (7)</td>
<td>$6.8 \pm 0.8$ (5)</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>$10^{-4}$ M probenecid</td>
<td>$23.3 \pm 4.0$ (6)</td>
<td>$17.7 \pm 2.3$ (6)</td>
<td>ns</td>
</tr>
</tbody>
</table>

Data are $\times 10^{-7}$ cm/sec (mean ± SEM).

* $P < 0.001$.

ns = not significant.

### Table 2. Inward (Pin) and outward (Pout) permeability of the blood-retinal barrier to fluorescein; comparison of reported values

<table>
<thead>
<tr>
<th>Source</th>
<th>Species</th>
<th>Pin (cm/sec)</th>
<th>Pout (cm/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cunha-Vaz and Maurice7</td>
<td>Rabbit</td>
<td>15</td>
<td>560</td>
</tr>
<tr>
<td>Palestine and Brubaker8</td>
<td>Human</td>
<td>15</td>
<td>570</td>
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<tr>
<td>Zeimer et al9</td>
<td>Human</td>
<td>1.2</td>
<td>35</td>
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<tr>
<td>Cantrill and Pederson10</td>
<td>Monkey</td>
<td>9.8</td>
<td>190</td>
</tr>
<tr>
<td>Tsuboi et al11</td>
<td>Human</td>
<td>1.0</td>
<td>17</td>
</tr>
<tr>
<td>Ogura et al12</td>
<td>Human</td>
<td>3.0</td>
<td>93</td>
</tr>
<tr>
<td>Present study</td>
<td>Dog</td>
<td>6.8</td>
<td>320</td>
</tr>
</tbody>
</table>

Data are $\times 10^{-7}$.
Acute Changes in RPE Apical Morphology After Retinal Detachment in Rabbit

A SEM Study

Joe Immel,* Akira Negi,† and Michael F. Marmor

The morphology of the apical surface of rabbit retinal pigment epithelium was studied by scanning electron microscopy from the first minute to several hours after making small non-rhegmatogenous retinal detachments (blebs). From 0 to 5 min, there were only slight changes in the homogenous, dense mat of filamentous microvilli. From 5 to 30 min, filamentous microvilli retracted exposing larger processes. From 30 to 60 min, blunt processes became completely exposed and sheet-like processes disappeared. At about 60 min, cone sheaths were no longer identifiable in most specimens. Between 60 min and the time of retinal reapposition (several hours), the apical surface became highly rounded. Colchicine and cytochalasin D had no effect on fluid resorption, but colchicine greatly accelerated and enhanced cell rounding, while cytochalasin D produced prominent apical tufts. Invest Ophthalmol Vis Sci 27:1770–1776, 1986

Changes in RPE apical morphology have been observed 24 hours to several weeks after experimental rhegmatogenous detachment of the retina,1,2,3,4,5 or within minutes after making the vitreous severely hyperosmotic.6 The aim of this study is to examine the apical surface immediately after relativelyatraumatic mechanical detachment in the rabbit eye, and to determine the time of onset and the sequence of changes in RPE surface morphology.

Materials and Methods. Our technique for making small non-rhegmatogenous retinal detachments (blebs) has been described in detail elsewhere.7 In brief, Dutch rabbits weighing approximately 1.5 kg were anesthetized with pentobarbital, and a glass micropipette filled with Hank's balanced salt solution (Gibco Chagrin Falls, OH), with a tip diameter of 40 to 50 μm, was advanced through a limbal incision to the retinal surface. Air pressure slowly expelled the fluid from the micropipette so that upon penetrating the retina and