Movement of Fluorescein and Its Glucuronide Across Retinal Pigment Epithelium-Choroid

Satoshi Koyano, Makoto Araie, and Shuichiro Eguchi

Purpose. To characterize movement of fluorescein and its glucuronide across the blood-retinal barrier.

Methods. Retinal pigment epithelium (RPE)-choroid preparations from New Zealand albino rabbit were sealed in an Ussing-type chamber in a stabilized condition for 3 hr, where movement of fluorescein and fluorescein glucuronide across the RPE-choroid was studied under a short circuit condition.

Results. The outward (vitreous-choroid) permeability to fluorescein determined at a concentration of 15 \(\mu\)mol/l was about 4 times greater than the inward (choroid-vitreous) permeability \((P < 0.01)\). The outward permeability was significantly decreased by 50–65% by metabolic or competitive inhibitors \((1 \mu\text{mol}/l\text{ ouabain, } 10 \mu\text{mol}/l\text{ 2,4-dinitrophenol, } 100 \mu\text{mol}/l\text{ probenecid, } 30 \mu\text{mol}/l\text{ hippurate, or } 5 \mu\text{mol}/l\text{ iodipamide})\), whereas the inward permeability was not affected by any of the above competitive inhibitors. As the fluorescein concentration was increased from 15 to 150 \(\mu\)mol/l, the net fluorescein movement across the tissue indicated saturation, and a Lineweaver-Burk plot gave an apparent \(K_m\) of 26 \(\mu\text{mol}/l\) and \(V_{\text{max}}\) of 1.56 nmol/hr/cm². The outward permeability to fluorescein glucuronide determined at 15 \(\mu\)mol/l was about double the inward permeability \((P < 0.01)\) and about \(\frac{1}{2}\) of the outward permeability to fluorescein \((P < 0.01)\). The outward permeability to fluorescein glucuronide was significantly decreased by about 50% by 1 \(\mu\text{mol}/l\text{ ouabain, } 10 \mu\text{mol}/l\text{ 2,4-dinitrophenol, or } 100 \mu\text{mol}/l\text{ probenecid, whereas the inward permeability was not affected by } 100 \mu\text{mol}/l\text{ probenecid.}

Conclusion. These results suggest that the majority of the outward fluorescein movement across the tissue and part of that of fluorescein glucuronide depends on an active transport mechanism, whereas the inward movement of both fluorescein and fluorescein glucuronide occurs by a passive mechanism. Invest Ophthalmol Vis Sci. 1993;34:531–538.

Fluorescein has been widely used as a biologic marker to study the integrity of the blood-retinal barrier (BRB). Fluorescein fundus angiography has been the conventional method and is now complemented by the recently developed vitreous fluorophotometry. In this method, the integrity of the BRB is calculated based on the intensity of fluorescence in the vitreous cavity after systemic administration of fluorescein. When administered systemically, fluorescein is rapidly metabolized; its principal metabolite, fluorescein glucuronide, also is fluorescent, although weaker than that of fluorescein itself. In the plasma, this metabolite reaches a much higher concentration than that of fluorescein and penetrates into the anterior chamber and the vitreous, where it represents a significant fraction of the total fluorescence. In body fluids, fluorescein, an organic anion, is thought to act as a substrate for an active transport system in ocular tissues. In vivo studies have indi-
cated that fluorescein is rapidly lost from the vitreous mainly by an active mechanism,11,16,18,19 whereas the loss rate of fluorescein glucuronide from the vitreous was much slower than that of fluorescein.11,19,20 In the isolated retinal pigment epithelium (RPE)-choroid of the dog, fluorescein was reported to be transported from the vitreous to the choroidal side by an active mechanism, which was suppressed by 1 mmol/L KCN or 0.1 mmol/L probenecid, but not by 1 mmol/L ouabain.21 However, no report is available concerning the movement of fluorescein glucuronide across the RPE-choroid.

Fluorescein is the only diagnostic tracer that is routinely used to evaluate the status of the BRB in clinical ophthalmology. Therefore, further characterization of the mode of movement of fluorescein across the RPE-choroid, including a saturation kinetic study, and that of fluorescein glucuronide, its principal metabolite in the blood, could be very useful. In the present report, we studied the movement of fluorescein and fluorescein glucuronide across the BRB by using the isolated rabbit RPE-choroid mounted in an Ussing-Zerahn-type chamber.22

METHODS

Drugs

Commercially available purified fluorescein (Fluoresite, Alcon Laboratories Inc., Fort Worth, TX) was used throughout the study. Fluorescein glucuronide was extracted from rabbit urine and purified using high performance liquid chromatography, according to the method of Sakai et al.23 Ouabain, hippocric acid, and probenecid were obtained from Sigma Chemical Co., St Louis, iodipamide was from Tokyo Kasei Kogyo Ltd., Tokyo, and 2,4-dinitrophenol was from Wako Chemical Co., Ltd., Osaka.

Tissue Preparation and Electrical Measurements

The procedures used in this study conformed to the ARVO Resolution on the Use of Animals in Research. Adult New Zealand albino rabbits of both sexes, weighing 2.5–3.0 kg, were killed with an overdose of pentobarbital (Nembutal, Abbott Laboratories, North Chicago, IL), and the eyes were enucleated immediately. The enucleated eyes were bisected at the equator by three meridional cuts, opened from the equator by three meridional cuts, and the vitreous and sensory retina were carefully peeled off with very delicate forceps. Then, the subchoroidal tissue was separated from the underlying sclera millimeter by millimeter with microscissors, and a rectangular sheet of RPE-choroid (5 mm × 12 mm) complex was isolated. The isolated RPE-choroid was placed, retinal side up, on a paraffin membrane immersed in the Krebs-Ringer solution. The isolated tissue was slid from the paraffin membrane onto a piece of nylon net stretched over the fenestration of a silicone disc, which was coated with high vacuum grease (Dow Corning, Medfield, MA) to protect the tissue. The tissue was fixed on the net by blotting the Ringer with a filter paper. Then, another silicone disc coated with the grease was placed on the retinal side of the tissue, and the whole preparation was fixed between two halves of an Ussing-Zerahn chamber. The exposed tissue surface area was 0.14 cm², and the volume of each chamber was 7.0 ml. Each chamber was filled with Krebs-Ringer physiological solution bubbled with 95% O₂ and 5% CO₂ for effective stirring, supply of O₂, and maintenance of the pH at 7.4. No hydrostatic pressure difference was present between the chambers. The temperature of the system was kept at 37°C by circulating warmed water around the chamber.

The transepithelial potential difference and the short-circuit current (SCC) were recorded through two pairs of 3.0% agar-3 mol/L KCl bridges and calomel electrodes using an automatic voltage clamp device. The transepithelial resistance was calculated from the transepithelial potential difference and SCC. The calomel electrodes with their agar bridges were equilibrated for at least 10 min, and the temperature of the electrodes was maintained at 37°C.

Fluorescein Movement Across the Isolated RPE-Choroid

After a 40 min stabilization period under short-circuit conditions, sodium fluorescein was added to one side to give a concentration of 15, 45, 75, or 150 μmol/L. A 50 μl sample was taken with a micropipette (Drummond Micropipettes, #350; Drummond Scientific Company, Broomall, PA) from the opposite chamber every 15 min and was replaced with an equal amount of Krebs-Ringer solution during the 2 hr experimental period. Samples taken were diluted 10X with a phosphate buffer at pH 7.4, and their fluorescein concentrations were determined using a Topcon slit-lamp fluorophotometer (Tokyo Optical Co. Ltd., Tokyo). When fluorescein was added to the vitreous side of the tissue, its movement to the choroidal side was taken as a measure of outward movement. Conversely, the movement of the dye from the choroidal to the vitreous side was defined as the inward movement. The
movement of fluorescein across the tissue was analyzed using Fick's first law:

\[ J = A \frac{P}{R} (C_1 - C_o) \]

where \( J \) is the rate of movement of a substance (nmol/sec), \( A \) is the area of the tissue (cm\(^2\)), \( P \) is the permeability of the tissue to the substance in cm/sec, \( C_1 \) is the substance's concentration in nmol/ml in chamber “1,” where fluorescein was added and \( C_o \) is that in the opposite chamber. As described later, \( C_o \) was negligible as compared to \( C_1 \) in the present experiments. The value of \( J \) was determined from the \( C_o \) time data using the least squares method and then the value of \( P \) was calculated.

Effects of various metabolic or competitive inhibitors and the temperature of the medium on the inward and outward movement of fluorescein across the isolated RPE-choroid were studied at a fluorescein concentration of 15 \( \mu \)mol/l. Simultaneous with the addition of fluorescein, a competitive inhibitor—probenecid at a concentration of 100 \( \mu \)mol/l, hippurate at a concentration of 30 mmol/l, or iodipamide at a concentration of 5.0 mmol/l—was added to both vitreous and choroidal sides of the tissue. A metabolic inhibitor—ouabain at a concentration of 1.0 \( \mu \)mol/l or 2,4-dinitrophenol at a concentration of 10.0 \( \mu \)mol/l—was also added to both sides. The effect of medium temperature on the outward movement also was studied by maintaining the temperature of the incubation system at 0.5–1.0°C by circulating chilled water around the apparatus.

Fluorescein Glucuronide Movement Across the Isolated Rabbit RPE-Choroid

The experiments were performed under the same experimental conditions as in the fluorescein experiment. Purified fluorescein glucuronide was added to one side at a final concentration of 15 \( \mu \)mol/l, and samples were taken from the opposite chamber as described. Fluorescein glucuronide concentrations in samples were determined according to the method of Chen et al. Effects of probenecid, metabolic inhibitors, and medium temperature were studied as in the fluorescein experiment, using probenecid at a concentration of 100 \( \mu \)mol/l, ouabain at a concentration of 1.0 \( \mu \)mol/l, or 2,4-dinitrophenol at a concentration of 10.0 \( \mu \)mol/l.

RESULTS

Electrical Properties

The potential on the vitreous side was consistently positive with respect to that on the choroidal side. The transepithelial potential difference and SCC decreased gradually and reached a virtually steady state in 40 min and were maintained for about 3 hr (Fig. 1). In the experiment carried out at 37°C without inhibitors, the transepithelial potential difference and SCC 40 min after the start of the experiment averaged 1.81 ± 0.08 mV and 10.5 ± 0.5 \( \mu \)A/cm\(^2\) (mean ± SE; \( n = 78 \)), respectively, and the transepithelial resistance calculated from these two parameters averaged 179.2 ± 6.0 ohm.cm\(^2\).

Fluorescein Movement Across the Isolated Rabbit RPE-Choroid

For outward and inward movement, the fluorescein concentration in the opposite chamber increased almost linearly until the end of the experiment. However, the rate of increase during the first 30 min was significantly smaller than after the first 30 min (paired t-test, \( P < 0.01 \)). It seemed possible that there was a time delay for fluorescein to pass across the RPE-choroid and be well mixed in the opposite chamber. Therefore, only data obtained at least 30 min after the addition of fluorescein were used for calculation. The fluorescein concentration in the opposite chamber, \( C_o \), was maximally only 0.3% of the concentration administered.

The outward permeability of the isolated RPE-choroid to fluorescein at a concentration of 15 \( \mu \)mol/l was about 4 times higher than the inward permeability (\( P < 0.01 \), unpaired t-test) (Tables 1 and 2). The outward and inward permeabilities obtained at 15, 45, 75, 150 \( \mu \)mol/l are shown in Table 1. As the concentration rose, the outward permeability decreased considerably (ANOVA, \( P < 0.01 \)), while the inward permeability...
TABLE 1. Outward and Inward Permeabilities of Isolated Rabbit RPE-Choroid to Fluorescein

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Outward Permeability (×10⁻⁵ cm/sec)</th>
<th>Inward Permeability (×10⁻⁵ cm/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 μmol/l</td>
<td>1.80 ± 0.20</td>
<td>0.39 ± 0.14</td>
</tr>
<tr>
<td>45 μmol/l</td>
<td>1.03 ± 0.22</td>
<td>0.38 ± 0.11</td>
</tr>
<tr>
<td>75 μmol/l</td>
<td>0.85 ± 0.17</td>
<td>0.34 ± 0.02</td>
</tr>
<tr>
<td>150 μmol/l</td>
<td>0.60 ± 0.06</td>
<td>0.34 ± 0.04</td>
</tr>
</tbody>
</table>

Figures are mean ± SE in five experiments.

remained unaltered. Figure 2 plots net outward movement of fluorescein against concentration and shows that it increased as the concentration rose, reaching saturation at 150 μmol/l. The outward permeability was significantly decreased to 39%, 41%, and 35% of the control on the average with 100 μmol/l probenecid, 30 mmol/l hippurate, and 5 mmol/l iodipamide, respectively. In contrast, the inward permeability was not affected by any of the above competitive inhibitors (multiple comparison test of Dunnett, P < 0.01) (Table 2). The outward permeability also was significantly decreased to 49%, 40%, and 3% of the control on the average with 1.0 μmol/l ouabain, 10 μmol/l 2,4-dinitrophenol, and incubation at 0.5–1.0°C, respectively (P < 0.05; Table 3).

**Fluorescein Glucuronide Movement Across the Isolated Rabbit RPE-Choroid**

In accordance with the results of the fluorescein experiment, only the data obtained at least 30 min after the addition of fluorescein glucuronide were used for calculations. Using the method of Chen et al., it was confirmed that change of fluorescein glucuronide to fluorescein during the experiment was negligible. The outward permeability of the isolated rabbit RPE-choroid to fluorescein glucuronide at a concentration of 15 μmol/l was about double the inward permeability (P < 0.01, unpaired t-test). One hundred micromolar probenecid significantly decreased the outward permeability (P < 0.01) but had no effect on the inward permeability (Table 4). One micromolar ouabain, 10 μmol/l 2,4-dinitrophenol, and incubation at 0.5–1.0°C all significantly decreased the outward permeability (P < 0.05–0.01, multiple comparison test of Dunnett (Table 5).

**DISCUSSION**

**Preparation of Rabbit RPE-Choroid**

The transepithelial transport properties of the mammalian RPE have been studied in the RPE-choroid preparation in various species. However, the RPE-choroid of the rabbit, which is relatively cheaper to obtain and is more suitable for extensive studies, had not been used until Frambach and associates re-
TABLE 4. Outward and Inward Permeabilities of Isolated Rabbit RPE-Choroid to Fluorescein Glucuronide and Effect of Probenecid

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Outward Permeability (×10^{-5} cm/sec)</th>
<th>Inward Permeability (×10^{-5} cm/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.63 ± 0.06</td>
<td>0.34 ± 0.03*</td>
</tr>
<tr>
<td>Probenecid (100 µmol/l)</td>
<td>0.30 ± 0.07†</td>
<td>0.34 ± 0.04</td>
</tr>
</tbody>
</table>

Figures are mean ± SE in eight experiments.
* Significantly smaller than outward permeability (unpaired t-test, \( P < 0.01 \)).
† Significantly smaller than control (unpaired t-test, \( P < 0.01 \)).

...ported observations on the rabbit RPE-choroid-sclera preparations. Although their method of preparation was useful for studying the effects of pharmacologic agents on the RPE-generated transepithelial electrical current, an analysis of substance movements across the RPE, as attempted in the present study, would surely be complicated by the presence of the sclera.

The transepithelial potential difference presently recorded in the rabbit RPE-choroid preparation averaged 1.81 mV (retinal side positive) and was lower than those recorded in the RPE-choroid preparation from other mammals or in the RPE-choroid-sclera preparation from rabbits. This may be explained by species difference or the fact that the present experimental conditions did not perfectly replicate the in vivo state of the RPE. However, the transepithelial electric resistance was similar to that previously reported for RPE-choroid preparations from various species and remained unchanged during the experimental period. In a study by Araie and Maurice, fluorescein was injected into a rabbit vitreous, the eye was enucleated, and the concentration distribution of the fluorophore was measured in the frozen section of the eye. By analyzing the concentration gradient of dye perpendicular to the retinal surface, the average outward permeability of the retinal layer to fluorescein in the living rabbit eye was estimated to be 2.3 \( \times 10^{-5} \) cm/sec, which is comparable to the value presently obtained for the isolated RPE-choroid, 1.8 \( \times 10^{-5} \) cm/sec. These facts indicate that tissue damage was not serious and that the present preparation could be used for characterization and comparison of fluorescein and fluorescein glucuronide movements across the RPE-choroid.

**Movement of Fluorescein and Fluorescein Glucuronide Across the Isolated Rabbit RPE-Choroid**

The inward permeability of the isolated rabbit RPE-choroid obtained in the absence of inhibitors of the outward movement of fluorescein averaged 0.42 \( \times 10^{-5} \) cm/sec, similar to that obtained in the isolated RPE-choroid of the dog. None of the competitive inhibitors that significantly inhibited the outward movement of fluorescein had any affect on the inward permeability. Furthermore, the measured value of inward permeability was independent of concentration of 15–150 µmol/l. Based on these results, the inward movement of fluorescein across the isolated rabbit RPE-choroid was considered to be mainly a passive process.

As discussed later, the present and previous results suggest that the outward movement of fluorescein across the RPE-choroid is carrier-mediated. Thus, it is possible that the above value of the inward permeability is an underestimate, because the outward transport of fluorescein counteracts the inward movement of fluorescein and it was not inhibited. However, the inward permeability values obtained after the addition of competitive inhibitors showed no significant difference from the value obtained in their absence, and the underestimate is thought to be not substantial.

The outward permeability was about 4 times greater than the inward permeability at 15 µmol/l, and the ratio of the former to the latter gradually decreased as the concentration rose from 15 to 150 µmol/l. The nonpassive component of the outward fluorescein movement across the RPE-choroid was given by the net outward fluorescein movement, because the inward fluorescein movement was considered to represent the passive component. Figure 2 shows a saturation curve, which suggests a carrier-mediated transport component in the outward movement of fluorescein. To further characterize this mechanism, a Lineweaver-Burk plot of the reciprocals of the net outward fluorescein movement against the reciprocals of fluorescein concentration was generated (Fig. 3). An apparent \( K_m \) value of 26 µmol/l and an apparent \( V_{max} \) (maximum velocity) of 1.56 nmol/hr/cm² were obtained.

**TABLE 5. Effect of Metabolic Inhibitors and Low Temperature on Outward Permeability of Isolated Rabbit RPE-Choroid to Fluorescein Glucuronide**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Outward Permeability (×10^{-5} cm/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.67 ± 0.08</td>
</tr>
<tr>
<td>Ouabain (1 µmol/l)</td>
<td>0.39 ± 0.07*</td>
</tr>
<tr>
<td>2,4-dinitrophenol (10 µmol/l)</td>
<td>0.39 ± 0.05*</td>
</tr>
<tr>
<td>0.5–1.0°C</td>
<td>0.08 ± 0.04†</td>
</tr>
</tbody>
</table>

Figures are mean ± SE in six experiments.
* Significantly smaller than control (multiple comparison test of Dunnet, \( P < 0.05 \)).
† \( P < 0.01 \).
If we assume the passive component of the permeability of the RPE-choroid to fluorescein to be 0.42 × 10^{-5} cm/sec, the nonpassive component of outward movement of fluorescein was calculated to be inhibited by 1.0 μmol/l ouabain, 10 μmol/l 2,4-dinitrophenol, and low temperature by about 36%, 48%, and 100%, respectively, suggesting that the outward transporting process of fluorescein observed here was linked to the function of the sodium pump and metabolic energy production. Probenecid is a well-known competitive inhibitor of outward fluorescein transport in the ocular tissues.11,16-19,21,35 One hundred micromolar probenecid in the present study inhibited the outward movement of fluorescein across the RPE-choroid by 62%. It has been suggested that there are several overlapping transport systems for organic anions in the anterior uvea. one is called the hippurate system and another is described as the liver-like system.36-38 Fluorescein uptake was reported to show a greater affinity to the hippurate system than the liver-like system in the rabbit iris-ciliary body.17 In the present study, however, a relatively high concentration of hippurate and iodiampide showed a similar effect on the outward movement of fluorescein across the RPE-choroid. Furthermore, the outward movement of fluorescein across the rabbit RPE-choroid was effectively inhibited by 1.0 μmol/l ouabain, whereas the outward movement of fluorescein across the rabbit iris-ciliary body35 or the fluorescein uptake by the rabbit iris-ciliary body17 were reportedly insensitive to much higher concentrations of ouabain. It also was reported that the outward transport of fluorescein across the dog RPE-choroid was insensitive to 1.0 μmol/l ouabain.51 Although we do not have good explanation for this discrepancy, it might be that the fluorescein transport system is not identical between the iris-ciliary body and the RPE-choroid nor between rabbits and dogs.

The inward permeability of the isolated rabbit RPE-choroid to fluorescein glucuronide averaged 0.34 × 10^{-5} cm/sec. This value is smaller than that for fluorescein, although the difference is not significant, which probably is attributable to larger molecular size and lower lipophilicity of fluorescein glucuronide.16 The outward permeability to fluorescein glucuronide was much smaller than that to fluorescein (P < 0.01, unpaired t-test), but significantly larger than the inward permeability to fluorescein glucuronide, suggesting that the fluorescein glucuronide movement across the rabbit RPE-choroid is not entirely by a passive process. In accordance with this, a competitive inhibitor—100 μmol/l probenecid—and metabolic inhibitors—1.0 μmol/l ouabain or 10 μmol/l 2,4-dinitrophenol—significantly lowered the outward permeability to fluorescein glucuronide. These findings indicate that roughly half of the outward movement of fluorescein glucuronide is by a nonpassive process, confirming previous in vivo findings.11,15 This characteristic of fluorescein glucuronide movement across the rabbit RPE-choroid is somewhat different from that across the rabbit iris-ciliary body, where 100 μmol/l probenecid or 10 μmol/l 2,4-dinitrophenol were found to have no effect.35

**In Vivo Application**

In clinical vitreous fluorophotometry, fluorescence intensity 3 mm from the retina 1 hr after fluorescein injection often is used as a simple measure of the integrity of the BRB. Thus, it would be interesting to calculate the fluorescein and fluorescein glucuronide concentrations in the vitreous 3 mm from the retina using the outward and inward permeabilities to fluorescein and fluorescein glucuronide presently obtained and the concentration change of fluorescein and fluorescein glucuronide after intravenous injection of fluorescein.12 Based on the result of Nishimura et al,39 the diffusion of fluorescein glucuronide is estimated to be about 4.1 × 10^{-6} cm²/sec. A simulation using the above results and the mathematical model of Ogura et al4 showed that fluorescein concentration in the vitreous 3 mm from the retina 1 hr after the injection was about 180% of the fluorescein glucuronide concentration at the same site. If a Fluotron Master (Coherent, Palo Alto, CA) is used,12 94% of the fluorescence intensity measured would be attributable to fluorescein. This result suggests that when the measurement was taken 1 hr after the injection, the influence of fluorescein glucuronide in the vitreous may be neglected in clinical vitreous fluorophotometry. In the living eye, however, the outward permeability for fluorescein is expected to be higher than the present value, because of the presence of retinal vessels.16 Thus, the fraction
Fluorescein and Fluorescein Glucuronide

of fluorescence intensity attributable to fluorescein may be somewhat lower than the above estimate.

Key Words
fluorescein, fluorescein glucuronide, isolated retinal pigment epithelium-choroid, Lineweaver-Burk plot, rabbit

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References
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