Accuracy of aqueous humor flow determination by fluorophotometry. Jonathan E. Pederson, Douglas E. Gaasterland, and Helen M. MacLellan.

After corneal iontophoresis of fluorescein, freshly enucleated rhesus monkey eyes were perfused at a known rate with glutathione-bicarbonate-Ringer's solution. Total fluorescein mass and aqueous fluorescein concentration were measured with a fluorophotometer every 15 min for 6 to 8 hr. The aqueous flow was calculated from the fluorophotometry data by the second method of Jones and Maurice. This flow was then compared to the known perfusion rate. The curve for total fluorescence decay was not precisely parallel to the curve for aqueous fluorescence decay. This affected the value of the ratio of total:aqueous fluorescence. The most accurate and consistent flow values were obtained with the decay constant from the aqueous fluorescence decay curve combined with the ratio of total:aqueous fluorescence extrapolated back to zero time. Under these two conditions, the calculated flow in five experiments was 5.4% ± 3.3 (± S.D.) lower than the known flow. The 99% confidence interval of the true mean percent difference between the known and calculated flows is −12% to +1%. A small effect of corneal fluorescein concentration on measured aqueous fluorescence was observed.

The measurement of the rate of fluorescein loss from the anterior chamber after corneal iontophoresis is a safe and convenient method to estimate the aqueous flow rate in man. Different investigators have obtained similar aqueous flow rates of 2.5 to 2.8 μl/min by this method in man, as summarized by Bloom et al. However, the aqueous flow rate calculated from tonography is much lower, about 1.0 to 1.5 μl/min. This discrepancy could be due to errors in tonography or fluorophotometry, or it could be due to the existence of unconventional aqueous outflow, as suggested by Bárany. To date, no published reports have assessed the accuracy of the fluorophotometric method. In this report, the anterior chambers of enucleated monkey eyes were perfused at a known rate after fluorescein iontophoresis. The known perfusion rate was then compared to the flow rate calculated from the fluorophotometry data, as described by Jones and Maurice.

Materials and methods. The fluorophotometer is a modified version of the Maurice design and is incorporated into a Haag-Streit slit lamp (Model 900; Haag-Streit AG, Berne, Switzerland). The standard tungsten lamp, supplied by a regulated power supply, illuminated the eye. A circle or slit beam was imaged on the eye; the largest standard circle size was increased to 12 mm by boring out the largest hole of the rotatable aperture in the lamp housing. In addition, the distance from the projecting lens to the eye was increased, and the dioptric power of the projecting lens was reduced slightly. A B-4 bandpass (Baird Atomic, Inc., Bedford, Mass.) exciting filter was placed within the light housing. For aqueous concentration measurements, one eyepiece of the slit lamp was modified to contain a plano glass surface with a small, central silvered area. From this area, a small circular segment of light was reflected from the eye and fed to a photomultiplier tube (EMI 9789B) via a fiber optic bundle. This eyepiece was similar, but not identical, to that described by Waltman and Kaufman. The photomultiplier tube housing contained a Corning 3-69 interference filter. The illuminating slit was aimed at the eye from a 60-degree angle for aqueous measurements. For total fluorescence (mass) measurements, the other eyepiece was replaced by a
Fig. 1. Total fluorescein mass (open circles) and aqueous fluorescein concentration (closed circles) as a function of time after iontophoresis in a typical perfusion experiment. Fluorescence reading is in arbitrary units.

A photomultiplier tube (RCA 4516) whose window size exactly matched the size of the corneal image projected onto it. A Wratten No. 15 filter was used as a barrier for this tube. The illuminating light was chopped at 600 Hz. The photomultiplier tube outputs were fed to a synchronous detector and read on a digital voltmeter. The machine was calibrated with an artificial chamber of known volume, filled with various fluorescein concentrations.

Freshly enucleated eyes were obtained from five rhesus monkeys being sacrificed for unrelated testing purposes. An eye holder was fashioned from Styrofoam, and the eye was surrounded by moist cotton. The cornea was moistened every few minutes with glutathione-bicarbonate-Ringer's solution delivered from a nebulizer bottle. Three needles were inserted into the anterior chamber, which included a 23-gauge needle with stirrer, a 27-gauge needle attached to a calibrated syringe on a Harvard infusion pump (Model 901; Harvard Apparatus Co., Inc., Millis, Mass.), and a 23-gauge needle attached to an exit tubing to hold the intraocular pressure at 5 mm Hg during the perfusion (to keep the anterior chamber volume constant). Iontophoresis was performed on the upper third of the cornea. A 3 mm cylinder of polyacrylamide gel was saturated with 10% fluorescein solution, and a current of 200 μamp was passed through it for 15 sec during iontophoresis. A copper wire had been attached to the optic nerve to complete the circuit. Glutathione-bicarbonate-Ringer's solution was perfused through the anterior chamber at 4.0 or 6.5 μl/min. This solution was chosen because it is known to best preserve corneal endothelial integrity. Fluorescence readings were taken every 15 min for 6 to 8 hr. The aqueous flow rate was then calculated according to method 2 of Jones and Maurice, namely, \( f = \frac{ASF_t}{F_a} \) where \( f \) is the aqueous flow rate, \( A \) is exponential decay constant, \( S \) is standardization constant (determined from an artificial chamber), \( F_t \) is total fluorescence reading, and \( F_a \) is aqueous fluorescence reading.

An attempt was made to measure \( S \) in the monkey eyes, but the inability to precisely measure the anterior chamber volume precluded this determination.

In two additional eyes, only total fluorescence readings were taken after corneal iontophoresis. In these eyes, no needles had been inserted, and the eyes were assumed to have zero aqueous outflow.

In another set of experiments, the effect of corneal fluorescein concentration on measured aqueous concentration was evaluated. Eyes were iontophoresed and perfused as above, but with fluorescein added to the perfusate to a concentration of \( 10^{-7} \) gm/ml. No readings were taken for 2 hr. At that time, paired corneal and aqueous
Fig. 2. Total fluorescein mass as a function of time after iontophoresis in an eye with zero perfusion rate. Fluorescence reading is in arbitrary units.

Table I. Comparison of calculated flow with known flow, using decay constant from aqueous or total fluorescence curves

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Known perfusion rate (µl/min)</th>
<th>Calculated from aqueous fluorescence decay curve</th>
<th>Calculated from total fluorescence decay curve</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Flow (µl/min)</td>
<td>Error (%)</td>
<td>Flow (µl/min)</td>
</tr>
<tr>
<td>A</td>
<td>3.97</td>
<td>3.87</td>
<td>4.41</td>
</tr>
<tr>
<td>B</td>
<td>6.50</td>
<td>6.19</td>
<td>6.52</td>
</tr>
<tr>
<td>C</td>
<td>6.50</td>
<td>6.30</td>
<td>6.72</td>
</tr>
<tr>
<td>D</td>
<td>6.50</td>
<td>6.11</td>
<td>5.94</td>
</tr>
<tr>
<td>E</td>
<td>6.50</td>
<td>5.79</td>
<td>6.64</td>
</tr>
<tr>
<td>Mean</td>
<td>-5.4</td>
<td>3.3</td>
<td>1.7</td>
</tr>
</tbody>
</table>

*F_t/F_a from extrapolation to time zero.
†F_t/F_a from time = 4 hr.

fluorescence readings were taken at multiple points along the vertical midline of the cornea, from areas of less intense to more intense corneal fluorescence. Each aqueous reading was taken just posterior to its paired corneal reading. The readings were obtained in less than 10 min, and the aqueous concentration was assumed to be fairly constant during that short time. Following that, the anterior chamber aqueous was aspirated, and its concentration was determined in an artificial chamber. A plot of corneal fluorescence vs. measured aqueous fluorescence was made for each eye. A linear regression analysis was performed, and the predicted aqueous concentration at zero corneal fluorescence was compared to the true aqueous concentration determined from the anterior chamber aspiration.

Results. The calibration curve of the fluorophotometer from the artificial chamber revealed linearity of the total and aqueous fluorescence readings from $2.5 \times 10^{-9}$ to $5 \times 10^{-8}$ gm/ml. Readings were reproducible within 1% on concentrations greater than $10^{-8}$ gm/ml. The standardization constant, S, was constant for concentrations between $5 \times 10^{-9}$ and $2.5 \times 10^{-8}$ gm/ml. The usual aqueous concentration during the perfusion experiments was $10^{-7}$ to $10^{-6}$ gm/ml. Approximately 1 µg of fluorescein was iontophoresed into the cornea.

The plot of a typical perfusion experiment is shown in Fig. 1. An aqueous fluorescence reading of 100 corresponds to an aqueous concentration of $6.5 \times 10^{-7}$ gm/ml, a reading of 10 to $6.5 \times 10^{-8}$ gm/ml, etc. A total fluorescence reading of 50 corresponds to 0.94 µg of fluorescein. The total and aqueous fluorescence curves did not become
exactly parallel in any of the experiments. The slope of the curves from 2 hr to the end of the experiment gave the decay constant, \( A \). The mean of the decay constants for the four eyes perfused at 6.5 \( \mu l/min \) calculated from the aqueous fluorescence decay curves is \( 3.5 \pm 0.8 \times 10^{-3} \text{ min}^{-1} \), and from the total fluorescence decay curves is \( 3.2 \pm 0.1 \times 10^{-3} \text{ min}^{-1} \). Since the ratio of \( F_t/F_a \) changes with time if the curves are not parallel, the calculated flow would depend on the time chosen to measure \( F_t/F_a \). It was discovered empirically that the most consistent results were obtained by using \( A \) from the slope of the aqueous fluorescence decay curve, combined with the ratio of \( F_t/F_a \) at zero time. This ratio of \( F_t/F_a \) was obtained by extrapolating all data points after 2 hr back to zero time by mathematical regression. The calculated flows by this method are consistently lower than the known perfusion rate by 5.4% ± 3.3 (±S.D.). From the \( t \) distribution, the 99% confidence limits of the mean error are —12% to +1%. A second method combines \( A \) from the slope of the total fluorescence decay curve with the ratio of \( F_t/F_a \) midpoint in the experiment, i.e. at 4 hr. The calculated flows from this method are higher than the true value by 1.7% ± 7.1 (±S.D.). The 99% confidence limits of the mean error are +14% to —11%. These calculated flows from the total or aqueous fluorescence curves are compared to the known perfusion rate in Table I.

A tolerance interval for future individual flow measurements can be determined, assuming a Gaussian distribution. With 95% confidence, 95% of future individual calculated flow determinations will be within —22% to +11% of the true (unknown) flow. The infusion pump variation was ±1% as measured by fluid collection into tared vials. The corneas did not swell noticeably during the perfusion period, although their thicknesses were not measured.

The two eyes with zero outflow had nearly identical results. A plot of one of these experiments is shown in Fig. 2. Theoretically, the plot should be a horizontally straight line from time zero. Instead, the total fluorescence increased until leveling off at 1½ hr, after which it declined slowly.

The effect of corneal concentration on measured aqueous concentration is shown in Fig. 3, a typical experiment. The predicted aqueous concentration, from extrapolating the line to zero corneal fluorescence, is very close to the actual concentration measured by anterior chamber aspiration. In

Fig. 3. Effect of corneal fluorescence on measured aqueous fluorescence. Data points (closed circles) are from paired corneal and aqueous fluorescence readings in a typical experiment 2 hr after iontophoresis. The open circle is the concentration of fluorescein in the aqueous obtained by aspiration. Fluorescence readings are in arbitrary units.

five eyes, the predicted aqueous concentration was 10.5% ± 4.0 (±S.E.) higher than the actual concentration obtained by aspiration. The mean of the slopes of the lines from the five eyes is 0.033 ± 0.001 (±S.E.), leading to a correction equation: true aqueous fluorescence = measured aqueous fluorescence - 0.033 corneal fluorescence. This correction equation was not used in the perfusion experiments because the effect of corneal concentration on measured aqueous concentration was not discovered until after four of the perfusion experiments were completed. If the correction equation had been applied to the fifth experiment, the calculated flow rate would have been 6.8 \( \mu l/min \), rather than 5.8 \( \mu l/min \). That particular eye had an unusually large corneal concentration, which led to the discovery of this phenomenon. The magnitude of the correction would be much less in most eyes.

Discussion. The agreement between the known and calculated aqueous flow rates indicates that the mathematical modeling in method 2 of Jones...
and Maurice is valid. As seen from Table I, the calculated flows using the slope, A, from the aqueous fluorescence curves combined with the ratio $F_t/F_a$ extrapolated to zero time have a smaller standard deviation than those calculated with A from the total fluorescence curve combined with the ratio $F_t/F_a$ midpoint in the experiment. However, combining A from the aqueous fluorescence curve with $F_t/F_a$ midpoint in the experiment would have caused an overestimate of flow of about 20%. In contrast, combining A from the total fluorescence decay curve with $F_t/F_a$ at zero time would have underestimated the flow by 8% but with a standard deviation of 30%. These findings are probably due to the fact that the decay curves are not parallel.

The initial rise in the total fluorescence curves has been attributed to iris reflectance from blue irides, but it is better explained by Beer's law and quenching effects. Initially, the fluorescein concentration at the iontophoresis site is very high, about $10^{-3}$ gm/ml. This concentration greatly exceeds that at which extinction begins to occur, as seen from the calibration curves. The light striking the deeper stromal area of the iontophoresis depot is highly attenuated, because of light absorption by fluorescein in the superficial area. Fluorescence will thus be nonlinear through the corneal depot, with greater fluorescence superficially. In addition, photons directed toward the instrument, from fluorescence of the deeper area of the corneal depot, will be quenched more than photons from the superficial area. However, as fluorescein diffuses away laterally from the site of iontophoresis, the fluorescein in the deeper layers of the cornea will be illuminated more and quenched less. As this occurs, the measured total fluorescence will increase. The initial rise in total fluorescence seen in Fig. 1 was noted in all experiments, including those with zero outflow. This phenomenon would be lessened if smaller amounts of fluorescein were iontophoresed into the cornea or if a very bright light were used. The slight decrease in total fluorescence after 1 hr in Fig. 2 can be attributed to fluorescein loss at the limbus or into the iris.

The presence of excessive fluorescein in the cornea was found to erroneously increase the measured aqueous fluorescence ($F_a$), which if uncorrected, would cause an underestimate of the aqueous flow rate. The accuracy of the flow measurements, without correction for this error, suggests that the error is of minor importance in most eyes. Iontophoresis of only a small quantity of fluorescein into the eye would also minimize the error. The correction factor would be important with longer iontophoresis times. This phenomenon is probably due to internal reflection of light within the cornea, illuminating that portion of the cornea through which the aqueous reading is taken. This error, as well as the nonparallel behavior of the total and aqueous fluorescence curves, has also been noted by Brubaker (personal communication).

In this experiment, the anterior chambers were stirred, since enucleated eyes would not be expected to have appreciable convection currents. Poor mixing of the anterior chamber is a potential source of error in human studies. However, poor mixing would probably only affect early aqueous fluorescence readings.

This work has been stimulated by the discrepancy between the aqueous flow rate measured by tonography vs. fluorophotometry. The results strongly suggest that errors in fluorophotometry cannot explain this discrepancy, especially if the data are analyzed as outlined. Errors identified in this work would cause an underestimate of aqueous flow measured by fluorophotometry, if anything. Tonography and episcleral venous pressure measurements would need to have very large errors to account for this difference. A final possibility is the presence of unconventional aqueous outflow, which would be only minimally detected by tonography but would be included in the flow measured by fluorophotometry.

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Key words: aqueous humor, fluorophotometry, fluorescein, anterior chamber, monkey eye, aqueous humor flow measurement, iontophoresis, aqueous humor dynamics

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