A New Method for Determining Corneal Epithelial Barrier to Fluorescein in Humans

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Purpose. To derive the value of corneal epithelial barrier to fluorescein in humans from experiments in which the fluorophore is instilled in a single drop.

Method. A commercial scanning fluorophotometer, the Fluorotron Master, was used to scan through the anterior segment. It could not resolve the tear film from the cornea, but in the early stages of measurement, the tear component predominated. After 20 minutes, the remaining fluorescein was washed out of the conjunctival sac, and the amount that penetrated into the cornea was estimated. Because the resolution was not sufficient to estimate the concentrations, the total masses in the tear film and in the cornea derived from the area under the profile were used to calculate the epithelial permeability. A value for the tear film thickness had to be assumed.

Results. The technique led to reproducible values for epithelial permeability—a range of 2:1 in a subject. High concentrations of fluorescein were required to achieve a sufficient penetration into the cornea, and this led to error in estimating the tear film concentration of the dye. A method is presented that corrects for this effect. For 17 normal subjects, the corrected permeability was 0.15 nm/second, and the tear turnover was 0.15/minute (±0.016, standard error).

Conclusions. This technique is convenient and yields two useful physiological parameters from a single clinical procedure. The permeability values are considerably higher than those found by previous workers, and the source of the discrepancy is discussed. Invest Ophthalmol Vis Sci. 1996;37:1008-1016.

In humans, measurement of the barrier opposed to the penetration of fluorescein (F) across the corneal epithelium could be of value in diagnosing or monitoring disease or in assessing trauma caused by toxic drugs and preservatives or by contact lenses. The penetration of F into the cornea from an instilled drop has been determined,1-3 making use of a commercially available instrument, the Fluorotron Master (OcuMetrics, Mountain View, CA). The instrument does not have sufficient spatial resolution to distinguish the fluorescence of the tear film from that of the cornea or to give a reading proportional to the absolute concentration level in either layer. Although these measurements may be of value in comparing the condition of a cornea under different circumstances, they are difficult to transfer to the absolute epithelial permeability values necessary to compare one person to another. This is because the dye penetration is not a function of permeability alone but also of the concentration in the precorneal tear film, and it varies with time. A method of deriving an absolute measure of the permeability coefficient has been developed by de Kruijf et al,4 in which the eye is dipped into a solution of F, thus fixing the concentration in contact with the cornea. To determine the amount that penetrated into the cornea, a correction factor had to be determined to allow for the poor resolution of the instrument. The cornea was assumed to have a standard thickness for each subject. This method is less convenient for a subject than the simple instillation of a drop, and it results in extensive staining of the skin around the eye.

We have developed an alternative to the method of de Kruijf et al4 in which the amounts, rather than the concentrations, of F in the tear film and the cor-
nea are estimated. This is achieved by taking the areas under the concentration-distance scans that the fluorophotometer provides rather than their maximum values. This method avoids the problems associated with the poor spatial resolution of the Fluorotron Master, but, as will be shown later, it requires a value for the tear film thickness to be assumed. For most of the experimental period, the fluorescent reading corresponds to the tear film only because the corneal fluorescence is relatively low. The F remaining in the tear film at the end of the period must be thoroughly washed away to measure how much has penetrated into the cornea. To determine the epithelial permeability, measurements of tear film concentration must be conducted only for 5 minutes; if they are continued for 20 minutes, the same experiment can provide an estimate of the tear turnover rate.

Because of the low permeability of the human corneal epithelium to F, a high concentration of the dye must be established in the tear film to achieve a measurable rise in fluorescence in the cornea. Such a high level can lead to a false estimate of the tear film concentration as a result of fluorescence quenching. A compromise between these effects is necessary, which sets narrow limits on the initial tear film concentration of F that is created.

**MATHEMATICAL DESCRIPTION**

The Fluorotron Master is fitted with its anterior segment adapter that increases the resolution of the instrument, and Figure 1 shows a typical scan under these conditions. The instrument will record any fluorophore present in its focal diamond—the volume at which the light paths of the excitation and detection systems intersect. The optical system of the instrument shifts the focal diamond so that it scans through the eye from behind the lens into the space outside the cornea. The resolution is insufficient to distinguish

![Crystalline Lens](image1)

**FIGURE 1.** Typical fluorescence scan.

the tear film from the cornea, and their combined fluorescence gives rise to single peak.

**Determination of Fluorescein Mass**

The basis for the mathematical treatment that allows the amount of F to be derived from a Fluorotron Master scan can be explained by the help of Figure 2, which shows a sheet of tissue of infinitesimal thickness, dq, perpendicular to the axis of the fluorometer, and an element of area Q, corresponding to the largest cross-sectional area of the focal diamond. The volume of the element is Qdq, and the mass of fluorescein in it, dm_q, is equal to \( Q_c dq \), where \( Q_c \) is the corresponding F concentration. When the focal diamond is at a position, x, close to the sheet, the fluorescence recorded, \( f_x \), will be proportional to \( dm_q \) and to a spread function, \( S_x \), associated with the optics of the instrument, so that:

\[
f_x = S_x dm_q
\]

(1)

As the focal diamond scans through the element, it will record a profile of the fluorescent reading corresponding in shape to the spread function, and the area under this profile will be \( da_q \), given by:

\[
da_q = \int f_x dx
\]

(2)

Substituting for \( f_x \), we get:

\[
da_q = \int S_x dm_q dx
\]

(3)
or

\[ da_q = d m_q \int S_q dx \]  

where \( \int S_q dx \) is a constant denoted by \( S \). Thus:

\[ da_q = S dm_q \]  

As the instrument scans through a finite thickness, \( q \), of the tissue, the area, \( a \), under the corresponding fluorescent profile is given by:

\[ a = \int da_q \]  

or:

\[ a = S \int dm_q \]  

Because \( \int dm_q \) is the total mass of \( F \), \( m \), in this thickness of tissue, we have:

\[ a = Sm \]  

Thus, the area under the scan is proportional to the mass of fluorescein in the cross-section of the focal diamond, regardless of its distribution.

**Epithelial Permeability.** The transfer of \( F \) across the epithelium is assumed to follow simple, first-order kinetics, so that:

\[ \frac{d m_e}{d t} = P_e Q_e (C_{e_i} - C_e) \]  

where \( m_e \) is the mass transferred into the cornea from the tears, \( P_e \) is the epithelial permeability, \( Q_e \) as before, is the area over which transfer is considered, and \( C_{e_i} \) and \( C_e \) are the concentrations, or more correctly the activities, of \( F \) in the tears and cornea. Because \( C_{e_i} \gg C_e \), this may be written as:

\[ \frac{d m_e}{d t} = P_e Q_e C_{e_i} \]  

The loss of \( F \) is slow, and from the value of its transfer coefficient across the endothelium, 0.0034/minute, it would amount in 20 minutes to only 7% of that in the stroma if the dye were evenly distributed across it. Under the experimental conditions, several minutes are needed for an important concentration to be built up at the endothelial surface so that the loss will be smaller and can be ignored.

Integration of equation 10 leads to:

\[ P_e = \frac{m_e}{Q \int C_e d t} \]  

in which \( m_e \) represents the total mass of \( F \) in the stroma. Since

\[ m_e = m_t - m_c \]  

where \( m_t \) is the total mass of \( F \) in the tear film, \( m_c \) is the mass in the cornea, and \( m_t \) is the mass in the cornea.

Thus, the permeability can be determined from measurements of the areas under the tear–cornea peak and a knowledge of the tear film thickness. This replaces the assumption of a standard corneal thickness in de Kruijf et al. by one of a standard tear film thickness that must be the same for all subjects.

If \( a_c \) is the preinstillation inherent corneal fluorescence and \( a_t^i \) is the final value when no \( F \) is left in the tears, then \( a_t \), the penetration of \( F \) into the cornea in equation 14, is experimentally determined by:

\[ a_t = a_t^i - a_c^0 \]  

The Fluorotron Master signal comprises contributions from the tear film and the cornea, and the \( F \) has to be thoroughly washed out of the tear film before the final corneal scan can be obtained.

Similarly, as the tear film level diminishes, it may become comparable to the signal from the cornea at the end of 20-minute measurement period. To obtain the true value of the tear film profile, \( a_t \), the final \( a_t^i \) may be subtracted from the Fluorotron Master signal at any time, \( a_t \), so that:

\[ a_t = a_t^i - a_t^f \]  

Because the value of \( a_c \) does not change appreciably...
Determination of Corneal Epithelial Permeability and Tear Flow

ably with time after the first few minutes (e.g., it levels off at a value of 0.4 to 0.5 U after 10 minutes in Fig. 4), a constant value of \( a_d^\prime \) is appropriate at longer times.\(^6\) In the early period, the reading from the tear film is much greater (e.g., 10 U or more from Fig. 3) than that from the cornea, so the value of the latter is irrelevant.

**Tear Turnover Rate.** The method is limited to measuring the tear turnover rate because, to establish the tear flow, it is necessary to estimate the standing volume of tears in the conjunctival sac.\(^7\) The tear film readings in the first few minutes after the instillation of a drop are unreliable because tear flow can be stimulated or the mixing of F into the tear film is incomplete\(^6\) and because the high initial concentration of the dye can lead to error. After this initial period, the dye concentration generally follows first-order kinetics:

\[
C_d = C_d^0 e^{-kd}
\]

With the assumption that the tear film thickness is constant, the tear turnover rate, \( k_d \), was determined by using the linear regression of \( \ln a_d \) against time, for time points between 5 minutes and the end of the experiment.

\[
a_d = a_d^0 e^{-kd}
\]

**MATERIALS AND METHODS**

**Fluorophotometer**

The measurements were made with the Fluorotron Master using the standard excitation and emission filters and scanning software. The anterior chamber adapter doubles the standard angle between the excitation and emission pathways to 28°, resulting in a focal diamond that is 50 \( \mu m \) wide by 1.9 mm high and slightly more than 0.50 mm deep.\(^8\) The automatic scans were performed with a 0.125-mm stepping motor.

**Method**

The tenets of the Declaration of Helsinki were followed; informed consent was obtained from the subjects after biomicroscopy and visual acuity testing determined that their eyes were normal; and Allergan’s human experimentation committee approved this research. Normative tear turnover and epithelial permeability values were determined in 17 healthy, non-contact lens-wearing subjects (men and women between 20 and 45 years of age). Two baseline scans were made to familiarize the subject with the procedure and to determine the background fluorescence. A sterile 2 \( \mu l \) drop of 0.25% to 1.5% F dissolved in 50 mM phosphate-buffered saline, pH 7.4, was instilled on the temporal bulbar conjunctiva, and the subjects were asked to blink vigorously and roll their eyes to distribute the dye. The fluorescence of the tear film was followed for 20 minutes, when it became comparable to that of the cornea. Scans were repeated as rapidly as possible for the initial 8 minutes, then every 2 minutes until 20 minutes. The F remaining in the conjunctival fluid was then flushed out with repeated irrigations of preservative-free sterile saline (Allergan [Irvine, CA] unit-dose Lens Plus Saline) for at least 1 minute. Two further corneal scans were made after this wash to estimate the fluorescein penetration. On some occasions, the scans were repeated after further washing to check that the process was complete.

It can be noted that the tear film measurements were made at the same point on the cornea as the measurement of corneal penetration. The permeability value, therefore, refers to this single, apical, position.

**Data Analysis**

The hard copy output of the fluorometer was used to identify the position of the peak of corneal fluorescence. An equal distance of 16-step increments, slightly greater than half the apparent corneal thickness, was set off in both directions from the peak, and the 33 fluorescent values were summed to estimate the area under the profile. The area, \( a_o \), represents the fluorescence of the tears combined with that of the cornea, which may be either \( a_o^0 \), the corneal baseline fluorescence, or \( a_o^\prime \), the post-irrigation corneal fluorescence, according to circumstances. The time integral of the tear mass, \( \int a_o dt \), was calculated by using the integration routine (trapezoidal rule) in a standard curve-fitting software.\(^9\) The permeability in units of nm/second was calculated using equation 14, in which the tear film thickness, \( q_o \), was assumed to be 8 \( \mu m \).\(^10\) The tear turnover rate was calculated using the method described earlier.

**RESULTS**

**Drop Concentration**

Drops varying in F concentration were instilled in one normal subject on different days. Figure 3 illustrates typical time courses of the tear film fluorescence after the instillation of 0.5% and 1.5% F concentrations. Lower concentrations showed a monotonic decay of \( a_o \). On the other hand, the higher concentrations demonstrated an early increase in fluorescence before the fall. The most plausible explanation of this behavior is that quenching depresses the fluorescence of these concentrated solutions, as suggested by Webber and Jones.\(^11\) The fluorescence intensity increases as fresh tears dilute the quenched layer. This phenomenon will be analyzed further in the Discussion.

The \( P_o \) values were calculated for the different F
TABLE 1. Epithelial Permeability of Subject 1 for Various Instilled Fluorescein Concentrations*

<table>
<thead>
<tr>
<th>Concentration (%)</th>
<th>Pdc (measured) (nm/s)</th>
<th>Pdc (corrected) (nm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.250</td>
<td>0.142</td>
<td>0.138</td>
</tr>
<tr>
<td>0.500</td>
<td>0.168</td>
<td>0.136</td>
</tr>
<tr>
<td>0.625</td>
<td>0.165</td>
<td>0.113</td>
</tr>
<tr>
<td>0.750</td>
<td>0.195</td>
<td>0.123</td>
</tr>
<tr>
<td>0.880</td>
<td>0.261</td>
<td>0.158</td>
</tr>
<tr>
<td>1.500</td>
<td>0.362</td>
<td>0.141</td>
</tr>
</tbody>
</table>

*Also tabulated are the permeability values after correction for self-absorption and quenching in the tear film.

concentrations by the standard routine and are shown in Table 1. It can be noted that the lower fluorescein concentrations provided an approximately constant value of \( P_{dc} \), whereas the higher concentrations led to markedly greater values. These data suggested that the maximum concentration that gives reliable results is in the neighborhood of 0.75% F.

**Repeatability**

The repeatability of the technique was determined by conducting experiments with 0.75% F in two subjects on five different days. The measurements were taken at the same time of day (between 3 and 5 PM) to avoid potential diurnal variations.

Results of these tests are shown in Table 2. There is a wide variability in the tear turnover rates, especially for subject 2, in whom there was a 4:1 range between the extremes. The epithelial permeabilities show less variability—within a 2:1 range for both subjects. There is no correlation between tear turnover and permeability, which suggests that no systematic error is introduced by the method of data reduction.

**Penetration Kinetics**

A test of the linearity of the F penetration, as expressed by equation 10, was attempted. A 2 \( \mu \)l drop of 0.75% F was instilled into the same eye of subject 1 on seven different days. The dye was flushed out with sterile, nonpreserved saline drops after 1, 2, 4, 6, 8, 10, or 20 minutes, respectively. The experimental results (Fig. 4) seem to be explicable in terms of the tear film kinetics. Equations 14 and 18 give:

\[
a_c = a_f(1 - e^{-kt})
\]

where \( a_f \) is the average of the experimentally determined values at 20 minutes. This curve is plotted on Figure 4 for \( k_t = 0.2/minute \) and gives a reasonable fit to the points.

Saturation kinetics, in which \( P_{dc} \) varies with time because of partitioning of F into the epithelium, might produce a similar curve, but it could be difficult to distinguish unless a very large number of experiments were performed.

**Normative Values**

The experimental values are summarized in Table 3. The mean apparent epithelial permeability, \( P_{dc} \), was 0.24 nm/second; the range of values, 13:1, is comparable with that of de Kruijf et al.,4 11:1, as is the standard deviation 56% compared to 45%. Previous studies of epithelial permeability suggested that the logarithm of the values should be symmetrical, and this was
found to be the case with the present data (Fig. 5). This further encourages the belief that there was no large methodological error, such as might have resulted from the use of high F concentrations.

The mean value of the tear turnover rate, \(k_t\), was 0.15/minute. This is in accord with the median value of 0.16/minute (range, 0.10 to 0.17/minute) found in seven previous investigations.\(^6\) As with the individual case, there was no correlation between permeability and tear turnover between subjects (Fig. 6).

**DISCUSSION AND CONCLUSION**

The single-drop method, described here, has the advantage of convenience. The subject’s face is not extensively stained with F, as it is in the de Kruijf\(^4\) technique. With a single procedure that is acceptable in the clinic, values of both epithelial permeability and tear turnover may be obtained in 30 minutes. If only the permeability value is needed, the time can be considerably reduced by washing out the dye after a few minutes, and there is little reduction in the corneal penetration (Fig. 4).

This method suffers from the disadvantage that a standard thickness for the tear film must be assumed for each subject. Variation in thickness between individuals has not been established, but it is unlikely to be as large as other sources of variability. This assumption should be less important in a comparison of the values from a single subject under different circumstances or from similar populations. Permeability values in an individual subject show good consistency (Table 2).

A practical constraint is that the permeability of the epithelium in a normal person can be so low that, to obtain a measurable increase in corneal fluorescence over the natural background, the fluorescein concentration in the tears must be increased to a level at which its value may be underestimated by the fluorometer reading.

**Fluorescence Self-Absorption—Quenching**

When fluorometric measurements are made on high concentrations of F, the readings can underestimate the concentration because of two mechanisms:

1. Self-absorption. This results when the excitation light is absorbed in the outer layers of the solution so that the illumination of the deeper layers is reduced.
2. Quenching. This occurs when the excitation light is absorbed by a fluorophore but is not re-emitted as fluorescent light and is converted to thermal energy instead.

The two mechanisms were not distinguished clearly by Webber and Jones,\(^1\) but they provided valuable experimental data on which to estimate their combined effect. Their experimental data were summarized in a figure, the relevant portion of which is reproduced in Figure 7. If only self-absorption occurs in the tear film as its concentration, \(C_0\), becomes greater, then the fluorescent emission, \(F_0\), should rise asymptotically to a level, \(F_\infty\), corresponding to the total absorption of the incident light in the film. This leads to a theoretical relationship:

\[
\frac{F_d}{F_i} = 1 - e^{-k_d x d}
\]

that, with an extinction coefficient,\(^1\) \(E_0\), of \(7.9 \times 10^4\ \text{M}^{-1}\text{cm}^{-1}\) and a tear film thickness, \(x_0\), of 8 \(\mu\text{m}\), is shown as the broken line in Figure 7. However, the actual fluorescent emission drops below this curve at higher concentrations, indicating that quenching is occurring in addition to self-absorption.

Regardless of the mechanism involved, the lack of

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**Figure 5.** Frequency plot of log epithelial permeability values from 17 subjects.

**Figure 6.** Relationship between epithelial permeability and tear turnover in 17 subjects.
Figure 7. Relationship between tear film concentration and apparent fluorescence. Thick, full line is taken from calibration curve of Webber and Jones. Values on the y-axis are the fluorometer output voltages from their figure. The thin, straight line represents a theoretical linear relationship between fluorescence and concentration. The dashed line is the relationship for the absorption of light by fluorescein calculated from equation 19. The points on the straight line correspond to the fluorescein levels to be expected in the tears if 2 μl drops of different concentrations were instilled and the dye was diluted three times in the tear fluid. Thus, a 1.5% drop should give rise to an initial fluorescence level corresponding to point A, but, because of absorption and—more seriously—quenching, its value is depressed to A'.

Similarly, if a 0.5% drop is instilled, a fluorescence of B should be achieved, but it will be read as B'.

Linearity of the relationship between concentration and emission will result in an underestimate of the area, Oₐ, under the curve relating tear concentration to time. Idealized curves are plotted in Figure 8, on the assumption that fluorescein is lost monoexponentially from the tear film with a kₐ of 0.2/minute, the average value for subject 2 in our experiments. The thin lines in Figure 8, corresponding to the similarly identified line in Figure 7, represent a linear relationship between emission and concentration. The thick lines in these figures, corresponding to the experimental curve of Webber and Jones, show the effect of quenching on the emission. The drop concentrations are positioned in Figure 7 so that the theoretical curves of Figure 8 have peak values at the same time as the experimental curve in Figure 3. The tear film concentration on the x-axis of Figure 7 then was calculated on the basis that the 2 μl drop initially would be diluted three times in the conjunctival sac. This results in values that are approximately half those of the experimental solutions used by Webber and Jones, but it is uncertain whether the film thickness they used in their calibration was the same as that of the precorneal tear film. Areas under the curves, as shown in Figure 8, provide an estimate of the error resulting from the depression of the fluorescence as a result of the high dye concentrations in the tear film. They range from 60%, for a drop concentration of 1.5%, to 3%, for a concentration of 0.25%; clearly, this method of compensating for the quenching rapidly becomes less reliable the further the tear concentrations ride up and over the peak of the calibration curve. However, when the quenching was corrected in this manner, as shown in the third column of Table 1, a value of epithelial permeability that was independent of concentration was arrived at; this lends support to the procedure. On the other hand, until the calibration of the tear film error is carried out with the Fluorotron Master instrument, the exact value of the correction must remain uncertain, although it is unlikely to be very different from that calculated above. Developing a method that avoid quenching is a preferable alternative.

Pₑ Values

The concentration of the drops used in these experiments (0.75%) would require a downward correction

Figure 8. Time course of apparent tear film fluorescence (thick line) resulting from effect of absorption and quenching on actual tear concentration (thin line) according to calibration in Figure 7. Idealized first-order tear film kinetics assumed with kₑ = 0.2/minute. A and B correspond to instilled fluorescein concentrations of 0.5% and 1.5%, respectively.
TABLE 4. P_{dc} Values in Subject 1 Obtained by the Bath Method\textsuperscript{a} for a Variety of F Concentrations and Exposure Times

<table>
<thead>
<tr>
<th>Concentration (%)</th>
<th>1.5</th>
<th>3.0</th>
<th>6.0</th>
<th>10.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>0.030</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.50</td>
<td>0.240</td>
<td>0.050</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>0.048</td>
<td>0.039</td>
<td>0.029</td>
<td>0.039</td>
</tr>
<tr>
<td>2.0</td>
<td>0.036</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each value, expressed in nm/s, represents one experiment.

of 37% to the mean apparent epithelial permeability of 0.24 nm/second found in our experiments for a normal population; this leads to a true value of 0.15 nm/second. This value is approximately four times greater than that (0.034 nm/second) of the bath method, when the F was dissolved in a balanced salt solution or in saline.\textsuperscript{4} This discrepancy was confirmed in subject 1 by carrying out measurements with the bath using an isotonic borate buffer, pH 7.4. Further, the validity of the de Kruijf method\textsuperscript{4} was tested by changing the bath concentration and its time of application over a considerable range. Apart from one aberrant result, the uniformity of the resultant matrix of the P_{dc} values, displayed in Table 4, support the underlying assumption that the penetration of F is related linearly to time and concentration. The discrepancy between de Kruijf’s and our method is too large to be accounted for by small errors in the assumptions, and it is necessary to search for some major methodological error in one or the other of them.

In the drop method, a false-high value of P_{dc} might arise because of a false estimate of one of the three terms in equation 14. Thus:

1. The amount of F in the cornea, a_{t}, is overestimated because not all of it in the tears is flushed away. This does not seem probable because the value did not change when the eye was washed between duplicate measurements at the end of the experiment.
2. The integral, \int a_{d} \, dt, is underestimated. This could be a result of self-absorption or quenching by the high concentrations of F initially in the tear film, as just discussed. This is undoubtedly taking place, to some extent, but the constancy of the value of P_{dc} at various low-drop concentrations (Table 1) suggests that it is not a serious error for the 0.75% drops that we were using. A considerable rise in P_{dc} for the more dilute solutions would be expected if the error was 600%, as is required to match the values to those of de Kruijf et al.\textsuperscript{4}

Depression of the fluorescence of the F as a result of binding to proteins in the tear film is another possible cause of an underestimate of a_{t}. However, this is unlikely to be a major source of error because blood serum reduces the fluorescence of F to approximately 50%,\textsuperscript{15,16} and the tears contain less than 10% of the protein content of the serum. On the other hand, binding of F to the tear film components would cause an overestimate of a_{t} and accentuate the discrepancy.

3. The thickness of the tear film, q_{t}, could be overestimated. However, if anything, the error is likely to be in the opposite direction,\textsuperscript{17} and a true thickness of 2 \mu m is most improbable.

4. The epithelial permeability may be raised by the phosphate in the drop. de Kruijf et al\textsuperscript{1} found that the permeability increased six times when the bath fluid was buffered with phosphate. A drop would not have such a prolonged effect, but it could shift the pH of the tears for the first few minutes,\textsuperscript{18} when the greater part of the F penetration is occurring. Phosphate might bind calcium ions and open tight junctions.

In the bath method, a false low estimate of P_{dc} might arise because:

1. The peak value of F in the cornea is underestimated as a result of the limited resolution of the Fluorotron Master. An empirical correction for this effect of approximately 1.56 was devised by de Kruijf et al.\textsuperscript{1} Though it may not be correct for every cornea, it is unlikely that it could be in error four times.

2. The value of the bath concentration, 1%, that was adopted by de Kruijf et al\textsuperscript{1} is an overestimate because the chemical activity of the F in high concentration is lowered. This could be caused by the following:

 Interaction between F molecules. This is suggested by the quenching of the fluorescence at concentrations greater than 0.1%. However, our experiments (Table 4) in which the calculated permeability remained constant over a concentration range of 0.25% to 2.0%, makes this unlikely.

 Interaction with hydrogen ions. F is a weak acid with a pKa of 6, and, whereas most of its molecules will be ionized at physiological pH, a small fraction will be associated with a proton in the acid form. However, this associated form is likely to be more lipid soluble than the ion and could cross the epithelial barrier more readily.

 Interaction between F and other ions in the bath solution. de Kruijf et al\textsuperscript{1} noted, without comment, that preparing the F bath solution with phosphate-buffered saline leads to a rise in P_{dc} to a value similar to our norm. It is
possible that the the F ions and additional positively charged monovalent and divalent ions of the phosphate-buffered saline compete for the available common solvent in the bath. This could result in reduction in the net activity of the dye and overestimation of the bath concentration in the bath method. A similar phenomenon, reduced solubility, is observed for sparingly soluble electrolytes when they are dissolved in a solution containing additional competing ions.19

3. Finally, there is a possibility that the tonicity of the bath solution could affect the epithelium or alter the apparent permeability of the layer by setting up a flow of water that could interact with the influx of F. Bathing of the rabbit’s cornea for 10 minutes with hypotonic solution caused an increase in the penetration of radiolabeled Na, whereas hypertonic solutions had no effect.13

In any event, it is surprising that the bath gives lower permeabilities than the drop. Prolonged contact with an artificial medium might be expected to disturb the cell layer and raise its permeability. The absence of specific ions, in particular K, has been noted to have a deleterious effect on the epithelium.20 However, there is no evidence that F itself is harmful, and the other components of the physiological medium used are unlikely to be toxic. This is a problem that requires further investigation.

Key Words
fluorescein, fluorophotometer, human corneal epithelium permeability, pharmacokinetics, tear turnover

References