Characterization of Paracellular Penetration Routes

To the Editor:

In a recent article in this journal, the authors reported measurements of the permeability of various ocular membranes to a series of polyethylene glycols (PEGs) of different molecular weights. They interpreted their data in terms of a pore theory that predicts that the measured permeability should be inversely proportional to the diameter of the probe molecule. Although these data were available for the PEGs, the relationship was not plotted, so that it is difficult to assess the model's validity. However, it must be questioned because it predicts that "the pores in the cornea allow paracellular permeation of only small molecules (molecular weight <500)," whereas Figure 2A of the article, unlike the results of Liaw and Robinson, shows no sign of a cutoff at that size but only a gentle decline in permeability from MWt 500 to MWt 940. Furthermore, in the development of the model it is not evident how a greater pore density, m, can be distinguished from a greater pore diameter, $a_h$, because only the product, $ma_h$, appears in the equation provided. It is hoped that this will be clarified in the future publication that is promised.

Apart from this, the article exemplifies a trend by investigators trained in the pharmaceutical sciences to be unaware of previous work published by physiologists and ophthalmologists in the field of pharmacokinetics. As detailed below, this can lead to (1) the acceptance of suboptimal experimental techniques, (2) the lack of awareness of established principles, leading to the misinterpretation of data, (3) a waste of effort in repeating previous similar experiments and reinterpreting them, and (4) the continued performance of studies on animals when the potential for more relevant human experiments has been shown to exist.

First, if any conclusions of clinical or pharmacokinetic significance are to be drawn from experiments on excised tissue, it seems wise to ensure that its in vivo characteristics are maintained as closely as possible. A traumatized preparation may have a limited value in comparing the properties of different drugs, but it could lead to uncertain conclusions when the results of different investigators are to be compared, because the extent of the insult created by each group may not be the same. It became apparent long ago that it was deleterious to the limiting cell layers of the cornea if, during its dissection, it was allowed to fold or its surfaces to make contact with a solid body. Several techniques for avoiding this were developed, and the one that has been most favored by the pharmaceutical community is that of O'Brien and Edelhauser, a modification of the system of Dikstein and Maurice. In its original form, the enucleated globe was secured by its conjunctiva and the cornea was stabilized in its normal configuration by suction during its excision, and normal intraocular pressure was maintained behind the tissue during its subsequent perfusion.

Time has eroded the advantageous features of this technique; the intraocular pressure is not maintained and the old method of isolating the cornea, leaving it free to fold, has been reintroduced. The latter change was introduced by Rojanasakul and Robinson, who claimed that it led to better preservation of the tissue than the suction method. However, there is evidence that both the epithelium and endothelium are harmed by these procedures.

The integrity of the barrier presented by the epithelium can most easily be assessed by its electrical properties. In vivo, the rabbit cornea develops a potential of approximately 25 mV, and this value has been reproduced in isolated preparations when the tissues are isolated freehand by corneal surgeons or when the cornea is stabilized during dissection. The corresponding epithelial resistance is about 6000 $\Omega \text{cm}^2$. Even greater values, approximately 9000 $\Omega \text{cm}^2$, can be achieved if the effect of injury caused by clamping the tissue around its edge is reduced by greasing the jaws of the clamp. The resistance determined by Rojanasakul and Robinson was less than one tenth of this, a peak of 700 falling to 400 $\Omega \text{cm}^2$ after 3 hours. The low values may be due in part to the absence of a normal intraocular pressure across the tissue, because other researchers have found that similarly low figures were obtained unless a pressure of 6 to 20 cm H$_2$O was maintained; in truth, not all physiologists working in the field are aware of these findings.

The preservation of the function of the corneal endothelium in these isolated corneas is also questionable. A water content of 76% to 80% (hydration, 3.2 to 4 g water/g dry wt) is considered normal, in accordance with the figures of Schoenwald and Huang, who cite Maurice and Riley in its support. However, these figures correspond to data published for all species, whereas the average hydrations found for the rabbit are limited to 2.95 to 3.50 g water/g wt. Examination of the original literature shows that in individual studies the normal range is quite tight, with a standard deviation of approximately 0.2 U. Thus a hydration of 4 could represent a swelling of 20% in the preparation, which suggests that the operation of the endothelial pump was compromised.
In this article the hydration was estimated from the full area of the cornea, including its rim, which was clamped in the apparatus, and the swelling must have been even greater in the circle of tissue exposed to the test solution, an area of 1.1 cm$^2$, only about one half of that of the complete cornea.

This discussion should be unnecessary because it is quicker to dissect and mount the cornea using the original technique (less than 10 minutes) than by the degraded one (25 minutes), and it is easier as well.

Second, it may seem that the condition of the endothelium is irrelevant to the measurement of a flux of a hydrophilic drug across the cornea, which is primarily controlled by the permeability of the epithelium. However, as I previously noted in a discussion of an article by Huang et al, the resistance of the endothelium can have an influence lasting an hour or two. This is because the stroma forms a reservoir for the drug that must be filled before the flux across the endothelium into the receiving chamber reaches its true steady-state value. On the other hand, if the endothelial permeability rises in the course of the experiment because of unphysiological treatment, there will be a temporary rise in the flux across it that will suggest a falsely high epithelial permeability.

A more extreme ignorance of these structural relations in the cornea has been shown in a recent publication by another group of pharmaceutical researchers who treat the tissue as a uniform monolayer of low diffusivity, a concept that was generally discarded many decades ago.

Third, I will not detail every failure in the present work to cite previous studies, because this has become a widespread phenomenon. However, I must note that the authors compared the results of their experiments on the permeability of the rabbit sclera with supposedly similar ones of Huang et al, who, however, made no such studies. On the other hand, they are apparently unaware that such experiments, with a much wider range of solutes, have previously been done in bovine and human sclera. Making allowance for differences in tissue thickness between the species, their permeability values are in accord with the earlier ones.

Again, in their investigation of the permeability of the rabbit conjunctiva to PEGs, the authors concluded that this is about 20 times greater than that of the cornea. They overlook that this is approximately in accordance with earlier findings for mannitol in vitro and for the Na ion in vivo. They claim further that they are the first to estimate the limiting size of solutes that can pass the conjunctival barrier, ignoring the findings by Huang et al that it is between 20,000 and 40,000 Da (which is improbably precise), and those of Kahn et al, who found it to be between 500 and 3500 Da.

Finally, I do not understand the purpose of doing such experiments on rabbits, in vitro or in vivo, when the information is already available for humans. Although there is some uncertainty in the value of the corneal permeability to fluorescein, the best estimate is 0.034 nm/sec, whereas the conjunctival permeability averages 4 nm/sec. Thus the human conjunctiva is 100 times more permeable to a hydrophilic solute than the cornea, rather than the 20 times reported in the rabbit.

The significance of this ratio is not evident, however. The authors’ conclusion that “bulbar conjunctiva and sclera may constitute a more viable route for ocular peptide and oligonucleotide delivery than would the cornea” invites the query “delivery to where?” In one case the drugs enter the anterior chamber, and in the other case they are exposed to the suprachoroidal circulation. Furthermore, the complicated issues presented by limbal penetration or uveoscleral flow are not addressed.

Another instance in which the use of rabbits’ eyes is questionable is in the study of the effect of “permeability enhancers” on drug penetration, which can readily be determined in the human eye using fluorescent tracers. In any case, most of these are too irritating to be of practical value, as can be established by cautious experimentation in animals and then humans.

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References

9. Ehlers N, Ehlers D. Effects of hydrostatic and colloid-osmotic pressure on electrical potential and short-cir-
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where. 2 In the model, we plot permeability versus the inverse of molecular radius, \( J_{H}/C = f(1/r_s) \), as was described in the article (p. 629). The model was valid and gave correlation coefficients of 0.96 to 0.99 for the rabbit cornea and conjunctiva.

Determination of the area of individual pores \( A_h \) and their number was clearly explained on p. 629. Porosity \( e \) is obtained from the slope of \( J_{H}/C = f(1/r_s) \) plot and the limiting pore radius from the intercept on the x-axis. Porosity \( e \) is \( A_h/A \), where \( A_h \) is the total area of hydrophilic pores and \( A \) is the total membrane area. Area of individual pores, \( A_h \), is obtained from the limiting pore radius, and then from the relationship \( A_h = ma_h \). It is easy to calculate the number of pores, \( m \). This should be evident from the original text.

Dr. Maurice is correct that on the basis of our data, the limiting molecular weight for paracellular corneal penetration is larger than 500. Based on the analysis, the limiting diameter of the solute is 2 nm (p. 631). For example, PEG\(_{942}\) has a diameter of 1.7 nm.

Dr. Maurice’s concern about damage to the corneal tissue is unfounded. In our procedure, the cornea does not fold. The epithelial and endothelial sides of the isolated cornea do not contact any solid body. Before its placement to the diffusion chambers, the tissue is covered with Ringer’s solution. The validity of our procedure is supported by the low solute permeabilities across the cornea. Permeabilities of PEGs (MWt 238 to 942) were 0.2 to 1 \( \times 10^{-6} \) cm/s using the suction method of O’Brien and Edelhauser. 3 Permeabilities of hydrophilic small molecular weight solutes were 0.7 to 2.4 \( \times 10^{-6} \) cm/s, 4,5 suggesting that there is no difference between the permeabilities obtained using these two tissue mounting procedures. We place the cornea to the diffusion chamber adapters so that the edge of the adapter is aligned as closely as possible with the limbus. Therefore, the hydration values are realistic for the whole cornea.

Corneal endothelium is not the rate-determining barrier for permeation of hydrophilic drugs. The most apical cell layers in the epithelium are the critical barriers, and it is improbable that endothelium would affect corneal permeability of PEGs. It is true that the steady-state flux across the endothelium is reached only after the drug concentration has achieved pseudo-equilibrium in the stroma. However, this equilibration is part of the time lag before steady-state flux across the whole cornea is achieved. Therefore, this equilibration does not affect the values of steady-state permeabilities. Furthermore, we did not see any accelerated flux peaks during the experiments, and, accordingly, the standard deviations of the permeability values are tight (see Fig. 2A).

The citation on permeability of rabbit sclera should have been to Ahmed et al (our reference 28) instead of to Huang et al (reference 9). We are sorry because of that mistake and for the fact that many papers in the field were not cited. However, the number of references (45) in the article was already high.

We stated on p. 632 that “no attempts to estimate the pore size or porosity of conjunctiva have been reported.” Huang et al 6 used different sized molecules.