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The effect of polyethylene glycol molecular weight on corneal transport and the related influence of penetration enhancers

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Summary

In order to understand the mechanism of molecular weight dependence on corneal transport of solutes, it is important to separate charge type and molecular size, two major parameters for peptide delivery The transport characteristics of a series of different molecular weight polyethylene glycols (PEGs), which are hydrophilic and uncharged, were measured in vitro. The absorption of PEGs exhibited a dependence on the molecular weight with a cutoff between PEG 400 and PEG 600. The effect of penetration enhancers, including a bile salt, digitonin, and cytochalasin B, on the molecular weight permeability profile can be used to identify mechanisms of penetration enhancement Bile salt did not alter the molecular weight permeability profile In contrast, digitonin greatly increased absorption of the PEGs and selective molecular weight effects were seen with cytochalasin B. Correlation of mucosal integrity with the effectiveness of the penentration enhancer indicates that cytochalasin B acts on the tight junction by opening intercellular spaces. These results indicate that the paracellular pathway may play an important role in hydrophilic, large molecule transport through the cornea

Introduction

There is a perception that the molecular size of peptides and proteins, which are one to three or more orders of magnitude larger than conventional drug molecules, is a major factor limiting their diffusion across biological membranes (Lee, 1990). The dependence of transmembrane permeability on molecular size has been studied using compounds of vastly different physicochemical properties (Stein, 1986). While a compound's molecular weight is often predictive of its degree of absorption across many biological membranes, additional molecular parameters, such as the partition coefficient or solubility, can significantly improve structure-permeability correlations. If molecular weight dependency of permeation is to be more clearly established, it should be helpful to use model compounds that are physicochemically similar in order to exclude these additional parameters.

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Chadwick et al. (1977) and Ma et al. (1990) have identified polyethylene glycols (PEGs), polydisperse polymeric mixtures available in a wide range of molecular weights, as a series of compounds useful in the investigation of molecular weight dependency of membrane permeability. As a polyoxyethylene polymer, the physicochemical properties, particularly the partition coefficient and water solubility of the PEGs, do not change as drastically with increasing molecular weight as happens within a series of alkyl homologues. Chadwick et al. (1977) used polyethylene glycol 400 as an absorbable marker compound, yet polyethylene glycol 4000 is commonly used as a nonabsorbable gastrointestinal marker. These observations strongly suggest a significant molecular weight effect, rather than physicochemical component, controlling the permeability of the PEGs in the gastrointestinal tract. The PEGs also appear to be good markers because of their high water solubilities and low toxicities (Rowe and Wolf, 1982).

In addition, Ma et al. (1990) perfused rat intestine with PEG 400 and permeation was found to occur via passive diffusion and a solvent drag effect along its concentration gradient. There was a linear relationship between permeabilities of PEG 400 and water, with increased lumenal osmolarity causing decreased absorption of both water and PEG. They suggested that PEG is strongly hydrophilic and thus would be expected to permeate through 'aqueous pathways' by passive diffusion and solvent drag. Using the PEGs as marker compounds, a direct comparison of the molecular weight sensitivity of permeability across corneal tissue can be made. Thus, the objective of the present work was to determine the molecular weight dependency of corneal permeation and the means by which this process may be improved, i.e., by use of penentration enhancers.

Experimental

Animal and materials

Male albino New Zealand rabbits (New Franken, WI) weighing between 2.5 and 3.0 kg were used. Sodium deoxycholate, digitonin, and cytochalasin B were obtained from Sigma Chemical Co. (St. Louis, MO). Glutaraldehyde (Ladd Industries, Burlinton, VT), used in the scanning electron microscopic study, was from a 70% stock solution and stored under argin in sealed ampoules. PEGs (low polydispersity) with average molecular weights of 200 ($M_w/M_n = 1.10$), 400 ($M_w/M_n = 1.07$), 600 ($M_w/M_n = 1.10$), 1000 ($M_w/M_n = 1.05$), and 1450 ($M_w/M_n = 1.05$) were obtained from Chemical Pressure Co. (Pittsburgh, PA). All other chemicals were either reagent or analytic grade and were used as received.

In vitro diffusion studies

Preparation of the cornea for in vitro diffusion studies was similar to that described in the preceding article (Liaw et al., 1992). A bathing donor solution (polymer dissolved in water) of either PEG 200, 400, 600, 1000, or 1450 was incubated on the epithelial donor side such that each cornea received a dose of 59.2, 67.1, 78.9, 100, or 120 mg/ml, respectively, with a final osmolarity of 300 mosM for each solution. The absorption of each series of oligomers was determined by measuring PEG in the receiver chamber over a 4-h interval following dosing. All experiments were carried out at 37 °C and a 0.67 cm^2 area of tissue was exposed to the donor and receiver compartments, each compartment having a volume of 7 ml. For analysis, 1 ml of test solution was sampled from the receiver chamber (endothelial side) before HPLC analysis and replaced with 1 ml of sample bathing medium (0.16 M NaCl). For the penetration enhancer studies, all PEG solutions consisted of either PEG 200, 400, 600, or 1000 with 1 mM sodium deoxycholate, digitonin, and cytochalasin B.

Assay of polyethylene glycol

High-performance liquid chromatography (HPLC) has been used to separate and quantify the individual oligomers of a broad spectrum of PEGs (Chadwick et al., 1977; Westrom et al., 1984; Donoval et al., 1990; Ma et al., 1990). A chromatographic system consisting of a Model 501 solvent delivery system (Waters Associates, Milford, MA), a Rheodyne 7125 (Rheodyne Inc,

Cotati, CA) injection valve with a sample volume of 100 μl, a μ-Bondapak column (150 mm × 3.9 mm i.d.) packed with silica C_{18} with a mean particle diameter of 10 μm (Phenomenex, Rancho Palos Verdes, CA) and a Model 410 differential refractometer were used for all PEG assays. The refractometer was programmed to maintain a constant temperature of 33 °C. Each diffusion sample was subjected to direct assay without extraction. Four different mobile phae compositions were used. For the analysis of PEG 200 the mobile phase contained 200 ml methanol in 1000 ml aqueous solution, whereas methanol/water ratios in the mobile phase of 3:7, 4:6, 46:54 and 54:46 were used for the analysis of PEG 400, 600, 1000 and 1450, respectively (Chadwick et al., 1977). Assay standard were prepared by spiking known quantities of PEG 200, 400, 600, 1000 or 1450 into 0.16 NaCl solution. Each oligomer peak height was plotted vs the known total polymer concentration, and linear regression equations were calculated for each oligomer. The peak for each oligomer in the unknown sample was calculated and converted to the relative amount of PEG present using the regression equation. The fraction of transport of PEG through the cornea was evaluated after correction for sampling and replacement at each time point. These values were then plotted vs time. Therefore, the apparent permeability coefficient, P (cm/s), was calculated from the slope with receiver compartment V (7 ml) and surface area of tissue A (0.67 cm^2).

Electrical measurement

The electrodes were prepared as described previously (Rojanasakul and Robinson, 1989). To measure the potential difference and resistance across the cornea, the four-electrode system described in the preceding paper was used (Liaw et al., 1992). All experiments were conducted at 37 °C within 4 h. O'Brien and Edelhauser (1977) and Rojanasakul and Robinson (1989) have shown that glutathione-bicarbonate Ringer's solution (GBR) can maintain corneal tissue integrity for as long as 6 h. After each study, both sides of the bathing solution were changed to GBR solution and membrane viability and integrity were assessed.

Scanning electron microscopy

After the completion of a diffusion study, the cornea was carefully removed and placed in a fixative solution containing 4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3 for 2 h. After washing in the same buffer, the tissues were post-fixed in 2% OsO_4 in 0.1 M phosphate buffer solution (pH 7.3) and were dehydrated using a graded series of ethanol solution at concentrations of 35, 50, 70, 90, 95, and 100%. Prior to viewing, the specimens wre critical-point dried in a pressure chamber using liquid carbon dioxide. Subsequently, a uniform 100 Å layer of gold was applied on the specimen surfaces by means of sputter coating (Balzers SCD 030) (Rojanasakul and Robinson, 1989). The specimens were finally viewed in a scanning electron microscope (Jeol JSM-35C).

Transmission electron microscopy

Tissue preparation up to the dehydration process was identical to that used in scanning electron microscopy. The dehydrated specimens were embedded in Durcupan ACM embedding medium, and sectioned using a glass knife on a microtome (Porter-Blum, MT2-B Ultramicrotome). Sections were placed on copper grids and viewed with a transmission electron microscope (Jeol 100 CX) (Rojanasakul and Robinson, 1989).

Results and Discussion

Molecular weight (molecular size) effect on corneal transport

The corneal permeability coefficient of polyethylene glycol in the molecular weight range 200–1051 is illustrated in Fig. 1. Overall, the PEGs can be divided into two groups on the basis of the extent of absorption, the molecular weight of the first group being between 206 and 415, apparent permeability coefficients remaining essentially constant ($1.13–1.04 \times 10^{-6}$ cm/s) and the second group, in the molecular weight range 459–1051, showing decreasing permeability with increasing molecular weight. No PEG above molecular weight 1051 was detected by HPLC after the 4 h study. As can be seen from Fig. 1,

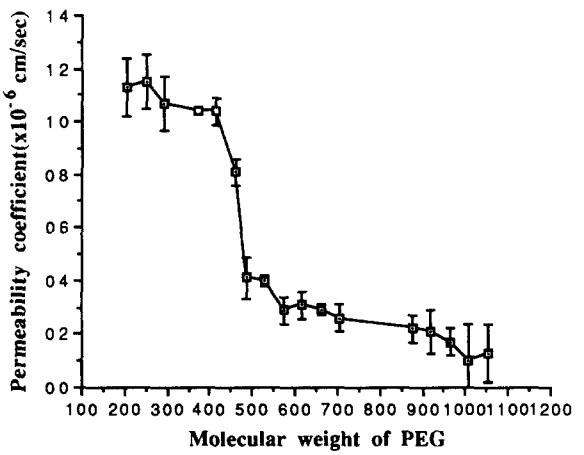

Fig 1. Permeability-molecular weight profiles for PEG 200–1000 in rabbit corneal tissue. Error bars represent the standard error of the mean; $n = 5$

the molecular weight dependency of absorption in the lower molecular weight region (PEG 400–600) following corneal transport is quite steep. The mean absorption of these oligomers over 4 h decreased from 0.18 to 0.05% of the amount incubated, as molecular weight increased from 400 to 600.

The molecular weight dependence of gastrointestinal permeability has been determined for lower molecular weight PEGs in human and various animal species (Chadwick et al., 1977; Philipsen et al., 1988; Hollander et al., 1989; Donovan et al., 1990). All of the investigators reported a progressively decreasing or constant relative urinary recovery of PEG between molecular weights 200 and 400. By using computerized molecular modeling, Hollander et al. (1988) showed that the molecular weight of the probes did not correlate with their rates of absorption in humans. In contrast, the calculated cross-sectional diameter of the probes correlated well with their relative absorption. They suggested that the molecular weight or surface area of probe molecules appear not to be important determinants of their intestinal permeability, especially for the analysis of individual PEG 400 polymers. Even though the molecular weight of the individual PEG 400 varies greatly, the cross-sectional diameter (0.53 nm) remains similar because of the linear structure of the molecule.

Taking into account molecular composition, weight, geometry, optimal conformation, and Van der Walls radii, Hollander et al. (1988) indicated that the PEG polymers began to fold when the molecules were longer than 12–14 units and such lengthy polymers of PEG would tend to become globular in shape. This could explain our absorption-molecular weight profiles. In addition, Falth-Magnusson et al. (1984) used a mathematical filter function to assess the intestinal permeability barrier and found that permeabilities of PEG between molecular weight 370 and 502 were similar, and above 502 permeation of PEG decreased with increasing molecular weight. Hollander et al. (1989) and Ma et al. (1990) observed a linearly progressive decrease in partition coefficient (K_d) with increasing molecular weight of the fractions between 200 and 1000 ($K_{d_{octanol/water}} = 0.01–0.0001$), which indicated increasing relative water solubility of individual molecular weight fractions between 200 and 1000. They indicated that REGs in the molecular weight range 200–1000 would require either protein channels for transport across the corneal membrane or paracellular transport across tight junctions. Philipsen et al. (1988) have attempted to determine the relationship between the octanol/water partition coefficients of PEGs 200–1000 and their absorption. They found a good correlation only for PEG molecular weight above 546. They suggested that partition coefficient is not a major controlling factor in the permeation of PEG. Our results agree with data reported by Philipsen et al. (1988).

In addition, changing the concentration of PEG allowed us to examine whether its transport is associated with saturation kinetics. The corneas were incubated with PEG 200 at concentrations of 1–100 mg/ml and the rate of absorption was found to be linearly related to time and PEG concentration (absorption rate ($\times 10^{-6}$ mg/cm^2 per s) = 1.02 × PEG concentration (mg/ml) + 10.1, $r = 0.99$). In order to confirm the passive diffusion per transport system, one experiment was performed in a cold room (0–4 °C) using PEG 200 and 600. Apparent permeability coefficients of PEG 200 and 600 were similar at 0 ° and 37 °C ($P = 0.89 \times 10^{-6} \pm 0.2$ for PEG 200, vs $1.04 \times 10^{-6} \pm 0.13$ cm/s at 37 °C; PEG 600, P =

$0.23 \times 10^{-6} \pm 0.05$, vs $0.29 \times 10^{-6} \pm 0.02$ cm/s at 37 °C; *t*-test: $P < 0.1$). These experiments as well as those of Hollander et al. (1989) provide more evidence for passive PEG transport probably through the paracellular pathway.

The effect of pH on transcorneal transport of PEG

Further evidence for paracellular transport as the major route for PEG absorption was provided by the study of its transport as the bathing medium pH was varied. As can be seen in Fig. 2, the absorption rates were similar at pH 7 and 10. At pH 3.2, the apparent permeability coefficients were lower than at pH 7 or 10 for PEG 200–400. The tight junction, which is known to present a negative surface charge toward its epithelium (p*I* = 3.2), allows the permeation of positively charged molecules or ions, such as sodium, at a higher rate than anions, such as chloride. At lower pH values, protons in the solution may adhere to the negatively charged surface of the paracellular pathway and decrease its negative surface charge, resulting in a decrease in sodium and water absorption (Davson, 1980). The consequence of this would be a lowered rate of PEG transport in conjunction with decreased sodium and water transport. A similar observation was made by Hollander et al. (1989) and Moreno and Diamond (1976), who reported that an increase in hydrogen ion content (more acidic) in the lumen decreased sodium and water transport across the tight junction of gallbladder epithelium and rat intestine. In general, polyethylene glycol has a structure favorable to the formation of hydrogen bonds with water molecules (Blow et al., 1978). Thus, the effect of pH on PEG absorption indicates a linkage to aqueous pores or paracellular transport.

Ultrastructural changes of the cornea by PEGs during in vitro diffusion were investigated using transmission electron microscopy. Tissues were obtained at time zero, and after a 4 h incubation in GBR solution or PEG isotonic solution, were processed and subjected to microscopic examination. Electron micrographs of these tissues are shown in Fig. 3. In all tissues, the epithelial cell layers remained intact. However, progressive cell separation, i.e., widening of the intercellular spaces was evident, except with fresh tissue. In Fig. 3c, there were no obvious morphologic changes that could be seen following exposure to PEG 600 or 1000. These photomicrographs indi-

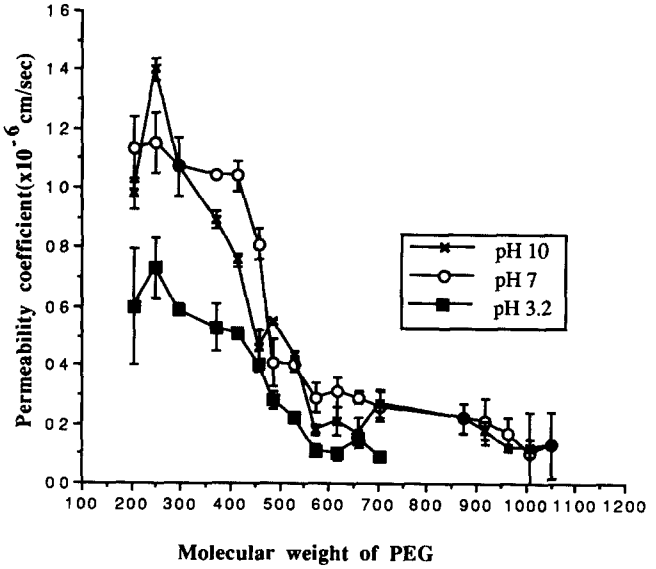

Fig. 2 Effect of perfusate pH on rabbit corneal uptake of PEG 200–1000 Error bars represent the standard error of the mean; $n = 5$

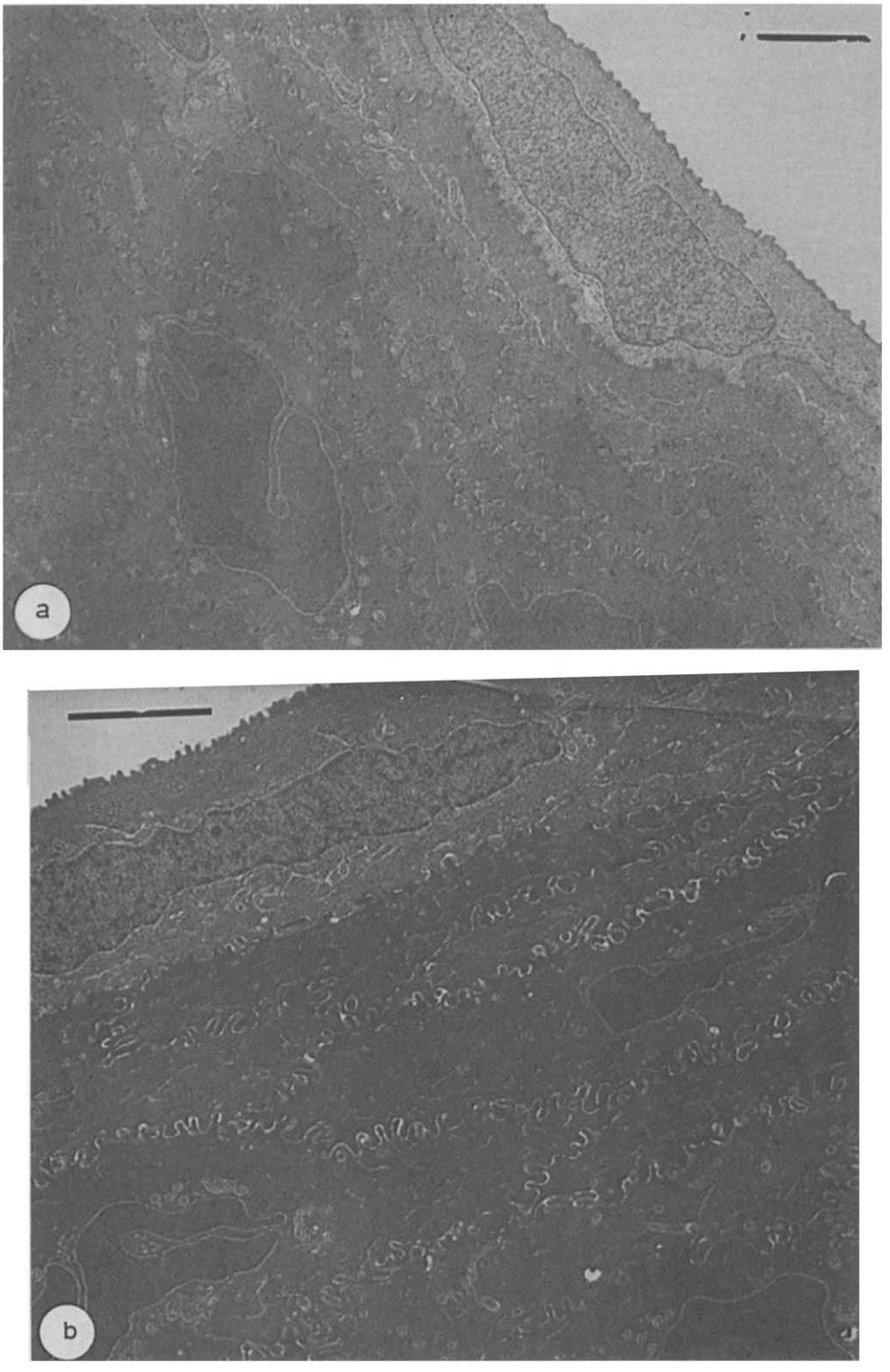

Fig 3 a, b

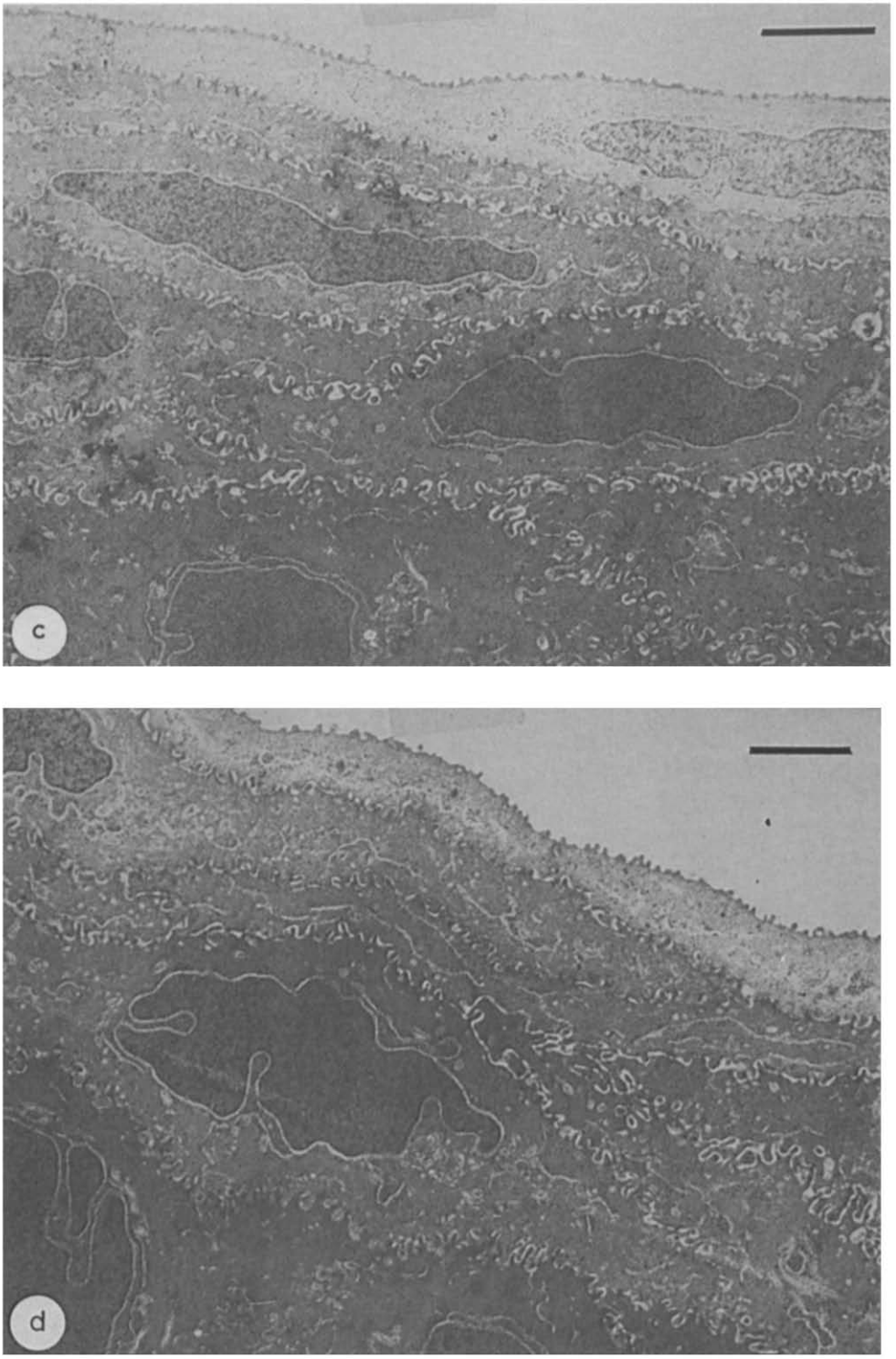

Fig 3. c, d.

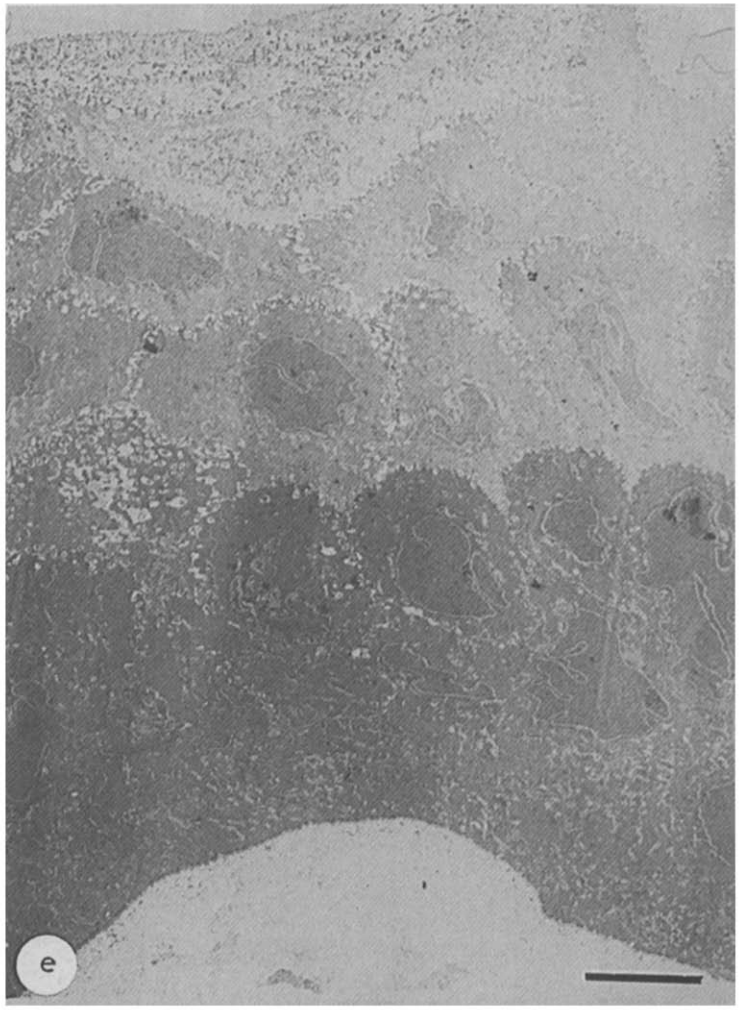

Fig. 3 Transmission electron micrograph of cornea after (a) 0 h, (b) 4 h, in GBR solution, (c) 4 h in pH 7 isotonic PEG 600 (78 9 mg/ml solution), (d) 4 h in pH 10 isotonic PEG solution, (e) 4 h in pH 3 isotonic PEG solution at 37 °C Cell separation as a result of expansion of the intercellular spaces is evident. Bars denote 2 μm

cate that PEG did not cause widening of the space between cells or cell fusion. Yamazaki and Ito (1990) have shown that PEGs above a critical concentration, 22% (w/w) for PEG 6000 and 28% (w/w) for PEG 1000, will convert the bilayer structure into a leaky membrane structure, and cause fusion. In the present study, the bathing solutions were always below this critical concentration. Thus, it appears that PEG did not cause membrane fusion in the cornea permeation study.

However, at pH 3 (Fig. 3e) the nuclei did not appear to be preserved as well as in the GBR bathing solution. The tissue viability and integrity were determined from the transmembrane potential differences and resistance (Figs 4 and 5). A potential difference, which develops when two identical solutions are placed on both sides of the membrane, indicates active ion transport and thus a viable state of the tissue. Membrane resistance, on the other hand, indicates membrane perme-

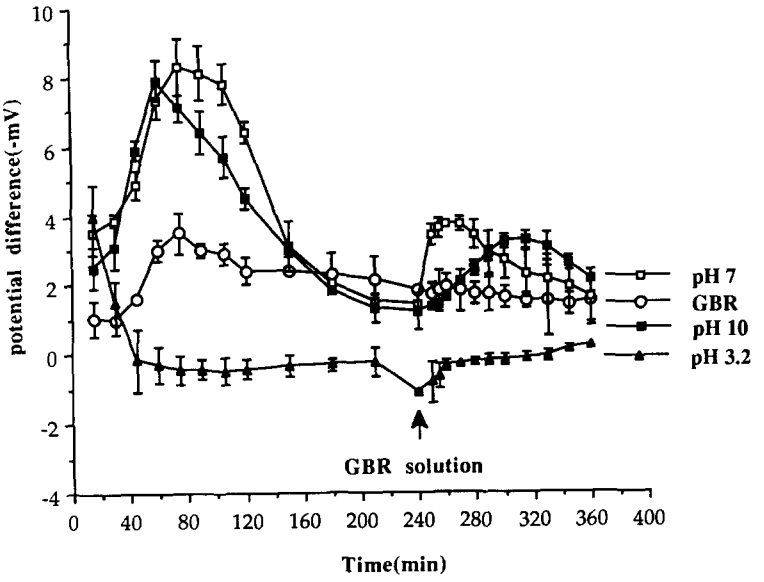

Fig. 4 Transmembrane potential difference in isotonic NaCl with three different pH and GBR solutions Bars indicate 1 SE; $n = 5$

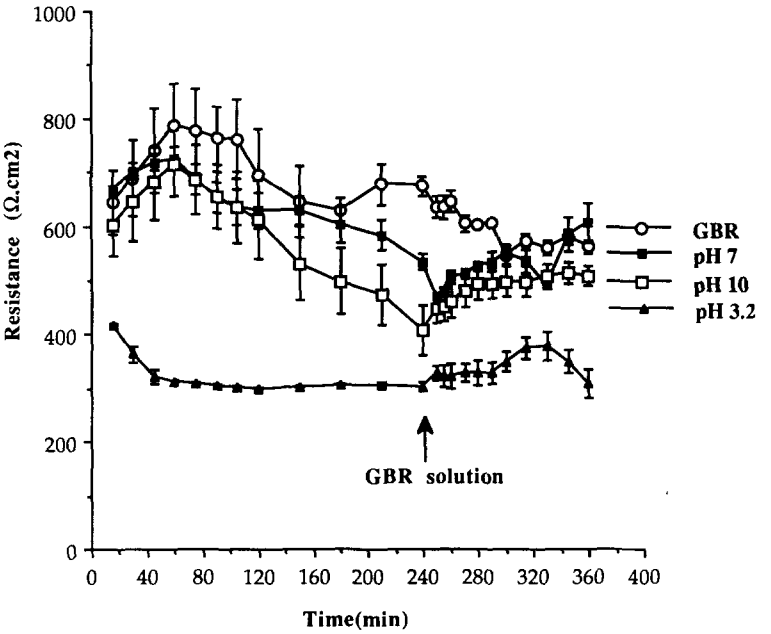

Fig. 5. A typical resistance-time profile of the cornea with different pH and GBR solutions The resistance was measured by applying direct current pulses of variable duration (1–5 s) and intensity (up to ± 10 μA cm^{-2}) and recording the voltage drop across the cornea. Bars indicate 1 SE; $n = 5$.

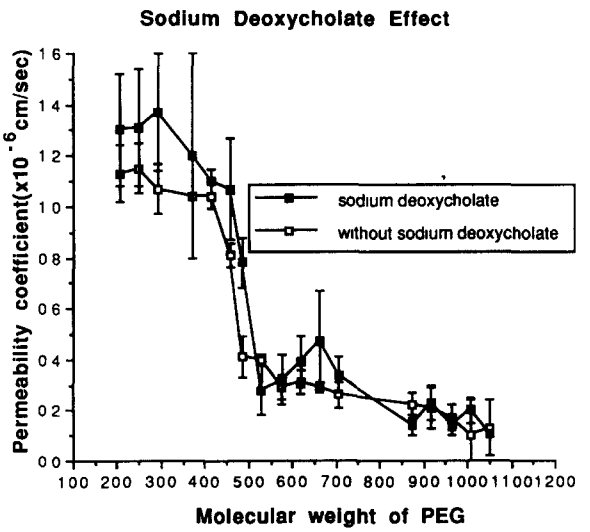

Fig. 6 Effect of sodium deoxycholate on rabbit corneal transport of PEG molecular weight dependence profile. The cornea was treated with sodium deoxycholate at 1 mM for 4 h. Error bars represent SE, $n = 5$

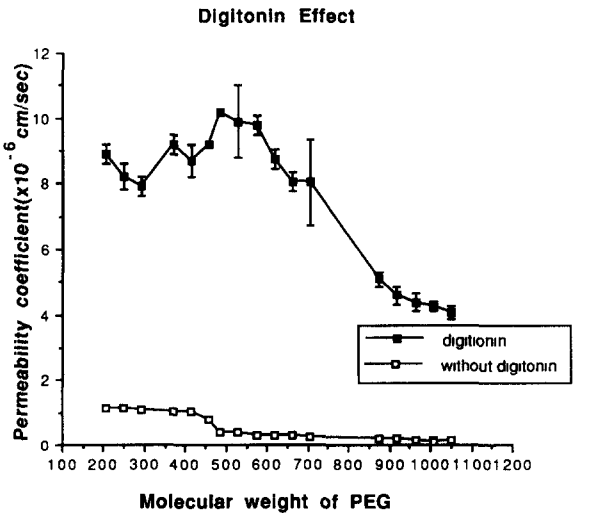

Fig. 8 Effect of digitonin on rabbit corneal transport of PEG molecular weight dependence profile. The cornea was treated with digitonin at 1 mM for 4 h. Error bars represent SE; $n = 5$

ability and thus was used as an indication of tissue integrity or damage. It can be seen that at pH 3, the potential difference and resistance profiles were different than at pH 7, pH 10 or in GBR solution. However, as soon as the solution at pH 3 was replaced with GBR solution, the potential difference began to partly recover and membrane resistance increased. At pH 3, the top layer of tissue was partly damaged and active ion transport through the cornea was partly inacti-

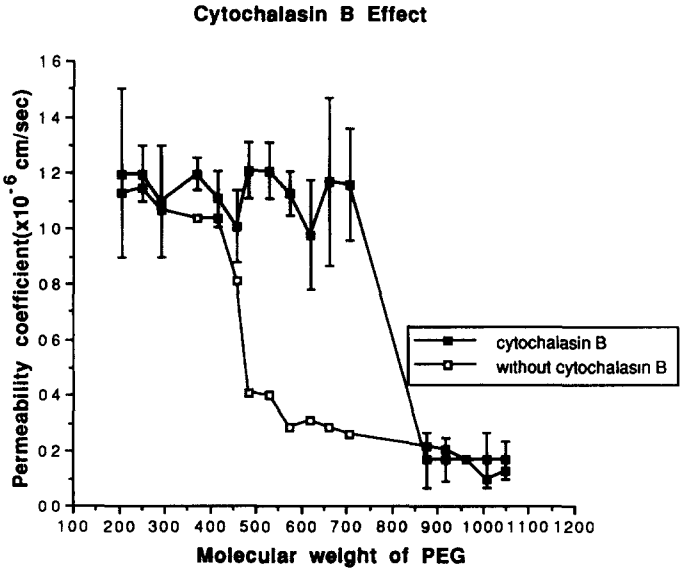

Fig 7 Effect of cytochalasin B on rabbit corneal transport of PEG molecular weight dependence profile. The cornea was treated with cytochalasin B at 1 mM for 4 h. Errors bars represent SE; $n = 5$

vated, so either an increase or no change in the permeability of PEG was expected. However, instead of an increase, the permeation of PEG decreased. This finding further indicates that the transport of PEG occurs via passive diffusion through the aqueous intercellular space.

The effect of penetration enhancers on the molecular weight dependence of corneal absorption

The use of a wide range of PEGs to investigate the effects of penetration enhancers, including a bile salt, cytochalasin B, and digitonin, on the molecular weight permeability profile through the cornea can be used to help identify a portion of the mechanism(s) of penetration enhancers. Previous studies (Rojanasakul et al., 1990) have shown that increasing the concentration of these enhancers from 0.1 to 1 mM results in a decrease in membrane resistance, and an increase in membrane permeability. Thus, all the penetration enhancers studied were incubated at a fixed (1 mM) concentration with PEG isotonic solution for a 4 h period.

Each of the penetration enhancers studied affected the PEG molecular weight permeability profile in a unique manner. Fig. 6 shows the

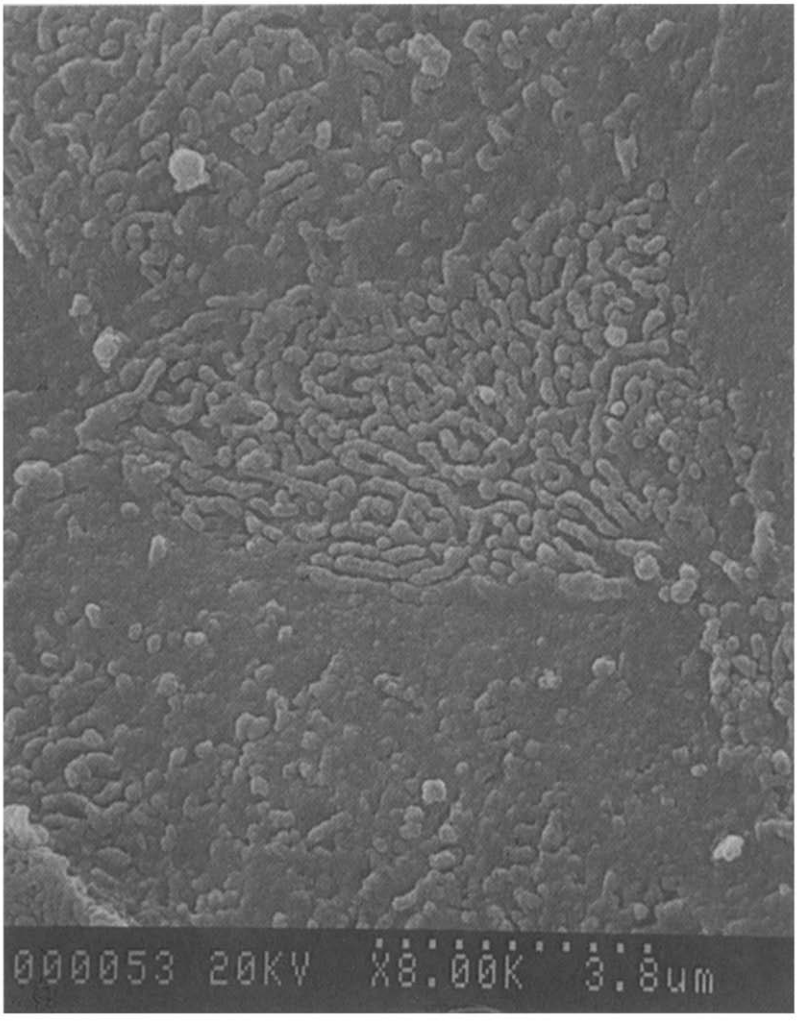

Fig. 9. Electron micrographs of the rabbit corneal epithelium in isotonic PEG solution. Note a typical epithelial surface showing a normal cell and one of the degenerating dark cell at left corner

effect of 1 mM sodium deoxycholate on PEG absorption. This bile salt caused virtually no absorption enhancement of PEG throughout the molecular weight range studied. Selective molecular weight effects were observed with 1 mM cytochalasin B (Fig. 7), a known actin cytoskeleton inhibitor. In this case, permeability coefficients of PEG 400 and 600 increased to values similar to that of PEG 200. However, the absorption of PEG 1000 did not change. Digitonin, a known membrane solubilizing agent (Fiskum, 1985), had a more dramatic enhancing effect (Fig. 8). The absorption of PEG 200, 400, and 600 increased approx. 10-fold and that of oligomers of PEG 1000 approx. 5-fold.

Representative scanning electron micrographs (SEM) of the corneal tissues are shown in Figs 9–12. Fig. 10 shows tissue samples following administration of 1 mM sodium deoxycholate. There were no significant morphologic changes in the top layers of the membrane compared with PEG solution of pH 7 (Fig. 9). Sodium deoxycholate has been shown to increase the absorption of insulin (Hirai et al., 1981; Gordon et al., 1985;

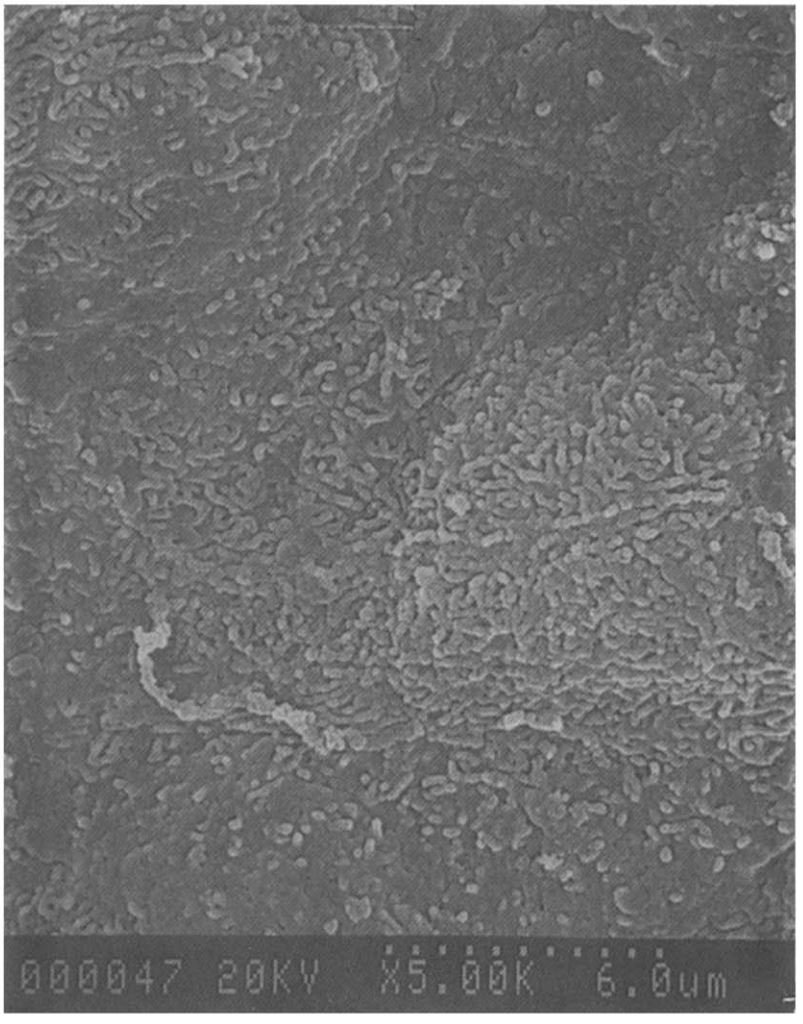

Fig 10 Effect of sodium deoxycholate on corneal epithelium. The degenerating cell is located at the upper-right corner, similar to that observed in the isotonic PEG solution

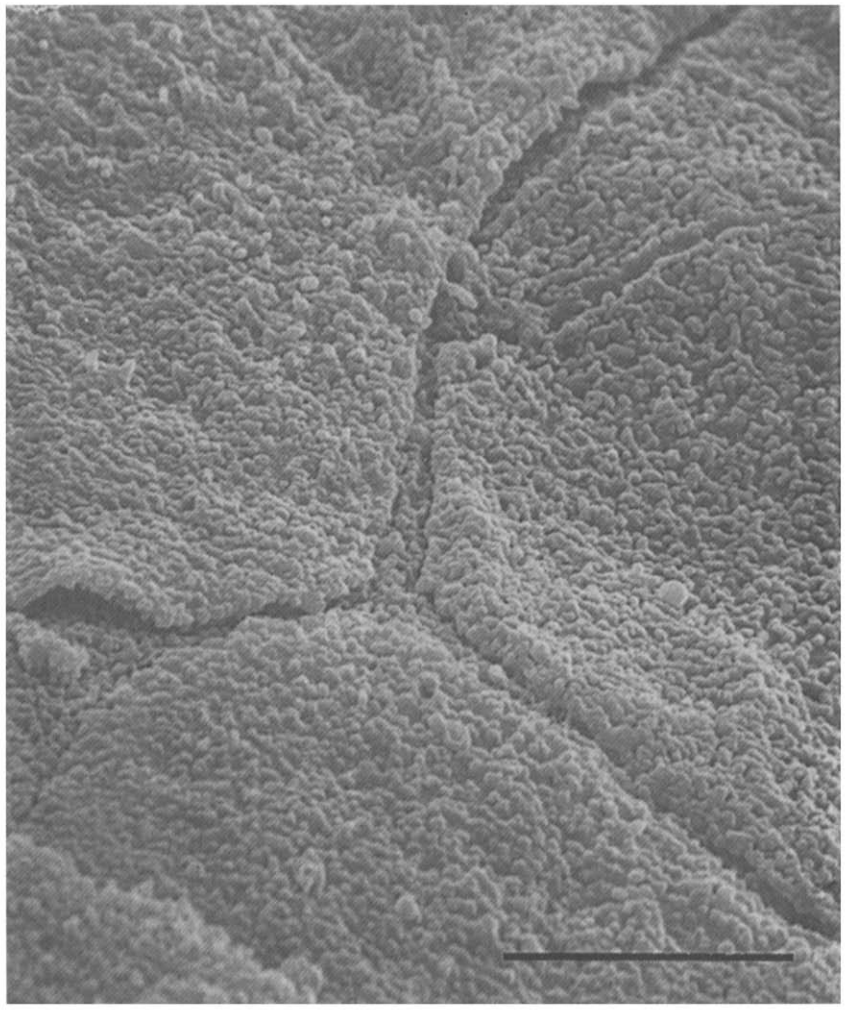

Fig 11 Effect of cytochalasin B on rabbit corneal epithelium. Note opening of the intercellular junction between two cells Bar indicates 6 μm.

Aungst et al., 1988). Nevertheless, there was no change in the PEG molecular weight permeability profile upon addition of the bile salt, and no significant changes in the SEM could be attributed to sodium deoxycholate. Minimal mucosal damage was also observed by Hirata et al. (1979) and Donovan et al. (1990), who noted slight damage to the surface microvilli following a 1 week treatment with an insulin/1% bile salt solution or with PEG/1% bile salt condition. In addition, Hollander et al. (1989) and Chadwick et al. (1977) have shown that either bile salts or oleic acid changed the permeation of PEG 900. They indicated that hydrophilic PEG molecules are absorbed primarily via the paracellular route in the intestine. One might be concerned that enhancement is dependent on the concentration of bile salt, yet the concentration of bile salt in the present study is higher than 1%, a level which has been used in other reported investigations. Lee and Kashi (1987) demonstrated that bile salts not only act by a direct solubilizing effect, but also have an inhibitory effect on cellular enzymatic activity. Using confocal microscopic studies,

with labelled membrane and nuclear probes. Rojanasakul et al. (1990) have shown that, despite the obvious membrane damage caused by the bile salt, loss of the plasma membrane was less than that observed when the membrane was exposed to digitonin. Thus, both the PEG absorption data and microscopic studies suggest that sodium deoxycholate, at this concentration, is not a very effective enhancer.

The effects of the PEGs in the combination with 1 mM cytochalasin B on the corneal tissue are shown in Fig. 11. The primary effect seen was disruption of the tight cell-cell packing present in normal epithelia (Fig. 9). Bentzel et al. (1980) have demonstrated that this enhancer affects the cell cytoskeleton and disorganzies the orderly arrangement of the junctional protein network. Also, Madara et al. (1986) and Meza (1980) have shown that cytochalasin D affects occluding junctions of intestinal absorptive cells influencing paracellular permeability and junctional charge selectivity. In this study, a broader range of molecular weight PEGs were used, to identify the effect of cytochalasin B on the moleclar weight cutoff

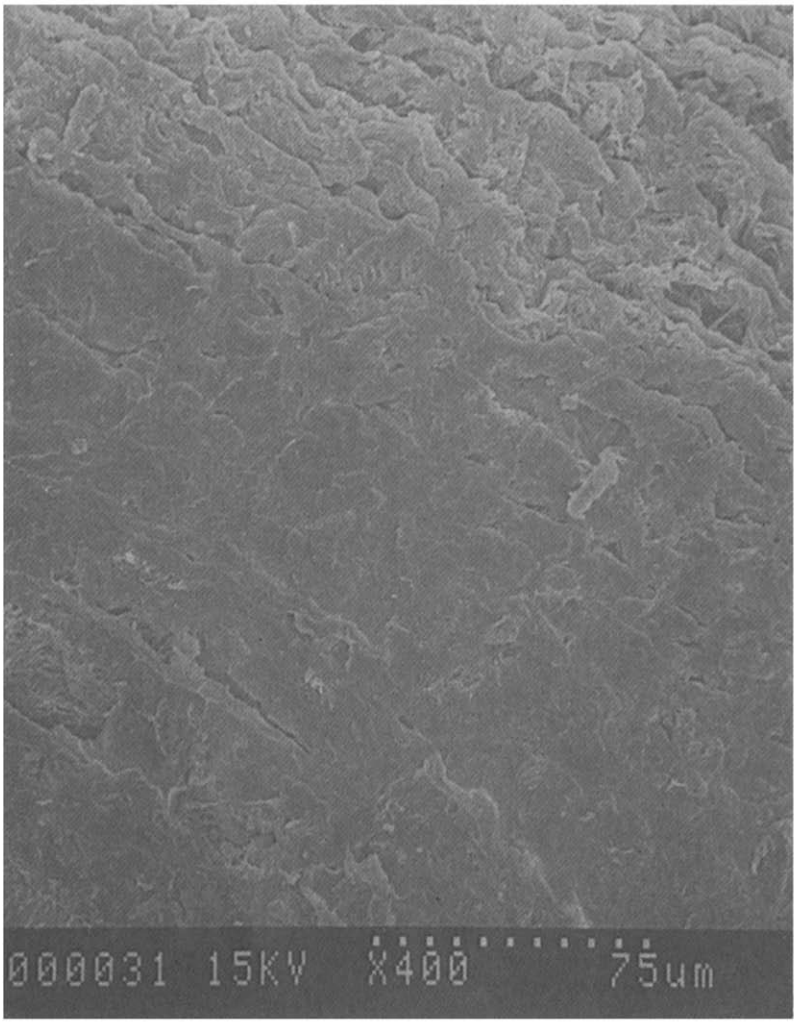

Fig 12. Effect of digitonin on rabbit corneal epithelium Typical stroma layers of corneal surface area without the epithelial layer are shown.

for absorption. The results obtained agree with other reported data showing cytochalasin B to infleunce the degree of opening of occluding junctions from molecular weights of PEG 400 to PEG 700. Above a molecular weight of PEG 700, there was insufficient change on permeation through the cornea to be sufficient. In addition, only a minimal drop in membrane capacitance was observed for cytochalasin B (Rojanasakul et al., 1990). Such observations are in good agreement with the present results from an SEM study. The effect of cytochalasin B appeared predominantly on the junctional portion of the epithelium and may be of potential use for safe and effective delivery of peptides, especially for those that permeate tissues paracellularly.

Digitonin is known to solubilize membrane protein and lipids, layer by layer (Wolosin, 1988). Hull et al. (1984) and Camber et al. (1987) have shown that in vitro tests, where the corneal epithelium is removed prior to drug administration, the permeation is dramatically increased by a factor of 2–15. As can be seen in Fig. 12, 1 mM digitonin caused significant alteration of the corneal epithelial layer, including epithelial layer detachment from the stroma, disruption of the cellular structure, and in some cases, complete removal of the epithelial layer (Fig. 12 shows the stroma layer). In this case, absorption of the oligomer of PEG 600 was increased approx. 20-fold and that of the oligomers of PEG 1000 approx. 10-fold. The permeability barrier of the cornea to hydrophilic molecules is thought to reside largely with the superficial surface of the epithelium, i.e., the top two cell layers. Klyce et al. (1972) showed that the apical surface of the corneal epithelium alone contributes over half of the total electrical resistance of the entire cornea. This is due to the presence of annular tight junctions or zonulae occludents, thus effectively sealing the epithelium to all but small hydrophilic molecules. Thus, a combination of SEM and molecular weight dependence studies suggest that digitonin solubilizes the membrane and destroys the epithelial layers. Nevertheless, it has yet to be resolved whether this enhancer will be useful in long-term studies.

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