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The effect of drug charge type and charge density on corneal transport

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Summary

In order to understand the effect of charge type on corneal transport of peptides, it is useful to first investigate the mechanism of transport of amino acids across corneal tissue. The transport characteristics of L-[^{14}C]lysine and L-[^{14}C]glutamic acid were based on in vitro flux measurements. Net flux of L-lysine occurs via a sodium-dependent cotransport mechanism (with a coupling ratio of 1:1). The transport of L-lysine involves the Na^+-K^+-ATPase pump and requires a stereospecific carrier-mediated transport system. The permeability coefficient (P) of L-lysine was decreased from 10.1 ± 0.80 to $2.34 \pm 0.75 \times 10^{-6}$ (cm/s) by blocking the active pump and carrier-mediated system. However, L-glutamic acid was apparently absorbed by a passive aqueous transport mechanism which was ouabain-insensitive, [Na^+]-independent, temperature-insensitive and non-stereospecific. Overall, the cornea was 2–3-fold more permeable to a cation (L-lysine) than an anion (L-glutamic acid). In addition, the negatively charged drug, salicylic acid, was 2 3-times less permeable than the cationic drug, benzylamine.

Introduction

Peptides and proteins have recently emerged as an important class of therapeutic agents and the study of these compounds has led to a growing understanding of their physiological roles. Development of these therapeutic agents presents an enormous challenge to scientists attempting to design an appropriate drug delivery system, due to several unique structural characteristics in comparison to conventional drugs. For example, most peptides are large, with numerous charged groups, which leads to problems associated with absorption through biological membranes.

Most epithelia are known to possess permselective properties, i.e., the ability to discriminate or show preference to passage of charged molecules. Barrier permselectivity is a complex phenomenon which combines not only a passive contribution from electrostatic shunt activity, probably due to membrane fixed charges, but also an active potential contribution from cell membrane activity. The latter is generally a reflection of carriers and pumps residing in the membrane.

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In rabbit cornea, the active potential arises from an inward sodium transport from tears to aqueous humor (Donn et al., 1959; Green, 1965) and an outward chloride transport (Klyce et al., 1973). This results in a negative transcorneal potential (epithelial with respect to endothelial side) with a magnitude of 25 mV (Maurice, 1967). As shown in Fig. 1, Na^+-linked symports, located in the apical (absorptive) domain of the plasma membrane, actively transport nutrients into the cell, building up substantial concentration gradients, while Na^+-independent transport proteins in the basal and lateral (basolateral) domain allow nutrients to leave the cell passively down these concentration gradients (modified from Albert et al., 1989). The Na^+-K^+-ATPase that maintains the Na^+ gradient across the plasma membrane of these cells is located in the basolateral domain. Related mechanisms are thought to be used by the kidney, intestine, and corneal epithelial cells to pump water from one extracellular space to another (Maurice, 1984; Albert et al., 1989).

With regard to passive permselectivity, previous studies have indicated that many peptides and proteins, including horseradish peroxidase (Tonjum, 1974), polylysine, and phalloidin (Rojanasakul et al., 1990) permeate the cornea predominantly through the paracellular pathway, with the apical layer of the epithelium contributing most to the permeability barrier. Like most other epithelia, the apical permeability barrier of the cornea has been shown to result from tight junctions between surface cells (Hogan, 1971; Maurice, 1984). Recent studies (Rojanasakul and

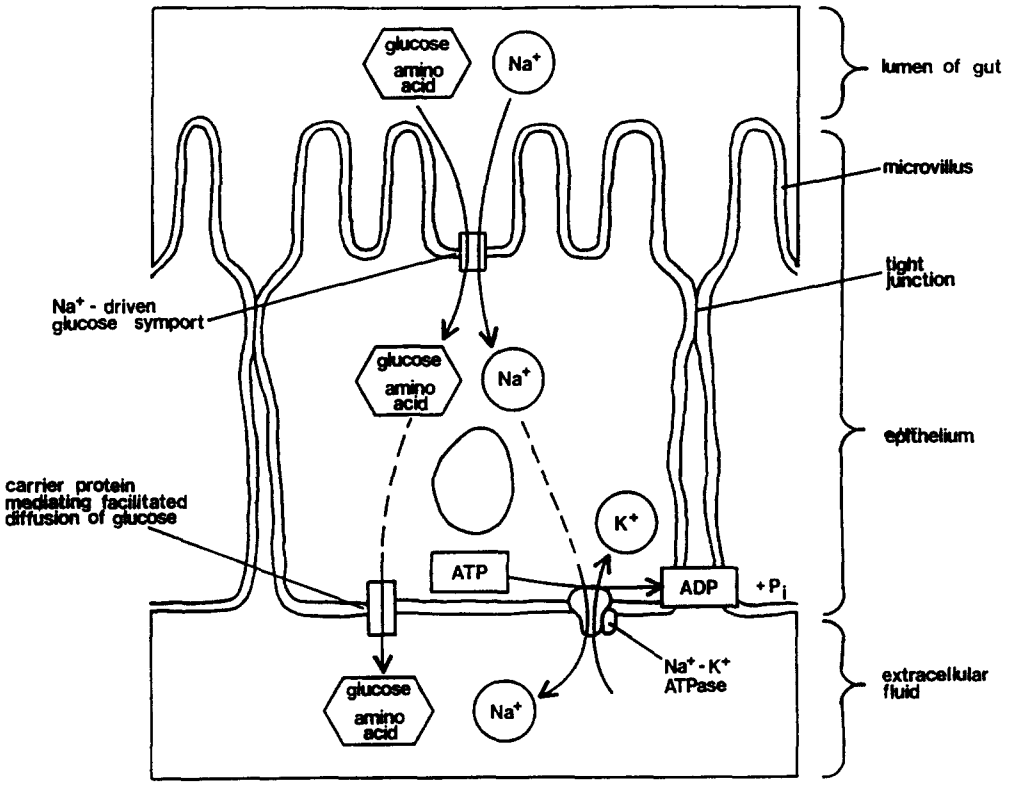


Fig. 1. The asymmetrical distribution of transport proteins in the plasma membrane of an epithelial cell results in the transcellular transport of glucose from the apical to the extracellular fluid. Adjacent cells are connected by tight junctions which prevent large molecules from crossing the epithelium paracellularly, allowing a concentration gradient of glucose to be maintained across the cell sheet

Robinson, 1989) have indicated that the cornea contains both positively and negatively charged groups, with the net magnitude and polarity depending on the degree of protonation. At a pH above the isoelectric point (p*I*) 3.2, the cornea carries a net negative charge and its selective to positively charged molecules. Below the p*I*, the reverse is valid. Based on these findings, it is anticipated that the type of charge and charge density carried by peptides/drugs will have a significant effect on their transport across the cornea.

To quantitatively evaluate this charge effect, a study based on in vitro flux measurements of lysine (Mol. Wt 146) and glutamic acid (Mol. Wt 147) was conducted. These two compounds were chosen because they have a similar size but opposite charge (lysine carries a net positive charge and glutamic acid a net negative charge at physiological pH), thus, the effect of charge selectivity can be directly interpreted without influence of the size effect. In addition, the charge density effect can be modified to promote absorption of these two amino acids by changing the pH of the medium and thus ionization of the permeant.

Experimental

Animal and materials

Male albino New Zealand rabbits (New Franken, WI) weighing between 2.5 and 3.0 kg were used. Radiolabelled L-[^{14}C]lysine and L-[^{14}C]glutamic acid were obtained from Research Product International Corp. (Mount Prospect, IL). [^{14}C]Salicylic acid, [7-^{14}C]benzylamine hydrochloride and *p*-amino[*carboxyl*-^{14}C]benzoic acid were purchased from NEN Research Products (Du Pont Co., Wilmington, DE). The purities of all radiolabelled compounds were between 98 and 99%, as ascertained with thin-layer chromatography. Ouabain, amiloride, L-lysine, D-lysine, L-glutamic acid, D-glutamic acid, salicylic acid, benzylamine hydrochloride, and *p*-aminobenzoic acid were obtained commercially (Sigma Chemical Co., St. Louis, MO). All other chemicals were either reagent or analytical grade and were used as received.

In vitro diffusion studies

The in vitro diffusion apparatus (Machine Shop, University of Wisconsin-Madison) was similar to that described by Schoenwald and Huang (1983) with slight modifications. Preparation of corneal membrane was performed as described by Rojanasakul et al. (1989). The two faces of the cornea were bathed with 0.16 M NaCl using NaOH or HCl to adjust pH. Aeration with a mixture of 95% O_2 and 5% CO_2 was initiated to provide oxygen and mixing in the tissue bath. All experiments were carried out at 37°C for a period of 2 h for the radioactive compounds. 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 at fixed intervals from the receiver chamber (endothelial side) into scintillation vials and replaced with 1 ml of sample bathing medium. Each 1 ml sample was then diluted with 10 ml of scintillation cocktail (Biosafe II, RPI, Mount Prospect, IL). The samples were analyzed by evaluation of the total radioactivity (dpm) in a liquid scintillation counter (Tric Cab 460 CD, Packard Instruments, Downers Grove, IL). In order to determine the initial donor concentration, a 1 ml sample of the test solution was incubated with 10 ml of scintillation cocktail and the total dpm counted.

Solution preparation

In most experiments related to charge type and charge density, isotonic NaCl solutions containing 160 mM NaCl were used. In some experiments, where the effect of sodium concentration was studied, an iso-osmotic amount of a nonelectrolyte, sucrose or mannitol, was added. The final osmolarity of all test solutions was maintained between 290 and 305 mosM as determined on a Wescor 5500 vapor pressure osmometer (Logan, UT). All labelled compounds were dissolved in 0.16 M NaCl at a fixed concentration of 0.6 μCi/ml. In a separate series of experiments, a solute or drug was added directly to the 0.16 M NaCl solution, bathing one or both sides of the tissue. These included 0.1–10 mM ouabain, up to 10 mM, L-lysine/or L-glutamic acid and D-lysine/or D-glutamic acid. The detailed composi-

tions of the test solutions are indicated in each section of Results. In the availability and tissue integrity experiments, glutathione-bicarbonate Ringer's solution (GBR), which is common bathing solution for cornea storage and preservation, according to O'Brien and Edelhauser (1977), was used. Solutions were stored in the refrigerator and used within 2 weeks.

Metabolism studies

To ensure that radiolabelled L-lysine and L-glutamic acid were not significantly metabolized or altered by the tissue, the following experiments were initiated (Kim et al., 1988). Excised rabbit corneal tissue was incubated for 2 h in 160 mM NaCl solution containing 0.05 mM L-[^{14}C]lysine or -glutamic acid. The incubation medium was continuously aspirated with 100% O_2 and any CO_2 evolved by oxidative metabolism was collected in a trap containing 2 N NaOH. Aliquots of the NaOH, after the incubation period, were assayed for radioactivity. There was no detectable $^{14}CO_2$ during 2 h incubation of rabbit corneal tissue with L-[^{14}C]lysine or L-[^{14}C]glutamic acid, indicating that the tissue did not oxidatively metabolize the radiolabelled lysine and glutamic acid. In addition, the tissue was then rinsed three times for 5 s each with fresh ice-cold NaCl solution and subsequently extracted in 0.1 N HNO_3 for 15 h. An aliquot of the tissue extract was precipitated with 10% trichloroacetic acid in order to determine whether L-[^{14}C]lysine or glutamic acid was incorporated into tissue protein during the experimental period. Results showed that all the radioactivity was recovered in the supernatant, indicating that no measurable amount of radiolabelled compounds was incorporated into the protein. The radioactivity associated with the incubated tissue was 0.78% for L-lysine and 0.08% for L-glutamic acid. The overall recovery of the radioactivity from these experiments was greater than 97% for both lysine and glutamic acid. Finally, aliquots of the tissue extract, the medium in which the tissue had been incubated, and the stock solution of L-[^{14}C]lysine or -glutamic acid were subjected to ascending paper chromatography using a sec-butanol/formate/H_2O (65:15:20, v/v) solvent system (Munck and Schultz, 1968; Kim et al., 1988). The radiochromatograms of the incubation medium, tissue extract, and stock solution each revealed one radioactivity peak at a similar location, indicating that there was no appreciable alteration of the radiolabelled L-lysine and L-glutamic acid during the 2 h experiment. Taken together, these data suggest that the radioactivities assayed in the reservoir solution in the transport studies represent intact L-[^{14}C]lysine and L-[^{14}C]glutamic acid.

Electrical measurements

Preparation of electrodes and measurement of potential differences and resistance were carried out as described by Rojanasakul et al. (1989). In all experiments, the anode was placed on the epithelial side and the cathode on the endothelial side. All resistances were calculated from the slope of the current and potential difference plots. To correct for the potential drop due to solution resistance between the sensing electrodes and the membrane, measurements were carried out before each membrane resistance determination using the same bathing solution but without the membrane in the diffusion chamber. The actual membrane resistance was then calculated by subtracting the resistance determined in the absence of the membrane from that in its presence.

Apparent permeability coefficient calculation

In vitro apparent permeability coefficients were calculated from:

$$P = \frac{dC \cdot V}{dt \cdot C \cdot A} = \frac{(\text{fraction of dose transported})}{dt} \frac{V}{A}$$

$$= \text{slope} \frac{V}{A}$$

where the fraction of dose transported through the cornea can be calculated after correction for sampling and solution replacement at each time point. These values were then plotted vs time. Therefore, the apparent permeability coefficient, P (cm/s), can be calculated from the slope using a receiver compartment V (7 ml) and surface area of tissue A (0.67 cm^2).

Results and Discussion

Effect of charge type on corneal transport of lysine and glutamic acid

Table 1 lists the structures and pK_a values of the two charged amino acids (Albert et al., 1989). At pH 7, lysine carries a net positive charge and glutamic acid has a net negative charge. According to electrophysiological measurements, at pH 7 and pH values above the isoelectric point (pI) 3.2, the cornea behaves as if it is negatively charged and is thus relatively less permeable to L-glutamic acid and functions as an ion-exchange membrane for L-lysine. Results from these experiments, shown in Fig. 2, indicate an approx. 10-fold difference in the permeability of the two compounds, with lysine being more absorptive. Evidently, most epithelia are cation selective, i.e., P_K and $P_{Na} > P_{Cl}$, and the difference in the permeability ratio between cation and anion is around 1.1–5-fold (Powell, 1981).

L-Lysine transport: an ouabain-sensitive, sodium-dependent and carrier-mediated system

Table 2 shows that 0.1–10 mM ouabain inhibited L-lysine transport by approx. 47–58%. It is known that the action of ouabain is due to its

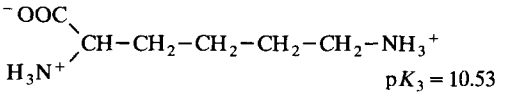
TABLE 1

Effect of pH on charge type and density of lysine and glutamic acid

	pK_1 (COOH)	pK_2 (NH_2)	pK_3 (NH_2)	Net charge
Lysine				
pH 3	−	+	+	+
pH 7	−	+	+	+
pH 10	−	neutral	+	0
Glutamic acid				
pH 3	−	+	neutral	0
pH 7	−	+	−	−
pH 10	−	neutral	−	=

Lysine

$pK_1 = 2.18$

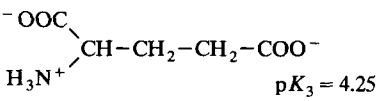


$pK_3 = 10.53$

$pK_2 = 8.95$

Glutamic acid

$pK_1 = 2.18$



$pK_3 = 4.25$

$pK_2 = 9\ 67$

TABLE 2

L-Lysine corneal permation an ouabain-sensitive, sodium-dependent and carrier-mediated system

	Apparent permeability coefficient $P \pm SE$ ($\times 10^{-6}$) (cm/s) ($n = 5$)			
(1) L-Lysine in 0.16 M NaCl [a].	10.10 ± 0.80			
(2) Ouabain effect·	5.3 ± 0.6, (0.1 mM)	4.5 ± 0.7, (1.0 mM)	4.4 ± 0 5, (5.0 mM)	4.2 ± 0.6 (10 mM)
(3) Amiloride (3 mM) effect [b].	5.14 ± 0 60			
(4) Cold temperature (0–4°C) effect.	0.12 ± 0.13			
(5) Reverse effect [c]:	0.34 ± 0.02			
(6) Na^+ dependent [d]:	6.4 ± 0.2, (0.12 M)	5.7 ± 0.5, (0.10 M)	5.3 ± 0.1, (0.08 M)	4 3 ± 0 1 (0.05 M)
(7) Mannitol (0.3 M) effect [e]:	2.34 ± 0.75			
(8) D-Lysine (10 mM) + L-lysine effect [b]:	11 2 ± 0.20			
(9) Ouabain, L-lysine + L-lysine [e]:	2.14 ± 0.61			

[a] L-Lysine: L-[^{14}C]lysine (0.6 μCi/ml)

[b] Reagent was incubated in the epithelial bathing medium

[c] Reverse effect incubation of L-[^{14}C]lysine was on the endothelial side.

[d] Both sides of the bathing solution were maintained around 300 mosM using sucrose to adjust tonicity.

[e] 0.3 M mannitol solution placed on both sides.

[f] 3 mM ouabain and 10 mM unlabelled L-lysine were incubated on both sides with L-[^{14}C]lysine in the donor solution

specificity in inhibiting the Na^+-K^+-ATPase pump and reducing the Na^+ pumping out of the cell against an electrochemical gradient. Maurice (1984) has shown that the epithelium of the cornea contains high Na^+-K^+-ATPase activities, 2000 mmol/kg wet weight, and that the associated pump is involved in Na^+ transport. Studies on the cornea using a short-circuit technique have indicated that the current is carried across the epithelium mainly by Na^+ transport from the tear to the aqueous side (Klyce and Marshall, 1982). Thus, a decrease in L-lysine transcorneal permeation by ouabain probably occurs in part via a decrease in the Na^+ gradient across the apical membrane of the cornea epithelial cells after Na^+-K^+-ATPase activity is inhibited by the agent (Riley et al., 1973; McGahan, 1981; Cooperstein, 1985; Kim et al., 1988; Wheel and Yudilevich, 1989).

In addition, amiloride induced a similar reduction (50%) in the transport of L-lysine to that in the case of ouabain (Table 2). In general, amiloride has been used as an inhibitor of Na^+-cotransporter and it interacts with a single set of sites on the apical surface (Balaban et al., 1979). The reduction of transcorneal movement suggests that transport of L-lysine through the cornea involves Na^+. In order to confirm the energy requirement of L-lysine transport, diffusion studies were conducted at a temperature between 0 and

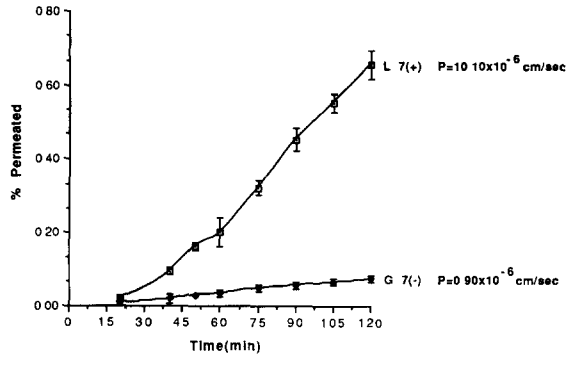


Fig. 2 Comparison of in vitro permeability profiles of charged amino acids across corneal tissue in 0.16 M NaCl. L 7(+) L-lysine at pH 7, with a net positive charge. G 7(−) L-glutamic acid which has a net negative charge at pH 7. Errors bars represent SE; $n = 5$.

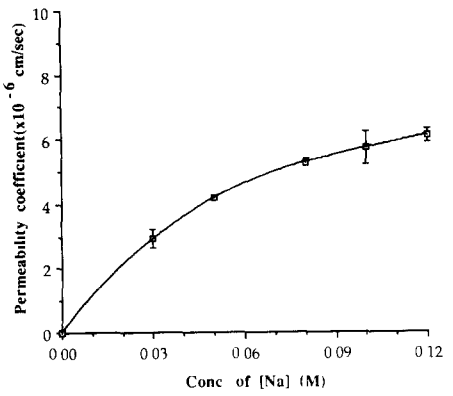


Fig 3. Net lysine absorption from epithelial donor side as a function of sodium concentration. Radiolabelled lysine concentration used was 0 6 μM

4°C. Indeed, the uptake and influx of L-[^{14}C]lysine were significantly decreased as compared to control studies (room temperature; Table 2). In the reverse permeation studies (endothelial-to-epithelial), there was a marked asymmetry in the two unidirectional fluxes of L-[^{14}C]lysine across the corneal tissue (Table 2). In the control studies, the transcorneal movement (epithelial-to-endothelial) of L-lysine was about 30-times greater in extent than that measured for reverse transport (endothelial-to-epithelial). The combined control data in the four different studies indicate that L-[^{14}C]lysine uptake is unidirectional and that influx was significantly greater at the epithelial surface. In addition, L-[^{14}C]lysine transport appears to involve Na^+ cotransport and some energy requirement.

The transport of L-[^{14}C]lysine in the absence of unlabelled lysine measured in the epithelial-to-endothelial direction decreased as a function of [Na^+] from 0.05 to 0.12 M (Fig. 3). The data shown in Fig. 3 were subjected to Hill analysis in order to estimate the coupling ratio between the radiolabelled L-lysine and Na^+ in a cotransport process (Kim and Crandall, 1988). The Hill plot of the data is depicted in Fig. 4. The slope of the theoretical line shown in Fig. 4 is 0.98 (with $r = 0.99$), indicating that one Na^+ was coupled to one substrate L-[^{14}C]lysine molecule in the cotransport process. Furthermore, the apparent permeability coefficient of L-[^{14}C]lysine, P, was

decreased to 2.34×10^{-6} cm/s (Table 2) by totally substituting sodium with mannitol (0.3 M). These data suggest that transcorneal movement of one L-lysine requires one Na^+ in the cotransport process.

Most tissues and cells appear to have a separate mechanism for transporting cationic amino acids (Lerner, 1978). The aforementioned author reported that the active transport of glycine and aminoisobutyric acid in the rat kidney cortex diminished as the sodium concentration of the medium was decreased. The lysine cotransport process found in this study, however, appears to have one unique feature, in that no other tissue barrier appears to require sodium for lysine transport as much as does the corneal epithelium. For example, lysine transport in intestinal cells and tissues has been shown to be mostly sodium-independent (Munck and Schultz, 1968). In rat enterocytes, only 35% of lysine uptake is sodium-dependent, compared with a strong dependence on sodium (80%) found in this study. In renal epithelium, Samarzija and Fromter (1982) utilized microelectrode techniques to show that

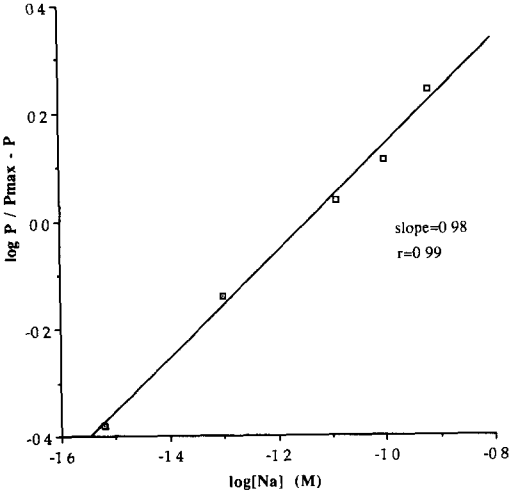


Fig 4. Hill plot of net radiolabelled lysine absorption as a function of sodium concentration. In this analysis, the same maximum apparent permeability coefficient (P_{max}) as that obtained in Fig 2 was used. Solid line was determined by linear regression. The slope of the line corresponds to a Hill coefficient of 0.98, with a correlation coefficient of 0 99, which indicates that the cotransport process requires a 1 1 coupling of lysine and sodium

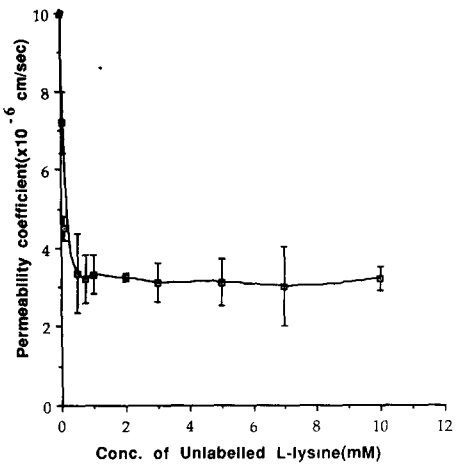


Fig. 5 Effect of unlabelled L-lysine concentration of L-[^{14}C]lysine transcorneal permeation

there may be sodium-dependent lysine transport. For Ehrlich cells, a sodium-dependent heterogeneous exchange mechanism, where the transport of lysine occurs via an exchange of neutral amino acid (A system) and sodium for the cationic amino acids (Lys^+ system), has been postulated (Christensen et al., 1969). It is not clear, however, whether lysine is transported via such an exchange mechanism in corneal tissue.

The D enantiomer of lysine did not reduce the apparent permeability coefficient of L-[^{14}C]lysine, $P = 11.2 \times 10^{-6} \pm 0.2$ vs $10.10 \times 10^{-6} \pm 0.80$ cm/s in control studies. In addition, Fig. 5 illustrates L-[^{14}C]lysine uptake as a function of unlabelled concentration L-lysine (up to 10 mM in the donor solution). The results show that L-[^{14}C]lysine uptake decreased with increasing unlabelled L-lysine until the concentration of unlabelled L-lysine reached 1 mM. These studies suggest that the mechanism of transport of L-lysine appears to involve a stereospecific carrier-mediated system as shown in Fig. 1. Na^+-linked symports, located in the apical domain of the plasma membrane, actively transport L-lysine into the cell, building up a substantial concentration gradient, while Na^+-independent transport proteins in the basal and lateral domain allow L-lysine to leave the cell passively down the concentration gradient. The Na^+-K^+-ATPase that maintains a gradient across the plasma membrane of the tis-

sue is located in the basolateral domain. Furthermore, the space between epithelial cells may be selectively permeable so that the transported molecule cannot easily diffuse back through the intercellular space down the concentration gradient created by the transepithelial transport process (as can be seen in the reverse experiment).

Using microelectrode measurements, Nagel (1981) and Diamond (1978) showed that small charged molecules can move through the intercellular space when the electrical resistance is reduced. The paracellular pathway is lined with negative charges that discriminate against passive movement of anions. In order to block L-lysine transport through the carrier-mediated system, L-[^{14}C]lysine uptake was assessed in the presence of 3 mM unlabelled L-lysine and 3 mM ouabain in the donor solution. The effect, both of saturating the carrier-mediated symport and of blocking the Na^+-K^+-ATPase pump, was to reduce the apparent permeability coefficient to 2.14×10^{-6} cm/s (Table 2). A similar degree of inhibition was observed even at a concentration of 10 mM unlabelled L-lysine, 10 mM ouabain, 3 mM amiloride and no sodium in the donor solution. After blocking the carrier-mediated pathway, the permeability of L-[^{14}C]lysine, with a net positive charge, was decreased about 80%.

Moreno and Diamond (1976) measured the permeability coefficients of 52 nitrogenous cations across the gallbladder epithelial of the frog and rabbit, the values obtained demonstrating a decrease with molecular size and an increase with number of donor protons available for hydrogen-bond formation. They suggested that the main permeation pathway for most hydrophilic cations is across the tight junction with a size sieve. In addition, by using scanning confocal microscopy, Rojanasakul and Robinson (1990) showed that the penetration of poly(L-lysine) (Mol. Wt 15 000–30 000; a net positive charge at pH 7) was localized in the intercellular spaces, as compared with that of insulin (Mol. Wt 5700; net negative charge at pH 7) which was shown to be through the cellular membrane. Thus, the above data suggest that the permeation of L-[^{14}C]lysine, with a net positive charge, uses the paracellular pathway after blocking the carrier-mediated and active pump system.

L-Glutamic acid transport: evidence of passive transport

The permeability of glutamic acid was not affected by the presence of 3 mM ouabain or Na^+-cotransport inhibitor – amiloride on the epithelial side, as seen from Table 3. Although it is

TABLE 3

Glutamic acid (GA). evidence of passive transport

	Apparent permeability coefficient $P \pm SE(\times 10^{-6})$ (cm/s) ($n = 5$)			
(1) L-GA in 0.16 M NaCl [a]	0.90 ± 0 22			
(2) Ouabain (3 mM) effect.	0.80 ± 0.31			
(3) Amiloride (3 mM) effect [b]:	0.83 ± 0.45			
(4) Cold temperature (0–4°C) effect	0.77 ± 0 21			
(5) Na^+ effect [c]	0.9 ± 0.1 (0.12 M)	0.8 ± 0.3 (0.10 M)	1.0 ± 0.2 (0.08 M)	0.9 ± 0.1 (0.05 M)
(6) Mannitol (0.3 M) effect [d]:	1.13 ± 0.25			
(7) D-GA (10 mM) + L-GA effect.	1.02 ± 0.26			
(8) L-GA (10 mM) + L-GA effect:	0.86 ± 0.75			
(9) Ouabain (3 mM), L-GA (10 mM) + L-GA effect [e]	0.85 ± 0.68			

[a] L-GA: L-[^{14}C]glutamic acid (0.6 μCi/ml)

[b] Reagent was incubated in the donor bathing medium.

[c] Sodium concentration was varied in both bathing solutions and sucrose was used to adjust the isotonicity.

[d] Diffusion solution was changed to 0.3 M mannitol instead of 0.16 M NaCl.

[e] 3 mM ouabain and 10 mM L-glutamic acid were incubated on both sides with L-[^{14}C]glutamic acid in the donor solution.

known that the epithelium of the cornea contains high Na^+-K^+-ATPase activity, the results indicate that permeation of L-[^{14}C]glutamic acid does not require energy. In order to confirm that the transport of L-glutamic acid was not an energy-requiring process, a further diffusion experiment was conducted at a temperature around 0–4°C. The result was similar to that of the L-[^{14}C]glutamic acid control studies (t-test: $P < 0.1$) (Table 3). The data indicate that transcorneal movement of L-glutamic acid does not use ATP enhancement or sodium cotransport. Herzberg and Lerner (1973) demonstrated that the transport of some basic and neutral amino acids is sodium-independent. The transport of radiolabelled L-glutamic acid in the absence of unlabelled L-glutamic acid in the epithelial-to-endothial direction did not change significantly as a function of [Na^+] from 0.05 to 0.16 M (Table 3). In addition, the effect of totally substituting sodium with mannitol solution (0.3 M) confirmed the sodium independence.

The transport of L-[^{14}C]glutamic acid appears to be unaffected by the D-enantiomer of glutamic acid (Table 3). Furthermore, Table 3 shows that L-[^{14}C]glutamic acid permeation was not reduced significantly by unlabelled L-glutamic acid (10 mM). Even in the presence of both 3 mM ouabain and 10 mM L-glutamic acid, no effect on the unidirectional permeation of L-[^{14}C]glutamic acid was observed. Thus, L-[^{14}C]glutamic acid transport from the cornea, epithelial-to-endothelial side, occurs probably by a simple diffusion process which is ouabain-insensitive, Na^+-independent and non-stereospecific. The net transport of L-lysine, after blocking the carrier-mediated pathway, was around 2-fold greater than L-glutamic acid permeation (Fig. 6). This difference is similar to that of typical relative permeabilities for cations and anions (permselectivities) of several epithelial tissues as discussed previously (Powell, 1981). Although the chemical identity of the membrane charges cannot yet be stated unequiv-

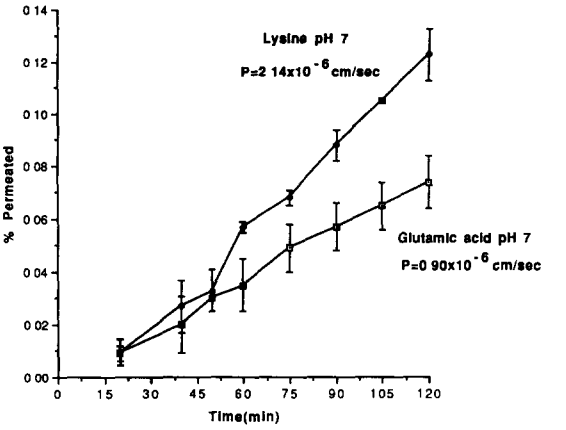


Fig 6. Effect of charge type on corneal transport of L-lysine and L-glutamic acid. After blocking the ATPase pump by ouabain and saturating the carrier-mediated protein by unlabelled L-lysine, the apparent permeability coefficient (P) of L-lysine is still statistically higher than that of L-glutamic acid at pH 7

TABLE 4

Influence of pH on the permeability of L-lysine and L-glutamic acid

	Charge density					Net charge	P ($\times 10^{-6}$) (cm/s) [a]	P with inhibitor [b]
	+ +	+	0	−	− −			
Glutamic acid								
pH 3		15	82	3		0	2.26 ± 0 47	
pH 7				100		−	0 90 ± 0.22	
pH 10				48	52	=	0.54 ± 0 09	
Lysine								
pH 3	8	92				+	2 54 ± 0.31	2.61 ± 0.68
pH 7		100				+	10 1 ± 0.80	2 14 ± 0.61
pH 10		8	76	16		0	5.85 ± 1 07	1.28 ± 0 13

[a] P· apparent permeability coefficient ($\times 10^{-6}$) expressed as cm/s.

[b] Diffusion studies were conducted with 3 mM ouabain and 10 mM unlabelled L-lysine on both sides of the diffusion chamber.

ocally, it has been suggested that (Davison, 1980; Hall and Pribnow, 1989) at physiological pH, this charge results from acidic and basic groups on stromal protein collagen and sulfate groups of the proteoglycans, the two major constituents of all the solid matter of the cornea.

The effect of charge density on corneal transport of lysine and glutamic acid

Both lysine and glutamic acid are zwitterionic, each having three pK_a values, and at pH 7, lysine carries a net positive charge and glutamic acid possesses a net negative charge. If the pH of the medium is changed to 10, lysine becomes neutral and glutamic acid should have two net negative charges. Similarly, if the medium is modified to pH 3, glutamic acid becomes neutral and lysine maintains one net positive charge. The effect of charge density on the corneal transport of lysine and glutamic acid can be assessed at different pH values, as shown in Table 4. At pH 3 and 7, L-lysine has a net positive charge, however, at pH 3 it has a lower permeability than at pH 7. L-Lysine has a neutral charge at pH 10 and has lower permeability than at pH 7. L-Glutamic acid at pH 3 has a net neutral charge and is transported to a greater extent than at pH 7 (has a net single negative charge), or at pH 10 (net double negative charge). This result suggests that the cornea behaves as if it is negatively charged and thus relatively less permeable to L-glutamic acid (pH 7 and 10) than to L-lysine. However, below the isoelectric point (3.2), functional groups become protonated so that the cornea possesses a net positive charge and has the reverse effect on the permeation of these amino acids. Wright and Diamond (1968) have shown that the gallbladder behaves as if it is negatively charged at physiological pH and above the isoelectric point 3.1, and is therefore selectively permeable to cations. However, it is positively charged and selectively permeable to anions at low pH. Furthermore, they suggested that increasing anion permeability at low pH is presumably due to protonation of an amine or some other nitrogen group.

The effect of charge density on L-lysine transport, after blocking the transcellular pathway, was examined. In these experiments, corneas were mounted in the diffusion cell with 3 mM ouabain and 10 mM unlabelled L-lysine, which served as an Na^+-K^+-ATPase inhibitor and saturated the stereospecific carrier-mediated enzyme. Table 4 demonstrates that at pH 10 L-lysine is neutral and has a lower apparent permeability coefficient ($P = 1.28 \times 10^{-6} \pm 0.13$ cm/s) than at pH 7 ($P = 2.14 \times 10^{-6} \pm 0.61$ cm/s). However, at pH 3 and pH 7, lysine contains a net positive charge, the permeability of L-lysine at pH 3 being similar to that at pH 7 (Table 4). A priori one might expect that the permeation should be lower at pH 3, since the cornea behaves as if it is positively charged (pI 3.2) and should be less permeable to a net single positive charge of L-lysine. The low pH may influence the activity of ouabain in the diffusion studies. Thus, the effect of pH 3 bathing medium on 3 mM ouabain was measured in the diffusion studies. The apparent permeability coefficient of L-[^{14}C]lysine decreased to $4.5 \times 10^{-6} \pm 0.61$ cm/s in comparison with that for the case where only L-[^{14}C]lysine was present in the donor solution studies ($P = 10.1 \times 10^{-6} \pm 0.80$ cm/s). These results suggest that the ouabain inhibitor was unaffected by low pH.

Thompson et al. (1970) showed that the transport of sarcosine was stimulated more by low pH than that of glycine and leucine in the rat jejunum. The sarcosine carrier seemed to be very sensitive to the stimulatory effects of low pH. The increased hydrogen ion concentration can act through changing the degree of ionization and hence the affinity either of the carrier for the amino acids or of the amino acids for the site, by increasing the supply of energy to the active transfer mechanisms or by affecting the sodium-binding site on the carrier. They suggested that a low pH had a specific effect on the jejunal transport mechanism (Thompson et al., 1970; Lerner 1978). Adibi et al. (1967) and Oldendorf et al. (1988) noted only a modest reduction of histidine absorption from a lumen tube in the human jejunum when the pH of the perfusate was reduced; the same conditions abolished the net absorption of water, sodium and chloride. However, it remains unclear whether lysine is transported via such an exchange mechanism in corneal tissue.

In summary, this study shows that cornea tissue favors uptake of L-lysine from the epithelial to endothelial direction. Flux of L-lysine occurs via an Na^+-dependent cotransport mechanism (with a coupling ratio of 1 : 1) and is ouabain-sensitive to some extent. However, L-glutamic acid is apparently absorbed by passive diffusion through the cornea. At physiological pH and pH above the isoelectric point (3.2), the cornea behaves as if it is negatively charged and thus relatively less permeable to L-glutamic acid (pH 7 and 10) than L-lysine (pH 7 and 10). Below the isoelectric point, functional groups become protonated so that the cornea possesses a net positive charge and has the reverse effect on the permeation of L-glutamic acid. However, low pH may have a specific effect on the corneal transport mechanism of lysine.

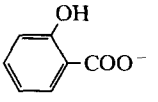
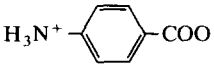
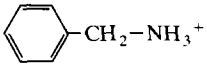
The effect of charge type on corneal transport of salicylic acid, p-aminobenzoic acid and benzylamine

In order to evaluate the corneal permselectivity to cations, three compounds were studied as shown in Table 5. The carboxyl groups of salicylic acid and *p*-aminobenzoic acid are completely ionized at pH 7, whereas the amino group of benzylamine exists 100% in the protonated form at pH 7. Since the three molecular weights are similar, if the cornea shows non-selective permeation, one would expect little difference in their permeabilities. As can be seen from Fig. 7, the apparent permeability coefficients of salicylic acid, *p*-aminobenzoic acid and benzylamine were found to be 0.74×10^{-5}, 1.08×10^{-5}, and 1.76×10^{-5} cm/s, respectively, and are statistically different ($P < 0.01$). Thus, the permeability coefficient of an anionic drug (salicylic acid) was 2.3-times less than that of the cationic drug (benzylamine). This is in agreement with the transport of ions, as measured in the electrophysiology studies. Since the corneal tissue is negatively charged at pH 7, it thus favors permeation of cationic molecules and is relatively less permeable to salicylic acid (anion). In addition, concentration dependence studies were performed separately with the perfusate containing additional unlabelled compound (10 mM) in the donor bathing solution with a constant concentration of radiolabelled compound (1.0 μCi/ml). Transport of the three radiolabelled compounds was not to be affected by the unlabelled compounds, and the apparent permeability coefficients of salicylic acid, *p*-aminobenzoic acid and benzylamine were $0.67 \times 10^{-5} \pm 0.28$, $1.11 \times 10^{-5} \pm 0.14$, and $1.56 \times 10^{-5} \pm 0.09$ (cm/s), respectively. These data suggest that these transport processes are not associated with saturation kinetics.

The donor solutions with various pH values (3, 7, and 10) are detailed in Table 5. At pH 10, salicylic acid and *p*-aminobenzoic acid both be-

TABLE 5

Influence of pH on the permeability of salicylic acid, p-aminobenzoic acid and benzylamine

	Apparent permeability coefficient, P ($\times 10^{-5}$) (cm/s)		
	pH 3	pH 7	pH 10
(1) Salicylic acid  pK_a 3 6, 13 4	2.93 ± 0 20 (0) [b]	0.74 ± 0 06 (–) [a]	0 27 ± 0 02 (–)
(2) *p*-Aminobenzoic acid  pK_a· 4.8, 4 2	2.31 ± 0 35 + / 0 / – [c] 92 7 6 0.4	1 08 ± 0 1 pH 4.5· (0) [d]	0 31 ± 0.24 (–)
(3) Benzylamine  pK_a· 9 6	0 60 ± 0.32 (+)	1 76 ± 0 20 (+)	1 69 ± 0 31 + / 0 [e] 28/72

[a] Representive net charge under different pH conditions

[b] Salicylic acid contains 22% of a single charge form and 78% of a neutral form at pH 3.

[c] *p*-Aminobenzoic acid has four composition charge forms at pH 3 a single positive charge (92%), a completely neutral (1 8%), a net neutral charge (5 8%) and a single negative charge form (0.4%).

[d] At pH 4.5, the molecule also has four different composition forms which are a single positive charge (21%), a completely neutral (11%), a net neutral charge (46%) and a single negative charge form (22%).

[e] Benzylamine contained 28% of a single charge form and 72% of neutral forms.

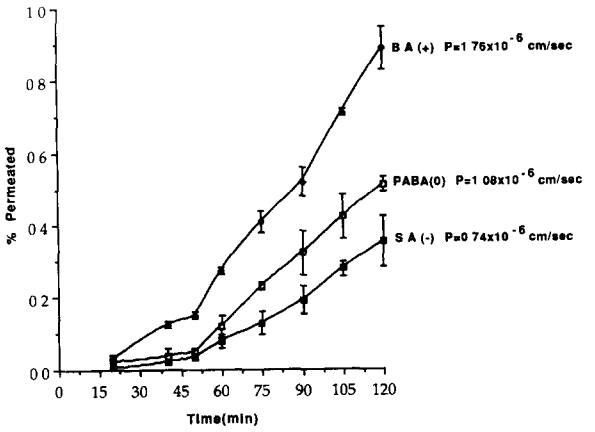


Fig. 7. Effect of charge type on transcorneal absorption of salicylic acid (S.A.), *p*-aminobenzoic acid (PABA) and benzylamine (B.A). Benzylamine has a higher apparent permeability coefficient (P) than that of *p*-aminobenzoic acid (a neutral form at pH 4.5) or salicylic acid (a single negative charge at pH 7 bathing medium). Error bars represent SE, $n = 5$.

come protonated and have a net negative charge. Benzylamine displays greater permeation than salicylic acid and *p*-aminobenzoic acid. On changing the bathing medium to pH 3, benzylamine (maintaining single positive charge form) was found to show less permeation than salicylic or *p*-aminobenzoic acid. *p*-Aminobenzoic acid has four different possible forms at pH 3, namely, a net single positive charge (92%), a neutral form (without any charges, 1.8%), a net neutral form (with a single positive and a single negative charge, 5.8%) and a net single negative charge (0.4%). The major form will be a single positive charge ($\sim 92\%$) and its permeability is higher than at pH 4.5, where the distribution of the forms is a net single positive charge (21%), a neutral form (11%), a net neutral form (46%) and a single negative charge (22%). The result was not expected since, at pH 3, the cornea should behave as if it is positively charged and thus relatively less permeable to positive molecules of *p*-aminobenzoic acid. The pH values of the bathing medium after the diffusion studies (0.16 M NaCl) were between 3.0 and 3.6. These studies indicated that the cornea readily buffers the *p*-aminobenzoic acid solutions from a pH of 3.0 to 3.6. On the other hand, the transport of *p*-aminobenzoic acid, whether it was stimulated more by low pH or involved a specific transport mechanism, is not yet clear.

The data on the effect of pH on the transcorneal permeation of salicylic acid are in agreement with the results on the transport of K^+ and Cl^- as determined via the transport number (K^+/Cl^- ratio, 6:4). The cornea behaves as if it is negatively charged at pH 7 and 10, and is relatively less permeable to anions. At pH below 3.2, it has the reverse effect on the permeation of ions. Benzylamine has a lower permeability coefficient ($P = 1.69 \times 10^{-5} \pm 0.31$ cm/s) at pH 10. This appears to be due to the fact that benzylamine contains a partial positive charge (28%) and partial neutral form (72%) at this pH, and that the permeability is not significantly different between pH 7 and pH 10. These permselectivity properties of the membrane are important from the viewpoint of drug delivery. In other words, depending on the pH of the environment and pI of the membrane, the transport of one species will be favored over that of the other.

Tissue viability and integrity test

Tissue viability and integrity were studied due to their importance in determining the overall transport characteristics of the membrane (Rojanasakul and Robinson, 1989). At the end of each experiment, the bathing solutions were replaced with freshly prepared GBR solutions, and the transmembrane potential difference and resistance were determined at 37°C (Figs 8 and 9). A potential difference, developed when two identical solutions are placed on both sides of a membrane, indicates active ion transport and thus a viable state of the tissue. Membrane resistance, on the other hand, indicates membrane permeability 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 from those at pH 7, 10 or that with GBR solution. However, as soon as the solution was replaced with GBR solution, the potential difference at pH 3 immediately recovered and the membrane resistance increased to a value similar to that of tissue incubated in a GBR solution. In addition, ultrastructural changes of

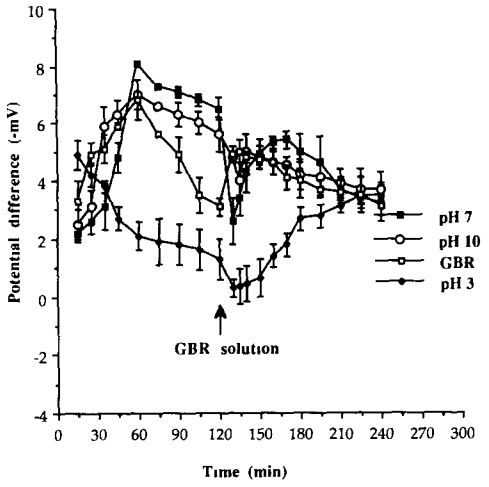


Fig. 8 Transmembrane potential difference in isotonic NaCl with three different pH and GBR solutions

the cornea during in vitro diffusion were also investigated using electron microscopy. Tissues obtained in GBR solution and after a 2 h study were processed for microscopic examination. The results indicate that, in all tissues, the epithelial cell layers remain intact.

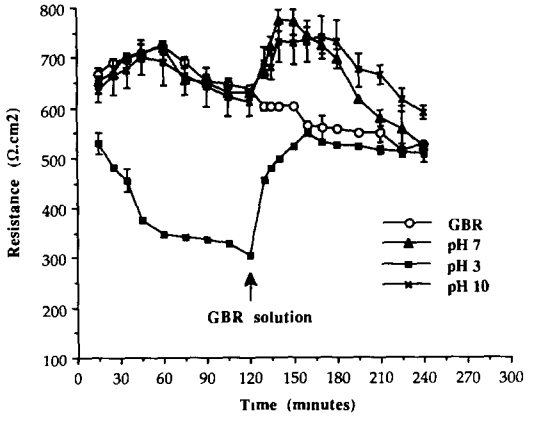


Fig 9 A typical resistance-time profile of the cornea with different pH and GBR solutions The resistance was measured by applying direct current pulses of variable duration (1–5 s) and intensity (up to $\pm 10\ \mu A\ cm^{-2}$) and recording the voltage drop across the cornea Bars indicate 1 SE; $n = 5$ At pH 3, the resistance decreased with decreasing membrane potential However, after changing to GBR solution after 2 h diffusion studies, membrane resistance immediately increased and attained a similar value at about 45 min

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