J. Membrane Biol. 66, 133–144 (1982)

The Journal of
Membrane Biology

Effects of Ag^+ on Ion Transport by the Corneal Epithelium of the Rabbit

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Summary. Exposure of the *in vitro* rabbit corneal epithelium to Ag^+ by the addition of $AgNO_3$ (10^{-7}–10^{-5} M) to the apical surface or by the use of imperfectly chlorided Ag/AgCl half-cells in Ussing-style membrane chambers, greatly increases short-circuit current and transepithelial potential. The early phase (the first 30 min) of the short-circuit current stimulation by Ag^+ is linearly dependent on tear-side sodium concentration, is largely a result of a tenfold increase in net Na^+ uptake and is incompletely inhibited by ouabain, suggesting that Ag^+ increases cation (primarily Na^+) conductance of the apical membrane. This mechanism for the Ag^+ effect is supported by microelectrode experiments, wherein Ag^+ depolarizes specifically the apical barrier potential and increases apical barrier conductance. A later phase in the effect (0.5–3 hr) is characterized by a gradual increase in $^{36}Cl^-$ and ^{14}C-mannitol unidirectional fluxes, by a decline in epithelial resting potential and short-circuit current, by complete ouabain inhibition and by fit to saturation kinetics with respect to Na^+ concentration in the bathing media. This phase of the effect apparently reflects a nonselective opening of the paracellular pathway in the epithelium and is rate-limited by Na^+ pump activity at the basolateral membrane. Both phases are associated with swelling of the corneal stroma and may be rapidly reversed using thiol agents (reduced glutathione and dithiothreitol). The results suggest that Ag^+ may be useful in the study of cation transport by epithelia and the work provides basic physiological information that is pertinent to the prophylactic use of $AgNO_3$ in clinical ophthalmology.

Key words silver ion · cation transport · epithelial transport · corneal epithelium · corneal hydration · *p*-chloromercuriphenyl sulfonic acid · rabbit cornea

Introduction

Sulfhydryl reagents affect membrane permeability in a variety of biological systems. In some cases, notably the erythrocyte membrane, specific actions on cation conductance have been demonstrated (Knauf & Rothstein, 1971). However, no studies involving such agents have been performed using the rabbit corneal epithelium. This tissue, an epithelium with tight apical intercellular junctions, actively secretes Cl^- at a low rate and has low sodium permeability (Klyce, Neufeld & Zadunaisky, 1973; Klyce & Wong, 1977; Marshall & Klyce, 1981*a*; Maurice, 1951). The corneal epithelium also actively absorbs Na^+ (Donn, Maurice & Mills, 1959) at a rate similar to the Cl^- secretion, such that the unstimulated tissue has no net solute transport in the presence of the resting potential (Klyce, 1975). When the Cl^- transport is stimulated (by epinephrine or theophylline) there is net Cl^-, Na^+ and fluid secretion in the presence of resting potential (Klyce, 1975). If, instead, Na^+ absorption is stimulated, as shown in this paper, the net fluxes of Na^+, Cl^- and fluid change to the uptake direction.

Silver ion is an effective sulfhydryl reagent, and one which is particularly important in corneal epithelial physiology. $AgNO_3$ (1% wt/vol) has long been used by pediatric ophthalmologists as a prophylactic agent in neonates (Barsham, 1966), yet little is known of the local impact of the treatment on corneal function.

The present work examines the effects of Ag^+ on ion transport and permeability of the rabbit corneal epithelium. Radioisotope flux experiments in Ussing-style membrane chambers using ^{36}Cl, ^{22}Na, ^{86}Rb, and ^{14}C-mannitol help to characterize the Ag^+ effects on cellular ion transport and on the permeability of the paracellular pathway. Corneal thickness measurements *in vitro* and *in vivo* demonstrate effects of Ag^+ on corneal hydration. Intracellular voltage and resistance measurements are used to establish the cellular location of Ag^+ action. Finally, the effects of Ag^+ are compared to those of specific sulfhydryl reagents and of amino and thiol reagents to investigate possible group-specific sites involved in the Ag^+ response.

Materials and Methods

New Zealand White rabbits (3.5–4.5 kg) were killed by injection of sodium pentobarbital. Eyes were enucleated, and the corneas dissected and mounted in Lucite® chambers (exposed corneal surface = 1.0 cm^2). The initial experiments (reported in Figs. 1 and 2) were carried out in a chamber that employed Ag/AgCl half-cells in direct contact with the bathing solutions (Klyce, 1971). Subse-

0022-2631/82/0066-0133 $02.40

quent experiments used chambers with intervening agar-Ringer's solution bridges (Klyce, 1972; Klyce et al., 1973). To enhance the penetration of ouabain from the endothelial side and for ^{14}C-mannitol fluxes, the endothelium was removed during the mounting procedure by carefully peeling off Descemet's membrane; otherwise, the experiments used corneas with the endothelium intact.

Because the present work involved the use of trace quantities of heavy metal ions to modify transport properties of the cornea, special care was taken to ensure that glassware, membrane chambers and associated materials were decontaminated of heavy metals.

Ringer's Solutions

The normal and low-Cl^- (sulfate) Ringer's solutions used in the Ussing-style chambers have been described previously (Klyce & Wong, 1977). Low-Na^+ Ringer's was prepared using TRIS-SO_4 and TRIS-Cl (pH 7.4) to replace Na_2SO_4 and NaCl, respectively; to make low-K^+ Ringer's, TRIS phosphate buffer (pH 7.4) was substituted for potassium phosphate buffer. The normal Ringer's solution used for the microelectrode experiments consisted of (in mM): 99.7 NaCl, 3.7 KCl, 26.0 glucose, 7.0 Na_2SO_4, 20 $NaHCO_3$,* 0.6 K_2HPO_4, 25 HEPES-Na (pH 7.4), 1.4 Ca-gluconate and 0.6 $MgSO_4$. Low-Cl^- Ringer's was similar except that NaCl and KCl were replaced by Na_2SO_4 and potassium gluconate, respectively, and sufficient sucrose was added to maintain a consistent osmolarity of 305 mOsm. All Ringer's solutions had a final pH of 7.4; tissues were incubated at 35 °C.

Pharmacological Agents

Stock solutions of the heavy metal ions Cu^{2+} and Cd^{2+} were prepared with Ringer's solution; that for $AgNO_3$ was made with distilled water. The sulfhydryl reagents [N-ethylmaleimide (NEM), *p*-chloromercuribenzoate (PCMB) and *p*-chloromercuriphenyl sulfonic acid (PCMBS)] and the thiol reagents [dithiothreitol (DTT; Cleland's reagent; Cleland, 1964) and reduced glutathione (GSH)] were obtained from Sigma Chemical Co. and were prepared in Ringer's solution. Dithio-bis-2-nitrobenzoate (DTNB; Sigma Chemical Co.) was dissolved in 10 µl of 95% ethanol and appropriate ethanol controls were performed. The amino reagent, 2-methoxy-5-nitrotropone (MNT; Vega Biochemicals), and amphotericin B (Fungizone, E.R. Squibb and Sons), which included sodium desoxycholate and a phosphate buffer, were dissolved in Ringer's solution. Unless otherwise specified, these agents were prepared fresh daily and were added only to the tear-side bathing solution. Ouabain (Sigma Chemical Co.) was prepared as a stock solution in Ringer's just prior to addition to the stromal-side bathing solution.

Radioisotope Fluxes

Unidirectional fluxes of radiotracers were measured by incorporating ^{36}Cl (3 µCi/ml), ^{86}Rb (0.8 µCi/ml), ^{22}Na (2 µCi/ml) or ^{14}C-mannitol (6 µCi/ml) into the Ringer's solution. Nonradioactive mannitol (2.0 mM), or RbCl (1.0 mM) were added to the Ringer's solutions for the respective fluxes of these substances. For the flux determinations, aliquots of 250 µl were removed from the less radioactive sides of the paired corneas at intervals of 30 min and, in each case, the sample volume was replaced with fresh Ringer's. Three samples (10 µl each) were taken from the more radioactive sides, at 30, 120 and 270 min. The samples were mixed with Bray's solution and counted in a liquid scintillation counter (Nuclear-Chicago, model Unilux III). Unidirectional and net fluxes were calculated as previously described (Klyce et al., 1973).

Corneal resting potential ($V_c = V_{stroma} - V_{tears}$) was clamped to zero mV using automatic voltage clamps (H.Fein, Yale University); short-circuit current (SCC) was monitored continuously. To measure V_c during the flux experiments, the corneas were released from the voltage clamp for 3 sec every 30 min.

Microelectrode Experiments

The microelectrode chamber and associated instrumentation and mounting techniques were similar to those reported previously (Klyce & Wong, 1977). Microelectrodes were filled with 1.0 M potassium-citrate and had tip resistances of 30–60 MΩ, measured in Ringer's solution. The microelectrodes were manipulated with the aid of a digitally operated hydraulic microdrive (David Kopf Industries); the microdrive was in turn controlled manually or with a control circuit (Marshall & Klyce, 1981*b*) which was devised to advance the microelectrode continuously through the bathing medium until a cell was impaled.

Most intracellular recordings were from basal cells of the corneal epithelium at open circuit. Impalements of these cells were identified by previously established criteria (Klyce, 1972) and were stable for long periods (15–30 min), during which the time course of the effect of Ag^+ (added to the tear-side bathing solution) and its reversal by a reducing agent could be monitored. The measured parameters were: transcorneal potential (V_c), apical barrier potential ($V_a = V_{cell} - V_{tears}$), basal barrier potential ($V_b = V_{cell} - V_{stroma}$) and the respective conductances G_c, G_a and G_b.

Corneal Thickness Measurements

The effect of Ag^+ on stromal thickness *in vitro* was examined using a specular microscope (Maurice, 1968; Dikstein & Maurice, 1972). Corneas were mounted in a Lucite® chamber (Klyce, 1972) and, to examine the influence of the epithelial barrier on corneal thickness, fluid transport across the endothelial side was blocked by bathing this surface with silicone oil, while the epithelial surface was bathed in Ringer's solution (Klyce, 1975). Under these conditions, the corneal stroma thins slowly, a result of active secretory transport of Cl^- (Klyce, 1975). The effects of Ag^+ and reduced glutathione were examined by adding these substances to the tear-side bathing solution while corneal thickness was monitored continuously with the specular microscope.

Measurements of corneal thickness *in vivo* in response to topical $AgNO_3$ were performed using a Maurice-Giardini pachometer (Maurice & Giardini, 1951) that was mounted on a slit lamp (Haag Streit, model 360). The rabbits were sedated with thorazine and, between measurements, their eyes were held closed by clamping the lashes. After a 6–7 hr control period, 3–4 drops of 1% $AgNO_3$ were applied topically to one eye for 1 min, and the eye rinsed thoroughly with normal Ringer's solution.

Results

Stimulation of Corneal Potential with Ag/AgCl Half-cells

Ag/AgCl half-cells are often used in Ussing-style membrane chambers to pass transepithelial currents; in some cases, they are placed in direct contact with the bulk solutions bathing the tissue. These electrodes are easily produced, are stable, and are particularly useful in multiple electrode experiments, such as voltage clamp studies of squid giant axon (Hodgkin, Hux-

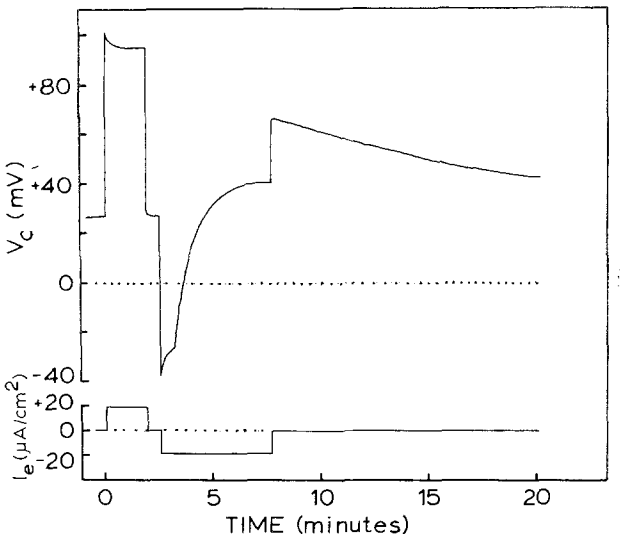

Fig. 1. Effect of sustained hyperpolarizing and depolarizing currents (I_e) on corneal potential (V_c). I_e pulses were appied via Ag/AgCl half-cells in the chamber; note the large biphasic response of V_c to the depolarizing pulse. (Redrawn from original)

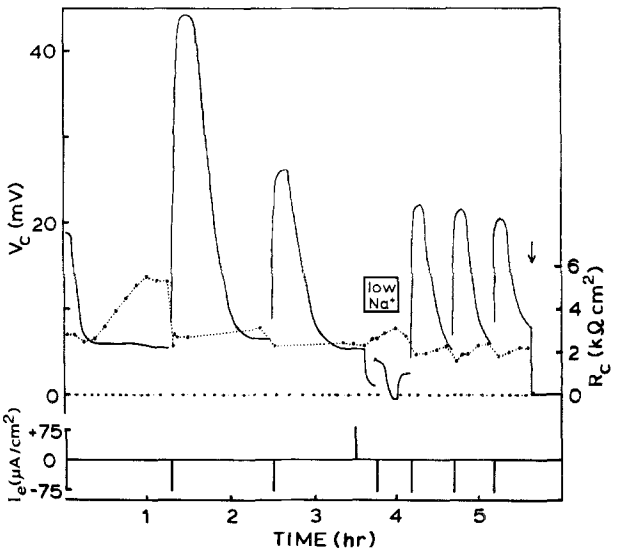

Fig. 2. Effect of repeated depolarizing (−) or hyperpolarizing (+) current pulses (I_e; 75 μA/cm² for 3 sec) on corneal potential (V_c, solid line) and resistance (R_c, broken line). Pulses were applied via Ag/AgCl half-cells; note the reduction in size and reversed polarity of the V_c response to depolarizing pulses when low-Na^+ Ringer's bathed the tear-side. V_c and R_c are primarily characteristics of the epithelium, as abrasion of the epithelium (arrow) reduced these parameters effectively to zero. (Redrawn from original)

ley & Katz, 1952) and of isolated epithelia (e.g., rabbit corneal epithelium: Donn et al., 1959; Klyce, 1971; bovine corneal epithelium: Lindemann, 1968; and frog skin: Curran, 1972).

Sustained hyperpolarizing currents, applied via Ag/AgCl half-cells, produced only minor polarization effects (*cf.* Kidder & Rehm, 1970) in transcorneal potential (time constant = 15 sec) and the resting potential was unchanged after the current pulse (Fig. 1). In contrast, depolarizing currents (greater than or equal to the short-circuit current) caused large biphasic increases in transcorneal potential (Fig. 1), and in transcorneal conductance and short-circuit current (*not shown*). The effect on the corneal potential commenced approximately 30 sec after application of the depolarizing current, was a first-order phenomenon and had a time constant of 50 sec (Fig. 1). Open-circuit potential after the depolarizing pulse approximately doubled to 45 mV (Fig. 1), and transcorneal conductance increased by 250%, yielding an increase of 450–500% in the calculated short-circuit current (*not shown*).

Repeatable transient increases in corneal potential were elicited using 3-sec 75 μA/cm² depolarizing current pulses (Fig. 2). These transient potential "spikes" were dependent on the presence of Na^+ in the tear-side bathing solution, as the substitution of TRIS for Na^+ on the tear-side greatly reduced the magnitude and reversed the polarity of the change in transcorneal potential (Fig. 2). This would suggest that the origin of the potential "spikes" was related to sodium conductance of the corneal epithelium.

Because depolarizing, but not hyperpolarizing, currents would tend to drive Ag^+ from the tear-side Ag/AgCl half-cell, thereby introducing small quantities of Ag^+ to the tear-side bathing solution, agar-Ringer's bridges were interposed between the half-cells and the bathing solutions. Under these conditions, depolarizing currents did not evoke the large potential, conductance and short-ciruit current responses seen previously (*above*). It was concluded that depolarizing currents *per se* were not responsible for the large Na-dependent changes; rather, the effects appeared to be associated with the exposure of the tear-side of the cornea to Ag^+. Therefore, the effects of controlled applications of Ag^+ (as $AgNO_3$) were examined, using corneas in membrane chambers with Ag/AgCl half-cells that were not in direct contact with the bathing solutions.

Effect of Ag^+ on Corneal Epithelial Ion Transport

Effect on Corneal Potential and SCC. The addition of Ag^+ (as $AgNO_3$, 10^{-7}–10^{-5} M) to the tear-side bathing solution initiated, within seconds, a stimulation of corneal SCC and V_c (Fig. 3). The threshold of the response was approximately 10^{-7} M, the ED_{50} was 2.0×10^{-6} M, and the maximally effective dose

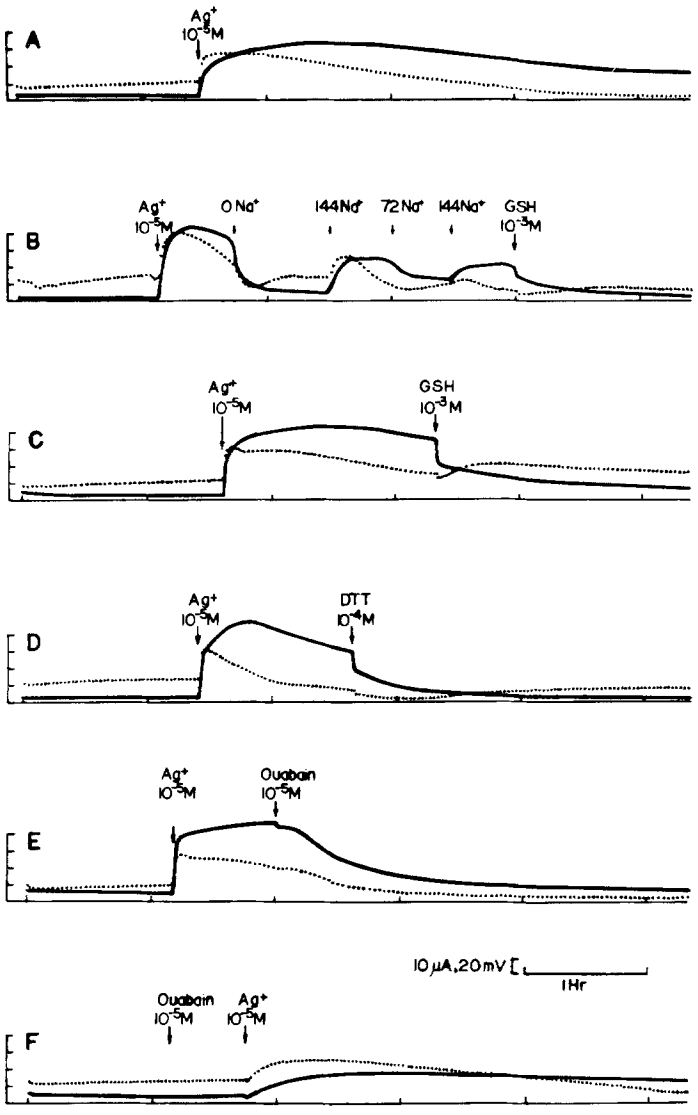

Fig. 3. Response of corneal potential (broken lines) and short-circuit current (solid lines) to 10^{-5} M Ag^+. Addition of Ag^+ to the tear-side increased short-circuit current from a resting level of 3–5 μA/cm² to 35–55 μA/cm² (Fig. 3*A–E*). The Ag^+ stimulation was abolished by removal of tear-side Na^+ (Fig. 3*B*) and by addition of reduced glutathione (GSH; Fig. 3*B, C*), dithiothreitol (DTT; Fig. 3*D*) and ouabain added during the late phase of the effect (Fig. 3*E*). Ouabain, added before Ag^+, did not entirely block the Ag^+ response (Fig. 3*F*). (Redrawn from original)

was 10^{-5} M (Klyce, 1976). The latter (highest) dose was used routinely in subsequent experiments.

The short-circuit current increased in a biphasic manner, reached a maximum of 35–55 μA/cm², and remained above control levels (3–5 μA/cm²) for several hours (Fig. 3*a*). Corneal potential increased for the first 20–30 min, but subsequently, 2 hr after Ag^+ addition, dropped to levels lower than the control period (Fig. 3*a*). The Ag-stimulated transport current and potential were sensitive to tear-side sodium concentration, as total or partial replacement of Na^+ with TRIS in the Ringer's solutions markedly reduced the stimulated SCC and V_c (Fig. 3*b*).

Agents that would reduce Ag^+ [reduced glutathione and Cleland's reagent (dithiothreitol)], reversed the SCC and V_c effects of Ag^+ treatment. Both reducing agents caused an initial rapid drop in SCC and V_c (Fig. 3*b, c, d*), with a time course of a few minutes; this was followed by a slower phase during which the corneal potential recovered completely. These agents, by themselves and at the concentrations used to reverse the Ag^+ response, had little effect on the resting corneal potential and short-circuit current.

Effect of Ouabain. Ouabain (10^{-5} M), added to the stromal-side bathing solution before or after the addition of Ag^+, reduced the V_c and SCC effects of Ag^+ (Fig. 3*e, f*). This suggested the involvement of (Na^+, K^+)-activated, Mg^{2+}-dependent, adenosinetriphosphatase [(Na^+, K^+)-ATPase, EC 3.6.1.3] in the ion transport effects of Ag^+. A comparison (Table 1) of the dose-response for ouabain inhibition of SCC during the early (*ca.* 0–30 min) and late (0.5–3.0 hr) phases of the Ag^+ biphasic effect suggested that the Na^+ pump, as inhibited by ouabain, was more impor-

Table 1. Effect of ouabain on the Ag^+-stimulated corneal short-circuit current.

Time	Ouabain (M)	Increase in short-circuit current ($\mu A/cm^2$)	Inhibition (%)
Peak response	0	$+39.0 \pm 2.3$[a] (10)	–
	10^{-7}	$+40.5 \pm 1.9$ (4)	0
	10^{-6}	$+25.0 \pm 2.5$* (13)	36
	10^{-5}	$+19.9 \pm 2.4$* (8)	49
	10^{-4}	$+10.4 \pm 2.0$* (6)	73
Late phase	0	$+23.1 \pm 1.0$ (10)	–
	10^{-7}	$+17.8 \pm 2.7$ (5)	22
	10^{-6}	$+ 6.0 \pm 2.6$* (5)	74
	10^{-5}	$+ 1.3 \pm 1.0$* (6)	94
	10^{-4}	$- 0.3 \pm 1.3$* (4)	100

[a] Values are expressed as the mean ± SEM (number of corneas). All corneas were treated with 10^{-5} M $AgNO_3$

* $P < 0.05$, t-test of ouabain-treated corneas *vs.* controls.

tant in the latter part of the Ag^+ effect. In the early phase of the Ag^+ response, ouabain (at concentrations as high as 10^{-4} M) only partially blocked the Ag^+-stimulated SCC or V_c (Table 1); the ID_{50} was approximately 1.0×10^{-5} M, a value much higher than is generally seen for ouabain inhibition of (Na^+, K^+)-ATPase. This suggested that the Na^+ pump was not rate-limiting during the early phase of the Ag^+ response. In contrast, the ID_{50} for ouabain inhibition of the latter part of the Ag^+ effect, 2.6×10^{-7} M, agreed with that determined for ouabain-induced corneal swelling (3×10^{-7} M; Mishima, Kaye, Takahashi, Kudo & Trenberth, 1969), a process that is dependent on the (Na^+, K^+)-ATPase activity in the endothelium. As would be expected for a process that is limited by the Na^+ pump, complete inhibition of the late phase of the Ag^+ effect on SCC was obtained with ouabain (Table 1).

Ionic Dependence of the Stimulated Current. The effects of bilateral removal of Na^+, Cl^-, or K^+ on the Ag^+-stimulated V_c and SCC (Table 2) suggested that Na^+ was the most important ion involved. In these experiments, the ion-substituted Ringer's solution was introduced into the half-chambers approximately 90 min before the addition of 10^{-5} M (final concentration) $AgNO_3$. Whereas low-Na^+ Ringer's solution greatly reduced the peak V_c and SCC following Ag^+ addition, low-K^+ and low-Cl^- Ringer's solutions had little effect (Table 2). Unlike the control tissues, those that were bathed in low-Cl^- or low-K^+ were not able to maintain for long periods (100–200 min) the high transport rate after Ag^+ treatment. In the case of low-Cl^-, this is probably related to a specific or nonspecific dependence of Na^+ transport on Cl^-. For the membranes that were bathed in low-K^+ Ringer's solution, it is likely that cell metabolism was compromised by partial depletion of intracellular K^+, so that the high transport rate could not be maintained.

These results, which suggested that the Ag^+ effect during the first 30 min primarily involved an opening of a Na^+-conductive pathway, were investigated further by means of a kinetic approach.

Kinetics of Na^+-Dependence of the Ag^+-Stimulated Current. It was found that, in the early phase of the Ag^+ response, the SCC was a linear function of the tear-side sodium concentration (Fig. 4). This result was consistent with the hypothesis that a Na^+ conductance change dominated the early part of the response to Ag^+. In contrast, the later part of the Ag^+ effect on the SCC exhibited saturation kinetics with respect to Na^+ concentration in the bathing solutions (Fig. 5). This phase of the Ag^+ response was therefore dependent on a saturable process involving Na^+, most likely the Na^+ pump. It is clear that Ag^+ evokes

Table 2. Effect of ionic substitution on the Ag^+-stimulated corneal potential and short-circuit current

Ringer's solution	Control (before treatment)		Maximum response		Time after Ag treatment			
					100 min		200 min	
	SCC ($\mu A/cm^2$)	V_c[a] (mV)	SCC ($\mu A/cm^2$)	V_c (mV)	SCC ($\mu A/cm^2$)	V_c (mV)	SCC ($\mu A/cm^2$)	V_c (mV)
Normal	3.5 ± 0.5[c]	20.3 ± 2.2	36.1 ± 1.2	53.5 ± 3.4	32.8 ± 1.1	29.8 ± 5.1	21.8 ± 1.0	8.5 ± 1.7
Low[b] Cl^-	1.9 ± 0.2*	27.1 ± 2.0*	36.6 ± 2.6	64.3 ± 2.3*	18.4 ± 2.0*	17.2 ± 3.9	15.6 ± 1.5*	16.5 ± 2.7*
Low K^+	2.5 ± 0.2	25.8 ± 3.1	29.6 ± 2.0*	59.8 ± 4.6	13.0 ± 1.0*	10.1 ± 1.9*	10.3 ± 1.4*	3.6 ± 1.1*
Low Na^+	2.2 ± 0.2*	10.7 ± 1.6*	6.4 ± 1.2*	18.6 ± 0.8*	5.4 ± 0.9*	6.4 ± 1.5*	–	–

[a] Abbreviations: V_c = resting corneal potential; SCC = short-circuit current.

[b] Ringer's solutions designated as "low" contained less than 10^{-4} M of the specified ion.

[c] Values are expressed as the mean ± SEM ($n = 6$).

* $p < 0.05$, t-test, compared to normal Ringer's.

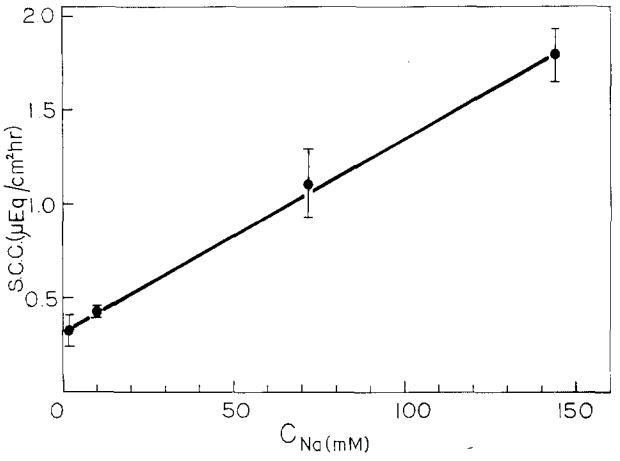

Fig. 4. Plot of maximal short-circuit current (SCC) reached after Ag^+ (10^{-5} M) treatment *vs.* Na^+ concentration in the tear-side bathing solution. Note the linear relationship, indicating a permeability limited, nonsaturable dependence of the early part of the Ag^+ response on tear-side Na^+ concentration. Drawn is the least-squares line of best fit. (Points are mean ± SEM; $n = 5$–7 for each)

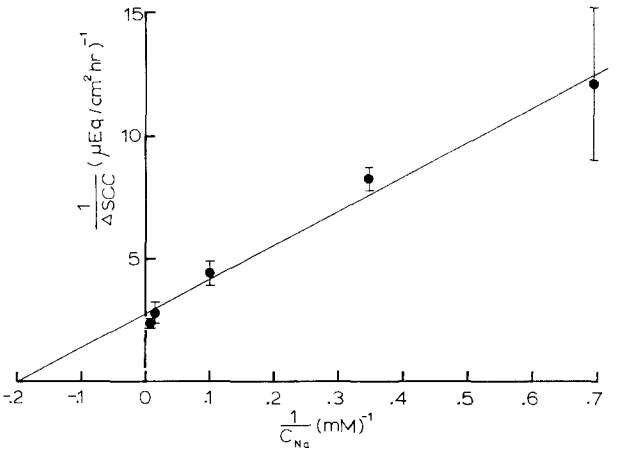

Fig. 5. Double-reciprocal plot of short-circuit current (SCC) response during the late phase of the Ag^+ effect *vs.* Na^+ concentration in the bathing solutions. During this phase, the SCC response is saturable, with an apparent half-maximal stimulation at 5 mM Na^+, suggesting that this phase is rate-limited by the Na^+ pump. Drawn is the least-squares line of best fit. (Points are mean ± SEM; $n = 5$–7 for each)

changes in sodium conductance of the epithelium, but could also have additional transport effects. Hence, the specificity of the Ag^+ effect and its relation to Cl^- transport were examined using radioisotope flux experiments.

Radioisotope Fluxes of Na^+, Cl^- and Rb^+. The unidirectional influx (tears-to-stroma) of $^{22}Na^+$ increased to approximately 16 times control levels within 1 hr after treatment of the tear-side bathing solution with

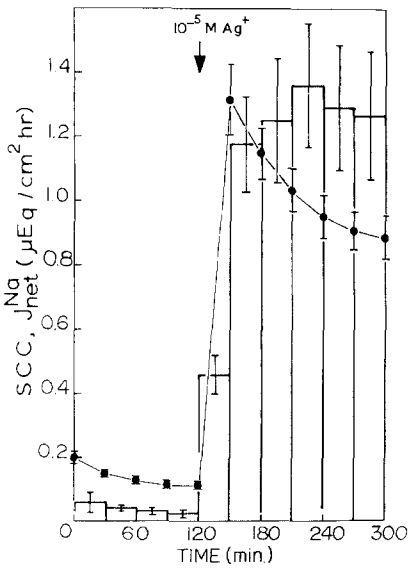

Fig. 6. Effect of Ag^+ (10^{-5} M) on net flux of $^{22}Na^+$ (histogram) and short-circuit current (SCC; solid line). Note the rapid, sustained increases in Na^+ net flux (uptake) and SCC. (Mean ± SEM; $n = 6$)

10^{-5} M $AgNO_3$. The Na^+ efflux increase during this period was less, approximately fourfold. The net flux (uptake) of sodium increased approximately 30-fold (Fig. 6), and accounted for a large part of the elevated SCC. Inasmuch as the enhanced Na^+ uptake continued for several hours in the absence of transcorneal electrochemical gradients (Fig. 6), it must have been maintained by cell metabolism. Whereas this experiment demonstrated clearly that the major constituent of the increased SCC after Ag^+ treatment was a result of Na^+ transport, the specificity of the effect was tested using $^{86}Rb^+$, a tracer for K^+.

The net flux of $^{86}Rb^+$ (uptake) under control conditions was significantly greater than zero, but was relatively small (0.001 μeq/cm² hr). Addition of $AgNO_3$ produced a rapid, marked increase in the stroma-to-tears unidirectional flux and in the net flux, yielding an apparently active Rb^+ secretion of 0.02–0.04 μeq/cm² hr (Fig. 7). The Rb^+ efflux reached a stable maximum approximately 30 min after Ag^+ addition; the respective influx increased more slowly. The stimulation of Rb^+ transport by Ag^+ demonstrated that the Ag^+ effect was not totally specific to Na^+ and that there may be a K^+-dependent component, assuming that $^{86}Rb^+$ is a representative tracer for K^+ in the cornea. Whereas the increase in Rb^+ conductance as a result of Ag^+ treatment was larger than the increment in Na^+ conductance, the total contribution of the Rb^+ and presumed K^+ transport to the SCC would be much smaller.

The corneal epithelium normally transports Cl^- in the secretory direction (Klyce et al., 1973). This

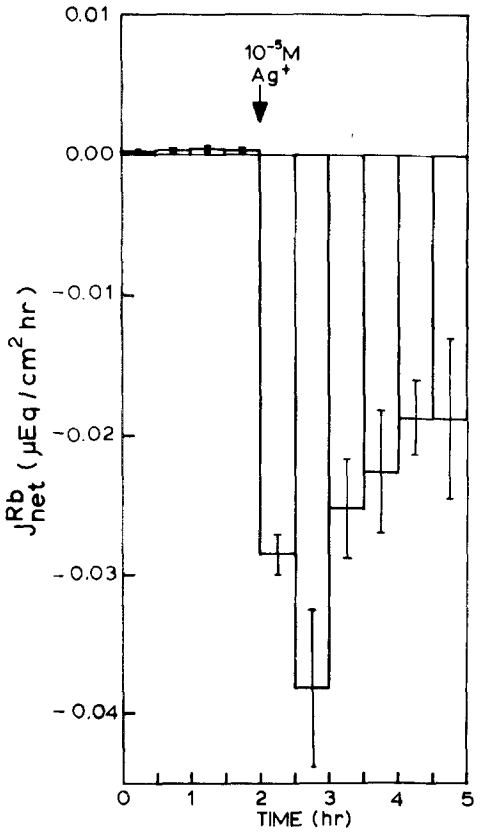

Fig. 7. Net flux of $^{86}Rb^+$ before and after addition of 10^{-5} M Ag^+; note that Ag^+ reversed the net flux and that the stimulated Rb^+ current (secretion) is relatively small (0.02–0.04 μeq/cm² hr), compared to the total Ag-stimulated increase in short-circuit current (*cf.* Fig. 6). (Mean ± SEM; $n=5$)

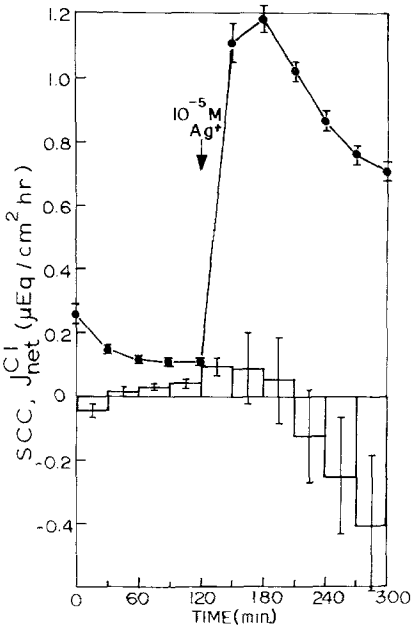

Fig. 8. Net flux of $^{36}Cl^-$ (histogram) and short-circuit current (SCC; solid line) before and after addition of 10^{-5} M Ag^+; note that the Cl^- net flux changes little during the first hour after Ag^+ treatment and that there is a trend to Cl^- uptake in the late phase of the Ag^+ effect. (Mean ± SEM; $n=5$)

net flux is slowly inhibited by the addition of Ag^+ (Fig. 8). In this study, unidirectional $^{36}Cl^-$ fluxes approximately doubled within the first 30 min after Ag^+ addition, from 0.06 ± 0.01 and 0.11 ± 0.01 to 0.11 ± 0.03 and 0.21 ± 0.04 μeq/cm² hr ($n=5$), for the influx and efflux, respectively. In the later phase of the Ag^+ effect, 2 hr after Ag^+ treatment, the Cl^- influx and efflux rose to 5–10 times control levels (0.80 ± 0.17 and 0.56 ± 0.21 μeq/cm² hr, influx and efflux, respectively), and there was a trend toward an inward net Cl^- flux (Fig. 8). The sum of the sodium and chloride currents from the flux experiments was approximately equal to the measured SCC. The time course of the Ag^+ effect on Cl^- transport was longer than that for the stimulation of Na^+ uptake (*above*), suggesting that the Cl^- transport effect could be related to a change in the permeability of the shunt.

Effect of Ag^+ on the Paracellular Pathway

The importance of intercellular "tight" junctions in the transport characteristics of epithelia is well-recognized (e.g., Frömter & Diamond, 1972). To examine the possible influences of Ag^+ on this pathway, ^{14}C-mannitol fluxes were measured. Mannitol is largely excluded from cells and may be used as an extracellular marker in studies of shunt pathways in epithelia (e.g., Dawson, 1977).

Ag^+ treatment slowly increased the permeability of the cornea to mannitol; after 3 hr both unidirectional mannitol fluxes were approximately ten times control levels (Fig. 9). In contrast, control corneas had essentially stable mannitol permeability for up to 5 hr (Fig. 9). This would suggest that Ag^+ slowly increases the permeability of the paracellular pathway. Because the increase in the Cl^- unidirectional fluxes followed a time course similar to that of the mannitol fluxes, it would appear that the Cl^- flux increases occurred in a shunt pathway. As the time courses for the increases in Na^+ and Rb^+ fluxes were much shorter than those of mannitol and Cl^-, these effects appeared to be associated with the transcellular pathway, rather than the shunt.

On the basis of the electrophysiological and flux experiments, we propose that Ag^+ has this dual effect: a rapid enhancement of cation conductance in the cellular pathway (early phase), and a slower, nonselective increase in the permeability of the paracellular pathway (late phase). Of primary interest in the response of the corneal epithelium to Ag^+ was the early phase, where cation fluxes (Na^+ and Rb^+) were enhanced, apparently as a result of increased apical

membrane conductance. In accord with this, the ouabain inhibition experiments (*above*) suggested a relative independence of the effect from the activity of the sodium pump. This early phase of the Ag^+ effect was studied using voltage-measuring microelectrodes to determine whether the effect was primarily associated with changes in the conductance of the apical barrier of the corneal epithelium.

Effect of Ag^+ on Membrane Voltage and Conductance

Voltage-measuring microelectrodes were used to trace intracellular potential and the conductance of the outer and inner barriers of six corneal epithelia during stimulation of the tissue with Ag^+ and the reversal of the Ag^+ effect with reduced glutathione. In this way, it was possible to determine the major cellular locus for the action of this ion. Addition of Ag^+ (2–10 μl of 10^{-3} M $AgNO_3$, to yield final concentrations of $0.2–1.0 \times 10^{-5}$ M) markedly depolarized the resting potential of the apical barrier (V_a), increased greatly the conductance of this barrier (G_a), and produced the typical changes in V_c and G_c (Fig. 10). No change was measured in V_b, and G_b increased only slightly. A specific increase in apical membrane Na^+ conductance would depolarize V_a, increase G_a, and decrease G_b, as observed. The decrease in G_b, the measured conductance across the basal barrier, does not necessarily imply that basolateral membrane conductance has decreased; rather, G_b decreased likely as a result of a larger fraction of the measuring current passing through the cellular pathway after Ag^+ treatment (*cf.* Klyce & Wong, 1977). V_a started to depolarize within 10 sec of the addition of Ag^+, suggesting that the ion acted very close to the tear-side surface of the epithelium. Therefore, during the early phase of the response, Ag^+ apparently acted at the apical membrane surface. The addition of reduced glutathione rapidly reversed the effects of Ag^+ (Fig. 10). The rapid initiation of the effect of reduced glutathione is also consistent with the notion that that Ag^+ acted at or near the apical membrane. Reversal of the effect could also be obtained, albeit more slowly, by thorough rinsing of the tear-side with Ringer's solution.

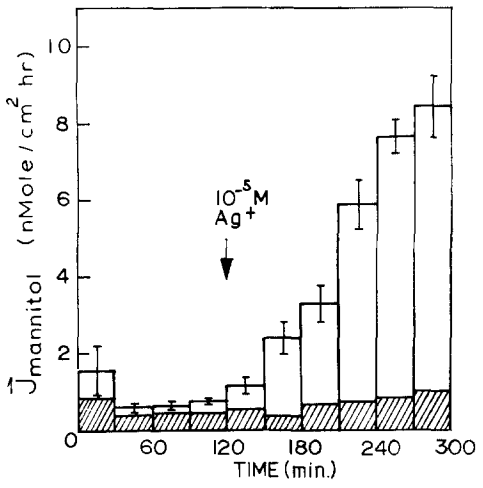

Fig. 9. Effect of Ag^+ (10^{-5}M) on ^{14}C-mannitol unidirectional fluxes (open histogram); note the gradual increase in mannitol fluxes after Ag^+ treatment, suggestive of an opening of the paracellular pathway. (The mean of six fluxes is shown: three effluxes and three influxes, ±SEM). A control cornea (hatched histogram) had relatively stable mannitol fluxes throughout the experimental period

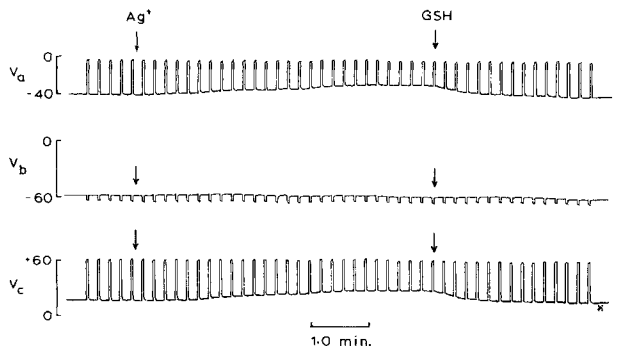

Fig. 10. Representative experiment with a microelectrode, showing outer barrier potential (V_a, upper panel), inner barrier potential (V_b, middle panel), and corneal resting potential (V_c, lower panel). Voltage deflections are responses to transcorneal hyperpolarizing currents of 5.0 μA/cm². Note that the addition of 10^{-5} M Ag^+ rapidly depolarized V_a and reduced outer barrier resistance. The Ag^+ response was totally reversed by the addition of 10^{-3} M reduced glutathione (GSH). (Redrawn from original)

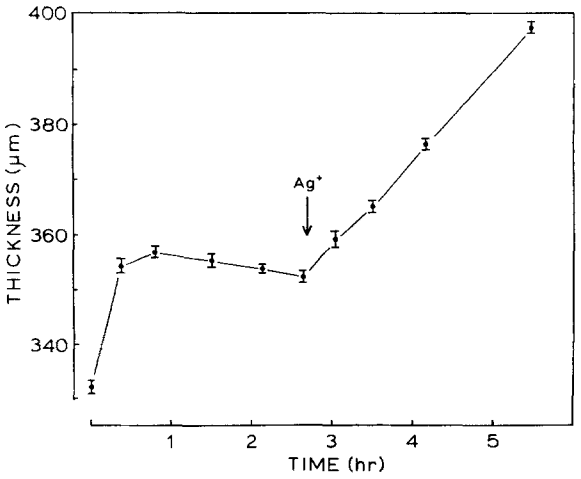

Fig. 11. Example of the effect of Ag^+ (10^{-5} M) on corneal stromal swelling rate *in vitro*. Under control conditions, the cornea thinned slowly; Ag^+ addition (arrow) initiated stromal swelling at a relatively constant rate. Error bars reflect measurement error

Ag^+ Effects on Corneal Thickness

Measurements in Vitro. It would be expected that Ag^+ treatment should lead to stromal edema because Ag^+ stimulates absorptive Na^+ transport and inhibits Cl^- secretion by the corneal epithelium (Figs. 6 and 8). Under control conditions, normal epithelial solute transport thinned the cornea at a rate of 1.7 ± 1.0 μm/hr ($n=6$). The addition of Ag^+ rapidly induced stromal swelling (Fig. 11), and the swelling rate was relatively constant (18.1 ± 1.5 μm/hr, $n=6$). The paired difference between control and Ag^+-treated corneas was 19.8 ± 1.2 μm/hr, which corresponds to a solute flow of approximately 0.6 μeq/cm² hr, assuming isotonic fluid transport. The corneal swelling induced by Ag^+ was reversed partially by the addition of reduced glutathione (*cf.* Klyce, 1975, Fig. 6).

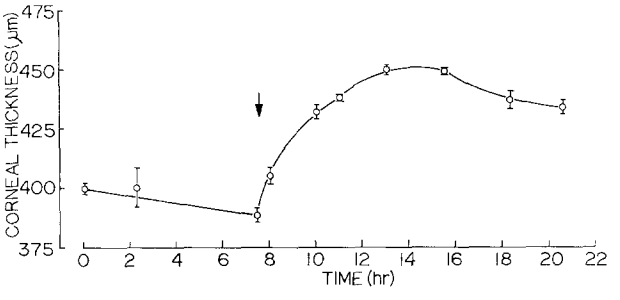

Fig. 12. Example of the effect of Ag^+ (3–4 drops of 1.0% $AgNO_3$ over a 1-min period, similar to the dose used clinically) on thickness of rabbit cornea *in vivo*; note that the Ag^+ treatment (arrow) induced moderate, sustained corneal edema. Error bars reflect measurement error

Measurements in Vivo. To substantiate the *in vitro* results with reference to the use of 1% $AgNO_3$ in pediatric ophthalmology, the thicknesses of the corneas of three rabbits were measured during a control period and after exposure to the same dose of $AgNO_3$ that is given neonates. In the example shown (Fig. 12), moderate stromal edema developed within 20 min of exposure to Ag^+, with an initial swelling rate of 12.5 μm/hr. The maximum stromal swelling of 50 μm was reached 6 hr after treatment, and the corneas were still edematous after 12 hr.

Effects of Other Reagents on Corneal Potential and Short-Circuit Current

To examine possible mechanisms for the action of Ag^+ on the cornea, other reagents, purportedly specific to sulfhydryl, disulfide, or amino groups, were tested for effects on corneal electrophysiology. Among the heavy metal ions tested, only Cu^{2+} mimicked the Ag^+ response (Table 3). At 10^{-4} M, Cu^{2+} increased the SCC from 3.23 ± 0.36 to 19.6 ± 3.8 μA/cm² ($n=4$) and the resting potential from 30.8 ± 1.1 to 45.8 ± 3.2 mV. NEM, a sulfhydryl reagent, also stimulated the short-circuit current (from 2.9 ± 0.3 to 5.6 ± 0.4 μA/cm²) and the resting potential (from 23 ± 2.6 to 49 ± 7.7 mV) across the cornea (Table 3). The NEM effect was similar to that of Ag^+ in that the enhancement of SCC was greatly reduced when the cornea was bathed in low-Na^+ Ringer's solution. The sulfhydryl reagents, PCMB and PCMBS, inhibited SCC and V_c, an effect opposite to that of Ag^+

Table 3. Effect of various agents on corneal epithelial short-circuit current (SCC) and resting potential (V_c)

Agent[a]	Reactive group(s)	Concentration (M)	SCC[b]	V_c[b]	n
Heavy metals:					
Ag^+	−SH (−PO_4, −NH_3, −COOH)	10^{-7} – 10^{-5}	+	+	(68)
Cu^{2+}	−SH	10^{-4}	+	+	(4)
NEM	−SH	10^{-3}	+	+	(6)
PCMB	−SH	10^{-3}	−	−	(2)
PCMBS	−SH	10^{-3}	−	−	(4)
−S-S-reagents:					
Cd^{2+}	−S−S−	10^{-4}	0	0	(4)
DTNB	−S−S−	2×10^{-3}	0	0	(8)
Thiol reagents:					
DTT	−SH protective	10^{-3}	0[c]	0	(5)
GSH	−SH protective	10^{-3}	0	0	(3)
Other:					
MNT	−NH_3	5×10^{-3}	−	−	(6)
Amphotericin B	membrane sterols	25 μg/ml	+	+	(7)

[a] NEM = N-ethylmaleimide, PCMB = *p*-chloromercuribenzene, PCMBS = *p*-chloromercuriphenyl sulfonate, DTNB = dithio-bis-2-nitrobenzoate, DTT = dithiothreitol, GSH = reduced glutathione, MNT = 2-methoxy-5-nitrotropone. [b] Stimulation (+), inhibition (−), no effect (0). [c] DTT stimulated SCC and V_c at concentrations of 2 mM or greater.

(Table 3). The thiol reagent GSH was without effect, and the amino reagent MNT inhibited SCC and V_c. DTT had little effect at up to 1.0 mM (a concentration sufficient to reverse the Ag^+ response) but, at high concentrations (2 mM), increased SCC and reduced corneal resistance. Amphotericin B was the most potent stimulator of SCC; it increased the current from 4.2 ± 0.8 to 61.1 ± 9.3 $\mu A/cm^2$, and augmented the resting potential from 21.4 ± 1.7 to 57.6 ± 1.9 mV ($n = 7$; Burstein & Klyce, 1977). This stimulation was dependent on the presence of tear-side Na^+ and its actions appear to be similar to those on cation transport by the frog corneal epithelium (Candia, Bentley & Cook, 1974).

Discussion

Silver Toxicity in Vivo

Previous reports on the pathological effects of silver nitrate in the eye indicate that, while the substance is useful and preferred as a prophylactic agent in newborns, there are substantial side effects. Under normal prophylactic treatment with 1% $AgNO_3$, a temporary (*ca.* 72 hr) chemical conjunctivitis usually occurs, and, in certain cases where 5–20% $AgNO_3$ was mistakenly applied, permanent corneal scarring and blindness has resulted (Barsham, 1966). Goldstein (1971) discusses chronic argyrosis (blue-black discoloration) of the conjunctiva, cornea, and lids, as well as persistent corneal keratitis in adults, as a result of topically or systemically applied silver nitrate. Acute silver toxicity of the cornea of an adult, accompanied by temporary but marked loss of visual acuity, has been reported in connection with the use of freshly-resilvered gonioprisms for gonioscopy (Grady, 1972). The latter study would suggest that induced silver burns of the cornea, accompanied by some desquamation, produce reversible corneal edema. This is substantiated by the present *in vitro* results, which demonstrate increased corneal hydration and corneal stromal edema after application of a smaller (10^{-5} M) dose of $AgNO_3$. It is clear that silver, even in low concentrations that should not cause significant desquamation of the epithelium, has substantial effects on the osmotic balance of the cornea. These effects appear to be a result of specific changes in the permeability of the corneal epithelium to solutes and a concomitant, marked stimulation of absorptive Na^+ transport.

Two Phases of the Ag^+ Effect in Vitro

The effect of Ag^+ on corneal transport parameters may be divided logically into two phases: an early phase which commences immediately after Ag^+ addition and continues until V_c reaches a maximum at about 30 min; and a late phase that includes the period from 30–270 min, during which V_c declines.

The combined results taken from the early phase of the Ag^+ response indicate that Ag^+ increases specifically the conductance of the apical membrane of the corneal epithelium to cations, primarily Na^+. This, in turn, evokes large increases in Na^+ uptake and, assuming isosmotic fluid transport, accounts for the observed increase in corneal stromal thickness. These results are in many ways similar to those obtained with 10^{-4} M $AgNO_3$ applied to the mucosal surface of frog skin (Curran, 1972; Li & de Sousa, 1976) and with Cu^{2+} (10^{-5} M) on frog skin (Koefoed-Johnsen & Ussing, 1958). In the previous studies, however, the Ag^+ effect was not examined using microelectrodes, nor were the kinetics of ouabain inhibition investigated. The major difference in the response of the cornea *vs.* that of the frog skin to Ag^+ is that, in the latter epithelium, mannitol (and sulfate) fluxes increase during the first 30 min of the effect (Curran, 1972), suggesting a rapid increase in the permeability of the shunt pathway. In this study, mannitol permeability of the cornea was not significantly increased until about 60 min after Ag^+ treatment, perhaps reflecting the lower dosage used (10^{-5} M) or species differences in the sensitivity of the apical tight junctions to Ag^+.

The efflux of Na^+ across frog skin after Ag^+ (10^{-4} M; Curran, 1972) or Cu^{2+} treatment (10^{-4} M; Ferreira, 1970) is enhanced much more than the Na^+ influx, whereas the present results using the cornea indicated that the Na^+ influx change was larger. This can be accounted for by the low initial Na^+ conductance of the cornea, compared to the frog skin, and the apparent lack of amiloride-sensitive Na^+ conductance in the former (Marshall & Klyce, *unpublished*). It is of interest, however, that the final Na^+ permeabilities of the two Ag^+-treated epithelia are similar to each other, which suggests that the Ag^+-induced Na^+ conductance is independent of the amiloride-sensitive Na^+ channel normally present in frog skin. Consistent with this hypothesis, Li and de Sousa (1976) report reduced amiloride sensitivity of the frog skin Na^+ transport after Ag^+ treatment.

The late phase of the Ag^+ response in the cornea is characterized by a nonselective increase in the overall solute permeability of the cornea. The increases, during this phase, in electrical conductance, Cl^- influx and both unidirectional mannitol fluxes point to an opening of the paracellular pathway. Although similar effects have been observed using Cu^{2+} or Ag^+ on the frog skin (Ferreira, 1970; Curran, 1972), the effect on the shunt in the latter is not temporally

separated from the cation conductance change at the apical membrane, as it is in the cornea. Hence, Ag^+ may be valuable as an agent to increase specifically the conductance of the apical membrane to cations. Preliminary experiments using the rabbit urinary bladder, an epithelium with normally low Na^+ permeability (Lewis & Diamond, 1976), suggested that Ag^+ may affect this tissue in a manner similar to the response seen using the corneal epithelium. (Klyce, *unpublished*). As such, Ag^+ could well be used effectively to increase sodium conductance in epithelia with low Na^+ permeability (cornea and rabbit urinary bladder) or in amiloride-blocked sodium-transporting epithelia (e.g., frog skin and toad urinary bladder).

On the Use of Ag/AgCl Electrodes

Considering the marked effects of low concentrations of Ag^+ on corneal ion transport and permeability, experimenters who would use exposed Ag/AgCl electrodes in *in vitro* membrane chambers should do so with caution. The possibility of artifacts associated with the use of these electrodes would likely be greater if the epithelium under investigation had low cation conductance. If such is the case, thiol reagents could be used to test for possible effects of Ag^+ contamination of the bathing medium. Problems associated with exposed Ag/AgCl half-cells may be easily avoided if the electrodes are isolated from the chamber using low-resistance agar-Ringer's bridges.

Mechanism of Ag^+ Action

The concentration of Ag^+ in the Ringer's solution would be expected to be on the order of 10^{-11} M. Considering the low concentration of Ag^+ in free solution, it is perhaps surprising that frog skin rapidly accumulates the metal (Curran, 1972). However, it is likely that AgCl neutral complex would remain in suspension and could enter epithelial cells in this form. Gutknecht (1981) has demonstrated that lecithin bilayer membranes are very permeable (1.3×10^{-2} cm/sec) to the neutral complex of mercury ($HgCl_2$) at micromolar concentrations and at physiological pH. In contrast, these membranes are much less permeable to charged forms of mercury (Hg^{2+}, $HgCl^+$, and $HgCl_3^-$). Assuming that biological membranes are as permeable to AgCl as they are to $HgCl_2$, added $AgNO_3$ could feasibly act on the membrane surface and/or on intracellular sites involved in ion transport.

Ionized silver, copper, cadmium, and other heavy metals are effective sulfhydryl agents. Ag^+ should react strongly with -SH groups to form hemi-silver sulfides, but could also react with amino, imidazole, carboxyl and phosphate moieties. The rapid onset of the Ag^+ effect and the equally rapid reversal by dithiothreitol and reduced glutathione suggest that Ag^+ acts at or near the apical surface of the cornea. Whereas Ag^+ could enter cells (as AgCl, *see above*), it is unlikely that relatively larger, more polar species such as dithiothreitol would enter cells easily to effect the rapid reversal of an intracellularly located Ag^+ response. As such, it would appear that the increased Na^+ conductance caused by Ag^+ may be associated with Ag^+ binding to superficial membrane proteins.

The effects of specific sulfhydryl reagents on some membrane systems are similar in several respects to those of Ag^+, suggesting that this ion may act primarily on sulfhydryl groups of membrane proteins in the corneal epithelium. It has been shown that impermeable and purportedly specific sulfhydryl reagents, notably PCMBS, increase cation conductance of toad urinary bladder (Spooner & Edelman, 1976) and human erythrocytes (Shapiro, Kollman & Martin, 1970; Knauf & Rothstein, 1971). PCMBS blocks sodium-dependent amino acid uptake across rabbit intestine (Schaeffer, Preston & Curran, 1973), presumably by enhancing a parallel pathway for Na^+ entry across the brush border membrane. PCMBS apparently acts on at least three different sulfhydryls at the membrane surface (Knauf & Rothstein, 1971), and its effects are rapidly reversed by dithiothreitol (Schaeffer et al., 1973) or reduced glutathione (Shapiro et al., 1970). This sulfhydryl reagent also stimulates SCC (Na^+uptake) across frog skin (Lindemann & Voute, 1977; Benos, Mandel & Simon, 1980) and the time courses of Ag^+ and PCMBS responses are comparable (Curran, 1972; Benos et al., 1980).

The fact that PCMB and PCMBS had inhibitory effects on corneal SCC and V_c does not necessarily rule out a sulfhydryl reaction as the cause of the Ag^+ response because NEM, also a sulfhydryl reagent, stimulated SCC and V_c in a manner similar to that of Ag^+. Because Ag^+, Cu^{2+} and NEM have lower molecular weights than PCMB and PCMBS, it is possible that the former agents may bind to membrane protein sulfhydryl moieties that are inaccessible to PCMB and PCMBS. As these reagents could be expected to produce different end products in their reactions with membrane groups, it is also possible that their effects on ion transport could differ for this reason. Because Cd^{2+} and DTNB have little effect on the cornea, a disulfide reaction in the Ag^+ response is unlikely. Also, the inhibitory effect of the amino reagent MNT indicates that Ag^+ probably does not act via interaction with amino groups. In summary, present evidence suggests that Ag^+ may act at sulfhydryl sites on intramembrane proteins at the apical surface of the epithelium.

Finally, with regard to the use of Ag^+ as a prophy-

lactic agent in pediatric ophthalmology, the results of this study would suggest that alternate bactericidal agents could well be used on the cornea without producing marked transport-related side effects. Hence, the testing of other possible reagents for efficacy in controlling gonococcal infections, the primary cause of ophthalmia neonatorum, is certainly called for, and may yield safer ophthalmic prophylaxis for neonates.

The expert assistance of O. Bernegger is gratefully acknowledged. This work was supported by N.I.H. grant EY03311 from the National Eye Institute.

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Received 14 August 1981; revised 30 October 1981