J. Membrane Biol. 83, 235–250 (1985)

The Journal of
Membrane Biology

Cl Transport in the Frog Cornea: An Electron-Microprobe Analysis

Roger Rick*, Franz X. Beck, Adolf Dörge, and Klaus Thurau
Department of Physiology, University of Munich, Munich, West Germany

Summary. The intracellular electrolyte concentrations of the bullfrog corneal epithelium have been determined in thin freeze-dried cryosections using the technique of electron-microprobe analysis. Under control conditions, transepithelial potential short-circuited and either side of the cornea incubated in Conway's solution, the mean intracellular concentrations (in mmol/kg wet weight) were 8.0 for Na, 18.4 for Cl and 117.3 for K. These values are in good agreement with ion activities previously obtained by Reuss et al. (*Am. J. Physiol.* **244:**C336–C347, 1983) under open-circuit conditions. From a comparison of the chemical concentrations and activities of Na and K a mean intracellular activity coefficient of 0.75 is calculated. For small ions no significant differences between nuclear and cytoplasmic concentration values were detectable. The Cl concentrations in the different epithelial layers were virtually identical and showed parallel changes at varying states of Cl secretion, suggesting that the epithelium represents a functional syncytium. For Na a concentration gradient between the outer and inner epithelial layer was observed, which can be accounted for by two different models of epithelial cooperation. The behavior of the intracellular Na and Cl concentrations after removal of Na, Cl or K from the outer or inner bathing medium provides support for a passive electrodiffusive Cl efflux across the apical membrane and a Na-coupled Cl uptake across the basolateral membrane. The results are inconclusive with regard to the exact mechanism of Cl uptake, indicating either a variable stoichiometry of the symporter or the presence of more than one transport system. Furthermore, a dependence of intracellular Cl on HCO_3 and CO_2 was observed. Extracellular measurements in corneal stroma demonstrated that ion concentrations in this space are in free equilibrium with the inner bath.

Key Words intracellular electrolytes · transepithelial transport · Cl secretion · corneal epithelium · electron-microprobe analysis

Introduction

The epithelium of the isolated bullfrog cornea transports Cl actively from the inner, stromal to the outer, tear-side bathing solution (Zadunaisky, 1966). As in many other secretory epithelia (for review *see* Frizzell, Field & Schultz, 1979), Cl secretion in the cornea is electrogenic and dependent on the presence of Na in the inner bath, although under short-circuited conditions Na is not transported transepithelially (Zadunaisky, 1966, 1972). Further characteristics shared by most Cl-secreting epithelia are a sensitivity to ouabain and loop diuretics (Candia, 1972, 1973) and an ability to be stimulated by agents which increase the intracellular levels of cAMP or Ca (Chalfie, Neufeld & Zadunaisky, 1972; Candia, Montoreano & Podos, 1977).

In the model of Cl secretion proposed by Silva et al. (1977) and Frizzell et al. (1979), transepithelial Cl transport is thought to involve two different transport steps. Cl uptake across the basolateral membrane of the transporting epithelial cells is a secondary active process, driven by an inwardly directed Na concentration gradient, whereas Cl efflux across the apical membrane is assumed to be passive. Two essential predictions of this hypothesis have recently been verified for the corneal epithelium of the bullfrog. Reuss et al. (1983) have demonstrated with ion-selective microelectrodes that the intracellular Cl activity is above electrochemical equilibrium and that the apical membrane has a high electrodiffusional Cl permeability.

In the present study the intracellular electrolyte concentrations in the bullfrog corneal epithelium were measured by electron-microprobe analysis. This technique allows the quantitative determination of element concentrations in individual epithelial cells and even in subcellular compartments. The dependence of the intracellular electrolyte concentrations on the ionic composition of the bathing medium and the effect of pH were investigated. The results demonstrate that the multilayered corneal epithelium can be regarded as a functional syncytium for transepithelial Cl transport. The mean intracellular electrolyte concentrations under control

* *Present address:* Department of Physiology and Biophysics, University of Alabama in Birmingham.

conditions were found to be in good agreement with previously reported ion activities (Zadunaisky, Spring & Shindo, 1979; Reuss et al., 1983). A preliminary account of this study has been presented at a meeting of the German Physiological Society (Rick et al., 1982*a*).

Materials and Methods

The experiments were performed on the isolated cornea of the American bullfrog, *Rana catesbeiana* (West Jersey Biological Supply Farm, Wenonah, N.J.). The animals were double pithed, the corneas excised and mounted between two Plexiglas® rings of 7 mm internal diameter. A rubber O-ring between the tear-side ring and the cornea gently clamped the tissue (Fig. 1). Silicone grease was used to electrically insulate the cut and compressed edge from the central part of the cornea. The rings were then inserted into Ussing-type incubation chambers. Throughout the experiment the half-chambers were continuously perfused with fresh bathing media, which were equilibrated with room air. Apart from the initial determination of the transepithelial potential difference the corneas were kept short-circuited using an automatic clamping device. At regular intervals the d-c resistance was measured by clamping the potential for short periods (500 msec) to + or −10 mV. The values for the short-circuit current (SCC) and transepithelial conductance (g_t) are given per nominally exposed area (38 mm²). The natural bulging of the cornea was maintained by applying a 1-cm H_2O hydrostatic pressure difference, anterior chamber-side positive.

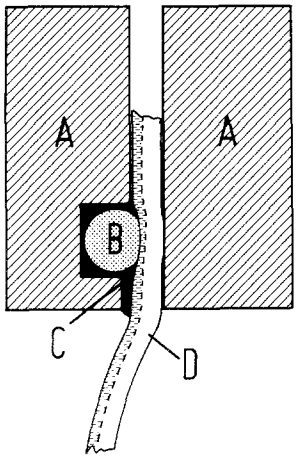

Fig. 1. Schematic cross-section illustrating the mounting of the cornea. *A* = Plexiglas® rings; *B* = rubber O-ring; *C* = silicone grease; *D* = cornea. For further details *see* text

During a control period of 30 to 50 min the corneas were incubated on both sides in Conway's or Ringer's solution until the SCC had reached a steady-state value. The incubation was then continued according to the experimental protocol described in Results. Usually one of the corneas remained untreated, serving as a control. The composition of the different bathing media is listed in Table 1. Bubbled with air, the pH of the 2.5 mM HCO_3-containing media varied between 8.1 and 8.3, and the pH of the 25 mM HCO_3-containing media between 8.3 and 8.5. CO_2 was applied as a 5% CO_2/95% air mixture.

At the end of the experiments the rings were quickly removed from the incubation chambers. The epithelial surface was gently blotted with filter paper and covered with a thin layer of an albumin-containing standard solution. The corneas were then plunged into a liquid nitrogen-cooled mixture of propane/isopentane (−196°C) to shock-freeze the tissue (Jehl et al., 1981). The time elapsing between removal of the rings from the chamber and freezing was between 5 and 15 sec.

From the frozen tissue 1-μm-thick sections were cut at a temperature between −80 and −100°C in a modified Reichert cryoultramicrotome (OmU2/FC150 or OmU3/FC3). The sections were mounted on thin formvar or collodion films and subsequently freeze-dried at −80°C and 10^{-6} mbar in a vacuum device. X-ray microanalysis of the freeze-dried sections was performed in a scanning electron microscope (Cambridge Stereoscan S4 or S150) to which an energy dispersive X-ray detecting system was attached (LINK Systems). The acceleration voltage used was 20 kV, and the probe current selected was between 0.2 and 0.5 nA. Small areas (1 to 2 μm²) within the cells or in the albumin standard layer were scanned for 100 sec and the emitted X-rays were analyzed in the energy range between 0 and 20 keV, which includes the K-lines of the biologically relevant light elements Na, Mg, P, CL, K and Ca. Discrimination between characteristic and noncharacteristic radiations (bremsstrahlung) was performed by a computer (Data General, Eclipse S230) using a specially designed deconvolution method (Bauer & Rick, 1978).

Quantification of the cellular element concentrations was achieved by comparing the intensities of the characteristic radiations obtained in the cells with those obtained in the adherent albumin standard layer. This quantification procedure directly

Table 1. Composition of incubation media (mM)

	NaCl	$NaHCO_3$	Na_2SO_4	Na_2HPO_4	Na-gluconate	KCl	$KHCO_3$
Conway's solution							
- normal	67.6	25.0	1.8	2.9	1.0	2.5	
- Na-free							2.5
- Cl-free		25.0	35.6	2.9	1.0		
- K-free	70.1	25.0	1.8	2.9	1.0		
- low-HCO_3	90.1	2.5	1.8	2.9	1.0	2.5	
Ringer's solution							
- normal	110.0						2.5
- high-HCO_3	87.5	22.5					2.5
- K-free	110.0	2.5					

provides concentrations in units of mmol/kg wet weight (w.w.). The cellular dry weight content was calculated from a comparison of the bremsstrahlung intensities of cell and standard and expressed as g% (g dry matter/100 g wet weight). The standard solutions were prepared by dissolving 1 g purified bovine serum albumin (Behringwerke, Marburg) in 4 ml incubation medium. Further details of the preparation of freeze-dried cryosections for X-ray microanalysis and of applied quantification method have been described earlier (Dörge et al., 1978; Rick, Dörge & Thurau, 1982*b*).

In the text mean values ±SD are given, with the number of measurements (n) in parentheses. In figures mean values ±SE are depicted, each point representing the mean of about 10 individual measurements. The Student's t-test was applied to determine whether the differences in the means attain statistical significance.

Results

Figure 2 shows a typical scanning electron transmission image of a 1-μm-thick freeze-dried cryosection of a snap frozen frog cornea incubated under control conditions. The layer on top, adherent to the outer epithelial surface corresponds to the albumin standard solution with which the cornea was coated immediately prior to freezing. On the bottom, the underlying stromal tissue is in part visible. The epithelium itself consists of four to five different layers of cells, which in conventional sections are usually referred to as surface, wing and basal cells (Hogan, Alvarado & Weddell, 1971). For the present study the cells of the epithelium were divided into four groups: the outermost and innermost cell layers were named layers 1 and 4, respectively. The remainder of the cells were grouped as layers 2 and 3 depending on whether they were located in the apical or basal half of the epithelium.

Compared to cryosections of the frog skin epithelium (Rick et al., 1978) the identification of the boundaries between the different epithelial cells is difficult, mainly because of the fact that the intercellular spaces are almost completely collapsed. Only during maximal inhibition of Cl secretion, for example after Cl removal from the inner bath or after addition of bumetanide (*own unpublished results*), did the spaces between the cells become visibly open.

Cellular measurements were performed separately in the nucleus and cytoplasm. In some cases, the scanning areas were positioned in such a way that about equal parts of each cellular compartment were excited. For small diffusible ions such as Na, K and Cl under none of the experimental conditions tested were significant differences between the nuclear and cytoplasmic concentration values detectable, suggesting that the two subcellular compartments form only one distributional space for these ions. This is demonstrated in Table 2 for a control cornea incubated in normal Conway's solution. The measurements are from one section and one cell

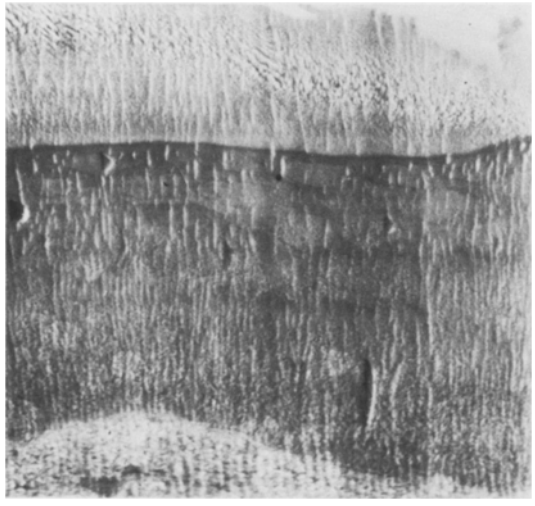

Fig. 2. Scanning transmission electron micrograph of a freeze-dried cryosection (1 μm thick) obtained from a control cornea. The height of the epithelium is about 30 μm. For further details *see* text

K_2SO_4	$MgCl_2$	$MgSO_4$	$CaCl_2$	$CaSO_4$	Ca-gluconate	Choline-Cl	Glucose	Sucrose
	1.2		1.0				26.0	
		1.2			1.0	80.0	26.0	40.0
1.25		1.2		1.0			26.0	20.0
	1.2			1.0			26.0	
	1.2		1.0				26.0	
			1.0				5.0	
			1.0				5.0	
			1.0				5.0	

Table 2. Cytoplasmic and nuclear element concentration and dry weight content (d.w.) in the cornea epithelium under control conditions (Conway)[a]

	Na	Cl	K	P	Mg	Ca	d.w.
			(mmol/kg wet wt)				(g/100g)
Nucleus	5.8 ± 2.1	17.5 ± 1.9	124.4 ± 5.5	117.2 ± 10.8	7.1 ± 4.0	0.4 ± 0.4	20.8 ± 1.3
Cytoplasm	6.7 ± 2.3	16.7 ± 3.6	119.0 ± 12.6	92.5[b] ± 17.6	10.5 ± 6.0	0.6 ± 0.5	21.1 ± 1.9

[a] Mean ± SD of nine paired measurements obtained in cells of the second epithelial layer under control conditions (only one section).
[b] Significantly different from the nuclear value ($2P < 0.05$).

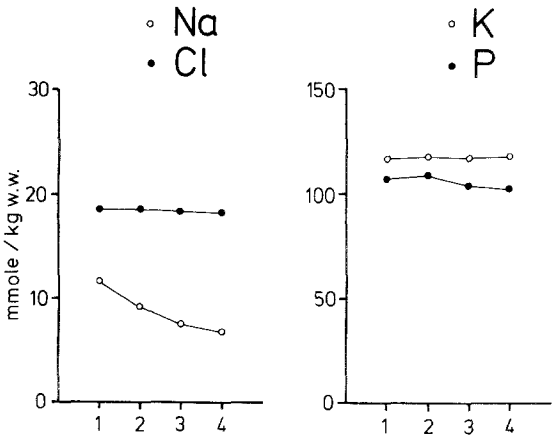

Fig. 3. Intracellular Na, Cl, K and P concentrations in the different epithelial cell layers of the frog cornea. Mean values obtained from 23 Conway control corneas. Cell layer 1 is the outermost, 4 the innermost layer of the epithelium

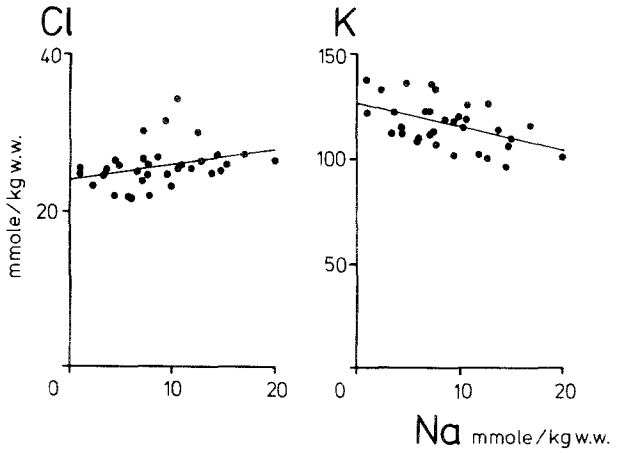

Fig. 4. Intracellular Cl and K concentrations as a function of the intracellular Na concentration. The points represent individual measurements obtained from one Ringer control cornea. The linear regression lines describe the functions [Cl] = 24.0 + 0.21 · [Na] and [K] = 126.6 − 1.09 · [Na], respectively. The corresponding r values are 0.35 and 0.48

type only (second cell layer). Apart from the higher P concentration in the nucleus no significant differences can be observed. However, the slightly elevated Ca and dry weight concentration in the cytoplasm was a regular finding in control corneas and could be observed under other experimental conditions as well.

The cells of the different epithelial layers had a very similar element composition, as shown in Fig. 3 for the Conway controls. Only for Na was an inwardly directed concentration gradient detectable, which also was present in most experimental corneas (Figs. 6–12). Figure 4 shows the dependence of the cellular Cl and K concentration on the cellular Na concentration in a Ringer control cornea which exhibited a marked Na concentration gradient. For K an inverse correlation is evident, suggesting an almost 1:1 exchange of Na for K, whereas for Cl no significant dependence is detectable. Under experimental conditions which resulted in major shifts of the cellular ion concentrations, usually parallel changes of similar magnitude were observed in all epithelial layers indicating that the epithelium forms a functional syncytium with regard to small ions.

Table 3 summarizes the results obtained from all 34 control corneas. The only major difference between corneas incubated in Conway's and Ringer's solution is the significantly higher Cl value in the Ringer controls. This difference seems to be largely due to the differential HCO_3 concentration of the two media employed (*see below*). The mean Cl values in the individual control experiments showed a negative dependence on the mean cellular Na concentration, as shown in Fig. 5 for the Conway controls. Similarly an inverse correlation between the K and Na concentration was detectable (*data not shown*). The cellular dry weight contents were at 21.5 ± 2.7 (Conway) and 21.9 ± 2.7 g% (Ringer) not significantly different.

The average SCC of the Ringer controls was slightly higher, a finding which likely reflects possible seasonal variations or unknown differences between different batches of frogs rather than a direct effect of the bathing media. In fact, in paired experiments or when the effect of the two media was tested in the same cornea, the SCC was usually slightly higher during incubation with Conway's so-

Table 3. Intracellular element concentrations and short-circuit current (SCC) of the cornea epithelium under control conditions using either Conway's (25 mM HCO_3) or normal frog Ringer's solution (2.5 mM HCO_3) as bathing medium[a]

	Na	Cl (mmol/kg wet wt)	K	P	SCC (μA/cm^2)
Conway	8.0 ± 3.0	18.4 ± 3.7	117.3 ± 8.0	105.4 ± 11.4	28.1 ± 10.9
Ringer	9.4 ± 2.2	25.9[b] ± 6.0	116.9 ± 6.4	106.3 ± 8.4	31.5 ± 10.8

[a] Mean ± SD of 23 (Conway) and 11 (Ringer) experiments.
[b] Significantly different from the Conway control ($2P < 0.01$). The total number of measurements is 787 (Conway) and 299 (Ringer), respectively.

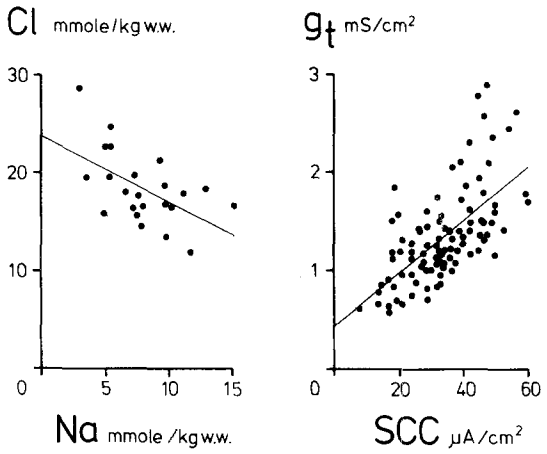

Fig. 5. Intracellular Cl concentration as a function of the intracellular Na concentration (left) and transepithelial conductance (g_t) as a function of the simultaneously measured short-circuit current (SCC, right). Data from 23 Conway controls (left), and 96 Conway and Ringer controls (right), respectively. The linear regression lines describe the functions [Cl] = 23.9 − 0.69 · [Na] and g_t = 0.43 + 0.027 · SCC, respectively. The corresponding r values are 0.56 and 0.65

lution. The initial transepithelial potential difference was on an average of all corneas 27.6 ± 7.6 mV (n = 96); the SCC and g_t measured at the end of the control period were 33.9 ± 11.6 μA/cm^2 and 1.35 ± 0.48 mS/cm^2, respectively. These parameters were not significantly different in the two control groups. The g_t and SCC values were found to be positively correlated as shown in the right part of Fig. 5. From the intercept with the y-axis the fraction of g_t which is unrelated to net Cl secretion can be estimated to be about 0.43 mS/cm^2. The apparent driving force of Cl secretion (E_{Cl}) as calculated from the slope of the regression line amounts to 37 mV. No significant correlations were observed between the SCC and the mean intracellular Na and Cl concentrations, respectively. If not stated otherwise, the corneas were incubated in Conway's solution.

Figure 6 shows the effect of removal of Cl from the outer, tear-side bathing solution on the SCC, g_t and intracellular Na and Cl concentrations. As expected for a passive Cl exit across the apical membrane the SCC is stimulated whereas g_t and the intracellular Cl concentration are lowered.[1] The drop in the Cl concentration is almost identical in all layers. In three similar experiments the mean intracellular Cl concentration decreased from 16.5 ± 4.8 (n = 102) to 12.3 ± 3.7 mmol/kg w.w. (n = 85, $2P < 0.001$) whereas the Na concentration increased slightly from 10.6 ± 4.7 to 12.1 ± 5.6 mmol/kg w.w. ($2P < 0.05$). No significant variations were observed in the K, P and dry weight values.

The effect of Cl removal from the inner, anterior chamber-side bathing medium is shown in Fig. 7. Within 10 min after removal of Cl, the SCC is reduced to about 5% of its original value and g_t to about 50%. In all epithelial layers a marked reduction in the Na and Cl concentrations can be observed. For Cl a large gradient is evident, the concentration in the outer layers being much higher than in the inner layers. Table 4 summarizes the results obtained from three similar experiments. The increase in the P concentration can be explained by some cellular shrinkage as also a small increase in the dry weight content from 21.7 ± 3.2 to 22.6 ± 4.0 g% was observed.

Figure 8 shows an experiment in which Na was removed from the inner bathing medium. Within 20 min the SCC reached a new steady-state value about 60% lower than the control, whereas g_t declined by only 20%. The Na and Cl concentrations are significantly reduced in all layers. Compared to

Table 4. Effect of Cl-free inner bathing solution (20 to 30 min) on the intracellular element concentrations and short-circuit current (SCC) of the cornea epithelium[a]

	Na	Cl (mmol/kg wet wet)	K	P	SCC (μA/cm^2)
Control	14.2 ± 5.8	14.8 ± 3.7	116.3 ± 17.1	99.6 ± 25.7	32.6 ± 1.8
I.s. Cl-free	7.0 ± 3.8	5.5 ± 2.2	118.7 ± 19.7	111.3 ± 29.4	−0.3 ± 5.5

[a] Mean ± SD of 81 (Control) and 89 (I.s. Cl-free) measurements obtained from three paired experiments.

[1] The change in g_t may be partially due to a parallel shift in the conductance of the paracellular shunt pathway. Similarly, because of the differences in the chemical composition of the bathing media the shunt may contribute to the SCC change, resulting in an overestimation of active Cl secretion.

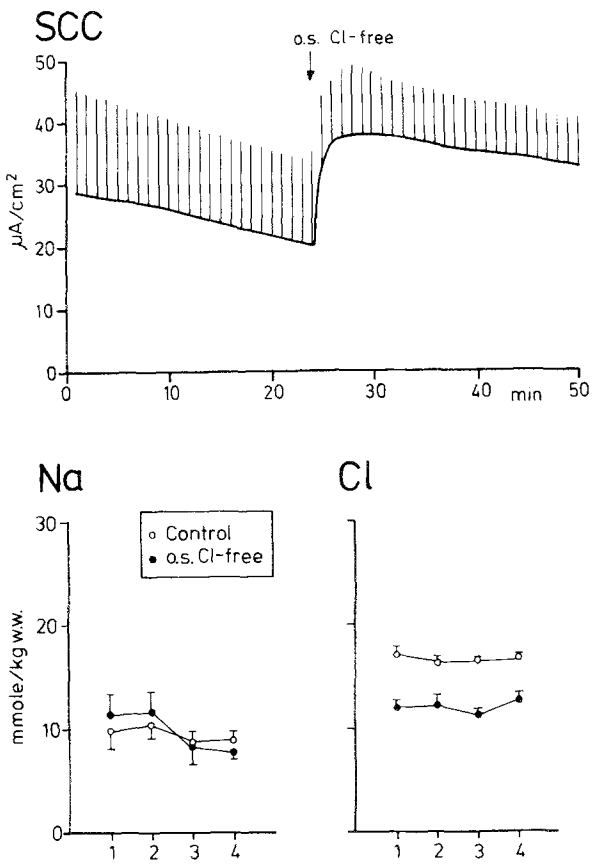

Fig. 6. Short-circuit current (SCC) and intracellular Na and Cl concentrations in the different epithelial cell layers in an experiment in which the outer bathing medium was replaced with a Cl-free solution (o.s. Cl-free). The heights of the current deflections are proportional to the transepithelial conductance g_t. Cell layer 1 is the outermost, 4 the innermost layer of the epithelium. Means ± SE

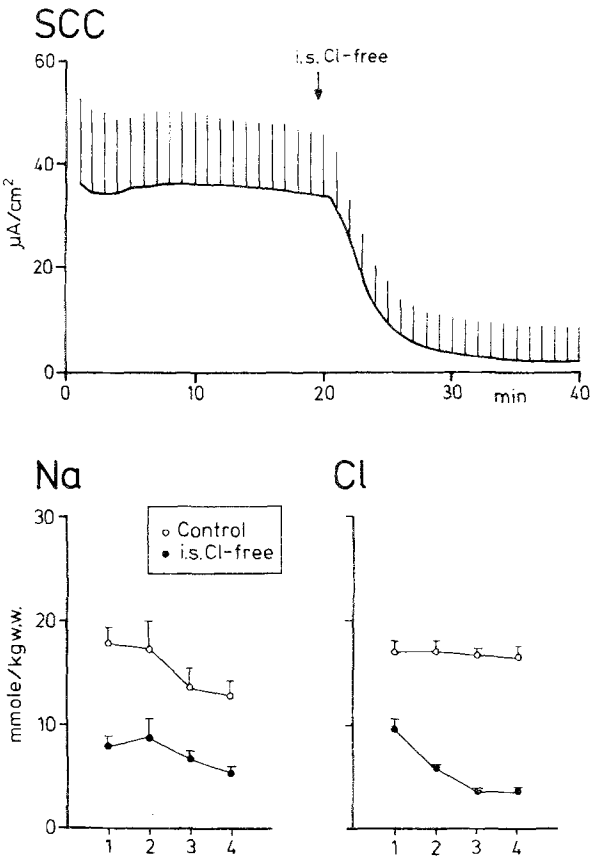

Fig. 7. Short-circuit current (SCC) and intracellular Na and Cl concentrations in the different epithelial cell layers in control and after removal of Cl from the inner bathing solution (i.s. Cl-free). For further explanations *see* legend to Fig. 6

Table 5. Effect of Na-free inner bathing solution (20 to 30 min) on the intracellular element concentrations and short-circuit current (SCC) of the cornea epithelium[a]

	Na	Cl (mmol/kg wet wt)	K	P	SCC (µA/cm²)
Control	12.3 ± 4.6	14.9 ± 3.8	117.1 ± 15.0	106.4 ± 20.7	29.7 ± 4.2
I.s. Na-free	6.5 ± 3.8	11.6 ± 3.1	124.6 ± 15.7	122.3 ± 19.3	13.2 ± 1.2

[a] Mean ± SD of 78 (Control) and 73 (I.s. Na-free) measurements obtained from three paired experiments.

the effect of Cl removal from the inner bath, the reduction in the Cl values is much less and no Cl concentration gradient is detectable. Table 5 summarizes the results obtained from three similar experiments. Again the increase in the P concentration is accompanied by an increase in the dry weight content from 22.0 ± 1.9 to 23.3 ± 2.4 g% indicating some cellular shrinkage after removal of Na from the inner bath. Furthermore a small rise in the Ca concentration from 0.4 ± 0.3 to 0.7 ± 0.8 mmol/kg w.w. ($P < 0.01$) was detectable.

Figure 9 illustrates the effect of K removal from the inner bathing medium. After 45 min the SCC declined to less than 50% of its original value, without yet reaching a new steady state. The g_t change is much faster though less pronounced. The Na concentration shows a marked increase in all epithelial layers, whereas the Cl concentration is slightly reduced. Table 6 lists the results of the same experiment. It is evident that the Na increase is matched by an equivalent drop in the K concentration. In this experiment the P concentration is practically unchanged, whereas the dry weight content is significantly increased from 18.6 ± 2.1 to 20.0 ± 2.4 g% ($P < 0.01$). Essentially the same effects were observed in a second experiment of this type using Ringer's solution as incubation medium.

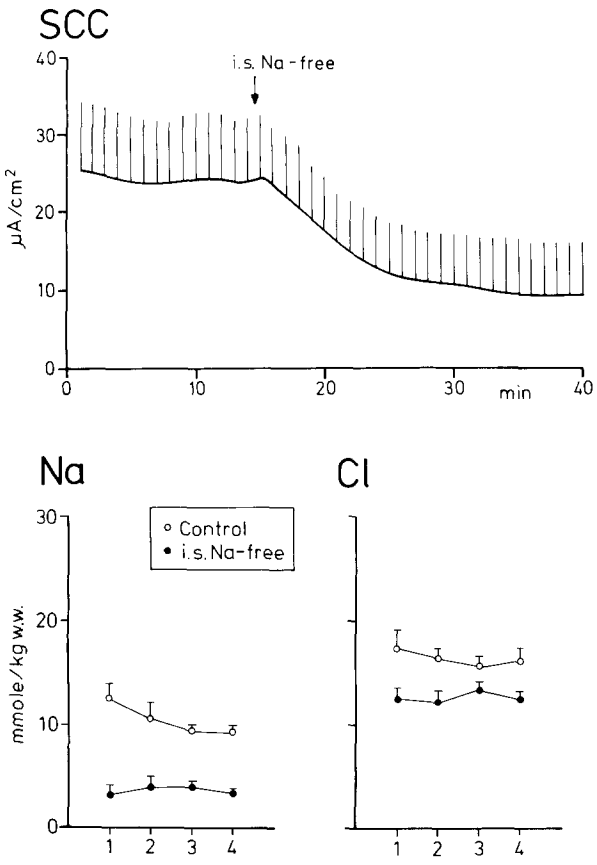

Fig. 8. Short-circuit current (SCC) and intracellular Na and Cl concentrations in the different epithelial cell layers in control and after removal of Na from the inner bathing solution (i.s. Na-free). For further explanations *see* legend to Fig. 6

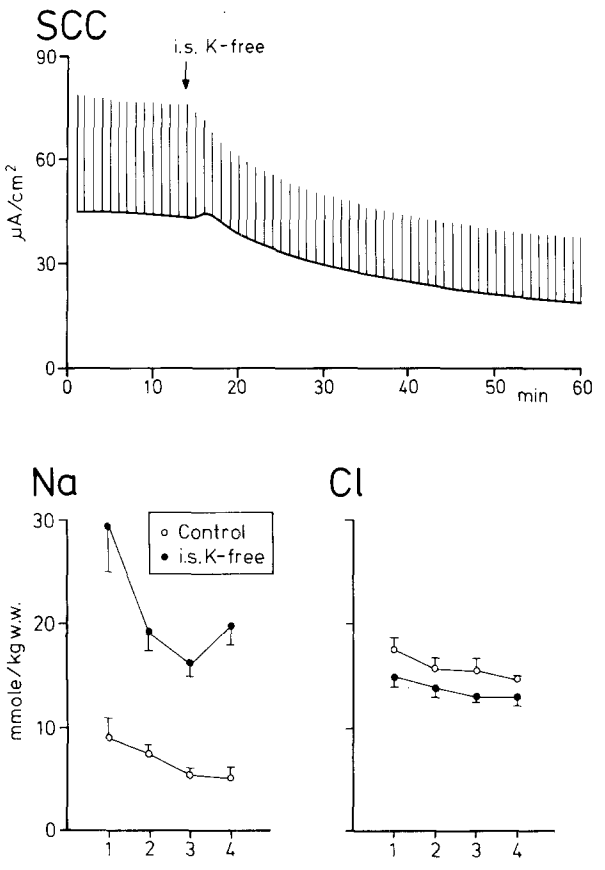

Fig. 9. Short-circuit current (SCC) and intracellular Na and Cl concentration in the different epithelial cell layers in control and after removal of K from the inner bathing solution (i.s. K-free). For further explanations *see* legend to Fig. 6

Table 6. Effect of K-free inner bathing solution (45 min) on the intracellular element concentrations and short-circuit current (SCC) of the cornea epithelium[a]

	Na	Cl (mmol/kg wet wt)	K	P	SCC (μA/cm^2)
Control	7.1 ± 5.8	16.4 ± 3.1	112.6 ± 17.0	104.5 ± 27.2	41.1
I.s. K-free	19.3[b] ± 8.8	13.0[b] ± 2.8	100.6[b] ± 16.1	101.5 ± 29.8	18.8

[a] Mean ± SD of 27 (Control) and 49 (I.s. K-free) measurements obtained from one paired experiment.
[b] Significantly different from control (for Na,Cl 2 $P < 0.001$; for K 2 $P < 0.01$).

Table 7. Effect of lowering the HCO_3 concentration in the inner bathing solution (2.5 mM, 40 min) on the intracellular element concentrations and short-circuit current (SCC) of the cornea epithelium[a]

	Na	Cl (mmol/kg wet wt)	K	P	SCC (μA/cm^2)
Control	9.8 ± 3.5	16.2 ± 2.6	109.7 ± 8.6	96.4 ± 19.6	20.8
I.s. low HCO_3	11.2 ± 3.9	20.3[b] ± 3.4	111.3 ± 11.9	95.8 ± 16.9	15.2

[a] Mean ± SD of 47 (Control) and 29 (I.s. low HCO_3) measurements obtained from one paired experiment.
[b] Significantly different from control (2 $P < 0.001$).

Figure 10 and Table 7 show the results of an experiment in which the HCO_3 concentration in the inner bath was lowered from 25 to 2.5 mM. Within a few minutes, both the SCC and g_t decreased by approximately 30%. In all layers an increase in the Cl concentration is evident. The small increase in the average Na concentration is not statistically significant. Lowering the HCO_3 concentration in the outer bath also resulted in a small reduction in the SCC by about 20% and a small increase in the Cl concentra-

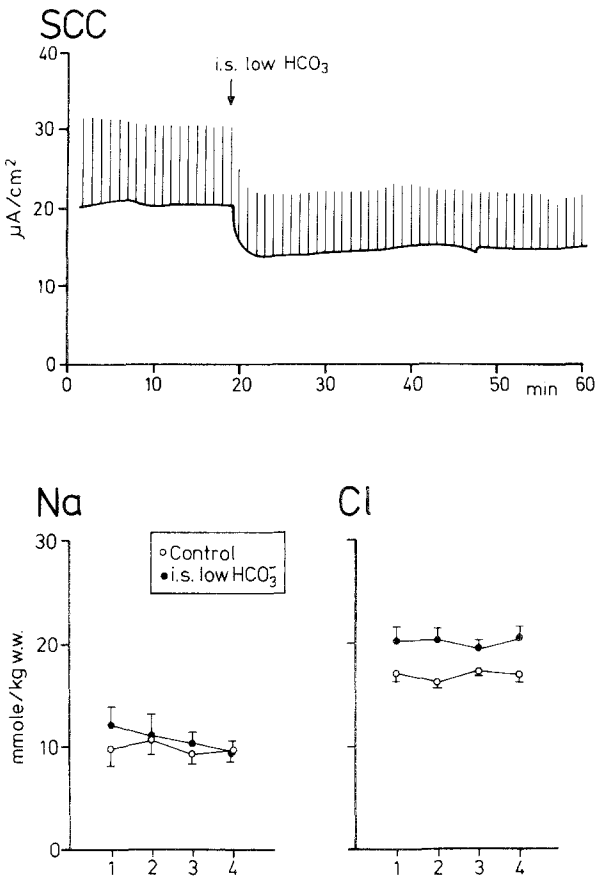

Fig. 10. Short-circuit (SCC) and intracellular Na and Cl concentrations in the different epithelial cell layers in control and after lowering the HCO_3 concentration in the inner bathing solution from 25 to 2.5 mM (i.s. low-HCO_3). For further explanations *see* legend to Fig. 6

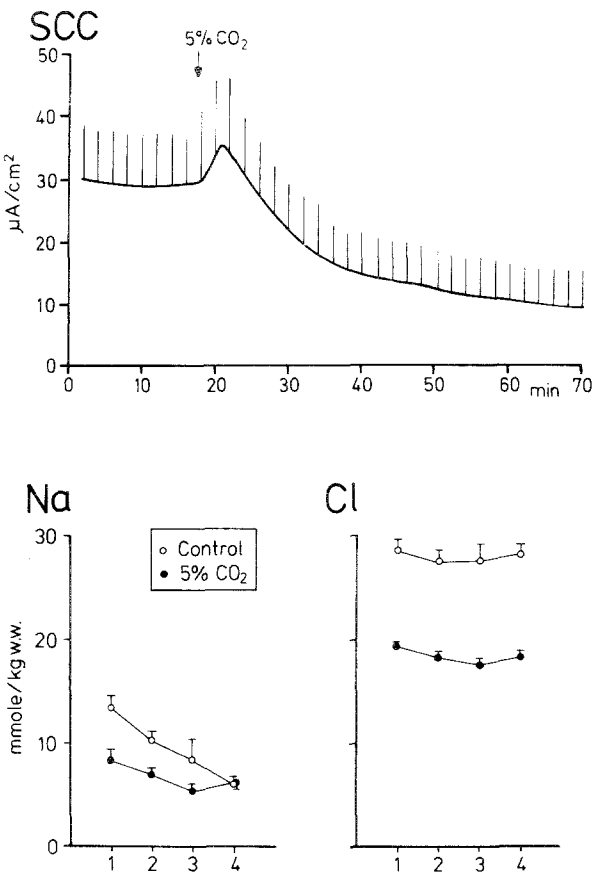

Fig. 11. Short-circuit current (SCC) and intracellular Na and Cl concentrations in the different epithelial cell layers in control and after bubbling the bathing solutions on either side of the cornea with a 5% CO_2/95% air mixture (5% CO_2). Experiment performed in Ringer's solution containing 2.5 mM HCO_3. For further explanations *see* legend to Fig. 6

Table 8. Effect of 5% CO_2 (at 2.5 mM HCO_3, 50 min) on the intracellular element concentrations and short-circuit current (SCC) of the cornea epithelium[a]

	Na	Cl (mmol/kg wet wt)	K	P	SCC (μA/cm²)
Control	8.3 ± 4.7	25.4 ± 4.5	123.0 ± 13.4	112.4 ± 19.1	26.3 ± 9.5
5% CO_2	6.7 ± 3.4	19.9 ± 2.7	102.5 ± 14.0	101.4 ± 20.7	6.7 ± 2.1

[a] Mean ± SD of 92 (Control) and 89 (5% CO_2) measurements obtained from three paired experiments.

tion from 21.2 ± 3.8 (n = 36) to 23.6 ± 4.5 mmol/kg w.w. (n = 34, 2 $P < 0.05$), which was accompanied by an increase in the Na concentration from 6.6 ± 3.1 to 9.3 ± 4.8 mmol/kg w.w. (2 $P < 0.01$).

The effect of HCO_3 was also tested in Ringer's solutions providing consistent results. Increasing the HCO_3 concentration in the inner bath from 2.5 to 25 mM effected a significant fall of the Cl concentration from 29.6 ± 3.9 (n = 38) to 24.3 ± 5.3 mmol/kg w.w. (n = 35, 2 $P < 0.001$). Increasing the HCO_3 concentration in the outer bath also resulted in a fall of the mean cellular Cl concentration from 27.9 ± 6.50 (n = 33) to 26.0 ± 5.1 mmol/kg w.w. (n = 28), which, however, did not attain statistical significance. In a third pair of corneas the HCO_3 concentration was increased on either side of the cornea simultaneously, resulting in a reduction of the Cl concentration from 24.8 ± 3.4 (n = 33) to 19.5 ± 3.3 mmol/kg w.w. (n = 36, 2 $P < 0.001$). The mean cellular Na concentration in this experiment was lowered from 7.9 ± 3.2 to 3.8 ± 2.7 mmol/kg w.w. (2 $P < 0.01$).

The pH effects were tested by equilibrating the bathing media with a 5% CO_2/air mixture. Figure 11 shows an experiment in which the Ringer's solutions

Table 9. Extracellular element concentrations and dry weight content (d.w.) in the corneal stroma under control conditions using either Conway's (25 mM HCO_3) or normal frog Ringer's solution (2.5 mM HCO_3) as bathing medium[a]

	Na	Cl (mmol/kg wet wt)	K	P	SCC (g/100g)
Conway	100.6 ± 3.3	56.4 ± 6.7	4.7 ± 2.4	5.7 ± 2.2	15.1 ± 1.6
Ringer	97.0 ± 14.8	75.5 ± 8.2	3.6 ± 2.7	2.5 ± 2.3	15.2 ± 3.1

[a] Mean ± SD of 24 (Conway) and 96 (Ringer) measurements.

bathing either side of the cornea were bubbled with CO_2 resulting in a pH drop of the medium from 8.3 to 6.5. Following a transient stimulation there is a marked reduction in both, the SCC and g_t. In all epithelial layers the Cl concentration is significantly reduced. The drop in the Na concentration is more pronounced in the outer layers. Table 8 summarizes the results obtained from three similar experiments. In one of the experiments the reduction in the Cl concentration was only minimal. In addition to the known Na and Cl changes a significant fall in the K and P concentration is evident. The cell swelling implied by the P change is also reflected by a small reduction in the dry weight content from 21.7 ± 2.3 to 20.9 ± 3.2 g% (*not statistically significant*).

In a further experiment the effect of CO_2 was tested in a cornea incubated in Conway's solution. Because of the much higher concentration of HCO_3 (25 mM versus 2.5 in the Ringer's solution) bubbling of the medium with CO_2 resulted in a pH drop from 8.5 to only 7.5. Figure 12 shows that under this condition the inhibition of the SCC is much less pronounced. The Na concentrations in the different cell layers are practically unchanged. The small decrease in the mean Cl concentration from 19.9 ± 2.8 (n = 30) to 18.7 ± 4.5 mmol/kg w.w. (n = 34) is not statistically significant. Also the K concentrations were at 122.9 ± 16.5 and 117.0 ± 15.7 mmol/kg w.w. not significantly different. On the other hand, the P concentration and dry weight content decreased by 16 and 11%, respectively, suggesting that the CO_2-induced cellular swelling is still present.

In some corneas measurements were also performed in the extracellular space of the stroma. Table 9 lists the mean values obtained under control conditions. The slightly elevated P and K concentrations indicate a minimal excitation of an intracellular compartment. Compared to the composition of the bathing media (Table 1) the main differences are

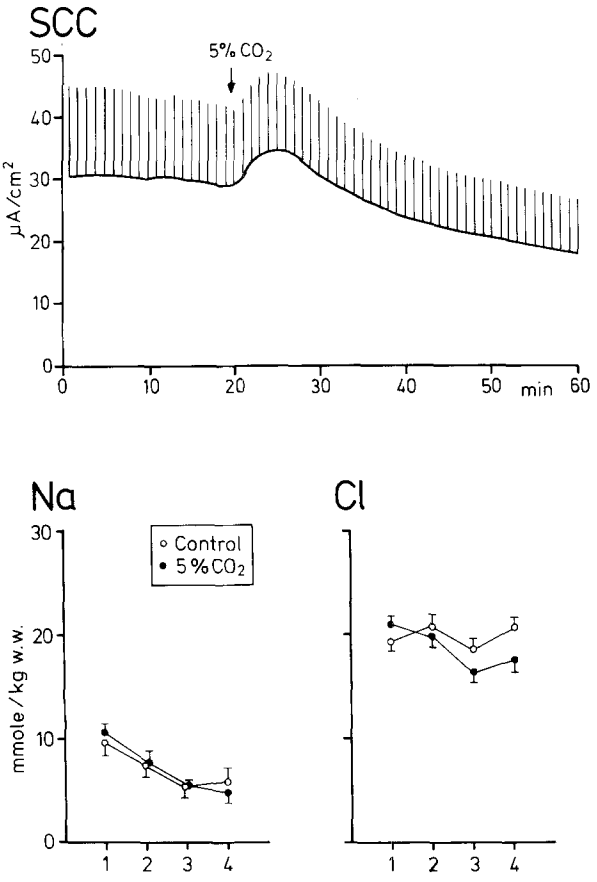

Fig. 12. Short-circuit (SCC) and intracellular Na and Cl concentrations in the different epithelial cell layers in control and after bubbling the bathing solutions on either side of the cornea with a 5% CO_2/95% air mixture (5% CO_2). Experiment performed in Conway's solution containing 25 mM HCO_3. For further explanations *see* legend to Fig. 6

the much lower Cl values, suggesting that the acid mucopolysaccharide and protein-rich ground substance (Anseth & Laurent, 1961; Laurent & Anseth, 1961) contains a large number of fixed negative charges. Allowing for a 1% contribution of intracellular spaces and recalculating the data into concentration per liter water the Na values are between 5 and 15% higher, whereas the Cl values are between 10 and 20% lower than in the inner, anterior chamber-side bathing medium. The results are consistent with a Donnan-type ion distribution between the inner bath and the extracellular space of the stroma. Similar Donnan effects have been observed for extracellular matrices in other epithelial tissues (*see* e.g. Dow et al., 1984).

After removal of Na from the inner bath the Na concentration in stroma was reduced to 3.4 ± 1.9 mmol/kg w.w., suggesting that the endothelium does not represent a significant diffusion barrier to small ions and that virtually no Na is fixed or bound

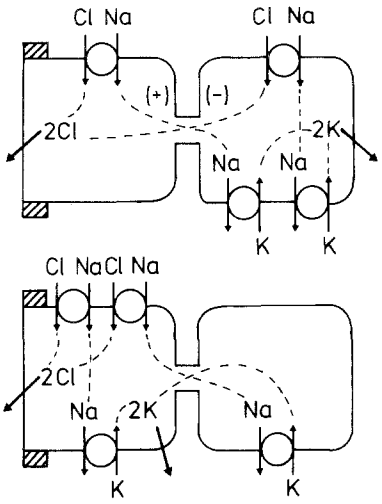

Fig. 13. Models of epithelial cooperation in Cl secretion. For further details *see* text

by the ground substance. Similarly, after removal of Cl from the inner bath the Cl concentration in the stroma was found to be reduced to very low values (1.9 ± 0.6 mmol/kg w.w., $n = 6$). Using a K-free incubation medium only a relatively small drop from 4.3 ± 2.6 to 3.0 ± 2.9 mmol/kg w.w. ($n = 6$) was observed, consistent with the notion that some of the K signal is due to stray excitation of an intracellular compartment. The concentrations of the substituted ion species in the effluent bath were in all cases less than 0.2 mM.

Discussion

Functional Organization of the Epithelium

The multilayered epithelium of the frog cornea can be regarded as a functional syncytium, similar to what has been shown for the Na-transporting frog skin (Rick et al., 1978). This view is based on the fact that the concentrations of small readily diffusable ions such as Na, K and Cl were almost identical in all epithelial layers and that experimental perturbations always resulted in parallel concentration shifts. The most clear-cut evidence for the existence of cell-to-cell communications between the different epithelial layers is the marked decrease in the Cl concentration of the intermediate and basal cell layers after removal of Cl from the outer bath (Fig. 6). Since the paracellular shunt pathway is sealed by tight junctions (zonule occludentes) at the level of the outermost epithelial cell layer (Hogan et al., 1971; *see also* Kaye, 1962), the response of the deeper-lying layers can only be explained, if there is some sort of coupling to the outermost layer of cells. Most likely, the morphological correlate of coupling are the gap junctions (maculae occludentes), which have been described in the epithelium of the human cornea (Hogan et al., 1971).

In control, a small inwardly directed Na concentration gradient was detectable (Fig. 3), suggesting a net diffusion of Na from the outer to the inner epithelial layers. The Na gradient is similar to that observed in the frog skin epithelium, where it can be accounted for by transepithelial Na transport (Rick et al., 1984). In the cornea, however, this explanation is rather unlikely, since the rate of Na reabsorption is only negligibly small (Candia & Askew, 1968). Also the fact that the Na gradient is dependent on the rate of Cl secretion argues against this possibility. When Cl secretion was inhibited, the Na gradient was usually reduced (Figs. 7, 8 and 11), whereas when Cl secretion was enhanced the gradient was increased (Fig. 6). Furthermore, the dependence of the intracellular Na on the availability of Na and Cl in the inner medium suggests that the cellular Na mainly originates from this side. In contrast, for Cl virtually no concentration gradient is detectable (Fig. 3), despite the fact that the rate of transepithelial Cl secretion greatly exceeds the rate of Na reabsorption (Zadunaisky, 1966; Candia & Askew, 1968). This finding seemingly indicates that the cell-to-cell communication is much more permeable to Cl than to Na. However, the large bore size of the cell-to-cell channel (Schwarzmann et al., 1981) precludes that this pathway could actually discriminate between Na and Cl.[2]

The two models of epithelial cooperation depicted in Fig. 13 provide two possible explanations for the observed intraepithelial Na concentration gradient. In either model it is assumed that the Cl influx across the basolateral membranes is in some way coupled to a Na influx. In the upper model it is further assumed that the cells of the outer epithelial layer have a lower capacity to actively extrude Na via the Na/K pump. Given the same rate of coupled NaCl influx into all layers, the Na in the outer layer therefore should rise above the level in the inner layers. Na can diffuse along its concentration gradient into the deeper cells from where it is finally extruded by the pump. In addition, because of the depolarizing Cl efflux across the apical membrane

[2] Apart from direct cell-to-cell communication indirect coupling via the extracellular (intercellular) spaces can be envisioned for ions to which the basolateral membrane is highly permeable and which are close to or at equilibrium distribution across this membrane. For Cl alone the latter requirement is certainly not fulfilled; however, if the Cl movement is coupled to other ions, like via a Na + K 2 Cl symporter or a Cl/HCO_3 exchanger, this possibility exists.

of the outer cells and the predominant pump activity of the inner cells, there should be a small electrical potential gradient across the cell-to-cell junction which may explain Cl diffusion from the inner to the outer cells in the absence of any driving chemical concentration gradient.

In the lower model it is assumed that the basolateral NaCl influx is confined to mainly the outermost epithelial layer, whereas the Na/K pump rates are the same in all layers. This would also lead to a rise in the Na concentration of the outer layer, Na diffusion towards the inner layers and active Na extrusion from the inner layers. In this model the inner epithelial layers are not engaged in the transepithelial transport pathway for Cl, obviating the need for any driving electrical or chemical gradient across the cell-to-cell junction. K, which is actively taken up in exchange for Na, diffuses from the inner to the outer layers, giving rise to a small outwardly directed K concentration gradient (*see* Figs. 3 and 4). The K efflux across the basolateral membrane of the outer cell layer electrically compensates for the Cl efflux across the apical membrane.

The present results do not allow to discriminate between the "pump gradient" and the "influx gradient" hypothesis; in fact, a combination of the two models is also conceivable. The finding of a lower ouabain binding capacity of the outer epithelial layers in the frog skin (Mills, Ernst & DiBona, 1977) may suggest that the first alternative is correct.

Intracellular Element Concentrations of the Corneal Epithelium

The intracellular electrolyte concentrations in the frog cornea epithelium (Tables 2 and 3) are strikingly similar to those observed in the Na-transporting frog skin under comparable conditions (Rick et al., 1978, 1984). The only major difference is the much lower Cl concentration in the cornea. In part, the difference is due to the higher HCO_3 concentration of the Conway's solution (*see below*); however, even if the same Ringer's solution is employed the Cl concentration in the cornea is still significantly lower. The difference is even more pronounced considering the electrochemical activity gradient across the epithelial cell membrane. In the frog skin, using a membrane potential of −73 mV (Nagel, 1976) and a chemical concentration of 37.5 mmol/kg w.w. (Rick et al., 1984) the intracellular Cl activity is 51 mV above equilibrium, whereas in the cornea, using a membrane potential of −54 mV (Nagel & Reinach, 1980) and the 25.9 mmol/kg w.w. obtained for the Ringer controls, the intracellular Cl is only 23 mV above equilibrium. It is of course tempting to speculate that the apparent discrepancy between the two epithelia is only due to the presence or absence of an additional Cl leak in the apical cell membrane.

The above-equilibrium distribution of the intracellular Cl in the frog cornea has already been observed by ion-selective electrode measurements. Zadunaisky et al. (1979) reported a Cl activity of 29 mM, corresponding to a 26-mV electrochemical potential difference. Recently, Reuss et al. (1983) have obtained a Cl activity of 22 mM, which under open-circuit conditions equals a 33-mV electrochemical potential difference across the basolateral membrane. They also measured the Na and K activities and obtained under open-circuit conditions mean values of 14 and 106 mM, respectively. Using the activity coefficient for the Ringer's solution of 0.76 and assuming a water content to be 100% minus dry weight content our present chemical concentrations can be converted into ion activities. The calculated Na, K and Cl activities are 7.7, 113.5 and 17.8 mM for the Conway controls, and 9.1, 113.7 and 25.2 mM for the Ringer controls, respectively. The values are close to the wet weight concentrations given in Table 3, as the corrections for the activity coefficient and water content almost cancel out. The comparison to the ion activities reported by Reuss et al. (1983) reveals a good agreement. Their Cl value lies right in the middle of our two control values: their Na value is higher by only 6 mM and their K value is lower by only 8 mM. The near identity of the two sets of data suggests that the assumption of the intracellular activity coefficient being identical to the extracellular one is essentially correct. In fact, the intracellular activity coefficient can be calculated from a comparison of chemical concentrations and ionic activities. Using the K values an intracellular activity coefficient of 0.71 is obtained; using the sum of the Na and K values the coefficient is 0.75. The justification for using the sum of Na and K is the apparent 1 : 1 exchange between the two cations observed between different cells of the same epithelium (Fig. 4), between different controls, and between different experimental conditions (Table 6). Thus, whatever the reason for the higher Na activity reported by Reuss et al. (1983), it should be compensated for by an equivalent drop in the K activity. Only after bubbling the bathing media with CO_2 was a significant change in the sum of Na and K detectable. The parallel drop in the Na and K concentrations observed under this condition (*see* Table 8) may be explained by a reduction in the number of fixed negative charges caused by the lowering of the intracellular pH.

For Na, K and Cl no significant differences between the cytoplasmic and nuclear concentration values were detectable, suggesting that the nuclear membrane does not constitute a significant barrier

to the movement of small ions. The Ca concentration in the cytoplasm was found to be higher than in the nucleus consistent with some sequestration of Ca by cytoplasmic organelles. A possible Na/Ca exchange at the basolateral membrane might explain the observed Ca increase after removal of Na from the inner bath.

Transport Steps Involved in Active Cl Secretion

The fact that the intracellular Cl concentration lies above the equilibrium value implies that the active step involved in transepithelial Cl secretion is located at the basolateral membrane, whereas the Cl exit across the apical membrane may be an entirely passive process. Thus the Cl efflux can be explained by simple electrodiffusion via a Cl-selective channel. Consistent with this assumption, a significant fall in the intracellular Cl concentration, a drop in g_t and simultaneously a marked stimulation of the SCC was observed, when the outer bathing medium was replaced with a Cl-free solution (Fig. 6). The existence of an apical Cl conductance has already been demonstrated by intracellular microelectrode studies in the rabbit (Klyce & Wong, 1977) as well as in the frog cornea (Nagel & Reinach, 1980; Reuss et al., 1983).

Similar to most Cl-secreting epithelia, Cl secretion in the frog cornea is dependent on the presence of Na in the inner bath (Zadunaisky, 1966, 1972) and can be inhibited by ouabain (Candia, 1972), suggesting that Cl uptake across the basolateral membrane is secondary active, driven by the Na gradient. Recently, Reuss et al. (1983) have shown that the intracellular Cl activity is lowered to the electrochemical equilibrium value, when the inner side of the cornea is bathed in a Na-free solution. The present study provides further evidences for a coupled NaCl uptake at the basolateral membrane. Removal of Cl from the inner bath not only resulted in a marked reduction of the intracellular Cl concentration, but also in a significant fall of the Na concentration (Fig. 7). Likewise, removal of Na from the inner bath resulted in both, a fall in Na and Cl (Fig. 8). Furthermore, the inverse relationship between the intracellular concentrations of Na and Cl observed in control corneas (Fig. 5) and after K removal from the inner bath (Fig. 9) suggests that the intracellular Cl value is determined by the height of the driving Na gradient.

Figure 14 depicts three different models which could account for the secondary active Cl uptake across the basolateral membrane. Cl influx could be directly coupled to the Na influx in a 1 : 1 fashion, as proposed by Silva et al. (1977) and Frizzell et al. (1979). Alternatively, a Na + K + 2 Cl symport system may be present, as described for red blood cells (Dunham, Stewart & Ellory, 1980), Ehrlich ascites cells (Bakker-Grunwald, 1978) and recently also for the thick ascending limb of Henle (Greger & Schlatter, 1983). Finally, the parallel operation of Na/H and Cl/HCO_3 exchangers is conceivable, as suggested for the NaCl reabsorption in the small intestine (Turnberg et al., 1970) and gallbladder (Petersen et al., 1981). The main difference between the first two models is the higher efficiency of the Na + K + 2 Cl transporter, since for each Na ion two Cl ions are pumped. On the other hand this entails that the available chemical driving force for the Cl uptake is significantly smaller. Using our values for the Conway controls, the net driving force for the Na + K + 2 Cl symporter is only 14 mV, or 7 mV per transported Cl ion, whereas for the Na + Cl symporter the gradient is 88 mV.

The quantitative analysis of the results of the ion substitution experiments should allow to distinguish between the first two alternatives, as the Na + K + 2 Cl symporter can be expected to be much more sensitive to changes in the chemical gradients of Na, K and Cl than the Na + Cl symporter. One critical assumption involved in this consideration is, however, that the corneal stroma freely equilibrates with the inner bath. To verify this assumption we measured the extracellular ion concentrations in the stroma close to the epithelium. The results show that the ion concentrations in the stroma are in equilibrium with the inner bath and can be readily exchanged within the applied incubation times, suggesting that the endothelium lining the inner surface of the cornea does not constitute a significant barrier to the movement of small ions. The same conclusion has already been reached by Kaye (1962). The results do not lend support to the notion that a significant fraction of the stromal Na is bound (Otori, 1967).

After removal of Na from the inner bath only a partial inhibition of SCC and g_t was observed, agreeing with the finding of Zadunaisky (1972) that net Cl secretion is not completely abolished under this condition. Calculating from the cellular ion concentrations listed in Table 5 the extracellular Na concentration for which the driving force of the cotransport system will be zero, we obtain values for the Na + K + 2 Cl and Na + Cl symporter of 16.6 and 1.5 mM, respectively. Thus at the actually observed Na concentrations of 3.4 mmol/kg w.w. only the Na + Cl symporter provides a positive driving force for Cl secretion. Also after removal of K from the inner bathing medium only a partial inhibition of the SCC and g_t was observed (*see* Fig. 9). For the

Na + K + 2 Cl model the extracellular K concentration at which the transporter will be in equilibrium is 1.4 mM. Unfortunately, most likely because of some stray excitation of an intracellular compartment it was not possible to exactly quantitate the reduction in the stromal K concentration under this condition. However, assuming that K behaves identically to Na and taking into account that an even longer equilibriation time was used, the K concentration in the subepithelial space can be expected to be much lower than 1.4 mM, implying that also in this case the Na + K + 2 Cl symporter can not provide a positive driving force. For the Na + Cl symporter the calculated chemical driving gradient is practically unchanged at 75 mV. In this model the inhibition of the SCC may be referred to the reduced K conductance of the basolateral membrane, providing a lower electrical driving force for Cl efflux across the apical membrane. After removal of Cl from the inner bath the extracellular Cl concentration for which the cotransport system is in equilibrium is 15.3 mM for the Na + K + 2 Cl symporter and 0.6 mM for the Na + Cl symporter (data from Table 4). Thus at a Cl concentration in the subepithelial space of 1.9 mmol/kg w.w., Cl secretion is still possible in the Na + Cl model, whereas in the Na + K + 2 Cl model Cl reabsorption should take place provided that the direction of transport can be reversed. Since the SCC under this condition is close to zero it is not possible to differentiate between the two alternative models. A small Cl secretion in the transcellular pathway may be masked by an opposite net Cl flux in the shunt pathway, whereas a small Cl reabsorption may be balanced by an active Na reabsorption.

Summarizing the evidences obtained from the ion substitution experiments it seems that the Na + Cl cotransporter provides a much better explanation for the observed behavior of the intracellular electrolyte concentrations. It should be stressed, however, that the above interpretation assumes that the ion concentration in the lateral intercellular spaces are identical to those in the adjacent subepithelial space, which need not be the case. In view of the fact that the lateral intercellular spaces in the freely transporting frog cornea are almost completely collapsed, it is conceivable that ions are trapped within these spaces. For K the washout to the stroma can be expected to be slowed down by an active K reuptake via the Na/K pump. Furthermore, the large cellular pool of K may replenish the K lost from the intercellular spaces for a long time, thus effectively buffering the K concentration. For Na the small transepithelial net reabsorption may be sufficient to maintain the Na concentration in the intercellular spaces significantly above the level in the subepithelial space. Finally, the results do not rule out the possibility of a heterogeneous Cl uptake mechanism. It is conceivable that under normal transport conditions Cl uptake is accomplished by the Na + K + 2 Cl symporter, whereas, when the driving force is too low, an otherwise quiescent Na + Cl symporter or an equivalent process is activated.

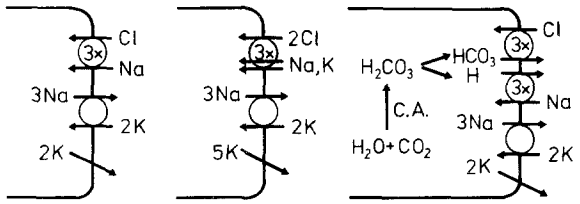

Fig. 14. Possible secondary active uptake mechanisms of Cl across the basolateral membrane of the frog cornea epithelium. For each cycle of the Na/K pump 3 Cl ions are taken up in the Na + Cl symport model (*left*) and in the Na/H plus Cl/HCO_3 exchange model (*right*), whereas in the Na + K + 2 Cl symport model (*middle*) 6 Cl ions are taken up. C.A. = carbonic anhydrase

pH Effects on Cl Secretion

Compared to Conway controls, the Ringer control corneas had a significantly higher intracellular Cl concentration. As evident from the experiments in which the HCO_3 content of the Ringer's or Conway's solution was varied, this difference is largely attributable to the differential HCO_3 concentration of the two media. The intracellular Cl concentration is mainly a function of the HCO_3 concentration in the inner bath, although some effect was also detectable when the HCO_3 in the outer bath was changed. The inverse relationship between the intracellular Cl and extracellular HCO_3 provides some evidences for a Cl/HCO_3 exchange at the basolateral cell membrane. The existence of an anion exchanger in the frog cornea epithelium has already been postulated by Candia (1980), based on the observation that the inhibitory effect of vanadate can be prevented by prior addition of DIDS.

The HCO_3 dependence of the intracellular Cl concentration is consistent with the third model depicted in Fig. 14, in which the NaCl uptake is accomplished by the parallel operation of an anion and cation exchanger. In two preliminary experiments we tested this hypothesis directly by applying the anion exchange inhibitor, SITS (10^{-3} M). Contrary to the prediction from the model, but consistent with the effect of DIDS (Candia, 1980), we observed a small stimulation of the SCC. The intra-

cellular Cl was only slightly reduced. Further evidence against a significant contribution of a Cl/HCO_3 exchange to the rate of Cl uptake across the basolateral membrane is the fact that we were unable to detect any significant changes in the SCC and intracellular Cl concentration after addition of the carbonic anhydrase inhibitor, acetazolamide (*own unpublished results*). It has to be pointed out, however, that the experiments were performed in the absence of exogenous CO_2. Under this condition the rate of Cl/HCO_3 exchange is most likely limited by the very low supply of cellular CO_2. Possibly the transient stimulation of the SCC observed after bubbling the bathing media with 5% CO_2 (*see* Figs. 11 and 12) reflects a stimulation of the Cl uptake via a Cl/HCO_3 exchange, which is normally quiescent.

The inhibition of the SCC and increase in the cellular Cl concentration after lowering the HCO_3 concentration of the medium may be explained by a possible intracellular pH change. A drop in the cell pH may either directly lower the apical Cl permeability, or indirectly lower the electrical driving force for the Cl efflux by reducing the K permeability of the basolateral membrane. A consistent pH dependence has been demonstrated for the Cl conductance in striated muscle (Hutter & Warner, 1967; Palade & Barchi, 1977) as well as for the K conductance in renal tubules (O'Neil, 1982; Stanton, Guggino & Giebisch, 1982).

Similarly, the inhibition of the SCC after bubbling the bathing media with CO_2 can be referred to an intracellular pH drop. However, the parallel decrease in the Na and Cl concentration observed with a low HCO_3-containing Ringer's solution (Fig. 11) implies, that in addition to the Cl efflux also the coupled NaCl uptake must be reduced. The effects of CO_2 were found to be greatly attenuated, when the cornea was incubated in the HCO_3-rich Conway's solution (Fig. 12), suggesting that either the intracellular pH change is blunted by an increased intracellular HCO_3 concentration or that the site of the pH action is, at least partially, extracellular. Candia (1973) concluded from transepithelial flux measurements that the pH mainly affects a series resistance in the transepithelial transport pathway.

Epithelial Cell Volume and Size of Lateral Intercellular Spaces

Under some of the experimental conditions tested parallel changes in the P concentration and dry weight content were observed. Assuming that the cellular dry matter and P content remains constant, their concentration changes are best explained by a variation of the cellular volume. According to these criteria some cellular shrinkage was evident after removal of Na or Cl from the inner bath. In K-free media only the increased dry weight content indicated cell shrinkage, whereas the P concentration was slightly lowered. Cellular swelling was observed after bubbling the bathing media with 5% CO_2.

The involvement of the epithelial cell volume changes in Cl transport is clear from the following consideration. Essentially, transepithelial Cl secretion is accomplished by two different processes with opposing effects on the epithelial cell volume. The electrogenic, mutually dependent effluxes of Cl and K will lower the intracellular solute content, thereby reducing the cell volume, whereas the presumably electroneutral NaCl uptake, or an equivalent mechanism, tends to increase the cell volume, because of the addition of intracellular solutes.[3] Thus the shrinkage observed after removal of Na or Cl may be taken as a further evidence for a primary action on the NaCl uptake mechanism, whereas the swelling observed after CO_2 suggests that the predominant effect is the inhibition of the Cl and K efflux. Viewed in this way, transepithelial Cl secretion is intimately related to the epithelial cell volume regulation. It is obvious that the epithelial cell volume can only be maintained when the rate of KCl efflux and NaCl influx are tightly coupled. In fact, considering the drastic perturbations employed in the present investigation, the observed cellular volume changes are surprisingly small.

In our freeze-dried cryosections of snap frozen corneas the lateral intercellular spaces were found to be almost completely collapsed (*see* Fig. 2). Only under conditions in which the Cl transport was fully inhibited, like after removal of Cl from the inner bathing medium, open intercellular spaces were detectable. This behavior is exactly opposite to that previously seen in the frog skin epithelium, where the intercellular spaces are generally wide open and only collapsed when the Na transport is inhibited (*own unpublished observation*). The apparent discrepancy may be explained by the different direction of salt transport in the two epithelia. In the skin Na (and Cl) is secreted into the intercellular spaces resulting in a dilation of the spaces, if water can follow. In the cornea NaCl is reabsorbed from the intercellular spaces, which therefore should shrink as water follows.

[3] The Na/K pump has very little direct effect on the cell volume. On the other hand by actively recycling the Na and K ions it provides the basis for either processes.

Transepithelial Electrical Parameters

Compared to previous investigations (e.g. Nagel & Reinach, 1980; Reuss et al., 1983) we obtained relatively high values for the SCC, g_t and open-circuit transepithelial potential difference. To some extent the differential g_t values may be due to hydrostatic pressure effects. In preliminary experiments we observed a significant g_t decrease, when the transcorneal hydrostatic pressure difference was raised from 1 to 10 cm H_2O, anterior chamber-side positive. The SCC was virtually unchanged under this condition, while the potential difference was further increased.

Under all experimental conditions which resulted in an inhibition of the SCC also a reduction in g_t was observed. An inverse relationship between the two parameters was only detectable after removal of Cl from the outer bath, where a stimulation of the SCC was accompanied by a fall in g_t (Fig. 6). The g_t change under this condition can be explained by a reduction in the apical Cl conductance due to the lowered Cl concentration on either side of the membrane. However, it is not possible to say to what extent the observed g_t variations actually reflect conductance changes in the transcellular transport pathway. The apparently SCC unrelated component of g_t as indicated by the nonzero intercept with the y-axis in Fig. 5 may be regarded as an upper estimate of the shunt conductance, since the transcellular conductance should attain a minimum value at zero transepithelial transport. Assuming that the linear correlation between the g_t and SCC values is primarily due to a variation of a series resistance, then the slope of the regression line equals the transepithelial driving force of Cl secretion, E_{Cl}. The value of 37 mV calculated in this way agrees well with the 45 mV obtained by others in the rabbit cornea (Klyce & Wong, 1977) and frog cornea (Nagel & Reinach, 1980) using entirely different approaches.

The authors wish to thank Brigitte Mayer, Birgit Sauer, Gertrud Spancken, Elborg von Arnim-Spellenberg, Erwin Strigl and Monika Weigel for their excellent technical assistance. Financial support was provided by grants from the Deutsche Forschungsgemeinschaft.

References

Anseth, A., Laurent, T.C. 1961. Studies on corneal polysaccarides. I. Separation. *Exp. Eye Res.* **1**:25–38

Bakker-Grunwald, T. 1978. Effect of anions on potassium self-exchange in ascites tumor cells. *Biochim. Biophys. Acta* **513**:292–295

Bauer, R., Rick, R. 1978. Computer analysis of X-ray spectra (EDS) from thin biological specimens. *X-Ray Spectrom.* **7**:63–69

Candia, O.A. 1972. Ouabain and sodium effects on chloride fluxes across the isolated bullfrog cornea. *Am. J. Physiol.* **233**:1053–1057

Candia, O.A. 1973. Effect of pH on chloride transport across the isolated bullfrog cornea. *Exp. Eye Res.* **15**:375–382

Candia, O.A. 1980. Use of stimulants and inhibitors for studying the mechanisms of Cl transport. *Ann. N.Y. Acad. Sci.* **34**:117–124

Candia, O.A., Askew, W.A. 1968. Active sodium transport in the isolated bullfrog cornea. *Biochim. Biophys. Acta* **163**:262–265

Candia, O.A., Montoreano, R., Podos, S.M. 1977. Effect of the ionophore A23187 on chloride transport across isolated frog cornea. *Am. J. Physiol.* **233**:F94–F101

Chalfie, M., Neufeld, H., Zadunaisky, J.A. 1972. Action of epinephrine and other cyclic AMP-mediated agents on the chloride transport of the frog cornea. *Invest. Ophthalmol.* **11**:644–650

Dörge, A., Rick, R., Gehring, K., Thurau, K. 1978. Preparation of freeze-dried cryosections for quantitative X-ray microanalysis of electrolytes in biological soft tissue. *Pfluegers Arch.* **373**:85–97

Dow, J.A.T., Gupta, B.L., Hall, T.A., Harvey, W.R. 1984. X-ray microanalysis of elements in frozen-hydrated sections of an electrogenic K^+ transport system: The posterior midgut of tobacco hornworm (*Manduca sexta*) *in vivo* and *in vitro*. *J. Membrane Biol.* **77**:223–241

Dunham, P.B., Stewart, G.W., Ellory, J.C. 1980. Chloride-activated passive potassium transport in human erythrocytes. *Proc. Natl. Acad. Sci. USA* **77**:1711–1715

Frizzell, R.A., Field, M., Schultz, S.G. 1979. Sodium coupled-chloride transport by epithelial tissues. *Am. J. Physiol.* **236**:F1–F8

Greger, R., Schlatter, E. 1983. Properties of the basolateral membrane of the cortical thick ascending limb of Henle's loop of rabbit kidney. *Pfluegers Arch.* **396**:325–334

Hogan, M.J., Alvarado, J.A., Weddell, J.E. 1971. The cornea. *In:* Histology of the Human Eye. An Atlas and Textbook. pp. 65–111. W.B. Saunders, Philadelphia

Hutter, O.F., Warner, A.E. 1967. The pH sensitivity of the chloride conductance of frog skeletal muscle. *J. Physiol. (London)* **189**:403–425

Jehl, B., Bauer, R., Dörge, A., Rick, R. 1981. The use of propane/isopentane mixtures for rapid freezing of biological specimens. *J. Microsc. (Oxford)* **123**:307–309

Kaye, G.I. 1962. Studies on the cornea. III. The fine structure of the frog cornea and the uptake and transport of colloidal particles by the cornea *in vivo*. *J. Cell Biol.* **15**:241–258

Klyce, S.D., Wong, R.K.S. 1977. Site and mode of adrenaline action on chloride transport across the rabbit corneal epithelium. *J. Physiol. (London)* **266**:777–799

Laurent, T.C., Anseth, A. 1961. Studies on corneal polysaccharides. II. Characterization. *Exp. Eye Res.* **1**:99–105

Mills, J.W., Ernst, S.A., DiBona, D.R. 1977. Localization of Na^+-pump sites in frog skin. *J. Cell Biol.* **73**:88–110

Nagel, W. 1976. The intracellular electrical potential profile of the frog skin epithelium. *Pfluegers Arch.* **365**:135–143

Nagel, W., Reinach, P. 1980. Mechanism of stimulation by epinephrine of active transepithelial Cl transport in isolated frog cornea. *J. Membrane Biol.* **56**:73–79

O'Neil, R.G. 1982. Effect of luminal H^+ and Ba^{++} on the apical

cell membrane K^+ conductance of the cortical collecting tubule (CCT). *Fed. Proc.* **41:**1006

Otori, T. 1967. Electrolyte content of rabbit corneal stroma. *Exp. Eye Res.* **6:**356–367

Palade, P.T., Barchi, R.L. 1977. Characteristics of the chloride conductance in muscle fibers of the rat diaphragm. *J. Gen. Physiol.* **69:**325–342

Petersen, K.-U., Wood, J.R., Schulze, G., Heintze, K. 1981. Stimulation of gallbladder fluid and electrolyte absorption by butyrate. *J. Membrane Biol.* **62:**183–193

Reuss, L., Reinach, P., Weinman, S.A., Grady, T.P. 1983. Intracellular ion activities and Cl^- transport mechanisms in bullfrog corneal epithelium. *Am. J. Physiol.* **244:**C336–C347

Rick, R., Dörge, A., Arnim, E. von, Thurau, K. 1978. Electron microprobe analysis of frog skin epithelium: Evidence for a syncytial sodium transport compartment. *J. Membrane Biol.* **39:**313–331

Rick, R., Dörge, A., Beck, F., Thurau, K. 1982*a*. Intracellular electrolyte concentration of frog cornea epithelium at different functional states of Cl-secretion. *Pfluegers Arch.* **Suppl. 392:**R20

Rick, R., Dörge, A., Thurau, K. 1982*b*. Quantitative analysis of electrolytes in frozen dried sections. *J. Microsc. (Oxford)* **125:**239–247

Rick, R., Roloff, C., Dörge, A., Beck, F.X., Thurau, K. 1984. Intracellular electrolyte concentrations in the frog skin epithelium: Effect of vasopressin and dependence on the Na concentration in the bathing media. *J. Membrane Biol.* **78:**129–145

Schwarzmann, G., Wiegant, H., Rose, B., Zimmerman, A., Ben-Haim, D., Loewenstein, W.R. 1981. Diameter of the cell-to-cell junctional membrane channels as probed with neutral molecules. *Science* **213:**551–553

Silva, P., Stoff, J., Field, M., Fine, L., Forrest, J.N., Epstein, F.H. 1977. Mechanism of active chloride secretion by shark rectal gland: Role of Na-K-ATPase in chloride transport. *Am. J. Physiol.* **233:**F298–F306

Stanton, B., Guggino, W., Giebisch, G. 1982. Acidification of the basolateral solution reduces potassium (K) conductance of the apical membrane. *Fed. Proc.* **41:**1006

Turnberg, L.A., Bieberdorf, F.A., Morawski, S.G., Fordtran, J.S. 1970. Interrelationships of chloride, bicarbonate, sodium, and hydrogen transport in the human ileum. *J. Clin. Invest.* **49:**557–567

Zadunaisky, J.A. 1966. Active transport of chloride in frog cornea. *Am. J. Physiol.* **211:**506–512

Zadunaisky, J.A. 1972. Sodium activation of chloride transport in the frog cornea. *Biochim. Biophys. Acta* **282:**255–257

Zadunaisky, J.A., Spring, K.R., Shindo, T. 1979. Intracellular chloride activity in the corneal epithelium. *Fed. Proc.* **38:**1059

Received 22 May 1984; revised 23 August 1984