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THE REGULATION OF VOLUME AND ION COMPOSITION IN FROG SKIN

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1. Chemical determinations of Cl^- in frog skin indicate that the intracellular concentration of this ion is above that corresponding to an equilibrium distribution. 2. Published data on Na^+ efflux from the cell compartments into the two external bathing solutions, and on unidirectional fluxes across the whole preparation suggest that there are large unidirectional fluxes across the basolateral membranes. 3. Cl^- uptake from the mucosal barrier is very small, and the removal of Cl^- from the mucosal bathing solution does not affect the intracellular Cl^- concentration. 4. Removal of Cl^- from the serosal bathing solution produces a drastic decrease in cell Cl^- together with a loss of water. 5. This is accompanied by a less marked effect on Na^+ and K^+ content of the cells. 6. The removal of Na^+ from the serosal bathing solution produces also a decrease in Cl^-, Na^+ and K^+ content of the cells with a marked loss of water. 7. It is suggested that the basolateral membrane of the frog skin is the site of mechanisms able to regulate volume and ion composition of the epithelial cells and that part of these mechanisms consists of a coupling between the movements of Na^+ and Cl^-.

Introduction

Most work done on epithelial systems aims at describing mechanisms capable of performing transepithelial transport. As shown recently [1] the two-membrane model originally proposed by Koefoed-Johnsen and Ussing [2] is capable of explaining a large number of electrophysiological observations. One should ask however whether the machinery responsible for transepithelial transport as postulated in that model can also regulate epithelial cell composition and volume. Two specific problems arise in this context.

The first relates to the regulation of cell chloride. Several groups have reported intracellular potentials measured in short-circuited frog skin ranging from −40 to −100 mV [3–5]. Even taking the lower value, should Cl^- be at equilibrium with the external solutions one should expect its intracellular concentration to be around 20 mM. Yet the values usually measured are higher [6–8]. In addition, a number of workers have identified an acetazolamide-sensitive Cl^- transport [9–12] and Kristensen [9,13] suggested that the active step was located in the external barrier. If that is so one should expect intracellular Cl^- to be sensitive to acetazolamide and to depend on mucosal chloride.

The second question relates to the Na^+ exchanges that taken place across the basolateral membranes. It is widely accepted that the serosal barrier is fairly impermeable to Na^+ and is the place where Na^+ pumping occurs. If that is so one should expect the short-circuit current not to be affected, the cell Na^+ content to be insensitive to the level of serosal Na^+ and

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the Na^+ influx across the basolateral membrane to be very small. Yet it has been repeatedly reported [14, 15] that the Na^+ efflux across the basolateral membrane is much larger than the net transepithelial Na^+ transport, implying a large Na^+ influx across this barrier.

The work to be described below was undertaken to study these problems.

Methods

Frogs of the species *Rana ridibunda* Pallas were used. They were kept in a cold room at 4–6°C. Experiments were performed during the whole year. The following Ringer's solution was used unless otherwise specified. Composition in mM: Na^+ 112; Cl^- 119; K^+ 2.4; Ca^{2+} 1; Mg^{2+} 1. The aerated Ringer's solution was buffered with Tris base to pH 8. The osmolality was 220 mosM/kg. Usually two symmetrical pieces of the same skin were mounted in Ussing type chambers with an exposed area of 3.14 cm^2, clamped between two neoprene O rings covered with silicone grease to avoid edge damage. The skins were continuously aerated.

The short-circuit current (I_{sc}) was measured by means of an automatic voltage clamp device and the experiments were always performed after a steady state was reached.

Transepithelial $^{36}Cl^-$ influxes and backfluxes were measured in symmetrical pieces of the same skin after incubation with the isotope for 1 h. 15-min periods were used. The volume used was measured with a pipette, and at the end of each period all the fluid was collected directly into a scintillation vial. Suitable amounts of radioactive Ringer's solution were also counted.

Ion contents and washout experiments were performed in isolated epithelia (epidermis). The technique of isolating the epidermis was described before [16] and was used here with some modifications. The skins were mounted in Ussing-type chambers and incubated at their chorion side with an Ringer's solution containing 80 U/ml colagenase type 1 from Sigma and 5 mM Ca^+ for 2 h at 22–25°C. For the determination of ion contents the skins remained in the chambers, the collagenase Ringer's solution was removed and rinsed several times, and finally the chambers were filled with the desired Ringer's solution for 1 h under short circuit conditions. During this hour the solution was renewed frequently. In the Ringer's solution without Cl^- this anion was substituted for gluconate, and in the Ringer's solution without Na^+ this cation was substituted cor choline. The osmolality was checked and when needed sucrose was added to preserve isotonicity at (220 mosM/kg). To measure the extracellular space ^{60}Co EDTA was used [17]. The Ringer's solution contained $0.5 \cdot 10^{-4}$ M unlabeled $CoCl_2$ and $1 \cdot 10^{-4}$ M EDTA and no Ca^{2+} and Mg^{2+}. After 1 h incubation the skins were removed, dissected in less than 1 min, rinsed in ice-cold isotonic sucrose for 1 s, blotted with filter paper, placed in tared aluminium foil boxes, weighed and dried at 100°C to constant weight and placed in vials for ^{60}Co counting. Then suitable amounts of 0.1 M HNO_3 were added to the vials, which were maintained for 24 hours with slow agitation at room temperature, for extraction of Na^+, K^+ and Cl^-. Na^+, K^+ and Cl^- contents of the Ringer's solution and the tissue extract were measured after appropriate dilution. In the case of washout experiments the skins were first incubated for 2 h with Ringer's containing collagenase and $^{36}Cl^-$ in chambers of 7 cm^2; they were then dissected and the isolated epidermis was mounted in smaller chambers (3.14 cm^2) and short-circuited and the washout procedure was performed. First the skins were washed on both sides three times in 1 min and the fluid was discarded. Then 2 ml of Ringer's solution were measured to each side with a calibrated pipette and after 10 min the solution from each side was collected directly into counting vials. The washouts were done during 1.5–2 h. At the end of the experiments, the skin was carefully cut out of the chamber, blotted, weighed, put into a vial with 2 ml of 0.1 M HNO_3, for extraction during 24 h, and the tissue extract counted. Suitable dilutions of the loading Ringer's solution were counted as well. Cl^- determinations of the tissue extracts were also performed, and it can be seen that after 2 h all the Cl^- of the preparation is marked with $^{36}Cl^-$ (Cl^- marked isotopically 0.356 ± 0.040 μequiv./cm^2 vs. Cl^- determined chemically 0.356 ± 0.033 μequiv./cm^2). The total amount of counts in the skin at the beginning of each collection period was calculated by adding back the counts lost in successive washout samples. A semilogarithmic plot of the total remaining counts in the skin versus time was made and the rate constants of

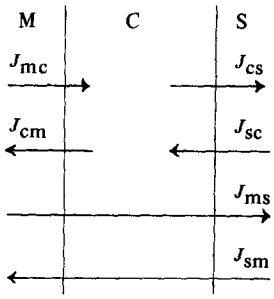
the exponential components were calculated by successive linear extrapolations. Knowing the specific activity of Cl^- in the loading solution, and the total initial activity in the skin the total Cl^- content can be calculated. The Cl^- content of each compartment was calculated from the extrapolated activity at zero time. The Cl^- concentration could then be calculated using extracellular and intracellular water determinations from previous experiments. The separated fractional losses of the inside and outside barriers were obtained by dividing the amount washed out during one period by the total amount of isotope in the skin in the middle of the same period. From the fractional losses and the Cl^- contents the fluxes can be calculated and from the fluxes and the intracellular Cl^- concentrations the apparent permeabilities can be evaluated. Finally, the permeabilities are obtained by using the Goldman-Hodgkin-Katz equation [18] and the potentials reported in the literature [4,5]:

$$P_R = P_A(1 - e^{-V})/V \text{ and } V = ZF\psi_{sc}/RT$$

where P_R is the real permeability, P_A is the apparent permeability, Z, F, R, T have their usual meaning and ψ_{sc} is the intracellular potential.

Most of the results are expressed as means ± S.E. To compare two means the Student's t-test has been used.

The radioactive fluxes will be discussed in terms of the following compartmental model:



The system is assumed to consist of two barriers (mucosal and basolateral) separating three compartments (mucosal (M), cellular (C), serosal (S)). Two of these compartments (M, S) are assumed to be sufficiently large for the radioactivity in them to remain constant (either A_0 or zero). Under steady state the radioactivity in the central compartment (A_c) is constant and can be computed from the unidirectional fluxes from the two following relationships, according to whether the mucosal or serosal compartment being labeled.

$$A_c^m = A_c/A_0^m = J_{mc}/(J_{cm} + J_{cs}) \quad (1)$$

$$A_c^s = A_c/A_0^s = J_{sc}/(J_{cm} + J_{cs}) \quad (2)$$

where A_0^m and A_0^s are the specific activities of the radioactive compartments (mucosal and serosal, respectively). The following relationships apply to the transepithelial fluxes:

$$J_{net} = J_{ms} - J_{sm} = J_{mc} - J_{cm} = J_{cs} - J_{sc} \quad (3)$$

and

$$J_{ms} = J_{mc}(J_{cs}/(J_{cm} + J_{cs})) = J_{mc}(1/(1 + (J_{cm}/J_{cs}))) \quad (4)$$

$$J_{sm} = J_{sc}(1/(1 + (J_{cs}/J_{cm}))) \quad (5)$$

Finally

$$A_c^m/A_c^s = J_{mc}/J_{sc} \quad (6)$$

The paracellular pathway is excluded from this analysis.

Results

In earlier work it was suggested that the transepithelial movements of Cl^- across the frog skin preparation in vitro are entirely passive [19–21]. If the cellular pathway is not impermeable to Cl^- this implies that, under short-circuit conditions, the intracellular Cl^- is at equilibrium with the extracellular [7,21] unless across both mucosal and serosal barriers there is an uphill transport of Cl^- exactly balanced by a leak flux. Recent measurements of intracellular Cl^- concentrations in scraped cells and in isolated epidermis by chemical analysis [7,8,22], or by potentiometric methods (ion-sensitive microelectrodes) [23–25], gave results ranging from 30 to 70 mM (expressed per l cell water).

Table III reports a set of determinations obtained in the isolated epidermis of *Rana ridibunda* Pallas using radioactive $^{60}Co \cdot EDTA$ as extracellular marker. The results obtained under short-circuit con-

TABLE I

TRANSEPITHELIAL CHLORIDE FLUXES IN SYMMETRICAL PIECES OF FROG SKIN MOUNTED IN USSING TYPE CHAMBERS AND SHORT-CIRCUITED

The Ringer's solution used in this set of experiments had the following composition in mM : Na^+ 112; Cl^- 1; gluconate 111; K^+ 2.4; HCO_3^- 2.4; Mg^{2+} 1; Ca^{2+} 1; SO_4^{2-} 2; sucrose to preserve isotonicity, 220 mosmol/kg. Each experiment consisted of three control periods of 15 min and three periods of 15 min after adding acetazolamide at 10^{-3} M to the serosal bathing solution. Results are expressed in pmol/cm² per s. Means ± S.E. of 12 periods out of four experiments.

	Influx	Backflux	Net flux
Control	15.1 ± 1.5	4.1 ± 0.4	11.1 ± 1.2
Acetazolamide (10^{-3} M)	4.3 ± 0.7	1.3 ± 0.2	3.0 ± 0.6

ditions range between 50 and 60 mequiv./kg cell water. Since the Cl^- concentration in the bathing solutions was 119 mM the equilibrium potential for this ion across both barriers was −17.5 mV (external solution used as reference). Although we did not measure the intracellular electrical potential, a number of authors have reported lately [3–5] intracellular potentials in frog skin and toad bladder ranging between −40 and −100 mV, to which should correspond a maximum equilibrium concentration of 20 mequiv./kg cell water. These results imply an uphill translocation process which 'pumps' Cl^- into the cell compartment. Such a mechanism might or might not be part of a transepithelial Cl^- transport system described in the skin of several different frog species [9–12,26,27]. When the isolated skin of *R. ridibunda* Pallas is bathed on both sides by low-Cl^- (1 mM) bicarbonate containing solutions a small net Cl^- transport towards the serosal bath can be demonstrated (Table I). In this set of results there was an inward flux of 15.1 pmol/cm² per s and an outward flux of 4.1 pmol/cm² per s. In the same preparations the short-circuit current was 4.4 μA/cm². Such low values of the short circuit currents, as we shall see later are due to the low Cl^- concentrations of the bathing solutions. The net Cl^- flux measured (11.1 pmol/cm² per s), if entirely electrogenic, corresponds to an outward current of about 1.04 μA/cm². This flux is almost completely abolished by acetazolamide (1 mM). When this drug is added to the serosal solution both unidirectional fluxes are inhibited as shown in Table I. In the skins where the effect of acetazolamide on the short-circuit current was monitored after the addition of the drug the current increased by 0.31 ± 0.08 μA/cm² ($n^0 = 6$).

TABLE II

THE EFFECT OF ACETAZOLAMIDE (10^{-3} AND 10^{-2} M) ON THE WATER AND CELLULAR CHLORIDE CONTENT OF THE ISOLATED FROG EPIDERMIS

Two symmetrical pieces of the same skin were mounted in Ussing type chambers and short circuited, one being used as control. Full-strength NaCl Ringer's solution was used. Results are expressed as means ± S.E. of ten experiments.

	Intracellular water (kg/kg d.w. [a])	Intracellular Cl^- content	
		mequiv./kg d.w.	mequiv./kg i.c.H_2O [b]
Control	2.77 ± 0.11	141.8 ± 7.6	51.6 ± 2.7
Acetazolamide (10^{-3} M)	2.80 ± 0.09	136.4 ± 4.1	49.0 ± 1.8
Difference	0.03	5.4	2.6
P	>0.5	>0.5	>0.4
Control	2.83 ± 0.14	134.4 ± 13.2	47.0 ± 2.9
Acetazolamide (10^{-2} M)	2.66 ± 0.09	129.0 ± 11.2	48.2 ± 3.1
Difference	0.17	5.4	1.2
P	>0.2	>0.5	>0.5

[a] Dry weight.
[b] Intracellular water.

TABLE III

WATER AND CHLORIDE CONTENTS

Water and Cl^- contents of the isolated frog epidermis as measured under the following experimental conditions: (1) Pairs of control skins bathed with normal NaCl-Ringer's solution on both sides of the preparation. (2) Gluconate substituted for Cl^- in the external bathing solution. (3) Gluconate substituted for Cl^- in the internal bathing solution. (4) Choline substituted for Na^+ in the internal bathing solution. Two symmetrical pieces of the same skin were mounted in Ussing type chambers and short-circuited, one of them being used as control. Results are expressed as means ± S.E. of eight experiments.

		H_2O total (kg/kg d.w.)	H_2O cell (kg/kg d.w.)	Cell chloride	
				mequiv./kg i.c. H_2O	mequiv./kg d.w.
(1)	Odd	4.31 ± 0.13	3.15 ± 0.09	53.0 ± 2.8	166.6 ± 8.4
	Even	4.38 ± 0.20	3.03 ± 0.08	58.1 ± 2.4	176.2 ± 10.1
	Diff.	0.07	0.12	5.1	9.6
	P	>0.5	>0.2	>0.1	>0.4
(2)	Control	3.92 ± 0.33	2.84 ± 0.23	53.2 ± 4.1	146.7 ± 10.4
	Exp.	3.90 ± 0.23	2.82 ± 0.21	48.2 ± 2.4	134.5 ± 7.1
	Diff.	0.02	0.02	5.0	12.2
	P	>0.9	>0.9	>0.2	>0.2
(3)	Control	4.42 ± 0.09	3.31 ± 0.07	59.0 ± 4.7	192.6 ± 12.2
	Exp.	3.71 ± 0.13	2.25 ± 0.08	14.4 ± 2.0	32.3 ± 3.8
	Diff.	0.71	1.06	44.6	160.3
	P	<0.001	<0.001	<0.001	<0.001
(4)	Control	3.82 ± 0.16	2.88 ± 0.14	59.8 ± 3.8	169.5 ± 7.0
	Exp.	3.25 ± 0.12	2.10 ± 0.10	19.5 ± 2.6	41.3 ± 6.6
	Diff.	0.57	0.78	40.3	128.2
	P	<0.02	<0.001	<0.001	<0.001

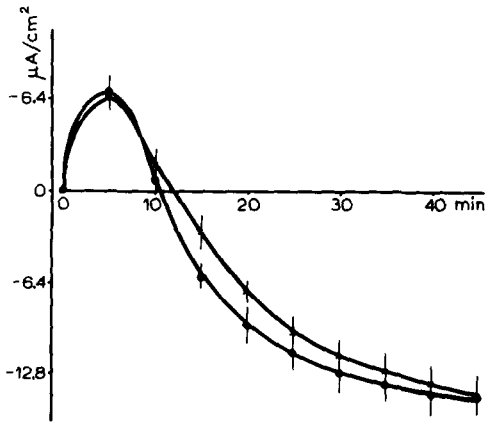


Fig. 1. Average variation of the short-circuit current compared with the control values, when gluconate was substituted for Cl^- (•), or choline was substituted for Na (X) at the internal bathing solution. Means ± S.E. of ten experiments. Results are expressed as $\mu A/cm^2$ vs. time (min).

If the mechanism responsible for the net transepithelial Cl^- transport is also responsible for maintaining the high concentration of Cl^- inside the cells one should expect the intracellular Cl^- content to be sensitive to acetazolamide. Table II displays the results obtained in a group of experiments in which the intracellular Cl^- and water content were measured in the isolated epidermis under control conditions and after one hour incubation with acetazolamide at two different concentrations (1 and 10 mM). The differences in both cases were not statistically significant. These results are not inconsistent with those reported by Kristensen [9], since the chloride transport pool described by this author is very small when compared to the total amount of Cl^- measured in the preparation bathed with solutions containing physiological concentrations of Cl^-.

As shown in Table III, removal of Cl^- from the

TABLE IV

WATER SODIUM AND POTASSIUM

Water, Na^+ and K^+ contents of isolated frog epidermis under control conditions, and when gluconate was substituted for Cl^- ('$-Cl^-$ ins.') or choline for Na^+ ('$-Na^+$ ins.') in the internal bathing solution. Three pieces of the same skin were used randomly, mounted in Ussing type chambers and short-circuited. Results are expressed as means and standard errors of the mean of ten experiments.

	H_2O total (kg/kg d.w.)	H_2O cell (kg/kg d.w.)	Na^+ cell (mequiv./kg i.c. H_2O)	K^+ cell (mequiv./kg i.c. H_2O)	Na^+ cell (mequiv./kg d.w.)	K^+ cell (mequiv./kg d.w.)
Control	3.72 ± 0.16	2.75 ± 0.13	53.0 ± 4.4	102.8 ± 5.2	145.2 ± 13.2	276.5 ± 13.1
$-Cl^-$ ins.	3.05 ± 0.13	1.87 ± 0.11	63.9 ± 4.3	127.9 ± 12.0	117.1 ± 11.7	232.8 ± 19.1
Diff.	0.67	0.88	10.9	25.1	28.1	43.7
P	<0.01	<0.001	<0.1	<0.1	<0.2	<0.1
$-Na^+$ ins.	3.02 ± 0.16	2.00 ± 0.11	33.0 ± 2.9	97.3 ± 4.6	65.8 ± 7.9	194.9 ± 11.9
Diff.	0.70	0.75	20.0	4.5	79.4	81.6
P	<0.01	<0.001	<0.01	<0.5	<0.001	<0.001

external bathing solution has practically no effect on intracellular Cl^- and water content. This confirms previous results reported by us and others [28,29] showing that the permeability of the external barrier of frog skin or toad bladder to Cl^- is very low. We have to conclude that the regulation of the intracellular Cl^- content is due to exchanges taking place across the basolateral membrane, and is insensitive to acetazolamide. In effect, removal of Cl^- from the internal bathing solution produces a marked reduction in the intracellular Cl^- content and in the cell water (Table III). The intracellular Cl^- content falls to 17% and the intracellular water to 68% of the control value. Similar results are obtained by removing Na^+ from the internal bathing solution, as also shown in Table III. Again, a decrease in the intracellular Cl^- content to 24% of its initial value is observed with a loss of water to 72% of its control value.

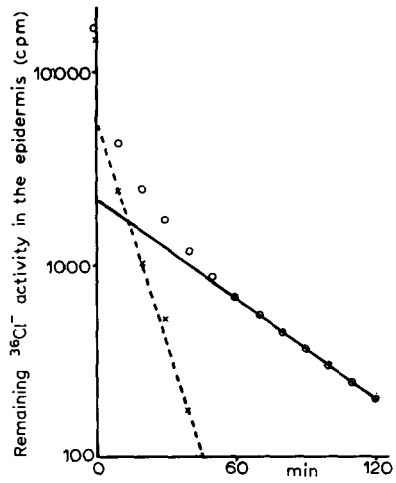


Fig. 2. Kinetics of $^{36}Cl^-$ release from the isolated epidermis of the frog skin mounted in Ussing type chambers and washed from both sides. A semilogarithmic plot of total remaining counts in the skin vs. time was made and the rate constants of the two exponential components were calculated by successive linear extrapolations.

Na^+ and Cl^- removal from the internal bathing solution seem to act by a common mechanism; this is also shown by comparing the transients induced in the short-circuit currents (Fig. 1). In both cases the short-circuit current rises first to reach a peak value 5 min after either of the two ions is removed and falls afterwards to reach a new steady-state value corresponding to 15–20% of the control value. The curves of Fig. 1 were obtained by calculating for each time point the average (ten paired experiments) of the currents expressed as a difference in relation to the control value. The fall in the short circuit current produced by removing Na^+ and/or Cl^- from the serosal bathing solution has been reported before [28,30]. But the mechanism of this effect remains unexplained.

On the other hand, as shown in Table IV there are simultaneously, marked changes in the intracellular

TABLE V

KINETICS OF ^{36}Cl WASHOUT TO BOTH SIDES FROM PRELOADED ISOLATED FROG SKIN EPIDERMIS

Preparations mounted in Ussing type chambers continuously short-circuited. (For details of the calculations see Methods.) Results are expressed as means ± S.E. for six experiments.

	Pool 1	Pool 2
Rate constant (k) (s^{-1}) ($\times 10^3$)	2.36 ± 0.30	0.422 ± 0.03
Total Cl^- (nequiv. · cm^{-2})	179.0 ± 20	176.0 ± 40
Cl^- concentration (mequiv · l^{-1})	107.4 ± 13.5	62.8 ± 3.4

FLUXES AND PERMEABILITIES COMPUTED FROM SEPARATED WASHOUTS INTO THE INTERNAL AND EXTERNAL CHAMBERS

	Internal barrier	External barrier
Fractional losses (s^{-1}) ($\times 10^3$)	0.345 ± 0.03	0.073 ± 0.012
Cl^- fluxes (pmol · s^{-1} · cm^{-2})	62.8 ± 16.7	14.2 ± 3.3
Apparent permeabilities (cm · s^{-1}) ($\times 10^6$)	0.967 ± 0.067	0.210 ± 0.034
Permeabilities [a] (cm · s^{-1}) ($\times 10^7$)	2.89 ± 0.20	0.63 ± 0.10

[a] These permeabilities are obtained from the apparent permeabilities (P_a) using the Goldman-Hodgkin-Katz equation:

$$P = P_a (1 - e^{-V})/V$$

where $V = (ZF/RT)\, V_{sc}$, and $Z = -1$ for Cl^-, $F = 96\,500$ C/g-equiv., $R = 8.315$ J · K^{-1} · mol^{-1}, $T = 295$ K and $V_{sc} = -80$ mV.

composition and volume. When the basolateral side of the preparation is bathed by either a Cl^--free or a Na^+-free solution, Na^+, K^+ and Cl^- are lost from the cells, but due to a simultaneous water loss, in one of the situations (Cl^- removal) the Na^+ concentration in fact increases, while in the other (Na^+ removal) it decreases.

These results imply that the internal barrier is permeable to Cl^-. To measure the Cl^- permeability of both barriers, isolated epidermis of frog skin were used and incubated for 2 h with $^{36}Cl^-$-containing Ringer's solution. Separate washouts to both sides were then studied. Fig. 2 shows one of such experiments. The radioactivity in the preparation falls exponentially following two rate constants. From the zero intercept in semilogarithmic graphs, from the specific activity of the loading solution and from the measurements of the intracellular and extracellular water, the Cl^- concentration in the two Cl^- pools can be computed. The Cl^- concentration corresponding to the pool that decays more quickly is 107 ± 13.5 mM, whilst for the other a concentration of 62.8 ± 3.4 mM is obtained. These values are in good agreement with those corresponding to the Cl^- concentrations in the external fluid and in the cell water.

Since the mucosal and serosal fluids were collected separately the efflux from either side can also be calculated (Table V). The Cl^- fluxes across any of the barriers is obtained from the fractional losses and from the intracellular Cl^- contents. From these fluxes and from the intracellular Cl^- concentrations the apparent permeabilities of both barriers to Cl^- can be calculated: 'Apparent' because they are probably a function of the intracellular potential, but since under short-circuit conditions the electrical potential across the external barrier is the same as that across the internal barrier, the ratio between the apparent permeabilities is a good estimate of the ratio between the two permeabilities. Using this estimate the serosal barrier is 5-times more permeable to Cl^- than the mucosal barrier. The permeabilities of the two barriers calculated using the Goldman-Hodgkin-Katz equation [18] and assuming that the intracellular potential is −80 mV [4,5], are $(2.89 \pm 0.20) \cdot 10^{-7}$ and $(0.63 \pm 0.10) \cdot 10^{-7}$ cm · s^{-1}, respectively, for the serosal and mucosal barrier (Table V).

The estimate of the apparent permeability of the serosal barrier is consistent with the rate at which the Cl^- leaks out of the cells when the serosal bath is Cl^- free. If we assume that the Cl^- efflux is proportional to the intracellular Cl^- concentration, the apparent permeability is then given by:

$$P_A = (V_c */t)\ln(Q_0/Q)$$

in which V_c is the cell volume expressed in cm^3/cm^2, Q_0 is the total amount of intracellular Cl^- at time zero in mol/cm^2, Q is the total amount of Cl^- at the end of the experiment (1 h) and t is the duration of the experiment in s. The value obtained is $1.49 \cdot 10^{-6}$ $cm \cdot s^{-1}$. The Cl^- permeability obtained by using the Goldman-Hodgkin-Katz equation is $4.47 \cdot 10^{-7}$ $cm \cdot s^{-1}$.

Discussion

Recent electrophysiological data suggest that intracellular Cl^- electrochemical potential is higher than that of the extracellular Cl^- [4–8] implying the existence of an uphill transport into the cells as part of the mechanism responsible for the regulation of the intracellular Cl^- content. Our results showed a net inwards transepithelial Cl^- transport sensitive to acetazolamide, in agreement with data described in the literature [9–12], but acetazolamide had no effect on the intracellular Cl^- concentration. This probably results from the fact that as shown by Kristensen [9] the Cl^- pool involved in the transepithelial Cl^- transport is very small when compared with the total intracellular Cl^- content.

The uptake of $^{36}Cl^-$ across the mucosal barrier is very small or negligible [28,29]. On the other hand, we showed that the removal of Cl^- from the mucosal bath does not affect the intracellular Cl^- concentration, while the removal of Cl^- from the internal bathing solution produces a substantial decrease in intracellular Cl^- content. We also showed that intracellular Cl^- exchanges completely with the serosal Cl^- and that the efflux across the serosal barrier is 5-times that across the mucosal barrier. These results are compatible with the transepithelial fluxes measured in minimally, edge-damaged preparations by Macchia and Helman [31]. In effect, using Eqns. 2, 5 and the values of J_{cm} and J_{cs} obtained by us, the estimated value of J_{sm} is around 13 $pmol/cm^2$ per s, similar to that reported by those authors. Other values reported in the literature are higher, ranging from 30 to 200 $pmol/cm^2$ per s [28,32,33] but until a method of measuring unequivocally the cellular and the paracellular pathways is found, it is difficult to interpret such results. We may therefore conclude that the regulation of the intracellular concentration of Cl^- takes place mainly by an acetazolamide-insensitive mechanism located at the serosal barrier.

The reduction of the intracellular Cl^- concentration upon removal of serosal Na^+ suggests that such mechanism requires serosal Na^+, probably because it is driven by the electrochemical gradient for Na^+ across the same barrier. Nagel and Moshagen [14] and Candia and Reinach [15] reported that under steady-state conditions, large unidirectional serosal Na^+ effluxes (twice the short-circuit current) and much larger than the K^+ effluxes measured under the same conditions (0.7 of the short-circuit current [16]). Since it is known that the serosal barrier behaves as a K^+ electrode [2] a large fraction of the serosal Na^+ fluxes must be electrically silent corresponding either to a Na^+/Na^+ exchange and/or to a Na^+-Cl^- coupled flux. This latter mechanism would explain the dependence of intracellular Cl^- on serosal Na^+.

In the absence of more detailed kinetic studies and of simultaneous measurements of intracellular potentials it is premature to discuss whether the coupling mechanism suggested by our observations is similar to that described in leaky epitheliae [34–40] or to the more recent one proposed by Geck et al. [41] for Ehrlich cells.

The effect of the short-circuit current cannot be explained by the gradients created across the preparation by the ion substitutions. If the diffusional fluxes contributed substantially to the net transepithelial current, one should expect a fall in one situation (Cl^- removal) and a rise in the other (Na^+ removal). Furthermore removal of Cl^- from the mucosal bath induces also a fall in the short circuit current [28] and the removal of Cl^- from both sides causes a fall quantitatively similar to that produced by removal of serosal Cl^- [42]. The effect cannot be due, either,

* In this formula V_c is assumed to be constant. If the change in V_c is included, the calculated P_A is smaller by less than 20%.

TABLE VI

Composition of the solution lost by the cells computed from the results reported on Table III and IV, assuming electroneutrality and that there are no osmotic gradients across the basolateral barrier. Results expressed in mmol/kg water lost by the cells. ('$-Cl^-$'), gluconate substituted for Cl^- in the internal bathing solution. ('$-Na^+$'), choline substituted for Na^+ in the internal bathing solution). O_r, osmolality of Ringer's solution bathing the serosal side of the preparation (220 mosM).

	Na^+	K^+	Cl^-	O_s $Na^+ + K^+ + Cl^-$	ΔO_s $O_s - O_r$	ΔChs $Na^+ + K^+ - Cl^-$
$-Cl^-$	32.1	49.9	152	235	15	−70
$-Na^+$	107.0	110.0	165	382	162	+52

to a fall in the intracellular Na^+ concentration. While gluconate substitution for Cl^- is followed by a fall in I_{sc} to 0.21 ± 0.074 of the control level without an appreciable change in intracellular Na^+ concentration (average ratio of concentration under experimental conditions over control conditions = 1.06 ± 0.053), substitution of choline for Na^+ induces a slightly smaller fall in the I_{sc} (0.30 ± 0.081) and a fall in the intracellular Na^+ concentration (0.63 ± 0.08). We cannot advance an explanation which will be valid for both sets of experiments except that it might be caused by cell shrinkage [43] through an unknown mechanism. It may be that the cell shrinkage causes uncoupling between different cell layers of the preparation so that while under control conditions several layers of cell participate in the transepithelial transport, after cell shrinkage only a third to a fifth of the cells are involved. But then it is less easy to understand the reversibility of the effect [28].

Although both Na^+ and Cl^- substitutions produced a loss of both Na^+ and K^+, the intracellular concentrations of these ions do not undergo very marked modifications, and this suggests that the Na^+ pump is still able to regulate them. On the other hand, the intracellular concentration of Cl^- falls in both types of experiment, suggesting that the mechanism of regulation of intracellular Cl^- is strongly affected. The Cl^- equilibrium potential falls from around 17 mV (inside negative), much less negative than the intracellular potential measured in microelectrode studies by many workers [4,5], to around 44 mV (in the case of Na^+ removal) much nearer to the reported values as if Cl^- were approaching a passive distribution.

Since the permeability of the basolateral barrier to water is probably much larger than to ions we have to assume that the cells lose water isotonically with the serosal medium. From Table VI it can be seen that when gluconate is substituted for Cl^- in the serosal medium the 'apparent' tonicity of the solution lost by the cells (i.e., assuming that the only solutes lost or gained by the cells were Na^+, K^+ and Cl^-) is 235 instead of 220 mosM. That implies that the cells must have gained 15 mmol (kg_{H_2O}) * of some other solute(s). We must also postulate an additional charge movement (loss of H^+, retention of HCO_3^-, uptake of gluconate) to accound for the apparent charge imbalance resulting from a loss of 70 mmol (kg_{H_2O}) of Cl^- in excess of the loss of $K^+ + Na^+$. A similar analysis applied to the experiments in which choline was substituted for Na^+ shows that 162 mmol (kg_{H_2O}) of solute must have been taken up by the cells and that in addition to the loss of $Na^+ + K^+ + Cl^-$ a movement of some 52 mmol (kg_{H_2O}) of charge must have occurred (H^+ gain, HCO_3^- loss, choline uptake) to offset the apparent charge imbalance.

Globally our results suggest that, while the external barrier plays an important role in the control of transepithelial transport, the internal barrier is the site of mechanisms responsible for the regulation of cell volume and composition.

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* (kg_{H_2O}) refers to kg of water lost.

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