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Cellular and Transepithelial Responses of Goldfish Intestinal Epithelium to Chloride Substitutions

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Summary. In goldfish intestine chloride was substituted by large inorganic anions (gluconate or glucuronate) either mucosally, serosally or bilaterally. Changes in intracellular activities of chloride (a_iCl^-), sodium (a_iNa^+) and potassium (a_iK^+), pH_i, relative volume, membrane and transepithelial potentials, transepithelial resistance and voltage divider ratio were measured. Control values were: $a_iCl^- = 35$ meq/liter, $a_iNa^+ = 11$ meq/liter and $a_iK^+ = 95$ meq/liter. During bilateral substitution the latter two did not change while a_iCl^- dropped to virtually zero.

Mucosal membrane potentials (ψ_{mc}) were: control, −53 mV; serosal substitution, −51 mV; bilateral substitution, −66 mV; while during mucosal substitution a transient depolarization occurred and the final steady state ψ_{mc} was −66 mV.

During control and bilateral substitution the transepithelial potentials (ψ_{ms}) did not differ from zero. During unilateral substitutions ψ_{ms} was small, in the order of magnitude of the errors in the liquid junction potentials near the measuring salt bridges.

During bilateral substitution pH_i increased 0.4 pH units. Cellular volume decreased during mucosal substitution to 88% in 40 min; after serosal substitution it transiently increased, but the new steady-state value was not significantly above its control value.

Three minutes after mucosal substitution an a_iCl^- of approx. 10 meq/liter was measured.

Chemical concentrations of Na, K and Cl were determined under control conditions and bilateral substitution. Cl concentrations were also measured as a function of time after unilateral substitutions.

The data indicate an electrically silent chloride influx mechanism in the brush border membrane and an electrodiffusional chloride efflux in the basolateral membrane. A substantial bicarbonate permeability is present in the basolateral membrane. The results are in agreement with the observed changes in membrane resistances, volume changes and pH changes.

Key Words intracellular ion activity · K^+ · Na^+ · Cl^- · HCO_3^- · intestinal epithelium · intercellular space · goldfish

Introduction

Henin and Smith [27] were the first to report that ψ_{mc} in rabbit colonic mucosa hyperpolarized after mucosal chloride omission. Later this effect was also found in other intestinal preparations, especially those bathed in bicarbonate salt solutions [14, 26, 30]. Hyperpolarizations in bicarbonate-free situations were smaller or insignificant [37, 43, 54]. In *Necturus* proximal tubule and gallbladder ψ_{mc} even depolarized when chloride was substituted in bicarbonate-free solutions. Guggino et al. [25], however, showed that after bilateral or serosal chloride substitution a hyperpolarization occurred which was dependent on bicarbonate and was abolished by serosal SITS (4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid) application.

The chloride conductances of both the apical and basolateral membrane of proximal tubule and gallbladder are usually estimated low [25, 39, 40, 46]. Some of the limitations of these studies were corrected for recently by Fisher [16] who carefully measured the transient diffusion potentials across the subepithelial connective tissue and minimized the influence of electrical changes at the apical barrier. His results support the earlier conclusions.

Baerentsen et al. [4], however, pointed out that the chloride permeability of the basolateral membrane is masked by the large potassium permeability and the rheogenic contribution of the Na/K pump and is therefore easily underestimated. Also subsequent changes in intracellular chloride activity tend to mask the depolarizing effects of chloride substitutions. Especially the latter effects may be important in intestinal epithelia, whose villus or fold structure gives rise to considerable unstirred layers and consequently delayed responses. Moreover, the columnar shape of the enterocytes and the long and narrow interspace bounding the basolateral membrane make it necessary to treat the interspace as a separate compartment in the analysis [15].

The purpose of the present study was to obtain information about the chloride conductance of the plasma membranes of goldfish intestinal epithelium. To this end we performed an analysis of potential changes and changes in intracellular activities in re-

sponse to chloride substitutions. In contrast to the aforementioned studies in gallbladder and proximal tubule we conclude that the basolateral membrane of goldfish enterocytes possesses a substantial chloride permeability.

Part of this study was presented at the 4th Conference of the European Society for Comparative Physiology and Biochemistry, 1982 [24].

Materials and Methods

Preparation and Experimental Set-up for Intracellular Measurements

Preparation of the tissue and experimental set-up have been described earlier [1, 48].[1]

Bathing Solutions

The standard salt solution had the following composition (in mM): NaCl, 117.5; KCl, 5.7; $NaHCO_3$, 25; NaH_2PO_4, 1.2; $CaCl_2$, 2.5; $MgSO_4$, 1.2; and mannitol, 27.8. Solutions were gassed with humidified 95% O_2 + 5% CO_2. In the chloride-free solutions the sodium salts of glucuronate (Sigma Chemicals) and gluconate (Merck-Schuchardt) were used as substitutes for chloride; in these solutions KCl and $CaCl_2$ were substituted by K_2SO_4 and $CaSO_4$, respectively, and balanced osmotically by addition of 14 mM of mannitol. No different results were found between solutions where gluconate or glucuronate were used as substitute.

Assays of Intracellular pH and Extracellular Space

Intracellular pH and extracellular space were determined as described by Groot [21, 22]. Free floating strips of intestinal mucosa were preincubated several times in fresh solutions of the desired composition for at least 30 min before incubation in radioactive solutions. The solutions were stirred by continuous flow of humidified gas (95% O_2 + 5% CO_2). After 30 to 45 min the strips were removed, gently blotted on Whatman (#1) filter paper and weighed in tarred aluminium weighing boats (Heraeus) on a Mettler ME 30 microbalance. Radioactivity was measured in a liquid scintillation counter (Packard Tricarb 2660) with Instagel® (Packard) as scintillation mixture. The extracellular space was calculated from the distribution ratio of ^{3}H-labeled PEG (4000 D) between tissue and medium and was normally 15% of the tissue volume [*cf.* 21, 36, 54, 55]. This value is assumed to be constant during the experiments. Intracellular pH was estimated from the distribution ratio of ^{14}C-labeled DMO (5,5-dimethyloxazolidine-2,4-dione) according to Waddell and Butler [53].

The radioactivity in the solutions was about 10^6 dpm/ml. Experiments were carried out at 20°C.

Assay of Intracellular Na, K, Cl Concentrations and Water Content[2]

Ion and water content in free-floating strips were analyzed as described by Groot [21]. For the determination of the intracellular Cl concentration and water content after unilateral Cl substitution mucosal strips were mounted as flat sheets on tissue holders, leaving an exposed area of 0.2 cm^2 [23]. The holders were clamped between two Lucite chambers with gassed solutions of the desired composition.

At chosen intervals the tissue was punched out of the holders, gently blotted with Whatman (#1) filter paper and weighed as described above. Dry weight was determined after drying for at least 2 hr at 105°C. The dried tissue was extracted in 0.1 N HNO_3 for at least 2 hr. Then chloride was determined with a Micro Chlor-o-counter (Marius, Utrecht, Netherlands).

Ion-Sensitive Microelectrodes[3]

Although the steady-state ψ_{mc} in this study varied from −40 to −70 mV, the differences between individual cells of the same preparation are small, normally less than 5 mV. In order to reduce the errors due to this variability, the average of 3 to 8 individually measured membrane potentials was taken as the reference value for calculation of the steady-state intracellular activities.

Construction, calibration and application of both normal and ion-sensitive microelectrodes are described elsewhere.[4] For the Cl^--sensitive microelectrodes a liquid membrane (WPI-IE 170) was used. A slope constant of 54.9 ± 0.2 mV/decade in Cl^--activity was found (n = 30), while the selectivity coefficients were K_{Cl,HCO_3} = 0.16 ± 0.01 (n = 12), $K_{Cl,gluconate}$ = 0.04 ± 0.01 (n = 12) and $K_{Cl,HPO_4} < 0.10$ [3, 34].

In the solutions used for chloride substitution experiments the apparent chloride ion activity was 6.8 meq/liter; as the salt solution contained 25 mM $NaHCO_3$ one can calculate that 3.2 meq/liter can be attributed to HCO_3^- taking into account an activity coefficient of 0.76.

The intracellular HCO_3^- activity is calculated from pH_i values obtained as described above with the assumption that pCO_2, CO_2 solubility and activity coefficients in the cell and the bathing solutions are the same.

Measuring Artifacts Due to the Use of Large Organic Anions

During calibration when control solutions were changed to glucuronate and gluconate salt solutions a shift of about −4.1 mV with respect to the reference salt bridge was measured with potassium-selective microelectrodes. This shift varied almost linearly with the concentration of the substituting organic anions, but slope constant or selectivity of the electrodes were not affected. The deviation can be caused by changes in the liquid junction potential near the reference salt bridge or by a reduction in single cation activities of sodium and potassium. On theoretical grounds it is impossible to discriminate between these two alternatives [8, 31, 34, 60]. A full account of the possible causes

[1] Zuidema, T., Kamermans, M., Siegenbeek van Heukelom, J. Basolateral potassium redistribution in goldfish intestine induced by glucose absorption. *Pfleugers Arch.* (*submitted*).

[2] Throughout this paper ionic activities are denoted $a_x I^z$, chemical concentrations $[I^z]_x$; I ionic species, z charge number and x intracellular compartment (i) or bathing solution (o).

[3] *See* footnote 2.

[4] *See* footnote 1.

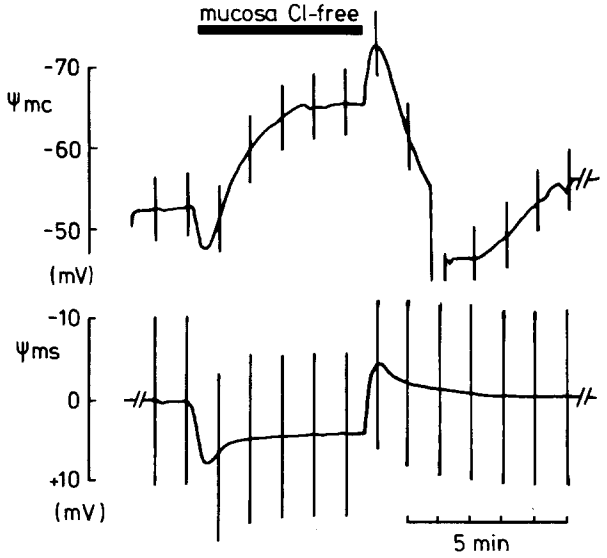

Fig. 1. Typical recordings of ψ_{mc} and ψ_{ms} during mucosal chloride-gluconate substitution. In these recordings a potassium-selective microelectrode was used as reference electrode

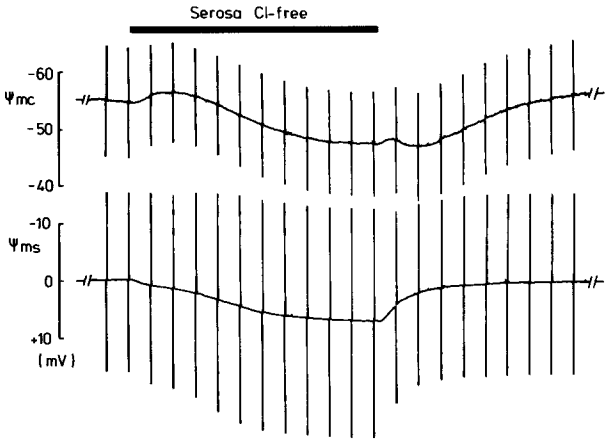

Fig. 2. Typical recordings of ψ_{mc} and ψ_{ms} during serosal chloride gluconate substitutions. Mucosal reference electrode and serosal voltage electrode were connected to the bath by Ringer agar bridges

and consequences of this deviation is given in a separate paper [60].

As normally ψ_{mc} is defined as the potential change sensed by the microelectrode upon penetration of the cell membrane, the reference potential taken is the one measured by the microelectrode in the mucosal bath solution. Accordingly, corrections are made when ψ_{mc}, measured during ion substitutions, is used for calculations of permeability ratios (*see* Table 5).

ψ_{ms} is normally measured with agar salt bridges, mostly 3 M KCl. The shift has no consequences for bilateral substitutions, when it occurred at both sides of the epithelium. Corrections are needed, however, for the shift during unilateral substitutions [8, 31, 60], in order to calculate the transepithelial potential changes.

As plasma membranes and the tight junctions are cation-selective, the reading of a cation-selective electrode in the mucosal bath was chosen as the reference. If the shift of −4.1 mV is, indeed, due to a change in cation activity in the bathing solutions, this is the best way to account in the analysis for the membrane responses to this change. A number of experiments was carried out even using the potassium-sensitive microelectrode directly as reference electrode (Fig. 1). By placing the tip of this electrode near the cells penetrated by the measuring microelectrode, potential artifacts by diffusional delays in unstirred layers are minimized [16].

Finally, the sodium-selective liquid membrane in the microelectrode had a high sensitivity to Ca^{2+} [12, 34], which was present in the solution in a concentration of 2.5 mM. As in gluconate and glucuronate solutions a substantial part of Ca^{2+} is associated to these anions [10], an average additional deviation was found of −6.3 mV, corresponding to approx. 70% of the calcium being associated. Since neither addition nor omission of $CaSO_4$ in chloride-free solutions alters the membrane potential significantly [*cf.* 16, 25] the chloride-free solutions were not adjusted by increasing the calcium activity.

Statistics

The presented steady-state values are the average of the measured values. Transient responses were measured at distinct time intervals with respect to the preceding steady-state values and averaged. All values are given ± SEM,

Results

Electrical Responses to Chloride Substitutions

Mucosal Substitutions

In Fig. 1 representative recordings are shown of the responses to mucosal chloride substitution of ψ_{mc} and ψ_{ms} both with respect to a potassium-sensitive microelectrode in the mucosal bath. The initial depolarization of ψ_{mc} is followed by a hyperpolarization. In eight identical determinations we found an average peak depolarization of ψ_{mc} of +4.7 ± 0.5 mV followed by a hyperpolarization of −10.0 ± 0.6 mV. In contrast, ψ_{sc} (defined as $\psi_{sc} = \psi_{mc} - \psi_{ms}$) did not depolarize at any time. The initial change in ψ_{ms} was +5.9 ± 0.5 mV, while the steady-state potential change was + 2.9 ± 0.5 mV (equivalent with −1.2 mV with respect to a 3 M KCl agar bridge).

Serosal Substitutions

Typical changes in ψ_{mc} and ψ_{ms} due to serosal chloride substitution are shown in Fig. 2. The serosal voltage electrode was formed by a salt bridge filled with standard salt solution, thus introducing a large liquid junction potential in series with the transepithelial potential. The small initial upward deflection in ψ_{mc} (2.8 ± 0.5 mV) is most likely induced by the shunt diffusion potential which develops more quickly and introduces, by voltage dividing, a hyperpolarization of several mV.

Table 1. Electrical responses to chloride substitutions

$N = 33$	Control	Mucosal	Serosal	Bilateral
ψ_{mc} (mV)	−52.4 ± 0.7	−65.7 ± 1.2	−51.4 ± 1.6	−66.6 ± 1.3
ψ_{ms} (mV)	0.0 ± 0.1	−1.2 ± 0.3	−2.5 ± 0.6	−0.2 ± 0.1
R_{ms} (Ωcm^2)	17.8 ± 0.5	16.9 ± 0.8	21.9 ± 1.1	20.3 ± 0.9
R_m/R_s	1.48 ± 0.08	1.49 ± 0.14	1.24 ± 0.16	1.11 ± 0.25
n	58	19	17	22

Steady-state membrane potentials and transepithelial potentials during control, mucosal, serosal or bilateral chloride substitution situations. Control values are pooled averages of the individual controls of the mucosal, serosal and bilateral substitutions. N is the number of preparations, n is the number of measurements.

Table 2. Intracellular ion activities

Ion	ψ_{mc} (mV)	a_iI (meq/liter)	$(a_iI)_{eq}$ (meq/liter)	N, m, n
Control				
Na^+	−50.6 ± 1.3	11.1 ± 1.1	800	11, 60, 61
K^+	−53.6 ± 1.7	95.1 ± 3.2	36	14, 68, 60
Cl^-	−55.7 ± 1.4	35.3 ± 1.4	10.4	15, 74, 78
Bilateral				
Na^+	−64.8 ± 1.4	15.5 ± 3.3	1240	5, 21, 24
K^+	−69.8 ± 2.8	93.1 ± 3.0	60	5, 21, 26
Cl^-	−70.2 ± 1.0	(7.0 ± 0.3)[a]	0	5, 21, 27

Steady-state membrane potentials (ψ_{mc}) and intracellular ion activities (a_iI) of goldfish enterocytes during perfusion with normal Ringer (control) and during bilateral perfusion with Cl^--free solutions. The ion activities are calculated as described in Materials and Methods.

[a] The apparent a_iCl^- under chloride-free conditions and normalized to 0.

$(a_iI)_{eq}$ is the intracellular activity expected if electrochemical equilibrium would exist across the cellular membrane.

Averages were taken over the number of epithelia investigated (N); m is total number of impalements with voltage-sensing electrodes; n is total number of impalements with ion-sensitive microelectrodes. ψ_{mc} averaged over all bilateral determinations was 68.5 mV.

Table 1 gives the steady-state values of ψ_{mc} and ψ_{ms} during control, mucosal, serosal and bilateral chloride substitution. In this table ψ_{ms} was taken with respect to a 3 M KCl salt bridge. The transepithelial resistance R_{ms}, simultaneously determined, is also presented. From the apparent voltage divider ratio in the cell and across the epithelium: $\Delta V_{mc}/\Delta V_{ms}$, that was measured simultaneously, the apparent ratio R_m/R_s (*see also* Table 1) was calculated as described earlier [1] using a correction for the different resistivity of the chloride-free solutions (112 Ωcm *vs.* 64 Ωcm). Both R_{ms} and R_m/R_s do not change significantly after mucosal replacement of chloride ($P > 0.1$). However, after serosal or bilateral chloride substitution R_m/R_s decreases by 20–30% and R_{ms} increases by approx. 15%.

Steady-State Membrane Potentials and Intracellular Ion Activities[5]

Whereas in the gluconate solutions an apparent a_oCl^- was measured of 6.8 meq/liter, in the cell an apparent a_iCl^- is measured of 7.0 ± 0.3 meq/liter (27 cells). Therefore, this a_iCl^- was calibrated to be zero.

In Table 2 the steady-state ψ_{mc} values are given under control conditions and during prolonged bilateral chloride substitution together with the average intracellular activities of Na^+, K^+ and Cl^-. In addition, the intracellular equilibrium ion activity (a_iI_{eq}) is given for each ion. Clearly the experimentally determined a_iK^+ and a_iCl^- are above electrochemical equilibrium while a_iNa^+ is far below. This is commonly found in leaky epithelia like gallbladder [20, 42], proximal tubule [51] and intestine [2, 11, 19, 28, 30, 49, 54, 56, 58, 59]. Under Cl^--free conditions the membrane potential hyperpolarizes so that the equilibrium activities of the cations increase by 75–80%, while the equilibrium activity of chloride is zero.

Moreover, the data show that replacement of chloride does not significantly affect the intracellular cation activities, which is in agreement with the cation activity measurements of Lee and Armstrong [32] (in bullfrog intestine) and White [54] (in *Amphiuma* intestine).

Change in Cell Water and Intracellular pH Induced by Bilateral Cl^- Substitution

As glucuronate is a relatively impermeable anion, the most likely candidate for an exchange with intracellular Cl^- is HCO_3^-. Therefore one must expect an increase in pH_i according to the Henderson-Hasselbalch equation:

$$pH = pK' + \log(HCO_3^-) - \log(CO_2). \quad (1)$$

Indeed, such an increase is found in goldfish intestine [22, 24].

[5] *See* footnote 2, p. 294.

Table 3. Ion content, cell water and pH

Salt solution	NaCl	Na-glucur	*P*
Cell water (kg H_2O/kg dry wt)	3.19 ± 0.06	2.97 ± 0.04	<0.01
Na^+ content	146 ± 7	140 ± 7	NS
Na^+ conc.	34 ± 2	47 ± 2	NS
K^+ content	479 ± 9	465 ± 6	NS
K^+ conc.	151 ± 3	157 ± 3	NS
Cl^- content	193 ± 6	30 ± 3	<0.001
Cl^- conc.	61 ± 2	10 ± 1	<0.001
pH_o	7.45 ± 0.02	7.53 ± 0.01	
pH_i	7.14 ± 0.03	7.64 ± 0.02	<0.001
$pH_i - pH_o$	−0.31 ± 0.04	+0.11 ± 0.02	<0.001

Influence of Cl^--free incubation solution on cell water, ion content, ion concentration, pH_i and difference between pH_i and pH_o in free floating strips of goldfish intestinal epithelium. Determinations were executed in 30 strips from 3 animals.

The effect of Cl^--free solutions on intracellular concentrations resembles the changes in intracellular activities measured with ion-selective microelectrodes (Tables 2 and 3). In paired experiments the cation content and concentration remained unchanged within experimental accuracy, while cell water decreased with 7% and ($pH_i - pH_o$) increased with 0.42 pH units.

From these data one can calculate that the HCO_3^- activity increased from 8.9 meq/liter to 23.5 meq/liter; these are the values used for correction of the chloride-selective microelectrodes for intracellular interference of HCO_3^-. With this information one can calculate that the total interference of all other anions in the cell under control conditions is equivalent with $7.0 - 0.16 \times (23.5 - 8.9) = 4.7$ meq/liter Cl^-.

Changes in Cell Water after Unilateral Chloride Substitutions

Figure 3 shows the changes in cell water (kg H_2O/kg dry wt) after mucosal Cl^- substitution (filled symbols) and serosal substitution (open symbols). After mucosal substitution cell water decreased by 12%, and after serosal substitution it increased transiently and returned to a value not significantly different from control.

Changes in Intracellular Chloride Activity and Concentration after Mucosal Chloride Substitution[6]

Figure 4 shows the changes in a_iCl^- after mucosal substitution. The average initial rate of change is approximately 0.25 meq/liter · sec. a_iCl^- decreased within 3 min to approximately 10 meq/liter, and was still decreasing. This value is already nearly the electrochemical equilibrium value for chloride across the basolateral membrane (6.5 meq/liter). After 3 min it became impossible to keep both microelectrodes in their respective cells, most likely because of cell shrinkage.

[6] *See* footnote 2, p. 294.

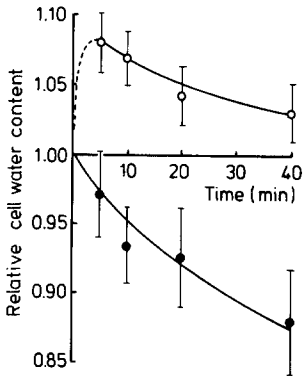

Fig. 3. Responses of relative cell water content to mucosal (●; $n = 9$) and serosal (○; $n = 18$) chloride substitutions as a function of time after the substitution

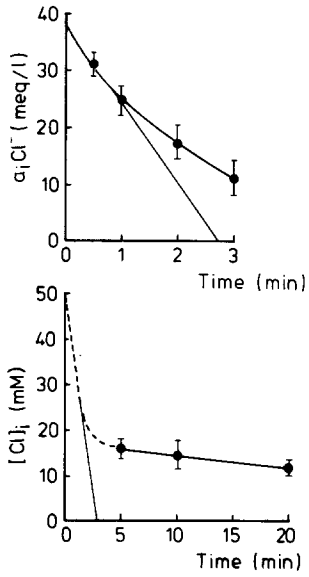

Fig. 4. Intracellular chloride activity (a_iCl^-) and concentration $[Cl^-]_i$ as a function of time after mucosal substitution by gluconate (at $t = 0$ min). Note the difference in time scale.

Upper curve: a_iCl^- averaged over recordings where ψ_{mc} and ΔE_{Cl} were simultaneously recorded in two cells closely apart. The slope of the straight line corresponds to a rate of change of 0.25 meq/liter · sec.

Lower curve: $[Cl^-]_i$ averaged over 8 to 12 mucosal strips from three fishes. The interrupted part of the curve is obtained by transformation of the upper curve using an apparent activity coefficient of 0.76 (*see* text) and allowing for the different time scale

Table 4. Influence of bicarbonate on membrane potential and pH_i

$[NaHCO_3]$ (mM)	ψ_{mc} (mV)	$(pH_i - pH_o)$	$a_iHCO_3^-$ (meq/liter)
4	−30.5 ± 0.5	0.10 ± 0.03	3.7
10	−45.2 ± 2.1	0.13 ± 0.02	9.8
25	−58.8 ± 2.3	−0.19 ± 0.04	11.8
62.5	−71.4 ± 1.6	−0.34 ± 0.03	32.5

Membrane potentials, intracellular to extracellular pH differences and estimated intracellular HCO_3^- activity as a function of $NaHCO_3$ concentration in the bathing solutions.

The decrease of the cellular chloride content was measured for longer times. During these determinations the chloride concentration in the extracellular space is assumed to be equal to that in the serosal bathing solution. The ratio of a_iCl^- and $[Cl^-]_i$ in control solutions was 0.76. Applying this ratio to the other determinations of $[Cl^-]_i$ one finds that the data of a_iCl^- and $[Cl^-]_i$ correspond reasonably and the final steady state in Cl^--free solutions is 9.2 meq/liter, which is nearly the equilibrium value of a_iCl^- across the basolateral membrane (6.5 meq/liter).

Membrane Potentials and Estimated Intracellular Bicarbonate Activity

In Table 4 measurements are presented of ψ_{mc} and transmembrane pH gradients in four different HCO_3^- buffers. These solutions were obtained by substituting 37.5 mM out of 127.1 mM Cl^- and the 25 mM HCO_3^- already present in the solution, by mixtures of HCO_3^- and gluconate having a total concentration of 62.5 mOsm. With increasing $NaHCO_3$ concentration the pH of the salt solution (pH_o) increased. ψ_{mc} hyperpolarized and pH_i increased, but less than pH_o. From $(pH_o - pH_i)$ $a_iHCO_3^-$ was calculated under the same assumptions as above using the Eq. (1).

Analysis and Discussion

For the analysis, the obtained data, corrected for liquid junction potentials as described in Materials and Methods, are lumped in one set in Table 5. Data in parentheses are estimates made as interpolations between experimentally determined values. The simplifying derivatives and assumptions which have been made for the analysis are discussed in paragraphs 1 to 4.

1. Equivalent Electrical Network

The equations of the equivalent electrical network related to our preparation [1] are:

$$\psi_{mc} = (R_s + R_l) \cdot E_m/R_T + R_m \cdot (E_s + E_l)/R_T \quad (2a)$$

and

$$\psi_{ms} = R_l \cdot (E_m - E_s)/R_T + (R_m + R_s) \cdot E_l/R_T \quad (2b)$$

where R refers to specific membrane resistances (Ωcm^2), E to electromotive forces (mV), subscript m to the brush border membrane, s to the basolateral membrane and l to the shunt pathway. R_T is defined as $R_T = R_s + R_m + R_l$. The theoretical zero-current potentials E are assumed to satisfy the Goldman-Hodgkin-Katz equation extended to include the phenomenological permeabilities of the four major ions (Na^+, K^+, Cl^- and HCO_3^-) and the contribution of a rheogenic Na/K pump [29, 57].

As the relative shunt conductance $g_{shunt} = (R_m + R_s)/R_T = 0.95$ [1] it can easily be shown[7] that by substitution of ψ_{ms} for E_l in Eq. (2*a*) an error is introduced of less than 2.5%. Therefore the influence of E_l in ψ_{mc} will be equated with the value of ψ_{ms}.

In control situation and during bilateral substitution $\psi_{ms} = 0$:

$$\psi_{mc} = (R_s + R_l)E_m/R_T + R_mE_s/R_T = \psi_{sc} = E_a. \quad (3)$$

With this equation one can relate the influence of

[7] From Eq. (2*b*) one derives that

$$E_l = \psi_{ms} - R_l(E_m - E_s - E_l)/R_T.$$

Insertion of ψ_{ms} instead of E_l in Eq. (2*a*) introduces an error of the magnitude

$$R_m \cdot R_l(E_m - E_s - E_l)/R_T^2.$$

Repeated insertion of ψ_{ms} for E_l leads to a series expansion (with $x = R_l/R_T$)

$$\psi_{mc} = E_m - (R_m/R_T) \cdot (E_m - E_s - E_l) \cdot (1 - x + x^2 - \ldots)$$
$$\psi_{mc} = E_m - (R_m/R_T) \cdot (E_m - E_s - E_l)/(1 + x).$$

The error is therefore

$$\varepsilon(\psi_{mc}) = x \cdot (R_m/R_T) \cdot (E_m - E_s - E_l)/(1 + x).$$

Insertion of $R_m/R_T = 0.55$, $x = R_l/R_T = 0.05$ and the estimate $E_m - E_s < 50$ mV gives $\varepsilon < 2.5\%$.

electrogenic events, occurring either only mucosally (ΔE_m) or basolaterally (ΔE_s), to a potential change of the enterocyte membrane potential ($\psi_{sc} = \psi_{mc}$) as if it were an unpolarized or apolar cell (E_a) provided that the voltage divider ratio is known. This applies also the rheogenic contribution of the Na/K pump in the basolateral membrane ($\Delta E_{s,pump}$) and to the unilateral electrodiffusional permeabilities (*see* Appendix B).

The rheogenic contribution of the Na/K pump to E_a ($\Delta E_{a,pump}$) is calculated with the equation that compares the Goldman-Hodgkin-Katz equation with a pump ratio $r = 3/2$ (Na : K = 3 : −2) and 1 (Na : K = 1 : −1) [*cf.* 57]:

$$\Delta E_{a,pump} = RT/F \cdot \ln\{L[M + (r-1)a_oK^+]/M[L + (r-1)a_iK^+]\}. \quad (4)$$

R, T and F have their usual meaning, L and M are defined in Appendix A. Consequently $\Delta E_{s,pump} = (R_T/R_m) \cdot \Delta E_{a,pump}$.

2. HCO_3^- Permeability

After bilateral chloride substitution the total cation activity did not diminish, though Cl^- is, within the accuracy of the experimental error, completely washed out of the cell (*see* Table 3). To preserve electroneutrality, other negative charges must have replaced Cl^- in the cell and the most likely substitute is HCO_3^-. The observed pH_i changes are in agreement with an $a_iHCO_3^-$ increase of 14.6 meq/liter. Since HCO_3^- can easily substitute for Cl^-, a substantial pathway through the membranes for HCO_3^- exists.

Bicarbonate changes in the media induce membrane potential changes that do not allow a calculation of P_{HCO_3}/P_K since they are more than Nernstian (Table 4). These results could be explained by a pH- or HCO_3^--sensitive P_K of the cell membrane as proposed by Reuss et al. [41] and Halm et al. [26]. Unilateral substitutions suggest that the basolateral membrane possesses mainly the HCO_3^- or H^+ permeability [*cf.* 9, 18], as in chloride-free media mucosal substitution of the bicarbonate buffer by a Tris-Hepes buffer causes a hyperpolarization while serosal substitution causes a depolarization.

3. P_{Na} in Basolateral Membrane is Negligible

The electrodiffusional sodium permeability of the basolateral membrane in leaky epithelia is generally estimated low [41, 44, 50]. The direct influence of unilateral sodium substitutions on ψ_{mc} are disturbed by large transepithelial diffusion potentials and possibly by changes in pH_i altering the permselectivity of the mucosal membrane [26, 41]. Bilateral substitutions of sodium by NMDG (N-methyl-D-glucamine) in our preparation led to a sharp increase in R_m/R_s ratio (from 1.36 ± 0.29 to 3.28 ± 0.35, $n = 5$). Therefore we assume that $(P_{Na}/P_K)_b$ is negligible.

Table 5. Data lumped for the analysis

	Control	Mucosal	Serosal	Bilateral
ψ_{mc} (mV)	−53	−64	−51	−66
ψ_{ms} (mV)	0	+2.9	−6.6	0
a_iK^+ (meq/liter)	95	(94)	(94)	93
a_iNa^+ (meq/liter)	11	(14)	(14)	16
a_iCl^- (meq/liter)	35	10[a]	26[a]	0
a_iHCO_3 (meq/liter)[b]	9	(20)[c]	(13)[c]	24
R_{ms} (Ωcm²)	18	18	21	21
R_m/R_s	1.4	1.4	1.0	1.1

Steady-state membrane potentials, transepithelial potentials, intracellular ion activities, under control conditions, and after mucosal, serosal and bilateral chloride substitution. The values in parentheses are interpolated between the values found in control situations and when bilaterally chloride was substituted.

[a] Values obtained by multiplying the intracellularly determined concentration with 0.76 (*see* text).

[b] Calculated from pH_i values (*see* text).

[c] Interpolation under the assumption that the increase is proportional to the decrease in chloride concentration.

Membrane resistances and shunt resistance under control condition are calculated under the assumption that the relative shunt conductance is 0.95, so that $R_m = 210$ Ωcm², $R_s = 150$ Ωcm² and $R_l = 18.9$ Ωcm².

4. The Influence of $E_l = 0$

In the bilaterally substituted situation the observation that $\psi_{ms} = 0$ and the assumption that $E_l = 0$ lead to the conclusion that E_m and E_s are equal (*cf.* Eq. (1)). As E_s includes the rheogenic contribution of the pump ($\Delta E_{s,pump}$), the potential in the basolateral membrane due to electrodiffusional fluxes (GHK equation) must be smaller than the potential calculated similarly for the mucosal membrane.

Equally, in control situation ψ_{ms} does not differ significantly from zero [1]. An increased osmolarity in the interspace with respect to the bathing media (e.g., 8 mOsm; [52], will only introduce a contribution of −0.55 mV. This value would introduce in the analysis an increase of about 4 mV for E_s and a decrease of 6 mV for E_m. This leads to a contradiction with experimental results, as will be discussed in paragraph 7.

Table 6. Membrane permeabilities and electromotive forces

A.	Brush border membrane	Basolateral membrane	Tight junction	
P_K (10^{-6} cm/sec)	37	34	124	
P_{Cl}/P_K	0.21	0.59	0.20	
P_{Na}/P_K	0.025	0	0.80	
P_{HCO_3}/P_K	0	0.23	ND	
B.	Control	Mucosal	Serosal	Bilateral
E_m (mV)	(−53)	−60	−56	(−66)
E_s[a] (mV)	(−53)	$-59 + \Delta E_{s,pump}$	$-38 + \Delta E_{s,pump}$	(−66)
R_m/R_s	(1.4)	1.4	1.1	1.1

A. Permeabilities for potassium of the three main barriers in the epithelium: The brush border membrane, basolateral membrane and tight junction are given in absolute values related to the serosal area (0.2 cm²). The permeabilities of Cl^-, Na^+ and HCO_3^- are with respect to the potassium permeability. ND: Not determined.

B. Electromotive forces across the mucosal and the serosal membrane, and voltage divider ratio calculated with the Goldman equations with permeabilities from Table 6*A* and activities from Table 5. Values used to obtain the permeabilities of Table 6*A* are placed in brackets.

[a] The value E_s contains a contribution of the Na/K pump ($\Delta E_{s,pump}$) which is estimated as −9 and −8 mV during resp. control and bilateral substitutions. The value for unilateral substitutions is estimated to be −8.5 mV.

5. Solution of the Goldman-Hodgkin-Katz Equation

Essentially the problem of the analysis is to solve two sets of Goldman-Hodgkin-Katz equations [45] to satisfy the condition that $E_m = E_s$ using the measured values for the ion activities under different conditions. The equations used are presented in Appendices A and B.

During bilateral substitutions the term representing the chloride contribution can be put to zero. This provides a possibility of calculating the relative sodium permeability in the mucosal membrane and for bicarbonate in the basolateral membrane. In order to estimate $\Delta E_{s,pump}$ the permeability ratios weighed by the ratios as described in Appendix B were substituted in the Goldman-Hodgkin-Katz equation for the apolar cell. Comparison of the GHK equations with and without the rheogenic contribution of the Na/K pump (Eq. (4); $r = 3/2$ and $r = 1$) yields another set of equations which permits the unknown parameters to be solved [57]. The resulting relative permeability ratios are presented in Table 6*A* (first and second column), $\Delta E_{s,pump}$ is approximately −9 mV in the control situation and approximately −8 mV in chloride-free media.

From the values of R_m and R_s (Table 5) the absolute potassium permeability of the membranes can be calculated (Table 6*A*) with the equation for the slope conductance (g) derived from the GHK equation (*cf.* [45]):

$$g = F^2P_K/RT\{[\xi \ln\xi(M - L)]/(1 - \xi)^2 + (M\xi - L)/(1 - \xi)\} \quad (5)$$

where $\xi = \exp(\psi F/RT)$; the meaning of M and L is described in Appendix A.

6. Unilateral Substitutions

With the values of Table 6*A* and reasonable estimates for the intracellular ion activities (presented in parentheses in Table 5) we can predict E_m, E_s and R_m/R_s for the unilateral substitutions. These are given in Table 6*B*. Assuming that $\Delta E_{s,pump}$ is not very different from the values found above the predicted values of the electrical parameters are rather accurate (*cf.* Table 5).

In previous papers [7, 47] the relative permeabilities of the tight junction for goldfish intestine were estimated as: $P_{Cl}/P_{Na}/P_K = 0.25 : 1 : 1.25$ (Table 6*A*, third column). Using these values to calculate E_l after unilateral chloride substitution one finds $\Delta E_l = 4.8$ mV assuming for the tight junction $(P_{gluc}/P_K)_{tj} = 0$. Whereas ψ_{ms} due to serosal substitutions can be fitted within experimental accuracy with this value the steady-state ψ_{ms} due to mucosal chloride substitution (+2.9 mV) cannot. The transient value at 40 sec (+5.9 ± 0.5 mV; *see* Fig. 1) might still be in the range.

On the other hand, R_{ms} does not change during

mucosal chloride substitutions, suggesting that the tight junction permeabilities for chloride and gluconate are not very different. As a tentative conclusion one may infer that initially $(P_{gluconate})_{tj}$ is still low (ψ_{ms} = 5.9 mV) but that it increases due to the substitution (ψ_{ms} = 2.9 mV). As yet there is no good explanation for this biphasic response of ψ_{ms}. A possible explanation might be that, though the osmolarity of the solutions used were alike, the difference in permeability introduced water flows, so that the diffusional junction between chloride and gluconate solutions does not occur exclusively in the tight junction. ψ_{ms} may be the result of liquid junction potentials due to a gradual change in composition of the solution in mucosal unstirred layer and in the interspace from the tight junction to the basement membrane. Especially during serosal substitution the high rate of transcellular chloride transport entering the interspace may enhance this effect.

The increase in R_{ms} during serosal or bilateral substitution is probably mainly due to the increase in resistivity of the interspace fluid which according to the dimensions of the interspace and the higher resistivity of the gluconate salt solution is expected to be 3–4 Ωcm^2.

7. Testing of the Assumptions

In the analysis it was assumed that P_{Na} and P_K of the apical membrane do not change during chloride substitutions. Earlier a $P_{Na}/P_K = 0.072$ was reported [47] with the assumption that the apical membrane is impermeable for chloride. Insertion of the presently found P_{Cl} in the old data shows then that $(P_{Na}/P_K)_m$ agrees with the value presented here. The present results are more likely as a fit, for the old value would require a decrease of $(P_{Na}/P_K)_m$ by a factor of three during bilateral chloride substitution.

According to Eq. (2*b*) small changes in E_l can only occur when considerable changes in E_m and E_s compensate for these changes in E_l, given the experimental fact that $\psi_{ms} = 0$ during control and bilateral substitutions. Consequently, the ion permeabilities determining E_s and E_m must also change considerably. For example, if, due to a hyperosmolarity of 8 mOsm in the interspace, $E_l = -0.55$ mV, the resultant increase in E_m of 4 mV can only be explained with a twofold increase of $(P_{Cl}/P_K)_m$ or a fourfold increase of $(P_{Na}/P_K)_m$. The decrease in E_s of 6 mV can then be explained with a smaller $(P_{Cl}/P_K)_s$. These different permeability values would cause an increase in R_m/R_s ratio after bilateral chloride substitution of 15–20%, whereas a decrease was observed (*cf.* Eq. (5)). So the assumption that E_l is negligible is not unwarranted.

8. Electroneutral Chloride Transport in the Brush Border Membrane

In control solutions a_iCl^- is above electrochemical equilibrium and $[Cl^-]_i$ is below chemical equilibrium. When Cl^--influx from the mucosal side was stopped by mucosal Cl^--omission, a_iCl^- dropped within 3 min to nearly the electrochemical equilibrium value over the basolateral membrane (10 *vs.* 6.5 meq/liter). As $[Cl^-]_i$ reduced further after 3 min, it is most likely that a_iCl^- reduces even below 10 meq/liter. Clearly, under control conditions most Cl^--influx through the mucosal membrane is electroneutral. Electroneutral entry mechanisms only exist when the chloride fluxes are coupled to those of other ions. This coupling is not necessarily completely electroneutral (i.e., may have a ratio differing from 1 : 1). If a direct coupling to Na^+ influx, as first proposed by Nellans et al. [35] (*see also* Refs. 13, 16), exists in goldfish intestine, it cannot be inhibited by furosemide or bumetanide. More likely Cl^- is coupled by countertransport to OH^- or HCO_3^- and via pH_i indirectly to Na^+, since not only chloride substitution causes alkalinization, but also Na^+ substitution causes cell acidification [22, 24]. The large transepithelial Cl fluxes (0.92 ± 0.14 nM/cm^2 sec) [6, 7] cannot occur paracellularly as the transepithelial potentials are small and the paracellular chloride conductance is not sufficient. Therefore an electroneutral transport mechanism is indispensable to explain the findings. Apparently in goldfish intestine this mechanism is located in the brush border membrane. It consists, most probably, of the dual-exchange mechanism (Na^+/H^+ + Cl^-/HCO_3^-) suggested by Turnberg et al. [52] for human ileum [5, 33].

One can get an impression of the ease with which Cl^- passes through the mucosal membrane by calculating the chloride conductance needed to explain the mucosal efflux after mucosal chloride substitution. The observed initial rate of decrease of 0.25 meq/liter · sec can only be explained with a value about four times as large as the total cell membrane chloride conductance.

9. The Basolateral Anion Conductances

The basolateral chloride conductance is high and is responsible for nearly all chloride permeability in the apolar cell. As mentioned above it still has a transport capacity which is lower than the electroneutral influx mechanism. This is supported by the observation that during serosal chloride substitutions the $[Cl^-]_i$ dropped less than during mucosal substitutions (*see* Table 5). It is also the reason why

during mucosal chloride substitution the relative cell volume decreases while during serosal substitutions it does not. The electrodiffusional permeability of the brush border membrane for chloride is so low that it is easily overlooked for two reasons. First: If 3 M KCl salt bridges are used as reference electrodes [60] the depolarization of the mucosal membrane is obscured by the shift of −4.1 mV to be expected for the cation-selective membrane in analogy to the shift observed with a potassium-sensitive microelectrode. Second, the reduction of a_iCl^- will lead to hyperpolarization overriding the depolarization. The initial depolarization after mucosal chloride substitution reflects the chloride permeability of both the mucosal membrane and the tight junction and is a weighed superposition of the depolarization of E_m, a hyperpolarization of E_s and a serosa-positive shunt diffusion potential.

The basolateral chloride permeability is approximately 20×10^{-6} cm sec^{-1} representing more than 30% of the total basolateral membrane conductance. This is the major reason why R_m/R_s decreases by serosal chloride substitution.

10. Conclusions for the Active Salt Transport of the Epithelium

In conclusion: the goldfish enterocyte possesses an electroneutral influx mechanism of chloride in the brush border membrane and a large chloride permeability in the basolateral membrane. The effective diffusional impedance of the basolateral membrane is higher than that of the brush border membrane, so that the cellular chloride activity is more influenced by the mucosal chloride activity than by the serosal activity.

If the stoichiometry of the mucosal coupling is 1 : 1 an extremely profitable configuration is present in the enterocyte since the energetics for uphill transport of chloride into the cell are minimized, as by this coupling no energy is necessary to overcome the electrical part (i.e., ψ_{mc}) of the electrochemical gradient. At the basolateral side this electrical part is used in a favorable way as here the chloride efflux is only coupled to other ions through the electrical gradient (principle of electroneutrality).

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Appendix A

E_m and E_s are both of the type $(RT/F) \cdot \ln(M/L)$ (according to the Goldman-Hodgkin-Katz equation). With the simplifying assumptions from the Discussion one can write:

$$M_m = a_oK^+ + (P_{Na}/P_K)_m \cdot a_oNa^+ + (P_{Cl}/P_K)_m \cdot a_iCl^- \quad (A1)$$

$$L_m = a_iK^+ + (P_{Na}/P_K)_m \cdot a_iNa^+ + (P_{Cl}/P_K)_m \cdot a_oCl^- \quad (A2)$$

$$\psi_{mc} = E_m = RT/F \cdot \ln(M_m/L_m) \quad (A3)$$

$$M_s = a_oK^+ + (P_{Cl}/P_K)_s \cdot a_iCl^- + (P_{HCO_3}/P_K)_s \cdot a_iHCO_3^- \quad (A4)$$

$$L_s = a_iK^+ + (P_{Cl}/P_K)_s \cdot a_oCl^- + (P_{HCO_3}/P_K)_s \cdot a_oHCO_3^- \quad (A5)$$

$$E_s - \Delta E_{s,pump} = RT/F \cdot \ln(M_s/L_s) \quad (A6)$$

$\Delta E_{s,pump}$ is the rheogenic contribution of the basolaterally located Na/K pump to E_s.

Appendix B

The membrane potential of the unpolarized or apolar cell, E_a, is, like E_m and E_s, of the type $(RT/F) \cdot \ln(M/L)$ and according to Eq. (3) E_a can also been written as:

$$E_a = p \cdot E_s + (1 - p) \cdot E_m \quad (B1)$$

where $p = R_m/R_T$.

Taking only the "M"-terms one obtains:

$$\ln M_a = p \ln M_s + (1 - p) \ln M_m. \quad (B2)$$

Taking $A = a_oK^+$, $B = (P_{Cl}/P_K)_s \cdot a_iCl^- + (P_{HCO_3}/P_K)_s \cdot a_iHCO_3^-$ and $C = (P_{Na}/P_K)_m \cdot a_oNa^+ + (P_{Cl}/P_K)_m \cdot a_iCl^-$ (*cf.* Eqs. (A1) and (A4)), one can rewrite this equation as

$$\ln M_a = \ln(A + C)\{(1 + (B - C)/(A + C)\}^p \quad (B3)$$

and series expansion of the last right-hand term gives

$$\ln M_a = \ln(A + C)\{(1 + p(B - C)/(A + C) + p(p - 1)[(B - C)/(A + C)]^2/2 + \ldots\}$$

which reduces in first approximation to

$$\ln M_a = \ln A(1 + pB/A + (1 - p)C/A). \quad (B4)$$

A similar equation for the "L" terms can be derived. This shows that in the overall equation $E_a = (RT/F)\ln M_a/L_a$ the contributions of the mucosal and serosal permeabilities in both the nominator (M_a) and denominator (L_a) should be weighed with the same ratios that also determine the contributions of E_m and E_s in E_a.