J. Physiol. (1986), **378**, *pp.* 195–211
With 6 text-figures
Printed in Great Britain

ACID SECRETION THROUGH THE *RANA ESCULENTA* SKIN: INVOLVEMENT OF AN ANION-EXCHANGE MECHANISM AT THE BASOLATERAL MEMBRANE

BY E. DURANTI, J. EHRENFELD AND B. J. HARVEY

From the Laboratoire Jean Maetz du Département de Biologie du C.E.A., B.P. 68, 06230 Villefranche-sur-Mer, France

(*Received 12 July 1985*)

SUMMARY

1. Kinetic and electrophysiological studies were carried out to characterize the efflux of HCO_3^- (or OH^-) across the basolateral membrane of the proton-secreting cells of the frog skin epithelium bathed with dilute saline mucosal solutions.

2. In control conditions, the acidification of the mucosal solution ($J_n^{H^+}$) was correlated directly with serosal alkalinization. Cl^- substitution in the serosal Ringer (by gluconate or methylsulphate ions) induced an inhibition of proton excretion (70 % inhibition).

3. Measurements of the basolateral membrane potential with conventional micro-electrodes and of cell Cl^- activity (a_{Cl}^i) and proton activity with double-barrelled ion-sensitive micro-electrodes recorded a basolateral membrane depolarization of $5{\cdot}1 \pm 0{\cdot}7$ mV ($n = 12$), a decrease in a_{Cl}^i from $14{\cdot}5 \pm 1{\cdot}6$ mequiv l^{-1} to $1{\cdot}8 \pm 0{\cdot}3$ mequiv l^{-1} ($n = 12$), and a cell pH increase from $7{\cdot}18 \pm 0{\cdot}04$ to $7{\cdot}32 \pm 0{\cdot}06$ ($n = 12$) after serosal Cl^- replacement.

4. 4,4′-diisothiocyanostilbene-2-2′-disulphonic acid (DIDS) (10^{-4} M) and meclofenamate (5×10^{-5} M) inhibit $J_n^{H^+}$ by 34 % and 53 % respectively whereas bumetanide did not block $J_n^{H^+}$.

5. Depolarization of the basolateral membrane (2 mM-Ba^{2+} addition to the serosal solution) did not block proton excretion.

6. We show that cell Cl^- activity is maintained at a higher level than that predicted by the equilibrium potential, by a mechanism located at the basolateral membrane of the epithelium since the apical solution was Cl^--free. This mechanism is not sensitive to potential changes at the basolateral membrane in the range tested. An electroneutral Cl^-–HCO_3^- exchange mechanism is the simplest hypothesis which can account for our results.

INTRODUCTION

Many transporting epithelia have the ability to excrete protons and to absorb Na^+. For the case of the frog skin epithelium we have previously demonstrated that the excretion of protons was achieved by the presence of a primary active electrogenic proton pump located on the apical side of the epithelium. In open-circuit conditions

in the presence of low external Na^+, the electrogenicity of the proton pump was shown to be the mechanism of the indirect electrical coupling between the excretion of protons and the electro-diffusion of external Na^+ to the cellular Na^+ compartment (Ehrenfeld & Garcia-Romeu, 1977; Ehrenfeld, Garcia-Romeu & Harvey, 1985). Since the amount of HCO_3^- (or OH^-) in the cells increases after the apical excretion of protons these anions must be eliminated to keep a stable intracellular pH. The excretion of excess HCO_3^- (or OH^-) can follow either the apical or the basolateral routes. In this paper we have devoted special attention to the basolateral exit of HCO_3^- (or OH^-) in the frog skin epithelium, blocking any possible Cl^-–HCO_3^- exchange on the apical side (Kristensen, 1972; Ehrenfeld & Garcia-Romeu, 1978) by using a Cl^--free mucosal solution.

By the use of serosal ion substitution, anionic transport inhibitors and changing the potential of the basolateral membranes (by Ba^{2+} addition), we attempt to characterize the mechanism responsible for the observed alkalinization of the serosal solution. For this purpose kinetic and ion-sensitive micro-electrode (Cl^-, pH) techniques were employed.

METHODS

Rana esculenta were obtained from Central Europe and were kept at 15 °C without food in running tap water which contained very low concentrations of NaCl (150 μequiv l^{-1}). The frogs were used within 10 days of arrival in the laboratory.

Kinetic studies

The ventral skin was mounted between two lucite chambers of 7 ml capacity and the exposed area was 7 cm^2. The skins were selected by their ability to maintain positive net fluxes of Na^+ from a dilute mucosal solution containing 1 mM-Na_2SO_4. Of all the skins studied 20–30% were found to lose Na^+ in these conditions and were discarded.

The mucosal solution contained 1 mM-Na_2SO_4 buffered at pH 7·34 with 2 mM-imidazole (pH adjustment with H_2SO_4); this solution was stagnant and continually aerated in order to eliminate any possible accumulation of CO_2 coming from the cells and/or the serosal solution. The serosal solution of Ringer contained HCO_3^- (20 mM) and was gassed with a mixture of 5% CO_2 and 95% O_2; its pH was 7·34 and contained (mM): NaCl, 85; $NaHCO_3$, 20; KCl, 2·5; $CaCl_2$, 2; $MgSO_4$, 2; Na_2HPO_4, 2·5; KH_2PO_4, 1·2; and glucose, 11. In chloride-free-Ringer experiments the NaCl, KCl and $CaCl_2$ salts were replaced by gluconate salts or methylsulphate salts.

The skins were studied either in open-circuit conditions, or at an imposed transepithelial potential (ψ_{ms}) of +50 mV. Clamping the skins at a definite potential was performed with a custom-built voltage-clamp apparatus. 3 M-KNO_3-agar salt bridges were used to pass transepithelial currents (I_t) or to measure the spontaneous potential (ψ_{ms}). The potential-measuring electrodes were placed as close as possible to the skin surfaces. The electrical potential across the skin was referred to the mucosal solution and mucosal-directed current was assigned a positive value. Samples of the mucosal solution were taken every 30 min and the analysis of Na^+ and proton concentrations in these samples permitted the calculation of net Na^+ and proton fluxes ($J_n^{Na^+}$ and $J_n^{H^+}$ respectively). Na^+ concentration was measured by flame photometry with an Eppendorf photometer and proton concentration was measured by titration using a Radiometer automatic titrator. The measured samples were aerated for 30 min before the determination of proton concentration in order to eliminate any volatile acidity arising from the large CO_2 gradients across the epithelium. Titration of the samples was performed with a 1 mM-HCl solution fixing a pH end-point 0·3 pH units lower than the initial pH of the samples. The volume of HCl added permits the calculation of the acid–base net fluxes.

Measurement of the alkalinization of the serosal solution presents some difficulty in a HCO_3^--containing Ringer gassed with 5% CO_2. The problem is in detecting small variations of HCO_3^- (or OH^-) which produce small pH changes in a well-buffered Ringer. However, reducing almost all

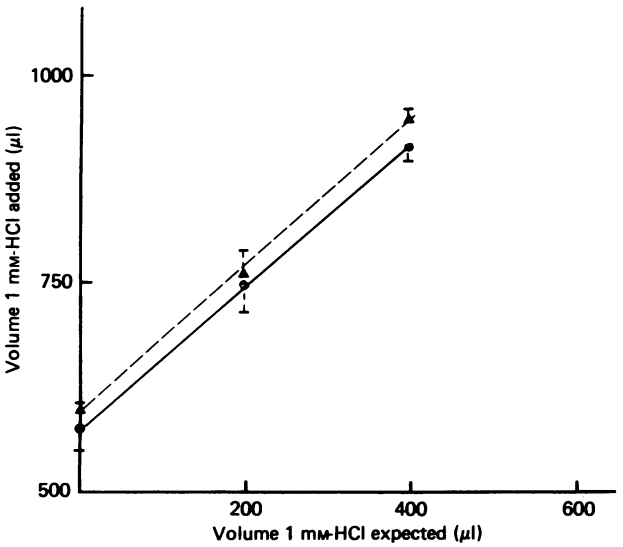

Fig. 1. Standard curve of Ringer solutions containing known amounts of base. In ordinate, volume in μl of HCl (1 mM) added to the Ringer; in abscissa, calculated volume of HCl (1 mM) expected for each standard solution. Triangles indicate $NaHCO_3$ addition; circles indicate NaOH addition.

the buffering power of the HCO_3^-–CO_2 system permitted a magnification of the difference in titratable acidity of the samples after base secretion by the skin. For this purpose an identical volume of 1 N-HCl (pipette error less than 0·1 μl) delivered by an automatic pipette (Microlab, Hamilton, Switzerland), was added to each serosal sample to bring the pH to a value close to 3·6, and the final pH thereby obtained was dependent on the rate of the alkalinization in the original sample. In order to eliminate volatile acidity the samples were then equilibrated with air for 30 min. Titration of the samples with a 1 mM-HCl solution was then performed with an automatic TTA 61 titrator (Radiometer, Copenhagen), fixing an end-point pH lower than that of the treated samples.

That this procedure permitted the detection of the original quantity of base secreted by the epithelium into the Ringer was tested by adding various known amounts of $NaHCO_3$ or NaOH (0–400 μequiv) to a normal Ringer solution and performing the titration procedure as described above. Fig. 1 shows that the quantity of base, whether in HCO_3^- or OH^- form, added to the Ringer solution, can be detected by the titration procedure.

The Na^+, proton and HCO_3^- fluxes were calculated in nequiv h^{-1} cm^{-2} and expressed as means $\pm$ S.E. of mean. Statistical analysis was carried out by the paired t test.

K^+ wash-out experiments were performed on the intact frog skin. The skins were mounted in a chamber of 3 ml volume with an exposed area of 7 cm^2 and skin cells were loaded with $^{42}K^+$ by exposing the serosal surface of the skin for 2 h with a Ringer containing $^{42}K^+$. After an initial washing of 1 min, 3 ml of Ringer solution was added to the serosal side and collected after 2 min directly into counting vials. The sampling procedure was repeated over a period of 1·5–2 h; at the end of the experiment the mucosal solution was collected for counting and analysed for net Na^+ or proton fluxes, and the skin was counted directly in an automatic γ-counting system (MR 252 KONTRON, France). The total amount of counts in the skin at the beginning of the collection period was calculated by accumulating the counts measured in the successive wash-out samples. The rate constant ($t_{\frac{1}{2}}$) of the exponential components were calculated by linear extrapolations of the semilogarithmic plot of the total remaining counts of the skin *versus* time.

Electrophysiological studies

The apical and serosal sides of the isolated frog skin epithelium (Aceves & Erlij, 1971) were continually perfused with solutions identical in composition to those described for kinetic studies.

The isolated epithelium was mounted apical side downwards in a lucite chamber of area 1 cm^2 and cell impalement with micro-electrodes was from the basolateral side. The tissue was supported on an ultra-thin steel mesh and immobilized by a negative hydrostatic pressure of 30 cmH_2O, which served also as the driving pressure for serosal Ringer perfusion. These procedures did not influence the electrical parameters of the epithelium but greatly facilitated the stability and maintenance of cellular impalements during constant perfusion and rapid solution changes. The chamber was secured in a heavy metal block supported on tennis balls to avoid mechanical vibrations.

Conventional micro-electrode techniques were used to measure apical membrane potential (ψ_{mc}) and basolateral membrane potential (ψ_{cs}). The electrical recording arrangements, circuit analysis, and impalement criteria are given in a previous paper (Ehrenfeld, Garcia-Romeu & Harvey, 1985).

In addition, experiments were performed on the isolated epithelium with Cl^-- and pH-sensitive double-barrelled micro-electrodes using the Cl^--selective liquid ion exchanger (Corning 477913) and the pH-selective ion exchanger (Fluka 82500) to measure intracellular chloride activity (a^i_{Cl}) and pH (pH_i) respectively. These double-barrelled micro-electrodes had the same tip size as conventional micro-electrodes and were constructed as previously described (Harvey & Kernan, 1984*a*). The Cl^--sensitive electrodes had a slope of 52–58 mV $decade^{-1}$ ($n = 32$ electrodes) in pure KCl solutions from 10 to 100 mM and the response time was of the order of 2 s. The selectivity coefficient of the Cl^- electrode with respect to HCO_3^- was 0·1 when measured in solutions of 10 mM-KCl and 10 mM-$NaHCO_3$. An intracellular HCO_3^- activity of 7·6 mM has been measured in frog skin (Harvey & Kernan, 1984*a*), thus under normal intracellular-pH conditions, an intracellular-Cl^- activity of 1 mM represents the minimum detection limit of our Cl^- electrodes. Corrections for intracellular interference by other anions were not applied. The Cl^--sensitive micro-electrodes had an impedance of $8 \times 10^9\ \Omega$ in the ion-sensitive barrel and 80 MΩ in the reference barrel. The pH-sensitive electrodes had a slope of 52–56 mV $decade^{-1}$ ($n = 12$ electrodes) with an impedance of $2 \times 10^{11}\ \Omega$ in the ion-sensitive barrel. The electrode response time to changes in pH from pH 7·0 to 6·0 was approximately 6 s. The output voltages from the two barrels of the micro-electrodes were amplified by a dual, differential high-impedance electrometer (Model FD 223 WPI) and displayed on a potentiometric pen recorder (Sefram, Paris) and dual-beam oscilloscope (Tektronix, Model 5-1037).

Drugs

Amiloride was purchased from Merck Sharp and Dohme Research Laboratories, West Point, PA, U.S.A; ethoxzolamide and DIDS (4,4′-diisothiocyanostilbene-2-2′-disulphonic acid) were purchased from Sigma, St. Louis, MO, U.S.A.; bumetanide was purchased from Leo Laboratories France, furosemide from Hoechst (RFA) and meclofenamate from Parke Davis and Company, U.S.A.

RESULTS

In a first approach we intended to measure the HCO_3^- (or OH^-) transferance through the basolateral membranes of the skin. (It must be noticed that HCO_3^- transport is not distinguishable from $OH^- + CO_2$ movements or proton entrance through the basolateral membranes in our experiments.) For this purpose a technique was developed to measure the variations of acidity of the serosal Ringer (with high HCO_3^- concentrations, see Methods). As can be observed in Table 1, the mean net proton excretion is not different from the mean HCO_3^- (or OH^-) appearance in the serosal solution; furthermore, ethoxzolamide (10^{-4} M), a known proton-excretion blocker, added to the mucosal side inhibits $J_n^{H^+}$ and $J_n^{HCO_3^-}$ (net HCO_3^- flux) to the same extent. Fig. 2 shows the 1:1 correlation found between the rate of acidification of the mucosal solution (expressed as $J_n^{H^+}$) and the rate of alkalinization of the serosal solution ($J_n^{HCO_3^-}$). However, from the experiments presented in Table 1, it can be seen that the S.E. of mean of $J_n^{HCO_3^-}$ is nearly two times larger than the S.E. of mean of $J_n^{H^+}$. This observation is explained by the difficulty in measuring small degrees of alkalinization in a Ringer containing high concentrations of HCO_3^-. The detection of small variations of HCO_3^- transfer to the serosal side of the skin appeared, in these

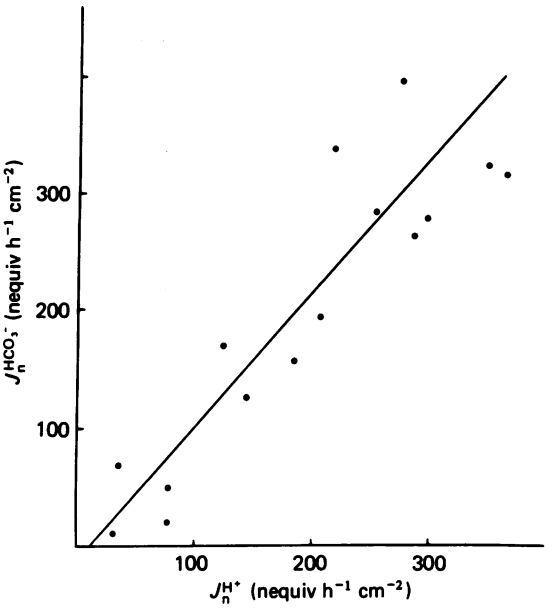

Fig. 2. Correlation existing between the acidification of the mucosal side ($J_n^{H^+}$ in abscissa) and the alkalinization of the serosal side ($J_n^{HCO_3^-}$ (or OH^-) in ordinate).

TABLE 1. Effects of ethoxzolamide (10^{-4} M) on mucosal acidification and serosal alkalinization of the solutions bathing the *R. esculenta* skin

	$J_n^{H^+}$	$J_n^{HCO_3^-}$
Control	-217 ± 46	$+207 \pm 78$
Ethoxzolamide	-69 ± 19	$+36 \pm 43$
Difference	-149 ± 36	-171 ± 45

Ethoxzolamide (10^{-4} M) was added to the mucosal solution. Na^+ transport was blocked by 5×10^{-6} M-amiloride and the skin was clamped to +50 mV. The sign of the net fluxes is negative for a proton excretion to the mucosal solution and positive for the HCO_3^- (or OH^-) excretion to the serosal solution. Fluxes are expressed in nequiv h^{-1} cm^{-2}. $n = 5$.

conditions, very problematic. So, taking into account the correlation found in Fig. 2 between $J_n^{H^+}$ and $J_n^{HCO_3^-}$, we decided to measure preferentially $J_n^{H^+}$ by titration of the mucosal solution and to consider this net flux as equivalent to the transfer of HCO_3^- (or OH^-) through the basolateral membranes.

Effect of Cl^- replacement in the serosal Ringer

We considered the possible mechanisms responsible for HCO_3^- movements through the basolateral membrane and among these the Cl^-–HCO_3^- exchange which has been proposed for many epithelia.

The dependence of $J_n^{H^+}$ on serosal Cl^- was tested using gluconate or methylsulphate as Cl^- substitutes, which are anions of relatively high molecular weight and unlikely to be involved in an eventual exchange mechanism.

TABLE 2. Effects of serosal Cl^- substitution on Na^+ and proton transports through *R. esculenta* skin mounted in open-circuit conditions

		Control	First period of Cl^- substitution	Second period of Cl^- substitution	Control
A	$J_n^{Na^+}$	207±29	159±21	89±14	137±26
	$J_n^{H^+}$	−237±20	−183±21	−111±14	−183±29
	ψ_{ms}	+52±13	+65±16	+54±17	+35±15
B	$J_n^{Na^+}$	236±27	153±21	76±29	157±46
	$J_n^{H^+}$	−260±30	−178±20	−99±32	−209±32
	ψ_{ms}	+53±9	+50±4	+45±9	+41±11

After a control period, the serosal side of the skin was perfused with a gluconate (A) or methylsulphate (B) Ringer during two successive periods of 45 min followed by a last reversal period. Fluxes are expressed in nequiv $h^{-1} cm^{-2}$ and ψ_{ms} in mV. Number of skins is six for A and for B. The mucosal solution contained 2 mequiv Na_2SO_4 buffered to pH 7·34.

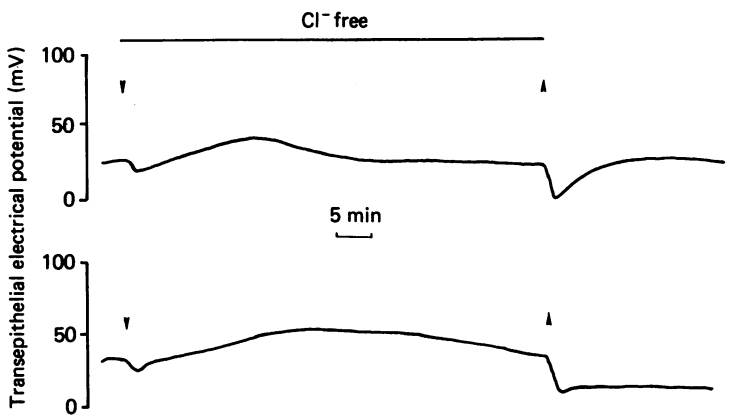

Fig. 3. Evolution of the transepithelial electrical potential after Cl^--substitution experiments. Upper trace, Cl^- replaced by gluconate; lower trace, Cl^- replaced by methylsulphate. The beginning and the end of the Cl^--free perfusion period is indicated by an arrow.

Preliminary experiments of substituting Cl^- in the Ringer with gluconate did not reveal any significant effect on $J_n^{H^+}$. The amount of Cl^- lost from the skin into the stagnant Cl^--free serosal solution (600–800 μequiv l^{-1}) was possibly sufficient to support a Cl^--dependent mechanism. Therefore, we decided to repeat these experiments while continuously perfusing the serosal side of the frog skin with a gluconate or methylsulphate Ringer. By this procedure, Cl^- in the serosal solution was reduced to 105 μequiv l^{-1}.

Table 2 summarizes the results obtained when gluconate (A) or methylsulphate (B) were used as Cl^- substitutes. The frog skin in these experiments was in open-circuit conditions. Cl^- substitution by either of the substituting anions produced a significant and similar inhibition of $J_n^{Na^+}$ and $J_n^{H^+}$; maximal inhibition after 90 min of Cl^--free serosal perfusion was 55 % using a gluconate Ringer and 65 % with a methylsulphate Ringer. Reversal of the inhibition on returning to Cl^- Ringer was observed in these

TABLE 3. Effect of serosal Cl^- substitution on proton excretion through the *R. esculenta* clamped skin

	Cl^- substituent used		Control period	First Cl^--free period (30 min)	Second Cl^--free period (30 min)	Third Cl^--free period (30 min)
A	Gluconate	$J_n^{H^+}$	-225 ± 46	-102 ± 24	-83 ± 26	-74 ± 31
		I_t	-190 ± 35	-86 ± 12	-80 ± 10	-65 ± 5
			Control	Cl^- free	Control	
B	Gluconate	$J_n^{H^+}$	-237 ± 38	-143 ± 35	-238 ± 31	
		I_t	-344 ± 70	-148 ± 43	-304 ± 53	
					Cl^--free Ringer +3 mM-NaCl	
			Control	Cl^- free		
C	Gluconate	$J_n^{H^+}$	-295 ± 38	-144 ± 21	-269 ± 47	
		I_t	-332 ± 54	-158 ± 59	-262 ± 43	
D	Methylsulphate	$J_n^{H^+}$	-147 ± 18	-88 ± 7	-121 ± 15	
		I_t	-172 ± 30	-86 ± 13	-146 ± 21	

The Cl^- substituent used was gluconate for A, B and C experiments and methylsulphate for D experiments. Cl^--free Ringer was perfused in the serosal side after a control period. The mucosal solution contained 2 mequiv Na_2SO_4 buffered to pH 7·34. The skin was clamped to 50 mV and Na^+ transport was blocked by 5×10^{-6} M-amiloride. In A experiments, the proton excretion ($J_n^{H^+}$) and the negative current (I_t) were followed as a function of time. In B the experiments demonstrate the reversal of the Cl^- substitution; in C and D experiments 3 mM-NaCl was added after the Cl^- substitution. Fluxes and current are expressed in nequiv h^{-1} cm^{-2}. $n = 6$ for each group of experiments.

experiments but was not complete. After a serosal Cl^--free-Ringer perfusion, a small (5–10 mV) and fast (1 min) depolarization of ψ_{ms} was observed (Fig. 3); this initial depolarization was followed within 30–40 min by a slow repolarization of ψ_{ms} by 10–30 mV. During the reversal period, a fast (maximum at 2–3 min) and large (20–30 mV) depolarization was observed followed by a slow repolarization of the skin lasting 15–30 min.

To avoid any additional complications due to potential changes, these experiments were repeated while clamping the preparation to a ψ_{ms} of +50 mV (a value close to that of the spontaneous potential); furthermore, Na^+ transport was blocked by the addition of 5×10^{-6} M-amiloride. We have previously shown that in this situation, $J_n^{Na^+}$ is slightly negative and the transepithelial current (I_t) reflects mainly the proton excretion $J_n^{H^+}$ (Ehrenfeld, Garcia-Romeu & Harvey, 1985). As observed for the open-circuit experiments, serosal Cl^--free-Ringer perfusion produced inhibition of $J_n^{H^+}$ and I_t (Table 3A). The inhibitory effect was similar using gluconate or methylsulphate Ringer. Complete reversal of $J_n^{H^+}$ inhibition was achieved when normal Ringer replaced Cl^--free Ringer (Table 3B) and partial reversal when 3 mM-NaCl was added to a Cl^--free Ringer (Table 3C and D). When these experiments were repeated under short-circuit conditions, without amiloride present, we found that both $J_n^{Na^+}$ and $J_n^{H^+}$ were inhibited in Cl^--free Ringer by 46 ± 7 and $49\pm10\%$ ($n = 5$) respectively. However, the subsequent addition of 3 mM-NaCl to an otherwise Cl^--free Ringer did not restore $J_n^{Na^+}$ despite the partial recovery of $J_n^{H^+}$ to $75\pm4\%$ of control.

TABLE 4. Effect of serosal Cl^- substitution on the electrical potentials of the apical and basolateral membranes, on the transepithelial potential, on the cell Cl^- activity and on the cell pH of the *R. esculenta* skin

	Normal Ringer	Cl^--free Ringer
ψ_{mc}	$-53 \pm 1{\cdot}2$	$-53 \pm 1{\cdot}6$
ψ_{cs}	$-82 \pm 1{\cdot}4$	$-77 \pm 1{\cdot}7$
ψ_{ms}	$+29 \pm 1{\cdot}5$	$+24 \pm 1{\cdot}9$
a^i_{Cl}	$14{\cdot}5 \pm 1{\cdot}6$	$1{\cdot}8 \pm 0{\cdot}3$
pH_i	$7{\cdot}18 \pm 0{\cdot}04$	$7{\cdot}32 \pm 0{\cdot}06$
α (R_a/R_b)	$2{\cdot}30 \pm 0{\cdot}05$	$2{\cdot}09 \pm 0{\cdot}09$
R_a	$3{\cdot}544 \pm 0{\cdot}15$	$3{\cdot}55 \pm 0{\cdot}16$
R_b	$1{\cdot}540 \pm 0{\cdot}05$	$1{\cdot}70 \pm 0{\cdot}08$
R_j	$13{\cdot}80 \pm 0{\cdot}50$	$17{\cdot}25 \pm 0{\cdot}70$
R_t	$3{\cdot}71 \pm 0{\cdot}11$	$4{\cdot}03 \pm 0{\cdot}11$

The epithelia ($n = 12$) were perfused on the serosal side by a normal Ringer ($a^o_{Cl} = 70$ mM) or a Cl^--free Ringer; gluconate was used as Cl^- substitute. The apical side was perfused with a 1 mM-Na_2SO_4 solution buffered to pH 7·34. Electrical potentials are expressed in mV. R_a, R_b, R_j, R_t are the equivalent resistances of the apical membrane, basolateral membrane, paracellular shunt and transepithelial resistance respectively estimated from circuit analysis and expressed in $k\Omega\ cm^{-2}$. α is the ratio of the apical to basolateral membrane resistances. a^i_{Cl} (in mM) and pH_i are measured 5 min after Cl^- substitution.

Therefore, under Cl^--free conditions, an additional mechanism of inhibition of Na^+ transport may be involved, possibly related to changes in cell volume.

From these experiments, we can conclude that: (1) Cl^- substitution produces reversible inhibitory effects on $J^{Na^+}_n$ and $J^{H^+}_n$; (2) transients in ψ_{ms} are observed; (3) the inhibition is similar using gluconate or methylsulphate as Cl^- substituent; (4) low concentrations of Cl^- added to a Cl^--free Ringer are sufficient to maintain proton transport. The latter result indicates that gluconate or methylsulphate *per se* are not inhibitory substances but that it is the absence of Cl^- which is responsible for the observed inhibitory effect.

Effects of Cl^- substitution on the cell electrical parameters and the intracellular Cl^- activity and pH

The effects of substitution of gluconate for Cl^- in the serosal Ringer on intracellular Cl^- activity (a^i_{Cl}) and the intracellular pH (pH_i) and on membrane potentials and resistances in the isolated epithelium are presented in Table 4.

Immediately after Cl^- substitution ψ_{cs} and ψ_{ms} depolarized slightly (mean depolarization $5{\cdot}1 \pm 0{\cdot}7$ mV); no change in the first minute was observed in the apical membrane potential (ψ_{mc}). Circuit analysis indicated that the decrease in the ratio of apical to basolateral membrane resistances (α, $2{\cdot}30 \pm 0{\cdot}05$ to $2{\cdot}09 \pm 0{\cdot}09$) was due to a slight increase in the latter resistance. The resistance of the paracellular pathway (R_j) and the total resistance (R_t) of the epithelium were also increased.

Measurements of a^i_{Cl} with double-barrelled micro-electrodes show that a^i_{Cl} decreased rapidly after perfusion with a Cl^--free Ringer. The mean a^i_{Cl} was $14{\cdot}5 \pm 1{\cdot}6$ and $1{\cdot}8 \pm 0{\cdot}3$ mequiv l^{-1} in the control period and the Cl^--free experiments respectively. The final value obtained is close to the detection limit of these electrodes and may therefore be influenced by interfering anions and may be over-estimated.

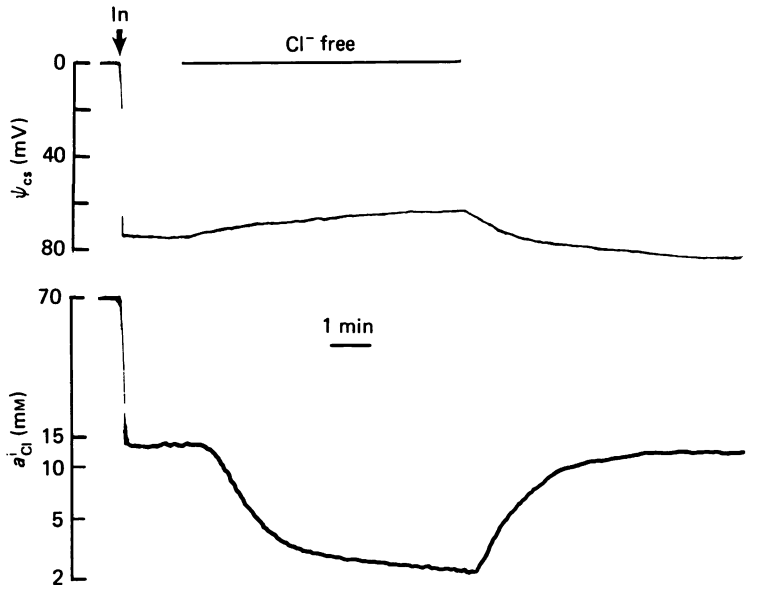

Fig. 4. Pen recording of an experiment showing the effects of Cl^--free Ringer (gluconate substitution) on ψ_{cs} and on a^i_{Cl} in the isolated epithelium of frog skin. An epithelial cell was impaled (arrow) from the basolateral side with a double-barrelled Cl^--sensitive micro-electrode. In the control condition with 1 mM-Na_2SO_4 bathing the apical side and normal Ringer on the basolateral side, the $\psi_{cs} = -74$ mV and $a^i_{Cl} = 15$ mM. On changing rapidly the perfusate on the basolateral side from normal Ringer to Cl^--free Ringer, the ψ_{cs} depolarized to -64 mV and a^i_{Cl} fell to 2·5 mM. These effects were reversible on return to normal Ringer.

Measurements of pH_i with double-barrelled micro-electrodes indicate that the pH_i was raised from $7{\cdot}18 \pm 0{\cdot}04$ to $7{\cdot}32 \pm 0{\cdot}06$ after a Cl^- substitution. Figs. 4 and 5 represent typical experiments performed on the isolated epithelium and show Cl^--activity changes (Fig. 4) and cell-pH changes (Fig. 5) occurring after perfusion with a Cl^--free Ringer. The results for twelve epithelia are summarized in Table 4.

The half-time ($t_{\frac{1}{2}}$) for a^i_{Cl} and pH_i changes on rapidly switching perfusion from normal Ringer to Cl^--free Ringer were $1{\cdot}5 \pm 0{\cdot}2$ min ($n = 9$) and $1{\cdot}2 \pm 0{\cdot}1$ min ($n = 9$) respectively.

After inhibition of acid secretion the magnitude of the change in pH_i will depend on the net amount of HCO_3^- entering the cell ($Q_{HCO_3^-}$) and upon the intracellular buffering power (B_i). $Q_{HCO_3^-}$ may be calculated from the formula:

$$Q_{HCO_3^-} = |HCO_3^-|^f_i - |HCO_3^-|^s_i + (pH^f_i - pH^s_i)B_i,$$

where superscripts f and s mean the measurement is made in Cl^--free Ringer and in control Ringer respectively. Cell HCO_3^- concentration ($[HCO_3^-]_i$) was calculated from the Henderson–Hasselbach equation. Using our results for pH_i changes and a B_i of 13·7 mM pH unit^{-1} (Harvey, 1982) we calculate $Q_{HCO_3^-}$ per unit time to be $3{\cdot}03 \pm 0{\cdot}3$ mM min^{-1} which compares with a change in a^i_{Cl} of $2{\cdot}82 \pm 0{\cdot}05$ mM min^{-1} under similar experimental conditions ($n = 12$).

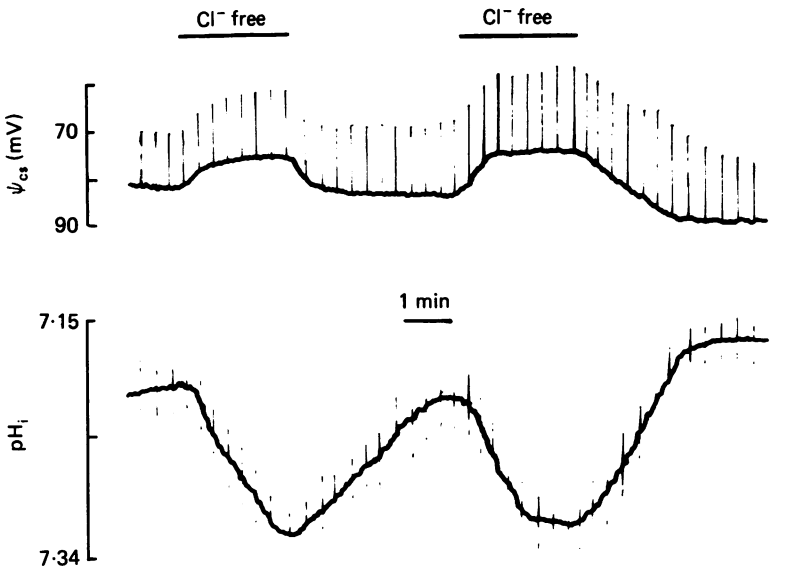

Fig. 5. Pen recording of an experiment showing the effects of Cl^--free Ringer (gluconate substitution) on ψ_{cs} and pH_i in the isolated epithelium of frog skin. ψ_{cs} and pH_i were recorded simultaneously from the same epithelial cell impaled from the basolateral side with a double-barrelled pH-sensitive micro-electrode. Under normal perfusion conditions (1 mM-Na_2SO_4 on the apical side, normal Ringer on the basolateral side) the $\psi_{cs} = -80$ mV and $pH_i = 7{\cdot}20$. When Cl^--free Ringer was rapidly substituted for normal Ringer during the two periods marked by a bar, ψ_{cs} depolarized by up to 10 mV and pH_i increased to 7·32. These effects were reversible on restoration of normal Ringer. The spikes on the ψ_{cs} trace are potential responses to the passage of transepithelial current pulses of 10 μA cm^{-2} strength. The spike height is indicative of basolateral-membrane resistance and is increased on perfusion with Cl^--free Ringer.

If basolateral Cl^- electrodiffusion is the current source linked in series with the apical rheogenic proton pump and both these current sources are the origin of the transepithelial current (I_t) associated with proton excretion ($J_n^{H^+}$), then the decrease in basolateral Cl^- current in Cl^--free media should equal the decrease in I_t (≈ 5 μA cm^{-2}). From this law, knowing the basolateral membrane resistance, (1–54 kΩ cm^{-2}) the expected depolarization of ψ_{cs} under these conditions would be ≈ 8 mV, which is in the range found experimentally.

Is there a K^+–HCO_3^- co-transport at the basolateral membrane?

A relatively large K^+ permeability has been reported to exist at the basolateral membrane of the frog skin (Koefoed-Johnsen & Ussing, 1958; Nagel, 1979); therefore, we have tested if the mechanism of proton excretion was coupled to basolateral K^+ transport, i.e. if a K^+–HCO_3^- co-transport was present at the inner barrier of the epithelium. Thus, after loading the epithelium with $^{42}K^+$, we followed the appearance of the isotope in the serosal solution by replacement of the total serosal solution every 2 min. Analysis of $^{42}K^+$ wash-out shows that after 15–20 min, the K^+ efflux is nearly linear during 2 h. A strict comparison of the first and second hours of wash-out reveals, however, a small increase ($\Delta t_{\frac{1}{2}} = 20$ min) in the $t_{\frac{1}{2}}$ of the second hour of

TABLE 5. Effect of serosal Cl^- substitution on the cell to serosal K^+ effluxes in *R. esculenta* skin

Serosal solution	$J_n^{Na^+}$	$J_n^{H^+}$	$t_{\frac{1}{2}}$ K^+
Cl^--free Ringer	114±17	−104±10	228±13
Normal Ringer	221±17	−175±40	343±33
Difference	107±33	71±31	115±23

The skins were first equilibrated on the serosal side with a Cl^--free Ringer and loaded for 2 h with $^{42}K^+$. Two successive periods of 45 min each were then followed, one with a Cl^--free Ringer and the other with a Cl^- Ringer as serosal solution. $J_n^{Na^+}$ and $J_n^{H^+}$ represent the net fluxes of Na^+ and protons expressed in nequiv h^{-1} cm^{-2}. $t_{\frac{1}{2}}$ K^+ is expressed in minutes. Number of skins: $n = 7$.

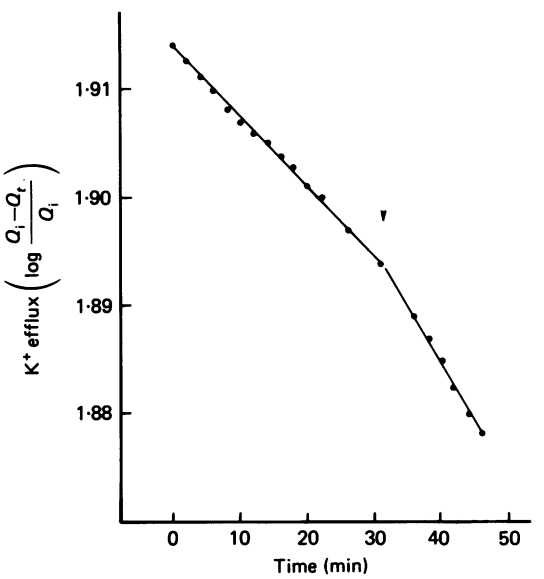

Fig. 6. K^+ efflux through the basolateral membrane of the *R. esculenta* skin as a function of time. At the arrow 10 mM-KCl was added to the serosal solution. K^+ efflux calculated using the expression $\log Q_i - Q_t/Q_t$ where Q_i and Q_t correspond to the initial and the t-time $^{42}K^+$-amounts in the skin respectively.

measurement. The K^+ efflux measured during these periods corresponds to a cellular compartment. Indeed, as can be seen in Fig. 6, the K^+ efflux was sensitive to KCl addition (10 mM) in the serosal solution; the mean $t_{\frac{1}{2}}$ was reduced from 381±40 min (control period) to 222±28 min ($n = 4$) (KCl addition) and the reduced $t_{\frac{1}{2}}$ can be interpreted as the result of a basolateral membrane depolarization. Furthermore, the cellular nature of this K^+ efflux was confirmed by the use of ouabain, which increased the K^+ efflux (E. Duranti, J. Ehrenfeld & B. J. Harvey, unpublished results) in agreement with the Helman & Cox experiments (1984). In Table 5 are summarized the $^{42}K^+$ effluxes (expressed by their $t_{\frac{1}{2}}$) to the serosal solution in a Cl^--free period (gluconate Ringer as serosal solution) followed by a normal Ringer period. $J_n^{Na^+}$ and $J_n^{H^+}$ were measured simultaneously in order to verify that the Cl^- substitution produced the effects described above. As can be seen the calculated $t_{\frac{1}{2}}$ is much lower in the gluconate period than in the Cl^--Ringer period which means that the K^+ efflux

TABLE 6. Effect of Ba^{2+} on Na^+ and proton transports across the frog skin

		Control open-circuit conditions	Ba^{2+} open-circuit conditions	Ba^{2+} clamp
A	$J_n^{Na^+}$	$+189 \pm 18$	$+170 \pm 21$	$+46 \pm 14$
	$J_n^{H^+}$	-213 ± 42	-222 ± 40	-184 ± 50
	ψ_{ms}	56 ± 12	8 ± 4	55 ± 12
		Control clamp	Ba^{2+} clamp	Control clamp
B	$J_n^{Na^+}$	-55 ± 35	-27 ± 18	-18 ± 15
	$J_n^{H^+}$	-238 ± 28	-269 ± 30	-247 ± 36

A, the two first periods were in open-circuit conditions, and in the last one ψ_{ms} was clamped to the control period ψ_{ms}. The second period is measured 15 min after the addition of Ba^{2+}. B, in this experiment the skin was clamped at +50 mV in the presence of 5×10^{-6} M-amiloride added to the mucosal bath. Ba^{2+} concentration was 2 mM (serosal addition). Fluxes expressed in nequiv h^{-1} cm^{-2} and ψ_{ms} in mV. Number of animals: $n = 6$ for each group of experiments.

was larger in the first period than in the latter period. This experiment demonstrates that as $J_n^{H^+}$ is inhibited, the K^+ effluxes are increased after Cl^- substitution, a result which argues against the likelihood of a K^+–HCO_3^- co-transport.

Ba^{2+} has been reported to reduce the K^+ conductance in frog skin (Nagel, 1979) and in isolated proximal straight tubules (Biagi, Kubota, Sohtell, & Giebisch, 1981; Bello-Reuss, 1982) causing a depolarization of the basolateral membranes. Therefore, if HCO_3^- exit is rheogenic, the transepithelial acidification rate would be inhibited by reducing the electrical driving force for HCO_3^- exit across the basolateral membrane. Secondly, if a K^+–HCO_3^- co-transport exists, Ba^{2+} would indirectly inhibit HCO_3^- exit by its primary effect on the K^+ permeability. The effects of Ba^{2+} (2 mM) addition to the serosal side of the frog skin on Na^+ and proton transports is shown in Table 6. When the skin is maintained in open-circuit conditions (Table 6A), Ba^{2+} induces a depolarization of ψ_{ms} within some minutes (maximal inhibition at 30 min) whereas $J_n^{Na^+}$ and $J_n^{H^+}$ were not affected by the divalent cation. However, clamping the preparation in the presence of Ba^{2+} to the ψ_{ms} of the open-circuit state (56 mV) leads to a considerable inhibition of J_n^{Na} while $J_n^{H^+}$ is not affected. The inhibition of $J_n^{Na^+}$ under the latter conditions may have resulted from the depolarization by the voltage clamp and the reduction of ψ_{mc} towards zero mV, an effect which greatly reduced the electrical gradient favouring passive Na^+ entry to the cells. The maintenance of a net Na^+ flux in open-circuit conditions can be due to the increased electrochemical driving force for transepithelial Na^{2+} transport, since ψ_{ms} decreased after Ba^{2+} application with only slight depolarization of ψ_{mc} (E. Duranti, J. Ehrenfeld & B. J. Harvey, unpublished results). Therefore Ba^{2+} does not seem to inhibit the two key phases of Na^{2+} transport, i.e. its entrance to its compartment or its extrusion through the Na^+–K^+-ATPase at the inner barrier of the Na^+ compartment.

The absence of effectiveness of Ba^{2+} on proton excretion is confirmed by the experiments presented in Table 6B where in the case of Na^+ transport was blocked with amiloride (5×10^{-6} M) and the preparation clamped to a ψ_{ms} of 50 mV. These

TABLE 7. Effects of DIDS, furosemide, meclofenamate and bumetanide on proton transports across the frog skin

Inhibitor used	DIDS (10^{-4} M)	Furosemide (10^{-3} M)	Meclofenamate (10^{-5} M)	Meclofenamate (5×10^{-5} M)	Bumetanide (5×10^{-4} M)
Control $J_n^{H^+}$	-153 ± 22	-295 ± 33	-220 ± 31	-293 ± 45	-118 ± 24
Test $J_n^{H^+}$	-100 ± 28	-232 ± 20	-145 ± 22	-139 ± 16	-142 ± 37
Difference	53 ± 23*	63 ± 17**	75 ± 12***	154 ± 33***	-24 ± 18 n.s.

The *R. esculenta* skins were clamped to a transepithelial potential of 50 mV and Na^+ transport was blocked with 5×10^{-6} M-amiloride. Fluxes ($J_n^{H^+}$) are expressed in nequiv h^{-1} cm^{-2}. Number of animals: $n = 6$ for each group of experiments. Significance: * $P < 0.05$; ** $P < 0.025$; *** $P < 0.005$; n.s., not significant.

results do not support the presence of a HCO_3^--electrodiffusion pathway nor a K^+–HCO_3^- co-transport as the mechanism of HCO_3^- exit across the basolateral membrane.

Effects of anion-transport inhibitors on proton excretion

We have tested the effects of some known inhibitors of anionic transport mechanisms on proton excretion. DIDS (10^{-4} M), furosemide (10^{-3} M) and meclofenamate (10^{-5} M) inhibited $J_n^{H^+}$ by 35, 21 and 34% respectively. Meclofenamate added at a higher concentration of 5×10^{-5} M blocked $J_n^{H^+}$ by 53%.

Bumetanide, which has been reported as a Na^+–K^+–Cl^- co-transport inhibitor had no effect on the proton excretion at the tested concentration of 5×10^{-4} M (the results are summarized in Table 7).

DISCUSSION

In a previous paper (Ehrenfeld, Garcia-Romeu & Harvey, 1985) we demonstrated the presence of an active electrogenic proton pump localized at the apical border of the epithelium which was responsible for the net proton excretion and for the negative current (I_t) observed in the absence of Na^+ transport. The present studies demonstrate that the proton excretion measured by titration of the mucosal solution is directly correlated to the alkalinization of the serosal solution and the question we posed at the outset of the present study concerned the nature of the mechanism of HCO_3^- (or OH^-) exit at the inner barrier of the epithelium.

At least three models have been proposed in the kidney and the urinary bladder for the basolateral exit of HCO_3^-. First, a passive rheogenic process via a conductive pathway has been described in the rat renal proximal tubule (Burckhardt, Sato & Frömter, 1984) and the rabbit proximal convoluted tubule (Sasaki & Berry, 1984). Secondly, an electrogenic Na^+–HCO_3^- transporter was suggested in the renal proximal tubule of the salamander (Boron & Boulpaep, 1983); thirdly, a Cl^--dependent HCO_3^- exit via an electroneutral mechanism has been postulated in the turtle urinary bladder (Fisher, Husted & Steinmetz, 1983) and in the peritubular cell membrane of Necturus proximal tubule (Edelman, Bouthier & Anagnostopoulos, 1981).

In the case of a rheogenic HCO_3^- (or OH^-) exit its rate will depend on the HCO_3^- electrochemical gradient across the basolateral membrane of the epithelium. Assuming

that the cell P_{CO_2} is in equilibrium with the serosal solution and that the CO_2 solubility coefficient and the pK_a for carbonic acid are the same intracellularly as in the Ringer solution, with a measured cell pH of 7·18, we can calculate an approximate intracellular HCO_3^- activity ($a^i_{HCO_3^-}$) of 9·8 mM. Since the measured basolateral membrane potential is −82 mV, the HCO_3^- electrochemical driving force would be +71 mV which is largely sufficient to account for a passive HCO_3^- (or OH^-) exit step through a conductive pathway. Varying the basolateral membrane potential will be expected to change the HCO_3^- movements through this membrane.

We found, however, that depolarization of the basolateral membrane using Ba^{2+} did not block acid secretion either in open-circuit conditions or when ψ_{ms} was voltage clamped at +50 mV. Several conclusions can be drawn from these experiments: (1) HCO_3^- (or OH^-) exit across the basolateral membrane of the epithelium is not coupled with K^+; such a conclusion is also strengthened from the observation of the simultaneous increase in the $t_{\frac{1}{2}}$ of K^+ wash-out and a decrease in serosal alkalinization after Cl^- substitution; (2) the mechanism of HCO_3^- (or OH^-) exit is not sensitive to the potential changes of the basolateral membrane induced by Ba^{2+}. Therefore rheogenic HCO_3^- exit or a K^+–HCO_3^- co-transport appear unlikely.

We also examined the possibility of a Na^+–HCO_3^+ co-transport. Such a mechanism was described in the renal proximal tubule of the salamander (Boron & Boulpaep, 1983). However, its presence in frog skin seems unlikely since: (1) acid secretion was normal under conditions (amiloride and voltage clamp) known to diminish considerably the cell Na^+ activity (Harvey & Kernan, 1984*b*); (2) Na^+ substitution of the serosal Ringer did not produce significant effects on the proton excretion (E. Duranti, J. Ehrenfeld & B. J. Harvey, unpublished results).

In the case of the coupling between HCO_3^- exit with Cl^- directed in the opposite direction, the driving force for such a system would be the Cl^- chemical gradient existing between the serosal solution and the cells ($a^i_{Cl}/a^o_{Cl} = 15/70$, where a^o_{Cl} is the external Cl^- activity) which is nearly threefold greater than the inwardly directed HCO_3^- chemical gradient. A reduction of this gradient would reduce the HCO_3^- exit. The Cl^- dependence of the proton excretion has been considered in a variety of other tight epithelia as an indication of the presence of a Cl^-–HCO_3^- exchange (Cohen, Mueller & Steinmetz, 1978; Fisher *et al.* 1983; Stone, Seldin, Kokko & Jacobson, 1983). Our results show that the proton excretion is sensitive to Cl^- substitution in the serosal solution. The two substituents used, gluconate and methylsulphate, both exert similar inhibitory effects on proton transport; inhibition of $J^{H^+}_n$ was also observed in Cl^--free conditions when the Na^+ transport was blocked with amiloride. The maximal inhibition of $J^{H^+}_n$ after Cl^- substitution varied between 60 and 70%. This lack of complete inhibition could be explained if the Cl^--dependent proton-excretion mechanism presents a high affinity (very low apparent Michaelis constant (K_m)) for Cl^-. This assumption is justified by the lack of inhibition of $J^{H^+}_n$ observed when the serosal side was not continuously perfused with a Cl^--free solution and by the recovery of $J^{H^+}_n$ to control values when 3 mM-NaCl was added to the Cl^--free serosal solution. In the turtle bladder, Fisher *et al.* (1983) report a Cl^-–HCO_3^--exchange mechanism with an apparent K_m for Cl^- of 0·13 mM, and in this respect Cl^--dependent proton excretion in *R. esculenta* skin shows a close similarity.

For the case of a Cl^-–HCO_3^- exchange at the inner barrier of the epithelium, Cl^-

replacement in the serosal solution would tend to block, or may reverse this mechanism and therefore lead to a cell Cl^- depletion and to a cell pH increase. The experiments reported in Table 4 are consistent with this hypothesis. The cell Cl^- decreases from $\simeq 15$ mM to very low values of < 2 mM (within the limits of measurements). Cassola, Mollenhauer & Frömter (1983) have reported that in the rat kidney proximal tubular cells, the cell Cl^- activity varies from 18 mM (control conditions) to 3·8 mM (Cl^--free conditions); these values are very close to our measurements in the frog-skin epithelium and agree with earlier measurements (Harvey & Kernan, 1984*a*). Such a cell Cl^- decrease was also described in the frog skin by Ferreira & Ferreira (1981) although the cell Cl^- concentrations measured analytically by these authors were much higher than the cell Cl^- activities reported in our study. A cell alkalinization of 0·14 pH units was observed after Cl^- replacement by gluconate; such an alkalinization has also been described, in similar experimental conditions, in rabbit proximal tubules (Kleinman, Ware & Schwartz, 1981). This rise in cell pH may be at the origin of decrease in proton supply to the apical proton pump leading to a reduction of its transport capacity.

The disulphonic acid stilbene derivatives are well established inhibitors of Cl^-–HCO_3^- exchange, first demonstrated in red blood cells (Knauf & Rothstein, 1971; Cabantchik & Rothstein, 1972) and later in the squid axon (Russell & Boron, 1976), snail neurones (Thomas, 1976), barnacle muscle (Boron, 1977) and mammalian skeletal muscle (Aickin & Thomas, 1977). Meclofenamate is also a powerful blocker of the electroneutral anion exchanger in red blood cells (Cousin & Motais, 1979). In epithelial tissues, SITS (4-acetamido-4′-isothiocyanostilbene-2,2′-disulphonic acid) has been reported to inhibit the acidification process of the turtle urinary bladder (Cohen *et al.* 1978) and of the renal proximal convoluted tubule (Ullrich, Capasso, Rumrich, Papavassiliou & Klöss, 1977; Chan, Lai, Biagi & Giebisch, 1982). In these epithelia the effects of SITS have been attributed to the inhibition of anion-transport mechanisms located at the basolateral membrane resulting in secondary pH alkalinization. In our study, DIDS (10^{-4} M) and meclofenamate (5×10^{-5} M) may block the proton excretion by the same mechanism. The larger inhibitory effect obtained with meclofenamate could be due to the greater hydrophobicity of this compound which could result in a better accessibility of this drug to its site of action.

Since we observe a transepithelial current associated with proton excretion it remains for us to determine the nature of the inward current at the basolateral membrane which must be coupled in series with the outward current produced by the proton pump at the apical membrane. Fisher *et al.* (1983) have suggested that Cl^- exit proceeds through a Cl^--conductive pathway and is recycled at the basolateral membrane of the turtle urinary bladder. This hypothesis could be applied to the frog skin since a Cl^--conductive pathway was suggested to exist in the frog skin (Ferreira & Ferreira, 1981) and has been demonstrated in the basolateral membranes of the cortical collecting tubule (Sansom, Weinman & O'Neil, 1983) and is implied in our present results.

The technical assistance of N. Gabillat, P. Lahitette and A. Giovagnoli is gratefully acknowledged. This work was supported by grants from the Centre National de la Recherche Scientifique (Physiologie des Membranes, UA 638 associée au C.E.A.). B. J. Harvey is a career investigator of the C.N.R.S., France.

REFERENCES

ACEVES, J. & ERLIJ, D. (1971). Sodium transport across the isolated epithelium of the frog skin. *Journal of Physiology* **212**. 195–210.

AICKIN, C. C. & THOMAS, R. C. (1977). An investigation of the ionic mechanism of intracellular pH regulation in mouse soleus muscle fibres. *Journal of Physiology* **273**, 295–316.

BELLO-REUSS, E. (1982). Electrical properties of the basolateral membrane of the straight portion of the rabbit proximal renal tubule. *Journal of Physiology* **326**, 49–63.

BIAGI, B., KUBOTA, T., SOHTELL, M. & GIEBISCH, G. (1981). Intracellular potentials in rabbit proximal tubules perfused *in vitro*. *American Journal of Physiology* **240**, F200–210.

BORON, W. F. (1977). Intracellular pH transients in giant barnacle muscle fibres. *American Journal of Physiology* **233**, C61–73.

BORON, W. F. & BOULPAEP, E. L. (1983). Intracellular pH regulation in the renal proximal tubule of the salamander basolateral HCO_3^- transport. *Journal of General Physiology* **81**, 53–94.

BURCKHARDT, B. C., SATO, K. & FRÖMTER, E. (1984). Electrophysiological analysis of bicarbonate permeation across the peritubular cell membrane of rat kidney proximal tubule. I. Basic observations. *Pflügers Archiv* **401**, 34–42.

CABANTCHIK, Z. I. & ROTHSTEIN, A. (1972). The nature of the membrane sites controlling anion permeability of human red blood cells as determined by studies with disulfonic stilbene derivatives. *Journal of Membrane Biology* **10**, 311–330.

CASSOLA, A. C., MOLLENHAUER, M. & FRÖMTER, E. (1983). The intracellular chloride activity of rat kidney proximal tubular cells. *Pflügers Archiv* **399**, 259–265.

CHAN, Y. L., LAI, L., BIAGI, B. & GIEBISCH, G. (1982). Control mechanisms of bicarbonate transport across the rat proximal convoluted tubule. *American Journal of Physiology* **242**, F532–543.

COHEN, L. H., MUELLER, A. & STEINMETZ, P. R. (1978). Inhibition of the bicarbonate exit step in urinary acidification by a disulfonic stilbene. *Journal of Clinical Investigation* **401**, 981–986.

COUSIN, J. L. & MOTAIS, R. (1979). Inhibition of anion permeability by amphiphilic compounds in human red cell: evidence for an interaction of niflumic acid with the band 3 protein. *Journal of Membrane Biology* **46**, 125–153.

EDELMAN, A., BOUTHIER, M. & ANAGNOSTOPOULOS, T. (1981). Chloride distribution in the proximal convoluted tubule of necturus kidney proximal tubule. *Pflügers Archiv* **351**, 69–83.

EHRENFELD, J. & GARCIA-ROMEU, F. (1977). Active hydrogen excretion and sodium absorption through isolated frog skin. *American Journal of Physiology* **233**, F46–54.

EHRENFELD, J. & GARCIA-ROMEU, F. (1978). Coupling between chloride absorption and base excretion in isolated frog skin of *Rana esculenta*. *American Journal of Physiology* **235**, F33–39.

EHRENFELD, J., GARCIA-ROMEU, F. & HARVEY, B. (1985). Electrogenic active proton pump in *Rana esculenta* skin and its role in sodium ion transport. *Journal of Physiology* **359**, 331–355.

FERREIRA, K. T. G. & FERREIRA, H. G. (1981). The regulation of volume and ion composition in frog skin. *Biochimica et biophysica acta* **66**, 193–202.

FISHER, J. L., HUSTED, R. F. & STEINMETZ, P. R. (1983). Chloride dependence of the HCO_3^- exit step in urinary acidification of the turtle bladder. *American Journal of Physiology* **245**, F564–568.

HARVEY, B. J. (1982). Ph.D. thesis. National University of Ireland.

HARVEY, B. J. & KERNAN, R. P. (1984*a*). Intracellular ion activities in frog skin in relation to external sodium and effects of amiloride and/or ouabain. *Journal of Physiology* **349**, 501–517.

HARVEY, B. J. & KERNAN, R. P. (1984*b*). Sodium-selective micro-electrode study of apical permeability in frog skin: effect of sodium, amiloride and ouabain. *Journal of Physiology* **356**, 359–374.

HELMAN, S. T. & COX, T. C. (1984). Basolateral membrane K^+ transport in frog skin. *Federation Proceedings* **43**, 2490–2492.

KLEINMAN, J. G., WARE, R. A. & SCHWARTZ, J. H. (1981). Anion transport regulates intracellular pH in renal cortical tissue. *Biochimica et biophysica acta* **648**, 87–92.

KNAUF, P. A. & ROTHSTEIN, A. (1971). Chemical modification of membranes. I. Effect of sulphydryl and amino reactive reagents on anion and cation permeability of the human red blood cell. *Journal of General Physiology* **268**, 35–49.

KOEFOED-JOHNSEN, V. & USSING, H. H. (1958). The nature of the frog skin potential. *Acta physiologica scandinavica* **42**, 298.

KRISTENSEN, P. (1972). Chloride transport across isolated frog skin. *Acta physiologica scandinavica* **84**, 338–346.

NAGEL, W. (1979). Inhibition of potassium conductance by barium in frog skin epithelium. *Biochimica et biophysica acta* **552**, 346–357.

RUSSELL, J. & BORON, W. (1976). Role of chloride transport in regulation of intracellular pH. *Nature* **264**, 73–74.

SANSOM, S. C. E., WEINMAN, J. & O'NEIL, R. G. (1983). Microelectrode assessment of chloride conductive properties of cortical collecting duct (CCD). *Federation Proceedings* **42**, 305–311.

SASAKI, S. & BERRY, C. A. (1984). Mechanism of bicarbonate exit across basolateral membrane of the rabbit proximal convoluted tubule. *American Journal of Physiology* **246**, F889–896.

STONE, D. K., SELDIN, D. W., KOKKO, J. P. & JACOBSON, H. R. (1983). Chloride dependence of medullary collecting duct acidification. *Kidney International* **23**, 239A.

THOMAS, R. C. (1976). Ionic mechanism of H^+ pump in a snail neurone. *Nature* **262**, 54–55.

ULLRICH, K. J., CAPASSO, G., RUMRICH, G., PAPAVASSILIOU, F. & KLÖSS, S. (1977). Coupling between proximal tubular transport processes: studies with ouabain, SITS and HCO_3^- free solutions. *Pflügers Archiv* **368**, 245–252.