Electroneutral, HCO_3^- -independent, pH gradient-dependent uphill transport of Cl^- by ileal brush-border membrane vesicles

Possible role in the pathogenesis of chloridorrhea

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By applying a rapid filtration technique to isolated brush border membrane vesicles from guinea pig ileum, ^{36}Cl uptake was quantified in the presence and absence of electrical, pH and alkali-metal ion gradients. A mixture of 20 mM-Hepes and 40 mM-citric acid, adjusted to the desired pH with Tris base, was found to be the most suitable buffer. Malate and Mes could be used to replace the citrate, but succinate, acetate and maleate proved to be unsuitable. In the absence of a pH gradient ($\text{pH}_{\text{out}}:\text{pH}_{\text{in}} = 7.5:7.5$), Cl^- uptake increased slightly when an inside-positive membrane potential was applied, but uphill transport was never observed. A pH gradient ($\text{pH}_{\text{out}}:\text{pH}_{\text{in}} = 5.0:7.5$) induced both a 400% increase in the initial Cl^- influx rate and a long-lasting (20 to 300 s) overshoot, indicating that a proton gradient can furnish the driving force for uphill Cl^- transport. Under pH gradient conditions, initial Cl^- entry rates had the following characteristics. (1) They were unaffected by *cis*- Na^+ and/or $-\text{K}^+$, indicating the absence of Cl^-/K^+ , Cl^-/Na^+ or $\text{Cl}^-/\text{K}^+/\text{Na}^+$ symport activity. (2) Inhibition by 20–100 mM-*trans*- Na^+ and/or $-\text{K}^+$ occurred, independent of the existence of an ion gradient. (3) Cl^- entry was practically unaffected by short-circuiting the membrane potential with equilibrated potassium and valinomycin. (4) Carbonyl cyanide *m*-chlorophenylhydrazone was strongly inhibitory and so, to a lesser extent, was 4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonic acid [(SITS)], independent of the sign and size of the membrane potential. (5) Cl^- entry was negligibly increased (< 30%) by either *trans*- Cl^- or $-\text{HCO}_3^-$, indicating the absence of an obligatory Cl^- /anion antiport activity. In contrast, the height of the overshoot at 60 s was increased by *trans*- Cl^- , indicating time-dependent inhibition of ^{36}Cl efflux. That competitive inhibition of ^{36}Cl fluxes by anions is involved here is supported by initial influx rate experiments demonstrating: (1) the saturability of Cl^- influx, which was found to exhibit Michaelis–Menten kinetics; and (2) competitive inhibition of influx by *cis*- Cl^- and $-\text{Br}^-$. Quantitatively, the conclusion is warranted that over 85% of the total initial Cl^- uptake energized by a pH gradient involves an electroneutral Cl^-/H^+ symporter or its physicochemical equivalent, a Cl^-/OH^- antiporter, exhibiting little Cl^- uniport and either Cl^-/Cl^- or $\text{Cl}^-/\text{HCO}_3^-$ antiport activities. We propose that the HCO_3^- -independent Cl^-/H^+ symporter described here may be at the molecular basis of the genetic disease chloridorrhea, which is characterized by a massive loss of chloride in the stools, accompanied by alkalosis.

INTRODUCTION

The genetic disease chloridorrhea is characterized by massive loss of chloride in the stools, accompanied by alkalosis (Turnberg, 1971; Bieberdorf *et al.*, 1972). This suggests that the role played in acid–base balance by the ileum and colon, organs to which the molecular lesion in this disease has been traced (Milla, 1982), is much greater than heretofore recognized (for a recent review, see Hopfer & Liedtke, 1987). It therefore seems reasonable to hypothesize that a key physiological role of the ileum and colon is to achieve a net reabsorption of H^+ in conjunction with that of Cl^- . As a corollary of this coupling, severe malfunctioning of the Cl^- reabsorption apparatus, as occurs in chloridorrhea, should be accompanied by a net loss of protons in the stools, the primary cause of alkalosis.

Recent work with isolated brush border membrane vesicles from rat and rabbit (Knickelbein *et al.* 1985;

Hopfer & Liedtke, 1987) has suggested the existence in the ileum of a Cl^-/OH^- antiporter purported to exchange luminal Cl^- for intracellular OH^- , Cl^- and/or HCO_3^- . It is believed that HCO_3^- is a particularly good substrate here, better even than OH^- , and that this transporter functions physiologically as a $\text{Cl}^-/\text{HCO}_3^-$ antiporter (Hopfer & Liedtke, 1987). Perusal of the literature, however, reveals that direct evidence for $\text{Cl}^-/\text{HCO}_3^-$ antiporter activity in the ileum consists mainly of a generalization to the ileum of knowledge derived from the better studied $\text{Cl}^-/\text{HCO}_3^-$ antiporter of the red blood cell (reviewed in Lowe & Lambert, 1983).

By using isolated brush border membrane vesicles from guinea pig ileum, we set out: (1) to investigate whether an electroneutral Cl^-/H^+ symporter (a Cl^-/OH^- antiporter) exists in the ileal brush border membrane and (2) if this is the case, to distinguish between: (i) a specific Cl^-/OH^- antiporter where the main substrate would indeed be OH^- , and (ii) a less specific Cl^- /anion

Abbreviations used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; SITS, 4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonic acid; r_t , peak/equilibrium substrate concn. ratio at time *t*. The subscripts 'out' and 'in' refer to the extra- and intravesicular media respectively.

antiporter which accepts equally well (or even preferentially) HCO_3^- as the substrate. (From a physicochemical point of view, translocation of H^+ in one direction cannot be distinguished from that of OH^- in the opposite one. Following current practice, in this paper we will use as equivalent the terms Cl^-/H^+ symporter and Cl^-/OH^- antiporter, although we emphasize that the second of these terms has the disadvantage of suggesting the presence of a $\text{Cl}^-/\text{HCO}_3^-$ antiporter, a conclusion not supported by our present work.)

We have also attempted to quantify the relative contribution to total Cl^- uptake of the electroneutral mechanism(s) just mentioned and of a conductance pathway or rheogenic Cl^- uniport.

Although most of our work has involved guinea pig brush borders, the key experiments have been repeated with rabbit vesicles to minimize the animal species problem. Taken together, our results warrant the conclusion that over 85% of the total Cl^- influx across the ileal brush border membrane involves an electroneutral Cl^-/OH^- antiporter exhibiting little Cl^-/Cl^- or $\text{Cl}^-/\text{HCO}_3^-$ antiporter activity.

Preliminary accounts of this work have been published (Colin *et al.*, 1984; Vasseur *et al.*, 1986, 1987).

MATERIALS AND METHODS

Materials

H^{36}Cl (0.4 mCi/mmol, Amersham) was neutralized with Tris base before use. Valinomycin, CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) and other chemicals of the highest purity were purchased from Sigma. SITS (4-acetamido-4'-isothiocyano stilbene-2,2'-disulphonic acid) was obtained from Fluka.

Buffer composition

Unless stated otherwise, all experiments were performed in a 20 mM-Hepes/40 mM-citric acid buffer, adjusted to the desired pH with Tris base. The extravesicular media were always pH-adjusted by taking into account the inevitable carry-over of a known fraction of intravesicular pH 7.5 medium at the beginning of the transport assay. The ionic strength of the various buffers was maintained as nearly constant as possible by adding, as needed, the appropriate amount of 'inert' base and/or acid (respectively, Tris base and gluconic acid). All media were adjusted with sorbitol to maintain an osmolarity ratio [out:in] of 600:500 mosmol/litre. Buffer capacities (in the range from pH 7.5 to 7.0) were computed from the slope of appropriate titration curves (Ives *et al.*, 1986). Valinomycin and CCCP dissolved in ethanol were allowed to evaporate to dryness before mixing with the membrane preparation, a practice made possible by the high lipid/water partition coefficient of both ionophores (Grzesiek & Dencher, 1986). We verified that the doses used (10 $\mu\text{g}/\text{mg}$ of protein in each case) were sufficient to achieve maximal effect (Supplisson & Alvarado, 1986). SITS was dissolved in dimethyl sulphoxide; the controls without SITS received an identical volume of dimethyl sulphoxide (final concn. 2%).

Membrane vesicle preparation

Brush border membrane vesicles were isolated by the

$\text{Mg}^{2+}/\text{EGTA}$ -precipitation method of Hauser *et al.* (1980) from the ileum of either guinea pigs or New Zealand white rabbits. Similar results were obtained when using either fresh tissue or frozen material which had been stored at -80°C for less than 12 months. To minimize the Cl^- content of the vesicle preparations, MgCl_2 was replaced (with identical results) with either MgSO_4 or magnesium gluconate. Compared with the homogenate, the membranes were enriched more than 16-fold in terms of the specific marker, sucrase. Functional tests based on the use of specific D-glucose transport inhibitors (Brot-Laroche *et al.*, 1987) indicated that our brush border membrane preparations were free of contamination by basolateral membrane vesicles.

After washing with an appropriate buffer, the vesicles were adjusted to about 20 mg of protein/ml and stored in liquid N_2 in batches of 200 μl until the day of the experiment. No difference was found in the transport activity of freshly prepared and frozen vesicles. Protein was measured by using the Bio-Rad assay kit.

To obtain vesicles preloaded with certain ions (K^+ , Cl^- , HCO_3^-), two distinct procedures were used with similar results: either entrapment during vesicle formation or application of two cycles of freezing and thawing.

Cl^- transport measurement

Cl^- uptake was assayed by using ^{36}Cl and a rapid filtration technique (Hopfer *et al.*, 1973). Initial uptake rates (0.5 to 2 s) were determined with a Short-Time Incubation apparatus (Innovativ Labor AG, Zürich, Switzerland) in a constant temperature room at $23^\circ\text{C} \pm 2^\circ\text{C}$. Briefly, 10 μl of a membrane suspension containing 200–300 μg of protein was mixed with either 40 or 90 μl of reaction medium containing ^{36}Cl as the substrate. Incubations were terminated automatically with 2.5 ml of an ice-cold stop solution consisting of 25 mM- MgSO_4 , 350 mM-KCl, 10 mM-Hepes and 0.7 mM-citric acid, adjusted to pH 7.4 with Tris (identical results were obtained when using potassium gluconate instead of KCl). The samples were immediately filtered through a 0.65 μm pore size Sartorius filter prewetted with distilled water. After washing twice with 5 ml of ice-cold stop solution, the filters were added to 4 ml of Aqualuma plus (Kontron) scintillation fluid and the radioactivity determined in a scintillation spectrometer. Non-specific isotope binding to the filters was determined separately and subtracted from the total radioactivity of each sample.

Expression of results

Results are expressed as either absolute uptakes (units of $\text{nmol} \cdot \text{mg}$ of protein $^{-1}$), absolute velocities ($\text{nmol} \cdot \text{s}^{-1} \cdot \text{mg}$ of protein $^{-1}$) or relative velocities ($\text{nmol} \cdot \text{s}^{-1} \cdot \text{mg}$ of protein $^{-1}$) (Brot-Laroche & Alvarado, 1984). The data are presented as the means ($\pm$ S.D.) from several experiments performed with at least two different membrane preparations. Occasionally, representative results obtained with a single membrane preparation are presented. Uptake data were compared by applying a one-way analysis of variance (Snedecor & Cochran, 1967).

Substrate overshoots are quantified in terms of the peak/equilibrium substrate concentration ratio r_t (where t is the time of the observation in s).

RESULTS AND DISCUSSION

pH gradient-dependent uphill transport of Cl^-

(a) **Search for the appropriate buffer: extravesicular citrate is inert.** To study pH gradient-dependent Cl^- transport, the choice of buffer is critical because the weak acids most commonly used for buffer preparations yield anions which may compete with Cl^- for the specific binding site(s) in the membrane. We therefore began by studying the time-dependent uptake of ^{36}Cl under pH gradient conditions ($\text{pH}_{\text{out}}:\text{pH}_{\text{in}} = 5.0:7.5$) in the presence of a single intravesicular buffer (20 mM-Hepes/Tris, pH 7.5) plus various extravesicular organic acids having appropriate pK values (Fig. 1). Both citrate and malate gave the highest initial Cl^- entry rates and both sustained an overshoot. Mes gave similar results (not shown) whereas maleate, succinate and acetate were found to be unsuitable for our purposes.

All of the progress curves illustrated in Fig. 1 pass through the origin, indicating the absence of any appreciable binding of Cl^- to the external surface of the vesicles. The absence of appreciable Cl^- binding to intravesicular binding sites was confirmed by demonstrating (separate experiments, results not shown) that the equilibrium Cl^- uptake values in Hepes/citrate/Tris buffer dropped to insignificant levels when the vesicles were lysed by adding 1% Triton X-100 to the stop solution.

In conclusion, because citrate behaves inertly (it gives identical results at either 10 or 40 mM extravesicular concentrations) and has a high buffer capacity, we have selected for routine work a 20 mM-Hepes/40 mM-citric

acid buffer, adjusted to the desired pH with Tris base. Nevertheless, all of our key experiments have been repeated, with similar results, in buffers where Mes fully substituted for the citrate.

(b) **Apparent activation of Cl^- uptake by vesicle preincubation in citrate buffer.** It is believed that H^+ is a highly permeable cation (Wright *et al.*, 1984; Grzesiek & Dencher, 1986), which suggests that pH gradient-dependent uphill transport of Cl^- should be short-lived, particularly if the buffering capacity of the intravesicular medium is small. In theory, the intravesicular buffer capacity should not affect the initial Cl^- entry rate, whereas both the height and the duration of the overshoots should be in proportion to the intravesicular buffering power. To address this question, we compared the uptake of Cl^- in the presence of a pH gradient ($\text{pH}_{\text{out}}:\text{pH}_{\text{in}} = 5.0:7.5$) by using vesicles equilibrated with 20 mM-Hepes/Tris buffer, supplemented or not with citric acid to yield, respectively, $[\text{citrate}]_{\text{out}}:[\text{citrate}]_{\text{in}}$ ratios of either 40:40 or 40:0 mM (the intravesicular buffering power, in the 7.0–7.5 pH range, increased from $16 \pm 8 \mu\text{mol of H}^+ \cdot \text{ml}^{-1} \cdot \text{pH unit}^{-1}$ in the absence of citrate to 42 ± 9 in its presence). For about half of the experimental points, a K^+ gradient ($[\text{K}^+]_{\text{out}}:[\text{K}^+]_{\text{in}} = 80:0 \text{ mM}$) was superimposed, allowing the generation of an inside-positive electrical membrane potential upon addition of valinomycin.

The results indicate that preincubation of the vesicles with 40 mM-citrate buffer, pH 7.5, has an apparent activating effect on Cl^- uptake since the initial Cl^- entry rate increased significantly (from 0.61 ± 0.06 to

Fig. 1. Time course of pH gradient-dependent Cl^- uptake in the presence of five different extravesicular organic acids

The uptake of 4 mM-Cl^{36} (zero-trans) was determined by using vesicles equilibrated with 20 mM-Hepes adjusted to pH 7.5 with Tris. The extravesicular media contained 20 mM-Hepes supplemented with 40 mM organic acid and adjusted to pH 5.0 with Tris. The organic acids used were acetic acid ($\circ$), succinic acid ($\square$) and maleic acid ($\triangle$). Since statistically indistinguishable results were obtained with either malic acid or citric acid, the corresponding data were pooled to yield the upper curve ($\blacktriangle$). Except for the data obtained in the presence of acetic acid, all curves converged at 2 h to give identical equilibrium values (arrow). Absolute Cl^- uptakes are shown in $\text{nmol} \cdot \text{mg of protein}^{-1}$ ($n = 6$ except for the upper curve where $n = 15$). To simplify the Figure, the S.D. is shown only for the upper two curves; in any case, for most of the remaining points, the S.D. was smaller than the symbol.

Fig. 2. Time course of pH gradient-dependent Cl^- uptake: effect of the intravesicular buffering power

The uptake of 4 mM-Cl^{36} was determined by using vesicles equilibrated with 20 mM-Hepes supplemented ($\bullet$) or not supplemented ($\circ$) with 40 mM-citric acid; the pH was adjusted to 7.5 with Tris. The extra-vesicular media contained 20 mM-Hepes and 40 mM-citric acid, adjusted to pH 5.0 with Tris. In addition, potassium gluconate was used to obtain $[\text{K}^+]_{\text{out}}:[\text{K}^+]_{\text{in}}$ gradients of either 0:0 ($n = 6$) or 80:0 mM ($n = 3$): the K^+ -containing series also received valinomycin to produce an inside-positive membrane potential. Because the results obtained in the presence or absence of a K^+ gradient and valinomycin were statistically indistinguishable, the two sets have been pooled to obtain the illustrated data points ($n = 9$ per point). The initial Cl^- entry rates were calculated from the influx during the first 2 s, which appears to be linear (broken lines). Other details were as described in Fig. 1 legend.

$0.94 \pm 0.09 \text{ nmol} \cdot \text{s}^{-1} \cdot \text{mg of protein}^{-1}$) when the vesicles had been preincubated with citrate (see Fig. 2). Such a result cannot be attributed to any differences in membrane potential due to the absence or presence of citrate gradients (out > in) because the uptakes were not affected by adding valinomycin in the presence of a superimposed K^+ gradient (out > in) which would create a membrane potential opposite to that presumably created by a citrate gradient. Because the effect of citrate can be extrapolated to time zero, it should not be attributed to the higher buffering capacity of citrate-treated vesicles, meaning that the 20 mM-Hepes provides a sufficiently large sink for the incoming protons.

Taken together, these results warrant the interpretation that the uphill Cl^- uptake driven by a pH gradient involves mainly an electrically silent Cl^-/H^+ symporter, a conclusion confirmed by evidence to be presented next.

Total Cl^- uptake involves negligible Cl^- uniporter activity

(a) Comparison of the effects of a pH gradient and an inside-positive membrane potential in the absence of a pH gradient. To quantify the contribution to total Cl^- uptake of a possible potential-sensitive conductance pathway, we studied the effect of valinomycin in the presence of a potassium gradient ($[\text{K}^+]_{\text{out}}:[\text{K}^+]_{\text{in}} = 80:0 \text{ mM}$), both with and without a pH gradient. Our results (Fig. 3, upper

Fig. 3. Uphill Cl^- transport requires the presence of an inside-alkaline pH gradient

The time course of 4 mM- Cl^{36} uptake was determined by using vesicles equilibrated with a mixture of: (1) 100 mM-Tris gluconate and (2) a 20 mM-Hepes/40 mM-citric acid buffer, adjusted to pH 7.5 with Tris. The extravesicular media were similar in composition except that (1) the Tris gluconate was substituted as needed by either sodium or potassium gluconate to give the alkali-metal ion gradients mentioned below and (2) the buffer was adjusted with Tris either to pH 7.5 (lower two curves) or to pH 5 (upper curve). In the upper curve, in the presence of a pH gradient ($\text{pH}_{\text{out}}:\text{pH}_{\text{in}} = 5.0:7.5$), statistically identical results ($n = 3-6$ determinations per condition, pooled under the symbol $\triangle$) were obtained in the presence and absence of either Na^+ or K^+ at concentration ratios (out:in) of 80:0, 40:0 or 0:0 mM, independent of the presence or absence of valinomycin ($20 \mu\text{g}/\text{mg}$ of protein). For the lower two curves, in the absence of a pH gradient ($\text{pH}_{\text{out}}:\text{pH}_{\text{in}} = 7.5:7.5$), a favourable K^+ gradient ($[\text{K}^+]_{\text{out}}:[\text{K}^+]_{\text{in}} = 80:0 \text{ mM}$) was imposed in the presence ($\blacksquare$) or in the absence ($\square$) of valinomycin ($20 \mu\text{g}/\text{mg}$ of protein). Further details in the text.

curve) show that, in the presence of both a pH gradient ($\text{pH}_{\text{out}}:\text{pH}_{\text{in}} = 5.0:7.5$) and a K^+ gradient, valinomycin does not affect Cl^- uptake significantly (initial rate = $0.93 \pm 0.07 \text{ nmol} \cdot \text{s}^{-1} \cdot \text{mg of protein}^{-1}$ and $r_{60} = 5.4$, independent of the presence or absence of valinomycin), as should be expected if the overshoots supported by the pH gradient involve mainly an electroneutral mechanism.

In the presence of an inward-directed K^+ gradient, but in the absence of a pH gradient ($\text{pH}_{\text{out}}:\text{pH}_{\text{in}} = 7.5:7.5$; lower two curves in Fig. 3), valinomycin increased significantly, but only slightly, the initial Cl^- entry rate (from 0.18 ± 0.02 to $0.24 \pm 0.02 \text{ nmol} \cdot \text{s}^{-1} \cdot \text{mg of protein}^{-1}$). The activity of the rheogenic Cl^- uniport demonstrated here (given by the difference of uptake in the presence and in the absence of valinomycin) represents less than 10% of the total initial uptake rate induced by the pH gradient. Moreover, we have never observed any Cl^- overshoots in the presence of an inside-positive electrical gradient when a pH gradient was absent. Quantitatively similar results (not shown) were found at pH 6.5 ($\text{pH}_{\text{out}} = \text{pH}_{\text{in}}$).

(b) Absence of effect of extravesicular alkali-metal ions.

Because the Cl^- overshoot illustrated in Fig. 3 was obtained in the presence of both H^+ and K^+ gradients, we investigated next the effects of modifying the alkali-metal ion gradient. Our findings (Table 1) show that identical results were obtained: (i) in the complete absence of alkali-metal ions; (ii) in the presence of either Na^+ or K^+ when present in the form of an inward-directed gradient (namely, at $[\text{cation}]_{\text{out}}:[\text{cation}]_{\text{in}}$ ratios of either 80:0 or 40:0), both with and without valinomycin; and (iii) when, only outside the vesicles, equal concentrations of each Na^+ and K^+ were present. When the K^+ gradient was increased further to $[\text{K}^+]_{\text{out}}:[\text{K}^+]_{\text{in}} = 200:0 \text{ mM}$, valinomycin caused a statistically significant increase in total Cl^- uptake. However, this increase was small (about 15%), which supports our conclusion that rheogenic Cl^- influx contributes very little to the total Cl^- uptakes observed in the presence of a pH gradient.

Taken together, these results indicate the absence of any Cl^-/Na^+ , Cl^-/K^+ and/or $\text{Cl}^-/\text{Na}^+/\text{K}^+$ symporter activity in our guinea pig ileal brush border membrane vesicle preparations. Similar conclusions have been reached by others working with brush border membrane vesicles from rabbit and necturus kidney (Warnock & Yee, 1981; Seifter *et al.*, 1984; Seifter & Aronson, 1984) or rabbit and rat small intestine (Liedtke & Hopfer, 1982a; Knickelbein *et al.*, 1985).

(c) Inhibitory effect of intravesicular K^+ . To quantify further the relative contribution of a rheogenic pathway to the total Cl^- uptake observed in the presence of a pH gradient, we short-circuited any possible electrical gradient by adding valinomycin in the presence of equilibrated K^+ concentrations. To ensure that true short-circuiting was achieved, these experiments were performed at high K^+ concentrations (Ives *et al.*, 1986; Supplisson & Alvarado, 1986). This work led to the unexpected observation that Cl^- uptake is inhibited by the presence of moderately high (at least 20 mM) intravesicular alkali-metal ion concentrations. For instance (compare the upper two curves in Fig. 4), when the vesicles contained 100 mM of either K^+ or Na^+ , both the initial Cl^- entry rate and the size of the overshoot caused

Table 1. Absence of effect of *cis*-Na⁺ and/or -K⁺ on pH gradient-dependent Cl⁻ uptake in the presence and absence of valinomycin and/or CCCP

Both the extra- and the intra-vesicular spaces contained: (1) the 20 mM-Hepes/40 mM-citric acid/Tris buffer giving a pH gradient (pH_{out}:pH_{in}) = 5.0:7.5; and (2) 100 mM of the gluconate salt of either Tris, Na, K or appropriate mixtures thereof, yielding the indicated alkali-metal ion concentration ratios. Valinomycin (Val) and/or CCCP were both at 10 µg/mg of protein. The initial Cl⁻ entry rate, *v*, is expressed in nmol·s⁻¹·mg of protein⁻¹; means ± s.d. (*n*); *r*₂₀ is the 20 s/equilibrium ³⁶Cl concentration ratio.

Alkali-metal ion	[Out]/[in] ratios (mM)	Val	CCCP	<i>v</i>	<i>r</i> ₂₀
None		—	—		
Na ⁺	80:0 or 40:0	—	—	1.95 ± 0.33 (18)	5.4
K ⁺	80:0 or 40:0	±	—		
Na ⁺ + K ⁺	(40 + 40):0	±	—		
Na ⁺ , K ⁺ or none	80:0 or 0:0	—	+	0.40 ± 0.06 (6)	3.0
Na ⁺ or K ⁺	100:100 or 20:100	—	—	1.23 ± 0.31 (15)	2.7
Na ⁺ + K ⁺	(50 + 50):(50 + 50)				
K ⁺	100:100 or 20:100				
Na ⁺ or K ⁺	100:100	±	+	0.27 ± 0.06 (15)	1.04

Fig. 4. Effect of CCCP on pH gradient-dependent Cl⁻ uptake in the presence and absence of *trans*-K⁺

The composition of the buffers was identical to that of the upper curve in Fig. 3, with a pH gradient pH_{out}:pH_{in} = 5.0:7.5. The cation accompanying the 100 mM-gluconate of both media was either Tris (Δ, ▲) or K⁺ (□, ■) to yield concentration ratios [K⁺]_{out}: [K⁺]_{in} of 0:0 or 100:100 mM respectively. For the lower two curves (▲, ■), CCCP was present (10 µg/mg of protein).

by a pH gradient dropped significantly. This inhibitory effect of *trans*-alkali-metal ions was not dependent on the existence of a gradient, since identical results were found at concentration ratios ([cation]_{out}: [cation]_{in}) of either 100:100 or 20:100 mM (see also Table 1).

Adding valinomycin in the presence of equilibrated K⁺ ([K⁺]_{out}/[K⁺]_{in} = 100:100 mM) had a small (10%), statistically insignificant inhibitory effect on the initial influx rate, a result fully supporting the tenet that only a small portion of the total Cl⁻ influx detected in our experiments involves a conductance pathway. The overshoot exhibited a statistically significant drop in *r*₂₀ from 2.7 to 1.5 (Table 1).

(d) Effect of the protonophore CCCP. We next investigated whether a proton gradient, by inducing an

inside-positive membrane potential, favours Cl⁻ influx through a rheogenic uniporter (Seifter *et al.*, 1984). If this mechanism existed in our vesicles, the protonophore CCCP should increase both the initial Cl⁻ entry rate and the height of the overshoots (Shiuan & Weinstein, 1984; Liedtke & Hopfer, 1977). Instead, we found that CCCP drastically inhibits the initial Cl⁻ influx, independent of the presence or absence of intravesicular K⁺ (80% inhibition under either condition; Fig. 4). This result would rule out the existence of a rheogenic uniporter, suggesting instead that CCCP acts by facilitating dissipation of the H⁺ gradient that acts as the driving force for an electroneutral Cl⁻/H⁺ symport. However, the alternative explanation cannot be excluded at present that CCCP inhibits Cl⁻ uptake directly (Liedtke & Hopfer, 1977). As Cl⁻ uptake and the effect of CCCP are both very rapid, other techniques (e.g. stopped-flow analysis) will have to be used to settle this question definitely.

In the absence of *trans*-K⁺, CCCP decreased the height of the overshoot by 44%, but did not abolish it completely (*r*₂₀ = 3.0, Table 1). The overshoot was fully abolished (*r*₂₀ = 1.04) only in the presence of CCCP plus 100 mM-*trans*-K⁺, independent of the presence or absence of valinomycin. It is unclear why: (1) CCCP appears not to abolish fully the pH gradient acting as the driving force for Cl⁻ uphill transport, and (2) the effects of CCCP and intravesicular K⁺ are additive. Nevertheless, preliminary observations with either ³⁶Cl (Vasseur *et al.*, 1987) or the fluorescent pH monitor pyranine (M. Vasseur, R. Frangne & F. Alvarado, unpublished work), indicate that intravesicular K⁺ facilitates pH gradient dissipation. Because they involve different mechanisms, the pH gradient-dissipating actions of CCCP and K⁺ can indeed be expected to be additive.

Unspecific Cl⁻/anion antiporter activity

The work so far presented (Figs. 1–4) shows that pH gradient-dependent Cl⁻ overshoots can be obtained in the complete absence of *trans*-Cl⁻ and/or -HCO₃⁻, indicating that obligatory Cl⁻/anion exchange plays no role in the electroneutral Cl⁻/OH⁻ antiporter under

study here. Nevertheless, we undertook additional experiments to distinguish between a truly specific Cl^-/OH^- antiporter and a possible, less specific Cl^-/anion antiporter, notably, one that could use either trans-Cl^- or $-\text{HCO}_3^-$ as substrates.

Fig. 5. Time course of Cl^- uptake in the presence and absence of pH- and/or Cl^- gradients

The uptake of 9 mM-Cl^{36} was determined by using vesicles equilibrated with (1) 50 mM of the potassium salt of either gluconate or Cl^- and (2) $20\text{ mM-Hepes}/40\text{ mM-citric acid}$ buffer, adjusted to $\text{pH } 7.5$ with Tris. The pH of the extravesicular medium was adjusted with Tris to obtain pH gradients ($\text{pH}_{\text{out}}:\text{pH}_{\text{in}}$) of $5.0:7.5$ ($\square, \blacksquare$), $6.5:7.5$ ($\triangle, \blacktriangle$) or $7.5:7.5$ ($\diamond, \blacklozenge$). At time zero, the total $[\text{Cl}^-]_{\text{out}}:[\text{Cl}^-]_{\text{in}}$ concentration ratios were either $9:0$ ($\square, \triangle, \diamond$) or $9:50$ ($\blacksquare, \blacktriangle, \blacklozenge$) mM ; for all points, $[\text{K}^+]_{\text{out}} = [\text{K}^+]_{\text{in}} = 50\text{ mM}$. In (b), the first 6 s are expanded to illustrate the initial Cl^- entry rates (first $1\text{--}2\text{ s}$). Because the results in presence of a pH gradient (upper four curves) are not affected by the presence of trans-Cl^- , they have been pooled to give the two upper (discontinued) straight lines.

(a) **The effect of trans-Cl^- .** The time course of pH gradient-dependent Cl^- uptake was studied at two different values of pH_{out} , in the presence and in the absence of 50 mM-trans-Cl^- (upper four curves, Fig. 5). The initial Cl^- entry rates were not significantly affected by the presence of trans-Cl^- (Fig. 5b), confirming the absence of obligatory Cl^-/Cl^- antiporter activity in our vesicle preparations. With other vesicle lots, 50 mM-trans-Cl^- was found to induce small but significant increases in the initial Cl^- influx rates. However, such positive results (e.g. 40% ; see Table 4) are quantitatively negligible when compared with the 400% increases constantly observed under pH gradient conditions, independent of the presence of trans-Cl^- (see below for further details on this point).

In contrast with its negligible effect on initial influx rates, trans-Cl^- caused the height of the overshoots to increase significantly (Fig. 5a), a time-dependent effect that can be interpreted most simply in terms of the inhibition of ^{36}Cl efflux by cold intravesicular Cl^- (see, for instance, Wright & Wunz, 1988). This conclusion is strengthened by additional observations (Table 2) indicating that: (1) net (total) Cl^- uptake increases as pH decreases, as is also true for the initial entry rates (Fig. 5b); (2) Cl^- uphill transport occurs in the presence of a pH gradient and is proportional to ΔpH ; and (3) trans-Cl^- induces the absolute Cl^- uptake to increase, and this effect is larger as ΔpH diminishes. These last results support particularly well our interpretation that cold intravesicular Cl^- inhibits ^{36}Cl efflux competitively. The effects are greater as pH increases because, at each given time, the concentration of the inhibited species, intravesicular ^{36}Cl , is smaller.

In sharp contrast with the above results, trans-Cl^- under pH-equilibrated conditions ($\text{pH}_{\text{out}} = \text{pH}_{\text{in}} = 7.5$, lower two curves in Fig. 5) has an activating effect on the initial rate of Cl^- influx and induces a small but quantitatively significant overshoot ($r_{60} = 1.2$, Table 2). Taken at face value, this result would appear to indicate the existence of a Cl^-/Cl^- antiport mechanism, but this interpretation has the disadvantage of failing to explain why such an antiport becomes evident only when a pH gradient is absent.

An alternative interpretation of the entire set of results in Fig. 5 is suggested by recent work from our laboratory (Vasseur *et al.*, 1989b). With our vesicles, a Cl^- gradient ($\text{out} < \text{in}$) should cause net proton efflux through the Cl^-/H^+ symport that is probably symmetrical or at least reversible (Vasseur *et al.*, 1989a). This means that, under

Table 2. pH gradient-dependent Cl^- uptake: effect of trans-Cl^- on the absolute Cl^- uptakes at 60 s

Both the extra- and the intra-vesicular spaces contained: (1) the $20\text{ mM-Hepes}/40\text{ mM-citric acid}/\text{Tris}$ buffer giving the indicated pH gradients; and (2) 50 mM of the potassium salt of either gluconate or Cl^- to yield the $[\text{Cl}^-]_{\text{out}}:[\text{Cl}^-]_{\text{in}}$ concentration ratios shown. Cl^- uptakes are expressed in $\text{nmol} \cdot 60\text{ s}^{-1} \cdot \text{mg}$ of protein $^{-1}$; means $\pm$ S.D. (n).

$[\text{Cl}^-]_{\text{out}}:[\text{Cl}^-]_{\text{in}}$ (mM)		$\text{pH}_{\text{out}}:\text{pH}_{\text{in}}$		
		5.0:7.5	6.5:7.5	7.5:7.5
9:0	Uptake	11.84 ± 0.64 (3)	4.37 ± 0.94 (10)	1.73 ± 0.18 (20)
	r_{60}	4.4	1.62	0.60
9:50	Uptake	16.53 ± 0.09 (3)	8.12 ± 0.65 (10)	3.29 ± 0.36 (15)
	r_{60}	6.12	3.00	1.24

Table 3. pH gradient-dependent Cl⁻ uptake: effect of either *trans*-HCO₃⁻ or *trans*-Cl⁻ on the initial Cl⁻ entry rates

The vesicles were pre-equilibrated with either (1) 50 mM of the potassium salt of either gluconate or HCO₃⁻ to yield the indicated [HCO₃⁻]_{out}: [HCO₃⁻]_{in} concentration ratios or (2) 50 mM of the potassium salt of either gluconate or Cl⁻ to yield the indicated (total) [Cl⁻]_{out}: [Cl⁻]_{in} concentration ratios. Both the extra- and the intra-vesicular spaces contained 50 mM-K⁺ and the 20 mM-Hepes/40 mM-citric acid buffer adjusted with Tris to give a pH gradient (pH_{out}:pH_{in}) = 5.0:7.5. Prior to transport assay, the vesicles (in 150–200 μl aliquots) were bubbled for 30 min with the indicated water-saturated gasses. Values for initial ³⁶Cl entry rate are means ± S.D.; n = 3.

Gassing	[Cl ⁻] _{out} : [Cl ⁻] _{in} (mM) ... [HCO ₃ ⁻] _{out} : [HCO ₃ ⁻] _{in} (mM) ...	³⁶ Cl ⁻ initial entry rate (nmol · s ⁻¹ · mg of protein ⁻¹)			
		4:0 0.2:0	4:0 0.2:47	9:0 0:0	9:50 0:0
100 % O ₂		0.32 ± 0.04	0.35 ± 0.05	—	—
100 % N ₂		0.27 ± 0.02	0.30 ± 0.04	—	—
5 % CO ₂ + 95 % O ₂		0.23 ± 0.04	0.29 ± 0.03	—	—
No gassing		0.36 ± 0.01	0.43 ± 0.03	0.44 ± 0.03	0.58 ± 0.02

the pH-equilibrated conditions of the lower two curves in Fig. 5, the 9/50 chloride gradient should lead to the formation of an inside-alkaline pH gradient, capable of driving ³⁶Cl influx uphill. Under pH gradient conditions (upper four curves), similar intravesicular alkalization may be expected to occur, but its effect on ³⁶Cl influx would be hard to detect because it is working in the same direction as the imposed proton gradients. Indeed, the small activations mentioned above induced by a Cl⁻ gradient in the presence of a pH gradient (e.g. 40 %; see Table 4) can be explained simply in this way.

(b) The effect of *trans*-HCO₃⁻. Cl⁻/HCO₃⁻ antiporter activity was studied with vesicles exposed simultaneously to a pH_{out}:pH_{in} = 5.0:7.5 gradient and the HCO₃⁻ gradients shown in Table 3. The necessity of gassing with CO₂-containing gas mixtures was investigated by comparing the transport behaviour of non-gassed vesicles and those gassed with 100 % N₂, 100 % O₂ or a mixture of 95 % O₂ plus 5 % CO₂. The results reveal that the effect of *trans*-HCO₃⁻ on the initial rate of pH gradient-dependent Cl⁻ uptake is negligible (increases of only 11–26 %), slightly lower than that elicited by *trans*-Cl⁻ (30 %), which was used as a reference with the same vesicle lot. Given the considerably larger [in]:[out] HCO₃⁻ gradients used (227-fold as compared with 5.6-fold for Cl⁻), the conclusion is warranted that our vesicle preparations possess no significant Cl⁻/HCO₃⁻ antiporter activity.

As we explain below, the results in Table 3 permit making the additional conclusion that, because of our using a strong buffer mixture in our experiments, gassing with CO₂ can be omitted altogether. One important advantage of omitting such gassing is practical: in spite of the fact that the gases were water-saturated, the small size of the vesicle preparations used determined that considerable evaporation of water occurred during gassing. Although this source of error was compensated for by determining the final protein concentration of the vesicles, an inordinate fluctuation in the control uptake results was impossible to avoid.

To test the effect of gassing, the 20 mM-Hepes/40 mM-citrate buffer (adjusted to pH 7.5 with Tris) was brought to pH 8.0 with KOH and supplemented with the potassium salt of either HCO₃⁻ or gluconate before being

titrated with HCl. Compared with gluconate and independent of gassing, bicarbonate increased the buffer capacity (in the 7.0–7.5 pH range) from 42.0 to 55.7 μmol of H⁺ · ml⁻¹ · pH unit⁻¹, but the starting pH was unaffected by either of these salts. Moreover, buffer gassing under the conditions listed in Table 3 did not significantly modify the shape of the titration curves in the 8.0–6.5 pH range. It is only below pH 6.5 that a progressive increase in total buffer capacity of the HCO₃⁻-containing buffers when gassed with 5 % CO₂ was observed. Our conclusion is that, although pCO₂ is a key pH-determining factor for buffers formed only of bicarbonate, gassing with CO₂ might be neglected when the main component of a mixture is another buffer having a suitable pK, as is the case for the Hepes/Tris used in the present study.

(c) A comment on the absence of effect of HCO₃⁻ on an electroneutral Cl⁻/OH⁻ antiporter. Unequivocally establishing whether or not a Cl⁻/HCO₃⁻ antiporter plays a role on pH gradient-dependent, electroneutral uphill transport of Cl⁻ in the ileum is a question of paramount importance to the present work. Cl⁻/HCO₃⁻ antiporter activity is known to occur in the red blood cell where it plays a key role in blood-to-tissue CO₂ exchange (for a review, see Lowe & Lambert, 1983). In contrast, perusal of the literature reveals that direct evidence for the existence of a similar Cl⁻/HCO₃⁻ antiporter mechanism in the ileum is very scarce. It appears to depend mainly (see p. 58 in Hopfer & Liedtke, 1987) on work of Aronson and colleagues purporting to show that, in rabbit ileum, 'Cl-HCO₃ exchange occurs more readily than Cl-OH exchange or H-Cl cotransport' (Knickelbein *et al.*, 1985). Closer examination of this particular piece of work, however, provides reasons for questioning the conclusion reached by these workers. From the key rabbit experiment (Fig. 1 of Knickelbein *et al.*, 1985) the following can be deduced. (1) A 158-fold proton gradient can induce a 400 % increase in the total Cl⁻ uptake at 15 s, but this stimulation is independent of the absence or presence of *trans*-HCO₃⁻. (2) Independent of the pH gradient, a HCO₃⁻ gradient ([HCO₃⁻]_{in}: [HCO₃⁻]_{out}) of either 175 (pH_{out}:pH_{in} = 5.5:7.7) or 10 (pH_{out}:pH_{in} = 7.7) increases total Cl⁻ uptake by about 200 % at 15 s (100 % at 60 s). We emphasize, however, that the shortest time used in these experiments was 15 s, too long to

provide a measure of the key parameter, the initial rate of Cl^- influx. (3) Even more significantly, in the absence of a pH gradient, a HCO_3^- gradient of 10 failed to induce any uphill transport of Cl^- , a result in discord with the conclusion that Cl^- transport in the rabbit ileum involves a $\text{Cl}^-/\text{HCO}_3^-$ antiporter.

In order to eliminate differences between animal species as a source of uncertainty, which would weaken the conclusions reached in this article, we have repeated the key guinea pig HCO_3^- experiments by using brush border membranes from rabbit ileum. Our results (not shown) reveal no qualitative differences with those described above for the guinea pig, and agree well with those of Knickelbein *et al.* (1985), summarized in the preceding paragraph. In particular, our rabbit results confirm that, in the absence of a pH gradient, a 10-fold bicarbonate gradient induces only a neglectably small ($r_{60} = 1.03$) Cl^- overshoot. Moreover, in contrast with Knickelbein *et al.* (1985), our work includes measurement of the initial rate of Cl^- influx (at 2 s). This initial rate is increased only slightly (about 40%) by *trans*- HCO_3^- .

Because of the reasons already explained, therefore, we conclude that the Cl^-/OH^- antiporter in the ileal brush border membrane of both guinea pigs and rabbits is specific and involves no quantitatively significant $\text{Cl}^-/\text{HCO}_3^-$ antiporter activity.

The effect of SITS

SITS is thought to act as a specific inhibitor of electroneutral Cl^- transport systems such as the anion/anion antiporter and Cl^-/H^+ symporter (Liedtke & Hopfer, 1982b; Shiuan & Weinstein, 1984; Knickelbein *et al.*, 1985; Hoffmann, 1986; Hopfer & Liedtke, 1987), although the information available in the literature is often contradictory. For instance, according to Aronson and co-workers, SITS inhibits electroneutral, not electrogenic, Cl^- uptake in the ileum (Knickelbein *et al.*, 1985) but has the opposite effect on renal brush border membranes (Seifter *et al.*, 1984). With our guinea pig brush border membrane vesicle preparations (Fig. 6), 5 mM-SITS has no effect on the small, rheogenic component of Cl^- uptake but inhibits pH gradient-dependent uptake. To clarify the mechanism of inhibition by SITS, we performed uptake experiments with equilibrated K^+ (with and without valinomycin), both in the presence and in the absence of *trans*- Cl^- . The results (Table 4) show that 5 mM-SITS inhibits the initial Cl^- entry rate by 58%, independent of either (1) the presence or absence of *trans*- Cl^- ; and (2) whether the membrane potential has been short-circuited with valinomycin. In addition, these results confirm that *trans*- Cl^- increases ^{36}Cl uptake only slightly (45% in this particular experiment), whereas short-circuiting the membrane potential with equilibrated K^+ and valinomycin causes a 20% inhibition, agreeing with the tenet that only a small fraction of the total Cl^- influx in the presence of a pH gradient involves either a Cl^- /anion antiporter or a rheogenic uniporter.

By using a spectrofluorimetric technique, Ives *et al.* (1986) have recently shown that, at 1–5 mM concentrations, SITS behaves as a protonophore and therefore may inhibit pH gradient-dependent transport by accelerating the spontaneous collapse of existing pH gradients. Our SITS results, which are reminiscent of those obtained with the protonophore CCCP (Fig. 4), can, most probably, be explained in identical terms. Nevertheless, the available information is insufficient to

Fig. 6. Effect of SITS on pH gradient-dependent Cl^- transport

The time course of zero-*trans*, 4 mM- Cl^{36} uptake was determined with vesicles containing 100 mM-Tris gluconate and the 20 mM-Hepes/40 mM-citric acid buffer, adjusted to pH 7.5 with Tris. The extravesicular media contained 20 mM-Tris gluconate plus 80 mM-potassium gluconate and the Hepes/citrate buffer adjusted either to pH 5.0 (Δ , $\blacktriangle$) or to pH 7.5 ($\square$, $\blacksquare$). When present, 5 mM-SITS ($\blacksquare$, $\blacktriangle$) was added both to the vesicles and to the extravesicular media.

Table 4. pH gradient-dependent Cl^- uptake: effect of *trans*- Cl^- and SITS on the initial Cl^- entry rates

Both the extra- and the intra-vesicular spaces contained: (1) the 20 mM-Hepes/40 mM-citric acid buffer adjusted with Tris to give a pH gradient ($\text{pH}_{\text{out}}:\text{pH}_{\text{in}} = 5.0:7.5$); (2) a 50 mM mixture of the potassium salts of either gluconate or Cl^- to give the indicated (total) $[\text{Cl}]_{\text{out}}:[\text{Cl}]_{\text{in}}$ concentration ratios; and (3) SITS as indicated. The initial ^{36}Cl entry rate is expressed in $\text{nmol} \cdot \text{s}^{-1} \cdot \text{mg}$ of protein $^{-1}$; means $\pm$ S.D. (*n*). In all cases, $[\text{K}^+]_{\text{out}} = [\text{K}^+]_{\text{in}}$.

$[\text{Cl}]_{\text{out}}:[\text{Cl}]_{\text{in}}$ (mM)	Valino- mycin	Cl ⁻ entry rate (nmol · s ⁻¹ · mg of protein ⁻¹)	
		Control	5 mM-SITS
9:0	—	0.93 ± 0.21 (6)	0.40 ± 0.09 (7)
9:50	—	1.30 ± 0.12 (7)	0.57 ± 0.09 (6)
9:0	+	0.77 ± 0.14 (3)	0.30 ± 0.02 (3)
9:50	+	1.09 ± 0.03 (3)	0.47 ± 0.04 (3)

exclude the possibility of a direct effect of SITS on Cl^- uptake. As in the case of CCCP, a clear-cut solution of this question would require using more rapid techniques.

Effect of *cis* anions on pH gradient-dependent Cl^- uptake

To test the hypothesis that the time-dependent activation of pH gradient-dependent Cl^- uptake by *trans* anions is due to an inhibition of ^{36}Cl efflux, saturation and competition experiments were performed. We used *cis* anions, based on the assumption that, at least semiquantitatively, the Cl^-/H^+ symport is symmetrical and therefore competition should occur similarly from either side of the membrane.

As expected (Fig. 7), *cis* anions inhibited initial Cl^- uptake according to hyperbolic kinetics, indicating com-

Fig. 7. pH gradient-dependent Cl^- uptake: inhibition by *cis*-anions

The 2-s, initial rate of uptake of zero-*trans*, 4 mM- ^{36}Cl , was determined by using vesicles equilibrated with 100 mM-Tris gluconate and the 20 mM-Hepes/40 mM-citrate buffer adjusted to pH 7.5 with Tris. The extravesicular media contained the same buffer adjusted to pH 5.0 with Tris, plus 100 mM of the potassium salt of either gluconate (control, v_0), or appropriate mixtures of this salt with either KCl ($\blacktriangle$) or KBr ($\blacklozenge$) to yield the indicated inhibitor concentrations. (a) The uncorrected, initial velocity data are plotted as a function of the inhibitor concentration. The solid lines represent the theoretical fits calculated by assuming fully competitive inhibition and the kinetic constants mentioned in the text. In the insert, the same data are plotted according to the Inui & Christensen (1966) transformation. (b) The Cl^- data, recalculated as explained in the text, are plotted as a function of substrate concentration. The solid lines represent the theoretical fits calculated by non-linear regression analysis, whereby the total uptake (upper curve) is decomposed into a saturable component (middle curve) plus diffusion (lower, straight line).

petition for (external) Cl^- binding site(s). A preliminary analysis of the specificity of this inhibition yielded the affinity sequence $\text{SCN}^- > \text{NO}_3^- > \text{I}^- > \text{Br}^- > \text{Cl}^- > \text{maleate} \gg \text{gluconate}$ (HCO_3^- was not tested because of the practical impossibility of using this anion at pH 5). Ignoring for the moment the possibly complex effects of the lipophilic anions at the beginning of this list, the following will focus only on the Cl^- and Br^- results.

First, linearization of these data (Fig. 7a) according to the Inui & Christensen (1966) transformation permits calculation of the fraction of Cl^- uptake (9% of the total) that is not inhibitable by excess anions and which represents Cl^- uptake due only to diffusion (Alvarado, 1970). From the y-axis intercept where the Cl^-

and Br^- lines converge (insert, Fig. 7a), we have calculated a Cl^- permeability coefficient or kinetic diffusion constant, K_{ds} , which, in relative uptake units, gives: $K_{ds} = 11.5 \text{ nl} \cdot \text{s}^{-1} \cdot \text{mg of protein}^{-1}$. Secondly, by assuming Cl^- to be the substrate (specific activity variable) and the inhibitor concentration to be nil, the Cl^- data indicate Cl^- uptake to be a saturable function of its own concentration (Fig. 7b). Non-linear regression analysis of these data according to the premise that total uptake is the sum of a hyperbolic (saturable) Michaelian component plus a linear, diffusive one (van Melle & Robinson, 1981), gave the following results: $V = 2.0 \pm 0.8 \text{ nmol} \cdot \text{s}^{-1} \cdot \text{mg of protein}^{-1}$; $K_m = 12.9 \pm 6.4 \text{ mM}$ and $K_{ds} = 13.3 \pm 9.0 \text{ nl} \cdot \text{s}^{-1} \cdot \text{mg of protein}^{-1}$. The agreement between this K_{ds} value and that derived from the inhibition experiments sustains the validity of the analysis. Thirdly, by multiplying K_{ds} by the substrate concentration (4 mM), the initial Cl^- influx rate due only to diffusion was calculated. At pH 5, it varied between 0.05 and $0.11 \text{ nmol} \cdot \text{s}^{-1} \cdot \text{mg of protein}^{-1}$, a value that is of the same order of magnitude as the Cl^- entry rate observed in the absence of a pH gradient at either pH 7.5 or pH 6.5 ($0.06\text{--}0.18 \text{ nmol} \cdot \text{s}^{-1} \cdot \text{mg of protein}^{-1}$; data derived from Fig. 3 and other, unpublished, results). Finally, by applying the estimated values of V and K_{ds} , the inhibition data in Fig. 7 were recalculated according to the $V/v_c = f$ ([inhibitor]) transformation (Thorn, 1953), where v_c is the initial velocity, v , corrected for the diffusion component ($v_c = v - K_{ds} \cdot [S]$). The data did fit two straight lines, from where inhibitory constants were calculated for both Br^- ($K_i = 5.8 \text{ mM}$) and Cl^- ($K_i = 14.0 \text{ mM}$). In further support of the validity of the analysis, the K_i for Cl^- is practically identical with the K_m for Cl^- of 12.9 mM estimated by iteration from the saturation curve in Fig. 7(b).

A mechanistic interpretation of the indirect exchange of NaCl for NaHCO_3 in the ileum: application to the case of chloridorrhea

Following the pioneering work of Turnberg and colleagues (Turnberg, 1971; Bieberdorf *et al.*, 1972), the hypothesis has acquired widespread acceptance that the ileum and colon possess a $\text{Cl}^-/\text{HCO}_3^-$ antiporter or direct anion-exchange mechanism whose malfunctioning may explain the two key symptoms of the genetic disease chloridorrhea: loss of Cl^- in the stools, and alkalosis. This hypothesis, however, is not supported by our present work, showing that *trans*- HCO_3^- plays no direct role in pH gradient-dependent Cl^- transport across the brush border membrane of the ileum.

If an alternative to Turnberg's hypothesis is desirable, our results suggest the following one. Physiologically, the Cl^-/H^+ symporter in the apical border of the ileum contributes to the overall acid-base balance by catalysing a net absorption of HCl in indirect exchange for NaHCO_3 . The following cycle can be envisaged (Fig. 8). (1) The main salt input to the ileum is NaCl. (2) The Cl^- ion is taken up together with one proton by means of the Cl^-/H^+ symporter, possibly favoured by the low pH prevailing at the external face of the brush border membrane (Lucas *et al.*, 1975; Shiau *et al.*, 1985). (3) The incoming H^+ combines with intracellular HCO_3^- to give carbonic acid which, catalysed by the cytoplasmic carbonic anhydrase, gives CO_2 and water, both of which can readily cross the brush border membrane. (4) Catalysed by the extracellular carbonic anhydrase (Wistrand, 1984;

Fig. 8. A model to explain the indirect exchange of luminal Cl^- for bicarbonate

This model is based on the brush border Cl^-/H^+ symport system described in the present paper. For an explanation, see the text.

Hopfer & Liedtke, 1987), H^+ and HCO_3^- are regenerated at the external face of the brush border membrane. (5) The HCO_3^- is excreted in the form of NaHCO_3 , the main ileal output. (6) The H^+ left behind closes the cycle.

Based on this cycle, chloridorrhea can be understood simply as involving a molecular lesion of the Cl^-/H^+ symporter. On top of the loss of Cl^- , this situation would lead directly to the net loss of H^+ and, indirectly, to HCO_3^- retention. We are aware that, based on the same cycle, chloridorrhea could also be interpreted as involving other, different molecular lesions, notably, a diminution in either intra- or extracellular carbonic anhydrase activity. However, the discussion of such alternatives exceeds the scope of the present paper and will be dealt with elsewhere.

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