J. Membrane Biol. 92, 75–89 (1986)

The Journal of
Membrane Biology

Modes of Cl^- Transport across the Mucosal and Serosal Membranes of Urodele Intestinal Cells

John F. White
Department of Physiology, Emory University, Atlanta, Georgia 30322

Summary. The characteristics of Cl^- movement across luminal and basolateral membranes of *Amphiuma* intestinal absorptive cells were studied using Cl^--sensitive microelectrodes and tracer ^{36}Cl techniques. Intracellular Cl^- activity (a^i_{Cl}) was unchanged when serosal Cl^- was replaced; when luminal Cl^- was replaced cell Cl^- was rapidly lost. Accordingly, the steady state a^i_{Cl} could be varied by changing the luminal [Cl]. As luminal [Cl] was raised from 1 to 86 mM, a^i_{Cl} rose in a linear manner, the mucosal membrane hyperpolarized, and the transepithelial voltage became serosa negative. In contrast, the rate of Cl^- transport from the cell into the serosal medium, measured as the SITS-inhibitable portion of the Cl^- absorptive flux, attained a maximum when a^i_{Cl} reached an apparent value of 17 mM, indicating the presence of a saturable, serosal transport step. The stilbene-insensitive absorptive flux was linear with luminal [Cl], suggestive of a paracellular route of movement. Intracellular a_{Cl} was near electrochemical equilibrium at all but the lowest values of luminal [Cl] after interference produced by other anions was taken into account. a^i_{Cl} was unaffected by Na replacement, removal of medium K, or elevation of medium HCO_3^-. Mucosae labeled with ^{36}Cl lost isotope into both luminal and serosal media at the same rate and from compartments of equal capacity. Lowering luminal [Cl] or addition of theophylline enhanced luminal Cl^- efflux. It is concluded that a conductive Cl leak pathway is present in the luminal membrane. Serosal transfer is by a saturable, stilbene-inhibitable pathway. Luminal Cl^- entry appears to be passive, but an electrogenic uptake cannot be discounted.

Key Words small intestine · Cl transport · *Amphiuma* · urodele · Cl^--sensitive microelectrode · SITS

Introduction

In a variety of animals Cl^- absorption by the small intestine is an active process. Transepithelial net transport requires an active transport step for Cl^- across at least one membrane. It is widely held that the active step occurs at the luminal membrane primarily because, when it has been measured with Cl-sensitive microelectrodes, the intracellular Cl^- activity (a^i_{Cl}) in the steady state, appears to be above the value expected for electrochemical equilibrium [1, 6, 31, 35, 37]. Four different modes of Cl^- uptake have been proposed for the luminal membrane. First, NaCl cotransport has been proposed for bullfrog [23], rabbit [22] and teleost small intestine [25]. According to this view, Cl^- uptake is coupled to the inward gradient for Na^+, i.e., a secondary active uptake of Cl^- [8]. Secondly, Cl^--HCO_3^- exchange operating in parallel with Na^+-H^+ exchange has been suggested [30]. Experimental evidence for both the cation and the anion exchange process has been reported in isolated brush borders of rat small intestine [19, 20]. NaCl cotransport, as such, was not demonstrable in brush border vesicles prepared from rat intestine [18]. Third, uptake of Cl^- linked to both Na and K has been proposed for flounder intestine [21]. Fourth, a primary, electrogenic Cl^- uptake has been proposed for *Amphiuma* small intestine, a preparation in which Cl^- absorption is linked to the secretion of bicarbonate as in human [30], rat [12] and canine [16] small intestine. Electrogenic uptake was proposed because net Cl^- transport is electrogenic, it is not accompanied by net Na^+ absorption [33], and the apparent accumulation of Cl^- across the luminal membrane to a value above electrochemical equilibrium did not require Na^+ in the luminal medium [35].

Less well studied is the serosal exit step for Cl^- from cytoplasm to blood. Since there appears to be a favorable electrochemical gradient for Cl^- movement from the cell into the serosal fluid, then Cl^- movement across the serosal membrane may be downhill if the conductance of the serosal membrane is sufficiently high [8]. This proposal has received little direct study. In contrast, in *Amphiuma*, Cl^- absorption requires HCO_3^- in the medium [31]; HCO_3^- in the serosal medium is sufficient to support Cl^- absorption [33]. Also, net Cl^- transport is completely blocked by stilbenes (e.g. SITS) added to the serosal medium [31]. More recently, it has been shown in separate studies that Na is required specifically in the serosal medium both for net Cl^- absorp-

tion [35] and for Cl^--dependent HCO_3^- secretion [36]. We have proposed that Cl^- exit is linked by cotransport to the entry of Na and HCO_3^- as reported for salamander renal tubule [4]. This is a secondary active transport process. The exchange is energetically feasible in urodele intestine [14] and provides an explanation for the inhibition of net Cl^- absorption by transported solutes which elevate intracellular Na activity [34]. Stilbene-sensitive anion exchange has been reported in rabbit ileum [17]. Also, stilbenes slowed SO_4^{2-} efflux from basolateral membrane vesicles prepared from rat and dog intestine, but did not block Cl^- efflux [9].

There are two pathways for Cl^- movement through the epithelium which can potentially counter the forces which absorb Cl^- into the blood. These are a paracellular leak pathway and a leak pathway in the luminal membrane. A high conductance for Cl^- in the paracellular path has been inferred from the high serosa-to-mucosa flux of labeled Cl^- under short-circuited conditions [28]. Unfortunately, it has not always been possible to rule out the involvement of the cellular pathway in this flux. A luminal leak path for Cl^- has been observed in brush border vesicles [19] and inferred from the effect on net Cl^- transport of exposing the tissue to actively transported solutes [34] or to agents that elevate cellular cyclic AMP [32]. A mucosal membrane leak will reduce the effectiveness of the luminal uptake mechanism in elevating a^i_{Cl}. Despite the obvious importance of this pathway on net Cl^- absorption, it has received little study. Clearly, both of the dissipative pathways as well as the uptake mechanism(s) may serve as points of control in the regulation of Cl^- absorption and water transport.

The present study was undertaken to define and quantitate the different pathways of Cl^- transport in the urodele intestine. Cl^- uptake across the luminal membrane appears to be passive; serosal transfer is carrier mediated.

Materials and Methods

Adult *Amphiuma* were maintained and anesthetized as described previously [35]. The duodenum was excised, stripped of its serosal smooth muscle layers and either opened as a sheet for measurement of intracellular Cl^- activity with microelectrodes or everted with the aid of a plexiglas rod for loading and washout of ^{36}Cl.

Bathing Media

The normal (control) bathing medium contained in mM: Na 105, Cl 86.3, K 2.5, HCO_3^- 25, Ca^{2+} 0.9, Mg^{2+} 1.0, and mannitol 20. Solutions were gassed with 95% O_2/5% CO_2. When the [Cl] was varied this was done by equimolar substitution with gluconate. This required further additions of calcium gluconate to the medium to maintain the [Ca^{2+}]. The [Cl] of the nominally Cl^- free medium, determined by the titrimetric method, was 0.11 and 0.13 meq/liter in two separate batches prepared on different days. In one series chloride and bicarbonate were substituted with gluconate. In this case the medium was buffered with 5 mM Tris (hydroxymethyl aminomethane) sulfate and gassed with oxygen. Sodium was substituted in one series with choline. When K^+ was removed no cation was substituted.

In one series Na^+ was replaced with lithium and Cl^- with sulfate. In this case the mannitol concentration was increased to 69.5 mM to maintain osmolarity. The osmolality of bathing media was routinely checked with a Wescor 5,100C vapor pressure osmometer (Logan, UT). All chemicals were reagent grade. SITS (4-acetamido-4′-isothiocyanostilbene-2-2′disulfonate) and theophylline were purchased from Sigma (St. Louis, MO).

Cl-Specific Microelectrodes

Double-barreled Cl^--sensitive microelectrodes (DBM) were prepared by the method reported previously [35]. The method of sensing and recording the voltage on each barrel of the microelectrode was identical to that described earlier [35]. The microelectrodes were calibrated in solution of 0.1, 1, 10, 20 and 100 mM NaCl. The slope (S) of the electrodes in the range from 10 to 100 mM, calculated from the regression line of voltage *vs.* the log of the Cl^- activity, was linear and averaged -54.4 ± 0.5 mV for 11 microelectrodes employed in one randomly selected series of calibrations. In the range from 1 to 10 mM NaCl the change in voltage averaged -47.1 ± 0.9 mV. The intracellular Cl^- activity ($a_{Cl}{}^i$) was calculated using Eq. (1):

$$a^i_{Cl} = a''_{Cl}/e^{2.303\,(v^i_{Cl}-v_m)-v''_{Cl}/S} \tag{1}$$

where S is the slope of the electrode in the appropriate range (1–10 or 10–100 mM NaCl) a''_{Cl} and v''_{Cl} are the chloride activity and voltage of the electrode in the highest NaCl calibrating standard used, and v^i_{Cl} is the voltage of the Cl barrel in the cell. The equilibrium potential for Cl^- (E_{Cl}) at the mucosal membrane was calculated using Eq. (2):

$$E_{Cl} = RT/F \ln a^i_{Cl}/a^m_{Cl} \tag{2}$$

where $RT/F = 25.4$ mV at room temperature (22°C) and a^m_{Cl} is the mucosal bath Cl^- activity.

The electrochemical driving force for Cl diffusion across the serosal membrane was calculated as ($\Delta\mu_{Cl}/F = RT/F \ln a^s_{Cl}/a^i_{Cl} - v_s$) where a^s_{Cl} is the Cl^- activity of the serosal medium and v_s is the voltage of the serosal medium relative to the cytoplasm.

The selectivity of the exchanger for Cl^- over gluconate, estimated with single salt solutions, was $k_{Cl,gluconate} = 0.016 \pm 0.002$. Since it is unlikely that gluconate reached concentrations in the cytoplasm exceeding 40 mM, the "gluconate error" should not exceed 0.6 mM.

The selectivity of the anion exchanger for Cl over HCO_3^- was evaluated with single and mixed salt solutions. Employing 100 mM NaCl or $NaHCO_3$, the selectivity of the Cl^- electrode for HCO_3^- (k_{Cl,HCO_3}) varied between 0.11 and 0.13 in different series. However, there was essentially no difference in electrode voltage between a 20-mM solution of NaCl and one containing 20 mM NaCl + 20 mM $NaHCO_3$. Comparing 20 mM Cl with 20 mM Cl +

40 mM HCO_3, $k_{Cl,HCO_3} = 0.03$. The mixed salt solution method of calibration more closely approximates the conditions under which cell Cl^- is estimated and is, therefore, a better measure of selectivity [2].

The protocol of experiments utilizing Cl^--sensitive DBM were as follows: tissues were incubated in normal medium and a^i_{Cl} and v_m were measured simultaneously in about six individual villus epithelial cells prior to calibration. Then the medium was changed, as necessary, and further impalements of villus cells made at selected intervals to obtain an estimate of the time course of changes in a^i_{Cl} and v_m. Simultaneously, the transepithelial voltage difference, v_{ms}, was sensed by a voltage clamp device through calomel cells connected to the chamber via saturated KCL-agar bridges. The voltage divider ratio was routinely measured. Changes in this variable were difficult to interpret and are not reported. As before, recordings were deemed acceptable when (i) the difference in voltage between the two barrels of the DBM, $v_{Cl} - v_m$, while in the cell was constant within 1 mV for 10 sec, (ii) the tip potential of the barrel sensing v_m did not change more than 3 mV from penetration to withdrawal, and (iii) the Cl exchanger responded to changes in a_{Cl} with a slope S greater than -45 mV.

Titrimetric Estimate of Cell Cl^-

The intracellular concentration of Cl^-, $[Cl]^i$, was estimated as follows: five adjacent segment of duodenum were stripped, pinned into paraffin, and incubated in normal, oxygenated medium. After 1 hr one tissue was removed for analysis, while the remaining segments were rinsed and then bathed in Cl^--free medium which was completely replaced when a segment was removed for analysis. This was done at regular intervals. Tissues were gently blotted with ash-free filter paper, weighed, and digested overnight in 0.1 N HNO_3^- acid. The ratio of wet to dry weight was estimated in other tissues. The ratio of cell/total tissue water was assumed to be 4 : 1 under all conditions based on previous measurements on tissues bathed in normal medium. Tissue Cl was assayed using an Aminco-Cotlove chloridimeter (American Inst. Co., Silver Springs, MD). In a related protocol, two adjacent segments were bathed in Cl^--free medium which was completely replaced with fresh medium every 15 min. After 90 min tissues were removed for analysis. One homogenate was treated with sodium perborate to oxidize sulfhydryl groups which can combine with silver ions and provide an erroneously high measure of tissue Cl^- [5].

Isotopic Estimate of Cell Cl^-

Duodenal segments were stripped, stretched, and pinned into individual dishes having a paraffin base. They were bathed in normal, oxygenated medium containing ^{36}Cl (New England Nuclear) at 1 μCi/ml and, in addition, 2.5 μM cold inulin. After 1 hr the tissues were rinsed 3 times in unlabeled 1 mM Cl^- medium and incubated in that medium. ^{36}Cl was added to the same specific activity (μCi/meq Cl) as in the first hr of exposure. In addition, 0.125 μCi/ml of ^3H inulin was added. Tissues were removed at 100 min. The mucosa was scraped from the underlying smooth muscle, weighed, and digested overnight in 0.1 N HNO_3. Aliquots were counted on a liquid scintillation spectrophotometer (Packard Inst., Downers Grove, IL) with appropriate correction of the ^3H cpm in the ^{36}Cl window. The % dry wt was estimated in duplicate in other tissues and averaged 17.2 ± 1.4% ($n = 6$). Cell water averaged 37 ± 6% in the 100-min samples.

Measurement of Cellular Cl^- Efflux

Segments of duodenal mucosa 6 cm long were stripped of smooth muscle, everted, and mounted on a tissue holder which consisted of two segments of hollow plexiglas tubing (5 mm diam.) attached to each other by a thin connector 5 cm in length. The tissue holder was threaded through the everted intestinal segment. The intestinal segments were tied to it at each end with surgical thread and the apparatus was mounted vertically in a bath. Bathing medium was perfused upward through the interior (serosal) compartment at a rate of 1 ml/min by means of a peristaltic pump. By collecting the serosal effluent at the fluid level of the bathing medium the presence of a hydrostatic pressure head across the mucosa was avoided. The design permitted perfusion of the serosal compartment of a 5-cm segment of the everted gut using Cl^--free medium while the mucosal surface was bathed in Cl^--containing medium. Intestinal segments were incubated for 1 hr in normal medium containing ^{36}Cl at 1 μCi/ml. During this time the serosal compartment was perfused continuously. Fractions of serosal medium were collected at 10-min intervals during the loading and washout periods. After isotopic loading the assembly was transferred to a vessel containing 40 ml of fresh (unlabeled) medium, and ^{36}Cl efflux into the luminal medium was monitored by collecting 100-μl samples at regular intervals over a 55-min period. By a process of back addition the μeq Cl in the tissue compartment at each time was calculated. Only the first 15 min of mucosal washout are reported.

Statistical Tests

Student's t-test for paired and unpaired comparisons were used to determine the significance of the difference between population means.

Results

In Fig. 1 it is seen that the average value of intracellular Cl^- activity (a^i_{Cl}) was constant over several hours for six tissues bathed bilaterally in normal Cl^- containing medium. In the same figure, five tissues exposed on their serosal surfaces to 0.1 mM Cl^- (Cl^- replaced with gluconate) continued to retain Cl^- within the cells at a normal level even though the driving force for Cl diffusion across the serosal membrane ($\Delta\mu_{Cl}/F$) increased markedly from -6.5 to -160 mV. If there was a finite Cl^- conductance at the serosal membrane then a decline in cell Cl^- would be expected to occur unless losses of Cl^- across the serosal membrane were matched by entry of Cl^- into the cell across the mucosal membrane. As seen in the inset of Fig. 1, the serosal membrane depolarized over the first 30 min but was essentially unchanged thereafter even when serosal Cl^- was reinfused. Since the effect on v_s was not

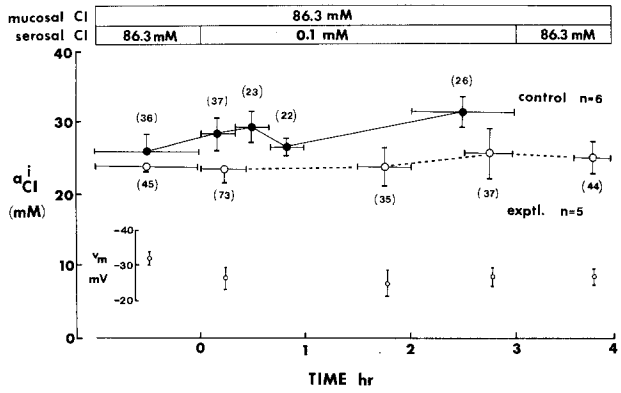


Fig. 1. The time course of intracellular Cl^- activity (a^i_{Cl}) in six tissues bathed bilaterally with normal medium (filled circles) and in five tissues exposed after a control period to medium in which all but 0.1 mM Cl^- was replaced with gluconate (open circles). The horizontal bars illustrate the period over which measurements were made with double-barreled microelectrodes. In parentheses is the number of measurements collected within the period. Also illustrated is the mucosal membrane potential (v_m) of the experimental series

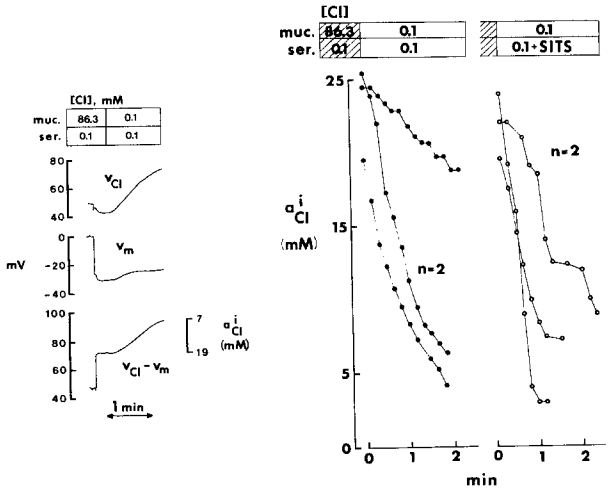


Fig. 2. Single cell response of a^i_{Cl} to luminal Cl^- replacement. Left: response of a single cell to mucosal Cl^- washout. v_{Cl} is the voltage on the Cl^--sensitive barrel, v_m the voltage of the membrane-potential sensing barrel of the double-barreled microelectrode. The difference voltage, subtracted electronically, is related to the intracellular Cl^- activity. Right: response of a^i_{Cl} in three different cells to replacement of all but 0.1 mM Cl^- in the mucosal medium (filled circles). The serosal medium contained Cl at 0.1 mM. Far right: washout of intracellular Cl^- after preexposure for at least 1 hr to SITS (1 mM) in the serosal medium. n is the number of animals examined

reversed upon readdition of Cl^- to the serosal medium, there is no support for a significant Cl^- conductance in the serosal membrane. It can be safely concluded from these measurements that $a_{Cl}{}^i$ is virtually independent of the electrochemical driving force on Cl^- at the serosal membrane. Hence it is unlikely that there is a significant diffusional efflux of Cl^- across the serosal membrane.

In contrast, cell Cl^- was very sensitive to changes in the mucosal bath [Cl]. In Fig. 2, left, is a recording from a single cell. a^i_{Cl} fell rapidly upon replacement of luminal Cl^-. On the right side of the figure it is seen that reduction of mucosal Cl^- to 0.1 mM (serosal [Cl] = 0.1 mM) caused a rapid drop in a^i_{Cl} in three different cells (filled circles). The steady-state value of a^i_{Cl} finally achieved is decribed below. In cells of other tissues pre-exposed on their serosal surface to 1 mM SITS for 1 hr, a^i_{Cl} declined at about the same rate (Fig. 2, far right) as untreated tissues. The latter observation was supported when, in another tissue pre-exposed on its serosal surface to SITS for 55 min, replacement of mucosal Cl was followed by a decline of a^i_{Cl} from an average control value of 24.5 ± 3.1 mM (n = 4) to a value of 10.0 ± 3.4 mM in the first 7 min (n = 4) and to 6.8 ± 0.6 mM during the next 8-min period (n = 6). If the decline in a^i_{Cl} was a consequence of the serosal transfer of Cl from the cell it would be expected that SITS would block the decline. Furthermore, the decline cannot be attributed solely to reduced Cl^- uptake, otherwise, with SITS blocking serosal exit, a^i_{Cl} would have remained constant. Hence, the decline observed is due to a leak of Cl^- into the mucosal medium. Further support for this conclusion is advanced below.

In other tissues, increasing the mucosal [Cl] from 0.1 mM resulted in a rise in a^i_{Cl} which was more rapid when higher concentrations of Cl were employed (86.3 mM) than with lower (e.g. 25 mM) concentrations of Cl. This is illustrated in Fig. 3. In summary, intracellular Cl^- responds rapidly and reversibly to changes in luminal [Cl].

In order to determine the lowest level that a^i_{Cl} will fall, tissues from four animals were exposed to nominally Cl^--free media on both mucosal and serosal surfaces. In Fig. 4 it is seen that a^i_{Cl} fell from an average value of 22.8 ± 2.5 mM in four tissues to 6.5 ± 0.4 mM in the period 1–2 hr after exposure to gluconate medium and did not decline further in the next hour. Hence, in the steady state considerable Cl^- appeared to remain within the cell. One explanation for the failure of cell Cl to fall further is that the Cl-sensitive microelectrode sensed anions other than Cl^-.

In an effort to test the yalidity of the value of a^i_{Cl} measured in gluconate medium, the time course of the decline in total tissue Cl^- was estimated by the titrimetric method in intestinal segments incubated in a similar manner, first in normal medium

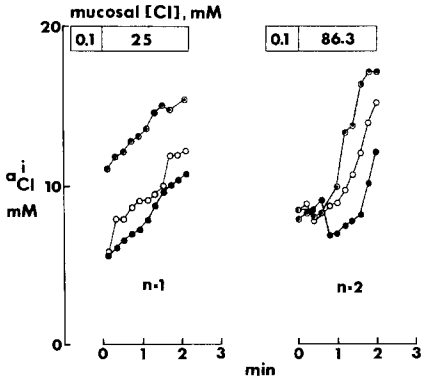


Fig. 3. Single cell response to elevation of luminal [Cl^-]. Left: response when luminal [Cl] was raised to 25 mM. Right: response when luminal [Cl] was raised to 86.3 mM. Serosal [Cl] was 86.3 mM. *n* is the number of animals studied

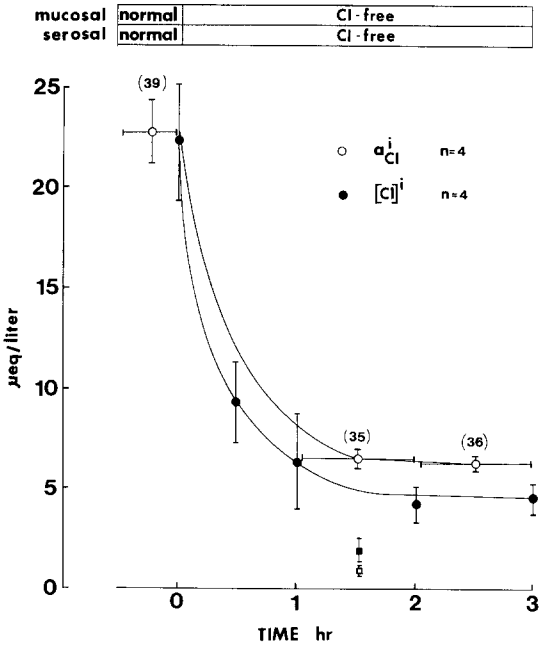


Fig. 4. Decline in intracellular Cl^- activity, a^i_{Cl} (open circles), and intracellular Cl^- concentration, $[Cl]^i$ (filled circles), in unpaired groups of tissues bathed in Cl^--free medium. $[Cl]^i$ was determined from titrimetric analysis of mucosal scrapes assuming cell water at 84% of total water and dry wt was 20% of wet wt. In parentheses is the number of estimates of a^i_{Cl}. *n* is the number of tissues examined. The point defined by the squares are explained in the text

then in several changes of gluconate medium to approximate the conditions (of perfusion) under which a^i_{Cl} was estimated. Intracellular Cl^- concentration ($[Cl]^i$), estimated from the Cl^- content of mucosal scrapes, fell over the same time course as a^i_{Cl} to 4.5 mM and was essentially unchanged after 90 min. Because it seemed possible that $[Cl]^i$ could be lowered further with a more extensive exposure to Cl-free medium, an additional series of four tissues were rinsed every 15 min over a period of 90 min with fresh gluconate medium. In these tissues $[Cl]^i$ was lower at 1.8 ± 0.5 mM. Furthermore, paired tissues treated with perborate to oxidize potentially interfering sulfhydryl groups [5] exhibited a still lower value of $[Cl]^i$ of 0.9 ± 0.3 mM. The latter value is the best estimate of the true $[Cl]^i$. The disparity between this estimate of $[Cl]^i$ and the estimate of a^i_{Cl} (6.5 mM) suggests that 5–6 mM of apparent Cl^- measured with Cl^--sensitive microelectrodes is actually due to interference from other anions.

The possibility was examined that HCO_3^- was interfering. The bicarbonate concentration of the cytoplasm of *Amphiuma* intestinal cells ($[HCO_3^-]^i$) bathed in normal medium, estimated directly with liquid ion exchange microelectrodes, was 15.6 mM [14]. While a comparable estimate of $[HCO_3^-]^i$ for tissues bathed in Cl^--free medium is not available, intracellular pH is increased to 7.1. Therefore $[HCO_3^-]^i$ may be as high as 27 mM. At this pH, HCO_3^- ions could account maximally for 3 mmol/liter of the apparent Cl (selectivity of Cl electrode for HCO_3^- in absence of Cl ≈ 0.12), particularly if the true value of a^i_{Cl} is near zero. In order to examine the possible interference by HCO_3^- more closely, four tissues were exposed to medium which was nominally HCO_3^- and Cl^- free. a^i_{Cl} fell from 30.2 ± 3.3 mM (n = 27) in normal medium to 8.1 ± 1.8 mM in the period 2–3 hr after exposure to a (Cl^- + HCO_3^-)-free medium. The latter value was not different ($P > 0.20$) from the value obtained in Cl^--free HCO_3^--containing medium (Fig. 4). Since there was, most likely, a large reduction in intracellular HCO_3^-, then it is safe to conclude that HCO_3^- ions contributed little interference in the measurement of a^i_{Cl}. Other anions must be responsible for the interference.

Intracellular a_{Cl} as a Function of Luminal [Cl]

In Fig. 5 it is seen that the steady-state value of a^i_{Cl} is related to the medium [Cl]. Tissues were bathed continually on their serosal surface with Cl^--free medium. Initially the mucosal chamber was perfused with normal medium. After a control series of measurements was obtained, the mucosal solution was changed to one containing either 1, 10, 20, 40 or 60 mM Cl^-. As bath [Cl] was reduced from 86.3 mM by replacement with gluconate, a^i_{Cl} declined the mucosal membrane depolarized and the serosa be-

came more positive, reflecting reduced net Cl absorption. When bath Cl^- was 1 mM, a^i_{Cl} was apparently 8.9 ± 1.6 mM. At this and all other values of a^i_{Cl}, Cl^- appeared to be above electrochemical equilibrium, i.e., $E_{Cl} - v_m > 0$, as seen in Fig. 5. After correction for the likely amount of interference by other unidentified anions in the cytoplasm (≈6 mM), a^i_{Cl} was 2–3 mM. Correcting the values of a^i_{Cl} measured at each medium [Cl] in a similar manner, the value of $E_{Cl} - v_m$ was markedly positive only at the bath [Cl] = 1 mM and exhibited negative values at higher [Cl]. This is illustrated in Fig. 5 with a dashed line.

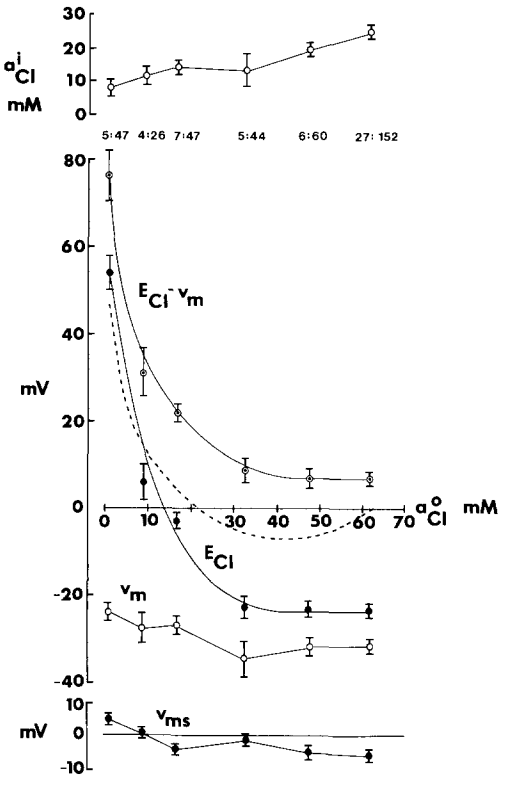
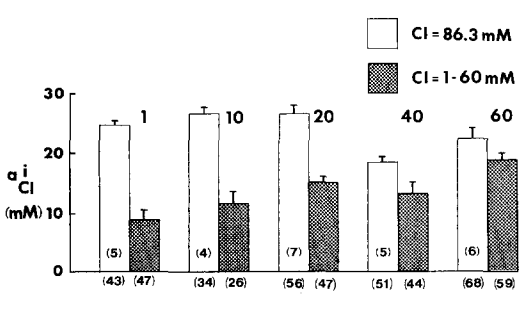


Fig. 5. a^i_{Cl} and mucosal electrical characteristics as a function of luminal Cl^- activity (a^o_{Cl}). Steady-state values of mucosal membrane potential (v_m), Cl^- equilibrium potential (E_{Cl}), Cl^- accumulation potential ($E_{Cl} - v_m$) and transepithelial potential (v_{ms}) are shown. Values of a^i_{Cl} were obtained in the period 1–2 hr after replacing normal medium with medium of lower [Cl]. a^i_{Cl}, E_{Cl} and $E_{Cl} - v_m$ are all uncorrected for interference by other anions. The serosal medium was Cl-free. In the upper part of the figure is number of tissues/number of measurements. The dashed line is explained in the text

Additional evidence for interference of the Cl^- electrode response by other anions was obtained when the intracellular Cl^- concentration was estimated by measuring the distribution of ^{36}Cl. Extracellular volume was estimated with ^3H-inulin. After a 90-min incubation period in medium containing 1 mM Cl (a period assumed sufficient for uniform labeling of all cell Cl), mucosal scrapes from three animals had an average cell Cl content of 3.6 ± 0.2 mM. This value is less than half of the value measured with Cl^--sensitive microelectrodes. Parenthetically, this value is also significantly ($P < 0.01$) above the bath [Cl] and therefore consistent with a modest Cl^- uptake.

In Fig. 6 the values of a^i_{Cl} reported in Fig. 5 are replotted. On the left are the values of a^i_{Cl} in normal medium (open bars) and in the period 1–2 hr after exposure to the medium of lower [Cl] (hatched bars). On the right side of the figure, the steady-state values of cell Cl are normalized as a fraction of the control value of cell Cl (in normal medium) to reduce the influence of the variation in the control



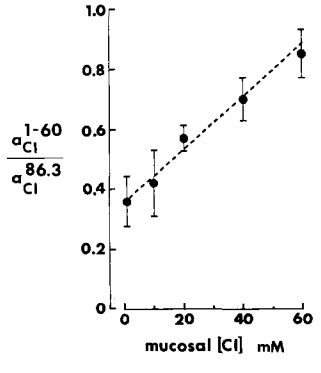


Fig. 6. Left: paired average values of a^i_{Cl} in normal mucosal medium (open bars) and 1–2 hr after exposure to low Cl medium (hatched bars). In parentheses at bottom are number of DBM recordings. Values within open bars are number of tissues examined. Right: normalized cell Cl activity as a function of bath [Cl]. Dotted line is least squares estimate of slope (correlation coefficient = 0.99)

values of a^i_{Cl}. Cell Cl declined in a linear manner (correlation coefficient = 0.99) with bath [Cl]. The correlation was highly significant ($P < 0.002$). In contrast, in *Necturus* renal proximal tubules a^i_{Cl} varied in a sigmoidal manner with luminal [Cl] [29].

Transserosal Cl^- Flux as a Function of Luminal [Cl]

It has been demonstrated that the disulfonic stilbene SITS completely blocks net transcellular Cl^- absorption by urodele intestine when added to the serosal medium. Luminal SITS was without effect [31]. Thus the exit of Cl across the serosal membrane is the specific step where inhibition occurs. The amount of reduction of the absorptive Cl^- flux produced by SITS is a direct measure of the rate of transserosal Cl^- flux. The relationship between the luminal [Cl] and the rate of transcellular Cl^- absorption was obtained in short-circuited tissues by measuring the *m* to *s* (*ms*) flux of $^{36}Cl^-$ into a Cl^--free serosal medium before and after exposure to SITS. Paired tissues were examined. The absorptive Cl^- flux (J^{Cl}_{ms}) from a luminal solution containing Cl^- at a concentration of 1, 2, 10, 20, 40 or 60 mM was compared with that of a paired adjacent tissue in which the luminal [Cl] was 86.3 mM. The stilbene-induced reduction in the mucosa-to-serosa flux (ΔJ^{Cl}_{ms}) is plotted in Fig. 7, left (open circles) against the mucosal [Cl]. Stilbene-sensitive Cl^- absorption approached a maximum at higher bath [Cl]. Similarly, the reduction in I_{sc} (the Cl^- current) also reached a maximum (closed circles). However, the relationship was obscured by the variability between tissues. In contrast, when the responses were normalized within tissues, by comparing the response when luminal [Cl] was 1–60 mM with the response of the paired tissue bathed in media of normal [Cl], as illustrated in the upper right hand portion of Fig. 7, then the stilbene-sensitive transserosal ^{36}Cl flux approached a maximum as luminal [Cl] was elevated. This is seen in the lower right-hand portion of Fig. 7. Cl^- absorption reached a maximum when the mucosal [Cl] was 40 mM.

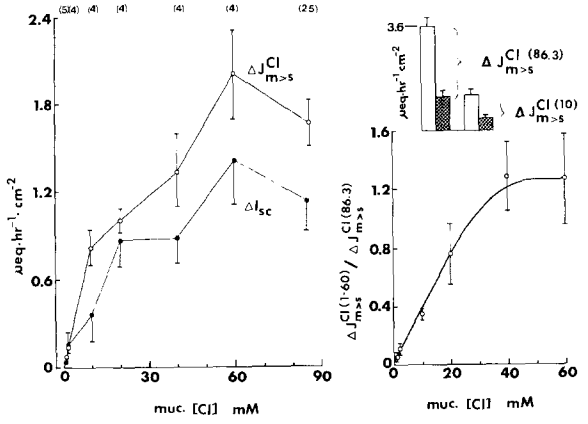


Fig. 7. Left: average stilbene-sensitive Cl flux (ΔJ^{Cl}_{ms}) and short-circuit current (ΔI_{sc}) as a function of mucosal [Cl]. In parentheses is the number of tissues studied. Paired tissues were studied, one in normal medium ([Cl] = 86.3 mM), the other in low Cl medium. All results are included. Right upper: the method of normalizing to factor out the variation in Cl flux between tissues is illustrated for the series in which the response in normal medium ([Cl] = 86.3 mM) was compared with that in media of mucosal [Cl] = 10 mM. Right lower: normalized serosal Cl^- transport at different mucosal [Cl]. The curve was drawn by eye

Relationship Between a^i_{Cl} and Transserosal Cl Transport

The relationship seen in Fig. 8 between the apparent a^i_{Cl} and the rate of Cl^- exit from the cell into the serosal medium, was derived from the separate, normalized relationships illustrated in Figs. 6 and 7. The curve reveals that there is an apparent threshold [Cl] of about 8 mM (most likely accounted for by interfering anions) above which a significant flux of Cl^- into the serosal medium begins. Serosal trans-

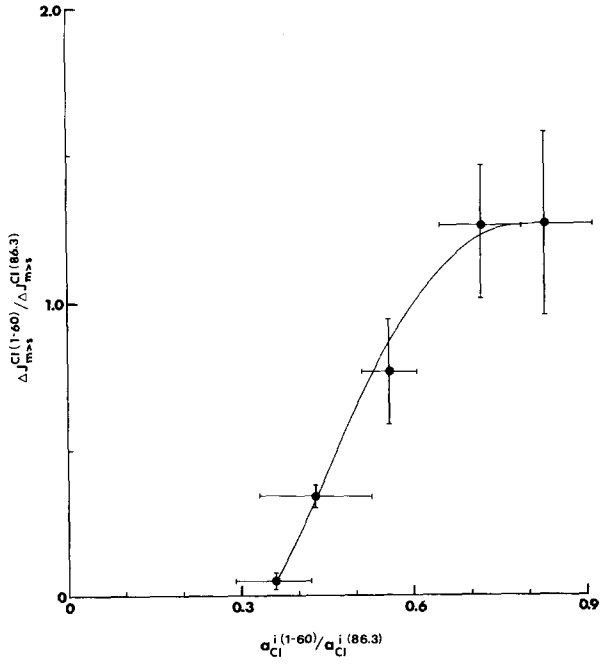


Fig. 8. The relationship between normalized intracellular Cl activity ($a^{i(1-60)}_{Cl}/a^{i(86.3)}_{Cl}$) and normalized serosal Cl^- transfer ($\Delta J^{Cl(1-60)}_{ms}/\Delta J^{Cl(86.3)}_{ms}$). The data points were taken from Figs. 6 and 7. The line was drawn by eye. The K_m was computed graphically as the value of $a^{i(1-60)}_{Cl}/a^{i(86.3)}_{Cl}$ corresponding to $\frac{1}{2}$ the maximal value of $\Delta J^{Cl(1-60)}_{ms}/\Delta J^{Cl(86.3)}_{ms}$

Table 1. Effect of SITS on cell Cl^- in low Cl^- medium

	Control	+SITS			
		0–15 min	15–30 min	30–60 min	1–2 hr
a^i_{Cl} (mM)	11.3 ± 1.3	11.0 ± 2.0	10.3 ± 2.8	11.8 ± 2.2	11.2 ± 1.8
v_m (mV)	−24.6 ± 2.2	−20.6 ± 1.4	−20.8 ± 1.4	−22.3 ± 1.8	−20.9 ± 2.0
$E_{Cl} - v_m$ (mV)	29.9 ± 3.1	24.1 ± 5.2	21.9 ± 6.9	28.0 ± 5.3	25.0 ± 3.0
v_{ms} (mV)	−0.8 ± 1.5	0.3 ± 1.1	0.2 ± 1.0	0.6 ± 0.6	1.0 ± 0.5
n	24	28	26	23	34

The luminal [Cl^-] was 10 mM; serosal [Cl^-] was 0.1 mM. SITS was employed in the serosal medium at 1 mM. The averages are from three animals. n is the number of measurements.

Table 2. Effect of replacing luminal Na^+ on cell Cl^-

	Control period	1–2 hr after Na replacement
a^i_{Cl} (mM)	8.4 ± 1.0	7.5 ± 0.2
v_m (mV)	−17.8 ± 1.6	−20.0 ± 2.7
$E_{Cl} - v_m$ (mV)	71.0 ± 5.3	71.1 ± 2.6
n	26	37

Cl was present at 1 mM. Values are the average from six tissues. n is the total number of impalements. Na was replaced with choline.

membrane Cl transport reached a maximal value when a^i_{Cl} was about 17 mM, corresponding to a bath [Cl] of about 40 mM (Fig. 6). The K_m of the serosal transporter, computed graphically, was 11.8 mM. If interference from other anions amounts to 6–8 mM then the true K_m = 4–6 mM. Saturation of transport is an expected consequence of the presence of a carrier-mediated transport process.

It is possible that the failure of stilbenes to elevate a^i_{Cl}, noted in a previous study [4], may have been because, when bathed in normal medium, a^i_{Cl} is considerably above the value needed to saturate the serosal carrier. For this reason the effect of serosal SITS on a^i_{Cl} was examined in tissues bathed in medium of 10 mM Cl. At this value of luminal [Cl], a^i_{Cl} is near the K_m of the serosal transporter. Inhibition of the transporter should elevate a^i_{Cl} if the transporter has an influence on cell [Cl]. As seen in Table 1, the normal, serosa-negative transepithelial voltage was reversed by SITS as occurs when electrogenic Cl^- absorption is blocked. However, SITS did not change a^i_{Cl}. There are several possible explanations. Luminal Cl^- entry may be downregulated by inhibition of serosal transport. Alternatively, the serosal transport process may not influence the steady-state intracellular Cl^- activity to a measurable degree. Lastly, blockage of Cl exit may cause cell swelling as Cl accumulates in the cell.

Mechanism of Mucosal Cl^- Uptake

As seen in Table 2, complete replacement of luminal Na with choline had no observable effect on a^i_{Cl} when this was examined in low Cl^- medium. Low Cl^- medium was used because Cl^- accumulation appears to be significant in this condition (Fig. 5). The lack of dependence of a^i_{Cl} on luminal Na^+ was also noted in a previous study in which media of normal [Cl] was employed [35].

In another series it was observed that replacement of medium Na^+ did not slow the rate of Cl^- uptake. The rate of entry of Cl^- into cells incubated initially in a Cl^- + Na^+-free medium was compared when Na^+ or Li^+ was the major cation. As seen in Table 3, a^i_{Cl} climbed rapidly whether NaCl- or LiCl-based medium was infused into the mucosal medium. The hyperpolarization of the luminal membrane when NaCl-based medium was infused is probably due to activation of electrogenic Na transport at the basolateral membrane. In contrast with these results, in *Necturus* renal proximal tubule, Cl^- activities were greatly reduced in LiCl-based medium as compared to NaCl-based medium [29]. This result argues against a role of Na-Cl cotransport as a mechanism for Cl^- entry into *Amphiuma* intestinal cells.

Linked K-Cl uptake is also an unlikely mechanism since removal of luminal K^+ (from medium of normal [Cl]) had no effect on cell Cl^-. The results are listed in Table 4.

Another possible route of Cl^- uptake is via Cl^--HCO_3^- exchange. To test for this mode of Cl^- uptake tissues were incubated on their luminal surface in medium containing 10 mM Cl^-, and 25 mM

Table 3. Effect of Na^+ *vs.* Li^+ on increase in cell Cl^-

	Control period	Mucosal Cl^- added		
		15–30 min	75–130 min	150–180 min
LiCl				
a^i_{Cl} (mM)	12.8 ± 2.5	26.8 ± 1.3	35.3 ± 1.1	32.6 ± 4.8
v_m (mV)	−11.6 ± 2.4	−11.1 ± 1.2	−8.9 ± 2.5	−10.6 ± 1.1
n	27	16	14	19
NaCl				
a^i_{Cl} (mM)	12.5 ± 3.6	37.9 ± 8.8	29.7 ± 6.6	30.5 ± 5.2
v_m (mV)	−12.1 ± 1.9	−16.5 ± 2.4	−17.0 ± 1.6	−18.4 ± 1.9
n	24	16	20	24

Results are the average of four unpaired tissues in each series. Tissues were bathed during the control period in a $LiSO_4$-based medium buffered with Tris. n is the total number of impalements.

Table 4. Effect of removing mucosal K^+ on cell Cl^-

	Control period	K^+-free		
		0–30 min	30–60 min	3–4 hr
a^i_{Cl} (mM)	26.0 ± 1.3	25.5 ± 2.2	26.5 ± 1.1	23.1 ± 1.5
v_m (mV)	−31.7 ± 1.9	−29.8 ± 2.3	−27.4 ± 1.2	−28.1 ± 2.5
$E_{Cl} - v_m$ (mV)	8.2 ± 1.2	5.6 ± 1.9	4.9 ± 1.4	1.7 ± 1.8
n	67	101	82	70

$[Cl^-]$ = 86.3 mM in mucosal and serosal fluids. n is the total number of impalements.

HCO_3^-. Since Cl and HCO_3^- are both present in the cytoplasm then the selectivity of the ion-exchange microelectrode for Cl over HCO_3^- should be high (e.g. $k_{Cl,HCO_3} = 0.03$). After a^i_{Cl} was determined the luminal $[HCO_3]$ was elevated to 75 mM (pH = 7.85) at the expense of gluconate, and a^i_{Cl} was measured again. If electroneutral exchange is responsible for Cl^- uptake, then the chemical gradients for Cl^- and HCO_3^- should be coupled. Thus the accumulation of Cl^- in the cell to a greater concentration than in the medium ($a^i_{Cl} > a^o_{Cl}$) should be accompanied by a chemical gradient for HCO_3^- in the same direction, i.e., $[HCO_3]^i > [HCO_3]^o$. In this case elevating mucosal $[HCO_3]$ to 75 mM should reverse the HCO_3^- gradient and favor Cl^- exit over the exchange pathway. A drop in a^i_{Cl} did not occur. In Table 5 it is seen that a^i_{Cl} was essentially unchanged in the short or the long term. Furthermore, there was no immediate change in a^i_{Cl} in single cells when this was monitored during the switch from normal to elevated HCO_3^- media (recordings not shown). These results argue against luminal anion exchange as a mechanism for Cl^- entry.

Table 5. Effect of elevating luminal HCO_3^- on cell Cl^-

	Control period	0–30 min	30–60 min
$[HCO_3^-]$ (mM)	25	75	75
a^i_{Cl} (mM)	14.9 ± 0.4	15.6 ± 0.9	13.5 ± 2.0
v_m (mV)	−30.5 ± 1.8	−27.7 ± 3.6	−23.5 ± 3.5
$E_{Cl} - v_m$ (mV)	42.6 ± 1.4	40.7 ± 2.5	32.4 ± 1.2
n	45	61	53

Mucosal $[Cl^-]$ was 10 mM; serosal medium was Cl^--free. Mucosal HCO_3^- was elevated at the expense of gluconate. n is the number of impalements.

The Paracellular Cl^- Flux

While the stilbene-sensitive Cl^- flux increased to a constant value as bath Cl was elevated, the flux remaining after SITS exposure (the SITS-insensitive Cl^- flux) did not reach a maximal value. This is seen in Fig. 9. The correlation coefficient between bath [Cl] and Cl flux was 0.99. It has been demonstrated in this tissue that stilbenes inhibit all net

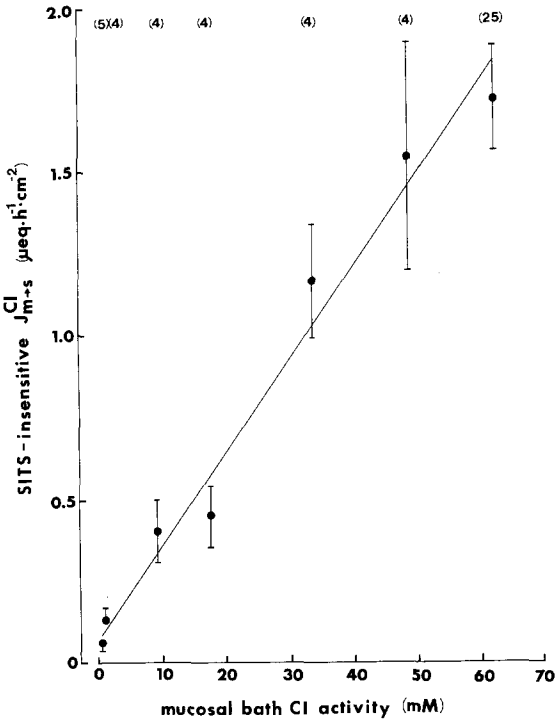


Fig. 9. Relationship between mucosal Cl^- activity and stilbene-insensitive Cl^- absorptive flux. In parentheses is the number of tissues studied. The line of linear regression intercepted the y axis at 0.083 μeq/hr · cm^2

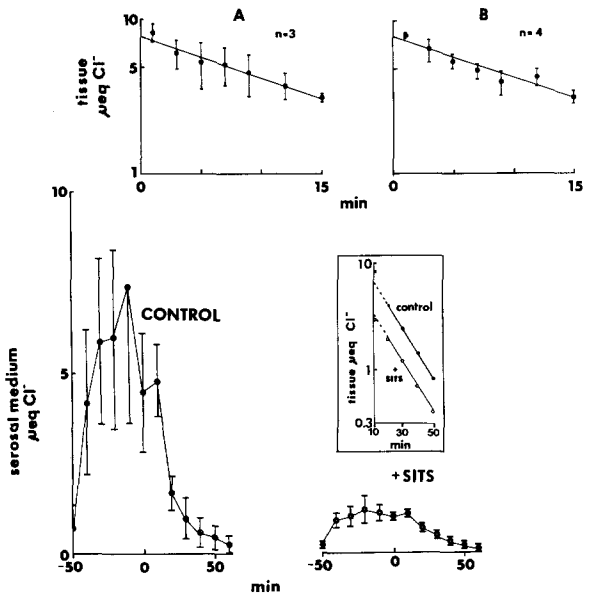


Fig. 10. ^{36}Cl efflux into mucosal and serosal media. (*A*) Cl washout from a tissue compartment calculated by back addition of cpm appearing in normal mucosal medium. (*B*) Washout with SITS (1 mM) present. n is the number of tissues examined. Bottom: appearance of Cl in serosal medium during loading period (before zero time) and during subsequent washout period. Inset: serosal Cl^- washout kinetics. Points were obtained by back addition of cpm appearing in the serosal medium in the period from 10–55 min after washout began. Lines were fitted to the data points from 20–50 min by linear regression

active Cl^- absorption by blocking the serosal anion transport process (*see* below and refs. 4, 7). The stilbenes do not alter tissue conductance, as would be expected if they blocked a Cl^- conductive pathway in the serosal membrane [31]. Since there is no direct support for Cl^- conductive pathways in the serosal membrane, the route of the stilbene-insensitive Cl^- flux is most likely paracellular. In agreement with this conclusion, the secretory (*sm*) Cl^- flux is equal to the absorptive flux in short-circuited tissues exposed to SITS [31].

Cl Efflux from Cell to Lumen

In an effort to assess the rate of Cl efflux from cell to lumen and the factors which influence it, tissue Cl was labeled with ^{36}Cl and the rate of efflux into both luminal and serosal compartments measured. As seen in Fig. 10*A*, the average Cl^- efflux (n = 3) into normal mucosal medium was log-linear (correlation coefficient = 0.97) over 15 min with a half-time of 11.4 min. The slope intercepted the y-axis at 7.7 μeq Cl^-. Simultaneously, there was a brisk transfer of Cl^- into the Cl-free serosal medium (lower part of figure). Analysis of the washout curve by the process of back addition revealed that, in the period from 10–40 min after washout began, the serosal efflux was loglinear (r = 0.99) with a $t_{1/2}$ = 12.7 min. This is illustrated in the inset of the figure. The slope intercepted the y-axis at 7.1 μeq. Clearly the movement of labeled Cl^- occurred across the mucosal and serosal membranes at very similar rates and from compartments of nearly identical Cl^- content.

When SITS was included in the serosal perfusion fluid (Fig. 10*B*), serosal Cl^- transfer was greatly reduced. Nevertheless, Cl^- efflux into the mucosal fluid was unchanged ($t_{1/2}$ = 11.8 min). The capacity of the mucosal compartment (7.5 μeq · Cl) was also unchanged, a result in agreement with the observations reported above (Table 1) that serosal stilbenes do not elevate a^i_{Cl}. If a sizeable fraction of the ^{36}Cl entering the mucosal fluid originated in the paracellular spaces, then the capacity of the mucosal efflux component should have been greater in Fig. 10*A* compared with Fig. 10*B* since, in the absence of SITS, serosal Cl transfer occurred and the amount of Cl in the lateral intracellular spaces would be larger. Instead, the capacity of the two compartments was identical. It is concluded that ^{36}Cl efflux into the mucosal medium originated largely from the cytoplasmic pool and was unaffected by inhibition of the mechanism of ^{36}Cl efflux

Table 6. ^{36}Cl efflux

Series	n	Mucosal washout medium	Serosal washout medium	C (μeq/min)	Slope (ln μeq/min)	r	$t_{1/2}$ (min)	Serosal efflux over 110 min (μeq)
1	3	Normal	Cl-free	7.7 ± 2.0	−0.061 ± 0.005	−0.97	11.4	28.3 ± 11.5
2	4	Normal	Cl-free + SITS	7.5 ± 0.5	−0.060 ± 0.001	−0.96	11.8	7.9 ± 0.5
3	4	Cl − 1 mM	Cl-free + SITS	9.8 ± 2.2	−0.082 ± 0.011	−0.96	9.1	7.4 ± 1.7
4	3	Normal + theophylline	Cl-free + SITS	9.8 ± 1.5	−0.088 ± 0.020	−0.95	8.6	9.5 ± 2.6

n is the number of tissues. C, the extrapolated intercept of the slope on the y-axis, is the capacity of the tissue compartment relative to the mucosal efflux. The slope is the rate of decline and cpm obtained by back addition of cpm in the mucosal medium. r is the correlation coefficient of cpm *vs.* time. SITS and theophylline were employed at 1 mM. Cl^- was replaced with gluconate.

across the serosal membrane. In the remaining studies SITS was present in the serosal medium.

When loaded tissues were washed out into mucosal medium containing only 1.0 mM Cl^- there was a significant ($P < 0.05$) increase in the rate of Cl^- loss ($t_{1/2} = 9.1$ min). The results of this and the previous studies are tabulated in Table 6 for comparison. The greater rate of Cl^- loss into low Cl^- medium would be predicted if transmembrane Cl efflux occurred through channels which are ordinarily partially blocked by luminal Cl^- ions [29]. On the other hand, a decrease should have occurred if Cl^- exit across the mucosal medium was a result of Cl-Cl exchange.

Elevation of tissue levels of cyclic AMP has been shown to inhibit net Cl^- absorption and enhance the secretory (*sm*) flux of Cl^- [31]. It was concluded that cyclic AMP elevated luminal Cl^- permeability. This finding permitted a test of the technique of measurement of $^{36}Cl^-$ efflux. Loaded tissues were washed out into medium containing 1 mM theophylline. The results are listed in Table 6. As expected, Cl^- efflux was enhanced ($t_{1/2} = 8.6$ min), although the slope of Cl efflux was not significantly greater ($P < 0.20$) than that of series #2. The enhanced efflux cannot be secondary to mucosal membrane hyperpolarization since theophylline causes depolarization of urodele intestinal mucosal cells [32]. Hence, the result supports the earlier conclusion that theophylline accelerates luminal Cl^- efflux and lends validity to the Cl^- efflux technique.

Discussion

The small intestine of *Amphiuma* absorbs Cl^- by a process which is electrogenic, Na-dependent, and linked to the secretion of HCO_3^-. The present study took advantage of the high permeability of the luminal membrane for Cl^- to control a^i_{Cl} and thereby to define the relationship between a^i_{Cl} and serosal Cl^- transport. In the process important information relevant to the uptake of Cl^- across the luminal membrane was forthcoming.

Validity of Estimate of a^i_{Cl}

An accurate estimate of a^i_{Cl} is necessary in order to estimate kinetic constants for Cl transport across the serosal membrane as well as to establish the degree of active Cl^- uptake across the mucosal membrane. Despite considerable effort, the true value of a^i_{Cl} remains somewhat uncertain because cell Cl^- only fell to an apparent value of 6.5 mM when tissues were exposed to nominally Cl^--free medium. Substantial Cl^- also appeared to remain in rabbit papillary muscle [3] and renal proximal tubules [29] exposed to Cl^--free medium. The continued Cl^- signal was attributed to interference from other anions. That Cl^- was not retained in the cells of *Amphiuma* is evidenced from the titrimetric measurement of $[Cl]^i$. HCO_3^- can be excluded as a significant interferent of the Cl microelectrode (i.e., >1 mM) because the HCO_3^- activity and the exchanger sensitivity to HCO_3^- are too low, and because a^i_{Cl} in gluconate medium was not different whether HCO_3^- was present in the medium or not. Likewise, gluconate anions probably did not contribute to the apparent Cl^- signal. Possibly phosphate and organic anions other than HCO_3^- react with the exchanger in the cytoplasm.

Because of the uncertainty in the estimate of a^i_{Cl}, the possibility cannot be discounted that Cl^- is actively accumulated across the luminal membrane. However, if it occurs, the uptake does not elevate a^i_{Cl} more than a few meq/liter. Supportive of the presence of luminal uptake is the recent observation [35] that luminal Cl^- stimulates a Cl^- current maxi-

mally at about 15 mM, a value considerably below the concentration (40 mM) at which the serosal Cl^- transport step is saturated (Fig. 8).

Saturable Serosal Efflux: Na-Cl-HCO_3 Exchange

Replacement of serosal Cl^- produced no detectable change in a^i_{Cl}. If the transport of Cl^- across the serosal membrane was strictly passive and occurred through conductive channels, then J^{Cl}_{ms} should increase proportionate to the increase in a^i_{Cl}. Instead serosal Cl^- transfer attained a maximal value when luminal [Cl] was 40 mM (Fig. 7). This finding is indicative of the presence of a saturable process underlying Cl transport from the cell into the serosal medium. Considerable evidence has been developed in recent years for the presence of an anion exchange process in the serosal membrane of *Amphiuma* intestinal cells [13, 31, 34]. For example, the value of a^i_{Cl} is normal when HCO_3^- is absent from the bathing medium [27], yet Cl^- absorption from the cell into the serosal medium is completely blocked [31]. Also, inhibitors of anion exchange such as SITS and DIDS, inhibit all net Cl absorption when added to the serosal medium [31, 35] and greatly reduce the secretion of HCO_3^-, a process which is dependent on the presence of Cl^- in the mucosal medium [13]. Despite the strong evidence in support of serosal anion exchange it is unlikely that a simple 1-for-1 exchange of Cl^- for HCO_3^- is occurring because it is energetically infeasible. The concentration gradients for these ions across the serosal membrane are such that linked exchange would tend to bring Cl into the cell across the serosal membrane in exchange for HCO_3^- ions [14], i.e., opposite the observed direction of transport. However, if the exchange is coupled to the transmembrane Na gradient at the serosal membrane, then Cl^- efflux from and HCO_3^- entry into the cell could occur. In support of this view, it has been shown that Cl^- absorption by *Amphiuma* intestine is dependent on the presence of sodium in the serosal fluid [35]. Likewise, the secretion of HCO_3^- by the same tissue is also dependent on Na^+ in the serosal medium [36]. We have proposed that there is a carrier which binds Na^+, Cl^- and HCO_3^- and, in the steady state, transports Cl^- out of the cell into the serosal medium as Na^+ and HCO_3^- enter into the cytoplasm, i.e., a secondary active transport of Cl^- [14]. This may be similar to the carrier systems operating in *Necturus* kidney [4, 10] and squid giant axon [27], which simultaneously transports Na^+, Cl^-, H^+ and HCO_3^- ions. This mechanism would ultimately rely on the energy of the sodium gradient developed by the operation of the Na^+-K^+ pump in the serosal membrane. In agreement with this, it was previously shown that ouabain completely blocks electrogenic Cl^- absorption by *Amphiuma* small intestine [31].

Another explanation for the constancy of a^i_{Cl} upon replacement of serosal Cl^- (Fig. 1) is that a conductive efflux of Cl^- was increased, but that the expected decline of a^i_{Cl} was offset by a simultaneous increase in mucosal Cl uptake. However, in a related study it was shown that the absorptive (*ms*) flux of tracer ^{36}Cl is not increased but rather is reduced following replacement of serosal Cl^- with gluconate [33]. The reduction is attributable to elimination of serosal Cl-Cl exchange, a process which normally accelerates the efflux of ^{36}Cl from the cell. Thus there is little direct support for a conductive pathway for Cl^- in the serosal membrane. Serosal Cl efflux occurs primarily, perhaps exclusively, by secondary active transport.

Mucosal Membrane Cl Leak Pathway

Cell Cl dropped rapidly when the mucosa was exposed to Cl-free media (Fig. 3). The fall could not be due to serosal pumping of Cl because the rate of decline was equally great when serosal transfer was blocked by the inclusion of SITS in the serosal bath. The decline must be due to a high luminal permeability for Cl^-. The rate of decline seen with microelectrodes was greater than seen with ^{36}Cl. The point is not important from a qualitative sense, i.e., the results by either approach show that Cl moves quickly from the cell. Possibly the diffusion of ^{36}Cl ions away from the mucosal surface into the bulk luminal medium may be slowed by barriers such as those produced by mucus. Alternatively, the slower ^{36}Cl washout may reflect, in part, a slower loss of Cl from nonvillus epithelial cells.

The efflux is rapid and behaves as though it occurs via single-file diffusion through a channel [11], since replacement of most of the Cl in the luminal medium with a nonabsorbed anion-accelerated Cl efflux, a result completely opposite that expected if there was Cl-Cl exchange. We have shown that when the [HCO_3] of the serosal medium is reduced to a very low value a *sm* flux of ^{36}Cl can be demonstrated, which is blocked by serosal SITS but which is completely unaffected by mucosal SITS, indicating that the flux is transcellular but does not involve anion exchange at the luminal membrane [33]. A mucosal conductive pathway for Cl is completely consistent with earlier observations on the effect of theophylline on Cl^- transport [32]. It was observed that theophylline blocked net Cl absorption and stimulated the secretory Cl flux (but not net Cl

transport) without changing a^i_{Cl}. These facts could be understood if elevation of cyclic AMP enhances luminal Cl permeability. The results in Table 6 furnish support for this conclusion. A luminal Cl^- leak pathway has also been proposed for rat [19] and rabbit small intestine [7].

Replacement of mucosal medium Cl produced depolarization of the mucosal membrane which was evident in acute measurements (Fig. 2) and in the steady-state values (Fig. 5). In contrast, replacement of Cl^- did not change v_m in rabbit ileum [26]. The depolarization cannot be attributed to a transepithelial Cl^- diffusion potential since the average value of v_m observed when a maximal Cl^- gradient was present across the epithelium (−32 mV) was identical to the value obtained in tissues bathed on both sides with medium of normal [Cl] (*cf.* Fig. 1). The depolarization was not detectable in steady-state measurements until the mucosal [Cl] was 20 mM or less (Fig. 5). Hence, the change in v_m may be due to a reduction in electrogenic Cl^- entry. In agreement, as recently reported, the ability of Cl^- ions added to the mucosal medium to induce a Cl^- current toward the serosal medium is maximal at about 15 mM Cl [35]. The nature of electrogenic Cl^- entry remains unclear because of the uncertainties in estimating a^i_{Cl}.

Mucosal Membrane Cl^- Entry Pathway

Since Cl^- leaves the cell very rapidly across the mucosal membrane (Table 6), it follows that in the steady state Cl^- uptake occurs even more rapidly to account for net Cl^- absorption. Cl^- uptake by the luminal membrane has been attributed to NaCl cotransport in bullfrog [2] and rabbit small intestine [22], to Cl^--HCO_3^- exchange in rat small intestine [19], and to Na-K-Cl cotransport in flounder small intestine [21]. Arguing against a luminal uptake mechanism for *Amphiuma* is the observation that a^i_{Cl} was not influenced by luminal Na^+, K^+, or HCO_3^- as would be expected if a cotransport process or anion exchange was responsible for Cl^- uptake. Also, the normalized a^i_{Cl} was a linear function of the luminal [Cl], when bath [Cl] exceeded 1 mM, as would be expected if passive diffusion determined a^i_{Cl}. When the measured values of a^i_{Cl} were corrected for interference by other anions Cl^- was distributed close to electrochemical equilibrium ($E_{Cl} - v_m > 0$) at all but the lowest luminal [Cl] (Fig. 5). In summary, the weight of evidence is for passive Cl^- movement across the luminal membrane.

Nevertheless, at the lower luminal Cl^- concentrations Cl^- appeared to be above electrochemical equilibrium after making a reasonable correction for interference by other anions. For this reason an active uptake mechanism for Cl^- cannot be discounted. If Cl^- uptake occurs by a rheogenic process and is in parallel with a Cl^- conductance then Cl^- might well appear to be in or near electrochemical equilibrium. The value of a rheogenic pump is that Cl^- would be distributed close to electrochemical equilibrium, thereby minimizing the driving force for Cl^- backflux.

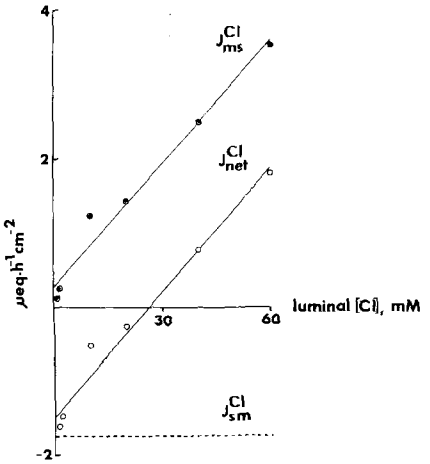


Fig. 11. Estimate of lowest luminal [Cl] achievable by Cl^- absorptive mechanism. Values of J^{Cl}_{ms} were taken from Fig. 7. The value of J^{Cl}_{sm} is taken as the value of J^{Cl}_{ms} measured after SITS addition. J^{Cl}_{net} is the difference. The lines through the points were obtained by linear regression. J^{Cl}_{net} is zero when luminal [Cl] = 26 mM

Theoretical Cl^- Gradient Achieved by Intestinal Mucosa

An analysis of the unidirectional Cl^- fluxes reported in Figs. 5 and 8, indicate that the short-circuited small intestinal mucosa should lower the luminal [Cl] to about 26 mM. The graphical analysis, illustrated in Fig. 11, is predicated on the assumption that the blood-to-lumen Cl^- flux is approximated by the lumen-to-blood flux remaining after exposure to SITS, i.e., the stilbene-insensitive Cl^- flux. In support, it was earlier observed that after exposure to stilbene the unidirectional Cl^- fluxes were equal [31]. In agreement with the view that the luminal [Cl] should decline substantially, Rabinovitch observed that the luminal [Cl] fell to as low as 34 mM (i.e., 0.2% NaCl) in in-vivo intestinal loops of dog duodenum [24]. The power of intestinal active Cl transport was dramatically illustrated in the work of Ingraham and Visscher who demonstrated that the luminal [Cl] fell to 0.4 mM (corresponding to a blood-to-lumen [Cl] gradient of 250 : 1 !) 90 min af-

ter an isotonic mixture of NaCl and Na_2SO_4 was instilled into loops of dog intestine [15]. While dramatic, these early studies do not define the extent to which the luminal [Cl] may fall below that of the blood [Cl] in the normal, unligated intestine. Although the mechanism of Cl transport has not been characterized in dog intestine it is noteworthy that, as in the urodele, Cl absorption by the intestine is linked to the secretion of HCO_3^- [16].

To the extent that another anion, HCO_3^-, replaces absorbed Cl^- in the lumen, then the osmolarity of the luminal fluid will remain constant and the lumen [Cl] will decline. Otherwise, the loss of an osmotic constituent will promote water absorption and concentrate the remaining solute. The partial coupling of Cl^- to HCO_3^- should render overall net Cl absorption less electrogenic and may permit a true reduction in the luminal Cl concentration.

Magnitude of the Cl^- Absorptive Flux

The existence of a large backleak of Cl^- across the mucosal membrane has heretofore made it very difficult to estimate the magnitude of the absorptive flux of Cl^- into the intestinal cell. However, the characterization of Cl^- transport provided by this study permits an estimate of this important variable. In the steady state, the flux of Cl^- into the cell from the mucosal (J_{mc}) and serosal (J_{sc}) media is equal to the efflux of Cl^- across the same membranes (J_{cm} and J_{cs}). For tissues incubated on their serosal surface in Cl^--free medium then, $J_{sc} = 0$ and $J_{mc} = J_{cm} + J_{cs}$. When the rate constants for labeled Cl^- loss from loaded tissue were measured within the same tissues (Fig. 10), they were found to be very similar and from compartments of similar capacity. Assuming that these fluxes were largely from the absorptive epithelial cells (the tissue-to-serosa flux was reduced more than 80% by SITS [Fig. 10]), then it follows that the influx of Cl^- across the mucosal membrane (J_{mc}) is twice that of Cl^- backflux (J_{cm}) and also twice that of serosal efflux (J_{cs}). Thus, when net Cl^- absorption is 2 μeq/hr · cm^2 then Cl^- uptake across the luminal membrane is about 4 μeq/hr · cm^2.[1]

[1] This conclusion is supported by the following: Assuming a cell volume of 30 pl and a density of 200,000 cells/cm^2 (cell surface area = 500 μm^2), then it can be shown, using a value for cytoplasmic [Cl] of 25 mM (Fig. 5) and the initial rate of Cl^- loss upon luminal washout (Fig. 2), that the rate of Cl^- transport from cell to serosa (7×10^{12} ions/cell/hr) is about equivalent to the rate of Cl^- loss from cell to mucosal medium (6×10^{12} ions/cell/hr) when net Cl^- transport is 2 μeq/hr · cm^2. That is, Cl^- ions in the cytoplasm leak into the luminal medium at the same rate as they are transported into the serosal fluid.

Paracellular Cl^- Transport Pathway

In the presence of SITS, the absorptive Cl^- flux was strictly proportional to mucosal bath [Cl] in accordance with simple diffusion. The serosal membrane of the villus absorptive cells does not appear to have a large permeability for Cl^-, and thus it is unlikely that these cells offer a significant transepithelial pathway for Cl^- diffusion. Most likely this flux occurs through paracellular pathways, but it cannot be discounted that nonvillus epithelial cells or goblet cells permit Cl^- movement. The role of the paracellular pathway in intestinal ion transport has been discussed by Schultz et al. [28].

In summary, these results indicate that Cl ions are transported out of the intestinal cell by a saturable, stilbene-inhibitable mechanism. The mechanism has been previously characterized tentatively as Na-Cl-HCO_3 exchange, a secondary active transport mechanism. There is little or no Cl-conductive flux across the serosal membrane. Mucosal Cl entry across the brush border occurs at a rate which is twice the rate of net Cl^- absorption, is independent of luminal Na and K, and does not occur as a result of Cl-HCO_3 exchange. Luminal membrane Cl uptake occurs in parallel with a passive Cl efflux which is enhanced by elevation of cell cyclic AMP. A parallel, stilbene-insensitive and nonsaturable component of the transepithelial Cl flux occurs most likely through the paracellular pathway.

This work was supported by U.S. Public Health Service Grants AM 17361 and AM 26870.

References

1. Armstrong, W.McD., Bixenmann, W.R., Frey, K.F., Garcia-Diaz, J.F., O'Regan, M.G., Owens, J.L. 1979. Energetics of coupled Na^+ and Cl^- entry into epithelial cells of bullfrog small intestine. *Biochim. Biophys. Acta* **551**:207–219
2. Armstrong, W.McD., Garcia-Diaz, J.F. 1980. Ion-selective microelectrodes: Theory and technique. *Fed. Proc.* **39**:2851–2859
3. Baumgarten, C.M., Fozzard, H.A. 1981. Intracellular Cl^- activity in mammalian ventricular muscle. *Am. J. Physiol.* **241**:C121–C129
4. Boron, W.F., Boulpaep, E.L. 1983. Intracellular pH regulation in the renal proximal tubule of the salamander. Basolateral HCO_3^- transport. *J. Gen. Physiol.* **81**:53–94
5. Cotlove, E. 1963. Determination of true chloride content of biological fluids and tissues. II. Analysis by simple, nonisotopic methods. *Anal. Chem.* **35**:101–105
6. Duffey, M.E., Thompson, S.M., Frizzell, R.A., Schultz, S.G. 1979. Intracellular chloride activities and active chloride absorption in the intestinal epithelium of the winter flounder. *J. Membrane Biol.* **50**:331–341
7. Field, M. 1971. Ion transport in rabbit ileal mucosa. II. Effect of cyclic 3'5'-AMP. *Am. J. Physiol.* **221**:992–997

8. Frizzell, R.A., Field, M., Schultz, S.G. 1979. Sodium-coupled chloride transport by epithelial tissue. *Am. J. Physiol.* **236:***F1–F8*
9. Grinstein, S., Turner, R.J., Silverman, M., Rothstein, A. 1980. Inorganic anion transport in kidney and intestinal brush border and basolateral membranes. *Am. J. Physiol.* **238:***F452–F460*
10. Guggino, W.B., London, R., Boulpaep, E.L., Giebisch, G. 1983. Chloride transport across the basolateral cell membrane of the *Necturus* proximal tubule: Dependence on bicarbonate and sodium. *J. Membrane Biol.* **71:**227–240
11. Hodgkin, A.L., Keynes, R.D. 1955. The potassium permeability of a giant nerve fiber. *J. Physiol. (London)* **128:**61–88
12. Hubel, K.A. 1969. Effect of luminal chloride concentration on bicarbonate secretion in rat ileum. *Am. J. Physiol.* **217:**40–45
13. Imon, M.A., White, J.F. 1981. Intestinal HCO_3^- secretion measured by pH stat in vitro: Relationship to metabolism and transport of Na^+ and Cl^-. *J. Physiol. (London)* **314:**429–443
14. Imon, M.A., White, J.F. 1986. Microelectrode measurement of intracellular pH and HCO_3 in intestinal cells. *Am. J. Physiol. (Submitted)*
15. Ingraham, R.C., Visscher, M.B. 1936. The production of chloride-free solutions by the action of the intestinal epithelium. *Am. J. Physiol.* **114:**676–680
16. Kinney, V.R., Code, C.F. 1964. Canine ileal chloride absorption: Effect of carbonic anhydrase inhibitor on transport. *Am. J. Physiol.* **207:**998–1004
17. Langridge-Smith, J.L., Field, M. 1981. Sulfate transport in rabbit ileum: Characterization of the serosal border anion exchange process. *J. Membrane Biol.* **63:**207–214
18. Liedtke, C.M., Hopfer, U. 1982. Mechanism of Cl translocation across small intestinal brush border membrane: I. Absence of Na^+-Cl cotransport. *Am. J. Physiol.* **242:***G263–G271*
19. Liedtke, C.M., Hopfer, U. 1982. Mechanism of Cl^- translocation across small intestinal brush-border membrane: II. Demonstration of Cl^--OH^- exchange and Cl^- conductance. *Am. J. Physiol.* **242:***G272–G280*
20. Murer, H., Hopfer, U., Kinne, R. 1976. Sodium/proton antiport in brush-border-membrane vesicles isolated from rat small intestine. *Biochem. J.* **154:**597–604
21. Musch, M.W., Orellana, S.A., Kimberg, L.S., Field, M., Halm, D.R., Krasny, E.J., Jr., Frizzell, R.A. 1982. Na^+-K^+-Cl^- cotransport in the intestine of a marine teleost. *Nature (London)* **300:**351–353
22. Nellans, H.N., Frizzell, R.A., Schultz, S.G. 1973. Coupled sodium-chloride influx across the brush border of rabbit ileum. *Am. J. Physiol.* **225:**467–475
23. Quay, J.F., Armstrong, W.McD. 1969. Sodium and chloride transport by isolated bullfrog small intestine. *Am. J. Physiol.* **217:**694–702
24. Rabinovitch, J. 1927. Factors influencing the absorption of water and chlorides from the intestine. *Am. J. Physiol.* **82:**279–289
25. Ramos, M.M.P., Ellory, J.C. 1981. Na and Cl transport across the isolated anterior intestine of the *Plaice pleuronectes* platessa. *J. Exp. Biol.* **90:**123–142
26. Rose, R.C., Schultz, S.G. 1971. Studies on the electrical potential profile across the rabbit ileum. *J. Gen. Physiol.* **57:**639–663
27. Russell, J.M., Boron, W.F. 1982. Intracellular pH regulation in squid giant axons. *In:* Intracellular pH: Its measurement, regulation and utilization in cellular function. R. Nuccitelli and D. Deamer, editors. pp. 221–237. Liss, New York
28. Schultz, S.G., Frizzell, R.A., Nellans, H.N. 1974. Ion transport by mammalian small intestine. *Annu. Rev. Physiol.* **36:**51–91
29. Spring, K.R., Kimura, G. 1978. Chloride reabsorption by renal proximal tubules of *Necturus*. *J. Membrane Biol.* **38:**233–254
30. Turnberg, L.A., Bieberdorf, F.A., Morawski, S.G., Fordtran, J.S. 1970. Interrelationships of chloride, bicarbonate, sodium and hydrogen transport in human ileum. *J. Clin. Invest.* **49:**557–567
31. White, J.F. 1980. Bicarbonate-dependent chloride absorption in small intestine: Ion fluxes and intracellular chloride activities. *J. Membrane Biol.* **53:**95–107
32. White, J.F. 1981. Chloride transport and intracellular chloride activity in the presence of theophylline in *Amphiuma* small intestine. *J. Physiol. (London)* **321:**331–341
33. White, J.F. 1986. Evidence against luminal 1 for 1 Cl -HCO_3 exchange. *Am. J. Physiol. (in press)*
34. White, J.F., Burnup, K., Ellingsen, D.E. 1986. Effect of sugars and amino acids on amphibian intestinal Cl transport and intracellular Na^+, K^+ and Cl^- activity. *Am. J. Physiol.* **250:***G109–G117*
35. White, J.F., Ellingsen, D., Burnup, K. 1984. Electrogenic Cl^- absorption by *Amphiuma* small intestine: Dependence on serosal Na^+ from tracer and Cl^- microelectrode studies. *J. Membrane Biol.* **78:**223–233
36. White, J.F., Imon, M.A. 1982. Intestinal HCO_3 secretion in *Amphiuma*: Stimulation by mucosal Cl and serosal Na^+. *J. Membrane Biol.* **68:**207–214
37. Zeuthen, T., Ramos, M., Ellory, J.C. 1978. Inhibition of active chloride transport by piretanide. *Nature (London)* **273:**678–680

Received 10 September 1985; revised 4 April 1986