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# Bicarbonate-Dependent Chloride Absorption in Small Intestine: Ion Fluxes and Intracellular Chloride Activities

John F. White

Department of Physiology, Emory University, Atlanta, Georgia 30322

**Summary.** Proximal, stripped segments of small intestine from the urodele *Amphiuma* were short-circuited in media containing $Na^+$, $Cl^-$ and $HCO_3^-$. Under these conditions there was a large net absorption of $Cl^-$, a small net absorption of $Na^+$ and a residual flux ($J^R_{Net}$) consistent with $HCO_3^-$ secretion. Net $Cl^-$ absorption correlated with the short-circuit current ($I_{sc}$); net $Na^+$ absorption correlated negatively with $J^R_{Net}$. Acetazolamide eliminated the $I_{sc}$, lowered $Cl^-$ absorption by 50%, and reduced net $Na^+$ absorption without altering $J^R_{Net}$. Benzolamide inhibited the $I_{sc}$ more rapidly when applied on the mucosal surface. Replacement of $Na^+$ or $HCO_3^-$ (and $CO_2$) in the media eliminated the $I_{sc}$, net $Cl^-$ absorption and the residual flux. Likewise, inclusion of the stilbene SITS in the serosal media eliminated the $I_{sc}$, net $Cl^-$ absorption and the residual flux. The cytoplasmic activity of $Cl^-$ ($a^i_{ci}$) was determined with single and double-barreled microelectrodes. The $a^i_{ci}$ of villus absorptive cells in normal media was 21.0 mM and in excess of that expected on the basis of electrochemical equilibrium of $Cl^-$ at the mucosal membrane. Active $Cl^-$ accumulation was also observed in the presence of acetazolamide but was eliminated upon replacement of media $Na^+$ with choline. The mucosal membrane potential was depolarized upon replacement of media $Na^+$. It is concluded that $Cl^-$ is actively absorbed into intestinal cells of *Amphiuma* by an electrogenic process located in the mucosal membrane. Depending on the level of intracellular $HCO_3^-$, accumulated $Cl^-$ may diffuse passively back into the mucosal media or undergo exchange with bath $HCO_3^-$ at the serosal membrane.

It has been known for several years that $Cl^-$ absorption is an active process in both mammalian [5, 19, 25] and amphibian [23] small intestine. Compelling evidence for this has developed from measurements of transepithelial fluxes of isotopically labeled $Cl^-$ in isolated, short-circuited intestinal segments. Some of these studies have pointed to a link between the absorption of $Cl^-$ and the transport of $Na^+$ and $Cl^-$ by a carrier residing in the mucosal (brush border) membrane [19, 23]. Simultaneously, evidence has accumulated linking ileal $Cl^-$ absorption and the presence of another ion, $HCO_3^-$ [13, 14, 25]. In regard to this, recent observations in flounder [9] indicate that the presence of $HCO_3^-$ in the media bathing the small intestine stimulates absorption of $Cl^-$. Taken together these reports illustrate the complex nature of the $Cl^-$ absorptive process in the small intestine and point to the need for more extensive study of gut electrolyte transport.

We have reported earlier that the absorptive cells lining the proximal small intestine of the urodele, *Amphiuma,* accumulate $Cl^-$ above electrochemical equilibrium [26]. It was also observed that inclusion of $HCO_3^-$ in the bathing media induces a serosa-negative transmural potential that depends on the presence of $Cl^-$ as well [10]. The study reported here was conducted to determine the influence of $Na^+$, $HCO_3^-$, and various agents which alter ion transport, on the absorption of $Cl^-$ in *Amphiuma* intestine. For this purpose, transmural fluxes of $^{22}Na$ and $^{36}Cl$ were measured in isolated, short-circuited intestinal segments. Also, intracellular $Cl^-$ activity was measured with $Cl^-$-specific microelectrodes.

## Materials and Methods

### *Animals*

Adult *Amphiuma* were maintained in fresh tap water at room temperature. The animals were anesthetized by incubating for 20 min in 0.2% chloretone. The small intestine was excised, rinsed with

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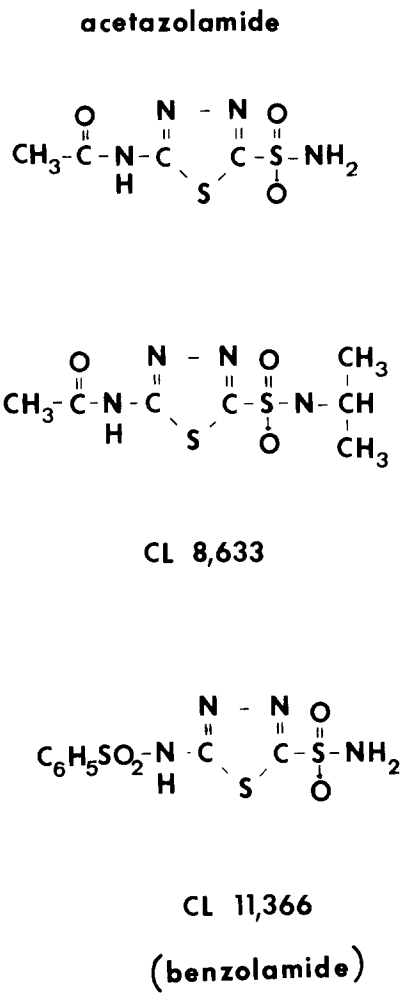


**Fig. 1.** The structure of acetazolamide and its analogues

buffer solution, and the proximal one-third (just distal to the attachment of the pancreas) was stripped of its serosal muscle layers by dissection with jeweler's forceps. Some of the stripped segments were opened and mounted as a sheet between two halves of Lucite chambers described previously [27] which were filled with a physiological media. These chambers exposed 31.5 $mm^2$ of tissue. Other segments were mounted as a sheet in a Lucite chamber described below which allowed measurement of intracellular chloride activities.

*Bathing Media*

The normal bathing media contained (in meq/liter) 95 $Na^+$, 2.5 $K^+$, 0.9 $Ca^{++}$, 1.0 $Mg^{++}$, 73.8–75.6 $Cl^-$, 25 $HCO_3^-$, and 20 mM mannitol buffered to pH 7.4 by gassing with 95% $O_2$, 5% $CO_2$. In one series ($HCO_3^-$-free) $HCO_3^-$ was replaced on an equimolar basis with $Cl^-$ and the pH (7.4) maintained with Tris hydroxymethylaminomethane. Acetazolamide was obtained from Sigma. Two analogs of acetazolamide were also used in these experiments. Benzolamide (Cl 11,366) was a gift of Lederle Laboratories (Pearl River, N.Y.) and the $N^5$-isopropyl derivative of acetazolamide (Cl 8,633) was a gift of Dr. Thomas Maren. The structure of acetazolamide and its analogues is shown in Fig. 1. SITS (4-acetamido-4′-isothiocyano-2′-disulfonic stilbene) was purchased from Polysciences (Warrington, Pa.). Sodium glycodiazine was a gift of Schering.

*Short-Circuiting Technique*

Transmural potential difference ($\psi_{ms}$) and short-circuit current ($I_{sc}$) were measured in paired tissues with voltage clamp devices which compensated automatically for the resistance of the solution between the potential-sensing electrode and the tissue. Current-passing bridges were formed from the same media used to bathe the tissue. Prior to sacrificing the animal, the tissue chambers were assembled and pre-equilibrated with the appropriate bathing media to reduce potential offsets. With the tissue in position, the media was frequently replaced with fresh solution pre-equilibrated with the gas mixture.

*Ion Flux Measurements*

Unidirectional fluxes of $Na^+$ and $Cl^-$ were determined simultaneously under short-circuit conditions in paired adjacent segments of small intestine using $^{22}Na$ and $^{36}Cl$. In one segment the mucosa-to-serosa flux of both isotopes was measured, while in the other segment the opposite flux was measured. Since this approach requires that the two segments are nearly identical in their transport behavior, flux measurements were terminated when ever adjacent segments had resistances differing by more than 25%. The tissue resistance ($R_t$) was calculated as the ratio of $\psi_{ms}$ and $I_{sc}$ and is expressed in $\Omega \cdot cm^2$. The value of $R_t$ is the mean of all flux periods unless stated otherwise.

Tissues were exposed to unlabeled solutions for 2–3 hr to allow the $I_{sc}$ to approach a steady state. $^{22}Na$ and $^{36}Cl$ were added to a final concentration of 1 μCi/ml. Since addition of $^{36}Cl$ (as NaCl) causes a significant increase in bath NaCl concentration, an aliquot of the bath was removed prior to isotope addition and substituted with an appropriate volume of NaCl-free buffer. Preliminary experiments established that the unidirectional isotope fluxes were constant within 1 hr. Aliquots of 0.1 ml were removed from the opposite bath at 20-min intervals beginning 1 hr after isotope addition. Removal of an aliquot was accompanied by replacement of an equal volume of unlabeled bathing media. In two series after seven flux periods, acetazolamide was added to both sides of both chambers. Thirty minutes later sampling recommenced. Since the addition of acetazolamide caused only a 2% dilution of the isotope, no correction of the flux calculation was made. Specific activity was determined from multiple samples of the half-chamber to which isotope was added.

The samples were first counted for $^{22}Na$ using a Packard gamma spectrometer then both $^{22}Na$ and $^{36}Cl$ were counted using a packard liquid scintillation spectrometer. The $^{36}Cl$ activity was calculated by subtraction of the $^{22}Na$ activity after determining the relative efficiency of the separate counting systems for $^{22}Na$. The counts were subsequently corrected for the change in chamber volume produced by the sampling procedure and the fluxes expressed as μeq/hr $cm^2$. In this way the unidirectional fluxes from mucosa to serosa ($J_{m\to s}$) and serosa to mucosa ($J_{s\to m}$) were calculated. Measured values of $I_{sc}$ were also converted to μeq/hr $cm^2$. The average $I_{sc}$ (mean of both paired segments) was compared to the net fluxes of Na ($J^{Na}_{net}=J^{Na}_{m\to s}-J^{Na}_{s\to m}$) and Cl ($J^{Cl}_{net}$) observed in the same flux period using the equation $I_{sc}=J^{Na}_{net}+J^{Cl}_{net}+J^{R}_{net}$. $J^{R}_{net}$, the residual flux, represents the inequality between $I_{sc}$ and the summed net $Na^+$ and $Cl^-$ fluxes and presumably then is the sum of all additional net ion fluxes occurring in the tissue.

**Table 1.** Ion transport in media containing $Cl^-$ and $HCO_3^-$ (normal media) and following addition of acetazolamide

| | $J^{Na}_{m\to s}$ | $J^{Na}_{s\to m}$ | $J^{Na}_{net}$ | $J^{Cl}_{m\to s}$ | $J^{Cl}_{s\to m}$ | $J^{Cl}_{net}$ | $I_{sc}$ | $J^{R}_{net}$ | $R_t$ | |
|---|---|---|---|---|---|---|---|---|---|---|
| Normal media (10:60) | 4.66±0.14 | 4.39±0.16 | 0.26±0.19 | 2.65±0.10 | 1.28±0.06 | 1.37±0.06 | −0.29±0.04 | 0.81±0.32 | 93.3±2.3 | [32] |
| +Acetazolamide (10:60) | 4.22±0.19 | 4.26±0.17 | −0.08±0.27 | 2.14±0.13 | 1.43±0.10 | 0.70±0.15 | 0.07±0.02 | 0.85±0.35 | 102.7±2.9 | [35] |

Fluxes and $I_{sc}$ are in μeq/hr cm² ± 1 SEM. In parentheses is number of animals:number of flux periods. Acetazolamide was present at $10^{-4}$ M. $R_t$ is in Ω cm² and is the mean value calculated for the number of flux periods in the adjacent brackets.

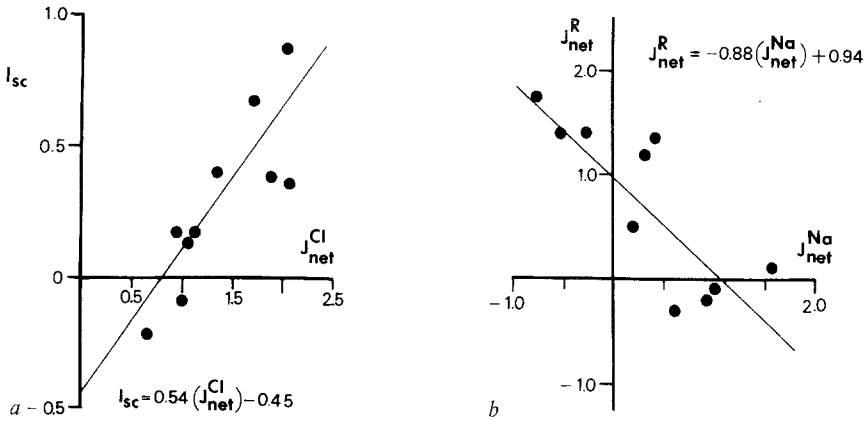


**Fig. 2.** Flux relationships for 10 animals in control media. Units are μeq/hr cm². Lines drawn are least squares fit of the data points. (*a*): Positive correlation between $I_{sc}$ and $J^{Cl}_{net}$. (*b*): Negative correlation between $J^{R}_{net}$ and $J^{Na}_{net}$

*Statistical Tests*

Students *t* test for paired or unpaired comparisons was used to determine the significance of the difference between population means.

*Intracellular Chloride Activity Measurements*

Intracellular chloride activity ($a^i_{Cl}$) was measured using Corning liquid ion exchanger in either single or double-barreled microelectrodes fabricated as described previously [26]. For double-barreled microelectrodes, one barrel was used to sense the mucosal membrane potential ($\psi_m$), while the other sensed the chloride potential ($\psi_{Cl}$). The output from the latter barrel was amplified with an Analog Devices 42J amplifier (input impedance $10^{13}$ Ω) and then displayed on a Keithley electrometer. The membrane potential was amplified with a WPI amplifier and displayed on a Digitec panel meter. Both potentials were also recorded on a Brush recorder. The difference in potential of the two barrels was measured with a Digitec digital voltmeter operating in the differential mode. The criteria for acceptable recordings is discussed in Results. The slope of each microelectrode was determined from its response to $HCO_3^-$-free solutions of NaCl of 10 and 100 mM. We have reported previously that the response of the $Cl^-$ exchanger in microelectrodes is linear between the range of 10 and 100 mM as exemplified by a correlation coefficient between the potential and log $a_{Cl}$ of greater than 0.999 [26]. Intracellular chloride activity was calculated using the equation:

$$a^i_{Cl} = a''_{Cl}/e^{2.303\{(\psi_i - \psi_m) - \psi''\}/s}$$

where $\psi_i$ is the electrode potential in the cell and $a''_{Cl}$ is the activity of chloride and $\psi''$ the potential of the chloride electrode in 100 mM NaCl. The method of calculating $a^i_{Cl}$ and the selectivity of the electrode for $Cl^-$ over $HCO_3^-$ and $H_2PO_4^-$ has been reported previously [26]. The electrode response was not altered in the presence of choline (95 mM) or acetazolamide ($10^{-4}$ M).

For intracellular activity measurements a simple Lucite chamber was used which allowed the intestinal sheet to be stretched and pinned to minimize motility. When necessary the segment could be stretched further during the course of the experiment. This design precluded simultaneous measurement of the transmural potential. The chamber also featured inlet and outlet ports for continued perfusion of fresh, oxygenated amphibian media.

Activity measurements commenced two hours after removal of the tissue from the animal. At this time the electrical properties of the tissue were nearly equilibrated as judged by previous experiments [10]. In those experiments in which the effect of acetazolamide was examined, the tissue was allowed to equilibrate two hours in normal buffer prior to exposure to buffer containing $10^{-4}$ M acetazoamide. Microelectrode measurements commenced one hour later.

## Results

### *Sodium and Chloride Fluxes*

*Normal buffer.* We have previously reported that proximal segments of small intestine from *Amphiuma*, when stripped of their muscle layers and incubated in a chloride-based media containing $HCO_3^-$, generate a transmural potential ($\psi_{ms}$) serosa negative to mucosa [10]. In order to define the ion transport underlying this behavior, paired adjacent segments of proximal intestine were maintained in the short-circuited state while the unidirectional mucosa-to-serosa flux ($J^{Na}_{m\to s}$) or serosa-to-mucosa flux ($J^{Na}_{s\to m}$) of both sodium and chloride were measured using $^{22}Na$ and $^{36}Cl$. In Table 1 it is seen that the short-circuit current

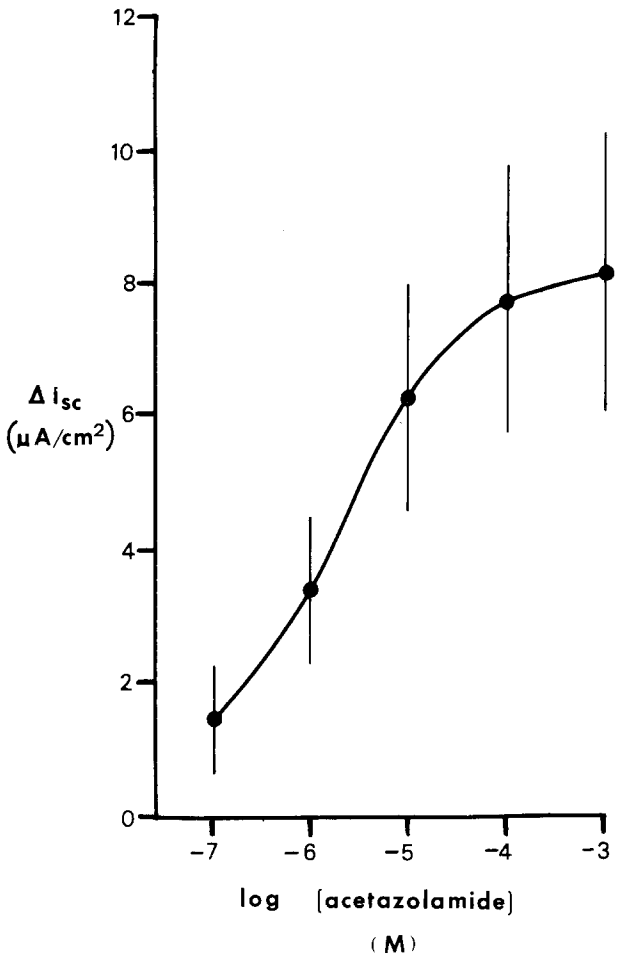


**Fig. 3.** Dose-response relationship for acetazolamide. Points are mean ±SEM of 5 tissues exposed to increasing concentrations of acetazolamide. The curve was fit by eye

($I_{sc}$) was negative under these conditions, as reported previously [10]. Examination of the ion fluxes reveals a significant ($P<0.005$) net flux of $Cl^-$ ($J^{Cl}_{net}$) from mucosa to serosa. The net absorption of $Cl^-$ was far in excess of net $Na^+$ absorption, which was itself not significant ($P>0.05$). However, there was a significant ($P<0.005$) residual flux ($J^R_{net}$) consistent with absorption of a cation or secretion of an anion.

The relationship between the transport of $Cl^-$ and the $I_{sc}$ for the experiments of this series was examined further. The correlation coefficient between the mean $I_{sc}$ and the average net $Cl^-$ flux for paired tissues from ten animals was 0.82. This correlation was highly significant ($P<0.01$) and is illustrated in Fig. 2*a*. Furthermore, the correlation coefficient between the mucosa-to-serosa flux ($J^{Cl}_{m\to s}$) and $I_{sc}$ was significant ($P<0.05$) at 0.76 indicating that this flux was a principal determinant of the $I_{sc}$. In contrast, the correlation coefficient between the mean $I_{sc}$ and the oppositely directed flux ($J^{Cl}_{s\to m}$) was only 0.15,

**Table 2.** Rate of decline of $\psi_{ms}$ (μV/min) following mucosal or serosal exposure to benzolamide ($10^{-4}$ M) under two sets of conditions

| Condition | *n* | Mucosal | Serosal | Mucosal / Serosal |
|---|---|---|---|---|
| Normal media | 5 | 61 ± 27 | 25 ± 9 | 2.2 ± 0.2 |
| +Theophylline | 5 | 21 ± 2 | 58 ± 14 | 0.4 ± 0.1 |

Paired adjacent segments of intestine bathed in media with or without theophylline were exposed to benzolamide applied on the mucosal or serosal side. Rates are calculated from the slope of the response in the period between 10 and 90% of the total response.

suggesting that this ion movement was independent of the $I_{sc}$.

No strong correlation was observed between the fluxes of $Na^+$ and $Cl^-$. For example, there was a poor correlation ($r=0.31$) between $J^{Na}_{net}$ and $J^{Cl}_{net}$. Also the correlation coefficient between the unidirectional absorptive fluxes ($J_{m\to s}$) of $Na^+$ and $Cl^-$ was 0.50, and this was not significant ($P>0.05$). These facts suggest that $Cl^-$ absorption is not strongly coupled to other ion movements and is electrogenic.

There was also a strong but negative correlation between the mean net $Na^+$ flux and the mean residual flux.[1] The relationship between the fluxes is illustrated in Fig. 2*b*. The correlation coefficient was significant ($P<0.05$) at $-0.87$.

*Effect of acetazolamide.* Since the carbonic anhydrase inhibitor acetazolamide nearly abolishes the $I_{sc}$ under these conditions as reported previously [10] its effect on the ion fluxes was measured in the same tissues. As seen in Table 1 at $10^{-4}$ M, this agent reduced the $I_{sc}$ to zero while significantly reducing the net absorption of $Cl^-$ by nearly 50% ($P<0.01$). The reduction in net $Cl^-$ absorption was due almost solely to a significant ($P<0.05$) decline in the $m\to s$ flux of $Cl^-$.

[1] The significance of this correlation is somewhat ambiguous. As indicated in Eq. (1), the short-circuit current is the sum

$$I_{sc}=J^{Na}_{net}+J^{Cl}_{net}+J^{R}_{net} \quad (1)$$

of the net fluxes of $Na^+$ ($J^{Na}_{net}$) and $Cl^-$ ($J^{Cl}_{net}$) plus any additional ion fluxes lumped together as the residual flux ($J^R_{net}$). Clearly, when

$$I_{sc}=J^{Cl}_{net} \quad (2)$$

then

$$J^{Na}_{net}=J^{R}_{net}. \quad (3)$$

This illustrates that for *Amphiuma* small intestine, in which there is a close correlation between $I_{sc}$ and $J^{Cl}_{net}$, the correlation between Na transport and the electrogenic movement of all other (residual) ions, noted here, may simply be a consequence of the required electroneutrality. Certainty about the physiological significance of this latter correlation then must await independent assessment of these two variables.

The opposite flux ($J^{Cl}_{s\to m}$) was not altered. This observation is consistent with the correlation noted previously between $J^{Cl}_{net}$ and $I_{sc}$.

Acetazolamide appeared to have a significant effect on net sodium transport as well when compared within the same tissue. The small net absorptive flux was reversed to secretion, and this was a significant change ($P<0.05$). The change was due principally to a decline in $J^{Na}_{m\to s}$ which was significant ($P<0.01$) when compared in the same tissue. The residual flux was not affected. Acetazolamide appeared to produce a small increase in tissue resistance of about 10%. In summary, the effect of acetazolamide to lower the $I_{sc}$ is accompanied by a large reduction in net $Cl^-$ absorption and a smaller reduction in net $Na^+$ transport.

In order to determine whether higher concentrations of acetazolamide might completely inhibit $Cl^-$, absorption $Cl^-$ fluxes were measured in intestinal segments from two animals upon exposure to 5 mM acetazolamide. Although the inhibitor produced the expected lowering of the $I_{sc}$, the mean net $Cl^-$ flux for 18 flux periods remained quite high in both animals, averaging $1.3\pm0.1$ μeq/hr·cm$^2$. Thus acetazolamide, even at high concentrations, will not completely inhibit active $Cl^-$ absorption.

Acetazolamide lowered the $I_{sc}$ in concentrations as low as $10^{-7}$ M. In most tissues a maximal effect was obtained at $10^{-4}$ M, although in some tissues $10^{-3}$ M produced further changes. This is reflected in the curve of Fig. 3. At maximal concentrations acetazolamide reduced the $I_{sc}$ to near zero within 20 min. The effect showed no reversal after a 20-min exposure to normal buffer. The effect of acetazolamide to inhibit $Cl^-$ absorption has been observed in rat [22], dog [16], rabbit [21], and human [25] intestine but, when examined, was not accompanied by electrical effects [16, 21].

The ability of analogues of acetazolamide to alter the $I_{sc}$ paralleled their ability to inhibit carbonic anhydrase. An analogue which does not inhibit carbonic anhydrase, namely Cl 8,633 [17] had small, variable effects on the $\psi_{ms}$, while subsequent addition of acetazolamide was fully effective. This is illustrated for a single tissue in Fig. 4*a*. In contrast, the analogue CL 11,366 (benzolamide), which is an effective inhibitor of carbonic anhydrase [18] was as effective as acetazolamide at reducing the $I_{sc}$. This is demonstrated in Fig. 4*b*.

Benzolamide was also useful in determining the sidedness of carbonic anhydrase inhibition. Acetazolamide itself lowered the $I_{sc}$ as rapidly whether applied on the mucosal or the serosal side. However, when the larger and more slowly diffusing analogue, benzolamide, was applied to the mucosal side of an intestinal segment at $10^{-4}$ M, $\psi_{ms}$ declined more than twice as rapidly as in an adjacent segment exposed to the inhibitor on the serosal side. As shown in Table 2, the rate of decline of $\psi_{ms}$ was twice as great upon mucosal addition as upon serosal addition of benzolamide. One possible explanation for these results is that the inhibitor-sensitive site is localized near the mucosal border of the epithelial cells. An alternative explanation is that the serosal muscle layers and subcellular tissue represent an effective barrier to diffusion of benzolamide. However, this explanation is not supported by the finding that $\psi_{ms}$ in tissues preexposed to theophylline (which causes $\psi_{ms}$ to become serosa positive [10]) is more rapidly inhibited by benzolamide applied on the serosal side of the tissue. The absolute rates of decline are compiled in Table 2 in which it is seen that benzolamide lowered the theophylline-stimulated $\psi_{ms}$ more than twice as rapidly when added on the serosal side. This would not result if the subcellular tissues were an effective barrier to benzolamide diffusion.

*Removal of $HCO_3^-$.* Since it was already demonstrated that the polarity of the basal $\psi_{ms}$ could be reversed by removal of $HCO_3^-$ [9], the fluxes of sodium chloride were measured in short-circuited segments in $HCO_3^-$-free solutions gassed with 100% $O_2$ and buffered with Tris. As seen in Table 3, in $HCO_3^-$-free media the $I_{sc}$ was opposite that observed in normal buffer and net chloride absorption was eliminated. Comparison with the unidirectional $Cl^-$ fluxes in the presence of $HCO_3^-$ (Table 1) suggests that inhibition of net $Cl^-$ absorption resulted in part from a decline in the absorptive flux of $Cl^-$ and, in part, from an increase in $Cl^-$ backflux. Possibly related to this observation, the residual flux was also not different from zero ($P>0.20$). Net $Na^+$ absorption was, again, quite small in this series and of borderline significance ($P<0.05$). Thus, in the absence of $HCO_3^-$ in the media, both $Cl^-$ absorption and the residual flux are inhibited whereas net $Na^+$ transport continues.

It is also seen in Table 3 that in the absence of $HCO_3^-$ in the bathing media acetazolamide had little effect on the unidirectional $Cl^-$ fluxes. In contrast, the unidirectional $Na^+$ fluxes were increased significantly when compared within the same tissue ($P<0.01$). This resulted in a significant lowering of $J^{Na}_{net}$ ($P<0.01$) when compared within the same tissue. Acetazolamide produced a similar effect on net $Na^+$ absorption in $HCO_3^-$-containing media (Table 1). In $HCO_3^-$-free media acetazolamide appeared to produce a slight lowering of resistance (Table 3).

*Organic anion substitution for $HCO_3^-$.* $HCO_3^-$ was replaced with either of three different organic anions.

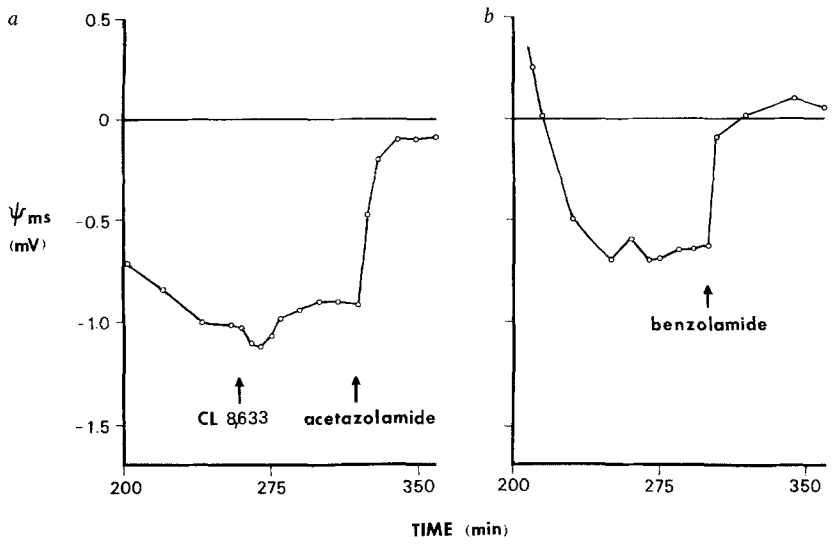


**Fig. 4.** Effect of acetazolamide analogues on transmural potential of separate tissues. The tissue was exposed on both surfaces to the agents at $10^{-4}$ M. (*a*): The analogue CL 8,633, which does not inhibit carbonic anhydrase, was added at the time indicated by the arrow followed by acetazolamide. (*b*): The effect of benzolamide

**Table 3.** Ion fluxes in $HCO_3$-free media and following addition of acetazolamide

| | $J^{Na}_{m\to s}$ | $J^{Na}_{s\to m}$ | $J^{Na}_{net}$ | $J^{Cl}_{m\to s}$ | $J^{Cl}_{s\to m}$ | $J^{Cl}_{net}$ | $I_{sc}$ | $J^{R}_{net}$ | $R_t$ |
|---|---|---|---|---|---|---|---|---|---|
| $HCO_3$-free [36] | 4.28±0.12 | 4.00±0.14 | 0.29±0.17 | 1.97±0.14 | 1.99±0.19 | −0.02±0.19 | 0.11±0.01 | −0.20±0.25 | 108.2±0.9 |
| +Acetazolamide [36] | 4.83±0.21 | 5.01±0.23 | −0.18±0.24 | 1.97±0.20 | 2.22±0.31 | −0.26±0.33 | 0.12±0.01 | 0.07±0.41 | 101.6±1.2 |

Fluxes were measured in six animals. Acetazolamide was used at a concentration of $10^{-4}$ M.

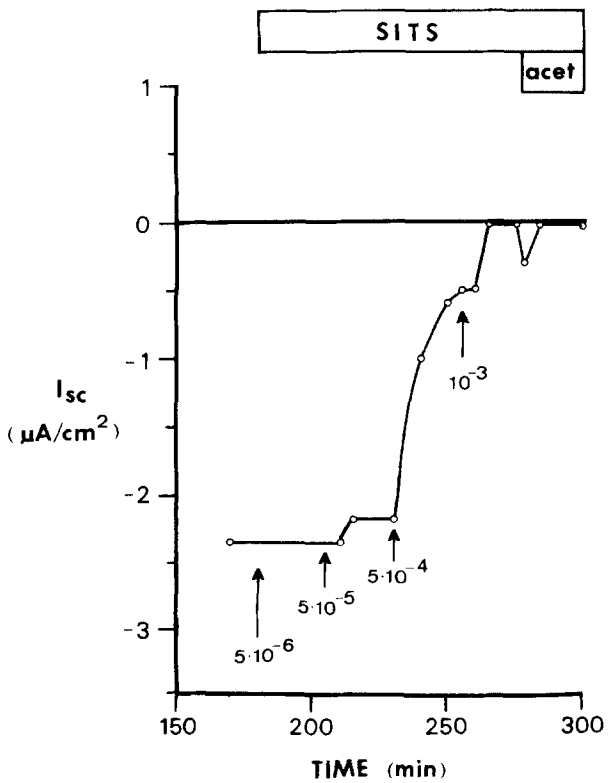


**Fig. 5.** (below left) Effect of SITS on $I_{sc}$. The agent was added in increasing concentrations at the times indicated by the arrow. Finally, acetazolamide was added at $10^{-4}$ M

**Table 4.** Comparison of basal transmural potential ($\psi_{ms}$) and response to acetazolamide ($\Delta\psi_{ms}$) in the presence of bicarbonate or another anion.

| Organic anion | *n* | $\psi_{ms}$ (mV) | $\Delta\psi_{ms}$ (mV) |
|---|---|---|---|
| Bicarbonate | 4 | −1.1±0.1 | 1.2±0.1 |
| Butyrate | 2 | 0.1±0.1 | 0.0 |
| Glycodiazine | 2 | 0.4±0.2 | 0.0 |
| Acetate | 4 | −0.2±0.2 | +0.2±0.1 |

All organic anions were used at 25 meq/liter. Acetazolamide was added at $10^{-4}$ M. The responses were recorded within 20 min after addition.

In Table 4 it is seen that, except for a slight effect of acetate, none of the anions, at 25 mM, could support the serosa-negative $\psi_{ms}$. Only in acetate buffer was $\psi_{ms}$ usually serosa-negative. In this case, addition of acetazolamide produced a slight but detectable increase in potential in every tissue. Clearly though, the average response was miniscule in comparison with that measured in normal media (Table 4). Therefore, $HCO_3^-$ appears rather specific in its role in stimulating $Cl^-$ absorption.

*Effect of SITS.* The stilbene, SITS (4-acetamido-4′-isothiocyano-2-2′ disulfonic stilbene), which inhibits $HCO_3^-/Cl^-$ exchange in red cells [4], was tested for

its effect on the small intestine. In normal media SITS rapidly reduced the $\psi_{ms}$ and $I_{sc}$ to zero when applied on the serosal side but was ineffective when added on the mucosal side of the tissue. The inhibitory effect of SITS is illustrated in Fig. 5. Serial addition of increasing amounts of SITS revealed a threshold dose of $5 \cdot 10^{-6}$ M and a maximal dose above 1 mM. Subsequent addition of acetazolamide produced no further electrical effects. After rinsing in SITS-free media, SITS inhibition was not reversed substantially in 30 min.

SITS eliminated the net $Cl^-$ and residual fluxes. This is seen in Table 5 in which the ion fluxes in the presence of $5 \times 10^{-4}$ M SITS can be compared to those observed in a control series conducted in the same time period on separate animals. The $m \rightarrow s$ flux of $Cl^-$ was reduced in the presence of SITS and equal to the backflux. Net $Cl^-$ absorption was insignificant ($P > 0.20$). The residual flux was also eliminated. There was no apparent effect of SITS on net $Na^+$ transport which was negligible in normal media (Tables 1 and 5) as well as in the presence of SITS. SITS did not influence $R_t$. Therefore, in contrast to acetazolamide, which only reduced net $Cl^-$ absorption and did not alter the residual flux, SITS eliminated net $Cl^-$ absorption and the residual flux as well.

It was noted above, in comparing Tables 1 and 3, that net $Cl^-$ absorption in the absence of exogenous $HCO_3^-$ was eliminated not only as a consequence of a reduction in the $m \rightarrow s$ flux but also an increase in the $s \rightarrow m$ flux of $Cl^-$. Since this suggested that $HCO_3^-$ normally inhibits the $s \rightarrow m$ flux of $Cl^-$ by successfully competing for a serosal carrier, the effect of SITS on the $s \rightarrow m$ flux of $Cl^-$ in $HCO_3^-$-free media was determined. The $s \rightarrow m$ flux of $^{36}Cl$ was measured in 6 intestinal segments bathed in media devoid of exogenous $HCO_3^-$. After 3 flux periods SITS was added to a final concentration of 0.5 mM and 3 additional flux periods were measured. During the control period $J^{Cl}_{s \rightarrow m}$ averaged $1.67 \pm 0.08$ μeq/hr·cm$^2$. Following exposure to SITS, $J^{Cl}_{s \rightarrow m}$ declined in every segment to a value averaging $1.21 \pm 0.11$ μeq/hr·cm$^2$. This observation supports the notion that a portion of $Cl^-$ backflux in $HCO_3^-$-free media is carrier-mediated.

*Replacement of $Na^+$.* One possible mode of $Cl^-$ transport is neutral coupled, co-transport with $Na^+$ [19]. It has already been demonstrated that the serosa-negative $\psi_{ms}$ in $HCO_3$-containing media depends on the bath $Na^+$ concentration [10]. Unidirectional $^{36}Cl^-$ fluxes were measured in media in which $Na^+$ was replaced with choline. The solutions were not completely free of sodium since the isotope of $Cl^-$ was added as NaCl. The calculated sodium concentration was 3.8 meq/liter. As seen in Table 5, substitution with choline lowered both unidirectional $Cl^-$ fluxes, elevated $R_t$, and abolished net $Cl^-$ absorption ($P > 0.05$) and the $I_{sc}$. Thus net $Cl^-$ absorption is dependent on the presence of $Na^+$ as well as $HCO_3^-$ in the media.

Sodium dependence was also demonstrable using ouabain. As seen in Table 5, after measuring ion fluxes in normal media the addition of ouabain abolished on-going net $Cl^-$ absorption and reduced net $Na^+$ absorption and the residual flux. While $J^{Na}_{net}$ was not significant in the control periods of this series, or in the presence of ouabain, $J^{Na}_{m \rightarrow s}$ was reduced significantly ($P < 0.05$) by ouabain when compared within the same tissue. Furthermore, the value of $J^{Na}_{m \rightarrow s}$, measured in the presence of ouabain, was lower than that observed in any other series (*cf.* Tables 1, 3 and 5). This is consistent with an effect of ouabain on the absorptive component of the Na flux. Ouabain also produced a significant ($P < 0.05$) decline in tissue resistance (Table 5).

### *Intracellular Chloride Activity*

*Normal buffer.* Single and double-barreled microelectrodes were used to measure the mucosal membrane potential ($\psi_{ms}$) and the intracellular $Cl^-$ potential ($\psi_{Cl}$) in cells lining the villi of *Amphiuma* small intestine. Microelectrode recordings were more difficult in $HCO_3^-$-containing media than encountered previously in $HCO_3^-$-free media [26]. This was due in part to enhanced spontaneous smooth muscle motility but also to a decreased ability of the mucosal membrane to withstand the electrode penetration in $HCO_3^-$-containing media. Therefore it became necessary to establish less rigorous criteria than used previously [26]. Recording were deemed acceptable if (i) the difference in potential between the two barrels ($\psi_{Cl} - \psi_m$), subtracted with a voltmeter, was constant within a millivolt for 10 sec; (ii) the tip potential of the barrel sensing $\psi_m$ did not change more than 3 mV from penetration to withdrawal; (iii) the chloride exchanger responded to changes in chloride activity with a slope ($S$) greater than $-47$ mV. The value of $S$ averaged about $-51$ mV. The reported values of $\psi_m$ are the highest values measured during the 10-sec interval. Some representative recordings with double-barreled microelectrodes are seen in Fig. 6. Measurements with these microelectrodes indicate $Cl^-$ is accumulated by the absorptive cell. Figure 7 shows the relationship between intracellular $Cl^-$ activity ($a^i_{Cl}$) and $\psi_m$ in 37

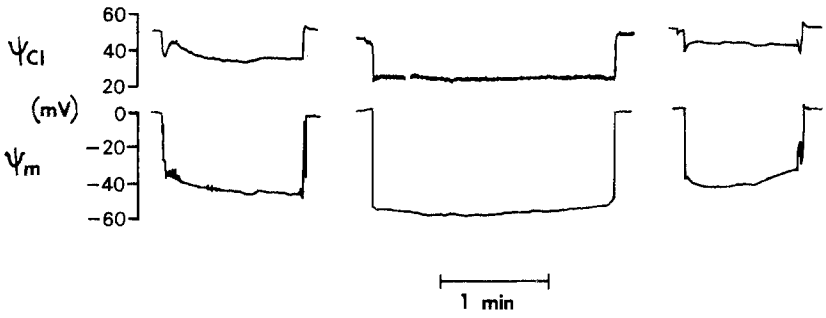


**Fig. 6.** Representative recordings with double-barreled microelectrodes of chloride potential ($\psi_{Cl}$) and the mucosal membrane potential ($\psi_m$) in 3 separate animals

**Table 5.** Ion fluxes under different conditions

| Series conditions | $J^{Na}_{m\to s}$ | $J^{Na}_{s\to m}$ | $J^{Na}_{net}$ | $J^{Cl}_{m\to s}$ | $J^{Cl}_{s\to m}$ | $J^{Cl}_{net}$ | $I_{sc}$ | $J^{R}_{net}$ | $R_t$ |
|---|---|---|---|---|---|---|---|---|---|
| a) In normal media and in media containing SITS ($5\times10^{-4}$ M) | | | | | | | | | |
| 1) Normal media (5:37) | 4.28±0.17 | 4.25±0.17 | 0.03±0.14 | 1.96±0.14 | 1.22±0.16 | 0.74±0.19 | −0.20±0.04 | 0.43±0.26 | 115.6±2.6 |
| 2) SITS (8:55) | 4.30±0.15 | 4.15±0.11 | 0.15±0.19 | 1.49±0.12 | 1.24±0.12 | 0.25±0.19 | 0.04±0.02 | −0.01±0.25 | 112.0±1.6 |
| b) In media in which $Na^+$ was replaced with choline | | | | | | | | | |
| 3) Low Na media (5:32) | – | – | – | 1.01±0.04 | 0.92±0.05 | 0.09±0.05 | 0.02±0.5 | 0.10±0.02 | 250.4±10.8 |
| c) In normal media and after addition of ouabain (1 mM) | | | | | | | | | |
| 4) Normal media (7:34) | 4.27±0.15 | 4.02±0.19 | 0.25±0.19 | 2.44±0.15 | 1.33±0.13 | 1.11±0.16 | −0.38±0.04 | 0.49±0.30 | 116.2±3.9 |
| $+10^{-4}$ M ouabain (7:30) | 3.84±0.20 | 4.12±0.23 | −0.28±0.24 | 1.56±0.17 | 1.63±0.18 | −0.07±0.20 | 0.30±0.04 | 0.50±0.30 | 105.9±3.4 |

Flux Units are μeq/hr cm². $R_t$ is in Ω cm². In parentheses is the number of animals/number of flux periods.

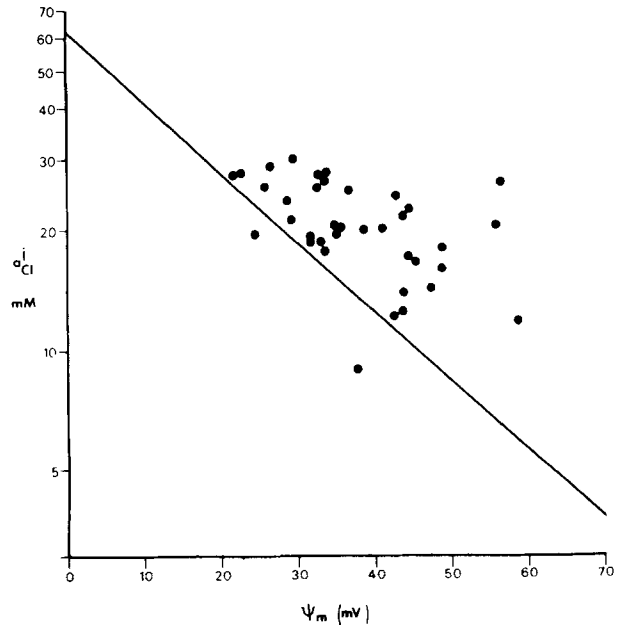


**Fig. 7.** Intracellular chloride activities ($a^i_{Cl}$) as a function of mucosal membrane potential in normal media using double-barreled microelectrodes. The solid line is the solution of the Nernst equation using $a^i_{Cl}=60\cdot2$ meq/liter

**Table 6.** Intracellular chloride activities ($a^i_{Cl}$), mucosal membrane potential ($\psi_m$), and calculated chloride equilibrium potential ($E_{Cl}$) under different conditions

| Series | Media | Microelectrode | $n$ | $a^i_{Cl}$ (mM) | $\psi_m$ (mV) | $E_{Cl}$ (mV) |
|---|---|---|---|---|---|---|
| 1 | Normal | DBM | 10 | 21.0±1.0 [36] | 38.6±1.6 [36] | 28.2±1.3 |
| 2 | +Acetazolamide | DBM | 5 | 23.3±1.2 [20] | 35.0±2.1 [20] | 24.7±1.3 |
| 3 | Na-free | DBM | 2 | 17.2±1.7 [5] | 27.1±2.6 [5] | 30.5±2.4 |
| | | SBM | 5 | 13.1±1.0 [23] | 29.0±1.8 [26] | 36.9 |

All measurements were made in the fall and winter. DBM represents double-barreled microelectrodes; SBM, single-barreled microelectrodes. $n$ is the number of animals; the number of impalements is in parentheses.

recordings from 12 animals. Taking bath chloride activity to be 60.2 meq/liter, the solid line illustrates the expected relationship between $\psi_m$ and $a^i_{Cl}$ if intracellular chloride were in electrochemical equilibrium with bath chloride. The position of most of

the points above the line indicates $Cl^-$ is accumulated above levels consistent with passive distribution.

These and other data are collected in Table 6. In normal $HCO_3^-$-containing media $a^i_{Cl} = 21.0$ mM using double-barreled microelectrodes (DBM). The mean chloride equilibrium potential ($E_{Cl}$) calculated with this value varies considerably from the mean mucosal membrane potential in keeping with active $Cl^-$ accumulation. The value of $a^i_{Cl}$ consistent with electrochemical equilibrium is about 13 mM, thus the cells are accumulating $Cl^-$ by a factor of about 1.6.

Chloride accumulation was also observed in the presence of acetazolamide ($10^{-4}$ M). This is seen in series 2 of Table 6 where there is again a considerable difference between $\psi_m$ and $E_{Cl}$ as calculated from measurements with the double-barreled microelectrode. Values for $\psi_m$ were slightly lower than in the first series in the absence of acetazolamide.

Chloride accumulation was abolished when $Na^+$ was removed from the bathing media. This is shown in series 3 of Table 6. A small number of measurements using double-barreled microelectrodes point to passive $Cl^-$ distribution since $\psi_m$ and $E_{Cl}$ were not different. Additional measurements with single-barreled microelectrodes (SEM) shown in the same Table, are even consistent with a small active $Cl^-$ extrusion since $E_{Cl}$ calculated from the mean $a^i_{Cl}$ was in slight excess of $\psi_m$. The differences between these sets of data are small. While the conclusions are contradictory in this regard, they are in agreement that upon replacement of media $Na^+$ with choline, active $Cl^-$ accumulation is abolished. This has also been reported for gallbladder [7] and renal proximal tubule [24].

It is also evident in Table 6 that $\psi_m$ is lower in Na-free (choline) media than in normal media. The difference between the mean $\psi_m$ in series 1 ($38.6 \pm 1.6$ mV) and series 3 ($29.0 \pm 1.8$ mV) was statistically significant ($P < 0.01$). This was examined further in a series of 4 experiments in which the mucosal membrane potential was measured first in normal media, then in Na-free choline media and then again in normal media. A 1-hr incubation in the respective media preceded a 1-hr interval of measurements. In normal media $\psi_m$ averaged $47.2 \pm 2.2$ mV ($n = 43$), a value somewhat higher than observed in earlier measurements (Table 5, series 1). After replacement of media Na with choline, $\psi_m$ was reduced in all four tissue and averaged $36.3 \pm 2.4$ ($n = 34$). After returning to normal media, the mean $\psi_m$ rose markedly in two tissues and remained unchanged in the other two for an overall mean increase of $\psi_m$ to $43.7 \pm 3.3$ mV ($n = 19$). These results lend further support to the conclusion that the mucosal membrane becomes depolarized in choline-substituted media.

## Discussion

These measurements indicate that the proximal small intestine of *Amphiuma* absorbs $Cl^-$ and that this process requires $Na^+$ and $HCO_3^-$. Incubation of segments in either low $Na^+$ (choline) media (Table 5), ouabain-containing media (Table 5), or $HCO_3^-$-free media (Table 3) reversed the sign of $\psi_{ms}$ and eliminated net $Cl^-$ transport.

We have previously shown that the polarity of $\psi_{ms}$ in *Amphiuma* intestine is dependent on the presence of $HCO_3^-$ and that the $I_{sc}$ is proportional to bath $HCO_3^-$ up to 25 meq/liter [10]. A serosa-negative $\psi_{ms}$ associated with $HCO_3^-$-dependent $Cl^-$ absorption has also been reported for flounder small intestine [9, 12]. Except for a slight ability of acetate to substitute for $HCO_3^-$ (Table 4), the requirement for $HCO_3^-$ (and/or $CO_2$) appears very specific in *Amphiuma* intestine.

The dependency on $Na^+$ for electrogenic $Cl^-$ absorption, as reported here, has been observed in flounder intestine as well. Field et al. [9] reported that incubation in Na-free media inhibited net $Cl^-$ absorption and the $I_{sc}$. In contrast, in flounder Huang and Chen [12] and Ando et al. [1] had earlier reported serosa-negative $\psi_{ms}$ in media in which most but not all $Na^+$ was replaced with choline. As pointed out by Field et al. [10], these earlier reports may be complicated by the presence of asymmetric diffusion potentials.

There was a significant residual flux ($J^R_{net}$) in *Amphiuma* small intestine under control conditions (Table 1), as has been reported for rabbit small intestine [6] but in contrast with flounder intestine in which no residual flux was observed [10]. In two smaller series in normal media $J^R_{net}$ was large but not significant at $P = 0.05$ (Table 5). In these two series $J^{Cl}_{net}$ was smaller, as well. Some evidence suggests that the residual flux reported here represents, at least in part, $HCO_3^-$ secretion. For example, the residual flux was absent in media devoid of exogenous $HCO_3^-$ (Table 3). More importantly, the agent SITS, which appears to inhibit a $Cl^- - HCO_3^-$ exchange process in erythrocytes [4], eliminated the residual flux (Table 5).

If the residual flux does indeed represent $HCO_3^-$ secretion, there is some evidence to indicate that the secretion of $HCO_3^-$ depends, in turn, on the presence of $Cl^-$. As reported earlier, the residual flux was oppositely directed ($J^R_{net}$ was negative) when $Cl^-$ was replaced with $SO_4^=$ [10]. This is consistent with $HCO_3^-$-absorption. In addition SITS, which inhibited net $Cl^-$ absorption, eliminated $J^R_{net}$ as well (Table 5). Indeed, a role for $Cl^-$ in $HCO_3^-$ secretion has been noted in the past. Hubel has demonstrated a positive correlation between the luminal $Cl^-$ concentration

and net $HCO_3^-$ secretion in rat ileum [13, 14]. Also, Turnberg et al. reported that $HCO_3^-$ secretion in human ileum was blocked by replacement of $Cl^-$ with $SO_4^=$ [25].

Net $Cl^-$ absorption observed in this study could be due to the operation of a transport mechanism located in either the mucosal or serosal membrane of the absorbing cells. If active $Cl^-$ transport occurs at the serosal membrane alone then entry into the cell across the mucosal membrane would be expected to be passive and the intracellular activity of $Cl^-$ would be no greater than that expected from a Nernstian distribution across the mucosal membrane. On the other hand, if the transport mechanism resides in the mucosal membrane then it would be expected that the intracellular $Cl^-$ activity would be elevated above levels consistent with passive distribution. The measurements with chloride-selective microelectrodes argue in favor of the latter since, for most of the cells impaled in normal media (Fig. 7), $\psi_m$ exceeded the calculated $E_{Cl}$. Following an earlier report of $Cl^-$ accumulation by *Amphiuma* intestine in $HCO_3$-free media [26], $Cl^-$ accumulation has been observed in bullfrog small intestine [2], rabbit gallbladder [7], and proximal tubule of *Necturus* [24] with ion-specific microelectrodes. For the present measurements, if the $Cl^-$ ions sensed by the microelectrode participate in transcellular transport, the only active step which need be invoked is that at the mucosal membrane of the absorbing cells.

This conclusion rests upon the assumption that the activity of $HCO_3^-$ in the cytoplasm is low relative to $Cl^-$. This seems reasonable. For example if the cytoplasmic $pCO_2$ is equal to that of the external media and the intracellular pH is as high as 7.4, the $HCO_3^-$ concentration in the cytoplasm would only be 25 meq/liter. This concentration of $HCO_3^-$ could not account for the observed anion accumulation of 8 mM in the cytoplasm since the ion exchanger has a selectivity for $Cl^-$ over $HCO_3^-$ of at least 11:1 [26]. Furthermore, a significant $HCO_3^-$ error could not easily account for the anion accumulation reported previously in the absence of exogenous $HCO_3^-$ [26]. For these reasons it is assumed that the ion exchanger is detecting $Cl^-$ for the most part and that $Cl^-$ is actively accumulated across the mucosal membrane.

The results with SITS suggest that $Cl^-$, thus accumulated, can move across the serosal membrane in exchange for $HCO_3^-$. When applied on the serosal side, SITS inhibited the $I_{sc}$, net $Cl^-$ absorption, and the residual flux (Table 5). SITS did not inhibit the $I_{sc}$ when applied on the mucosal side. This indicates that SITS does not penetrate either the mucosal or basolateral membrane but produces its effect at the outer surface of the basolateral membrane. This conclusion, and the observation that SITS inhibition was not reversible, is consistent with its observed inhibitory effects on $HCO_3^- - Cl^-$ exchange in erythrocytes [4]. Also, Ehrenspeck and Brodsky have shown that serosally applied SITS inhibits the $I_{sc}$ in turtle bladders under conditions (Na-free) in which $HCO_3^-$ and $Cl^-$ are being actively absorbed [8]. SITS was also shown to inhibit carbonic anhydrase but with much less potency ($1/1{,}000^{th}$) than acetazolamide. The same agent also inhibited $(Na^+ + K^+)$-ATPase but had no effect on the $I_{sc}$ under conditions in which $Na^+$ alone is actively transported by turtle bladder. Ehrenspeck and Brodsky concluded that SITS acts principally to inhibit an anion pathway in the serosal membrane. The effect of SITS to eliminate both net $Cl^-$ and residual fluxes in *Amphiuma* intestine cannot be attributed to inhibition of carbonic anhydrase. Acetazolamide, itself a carbonic anhydrase inhibitor, did not alter the residual flux and only lowered $Cl^-$ absorption. The effect of SITS on *Amphiuma* intestine is not apparently related to inhibition of $(Na^+ + K^+)$-ATPase either since no alteration of the unidirectional $Na^+$ fluxes was observed. This contrasts with the effects produced by ouabain, the classical inhibitor of $(Na + K^+)$-ATPase (Table 5). Ouabain inhibited $Cl^-$ absorption but also lowered the absorptive flux of $Na^+$. Therefore the simplest interpretation for the effect of SITS is inhibition of a serosally-located $Cl^- - HCO_3^-$ exchange mechanism.

$Cl^-$ transport in *Amphiuma* small intestine generates a serosa-negative PD and is, therefore, electrogenic. This is supported by the strong correlation between $J_{net}^{Cl}$ and $I_{sc}$ (Fig. 2*a*). Assuming $Cl^-$ movement across the serosal membrane occurs in one-for-one exchange with $HCO_3^-$ then the simplest interpretation of these observations is that $Cl^-$ uptake at the mucosal membrane is rheogenic, i.e., it occurs as uncoupled active $Cl^-$ uptake. However, earlier reports by others support the view that $Cl^-$ uptake at the mucosal membrane is linked to $Na^+$ uptake as a electrically neutral process. For example, active $Cl^-$ absorption in rabbit intestine is reduced in the absence of $Na^+$ [20]. In the same way active $Na^+$ absorption is reduced in the absence of $Cl^-$ [20, 23]. In addition, a saturable portion of $Cl^-$ influx is coupled one-for-one to $Na^+$ influx in rabbit ileum [19].

Indeed some of the observations in the present study are consistent with coupled cotransport of $Na^+$ and $Cl^-$. For example, in the absence of $Na^-$ active $Cl^-$ absorption was eliminated (Table 5), and $Cl^-$ accumulation, as measured with $Cl^-$-specific microelectrodes, was eliminated as well (Table 6). In addition, acetazolamide, which reduced net $Cl^-$ absorption, also reduced the unidirectional absorptive flux

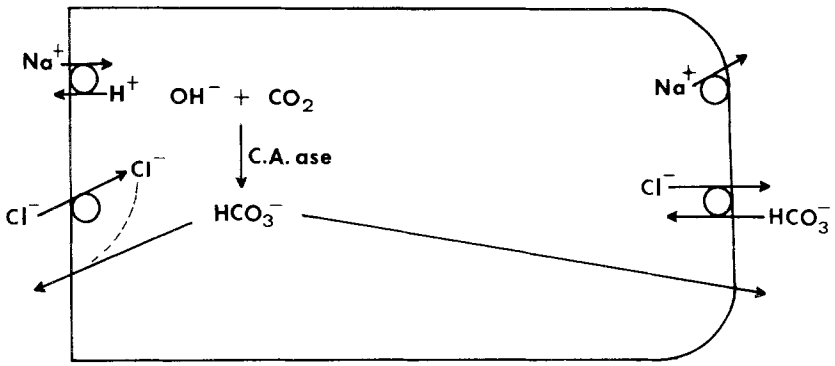


**Fig. 8.** Model for $Cl^-$ absorption in urodele intestine. Details are described in the text

of $Na^+$ (Table 1). Also, ouabain inhibited both net $Na^+$ and $Cl^-$ absorption. In spite of the apparent conformity of these observations with a coupled cotransport of sodium and chloride, there are difficulties which must be considered. Most importantly, if $Cl^-$ is absorbed by a coupled neutral process, where does the electrical potential associated with the absorption of $Cl^-$ arise? It is not likely to develop at the basolateral membrane since at this site $Cl^- - HCO_3^-$ exchange, a presumptively neutral process, appears to occur. Field et al. [9] have argued that the potential arises from a salt diffusion potential across the tight junction following active $Na^+$ transport into the lateral intercellular space and passive diffusion of $Cl^-$ from the cell. This proposal, however, does not provide a role for $HCO_3^-$ (and/or $CO_2$) which is essential for $Cl^-$ absorption in *Amphiuma* intestine.

Another difficulty with the notion of NaCl cotransport concerns the labeled fluxes of $Na^+$ and $Cl^-$ in this study. A correlation between these fluxes would be expected if cotransport constituted the principal mode of transcellular transport. However, no significant correlation was noted between the unidirectional absorptive fluxes of these ions or their net fluxes within a control series. Rather, the net $Cl^-$ flux was strongly correlated with the $I_{sc}$ (Fig. 2). Furthermore, inspection of Table 3 reveals that after exposure to acetazolamide the absorptive flux of $Na^+$ was elevated while that for $Cl^-$ was unchanged. Similarly, as apparent in Table 5 in the presence of SITS, the $Cl^-$ absorptive flux was reduced considerably while the value of the $Na^+$ absorptive flux (4.3 μeq/hr $cm^2$) was similar to that measured in untreated segments examined concurrently or in the $HCO_3$-free series (Table 3). Lastly, the correlation between net $Na^+$ transport and the residual flux in the control series (Fig. 2) indicates $Na^+$ transport may be linked to other ions. It is concluded that $Cl^-$ transport does not occur in cotransport with $Na^+$ *per se*, but instead is uncoupled and electrogenic.

The model in Fig. 8 is presented as a working hypothesis and envisions an electrogenic $Cl^-$ uptake at the mucosal membrane and serosal $Cl^- - HCO_3^-$ exchange. $H^+$ is exchanged for $Na^+$ by a neutral process at the mucosal membrane. It is proposed that intracellular $HCO_3^-$, formed enzymatically by hydroxylation of $CO_2$, and arising from exchange with $Cl^-$ at the basolateral membrane, competes with $Cl^-$ for exit across the mucosal membrane in some as yet undetermined manner. Thus any treatment which lowers intracellular $HCO_3^-$ will accelerate $Cl^-$ backflux across the mucosal membrane and inhibit net $Cl^-$ absorption. For example, inhibition of carbonic anhydrase will limit the availability of intracellular $HCO_3^-$ and permit accumulated intracellular $Cl^-$ to move back into the luminal fluid. Likewise, removal of bath $Na^+$ or elevation of intracellular $Na^+$ with ouabain would lower intracellular $HCO_3^-$ by inhibiting mucosal $Na^+ - H^+$ exchange. Finally, incubation in media devoid of exogenous $HCO_3^-$ would inhibit basolateral $Cl^- - HCO_3^-$ exchange directly.

The model is in conformity with the correlations noted in the isotope flux experiments. If the residual flux ($J^R_{net}$) is largely attributable to $HCO_3^-$ secretion then greater activity of parallel $Na^+ - H^+$ exchange processes should, by enhancing $H^+$ secretion, reduce the effective net secretion of $HCO_3^-$. The same tissues which exhibit greater $Na^+ - H^+$ exchange would be expected to effect greater net $Na^+$ transport as well since mucosal $Na^+$ entry would be elevated. This could account for the negative correlation between the net $Na^+$ and residual fluxes (Fig. 2).

The isotope flux measurements also indicate that, in the absence of available $HCO_3^-$, $Cl^- - HCO_3^-$ exchange is replaced by $Cl^- - Cl^-$ exchange. This would explain the inhibition of net $Cl^-$ transport in the absence of exogenous $HCO_3^-$ (Table 3) if it is assumed that $Cl^-$ is able to permeate the serosal membrane only by mediation of the carrier. Upon removal of media $HCO_3^-$ the $s \rightarrow m$ flux of $Cl^-$ was elevated and was sensitive to SITS. On the other hand, SITS did not alter this flux when $HCO_3^-$ was present in high concentrations in the media (Table 5). Support for this interpretation is given in recent studies with

muscle fibers of the barnacle. Boron et al. [3] observed that cyclic AMP stimulates a $HCO_3^- - Cl^-$ exchange mechanism which controls intracellular pH. This mechanism is inhibited by SITS and also promotes $Cl^- - Cl^-$ exchange.

The measurements of intracellular chloride activity are reasonably consistent with the proposed model. Thus $Cl^-$ was actively accumulated across the mucosal membrane equally well in normal media and in the presence of acetazolamide (Table 6), conditions under which net $Cl^-$ absorption was observed (Table 1). In both cases the energy invested in accumulation ($\psi_m - E_{Cl}$) was 10 mV. $Cl^-$ accumulation was previously observed in $HCO_3$-free media as well, although the accumulation was less at 5 mV [26]. As long as $HCO_3^-$ is available from metabolism, some accumulation of $Cl^-$ across the mucosal membrane would be expected to occur even though net transcellular transport was blocked by the lack of bicarbonate at the outer border of the basolateral membrane. Contrasting with these conditions, when $Na^+$ was replaced in the bathing media no accumulation of $Cl^-$ was observed (Table 6), suggesting that this condition strongly depresses intracellular levels of $HCO_3^-$. Indeed, it would be expected that $H^+$ efflux would be reduced following inhibition of $Na^+ - H^+$ exchange in choline media. As noted above, lowering intracellular $HCO_3^-$ in this way would, in turn, allow $Cl^-$ to move back into the mucosal media.

If mucosal $Cl^-$ uptake is electrogenic, as suggested here, it would be expected that $\psi_m$ would be depolarized under conditions of reduced $Cl^-$ accumulation. The only condition which resulted in a measurably reduced accumulation of $Cl^-$ was the $Na^+$-free condition. In choline-substituted media $\psi_m$ was depolarized significantly (Table 6). Assuming that the depolarization is due to $Na^+$ removal rather than addition of choline, this observation supports the conclusion of electrogenic $Cl^-$ entry at the mucosal membrane of the absorptive cell. Substitution of $Na^+$ with other ions will provide a more definitive test of electrogenic $Cl^-$ absorption. The energy for mucosal $Cl^-$ uptake may be derived from ATP. Recently Humphreys and Chou [15] reported strong new evidence for the presence of anion ($Cl^-$ and $HCO_3^-$)-stimulated ATPase activity in the brush border of rat small intestine. Such an enzyme would be well suited to energize $Cl^-$ absorption and $HCO_3^-$-secretion.

The model presented above assumes that all of the net $Cl^-$ transport capacity of the tissue resides in the villus absorptive cells which have been shown here to accumulate $Cl^-$. In fact, we have demonstrated that only these cells generate a serosa negative potential difference like that observed in the whole tissue; the intervillus epithelial cells (between the villi) do not contribute to the transepithelial potential difference under these conditions [11]. Since the present study has demonstrated that the serosa-negative transmural potential reflects electrogenic $Cl^-$ absorption, it appears safe to conclude that this transport activity is localized in the villus absorptive cells alone. Nevertheless, it cannot be ruled out that, in addition, an electrically neutral $Cl^-$ absorptive process resides in the intervillus epithelium.

It is instructive to compare these observations with those of Turnberg et al. [25] on normal human ileum *in situ*. They observed active $Cl^-$ absorption in excess of bicarbonate secretion using a triple-lumen perfusion technique. In addition, the absorption of sodium was associated with a decrease in bicarbonate secretion. When media $Cl^-$ was completely replaced with $SO_4^=$ bicarbonate secretion was inhibited and bicarbonate absorption ensued. Finally, acetazolamide markedly inhibited both $Na^+$ and $Cl^-$ absorption. The results described in this and a previous report [10] are in qualitative agreement with the human data of Turnberg et al. The $Cl^-$ absorptive process in the human ileum is apparently electrically neutral although, owing to the nature of their preparation, this could not be examined in the manner of the present study. Their observations led Turnberg et al. to propose the existence within the epithelial cell of parallel neutral $Na^+ - H^+$ and $Cl^- - HCO_3^-$ exchange processes. Even earlier evidence had suggested the existence of parallel acid and base secretory processes in the small intestine [22]. The present study has pointed to a model containing similar processes but now localized to the individual membranes of the absorptive cell and containing additional features consistent with the electrogenic nature of the $Cl^-$ transport process in the urodele.

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