

## EFFECTS OF BICARBONATE ON SALT AND WATER TRANSPORT ACROSS THE INTESTINE OF THE SEAWATER EEL

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### Summary

To elucidate whether the  $\text{Na}^+/\text{K}^+/\text{Cl}^-$  cotransport system depends on  $\text{HCO}_3^-$  in the seawater eel intestine, the effects of  $\text{HCO}_3^-$  on the transepithelial potential difference (PD) and on net water and ion fluxes were examined. When  $\text{HCO}_3^-$  buffer was replaced with phosphate buffer, the serosa-negative PD and net  $\text{Na}^+$ ,  $\text{Cl}^-$  and water fluxes from mucosa to serosa were inhibited, indicating that the  $\text{Na}^+/\text{K}^+/\text{Cl}^-$  cotransport system is inhibited in phosphate-buffered solutions. Similar inhibitory effects were also observed in solutions buffered with Hepes, Tris or Tes, indicating that the inhibitory effects are not specific for the phosphate buffer but are caused by omission of the  $\text{HCO}_3^-$  buffer system. Although the  $\text{HCO}_3^-$  buffer system consists of  $\text{HCO}_3^-$  and  $\text{CO}_2$ , higher  $\text{CO}_2$  pressure with constant  $\text{HCO}_3^-$  concentration did not enhance, but inhibited, the PD and the net water flux: this indicates that the inhibition observed after removal of the  $\text{HCO}_3^-$  buffer system is due to omission of  $\text{HCO}_3^-$  rather than  $\text{CO}_2$ . The inhibition of PD and the net water flux was greater after removal of  $\text{HCO}_3^-$  from the serosal side than from the mucosal side. Similarly, the inhibitory effects of 4,4'-diisothiocyanostilbene-2,2'-disulphonic acid (DIDS), an inhibitor of  $\text{HCO}_3^-$  transport, were more pronounced on the serosal side than on the mucosal side. Mucosal  $\text{Ba}^{2+}$  also inhibited PD and the short-circuit current ( $I_{sc}$ ) and enhanced the tissue resistance ( $R_t$ ), presumably through partially blocking the apical  $\text{K}^+$  channels. However, these effects of  $\text{Ba}^{2+}$  were completely abolished after pretreatment with serosal DIDS, suggesting that  $\text{Ba}^{2+}$  and DIDS evoked the same effect. These results are combined and a possible role for  $\text{HCO}_3^-$  in the  $\text{Na}^+/\text{K}^+/\text{Cl}^-$  cotransport system is discussed.

### Introduction

The seawater eel intestine is typical of epithelia that absorb  $\text{NaCl}$  via a  $\text{Na}^+/\text{K}^+/\text{Cl}^-$  cotransport system (Ando and Utida, 1986). In this system,  $\text{K}^+$  is taken up into the epithelium by  $\text{Na}^+/\text{K}^+/\text{Cl}^-$  cotransport, leaks out into the luminal fluid, and then is taken up again into the cell. During this recycling of  $\text{K}^+$ ,

Key words:  $\text{HCO}_3^-$ ,  $\text{Na}^+/\text{K}^+/\text{Cl}^-$  cotransport, pH, water transport, eel intestine.

NaCl is transported to the serosal side, and water follows the NaCl flow. The K<sup>+</sup> leakage from the cell to the mucosal fluid causes the serosa-negative PD. A similar cotransport system has also been reported in the flounder intestine (Halm *et al.* 1985) and the cortical thick ascending limb of Henle's loop of rabbit kidney (Greger *et al.* 1983). However, in these epithelia the Na<sup>+</sup>/K<sup>+</sup>/Cl<sup>-</sup> cotransport system is independent of HCO<sub>3</sub><sup>-</sup> (Greger *et al.* 1983; Halm *et al.* 1985). In contrast, Field *et al.* (1978) found that net Cl<sup>-</sup> absorption in the flounder intestine is more than twice as great at a HCO<sub>3</sub><sup>-</sup> concentration of 20 mmol l<sup>-1</sup> than it is at 5 mmol l<sup>-1</sup>. To elucidate whether the cotransport system depends on HCO<sub>3</sub><sup>-</sup> in the seawater eel intestine, I examined the effects of HCO<sub>3</sub><sup>-</sup> on PD and the net water and ion fluxes. The results indicate that, at least in the seawater eel intestine, the Na<sup>+</sup>/K<sup>+</sup>/Cl<sup>-</sup> cotransport system depends on HCO<sub>3</sub><sup>-</sup>.

### Materials and methods

Japanese cultured eels *Anguilla japonica*, weighing 200–240 g, were obtained from a commercial supplier and kept in seawater aquaria at 20°C for more than 1 week before use. After decapitation, the intestine was excised and stripped of its serosal muscle layers. The intestine was everted and bathed at 20°C in various Ringer's solutions. The serosal side was perfused with various Ringer's solutions at a constant rate (around 170 μl min<sup>-1</sup>), and the effluent collected every 10 min. The net water flux was calculated directly from the difference between the rates of effluent and perfusate flow. Details for simultaneous measurement of net water flux and PD were as described previously (Ando *et al.* 1986). In some preparations, net Na<sup>+</sup>, K<sup>+</sup> and Cl<sup>-</sup> fluxes were also measured simultaneously from the collected volume and ionic concentrations, as described previously (Ando, 1983). Sodium and potassium concentrations were measured by flame photometry (Hiranuma, FPF-2A) and chloride concentration was determined with a chloride counter (Hiranuma, CL-5M).

Table 1 shows the compositions of the various Ringer's solutions. Bicarbonate Ringer is the normal solution and is bubbled with a 95% O<sub>2</sub>/5% CO<sub>2</sub> gas mixture (pH 7.4). In other solutions, NaHCO<sub>3</sub> was replaced with Na<sub>2</sub>HPO<sub>4</sub>, Hepes, Tris or Tes. These solutions were gassed with 100% O<sub>2</sub>, and the pH was adjusted to 7.4 with HCl or NaOH. The osmolarity was adjusted with NaCl or Na<sub>2</sub>SO<sub>4</sub>. All solutions contained 5 mmol l<sup>-1</sup> L-alanine and 5 mmol l<sup>-1</sup> D-glucose to maintain higher PD and water transport (see Ando, 1987, 1988). The extracellular fluid pH was monitored with a pH meter (TOA, HM-5B), a pH electrode (TOA, GS-195C) and a recorder (TOA, EPR-200A).

When the ratio of O<sub>2</sub> and CO<sub>2</sub> gases was altered, the flow rates of O<sub>2</sub> and CO<sub>2</sub> were controlled with purge meter (Ueshima, 1355V) and mass flow systems (STEC, SEC-400 Mark 3), respectively.

Some stripped intestines were mounted in the Ussing chamber, and the PD, the short-circuit current ( $I_{sc}$ ) and the tissue resistance ( $R_t$ ) were measured simultaneously every 10 min. In this preparation, the serosal fluid was the normal

Table 1. *Composition of experimental solutions (mmol l<sup>-1</sup>)*

|                                  | HCO <sub>3</sub> <sup>-</sup> | Phosphate | Hepes | Tris  | Tes   |
|----------------------------------|-------------------------------|-----------|-------|-------|-------|
| NaCl                             | 118.5                         | 137.4     | 118.5 | 118.5 | 118.5 |
| KCl                              | 4.7                           | 4.7       | 4.7   | 4.7   | 4.7   |
| CaCl <sub>2</sub>                | 3.0                           | 3.0       | 3.0   | 3.0   | 3.0   |
| KH <sub>2</sub> PO <sub>4</sub>  | 1.2                           | 0.6       | 1.2   | 1.2   | 1.2   |
| MgSO <sub>4</sub>                | 1.2                           | 1.2       | 1.2   | 1.2   | 1.2   |
| NaHCO <sub>3</sub>               | 24.9                          |           |       |       |       |
| Na <sub>2</sub> HPO <sub>4</sub> |                               | 2.5       |       |       |       |
| Hepes                            |                               |           | 15.5  |       |       |
| Tris                             |                               |           |       | 10.7  |       |
| Tes                              |                               |           |       |       | 15.5  |
| NaOH                             |                               |           | 6.2   |       | 6.2   |
| HCl                              |                               |           |       | 9.2   |       |
| Na <sub>2</sub> SO <sub>4</sub>  |                               |           | 9.3   | 9.9   | 9.3   |
| D-Glucose                        | 5.0                           | 5.0       | 5.0   | 5.0   | 5.0   |
| L-Alanine                        | 5.0                           | 5.0       | 5.0   | 5.0   | 5.0   |

HCO<sub>3</sub><sup>-</sup> Ringer's solution, while the mucosal side was bathed with unbuffered Ringer's solution (mmol l<sup>-1</sup>): NaCl, 118.5; sodium gluconate, 24.3; KCl, 2.3; potassium acetate, 3.6; CaCl<sub>2</sub>, 3.0; MgCl<sub>2</sub>, 1.2 (bubbled with 100 % O<sub>2</sub>). The pH in the mucosal fluid was clamped at 7.4 with a pH-stat (TOA, HSM-10A) by titrating automatically with 20 mmol l<sup>-1</sup> HCl. From the amount of HCl titrated, the rate of mucosal alkalization (*J<sub>m</sub><sup>OH</sup>*) was calculated. Details of the pH-stat method are described in the accompanying paper (Ando and Subramanyam, 1990).

### Results

#### *Effects of replacement of bicarbonate buffer with other buffers*

Fig. 1 shows the effects of replacement of HCO<sub>3</sub><sup>-</sup> buffer with other buffers on the PD and net water flux. When the bicarbonate-buffered solutions bathing both sides of the intestine were replaced with phosphate-, Hepes-, Tris- or Tes-buffered solutions, the serosa-negative PD decreased gradually with time, accompanied by a decrease in the net water flux. After reintroduction of HCO<sub>3</sub><sup>-</sup> buffer, the PD and the net water flux tended to return to their original levels, but did not recover completely. These results suggest that the serosa-negative PD and the net water flux can be maintained at a high level only in the presence of the HCO<sub>3</sub><sup>-</sup> buffer system. It is also possible that all these four buffers could have some toxic effects on this tissue. In the next experiment, therefore, without using other buffer systems, HCO<sub>3</sub><sup>-</sup> concentration was decreased in steps, keeping the pH at 7.4 by simultaneously reducing CO<sub>2</sub> pressure (Fig. 2). When the HCO<sub>3</sub><sup>-</sup> concentration was reduced, both PD and the net water flux decreased.

Table 2 demonstrates that the net Na<sup>+</sup> and Cl<sup>-</sup> fluxes from mucosa to serosa are almost halved in phosphate Ringer's solution. Since net NaCl absorption across

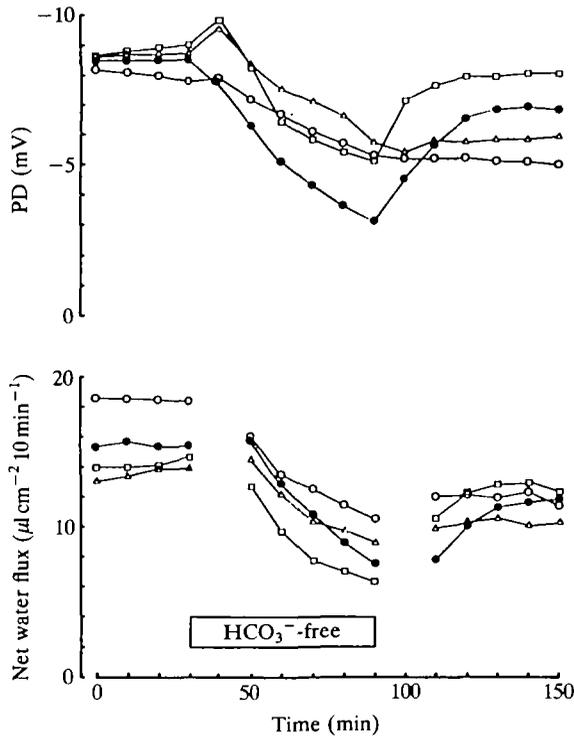


Fig. 1. Effects of replacing the  $\text{HCO}_3^-$  buffer with various buffer solutions on the transepithelial potential (PD) and the net water flux. After bathing both sides of the intestine with  $\text{HCO}_3^-$  Ringer's solution, the  $\text{HCO}_3^-$  buffer in both fluids was replaced with phosphate (●), Hepes (Δ), Tris (□) or Tes (○) buffer (30 min). It takes 10 min to replace the serosal fluid with new test solution by perfusion. At 90 min, the  $\text{HCO}_3^-$  buffer was reintroduced in both mucosal and serosal fluids. Each point indicates the mean value. The sample sizes were: phosphate, 6; Hepes, 5; Tris, 5; Tes, 3.

the seawater eel intestine is due to  $\text{Na}^+/\text{K}^+/\text{Cl}^-$  cotransport (Ando and Utida, 1986), this result indicates that the  $\text{Na}^+/\text{K}^+/\text{Cl}^-$  cotransport system depends on the  $\text{HCO}_3^-$  buffer system. However, since the  $\text{HCO}_3^-$  buffer system consists of  $\text{HCO}_3^-$  and  $\text{CO}_2$  and the latter is also omitted in the  $\text{HCO}_3^-$ -free Ringer's solution, I performed the following experiments to discriminate between the effects of  $\text{HCO}_3^-$  and  $\text{CO}_2$ .

#### *Effects of $\text{CO}_2$*

Whilst keeping  $\text{HCO}_3^-$  concentration at  $24.9 \text{ mmol l}^{-1}$ ,  $\text{CO}_2$  pressure was altered in stepwise increments (Fig. 3). The pH in the bathing solution was also monitored simultaneously together with the PD and the net water flux. When mucosal  $\text{CO}_2$  pressure was raised (20.9%) and the serosal fluid was gassed with 95%  $\text{O}_2/5\% \text{CO}_2$  (pH 7.4), the serosa-negative PD and the net water flux decreased, accompanied by an acidification (pH 6.8) in the mucosal fluid

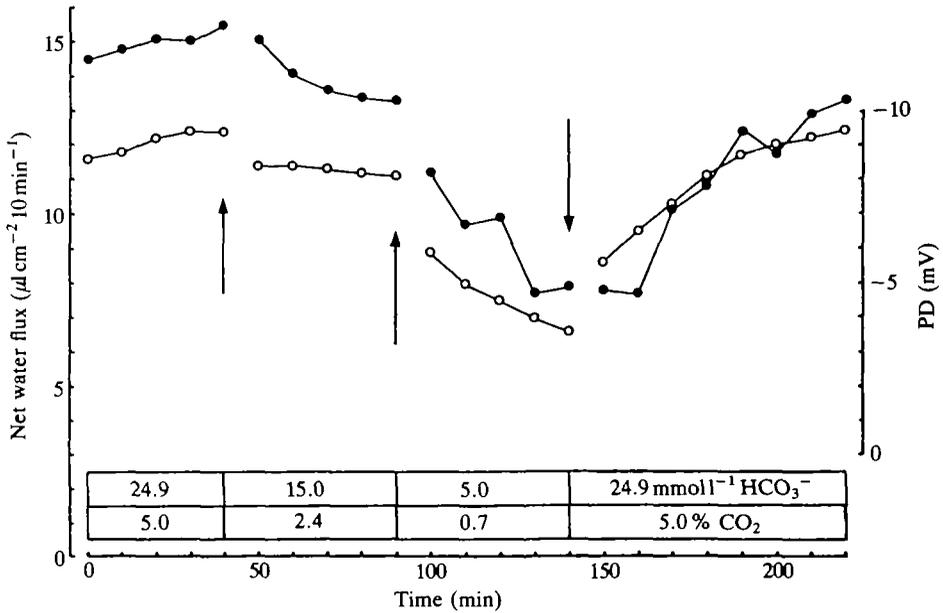


Fig. 2. The concentration-dependence of the effects of HCO<sub>3</sub><sup>-</sup>. After measuring transepithelial potential (PD) (○) and net water flux (●) in normal Ringer's solution ([HCO<sub>3</sub><sup>-</sup>]=24.9 mmol l<sup>-1</sup>), HCO<sub>3</sub><sup>-</sup> concentration was reduced stepwise by substituting with Cl<sup>-</sup>, as shown in the lower panel at the times indicated by arrows. On reduction of the HCO<sub>3</sub><sup>-</sup> concentration, CO<sub>2</sub> pressure was lowered simultaneously to maintain the pH at 7.4. Discontinuous lines show that measurements were interrupted for 10 min, the time required to replace the serosal fluid with the test solution by perfusion.

Table 2. Effects of the HCO<sub>3</sub><sup>-</sup> buffer system on the transepithelial potential difference (PD), net ion fluxes (J<sub>net</sub><sup>Na</sup>, J<sub>net</sub><sup>K</sup>, J<sub>net</sub><sup>Cl</sup>) and net water flux (J<sub>net</sub><sup>H<sub>2</sub>O</sup>) across the seawater eel intestine

| Buffer                        | PD (mV)    | J <sub>net</sub> (µequiv cm <sup>-2</sup> 10 min <sup>-1</sup> ) |                               |                                | J <sub>net</sub> <sup>H<sub>2</sub>O</sup> (µl cm <sup>-2</sup> 10 min <sup>-1</sup> ) |
|-------------------------------|------------|--|-------------------------------|--------------------------------|--|
|                               |            | J <sub>net</sub> <sup>Na</sup>                                   | J <sub>net</sub> <sup>K</sup> | J <sub>net</sub> <sup>Cl</sup> |  |
| HCO <sub>3</sub> <sup>-</sup> | -8.5±0.6   | 3.3±0.3  | -0.1±0.1                      | 3.0±0.2                        | 15.4±1.4   |
| Phosphate (60 min)            | -2.9±0.5** | 1.5±0.3*   | 0.0±0.0                       | 1.3±0.2**                      | 7.5±0.9**  |
| Difference†                   | -5.6±0.3   | 1.8±0.3  | -0.1±0.1                      | 1.7±0.3                        | 7.9±0.5  |

After bathing both sides of the intestine with the normal Ringer's solution (HCO<sub>3</sub><sup>-</sup>), the HCO<sub>3</sub><sup>-</sup>-buffered solutions were replaced with phosphate-buffered solutions (Phosphate).

Values are mean±s.e. for six fishes.

Difference from control value, \* P<0.01, \*\* P<0.001 (paired t-test).

† Difference=HCO<sub>3</sub><sup>-</sup>-Phosphate.

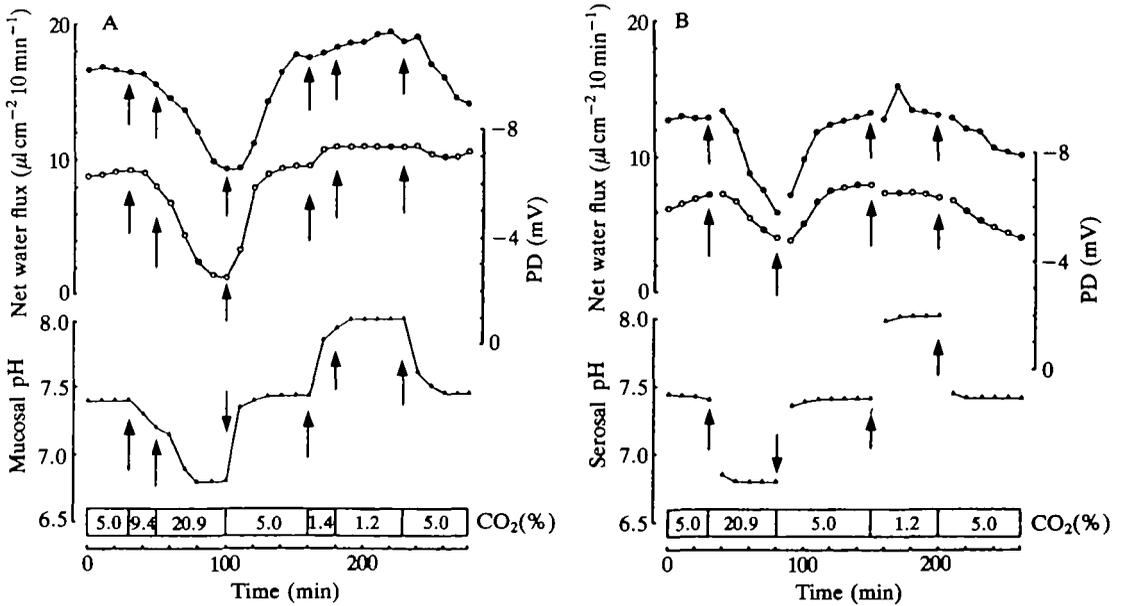


Fig. 3. Effects of CO<sub>2</sub> pressure on transepithelial potential (PD) (○) and net water flux (●). The pH change (▲) in the mucosal or serosal fluid was monitored simultaneously. (A) Keeping the HCO<sub>3</sub><sup>-</sup> concentration at 24.9 mmol l<sup>-1</sup>, the mucosal CO<sub>2</sub> pressure was altered in steps, as shown in the lower column, while the serosal CO<sub>2</sub> pressure was maintained at 5% (pH 7.4). After gassing with 5% CO<sub>2</sub>/95% O<sub>2</sub>, the CO<sub>2</sub> pressure was increased to 9.4% (first arrows), then to 20.9% (second arrows). At the third arrows, 5% CO<sub>2</sub> was reintroduced. After the control period, the CO<sub>2</sub> pressure was reduced to 1.4% (fourth arrows), then to 1.2% (fifth arrows). At the sixth arrows, 5% CO<sub>2</sub> was reintroduced. (B) Keeping HCO<sub>3</sub><sup>-</sup> concentration at 24.9 mmol l<sup>-1</sup>, serosal CO<sub>2</sub> pressure was altered in steps, while mucosal CO<sub>2</sub> pressure was maintained at 5% (pH 7.4). The altered CO<sub>2</sub> pressure is shown in the lower column. It takes 10 min to replace the serosal fluid, and this is indicated by a discontinuous line.

(Fig. 3A). PD and the net water flux increased on reducing CO<sub>2</sub> pressure (1.2%), when the mucosal fluid was alkalinized (pH 8.0). These results conflict with the idea that the lowered CO<sub>2</sub> pressure might inhibit the PD and the net water flux.

A similar experiment was performed by altering serosal CO<sub>2</sub> pressure (Fig. 3B). When serosal CO<sub>2</sub> pressure was raised (20.9%), accompanied by serosal acidification (pH 6.8), PD and the net water flux decreased. After reducing CO<sub>2</sub> pressure, accompanied by serosal alkalinization (pH 8.0), however, PD decreased slightly and the net water flux did not increase significantly. Strangely, after bubbling the serosal fluid with a lower CO<sub>2</sub> pressure (pH 8.0), PD and the net water flux could not be maintained but decreased gradually, even in normal Ringer's solution. It is not clear why serosal alkalinization inhibits PD and the net water flux.

'Sidedness' of the effects of  $\text{HCO}_3^-$ 

Since  $\text{CO}_2$  itself does not enhance the PD and the net water flux,  $\text{HCO}_3^-$  in the buffer system appears to have a major role in ion and water transport. To elucidate which surface of the intestine requires  $\text{HCO}_3^-$ , mucosal and serosal  $\text{HCO}_3^-$  were omitted separately (Fig. 4). When mucosal  $\text{HCO}_3^-$  Ringer's solution was replaced with phosphate Ringer's solution, while the serosa was being bathed with  $\text{HCO}_3^-$  Ringer's solution, the PD and the net water flux decreased only slightly. Omission of  $\text{HCO}_3^-$  from the serosal side decreased these two parameters more distinctly. However, this inhibition was still much smaller than that observed after omitting

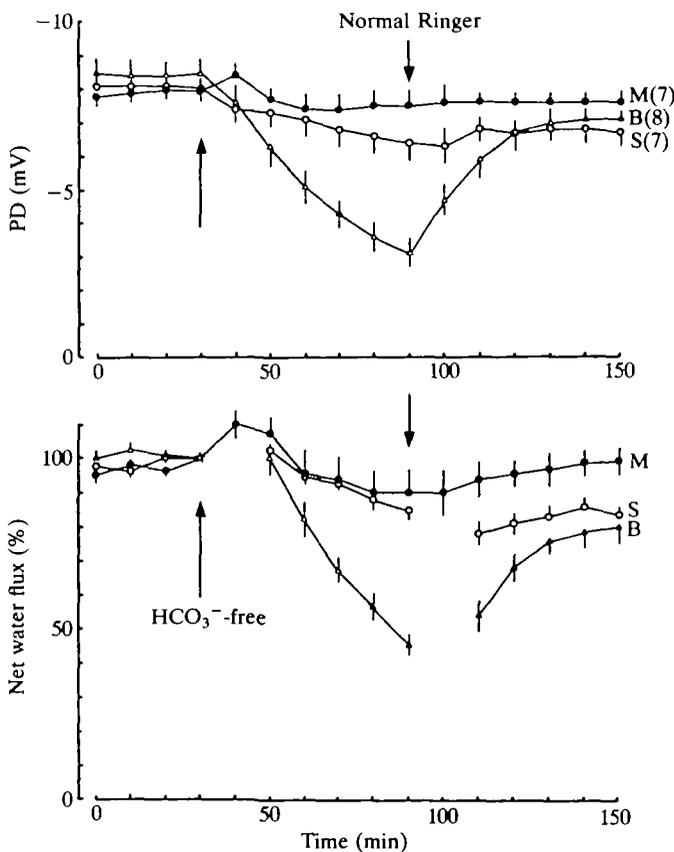


Fig. 4. 'Sidedness' of the effects of  $\text{HCO}_3^-$  on transepithelial potential (PD) and net water flux. After bathing both sides of the intestine with normal  $\text{HCO}_3^-$  Ringer's solutions, the  $\text{HCO}_3^-$  buffer was replaced with phosphate buffer in either the mucosal (M, ●) or serosal (S, ○) fluid (first arrows). For comparison, both sides were also bathed with phosphate Ringer's solutions (B, △). At the second arrows,  $\text{HCO}_3^-$  Ringer's solution was reintroduced. The net water flux is expressed as a percentage of the control flux obtained immediately before the replacement. Values are mean  $\pm$  s.e.: sample sizes are given in parentheses.

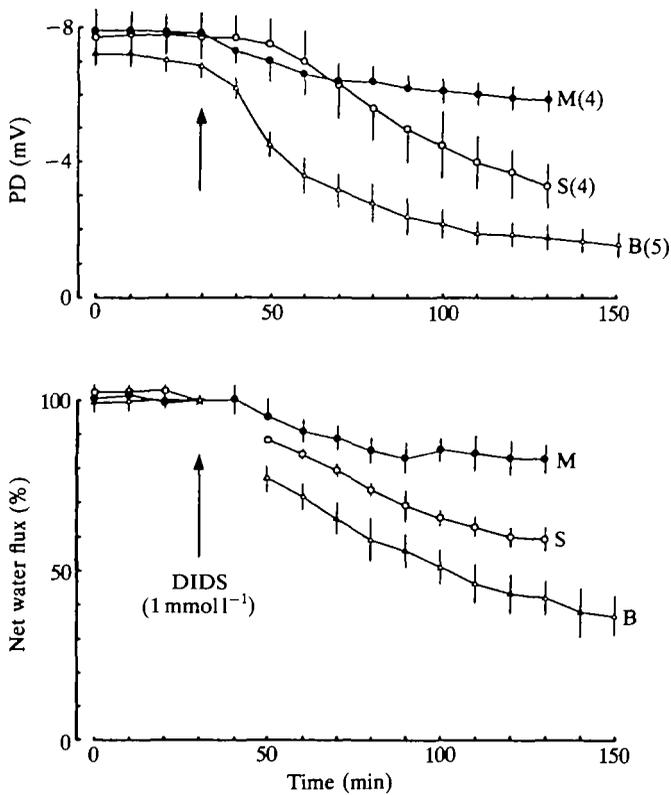


Fig. 5. 'Sidedness' of the effects of DIDS on the transepithelial potential (PD) and the net water flux. After bathing both sides with normal  $\text{HCO}_3^-$  Ringer's solution,  $1 \text{ mmol l}^{-1}$  DIDS was applied to the mucosal (M, ●) or serosal (S, ○) side or to both sides (B, △). The net water flux is expressed as a percentage of the control flux obtained immediately before addition of DIDS. Values are mean  $\pm$  s.e.: sample sizes are given in parentheses.

$\text{HCO}_3^-$  from both sides, indicating that both surfaces of the intestinal epithelium require  $\text{HCO}_3^-$ , but that the serosal side is more sensitive than the mucosal side. In other words, salt and water transport across the seawater eel intestine may be maintained by  $\text{HCO}_3^-$  supply from both the serosa and the mucosa.

To determine whether a specific  $\text{HCO}_3^-$  uptake mechanism exists, the effects of DIDS, a well-known inhibitor of  $\text{HCO}_3^-$  transport, were examined in the next experiment. When  $1 \text{ mmol l}^{-1}$  DIDS was added to the mucosal fluid, the PD and the net water flux decreased gradually (Fig. 5). More distinct effects were observed after addition of this drug to the serosal fluid. The fastest effect was obtained after application of the drug to both sides. The PD and net water flux were not restored to their original levels even after washing out the DIDS. These results suggest that DIDS-sensitive  $\text{HCO}_3^-$  uptake processes exist on both brush border and basolateral membranes of the epithelium in the seawater eel intestine,

and that they are more active on the basolateral membrane. Similar inhibitory effects on the PD and the net water flux were also observed after treatment with 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulphonic acid (SITS), another inhibitor of  $\text{Cl}^-/\text{HCO}_3^-$  exchange, or with acetazolamide, an inhibitor of carbonic anhydrase, but the effects of these drugs were smaller than those of DIDS. Addition to the mucosal side of amiloride ( $1 \text{ mmol l}^{-1}$ ), an inhibitor of  $\text{Na}^+/\text{H}^+$  exchange, also inhibited the net water flux, though only slightly (data not shown).

#### Effects of $\text{Ba}^{2+}$

It is well known that  $\text{Ba}^{2+}$  inhibits  $\text{K}^+$  channels in various epithelial cells (Nagel, 1979; Reuss *et al.* 1981; Smith and Frizzell, 1984; Wills and Biagi, 1982). Therefore, evidence for recycling of the leaked  $\text{K}^+$  comes from the inhibitory effects of mucosal  $\text{Ba}^{2+}$  on  $\text{Na}^+/\text{K}^+/\text{Cl}^-$  cotransport in the flounder intestine (Halm *et al.* 1985) and the thick ascending limb of Henle's loop of rabbit kidney (Greger *et al.* 1983). In these epithelia,  $\text{Ba}^{2+}$  was added to the Hepes-buffered Ringer's solution. However, since  $\text{Ba}^{2+}$  cannot be added directly to the  $\text{HCO}_3^-$  Ringer's solution ( $\text{Ba}^{2+}$  is precipitated in the  $\text{HCO}_3^-$  Ringer's solution used in the present study), and since the  $\text{HCO}_3^-$  buffer system cannot be replaced with other buffer systems in the seawater eel intestine, I looked for another experimental system in which to investigate the effect of  $\text{Ba}^{2+}$ . After several trials, I found that PD and  $I_{\text{sc}}$  could be maintained at high levels when the mucosal  $\text{HCO}_3^-$  was replaced with gluconate and acetate and the mucosal pH was clamped at 7.4 with a pH-stat, while the serosa was bathed with the normal  $\text{HCO}_3^-$  Ringer's solution (see Materials and methods). When PD,  $I_{\text{sc}}$ ,  $R_t$  and  $J_m^{\text{OH}}$  reached a steady level under such conditions,  $\text{BaCl}_2$  ( $5 \text{ mmol l}^{-1}$ ) was added to the mucosal fluid (Fig. 6A). After treatment with  $\text{Ba}^{2+}$ , PD and  $I_{\text{sc}}$  decreased immediately, accompanied by a small increase in  $R_t$ , suggesting that  $\text{Ba}^{2+}$  partially inhibits the  $\text{K}^+$  leakage through the brush-border membrane. After a latent period (about 10 min),  $J_m^{\text{OH}}$  also increased.

To compare the effects of DIDS and  $\text{Ba}^{2+}$ ,  $5 \text{ mmol l}^{-1}$   $\text{BaCl}_2$  was added to the mucosal fluid after pretreatment with DIDS (Fig. 6B). When DIDS ( $0.5 \text{ mmol l}^{-1}$ ) was added to the serosal fluid, PD and  $I_{\text{sc}}$  decreased gradually, as shown in Fig. 5. At the same time,  $R_t$  increased gradually, indicating an inhibition of ionic conduction. During this period,  $J_m^{\text{OH}}$  decreased gradually, suggesting that  $\text{HCO}_3^-$  transport from serosa to mucosa is inhibited by DIDS (see Ando and Subramanyam, 1990). In the presence of DIDS, addition of  $\text{Ba}^{2+}$  evoked no change in any of these four parameters, suggesting that the effects of  $\text{Ba}^{2+}$  are identical to those of DIDS.

#### Discussion

The present study is the first report of  $\text{HCO}_3^-$ -dependence in a  $\text{Na}^+/\text{K}^+/\text{Cl}^-$  cotransport system. When the  $\text{HCO}_3^-$  buffer system was replaced with a phosphate buffer, the serosa-negative PD and the net water flux were inhibited,

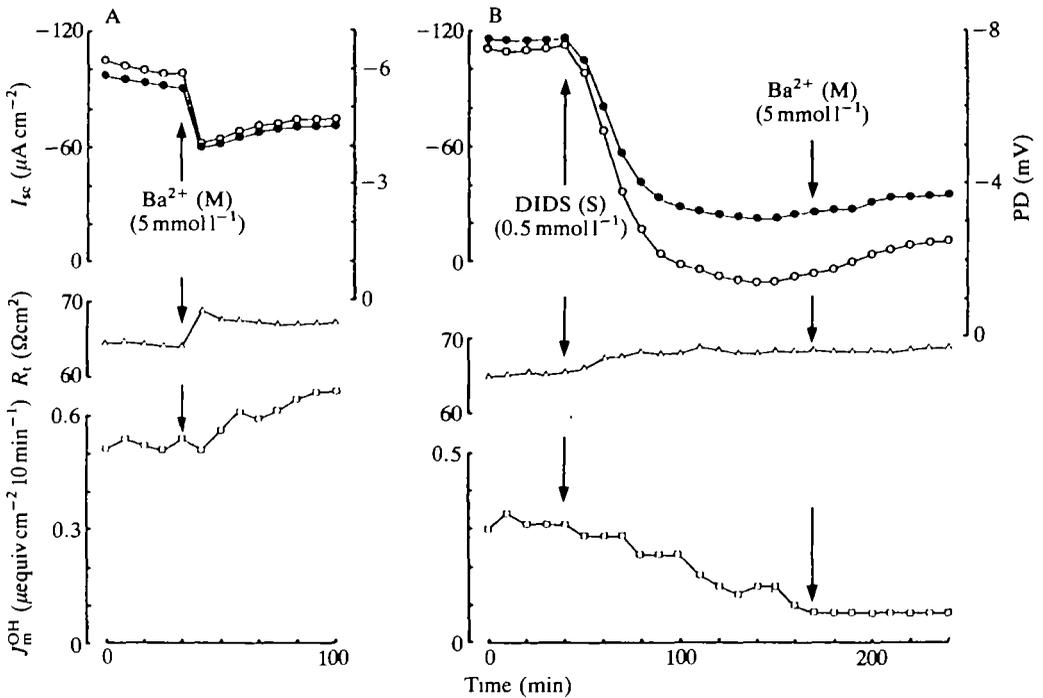


Fig. 6. The effects of  $Ba^{2+}$  on transepithelial potential (PD,  $\circ$ ), short-circuit current ( $I_{sc}$ ,  $\bullet$ ), tissue resistance ( $R_t$ ,  $\Delta$ ) and the rate of mucosal alkalization ( $J_m^{OH}$ ,  $\square$ ) in the absence or presence of DIDS. (A) Effects of  $Ba^{2+}$  in the absence of DIDS. After bathing the mucosa with unbuffered Ringer's solution, the pH was clamped at 7.4 with a pH-stat, the serosa was bathed with normal  $HCO_3^-$  Ringer's solution (pH 7.4), and  $5 \text{ mmol l}^{-1}$   $BaCl_2$  was added to the mucosal fluid (arrows). (B) Effects of  $Ba^{2+}$  in the presence of DIDS. After incubating the intestine under the same conditions as in A,  $0.5 \text{ mmol l}^{-1}$  DIDS was added to the serosal fluid (first arrows). At the second arrows,  $5 \text{ mmol l}^{-1}$   $BaCl_2$  was added to the mucosal fluid. M or S in parentheses denotes the side to which the drug was applied (mucosal or serosal).

accompanied by an attenuation in net  $Na^+$  and  $Cl^-$  fluxes from mucosa to serosa. Since the net  $Na^+$  and  $Cl^-$  fluxes are due to  $Na^+/K^+/Cl^-$  cotransport and the serosa-negative PD is due to  $K^+$  leakage coupled to that cotransport, these results indicate that the  $Na^+/K^+/Cl^-$  cotransport system is inhibited in phosphate-buffered solutions. However, these inhibitory effects were not specific for phosphate. Similar inhibition of PD and net water flux was also observed in other buffer solutions such as Hepes, Tris and Tes. In all these treatments, the  $HCO_3^-$  buffer system was omitted. Moreover, PD and the net water flux were dependent on the presence of  $HCO_3^-$  in the bathing media. All these results indicate that the  $Na^+/K^+/Cl^-$  cotransport system depends on the  $HCO_3^-$  buffer system. Although the  $HCO_3^-$  buffer system consists of  $HCO_3^-$  and  $CO_2$ , higher  $CO_2$  pressure at constant  $HCO_3^-$  concentration did not enhance, but inhibited, the PD and the net water flux, indicating that the inhibitory effects observed after removal of the

$\text{HCO}_3^-$  buffer system are not the result of the omission of  $\text{CO}_2$ . With increased  $\text{CO}_2$  pressure, the pH in the mucosal fluid was lowered. In contrast, lowered  $\text{CO}_2$  pressure caused mucosal alkalization and enhanced the PD and the net water flux. These effects of  $\text{CO}_2$  will be due to pH change, since similar pH-dependence of PD and  $I_{sc}$  has been reported in the seawater eel intestine in Tris Ringer's solution without  $\text{CO}_2$  (Hirano *et al.* 1976); the lower the mucosal pH, the smaller are the PD and  $I_{sc}$ . Recently, Charney *et al.* (1988) have also demonstrated in the flounder intestine that the  $I_{sc}$  and the net  $\text{Cl}^-$  flux from mucosa to serosa depend on the bathing solution pH; the higher the pH, the greater are the  $I_{sc}$  and the  $\text{Cl}^-$  transport.

Because  $\text{CO}_2$  itself does not enhance the PD and the net water flux,  $\text{HCO}_3^-$  appears to play a major role in ion and water transport. However, it is unlikely that  $\text{HCO}_3^-$  absorption itself contributes to the serosa-negative PD and water absorption, because the effects of mucosal  $\text{HCO}_3^-$  are smaller than those of serosal  $\text{HCO}_3^-$ . In addition, the inhibitory effects of DIDS were more pronounced from the serosal side. Since DIDS is a known inhibitor of  $\text{HCO}_3^-$  transport in red blood cells (Cabantchik and Rothstein, 1972), *Necturus* gallbladder (Marsh and Spring, 1985) and bovine corneal endothelial cells (Jentsch *et al.* 1988), this result suggests that the PD and the net water flux are maintained by a DIDS-sensitive  $\text{HCO}_3^-$  uptake process, mainly through the basolateral membrane of the epithelium, but with a minor part through the brush-border membrane. All the present results support the idea that  $\text{Na}^+/\text{K}^+/\text{Cl}^-$  cotransport in the seawater eel intestine is controlled by intracellular  $\text{HCO}_3^-$  concentration.

Mucosal  $\text{Ba}^{2+}$  also inhibited PD and  $I_{sc}$  and enhanced  $R_t$ . All these effects can be explained by a blocking action of  $\text{Ba}^{2+}$  on the  $\text{K}^+$  channels that exist on the brush-border membrane of the eel intestinal epithelium. However, these effects of  $\text{Ba}^{2+}$  were completely abolished after pretreatment with serosal DIDS, suggesting that these two drugs evoke the same result. Since  $\text{Ba}^{2+}$  inhibits apical  $\text{K}^+$  channels and serosal DIDS inhibits  $\text{HCO}_3^-$  transport from serosa to mucosa (Ando and Subramanyam, 1990), these results suggest that the apical  $\text{K}^+$  channels are controlled by the intracellular  $\text{HCO}_3^-$  concentration. Although the inhibitory effects of  $\text{Ba}^{2+}$  are not complete, this may be due to the presence of  $\text{K}^+$  in the mucosal fluid, since it is reported in the mouse thick ascending limb of Henle that blockade of apical  $\text{K}^+$  channels is complete only at  $0 \text{ mmol l}^{-1} \text{ K}^+$  and high  $\text{Ba}^{2+}$  concentration (Hebert *et al.* 1984; Hebert and Andreoli, 1986).

Intracellular  $\text{HCO}_3^-$  concentration may control cytoplasmic  $\text{H}^+$  concentration (pHi). Such a regulatory role of  $\text{HCO}_3^-$  in pHi homeostasis has recently been demonstrated in bovine corneal endothelial cells (Jentsch *et al.* 1988). Furthermore, it has been reported in pancreatic  $\beta$ -cells (Cook *et al.* 1984), liver cells (Henderson *et al.* 1988) and amphibian kidney tubules (Oberleithner *et al.* 1987) that intracellular acidification inhibits  $\text{K}^+$  channels. Therefore, it is likely that similar pHi-sensitive  $\text{K}^+$  channels also exist in the seawater eel intestine. The present results demonstrate that lowered extracellular pHi caused inhibition of both the PD and the net water flux. Although pHi was not measured directly in

this study, it can be stated qualitatively that increased  $\text{CO}_2$  pressure ( $P_{\text{CO}_2}$ ) reduces  $\text{pHi}$  according to the Henderson–Hasselbalch equation,  $\text{pH}=6.1+\log([\text{HCO}_3^-]/0.03P_{\text{CO}_2})$ , since  $\text{CO}_2$  diffuses very easily across the plasma membrane (Malnic, 1980). In addition, the inhibitory effect of amiloride on the net water flux also supports the hypothesis that intracellular acidification lowers the rate of water absorption, since amiloride is known to lower  $\text{pHi}$  by inhibiting  $\text{Na}^+/\text{H}^+$  exchange. However, in the presence of  $\text{HCO}_3^-$ , the contribution of  $\text{Na}^+/\text{H}^+$  exchange to  $\text{pHi}$  homeostasis seems to be smaller, at least in the eel intestine, than that of the  $\text{HCO}_3^-$  transport system(s), since the effect of amiloride is smaller than that of DIDS or  $\text{HCO}_3^-$  removal. If the  $\text{Ba}^{2+}$ -sensitive  $\text{K}^+$  leakage in the brush-border membrane is due to such a  $\text{pHi}$ -sensitive  $\text{K}^+$  channel, intracellular acidification induced by  $\text{HCO}_3^-$  removal from the bathing media or by diminishing  $\text{HCO}_3^-$  transport with DIDS may inhibit the  $\text{K}^+$  leakage into the mucosal fluid. Inhibition of this  $\text{K}^+$  leakage will diminish the serosa-negative PD, secondarily reducing  $\text{Na}^+/\text{K}^+/\text{Cl}^-$  cotransport, and thus inhibiting water transport.

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