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NO_2^- UPTAKE AND HCO_3^- EXCRETION IN THE INTESTINE OF THE EUROPEAN FLOUNDER (*PLATICHTHYS FLESUS*)

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Summary

Ion transport across isolated intestinal segments from the European flounder (*Platichthys flesus*) was studied with the primary aim of evaluating the mechanisms of nitrite (NO_2^-) uptake and HCO_3^- excretion. A double-radiolabelling technique was applied to monitor unidirectional Cl^- and Na^+ influx. Furthermore, net fluxes of NO_2^-, HCO_3^-, Cl^-, Na^+ and water were recorded. NO_2^- uptake was inhibited by mucosal application of bumetanide (10^{-4} mol l^{-1}) but not DIDS (10^{-3} mol l^{-1}), suggesting that NO_2^- is transported across the intestine *via* the $Na^+/K^+/2Cl^-$ cotransporter rather than *via* a Cl^-/HCO_3^- exchanger. In addition to transport *via* the $Na^+/K^+/2Cl^-$ cotransporter, NO_2^- uptake may also occur through the Na^+/Cl^- cotransporter and by conductive transport. NO_2^- and Cl^- influx rates seemed to reflect their mucosal concentrations, and NO_2^- did not influence unidirectional influx or net flux of Cl^-. HCO_3^- efflux was significantly reduced in the presence of 10^{-3} mol l^{-1} DIDS in the mucosal solution. This may indicate the presence of an apical Cl^-/HCO_3^- exchanger in the intestinal epithelium, which would not comply with the current model of HCO_3^- excretion in the intestine of marine teleost fish. An alternative model of HCO_3^- excretion across the intestinal epithelium is proposed.

Key words: nitrite, NO_2^-, Cl^-/HCO_3^- exchange, DIDS, $Na^+/K^+/2Cl^-$ cotransport, bumetanide, base excretion, marine teleost, flounder, *Platichthys flesus*, ion transport.

Introduction

Nitrite (NO_2^-) is a potential contaminant in aquatic environments that receive nitrogenous waste and in recirculated aquaculture systems. Several studies have examined the toxicity and physiological effects of NO_2^- in fish (for reviews, see Lewis and Morris, 1986; Eddy and Williams, 1987; Jensen, 1995, 1996).

Most investigations have concerned freshwater teleosts, because NO_2^- is generally more toxic to freshwater than to marine species. However, we have shown that a marine teleost, the European flounder *Platichthys flesus*, accumulates NO_2^- in the plasma during exposure to 1 mmol l^{-1} ambient NO_2^- and that this accumulation is associated with an increased blood methaemoglobin content (haemoglobin not available for oxygen transport) (M. Grosell and F. B. Jensen, in preparation). Whereas freshwater fish take up NO_2^- primarily across the gills, marine fish may be expected to have significant intestinal uptake as a result of the high rate of drinking required for osmoregulation (Jensen, 1995). Indeed, *in vivo* studies have demonstrated that the European flounder has high NO_2^- concentrations in the intestinal fluids during exposure to elevated ambient NO_2^- concentrations (M. Grosell and F. B. Jensen, in preparation). Furthermore, by using *in situ* perfusion of the intestine (M. Grosell, G. Deboeck, O. Johannsson and C. M. Wood, in preparation), we have demonstrated that intestinal NO_2^- uptake accounts for approximately two-thirds of the total whole-body uptake (M. Grosell and F. B. Jensen, in preparation).

Having established that the intestine is the primary site of NO_2^- uptake in the European flounder, we set out to investigate the mechanism of NO_2^- transport across the intestinal epithelium. In freshwater fish, it has been suggested that the branchial Cl^-/HCO_3^- exchanger is the carrier mediating the apparent active uptake of NO_2^- (Bath and Eddy, 1980), but no attempts have been made to document this.

In the present study, insight into transport mechanisms was obtained by using preparations of freshly isolated intestinal segments, a modification of the technique described by M. Grosell, G. Deboeck, O. Johannsson and C. M. Wood (in preparation), which allowed us to manipulate the composition of both the apical (mucosal) and basolateral (serosal) media.

To evaluate the possible uptake of NO_2^- *via* an intestinal Cl^-/HCO_3^- exchanger, we applied 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid (DIDS), a well-known blocker of Cl^-/HCO_3^- exchange in erythrocytes and other cells

(Nikinmaa, 1990). In a recent review of intestinal ion transport in marine teleost fish, Loretz (1995) proposed a model involving a Cl^-/HCO_3^- exchanger in the basolateral membrane. However, only two studies (Dixon and Loretz, 1986; Ando, 1990) provide evidence for a basolateral Cl^-/HCO_3^- exchanger, and Ando (1990) also reports evidence for apical Cl^-/HCO_3^- exchange. Consequently, we applied DIDS both in the mucosal solution and in the mucosal and serosal solutions in combination to assess the potential importance of apical and basolateral Cl^-/HCO_3^- exchangers for intestinal NO_2^- uptake.

A further possible route of uptake for NO_2^- would be the $Na^+/K^+/2Cl^-$ cotransporter in the apical membrane of the intestine (Loretz, 1995). Accordingly, we have also used bumetanide in the mucosal solution to target this transporter and its possible role in NO_2^- uptake.

During the studies, double radiolabelling of Na^+ and Cl^- allowed us to measure not only NO_2^- uptake but also unidirectional Na^+ and Cl^- fluxes as indicators of the viability of the freshly isolated intestinal segments and of the action of pharmacological agents. These isotopic techniques, in combination with recordings of net transport of Na^+, Cl^-, HCO_3^- and water, gave insight into the general ion-transport properties of the intestinal epithelium of the European flounder.

Materials and methods

European flounder (*Platichthys flesus*) were obtained from a local fisherman and brought to the Aquatic Research Center of Odense University in Kerteminde, Funen, Denmark. The fish were held in a 400 l polyvinylchloride tank with a flow of aerated Kerteminde sea water for at least 6 days prior to experiments. The fish were not fed during holding. Experimental fish were transferred to glass aquaria with a bottom surface area of 0.25 m^2 containing 25 l of Kerteminde sea water (static, 24 h renewal) adjusted to 20 ‰ salinity with sea salt (three fish per tank) at 6 °C and allowed to acclimate to this salinity and temperature for a minimum of 3 days prior to experiments.

General experimental protocol

Preparations were made from a total of 36 fish, ranging in body mass from 105 to 283 g. Fish were killed with an overdose of neutralized MS-222 (200 mg l^{-1}). One side of the body cavity was removed, exposing the gastrointestinal tract. The intestine was obtained by dissection, placed on a glass plate, and put on ice. An inflow catheter (a 10 cm length of PE90 tubing flared at the tip) was inserted at the anterior end of the intestine and tied in place using two silk ligatures. This catheter was used to flush the intestine gently with 20 ml of the gut saline to be used in the following experiments (see below) to displace any intestinal fluid and solids. The anterior, mid and posterior parts of the intestine were then separated. The mid and posterior parts were fitted with a filling catheter (PE90) at the anterior end as described above, and all three 'gut bags' were then closed at the posterior end using two silk ligatures. Subsequently, these 'gut bags' were filled with gut saline containing ^{22}Na and ^{36}Cl as well as NO_2^- (see below for composition) and transferred to glass vials containing 18 ml of aerated L-15 medium (Leibovitz's L-15 medium, containing L-glutamine; Gibco). The L-15 medium was supplemented with 10 mmol l^{-1} $NaHCO_3$, giving 10 mmol l^{-1} total CO_2 and a pH of approximately 8.00 to mimic the *in vivo* situation for these fish at a similar temperature (M. Grosell and F. B. Jensen, in preparation). The vials were placed in a water bath maintained at a constant temperature of 6 °C. All solutions used in the experiments were at 6 °C. Immediately after filling the gut bag with the appropriate gut saline, a sample of 300–400 µl of gut saline was obtained *via* the catheter, which was subsequently plugged. The gut bags were carefully lifted out of the surrounding L-15 medium, blotted dry, weighed and again placed in the vial (time zero). Samples (0.5 ml) of the external L-15 medium were taken (in triplicate) at 0, 2 and 4 h and transferred to scintillation vials for analysis of ^{36}Cl and ^{22}Na. A fourth sample of L-15 medium was taken at the same times in the NO_2^--treated preparations and used for analysis of $[NO_2^-]$. The L-15 medium removed by sampling was replaced with fresh medium. After 4 h, the gut bags were blotted dry and reweighed. Finally, a 300–400 µl sample of the internal gut saline was collected from the catheter. The gut saline samples taken at the start and at the end of the experiment were analysed for pH, total CO_2 content, $[Na^+]$, $[Cl^-]$, $[NO_2^-]$ and ^{36}Cl and ^{22}Na radioactivity. Each gut bag was opened using a longitudinal incision, the remaining gut saline was removed, and the intestinal tissue was blotted dry and reweighed. The exposed net gross surface area of each gut bag was measured using graph paper.

The internal volume of gut saline in each individual gut bag was determined by subtracting the mass of the empty gut bag from the corresponding initial mass. Net water transport was calculated from the difference in total mass over the experimental period, with the surface area of each gut bag and the time elapsed taken into account. Net fluxes of Na^+, Cl^- and HCO_3^- were calculated by dividing the difference in the total number of moles of the given ion in the gut saline between the beginning and end of the experiment by the surface area and elapsed time.

Unidirectional mucosal-to-serosal Na^+ and Cl^- fluxes were calculated from the increase in ^{22}Na and ^{36}Cl radioactivity in the surrounding L-15 medium during two successive 2 h periods using the means of corresponding specific activities at the start and at the end of the flux period. Unidirectional NO_2^- flux was calculated from the appearance of NO_2^- in the surrounding L-15 medium during the two successive 2 h periods. Unidirectional Na^+ and Cl^- fluxes and net NO_2^- fluxes were related to corresponding gut bag surface area and elapsed time.

Pharmacology

The gut saline used for flushing the intestine prior to experiments consisted of the following (in mmol l^{-1}); 24 NaCl,

15 $MgCl_2$, 2 $CaCl_2$, 1 KCl and 1 $NaHCO_3$, pH 7.4. The composition of the gut saline was based on actual measurements of gut fluid composition (for details, see M. Grosell and F. B. Jensen, in preparation). During experiments, 11.1 kBq of ^{36}Cl (Amersham) and 7.4 kBq of ^{22}Na (Amersham) were added for unidirectional measurements of Cl^- and Na^+ fluxes, respectively. For the studies of NO_2^- transport, 1 mmol l^{-1} NO_2^- (as $NaNO_2$) was added. Experimental treatments consisted of the following four series with control L-15 medium on the serosal side of the gut bags: (1) control gut saline with 0.1 % dimethylsulphoxide (DMSO) (vehicle control), (2) gut saline with 10^{-4} mol l^{-1} bumetanide, an inhibitor of the $Na^+/K^+/2Cl^-$ cotransporter, dissolved in a final concentration of 0.1 % DMSO; (3) control gut saline with 1 % DMSO (vehicle control) and (4) 10^{-3} mol l^{-1} DIDS, a blocker of Cl^-/HCO_3^- exchange, dissolved in 1 % DMSO. Two additional experimental series were as follows: (5) 1 % DMSO in both the L-15 medium and the gut saline (vehicle control); and (6) 10^{-3} mol l^{-1} DIDS dissolved in a final concentration of 1 % DMSO in both the L-15 medium (serosal) and the gut saline (mucosal).

Appropriate gut bag saline was made up freshly just prior to experiments from a stock solution of gut saline by adding ^{36}Cl, ^{22}Na, $NaNO_2$ (where appropriate) and bumetanide or DIDS with corresponding DMSO vehicle controls. L-15 medium containing DIDS and the corresponding DMSO control (experimental series 5 and 6) were also made up freshly prior to experiments.

Analytical techniques

γ-Radioactivity from ^{22}Na was measured in samples of the external L-15 medium and in samples of gut bag saline from the start and from the end of the flux period using a gamma-counter (1480 Wizard 3 Automatic, Wallac, Turku, Finland). Total β-radioactivity from ^{36}Cl and ^{22}Na was also measured in the same external L-15 medium samples and in the initial and terminal internal gut bag saline samples using a scintillation counter (Packard 2200 CA Tri-Carb, Packard, Groningen, Netherlands). The two counters were cross-calibrated for ^{22}Na radioactivity, and the contribution of ^{22}Na to the total β-radioactivity was subtracted from the total β-radioactivity to determine the ^{36}Cl β-radioactivity. NO_2^- concentrations in gut saline samples and in samples of L-15 medium were determined spectrophotometrically following the method of Shechter et al. (1972). For NO_2^- measurements in L-15 medium, the corresponding standard curve was obtained from known concentrations of NO_2^- in L-15 medium, since the red colour of the medium interfered with the absorbance of the NO_2^- assay. Similarly, at the relatively high concentrations used in the present investigation, DIDS was found to interfere with the NO_2^- assay. Consequently, the standard curves for NO_2^- measurements in experimental series 5 and 6 were derived from known NO_2^- concentrations in L-15 medium containing the appropriate DIDS concentration. Gut bag saline and L-15 medium pH was measured using Radiometer (Copenhagen, Denmark) BMS3 electrodes (BMS3 system) with the signal displayed on a PHM 73 blood gas monitor and REC 80 recorders. The total CO_2 concentrations of gut bag saline and L-15 medium were measured using the method of Cameron (1971). The Cl^- concentrations of gut bag saline samples were measured using a Radiometer CMT 10 chloride titrator, and $[Na^+]$ was measured by flame photometry (Instrumentation Laboratory 243).

Statistical evaluation and data presentation

All values are expressed as absolute values, means ± S.E.M. (*N*). Significant differences between values obtained from preparations with either bumetanide or DIDS and their corresponding vehicle controls were evaluated using unpaired Student's *t*-test (two-tailed). Because of day-to-day variations in absolute flux values, experiments involving preparations from two control fish (vehicle control) and two fish from one of the above treatments were conducted simultaneously. Statistical comparisons with the corresponding control values were conducted on relative flux rates (percentage of the corresponding control value for the same day) using an ASIN conversion to ensure normal distribution of the data. Vehicle controls (0.1 % and 1 % DMSO) were compared with control (no DMSO) values by two-tailed *t*-test with Bonferroni multi-sample comparison correction. One-way analysis of variance (ANOVA) was used to compare both unidirectional and net flux rates between different segments of the intestine. In all cases, groups were considered significantly different at $P<0.05$.

Results

The presence of NO_2^- in the gut lumen led to a significant NO_2^- uptake across the gut epithelium. The rate of NO_2^- influx correlated well with the rate of Cl^- influx ($y=0.0035+0.0071x$; $P<0.05$, $r^2=0.566$) (Fig. 1). The ratio between unidirectional

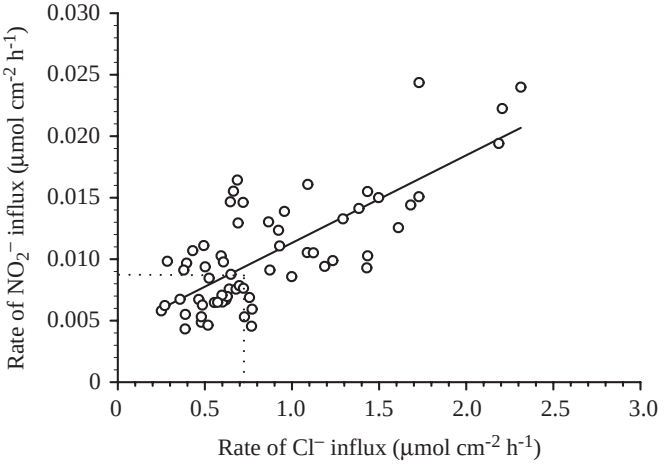

Fig. 1. Control rates of NO_2^- influx (μmol cm^{-2} h^{-1}) in all intestinal segments plotted as a function of the corresponding rates of Cl^- influx (μmol cm^{-2} h^{-1}) (*N*=54). The regression line has the equation: $y=0.0035+0.0071x$ ($P<0.05$, $r^2=0.566$). The dotted lines indicate mean rates of Cl^- and NO_2^- influx.

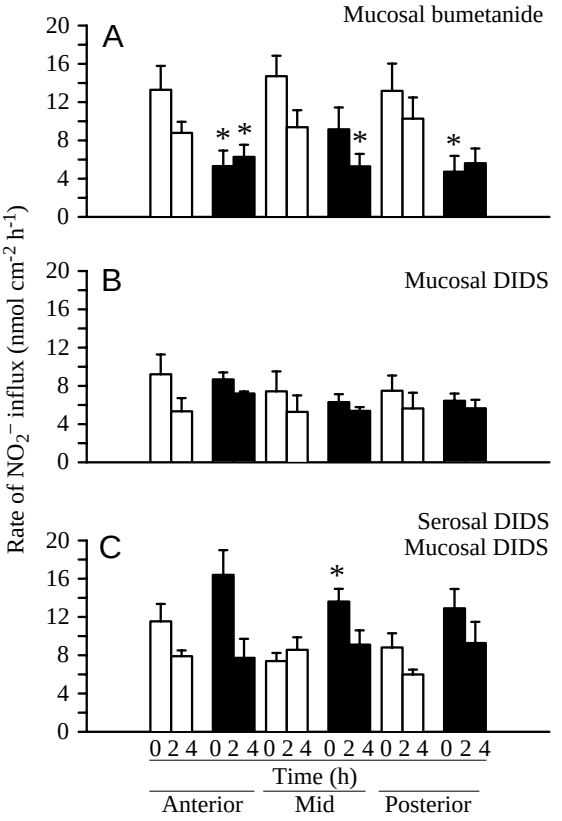

Fig. 2. Rates of NO_2^- influx (nmol cm^{-2} h^{-1}) in the anterior, mid and posterior segments of freshly isolated flounder intestine during two successive 2 h flux periods. Open columns are vehicle control (dimethylsulphoxide) values. Filled columns are values obtained in the presence of 10^{-4} mol l^{-1} bumetanide in the mucosal solution (A), 10^{-3} mol l^{-1} DIDS in the mucosal solution (B) and 10^{-3} mol l^{-1} DIDS in both the mucosal and serosal solutions (C). Values are means + S.E.M. (N=6 in all cases). An asterisk indicates a statistically significant difference from the corresponding control value (P<0.05).

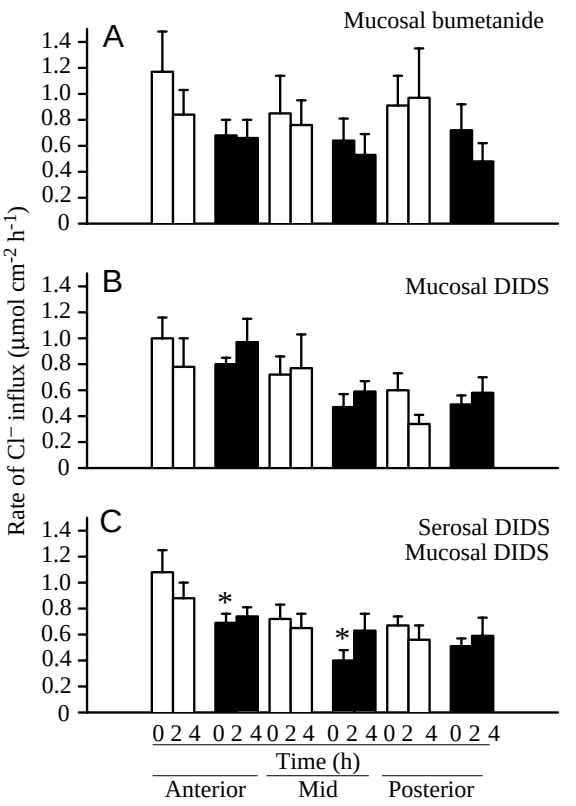

Fig. 3. Rates of Cl^- influx (μmol cm^{-2} h^{-1}) in the anterior, mid and posterior segments of freshly isolated flounder intestine during two successive 2 h flux periods. Other details as in Fig. 2.

NO_2^- and Cl^- flux rates was approximately 1:100 at mean flux rates. Considering the change in concentration of these two anions in the gut saline over time, this ratio reflects the different luminal concentrations of NO_2^- and Cl^- ($[NO_2^-]$:$[Cl^-]$ being 1:59 initially and 0.7:116 finally).

The rate of NO_2^- influx did not differ among the different segments of the intestine, but tended to be lower during the second 2 h flux period than during the first 2 h flux period (Fig. 2). Overall, the unidirectional rate of influx of NO_2^- was 8.9 nmol cm^{-2} h^{-1} in the control series. NO_2^- uptake was significantly inhibited by 10^{-4} mol l^{-1} bumetanide added to the gut saline and thus contacting the mucosal side of the intestine (Fig. 2). Bumetanide (10^{-4} mol l^{-1}) caused an overall 47 % inhibition of NO_2^- uptake in all segments of the intestine compared with corresponding control values. DIDS (10^{-3} mol l^{-1}) applied to the mucosal side of the intestine did not influence NO_2^- influx (Fig. 2). When applied simultaneously to both the gut saline and the L-15 medium and thus acting on both the mucosal and serosal sides of the intestine, DIDS (10^{-3} mol l^{-1}) caused a slight increase in the initial rate of NO_2^- influx. This initial increase was only statistically significant in the mid part of the intestine (Fig. 2).

Vehicle control (saline + DMSO) unidirectional Cl^- (Fig. 3) and Na^+ (Fig. 4) flux rates and net flux rates of HCO_3^- (Fig. 5) did not differ from corresponding control flux rates in preparations exposed to gut saline without DMSO and NO_2^- (data not shown).

Overall, the unidirectional rate of Cl^- influx was 0.72 μmol cm^{-2} h^{-1}. This rate tended to be slightly higher in the anterior part than in the mid and posterior parts of the intestine, but did not differ significantly between the first and the second 2 h flux periods (Fig. 3). Neither bumetanide (10^{-4} mol l^{-1}) nor DIDS (10^{-3} mol l^{-1}) applied to the mucosal side of the intestine caused significant changes in unidirectional Cl^- fluxes. Rates of Cl^- influx in the bumetanide-treated preparations, however, showed a general trend to be lower than their corresponding controls, but these differences were not significant (Fig. 3A). DIDS (10^{-3} mol l^{-1}) applied to both the mucosal and serosal sides of the preparation caused a significant 22 % inhibition of Cl^- influx in the anterior and posterior parts of the intestine in the first 2 h flux period (Fig. 3C).

The mean overall Na^+ influx in control preparations was 0.66 μmol cm^{-2} h^{-1}. As for Cl^-, the rate of Na^+ influx tended to be slightly higher in the anterior than in the mid and posterior parts of the intestine, but did not differ significantly between the first and the second 2 h flux period (Fig. 4).

Table 1. *Net Na^+, Cl^- and water fluxes in the anterior, mid and posterior intestinal segments of the control, vehicle control (0.1 % and 1 % dimethylsulphoxide), 10^{-4} mol l^{-1} bumetanide, 10^{-3} mol l^{-1} DIDS (mucosal) and 10^{-3} mol l^{-1} DIDS (mucosal + serosal) samples*

Net flux	Control	Series 1 Vehicle control, 0.1 % DMSO	Series 2 0.1 % DMSO + bumetanide	Series 3 Vehicle control, 1 % DMSO	Series 4 1 % DMSO + DIDS, mucosal	Series 5 Vehicle control 1 % DMSO	Series 6 1 % DMSO+ DIDS, mucosal + serosal
Na^+ flux (μm cm^{-2} h^{-1})							
Anterior	–0.49±0.19	–0.28±0.07	–0.36±0.07	–0.70±0.15	–0.42±0.11	–0.41±0.10	–0.36±0.04
Mid	–0.75±0.19	–0.29±0.05	–0.34±0.07	–0.61±0.14	–0.34±0.17	–0.57±0.09	–0.16±0.06*
Posterior	–0.81±0.08	–0.63±0.18	–0.46±0.09	–0.58±0.09	–0.42±0.08	–0.53±0.08	–0.30±0.11
Cl^- flux (μm cm^{-2} h^{-1})							
Anterior	–0.16±0.12	0.25±0.19	–0.38±0.07	–0.62±0.20	–0.61±0.21	–0.43±0.16	–0.40±0.05
Mid	–0.19±0.12	–0.35±0.20	–0.35±0.07	–0.53±0.12	–0.32±0.28	–0.59±0.11	–0.24±0.16
Posterior	–0.36±0.12	–0.50±0.14	–0.37±0.11	–0.49±0.10	–0.41±0.09	–0.49±0.05	–0.31±0.18
Water flux (μl cm^{-2} h^{-1})							
Anterior	8.04±2.19	18.40±3.21‡	8.98±1.32*	12.07±2.35	11.14±1.98	11.23±2.07	15.17±1.90
Mid	11.63±4.55	12.63±2.81	7.27±1.61	6.90±1.53	9.54±2.02	6.17±0.73	13.81±1.74*
Posterior	6.41±2.86	9.35±2.69	4.74±1.83	4.29±1.26	6.32±0.70	3.42±0.86	10.89±2.52*

*Significantly different from the corresponding vehicle control; ‡significant difference between the control and vehicle control (both at $P<0.05$).

Overall, water flux was greatest in the anterior part of the intestine, intermediate in the mid intestine and lowest in the posterior intestine (ANOVA, $P<0.05$).

Bumetanide (10^{-4} mol l^{-1}) caused an overall 40 % inhibition of unidirectional Na^+ influx. DIDS (10^{-3} mol l^{-1}) applied to the mucosal side of the intestine did not influence Na^+ influx rates. When DIDS was applied to both the mucosal and the serosal sides, however, Na^+ influx was significantly increased by 38 % compared with control values (Fig. 4C).

The net HCO_3^- flux averaged for all control flux periods was –0.28 μmol cm^{-2} h^{-1} (i.e an efflux) (Fig. 5). The rate of HCO_3^- efflux to the gut lumen, however, tended to be highest in the anterior part of the intestine (Fig. 5). All the blockers applied caused inhibition of HCO_3^- efflux (Fig. 5). Mucosal bumetanide (10^{-4} mol l^{-1}) resulted in an overall inhibition of 24 %, mucosal DIDS (10^{-3} mol l^{-1}) caused a 39 % inhibition, while DIDS applied to both the serosal and the mucosal sides resulted in 28 % inhibition. For both DIDS treatments, the inhibition was only significant in the anterior and posterior parts of the intestine (Fig. 5).

Net flux rates of Na^+, Cl^- and water in control, vehicle control, bumetanide- and DIDS-treated preparations are given in Table 1. With the exception of water flux in the anterior segment of series 1, none of the net flux rates differed significantly between the control and the vehicle control (two different concentrations of DMSO). Overall, net flux rates of both Na^+ and Cl^- were slightly negative (i.e. a net efflux of both these ions). For both Na^+ and Cl^- fluxes, there were no apparent differences in net flux rates among the different intestinal segments. For water flux, however, the anterior part of the intestine exhibited the highest flux rate, and there was a progressive decrease in the rate of water uptake in the mid and posterior parts of the gut. DIDS applied to both the mucosal and serosal sides of the intestine caused an increased net flux of Na^+ in the mid section (less negative). This is consistent with the effect of DIDS on unidirectional Na^+ flux (Fig. 4) and was apparently associated with increased rates of water transport (Table 1).

Bumetanide appeared to have a significant effect on the rate of water transport in the anterior part of the intestine. It should be noted, however, that the control value for this treatment is unusually high (compared with the other control values) (Table 1).

Discussion

The relatively high concentrations of DMSO needed to dissolve the high concentrations of both bumetanide and DIDS used in the present study did not influence net flux rates of Na^+, Cl^- or water, indicating that there was no apparent effect of DMSO on membrane permeability at these concentrations. The intestinal epithelium is highly convoluted and rich in microvilli, and this probably explains the relatively high concentration of blockers needed to provide a local concentration sufficient to inhibit carrier-mediated ion fluxes. Typical concentrations of DIDS needed to inhibit Cl^-/HCO_3^- exchange by 50 % (I_{50}) in erythrocytes are between 10^{-7} and 0.5×10^{-6} mol l^{-1} (Nikinmaa, 1990). In comparison, Wilson et al. (1996) reported no effect of mucosal DIDS at 10^{-5} mol l^{-1} on HCO_3^- excretion in seawater-adapted rainbow trout during *in situ* perfusion of the intestine, and Dixon and Loretz (1986)

observed no effect of mucosal DIDS at 10^{-4} mol l^{-1} on HCO_3^- transport in the goby (*Gillichthys mirabilis*). In the present study, 10^{-3} mol l^{-1} DIDS applied to the mucosal medium caused a significant inhibition of the rate of HCO_3^- transport. The same concentration of mucosal DIDS was effective in reducing transepithelial potential and water flux in seawater-adapted Japanese eel (*Anguilla japonica*) (Ando, 1990). The value of I_{50} for bumetanide is typically betweeen 10^{-7} and 0.5×10^{-6} mol l^{-1} (Nikinmaa, 1990). Various isoforms of the $Na^+/K^+/2Cl^-$ cotransporter, however, have different sensitivities to bumetanide; in the basolateral cotransporter of the operculum epithelium, for instance, the sensitivity can be low (Marshall and Bryson, 1998). We chose a concentration of 10^{-4} mol l^{-1} in the present study. Similar mucosal concentrations of bumetanide have previously been used to study the apical $Na^+/K^+/2Cl^-$ cotransporter in marine teleost intestines (Charney et al., 1988; Musch et al., 1982) and to study branchial $Na^+/K^+/2Cl^-$ cotransport (Flik et al., 1997).

NO_2^- uptake by the intestine

The present data suggest that NO_2^- is taken up across the intestine in the European flounder *via* the $Na^+/K^+/2Cl^-$ cotransporter rather than *via* the Cl^-/HCO_3^- exchanger. The mucosal $Na^+/K^+/2Cl^-$ cotransporter was targeted with bumetanide, which caused a similar inhibition of NO_2^- and Na^+ influx, and a tendency to reduce Cl^- influx. In contrast, DIDS applied to the mucosal medium did not inhibit NO_2^- uptake rates, although the rate of HCO_3^- excretion was significantly reduced. When DIDS was applied to both the mucosal and serosal sides of the epithelium, NO_2^- uptake was slightly elevated compared with controls. A similar effect was observed on unidirectional Na^+ influx and net Na^+ flux rates. The effects of DIDS when applied to both solutions differ from the situation where DIDS was applied to the mucosal solution only and can therefore be regarded as an effect of DIDS in the serosal solution. The mechanism(s) involved in the stimulation of unidirectional Na^+ influx and net Na^+ flux by serosal DIDS is unknown.

NO_2^- did not influence unidirectional Cl^- influx or net Cl^- flux. This contrasts with the situation in freshwater teleost fish and crustaceans, in which NO_2^- uptake across the gills induces net Cl^- loss with a consequent reduction in extracellular Cl^-

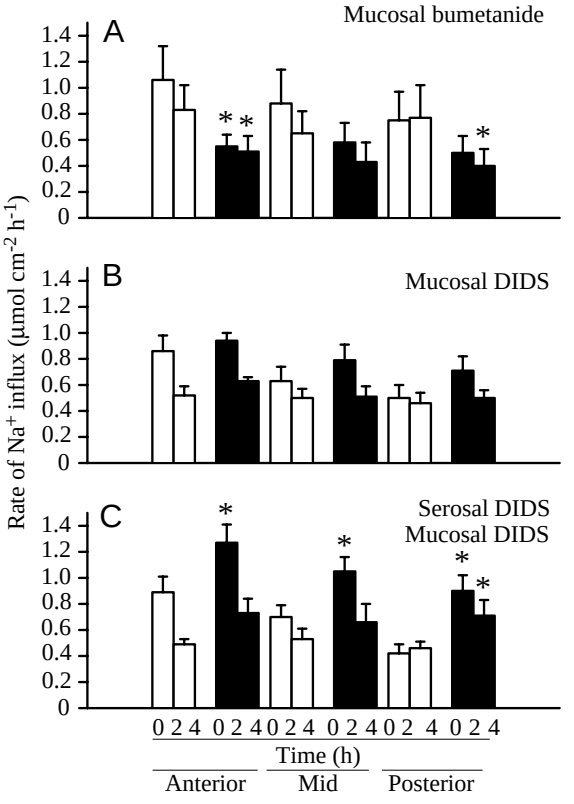

Fig. 4. Rates of Na^+ influx (μmol cm^{-2} h^{-1}) in the anterior, mid and posterior segments of freshly isolated flounder intestine during two successive 2 h flux periods. Other details as in Fig. 2.

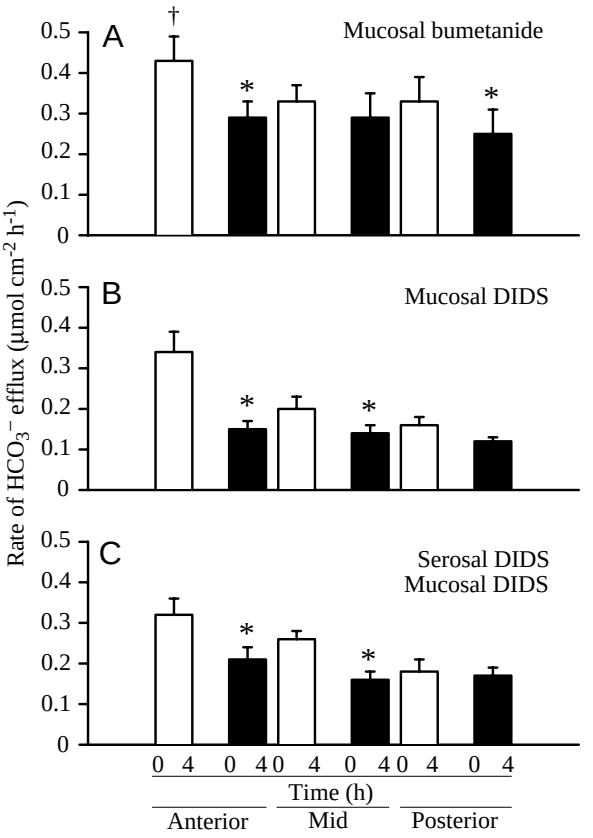

Fig. 5. Net rates of flux of HCO_3^- (μmol cm^{-2} h^{-1}) in the anterior, mid and posterior segments of freshly isolated flounder intestine during one 4 h flux period. Open columns are vehicle control (dimethylsulphoxide) values. Filled columns are values obtained in the presence of 10^{-4} mol l^{-1} bumetanide in the mucosal solution (A), 10^{-3} mol l^{-1} DIDS in the mucosal solution (B) and 10^{-3} mol l^{-1} DIDS in both the mucosal and serosal solutions (C). Values are means + S.E.M. (N=6 in all cases). An asterisk (*) indicates a statistically significant difference from the corresponding control value. A double dagger (‡) indicates a statistically significant difference between the vehicle control and the true control (data not shown). In both cases, P<0.05.

concentration (Jensen et al., 1987; Jensen, 1990; Harris and Coley, 1991). In freshwater fish, increased external Cl^- concentrations reduce toxicity by reducing the accumulation of NO_2^- in the plasma (Eddy et al., 1983; Bath and Eddy, 1980). Williams and Eddy (1986) demonstrated that NO_2^- acts as a competitive inhibitor of Cl^- uptake and *vice versa*, which explains the protective effect of ambient Cl^- against NO_2^- uptake and toxicity. The transport affinities (K_m) for NO_2^- and Cl^- were comparable. Taken together, these observations indicate that Cl^- and NO_2^- share the same uptake mechanism across the gills of freshwater fish and that the carrier involved has a similar affinity for both anions (i.e. does not distinguish between them). This means that the ratio of NO_2^- to Cl^- uptake across gills in freshwater fish will reflect the ratio of the ambient concentrations of the two anions. In the present study, the Cl^- concentration in the gut saline applied on the mucosal side was 59 mmol l^{-1}, and this increased during the 4 h of experimentation to approximately 116 mmol l^{-1} (negative net Cl^- flux), whereas the corresponding initial NO_2^- concentration was 1 mmol l^{-1}, decreasing to approximately 0.7 mmol l^{-1} during the experimental period (uptake from the lumen). The slope of the regression line in Fig. 1 (approximately 0.01) therefore suggests that Cl^- and NO_2^- transport rates across the intestine reflect the available concentrations of the two anions, as is the case for branchial transport in freshwater fish. However, experiments involving different NO_2^- and Cl^- concentrations are needed to determine conclusively whether the ratio of the concentrations of the two ions is determining the rate of uptake of NO_2^- in the intestine of marine fish. Considering the high $[Cl^-]$:$[NO_2^-]$ ratio in the mucosal solution, a similar affinity of the $Na^+/K^+/2Cl^-$ cotransporter for NO_2^- and Cl^- would imply that Cl^- influx should be inhibited by approximately by 1 % in the presence NO_2^-. Such a low level of inhibition would not be detectable.

The $Na^+/K^+/2Cl^-$ cotransporter is also present in the gills of freshwater teleosts (Cutler et al., 1998; Flik et al., 1997) and, in the light of the present study, it seems relevant to investigate the relative roles of this transporter and of the Cl^-/HCO_3^- exchanger (which was implicated by the study of Bath and Eddy, 1980) in NO_2^- uptake across the gills of freshwater fish.

Na^+ and Cl^- influx in the presence of bumetanide was not completely inhibited. This could be due to an insufficient bumetanide concentration in the microenvironment of the transporter, but it could also be due to transport across the apical membrane by the bumetanide-insensitive Na^+/Cl^- cotransporter, which is present at the apical membrane of the intestine of marine teleosts (Loretz, 1995). Whether NO_2^- is also transported across the apical membrane *via* the Na^+/Cl^- cotransporter must be determined in further studies. However, assuming NO_2^- concentrations of 1 mmol l^{-1} in the mucosal solution and 0.01 mmol l^{-1} (typical NO_2^- concentration in the L-15 medium at the end of an experiment) in the serosal solution, the equilibrium potential of NO_2^- would be approximately −120 mV. This is much lower than the transepithelial potential recorded in asymmetrical *in vivo* conditions (−20 mV; Loretz, 1995), indicating that part of the NO_2^- uptake could be by simple diffusion.

Base excretion by the intestine

The intestine of seawater-adapted rainbow trout has recently been identified as an important site for base excretion (Wilson et al., 1996). Most of the base equivalents transported across the intestinal epithelium are in the form of HCO_3^- (Dixon and Loretz, 1986; Wilson et al., 1996). Even though a significant part of the excreted HCO_3^- forms carbonate deposits within the intestinal lumen of various marine teleost fish (Walsh et al., 1991; Wilson et al., 1996; Wilson, 1997), high concentrations of HCO_3^- in the intestinal fluids have frequently been reported from a wide range of marine teleosts (Walsh, 1991; Wilson et al., 1996; Wilson, 1997; M. Grosell, G. Deboeck, O. Johannsson and C. M. Wood, in preparation; M. Grosell and F. B. Jensen, in preparation). In the European flounder, we have recently shown that HCO_3^- is the major anion in the fluids of at least the mid and posterior parts of the intestine (M. Grosell and F. B. Jensen, in preparation). The lumen-to-blood gradient of $[HCO_3^-]$ in the European flounder (M. Grosell and F. B. Jensen, in preparation) would require an equilibrium potential of −47 mV (lumen negative); in seawater-adapted rainbow trout the corresponding equilibrium potential is −63 mV (Wilson, 1997). Considering that the transepithelial potential is only a few millivolts serosa-negative even in asymmetrical conditions (Loretz, 1995), this means that HCO_3^- is not passively distributed and that HCO_3^- excretion must be active. An active Cl^-/HCO_3^--ATPase was suggested by Wilson (1997). However, considering transmembrane potentials and electrochemical Cl^- gradients, HCO_3^- excretion can be accounted for by Cl^-/HCO_3^- exchange. A strong electrochemical gradient for Cl^- favours Cl^- efflux across the basolateral membrane, and part of this efflux has been attributed to a basolateral non-conductive exchange with HCO_3^- (Dixon and Loretz, 1986). This exchange, driven by the electrochemical gradient for Cl^-, could allow HCO_3^- to be taken up across the basolateral membrane against its electrochemical gradient. Subsequent HCO_3^- efflux across the apical membrane would be favoured by the membrane potential (−100 mV, cytosol negative; Loretz, 1995), which can potentially support a $[HCO_3^-]$ gradient of approximately 1:60 (cytosol:lumen). The apical HCO_3^- efflux could be *via* a conductive pathway, as suggested by Dixon and Loretz (1986). This suggestion, as well as the proposed involvement of a Cl^-/HCO_3^--ATPase (Wilson, 1997), is, however, based on the absence of an effect of mucosal DIDS at concentrations of 10^{-4} and 10^{-5} mol l^{-1}, respectively. In the present study, and in the study of Ando (1990), mucosal DIDS at a concentration of 10^{-3} mol l^{-1} (10–100 times more concentrated than in the previous studies) did reduce the rate of HCO_3^- transport, suggesting the presence of an apical Cl^-/HCO_3^- exchanger.

Transepithelial HCO_3^- transport could therefore include both a basolateral Cl^-/HCO_3^- exchanger, favoured by the electrochemical gradient for Cl^- (as reported by Dixon and Loretz, 1986), and an apical DIDS-sensitive carrier-mediated

HCO_3^- transport, favoured by the electrochemical gradient for HCO_3^- (as suggested by the results of the present study). This model is supported by Ando (1990), who reported additional effects of mucosal and serosal DIDS. In contrast to Ando (1990), we found no effect of serosal DIDS in the present study. This discrepancy could be due to different experimental approaches. Unlike Ando (1990), we worked on intact intestinal segments, in which the serosal muscle layer could have reduced the access of DIDS to the serosal membrane and thus have caused the apparent lack of effect of serosal DIDS. Finally, species-specific differences in the mechanisms of HCO_3^- excretion cannot be excluded. Clearly, more studies of transepithelial HCO_3^- transport in either isolated epithelia or double-perfused (luminal and vascular) whole-intestine preparations are required to determine the mechanism of base excretion by marine teleost fish.

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References

Ando, M. (1990). Effect of bicarbonate on salt and water transport across the intestine of seawater eel. *J. Exp. Biol.* **150**, 367–379.

Bath, R. N. and Eddy, F. B. (1980). Transport of nitrite across fish gills. *J. Exp. Zool.* **214**, 119–121.

Cameron, J. N. (1971). Rapid method for determination of total carbon dioxide in small blood samples. *J. Appl. Physiol.* **31**, 632–634.

Charney, A. N., Scheide, J. I., Ingrassia, P. M. and Zadunaisky, J. A. (1988). Effect of pH on chloride absorption in the flounder intestine. *Am. J. Physiol.* **255**, G247–G252.

Cutler, C., Comie, M., Bekir, S., Brezillon, S., Hazon, N. and Cramb, G. (1998). Molecular biology of the transporters in the European eel (*Anguilla anguilla*). The role of changes in gene expression in salinity adaptation and developmental maturation. *XIX ESCPB Congress. European Society for Comparative Physiology and Biochemistry. Cellular and Molecular Responses to Environmental Changes*. 23–26 August. Turku, Finland.

Dixon, J. M. and Loretz, C. A. (1986). Luminal alkalinization in the intestine of the goby. *J. Comp. Physiol.* **156**, 803–811.

Eddy, F. B., Kunzlik, P. A. and Bath, R. N. (1983). Uptake and loss of nitrite from the blood of rainbow trout, *Salmo gairdneri* Richardson and Atlantic salmon, *Salmo salar* L. in fresh water and in dilute sea water. *J. Fish Biol.* **23**, 105–116.

Eddy, F. B. and Williams, E. M. (1987). Nitrite in freshwater fish. *Chem. Ecol.* **3**, 1–38.

Flik, G., Kaneko, T., Greco, A. M., Li, J. and Fenwick, J. C. (1997). Sodium dependent ion transporters in trout gills. *Fish Physiol. Biochem.* **17**, 385–396.

Harris, R. R. and Coley, S. (1991). The effect of nitrite on chloride regulation in the crayfish *Pacifastacus leniusculus* Dana (Crustacea: Decapoda). *J. Comp. Physiol.* **161**B, 199–206.

Jensen, F. B. (1990). Sublethal physiological changes in freshwater *Astacus astacus*, exposed to nitrite: haemolymph and muscle electrolyte status and haemolymph acid–base balance and gas transport. *Aquat. Toxicol.* **18**, 51–60.

Jensen, F. B. (1995). Uptake and effects of nitrite and nitrate in animals. In *Nitrogen Metabolism and Excretion* (ed. P. J. Walsh and P. Wright), pp. 289–303. Boca Raton, FL: CRC Press.

Jensen, F. B. (1996). Physiological effects of nitrite in teleost and custaceans. In *Toxicology of Aquatic Pollution. Physiological, Cellular and Molecular Approaches* (ed. E. W. Taylor), pp. 169–186. Cambridge: Cambridge University Press.

Jensen, F. B., Andersen, N. A. and Heisler, N. (1987). Effects of nitrite exposure on blood respiratory properties, acid–base and electrolyte regulation in the carp (*Cyprinus carpio*). *J. Comp. Physiol.* B **157**, 533–541.

Lewis, W. M. and Morris, D. P. (1980). Toxicity of nitrite to fish: a review. *Trans. Am. Fish. Soc.* **115**, 183–195.

Loretz, C. A. (1995). Electrophysiology of ion transport in teleost intestinal cells. In *Fish Physiology*, vol. 14, *Cellular and Molecular Approaches to Fish Ionic Regulation* (ed. C. M. Wood and T. J. Shuttleworth), pp. 25–99. New York: Academic Press.

Marshall, W. S. and Bryson, S. E. (1998). Transport mechanisms of seawater teleost chloride cells: An inclusive model of a multifunctional cell. *Comp. Biochem. Physiol.* **119**A, 97–106.

Musch, M. W., Orellana, S. A., Kimberg, L. S., Field, M., Halm, D. R., Krasny, E. J. and Frizzell, R. A. (1982). Na^+–K^+–Cl^- cotransport in the intestine of a marine teleost. *Nature* **300**, 351–353.

Nikinmaa, M. (1990). *Zoophysiology: Vertebrate Red Blood Cells, Adaptations of Function to Respiratory Requirements*. Berlin: Springer Verlag.

Shechter, H., Gruener, N. and Shuval, H. I. (1972). A micromethod for the determination of nitrite in blood. *Analyt. Chim. Acta* **60**, 93–99.

Walsh, P. J., Blackwelder, P., Gill, K. A., Danulat, E. and Mommsen, T. P. (1991). Carbonate deposits in marine fish intestines: a new source of biomineralization. *Limnol. Oceanogr.* **36**, 1227–1232.

Williams, E. M. and Eddy, F. B. (1986). Chloride uptake in freshwater teleosts and its relationship to nitrite uptake and toxicity. *J. Comp. Physiol.* B **156**, 867–872.

Wilson, R. W. (1997). A novel role for the gut of seawater teleosts in acid–base balance. In *Acid–Base Status in Animals and Plants* (ed. E. W. Taylor, J. A. Raven and S. Eggiton), pp. 257–274. SEB Seminar Series **68**. Cambridge: Cambridge University Press.

Wilson, R. W., Gilmour, K. M., Henry, R. P. and Wood, C. M. (1996). Intestinal base excretion in the seawater-adapted rainbow trout: a role in acid–base balance? *J. Exp. Biol.* **199**, 2331–2343.