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Na^+ and Cl^- Transport by the Urinary Bladder of the Freshwater Rainbow Trout (*Oncorhynchus mykiss*)

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ABSTRACT Freshwater (FW) rainbow trout (*Oncorhynchus mykiss*) urinary bladders mounted in vitro under symmetrical saline conditions displayed electroneutral active absorption of Na^+ and Cl^- from the mucosal side; the transepithelial potential (V_t) was 0.1 mV, and the short-circuit current was less than 1 μA cm^{-2}. Removal of Na^+ from mucosal saline decreased Cl^- absorption by 56% and removal of Cl^- decreased Na^+ absorption by 69%. However, active net absorption of both Na^+ and Cl^- was not abolished when Cl^- or Na^+ was replaced with an impermeant ion (gluconate or choline, respectively). Under physiological conditions with artificial urine ($[Na^+]$ = 2.12 mM, $[Cl^-]$ = 3.51 mM) bathing the mucosal surface and saline bathing the serosal surface, transepithelial potential (V_t) increased to a serosal positive ~+7.6 mV. Unidirectional influx rates of both Na^+ and Cl^- were 10–20-fold lower but active absorption of both ions still occurred according to the Ussing flux ratio criterion. Replacement of Na^+ with choline, or Cl^- with gluconate, in the mucosal artificial urine yielded no change in unidirectional influx of Cl^- or Na^+, respectively. However, kinetic analyses indicated a decrease in maximum Na^+ transport rate (J_{max}) of 66% with no change in affinity (K_m) in the low Cl^- mucosal solution relative to the control solution. Similarly, there was a 79% decrease in J_{max} values for Cl^-, again with no change in K_m, in the low-Na^+ mucosal bathing. The mucosal addition of DIDS, amiloride or bumetanide (10^{-4} M) had no effect on either Na^+ or Cl^- transport, under either symmetrical saline or artificial urine/saline conditions. Addition of the three drugs simultaneously (10^{-4} M), or chlorothiazide (10^{-3} M), under symmetrical saline conditions also had no effect on Na^+ or Cl^- transport rates. Cyanide (10^{-3} M) addition to mucosal artificial urine caused a slowly developing decrease of Na^+ influx to 59% and Cl^- influx to 50% in the period after drug addition. Na^+ and Cl^- reabsorption appears to be a partially coupled process in the urinary bladder of *O. mykiss*; transport mechanisms are both dependent upon and independent of the other ion. *J. Exp. Zool. 287:1–14, 2000.*

The transport functions of the urinary bladder in freshwater teleosts have been studied extensively in vitro, particularly in the European rainbow trout (*Salmo irideus*, generally considered a strain of *Oncorhynchus mykiss*) (Lahlou and Fossat, '71, '84; Fossat et al., '74; Fossat and Lahlou, '77, '79a,b, '82), brook trout (*Salvelinus fontinalis*) (Marshall, '86, '88; Marshall and Bryson, '91), and North American rainbow trout (*O. mykiss*) (Hirano et al., '73; Demarest and Machen, '82; Harvey and Lahlou, '86). The urinary bladder of a teleost fish is an enlargement of the paired mesonephric ducts which leave the kidney and unite to form the urinary bladder (Hickman and Trump, '69). It is a single-layered epithelium containing mitochondria-rich cells, microvilli, and cilia surrounded by a contractile muscular wall (Lahlou and Fossat, '84). In vivo studies have shown that ureteral urine is modified during residence in the bladder, yielding a urine with lower Na^+ and Cl^- concentrations (Curtis and Wood, '91). Therefore, the urinary bladder functions as an accessory osmoregulatory organ to the kidney in the FW teleost, facilitating hyperosmotic regulation.

The mechanism of Na^+ and Cl^- transport in the urinary bladder of *Salmo irideus* has been characterized as electroneutral co-transport (Fossat and Lahlou, '79a). Thus the removal of either ion from the mucosal side induced the disappearance of the net flux of the other ion. This coupled NaCl transport is associated with an undetectable transepithelial potential difference (V_t) and short-

Abbreviations used: FW, freshwater; SW, seawater; G_t, transepithelial conductance; V_t, transepithelial potential; J_{ms}, influx; J_{sm}, efflux; J_{net}, $J_{ms} - J_{sm}$.

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circuit current (I_{sc}). The urinary bladder is termed a "leaky" epithelium due to its low transepithelial resistance R_t (~200 $\Omega \cdot cm^{-2}$) and the paracellular pathway does not exhibit cation-selective properties, in contrast to other low-resistance epithelia (Fossat and Lahlou, '79b).

In urinary bladder of brook trout, *Salvelinus fontinalis*, by contrast, uptake of NaCl is again electroneutral, but Na^+ or Cl^- transport continue when Cl^-- or Na^+-free solutions, respectively, are placed on the mucosal surface (Marshall, '86, '88). There is also evidence of independent Cl^-/HCO_3^- and Na^+/H^+ exchange mechanisms, and there is no response of Na^+ or Cl^- movement to the mucosal addition of the cotransporter antagonist bumetanide (Na^+–K^+–$2Cl^-$ transport blocker). This "independent model" of mucosa to serosa absorption appears very similar to that normally presented for the freshwater teleost gill (Wood, '91; Perry, '97).

It is of interest that two related salmonid species, albeit in different genera, demonstrated two very different methods of urinary bladder Na^+ and Cl^- transport. Marshall ('88) has speculated that discrepancies between work done on *Salvelinus fontinalis* and previous work done on the urinary bladder of *Salmo irideus* reflected different techniques, holding conditions or genetic variations among the fish.

The aim of the present study was to establish the mechanism(s) of Na^+ and Cl^- transport in vitro in the urinary bladder of the North American strain of freshwater rainbow trout (*Oncorhynchus mykiss*). Isolated bladders were set up in Ussing chambers under open- or short-circuit conditions and Na^+ and Cl^- transport was measured under a variety of conditions. In particular we wished to determine whether the transport of these ions was coupled or independent. First, the urinary bladder was bathed in symmetrical saline, either with both ions present, or with one removed, and flux rates were measured using radiolabeled Na^+ and/or Cl^-. Second, artificial urine was used to more closely mimic the conditions found in vivo and similar experiments were performed as above. Third, with saline or artificial urine bathing the mucosal surface, a number of drugs known to affect Na^+ and Cl^- transport in other transporting epithelia were applied. Finally, electrophysiological and kinetic transport properties of the epithelium were determined. Our results suggest that Na^+ and Cl^- transport in the urinary bladder of *O. mykiss* is a partially coupled process, where uptake processes are both dependent on and independent of Cl^- and Na^+, respectively.

MATERIALS AND METHODS

Animals

Adult rainbow trout (*Oncorhynchus mykiss*; 300–750 g) were obtained from Humber Springs Hatchery (Orangeville, ON). Fish were maintained in a 500-l flow-through system, supplied with dechlorinated, aerated Hamilton tap water at a flow rate of 900 ml $\cdot$ min^{-1} and with an average composition (in mmol $litre^{-1}$) of Na^+, 0.6; Cl^-, 0.7; Ca^{2+}, 1.05; pH 7.5–8.0. Photoperiod was seasonal, and water temperature varied according to ambient temperature during September to May (5–14°C). The fish were fed commercial trout pellets (Zeigler, Hazelton, PA) at a rate of 1% of their body mass per day.

Bathing solutions

All salts were obtained from Sigma Chemical Co. (St. Louis, MO). The Ringer's solution used for dissection and for the basolateral bathing solution was a modified Cortland saline composed of (in mmol l^{-1}) NaCl, 129.9; KCl, 2.55; $CaCl_2 \cdot H_2O$, 1.56; $MgSO_4 \cdot 7H_2O$, 0.93; $NaHCO_3$, 13.00; $NaH_2PO_4 \cdot H_2O$, 2.97; glucose, 5.55; NH_4Cl, 0.30. Saline pH was 7.8–7.9 when equilibrated with a 0.3% CO_2, balance O_2.

The artificial urine solution for the mucosal surface of the membrane was formulated from the results of "spot-sampling" of urine from the bladder of undisturbed, non-cannulated rainbow trout, as reported by Curtis and Wood (1991). Artificial urine consisted of (in mmol $litre^{-1}$) KCl, 0.81; $CaCl_2 \cdot H_2O$, 1.35; $MgSO_4 \cdot 7H_2O$, 0.79; $NaHCO_3$, 1.66; $NaH_2PO_4 \cdot H_2O$, 0.46; urea, 0.55; $Ca(NO_3)_2 \cdot 4H_2O$, 0.045; $(NH_4)_2SO_4$, 0.235. The Ringer's and artificial urine solutions were each modified for ion replacement experiments. In the Cl^--free Ringer's, sodium, potassium, and calcium gluconates and $(NH_4)_2SO_4$ were substituted for NaCl, KCl, $CaCl_2 \cdot 2H_2O$, and NH_4Cl, respectively. The Na^+-free Ringer's substituted choline chloride, choline bicarbonate, and KH_2PO_4 for NaCl, $NaHCO_3$, and $NaH_2PO_4 \cdot H_2O$, respectively. The osmolality of Ringer's, Cl^--free and Na^+-free Ringer's was 288, 279, and 281 mosmol/kg, respectively. In Cl^--free artificial urine, KCl and $CaCl_2 \cdot 2H_2O$ were replaced with K^+ gluconate and Ca^{2+} gluconate, respectively. In Na^+-free artificial urine, $NaHCO_3$ and $NaH_2PO_4 \cdot H_2O$ were replaced with choline bicarbonate and KH_2PO_4, respectively.

For the kinetic experiments, in the initial mucosal "urine," Cl^- was adjusted to equal 2.16 mM and Na^+ was set initially to 2.12 mM, so that Cl^-

and Na^+ concentrations were roughly equal. The Na^+ and Cl^- concentrations in artificial urine were then adjusted to approximately 5, 10, 50, and 150 mM by addition of NaCl, Na^+ gluconate, or choline chloride as appropriate.

The pH of artificial urine when equilibrated with 0.3% CO_2, balance O_2, was 7.2–7.5. Prior to the start of the experiment and at the end of each 60-min flux period (see below) except in the kinetic experiment, the mucosal solution was rinsed extensively to maintain the original ion composition of the artificial urine (Na^+ = 2.12, Cl^- = 3.51, and Ca^{2+} = 1.4 mmol litre^{-1}).

Isolated urinary bladder preparation

Fish were anaesthetized using 0.1 g/litre MS-222 and a flanged PE tubing was inserted into the bladder and tied to the urinary papilla. The bladder was filled with Ringer's to aid in differentiation of its structure from the peritoneum during dissection. Both ureters were ligated just anterior to the urinary bladder with surgical thread, and the urinary bladder was excised from surrounding tissues. Following careful removal of adhering fat and connective tissue, the bladder was cut into equal anterior and posterior portions and then both portions were opened by a longitudinal cut. The area of each portion of the bladder was approximately 1–2 cm^2. Preparations were kept moist by the addition of saline throughout the dissection.

The Ussing membrane apertures (0.125 cm^2) were coated with stopcock grease (Dow Corning, Midland, MI), and a thin vinyl mesh was placed over each aperture to support the tissue. Each portion of urinary bladder was stretched gently (to remove macroscopic folds) and carefully pinned mucosal side up to the aperture; the second half of the aperture then sandwiched the epithelium. After this mounting, the mucosal surfaces were thoroughly rinsed with the appropriate solution to remove the saline and any mucus that had accumulated during the dissection. The aperture sandwich was then placed between the two hemi-chambers and tightened into place. Hemi-chambers were filled with the appropriate solutions: Ringer's or artificial urine or ion replacement solution for the mucosal side, and saline for the serosal side. Each hemi-chamber was filled at an equal rate to prevent damage by unequal hydrostatic pressure. Membranes were then given a 30-min period to adjust to the in vitro conditions. Appropriate gases were passed across the surface of each hemi-chamber's media, and mucosal and serosal solutions were mixed by magnetic stirrers.

Electrophysiology

Polyethylene 4% agar/Ringer bridges were used to measure transepithelial potential (V_t, mucosal side grounded) and membrane conductance (G_t). Each bridge was connected to the voltage/current clamp (DVC-1000, WP Instruments, New Haven, CT) by Hg/HgCl calomel half-cells. Membrane conductance was corrected for solution resistances. In asymmetrical solutions, corrections for liquid junction potentials were accomplished by measurements against a free flowing 3 M KCl half-cell. Ussing chambers were maintained at 15°C using water jackets. In flux experiments with saline bathing the mucosal surface, the preparation was short-circuited to negate any effect of V_t on ion transport. G_t was determined by clamping the membrane to a set voltage every 10 min, and V_t was recorded at the beginning and end of each 60-min short-circuited flux period.

Experimental protocols

One chamber was set up for each portion of the urinary bladder for independent measurements of either influx or efflux. Therefore either the anterior or posterior portion yielded a mucosal to serosal unidirectional influx (J_{ms}) measurement while the other portion yielded the serosal to mucosal unidirectional efflux (J_{sm}) measurement. These were alternated to obtain equal numbers of both approaches within each treatment group. For data analyses, posterior and anterior portions of the membranes were matched according to G_t. After the initial 30-min adjustment period, the mucosal hemi-chamber volume was gently rinsed again with the appropriate solution (20 times the chamber volume) and isotope was added to the appropriate side.

Dual-flux experiments were performed with ^{36}Cl ($Na^{36}Cl$ from ICN Radiochemicals, Irvine, CA) and ^{22}Na ($^{22}NaCl$ from NEN-Dupont, Boston, MA) which were added to either the mucosal side (final specific activity of 300,000 CPM μmol^{-1}, artificial urine) for unidirectional influx or serosal side (8,000 CPM μmol^{-1}, Ringer) to monitor unidirectional efflux. Upon first addition of the radioisotope(s), a 45-min equilibration period was employed. Each experiment consisted of three 60-min periods; within each period samples were taken from the unlabeled bath every 20 min for radioactivity analyses. Flux values for each 20-min period were averaged to produce a final flux rate for each 60-min experimental period. At the start and end of each 60-min period, samples were

taken from the labeled side to determine the specific activity and ionic concentrations and the solutions were replaced at the end. Unidirectional fluxes were determined by measuring the specific activity on the labeled side and the appearance of isotope on the unlabeled side. Three 60-min periods were employed to check whether steady-state conditions were achieved, and to compare the effects of various drugs applied in period 2 against control treatments in periods 1 and 3.

For amiloride, DIDS, and bumetanide experiments in Ussing chambers, only the solvent (DMSO) used to dissolve the drugs was present for the first 60-min control period. DMSO was added at the same concentration as in the following drug period. The chambers were then emptied, new isotope along with the drug and DMSO solution was added, and the resulting solution was allowed to equilibrate for at least 45 min. The drug experimental flux period was 60-min in duration and was followed by a final 60-min control period (DMSO alone again present). Prior to the final control period, the chambers were emptied and the membrane was removed. The hemi-chambers were flushed extensively with distilled H_2O, the membrane was then replaced, and new solutions with isotope were added and equilibrated for 45 min. The procedure for chlorothiazide and cyanide experiments was similar except that DMSO was not employed.

Each kinetic flux experiment consisted of 5 measurement periods. Each of the 5 periods represented a different mucosal artificial urine concentration of Na^+ and/or Cl^- at approximately 2, 5, 10, 50, or 150 mM, in an increasing sequence. Appropriate isotope was added and followed by a 30-min equilibration period prior to the beginning of each 40-min flux period. At the end of each flux period, both the serosal and mucosal sides were emptied from each hemi-chamber, and new solutions were added to each side, using an increasing mucosal concentration of Na^+ and/or Cl^- throughout the kinetic flux experiment.

The observed flux ratio (J_{ms}/J_{sm}) was compared to the predicted flux ratio using the Ussing flux ratio equation (Ussing, '49). Disagreement between the observed and predicted value indicated the presence of non-diffusive transport. The predicted flux ratio equation was calculated as follows:

$$J_{ims}/J_{ism} = (a_i^m/a_i^s)\exp(Z_iFV_t/RT).$$

The activities of ion i are a_i^s and a_i^m on the serosal and mucosal sides, respectively; valency of the ion is Z_i, V_t is the transepithelial potential, and F, R, and T have their usual thermodynamic meanings. The ionic activity of Na^+ (109.5 mmol $litre^{-1}$) in Cortland saline was taken from measurements with microelectrodes filled with the appropriate ionophore (Steiner et al., '79), while Cl^- was predicted to have the same relative activity (on a percentage basis) as Na^+ from theory for a solution of this ionic strength (Lee, '81). The activities of Na^+ and Cl^- in artificial urine were taken as equal to their measured concentrations.

Pharmaceuticals

DIDS (4,4-diisothiocyanatostilbene-2,2-disulfonic acid) was dissolved in dimethyl sulfoxide (DMSO, 20 $\mu l \cdot mg^{-1}$) and added to the mucosal side at a final concentration of 10^{-4} M. Amiloride (10^{-4} M) and bumetanide (10^{-4} M) were dissolved using the same protocol as DIDS. DMSO concentrations in each drug experiment were ≤0.1%. The same DMSO concentration was added to the mucosal side in both control periods. Chlorothiazide was dissolved in Cortland saline at a concentration of 10^{-3} M in the mucosal bathing medium. NaCN (sodium cyanide) was dissolved in artificial urine at a concentration of 10^{-3} M on the mucosal side. All drugs were obtained from Sigma Chemical Co.

Analytical techniques

Saline Cl^- concentrations were measured by coulometric titration (model CMT10, Radiometer, Copenhagen), and artificial urine Cl^- concentrations were determined by colorimetric assay (Zall et al., '56). Na^+ concentrations in both media were analyzed by atomic absorption spectrophotometry (model AA-1275, Varian, Springvale, Australia). Ammonia was determined by a micro-modification of the colorimetric assay of Verdouw et al. ('78). Net titratable acid flux was determined by removing 3 ml from each mucosal hemi-chamber at the beginning and end of each flux period, equilibrating with air for 30 min, and then titrating down through a pH of 4, using a burette filled with 0.02 N HCl. Net titratable acid flux was calculated by subtracting the final titration value ($\mu mol \cdot cm^{-2} \cdot hr^{-1}$) from the initial titration value and taking into account aperture area and flux duration. A positive flux value represented movement of acid from mucosa to serosa.

Samples of 40 μl from the labeled side and 250 μl from the unlabeled side of the Ussing chamber were added to 4.0 ml of Readysafe fluor (Beckman, Fullerton, CA). Radioactivity of ^{22}Na was determined on a Minaxi Autogamma 5000 counter

(Packard Instrument Co., Downers Grove, IL), that of ^{22}Na and ^{36}Cl on a Rackbeta 1217 liquid scintillation counter (LKB, Wallac, Turku, Finland), and that of ^{36}Cl by a count subtraction procedure (see Wood et al., '84).

Data are presented as means ± 1 standard error unless indicated otherwise. Comparisons within and between treatments were analyzed by paired or un-paired *t*-tests (two-tailed and one-tailed), as appropriate, at $P < 0.05$ after checking for homogeneity of variance using *F*-tests with standard transformations applied as needed. In cases where these transformations were unsuccessful in normalizing the variance, the non-parametric Wilcoxon signed rank sum test was employed to test for significance. Regressions were performed by the method of least squares, and correlation evaluated by Pearson's linear correlation coefficient. Kinetic values of J_{max} (maximum transfer rate) and K_m (substrate concentration at 50% of J_{max}) were determined by Eadie–Hofstee plots, and kinetic plots were generated according to the Michaelis–Menten relationship.

RESULTS

Mucosal saline/serosal saline condition

V_t and G_t of the isolated urinary bladder bathed in symmetrical saline were 0.10 ± 0.04 mV, serosa-positive, and 9.15 ± 1.50 mS · cm^{-2}, respectively, under open-circuit conditions. Short-circuit current (I_{sc}) of the preparation was <1 μA · cm^{-2}. Comparison of both Na^+ and Cl^- observed flux ratios with their respective predicted flux ratios indicated active uptake of both ions under symmetrical conditions ($P < 0.05$) (Fig. 1). Both unidirectional flux values and net flux values (all periods) of Na^+ were less than those for Cl^- ($P < 0.05$).

When Na^+ was removed by replacement with choline, V_t increased slightly to 0.7 ± 0.2 mV ($P < 0.05$) and G_t decreased to 2.26 ± 0.33 mS · cm^{-2} ($P < 0.05$). For both control and experimental treatments, there was net uptake of Cl^- for all three periods. Net movement of Cl^- was not different between the two treatments ($P > 0.05$) (Fig. 2). However, unidirectional influx ($P < 0.05$) of Cl^- decreased in the Na^+-free mucosal saline treatment by about 56%; the accompanying decrease in efflux was not significant. The observed flux ratios were different from their predicted ratios ($P < 0.05$), indicating active uptake of Cl^- in the absence of Na^+. Observed Cl^- flux ratios measured under Na^+-free conditions in the mucosal bath were not statistically different from those in the control treatments ($P > 0.05$).

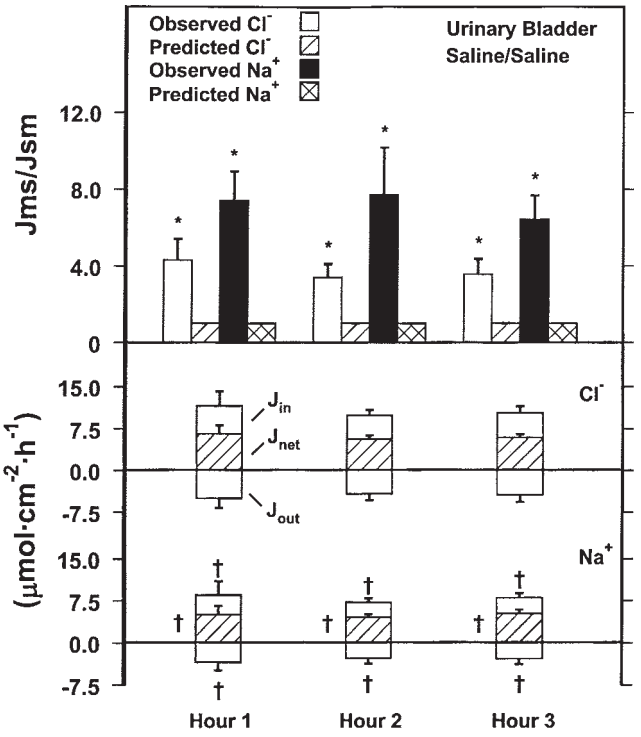

Fig. 1. Observed and predicted flux ratios for the unidirectional movements of Cl^- and Na^+ in urinary bladders of FW *O. mykiss* under symmetrical saline conditions. Details of the experimental protocol given in Materials and Methods. Open bars and solid bars represent the observed flux ratios for Cl^- and Na^+, respectively, and hatched and crossed bars represent the predicted flux ratios for Cl^- and Na^+, respectively, based on the Ussing flux ratio criterion. Means ± 1 SEM ($n = 6$). Asterisk indicates significant difference from predicted flux ratio ($P < 0.05$, paired *t*-test, two-tailed), indicating non-diffusive transport. Unidirectional influx (J_{in}), efflux (J_{out}), and net flux (J_{net}) are shown for Cl^- and Na^+. Cross indicates significant differences from unidirectional influx (above bar), efflux (below bar), or net flux (beside bar) ($P < 0.05$, paired *t*-test, two-tailed) between Cl^- and Na^+.

When Cl^- was removed from the mucosal saline by replacement with gluconate, V_t increased to 11.6 ± 0.2 mV ($P < 0.05$) and G_t decreased to 3.26 ± 0.4 mS · cm^{-2} ($P < 0.05$) from their respective control values (see above). Net mucosa→serosa flux of Na^+ remained positive but was smaller ($P < 0.05$; 2 of 3 periods) in the Cl^--free treatment group compared to control values (Fig. 3). Na^+ influx was also significantly lower ($P < 0.05$) than control values by about 69% in all three periods of the Cl^--free treatment; the accompanying decrease in efflux was not significant (Fig. 3). Observed flux ratios for Na^+ in the Cl^--free solution remained different from the predicted ratios ($P < 0.05$) as in the

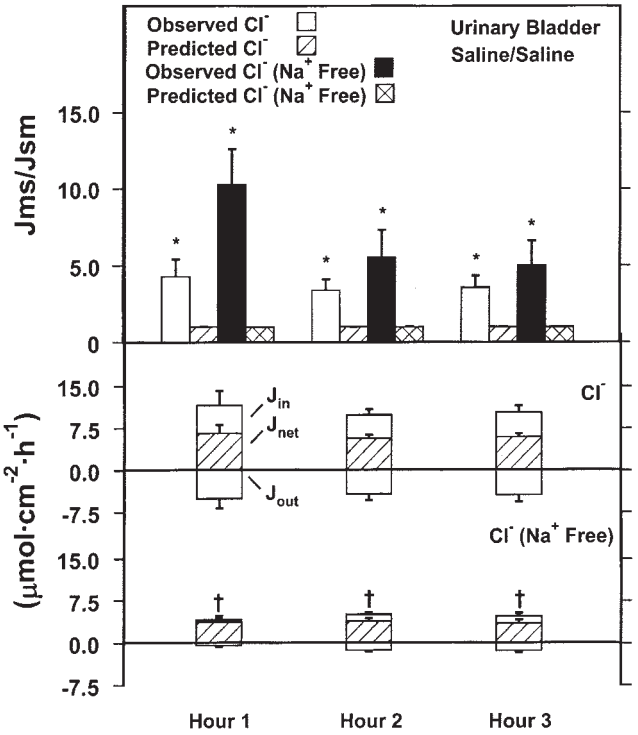

Fig. 2. Observed and predicted flux ratios for the unidirectional movements of Cl^- with control or Na^+-free saline bathing the mucosal surface of urinary bladder epithelia of FW *O. mykiss*. Open bars and solid bars represent the observed flux ratios for Cl^- in control and in Na^+-free mucosal saline, respectively, and hatched and crossed bars represent the predicted flux ratios for Cl^- in control and in Na^+-free mucosal saline, respectively, based on the Ussing flux ratio criterion. Means ± 1 SEM (n = 4). Asterisk indicates significant differences from predicted flux ratio ($P < 0.05$, paired t-test, two-tailed), indicating non-diffusive transport. Unidirectional influx (J_{in}), efflux (J_{out}), and net flux (J_{net}) are shown for Cl^- in control and in Na^+-free mucosal saline. Cross indicates significant difference from unidirectional influx (above bar), efflux (below bar), or net flux (beside bar) ($P < 0.05$, unpaired t-test, two-tailed) of Cl^- between the two treatment groups.

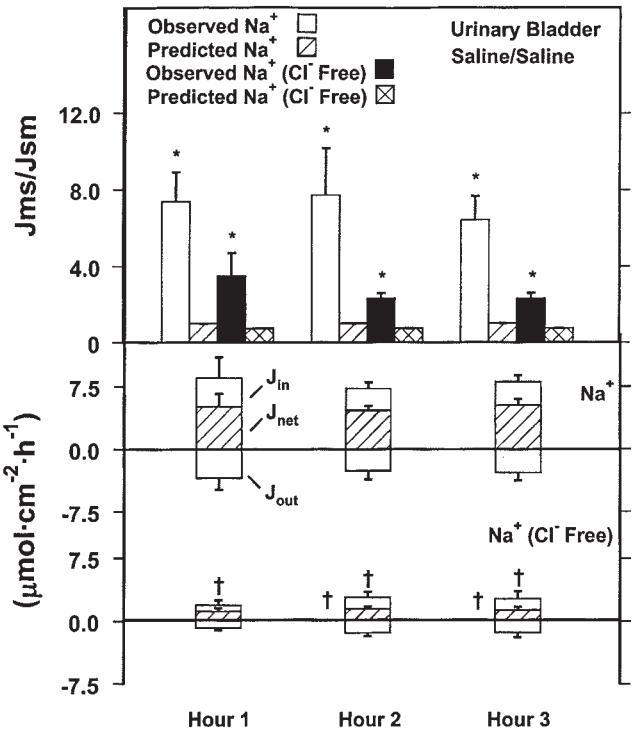

Fig. 3. Observed and predicted flux ratios for the unidirectional movements of Na^+ with control or Cl^--free saline bathing the mucosal surface of urinary bladder epithelia of FW *O. mykiss*. Open bars and solid bars represent the observed flux ratios for Na^+ in control and in Cl^--free mucosal saline, respectively, hatched and crossed bars represent the predicted flux ratios for Na^+ in control and in Cl^--free mucosal saline, respectively, based on the Ussing flux ratio criterion. Means ± 1 SEM (n = 4). Asterisk indicates significant difference from predicted flux ratio ($P < 0.05$, paired t-test, two-tailed), indicating non-diffusive transport. Unidirectional influx (J_{in}), efflux (J_{out}), and net flux (J_{net}) are shown for Na^+ in control and in Cl^--free mucosal saline. Cross indicates significant difference from unidirectional influx (above bar), efflux (below bar), or net flux (beside bar) ($P < 0.05$, unpaired t-test, two-tailed) of Na^+ between the two treatment groups.

control treatment, indicating continued active transport of Na^+. For each of the one hour flux periods, Na^+ flux ratios in the Cl^--free solution were not significantly different from those in the control treatment ($P > 0.05$).

The addition of either DIDS (10^{-4} M) or amiloride (10^{-4} M) or bumetanide (10^{-4} M) to the mucosal saline had no effect on Cl^- or Na^+ observed flux ratios or unidirectional flux of either ion (Table 1). There were no effects on V_t or G_t with any of the drug treatments. Treatment of the bladder with the three drugs simultaneously, under mucosal saline/serosal saline conditions, also had no effect on flux rates of Na^+ or Cl^- (data not shown; n = 4). Unidirectional influx of Na^+ was 10.8 ± 1.8 μmol · cm^{-2} · hr^{-1} in the presence of the three drugs simultaneously, relative to control values of 10.7 ± 1.6 and 9.5 ± 3.1 μmol · cm^{-2} · hr^{-1}. For Cl^- the unidirectional influx was 7.4 ± 1.4 μmol · cm^{-2} · hr^{-1} in the presence of the three drugs, relative to control values of 7.7 ± 1.5 and 8.1 ± 2.3 μmol · cm^{-2} · hr^{-1}. Chlorothiazide (10^{-3} M) treatment caused no change in unidirectional influx rates (data not shown; n = 6) of either Na^+ (Hour 1, 6.71 ± 2.36 and Hour 3, 5.59 ± 2.19 μmol · cm^{-2} · hr^{-1}, controls; Hour 2, 6.25 ± 2.17 μmol · cm^{-2} · hr^{-1} chlorothiazide), or Cl^- (Hour 1, 8.64 ± 2.96 and Hour 3, 7.69 ± 2.82 μmol · cm^{-2} · hr^{-1} controls; Hour 2, 7.80 ± 2.59 μmol · cm^{-2} · hr^{-1} chlorothiazide).

TABLE 1. Effect of the 10^{-4} M mucosal addition of DIDS, amiloride, or bumetanide on unidirectional efflux (J_{sm}), influx (J_{ms}), and flux ratios of Cl^- and Na^+ and on V_t and G_t in urinary bladder of O. mykiss *(n = 4) bathed on mucosal side with Cortland saline under open-circuit conditions*[†]

Experimental period	V_t(mV)[a]	G_t(mS · cm^{-2})[b]	J_{sm} (μmol · cm^{-2} · hr^{-1})	J_{ms} (μmol · cm^{-2} · hr^{-1})	J_{ms}/J_{sm} Observed	J_{ms}/J_{sm} Predicted
Cl^-						
Control (hour 1)	0.1 ± 0.1	8.39 ± 2.1	3.5 ± 1.0	12.3 ± 4.6	5.8 ± 2.1	1.00 ± 0.003*
DIDS	0.1 ± 0.2	9.20 ± 1.7	3.5 ± 0.6	8.7 ± 2.3	2.7 ± 0.7	1.00 ± 0.008*
Control (hour 3)	–0.1 ± 0.2	8.23 ± 1.9	3.4 ± 0.7	12.9 ± 3.5	5.0 ± 1.6	1.00 ± 0.006*
Na^+						
Control (hour 1)			0.9 ± 0.3	2.9 ± 1.0	11.1 ± 3.0	1.00 ± 0.003*
DIDS			1.3 ± 0.4	5.8 ± 0.9	10.1 ± 2.5	1.00 ± 0.008*
Control (hour 3)			1.2 ± 0.5	3.5 ± 0.3	13.5 ± 2.3	1.00 ± 0.006*
Cl^-						
Control (hour 1)	0.2 ± 0.1	3.49 ± 0.9	1.8 ± 0.6	5.0 ± 1.6	3.1 ± 0.5	1.01 ± 0.005*
Amiloride	0.2 ± 0.1	3.36 ± 1.1	2.4 ± 0.8	5.6 ± 0.8	3.1 ± 0.6	1.01 ± 0.004*
Control (hour 3)	0.1 ± 0.1	3.56 ± 1.2	1.8 ± 0.6	5.8 ± 0.8	4.2 ± 0.9	1.00 ± 0.005*
Na^+						
Control (hour 1)			0.9 ± 0.3	3.3 ± 1.5	4.2 ± 1.8	0.99 ± 0.005*
Amiloride			1.0 ± 0.3	3.9 ± 0.8	4.3 ± 0.6	0.99 ± 0.004*
Control (hour 3)			1.0 ± 0.4	4.2 ± 0.4	6.0 ± 1.6	1.00 ± 0.005*
Cl^-						
Control (hour 1)	0.2 ± 0.1	6.8 ± 1.5	2.7 ± 0.4	5.6 ± 0.6	2.2 ± 0.1	1.01 ± 0.003*
Bumetanide	0.3 ± 0.1	4.6 ± 1.0	2.4 ± 0.6	5.2 ± 0.6	2.7 ± 0.6	1.02 ± 0.003*
Control (hour 3)	0.1 ± 0.1	4.3 ± 0.6	2.1 ± 0.4	4.3 ± 0.5	2.2 ± 0.3	1.00 ± 0.005*
Na^+						
Control (hour 1)			1.8 ± 0.8	4.1 ± 0.8	4.6 ± 1.6	0.99 ± 0.003*
Bumetanide			1.3 ± 0.6	3.4 ± 0.6	4.7 ± 1.4	0.98 ± 0.003*
Control (hour 3)			1.1 ± 0.2	3.6 ± 0.5	3.9 ± 0.9	1.00 ± 0.005*

[†]There were no significant effects of any of the drug treatments on the parameters measured.
[a]Transepithelial potential (mucosal ground) corrected for junction potentials; $n = 8$.
[b]Tissue conductance corrected for solution resistance; $n = 8$.
*Paired t-test, two-tailed, observed versus predicted values of J_{ms}/J_{sm}. Comparison of all observed and predicted ratios were significantly different, $P < 0.05$.

Mucosal urine/serosal saline conditions

When the isolated urinary bladder was bathed on the mucosal side with artificial urine, the V_t increased to 7.6 ± 0.4 mV and the G_t decreased to 1.47 ± 0.1 mS · cm^{-2}, both significant relative to the saline/saline condition ($P < 0.05$). Net fluxes of Cl^- and Na^+ were serosa → mucosa, and unidirectional influx rates were reduced to 5–10% of the rates under saline/saline conditions.

With artificial urine on the mucosal surface, the net Cl^- flux rates were similar to net Na^+ flux rates except in Hour 1 ($P < 0.05$) (Fig. 4). Cl^- unidirectional influx was larger than Na^+ influx ($P < 0.05$) in two of the three hours, and Cl^- unidirectional efflux was greater than Na^+ efflux in all periods ($P < 0.05$). Of interest was the significant increase in the observed Cl^- flux ratio over time; the observed Na^+ flux ratio did not display any trend over time. The Na^+ and Cl^- observed flux ratios were significantly different ($P < 0.05$) from each of their respective predicted flux ratios suggesting non-diffusive uptake of both ions.

With Na^+-free artificial urine bathing the mucosal surface, V_t (9.7 ± 0.4 mV) and G_t (1.1 ± 0.1 mS · cm^{-2}) were not different from control, Na^+-replete artificial urine ($P > 0.05$). Unidirectional influx and efflux of Cl^- were also the same in both treatments ($P > 0.05$) (Fig. 5). The net movement of Cl^- was still serosa → mucosa, and there was no difference between the net flux rates in the two treatments. In all three periods, the observed Cl^- flux ratios in the Na^+-free treatment were not significantly different ($P > 0.05$) from the observed ratios in the control treatment. The observed Cl^- flux ratios were different from their respective predicted values for each bathing solution ($P < 0.05$), indicating that non-diffusive transport was maintained. Again, the observed Cl^- flux ratios increased significantly over time.

When gluconate was substituted for Cl^- in the mucosal artificial urine solution, V_t decreased to 3.3 ± 0.4 mV and G_t increased to 3.0 ± 0.2 mS · cm^{-2}, respectively ($P < 0.05$). The unidirectional and net flux rates of Na^+ were similar for both treatments ($P > 0.05$) (Fig. 6). The observed ratios for the con-

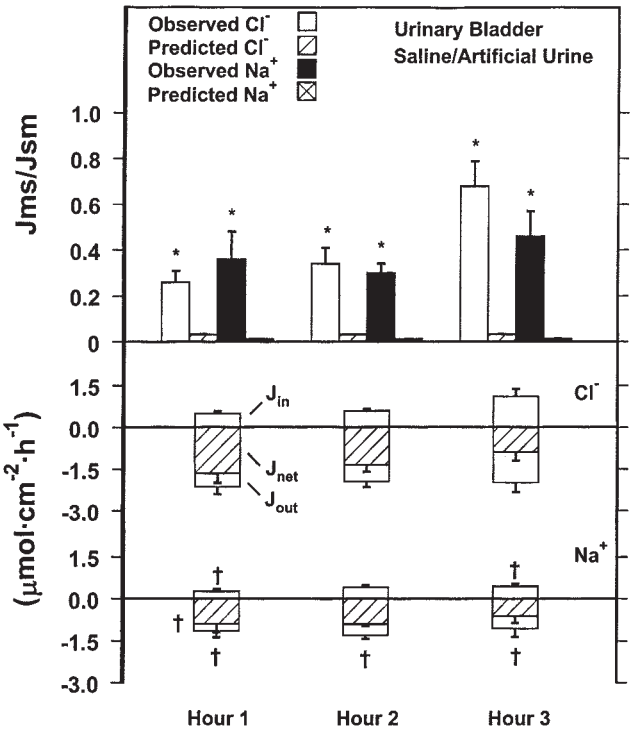

Fig. 4. Observed and predicted flux ratios for the unidirectional movements of Cl^- and Na^+ in urinary bladders of FW *O. mykiss* bathed with artificial urine on the mucosal surface. Open bars and solid bars represent the observed flux ratios for Cl^- and Na^+, respectively, and hatched and crossed bars represent the predicted flux ratios for Cl^- and Na^+, respectively, based on the Ussing flux ratio criterion. Means ± 1 SEM (n = 6). Asterisk indicates significant difference from predicted flux ratio ($P < 0.05$, paired t-test, two-tailed), indicating non-diffusive transport. Unidirectional influx (J_{in}), efflux (J_{out}), and net flux (J_{net}) are shown for Cl^- and Na^+. Cross indicates significant difference from unidirectional influx (above bar), efflux (below bar), or net flux (beside bar) ($P < 0.05$, paired t-test, two-tailed) between Cl^- and Na^+.

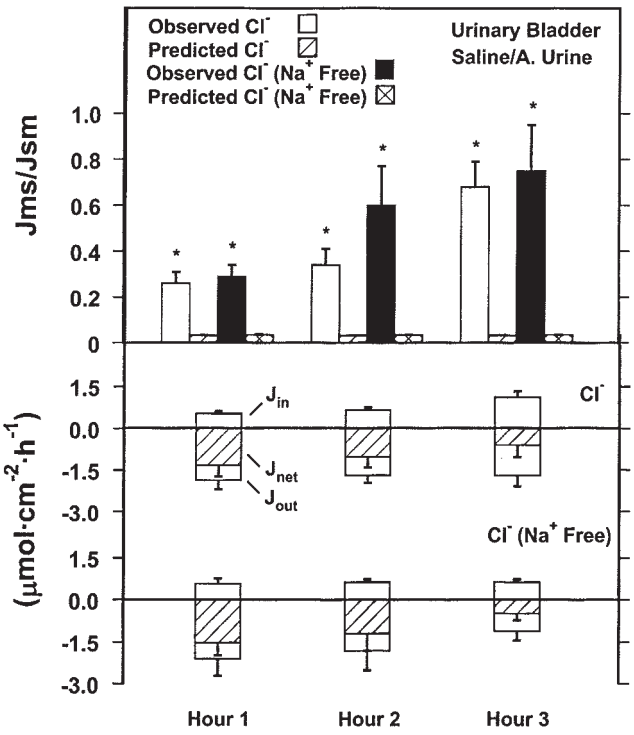

Fig. 5. Observed and predicted flux ratios for the unidirectional movements of Cl^- with control or Na^+-free artificial urine bathing the mucosal surface of urinary bladder epithelia of FW *O. mykiss*. Open bars and solid bars represent the observed flux ratios for Cl^- in control and in Na^+-free mucosal artificial urine, respectively, and hatched and crossed bars represent the predicted flux ratios for Cl^- in control and in Na^+-free mucosal artificial urine, respectively, based on the Ussing flux ratio criterion. Means ± 1 SEM (n = 4). Asterisk indicates significant difference from predicted flux ratio ($P < 0.05$, paired t-test, two-tailed), indicating non-diffusive transport. Unidirectional influx (J_{in}), efflux (J_{out}), and net flux (J_{net}) are shown for Cl^- in control and in Na^+-free mucosal artificial urine. There were no significant differences ($P > 0.05$) between the two treatment groups.

trol and mucosal Cl^--free solution were very similar ($P > 0.05$), indicating there was no change in the non-diffusive transport of Na^+.

With artificial urine bathing the mucosal surface of the urinary bladder, net NH_4^+ flux was 0.56 ± 0.87 μmol · cm^{-2} · hr^{-1} mucosa → serosa. Net titratable acid flux was 0.91 ± 0.40 μmol · cm^{-2} · hr^{-1}, mucosa → serosa. When Cl^- free or Na^+ free artificial urine bathed the mucosal side, net NH_4^+ flux was –0.60 ± 0.20 and –0.22 ± 0.40 μmol · cm^{-2} · hr^{-1}, respectively (serosa→mucosa), and net titratable acid flux was 0.22 ± 0.30 and –1.96 ± 0.90 μmol · cm^{-2} · hr^{-1} (mucosa → serosa and serosa → mucosa), respectively. None of the above net acid or net NH_4^+ fluxes were significantly different from zero ($P > 0.05$).

The addition of either DIDS (10^{-4} M) or amiloride (10^{-4} M) or bumetanide (10^{-4} M) to the mucosal artificial urine had no effect on Cl^- or Na^+ observed flux ratios or unidirectional flux of either ion (Table 2). There were no effects on V_t or G_t with any of the drug treatments.

There were no immediate, significant decreases in either Na^+ or Cl^- influx (m → s) rates upon addition of 10^{-3} M NaCN (CN^-) to the mucosal bathing solution (Hour 2, Fig. 7). However, there was a decreasing trend after the addition of CN^- and both Na^+ and Cl^- influx rates were significantly reduced in the second control period (Hour 3, $P < 0.05$). Cl^- influx was reduced by 50% from 0.55 ± 0.15 to 0.28 ± 0.03 μmol · cm^{-2} · hr^{-1}, and Na^+ influx by 41% from 0.37 ± 0.10 to 0.22 ± 0.05 μmol · cm^{-2} · hr^{-1}. Concomitant with the reduced influx, G_t decreased in Hour 3 (2.13 ± 0.36 to 1.93 ± 0.30 mS · cm^{-2}, $P <$

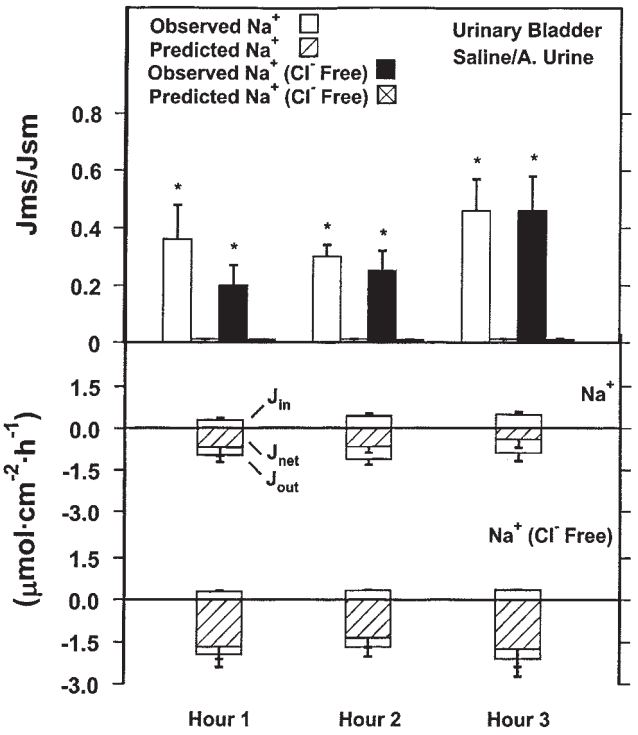

Fig. 6. Observed and predicted flux ratios for the unidirectional movements of Na^+ with control or Cl^--free artificial urine bathing the mucosal surface of urinary bladder epithelia of FW *O. mykiss*. Open bars and solid bars represent the observed flux ratios for Na^+ in control and in Cl^--free mucosal artificial urine, respectively, and hatched and crossed bars represent the predicted flux ratios for Na^+ in control and in Cl^--free mucosal artificial urine, respectively based on the Ussing flux ratio criterion. Means ± 1 SEM (n = 4). Asterisk indicates significant difference from predicted flux ratio (P < 0.05, paired t-test, two-tailed) implying evidence for nondiffusive transport. Unidirectional influx (J_{in}), efflux (J_{out}), and net flux (J_{net}) are shown for Na^+ in control and in Cl^--free mucosal artificial urine. There were no significant differences (P > 0.05) between the two treatment groups.

0.05), and V_t increased in both Hours 2 and 3 (4.09 ± 0.51 mV to 4.94 ± 0.51 and 6.14 ± 0.50 mV, respectively; P < 0.01). Note that while CN^- caused a significant reduction in the observed flux ratios of Cl^- in both Hours 2 and 3 (P < 0.05), the observed ratios for both Na^+ and Cl^- remained significantly higher than the predicted values, indicating that active transport persisted to some degree.

Na^+ influx rates (i.e., "Na^+ kinetics") in response to increases in Na^+ concentration in the artificial urine (control) and low Cl^- artificial urine solutions bathing the mucosal surface are shown in Fig. 8. Analysis of the kinetic curves by Eadie–Hofstee plots indicated J_{max} and K_m under control conditions were 6.1 ± 2.3 μmol · cm^{-2} · hr^{-1} and 34 ± 20 mM, respectively. The solution low in Cl^- decreased (P < 0.05) the J_{max} (2.1 ± 0.56 μmol · cm^{-2} · hr^{-1}) of the Na^+ influx but did not change K_m (27 ± 12 mM) (P > 0.05). Comparison of Na^+ influx rates between the two mucosal solutions demonstrated significant differences in the 40–50 and 110–150 mM range (P < 0.05). Unidirectional influx of Na^+ decreased 80%, and net uptake was abolished (not shown) in the 110–150 mM range when the solution low in Cl^- bathed the mucosal surface.

Cl^- influx rates in response to increases in Cl^- concentration (i.e., "Cl^- kinetics") in the artificial urine and low Na^+ mucosal solutions are shown in Fig. 9. The control and low-Na^+ curves had different (P < 0.05) J_{max} values of 11.4 ± 5.1 and 2.4 ± 0.16 μmol · cm^{-2} · hr^{-1} and similar (P > 0.05) K_m values of 37 ± 26 and 16 ± 2 mM, respectively. Control Cl^- influx rates were significantly greater than those in the low-Na^+ treatment at mucosal Cl^- concentration ranges of 5–10, 40–50, and 110–150 mM (P < 0.05). In the range of 110–150 mM of Cl^- in the mucosal low-Na^+ artificial urine solution, unidirectional influx of Cl^- decreased 85% from control values and the net uptake of Cl^- was abolished (not shown). There were no significant differences between the K_m and J_{max} values of the Na^+ and Cl^- control curves.

DISCUSSION

Urinary bladder Na^+ and Cl^- transport

The negligible transepithelial potential (~0.1 mV) and apparent active absorption of Na^+ and Cl^- (Fig. 1) suggests an electrically neutral uptake mechanism for these ions in the rainbow trout urinary bladder. Electro-neutral transport is a well-documented characteristic of many fish transporting epithelia such as the flounder intestine (Field et al., '78), sculpin intestine (House and Green, '65), marine eel intestine (Skadhauge, '74), SW-adapted (Renfro, '77) and FW-adapted flounder urinary bladder (Demarest and Machen, '84), and the urinary bladder of the European strain of rainbow trout (Lahlou and Fossat, '71).

Replacement of either Na^+ or Cl^- in the mucosal saline significantly decreased influx of the cotransport ion by 56% and 69%, respectively (Figs. 2 and 3). In the kinetics experiments, the absorption of Na^+ and Cl^- appeared saturable at high mucosal concentrations of substrate. However, under these conditions, removal of the Cl^- or Na^+ from the mucosal bath caused 80–85% inhibition of the absorption rate of the other ion (Na^+ or Cl^-) (Figs. 8 and 9). These results suggest that a Na^+–Cl^--dependent, carrier-mediated uptake mecha-

TABLE 2. Effect of the 10^{-4} M mucosal addition of DIDS, amiloride, or bumetanide on unidirectional efflux (J_{sm}), influx (J_{ms}), and flux ratios of Cl^- and Na^+ and on V_t and G_t in urinary bladder of O. mykiss *(n = 4) bathed on mucosal side with artificial urine under open-circuit conditions*[†]

Experimental period	V_t(mV)[a]	G_t(mS · cm^{-2})[b]	J_{sm} (μmol · cm^{-2} · hr^{-1})	J_{ms} (μmol · cm^{-2} · hr^{-1})	J_{ms}/J_{sm} Observed	J_{ms}/J_{sm} Predicted
Cl^-						
Control (hour 1)	6.5 ± 0.4	1.45 ± 0.2	2.3 ± 0.3	0.3 ± 0.1	0.14 ± 0.02	0.03 ± 0.0004*
DIDS	5.2 ± 0.6	1.31 ± 0.2	2.3 ± 0.3	0.3 ± 0.1	0.16 ± 0.06	0.03 ± 0.0004*
Control (hour 3)	5.2 ± 0.6	1.34 ± 0.3	2.7 ± 0.4	0.3 ± 0.1	0.11 ± 0.03	0.03 ± 0.0004*
Na^+						
Control (hour 1)			1.3 ± 0.2	0.1 ± 0.01	0.12 ± 0.03	0.01 ± 0.0001*
DIDS			1.3 ± 0.3	0.2 ± 0.03	0.13 ± 0.03	0.01 ± 0.0002*
Control (hour 3)			2.0 ± 0.2	0.1 ± 0.02	0.06 ± 0.01	0.01 ± 0.0001*
Cl^-						
Control (hour 1)	4.4 ± 1.0	2.38 ± 0.8	0.7 ± 0.3	0.3 ± 0.1	0.53 ± 0.11	0.03 ± 0.002*
Amiloride	4.7 ± 1.1	2.10 ± 0.8	2.2 ± 0.6	0.5 ± 0.2	0.33 ± 0.16	0.03 ± 0.001*
Control (hour 3)	5.1 ± 0.8	2.70 ± 0.9	1.0 ± 0.1	0.5 ± 0.1	0.56 ± 0.17	0.03 ± 0.001*
Na^+						
Control (hour 1)			0.6 ± 0.2	0.1 ± 0.03	0.23 ± 0.09	0.01 ± 0.0001*
Amiloride			1.4 ± 0.5	0.3 ± 0.05	0.34 ± 0.08	0.01 ± 0.0002*
Control (hour 3)			0.6 ± 0.1	0.2 ± 0.02	0.45 ± 0.13	0.01 ± 0.0001*
Cl^{-*}						
Control (hour 1)	4.9 ± 1.0	1.41 ± 0.3	1.0 ± 0.2	0.4 ± 0.1	0.40 ± 0.06	0.03 ± 0.002*
Bumetanide	4.0 ± 1.3	1.51 ± 0.2	0.8 ± 0.2	0.4 ± 0.1	0.60 ± 0.17	0.03 ± 0.002*
Control (hour 3)	3.8 ± 1.2	1.36 ± 0.4	1.0 ± 0.2	0.4 ± 0.1	0.43 ± 0.09	0.03 ± 0.002*
Na^{+*}						
Control (hour 1)			0.4 ± 0.1	0.1 ± 0.02	0.46 ± 0.17	0.01 ± 0.001*
Bumetanide			0.3 ± 0.1	0.2 ± 0.03	0.53 ± 0.24	0.01 ± 0.001*
Control (hour 3)			0.6 ± 0.2	0.2 ± 0.03	0.44 ± 0.13	0.01 ± 0.001*

[†]There were no significant effects of any of the drug treatments on the parameters measured.
[a]Transepithelial potential (mucosal ground) corrected for junction potentials; $n = 8$.
[b]Tissue conductance corrected for solution resistance; $n = 8$.
*Paired t-test, two-tailed, observed versus predicted values of J_{ms}/J_{sm}. Comparison of all observed and predicted ratios were significantly different, $P < 0.05$.

nism is present in the FW *O. mykiss* urinary bladder. Previous studies using the urinary bladder of the closely related *S. irideus* by Fossat and Lahlou ('77, '79a,b, '82) report a tight coupling of Na^+ and Cl^- transport from mucosa to serosa, whereby the removal of one ion completely abolished the net transport of the other ion. Noteworthy, however, is that in the present study, net active absorption of either ion, albeit reduced, persisted upon the removal of the other ion (Figs. 2 and 3). Therefore, the lumenal absorption of Na^+ and Cl^- in the FW trout urinary bladder may only be a partially coupled process. These observations are in agreement with studies performed on urinary bladders of FW (Demarest and Machen, '84) and SW-adapted flounder (Renfro, '77), both of which display a partial coupling (60% and 75%, respectively) of Na^+ and Cl^- transport. In the present study, the mucosal bath in each of the ion replacement experiments contained a small concentration of the "removed" ion (approximately 3 mM) due to either a "rinse-off" effect of the epithelium or passive unidirectional efflux that appeared to increase throughout the experimental period (data not shown). Therefore, the 56% and 69% decrease in influx of Cl^- and Na^+, respectively (as mentioned above) would most likely be greater if their respective co-transport ions were completely absent from the mucosal bath, assuming that a coupled transport mechanism is actually present. Further evidence for a coupled transport system in the urinary bladder of *O. mykiss* was observed when net transport of Na^+ or Cl^- was abolished upon the removal of the co-transport ion at higher levels (150 mM) of Na^+ or Cl^- in the mucosal artificial urine.

Under more realistic conditions representative of those occurring in the animal in vivo, with artificial urine bathing the mucosal surface, the active unidirectional influx of Na^+ and Cl^- continued, though the net balance was negative (Fig. 4). However, unlike results reported under symmetrical saline conditions, there was no effect on the rate of transport of Na^+ or Cl^- upon the removal of the Cl^- or Na^+, respectively (Figs. 5 and 6). These data suggest that at lower mucosal concentrations of

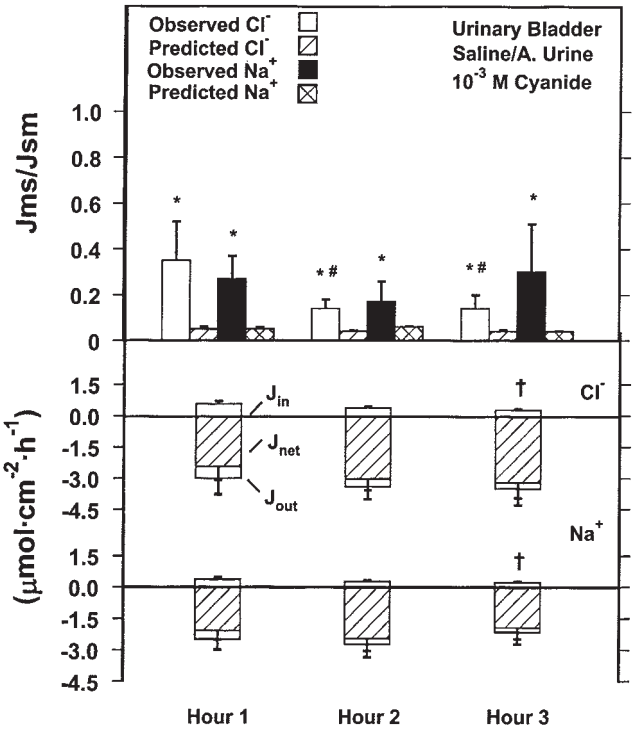

Fig. 7. Observed and predicted flux ratios for the unidirectional movements of Cl^- and Na^+ in urinary bladders of FW *O. mykiss* bathed with artificial urine on the mucosal surface with addition of 10^{-3} M cyanide (NaCN) during the second period (Hour 2). Open bars and solid bars represent the observed flux ratios for Cl^- and Na^+, respectively, and hatched and crossed bars represent the predicted flux ratios for Cl^- and Na^+, respectively, based on the Ussing flux ratio criterion. Means ± 1 SEM (n = 6). Asterisk indicates significant difference from predicted flux ratio ($P < 0.05$, Wilcoxon signed rank sum test), indicating nondiffusive transport. Pound sign (#) indicates observed flux ratio significantly lower than Hour 1 ($P < 0.05$, Wilcoxon signed rank sum test). Unidirectional influx (J_{in}), efflux (J_{out}), and net flux (J_{net}) are shown for Cl^- and Na^+. Cross indicates significant difference from unidirectional influx of Hour 1 ($P < 0.05$, paired t-test, one-tailed).

Na^+ and Cl^-, coupled transport did not occur. These results are more consistent with data reported by Marshall ('86) which indicated active independent transport of Na^+ and Cl^- in the urinary bladder of brook trout (*Salvelinus fontinalis*). However unlike results from Marshall's ('86) study, the removal of either Na^+ or Cl^- at higher levels of Na^+ and Cl^- substantially reduced the absorption rates of Cl^- and Na^+, respectively. Furthermore, analyses of net acid and NH_4^+ fluxes in the present study argue against acid–base linkage of an independent transport mechanism.

Under these "more realistic" conditions, bladder epithelia were also in negative balance (net loss of Na^+ and Cl^-) (Fig. 4). While this might indicate that the bladder was not behaving as efficiently as in vivo, alternately it is possible that the bladder may be "poised" kinetically to transport at the concentrations of Na^+ and Cl^- present in ureteral urine (7.12 mM and 5.21 mM, respectively; Curtis and Wood, '91, '92) and not for the concentrations in the final "polished" urine (2.12 mM and 3.51 mM, respectively) which we supplied. Indeed, comparison of the kinetic curves for influx (Figs. 8, 9) with the unidirectional flux data of Fig. 4, suggests that both Na^+ and Cl^- influx would be markedly higher at 5.2–7.1 mM than at 2.1–3.5 mM, and that influx and efflux would be in approximate balance at these mucosal concentrations.

Effects of pharmaceuticals

Mucosal addition of DIDS (Cl^-/HCO_3^- exchange blocker), amiloride (Na^+/H^+ exchange and Na^+ channel inhibitor) or bumetanide (Na^+–Cl^- and Na^+–K^+–$2Cl^-$ co-transport blocker) all had no effect on the transport (absorption/extrusion) of Na^+ and/or Cl^- (Tables 1 and 2) in either symmetrical saline or mucosal artificial urine conditions. A combination of the three drugs was also ineffective in blocking Na^+ or Cl^- transport under symmetrical saline conditions. Non-diffusive, apparent active absorption of Na^+ and Cl^- continued in the presence of each drug, which reinforces the suggestion of a dual mechanism type of transport. Thus, for example, if two transport systems were operating in parallel (Na^+ and Cl^- independent and dependent mechanisms), addition of DIDS would impair anion exchange but co-transport mechanisms would be unaffected. In fact in saline/saline conditions, DIDS decreased Cl^- absorption and bumetanide decreased Cl^- and Na^+ absorption, but not significantly. However, in each case active transport persisted. The use of the diuretic chlorothiazide at 10^{-3} M was also ineffective in blocking Na^+ and/or Cl^- influx. The thiazide diuretics (hydrochlorothiazide, chlorothiazide) are known Na^+/ Cl^- co-transporter blockers as well as inhibitors of carbonic anhydrase (Weiner, '90). Winter flounder urinary bladder studies have shown that 10^{-4} M hydrochlorothiazide or metolazone (also a diuretic) were sufficient to inhibit Na^+ and Cl^- cotransport (Stokes, '84). Both diuretics were equally effective in reducing Na^+ and Cl^- absorption except that full hydrochlorothiazide effects were witnessed in 2–3 min. whereas metolazone appeared to take up to 60 min. (Stokes, '84). The present studies have shown that 10^{-3} M chlorothiazide was ineffective in inhibiting co-transport after a 45-min pre-incubation period

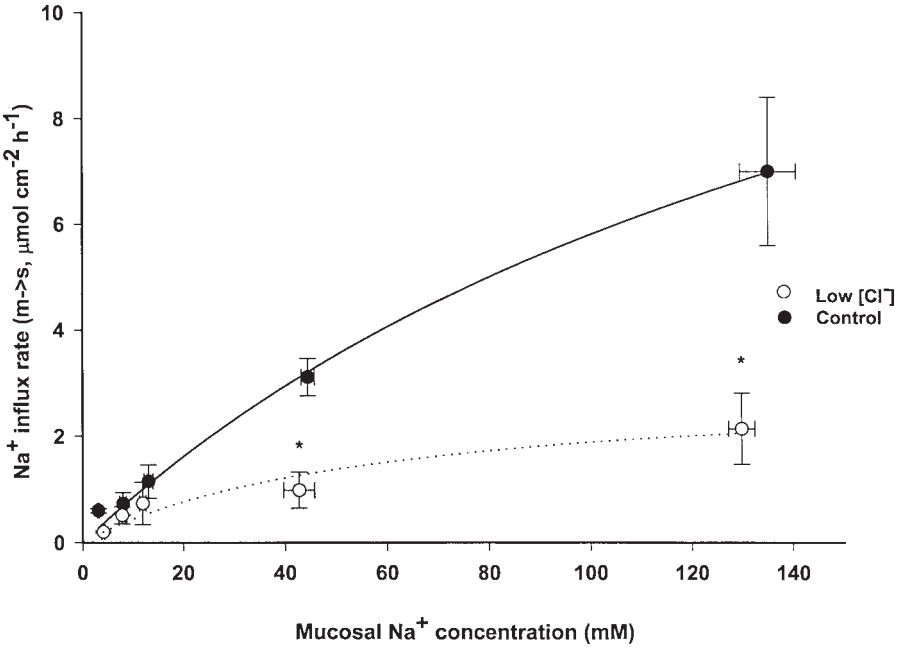

Fig. 8. Relationship of mucosal to serosal Na^+ flux (Na^+ influx) to the concentration (mM) of Na^+ in control solutions (closed circles) and low Cl^- solutions (open circles). Serosal medium was Cortland's saline, and mucosal medium was artificial urine with varying concentrations of Na^+ and/or Cl^-. Vertical and horizontal lines represent ± SEM; n = 4–8 for each concentration range (0–5, 5–10, 10–20, 40–50, and 110–150 mM). Curves were best fit for Michaelis–Menten kinetics. Asterisk indicates significant difference between concentration range of each treatment ($P < 0.05$, unpaired t-test, two-tailed).

(see Materials and Methods), even though the same concentration of chlorothiazide was responsible for the complete inhibition of Na^+ and Cl^- absorption in rat early distal tubule (Ellison et al., '87). The carbonic anhydrase inhibition by thiazide diuretics could complicate interpretation, but lack of any effect of the chlorothiazide made this an immaterial consideration in the present study.

The existence of a dual dependent/independent transport mechanism is a possible explanation for the lack of effect with use of amiloride, bumetanide, chlorothiazide, and DIDS, but the results of the combination of amiloride, bumetanide, and DIDS experiment make this difficult to accept. However, physical explanations may be responsible for the lack of change in Na^+ or Cl^- transport. The bladder preparation may be such that effective penetration by the drugs does not occur. This would occur if the sites that are primarily acted on by the drugs were obscured or not readily accessible to the drug molecules. The results of the cyanide experiment were informative in this regard, and indicate that any conclusions based on pharmacological results must be tempered by recognition of this slow penetration problem.

Cyanide was employed as a means to establish that active transport did indeed occur under asymmetric artificial urine and saline conditions, and to evaluate the time course of drug penetration. Cyanide was tested under the worst-case scenario of asymmetric conditions, rather than under saline/saline conditions. In the latter, the evidence for active transport was obvious—i.e., net mucosal-to-serosal transport of both Na^+ and Cl^- in the absence of any driving gradients. However, under artificial urine/saline conditions, the evidence for the occurrence of active transport based solely on the Ussing flux ration equation might be considered weaker, since concentration, electrical, and osmotic gradients all existed across the preparation, and net mucosal-to-serosal transport was not occurring. Although cyanide was not immediately effective, there were decreasing trends in the influx rates of both Na^+ and Cl^- which became significant during the final control period, even though cyanide had been removed from the mucosal bath by this time. Cyanide, therefore, appeared to cause a slowly developing inhibition of Na^+ and Cl^- unidirectional influx at low mucosal concentrations of Na^+ and Cl^- when net efflux was occurring. This, in conjunction with the Ussing flux ratio criterion (Fig. 7), indicates that even during low mucosal Na^+ and Cl^- concentrations,

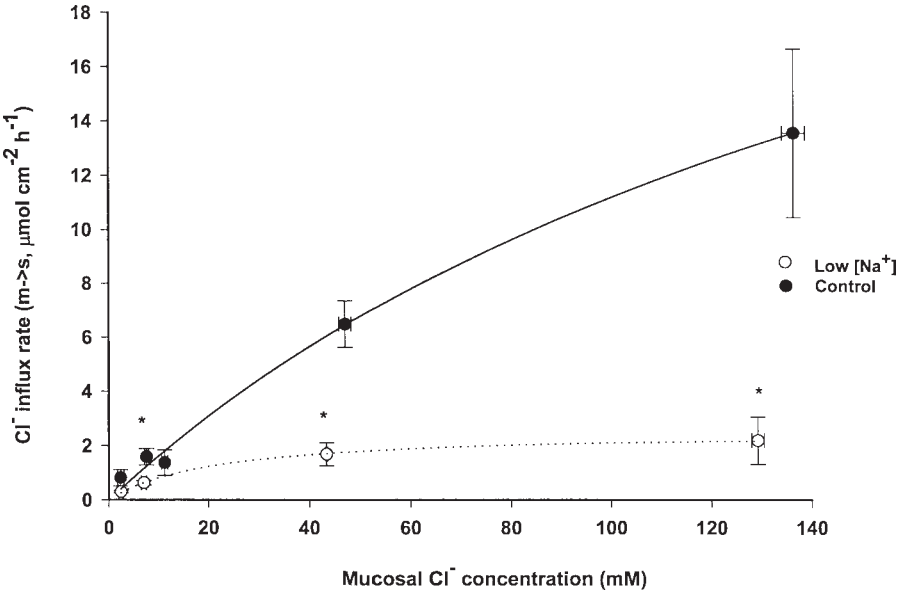

Fig. 9. Relationship of mucosal to serosal Cl^- flux to the concentration (mM) of Cl^- in control solutions (closed circles) and low Na^+ solutions (open circles). Serosal medium was Cortland's saline, and mucosal medium was artificial urine with varying concentrations of Na^+ and/or Cl^-. Vertical and horizontal lines represent ±SEM; n = 4–8 for each concentration range (0–5, 5–10, 40–50, and 110–150 mM, excluding the 10–20 mM range for low Na^+ artificial urine where no observations were recorded). Curves were best fit for Michaelis–Menten kinetics. Asterisk indicates significant difference between concentration range of each treatment ($P < 0.05$, unpaired t-test, two-tailed).

the low, unidirectional influx is indeed active. Although the influx of both ions was reduced by cyanide, the Ussing flux ratio shows that active transport was not completely abolished during the second and third hour (cyanide and control/post cyanide periods, respectively). The length of time for cyanide effectiveness along with the retention of some active transport seems to indicate that accessibility may indeed be hampering the action of the other drugs. It must also be remembered, however, that anaerobic ATP supply to transport systems can persist in the presence of cyanide.

Further conclusions

The present study observed electroneutral co-transport of Na^+ and Cl^- in the urinary bladder of *O. mykiss* under symmetrical saline conditions, thus reinforcing previous studies performed on the urinary bladder of the closely related European strain of rainbow trout (*Salmo irideus*) (Fossat and Lahlou, '79a, '82). However, there was not 100% coupling of Na^+ and Cl^- transport as reported in *S. irideus* (Fossat and Lahlou, '79a), and relevant pharmacological agents (bumetanide, chlorothiazide) did not provide supporting evidence for the presence of a co-transport system. Experiments performed on *S. irideus* did not use an artificial urine, and Na^+ and Cl^- transport measured at these more realistic levels of mucosal NaCl in the present study appeared to be independent processes. Therefore, the NaCl transport process in *O. mykiss* appears to be only partially coupled; transport mechanisms are both dependent upon and independent of the other ion. Electrical parameters of *S. irideus* (Fossat and Lahlou, '79a, '82) and *O. mykiss* (present study) were very similar under symmetrical saline conditions (negligible V_t, $I_{sc} < 1\ \mu A \cdot cm^{-2}$ and G_t = 3–4 $mS \cdot cm^{-2}$). In contrast, the urinary bladder of the brook trout (*Salvelinus fontinalis*), exhibited independent transport of Na^+ and Cl^- at both higher and lower levels of mucosal NaCl (Marshall, '86). The transport of NaCl, under saline/saline conditions, was reported to be electroneutral; however, V_t (6.7 mV) and G_t (0.23 $mS \cdot cm^{-2}$) were rather different from those of the present study. It appears, therefore, that the brook trout transports Na^+ and Cl^- in a very different manner than the rainbow trout (*O. mykiss*).

ACKNOWLEDGMENTS

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