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TRANSINTESTINAL ACETATE TRANSPORT IN A HERBIVOROUS TELEOST: ANION EXCHANGE AT THE BASOLATERAL MEMBRANE

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Summary

Purified basolateral membrane vesicles were prepared from the intestinal epithelium of the tilapia *Oreochromis mossambicus*, a herbivorous teleost. Characteristics of volatile fatty acid (VFA) transport were investigated using [^{3}H]acetate as a representative anion. No significant change in [^{3}H]acetate influx was observed in the presence of a cation gradient (K^+ or Na^+) compared to the influx observed in the absence of a cation gradient, indicating the lack of cation-dependent coupling for acetate transport. The time course of [^{3}H]acetate uptake into vesicles preloaded with acetate or bicarbonate was enhanced compared to [^{3}H]acetate uptake into vesicles preloaded with gluconate. A series of trans-stimulation and cis-inhibition studies involving both organic and inorganic anions indicated the presence of a highly specific anion-exchange carrier which readily exchanged [^{3}H]acetate with the volatile fatty acids (formate, acetate, propionate and butyrate) and bicarbonate. Kinetic analysis of [^{3}H]acetate influx as a function of external acetate concentration yielded a biphasic uptake curve which was interpreted as carrier-mediated transfer (J_{max}=21.77±2.05 nmol mg^{-1} protein 10 s^{-1}; K_m=12.70±2.95 mmol l^{-1}) plus apparent diffusion (P=0.17±0.02 nmol mg^{-1} protein 10 s^{-1} mmol^{-1} acetate). [^{3}H]Acetate uptake was also a hyperbolic function of internal bicarbonate concentration, displaying a relatively low HCO_3^- half-saturation constant (K_m=0.41 mmol l^{-1}). Intact intestinal sheets mounted in Ussing chambers demonstrated net absorptive fluxes of [^{3}H]acetate when serosal acetate concentration was maintained at 1.0 mmol l^{-1} and the mucosal acetate concentration was varied from 1.32 to 10.0 mmol l^{-1}. At mucosal acetate concentrations lower than 1.32 mmol l^{-1}, a net secretion of VFAs was observed. Transepithelial transport of [^{3}H]acetate was significantly inhibited by the presence of acetazolamide. A transintestinal transport model for volatile fatty acids is proposed in which specific anion antiporters, located on the brush-border and basolateral poles of the cell, exchange luminal VFAs for serosal and intracellular bicarbonate, resulting in net transepithelial uptake of VFAs. This process is driven by a downhill lumen-to-blood VFA concentration gradient and the intracellular generation of bicarbonate by carbonic anhydrase.

Key words: acetate, volatile fatty acids, anion exchange, intestine, transport, basolateral, tilapia, herbivore, *Oreochromis mossambicus*.

Introduction

Volatile fatty acids (VFAs) are nutritive solutes produced as by-products of microbial fermentation of nondigestible carbohydrates, including such dietary constituents as pectins, celluloses and hemicelluloses. In many vertebrates VFAs constitute the major fraction of the contents of the intestine, caecum and colon (Engelhardt and Rechkemmer, 1985). The extent of VFA generation and assimilation varies greatly among the vertebrates. Ruminants possess specialized, highly efficient gastrointestinal tracts for the production of VFAs, from which they obtain the majority of their metabolic requirements. Non-ruminant mammals utilize the large intestine as the primary site of fermentation and subsequent VFA assimilation (Engelhardt and Rechkemmer, 1985). VFA production/assimilation has also been reported to some degree in birds (Clemens *et al.* 1975; Rice and Skadhauge, 1982), reptiles (Gossling *et al.* 1982) and amphibians (Guard, 1980; Hollander *et al.* 1986).

Only one study has reported any aspect of VFA production/assimilation in the largest class of vertebrates, the fishes (Titus and Ahearn, 1988). Transport of VFAs across the brush-border membrane of a herbivorous teleost was reported to be a facilitated exchange mechanism whereby intracellular bicarbonate (HCO_3^-) was exchanged with extracellular VFAs in a one-to-one antiport. Prior to this, absorption of VFAs has always been reported as simple diffusion with the transport rate increasing in a linear fashion with concentration. No previous evidence for a saturable carrier mechanism has been presented (Ruppin *et al.* 1980; Rechkemmer and Engelhardt, 1988; Rechkemmer *et al.* 1988). Based upon the findings of Titus and Ahearn (1988) for VFA/HCO_3^- antiport at the intestinal brush-border membrane, three potential models for cell-to-blood transport across the basolateral membrane of fish intestine are (1) a passive or facilitated diffusion process down a concentration gradient, similar to that found in basolateral glucose transport in teleost intestine (Reshkin and Ahearn, 1987), (2) a cationic symport mechanism whereby VFAs cross the basolateral membrane *via* a cation-dependent carrier, as described for glutamate and proline transport in the gut of the carnivorous eel *Anguilla anguilla* (Reshkin *et al.* 1988) or (3) an antiport mechanism similar to that described above for the brush border, in which VFAs cross the basolateral membrane in exchange for bicarbonate or other anions located extracellularly.

At present, VFA transport across the intestinal basolateral membrane remains unstudied. This investigation examines the characteristics of basolateral VFA transport in an effort to determine which of the aforementioned mechanisms actually occurs at this epithelial pole. In addition, this study also presents a transepithelial transport analysis of VFA transfer from gut lumen to blood. The results allow us to propose a transintestinal transport model for VFAs combining the previously proposed brush-border VFA transport characteristics with current findings. This model should be useful for elucidating a mechanism by which a herbivorous teleost can assimilate VFAs, a potentially valuable nutrient source,

which have been shown to exist in luminal contents of these animals in significant concentrations (Titus and Ahearn, 1988).

Materials and methods

Preparation of basolateral membrane vesicles

Adult tilapia, *Oreochromis mossambicus* Peters, were stored in a flow-through seawater tank for 1 week to 2 months prior to experimentation. Basolateral membrane vesicles (BLMV) were prepared by a modification of the techniques of Brasitus and Keresztes (1983) as described in Reshkin and Ahearn (1987). Animals were killed by a blow to the head. Five to ten animals were used per preparation. The proximal half of the intestine following the stomach was removed, slit lengthwise and stored in 300 mmol l^{-1} mannitol and 12 mmol l^{-1} Hepes–Tris (pH 7.4) at 0°C.

Epithelial cells were isolated from the intestine using the sodium citrate removal technique of Murer *et al.* (1974), in which the slit intestines were agitated for 5 min in a releasing buffer of the following constituents (in mmol l^{-1}): 27 sodium citrate, 96 NaCl, 5.6 NaH_2PO_4, 1.5 KCl, 8 KH_2PO_4 (pH 7.4). Intestines were discarded and the resulting cell suspension was strained through a double layer of cheesecloth to remove debris. The clean suspension was centrifuged for 1 min at 9700 ***g*** (Sorvall RC-5C with an SS 34 rotor) to pellet the cells. The resulting pellet was resuspended in 30 ml of 25 mmol l^{-1} NaCl, 5 mmol l^{-1} Hepes–Tris, 2 mmol l^{-1} phenylmethylsulfonylfluoride (PMSF) (pH 8.0) and homogenized 10 strokes with a Potter–Elvehjem homogenizer. This suspension was centrifuged for 13 min at 500 ***g***, followed by a spin at 1000 ***g*** for 2 min. The supernatant was decanted and further centrifuged for 30 min at 100 000 ***g*** in a Beckman L8–55 ultracentrifuge equipped with an SW 28 swinging bucket rotor. The creamy upper portion of the resulting pellet was removed by pipet and resuspended in 9 ml of 50 % sucrose in 12.5 mmol l^{-1} NaCl, 10 mmol l^{-1} Hepes–Tris and 0.5 mmol l^{-1} EDTA (pH 7.5). This 9 ml suspension was placed in the bottom of an ultracentrifuge tube and overlaid by 9 ml layers of 40 %, 30 % and 20 % sucrose solutions, resulting in a four-layer discontinuous gradient. This gradient was centrifuged for 90 min at 100 000 ***g*** in a Beckman ultracentrifuge. The fraction accumulated at the 30 %–40 % interface was collected and the rest discarded. The collected fraction was diluted in 35 ml of the final working buffer and centrifuged for 30 min at 100 000 ***g***. The final pellet was resuspended in the final working buffer to a protein concentration of 8–10 mg ml^{-1} and passed through a 22 gauge syringe needle. Protein concentrations were determined with the Bio Rad protein assay kit.

Enzyme assays

Relative purities of basolateral membrane vesicle preparations were determined using spectrophotometric assays of known marker enzymes. Enzyme concentrations from the final vesicle pellet were compared with concentrations from the initial cellular homogenate pellet to determine the degree of purity. Alkaline

phosphatase activity was determined using Sigma kit no. 104. Na^+/K^+-ATPase activity was measured using modifications of the technique of Ames (1960). K^+-stimulated *p*-nitrophenyl phosphatase activity was determined using the method of Arvan and Castle (1982). Basolateral vesicle orientation has previously been determined by modifications of a Na^+/K^+-ATPase latency technique by Lee and Pritchard (1985) and was determined to be 70% leaky (nontransporting), 19% inside out and 11% right side out (Reshkin and Ahearn, 1987).

Transmembrane transport measurements

Transport of [^{3}H]acetate into BLMV was measured using modifications of the Millipore filtration technique of Hopfer *et al.* (1973). Vesicle suspensions were stored on ice for the duration of the transport experiments (up to 3 h). A known volume of vesicle suspension (5 μl) was automatically combined with 10 or 20 volumes of radiolabelled incubation medium at 22°C using a rapid uptake apparatus (Innovativ Labor. AG; Adliswil, Switzerland). At the end of the incubation period, the uptake was terminated by automatic addition of 2 ml of stop solution (0°C). Vesicle suspensions were immediately suction-filtered through 0.65 μm Millipore filters and washed with an additional 8 ml of stop solution at 0°C. Filters were placed in vials containing Beckman Ready Solv scintillation cocktail and activity was measured in a Beckman LS-8100 liquid scintillation counter. Each experiment was run at least in duplicate and data points represent the means of 4–5 replicates±standard error.

Transepithelial fluxes

Unidirectional transepithelial [^{3}H]acetate fluxes across intestinal sheets were determined using modified Ussing flux chambers. The upper intestine was excised from a test animal, sectioned into 1 cm pieces, slit lengthwise and mounted in an Ussing flux apparatus with a 0.5 cm diameter aperture (0.196 cm^2) and 5 ml reservoirs. [^{3}H]Acetate was added to the cis side of the apparatus to initiate transmural flux. Total activity measurements were monitored by removing 10 μl samples from the initially unlabelled trans side at 5 min intervals for 75 min, a period for which there was no apparent time-dependent decrease in transepithelial flux rates. Experimental design was such that four adjacent sections of tissue from the same length of upper intestine were mounted in four separate flux chambers to determine separate mucosa-to-serosa and serosa-to-mucosa fluxes, for both the control and experimental treatments. All four treatments were run concurrently. Net fluxes were determined by subtracting one unidirectional flux from the other. The incubation medium had the following salt concentrations (in mmol l^{-1}): NaCl, 150; KCl, 4; $CaCl_2$, 2; $NaHCO_3$, 1; $MgSO_4$, 1; NaH_2PO_4, 0.5; Hepes, 30; pH was adjusted to 7.2 with NaOH.

Blood bicarbonate determination

Quantitative analysis of bicarbonate concentrations in the blood plasma of healthy adult tilapia was undertaken utilizing the method of Cameron (1971).

Samples of blood were extracted from the caudal vein of fish sedated in an ice bath. The samples were centrifuged for 5 min at 1000 *g* and the resulting plasma was analysed directly for [HCO_3^-].

Materials

[^{3}H]Acetate was procured from New England Nuclear Corporation (Boston, MA). Propionic acid, pyruvic acid and L-lactic acid were purchased from United States Biochemical Corporation (Cleveland, OH). Butyric acid was obtained from MCB Chemical (Los Angeles, CA). The ionophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), the metabolic inhibitor acetazolamide and all other reagent grade chemicals were procured from Sigma Chemical (St Louis, MO).

Test animals were obtained from Mariculture Research and Training Center (MRTC), Oahu, Hawaii.

Results

Purity of membrane vesicles

Purity of the BLMV preparation was assessed by comparing characteristic marker enzyme activities of the homogenate with those of the final vesicle preparation. From previous studies using an identical vesicle preparation it was determined that membranes recovered from the 30 %–40 % sucrose interface were the most enriched in basolateral membrane marker enzymes (Reshkin and Ahearn, 1987; Reshkin *et al.* 1988). Enzyme assays for the basolateral markers Na^+/K^+-ATPase and K^+-stimulated *p*-nitrophenylphosphatase determined enrichment factors (vesicle activity/homogenate activity) of 13.5 and 6.9, respectively, indicating a purified basolateral membrane preparation. Enzyme assays for alkaline phosphatase, a brush-border marker enzyme, gave an enrichment value of 0.3, indicating no significant contamination by brush border membranes. In addition, Reshkin and Ahearn (1987) reported enrichment values for the mitochondrial marker cytochrome *c* oxidase to be 0.9, and for the endoplasmic reticulum marker NADPH cytochrome *c* reductase to be 0.7, indicating no measurable contamination by these organelle membranes.

Time course of [^{3}H]acetate uptake: cation independence and effect of preloaded anions

Fig. 1 shows the effect of inwardly directed gradients of sodium and potassium on 2.5 mmol l^{-1} [^{3}H]acetate uptake by basolateral membrane vesicles. Vesicles were preloaded with 25 mmol l^{-1} potassium acetate and 100 mmol l^{-1} tetramethylammonium (TMA^+) gluconate and exposed to inward 100 mmol l^{-1} gradients of sodium and potassium, as well as equimolar concentrations of TMA^+ gluconate, which represented the absence of a cation gradient. Uptake values were reported as total uptake minus nonspecific external binding of [^{3}H]acetate, as determined by the amount of [^{3}H]acetate activity measured in vesicles plunged directly into

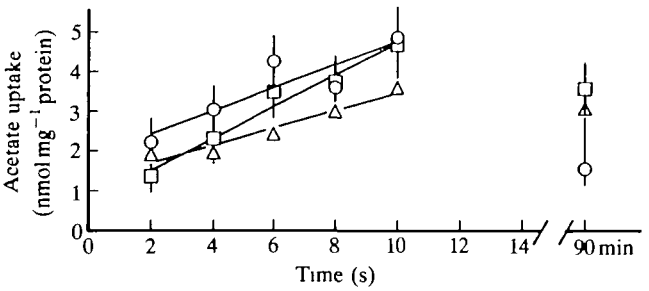

Fig. 1. Cationic independence of 2.5 mmol l^{-1} [^{3}H]acetate uptake into tilapia basolateral membrane vesicles (BLMV). Vesicles were loaded with 100 mmol l^{-1} TMA gluconate, 25 mmol l^{-1} potassium acetate, 12 mmol l^{-1} Hepes–Tris, pH 7.4 and 20 μmol l^{-1} CCCP. External media were 125 mmol l^{-1} potassium gluconate (○); 25 mmol l^{-1} potassium acetate and 100 mmol l^{-1} sodium gluconate (□); 100 mmol l^{-1} potassium gluconate and 25 mmol l^{-1} TMA gluconate (△). External media also contained 2.5 mmol l^{-1} [^{3}H]acetate and 12 mmol l^{-1} Hepes–Tris, pH 7.4. Uptake rates for the three treatments were not significantly different from each other at $P>0.05$. Results are shown as the mean and S.E. of 4–5 replicates.

labelled incubation media plus stop solution (i.e. 0 s incubation time). The results show little difference in initial rates (2–10 s) for the uptake of [^{3}H]acetate in sodium, potassium or TMA media by potassium-loaded BLMV. This indicates the probable lack of coupling between acetate and sodium or potassium in the transport of acetate across the intestinal basolateral membrane of tilapia. The similarities in the equilibrium values (90 min) indicate that the vesicle volumes were not significantly affected by variations in the cationic composition of the incubation media.

Fig. 2 shows the effects of preloading BLMV with different anions on [^{3}H]acetate uptake. Uptake of [^{3}H]acetate (2.5 mmol l^{-1}) was measured into vesicles preloaded with 50 mmol l^{-1} sodium gluconate and 50 mmol l^{-1} acetate, bicarbonate or gluconate (all sodium salts). The results indicate an enhanced rate of uptake into vesicles preloaded with acetate and HCO_3^- compared with that into vesicles preloaded with gluconate. This suggests that uptake of [^{3}H]acetate into BLMV may occur as a result of exchange with internally located anions. Again, the similarity in equilibrium values indicates that the different treatments did not affect the intravesicular volumes.

The flat uptake curve for [^{3}H]acetate uptake into gluconate-loaded vesicles indicates that transfer under these conditions was little more than external binding and passive diffusion. The apparent trans stimulation of [^{3}H]acetate uptake into HCO_3^-- and acetate-loaded vesicles showed transient concentrative accumulation (overshoot) at 30 s, which exceeded final equilibrium values twofold and sixfold, respectively. This suggests an anion antiport mechanism in the basolateral membrane capable of transporting its substrate against a concentration gradient. All transport was measured under short-circuit conditions (addition of CCCP) to

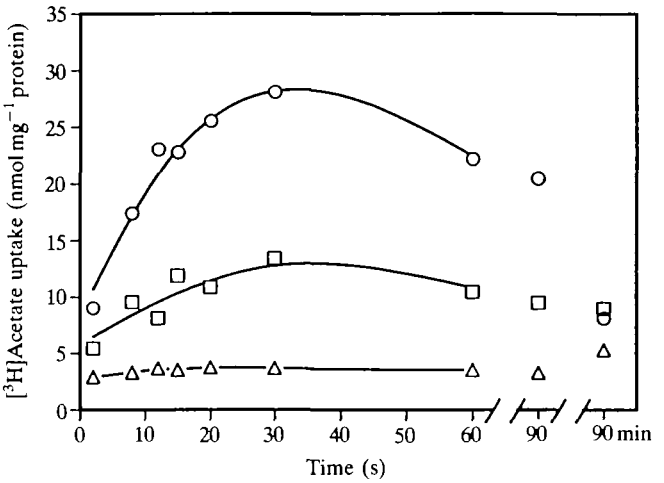

Fig. 2. Time course 2.5 mmol l^{-1} [^{3}H]acetate uptake into upper intestinal tilapia BLMV. Vesicles were preloaded with (in mmol l^{-1}) 50 sodium acetate, 50 sodium gluconate (○); 50 $NaHCO_3$, 50 sodium gluconate (□) or 100 sodium gluconate (△). In addition, all media inside the vesicles contained 12 mmol l^{-1} Hepes–Tris, pH 7.4, and 20 μmol l^{-1} CCCP. External media contained (in mmol l^{-1}) 100 sodium gluconate; 2.5 [^{3}H]acetate and 12 Hepes–Tris, pH 7.4. Symbols represent the means of three replicates. Results are shown as the mean of 4–5 replicates. Error bars are obscured by symbols.

rule out electrical coupling by way of diffusion potentials as a reason for enhanced [^{3}H]acetate uptake.

Anion specificity: trans stimulation and cis inhibition

Fig. 2 suggests an anion exchange mechanism in the BLMV of tilapia whereby [^{3}H]acetate uptake is enhanced by certain internally located anions. To characterize this antiport mechanism further we examined trans stimulation and cis inhibition of [^{3}H]acetate uptake into BLMV by a number of organic and inorganic anions. The incubation period for [^{3}H]acetate uptake in these experiments was 10 s, based on the linearity for uptake at that point as shown in Fig. 1. Fig. 3 illustrates trans stimulation of 2.5 mmol l^{-1} [^{3}H]acetate uptake into BLMV preloaded with 25 mmol l^{-1} test inorganic anion (sodium salts) and 75 mmol l^{-1} sodium gluconate. Incubation media contained 100 mmol l^{-1} sodium gluconate in addition to the labelled acetate. The gluconate-loaded treatment (far right) represents unstimulated acetate influx as a result of passive diffusion and binding to the vesicle membrane. The results show stimulated [^{3}H]acetate uptake into those vesicles loaded with HCO_3^- and thiocyanate (SCN^-), indicating a specificity of acetate exchange for these anions. All other anions exhibited uptakes that were not significantly different from that of the gluconate-loaded control treatment, indicating the absence of acetate exchange with these anions.

Fig. 4 shows trans stimulation of 2.5 mmol l^{-1} [^{3}H]acetate uptake into vesicles

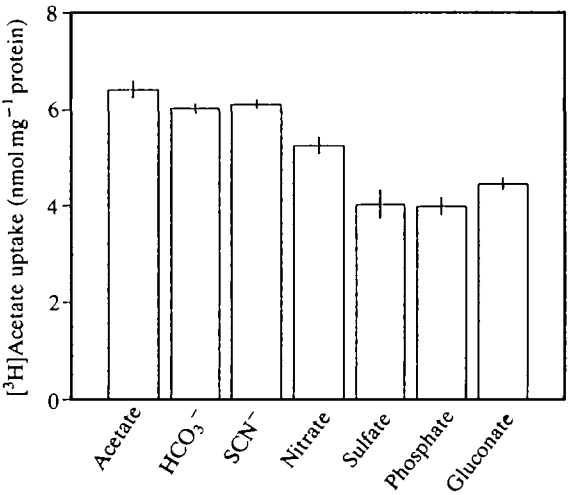

Fig. 3. Trans stimulation of 2.5 mmol l^{-1} [^{3}H]acetate uptake by various inorganic anions in tilapia upper intestinal BLMV. Vesicles were preloaded with 75 mmol l^{-1} sodium gluconate and 25 mmol l^{-1} test anion. External media contained 100 mmol l^{-1} sodium gluconate and 2.5 mmol l^{-1} [^{3}H]acetate. All media contained 12 mmol l^{-1} Hepes–Tris, pH 7.4, and 20 μmol l^{-1} CCCP. The gluconate-loaded treatment (right) represents the control (diffusion) for [^{3}H]acetate transport. Results are shown as the mean of five 10-s replicates and error bars represent S.E.M.

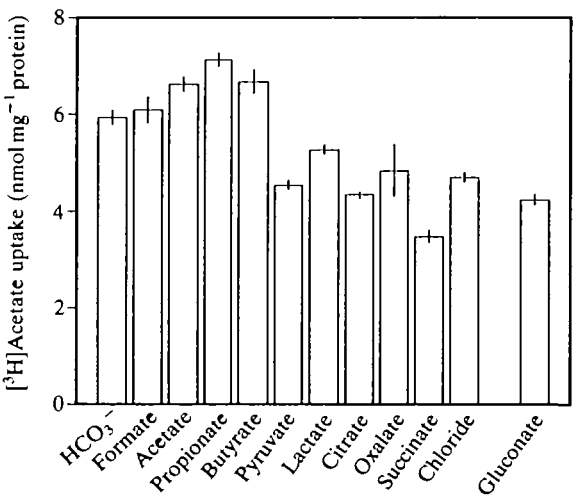

Fig. 4. Trans stimulation of 2.5 mmol l^{-1} [^{3}H]acetate uptake by various organic anions in BLMV of tilapia anterior intestine. Vesicles were preloaded with 75 mmol l^{-1} sodium gluconate and 25 mmol l^{-1} test anion. External media contained 100 mmol l^{-1} sodium gluconate and 2.5 mmol l^{-1} [^{3}H]acetate. All media contained 12 mmol l^{-1} Hepes–Tris, pH 7.4, and 20 μmol l^{-1} CCCP. The gluconate-loaded treatment (far right) represents the control (diffusion) for [^{3}H]acetate transport. Results are shown as the mean of five 10-s replicates. Error bars represent S.E.M.

preloaded with various biologically relevant organic anions. Vesicles were preloaded with 25 mmol l^{-1} test organic anion (sodium salts) and 75 mmol l^{-1} sodium gluconate, as in the experiment shown in Fig. 3. Again, the gluconate-loaded treatment represented the unstimulated control. Results show a marked stimulation (29–41 %) of [^{3}H]acetate influx into BLMV preloaded with HCO_3^-, formate and the volatile fatty acids (VFAs) acetate, propionate and butyrate. The other anions tested showed nonsignificant trans stimulation of [^{3}H]acetate uptake. This further suggests the occurrence of an antiport mechanism by which there is a high degree of specificity of exchange of labelled acetate, not only for HCO_3^-, but also for other VFAs and VFA-like anions.

Cis inhibition of [^{3}H]acetate uptake into BLMV as a result of inorganic anions present in the reaction media is shown in Fig. 5. Vesicles containing either acetate as an exchanger or gluconate as a control were exposed to reaction media containing 2.5 mmol l^{-1} [^{3}H]acetate, 25 mmol l^{-1} test anion (sodium salts) and 75 mmol l^{-1} sodium gluconate. The gluconate-loaded treatments represent simple diffusion and label binding. The gluconate test anion treatment represents uninhibited exchange of [^{3}H]acetate for the internal anion. The results show that HCO_3^- and acetate are the strongest inhibitors of [^{3}H]acetate uptake, although there are limited inhibitory effects elicited by the presence of other test anions in the incubation media.

Fig. 6 illustrates the effects of various organic anions as potential cis inhibitors of [^{3}H]acetate uptake into BLMV. The same experimental design was used as in

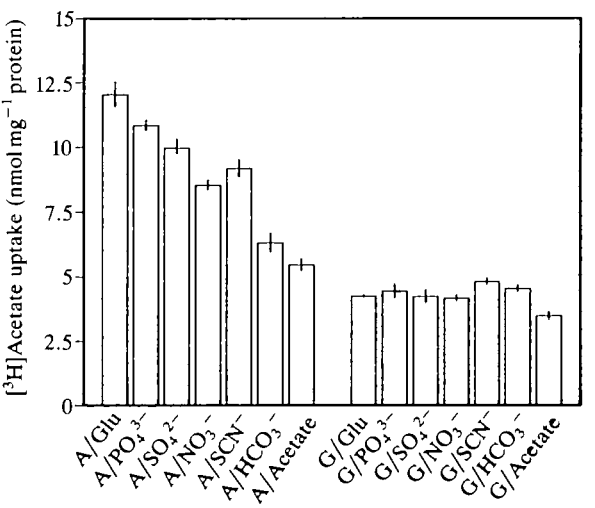

Fig. 5. Cis inhibition of 2.5 mmol l^{-1} [^{3}H]acetate uptake by various inorganic anions in tilapia BLMV. Vesicles were preloaded with 50 mmol l^{-1} sodium acetate and 50 mmol l^{-1} sodium gluconate (A/) or 100 mmol l^{-1} sodium gluconate (G/). External media contained 75 mmol l^{-1} sodium gluconate, 25 mmol l^{-1} test anion and 2.5 mmol l^{-1} [^{3}H]acetate. All media contained 12 mmol l^{-1} Hepes–Tris, pH 7.4, and 20 μmol l^{-1} CCCP. Acetate-loaded (A/Glu) and gluconate-loaded vesicles (G/Glu) represent stimulated and non-stimulated controls, respectively. Results are shown as the mean of five 10-s uptakes. Error bars indicate S.E.M.

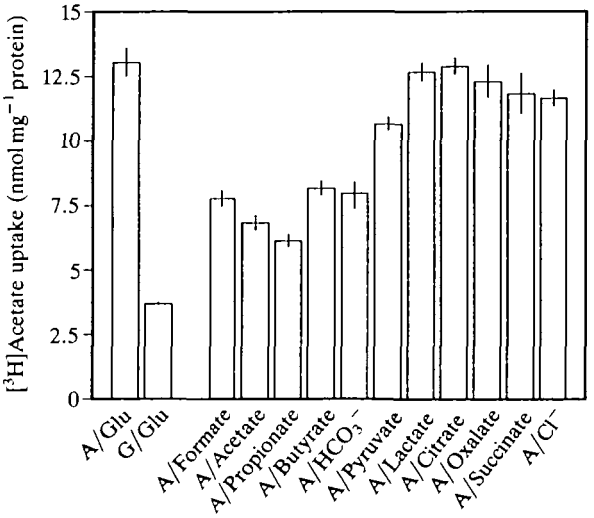

Fig. 6. Cis inhibition of 2.5 mmol l^{-1} [^{3}H]acetate uptake by various organic anions in upper intestinal BLMV of tilapia. Vesicles were preloaded with 50 mmol l^{-1} sodium acetate and 50 mmol l^{-1} sodium gluconate (A/) or 100 mmol l^{-1} sodium gluconate (G/). External media contained 75 mmol l^{-1} sodium gluconate, 25 mmol l^{-1} test anion and 2.5 mmol l^{-1} [^{3}H]acetate. All media contain 12 mmol l^{-1} Hepes–Tris, pH 7.4, and 20 μmol l^{-1} CCCP. A/Glu and G/Glu represent stimulated and non-stimulated controls. [^{3}H]Acetate uptake into gluconate-loaded vesicles inhibited by test anions was not significantly different from (G/Glu), the non-stimulated control (not shown). Results are shown as the mean of five 10-s uptakes. Error bars indicate S.E.M.

Fig. 5. The results show that [^{3}H]acetate uptake is strongly inhibited by HCO_3^- as well as by formate and the VFAs (acetate, propionate, butyrate). As in Fig. 5 there was limited cis inhibition by some of the test anions, suggesting a possible low-affinity exchange. No apparent exchange occurred involving gluconate-loaded vesicles, as shown by the consistently low measurements of [^{3}H]acetate uptake for these treatments. The combined results of Figs 3–6 indicate the presence of an anion exchange mechanism in the basolateral membrane of the tilapia intestine that is highly specific for HCO_3^-, formate and the volatile fatty acids.

Kinetic characteristics of [^{3}H]acetate transport

Carrier-mediated and diffusional influx kinetics of acetate were determined by computer fitting influx rates for 10 s to a Michaelis–Menten plus diffusion equation:

$$J_{oi} = (J_{max}[S])/(K_m + [S]) + P[S], \quad (1)$$

where J_{oi} is acetate influx rate, $[S]$ is external acetate concentration in mmol l^{-1}, J_{max} is the maximal rate of acetate influx, K_m is the concentration of acetate at which half J_{max} is attained, and P is the apparent diffusional coefficient.

Kinetic parameters for transmembrane [^{3}H]acetate transfer were determined by measuring 10-s influx as a function of increasing external concentration of acetate. Fig. 7 shows the effect of increasing acetate concentration (0.1–25 mmol l^{-1}) on

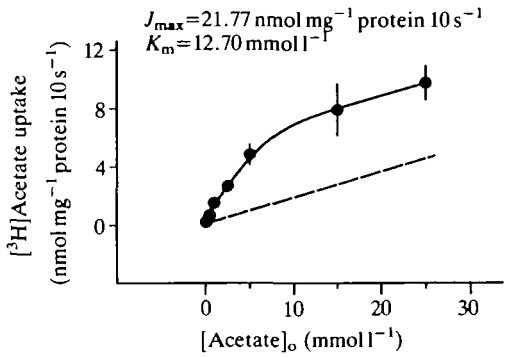

Fig. 7. Effect of external acetate concentration on [^{3}H]acetate influx (10 s) into tilapia BLMV. Vesicles were preloaded with 25 mmol l^{-1} sodium bicarbonate and 75 mmol l^{-1} sodium gluconate. External media contained n mmol l^{-1} [^{3}H]acetate and 100n mmol l^{-1} sodium gluconate. All media contain 12 mmol l^{-1} Hepes–Tris, pH 7.4, and 20 μmol l^{-1} CCCP. The dashed line indicates the rate of apparent diffusive entry of acetate (i.e. total uptake minus the carrier-mediated component). Data are presented as the mean of five 10-s replicates. Error bars indicate S.E.M. Kinetic parameters were determined from an iterative non-linear curve-fitting program.

the influx rate of [^{3}H]acetate. Influx values reflect total uptake minus a 0 s uptake measurement estimated as described above. The results indicate that acetate influx is a curvilinear function at low acetate concentrations and a linear function at higher concentrations. These findings are characteristic of a two-component transport system in which two independent processes are operating concurrently: a saturable Michaelis–Menten carrier and a diffusional process that has a rate directly proportional to the concentration. The kinetic parameters determined by iterative computer fitting were J_{max}=21.77±2.05 nmol mg^{-1} protein 10 s^{-1}, K_m= 12.70±2.95 mmol l^{-1} and P=0.17±0.02 nmol mg^{-1} protein 10 s^{-1} mmol^{-1} acetate.

Effect of intravesicular [HCO_3^-] on [^{3}H]acetate influx kinetics

[^{3}H]Acetate influx (2.5 mmol l^{-1}) was measured, during a 10 s exposure, into vesicles of varying internal HCO_3^- concentrations ([HCO_3^-]$_i$). Fig. 8 shows a hyperbolic curve for [^{3}H]acetate influx as a function of increasing [HCO_3^-]$_i$. The hyperbolic curve suggests that HCO_3^-/acetate exchange rate follows Michaelis–Menten kinetics according to the equation:

$$J_{oi} = (J_{max}[HCO_3^-]_i)/K_i + [HCO_3^-]_i, \quad (2)$$

where [HCO_3^-]$_i$ is the internal concentration of HCO_3^-, and K_i is the [HCO_3^-]$_i$ at which half-maximal acetate influx occurs. J_{oi} and J_{max} are defined above. At 0 mmol l^{-1} [HCO_3^-]$_i$ an [^{3}H]acetate influx rate of 0.24 nmol mg^{-1} protein 10 s^{-1} was observed, which probably represented diffusional entry of [^{3}H]acetate. The kinetic constants, determined by iterative computer fitting, were J_{max}= 2.11±0.13 nmol mg^{-1} protein 10 s^{-1} and K_m=0.41±0.02 mmol l^{-1}.

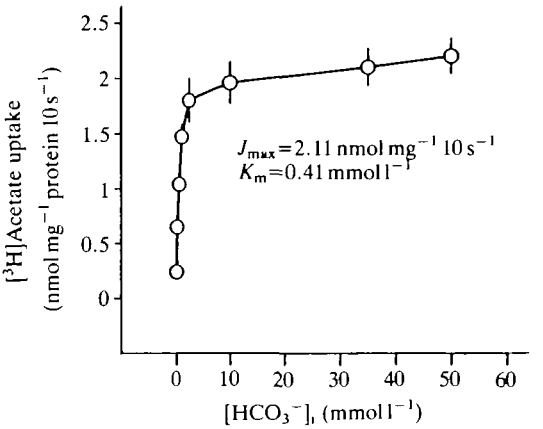

Fig. 8. Effect of internal bicarbonate concentration on 2.5 mmol l^{-1} [^{3}H]acetate influx (10 s) into tilapia BLMV. Vesicles were preloaded with n mmol l^{-1} sodium bicarbonate and 100n mmol l^{-1} sodium gluconate. External media contained 100 mmol l^{-1} sodium gluconate and 2.5 mmol l^{-1} [^{3}H]acetate. All media contained 12 mmol l^{-1} Hepes–Tris, pH 7.4, and 20 μmol l^{-1} CCCP. Data are presented as the mean of 5–10-s replicates. Error bars indicate S.E.M. Kinetic parameters were determined using an iterative non-linear curve-fitting program.

Transepithelial [^{3}H]acetate transport

Unidirectional (mucosa to serosa and serosa to mucosa) and net fluxes of [^{3}H]acetate were measured over time as a function of varying mucosal acetate concentrations (0.5–10 mmol l^{-1}) at a fixed serosal acetate concentration of 1.0 mmol l^{-1} (Table 1). Unidirectional transepithelial acetate fluxes were monitored at 5 min intervals over an incubation period of 75 min. Unidirectional mucosa-to-serosa flux increased proportionally with the mucosal acetate concentration, whereas unidirectional serosa-to-mucosa flux remained essentially unchanged over the range of mucosal acetate concentrations selected. Net flux ($J_{ms}-J_{sm}$) ranged from -0.36 nmol acetate cm^{-2} tissue min^{-1} (net secretion) at a mucosal acetate concentration of 0.5 mmol l^{-1} to 4.50 nmol acetate cm^{-2} tissue min^{-1} (net absorption) at a mucosal acetate concentration of 10 mmol l^{-1}. Fig. 9 is a plot of these net flux rates as a function of their corresponding mucosal acetate concentrations. Interpolation of this line ($y=0.52x-0.66$) to the point at which net acetate flux is equal to 0 nmol cm^{-2} tissue min^{-1} (equal flux in both directions) reveals that a mucosal concentration above 1.32 mmol l^{-1} will result in a net unidirectional transmural uptake of acetate across the epithelium, whereas a mucosal acetate concentration below 1.32 mmol l^{-1} will result in a net secretion of acetate.

The carbonic anhydrase inhibitor acetazolamide (1.0 mmol l^{-1}) was tested for its influence on net [^{3}H]acetate flux (Table 2). The experimental methods were the same as described above. Acetazolamide significantly inhibited unidirectional flux (J_{ms}) and net flux at mucosal acetate concentrations of 5.0 and 10.0 mmol l^{-1}

Table 1. *Unidirectional and net fluxes of [^{3}H]acetate across tilapia upper intestine*

Mucosal [acetate] (mmol l^{-1})	Transepithelial fluxes (nmol acetate cm^{-2} tissue min^{-1})		
	J_{ms}	J_{sm}*	J_{net}
0.5	0.16±0.06	0.51±0.22	−0.36±0.06
1.0	0.82±0.13	1.06±0.29	−0.25±0.18
2.5	1.75±0.51	1.02±0.20	0.72±0.31
5.0	2.28±0.78	0.41±0.12	1.87±0.68
10.0	5.37±0.72	0.87±0.16	4.50±0.54

Values are means±S.E.M. (N=3).
J_{ms}, flux from mucosa to serosa; J_{sm}, flux from serosa to mucosa; J_{net}, net flux ($J_{ms}-J_{sm}$).
* Serosal [acetate] fixed at 1.0 mmol l^{-1}.

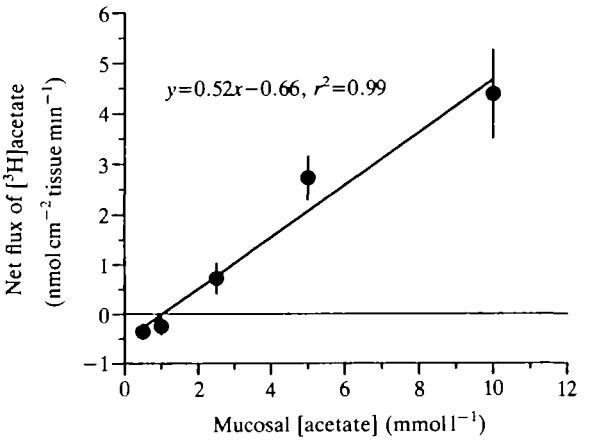

Fig. 9. Transepithelial net fluxes of [^{3}H]acetate as a function of mucosal acetate concentration. Mucosal acetate concentration ranged from 0.5 to 10.0 mmol l^{-1}. Serosal acetate concentration was 1.0 mmol l^{-1}. Net fluxes (J_{net}) were calculated as the difference ($J_{ms}-J_{sm}$) from Table 1. Error bars represent the standard error of the mean (N=4). The intercept of the curve (r=0.99) with y=0 represents the mucosal concentration at which no net flux occurs (1.32 mmol l^{-1}).

($P<0.05$). The inhibitory effect of acetazolamide is suggestive of a transmural acetate flux pathway which is partially dependent on the intracellular generation of bicarbonate by the carbonic-anhydrase-mediated hydration reaction of CO_2 and H_2O.

Blood bicarbonate determinations

Analysis of blood bicarbonate levels for the tilapia revealed P_{CO_2} values of 9.52±0.10 mmol l^{-1} (N=8). At the pH value of 7.6 determined for the blood (E. Titus and G. A. Ahearn, unpublished data) the fraction of P_{CO_2} in the form of HCO_3^- is more than 90%.

Table 2. *Effect of bilateral addition of 1.0 mmol l^{-1} acetazolamide on unidirectional fluxes of [^{3}H]acetate across upper intestine of tilapia*

Mucosal [acetate] (mmol l^{-1})	Transepithelial fluxes (nmol acetate cm^{-2} tissue min^{-1})		
	J_{ms}	J_{sm}*	J_{net}
Control			
1.0	0.82±0.13	1.06±0.29	−0.25±0.18
5.0	3.13±0.55	0.41±0.12	2.72±0.43
10.0	5.37±0.72	0.87±0.16	4.50±0.54
Plus acetazolamide			
1.0	0.35±0.08	0.40±0.04	−0.05±0.04
5.0	1.34±0.06	0.34±0.08	1.00±0.02†
10.0	2.62±0.22	0.63±0.23	1.99±0.01†

Values are means±S.E.M. (N=3).

J_{ms}, acetate flux from mucosa to serosa; J_{sm}, acetate flux from serosa to mucosa; J_{net}, net acetate flux ($J_{ms}-J_{sm}$).

* Serosal acetate concentration fixed at 1.0 mmol l^{-1}.

† Significantly different at $P<0.05$.

Discussion

Characteristics of basolateral anion antiport

The current study focuses on the basolateral step of transepithelial VFA transport across the upper intestine of a euryhaline teleost. This investigation complements our earlier study involving the brush-border step in VFA transepithelial transport in fish intestine (Titus and Ahearn, 1988). The two studies were performed using purified vesicles from the respective membranes with the intention of characterizing the transepithelial transport process for this group of organic solutes. This study also represents the first reported investigation of VFA transport across the basolateral membrane of any vertebrate intestinal epithelium.

As in the brush-border membrane vesicle (BBMV) study, the characteristics of VFA transport in basolateral membrane vesicles (BLMV) were investigated using labelled acetate as a test substrate. Because the majority of transporting BLMV produced by the method used in this study were oriented inside out (Reshkin and Ahearn, 1987), our transport data have been interpreted as representing transfer from cell to blood.

Fig. 1 illustrates initial rates of acetate uptake into basolateral membrane vesicles in the presence of a variety of cation gradients. The rates of [^{3}H]acetate entry into BLMV were not significantly different ($P<0.05$) in the presence of Na^+, K^+ or TMA^+ gradients, indicating no cation dependency. This is in agreement with the cation-independent acetate influx we found for the brush border (Titus and Ahearn, 1988). Sodium-independent, carrier-mediated acetate transport has

not been reported in any vertebrate species, although it has been observed in the cockroach hindgut and the earthworm integument (Richards and Arme, 1980; Hogan *et al.* 1985). Hagenbuch *et al.* (1985) reported sodium-independent antiport for a different anion, sulfate, in both renal and intestinal BLMV of rat, which parallels the situation we have described here for [^{3}H]acetate transport in BBMV and BLMV of the teleost intestine.

Fig. 2 shows the time course of [^{3}H]acetate uptake in BLMV preloaded with various anions as potential exchangers. Notable enhancements of [^{3}H]acetate influx occurred in vesicles preloaded with acetate (sixfold) and bicarbonate (twofold) when compared with vesicles preloaded with gluconate, a non-exchangeable compound. These results are similar to those found for anion exchange specificity in brush-border acetate transport; however, a longer time was required to attain a transient uptake overshoot in the BLMV (30 s) than in the BBMV (15 s).

The trans stimulation and cis inhibition experiments (Figs 3–6) enabled a thorough investigation of anion specificity for the exchange mechanism by a variety of inorganic and organic anions. Figs 3 and 4 show significantly enhanced uptake of [^{3}H]acetate into BLMV preloaded with bicarbonate and the C1–C4 configurations of volatile fatty acids. In addition, there was also an enhancement of [^{3}H]acetate uptake into vesicles preloaded with thiocyanate (SCN^-). Fig. 5 indicates inhibition of [^{3}H]acetate influx into vesicles by bicarbonate and acetate, but only limited inhibition by the other inorganic anions, including thiocyanate. Fig. 6 shows strong inhibition of [^{3}H]acetate influx by the C1–C4 VFAs, with either limited inhibition (pyruvate) or no inhibition by other cis-oriented organic anions. The general pattern for these four experiments indicates a specificity of the anion exchange mechanism for bicarbonate and volatile fatty acids. This carrier is qualitatively similar to that previously reported for the brush-border membrane (Titus and Ahearn, 1988), being cis inhibited and trans stimulated by the same substrates. One notable dissimilarity between the carriers of the two membranes lies in the apparently higher affinity of the basolateral carrier for formate than that exhibited by the brush-border carrier. A conclusion that can be made at this point is that the brush-border and basolateral membranes in teleost intestinal epithelium both contain a qualitatively similar anion exchange mechanism that is specific for bicarbonate and volatile fatty acids.

Anion exchange mechanisms have been suggested or reported for various epithelia in mammals and other vertebrates. Hugentobler and Meier (1986) described a multispecific anion exchanger for sulfate in rat liver BLMV where sulfate, thiosulfate, succinate and oxalate had both cis-inhibitory and trans-stimulatory effects on the influx of sulfate. However, they found no evidence for exchange in the presence of chloride, bicarbonate, acetate and a host of other organic anions.

Anion exchange mechanisms have been extensively investigated in rat renal cortex BLMV. Pritchard and Renfro (1983) described a mechanism for sulfate transport where sulfate uptake was stimulated by trans-oriented bicarbonate,

thiosulfate, hydroxyl and sulfate itself, the most effective of these anions being bicarbonate. Trans-oriented chloride and thiocyanate had no effect on sulfate uptake. Known inhibitors of anion transport, such as SITS and phloretin, significantly decreased bicarbonate-stimulated sulfate uptake, as did probenicid and the carbonic anhydrase inhibitor acetazolamide. A subsequent independent study by Low *et al.* (1984) expanded on the work of Pritchard and Renfro (1983) on sulfate transport in rat renal cortex BLMV. They described a common anion exchanger for sulfate which displayed reciprocating cis inhibition and trans stimulation of sulfate uptake by a variety of organic and inorganic anions. It was suggested that this antiport mechanism is available for anion secretion and reabsorption in the rat kidney proximal tubules.

In comparison with the studies in vertebrate kidney there has been relatively little investigation into anion exchange in the vertebrate intestine. Hagenbuch *et al.* (1985), in a direct comparison of basolateral sulfate transport between rat renal BLMV and jejunal BLMV, reported qualitatively different carriers in the two epithelia. Whereas sulfate influx in both preparations was trans stimulated by sulfate or hydroxyl ions, renal BLMV sulfate influx was also trans stimulated by bicarbonate, while intestinal BLMV sulfate transport was trans stimulated only by chloride. This suggests there are two qualitatively different antiporters for transport of the same anion in the two types of epithelia. The sulfate/chloride exchanger reported in the rat jejunal BLMV is similar to that found in rabbit ileal BLMV (Schron *et al.* 1987). This carrier was described as stimulating sulfate uptake by an outwardly directed chloride gradient. Sulfate uptake was inhibited competitively by cis-oriented oxalate, nitrate, iodide and bromide. Cis-oriented lactate, β-hydroxybutyrate, phosphate and para-aminohippurate (PAH) had no inhibitory effect on chloride-stimulated sulfate uptake. Interestingly, there was no experimental treatment presented testing the effect of bicarbonate on the transport of sulfate into the rabbit BLMV. Additionally, the anion exchange inhibitors DIDS and SITS had significant inhibitory effects on sulfate uptake. Another characteristic of the rabbit ileal sulfate transport system is the presence of a brush-border-localized carrier by which sulfate is exchanged for bicarbonate (Schron *et al.* 1987). Transepithelial sulfate transport in rabbit ileum, therefore, occurs by two qualitatively different anion exchangers working in conjunction to attain a net transmural flux. In contrast to these results in mammals, our findings suggest that transepithelial transport of acetate in fish intestine is accomplished by qualitatively similar anion exchangers, oriented at opposite poles of the cell, displaying quantitative differences in apparent substrate affinities.

Transepithelial VFA flux

Our previous characterization of the brush-border membrane VFA/bicarbonate exchanger (Titus and Ahearn, 1988) and the present description of qualitative and quantitative properties of VFA/bicarbonate exchange in the basolateral membrane provide the basis for a tentative model for transepithelial flux of VFA

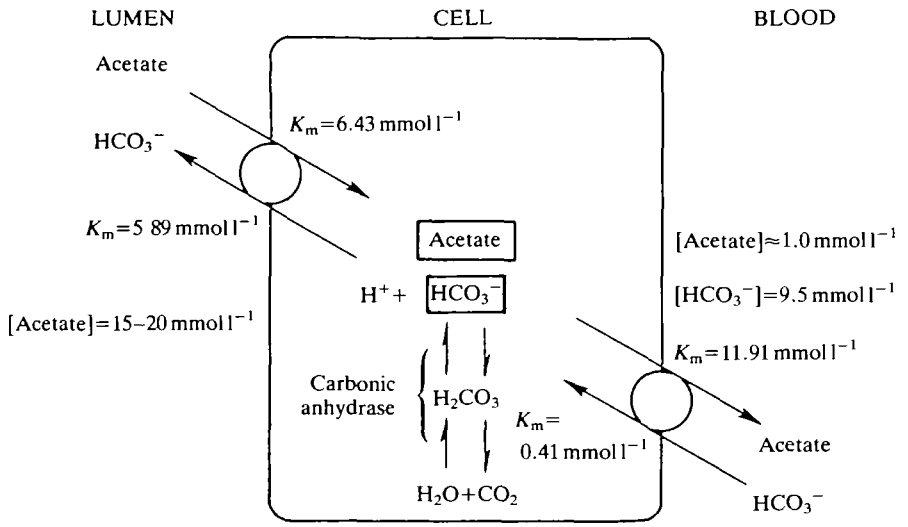

Fig. 10. Proposed model of transcellular acetate transport in the intestinal epithelium of a herbivorous teleost. Luminal acetate enters across the brush-border membrane, down a chemical gradient, in exchange for intracellular bicarbonate. At the basolateral membrane acetate leaves the cell by way of a low-affinity acetate/high-affinity bicarbonate exchanger which transports bicarbonate against a concentration gradient into the cell. Blood-to-cell bicarbonate flux provides a substrate for the brush-border acetate/bicarbonate antiport mechanism. Bicarbonate is also generated intracellularly by the carbonic-anhydrase-catalyzed hydration of carbon dioxide and water to provide additional substrate for the brush-border antiporter.

and bicarbonate in fish intestine (Fig. 10). The luminal acetate concentration, as reported in Titus and Ahearn (1988), is approximately 15–20 mmol l^{-1}. Although the acetate concentration in tilapia blood *in vivo* has not been determined, acetate concentrations of approximately 0.45 mmol l^{-1} in goat blood (de Jong, 1984) and 1.77 mmol l^{-1} in rabbit blood have been reported (Marty *et al.* 1985). The assumption for the present model is that blood acetate concentration in tilapia is similar to the values observed in other vertebrates.

Ruppin *et al.* (1980), using *in vivo* perfusions of the human colon, have proposed a model in which ionized short-chain fatty acids (SCFAs) exchange with intracellular bicarbonate. Once inside the cell, they suggest that the SCFAs are protonated and leave the serosal side by diffusion. We have shown that lumen-to-cell entry of acetate across the tilapia intestinal brush border occurs by way of anion exchange with intracellular bicarbonate (Titus and Ahearn, 1988). This is an electroneutral, one-for-one antiport mechanism specific for bicarbonate and the volatile fatty acids acetate, propionate and butyrate. The K_m for acetate of this

carrier is 6.43 mmol l^{-1} and the K_m for bicarbonate is approximately the same at 5.89 mmol l^{-1} (E. Titus and G. A. Ahearn, unpublished data).

The present study illustrates that a qualitatively similar anion exchange mechanism may facilitate the transport of intracellular acetate across the basolateral membrane in exchange for serosal bicarbonate. We propose that these two carriers work in conjunction to facilitate a net transmural uptake of acetate across the intestinal epithelium of this teleost. The kinetic parameters determined for [^{3}H]acetate uptake in the basolateral membrane indicate an apparently lower-affinity carrier (K_m=11.91 mmol l^{-1}) than that found for [^{3}H]acetate transport in the brush-border membrane. However, the affinity constant determined for [^{3}H]acetate influx as a function of varying bicarbonate concentration (Fig. 8) indicates an apparently high-affinity carrier (K_m=0.41 mmol l^{-1}) for bicarbonate on the basolateral membrane. The physiological concentrations of bicarbonate in the blood of tilapia have been determined to be approximately 9.5 mmol l^{-1}. The relatively large disparity in affinity constants for bicarbonate between the brush-border and basolateral membranes (approximately 14-fold) coupled with the steep lumen-to-blood acetate gradient (approximately 40-fold) suggest that the basolateral carrier transports bicarbonate against a concentration gradient in the blood-to-cell direction, in exchange for acetate moving down a transcellular concentration gradient in the opposite direction. The high-affinity binding properties of the basolateral carrier for bicarbonate (K_m=0.41 mmol l^{-1}) suggest a system in which acetate exchange at the basolateral site is initiated by bicarbonate binding, with the high-affinity binding site ensuring constant saturation by bicarbonate, thus enabling the downhill transmembrane flow of acetate to occur continuously.

To investigate the intracellular generation of bicarbonate as a possible driving force for the bilateral VFA/bicarbonate antiporters, the role of carbonic anhydrase was investigated using intact sheets of excised tilapia intestine mounted in Ussing flux chambers. Carbonic anhydrase, which catalyzes the hydration reaction:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+, \quad (3)$$

is commonly observed in vertebrates, and has been studied in renal and, to a lesser extent, intestinal systems. Its presence has been described in mouse gastric mucosa as well as in the stomach and intestine of the monkey (Wistrand, 1984). It has been experimentally localized in the rat small intestine, where it has been found to occur in the brush-border and basolateral membranes of the duodenum and ileum, as well as in the cytoplasm of the duodenum and the goblet cells of the jejunum. Additionally, carbonic anhydrase was found to occur in the basolateral membranes of the guinea pig duodenum (Sugai, 1984).

We observed significant decreases in unidirectional and net fluxes of acetate as the result of the addition of 1.0 mmol l^{-1} acetazolamide at mucosal acetate concentrations of 5.0 and 10.0 mmol l^{-1} (Table 2). This suggests that the action of carbonic anhydrase plays a significant role in acetate transport, probably by

providing the antiport proteins with bicarbonate from the hydration reaction. The proportion of unidirectional acetate flux that is not affected by the addition of acetazolamide is likely to be the sum of facilitated exchange involving extracellular bicarbonate, as provided by the blood *in vivo*, and passive or paracellular diffusion. There is an apparently lower proportional inhibition of net acetate flux by acetazolamide as mucosal acetate concentrations are increased from 5.0 to 10.0 mmol l^{-1}, suggesting a greater degree of passive acetate transport in the presence of higher acetate concentrations.

Table 1 illustrates that, in the presence of equal concentrations of mucosal and serosal acetate (1.0 mmol l^{-1}; also 10 mmol l^{-1}, E. Titus and G. A. Ahearn, unpublished data), there is a net secretory flux of [^{3}H]acetate. This same effect was found to be true for both acetate and propionate in the rabbit caecum (Hatch, 1987) where 20 mmol l^{-1} trans-oriented solute resulted in significant net secretion across intact caecal sheets mounted in Ussing chambers. However, as 10 mmol l^{-1} acetate in the blood of the teleost is not recognized as a physiologically realistic concentration, we chose 1.0 mmol l^{-1} as a representative serosal concentration to simulate the *in vivo* intestinal environment. The mucosal concentrations were varied over a range of 0.5–10 mmol l^{-1}, and net mucosa-to-serosa flux was found to occur at mucosal concentrations of acetate above 1.32 mmol l^{-1}. As mentioned, the acetate concentrations in the tilapia gut lumen were found to be 15–20 mmol l^{-1}, a range which would result in a significant net uptake of acetate.

In considering non-ionic diffusion of acetate, the pKa for acetate is 4.8, so that at physiological pH for the fish gut lumen (6.8–7.4) more than 99 % of the acetate available for uptake will be present in the ionic form. Non-ionic diffusion, therefore, does not figure significantly into this model at this physiological pH range. However, based on our reported P values for the brush-border and basolateral membranes from our vesicle studies, we have calculated relative diffusion rates for acetate concentrations of 15 and 5 mmol l^{-1}. For the brush border, diffusion accounts for 60 % of uptake at 15 mmol l^{-1} and 45 % at 5 mmol l^{-1}. These percentages approximate those Ruppin *et al.* (1980) reported for the short-chain fatty acids in human colon. They determined that 55 % of acetate and 35 % of propionate were taken up by means other than association with bicarbonate secretion into the lumen, presumably diffusion. For the basolateral membrane, we have determined that diffusion accounts for 21 % of total uptake at 15 mmol l^{-1} and 12 % at 5 mmol l^{-1}. This suggests that the anion exchange mechanism proposed here accounts for the majority of acetate uptake in the presence of relatively low luminal acetate concentrations and acts as an accessory mechanism for transcellular acetate transport in the presence of high luminal acetate concentrations.

The model we have presented here depicts a unique pathway for transmural uptake of acetate based on bilaterally oriented anionic exchangers, the intracellular action of carbonic anhydrase and the extracellular concentrations of the exchangeable substrates. This pathway is apparently specific for a class of compounds, the volatile fatty acids, that are normally generated by microbial

catabolic activity of plant matter, and also for bicarbonate, a metabolic waste product. The significance of this specific exchange pathway lies in its ability to transport and assimilate potentially nutritive anions in exchange for waste products, while expending little metabolic energy. This model further supports our earlier proposal that the teleost intestine is capable of plant fiber fermentation (Titus and Ahearn, 1988), a process that remains unsubstantiated in fish systems.

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