ELSEVIER

Biochimica et Biophysica Acta 1372 (1998) 244–254

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

# pH-dependent fluoride transport in intestinal brush border membrane vesicles

Han He [a], Vadivel Ganapathy [b], Carlos M. Isales [c], Gary M. Whitford [a,*]

[a] *Department of Oral Biology-Physiology, Medical College of Georgia, Augusta, GA 30912-1129, USA*
[b] *Biochemistry and Molecular Biology, Medical College of Georgia, Augusta, GA 30912-1129, USA*
[c] *Institute of Molecular Medicine and Genetics, Medical College of Georgia, Augusta, GA 30912-1129, USA*

Received 26 January 1998; accepted 3 April 1998

**Abstract**

Fluoride (F) absorption from the rat stomach and urinary bladder, hamster cheek pouch, and the renal tubules of several species are pH gradient-dependent. These observations led to the hypothesis that F crosses these epithelia in the form of the undissociated acid, HF. Several recent reports, however, have provided evidence that F absorption from the rat small intestine is insensitive to the lumenal pH. We report here our evidence that F uptake by rabbit intestinal brush border membrane vesicles (BBMV) occurred rapidly and with an overshoot only in the presence of an inward-directed proton gradient. In the absence of a proton gradient or in the presence of an outward-directed gradient, F uptake was slow and without an overshoot. In the presence of an inward-directed proton gradient, F uptake was partially inhibited by DIDS and DEP but not by diBAC. PCMBS inhibited F uptake by up to 83% in a dose-response manner. DiBAC appeared to reduce intravesicular pH slightly but the other reagents had no effect. When the uptake buffer contained chloride or nitrate, F uptake was partially inhibited compared to the mannitol or gluconate controls. It was concluded that F transport across the rabbit intestinal BBMV occurs via a carrier-mediated process which may involve cotransport of F with $H^+$ or exchange of F with $OH^-$. The inhibitory effects of DIDS, DEP and PCMBS may occur by affecting this carrier-mediated transport. 

*Keywords:* Fluoride; Absorption; pH gradient; 4,4′-Diisothiocyanostilbene-2,2′-disulfonate; Diethylpyrocarbonate; *p*-Chloromercuribenzene sulfonate

## 1. Introduction

Fluoride is absorbed from the oral cavity, stomach and small intestine [1]. Whitford and Pashley [2] found that the rate of fluoride absorption from the rat stomach was directly related to the acidity of the lumenal solution. The same relationship was noted in studies with the rat urinary bladder [3], hamster cheek pouch [4], and renal tubules of the rat [1,5], rabbit [6], dog [7], and human [8–10]. These findings formed the basis for the hypothesis that fluoride crosses epithelia in the form of the undissociated acid, HF ($pK_a = 3.4$). Messer and Nopakun confirmed the pH dependence of fluoride absorption

Abbreviations: BBMV, brush border membrane vesicles; BCECF acid, 2′,7′-bis(2-carboxyethyl)-5-(and 6-)-carboxyfluorescein; DEP, diethylpyrocarbonate; diBAC, bis(1,3-dibutylbarbituric acid)pentamethine oxonol; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; PCMBS, *p*-chloromercuribenzene sulfonate

* Corresponding author. Fax: (706) 7216252;
E-mail: gwhitfor@mail.mcg.edu

0005-2736/98/$19.00 
PII: S0005-2736(98)00064-9

from the stomach but reported [11–13] that the majority of fluoride absorption occurred from the proximal small intestine and was not pH-dependent. Their findings prompted the present study which further investigated the effects of pH and pH gradients on the intestinal transport of fluoride.

There are conflicting reports concerning the effects of the disulfonic stilbene, 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS), on the transmembrane or transepithelial transport of fluoride. DIDS is a potent inhibitor of the cell membrane band 3 protein anion exchanger. Whitford et al. [14] reported that DIDS almost completely inhibited the efflux of chloride from red blood cells but had little or no effect on the efflux of fluoride. Similarly, Rouch et al. [6] found no effect of DIDS on the transport of fluoride from the isolated, perfused cortical collecting duct of the rabbit. Melvin et al. [15], however, used rat submandibular gland cell aggregates and found that fluoride uptake was partially inhibited by both bumetanide, an inhibitor of the Na/K/Cl cotransporter, and DIDS. They concluded that fluoride movement occurred primarily via chloride pathways and that passive fluoride fluxes were of minor importance. Along with the lack of a pH gradient effect on the intestinal absorption of fluoride, these divergent results stemming from studies with DIDS and red blood cells, renal tubules and submandibular cells suggest the possibility that fluoride transport may share a common pathway with chloride that is peculiar to certain cells, particularly glandular cells.

In the present study, we investigated the effects of pH, pH gradients, DIDS, diethylpyrocarbonate (DEP), *p*-chloromercuribenzene sulfonate (PCMBS) and bis(1,3-dibutylbarbituric acid)pentamethine oxonol (diBAC) on fluoride uptake by rabbit small intestinal brush border membrane vesicles (BBMV). The results showed that fluoride uptake was dependent on an inward-directed pH gradient and that it was partially inhibited by DIDS, DEP and PCMBS.

## 2. Materials and methods

### 2.1. Preparation of BBMV

The BBMV were prepared from the small intestines of adult, female New Zealand white rabbits by $Mg^{2+}$ precipitation in the presence of EGTA [16]. After sacrifice of the animal with carbon dioxide, the proximal half of the small intestine was removed and rinsed with ice-cold 0.9% saline. The mucosa was gently scraped off and homogenized in 20 vols. (v/w) of ice-cold 75 mM mannitol, 5 mM EGTA, 20 mM Tris/NaOH buffer, pH 7.5, for 90 s using a INST Blender. One mole/l $MgCl_2$ was added to the homogenate to adjust the $MgCl_2$ concentration to 10 mM. After incubation for 30 min at 4°C, the mixture was centrifuged at $3000 \times g$ for 15 min. The supernatant fraction was then centrifuged at $60\,000 \times g$ for 30 min. The pelleted material containing the BBMV was suspended in preloading buffer, consisting of 320 mM mannitol, 20 mM HEPES/Tris pH 7.5. This suspension was again centrifuged at $60\,000 \times g$ for 30 min and the pellet was resuspended in the same buffer using a syringe with a 25-gauge needle. The protein concentration of the final brush border membrane preparation was determined [17] and adjusted to 10 mg/ml with preloading buffer. The BBMV were then ready for transport studies.

### 2.2. Transport study

The fluoride uptake measurements were carried out using a rapid filtration technique [16]. In general, uptake was initiated by addition of 40 μl of the membrane suspension containing 0.4 mg of membrane protein to 160 μl of uptake buffer containing a known concentration of NaF. The mixture was shaken gently at room temperature in a gyratory shaker. At the end of the incubation period, the uptake process was stopped by adding 5 ml of ice-cold 'stop' buffer which was filtered immediately through a Millipore filter (pore size, 0.65 μm). The filter was washed four times with 5 ml of stop buffer to remove the last traces of uptake buffer while retaining the vesicles. Finally, the filter was transferred to a petri dish for fluoride analysis using the ion-specific electrode after diffusion using the hexamethyldisiloxane (HMDS)-facilitated method [1]. The amounts of fluoride analyzed typically ranged from 1 to 3 nmoles which were within the linear portion of the standard curve. The coefficient variation for these amounts of fluoride is $< 7\%$. Appropriate stop buffers were used based on the design of the different studies. The up-

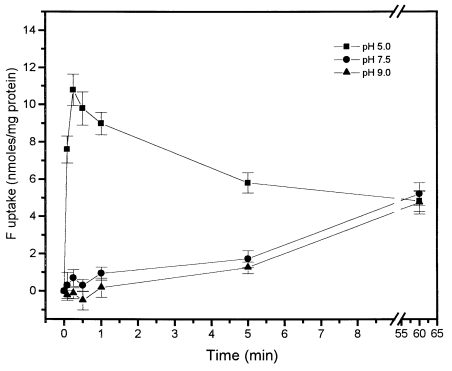


Fig. 1. Time course of fluoride uptake by small intestinal BBMV. BBMV were preloaded with 320 mM mannitol and 20 mM HEPES/Tris (pH 7.5). The uptake medium contained 150 mM NaCl, 10 mM NaF and 20 mM MES/Tris (pH 5.0), 20 mM HEPES/Tris (pH 7.5) or 20 mM Tris/HEPES (pH 9.0). Final pH values of the uptake media after mixing with the BBMV were 5.5, 7.5 and 8.5, respectively.

take rate for each time point or group was calculated from the analysis of BBMV on six separate filters.

The uptake buffers, each of which contained 10 mM NaF (8 mM NaF after mixing with the vesicle suspension), usually consisted of (1) 20 mM MES/Tris, 150 mM NaCl, pH 5.0, (2) 20 mM HEPES/Tris, 150 mM NaCl, pH 7.5, or (3) 20 mM Tris/HEPES, 150 mM NaCl, pH 9.0. Mannitol, KCl or other salts replaced NaCl in some experiments as required.

### 2.3. Measurement of intravesicular pH

The dissipation rate of the pH gradient across brush border membrane was estimated using a recording spectrofluorimeter and BCECF acid, a pH-sensitive fluorescent dye commonly used to estimate transmembrane pH gradients. Since BCECF acid does not cross biological membranes, the following procedure was used to load BCECF acid into the vesicles: the vesicles mixed with BCECF acid were frozen in liquid nitrogen for 20 min, then thawed with warm water. The freeze-thaw procedure was repeated two more times. It was assumed that during the freeze-thaw process, the vesicles would be broken and resealed so that BCECF acid would be loaded into the vesicles. We obtained good BCECF signals using this method. Extravesicular BCECF was removed by centrifugation at $60\,000\times g$ for 30 min. The pellets were resuspended in preloading buffer and centrifuged again. The resulting membrane pellets were washed twice with preloading buffer and finally suspended in preloading buffer. The membrane vesicles were then transferred to a cuvette to obtain a baseline fluorescence signal. DIDS, DEP, PCMBS or diBAC were then added to the cuvette at final concentrations of 5 mM, 1 mM, 1 mM and 50 μM, respectively. Fluorescence signals were measured with a dual-wavelength spectrofluorimeter (Photon Technologies International, South Brunswick, NJ) and using excitation wavelengths of 440 and 490 nm and an emission wavelength of 530 nm [18].

### 2.4. Materials

DIDS, DEP, PCMBS, MES, HEPES, Tris and valinomycin were purchased from Sigma (St. Louis, MO). BCECF and diBAC were obtained from Molecular Probes (Eugene, OR). All chemicals used were of analytical grade.

Table 1
Influence of pH per se and a transmembrane pH gradient on fluoride uptake in small intestinal BBMV

| pH | | | F uptake (nmoles/mg of protein/15 s) |
|---|---|---|---|
| Intravesicular pH | Extravesicular pH | Gradient | |
| 9.0 | 9.0 | 0 | −1.36 ± 0.25 |
| 7.5 | 7.5 | 0 | −0.09 ± 0.62 |
| 5.5 | 5.5 | 0 | 0.26 ± 0.47 |
| 7.5 | 5.5 | 2.0 | 4.59 ± 0.73* |

Membrane vesicles were preloaded with 320 mM mannitol and 20 mM HEPES/Tris (pH 7.5), 20 mM Tris/HEPES (pH 9.0) or 20 mM MES/Tris (pH 5.5). Uptake of fluoride (10 mM in uptake medium) was measured after a 15 s incubation. Extravesicular pH values are the final pH values of the uptake media after mixing the membrane vesicles with the uptake buffer.
*$P < 0.01$ compared to other three groups.

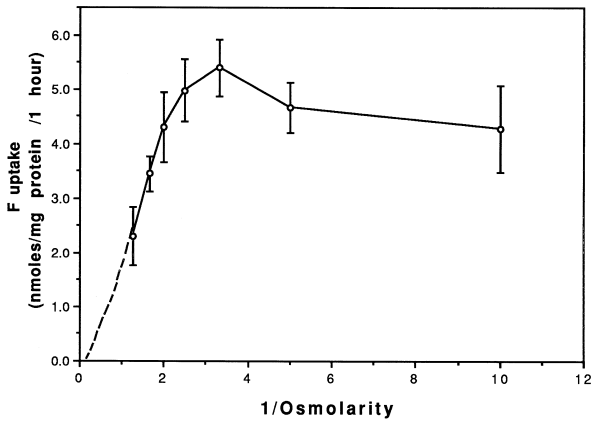


Fig. 2. Effect of medium osmolarity on fluoride uptake. BBMV were preloaded with 320 mM mannitol and 20 mM HEPES/Tris (pH 7.5). Membrane vesicles were incubated for 60 min at room temperature in uptake media containing 10 mM NaF, 20 mM MES/Tris (pH 5.5) and graded mannitol concentrations (100–800 mM).

### 2.5. Preparation of solutions

DIDS was dissolved in the preloading buffer and adjusted to 2 or 10 mM.

Fresh DEP stock solution (60 mM) was prepared just prior to use by mixing 0.1 ml of commercially available DEP solution (6.91 M) with 0.1 ml of ethanol and diluting the mixture with 11.3 ml of preloading buffer.

PCMBS solutions were made by dissolving the inhibitor in preloading buffer.

DiBAC was dissolved in dimethyl sulfoxide (DMSO) to 9.2 mM as stock solution.

2′,7′-bis(2-Carboxyethyl)-5-(and 6-)-carboxyfluorescein (BCECF) acid stock solution was prepared by dissolving 1 mg BCECF in 2 ml anhydrous DMSO. This stock solution was divided into small aliquots (200 μl/tube) and stored at −20°C.

Valinomycin was dissolved in ethanol.

### 2.6. Statistical analysis

Uptake measurements under each condition were usually done by analyzing the fluoride on each of six different filters. The results are expressed as the mean ± S.E. and analyzed for statistically significant differences using the unpaired Student's *t*-test or analysis of variance and Fisher's PLSD post hoc test. An α-value of 0.05 was selected a priori as the indicator for significance.

## 3. Results

### 3.1. Time course of fluoride uptake

The time course of fluoride uptake by BBMV was studied under three different experimental conditions: (1) in the presence of an inward-directed $H^+$ gradient ($pH_i = 7.5$, $pH_o = 5.0$); (2) in the absence of $H^+$ gradient ($pH_i = pH_o = 7.5$); and (3) in the presence of an outward-directed $H^+$ gradient ($pH_i = 7.5$, $pH_o = 9.0$). The uptake of fluoride was rapid in the presence of an inward-directed $H^+$ gradient and it exhibited the overshoot phenomenon, indicating transient concentrative accumulation of fluoride inside the vesicles (Fig. 1). The uptake peaked at 15 s and then decreased toward an apparent equilibrium or steady state concentration at 60 min. The intravesicular fluoride concentration at the overshoot was 2.3 times the equilibrium concentration observed at

Table 2
The effect of DIDS and DEP on fluoride uptake in small intestinal BBMV

| Group | F uptake (nmoles/mg of protein/15 s) | % Reduction |
|---|---|---|
| Ethanol control | 6.42 ± 0.47 | 0 |
| DIDS (10 mM) | 3.82 ± 0.30* | 40 |
| DIDS (2 mM) | 5.27 ± 0.30* | 18 |
| DEP (5 mM) | 3.89 ± 0.55* | 39 |
| DEP (1 mM) | 3.74 ± 0.08* | 42 |
| DIDS (2 mM)+DEP (1 mM) | 3.22 ± 0.29* | 50 |

Membrane vesicles were preloaded with 320 mM mannitol and 20 mM HEPES/Tris (pH 7.5) and treated with DIDS for 60 min or DEP for 10 min. Uptake of fluoride was measured after 15 s. The uptake buffer contained 20 mM MES/Tris (pH 5.0), 150 mM NaCl and 10 mM NaF.

*$P < 0.05$ compared with the ethanol control group.

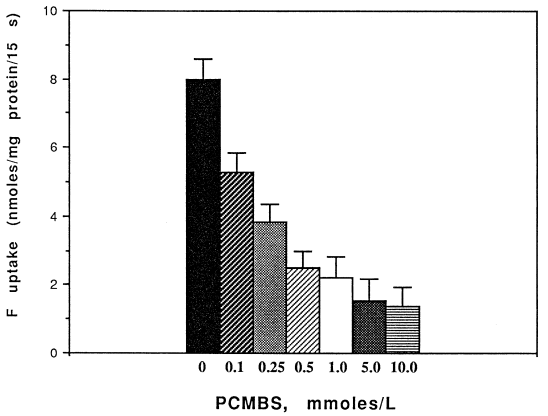


Fig. 3. Effect of PCMBS on fluoride uptake in the presence of pH gradient ($pH_i = 7.5$, $pH_o = 5.5$). Membrane vesicles prepared from small intestine were treated with PCMBS for 30 min at room temperature before adding the uptake buffer.

1 h. In the absence of a $H^+$ gradient or the presence of an outward-directed $H^+$ gradient, fluoride uptake was slow, did not demonstrate an overshoot, and reached an equilibrium or steady state concentration at some time between 5 and 60 min.

### 3.2. Influence of pH and transmembrane pH gradients on fluoride uptake

In order to obtain specific information on the role of $H^+$ concentrations per se, fluoride uptake was compared in the presence and absence of a transmembrane proton gradient. The fluoride uptake rates were determined after adjusting the pH of both the intravesicular and extravesicular solutions to the same values (pH = 9.0, 7.5 or 5.5). As shown in Table 1, the uptake rates under these conditions were close to zero and not significantly different from each other. The rate of uptake in the presence of an inward-directed $H^+$ gradient ($pH_i = 7.5$, $pH_o = 5.5$) was greater than the rates in the absence of an inward-directed $H^+$ gradient ($P < 0.01$). The results indicated that $pH_i$ or $pH_o$ had little effect on fluoride uptake when there was no pH gradient.

### 3.3. Effect of medium osmolarity on fluoride uptake

The effect of medium osmolarity on fluoride uptake by intestinal BBMV was studied by measuring the uptake of fluoride after 60 min incubations. The extravesicular medium osmolarity was varied by changing mannitol concentrations. A 60 min incubation period was chosen because the uptake of fluoride into the vesicles reaches an apparent equilibrium or steady state distribution after that amount of time (Fig. 1). Increasing concentrations of mannitol, to which the vesicles are impermeant, were correlated with decreasing uptakes of fluoride (Fig. 2). Extrapolation to infinite medium osmolarity (zero intravesicular space) indicated no uptake. Thus, fluoride uptake was completely due to transport into intravesicular fluid space rather than binding to the membrane.

### 3.4. Effects of DIDS and DEP on fluoride uptake

In these experiments, there was an inward-directed $H^+$ gradient ($pH_i = 7.5$, $pH_o = 5.5$). The vesicles were pretreated with DIDS and DEP for 60 and 10 min, respectively. The results (Table 2) showed that uptake of fluoride into vesicles treated with 2 mM or 10 mM DIDS decreased 18 and 40% compared with control group. Fluoride uptake into vesicles treated with 1 mM or 5 mM DEP decreased 39 and 42% compared with control group. The fluoride uptake was decreased by 50% when the vesicles were treated with a combination of 2 mM DIDS and 1 mM DEP. Uptake by each of the treatment groups was significantly lower than that of the control group.

### 3.5. Effect of PCMBS on fluoride uptake

Small intestinal BBMV were treated with different concentrations of PCMBS for 30 min and then washed free of the reagent. Fluoride uptake was measured in the presence of a $H^+$ gradient ($pH_i = 7.5$, $pH_o = 5.5$). The uptake was inhibited by PCMBS in a dose-dependent manner (Fig. 3). The rate of fluoride uptake was inhibited by 34% when the PCMBS concentration was 0.1 mM and by 81% when the PCMBS concentration was 5.0 mM. The inhibition of uptake when the PCMBS concentration was 10.0 mM was not significantly different from that at 5.0 mM.

### 3.6. Effect of diBAC on fluoride uptake

The BBMV were treated on ice with 1, 10, and 50 μM diBAC for 10 min. The excess unreacted re-

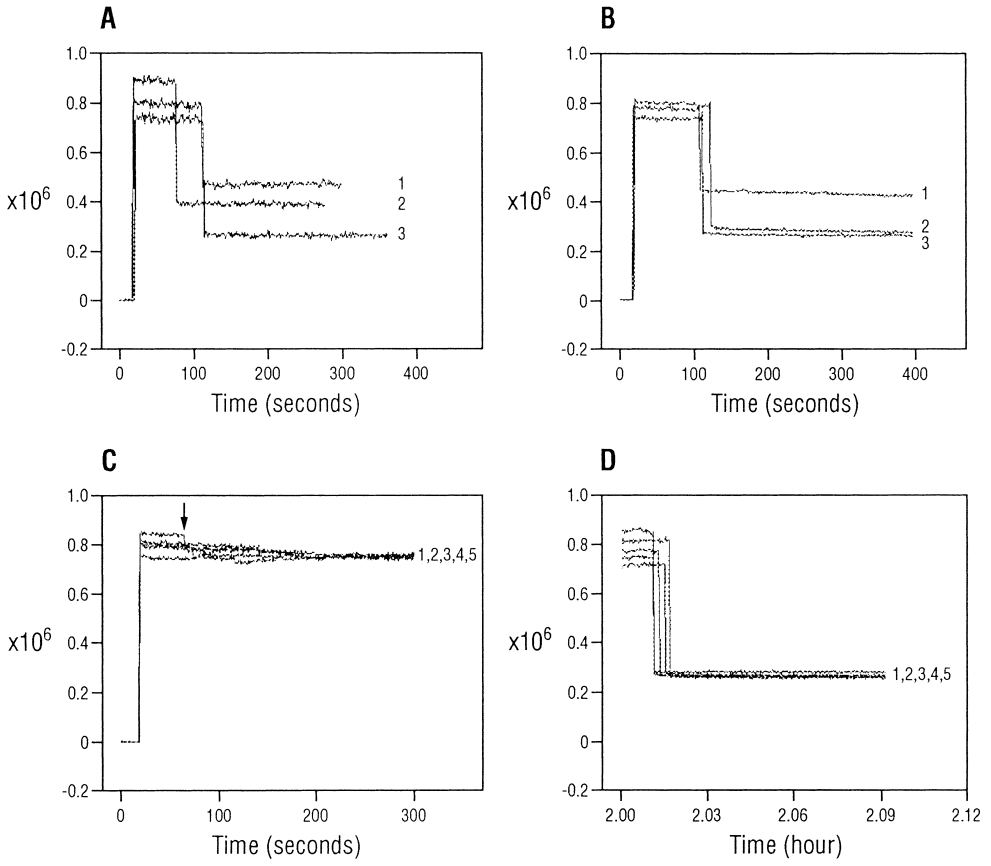


Fig. 4. Effects of DIDS, DEP, PCMBS and diBAC on the transmembrane pH gradient. BBMV were preloaded with 320 mM mannitol and 20 mM HEPES/Tris (pH 7.5). The vesicles were then loaded with the pH-sensitive dye BCECF (96 μM) and placed in a cuvette. The membrane vesicles were then exposed to pH 5.0 uptake buffer (10 mM NaF, 20 mM MES/Tris, 150 mM NaCl). Fluorescence signals were measured with a dual-wavelength spectrofluorimeter using excitation wavelengths of 440 and 490 nm and an emission wavelength of 530 nm. Panel A shows the standard curve. Tracks 1, 2 and 3 represent the pH 7.5, 6.0 and 5.0 standard, respectively. In panel B, the drop of fluorescence signal of track 1 is the dilution effect caused by adding 1.0 ml of preloading buffer (pH 7.5) to 1.0 ml of the BBMV suspension (pH 7.5). Tracks 2 and 3 show the effects of fluoride and non-fluoride uptake buffers (pH 5.0) on the fluorescence signal. Panel C shows the effects of DIDS, DEP, PCMBS and diBAC (20 μl of inhibitor solution was added to the cuvettes at arrow) on the fluorescence signal. Two hours later (D), the same vesicles were exposed to pH 5.0 uptake buffer containing 10 mM fluoride.

agent was separated from the BBMV after centrifugation at $60\,000 \times g$ for 30 min. Measurements of fluoride uptake were carried out in the presence of an inward-directed $H^+$ gradient ($pH_i = 7.5$, $pH_o = 5.5$). The results indicated that diBAC had no effect on F uptake (data not shown).

### 3.7. *Effect of DIDS, DEP, PCMBS and diBAC on transmembrane pH gradient*

To test the possibility that the inhibitors may have reduced fluoride uptake by reducing the transmembrane $H^+$ gradient, the effects of DIDS, DEP, PCMBS and diBAC on transmembrane pH gradient were done. The membrane vesicles were loaded with BCECF and placed in cuvettes to determine the baseline signals. DIDS, DEP, PCMBS and diBAC were then added separately to the cuvettes to achieve final concentrations of 5 mM, 1 mM, 1 mM and 50 μM, respectively. The results are shown in Fig. 4.

Fig. 4A shows standard curve. BBMV were added to buffers at pH 7.5 (track 1), 6.0 (track 2) or 5.0 (track 3). Fig. 4B shows that fluoride in the uptake buffer had no effect on the fluorescence signal compared to uptake buffer without fluoride. Fig. 4C and D show the virtual lack of effects of the inhibitory

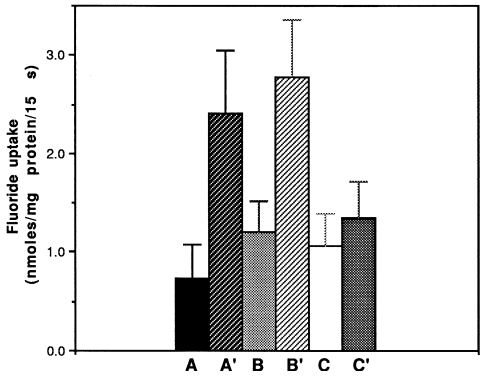


Fig. 5. Effect of an inwardly redirected $K^+$ diffusion potential on fluoride uptake. BBMV were preincubated as follows: A, 20 mM HEPES/TMA-OH (tetramethylammonium hydroxide), 100 mM potassium gluconate, 120 mM mannitol and valinomycin (no $K^+$ gradient); A′, 20 mM HEPES/TMA-OH, 320 mM mannitol and valinomycin ($K^+$ gradient); B, 20 mM HEPES/TMA-OH, 100 mM potassium gluconate, 120 mM mannitol, 2 mM DIDS and valinomycin (DIDS without $K^+$ gradient); B′, 20 mM HEPES/TMA-OH, 320 mM mannitol, 2 mM DIDS and valinomycin (DIDS with $K^+$ gradient); C, 20 mM HEPES/TMA-OH, 100 mM potassium gluconate, 120 mM mannitol (no $K^+$ gradient or valinomycin); C′, 20 mM HEPES/TMA-OH and 320 mM mannitol ($K^+$ gradient without valinomycin). Valinomycin was added to the vesicle suspension (10 μg/mg protein). The composition of the uptake buffers for all groups was the same: 20 mM HEPES/TMA-OH, 10 mM NaF, 100 mM potassium gluconate and 100 mM mannitol. There was no pH gradient in any of these experiments.

agents on the fluorescence signal. The signal of the diBAC group was slightly reduced when diBAC was added to the cuvette (Fig. 4C, track 1) and continued declining for 2 h (Fig. 4D, track 5). The signals of DIDS, DEP and PCMBS cuvettes were unchanged or only slightly decreased. The magnitude of fluorescence change induced by adding pH 5.0 uptake buffer after 2 h was similar in the control and DIDS-, DEP-, PCMBS- and diBAC-treated membrane vesicles (Fig. 4D). These results indicated that diBAC slightly reduced intravesicular pH prior to adding pH 5.0 uptake buffer and that DIDS, DEP and PCMBS had little or no effect on intravesicular pH prior to adding pH 5.0 uptake buffer, and did not facilitate dissipation rate of imposed pH gradient.

### 3.8. $F^-$-$Cl^-$ and $F^-$−$HCO_3^-$ exchange studies

To determine whether an anion exchange pathway for fluoride was present in the BBMV, two anions ($Cl^-$ and $HCO_3^-$ added as sodium salts) that might exchange with fluoride were tested in the absence of a pH gradient ($pH_i = pH_o = 7.5$). An outward $Cl^-$ or $HCO_3^-$ gradient (50 mM) did not accelerate fluoride influx (data not shown), indicating that there was no $F^-$-$Cl^-$ or $F^-$−$HCO_3^-$ exchange.

### 3.9. Fluoride conductance pathway study

To test for the presence of a membrane potential-sensitive transport of fluoride ion in the BBMV, fluoride uptake was measured in response to a $K^+$ diffusion potential induced by valinomycin which increases the $K^+$ conductance of biological membranes. The experiments were carried out in the absence of a transmembrane pH gradient ($pH_i = pH_o = 7.5$).

As shown in Fig. 5, fluoride uptake by the BBMV

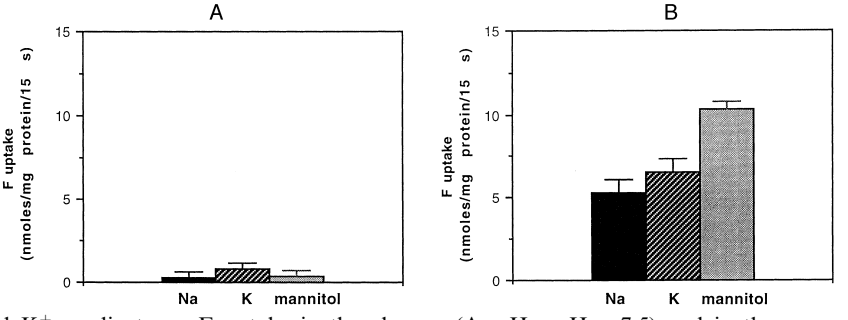


Fig. 6. Effect of $Na^+$ and $K^+$ gradients on F uptake in the absence (A, $pH_i = pH_o = 7.5$) and in the presence of a pH gradient (B, $pH_i = 7.5$, $pH_o = 5.5$). BBMV were preloaded with 320 mM mannitol and 20 mM HEPES/Tris (pH 7.5). (A, no pH gradient) The uptake medium consisted of 10 mM NaF, 20 mM HEPES/Tris (pH 7.5), and 300 mM mannitol or 150 mM NaCl or 150 mM KCl. (B, pH gradient) The uptake medium consisted of 10 mM NaF, 20 mM MES/Tris (pH 5.5), and 300 mM mannitol or 150 mM NaCl or 150 mM KCl.

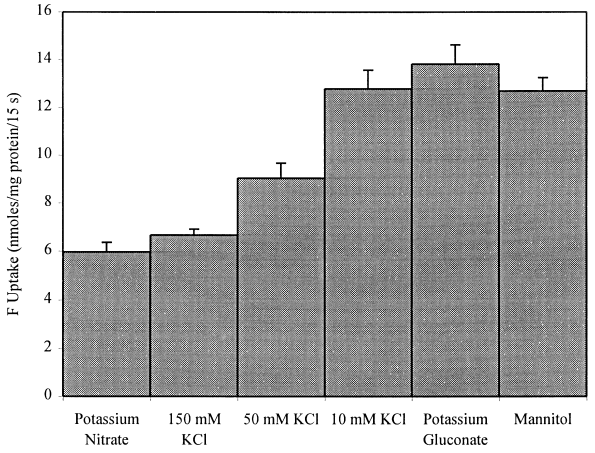


Fig. 7. Effect of $Cl^-$ and $NO_3^-$ on F uptake in the presence of a pH gradient ($pH_i = 7.5$, $pH_o = 5.5$). BBMV were preloaded with 320 mM mannitol and 20 mM HEPES/Tris (pH 7.5). The uptake media consisted of 10 mM NaF, 20 mM HEPES/Tris (pH 7.5), and 300 mM mannitol, 150 mM potassium gluconate, 150 mM $KNO_3$, or 10, 50 or 150 mM KCl. Potassium gluconate was added where necessary to achieve the same osmolarities among the uptake solutions.

in the presence of an inward-directed potassium gradient (group A′) was higher than that in the absence of a potassium gradient (group A, $P < 0.05$). Since the uptake of group B′ was higher than that of group B ($P < 0.05$), the increased uptake (group B′) due to an inward potassium gradient was not inhibited by 5 mM DIDS. Comparing group C to A, we found that valinomycin itself had no effect on fluoride uptake ($P > 0.05$). Comparing group C′ to C, we found that potassium gradient itself did not increase fluoride uptake ($P > 0.05$).

### 3.10. Effect of a sodium or potassium gradient on fluoride uptake

The effects of a $Na^+$ or $K^+$ gradient on F uptake by the BBMV were examined in the presence and absence of a pH gradient (Fig. 6). Under pH gradient conditions (Fig. 6B, $pH_i = 7.5$, $pH_o = 5.5$), uptake by the $Na^+$, $K^+$ or mannitol groups was higher than that of the corresponding groups in the absence of a pH gradient shown in Fig. 6A ($P < 0.01$). Fluoride uptake in BBMV in the $Na^+$ and $K^+$ groups were not different with statistical significance ($P > 0.05$), but fluoride uptake in the mannitol group was higher than that of $Na^+$ and $K^+$ groups ($P < 0.01$). This may have been due to chloride in the $Na^+$ and $K^+$ groups competing with fluoride for the same transport system. In the absence of a pH gradient (Fig. 6A, $pH_i = pH_o = 7.5$), uptake values were lower and were not different with statistical significance ($P > 0.05$). The results showed that fluoride uptake was pH-dependent but not $Na^+$- or $K^+$-dependent.

### 3.11. Effect of $NO_3^-$ and $Cl^-$ on fluoride uptake

To further examine the possibility that chloride competes with fluoride for the same transport system, chloride and nitrate were tested for the ability to interfere with the uptake of fluoride in the BBMV. The experiments were done in the presence of an inward-directed proton gradient ($pH_i = 7.5$, $pH_o = 5.5$). The uptake buffers contained mannitol, potassium gluconate, different chloride concentrations (10, 50 and 150 mM KCl) or 150 mM $KNO_3$ and 10 mM sodium fluoride. Fig. 7 shows that the uptake of fluoride was inhibited by 150 mM $NO_3^-$ and by 50 and 150 mM $Cl^-$ compared to mannitol or gluconate control ($P < 0.05$). This suggested that $NO_3^-$ and $Cl^-$ can compete with fluoride for the same transport system.

## 4. Discussion

Our data show that a proton gradient is required for fluoride transport into the rabbit small intestinal BBMV. In the presence of a proton gradient (outside > inside), fluoride rapidly crossed the membranes and transiently accumulated within the vesicles with an overshoot concentration that was more than twice the equilibrium or steady state concentration. The reduced uptake after 15 s appeared to be due to the subsequent fluoride efflux from the vesicles due to the progressive collapse of the pH gradient. Whitford et al. [19] reported a similar pH-dependent uptake and overshoot in their study of fluoride uptake by *Streptococcus mutans* 6715.

On the other hand, fluoride uptake in the absence of an $H^+$ gradient or in the presence of an outward-directed $H^+$ gradient was very slow and did not demonstrate an overshoot. Instead, fluoride uptake increased slowly and reached what appeared to be an

equilibrium value sometime between 5 and 60 min. The equilibrium concentrations of fluoride were nearly identical (Fig. 1), suggesting that the observed differences in the initial uptake rates were not due to pH-induced alterations in the volume or integrity of the vesicles.

We evaluated the role of $H^+$ concentration on BBMV fluoride uptake (Table 1). In the absence of a proton gradient, the initial uptake rates of fluoride in three different proton concentration groups (pH 5.5, 7.5 and 9.0) were not significantly different. This indicated that it was the transmembrane proton gradient, not the internal or external $H^+$ concentration per se, that influenced fluoride transport.

DIDS has been found to covalently link to the band 3 protein, an anion transport system [20]. DIDS can also inhibit $Cl^- - HCO_3^-$ exchange [21,22], $SO_4^- - OH^-$ [23], and Cl-oxalate exchange [24,25]. Studies have shown that DIDS has no effect on fluoride efflux from red blood cells [14] nor fluoride reabsorption from the cortical collecting duct of the rabbit [6]. Uptake by rabbit submandibular cells, however, was partially inhibited by DIDS [15]. In the present study, 10 mM DIDS inhibited fluoride uptake by intestinal BBMV by 40% (Table 2). This suggests that fluoride transport in these glandular systems involves a transport protein which is sensitive to DIDS.

DEP, a histidine-specific reactive reagent, has been used to characterize proton-coupled transport systems. Histidyl residues have been shown to be essential for the function of many transport systems that are driven by $H^+$ gradients. This includes the transport systems responsible for the uptake of lactose, lactate and proline into *Escherichia coli* membrane vesicles [26], the uptake of $Na^+$ into renal BBMV [27] and *E. coli* membrane vesicles [28], the uptake of peptide into renal BBMV [29] and the uptake of cephradine via dipeptide carriers into small intestinal BBMV [30]. The uptake of fluoride was inhibited by DEP in the presence of a proton gradient (Table 2). These results, like those from the studies with DIDS, provide evidence that proton gradient-dependent fluoride transport can use a transport protein in which histidyl residues play an important role.

PCMBS inhibits the uptake of *p*-aminohippurate [31], tetraethylammonium in renal brush border membrane vesicles [32] and the uptake of tetraethylammonium in rat renal basolateral membrane [33]. In addition to that, PCMBS also inhibits membrane water channels [34,35]. In the present studies, fluoride uptake was inhibited by PCMBS and the inhibition was dose-dependent (Fig. 3). This indicated that PCMBS is an important inhibitor of fluoride transport and that sulfhydryl groups are involved in fluoride transport system.

Grillo and Aronson [27], Sokol et al. [36] and Kato et al. [30] indirectly demonstrated that DEP did not facilitate dissipation of the $H^+$ gradient. Hori et al. [32] concluded that PCMBS had no effect on the dissipation rate of the $H^+$ gradient in BBMV. Kato et al. [30] found that the uptake of cephradine into intestinal BBMV was inhibited by DEP but was not inhibited by PCMBS in the presence of a proton gradient. These results indicated that histidyl groups, not sulfhydryl groups, are essential for the transport of cephradine and indirectly demonstrated that PCMBS did not facilitate dissipation of a $H^+$ gradient.

DiBAC slightly reduced intravesicular pH prior to adding pH 5.0 uptake buffer (Fig. 4). DIDS, DEP and PCMBS had little or no effect on intravesicular pH prior to adding pH 5.0 uptake buffer and did not facilitate dissipation of the imposed pH gradient, consistent with previous reports. Thus, it is likely that the inhibitions of proton gradient-dependent fluoride transport by DIDS, DEP and PCMBS were due to specific interactions with one or several transporters, rather than due to the decrease of the intravesicular space or to the collapse of the proton gradient.

DiBAC is the most potent known inhibitor of band 3-mediated anion exchange in the red blood cell [37]. Under our experimental conditions, fluoride transport was inhibited by DIDS but not by diBAC. These results suggest that DIDS and diBAC may modify different parts of the band 3 protein or modify two different anion exchanger proteins. Other interpretations of these discrepant results are species differences (rabbit vs. humans) or different properties of the human erythrocytic anion exchanger and that of the brush border membranes used in the present studies. Further studies are needed to clarify this subject.

We found (Fig. 5) that the fluoride uptake rate in

the $K^+$ diffusion potential groups (A′, B′) were slightly higher than occurred in the absence of a $K^+$ diffusion potential (groups A, B, C, C′). The increased uptake rate was not inhibited by DIDS. This may indicate that an inwardly directed $K^+$ diffusion potential in small intestinal BBMV provides the energy for fluoride uptake by establishing an inside-positive potential. This conductance pathway appears to exist separately from the $F^-$-$H^+$ cotransporter (or $F^-$-$OH^-$ exchanger), since the $F^-$-$H^+$ cotransporter (or $F^-$-$OH^-$ exchanger) was proton gradient-dependent and was partially inhibited by DIDS, DEP and PCMBS.

The transport systems for many amino acids and sugars are sodium-dependent [38]. The results of our studies of the effects of a sodium or potassium gradient on fluoride uptake by the BBMV showed that the process was $Na^+$- or $K^+$-independent. However, our results demonstrated that high concentrations of chloride and nitrate can compete with fluoride for the same transport system(s) in small intestinal BBMV (Fig. 7). These data suggest that fluoride, chloride and nitrate can share the same transport system. It is possible, however, that the rapid influx of these ions may have interfered with fluoride uptake via a conductance pathway.

Our findings suggest that fluoride absorption from the intact rabbit intestine should be related to the transmucosal pH gradient. In their studies of fluoride absorption from the rat intestine, however, Messer and Nopakun found no such relationship. The reason for this discrepancy is not clear but may be related to species-specific transport mechanisms.

Taken together, the experimental evidence from our studies suggests that there are several pH gradient-dependent, carrier-mediated mechanisms for fluoride transport into rabbit small intestinal BBMV. One apparent mechanism is not inhibited by DIDS, DEP or PCMBS and may involve fluoride uptake in the form of HF by diffusion. Other mechanisms are inhibitable and sensitive to DIDS, DEP and PCMBS. In these cases, fluoride appears to cross the brush border membrane by a $F^-$-$H^+$ cotransporter or $F^-$-$OH^-$ exchangers but only in the presence of an inward-directed proton gradient. Sulfhydryl and histidyl groups appear to play important roles in this transport system.

## Acknowledgements

This work was supported by research grants DE-06429 and DE-06113 from the National Institute of Dental Research, National Institutes of Health, Bethsda, MD.

## References

[1] G.M. Whitford, The Metabolism and Toxicity of Fluoride, H.M. Myers (Ed.), S. Karger, Basel, 1996, pp. 10–18.
[2] G.M. Whitford, D.H. Pashley, Calcif. Tissue Int. 36 (1984) 302–307.
[3] G.M. Whitford, D.H. Pashley, K.E. Reynolds, Am. J. Physiol. 232 (1977) F10–F15.
[4] G.M. Whitford, R.S. Callan, H.S. Wang, J. Appl. Toxicol. 2 (1982) 303–306.
[5] G.M. Whitford, D.H. Pashley, G.I. Stringer, Am. J. Physiol. 230 (1976) 527–532.
[6] A.J. Rouch, G.M. Whitford, H.T. Campbell, Kidney Int. 41 (1992) 342–349.
[7] G.M. Whitford, D.H. Pashley, Proc. Soc. Exp. Biol. Med. 196 (1991) 178–183.
[8] J. Ekstrand, M. Ehrnebo, G.M. Whitford, P.O. Jarnberg, Eur. J. Clin. Pharmacol. 18 (1980) 189–194.
[9] J. Ekstrand, C.J. Spak, M. Ehrnebo, Acta Pharmacol. Toxicol. 50 (1982) 321–325.
[10] P.O. Jarnsberg, J. Ekstrand, L. Irestedt, Anesthesiology 54 (1981) 48–52.
[11] J. Nopakun, H.H. Messer, V. Voller, J. Nutr. 119 (1989) 1411–1417.
[12] J. Nopakun, H.H. Messer, Nutr. Res. 10 (1990) 771–780.
[13] H.H. Messer, R.H. Ophaug, J. Dent. Res. 72 (1993) 619–622.
[14] G.M. Whitford, N.B. Allison, D.E. Pearson, J. Dent. Res. 64 (Abst. 400) (1985) 219.
[15] J.E. Melvin, W.H. Bowen, R.J. Turner, J. Dent. Res. 69 (Abst. 1) (1990) 824.
[16] C. Tiruppathi, Y. Miyamoto, V. Ganapathy, F.H. Leibach, Biochem. Pharmacol. 37 (1988) 1399–1405.
[17] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265–275.
[18] J. Gasalla-Herraiz, S. Rhee, C.M. Isales, Biochem. Biophys. Res. Commun. 214 (1995) 373–388.
[19] G.M. Whitford, G.S. Schuster, D.H. Pashley, P. Venkateswarlu, Infect. Immun. 18 (1977) 680–687.
[20] Z.I. Cabantchik, A. Rothstein, J. Membrane Biol. 15 (1974) 207–226.
[21] R. Knickelbein, P.S. Aronson, C.M. Schron, J. Seifter, J.W. Dobbins, Am. J. Physiol. 249 (1985) G236–G245.
[22] G. Lamprecht, U. Seidler, M. Classen, Am. J. Physiol. 265 (1993) G903–G910.

[23] C.M. Schron, R. Knickelbein, P.S. Aronson, Am. J. Physiol. 249 (1985) G607–G613.
[24] R. Knickelbein, P.S. Aronson, J.D. Dobbins, J. Membrane Biol. 88 (1985) 199–204.
[25] K.R. McConnell, P.S. Aronson, J. Biol. Chem. 269 (1994) 21489–21494.
[26] E. Padan, L. Patel, H.R. Kaback, Proc. Natl. Acad. Sci. USA 76 (1979) 6221–6225.
[27] F.G. Grillo, P.S. Aronson, J. Biol. Chem. 261 (1986) 1120–1125.
[28] E. Damiano, M. Bassilana, G. Leblanc, Eur. J. Biochem. 148 (1985) 183–188.
[29] Y. Miyamoto, V. Ganapathy, F.H. Leibach, J. Biol. Chem. 261 (1986) 16133–16140.
[30] M. Kato, H. Maegawa, T. Okano, K. Inui, R. Hori, J. Pharmacol. Exp. Ther. 251 (1989) 745–749.
[31] P.P. Sokol, P.D. Holohan, C.R. Ross, Biochim. Biophys. Acta 862 (1986) 335–342.
[32] R. Hori, H. Maegawa, T. Okano, M. Takano, K. Inui, J. Pharmacol. Exp. Ther. 241 (1987) 1010–1016.
[33] T. Katsura, M. Takano, Y. Tomita, M. Yasuhara, K. Inui, R. Hori, Biochim. Biophys. Acta 1146 (1993) 197–202.
[34] R.I. Macey, R.E.L. Farmer, Biochim. Biophys. Acta 211 (1970) 104–106.
[35] A.K. Solomon, B. Chasan, J.A. Dix, M.P. Lukacovic, M.R. Toon, A.S. Verkman, Ann. NY Acad. Sci. 414 (1983) 97–124.
[36] P.P. Sokol, P.D. Holohan, C.R. Ross, J. Biol. Chem. 263 (1988) 7118–7123.
[37] P.A. Knauf, F.Y. Law, K. Hahn, Am. J. Physiol. 269 (1995) C1073–C1077.
[38] V. Ganapathy, M. Brandsch, F.H. Leibach, Intestinal transport of amino acids and peptides, in: L.R. Johnson (Ed.), Physiology of the Gastrointestinal Tract, Raven Press, New York, 1994, pp. 1773–1793.