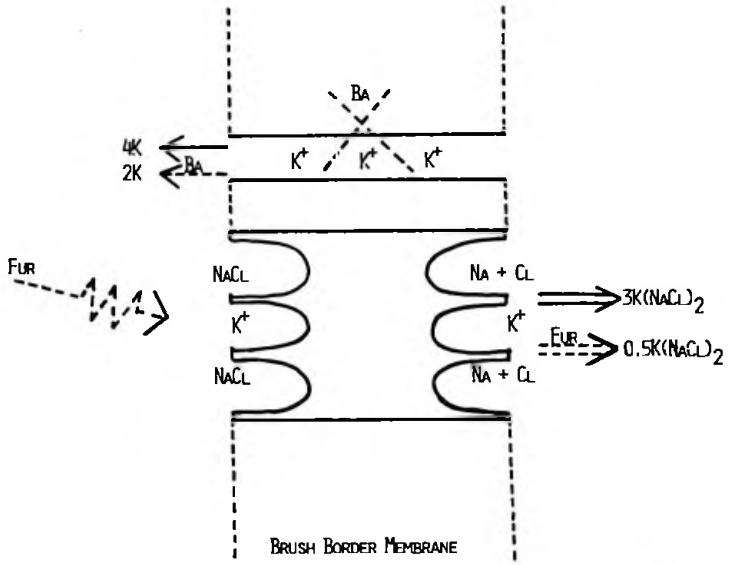

Figure 1.--Model for K^+ Transport Across Flounder Intestinal Brush Border. The K^+ channel shows a single file diffusion properties and has 3 or more K^+ sites. Ba partially inhibits the K^+ current through this channel. The cotransport complex is positively charged with K:2Na:2Cl stoichiometry. Translocation of this complex is inhibited by furosemide.

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SODIUM PROTON ANTIPORT IN INTESTINAL BRUSH BORDER VESICLES OF THE FLOUNDER, PSEUDOPLEURONECTES AMERICANUS

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INTRODUCTION

In intact, short-circuited flounder intestine, unidirectional Na uptake across the brush border appears to be coupled to that of Cl (Frizzell et al., J. Membr. Biol. 46:27, 1979) and also K (Musch et al., Bull. MDIBL, 1981). Working with brush border vesicles isolated from flounder intestine, Eveloff et al (J. Comp. Physiol. 135: 175, 1980) found evidence for two electrically neutral Na transport systems - NaCl symport and Na/H antiport. In intact rabbit ileum, Cl-coupled Na uptake across the brush border has also been demonstrated (Nellans et al., Am. J. Physiol. 225:467, 1973). However, in recent studies with rabbit ileal brush border vesicles only Na/H^+ antiport could be demonstrated (Misanko, B.S., Dubinsky, W., and Frizzell, R.A., submitted for publication). Na/H antiport has also been demonstrated in rat intestinal and renal proximal tubule membrane vesicles (Murer et al., Biochem. J. 154:597, 1976).

In the present study we have further analyzed Na uptake by brush border vesicles from flounder intestine with respect to (1) effect of buffer strength, (2) dependence on H^+ gradients and (3) anion dependence.

METHODS

Flounder intestinal mucosa was isolated and brush border vesicles prepared from it by modifications or previously described procedures (Eveloff et al., J. Comp. Physiol., 135:175, 1980; Schmitz et al., Biochim. Biophys. Acta 323:98, 1975). Briefly, flounder intestinal mucosa was homogenized for 2 min in a Waring

blender (7% w/v in ice cold 50 mM mannitol, 1mM Tris-Hepes, pH 7.0) and the homogenate precipitated with solid $CaCl_2$ (to 30 mM) over 20 min. The homogenate was centrifuged at 5000 x g for 15', and the supernate recentrifuged at 27,000 x g for 30'. The high speed pellet, which contained brush border vesicles, was resuspended in 100 mM mannitol 2 mM Tris-driven Potter-Elvejhem homogenizer. This suspension was centrifuged at 27,000 x g for 30' and washed once in the same buffer. The final pellet (P_1) was suspended in 50 mM mannitol 50 mM Tris-Hepes pH 7.5 to a final protein concentration of = 5 mg/ml and stored in the refrigerator.

For treatment with EDTA, P_1 was resuspended in 100 mM mannitol 2 mM Tris-Hepes (pH 7.0) and EDTA was added to a final concentration of 2 mM. After stirring for 20 min at 4°C, the suspension was centrifuged (27,000 x g, 30') and the pellet washed twice in 100 mM mannitol, 2mM Tris-Hepes pH 7.0. Unless otherwise indicated, the final pellet was resuspended and stored as described in the preceding paragraph.

Uptake of ^{22}Na by vesicles was measured in different incubation media (see individual experiments) care being taken that the media were isoosmolar with respect to the vesicles. Assays were carried out at 15°C in the presence of 1 mM $^{22}NaCl$ by a modification of the procedure of Gasko et al (Analyt. Biochem. 72:57, 1976). Disposable Pasteur pipettes (5 1/4") were packed with 15 ml of cation exchange resin (Dowex 50 x 8 (Tris) 100 mesh) over a glass wool plug. The columns were rinsed twice with 3 ml of sucrose made isoosmotic with the incubation buffer (generally 0.1 M). The reaction was initiated by the addition of 25 µl membrane suspension to 175 µl of radiolabelled medium. At the end of the incubation period the entire reaction mixture was transferred onto the Dowex column and rinsed (x2) with 1.0 ml of 0.1 M (or isoosmotic) sucrose. The entire effluent was collected in a scintillation vial, dissolved in Bray's scintillation fluid, and counted. Proteins were determined by the method of Lowry (Lowry et al., J. Biol. Chem. 193:265, 1951).

RESULTS AND DISCUSSION

To establish that the observed sequestration of Na was due to influx and not binding, vesicle Na was determined in the presence of increasing medium osmolarity, and constant intravesicular osmolarity. Under 50 mM buffer conditions, there was a linear inverse correlation between medium osmolarity and Na uptake indicating that uptake is occurring into osmotically active space. In addition, the binding component determined by extrapolating the above relationship to infinite osmolarity (y-intercept of $\frac{1}{osm}$ vs uptake) represented only 18% of the total uptake.

The time course of ^{22}Na uptake was studied under various conditions. Uptake was found to be linear for 5 min, maximal uptake being achieved in 30-60 min. Specific activity of uptake appeared to be independent of protein concentration in the range of 0.6 - 2 mg/ml.

As previously observed for rabbit ilea brush borders (Misanko et al., submitted for publication), prewashing flounder brush border vesicles with EDTA increased Na uptake 2-fold (Table 1). This change can be attributed to

Table 1.--Effects of EDTA Wash on Na Uptake

Time (min.)	Control Vesicles	+0.5 mM Amiloride	EDTA Vesicles	+0.5 mM Amiloride
2.5	0.6	0.4	2.2	1.35
30	1.6	0.9	3.5	1.90
360	1.4	0.75	2.5	1.5

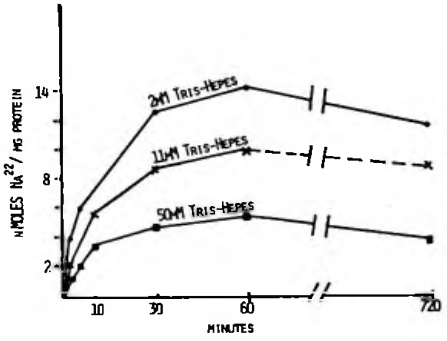
Vesicles were prepared by Ca precipitation. Intravesicular and extravesicular buffer concentrations were 50 mM Tris-Hepes 50 mM mannitol pH 7.0.

the removal of membrane-bound Ca by EDTA. It remains to be established, however, that EDTA did not increase the amount of Na bound to the vesicles (vs Na transported to the intravesicular space). Table 1 also shows that Na

uptake was inhibited by 0.5 mM amiloride. Even at 30 min amiloride-treated and control vesicles exhibited different uptake values. One possible explanation for the inhibitory effect of amiloride is that it is taken up into the vesicles by non-ionic diffusion resulting in an alkalinization of the vesicle interior. The reason for the lack of equilibration after 6 hours (rabbit vesicles at 25° equilibrate more rapidly; Misanko et al) is uncertain but suggests very slow dissipation of ion gradients in flounder vesicles at 15°.

Since Ca is a known activator of phospholipase A_2, we explored the use of Mg in the preparation of the vesicles. Vesicles precipitated with Mg and washed with EDTA exhibited essentially the same time course for Na uptake as did the Ca precipitated - EDTA-washed vesicles. However, the uptake was consistently lower in the Mg precipitated vesicles (data not shown).

In rabbit brush border vesicles, Na uptake is inversely related to buffer strength (Misanko et al; initial uptake in 1 mM Tris-Hepes >> 10 mM Tris-Hepes > equilibrium values). This "overshoot" is not seen in the presence of 50 mM Tris-Hepes, and with all three buffer strengths uptake reaches the same equilibrium value in 200 min. To determine whether Na uptake in flounder vesicles exhibits a similar response to different ionic strengths, we examined uptake in 1, 10, and 50 mM Tris-Hepes (isoosmolarity maintained with mannitol). As shown in Figure 1, Na uptake decreases with buffer strength. However, even at 720 min, we were unable to



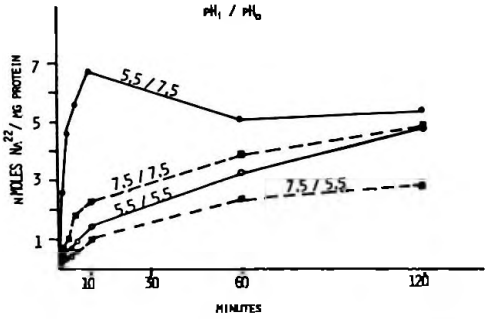


Figure 1.--Effects of buffer strength on Na uptake. Ca precipitated, EDTA washed vesicles were pre-equilibrated with either 2, 11 or 50 mM Tris-Hepes pH 7.0, the three media being made isoosmotic (102 m osmoles) with appropriate concentrations of mannitol.

Figure 2.--Effects of pH gradient on Na uptake. Ca precipitated vesicles were pre-equilibrated in 50 mM Tris-MES buffer either at pH 5.5 or pH 7.5. Na uptake into each of these vesicular preparations was studied in the presence or absence of a pH gradient. pH_i/pH_o = intravesicular pH/extravesicular pH.

achieve the same equilibrium value under all buffer conditions. This difference may be due to the establishment of a Donnan equilibrium in which Tris may replace Na as a permeant cation. The percent inhibition of Na uptake by amiloride ($\simeq$ 50%) is unaffected by buffer strength. Finally, this ionic strength effect is not restricted to Tris-buffers; Na uptake in various concentrations of TMA-Hepes also showed dependence on buffer strength. To circumvent any effects due to low buffer concentrations all subsequent experiments were conducted in 50 mM Tris-Hepes.

The presence of a Na^+/H^+ antiporter has been demonstrated in renal and intestinal brush border membranes (Murer et al., Biochem. J. 154:597, 1976). More recently an inwardly directed H^+ gradient was shown to inhibit Na uptake in flounder vesicles (Eveloff et al., J. Comp. Physiol. 135:175, 1980). To examine this mechanism further, vesicles were allowed to pre-equilibrate in 50 mM mannitol, 50 mM Tris-MES (2 N - morpholino - ethane sulofonic acid), pH 5.5 or 50 mM mannitol 50 mM Tris-MES pH 7.5 and then uptake of Na into each of these vesicle preparations was measured in the presence of pH 5.5 or pH 7.5 buffers. As shown in Figure 2, an outwardly

directed proton gradient ($pH_i < pH_o$) causes an "overshoot" in Na uptake. This was not seen in the absence of a proton gradient ($pH_i = pH_o$). An inwardly directed proton gradient, on the other hand, inhibits Na uptake. Under all conditions tested an equilibrium value is reached around 120 min, suggesting slow dissipation of the H^+ ion gradient. Although they reach the same equilibrium value, Na uptake in the absence of a H^+ gradient is slower in pH 5.5 buffer than in pH 7.5 buffer, suggesting that H^+/H^+ exchange can compete with Na^+/H^+ exchange at higher H^+ concentrations.

To test for the presence of NaCl symport, the anion dependence of Na uptake was examined. Furosemide (0.5 mM) and bumetanide (0.5 mM) did not inhibit Na uptake in either EDTA-washed or unwashed vesicles. In the presence of 1 mM Na salt, Na uptake was independent of the anion present (Cl=SCN=SO_4=gluconate, data not shown). The anion dependence could be due to Na^+/H^+ exchange operating at a much faster rate than Cl^-/OH^- exchange. Thus a higher $[Cl^-]_o$ may be required to maximize Cl^-/OH^- exchange and create anion-dependence. In the presence of 100 mM anion (with K as accompanying cation) and 1 mM Na, anion effects on Na uptake were observed (Figure 3A; SCN > Cl > gluconate > SO_4). This anion dependence could be due to either coupled Na-

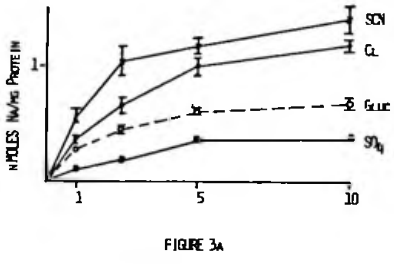


FIGURE 3A

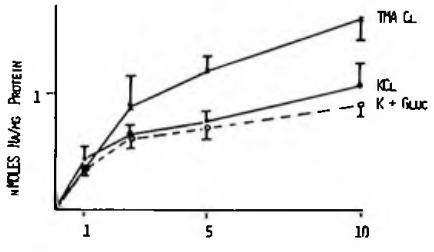


FIGURE 3B

Figure 3a.--Anion dependence of Na uptake. Mg precipitated, EDTA washed vesicles were pre-equilibrated in 300 mM mannitol, 50 mM Tris-Hepes, pH 7.0. For the assay, extravesicular buffer contained 100 mM K salt, 1 mM Na salt, 50 mM Tris-Hepes and mannitol to make it isoosmotic with intravesicular buffer. Values represent mean ± SEM for 3 preparations.

Figure 3b.--Effect onNa uptake of pre-equilibrating with high salt: Mg precipitated, EDTA washed vesicles were pre-equilibrated for > 12 hrs in 100 mM salt (TMA Cl, K Cl or K gluconate), 100 mM mannitol and 50 mM Tris-Hepes, pH 7.0. ^{22}Na uptake was then assessed in these vesicles by addition of 1 mM ^{22}Na salt to the same buffers. Values represent means ± S.E.M.

Table 2.--Comparison of Effects of TMA and K Salts on Na Uptake

Time	TMA Cl	TMA Gluc	K Cl	K Gluc
1	0.93	0.8	0.46	0.29
2.5	1.64	1.28	0.89	0.63
5	2.2	1.66	1.08	0.76
10	2.82	2.0	1.26	0.98

Vesicles were prepared by Mg precipitation followed by an EDTA wash. Intravesicular buffer concentrations at beginning of the experiment were: 300 mM mannitol 50 mM Tris-Hepes, pH 7.0. Vesicles were incubated in 100 mM mannitol, 100 mM K or TMA salt 50 mM Tris-Hepes and 1 mM NaCl, pH 7.0.

anion entry or a diffusion potential generated by differing permeability ratios between K^+ and each anion. To distinguish between these two possibilities, we measured ^{22}Na uptakes in the presence of 100 mM KCl or 100 mM

K gluconate into vesicles pre-equilibrated for more than 12 hours in the same media. If Na entry occurred by a Cl-coupled process, then uptake should have been greater in KCl than in K gluconate. However, as shown in Figure 3b, Na uptake was the same in KCl and K gluconate. Therefore, the observed anion dependence cannot be explained by an obligatory NaCl symport. It is not clear why the previously observed (Eveloff et al., J. Comp. Phyiol. 135:175, 1980) furosemide-inhibitable Cl dependent Na influx was not observed in the present study. Differences in assay conditions may have been responsible; in the earlier study Na uptake was measured in the presence of 100 mM mannitol, 20 mM Tris-Hepes, 2 mM Ca gluconate at pH 8.2.

To determine if K is essential to NaCl symport (i.e., Na, K, Cl symport; see Musch et al., Bull. MDIBL, 1981), we compared Na uptake into TMACl (TMA is an impermeant cation) and KCl pre-equilibrated vesicles. If K facilitated NaCl transport a higher Na uptake would have been expected in Kcl. However, as shown in Figure 3b this is not the case; in contrast, there is higher Na uptake into TMACl vesicles, suggesting that K competes for either the H^+ gradient and/or the same transporter. The observation that Na uptake is higher in the presence of TMACl than TMA gluconate (Table 2) suggests that there is an anion-dependent, K-independent uptake but it remains to be established whether this is due to Cl/OH exchange or a Cl diffusion potential.

EFFECT OF STIMULATION OF THE RECTAL GLAND ON OUABAIN BINDING: EVIDENCE FOR A DIRECT ACTION OF CYCLIC AMP

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Chloride secretion by the rectal gland of the dogfish depends on the activity of Na-K-ATPase located in the basolateral membrane of the cells lining the tubules of the gland. When chloride secretion is stimulated by theophylline and cyclic AMP there is indirect evidence that Na-K-ATPase is simultaneously activated. First, ouabain inhibitable oxygen consumption increases six-fold; second, the intracellular concentration of sodium decreases while that of potassium rises. Both these findings are consistent with primary activation of Na-K-ATPase. However, the activity of Na-K-ATPase measured in homogenates in vitro was the same whether the glands were previously perfused with or without theophylline and cyclic AMP. Shuttleworth and Thompson (J. Exper. Zool., 206:297-302, 1978), reported that the binding of 2.2×10^{-6}M ouabain to the rectal gland of the dogfish Scyliorhinus canicola was increased after incubation with theophylline and cyclic AMP. Previous attempts in our laboratory to demonstrate increased binding of 5×10^{-6}M or 10^{-4}M ouabain to rectal gland slices of Squalus acanthias were unsuccessful (Bull. MDIBL 18:16-19, 1978; J. Cell Biol. 83:16-32, 1979). The present experiments were designed to investigate further the nature of the binding of ouabain to rectal gland cells.

All experiments were done using rectal glands from spiny dogfish, Squalus acanthias. The glands were removed via an abdominal incision and perfused by gravity with shark-Ringers for ten minutes to remove all red cells.

Coronal slices of rectal gland were prepared using a Stadie-Riggs microtome. The slices were kept in ice cold shark-Ringers until used, usually within fifteen minutes. The binding of ouabain to slices of rectal gland was measured in a solution containing 10^{-9}M carrier free ^{3}H-ouabain. A parallel incubation containing 10^{-3}M theophylline and 10^{-3}M dibutyrylcyclic AMP was run to determine the effect of stimulation of rectal gland secretion on ouabain binding. Additional incubations were done with varying concentrations of cold ouabain in order to determine the kinetics of ouabain binding. Nonspecific binding of ouabain to the slices was measured using 10^{-4}M cold ouabain. Extracellular space was labelled using ^{14}C-inulin, 0.1 microCi/ml. After varying times of incubation, the slices were removed from the incubation medium and transferred through