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Functional Roles of Na^+ and H^+ in SO_4^{2-} Transport by Rabbit Ileal Brush Border Membrane Vesicles

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Summary. Sulphate uptake by rabbit ileal brush border membrane vesicles was stimulated by a transmembrane sodium gradient ($[Na^+]_o > [Na^+]_i$), but not by a similar potassium gradient. $^{35}SO_4^{2-}$ influx ($J_{oi}^{SO_4}$) from outside (*o*) to inside (*i*) these vesicles was a hyperbolic function of $[SO_4^{2-}]_o$ and the affinity constant for anion transport was strongly influenced by $[Na^+]_o$ (100 mM Na^+, $K_t^{SO_4} = 0.52$ mM SO_4^{2-}; 10 mM Na^+, $K_t^{SO_4} = 4.32$ mM SO_4^{2-}). $J_{oi}^{SO_4}$ was a sigmoidal function of $[Na^+]_o$ at pH 7.4 for both low (0.2 M) and high (4.0 mM) $[SO_4^{2-}]_o$. The Na^+-dependency of $J_{oi}^{SO_4}$ was examined at pH 6.0, 7.4, and 8.0 (same pH inside and outside). At pH 6.0 and 7.4 sigmoidal Na^+-dependent $J_{oi}^{SO_4}$ exhibited nonlinear Eadie-Hofstee plots indicative of a transport mechanism capable of binding a variable number of sodium ions over the $[Na^+]_o$ range used. Hill plots of anion transport under these conditions displayed slopes near unity at low $[Na^+]_o$ and slopes approximating 2.0 at higher cation concentrations. At pH 8.0, Na^+-dependent $J_{oi}^{SO_4}$ was hyperbolic and showed linear Eadie-Hofstee and Hill plots, the latter with a single slope near 1.0. When a H^+ gradient was imposed across the vesicle wall ($pH_i = 8.0$, $pH_o = 6.0$), Na^+-dependent $J_{oi}^{SO_4}$ was hyperbolic and significantly increased at each $[Na^+]_o$ over values observed using bilateral pH 8.0. In contrast, a H^+ gradient oriented in the opposite direction ($pH_i = 6.0$, $pH_o = 8.0$) led to Na^+-dependent $J_{oi}^{SO_4}$ that was sigmoidal and significantly lower at each $[Na^+]_o$ than values found using bilateral pH 6.0. Electrogenicity of $J_{oi}^{SO_4}$ at pH 8.0 for both high and low $[Na^+]_o$ was demonstrated by using a valinomycin-induced transmembrane electrical potential difference. At pH 6.0, electrogenic $J_{oi}^{SO_4}$ occurred only at low $[Na^+]_o$ (5 mM); anion transfer was electroneutral at 50 mM Na^+. A model is proposed for proton regulation of sodium sulphate cotransport where flux stoichiometry is controlled by $[H^+]_i$ and sodium binding affinity is modified by $[H^+]_o$. Preliminary experiments with rabbit proximal tubular brush border membrane vesicles disclosed similar $J_{oi}^{SO_4}$ kinetic properties and a common transport mechanism may occur in both tissues.

Key Words membrane vesicles · rabbit ileum · rabbit proximal tubule · brush border · sulphate transport · Na^+ dependence · H^+ dependence · cooperativity · sigmoidal transport · cotransport

Introduction

Inorganic sulphate transport in mammalian intestine has been extensively studied in rabbit ileum using classical Ussing chamber techniques, which allow the tissue to be short-circuited and facilitate the measurement of bidirectional transepithelial ion fluxes (Langridge-Smith & Field, 1981; Smith, Orellana & Field 1981). In addition, influx characteristics of SO_4^{2-} transport from luminal solution to epithelial cell in this same tissue were recently disclosed by employing identical experimental techniques (Langridge-Smith, Sellin & Field 1983). These investigations have shown that transepithelial SO_4^{2-} transport in rabbit ileum involves Na^+-dependent uptake across the brush border membrane and Cl^--dependent efflux across the serosal membrane. Transport specificities of Na^+-dependent SO_4^{2-} influx and transmural transfer processes were defined by using a variety of potentially inhibitory ionic species and drugs. A model was proposed by Langridge-Smith et al. (1983) for Na^+-dependent SO_4^{2-} influx in rabbit ileum that included a variable flux stoichiometry between the binding ligands. At low luminal $[SO_4^{2-}]$, Na^+/SO_4^{2-} influx stoichiometry was 1.0, while at higher $[SO_4^{2-}]$ the ratio approached 2.0. Under both conditions the authors suggested an electroneutral anion transport; at low $[SO_4^{2-}]$ electroneutrality was established by a Na^+-H^+-SO_4^{2-} transfer and at high $[SO_4^{2-}]$ two sodium ions were coupled with sulphate flux.

Sulphate transport by brush border membrane vesicles of rat ileum illustrates properties that are in general agreement with those mentioned above for rabbit ileum (Lucke, Stange & Murer, 1981). Using a technique that allows independent manipulation of solutions bathing both surfaces of the epithelial apical border so that a transmembrane electrical gradient could be established, those authors suggested that in the rat, sodium sulphate cotransport is also electroneutral. However, they only examined the effects of a transmembrane electrical gradient on SO_4^{2-} transport under a limited

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set of experimental conditions, and in light of the variable transport stoichiometry for this anion shown in rabbit ileum when a broader range of treatments was used, a more complete analysis of this transfer mechanism seems necessary.

The present investigation is a detailed study of SO_4^{2-} transport in rabbit ileal brush border membrane vesicles, specifically examining factors which affect Na^+-sulphate flux stoichiometry and whether these conditions lead to electrogenic anion movement across the vesicle wall. Results confirm previous general findings discussed above, but indicate that sodium sulphate cotransport probably does not involve simultaneous carrier-mediated proton fluxes and is electrogenic when flux stoichiometry is 1 Na^+/1 SO_4^{2-}. Preliminary findings of this report have already appeared in print (Ahearn & Murer, 1983; Murer et al., 1983).

Materials and Methods

Ilea were dissected from freshly sacrificed male New Zealand white rabbits purchased from a farm in Zaziwil, Switzerland. Immediately upon removal from the animals, the ilea were rinsed thoroughly with 0.9% NaCl (4 °C), everted on a plastic rod to expose the mucosal epithelium, and frozen on dry ice. The frozen tissue was kept at −80 °C for up to 4 months before experimental use.

When brush border membrane vesicles were prepared from this frozen tissue, 20 g were thawed at room temperature in 60 ml of a buffer containing 300 mM mannitol, 5 mM EGTA, and 12 mM TRIS, which was adjusted to pH 7.1 with HCl. Upon thawing, the tissue was strongly agitated with a Vibromixer® (Chemap AG, Mannedorf, Switzerland) to release the epithelium from the underlying muscle and connective tissue layers. The resulting cell suspension was filtered through a Buchner funnel, diluted with 240 ml distilled water, and homogenized at high speed with a blender for 3 min. Three ml of 1-M $MgCl_2$ were added to the 300 ml homogenate to give a final $MgCl_2$ concentration of 10 mM, and this solution was allowed to settle on ice for 15 min. Two centrifugations of this solution were performed using a Sorvall RC-5B centrifuge fitted with an SS-34 fixed-angle rotor, the first at $3{,}000 \times g$ (15 min) and the second at $27{,}000 \times g$ (30 min). The resulting pellet was resuspended in 35 ml of a buffer containing 60 mM mannitol and 5 mM EGTA with the pH adjusted to 7.1 with TRIS. The Mg precipitation was repeated on this mixture followed by two additional centrifugations as mentioned previously. The pellet recovered from the second centrifugation was resuspended in different buffers that were appropriate for each experiment (*see* figure legends) and centrifuged a final time at $27{,}000 \times g$ for 30 min. The final pellet contained purified brush border membrane vesicles, which were resuspended in a small volume of the same buffer used in the last centrifugation.

Previous experiments (G. Danisi, H. Murer, and R.W. Strau, *in preparation*) have shown that purified rabbit ileal brush border membranes prepared as discussed above from frozen tissue exhibit high enrichment (10–15 times that in the original homogenate) of known brush border enzyme markers (leucine amino peptidase, maltase, alkaline phosphatase) and only slight enrichment of Na^+/K^+-ATPase (basolateral enzyme marker). In addition, this study showed that the freezing process did not have any apparent adverse effects on the phosphate transport properties of this tissue. In the present series of experiments, brush border membrane purification was assessed by the enrichment of alkaline phosphatase and Na^+/K^+-ATPase over that shown by the concentration of these enzymes in the tissue homogenate. Triplicate analyses of both enzymes yielded average enrichments of 12.25 (alkaline phosphatase) and 1.16 (Na^+/K^+-ATPase), indicating a minimal contamination of these brush border vesicles by basolateral membranes. Throughout the present study vesicle protein content was determined using the Bio Rad protein assay.

$^{35}SO_4^{2-}$ uptake ($H_2{}^{35}SO_4^{2-}$; New England Nuclear, Corp.) was measured at 25 °C using a rapid filtration technique (Berner, Kinne & Murer, 1976; Evers, Murer & Kinne, 1976) that employed Sartorius filters (pore diameter, 0.65 μm) soaked in distilled water. During most incubations 10 μl of membrane suspension was mixed for a predetermined time interval with 10 μl of buffer containing the isotope, using a rapid-exposure uptake apparatus (Inovativ Labor AG, Adliswil, Switzerland). In pH gradient experiments the ratio of membranes/isotope buffer was changed to 5:45 μl to minimize dissipation of desired gradients by the mixing process. Following isotope incubation an ice-cold stop solution having a composition appropriate for each experiment (*see* figure legends) was injected into the membrane-isotope mixture. Each experiment was repeated at least twice using membranes prepared from different frozen rabbit ilea. The same experimental findings were consistently obtained in the reptition of experiments. Within a given experiment, each point was analyzed in quadruplicate. The individual values generally showed an experimental scatter of about 5%. Thus, throughout this study mean values obtained in an individual experiment will be presented. Filters containing radioactive vesicles were placed in 3 ml of Beckman Ready-Solv EP liquid scintillation cocktail and counted in a Packard Tri-Carb scintillation counter. All chemicals were of analytical grade and valinomycin was purchased from Sigma Chemical Company.

Results

Effect of Transmembrane Cation Gradients on SO_4^{2-} Uptake

Figure 1 illustrates the effects of 100 mM NaCl or 100 mM KCl transmembrane concentration gradients on the time course of 0.06 mM SO_4^{2-} uptake by rabbit ileal brush border membrane vesicles. In the presence of a Na^+ gradient, anion uptake transiently exceeded the accumulation at equilibrium (90 min) by approximately 2.5 times (overshoot phenomenon). In contrast, SO_4^{2-} uptake using a K^+ gradient was considerably slower and did not exhibit an overshoot. The initial rate of SO_4^{2-} uptake at 20 sec with a Na^+ gradient (1.73 pmol mg protein^{-1} sec^{-1}) was 25 times more rapid than that observed when K^+ was employed (0.07 pmol mg protein^{-1} sec^{-1}). Similar equilibrium values for anion uptake were observed for both cations ($Na^+ = 22.8$; $K^+ = 25.7$ pmol mg protein^{-1}).

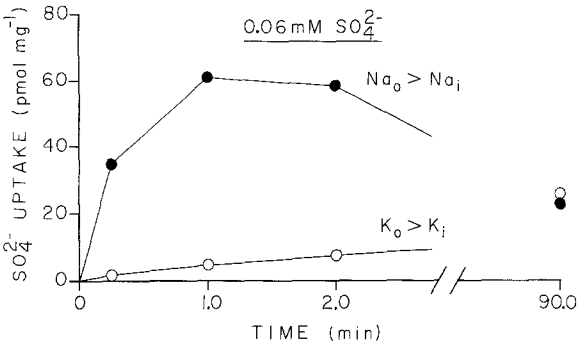

Fig. 1. Effects of transmembrane Na^+ or K^+ concentration gradients on the time course of SO_4^{2-} uptake by rabbit ileal brush border vesicles. Membranes were preloaded with buffer containing 300 mM mannitol and 20 mM HEPES-TRIS at pH 7.4. The final incubation medium after mixing membranes and buffer contained (in mM): 100, mannitol; 100, NaCl (or 100, KCl); 0.06, Na_2SO_4 (or 0.06, K_2SO_4); 20, HEPES-TRIS, at pH 7.4. Stop solution contained (in mM): 100, mannitol; 150, NaCl; 20, HEPES-TRIS, at pH 7.4

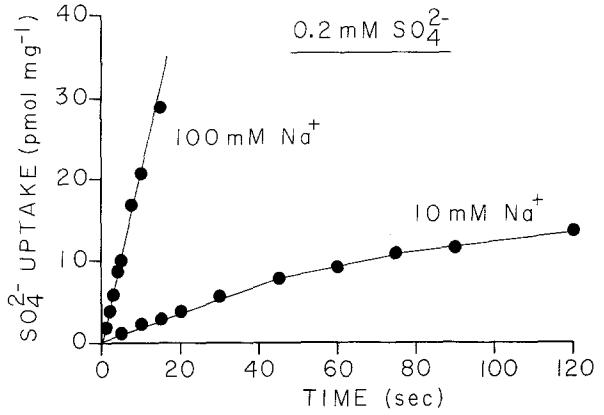

Fig. 2. Time course of SO_4^{2-} uptake at 10 and 100 mM Na^+. Membranes were preloaded the same as in Fig. 1. Final incubation media after mixing membranes and buffer contained (in mM): (a) 10, NaCl; 140, choline chloride; 0.2, K_2SO_4; 10, HEPES-TRIS, at pH 7.4; (b) 100, NaCl; 50, choline chloride; 0.2, K_2SO_4; 10, HEPES-TRIS, at pH 7.4. Stop solution for both $[Na^+]$ consisted of 150 mM choline chloride, 10 mM HEPES-TRIS at pH 7.4

Time Course of SO_4^{2-} Uptake at Two Sodium Concentrations

In order to estimate unidirectional anion uptake (initial linear uptake) from extravesicular to intravesicular space, the time course of 0.2 mM SO_4^{2-} accumulation at two Na^+ concentrations was assessed. In this experiment the intravesicular medium contained only mannitol, while different isosmotic solutions of NaCl and choline chloride were placed extravesicularly. Figure 2 shows that SO_4^{2-} uptake in 100 mM NaCl was rapid and remained linear for at least 15 sec, while anion accumulation at 10 mM NaCl was considerably slower and only remained a linear function of time for 45 sec. Anion influxes calculated at these two sodium concentrations were 3.0 pmol mg protein^{-1} sec^{-1} (100 mM Na^+) and 0.2 pmol mg protein^{-1} sec^{-1} (10 mM Na^+), respectively. In subsequent experiments 15-sec incubations were used to establish SO_4^{2-} influx when extravesicular Na^+ concentrations of 150, 100, 75, 50, 37.5, 25, and 15 mM were employed, while 45-sec exposures were chosen for all other cation concentrations.

Effect of Extravesicular $[SO_4^{2-}]$ on Sulphate Influx

Sulphate influx ($J_{oi}^{SO_4}$) across rabbit ileal brush border membrane vesicles was a hyperbolic function of extravesicular $[SO_4^{2-}]$ (10, 5, 2.5, 1.75, 1.0, 0.5, 0.35, and 0.20 mM SO_4^{2-}) at both 10 and

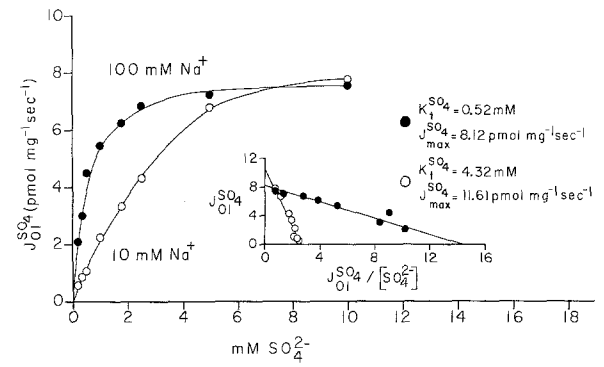

Fig. 3. Effects of extravesicular $[SO_4^{2-}]$ on sulphate influx. All membranes were preloaded with 450 mM mannitol, 10 mM HEPES-TRIS at pH 7.4. Final incubation media after mixing membranes and buffer contained (in mM): (a) 10, NaCl; 190, choline chloride; 10, 5, 2.5, 1.75, 1.0, 0.5, 0.35, or 0.2, K_2SO_4; 10, HEPES-TRIS at pH 7.4, and additional choline chloride to osmotically balance the various $[K_2SO_4]$; (b) 100, NaCl; 100, choline chloride; same $[K_2SO_4]$ as in (a); 10, HEPES-TRIS at pH 7.4, and additional choline chloride for osmotic balance. Stop solution for both $[Na^+]$ contained 230 mM choline chloride, 10 mM HEPES-TRIS at pH 7.4

100 mM external sodium concentrations (Fig. 3). Maximal influx velocity was reached at 5 mM SO_4^{2-}; further increases in anion concentration did not appreciably elevate its entry rate. The inset in Fig. 3 suggests that the main effect of extravesicular Na^+ on $J_{oi}^{SO_4}$ was on the affinity constant for anion entry (10 mM Na^+, $K_t^{SO_4} = 4.32$ mM SO_4^{2-}; 100 mM Na^+, $K_t^{SO_4} = 0.52$ mM SO_4^{2-}. Increasing the external $[Na^+]$ from 10 to 100 mM elevated the binding affinity of the membranes for SO_4^{2-} by a factor of 8.3, while maximal influx velocity was

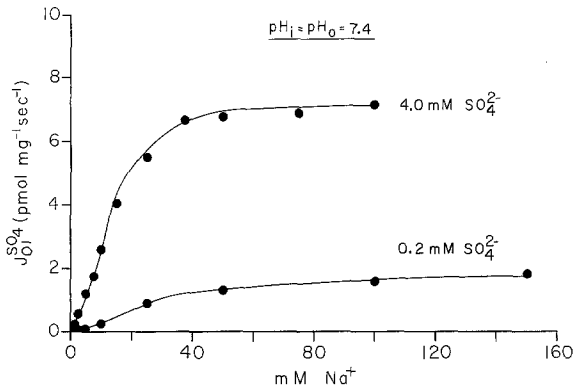

Fig. 4. Influence of extravesicular [Na^+] on 0.2 and 4.0 mM sulphate influx at pH 7.4. (a) *0.2 mM SO_4^{2-} conditions:* Membranes were loaded with 300 mM mannitol, 10 mM HEPES-TRIS at pH 7.4. Final incubation media after mixing contained 0.2 mM K_2SO_4, 150, 100, 50, 25, 10, 5, 2.5, and 1.25 mM NaCl, 10 mM HEPES-TRIS at pH 7.4, and additional choline chloride for osmotic adjustment. Stop solution: 150 mM choline chloride and 10 mM HEPES-TRIS at pH 7.4. (b) *4.0 mM SO_4^{2-} conditions:* Membranes preloaded with 240 mM mannitol, 10 mM HEPES-TRIS at pH 7.4. Final incubation media consisted of 4.0 mM K_2SO_4, 100, 75, 50, 37.5, 25, 15, 10, 7.5, 5, 2.5, and 1.25 mM NaCl, 10 mM HEPES-TRIS to pH 7.4, and additional choline chloride for osmotic balance. Stop solution: 210 mM choline chloride and 10 mM HEPES-TRIS to pH 7.4

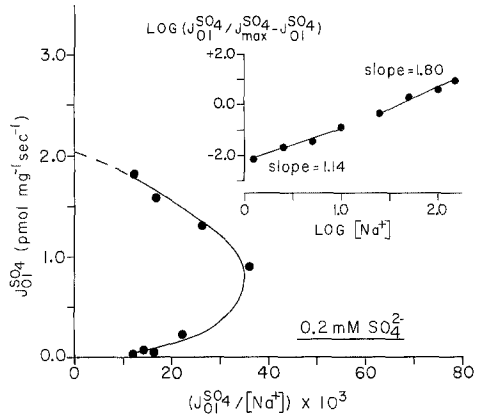

Fig. 5. Kinetic analysis of 0.2 mM sulphate influx as a function of [Na^+]$_o$ at pH 7.4. Eadie-Hofstee plot of data from lower curve on Fig. 4. $J_{max}^{SO_4}$ estimated as extrapolation of curve to vertical axis (2.04 pmol mg^{-1} sec^{-1}). Inset is a Hill plot of data in main body of figure

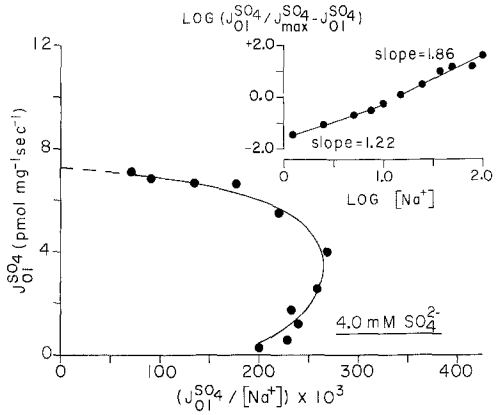

Fig. 6. Kinetic analysis of 4.0 mM sulphate influx as a function of [Na^+]$_o$ at pH 7.4. Eadie-Hofstee plot of data from upper curve on Fig. 4. $J_{max}^{SO_4}$ estimated as extrapolation of curve to vertical axis (7.30 pmol mg^{-1} sec^{-1}). Inset is a Hill plot of data in main body of figure

only slightly altered under these conditions. Both Figs. 2 and 3 suggest that $J_{oi}^{SO_4}$ depends on external Na^+ and that this dependence is, at least partly, exerted through anion association with carrier sites in the brush border membrane.

Influence of Extravesicular [Na^+] on $J_{oi}^{SO_4}$ at pH 7.4

Influxes of 0.2 and 4.0 mM SO_4^{2-} were both sigmoidal functions of extravesicular [Na^+] when the solutions on each side of the vesicular membranes were at pH 7.4 (Fig. 4). Under these conditions $J_{oi}^{SO_4}$ at both anion concentrations was very slow below external Na^+ concentrations of 5–10 mM. Between 10–50 mM Na^+, sulphate entry increased rapidly and reached maximal influx velocity at 50 mM Na^+ or greater. Influx kinetics of sulphate transport as a function of external [Na^+] at pH 7.4 were analyzed using Eadie-Hofstee and Hill transformations of the data shown in Fig. 4.

Figure 5 is a kinetic analysis of 0.2 mM $J_{oi}^{SO_4}$, while Fig. 6 presents the data transformation for 4.0 mM $J_{oi}^{SO_4}$. When media inside and outside the vesicles were at pH 7.4, Eadie-Hofstee plots of $J_{oi}^{SO_4}$ as a function of [Na^+]$_o$ were nonlinear at both high and low [SO_4^{2-}]$_o$. Segel (1975) indicates that nonlinear kinetics under these conditions suggest the occurrence of a sulphate transport process capable of binding a variable number of sodium ions over a wide range of [Na^+]$_o$. Slopes of the Hill plots shown in each figure indicate that at low [Na^+]$_o$ a single cation accompanied SO_4^{2-} across the vesicle wall (slopes approximately = 1.0), while at high [Na^+]$_o$ the transport stoichiometry changed to 2 Na^+/1 SO_4^{2-} (slopes approximately = 2.0).

Effect of pH on Na^+-Dependent $J_{oi}^{SO_4}$

Figure 7 illustrates the influence of pH on 4.0 mM $J_{oi}^{SO_4}$ at [Na^+]$_o$ from 1.25 to 100 mM. Sulphate influx was a sigmoidal function of [Na^+]$_o$ when pH 6.0 was present on both sides of the vesicular membrane (Fig. 7*A*). The sigmoidal nature of $J_{oi}^{SO_4}$ at varying external [Na^+] did not change when a pH gradient was placed across the vesicular

membrane ($pH_i = 6.0$; $pH_o = 8.0$), but the entire anion entry curve was shifted to the right of that exhibited when bilateral pH 6.0 was employed. In addition, sulphate influxes were identical under the two pH conditions at high $[Na^+]_o$. These results suggest that an increase in external proton concentration (reduced pH) increased the sodium binding affinity of the cotransport system without appreciably altering the maximal anion entry rate. Eadie-Hofstee and Hill plots of the data in Fig. 7*A* were similar to those described for $pH_i = pH_o = 7.4$ conditions in Figs. 5 and 6.

Figure 7*B* shows that the basic character of $J_{oi}^{SO_4}$ as a function of $[Na^+]_o$ was altered significantly when either bilateral pH 8.0 conditions or a pH gradient where $pH_i = 8.0$, $pH_o = 6.0$ were employed. Under these conditions sulphate influx became a hyperbolic function of sodium concentration and $J_{oi}^{SO_4}$ in the presence of the pH gradient significantly exceeded the anion entry at each $[Na^+]_o$ when $pH_i = pH_o = 8.0$.

Eadie-Hofstee and Hill plots for Na^+-dependent $J_{oi}^{SO_4}$ when $pH_i = pH_o = 8.0$ or when $pH_i = 8.0$, $pH_o = 6.0$ are illustrated in Fig. 8. In contrast to previous experiments, a linear Eadie-Hofstee plot was exhibited by $J_{oi}^{SO_4}$ at each pH condition. A pH gradient across the brush border membrane vesicle wall ($pH_i = 8.0$, $pH_o = 6.0$) resulted in a significant increase in sodium binding affinity (decrease in slope) and a significant increase in $J_{max}^{SO_4}$ when compared to the control condition ($pH_i = pH_o = 8.0$). Hill plots of $J_{oi}^{SO_4}$ as a function of external $[Na^+]$ under these two pH conditions are illustrated as an inset to Fig. 8. Hill slopes of 1.0 were disclosed for anion entry for both $pH_i = pH_o = 8.0$ and $pH_i = 8.0$, $pH_o = 6.0$.

It is clear by comparing the $J_{oi}^{SO_4}$ kinetics in Figs. 4, 5, 6 and 7 with those in Fig. 8 that vesicles having an internal pH of either 6.0 or 7.4 are capable of transporting the anion with a variable number of cotransported sodium ions. In contrast, vesicles with $pH_i = 8.0$ are restricted to a transport stoichiometry of 1 Na^+/1 SO_4^{2-} over the entire range of $[Na^+]_o$.

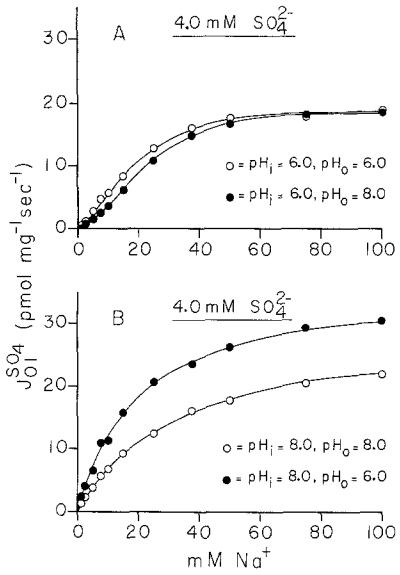

Fig. 7. Effect of pH on Na^+-dependent sulphate influx. (*A*): Membranes were preloaded with 420 mM mannitol and 10 mM MES-TRIS to pH 6.0. Final incubation media were the same as those in Fig. 4, part (b), except that Na^+-gluconate was used in this instance, and pH was adjusted to either 6.0 (10 mM MES-TRIS) or 8.0 (10 mM HEPES-TRIS). Stop solution: 210 mM tetramethylammonium gluconate at either pH 6.0 or 8.0. (*B*): Membranes preloaded the same as in *A* except that pH was adjusted to 8.0 with 10 mM HEPES-TRIS. Final incubation media and stop solution were prepared as in *A*

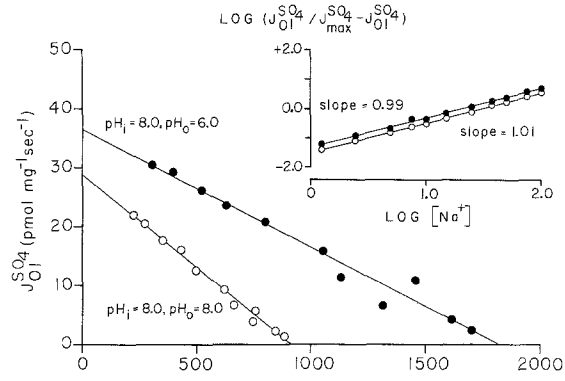

Fig. 8. Kinetic analysis of 4.0 mM sulphate influx as a function of $[Na^+]_o$ at intravesicular pH of 8.0. Data for the Eadie-Hofstee and Hill plots were taken from Fig. 7*B*

Effect of a Transmembrane Electrical Potential on $J_{oi}^{SO_4}$

In order to estimate the extent to which $J_{oi}^{SO_4}$ was electrogenic, a transmembrane electrical potential across the rabbit ileal brush border vesicle wall was established using a 50-mM potassium gluconate gradient in combination with bilateral addition of the K-selective ionophore, valinomycin. Sulphate influx under these conditions was measured at 5 and 50 mM external $[Na^+]$ and at two bilateral pH values, pH 8.0 and 6.0. In experiments with 50 mM Na^+, a 15-sec uptake period was used, while a 45-sec incubation was employed for 5 mM Na^+ tests.

Figure 9 (left panel) indicates that in the presence of valinomycin, when pH 8.0 was used on both sides of the vesicle wall, an outwardly directed potassium-induced diffusion potential (electrically negative vesicle interior) resulted in a significant

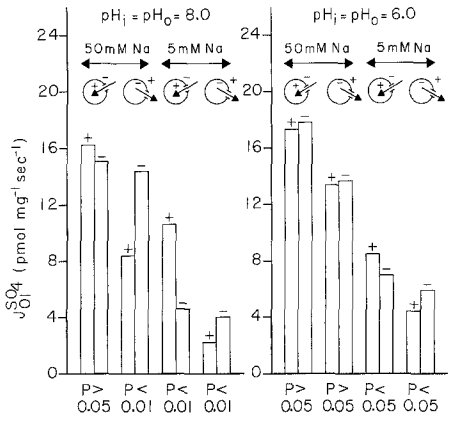

Fig. 9. Effect of a transmembrane K^+ diffusion potential on 4 mM $J_{oi}^{SO_4}$ at pH 6.0 and 8.0. Membranes were preloaded with either: (a) 200 mM mannitol and 10 mM HEPES-TRIS at pH 8.0 (or 10 mM MES-TRIS at pH 6.0), or (b) 100 mM mannitol, 66 mM K^+-gluconate, and 10 mM HEPES-TRIS at pH 8.0 (or 10 mM MES-TRIS at pH 6.0). Valinomycin, in 95% ethanol (50 μg/ml), was added to the internal media of one-half of these vesicles, the remaining half was given only 95% ethanol. Final incubation media after mixing membranes and buffer contained one of the four following combinations (in mM): (a) 50, Na^+-gluconate; 42, K^+-gluconate; 4, K_2SO_4; (b) 5, Na^+-gluconate; 42, K^+-gluconate; 90, mannitol; 4, K_2SO_4; (c) 50, Na^+-gluconate; 100, mannitol; 4, K_2SO_4; and (d) 5, Na^+-gluconate; 190, mannitol; 4, K_2SO_4. Each of these four solutions were adjusted to either pH 6.0 or 8.0 as above, making a total of eight combinations of external conditions. Valinomycin, in ethanol, was added at the same concentration as mentioned above to one-half of these incubation media, while ethanol alone served as the control. Stop solution was 150 mM tetramethylammonium gluconate adjusted to either pH 6.0 or 8.0 as above. Models shown in the figure illustrate the direction of the imposed K^+ diffusion potential for each experimental condition, while + and − signs refer to the presence or absence of valinomycin, respectively. Columns are means of four replicates, and significance figures refer to each column pair

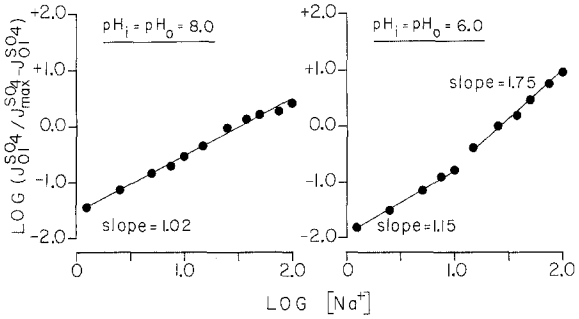

Fig. 10. Hill plots of 4.0 mM $J_{oi}^{SO_4}$ as a function of $[Na^+]_o$ in rabbit proximal tubule brush border membrane vesicles at two transmembrane pH conditions. Data were calculated from Eadie-Hofstee transformations of original influx values. Incubation conditions were similar to those described in Fig. 7

drop in $J_{oi}^{SO_4}$ as compared to the control condition lacking the ionophore. When the K-diffusion potential was reversed at pH 8.0 (inside positive), a stimulation of $J_{oi}^{SO_4}$ was observed at 5 mM Na^+ if valinomycin was present, but no effect on anion entry was recorded at the higher $[Na^+]$ (50 mM).

Figure 9 (right panel) presents the results of K^+-induced diffusion potentials on $J_{oi}^{SO_4}$ when pH 6.0 occurred on both vesicle surfaces. At the higher sodium concentration (50 mM), neither an inward nor outward flow of K^+ across the vesicle membrane induced a change in sulphate influx as compared to that of the controls (no valinomycin). In contrast, at 5 mM Na^+, a small stimulation of $J_{oi}^{SO_4}$ was observed when the vesicle interior was made electrically positive relative to the exterior, and a small inhibitory effect on anion entry occurred with a negative vesicle interior.

These results suggest that sulphate influx across rabbit ileal brush border membrane is electrogenic, bearing a net negative charge, at both high (50 mM) and low (5 mM) sodium concentrations when $pH_i = pH_o = 8.0$. However, under conditions when $pH_i = pH_o = 6.0$, electrogenic flow of sulphate only occurs at low $[Na^+]_o$; at high $[Na^+]_o$, $J_{oi}^{SO_4}$ is electroneutral.

$J_{oi}^{SO_4}$ in Rabbit Proximal Tubule Brush Border Membrane Vesicles

Sulphate influx was examined in proximal tubule brush border membrane vesicles prepared using thin slices of rabbit kidney cortex and the same experimental procedures outlined previously for the ileum. After determining appropriate incubation intervals to ensure initial SO_4^{2-} entry rates for this tissue, 4 mM $J_{oi}^{SO_4}$ was investigated as a function of $[Na^+]_o$ at bilateral pH values of 6.0 and 8.0. When $pH_i = pH_o = 6.0$, $J_{oi}^{SO_4}$ was a sigmoidal function of $[Na^+]_o$, but at $pH_i = pH_o = 8.0$ a hyperbolic relationship occurred between the variables.

A nonlinear Eadie-Hofstee transformation resulted from the sigmoidal data, while a linear relationship between the variables was obtained with the hyperbolic function. Figure 10 presents Hill plots of the $J_{oi}^{SO_4}$ data from the experiments with kidney vesicles at different pH conditions. As with vesicles from the ileum, at $pH_i = 8.0$, a Hill plot with a single slope near 1.0 was obtained; while at $pH_i = 6.0$, the data were characterized by two different slopes at opposite ends of the external sodium concentration range.

Discussion

General Characteristics of $J_{oi}^{SO_4}$

Inorganic sulphate transport across rabbit ileal brush border membranes occurs by a carrier-mediated transfer mechanism shared by sodium ion and capable of accumulating the anion above its electrochemical equilibrium (Smith et al., 1981). Total replacement of incubation medium Na^+ by $choline^+$ abolishes sulphate transport across this membrane and suggests the absence of significant Na^+-independent SO_4 transfer. Potassium (Fig. 1) and lithium (Langridge-Smith et al., 1983) only appear to provide weak support for the Na^+-dependent process. Kinetics of $J_{oi}^{SO_4}$ as a function of $[SO_4^{2-}]$ are hyperbolic at 10 and 100 mM Na^+ (Fig. 3), suggesting a single binding site for the anion on the membrane carrier. A considerable stereospecificity has been demonstrated for SO_4^{2-} binding to its transport site; thiosulphate ($S_2O_3^{2-}$) markedly reduced sulphate influx, but phosphate, methylsulphate, vanadate, and taurocholate were without effect (Langridge-Smith et al., 1983). The role of sodium in SO_4^{2-} influx is complex, but appears to be that of an affinity modifier (Fig. 3), enhancing anion binding to the carrier mechanism and subsequently accelerating its movement across the membrane.

Previous studies using sheets of rabbit ileum clamped in Ussing chambers have suggested a variable stoichiometry between Na^+ and SO_4^{2-} binding to the cotransport carrier; at low $[SO_4^{2-}]$ a flux ratio of $Na^+/SO_4^{2-}=1.0$ was proposed and at high $[SO_4^{2-}]$ the value increased to 2.0 (Langridge-Smith et al., 1983). Even though the number of Na^+ transported per SO_4^{2-} appeared to be variable in this investigation, it was suggested that electroneutrality was maintained under all conditions as a result of proton coupling when $Na^+/SO_4^{2-}=1.0$ and the lack of such coupling when two Na^+ accompanied the anion across the membrane. In this study it was not possible to measure a proton flux across the brush border to substantiate its involvement with anion movements, nor was the proposed electroneutral model tested by measuring SO_4^{2-} influx after imposing an electrical field across the membrane.

In rat ileum, Lucke et al. (1981) also proposed an electroneutral Na^+-sulphate cotransport for the brush border membrane, but in this instance membrane vesicles were used where direct control over the ionic composition bathing both membrane surfaces was possible. Those authors imposed a 50-mM potassium-gluconate gradient across the vesicle walls and rendered them selectively permeable to this cation by adding the ionophore valinomycin to the incubation medium. Using a 100-mM Na^+-gradient to facilitate Na^+-sulphate cotransport and a bilateral pH of 7.4, they were unable to show an effect of the K^+-induced transmembrane diffusion potential on SO_4^{2-} transfer in these vesicles and therefore concluded that these anion fluxes occurred by an electroneutral process. Several lines of evidence from the present investigation suggest that assigning an electroneutral model to SO_4^{2-} transport across mammalian intestinal brush border membranes under all conditions may be premature and that electrogenic anion flux may occur whenever flux stoichiometry of $Na^+/SO_4^{2-}=1.0$ takes place.

Role of H^+ in Modifying Na^+-Sulphate Cotransport

At $pH_i=pH_o=7.4$, $J_{oi}^{SO_4}$ was a sigmoidal function of external $[Na^+]$ at both high (4.0 mM) and low (0.2 mM) sulphate concentrations (Fig. 4). The sigmoidal shape of the influx curve suggests cooperativity between binding sodium ions in their influence on anion transport where the association of the first Na^+ to the membrane carrier would enhance the ability of the transfer mechanism to accommodate a second cation and in the process accelerate the movement of SO_4^{2-} across the vesicle wall. Hill plots displayed in Figs. 5 and 6 support the concept of sodium binding cooperativity under this set of pH conditions. At $[Na^+]_o$ ranging from 1.25 to 10 mM, Hill plots for $J_{oi}^{SO_4}$ at both $[SO_4^{2-}]_o$ exhibited slopes approximating unity, suggesting 1 Na^+/1 SO_4^{2-} cotransport. In contrast, higher $[Na^+]_o$ (25 to 150 mM) led to Hill plots with slopes near 2.0, reflecting a change in transport stoichiometry to 2 Na^+/1 SO_4^{2-}.

The experiments outlined in Figs. 7 and 8 indicate that altering the proton concentration on the two vesicle surfaces had marked effects upon the kinetics of Na^+-dependent SO_4^{2-} transport. Two specific changes in Na^+-sulphate cotransport were induced by pH manipulations: (1) alteration of the flux stoichiometry between Na^+ and SO_4^{2-} transport; and (2) modification of sodium binding affinity ($K_{Na}^{SO_4}$, $[Na^+]_o$ resulting in $^1/_2$ maximal $J_{oi}^{SO_4}$) and maximal anion influx rate ($J_{max}^{SO_4}$).

As indicated in Fig. 7*A*, when $pH_i=6.0$ and the external pH was either 6.0 or 8.0, $J_{oi}^{SO_4}$ was a sigmoidal function of external $[Na^+]$ and displayed Hill coefficients similar to those at $pH_i=pH_o=7.4$ (Figs. 5 and 6). Altering the internal pH to 8.0, at $pH_o=6.0$ or 8.0, markedly changed the

character of the relationship between $J_{oi}^{SO_4}$ and $[Na^+]_o$ to a hyperbolic function (Fig. 7*B*). Figure 8 shows that the change in $J_{oi}^{SO_4}$ from a sigmoidal to a hyperbolic function of $[Na^+]_o$ resulted from the loss of one externally binding sodium ion when internal proton concentrations were lowered. Therefore, the association of this particular Na^+ to its specific binding site on the external surface of the vesicle membrane may be regulated by protons binding to specific locations on the vesicular inner face. Flux stoichiometry between Na^+ and SO_4^{2-}, therefore, ranged between 1.0 and 2.0 and was determined over the entire $[Na^+]_o$ range by the pH occurring adjacent to the vesicle inner surface. Control of this flux stoichiometry appears possible over the relatively narrow internal pH range between 7.4 and 8.0, as these are the points where $J_{oi}^{SO_4}$ kinetics changed from sigmoidal to hyperbolic. In renal microvillus membrane vesicles, internal protons were shown to function as an allosteric activator of the Na^+/H^+ exchange carrier (Aronson, Nee & Suhm, 1982). It was suggested in this study that the Na^+/H^+ exchanger is a transmembrane protein with one or more inwardly facing titratable groups to which internal H^+ may bind and thereby induce a conformational change resulting in increased transport activity.

The second change in Na^+-sulphate cotransport induced by pH modifications concerned the relative values of the transport constants $K_{Na}^{SO_4}$ and $J_{max}^{SO_4}$. Figure 7*A* and *B* show that regardless of the internal pH conditions or the general shape of the transport curve, $J_{oi}^{SO_4}$ was greater at almost every external $[Na^+]$ when $pH_o = 6.0$ than when $pH_o = 8.0$. Two possible effects on $J_{oi}^{SO_4}$ by a high external concentration of protons might be expected. First, if conditions were appropriate for electrogenic sulphate transport (e.g., cotransport stoichiometry = 1 Na^+/1 SO_4^{2-}), and if the membrane exhibited a significant proton conductance, $J_{max}^{SO_4}$ might be influenced by a proton-generated transmembrane diffusion potential. G. Cassano, B. Stieger, and H. Murer (*unpublished observations*) have recently demonstrated proton transfer across rabbit ileal brush border membranes which was independent of Na^+/H^+ exchange and appeared conductive in character. The significant increase in $J_{max}^{SO_4}$ when $pH_i = 8.0$, $pH_o = 6.0$ over that occuring at bilateral pH = 8.0 (Figs. 7*B* and 8) argues for electrogenically elevated anion transport under the first condition in response to an electrically positive vesicle interior. Apparent lack of a proton gradient effect on $J_{max}^{SO_4}$ when the vesicle interior was at pH 6.0 (Fig. 7*A*) most likely was the result of electroneutral anion transport (e.g., cotransport stoichiometry = 2 Na^+/1 SO_4^{2-}) over much of the $[Na^+]_o$ range used.

The second possible effect of high external $[H^+]$ on $J_{oi}^{SO_4}$ was that protons may alter SO_4^{2-} transport by influencing the binding properties of the cotransport substrate, sodium. Significant increases in sodium binding affinity (decreases in $K_{Na}^{SO_4}$) were observed when $pH_o = 6.0$ as compared to those found at $pH_o = 8.0$ (Figs. 7*A*, *B* and 8). Since these external H^+ effects on Na^+ binding occurred whether one or two sodium ions were associated with the cotransport system, a modifier role of protons for the attachment of the Na^+ that is spatially closest to the anion binding site on the shared carrier is most likely. Binding of the second Na^+ may not be appreciably influenced by external protons.

Electrical Character of $J_{oi}^{SO_4}$

Apparent electrogenic movement of SO_4^{2-} in response to a transmembrane pH gradient does not, by itself, disprove an electroneutral transport model for this anion as proposed for rat ileum by Lucke et al. (1981) and for rabbit ileum by Langridge-Smith et al. (1983). However, the results shown in Fig. 9 indicate that, under certain conditions, $J_{oi}^{SO_4}$ is electrogenic and under others it is electroneutral. The key factor in determining the electrical character of this carrier mechanism is whether one or two sodium ions are being accommodated for each sulphate ion. At a bilateral pH of 8.0, Na^+ only associates with a single binding site over the entire $[Na^+]_o$ range used. Therefore, under these conditions electrogenic SO_4^{2-} transport occurs at both 5 and 50 mM Na^+ as the result of a constant flux stoichiometry of 1 Na^+/1 SO_4^{2-}. When pH 6.0 is present on both membrane surfaces, electrogenic SO_4^{2-} transport takes place at 5 mM Na^+ because only a single sodium ion is associated with the carrier system. At 50 mM Na^+ both cation sites are filled with sodium ions, resulting in electroneutral anion movement. Therefore, transmembrane SO_4^{2-} transport in rabbit ileal brush border vesicles is electrogenic when the flux stoichiometry is 1 Na^+/1 SO_4^{2-} and is electroneutral when this ratio approaches 2.0. External protons do not substitute for sodium at the Na^+ binding sites and are apparently not transferred across the membrane by this carrier mechanism.

A Model for SO_4^{2-} Transport

A model for carrier-mediated sulphate transport by rabbit ileal brush border membranes was pro-

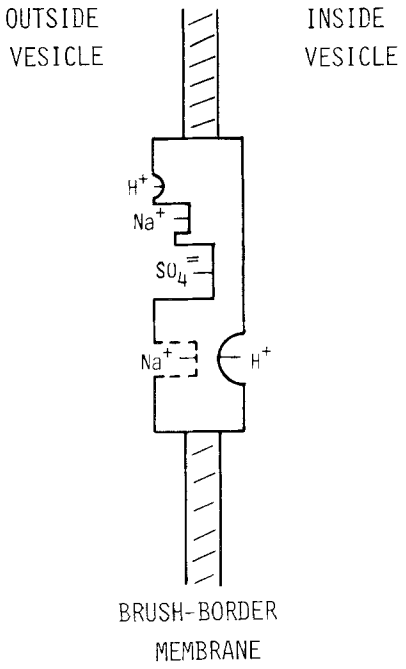

Fig. 11. Model for H^+-regulation of Na^+-dependent sulphate transport. A single transmembrane transport protein is shown illustrating two types of binding sites on the extravesicular carrier surface. Rectangular sites lead to $Na^+ + SO_4^{2-}$ movements or Na^+ movements alone across the membrane. The external circular binding site represents a modifier site that does not transfer its ligand to the vesicle interior, but instead, induces affinity modifications for Na^+ binding at the $Na^+ + SO_4^{2-}$ site. The second sodium transport binding locality is shown as a dashed rectangle, signifying that Na^+ may bind and be subsequently transferred across the membrane if the site is open as the result of protons binding to an internal, nontransporting, modifier site. This model suggests that protons regulate Na^+-dependent sulphate transport by modifying Na^+/SO_4^{2-} flux stoichiometry and by influencing the external binding affinity of the carrier for Na^+

posed by Langridge-Smith et al. (1983) which indicated two separate binding sites on the membrane carrier. The first site accommodated Na^+ alone, and the second either $NaSO_4^-$ or HSO_4^-. Under all experimental conditions this model predicted electroneutral SO_4^{2-} transport either as $Na^+ - H^+ - SO_4^{2-}$ or as $Na^+ - Na^+ - SO_4^{2-}$. Figure 11 of the present investigation is a modification of this previous model, considering the results obtained on SO_4^{2-} transport in membrane vesicles from the rabbit ileal brush border. As with the model by Langridge-Smith et al. (1983), two distinct transport binding sites occur on the SO_4^{2-} carrier, one accommodating $Na^+ + SO_4^{2-}$ and the other filled by Na^+ alone. The new model indicates the presence of two modifier binding sites for H^+ which do not lead to their transmembrane movement, but rather to the conformational alteration of the transport protein so that Na^+ binding is enhanced. The first H^+ modifier site is on the inside of the vesicle wall, and when activated (filled with H^+) by an internal pH lower than 8.0, leads to the opening of a specific Na^+ binding site on the external membrane surface. When this internal H^+ site is unfilled (e.g., at pH 8.0), the Na^+ binding site is closed and does not accommodate its cation. The second H^+ modifier site is on the outside surface of the membrane, close to the $Na^+ + SO_4^{2-}$ binding site, and when activated leads to enhanced Na^+ binding affinity (decreased $K_{Na}^{SO_4}$) at this location. Electrogenic SO_4^{2-} transport occurs whenever the inner H^+ modifier site is unfilled or, if filled, whenever insufficient external $[Na^+]$ occurs to accommodate both cation transport sites.

Mechanism of SO_4^{2-} Transport in Other Cell Types and Membranes

In order to establish the possible occurrence of the SO_4^{2-} transport mechanism illustrated in Fig. 11 in cells other than those of the intestine, $J_{oi}^{SO_4}$ was examined in rabbit kidney proximal tubule brush border membrane vesicles. Figure 10 shows the findings of this preliminary study and a comparison of the Hill slopes in this figure with those presented for the intestine in Figs. 5, 6, and 8 indicates that a similar anion transport system is most likely present in both locations.

Previous studies examining sulphate transport in rat kidney cortex proximal tubule brush border membrane using both microperfusion and vesicle techniques suggest that the relationship between luminal $[Na^+]$ and $J_{oi}^{SO_4}$ is hyperbolic and that cotransport between Na^+ and SO_4^{2-} is electroneutral in this species (Lucke, Stange & Murer, 1979; Ullrich, Rumrich & Kloss, 1980). In addition, an electroneutral SO_4^{2-} transport mechanism was previously proposed for rabbit proximal tubule membrane vesicles in a recently published abstract (Schneider, Durham & Sacktor, 1980). None of these investigations specifically examined the possible role of protons as modifiers of Na^+-sulphate cotransport stoichiometry, and they therefore were unable to observe possible variations in the electrical character of anion movements.

While a common H^+-regulated, Na^+-sulphate cotransport mechanism may occur in more than one type of mammalian epithelial brush border membrane, it is clear that other carrier systems for this anion are present in membranes from other cell types or organelles. Both erythrocyte and mitochondrial sulphate transport processes involve H^+-sulphate cotransport or sulphate/hydroxyl antiport (Jennings, 1976; Saris, 1980; Milanick & Gunn, 1982). In addition, a recent paper examining SO_4^{2-}

transport across the basolateral membrane of a marine teleost renal tubule has also presented evidence for proton-coupled anion transfer (Renfro & Pritchard, 1982). While it is conceivable that the differences observed in SO_4^{2-} transport in this variety of membranes may be due to markedly dissimilar carrier properties, a likely possibility is that a common anion transfer system, not unlike that illustrated in Fig. 11, may be present in all these locations and only differ substantially in its cation binding properties.

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