ANION TRANSPORT IN BRUSH BORDER MEMBRANES ISOLATED FROM RAT SMALL INTESTINE

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SUMMARY. Addition of sodium salts to isolated membrane vesicles induced a rapid release of protons from the vesicles, followed by a slower reuptake. As proton reuptake under the experimental conditions is dependent on simultaneous anion translocation, the rates of anion transport could be calculated from the changes in medium pH. Anion transport was measured in the absence and presence of carbonyl cyanide p-trifluoromethoxyphenylhydrazone, a proton carrier. It is concluded that the brush border membrane is relatively impermeable to cyclamate and that it contains conductance pathways for thiocyanate and nitrate as well as a chloride transport system catalyzing an electroneutral chloride-cation co-transport (chloride-hydroxyl exchange). For acetate, the protonated form rather than the anion appears to be the permeant species.

INTRODUCTION. Anion transport has been studied in mitochondria (1, 2), intact erythrocytes (3, 4, 5) and recently in resealed erythrocyte ghosts (6). The results suggest the existence of specific transport systems and reactions for many monovalent and multivalent anions. Mechanisms of anion translocation across the brush border membrane of the small intestine remain undefined; yet, information on these processes is essential for an understanding of electrolyte or sodium dependent non-electrolyte transport in intact enterocytes or isolated plasma membrane preparations. For example, it has been suggested that thiocyanate crosses the brush border membrane in the ionized form and, therefore, can support active D-glucose transport in vitro (7). However, direct evidence on the mode of translocation is missing.

We have approached the problem of measuring anion flow across the isolated intestinal brush border membrane by relying on the overall electroneutrality of salt transport and the existence of a rapid Na^+/H^+ exchange across this membrane (8). Due to the Na^+/H^+ antiporter, addition of sodium salts to suspensions of brush border membrane vesicles results in a rapid acidification of the medium. Since reuptake of protons depends on the anion translocation, rates of anion transport can be obtained from subsequent changes in medium pH back to equilibrium.

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METHODS AND MATERIALS. Brush border membranes of rat small intestine were prepared from male Sprague-Dawley rats weighing 150-250 g as reported previously (9). The purified brush border membranes were suspended in 0.1 M D-mannitol, 0.1 M $(Tris^+)_2SO_4$, pH 6.6, 0.1 mM $MgSO_4$, rehomogenized and collected by centrifugation at 25,000 x g for 10 min. The final membranes were resuspended in the same buffer containing, in addition, 5 mM $MgSO_4$. Salt solutions were added to aliquots of this suspension to initiate transport.

Salt solutions (1 M) were prepared in 0.1 M $(Tris^+)_2SO_4$, pH 6.6. Prior to salt additions, the pH of the salt solution was adjusted with Tris base or H_2SO_4 to the same pH as the suspension buffer for the membranes. In order to test for anion conductance, the proton carrier CF_3-CCP[1] was added to membranes before addition of salt. CF_3-CCP was prepared as 10 mM stock solution in 50% ethanol. The amount of CF_3-CCP in experiments was 3.4 nmoles/mg protein. The ethanol concentration in these experiments never exceeded 0.5%. Controls showed that ethanol up to 3% did not alter the rate of proton ejection or uptake by the membranes.

pH measurements were performed in a temperature-controlled (25°C) mixing chamber. 15 µl salt solutions were added to 285 µl membrane suspension (6-7 mg protein/ml) with a Hamilton syringe through a lateral inlet. The sample was continuously mixed by a magnetic stirrer. A planar glass electrode connected to a Radiometer pH meter (Model 64) and a Honeywell strip-chart recorder, was used to monitor the pH of the suspension. pH changes after addition of acid were complete within 1 sec in all experiments. The fresh pH-electrode did not show any detectable sodium error or transients at the pH used. However, over the course of several months, the electrode developed a transient response to the addition of sodium with a maximal amplitude of about 3 mV (equivalent to 0.05 pH units) and a time constant for decay of about 0.08 min. To correct for the sodium transients, salt solutions were added to vesicle suspensions pretreated with 0.3% Triton X-100 and the apparent pH changes subtracted from the observed values with intact membranes. Because of this experimental limitation the rate of acetate transport (Table 1) may be underestimated.

The planar glass electrode was a gift of Dr. D. Gray, Owens-Illinois, Toledo, Ohio. CF_3-CCP was kindly given to us by Dr. Heytler, DuPont de Nemours and Co., Wilmington, Delaware. Other chemicals were of the highest grade available.

RESULTS AND DISCUSSION. Figure 1 illustrates typical recordings of pH changes after addition of sodium salts to a final concentration of 50 mM, to brush border membranes. The initial response was an acidification of the medium presumably due to Na^+/H^+ exchange across the membrane (8). To ensure sufficient intravesicular buffer capacity during this initial proton loss from the vesicles, 100 mM $Tris^+$ is used at about 1.5 pH units below its pK. Figure 1 also demonstrates a subsequent relaxation of the medium pH to a new more alkaline equilibrium. Addition of Triton X-100 (final concentration 0.3% w/v) to destroy the barrier property of the membranes did not shift the equilibrium pH for sodium chloride, acetate, nitrate, and thiocyanate, but did increase the pH for vesicles exposed to sodium cyclamate.

The initial amount of protons ejected from the vesicles was proportional to protein concentration, at least up to about 17 mg/ml, and also increased with increasing sodium

[1] CF_3-CCP = carbonyl cyanide p-trifluoromethoxyphenylhydrazone

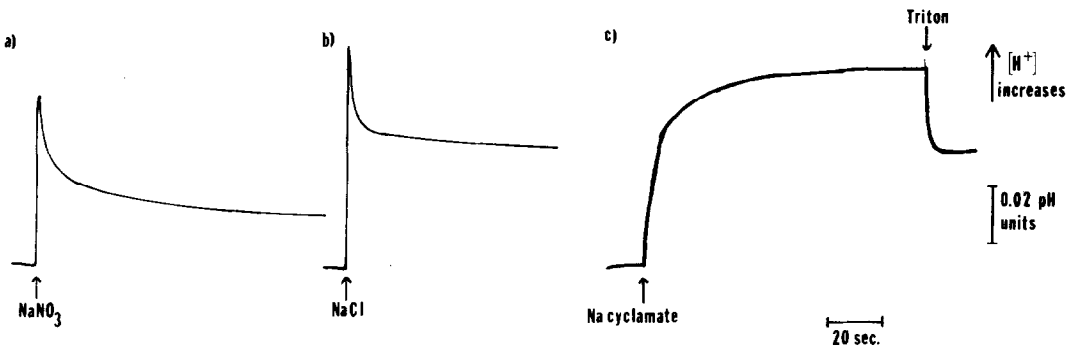
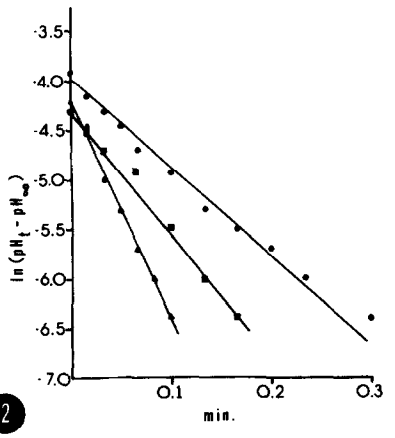


Fig. 1. Anion-dependent pH-changes after sodium salt addition to intestinal brush border membrane. 15 µl of a 1 M salt solution was added to 285 µl membranes (6-7 mg protein/ml) where indicated: a) $NaNO_3$, b) NaCl, and c) Na cyclamate. In the case of Na cyclamate, Triton X-100 (final concentration 0.3% w/v) was added after the quasi-equilibrium state had been attained. Note the pH -shift after Triton treatment.

concentration. The response appeared to be specific for sodium as it was not seen with potassium salts. These three observations support the interpretation of the initial medium acidification as a result of rapid Na^+/H^+ exchange across the vesicle membrane.

The reuptake of protons by the membranes can be described by an exponential curve as illustrated in Figure 2. The slope of the curve calculated from the regression line, has the dimensions of a rate constant (τ^{-1}) characteristic for each anion. τ^{-1} was found to be independent of protein concentration up to about 30 mg/ml.

As the overall ion uptake by membrane vesicles is electrically neutral, the reuptake of protons depends on concomitant transport of anions. The uptake of protons can be coupled to anion uptake either on a molecular level by co-transport via a specific "carrier" or by permeation of the undissociated acid if present in sufficient concentration (Figure 3A). Obviously, in membranes with high water permeability anion-proton co-transport cannot be distinguished from and is equivalent to anion-hydroxyl exchange across the membrane. An alternative to a molecular coupling mechanism is the presence of separate conductance pathways for protons and anions and coupling of their fluxes by the electric field (Figure 3Bα and β). It is possible to differentiate between these modes of transport by specifically increasing the proton conductance of the membrane; this was achieved by preincubating vesicles with the proton carrier CF_3-CCP (10). The



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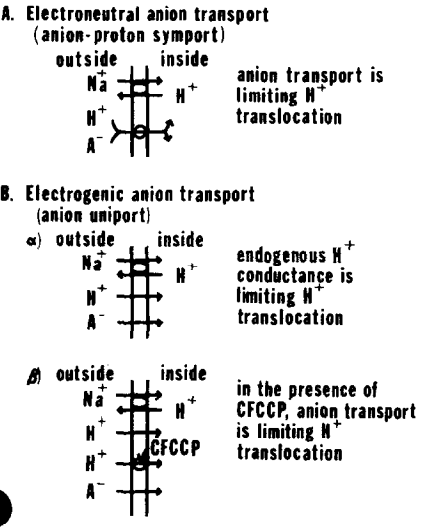

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Fig. 2. Decay of the pH-gradient generated by the Na^+/H^+ antiporter upon addition of sodium salts to membranes. Ln (pH_t - pH_∞) is calculated from recordings as shown in Fig. 1. CF_3-CCP (3.4 nmoles/mg protein), when present, was added to membranes before salt addition. ● NaSCN, ▲ NaSCN in presence of CF_3-CCP, ■ NaCl.

Fig. 3. Relationship of pH-changes to anion transport. The direction of ion flow is given for the experimental condition of salt addition to the outside. A^- represents permeant monovalent anions.

rate of anion-proton co-transport should not be influenced by an increase in proton conductance. However, the rate of electrogenic anion fluxes under the same experimental conditions should increase (Figure 3Bβ) if endogenous proton conductance was limiting anion uptake in the absence of CF_3-CCP.

Table 1 presents the relative rate constants (τ^{-1}) for monovalent anions in the absence (column 1) and the presence of CF_3-CCP (column 2). Column 3 is the calculated CF_3-CCP-dependent anion uptake. The following order for rates of uptake without added proton carrier was obtained: acetate > chloride > nitrate > thiocyanate > cyclamate. Note that for thiocyanate and nitrate, τ^{-1} increases with an increase in proton conductance (Table 1, column 2), suggesting that the endogenous proton conductance limits the transport of these anions in the absence of CF_3-CCP (Figure 3Bα). With the proton carrier present, about half of the nitrate and most of the thiocyanate translocation appears to occur via a conductance pathway (Table 1, column 3). Thus, the

Table 1. Rate of Anion Uptake in Intestinal Brush Border Membranes

Anion	Rate of Translocation (min^{-1}) *	with CF_3-CCP	CF_3-CCP-Dependent Anion Uptake #
Chloride	9.8	4.5	
Acetate	29.9	21.1	
Nitrate	4.8	9.5	49%
Thiocyanate	3.9	13.0	70%
Cyclamate	0.08	0.09	

*obtained from pH-changes with time plotted as in Fig. 2.

$\# \equiv 100 \times (\tau^{-1}_{CF_3\text{-CCP}} - \tau^{-1}) \div \tau^{-1}_{CF_3\text{-CCP}}$

rate of thiocyanate transport without CF_3-CCP provides an estimate for the upper limit of the endogenous proton conductance. In contrast to thiocyanate and nitrate, the slow uptake rate of cyclamate is not significantly enhanced by CF_3-CCP, indicating that the brush border membrane is relatively impermeable to this anion.

Interestingly, the rates of chloride and acetate transport in the absence of a proton carrier are already greater than can be explained by the endogenous proton conductance, and, hence, an electroneutral co-transport of these anions with a cation (proton or sodium) must occur. Quantitatively, a minimum of 60% of chloride uptake and 87% of acetate uptake appears to proceed via an electroneutral mechanism under our conditions, as calculated from the ratios of 2.5 and 7.7 for chloride to thiocyanate and acetate to thiocyanate transport, respectively. It is noteworthy that CF_3-CCP slowed down the rate of chloride transport and, to some extent, that of acetate (Table 1, column 2). This decrease in rate cannot be explained on the basis of a CF_3-CCP-induced proton conductance, thus suggesting a secondary effect which may involve interaction of CF_3-CCP with an anion carrier or a change in surface charge.

The observed electroneutral acetate transport is probably due to the high permeability of lipid membranes to undissociated acetic acid (11). In contrast, for chloride a

"carrier" has to be postulated since the concentration of undissociated HCl is negligible at pH 6.6.

The transport rates for the various anions were quite reproducible for different membrane preparations of animals from the same batch and similar age. However, there was variation with animals of differing age and from different batches. Nevertheless, the effect of CF_3-CCP was consistent in all experiments. No attempt was made to systematically investigate the effect of age and strain of rats and diets on anion transport.

The results of these experiments with membrane vesicles are in general agreement with conclusions about ion permeabilities inferred from the anion dependence of Na^+-nonelectrolyte co-transport in membrane vesicles and electrolyte transport in intact epithelial sheets. For example, gradients of anions for which the membrane possesses appreciable conductance would be expected to enhance sugar or amino acid uptake via electrogenic transport systems in brush border membrane vesicles. Thus, the order of efficacy in supporting electrogenic non-electrolyte uptake predicted from anion conductance as measured in Table 1 would be: $SCN^- \geq NO_3^- >> Cl^- \geq CH_3COO^- \geq$ cyclamate . This sequence actually has been observed for Na^+-dependent D-glucose and L-alanine transport (7, 9, 12, 13, and[2]).

Nellans, Frizzell and Schultz have measured Na^+ and Cl^- entry into enterocytes across the brush border in intact epithelial sheets (14). They concluded that a large proportion of sodium and chloride transport across this membrane is electroneutral and tightly coupled, therefore, suggesting a carrier catalyzing NaCl symport. The electroneutrality of NaCl transport is confirmed in this study with isolated membrane vesicles. However, it is important to point out that sodium-chloride symport is not necessarily the only way to achieve electroneutral ion transfer. A Na^+/H^+ exchange reaction in combination with a Cl^--H^+ symport would also be electroneutral. The pattern of pH changes after sodium salt addition to the membranes actually favors the latter interpretation and offers the additional information that sodium uptake is much faster

[2]Unpublished observation for nitrate and cyclamate with intestinal brush border membranes.

than that of chloride. This method of using pH as an indicator of anion transport is very convenient and should prove useful in further investigations of anion transport across the brush border membrane.

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