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Characterization of a Na : K : 2Cl Cotransport System in the Apical Membrane of a Renal Epithelial Cell Line (LLC-PK$_1$)

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Summary. ^{86}Rb uptake into LLC-PK$_1$ cells (an established renal epithelial cell line) was found to be comprised of an active ouabain-sensitive component, a loop diuretic-sensitive component which was passive and strictly dependent upon the presence of extracellular Na^+ and Cl^- for activity, and a "leak" component. The diuretic-sensitive component of influx was investigated further in apical membrane vesicles derived from these cells. A large fraction of ^{86}Rb, ^{22}Na and ^{36}Cl flux into these vesicles was sensitive to inhibition by furosemide and dependent upon the presence of the other two co-ions, in keeping with the presence of a loop diuretic-sensitive $Na^+ : K^+ : Cl^-$ cotransport system. The kinetic parameters for Na^+ and K^+ interaction have been analyzed under initial linear zero *trans* conditions. The following values were obtained: $K_{mNa^+} = 0.42 \pm 0.05$ mmol/liter, $V_{max} = 303 \pm 24$ pmol/mg/6 sec; $K_{mK^+} = 11.9 \pm 1.0$ mmol/liter, $V_{maxK^+} = 307 \pm 27$ pmol/mg/6 sec. For Cl^- interaction evidence for two cooperative binding sites with different affinities and different specificities were obtained. Thus, a stoichiometry of $1Na^+ : 1K^+ : 2Cl^-$ can be calculated. It is concluded that the apical membrane of LLC-PK$_1$ cells contains a $Na^+ : K^+ : 2Cl^-$ cotransport system with properties similar to those described for the thick ascending limb of the loop of Henle.

Key Words Na : K : 2Cl cotransport · furosemide/piretanide · renal epithelium · epithelial tissue culture · thick ascending limb · membrane vesicles

Introduction

During the last few years, loop diuretic-sensitive coupled Na : K : Cl cotransport systems have been identified in a number of tissues of diverse origins (Aiton et al., 1981; Saier & Boydon, 1984). In epithelial tissue, the transport system has been implicated in both the absorption and the secretion of NaCl (Saier & Boydon, 1984), and in consequence the location of the cotransport system to either the apical or basolateral cell membrane has been found to be dependent primarily upon the functional role of the epithelium. Thus in NaCl absorptive tissue such as flounder small intestine (Musch et al., 1982) or the thick ascending limb of the loop of Henle (TALH) cells (Greger & Schlatter, 1983) the cotransport system appears to be located on the apical cell membrane. In contrast to this, in secretory epithelia such as shark rectal gland (Hannafin et al., 1983) or canine tracheal epithelia (Welsh, 1983*a*) the cotransport system occupies an exclusively basolateral location. In both absorptive and secretory epithelia electrophysiological data, derived from both conventional and ion-selective microelectrodes, suggest that the cotransport system mediates a large part of the energetically uphill movement of Cl^- into the cell (Welsh et al., 1980; Greger et al., 1983; Welsh, 1983*b*). The energy necessary for this step is thought to be derived from the energy inherent in the sum of the individual ion gradients for Na^+, K^+ and Cl^- (Haas et al., 1982; Saier & Boydon, 1984). More recently, data concerning the kinetics and the coupling ratios between Na^+, K^+ and Cl^- have been reported for plasma membrane vesicles derived from cortical TALH cells (Koenig et al., 1983) and from basolateral membrane vesicles from shark rectal glands (Hannafin et al., 1983).

In this report, we describe the properties of a loop diuretic-sensitive cotransport system in the apical membrane of a renal derived cell line: LLC-PK$_1$. The LLC-PK$_1$ cell line has been used extensively in the past as a model to study the development and cellular control of a number of nephronal transport systems, e.g. Na : glucose (Amsler & Cook, 1982; Moran et al., 1983) and Na : phosphate cotransport (Brown et al., 1984; Biber & Murer, 1985; Caverzasio et al., 1985). The identification of a diuretic-sensitive coupled Na : K : Cl cotransport system in the apical membrane of this cell line provides a model to study the properties and the regulation of this system by various factors.

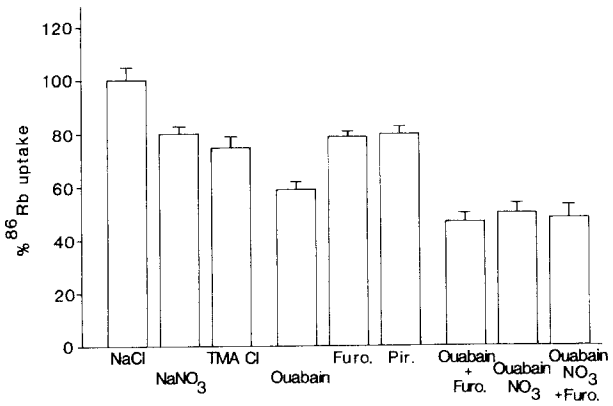

Fig. 1. ^{86}Rb uptake into LLC-PK_1 cell monolayers. K^+ influx into confluent LLC-PK_1 monolayers was measured using ^{86}Rb as a tracer for K^+ and a 5-min influx period. Tracer influx under control conditions (137 mmol/liter NaCl) is compared with uptake in the absence of Na^+ (TMA replacement) and of Cl^{-1} (NO_3^- replacement). Similarly, ^{86}Rb uptake was measured in the presence of ouabain (1.0 mmol/liter), furosemide (0.1 mmol/liter), piretanide (0.1 mmol/liter) and ouabain + furosemide, ouabain with NO_3^- replacement and ouabain with NO_3^- replacement + furosemide. The results are the mean ± SD of three determinations

Materials and Methods

Cell Culture and Membrane Isolation

LLC-PK_1 cells were originally purchased from Flow Laboratories (Irvine, Scotland) at 148 serial passages. The cells were maintained in culture as previously described (Biber et al., 1983). For cellular uptake cells were seeded onto tissue culture dishes (35 mm diam) at a density of approximately 1×10^4 cells/cm^2 and the dishes used for experimental purposes after 4 to 5 days in culture. At this point the monolayers were confluent and exhibited dome formation. For isolation of apical membrane vesicles, cells were grown to confluence in 890 cm^2 growth area glass roller bottles. In this case the cells were harvested after 8 to 10 days in culture. Confluence was generally obtained by day 6. Apical membrane vesicles were prepared as previously described (Brown et al., 1984) except that the initial cell suspension was obtained by scraping the cells from the roller bottles with a rubber policeman. The isolation procedure was essentially identical to the $MgCl_2$ precipitation method of Biber et al. (1981). In the final membrane pellet the apical membrane markers alkaline phosphatase and leucine aminopeptidase were enriched 7 to 8 times as compared to the homogenate; in contrast the membranes were not enriched of $Na^+ + K^+$-ATPase as we reported previously (Brown et al., 1984).

Measurement of Na^+, K^+ and Cl^- Tracer Uptake

Cellular uptake of ^{86}Rb, as a tracer for K^+, was measured as previously described (Aiton et al., 1982). A 5-min uptake point was used and the incubation solution contained (in mmol/liter): 137 NaCl, 5.4 KCl, 2.8 $CaCl_2$, 1.2 $MgSO_4$, 0.6 NaH_2PO_4, 10 HEPES/Tris and 10 glucose at pH 7.4 and 37°C. Na^+-free solution was prepared by replacing NaCl and NaH_2PO_4 by choline chloride and KH_2PO_4. A Cl^--free solution was prepared by replacing NaCl and KCl by $NaNO_3$ and KNO_3. The uptake was stopped by adding 2 ml of cold incubation media and by washing the plates 2 times with 2 ml of this solution. The radioactivity was then extracted in 1 ml of a 2% SDS solution and a sample prepared for liquid scintillation counting.

The uptake of ^{22}Na, ^{36}Cl and ^{86}Rb into apical membrane vesicles from LLC-PK_1 cells was measured using a rapid filtration technique as previously described (Berner et al., 1976). For initial time points (0 to 6 sec) a semi-automatic uptake device was used (Kessler et al., 1978). All uptake experiments with vesicles were performed at room temperature (22 to 23°C). The membrane vesicles were usually suspended in a membrane buffer containing (in mmol/liter): 150 $TMANO_3$, 1 $MgSO_4$ and 10 HEPES/Tris at pH 7.4. For the study of the Cl^--dependence of ^{86}Rb uptake, $TMANO_3$ was occasionally replaced by TMA gluconate (*see* Results section for details). The composition of the incubation media is given in the appropriate figure legend. Uptake was initiated by mixing 10 μl of membrane suspension (50 μg prot) with 10 μl of labeled incubation media (containing 1 μCi tracer). The reaction was terminated after the appropriate time by the addition of 1 ml of stop solution at 4°C followed by rapid filtration through a 0.6 μm cellulose nitrate filter. The membranes were then washed rapidly twice with 3 ml of cold stop solution. The stop solution contained (in mmol/liter): 150 $TMANO_3$, 150 KNO_3, 1 $MgSO_4$, 10 HEPES/Tris and 0.1 furosemide at pH 7.4. Nonspecific binding was determined by the addition of labeled incubation media to 1 ml of stop solution containing 50 μg of membranes followed immediately by filtration and washing of the membranes as described above. Nonspecific binding was less than 10% of the total equilibrium uptake at 120 min. ^{86}Rb uptake through the cotransport system was calculated from the total ^{86}Rb uptake minus ^{86}Rb uptake measured in the absence of extracellular Cl^-. This maneuver gave identical results as calculating the flux from the total uptake minus uptake in the presence of 0.1 mmol/liter furosemide (*see*, for example, Figs. 2–5). Protein was measured by the method of Bradford (1976) using a commercially available kit (BioRad reagents).

All experimental data are expressed as the mean ± standard deviation of three or more separate experiments. Within an experiment all points were made in quadruplicate, unless otherwise stated.

Materials

All cell culture materials were purchased from Flow Laboratories, Baar, Switzerland. ^{22}Na and ^{86}Rb (both carrier free) were obtained from New England Nuclear (Boston). ^{36}Cl as HCl was purchased from Amersham International. The semi-automatic uptake device was built by Innovativ A.G., Adliswil, Switzerland. All other chemicals were of the purest grades available and were purchased either from Merck, Darmstadt, FRG or from Fluka A.G., Buchs, Switzerland. Furosemide and piretanide were gifts from R. Greger, Frankfurt, FRG.

Results

Cellular Uptake of ^{86}Rb by LLC-PK_1 Cells

^{86}Rb tracer fluxes were used for the analysis of the physiologically occurring K^+ uptake into LLC-PK_1

cells. As will be shown below Rb is apparently accepted instead of K by the transport system studied in detail. Figure 1 shows that similar to a number of other cell systems (Aiton et al., 1981), ^{86}Rb uptake into LLC-PK_1 cells can be attributed to at least three pharmacologically distinct uptake pathways: A large proportion of the ^{86}Rb uptake (43%) into LLC-PK_1 cells was sensitive to inhibition by the cardiac glycoside ouabain at a concentration of 1.0 mmol/liter. This component of influx probably represents active K^+ influx across the basolateral membrane via the $Na^+ + K^+$-ATPase. A second component of ^{86}Rb uptake (20%) was sensitive to inhibition by the loop diuretics furosemide and piretanide (at 0.1 mmol/liter). This component was passive and independent of inhibition of the $Na^+ + K^+$-ATPase by ouabain. As has been shown previously for MDCK-cells (Aiton et al., 1982) a similar inhibition of ^{86}Rb uptake as with furosemide was observed when either Na^+ or Cl^- ions were removed from the uptake media (Fig. 1). That the loop diuretic-sensitive component of uptake and the Na^+ and Cl^--dependent component of uptake were identical was inferred from the result that addition of furosemide to a Na^+ or Cl^--free uptake media resulted in no further inhibition of uptake. This is in contrast to the additivity of inhibition observed when furosemide is added to a ouabain-containing uptake media (Fig. 1). The furosemide sensitivity and strict dependence of a large part of the passive ^{86}Rb uptake upon both the presence of Na^+ and Cl^- ions support the presence of a $Na^+ : K^+ : Cl^-$ cotransport system in these cells. A third component of influx is insensitive to inhibition by either ouabain or furosemide (Fig. 1) and probably represents the sum of all other passive permeability pathways for ^{86}Rb influx into the cell.

Uptake of ^{86}Rb, ^{36}Cl and ^{22}Na into Apical Membrane Vesicles of LLC-PK_1 Cells

In light of the findings mentioned above it was decided to investigate whether the cotransport system was present in the apical membrane of LLC-PK_1 cells. Figure 2 shows that ^{86}Rb uptake into LLC-PK_1 apical membrane vesicles is equilibrating; uptake in the first 3 min of measurement is over 65% of the final equilibrium value. Furthermore ^{86}Rb uptake is markedly stimulated in the presence of inwardly directed Na^+ and Cl^- gradients: Over the first 3 min of measurement ^{86}Rb uptake in the presence of a 100-mmol/liter NaCl gradient is nearly twice as great as in the presence of similar gradients of either TMACl or of $NaNO_3$ (Fig. 2). Similarly, addition of 0.1 mmol/liter furosemide to the membranes 60 sec before initiating uptake reduced the rate of ^{86}Rb influx by a similar amount as found with

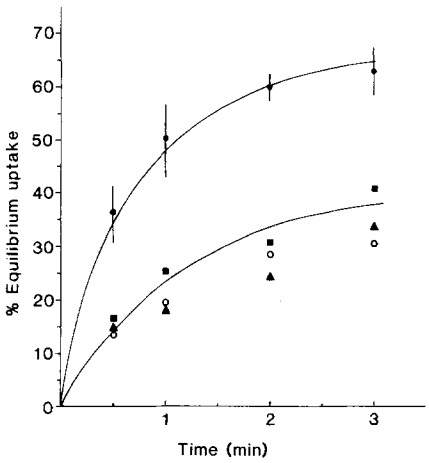

Fig. 2. Time course of ^{86}Rb uptake into LLC-PK_1 apical membrane vesicles. The vesicles contained (in mmol/liter): 150 $TMANO_3$, 1 $MgSO_4$ and 10 HEPES/Tris at pH 7.4. The incubation media contained in addition 0.5 mmol/liter $^{86}RbCl$ (2 μCi/point) and either 100 mmol/liter NaCl (●), 100 mmol/liter $NaNO_3$(▲), 100 mmol/liter TMACl (■) or 100 mmol/liter NaCl + 0.1 mmol/liter furosemide (○). The results are expressed as a % of the NaCl equilibrium uptake at 90 min (330 ± 12 pmol/mg prot)

the removal of Na^+ or Cl^- from the incubation media. These initial observations show the presence of a diuretic-sensitive cotransport system in the apical membrane of LLC-PK_1 cells.

A feature of a cotransport system is that the fluxes of the transported substrates are tightly coupled so that it should be possible to demonstrate not only the Na^+ and Cl^- dependence of a large part of the ^{86}Rb uptake (Fig. 2) but also that a similar proportion of both Na^+ and Cl^- uptake into these vesicles is dependent upon the presence of the two other ions. For ^{22}Na uptake this is shown in Fig. 3, where ^{22}Na uptake in the presence of an inwardly directed 100-mmol/liter KCl gradient is nearly twice as great as ^{22}Na uptake measured in the presence of similar gradients of either KNO_3, TMACl, or NaCl + 0.1 mmol/liter furosemide over the first 3 min of measurement. In view of the recent report of a $Na^+ : H^+$ exchange mechanism in these cells (Chaillet et al., 1985) at least part of the furosemide-insensitive ^{22}Na uptake is probably mediated through this mechanism. Figure 4 shows similar data for ^{36}Cl uptake by LLC-PK_1 apical membrane vesicles. In this case however, ^{36}Cl uptake was greatest when a combined 50 mmol/liter $NaNO_3$/50 mmol/liter KNO_3 gradient was present compared to similar gradients of $KNO_3/TMANO_3$ or $NaNO_3/TMANO_3$ or to a 100-mmol/liter gradient of $NaNO_3/KNO_3$ plus 0.1 mmol/liter furosemide. Calculation of the equilibrium volume of the membrane vesicles gave values of 0.66, 0.68 and 0.64 μl/mg/prot, respectively, from the equilibrium uptake values for ^{86}Rb, ^{22}Na and ^{36}Cl. Taken together these results clearly

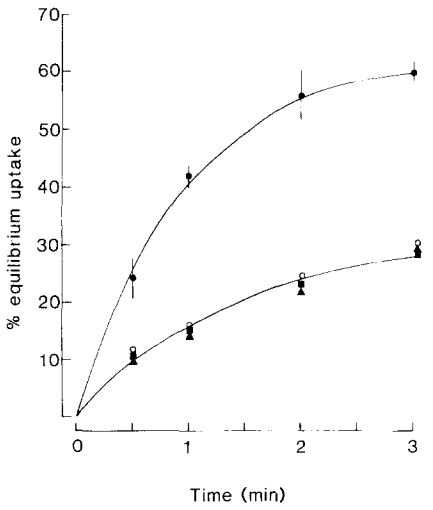

Fig. 3. Time course of ^{22}Na uptake into LLC-PK$_1$ apical membrane vesicles. The vesicles contained (in mmol/liter): 150 $TMANO_3$, 1 $MgSO_4$ and 10 HEPES/Tris at pH 7.4. The incubation media contained in addition 0.25 mmol/liter ^{22}Na (2 μCi/point) and either 100 mmol/liter KCl (●), 100 mmol/liter TMACl (■), 100 mmol/liter KNO_3 (▲) or 100 mmol/liter KCl + 0.1 mmol/liter furosemide (○). The results are expressed as a % of the KCl equilibrium value at 90 min (170 ± 9 pmol/mg prot)

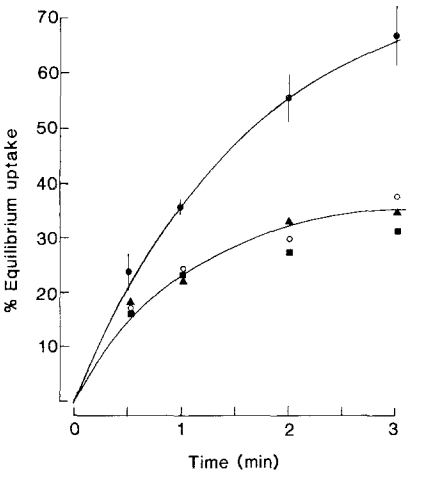

Fig. 4. Time course of ^{36}Cl uptake into LLC-PK$_1$ apical membrane vesicles. The vesicles contained (in mmol/liter): 150 $TMANO_3$, 1 $MgSO_4$ and 10 HEPES/Tris at pH 7.4. The incubation media contained in addition 13 mmol/liter $Cs^{36}Cl$ (2 μCi/point) and either 50 mmol/liter $NaNO_3$ + 50 mmol/liter KNO_3 (●), 50 mmol/liter $NaNO_3$ + 50 mmol/liter $TMANO_3$ (▲), 50 mmol/liter KNO_3 + 50 mmol/liter $TMANO_3$ (■) or 50 mmol/liter $NaNO_3$ + 50 mmol/liter KNO_3 + 0.1 mmol/liter furosemide (○). The results are expressed as a % of the equilibrium value at 90 min in the presence of both Na^+ and K^+ (8.4 nmol/mg prot)

demonstrate that a large part of the influx of Na^+, K^+ and Cl^- into apical membrane vesicles is tightly coupled and sensitive to inhibition by the loop diuretic furosemide. These findings are consistent

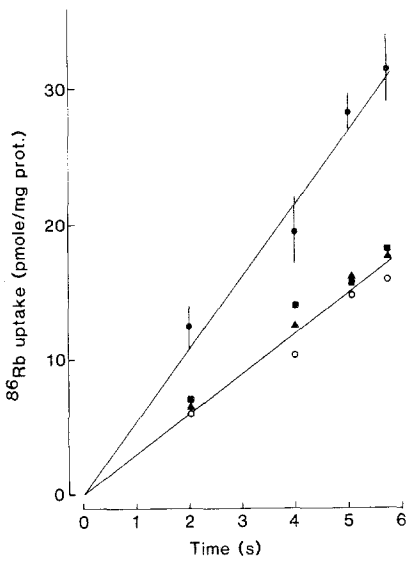

Fig. 5. Initial linear uptake of ^{86}Rb into apical membrane vesicles. Initial linear uptake was measured at an ^{86}Rb concentration of 0.4 mmol/liter. Uptake conditions were as described in Fig. 2 with 100 mmol/liter gradients of either NaCl (●), $NaNO_3$ (▲), TMACl (■) or NaCl + 0.1 mmol/liter furosemide (○). In all cases uptake remained linear for at least the first 6 sec of uptake (r^2 = 0.95)

with the presence of a cotransport system for Na^+, K^+ and Cl^-.

Kinetic Analysis of ^{86}Rb Uptake by LLC-PK$_1$ Apical Membrane Vesicles

Since measurement of either ^{22}Na, ^{86}Rb or ^{36}Cl uptake gave qualitatively the same result (Figs. 2–4) we decided to concentrate upon the uptake of ^{86}Rb as a measure of cotransport activity. Figure 5 shows the initial linear uptake of ^{86}Rb into LLC-PK$_1$ apical membrane vesicles. As we described in Fig. 2, ^{86}Rb uptake in the presence of a 100-mmol/liter NaCl gradient is twice that measured if either Na^+ or Cl^- ions are not present or if the membranes are exposed to furosemide (0.1 mmol/liter). Under all conditions, ^{86}Rb uptake was linear for at least the first 10 sec of measurement (linear regression; r^2 = 0.95).

K^+ Dependence of the Cl^--Dependent Component of ^{86}Rb Uptake

Figure 6*a* shows the effect of increasing the extravesicular K^+ upon the Cl^--dependent component of ^{86}Rb uptake by LLC-PK$_1$ apical membrane vesicles. Under V_{max} driving conditions for Na^+ and Cl^- (a 100 mmol/liter NaCl gradient) increasing extravesicular K^+ led to a saturable increase in ^{86}Rb up-

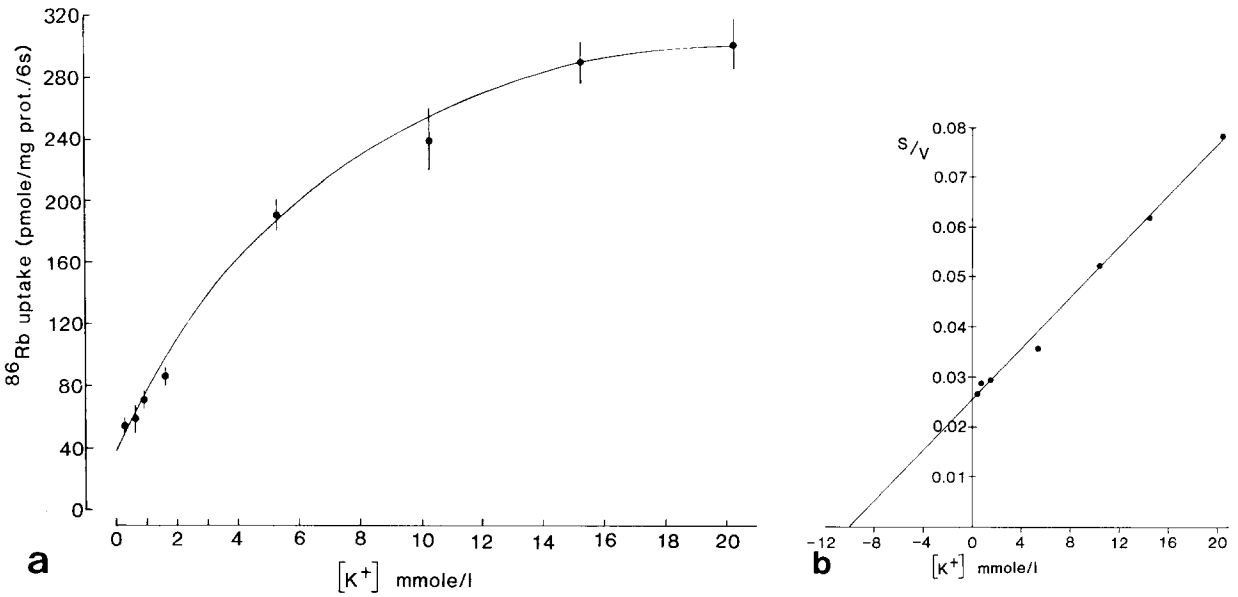

Fig. 6. (*a*) K^+ dependence of the Cl^--dependent component of ^{86}Rb [K^+] uptake. The ability of K^+ ions to stimulate Cl^--dependent ^{86}Rb uptake was measured using 6-sec uptake point. The vesicles contained (in mmol/liter): 150 $TMANO_3$, 1 $MgSO_4$ and 10 HEPES/Tris at pH 7.4. The incubation media contained in addition 100 mmol/liter NaCl and various amounts of KCl or TMACl to vary K between 0.5 and 20 mmol/liter. In the calculations we have assumed, that ^{86}Rb would be a perfect substitute for K^+ in the transport process, i.e. ^{86}Rb uptake stands for K^+ uptake. (*b*) A Hanes-Woolf plot of K^+ stimulation of the Cl^--dependent component of ^{86}Rb [K^+] uptake. The data presented in Fig. 6*a* were analyzed using a Hanes-Woolf plot. The regression line ($r^2 = 0.98$) gave an apparent K_m of 11.9 ± 1.0 mmol/liter K^+ and an apparent V_{max} of 307 ± 27 pmol/mg prot/6 sec

take. An identical curve was obtained when Rb^+ was substituted for K^+ (*data not shown*). Kinetic analysis of the data presented in Fig. 6*a* using a Hanes-Woolf plot (Fig. 6*b*) gave an apparent K_m of 11.9 ± 1.0 mmol/liter K^+ and a V_{max} of 307.4 ± 27.6 pmol/mg prot/6 sec. Hill analysis of the data presented in Fig. 6*a* gave a Hill coefficient of 0.97 ± 0.06 (Table) consistent with the interaction of 1 K^+ ion with the transport system. For the measurement of the Na^+ and Cl^- dependence of ^{86}Rb uptake near V_{max} driving conditions were chosen. These conditions were a 100-mmol/liter gradient of either Na^+ or Cl^- and the highest dilution of the isotope with K^+ as was feasible to work with (10 mmol/liter K^+). Under these conditions uptake was still linear over the first 6 sec of measurement (*data not shown*).

The Na^+ Dependence of the Cl^--Dependent Component of ^{86}Rb Uptake

The Na^+ dependence of the Cl^--dependent component of ^{86}Rb uptake is shown in Fig. 7*a*. Again, under near V_{max} conditions (a 100-mmol/liter Cl^- and a 10-mmol/liter K^+ gradient) Na^+ ions stimulate ^{86}Rb uptake in a saturable manner. Analysis of this data, using a Hanes-Woolf plot (Fig. 7*b*) and by Hill analysis (Table) gave an apparent K_m of 0.42 ± 0.05 mmol/liter Na^+, a V_{max} of 303 ± 23.9 pmol/mg prot/6 sec and a Hill coefficient of 1.05 ± 0.06.

The Cl^- Dependence of the Furosemide-Sensitive Component of ^{86}Rb Uptake

Initial attempts to determine the kinetics and stoichiometry of the Cl^- dependence of the cotransport system were, similar to the experiments described above, performed using NO_3^- as the replacement anion for Cl^-. The results of these experiments are shown in Fig. 8 and summarized in the Table. Under near V_{max} conditions for the other 2 co-ions (100 mmol/liter Na^+ and 10 mmol/liter K^+) Cl^- ions were able to stimulate ^{86}Rb uptake in a saturable manner. In contrast to expectations based on previous publications on similar systems (Saier & Boydon, 1984), where a sigmoidal relationship between uptake and Cl^- concentration was observed, we found that our data followed simple Michaelis-Menten kinetics and the best fit was with a hyperbolic curve (Fig. 8). Kinetic analysis of the data presented in Fig. 8 using a Hanes-Woolf plot gave an apparent K_m of 119 ± 0.2 mmol/liter Cl^- and an apparent V_{max} of 220 ± 6 pmol/mg prot/6 sec. Replotting the Hanes-Woolf plot with the Cl^- concentration raised to different powers, e.g. $[Cl^-]^1$, $[Cl^-]^{1.5}$ or $[Cl^-]^2$, reduced the correlation coefficient of the regression line from 0.98 ± 0.06 to 0.43 ± 0.04. Taken together with a Hill coefficient of 0.96 ± 0.04 (Table), these data suggest the interaction of only 1 Cl^- ion with the cotransport system in the presence of a high nitrate concentration.

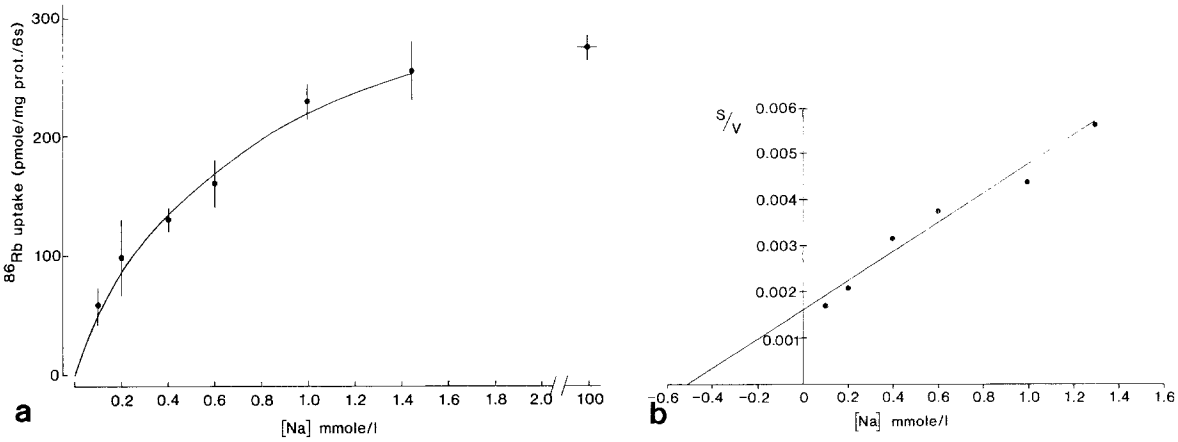

Fig. 7. (*a*) Na^- dependence of the Cl^--dependent component of ^{86}Rb uptake. The ability of Na^+ ions to stimulate ^{86}Rb uptake through the cotransport system was measured using a 6-sec uptake point. The vesicles contained (in mmol/liter): 150 $TMANO_3$, 1 $MgSO_4$ and 10 HEPES/Tris at pH 7.4. In addition the incubation media contained various amounts of NaCl and TMACl to vary the Na^+ concentration from 0 to 100 mmol/liter while maintaining a Cl^- gradient of 100 mmol/liter. The K^+ concentration was 10 mmol/liter. (*b*) A Hanes-Woolf plot of Na^+ stimulation of the Cl^--dependent component to ^{86}Rb uptake. The data presented in Fig. 7*a* was analyzed using a Hanes-Woolf plot. The regression line ($r^2 = 0.99$) gave an apparent K_m of 0.42 ± 0.05 mmol/liter Na^+. The apparent V_{max} was 303 ± 23.9 pmol/mg prot/6 sec

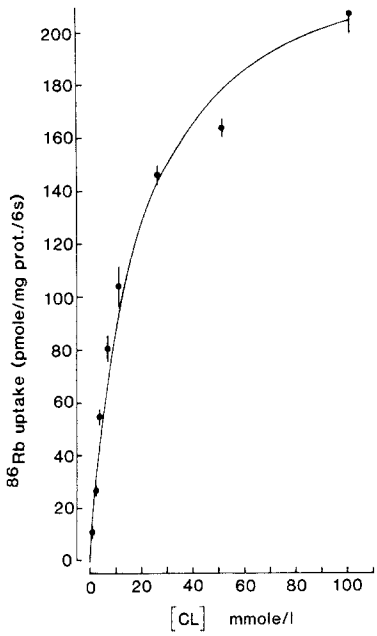

Fig. 8. Cl^- stimulation of the furosemide-sensitive component of ^{86}Rb uptake. The ability of Cl^- ions to stimulate furosemide-sensitive ^{86}Rb uptake was measured after a 6-sec uptake period. The vesicles contained (in mmol/liter): 150 $TMANO_3$, 1 $MgSO_4$ and 10 HEPES/Tris at pH 7.4. In addition the incubation media contained various amounts of NaCl or $NaNO_3$ to vary the Cl^- concentration between 0 and 100 mmol/liter, while keeping Na^+ at 100 mmol/liter. K^+ was at a concentration of 10 mmol/liter

Table. Summary of the apparent kinetic constants for the Cl - dependent component of ^{86}Rb uptake[a]

Ion	K_m (mmol/liter)	V_{max} (pmol/mg prot/6 sec)	Hill coefficient
Na^+ (at 100 mM Cl 10 mM K)	0.42 ± 0.05	303 ± 24	1.05 ± 0.06
K^+ (at 100 mM Cl 100 mM Cl)	11.9 ± 1.0	307 ± 27	0.97 ± 0.05
Cl^- No_3 replacement (at 100 mM Na 10 mM K)	11.9 ± 0.2	220 ± 6	0.96 ± 0.04
Cl^- Gluconate replacement (at 100 mM Na 10 mM K)	5.14[b] 55.2[c]	53 310	1.63 ± 0.4

[a] The values were obtained from Hanes-Woolf plots and Hill plots except for Cl^--interaction determined by gluconate replacement where an Eadie-Scatchard plot was used.
[b] "High" affinity K_m.
[c] "Low" affinity K_m.

In a series of further experiments to clarify the stoichiometry of the cotransport system a similar experiment to the one described above was performed. In this case, however, Cl^- was replaced by gluconate. The data are presented in Fig. 9*a*. In contrast to the hyperbolic curve obtained when NO_3^- was used as a replacement for Cl^-, a sigmoidal relationship between ^{86}Rb uptake and Cl^- concentration was obtained. The sigmoidicity of the Cl^- dependence is indicative for the involvement of at least two Cl^- ions in the transport process. In addition, it must be assumed that the two Cl^- ions are interacting at two separate sites with cooperative interactions. This conclusion is further supported

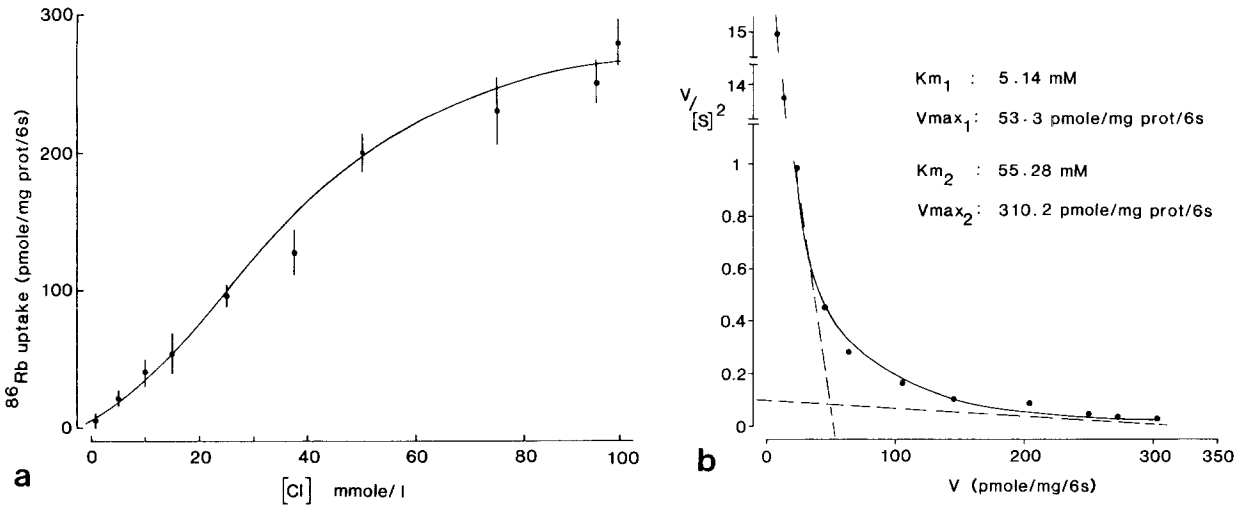

Fig. 9. (*a*) Cl^- stimulation of ^{86}Rb uptake when gluconate is used as replacement for Cl^-. The experimental protocol was identical to that described in Fig. 8 except that the vesicles contained TMA gluconate instead of $TMANO_3$ and Na gluconate was used instead of $NaNO_3$ as a substitute for Cl . (*b*) An Eadie-Scatchard plot of the data presented in Fig. 9*a*. The data from Fig. 9*a* was replotted using an Eadie-Scatchard plot. Two regression lines could be fitted to the curve. The first ($r^2 = 0.99$) gave an apparent K_m of 5.14 mmol/liter and an apparent V_{max} of 53.3 pmol/mg prot/6 sec. The second line ($r^2 = 0.98$) gave an apparent K_m of 55.2 mmol/liter and an apparent V_{max} of 310 pmol/mg prot/6 sec

by a Hill plot of the data presented in Fig. 9*a* which gave a Hill number of 1.63 ± 0.34 (Table). A comparison of the results obtained with nitrate and gluconate suggest that the difference in the apparent stoichiometry obtained with the two anion replacements may be the result of an interaction of NO_3 with one of the two Cl^- binding sites (*see below*).

The existence of two separate and cooperative sites for chloride interaction with different specificities makes a determination of the kinetic constants difficult. A kinetic approach assuming identical affinities for the two chloride sides, e.g. by expressing the Cl^- concentration as $[Cl^-]^2$ in a Hanes-Woolf plot cannot result in a reliable affinity constant but rather indicates some median value between the two sites. If the data presented in Fig. 9*a* are replotted using an Eadie-Scatchard plot, commonly used to evaluate the kinetic parameters of two enzymes using the same substrate (Segal, 1975), a curve is obtained (Fig. 9*b*). Using an iterative curve-stripping program (Spears et al., 1971), two regression lines were fitted to the curve. The first line ($r^2 = 0.997$) yielded a high-affinity site with an apparent K_m of 5 mmol/liter Cl^- and an apparent V_{max} of 54 pmol/mg prot/6 sec. The second regression line ($r^2 = 0.988$) gave a low-affinity site with an apparent K_m of 55 mmol/liter Cl^- and an apparent V_{max} of 310 pmol/mg prot/6 sec. However, the data obtained by this procedure do not take into account the cooperative effects between the two sites and can therefore provide only indirect evidence that the two sites have largely different affinities for chloride.

When the data of Fig. 9 was analyzed using a Hanes-Woolf plot a linear regression was obtained ($r^2 = 0.63$). Replotting this data with the Cl^--concentration expressed as $[Cl^-]^2$ gave the best fit with a correlation coefficient of 0.98 ± 0.03. From this regression line an apparent K_m of 28.2 ± 2.8 mmol/liter and a V_{max} of 280 ± 15 pmol/mg protein was calculated. This value is similar to that reported for the *in vitro* perfused thick ascending limb of Henle's loop (49 ± 31 mmol/liter; Greger et al., 1983) and to that reported for vesicles isolated from cells of the same nephron segment (15.3 ± 0.7 mmol/liter; Koenig et al., 1983).

Discussion

In this report we have presented strong evidence in favor of the presence of a $Na^+ : K^+ : 2Cl^-$ cotransport system in the LLC-PK_1 cell line. In both whole cells and in isolated apical membrane vesicles we have shown that a large part of the ^{86}Rb uptake is sensitive to inhibition by the loop diuretic furosemide and a related drug piretanide. Similarly, we have demonstrated that this component of influx is strictly dependent upon the presence of both extracellular Na^+ and Cl^- ions; removal of either led to a similar inhibition of uptake as was measured in the presence of the loop diuretics. Further evidence for the presence of a tightly coupled uptake mechanism for Na^+, K^+ and Cl^- was the demonstration of similar uptake rates (V_{max}), furosemide sensitivity and dependence upon the presence of the other two putative co-ions of a large component of ^{22}Na, ^{36}Cl and ^{86}Rb influx into apical membrane vesicles from LLC-PK_1 cells.

Comparison of the Properties of the Cotransport System in LLC-PK$_1$ Cells with Those of Na$^+$: K$^+$: 2Cl$^-$ Cotransport Systems in Other Tissues

Our study documents the presence of the transport system in the apical membrane of LLC-PK$_1$ cells. However, as we have not analyzed basolateral membranes our study does not allow to conclude that the transport mechanism is expressed only in the apical membrane, i.e. in a polarized manner similar to other epithelia (*see below*).

A preferential location of the transport system to the apical membrane of LLC-PK$_1$ cells would be similar to the location of the cotransport system in chloride-absorptive epithelia, e.g. thick ascending limb of the loop of Henle (Greger & Schlatter, 1981) and in flounder small intestine (Musch et al., 1982), and in contrast to the location of the transport system in Cl$^-$ secretory epithelia: e.g. high resistance MDCK cells (Brown & Simmons, 1981), shark rectal gland (Hannafin et al., 1983), and canine tracheal epithelial cells (Welsh, 1983*a*). An apical location of the Na$^+$: K$^+$: 2Cl$^-$ cotransport system would be indicative for NaCl-absorption by LLC-PK$_1$ cells. This assumption is in parallel with the data documenting a number of absorptive Na : solute cotransport systems in the apical membrane of LLC-PK$_1$ cells (Misfeldt & Sanders, 1981; Biber et al., 1983; Moran et al., 1983).

The kinetic parameters of the cotransport system in LLC-PK$_1$ apical membrane vesicles are summarized in the Table. The K_m values for Na$^+$ and K$^+$ (0.4 and 28.8 mmol/liter, respectively) are in the range of published values for TALH membrane vesicles (1.8 and 29.8 mmol/liter for Na$^+$ and K$^+$, respectively) (Koenig et al., 1984) and for a wide range of intact epithelial cells (Aiton et al., 1981; Musch et al., 1982; Greger & Schlatter, 1983; Greger et al., 1983; McRoberts et al., 1983). Some of the variation in the reported values probably reflects the different conditions under which the values were ascertained. In our case, we tried always to provide V_{max} driving condition for two of the three ions under study. That this indeed was the case, and that the uptake of the third ion was not limited by the concentration of the other two ions, is suggested by the observation that under these conditions the V_{max} value calculated for each of the three ions were very close.

In all cases an apparent stoichiometry of 1 was reported for the interaction of Na$^+$ and K$^+$ ions with the cotransporter. A study of the Cl$^-$ dependence of ^{86}Rb uptake through the cotransport system in LLC-PK$_1$ cells showed that the apparent stoichiometry of Cl$^-$ activation of ^{86}Rb uptake was dependent upon the anion used as a Cl$^-$ substitute. Using NO_3^- as a replacement for Cl$^-$, consistently supported an interaction of 1 Cl$^-$ ion with the cotransport system. However, when gluconate was substituted for NO_3^- a stoichiometry of 2 for Cl^{-1} was found. Similar data have been reported for the cotransporter in shark rectal glands (Epstein & Silva, 1984). These results may be explained in two ways: either in the presence of NO_3^- only 1 Cl$^-$ anion interacts with the system and it would operate electrogenically, or as we think more likely: NO_3^- itself is a weak substrate for the cotransport system. In this scheme Cl$^-$ and NO_3^- compete for one of the two Cl$^-$ binding sites. To explain our observation we propose that one of the two Cl$^-$ binding sites of the cotransporter has a high affinity for Cl$^-$ and no or low affinity for NO_3^- and the second binding site has a low discrimination between the two anions. From the results of our studies in which NO_3^- was decreased from 250 to 100 mmol/liter (NO_3^- replacement) and Cl$^-$ was increased from 0.63 to 100 mmol/liter it appeared that while Cl$^-$ occupied the high-affinity site, the low-affinity site seemed to be always occupied by NO_3^-. These results are expressed in a simple hyperbolic relationship between Cl$^-$ concentration and ^{86}Rb uptake and in a Hill coefficient of 1. In contrast, gluconate seems to have no or little affinity for either Cl$^-$ site and the Cl$^-$ dependence of ^{86}Rb uptake under gluconate replacement reflects the interaction of Cl$^-$ with two sites. Due to the problems related to the kinetic treatment of a transport system with two cooperative sites for the same substrate (*see above*) the apparent K_m values for Cl$^-$ are at best of a semiquantitative value. The lower affinity for chloride in the NO_3^- replacement experiment as compared to the "high"-affinity site in the gluconate replacement experiment can be related to several factors: *i*) some competitive effects of NO_3^- on the interaction of chloride with the high-affinity site; *ii*) methodological differences, e.g. related to the different procedures used for obtaining the kinetic constants. The data on V_{max} under different anion replacement suggest that a cotransporter with one Cl$^-$ binding site occupied by NO_3^- shows a lower rate of transport than one with both Cl$^-$ sites occupied with Cl$^-$.

In this paper we have described the existence of a Na$^+$: K$^+$: 2Cl$^-$ cotransport system in the apical membrane of a cultured renal cell line. Our observations suggest that the two Cl$^-$ sites which have been reported for Na$^+$: K$^+$: 2Cl$^-$ cotransport systems in a number of epithelia (e.g. McRoberts et al., 1983; Epstein & Silva, 1984; Greger & Schlatter, 1984) may differ not only in their affinity for Cl$^-$ but in their anion specificity. The kinetic parameters of the transport system in LLC-PK$_1$ cells are similar to those of the cotransport system described in the

thick ascending limb of the loop of Henle of the renal tubule. This does not of course mean that LLC-PK$_1$ cells are derived from the TALH, for there is substantial evidence of transport systems in LLC-PK$_1$ that are characteristic of other (particular proximal) nephron segments (Misfeldt & Sanders, 1981; Biber et al., 1983). What it does imply is that LLC-PK$_1$ cells have retained (or gained) the ability to express renal transport functions. So that, at the level of the individual transport system, the LLC-PK$_1$ cell line provides a good experimental model to study the regulation of these transport systems.

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References

Aiton, J.F., Brown, C.D.A., Ogden, P., Simmons, N.L. 1982. K^+ transport in "tight" epithelial monolayers of MDCK cells. *J. Membrane Biol.* **65:**99–109

Aiton, J.F., Chipperfield, A.R., Lamb, J.F., Ogden, P., Simmons, N.L. 1981. Occurence of passive furosemide-sensitive transmembrane K transport in cultured cells. *Biochim. Biophys. Acta* **646:**389–398

Amsler, K., Cook, J.S. 1982. Development of Na^+-dependent hexose transport in a cultured line of porcine kidney cells. *Am. J. Physiol.* **242:**C94–C101

Berner, W., Kinne, R., Murer, H. 1976. Phosphate transport into border brush membrane vesicles isolated from rat small intestine. *Biochem. J.* **160:**467–474

Biber, J., Brown, C.D.A., Murer, H. 1983. Sodium dependent transport of phosphate in LLC-PK$_1$ cells. *Biochim. Biophys. Acta* **735:**325–330

Biber, J., Murer, H. 1985. Na/P_i cotransport in LLC-PK$_1$ cells: Fast adaptive response to P_i deprivation. *Am. J. Physiol.* (*in press*).

Biber, J., Stieger, B., Haase, W., Murer, H. 1981. A high yield preparation for rat kidney brush border membranes. Different behaviour of lysosomal enzymes. *Biochim. Biophys. Acta* **647:**169–176

Bradford, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principle of protein dye binding. *Anal. Biochem.* **72:**248–254

Brown, C.D.A., Bodmer, M., Biber, J., Murer, H. 1984. Sodium-dependent phosphate transport by apical membrane vesicles from a cultured renal epithelial cell line (LLC-PK$_1$). *Biochim. Biophys. Acta* **769:**471–478

Brown, C.D.A., Simmons, N.L. 1981. Catecholamine stimulation of Cl secretion in MDCK cell epithelium. *Biochim. Biophys. Acta* **649:**427–435

Caverzasio, J., Brown, C.D.A., Biber, J., Bonjour, J.-P., Murer, H. 1985. Na-dependent phosphate transport in phosphate deprived LLC-PK$_1$ cells. *Am. J. Physiol.* **248:**F122–F127

Chaillet, J.R., Amsler, K., Boron, W.F. 1985. Optical measurements of intracellular pH in single LLC-PK$_1$ cells: Demonstration of Cl-HCO_3 exchange. *Am. J. Physiol.* (*Submitted*)

Epstein, F.H., Silva, P. 1984. Na/K/Cl cotransport in chloride transporting epithelia. *Ann. N.Y. Acad. Sci.* (*in press*)

Greger, R., Schlatter, E. 1981. Presence of luminal K^+, a prerequisite for active NaCl transport in the cortical thick ascending limb of Henle's loop of rabbit kidney. *Pfluegers Arch.* **392:**92–94

Greger, R., Schlatter, E. 1983. Properties of the lumen membrane of the cortical thick ascending limb of Henle's loop of rabbit kidney. *Pfluegers Arch.* **396:**315–324

Greger, R., Schlatter, E. 1984. Mechanism of NaCl secretion in the rectal gland of the spiny dogfish (*Squalus acanthias*). 1. Experiments in isolated *in vitro* perfused rectal gland tubules. *Pfluegers Arch.* **402:**63–75

Greger, R., Schlatter, E., Lang, F. 1983. Evidence for electroneutral sodium chloride cotransport in the cortical thick ascending limb of Henle's loop of rabbit kidney. *Pfluegers Arch.* **396:**308–314

Haas, M., Schmidt, W. F., McManus, T.J. 1982. Catecholamine stimulated ion transport in duck red cells. Gradient effects in electrically neutral (Na + K + 2Cl) cotransport. *J. Gen. Physiol.* **80:**125–147

Hannafin, J., Kinne-Saffran, E., Friedman, D., Kinne, R. 1983. Presence of a sodium-potassium-chloride cotransport system in the rectal gland of *Squalus acanthias*. *J. Membrane Biol.* **75:**73–83

Kessler, M., Tannenbaum, V., Tannenbaum, C. 1978. A simple apparatus for performing short-time (1–2 seconds) uptake measurements in small volumes; its application to D-glucose transport studies in brush border vesicles from rabbit jejenum and ileum. *Biochim. Biophys. Acta* **509:**348–359

Koenig, B., Ricapito, S., Kinne, R. 1984. Chloride transport in the thick ascending limb of Henle's loop: Potassium dependence and stoichiometry of the NaCl cotransport system in plasma membrane vesicles. *Pfleugers Arch.* **399:**173–179

McRoberts, J.A., Tran, C.T., Saier, M.H. 1983. Characterisation of low potassium-resistant mutants of the Madin Darby canine kidney cell line with defects in NaCl/KCl symport. *J. Biol. Chem.* **258:**12320–12326

Misfeldt, D.S., Sanders, M.J. 1981. Transepithelial glucose transport in cell culture. *Am. J. Physiol.* **240:**C92–C95

Moran, A., Turner, R.J., Handler, J.S. 1983. Regulation of sodium-coupled glucose transport by glucose in a cultured epithelium. *J. Biol. Chem.* **258:**15087–15090

Musch, M.W., Orellana, S.A., Kimberg, S.A., Field, M., Halm, D.R., Krasny, E.J., Frizzell, R.A. 1982. Na^+–K^+–Cl^- cotransport in the intestine of a marine teleost. *Nature* (*London*) **300:**351–353

Saier, M.H., Boyden, D.A. 1984. Mechanisms, regulation and physiological significance of the loop diuretic-sensitive NaCl/KCl symport systems in animal cells. *Mol. Cell. Biochem.* **59:**11–32

Segal, I.H. 1975. Enzyme Kinetics. John Wiley, New York

Spears, G., Sneyd, J.T.D., Loten, E.G. 1971. A method for deriving kinetic constants for two enzymes acting on the same substrate. *Biochem. J.* **125:**1149–1151

Welsh, M.J. 1983*a*. Inhibition of chloride secretion by furosemide in canine tracheal epithelium. *J. Membrane Biol.* **71:**219–226

Welsh, M.J. 1983*b*. Intracellular chloride activities in canine tracheal epithelium. Direct evidence for sodium coupled intracellular chloride accumulation in a chloride secreting epithelium. *J. Clin. Invest.* **71:**1392–1401

Welsh, M.J., Smith, P.L., Frizzell, R.A. 1980. Intracellular chloride activities in dogfish rectal gland. *Bull. Mt. Desert Isl. Biol. Lab.* **20:**101–103

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