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Sodium-Dependent Ion Cotransport in Steady-State Ehrlich Ascites Tumor Cells

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Summary. The Ehrlich tumor cell possesses an anion-cation cotransport system which operates as a bidirectional exchanger during the physiological steady state. This cotransport system, like that associated with the volume regulatory mechanism (i.e. coupled net uptake of Cl^- + Na^+ and/or K^+), is Cl^--selective and furosemide-sensitive, suggesting the same mechanism operating in two different modes. Since Na^+ has an important function in the volume regulatory response, its role in steady-state cotransport was investigated. In the absence of Na^+, ouabain-insensitive K^+ and DIDS-insensitive Cl^- transport (KCl cotransport) are low and equivalent to that found in 150 mM Na^+ medium containing furosemide. Increasing the [Na^+] results in parallel increases in K^+ and Cl^- transport. The maximum rate of each (18 to 20 meq/(kg dry wt) · min) is reached at about 20 mM Na^+ and is maintained up to 55 mM. Thus, over the range 1 to 55 mM Na^+ the stoichiometry of KCl cotransport is 1 : 1. In contrast to K^+ and Cl^-, furosemide-sensitive Na^+ transport is undetectable until the [Na^+] exceeds 50 mM. From 50 to 150 mM Na^+, it progressively rises to 7 meq/(kg dry wt) · min, while K^+ and Cl^- transport decrease to 9 and 16 meq/(kg dry wt) · min, respectively. Thus, at 150 mM Na^+ the stoichiometric relationship between Cl^-, Na^+ and K^+ is 2 : 1 : 1. These results are consistent with the proposal that the Cl^--dependent cation cotransport system when operating during the steady state mediates the exchange of KCl for KCl or NaCl for NaCl; the relative proportion of each determined by the extracellular [Na^+].

Key Words Na^+, K^+, Cl^- cotransport · furosemide · DIDS · Ehrlich cells

Introduction

Ehrlich ascites tumor cells, like a number of other mammalian cells, regulate their volume in response to changes in the osmolality of the extracellular medium (McManus & Schmidt, 1978; Kregenow, 1981). For example, incubation in hypotonic medium results in rapid osmotic swelling followed by the loss of KCl and water, thereby re-establishing the normal resting volume. Recently it was proposed that the mechanism responsible for regulatory volume decrease (RVD) involved the activation of a Ca^{++}-dependent conductive K^+ channel and a separate Cl^- conductive pathway (Hoffmann et al., 1984). A similar mechanism has been suggested for RVD in human lymphocytes (Grinstein et al., 1982, 1983).

In contrast to RVD, regulatory volume increase (RVI) apparently involves a different transport mechanism. Pretreatment of cells in hypotonic medium followed by restoration of the tonicity (300 mosmolal) results in a rapid osmotic shrinkage. Cell volume returns to normal due to the activation of an electrically silent cotransport system that mediates the net uptake of Na^+ and Cl^-. This transporter has an obligatory requirement for Na^+ and Cl^-, since replacement of Na^+ by choline or Cl^- by NO_3^- prevents volume recovery. Moreover, bumetanide and furosemide are potent inhibitors of RVI while DIDS is without effect (Hoffmann, 1982; Hoffmann et al., 1983).

The relationship between ion cotransport during RVI and that which occurs in the steady state is unclear. In the absence of net ion and water movements approximately 50% of K^+ transport is ouabain insensitive (Tupper, 1975; Geck et al., 1978) while about 45% of Cl^- transport persists in the presence of DIDS, an inhibitor of the anion exchanger (Aull et al., 1977; Levinson, 1978, 1984). The observations that ouabain-insensitive K^+ transport is abolished when Cl^- is replaced by NO_3^- and that both K^+ and Cl^- transport are inhibited by furosemide suggests that K^+ and Cl^- are cotransported (Aull, 1982; Bakker-Grunwald, 1978; Bakker-Grunwald et al., 1980). During the physiological steady state this system apparently functions as a bidirectional exchanger, since in the presence of furosemide the cells maintain normal levels of Na^+, K^+ and Cl^- and exhibit little or no change in resting volume (Bakker-Grunwald, 1978; Aull, 1982). There is, however, evidence that even during the steady state the activity of the cotransport pathway is influenced by cell volume (Geck et al., 1981).

It has been suggested that in the absence of net

ion and water movements $Na^+ + Cl^-$ cotransport does not occur. This is based to large extent on the findings that the Cl^- exchange flux is insensitive to bumetanide (however, *see* Aull, 1981) and that the substitution of choline for Na^+ is without effect on the steady-state transfer of Cl^- (Hoffmann et al., 1983). The dismissal of Na^+ as a participant in the steady-state cotransport of ions, however, may be premature. It has been demonstrated that a component of Na^+ transport, about 25% of the total flux, is inhibited by low concentrations of furosemide (Tupper, 1975). Thus, it is possible that the cotransporter has two different modes of operation. In the steady state it functions primarily as an exchanger mediating the simultaneous, bidirectional transfer of Na^+, Cl^- and K^+. However, when cell electrolytes and/or volume deviates from the resting level, the cotransport is "activated" by unknown factors to mediate the net uptake of Na^+ and Cl^-.

Since Cl^--dependent Na^+ transport appears to play a central role in RVI and as such could provide a common link between the two modes of operation of the cotransporter, its role in steady-state ion cotransport requires clarification. Therefore, a major goal of the present study was to determine in steady-state cells whether: (1) a component of Na^+ transport was dependent on Cl^- and sensitive to furosemide and (2) a relationship exists between Cl^-, K^+ and Na^+ cotransport. The results suggest that the Cl^--dependent cation cotransport system of the Ehrlich tumor cell behaves functionally as a single entity that mediates the transfer of both NaCl and KCl. At low extracellular Na^+ concentrations (1 to 55 mM) the cotransport system mediates the exchange of KCl but not NaCl across the membrane. However, with increasing Na^+ (60 to 150 mM) progressively less KCl is cotransported. The decrease in K^+ transport is offset by an increase in NaCl cotransport such that the sum (KCl + NaCl) is maintained relatively constant.

Materials and Methods

Cell Suspensions

Ehrlich-Lettré ascites tumor cells (hyperdiploid strain) grown in Ha/ICR white male mice were harvested and washed as previously described (Levinson, 1972). The standard saline solution had the following composition (mM): 150 NaCl, 6 KCl, 10 HEPES-NaOH (pH 7.3; 285 to 300 mOsm). This, as well as all other media was Millipore filtered (0.45 μm) prior to use. Cells were washed twice in this solution and subsequently incubated at a density of 15 to 20 mg dry weight/ml for 30 min at 21 to 23°C. Following the preincubation period aliquots of cell suspensions were centrifuged, washed in the appropriate experimental medium and incubated as a dilute cell suspension (3 to 5 mg dry weight/ml) for 60 min. Two types of media were prepared. In experiments designed to investigate the dependency of Cl^-, Na^+ and K^+ transport on the extracellular [Na^+], choline in equimolar amounts replaced Na^+; while in studies aimed at establishing the anion dependence of Na^+ transport, NO_3^- was substituted for Cl^-. All media contained 5 to 7 mM K^+, 10 mM HEPES buffer and were pH 7.3 to 7.4, 290 to 300 mOsm.

After incubation the suspensions were centrifuged and the cells resuspended in the same medium at a density of 18 to 20 mg dry wt/ml. They were subsequently placed in a 37°C water bath under an atmosphere of 100% O_2 for at least 5 min before the start of an experiment.

Reagents

Furosemide was kindly supplied by Hoechst Pharmaceuticals, Inc. Ouabain and DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonic acid) were obtained from Sigma Chemical Co., St. Louis, Mo. Radioisotopes, ^{22}Na and ^{86}Rb, were purchased from New England Nuclear, Boston, Mass., while ^{36}Cl was a product of ICN, Irvine, Calif.

Steady-State Fluxes of Na^+, K^+ and Cl^-

K^+ Influx

The unidirectional influx of K^+ was determined from the uptake of ^{86}Rb. In the Ehrlich cell ^{86}Rb serves as an analog of K^+ and as such faithfully traces K^+ movements (Tupper, 1975), an observation consistent with numerous other cell types. Cell suspensions were incubated in the presence and absence of inhibitors (e.g., ouabain, furosemide, DIDS) and at time zero a tracer amount of isotope (0.30 μCi/ml cell suspension) was added. Periodically during the next 6 min, 0.5-ml aliquots of cell suspension were removed and centrifuged 30 sec at 15,000 × g through 0.8 ml ice-cold isosmotic choline dihydrogen citrate solution (CDHC; Villereal & Levinson, 1977). The supernatant was removed and the cell pellets mixed with 1 ml of 1% (vol/vol) cold perchloric acid (PCA). Additional samples of cell suspension and medium were removed for the determination of wet and dry cell weight and intracellular water (Bowen & Levinson, 1982) and for the analysis of ^{86}Rb, Na^+, K^+ and Cl^-. Correction for ^{86}Rb trapped in the extracellular space was made by the addition of 0.5 ml cell suspension (before the addition of isotope) to 0.8 ml cold CDHC solution containing the appropriate amount of tracer and by centrifuging immediately. ^{86}Rb trapped within the extracellular space amounted to between 3 and 7% of the radioactivity accumulated within 5 min.

The initial steady-state influx of $K^+(Rb^+)$, expressed as meq/(kg dry wt) · min, was calculated from the uptake of ^{86}Rb which was in all cases, linear over the period of observation. Influx was determined from: $(d^{86}Rb/dt)/SA$, where $d^{86}Rb/dt$ is the incorporation of ^{86}Rb per kg dry wt per min, that is, the slope of the time-dependent uptake, and SA is the specific activity (cpm/μeq) of the extracellular $K^+(Rb^+)$.

Na^+ Influx

Measurements of the uptake of ^{22}Na by the procedure described above were highly variable. The lack of success was attributed to two factors. First, the turnover of intracellular Na^+ is faster than

that of either K^+ or Cl^-. Second, the Na^+ compartment size is only about 15 and 40% of that of Na^+ and Cl^-, respectively. Because of this a technique was developed that permitted the rapid sampling of cell suspension after the introduction of ^{22}Na. A small volume of solution containing ^{22}Na (10 μl; 20 μCi/ml) was added to each of 6 tubes (1.5-ml capacity) maintained at 37°C. At intervals (0.25 to 1 min) a 0.5-ml aliquot of cell suspension was then added to one of the tubes. After the last addition, 0.8 ml ice-cold CDHC was rapidly injected into each sample by a 4.8-ml automatic pipetor connected to a manifold containing 6 ports. Less than 5 sec is required to deliver the CDHC solution simultaneously to each tube. The samples were then centrifuged 30 sec at 15,000 × g, the supernatants removed and the cell pellets mixed with 1% cold PCA. Preliminary experiments showed that the addition of cold CDHC solution followed by rapid centrifugation effectively stops uptake of ^{22}Na by the cells. Estimates of ^{22}Na trapped within the extracellular space were made by adding 0.5 ml of cell suspension to 0.8 ml ice-cold CDHC solution containing ^{22}Na and centrifuging immediately. Although somewhat variable, the ^{22}Na trapped within the extracellular space was between 20 and 25% of the radioactivity accumulated within 3.5 min.

At the end of the experiment additional samples of cell suspension and medium were collected as described above. The initial steady-state influx of Na^+ was calculated from the uptake of ^{22}Na, which was linear for at least 3 min. Influx was calculated from: $(d^{22}Na/dt)/SA$, where $d^{22}Na/dt$ represents ^{22}Na incorporated per kg dry wt per min and SA is the specific activity of the extracellular Na^+.

Cl^- Influx

The unidirectional Cl^- influx was calculated from the time-dependent uptake of ^{36}Cl as previously described (Levinson, 1984). In the present experiments the sampling procedure, as well as the measurement of ^{36}Cl trapped within the extracellular space, was identical to that described for ^{86}Rb uptake.

Analytical Procedures

The cell pellets which were extracted with PCA were kept in an ice bath for 60 min and subsequently centrifuged at 15,000 × g for 2 min to remove the PCA-insoluble residue. Aliquots of the PCA extracts and cell medium were used to determine Na^+ and K^+ by emission flame photometry using 15 mM Li^+ as an internal standard and Cl^- with an autotitrator (Levinson & Villereal, 1976). Aliquots of the PCA extracts and media were assayed for ^{86}Rb and ^{22}Na radioactivity with a Packard gamma counter, while ^{36}Cl activity was measured with the liquid scintillation counter (Beckman LS 355).

Results

DIDS- and Furosemide-Sensitive Cl^- Fluxes

Although as much as 50% of the steady-state Cl^- flux is believed to be cotransported with cations (Aull, 1981, 1982), it was recently reported that Cl^- transport was unaffected when Na^+ was replaced by choline (Hoffmann et al., 1983). The lack of effect of Na^+ removal suggests that this cation is not transported by the furosemide-sensitive, Cl^--dependent cotransporter under normal steady-state conditions. If this were the case then it is reasonable to expect that the furosemide-sensitive fraction of the Cl^- flux would be the same both in the presence and absence of Na^+. This was tested in a series of experiments, the results of which are shown in Figs. 1 and 2 and summarized in Table 1.

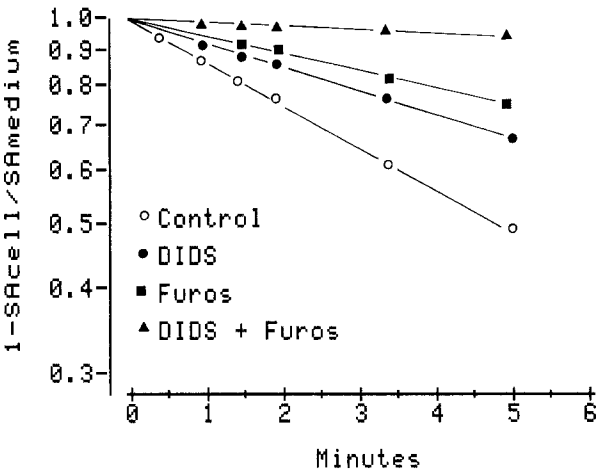

Fig. 1. Inhibition of steady-state Cl^- exchange by DIDS and furosemide in Na^+-containing medium. At time zero ^{36}Cl was added to cell suspensions: (1) incubated in the standard HEPES-buffered saline solution (150 NaCl/6 KCl; ○) (2) 30 sec after the addition of DIDS (0.2 mM; ●) or furosemide (0.5 mM; ■) and (3) 30 sec after the addition of DIDS + furosemide (▲). Ln (1 − relative specific activity of Cl^-) is plotted as a function of time (Levinson, 1984). The efflux rate coefficients (ke; min^{-1}) which were determined from the slope of the linear regressions are: Control, 0.143; DIDS, 0.078; furosemide, 0.066; and DIDS + furosemide, 0.017. Correlation coefficients (r) exceeded 0.98 in all cases. Five such experiments are summarized in Table 1

Figure 1, which displays data from a representative experiment, compares the inhibitory effects of DIDS and furosemide on Cl^- transport in normal Na^+-containing medium (150 mM NaCl, 6 mM KCl). DIDS (0.2 mM) and furosemide (0.5 mM) reduce the Cl^- efflux rate coefficient (ke; min^{-1}) by 44 and 54%, respectively. In preliminary experiments we found the concentration of DIDS which causes maximal inhibition lies between 0.1 and 0.2 mM. In contrast to DIDS, increases in the concentration of furosemide result in progressive inhibition of Cl^- transport due most likely to inhibition of the cotransport pathway, as well as to partial inhibition of the DIDS-sensitive, self-exchange pathway. There is evidence, however, that the inhibitory action of furosemide at 0.5 mM is confined exclusively to DIDS-insensitive Cl^- transport (Aull, 1982). The addition of 0.2 mM DIDS plus 0.5 mM fuorsemide

Table 1. Effect of DIDS and furosemide on chloride transport[a]

Medium	Cl^- Flux: meq/(kg dry wt) · min			
	Control	DIDS	Furos.	DIDS + Furos.
(*A*) 150 mM Na^+	32.1 ± 1.5	16.9 ± 2.1	14.8 ± 1.2	3.8 ± 1.4
DIDS-sensitive (Cl^- self-exchange)		= 15.2		
Furosemide-sensitive (cotransport)		= 17.3		
(*B*) 0.3–1.2 mM Na^+	34.3 ± 1.6	7.4 ± 1.1	30.5 ± 1.7	4.1 ± 0.9
DIDS-sensitive (Cl^- self-exchange)		= 26.9		
Furosemide-sensitive (cotransport)		= 3.8		

[a] Ehrlich cells were incubated in either Na^+-containing (*A*) or choline (*B*) media in the presence and absence of inhibitors as described in Fig. 1. Fluxes were calculated from the product: *ke* (min^{-1}) × Cl content (meq/kg dry wt) as previously described (Levinson, 1984). Mean values ± 1 SE for five separate experiments in each medium are shown. Inhibitor-sensitive fractions are calculated from the difference: Cl^- flux in the absence—Cl^- flux in the presence of the inhibitor.

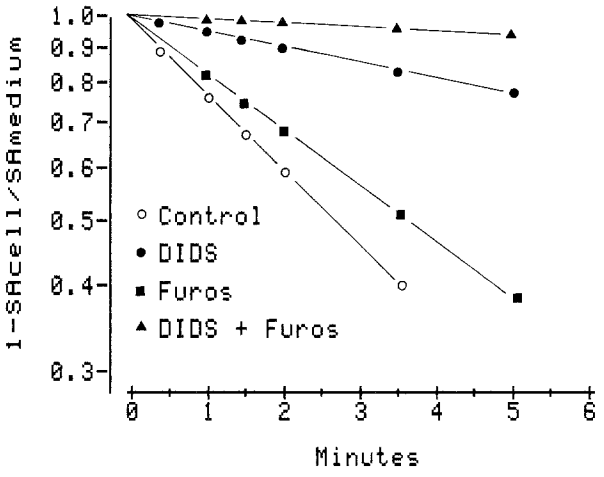

Fig. 2. Inhibition of steady-state Cl^- exchange by DIDS and furosemide in Na^+-free, choline medium. At time zero ^{36}Cl was added to cell suspensions: 1) incubated in HEPES-buffered choline medium (150 choline Cl/6 KCl; ○), (2) 30 sec after the addition of DIDS (0.2 mM; ●) or furosemide (0.5 mM; ■) and (3) 30 sec after the addition of DIDS + furosemide (▲). The efflux rate coefficients which were determined as described in Fig. 1 are: Control, 0.229; DIDS, 0.055; furosemide, 0.195 and DIDS + furosemide, 0.025. Five such experiments are summarized in Table 1

results in an 89% reduction in *ke* which is nearly equivalent to the sum observed by DIDS and furosemide alone. Despite the significant inhibitory effects of these agents, intracellular Cl^- does not change (69 meq/kg cell water; 224.5 meq/kg dry wt).

The data shown in Fig. 2 were obtained from cells made Na^+-free by incubation in choline medium (150 mM choline Cl^-, 6 mM KCl). In the absence of extracellular Na^+ the cells lose, in addition to Na^+ and Cl^-, a small amount of K^+ and water. After about 45 to 60 min a new steady state is established in which the intracellular [Cl^-] is similar to that of normal Na^+-containing cells but the Cl^- content is reduced (60 meq/kg cell water; 163.5 meq/kg dry wt). In the absence of Na^+, the effects of DIDS and furosemide are markedly different. The *ke* is depressed 78% by DIDS but only 11% by furosemide. The combination of DIDS plus furosemide results in an 88% reduction in *ke* which is equivalent to the sum of the individual effects. As in the case of Na^+-containing cells, the presence of DIDS, furosemide or both does not alter the intracellular [Cl^-] or content.

Table 1 summarizes the results of 10 separate experiments. In normal Na^+-containing medium the steady-state Cl^- exchange flux can be divided into two functionally distinct fractions: one that is inhibited by DIDS and a second that is inhibited by furosemide. There is compelling evidence obtained from numerous studies, in this as well as other cell types, that the DIDS-sensitive fraction represents Cl^- transport mediated by the anion exchanger or self-exchange pathway. The DIDS-insensitive or furosemide-sensitive component, as noted above, represents the movement of Cl^- coupled to the transfer of cation. A small fraction of the Cl^- flux, about 12% in our experiments, is not inhibited by the combination of DIDS plus furosemide and most likely represents movement through the conductive or diffusional pathway (Hoffmann et al., 1979). The response of the Cl^- transport system to the removal of Na^+ is noteworthy. Although the steady-state exchange flux (34.3 meq/(kg dry wt) · min) is not significantly different from that measured in Na^+-containing medium (32.1 meq/(kg dry wt) · min), the effects of DIDS and furosemide are markedly different. While about 44% of the Cl^- flux is inhibited by DIDS in Na^+ medium, this increases to 78% when

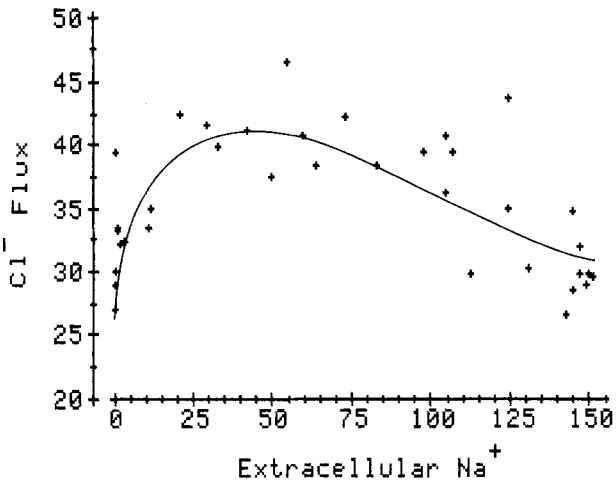

Fig. 3. Dependence of the steady-state Cl^- exchange flux (meq/(kg dry wt) · min) on the extracellular $[Na^+]$. Tumor cells were incubated in media containing varying concentrations of Na^+ and the Cl^- flux measured as described in Fig. 1 and Table 1. Results from nine experiments are shown

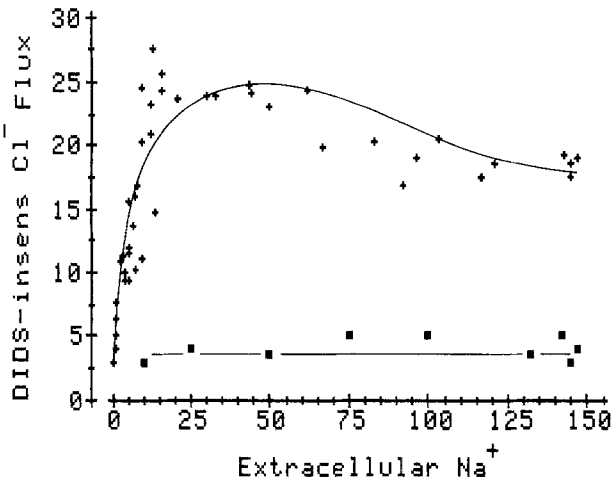

Fig. 4. Dependence of DIDS-insensitive Cl^- transport on the extracellular $[Na^+]$ and the effect of furosemide. Tumor cells were incubated in media containing varying concentrations of Na^+. DIDS (0.2 mM) was added 30 sec before the addition of ^{36}Cl, while furosemide (0.5 mM) when present, was added 15 sec after the addition of DIDS. Results from 17 experiments are shown: 14 DIDS alone (+) and 3 DIDS + furosemide (■)

Na^+ is removed. Furosemide, on the other hand, inhibits Cl^- transport by 11% in the absence of Na^+, but in its presence by 54%.

The simplest interpretation of these results is that Na^+ has two effects on steady-state Cl^- transport. First, since the removal of Na^+ increases the fraction of the total flux susceptible to inhibition by DIDS, the self-exchange pathway transports Cl^- at a higher rate in the absence of Na^+ than in its presence. Second, the finding that furosemide, in the absence of Na^+ has only a small effect on Cl^- transport, suggests that Na^+ is required either directly or indirectly by the cotransport pathway. Thus, although the removal of Na^+ would be expected to reduce Cl^- transport by an amount equal to that contributed by the cotransporter this would be offset by an increase in Cl^- self-exchange. This would explain why steady-state Cl^- transport is apparently unaffected by the replacement of Na^+ by choline (Hoffmann et al., 1983).

Na^+-Dependence of the Cl^- Flux

Since the removal of Na^+ significantly reduced the effect of furosemide on Cl^- transport, it was reasonable to suspect as a working hypothesis, that Na^+ plays an important role in ion cotransport. Because of this we systematically investigated the effect of varying the extracellular $[Na^+]$ on the steady-state Cl^- flux both in the presence and absence of DIDS and furosemide.

The relationship between the Cl^- flux and extracellular $[Na^+]$ is shown in Fig. 3. In media nominally free of Na^+ (0.1 to 1 mM) the flux ranges between 27 and 33 meq/(kg dry wt) · min. With increasing concentrations of Na^+ the flux increases, reaches and maintains a maximum value of approximately 41 meq/(kg dry wt) · min between 25 and 60 mM Na^+ but then progressively declines. At 150 mM Na^+ the flux averages 30.5 ± 1.1 meq/(kg dry wt) · min.

Figure 4 illustrates the dependence of the cotransport component on the extracellular $[Na^+]$. In these experiments, like those described in Figure 3, Ehrlich cells were equilibrated in media containing 5 to 7 mM K^+, 150 mM Cl^- and varying concentrations of Na^+/choline. DIDS (0.2 mM) was added to block the anion exchanger and the uptake of ^{36}Cl measured during the next 6 min. In the absence of Na^+ the steady-state Cl^- flux is 3.0 ± 0.41 meq/(kg dry wt) · min but rapidly increases to a maximal value of 23 meq/(kg dry wt) · min at about 20 mM Na^+. Between 20 and 55 mM Na^+ the flux is relatively constant but then decreases to 19 ± 1.8 meq/(kg dry wt) · min when the $[Na^+]$ increases to 150 mM. Note that furosemide (0.5 mM) abolishes the response to Na^+ and that the addition of this agent is equivalent to the removal of Na^+. These data clearly demonstrate that the cotransport pathway has a strict requirement for Na^+.

Na^+-Dependent K^+ Transport

Our results to this point indicate that Na^+ is required by the furosemide-sensitive component of the Cl^- transport system. Since a fraction of K^+

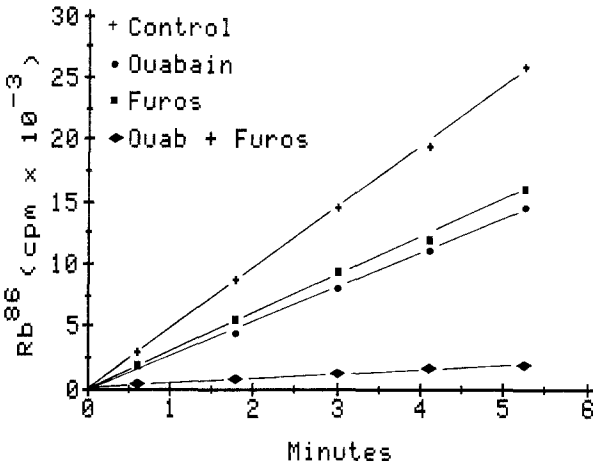

Fig. 5. Time-dependent uptake of ^{86}Rb and the effect of ouabain and furosemide. Tumor cells were incubated in Na^+-containing medium, centrifuged and resuspended in the same medium containing 2 mM ouabain (●) or 0.5 mM furosemide (■) or the combination of both inhibitors (◆). After a brief incubation at 37°C (about 5 min), ^{86}Rb was added and the uptake measured during the next 6 min. The unidirectional influxes (meq/kg dry wt) · min, calculated as described in Materials and Methods, for this as well as three additional experiments are: Control, 19.2 ± 2.3; furosemide, 9.9 ± 1.3; ouabain, 10.4 ± 1.4 and furosemide + ouabain, 1.54 ± 0.6

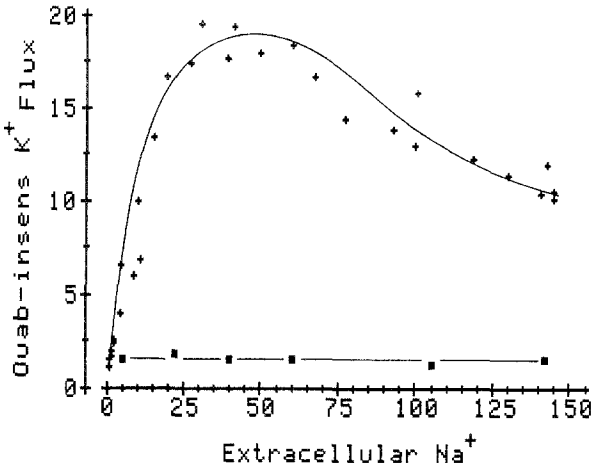

Fig. 6. Dependence of ouabain-insensitive K^+ transport (meq/(kg dry wt) · min) on the extracellular $[Na^+]$ and the effect of furosemide. Tumor cells were incubated in media containing varying concentrations of Na^+, centrifuged and resuspended in the same medium containing 2 mM ouabain. After a brief incubation at 37°C (about 5 min), ^{86}Rb was added and its uptake measured during the next 6 min. Furosemide (0.5 mM), when present, was added 30 sec before the addition of ^{86}Rb. Results from seven experiments: five ouabain alone (+), two ouabain + furosemide (■) are shown

transport, linked to the transport of Cl^- and inhibited by furosemide, has been identified in steady-state Ehrlich cells (Bakker-Grunwald, 1978; Aull, 1981, 1982), it was of interest to examine the effect of Na^+ on K^+ cotransport. The data shown in Fig. 5, taken from a representative experiment, demonstrate the inhibitory effects of ouabain and furosemide on the uptake of ^{86}Rb in normal Na^+ medium. Approximately 48% of the total unidirectional ^{86}Rb (K^+) influx (19.2 ± 2.3 meq/(kg dry wt) · min) is inhibited by 0.5 mM furosemide. We found that the concentration of furosemide at which inhibition is maximal lies between 0.4 and 1 mM. The ouabain-sensitive component is maximally inhibited between 1 and 2 mM ouabain and represents about 46% of the total unidirectional influx. These values agree favorably with those reported by others (Mills & Tupper, 1975; Tupper, 1975; Geck et al., 1980; Aull, 1982). The addition of 2 mM ouabain plus 0.5 mM furosemide leads to 92% reduction in ^{86}Rb influx which is nearly equivalent to the sum of the reductions observed with ouabain and furosemide alone. DIDS, however, has no effect on either ouabain-sensitive or insensitive K^+ transport.

Figure 6 shows the dependence of ouabain-insensitive K^+ transport on the extracellular $[Na^+]$. This component of K^+ transport increases sharply with increasing Na^+ and attains a maximal value (19 meq/(kg dry wt) · min) at about 25 mM Na^+. Between 25 and 55 mM Na^+ the flux is relatively constant, but then steadily decreases to 10.1 ± 0.3 meq/(kg dry wt) · min as the $[Na^+]$ increases to 150 mM. The addition of 0.5 mM furosemide completely abolishes the stimulation of K^+ transport by Na^+. In fact, the residual flux (1.20 to 1.51 meq/(kg dry wt) · min) is identical to that measured in the absence of Na^+.

It is useful at this point to summarize the effects of Na^+ on the furosemide-sensitive Cl^- and K^+ fluxes. In the absence of Na^+ both are minimal and most likely represent nonmediated transport. With an increase in the concentration of Na^+ the fluxes increases in parallel. The maximal flux of each is reached and maintained at between 25 and 55 mM Na^+. In the case of Cl^- the flux is 20 meq/(kg dry wt) · min (23 − 3.0; Fig. 4), while that for K^+ is 17.8 meq/(kg dry wt) · min (19 − 1.20; Fig. 6). This is consistent with the idea that between 1 and 55 mM Na^+ the cotransport system mediates the simultaneous transfer of K^+ and Cl^- with a stoichiometry of approximately 1 Cl^- : 1 K^+. With increasing concentrations of Na^+ the transport of both K^+ and Cl^- decreases, although not in parallel. For example, at 150 mM Na^+ the corresponding fluxes of K^+ and Cl^- are 8.8 meq/(kg dry wt) · min and 16 meq/(kg dry wt) · min (19 − 3.0), respectively; a finding consistent with transfer of 1 K^+ : 2 Cl^-.

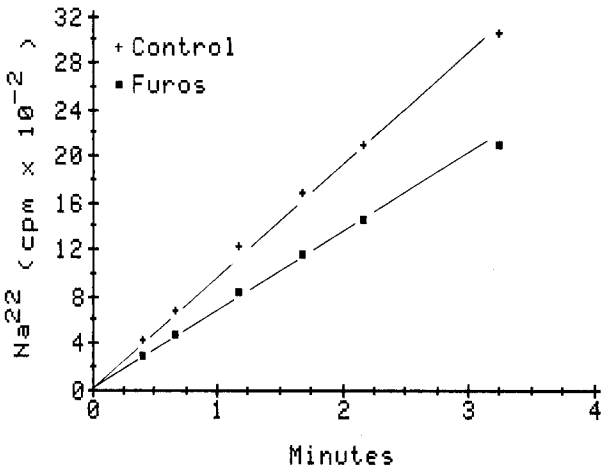

Fig. 7. Time-dependent uptake of ^{22}Na in Na^+-containing medium and the effect of furosemide. This experiment which is representative of three others was carried out as described in Table 2. The unidirectional Na^+ influxes (meq/(kg dry wt) · min) for the four experiments are: Control, 23.1 ± 2.6 (+) and furosemide, 16.6 ± 1.9 (■)

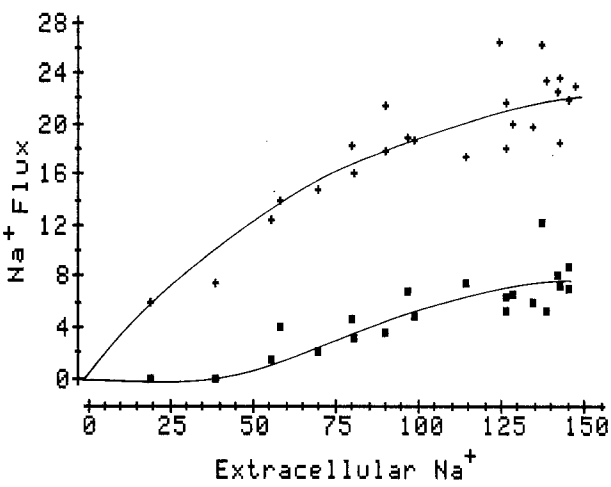

Fig. 8. Dependence of the Na^+ influx and furosemide-sensitive component on the extracellular [Na^+]. Tumor cells were incubated in media of varying concentrations of Na^+ and the Na^+ influx (meq/(kg dry wt) · min) determined from the uptake of ^{22}Na as described in Table 2. The results of 12 experiments are shown: six Control (+) and six furosemide-sensitive (■; determined from the difference between Na^+ influx in the absence and presence of 0.5 mM furosemide)

Furosemide-Sensitive Na^+ Transport

The results described in the preceding section show that increasing the [Na^+] from 60 to 150 mM results in almost 50% inhibition of ouabain-insensitive K^+ movement but only a 30% inhibition of the corresponding Cl^- flux. This raised the possibility that, while at low [Na^+] (e.g., 1 to 60 mM) the cotransporter mediates the simultaneous transfer of K^+ + Cl^-, with increasing Na^+ a fraction of Cl^- transfer is accompanied by Na^+. In order to investigate this point the dependence of steady-state Na^+ transport on the extracellular [Na^+] and how furosemide effects this process was investigated.

The inhibitory effect of furosemide on the uptake of ^{22}Na in Na^+-containing medium is shown in Fig. 7. In this experiment 30% of the total unidirectional influx (23.1 meq/(kg dry wt) · min) is inhibited by 0.5 mM furosemide. As in the case of K^+ transport, maximal inhibition occurred at between 0.4 and 1 mM furosemide, confirming Tupper's original finding (Tupper, 1975). Table 2 shows that replacement of Cl^- by NO_3^- inhibits Na^+ influx by 34%. The addition of 0.5 mM furosemide to NO_3^--containing medium has no additional inhibitory effect; although furosemide does depress influx by 31% in Cl^--containing medium. These results support the idea that a fraction of the Na^+ influx is dependent on Cl^- and that this represents, at least functionally, the furosemide-sensitive component. Figure 8, which summarizes this series of experiments, shows the relationship between the extracellular [Na^+], steady-state Na^+ influx and the furosemide-sensitive component, calculated as the difference between transport in the presence and absence of furosemide. In the absence of furosemide, Na^+ influx rises curvilinearly in response to an increasing [Na^+] and at 150 mM is 23.5 ± 2.6 meq/(kg dry wt) · min. The furosemide-sensitive component on the other hand, is essentially zero until the [Na^+] exceeds about 50 mM and then rises progressively to 7 meq/(kg dry wt) · min.

Table 2. Effect of NO_3^- and furosemide on Na^+ influx[a]

Medium	Na^+ influx	Inhibition relative to control	
(*A*) Cl^-	23.6 ± 2.2	0	(n = 4)
(*B*) Cl^- + furos.	16.1 ± 1.6	31.7	(n = 5)
(*C*) NO_3^-	15.4 ± 2.1	34.7	(n = 3)
(*D*) NO_3^- + furos.	15.8 ± 1.1	33.1	(n = 3)

[a] Ehrlich cells were incubated in either Cl^--containing medium (150 NaCl/6 KCl) or Cl^--free, NO_3^- medium (150 $NaNO_3$/6 KNO_3) in the presence and absence of 0.5 mM furosemide. At time zero ^{22}Na was added and the uptake measured for 3 min. Na^+ influx (meq/(kg dry wt) · min) was calculated from the uptake of ^{22}Na as described in Materials and Methods. Mean values ± 1 SE for between three and five experiments are reported.

The results of these experiments, when considered in the context of those described above, show that although the cotransport system requires Na^+

for optimal activity, Na^+ is not cotransported until its concentration exceeds 50 mM. Since K^+ cotransport decreases when the [Na^+] exceeds about 55 mM, it appears that decrement in K^+ transport is compensated for by an increase in Na^+ cotransport. Although the total cation influx through the cotransport pathway is almost constant, the relative fraction of KCl to NaCl movement decreases when [Na^+] exceeds about 55 mM. Therefore, in the normal physiological medium the cotransport system facilitates NaCl and KCl exchange with an overall stoichiometric relationship of 2 Cl^- : 1 K^+ : 1 Na^+.

Discussion

In the Ehrlich tumor cell restoration of normal volume and electrolyte content following a variety of experimental manipulations is associated with the activation of an electrically silent cotransport of $Na^+ + Cl^-$ (Hoffmann et al., 1983) or $Na^+ + K^+ + Cl^-$ (Geck et al., 1980). Volume-dependent, as well as catecholamine-activated cotransporters, from a variety of cell types share at least two features in common. In addition to the requirement for Na^+ and/or K^+, they are sensitive to the "loop" diuretics, bumetanide or furosemide, and exhibit selectivity of Cl^- over NO_3^- and most other anions (e.g., McManus & Schmidt, 1978; Kregenow, 1981; Hoffmann et al., 1983; Dunham & Benjamin, 1984).

In contrast to volume-dependent cotransport which effects net ion uptake, the Ehrlich cell membrane during the normal physiological steady state, mediates a furosemide-sensitive, Cl^--dependent cation transport which operates as a bidirectional exchanger.

Although both processes are furosemide-sensitive and require Cl^-, the lack of effect of replacement of Na^+ by choline on the steady-state Cl^- flux suggested the absence of $Na^+ + Cl^-$ cotransport (Sjoholm et al., 1981; Hoffmann et al., 1983). Our data (Fig. 3, Table 1) confirms this and in addition shows that in media nominally free of Na^+ the response to furosemide is almost completely abolished. If we accept furosemide sensitivity as an index of cotransport activity, then in the absence of Na^+, cotransport ceases or is greatly reduced. Since about 50% of Cl^- transport is mediated by the furosemide-sensitive pathway, one would expect the removal of Na^+ to result in a significant reduction in Cl^- transport. This, however, is not the case. The apparent inconsistency is resolved when the effect of the anion exchange exhibitor, DIDS, is considered. In normal Na^+ medium DIDS inhibits Cl^- transport by about 45%; this, however, increases to 78% in the absence of Na^+ suggesting an increase in the turnover of the self-exchange pathway. Since the DIDS-sensitive component of Cl^- transport exhibits self-inhibition (Hoffmann et al., 1979; Levinson, 1984), it is tempting to speculate that the removal of Na^+, like an increase in [H^+], partially reduces the inhibitory effect of the modifier site, thereby resulting in an increase in transport through the self-exchange pathway. This possibility is currently under investigation.

The dependence of steady-state cotransport on Na^+ is perhaps best seen under conditions where the anion exchanger is inhibited by DIDS (Fig. 4). Note that the removal of Na^+ is equivalent to the addition of furosemide in that the Cl^- flux is almost completely inhibited. The residual flux most likely represents nonmediated, diffusional transport. However, with increasing concentrations of Na^+, furosemide-sensitive Cl^- transport increases and attains a maximal rate of 20 meq/(kg dry wt) · min. This rate is maintained between 20 and 55 mM Na^+. Since Na^+ and K^+ were present in the medium, it was conceivable both were cotransported with Cl^-. It is clear, however, that over the range of 1 to 55 mM Na^+ only K^+ is cotransported (Fig. 6). In the absence of Na^+ the ouabain-insensitive component of K^+ transport is inhibited by between 92 and 94%, which is equivalent to the effect of furosemide at all concentrations of Na^+ tested. Ouabain-insensitive transport increases with an increase in Na^+ and like that of Cl^- reaches and maintains a maximal rate of 17.8 meq/(kg dry wt) · min at about 20 to 55 mM. Over the range of 1 to 55 mM Na^+ the stoichiometric relationship between furosemide-sensitive Cl^- and K^+ transport is with minor variation, 1 : 1; while the maximal rate of KCl cotransport is between 18 to 20 meq/(kg dry wt) · min. Dependence of KCl transport on Na^+ is also characteristic of the cotransport system of human and avian red blood cells. However, in contrast to the erythrocyte where Na^+ is transported along with K^+ (Wiley & Cooper, 1974; Schmidt & McManus, 1977; Chipperfield, 1980; Dunham et al., 1980) our data indicate at [Na^+] below 50 mM, KCl is the only ion-pair transported. This is suggested by the finding that although Na^+influx increases when the [Na^+] is increased from 5 to 50 mM, a furosemide-sensitive, Cl^--dependent component is not detectable (Fig. 8, Table 2), However, at [Na^+] greater than about 50 mM there is a progressive increase in the furosemide-sensitive Na^+ influx. At 150 mM Na^+ this amounts to 7 meq/(kg dry wt) · min or 30% of the total Na^+ flux. The increase in the furosemide-sensitive Na^+ influx which occurs over the range of Na^+ concentrations, 50 to 150 mM, correlates well with the decrease in the ouabain-insensitive K^+ influx. Thus, while K^+ transport decreases from 17.8 meq/(kg dry

wt) · min at 60 mM Na^+ to about 9 meq/(kg dry wt) · min at 150 mM Na^+, Na^+ cotransport increases by a similar amount.

Furosemide-sensitive Cl^- transport, on the other hand, decreases about 20%, from 20 meq/(kg dry wt) · min at 55 mM Na^+ to 16 meq/(kg dry wt) · min at 150 mM Na^+. Thus, while the stoichiometric relationship between furosemide-sensitive K^+ and Cl^- transport is 1 : 1 at 55 mM Na^+ and lower, at 150 mM it is 2 Cl^- : 1 K^+ : 1 Na^+.

The change in stoichiometry may be interpreted in at least two ways. First, although the cotransport system may mediate the transmembrane movement of the quarternary complex, 2 Cl^- : 1 K^+ : 1 Na^+ at physiological concentration of Cl^-, K^+ and Na^+, the coupling ratio is not fixed. Rather, the relationship between Cl^-, K^+ and Na^+ is variable and as our results suggest is dependent on Na^+. Thus, at low [Na^+] 2 Cl^- : 2 K^+ are transported but at high [Na^+], 2 Cl^- : 1 K^+ : 1 Na^+. This of course, does not preclude a dependence on other variables including K^+, Cl^-, and cell volume (Geck et al., 1981). Second, it is also possible that depending on the condition, the cotransport system mediates the transfer of only KCl or NaCl ion pairs; the relative proportion of each dependent on the [Na^+]. In both cases Na^+ may have two roles. At low concentration, up to about 20 mM, Na^+ is required for KCl cotransport but is not transported with Cl^-. This suggests that Na^+ serves as an activator and may be related to an observation made in LK sheep red cells that, in addition to K^+ and Cl^-, Na^+ is required for inhibition of KCl transport by furosemide even though Na^+ is not transported (Lauf, 1984). Na^+ could facilitate furosemide inhibition by binding to a functionally inoperative site or alternatively compete for K^+ on the KCl cotransporter. A distinction between these and other possibilities cannot presently be made. At higher [Na^+], 50 to 150 mM, there is a decrease in KCl and a concomitant increase in NaCl cotransport. This suggests that although K^+ is the preferred substrate, it can be partially displaced by Na^+. The implication is that the cation binding site(s) is not absolutely specific for K^+. It is important to note that the total flux through the cotransport pathway decreases somewhat when the transport of NaCl relative to KCl increases (Fig. 4). This raises the possibility that KCl and NaCl are not strictly equivalent and may signify that the transporter cycles slower when transferring NaCl or the complex, 2 Cl^- : 1 K^+ : 1 Na^+.

We can only speculate as to the relationship between steady-state ion cotransport and that which occurs during nonsteady-state conditions, such as volume regulation. Both are Cl^--selective, furosemide-sensitive and involve, under certain conditions, the same ions. Thus, it seems reasonable to suspect, at least superficially, that both processes are accomplished by the same membrane transport system. During the physiological steady state the driving forces on Na^+, K^+, and Cl^-, as well as the symmetry of the system, are such that the cotransporter effects a transmembrane exchange of KCl for KCl and NaCl for NaCl. Activation of anion-cation cotransport during volume regulation most likely involves the conversion of the cotransporter from an exchanger to a net pathway. Although the factors responsible are as yet unknown, the suggestion has been made that cellular Cl^- may play an important role in the volume regulatory response of frog skin epithelial cells (Ussing, 1982). Since volume regulation (RVI) in Ehrlich cells is studied in preparations that have experienced a significant loss of Cl^-, about 35% in Hoffmann's experiments (Hoffmann et al., 1983), it is conceivable that this contributes to a selective inhibition of ion efflux mediated by the cotransport system.

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References

Aull, F. 1981. Potassium chloride cotransport in steady-state ascites tumor cells. Does bumetanide inhibit? *Biochim. Biophys. Acta* **643**:339–345

Aull, F. 1982. Specific drug sensitive transport pathways for chloride and potassium ions in steady-state Ehrlich mouse ascites tumor cells. *Biochim. Biophys. Acta* **688**:740–746

Aull, F., Nachbar, M., Oppenheim, J.D. 1977. Chloride self-exchange in Ehrlich ascites cells. Inhibition by furosemide and 4-actamido-4′-isothiocyanostilbene-2,2′-disulfonic acid. *Biochim. Biophys. Acta* **471**:341–347

Bakker-Grunwald, T. 1978. Effect of anions on potassium self-exchange in ascites tumor cells. *Biochim. Biophys. Acta* **513**:292–295

Bakker-Grunwald, T., Andrew, J.S., Neville, M.C. 1980. K^+ influx components in ascites cells: The effects of agents interacting with the (Na^+ + K^+)-pump. *J. Membrane Biol.* **52**:141–146

Bowen, J.W., Levinson, C. 1982. Phosphate concentration and transport in Ehrlich ascites tumor cells: Effect of sodium. *J. Cell. Physiol.* **110**:149–154

Chipperfield, A.R. 1980. An effect of chloride on (Na + K) cotransport in human red blood cells. *Nature (London)* **286**:281–282

Dunham, P.B., Benjamin, M. 1984. Cl^- dependent cation transport in mammalian erythrocytes. *Fed. Proc.* **43**:2476–2478

Dunham, P.V., Stewart, G.W., Ellory, J.C. 1980. Chloride-activated potassium transport in human erythrocytes. *Proc. Natl. Acad. Sci. USA* **77**:1711–1715

Geck, P., Heinz, E., Pfeiffer, B. 1981. Influence of high ceiling diuretics on ion fluxes and cell volume of Ehrlich ascites tumour cells. *In:* Ototoxic Side Effects of Diuretics. R. Klinke, H. Querfurth, and J.S. Scholtholt, editors. pp. 25–37. *Scand. Audio. Suppl.* 14.

Geck, P., Heinz, E., Pietrzyk, C., Pfeiffer, B. 1978. The effect of furosemide on the ouabain-insensitive K^+ and Cl^- movements in Ehrlich cells. *In:* Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach. R.W. Straub and I. Bolis, editors, pp. 301–307. Raven, New York

Geck, P., Pietrzyk, C., Burckhardt, B.C., Pfeiffer, B., Heinz, E. 1980. Electrically silent cotransport of Na^+, K^+ and Cl^- in Ehrlich cells. *Biochim. Biophys. Acta* **600:**432–437

Grinstein, S., Clarke, C.A., DuPre, A., Rothstein, A. 1983. Volume-induced increase of anion permeability in human lymphocytes. *J. Gen. Physiol.* **89:**801–823

Grinstein, S., DuPre, A., Rothstein, A. 1982. Volume regulation by human lymphocytes. *J. Gen. Physiol.* **79:**849–868

Hoffmann, E.K. 1982. Anion exchange and anion-cation cotransport systems in mammalian cells. *Philos. Trans. R. Soc. London, B* **299:**519–539

Hoffmann, E.K., Simonsen, L.O., Lambert, I.H. 1984. Volume-induced increase of K^+ and Cl^- permeabilities in Ehrlich ascites tumor cells. Role of internal Ca^{2+}. *J. Membrane Biol.* **78:**211–222

Hoffmann, E.K., Simonsen, L.O., Sjøholm, C. 1979. Membrane potential, chloride exchange and chloride conductance in Ehrlich mouse ascites tumor cells. *J. Physiol. (London)* **296:**61–84

Hoffmann, E.K., Sjøholm, C., Simonsen, L.O. 1983. Na^+, Cl^- cotransport in Ehrlich ascites tumor cells activated during volume regulation (regulatory volume increase). *J. Membrane Biol.* **76:**269–280

Kregenow, F.M. 1981. Osmoregulatory salt transporting mechanisms: Control of cell volume in anisotonic media. *Annu. Rev. Physiol.* **43:**493–505

Lauf, P.K. 1984. Thiol-dependent passive K/Cl transport in sheep red cells: IV. Furosemide inhibition as a function of external Rb^+, Na^+, and Cl^-. *J. Membrane Biol.* **77:**57–62

Levinson, C. 1972. Phosphate transport in Ehrlich ascites tumor cells and the effect of arsenate. *J. Cell. Physiol.* **79:**73–78

Levinson, 1978. Chloride and sulphate transport in Ehrlich ascites tumor cells: Evidence for a common mechanism. *J. Cell. Physiol.* **95:**23–32

Levinson, 1984. Self-inhibition of chloride transport in Ehrlich ascites tumor cells. *J. Cell. Physiol.* **121:**442–448

Levinson, C., Villereal, M.V. 1976. The transport of chloride in Ehrlich ascites tumor cells. *J. Cell. Physiol.* **88:**181–192

McManus, T.J., Schmidt, W.F., III 1978. Ion and co-ion transport in avian red cells. *In:* Membrane Transport Processes. J.F. Hoffman, editor. Vol. 1, pp. 79–106. Raven, New York

Mills, B., Tupper, J.T. 1975. Cation permeability and ouabain-insensitive cation flux in the Ehrlich ascites tumor cell. *J. Membrane Biol.* **20:**75–97

Schmidt, W.F., III, McManus, J. 1977. Ouabain-insensitive salt and water movements in duck red blood cells. II. Norepinephrine stimulation of sodium plus potassium co-transport. *J. Gen. Physiol.* **79:**81–97

Sjøholm, C., Hoffmann, E.K., Simonsen, L.O. 1981. Anion cation co-transport and anion exchange in Ehrlich ascites tumour cells. *Acta Physiol. Scand.* **112:**24A

Tupper, J.T. 1975. Cation flux in the Ehrlich ascites tumor cell. Evidence for Na-for-Na and K-for-K exchange diffusion. *Biochim. Biophys. Acta* **394:**586–596

Ussing, H.H. 1982. Volume regulation of frog skin epithelium. *Acta Physiol. Scand.* **114:**363–369

Villereal, M.L., Levinson, C. 1977. Chloride-stimulated sulfate efflux in Ehrlich ascites tumor cells: Evidence for 1 : 1 coupling. *J. Cell. Physiol.* **90:**553–564

Wiley, J.S., Cooper, R.A. 1974. A furosemide-sensitive cotransport of sodium plus potassium in the human red cell. *J. Clin. Invest.* **53:**745–755

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