J. Membrane Biol. 76, 269–280 (1983)

The Journal of
Membrane Biology

Na^+,Cl^- Cotransport in Ehrlich Ascites Tumor Cells Activated During Volume Regulation (Regulatory Volume Increase)

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Summary. Ehrlich ascites cells were preincubated in hypotonic medium with subsequent restoration of tonicity. After the initial osmotic shrinkage the cells recovered their volume within 5 min with an associated KCl uptake. 1. The volume recovery was inhibited when NO_3^- was substituted for Cl^-, and when Na^+ was replaced by K^+, or by choline (at 5 mM external K^+). 2. The volume recovery was strongly inhibited by furosemide and bumetanide, but essentially unaffected by DIDS. 3. The net uptake of Cl^- was much larger than the value predicted from the conductive Cl^- permeability. The unidirectional ^{36}Cl flux, which was insensitive to bumetanide under steady-state conditions, was substantially increased during regulatory volume increase, and showed a large bumetanide-sensitive component. 4. During volume recovery the Cl^- flux ratio (influx/efflux) for the bumetanide-sensitive component was estimated at 1.85, compatible with a coupled uptake of Na^+ and Cl^-, or with an uptake via a $K^+,Na^+,2Cl^-$ cotransport system. The latter possibility is unlikely, however, because a net uptake of KCl was found even at low external K^+, and because no K^+ uptake was found in ouabain-poisoned cells. 5. In the presence of ouabain a bumetanide-sensitive uptake during volume recovery of Na^+ and Cl^- in nearly equimolar amounts was demonstrated. It is proposed that the primary process during the regulatory volume increase is an activation of an otherwise quiescent, bumetanide-sensitive Na^+,Cl^- cotransport system with subsequent replacement of Na^+ by K^+ via the Na^+/K^+ pump, stimulated by the Na^+ influx through the Na^+,Cl^- cotransport system.

Key Words Na^+,Cl^- cotransport · volume regulation · regulatory volume increase · chloride fluxes · Ehrlich mouse ascites tumor cells · bumetanide · furosemide · DIDS

Introduction

The ion distribution across the cell membrane of Ehrlich ascites tumor cells is generally described, following the concept originally developed by Leaf (1959), Ussing (1960) and Tosteson and Hoffman (1960), as a pump-leak steady state for Na^+ and K^+ (Grobecker, Kromphardt, Mariani & Heinz, 1963; Hempling, 1966), with some evidence of an exchange diffusion component (Mills & Tupper, 1975; Tupper, 1975). Under steady-state conditions Cl^- was found to be close to electrochemical equilibrium across the cell membrane (Lassen, Nielsen, Pape & Simonsen, 1971). The volume of Ehrlich cells is determined by the cell content of osmotically active solutes, and the cell volume is precisely regulated via dynamic and controlled changes of the leak pathways (Hoffmann, 1978; for other cell types *see* review by Kregenow, 1981).

Several pathways for passive anion and cation transport have been demonstrated in Ehrlich cells during recent years. The conductive Cl^- permeability is low and of a magnitude similar to that of the conductive Na^+ and K^+ permeabilities (Heinz, Geck & Pietrzyk, 1975; Simonsen, Hoffmann & Sjøholm, 1976; Hoffmann, Simonsen & Sjøholm, 1979). This means that in Ehrlich cells the Cl^- permeability may become rate limiting for loss and uptake of salt and water during volume regulation. This is at variance with the findings in human red cells (*see* Knauf, Fuhrmann, Rothstein & Rothstein, 1977), where the conductive Cl^- permeability is of an absolute magnitude similar to that found in Ehrlich cells but about two orders of magnitude larger than the conductive Na^+ and K^+ permeabilities.

An electrically silent Cl^- exchange with properties similar to that of the anion exchange system found in red cells and some other cell types (*see* review by Wieth & Brahm, 1983) has been demonstrated in Ehrlich cells (Levinson & Villereal, 1976; Aull, Nachbar & Oppenheim, 1977; Hoffmann et al., 1979). Moreover, cotransport systems for K^+,Cl^- (Aull, 1981) or $K^+,Na^+,2Cl^-$ (Geck et al., 1980) have recently been described. Furthermore, a Ca^{2+}-dependent K^+ channel similar to that found in red cells (*see* Lew & Ferreira, 1978) has recently been demonstrated (Valdeolmillos, Garcia-Sancho & Herreros, 1982). The net loss of KCl seen in several cell types during regulatory volume decrease in hypotonic media (*see* reviews by

McKnight & Leaf, 1977, and Hoffmann, 1977, 1983) has been proposed to involve activation of both the Ca^{2+}-dependent K^+ channel and a separate Cl^- channel in human lymphocytes (Grinstein, DuPre & Rothstein, 1982; Grinstein, Clarke, DuPre & Rothstein, 1983) and in Ehrlich cells (Hoffmann, Simonsen & Lambert, 1983). The pathways of anion transport in Ehrlich cells have recently been reviewed by Hoffmann (1982).

In hypertonic media Ehrlich cells behave as osmometers with no sign of volume recovery (Hempling, 1960; Hendil & Hoffmann, 1974). At low Cl^- concentration, however, an increase in K^+ and Cl^- fluxes in osmotically shrunken cells has been demonstrated (Hoffmann, 1978), suggesting a possible role in volume regulation. The present study demonstrates a volume recovery in Ehrlich cells when hypotonic pretreatment, resulting in a substantial net loss of KCl, is followed by restoration of tonicity. After the initial osmotic shrinkage a net uptake of KCl could be deomonstrated which was found to be Na^+- and Cl^--dependent and bumetanide- and furosemide-sensitive. It is proposed that the primary process during the regulatory volume increase is an activation of an otherwise quiescent bumetanide-sensitive Na^+,Cl^- cotransport system with subsequent replacement of Na^+ by K^+ via the Na^+/K^+ pump, stimulated by the Na^+ influx through the Na^+,Cl^- cotransport system. A similar mechanism has quite recently been proposed for volume regulation in frog skin epithelial cells (Ussing, 1982; Kristensen & Ussing, 1983). A $K^+,Na^+,2Cl^-$ cotransport system activated during regulatory volume increase has been described in nucleated avian red cells (*see* reviews by McManus & Schmidt, 1978, and Kregenow, 1981). The trigger mechanism for activation of the proposed Na^+,Cl^- cotransport system during regulatory volume increase in Ehrlich cells is discussed. Preliminary reports of this study have previously been presented (Hoffmann, Sjøholm & Simonsen, 1981; Sjøholm, Hoffmann & Simonsen, 1981).

Materials and Methods

Cell Suspensions

Ehrlich mouse ascites tumor cells (hyperdiploid strain) were maintained by weekly intraperitoneal transplantation in white female NMRI mice, and 8 days after transplantation harvested and suspended in a standard saline solution containing heparin (2.5 IU/ml). This solution (A) had the following composition (mM): Na^+, 150; K^+, 5; Mg^{2+}, 1; Ca^{2+}, 1; Cl^-, 150; sulfate, 1; inorganic phosphate, 1; MOPS (morpholinopropane sulfonic acid), 3.3; TES (N-tris-(hydroxymethyl)-methyl-2-amino-ethane sulfonic acid), 3.3; and HEPES (N-2-hydroxyethylpiperazine-N′-2-ethane-sulfonic acid), 5; pH 7.40 (*cf.* Eagle, 1971). The cells were washed by centrifugation (45 sec, $700 \times g$) once with the standard saline solution and once with the appropriate experimental incubation medium (*see below*). The cytocrit was adjusted to 8%. [^{3}H] inulin (The Radiochemical Centre, Amersham, England; 5×10^6 dpm/ml) was added as marker of extracellular space.

Incubation Media

The following media were used: (A) Standard saline solution, 300 mOsm (*see above*); (B) Hypotonic 225 mOsm and 150 mOsm saline solutions, prepared by diluting the standard saline solution (A) with distilled water containing MOPS, TES, and HEPES in concentrations as in solution A, in order to lower both ion concentrations and total osmolarity; (C) Double-strength saline solution with double ion concentrations compared to solution A, but with the same buffer concentration; (D) Isotonic, hypotonic or double-strength nitrate or choline incubation media in which $NaNO_3$ or choline chloride, respectively, was substituted for NaCl in equimolar amounts. The choline media contained the same K^+ concentration as the standard media; (E) Potassium incubation medium in which KCl was substituted for NaCl in equimolar amounts.

Reagents

Bumetanide, furosemide and trifluoperazine were gifts from Leo Pharmaceutical Products, Copenhagen; Hoechst, Frankfurt (M); and Lundbeck & Co., Copenhagen, respectively. Quinine hydrochloride was obtained from Sigma, St. Louis, Mo. 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) was the kind gift of Dr. J.O. Wieth, University of Copenhagen. All reagents were analytical grade.

Pretreatment of the Cells by Exposure to Low External Osmolarity

The cells were initially incubated in 300 mOsm saline solution for 30 to 40 min. The cells were then packed by centrifugation, resuspended in hypotonic saline solution (225 or 150 mOsm) and incubated for 20 to 40 min. At zero time a tonicity of 300 mOsm was restored by addition of 1/4 or 1/2 volume, respectively, of double-strength saline solution. Duplicate samples for determination of water and ion content were taken before exposure to the hypotonic saline solution and again before restoration of tonicity. In the experiments in K^+ medium the hypotonic pretreatment followed the protocol for control cells, and at zero time the cells were washed and resuspended in 300 mOsm K^+ incubation medium.

Measurements of Cellular and Extracellular Ion Concentrations

Two 1-ml samples of the cell suspension were transferred to preweighed vials for determination of ion content and cell water, respectively. The vials were centrifuged ($14{,}000 \times g$, 60 sec), the supernatant removed by suction and the samples weighed. In one sample cell water was determined by reweighing after drying for 48 hr at 90 °C. In the other sample the packed cells were lysed in 800 µl distilled water and deproteinized with addition of 100 µl perchloric acid (70%). Two 100-µl samples of the supernatant were saved and processed in parallel with the cell pellets for determination of ion content.

K^+ and Na^+ were determined by emission flame photometry (Pye/Unicam SP90B Atomic Absorption Photometer, Pye/Unicam, Ltd., England) after dilution of samples and stan-

dards with 1 mM CsCl in order to increase the linearity of standard curves and to eliminate the interference of Na^+ in K^+ measurements and vice versa. Cl^- concentration was assessed by coulometric titration (CMT 10 Chloride Titrator, Radiometer, Copenhagen, Denmark). ^3H and $^{36}Cl^-$ (*see below*) were measured by liquid scintillation counting using Pico-Flour-15 (Packard) as scintillation cocktail and a Packard CD 460 spectrometer.

The cellular ion concentrations are given as the concentration in cell water after correction for trapped volume ([^3H] inulin space) in the cell pellets.

^{36}Cl Flux Experiments

The chloride tracer exchange flux under steady-state conditions was measured as unidirectional efflux, as previously described (Hoffmann et al., 1979). Briefly, the efflux from ^{36}Cl preloaded cells into the medium was followed with time by a rapid filtration technique.

The ^{36}Cl influx during regulatory volume increase was measured by adding ^{36}Cl to the external medium and monitoring the cell ^{36}Cl activity with time (*see* Simonsen & Nielsen, 1971). In the present experiments the unidirectional influx was measured as the initial rate of ^{36}Cl uptake, as determined from the slope of the first part of the influx curves. ^{36}Cl was obtained from Risø, Denmark.

Cell Volume Measurements

An aliquot of the cell suspension was diluted to 50,000 cells per ml with filtered medium (3 times, 0.45 μm filter). Cell volume distribution curves were obtained from a Coulter Counter model Z with Coulter Channelyser (C-1000) and recorder (HR 2000). Orifice diameter was 100 μm to ensure linearity between cell volume and pulse height. The mean cell volume (arbitrary units) was calculated as the median of the distribution curves. To obtain absolute cell volumes polystyrene latex beads (18.71 μm and 12.6 μm diameter) were used as standards. Control experiments with polystyrene latex beads have shown that the instrument readings are independent of the ionic composition and osmolarity of the media within the range used here (Hendil & Hoffmann, 1974).

Statistical Evaluation

All values are given as the mean ±SEM with the number of experiments in brackets. Student's *t*-test was used to evaluate statistical significance.

Results

Regulatory Volume Increase

In the present experiments Ehrlich cells were exposed to hypotonic pretreatment for 20 to 40 min (*see* Materials and Methods) resulting, as previously reported (Hendil & Hoffmann, 1974; Hoffmann, 1978), in a regulatory volume decrease associated with a substantial net loss of KCl. The pretreatment was followed by restoration of tonicity, and under these conditions a volume recovery (regulatory volume increase) could be observed following the initial osmotic shrinkage. Similar protocols have been used for experiments with flounder red blood cells (Cala, 1977) and frog skin epithelium (Ussing, 1982).

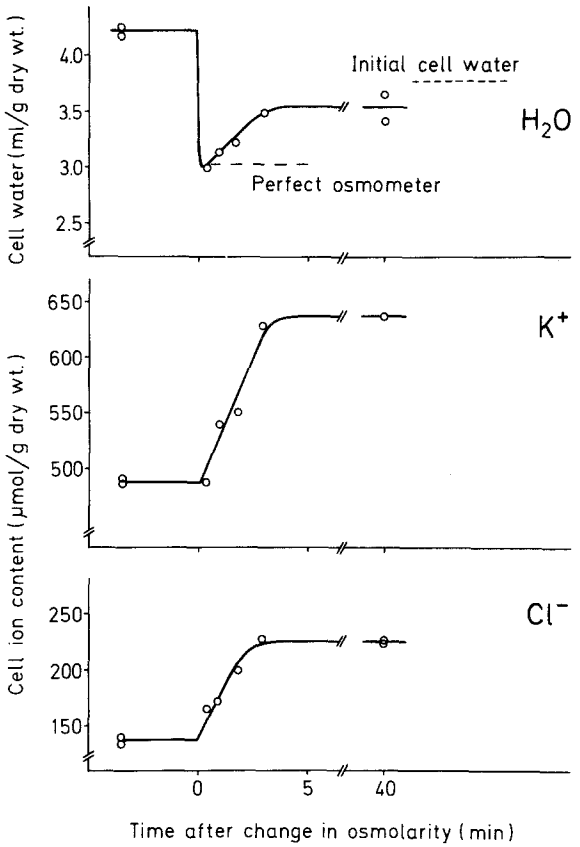

Fig. 1. Regulatory volume increase in Ehrlich ascites cells after increase in external osmolarity. The cells were pretreated by exposure to low external osmolarity (225 mOsm) for 20 to 40 min (*see* Materials and Methods). At zero time a tonicity of 300 mOsm was restored by addition of 1/4 volume of a double-strength saline solution, and cell water, K^+, and Cl^- content were followed with time. The cell water content calculated for a perfect osmometer is indicated by the broken line. The original cell water content in 300 mOsm saline solution before the hypotonic pretreatment is indicated on the Figure for reference. The Figure shows a typical experiment from a total of 8 experiments, summarized in Table 1 (control)

Figure 1 shows that restoration of 300 mOsm tonicity after hypotonic pretreatment induced an initial osmotic shrinkage to a cell water content close to that predicted for a perfect osmometer, followed by a net uptake of water, K^+, and Cl^-. This net uptake resulted within 3 to 5 min in a recovery of cell volume up to a level near the original cell volume in the 300 mOsm saline solution. The rate of uptake was calculated from the slope of the approximately linear first part of the uptake curves. The values from several experiments are given in Table 1. The net uptake of Na^+ was insignificant [5.6±2.7 μmol/g dry wt × min (7 independent experiments)], and the increase in the Na^+

Table 1. Rate of uptake of water, K^+, Cl^- during regulatory volume increase in Ehrlich ascites cells[a]

Exptl. condition	H_2O	K^+	Cl^-
	(ml/g dry wt × min)	(μmol/g dry wt × min)	
Control	0.18±0.04 (6)[b]	28±4 (7)	25±3 (8)
Nitrate medium[c]	0.04±0.02 (3)	7±3 (3)	–
Choline medium	−0.01±0.03 (3)	−11±4 (2)	−3±3 (3)
Potassium medium	0.12±0.03 (2)	17±5 (2)	15±3 (2)
Furosemide, 1 mM	0.00±0.03 (3)	8±2 (3)	6±1 (3)
Bumetanide, 25 μM	0.03±0.01 (4)	9±1 (4)	3±1 (4)

[a] The cells were pretreated at low external osmolarity (225 mOsm) for 20 to 40 min (*see* Materials and Methods). A 300-mOsm tonicity was restored at zero time, and cell water, K^+ and Cl^- content were followed with time during the regulatory volume increase. For experimental details *see* legend to Fig. 1. The choline media contained the same K^+ concentration as the standard media. In the experiments in K^+ medium the hypotonic pretreatment followed the protocol for control cells. At zero time the cells were washed and resuspended in 300 mOsm K^+ incubation medium. Furosemide and bumetanide were added with the double-strength saline solution used for restoration of tonicity at zero time. The rate of uptake was determined as the slope of the first part of the uptake curves. Typical experiments are shown in Figs. 1 through 4.

[b] The values are given as mean ± SEM with the number of independent experiments in parentheses.

[c] In one experiment the cells were pretreated in 150 mOsm nitrate medium.

concentration in cell water during regulatory volume increase was within the experimental error. The ratio of the uptake of $(K^+ + Na^+ + Cl^-)$ to the uptake of water corresponds to a calculated osmolarity of about 300 mOsm.

Ion Substitution

Substitution of nitrate for Cl^- during the hypotonic pretreatment and during the restoration of tonicity resulted in inhibition of the regulatory volume increase (*see* Fig. 2 and Table 1). Substitution of choline for Na^+ (at unchanged external K^+) also inhibited the volume recovery (*see* Fig. 3 and Table 1). If K^+ is substituted for Na^+ in the restoration of tonicity (for details *see* Materials and Methods) the regulatory volume increase was inhibited about 40% (*see* Table 1). Thus, the uptake of KCl seems to be Cl^-- and Na^+-dependent.

Inhibitors

Both furosemide and bumetanide, which have been reported to inhibit cotransport systems in Ehrlich cells (Geck, Heinz, Pietrzyk & Pfeiffer, 1978; Geck et al., 1980; Aull, 1981), are strong inhibitors of the ion and water uptake during the regulatory volume increase (Fig. 4. Table 1). DIDS, however, which inhibits the anion exchange in Ehrlich cells (Levinson, 1978; Sjøholm et al., 1981) has no effect on the regulatory volume increase: In the presence of 200 μM DIDS (in one experiment 100 μM) the net uptake of Cl^- was 18.3±2.5 μmol/g dry wt × min (3 experiments) or 91±3% of the uptake in parallel control cells ($n=3$, paired analysis). In some experiments (*data not shown*) the regulatory volume increase was monitored using a Coulter Counter (*see* Materials and Methods). In these experiments the volume recovery was accelerated in the presence of quinine (0.5 or 1 mM) which inhibits the Ca^{2+}-dependent K^+ channel in red cells (Lew & Ferreira, 1978) and in Ehrlich cells (Valdeolmillos et al., 1982). The regulatory volume increase was not affected by the anti-calmodulin drug trifluoperazine (Weiss et al., 1980) at a concentration of 40 μM (*data not shown*).

Activation of Cl^- Transport During Regulatory Volume Increase

The data presented in Table 2 show that the bumetanide-sensitive Cl^- transport is negligible under physiological steady-state conditions, but strongly activated during the regulatory volume increase: The unidirectional ^{36}Cl influx under steady-state conditions was neither affected by bumetanide nor (*data not shown*) by substitution of Na^+ with choline. During the regulatory volume increase, however, the ^{36}Cl influx was significantly increased, and the additional ^{36}Cl influx was completely inhibited by bumetanide.

Flux Ratio Analysis

In the experiments presented in Table 3 the unidirectional ^{36}Cl influx was measured concurrently with the net Cl^- uptake during the regulatory volume increase in the presence or absence of either DIDS or bumetanide. The DIDS-sensitive and bumetanide-sensitive components were determined from the difference in paired controls. It can be seen that the unidirectional Cl^- influx has both a DIDS- and a bumetanide-sensitive component, whereas the Cl^- net flux is essentially bumetanide-sensitive and DIDS-insensitive. The finding that the sum of the DIDS- and bumetanide-sensitive components of the unidirectional ^{36}Cl influx is only about 85% of the control is probably due to incomplete DIDS inhibition of the anion exchange system: The $K_{1/2}$ for DIDS inhibition of ^{36}Cl self-exchange is about 50 μM and at 100 to

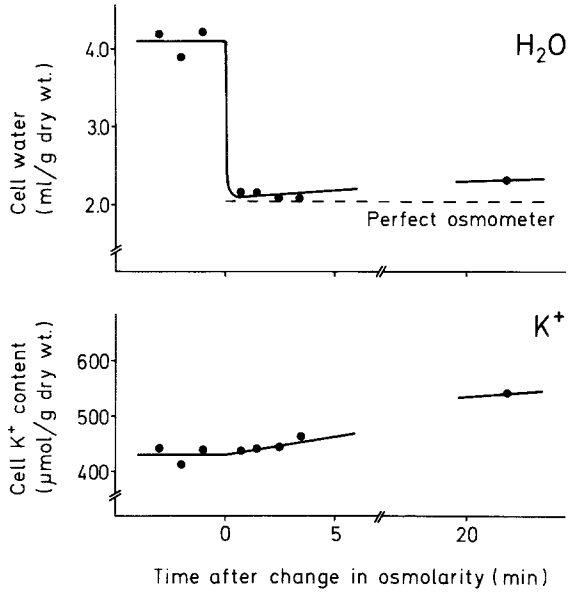

Fig. 2. Effect of chloride substitution on regulatory volume increase in Ehrlich ascites cells. Experimental protocol as in Fig. 1, except that nitrate was substituted for Cl^- during hypotonic pretreatment of the cells in 150 mOsm saline solution and in the double-strength saline solution used for restoration of 300 mOsm tonicity at zero time. Two experiments with hypotonic pretreatment in 225 mOsm nitrate medium gave similar results, summarized in Table 1

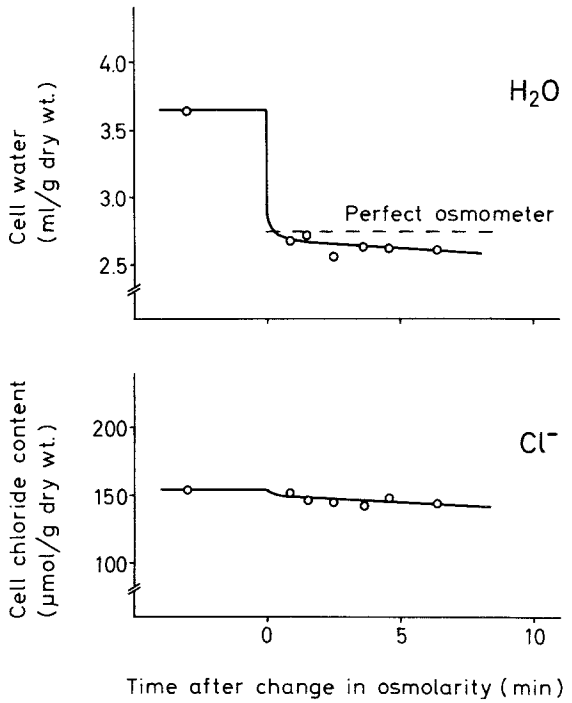

Fig. 3. Effect of sodium substitution on regulatory volume increase in Ehrlich ascites cells. Experimental protocol as in Fig. 1, except that choline was substituted for Na^+ in the 225 mOsm saline solution used for hypotonic pretreatment of the cells, and in the double-strength saline solution used for restoration of 300 mOsm tonicity at zero time. The K^+ concentration of the choline media was equal to that of the standard media. The Figure shows a typical experiment from a total of 3 experiments summarized in Table 1

200 μM DIDS the inhibition is only 65 to 80% of the maximal DIDS inhibition (C. Sjøholm & E.K. Hoffmann, *to be submitted*). Extrapolating to maximal DIDS inhibition, the DIDS-sensitive component is calculated to be 25 ± 4 μmol/g dry wt × min ($n = 3$). The sum of this value and the bumetanide-sensitive component is 64 ± 6 μmol/g dry wt × min which is close to the control value. The small difference may be accounted for by the conductive Cl^- flux which in the absence of inhibitors is estimated at about 5 μmol/g dry wt × min (Hoffmann et al., 1979).

The unidirectional Cl^- efflux was calculated as the difference between the simultaneously measured unidirectional ^{36}Cl influx and the Cl^- net flux, and the flux ratio was calculated as the ratio between unidirectional influx and efflux. The fluxes were measured as the initial rate of uptake after restoration of tonicity, and consequently the calculated flux ratio is extrapolated to zero time. The flux ratio can be analyzed also under non-steady-state conditions, and the extrapolated value should represent the true flux ratio at that time

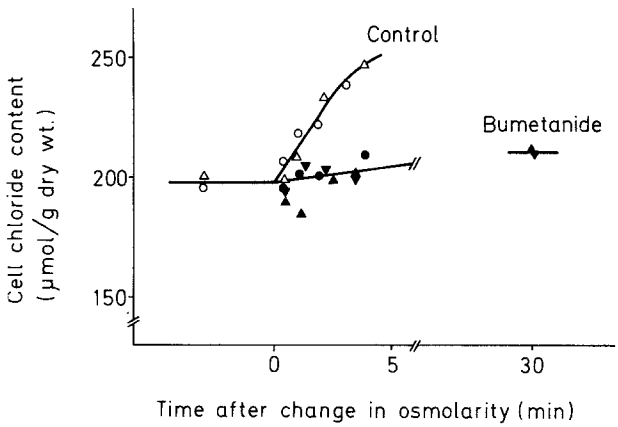

Fig. 4. Inhibition by bumetanide of regulatory volume increase in Ehrlich ascites cells. For control cells (open symbols) the experimental protocol was the same as that in Fig. 1. In the parallel bumetanide groups (closed symbols) bumetanide, 25 μM (●, ▲) or 100 μM (▼) was added at zero time together with the double-strength saline solution. Only the cell chloride content is shown on the Figure. The Figure shows two independent experiments, marked by circles and triangles, respectively. Two further experiments (25 μM bumetanide) gave similar results (*see* Table 1)

Table 2. Activation of bumetanide-sensitive unidirectional chloride fluxes during regulatory volume increase in Ehrlich ascites cells[a]

Exptl. condition	Unidirectional ^{36}Cl influx (μmol/g dry wt × min)		
	Control	Bumetanide (25 μM)[c]	Bumetanide-sensitive component
Physiological steady state	43.6±2.0 (7)[b]	44.4±1.1 (3)	−0.8±2.3[d]
Regulatory volume increase (initial phase)	67.5±3.1 (8)	31.7±2.3 (4)	38.9±3.4 (3)[e]

[a] The cells in physiological steady state were incubated in 300 mOsm saline solution and the unidirectional Cl^- influx was determined as the tracer exchange flux under steady-state conditions (*see* Materials and Methods). Bumetanide was added 20 min before the ^{36}Cl addition for the flux measurements. For the measurements during regulatory volume increase the cells were pretreated at low external osmolarity, with restoration of 300 mOsm tonicity at zero time as described in legends to Figs. 1 and 4. ^{36}Cl was added with the double-strength saline solution at zero time, and the cell ^{36}Cl activity was followed with time during the regulatory volume increase. The initial rate of ^{36}Cl influx was determined as the slope of the first part of the influx curve. Bumetanide was added at zero time with the double-strength saline solution used for restoration of tonicity.

[b] The values are given as mean ±SEM with the number of independent experiments in parentheses.

[c] In one experiment the bumetanide concentration was 100 μM.

[d] Calculated as the difference between control and bumetanide columns.

[e] Paired analysis.

Table 3. Flux ratio analysis of unidirectional chloride fluxes during regulatory volume increase in Ehrlich ascites cells[a]

	n	Unidirectional ^{36}Cl influx	Net Cl^- flux	Unidirectional Cl^- efflux	Flux ratio Influx/efflux[d]
		μmol/g dry wt × min			
Control	8	67.5±3.1[b]	22.8±2.7	44.7±2.4	1.53±0.08
DIDS-sensitive component	3	18.3±2.4[c]	1.9±0.7	16.4±2.6	1.13±0.06
Bumetanide-sensitive component	3	38.9±3.4	17.3±0.3	21.6±3.6	1.85±0.15

[a] The cells were pretreated at low external osmolarity prior to restoration of 300 mOsm tonicity, and net Cl^- flux and unidirectional ^{36}Cl influx were measured during regulatory volume increase (*see* Tables 1 and 2). The unidirectional Cl^- efflux was determined in each experiment as the difference between the concurrently measured ^{36}Cl influx and net Cl^- flux. DIDS and bumetanide were added with the double-strength saline solution used for restoration of tonicity at zero time.

[b] The values are given as mean±SEM, *n* is the number of independent experiments with concurrent measurements of unidirectional ^{36}Cl influx and net Cl^- flux. The DIDS and bumetanide-sensitive flux components were determined as the difference between the fluxes in parallel groups with and without addition of inhibitor. The DIDS concentration was 100 μM in one experiment and 200 μM in two experiments. The bumetanide concentration was 25 μM in two experiments and 100 μM in one experiment.

[c] 25±4 μmol/g dry wt × min when extrapolated to infinite DIDS concentration (*see* text).

[d] The values are given as the mean±SEM of the flux ratios calculated in each independent experiment.

(Sten-Knudsen & Ussing, 1981). As seen in Table 3 the DIDS-sensitive component has a flux ratio close to unity, as expected for an exchange system. On the other hand, the bumetanide-sensitive component, which essentially accounts for the net uptake induced during regulatory volume increase, has a flux ratio of 1.85±0.15. This means that for the transport system involved in regulatory volume increase, the minimum ratio between the driving forces for influx and efflux must be 1.85.

Transport Pathways, Involvement of the Na^+/K^+ Pump

The observed Cl^- uptake during regulatory volume increase could conceivably be mediated either by a conductive Cl^- pathway or by anion-cation cotransport systems, such as K^+,Cl^-, or Na^+,Cl^-, or $K^+,Na^+,2Cl^-$ cotransport. The ratio between the driving forces for unidirectional influx and efflux for the above transport systems was calculated from the internal and external ion concentrations measured at the first data points after restoration of tonicity (Table 4). In these calculations the activity coefficients were assumed to be equal in cell water and in the extracellular medium. The flux ratio would be 0.96 for an electrodiffusional Cl^- transport, assuming a membrane potential of −27 mV as found in unperturbed Ehrlich cells (Hoffmann et al., 1979). The ratio between the driving forces for a K^+,Cl^- cotransport system is well below unity, which means that a cotransport of K^+,Cl^- would result in a net loss of KCl rather than the observed net uptake. A cotransport of Na^+,Cl^- and of $K^+,Na^+,2Cl^-$ could both account for a net uptake of Cl^-. A Na^+,Cl^- cotransport could easily account for the observed flux-ratio of 1.85±0.15, whereas the ratio of the driving

Table 4. Ion concentration ratio and the ratio between the driving forces for unidirectional influx and efflux during regulatory volume increase for a conductive Cl^- pathway and for various anion-cation cotransport systems[a]

$[Cl^-]_o/[Cl^-]_i$	2.63 ± 0.083 (8)[c]
$([Cl^-]_o/[Cl^-]_i) \exp(FV_m/RT)$[b]	0.96
$[K^+]_o[Cl^-]_o/[K^+]_i[Cl^-]_i$	0.079 ± 0.006 (8)
$[Na^+]_o[Cl^-]_o/[Na^+]_i[Cl^-]_i$	18 ± 1.6 (8)
$[K^+]_o[Na^+]_o[Cl^-]_o^2/[K^+]_i[Na^+]_i[Cl^-]_i^2$	1.5 ± 0.13 (8)

[a] The ion concentration ratios were calculated for each of the 8 experiments summarized in Table 1 from the internal and external ion concentrations measured at the first data point taken about 1/2 min after restoration of tonicity. The mean value of the measured internal ion concentrations were (mmol/liter cell water): K^+, 183 ± 7.1 (8); Na^+, 22 ± 1.9 (8); Cl^-, 59 ± 2.6 (8). The external ion concentrations were (mM): K^+, 5.6 ± 0.20 (8); Na^+, 150 ± 2.9 (8); Cl^-, 150 ± 2.2 (8).

[b] Calculated assuming a membrane potential of $V_m = -27$ mV.

[c] The values are given as mean ± SEM with the number of independent experiments in parentheses.

forces for a K^+,Na^+,$2Cl^-$ cotransport system (1.5 ± 0.13) is slightly below the observed flux ratio. The significance of the difference between these values is only marginal, however.

At low external K^+ concentration the ratio between the driving forces for influx and efflux via a K^+,Na^+,$2Cl^-$ cotransport system will be reduced. Figure 5 shows that a net uptake of KCl during regulatory volume increase was found even at low external K^+ concentration. Under these conditions the calculated ratio of the ion concentration products (shown in the lower panel) is below unity. Therefore, a cotransport of K^+,Na^+,$2Cl^-$ cannot account for the observed net uptake of KCl at low external K^+ concentration.

An activation during regulatory volume increase of a Na^+,Cl^- cotransport system would result in a net uptake of NaCl. The observed net uptake of K^+ could then be the result of a subsequent replacement of Na^+ by K^+ via the Na^+/K^+ pump, stimulated by the Na^+ influx through the Na^+,Cl^- cotransport system.

To test the involvement of the Na^+/K^+ pump, the ion uptake during regulatory volume increase was measured under conditions where the Na^+/K^+ pump was inhibited with ouabain. Figure 6 shows that a net uptake of NaCl during regulatory volume increase could be demonstrated in ouabain-poisoned cells whereas the K^+ uptake was strongly inhibited. In control cells the Na^+ net uptake was negligible. These findings strongly suggest that the K^+ uptake during regulatory volume increase is secondary, being the result of an activation of the Na^+/K^+ pump.

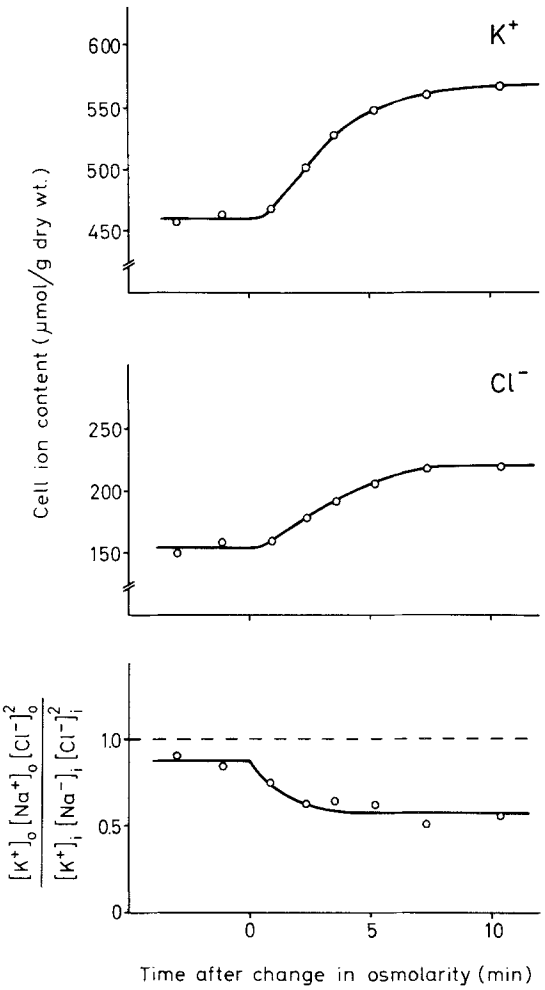

Fig. 5. Regulatory volume increase in Ehrlich ascites cells at low external K^+ concentration. Experimental protocol as in Fig. 1, except that Na^+ was substituted for K^+ in the double-strength saline solution used for restoration of tonicity at zero time (the final K^+ concentration during regulatory volume increase was 3.5 to 4 mM). The lower panel shows the ratio of the ion concentration products $[K^+]$ $[Na^+]$ $[Cl^-]^2$ calculated for the external medium (*o*) and cell water (*i*), respectively. A ratio below unity would indicate a net efflux by a hypothetical Na^+, K^+, $2Cl^-$ cotransport system. The Figure is representative of 2 experiments

In the presence of ouabain it should, moreover, be possible to distinguish between a Na^+,Cl^- and a Na^+,K^+,$2Cl^-$ cotransport, because a bumetanide-sensitive K^+ entry in ouabain-poisoned cells should be found together with the Na^+ entry only if the second process is operating. Figure 6 shows that the K^+ uptake in ouabain-poisoned cells during regulatory volume increase is negligible. In addition, Fig. 7 shows that the Na^+ uptake seen in the presence of ouabain during regulatory volume increase is bumetanide-sensitive and, moreover, that the bumetanide-sensitive Na^+ and Cl^- up-

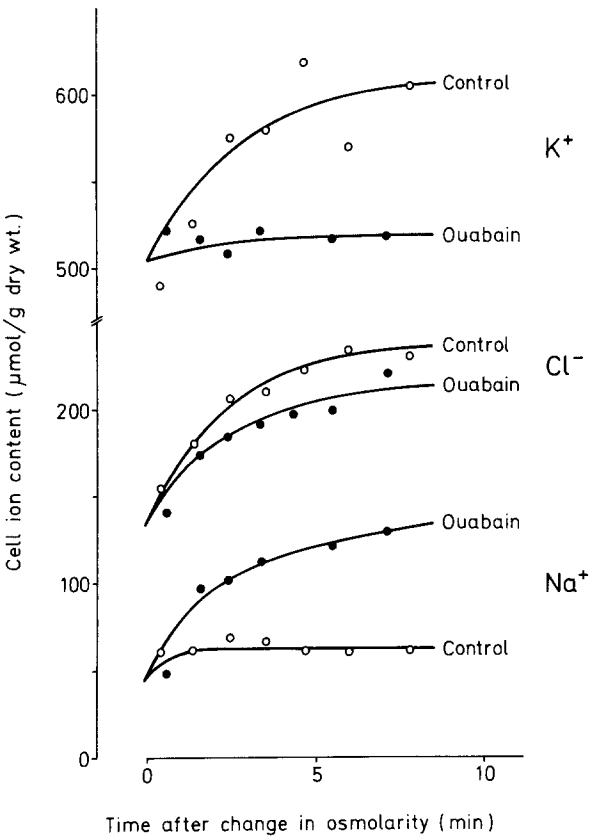

Fig. 6. Effect of ouabain on ion uptake during regulatory volume increase in Ehrlich ascites cells. Experimental protocol for the control group (open symbols) as in Fig. 1. In a parallel group (closed symbols) ouabain (1 mM) was added at zero time with the double-strength saline solution used for restoration of tonicity. The Figure is representative of 3 experiments

takes are of about equal magnitude. These findings provide evidence for the involvement of a Na^+,Cl^- cotransport system during regulatory volume increase rather than a Na^+,K^+,$2Cl^-$ cotransport system.

Discussion

In the present experiments Ehrlich ascites cells were exposed to hypotonic pretreatment followed by restoration of tonicity. As previously reported (Hendil & Hoffmann, 1974) Ehrlich cells initially swell in the hypotonic medium but subsequently regulate their volume (regulatory volume decrease) and reach a new steady state with a reduced KCl content and with a cell volume only slightly larger than the original cell volume. Following the restoration of tonicity the cells initially shrank but subsequently recovered their volume (regulatory volume increase) with an associated net uptake of KCl (*see* Fig. 1). We propose as a simple interpretation that the primary process during the observed regulatory volume increase is an activation of an otherwise quiescent, bumetanide-sensitive coupled uptake of Na^+ and Cl^-, with subsequent replacement of Na^+ by K^+ via the Na^+/K^+ pump, stimulated by the Na^+ influx through the Na^+,Cl^- cotransport system. The evidence for this hypothesis can be summarized as follows:

(i) The net K^+ and water uptake was found to be Cl^- dependent as seen from the inhibition of the uptake following substitution of NO_3^- for

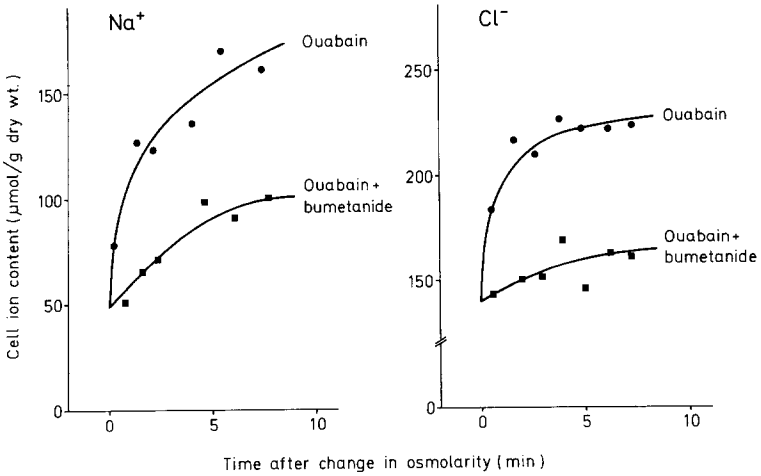

Fig. 7. Effect of bumetanide on Na^+ and Cl^- uptake during regulatory volume increase in the presence of ouabain. Experimental protocol as in Fig. 1. The volume recovery and the ion uptake was monitored in the presence of ouabain with and without addition of bumetanide. The data presented are from two independent experiments both showing inhibition of volume recovery (*data not shown*) and of Na^+ uptake (left frame) and Cl^- uptake (right frame). Ouabain (1.5 mM) was added at zero time in the experiment shown in the right panel and 2 min before the addition of the double-strength saline solution used for restoration of tonicity in the experiment shown in the left panel. In both experiments bumetanide (50 μM) was added with the double-strength saline solution at zero time. No significant net movements of K^+ were found in the presence of ouabain (*cf.* Fig. 6) nor in the presence of ouabain plus bumetanide (*data not shown*)

Cl^-. Moreover, the net uptake of KCl and water is Na^+ dependent: The uptake was abolished in media where Na^+ had been replaced by choline (at 5 mM external K^+), and inhibited in media where K^+ had been substituted for Na^+. It may be noted that the membrane potential is hyperpolarized in the choline medium but depolarized in the high K^+ medium. Thus, the inhibition of the net Cl^- uptake appears to be caused directly by the absence of external Na^+ rather than by a change in the membrane potential.

(ii) The volume recovery was strongly inhibited by furosemide and bumetanide which have been reported to inhibit cotransport systems in Ehrlich cells (Geck et al., 1978; Aull, 1981, 1982).

(iii) The observed net Cl^- flux during regulatory volume increase was more than 10-fold larger than the conductive net Cl^- flux as calculated from the known Cl^- concentrations, assuming a membrane potential of -27 mV and a conductive Cl^- permeability of 4×10^{-8} cm/sec as previously reported under steady-state conditions (Hoffmann et al., 1979). The bumetanide-sensitive component of the unidirectional ^{36}Cl flux was negligible under steady-state conditions and the unidirectional ^{36}Cl flux was unaffected by substitution of choline for Na^+. In contrast, during regulatory volume increase the bumetanide-sensitive component of the unidirectional ^{36}Cl flux was activated and amounted to about half the total unidirectional ^{36}Cl flux.

(iv) The flux ratio for unidirectional Cl^- influx and efflux was 1.85. An electrodiffusional Cl^- transport could not give a flux ratio of the observed magnitude, unless the membrane potential were depolarized to between -8 and -10 mV; such a depolarization might hypothetically result from simultaneous activation of conductive Na^+ and Cl^- channels. For an electroneutral anion, cation cotransport the ratio of the products of the concentrations of the transported ions will indicate the direction of a net flux and, moreover, give a maximal value for the flux ratio. As the transport system becomes saturated the flux ratio will decrease from this value towards unity. The analysis shows (*see* Table 4) that a cotransport of Na^+,Cl^- could easily account for the observed flux ratio. In this case the observed ratio of 1.85 would suggest a fairly high degree of saturation of the cotransport system. A cotransport of K^+,Na^+, $2Cl^-$ could account for a flux ratio slightly lower than the observed value. At low external K^+ concentration, however, the K^+,Na^+, $2Cl^-$ gradient was found to be insufficient to account for the observed net uptake of KCl (*see* Fig. 5).

(v) Under conditions where the Na^+/K^+ pump was inhibited with ouabain a bumetanide-sensitive uptake of Na^+ and Cl^- in about equimolar amounts could be demonstrated, whereas the uptake of K^+ was negligible. This provides direct experimental evidence for the involvement of the Na^+/K^+ pump, and supports the conclusion that a Na^+,Cl^- cotransport system is involved rather than a K^+,Na^+, $2Cl^-$ cotransport system. The question how the activation of the Na^+/K^+ pump is effected cannot be answered at present. Only a small increase in the Na^+ concentration in cell water was observed comparing the measured values for the last data points during hypotonic pretreatment (17 ± 2.2 mM, 7 experiments) and for the first data points taken about 1/2 min after restoration of tonicity (22 ± 1.9 mM, 8 experiments; *cf.* legend to Table 4), and the further increase during regulatory volume increase was insignificant as mentioned previously. A net uptake of Na^+ and Cl^- during regulatory volume increase in the presence of ouabain has also been reported in flounder red cells (Cala, 1977).

The regulatory volume increase in Ehrlich cells is, based on the above evidence, presumed to be effected by activation of an electrically silent cotransport of Na^+ and Cl^- which is sensitive to furosemide and bumetanide, and specific to Na^+ and Cl^-. A different model has recently been proposed by Cala (1980) and Kregenow (1981) for regulatory volume increase in *Amphiuma* red blood cells, involving a Na^+/H^+ exchange functionally coupled to a Cl^-/HCO_3^- (or OH^-) exchange with a strict Cl^- requirement. This mechanism cannot, however, explain the coupled uptake of Na^+ and Cl^- found in the present experiments since, in contrast to the findings in *Amphiuma* red cells, the net Cl^- uptake was found to be essentially unaffected by the anion exchange inhibitor DIDS.

An alternative interpretation of the present results would be a simultaneous activation of separate conductive pathways specific to Na^+ and Cl^-, with one or both of the pathways being sensitive to furosemide and bumetamide. The bumetanide-sensitivity and the anion selectivity of such a Cl^- pathway would be in sharp contrast to the properties of the separate Cl^- pathway which has been proposed to be involved in the regulatory volume decrease in Ehrlich cells (Hoffmann, 1978; Hoffmann et al., 1983) and in human lymphocytes (Grinstein et al., 1982, 1983). This suggests the involvement of a Na^+,Cl^- cotransport system rather than separate Na^+ and Cl^- pathways in the regulatory volume increase. A Na^+,Cl^- cotransport has recently been proposed in the basolateral mem-

brane of epithelial cells in dogfish rectal gland (Eveloff et al., 1978), frog skin (Ussing, 1982; Kristensen & Ussing, 1983) and salivary glands (Poulsen, Laugesen & Nielsen, 1982). This Na^+,Cl^- cotransport is of the type originally proposed by Nellans, Frizzell and Schultz (1973) for the mucosal membrane of intestinal epithelial cells.

The sensitivity to bumetanide and furosemide, and selectivity to Cl^- as compared to NO_3^- found for the cotransport system reported here is also found for the volume-dependent and catecholamine-stimulated cotransport system reported in duck red cells (Kregenow, 1981; Haas, Schmidt & McManus, 1982), and for the volume-dependent cotransport systems described in LK sheep red cells (Lauf & Theg, 1980; Dunham & Ellory, 1981), human red cells (Dunham, Stewart & Ellory, 1980; Chipperfield, 1981), some fish erythrocytes (Lauf, 1982), and dog red cells (Parker, 1983). The Na^+,K^+ cotransport system in turkey erythrocytes is also anion-dependent and inhibited by bumetanide and furosemide (Palfrey, Feit & Greengard, 1980). Anion-cation cotransport has previously been reported in Ehrlich cells (Geck et al., 1980; Aull, 1981). It is unclear whether or not the K^+,Cl^- cotransport seen by Aull (1981), the $Na^+,K^+,2Cl^-$ cotransport seen by Geck et al. (1980), and the coupled uptake of Na^+ and Cl^- found in the present experiments can all be mediated by the same system operating under different conditions.

The contribution in the present experiments of the cotransport system to the unidirectional Cl^- flux under steady-state conditions cannot be given with precision. In the present experiments the unidirectional Cl^- flux was found to be insensitive to bumetanide and to substitution of choline for Na^+, suggesting a negligible cotransport component. On the other hand, Sjøholm and Hoffmann (*to be submitted*) found a DIDS-insensitive component of the unidirectional Cl^- flux of 25 to 30% which is significantly higher than the conductive flux at about 6% (Hoffmann et al., 1979). In those experiments, however, the DIDS inhibition of the anion exchange may have been incomplete.

Bumetanide and DIDS can effectively be used to discriminate between anion-cation cotransport and anion exchange in Ehrlich cells: The coupled uptake of Na^+ and Cl^- during regulatory volume increase was strongly inhibited by furosemide (1 mM) or bumetanide (25 μM) (*see* Table 1), but essentially unaffected by DIDS (*see* Results). On the other hand, the anion exchange in Ehrlich cells is inhibited by DIDS (Levinson, 1978; Sjøholm et al., 1981), but insensitive to bumetanide (25 or 100 μM) (*see* Table 2). In human red cells inhibition of Cl^- exchange has been demonstrated at high bumetanide concentrations, with 50% inhibition at 400 μM bumetanide (*see* Wieth & Brahm, 1983). Furosemide inhibits anion exchange in Ehrlich cells with 50% inhibition at about 5 mM furosemide (Geck et al., 1978). Aull (1982 has recently demonstrated that at low furosemide concentrations (0.5 mM) the effects of DIDS and furosemide on steady-state Cl^- fluxes in Ehrlich cells were additive whereas 1.0 mM furosemide and DIDS had overlapping inhibitory actions.

Assuming that the coupled uptake of Na^+ and Cl^- is mediated by a Na^+,Cl^- cotransport system, the Cl^- influx is a secondary active transport driven by the Na^+ gradient, and the intracellular Cl^- concentration will be increased to a level above the equilibrium concentration (*see* Geck et al., 1980). Under steady-state conditions the Nernst potential for Cl^- (-23 ± 1.2 mV) is only slightly above the membrane potential of -27 ± 1.0 mV measured with microelectrodes (Hoffmann et al., 1979), or the potential of -34 mV measured with a potential-sensitive fluorescent probe (Hoffmann & Lambert, 1983). This is consistent with the low Cl^- influx via the cotransport system under steady-state conditions compared to the conductive Cl^- leak. During regulatory volume increase the Nernst potential for Cl^- increases from -26 mV to about -20 mV, consistent with an activation of the Cl^- influx via the Na^+,Cl^- cotransport system.

Activation of anion, cation cotransport during regulatory volume increase was first demonstrated for the $K^+,Na^+,2Cl^-$ cotransport system in duck red cells (*see* reviews by McManus & Schmidt, 1978, and by Kregenow, 1981), and recently also for the Na^+,Cl^- cotransport system in frog skin epithelium (Ussing, 1982). The nature of the trigger mechanism for activation of the coupled Na^+,Cl^- uptake during regulatory volume increase in Ehrlich cells is unclear. It cannot be the cell volume *per se* which controls the activation since no volume recovery was seen in Ehrlich cells shrunken by addition of hypertonic NaCl (Hempling, 1960) or sucrose (Hendil & Hoffmann, 1974, Fig. 1), although the sum of the chemical potentials for Na^+ or Cl^- still provided the necessary driving force for NaCl uptake. Ussing (1982) has recently proposed that the cell Cl^- plays a critical role in frog skin epithelial cells with the Na^+,Cl^- cotransport system being activated when the cellular Cl^- concentration drops below a critical level. In Ehrlich cells shrunken by addition of sucrose at low Cl^- concentration, an increase in unidirectional K^+

and Cl^- fluxes could be observed (Hoffmann, 1978, Fig. 5, Table 4; Hoffmann, 1983, Fig. 13). Moreover, at low Cl^- concentration, a DIDS-resistant, furosemide-sensitive component of the unidirectional ^{36}Cl flux could be demonstrated (Sjøholm & Hoffmann, *to be submitted*). This might suggest that in Ehrlich cells the cellular Cl^- concentration has to be low but that some additional factor related to cell volume changes is also involved in the activation of the coupled Na^+,Cl^- uptake.

We wish to thank Professor H.H. Ussing for discussions and advice at various stages of the work and for critical reading of the manuscript, and to thank our colleagues for stimulating discussions and helpful comments. Karen Stentoft and Marianne Schiødt are acknowledged for their careful technical assistance. Carsten Sjøholm was supported by a research fellowship from the Danish Natural Science Research Council.

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Received 9 December 1982; revised 6 April 1983