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Anion Exchange and Anion-Cation Co-Transport Systems in Mammalian Cells

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Anion exchange and anion–cation co-transport systems in mammalian cells

By Else K. Hoffmann

Institute of Biological Chemistry A, August Krogh Institute, University of Copenhagen, 13 Universitetsparken, DK-2100 Copenhagen Ø, Denmark

Electroneutral anion transfer in the Ehrlich ascites tumour cell has been found to occur by two separate mechanisms. One is an exchange diffusion system with many similarities to that found in erythrocytes, e.g. saturation kinetics with 'self-inhibition', a relatively pronounced temperature dependence, competitive interactions of Br^-, NO_3^- and SCN^-, and a low conductive P_{Cl^-} of 4×10^{-8} cm s^{-1}. The main differences are that the Cl^- flux in Ehrlich cells at 38 °C is one thousandth of the flux in red cells, and that the specificity of the system is less pronounced. It is suggested that the density of anion exchange sites in Ehrlich cells could be the same as in red blood cells, but with a lower turnover rate. The other system is an anion–cation co-transport system capable of mediating a secondary active Cl^- influx. This system has a volume-regulatory function and is activated by a reduction in cell volume and intracellular [Cl^-]. The two transport systems can be separated by using DIDS as an inhibitor of anion exchange and bumetanide as an inhibitor of co-transport. Under normal steady-state conditions Cl^- flux is dominated by the exchange system. It is suggested that intracellular pH regulation can be achieved by the two systems operating in parallel, because the chloride disequilibrium maintained by the co-transport system can drive an influx of bicarbonate through the exchange mechanism.

1. Introduction

Anion transport in mammalian cells can primarily be subdivided into two components; one that does not contribute to the conductance of the membrane and another that does. In the red blood cell the former exceeds the latter by about 10^4-fold (Hunter 1971, 1977) in other mammalian cells the difference is probably much smaller. In the Ehrlich ascites tumour cell the apparent permeability has been measured as 20 times higher than the permeability estimated from conductance measurements (Hoffmann *et al.* 1979), and a similar difference is found in mammalian heart muscle cells (Page *et al* 1980). The electrically silent anion transfer in mammalian red blood cells is completely dominated by the tightly coupled exchange of anion across the membrane (see papers by J. O. Wieth and A. Rothstein in this symposium).

In recent years membranes of many other mammalian cells have been found to mediate anion exchange processes. These include Ehrlich ascites tumour cells, hepatocytes (Cheng & Levy 1980), heart muscle cells (Page *et al.* 1980) and neuroglial cells (Kimelberg *et al.* 1979). The best described system is the Ehrlich ascites tumour cell, and the structure and function of the anion exchange system in these cells will be discussed below with a reference background of the transport properties known from studies of erythrocytes.

In the Ehrlich cell and in the glial cells electrically silent anion transfer has been found to occur by two separate mechanisms. One is the exchange diffusion system, the other is a co-transport system for Na^+, Cl^- and probably K^+, capable of mediating a secondary active

chloride influx. In both cell types it has been shown that this secondary active influx of Cl^- is involved in regulatory processes. The co-transport system in glial cells is described by Bourke *et al.* (1976, 1978). Also in heart muscle it is likely that a co-transport mechanism exists that is involved in the regulation of intracellular pH; a discussion of Cl^- and pH regulation in muscles can be found in the paper by R. D. Vaughan-Jones in this symposium. The present paper will mainly discuss these regulatory functions for the Ehrlich cell. A discussion of the biological significance of the anion transport mechanisms in mammalian heart muscle cells and in glial cells can be found in a review by Wieth & Brahm (1982), and a description of the Cl^- HCO_3^- exchange in the brain can be found in Ahmad *et al.* (1976, 1978).

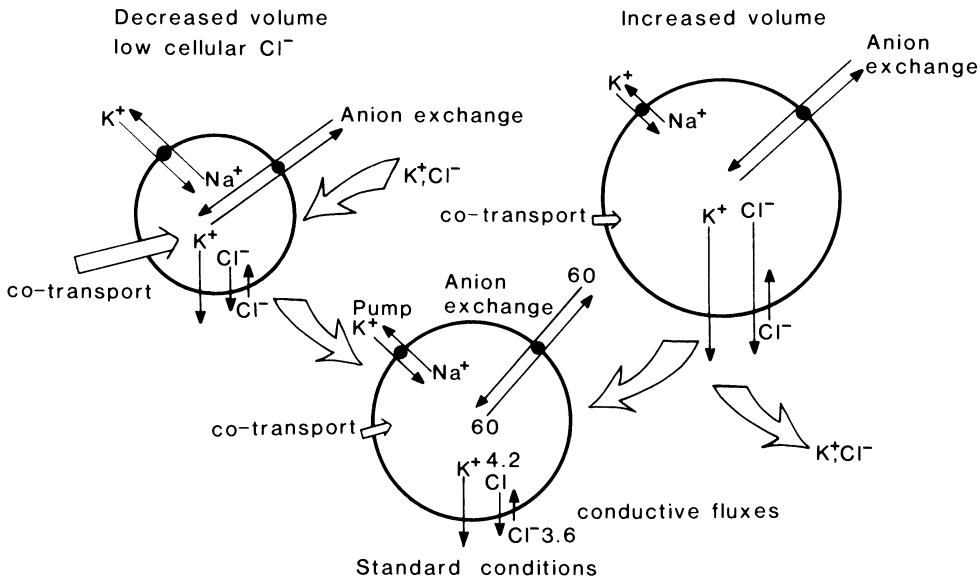

FIGURE 1. Schematic drawing of the anion transport components in Ehrlich cells and the responses to changes in cell volume and chloride concentration. The values of the exchange flux and the conductive flux (both in picomoles per square centimetre per second) are from Hoffmann *et al.* (1979). The range for the co-transport component is given on the basis of the following: (i) in § 4 it is shown that the bumetanide (25 μM) sensitive unidirectional $^{36}Cl^-$ flux is 0 ± 3 pmol cm^{-2} s^{-1}, and (ii) the electroneutral DIDS insensitive flux is 10 pmol cm^{-2} s^{-1} (Sjøholm & Hoffmann 1982).

2. Anion conductance, anion exchange and anion–cation co-transport

Figure 1 is a schematic drawing of the anion transport components in the Ehrlich cells and the responses to changes in cell volume and chloride concentration. The experimental evidence for this model will be given below. It can be seen that 95 % of chloride transfer through the Ehrlich cell membrane is non-conductive and that this electrically silent chloride flux is completely dominated by the coupled exchange of anions under normal experimental conditions. It appears, however, that a variable fraction of the chloride fluxes during various experimental conditions is mediated not by the exchange diffusion system but by a regulated co-transport mechanism coupling the simultaneous transfer of cations and anions. This transport mechanism is responsible for the previously observed increase of KCl flux in osmotically shrunk Ehrlich cells (Hoffmann 1978) and is a volume-regulatory co-transport system activated in shrunken cells as described for the Ehrlich cell (Hoffmann *et al.* 1981), the nucleated avian red

cell (see Kregenow 1981) and frog skin epithelial cells (Ussing 1982). The chloride influx is a secondary active transport driven by the sodium gradient. The intracellular chloride concentration seems, however, only to be slightly increased above the equilibrium concentration. Under the normal experimental conditions the Nernst potential for Cl^- is -23 ± 1.2 mV, which is only slightly above the membrane potential of -27 ± 1.0 mV measured by a microelectrode (Hoffmann *et al.* 1979) or of -34 mV measured by a fluorescent technique (Hoffmann & Lambert 1982). Geck *et al.* (1980) also find that the co-transport system may increase the intracellular chloride concentration above the equilibrium concentration.

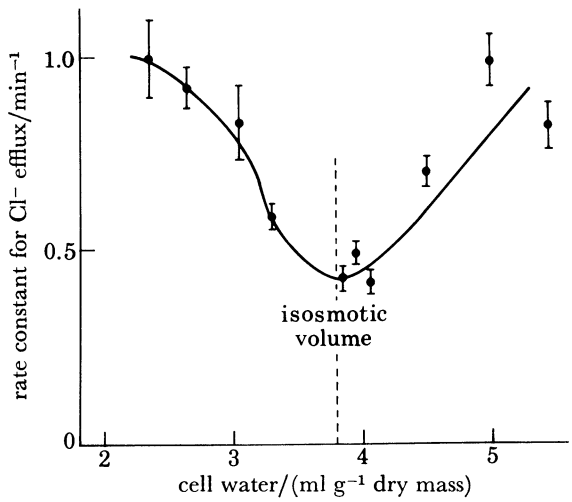

FIGURE 2. Volume dependence of the rate constant for $^{36}Cl^-$ efflux measured as unidirectional steady-state flux. The NaCl concentration is in all cases 75 mM, and differences in osmolarity are obtained by addition of different amounts of sucrose.

As discussed by Wieth & Brahm (1982) such a co-transport system may be involved in the control of intracellular pH by a chloride-driven anion exchange, where the increase in the ratio of $[Cl^-]_i/[Cl^-]_o$ above the ratio of $[HCO_3^-]_i/(HCO_3^-]_o$ provides the necessary conditions for an exchange-coupled influx of bicarbonate against an electrochemical gradient thus given an intracellular neutralization of acid equivalents. It was suggested (Hoffmann *et al.* 1979) that the anion exchange system may be involved in pH regulation. Ehrlich cells seem very suitable for testing this mechanism, but it has not yet been done.

In addition, the conductive chloride flux seems to be a variable fraction of the chloride fluxes during various experimental conditions. An increase in this transport seems to be responsible for the previously observed increase in chloride flux in osmotically swollen tumour cells (Hoffmann 1978).

The rate constant for steady-state chloride flux is increased with both increasing and decreasing volume (Hoffmann 1978) (see figure 2). It is therefore suggested that the increase in chloride flux after cell swelling reflects an increase in the conductive Cl^- flux, whereas the increase seen in shrunken cells with low intracellular $[Cl^-]$ is an increase in the co-transport of anions and cations. Experimental evidence for this model is given below.

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3. Anion conductance

The conductive Cl^- permeability accounts for only about 5 % of the value deduced from tracer flux measurements. This was shown by Heinz *et al.* (1975), who used the valinomycin technique of Hunter (1971). We reached the same conclusion for the conductive Cl^- permeability by a quite different electrophysiological method (Simonsen *et al.* 1976; Hoffmann *et al.* 1979).

The membrane potential (V_m) was measured as a function of the concentration of external K^+, substituting K^+ for Na^+ as shown in figure 3. The transference number for K^+ (t_{K^+}) was estimated from the slope of V_m against $\log_{10}[K^+]$, and t_{Cl^-} and t_{Na^+} were calculated, neglecting

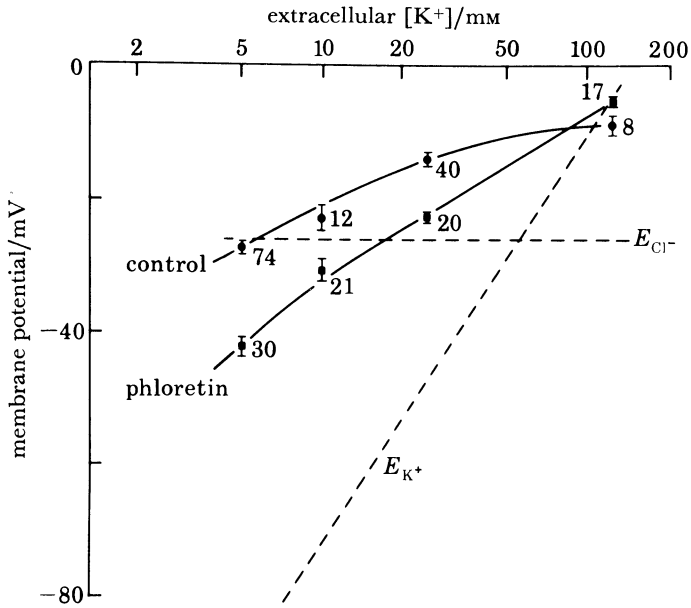

Figure 3. Dependence of the membrane potential with and without the presence of phloretin on the K^+ concentration of the suspending medium, K^+ being substituted for Na^+. After preincubation in basic Ringer solution an aliquot of cells was washed and resuspended in medium with the K^+ concentration indicated, with and without the addition of phloretin (0.25 mM) and 10–20 potential recordings were obtained from 2–12 min after the change in suspending medium. Note logarithmic scale on abscissa. The potentials are given as mean values ± s.e. of mean, the number of measurements being indicated by each point. The Nernst potentials for Cl^- (E_{Cl^-}), and K^+ (E_{K^+}) are indicated as broken lines. (From Hoffmann *et al.* (1979).)

current carried by ions other than Cl^-, K^+ and Na^+. The diffusional net flux of K^+ was calculated from the steady-state flux of $^{42}K^+$, assuming the flux ratio equation to be valid. From this value the K^+ conductance and the Na^+ and Cl^- conductances were calculated. They were all measured as about 14 μS cm^{-2}. The conductive fluxes and the conductive permeability derived from the Cl^- conductance are given in table 1, compared with the Cl^- tracer exchange flux and with the apparent permeability calculated from this flux. For further details of the methods, see Hoffman *et al.* (1979). These data show that the conductive Cl^- permeability accounts for about 5 % of the value deduced from tracer exchange measurements. The conductive Cl^- permeability of 4×10^{-8} cm s^{-1} in Ehrlich cells at 38 °C is similar to that in the human red blood cell (see Knauf 1979), the sheep red blood cell (Tosteson *et al.* 1973) and in the dog red blood cell (Parker *et al.* 1977). It must be noted that the permeability to Cl^- of human

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red cells is still two orders of magnitude larger than the Na^+ and K^+ permeabilities, whereas they are all of the same order of magnitude in the Ehrlich cell (see Hoffmann *et al.* 1979).

It is likely that a fraction of the conductive Cl^- flux is transported through the exchange mechanism. The finding that phloretin (0.25 mM) inhibits Cl^- conductance and exchange to about the same extent (about 80 %) might indicate that the exchange and conductance pathways are not completely separate and distinct modes of transport, but may involve common elements.

TABLE 1. UNIDIRECTIONAL Cl^- FLUXES AND ESTIMATED MEMBRANE PERMEABILITIES TO Cl^- (P_{Cl^-}) IN EHRLICH ASCITES CELLS

(The conductive unidirectional flux and the conductive P_{Cl^-} are derived from the membrane conductance to Cl^-. The apparent P_{Cl^-} is calculated from the measured Cl^- tracer exchange flux.)

unidirectional Cl^- fluxes / pmol cm^{-2} s^{-1}			Cl^- permeabilities / 10^{-8} cm s^{-1}	
conductive influx	conductive efflux	tracer exchange flux	conductive P_{Cl^-}	apparent P_{Cl^-}
3.6	4.2	63	3.9	62

Data from Hoffmann *et al.* (1979).

4. ANION EXCHANGE

Studies of Cl^- exchange kinetics under steady-state conditions are carried out under conditions with a minimum activation of the co-transport mechanism. This is suggested by the following results: (i) bumetanide (25 μM) which is an effective co-transport inhibitor in Ehrlich cells (Hoffmann *et al.* 1982), does not inhibit the unidirectional Cl^- flux under steady-state conditions; (ii) the unidirectional steady-state flux was also unchanged after substitution of choline for Na^+.

The characteristic properties observed for the exchange system include saturation kinetics with 'self-inhibition' at high Cl^- concentration, a relatively pronounced temperature dependence (activation energy of 67 kJ mol^{-1}, constant in the temperature range from 38 to 7 °C) (see figure 4) and evidence of competitive interaction of other anions.

Saturation kinetics can be seen in figure 5, which shows the steady-state tracer exchange flux of Cl^- measured at 10–150 mM external Cl^- concentration, substituting sucrose for Cl^-. The results are consistent with a saturable carrier model for Cl^- transport. $K_{\frac{1}{2}}$ is estimated as 15 mM for extracellular [Cl^-] and 13 mM for intracellular [Cl^-]. The relation between extracellular and intracellular $K_{\frac{1}{2}}$ has been discussed by Dalmark (1975). The particular shape of the curve could indicate that the transport is regulated by two anion-binding sites, a transport site and a modifier site, as has been suggested for the red blood cell (Dalmark 1976*a*). In agreement with that, Levinson (1978) has proposed a two-site model for Cl^- and SO_4^{2-} transport in the Ehrlich cell. An attempt has been made to fit the present saturation curves to a model like that used for red blood cells by Dalmark (1976*a*, *b*), but it does not completely fit the particular shape of the curves. It might be that a decrease in the intracellular Cl^- concentration is a stimulus to the co-transport mechanism, which thus at lower [Cl^-] becomes a larger fraction of the Cl^- transport. The first demonstration of saturable anion transport for Ehrlich cells was given by Levinson & Villereal (1975, 1976). Under the experimental conditions that they employed they found a major fraction of the Cl^- transport to be mediated by a different pathway, which could be the co-transport system.

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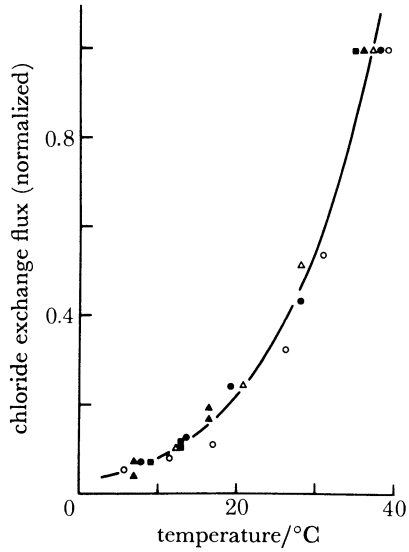

FIGURE 4. The temperature dependence of the chloride steady-state exchange flux. The chloride flux at the experimental temperature is given as fraction of the flux measured at 38 °C in the same experiment. The figure shows the normalized flux plotted against the temperature. The equation $J = A \exp(-E_a/RT)$ was fitted by an iterative nonlinear unweighted least-squares analysis. The continuous line shows the best fit corresponding to $E_a = 67 \pm 6.2$ kJ mol^{-1}. The cells were equilibrated at the experimental temperature between 40 min (38 °C) and 100 min (7 °C), before starting the efflux experiments. At 7 °C the cell Na^+ content increased by about 22% per hour, and cell K^+ decreased by about 13% per hour. At higher temperatures the changes were less. Cell $[Cl^-]$ changed by less than 5% in all experiments. The measurements are from five separate experiments as indicated. (Values are from Hoffmann *et al.* (1979).)

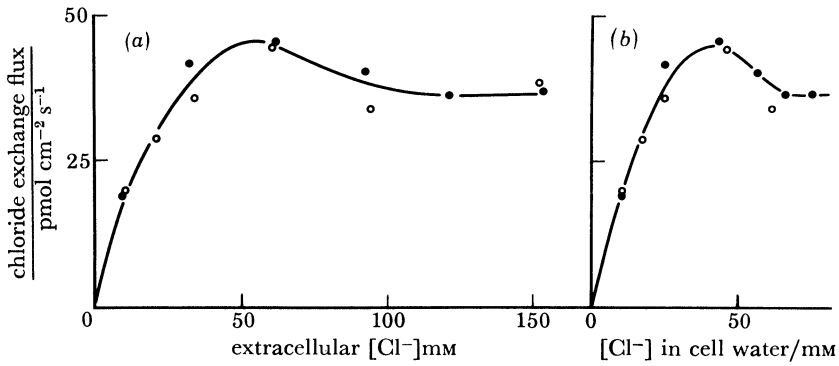

FIGURE 5. Chloride steady-state exchange flux at 38 °C as a function of extracellular (*a*) or intracellular (*b*) chloride concentration. The chloride concentration in the medium was varied by substituting sucrose for sodium chloride. The cell volume was constant. The chloride distribution ratio between cell water and medium (r_{Cl^-}) was increased with increasing sucrose concentration from 0.6 to 1.1. The measurements are from two separate experiments as indicated by the different symbols. Other experiments gave similar results, but were quantitatively slightly different. (From Hoffmann *et al.* (1979).)

The Cl^- flux is inhibited by several other anions. As an example, figure 6 shows the Cl^- steady-state exchange flux as a function of extracellular Cl^- concentration when NO_3^- was substituted for Cl^- in equimolar amounts. The flux was found to be progressively inhibited by the substitution of increasing amounts of NO_3^- for Cl^-, indicating a higher affinity for NO_3^- than for Cl^-. It is shown that for a competitive inhibitor a plot of $[Cl^-]/J_{Cl^-}$ should be linearly dependent on $[Cl^-]$. In contrast, for a non-competitive inhibitor $[Cl^-]/J_{Cl^-}$ should be dependent on $[Cl^-]^2$. Figure 7 shows such a plot of a typical NO_3^- substitution experiment. NO_3^- is

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seen to be a competitive inhibitor of the Cl^- exchange flux. From the slope and the intercept the K_i/K_m ratio corresponding to a K_m of 15 mM was calculated to be 0.5 ± 0.06. For Br^- and SCN^- the K_i/K_m ratios were calculated to be 1.4 ± 0.05 and 0.5 ± 0.02 (Hoffmann *et al.* 1979), suggesting that the sequence of increasing affinity is $Br^- < Cl^- < SCN^- = NO_3^-$. In addition, Levinson & Villereal (1976), Grobecker *et al.* (1963), Aull (1972), and Aull *et al.* (1977) have shown an inhibition of Cl^- flux by other anions. In human cells the corresponding sequence is $Cl^- < Br^- < NO_3^- < SCN^-$ (Dalmark & Wieth 1972). The inverted sequence of Cl^- and Br^- could indicate that the electrical field strength of a positively charged transport site is higher in Ehrlich cells than in red cells (see, for example, Wright & Diamond 1977). The sequence for flux rates is quite different in the red blood cell (Wieth 1972; Wieth *et al.* 1973); in

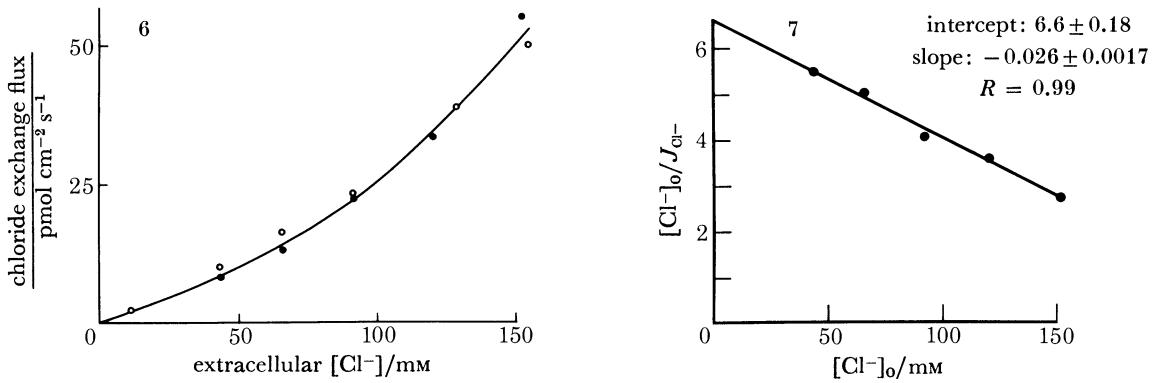

FIGURE 6. Chloride steady-state exchange flux at 38 °C as a function of extracellular chloride concentration. Nitrate was substituted for chloride in equimolar amounts, the sum being kept constant at 150 mM. The cell volume and r_{Cl^-} were constant. The measurements are from two separate experiments as indicated. Other experiments gave similar results, but were quantitatively slightly different. (From Hoffmann *et al.* (1979).)

FIGURE 7. A plot of $[Cl^-]_o/J_{Cl^-}$ against $[Cl^-]_o$ in the extracellular medium. J_{Cl^-} is the chloride steady-state exchange flux (expressed as pmol $cm^{-2}\ s^{-1}$) and $[Cl^-]_o$ the extracellular chloride concentration. Data from a typical experiment in which NO_3^- is substituted for Cl^-. The straight lines indicate a competitive inhibition, whereas non-competitive inhibition would give a parabolic curve (convex upwards).

Ehrlich cells it is not known. The only data permitting comparison between the relative anion flux rates of the Ehrlich cell system and the red blood cell system are those on Cl^- and SO_4^{2-} transport. The maximum Cl^- fluxes in Ehrlich cells at 38 °C are 63 pmol $cm^{-2}\ s^{-1}$, or one thousandth of the red cell exchange flux (Brahm 1977). The rate of sulphate exchange is, however, of similar magnitude in red blood cells (Brahm 1977; Schnell *et al.* 1977) and in Ehrlich cells (Levinson & Villereal 1975, 1976). Since SO_4^{2-} flux in both cell types is probably mediated by the same transport system as Cl^- exchange (Villereal & Levinson 1977; Levinson 1978; Schnell *et al.* 1977) it has been suggested by Hoffmann *et al.* (1979) that the density of anion-exchange sites in Ehrlich cells could be the same as in red blood cells, but that the red blood cell sites have an extraordinarily high turnover rate, three orders of magnitude higher than in the Ehrlich cell. In agreement with this idea we find the same density of DIDS-binding sites in Ehrlich cells as in the red blood cells (Sjøholm & Hoffmann 1982) (see below).

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5. Effect of inhibitors on Cl^- transport

Defining transport systems by the effect of inhibitors is a risky thing, but nevertheless they may give some help. On the basis that phloretin (0.25 mM) has parallel effects on conductance and exchange Cl^- flux (Hoffmann *et al.* 1979), it was suggested that some conductive Cl^- flow might take place via the exchange system, but this is not at all conclusive since the effects of other inhibitors on the conductance are not well described; neither has it been shown by its kinetic characteristics. The transport pathway through which net movements of anions can occur can be separated from the exchange transport pathway because of the marked difference in the sensitivity of the two types of anion flow to DIDS (4,4-diisothiocyano-2,2′-stilbene disulphonate) and bumetanide. DIDS is a reversible competitive inhibitor of Cl^- exchange, and the total steady-state Cl^- flux can be inhibited 80 % both by acute reversible and later slow irreversible binding of DIDS (Sjøholm & Hoffmann 1980, 1982), but it is completely insensitive to bumetanide (25 μM). On the other hand, the sodium-dependent KCl net uptake during volume regulatory increase is not inhibited by DIDS (100 μM) (Hoffmann *et al.* 1981), but strongly reduced by bumetanide (25 μM) or furosemide (1 mM) (see figure 12). It thus seems that an almost perfect discrimination between two of the transport systems can be obtained with bumetanide which inhibits co-transport without affecting exchange, and DIDS, which inhibits exchange without affecting co-transport.

Because net movements (which include conductive Cl^- flux as well as co-transport) are not inhibited by DIDS, it is likely that Cl^- conductance is not very sensitive to DIDS either. This would indicate that at least part of the conductance is likely to be an independent pathway. This is probably also so in red blood cells (Hoffmann *et al.* 1980; Knauf & Law 1980; Knauf *et al.* 1977; Kaplan *et al.* 1980). Furosemide is less specific than bumetanide. Furosemide (1 mM) will inhibit co-transport by 76 % (see figure 12) and the unidirectional Cl^- flux by 21 % (Geck *et al.* 1978), the apparent K_i for net Cl^- flux being about 2×10^{-4} M furosemide and for steady-state Cl^- flux 5×10^{-3} M (Geck *et al.* 1978). In red blood cells the apparent K_i for the exchange system is much lower, i.e. 1.5×10^{-4} (Brazy & Gunn 1976).

The sensitivity of the exchange diffusion system of the Ehrlich cells to DIDS is also much lower than that of the red blood cell. In normal Ringer the apparent K_i for H_2DIDS is 0.3 μM in red blood cells (Shami *et al.* 1978) and the apparent K_i for DIDS is 55 μM in Ehrlich cells. This can be seen in figure 8, which shows the Cl^- steady-state exchange flux as a function of the DIDS concentration at high and low Cl^- concentrations. The Cl^- concentration was found to have a pronounced effect on the inhibition of the Cl^- flux by DIDS. The DIDS concentration for half-maximal inhibition was 6 ± 1 μM in low-Cl^- medium and 55 ± 6 μM in high Cl^- medium. This suggests an interaction of the inhibitor with a Cl^--binding site. A kinetic analysis of the DIDS-sensitive Cl^- fluxes (see figure 8) shows that DIDS appears to be a competitive inhibitor of Cl^- transport with an apparent K_i at zero Cl^- concentration in the range 0.5–2 μM (Sjøholm & Hoffmann 1982). This value is 5–20 times higher than that found for H_2DIDS in red blood cells (Shami *et al.* 1978), indicating a lower DIDS affinity in the Ehrlich cell. The above analysis indicates a K_S of about 8 mM for the site where DIDS competes with Cl^-. This corresponds reasonably well with the apparent K_m for the transport site (see figure 5), which might indicate that DIDS competes with Cl^- for the transport site.

The experiments described above were done under conditions in which the binding of DIDS is reversible. After a longer exposure DIDS becomes covalently bound. Figure 9 shows

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the irreversible DIDS inhibition as a function of time. It can be seen that Ehrlich cells have a very slow irreversible binding of DIDS depending on the Cl^- concentration.

The DIDS-insensitive Cl^- movements, which are around 20% of the total, are inhibited by 88% by 2 mM furosemide and correspond probably to Cl^- movements mediated by the

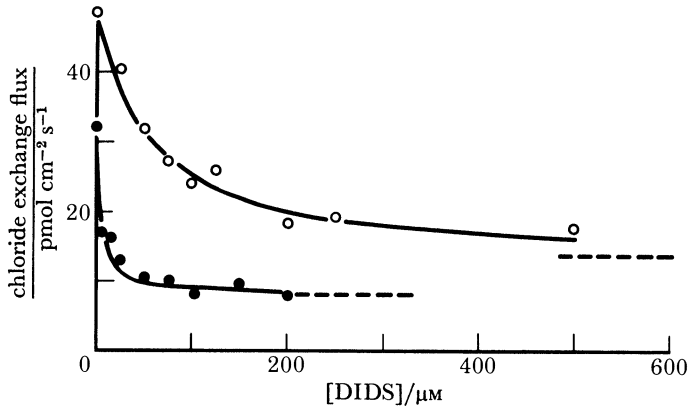

FIGURE 8. The chloride exchange flux ($^{36}Cl^-$ efflux) as a function of the DIDS concentration for 150 mM chloride (○) and 15 mM chloride (●). The chloride concentration in the medium was varied by substituting sucrose for sodium chloride. The efflux was determined as described by Hoffmann *et al.* (1979). The efflux measurements were based on five time points taken during the first 2 min in the low-chloride medium and during the first 5 min in the high-chloride medium. During this period the irreversible inhibition did not exceed 10%. It was found (Sjøholm & Hoffmann 1982) that the DIDS-inhibited part of the chloride flux when plotted against the DIDS concentration could be fitted by a simple expression of the Michaelis–Menten type, and the curves shown on the figure are calculated based on the best fit obtained by least-squares analysis. The broken lines indicate the DIDS-insensitive flux extrapolated to infinite DIDS concentration. The apparent K_i or DIDS can be calculated as 55 ± 6 μM at 150 mM chloride and 6 ± 1 μM at 15 mM chloride.

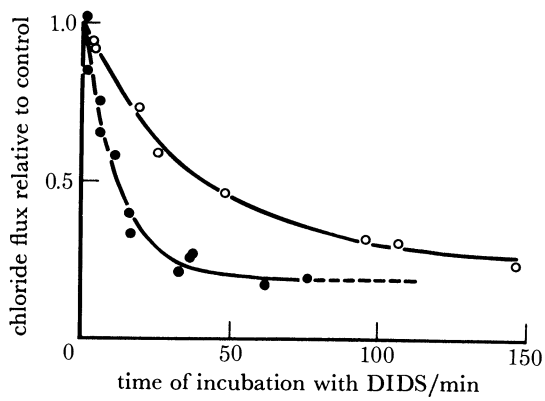

FIGURE 9. $^{36}Cl^-$ influx as a function of the preincubation period with 100 μM DIDS, in 150 mM Cl^- and in 15 mM Cl^-. The preincubation was terminated by washing twice with albumin (5 mg ml^{-1}) and the flux measurements were carried out as described by Hoffmann *et al.* (1979). The curve was fitted by an iterative non-linear unweighted least-squares analysis. The value for the residual $^{36}Cl^-$ flux at infinite time is indicated as a dotted line (From Sjøholm & Hoffmann (1982).)

co-transport system and by the conductance. It has been reported that SITS and H_2DIDS at high concentrations always inhibit sulphate fluxes by more than 95% (Villereal & Levinson 1977; Levinson 1978), whereas Cl^- fluxes were not. This may be explained if sulphate is transported by the exchange mechanism, whereas Cl^- under their experimental conditions is transported as co-transport. It might also be that H_2DIDS and SITS irreversible inhibition with Cl^- exchange is very slow (see later).

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The Cl^- concentration-dependent variations in the irreversible inhibitory effects of DIDS are consistent with the hypothesis that the site of covalent binding and the site of reversible binding are the same. It therefore seems that labelled DIDS can serve as a marker for the transport site. If 20 μM tritiated DIDS is added to cells in a medium with 15 mM Cl^-, a number of 50–90 million binding sites per cell is found (Sjøholm & Hoffmann 1982). The number of binding sites in the red blood cell is 1.2 million (Lepke *et al.* 1976; Ship *et al.* 1977) binding sites per cell. Since the surface area of the Ehrlich cell (Hoffmann *et al.* 1979) is at least 10 times the surface area of the red blood cell, the density of sites is of the same order of magnitude in the two cells, supporting the idea (Hoffmann *et al.* 1979) that it is the turnover rate in red cells that is extraordinarily high, three orders of magnitude higher than in the Ehrlich cell. Caution must of course be used when evaluating the number of sites in the Ehrlich cell. After the cells have been labelled with 20 μM tritiated DIDS in 30 min in a low-Cl^- medium we only see one labelled region by polyacrylamide gel electrophoresis of membrane peptides solubilized with sodium dodecyl sulphate (SDS) plus urea (6 M) (C. Sjøholm, E. Hoffmann & W. Uerkvitz, unpublished), but it is impossible to exclude the possibility that this region on the gel contains DIDS-binding protein molecules, not engaged in anion transport. Levinson (1980), using H_2-DIDS (25 μM, 30 min) in normal 150 mM Cl^-, found that the label binds rather indiscriminately to many protein fractions of the membrane. The biggest difference between the two sets of experiments is the difference in Cl^- concentration. With high-Cl^- medium as used by Levinson, the apparent K_i for DIDS affinity to the transport site is 50 μM (see figure 8) and at 25 μM the irreversible inhibition in 30 min is only about 15 %. In the low-Cl^- medium that we use, the irreversible inhibition obtained in 30 min was 85 %. Levinson sees an irreversible inhibition of SO_4^{2-} transport but not of Cl^- transport, and he has suggested (Levinson 1978) that both the transport site and the modifier site are necessary for SO_4^{2-} transport, whereas only the transport site is required for Cl^- transport. If this is true, the following can be suggested: in the low-Cl^- medium there is binding to the transport site with a high affinity (K_i at 15 mM Cl^- is 5 μM), which is higher than for any of the other proteins, and this brings about the selective labelling. In the high-Cl^- medium the apparent K_i is now 50 μM for the transport site, which is higher than the affinity for many other sites including the second site on the exchange protein. This would explain the rather indiscriminate binding and the inhibition of SO_4^{2-} flux with no inhibition of the Cl^- flux. In agreement with this, preliminary results show that our selective labelling of one region on the gel disappears if the Cl^- concentration is increased.

Conclusive attempts to identify the anion exchange component in Ehrlich cells by binding studies with tritium-labelled DIDS are encouraging. SITS is shown to be an inhibitor of Cl^- transport in glial cells (Kimelberg *et al.* 1979) and in sheep heart muscle fibres (Vaughan-Jones 1978).

5. The regulated co-transport mechanism

A variable fraction of the Cl^- fluxes during various experimental conditions are mediated by a regulated co-transport mechanism coupling the simultaneous transfer of one Na^+ and one Cl^-, or maybe even 1 Na^+, 1 K^+, and 2 Cl^- as suggested by Geck *et al.* This system seems to be a volume regulatory system (Hoffmann *et al.* 1981) and it is probably related to the volume regulatory co-transport described in nucleated avian red cells (see Kregenow 1981) and in frog skin epithelial cells (Ussing 1982). The system might also be involved in regulation of intracellular Cl^- and in cellular pH regulation.

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Ehrlich cell can regulate their volume in anisotonic media (Hendil & Hoffmann 1974); corrective changes in cell size result from shifts in cell water brought about by changes in salt content. The volume-controlling mechanism were found to use ouabain-insensitive transport processes to move Na^+ and K^+ across the membrane in a controlled fashion (see, for example, Hoffmann 1977, 1978). At that time these transport processes were believed to be conductive transport through leak pathways. Because the leak transport during the volume regulatory responses were dynamic and regulated, the static nature of the leak had to be revised.

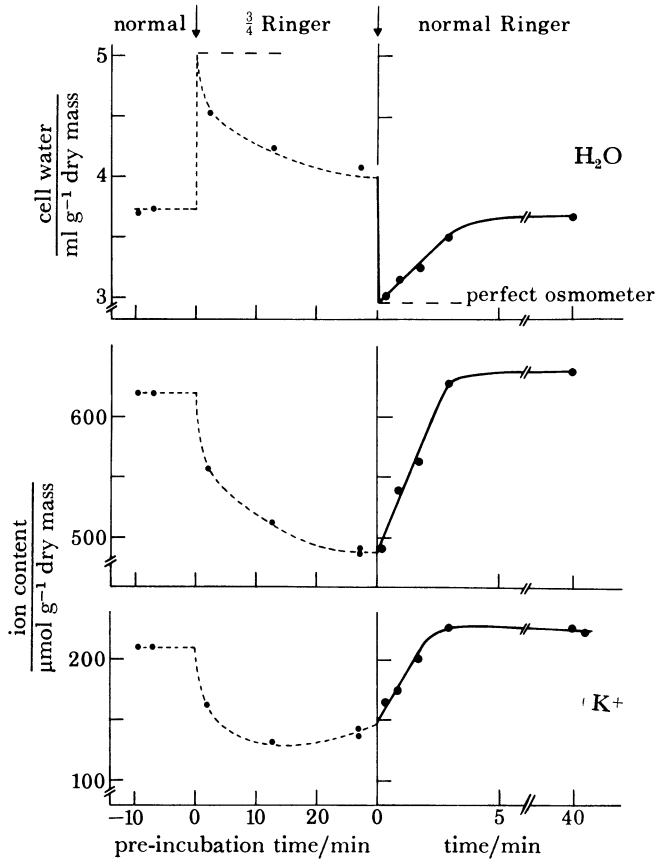

FIGURE 10. Cell volume, K^+ and Cl^- content in Ehrlich ascites cells as a function of time after changes in medium osmolarity. Osmolarity was changed at $t = 0$ from 300 to 225 mosmol l^{-1} by dilution with distilled water and at second $t = 0$ from 225 to 300 mosmol l^{-1} by addition of the salts present in the normal Ringer solution.

Figure 10 shows that when Ehrlich cells are enlarged, an ouabain-insensitive transport process is activated that controls the egress of KCl from the cell (Hendil & Hoffmann 1974; Hoffmann 1978). Other mammalian cell types, e.g. mouse leukaemic cells (Roti Roti & Rothstein 1973) and human lymphocytes (Benn-Sasson *et al.* 1975), have an analogous response. For a number of red blood cell types the activation of a K^+–Cl^- co-transport is suggested as a component of this hypotonic response. This is true for duck (Kregenow 1981; McManus & Haas 1981), sheep (Dunham & Ellory 1981; Lauf & Theg 1980), fish (Lauf 1982) and dog (J. C. Parker, personal communication) red blood cells.

In other cells like frog skin epithelium (Ussing 1982) and Ehrlich cells (Hoffmann *et al.* 1982), furosemide or bumetanide do not inhibit the KCl loss. Figure 11 (left) shows the loss of Cl^-

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with and without addition of bumetanide. Bumetanide has no effect on the initial loss of K^+ and Cl^-. The passive K^+ flux is independent of the dominating anion and is not inhibited after NO_3^- substitution (Hoffmann *et al.* 1982). It is thus more likely that swelling increases separated leak pathways. This is supported by the observation that quinine, an inhibitor of the Ca^{2+}-activated K^+ pathway in other systems (Armando-Hardy *et al.* 1975), blocks the KCl loss in Ehrlich cells (E. Hoffmann unpublished observations) and in lymphocytes (Grinstein *et al.* 1982). These results suggest that it is not the co-transport that increases with increased volume in Ehrlich cells or lymphocytes, but more probably the conductive flux.

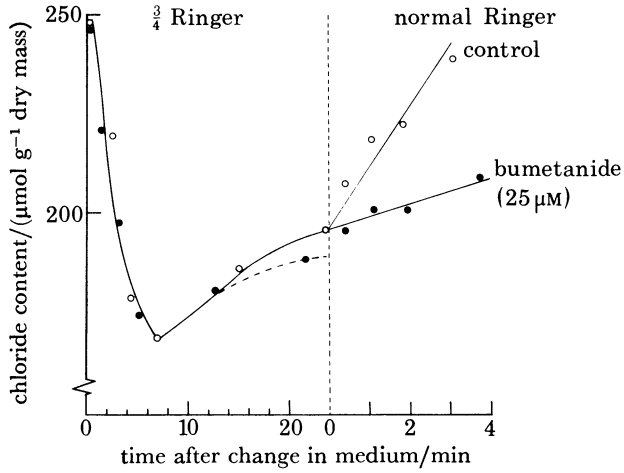

FIGURE 11. Cl^- content in Ehrlich ascites cells as a function of time after changes in medium osmolarity. Osmolarity was changed at $t = 0$ from 300 to 225 mosmol l^{-1} by dilution with distilled water and at second $t = 0$ from 225 to 300 mosmol l^{-1} by addition of the salts present in the normal Ringer solution. Bumetanide (25 μM) was in both cases added at time zero.

Ehrlich cells that have reached a steady state upon preincubation in hypotonic media (Hoffmann 1978) shrink when transferred to a standard medium (now hypertonic compared with the cells) but return to their original volume within 10 min, with an associated net uptake of KCl (see figure 10). Osmotic shrinkage stimulates an otherwise quiescent ouabain-insensitive Na^+ and Cl^- transport system.

The assumption that the primary process is a coupled uptake of Na^+ and Cl^- followed by a replacement of Na^+ by K^+ by the Na^+–K^+ pump is supported by the following findings.

(i) Furosemide (1 mM), which is known to inhibit co-transport systems (Geck *et al.* 1978), reduces water uptake and Cl^- uptake by 74 %. Bumetanide (25 μM), which is shown to inhibit K^+, Cl^- co-transport in Ehrlich cells (Aull 1981), reduces water uptake and Cl^- uptake by 88 % (see figure 12).

(ii) On replacing Cl^- with NO_3^-, net K^+ and water uptake is reduced by 60 % and in media where Na^+ had been replaced by choline (at 5 mM K^+), water and KCl uptake are abolished (figure 13). The uptake of water and KCl are also strongly inhibited in media were K^+ had been substituted for Na^+. The cells are hyperpolarized in choline medium but depolarized in K^+ medium. Thus, the net Cl^- uptake seems to be directly dependent on the Na^+ concentration (Hoffmann *et al.* 1981, 1982).

(iii) K^+ influx is stimulated and increases by about the same amount as the chloride influx.

(iv) Water and KCl uptake are insensitive to DIDS (200 μM), which has been shown to inhibit the self-exchange flux of Cl^- by 85 % (Sjøholm *et al.* 1981).

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(v) The net Cl^- flux is more than ten times higher than expected from the conductive Cl^- permeability previously reported (Hoffmann *et al.* 1979) (see figure 13).

Taken together, these results demonstrate the presence of an electrically silent, bumetamide and furosemide-sensitive co-transport of Cl^- and Na^+. It could, however, also be a mechanism

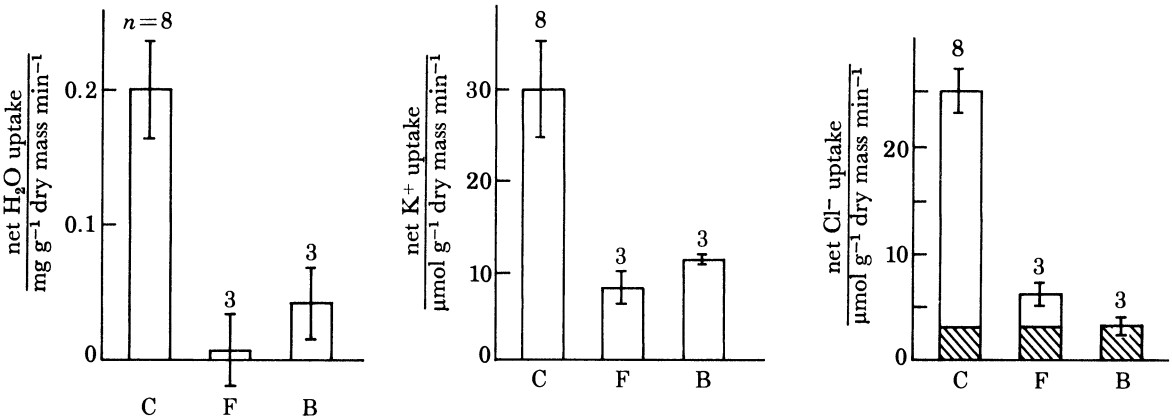

FIGURE 12. Effect of furosemide (F,) (1 mM) and bumetanide (B,) (25 µM) on the net uptake of water, K^+ and Cl^- during volume regulatory increase. C, control. The experimental conditions were identical to those of figure 11. Values are given ±s.e.m. with the number of separate experiments indicated. Hatched columns, conductive flux. (Values from Hoffmann *et al.* (1982).)

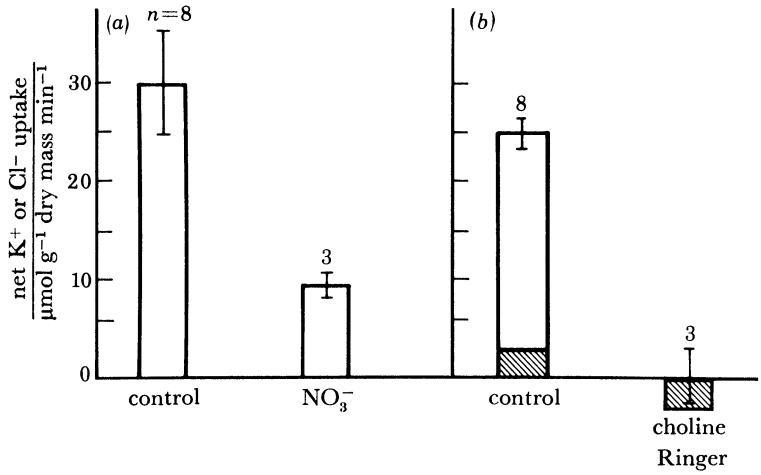

FIGURE 13. Effects of replacing Cl^- with NO_3^- (*a*), or Na^+ with choline (*b*), on the net uptake of KCl during volume regulatory increase. The experimental conditions were identical to those in figure 11. Values are given ±s.e.m. with the number of separate experiments indicated. Hatched columns, conductive flux. (Values from Hoffmann *et al.* (1982).)

that transports NaCl and KCl together in the ratio 1:1, like the mechanism proposed by Geck *et al.* (1980). Our present data do not permit a distinction between the two models. In duck red blood cells a requirement for all three ions is clearly demonstrated, and it is proposed that all three ions cross the membrane as an entity (Kregenow 1981). Evidence for a furosemide-sensitive Na^+–K^+ co-transport has been presented by Kregenow (1976, 1977, 1978) and by Schmidt & McManus (1977*a*, *b*, *c*) and McManus & Schmidt (1978). Evidence for the concept of Cl^-–cation co-transport has been summarized by Kregenow (1981).

Under physiological steady-state conditions the Ehrlich cells are relatively tight to Cl^- (Hoffmann *et al.* 1979) and there is very little co-transport of NaCl into the cells (Sjøholm *et al.*

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1981; Hoffmann *et al.* 1981). This is also indicated by the finding that the cells retain their volume for relatively long periods of time after addition of the co-transport inhibitors furosemide or bumetanide (E. Hoffmann, unpublished observation). The unidirectional Cl^- flux in the steady state is also nearly unaffected when Na^+ is replaced by choline, or the co-transport inhibitor bumetanide is added. During the volume regulatory increase, however, the Na^+ dependent co-transport system seems to be activated and to play an important role. When the cells have attained their normal volume the co-transport mechanism is again inactivated. Activation of anion–cation co-transport in shrunken cells has recently been suggested for duck red cells (Kregenow 1981) and for epithelial cells under conditions similar to those described in figure 10 (Ussing 1982).

The trigger for activation of the Na^+–Cl^- co-transport is not clear. It cannot just be the cell volume, which controls the activation of the co-transport system, because Ehrlich cells shrunken by addition of sucrose do not regulate volume (Hendil & Hoffmann 1974), although the sum of the chemical potentials for Na^+ and Cl^- still provides the necessary driving force for NaCl uptake. It is conceivable that cellular Cl^- concentration is a critical factor. Ussing (1982) has recently suggested that in frog skin the co-transport system is activated when the cellular Cl^- concentration decreases below a critical level. For Ehrlich cells the available evidence suggests that the intracellular Cl^- concentration has to be low, but that some additional factor connected with cell volume changes is also involved in the activation of the anion–cation co-transport system.

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