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Anion-Dependent Cation Transport in Erythrocytes [and Discussion]

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Anion-dependent cation transport in erythrocytes

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A selective survey of the literature reveals at least three major anion-dependent cation transport systems, defined as $Na^+ + Cl^-$, $K^+ + Cl^-$ and $Na^+ + K^+ + Cl^-$ respectively. In human red cells, kinetic data on the fraction of K^+ and Na^+ influx inhibitable by bumetanide are presented to indicate an Na^+:K^+ stoichiometry of 1:2. For LK sheep red cells the large Cl^--dependent K^+ leak induced by swelling is shown to share many characteristics with that induced by *N*-ethyl maleimide (NEM) treatment. NEM has complex effects, both inhibiting and then activating Cl^--dependent K^+ fluxes dependent on NEM concentration. The alloantibody anti-L can prevent the action of NEM.

In human red cells NEM induces a large Cl^--dependent specific K^+ flux, which shows saturation kinetics. Its anion preference is $Cl^- > Br^- > SCN^- > I^- > NO_3^- > MeSO_4^-$. This transport pathway is not inhibited by oligomycin or SITS, although phloretin and high concentrations of furosemide and bumetanide (over 0.3 mM) do inhibit. Quinine (0.5 mM) is also an inhibitor.

It is concluded that at least two distinct Cl^--dependent transport pathways for K^+ are inducible in mammalian red cells, although the evidence for their separation is not absolute.

Introduction

Chloride-dependent transport of Na^+, and more recently of K^+, has been described for a wide variety of tissues, including many epithelia, ascites tumour cells, nerve, muscle and red cells (see table 1 for references). An obvious question is therefore whether these fluxes are conservative, and represent a rather widespread distribution of one or two definite transport systems akin to the sodium pump or the Ca^{2+}-activated K^+ channel. Certainly the idea of coupled NaCl transport via a specific transport system has gained wide acceptance as a general mechanism involved in salt transport in epithelia (Frizzell *et al.* 1979; Schultz 1981). In this context the pharmacological agents known as the loop or group 3 diuretics, and in particular furosemide, have often been used as specific inhibitors to define chloride transport systems.

The present aim is to try and identify the possible number, and specificities of transport systems for Na^+ or K^+, or both, linked to Cl^-, initially from a selective survey of the literature (table 1) and subsequently from our detailed studies on sheep and human erythrocytes. Classification of Cl^--dependent cation transport can be made into $Na^+ + Cl^-$, $Na^+ + K^+ + Cl^-$ and $K^+ + Cl^-$ systems, and there is good evidence for the individual existence, or at least separation on kinetics and pharmacological grounds, of these three systems in some tissues. However, it is likely that published data may not always have been rigorous in the sense of having examined the interaction of all three ions. A case in point is salt transport in the thick

ascending limb of the loop of Henle of the mammalian kidney, the site of action of furosemide in its role as a diuretic. Initial work indicated electrogenic chloride transport which was not Na^+-dependent in this tissue (Burg & Green 1973); subsequently coupled NaCl transport was proposed (Jørgensen 1976; Greger 1981) and recently Na^+, K^+, Cl^- interactions have been established (Greger & Schlatter 1981). Again, for flounder intestine, one of the key model systems for establishing coupled NaCl transport in epithelia, a role for K^+ has recently been reported (Musch *et al.* 1982). In like manner NaCl cotransport shown for squid axon (Russell

TABLE 1. HOW MANY COUPLED TRANSPORT SYSTEMS?

furosemide-sensitive NaCl *co-transport*	*furosemide-sensitive* Na^+, K^+, Cl^-*-dependent transport*†	*relatively furosemide-insensitive* KCl*-dependent transport*†
many secretory epithelia[1], skeletal[2] and smooth muscle[3] (or is it Na^+–H^+ and Cl^-–HCO_3^- exchange?)[4]	red cells (human[8], nucleated[9, 10]), kidney (ThAL[11], MDCK[12]), small intestine (flounder[13]), ascites tumour cells[14], nerve[15]	red cells (human[18], sheep[19]), lymphocytes[20], leukaemic cells (L5178Y)[21], ? ascites cells[22]
carbonate–cation transport	Na^+, Cl^-*-linked glycine transport*	*anion-sensitive ATPase* (*also* K^+*-sensitive*[23])
red cells[5], epithelia[6], squid axon[7]	avian[16] and mammalian[17] red cells	stomach, gill, kidney

References: 1, Frizzell *et al.* (1979); 2, Russell & Brodwick (1979); 3, Brading (1980); 4, Schultz (1981); 5, Funder & Wieth (1980); 6, Ellory (unpublished); 7, Russell & Boron (1976); 8, Dunham *et al.* (1980); 9, Kregenow (1981); 10, Bourne & Cossins (1982); 11, Greger & Schlatter (1981); 12, Aiton *et al.* (1981); 13, Musch *et al.* (1982); 14, Geck *et al.* (1980); 15, Russell (1982); 16, Vidaver & Shepherd (1968); 17, Ellory *et al.* (1981); 18, this paper; 19, Ellory & Dunham (1980); 20, Doljanski *et al.* (1974); 21, Roti Roti & Rothstein (1973); 22, Aull (1981); 23, De Pont & Bonting (1981).

These references are selective, and not exhaustive.

† The word co-transport has been avoided since Cl^--dependence, but not Cl^- transport, has been shown for many systems, e.g. red cells.

1979) has recently been demonstrated to be dependent on K^+ (Russell 1981), and is probably electroneutral Na^+, K^+, Cl^- co-transport with a complex stoichiometry ($2Na^+:1K:3Cl^-$) (J. M. Russell, personal communication).

At the outset it is important to consider some of the problems involved in studying linked anion–cation transport. Aside from the difficulty of choosing inert cation or anion substitutes that do not act as inhibitors (e.g. Mg^{2+}, NO_3^-), or partial substrates (I^-, SCN^-), there are often particular problems in terms of electrical potential effects giving apparent coupling. For example, it has been proposed that resolution between coupled NaCl transport on a single discrete carrier, and Na^+–H^+ and Cl^-–HCO_3^- exchange coupled electrically and by intracellular buffering is extremely difficult (Schultz 1981). Similar arguments implicating K^+–H^+ and Cl^-–HCO_3^- exchange in *Amphiuma* red cells have also been made (Kregenow 1981). On the other hand one may be able to resolve the two systems in squid axon. There is a cation–Cl^- co-transport system that is inhibited by stilbene derivatives (inhibitors of anion exchange in red cells) (Russell & Boron 1976). This system, probably independent of K^+, may represent electrical coupling between Na^+–H^+ and HCO_3^-–Cl^- exchange pathways. The Na^+, K^+,Cl^- co-transport, not inhibited by stilbenes, is probably a separate pathway with direct rather than electrical coupling between anions and cations.

Further problems may arise from total reliance on furosemide as a specific inhibitor, because it can affect a variety of systems including the band 3 anion exchanger, amino acid transport, the sodium pump and adenyl cyclase. Recently the high-affinity furosemide analogues bumetanide and piretanide have been preferred for defining co-transport fluxes (Ellory & Stewart 1982).

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Other anion-dependent Na^+ fluxes, which are listed in table 1 for completeness, include transport on the band 3 anion protein as $NaCO_3^-$ (Funder & Wieth 1980) and via the glycine transport system, which requires both Na^+ and Cl^- to be present (Ellory *et al.* 1981). Experimentally, single cell systems, particularly ascites tumour cells and erythrocytes (mammalian, and also avian and fish) represent attractive preparations for studying membrane transport since they can eliminate extracellular space and cell inhomogeneity problems. The very high Cl^- permeability of red cells, and large Na^+ and K^+ pump rates of ascites cells, make Na^+:K^+:Cl^- stoichiomety measurements difficult, but nevertheless these preparations have yielded important results on Cl^--dependent cation transport. Work on ascites cells has been recently summarized by Hoffman and others and is presented at this symposium, so in the succeeding sections we intend to concentrate on erythrocytes.

Na^+, K^+, Cl^- co-transport

The initial observations of ouabain-insensitive mediated Na^+ transport, which produced the concept of pump II (Hoffman & Kregenow 1966; Sachs 1971) in human red cells, were consolidated by the demonstration of Na^+–K^+ co-transport by Wiley & Cooper (1974) that was furosemide-sensitive. Subsequently two groups have shown that this system is Cl^--dependent (Dunham *et al.* 1980; Chipperfield 1980). Kinetic considerations led to the idea that this flux might be mediated by the sodium pump even in the presence of ouabain (Beaugé & Lew 1977) but experiments on liposomes failed to confirm this (Karlish *et al.* 1981).

Recently it has been proposed that this co-transport system is altered in red cells from hypertensive patients (Garay *et al.* 1980). This is controversial. However, it has generated a lot of interest in the properties of the system, although there are still unresolved problems, particularly with regard to the stoichiometry of transport of the three ions involved, both in efflux and influx studies. Na^+ and K^+ transport have been measured by both net and radio-isotope fluxes. For tracer uptake the system shows simple Michaelis–Menten-type kinetics for both Na^+ and K^+ influx (figure 1), with respective apparent K_m values around 5 and 10 mM (see also Beaugé & Lew 1977; Dunham *et al.* 1980). Anion substitution experiments reveal a low apparent affinity for Cl^-. Using PCMBS-loading techniques, Garay *et al.* (1981) looked at internal activation of efflux by Na^+ and K^+. The kinetics were not simple, but half-maximal fluxes were obtained at Na^+ and K^+ concentrations around 13 and 10 mM respectively. In their experiments, where net efflux was measured by atomic absorption analyses, there was a 1:1 stoichiometry for Na^+ and K^+ efflux. In contrast, comparison of the apparent V_{max} for influx in the three subjects in figure 1 gives a ratio of bumetanide-sensitive Na^+/K^+ influx of 1:2. Such differences in stoichiometry may be due to a fraction of the flux representing an exchange component, i.e. as a bumetanide-sensitive tracer K^+–K^+ exchange, which may not participate in net fluxes. A further obvious possibility is that there are two independent systems, rather than a single coupled Na^+, K^+, Cl^- transporter. A two-system approach, involving independent NaCl and KCl transport, has been proposed for ascites tumour cells (Hoffman, this symposium; see also the comments on avian erythrocytes by Kregenow 1981), and recently rat erythrocytes (Duhm *et al.* 1982). In this vein Adragna *et al.* (1981) have suggested that the Na^+–K^+ stoichiometry varies with changes in cell volume. This observation is consistent with two separate pathways: a $Na^+ + K^+ + Cl^-$ system insensitive to volume changes and a $K^+ + Cl^-$ system sensitive to volume changes. In these cases there are volume-dependent fluxes that

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explain differences in K^+/Na^+ stoichiometry. It is therefore important to look at volume-sensitive KCl fluxes in more detail.

Cl^--dependent K^+ fluxes in sheep erythrocytes

Volume-dependent K^+ fluxes have been demonstrated in the erythrocytes of a variety of species and in various types of mammalian cells (see Dunham & Ellory (1981) for a review, and also the contributions of Hoffman and Grinstein *et al.* in this symposium). In this context we have worked with LK sheep red cells, because their volume-dependent K^+ flux is large,

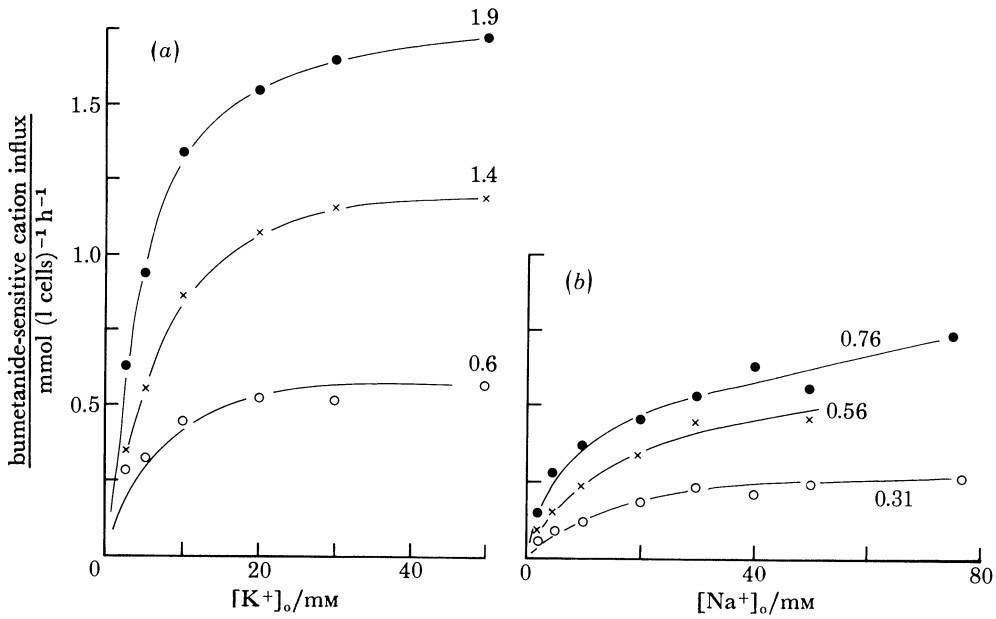

FIGURE 1. Concentration dependence of bumetanide-sensitive K^+ (*a*) and Na^+ (*b*) influx in red cells from three different human subjects. The numbers on the curves refer to the calculated V_{max} values for the fitted curves by using Michaelis–Menten kinetics. For varying $[Na]_o$, $[K]_o$ was constant at 75 mM, with choline replacement; for varying $[K]_o$, $[Na]_o$ was constant at 100 mM, with choline replacement. Ouabain (0.1 mM) was always present.

Cl^--dependent and genetically determined. By contrast, passive K^+ influx in HK-type cells is relatively insensitive to changes in cell volume (figure 2). Further, the K^+ flux induced by swelling can be modified by the specific blood group alloantibody anti-L, in LK cells. This antibody was originally identified serologically by the usual complement lysis methods, but has subsequently been shown to stimulate the Na^+ pump in LK cells by altering their internal cation affinity (Sachs *et al.* 1974; Cavieres & Ellory 1977). Recently anti-L has been fractionated into two components, anti-L_1 and L_P (Smalley *et al.* 1982), defined on the basis of lytic or pump-stimulating ability. It is the anti-L_1 antibody that modifies the volume response in LK cells (Dunham & Ellory 1981), by markedly decreasing the sensitivity of passive K^+ influx to cell swelling or shrinking. In an attempt to characterize this KCl passive transport route by using various pharmacological agents, Ellory & Dunham (1980) showed that it is not Ca^{2+}-dependent or quinine-inhibitable, and is thus not mediated via a Gardos-type channel (Lew & Ferreira 1978). It is insensitive to piretanide and bumetanide, and shows a weak inhibition by high concentrations of furosemide ($I_{50\%} = 2$ mM), and phloretin ($I_{50\%} = 0.16$ mM). Recently

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Lauf & Theg (1980) reported that treating LK sheep red cells with *N*-ethylmaleimide (NEM) increased passive K^+ permeability via a Cl^--dependent system. The magnitude of the reported fluxes was of the same order as those induced by swelling the cells by 15%. It is therefore important to establish whether these two treatments are inducing the same or parallel transport systems, and so we have investigated the interactions of volume changes, NEM treatment and anti-L sensitization on Cl^--dependent K^+ permeability in LK sheep cells. Figure 3*a* shows a dose–response curve for NEM treatment affecting passive Cl^--dependent K^+ influx (measured in the presence of Cl^- or NO_3^-). The effect of NEM on the anion-dependent K^+ flux is clearly biphasic, giving an initial inhibition and subsequent large stimulation as the NEM dose is increased. (It is not really meaningful to talk about the *concentration* of NEM since the red cell –SH

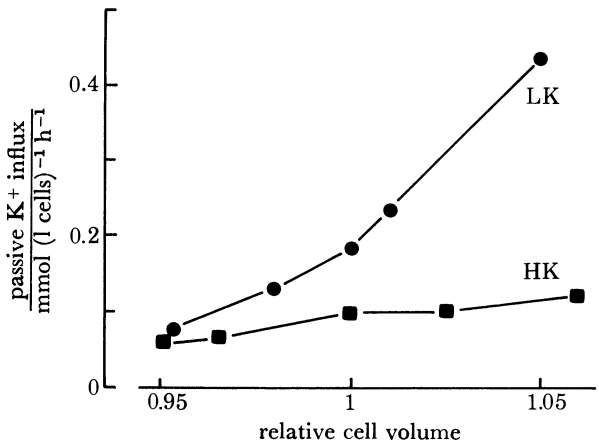

FIGURE 2. Passive K^+ influx into red cells from one HK (■) and one LK (●) sheep as a function of cell volume. The cell volumes were altered with sucrose or water addition and measured as described previously (Dunham & Ellory 1981).

groups (especially haemoglobin) will titrate all the NEM as its concentration is raised until NEM is in excess. Furthermore, effectiveness depends critically on time of exposure and particularly on haematocrit.) This biphasic effect was consistently obtained, and may reflect a difference in the site of action, inhibiting initially by titrating superficial sites, and subsequently stimulating via less exposed –SH groups. In the absence of NEM, replacement of Cl^- with NO_3^- caused a reduction in K^+ influx, as reported before (Dunham & Ellory 1981). Furthermore, the NEM-sensitive pathways (both the inhibitable pathway and the stimulable pathway) are Cl^--dependent because NEM had no effect at any concentration in NO_3^- medium.

In the lower panel (figure 3*b*) the interactions of the anti-L antibody with the NEM effect on passive K^+ uptake are demonstrated. Pretreatment with anti-L, and subsequent washing, prevents the NEM stimulation of flux, whereas the addition of anti-L after NEM has been reacted with the cells fails to reverse the effect, although it somewhat reduces its overall magnitude. The effects of anti-L are consistent with two separate sites of action of NEM: anti-L prevented the stimulation by NEM (at sites less exposed, or perhaps with lower affinity), but did not prevent inhibition by NEM. Even after NEM treatment, there is still a volume dependence of K^+ flux in LK cells: figure 4 shows that the passive K^+ flux in NEM-treated cells is doubled by a 20% change in cell volume. Finally, the passive K^+ flux induced in LK-sheep cells by NEM shows a low affinity for Cl^- (figure 5) consistent with the flux induced by swelling;

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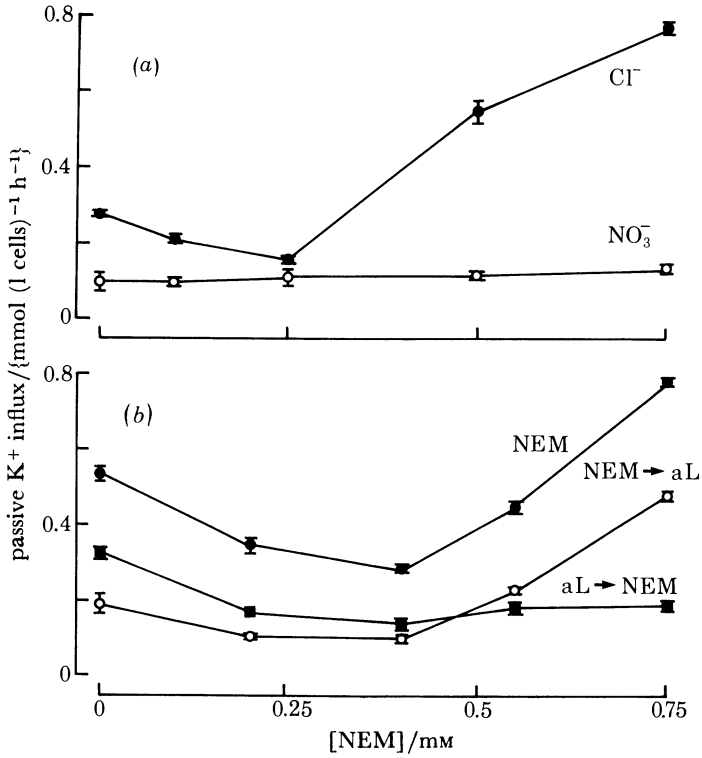

FIGURE 3. (*a*) The concentration-dependence of the NEM effect on passive K^+ influx in LK sheep red cells in Cl^- or NO_3^- media. Cells were incubated with NEM at 5% haematocrit for 5 min, and then washed by centrifugation. Some aliquots were then equilibrated and washed in Cl^--free medium with NO_3^- substitution. (*b*) Comparison of the effect of anti-L (aL) treatment before or after NEM treatment on passive K^+ influx in LK sheep red cells. Cells were treated with NEM, as in (*a*), either before or after anti-L incubation.

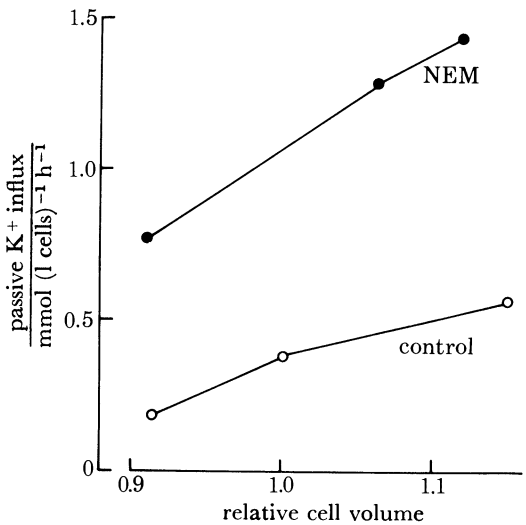

FIGURE 4. The effect of cell volume on passive K^+ influx in control (○) and NEM-treated (●) LK sheep red cells. Cells were treated with NEM, (2 mM) at 5% haematocrit for 10 min.

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taken together with K^+ affinity experiments this suggests that the stimulation is an increased velocity (V_{max}) rather than altered affinity as the flux is activated. These results suggest that NEM treatment and swelling are inducing the same Cl^--dependent K^+-transport system, the titration of membrane –SH groups effectively mimicking the volume sensor.

NEM effects on other erythrocytes

Davson (1937) showed that erythrocytes from several mammalian species showed an increased K^+ permeability when swollen. Although Lauf & Theg (1980) found that NEM stimulation of K^+ flux was confined to LK-type sheep erythrocytes (HK sheep being insensitive), we have

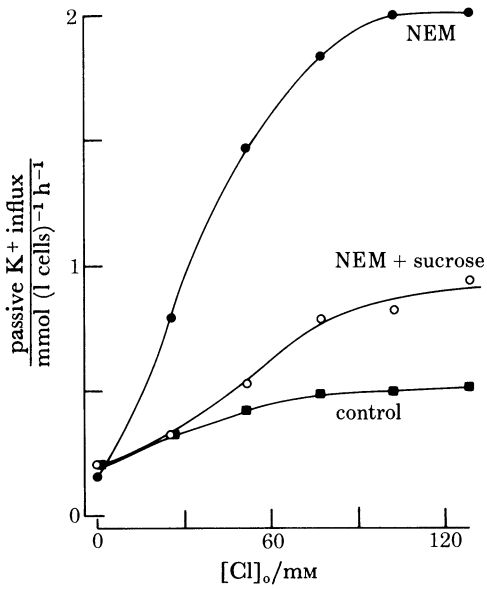

Figure 5. The effect of varying external Cl^- concentrations on the passive K^+ influx in LK sheep red cells, with (●) or without NEM (■) treatment (2 mM, 10 min, 5% haematocrit). Data for NEM cells subsequently shrunken by the addition of sucrose are also shown (○).

surveyed a number of other species' red cells, and found NEM stimulation of K^+ influx in all cases. Species examined so far include LK goat, cow, barbary sheep, rabbit, hamster, hedgehog, rat and human. Table 2 presents data on passive K^+ influx in human red cells, measured in Cl^- or methylsulphate ($MeSO_4^-$) substituted media, in the presence or absence of ouabain and bumetanide (each 0.1 mM). It is clear that under these conditions that NEM causes a small inhibition of the sodium pump, has no effect on the bumetanide-sensitive Na^+–K^+ co-transport system, but gives a large Cl^--dependent increase in passive K^+ influx (see also Duhm 1982). Time-course experiments confirmed that the NEM effect was maximal after 10 min treatment, and the measured fluxes were linear over 30 min. To eliminate the possibility that Ca^{2+} activation via a Gardos channel was involved, we showed that external addition of 1 mM EGTA + 1 μM A23187 failed to affect the flux. However, experiments with quinine (0.5 mM) revealed a significant inhibition. Thus, although direct activation via Ca^{2+} can be ruled out, the NEM-induced flux is sensitive to the Gardos-channel inhibitor quinine (but see Lew & Ferreira (1978) for comments on the specificity of this inhibitor). Anion replacement experiments revealed the series of effectiveness $Cl^- > Br^- > SCN^- > I^- > NO_3^- > MeSO_4^-$ (i.e.

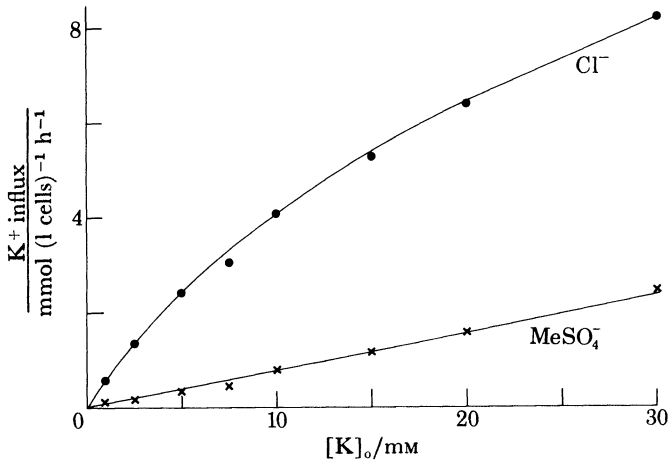

FIGURE 6. Concentration dependence of passive K^+ influx in NEM (2 mM) treated human red cells, measured in Cl^- (●) or $MeSO_4^-$ (×) medium. The fitted line for Cl^- medium represents $v = 8.93/\{1 + (17.2/[K])\} + 0.0077[K]$.

TABLE 2. THE EFFECT OF NEM ON K^+ INFLUX IN HUMAN RED CELLS

(Units are millimoles K^+ per litre of cells per hour.)

	control cells		NEM-treated cells	
	NaCl medium	$NaMeSO_4$ medium	NaCl medium	$NaMeSO_4$ medium
control	2.132	1.354	4.758	1.509
+ ouabain (0.1 mM)	0.833	0.121	3.662	0.520
+ ouabain, bumetanide (0.1 mM)	0.143	0.148	2.988	0.634
Na^+ pump	1.299	1.233	1.096	0.989
Na^+–K^+ co-transport	0.690	0	0.674	0
Cl^--dependent residual component	−0.003		2.354	

External $[K^+]$, 7.5 mM; pH 7.5; [NEM], 2 mM, 15 min; flux time, 30 min.

TABLE 3. CONCENTRATION DEPENDENCE OF FUROSEMIDE AND BUMETANIDE INHIBITION OF NEM-STIMULATED K^+ UPTAKE IN HUMAN RED CELLS

inhibitor concentration/M	furosemide K^+ flux mmol(l cells^{-1}) h^{-1}	I(%)	bumetanide K^+ flux mmol(l cells^{-1})h^{-1}	I(%)
0	5.49	0	5.42	0
10^{-3}	3.41	38	3.42	40
3×10^{-4}	3.76	31	4.31	20
10^{-4}	4.54	17	4.67	14
3×10^{-5}	5.40	2	5.04	7
10^{-5}	—	—	5.28	2

Flux conditions: $[K^+]$, 7.5 mM; choline-chloride medium; 20 min incubation; NEM pretreatment, 2 mM 10 min.

the K^+ influx was lowest in $MeSO_4^-$). Kinetic analysis of the NEM-induced K^+ flux (figure 6) indicated simple Michaelis–Menten kinetics, with a K_m around 16 mM K^+, and a V_{max} of 9 mmol (l cells)$^{-1}$ h^{-1}. Inhibitor studies indicated no effect of oligomycin (10 μg ml^{-1}) or SITS (0.2 mM), although phloretin did show significant inhibition (50 % at 0.2 mM). Since the loop diuretics have often been used to characterize these anion-dependent cation fluxes, it is important to define the sensitivity of this NEM-induced flux to these pharmacological agents.

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Table 3 shows that both furosemide and bumetanide inhibit this NEM-induced K^+ flux at high (over 0.3 mM) concentration. However, the human Na^+–K^+ co-transport system shows a $K_{\frac{1}{2}}$ of 9 and 0.16 μM respectively for these two agents (Ellory & Stewart 1982), and Cl^- transport via band 3 has a $K_{\frac{1}{2}}$ of 0.12–0.17 μM for furosemide (Lambert & Lowe 1980). These effects of loop diuretics at high concentrations are therefore probably non-specific, reflecting a very low-affinity interaction.

In Na^+-substitution experiments, with the use of choline to replace Na^+, the NEM-enhanced K^+ flux in human red cells was increased in the Na^+-free medium, indicating no Na^+-dependence but rather a Na^+ inhibition. A similar Na^+ effect has been demonstrated for the LK sheep volume-sensitive K^+ transport system (Dunham & Ellory 1981).

TABLE 4. RESOLUTION OF RED CELL ANION-DEPENDENT TRANSPORT SYSTEMS?

	human red cell		LK sheep red cell
	Na^+, K^+ transport	NEM-induced K^+ transport	volume-dependent K^+ transport
cell swelling	no effect†	no effect	stimulates
hydrostatic pressure	inhibits	n.t.	stimulates
$K_m[K]_o$	*ca.* 5 mM	*ca.* 16 mM	*ca.* 40 mM
anion selectivity	$Cl^- > Br^-$	$Cl^- > Br^-$	$Br^- > Cl^-$
NEM	no effect	stimulates	stimulates
$[Na]_o$	required	inhibits	inhibits
quinine (0.5 mM)	no effect	inhibits	no effect
furosemide I_{50}	0.009 mM	2 mM	2 mM
bumetanide I_{50}	0.0002 mM	2 mM	2 mM

† Adragna *et al.* (1980) do report a volume effect, but this is controversial.

DISCUSSION

One aim of the present contribution (and, indeed, this symposium) is to try and establish the number and identity of anion-dependent Na^+ and K^+ transport systems in mammalian cells. We have defined in some detail the properties of three transport systems, the human red cell Cl^--dependent Na^+–K^+ co-transport system, the LK sheep red cell volume-dependent KCl system, and the human red cell NEM-induced passive K^+ transport pathway. What is the evidence for their separate identity? Table 4 compares some kinetic properties and pharmacological data to indicate points of difference. One additional piece of evidence included in table 4 is the effect of high hydrostatic pressure (100–400 atm (*ca.* 10–40 MPa)) which causes a marked stimulation of Cl^--dependent K^+ flux in human red cells, while inhibiting Na^+–K^+ co-transport (Hall *et al.* 1982). It is tempting to suggest that pressure rearranges the membrane in the same way that NEM titration of membrane –SH groups may do.

Taken overall, most of the evidence presented in table 4 is weak in the sense that the differences between the three systems are relative, or quantitative rather than absolute. Such changes in affinity could reflect different activation states of the same physiological system, for instance as has been clearly described for the red cell Ca^{2+} pump (Schatzmann 1982).

An important caveat concerns the use of furosemide, particularly at concentrations in the millimolar range, as a specific inhibitor of Cl^--dependent transport systems. As can be seen in table 4, at high concentrations the loop diuretics start to inhibit all three systems, but a 1000-fold difference in affinities argues strongly for a non-specific effect at high concentrations, and low (less than 0.1 mM) concentrations of bumetanide and piretanide are to be preferred as specific pharmacological agents.

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If, as seems likely, the LK volume-sensitive and NEM-sensitive systems are in fact identical, the role of NEM in inducing the transport system may define the site of the volume-sensor in terms of membrane –SH groups. However, particularly in the erythrocyte it is difficult to see how a change in cell volume is transmitted to the cell membrane. The demonstration of inhibition by anti-L_1 of the sheep Cl^--dependent K^+ transport system argues for a specific effect, with rather few membrane sites involved. In contrast with the huge number of NEM-binding residues in a sheep red cell, 5×10^6 sites in the membrane alone (Tosteson 1966), there are only *ca.* 850 L_1 sites per cell (Smalley *et al.* 1982).

A major uncertainty at present is the status of Na^+ transport (particularly Na^+ influx) via the Na^+–K^+ co-transport system. The variable stoichiometry of Na^+–K^+ transport reported is difficult to explain and suggests either that a fraction of the K^+ flux may be via an exchange mechanism (i.e. an isotope effect) or that there is a separate, superimposed K^+–Cl^- flux being measured in these experiments. Functionally, these transport systems have been proposed to play a role in NaCl transport and volume regulation in both red cells (Sachs *et al.* 1975; Kregenow 1981) and epithelia (Schultz 1981). Certainly the concept of secondary active transport, where the energy derived from the electrochemical gradient for one ion can drive another uphill, is now firmly established in the scientific orthodoxy. It is to be hoped that these anion-coupled fluxes will soon be defined and separated in sufficient detail for their biological role to be determined.

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References

Adragna, N., Canessa, M., Bize, I., Garay, R. & Tosteson, D. C. 1980 (Na+K) co-transport and cell volume in human red blood cells. *Fedn. Proc. Fedn. Am. Socs. exp. Biol.* **39**, 1842.

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

Aull, F. 1981 Potassium chloride cotransport in steady-state ascites tumor cells. *Biochim biophys. Acta* **643**, 339–345.

Beaugé, L. A. & Lew, V. L. 1977 Passive fluxes of sodium and potassium across cell membranes. In *Membrane transport in red cells* (ed. J. C. Ellory & V. L. Lew), pp. 39–51. Academic Press.

Bourne, P. K. & Cossin, A. R. 1982 On the instability of K influx in erythrocytes of the rainbow trout *Salmo gairdari* and the role of catecholamine hormones in maintaining *in vivo* influx activity. *J. exp. Biol.* (In the press.)

Brading, A. F. 1981 Ionic distribution and mechanisms of transmembrane ion movements in smooth muscle. In *Smooth muscle: an assessment of current knowledge* (ed. E. Bülbring, A. F. Brading, A. W. Jones & T. Tomita), pp. 65–92. London: Edward Arnold.

Burg, M. & Green, N. 1973 Function of the thick ascending limb of Henle's loop. *Am. J. Physiol.* **224**, 659–668.

Cavieres, J. D. & Ellory, J. C. 1977 The interaction of monovalent cations with the sodium pump of low potassium goat erythrocytes. *J. Physiol., Lond.* **271**, 289–318.

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

Davson, H. 1937 The loss of potassium from the erythrocyte in hypotonic saline. *J. cell Physiol.* **10**, 247–264.

De Pont, J. J. H. H. M. & Bonting, S. L. 1981 Anion-sensitive ATPase and (K^++H^+)-ATPase. In *Membrane transport* (ed. S. L. Bonting & J. J. H. H. M. de Pont), pp. 209–234. Amsterdam: Elsevier.

Daljanski, F., Ben-Sasson, S., Reich, M. & Grover, N. B. 1974 Dynamic osmotic behaviour of chick blood lymphocytes. *J. Cell Physiol.* **84**, 215–224.

Dunham, P. B. & Ellory, J. C. 1981 Passive potassium transport in LK sheep red cells: dependence upon cell volume and chloride. *J. Physiol., Lond.* **318**, 511–530.

Dunham, P. B., Stewart, G. W. & Ellory, J. C. 1980 Chloride-activated passive potassium transport in human erythrocytes. *Proc. natn. Acad. Sci. U.S.A.* **77**, 1711–1715.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Duhm, J. 1982 Lithium transport pathways in erythrocytes. In *Symposium on basic mechanisms in the action of lithium* (ed. H. D. Lux & H. M. Emrich), pp. 1–20. Excerpta Medica.

Duhm, J., Göbel, B. O., Lorenz, R. & Weber, P. C. 1982 Sodium lithium exchange and sodium potassium cotransport in human erythrocytes. Part II. *Hypertension* **4**. (In the press.)

Ellory, J. C. & Dunham, P. B. 1980 Volume-dependent passive potassium transport in LK sheep red cells. In *Membrane transport in erythrocytes* (*Alfred Benzon Symposium* no. 14) (ed. U. Lassen, H. H. Ussing & J. O. Wieth), pp. 409–427. Copenhagen: Muncksgaard.

Ellory, J. C., Hall, A. C., Klein, R. A. & Macdonald, A. G. 1982 The effect of pressure on passive K^+ uptake by human erythrocytes and the influence of counter ions. *J. Physiol., Lond.* **324**, 50–51P.

Ellory, J. C., Jones, S. E. M. & Young, J. D. 1981 Glycine transport in human erythrocytes. *J. Physiol., Lond.* **320**, 403–422.

Ellory, J. C, & Stewart, G. W. 1982 The human erythrocyte Cl^-dependent Na–K cotransport system as a possible model for studying the action of loop diuretics. *Br. J. Pharmac.* **75**, 183–188.

Frizzell, R. A., Field, M. & Schultz, S. G. 1979 Sodium-coupled chloride transport by epithelial tissues. *Am. J. Physiol.* **236**, F1–F8.

Funder, J. & Wieth, J. O. 1980 Passive cation transport by ion pair formation. In *Membrane transport in erythrocytes* (*Alfred Benzon Symposium*, no. 14) (ed. U. V. Lassen, H. H. Ussing & J. O. Wieth), pp. 520–530. Copenhagen: Muncksgaard.

Garay, R., Adragna, N., Canessa, M. & Tosteson, D. 1981 Outward sodium and potassium cotransport in human red cells. *J. Membrane Biol.* **62**, 169–174.

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**, 432–447.

Greger, R. 1981 Coupled transport of Na^+ and Cl^- in the thick ascending limb of Henle's loop of rabbit nephron. *Scand. J. Audiol. Suppl.* **14**, 1–15.

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

Hall, A. C., Ellory, J. C. & Klein, R. A. 1982 Pressure and temperature effects on human red cell cation transport. *J. Membrane Biol.* **68**, 47–56.

Hoffman, J. F. & Kregenow, F. M. 1966 The characterization of new energy dependent cation transport processes in red blood cells. *Ann. N.Y. Acad. Sci.* **137**, 566–576.

Jørgensen, P. L. 1976 The function of (Na^+, K^+)-ATPase in the thick ascending limb of Henle's loop. In *Current problems in clinical biochemistry*, vol. 6 (*Renal metabolism in relation to renal function*) (ed. U. Schmidt & U.C. Dubach), pp. 190–199. Berne, Stuttgart and Vienna: Hans Huber.

Karlish, S. J. D., Ellory, J. C. & Lew, V. L. 1981 Evidence against Na^+-pump mediation of Ca^{2+}-activated K^+ transport and diuretic-sensitive (Na^+/K^+)-cotransport. *Biochim. biophys. Acta* **646**, 353–355,

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

Lambert, A. & Lowe, A. G. 1980 Chloride–bicarbonate exchange in human red cells measured using a stopped flow apparatus. *J. Physiol., Lond.* **306**, 431–443.

Lauf, P. K. & Theg, B. E. 1980 A chloride dependent K^+ flux induced by *N*-ethylmaleimide in genetically low K^+ sheep and goat erythrocytes. *Biochim. biophys. Res. Commun.* **92**, 1422–1428.

Lew, V. L. & Ferreira, H. G. 1978 Calcium transport and the properties of a calcium-activated potassium channel in red cell membranes. *Curr. Top. Membranes Transport* **10**, 217–277.

Musch, M. W., Field, M. & Frizzell, R. A. 1982 Active K^+ transport by the intestine of the flounder, *Pseudopleuronectes americanus*: evidence for cotransport with Na and Cl. *Bull. MDIBL* (in the press.)

Roti Roti, L. W. & Rothstein, A. 1973 Adaptation of mouse leukemic cells (L 5178 Y) to aniosotonic media. *Expl Cell. Res.* **79**, 295–310.

Russell, J. M. 1979 Chloride and sodium influx: a coupled uptake mechanism in the squid giant axon. *J. gen. Physiol.* **73**, 801–818.

Russell, J. M. 1981 K^+-dependent Na + Cl uptake by squid giant axon. *Biophys. J.* **33**, 40a.

Russell, J. M. & Boron, W. F. 1976 Role of chloride transport in regulation of intracellular pH. *Nature, Lond.* **264**, 73–74.

Russell, J. M. & Brodwick, M. S. 1979 Properties of chloride transport in barnacle muscle fibres. *J. gen. Physiol.* **73**, 343–368.

Sachs, J. R. 1971 Ouabain insensitive sodium movements in the human red blood cell. *J. gen. Physiol.* **57**, 259–282.

Sachs, J. R., Ellory, J. C., Kroop, D. L., Dunham, P. B. & Hoffman, J. F. 1974 Antibody-induced alterations in the kinetic characteristics of the Na:K pump in goat red cells. *J. gen. Physiol.* **63**, 389–414.

Sachs, J. R., Knauf, P. A. & Dunham, P. B. 1975 Transport through red cell membranes. In *The red blood cell* (ed. D. M. Surgenor), pp. 613–703. Academic Press.

Schultz, S. G. 1981 Salt and water absorption by mammalian small intestine. In *Physiology of the gastrointestinal tract* (ed. L. R. Johnson), pp. 983–989. New York: Raven Press.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Schatzmann, H. J. 1982 The plasma membrane calcium pump of erythrocytes and other animal cells. In *Membrane transport of calcium* (ed. E. Carafoli), pp. 41–108. Academic Press.
Smalley, C. E., Tucker, E. M., Dunham, P. B. & Ellory, J. C. 1982 Interaction of L antibody with low potassium type sheep red cells: resolution of two separate functional antibodies. *J. Membrane Biol.* **64**, 167–174.
Tosteson, D. C. 1966 Some properties of the plasma membranes of high potassium and low potassium sheep red cells. *Ann. N.Y. Acad. Sci.* **137**, 577–590.
Vidaver, G. A. & Shepherd, S. L. 1968 Transport of glycine by haemolyzed and restored pigeon red blood cells. *J. biol. Chem.* **243**, 6140–6150.
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.

Discussion

C. Claire Aickin (*University Department of Pharmacology, Oxford, U.K.*). Dr Ellory has suggested a classification for K^+–Cl^- co-transport by its insensitivity to furosemide. However, we have evidence for a K^+–Cl^- co-transport system that is inhibited by both furosemide and DIDS and in addition does not require an osmotic challenge for its activation (Aickin *et al.* 1982). This is an outwardly directed mechanism responsible for the low intracellular Cl^- activity ($a^i_{Cl^-}$) in the crayfish stretch receptor neuron, thus providing the driving force for hyperpolarizing postsynaptic inhibition. We have investigated the mechanism by using the reversal potential of the i.p.s.p., or in Na^+-free conditions the response to ionophoretically applied GABA, to monitor changes in $a^i_{Cl^-}$ (Deisz & Lux 1982). This mechanism does not appear to be analogous to a furosemide-sensitive Na^+, K^+, Cl^- co-transport since total removal of extracellular Na^+ (choline substitution) has no effect on the reversal potential, whereas total removal of extracellular K^+ (K^+_o) causes a hyperpolarizing shift (Aickin *et al.* 1982). In addition, the reversal potential is shifted to less negative values when intracellular [Na^+] would be expected either to decrease (increasing K^+_o concentration or applying NH^+_4) or to increase (prolonged removal of K^+_o or ouabain application). Another aspect in which this K^+–Cl^- co-transport differs from that activated by volume changes is its higher affinity for Rb^+ than for K^+ (Aickin *et al.* 1980, 1982). This mechanism may not be confined to the crayfish because the abolition of the hyperpolarizing Cl^- gradient by application of NH^+_4, resulting from interference with the mechanism, has often been demonstrated in mammalian neurons (for review see Iles & Jack 1980).

References

Aickin, C. C., Deisz, R. A. & Lux, H. D. 1980 *J. Physiol., Lond.* **300**, 40P.
Aickin, C. C., Deisz, R. A. & Lux, H. D. 1982 *J. Physiol., Lond.* **329**. (In the press.)
Deisz, R. A. & Lux, H. D. 1982 *J. Physiol., Lond.* **326**, (In the press.)
Iles, J. F. & Jack, J. J. B. 1980 *Brain* **103**, 555–578.

J. A. Young (*Department of Physiology, Sydney University, Australia*). It is not a universal observation that furosemide inhibits only Na^+–Cl^- co-transport mechanisms. In the perfused rabbit mandibular gland, where we find that 1 mM furosemide reduces secretory rate by about 30–45%, the blocker causes the composition of the secreted saliva to switch from a predominantly Cl^--containing fluid ([Cl^-] = 30–60 mM and [HCO^-_3] = 13–26 mM) to a markedly HCO^-_3-rich secretion ([Cl^-] = 1–12 mM, [HCO^-_3] = 34–80 mM) (Novak & Young 1982). If, as we suppose, the secretory process is dependent on the activity of a double Na^+–H^+, Cl^-–OH^- countertransport system located in the basal plasma membrane, then it follows that furosemide specifically inhibited the Cl^-–OH^- exchange mechanism, so that HCO^-_3 entry into the endpiece lumen across the apical cell membrane replaced Cl^- entry as the prime driver of secretion. It

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must be admitted, however, that an alternative secretory model involving two separate systems operating in parallel, one for Na^+–Cl^- co-transport, sensitive to furosemide, and another for Na^+–H^+ exchange, would also account for our observations and preserve intact the idea that furosemide blocks cation–Cl^- co-transport mechanisms rather than anion exchangers.

Reference

Novak, I. & Young, J. A. 1982 Is salivary secretion dependent on a double Na/H, Cl/OH countertransport system? *Proc. Aust. physiol. pharmac. Soc.* **12**, no. 2.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES